Regulation of Eukaryotic Translational Initiation in Transformed and Differentiation Inducible Haemopoietic Cell Lines.

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by

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ABSTRACT

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Mechanisms which regulate gene expression at the level of protein synthesis play an important role in the control of cell proliferation, and de-regulation of translation has been shown to cause cell transformation. Haemopoiesis is a complex and highly regulated process of cell proliferation, differentiation and apoptosis which is subject to a variety of neoplastic disorders. Control of translation is usually effected through modulation of the eukaryotic initiation factors (eIFs) and cultured cells of haemopoietic origin were chosen to study eIFs and their regulation.

The eIFs were examined in cell lines derived from patients with the B-cell tumours Burkitt's lymphoma and Multiple Myeloma. Study of the expression of the proto-oncogene c-myc and its transcriptional targets, eIF4E and eIF2α demonstrated no correlation between the eIFs and c-myc expression. Investigation of another component of the eIF4F complex revealed that eIF4G is cleaved into specific N- and C-terminal fragments in the Multiple Myeloma cell line GM2132. This suggests that in these cells there may be an advantage for mRNAs translated by internal ribosome entry.

HL60, a differentiation inducible leukaemic cell line, was used to investigate alterations in protein synthesis during granulocytic differentiation. A 2-fold increase in eIF4E phosphorylation was observed which does not affect the protein synthesis rate. This implies that the increase in eIF4E phosphorylation may specifically affect the translation of a sub-set of mRNAs. Differentiating HL60 cells were also used to study the relationship between c-myc, eIF4E and eIF2α expression. No correlation between expression of c-myc and eIF4E was found in this system. However, eIF2α expression did appear to be responsive to c-Myc during the initial stages of granulocytic differentiation.

Investigation of a serum response in HL60 cells demonstrated that association of eIF4G with eIF4E is modulated by alterations in eIF4G protein levels. This suggests that eIF4E is not a limiting factor in the initiation of translation in HL60 cells.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

4E-BP  eIF4E binding protein
ATP   adenosine triphosphate
BL    burkitt's lymphoma
bp    base pairs
BR    basic region
CAT   chloramphenicol acetyl transferase
CSF   colony stimulating factor
DMSO  dimethyl sulphoxide
DNA   deoxyribonucleic acid
dNTP  deoxyribonucleoside triphosphate
eEF   eukaryotic elongation factor
EGF   epidermal growth factor
eIF   eukaryotic initiation factor
ER    oestrogen receptor
eRF   eukaryotic release factor
Erk   extracellular signal-regulated protein kinase
FCS   foetal calf serum
FGF-2 fibroblast growth factor-2
FKBP  FK506-binding protein
FMDV  foot and mouth disease virus
FRAP  FKBP rapamycin-associated protein
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GDP   guanosine diphosphate
GSK-3 glycogen synthase kinase-3
GTP   guanosine triphosphate
HCR   haem controlled repressor
HLH   helix-loop-helix
IEF   isoelectric focusing
IGF-II insulin-like growth factor-II
IL    interlukin
IRE   iron responsive element
IRES  internal ribosome entry segment
IRP   iron regulatory protein
kDa   kilodaltans
LCL   lymphoblastoid cell line
LZ    leucine zipper
m7G   7-methyl guanosine
MAP kinase mitogen activated protein kinase
<table>
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<th>Description</th>
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<tr>
<td>met-tRNA_i</td>
<td>initiator-methionyl-transfer RNA</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td>Mnk1</td>
<td>MAP kinase-interacting kinase 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD gene</td>
<td>myeloid differentiation gene</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>p70S6k</td>
<td>70 kDa ribosomal protein S6 kinase</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>Pablp/PABP</td>
<td>poly(A) binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase activated by double-stranded RNA</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>polyadenosine</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
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<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetra-decanoarylphorbol-13-acetate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxy methyl) aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>uORF</td>
<td>upstream open reading frame</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>Vit D_3</td>
<td>1,25 dihydroxy vitamin D_3</td>
</tr>
<tr>
<td>VRC</td>
<td>vanadyl ribonucleoside complexes</td>
</tr>
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CHAPTER 1

INTRODUCTION

1.1 Overview

Regulation of protein synthesis is an important component of the mechanisms which control gene expression in eukaryotes. Translational regulators play a significant role in the control of cell proliferation and, as with any modulator of gene expression, de-regulation of translation can lead to neoplastic transformation. Most of the translational control mechanisms characterised to date target the initiation of translation; the limiting stage of protein synthesis. These controls are exerted through alterations in the activity, or expression, of a selection of the protein factors which mediate initiation of translation, the eukaryotic initiation factors.

Haemopoietic differentiation is a complex and highly regulated process leading to the formation of the whole range of cell types found in the circulatory and immune systems. Haemopoiesis is also subject to a variety of neoplastic disorders and many cell lines have been established from patients with such malignancies. These provide a valuable resource for the study of differentiation and a number of aspects of carcinogenesis.

Two main lines of investigation have been pursued in this study. Transformed B-cell lines that overexpress the oncogene c-myc have been examined for alterations in the initiation factors, when compared to control cell lines. Additionally, a differentiation inducible cell line has been used to study the initiation factors during haemopoietic differentiation and how alterations in these might affect the changes in gene expression which are a fundamental part of the differentiation process.

1.2 Eukaryotic Translation

Protein synthesis is one of the final stages in the chain of events leading from transcription of a gene to the presence of functional protein in the cell. Control of translation is fundamental to cell cycle progression and alterations in growth rates are accompanied by corresponding changes in the rate of protein synthesis (reviewed in Morris, 1995 and Flynn & Proud, 1996c). In addition to modulating global protein synthesis rates, translational regulation can target individual genes, or groups of messenger RNAs (mRNAs), and provides a mechanism for controlling gene expression in circumstances where nuclear functions are absent (reviewed in Hentze,
Such a pivotal cellular process is necessarily subject to a number of controls in response to a variety of stimuli (reviewed in Redpath & Proud, 1994).

In order for a mRNA molecule to be decoded and its specified polypeptide chain synthesised, a well defined sequence of events must occur. Three phases can be distinguished in this process; initiation of translation, elongation of the polypeptide chain and termination (Figure 1.1). Each of these phases requires protein factors that interact with the ribosomal subunits, the mRNA and each other (reviewed in Hershey, 1991 and Pain, 1996).

1.2.1 Initiation

Initiation of translation involves the dissociation of the 80S ribosome into its 40S and 60S subunits, charging of the 40S subunit with initiator-methionyl-transfer RNA (met-tRNA\textsubscript{i}) and binding of this 43S pre-initiation complex to the mRNA to form the 48S initiation complex. In the case of most cellular mRNAs, the initiation complex then scans along the message until it recognises an initiation codon (most commonly AUG, although CUG, GUG and ACG may also be used) at which stage the 60S ribosomal subunit joins the complex to form a translation competent 80S ribosome (reviewed in Hershey, 1991). Five groups of eukaryotic initiation factors (eIFs) are required for this process (Figure 1.1), consisting of nine proteins, three of which have multiple subunits (Table 1.1).

**Formation of the 43S Pre-Initiation Complex**

The equilibrium between the 80S ribosome and its component subunits, under normal intracellular conditions, lies in favour of the former. In order for a 40S subunit to be recruited to initiation, newly dissociated subunits must be prevented from reassociating. This function is believed to be carried out by eIF3 and eIF1A binding to the 40S subunit, although recent findings question this role for eIF1A (Chaudhuri e\,\textit{a}l., 1997). eIF3 is a multimeric complex consisting of at least eight polypeptide chains in mammals. RNA binding activity has been identified in some of these subunits; cross-linking experiments with 48S complexes show that eIF3 interacts with both the mRNA and the 18S rRNA (Bommer e\,\textit{a}l., 1991).

eIF2 is the factor responsible for charging the 40S ribosomal subunit with the initiator-methionyl-tRNA. Met-tRNA\textsubscript{i} can only bind to eIF2 that is complexed with GTP, thus forming the ternary complex which binds the 40S ribosome. eIF2 is a heterotrimer composed of \(a\), \(b\) and \(\gamma\) polypeptides. The \(\gamma\) subunit contains consensus GTP binding elements and shows similarity to other GTP binding proteins (Gaspar e\,\textit{a}l., 1994). Cross-linking studies show that met-tRNA\textsubscript{i} binds in the region of the \(\gamma\) subunit N-terminus and the \(\beta\) subunit C-terminus (Gaspar e\,\textit{a}l., 1994). The \(\alpha\) subunit
Figure 1.1: Scheme outlining protein synthesis; showing the factors required for initiation, elongation and termination. eIF: eukaryotic initiation factor, eEF: eukaryotic elongation factor, eRF: eukaryotic release factor, AUG: initiation codon, UAA: stop codon.
Table 1.1: The eukaryotic initiation factors (eIFs); indicating which consist of multiple subunits and their molecular masses.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Subunits</th>
<th>$M_r$ (kDa)</th>
<th>Former Name(s)</th>
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<tr>
<td>eIF1A</td>
<td></td>
<td>17</td>
<td>eIF-4C</td>
</tr>
<tr>
<td>eIF2</td>
<td>$\alpha$</td>
<td>130</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\gamma$</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>eIF2B</td>
<td>$\alpha$</td>
<td>270</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\gamma$</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\delta$</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\varepsilon$</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>eIF3</td>
<td>eight ($\alpha-\varepsilon$)</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>eIF4A</td>
<td></td>
<td>46</td>
<td>eIF-4$\beta$</td>
</tr>
<tr>
<td>eIF4B</td>
<td></td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>eIF4E</td>
<td></td>
<td>25</td>
<td>CBP1, eIF-4$\alpha$</td>
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<tr>
<td>eIF4G</td>
<td></td>
<td>154</td>
<td>p220, eIF-4$\gamma$</td>
</tr>
<tr>
<td>eIF5</td>
<td></td>
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</tr>
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does not appear to be involved in ligand binding but is a target for regulation of the complex.

At the end of the initiation process, eIF2, bound to GDP, is released from the initiation complex, following GTP hydrolysis, and is recycled via a guanine nucleotide exchange reaction catalysed by eIF2B (reviewed in Price & Proud, 1994). eIF2B consists of five subunits and is more complex than many other guanine nucleotide exchange proteins. Although no consensus GTP binding domains have yet been identified in any of the eIF2B polypeptides there is evidence to suggest that the $\varepsilon$ subunit may be responsible for guanine nucleotide exchange activity (Oldfield et al., 1994). In addition to eIF2$\alpha$, eIF2B is involved in the complex regulation that can be exerted over this phase of protein synthesis, which is discussed further in section 1.2.4.

**mRNA Binding to the Ribosome**

The eIF4 initiation factors catalyse the binding of mRNA to the 43S pre-initiation complex and migration to the initiation codon. For the majority of eukaryotic mRNAs this is accomplished by binding of the 43S pre-initiation complex to the 7-methyl guanosine cap (m$^\text{7}$G cap) structure at the 5' end of the message and scanning, in a linear fashion, to the initiation codon (Kozak, 1989b). However, an alternative model, originally identified as operating for picornaviral transcripts, has been shown
to apply to some cellular messages (Jackson & Kaminski, 1995). In these cases, the 43S pre-initiation complex binds directly to an internal ribosome entry segment (IRES) in the 5' untranslated region of the mRNA which directs it to the initiation codon (reviewed in Sachs et al., 1997).

In the scanning model of initiation of translation, eIF4F specifically binds to the m^7G cap and unwinds any secondary structure in the immediate vicinity of the cap in conjunction with eIF4B. This permits the 43S pre-initiation complex to be recruited, via interaction of eIF3 with both the 40S ribosomal subunit and eIF4F. RNA secondary structure in the 5' untranslated region (5' UTR) is unwound by eIF4F and eIF4B allowing the ribosome to scan along the mRNA to the initiation codon (reviewed in Rhoads et al., 1994). eIF4F is also essential in initiation directed by an IRES (Pestova et al., 1996a). eIF4F consists of 3 separate initiation factors eIF4E, eIF4G and eIF4A.

**eIF4E**

eIF4E is the 25 kDa component of eIF4F which binds the m^7G cap (Sonenberg et al., 1978). The structure of eIF4E in complex with m^7GDP has been solved for the murine protein by X-ray crystallography (Marcotrigiano et al., 1997) and for yeast eIF4E by NMR imaging (Matsuo et al., 1997). Although there are some differences between these two structural determinations they are broadly similar, revealing a molecule shaped like a cupped hand with the m^7GDP binding site in a narrow slot on the protein's concave surface. The binding of eIF4E to m^7GDP involves three highly conserved tryptophan residues. eIF4E binds to amino acids 409 to 457 of eIF4G (Mader et al., 1995) a motif that eIF4G shares with the eIF4E binding proteins (4E-BPs). The 4E-BPs regulate the activity of eIF4E by competing with eIF4G for eIF4E binding (Haghighat et al., 1995) and are discussed in further detail in section 1.2.4.

In addition to its function in the initiation of translation, an eIF4E has been implicated in transport of mRNA from the nucleus to the cytoplasm. In cells overexpressing eIF4E, increased levels of cyclin D1 expression have been observed; this was found to be due to increased nucleocytoplasmic mRNA transport rather than enhanced initiation of translation (Rosenwald et al., 1993a; Rousseau et al., 1996b).

**eIF4G**

eIF4G provides an ‘assembly platform’ for the formation of the initiation complex; eIF4E binds a region in the N-terminus of eIF4G, eIF3 interacts with the central region of the molecule (Lamphear et al., 1995; Mader et al., 1995) and eIF4A has two
binding sites, in the central and C-terminal parts of eIF4G (Figure 1.2) (Imataka & Sonenberg, 1997). eIF4G has also been shown to bind the poly(A) binding protein Pab1p in *Saccharomyces cerevisiae* (Tarun & Sachs, 1996) however, it has been reported that a similar interaction does not to occur between mammalian eIF4G and PABP (Craig *et al.*, 1998).

eIF4G is a target for proteolytic cleavage during picornaviral infection. Both the 2A protease of entero- and rhinoviruses and the L protease of the aphthovirus FMDV (foot and mouth disease virus) cleave eIF4G (reviewed in Jackson & Kaminski, 1995). Proteolytic cleavage occurs at amino acids 479-480 or 486-487, a flexible hinge region; this results in a smaller N-terminal fragment which retains eIF4E binding capacity and a larger C-terminal fragment which can bind both eIF3 and eIF4A (Figure 1.2) (Lamphear *et al.*, 1995). The proteolysis of eIF4G eliminates cap-dependent translational initiation but allows initiation of translation mediated by the viral IRESes to continue (Pestova *et al.*, 1996b).

**eIF4A and eIF4B**

eIF4A possesses RNA dependent ATPase activity (Grifo *et al.*, 1984) and is an archetypal member of the ‘DEAD’ box family of RNA helicases (Linder *et al.*, 1989). However another initiation factor, eIF4B, is required for maximal helicase activity; eIF4F in conjunction with eIF4B exhibits helicase activity 6 times greater than that of eIF4A and eIF4B alone (Rozen *et al.*, 1990). Study of dominant negative mutants of eIF4A, which abolish translation, has suggested that free eIF4A is required to recycle through the eIF4F complex during the scanning phase of initiation (Pause *et al.*, 1994b).

eIF4A is one of the most abundant initiation factors found in the cell and can be isolated as free protein as well as part of the eIF4F complex (Duncan & Hershey, 1983). Inclusion of eIF4A as a member of the core eIF4F complex has been disputed, since eIF4E-eIF4G complexes have been purified which are not associated with eIF4A (reviewed in Pain, 1996 and Rhoads *et al.*, 1994). Recent identification of two eIF4A binding domains in mammalian eIF4G (Imataka & Sonenberg, 1997) raises the possibility that eIF4G may bind more than one molecule of eIF4A, although equally eIF4G could have two separate contacts with one eIF4A molecule. Hence, questions remain to be resolved about the exact interactions of eIF4A with other eIF4 factors, however, its requirement in the initiation of translation (both cap-dependent and cap-independent) has been clearly demonstrated (Pause *et al.*, 1994b; Pestova *et al.*, 1996b).

eIF4B contains two RNA binding regions; a consensus RNA recognition motif (RRM) near the N-terminus (Milburn *et al.*, 1990) and a second RNA binding region in the C-
Figure 1.2: Human eIF4G protein. Showing the domains which bind eIF4A, eIF4E and eIF3, the sites at which picornavirus proteases cleave and the region to which p97 is homologous (not to scale).
terminal half of the protein (Méthot et al., 1994). The C-terminal RNA binding activity is non-specific, whereas the RRM, whilst inefficient at binding random RNA sequences, has been shown to bind specifically to the 18S rRNA (Méthot et al., 1996). In addition, Méthot et al. have shown that eIF4B can interact with two different RNA molecules simultaneously, which leads them to hypothesise that eIF4B participates in the binding of mRNA to the 40S ribosomal subunit via association of eIF4B with the 18S ribosomal RNA (rRNA). How eIF4B stimulates the RNA helicase activity of eIF4A is yet to be determined, although it has been shown that the helicase stimulatory activity maps to the C-terminal half of eIF4B (Méthot et al., 1994).

Assembly of the 80S Ribosome at the Initiation Codon

The scanning process by which the 40S ribosomal subunit migrates from the m7G cap, along the 5' UTR, to the initiation codon has so far proved difficult to examine. Scanning can be impeded by stable secondary structures (with a free energy greater than -60 kcal/mol); however, it is not as sensitive to inhibition by less stable structures (-30 kcal/mol) which are sufficient to inhibit the initial association between the mRNA and the 43S pre-initiation complex (Kozak, 1989a). Arrest of the scanning 40S subunit is mediated by codon-anticodon recognition between the met-tRNA\textsubscript{j} and the AUG initiation codon. However, other factors also influence this event, including the immediate context of the initiation codon, and these are discussed further in section 1.2.3.

eIF5 is the final factor to participate in initiation; it catalyses hydrolysis of the GTP molecule bound to eIF2, which leads to release of initiation factors from the initiation complex, followed by association of the 60S ribosomal subunit. Sequence analysis of eIF5 reveals consensus GTPase motifs (Das et al., 1993), although eIF5 GTPase activity is only operative in the presence of 40S subunits, and the factor interacts specifically with eIF2 (Chaudhuri et al., 1994). Thus, the current model is that eIF5 interacts with eIF2 on the surface of the 40S subunit which results in activation of the GTPase, hydrolysis of GTP and release of eIF2-GDP, eIF3 and probably other eIFs from the initiation complex (reviewed in Pain, 1996). Once these processes have occurred, the 60S subunit associates with the 40S subunit to form a translation-competent 80S ribosome.

