Analysis of the Canonical Initiation and *Trans*-acting Factor Requirements of 5'TOP Containing mRNAs

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Abstract

All eukaryotic mRNAs process a cap structure ($m^7G(5')ppp(5')N$) at the 5' end of their message and most have an A as the first nucleotide after the cap. However, 30% of messages within eukaryotic cells have a C ($m^7G(5')ppp(5')C$) as the first nucleotide followed by a short polypyrimidine tract. These mRNAs are termed TOP (Terminal Oligopyrimidine tract) messages and are co-ordinately regulated by mitogenic, growth and nutritional stimuli. This work describes the construction of a reporter vector that encodes mRNA containing the TOP motif, and its use in a series of systematic experiments to further investigate the translational regulation of TOP messages. Given that TOP containing mRNAs are known to encode proteins involved in the translational machinery, these findings have important implications with regard to translational control and translation related disease.

In this study, reporter vectors have been used to investigate the role of the mTOR and PI3K signalling pathways, which have previously been implicated in the translational regulation of TOP containing mRNAs. The data obtained suggests that the mTOR signalling pathway may be involved in the regulation of TOP containing mRNAs. The canonical initiation and *trans*-acting factor requirements of TOP mRNAs were also investigated using a combination of protein over-expression and affinity purification of TOP-containing-mRNA:protein complexes. The data obtained raises the possibility that eIF4E may not be required in the initiation of TOP containing mRNA translation. The candidate *trans*-acting factors that were identified include La, ILF2 and EBP1, the latter of which has previously been shown to associate with mature ribosomes in the cytoplasm. Finally, affinity purification of TOP-containing-mRNA:microRNA complexes was carried out. Candidate microRNAs which may be involved in the regulation of TOP containing mRNAs were identified. The data obtained was consistent with a previous study, which suggested that microRNA-10a may bind to the 5'UTR of TOP containing mRNAs.

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Abbreviations

4E-BP	eIF4E-binding protein
AGO1/2/3/4	argonaute 1/2/3/4
Akt /PKB	murine thymoma viral oncogene homolog 1/protein kinase B
Apaf-1	apoptotic protease activating factor 1
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphate
Bag-1	BCl-2 associated anthanogene 1
BCl-2	B-cell leukaemia gene 2
Bip	immunoglobulin heavy chain binding protein
bp	base pairs
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatise
CMV	cytomegalovirus
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
cpm	counts per minute
CrPV	cricket paralysis virus
CSFV	classic swine fever virus
CTP	cytidine 5'triphosphate
dATP	deoxyadenosine 5'-triphosphate

DAP5	death associated protein 5
dCTP	deoxycytidine 5'triphosphate
ddNTP	dideoxynucleotide
DEAD box	Asp-Glu-Ala-Asp box protein
dGTP	deoxyguanosine 5'triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
dTTP	deoxythymidine 5'triphosphate
EBP1	ErbB3-binding protein 1
EBV	Epstein-Barr virus
E.Coli	*Escherischia coli*
EDTA	diaminoethanetetra-acetic acid
eIF	eukaryotic initiation factor
eEF	eukaryotic elongation factor
EMCV	encephalomyocarditis virus
FCS	fetal calf serum
Fluc	firefly luciferase
GAPDH/G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor

GST	glutathione-S-transferase
GTP	guanosine 5'-triphosphate
GTPase	guanidine triphosphatase
HCV	hepatitis C virus
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hnRNP	heteronuclear ribonuclearprotein
Hr	hour
HSP	heat shock protein
IGF-II	insulin-like growth factor II
ILF2/3	interleukin enhancer binding factor 2/3
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRE	iron response element
IRES	internal ribosome entry site
ITAF	IRES *trans*-acting factor
kB	kilobase
kcal	kilocalorie
kDa	kilodalton
La	lupus antigen
LARP	lupus antigen related protein
LB	Luria-Bertani broth
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
Met	methionine

Met-tRNA	initiator methionyl tRNA
miRNA	microRNA
miRNP	miRNA-containing-ribonucleoprotein particle
Mnk1	MAP kinase interacting kinase 1
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid
Ni-NTA	nickel-nitrilotriacetic acid
NLS	nuclear localisation signal
NPM	nucleophosmin
nt	nucleotide
ORF	open reading frame
PA2G4	proliferation-associated protein 2G4
PABP	poly(A) binding protein
PAIP-1/2	poly(A) binding protein interacting protein 1/2
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCBP1/2	poly(rC) binding protein 1/2
PCR	polymerase chain reaction
PKR	protein kinase A
PTB	polypyrimidine tract binding protein
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

RNase	ribonucleic acid hydrolyase
RNasin	ribonucleic acid hydrolyase inhibitor
RRL	rabbit reticulocyte lysate
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
s.d.	sample standard deviation
SDS	sodium dodecyl sulphate
siRNA	short interfering RNA
shRNA	short hairpin RNA
SV40	simian virus 40
TAE	tris-acetate EDTA
Taq	*Thermophilus aquaticus*
TBE	tris-borate EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TMK	Tris-$MgCl_2$-KCl Buffer
TMN	Tris-$MgCl_2$-NaCl Buffer
TOP	terminal oligopyrimidine tract
tRNA	transfer ribonucleic acid
uORF	upstream open reading frame
UTR	untranslated region
UTP	uridine 5'-triphosphate

Chapter One

Introduction

1.1 Gene Expression and Protein Synthesis: An Overview

The encoded genetic information contained within DNA is used to synthesise proteins in a multi-stage and highly regulated process known as gene expression. The regulation required to ensure normal cell growth can be achieved at multiple stages along the gene expression pathway. In transcription, the first stage of gene expression, RNA polymerase produces a complementary RNA copy of the DNA template, adding one nucleotide at a time. Post-transcriptional modifications, convert the pre-mRNA into mature mRNA. These events also occur in the nucleus, which is the site of transcription in most eukaryotic cells. In one such event, a 7-methylguanosine (m^7G) cap structure is added to the 5' end of the pre-RNA. This process involves the formation of a triphosphate bond, in a 5' to 5' orientation between the pre-mRNA and guanine residue. Methylation of the guanine ring at the N-7 position then occurs in a reaction catalysed by S-adenosyl methionine. The presence of a correctly orientated 5' cap structure ensures that the mRNA is protected from ribonucleases, whose action is specific to phosphodiester bonds, which are in a 3' to 5' orientation. At the 3' end of the mRNA, the molecule is cleaved at a site which lies downstream of a polyadenylation signal sequence. Following cleavage a poly(A) tail is attached in a process catalysed by the enzyme Poly(A) polymerase. In the final post transcriptional modification, splicing occurs, removing non-coding regions called introns. This process is catalysed by a large protein complex called the spliceosome and in some instances can occur co-transcriptionally. The remaining exons are reconnected to form a single, continuous mature mRNA. In a process

known as alternate splicing, many different mature mRNAs that encode different protein sequences may be obtained from a single pre-mRNA as a result of multiple ways that splicing may occur. Following post transcriptional modification, the mature mRNA is exported from the nucleus into the cytoplasm.

An intricate process is required to decode the triplet codons of the mature mRNA following transportation from the nucleus. This process comprises three sequential events and is termed translation (Figure 1.1). Initiation is the first of these events and encompasses the processes required for the formation of the 80S initiation complex, with a ribosome located at the appropriate translation start site. In the elongation phase, each codon is recognised by tRNAs charged with amino acids. As the ribosome translocates the mRNA in a 5' to 3' direction, anticodon recognition and peptide bond formation occur, adding each successive amino acid to the growing polypeptide chain. This process is aided by elongation factors. The final termination phase of translation occurs when the ribosome reaches the stop codon. At this stage, eukaryotic release factor eRF1 binds and hydrolyses the peptidyl-tRNA, resulting in the release of the polypeptide chain along with the ribosome form the mRNA. The entire process of translation is highly regulated, with regulation at the initiation stage being of particular important.

1.2 The Initiation Of Protein Synthesis

Initiation is the first step in the process of translation and is believed to be the rate-limiting step. It is therefore a highly regulated process and as a result has been a subject of great interest for many years (Pain 1996). One of the main advantages of controlling gene expression at the translational level is that the cell can respond

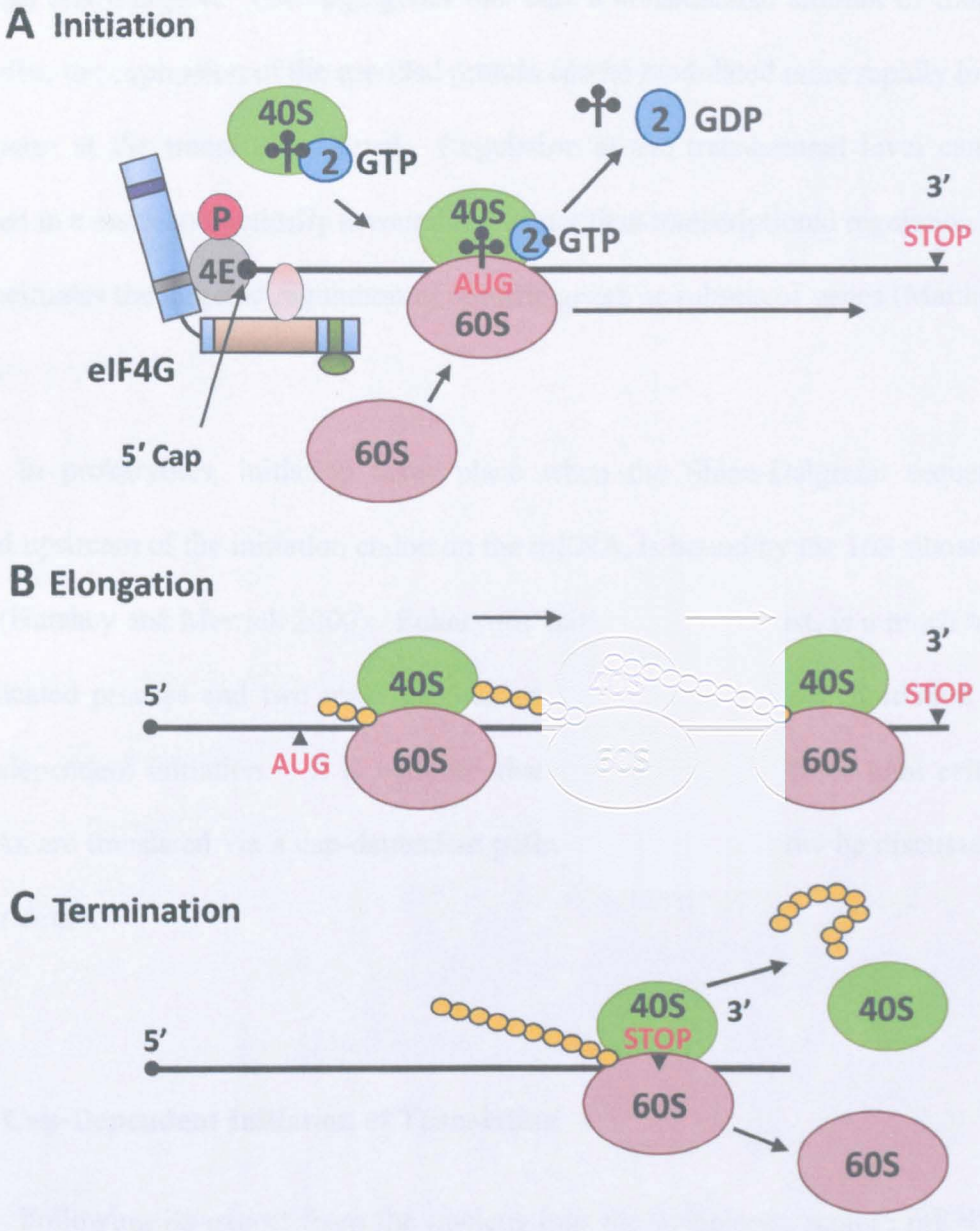


Figure 1.1 Overview of Protein Synthesis

The process of Translation involves sequential events. Following recognition of the cap-structure, initiation (A) takes place. The 40S subunit is recruited to the mRNA and scans to the initiation codon, where formation of the 80S ribosome occurs. During elongation (B), the ribosome translocates the mRNA in a 5' to 3' direction. Anticodon recognition and peptide bond formation occur, adding successive amino acids to the growing polypeptide chain. When the ribosome reaches the stop codon, termination occurs (C), resulting in the release of the polypeptide chain and dissociation of the 40S and 60S subunits.

rapidly to external stimuli, without the need for the nuclear pathways of mRNA synthesis and transport. For large genes that take a considerable amount of time to transcribe, the expression of the encoded protein can be modulated more rapidly in the short term at the translational level. Regulation at the translational level can be reversed in a more energetically favourable manner than transcriptional regulation and also facilitates the targeted regulation of specific genes or subsets of genes (Matthews 2000).

In prokaryotes, initiation takes place when the Shine-Dalgrano sequence, located upstream of the initiation codon on the mRNA, is bound by the 16S ribosomal RNA (Hershey and Merrick 2000). Eukaryotic initiation by contrast, is a much more complicated process and two main mechanisms exist: cap-dependent initiation and cap-independent initiation. It is believed that approximately 85% of total cellular mRNAs are translated via a cap-dependent pathway, which will now be discussed in greater detail.

1.2.1 Cap-Dependent Initiation of Translation

Following its export from the nucleus into the cytoplasm, mature mRNA is packaged into a messenger ribonucleoprotein (mRNP) complex. This highly structured complex requires a sophisticated recognition process. The scanning model proposed by Kozak describes the recognition of the 5' terminal m^7G-cap structure, resulting in recruitment of the 40S ribosomal subunit. The 40S ribosomal subunit scans the mRNA strand in the 5' to 3' direction, until an AUG codon is reached, which is in the correct context (Kozak 1987). In addition for the requirement of 5' m^7G-cap recognition, methylated guanine and a large number of proteins known as

eukaryotic initiation factors (eIFs) are required in order for the initiation to proceed. Many of these factors are proteins which are regulated by signalling resulting in changes in their phosphorylation status (Hershey and Merrick 2000). In simplistic terms, the initiation of protein synthesis can be broken down into four sequential phases: 1) formation of the ternary complex; 2) formation of the 43S preinitiation complex; 3) the formation of the 48S preinitiation complex; 4) formation of the 80S initiation complex.

1.2.2 The Ternary Complex

The Ternary complex consists of eIF2 bound to both GTP and Met-tRNA$_i^{Met}$, is the first step in the process of initiation. The sequence and structural characteristics of eukaryotic tRNA$_i^{Met}$ allow it to be distinguished from elongator tRNAs by eIF2 (Hinnebusch 2000). These distinguishing features, which are implicated in eIF2 binding, include the A1:U72 base-pair at the 5' TOP of the acceptor stem and a region of G:C binding pairs in the anti-codon stem (Farruggio et al. 1996).

Between rounds of initiation, ribosomal sub-units are maintained in a dissociated state through their interaction with initiation factors. In particular, the 60S ribosomal sub-unit has been shown to interact with eIF6 (Ceci et al. 2003), whilst the 40S sub-unit is associated with eIF1A and eIF3 (Goumans et al. 1980). The ternary complex is responsible for delivering Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit and its formation is essential for the translation of all mRNAs. As a result, the assembly of this complex is an early stage at which translation can be regulated.

1.2.2.1 eIF2

eIF2 is a heterotrimer, which is comprised of three non-identical subunits α, β and γ sub-units (Figure 1.2). During a round of initiation, the three subunits remain associated with the eIF2 complex and the GTP is hydrolysed to GDP. The eIF2γ subunit has binding sites for both GTP and Met-tRNA$_i^{Met}$ and is homologous to eEF1A (eukaryotic elongation factor 1A), which is also a translational GTPase. It is thought that the 3' end of the Met-tRNA$_i^{Met}$ binds to cleft region of eIF2γ, which is formed from a region of the GTPase active site (Roll-Mecak et al. 2004; Yatime et al. 2005). Structural changes in eIF2γ caused by GTP hydrolysis are likely to account for a decrease in its affinity for Met-tRNA$_i^{Met}$ (Yatime et al. 2006). This model is supported by the observation that replacement of GTP with GDP, results in a breakage of the interactions between eIF2γ and the methionine moiety at the 3' end of Met-tRNA$_i^{Met}$ (Kapp and Lorsch 2004).

Although the role of the eIF2β subunit is not fully understood, it is known that eIF2β is comprised of three distinct regions. A zinc finger located within the carboxy-terminal region is responsible for eIF2β's RNA-binding activity (Cho and Hoffman 2002). It has been suggested that this region is important for start codon recognition, as mutations around the zinc finger allow initiation at UUG codons (Donahue et al. 1988). The amino-terminal region is believed to contribute to RNA-binding and also interacts with eIF2B and eIF5 (which will be introduced in more detail later). The central region of eIF2β is known to interact with eIF2γ.

The eIF2α subunit does not appear to play a critical role in the binding of either GTP or Met-tRNA$_i^{Met}$ and has been shown to be dispensable in yeast, when eIF2β and eIF2γ are over-expressed (Erickson et al. 2001). Crystal structure data

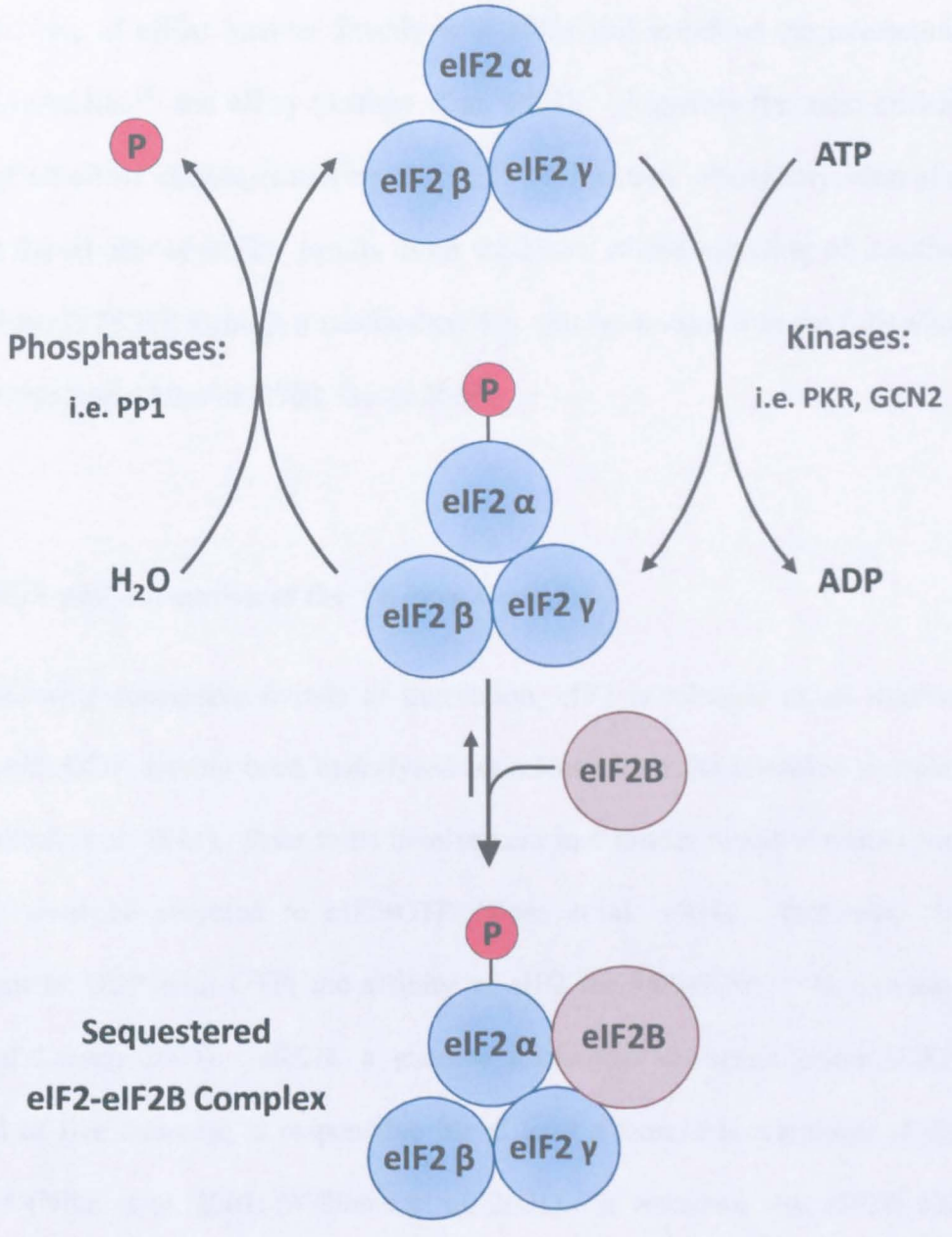


Figure 1.2 The Role of eIF2 Availability in Translational Regulation

Phosphorylation of the α subunit of eIF2, prevents the dissociation of eIF2 from eIF2B and inhibits the recycling of eIF2-GDP to eIF2-GTP. Consequently, translation is negatively regulated by this process. Translational is allowed to commence following dephosphorylation of eIF2α.

shows that eIF2α and eIF2β bind at independent sites on eIF2γ and do not appear to interact with each other (Schmitt et al. 2002). It has been proposed that the carboxy-terminal domain of eIF2α interact directly with eIF2γ and stabilises the interaction between Met-tRNA$_i^{Met}$ and eIF2γ (Yatime et al. 2004). Arguably the most critical function of the eIF2α subunit, is as a regulator of eIF2 function. Phosphorylation of a conserved Ser-51 site of eIF2α, results in an inhibition of the recycling of inactive eIF2•GDP to eIF2•GTP through a mechanism that will be discussed in the following section (Hershey and Merrick 2000; Dever 2002).

1.2.2.2 eIF2B and Formation of the Ternary Complex

Following successive rounds of translation, eIF2 is released as an inactive complex with GDP, having been hydrolysed on release from the initiation complex (Raychaudhuri et al. 1985). Prior to its involvement in a further round of translation, eIF2•GDP must be recycled to eIF2•GTP (Goss et al. 1984). Following the replacement of GDP with GTP, the affinity of eIF2 for Met-tRNA$_i^{Met}$ is increased (Kapp and Lorsch 2004). eIF2B, a guanine nucleotide exchange factor (GEF) comprised of five subunits, is responsible for at least a tenfold acceleration of this conversion (Nika et al. 2001; Williams et al. 2001). It is known that eIF2B bio-availability is directly influenced by the phosphorylation of Ser-51 site on eIF2α (Figure 1.2). In its phosphorylated state, the interaction between eIF2 and eIF2B is strengthened, resulting in the inhibition of the recycling of inactive eIF2•GDP to eIF2•GTP (Hershey and Merrick 2000; Dever 2002). Ser-51 is the target for multiple protein kinases, which include PERK (Harding et al. 2000), PKR and GCN2 (Hershey

and Merrick 2000) and as a result, it plays a key role in the control of translation initiation.

1.2.3 Assembly of the 43S Preinitiation Complex

The formation of the 43S preinitiation complex, involves the interaction of the ternary complex with a 40S subunit in a process facilitated by eIF1, eIF1A and eIF3 (Figure 1.3) (Trachsel et al. 1977; Benne and Hershey 1978) . The importance of these factors in 43S complex formation has been confirmed genetically, biochemically, and through analysis of the involvement of the corresponding yeast factors in 43S complex formation. It is through this analysis that eIF5 has also been implicated in 43S complex formation. In a reconstituted yeast system, it has been shown that the binding of the ternary complex to 43S-mRNA, is stimulated by both eIF1 and eIF1A in the absence of eIF3 (Algire et al. 2002; Maag et al. 2005). However, the binding of ternary complex to the 43S preinitiation complex, which has a 50-fold lower affinity for ternary complex than 43S-mRNA, is enhanced by the presence of both eIF5 and eIF3 (Maag et al. 2005). Perhaps the most compelling evidence for the involvement of eIF5 comes from the observation that eIF1, eIF3, eIF5 and the ternary complex can be isolated in a multifactor complex (Asano et al. 2000). This suggests that these factors are recruited to the 40S subunit as a preformed unit, possibly through the known interaction of eIF5 with both eIF2 and eIF3 (Das and Maitra 2000).

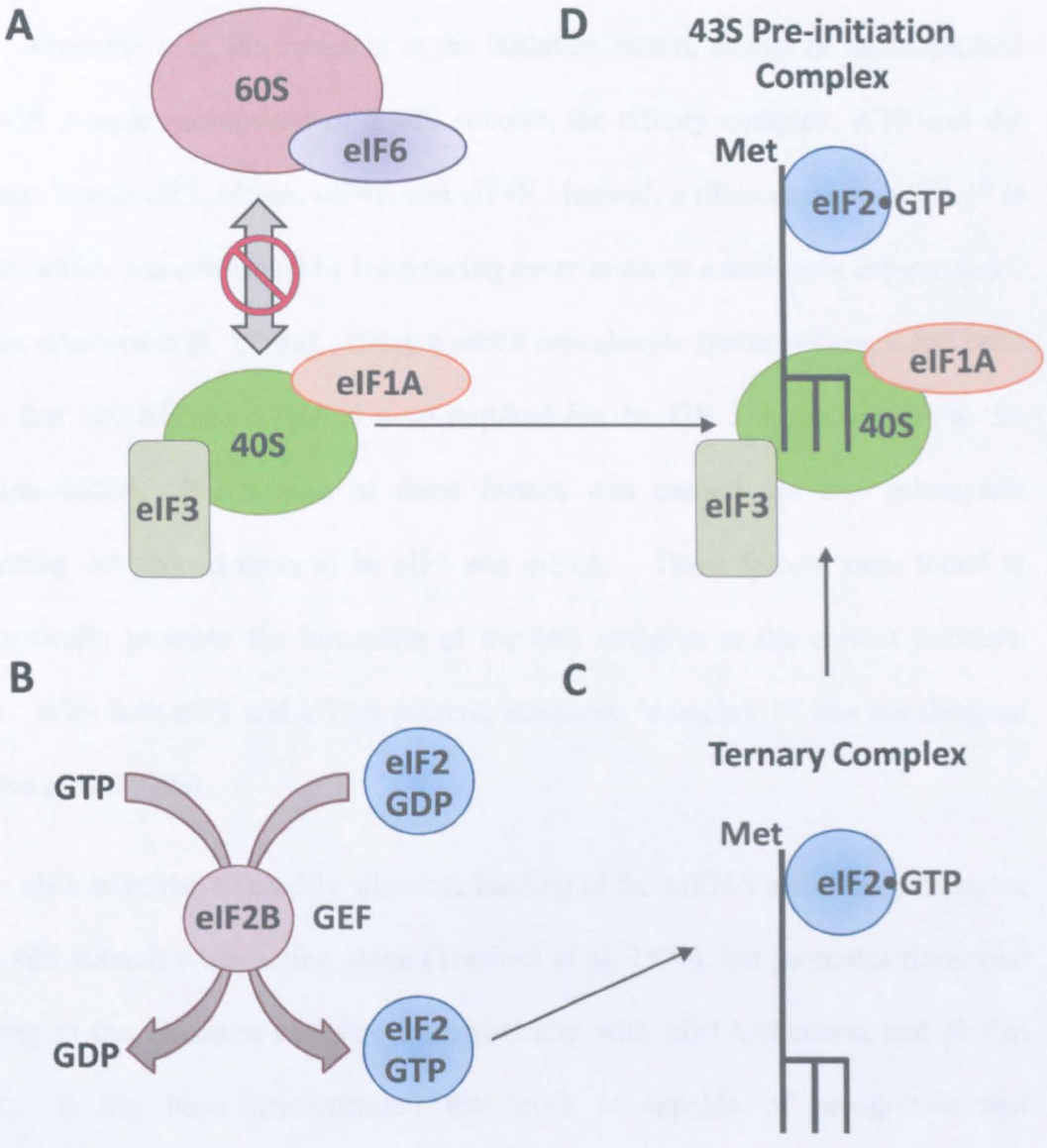


Figure 1.3 Formation of the 43S Pre-initiation Complex

A) The interaction of eIF6 with the 60S subunit and eIF1A and eIF3 with the 40S subunit, ensure that the subunits remain dissociated.

B) The guanine nucleotide exchange factor eIF2B, recycles eIF2-GDP to eIF2-GTP.

C) eIF2-GTP binds to the initiator methionine (Met-tRNA$_i^{Met}$), resulting in formation of the ternary complex.

D) The 43S pre-initiation complex forms, following the interaction of the ternary complex with the 40S subunit.

1.2.3.1 eIF1 and eIF1A

Assembly of a 48S complex at the initiation codon, cannot be accomplished by a 43S complex comprised of a 40S subunit, the ternary complex, ATP and the initiation factors eIF3, eIF4A, eIF4B and eIF4F. Instead, a ribosomal "complex 1" is formed, which was confirmed by toe-printing assay to adopt a stationary cap-proximal position (Pestova et al. 1998a). Using a rabbit reticulocyte lysate system, it has been shown that two additional factors were required for the 43S complex to scan to the initiation codon. Purification of these factors was carried out and subsequent sequencing determined them to be eIF1 and eIF1A. These factors were found to synergistically promote the formation of the 48S complex at the correct initiation codon. With both eIF1 and eIF1A present, ribosome "complex 1" was not detected (Pestova et al. 1998a).

eIF1 is known to weakly stimulate binding of the mRNA and ternary complex to the 40S subunit when acting alone (Trachsel et al. 1977), but promotes ribosomal scanning to the initiation complex synergistically with eIF1A (Pestova and Hellen 2001). It has been demonstrated that eIF1 is capable of recognition and destabilisation of stalled complexes within the 5' UTR and those that have assembled aberrantly at an incorrect AUG codon in internal ribosomal entry (discussed in further detail in section 1.6) (Pestova et al. 1998a). It has not yet been determined whether eIF1 and eIF1A are required for scanning *per se*, to stabilise correctly positioned complexes or to destabilise those, which are not located at an AUG codon (Hinnebusch 2000).

eIF1A is a single polypeptide that is one of the most conserved initiation factors in eukaryotes (Kyrpides and Woese 1998). It has been implicated in the

processes of ribosome dissociation, ternary complex binding and mRNA binding to the ribosome. More specifically, it has been shown to increase the binding affinity of 43S complexes for mRNA and increases the processing capability of scanning ribosomal complexes (Pestova et al. 1998b). In the absence of mRNA, it has been shown that eIF1A stabilises the interaction between the 40S subunit and the ternary complex (Chaudhuri et al. 1999).

1.2.3.2 eIF3

One of the main functions of eIF3 is to stabilise the interaction between the 40S subunit and Met-tRNA$_i^{Met}$, therefore promoting the recruitment of the ternary complex (Hinnebusch 2000). eIF3 also plays an important role in ensuring that this recruitment can occur in the presence of 60S ribosomal subunits and indeed stimulates the recruitment of the ternary complex to the 40S subunit under conditions that favour 40S-60S subunit joining (Trachsel et al. 1979). It has been shown that this stimulation does not occur when an AUG triplet is present (Chaudhuri et al. 1999). In contrast, the stimulating affect of eIF1A, which has been shown to be more effective than eIF3, was found to be absent in conditions that promoted 40S and 60S subunit joining and when mRNA was present (Chaudhuri et al. 1999). These data suggests that eIF3 and eIF1A are both required to form a stable 43S preinitiation complex. It has also been proposed that prior to mRNA binding, eIF3 is required to protect the 43S complex from disruption by the 60S ribosomal subunit. However, following base pairing of the Met-tRNA$_i^{Met}$ to the AUG start codon, eIF3 is required for the disruption of the 43S complex (Chaudhuri et al. 1999). eIF3 also appears to be required for mRNA binding and strongly stimulates mRNA binding by the 40S initiation complex (Trachsel et al. 1977). This is generally believed to be due to eIF3 acting as a bridge

between the 40S subunit and the eIF4F complex, via eIF4G (Figure 1.5A) (Lamphear et al. 1995; Hershey and Merrick 2000). The implications of this bridging interaction will be discussed in more detail in section 1.2.4.3.

1.2.3.4 eIF2C / Co-eIF-2A / AGO2

At low eIF2 concentrations it has been shown *in vitro* that mRNA destabilises the ternary complex. The presence of eIF2C (also known as Co-eIF-2A and AGO2), a 94 kDa polypeptide, is sufficient to relieve this destabilisation (Roy et al. 1988). eIF2C has also been shown to stimulate and stabilise Met-tRNA$_i^{Met}$ binding to eIF2 (Roy et al. 1981; Chakravarty et al. 1985). eIF2C/AGO2 is known to have other important functions in post-transcriptional regulation and these will be discussed in more detail in section 1.8.3.

1.2.4 Formation of the 48S Preinitiation Complex

It has been shown that 43S complexes are intrinsically capable of binding to the 5' terminus of mRNAs which posses an unstructured 5' UTR (Pestova and Kolupaeva 2002). However, most mRNAs are known to possess some secondary structure in their 5' UTR and additional initiation factors are therefore required. These factors are believed to be involved in the recognition and binding of the m^{7}G cap structure at the 5' terminus of the mRNA. This structure, which results from a post transcriptional modification, is therefore an essential requirement of cap-dependent initiation. The role of each of these initiation factors in the recruitment of the 43S subunit to the mRNA, will be discussed in more detail in the following sections.

1.2.4.1 eIF4E: Cap Recognition and mRNA Binding

The 5' m^7G cap is recognised by the cap-binding protein eIF4E, which is part of the eIF4F complex. The eIF4F (Figure 1.4) complex is a heterotrimer that also contains eIF4A and eIF4G, whose functions will be discussed later. Previously, as a result of studies in several cell lines, it was thought that the abundance of eIF4E was limited in comparison to other members of the translational machinery (Hiremath et al. 1985; Duncan et al. 1987). However, additional data have shown that this may not be the case for many systems (Rau et al. 1996) and eIF4E may not be the rate limiting factor in the initiation of translation (von der Haar et al. 2004). The mRNA cap structure has been shown to stack between two tryptophan residues, which are located on the concave surface of eIF4E (Tomoo et al. 2002). The affinity of eIF4E for the cap structure is increased when eIF4E is bound to eIF4G (Haghighat and Sonenberg 1997). A structural study of the interaction of yeast eIF4E and an eIF4G fragment have revealed that eIF4G forms a ring like structure as a result of a coupled folding transition. This ring wraps around a narrow protrusion of eIF4E formed by induced folding of its amino terminus (Gross et al. 2003). Dissociation rate of eIF4E from the cap is reduced and the complex is stable with a higher affinity for the cap than eIF4E alone (Ptushkina et al. 1998; von Der Haar et al. 2000). The corresponding region of mammalian eIF4G is homologous to the folded domain of yeast eIF4G, suggesting that stabilisation of eIF4F occurs via a similar mechanism. The binding of eIF4E to eIF4G appears to be stabilised by additional contacts between eIF4E and the nucleotide adjacent to the cap (Slepenkov et al. 2006).

In resting cells eIF4E remains tightly bound to hypo-phosphorylated members of the eIF4E-binding protein (4E-BP) family (Figure 1.4B) (Gingras et al. 2001a; Gingras et al. 2001b). The 4E-BPs compete with eIF4G for a common binding site on

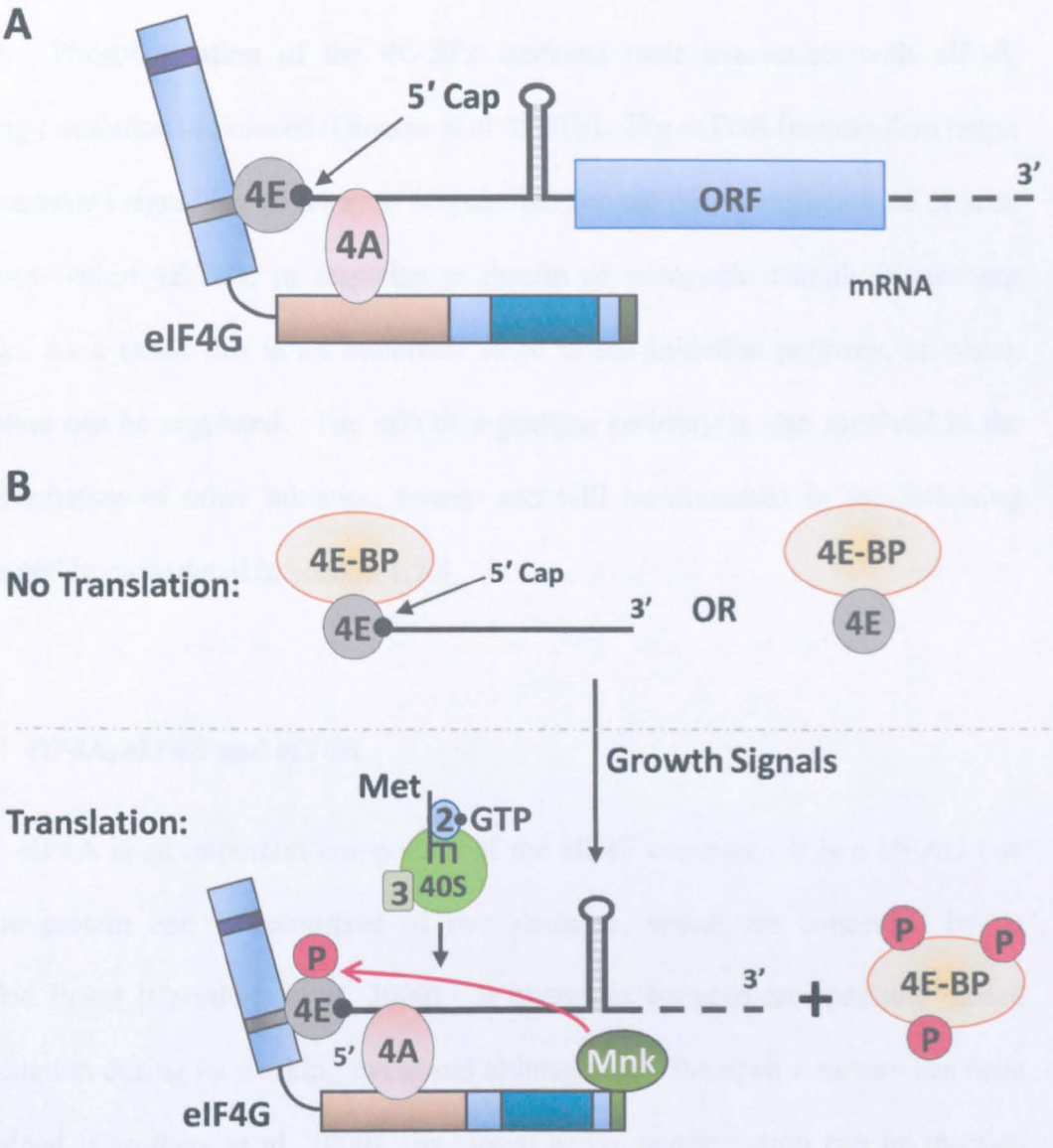


Figure 1.4 The eIf4F Complex

A) eIF4E recognises the 5' cap-structure and recruits the scaffold protein eIF4G. eIF4G has binding sites for many initiation factors and is shown bound the helicase eIF4A.

B) 4E-BPs sequester eIF4E, preventing its association with eIF4G and the initiation of translation. Growth signals result in the phosphorylation of 4E-BP, thus releasing eIF4E. eIF4E can also be phosphorylated in response to growth signals by the MAP kinase interacting kinase/MAP kinase signal integrating kinase, Mnk1. This alters the affinity of eIF4E for the cap-structure.

eIF4E and as a result, impede assembly of the cap-binding complex (Sonenberg 1993a). Phosphorylation of the 4E-BPs weakens their interaction with eIF4E, allowing translation to proceed (Gingras et al. 2001b). The mTOR (mammalian target of Rapamycin) signalling pathway is responsible for the phosphorylation of at least two sites within 4E-BPs, in response to insulin or mitogenic stimuli (Sonenberg 1993a). As a result this is an important stage in the initiation pathway, at which translation can be regulated. The mTOR signalling pathway is also involved in the phosphorylation of other initiation factors and will be discussed in the following section and in more detail in section 1.7.4.

1.2.4.2 eIF4A, eIF4B and eIF4H

eIF4A is an important component of the eIF4F complex. It is a DEAD box helicase protein and is comprised of two domains, which are connected by an extended linker (Caruthers et al. 2000). It alternates between an open and closed conformation during its working cycle and although only the open structure has been determined (Caruthers et al. 2000), the closed active conformation can be mapped onto the structure of other similar helicases (Oberer et al. 2005; Sengoku et al. 2006). eIF4A is involved in the binding and hydrolysis of ATP, binding RNA, and with coupled ATP hydrolysis and helicase activity (Cordin et al. 2006). A thermodynamic investigation of the binding and hydrolysis of ATP has revealed that the conformational changes, which result, may be sufficient to fulfil the energy requirements for the disruption of base-pairing in RNA helices (Lorsch and Herschlag 1998). It is believed that eIF4A unwinds the secondary structure within the 5' UTR, facilitating the binding of the 43S ribosomal subunit and subsequent scanning to an AUG codon.

Acting alone, eIF4A possesses only a weak helicase function. As a component of the eIF4F complex, its functionality is enhanced more than 20-fold and can be further activated by the cofactor eIF4B (Rozen et al. 1990; Korneeva et al. 2005). It is believed that eIF4B increases the affinity of eIF4A for RNA (Abramson et al. 1988). The factor eIF4H has also been shown to activate and enhance the functionality of eIF4A. Despite being related to eIF4B and possessing a homologous amino-terminal region, it cannot substitute eIF4B in all conditions and has been shown to be less effective than eIF4B in the activation of eIF4A (Rogers et al. 2001; Dmitriev et al. 2003). It has not yet been determined whether the action of eIF4H and eIF4B is mRNA-specific, or whether the differences observed are due to physiological conditions.

The mTOR signalling pathway (which will be discussed in more detail in section 1.7.4), is a downstream Ser/The kinase in the PI3K/Akt signalling pathway and is activated in response to insulin or mitogenic stimuli (Kim et al. 2002). The S6 kinases (S6Ks) are involved in the mTOR signalling pathway and are downstream targets for phosphorylation by the mTOR Complex 1 (mTORC1) (Kim et al. 2002). The S6Ks phosphorylate the tumour suppressor processor Pdcd4, which in its unphosphorylated state binds to and consequently inhibits eIF4A (Dorrello et al. 2006). Phosphorylation of Pdcd4 results in its ubiquitination and degradation by the protesomome, thus alleviating the inhibition of eIF4A. It has been shown that the S6Ks also phosphorylate eIF4B on Ser-422, resulting in an enhanced interaction of eIF4B with eIF3 (Holz et al. 2005). Phosphorylation at Ser-422 has also been shown to occur as a result of signalling through the RAS-MAPK pathway (Holz et al. 2005). eIF4A and eIF4B are therefore involved in regulation at the level of translation initiation.

1.2.4.3 eIF4G: Role and Regulation

The initiation factor eIF4G acts a molecular scaffold and plays an integral part in bringing together many of the canonical initiation factors. It has been shown that the two isoforms of eIF4G, known as eIF4GI and eIF4GII, share 46% identity at the amino acid level (Gradi et al. 1998). Both isoforms bind a number of initiation factors. It has also been determined that alternative start codons may be used, resulting in multiple isoforms of eIF4GI. The function of these isoforms are still currently unclear (Byrd et al. 2002). eIF4G has several important functions during ribosomal recruitment, including coordinating the cap-binding activity of eIF4E and the RNA helicase activity of eIF4A. The cross linking of RNA to eIF4A is also enhanced by the central HEAT-repeat domain of eIF4G (Pestova et al. 1996; Oberer et al. 2005). The crystal structure of the yeast eIF4A-eIF4G complex has revealed that when bound to eIF4G, the two lobes of eIF4A are held in an orientation that results in the alignment of the DEAD-box helicase motifs. This alignment is necessary to facilitate the ATP-dependent helicase activity (Schutz et al. 2008).

An important regulatory mechanism that is utilised during cellular stress, involves the cleavage of eIF4G. Following cleavage, eIF4G is no longer available to function within the eIF4F complex. Consequently, a new round of cap-dependent initiation cannot occur and translation is shut down. Caspase-3 is responsible for eIF4G cleavage during apoptosis, at a location close to the eIF4E binding site (Clemens et al. 1998; Marissen and Lloyd 1998; Morley et al. 1998). Shut down of host cell protein synthesis by picornavirus infection, involves a similar mechanism at a site amino acids away from the caspase 3 site (Etchison and Fout 1985; Lloyd et al. 1987). During heat shock, eIF4G is localised into insoluble granules (stress granules) in a process chaperoned by hsp27 (heat shock protein 27). In this instance eIF4G is

not cleaved and this process is rapidly reversible following the release of hsp27 (Cuesta et al. 2000). This mechanism ensures that restoration of translation can be effected both quickly and energetically efficiently following recovery from stress.

The phosphorylation of eIF4G is believed to correlate, in most cases, with an increase in the rate of protein synthesis. Although the mechanism has not been fully determined, a number of sites on eIF4G have been implicated as phosphorylation targets of kinases such as PKA, PKC and GSK2 (Morley and Traugh 1990; Raught et al. 2000).

The MAP kinase interacting kinase/MAP kinase signal integrating kinase, Mnk1, interacts with the C-terminus of eIF4G. When bound to eIF4G, Mnk1 can phosphorylate eIF4E on Ser-209 in response to signalling pathways (Raught et al. 2000). Phosphorylation of eIF4E is increased by serum treatment of cells and addition of growth factors in a process, which appears to be mediated via the MEK/ERK pathway (Flynn and Proud 1996). It has been the subject of considerable debate as to whether the phosphorylation state of eIF4E plays a role in the regulation of translation initiation (Scheper and Proud 2002). Interestingly, it has been shown that mice, which have both Mnk1 and Mnk2 genes deleted are apparently normal, leading to the suggestion that eIF4E phosphorylation is not significantly important (Ueda et al. 2004). It has also been shown, that chemical inhibition of eIF4E phosphorylation does not hinder de novo initiation of translation, during the recovery from hypertonic stress (Morley and Naegele 2002). However, other data appears to support the view that eIF4E phosphorylation is a positive regulator of translation, including the observation that mutation of the eIF4E phosphorylation site of *Drosophila* results in a reduction in body size and viability (Lachance et al. 2002). Despite the uncertainty as to the exact role of eIF4E phosphorylation, two models

have been proposed for the role of eIF4E phosphorylation in translation initiation, which differ only at the proposed stage at which phosphorylation occurs (Scheper and Proud 2002). In both models, phosphorylation of eIF4E increases its affinity for capped RNA, which is consistent with the observation that high levels of eIF4E phosphorylation can impair cap dependent translation (Scheper et al. 2002).

As previously introduced in section 1.2.3.2, the central domain of eIF4G can interact with eIF3 and act as a bridge between the 40S subunit and the eIF4F complex (Figure 1.5A) (Lamphear et al. 1995; Hershey and Merrick 2000). To date, the subunit(s) involved in the binding of eIF3 to eIF4G have not been identified. In yeast it has been observed that eIF3 does not bind to eIF4G directly. Instead, interaction has been shown to occur via eIF1 and eIF5, which are components of a yeast eIF3C-eIF5-eIF1 complex (Yamamoto et al. 2005).

Poly(A)-binding protein (PABP) can interact with eIF4G (Figure 1.5B), resulting in circularisation of the mRNA. The resultant "closed loop" structure links the 5' cap and the 3' poly(A) tail of the mRNA. It is thought that PABP is able to stimulate the association of the 43S preinitiation complex with the mRNA, by enhancing the interaction of eIF4F and the cap structure (Pestova et al. 2007). This enhancement is likely to be due to induced conformational changes exerted on eIF4E (Borman et al. 2000; von Der Haar et al. 2000). PABP is involved in coupling the different stages of protein synthesis, as it known to associates with eukaryotic release factor eRF3 (Cosson et al. 2002)

PABP-interacting protein 1 (PAIP-1) is a homologue of eIF4G, which shares 25% sequence identity. It is able to bind both eIF3 and PABP, resulting in a stimulation of translation, which may be due to mRNA circularisation

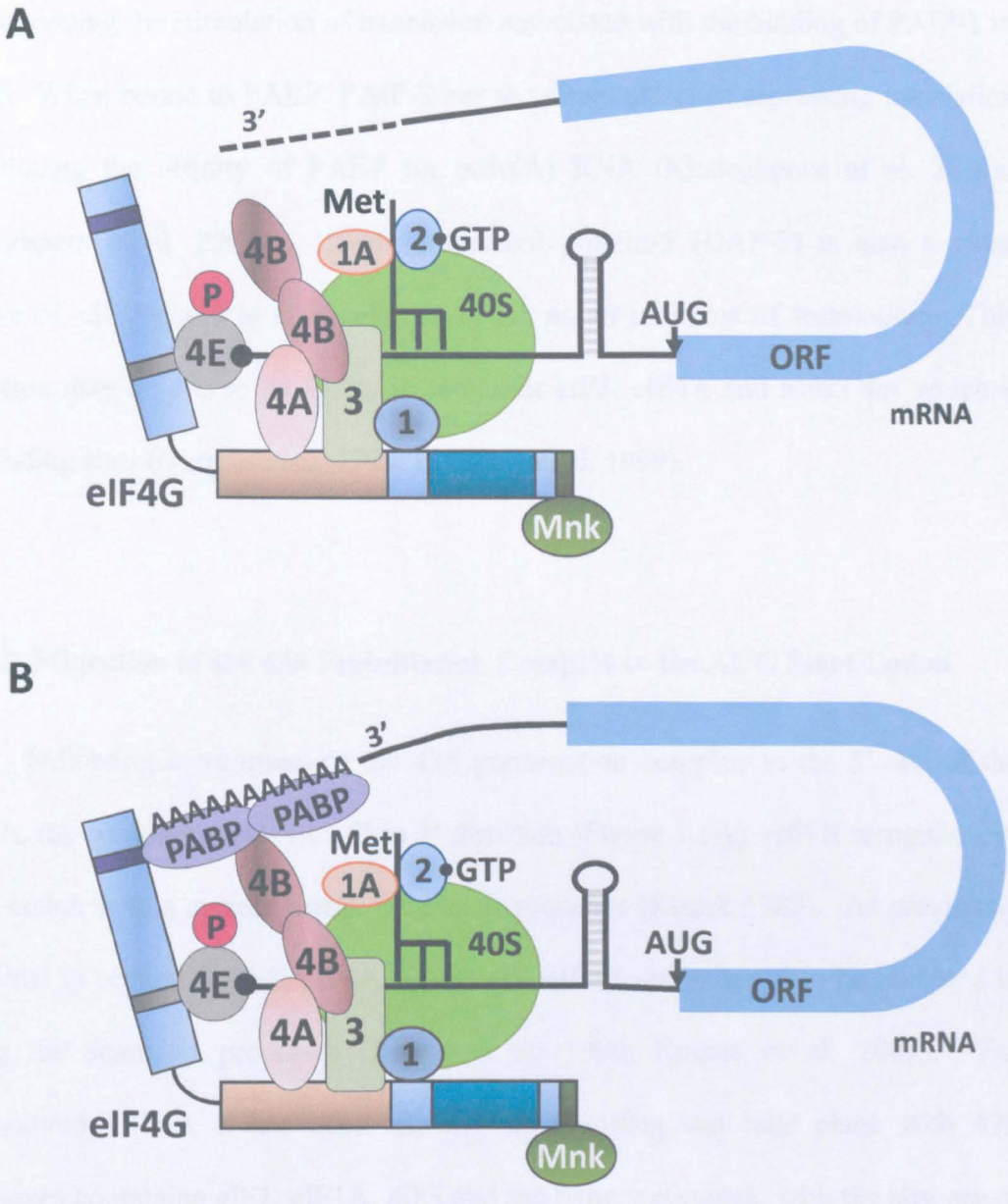


Figure 1.5 The 48S Preinitiation Complex

A) The 43S pre-initiation complex is recruited to the mRNA through the interaction between eIF3 and eIF4G.

B) The binding of Poly(A)-binding protein (PABP) to both the poly(A) tail and eIF4G, results in circularisation of translation. The resultant "closed loop" structure stabilises the complex and enhances translation.

(Craig et al. 1998; Martineau et al. 2008). PAIP-2 is a binding competitor of PABP-1, thus repressing the stimulation of translation associated with the binding of PAIP-1 to PABP. When bound to PABP, PAIP-2 has the direct effect of repressing translation by reducing the affinity of PABP for poly(A) RNA (Khaleghpour et al. 2001a; Khaleghpour et al. 2001b). Death associated protein-5 (DAP-5) is also a close relative of eIF4G, and is also believed to act as an inhibitor of translation. This inhibition may be due to its ability to sequester eIF3, eIF4A and Mnk1 for which it has binding sites (Gingras et al. 1999; Pyronnet et al. 1999).

1.2.4.3 Migration of the 43S Preinitiation Complex to the AUG Start Codon

Following recruitment of the 43S preinitiation complex to the 5' end of the mRNA, the complex scans in a 5' to 3' direction (Figure 1.6A) until it recognises an AUG codon with a correct Kozak consensus sequence (Kozak 1987). As previously described in section 1.2.4.2, eIF4A, eIF4B and eIF4H are believed to be involved in aiding the scanning processes (Rozen et al. 1990; Rogers et al. 2001). For unstructured mRNA it has been shown that scanning can take place with 43S complexes containing eIF1, eIF1A, eIF3 and the ternary complex, with the absence of ATP, eIF4A, eIF4B, and eIF4F (Pestova and Hellen 2001). The intrinsic ability of the 43S complex to move on the mRNA is therefore clearly reflected by the scanning process. During scanning, successive triplets on the mRNA enter the P site of the preinitiation complex. These triplets are inspected for complementary to the Met-$tRNA_i^{Met}$, which is anchored to the GTP bound form of eIF2 (Pestova et al. 2007). A perfect match with an AUG codon in the correct context triggers the irreversible hydrolysis of GTP and the subsequent release of eIF2•GDP, eIF3 and eIF1 (Paulin et

al. 2001). The eIF2B mediated recycling of eIF2•GDP to eIF2•GTP was previously discussed in section 1.2.2.2.

The context of the start codon is important in the selection of the AUG codon which is utilized for translation. In higher eukaryotes it has been determined the ideal context is CCRCCAUG**G**. The most important nucleotides in terms of AUG selection are highlighted in bold. Without the presence of a guanosine at the +4 position (where the A of the AUG is defined as the +1 position), and a purine at the -3 position, initiation efficiency is reduced and "leaky scanning" can occur (Kozak 1989b; Kozak 1991a; Kozak 1997). When this happens, the 43S subunit continues to scan until it reaches an AUG codon in better context (Figure 1.6B), where it may more successfully initiate translation (Kozak 1995). Leaky scanning can produce peptides of different lengths from a single mRNA. In 95-97% of cases, the AUG codons, which initiate the main open reading frame of the mRNA, are in a good or adequate context (Meijer and Thomas 2002). In some cases, GUG, CUG and ACG are also utilized for initiation, although at lower efficiency that AUG (Kozak 1989b). Selection of alternative initiation codons is dependent upon the correct surrounding consensus sequence (Kozak 1997). Recognition of an AUG codon can also be stimulated by a hairpin loop structure in the mRNA, approximately 14-18nt downstream of the AUG (Kozak 1990). This structure is believed to increase the efficiency of initiation by reducing the scanning rate of the 43S complex, thus aiding start site selection.

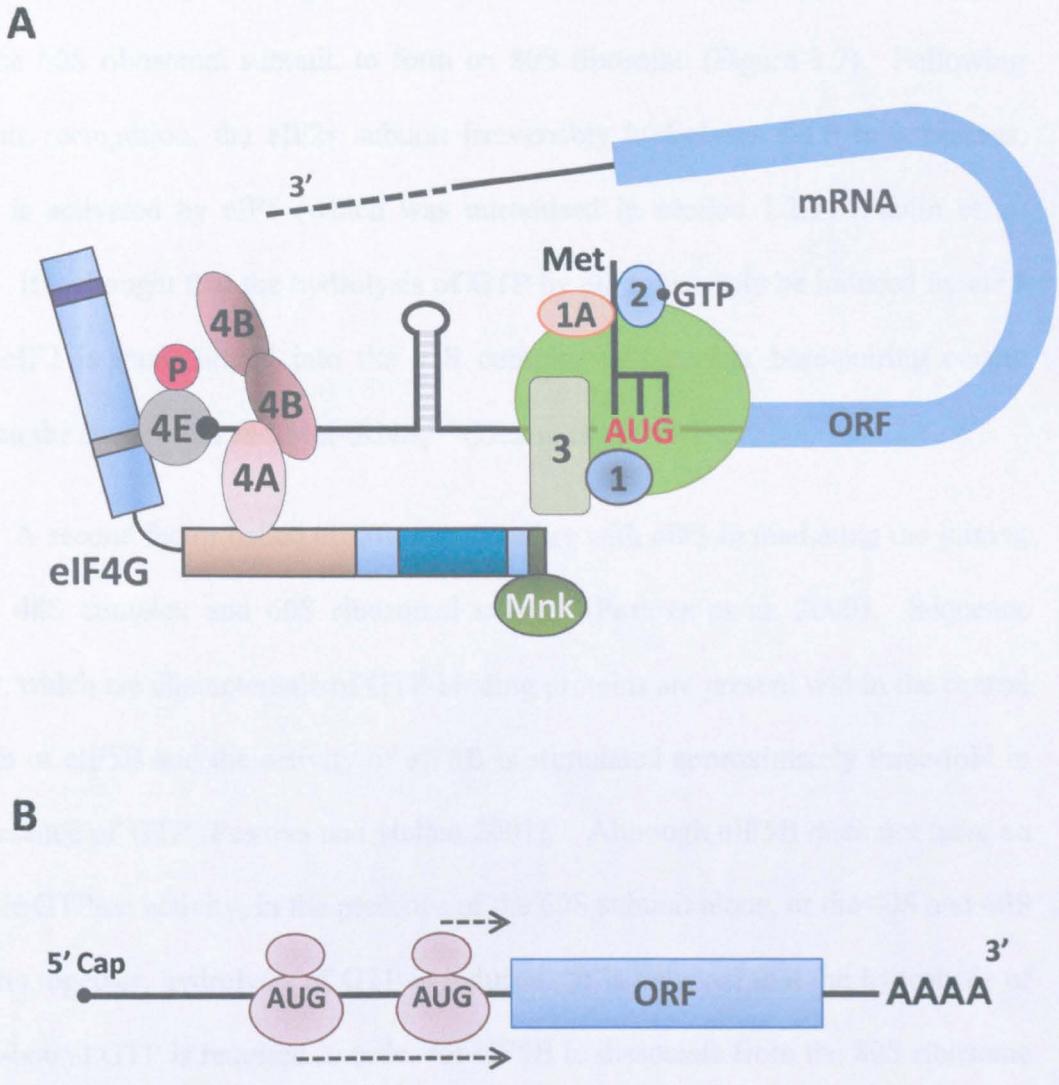


Figure 1.6 The Scanning Mechanism

A) Following its recruitment to the mRNA, the 43S complex scans in a 5' to 3' direction until it recognises an AUG codon with a Kozak consensus sequence. The scanning process is aided by the factors eIF4A, eIF4B, eIF4H, eIF1 and eIF1A.

B) If the first initiation codon reached is in poor context, the 43S subunit may continue to scan until it reaches an AUG codon in better context. This process is called leaky scanning (Kozak 1995) and may produce peptides of different lengths from a single mRNA.

1.2.5 Formation of the 80S Initiation Complex

The final stage in the process of initiation is the joining of the 48S complex with the 60S ribosomal subunit, to form an 80S ribosome (Figure 1.7). Following start site recognition, the eIF2γ subunit irreversibly hydrolyses GTP in a process, which is activated by eIF5 (which was introduced in section 1.2.3) (Paulin et al. 2001). It is thought that the hydrolysis of GTP by eIF2, can only be induced by eIF5 when eIF2 is incorporated into the 43S complex and perfect base-pairing occurs between the start codon and Met-tRNA$_i^{Met}$ (Huang et al. 1997).

A second factor called eIF5B, acts together with eIF5 in mediating the joining of the 48S complex and 60S ribosomal subunit (Pestova et al. 2000). Sequence motifs, which are characteristic of GTP-binding proteins are present within the central domain of eIF5B and the activity of eIF5B is stimulated approximately three-fold in the presence of GTP (Pestova and Hellen 2001). Although eIF5B does not have an intrinsic GTPase activity, in the presence of the 60S subunit alone, or the 40S and 60S subunits together, hydrolysis of GTP is induced. It is believed that the hydrolysis of eIF5B-bound GTP is required in order for eIF5B to dissociate from the 80S ribosome (Pestova and Hellen 2001). This hydrolysis reaction, which triggers eIF5B release for the initiation complex, is stimulated by the interaction of eIF5B with the C terminus of eIF1A (Shin et al. 2002; Acker et al. 2006). Evidence suggests that when eIF5B is bound to the ribosome, elongation factor 1, which is required for the next stage of translation, is unable to bind (Wilson et al. 2000). It appears that eIF5B is able to catalyse multiple rounds of ribosomal subunit joining without the requirement of a guanidine nucleotide exchange factor to exchange eIF5B bound GDP for GTP (Pestova and Hellen 2001).

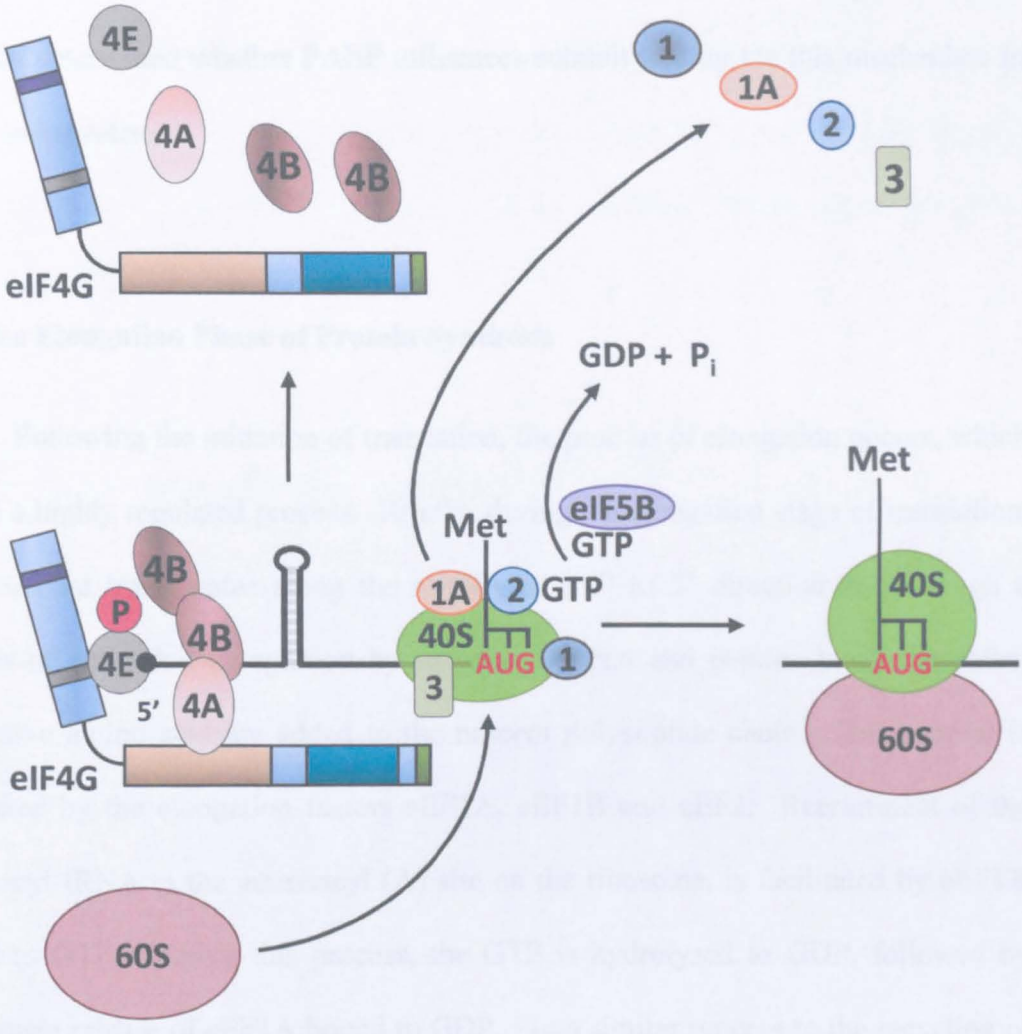


Figure 1.7 The Joining of the 60S Subunit

The joining of the 60S subunit results in the formation of the 80S ribosome. Following recognition of the AUG start codon, the 60S subunit joins and GTP hydrolysis occurs on both eIF5B and eIF2. Initiation factors are also released and the competent 80S complex is able to commence elongation.

PABP, previously introduced in section 1.2.4.3, has also been shown to stimulate ribosomal subunit joining (Kahvejian et al. 2005). Following experiments carried out in yeast, it has been suggested that the poly(A) tail is required to block the effects of two RNA helicases, which inhibit eIF5B (Searfoss et al. 2001). It has not yet been determined whether PABP influences subunit joining via this mechanism in higher eukaryotes.

1.3 The Elongation Phase of Protein Synthesis

Following the initiation of translation, the process of elongation occurs, which is also a highly regulated process. Briefly, during the elongation stage of translation, the ribosome translocates along the mRNA in a 5' to 3' direction and through a process of anticodon recognition by aminoacyl-tRNA and peptide bond formation, successive amino acid are added to the nascent polypeptide chain. This process is facilitated by the elongation factors eEF1A, eEF1B and eEF2. Recruitment of the aminoacyl-tRNA to the aminoacyl (A) site on the ribosome, is facilitated by eEF1A bound to GTP. During this process, the GTP is hydrolysed to GDP, followed by subsequent release of eEF1A bound to GDP. In a similar process to the recycling of eIF2 by the guanine nucleotide exchange factor eIF2B, eIF1A in its GDP-bound form is recycled to its GTP-bound form using eIF2B.

In the next stage of elongation, eEF2 mediates the transfer of aminoacyl-tRNA from the ribosomal A-site, to the P-site. In doing so, the next codon is exposed to the A-site and the nascent polypeptide (or the initiator methionine) is transferred from the tRNA located in the P-site to the new aminoacyl-tRNA located in the A-site. The formation of a new peptide bond can then occur, increasing the length of the growing

polypeptide chain. The new peptidyl-tRNA is then transferred to the P-site while the anticodon remains in the A-site. The deacylated tRNA is then switched to the ribosomal E-site. During this cycle, eEF2 which is bound to GTP is hydrolysed. The process can be regulated through phpshphorylation events effected by the eEF2-kinase. Phosphorylation of eEF2 has been shown to inhibit the activity of eEF2 (Redpath and Proud 1993). De-phosphorylation of eEF2 as a result of insulin treatment has been shown to increase the rate of translation at the elongation phase (Redpath et al. 1996).

1.4 The Termination of Protein Synthesis

The final termination phase of translation occurs when the ribosome reaches one of three termination codons (UAA, UAG or UGA). At this stage, eukaryotic release factor eRF1 binds and hydrolyses the peptidyl-tRNA, resulting in cleavage of the last aminoacyl bond and the release of the polypeptide chain along with the ribosome form the mRNA. eRF3 (bound to GTP) stimulates the activity of eRF1 in a GTP-dependent manner. In some circumstances the 40S ribosomal subunit may remain associated with the mRNA and can re-initiate translation at a downstream initiation codon (Kozak 1992).

1.5 Regulation of Cap-dependent Initiation of Protein Synthesis

As previously discussed, cellular signalling pathways involving the subsequent phosphorylation or dephosphorylation of various initiation factors, are important components in the regulation of translation. In addition to these control

mechanisms, there are many structural elements within the mRNA that are involved in the regulation of translation. Features installed during post-transcriptional modification (refer to section 1.1), including the 5' m^7G cap and the poly(A) tail are known to enhance translation. The 5' UTR is an important region in the control of translation and up to 90% of all eukaryotic mRNAs contain a 5' UTR of between 20-100 nucleotides (Kozak 1997). There are several different types of elements within both the 5' UTR and 3' UTR which contribute to translational regulation and these will be described in more detail in the following sections.

1.5.1 Secondary Structure

In conjunction with eIF4B and eIF4H, the RNA helicase eIF4A is able to unwind secondary structure within the 5' UTR under normal conditions (Rogers et al. 2001). The presence of stronger secondary structure in the 5' UTR drastically inhibits translation efficiency of the mRNA, as they cannot be unwound by the helicase action of eIF4A (Figure 1.8A). Increasing eIF4F complex formation through the over-expression of eIF4E, results in the binding of more eIF4A and has been shown to overcome the repressive effect (Rogers et al. 2001). Translational efficiency is also affected by the position of the secondary structure in relation to the 5' cap. For example, a stem-loop structure of -30 kcal/mol free energy 12 nucleotides downstream of the cap, inhibited translation, as the position of the structure prevents the 43S complex from binding to the mRNA. However, the same structure positioned 52 nucleotides downstream of the cap, does not inhibition translation (Kozak 1989a). Complete blockage of translation can be achieved by the insertion of higher free energy stem-loop structures (-61 kcal/mol) approximately 71 nucleotides downstream

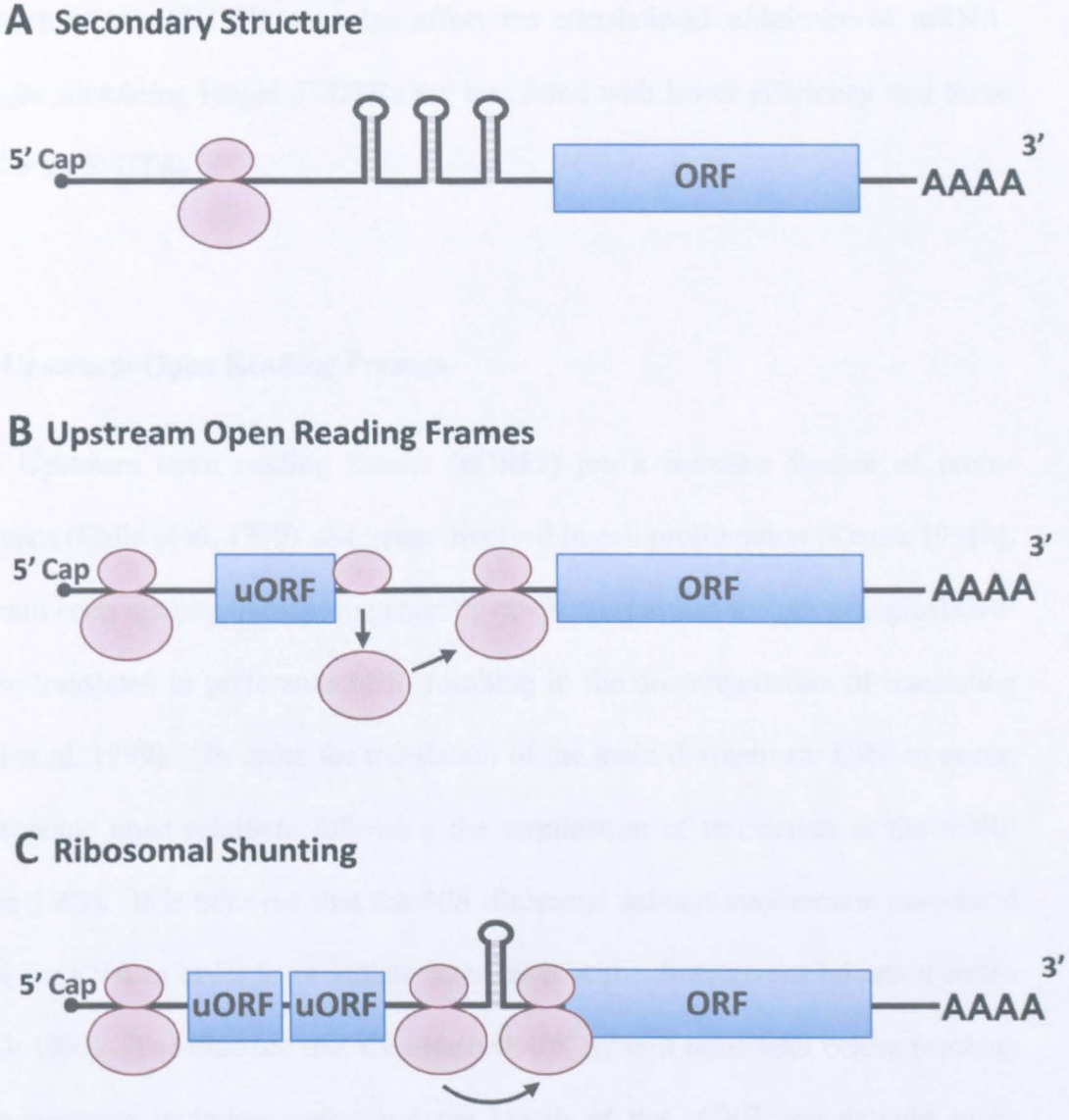


Figure 1.8 Translational Regulation by Elements Within the 5' UTR

A) Translation is inhibited by the presence of secondary structure, such as hairpins, with the 5' UTR.

B) The presence of upstream open reading frames (uORFs) can reduce the translational efficiency of downstream open reading frames (ORFs).

C) The ribosome can by-pass areas of secondary structure within the 5' UTR by a process called shunting.

of the cap structure (Kozak 1989a; Gray and Hentze 1994). It has been shown that the length of the 5' UTR can also affect the translational efficiency of mRNA. Messages containing longer 5' UTRs are translated with lower efficiency that those with shorter 5' UTRs.

1.5.2 Upstream Open Reading Frames

Upstream open reading frames (uORFs) are a common feature of proto-oncogenes (Child et al. 1999) and genes involved in cell proliferation (Kozak 1991b). Upstream open reading frames are generally much shorter than the physiological ORF and are translated in preference to it, resulting in the downregulation of translation (Child et al. 1999). In order for translation of the main downstream ORF to occur, the ribosome must reinitiate following the termination of translation at the uORF (Figure 1.8B). It is believed that the 40S ribosomal subunit may remain associated with the mRNA in order to re-initiate translation at the downstream initiation codon (Kozak 1992). The distance that the attached 40S subunit must scan before reaching the downstream initiation codon and the length of the uORF, are thought to be important factors in determining whether a re-initiation event is likely to occur. Before re-initation can take place, the scanning 40S subunit must acquire ternary complex and other initiation factors in order for the 80S ribosome to reform. As the distance between the uORF termination codon and the downstream ORF initiation codon is increased, the scanning time and consequently the chance of re-initiation is also increased (Hinnebusch 1997). The time taken to translate a short uORF appears to be more important than uORF length in determining whether re-initiation occurs (Morris and Geballe 2000). Although the mechanism of reinitiation is not fully

understood, it has been shown that in mammalian systems efficient reinitiation following uORF translation only occurs if the complete eIF4F complex, or eIF4A, 4B and central domain of eIF4G participated in the original initiation event (Poyry et al. 2004). Perhaps unsurprising when the ribosome is paused during uORF translation by the presence of a pseudo-knot, downstream re-initiation does not occur (Kozak 2001).

1.5.3 Ribosomal Shunting

Although little is known about the mechanism of ribosomal shunting, it is believed that it allows secondary structure present in an mRNA, to be bypassed by a 40S ribosomal subunit under conditions, which shut down cap-dependent translation (Figure 1.8C). It is thought that the process is mediated by *trans*-acting factors and the 18S ribosomal subunit (Yueh and Schneider 2000). The 35S RNA of the cauliflower mosaic virus (CaMV) contains a hairpin and five uORFs within its 5' UTR. It is believed that ribosomal shunting is responsible for translation of this mRNA, since the ribosome is able to bypass these inhibitory features and continue scanning (Futterer et al. 1993; Hemmings-Meiszczak and Hohn 1999)

1.5.4 The Iron Response Element

A structural motif known as the iron response element (IRE) and located in the 5' UTR is responsible for the translational regulation of a number of proteins involved in the storage and utilisation of iron (Hentze et al. 1987). The binding of iron regulatory proteins (IRP-1 and IRP-2) to the IRE co-ordinate the translational control, resulting in binding and translational repression when cellular iron levels are low.

This translation repression results from the proximity of the IRE stem loop to the cap. When the IRE is bound by IRP-1 or IRP-2, the 43S pre-initiation complex is prevented from binding to the mRNA, interfering with translation initiation (Goossen and Hentze 1992; Muckenthaler et al. 1998). When the IRE is positioned further away from the cap structure, the regulatory effect of IRP-1 and IRP-2 are reduced (Goossen and Hentze 1992). When no iron is present, the IRPs dissociate or degrade and therefore do not function to repression of translation.

1.5.5 Elements within the 3' UTR

The 3' UTR of an mRNA has many important roles in the control of expression of specific proteins. As previously mentioned in section 1.2.4.3, Poly(A)-binding protein (PABP) which binds to the poly(A) tail at the 3' end of the mRNA can interact with eIF4G forming a closed loop complex. This results in circularisation of the mRNA, stimulating the association of the 43S preinitiation complex with the mRNA, and promoting re-initiation on an mRNA (Pestova et al. 2007).

In oocytes of *Xenopus Laevis*, translational repression occurs through storage of mRNAs, which posses short poly(A) tails. During maturation, polyadenylation by the cytoplasmic poly(A)-polymerase GLD2 occurs, resulting in a long poly(A) tail and translational stimulation via the closed loop mechanism described above (Kim and Richter 2006). An element within the 3' UTR called a cytoplasmic polyadenylation element (CPE) is a Uridine-rich sequence, which is involved in both the activation and repression of polyadenylation (Figure 1.9A) (Richter 2000). Activation of polyadenylation during oocyte maturation is facilitated by the binding of Cytoplasmic-polyadenylation-element-binding protein (CPEB) to the CPE within

the target mRNA. Through the binding of CPEB, several additional proteins including CPSF, GLD2 and the deadenylase PARN become associated with the target mRNA, forming a protein complex. Phosphorylation at several sites on CPEB, results in rearrangement of the complex and subsequent ejection of PARN, allowing elongation of the poly(A) tail by GLD2 (Radford et al. 2008). Two models have been proposed to account for the repression of translation through CPE. The first involves Maskin binding to the CPE and preventing the binding of eIF4G. It has been shown that this binding event can silence a target mRNA by preventing polyadenylation from occurring prior to oocyte maturation (Stebbins-Boaz et al. 1999; Charlesworth et al. 2000; Mendez and Richter 2001). An alternative model proposes that PARN is recruited to the CPE and its proximity to the cap prevents eIF4E from binding, thus preventing initiation from occurring. The presence and binding of Maskin is not a requirement of this model (Kim and Richter 2006).

Another structural feature, which has shown to be involved in translational regulation, is the bicoid response element (BRE) (Figure 1.9B). Bicoid protein has a role in organising anterior development in *Drosophila* and has been shown to bind direction to the BRE element and eIF4E, resulting in the repression of translation (Niessing et al. 2002). The work detailed in this thesis concentrates mainly on the 5' UTR and as a result, the elements described above merely serve as an example of the numerous regulation pathways which involve elements within the 3' UTR.

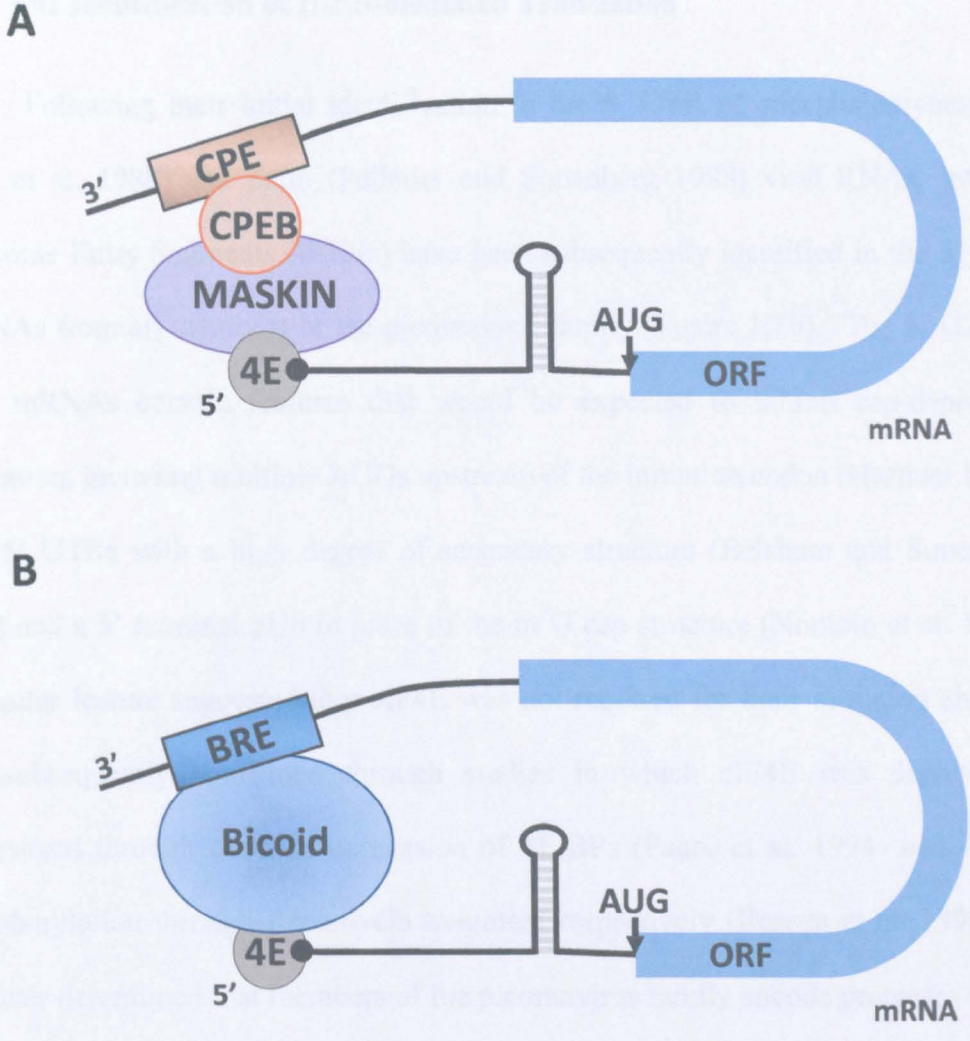


Figure 1.9 Translational Regulation by Elements Within the 3' UTR

A) The binding of cytoplasmic polyadenylation element binding protein (CPEB) to the CPE within the 3' UTR, results in the binding of Maskin. This prevents the formation of eIF4F, thus negatively regulating translation.

B) The binding of Bicoid to the Bicoid response element (BCR) within the 3' UTR, also prevents the formation of eIF4F and negatively regulates translation.

1.6 Internal Ribosome Entry Segments

1.6.1 The Identification of IRES-mediated Translation

Following their initial identification in the 5' UTR of encephalomyocarditis (Jang et al. 1988) and polio (Pelletier and Sonenberg 1988) viral RNAs, Internal Ribosome Entry Segments (IRESs) have been subsequently identified in the 5' UTR of RNAs from all members of the picornaviral family (Figure 1.10). The 5' UTR of these mRNAs contain features that would be expected to inhibit cap-dependent translation, including multiple AUGs upstream of the initiation codon (Herman 1989), long 5' UTRs with a high degree of secondary structure (Belsham and Sonenberg 1996) and a 5' terminal pUp in place of the m^7G cap structure (Nomoto et al. 1977). The latter feature suggested that eIF4E was not required for their initiation and this was subsequently confirmed through studies in which eIF4E was depleted or sequestered through the over-expression of 4E-BPs (Pause et al. 1994) and 4E-BP phosphorylation through Rapamycin treatment respectively (Beretta et al. 1996). It was later determined that members of the picornavirus family encode proteases which cleave eIF4G, uncoupling the cap-binding and ribosome recruitment functions of eIF4F. The consequence of this cleavage is that the cap-dependent translation of the host cell is shut down in order to make way for viral particle synthesis (Lamphear et al. 1995). Using dicistronic reporter constructs it has been definitively determined that IRESs are capable of internal initiation, which is independent of the cap structure or the eIF4F complex. When the 5' UTR of EMCV or poliovirus mRNA was cloned into the intercistronic space of dicistronic reporter constructs, these sequences were able to initiate translation of the second cistron (Jang et al. 1988; Pelletier and Sonenberg 1988).

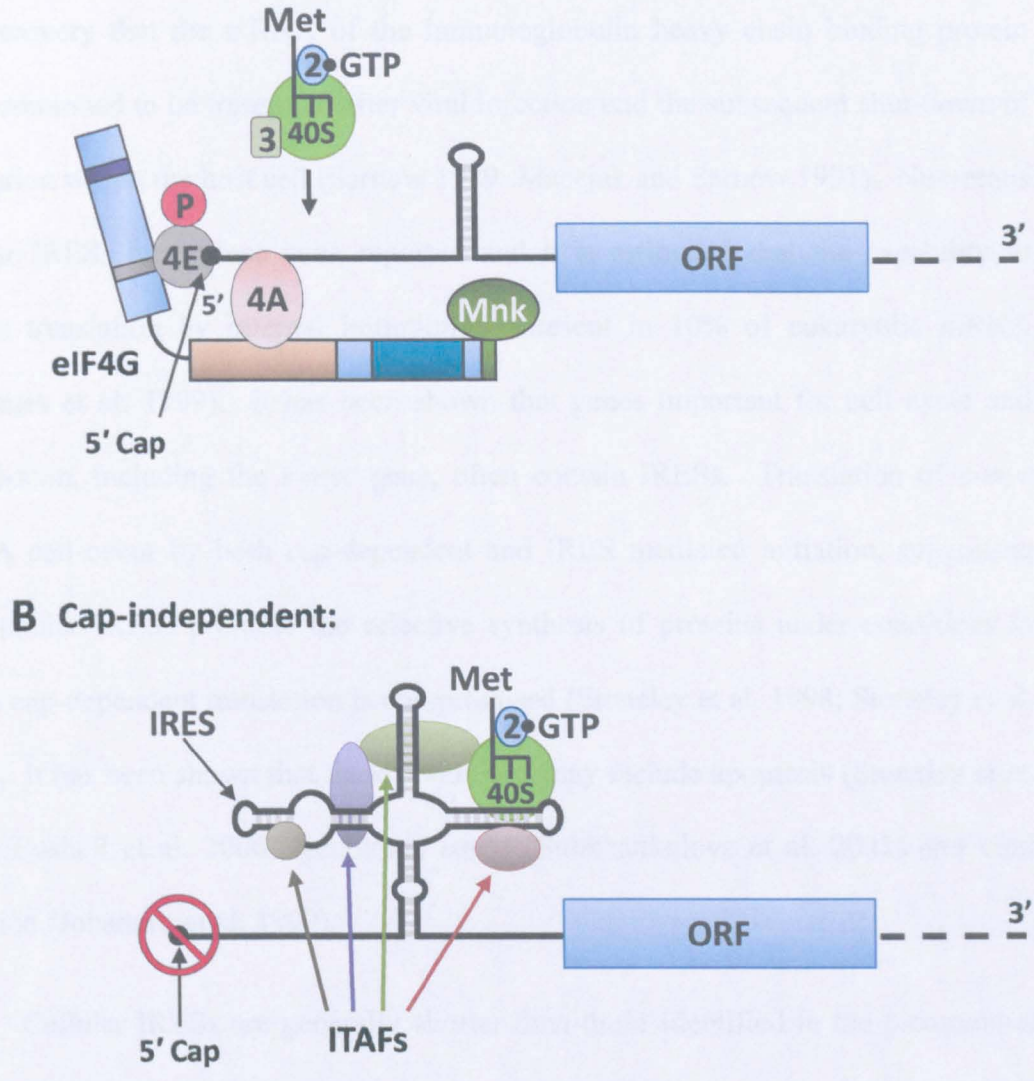


Figure 1.10 Cap-dependent and Cap-independent Initiation

A) Cap-dependent initiation of translation requires the recognition of the 5' cap-structure and formation of an initiation complex. This subsequently results in the recruitment of the 40S ribosomal subunit, scanning and the formation of the 80S ribosome.

B) A 5' cap-structure is not required for cap-independent initiation of translation. Instead, an internal ribosome entry segment (IRES) directly recruits subunits to the mRNA, in a process aided by IRES *trans*-acting factors (ITAFs).

1.6.2 Cellular IRESs

The first identification of an IRES element within cellular mRNA, followed the discovery that the mRNA of the immunoglobulin heavy chain binding protein (BiP) continued to be translated after viral infection and the subsequent shut-down of translation within the host cell (Sarnow 1989; Macejak and Sarnow 1991). Numerous cellular IRESs have since been reported and it is estimated that the capability to initiate translation by internal initiation, is present in 10% of eukaryotic mRNA (Johannes et al. 1999). It has been shown that genes important for cell cycle and progression, including the *c-myc* gene, often contain IRESs. Translation of *c-myc* mRNA can occur by both cap-dependent and IRES mediated initiation, suggesting that cellular IRESs promote the selective synthesis of proteins under conditions in which cap-dependent translation is compromised (Stoneley et al. 1998; Stoneley et al. 2000). It has been shown that these conditions may include apoptosis (Stoneley et al. 2000; Bushell et al. 2006), genotoxic stress (Subkhankulova et al. 2001) and viral infection (Johannes et al. 1999).

Cellular IRESs are generally shorter than those identified in the picornaviral mRNAs and harbour fewer AUGs. They are known to contain a high degree of secondary structure, although few similarities with the secondary structure have been shown between different cellular IRES elements. In contrast to viral IRESs, it is thought that the structural mechanisms for ribosomal recruitment by cellular IRESs are more complex. This is supported by the observation that the function of viral IRESs can be significantly inhibited by single point mutations, however by comparison the effect of single point mutations carried on cellular IRES activity is much less (Yang and Sarnow 1997; Huez et al. 1998; Stoneley et al. 1998). It has also been shown that in several instances, including the *c-myc* IRES, cellular IRESs

are often modular with sections working in concert to recruit the ribosome (Le Quesne et al. 2001). Direct recruitment of the ribosome by mRNA-rRNA base pairing has also been shown to be an important mechanism in cellular IRESs and it has been suggested that many cellular IRESs contain modules that utilise this mechanism (Chappell et al. 2000). It has been observed that some IRESs are more active in the presence of specific cellular proteins, which include La autoantigen and poly(rC)-binding protein (PCBP1) (Yueh and Schneider 2000). These proteins are described as IRES-trans acting factors (ITAFs) and it is possible that they acts as RNA chaperones and that the activity of certain mRNAs may correlate with their cellular distribution (Pilipenko et al. 2000b). The examples described above provide an insight into cap-independent translation, however, a more detailed analysis beyond the scope of this thesis. La and PCBP1 will be revisited in greater detail in sections 1.7.5.1 and 1.7.5.2 respectively.

1.7 5' TOP Containing mRNAs

1.7.1 *Cis*-Regulatory Element of 5' TOP Containing mRNAs

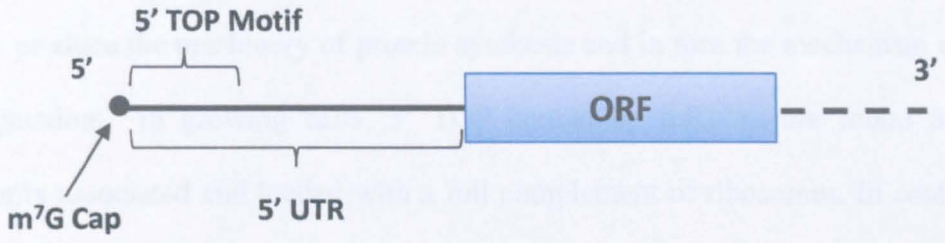
Many of the mRNAs that encode the components of the translational machinery, including ribosomal proteins and elongation factors, possess a characteristic structural hallmark in their 5' untranslated region (UTR). This feature is known as a 5' terminal oligopyrimidine tract (5' TOP) and has been identified in all currently sequenced mRNAs that encode ribosomal proteins (RPs) (Meyuhas 2000). Its presence has also been confirmed in the mRNAs encoding the eukaryotic elongating factors (eERFs), eEF1a and eEEF2 and in the mRNAs that encode the e, f and h subunits of eIF3 (Iadevaia et al. 2008). PABP, which has been implicated in the

initiation of translation (Galili et al. 1988) and mRNA stability (Coller et al. 1998), is also encoded by mRNA that is known to contain the 5'TOP motif. As will be discussed in more detail in section 1.7.3, there is evidence to suggest that the common presence of the 5'TOP motif within mRNAs encoding the translational apparatus maybe associated with the coordinated regulation of these mRNAs at the translational level.

5' TOP messages are characterized by the presence of a C residue followed by 4-14 uninterrupted pyrimidine residues immediately after the cap structure at their 5' end (Schibler et al. 1977). This uninterrupted pyrimidine sequence normally contains a roughly equal distribution of U and C residues (Meyuhas 2000). The presence of a C residue immediately after the cap is relatively uncommon and is only found in about 17% of mammalian transcripts (Schibler et al. 1977). In the majority of mRNAs the 5' cap structure is usually followed by an A residue (Bucher 1990). The 5' terminus of many mammalian 5'TOP containing mRNAs has been determined by primer extension and S1 nuclease mapping techniques (Meyuhas 2000). Comparative analysis of these mRNA sequences, clearly shows the common occurrence of a C residue immediately after the cap structure followed by a region of uninterrupted pyrimidine residues with no significant consensus sequence (Figure 1.11).

The first 30 nucleotides of the 5' TOP motif are believed to contain the activity of this major cis-regulatory element (Avni et al. 1994). Additional features which are common in 5' TOP containing mRNAs are CG rich sequences following the 5' TOP motif and short 5' UTRs, which lack upstream AUG codons (Meyuhas 2000).

A



B

Protein	Sequence	5' TOP (nt)	5' UTR (nt)
rpS6	CCUCUUUUCCGUGGCGCCUC	10	42
rpS8	CUCUUUCCAGCCAGCGCCGA	8	23
rpS11	CUUUUUUUCAGGCGGCCGGG	9	24
rpS14	CUCUUUCCGGUGUGGAGUCU	8	37
rpS17	CCUCUUUUACCAAGGACCCG	8	25
rpS24	CUUUUCCUCCUUGGCUGUCU	12	37
rpL7a	CUCUCUCCUCCCGCCGCCCA	12	22
rpL13a	CUUUUCCAAGCGGCUGCCGA	7	27
rpS4Y	CUCUUCCGUCGCAGAGUUUC	7	23
eEF1A	CUUUUUCGCAACGGGUUUGC	7	63
eEF1B	CUUUUUCCUCUCUUCAGCGT	15	84
PABP	CCCCUUCUCCCCGGCGGUUA	12	505
QM	CUCUUUCCCUUCGGUGUGGU	12	143
B23	CUUUCCCUGGUGUGAUUCCG	8	100
P23	CUUUUCCGCCCGCUCCCCCC	4	90

Figure 1.11 5'TOP Containing mRNAs

A) Diagram showing the structure of a typical TOP containing mRNA. The TOP motif if located within the 5'UTR immedioately succeeding the m^7G cap structure.

B) Typical sequence and alignment of the TOP motif from fifteen known TOP containing human mRNAs. The first twenty nucleotides immediately succeeding the cap structure are shown (column 2), along with the length of the 5' TOP motif (column 3) and the total 5'UTR length (column 4). Notable features are the uninterrupted polypyrimidine tract and the high C / U ratio within the first twenty nucleotides.

1.7.2 Growth Dependent Regulation of 5' TOP Containing mRNAs

The ability of a cell to modify its rate of protein synthesis is linked to its ability to produce the machinery of protein synthesis and in turn the mechanism of 5' TOP regulation. In growing cells, 5' TOP containing mRNAs are found to be polysomally associated and loaded with a full complement of ribosomes. In contrast, in cells that are growth-arrested, the 5' TOP containing mRNAs are found to be shifted into the sub-polysomal region and stored in RNP (Loreni and Amaldi 1992). These two contrasting states suggest that 5' TOP mRNA translation can be activated or repressed in a binary control mechanism, which alternates between the two extremes. Analysis of the polysomal association of mRNAs, which encode the translational machinery, such as eIF1A and PABP, supports this data (de Melo Neto et al. 1995; Wu and Bag 1998). The levels of mRNAs encoding ribosomal proteins were found to be constant during the shift from polysomes to RNPs. This suggests that mRNA stability was not affected and that a reversible transfer of mRNA encoding ribosomal proteins between polysomes and RNP, was responsible for the observed shift (Loreni and Amaldi 1992). *In vivo* studies have shown that the 5' TOP containing mRNAs extracted from mRNPs, have a similar translational efficiency to those extracted from translationally active polysomes (Shama and Meyuhas 1996). This suggests that mRNAs are not modified structurally during sequestration.

Interestingly, this binary 'all-or-none' control mechanism only occurs when the 5' TOP is positioned immediately after the cap structure (Hornstein et al. 1999b). It has been suggested that the binomial distribution of 5' TOP containing mRNAs between polysomes and mRNP, is indicative of block at their initiation stage of protein synthesis (Meyuhas 2000). The characteristic growth-dependent expression of

5' TOP containing mRNAs thereby facilitates a mechanism for their co-ordinated expression. This is likely to be particularly important in the context of ribosome biogenesis, which accounts for a large proportion of cellular energy expenditure (Warner et al. 2001).

1.7.3 5' TOP Containing mRNAs and the Translational Apparatus

The mRNAs encoding ribosomal proteins (RPs) were the first 5' TOP containing mRNAs to be identified in vertebrates. Their translation was found to be regulated in a growth dependent manner and their discovery confirmed the existence of an abundance of mRNAs with a 5' terminal m7GpppPy (Schibler et al. 1977; Geyer et al. 1982). Random selection of almost forty vertebrate RP mRNAs, have yet to identity a single RP which is not encoded by an mRNA containing a 5' TOP motif (Meyuhas 2000). As will be discussed in more detail in section 1.7.4, the mTOR signalling pathway has been implicated in translational regulation of 5'TOP containing mRNA (Jefferies et al. 1997). Disruption of the mTOR signalling pathway can be achieved using Rapamycin, a macrocyclic metabolite isolated from *Streptomyces hygroscopicus*, which form an inhibitory complex which targets mTOR (Vezina et al. 1975; Pullen et al. 1998; Mothe-Satney et al. 2000). Separate experiments in which cDNA microarray analysis was carried out following Rapamycin treatment, have shown that translation of the mRNAs encoding ribosomal proteins and eukaryotic elongating factors (eERFs) are concurrently repressed (Grolleau et al. 2002). It is therefore likely that all ribosomal protein mRNAs contain the 5' TOP motif and are consequently regulated in a growth-dependent manner.

Growth-dependent regulation, similar to that observed for RP mRNAs, has also been observed for the mRNA encoding elongation factor 1. An increase in the synthesis rate of eEF1A without a concomitant increase in eEF1A mRNA abundance, was detected following the growth stimulation of resting Swiss 3T3 cells (Thomas and Luther 1981; Thomas 1986). Further analysis indicated that eEF1A mRNAs shifted from mRNP particles to polysomes following mitogenic stimulation (Rao and Slobin 1987; Jefferies and Thomas 1994). Subsequent growth stimulation experiments and detailed sequencing at the transcription start site, have revealed that like eEF1A, all the translation elongation factors are encoded by 5' TOP containing mRNAs (Iadevaia et al. 2008). Interestingly, mRNAs encoding additional proteins that are involved in the translational machinery, including the e, f and h subunits of eIF3, also contain the 5' TOP motif (Iadevaia et al. 2008). Similarly, growth-dependent translational regulation has also been confirmed for mRNA encoding PABP (Hornstein et al. 1999a). PABP has been implicated in the initiation of translation (Galili et al. 1988) and mRNA stability (Coller et al. 1998).

1.7.4 Signalling Pathways Involved in the Translational Control of 5' TOP Containing mRNAs

An apparent correlation between translational activation of 5' TOP messages in response to mitogenic stimuli and the phosphorylation of RPS6 (ribosomal protein S6) by the kinases S6K1 and S6K2 was an initial focus of research involving signalling pathways and their role in 5' TOP mRNA translational regulation (Thomas et al. 1980). Mobilization into polysomes appeared to be higher in ribosomes with a higher proportion of phosphorylated RPS6 (Thomas et al. 1980). This led to the

proposal that selective 5' TOP message initiation was a result of S6K activation, leading to RPS6 phosphorylation (Jefferies et al. 1997).

Both S6K1 and S6K2 are known to be involved in the mTOR (mammalian target of Rapamycin) signalling pathway. They have been shown to be downstream targets of mTORC1 (mTOR Complex 1) and are phosphorylated in response to insulin, growth factors, serum and amino acids (Kim et al. 2002). mTORC1, comprised of mTOR, Raptor, mammalian LST8 and PRAS40, is one of two catalytic complexes through which mTOR functions (Kim et al. 2002). The function of mTOR1 and hence the phosphorylation of S6K1 and S6K2, has been shown to be sensitive to inhibition by Rapamycin. Interestingly, when S6K activity was blocked using Rapamycin as an inhibitor of mTOR, the translation of 5' TOP containing mRNAs was initially repressed (Jefferies et al. 1997). However, in several different cell lines only a moderate effect on 5' TOP mRNA translation was observed following Rapamycin treatment, despite the complete inhibition of S6K activity (Tang et al. 2001; Stolovich et al. 2002), suggesting that the effect of S6K inhibition is dependent upon cell type. The direct relationship between RPS6 phosphorylation and 5' TOP mRNA translation has been further called into question by several interesting observations. 5' TOP containing mRNAs were found to undergo translational repression during mouse erythroleukaemia (MEK) cell differentiation, despite RPS6 being constitutively dephosphorylated in these cells (Barth-Baus et al. 2002). It has also been shown that the translational regulation of 5' TOP containing mRNAs is unaffected by the targeted disruption of the S6K alleles (Tang et al. 2001) or by the disruption of S6K alleles, which is present in embryonic stem cells (Pende et al. 2004). Similarly, the combined knockout of both S6K1 and S6K2 has been shown to have no effect on the translational regulation of 5' TOP containing mRNAs in

response to mitogenic stimuli (Pende et al. 2004). It would appear therefore, that the translational regulation of 5' TOP containing mRNAs is not dependent upon the phosphorylation of RPS6 phosphorylation or on S6K.

Signalling through the mTOR signalling pathway in response to growth factors and nutritional stimuli make it ideally positioned for a regulatory role in translation initiation. It is, therefore, possible that an additional downstream target of mTOR, 4E-BP1 (eIF4E-binding protein 1) plays a role in the regulation of 5' TOP containing mRNAs. Phosphorylation of 4E-BP1, facilitated by mTORC1 results in the dissociation of 4E-BP1 from the initiation factor eIF4E (Hay and Sonenberg 2004). It is known that phosphorylation by mTOR can occur on at least two sites within 4E-BPs and can be prevented by the use of Rapamycin (Pullen et al. 1998; Mothe-Satney et al. 2000). Following the phosphorylation of 4E-BP1, dissociation of eIF4E facilitates the formation of the cap-binding complex and translational activation can then take place (Sonenberg 1993a).

Whilst the mTOR signalling pathway is clearly implicated in the translational regulation of 5' TOP containing mRNAs, the contradictory data surrounding the response to inhibition by Rapamycin and the apparent dependence upon cellular context indicate that other signalling pathways may also be involved in 5' TOP mRNA regulation. The PI3K (phosphoinositide 3-kinase) signalling pathway has recently been implicated in translational regulation of 5' TOP containing mRNAs. It has been shown that activation of PI3K signalling occurs upon insulin or mitogenic stimuli and results in the downstream activation of PKB (protein kinase B) through PDK1 (phosphoinositide dependent kinase 1) (Burgering and Coffer 1995; Kohn et al. 1995). When cells are treated with LY294002, a specific inhibitor of the PI3K signalling pathway (Vlahos et al. 1994), the translational activation of 5' TOP

containing mRNAs is completely blocked (Tang et al. 2001). This result is also obtained when cells are treated with the PI3K inhibitor Wortmannin (Caldarola et al. 2004). The tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), is known to dephosphorylate PI-3,4,5-triphosphate and PI-3,4-diphosphate, which are both upstream of PKB within the PI3K pathway (Cantley and Neel 1999). Over-expression of PTEN has been shown to inhibit the recruitment of 5' TOP containing mRNAs to polysomes upon serum stimulation (Tang et al. 2001). Furthermore, the same result was obtained upon over-expression of a dominant-negative mutant version of the PI3-kinase regulatory subunit, p85, which lacks the binding site for the p110 catalytic subunit. Together, these data strongly suggest that that both PI3K and PKB are involved in the growth regulated recruitment of 5' TOP containing mRNAs into polysomes.

Precisely how signalling via PI3K mediates the recruitment of 5' TOP containing mRNAs into polysomes is still not clearly defined. It has been proposed that PKB is a mediator conveying signals from PI3K to S6K1, and that S6K1 is involved in the translational activation of 5' TOP containing mRNAs (Meyuhas 2000). Supporting this model, the over-expression of PKB has been shown to result in the activation of S6K1 in unstimulated cells (Pullen et al. 1998; Romanelli et al. 1999). However, several lines of evidence (reviewed above) have indicated that the translational regulation of 5' TOP containing mRNAs is not dependent upon the phosphorylation of RPS6 phosphorylation or on S6K. Evidence also exists, which indicates that the mTOR signalling pathway can be directly activated by PI3K (Hay and Sonenberg 2004). However, it has been observed that the translational activation of 5' TOP containing mRNAs is only moderately affected by mTOR inhibition but fully dependent upon PI3K signalling (Stolovich et al. 2002). This indicates that the

direct activation of mTOR by PI3K signalling does not significantly contribute to the regulation of 5' TOP containing mRNAs. Despite a large volume of evidence, which implicates both the mTOR and PI3K signalling pathways in the translational regulation of 5' TOP containing mRNAs, the precise role of each pathway remains undetermined.

1.7.5 *Trans*-acting Factors implicated in the Translational Regulation of 5' TOP Containing mRNAs

1.7.5.1 La and CNBP

It has been suggested that the translational regulation of 5' TOP containing mRNAs may be mediated through the recognition of the 5' TOP motif by specific *trans*-acting factors. Without specifically identified proteins, which recognise the 5' TOP motif, several lines of evidence have emerged which support this proposal. It has been shown that the translational of 5' TOP containing mRNAs is selectively repressed in reticulocyte lysate derived from non growing cells (Shama and Meyuhas 1996). Interestingly, this repression can be relieved by the pre-incubation of the translational extracts with a synthetic 5' TOP RNA oligonucleotide (Biberman and Meyuhas 1999). This suggests that the accumulation of a titratable repressor, as opposed to the loss of a translational activator, may be involved in the translational repression of 5' TOP containing mRNAs. One such protein implicated in the translational regulation of 5' TOP containing mRNAs, is La autoantigen.

It has been shown using sucrose density gradient analysis, that La co-sediments with polysomes in an RNA-dependent manner and is specifically

associated with 5' TOP containing mRNAs in the polysome fraction (Cardinali et al. 2003). This data is supported by the observed shift of RPL4 mRNA from sub-polysomes to polysomes, when wild-type La is over-expressed in growth-arrested cells (Crosio et al. 2000). These experiments have led to a model, in which the specific interaction of La with 5' TOP containing mRNAs results in translational up-regulation of these mRNAs. Further evidence, which supports the involvement of La, has been provided by *in vitro* binding studies and has shown that La and cellular nucleic acid binding protein (CNBP) can interact with the 5' UTR of both *Xenopus Laevis* and Human 5' TOP containing mRNAs (Pellizzoni et al. 1996; Crosio et al. 2000). It has been shown in *Xenopus,* that both La and CNBP compete for interaction with the autoantigen Ro60, whose co-operation is required in order to bind the 5' UTR of ribosomal protein-L4 mRNA (Pellizzoni et al. 1998; Crosio et al. 2000). The interaction between the 5' UTR and Ro60 associated La and CNBP was found to be mutually exclusive, leading to the proposal that CNBP acts a translational repressor, while La exerts a positive effect on translation through competitive binding (Pellizzoni et al. 1998).

Recently, conflicting evidence has emerged which has challenged the proposed roles of La and CNBP in the regulation of 5' TOP containing mRNAs. It has been shown *in vivo* that 5' TOP containing mRNAs preferentially associate with La which is not phosphorylated on Ser^{366} (Intine et al. 2004). The over-expression of a mutant form of La protein, unable to undergo phosphorylation on Ser^{366}, resulted in an increase in La association with RPL37 mRNA and the proportion of RPL37 mRNA associated with sub-polysomal particles (Schwartz et al. 2004). Studies carried out *in vitro,* have also shown that La can inhibit 5' TOP mRNA translation (Zhu et al. 2001). Therefore, these data suggest that La regulates 5' TOP mRNA

translation in a manner which is opposite to that proposed by the earlier model. Interestingly, in contrast to the proposed role of CNBP in 5' TOP regulation, it has been shown that CNBP promotes cap-independent translation as part of an IRES-trans acting factor (ITAF) complex (Gerbasi and Link 2007). To date, no direct *in vivo* or *in vitro* evidence has emerged, which has demonstrated a direct relationship between the growth-dependent translational regulation of 5' TOP containing mRNAs and the binding of LA or CNBP. Whilst it is clear that both La and CNBP can interact with 5' TOP containing mRNAs, the precise role of these proteins in the regulation of 5' TOP containing mRNAs is still not well defined.

1.7.5.2 Pyrimidine-Binding Proteins

It has been suggested that several pyrimidine-binding proteins may associate with the polypyrimidine tract, which is present in all 5' TOP containing mRNAs (Meyuhas 2000). PTB (polypyrimidine tract binding protein) is a multi-function protein, which preferentially binds polypyrimidine-rich regions of mRNA (Garcia-Blanco et al. 1989). It is known that PTB often functions in combination with other proteins to initiate translation (Sawicka et al. 2008). For example, the combined action of PTB and PCBP1 (poly(rC)-binding protein 1) is required in the stimulation of the BAG-1 (Bcl-2-associated anthanogene 1) IRES. PCBP1 also known as heterogeneous nuclear ribonuclear protein E1 (hnRNP E1) is involved in remodelling the RNA structure to allow PTB to bind (Pickering et al. 2004). hnRNP E1 has been shown to operate in conjunction with hnRNP K as a translational silencer (Ostareck et al. 1997). The latter selectively binds poly(C) homopolymer and has, therefore, been implicated in the translational regulation of 5' TOP containing mRNAs (Matunis et al.

1992). It has been suggested that the over-expression of hnRNP E1, hnRNP K or PTB does not repress the translational efficiency of reporter 5' TOP mRNA in growing cells and does not further repress the efficiency in amino acid starved cells (Meyuhas 2000). However, it should be noted that this suggestion originated in a review publication and was largely based upon unpublished data. To date, no direct evidence has been published, which has definitively proven or disproven the involvement of PTB in the regulation of 5' TOP containing mRNAs. Whilst this data appears to confirm that hnRNP E1, hnRNP K and PTB are not repressors of 5' TOP mRNA translation, it remains unclear as to whether they are specifically required to initiate translation of 5' TOP containing mRNAs.

1.8 Regulation of Gene Expression by MicroRNAs

1.8.1 MicroRNA Function and Biogenesis

MicroRNAs (or miRNAs) are a class of short non-coding RNAs, which are typically 21 to 25 nucleotides in length (Cannell et al. 2008). They are genomically encoded as monocistronic and polycistronic gene clusters, which are located within intronic regions of genes, or as independent transcriptional units (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee et al. 2002). MicroRNAs are involved in post transcriptional regulation of gene expression through a mechanism, which requires them to bind with imperfect complimentary to their target mRNA (Pillai et al. 2007). Nucleotides 2 to 8 of the miRNA are known as the seed region and share Watson-Crick base-pairing complementarity with a region that is generally located within the 3' UTR of the target mRNA. Binding of the miRNA to the target mRNA results in the repression of protein production by mRNA destabilisation and translational

silencing (Lewis et al. 2005). Individual miRNAs may regulate a cohort of mRNAs, and miRNA binding sites may be conserved in several different species (Lewis et al. 2005; Pillai et al. 2007).

RNA polymerase II transcribes miRNA genes, resulting in the production of primary miRNA (pri-miRNA) precursors (Cai et al. 2004; Lee et al. 2004). The functional miRNAs are contained within pri-miRNA hairpin structures, which are rapidly cleaved in the nucleus by the RNase III enzyme Drosha to form pre-miRNAs (Kim 2005). Pre-miRNAs are stem loop structures, approximately 70 nucleotides in length (Figure 1.12A), which are exported from the nucleus by Exportin 5, prior to cleavage by a second RNAse III enzyme called Dicer (Kim 2005). The resultant miRNA duplexes, which are approximately 22 nucleotides in length, then undergo strand selection. It is currently believed that the RNA strand, which possesses the weakest thermodynamic stability at its 5' terminus, is the one selected for incorporated into a miRNA-containing-ribonucleoprotein particle (miRNP) (Khvorova et al. 2003). The resultant miRNP, also known as a miRNA-containing RNA-induced silencing complex (RISC), contains a number of protein components in addition to the miRNA guide strand (Figure 1.12B). This complex targets and inhibits proteins expression from specific mRNAs (Hutvagner and Zamore 2002) (Figure 1.12C).

The RISC complex is also involved in the targeted repression of mRNAs by small interfering RNAs (siRNAs). siRNAs share Watson-Crick base-pairing complementarity with a region located within the 3' UTR of their target mRNA (Elbashir et al. 2001). Unlike miRNAs, the entire siRNA binds to its target mRNA, resulting in the degradation rather than the repression of the target mRNA mediated by the RISC complex (Bernstein et al. 2001). Synthetic siRNAs that have complete

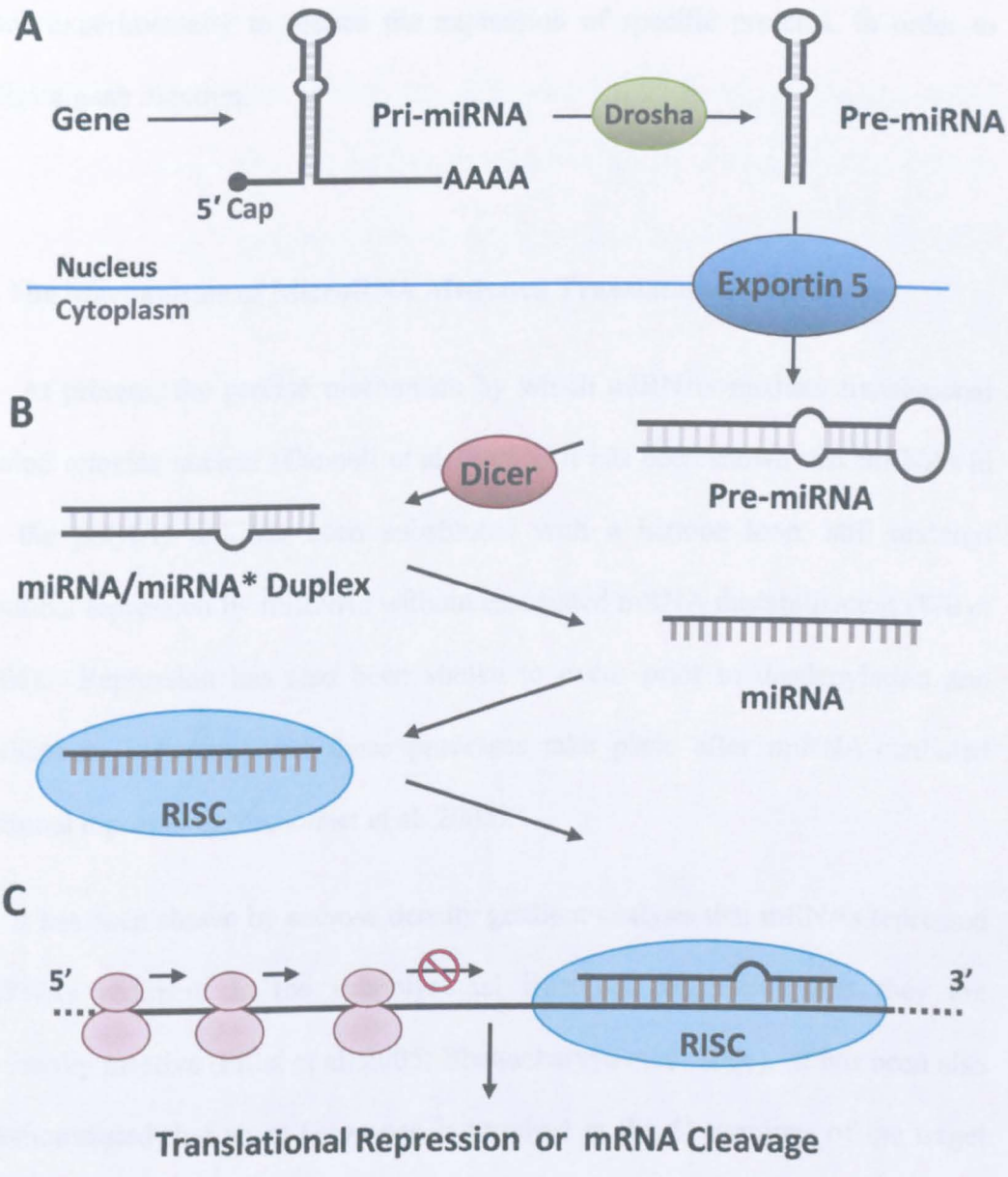


Figure 1.12 MicroRNA Biogenesis and Function

A) Transcription of miRNAs as pri-miRNAs occurs in the nucleus. The nuclease Drosha, cleaves pri-miRNAs into pre-miRNAs, which are exported into the cytoplasm by Exportin 5.

B) In the cytoplasm, Dicer cleaves pre-miRNAs into mature miRNAs. After the process of strand selection has taken place, mature miRNAs form part of the RNA-induces silencing complex (RISC).

C) The miRNA within the RISC, binds to its target mRNA. This results in either mRNA cleavage or translational repression, depending upon the level of sequence complementarity.

sequence complementarity with a region located in the 3' UTR of a target mRNA can be used experimentally to reduce the expression of specific proteins, in order to investigate gene function.

1.8.2 The Mechanisms of MicroRNA Mediated Translational Repression

At present, the precise mechanism by which miRNAs mediate translational repression remains unclear (Cannell et al. 2008). It has been shown that mRNAs in which the poly(A) tail has been substituted with a histone loop, still undergo translational repression by miRNAs without associated mRNA destabilization (Wu et al. 2006). Repression has also been shown to occur prior to deadenylation and destabilisation, indicating that these processes take place after miRNA-mediated translational repression (Mathonnet et al. 2007).

It has been shown by sucrose density gradient analysis that mRNAs repressed by miRNAs sediment in the subpolysomal fractions, suggesting that they are translationally inactive (Pillai et al. 2005; Bhattacharyya et al. 2006). It has been also been demonstrated that an m^7Gppp cap is required at the 5' terminus of the target mRNA, in order for repression to occur (Bhattacharyya et al. 2006; Wang et al. 2006; Mathonnet et al. 2007; Wakiyama et al. 2007). Together these data led to the proposal that miRNAs mediate translational repression at the initiation phase of protein synthesis. However, this model has been challenged by experiments carried out using different reporter constructs. These experiments indicated that miRNA targeted mRNAs were associated with polysomes, despite synthesis of their corresponding proteins being repressed (Nottrott et al. 2006; Petersen et al. 2006). This suggests that miRNA mediated repression occurs post-initiation and is supported

by the observation that large numbers of miRNAs co-sediment with polysomes (Kim et al. 2004b; Nelson et al. 2004; Maroney et al. 2006). Analysis of the reporter constructs used in the experiments, which proposed contrasting models, led to the discovery that these constructs employed a different promoter element (Kong et al. 2008). Subsequently, it has been suggested that the mechanism of miRNA mediated repression at the translational level, is dependent on the promoter element (Kong et al. 2008). Specifically, it has been proposed that miRNA target mRNAs derived from the SV40 (Simian vacuolating virus 40) promoter are repressed at initiation and those derived from the TK promoter are repressed post initiation (Kong et al. 2008). MicroRNA targeted mRNAs derived from the SV40 promoter were found to shift to sub-polysomal fractions. However, when ribosome translocation rate was reduced by treatment with low dose of cycloheximide, mRNAs were found to return to polysomes (Kong et al. 2008). This suggests that while an initiation block in translation is likely to occur, it is not complete and elongation may still occur at a reduced rate. In contrast, miRNA targeted mRNAs derived from the TK promoter were shown to be polysomally associated, indicating that miRNA mediated translational repression occurs at a post-initiation stage (Kong et al. 2008).

1.8.3 The Argonaute Family of Proteins

Investigations carried out to identify proteins associated with miRNPs, found that four Argonaute (AGO) proteins co-immunoprecipitate with Dicer (Sasaki et al. 2003; Gregory et al. 2004; Chendrimada et al. 2005). Although the roles of AGO1, AGO3 and AGO4 have not been specifically determined, AGO2 appears to be a component of the mature miRNA (Gregory et al. 2004; Chendrimada et al. 2005).

AGO2 has also been implicated in miRNA mediated cleavage which occurs when the miRNA has perfect complementarity with its target mRNA (Liu et al. 2004; Meister et al. 2004). Reporter mRNA derived from the TK promoter was found to shift into the subpolysomal region following artificial tethering of AGO2 to its 3' UTR, without a requirement for mRNA (Pillai et al. 2005). This suggests that AGO2 may be involved in the imposition of an initiation block on mRNA, which is normally repressed at a post-initiation stage. It has also been suggested that AGO2 interacts with the m^7Gppp mRNA cap, following its detection by affinity chromatography with m^7G-Sepharose (Kiriakidou et al. 2007). More recently however, it has been reported that human AGO2 does not contain an eIF4E-like mRNA cap binding motif and that the positions of the AGO2 aromatic residues are inconsistent with analogous cap-binding modes (Eulalio et al. 2008; Kinch and Grishin 2009).

1.8.4.1 Translational Up-regulation by Micro-RNAs

AGO2 is also known as Co-eIF-2A, and has previously been implicated in stabilising the ternary complex and performs an essential role in translation. In direct contrast to its role in miRNA mediated translational repression, AGO2 has been shown to stimulate and stabilise Met-$tRNA_i^{met}$ binding to eIF2 (Roy et al. 1981; Chakravarty et al. 1985), and antibodies prepared against homogenous Co-eIF2A strongly inhibited protein synthesis in rabbit reticulocytes (Chakravarty et al. 1985). Interestingly, despite the apparent dual function of AGO2, miRNA-mediated up-regulation of translation appears relatively uncommon. In the first reported instance, miR-122 was shown to regulate hepatitis C virus replication (Jopling et al. 2005). It has also been shown that miRNAs can stimulate translation when cells enter

quiescence. It has been determined that translational stimulation is mediated by the binding of AGO2, and FXR1, a second RISC associate protein, to the 3' UTR of the target mRNA (Vasudevan et al. 2007).

1.8.4.2 MicroRNA-10a Enhances the Translation of 5' TOP containing mRNAs

More recently, it has been reported that miR-10a binds to the 5' UTR of mRNAs that encode ribosomal proteins and enhances their translation (Orom et al. 2008). In this study, biotin-tagged miR-10a was transfected into cells and affinity purification was carried out using Streptavidin beads and purified mRNAs were then identified using cDNA microarray analysis. It was found that miR-10a co-purified with a specific subset of mRNAs, which were not found to be enriched by any of the control miRNAs used in the experiment. In particular, 55% of the most enriched mRNAs were known 5' TOP containing mRNAs, which encoded proteins involved in protein biosynthesis including the RPs (Orom et al. 2008). Subsequent analysis of the radio-labelled methionine incorporation following miR-10a transfection revealed that miR-10a significantly increased the synthesis of ribosomal proteins. Using a primer extension assay to map the site at which miR-10a interacted with mRNAs that encode RPs, it was determined that this site was located within the 5' UTR of the target mRNA. Interestingly, it was found that complete binding of the miR-10a seed region to the 5' UTR of the target mRNA was not required to achieve translational regulation (Orom et al. 2008).

In the context of 5' TOP mRNA translation, it has been shown that miR10-a can alleviate the translational repression imposed upon 5' TOP containing mRNAs following amino acid starvation (Orom et al. 2008). The requirement for the 5' TOP motif has also been specifically demonstrated, by the observation that miR-10a

mediated translational enhancement does occur when mutagenesis is carried out within the 5' TOP region of reporter mRNAs. It has also been shown that the translational enhancement imposed by miR-10a is rapamycin sensitive, which suggests that mTOR signalling pathway may also be involved in the miR-10a mediated translational regulation of 5' TOP containing mRNAs (Orom et al. 2008). This is in agreement with data which has also implicated the mTOR signalling pathway in the translational regulation of 5' TOP containing mRNAs (Jefferies et al. 1997).

1.9 Project Aims

Since the discovery almost three decades ago of the 5' TOP containing mRNAs that are regulated in a growth dependent manner, only limited progress has been made towards identifying the mechanism by which their regulation occurs at the translational level. Although it has been proposed that the PI3K and mTOR signalling pathways are involved in the translational regulation of 5' TOP containing mRNAs, the precise mechanism by which these pathways signal to these containing mRNAs remains undefined. Furthermore, several *trans*-acting factors have also been implicated in the regulation of 5' TOP containing mRNAs, without a clearly defined mechanism of how they are involved, or indeed the location at which they bind.

The aims of this project were:

1) to develop a 5' TOP mRNA reporter vector that would facilitate the direct investigation of the proteins that are associated with 5' TOP containing mRNAs;

2) to use affinity purification to obtain 5' TOP containing mRNAs and associated proteins and to directly identify these proteins using both mass spectroscopic analysis and western blotting;

3) to investigate the role of the mTOR and PI3K signalling pathways in this experimental system;

4) to use the 5' TOP reporter constructs developed in this study, to investigate the canonical initiation factors requirements of 5' TOP containing mRNAs. To date, there have been no direct attempts to characterise the involvement of the canonical initiation factors in the translation of 5' TOP containing mRNAs.

Chapter Two

Materials and Methods

2.1. General Reagents

2.1.1 Reagents and Equipment Suppliers

Unless otherwise stated, all chemical reagents were of analytical grade and were obtained from BDH laboratory supplies (Lutterworth, Leicestershire), Fisher Scientific (Loughborough, Leicestershire), ICN Flow Ltd (Thame, Oxfordshire), Oxoid (Unipath, Basingstoke, Hampshire) or Sigma Chemical Company Ltd (Poole, Dorset). Products for molecular biological techniques were routinely purchased from Ambion (Huntingdon, Cambridgeshire), Calbiochem (c/o CN Biosciences UK, Beeston, Nottingham), Gibco-BRL (Paisley, Scotland), MBI Fermentas (c/o Helena Biosciences, Sunderland, Tyne and Wear), New England Biolabs (NEB)(C/o CP Labs, Bishops Stortford, Hertfordshire), Perbio (Tattenhall, Cheshire), Pharmacia Biotech (Milton Keynes, Buckinghamshire), Promega (Southampton), QIAGEN (Crawley, West Sussex), Roche UK Ltd (Lewes, East Sussex), Sigma-Aldrich UK (Cambridge), Stratagene Ltd (Cambridge), and USB (Cleveland, Ohio, USA). Radiolabelled chemicals were purchased from Amersham International (Little Chalfont, Buckinghamshire) and NEN Dupont (Hounslow).

2.1.2 Antibodies

Actin was detected with a mouse monoclonal antibody at a dilution of 1:2000 (Sigma). Polyclonal rabbit antibody against PTB was raised in the Jackson laboratory. Rabbit antibodies to eIF4E, eIF4G (dilution 1:5000) and eIF4A were kindly provided by Dr. Simon Morley of the University of Sussex. 4E-BP, 4E-BP-Phospho, hnRNP-K, AGO2 and La antibodies were purchased from Cell Signaling Technology (New England Biolabs). Monoclonal anti-Flag-M2 antibody produced in mouse clone M2 was purchased from Sigma-Aldrich. All primary antibodies were used at a dilution of 1:1000 unless otherwise stated. Secondary antibodies raised against mouse and rabbit IgG and conjugated to horseradish peroxidase were obtained from Sigma and used at a dilution of 1:5000.