1.2.2 Elongation and Termination

The elongation phase of translation results in the decoding of the mRNA and synthesis of the corresponding polypeptide chain. Elongation in mammals requires two elongation factors, multimeric eEF1 and monomeric eEF2, both of which are active as complexes with GTP. The cycle of addition of each amino acid to the growing polypeptide chain has four features (Figure 1.1.). Initially, an amino acyl
tRNA, complexed with eEF1α-GTP, binds to the A site of the ribosome and this leads to release of the tRNA occupying the E site. GTP hydrolysis then occurs and eEF1α-GDP is released from the ribosome. Recycling of eEF1α to its active form requires guanine nucleotide exchange mediated by the β, γ and δ subunits of eEF1. Peptide bond formation between the incoming amino acid and the polypeptide chain is catalyzed by the peptidyl transferase activity of the 60S ribosome. Following this, the 3’ ends of the tRNAs shift into the adjacent sites of the ribosome. Finally, eEF2 complexed with GTP binds the ribosome and catalyzes complete shift of the deacylated-tRNA into the E site and the peptidyl-tRNA into the P site. As a consequence of the association of the mRNA with the tRNA anticodons, the mRNA moves relative to the ribosome. (Reviewed in Proud, 1994 and Nygård & Nilsson, 1990).

eEF2 has ribosome-dependent GTPase activity which is activated following the translocation step (Nygård & Nilsson, 1984; Carlberg et al., 1990). Unlike eEF1α, eEF2 has no requirement for a guanine nucleotide exchange factor. The ribosome is returned to the start of the elongation cycle when eEF2 departs from the ribosome and a new eEF1α ternary complex enters the A site (reviewed in Nygård & Nilsson, 1990).

One of three stop codons, UAA, UAG or UGA, is used to signal termination of translation. The eukaryotic release factors (eRFs) play an essential role in this process. Although termination of translation in prokaryotes is more thoroughly characterized, greater detail about the eukaryotic process is emerging (reviewed in Tuite & Stansfield, 1994 and Buckingham et al., 1997). eRF1 mimics an amino acyl tRNA and binds to the ribosome at the A site where it promotes hydrolysis of the polypeptide-tRNA bond (Frolova et al., 1994). eRF1 activity is stimulated by the recently discovered factor eRF3, an eRF1 and ribosome dependent GTPase (Kisselev & Frolova, 1995; Frolova et al., 1996). Once the polypeptide chain has been separated from the last tRNA these components are released from the ribosome which is then free to participate in a new round of protein synthesis.

1.2.3 Regulation of Protein Synthesis

The amount of protein produced from any individual mRNA is dependent upon a number of factors. Broadly these include: the half life of the mRNA, the global rate of protein synthesis in the cell, the length and sequence of the 5’ UTR, positioning and context of the initiation codon, the existence of regulatory motifs in the 5’ and 3’ UTRs and any protein factors which may interact with them. Mechanisms exist to regulate each of these aspects of translation, allowing diverse controls over global
Initiation of translation is the limiting stage of protein synthesis and thus the majority of control events occur here. However, elongation has also been shown to be regulated and some specific examples of gene expression modulated in this way exist (reviewed in Ryazanov et al., 1991 and Redpath & Proud, 1994).

**Determinants of Translational Efficiency**

Five structural features of mRNAs have been found to contribute to the fidelity and efficiency of translational initiation. These include the m7G cap, the context and positioning of the initiation codon, length of the 5' UTR and the secondary structures it forms (Kozak, 1991a, b). In addition, the discovery that eukaryotic cellular messages can contain IRESes, allowing initiation to occur in a cap-independent manner, presents the possibility that a different set of constraints may govern the translation of these messages.

**The m7G Cap**

All eukaryotic cellular mRNAs, except those synthesised in organelles, are post-transcriptionally modified by the addition of a methylated guanosine residue at the 5' end. The presence of an m7G cap structure has a major effect on the efficiency of translation of a message and cap analogues can inhibit protein synthesis (reviewed in Jackson et al., 1995). Reduced accessibility to the cap, for example caused by secondary structure at the extreme 5' end of a message, significantly reduces the efficiency of translation (Kozak, 1989a).

**The Initiation Codon**

In the majority of mRNAs the initiation codon is AUG which is surrounded by the consensus sequence: GCCGCA/CCCAUGG (Kozak, 1987). Mutational analysis has shown that the purine at position -3 (where the A of the initiation codon is +1) and the G at +4 are crucial to recognition of the AUG as a start site (Kozak, 1986). A small number of mRNAs initiate translation at non-AUG codons, namely GUG, CUG or ACG and in these cases positions +5 and +6 are also major determinants of translational efficiency (Boeck & Kolakofsky, 1994).

Over 95% of mRNAs that have been analysed use the AUG closest to the 5' end of the message as the initiation codon, in accordance with the scanning mechanism of initiation. However, there are some notable exceptions to this rule. Leaky scanning occurs when the first AUG is in poor context and many of the scanning 40S subunits fail to recognise it. Initiation then takes place at an AUG codon in better context.
further downstream; this mechanism allows some mRNAs to encode more than one product (Kozak, 1991a).

Some mRNAs have one or more AUG codons upstream of the authentic initiation codon. Such an upstream AUG in a strong context severely inhibits translation since it intercepts the scanning 40S subunit. Upstream AUGs can be followed closely by a termination codon, and as a result, encode a small upstream open reading frame (uORF). After translation of the uORF, a small proportion of the 40S subunits remain associated with the mRNA and are able to reinitiate at the authentic initiation codon (reviewed in Kozak, 1992). Successful reinitiation appears to depend on the uORF being short; this is thought to be due to the retention of some essential initiation factor on the 40S ribosomal subunit for a short time after elongation commences. Reinitiation is also more efficient when the distance between the first and second cistrons is greater, which may reflect the time needed for the 40S subunit to re-acquire a met-tRNA_i (reviewed in Geballe & Morris, 1994).

Although uORFs, leaky scanning and use of non-AUG initiation codons are all relatively rare, when they do occur it is almost always in messages which encode proto-oncogenes, growth factors and other growth regulatory genes (Kozak, 1987, 1991a). Since all of these mechanisms serve to repress translation to some extent, it is widely hypothesised that translational repression of genes central to the regulation of cell growth and division is an important part of the necessary control of these proteins.

**The 5' Untranslated Region**

Two characteristics of the 5' UTR affect the efficiency of protein synthesis; its length and the degree of secondary structure it forms. The majority of mRNAs have a 5' UTR in the range of 20 to 100 nucleotides long (Kozak, 1987). A minimal 5' UTR length of 10 to 20 nucleotides is required for initiation; if the first AUG is closer to the cap than 10 nucleotides it is not usually used as an initiation codon. The efficiency of initiation can increase as the leader length increases, up to 60 nucleotides, providing it does not contain any secondary structure (reviewed in Kozak, 1994). There is no evidence that longer 5' UTRs are themselves inhibitory to translation but the proviso that they contain no secondary structure or upstream AUGs is one rarely observed in natural messages. Indeed, in the small proportion of mRNAs that have long 5' UTRs, these tend to be GC rich, increasing the potential for secondary structure formation (Kozak, 1987). Once again, genes whose mRNAs have such potentially inhibitory, long, structured 5' UTRs, fall into the class of proto-oncogenes and growth regulatory genes described above (reviewed in Gray & Hentze, 1994b).
Regions of structure in the 5' UTR can have functions other than inhibiting the scanning 40S ribosomal subunit, they can also provide specific structural motifs to which regulatory proteins may bind. The paradigm of such a system is the translational control of Ferritin expression details of which are given in section 1.2.5. Additionally, secondary and tertiary structure motifs are fundamental to the function of IRESes, where protein-RNA interactions mediate 40S ribosomal subunit binding without involvement of the 5' cap (Jackson & Kaminski, 1995; Belsham & Sonenberg, 1996; Le & Maizel, 1997).

1.2.4 Mechanisms for the Control of Initiation of Translation

Apart from the inherent features of any individual mRNA which govern the efficiency of its translation, control is exerted over protein synthesis through the modulation of initiation factor activity. Regulation of initiation can influence both the overall global rate of protein synthesis and the relative rates of synthesis of different proteins. Two particular steps of the initiation pathway appear to be the focus of regulation; the binding of met-tRNA\textsubscript{\text{\text{\text{}}}} to the 40S ribosomal subunit and the initial binding of the 43S pre-initiation complex to the mRNA. This regulation is mediated through eIF2 factors, which almost exclusively affect global protein synthesis, and the eIF4F complex, which alters both global protein synthesis and the translation of specific messages.

Alteration of eIF2 Activity

Many physiological conditions that inhibit initiation of protein synthesis have been shown to decrease the activity of eIF2 (reviewed in Redpath & Proud, 1994). This control affects the recycling of eIF2-GDP to eIF2-GTP and involves the guanine nucleotide exchange factor eIF2B. Two different mechanisms are known to regulate this process; phosphorylation of the eIF2\textsubscript{\alpha} subunit and phosphorylation of eIF2B itself.

\textit{eIF2\textsubscript{\alpha} Phosphorylation}

Phosphorylation of eIF2\textsubscript{\alpha} on Ser51 results in increased affinity of eIF2 for eIF2B which leads to formation of inactive eIF2-GDP-eIF2B complexes (Rowlands \textit{et al.}, 1988). This causes a reduction in the rate of guanine nucleotide exchange on unphosphorylated eIF2, particularly since eIF2B is present in cells at less than stoichiometric levels with respect to eIF2 (Hershey, 1989). Phosphorylation of eIF2\textsubscript{\alpha} has been shown to be increased in response to a variety of physiological stresses which depress protein synthesis, including heat shock, amino acid starvation and viral infection (Scorsone \textit{et al.}, 1987; Clemens, 1994).
Two mammalian kinases which phosphorylate eIF2α have been characterised; HCR (the Haem Controlled Repressor) and PKR (Protein Kinase activated by double-stranded RNA) (reviewed in Rhoads, 1993 and Hershey, 1989). HCR is principally expressed in erythroid cells and is activated by a lack of haem prosthetic groups. Activated HCR phosphorylates eIF2α, resulting in reduced protein synthesis and preventing the production of globin polypeptides in the absence of haem (reviewed in Clemens, 1994). HCR has also been shown to be activated by heat shock, probably as a result of interactions with heat shock proteins (Matts et al., 1993).

PKR is expressed in most mammalian cells under normal conditions and plays an important role in defence of a cell against viral attack. Expression of PKR is up-regulated in response to interferons α and β and upon viral infection it is activated by the presence of double stranded RNA (reviewed in Hovanessian, 1991). The resulting phosphorylation of eIF2α and inhibition of translation prevents synthesis of viral proteins and thus restricts viral replication (Meurs et al., 1992).

**eIF2B Phosphorylation**

A number of examples exist where the activity of eIF2B is regulated in the absence of alterations in eIF2α phosphorylation, and these led to a search for a kinase which could modify the activity of eIF2B (reviewed in Price & Proud, 1994 and Proud & Denton, 1997). The best candidate kinase identified was glycogen synthase kinase-3 (GSK-3) which phosphorylates the ε subunit of eIF2B (Figure 1.3). Purified GSK-3 inhibits eIF2B activity when added to crude cell extracts and GSK-3 activity is down-regulated in response to insulin, a well known stimulator of protein synthesis (Welsh & Proud, 1993).

**Alteration of eIF4F Activity**

Regulation of the factors which mediate binding of the 43S pre-initiation complex to the mRNA can be exerted in a number of different ways. For a long time the focus of research was on eIF4E, which was identified as a limiting component of eIF4F in HeLa cells (Duncan et al., 1987) and the activity of which can be regulated by phosphorylation. The discovery of a family of eIF4E binding proteins (4E-BPs) that regulate eIF4E activity and which are themselves regulated by phosphorylation (reviewed in Proud & Denton, 1997), added further weight to the regulatory function of eIF4E. More recently however, it has become apparent that eIF4G may also have a role to play in modulating eIF4F function (reviewed in Morley et al., 1997).

Whilst changes in eIF4F activity can alter global protein synthesis rates, it has also been shown that a specific subset of mRNAs are particularly sensitive to the availability of active eIF4F complexes. mRNAs which have long, structured 5’ UTRs
Figure 1.3: A simplified outline of signaling pathways regulating phosphorylation of initiation factors. Dashed arrows indicate that other signaling components are, or may be, involved.
have a large requirement for the helicase activity of eIF4A and compete poorly with other messages in the process of initiation (Gray & Hentze, 1994b). It has been shown that the inhibition of translation of such messages can be relieved by overexpression of eIF4E (Koromilas et al., 1992a). This is presumably because increased amounts of the limiting component of eIF4F result in sufficient eIF4F complexes for the efficient translation of even those messages which compete most poorly.

**eIF4E Phosphorylation**

Increased phosphorylation of eIF4E correlates with increased initiation of translation in response to mitogens and growth factors (Morley & Traugh, 1989; Kaspar et al., 1990; Donaldson et al., 1991) and de-phosphorylation of eIF4E is observed during heat shock and in mitosis, when protein synthesis rates fall (Bonneau & Sonenberg, 1987; Duncan et al., 1987). Phosphorylation has been shown to have some effect on the ability of eIF4E to bind to m7G cap analogues; phosphorylated eIF4E, isolated by rRNA-sepharose chromatography, has a 3- to 4-fold greater affinity for cap analogues than the unphosphorylated protein (Minich et al., 1994). However, this is not a completely discriminating modification of eIF4E since both phosphorylated and unphosphorylated forms bind to m7GTP-sepharose (Bonneau & Sonenberg, 1987; Duncan et al., 1987).

Several studies have also indicated that eIF4E phosphorylation affects the formation of eIF4F complexes. Isolation of eIF4E present in eIF4F complexes or associated with ribosomes has shown that it is more highly phosphorylated than free eIF4E (Joshi-Barve et al., 1990; Lamphear & Panniers, 1990; Rau et al., 1996). It has also been demonstrated, in a number of systems, that increased phosphorylation of eIF4E correlates with increased association of eIF4G with eIF4E (Bu et al., 1993; Morley & Pain, 1995a, b). However, phosphorylation of several other initiation factors also correlates with these events, including eIF4G (see below), and in reticulocyte lysates unphosphorylated eIF4E, as well as the phosphorylated isoform, is found associated with ribosomes (Rau et al., 1996). These data suggest that while eIF4E phosphorylation enhances its function, it is not necessary for the inclusion of eIF4E in eIF4F complexes.

The major site of phosphorylation of eIF4E is a single serine residue, previously thought to be Ser53 but more recently identified as Ser209 (Flynn & Proud, 1995; Joshi et al., 1995). There have been conflicting reports as to which signalling pathways are involved in eIF4E phosphorylation. In CHO cells overexpressing the insulin receptor, protein kinase C (PKC) was shown to be required for phorbol ester stimulated phosphorylation of eIF4E but not for stimulation by serum or insulin (Flynn & Proud, 1996a). Further studies with these cells indicated that the MAP
kinase (Mitogen Activated Protein kinase) pathway is required for eIF4E phosphorylation in response to insulin (Flynn & Proud, 1996b). In contrast, in 32D myeloid precursor cells transfected with both the insulin receptor and IRS-1 (insulin receptor substrate), eIF4E phosphorylation in response to insulin appears to be independent of the MAP kinase pathway (Mendez et al., 1996).

Many proposals have been made as to which kinase or kinases phosphorylate eIF4E, but the discovery of a new target of MAP kinase, Mnk1 (MAP kinase-interacting kinase 1) has provided one of the best candidates to date. Mnk1 binds to and is activated by the growth factor regulated MAP kinases Erk1 and Erk2 and the stress activated MAP kinase p38 (Fukunaga & Hunter, 1997; Waskiewicz et al., 1997). In vivo, Mnk1 is activated by mitogens and stresses which cause increases in eIF4E phosphorylation and in vitro Mnk1 rapidly phosphorylates eIF4E on Ser209 (Waskiewicz et al., 1997). Furthermore, in NIH 3T3 cells, eIF4E phosphorylation in response to serum stimulation is prevented by the inhibitor PD98059, which is specific to the classical (Erk) MAP kinase pathway, and anisomycin induction of eIF4E phosphorylation is inhibited by SB203580, a specific p38 (stress activated) MAP kinase pathway inhibitor (Morley & McKendrick, 1997).

These data indicate that, in some systems at least, eIF4E phosphorylation is mediated by MAP kinase signalling pathways and suggest that Mnk1 could be a kinase responsible for directly phosphorylating eIF4E in vivo (Figure 1.3). However, conclusively identifying all the regulators of eIF4E phosphorylation and the precise way in which this modification mediates eIF4E activity, will require much further research.

**eIF4E Binding Proteins**

An alternative mechanism for regulating eIF4E was delineated by the discovery of two eIF4E binding proteins, 4E-BP1 and 4E-BP2 (Pause et al., 1994a). 4E-BP1 and 4E-BP2 have 56% sequence identity and 4E-BP1 was found to share extensive sequence similarity with the protein PHAS-I, which had previously been identified in rat adipocytes as a heat and acid stable protein, that was rapidly phosphorylated in response to insulin treatment (Lin et al., 1994). The binding of 4E-BP1 or 4E-BP2 to eIF4E inhibits cap-dependent (but not IRES mediated) translation in HeLa cell lysates and in cultured cells (Pause et al., 1994a). This inhibition is not a result of any decrease in cap-binding ability of eIF4E but is due to competition of 4E-BP1 with eIF4G for eIF4E (Haghighat et al., 1995). Indeed, it has been shown that eIF4G, 4E-BP1 and 4E-BP2 share a conserved motif essential for eIF4E binding (Mader et al., 1995). The 4E-BPs therefore inhibit translation by sequestering eIF4E and preventing the formation of eIF4F complexes.
Phosphorylation of 4E-BP1 in response to insulin stimulation results in the dissociation of 4E-BP1 from eIF4E, as indicated by a reduction in the amount of eIF4E co-immunoprecipitated with 4E-BP1 and by decreased recovery of 4E-BP1 by m7GTP affinity chromatography (Lin et al., 1994, 1995; Pause et al., 1994a; Graves et al., 1995; Diggle et al., 1996). PKC, which phosphorylates eIF4E in vitro, has been used to show that eIF4E binding by 4E-BPs inhibits eIF4E phosphorylation, suggesting a two-step model for eIF4E activation: dissociation from 4E-BPs followed by phosphorylation (Whalen et al., 1996). However, studies which employed cultured cell lines demonstrated that 4E-BP1 and eIF4E can independently regulate cap-dependent translation; eIF4E can be dephosphorylated irrespective of its interaction with 4E-BP1, and 4E-BP1 is associated with eIF4E regardless of the state of eIF4E phosphorylation (Feigenblum & Schneider, 1996).