2.2. Tissue Culture Techniques

2.2.1 Tissue Culture Solutions

Phosphate buffered saline (PBS): 4.3mM Na_2HPO_4, 1.5mM KH_2PO_4, 137mM NaCl, 2.7mM KCl, pH 7.4.

2.2.2 Cell Lines

Cell lines were purchased from the American Type Culture Collection (ATCC). Details of the cell lines used can be found in table 2.1.

Name	Cell Type
HeLa	Human cervical epithelioid carcinoma
HEK-293	Human embryonic kidney cell line Immortalised with adenovirus DNA
HEK-293T	Human embryonic kidney cell line Immortalised with adenovirus DNA and containing the SV40 Large T-antigen

Table 2.1. Descriptions of cell lines used.

2.2.3 Maintenance of Cell Lines

Cells were typically cultured in gamma sterilised plasticware (TPP c/o Helena Biosciences) in Dulbecco's modified Eagle's medium, without sodium pyruvate (GIBCO-BRL), supplemented with 10% fetal calf serum (FCS) (Helena Biosciences) and 2mM L-glutamine (Sigma), under a humidified atmosphere containing 5% CO_2. Cells were grown to confluence in 75cm^2 flasks, washed with phosphate buffered saline (PBS) and treated with 1x trypsin-0.5mM EDTA solution (GIBCO-BRL). Cells were resuspended in fresh medium pre-incubated at 37°C, and approximately 1 x 10^6 cells re-seeded into new 75cm^2 flasks.

2.2.4 Transfection of Cell Lines

Transfections were routinely carried out in 6 or 24 well plates. The following quantities are stated per well for 6 well plates, a six-fold reduction of all reagents and DNA was used for each well in 24 well plates.

2.2.4 Fugene-6 Mediated DNA Transfection

Cells were transfected using FuGene 6 (Roche) as specified by the manufacturer. Approximately 20 hours prior to transfection, 1 X 10^5 cells were seeded into each well of a 6-well plate in 1 mL of complete media. 3 μL of Fugene-6 was added to 97 μL of serum-free DMEM media per well and incubated at room temperature for five minutes, following which the solution was added to 1 μg of TOP plasmid DNA in combination with other plasmids as described in the results chapters. The DNA/Fugene-6 reaction mixture was incubated for a further fifteen minutes at room temperature and added drop wise to the cells. Cells were harvested 40 hours after transfection. Unless otherwise indicated, all transfections were performed in duplicate. Error bars represent standard deviations of three independent experiments.

2.2.5 Serum Starvation

To induce growth inhibition by serum starvation, cells were transfected as before. 24 hours post-transfection, the medium was aspirated and cells washed extensively with PBS to remove serum. The medium was replaced with fresh medium containing 10%, or 0.1% or serum. Cells were grown for a further 18 hours (unless

otherwise stated) and the medium was replaced with fresh medium containing 10% serum for 4 hours (unless otherwise stated) prior to harvesting.

2.3. Bacterial Methods

The bacterial strain used for most manipulations was Escherichia coli strain JM109: e14$^-$(mrcA)recA1, endA1, gyr A96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB), F', traD36, proAB, lacZΔM15

The BL21(DE3) strain of *E.coli* was used for production of recombinant proteins: F$^-$, ompT, hsdS$_B$, (r_B^-, m_B^-), dcm, gal

The INV110 strain of *E.coli* (Invitrogen) was used for transformation of plasmids containing *Xba*I restriction sites which were used for cloning: F′ (*traD36 proAB lacI^q lacZDM15*) *rps*L (Str^R) *thr leu end*A *thi*-1 *lacY galK galT ara ton*A *tsx dam dcm supE44 D(lac-proAB) D(mcr*C-*mrr)102*::Tn*10* (Tet^R)

2.3.1. Preparation of Competent Cells

A single bacterial colony was inoculated into 2.5 mL of LB medium and incubated overnight at 37°C in a shaking incubator. The entire overnight culture was used to inoculate 250 mL of LB medium supplemented with 20mM $mgSO_4$. The culture was incubated at 37°C in a shaking incubator until A_{595} = 0.4-0.6. Cells were pelleted by centrifugation at 4500x *g* for 5 min at 4°C. The pellet was resuspended in 100 mL ice-cold filter sterile TFB1 (30mM Kac, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% (v/v) glycerol, adjusted to pH 5.8 with 1M acetic acid). The solution was incubated on ice for 5 min. Cells were pelleted as before and

resuspended in 10 mL of ice-cold filter-sterile TFB2 solution (1mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% (v/v) glycerol, adjusted to pH 6.5 with 1M KOH), incubated on ice for 1 hour. 200 µL aliquots of cells were snap frozen in liquid nitrogen or dry ice/isopropanol and stored at -80°C.

2.3.2. Transformation of Competent Cells

10ng of plasmid DNA or ligation products were incubated on ice. Competent cells were thawed on ice and 50 µL added to the DNA. Incubation was continued for 15-20 minutes. Cells were heat-shocked at 42°C for 1 minute and 150 µL LB medium added. Cells were incubated in a shaking incubator at 37°C for 45-60 minutes and spread on a pre-warmed LB agar plate containing ampicillin. Plates were incubated overnight at 37°C.

2.4. Molecular Biology Techniques

2.4.1. Buffers and solutions

TE: 10mM Tris-HCl pH 8.0, 1mM EDTA

1 X TAE: 40mM Tris, 40mM acetic acid, 1mM EDTA, pH 8.0.

1 X TBE: 89mM Tris base, 89mM boric acid, 2.5mM EDTA, pH 8.0.

5 X TBE loading buffer: 50% (v/v) glycerol, 200mM Tris, 200mM acetic acid, 5mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol.

DNA sequencing formamide loading dye: 100% deionised formamide, 0.1%(w/v) xylene cyanol FF, 0.1%(w/v) bromophenol blue, 1mM EDTA.

2.4.2 Plasmids Used

The vector pcluc-MS2x3 was kindly supplied by Dr. Mark Stoneley, University of Leicester. pSilencer31, pSilencer-31M, pcDNA3.1-eIF4G1f-WT, pcDNA3.1-eIF4G1f-M, pcDNA3.1-eIF4G1f-eIF4E were the kind gift of Dr. M. J. Coldwell (University of Southampton) and Dr. S. J. Morley (University of Sussex). The construct pSL-MS2-12X was a kind gift from Prof. Robert H. Singer, Albert Einstein College of Medicine, Yeshiva University. Flag-MS2 mammalian expression vector was a kind gift from Dr A. Marcello, Laboratory of Molecular Biology, ICGEB, Trieste, Italy. pGL3-control vector was purchased from Promega.

2.4.3 Purification of Nucleic Acids

2.4.3.1. Determination of Nucleic Acid Concentrations

The concentration of RNA, DNA and oligonucleotides were determined spectrophotometrically by measuring absorbance at 260nm or alternatively, in the case of DNA, by comparison to a known concentration of DNA separated on an agarose gel.

2.4.3.2. Phenol-Chloroform Extraction

To separate nucleic acids from contaminating proteins, phenol-chloroform extraction was performed. An equal volume of phenol was added to nucleic acid solution, followed by an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 12,000x *g* for 15 minutes. The aqueous phase was removed to a new eppendorf and an equal volume of chloroform:isoamyl alcohol (24:1) added. The mixture was centrifuged at 12,000x *g* for 3 minutes and the aqueous layer removed to a new tube prior to precipitation of the nucleic acid.

2.4.3.3. Ethanol Precipitation of Nucleic Acids

Nucleic acids were precipitated by addition of 0.1 volumes of 3M NaAc, pH 5.2 and 2 volumes of absolute ethanol, incubated on ice for 15 minutes. DNA was pelleted by centrifugation at 12,000x *g* for 15 minutes. The pellet was washed with 70% ethanol to remove excess salt, air-dried and re-suspended in sterile deionised water.

2.4.4.1 Agarose Gel Electrophoresis

Electrophoresis through agarose gels was carried out to separate nucleic acids according to molecular weight. For the majority of manipulations, agarose was melted in 1 X TBE buffer, unless DNA fragments were to be eluted, in which case 1 X TAE gels were prepared. 2 μL of 10 mg/ mL ethidium bromide was added to the melted

agarose gel solution and the gel was cast in a gel tray. Samples were mixed with 5 X TBE loading buffer and loaded into the gel. Gels were run submerged in 1 X TBE or 1 X TAE buffer in a horizontal electrophoresis tank, typically at 100V, and visualised on a UV trans-illuminator.

2.4.4.2 Purification of DNA from Agarose Fragments Using Glass Wool

To obtain purified DNA from Agarose gel fragments, the fragment was placed on top of a small amount of glass wool in a 0.5 mL Eppendorf tube. A hole was made in the bottom of a 0.5 mL Eppendorf using a needle, and this Eppendorf placed into a large 1.5 mL Eppendorf. The lid was closed on the small Eppendorf and the lid was removed from the larger Eppendorf. Following centrifugation at 12,000x *g* 10 min, the supernatant was transferred to a 1.5 mL tube and the 0.5 mL Eppendorf was discarded. The DNA was extracted by phenol-chloroform extraction and ethanol precipitated. This method was used to obtain DNA that was sent for sequencing and as the method of first choice for obtaining DNA to be used in ligation reactions.

2.4.4.3 Purification of DNA from Agarose Gels

DNA fragments were excised from agarose gels and purified using a Qiaquick spin column (Qiagen). 3 volumes of buffer QG were added to the gel slice and the solution melted at 55°C. The gel solution was added to a Qiaquick spin column and bound by centrifugation at 12,000x *g* for 1 minute. DNA fragments were washed with buffer PE by centrifugation and last traces of buffer removed by an additional centrifugation as before. DNA was eluted in 30 µL of sterile deionised water by

incubation on the column for one minute prior to centrifugation. Due to expense, this method was only used to obtain DNA for use in ligation reactions when poor yield was obtained following purification using the glass wool method (section 2.4.4.2).

2.4.5.1 Oligonucleotide Synthesis and Purification

Oligonucleotides (Table 2.2) were synthesised by Promega at a scale of 0.2μM. Concentration determined by absorbance at 260nm (section 2.4.3.1). Oligonucleotides were then resuspended in 100 μL H_2O.

Name	Sequence
Primers Used For Cloning	
rpS6 F	5' - CGTTCAGGTGCTTCAAGATG - 3'
rpS6 RV	5' - GGAAAGTCTGCGTCTCTTCG - 3
3xMS2 F	5' - TAGGTGACACTATAGAATAGGGCCC - 3'
3xMS2 RV	5' - GGAATTCTAGAGATATCGTACACC - 3'
Primers Used For Mutagenesis	
Mout F	5' - ATCGATAGGTACCGAGCTCTT - 3'
Mout RV	5' - AACGGACATTTCGAAGTACTCA - 3'
Mtrct F	5' - AGCGGAAGTTGGCCGACTTTTCCGTGGCGG - 3'
Mtrct RV	5' - GCGCCACGGAAAAGTCGGCCAATTCCGCT - 3'
Mmir F	5' - CGCCTCGGTCCCGTTCAGCT - 3'
MmirRV	5' - AGCTGAACGGGACCGAGGCG - 3'
Primers Used For Quantification of 5' mRNA End Using qPCR	
qRLM all F	5' - CGTTTGCTGGCTTTGATGAA - 3'
qRLM top F	5' - TTGCTGGCTTTGATGAAACCT - 3'
qRLM mut F	5' - TGCTGGCTTTGATGAAACGA - 3'
qRLM top F *	5' - GTTTGCTGGCTTTGATGAAACTC - 3'
qRLM mut F *	5' - CGTTTGCTGGCTTTGATGAAAGA - 3'
Primers Used For PCR Prior To T7 RNA Transcription	
T7 F	5' - TAATACGACTCACTATAGGGAAGTTGGCCC - 3'
Control RV	5' - GCGGCCGTTACTAGTGGAT - 3'
MS2 RV	5' - ATGTATCTTATCATGTCTG - 3'

Table 2.2 Oligonucleotides used for Cloning and Sequencing

2.4.5.2 Polymerase Chain Reaction (PCR)

PCR reactions were performed using 100 ng of template in a reaction containing 1X KOD buffer 1 (Novagen), 125 ng of each primer, 0.2mM dNTPs, 1M Betaine, 2% (v/v) DMSO, 2 mM $mgCl_2$ and 2 units KOD DNA polymerase (Novagen). Reactions were performed in a Techne TC512 Temperature Gradient thermal cylinder. DNA was denatured at 94°C for three minutes and then subjected to cycling by denaturing at 94°C for 1 minute, annealing at a gradient of the oligonucleotide melting temperature +/- 5°C for 2 minutes and extending at 72°C for 1 minute. The reaction was cycled for 35 cycles and finally extended for 10 minutes at 72°C.

2.4.5.3 PCR Mutagenesis

Mutagenic PCRs were carried out by performing two half-reactions using the standard PCR procedure (section 2.4.5.2). The regions 5' and 3' of the mutation to be introduced into the rpS6 5' UTR were amplified using the primer pairs Mout F/ Mtrct RV and Mtrct F/ Mout RV. PCR products were gel isolated and 1 µL of a 1/500 dilution of each product was used as the template for a PCR reaction using the primer pair Mout F/ Mout RV. The mutant rpS6 5' UTR produced was digested with *Sac*I and *BstB*I and ligated into the vector pRF.

2.4.6 Restriction Enzyme Digestion

DNA was digested with restriction enzymes using buffers and quantities recommended by the manufacturers. The reaction volume was typically 10-50 µL and

the reaction mixture was incubated at the recommended temperature for 1-2 hours. For digests with restriction sites occurring close to one another or requiring different buffers, the DNA was incubated with one enzyme, the enzyme deactivated by heat inactivation at 65°C for 10 min and the DNA extracted by phenol-chloroform extraction, ethanol precipitated and incubated with the second enzyme. Following the digestion period, the restriction enzymes were deactivated as before.

2.4.7 Alkaline Phosphatase Treatment of DNA for Subcloning

Prior to ligation, linearised plasmids were treated with calf intestinal alkaline phosphatase (CIAP) to remove the 5' phosphate in order to prevent re-annealing of the plasmid vector. Dephosphorylation was performed in a final reaction volume of 50 µL with 1 unit of CIAP and 1 X CIAP buffer (MBI Fermentas). The reaction mixture was incubated at 37°C for 15 min followed by 15 min incubation at 56°C. The reaction was terminated by heat inactivation at 75°C for 10 min.

2.4.8 Ligations

1 µL of restriction digested, phosphatased vector was added to differing concentrations of insert with corresponding sticky ends. 2µL "Ligafast"™ rapid ligation buffer (Promega) and 2.5 units of T4 DNA ligase (Promega) were added to a final volume of 10 µL with sterile water. Ligations were incubated at 16°C for 3-16 hours. Reactions were terminated by heat inactivation at 65°C for 10 minutes. 5 µL of the ligation reaction mixture was used to transform competent *E.coli*.

2.4.9 Fill-in of Recessed 3'-termini of Double-stranded DNA Using Klenow

Restriction digested DNA (0.1-4μg) was dissolved in 10-15 μL of water. Klenow fragment (1μL), 2mM dNTP mixture (0.5μL) and 10x Klenow Reaction Buffer (2μL) were added to the dissolved DNA. The volume was made up to 20 μL with water, and the mixture incubated at 37°C for 10 minutes. The reaction was stopped by heating at 70°C for 10 minutes. The DNA was purified as necessary.

2.4.10 Preparation of Plasmid DNA

2.4.10.1 Mini-preparations

A single colony of *E.coli* transformed with plasmid DNA was grown in 5 mL of LB medium containing ampicillin for 12-16 hours in a 37°C shaking incubator. 1.5 mL was subsequently pelleted by centrifugation and the pellet resuspended in 100 μL of ice-cold solution I (25mM Tris-HCl, 10mM EDTA, 50mM glucose, pH 8.0). The mixture was incubated for 5 minutes at room temperature after which 200 μL of fresh solution II (1% (w/v) SDS, 0.2M NaOH) was added and the solution mixed gently prior to incubation on ice for 5 minutes. 150 μL of solution III (7.5M NH_4Ac, pH 7.6) was added and the mixture vortexed before incubation on ice for 5 minutes. Precipitated cell debris was pelleted by centrifugation at 12,000x *g* for 5 minutes and the supernatant removed to a fresh tube. Plasmid DNA was ethanol precipitated, washed in 70% ethanol, air-dried and re-suspended in 30 μL sterile deionised water. 5 μL of prepared plasmid DNA was digested with restriction enzymes to identify successful clones.

2.4.10.2 Maxi-preparations

To prepare larger quantities of plasmid DNA, a 5 mL culture of *E.coli* transformed with plasmid DNA was inoculated into 250 mL of LB plus ampicillin medium and grown for 12-16 hours in a 37°C shaking incubator. Cells were subsequently pelleted by centrifugation at 5,000x *g* for 10 minutes at 4°C. The pellet was re-suspended in 6 mL of ice-cold solution I and incubated at room temperature for 5 minutes. 12 mL of solution II was added and the mixture incubated on ice for 10 minutes. Neutralisation was carried out by addition of 9 mL of 7.5M NH_4Ac, pH 7.6 with a further 10-minute incubation on ice. Cell debris was pelleted by centrifugation at 10,000x *g* for 10 minutes at 4°C and the supernatant removed to a new tube. The solution was incubated with 0.6 volumes of isopropanol for 10 minutes at room temperature and the pellet resuspended in 2M NH_4Ac, pH 7.4. The insoluble matter was pelleted and the supernatant removed to a fresh tube upon which an equal volume of isopropanol was added and the solution incubated at room temperature for 10 minutes. Plasmid DNA was then pelleted by centrifugation as before and resuspended in 1 mL of sterile water. RNA was removed by addition of 100 µg of RNase A and incubation for 15 minutes at 37°C. Contaminating proteins were removed by precipitation with 0.5 volumes of 7.5M NH_4Ac, pH 7.6 at room temperature for 5 minutes and pelleted by centrifugation. The supernatant was removed to a fresh tube and plasmid DNA was precipitated with an equal volume of isopropanol and centrifugation as before. The pellet was washed with 70% ethanol, air-dried and resuspended in 0.5 mL sterile deionised water.

2.4.10.3 Preparations by Qiagen/Wizard® Columns

To produce plasmid DNA suitable for transfection, the Wizard® plasmid purification kit (Promega) was used to produce up to 50 μg of DNA. Alternatively, the Qiagen midi/mega kit was used to produce larger quantities of plasmid DNA according to the manufacturer's instructions.

2.5 RNA Methods

All solutions used for RNA methods were purified by passage through a 0.2μM filter prior to use, unless purchased sterile and used only for RNA purposes.

2.5.1 *In vitro* Run-off Transcription

Template DNA was linearised from the appropriate plasmid by restriction digestion using a site downstream of the region of interest. Uncapped transcripts were synthesised in a reaction mixture containing 1 X transcription buffer (MBI Fermentas), 40 U RNAguard or RNasin, 1mM ATP, 1mM UTP, 1mM CTP, 1mM GTP, 1 μg DNA template and 20 U T7 or T3 RNA polymerase to a final volume of 50 μL. The reaction mixture was incubated at 37°C for 1 hour, following which a further 10 units of RNA polymerase were added and the incubation continued for 30 minutes. The DNA template was digested with 10 units of RNase-free DNase I for 15 min at 37°C. The RNA was purified by phenol/chloroform extraction and passed through a Sephadex G-50 column (Amersham) to remove unincorporated nucleotide. The RNA was ethanol precipitated and resuspended in 50 μL of sterile deionised

water. 5 µL of the RNA was subjected to agarose gel electrophoresis to ensure the integrity of the product.

For radiolabelled RNAs, CTP was replaced with 50 µCi [α-^{32}P]CTP. For electrophoretic mobility shift assays, with the exception of the inclusion of radiolabelled nucleotide and a decrease of unlabelled CTP to a final concentration of 0.5mM, the reaction mixture was identical to that described for unlabelled transcripts.

2.5.2 Folding RNA

5 µg RNA was combined with 5 µL standard structure probing buffer, SSPB (100mM Tris-HCl pH 7.0, 1M KCl). 5 µL of 100mM MgOAc was added and the mixture brought to 50 µL with sterile deionised water. The RNA was heated to 80°C for 3 minutes then cooled to 4°C over a one-hour period in a PCR machine. The mixture was then incubated at 0°C for 10 minutes to allow structural equilibrium.

2.6. Biochemical techniques

2.6.1. Buffers and solutions

1 X SDS sample buffer: 50mM Tris pH 6.8, 10% (v/v) glycerol, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) β-mercaptoethanol, 1mM EDTA

SDS-PAGE resolving buffer: 1.5M Tris, 0.24% (v/v) TEMED, 1% (v/v) SDS pH 8.8

SDS-PAGE stacking buffer: 0.25M Tris, 0.12% (v/v) TEMED, 0.2% (v/v) SDS pH 6.8

1 X SDS running buffer: 25mM Tris, 192mM glycine, 0.1% (v/v) SDS pH 8.3

TBST (Tris buffered saline, Tween): 10mM Tris pH 8.0, 0.9% (w/v) NaCl, 0.1% (v/v) Tween

Destaining solution: 5:1:5 methanol: acetic acid: water

Protease inhibitors: 19 μg/ mL Aprotinin, 1 μg/ mL Leupeptin, 1 μg/ mL TLCK, 20 μg/ mL PMSF, 20 μg/ mL pepstatin

2.6.2 Harvesting Lysates from Transfected Cells

After transfection, the medium was aspirated and cells washed twice with phosphate buffered saline (PBS). Lysates were prepared by incubating each well of a 6-well plate of cells with 200 μL of 1 X passive lysis Buffer (Promega) for five minutes (50 μL of passive lysis buffer was added per well of a 24-well plate). The cells were subjected to a freeze-thaw cycle at -20°C to disrupt cell membranes and the wells scraped with a cell scraper. Cells were removed to a tube, insoluble matter pelleted by centrifugation and the supernatant removed to a fresh tube. In cells that had been subjected to cell stress/ induction of apoptosis, detached cells were harvested from the aspirated medium by centrifugation and combined with the cell lysate from the same well.

2.6.3 Luciferase Assays

5 μL of lysate from transfected cells was used to assay enzyme activity. In the case of monocistronic constructs, the luciferase reporter assay system (Promega) was

used. 25 μL of luciferase assay reagent was added to the lysate and light emission measured over 10s using an Optocomp I luminometer (MGM Instruments).

For cells co-transfected with vectors expressing both *firefly* and *Renilla* luciferase, activities of cell lysates were measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions with the exception that only 25 μL of each reagent was used. Light emission was measured as before.

2.6.4 β-galactosidase Assays

β-galactosidase activity produced from the transfection control plasmid pJ7*lac*Z in cell lysates was measured using a Galactolight Plus assay system (Tropix). 5 μL of cell lysate was added to 25 μL of Galacton Plus reaction buffer (Galacton-Plus substrate diluted 1:100 with reaction buffer diluent) and the reactions incubated at room temperature for 1 hour. 37.5 μL of Light Emission Accelerator II was added and enzyme activity measured immediately by light emission over 10s using a luminometer as described previously.

2.6.5 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast and run in a Bio-Rad Protean II system. Depending on resolution required, the following gel mixtures were prepared:

	Resolving gel		Stacking gel
%	10%	12.5%	6%
distilled water	2.1 mL	1.7 mL	1.8 mL
resolving/stacking buffer	1.25 mL	1.25 mL	2.5 mL
30%:0.8% acrylamide:bisacylamide	1.67 mL	2.1 mL	0.67 mL
25% ammonium persulphate	50 µL	50 µL	50 µL
Tetramethylethylenediamine (TEMED)	20 µL	20 µL	20 µL

Table 2.3 Recipes for SDS-polyacrylamide gels

25% ammonium persulphate and TEMED were added last to polymerise the gel. Samples were denatured prior to loading by boiling at 95°C for 5 minutes. Gels were run in SDS running buffer at a constant voltage of 150V for approximately 1-2 hours.

To analyse endogenous protein levels, cell lysates were prepared in 1 x SDS buffer supplemented with protease inhibitors and solubilised by passage through a 25G needle. Protein extracts were heated to 95°C for 2 min prior to loading and

analysed by SDS-polyacrylamide gel electrophoresis (PAGE) as described above. Gels were typically run at a constant voltage of 150V in SDS running buffer until the bromophenol blue dye front reached the bottom of the gel, typically between 1-2 hours.

2.6.6 Transfer of Proteins onto Nitrocellulose Membranes.

Gels were electroblotted and probed as described previously (West *et al.*, 1998). Briefly, cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell) by semi-dry blotting in transfer buffer (50mM Tris, 192mM glycine, 20% (v/v) methanol) for between 30 and 90 min at 10V. Protein transfer was confirmed by visualising with Ponceau-S solution (0.5% w/v in 5% w/v trichloroacetic acid [TCA]).

2.6.7 Western blotting / Immunodetection

Proteins immobilized on nitrocellulose membranes (Section 2.6.6) were detected immunologically using antibodies to PTB, hnRNP-K, eIF4G, eIF4E, eIF4B, AGO2, actin or anti-Flag-M2. Binding at non-specific sites was first blocked by incubation of the nitrocellulose membranes in a 5% dried milk in TBST solution for approximately 1 hour at room temperature, with agitation. The blocking solution was aspirated and replaced with 10-20 mL of appropriately diluted primary antibody (Section 2.1.2) in 5% milk TBST solution and incubated overnight at 4°C with agitation. Excess antibody was removed by washing for 10 minutes with TBST, repeated 3 times. Membranes were incubated with peroxidase-conjugated secondary

antibodies raised against mouse immunoglobulins (Dako A/S) or rabbit immunoglobulins (Sigma) diluted 1:2000 and 1: 10,000 respctively, for 1 hour at room temperature with agitation. Excess secondary antibody was removed by washing for 10 minutes with TBST, repeated 3 times. Protein-antibody complexes were detected by enhanced chemiluminescence (ECL) according to a recipe kindly provided by Professor Ken Siddle (University of Cambridge). 1 mL of luminol solution (50 mg luminol [5-amino-2,3-dihydro-1,4-phyhalazinedione] in 0.1M Tris-HCl pH 8.6), 10 μL enhancer (11 mg para-coumaric acid in 10 mL DMSO) and 3.1 μL 3% hydrogen peroxide were mixed and applied to the membrane for 60s with agitation.

2.6.8 Stripping and Re-probing of Western Blots

Nitrocellulose membranes were stripped of existing protein-antibody interactions by incubation in a solution of 100mM β-mercaptoethanol, 2% (v/v) SDS and 62.5mM Tris-HCl pH 6.7 for 10 min at 50°C. Membranes were washed in TBST for 15 min at room temperature with agitation. The membranes were then re-probed with a different antibody.

2.6.9 Measuring Protein Synthesis Rates by Incorporation of ^{35}S Methionine

Approximately 1 X 10^5 cells were seeded into each well of a 6-well plate and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The media was aspirated and the cells washed 3 X with PBS before adding 1 mL of methionine-free media supplemented with 10% (v/v) dialysed fetal calf serum. 10 μCi of ^{35}S-

Methionine was added to each well, in addition to the appropriate concentration of vincristine or cisplatin, as denoted in the text, as required. For concentration-dependence experiments, cells were incubated for a further 24 hours. Cells were harvested by washing 3 X with PBS, scraped using a cell scraper and resuspended in 1 mL of 10% TCA. Cell suspensions were stored at -80°C. To assay methionine incorporation, cell suspensions were filtered onto Whatman glass fibre filter paper circles, washed thoroughly with ice-cold 10% TCA and air-dried. Dried papers were placed in scintillation vials with 5 mL scintillation fluid and methionine incorporation was measured by counting for 1 minute using a scintillation counter.

2.6.10 Affinity Purification of mRNA Containing MS2 Binding Sites Using Flag-MS2 Protein and Anti-Flag M2 Agarose Beads

Lysis of HEK-293T cells was carried out using a modified lysis buffer (Tris-HCl pH 8.0, 50 mM; Igepal (Sigma) 0.5%; NaCl 150 mM; EDTA 1 mM; $MgCl_2$ 5 mM; protease inhibitor mix (PIM) (Roche); DTT 1 mM) and by passing through a 27 gauge needle. In order to reduce background non-specific binding, the lysate was first incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotech), supplemented with normal mouse serum (Santa Cruz Biotech). The cleared lysate was then incubated for 4 hours at 4°C with anti-Flag-M2 affinity resin. The anti-Flag-M2 affinity resin was then washed three times with wash buffer (Tris-HCl pH 8.0, 50 mM; NaCl 150 mM; EDTA 1 mM; $MgCl_2$ 5 mM; protease inhibitor mix (PIM) (Roche); DTT 1 mM). Samples were eluted either using TRI reagent or SDS loading buffer (40 µl). For protein analysis experiments, samples were eluted in SDS loading buffer. The fractions obtained from the three successive washes, were combined and

subjected to TCA precipitation. Following centrifugation, the protein pellets obtained were also re-suspended in SDS loading buffer (40 µl).

2.6.11 Microarray Analysis of Micro-RNAs Associated with Affinity Purified Reporter mRNAs

Following RNA extraction using TRI reagent (Sigma), the concentration of both total and affinity purified mRNA was obtained by UV-Vis spectroscopy. A Genisphere FlashTag microarray labelling kit was used to label RNA, in accordance with the manufacturer's instructions. Total RNA (2.5 µg) was labelled using Cy5 fluorescent dye (~650 nm excitation /670 nm emission) and affinity purified RNA was labelled using Cy3 dye (~550/570 nm). Labelled samples were denatured, loaded microRNA microarray chips (obtained from Dr T.W. Gant, MRC Toxicology Unit, University of Leicester) prior to hybridisation at 45°C for 16 hours. Following hybridisation, the chips were scanned using an Axon Genepix 4000b scanner and a grid containing probe definitions was assigned by using the gridding algorithm within the Genepix Pro software (Molecular Devices). The algorithm assigned grid was visually verified and assignments corrected as necessary. The fluorescence intensities of the Cy5 and Cy3 dyes and the respective background were then recorded in preparation for data analysis.

Chapter Three

Construction of a 5' terminal oligopyrimidine tract (TOP) Containing mRNA Luciferase reporter system

3.1 Introduction

A 5' terminal oligopyrimidine tract (TOP) containing mRNA is defined in terms of its sequence by the presence of a C residue immediately preceding the 5' terminal m^7G cap structure of the mRNA and an uninterrupted 4-14 nucleotide polypyrimidine tract (Meyuhas 2000; Hamilton et al. 2006). Transcription initiation at a C residue occurs in 17% of eukaryotic mRNAs, although it has yet to be determined what proportion of these mRNAs also possess a 5' terminal oligopyrimidine tract (Meyuhas 2000). Despite this, many individual mRNAs have been identified which contain the 5'TOP motif, including those which encode proteins involved in ribosome biogenesis. The mRNAs encoding Ribosomal Proteins (RPs), which comprise a significant proportion of this group, were the first TOP containing mRNAs to be identified in vertebrates (Schibler et al. 1977; Geyer et al. 1982). Experimentally, sucrose density gradient analysis has been used to determine the translational characteristics of this group of mRNAs. Two distinct translational modes have been identified, which operate in a growth dependent manner. In growing cells, TOP containing mRNAs are loaded with a full complement of ribosomes and are, therefore, not only part of the translationally active population of mRNAs, but are also translated with full efficiency. In growth arrested cells however, TOP containing mRNAs are entirely shifted into the sub-polysomal, non-translating population (Loreni and Amaldi 1992). This distinct growth-associated bimodal distribution is a characteristic unique to TOP containing mRNAs and has proven dependency on both

the integrity of the *cis*-acting TOP motif and its immediate Cap-proximal position (Avni et al. 1994). The mechanism is common to all TOP containing mRNAs and thereby facilitates a mechanism for their co-ordinated expression. This is particularly important in the aforementioned case of ribosome biogenesis, which accounts for a large proportion of cellular energy expenditure (Warner et al. 2001).

3.2 TOP and Mutated-TOP Constructs

The first aim of this project was to construct a model, which accurately reproduces the structural and translational characteristics of TOP containing mRNAs. This model can then be manipulated by mutagenesis to deduce the location and length requirements of the TOP motif. Additionally, such a model would present an opportunity for the identification of *trans*-acting, factors, which are specifically required for the growth dependent regulation of TOP containing mRNAs. This chapter describes the development of such a model system using reporter plasmids containing a firefly luciferase open reading frame. Upon transfection, mRNA is encoded *in vivo* facilitating the quantitative measurement of translational efficiency, by the observation of firefly luciferase expression. The use of reporter constructs alleviates the problem associated with exogenous transcription reactions using T3, T7 or Sp6 RNA polymerases, which have low levels of efficiency when transcribing mRNA with a C residue immediately preceding the 5' terminal m^7G cap structure (Milligan and Uhlenbeck 1989).

The pGL3 Control vector, which contains the firefly luciferase open reading frame, was chosen as a starting point for the cloning of a TOP containing construct. A cloning strategy was devised in which the completed construct would be under the

control of a promoter found within an endogenous 5'TOP containing mRNA. By incorporating this promoter and the 5'UTR from the same TOP containing mRNA, the completed construct would be expected to encode mRNA with a C residue immediately succeeding the 5' terminal m^7G cap structure and the characteristic uninterrupted polypyrimidine tract TOP motif. The promoter element and 5' UTR sequence of Ribosomal Protein S6 (rpS6) was chosen as the basis of the TOP construct, since rpS6 mRNA is known to contain the 5'TOP motif and this promoter region has been the focus of prior characterisation and analysis (Antoine and Kiefer 1998).

The full length rpS6 promoter has previously been shown to be approximately 900bp in length and has been shown to include a core 40bp promoter region upstream of the transcription start site and initiation region (Antoine and Kiefer 1998). An initial attempt was made to clone the full length 900bp promoter region and rpS6 5'UTR into the pGL3 control vector. A polymerase chain reaction (PCR) was carried out on cDNA obtained from HeLa cells in order to obtain a DNA fragment. However, despite attempts at optimising the reaction conditions, the PCR was repeatedly unsuccessful. As a result of this failure, the literature relating to the rpS6 promoter was reviewed, in order to ascertain whether a modification to the cloning strategy was possible.

It has previously suggested that different transcription factors from different cell types, may be functionally interchangeable and bind to common regions within the promoter (Antoine and Kiefer 1998). This would ensure high-level expression of housekeeping genes independently of the cell type. An experiment has been reported in which an attempt was made to determine the length of promoter required in order for transcriptional activity to be retained. Reporter constructs were made, with full-

length and truncated rpS6 promoter regions of differing size. The relative activity of these promoter regions was then determined in a variety of cell types (Antoine and Kiefer 1998). This study indicated that a region of 400bp immediately preceding the transaction start site, is the minimum promoter length required to achieve activity in excess of 95%, when compared to the full-length promoter (Antoine and Kiefer 1998). For this reason, the PCR reaction was repeated with a set of primers which had been redesigned to amplify a 400bp promoter region and the rpS6 5'UTR. The primary considerations in this cloning strategy were to maintain a system which is similar to endogenous conditions and to retain an unaltered transcription start site.

The DNA fragment containing the 400bp rpS6 promoter element and 5' UTR, was then cloned into the pGL3 Control vector between the HindIII and the NcoI restriction sites, by restriction digest and ligation. The NcoI site contains the ATG sequence which corresponds to the AUG start codon of the *in vivo* transcribed mRNA, ensuring in-frame cloning of the 5' UTR with the firefly luciferase open reading frame. The simian virus 40 (SV40) promoter was then removed by restriction digest using XhoI and HindIII followed by blunt end ligation. (Figure 3.1.1). Following cloning, sequencing was carried out, which confirmed that the construct contained both the 400bp rpS6 promoter region and 5' UTR, and that the SV40 promoter was no longer present (Figure 3.1.2).

The main considerations when designing a suitable control construct were to maintain an unaltered transcription start site and make only the minimum required modification to disrupt the 5'TOP motif. For this reason the 400bp rpS6 promoter element was maintained along with the 1st nucleotide downstream of the 5' terminal m^7G cap position. Carrying out mutagenesis at the 1st nucleotide downstream of the 5' terminal m^7G cap could potentially disrupts the transcription start site, thus

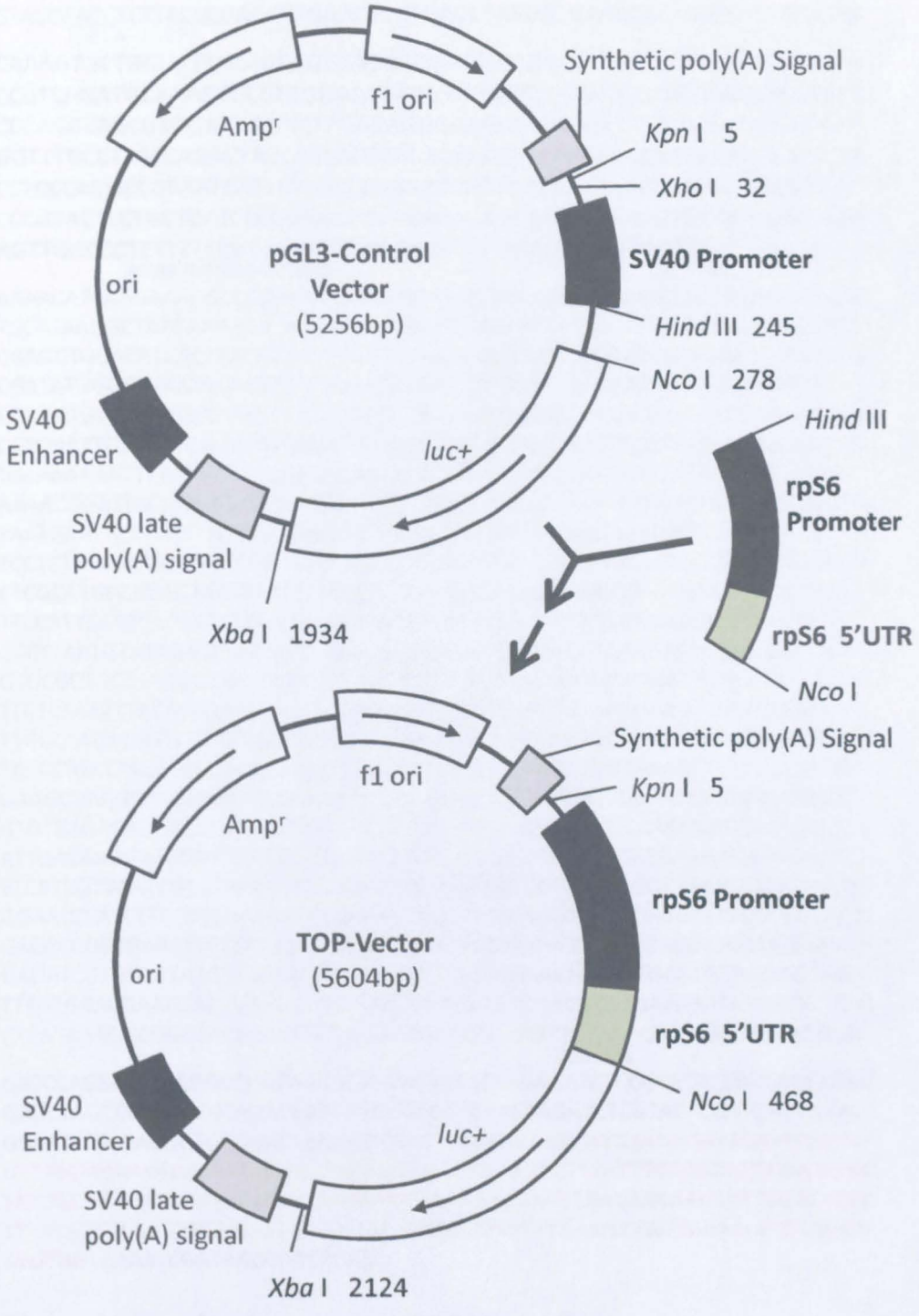


Figure 3.1.1: Construction of TOP Vector

Step 1: The PCR fragment containing the rpS6 promoter and 5'UTR was digested with *Nco*I and *Hind*III. This fragment was ligated into the pGL3 vector, which had been digested with *Nco*I and *Hind*III and dephosphorylated.

Step 2: The vector was digested with Hind III and *Xho*I. Blunt end ligation was carried out to produce the pGL3-rpS6 construct.

Step 3: A final cloning step was carried out to obtain the TOP construct (refer to Section 5.2.1 & Figure 5.1.1 for more details).

GTACCGAGCTCTTACGCGAGCTTGAACTCCTGACCTAAGATGATCCGCCCGCCTCGGCCTCC
Kpn I 5
CAAAGTGCTGGGATTACAGGCGTGACGACCGCGCCCGGCCTTAGCCCTCCTATTTTGGTA
CCGTCAGATGCAAAGTGCCTGGGACAGAAGTGGGGCTCCGCTGGCGCCCAGCTCCAAAA
CCCAGGCAGCGTGGAAAAGACTAGACAGGGAAGGGGTTAGCCCTCAGAATTACACGCG
GGTTTGCCTTACCAGACTACCACCACTGGCACAACCTCAGACCCACACCCAACCGACTTTA
CCTCCCAGGCCCTGATTAATCTCGCCCGGAAGTACCGCCCACCCATGCTCACTTCCGCTAT
CCCGTACTTCTGCTCATCTCGCGAGAACTGAAAGCGCCTATGTGACCTGCGCTAAGCGGA
AGTTGGCCCTCTTTTCCGTGGCGCCTCGGAGGCGTTCAGCTGCTTCACCATGGAAGACGCCA
AG for TOP-trctMut vector *Nco* I 468
AAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAAC
TGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATAT
CGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCGGTTGGCAGAAGCTATGAAA
CGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTAT
GCCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGAA
CGTGAATTGCTCAACAGTATGGGCATTTCGCAGCCTACCGTGGTGTTCGTTTCCAAAAAGGGGT
TGCAAAAAATTTTGAACGTGCAAAAAAAGCTCCCAATCATCCAAAAAATTATTATCATGGATTCT
AAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTACCTCCCGGTTT
TAATGAATACGATTTTGTGCCAGAGTCCTTCGATAGGGACAAGACAATTGCACTGATCATGAAC
TCCTCTGGATCTACTGGTCTGCCTAAAGGTGTCGCTCTGCCTCATAGAACTGCCTGCGTGAGATT
CTCGCATGCCAGAGATCCTATTTTTGGCAATCAAATCATTCCGGATACTGCGATTTTAAGTGTTG
TTCCATTCCATCACGGTTTTGGAATGTTTACTACACTCGGATATTTGATATGTGGATTTCGAGTC
GTCTTAATGTATAGATTTGAAGAAGAGCTGTTTCTGAGGAGCCTTCAGGATTACAAGATTCAAA
GTGCGCTGCTGGTGCCAACCCTATTCTCCTTCTTCGCCAAAAGCACTCTGATTGACAAATACGAT
TTATCTAATTTACACGAAATTGCTTCTGGTGGCGCTCCCCTCTCTAAGGAAGTCGGGGAAGCGG
TTGCCAAGAGGTTCCATCTGCCAGGTATCAGGCAAGGATATGGGCTCACTGAGACTACATCAGC
TATTCTGATTACACCCGAGGGGGATGATAAACCGGGCGCGGTCGGTAAAGTTGTTCCATTTTTT
GAAGCGAAGGTTGTGGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAAAGAGGCGAACT
GTGTGTGAGAGGTCCTATGATTATGTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCTTG
ATTGACAAGGATGGATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTC
TTCATCGTTGACCGCCTGAAGTCTCTGATTAAGTACAAAGGCTATCAGGTGGCTCCCGCTGAATT
GGAATCCATCTTGCTCCAACACCCCAACATCTTCGACGCAGGTGTCGCAGGTCTTCCCGACGAT
GACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAA
GAGATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGCGCGGAGGAGTTGTG
TTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTCGACGCAAGAAAAATCAGAGAGATC
CTCATAAAGGCCAAGAAGGGCGGAAAGATCGCCGTGTAATTCTAGAGTAATACTCTAGGGGG
*Xba*I 2124
GATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTAGAGATATCCGTACACCATCAGGGTAC
GAGCTAGCCCATGGCGTACACCATCAGGGTACGACTAGTAGATCTCGTACACCATCAGGGTAC
GGAATTCTCTAGAGTCGGGGCGGCCGGCCGCTTCGAGCAGACATGATAAGATACATTGATGA
GTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGC
TATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCAT
TTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAA
ATGTGGTAAAATCGATAAGGATCTGAA...

Figure 3.1.2: DNA Sequence of TOP Vector Construct

DNA sequence of TOP vector construct from base-pair 1 (refer to Figure 3.1.1) up to and including the SV40 late poly(A) signal. Vector is unchanged from pGL3-Control vector outside of this region. Mutation in case of TOP-trctMut is indicated.

Key:

Ribosomal Protein S6 promoter - **Purple**
Ribosomal Protein S6 5'UTR - **Cyan**
Firefly Luciferase coding region - **Green**
Start (ATG) and Stop (TAA) codons - **Red**
SV40 late poly(A) signal - **Orange**
Restriction enzyme sites - Underlined

producing mRNA with an entirely different length and 5'UTR. This would not be suitable as a control, construct since any differences observed could not be singularly attributed to disruption of the 5'TOP motif. Similarly, introducing a large number of mutations within the 5'UTR could also result in unforeseen effects such as differences in binding of other non TOP-specific *trans*-acting factors. It was therefore decided that the polypyrimidine tract element of the TOP motif should be disrupted at only two adjacent nucleotides. Mutagenesis was employed at the 2nd and 3rd nucleotide downstream of the 5' terminal m^{7}G cap (Figure 3.2). This mutation pair was chosen because it interrupts the polypyrimidine tract, thus disrupting the integrity of the 5'TOP motif, with the minimum possible modification (Figure 3.3A). The expected consequence of this mutation is the alleviation of the growth-dependent translational control that is unique to TOP containing mRNAs.

To confirm that that new constructs were translationally active, TOP and TOP control (TOP-trctMut) constructs were transfected into HEK-293 cells in conjunction with *Renilla* luciferase expression vector (transfection control). This cell line was chosen because it has previously been used for a large range of experiments to investigate the characteristics of TOP containing mRNAs (reviewed in Meyuhas 2000). The cells were harvested 48 hours after transfection and northern blotting was carried out to detect *actin* and *firefly* (reporter) mRNAs (Figure 3.3B). The cells were grown under control conditions throughout and were not subject to serum starvation or re-feeding. A dual luciferase assay was also carried out and *Firefly* (reporter) activity was normalised using the *Renilla* luciferase transfection control (Figure 3.3C). These data confirmed that both constructs expressed the *Firefly* luciferase gene and were translationally active.

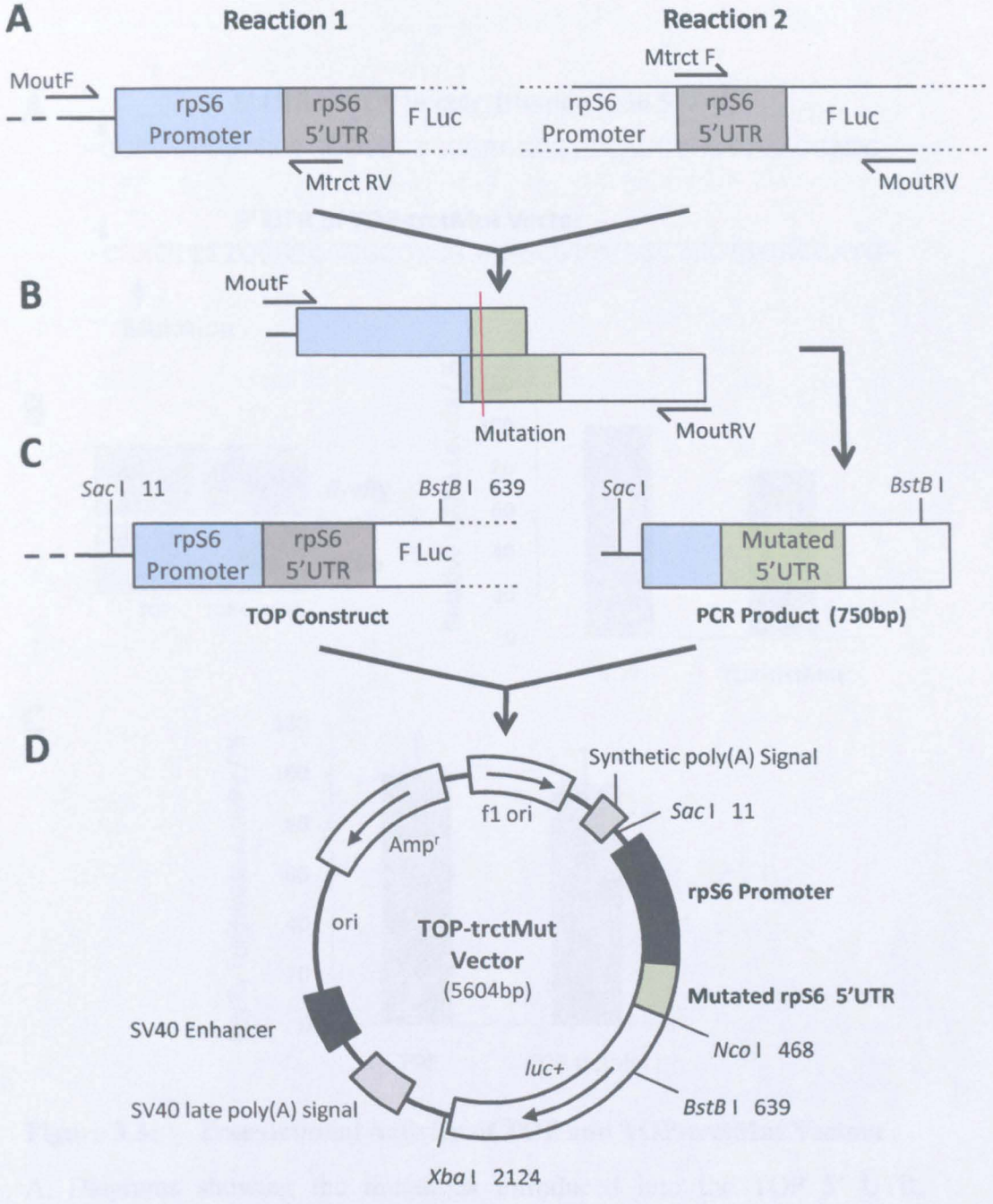


Figure 3.2: Construction of TOP-trctMut Vector

A. Two PCR reactions were carried out to perform the mutation using the TOP vector as a template. Reaction 1 used the MoutF primer and the reverse primer Mtrct RV, which contained the mutated sequence. Reaction 2 used the primer MoutRV and the forward primer Mtrct F, which contained the mutated sequence.

B. A second PCR reaction was carried out, which used the MoutRF and MoutF primers and the amplified fragments as a PCR template, to produce a fragment with a mutated 5'UTR.

C. A restriction digest was carried out on both the TOP vector and PCR fragment, using Sac I and BstB I. The digested TOP vector was then dephosphorylated.

D. The digested TOP vector and PCR fragment were ligated to produce the TOP-trctMut vector which contains two point mutations in the polypyrimidine tract of the 5'UTR.

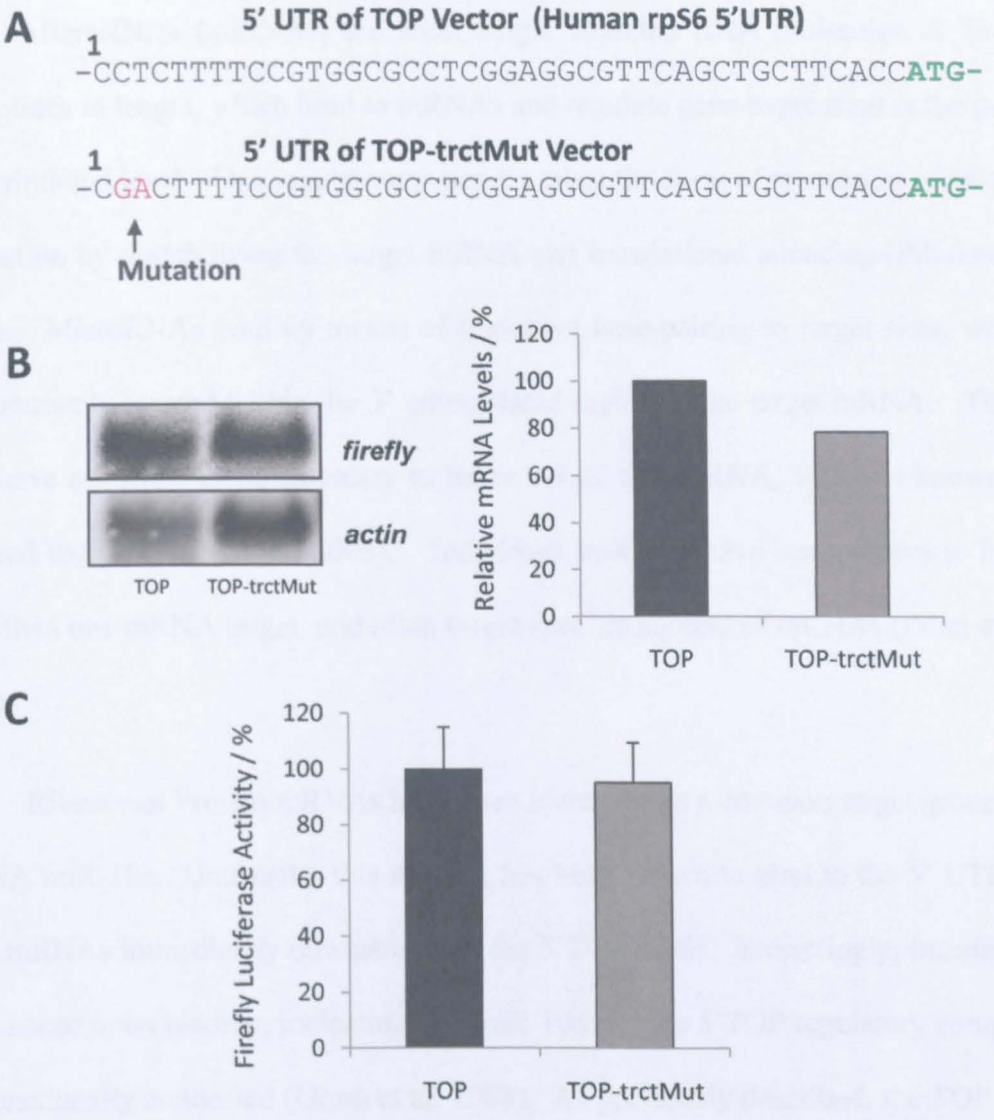


Figure 3.3: Translational Activity of TOP and TOP-trctMut Vectors

A. Diagrams showing the mutations introduced into the TOP 5' UTR. Mutated sequence in the TOP-trctMut vector is shown in orange. The initiation codons of each vector are shown in green.

B. TOP and TOP-trctMut vectors were transfected in triplicate into HEK-293 cells. The cells were harvested 48 hours after transfection and RNA was extracted from each replicate and combined. Northern blotting and phosphor-imaging was carried out and *firefly* mRNA levels from one single experiment were normalised to *actin* mRNA levels.

C. Luciferase activity was recorded in counts s^{-1} from each replicate and normalised to TOP activity (100%). The standard deviation of the three replicates was used to calculate error values.

3.3 MicroRNA-10a and the regulation of TOP and TOP-trctMut mRNAs

MicroRNAs (miRNAs) are short single stranded RNA molecules of 21-23 nucleotides in length, which bind to mRNAs and regulate gene expression at the post-transcriptional level. This regulation generally takes the form of repression of protein production by destabilizing the target mRNA and translational silencing (Pillai et al. 2005). MicroRNAs bind by means of imperfect base-pairing to target sites, which are commonly located within the 3' untranslated region of the target mRNA. These sites have sequence complementary to bases 2-8 of the miRNA, which is known as the seed region (Pillai et al. 2005). Individual miRNAs have been shown to have more than one mRNA target, and often target specific subsets of mRNAs (Pillai et al. 2005).

Ribosomal Protein mRNAs have been identified as a common target group for miRNA miR-10a. Unusually, this miRNA has been shown to bind to the 5' UTR of these mRNAs immediately downstream of the 5'TOP motif. Interestingly, translation is enhanced upon binding, indicating that miR-10a and the 5'TOP regulatory complex are functionally connected (Orom et al. 2008). As previously described, the TOP and TOP-trctMut constructs transcribe mRNAs, which contain the 5' UTR sequence of ribosomal protein S6. For this reason, the sequence of both 5'UTRs was analysed to identify potential miRNA-10a binding sites. A region common to both TOP and TOP-trctMut was found to contain a potential miRNA-10a binding site, with base pairing consistent to that previously predicted for this miRNA (Orom et al. 2008) (Figure 3.4A). Mutagenesis was carried out on three nucleotides within the potential binding site of both constructs but outside of the 5'TOP motif, to facilitate future investigation of the functionality of this site (for details of the method, refer to chapter 2, section 2.4.5.3). Since the potential binding site did not contain a readily

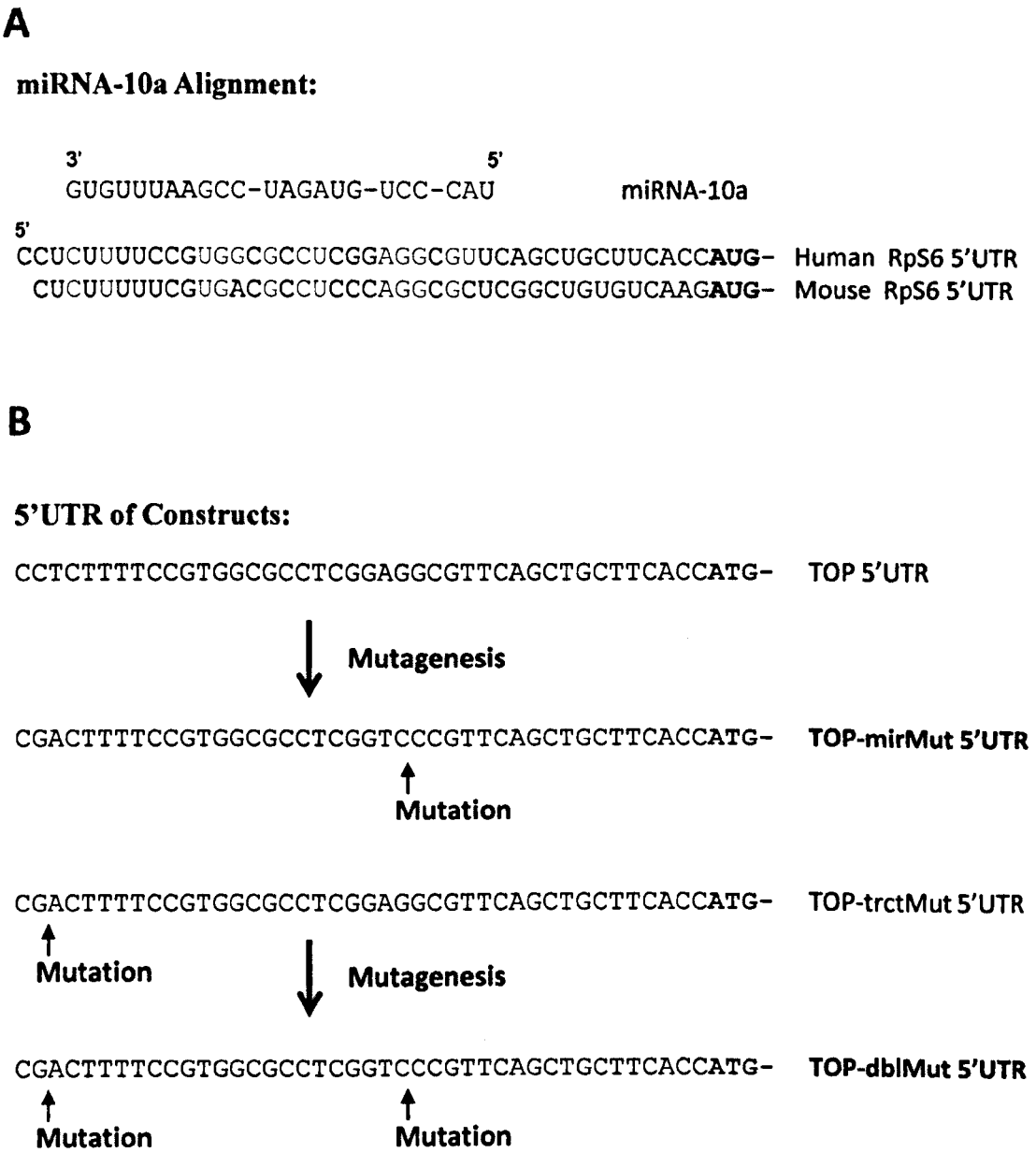


Figure 3.4: Construction of TOP-mirMut and TOP-dblMut Vectors

A. Diagram showing the most probable alignment of miRNA-10a with the 5'UTR of rpS6 mRNA. The aligned sequence is conserved in both Human and Mouse rpS6 mRNA and proposed base-pairing is indicated by purple and blue text.

B. Schematic diagram showing the mutations introduced into the TOP 5'UTR and TOP-trctMut constructs. Mutated sequences in the TOP-mirMut and TOP-dblMut vectors are shown in orange. The initiation codons of each vector are shown in green.

identifiable seed region, the point mutations were carried out at neighbouring positions to produce the longest possible region of non-complementarily. Mutagenesis of the TOP and TOP-trctMut constructs produced the new constructs TOP-mirMut and TOP-dblMut respectively (Figure 3.4B).

To confirm that that new constructs were translationally active, TOP, TOP-trctMut, TOP-mirMut and TOP-dblMut vectors were transfected into HEK-293 cells in conjunction with *Renilla* luciferase expression construct (transfection control). The cells were harvested 48 hours after transfection and northern blotting was carried out to detect *actin* and *firefly* (reporter) mRNAs (Figure 3.5A). Quantification of the northern blots was carried out and the detected *firefly* (reporter) mRNA was normalised to the *actin* mRNA levels. A dual luciferase assay was also carried out and *Firefly* (reporter) activity was normalised using the *Renilla* luciferase transfection control (Figure 3.5C).

These data confirmed that both TOP and TOP-trctMut constructs expressed the *Firefly* luciferase gene and were translationally active. Interesting, the data show that the mRNA levels and luciferase activity obtained from the TOP-mirMut and TOP-dblMut vectors, were approximately 10% of those obtained from the TOP vector. Whilst it is clear that both TOP-mirMut and TOP-dblMut constructs transcribe mRNA, they behave significantly different to the TOP-trctMut which does not possess the miRNA-10a binding site. DNA sequencing of all four constructs confirmed that no mutations were present other than those expected. It is therefore possible that the relatively low mRNA levels and translational activity obtained from the TOP-mirMut and TOP-dblMut constructs, is related to the miRNA-10a binding site mutation. It is possible that this mutation may have resulted in reduced mRNA stability, either as a result of abolition of the potential miRNA-10a binding or through

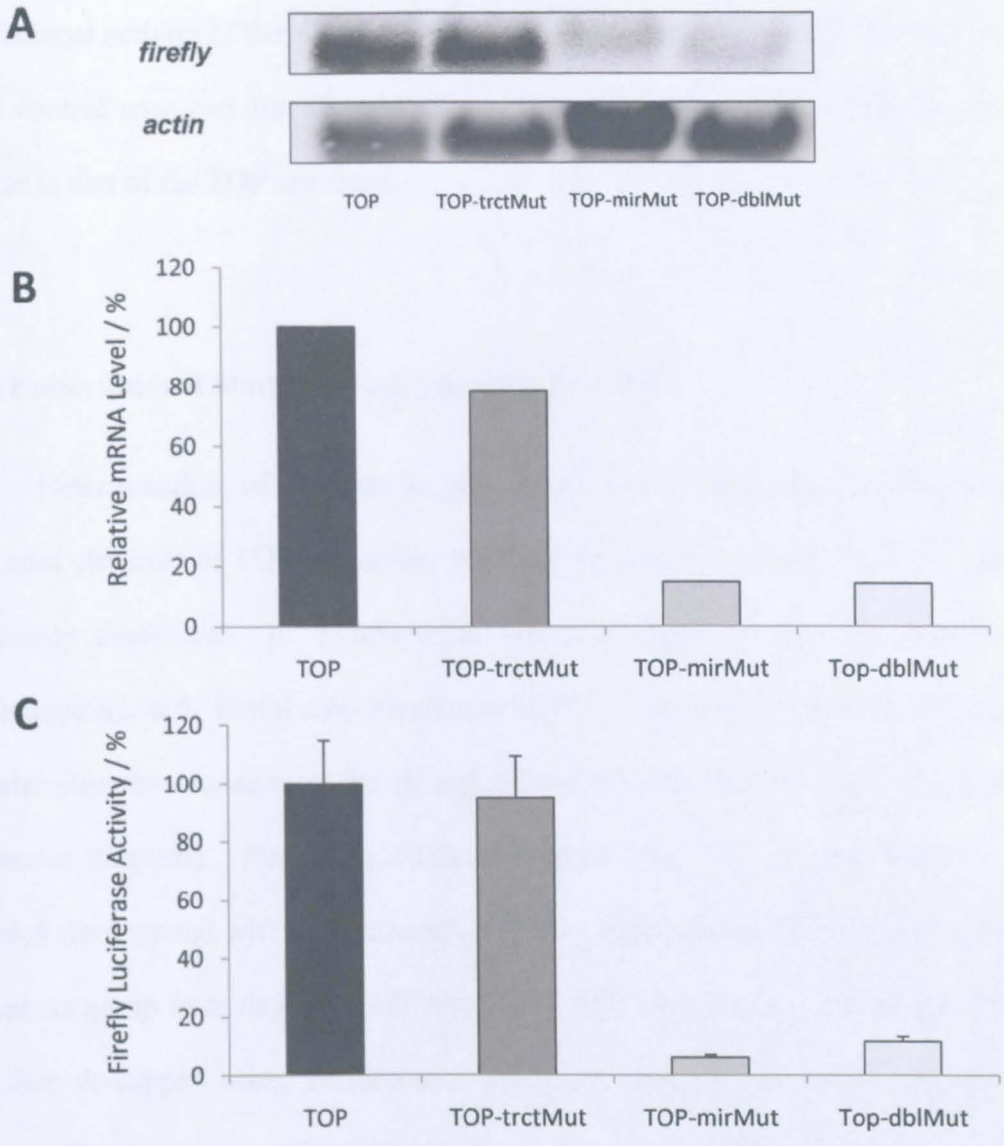


Figure 3.5: Translational Activity of TOP, TOP-trctMut, TOP-mirMut and TOP-dblMut Vectors

A. TOP, TOP-trctMut, TOP-mirMut, and TOP-dblMut vectors were transfected in triplicate into HEK-293 cells. The cells were harvested 48 hours after transfection and RNA was extracted from each replicate and combined. Northern blotting was carried out on the combined extract.

B. Quantification of *firefly* mRNA was carried out following detected by Northern blotting and phosphor-imaging. Values from one single experiment were normalised to *actin* mRNA levels.

C. Luciferase activity was recorded in counts s^{-1} from each replicate and normalised to TOP activity (100%). The standard deviation of the three replicates was used to calculate error values.

a loss of secondary structure within the mRNA. As a result of the relatively low translational activity of these constructs, it was decided to use the TOP-trctMut as the main control construct for all subsequent experiments, since its activity was more similar to that of the TOP construct.

3.4 Transcriptional Start Site Analysis using 5' RACE

Determination of the transcription start site is important, since the key structural elements of TOP containing mRNAs are directly related to their 5' end, as previously described. In collaboration with Dr Mark Coldwell (University of Southampton), a 5' Rapid amplification of cDNA ends (RACE) technique was used to determine the sequence at the 5' end of the mRNA (refer to Figure 3.6.1 for a schematic diagram). Following RNA extraction using TRI reagent (Sigma), the samples were treated with calf intestinal alkaline phosphatase (CIP) to remove the 5'-phosphate group from degraded mRNA, tRNA, rRNA and DNA. Full length mRNA was then de-capped using tobacco acid pyrophosphatase (TAP). A synthetic RNA adapter of known sequence was then ligated to the exposed 5'-phosphate group, which was only present on the full length-de-capped transcripts. Random-primed reverse transcription reactions and nested PCRs were then carried out to amplify a PCR product containing the sequence at the 5'-end of the mRNA transcript (Figure 3.6.2). The PCR products (450bp) were then cloned into pSC-B (Stratagene) and positive clones were sequenced with a T7 primer (Figure 3.6.3A, Figure 3.6.3B). Using the sequencing results, the percentage of transcripts containing the expected TOP and TOP-trctMut 5' UTR sequence was calculated.

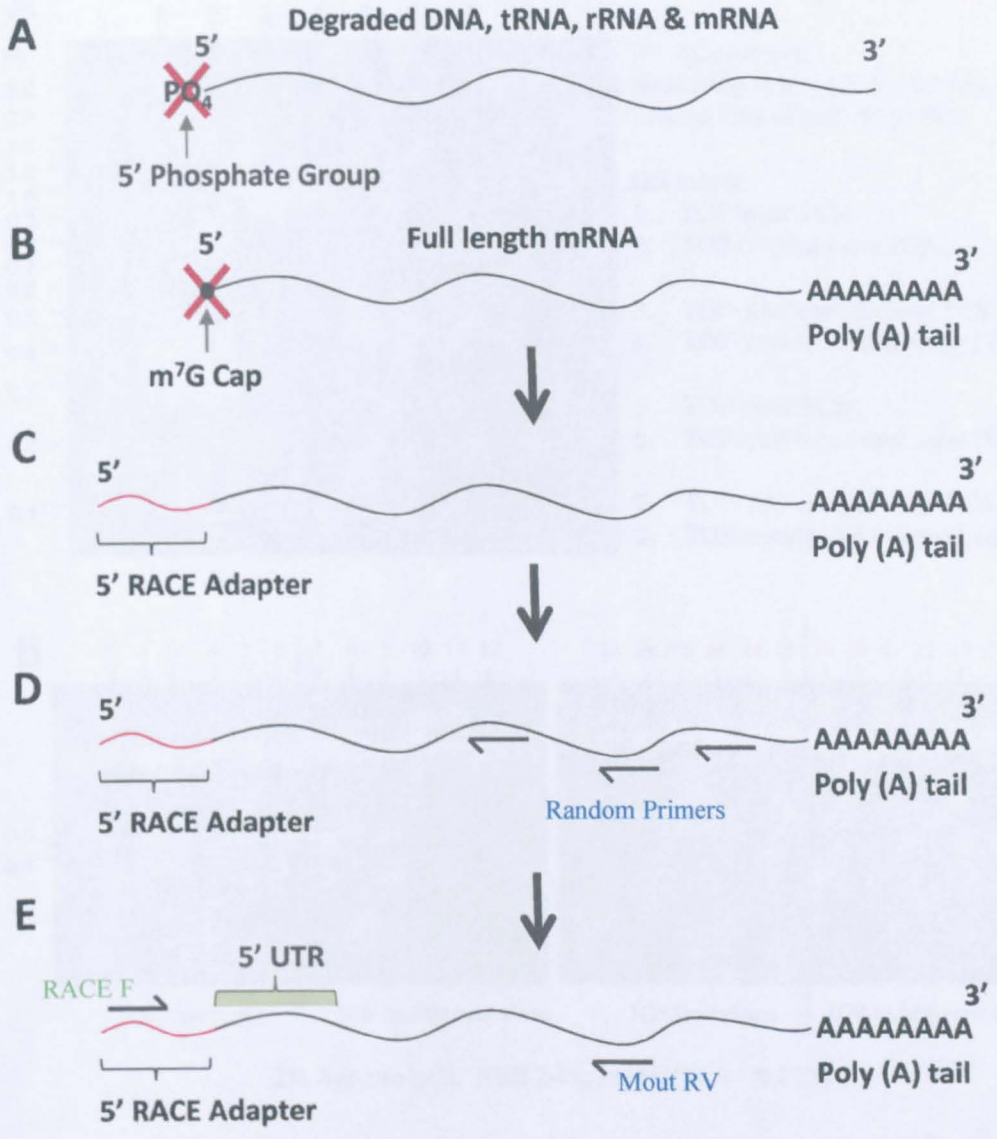


Figure 3.6.1: Schematic Diagram of 5'RACE (5' Rapid amplification of cDNA ends)

A. Following RNA extraction, samples were treated with calf intestinal alkaline phosphatase (CIP) to remove the 5'-phosphate group from degraded mRNA, tRNA, rRNA and DNA.

B. Full length mRNA was de-capped using tobacco acid pyrophosphatase (TAP).

C. A synthetic RNA adapter of known sequence was ligated to the exposed 5'-phosphate group (only present on full length-de-capped transcripts).

D. A random-primed reverse transcription reaction was carried out to obtain cDNA.

E. A PCR was carried out to amplify a product containing the sequence at the 5'-end of the mRNA transcript. (RACE F primer supplied with kit.)

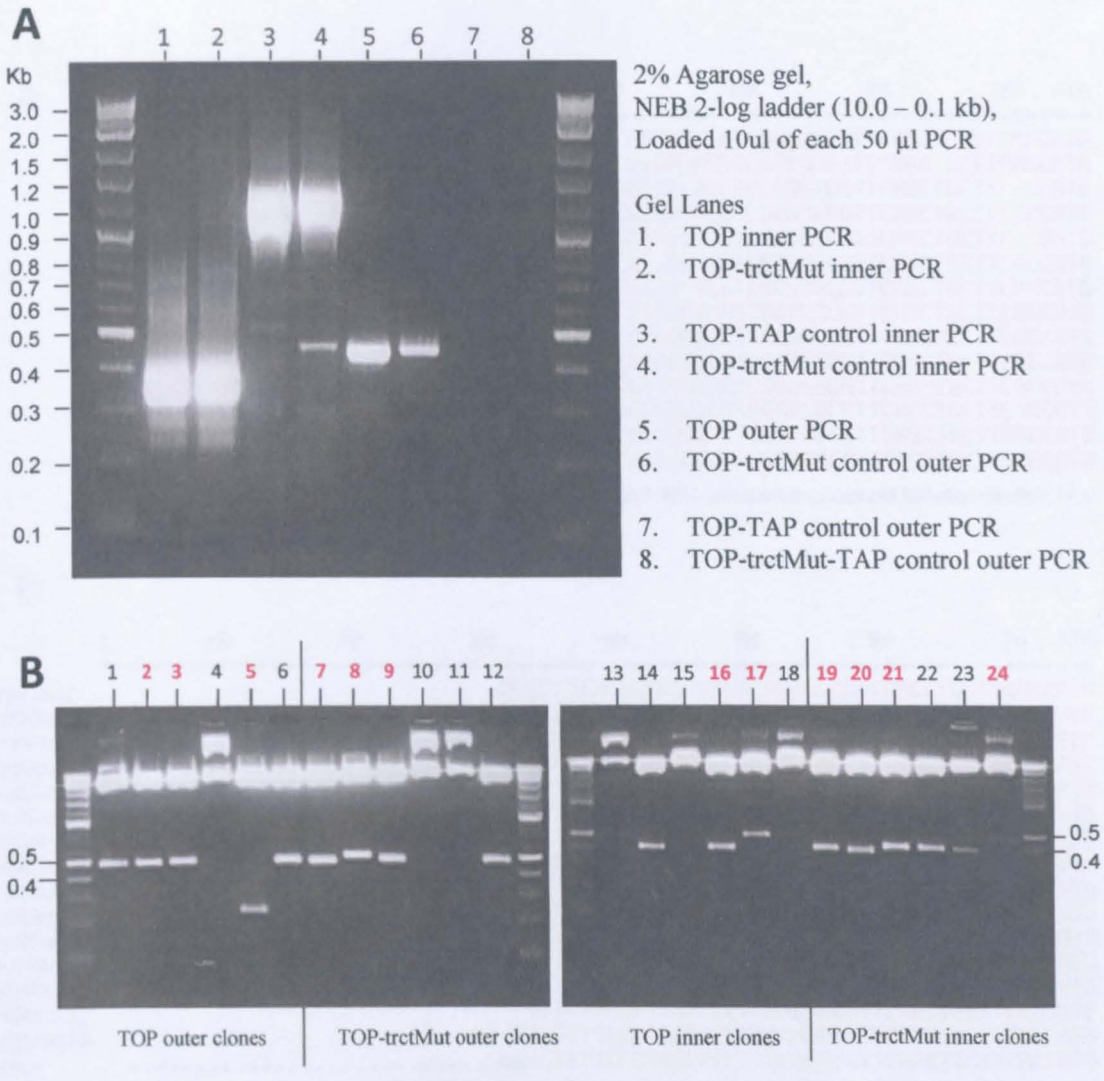


Figure 3.6.2: RLM-RACE PCRs of TOP and TOP-trctMut Vectors

A. 2% Agarose gel of PCR reactions using Ambion RLM-RACE kit. An Improm-II kit (Promega) was used for reverse transcription and Pfu Ultra HS (Stratagene) for amplification. Primers were designed to produce a 450bp product for Outer PCRs and a 350bp PCR product for inner PCR reactions. TAP (tobacco acid pyrophosphatase) PCR reactions using template without ligated adapter produced a negative result, indicating specific priming from within RACE adapter. (NEB 2-log ladder 10.0-0.1kb, 10µl of 50µl PCR reactions were loaded.)

B. Presence of clones in pSC-B (Stratagene) was confirmed by *EcoR*I restriction digest. Positive clones from the lanes labelled in red, were sequenced with a T7 primer. (2% Agarose gels, NEB 2-log ladder 10.0-0.1kb.)

Work carried out in collaboration with Dr. M. Coldwell, University of Southampton

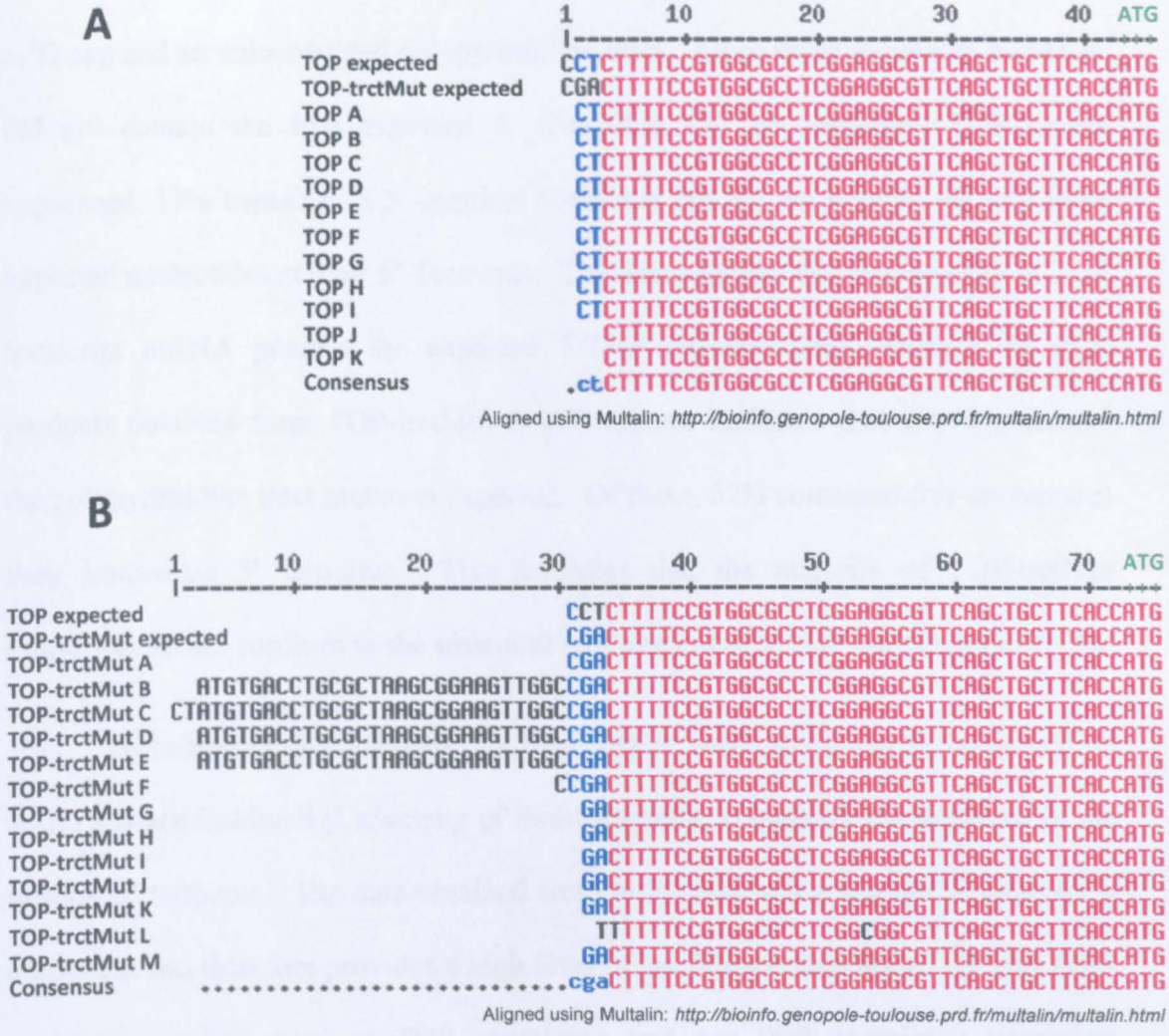


Figure 3.6.3: Sequencing Analysis of TOP and TOP-trctMut Vectors

Positive TOP clones (A) and TOP-trctMut clones (B) were sequenced with a T7 primer. The sequence from the host vector and the RNA linker was removed prior to sequence alignment. Sequence in red is the 5'UTR and ATG start codon downstream of the mutated region. Sequence shown in blue is the expected first nucleotide succeeding the cap structure and/or the correctly mutated (TOP-trctMut) or un-mutated (TOP) second and third nucleotides.

Sequencing data from the PCR products obtained from TOP RACE clones, indicated that 75% contained a C-residue immediately downstream of the 5' terminal m^7G cap and an uninterrupted polypyrimidine tract. All of these sequences however, did not contain the first expected 5' C-residue. Of the remaining TOP clones sequenced, 17% contained a 5' terminal C-residue but did not contain the first three expected nucleotides at their 5' Terminus. The data suggests that the majority of TOP transcript mRNA possess the expected 5'TOP characteristics. Analysis of PCR products obtained from TOP-trctMut RACE clones, indicated that 100% contained the polypyrimidine tract mutation expected. Of these, 62% contained this sequence at their immediate 5' terminus. This indicates that the majority of TOP-trctMut transcripts do not conform to the structural requirement of 5'TOP containing mRNAs.

To independently validate this data, qPCR was carried out in collaboration with Dr Mark Coldwell (University of Southampton), to quantify the sequence of the mRNA 5' terminus. The data obtained from both sequencing and qPCR analysis is consistent and therefore provides a high level of confidence, that both TOP and TOP-trctMut constructs produce TOP containing and non-TOP containing transcripts respectively (personal communication, Dr M. Coldwell).

3.5 Determination of protein synthesis rate using *in vivo* ^{35}S-methionine radio-labelling

In response to serum starvation and re-feeding, it is known that TOP containing mRNAs move from sub-polysomes onto polysomes (reviewed in Chapter 1). It is therefore important to determine the time-frame in which serum-starved cells respond to serum re-feeding, so that experimental conditions can be optimised. A

common technique employed to obtain this information is to monitor the level of protein synthesis within cells after a period of nutrient stimulation. This data is obtained by recording the amount of radio-labelled ^{35}S-methionine that is incorporated into total cellular protein *in vivo*.

Previous experiments investigating the response of 5'TOP containing mRNAs to cell starvation and re-feeding have utilised HEK-293T cells and the HEK-293 cell-line from which HEK-293T cells are derived (Meyuhas et al. 1997; Stolovich et al. 2002). Unlike HEK-293 cells, HEK-293T cells contain the SV40 Large T-antigen which facilitates episomal replication of plasmids containing the SV40 origin of replication. This SV40 origin of replication is present in both TOP and Mut-TOP, facilitating their amplification and extended temporal expression (Dean, Bullock, 1987). For this reason, the HEK-293T cell-line was chosen for use in this and all future experiments.

HEK-293T cells were plated onto 6 well plates and serum starved for 18 hours in DMEM supplemented with 0.1% Foetal calf serum (FCS). It has previously been shown that a serum re-feeding period of 3 hours is sufficient for HEK-293 cells to recover from serum starvation (Stolovich et al. 2002). For this reason HEK-293T cells in this experiment were re-fed over a 4 hour time course, with DMEM supplemented with 10% FCS. This time course incorporates the previously described conditions with an additional 1 hour extension to accommodate any differences, either in the incubation conditions, or between the HEK-293 and HEK-293T cell lines. For the final 30 minutes of each incubation 10 μCi ^{35}S-methionine was added in DMEM supplemented with 10% FCS. On completion of the time course, cells were harvested, precipitated in 25% Trichloroacetic Acid (TCA), washed and transferred to

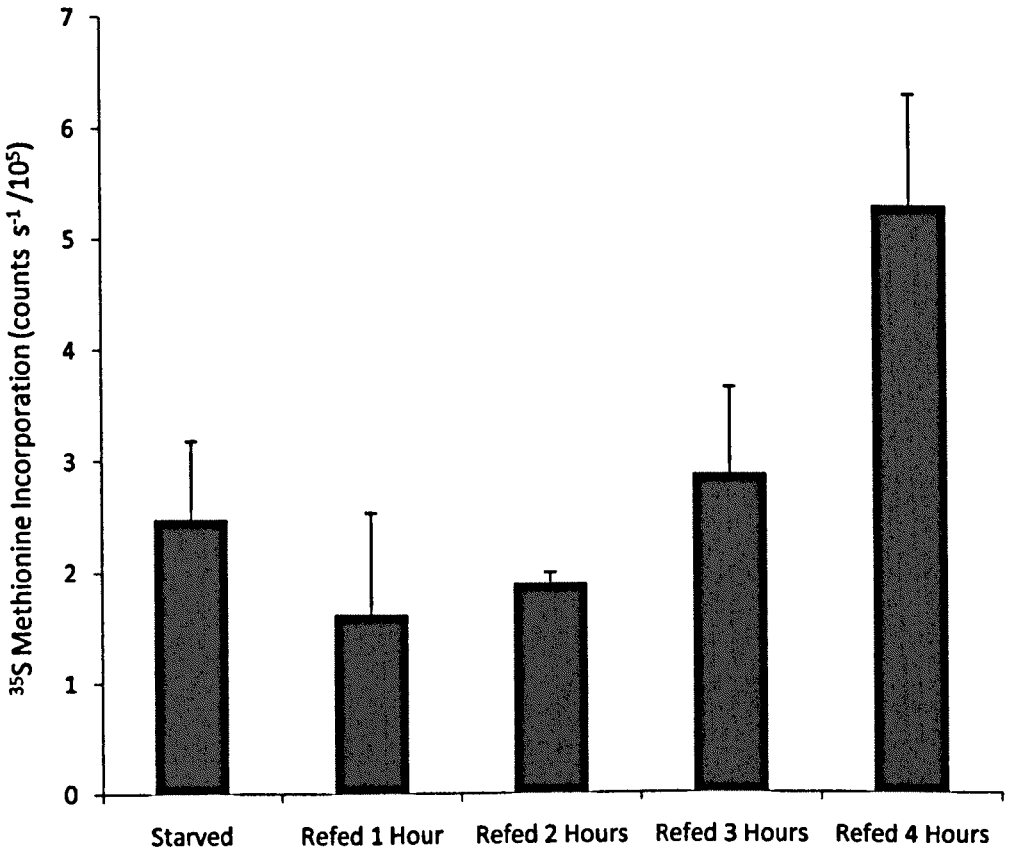


Figure 3.7: ^{35}S Methionine Incorporation in HEK-293T Cells

HEK-293T cells were plated onto 6 well plates and serum starved for 18 hours in DMEM supplemented with 0.1% Foetal calf serum (FCS). Following starvation, cells were re-fed over a time course with DMEM supplemented with 10% FCS. For the final 30 minutes of each incubation, ^{35}S-methionine was added to DMEM supplemented with 10% FCS. The cells were harvested and precipitated with 25% Trichloroacetic Acid (TCA). After washing with 10% TCA containing non-radio-labelled methionine, followed by IMS and Acetone, the samples were transferred to filter paper and dried. The samples were then placed in scintillation count bottles, scintillation fluid was added and a continuous count was carried out for 60 seconds per sample. The experiment was carried out in triplicate and standard deviation of the three replicates was used to calculate error values.

filter paper. After drying, scintillation fluid was added and a continuous count carried out using a scintillation counter for 60 seconds per sample (Figure 3.7).

The data shows that when HEK-293T cells are re-fed with DMEM supplemented with 10% FCS, the recovery from serum-starvation is not complete after 3 hours. The difference observed in the ^{35}S-methionine incorporation between the starved and 3 hour time-point is within experimental error. It is likely that the differences between the HEK-293 and HEK-293T cell lines, or within the incubation conditions themselves, are responsible for the increased recovery time compared to the previously described conditions. After 4 hours, the rate of protein synthesis was approximately double that of starved cell conditions. For this reason the 4 hour time-point was chosen as the re-feeding period for use in further experiments.

3.6 Translational Profiling Using TOP and TOP-trctMut Constructs

Growth dependent expression has already been introduced as a key characteristic of TOP containing mRNAs. For this reason, it was necessary to investigate whether the TOP construct produced mRNAs that were regulated in a growth dependent manner. Similarly it was also important to confirm that the mutations used to generate the TOP-trctMut construct were sufficient to relieve the growth dependent translation control. TOP and TOP-trctMut constructs were transfected into HEK-293T cells. After a period of 24 hours following transfection, the cells were starved for 18 hours, with or without re-feeding for 4 hours prior to harvesting. The cells were lysed and cytoplasmic extracts were loaded onto sucrose density gradients (50-10%) and after ultra-centrifugation (153000 x g for 2 hrs at 4°C in a Sorvall TH-641 rotor and stored at 4°C) the samples were fractionated, treated

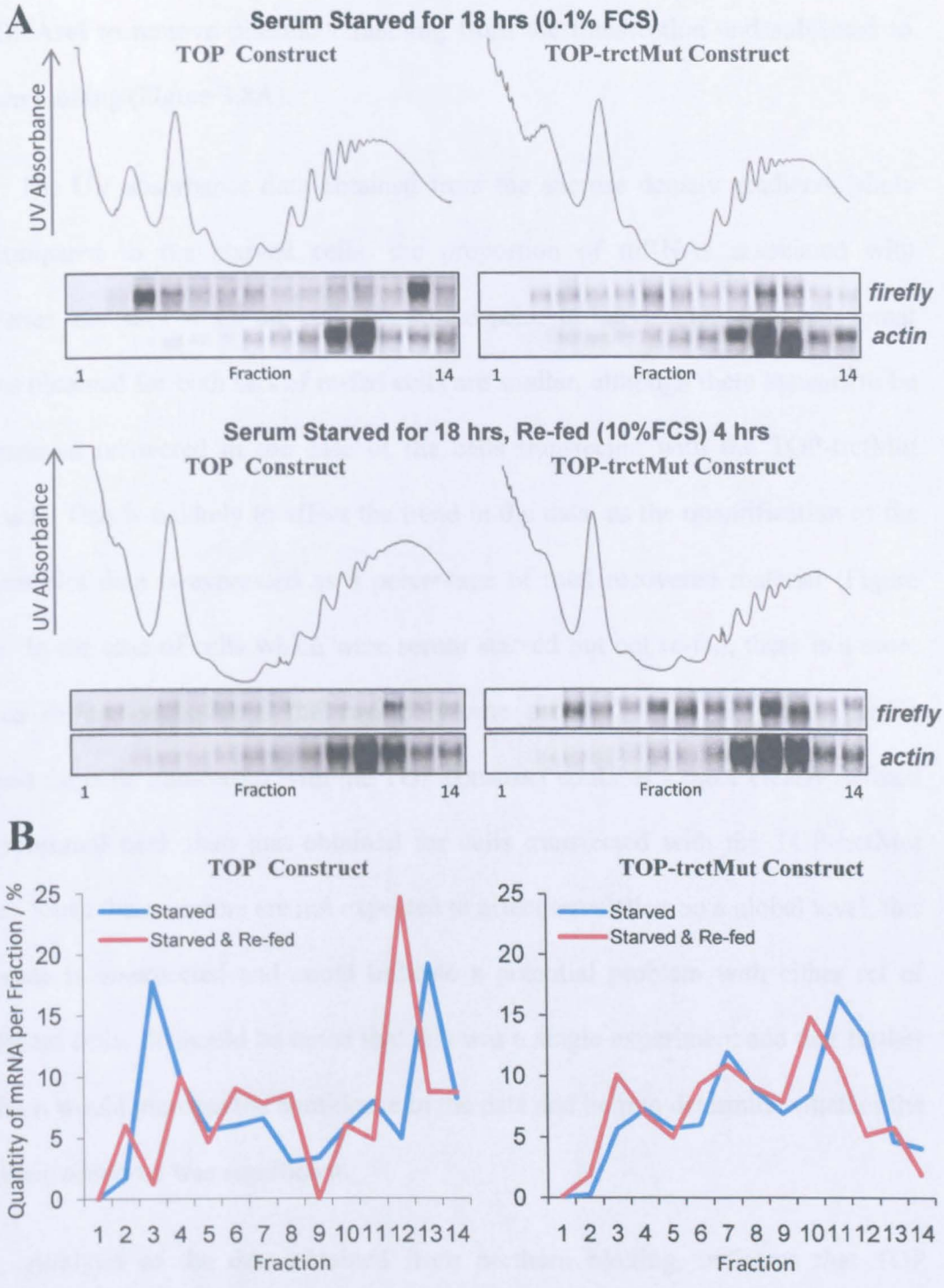


Figure 3.8: Translational Profiling of Starved/Re-fed HEK-293T Cells

A. HEK 293T Cells were transfected with either TOP or TOP-trctMut constructs and incubated for either 18hr in conditions of serum starvation (0.1% FCS), or 18hr serum starvation (0.1% FCS) followed by 4hrs of serum replenishment (10% FCS). Graphs show UV absorbance data as recorded during fractionation of sucrose density gradients. Northern blotting for *firefly* and *actin* mRNA was carried out.

B. Quantification of *firefly* mRNA, as detected by Northern blotting and phosphor-imaging.

with DNAseI to remove plasmid remaining from the transfection and subjected to northern blotting (Figure 3.8A).

The UV absorbance data obtained from the sucrose density gradients, show that compared to the starved cells, the proportion of mRNAs associated with polysomes increases when the cells are re-fed prior to harvesting. The polysomal profiles obtained for both sets of re-fed cells are similar, although there appears to be less material recovered in the case of the cells transfected with the TOP-trctMut construct. This is unlikely to affect the trend in the data, as the quantification of the northern blot data is expressed as a percentage of total recovered material (Figure 3.8B). In the case of cells which were serum starved but not re-fed, there is a more obvious difference between the two polysome profiles. The polysome profile obtained for cells transfected with the TOP construct contains a more clearly defined 40S ribosomal peak than that obtained for cells transfected with the TOP-trctMut vector. Since these vectors are not expected to affect translation on a global level, this difference is unexpected and could indicate a potential problem with either set of transfected cells. It should be noted that this was a single experiment and that further repetition would increase the confidence in the data and help to determine whether the difference observed was significant.

Analysis of the data obtained from northern blotting, indicates that TOP containing mRNA in the nutrient stimulated cells is predominantly associated with polysomes. In serum-starved cells TOP mRNA is almost entirely associated with sub-polysomal particles (Figure 3.8B). Previous experiments investigating the response of TOP containing mRNAs to serum-stimulation have considered the shift from combined sub-polysomal fraction to combined polysomal fractions (Meyuhas 2000). If it is therefore assumed that the clearly defined 40S ribosomal peak of the

serum-starved cell has little influence on the trend outside of the sub-polysomal region, this result is consistent with the characteristic growth-dependent expression of TOP containing mRNAs. The TOP-trctMut mRNA shows a more gradual shift from lighter to heavier polysomal fractions upon re-feeding and behaves more similarly to the non-TOP containing mRNA actin. It would therefore appear that both TOP and TOP-trctMut constructs produce TOP and non-TOP containing mRNAs respectively. Whilst repetition would increase the confidence in the result, the data from this single experiment indicates that both constructs are suitable for use in a model for investigating of the translation of TOP containing mRNAs.

3.7 Signalling Pathway Response

The mTOR (mammalian target of Rapamycin) signalling pathways have been implicated in the translational activation of TOP containing mRNAs by a number of studies (Grolleau et al. 2002)(Figure 3.9.1). mTOR functions as a subunit of two catalytic complexes, mTOR Complex 1 (mTORC1) and (mTOR Complex 2) mTORC2. mTORC1 is ideally placed in the signalling pathway to effect control of TOP containing mRNAs, as it has been shown to be activated by stimuli including insulin, growth factors, serum and amino acids (Kim et al. 2002). Inhibition of mTORC1 has been shown to occur in response to growth factor depletion, low nutrient levels and treatment with Rapamycin (Kim et al. 2002). mTORC1 is comprised of mTOR, regulatory associated protein of mTOR (Raptor), mammalian LST8 and PRAS40 (Kim et al. 2002). mTORC1 has been shown to target p70-S6 Kinase 1 (S6K1) and eIF4E binding protein 1 (4E-BP1), resulting in their phosphorylation (Hay and Sonenberg 2004).

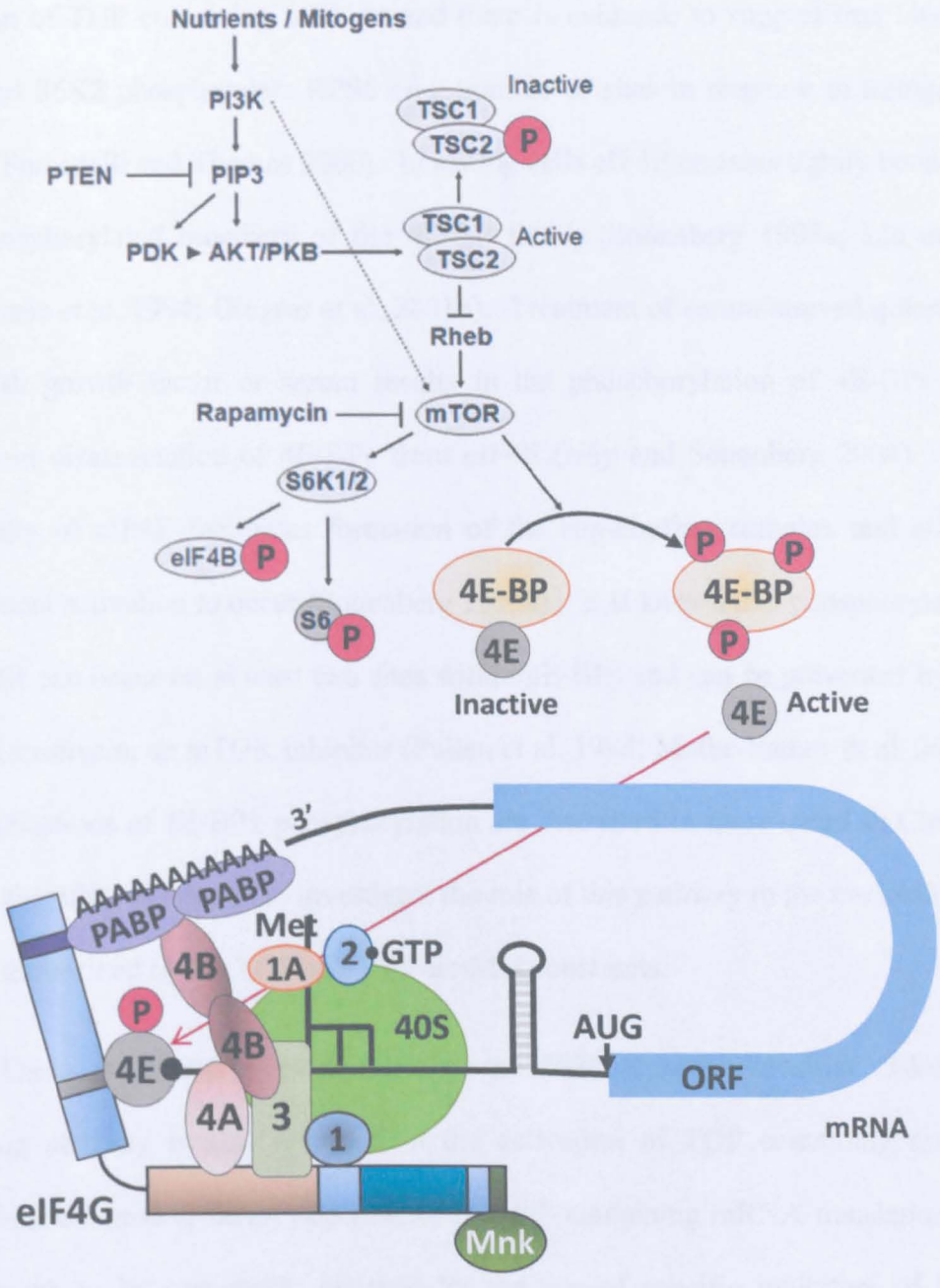


Figure 3.9.1: The PI3K/mTOR Signalling Pathway

The PI3K/mTOR signalling pathway has been previously shown to selectively mediate translation initiation of 5'TOP mRNA translation. The role of the mTOR signalling pathway in the phosphorylation of ribosomal protein S6, 4E-BP1, eIF4B and eIF4G, makes it ideally positioned for a regulatory role in the translation initiation of TOP mRNAs.

Figure partially adapted from: Hamilton et al. 2006.

Phosphorylation of RPS6 has been shown to correlate with the translational activation of TOP containing mRNAs and there is evidence to suggest that kinases S6K1 and S6K2 phosphorylate RPS6 on a number of sites in response to mitogenic stimuli (Fumagalli and Thomas 2000). In resting cells eIF4E remains tightly bound to hypo-phosphorylated members of the 4E-BP family (Sonenberg 1993a; Lin et al. 1994; Pause et al. 1994; Gingras et al. 2001b). Treatment of serum-starved quiescent cells with growth factor or serum results in the phosphorylation of 4E-BPs and subsequent disassociation of 4E-BPs from eIF4E (Hay and Sonenberg 2004). The availability of eIF4E facilitates formation of the cap-binding complex and allows translational activation to occur (Sonenberg 1993a). It is known that phosphorylation by mTOR can occur on at least two sites within 4E-BPs and can be prevented by the use of Rapamycin, an mTOR inhibitor (Pullen et al. 1998; Mothe-Satney et al. 2000). The implications of 4E-BP1 phosphorylation are discussed in more detail in Chapter 4. It is therefore of interest to investigate the role of this pathway in the translation of mRNA transcribed by the TOP and TOP-trctMut constructs.

There is mounting evidence that the PI3K (phosphoinositide 3-kinase) signalling pathway is also involved in the activation of TOP containing mRNA translation and growth factor dependence of TOP containing mRNA translation has been shown to be completely relieved by the use of specific inhibitors of PI3K (Hamilton et al. 2006). Sucrose density gradient analysis has also been carried out in cells in which proteins that interfere with PI3K signalling were over-expressed. Upon serum stimulation in these cells, the recruitment of TOP containing mRNAs to polyribosomes was inhibited. In growth inhibited cells, the use of constitutively active forms of PI3K or PKB was shown to relieve this repression and allow the recruitment of TOP containing mRNAs to polysomes (Hamilton et al. 2006).

It was of interest to determine whether the mTOR and PI3K signalling pathways play a role in the translation of mRNAs transcribed from the TOP and TOP-trctMut construct. Rapamycin was used to investigate the effect of inhibiting the mTOR signalling pathway, whereas the PI3K signalling pathway was investigated using the known PI3K inhibitors Wormannin and LY294002 (Vlahos et al. 1994). HEK-293T cells were co-transfected (in triplicate) with either TOP or TOP-trctMut construct, along with a transfection control vector, which expresses β-galactosidase. The cells were grown under control conditions and were not subjected to serum-starvation or re-feeding. After 24 hours cells were treated with either: Rapamycin (50nM); Wortmannin (100nM); or LY294002 (10μM). Following 8 hours of incubation, both control and treated cells were harvested and luciferase activity was determined (Figure 3.9.2).

The data show that treatment with either LY294002 or Wortmannin, resulted in the translational inhibition of the mRNA transcribed by both TOP and TOP-trctMut constructs. The difference observed between the inhibition of TOP and TOP-trctMut does not appear to be statistically significant as it falls within the bounds of experimental error. The data obtained from the LY294002 and Wortmannin treatment are not consistent with the PI3K signalling pathway having a significant effect on the translational activation of TOP containing mRNAs. In the case of Rapamycin treatment, the protein expression from the TOP mRNA does appear to have been inhibited more than that of TOP-trctMut mRNA. The translational activity of TOP-trctMut mRNA itself is not statistically significant from the control, suggesting that it is not affected by the mTOR signalling pathway. It is therefore possible that the mTOR signalling pathway is involved in the translational activation of TOP containing mRNAs. It should be noted however that the difference observed between

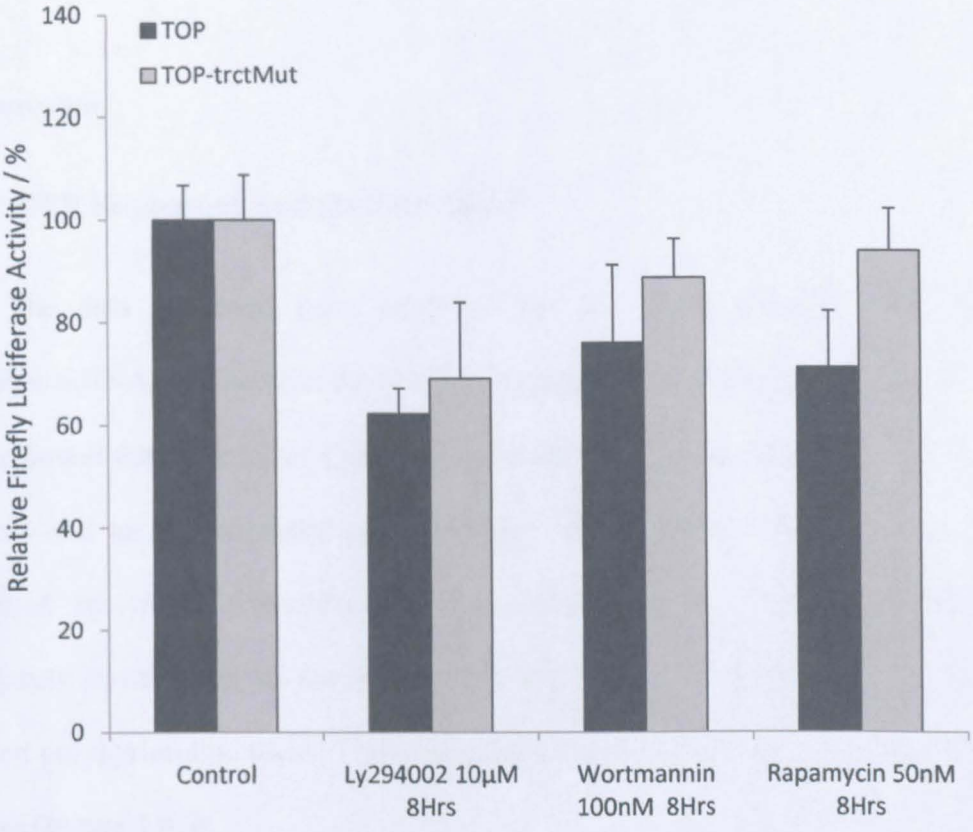


Figure 3.9.2: PI3K/mTOR Signalling Pathway Inhibition

HEK-293T cells were transfected in triplicate with either TOP or TOP-trctMut construct. After 24 hours non-control cells were treated with either: Rapamycin (50nM); Wortmannin (100nM); or LY294002 (10μM). The cells were grown under control conditions and were not subjected to serum-starvation or re-feeding. Following 8 hours of incubation, both control and treated cells were harvested and a luciferase assay was carried out. Data was normalised to control (untreated) cell values which was set at 100% for both constructs. Standard deviation of three replicates was used to calculate error values.

the translation activity of TOP and TOP-trctMut mRNA following Rapamycin treatment, lies only just outside the bounds of experimental error. Further repetition of this experiment may be required in order to increase the confidence in this result.

3.8 Discussion

3.8.1 5' UTR Sequencing and the TOP Motif

The data presented here indicates that the TOP construct successfully transcribes mRNA that contains the 5'TOP structural motif. Analysis of the 5' RACE data confirmed the presence of C-residue immediately downstream of the 5' terminal m^7G cap and an uninterrupted polypyrimidine tract (Figure 3.6.3). The mRNA transcribed by the TOP-trctMut construct was found to contain a G-residue immediately downstream of the 5' terminal m^7G cap in 35% of transcripts and a disrupted polypyrimidine tract. The remaining transcripts were also non-TOP like in structure (Figure 3.6.3).

It is noteworthy that the first C-residue, which was expected, based upon the NCBI database data for rpS6 mRNA, is not present in the TOP transcribed mRNA and consequently in the TOP-trctMut transcribed mRNA. It is possible that the sequence data contained within the database may have incorrectly predicted the 5' terminus of the transcript. A common method used to determine the transcription start site involves DNA sequencing of the gene in combination with the extension of the primer of the corresponding mRNA (Sambrook 1989). In this process, reverse transcriptase of the annealed DNA primer takes place on mRNA transcribed from the gene of interest. When the 5' terminus of the mRNA is reached, the reverse

transcription reaction stops. If identical primers are used for gene sequencing and primer extension, PAGE (polyacrylamide gel electophoresis) can be employed to determine the nucleotide at which the primer extension terminated. This nucleotide coincides with the mRNA transcription site. There are many reported instances of inconsistent transcription start sites being reported for many families of genes. Examples include the Euplotes genes (Kaufmann and Klein 1992; Ghosh et al. 1994; Bender and Klein 1997) and tubulin genes in *S. Lemnae* minichromosomes (Skovorodkin and Günzl A. 1999). It has been suggested that the differing processes of gene sequencing and primer extension produce DNA with slightly different electorphoretic mobility. This could result in an incorrect alignment between the primer extended DNA and the reference DNA produced from the gene sequencing reaction (Pimenov 2008). This could potentially explain the differences in the observed transcription start site of the TOP and TOP-trctMut, when compared to the transcription start site of rpS6 mRNA contained in the NCBI database.

The difference between the results obtained for the TOP and TOP-trctMut constructs from the sucrose density gradient analysis, is consistent with the proven dependency on both the integrity of the *cis*-acting TOP motif and its immediate Cap-proximal position. Detailed analysis of the polyribosome profiles obtained from sucrose density gradient analysis, indicates that fractions 12 and 13 were the highest occupied fractions containing significant levels of mRNA for both TOP and TOP-trctMut constructs (Figure 3.8B). From analysis of fraction 12 in serum starved and re-fed cells (as an example), it is evident from the UV absorbance data that this fraction corresponds to approximately 6 to 8 translocating ribosomes per mRNA. Division of the number of bases in the Firefly Luciferase coding region (1652nt) by the number of ribosomes in the peak polysome fractions, results in an estimated

ribosome spacing of 207-275nt. Since Ribosomes are approximately 300A in diameter which is equivalent to approximately 90nt, the calculated spacing seems reasonable (Pe'ery and Mathews 2000).

It is interesting to note that the predicted miRNA-10a binding site in ribosomal protein S6 does not contain a classical microRNA seed region, sequence complementary to bases 2-8 of the miRNA (Pillai et al. 2005). Instead, miRNA-10a is predicted to bind along its entire length, by means of conventional base pairing along with relatively weaker U-G interactions. This proposed binding is however consistent with that predicted for ribosomal protein L13 mRNA, based upon mapping of the binding between miRNA-10a and the 5' UTR of this mRNA. More detailed mapping of the miRNA-10a binding site in the 5' UTR of rpS16, suggests that this type of microRNA-RNA interaction is common in a wide variety of 5'TOP containing mRNAs (Orom et al. 2008).

3.8.2 The mTOR and PI3K Signalling Pathways

Treatment of TOP and TOP-trctMut transfected cells with the PI3K inhibitors Wortmannin and LY294002 resulted in the translational down regulation of both TOP and TOP-trctMut transcribed mRNA. No significant difference was observed between TOP and TOP-trctMut mRNA under these conditions. This suggests that the PI3K signalling pathway does not have a significant involvement in the translational activation of TOP containing mRNAs. Interestingly, previous experiments using the HEK-293T cell-line have revealed selective and pronounced repression of TOP containing mRNAs encoding rpL32 and eEF1A when the cells were treated with LY294002, compared to only mild repression upon Rapamycin treatment (Stolovich

et al. 2002). The data reported in the literature does not appear to be consistent with that obtained in this study. A possible explanation for this finding is that the response to the mTOR and/or PI3K signalling pathways differs between TOP containing mRNAs. It would therefore be of interest to repeat the experiments performed here using constructs containing the rpL32 5' UTR, and the 5'UTRs of a wide variety of different TOP containing mRNAs.

Interestingly, the only difference observed between mRNAs transcribed from the TOP and TOP-trctMut constructs was with the use of Rapamycin, an mTOR pathway inhibitor. Whilst this difference was only just outside the bounds of experimental error, it suggests that the mTOR signalling pathway may affect the translation of TOP containing mRNAs. It is important to note that the HEK-293T cell line was used in this experiment and there is evidence that the signalling pathway response of TOP containing mRNAs is dependent upon cell type (Stolovich et al. 2002).

Early evidence suggested that the phosphorylation of RPS6 resulted in the selective translation of TOP containing mRNAs. When the mTOR inhibitor Rapamycin was used to block S6K activity in Swiss 3T3 cells, the translational activation of TOP containing mRNAs was almost completely repressed (Jefferies et al. 1994). However, when a similar experiment was carried out using the HEK-293 cell-line, the translational activiation of 5'TOP containing mRNAs was only partially repressed (Tang et al. 2001). A study using *S6K1*$^{-/-}$*/S6K2*$^{-/-}$ mice has previously shown that the combined knockout of both S6K1 and S6K2 has no effect on the translational regulation of TOP containing mRNAs in response to mitogens (Pende et al. 2004). Drawing comparisons between the effect observed in this experiment and those observed in previous studies is not straightforward. The partial effect observed

following Rapamycin treatment in this study appears consistent with that previously observed in the HEK-293 cell-line. However, it is important to note that the earlier study specifically investigated the effect of Rapamycin on the translational activation of TOP containing mRNAs following serum starvation and re-feeding. In this experiment, HEK-293T cells were grown under control conditions. Interestingly, in the human BJAB B cell line, which was not subjected to serum-starvation or re-feeding, TOP containing mRNAs encoding ribosomal proteins and elongation factors were selectively repressed (Zhu et al. 2003). In order to better compare the effect between this and previous studies, it may of interest to repeat this experiment under conditions of serum starvation and re-feeding.

It has been suggested that functions of mTOR Complex 1 (mTORC1) are Rapamycin resistant and a model had been proposed whereby Rapamycin blocks access to only a specific subset of mTORC1 substrates. In particular, it has been proposed that mTORC1, or a raptor containing mTOR complex, regulates 4E-BP1 phosphorylation through a kinase-dependent mechanism, which is Rapamycin-insensitive (Thoreen et al. 2009). Since a slight effect was observed following Rapamycin treatment, it remains possible that 4E-BP1 is not involved in the regulation of TOP reporter mRNA.

3.8.3 Canonical Initiation and *Trans*-acting Factor Requirements

The potential involvement of the mTOR signalling pathway in the control of TOP containing mRNAs gives rise to the possibility that 4E-BP1 and perhaps its binding partner eIF4E, may be involved in the regulation of TOP containing mRNAs. Indeed it would be interesting to determine the roles, if any, of the canonical initiation

factors that are required for cap-dependent translation. To date there have been no reported studies to determine the canonical initiation factor requirements 5'TOP containing mRNAs. In addition to the canonical initiation factors, other *trans*-acting factors may be involved in 5'TOP containing mRNA regulation. As is particularly evident from the 5' RACE analysis, the polypyrimidine tract is a key structural component of TOP containing mRNAs. Polypyrimidine-tract binding protein (PTB) is a multi-function protein, which preferentially binds polypyrimidine-rich regions of mRNA (Garcia-Blanco et al. 1989). It functions are dependent on its location within the cell and are known to include involvement in translation initiation (Jang and Wimmer 1990). It would therefore be of interest to investigate whether or not it is involved in the mechanism of 5'TOP translational regulation. Another potential candidate for investigation is the La autoantigen. Previous research has suggested that La acts a specific *trans*-acting factor, involved in the translational regulation of TOP containing mRNA (Pellizzoni et al. 1996).

Chapter Four

Canonical Initiation and *Trans*-acting Factor Requirements of 5' TOP Containing mRNAs

4.1 Canonical Initiation factors in Cap-dependent Translation

Initiation of protein synthesis in eukaryotes requires recruitment of the ribosome to the mRNA and its subsequent translocation to the AUG start codon. Cap-dependent translation is the major translation pathway, with estimates suggesting up to 97% of total cellular mRNAs are translated via this route (Merrick 2004). The mechanism requires the assembly of the 43S complex, which is comprised of the 40S ribosomal sub-unit bound to eIF2-GTP/Met-tRNA$_i$ and the canonical initiation factors eIF1A and eIF3 (previously reviewed in Chapter 1). This complex is recruited to the mRNA in a process which requires recognition of the 5' terminal m^{7}G cap by the cap-binding complex eIF4F though its sub-unit eIF4E. Once recruited, the 43S sub-unit traverses the mRNA in a 5' to 3' direction in a process known as scanning, stopping at the first AUG codon in favourable context for the initiation of translation to occur (Kozak 1987)

The cap binding-complex eIF4F is comprised of eIF4E, eIF4A and the scaffold protein eIF4G. eIF4A is an RNA helicase, whose function is enhanced by its association with eIF4B and/or eIF4H. In addition to binding eIF4E and eIF4A, eIF4G also binds to eIF3 and poly(A)-binding protein (PABP), which also have important roles in translation initiation (refer to Chapter 1 for a more detailed discussion). The initiation factors therefore comprise a functional ensemble, which is integral to the cap dependent translation mechanism (Table 4.1). Despite the importance of the cap dependent mechanism for the majority of cellular messages, to

Name	Function
eIF1	Start codon recognition
eIF1A	Interaction between 40S subunit and Met-tRNA$_i$, 40S dissociation
eIF2	α subunit binds eIF2B β subunit binds eIF2B and eIF5 γ subunit binds GTP and Met-tRNA$_i$, GTPase activity
eIF2B	5 subunits, guanosine nucleotide exchange factor (GNEF) for eIF2
eIF3	11 subunits, interacts with 40S subunit as well as RNA, eIF1, eIF4B, eIF4G and eIF5
eIF4AI	ATP-dependent RNA helicase activity
eIF4AII	ATP-dependent RNA helicase activity
eIF4B	Stimulates eIF4A and binds RNA
eIF4E	Cap-binding protein
eIF4GI	Binds via eIF4E and interacts with eIF4A, eIF3, PABP and RNA
eIF4GII	Binds via eIF4E and interacts with eIF4A, eIF3, PABP and RNA
eIF5	GTPase activating protein (GAP) of eIF2
eIF5B	60S subunit joining at initiation AUG, GTPase activity
eIF6	60S subunit joining and dissociation

Table 4.1: Names and Functions of the Eukaryotic Initiation Factors

date there have been no reported studies to determine the canonical initiation factor requirements 5'TOP containing mRNAs. It is therefore of great interest to determine whether 5'TOP containing mRNAs have specific canonical initiation factor requirements, and if their general requirements differ from those of non-TOP containing mRNAs.

4.2 Over-expression of 4E-BP1

In order for the cap-binding complex (eIF4F) to assemble and cap recognition to take place, eIF4E must be available to bind to the scaffold protein eIF4G. The eIF4E-binding protein family, the 4E-BPs (1-3), compete with eIF4G for a common binding site on eIF4E. When this site is occupied by a member of the 4E-BP family, the cap-binding complex is unable to assemble and consequently translation is repressed. In resting cells eIF4E remains tightly bound to hypo-phosphorylated members of the 4E-BP family (Sonenberg 1993a; Lin et al. 1994; Pause et al. 1994; Gingras et al. 2001b). Treatment of serum-starved quiescent cells with growth factor or serum results in the phosphorylation of 4E-BPs and subsequent disassociation of 4E-BPs from eIF4E. The availability of eIF4E facilitates formation of the cap-binding complex and translational activation to occur (Sonenberg 1993b). It is known that phosphorylation by mTOR can occur on at least two sites within 4E-BPs and can be prevented by the use of Rapamycin, an mTOR inhibitor (Pullen et al. 1998; Mothe-Satney et al. 2000). The mTOR signalling pathway has previously been implicated in the regulation of TOP containing mRNAs (as described in Chapter 1 and Chapter 3), indicating a potential connection between the 4E-BPs and the translation regulation of TOP containing mRNAs.

In order to investigate the potential role of 4E-BPs in the regulation of TOP containing mRNA translation, 4E-BP1 was over-expressed in HEK-293T cells that were transfected with the TOP or TOP-trctMut constructs and a transfection control vector expressing Renilla. To reduce the number of variables in each transfection, it was necessary to ensure that a constant amount of DNA was transfected and each transfection contained the same ratio of DNA to transfection reagent. This was achieved by supplementing the pcDNA3.1 vector containing the 4E-BP1 coding region (pcDNA3.1-4EBP1) with empty pcDNA3.1 vector, so that the total quantity of these two constructs was constant in each transfection. Thus, in addition to the reporter constructs, HEK-293T cells were also transfected with either a) 0.4 μg pcDNA3.1 vector; b) 0.1 μg pcDNA3.1-4EBP1 & 0.3 μg pcDNA3.1; c) 0.2 μg pcDNA3.1-4EBP1 & 0.2 μg pcDNA3.1; or d) 0.4 μg pcDNA3.1-4EBP1. After a period of 24 hours had elapsed, the cells were serum starved for 18 hours in DMEM supplemented with 0.1% Foetal calf serum (FCS). These conditions were chosen after consideration of previous data, which determined the quantity of radio-labelled ^{35}S-methionine incorporated into total cellular protein *in vivo*, following starvation and re-feeding (Chapter 3, Figure 3.8). To confirm over-expression of 4E-BP1, western blotting was carried out (Figure 4.2B). Luciferase activity was then determined to investigate the translational efficiency of both the TOP and TOP-trctMut reporters (Figure 4.2A).

The western blotting data shows that as the amount of transfected pcDNA3.1-4E-BP1 vector increased, an increase in 4E-BP1 protein expression did not occur. Whilst the data obtained for the Actin loading control appears constant for across the pcDNA3.1-4EBP1 titration, it is possible that the high relative abundance of Actin made it difficult to detect small changes between samples. An improved control for

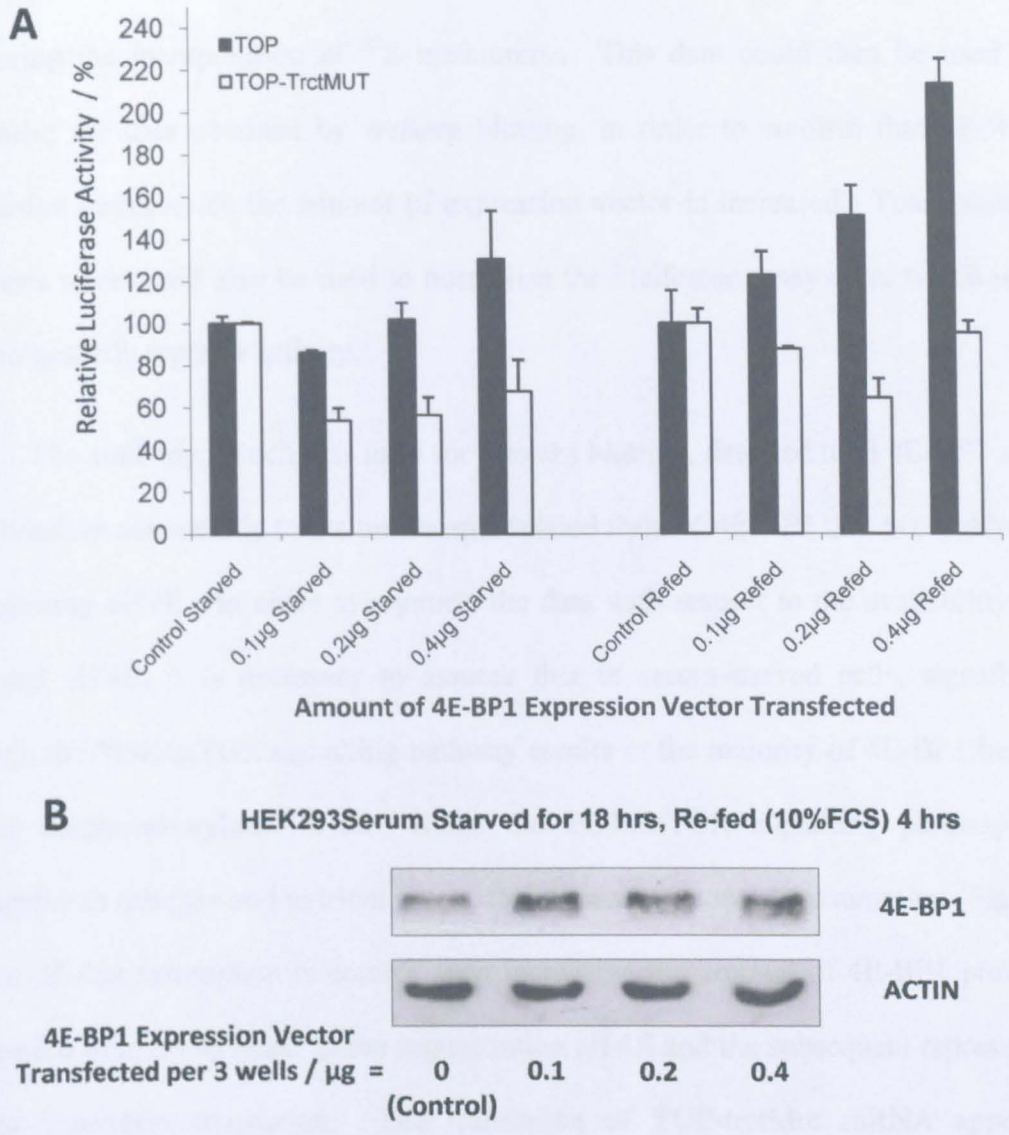


Figure 4.2: Translational Activity of TOP and TOP-TrctMut upon 4E-BP1 Over-expression in HEK-293T Cells

A. TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with *Renilla* luciferase expression vector (transfection control) and either a pcDNA3.1 empty vector (control) or increasing concentrations of pcDNA3.1-4E-BP1 expression vector. Cells were serum starved (0.1% FCS) for 18 hrs or serum starved (0.1% FCS) for 18 hrs and serum stimulated (10% FCS) for 4 hours immediately prior to harvesting 48 hours after transfection. A luciferase assay was carried out and the ratio of *Firefly* to *Renilla* luciferase activity was calculated. Error bars were calculated from the standard deviation of three replicates.

B. Western blotting was then carried out to determine the levels of total 4E-BP1 and Actin (loading control).

this experiment would be to determine the total cellular protein synthesis rate by measuring the incorporation of ^{35}S methionine. This data could then be used to normalise the data obtained by western blotting, in order to confirm that 4E-BP1 expression increases as the amount of expression vector is increased. Total protein synthesis rates could also be used to normalise the luciferase assay data, which was used to quantify reporter activity.