Initial indications were that 4E-BP1 phosphorylation is mediated by the MAP kinase pathway (Lin et al., 1994). In vitro MAP kinase phosphorylates 4E-BP1 on 5 conserved Serine and Threonine residues which are also phosphorylated in vivo in response to insulin (Fadden et al., 1997). However, MAP kinase may not be important in the phosphorylation of 4E-BP1 in vivo since 4E-BP1 is a poor substrate for MAP kinase when bound to eIF4E (Lin et al., 1995; Diggle et al., 1996). Additionally, in both 3T3-L1 adipocytes and smooth muscle cells, phosphorylation of 4E-BP1 is inhibited by rapamycin, an inhibitor of the p70s6k pathway, but not by PD98059, a MAP kinase inhibitor. This demonstrates that the FRAP/mTOR* rather than a MAP kinase signalling pathway mediates 4E-BP1 phosphorylation, in these systems (Graves et al., 1995; Lin et al., 1995; Beretta et al., 1996; Lin & Lawrence, 1996; Arnott et al., 1997; Fleurent et al., 1997).

With respect to the FRAP/mTOR signalling pathway it has been shown that 4E-BP1 is not phosphorylated by p70S6k but that this pathway bifurcates at a rapamycin-sensitive point immediately upstream of p70S6k (Von Manteuffel et al., 1997). Neither the kinase responsible for activating both p70S6k and the 4E-BP1 kinase, nor the 4E-BP1 kinase itself, have yet been identified (Figure 1.3). In addition it has been demonstrated that 4E-BP1 can be phosphorylated by a rapamycin insensitive pathway, on a different residue to the five already identified (Diggle et al., 1996). This phosphorylation event occurs only on 4E-BP1 bound to eIF4E and does not initiate dissociation. It has been proposed that the rapamycin insensitive phosphorylation of 4E-BP1 is a ‘priming’ event which allows phosphorylation of the other sites, and as a result dissociation, to occur (Proud & Denton, 1997).

*FKBP (FK506-binding protein) Rapamycin-Associating Protein / mammalian Target Of Rapamycin
As a central component of eIF4F, eIF4G would seem to be an ideal target for the regulation of the initiation of translation. Although little is yet known about regulation of eIF4G, evidence is beginning to accumulate that this is indeed the case (reviewed in Morley et al., 1997).

Phosphorylation of eIF4G at multiple, and as yet undefined, sites has been shown to correlate with increased rates of translation and often occurs in parallel with eIF4E phosphorylation. Treatment of rabbit reticulocytes or porcine peripheral blood lymphocytes (PBLs) with phorbol ester, results in increased phosphorylation of eIF4G and eIF4E (Morley & Traugh, 1989; Morley & Pain, 1995b) and this correlates with enhanced eIF4F formation in the PBLs (Morley & Pain, 1995b). Likewise, phosphorylation of both factors is increased in epithelial cells treated with epidermal growth factor or okadaic acid (Donaldson et al., 1991); on treatment of 3T3-L1 cells with insulin or phorbol ester (Morley & Traugh, 1993); and during meiotic maturation of *xenopus* oocytes, when increased eIF4F formation is also observed (Morley & Pain, 1995a). Additionally, it has been shown that phosphorylation of eIF4G increases cross-linking of eIF4F to the mRNA cap (Morley et al., 1991; Bu et al., 1993).

This evidence suggests that eIF4G phosphorylation may play a role in enhancing formation of eIF4F, in concert with a similar function attributable to eIF4E phosphorylation. The recent demonstration that the cap binding activity of eIF4E is enhanced approximately 7-fold when bound to eIF4G (Haghighat & Sonenberg, 1997), provides a possible mechanism by which formation of eIF4F leads to increased cap-binding by initiation complexes and thus stimulation of translational initiation. However, much remains to be determined about eIF4G phosphorylation and its role in the regulation of protein synthesis.

Two other possibilities present themselves for eIF4G mediated regulation of protein synthesis. One of these is regulation of the stability of the eIF4G protein, suggested by the observation that the N-terminal portion of eIF4G contains five high-scoring PEST regions (Yan et al., 1992). The other is that there may be cellular proteases that are equivalent to the picornaviral proteases which cleave eIF4G to form a functional C-terminal fragment (reviewed in Morley et al., 1997).

The recent discovery of a new group of proteins which have sequence similarity to the central and C-terminal domains of eIF4G has suggested yet another mechanism by which translation may be regulated (reviewed in Morley et al., 1997). The gene encoding a 97 kDa protein was independently cloned by four different research groups. ‘p97’ was cloned from a human embryo brain cDNA library (Imataka et al.,
1997) and Eif4g2, encoding an identical protein, was cloned from a screen of retroviral integration sites in murine myeloid leukaemias (Shaughnessy et al., 1997). DAP-5 is also identical to p97 and was cloned as a cDNA whose expression conveyed resistance to interferon γ induced apoptosis (Levy-Strumpf et al., 1997). NAT1 is 99% identical to p97 and was found to be extensively edited in the livers of transgenic mice overexpressing the apolipoprotein B mRNA editing enzyme (APOBEC-1); the editing of NAT1 leads to reduced expression and the transgenic mice suffer from liver dysplasia and carcinoma (Yamanaka et al., 1997).

The 97 kDa proteins exhibit about 30% sequence identity with the C-terminal two-thirds of eIF4G (Figure 1.2). The N-termini of these proteins equate with a position close to the cleavage site of picornaviral proteases (Imataka et al., 1997; Levy-Strumpf et al., 1997; Shaughnessy et al., 1997; Yamanaka et al., 1997). The 97 kDa proteins do not contain the eIF4E binding motif found in eIF4G and it has been demonstrated that p97 does not co-immunoprecipitate with eIF4E (Imataka et al., 1997). The proteins do have homology to the region of eIF4G which binds eIF3 and it has been shown that p97 interacts with eIF3 in vivo (Imataka et al., 1997). Both NAT1 and p97 have been shown to associate with eIF4A (Imataka et al., 1997; Yamanaka et al., 1997) although, in contrast to eIF4G, they only possess one eIF4A binding site (Imataka & Sonenberg, 1997).

It has been suggested that the 97 kDa proteins may act as negative regulators of initiation in a manner similar to the 4E-BPs by competing with eIF4G for eIF4A and eIF3 (Imataka et al., 1997; Yamanaka et al., 1997). NAT1 and p97 can inhibit cap-dependent and cap-independent translation both in vitro and in intact cells (Imataka et al., 1997; Yamanaka et al., 1997). Additionally, DAP-5 has been shown to inhibit cell growth when expressed at high levels (Levy-Strumpf et al., 1997). However, the data of Levy-Strumpf et al. suggest that the growth regulatory effects of DAP-5 are attributable, at least in part, to the C-terminal domain of the protein which bears least similarity to eIF4G.

**The mRNA 3' End in Initiation of Translation**

The 3' UTR and poly(A) tail of a message have been found to affect the mRNA in several ways; in addition to mediating mRNA localisation and controlling mRNA stability they can, perhaps surprisingly, affect the initiation of translation (reviewed in Jackson & Standart, 1990 and Decker & Parker, 1995). The poly(A) tail has been shown to enhance mRNA translation and acts synergistically with the m7G cap (reviewed in Sachs et al., 1997). In yeast, the essential poly(A) tail binding protein, Pab1p, has been demonstrated to stimulate the binding of the 40S ribosomal subunit to mRNA (Tarun & Sachs, 1995). More recently it has been demonstrated that the poly(A) tail, in the absence of a cap structure, is capable of directing translational
initiation in a yeast in vitro system, although this effect is extremely inefficient in vivo (Preiss & Hentze, 1998).

These data have led to the proposal of models in which the 3' and 5' ends of an mRNA molecule interact during the initiation of translation. In support of these proposals, association of the poly(A) tail with eIF4B and eIF4F has been reported in the wheat germ lysate in vitro translation system (Gallie & Tanguay, 1994) and more recently, Pab1p has been shown to interact with eIF4G in Saccharomyces cerevisiae (Tarun & Sachs, 1996). Pab1p was found to be associated with eIF4F isolated by m7GTP sepharose chromatography and it co-immunoprecipitates with both of the yeast eIF4G homologues, Tif4632p and Tif4631p (Tarun & Sachs, 1996). Disruption of the eIF4G-Pab1p interaction by mutagenesis abolishes the ability of the poly(A) tail to stimulate initiation, whilst leaving the ability of the m7G cap to do so unaffected (Tarun et al., 1997).

In yeast, the interactions of eIF4G with both eIF4E and the poly(A) binding protein could explain the synergism between the poly(A) tail and m7G cap in stimulating translation. However, a recent report suggests that such an interaction between mammalian eIF4G and PABP does not occur (Craig et al., 1998). This report identifies a protein that has homology to the central region of eIF4G and which interacts with both PABP and eIF4A (Craig et al., 1998). Craig et al. propose that this protein, PABP-interacting protein (PAIP-1), provides the link between the poly(A) tail and the 5' of an mRNA in mammals, instead of the direct interaction with eIF4F seen in yeast and plants. In whichever way interaction between the 5' and 3' ends occurs, it would seem possible that some 3' UTR sequences, and their binding proteins, could produce their effects on the initiation of translation by modulating the function of the poly(A) binding protein (discussed in Sachs et al., 1997). In summary, the existence of a role for the 3' end of a message in the initiation of translation has been established, but much still remains to be determined about the process of 5'-3' 'cross-talk' and how it affects translation.

**Expression of Initiation Factor Genes**

A common mechanism for regulating proteins with important roles in growth control is to alter their cellular concentration. However, very few examples of regulated initiation factor gene expression have been described to date (reviewed in Hershey, 1994 and Morris, 1995). One system where a number of experiments have demonstrated regulation of initiation factor gene expression, is the activation or mitogen stimulation of T-cells, when large increases in the rate of protein synthesis are observed (reviewed in Hershey, 1994). Rat T-cells treated with concanavalin A display increased levels of eIF2 and eIF2B proteins in parallel with enhanced ribosome production (Jedlicka & Panniers, 1991). Activation of human T-cells by
cross-linking the TCR-CD3 complex with monoclonal antibody results in increased levels of eIF2α, eIF4E and eIF4A mRNAs (Mao et al., 1992; Boal et al., 1993) and increased eIF4E phosphorylation (Boal et al., 1993). Since in each of these studies, up-regulated expression was observed for all of the initiation factors examined, it is not clear whether there is increased expression of only certain components of the initiation machinery or, cellular levels of all the initiation factors are raised concomitantly with increased ribosome production.

A second example of altered initiation factor gene expression which more clearly involves regulation of individual factors was identified in NIH 3T3 fibroblasts stimulated with serum or platelet derived growth factor (PDGF). In this case, mRNA levels of eIF4E and eIF2α, but not eEF1α or eIF4A, were elevated in response to growth factor stimulation; furthermore, these increases in expression correlated with enhanced expression of c-myc (Rosenwald et al., 1993b). Rosenwald et al. also showed that transcription rates of eIF4E and eIF2α were regulated by oestrogen in BALB/c 3T3 cells expressing a c-Myc-oestrogen receptor fusion protein (Myc-ER) and that the eIF2α promoter region contained a potential c-Myc regulated sequence. Later work identified a conserved sequence in the eIF4E promoter that is a target for activation by c-Myc (Jones et al., 1996).

Expression of antisense RNA against eIF4E in HeLa cells not only results in decreased levels of eIF4E but also of eIF4G, implying that the two genes may be coordinately regulated (De Benedetti et al., 1991). eIF4G expression may itself be regulated at the level of translation, as it has been demonstrated that the 5' UTR of eIF4G is capable of directing internal initiation (Gan & Rhoads, 1996). It has been proposed that this IRES allows synthesis of eIF4G to continue under conditions where normal cap-dependent translation is inhibited, e.g. during picornaviral infection (Gan & Rhoads, 1996).

1.2.5 Translational Regulation of Specific Genes

Many genes, the expression of which can be regulated by translational control, have now been characterised. A selection of these are discussed below, providing detailed examples of many of the mechanisms outlined in the previous two sections.

Cellular mRNAs Which Contain Internal Ribosome Entry Segments

The presence of an IRES within an mRNA allows it to be translated when cap-dependent initiation of translation is reduced or abolished, e.g. under such conditions as heat shock, picornaviral infection and during mitosis. A small number of cellular mRNAs which contain IRESes have been described to date, but the significance of the IRES to the translational regulation of these genes has not yet been
established. The first mRNA identified which contains an IRES was that of the immunoglobulin heavy-chain binding protein, BiP (Macejak & Sarnow, 1991). Other examples include Drosophila melanogaster Antennapedia mRNA (Oh et al., 1992) and, as mentioned in section 1.2.4, eIF4G (Gan & Rhoads, 1996).

Perhaps the most interesting examples of cellular IRESes are those identified in the 5' UTRs of three growth factors and a proto-oncogene, which are members of the class of messages whose long 5' UTRs are known to be burdened with upstream AUGs and potentially inhibitory secondary structure. The human Fibroblast Growth Factor 2 (FGF-2) IRES was the first of these to be discovered. Translation of this protein is extremely complex; four functionally distinct isoforms are produced from one mRNA, by initiation at an AUG and three alternative CUG start codons. Translation of two of these isoforms (the CUG-1 and AUG initiated forms) is enhanced by increased eIF4E (Kevil et al., 1995). In addition to this cap-dependent regulation of FGF-2, translation can occur in a cap-independent manner, mediated by an IRES located just upstream of the first CUG (Vagner et al., 1995). The insulin-like growth factor II (IGF-II) is encoded by four different mRNAs which have identical coding regions but unique 5' UTRs; an IRES has been identified in the transcripts produced from promoter 1 (Teerink et al., 1995).

The 5' UTR of Platelet-Derived Growth Factor 2 (PDGF-2) was shown to be extremely inhibitory to translation, in various constructs transfected into a number of different cell types (Rao et al., 1988). However, this translational repression is relieved during megakaryocytic differentiation of K562 cells (Bernstein et al., 1995). The relief of translational repression was shown to be due, at least in part, to an IRES in the 5' UTR of PDGF-2 mRNA, which is activated in differentiating cells, leading to increased internal initiation of translation (Bernstein et al., 1997). Finally, the message of the proto-oncogene c-myc contains an IRES (Nanbru et al., 1997; Stoneley et al., 1998) which is discussed further in section 1.3.2.

Translational Regulation by the Iron Regulatory Protein

Regulation of translation of ferritin mRNA is the paradigm for translational control by interaction between a motif in the 5' UTR and a regulatory protein (reviewed in Klausner et al., 1993). The translation of ferritin is regulated coordinately with iron availability; a 90 kDa Iron Regulatory Protein (IRP) binds specifically to a sequence in the 5' UTR termed the Iron Responsive Element (IRE) (Brown et al., 1989; Walden et al., 1989). The consensus IRE consists of a stem-loop structure with a 6 base loop and an unpaired C 'bulge' in the stem (reviewed in Klausner et al., 1993). Binding of IRP to the IRE, which is located about 30 nucleotides from the cap, prevents binding of the 43S pre-initiation complex to the mRNA (Gray & Hentze, 1994a). IRP activity is
modulated by iron levels such that it has high affinity for the IRE under iron starvation conditions and low affinity when iron is plentiful (Constable et al., 1992).

In addition to regulating levels of the iron storage protein, ferritin, IRP has also been shown to regulate translation of the rate limiting enzyme in haem biosynthesis, erythroid 5-aminolevulinate synthase (eALAS), via an IRE in its 5’ UTR (Gray & Hentze, 1994a). In contrast to these two proteins which are down-regulated when intracellular iron levels are low, expression of the transferrin receptor, which mediates iron uptake, is increased. This increase is also post-transcriptionally regulated by IRP, but in this case IRP binding to five IREs in the 3’ UTR of the transferrin receptor mRNA increases the stability of the mRNA. This increased stability leads to accumulation of the mRNA and as a result to increased synthesis of transferrin receptor (reviewed in Klausner et al., 1993).

**Ornithine Decarboxylase**

Studies of translationally repressed mRNAs often reveal inhibition mediated by the 5’ UTR, which can be relieved by overexpression or increased phosphorylation of eIF4E. One of the most thoroughly characterised examples of a gene regulated in this way is ornithine decarboxylase (ODC). Other examples of this type of regulation include vascular permeability factor and the proto-oncogene *pim-1*, a serine/threonine kinase (Kevil et al., 1996; Hoover et al., 1997).