The antibody, which was used for western blotting, detected total 4E-BP1 and was therefore not specific to the un-phosphorylated form of 4E-BP1 that is capable of sequestering eIF4E. In order to interpret the data with respect to the availability of unbound eIF4E, it is necessary to assume that in serum-starved cells, signalling through the PI3K/mTOR signalling pathway results in the majority of 4E-BP1 being in the un-phosphorylated form. Since the PI3K/mTOR signalling pathway is responsive to mitogen and nutrient levels, this seems a reasonable assumption (Figure 3.9.1). If this assumption is correct, then increasing the amount of 4E-BP1 protein expression is likely to result in the sequestration eIF4E and the subsequent repression of cap-dependent translation. The translation of TOP-trctMut mRNA appears inhibited by 4E-BP1 over-expression, dropping to approximately 50% of control levels with limited dose dependence (Figure 4.2A). This is consistent with TOP-trctMut mRNA being under cap-dependent translational control. Interestingly, TOP containing mRNA translation initially drops by just 15%, but then increases to over 100% of control levels with increasing 4E-BP1 expression. It is therefore possible, that the translation activation of TOP containing mRNA does not require eIF4E. The increase in translation above control levels, may be due to TOP mRNAs being able to use the remaining translationally machinery with reduced competition from mRNA that require eIF4E.

Serum re-feeding is expected to result in signalling through the PI3K/mTOR pathway and the downstream effect of the phosphorylation of 4E-BP1 (refer to section 3.7). In its phosphorylated form, 4E-BP1 is unable to bind eIF4E, therefore increasing the amount of eIF4E which is available for cap-dependent translation (Figure 3.9.1). With the absence of western blot data to confirm the levels of non-phosphorylated 4E-BP1, it must be assumed that a quantity of 4E-BP1 that is expressed from the transfected pCDNA3.1-4EBP1 vector is also phosphorylated in this manner. However, it is important to realise the balance of how much of the additionally expressed 4E-BP1 is phosphorylated could has an important bearing on the interpretation of the data.

The translation of TOP-trctMut, mRNA following serum stimulation, appears to be less inhibited by 4E-BP1 over-expression than in the case of un-stimulated cells. Translation of TOP-trctMut, drops by only 20-40% compared to control levels (Figure 4.2A). This is consistent with eIF4E being required for the translational activation of TOP-trctMut mRNA. The fact that repression is still occurring following serum stimulation, suggests that the over-expression of 4E-BP1 is still resulting in the sequestration of eIF4E. This implies that not all the additionally expressed 4E-BP1 is phosphorylated following serum stimulation.

The increase in translation of TOP mRNA, which was observed following serum-stimulation, is much greater than that observed in serum-starved cells. This is consistent with the known response of TOP containing mRNAs under conditions of serum-stimulation. Interestingly, TOP mRNA translation increased when more 4E-BP1 expression vector was transfected (Figure 4.2A) suggesting that sequestration of eIF4E was a still a factor despite the serum stimulation. If this was the case, the increase in translational activity with increasing 4E-BP1 expression, is consistent with

that observed in serum-starved cells. Carrying out western blotting to determine the levels of unphosphorylated 4E-BP1 may provide a greater insight into the mechanism at work.

The transfections were repeated using and $2x10^6$ cells and 2.5 μg of 4E-BP1 expression vector, which was determined above to be a suitable ratio for examining the differences in translation activity of the mRNA transcribed by both constructs. After a period of 24 hours had elapsed, the cells were serum starved for 18 hours in DMEM supplemented with 0.1% Foetal calf serum (FCS). Following starvation, cells were either re-fed for 4 hours with DMEM supplemented with 10% FCS and then harvested or harvested without re-feeding. Sucrose density gradients analysis was carried out and polyribosome profiles were obtained (Figures 4.3.1A & 4.3.1B). These data show that when 4E-BP1 was over-expressed, polysomally located mRNAs shifted to lower fractions. The most notable difference when 4E-BP1 is over-expressed, is that the 60S ribosomal peak formed a broad shoulder with the 80S peak, and there was an increased proportion of material in the sub-polysomal fractions. This is consistent with translational repression taking place when 4E-BP1 is over-expressed.

Northern blotting was carried out for *firefly* (reporter) and *actin* (control) mRNAs, on the fractions obtained from the sucrose density gradients. Quantification was then carried out to determine the percentage of reporter and control mRNA in each fraction (Figure 4.3.2). The *actin* control remains relatively consistent for both TOP and TOP-trctMut transfected cells regardless of 4E-BP1 over-expression (Figure 4.3.2). In the case of TOP-trctMut, quantification of the *firefly* northern blots indicates that there is little change between control and 4E-BP1 over-expression (Figure 4.3.2). The apparent shift of mRNA from fraction 4 to fraction 6 upon

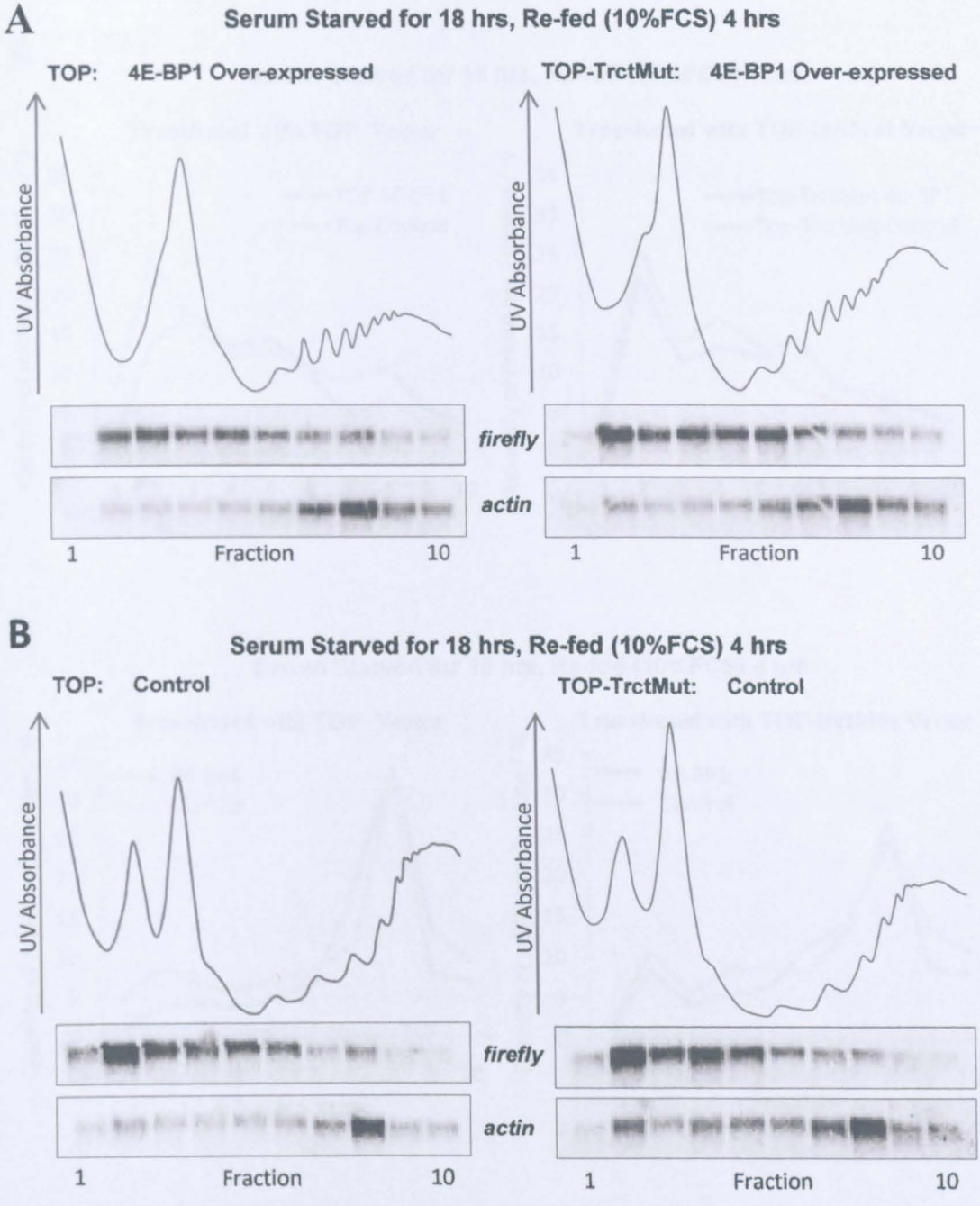


Figure 4.3.1: 4E-BP1 Over-expression in HEK-293T Cells

TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with either A) pcDNA3.1-4E-BP1 expression vector or B) empty pcDNA3.1 vector. The cells were incubated for either 18hr in conditions of serum starvation (0.1% FCS) followed by 4hrs of serum replenishment (10% FCS). Graphs show UV absorbance data as recorded during fractionation of sucrose density gradients. Northern blotting for *firefly* mRNA was carried out on the 10 fractions obtained from each gradient.

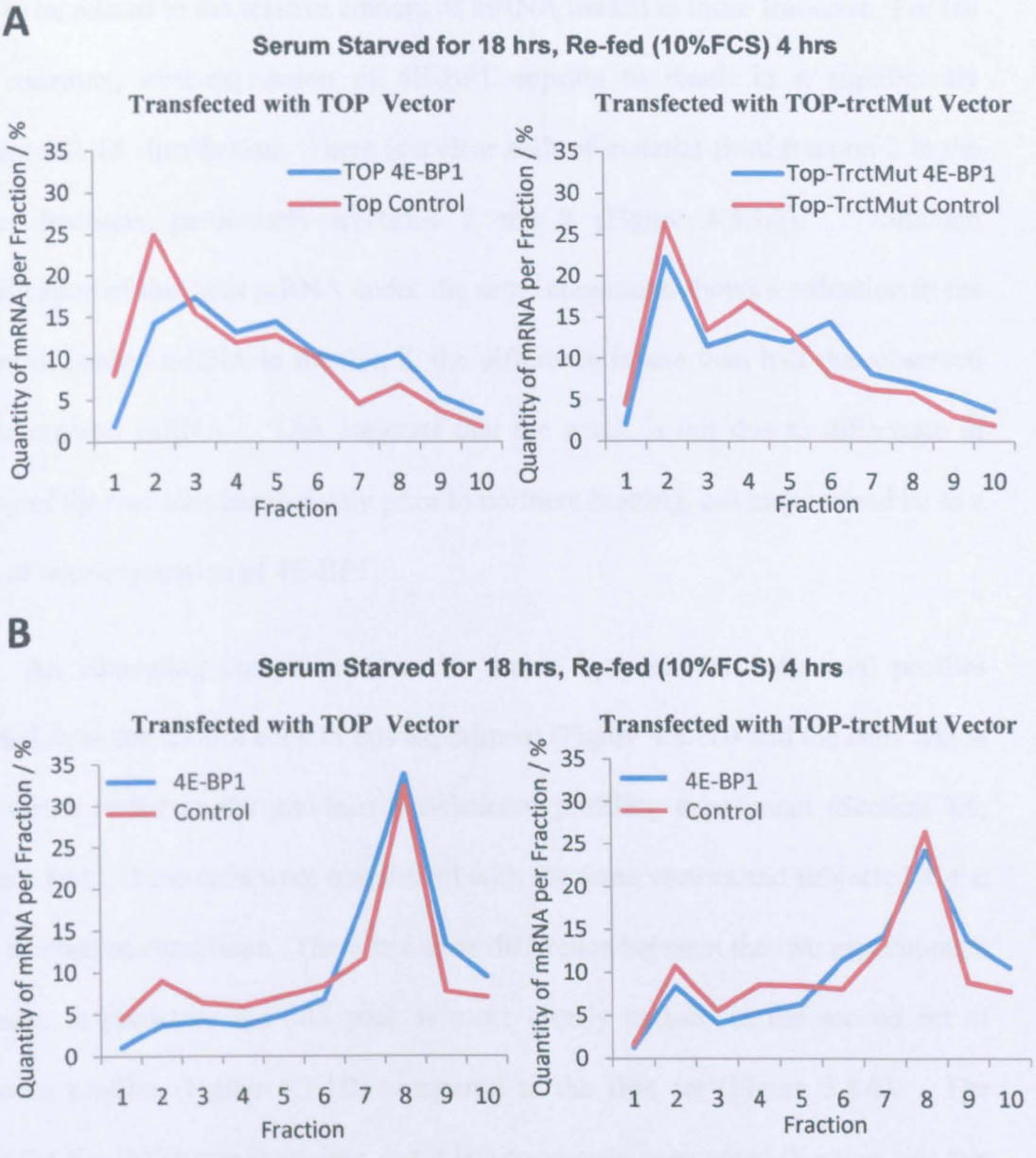


Figure 4.3.2: 4E-BP1 Over-expression in HEK-293T Cells

A. Quantification of *firefly* (reporter) mRNA, as detected by Northern blotting and phosphor-imaging.

B. Quantification of *actin* (control) mRNA, as detected by Northern blotting and phosphor-imaging.

4E-BP1 over-expression was also observed for *actin* mRNA, indicating that it is likely to be related to the relative amount of mRNA loaded in those fractions. For the TOP construct, over-expression of 4E-BP1 appears to result in a significantly different mRNA distribution. There is a clear shift of material from fraction 2 to the heavier fractions, particularly fractions 7 and 8 (Figure 4.3.1A). Although quantification of the *actin* mRNA under the same conditions shows a reduction in the amount of control mRNA in fraction 2, the difference is less than half that observed for the reporter mRNA. This suggests that the result is not due to difference in loading of the fractions immediately prior to northern blotting, but may instead be as a result of over-expression of 4E-BP1.

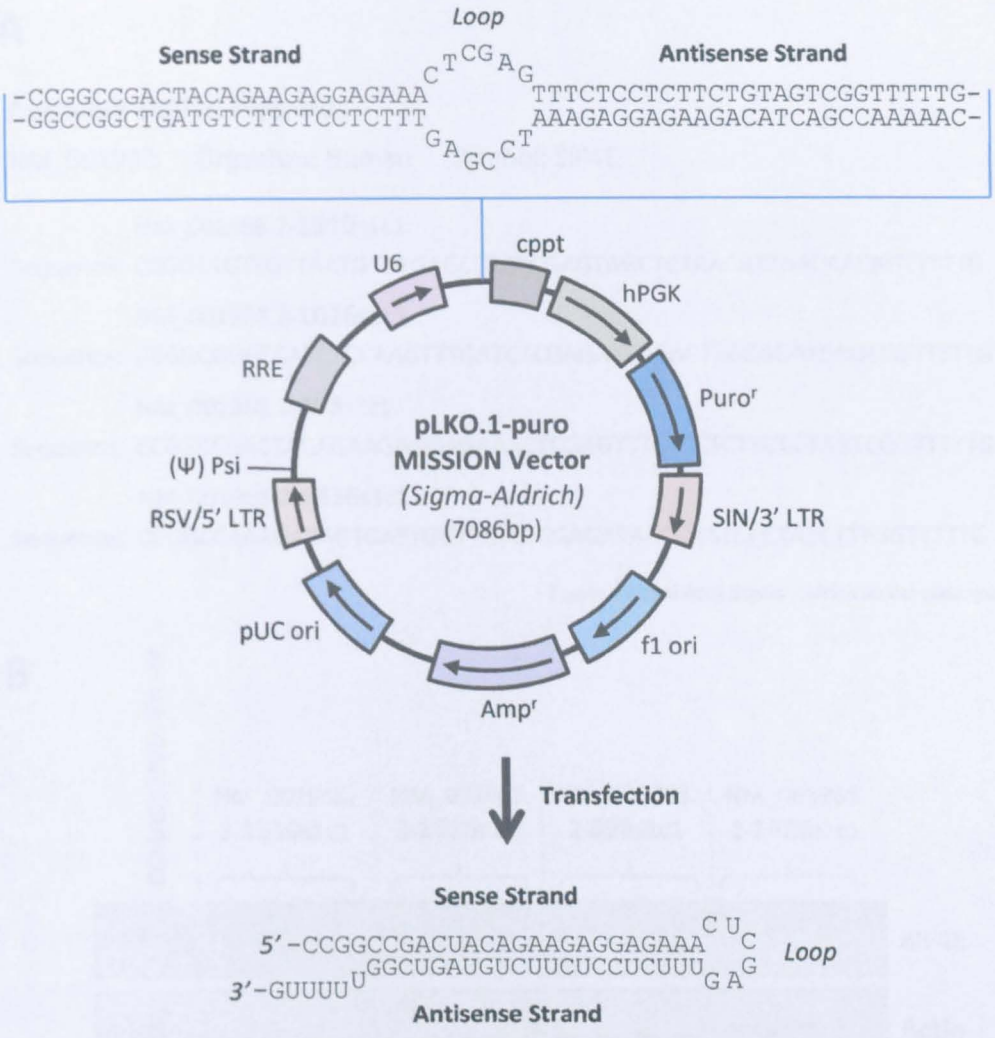
An interesting comparison can be drawn between the polysomal profiles obtained from the control cells in this experiment (Figure 4.3.1B) and the cells which were serum re-fed in the previous translational profiling experiment (Section 3.6, Figure 3.8A). These cells were transfected with the same vectors and subjected to the same incubation conditions. There is a clear difference between the two experimental data sets, in particular the 40S peak is more clearly defined in the second set of polysome profiles (Figure 4.3.1B) compared to the first set (Figure 3.8A). The reason for this difference is unclear, but it has previously been noted (Section 3.6) that a more distinguishable 40S peak was also obtained in polysomal profiles carried out on serum-starved cells, which had been transfected with the TOP construct. When the data obtained from northern blotting is compared, there also appears to be a difference between the two data sets. Comparison of the TOP construct (Figure 3.8B and Figure 4.3.2A - left panels, red lines), indicates that the mRNA distribution is not the same between data sets. In the first experiment, TOP mRNA is predominantly located in the sub-polysomal fractions, whereas in the second experiment TOP mRNA

is associated with polysomes. This is also found to be the case when an equivalent comparison is carried out for the TOP-trctMut construct (Figure 3.8B and Figure 4.3.2A - right panels, red lines). The difference could possibly be due to the unique conditions each experiment was performed under or be related to cell cycle effects. Within each experimental data set, comparison of the TOP and TOP-trctMut mRNA indicates that a clear difference remains in behaviour the behaviour of these mRNAs under identical conditions.

The data obtained from the sucrose density gradient analysis performed in this experiment and the luciferase assay to determine translational activity (Figure 4.2A), appears to be consistent. The TOP-trctMut construct transcribed mRNA that does not appear to be significantly affected by 4E-BP1 over-expression, whereas the mRNA transcribed from the TOP construct appears to be up-regulated under the same conditions. These data suggest that TOP containing mRNAs may not require eIF4E. The apparent up-regulation of TOP containing mRNAs when the availability of eIF4E is expected to be diminished, suggests that an alternative mechanism of translation initiation could be favoured.

4.3 eIF4E Knockdown

To independently validate the requirements of TOP containing mRNAs for eIF4E, a MISSION plasmid (Sigma-Aldrich) based short hairpin RNA (shRNA) expression vector was used to induce gene silencing of eIF4E. This vector produces shRNA transcripts upon transfection, which specifically targets eIF4E transcripts for degradation utilising the endogenous RNAi machinery (Figure 4.4.1). Four different MISSION shRNA plasmid clones were available, all of which specifically target



Abbreviations:

cppt	Central polypurine tract	f1 ori	f1 origin of replication
hPGK	Human phosphoglycerate kinase eukaryotic promoter	pUC ori	pUC origin of replication
Puroʳ	Puromycin resistance gene for mammalian selection	5' LTR	5' long terminal repeat
SIN/LTR	3' self inactivating long terminal repeat	(Ψ) Psi	RNA packaging signal
Ampʳ	Ampicillin resistance gene for bacterial selection	RRE	Rev response element

Figure 4.4.1: Vector Diagram of pLKO.1-puro (sigma) eIF4E Knockdown Plasmid

A plasmid based short hairpin RNA (shRNA) expression vector was used to induce gene silencing. The MISSION shRNA clones obtained from Sigma (NM_001968.2-898s1c1 is shown) produce shRNA transcripts upon transfection, which specifically target eIF4E transcripts for degradation utilising the endogenous RNAi machinery. MISSION Non-target shRNA vector (sequence not shown) was used in control transfections.

Figure adapted from Sigma-Aldrich online catalogue

A

Available shRNA Plasmids

NM_001968 Organism: Human Symbol: EIF4E

NM_001968.2-**1310**s1c1
Sequence: CCGGCTGTTGTTAATGTTAGAGCTACTCGAGTAGCTCTAACATTAACAACAGTTTTTG

NM_001968.2-**1036**s1c1
Sequence: CCGGCGGCTGATCTCCAAGTTTGATCTCGAGATCAAACTTGGAGATCAGCCGTTTTTG

NM_001968.2-**898**s1c1
Sequence: CCGGCCGACTACAGAAGAGGAGAAACTCGAGTTTCTCCTCTTCTGTAGTCGGTTTTTG

NM_001968.2-**1426**s1c1
Sequence: CCGGCCAAAGATAGTGATTGGTTATCTCGAGATAACCAATCACTATCTTTGGTTTTTG

Figure adapted from Sigma-Aldrich online catalogue

B

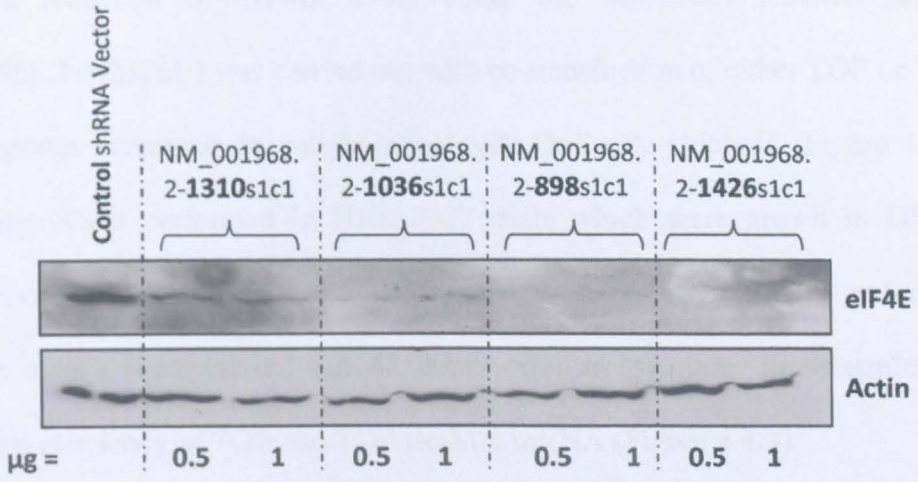


Figure 4.4.2: Selection of shRNA Plasmid

A. The catalogue number (Sigma-Aldrich) and sequence of the shRNA region of four available plasmids that target human eIF4E are shown.

B. Western blots confirming the knockdown of eIF4E compared to Actin (loading control) are shown for transfections carried out with either 0.5µg or 1µg of the candidate shRNA plasmid. The greatest reduction of eIF4E levels was observed for the plasmid with catalogue number 898 (column 3).

Work carried out in collaboration with Dr. L. Cobbold.

eIF4E (Figure 4.4.2A). In collaboration with Dr Laura Cobbold, transfections were carried out in HEK-293T cells, using 0.5 or 1 μg quantities of each MISSION eIF4E shRNA vector. MISSION Control non-target shRNA vector was used as a control. Transfected cells were incubated in DMEM supplemented with 10% FCS and cells were harvested 48 hours after transfection. Western analysis was then carried out to determine which MISSION shRNA plasmid produced the greatest reduction in eIF4E levels (Figure 4.4.2B). The data indicated that the NM_001968.2-898s1c1 clone produced the greatest reduction in eIF4E levels. This clone was therefore chosen for use in this experiment.

The reduction of eIF4E levels using the MISSION shRNA plasmid (NM_001968.2-898s1c1) was carried out with co-transfection of either TOP or TOP-trctMut reporter constructs in collaboration with Dr Laura Cobbold (Figure 4.4.3). Transfections were performed in HEK-293T cells which were grown in DMEM supplemented with 10% FCS and not subjected to serum starvation or re-feeding. Luciferase assays were carried out 48 hours after transfection, to determine the translational efficiency of TOP and TOP-trctMut mRNA (Figure 4.4.3).

TOP containing mRNA translational efficiency was found to increase by 100%, when eIF4E levels were reduced. During 4E-BP1 over-expression, translationally efficiency also increased to a level above that of the control (Figure 4.2A). In both cases the availability of unbound eIF4E is expected to be reduced and the two data sets obtained for the TOP construct are consistent. Interesting the data obtained from transfection of the TOP-trctMut construct also appears to show an increase in translational efficiency when the levels of eIF4E were reduced. This result was unexpected based upon the data obtained when 4E-BP1 was over-expressed. A reduction in the availability of eIF4E by the over-expression of 4E-BP1, previously

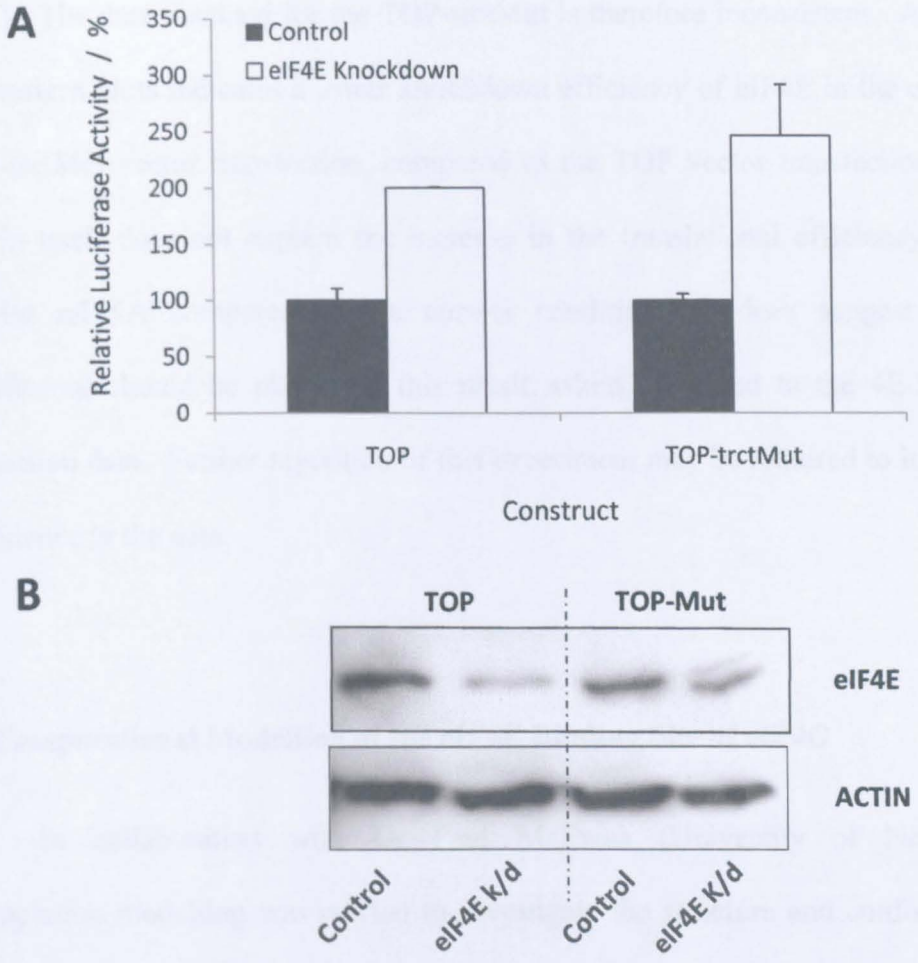


Figure 4.4.3: Translational Activity of TOP and TOP-trctMut Vectors upon eIF4E Knock-down in HEK293T Cells

A. TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with β-galactosidase expression vector (transfection control) and either a Non-Target Mission shRNA Control vector (Sigma) or an eIF4E-Target Mission shRNA vector (Sigma). The cells were harvested 48 hours after transfection and a luciferase assay was carried out. The ratio of Firefly luciferase to β-galactosidase (transfection control) activity was calculated and expressed as a percentage relative to control cells. Error bars were calculated from the standard deviation of three replicates and data was normalised to control cells.

B. Western blots confirming the knockdown of eIF4E compared to Actin (loading control) which appears evenly loaded under control and eIF4E knockdown conditions. Successful knockdown is shown for both TOP and TOP-Mut transfections.

Work carried out in collaboration with Dr. L. Cobbold.

resulted in a decrease in the translational efficiency of TOP-trctMut mRNA (Figure 4.2A). The data obtained for the TOP-trctMut is therefore inconsistent. Analysis of the western blots indicates a lower knockdown efficiency of eIF4E in the case of the TOP-trctMut vector transfection, compared to the TOP vector transfection. Whilst this in itself does not explain the increase in the translational efficiency of TOP-trctMut mRNA compared to the control conditions, it does suggest that less significance should be placed on this result, when compared to the 4E-BP1 over-expression data. Further repetition of this experiment may be required to increase the confidence in the data.

4.4 Computational Modelling of the eIF4E Binding Site of eIF4G

In collaboration with Dr Paul McEwan (University of Nottingham), computation modelling was carried to investigate the structure and conformation of the eIF4E binding site and its interaction with the 5' terminal m^7G cap and 5' UTR of mRNA. It was determined that the m^7G cap binding region of eIF4E contains a cleft, which allows eIF4E to interact with the first and second nucleotide adjacent to the mRNA cap structure (personal communication, data not shown). Whilst it has not been determined whether or not molecular recognition of these sites influences the binding of eIF4E to the cap structure, it is nevertheless interesting when considering the difference between TOP and TOP-trctMut mRNA. The 5'RACE procedure (detailed in Chapter 3) determined that the first and second nucleotides differ between mRNA transcribed by these constructs, and in a broader context, the first nucleotide adjacent to the cap structure differs between 5'TOP containing mRNAs and other cellular mRNAs.

4.5 eIF4G Knockdown and expression of eIF4G Mutants

To further investigate the canonical initiation factor requirements of TOP containing mRNAs, the level of eIF4GI was reduced in HeLa cells using a plasmid based RNAi system (Coldwell and Morley 2006). The RNAi plasmid (pSilencer31) was co-transfected with TOP or TOP-trctMut reporter constructs and one of two plasmids, which contained the coding region of eIF4GIf. This isoform was chosen as it is the longest form (1600 amino acids) and contains the poly(A)-binding protein (PABP) binding site (Coldwell and Morley 2006). The eI4G1f-WT plasmid was used to re-introduce eIF4G and the eIF4G1f-eIF4E plasmid was used to introduce a mutant form of eIF4G, which contained a deletion of the eIF4E binding site (Hinton et al. 2007) (Figure 4.5.1B). The mRNAs transcribed from these plasmids were resistant to targeting by the pSilencer31 siRNA, due to silent point mutations located within their coding sequence. These mutations were designed to reduce the base-complementarity between the target region of the eIF4G1f mRNA and the seed region of the targeting pSilencer31 siRNA. Successful reduction of eIF4GI expression and relative re-expression from eIF4GIf and its associated mutants, were confirmed by western blotting (Figure 4.5.2B). The translational efficiency of reporter mRNAs was then determined for each experiment by carrying out an assay to determine luciferase activity (Figure 4.5.2A). This enabled the requirement of eIF4GI and those factors prevented from binding to eIF4GI, to be identified for both TOP and non-TOP containing mRNA.

When eIF4GI levels were reduced without concurrent re-expression using the 4G1f vector, the translational efficiency of TOP containing mRNA appears to have increased by 20%. This increase falls within experimental error and therefore may not be significant. Under the same experimental conditions, the translational

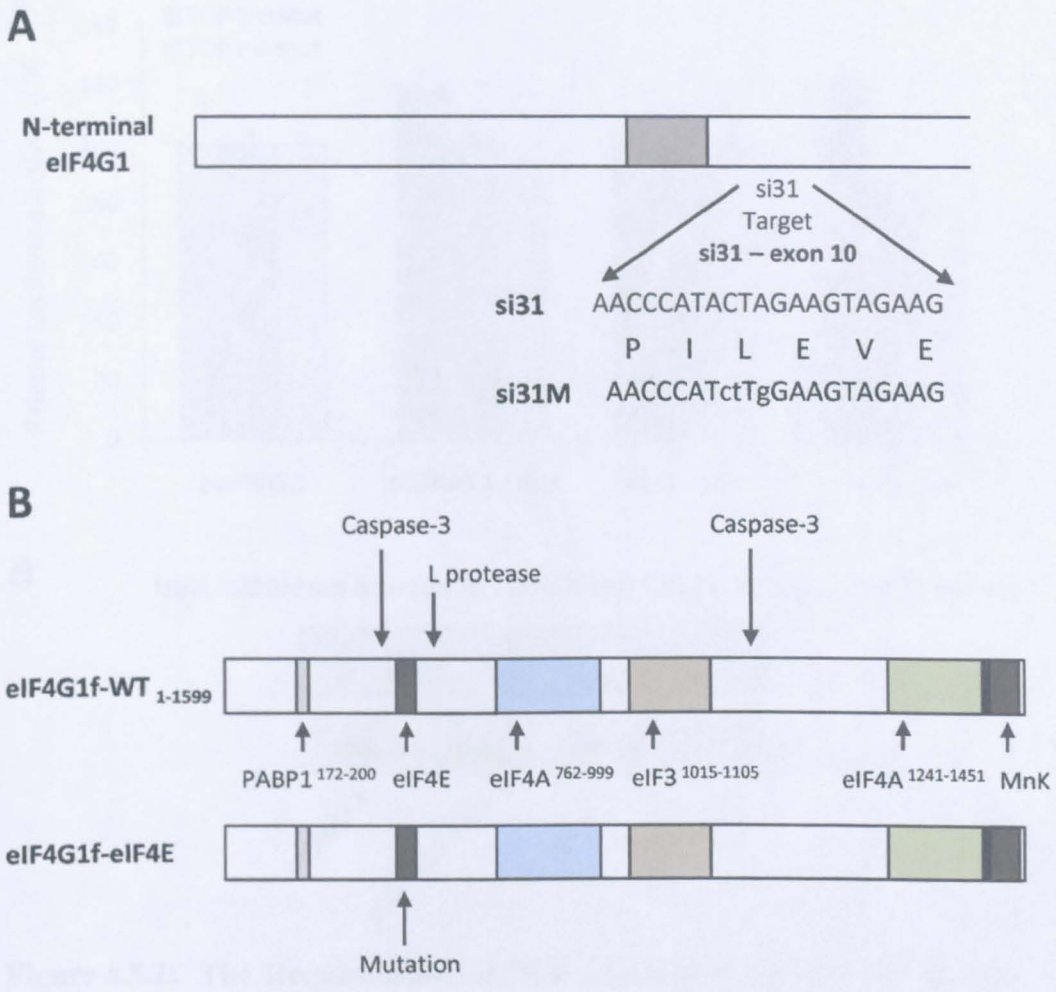


Figure 4.5.1: Schematic Diagrams Showing siRNA Target Site in the N terminus of eIF4G and eIF4G Mutant Construct.

Constructs were the kind gift of Dr. M. J. Coldwell (University of Southampton) and Dr. S. J. Morley (University of Sussex).

A. Oligonucleotide 31 corresponding to the siRNA target site was cloned into pSilencer 3.0 H1 plasmid (Ambion) to create a new plasmid. A mutated control version (si31M) was also created with three mismatches to the target site (Coldwell and Morley 2006).

B. Site directed mutagenesis was used to introduce mutations in the eIF4G nucleotide sequence as shown to disable the binding of eIF4E. Constructs were inserted into the pCDNA3.1-myc plasmid (Hinton et al. 2007).

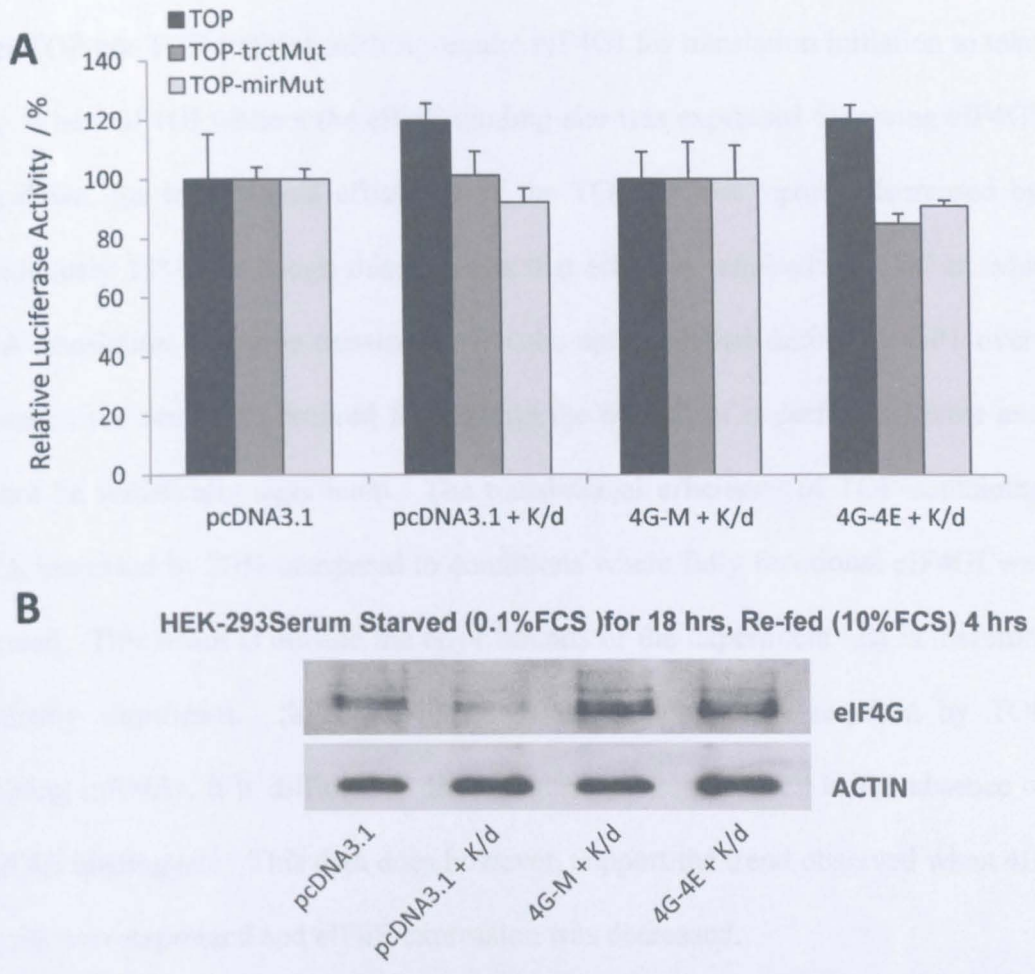


Figure 4.5.2: The Requirements of TOP Containing mRNAs for eIF4G and eIF4E

A. TOP, TOP-trctMut or TOP-mirMut vectors were co-transfected into HEK-293 cells with *Renilla* luciferase expression vector (transfection control) and either: 1) pcDNA3.1 and pSilencer31M (control); 2) pcDNA3.1 and pSilencer31; 3) pcDNA3.1-eIF4G1f-M and pSilencer31; 4) pcDNA3.1-eIF4G1f-eIF4e and pSilencer31. The eIF4G knockdown and replacement system has been described previously (Hinton, 2007). 4G-M corresponds to pcDNA3.1-eIF4G1f-M, which is resistant to the pSilencer31 knockdown plasmid. 4G-4E corresponds to the pcDNA3.1-eIF4G1f-eIF4E plasmid. Cells were serum starved (0.1% FCS) for 18 hrs and serum stimulated (10% FCS) for 4 hours immediately prior to harvesting (48 hours after transfection). A luciferase assay was carried out and the ratio of *Firefly* to *Renilla* luciferase activity was calculated. Error bars were calculated from the standard deviation of three replicates.

B. Western blotting was then carried out to determine the levels of eIF4G and Actin (loading control).

efficiency of TOP-trctMut mRNA remained relatively unchanged. This suggests that neither TOP nor TOP-trctMut mRNA, require eIF4GI for translation initiation to take place. When eIF4GI without the eIF4E binding-site was expressed following eIF4GI knock-down, the translational efficiency of the TOP-trctMut reporter decreased by approximately 15%. Although this suggests that eIF4E is required for TOP-trctMut mRNA translation, which is consistent with the data obtained during 4E-BP1 over-expression, the decrease observed falls within the bounds of experimental error and may not be statistically significant. The translational efficiency of TOP containing mRNA increased by 20% compared to conditions where fully functional eIF4GI was expressed. This result is outside the error bounds of the experiment and is therefore statistically significant. Since eIF4GI does not appear to be required by TOP containing mRNAs, it is difficult to directly attribute this increase to the absence of the eIF4E binding site This data does however, support the trend observed when 4E-BP1 was over-expressed and eIF4E expression was decreased.

Significant repression of translation for TOP-trctMut was not observed when eIF4GI levels were reduced or when the eIF4E binding-site deletion mutant was expressed. This data appears to suggest that cap-dependent initiation is not involved in the translational activation of TOP-trctMut mRNA and is therefore inconsistent with the result obtained when 4E-BP1 was over-expressed (Figure 4.2). Several limitations within the experimental method may be the cause of this inconsistency. Firstly, the reduction in the level of eIF4GI by the transfection of the pSilencer31 vector, should have no effect on the levels of the eIF4GII isoform of eIF4G. Western blotting was expected to detect both isoforms of eIF4G, which may explain why the reduction in eIF4G levels appears incomplete (Figure 4.5.2B). It is therefore possible that initiation of translation of TOP-trctMut mRNA may have occurred via a cap-

dependent pathway that utilised eIF4GII. Secondly, the TOP-trctMut mRNA contains only two adjacent nucleotide mutations compared to TOP containing mRNA. As a result of this, the TOP-trctMut mRNA may still possess some characteristics of TOP translation. To determine whether the data was specific to the non-TOP containing mRNA reporter, transfections were also carried out in parallel using the TOP-mirMut vector (Figure 4.5.2A). The data obtained for the TOP-mirMut was within 8% of that obtained for the TOP-trctMut construct and followed the same trend. The data obtained for TOP-mirMut translation following eIF4GI knockdown or eIF4GI mutant expression, was not therefore significantly different to control levels.

4.6 Candidate *Trans*-acting Factors

4.6.1 La Autoantigen

Previous research has suggested that the La autoantigen acts a specific *trans*-acting factor, involved in the translational regulation of TOP containing mRNA. La has been shown to interact with 5'TOP containing mRNAs from both humans and *Xenopis laevis*, including specific association with TOP containing mRNAs in the polysomal fraction (Pellizzoni et al. 1996; Cardinali et al. 2003). Although it is clear that La can modulate the translational behaviour of TOP containing mRNAs, its precise role and mode of action remains unresolved.

An experiment was therefore performed, to test the role of La in this system. HEK-293T cells were co-transfected with either an empty pcDNA3.1 vector or a titration of pcDNA3.1 containing the La coding region, alongside the TOP or TOP-trctMut constructs and a transfection control vector, which expressed Renilla

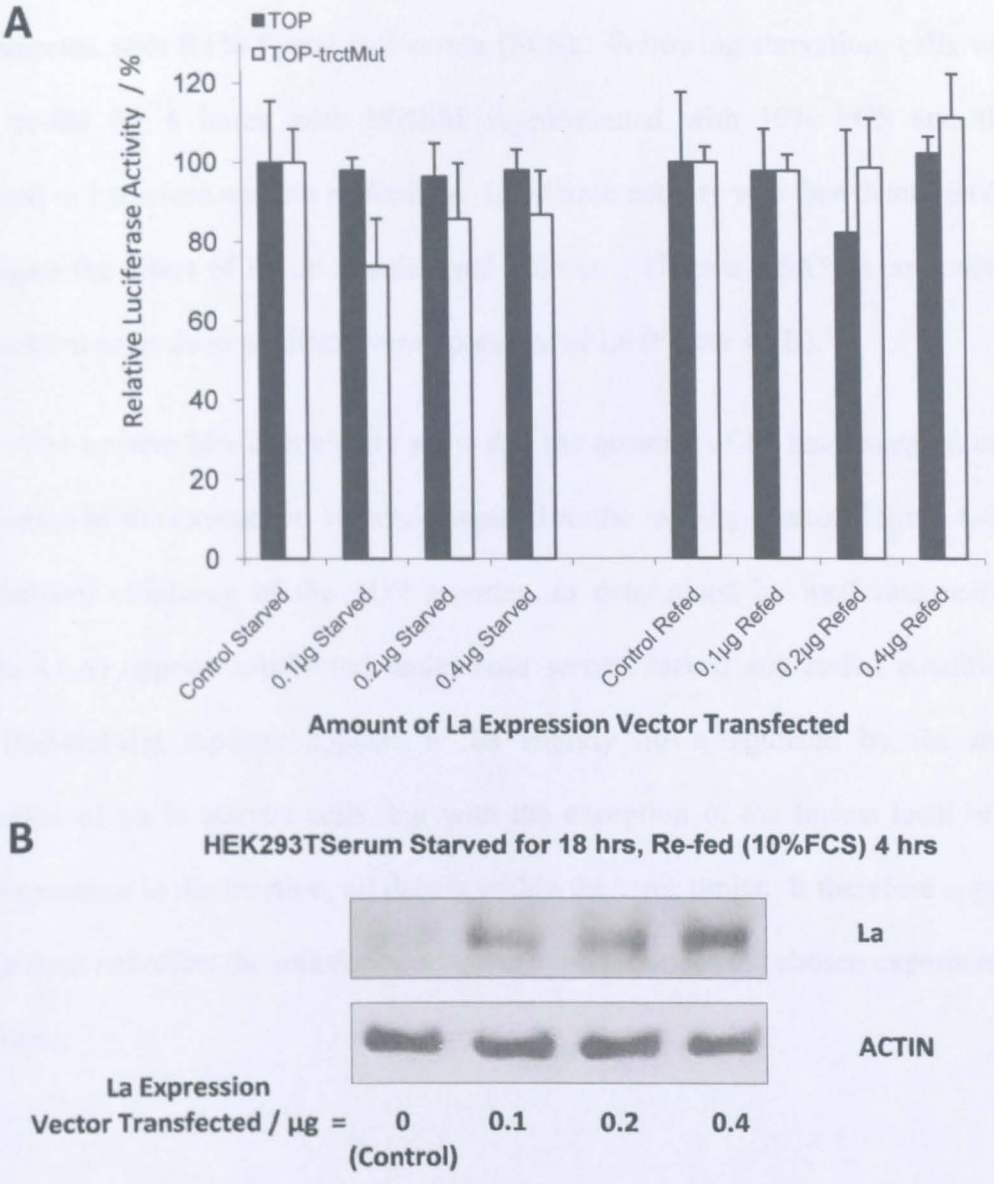


Figure 4.6: Translational Activity of TOP and TOP-TrctMut upon La Over-expression in HEK-293T Cells

A. TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with *Renilla* luciferase expression vector (transfection control) and either a pcDNA3.1 empty vector (control) or increasing concentrations of pcDNA3.1-La expression vector. Cells were serum starved (0.1% FCS) for 18 hrs or serum starved (0.1% FCS) for 18 hrs and serum stimulated (10% FCS) for 4 hours immediately prior to harvesting (48 hours after transfection). A luciferase assay was carried out and the ratio of *Firefly* to *Renilla* luciferase activity was calculated. Error bars were calculated from the standard deviation of three replicates.

B. Western blotting was then carried out to determine the levels of La and Actin (loading control).

luciferase. After 24 hours the cells were serum starved for 18 hours in DMEM supplemented with 0.1% Foetal calf serum (FCS). Following starvation, cells were either re-fed for 4 hours with DMEM supplemented with 10% FCS and then harvested or harvested without re-feeding. Luciferase activity was then determined to investigate the effect of La on translational efficiency (Figure 4.6A), in conjunction with western analysis to confirm over-expression of La (Figure 4.6B).

The western blot data clearly show that the quantity of La has increased upon transfection of the expression vector, compared to the loading control (Figure 4.6B). Translational efficiency of the TOP reporter, as determined by luciferase activity (Figure 4.6A) appears unaffected under both serum starved and re-fed conditions. The TOP-trctMut reporter appears to be slightly down-regulated by the over-expression of La in starved cells, but with the exception of the lowest level of La over-expression in the titration, all data is within the error limits. It therefore appears that La does not affect the translation of either reporter under the chosen experimental conditions.

4.6.2 PTB (Polypyrimidine-Tract-Binding Protein) and hnRNP-K

Polypyrimidine-tract binding protein (PTB) is a multi-function protein, which preferentially binds polypyrimidine-rich regions of mRNA (Garcia-Blanco et al. 1989). The functions of PTB are dependent on its location within the cell and are known to include involvement in translation initiation (Jang and Wimmer 1990). The four distinct RNA recognition motifs (RRMs) of PTB, have the ability to bind a different polypyrimidine-rich consensus sequence simultaneously within the same RNA molecule (Auweter et al. 2007). PTB is therefore a potential candidate for

ribosome recruitment in 5'TOP containing mRNAs, which by definition also possess a polypyrimidine tract.

To investigate the role of PTB in this system, HEK-293T cells were co-transfected with either an empty pcDNA3.1 vector or a titration of pcDNA3.1 containing the PTB coding region, in conjunction with the TOP or TOP-trctMut constructs and a transfection control vector, which expressed Renilla luciferase. After 24 hours the cells were serum starved for 18 hours in DMEM supplemented with 0.1% FCS. Following starvation, cells were either re-fed for 4 hours with DMEM supplemented with 10% FCS and then harvested, or harvested without re-feeding. Luciferase activity was then determined to investigate the effect of PTB on translational efficiency (Figure 4.7.1A), in conjunction with western analysis to confirm over-expression of PTB (Figure 4.7.1B).

When PTB is over-expressed luciferase activity derived from either TOP or TOP-trctMut vector, shows a similar trend regardless of whether the cells are serum starved or re-fed (Figure 4.7.1A). Both TOP and TOP-trctMut reporter transcribed mRNAs appear to be translationally activated by PTB over-expression, with the effect increasing with the amount of PTB expression vector transfected. Under both serum starved and re-fed conditions, transfection of the largest quantity of PTB expression vector resulted in an approximate increase in translational activity of 80% for the TOP reporter and 40% for the TOP-trctMut reporter. Since both reporter mRNAs posses a polypyrimidine-tract in their 5' UTR, it is perhaps unsurprising that both behave in a similar manner (5' UTR confirmed by RACE analysis in Chapter 3). The greatest effect is observed for the TOP construct which possesses an uninterrupted polypyrimidine tract that is two nucleotides longer. The data therefore suggests that

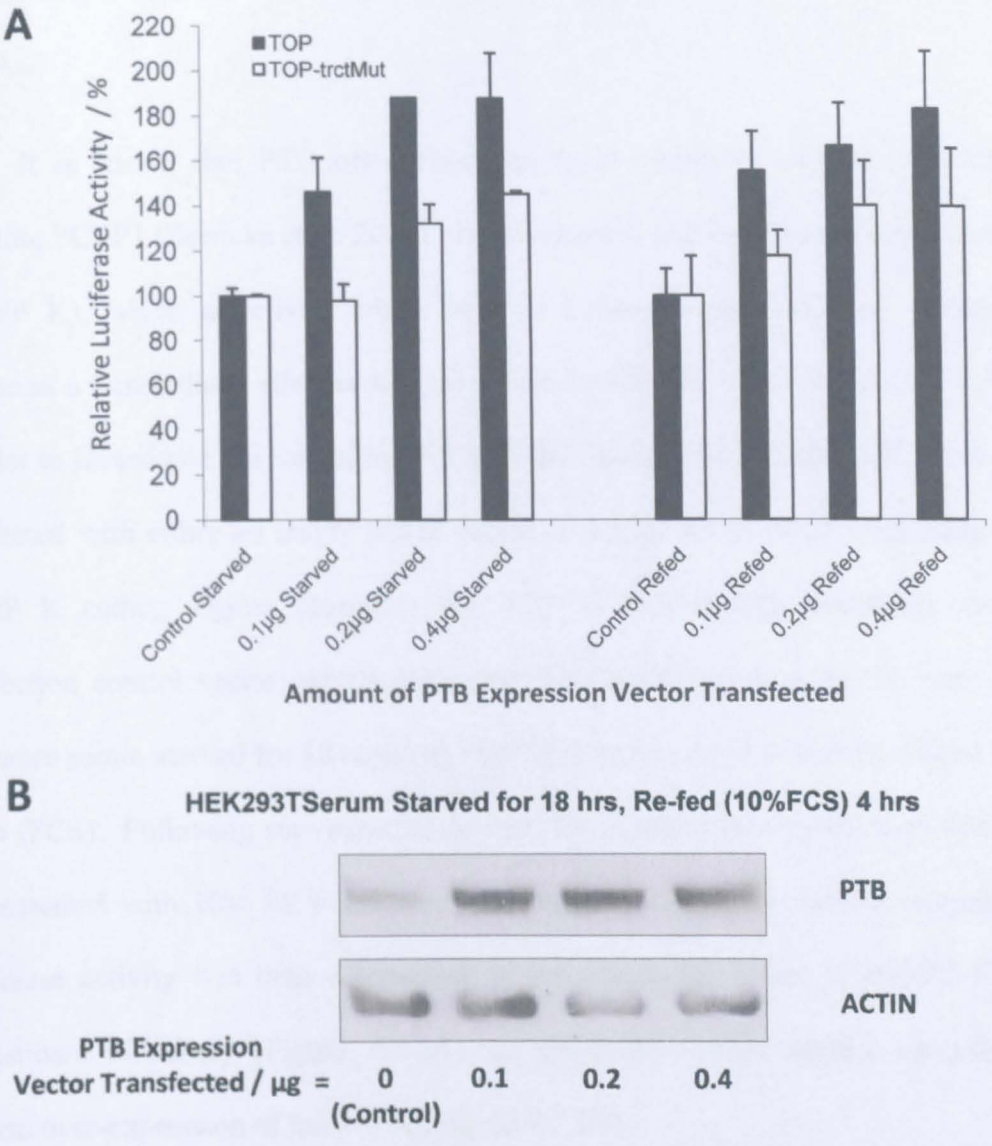


Figure 4.7.1: Translational Activity of TOP and TOP-TrctMut upon PTB Over-expression HEK-293T Cells

A. TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with *Renilla* luciferase expression vector (transfection control) and either a pcDNA3.1 empty vector (control) or increasing concentrations of pcDNA3.1-4E-BP1 expression vector. Cells were serum starved (0.1% FCS) for 18 hrs or serum starved (0.1% FCS) for 18 hrs and serum stimulated (10% FCS) for 4 hours immediately prior to harvesting (48 hours after transfection). A luciferase assay was carried out and the ratio of *Firefly* to *Renilla* luciferase activity was calculated. Error bars were calculated from the standard deviation of three replicates.

B. Western blotting was then carried out to determine the levels of PTB and Actin (loading control).

under the experimental conditions, PTB does up-regulate the translation of both these mRNAs.

It is known that PTB often functions in co-operation with other proteins including PCBP1 (Sawicka et al. 2008). Heterogeneous nuclear ribonuclear protein K (hnRNP K), which selectively binds poly(C) homopolymer, has been shown to operate as a translational silencer in conjunction with PCBP1 (Ostareck et al. 1997). In order to investigate the role of hnRNP K in this system, HEK-293T cells were co-transfected with either an empty pSG5 vector or a titration of pSG5 containing the hnRNP K coding region, alongside the TOP or TOP-trctMut constructs and a transfection control vector, which expressed Renilla luciferase. After 24 hours the cells were serum starved for 18 hours in DMEM, supplemented with 0.1% Foetal calf serum (FCS). Following starvation, cells were either re-fed for 4 hours with DMEM supplemented with 10% FCS and then harvested, or harvested without re-feeding. Luciferase activity was then determined to investigate the effect of hnRNP K on translational efficiency (Figure 4.7.2A), in conjunction with western analysis to confirm over-expression of hnRNP K (Figure 4.7.2B).

The over-expression of hnRNP K also shows a similar trend for both serum starved and serum starved and re-fed cells. The data appears to show an approximate 10% average decrease in the luciferase activity of the TOP-trctMut reporter. Translational activity of the TOP reporter mRNA appears to increase with the amount of hnRNP K expression vector transfected, reaching a maximum increase of 30%. However, this increase is within the error limits of the experiment and is therefore, not statistically significant.

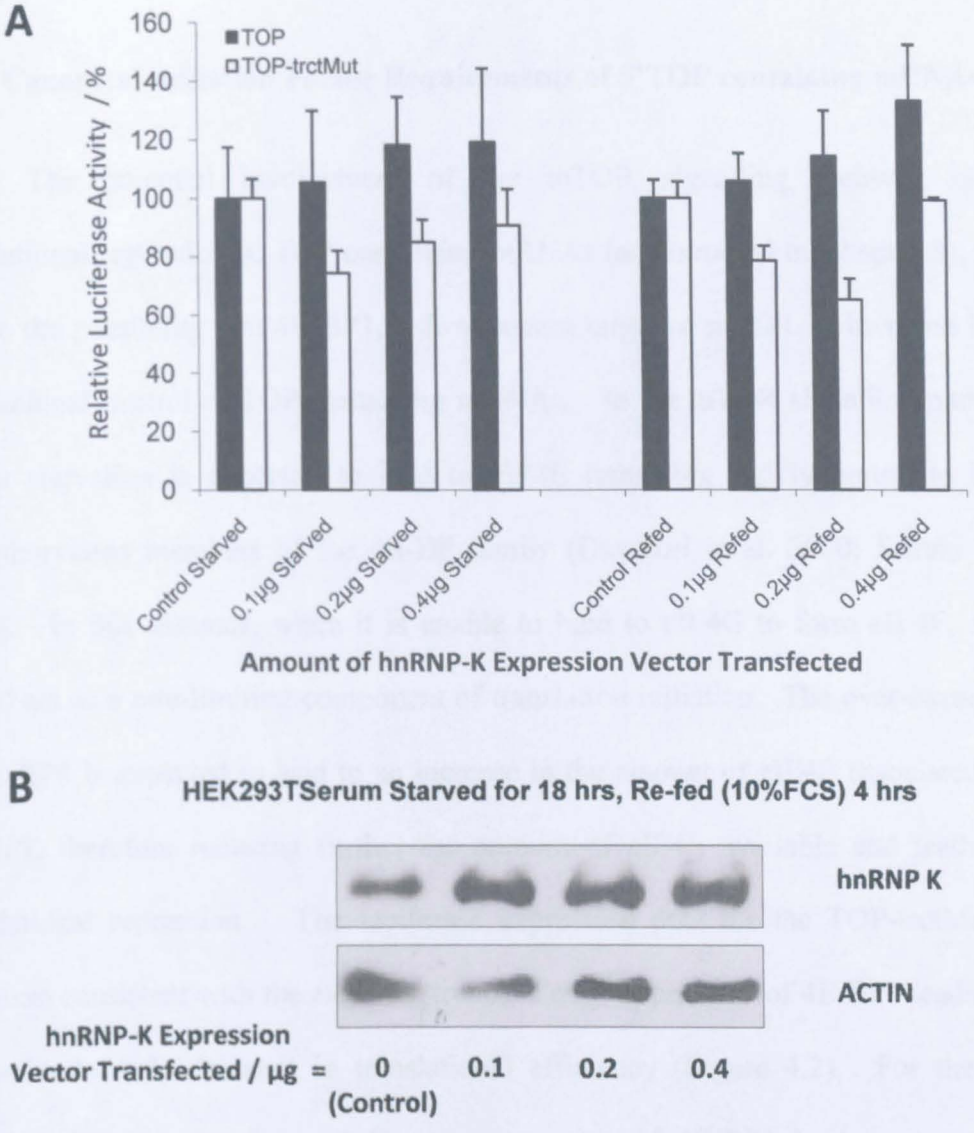


Figure 4.7.2: Translational Activity of TOP and TOP-TrctMut upon hnRNP-K Over-expression in HEK-293T Cells

A. TOP or TOP-trctMut vectors were co-transfected into HEK-293T cells with *Renilla* luciferase expression vector (transfection control) and either a pSG5 empty vector (control) or increasing concentrations of pSG5-hnRPP-K expression vector. Cells were serum starved (0.1% FCS) for 18 hrs or serum starved (0.1% FCS) for 18 hrs and serum stimulated (10% FCS) for 4 hours immediately prior to harvesting (48 hours after transfection). A luciferase assay was carried out and the ratio of *Firefly* to *Renilla* luciferase activity was calculated. Error bars were calculated from the standard deviation of three replicates.

B. Western blotting was then carried out to determine the levels of hnRNP-K and Actin (loading control).

4.7 Discussion

4.7.1 Canonical Initiation Factor Requirements of 5'TOP containing mRNAs

The potential involvement of the mTOR signalling pathway in the translational regulation of TOP containing mRNAs (as discussed in Chapter 3), gives rise to the possibility that 4E-BP1, a downstream target of mTOR, is involved in the translational control of TOP containing mRNAs. In the mTOR signalling pathway, serum starvation is expected to lead to eIF4E remaining tightly bound to hypo-phosphorylated members of the 4E-BP family (Davuluri et al. 2000; Suzuki et al. 2000). In this instance, when it is unable to bind to eIF4G to form eIF4F, eIF4E would act as a rate-limiting component of translation initiation. The over-expression of 4E-BP1 is expected to lead to an increase in the amount of eIF4E associated with 4E-BP1, therefore reducing further the amount of eIF4E available and leading to translational repression. The luciferase expression data for the TOP-trctMut are therefore consistent with the expected trend, as over-expression of 4E-BP1 leads to an approximate 40% decrease in translational efficiency (Figure 4.2). For the TOP construct however, an increase in over-expression of 4E-BP1 leads to an overall increase in translational efficiency. This is contrary to the expected trend and raises the possibility that the availability of eIF4E is not a rate-limiting factor in the translation initiation of 5'TOP containing mRNAs.

Analysis of the data obtained from sucrose density gradient analysis (Figures 4.3.1 and 4.3.2), appears to corroborate the trend that reduced eIF4E availability does not result in a down-regulation of TOP reporter mRNA as expected, but instead results in the apparent up-regulation of translation. This data also appears to be supported by the observation that the translational efficiency of the mRNA

transcribed by the TOP construct, was found to increase by 100% when eIF4E levels were reduced (Figure 4.4.2). A possible explanation for the observed trend is that TOP containing mRNA translation may utilise an alternative mechanism of translation initiation, which does not require eIF4E. When the availability of unbound eIF4E is reduced, either by knock-down or 4E-BP1 over-expression, the translation efficiency of non-TOP containing mRNAs also reduces. As this occurs, it is possible that other factors bound to non-TOP containing mRNAs may be released. This newly available pool of factors could then be utilised by TOP containing mRNAs, resulting in an increase in their translational efficiency. Surprisingly, the data obtained from transfection of the TOP-trctMut construct also appears to show an increase in translational efficiency when the levels of eIF4E were reduced. This result was inconsistent with the data obtained when 4E-BP1 was over-expressed and was not expected based upon the known involvement of eIF4E in cap-dependent initiation. Analysis of the western blots indicated a low knockdown efficiency of eIF4E in the case of the TOP-trctMut vector transfection compared, which may explain this result. It is also possible that the mutations introduced into the TOP-trctMut mRNA were insufficient to distinguish it from TOP mRNA and render it under cap-dependent translational control. Repetition of this experiment, with improved reduction of eIF4E levels in the case of the TOP-trctMut transfection, would help to increase the confidence in this data.

This potential lack of involvement of eIF4E in the translation initiation of TOP containing mRNAs, appears to be consistent with the result of an earlier study, in which eIF4E was over-expressed. It was found that in quiescent cells, over-expression of eIF4E did not relieve the translational expression of ribosomal protein mRNAs, which are a subset of TOP containing mRNAs (Mamane et al. 2007).