ODC is the rate limiting enzyme in the polyamine biosynthetic pathway; it converts ornithine into putrescine, releasing carbon dioxide (reviewed in Pegg, 1986). Polyamine biosynthesis is required for cell cycle progression and increased ODC activity has been implicated in malignant transformation (reviewed in Flynn & Proud, 1996c). Regulation of ODC activity occurs through alterations in the level of protein, which can be controlled at three levels; transcription, mRNA stability and translation. Insulin treatment of NIH 3T3 fibroblasts transfected with the insulin receptor results in a 50-fold increase in ODC enzyme activity. This increase was shown to be due to relief of translational repression conferred by the 5’ UTR and correlated with increased phosphorylation of eIF4E (Manzella et al., 1991). The translational inhibition was localised to the 5’-most 115 bases of the 5’ UTR which are highly conserved, GC rich and predicted to form secondary structure (Grens & Scheffler, 1990; Manzella et al., 1991). Point mutations, or alterations in the length of the 5’ UTR lead to increased translation of ODC (Pyronnet et al., 1996; Shantz et al., 1996b). A protein has been identified which binds to a conserved sequence about 60 bases upstream of the initiator AUG, but the function of this protein has not been determined and its role, if any, in translational regulation of ODC remains obscure (Manzella & Blackshear, 1992).
Cells overexpressing eIF4E have been shown to have increased ODC activity (in the absence of any alterations in mRNA levels or stability) and there is increased association of ODC mRNA with the polysomes, indicating enhanced initiation of translation (Rousseau et al., 1996b; Shantz et al., 1996b). Conversely, depletion of eIF4E in ras-transformed rat embryo fibroblasts suppresses ODC translation (Graff et al., 1997). In addition to the regulation of translation by the 5′ UTR, the 3′ UTR also appears to be involved; experiments with reporter constructs carrying both the 5′ and 3′ UTRs indicate that the 3′ UTR can relieve some of the translational inhibition imposed by the 5′ UTR (Grens & Scheffler, 1990). However, alone the 3′ UTR is inhibitory to translation and its ability to relieve translational repression mediated by the 5′ UTR has been shown to require the binding of, as yet unidentified, protein factors (Lorenzini & Scheffler, 1997).

1.2.6 Translational Control and Oncogenesis

Two lines of evidence indicate that de-regulation of protein synthesis can play a role in the transformation of cells and in carcinogenesis (reviewed in Rosenwald, 1996a). The first of these comes from experiments using cultured cells, which have shown that some components of the translational machinery can cause, or contribute to, cell transformation. The second is that surveys of various neoplastic tissues show that de-regulation of translation occurs in some human malignancies.

Transformation of Cultured Cells by Initiation Factors

The first initiation factor to be implicated in malignant transformation was eIF4E. Overexpression of eIF4E in NIH 3T3 cells and rat 2 fibroblasts resulted in anchorage independent growth, formation of transformed foci and tumour formation in nude mice (Lazaris-Karatzas et al., 1990). Likewise, overexpression of eIF4E in HeLa cells resulted in aberrant growth and morphology (De Benedetti & Rhoads, 1990). De-regulation of eIF2 function has also been shown to cause transformation. Overexpression of a dominant negative mutant of PKR, in NIH 3T3 cells, resulted in greatly reduced phosphorylation of eIF2α, and the cells displayed a transformed phenotype (Koromilas et al., 1992b). Most recently, it has been demonstrated that eIF4G also has oncogenic potential; overexpression of eIF4G in NIH 3T3 cells results in malignant transformation, in the absence of any increase in eIF4E expression (Fukuchi-Shimogori et al., 1997).

eIF4E in Transformation and Carcinogenesis

In addition to its ability to transform cultured cells, several other observations delineate an important role for eIF4E in neoplasia. Increased expression of eIF4E has been documented in a variety of rat tumour lines transformed by chemical, viral and oncogenic means (Miyagi et al., 1995). Additionally, expression of eIF4E and eIF2α is
elevated in cells transformed with c-myc, v-src and v-abl (Rosenwald, 1996b). Cells transformed by the oncogenes c-src or Ha-ras both display increased phosphorylation of eIF4E (Frederickson et al., 1991; Rinker-Schaeffer et al., 1992). Reduction of eIF4E levels, using antisense RNA, in ras transformed cells diminishes the transformed phenotype (Graff et al., 1995). The regulators of eIF4E, 4E-BP1 and 4E-BP2, have been shown to be negative regulators of cell proliferation and to cause a significant reduction in the malignant phenotype when overexpressed in cells transformed by eIF4E or by v-src (Rousseau et al., 1996a). These data indicate that eIF4E activation is common in transformed cells and it appears to be a target of a number of oncogenes; in particular, eIF4E (along with eIF2α) has been shown to be a transcriptional target of c-Myc, as described in section 1.2.4.

A study of 38 carcinomas of the breast revealed a 3- to 10-fold elevation in eIF4E protein levels when compared to normal tissue and benign adenomas (Kerekatte et al., 1995). Examination of five breast carcinoma cell lines produced a similar observation and the 10 fold increase in eIF4E was shown to occur at the level of transcription (Anthony et al., 1996). More recently, a larger study of 112 mammary tissue specimens has shown that the degree of eIF4E overexpression correlates with clinical outcome; the group of patients with 7-fold or greater eIF4E overexpression had higher incidence of breast carcinoma recurrence or death than the group with lower eIF4E levels (Li et al., 1997). In this context it is also interesting to note that eIF4G has been implicated in squamous cell lung carcinoma. Serum from a lung cancer patient was shown to contain anti-eIF4G antibodies, indicating that eIF4G had become a tumour antigen, and in three independent tumours the eIF4G gene was shown to be amplified (Brass et al., 1997).

**How Does De-Regulation of Protein Synthesis Contribute to Neoplastic Transformation?**

It is widely hypothesised that transformation by increased eIF4F function, i.e. eIF4E overexpression, increased eIF4E phosphorylation and perhaps eIF4G overexpression, is due to relief of the translational inhibition imposed by the 5' UTRs of a variety of proto-oncogenes and growth regulatory genes (reviewed in Sonenberg, 1994). In support of this hypothesis, mRNAs with extensive secondary structure in their 5' UTRs have been shown to be translated efficiently in cells overexpressing eIF4E (Koromilas et al., 1992a). Although the focus of research into carcinogenesis has been on transcriptional activation of proto-oncogenes, if the message of such a gene is translationally repressed then increased mRNA levels will not lead to a large increase in protein, unless translational controls are also abrogated. However, relatively few examples of proto-oncogenes or growth regulatory genes up-regulated in this way have been documented (reviewed in Gray & Hentze, 1994b and Willis, 1998).
Some recent examples of translational de-regulation of specific genes in transformed cells include: increased translation of c-myc in the plasma cell disorder Multiple Myeloma (Paulin et al., 1996 see also section 1.3.1); altered patterns of expression of FGF-2 isoforms in transformed cells, due to increased translation from CUG start codons (Vagner et al., 1996); and increased translation of vascular permeability factor (which is believed to be involved in the recruitment of new blood vessels to tumours) in eIF4E transformed cells (Kevil et al., 1996). Another target of eIF4E mediated translational up-regulation was believed to be cyclin D1 (Rosenwald et al., 1993a, 1995), however, it was finally demonstrated that the increase in cyclin D1 in eIF4E overexpressing cells is due to enhancement of nucleocytoplasmic mRNA transport (Rousseau et al., 1996b).

One well documented example of a gene the translation of which can be affected by eIF4E is ODC (see section 1.2.5). In addition to the observation that increased ODC translation occurs in eIF4E transformed cells (Rousseau et al., 1996b; Shantz et al., 1996b), ODC alone can cause cell transformation (Auvinen et al., 1992, 1997). Studies of rat mammary tumours show that increased ODC activity correlates with development of both hormone-independence and a more aggressive phenotype (Manni et al., 1995). If cells transformed by eIF4E are treated with the ODC inhibitor, α-difluoromethylornithine, a significant reduction of the transformed phenotype is observed (Shantz & Pegg, 1994). Additionally, expression of an ODC dominant negative mutant in eIF4E overexpressing cells completely abolishes the transformed phenotype (Shantz et al., 1996a). Thus, increased eIF4F activity can lead not only to increased rates of global protein synthesis, but also to increased polyamine biosynthesis, which in itself is capable of inducing a malignant phenotype.

1.3 The c-myc Proto-Oncogene

c-myc is the founder member of a small family of proto-oncogenes which encode short-lived nuclear phosphoproteins. c-myc plays a pivotal role in the regulation of cell growth, differentiation and programmed cell death (apoptosis). c-myc is also widely involved in cell transformation and has been implicated in a variety of human malignancies. Regulation of c-myc expression and function is a highly complex process involving control at multiple levels.

1.3.1 Overview of c-myc Function

The majority of work on the myc gene family has focused on three members which have been shown to be activated in various malignancies: c-myc, L-myc and N-myc. c-myc was discovered as the cellular homologue of v-myc, the oncogene identified in avian leukaemia virus, and is expressed in almost all proliferating cell types. N- and
L-myc were characterised as amplified sequences in human neuroblastoma and small cell lung carcinoma respectively. N-myc and L-myc display a high degree of homology to c-myc but their expression is limited to specific stages and tissues during embryonic development (reviewed in Marcu et al., 1992).

The human c-myc gene encodes two polypeptides, Myc-1 and Myc-2, with apparent molecular weights of 67 and 64 kDa respectively (Hann & Eisenman, 1984; Hann et al., 1988). Both proteins are translated from the same mRNA through initiation at alternative start codons; the major product, Myc-2, is initiated from an AUG codon; Myc-1, the larger isoform, is less abundant and is produced by initiation at an upstream CUG codon (Hann et al., 1988). The c-Myc proteins are both phosphoproteins with a half life of 20 to 30 minutes and are localised to the nucleus (Hann & Eisenman, 1984).

Roles of c-myc

Early research into the c-Myc proteins showed that their expression is associated with cell-cycle progression (Eilers et al., 1991) and is incompatible with terminal differentiation in a variety of cell types (Coppola & Cole, 1986; Miner & Wold, 1991). c-myc is an immediate early response gene, i.e. its expression is activated, independently of de novo protein synthesis, during the G0 to G1 transition undergone by mitogen stimulated quiescent cells (Kelly et al., 1983; Rabbitts et al., 1985). Levels of c-myc mRNA and protein do not vary throughout the cell cycle (Hann et al., 1985; Rabbitts et al., 1985; Thompson et al., 1985). Overexpression of exogenous c-myc in quiescent cells can, under certain conditions, drive them into the cell cycle in the absence of expression of any other immediate early genes (Eilers et al., 1991).

Expression of antisense c-myc RNA causes a block in cell cycle progression, at G1, in both serum stimulated and exponentially growing cells (Heikkila et al., 1987; Prochownik et al., 1988; Wickstrom et al., 1988). All of these data indicate that c-myc expression is essential for cell proliferation.

A rapid down-regulation of c-myc expression is observed in many cell lines which have been stimulated to differentiate (Grosso & Pitot, 1984; Spotts & Hann, 1990; Larsson et al., 1994). Moreover, reduction of c-Myc levels using antisense RNA in proliferating HL60, F9 and MEL cells leads to growth arrest and terminal differentiation (Griep & Westphal, 1988; Holt et al., 1988; Prochownik et al., 1988). Conversely, constitutive expression of c-myc prevents cells from leaving the cell cycle and has been shown to inhibit differentiation in a number of cell lines, including MEL cells and MyoD transfected NIH 3T3 cells (Coppola & Cole, 1986; Dmitrovsky et al., 1986; Miner & Wold, 1991). However, recent evidence suggests that the block to differentiation caused by c-Myc is not merely an indirect outcome of the stimulation of cell cycle progression. A differentiation-defective variant of the U937 cell line has
been isolated which does not down-regulate \textit{c-myc} expression on treatment with differentiation agents, however, this stimulus does cause growth arrest. Use of antisense RNA mediated reduction of c-Myc, in addition to treatment with a differentiation agent, results in complete differentiation of these cells (Ryan & Birnie, 1997). In summary, \textit{c-myc} appears to function as a control point in the selection of the mutually exclusive pathways for either proliferation or differentiation (reviewed in Henriksson & Lüscher, 1996).

In contrast to its role in cell proliferation, a number of observations have suggested that levels of c-Myc correlate with the susceptibility of a cell to apoptosis. Use of the Myc-ER fusion protein, transfected into in Rat1 cells, showed that under conditions of serum-starvation, activation of c-Myc function, by treatment with oestrogen or 4-hydroxy-tamoxifen, caused the cells to undergo apoptosis (Evan et al., 1992; Harrington et al., 1994). Likewise, constitutive expression of \textit{c-myc} in myeloid 32D cells results in apoptosis when they are deprived of IL-3 (Askew et al., 1991) and c-Myc has been shown to be required for T-cell receptor stimulated apoptosis in T-cell hybridomas (Shi et al., 1992). Reconciliation of these two apparently contradictory functions of c-Myc has led to the 'dual signal' hypothesis, which states that survival factors are required for \textit{c-myc} expression to mediate cell proliferation and that in the absence of these factors \textit{c-myc} expression results in apoptosis (Evan & Littlewood, 1993; Harrington et al., 1994).

\textbf{\textit{c-myc} in Carcinogenesis}

Oncogenic activation of \textit{c-myc} occurs primarily through de-regulation of expression, rather than through mutations in the coding region. \textit{c-myc} is capable of immortalising primary cells in culture, but co-operation with other oncogenes such as \textit{ras} is necessary for the transformed phenotype (reviewed in Marcu et al., 1992). The essential feature of \textit{c-myc} de-regulation is that it results in an increased potential for cells to enter or remain in the cell cycle and an inability to respond to differentiating agents. Increased expression of \textit{c-myc} has been observed in a wide variety of malignancies, including: small cell lung carcinoma, breast carcinomas, osteosarcoma, colon carcinomas, glioblastoma, cervical carcinoma, myeloid leukaemia and most commonly in lymphoid tumours, particularly; Burkitt's lymphoma, B and T cell acute lymphocytic leukaemia (ALL) and Multiple Myeloma (Paulin et al., 1996 and reviewed in Henriksson & Lüscher, 1996 and Potter, 1990).

A common mechanism for \textit{c-myc} activation is chromosomal translocation; Burkitt's lymphoma is the classic example of this mechanism. All cases of this disease display increased \textit{c-myc} expression; the \textit{c-myc} gene, located on chromosome 8, is translocated to chromosomes 2, 14 or 22 (Dalla-Favera et al., 1982a). This translocation results in truncation of sequences 5' of the coding region and places the gene in an
immunoglobulin locus, consequently levels of c-Myc increase (Battey et al., 1983; Bernard et al., 1983). A second mechanism for c-myc de-regulation is another type of chromosomal rearrangement which results in gene amplification, and is common in some of the examples mentioned above (reviewed in Potter, 1990).

More recently, evidence for a third mechanism of de-regulation of c-myc expression has been presented, one of aberrant translational control. Increases in c-Myc protein, in the absence of increased mRNA or protein stability, have been detected in cell lines established from patients with Bloom's syndrome or Multiple Myeloma (MM) (West et al., 1995; Paulin et al., 1996). MM is a B-cell malignancy in which a plasma cell type proliferates in the bone marrow; unlike most other B-cell tumours, no consistent chromosomal rearrangements involving c-myc, or any other oncogene, have been identified in MM patients (reviewed in Epstein et al., 1995). However, involvement of c-myc in MM has long been postulated and some data do exist to support this (Selvanayagam et al., 1988; Greil et al., 1991), including an immunohistochemical screen of 180 MM cases which showed a correlation between levels of c-Myc and grade of malignancy, (Skopelitou et al., 1993). The discovery of a translational mechanism for the de-regulation of c-myc, and furthermore, a consistent point mutation in the c-myc 5' UTR in MM cell lines provide the best link to date between this oncogene and the disease state (Paulin et al., 1996).

c-myc Encodes a Transcription Factor

Characterisation of the c-Myc protein revealed a number of domains which are required for its different functions (Figure 1.4). The C-terminal region contains features which are homologous to those identified in other transcriptional regulators; these include the basic region (BR), which mediates sequence-specific DNA binding, and the helix-loop-helix (HLH) and leucine zipper (LZ) motifs, which can promote protein-protein interaction (reviewed in Lüscher & Eisenman, 1990). A region in the N-terminus of the protein was identified through its ability to act as a transcriptional transactivator in fusion proteins with GAL 4 (Kato et al., 1990). The c-Myc protein therefore has many characteristics of a transcription factor, but the fact that c-Myc only homodimerises at unphysiologically high protein concentrations led to a search for a c-Myc binding partner (Dang et al., 1989).

Max is a small protein, isolated in a screen for c-Myc binding proteins, which also contains the BR-HLH-LZ motifs found in Myc (Figure 1.4) (Blackwood & Eisenman, 1991). Max can heterodimerise with Myc through the HLH-LZ domains and also forms homodimers (Blackwood & Eisenman, 1991; Kato et al., 1992); both of these complexes bind to a consensus DNA sequence CACGTG, referred to as an E-box, as well as noncanonical DNA sequences (Blackwell et al., 1993). Use of reporter constructs containing E-boxes demonstrated that Myc/Max complexes activate
Figure 1.4: Structural and functional domains of c-Myc and Max proteins. TAD: Transcriptional activation domain, NLS: Nuclear localisation signal, BR: Basic region, HLH: Helix-loop-helix motif, LZ: Leucine zipper domain (not to scale).
transcription in a sequence specific manner whereas Max/Max dimers do not, since Max lacks a transactivation domain (Amati et al., 1992; Kato et al., 1992; Kretzner et al., 1992). The function of Max itself is regulated by binding proteins, which also have the BR-HLH-LZ motif. Mad, Mad3, Mad4 and Mxi1 each dimerise with Max; these dimers can all bind the E-box motif and in conjunction with mSin3, form ternary complexes which repress transcription (Figure 1.5) (reviewed in Henriksson & Lüscher, 1996). Evidence suggests that the Mad family proteins induce opposite effects to c-Myc and they appear to antagonise its functions (Ayer et al., 1993; Lahoz et al., 1994; Hurlin et al., 1995).

1.3.2 Regulation of c-myc Expression

Regulation of c-myc expression is a complex process involving a number of transcriptional and post-transcriptional controls. Transcriptional control operates at both the level of initiation and of elongation; c-myc also autoregulates its transcription. The stability of c-myc mRNA and intracellular targeting of the message can be regulated. Translational control mechanisms mediated by the 5' UTR, which encodes an IRES, affect synthesis of c-Myc protein and can alter the ratio of production of the two isoforms.

Transcriptional Regulation

The c-myc gene contains 3 exons and has four promoters, P0, P1, P2 and P3, which result in production of transcripts with sequentially shorter 5' UTRs. Of these, P1 and P2 are the major promoters responsible for 75-90% and 10-25% of transcripts respectively (Battey et al., 1983; Bernard et al., 1983; Bentley & Groudine, 1986). Differential promoter usage has been observed in a variety of cell types and a number of control sequence elements have been identified through mapping of DNase I hypersensitive sites (reviewed in Marcu et al., 1992). c-Myc can also autorepress its own promoter, as observed in the silencing of the untranslocated c-myc allele in Burkitt's lymphoma and repression of endogenous c-myc genes by overexpression of exogenous c-myc (reviewed in Henriksson & Lüscher, 1996).

Reduction of mRNA levels through a block to transcriptional elongation has been demonstrated for c-myc and a number of other proto-oncogenes (reviewed in Spencer & Groudine, 1990). The sequence responsible for this premature termination of transcription was originally mapped to a 95 base pair region upstream of the exon 1 - intron 1 boundary, however, this sequence was found to have variable ability to block elongation of transcripts initiated by other promoters (Bentley & Groudine, 1988). Subsequently, sequences proximal to the P2 promoter have been shown to be necessary for the elongation block (Krumm et al., 1992).
Figure 1.5: Interactions of Myc and Max family proteins; indicating the transcriptional effect when each complex binds the E-box.
**mRNA Stability**

Post-translational control mechanisms play a major part in the regulation of c-*myc* expression. Both the c-Myc protein and its mRNA have extremely short half lives, of 20 to 30 minutes and 15 minutes respectively (Hann & Eisenman, 1984; Rabbitts et al., 1985). This rapid turnover accounts for the extremely low steady state levels of c-Myc in quiescent cells and allows for swift response to alterations in c-*myc* expression. The instability of c-*myc* mRNA is mediated by at least three different sequence elements, although the relative contribution of each of these to the mRNA half life has not yet been thoroughly assessed. AU rich sequences in the 3' UTR of c-*myc* mRNA have the ability to destabilise reporter genes (Jones & Cole, 1987) and a second element, located in the 3' region of the coding sequence, has been shown to function maximally only when the message is being actively translated (Wisdom & Lee, 1991). An instability determinant has also been identified in exon 2 of c-*myc*, which is independent of translation of the message (Pistoi et al., 1996).

**Translational Control**

The proposal that c-*myc* expression could be translationally controlled by its 5' UTR was first made in 1983, when characterisation of chromosomal translocations revealed different 5' UTRs with altered hypothetical secondary structures (Saito et al., 1983). Investigation of the ability of the c-*myc* 5' UTR to regulate both reporter constructs and the endogenous gene has yielded conflicting results.

*In vitro* analysis, using the rabbit reticulocyte lysate system, of two murine c-*myc* transcripts, one containing almost all of exon 1 (i.e. equivalent to the P1 transcript 5' UTR) and one containing only 46 nucleotides of exon 1, showed a 10-fold increase in translation of the construct without the 5' UTR (Darveau et al., 1985). Most of the translational repression by the murine 5' UTR was shown to reside in a 240 nucleotide sequence, which has the ability to act in cis to repress translation of a heterologous mRNA (Parkin et al., 1988). Likewise, this sequence was shown to repress translation of reporter genes in wheat-germ extracts and when injected into *Xenopus* oocytes. However, this repression was not observed when the construct was translated in HeLa cell extracts or when corresponding plasmid DNA constructs were transfected into COS cells, murine pre-B cells, U937 cells, 3T3 fibroblasts and P19 cells (Parkin et al., 1988). This disparity was hypothesised to be due to non-canonical factors, capable of relieving translational repression mediated by the 5' UTR, that are lacking from rabbit reticulocyte lysate and wheat germ extracts (Parkin et al., 1988).

A different approach to analysing translational repression supports the idea that c-*myc* expression *in vivo* is suppressed by a cap-dependent translational mechanism.
Overexpression of eIF4E in CHO cells causes increased expression of endogenous c-Myc and if Max is also overexpressed then cell transformation results (De Benedetti et al., 1994). More recently, it has been demonstrated that the 5' UTR of human c-myc contains an IRES that can stimulate translation of the downstream cistron in a dicistronic reporter construct 50-fold in HeLa and HepG2 cells (Stoneley et al., 1998) and which functions similarly in different dicistronic constructs in COS-7 cells (Nanbru et al., 1997). In reconciliation of these two observations it appears that at least some isoforms of the endogenous c-myc 5' UTR are capable of directing both cap-dependent and IRES mediated initiation of translation (Stoneley & Willis, unpublished observations).

1.3.3 Transcriptional Targets of c-myc

In order to determine how c-Myc functions to regulate the cell cycle, differentiation and apoptosis, identification of the genes transcriptionally activated by c-Myc is paramount. A number of criteria have been established that should be fulfilled in order for a gene to be considered a direct target of c-Myc (Henriksson & Lüscher, 1996). These are that expression of the target gene should correlate with the expression pattern of c-myc, i.e. its expression should be altered in concert with that of c-myc during the G0 - G1 transition, in differentiating cells and in tumours with overexpressed c-myc. The gene should also be expressed at a constant rate in cycling cells, because c-myc is expressed throughout the cell cycle. c-Myc regulated genes should be sensitive to mutations in c-myc which are known to reduce or eliminate its function. Finally, a promoter element should be present in the putative target gene which can bind Myc-Max complexes. This promoter should be activated in intact cells when c-Myc is supposedly regulating the gene and transcription should occur when protein synthesis is inhibited (Henriksson & Lüscher, 1996).

However, fulfilling these criteria may well be complicated by the fact that promoters are regulated by multiple elements and, as a result, expression of a gene can be altered by different transcription factors acting together or at separate times. Hence, even the most well defined targets of c-Myc activity, do not all display regulation by c-Myc under every circumstance and in each system examined (reviewed in Henriksson & Lüscher, 1996). The search for c-Myc target genes has been further hampered by the fact that the E-box, defined as the Myc-Max binding site, is also recognised by a number of other members of the BR-HLH-LZ class of transcription factors, including USF, TFE3 and TFEB (Blackwell et al., 1993). Additionally, Myc-Max can bind to non-canonical DNA sequences; indeed, an identification of genomic sites occupied by Myc-Max in vivo showed that the majority were non-canonical (Grandori et al., 1996).
A tool that has been widely used in the identification and/or authentication of c-Myc target genes is the Myc-ER fusion protein, which consists of the hormone-binding domain of the human oestrogen receptor fused to the carboxy terminus of the c-Myc protein (Eilers et al., 1989, 1991; Rosenwald et al., 1993b; Solomon et al., 1995; Galaktionov et al., 1996). This construct effectively provides inducible c-Myc activity when transfected into cells. On treatment of such cells with oestrogen or 4-hydroxy-tamoxifen, Myc-ER translocates to the nucleus, binds Max and activates transcription of c-Myc target genes (Eilers et al., 1989, 1991).

There is however, a caveat to the use of this system. In attempting to explain reports that c-Myc both activated and repressed the expression of cyclin D1 (Daksis et al., 1994; Philipp et al., 1994), Solomon et al. demonstrated that a cryptic transcriptional activation domain in the oestrogen receptor portion of Myc-ER, which is responsive to oestrogen but not 4-hydroxy-tamoxifen, can transactivate Cyclin D1 (Solomon et al., 1995). They concluded that this activity could have produced artifactual data leading to the suggestion that Cyclin D1 is a direct target of c-Myc.

A combination of experimental approaches has succeeded in characterising a number of candidate c-Myc target genes thoroughly enough that they are generally accepted as such (reviewed in Henriksson & Lüschter, 1996 and Grandori & Eisenman, 1997).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODC</td>
<td>Polyamine biosynthesis, necessary for S phase</td>
<td>Myc-ER, CHX, mediator of apoptosis, transforming</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Translation</td>
<td>Myc-ER</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Translation</td>
<td>Myc-ER, transforming</td>
</tr>
<tr>
<td>cdc25A</td>
<td>Activator of cyclin-dependent kinases</td>
<td>Myc-ER, CHX, mediator of apoptosis, transforming</td>
</tr>
<tr>
<td>CAD</td>
<td>Pyrimidine biosynthesis</td>
<td>Expression blocked by dominant negative c-myc mutants</td>
</tr>
<tr>
<td>p53</td>
<td>Tumour suppressor gene</td>
<td>Myc-ER, promoter activated by c-Myc</td>
</tr>
<tr>
<td>α-prothymosin</td>
<td>Chromatin remodelling?</td>
<td>Myc-ER, CHX</td>
</tr>
<tr>
<td>ECA-39</td>
<td>Role in growth regulation?</td>
<td>Elevated in tumours of c-myc transgenic mice</td>
</tr>
<tr>
<td>MrDb</td>
<td>Potential RNA helicase</td>
<td>Myc-ER, CHX</td>
</tr>
</tbody>
</table>

Table 1.2: Candidate c-Myc regulated genes. The criteria listed are the experimental evidence that the gene is a direct c-Myc target. CHX: transcription occurs in the presence of cycloheximide, Myc-ER: activated by Myc-oestrogen-receptor fusion protein on addition of oestrogen or 4-hydroxy-tamoxifen.
These include eIF2α and eIF4E (see section 1.2.4); ornithine decarboxylase; cdc25, a CDK-activating phosphatase; the tumour suppressor gene p53 and α-prothymosin (Eilers \textit{et al}, 1991; Bello-Fernandez \textit{et al}, 1993; Reisman \textit{et al}, 1993; Rosenwald \textit{et al}, 1993b; Galaktionov \textit{et al}, 1996; Jones \textit{et al}, 1996). These, and the other examples listed in Table 1.2, all have characterised Myc-Max binding sites and meet some, if not all, of the criteria outlined above (reviewed in Henriksson & Lüscher, 1996 and Grandori & Eisenman, 1997). Two of these genes, which exemplify the complexities of c-Myc regulated expression, are discussed in further detail below.

\textbf{Ornithine Decarboxylase}

ODC is one of the most thoroughly characterised c-Myc target genes to date. As described in sections 1.2.5 and 1.2.6, ODC is a rate limiting enzyme in the polyamine biosynthetic pathway and is capable of transforming cultured cells. Both of these functions are compatible with the effects of \textit{c-myc} expression, or overexpression. However, expression of ODC does not appear to be responsive to c-Myc in differentiating cells; HL60 cells treated with retinoic acid down-regulate \textit{c-myc} mRNA within 8 hours, but ODC message levels remain elevated until 48 hours after treatment (Rius & Aller, 1989). Indeed, a study in M1 myeloblastic cells has shown that constitutive expression of ODC does not prevent differentiation, nor does inhibition of ODC interfere with the block to differentiation imposed by constitutive expression of \textit{c-myc}; implying that ODC is not an effector of c-Myc function in differentiating cells (Selvakumaran \textit{et al}, 1996).

The ODC promoter contains two E-boxes that are conserved amongst mammals (Bello-Fernandez \textit{et al}, 1993). Expression of exogenous \textit{c-myc} has been shown to transactivate a murine ODC-CAT fusion construct and the two E-boxes confer c-Myc responsiveness to a heterologous promoter. Binding of Myc-Max to these sequences has been demonstrated \textit{in vitro} and the BR-HLH and transactivation domains shown to be essential for activation of the CAT reporter constructs (Bello-Fernandez \textit{et al}, 1993). It has also been shown that an E-box sequence in the endogenous human ODC promoter is occupied \textit{in vivo} at times which correlate with growth-associated ODC expression and c-Myc and Max have been identified as the proteins bound to this site. Furthermore, reduction of endogenous c-Myc by antisense oligodeoxynucleotide treatment reduces ODC expression in cultured human cells (Peña \textit{et al}, 1993).

The Myc-ER system has also been used to show that endogenous ODC can be activated in serum-starved cells, by addition of hormone. This transcription occurs in the presence of protein synthesis inhibitors indicating that ODC is a direct target for c-Myc (Wagner \textit{et al}, 1993). Most recently it has been demonstrated that
overexpression of Mxi1, the Max binding partner which prevents the formation of Myc-Max complexes, inhibits the transcription of endogenous ODC (Wu et al., 1996).

**α-prothymosin**

α-prothymosin was identified as a potential c-Myc target gene when it was cloned from subtractive hybridisation libraries made from cells expressing the Myc-ER construct (Eilers et al., 1991). α-prothymosin mRNA is induced within 2 hours of hormone treatment of Myc-ER cells, and this induction occurs in the presence of cycloheximide (Eilers et al., 1991). A consensus E-box element is present in the first intron of the α-prothymosin gene which is both necessary and sufficient for regulation by c-Myc (Gaubatz et al., 1994). For a long time, the function of α-prothymosin was unknown and as a result, how it may mediate the effects of c-Myc is not yet clear. However, recent evidence suggests that α-prothymosin may play a role in chromatin remodelling, which could be consistent with a function in cell proliferation (Díaz-Jullien et al., 1996).

α-prothymosin regulation by c-Myc is not, however, straight forward. Whilst α-prothymosin is induced when cells are stimulated to proliferate, the degree of responsiveness to c-Myc by reporter constructs bearing the α-prothymosin promoter varies in different cell lines (Eilers et al., 1991; Gaubatz et al., 1994). Additionally, α-prothymosin is not responsive to constitutive expression of c-myc or to activation of Myc-ER in growing cells (Eilers et al., 1991; Mol et al., 1995). In contrast, α-prothymosin is down-regulated in a manner which closely follows the reduction in c-myc expression in differentiating cells (Smith et al., 1993) and its expression has been found to correlate with that of c-myc in human colorectal cancer (Mori et al., 1993).

**1.4 Haemopoietic Differentiation**

Haemopoiesis is the process whereby pluripotent stem cells in the bone marrow proliferate and differentiate to form the whole range of cells found in the circulatory and immune systems. This process begins during embryonic development and continues throughout the life of an individual. Terminally differentiated blood cells lose the capacity to proliferate and their continued viability is dependent upon the presence of certain cytokines. A variety of haematological malignancies can arise, including various leukaemias and non-leukaemic lymphoproliferative or myeloproliferative disorders (reviewed in Sachs, 1996 and Potter, 1990).
1.4.1 Control of Haemopoiesis

The pathway of development of the various haemopoietic lineages is outlined in Figure 1.6. Briefly, a pluripotent self renewing stem cell divides to give rise to a new stem cell and a progenitor cell; this progenitor is another type of stem cell that is committed to either the lymphoid or myeloid differentiation pathways. The lymphoid pathway ultimately produces B and T lymphocytes. The myeloid pathway leads to the production of erythrocytes, macrophages, neutrophils, basophils, eosinophils and platelet producing megakaryocytes (reviewed in Pallister, 1994a).

The process of haemopoietic differentiation is initiated and controlled by a complex interacting network of cytokines. These cytokines include: i) factors that induce cell growth and initiate commitment to a particular differentiation pathway (colony stimulating factors - CSFs), ii) factors that promote differentiation and the associated growth arrest (interlukins - ILs) and iii) negative regulators of cell proliferation, such as tumour necrosis factor (TNF) and transforming growth factor β1 (TGF-β1). Many of these cytokines have overlapping functions and regulate the expression of other cytokines and their receptors (reviewed in Sachs, 1996).

Regulation of Gene Expression in Haemopoietic Differentiation

Whilst the terminally differentiated phenotypes of haemopoietic cells are well characterised, the events which lead to this state, through the successive restriction in the differentiation potential of a haemopoietic progenitor cell, in response to cytokines, have not yet been fully elucidated (reviewed in Sachs, 1996). However, multiple and sequential alterations in the pattern of gene expression are clearly involved in this process and in many cases crucial transcription factors have been identified (reviewed in Elefanty et al., 1997). These include transcriptional regulators which either play a general role in the differentiation process, e.g. myc family proteins (Larsson et al., 1994 and see section 1.3.1); are specific to haemopoietic differentiation, e.g. c-myb (Friedman, 1996); or are required for development along a particular pathway, such as GATA-1, which is required for erythroid differentiation (Sposi et al., 1992) and Oct-2, which is involved in B-cell differentiation (Corcoran et al., 1993).

Most studies of regulation of gene expression in differentiating haemopoietic cells have focused on transcriptional control. For example, study of the M1 myeloid leukaemic cell line has led to identification of a number of myeloid differentiation primary response (MyD) genes, which are induced within 30 minutes of treatment with IL-6, in the absence of de novo protein synthesis (reviewed in Liebermann & Hoffman, 1994). The MyD genes encode transcription factors (including known
Figure 1.6: Outline of the developmental stages of differentiating haematopoietic cells. Semi-circular arrows indicate that the stem cell is capable of self-renewal, dashed arrows indicate the existence of intermediate cell types which have been omitted. BFU: burst forming unit, CFU: colony forming unit, -E: erythroid, -GM: granulocyte macrophage, -MEGA: megakaryocyte, -EO: eosinophilic, -BAS: basophilic, NK-cell: natural killer cell.
Lymphoid Stem Cell

- Prothymocyte
  - Pre NK-Cell
  - Pro B-Lymphocyte
    - T-Lymphoblast
      - T-Lymphocyte (T-Cell)
      - NK-Cell
        - Mature B-Lymphocyte (B-Cell)
          - Plasma Cell
          - Memory B-Cell

- B-Lymphocyte
  - T-Lymphocyte (T-Cell)
  - Antigen Dependent
    - Erythrocyte

- NK-Cell
  - Antigen Dependent
    - Erythrocyte

Myeloid Stem Cell

- BFU-E
- CFU-GM
- CFU-MEGA
- CFU-EO
- CFU-BAS

- Monoblast
  - Monocyte
  - Granulocyte
    - Neutrophil
    - Eosinophil
    - Basophil

- Myeloblast
  - Promonocyte
  - Promyelocyte
  - Megakaryocyte
  - Macrophage (mature granulocyte)

- BFU-E
  - CFU-E
  - CFU-GM
  - CFU-MEGA
  - CFU-EO
  - CFU-BAS

- Erythrocyte
  - Antigen Dependent
    - Erythrocyte

- Macrophage
  - Neutrophil
  - Eosinophil
  - Basophil

- Megakaryocyte
  - Platelets
proto-oncogenes); histone variants; cell surface markers; proteins implicated in the regulation of apoptosis and a number of novel genes (Liebermann & Hoffman, 1994).