It remains uncertain as to whether the phosphorylation state of eIF4E plays a role in the regulation of translation initiation and this has been the subject of considerable debate (Scheper and Proud 2002). It is known that eIF4E is phosphorylated by MAP-kinase signal-integrating kinases Mnk1 and Mnk2 (Flynn and Proud 1995). However, it has been shown than mice which have both Mnk1 and Mnk2 genes deleted, are apparently normal (Ueda et al. 2004). This suggests that both Mnk genes function in adaptive pathways during conditions of cellular stress. More interestingly in the context of TOP containing mRNAs, the phosphorylation of eIF4E is increased by serum treatment of cells and addition of growth factors, in a process which appears to be mediated via the MEK/Erk pathway (Flynn and Proud 1996). Two distinct models have been proposed for the role of eIF4E phosphorylation in translation initiation (Scheper and Proud 2002). In both models phosphorylation of eIF4E decreases its affinity for capped RNA, which is consistent with observations in which high levels of eIF4E phosphorylation impair cap dependent translation (Scheper et al. 2002). The point in the initiation process at which eIF4E phosphorylation occurs differs between the two models. Another important difference is whether eIF4E phosphorylation is required to enhance initiation on the same mRNA or on another message (Scheper and Proud 2002). Neither model appears to explain the apparent lack of eIF4E involvement in the translation initiation of TOP containing mRNAs in this system.

It is interesting that TOP containing mRNAs, which generally have relatively short 5'UTRs, may potentially have little or no requirement for eIF4E. It has been shown that the m^7G cap structure does not significantly contribute to the binding of eIF4F to mRNAs, which have 5'UTRs greater than 60 nucleotides in length and intrinsic RNA binding by eIF4F occurs for these mRNAs (Kaye et al. 2009). With a

5' UTR length of 41 nucleotides, the mRNA transcribed by the TOP construct does not fulfil the criteria for intrinsic RNA binding by eIF4F. The model would instead predict a requirement for cap recognition by eIF4E in this system, which does not appear to be supported by the data obtained. While this difference lends supports the suggestion that TOP containing mRNAs do not require eIF4E, it also raises the question as to whether or not eIF4F is required.

The data obtained for the experiments in which eIF4GI levels were reduced, raises the possibility that TOP containing mRNAs do not require eIF4GI for the initiation of translation. The translational efficiency of TOP mRNA increased by 20% under conditions in which eIF4GI levels were reduced without the concurrent re-expression using the 4G1f vector. It is possible that this increase in translational efficiency is due to TOP containing mRNAs utilising other factors for translation initiation, which prior to the reduction in eIF4GI levels, were bound to non-TOP containing mRNAs. However, the data also shows that the translation activity of TOP-trctMut and TOP-mirMut transcribed mRNAs remained unaffected by the reduction in eIF4GI levels, challenging this proposal. The translational activity of TOP-trctMut and TOP-mirMut mRNAs also remained unchanged when eIF4GI without the eIF4E binding-site was expressed following eIF4GI knock-down. As previously discussed in section 4.5, the reduction in the level of eIF4GI by the transfection of the pSilencer31 vector, should have no effect on the levels of the eIF4GII isoform of eIF4G. It is therefore possible that initiation of translation of TOP-trctMut mRNA may have occurred via a cap-dependent pathway that utilised eIF4GII. An increase in translational activity of TOP mRNA was observed when eIF4GI without the eIF4E binding-site was expressed following eIF4GI knock-down. This result appears to be consistent with the broader suggestion that neither eFI4GI

nor eIF4E are required for the initiation of translation of TOP containing mRNAs. However, before drawing a firm conclusion it may be necessary to repeat this experiment with the simultaneous reduction in the level of both eIF4GI and eIF4GII. This would provide a clearer point of comparison with TOP-trctMut mRNA and would also reduce a critical variable in the reduction of eIF4G levels.

Consideration of the similarity in behaviour of the TOP-trctMut and TOP-mirMut transcribed mRNAs in this experiment raises interesting questions about how the 5'TOP motif is recognised. Despite the mutations being located in different regions of the 5' UTR, both constructs behaved in a similar manner, which was not the same as that observed for TOP construct transcribed mRNA. From the data available, it is not possible to determine whether the mutations affect the same or different processes within the TOP translation pathway. The data raises the possibility however, that both the mutated region within the potential miR-10a binding site and the two nucleotides immediately adjacent to the m^7G cap structure, play a pivotal role in the regulation of TOP containing mRNAs.

4.7.2 *Trans*-acting Factor Requirements of 5' TOP containing mRNAs

The data obtained upon over-expression of La, suggests that La has no effect on the translation of mRNA transcribed by both TOP and TOP-trctMut constructs, under the experimental conditions used in this system. It has previously been shown using sucrose density gradient analysis, that over-expression of wild-type La causes RPL4 mRNA to shift from sub-polysomes to polysomes in growth-arrested cells (Crosio et al. 2000). These experiments and others (more thoroughly reviewed in Chapter 1) have led to a model being proposed, in which the specific interaction of La

with TOP containing mRNAs results in translational up-regulation of these mRNAs. However, this model has been challenged and conflicting evidence exists. TOP containing mRNAs have been found to associate preferentially with phosphorylated La (reviewed in Chapter 1), and over-expression of a non-phosphorylatable La has been shown to increase the abundance of subpolysomal TOP containing mRNAs (Schwartz et al. 2004). Studies carried out *in vitro*, have also shown that La can inhibit TOP containing mRNA translation (Zhu et al. 2001). The data obtained in this study, does not appear to resolve this dichotomy. It is possible that the behaviour observed is related to the sequence of the 5' UTR of the TOP under study, rather that all TOP containing mRNAs as a group.

It has been suggested that La may act in competition with cellular nucleic acid binding protein (CNBP). Both La and CNBP have been shown in *Xenopus* to bind 5'TOP containing mRNA in a mutually exclusive manner with the co-operation of the autoantigen Ro60 (Pellizzoni et al. 1998; Crosio et al. 2000). This has led to the proposal that La exerts a positive effect on translation through competitive binding with CNBP, which potentially acts a translational repressor (Crosio et al. 2000). It may therefore be of interest to determine whether or not CNBP plays a role in 5'TOP containing mRNA translation in this system, especially since it appears that the cap-recognition complex may not be required for TOP containing mRNA translation.

The data obtained for PTB over-expression shows that both TOP and TOP-trctMut constructs, were translationally up-regulated under the conditions used in this system. This suggests that PTB acts by binding to the polypyrimidine tract as expected. This data appears inconsistent with other studies, which have shown that when vectors expressing TOP containing mRNAs were co-transfected alongside a vector expressing PTB, over-expression of PTB did not affect the translational

efficiency of TOP containing mRNAs (Meyuhas 2000). Translational up-regulation was greatest for mRNA transcribed by the TOP constructs. This may be due to PTB binding to the relatively longer polypyrimidine tract of the TOP transcribed mRNA, compared to the TOP-trctMut mRNA. It is possible that this increased polypyrimidine track length of 2 nucleotides is sufficient to increase the likelihood of PTB binding the same mRNA multiple times. It is known that when multiple binding of PTB occurs, this may give rise to the formation of RNA loop structures, especially when these sites are separated within the RNA molecule (Petoukhov et al. 2006). Ribosomal recruitment in internal ribosome entry site (IRES)-mediated translation initiation, is believed to require such conformational changes (Mitchell et al. 2003). Indeed, it has been shown under conditions that inhibit cap-dependent translation, most cellular IRESs require PTB to function, leading to the proposal that PTB is a general IRES *trans*-acting factor (ITAF)(Mitchell et al. 2005). Ribosome recruitment mediated by PTB, has been found to require the consensus sequence $(CCU)_n$ as part of a polypyrimidine tract (Mitchell et al. 2005). This may provide a potential explanation for the increase in translational efficiency of the mRNA transcribed by both the TOP and TOP-trctMut constructs.

An interesting follow-up experiment would be to investigate the effect upon the translation of TOP and TOP-trctMut mRNAs, following a directed reduction in PTB expression levels using siRNA. If PTB is required for the translation of TOP containing mRNAs, the data would be expected to indicate a reduction of TOP mRNA translation following PTB knockdown. A similar experiment has been carried out in which the translational of endogenous TOP containing mRNAs was examined following a reduction in PTB expression, which was mediated by siRNA transfection (personal communication from K. Sawicka, University of Nottingham, data not

shown). The data obtained from sucrose density gradients and northern blotting, indicated that ribosomal proteins RPS6 and RPS28 were translationally down-regulated when PTB expression was reduced. Both endogenous RPS6 mRNA and the mRNA transcribed by the TOP construct share a common 5'UTR sequence. It would therefore be interesting to repeat this experiment with co-transfections of both the TOP and TOP-trctMut constructs.

Investigation of the effect of hnRNP K over-expression on TOP and TOP-trctMut construct transcribed mRNAs, suggests that the TOP containing mRNA is relatively unaffected in both serum starved and serum starved and re-fed cells. These data are consistent with review literature, which citing unpublished data, indicates that hnRNP K does not act to repress the translation of TOP containing mRNAs (Meyuhas 2000). It would perhaps be interesting to repeat this experiment with simultaneous expression of hnRNP E1, which has been shown to operate in conjunction with hnRNP K as a translational silencer (Ostareck et al. 1997).

4.7.3 Translational Differences between Sub-sets of 5' TOP containing mRNAs

It is apparent from previous studies carried out involving La and CNPB, that conflicting models exist regarding the role of these *trans*-acting factors in the translational regulation of 5'TOP containing mRNAs. In these studies (discussed in more detail in Chapter 1), the differing results were obtained from experiments, which investigated the regulation of different TOP containing mRNAs. This raises the possibility that different TOP containing mRNAs respond differently to different factors and that sub-sets of 5'TOP containing mRNAs may exist. Further evidence for the potential existence of TOP containing mRNAs sub-sets, was revealed in the

microarray analysis of polysomal mRNA obtained from an eIF4E-inducible cell line (Mamane et al. 2007). Novel eIF4E targets were identified that were involved in ribosome biogenesis, cell proliferation and survival. Despite earlier studies suggesting that eIF4E could stimulate ribosomal protein mRNA translation both in vivo and in vitro (Kaspar et al. 1990; Hammond et al. 1991), eIF4E over-expression affected the translation of only a subset of TOP containing mRNAs. It would therefore be of interest to repeat many of the experiments carried out thus far, with a selection of constructs which contain the 5'UTRs of other known 5'TOP containing mRNAs.

4.7.4 Direct Determination of the *Trans*-acting Factor Requirements of 5'TOP containing mRNAs

In the experiments described thus far, the activity of TOP and non-TOP containing mRNA was compared under a variety of conditions. No direct attempt has been made to directly identify the specific factors which bind to 5'TOP containing mRNAs. The recent identification of microRNA-10a as a potential enhancer of TOP containing mRNA translation was achieved using an affinity purification approach. This method utilised biotin-tagged miRNA, which after binding to its mRNA target, was purified using Streptavidin beads (Orom et al. 2008). The application of a similar technique to 5'TOP containing mRNAs may facilitate the purification of such mRNAs and any associated protein complexes that may be required for their translation.

Chapter Five

Identification of Factors Which Bind and Control TOP Containing mRNAs

5.1 Introduction

Recently, through the use of affinity purification using biotin-tagged miRNA, it has been shown that miRNAs associate with 5'TOP containing mRNAs (Orom et al. 2008). The experiment itself was not designed to specifically identify 5'TOP containing mRNAs, but instead utilised affinity purification of a microRNA-10a to specifically identify all mRNAs, which were directly associated with it. There are however no reported instances of the application of this technique being reversed to specifically identify factors, which bind to 5'TOP containing mRNAs. It would therefore be of great interest to develop a technique in which 5'TOP containing mRNAs are specifically purified whilst bound to factors, which are potentially involved in their regulation. These factors could then be identified using mass spectrometry, thus providing a list of candidates that are potential regulators of 5'TOP containing mRNA translation.

5.2 Development of an MS2 Fusion Protein Pull-down System

5.2.1 Purification of 6xHis-MS2 Fusion Protein

Affinity purification of mRNAs can be achieved by the use of tagged-fusion proteins, which specifically bind to sites that have been deliberately incorporated into the mRNA. Fusion proteins of MS2 bacteriophage may be used to pull-down mRNAs that contain MS2 binding sites. Indeed, it has been shown that when two MS2

binding sites are incorporated at the 3' end of mRNA splicing substrates, purified MS2-GST (glutathione *S*-transferase) fusion protein can be used to specifically pull-down *in vitro*-assembled spliceosomes (Zhang and Krainer 2007). In order to utilise a similar technique in this system, the TOP and TOP-trctMut constructs were designed to transcribe mRNAs that contain binding sites for the bacteriophage MS2 coat protein within their 3' UTR (Witherell et al. 1991).

Three MS2 binding sites were inserted into the XbaI site of the TOP and TOP-trctMut reporter constructs (Figure 5.1.1). Each binding site contains a 19 nucleotide stem-loop which is specifically bound by the coat protein of the RNA bacteriophage MS2 (Witherell et al. 1991). The MS2 binding sites were obtained by PCR from the construct pcDNA3.1-MS2x3 (previously created in the lab). The sites were spaced 13 nucleotides apart to reduce steric hindrance that may prevent multiple protein binding. The sites were also located 70 nucleotides downstream of the firefly luciferase stop codon, in order to reduce interference with termination of translation (Figure 5.1.2A). The endogenous MS2 binding site contains adjacent uracil residues within the hairpin loop, whereas the TOP and TOP-trctMut transcribed mRNAs have been designed to contain hairpin structures in which one uracil is replaced with cytosine (Figure 5.1.2B). This modification has been shown to result in at least a 50-fold increase in the binding affinity of the MS2 protein (Lowary and Uhlenbeck 1987).

A 6xHis-MS2 fusion protein was selected for use in the mRNA pull-down experiment. The presence of polyhistidine-tags enables the MS2 fusion protein to be affinity purified following expression in *E.coli.* through the use of metal chelate chromatography. It has been shown that the relatively high affinity of the polyhistidine-tags for both Co^{2+} and Ni^{2+} metal ions, enables the fusion protein to be

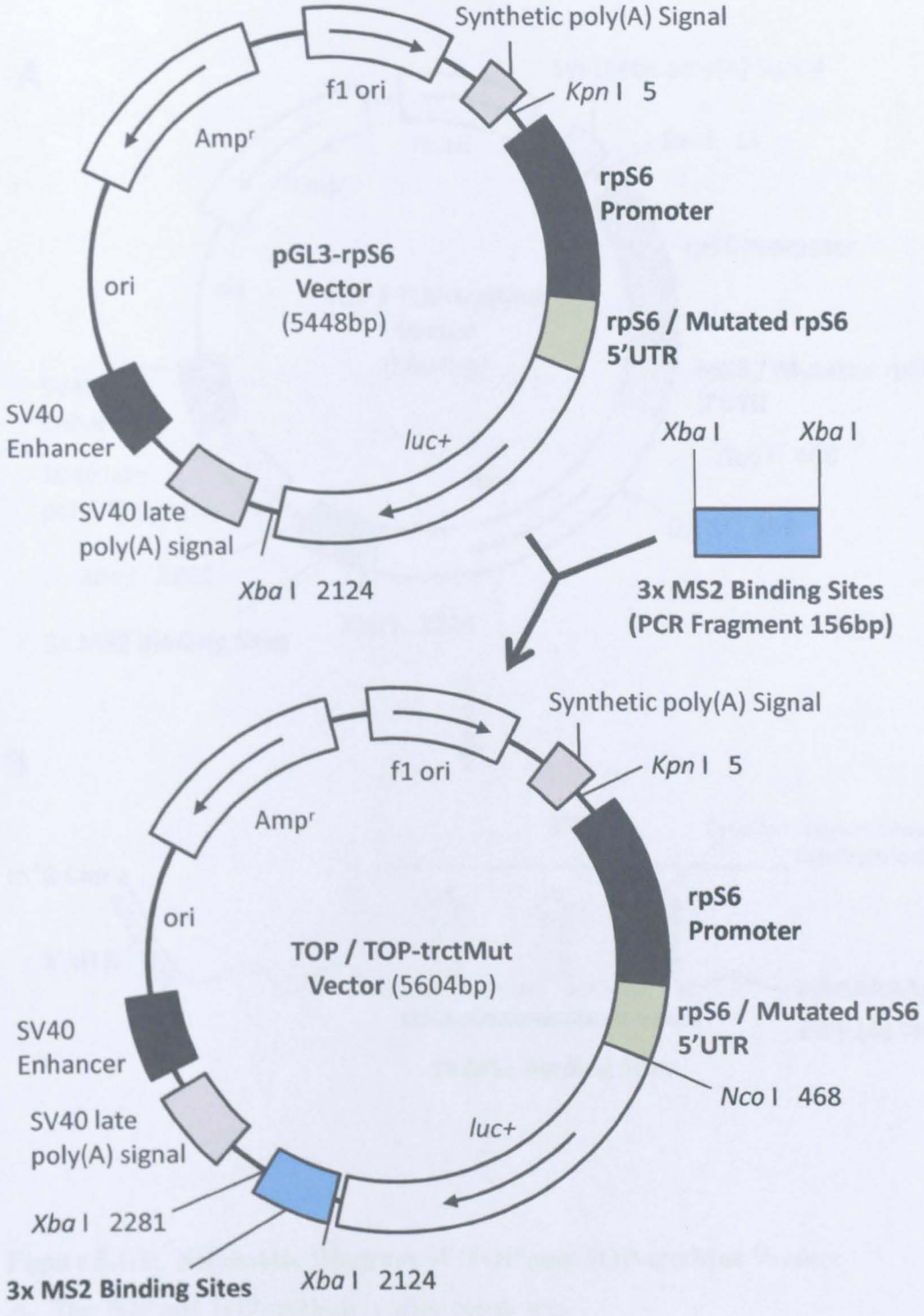


Figure 5.1.1: Final Construction of TOP and TOP-trctMut Vectors

The MS2 binding site fragment (obtained by PCR from pcLuc-mS2x3) was digested with *Xba*I. This was then ligated into the pGL3-rpS6 vector, which had been retransformed in INV110 competent cells, digested with *Xba*I and dephosphorylated. DNA sequencing was carried out to select clones with the correct fragment orientation.

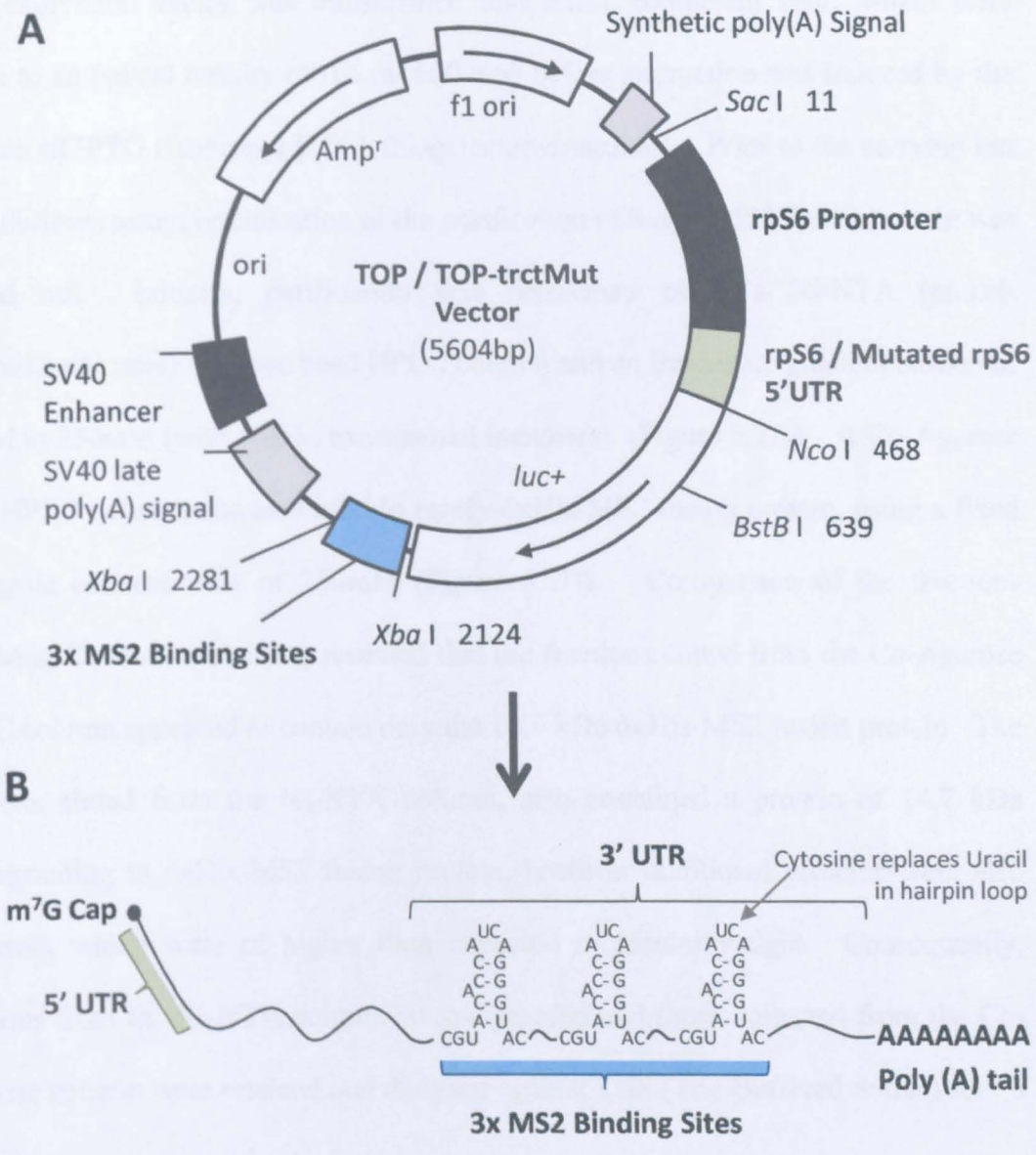


Figure 5.1.2: Schematic Diagram of TOP and TOP-trctMut Vectors

A. The TOP and TOP-trctMut vector constructs.

B. The transcription products of both the TOP and TOP-trctMut vector constructs, contain three MS2 binding sites within their 3' UTR. The MS2 binding sites contain hairpin structures in which one uracil is replaced with cytosine. This modification has been shown to result in at least a 50-fold increase in the binding affinity of the MS2 protein (Lowary and Uhlenbeck 1987).

separated from bacterial proteins with up to 95% purity (Hengen 1995) A 6xHis-MS2 expression vector was transformed into BL21 competent cells, which were grown to an optical density of 0.6 (at 600 nm) before expression was induced by the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Prior to the carrying out the pull-down assay, optimisation of the purification of 6xHis-MS2 fusion protein was carried out. Initially, purification was performed using a Ni-NTA (nickel-nitrilotriacetic acid) Agarose bead HPLC column and an Imidazole gradient elution of 50mM to 250mM (with 50mM incremental increases) (Figure 5.2A). A Co-Agarose bead HPLC column was also used to purify 6xHis-MS2 fusion protein, using a fixed Imidazole concentration of 250mM (Figure 5.2B). Comparison of the fractions following Coomassie staining revealed that the fractions eluted from the Co-Agarose HPLC column appeared to contain only the 14.7 kDa 6xHis-MS2 fusion protein. The fractions eluted from the Ni-NTA column, also contained a protein of 14.7 kDa corresponding to 6xHis-MS2 fusion protein, however additional proteins were also observed, which were of higher than expected molecular weight. Consequently, fractions from the Ni-NTA column were discarded and those collected from the Co-Agarose column were retained and dialysed against TBS (Tris Buffered Saline) for 18 hours.

5.2.2 Optimisation of 6xHis-MS2 Protein-mRNA Binding Conditions

The mRNA pull-down procedure requires the 6xHis-MS2 fusion proteins to bind to the MS2 binding sites of mRNA transcribed from the TOP (or TOP-trctMut) construct, following addition of the protein to the lysate of transfected cells. In addition, the 6xHis-MS2 protein must remain bound to the mRNA until the final

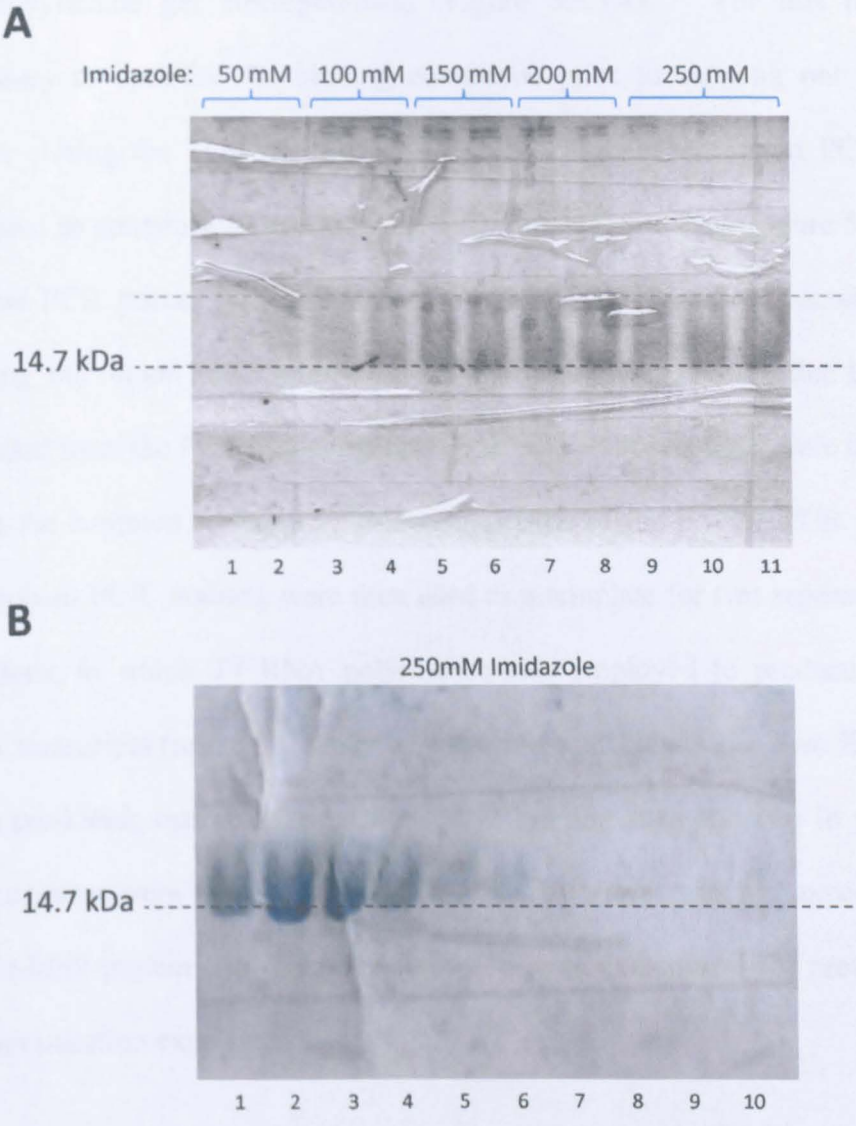


Figure 5.2: Optimisation of 6xHis-MS2 Protein Purification Conditions

A. Purification of 6xHis-MS2 fusion protein (14.7 kDA) performed using a Ni-NTA (nickel-nitrilotriacetic acid) Agarose bead HPLC column and an Imidazole gradient elution of 50mM to 250mM (with 50mM incremental increases). Additional proteins were also observed which were of higher than expected molecular weight (indicated by light grey dotted line).

B. Purification of 6xHis-MS2 fusion protein (14.7 kDA) performed using a Co-Agarose bead HPLC column with a fixed Imidazole concentration of 250mM.

elution in order for proteins of interest to be detected and separated using PAGE (polyacrylamide gel electophoresis) (Figure 5.3.1A). For this reason, it was necessary to optimise the binding conditions prior to carrying out the pull-down assay. Using the TOP construct as a PCR template, a forward PCR primer was designed to contain a 5' overhanging T7 promoter sequence (Figure 5.3.1B). One reverse PCR primer was designed to produce a PCR product containing the MS2 binding site region and a second primer was designed to ensure that this region was excluded from the PCR product (Figure 5.3.1B). PCR reactions were then carried out using the common forward T7 primer and each of the reverse PCR primers. The subsequent PCR products were then used as a template for two separate transcription reactions, in which T7 RNA polymerase was employed to produce radio-labelled RNA transcripts (refer to Chapter 2: Materials and Methods). Two RNA transcripts were produced; one containing three MS2 binding sites and one in which no MS2 binding sites were present. The latter RNA transcript was not expected to bind to 6xHis-MS2 protein and therefore acted as a negative-control for protein binding, in the optimisation experiments.

In order to determine the optimal protein-RNA binding conditions, a common binding buffer was used for each optimisation experiment. The protein-RNA binding conditions are therefore directly related to the choice of lysis buffer used in the experiment. Two candidate lysis buffers were selected to determine the optimal binding conditions. It has previously been shown that MS2 protein binds to mRNA, which contains MS2 binding sites, under conditions in which TMK (Tris-$MgCl_2$-KCl) buffer is used (Talbot et al. 1991). A TMK based lysis buffer was therefore selected as a potential candidate for use in MS2 pull-down experiments. A second TMN

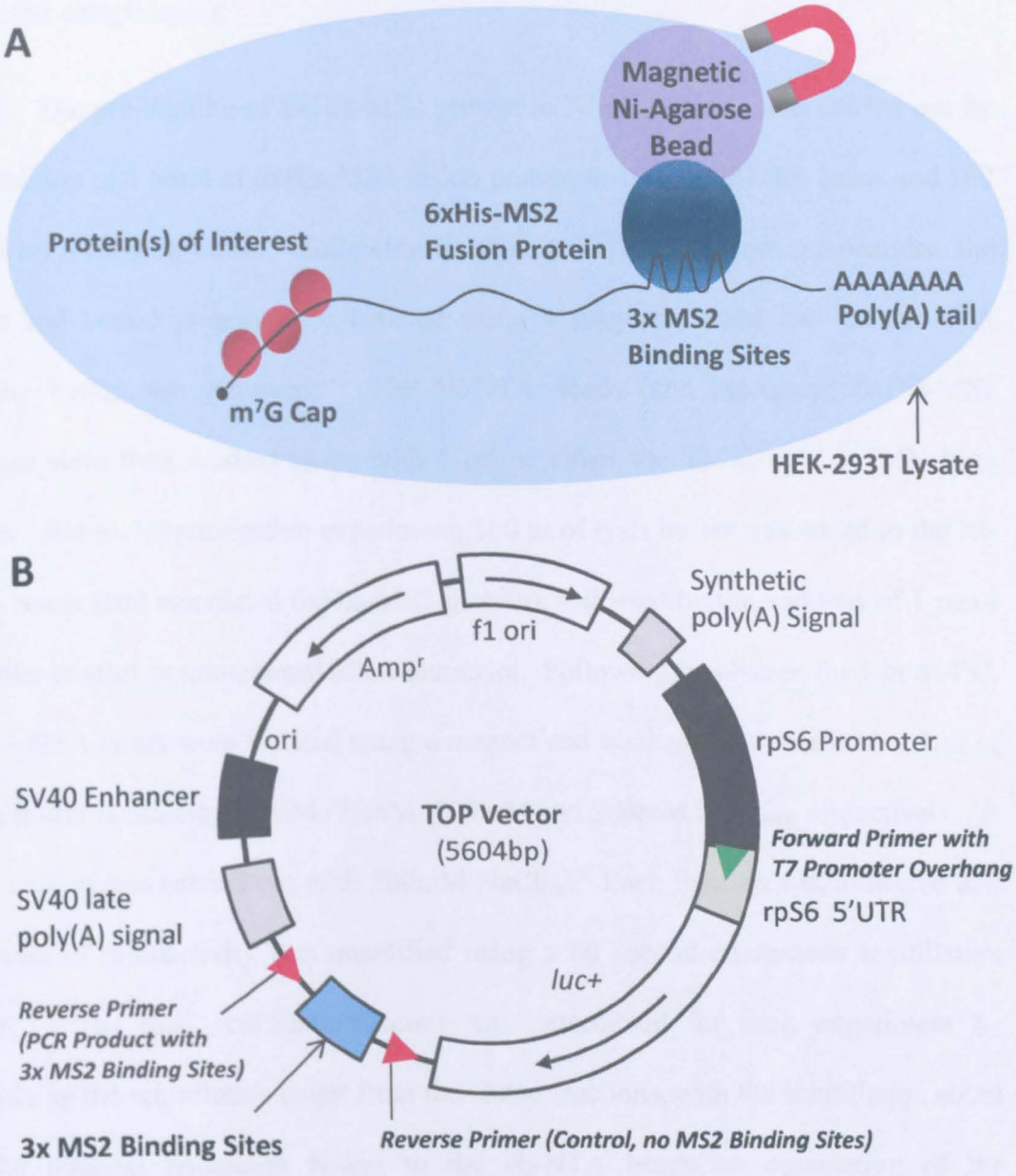


Figure 5.3.1: Optimisation of 6xHis-MS2 Protein-mRNA Binding Conditions

A. The mRNA pull-down procedure requires the 6xHis-MS2 fusion protein to bind to the MS2 binding sites of mRNA transcribed from the TOP (or TOP-trctMut) construct, following addition of the protein to the lysate of transfected cells.

B. Location of PCR priming sites within the 5' and 3' UTRs of the TOP Vector. The two PCR products were then used as a template for two separate transcription reactions, in which T7 RNA polymerase was employed to produce radio-labelled RNA transcripts.

(Tris-$MgCl_2$-NaCl) lysis buffer was also chosen for investigation (refer to Chapter 2 for buffer components).

The pre-binding of 6xHis-MS2 protein to Ni-NTA beads was carried out by the addition of 1 pmol of 6xHis-MS2 fusion protein to 100 µl Ni-NTA beads and 100 µl Ni-NTA binding buffer. Following incubation for 1 hr at room temperature, the beads and bound protein were isolated using a magnetic stand and the Ni-NTA binding buffer was removed. The Ni-NTA beads (and associated 6xHis-MS2 protein) were then washed twice with 1 ml of either the TMK, or the TMN lysis Buffer. For each optimisation experiment, 500 µl of lysis buffer was added to the Ni-NTA beads (and associated 6xHis-MS2 protein), followed by the addition of 1 pmol of either control or non-control RNA transcript. Following incubation for 1 hr at 4°C, the Ni-NTA beads were isolated using a magnet and washed, four times using 1ml of wash buffer containing 50mM, 75mM, 100mM and 200mM $NaCl_{(aq)}$ respectively. A final elution was carried out with 300mM $NaCl_{(aq)}$. Each fraction was collected and the level of radioactivity was quantified using a 60 second continuous scintillation count. The total scintillation count was determined for each experiment by combining the scintillation count from the eluted fractions, with the scintillation count of the material remaining bound to the Ni-NTA beads on completion of the experiment. This total count was then used to determine the percentage of radio-labelled RNA eluted following each wash. In addition, a scintillation count was also obtained for the un-bound flow through material. This was used to determine the percentage of RNA, which initially bound to the 6xHis-MS2 protein.

The data obtained for the experiment in which TMN lysis buffer was used (Figure 5.3.2A) show that 8% of both the control RNA and the RNA containing three MS2 binding sites remained bound to the 6xHis-MS2 protein following incubation.

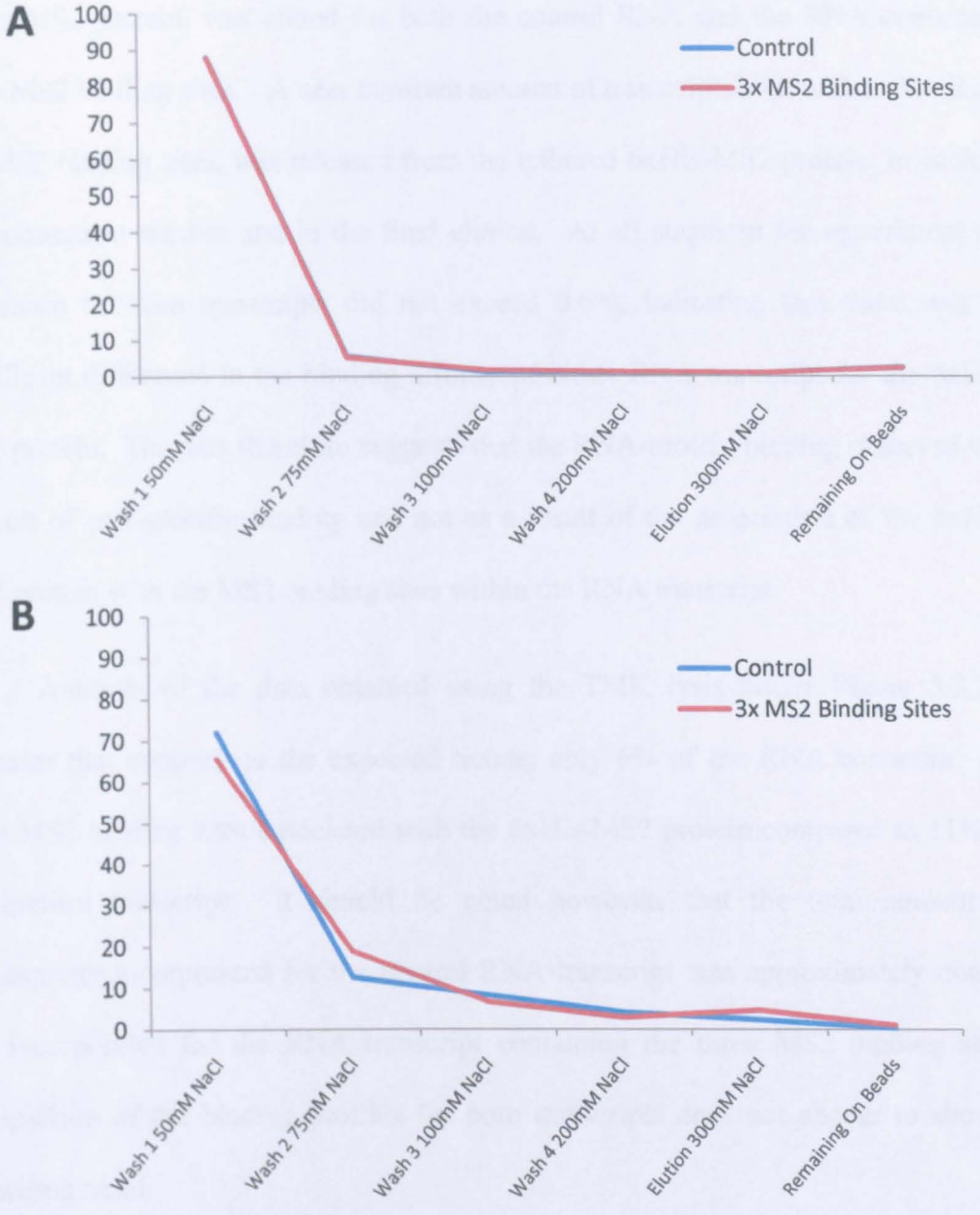


Figure 5.3.2: Optimisation of 6xHis-MS2 Protein-mRNA Binding Conditions

A. Optimisation of binding conditions using the TMN (Tris-$MgCl_2$-NaCl) buffer. Two radio-labelled RNA transcripts were used one containing three MS2 binding sites and a control transcript in which no MS2 binding sites were present. The total scintillation count was determined for each experiment and was used to determine the percentage of radio-labelled RNA eluted following each NaCl(aq) wash.

B. This process was then repeated using the TMK (Tris-$MgCl_2$-KCl) buffer.

Following the initial 50 mM NaCl wash, 88% of the RNA that had associated with the 6xHis-MS2 protein, was eluted for both the control RNA and the RNA containing three MS2 binding sites. A near constant amount of transcript, both with and without the MS2 binding sites, was released from the tethered 6xHis-MS2 protein, in each of the subsequent washes and in the final elution. At all stages in the experiment the difference between transcripts did not exceed 0.6%, indicating that there was no significant difference in the binding affinity of either RNA transcript for the 6xHis-MS2 protein. The data therefore suggests that the RNA-protein binding observed was a result of non-specific binding and not as a result of the association of the 6xHis-MS2 protein with the MS2 binding sites within the RNA transcript.

Analysis of the data obtained using the TMK lysis buffer Figure 5.3.2B, indicates that contrary to the expected trends, only 6% of the RNA containing the three MS2 binding sites associated with the 6xHis-MS2 protein compared to 11% of the control transcript. It should be noted however, that the total amount of radioactivity incorporated for the control RNA transcript was approximately double that incorporated for the RNA transcript containing the three MS2 binding sites. Comparison of the binding profiles for both transcripts does not appear to show a promising trend.

Following the initial 50mM NaCl wash, 6% more RNA remained associated with the 6xHis-MS2 protein when three MS2 binding sites were present. This suggests that the binding specificity is greater for the RNA containing the MS2 binding sites, compared to the control RNA. With the increase of NaCl concentration to 75mM, a greater percentage of RNA containing the MS2 binding sites was eluted. The next significant difference occurs at the 300mM NaCl elution, when 4.7% of RNA containing the MS2 binding sites was eluted, compared to 2.5% of control

RNA. While it was the case that less RNA containing the MS2 binding sites initially associated with the tethered 6xHis-MS2 protein, it appears that the specificity of binding was slightly higher compared to the control RNA, although the difference may not be statistically significant.

The data show that when either TMK or TMN lysis buffers were used as a protein-RNA binding medium, there was not a significant difference in the association of 6xHis-MS2 protein with either the control RNA transcript or the RNA containing the MS2 binding sites. For this reason, it was decided that a new approach should be investigated, in which an MS2 fusion protein is co-expressed *in vivo* with the TOP or TOP-trctMut construct. It is also possible that the interaction between the MS2 protein and the MS2 binding sites located within the RNA is too weak to result in any significant specific association of the protein and RNA. For this reason, it was decided that both TOP and TOP-trctMut constructs should be re-designed to incorporate an increased number of MS2 binding sites.

5.3 Modification of TOP and TOP-trctMut Constructs

In order to increase the number of MS2 binding sites within both TOP and TOP-trctMut constructs, additional cloning was required within the region of each construct which encodes the mRNA 3' UTR. The key consideration in the cloning strategy was to maintain the spacing between the binding sites in order to reduce steric hindrance, which would prevent multiple binding of the MS2 protein. Previously, tethering assays have been carried out using MS2-EGFP fusion protein and mRNAs containing twelve MS2 binding sites within their 3' UTR (Chen et al. 2008). It was therefore decided that at least twelve MS2 binding sites should be

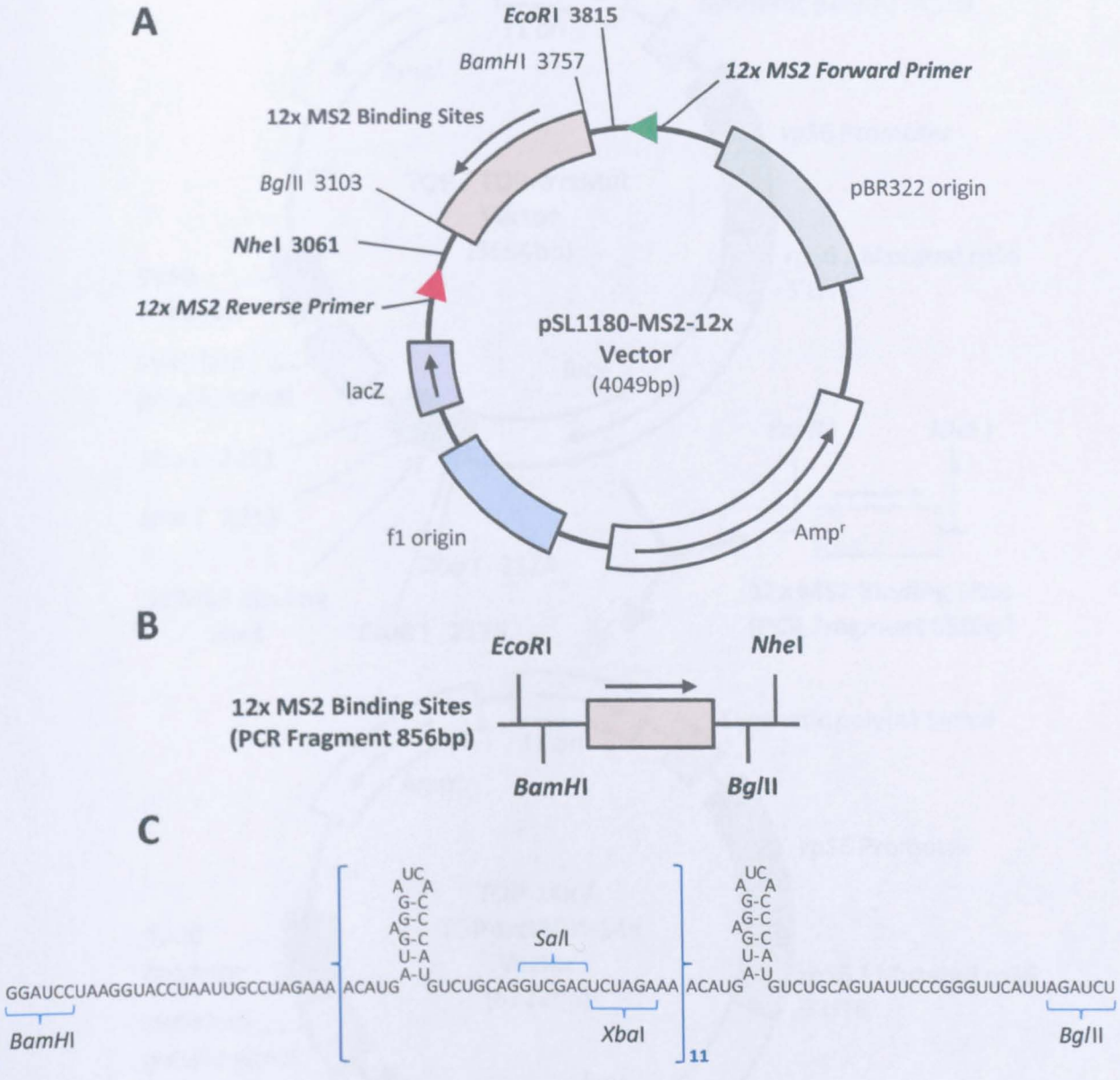


Figure 5.4.1: Construction of TOP-14x / TOP-trctMut-14X Vectors

A. Location of PCR priming sites within the 5' and 3' UTRs of the pSL1180-MS2-12x Vector (A kind gift from Prof. Robert H. Singer, Albert Einstein College of Medicine, Yeshiva University).

B. A PCR product was obtained which contained 12x MS2 binding sites.

C. Diagram showing the sequence, orientation and location of the 12x MS2 binding sites in the transcribed mRNA, relative to the restriction sites within the vector.

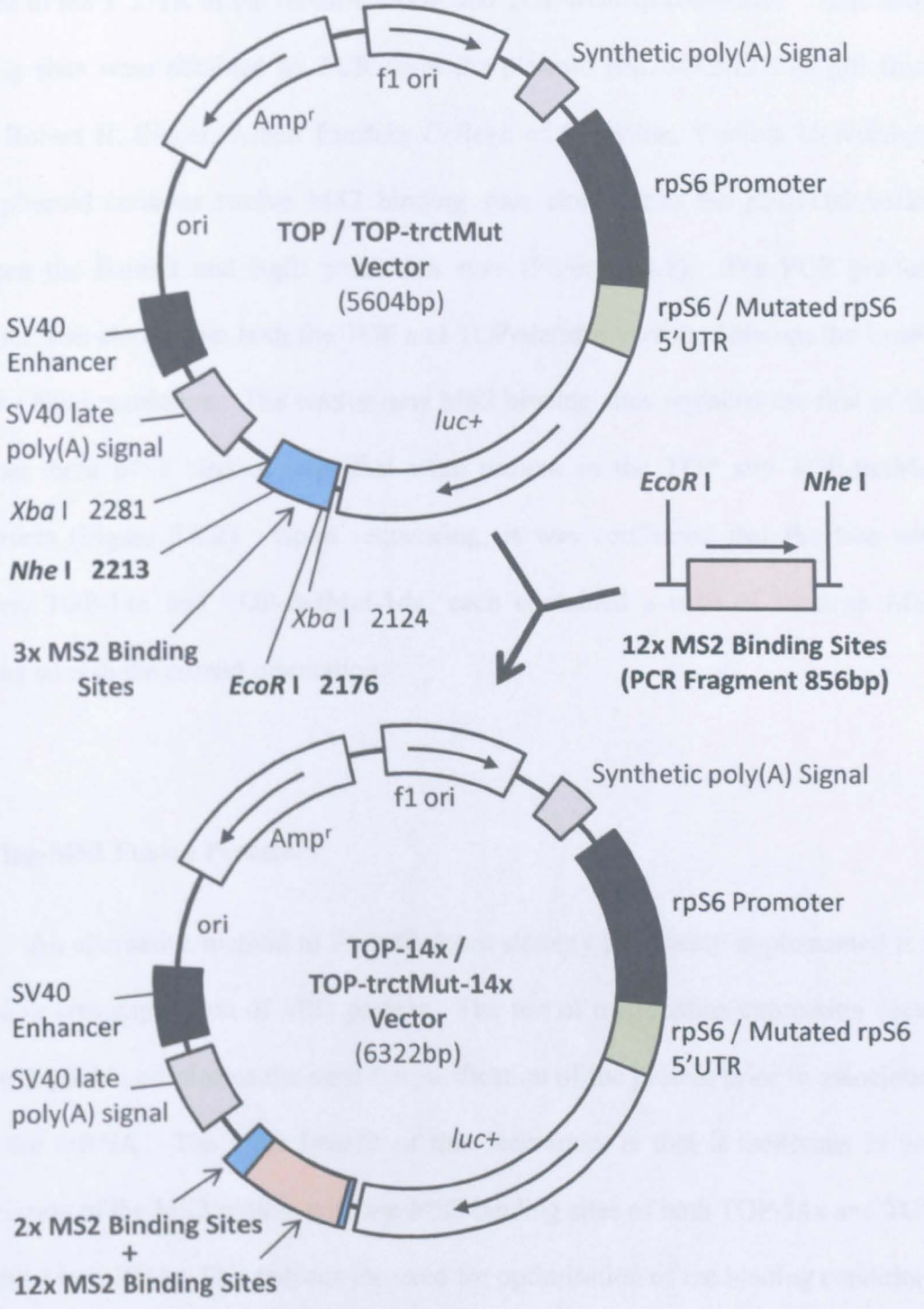


Figure 5.4.2: Construction of TOP-14x / TOP-trctMut-14X Vectors

The PCR fragment containing the 12x MS2 binding sites was digested with *EcoR*I and *Nhe*I. This fragment was ligated into the TOP or TOP-trctMut vector, which had been digested with *EcoR*I and *Nhe*I (removing 1x MS2 binding site) and dephosphorylated. The resultant TOP-14x and TOP-trctMut-14x vector constructs contain 2x MS2 binding sites from the original vector, and 12x additional MS2 binding sites.

present in the 3' UTR of the modified TOP and TOP-trctMut constructs. The MS2 binding sites were obtained by PCR from the plasmid pSL-MS2-12X (a gift from Prof. Robert H. Singer, Albert Einstein College of Medicine, Yeshiva University). This plasmid contains twelve MS2 binding sites cloned into the pSL1180 vector between the BamHI and BglII restriction sites (Figure 5.4.1). The PCR product obtained was cloned into both the TOP and TOP-trctMut vectors, between the EcoRI and the NheI restriction. The twelve new MS2 binding sites replaced the first of the original three MS2 binding sites that were present in the TOP and TOP-trctMut constructs (Figure 5.4.2). Upon sequencing, it was confirmed that the two new vectors, TOP-14x and TOP-trctMut-14x, each contained a total of fourteen MS2 binding sites in the correct orientation.

5.4 Flag-MS2 Fusion Protein

An alternative method to the pull-down strategy previously implemented is to utilise *in vivo* expression of MS2 protein. The use of mammalian expression vector for MS2 protein eliminates the need for purification of the protein prior to association with the mRNA. The main benefit of this technique, is that it facilitates *in vivo* association of the MS2 protein with the MS2 binding sites of both TOP-14x and TOP-trctMut-14x mRNA. This reduces the need for optimisation of the binding conditions, although it is still necessary to ensure that the wash and elution conditions are optimised to recover the protein-mRNA complex. In its dimeric form, MS2 protein contains two head-to-tail molecules, which may be synthesised from a single mRNA following genetic fusion of the two open reading frames (Peabody and Lim 1996). It has been reported that the in-frame insertion of octa-peptide sequence called Flag

within the reading frame of this dimer MS2 coat protein, can be achieved without significant functional impairment and with an increase in structural stability compared to the wild-type protein (Peabody 1997). Flag-MS2 fusion protein has previously been used in RNA tethering experiments (Keryer-Bibens et al. 2008) and has been successfully purified from whole-cell lysates HEK-293T cells using anti-Flag-M2 affinity resin (SIGMA) (Pellizzoni et al. 2002). The successful combination of these proven techniques should enable the isolation of mRNAs, which contain MS2 binding sites. This process would remove the need for bacterial expression of 6xHis-MS2 and the subsequent optimisation of the binding conditions, which is required for affinity purification using Ni-NTA beads.

5.5 Isolation of TOP-14x & TOP-trctMut-14x mRNAs using Flag-MS2 Affinity Purification

An investigation was carried out to determine the efficacy of the Flag-MS2 affinity purification technique described above. A co-transfection of $5x10^6$ HEK-293T cells was carried out in duplicate, using 9 μg of mammalian Flag-MS2 expression vector (a kind gift from Dr A. Marcello, Laboratory of Molecular Biology, ICGEB, Trieste, Italy) and 3 μg of either TOP-14x or Top-trctMut-14x construct. After a period of 48 hours had elapsed following the transfection, the cells were serum starved for 4 hours in DMEM supplemented with 0.1% FCS. Following serum starvation, the cells were re-fed for 45 minutes with DMEM supplemented 10% FCS. Using one set of the duplicate plates, TRI reagent (Sigma) was used to isolate total RNA from the samples. HEK-293T cells from the second set of transfected plates were subjected to lysis and affinity purification using anti-Flag-M2

resin (Sigma), using the procedure described in the material and methods chapter (Section 2.6.1). TRI reagent (Sigma) was then added to the anti-Flag-M2 affinity resin to extract the bound RNA. Northern blotting was carried out for *firefly* (reporter) mRNA, on the fractions obtained from the total lysate and the fractions obtained following anti-Flag-M2 affinity purification (Figure 5.5A). The data clearly show that correctly sized reporter mRNA was present in the total cell lysate of TOP-14x and TOP-trctMut-14x transfected cells (Figure 5.5A). The northern blot data that obtained for the affinity purified samples shows that these too contain correctly sized reporter mRNA. Thus, the affinity purification process appears to have successfully isolated reporter mRNA. The ratio of the transfected reporter and protein expression vectors appears to have been within a suitable range to facilitate in vivo binding of mRNA and protein. Additionally, it appears that the wash-buffers used are suitable to allow the Flag-MS2 protein to remain bound to the reporter mRNA during the purification process.

5.6 Western Analysis of Proteins Associated with Affinity Purified mRNAs

The successful isolation of reporter mRNAs using the anti-Flag-M2 affinity purification technique, opens the possibility for experiments to be carried out, which attempt to identify the proteins associated with TOP and TOP-trctMut mRNAs. Once isolated, these proteins could then be separated using PAGE, and specific proteins of interest could then be detected by western blot. Potential differences between the specific proteins which are associated with TOP containing mRNAs and not-TOP containing mRNAs could then be determined.

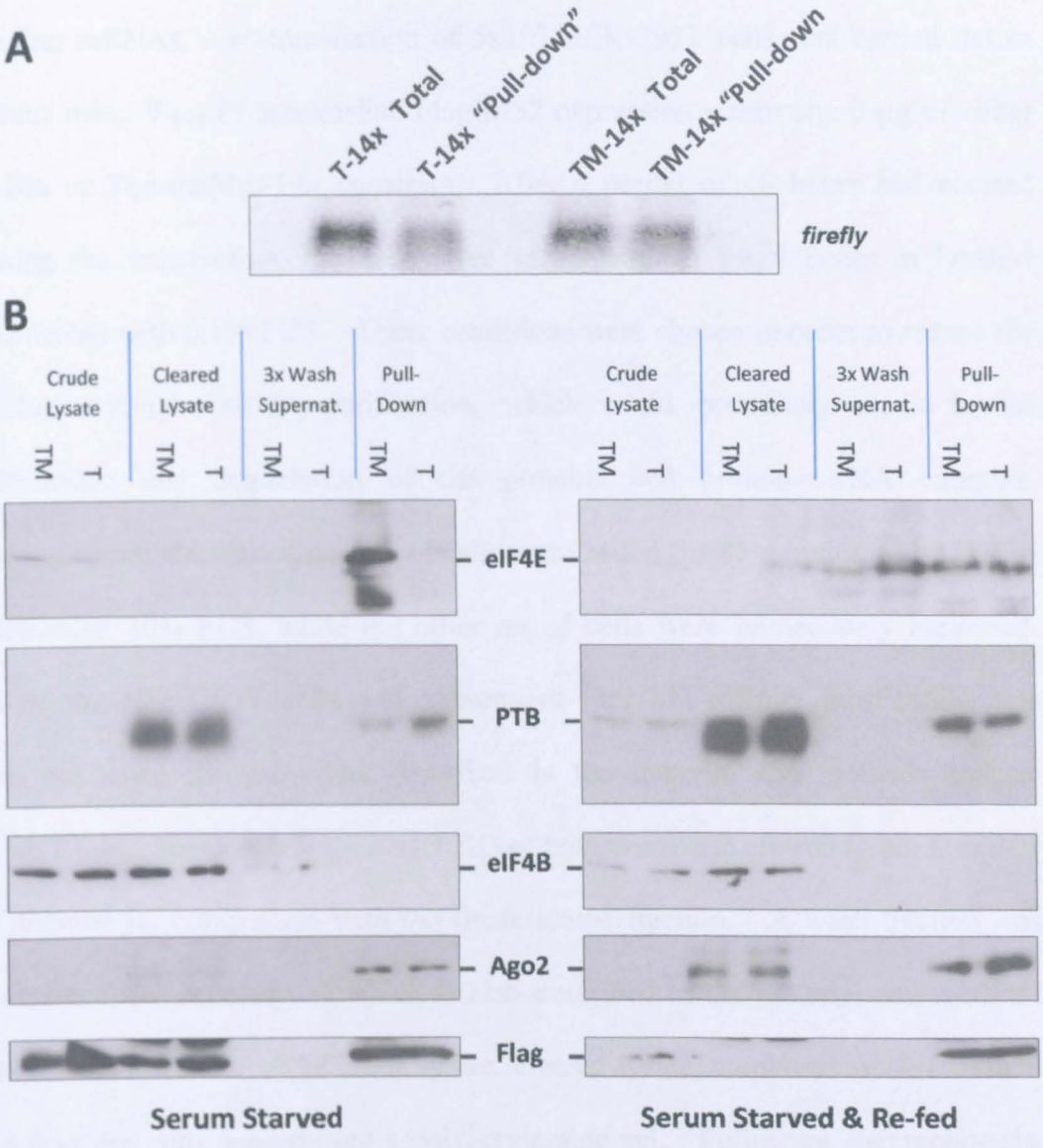


Figure 5.5: Affinity Purification of TOP-14x and TOP-trctMut-14x mRNA

A. Northern blotting was carried out for *firefly* (reporter) mRNA, on the fractions obtained from the total lysate and the fractions obtained following anti Flag-M2 affinity purification. "T" corresponds to TOP-14x and "TM" corresponds to TOP-trctMut-14x.

B. HEK-293T cells was carried out in duplicate, using 9 μg of mammalian Flag-MS2 expression vector and 3 μg of either TOP-14x or Top-trctMut-14x construct. After a period of 48 hours had elapsed following the transfection, cells were serum starved (0.1% FCS) for 4 hrs or serum starved (0.1% FCS) for 4 hrs and serum stimulated (10% FCS) for 45 min immediately prior to harvesting Affinity purification followed by western blotting was then carried out on the fractions obtained.

In order to investigate which proteins are bound to TOP and non-TOP containing mRNAs, a co-transfection of $5x10^6$ HEK-293T cells was carried out in duplicate, using 9 μg of mammalian Flag-MS2 expression vector and 3 μg of either TOP-14x or Top-trctMut-14x construct. After a period of 48 hours had elapsed following the transfection, the cells were serum starved for 4 hours in DMEM supplemented with 0.1% FCS. These conditions were chosen in order to reduce the need for overnight affinity-purification, which could potentially result in the destabilisation and degradation of the proteins and protein-mRNA complex. Following serum starvation, one set of cells were re-fed for 45 minutes with DMEM supplemented 10% FCS, while the other set of cells were immediately harvested. Lysis of the HEK-293T cells and subsequent Flag-M2 affinity purification was carried out using the procedure described in the material and methods chapter (Section 2.6.1). Samples (comprising 10%) of both crude and cleared lysate fractions were retained for comparison with the finale eluted fraction. A wash fraction was also obtained, the recovery of which is also described in the material and methods chapter (Section 2.6.1). The crude lysate, cleared lysate, combined wash and final eluted fractions were loaded onto a polyacrylamide gel. Following electrophoresis, the proteins were transferred to a PVDF (Polyvinylidene Fluoride) membrane.

It was first necessary to confirm that the Flag-MS2 protein had remained associated with the anti-Flag-M2 beads throughout the purification process. Western blotting was carried out using anti-bodies specific to the Flag-tag of the MS2 protein (Figure 5.5B). A 15 kDa band corresponding to Flag-MS2 was clearly visible in the Pull-down lanes of both serum-starved and serum-starved and re-fed cells. Thus the Flag-MS2 protein remained associated with the anti-Flag-M2 beads until the final elution. This supports the earlier data obtained by northern blotting, which confirmed

the presence of TOP and TOP-trctMut mRNAs in the eluted fractions. The western data from the starved then re-fed cells does not appear to confirm the presence of the Flag-MS2 proteins in the cleared lysate fraction, and only moderate amount in the crude lysate. It is possible that the protein had migrated off the bottom of the gel or did not transfer onto the PVDF membrane efficiently. The combined wash fractions from both starved and re-fed cells do not appear to contain sufficient Flag-MS2 protein to be visible on the western blot. This suggests that the majority of the protein remained associated with the beads, and suggests that the affinity purification process has a high degree of efficiency.

With confirmation that the technique is capable of purifying Flag-MS2 protein and associated mRNAs, it is now possible to determine whether specific proteins are associated with either TOP or non-TOP reporter mRNAs. The requirement of eIF4E in the translation of TOP containing mRNAs has previously been challenged by several observations, including the result that mRNA transcribed by the TOP construct was found to increase by 100% when eIF4E levels were reduced (Figure 4.4.2). It has previously been suggested that a possible explanation for the observed trend is that TOP containing mRNA translation may utilise an alternative mechanism of translation initiation, which does not require eIF4E. A western blot was carried out to determine whether or not eIF4E was associated with TOP-14x and TOP-trctMut-14x mRNAs (Figure 5.5B). In serum-starved cells, eIF4E was found to be associated with TOP-trctMut-14x mRNA, but not TOP-14x mRNA. Analysis of the data for serum starved and re-fed cells indicates that more un-associated eIF4E was removed from the anti-Flag-M2 beads prior to the final elution. This suggests that levels of eIF4E were higher in serum re-fed cells, as would be expected. It appears that eIF4E is associated approximately equally with both TOP-trctMut-14x

and TOP-14x mRNAs. It should, however, be noted that comparison of the un-bound wash and cleared lysate fractions suggests that eIF4E are slightly higher in cells transfected with the TOP-14x construct compared to TOP-trctMut-14x transfected cells. However, this does not affect the trend that eIF4E is associated with TOP-14x mRNA in serum re-fed cells but not in serum starved cells.