Little work has been done to investigate regulation of gene expression at the translational level in differentiating cells. However, given the increasing evidence that translational control is an integral part of the mechanisms regulating gene expression, a role for translational regulation in differentiating cells can be postulated. Indeed, the only studies carried out to date of the regulators of translation in differentiating cells provide some support for this hypothesis.

eIF4E regulation has been studied in murine P19 embryonal carcinoma cells. Differentiation of these cells initiated by treatment with retinoic acid (RA) results in a transient increase in protein synthesis, peaking at 150% of basal rates 30 minutes after RA treatment and returning to normal within 90 minutes. However, no alteration in the phosphorylation state of eIF4E occurs during this time (Kleijn et al., 1995), indicating that the transient stimulation of translation must be mediated by some other component of the translational machinery.

eIF4E has also been examined in rat pheochromocytoma (PC12) cells which, when treated with nerve growth factor (NGF), differentiate into sympathetic neurone-like cells. A rapid phosphorylation of eIF4E is observed in this system, detectable 3.5 minutes after NGF treatment and reaching a 6-fold induction by one hour (Frederickson et al., 1992). In contrast, treatment of the cells with epidermal growth factor (EGF) which has a weak mitogenic effect on these cells (and does not stimulate differentiation) causes no alteration in the phosphorylation state of eIF4E. Expression of a dominant negative ras mutant in PC12 cells abolishes eIF4E phosphorylation in response to NGF and also causes a marked inhibition of the morphological characteristics of differentiation (Frederickson et al., 1992). The increase in eIF4E phosphorylation correlates with a 50% increase in the rate of protein synthesis observed in NGF stimulated PC12 cells (Kleijn et al., 1995). It has also been shown that synthesis of subsets of the proteins expressed in PC12 cells are differentially modulated upon treatment with NGF (Greene & Tischler, 1976; Garrels & Schubert, 1979). These data led to the proposal that the increase in eIF4E phosphorylation regulates the expression of mRNAs with translationally repressive 5' UTRs during the differentiation of PC12 cells (Frederickson et al., 1992).

Further work on the regulation of eIF4E in PC12 cells has focused on 4E-BP1. Increased phosphorylation of 4E-BP1 is observed in PC12 cells treated with both NGF and EGF (Kleijn et al., 1996). The cellular response to EGF treatment peaked at 15 to 30 minutes and then returned to basal levels, whilst the response to NGF was still maximal 60 minutes after treatment (Kleijn et al., 1996). This indicates that
increased phosphorylation of 4E-BP1 may play a role in the translational effects observed in NGF treated PC12 cells. However, unlike the increase in eIF4E phosphorylation, this effect is also observed in response to mitogenic stimulation by EGF.

1.4.2 Cell Culture Models for the Study of Haemopoietic Differentiation and Neoplasia

Analysis of the molecular events mediating haemopoietic differentiation requires homogenous samples of cells at various stages of development along the pathways outlined in Figure 1.6. Although some populations of cells can be isolated via cell- and stage- specific surface markers, such samples are, on the whole, difficult to obtain, limited in their degree of homogeneity and restricted in the cell types available. The establishment of clonal cell lines which can be cultured consistently \textit{in vitro}, has therefore been an essential part of the study of haemopoiesis. The majority of these cell lines have been established from patients suffering from one of the many haematological malignancies that can develop in humans (reviewed in Miller & Koeffler, 1985).

\textit{Malignancies of the Haemopoietic System}

Haematological malignancies are classified in a number of ways, into acute and chronic leukaemias and non-leukaemic disorders which can be of either lymphoid or myeloid origin. Acute leukaemias are characterised by the proliferation of a poorly differentiated cell type, whilst in chronic leukaemias the predominant cell type shows some characteristics of maturity. Non-leukaemic lymphoproliferative malignancies include Multiple Myeloma and other plasma cell disorders, Hodgkin's lymphoma and non-Hodgkin's lymphomas. Non-leukaemic myeloproliferative malignancies include various types of polycythaemia, primary thrombocythaemia and myelofibrosis (reviewed in Pallister, 1994b).

\textit{Differentiation Inducible Cell Lines}

Leukaemias are characterised by uncontrolled clonal proliferation and accumulation of cells in the bone marrow. Both acute and chronic leukaemias are thought to originate from stem cells. In the case of acute leukaemia, the clonal progenitor is a pluripotent stem cell, producing poorly differentiated blast cells. Chronic leukaemia arises from either myeloid or lymphoid stem cells and the resultant cell types can be classified accordingly (reviewed in Pallister, 1994b). Cell lines established from leukaemic cells therefore represent clonal populations of a relatively un-differentiated cell type and many of these can be induced to differentiate into a recognisable mature cell type. Differentiation can be induced either by natural differentiation inducing cytokines, or by other compounds, which have often been
identified by their ability to stimulate differentiation of cell lines not responsive to cytokines (reviewed in Sachs, 1996).

A huge number of cell lines now exist which provide models of various pathways in haemopoiesis (reviewed in Miller & Koeffler, 1985). These include cells which have the potential to differentiate into only one cell type, e.g. murine erythroleukaemia (MEL) cells, which become erythroid cells; the human U937 cell line which differentiates into monocytes and human THP-1 cells which become well differentiated macrophage-like cells (Harris & Ralph, 1985; Auwerx, 1991). Other cell lines represent precursors at an earlier stage in the differentiation pathways and are capable of differentiating into more than one cell type, depending on the stimulus used. Examples of such cell lines include K562, which differentiates into erythroid, monocytic or granulocytic cell types and the HL60 cell line which can differentiate into either granulocytic or monocytic cells (Collins, 1987; Alitalo, 1990).

**The HL60 Cell Line**

The HL60 cell line was established from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia (Collins *et al.*, 1977). The cells display distinct morphological and histochemical myeloid characteristics and 88 to 90% are clearly identifiable as promyelocytes similar to those of the patient's uncultured leukaemic blood cells (Collins *et al.*, 1977). Approximately 5% of cells exhibit spontaneous differentiation into a more mature myeloid or granulocytic cell type under some culture conditions (Collins *et al.*, 1977).

A greater percentage of HL60 cells can be induced to differentiate with a variety of compounds. Dimethyl sulfoxide (DMSO) and other polar compounds induce the formation of morphologically mature myelocytes and banded and segmented neutrophils (Collins *et al.*, 1978). These cells display functional characteristics commonly associated with normal peripheral blood granulocytes, including response to chemoattractants, production of complement receptors and superoxide anion production (Collins *et al.*, 1978, 1979). Differentiation into this granulocytic cell type can also be induced by treatment with retinoic acid (Breitman *et al.*, 1980) and a number of other compounds (reviewed in Collins, 1987). Whilst the terminally differentiated cells acquire many morphological, functional, enzymatic and surface membrane antigen characteristics of neutrophils, the phenotype is not an exact match for that of normal mature granulocytes and suggests that the differentiation is to a certain extent incomplete (reviewed in Collins, 1987).

In contrast, phorbol esters, in particular 12-O-tetra-decanoylphorbol-13-acetate (TPA, otherwise known as phorbol 12-myristate 13-acetate (PMA)), induce differentiation of HL60 cells along a different pathway. Treatment of HL60 cells with TPA results in
loss of typical promyelocytic characteristics and development of a macrophage-like morphology (Rovera et al., 1979). A similar, but not identical, monocytic phenotype is developed by HL60 cells treated with 1,25 dihydroxy vitamin D₃ (Vit. D₃) and other naturally occurring compounds (McCarthy et al., 1983). The main differences between these two differentiated cell types are: i) that the macrophage-like cells (TPA treated) exhibit greater adherence than the monocytes (Vit. D₃ treated); ii) that there are some differences in the cell surface antigens expressed and iii) that the macrophage-like cells do not induce superoxide anion production (reviewed in Collins, 1987). Additionally, monocytic HL60 cells do not lose their proliferative capacity, in contrast to both macrophage-like HL60 cells and cells with the granulocytic phenotype (reviewed in Collins, 1987).

The HL60 cell line provides a useful model for studying the changes involved in two different lineages of haemopoietic differentiation and can provide relatively homogenous populations of four different cell types: the promyelocyte, the granulocyte, partially differentiated monocytes and the macrophage. As a result, the cell line has been widely used to study the morphological changes in haemopoietic differentiation and to investigate the control of, and alterations in, gene expression during this process (reviewed in Collins, 1987 and Harris & Ralph, 1985). A number of mutations in proto-oncogenes have been identified in HL60 cells that are implicated in the transformed phenotype of the cell line. These include an 8- to 30-fold amplification of the c-myc gene, which is highly expressed (Collins & Groudine, 1982; Dalla-Favera et al., 1982b); activation of the N-ras gene (Murray et al., 1983) and deletion of p53 (Wolf & Rotter, 1985).

Study of c-myc expression in HL60 cells has focused both on its role in leukaemogenesis and on its regulation during differentiation. As described in section 1.3.1, rapid down-regulation of c-myc expression is a feature of the differentiation process and this event is observed in HL60 cells, whichever agent is used to induce differentiation (Reitsma et al., 1983; Grosso & Pitot, 1984; Filmus & Buick, 1985; Mitchell et al., 1992). Likewise, reduction of c-Myc levels, by the use of an antisense oligodeoxynucleotide, inhibits proliferation and induces differentiation of the cells (Holt et al., 1988; Wickstrom et al., 1988). The fact that differentiation of HL60 cells, and other leukaemic cell lines, results in a loss of their capacity to proliferate, has given rise to the possibility that a therapy based on stimulating the differentiation of leukaemia cells could be developed (reviewed in Sachs, 1996).

1.5 Research Aims

This study has set out to address a number of questions relating to the role of the translation initiation factors in both the differentiation of haemopoietic cells and in

-37-
malignancies that arise in this system. Transcriptional regulation of eIF4E and eIF2α by c-myc has been investigated; to date this has only been described in response to growth stimulation, so the expression of these initiation factors has been examined in B-cell tumour derived lines which overexpress c-myc and in differentiating HL60 cells when c-myc expression is down-regulated. The translational environment in Multiple Myeloma cell lines, which have a documented translational up-regulation of c-myc, has also been examined, to determine whether any regulators of initiation of translation play a role in the c-myc overexpression. Finally, the HL60 cell line has been used to investigate alterations in translational control, in particular the regulation of eIF4F, during the differentiation process.
CHAPTER 2

MATERIALS AND METHODS

2.1 General Reagents

Unless otherwise stated, all chemical reagents were of analytical grade and were obtained from BDH laboratory supplies, Calbiochem, ICN Flow Ltd., Fisons or Sigma. Enzymes and other reagents for molecular biological techniques were purchased from Boehringer Mannheim UK Ltd, Gibco-BRL, MBI Fermentas, New England Biolabs, Pharmacia Biotech, Promega or Stratagene Ltd. SeaKem GTG agarose for general purposes was purchased from FMC Bioproducts and RNase free agarose for RNA analysis from Flowgen. Radio-labelled compounds were obtained from Du Pont NEN or Amersham Life Sciences.

2.2 Cell Culture Techniques

2.2.1 Cell Culture Media and Stock Solutions

RPMI 1640 medium: Rose Park Memorial Institute 1640 medium, with L-glutamine (Gibco-BRL) was supplemented with either 10 or 15% foetal calf serum (FCS) and 10 mg/ml gentamicin (Sigma). FCS was supplied by MB Meldrum Ltd., except in the case of HL60 cell culture where FCS purchased from Gibco-BRL was used.

PBS (Phosphate buffered saline): 150 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.2. PBS tablets (Oxoid) were dissolved in milli-Q water and the solution sterilised by autoclaving for 20 minutes at 15 psi, 120 °C.

DMSO: Tissue culture grade dimethyl sulphoxide (Sigma) was stored at room temperature in the absence of light.

Retinoic Acid: A stock solution of 1 mM retinoic acid (Sigma) in DMSO was stored at 4 °C.

TPA: A stock solution of 1 mg/ml 12-O-tetra-decanoylphorbol-13-acetate (Sigma) in DMSO was prepared and stored in aliquots at -20 °C.

Giemsa Stain: Giemsa solution (BDH laboratory supplies) was diluted 1:9 in sodium phosphate buffer pH 6.8.

Nitroblue Tetrazolium: A stock solution of 5% nitroblue tetrazolium (NBT) (Sigma) in 50% dimethylformamide was stored at 4 °C in the absence of light.

Rapamycin: A solution of rapamycin (Calbiochem) in DMSO at a concentration of 20 μM was stored in aliquots at -20 °C in the absence of light.

PD98059: PD98059 (Calbiochem) was dissolved in DMSO at a concentration of 50 mM and stored in aliquots at -20 °C in the absence of light.
### 2.2.2 Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Growth Medium</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM2132</td>
<td>Multiple Myeloma</td>
<td>RPMI 1640 (15% FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
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<td>Multiple Myeloma</td>
<td>RPMI 1640 (15% FCS)</td>
<td>NIGMS</td>
</tr>
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<td>Multiple Myeloma</td>
<td>RPMI 1640 (15% FCS)</td>
<td>NIGMS</td>
</tr>
<tr>
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<td>Multiple Myeloma</td>
<td>RPMI 1640 (15% FCS)</td>
<td>NIGMS</td>
</tr>
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<td>NIGMS</td>
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<td>ATCC</td>
</tr>
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<td>HL60</td>
<td>Promyelocytic Leukaemia</td>
<td>RPMI 1640 (10% FCS (Gibco-BRL))</td>
<td>Gift from Dr C. Bunce, Birmingham University.</td>
</tr>
</tbody>
</table>

Table 2.1: Cell lines cultured, giving details of their origin and *in vitro* growth requirements. Except for the HL60 cell line, cell lines were purchased from either the American type culture collection (ATCC) or the national institutes of general medical sciences human genetic mutant cell repository (NIGMS).

#### 2.2.3 Maintenance of Cell lines

Each of the cell lines listed in Table 2.1 was cultured in the indicated medium in sterile flasks (Nunclon, Gibco-BRL) at 37 °C in a humidified atmosphere containing 5% CO₂. Cell manipulations were carried out in a class II microbial safety cabinet. With the exception of the HL60 cell line, cultures were passaged twice a week. The Multiple Myeloma (MM) cell lines and LCLs were maintained at a density between 4x10⁵ and 1x10⁶ cells/ml. The Burkitt's Lymphoma (BL) cell lines were maintained at a density between 5x10⁵ and 2x10⁶ cells/ml.

#### 2.2.4 Culture of the HL60 Cell Line

The HL60 Cell line was cultured under the conditions described above, with two exceptions. The FCS used to supplement the RPMI 1640 medium was obtained from Gibco-BRL, rather than MB Meldrum Ltd., since the latter caused lysis of the HL60 cells. The HL60 cells were passaged three times a week in order to maintain a density between 2.5x10⁵ and 1.5x10⁶ cells/ml; the cell density was not allowed to exceed 1.5x10⁶ cells/ml.
2.2.5 Induction of Differentiation of HL60 Cells

Granulocytic differentiation of HL60 cells was induced by treatment with either 1.25% DMSO or 1 μM retinoic acid. Cells were counted, using a haemocytometer, then diluted to a density of 2.5x10⁵ cells/ml with pre-warmed RPMI 1640 (supplemented with 10% FCS). This point was designated t₀, the differentiation agent added and the cells returned to incubation. Cell counts were monitored on a daily basis. Control experiments were performed in parallel, in which HL60 cells were diluted as described but no differentiation agent added. Samples were taken at the indicated times by harvesting a determined number of cells which were subjected to centrifugation for 5 minutes at 1,500 rpm in a bench-top centrifuge, washed with PBS and the resultant cell pellets either used immediately or frozen at -80 °C for later use.

2.2.6 Monitoring of Morphological Changes in Differentiating HL60 Cells

To assess the morphological changes in differentiating HL60 cells, cytocentrifuge slide preparations of 300 μl of cells at 5x10⁴ cells/ml were made using a Shandon Cytospin 2. Centrifugation was for 8 minutes at 500 rpm using low acceleration. Slides were fixed in absolute methanol for 10 minutes at -20 °C, air dried and stained with giemsa stain for 1 to 2 hours at room temperature. The slides were rinsed three times in milli-Q water, allowed to dry and examined by light microscopy.

2.2.7 NBT Reduction Assay

Samples of 1x10⁶ HL60 cells were harvested at 0 and 96 hours, centrifuged for 5 minutes at 1,500 rpm in a bench-top centrifuge and resuspended in 0.5 ml of RPMI medium (supplemented with 10% FCS). To this was added 0.5 ml of 0.1% NBT diluted in PBS, containing 200 ng/ml of freshly diluted TPA. The cell suspension was incubated at 37 °C for 30 to 60 minutes and then used for cytocentrifuge slide preparations as detailed in section 2.2.6. The slides were fixed in absolute methanol for 10 minutes at -20 °C then allowed to air dry. The percentage of cells containing intracellular blue-black formazan deposits was determined under light microscopy by counting a minimum of 200 cells.

2.3 Protein Detection Methods

2.3.1. Stock Solutions and Buffers

**Extraction buffer:** 10% glycerol, 1% Triton X-100, 1 mM EDTA (diaminoethanetetra-acetic acid), 1 mM EGTA (ethylene glyco-bis(β-aminoethyl ether) N,N,N',N'-tetra-
acetic acid), 50 mM β-glycerophosphate, 1 mM DTT (dithiothreitol), 0.5 mM sodium orthovanadate, pH 7.5. Stored at -20 °C.

**Homogenisation Buffer**: 50 mM MOPS (3-[N-morpholino] propanesulphonic acid) - KOH pH 7.2, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM NaF, 50 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, 14 mM β-mercaptoethanol.

**RIPA Buffer**: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 5 mM EDTA. Stored at 4 °C.

**Yeast tRNA**: A stock solution of 10 mg/ml yeast tRNA (Sigma) was prepared in filter sterilised milli-Q water and stored in aliquots at -20 °C.

**SDS-PAGE Electrophoresis Buffer (1x)**: 50 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue, 1 mM EDTA. Stored at 4 °C.

**SDS-PAGE Running Buffer**: 25 mM Tris, 192 mM glycine, 0.1% SDS (approximate pH 8.3).

**SDS-PAGE Resolving Gel**: 375 mM Tris-HCl pH 8.8, 0.25% SDS, 7.5, 8, 10 or 15% acrylamide (30:0.8 acrylamide : bisacrylamide). Polymerisation was catalysed by addition of 0.06% TEMED (N,N,N′,N′-Tetramethyl-ethylenediamine) (Sigma) and 0.125% ammonium persulphate (APS) (Sigma).

**SDS-PAGE Stacking Gel**: 125 mM Tris-HCl pH 6.8, 0.1% SDS, 4% acrylamide (30:0.8 acrylamide : bisacrylamide). Polymerisation was catalysed by addition of 0.06% TEMED and 0.3% APS.

**7x IEF Sample Buffer**: 14% β-mercaptoethanol, 35% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate), 21% ampholytes pH 3.9-9.4 (Bio-Rad). Stored in aliquots at -20 °C.

**IEF Gel**: 6% acrylamide (17.8:1 acrylamide : bisacrylamide), 9 M urea, 7.5% ampholytes pH 3.9-9.4, 2% CHAPS. Polymerisation was catalysed by addition of 0.6% TEMED and 0.03% APS.

**Blotting Buffer**: 25 mM Tris, 160 mM glycine, 20% methanol (approximate pH 8.0).

**Ponceau Solution**: 1% ponceau S, 3% trichloroacetic acid, in de-ionised water.

**TBST (Tris Buffered Saline plus Tween)**: 10 mM Tris-HCl pH 8.0, 0.1% Tween-20 (polyoxyethylene(20)sorbitan monolaurate), 0.9% NaCl.

**Stripping Buffer**: 62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β-mercaptoethanol.

**Chemiluminescence Reagents**: The chemiluminesence reagents "Ilumin8" were a kind gift from Dr M. Murray, MRC Technology Transfer Centre, London.

2.3.2 Antibodies

**Primary Antibodies**: See Table 2.2. The eIF4G (N) and (C) antibodies were raised against a peptide corresponding to amino acids from the N- or C- terminal regions of eIF4G respectively. The 4E-BP1/BP2 antibody was raised against a peptide common
Table 2.2: Primary antibodies used for western blot analyses.

to both eIF4E binding proteins, whereas the 4E-BP2 antibody was raised against a peptide unique to 4E-BP2.

Secondary Antibodies: Horseradish-peroxidase-conjugated antibodies raised against either mouse or rabbit immunoglobulins (Sigma) were used at a dilution of 1:2,000.

2.3.3 Preparation of eIF4E Antibody

The following peptide corresponding to the indicated amino acids in the N-terminal region of eIF4E was synthesised by the Protein and Nucleic Acid Laboratory, Leicester University:

\[5\text{EPETTPTPNNPTTEEEKTSN}_{25}\]

The same facility conjugated the peptide to Keyhole Limpet Haemacyanin (KLH) and provided this at a concentration of 1.12 mg/ml in ammonium bicarbonate. An approximately 10 week old, female, New Zealand White rabbit was used for the production of the antiserum. The rabbit was immunised with 1 mg of conjugated peptide mixed with an equal volume of Freund's complete adjuvant. On days 28 and 42 further immunisations of 0.5 mg conjugated-peptide mixed with Freund's...
incomplete adjuvant were administered. Samples of blood were collected before the first immunisation, on day 35 and on day 49, the serum harvested and used to test for specific reactivity to eIF4E. After 49 days, significant specific eIF4E reactivity was detected, the rabbit exsanguinated and the serum harvested.

In order to remove cross-reactivity to non specific proteins, the eIF4E antiserum was affinity purified. The remaining un-conjugated peptide was resuspended at a concentration of 5 mg/ml in PBS and a portion of this conjugated to an equal volume of the agarose matrix Affi-Gel 15 (Bio-Rad). Conjugation was carried out according to manufacturer's instructions; the resultant 1 ml matrix was packed into a column and equilibrated with PBS. 4.5 ml of rabbit serum plus 0.5 ml of PBS was applied to the column, at a constant rate, over a 1 hour period. The column was then washed with 10 column volumes of PBS, at which point the flow-through had a protein content < 0.1 mg/ml. Bound immunoglobulin was eluted with 5 column volumes of 0.1 M glycine pH 2.5 plus 150 mM NaCl and the fractions immediately neutralised with 1M Tris. Further elution was carried out with 5 column volumes of 50 mM diethylamine and the fractions immediately neutralised with 1M HCl. Protein concentration in each fraction was determined using the Bradford assay (to be detailed in section 2.3.6) and the fractions with the greatest protein concentrations pooled. Aliquots of the purified antiserum were stored at -20 °C.

2.3.4 Preparation of Cell Extracts

Several methods were used for preparing solutions of protein from harvested cultured cells and details of these are given with individual methods, where relevant. The following two methods were commonly used to prepare cell extracts for western blot analysis and other analyses.

a) Lysates Made in Extraction Buffer: Freshly harvested cells, or cell pellets that had been stored at -80 °C, were resuspended in extraction buffer (see section 2.3.1) supplemented with 1% aprotinin, 1 μg/ml leupeptin and 1 μg/ml TLCK (N-α-p-Tosyl-l-lysine chloromethylketone) and, in some instances, 1 μM microcystin. The cell suspensions were kept on ice for 10 minutes to allow lysis to occur and then centrifuged for 10 minutes at 13,000 rpm in a micro-centrifuge at 4 °C, to pellet cell debris. The supernatant was removed and either mixed with an equal volume of 2x electrophoresis buffer, heated to 100 °C for 2 minutes and used for SDS-PAGE, or used for other analyses as detailed in following sections.

b) Lysates Made Directly in Electrophoresis Buffer: Freshly harvested cells, or cell pellets that had been stored at -80 °C, were resuspended in 1x electrophoresis buffer supplemented with 1% aprotinin, 1 μg/ml leupeptin and 1 μg/ml TLCK. DNA in
the lysate was sheared by passing the solution through a 27 gauge needle 5 to 10 times. These extracts were then heated to 100 °C for 2 minutes and used for SDS-PAGE.

2.3.5 Preparation of Mixed Cell Extracts

Mixed cell extracts, were prepared in the following way. Pre-determined numbers of each of the two cell types were harvested, resuspended in a fixed volume of PBS and variable proportions of the two suspensions mixed together. The cells were kept on ice and manipulations carried out at 4 °C. The cell suspensions were mixed such that the total number of cells in each preparation was equal but the ratio of one cell type to the other varied. The cells in PBS were then centrifuged for 20 seconds at 13,000 rpm in a micro-centrifuge at 4 °C and the supernatant removed. The cells were resuspended in extraction buffer plus protease inhibitors and the cell lysates then processed as described in section 2.3.4(a).

2.3.6 Determination of Protein Concentration (Bradford Assay)

To determine the concentration of protein in a solution, either neat sample or a dilution in PBS was assayed. To produce a standard curve, stock BSA (2 mg/ml) was diluted in PBS to concentrations between 0.1 and 1.5 mg/ml. Bradford reagent was added according to the manufacturer's instructions (Pierce and Warriner) and absorbance at 630 nm was monitored using a microtitre plate reader (Bio-tek instruments). Both the sample and the standards were assayed in triplicate and the averaged values used to determine the sample concentration from the standard curve.

2.3.7 m7GTP-sepharose Affinity Chromatography

Isolation of eIF4E and associated proteins was performed using an m7GTP affinity matrix. \(7 \times 10^6\) cells were resuspended in 500 µl of homogenisation buffer (see section 2.3.1) supplemented with 100 µM GTP (Sigma), 0.5% NP-40 (nonylphenoxy polyethoxy ethanol), 1% aprotinin, 1 µg/ml leupeptin and 1 µg/ml TLCK. To ensure complete lysis, the suspension was vortexed and left on ice for 10 minutes. Cell debris was removed by centrifugation for 5 minutes at 13,000 rpm in a micro-centrifuge at 4 °C. The supernatant was loaded onto a 250 µl column of 7-methyl-GTP sepharose 4B (Pharmacia Biotech), equilibrated with homogenisation buffer. The column was washed with 500 µl of buffer supplemented with 100 µM GTP and bound complexes were then eluted with 500 µl of buffer supplemented with 200 µM m7GTP (Sigma). The eluted protein was precipitated by the addition of 500 µl of 7% trichloroacetic acid (TCA) on ice. Precipitates were collected by centrifugation for 15 minutes at 13,000 rpm in a micro-centrifuge, washed 3 times with acetone and
allowed to dry. The pellets were dissolved in 1x electrophoresis buffer and used for SDS-PAGE.

2.3.8 Immunoprecipitation

$10^7$ cells were used for each immunoprecipitation reaction; cell pellets were resuspended in 1 ml of RIPA buffer supplemented with 1% aprotinin, 1 µg/ml leupeptin, 1 µg/ml TLCK and 10 mM VRC (vanadyl ribonucleoside complexes). Suspensions were left on ice for 10 minutes and DNA in the lysate was sheared by passing the solution through a 27 gauge needle 5 to 10 times. Cell debris was removed by centrifugation for 15 minutes at 13,000 rpm in a micro-centrifuge. All further manipulations were carried out at 4 °C or on ice.

To clear the lysate of non-specific binding proteins, 20 µl of normal rabbit serum (Sigma) and additional VRC was added and the mixture incubated on ice. After 1 hour, 40 µl of Protein A sepharose CL-4B (Sigma) was added and the suspension tumbled for a further hour. The Protein A sepharose complexes were collected by centrifugation for 20 seconds at 13,000 rpm in a micro-centrifuge and the supernatant harvested.

For the immunoprecipitation reaction, 15 µl of eIF4E antiserum (produced as described in section 2.3.3) was added to the pre-cleared lysate; for the negative control this was omitted. The lysate was also supplemented with 0.1 mg/ml yeast tRNA and further VRC. After an hour’s incubation on ice, 20 µl of Protein A sepharose, that had been pre-bound with yeast tRNA in RIPA buffer, was added and the suspension tumbled for 1 hour. The Protein A sepharose complexes were harvested by centrifugation for 20 seconds at 13,000 rpm in a micro-centrifuge, they were then washed with RIPA buffer three times and resuspended in either PBS (for RNA extraction, to be detailed in section 2.5.4) or electrophoresis buffer (for SDS-PAGE).

2.3.9 SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Briefly, 7.5, 8, 10 and 15% SDS-polyacrylamide gels were cast and run in a Bio-Rad Protean II apparatus. Samples were either prepared in 1x electrophoresis buffer, or added to an equal volume of 2x electrophoresis buffer prior to heating to 100 °C for 2 minutes and loading onto the gel. Gels were run in SDS running buffer for 6 to 20 hours at a current between 8 and 40 mA depending on the resolution required.
2.3.10 Isoelectric Focusing

Isoelectric Focusing (IEF) was performed using a method based on that of Jagus et al. (1993). Gels were cast and run in a Bio-Rad Protean II mini-gel apparatus; the running buffer at the cathode was 50 mM histidine and at the anode was 10 mM glutamic acid. Once the gel was cast, the wells were rinsed out with de-ionised water and loaded with 1x IEF sample buffer (see section 2.3.1), this was overlaid with 10 μl of 6 M urea followed by 10 mM glutamic acid. The gel was pre-focused for 20 minutes each at 200, 300 and 400 V, at reverse polarity. Samples were prepared from cell extracts made at a concentration of 10^6 cells per 20 μl in extraction buffer plus protease inhibitors and 1 μM microcystin (see section 2.3.4). To 20 μl of cell extract 0.022 g of urea and 5.7 μl of 7x IEF sample buffer were added, producing a 40 μl solution containing 9 M urea and 1x IEF sample buffer. The wells of the pre-focused gel were washed out with de-ionised water, 20 μl of each sample loaded (equivalent to approximately 5x10^5 cells) and overlaid with 6 M urea and 10 mM glutamic acid as above. The gel was focused at 500 to 750 V, at reverse polarity, increasing the voltage every 20 minutes in 50 V steps, followed by 20 minutes at 1000 V.

2.3.11 Electro-transfer of Proteins to Nitrocellulose

Proteins separated by SDS-PAGE or IEF were transferred onto a nitrocellulose membrane (Schleicher and Schuell) by electro-transfer in blotting buffer at 85 V. Mini-gels were transferred for 30 minutes, larger gels were transferred for 2 hours. When eIF4G was to be detected, the gel was transferred for 7 hours, during which time the blotting apparatus was stood on ice and the blotting buffer was discarded and replaced with fresh buffer after the first 3 to 4 hours. After transfer, protein on the membrane was temporarily visualised by staining with ponceau solution.

2.3.12 Immunodetection (Western Blotting)

Proteins immobilised on nitrocellulose were detected immunologically using the antibodies listed in section 2.3.2. Non-specific binding sites were first blocked by incubating the membrane in a solution of 5% dried, skimmed milk in TBST for 1 to 3 hours at room temperature. The membrane was then incubated in a minimum volume of 5% milk in TBST (typically 5 to 20 ml) containing antibody diluted to the appropriate concentration (see Table 2.2), for 1 to 2 hours, at room temperature, with constant agitation. The membrane was washed with two changes of TBST for 30 minutes and then incubated with the relevant horseradish peroxidase-conjugated secondary antibody, at a dilution of 1:2,000 in 5% milk in TBST, for 30 minutes, with constant agitation. The membrane was finally washed with three changes of TBST for 1 to 2 hours and immune complexes visualised using the "Ilumin8"
chemiluminescence reagents. Developed membranes were wrapped in saran wrap and exposed to X-ray film (Fuji) for 15 seconds to 30 minutes.

In order to further reduce non-specific background binding, this protocol was modified for use with the eIF4G antisera (both N- and C-terminal). 10% dried, skimmed milk in TBST was used for blocking and incubation with the antisera. The incubation with primary antibody was carried out at 4 °C for 14 to 20 hours, with constant agitation, and the following wash in TBST was extended to 3 changes over 1 to 2 hours. The remainder of the immunodetection was carried out as detailed above.

To enhance the reproducibility of data obtained from western blots, the primary antibodies used were each tested to determine the linear range of the signal detected from them. A serial dilution of cell extract was used for SDS-PAGE followed by western blot analysis using each of the antibodies listed in Table 2.2. From this, the amount of cell extract (in terms of cell numbers loaded) which resulted in a signal within the linear range was assessed and used for all subsequent determinations. Hence, detection with the 9E10 c-Myc antibody, the 4E-BP1/BP2 antibody and the 4E-BP2 antibody was performed using a loading of $10^6$ cells per lane, the eIF4E and eIF2α antibodies were used with a loading of $0.5 \times 10^6$ cells per lane and both the eIF4G antibodies were used with $1.5 \times 10^6$ cells loaded per lane.

Variations in the loading of SDS-PA gels used for western blot analyses were accounted for by probing blots with α-tubulin antibody. Numerical data calculated from western blots (as described in section 2.3.14) were adjusted relative to the α-tubulin levels. All experiments performed using this technique were carried out a minimum of twice.

2.3.13 Re-probing Western Blots

Existing protein-antibody interactions were removed from nitrocellulose membranes by incubation in stripping buffer at 50 °C for 10 minutes. The membrane was washed for 30 minutes in two changes of TBST and then probed with a different antibody as described in section 2.3.12.

2.3.14 Laser Densitometry

Proteins visualised by immunodetection were quantitated by laser densitometric scanning of the developed X-ray film. The laser densitometer was supplied by Molecular Dynamics and Image Quant software was used for numerical analysis of the scanned image.
2.4 Protein Analysis Techniques

2.4.1 Radioisotopes

**35S-Labelled Amino Acid Mix:** A mixture of 35S-labelled amino acids (including approximately 73% methionine and 22% cysteine) at a concentration of 11 μCi/μl was supplied by Du Pont NEN and stored at -80 °C in aliquots.

**14C-Labelled L-Ornithine Hydrochloride:** A solution of L-[1-14C] ornithine hydrochloride, at a concentration of 50 μCi/ml, was supplied by Amersham Life Sciences and stored at 4 °C.

2.4.2 Assay of Protein Synthesis Rate Over 10 Hours

The protein synthesis rate in HL60 cells was measured over a 10 hour period. Differentiating and control cell cultures were prepared as described in section 2.2.5, except that when the cells were diluted with fresh media, 5 μCi/ml of 35S-labelled amino acid mix was added. The cells were then returned to incubation for 30 minutes before the to samples were taken and the differentiation agent added. Duplicate samples of 5x10⁵ cells were taken at 0, 0.5, 1, 2, 4, 6 and 10 hours, filtered onto glass fibre filters, washed with PBS and treated with ice cold, 5% TCA solution. The filters were then washed three times with methanol, allowed to dry, placed in scintillation fluid (Emulsifier-Safe; Packard) and subjected to scintillation counting in a liquid scintillation analyser (TRI-CARB 2000CA; Packard).

2.4.3 Assay of Protein Synthesis Rates During 96 Hours

Protein synthesis rates in HL60 cultures were measured over 1 hour intervals throughout a 96 hour period. Cells for both control and differentiation experiments were prepared as described in section 2.2.5, removing to samples before addition of the differentiation agent. The duplicate to samples and further duplicate samples of 10⁶ cells (plus differentiation inducer) for 2, 4 and 10 hour time points were removed to a 6-well culture plate. Each sample was treated with 5 mCi/ml 35S-labelled amino acid mix 30 minutes before the designated time point and harvested 1 hour later. At 24, 48, 72 and 96 hours, the cells were counted, duplicate 10⁶ cell samples removed and labelled in the same way. All the labelled cell samples were processed as described in section 2.4.2 and similarly subjected to scintillation counting.

2.4.4 Assay of Ornithine Decarboxylase Enzyme Activity

Ornithine decarboxylase (ODC) enzyme activity was determined using an assay based on that of Seely & Pegg (1983). 30x10⁶ cells were lysed in sodium phosphate buffer (100 mM, pH 7.2) supplemented with 5 mM DTT, by 4 rounds of freeze-thawing followed by passage through a 27 gauge needle. Cell debris was removed.
by centrifugation for 30 minutes at 13,000 rpm in a micro-centrifuge at 4 °C. Protein concentration of each extract was determined using the Bradford assay (see section 2.3.6) and extracts were either used immediately or stored for not more than 24 hours at -80 °C before use. Reactions containing 600 to 800 µg of extract, 0.1 mM pyridoxal phosphate, 0.2 mM L-ornithine hydrochloride and 0.5 µCi (0.02 mM) $^{14}$C-L-ornithine hydrochloride were assembled in a vial. Within the vial was suspended an eppendorf containing a glass fibre filter soaked in 100 mM NaOH. The sealed vials were incubated at 37 °C, with agitation, for precisely 1 hour. The reaction was then stopped by the addition of 1 M perchloric acid and the vials incubated for a further hour at 37 °C. The filters were then removed, placed in scintillation fluid and counted. All samples were assayed in duplicate and activity was calculated as pmol CO$_2$ produced per hour per mg of protein.