The eukaryotic initiation factor 4B (eIF4B) plays a crucial role in the recruitment of the 40S ribosomal subunit to the mRNA. It has been shown to be phosphorylated in response to insulin in a process controlled by the PI3K/mTOR signalling pathway (Shahbazian et al. 2006). This makes eIF4B ideally positioned to play a role in the regulation of TOP containing mRNAs. A western blot was therefore carried out, in order to determine whether or not eIF4B is associated with TOP containing mRNAs in conditions of serum starvation, or stimulation with nutrients following serum re-feeding (Figure 5.5B). The data clearly shows that eIF4B was not associated with either TOP-14x or TOP-trctMut-14x mRNA under the conditions investigated. It would therefore appear that eIF4B is not an essential requirement of translation of both TOP-14x or TOP-trctMut-14x mRNA, perhaps due to the relatively short unstructured 5' UTR. It is also possible that eIF4E was removed during the washing stage and did not remain bound until the final elution.

As previously discussed (Chapter 4), Polypyrimidine-tract binding protein (PTB) is a multi-function protein, which preferentially binds polypyrimidine-rich regions of mRNA (Garcia-Blanco et al. 1989). It was previously observed that both TOP and TOP-trctMut reporter transcribed mRNAs appear to be translationally activated by PTB over-expression, with the observed activation increasing with the amount of PTB expression vector transfected (Figure 4.7.1). It was, therefore, of interest to investigate whether PTB was associated with TOP-14x and TOP-trctMut-

14x mRNAs under similar experimental conditions. The data show that the majority of PTB is removed in the cleared lysate fractions, and that the subsequent washing of the anti-Flag-M2 beads does not result in the elution of more PTB protein (Figure 5.5B). This suggests that PTB, which is present in the eluted pull-down fractions, has remained specifically bound to the mRNA associated with the Flag-MS2 protein. The data for both the serum-starved and serum starved and re-fed cells, show that PTB is associated with both TOP-14x and TOP-trctMut-14x mRNA under both conditions. In the serum starved cells, PTB appears more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA, a trend which appears to be reversed in cells which were serum starved then re-fed.

The potential association of microRNAs within the 5' UTR of TOP containing mRNAs raises the possibility that components normally involved in miRNA repression are also associated with TOP containing mRNAs. Argonaute 2 (AGO2) is a critical component of the RNA-induced silencing complex (RISC) and is, therefore, essential for miRNA repression (Cannell et al. 2008)]. Interestingly, AGO2 possesses a cap binding motif, which is similar to that found in eIF4E and has been detected by affinity chromatography with m^7GTP-Sepharose (Kiriakidou et al. 2007). Western blotting shows that in serum starved cells and in cells which have been serum starved then re-fed, AGO2 is associated with both TOP-14x and TOP-trctMut-14x mRNAs (Figure 5.5B). In the serum re-fed cells however, it appears that more AGO2 is associated with TOP-14x mRNA than TOP-trctMut-14x mRNA. This data suggests that AGO2 potentially plays a role in the translation of TOP containing mRNAs following nutrient stimulation.

5.7 Mass Spectrometry Analysis of Proteins Associated with Affinity Purified mRNAs

The western blot data obtained in the previous experiment confirm that it is possible to determine whether or not specific proteins are with TOP and TOP-trctMut mRNAs. It was decided to expand upon this technique and attempt to identify unknown proteins which may be associated with TOP and TOP-trctMut mRNAs. In order to identify unknown proteins, mass spectrometry was employed to compare the proteins obtained from the affinity purification process. As before, co-transfection of $5x10^6$ HEK-293T cells was carried out in duplicate, using 9 μg of mammalian Flag-MS2 expression and 3 μg of either TOP-14x or Top-trctMut-14x construct. After a period of 48 hours had elapsed following the transfection, the cells were serum starved for 4 hours in DMEM supplemented with 0.1% FCS. Following serum starvation, one set of cells were re-fed for 45 minutes with DMEM supplemented 10% FCS, while the other set of cells were immediately harvested. The anti-Flag-M2 affinity purification technique (described Section in 2.6.1) was then carried on both TOP and TOP-trctMut transfected cells. SDS loading buffer (40 μl) was used in the final elution to recover the proteins which had associated with the Flag-MS2 bound mRNA. Four samples were obtained: TOP-14x transfected serum-starved cells, TOP-trctMut-14x transfected serum-starved cells, TOP-14x transfected serum-starved and re-fed cells, and TOP-trctMut-14x transfected serum-starved and re-fed cells. The four protein samples were loaded onto a polyacrylamide gel. Following electrophoresis, the gel was then sliced into its constituent lanes, which were in turn sub-divided into eight equal sections. These gel segments were then subjected to

Mass Spectrometry analysis (carried out at the Cambridge Centre for Proteomics, School of the Biological Sciences, University of Cambridge).

The data obtained was compared to a known protein database using Mascot database search engine software (Perkins et al. 1999) and the complete dataset can be found in the Appendix section. Comparison of the proteins associated with TOP and TOP-trctMut mRNAs was then carried out (Figure 5.6). An important consideration to be made when analysing the data, is the lack of experimental validation of the data obtained following mass spectrometry. There are a large number of different proteins, which are purportedly co-purified with TOP and TOP-trctMut mRNAs. However, the difference between these two mRNAs is limited to a mutation of just two nucleotides within the 5'UTR. This suggests that there are potentially a large number of false-positives within the dataset. It is apparent from examining the data, that a wide variety of ribosomal proteins of both the 40S and 60S ribosomal subunits are associated with both TOP-14x and TOP-trctMut-14x mRNA. This is consistent with ribosomes being associated with both types of mRNA during translation.

La autoantigen has previously been implicated in the translational control of 5'TOP containing mRNAs (discussed in Chapter 4). The data obtained by mass spectrometry indicates that La and La related proteins (including LARP1, LARP2 and LARP7) were found to be associated with both TOP-14x and TOP-trctMut-14x mRNAs under both sets of experimental conditions. This is consistent with the trend observed when La was over-expressed, which indicated that there was no significant difference between the translational activity of TOP and TOP-trctMut mRNAs under serum starved and serum re-fed conditions (Figure 4.6).

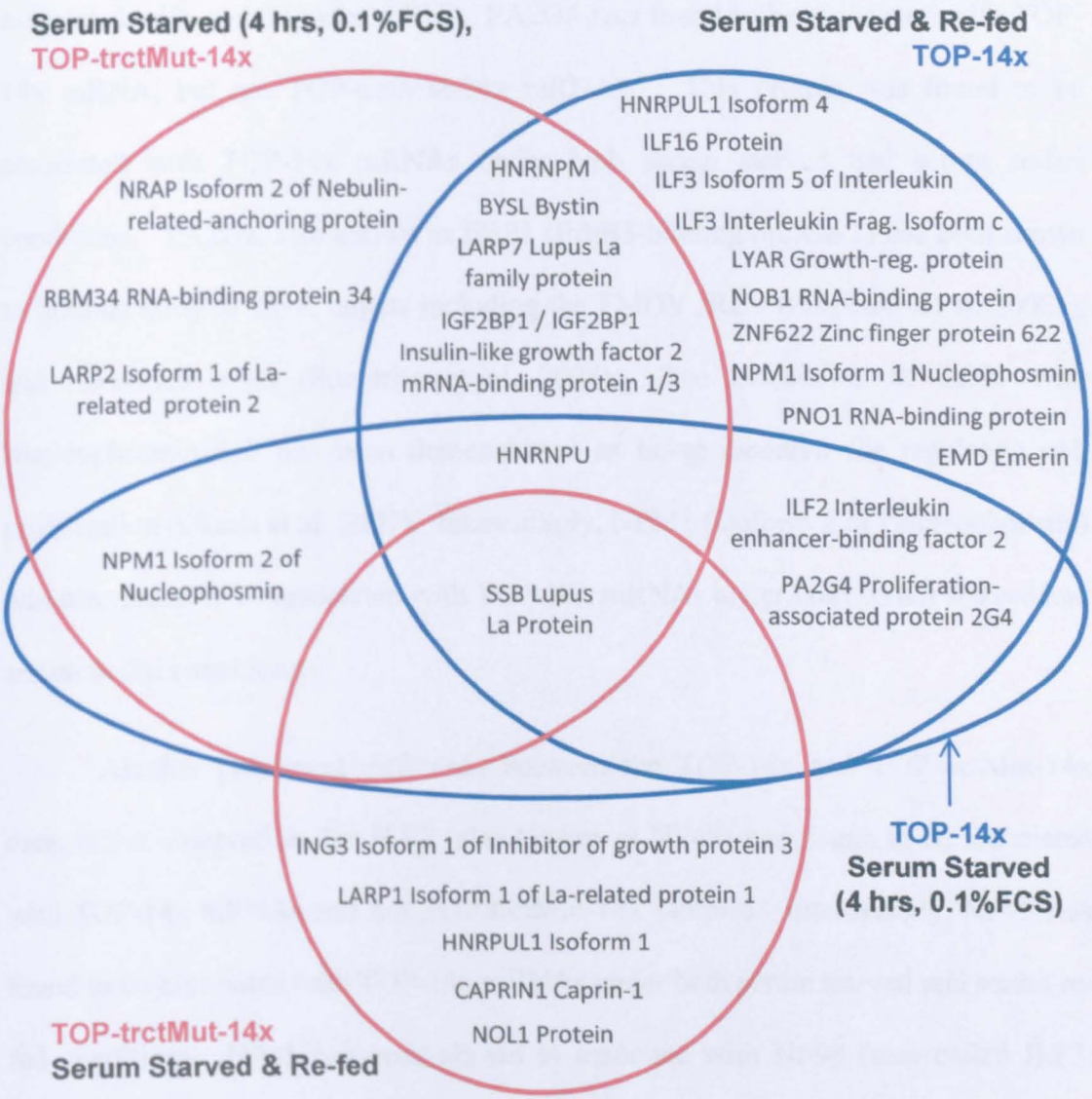


Figure 5.6: Analysis of Proteins Detected by Mass Spectrometry

Following repetition of the affinity purification technique, mass spectrometry was performed on the samples obtained (carried out at the Cambridge Centre for Proteomics, School of the Biological Sciences, University of Cambridge). The data was compared to a known protein database using Mascot database search engine software (Perkins & Pappin 1999) and a comparison of the proteins associated with TOP and TOP-trctMut mRNAs was then carried out. This Venn diagram highlights key differences between the samples and previously identified proteins of interest. Please refer to the appendix section for the complete dataset.

When a direct comparison was carried out on the proteins found to be associated with each reporter mRNA, PA2G4 was found to be associated with TOP-14x mRNA, but not TOP-trctMut-14x mRNAs. This protein was found to be associated with TOP-14x mRNAs under both serum starved and serum re-fed conditions. PA2G4, also known as EBP1 (ErbB3-binding protein 1) has been shown to bind an array of RNA targets including the FMDV IRES (Pilipenko et al. 2000a), and ribosomal RNA (Squatrito et al. 2004). The association of EBP1 with Nucleophosmin/B23 has been demonstrated as being essential for regulating cell proliferation (Okada et al. 2007). Interestingly, NPM1 (isoform 2 of Nucleophosmin) was also found to be associated with TOP-14x mRNAs under both serum starved and serum re-fed conditions.

Another prominent difference between the TOP-14x and TOP-trctMut-14x data, is the observation that ILF2 (also known as NF45) was found to be associated with TOP-14x mRNAs and not TOP-trctMut-14x samples. Interestingly, NF45 was found to be associated with TOP-14x mRNAs under both serum starved and serum re-fed conditions. NF45 has been shown to associate with NF90 (also called ILF3: Interleukin enhancer binding factor 3) in a variety of different cell types (Guan et al. 2008). Other binding partners have also been identified, including RNA helicase A, nucleolin and Heterogeneous nuclear ribonuclear proteins M and U (hnRNP-M & hnRNP-U) (Guan et al. 2008). These proteins along with NF90, were all identified as being associated with TOP-14x mRNAs under serum starved conditions.

5.8 Microarray Analysis of Micro-RNAs Associated with Affinity Purified Reporter mRNA

The transfections and anti Flag M2 affinity purification described above were repeated exactly. Following RNA extraction using TRI reagent (Sigma), northern blotting was carried out to confirm the presence of affinity purified reporter mRNA. The concentration of both total and affinity purified mRNA was then obtained by UV-Vis spectroscopy. Total RNA (2.5 μg) was labelled using Cy5 fluorescent dye (~650 nm excitation/670 nm emission) and affinity purified RNA was labelled using Cy3 dye (~550/570 nm). Labelled samples were denatured, loaded microRNA microarray chips (obtained from Dr T.W. Gant, MRC Toxicology Unit, University of Leicester) prior to hybridisation at 45°C for 16 hours. Following hybridisation, the chips were scanned using an Axon Genepix 4000b scanner (Figure 5.7.1). In order to extract data from the images obtained, a grid containing probe definitions was assigned by using the gridding algorithm within the Genepix Pro software (Molecular Devices). The algorithm assigned grid was visually verified and assignments corrected as necessary. The fluorescence intensities of the Cy5 and Cy3 dyes and the respective background were then recorded in preparation for data analysis.

Normalisation of the microarray sample data was performed using the LIMMA package (Smyth and Speed 2003) running in R statistical environment (http://www.r-project.org). The data sets obtained, contained Log_2 of the expression ratio and media spot intensity at the measured wavelengths. Global locally weighted scatter-point smoothing (LOWESS) was carried to normalise the data. It is documented that this method provides a balance between the dependence of log-ratio on measured spot intensity and the centre of the log-ratio distribution relative to zero (Leung and Cavalieri 2003). Following normalisation, the RankProd package within

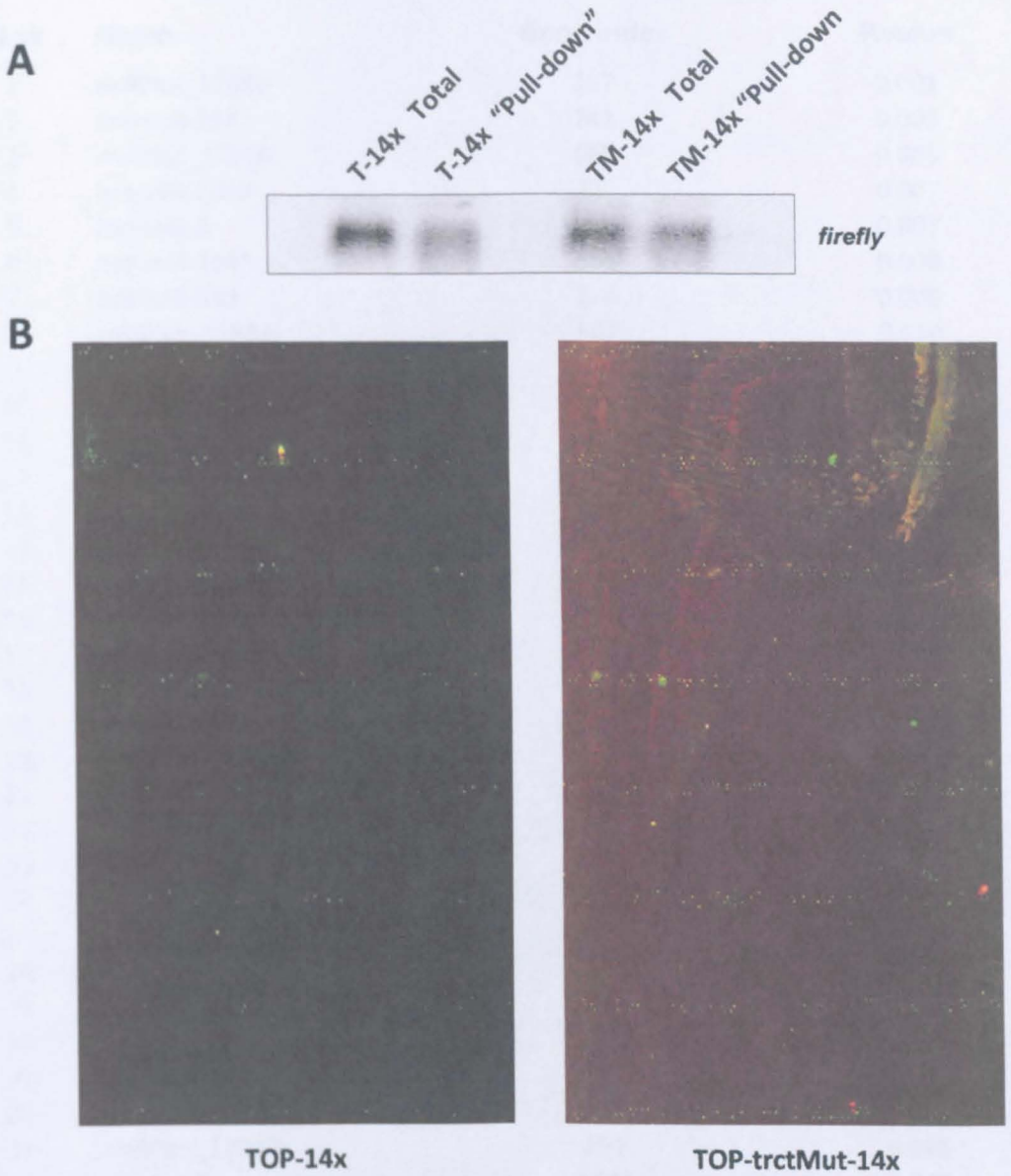


Figure 5.7.1: Microarray Analysis of Micro-RNAs Associated with Affinity Purified Reporter mRNAs

A. Northern blotting was carried out for *firefly* (reporter) mRNA, on the fractions obtained from the total lysate and the fractions obtained following anti Flag-M2 affinity purification. "T" corresponds to TOP-14x and "TM" corresponds to TOP-trctMut-14x.

B. Dye labelled RNA samples were denatured, loaded MicroRNA microarray chips (obtained from *Dr T.W. Gant, MRC Toxicology* Unit, University of Leicester) prior to hybridisation at 45°C for 16 hours. Following hybridisation, the chips were scanned using an Axon Genepix 4000b scanner.

MicroRNAs Differentially Associated with TOP-14x mRNA ($P \leq 0.05$)

Rank	Name	Gene Index	P.value
1	miRPlus_17960	237	0.001
2	hsa-miR-197	741	0.003
3	miRPlus_17820	665	0.005
4	hsa-miR-200a	65	0.007
5	hsa-miR-9	84	0.007
6	hsa-miR-154*	654	0.008
7	hsa-miR-335	274	0.009
8	miRPlus_17863	507	0.010
9	hsa-miR-583	655	0.014
10	miRPlus_17880	71	0.015
11	hsa-miR-642	72	0.019
12	miRPlus_17953	239	0.019
13	hsa-miR-625	342	0.020
14	hsa-miR-585	223	0.021
15	miRPlus_17928	671	0.021
16	hsa-miR-365	220	0.023
17	hsa-miR-519e*	656	0.028
18	hsa-miR-454	238	0.029
19	hsa-miR-148a	781	0.029
20	miRPlus_17932	70	0.031
21	hsa-miR-409-5p	117	0.033
22	hsa-miR-453	57	0.034
23	hsa-miR-616*	564	0.036
24	miRPlus_17848	285	0.037
25	hsa-miR-30c	544	0.038
26	hsa-miR-657	234	0.039
27	miRPlus_17875	22	0.040
28	hsa-miR-205	692	0.041
29	miRPlus_17952	1689	0.041
30	hsa-miR-130a	114	0.045
31	miRPlus_17910	292	0.045
32	hsa-let-7a	1686	0.047
33	hsa-miR-195	308	0.049
34	hsa-miR-181b	173	0.050

Figure 5.7.2: Analysis of MicroRNA Microarray Data

Normalisation of the microarray data was performed and identification of the differentially associated microRNAs between the TOP-14x and TOP-trctMut-14x mRNA, was carried out. Those microRNAs which are more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA following serum re-feeding, are located towards the top of the summary list. The complete dataset obtained following normalisation and ranking can be found in the appendix section. (* refers to the strand of the microRNA duplex that is not normally used and undergoes degradation.)

the R environment was used to identify differentially associated microRNAs between the TOP-14x and TOP-trctMut-14x mRNA, based on the estimated percentage of false predictions. Those microRNAs, which are more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA following serum re-feeding are located towards the top of the list which was produced. A summary of the most differentially associated miRNAs can be found in Figure 5.7.2. The complete dataset can be found in the Appendix section.

An alignment was then carried out between the TOP-14x mRNA sequence and the sequences of the fifteen microRNAs, which were found to be more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA (Figure 5.7.3). Of these, five example sequence alignments are shown, all of which were found to align to the TOP-14x mRNA sequence with mis-matched loop regions of less than three nucleotides in length (Figure 3.10). This alignment criterion is therefore consistent with that used in the identification of the microRNA-10a (Orom et al. 2008). The data indicates that all five miRNAs potentially have a high degree of base-pairing with the 5' UTR of TOP-14x mRNA (sequence derived from Human rpS6 mRNA). Of the five chosen miRNAs, microRNA-200a was found to be have the largest difference in association between TOP-14x and TOP-trctMut-14x mRNA. Analysis of the TOP-14x mRNA sequence confirms that it does not contain a complementary sequence to the microRNA-200a seed-region, which it usually a requirement of microRNA binding (Pillai et al. 2005). This suggests that the isolation of this microRNA is therefore a result of binding to the target mRNA at the position indicated within the 5' UTR. The presence of these miRNAs should also be confirmed by northern blotting in order to validate the microarray data.

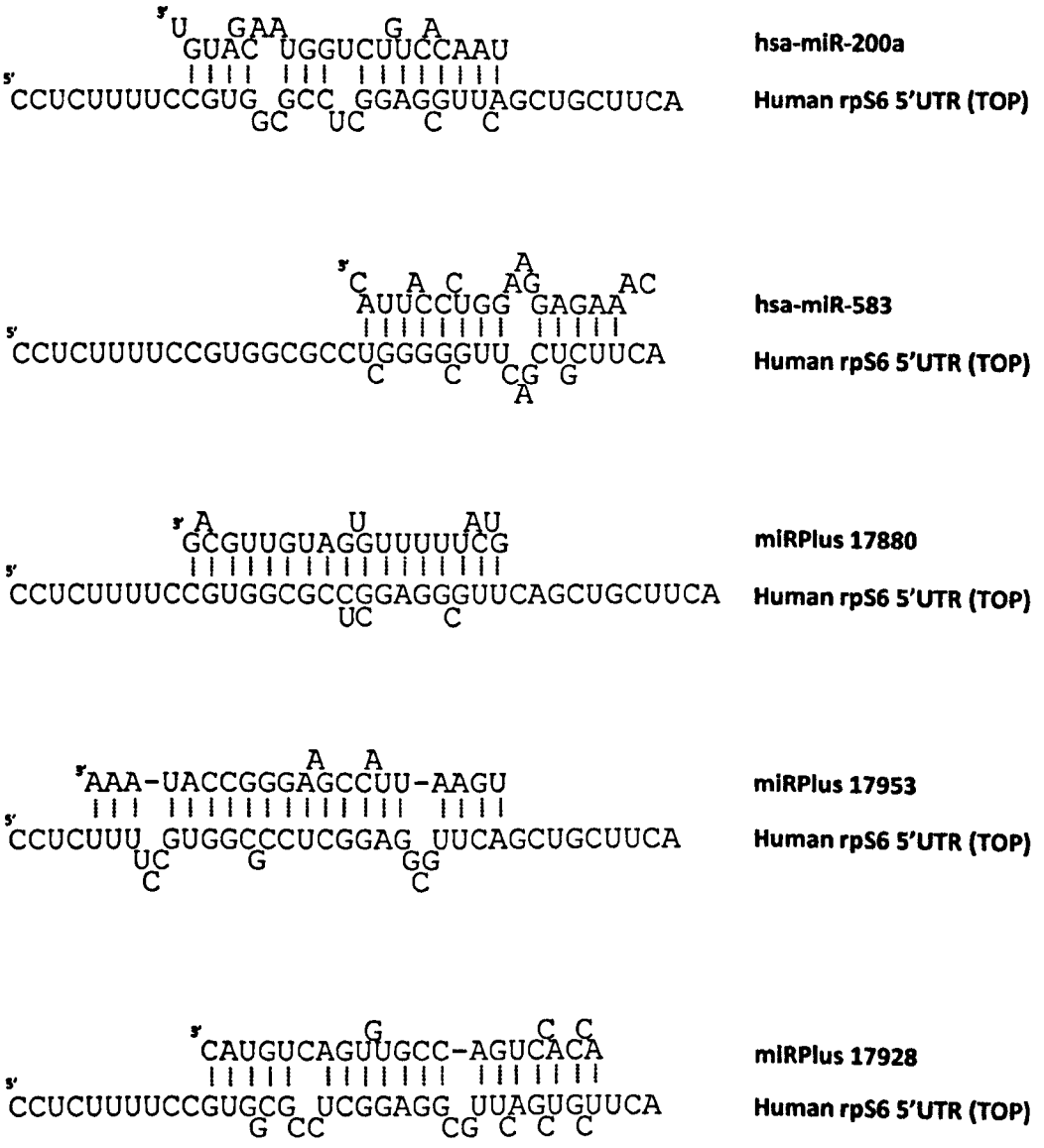


Figure 5.7.3: Alignment of MicroRNAs with TOP Containing mRNA

An alignment was then carried out between the TOP-14x mRNA 5' UTR sequence and the sequences of the fifteen microRNAs which were found to be more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA. Five example sequence alignments are shown, all of which were found to align to the TOP-14x mRNA sequence with mis-matched loop regions of less than three nucleotides in length.

5.9 Discussion

5.9.1 Analysis of the Proteins Associated with TOP and non-TOP Reporter mRNAs

PTB was found to be associated with both TOP-14x and TOP-trctMut-14x mRNA under both the serum-starved and serum starved and re-fed cells. This result supports the earlier observation that PTB is involved in the translational up-regulated of TOP and TOP-trctMut mRNAs, which both possess a polypyrimidine tract (refer to Chapter 4). In the serum starved cell, PTB is more associated with TOP-14x mRNA than TOP-trctMut-14x mRNA, which is consistent with the previous data in which TOP containing mRNA was up-regulated to a greater extent than Top-trctMut mRNA, when PTB was over-expressed (Figure 4.7.1). Surprising, in cells that were serum starved then re-fed, PTB appears to be more associated with TOP-trctMut-14x mRNA than TOP-14x mRNA. It is therefore possible that in cells that are serum starved then re-fed, PTB may bind to TOP containing mRNAs in competition with an as yet undetermined protein. Repetition of this experiment may be necessary in order to confirm the observed trend.

It has previously been observed that eIF4E does not appear to be involved the translation initiation of TOP containing mRNAs. For the TOP construct, over-expression of 4E-BP1 resulted in an overall increase in translational efficiency (Figure 4.2). It was also found that translational efficiency of the mRNA transcribed by the TOP construct was found to increase by 100% when eIF4E levels were reduced (Figure 4.4.2). In serum starved and re-fed cells, eIF4E was found to be associated approximately equally with both TOP-trctMut-14x and TOP-14x mRNAs. This data does not appear to fit the previously observed trend, which suggested that eIF4E was

not required by 5'TOP containing mRNAs. This data could be skewed however, as comparison of the un-bound wash and cleared lysate fractions suggests that eIF4E levels are slightly higher in cells transfected with the TOP-14x construct compared to TOP-trctMut-14x transfected cells. When this difference is taken into account, it is possible that TOP-14x mRNAs were associated with less of the available eIF4E than TOP-trctMut-14x mRNAs. Repetition of this experiment is required to confirm this trend. In serum-starved cells, eIF4E was found to be associated with TOP-trctMut-14x mRNA, but not TOP-14x mRNA (Figure 5.5B). This data appears to be consistent with an earlier study in which eIF4E was over-expressed. It was found that in quiescent cells, over-expression of eIF4E did not relieve the translational expression of ribosomal protein mRNAs, which are a subset of TOP containing mRNAs (Mamane et al. 2007).

Argonaute 2 (AGO2) was found to be associated with both TOP-14x and TOP-trctMut-14x mRNAs. Following serum re-feeding it was found that more AGO2 was associated with TOP-14x mRNA than TOP-trctMut-14x mRNA. This data suggests that AGO2 potentially plays a role in the translation of TOP containing mRNAs following nutrient stimulation. AGO2 is a critical component of the RNA-induced silencing complex (RISC) and it is therefore possible that its detection in serum-refed cells is related to the binding of miRNAs within the 5' UTR, as previously confirmed by microarray analysis. The potential involvement of Ago2 at the 5' terminus of mRNA is supported by the fact that AGO2 possesses a cap binding motif, which is similar to that found in eIF4E (Kiriakidou et al. 2007). Interestingly, it has also been shown that AGO2, which is normally thought of as being involved in translational repression by its involvement with miRNAs, can also be involved in translational up-regulation. It has been shown that fragile-X-mental-retardation-

related protein 1 (FXR1) and AGO2 associated with AU-rich elements (AREs), present in mRNA 3'-UTRs, exclusively during translation activation (Vasudevan and Steitz 2007). It would therefore be interesting to carry out a western blot to determine if FXR1 is also associated with TOP containing mRNAs under serum re-fed conditions.

The conditions of serum-starvation and re-feeding in this experiment were considerably shorter than in the previous experiments, in which the translation of TOP and TOP-trctMut mRNAs was investigated. This decision was made in an attempt to reduce the need for overnight affinity-purification, which may have resulted in the destabilisation and degradation of the proteins and protein-mRNA complex. Although the 4 hour serum-starvation period has previously been used for investigations involving TOP reporter constructs, stimulation for 45 minutes has only previously been reported utilising amino acid addition rather than the re-addition of serum (Stolovich et al. 2002). It is possible that the serum re-feeding conditions may not have been sufficient to affect translation activation of TOP containing mRNA. In order to confirm the effectiveness of the serum re-feeding, the experiment to determine 35S methionine incorporation (described in section 3.5) could be repeated with the new conditions. This would help to improve the confidence in the data obtained by western blotting. Additional negative controls could also be incorporated into this experiment and the subsequent experiment which detected differentially associated proteins using mass spectrometry. Constructs which did not encode MS2 binding sites could also be used to confirm the specificity of the binding of the mRNA-protein complex with the anti-Flag M2 beads. An mRNA transcript could also be transfected which did not contain a 5'UTR, but did contain the MS2 binding sites. This could be used to potentially eliminate from consideration, proteins that did

not bind within the vicinity of the 5'TOP motif. Whilst this may eliminate the detection of proteins that bound within the 5'UTR and also elsewhere on the mRNA, it may substantially reduce the number of false-positive results.

5.9.2 Analysis of the Proteins Detected by Mass Spectrometry

The data obtained following mass spectroscopy suggests that there are large differences between the number and type of proteins which bound to both TOP and TOP-trctMut mRNAs under serum-stimulated and serum-deprived conditions. In the case of the longest list (TOP, serum starved conditions), 310 proteins were detected, compared to just 74 proteins in the shortest list (TOP, serum re-fed conditions). A large number of differences also exist between the proteins purportedly co-purified with TOP and TOP-trctMut mRNAs. This result is particularly surprising considering that the difference between these two mRNAs is limited to a mutation of just two nucleotides within the 5'UTR. As previously suggested, it is therefore likely that these lists contain a large number of false-positive results. In order to draw firm conclusions, it would first be necessary to independently validate the data obtained using at least one different experimental technique. This could be achieved by repeating the affinity purification process and carrying out directed western blotting to attempt to detect specific proteins of interest. Experiments could also be carried out to determine the effect of over-expression or a reduction in levels of the candidate proteins. With these limitations in mind, considered analysis of the data can used to suggest candidate proteins which may be involved in the translation of TOP containing mRNAs.

La autoantigen and La related proteins (including LARP1, LARP2 and LARP7) were found to be associated with both TOP-14x and TOP-trctMut-14x mRNAs under both sets of experimental conditions. Previously, it was shown that the over-expression of La had no effect on the translation of mRNA transcribed by both TOP and TOP-trctMut constructs under similar experimental conditions (Figure 4.6). It is therefore likely that La is a polypyrimidine tract binding protein and does not contribute significantly to the translational regulation of 5'TOP containing mRNAs.

NF45 (ILF2) was found to be associated with TOP-14x mRNAs and not TOP-trctMut-14x samples. Interestingly, other known binding partners of NF45 including NF90 (ILF3), RNA helicase A, nucleolin and Heterogeneous nuclear ribonuclear proteins M and U (hnRNP-M & hnRNP-U) were also found to be associated with TOP-14x mRNA under serum stimulated conditions. NF90 is known to contain two double-stranded RNA (dsRNA) binding domains, allowing it to bind to double-stranded and structured single-stranded RNAs (Guan et al. 2008). When either NF45 or NF90 are knocked-down, the rate of cell division has been shown to decrease. NF45, with a half life of 1.5 hrs in Hela cells, is relatively unstable and is protected by NF90 (Guan et al. 2008). This rapid turnover raises the possibility that NF45 could have a potential role as a regulatory subunit.

Like AGO2, NF90 has also been shown to bind to AU-rich elements (AREs), present in mRNA 3'-UTRs (Guan et al. 2008). AGO2 and miRNAs have been shown to migrate together with messenger ribonucleoproteins (mRNPs) (Kim et al. 2004a; Nelson et al. 2004). AGO2 was found to sediment with three distinct AGO complexes (I-III). Interestingly, RNA helicase A, hnRNP-U, NF90 and NF45 were all found to be associated with AGO2 complex II, and with the exception of NF90,

AGO2 complex III (Hock et al. 2007). It is therefore possible that AGO2 functions with NF90 and its binding partners in the control of 5'TOP regulation.

The discovery that EBP1 was only found to be associated with TOP-14x mRNA, gives rise to the possibility that it is involved in the translational regulation of TOP containing mRNAs. It has been previously shown that EBP1 appears to act synergistically with PTB in stimulating formation of 48S initiation complexes (Pestova et al. 2001). It has been demonstrated using mass spectrometry that EBP1 is part of ribonucleoprotein complexes and associates with different rRNA species. This has led to speculation that EBP1 may provide a link between ribosome biosynthesis and cell proliferation (Squatrito et al. 2004).

It has been shown that EBP1 is localized to both the cytoplasm and to the nucleolus (Squatrito et al. 2004). Nucleophosmin (NPM), is one of the most abundant proteins in the nucleolus (Schmidt-Zachmann et al. 1987), and was also found to be associated with TOP-14x mRNA. EBP1 has been shown to form a complex with NPM, which plays a critical role in cell proliferation and survival (Okada et al. 2007). When levels of NPM or EBP1 are reduced it has been shown that both ribosome biogenesis and cell survival substantially decrease (Okada et al. 2007). Interestingly, EBP1 has been shown to be associated with mature ribosomes in the cytoplasm. It has also been shown that EBP1 is capable of inhibiting the phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Squatrito et al. 2006). It has been previously suggested that the phosphorylation eIF2α may be connected to the mTOR signalling pathway (Caldarola et al. 2004). EBP1 is highly conserved and it had been suggested that EBP1 could be involved downstream of multiple signal transduction pathways (Xia et al. 2001). EBP1 therefore appears to be perfectly positioned to regulate the translation 5'TOP containing mRNAs. It would therefore be of great interest to

further investigate the role of this protein in this system. Carrying out both over-expression and reduction of EBP1 levels and investigating the effect on endogenous and reporter 5'TOP containing mRNAs may yield useful information on the depth of involvement of this protein.

Although it is difficult to draw firm conclusions from the mass spectrometry data due to the lack of experimental validation, it should be possible to use these data to validate the results which were obtained following western blotting. Surprisingly, none of the proteins (specifically PTB, AGO2 and eIF4B), which were identified by western blotting as being associated with TOP and TOP-trctMut mRNA, are present in the mass spectrometry results. Since there is no obvious explanation for this, it would therefore be of interest to repeat the mass spectrometry and western blotting, using samples obtained from the same experiment.

5.9.3 Micro-RNAs Associated with Affinity Purified Reporter mRNA

It is interesting that of the sequence alignments carried out using the list of TOP-14x associated microRNAs, all five proposed binding sites of the microRNAs were outside of the region that differs between the TOP-trctMut-14x mRNA and TOP-14x mRNA (Figure 5.7.3A, indicated in red). This result calls into question the significance of the data, since the differential association of miRNAs cannot directly be associated with the differences in the mRNA sequences. It has been previously proposed that microRNA-10a specifically associates with the 5'UTR of TOP containing mRNAs (Orom et al. 2008). The mRNA transcribed by the TOP-mirMut-14x construct contains a mutation within the proposed binding site of microRNA-10a. Therefore, a more direct experimental approach would have been to compare the

differential association of microRNAs between the mRNA transcribed by the TOP-14x construct and a new TOP-mirMUT-14x construct.

If it is assumed that the difference between TOP-14x mRNA and TOP-trctMut-14x mRNA is unlikely to affect miRNA binding, then it may be possible to treat the data obtained from the TOP-trctMut mRNA as a replicate. The comparative list (refer to Appendix) was ranked with those microRNAs most differentially associated with the TOP-14x mRNA at the top of the list, and those most differentially associated with the TOP-trctMut-14x mRNA at the bottom of the list. Therefore, the microRNAs which are located towards the centre of the list are those which were found to associate with similar affinity to both TOP mRNA and its replicate. The comparative list contains 720 microRNAs, meaning that the microRNAs with the greatest affinity to TOP mRNA are clustered around rank 370. Interestingly, Micro-RNA-10a is located at rank 372 on the comparative list. If the earlier assumption is correct and the TOP-trctMut data may be treated as a replicate, then this data is consistent with the earlier study in which microRNA-10a was shown to be associated with the 5' UTR of TOP containing mRNAs (Orom et al. 2008). Confirmation of this data could be carried out by repeating the affinity purification experiment and comparing the differential association of microRNAs between the mRNA transcribed by the TOP-14x construct and a new TOP-mirMut-14x construct. Despite the evidence which suggests that microRNA-10a associates with TOP containing mRNA, it remains unclear what functional role is performed by this association.

Chapter Six

Discussion

6.1 5' TOP Containing mRNAs

As previously discussed the critical structural features of mRNAs containing the 5'terminal oligopyrimidine tract motif, are the presence of a C residue immediately preceding the 5' terminal m^7G cap structure of the mRNA and an uninterrupted 4-14 nucleotide polypyrimidine tract (reviewed in Chapter 1). Two distinct translational modes, which operate in a growth dependent manner, are common to all TOP containing mRNAs thereby facilitating a mechanism for their co-ordinated expression. In growing cells, TOP containing mRNAs are loaded with a full complement of ribosomes and are also translated with full efficiency. In growth arrested cells however, TOP containing mRNAs are entirely shifted into the sub-polysomal, non-translating population (Loreni and Amaldi 1992).

One of the primary aims of this project was to develop a 5' TOP containing mRNA reporter vector construct. Analysis of the 5' RACE data (Figure 3.6.3) confirms that the TOP construct transcribes mRNA that contains the 5'TOP motif with a C-residue immediately downstream of the 5' terminal m^7G cap and an uninterrupted polypyrimidine tract. Sucrose density gradient analysis also confirmed that mRNAs transcribed by the TOP construct were predominantly associated with polysomes in nutrient stimulated cells, while in starved cells the mRNA was almost entirely associated with sub-polysomal particles (Figure 3.8B). This is consistent with the characteristic growth-dependent expression of TOP containing mRNAs and therefore suggests that the first objective of the project was successfully achieved.

6.2 Signalling Pathways and 5' TOP containing mRNAs

An initial focus of research involving signalling pathways and their role in 5' TOP containing mRNA translational regulation was the observation that the translational activation of 5' TOP messages in response to mitogenic stimuli correlated with the phosphorylation of RPS6 (Thomas et al. 1980). RPS6 is a downstream target of S6K1 and S6K2, and as previously discussed, both of which are known to be involved in the mTOR signalling pathway and are themselves downstream targets of mTORC1 (Kim et al. 2002). mTOR1 has been shown to be sensitive to inhibition by Rapamycin and the repression of translation of 5' TOP containing mRNAs has been observed following Rapamycin treatment (Jefferies et al. 1997). In this study, the treatment of TOP and TOP-trctMut transfected HEK-293T cells with Rapamycin resulted in the translational down-regulation of mRNA transcribed from the TOP construct. Interestingly, translation of the TOP-trctMut remained relatively unaffected when compared to the control (Figure 3.10B). Whilst this data is only just outside the bounds of experimental error, it suggests that the regulation of 5' TOP containing mRNAs may be mediated by the mTOR signalling pathway. However, as previously discussed, evidence exists which suggest that this inhibition may be cell type specific (Tang et al. 2001; Stolovich et al. 2002). Repetition of this experiment in a wider variety of cells lines would help to determine if the observed trend is specific to the HEK-293T cell-line.

The PI3K (phosphoinositide 3-kinase) signalling pathway has also been implicated in translational regulation of 5' TOP containing mRNAs. It has been shown that activation of PI3K signalling occurs upon insulin or mitogenic stimuli

(Burgering and Coffer 1995; Kohn et al. 1995). The translational activation of 5' TOP containing mRNAs is completely blocked when cells are treated with LY294002 and Wortmannin, which are both inhibitors of the PI3K signalling pathway (Vlahos et al. 1994; Tang et al. 2001; Caldarola et al. 2004). This result was also obtained when cells were treated with the PI3K inhibitor Wortmannin (Caldarola et al. 2004). As discussed in section 3.8.2, studies in which HEK-293 cells were treated with LY294002, have revealed selective and pronounced repression of protein synthesis directed by TOP containing mRNAs encoding rpL32 and eEF1A. (Stolovich et al. 2002). Following the treatment of HEK-293T cells with both Wortmannin and LY294002 in this study (Figure 3.10B), no significant difference was observed between TOP and TOP-trctMut transcribed mRNAs. Luciferase activity from both constructs was reduced, suggesting that the effect observed was not specific to 5' TOP containing mRNAs. This data is therefore inconsistent with that obtained in previous studies. It is possible that the effects previously observed were specific to the particularly TOP containing mRNA that was investigated. As previously discussed, it would be of interest to repeat the experiments performed here using constructs containing the 5'UTRs of a wide variety of different TOP containing mRNAs.

6.3 Canonical Initiation Factors and 5' TOP Containing mRNAs

4E-BP was identified as a downstream target of mTOR and provides a potential link between this signalling pathway and the translational regulation of 5' TOP containing mRNAs. Under conditions of serum starvation, eIF4E is expected to remain tightly bound to hypo-phosphorylated members of the 4E-BP family and therefore act a rate limiting component in the cap-dependent initiation of translation

(Davuluri et al. 2000; Suzuki et al. 2000). The luciferase expression data (Figure 4.2) obtained following transfection of the TOP-trctMut construct is consistent with this hypothesis, with an observed decrease in translational efficiency of between 20-40% following over-expression of 4E-BP1. The fact that repression is still occurring following serum stimulation, suggests that the over-expression of 4E-BP1 is still resulting in the sequestration of eIF4E. This implies that not all the additionally expressed 4E-BP1 is phosphorylated following serum stimulation.

Contrary to the expected trend for cap-dependent initiation of translation, an increase in translational efficiency was observed following 4E-BP1 over-expression in the case of the TOP construct. This data raised the possibility that eIF4E availability may not be a rate-limiting factor in the translation initiation of 5' TOP containing mRNAs. The increase in translation of TOP mRNA, which was observed following serum-stimulation, was much greater than that observed in serum-starved cells. This is consistent with the known response of TOP containing mRNAs under conditions of serum-stimulation.

As previously discussed, the western blotting data shows that as the amount of transfected pcDNA3.1-4E-BP1 vector increased, an increase in 4E-BP1 protein expression was not observed. The loading control (Actin) appears relatively constant, although the relatively high abundance of Actin may have made it difficult to detect small changes between samples. It is also relevant the antibody, which was used for western blotting, detected total 4E-BP1 and was therefore not specific to the un-phosphorylated form of 4E-BP1 that is capable of sequestering eIF4E. An improved control for this experiment would be to determine the total cellular protein synthesis rate by measuring the incorporation of ^{35}S methionine. Carrying out western blotting

to determine the levels of unphosphorylated 4E-BP1 may also provide a greater insight into the mechanism at work.

The data obtained from sucrose density gradient analysis (Figures 4.3.1 and 4.3.2) also indicated that the translational efficiency of mRNA transcribed from the TOP construct increased following the over-expression of 4E-BP1. A similar trend was also observed following the reduction in eIF4E levels, which carried out using a plasmid expressing shRNA (Figure 4.4.2). However, when this experiment was repeated using the TOP-trctMut construct, the data obtained casts doubt on the significance of this experiment. Surprisingly, an increase in the translational efficiency of TOP-trctMut mRNA was observed when the levels of eIF4E were reduced. This result was inconsistent with that obtained when 4E-BP1 was over-expressed (Figure 4.2A). This anomalous result may potentially be explained by the subsequent analysis of the western blot data, which indicated a lower knockdown efficiency of eIF4E in the case of the TOP-trctMut vector transfection. Further repetition of this experiment may therefore be required to increase the confidence in the data.

It is difficult to draw firm conclusions from these experiments due to the limitations, which have been described. However, when these data are considered collectively, the possibility is raised that eIF4E may not be required for the initiation of translation of 5' TOP containing mRNAs. This proposal is challenged however, by the data obtained following the affinity purification of TOP-trctMut-14x and TOP-14x mRNAs, which indicated that eIF4E was associated approximately equally with both mRNAs. It should however be noted, that eIF4E levels were slightly higher in cells transfected with the TOP-14x construct compared to TOP-trctMut-14x transfected

cells. In serum-starved cells, eIF4E was found to be associated with TOP-trctMut-14x mRNA, but not TOP-14x mRNA (Figure 5.5B).

With the requirement of eIF4E by 5' TOP containing mRNAs called into question, investigations were carried out to determine whether its binding partner eIF4G (whose function is reviewed in Chapter 1) was also required. The translational efficiency of TOP mRNA increased by 20% under conditions in which eIF4GI levels were reduced without the concurrent re-expression using the 4G1f vector. An in increase observed in the translational activity of TOP mRNA was also observed when eIF4GI without the eIF4E binding-site was expressed following eIF4GI knock-down. These data appears consistent with the broader suggestion that neither eFI4GI nor eIF4E are required for the initiation of translation of TOP containing mRNAs. It is possible that as the translation of other mRNAs that require eiF4GI is reduced, TOP containing mRNAs can utilise the newly available pool of other initiation factors. In both of the above cases, the translation activity of TOP-trctMut and TOP-mirMut transcribed mRNAs remained unaffected. It is possible that initiation of translation of TOP-trctMut and TOP-mirMut mRNA may have occurred via a cap-dependent pathway that utilised eIF4GII, which was not expected to be affected by eIF4GI knockdown. The continued presence of eIF4GII as a variable in the data makes is impossible to draw a firm conclusion. Simultaneous reduction in the levels of both eIF4GI and eIF4GII would provide a clearer point of comparison. Real-time PCR could also be performed to confirm that the observed trends are related to translation effects and are not merely a function of cellular mRNA levels.

6.4 MicroRNAs

Recently, it has been reported that miR-10a binds to the 5' UTR of TOP containing mRNAs that encode ribosomal proteins and enhances their translation (Orom et al. 2008). It appears that Mir-10a is capable of alleviating the translational repression imposed upon 5' TOP containing mRNAs following amino acid starvation and that the 5' TOP motif is specifically required for this to occur (Orom et al. 2008). The mTOR signalling pathway has also been implicated in the regulation by miR-10a, since it has been determined that the translational enhancement imposed by miR-10a is Rapamycin sensitive (Orom et al. 2008). As a result of these observations, the affinity purification of TOP-14x and TOP-trctMut-14x was coupled microRNA microarray analysis to determine whether any microRNAs were specifically associated with either reporter mRNA.

Interestingly, the data revealed that all five proposed microRNA binding sites were outside of the region that differs between the TOP-trctMut-14x mRNA and TOP-14x mRNA (Figure 5.7.3). As a result, it was decided that it may have been more appropriate to treat the data obtained using the TOP-trctMut construct as an experimental replicate. Those microRNAs which were found to be associated with both TOP-14x and TOP-trctMut-14x would therefore be located towards the centre of a ranked list of differential association. Micro-RNA-10a was found to be located at a mid-way position on the list. This is consistent with the earlier study in which microRNA-10a was shown to be associated with the 5' UTR of TOP containing mRNAs (Orom et al. 2008). This data does not however provide any indication as to whether microRNA-10a binds within the earlier predicted location within the 5'UTR of TOP-14x mRNA. Repetition of the experiment using mRNA transcribed by the TOP-14x construct and a new TOP-mirMUT-14x construct, would more directly

address this question. Despite the evidence which suggests that microRNA-10a associates with TOP containing mRNAs, it remains unclear what functional role is performed by this association.

6.5 *Trans*-acting Factors

It has been suggested that the translation of 5' TOP containing mRNAs may be regulated by a variety of *trans*-acting factors (reviewed extensively in Chapter 1). To date, there have been no direct attempts to specifically purify factors that are bound to 5' TOP containing mRNAs. For this reason, a method was devised in which reporter mRNAs containing the 5' TOP motif, could be specifically purified whilst still bound to factors which are involved in their regulation. Following affinity purification, western blotting was carried out to investigate the association of previously identified candidate proteins with TOP containing mRNA. In addition, mass spectrometry was also carried out in an attempt to identify proteins which specifically associated with TOP mRNAs. As previously discussed in section 5.7, the lack of independent validation of the data obtained following mass spectrometry is a limitation to consider when evaluating the data. Without western blotting to confirm association of the proteins with the TOP-14x and TOP-trctMut-14x mRNAs, it is not possible to conclude that the proteins identified are not false-positive results. Indeed, the large number of different between TOP-14x and TOP-trctMut-14x associated proteins, for just a two nucleotide mutation, suggests that there are potentially a large number of false-positives within the dataset. Using the mass-spectrometer data to independently validate the western blots obtained in the first affinity purification also raises an interesting point to consider. PTB, AGO2 and eIF4B, which were identified

by western blotting as being associated with TOP and TOP-trctMut mRNA, are not present in the mass spectrometry results. Further repetition of the experiment is therefore required to increase confidence in the data. The limitations discussed here should be applied to the analysis of the candidate *trans*-acting factors discussed below.

The protein La has been linked to the regulation of 5' TOP containing mRNAs in numerous publications (reviewed in Chapter 1). It have been observed that La co-sediments with polysomes in an RNA-dependent manner and is specifically associated with 5' TOP containing mRNAs in the polysome fraction (Cardinali et al. 2003). Other research has proposed that La exerts a positive effect on translation through competitive binding with another protein called CNBP, which potentially acts a translational repressor (Crosio et al. 2000).

The data obtained upon over-expression of La, suggests that La has no effect on the translation of mRNA transcribed by both TOP and TOP-trctMut constructs under the experimental conditions used in this system (Figure 4.6). Interestingly, despite this observation, mass spectrometry carried out following the affinity purification of TOP-14x and TOP-trctMut-14x mRNAs indicated that La autoantigen and La related proteins (including LARP1, LARP2 and LARP7) were associated with both under both serum starved and serum re-fed conditions. Together these data suggest that La may be a general polypyrimidine tract binding protein and although it associates with 5' TOP containing mRNAs, it does not contribute significantly to the regulation of their translational. CNBP, which has been proposed as a translational repressor of 5' TOP containing mRNAs, was not found to be associated with either the TOP-14x or TOP-trctMut-14x mRNA. It may be of interest in the future to carry out an experiment in which CNBP is over-expressed under the same experimental

conditions carried out in this study. If CNBP is indeed a translational repressor of mRNAs containing the 5' TOP motif, then under conditions in which CNBP was over-expressed a reduction in the translational efficiency of 5' TOP containing mRNAs would be expected.

PTB (polypyrimidine tract binding protein) is a multi-function protein, which preferentially binds polypyrimidine-rich regions of mRNA (Garcia-Blanco et al. 1989). It is known that PTB often functions in collaboration with other proteins, including hnRNP K, to initiate translation (Sawicka et al. 2008). To date, no direct evidence has been published, which has definitively proven or disproven the involvement of PTB in the regulation of 5' TOP containing mRNAs. Following PTB over-expression in this study, both TOP and TOP-trctMUT constructs were translationally up-regulated. It is likely that PTB was binding to the polypyrimidine tract, which is a common feature of both mRNAs. Affinity purification and subsequent western blotting confirmed that PTB associated with both TOP-14x and TOP-trctMut-14x mRNA under both the serum-starved and serum starved and re-fed cells. As previously discussed, PTB was not detected by mass spectrometry. Investigation of the effect of hnRNP K over-expression on TOP and TOP-trctMut construct transcribed mRNAs, suggests that the 5' TOP containing mRNAs are relatively unaffected in both serum starved and serum starved and re-fed cells. It would perhaps be interesting to repeat this experiment with simultaneous expression of hnRNP E1, which has been shown to operate in conjunction with hnRNP K as a translational silencer (Ostareck et al. 1997).

Perhaps the most interesting candidate *trans*-acting factor to be identified following affinity purification and mass spectrometry is NF45 (ILF2). Interestingly, NF45 was found to be associated with TOP-14x mRNAs, but not TOP-trctMut-14x

mRNAs. As described in more detail in Chapter 5, additional binding partners of NF90 including RNA helicase A, nucleolin and Heterogeneous nuclear ribonuclear proteins M and U (hnRNP-M & hnRNP-U) were also found to be associated with TOP-14x mRNA under serum stimulated conditions. Repetition of this experiment could be carried out to confirm the association of NF45 and its known binding patterns with TOP-14x mRNAs. It may also be interesting to investigate the effect of over-expression or siRNA mediated reduction of NF45 and its associated proteins upon 5' TOP containing mRNAs. Preliminary data from experiments in which the TOP and TOP-trctMut vectors were transfected into NF45 deficient cell lines, showed relative translational up-regulation of TOP containing mRNA (personal communication from Dr. L. Cobbold).

Interestingly, NF90, NF45, RNA helicase A, and hnRNP-U have all been found to associate with known AGO2 complexes (Hock et al. 2007). AGO2 and microRNAs have been shown to migrate together with messenger ribonucleoproteins mRNPs (Kim et al. 2004a; Nelson et al. 2004). Thus, it is possible that AGO2 functions with NF90 and its binding partners in the control of 5' TOP regulation and that microRNAs are potentially involved in this process. AGO2 was found to be associated with both TOP-14x and TOP-trctMut-14x mRNAs. Following serum re-feeding it was found that more AGO2 was associated with TOP-14x mRNA than TOP-trctMut-14x mRNA. This data suggests that AGO2 potentially plays a role in the translation of 5' TOP containing mRNAs following nutrient stimulation.

EBP1 was also found to be associated specifically with TOP-14x mRNAs and not TOP-trctMut-14x. This is also an interesting result since EBP1 has been shown to act synergistically with PTB in stimulating formation of 48S initiation complexes (Pilipenko et al. 2000b). It has been demonstrated using mass spectrometry, that

EBP1 is part of ribonucleoprotein complexes and associates with different rRNA species. This has led to speculation that EBP1 may provide a link between ribosome biosynthesis and cell proliferation (Squatrito et al. 2004). Thus, EBP1 appears perfectly positioned for a role in the regulation of 5' TOP containing mRNAs.

6.6 Future Work

After addressing the limitations and implementing the experimental improvements discussed above, there are several research areas identified in this project which are likely to be worthy of future experimental work. If the additional data obtained does indeed suggest that TOP containing mRNAs do not require eIF4E for the initiation of translation, this study could be extended to include the over-expression of eIF4E. If the proposal is correct, the translation of TOP containing mRNAs should remain largely unaffected by eIF4E over-expression. Sucrose density gradient analysis could also be carried out following the over-expression of 4E-BP1, with the addition of Northern blotting to determine the polysomal distribution of known endogenous 5' TOP containing mRNAs. This may provide an insight as to whether all TOP containing mRNAs behave in the same way when 4E-BP1 is over-expressed.

If the results of the affinity purification and mass spectrometry are repeatable and can be validated by western blotting, the potential role of EBP1 could also be investigated further. A series of experiments involving over-expression of EBP1 and siRNA mediated reduction in EBP1 proteins levels could be carried out. It would be interesting to observe the effect on the translation of endogenous TOP containing mRNAs and also TOP and TOP-trctMut mRNA.

The data discussed above, suggests that AGO2 may play a role in the translation of 5' TOP containing mRNAs following nutrient stimulation. It has been shown that fragile-X-mental-retardation-related protein 1 (FXR1) and AGO2 associated with AU-rich elements (AREs), present in mRNA 3'-UTRs, exclusively during translation activation (Vasudevan and Steitz 2007). It would therefore be interesting to determine if FXR1 is also associated with TOP containing mRNAs under serum re-fed conditions. The over-expression of AGO2 or carrying out an siRNA mediated reduction in AGO2 proteins levels, may also provide further insight into the role (if any) of AGO2 in the regulation of 5' TOP containing mRNAs.

As previously discussed, the anti-Flag M2 affinity purification procedure could be repeated using a TOP-mirMut-14x construct and a new list of differentially associated microRNAs could be generated. Validation of any individual microRNA that is ranked highly on the list could then be carried out through the transfection of a 2'-O-methyl RNA oligonucleotide that has a complementary sequence to the miRNA of interest. The complementary 2'-O-methyl RNA oligonucleotide should sequester the candidate miRNA and prevent it from interacting with TOP containing mRNAs. Observing the translational effect on TOP mRNA may then provide an indication as to the likely significance of that miRNA in the translation of TOP containing mRNAs.

The constructs that contain 14x MS2 binding sites may also be useful for future studies involving the localisation of 5' TOP containing mRNAs. Co-transfection of these vectors with a vector expressing MS2-GFP fusion protein could be carried out. Following the association of the MS2-GFP fusion protein with the MS2 binding sites located in the 3' UTR of mRNAs, confocal microscopy could be used to visualise the location of these mRNAs. This is a proven technique for the visualisation of mRNAs and also has the advantage of being able to detect mRNA

mobility under a selection of cellular conditions (Fusco et al. 2003). Carrying out this technique following serum starvation and serum re-feeding may reveal new information about the location and movement of 5' TOP containing mRNAs under these conditions.

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Appendix

7.1.1 Top-trctMut-14x Affinity Purification: Mass Spectrometry Results (Serum Starved)

Anti-colorectal carcinoma heavy chain
CDNA FLJ46155 fis, clone TESTI4001517, moderately similar to Keratin, type I cytoskeletal 18
Kappa light chain variable region (Fragment)
Myosin-reactive immunoglobulin light chain variable region (Fragment)
Similar to 40S ribosomal protein S18
Similar to ribosomal protein S27
WUGSC:H_RG054D04.1 protein
ANK2 Isoform 1 of Ankyrin-2
APOB Apolipoprotein B-100 precursor
ARID4B Isoform 4 of AT-rich interactive domain-containing protein 4B
ARS2 Arsenite-resistance protein ARS2
ATG2A Isoform 1 of Autophagy-related protein 2 homolog A
C1QBP Complement component 1 Q subcomponent-binding protein, mitochondrial precursor
C20orf74 Isoform 3 of 250 kDa substrate of Akt
CAPRIN1 Caprin-1
CEP110 Centrosomal protein 110kDa
DCD Dermcidin precursor
DHX30 Isoform 1 of Putative ATP-dependent RNA helicase DHX30
DHX9 ATP-dependent RNA helicase A
EFCAB5 Isoform 3 of EF-hand calcium-binding domain-containing protein 5
EIF3B Isoform 1 of Eukaryotic translation initiation factor 3 subunit B
EIF3C Eukaryotic translation initiation factor 3 subunit C
GADD45GIP1 Growth arrest and DNA-damage-inducible proteins-interacting protein 1
GNB2L1 Lung cancer oncogene 7
hCG_2033311 Uncharacterized protein ENSP00000375173
hCG_2040224 Uncharacterized protein ENSP00000375262
hCG_2040224 Uncharacterized protein ENSP00000375262
hCG_21078 hypothetical protein LOC389435
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U
HNRPUL1 Isoform 1 of Heterogeneous nuclear ribonucleoprotein U-like protein 1
ICT1 Immature colon carcinoma transcript 1 protein precursor
ING3 Isoform 1 of Inhibitor of growth protein 3
KIF11 Kinesin-like protein KIF11
KRT1 Keratin, type II cytoskeletal 1
KRT10 Keratin, type I cytoskeletal 10
KRT12 Keratin, type I cytoskeletal 12
KRT2 Keratin, type II cytoskeletal 2 epidermal
KRT6B Keratin, type II cytoskeletal 6B
KRT77 Keratin 77
KRT9 Keratin, type I cytoskeletal 9
LARP1 Isoform 1 of La-related protein 1
LOC284393 similar to ribosomal protein L10 isoform 1
LOC389342 similar to ribosomal protein L10 isoform 6
LOC390998 similar to 60S ribosomal protein L10
LOC391370 similar to ribosomal protein S12
LOC402057 Uncharacterized protein ENSP00000375215
LOC644511 Uncharacterized protein ENSP00000375499
LOC644937 similar to 60S ribosomal protein L10
LOC729293 similar to ribosomal protein S18
LOC729611 similar to 60S ribosomal protein L29
LOC730070 similar to 60S ribosomal protein L26
LOC731567;RPL21;LOC729402 60S ribosomal protein L21
LYZ Lysozyme C precursor
MRPL12 39S ribosomal protein L12, mitochondrial precursor

MRPL2 39S ribosomal protein L2, mitochondrial precursor
MRPL23 Mitochondrial 39S ribosomal protein L23
MRPL28 39S ribosomal protein L28, mitochondrial precursor
MRPL43 mitochondrial ribosomal protein L43 isoform d
MRPS23 Mitochondrial ribosomal protein S23
MRPS26 28S ribosomal protein S26, mitochondrial precursor
MRPS34 Mitochondrial 28S ribosomal protein S34
MRPS7 28S ribosomal protein S7, mitochondrial precursor
NBEAL2 SQFE253
NCL Isoform 1 of Nucleolin
NDST3 Isoform 1 of Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 3
NOL1 NOL1 protein
PDAP1 28 kDa heatand acid-stable phosphoprotein
POP1 Ribonucleases P/MRP protein subunit POP1
PRSS3 Isoform A of Trypsin-3 precursor
PSMD1 Isoform 2 of 26S proteasome non-ATPase regulatory subunit 1
RPL10A 25 kDa protein
RPL10L 60S ribosomal protein L10-like
RPL11 Isoform 1 of 60S ribosomal protein L11
RPL12 60S ribosomal protein L12
RPL13 60S ribosomal protein L13
RPL13A 60S ribosomal protein L13a
RPL14 RPL14 protein
RPL15 60S ribosomal protein L15
RPL15;LOC653232 Ribosomal protein L15 pseudogene 3
RPL17 60S ribosomal protein L17
RPL18 60S ribosomal protein L18
RPL18A 60S ribosomal protein L18a
RPL19 60S ribosomal protein L19
RPL22 60S ribosomal protein L22
RPL23 60S ribosomal protein L23
RPL23A;hCG_16001 60S ribosomal protein L23a
RPL24 60S ribosomal protein L24
RPL26 60S ribosomal protein L26
RPL27 60S ribosomal protein L27
RPL28 60S ribosomal protein L28
RPL3 31 kDa protein
RPL30 60S ribosomal protein L30
RPL31 60S ribosomal protein L31
RPL34 60S ribosomal protein L34
RPL35 60S ribosomal protein L35
RPL35A 60S ribosomal protein L35a
RPL36 60S ribosomal protein L36
RPL7 60S ribosomal protein L7
RPL7A 60S ribosomal protein L7a
RPL8 60S ribosomal protein L8
RPL9 60S ribosomal protein L9
RPLP1 60S acidic ribosomal protein P1
RPLP2 60S acidic ribosomal protein P2
RPS10 40S ribosomal protein S10
RPS11 40S ribosomal protein S11
RPS12 ribosomal protein S12
RPS13 40S ribosomal protein S13
RPS14 40S ribosomal protein S14
RPS15A 40S ribosomal protein S15a
RPS16 40S ribosomal protein S16
RPS17 40S ribosomal protein S17

RPS18 40S ribosomal protein S18
RPS19 40S ribosomal protein S19
RPS2 40S ribosomal protein S2
RPS20 40S ribosomal protein S20
RPS23 40S ribosomal protein S23
RPS24 Isoform 1 of 40S ribosomal protein S24
RPS25 40S ribosomal protein S25
RPS26 OTTHUMP00000018641
RPS3 40S ribosomal protein S3
RPS3 40S ribosomal protein S3
RPS3A 40S ribosomal protein S3a
RPS4X 40S ribosomal protein S4, X isoform
RPS5 40S ribosomal protein S5
RPS6 40S ribosomal protein S6
RPS7 40S ribosomal protein S7
RPS8 40S ribosomal protein S8
RPS9 40S ribosomal protein S9
RPSA Ribosomal protein SA
SAMD9 Isoform 1 of Sterile alpha motif domain-containing protein 9
SLC25A5 ADP/ATP translocase 2
SMC4 SMC4 protein (Fragment)
SND1 Staphylococcal nuclease domain-containing protein 1
SSB Lupus La protein
THOC4 THO complex subunit 4
TMEM63C Transmembrane protein 63C
TPX2 Targeting protein for Xklp2
TSR1 Pre-rRNA-processing protein TSR1 homolog
UPF1 Isoform 1 of Regulator of nonsense transcripts 1
ZC3HAV1 Isoform 2 of Zinc finger CCCH type antiviral protein 1
ZFR 115 kDa protein
ZNF571 HERV-T_19q13.11 provirus ancestral Env polyprotein precursor

7.1.2 Top-14x Affinity Purification: Mass Spectrometry Results (Serum Starved)

Anti-colorectal carcinoma heavy chain
Kappa light chain variable region (Fragment)
Keratin-8-like protein 1
Myosin-reactive immunoglobulin light chain variable region (Fragment)
Putative p150
RcNSEP1 (Fragment)
Similar to 40S ribosomal protein S18
Similar to N-acetylgalactosaminyltransferase 7
Similar to ribosomal protein S27
WUGSC:H_RG054D04.1 protein
ACBD3 Golgi resident protein GCP60
AHSA1 Activator of 90 kDa heat shock protein ATPase homolog 1
AKAP12 A-kinase anchor protein 12 isoform 2
AMPH Amphiphysin I variant CT4 (Fragment)
ARHGEF18 Rho guanine nucleotide exchange factor 18
ATP1B1 Isoform 2 of Sodium/potassium-transporting ATPase subunit beta-1
ATP5B ATP synthase subunit beta, mitochondrial precursor
BYSL Bystin
C20orf23 Isoform 1 of Kinesin-like motor protein C20orf23
C4orf14 Uncharacterized protein C4orf14
C7orf50 Uncharacterized protein C7orf50
CCDC113 Coiled-coil domain-containing protein 113
CCDC50 Isoform 2 of Coiled-coil domain-containing protein 50
CCNT1 Cyclin-T1
CHMP2A Charged multivesicular body protein 2a
CIT Isoform 1 of Citron Rho-interacting kinase
CLASP2 CLIP-associating protein 2
CSDA Isoform 1 of DNA-binding protein A
CTAGE1 Protein cTAGE-2
DAP3 Mitochondrial 28S ribosomal protein S29
DAP3 Mitochondrial 28S ribosomal protein S29
DCD Dermcidin precursor
DCD Dermcidin precursor
DDX1 ATP-dependent RNA helicase DDX1
DDX18 ATP-dependent RNA helicase DDX18
DDX21 Isoform 1 of Nucleolar RNA helicase 2
DDX28 Probable ATP-dependent RNA helicase DDX28
DDX3X ATP-dependent RNA helicase DDX3X
DDX5 Probable ATP-dependent RNA helicase DDX5
DHX15 Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
DHX30 Isoform 1 of Putative ATP-dependent RNA helicase DHX30
DHX9 ATP-dependent RNA helicase A
DHX9 ATP-dependent RNA helicase A
DIAPH3 Isoform 2 of Protein diaphanous homolog 3
DMAP1 DNA methyltransferase 1 associated protein 1
DNAJA1 DnaJ homolog subfamily A member 1
DNAJA2 DnaJ homolog subfamily A member 2
DUSP27 Inactive dual specificity phosphatase 27
EEF1A1 Elongation factor 1-alpha
EEF2 Elongation factor 2
EFCAB5 Isoform 3 of EF-hand calcium-binding domain-containing protein 5
EFTUD2 116 kDa U5 small nuclear ribonucleoprotein component
EIF3EIP Eukaryotic translation initiation factor 3 subunit 6 interacting protein
EIF3F Eukaryotic translation initiation factor 3 subunit 5
EIF3H Eukaryotic translation initiation factor 3 subunit 3
EMD Emerin
ERAL1 Isoform HERA-A of GTP-binding protein era homolog

GFPT1 Isoform 1 of Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1
GNB2L1 Lung cancer oncogene 7
GTPBP4 Nucleolar GTP-binding protein 1
GTPBP4 Nucleolar GTP-binding protein 1
hCG_1644323 similar to 60S ribosomal protein L32
hCG_2023776 similar to Heterogeneous nuclear ribonucleoprotein A1
hCG_2033311 Uncharacterized protein ENSP00000375173
hCG_2040224 Uncharacterized protein ENSP00000375262
hCG_2040224 Uncharacterized protein ENSP00000375262
hCG_21078 hypothetical protein LOC389435
HEJ1 HEJ1
HIST1H1C Histone H1.2
HLA class I histocompatibility antigen, B-7 alpha chain precursor
HNRNPA0 Heterogeneous nuclear ribonucleoprotein A0
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1
HNRNPC Isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2
HNRNPR Heterogeneous nuclear ribonucleoprotein R
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B
HNRPCL1 Heterogeneous nuclear ribonucleoprotein C-like 1
HNRPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0
HNRPDL Isoform 3 of Heterogeneous nuclear ribonucleoprotein D-like
HNRPH1 Heterogeneous nuclear ribonucleoprotein H
HNRPK Isoform 1 of Heterogeneous nuclear ribonucleoprotein K
HNRPM Isoform 1 of Heterogeneous nuclear ribonucleoprotein M
HNRPUL1 Isoform 4 of Heterogeneous nuclear ribonucleoprotein U-like protein 1
HSP90AB1 Heat shock protein HSP 90-beta
HSP90AB2P Heat shock protein 90Bb
HSPA1B;HSPA1A Heat shock 70 kDa protein 1
HSPA2 Heat shock-related 70 kDa protein 2
HSPA5 HSPA5 protein
HSPA7 Putative heat shock 70 kDa protein 7
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein
HSPA9 Stress-70 protein, mitochondrial precursor
HSPD1 60 kDa heat shock protein, mitochondrial precursor
IGF2BP1 Insulin-like growth factor 2 mRNA-binding protein 1
IGF2BP3 Isoform 1 of Insulin-like growth factor 2 mRNA-binding protein 3
IGHV3OR16-13;IGHA1 IGHA1 protein
IL16 IL16 protein
ILF2 Interleukin enhancer-binding factor 2
ILF3 Interleukin enhancer binding factor 3 isoform c variant (Fragment)
ILF3 Isoform 5 of Interleukin enhancer-binding factor 3
ITPR2 Isoform Long of Inositol 1,4,5-trisphosphate receptor type 2
KCNK1 Potassium channel, subfamily K, member 1
KIF11 Kinesin-like protein KIF11
KIF13A Isoform 3 of Kinesin-like protein KIF13A
KIF27 Isoform 1 of Kinesin-like protein KIF27
KRT1 Keratin, type II cytoskeletal 1
KRT10 Keratin, type I cytoskeletal 10
KRT12 Keratin, type I cytoskeletal 12
KRT14 Keratin, type I cytoskeletal 14
KRT18 Keratin, type I cytoskeletal 18
KRT2 Keratin, type II cytoskeletal 2 epidermal
KRT4 keratin 4
KRT5 Keratin, type II cytoskeletal 5
KRT6B Keratin, type II cytoskeletal 6B

KRT73 Isoform 1 of Keratin, type II cytoskeletal 73
KRT77 Keratin 77
KRT9 Keratin, type I cytoskeletal 9
LARP1 Isoform 1 of La-related protein 1
LARP7 Lupus La protein family protein
LDHC L-lactate dehydrogenase C chain
LILRB3 leukocyte immunoglobulin-like receptor, subfamily B, member 3 isoform 2
LOC388720 similar to ubiquitin and ribosomal protein S27a precursor
LOC389342 similar to ribosomal protein L10 isoform 6
LOC390998 similar to 60S ribosomal protein L10
LOC402057 Uncharacterized protein ENSP00000375215
LOC440055 similar to ribosomal protein S12
LOC440577 similar to nucleophosmin 1 isoform 1
LOC644511 Uncharacterized protein ENSP00000375499
LOC644937 similar to 60S ribosomal protein L10
LOC646195;RPS28;LOC645899 40S ribosomal protein S28
LOC649946 LOC649946 protein
LOC729293 similar to ribosomal protein S18
LOC729611 similar to 60S ribosomal protein L29
LOC729611 similar to 60S ribosomal protein L29
LOC730037 similar to Collagen alpha-2(XI) chain precursor
LOC730070 similar to 60S ribosomal protein L26
LYAR Cell growth-regulating nucleolar protein
MAST1 Microtubule-associated serine/threonine-protein kinase 1
MEPCE 7SK snRNA methylphosphate capping enzyme
MLL3 Isoform 1 of Myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog
MRPL10 mitochondrial ribosomal protein L10 isoform b
MRPL11 39S ribosomal protein L11, mitochondrial precursor
MRPL2 39S ribosomal protein L2, mitochondrial precursor
MRPL21 39S ribosomal protein L21, mitochondrial precursor
MRPL28 39S ribosomal protein L28, mitochondrial precursor
MRPL3 Mitochondrial 39S ribosomal protein L3
MRPL37 39S ribosomal protein L37, mitochondrial precursor
MRPL38 39S ribosomal protein L38, mitochondrial precursor
MRPL39 Isoform 2 of Mitochondrial 39S ribosomal protein L39
MRPL43 mitochondrial ribosomal protein L43 isoform d
MRPL44 39S ribosomal protein L44, mitochondrial precursor
MRPL45 39S ribosomal protein L45, mitochondrial precursor
MRPL48 Isoform 1 of 39S ribosomal protein L48, mitochondrial precursor
MRPL49 Mitochondrial 39S ribosomal protein L49
MRPL50 Mitochondrial 39S ribosomal protein L50
MRPL9 Mitochondrial ribosomal protein L9
MRPS16 28S ribosomal protein S16, mitochondrial precursor
MRPS18A 28S ribosomal protein S18a, mitochondrial precursor
MRPS18B 28S ribosomal protein S18b, mitochondrial precursor
MRPS22 Mitochondrial 28S ribosomal protein S22
MRPS23 Mitochondrial ribosomal protein S23
MRPS26 28S ribosomal protein S26, mitochondrial precursor
MRPS27 Mitochondrial 28S ribosomal protein S27
MRPS30 Mitochondrial 28S ribosomal protein S30
MRPS31 28S ribosomal protein S31, mitochondrial precursor
MRPS34 Mitochondrial 28S ribosomal protein S34
MRPS7 28S ribosomal protein S7, mitochondrial precursor
MRPS9 mitochondrial ribosomal protein S9
MTDH Protein LYRIC
NCL CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin
NCL Isoform 1 of Nucleolin
NDST3 Isoform 1 of Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 3
NFE2L1 Isoform 1 of Nuclear factor erythroid 2-related factor 1

NOB1 RNA-binding protein NOB1
NPM1 Isoform 1 of Nucleophosmin
PA2G4 Proliferation-associated protein 2G4
PABPC1 Isoform 1 of Polyadenylate-binding protein 1
PABPC1L Polyadenylate-binding protein 1-like
PABPC1L2A;PABPC1L2B Polyadenylate-binding protein 1-like 2
PABPC3 Polyadenylate-binding protein 3
PABPC4 Poly
PCBP2 poly(rC)-binding protein 2 isoform b
PCCA Propionyl Coenzyme A carboxylase, alpha polypeptide
PCDH15 Isoform 1 of Protocadherin-15 precursor
PLA2G12A Group XIIA secretory phospholipase A2 precursor
PNO1 RNA-binding protein PNO1
POP1 Ribonucleases P/MRP protein subunit POP1
POP1 Ribonucleases P/MRP protein subunit POP1
PPFIA2 7 kDa protein
PPM1G Protein phosphatase 1G
PRKDC Isoform 1 of DNA-dependent protein kinase catalytic subunit
PRPF19 Pre-mRNA-processing factor 19
PRPF3 Isoform 1 of U4/U6 small nuclear ribonucleoprotein Prp3
PRSS1 Trypsin-1 precursor
PRSS2 Protease serine 2 isoform B
PRSS3 Isoform A of Trypsin-3 precursor
PSMC5 26S protease regulatory subunit 8
PTCD1 Pentatricopeptide repeat-containing protein 1
PTCD3 Isoform 1 of Pentatricopeptide repeat-containing protein 3, mitochondrial precursor
RBM26 Isoform 1 of RNA-binding protein 26
RBMX Heterogeneous nuclear ribonucleoprotein G
RCN2 Reticulocalbin-2 precursor
RNMTL1 RNA methyltransferase-like protein 1
RPAP3 Isoform 1 of RNA polymerase II-associated protein 3
RPL10A 25 kDa protein
RPL10L 60S ribosomal protein L10-like
RPL11 Isoform 1 of 60S ribosomal protein L11
RPL11 Isoform 1 of 60S ribosomal protein L11
RPL12 60S ribosomal protein L12
RPL13 60S ribosomal protein L13
RPL13A 60S ribosomal protein L13a
RPL14 RPL14 protein
RPL15 60S ribosomal protein L15
RPL17 60S ribosomal protein L17
RPL18 60S ribosomal protein L18
RPL18A 60S ribosomal protein L18a
RPL19 60S ribosomal protein L19
RPL22 60S ribosomal protein L22
RPL23 60S ribosomal protein L23
RPL23A;hCG_16001 60S ribosomal protein L23a
RPL24 60S ribosomal protein L24
RPL26 60S ribosomal protein L26
RPL27 60S ribosomal protein L27
RPL28 60S ribosomal protein L28
RPL3 31 kDa protein
RPL3 60S ribosomal protein L3
RPL30 60S ribosomal protein L30
RPL31 60S ribosomal protein L31
RPL34 60S ribosomal protein L34
RPL35 60S ribosomal protein L35
RPL35A 60S ribosomal protein L35a
RPL36 60S ribosomal protein L36

RPL3L 60S ribosomal protein L3-like
RPL4 60S ribosomal protein L4
RPL5 60S ribosomal protein L5
RPL6 60S ribosomal protein L6
RPL7 60S ribosomal protein L7
RPL7A 60S ribosomal protein L7a
RPL8 60S ribosomal protein L8
RPL9 60S ribosomal protein L9
RPLP0 60S acidic ribosomal protein P0
RPLP1 60S acidic ribosomal protein P1
RPLP2 60S acidic ribosomal protein P2
RPS10 40S ribosomal protein S10
RPS11 40S ribosomal protein S11
RPS13 40S ribosomal protein S13
RPS14 40S ribosomal protein S14
RPS15A 40S ribosomal protein S15a
RPS16 40S ribosomal protein S16
RPS17 40S ribosomal protein S17
RPS18 40S ribosomal protein S18
RPS19 40S ribosomal protein S19
RPS19BP1 40S ribosomal protein S19-binding protein 1
RPS2 40S ribosomal protein S2
RPS2 40S ribosomal protein S2
RPS20 40S ribosomal protein S20
RPS24 Isoform 1 of 40S ribosomal protein S24
RPS25 40S ribosomal protein S25
RPS26 OTTHUMP00000018641
RPS3 40S ribosomal protein S3
RPS3A 40S ribosomal protein S3a
RPS3A 40S ribosomal protein S3a
RPS4X 40S ribosomal protein S4, X isoform
RPS5 40S ribosomal protein S5
RPS6 40S ribosomal protein S6
RPS7 40S ribosomal protein S7
RPS7 40S ribosomal protein S7
RPS8 40S ribosomal protein S8
RPS9 40S ribosomal protein S9
RPSA Ribosomal protein SA
RPSA Ribosomal protein SA
RPSAP15 Laminin receptor-like protein LAMRL5
SAMD9 Isoform 1 of Sterile alpha motif domain-containing protein 9
SCN1A Isoform 1 of Sodium channel protein type 1 subunit alpha
SERBP1 Isoform 1 of Plasminogen activator inhibitor 1 RNA-binding protein
SF3B2 splicing factor 3B subunit 2
SFRS4 Splicing factor, arginine/serine-rich 4
SLC25A3 Isoform A of Phosphate carrier protein, mitochondrial precursor
SLC25A5 ADP/ATP translocase 2
SMC4 SMC4 protein (Fragment)
SMC6 Isoform 1 of Structural maintenance of chromosomes protein 6
SRP68 Isoform 1 of Signal recognition particle 68 kDa protein
SRP72 Signal recognition particle 72 kDa protein
SSB Lupus La protein
STAU1 Isoform Long of Double-stranded RNA-binding protein Staufen homolog 1
THOC4 THO complex subunit 4
TIE1 Tyrosine-protein kinase receptor Tie-1 precursor
TIMM50 Import inner membrane translocase subunit TIM50, mitochondrial precursor
TLN2 Talin-2
TROVE2 Isoform Long of 60 kDa SS-A/Ro ribonucleoprotein
TSEN34 tRNA-splicing endonuclease subunit Sen34

TSR1 Pre-rRNA-processing protein TSR1 homolog
TTN Isoform 4 of Titin
TTN titin isoform novex-1
TUBA1A Tubulin alpha-1A chain
TUBA1B Tubulin alpha-1B chain
TUBA4B Tubulin alpha-4 chain
TUBB Tubulin beta chain
TUBB Tubulin beta chain
TUBB2C Tubulin beta-2C chain
TUBB2C Tubulin beta-2C chain
TUFM Tu translation elongation factor, mitochondrial
UBC;UBB;RPS27A ubiquitin and ribosomal protein S27a precursor
WBP11 WW domain-binding protein 11
YBX1 Nuclease sensitive element-binding protein 1
YSK4 79 kDa protein
ZC3HAV1 Isoform 2 of Zinc finger CCCH type antiviral protein 1
ZNF571 HERV-T_19q13.11 provirus ancestral Env polyprotein precursor
ZNF622 Zinc finger protein 622

7.1.3 Top-trctMut-14x Affinity Purification: Mass Spectrometry Results (Serum Starved & Re-fed)

Anti-colorectal carcinoma heavy chain
CDNA FLJ46155 fis, clone TESTI4001517, moderately similar to Keratin, type I cytoskeletal 18
Kappa light chain variable region (Fragment)
Keratin-8-like protein 1
Myosin-reactive immunoglobulin light chain variable region (Fragment)
OTTHUMP00000028841
RcNSEP1 (Fragment)
Short heat shock protein 60 Hsp60s2
Similar to Ribosomal protein S2
WUGSC:H_RG054D04.1 protein
ABCF1 Isoform 2 of ATP-binding cassette sub-family F member 1
ADCY6 Isoform 1 of Adenylate cyclase type 6
AFF1 Putative FelC
ARHGAP28 Isoform 2 of Rho GTPase-activating protein 28
BYSL Bystin
C1QBP Complement component 1 Q subcomponent-binding protein, mitochondrial precursor
CCDC12 Coiled-coil domain-containing protein 12
CCDC15 Coiled-coil domain-containing protein 15
CCDC40 Isoform 3 of Coiled-coil domain-containing protein 40
CCDC66 CCDC66 protein
CENPJ Centromere protein J
CENTD1 Centaurin-delta-1
CEP110 Centrosomal protein 110kDa
CIT Isoform 1 of Citron Rho-interacting kinase
CSDA Isoform 1 of DNA-binding protein A
DAP3 Mitochondrial 28S ribosomal protein S29
DCD Dermcidin precursor
DDX3X ATP-dependent RNA helicase DDX3X
DDX5 Probable ATP-dependent RNA helicase DDX5
DIAPH3 Isoform 2 of Protein diaphanous homolog 3
DNA2 Isoform 3 of DNA2-like helicase
DNAH11 Ciliary dynein heavy chain 11
DUSP9 Similar to dual specificity phosphatase 9
ECT2 epithelial cell transforming sequence 2 oncogene protein
FCHSD2 Isoform 2 of FCH and double SH3 domains protein 2
FLJ46111 FLJ46111 protein
FRY WUGSC:H_2G3A.1 protein
GALK1 Galactokinase
GNB2L1 Lung cancer oncogene 7
GNL3 Isoform 2 of Guanine nucleotide-binding protein-like 3
GTPBP4 Nucleolar GTP-binding protein 1
HIST1H1C Histone H1.2
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1
HNRNPL heterogeneous nuclear ribonucleoprotein L isoform a
HNRNPR Heterogeneous nuclear ribonucleoprotein R
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U
HNRPCL1 Heterogeneous nuclear ribonucleoprotein C-like 1
HNRPH1 Heterogeneous nuclear ribonucleoprotein H
HNRPK Isoform 1 of Heterogeneous nuclear ribonucleoprotein K
HNRPM Isoform 1 of Heterogeneous nuclear ribonucleoprotein M
HSPA1B;HSPA1A Heat shock 70 kDa protein 1
HSPA1L Heat shock 70 kDa protein 1L
HSPA6 Heat shock 70 kDa protein 6

HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein
HYDIN;HYDIN2 Uncharacterized protein HYDIN2
IGF2BP1 Insulin-like growth factor 2 mRNA-binding protein 1
IGF2BP3 Isoform 2 of Insulin-like growth factor 2 mRNA-binding protein 3
ITPR2 Isoform Long of Inositol 1,4,5-trisphosphate receptor type 2
KIF13A Isoform 3 of Kinesin-like protein KIF13A
KRT1 Keratin, type II cytoskeletal 1
KRT10 Keratin, type I cytoskeletal 10
KRT12 Keratin, type I cytoskeletal 12
KRT13 keratin 13 isoform b
KRT14 Keratin, type I cytoskeletal 14
KRT16 Keratin, type I cytoskeletal 16
KRT18 Keratin, type I cytoskeletal 18
KRT19 Keratin, type I cytoskeletal 19
KRT2 Keratin, type II cytoskeletal 2 epidermal
KRT3 Keratin, type II cytoskeletal 3
KRT4 keratin 4
KRT4 keratin 4
KRT4 keratin 4
KRT5 Keratin, type II cytoskeletal 5
KRT6A Keratin, type II cytoskeletal 6A
KRT6B Keratin, type II cytoskeletal 6B
KRT73 Isoform 1 of Keratin, type II cytoskeletal 73
KRT75 Keratin, type II cytoskeletal 75
KRT76 Keratin, type II cytoskeletal 2 oral
KRT77 Keratin 77
KRT8 Keratin, type II cytoskeletal 8
KRT80 Isoform 3 of Keratin, type II cytoskeletal 80
KRT85 Keratin type II cuticular Hb5
KRT9 Keratin, type I cytoskeletal 9
LARP2 Isoform 1 of La-related protein 2
LARP7 Lupus La protein family protein
LOC343851 similar to 40S ribosomal protein S10
LOC387867 similar to 40S ribosomal protein SA
LOC729611 similar to 60S ribosomal protein L29
LTV1 Protein LTV1 homolog
LYAR Cell growth-regulating nucleolar protein
METTL3 Isoform 1 of N6-adenosine-methyltransferase 70 kDa subunit
MRPL1 mitochondrial ribosomal protein L1 precursor
MRPL19 39S ribosomal protein L19, mitochondrial precursor
MRPL2 39S ribosomal protein L2, mitochondrial precursor
MRPL28 39S ribosomal protein L28, mitochondrial precursor
MRPL39 Isoform 2 of Mitochondrial 39S ribosomal protein L39
MRPL4 Isoform 1 of Mitochondrial 39S ribosomal protein L4
MRPL44 39S ribosomal protein L44, mitochondrial precursor
MRPL9 Mitochondrial ribosomal protein L9
MRPS22 Mitochondrial 28S ribosomal protein S22
MRPS34 Mitochondrial 28S ribosomal protein S34
NCL CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin
NPM1 Isoform 2 of Nucleophosmin
NRAP Isoform 2 of Nebulin-related-anchoring protein
PABPC1 Isoform 1 of Polyadenylate-binding protein 1
PABPC1L Polyadenylate-binding protein 1-like
PABPC4 Isoform 2 of Polyadenylate-binding protein 4
PACSIN3 Protein kinase C and casein kinase substrate in neurons protein 3
PRSS3 Isoform A of Trypsin-3 precursor
PSMC5 26S protease regulatory subunit 8

PSMC5 26S protease regulatory subunit 8
RBJ Rab-related GTP-binding protein RabJ
RBM34 RNA-binding protein 34
RPL10A 25 kDa protein
RPL13 60S ribosomal protein L13
RPL13A 60S ribosomal protein L13a
RPL14 RPL14 protein
RPL15 60S ribosomal protein L15
RPL17 60S ribosomal protein L17
RPL18 60S ribosomal protein L18
RPL18A 60S ribosomal protein L18a
RPL19 60S ribosomal protein L19
RPL3 60S ribosomal protein L3
RPL35 60S ribosomal protein L35
RPL4 60S ribosomal protein L4
RPL5 60S ribosomal protein L5
RPL6 60S ribosomal protein L6
RPL7 60S ribosomal protein L7
RPL7A 60S ribosomal protein L7a
RPL8 60S ribosomal protein L8
RPL9 60S ribosomal protein L9
RPLP0 60S acidic ribosomal protein P0
RPS2 40S ribosomal protein S2
RPS3 40S ribosomal protein S3
RPS3A 40S ribosomal protein S3a
RPS4X 40S ribosomal protein S4, X isoform
RPS5 40S ribosomal protein S5
RPS6 40S ribosomal protein S6
RPS7 40S ribosomal protein S7
RPS8 40S ribosomal protein S8
RSL1D1 Ribosomal L1 domain-containing protein 1
SERBP1 Isoform 4 of Plasminogen activator inhibitor 1 RNA-binding protein
SLC25A13 Mitochondrial aspartate-glutamate carrier protein
SLC25A5 ADP/ATP translocase 2
SMC6 Isoform 1 of Structural maintenance of chromosomes protein 6
SRP68 Isoform 2 of Signal recognition particle 68 kDa protein
SSB Lupus La protein
SYNCRIP Isoform 1 of Heterogeneous nuclear ribonucleoprotein Q
THAP1 THAP domain-containing protein 1
TMEM63C Transmembrane protein 63C
TROVE2 Isoform Long of 60 kDa SS-A/Ro ribonucleoprotein
TTBK2 Tau-tubulin kinase
TTN 2268 kDa protein
TUBA1A Tubulin alpha-1A chain
TUBA1B Tubulin alpha-1B chain
TUBB Tubulin beta chain
TUBB2C Tubulin beta-2C chain
TUBB3 Tubulin beta-3 chain
TUBGCP6 TUBGCP6 protein
YBX1 Nuclease sensitive element-binding protein 1
ZBTB33 Transcriptional regulator Kaiso
ZNF571 HERV-T_19q13.11 provirus ancestral Env polyprotein precursor

7.1.4 Top-14x Affinity Purification: Mass Spectrometry Results
(Serum Starved & Re-fed)

Anti-colorectal carcinoma heavy chain
Kappa light chain variable region (Fragment)
Myosin-reactive immunoglobulin light chain variable region (Fragment)
RcNSEP1 (Fragment)
DAP3 Mitochondrial 28S ribosomal protein S29
DNAJA1 DnaJ homolog subfamily A member 1
EEF1A2 Elongation factor 1-alpha 2
ERAL1 Isoform HERA-A of GTP-binding protein era homolog
GRIA3 Isoform Flip of Glutamate receptor 3 precursor
hCG_2033311 Uncharacterized protein ENSP00000375173
hCG_21078 hypothetical protein LOC389435
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1
HNRNPC Isoform C1 of Heterogeneous nuclear ribonucleoproteins C1/C2
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B
HNRPCL1 Heterogeneous nuclear ribonucleoprotein C-like 1
HNRPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0
ILF2 Interleukin enhancer-binding factor 2
KRT1 Keratin, type II cytoskeletal 1
KRT10 Keratin, type I cytoskeletal 10
KRT12 Keratin, type I cytoskeletal 12
KRT13 Isoform 1 of Keratin, type I cytoskeletal 13
KRT16 Keratin, type I cytoskeletal 16
KRT2 Keratin, type II cytoskeletal 2 epidermal
KRT4 keratin 4
KRT77 Keratin 77
KRT9 Keratin, type I cytoskeletal 9
LOC440577 similar to nucleophosmin 1 isoform 1
MRPL37 39S ribosomal protein L37, mitochondrial precursor
MRPL38 39S ribosomal protein L38, mitochondrial precursor
MRPL39 Isoform 2 of Mitochondrial 39S ribosomal protein L39
MRPS22 Mitochondrial 28S ribosomal protein S22
MRPS27 Mitochondrial 28S ribosomal protein S27
MRPS27 Mitochondrial 28S ribosomal protein S27
MRPS31 28S ribosomal protein S31, mitochondrial precursor
MRPS9 mitochondrial ribosomal protein S9
NPM1 Isoform 2 of Nucleophosmin
PA2G4 Proliferation-associated protein 2G4
PCBP2 poly(rC)-binding protein 2 isoform b
PRSS3 Isoform A of Trypsin-3 precursor
RBMX Heterogeneous nuclear ribonucleoprotein G
RPL22 60S ribosomal protein L22
RPL23 60S ribosomal protein L23
RPL27 60S ribosomal protein L27
RPL3 60S ribosomal protein L3
RPL30 60S ribosomal protein L30
RPL31 60S ribosomal protein L31
RPL35 60S ribosomal protein L35
RPL35A 60S ribosomal protein L35a
RPL3L 60S ribosomal protein L3-like
RPL4 9 kDa protein
RPL6 60S ribosomal protein L6
RPLP0 60S acidic ribosomal protein P0
RPLP1 60S acidic ribosomal protein P1
RPLP2 60S acidic ribosomal protein P2

RPS10 40S ribosomal protein S10
RPS13 40S ribosomal protein S13
RPS14 40S ribosomal protein S14
RPS15A 40S ribosomal protein S15a
RPS16 40S ribosomal protein S16
RPS17 40S ribosomal protein S17
RPS18 40S ribosomal protein S18
RPS19 40S ribosomal protein S19
RPS2 40S ribosomal protein S2
RPS20 40S ribosomal protein S20
RPS23 40S ribosomal protein S23
RPS25 40S ribosomal protein S25
RPS26 OTTHUMP00000018641
RPS8 40S ribosomal protein S8
RPSA Ribosomal protein SA
RPSAP15 Laminin receptor-like protein LAMRL5
SFRS5 Isoform SRP40-1 of Splicing factor, arginine/serine-rich 5
SSB Lupus La protein
UBC;UBB;RPS27A ubiquitin and ribosomal protein S27a precursor
USP20 Ubiquitin carboxyl-terminal hydrolase 20
YBX1 Nuclease sensitive element-binding protein 1

Rank Ordered List of MicroRNAs Most Differentially Associated with TOP-14x mRNA

Rank	Name	Gene Index	P.value	Rank	Name	Gene Index
1	miRPlus_17960	237	9.00E-04	61	hsa_SNORD14B	110
2	hsa-miR-197	741	0.0032	62	hsa-miR-424	722
3	miRPlus_17820	665	0.0049	63	hsa-miR-34b*	1516
4	hsa-miR-200a	65	0.0067	64	hsa-miR-492	651
5	hsa-miR-9	84	0.0072	65	hsa-miR-552	547
6	hsa-miR-154*	654	0.0084	66	hsa-miR-331-3p	490
7	hsa-miR-335	274	0.009	67	hsa-miR-451	705
8	miRPlus_17863	507	0.0095	68	hsa-miR-634	78
9	hsa-miR-583	655	0.0142	69	miRPlus_17870	561
10	miRPlus_17880	71	0.0148	70	hsa-miR-454	204
11	hsa-miR-642	72	0.0188	71	hsa-miR-342-3p	658
12	miRPlus_17953	239	0.0194	72	hsa-miR-484	1089
13	hsa-miR-625	342	0.02	73	hsa-miR-29a	166
14	hsa-miR-585	223	0.0205	74	hsa-miR-151-3p	1518
15	miRPlus_17928	671	0.0211	75	hsa-miR-92a	1691
16	hsa-miR-365	220	0.0234	76	miRPlus_17840	291
17	hsa-miR-519e*	656	0.0275	77	hsa-miR-206	1523
18	hsa-miR-454	238	0.0286	78	hsa-miR-20a	281
19	hsa-miR-148a	781	0.0292	79	miRPlus_17942	1157
20	miRPlus_17932	70	0.031	80	miRPlus_17864	1479
21	hsa-miR-409-5p	117	0.0333	81	hsa-miR-524-3p/525-3p	1681
22	hsa-miR-453	57	0.0339	82	hsa-miR-665	235
23	hsa-miR-616*	564	0.0356	83	miRPlus_17893	1150
24	miRPlus_17848	285	0.0373	84	hsa-miR-19b-1*	295
25	hsa-miR-30c	544	0.0379	85	hsa-miR-429	1143
26	hsa-miR-657	234	0.0391	86	hsa-miR-181a	1037
27	miRPlus_17875	22	0.0396	87	hsa-miR-574-3p	1045
28	hsa-miR-205	692	0.0408	88	hsa-miR-147	876
29	miRPlus_17952	1689	0.0414	89	hsa-miR-651	1530
30	hsa-miR-130a	114	0.0448	90	hsa-miR-221*	156
31	miRPlus_17910	292	0.0454	91	hsa-miR-129-5p	330
32	hsa-let-7a	1686	0.0466	92	hsa-miR-493	219
33	hsa-miR-195	308	0.0489	93	hsa-miR-519d	1088
34	hsa-miR-181b	173	0.0501	94	hsa-miR-302c	118
35	hsa-miR-137	66	0.0506	95	hsa-miR-192	335
36	hsa-miR-154	870	0.0518	96	hsa-miR-626	126
37	hsa_negative_control_1	1258	0.053	97	hsa-miR-487b	9
38	hsa-miR-486-5p	441	0.0547	98	hsa-miR-21	875
39	hsa-miR-617	348	0.0553	99	hsa-let-7b	1470
40	miRPlus_17950	934	0.0611	100	hsa-miR-560	541
41	hsa-miR-325	64	0.0628	101	hsa-miR-448	63
42	hsa-miR-326	1570	0.0663	102	miRPlus_17892	718
43	hsa-miR-148a	660	0.068	103	hsa-miR-489	1299
44	hsa-miR-92b	1475	0.0686	104	hsa-miR-409-3p	333
45	hsa-miR-641	288	0.0697	105	hsa-miR-612	1428
46	miRPlus_17946	1097	0.0709	106	miRPlus_17925	455
47	hsa-miR-517c	494	0.0715	107	hsa-miR-606	1044
48	miRPlus_17927	886	0.0732	108	miRPlus_17845	1421
49	U6-snRNA-2	1416	0.0738	109	miRPlus_17923	880
50	hsa-miR-519e	872	0.0749	110	hsa-miR-520e	866
51	hsa-miR-18a	1375	0.0755	111	hsa-let-7i	174
52	hsa-miR-577	271	0.0761	112	hsa-miR-132	1362
53	hsa-miR-151-5p	630	0.0767	113	miRPlus_17872	503
54	spike_control_g	421	0.0773	114	hsa-miR-99a	395
55	hsa-miR-371-3p	1634	0.0778	115	hsa-miR-330-3p	74
56	hsa-miR-10b	768	0.0784	116	hsa-miR-29c*	994
57	hsa-miR-503	1676	0.0796	117	hsa-miR-142-5p	492
58	hsa-miR-219-5p	653	0.0807	118	miRPlus_17904	23
59	hsa-miR-518d-5p/518f*/5	272	0.0819	119	hsa-miR-32	1144
60	hsa-miR-578	55	0.0836	120	hsa-miR-15a	146

Rank	Name	Gene Index	P.value	Rank	Name	Gene Index	P.value
121	miRPlus_17924	615	0.1571	181	hsa-miR-769-3p	1581	0.2497
122	hsa-miR-574-3p	1477	0.1577	182	miRPlus_17849	393	0.2503
123	hsa-miR-199b-5p	1145	0.1618	183	miRPlus_17916	21	0.2509
124	hsa-miR-548b-3p	379	0.1623	184	hsa_SNORD6	596	0.252
125	hsa-miR-143	276	0.1629	185	hsa-miR-17*	824	0.2543
126	hsa-miR-596	1482	0.1635	186	hsa-miR-18a	1253	0.2555
127	hsa_negative_control_:	1042	0.1641	187	hsa-miR-628-3p	1374	0.2567
128	hsa-miR-9*	890	0.1681	188	hsa-miR-202	1327	0.2584
129	hsa-miR-518a-5p/527	62	0.171	189	miRPlus_17947	664	0.2601
130	hsa-miR-25	388	0.1733	190	hsa-miR-20a	497	0.2613
131	hsa-miR-95	1043	0.1745	191	hsa-miR-148b	444	0.2619
132	hsa-miR-19b	713	0.1756	192	hsa-miR-490-3p	1083	0.2648
133	hsa-miR-569	277	0.1768	193	miRPlus_17935	773	0.2659
134	hsa-miR-591	649	0.1797	194	hsa-miR-297	775	0.2671
135	hsa-miR-605	1260	0.1837	195	hsa-miR-649	240	0.2705
136	hsa-miR-153	349	0.1866	196	miRPlus_17957	1480	0.2746
137	hsa-miR-299-5p	598	0.1889	197	hsa-miR-520b/520c-3p	8	0.2763
138	hsa-miR-24-1*	1505	0.1913	198	miRPlus_17926	669	0.2769
139	hsa-miR-581	1087	0.193	199	miRPlus_17819	454	0.2775
140	hsa-miR-328	1354	0.1936	200	hsa-miR-542-5p	1459	0.2798
141	hsa-miR-126	245	0.1942	201	hsa-miR-671-5p	1419	0.281
142	hsa-miR-517a/517b	710	0.1947	202	miRPlus_17874	1365	0.2815
143	hsa-miR-135b	355	0.1953	203	hsa-miR-139-5p	139	0.2833
144	hsa-miR-129*/129-3p	725	0.1959	204	hsa-miR-19a	929	0.2844
145	hsa-miR-488	1255	0.1965	205	hsa-miR-518c	1352	0.2856
146	miRPlus_17903	933	0.197	206	hsa-miR-7-1*	1473	0.2891
147	hsa-miR-149	228	0.1976	207	miRPlus_17869	1211	0.2914
148	hsa-miR-15a	1721	0.1982	208	hsa-miR-485-5p	657	0.292
149	miRPlus_17917	177	0.1994	209	hsa-miR-92b	26	0.2925
150	miRPlus_17871	1534	0.2005	210	hsa-miR-505	391	0.2931
151	hsa-miR-574-3p	919	0.2023	211	hsa-miR-598	1050	0.2972
152	hsa-miR-215	44	0.204	212	hsa-miR-20b	1091	0.2983
153	hsa-miR-621	1206	0.2046	213	hsa-miR-506	1628	0.3006
154	hsa-miR-93	1259	0.2069	214	hsa_SNORD15A	164	0.3012
155	hsa-miR-564	1357	0.2086	215	miRPlus_17852	1311	0.3018
156	hsa-miR-7	1278	0.2092	216	hsa-miR-449a	1569	0.3041
157	hsa-miR-27a	1506	0.2098	217	hsa-miR-9*	1501	0.307
158	hsa-miR-205	59	0.2109	218	miRPlus_17876	941	0.3087
159	hsa-miR-432*	495	0.2115	219	hsa-miR-521	434	0.3093
160	hsa-miR-632	510	0.2133	220	hsa-miR-18b	1199	0.3099
161	hsa-miR-373	387	0.2185	221	hsa-let-7c	1254	0.3105
162	hsa-miR-643	1536	0.219	222	miRPlus_17843	1474	0.3122
163	hsa-miR-137	530	0.2208	223	hsa-miR-519c-5p	1082	0.3134
164	miRPlus_17885	69	0.2219	224	hsa-miR-210	659	0.3151
165	hsa-miR-140-5p	1140	0.2266	225	miRPlus_17941	1529	0.3157
166	hsa-miR-220	221	0.2289	226	hsa-miR-561	325	0.3168
167	hsa-miR-556-5p	1405	0.2295	227	hsa-miR-17*	1469	0.3186
168	hsa-miR-296-5p	814	0.23	228	hsa-miR-192	1208	0.3192
169	hsa-miR-200a*	1571	0.2306	229	miRPlus_17813	347	0.3215
170	hsa-miR-222	1684	0.237	230	hsa-miR-633	294	0.3238
171	hsa-miR-30a	976	0.2405	231	hsa-miR-512-5p	116	0.3244
172	miRPlus_17821	1257	0.2428	232	hsa-miR-380	1629	0.3261
173	hsa-let-7f	606	0.2433	233	hsa-miR-575	703	0.3273
174	hsa-miR-599	834	0.2439	234	miRPlus_17907	448	0.329
175	hsa-miR-192	119	0.2445	235	hsa-miR-624*	558	0.3302
176	hsa-miR-301b	642	0.2451	236	hsa-miR-647	672	0.3307
177	hsa-miR-191*	551	0.2457	237	miRPlus_17823	609	0.3325
178	hsa-miR-27b	458	0.2462	238	hsa-miR-545	811	0.333
179	miRPlus_17920	1312	0.2486	239	hsa-miR-496	1466	0.3348
180	miRPlus_17859	17	0.2491	240	hsa-miR-196b	727	0.3354

Rank	Name	Gene Index	P.value	Rank	Name	Gene Index	P.value
241	hsa-miR-500*/502-3p	602	0.3377	301	hsa-miR-208	1307	0.4054
242	hsa-miR-558	973	0.3382	302	hsa-miR-105	816	0.4077
243	hsa-miR-562	109	0.3388	303	hsa-miR-145	1524	0.41
244	hsa-miR-198	113	0.34	304	hsa-miR-125b	187	0.4106
245	hsa-miR-302d	1408	0.3406	305	hsa-miR-410	1623	0.4112
246	hsa-miR-150	565	0.3423	306	hsa-miR-638	936	0.4141
247	hsa-miR-512-3p	332	0.3435	307	miRPlus_17853	130	0.4146
248	hsa-miR-30a*	1192	0.344	308	hsa-miR-96*	791	0.4193
249	hsa-miR-514	1406	0.3458	309	hsa-miR-146a	1213	0.4204
250	hsa-miR-137	1472	0.3463	310	hsa-miR-520g/520h	650	0.4222
251	hsa-miR-520a-3p	440	0.3469	311	hsa-miR-187	383	0.4233
252	hsa-miR-127-3p	978	0.3475	312	miRPlus_17958	232	0.4285
253	miRPlus_17879	1426	0.3516	313	hsa-miR-425*	1575	0.4291
254	hsa-miR-567	709	0.3533	314	hsa-miR-33a	470	0.4308
255	hsa-miR-511	548	0.3539	315	miRPlus_17841	1096	0.4314
256	miRPlus_17883	233	0.3573	316	hsa-miR-553	331	0.4332
257	hsa-miR-185	815	0.3585	317	hsa-miR-620	1422	0.4349
258	hsa-miR-27b	1246	0.3591	318	hsa-miR-630	942	0.4355
259	miRPlus_17898	1095	0.3597	319	hsa-miR-199a-5p	1577	0.4378
260	miRPlus_17911	1535	0.3602	320	hsa-miR-183	1247	0.4389
261	hsa-miR-155	438	0.3614	321	spike_control_b	847	0.4395
262	hsa-miR-346	226	0.362	322	hsa-miR-549	1411	0.4401
263	hsa-miR-30d	328	0.3626	323	hsa-miR-338-3p	1522	0.4407
264	hsa_SNORD118	974	0.3631	324	hsa-miR-645	1104	0.4416
265	hsa-miR-106b	384	0.3637	325	hsa-miR-375	1461	0.4442
266	hsa-miR-501-5p	386	0.3666	326	hsa-miR-302c*	1154	0.4447
267	hsa-miR-507	1412	0.3672	327	hsa-miR-148a	258	0.4482
268	hsa-miR-656	450	0.3689	328	hsa-miR-146a	1715	0.4499
269	hsa-miR-636	1368	0.3695	329	hsa-miR-374b	286	0.4517
270	hsa-miR-568	493	0.3704	330	hsa-miR-650	24	0.4523
271	miRPlus_17347	1587	0.3712	331	hsa-miR-379	165	0.4528
272	hsa-miR-548c-3p	163	0.3718	332	hsa-miR-297	554	0.4546
273	hsa-miR-182*	1463	0.373	333	hsa-miR-30e*	112	0.4551
274	hsa-miR-215	1517	0.3736	334	miRPlus_17842	1367	0.4557
275	miRPlus_17866	1372	0.3747	335	hsa-miR-141	924	0.4563
276	miRPlus_17936	1103	0.3759	336	miRPlus_17918	1690	0.458
277	miRPlus_17944	940	0.3764	337	hsa_SNORD10	326	0.4586
278	hsa-miR-133a/133b	1146	0.3782	338	hsa-miR-30e	1576	0.4621
279	hsa-miR-33a	423	0.3793	339	hsa-miR-369-3p	1467	0.4661
280	hsa-miR-125b	1626	0.3805	340	miRPlus_17854	1589	0.4667
281	hsa-miR-198	527	0.3811	341	hsa-miR-107	168	0.4679
282	hsa-miR-639	720	0.3834	342	hsa-miR-888*	1596	0.4696
283	hsa-miR-613	1212	0.384	343	miRPlus_17851	1696	0.4725
284	hsa-miR-15a	222	0.3869	344	hsa-miR-519b-3p	1520	0.4742
285	hsa-miR-644	1320	0.388	345	hsa-miR-214	11	0.4748
286	hsa-miR-362-5p	868	0.3886	346	hsa-miR-631	726	0.476
287	hsa-miR-515-5p	1574	0.3892	347	hsa-miR-548d-3p	1627	0.4771
288	miRPlus_17838	778	0.3903	348	miRPlus_17894	1528	0.4783
289	miRPlus_17913	1205	0.3909	349	hsa-miR-580	1303	0.4789
290	hsa-miR-9*	371	0.3915	350	hsa-miR-211	443	0.48
291	hsa-miR-325	506	0.3932	351	hsa-miR-629*	1158	0.4824
292	hsa-miR-138	1572	0.3938	352	miRPlus_17830	1209	0.4858
293	hsa-miR-302c*	1624	0.395	353	hsa-miR-485-3p	873	0.4887
294	hsa-miR-193a-3p	1625	0.3979	354	hsa-miR-654-5p	882	0.4899
295	hsa-miR-497	1250	0.3984	355	hsa-miR-106a/17	600	0.4905
296	miRPlus_17811	125	0.399	356	hsa-miR-218	869	0.4939
297	hsa-miR-520d-3p	1298	0.4013	357	hsa-miR-450a	921	0.4945
298	hsa-miR-325	1178	0.4031	358	hsa-miR-587	1513	0.4968
299	hsa-miR-590-5p	865	0.4036	359	hsa-miR-302c	1451	0.4986
300	hsa-miR-450b-5p	878	0.4042	360	hsa-miR-544	1243	0.4991

Rank	Name	Gene Index	P.value
361	hsa-miR-518b	1568	0.4997
362	hsa-miR-34c-5p	1300	0.5009
363	hsa-miR-421	975	0.502
364	hsa-miR-607	828	0.5032
365	hsa-miR-380	884	0.5061
366	miRPlus_17889	719	0.5072
367	hsa-miR-378	543	0.5095
368	hsa-let-7g	390	0.5107
369	hsa-miR-526b*	817	0.513
370	hsa-miR-370	1035	0.5136
371	hsa-miR-302a	982	0.5148
372	hsa-miR-10a	984	0.5171
373	hsa-miR-615-3p	780	0.5182
374	hsa-miR-24-2*	1340	0.5188
375	hsa-miR-424*	938	0.52
376	hsa-miR-1	1062	0.5205
377	miRPlus_17921	1641	0.5211
378	hsa-miR-640	504	0.5234
379	hsa-miR-412	1191	0.5281
380	hsa-miR-487a	225	0.5292
381	hsa-miR-595	1698	0.5304
382	hsa-miR-652	1314	0.531
383	hsa-miR-376c	1683	0.5344
384	hsa-miR-345	442	0.535
385	hsa-miR-603	1692	0.5367
386	hsa-miR-202*	908	0.5379
387	hsa-miR-193b	1409	0.5385
388	hsa-miR-151-3p	1256	0.5391
389	miRPlus_17884	508	0.5396
390	miRPlus_17914	1635	0.5408
391	hsa_SNORD2	542	0.5425
392	hsa-miR-573	1135	0.5431
393	hsa-miR-432	711	0.5443
394	hsa-miR-23b	820	0.5448
395	hsa-miR-204	1424	0.5454
396	miRPlus_17949	1313	0.546
397	hsa-miR-423-3p	327	0.5466
398	hsa-miR-589*	1081	0.5486
399	hsa-miR-135a	714	0.5495
400	hsa-miR-194	1193	0.5501
401	hsa-miR-489	576	0.5512
402	hsa-miR-29b	1630	0.5518
403	hsa-miR-510	764	0.5547
404	hsa-miR-565	1141	0.5558
405	miRPlus_17837	449	0.557
406	hsa-miR-708	1610	0.5628
407	miRPlus_17922	1582	0.5634
408	hsa-miR-431	927	0.5639
409	hsa-miR-493*	435	0.568
410	hsa-miR-16	1685	0.5697
411	hsa-miR-126*	1194	0.5709
412	hsa-miR-26a	1678	0.572
413	miRPlus_17873	75	0.5726
414	hsa-miR-655	666	0.5807
415	hsa-miR-614	996	0.5813
416	hsa-miR-124	336	0.5825
417	hsa-miR-31	1108	0.5848
418	hsa-miR-377	597	0.5871
419	hsa-miR-195	1165	0.5883
420	hsa-miR-16	1236	0.5894
421	hsa-miR-223	1468	0.5923
422	hsa-miR-519c-3p	1304	0.594
423	hsa-miR-495	1682	0.5958
424	hsa-miR-518c*	1136	0.5987
425	hsa-miR-516a-3p/516b'	1358	0.6004
426	hsa-miR-380*	1413	0.6016
427	miRPlus_17834	779	0.605
428	hsa-miR-200b	1355	0.6073
429	hsa-miR-660	1481	0.6079
430	hsa-miR-339-5p	1306	0.6091
431	miRPlus_17833	1425	0.6097
432	hsa-miR-196b	545	0.6108
433	hsa-miR-101	1267	0.6114
434	hsa-miR-212	227	0.612
435	miRPlus_17822	1102	0.6131
436	miRPlus_17411	777	0.6155
437	miRPlus_17855	616	0.6172
438	hsa-miR-637	1152	0.6178
439	hsa-miR-7	617	0.6189
440	miRPlus_17836	346	0.6195
441	hsa-miR-196b	1124	0.6207
442	hsa-miR-433	279	0.6212
443	hsa-miR-619	1638	0.6224
444	hsa-miR-19a	79	0.623
445	miRPlus_17895	1588	0.6236
446	hsa-miR-329	1138	0.6241
447	hsa-miR-22*	400	0.6253
448	hsa-miR-204	275	0.6288
449	hsa-miR-182	1679	0.6293
450	hsa-miR-611	1644	0.6305
451	miRPlus_17934	1366	0.6311
452	miRPlus_17808	293	0.6322
453	hsa-miR-422a	759	0.6334
454	hsa-miR-99b	179	0.6351
455	hsa-miR-142-5p	1645	0.6357
456	hsa_SNORD4A	812	0.6369
457	hsa-miR-374a	1677	0.638
458	hsa-miR-20a	31	0.6386
459	hsa-miR-216b	889	0.6415
460	hsa-miR-31	1538	0.6421
461	hsa-miR-26a	172	0.6438
462	hsa-miR-199a-5p	608	0.6467
463	hsa-miR-659	1697	0.6473
464	hsa-miR-135b	571	0.649
465	miRPlus_17888	995	0.6502
466	hsa-miR-183	1214	0.6508
467	hsa-miR-635	1584	0.6513
468	miRPlus_17905	1427	0.6531
469	miRPlus_17930	1041	0.6542
470	hsa-miR-516b	1142	0.6548
471	miRPlus_17931	16	0.6565
472	hsa-miR-135b	498	0.6589
473	miRPlus_17814	123	0.66
474	hsa-miR-520a-5p	224	0.6606
475	miRPlus_17902	1319	0.6612
476	hsa-miR-134	930	0.6617
477	miRPlus_17827	1264	0.6635
478	hsa-miR-518e	704	0.6641
479	hsa-miR-33a	922	0.6646
480	hsa-miR-139-5p	1356	0.6664

Rank	Name	Gene Index	P.value	Rank	Name	Gene Index	P.value
481	miRPlus_17847	1047	0.6675	541	hsa-miR-563	1573	0.7555
482	miRPlus_17899	129	0.6704	542	hsa-miR-523	2	0.7561
483	hsa-miR-122	552	0.671	543	miRPlus_17878	881	0.7567
484	hsa-miR-27a	1462	0.6745	544	hsa-miR-195	977	0.7578
485	hsa-miR-572	1351	0.6751	545	hsa-miR-30e*	686	0.7613
486	miRPlus_17919	1527	0.6768	546	hsa-miR-559	757	0.7619
487	hsa-miR-302b	550	0.6803	547	hsa-let-7d	1038	0.763
488	miRPlus_17862	1151	0.6808	548	hsa-miR-27b	1074	0.7636
489	hsa-miR-557	1189	0.6814	549	hsa-miR-627	1590	0.7648
490	hsa-miR-361-5p	1084	0.682	550	hsa-miR-10b	1281	0.7682
491	hsa-miR-543	825	0.6826	551	hsa-miR-504	1460	0.7688
492	hsa-miR-132*	1219	0.6837	552	hsa-miR-623	774	0.7694
493	hsa-miR-449a	1235	0.6843	553	hsa-miR-301a	635	0.7705
494	hsa-miR-455-5p	1521	0.6872	554	hsa-miR-532-5p	385	0.7711
495	hsa-miR-181a*	821	0.6901	555	hsa-miR-542-3p	1675	0.7729
496	hsa-miR-181b	605	0.6947	556	hsa-miR-100	1464	0.774
497	miRPlus_17860	670	0.6953	557	hsa-miR-1	1680	0.7763
498	hsa-miR-153	1086	0.6965	558	hsa-miR-571	1567	0.7786
499	hsa-miR-190	983	0.697	559	miRPlus_17826	935	0.7792
500	hsa-miR-302b*	1370	0.6976	560	miRPlus_17857	989	0.7798
501	hsa-miR-30b	760	0.6982	561	hsa-miR-515-3p	1190	0.7804
502	hsa-miR-586	7	0.6988	562	hsa-miR-297	1704	0.7821
503	hsa-miR-449b	1353	0.6999	563	hsa-miR-24-1*/24-2*	1631	0.7833
504	miRPlus_17891	131	0.7017	564	miRPlus_17653	77	0.7879
505	hsa-miR-508-3p	1196	0.7023	565	hsa-miR-20a*	414	0.7885
506	miRPlus_17858	723	0.708	566	hsa-miR-662	1049	0.7891
507	miRPlus_17831	1533	0.7086	567	hsa-miR-31	1716	0.7902
508	hsa-miR-367	4	0.7092	568	hsa-miR-554	115	0.7937
509	hsa-miR-324-5p	280	0.7115	569	hsa-miR-499-5p	803	0.7943
510	hsa-miR-18a*	1415	0.7121	570	miRPlus_17954	987	0.7948
511	hsa-miR-425	1359	0.7127	571	hsa-miR-369-5p	1251	0.796
512	miRPlus_17961	399	0.7144	572	hsa-miR-483-3p	1305	0.7983
513	hsa-miR-202*	1111	0.7161	573	hsa-miR-301a	1198	0.7995
514	hsa-miR-196a	761	0.7167	574	hsa-miR-34b*	1648	0.8001
515	hsa-miR-592	433	0.7179	575	hsa-miR-33b	1090	0.8041
516	hsa-miR-31	1360	0.7185	576	U6-snRNA-1	1632	0.8064
517	hsa-miR-202	176	0.7208	577	hsa-miR-610	180	0.807
518	hsa-miR-363	652	0.7266	578	hsa-miR-345	1148	0.8076
519	hsa-miR-411	1407	0.7271	579	miRPlus_17868	555	0.8082
520	miRPlus_17890	345	0.73	580	hsa-let-7d*	563	0.8087
521	hsa-miR-551b	763	0.7306	581	miRPlus_17906	124	0.8093
522	hsa-miR-199a-3p/199b-	1361	0.7318	582	hsa-miR-488*	1515	0.8105
523	hsa-miR-376a	1245	0.7323	583	hsa-miR-29a	1118	0.8111
524	hsa-miR-505	1244	0.7329	584	miRPlus_17908	509	0.8116
525	miRPlus_17929	1156	0.7335	585	hsa-miR-10b*	403	0.8128
526	hsa-miR-155	1040	0.7341	586	hsa-miR-299-3p	382	0.8163
527	hsa-miR-593*	217	0.7376	587	hsa-miR-566	925	0.8168
528	hsa-miR-489	1014	0.7381	588	hsa-miR-555	1621	0.818
529	hsa-miR-133a	787	0.741	589	hsa_SNORD13	1028	0.8197
530	hsa-miR-142-3p	708	0.7433	590	miRPlus_17940	1642	0.8209
531	hsa-miR-499-5p	818	0.7445	591	miRPlus_17829	1637	0.8215
532	hsa-miR-22	437	0.7457	592	hsa-miR-200c	1139	0.822
533	hsa-miR-381	1586	0.7462	593	hsa-miR-381	1197	0.8267
534	hsa-miR-193a-3p	1499	0.7468	594	hsa_SNORD3	758	0.829
535	miRPlus_17938	76	0.748	595	hsa-miR-584	439	0.8313
536	hsa-miR-146a	1308	0.7497	596	hsa-miR-517*	926	0.833
537	miRPlus_17815	1420	0.7509	597	hsa-miR-23a	1036	0.8342
538	hsa-miR-373*	171	0.7514	598	hsa-miR-130a	1494	0.8354
539	hsa-miR-34a	10	0.752	599	hsa-let-7e	822	0.8377
540	hsa-miR-98	611	0.7526	600	hsa-miR-522*	601	0.8388

Rank	Name	Gene Index	P.value	Rank	Name	Gene Index	P.value
601	hsa-miR-582-5p	871	0.8411	661	hsa-miR-203	491	0.9158
602	hsa-miR-9	401	0.8417	662	miRPlus_17882	1048	0.917
603	miRPlus_17897	339	0.8423	663	hsa-miR-618	132	0.9175
604	miRPlus_17818	394	0.844	664	hsa-miR-103	1032	0.9181
605	hsa-miR-216a	1301	0.8446	665	hsa-miR-622	990	0.9193
606	miRPlus_17887	183	0.8452	666	miRPlus_17812	887	0.9222
607	hsa-miR-601	402	0.8458	667	hsa-miR-646	888	0.9239
608	hsa-miR-9	1542	0.8492	668	hsa-miR-126	1410	0.9256
609	hsa-miR-29c	1414	0.8504	669	miRPlus_17824	1263	0.928
610	hsa-miR-9*	185	0.8545	670	hsa-miR-31	836	0.9291
611	miRPlus_17861	1149	0.855	671	hsa-miR-96*	1447	0.9303
612	miRPlus_17943	1155	0.8556	672	hsa-miR-382	981	0.9326
613	spike_control_d	637	0.8562	673	hsa-miR-367	254	0.9337
614	hsa-miR-320	928	0.8568	674	hsa-miR-539	169	0.9349
615	hsa-miR-525-5p	1249	0.8573	675	miRPlus_17896	832	0.9355
616	hsa-miR-518a-3p	278	0.8602	676	hsa_SNORD12	380	0.9395
617	miRPlus_17900	771	0.8614	677	hsa-miR-494	3	0.9407
618	hsa-miR-609	396	0.8631	678	hsa-miR-384	549	0.9418
619	miRPlus_17850	1101	0.8637	679	hsa-miR-383	668	0.9436
620	hsa-miR-637	1418	0.8643	680	hsa-miR-15b	6	0.9453
621	hsa-miR-217	1085	0.8649	681	hsa-miR-191	767	0.947
622	hsa-miR-450b-3p	662	0.8678	682	miRPlus_17865	717	0.9488
623	hsa-miR-509-3p	980	0.8695	683	miRPlus_17856	453	0.9494
624	hsa-miR-146b-5p	1092	0.8701	684	miRPlus_17937	885	0.9534
625	hsa-miR-136	282	0.8712	685	miRPlus_17951	1371	0.954
626	hsa-miR-302a	98	0.8718	686	hsa-miR-181c	389	0.9552
627	hsa-miR-602	186	0.8724	687	miRPlus_17828	184	0.9563
628	hsa-miR-491-5p	867	0.8736	688	hsa-miR-424	111	0.958
629	hsa-miR-524-5p	1465	0.8747	689	miRPlus_17955	1643	0.9604
630	hsa-miR-383	765	0.8759	690	hsa-miR-551a	979	0.9638
631	miRPlus_17915	1203	0.8764	691	hsa-miR-574-5p	102	0.9644
632	miRPlus_17909	1636	0.877	692	hsa-miR-297	338	0.9656
633	hsa-miR-550*	1195	0.8782	693	miRPlus_17877	1318	0.9679
634	miRPlus_17886	562	0.8793	694	hsa-miR-498	1034	0.9685
635	hsa-miR-502-5p	170	0.8822	695	hsa-miR-608	612	0.969
636	hsa-miR-518d-3p	920	0.8828	696	hsa-miR-146b-5p	997	0.9696
637	hsa-miR-588	1297	0.8845	697	hsa-miR-604	1476	0.9702
638	hsa-miR-152	1302	0.8851	698	miRPlus_17912	557	0.9714
639	hsa-miR-124	425	0.8863	699	hsa-miR-330-3p	706	0.9725
640	hsa-miR-376b	813	0.8874	700	miRPlus_17956	178	0.9742
641	hsa-miR-548a-3p	595	0.8903	701	hsa-miR-101	1248	0.9748
642	miRPlus_17825	724	0.8909	702	hsa-miR-221	5	0.9777
643	hsa-miR-663	833	0.8915	703	hsa-miR-130b	1578	0.9783
644	hsa-miR-10b	835	0.8927	704	miRPlus_17933	340	0.9789
645	miRPlus_17817	1317	0.8932	705	hsa-miR-526b	1033	0.98
646	hsa-miR-372	603	0.8938	706	miRPlus_17945	501	0.9806
647	hsa-miR-802	588	0.8944	707	hsa-miR-648	456	0.9812
648	let-7a-e	788	0.895	708	hsa-miR-28-5p	1030	0.9829
649	hsa-miR-125a-5p	120	0.8961	709	hsa-miR-378*	381	0.9835
650	hsa-miR-597	1266	0.899	710	miRPlus_17867	939	0.9841
651	hsa-miR-550	87	0.8996	711	hsa-miR-197	329	0.9847
652	hsa-miR-10a	619	0.9002	712	miRPlus_17881	831	0.9852
653	hsa-miR-661	1265	0.9013	713	miRPlus_17816	502	0.9881
654	hsa-miR-96	827	0.9048	714	hsa-miR-188-5p	167	0.9887
655	miRPlus_17832	993	0.9071	715	hsa-miR-150	12	0.9933
656	miRPlus_17844	341	0.9089	716	hsa-miR-600	618	0.9939
657	hsa-miR-520c-3p/520f	1514	0.9117	717	hsa-miR-184	1031	0.9968
658	miRPlus_17959	1373	0.9129	718	miRPlus_17810	1210	0.9974
659	hsa-miR-24	604	0.9141	719	miRPlus_17948	287	0.998
660	hsa-miR-371-3p	819	0.9152	720	hsa-miR-658	18	0.9991