2.5 Nucleic Acid Techniques

2.5.1 Stock Solutions and Buffers

All reagents for use with RNA were purchased RNase free and all solutions were passed through a 0.2 µm filter to remove potential RNase contamination. The water used in all procedures was milli-Q water which had been filtered in the same way. 
Tri Reagent: A phenol guanidine thiocyanate mono-phase solution (Sigma) used for isolation of RNA.

Phenol: Phenol for use with RNA was saturated with 0.1 M citrate buffer pH 4.3 (Sigma).

Northern 10x Gel Buffer: 200 mM MOPS-KOH pH 7.0, 50 mM sodium acetate, 10 mM EDTA.

Northern Gel Loading Buffer: 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol.

Formaldehyde Agarose Gel: 0.73% agarose, 1x northern gel buffer, 2.2 M formaldehyde.

20x SSC (Salt Sodium Citrate): 3 M NaCl, 0.3 M tri-sodium citrate.

Church-Gilbert Buffer: 250 mM sodium phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA pH 8.0.

Church I Solution: 20 mM sodium phosphate buffer pH 7.2, 5% SDS.

Church II Solution: 20 mM sodium phosphate buffer pH 7.2, 1% SDS.

$^{32}$P-Labelled α-dCTP: $^{32}$P-labelled α-dCTP at a concentration of 10 mCi/ml was supplied by Du Pont NEN and stored at -20 °C.

$^{35}$S-Labelled α-dATP: $^{35}$S-labelled α-dATP at a concentration of 12.5 mCi/ml was supplied by Du Pont NEN and stored at -20 °C.

Stock dNTP solution: 10 mM dATP, 10 mM dCTP, 10 mM dTTP, 10 mM dGTP. Stored at -20 °C.
Dynabeads Binding Buffer: 100 mM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% LiDS (Lithium dodecyl sulphate), 5 mM DTT.

Dynabeads Washing Buffer: 10 mM Tris-HCl pH 8.0, 150 mM LiCl, 1 mM EDTA.

TAE (Tris Acetate EDTA): 40 mM Tris-acetate, 1 mM EDTA.

TBE (Tris Borate EDTA): 89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA.

OLB: In a ratio of 2:5:3, [1.25 M Tris-HCl pH 8.0, 0.125 M MgCl₂, 1.8% 0-mercaptoethanol, 0.5 M each of dATP, dTTP and dGTP] : [2 M HEPES-NaOH pH 6.6] : [0.9 μM random hexadeoxyribonucleotides in 3 mM Tris-HCl pH 7.0 and 0.2 mM EDTA]. Stored at -20 °C in aliquots.

RT (Reverse Transcription) Buffer: 100 mM Tris-HCl pH 8.3, 500 mM KCl, 50 mM MgCl₂.

2.5.2 Oligonucleotide primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'→3')</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc (exon 2)</td>
<td>GSF: AGAAACTTCTACCAGCAGCAGCA</td>
<td>221 bp</td>
</tr>
<tr>
<td></td>
<td>GSR: TCCATCTCCAGCTGTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CF: AGCAGCAGCAGCATGTCTTT</td>
<td>443 bp</td>
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<tr>
<td></td>
<td>CR: TCCAGCTGGTGCCGCAGCCAC</td>
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</tr>
<tr>
<td>GAPDH</td>
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<tr>
<td></td>
<td>CF: CAGCGTCCACCATGTCTTT</td>
<td>443 bp</td>
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<tr>
<td></td>
<td>CR: GACAACCTGGGCGCGCCACC</td>
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</tr>
</tbody>
</table>

Table 2.3: Oligonucleotide primers used for PCR reactions. The product size for each primer pair is given. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GSF: gene specific forward primer; GSR: gene specific reverse primer; CF: composite forward primer; CR: composite reverse primer.

2.5.3 Preparation of Total Cellular RNA

Total cellular RNA was prepared from cultured cells using Tri reagent, according to the manufacturer's instructions (Sigma). Up to $10^7$ cells were harvested, washed with PBS, then lysed in Tri reagent at a concentration of $10^6$ cells per 150 μl and vortexed for 30 seconds. 200 μl of chloroform per 1 ml of Tri reagent was added to the solution, the solution then mixed and allowed to stand for 5 minutes at room temperature. The mixture was then centrifuged for 15 minutes at 13,000 rpm in a micro-centrifuge at 4 °C. The upper, aqueous phase was transferred to a fresh tube and 500 μl of isopropanol added per 1 ml of Tri reagent used. This was mixed, left on ice for 15 to 30 minutes and then centrifuged for 10 minutes at 13,000 rpm in a micro-centrifuge at 4 °C. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol. The resultant RNA pellet was allowed to dry and then resuspended in 10 to 30 μl of filtered water.
2.5.4 Preparation of Poly(A)+ RNA

Poly(A)+ RNA was prepared directly from cultured cells using Dynabeads oligo (dT)_{25} according to the manufacturer’s instructions. Briefly, \(10^7\) cells were resuspended in 2.5 ml of binding buffer and passed through a 27 gauge needle 10 times. The lysate was added to 200 \(\mu\)l of oligo (dT)_{25} beads and tumbled for 5 minutes at room temperature. The supernatant was removed from the beads, which were then washed twice with 1 ml washing buffer supplemented with 0.1% LiDS followed by a final wash with 1 ml un-supplemented washing buffer. RNA was eluted from the beads into 10 \(\mu\)l 2 mM EDTA pH 8.0 at 65 °C for 2 minutes.

2.5.5 Preparation of RNA from Immunoprecipitates

To purify RNA isolated by immunoprecipitation of eIF4E (see section 2.3.8), the Protein A sepharose complexes were resuspended in PBS and an equal volume of Phenol added. The mixture was vortexed and then centrifuged for 15 minutes at 13,000 rpm in a micro-centrifuge at 4 °C. The upper, aqueous phase was removed to a new tube and an equal volume of chloroform added. This mixture was also vortexed and centrifuged as above. The upper, aqueous phase was removed to a new tube and 0.1 volumes 3 M NaAc pH 5.2 and 3 volumes of ethanol added. After 15 to 30 minutes on ice, the precipitate was collected by centrifugation for 10 minutes at 13,000 rpm in a micro-centrifuge and the pellet was washed with 75% ethanol. The resultant pellet was dried and then resuspended in 10 \(\mu\)l of filtered water.

2.5.6 Formamide Agarose Gel Electrophoresis of RNA

Samples of 5 to 20 \(\mu\)g total cellular RNA or 1 to 2 \(\mu\)g poly(A)+ RNA were treated with 2.4 M formaldehyde and 11.0 M de-ionised formamide in 1x gel buffer for 15 minutes at 55 °C. Loading buffer was then added to each sample and they were loaded onto a 0.73% horizontal slab formaldehyde agarose gel (see section 2.5.1). Electrophoresis was carried out in 1x gel buffer at a constant voltage of 100 V. Molecular weight markers were treated in the same way and loaded alongside the samples; after electrophoresis the marker track was cut from the gel, stained in 10 \(\mu\)g/ml ethidium bromide solution and the markers visualised and photographed on a UV transilluminator.

2.5.7 Capillary Transfer of RNA to Nylon Membrane

After electrophoresis, the formaldehyde agarose gel was washed in two changes of water for 20 minutes to remove the formaldehyde. The gel was then soaked in 20x SSC for 45 minutes. Capillary transfer of RNA from the gel to a positively charged
nylon membrane (Zeta-Probe GT; Bio-Rad) was performed with a gradient of 20x SSC to 2x SSC for approximately 15 hours. After transfer the membrane was baked at 80 °C for 1 to 2 hours to permanently affix the RNA.

2.5.8 Preparation of Radio-labelled DNA Probes

Probes for the detection of immobilised mRNA were made from DNA isolated from the following sources.

c-myc: a 1.1 kb \(Pvu\)II-NsI fragment of human cDNA; eIF4E: a 465 bp fragment of human cDNA amplified by PCR and cloned into pSK+-bluescript; eIF2α: a 1.1 kb \(EcoRI\)-\(EcoRI\) fragment of the plasmid pUKC552 (Green et al., 1991); eIF4G: a 1.4 kb \(EcoRI\)-BamHI fragment from the plasmid pSK-HFCl (Yan et al., 1992); ODC: a 1.3 kb \(EcoRI\)-NcoI fragment from pGEMODC (a gift from Prof. M. Clemens, St George's Hospital Medical School, London); GAPDH: a 1.25 kb \(EcoRI\)-\(EcoRI\) fragment of chicken cDNA (Dugaiczyk et al., 1983).

DNA fragments were produced by digesting approximately 1 \(\mu g\) of plasmid DNA with the indicated restriction enzymes under the conditions recommended by the manufacturer. The digested DNA was then subjected to horizontal slab agarose gel electrophoresis in TAE buffer, in the presence of ethidium bromide. The DNA was visualised on a UV transilluminator and the required fragment excised from the agarose gel. DNA was purified from agarose gel slices using Nal and glassmilk according to the manufacturer’s instructions (Geneclean II kit; BIO 101, supplied by Anachem). Concentration of the purified DNA was determined spectrophotometrically by reading absorbance at 260 nm and a stock solution of 10 ng/\(\mu l\) prepared and stored at -20 °C.

Radio-labelled probes were prepared by incubating 30 ng of heat denatured DNA with 2.5 units of Klenow fragment of \(E.coli\) DNA polymerase I, 0.3 mg/mL BSA and 25 \(\mu Ci\) \(^{32}P\) \(\alpha\)-dCTP in a 1 in 5 dilution of OLB for 1 hour at 37 °C. Unincorporated radionucleotide was removed by centrifugation through a 1 ml Sephadex G-50 column.

2.5.9 Detection of RNA with Radio-labelled DNA probes (Northern Blotting)

Northern blot nylon membranes were pre-hybridised for 1 hour in a sealed chamber at 65 °C in 10 ml of Church-Gilbert buffer supplemented with 200 \(\mu g/ml\) heat denatured salmon sperm DNA and 50 \(\mu g/ml\) yeast tRNA. The required \(^{32}P\)-labelled DNA probes were prepared on the day of use and heated to 100 °C for 2 minutes before addition to the pre-hybridisation solution. Hybridisation was carried out at 65 °C for a minimum of 14 hours. The membrane was then removed from the hybridisation solution and washed for two 30 minute periods in an excess of Church I solution at 65 °C. Further washing in Church II solution was performed at 65 °C.
until < 10 cpm of activity was detectable on the membrane. The membrane was then sealed in saran wrap and exposed to a phosphorimager plate (Molecular Dynamics) for 5 to 96 hours.

2.5.10 Reverse Transcription

Reverse transcription of RNA was performed with 1 μg of total cellular RNA or the total products of an immunoprecipitation reaction (see sections 2.5.5 and 2.3.8). The RNA was heated to 95 °C for 5 minutes and then cooled on ice, to remove inhibitory secondary structure. The RNA was then incubated with 0.5 mM dNTPs, 100 pmole of random hexadeoxyribonucleotides, 20 units of Rnasin and 100 units of MMLV (Moloney-murine leukaemia virus) reverse transcriptase in 1x RT buffer at 37 °C for 1 hour. In the case of the RNA isolated by immunoprecipitation, potential DNA contamination was first removed by addition of 5 units of RNase free DNase to the reaction. The reaction was incubated at 37 °C for 30 minutes and the enzyme then heat inactivated at 95 °C for 5 minutes. After this, reverse transcriptase was added to the reaction which was treated as described above.

2.5.11 Polymerase Chain Reaction

Amplification of fragments of \textit{c-myc} exon 2 and of GAPDH, from cDNA, were performed using the GS primers specified in Table 2.3. PCR reactions contained 6 μl of the 20 μl RT reaction, 1x PCR buffer (Gibco-BRL), 3.0 mM MgCl$_2$ (GAPDH) or 1.5 mM MgCl$_2$ (\textit{c-myc}), 1 unit of Taq polymerase (Gibco-BRL), 0.1 mM dNTPs and 20 mM of each oligonucleotide primer; in a 100 μl reaction. The mixture was overlaid with paraffin oil to minimise evaporation. Reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler at 94°C for 10 minutes followed by 35 cycles of [94 °C for 1 minute, 63 °C (GAPDH) or 55 °C (\textit{c-myc}) for 3 minutes, 72 °C for 1 minute] followed by 72 °C for 10 minutes. After the reaction was complete a portion of the reaction was analysed on a 1.5% TBE agarose gel in the presence of ethidium bromide, visualised and photographed on a UV transilluminator.

2.5.12 Preparation of Competitor DNA Fragments

DNA for use in the competitive PCR reactions was prepared from a 415 bp fragment of pSK+-bluescript produced by restriction enzyme digestion with \textit{A}flIII and \textit{N}otI, as described in section 2.5.8. This fragment had 5' overhangs of 4 nucleotides at each end which were filled in by treatment with 15 units of T4 DNA polymerase in the presence of 200 mM dNTPs, 1 μg/ml BSA and the manufacturer’s buffer (MBI Fermentas) at 37 °C for 15 minutes. The enzyme was then heat-inactivated at 75 °C for 10 minutes. Sequences complementary to the GS primers for \textit{c-myc} or GAPDH were added to the ends of the 423 bp fragment by two rounds of PCR, as outlined in
Figure 2.1: Scheme outlining preparation of competitor DNA fragments. Grey bars represent sequences corresponding to pSK⁺ bluescript DNA, black bars represent sequences corresponding to the gene specific primers.
Figure 2.1. The first round of PCR was carried out using the composite primers (see Table 2.3) which consist of 10 bp complementary to the 423 bp fragment plus 10 bp of the GS primers. The PCR was performed as specified in section 2.5.11 using 1 ng of fragment as template, 1.5 mM MgCl$_2$ and 30 cycles of [94 °C for 30 seconds, 30 °C for 30 seconds, 72 °C for 1 minute]. The 443 bp product of this reaction was isolated by electrophoresis through a 1.5% TAE agarose gel and then purified from a gel slice as detailed in section 2.5.8. The second round of PCR was performed using 0.02% of the first round product as template, with the GS primers, under the conditions outlined above, then isolated and purified in the same way.

2.5.13 Competitive PCR

Competitive PCR was carried out using $^{35}$S-labelled α-dATP and a stock dNTP mix consisting of 1.25 mM dATP, 10 mM dCTP, 10 mM dTTP and 10 mM dGTP; otherwise the reaction conditions were as outlined in section 2.5.11. The template used was a fixed amount of cDNA (2 µl of a 20 µl RT reaction) and 10-fold serial dilutions of competitor DNA, ranging from 2.5 pM to 25 aM. When the PCR reactions were complete, the products were subjected to electrophoresis through a 1.5% TBE agarose gel, the gel dried under vacuum at 60 °C for 2 hours followed by 80 °C for 1 hour and then exposed to a phosphorimager plate.
CHAPTER 3

ANALYSIS OF THE REGULATORS OF TRANSLATIONAL INITIATION IN A PANEL OF B-CELL LINES

3.1 Introduction

It is now evident that de-regulation of the factors which control translational initiation has the potential to play an important part in the process of tumourigenesis. Indeed, de-regulation of individual initiation factors has been shown to be sufficient to cause transformation of cultured cells. Studies have revealed aberrant expression or phosphorylation of initiation factors in various tumours and transformed cell types. Links have also been demonstrated between recognised oncogenes and altered expression or regulation of initiation factors. There is growing evidence that the effects of such translational de-regulation have the potential to contribute to human neoplasia. Consequently, alterations in one or more of these initiation factors might be expected in cells of tumour origin.

To explore this hypothesis, the factors which contribute to the control of eIF4F activity have been studied in human B-lymphoid malignancies. Two types of cell line, derived from different B-cell tumours, have been employed for this work. Four of these lines, GM2132, GM1311, GM06923 and GM1500C, were derived from patients with Multiple Myeloma (MM). Two lines, Raji and BL29, were derived from Burkitt’s lymphoma (BL) patients; Raji from a patient with endemic BL and BL29 from a sporadic BL patient. As a control, three lymphoblastoid cell lines (LCLs), GM1953, GM03201 and GM0892A, established from normal healthy donors, were also used.

This panel of B-cell lines also offers the opportunity to investigate the transcriptional regulation of eIF4E and eIF2α by c-Myc. Evidence for such regulation includes a correlation between eIF4E, eIF2α and c-myc expression, observed in growth stimulated NIH 3T3 cells, c-myc transformed rat embryo fibroblasts and in response to induction of the Myc-ER construct with oestrogen (Rosenwald et al., 1993b). Characterisation of the promoters of eIF4E and eIF2α has also revealed potential c-Myc regulated elements (Rosenwald et al., 1993b; Jones et al., 1996) and in the case of eIF4E, this sequence has been shown to be essential to transcription of the gene and to bind Myc-Max complexes at times when increased c-myc expression correlates with increased eIF4E expression (Jones et al. 1996).
The MM cell lines overexpress c-myc through a translational up-regulation (Paulin et al., 1996), particularly GM2132, which has been shown by FACS analysis to consist of a cell type that is most representative of those found in MM (Paulin & Willis, unpublished observations). Raji and BL29 are typical BL cell lines, displaying a transcriptionally mediated overexpression of c-myc through a t(8:14) translocation (Lenoir et al., 1982). In the case of the Raji cell line the entire 5' UTR of c-myc is left intact by this translocation; in BL29 cells the 5' UTR is lost (Pelicci et al., 1986). In contrast, the LCLs, which were established by Epstein Barr Virus transformation, have low c-myc expression, although it is comparable to that in some of the MM cell lines.

Levels of c-myc mRNA, and eIF4E and eIF2α mRNA and protein have been measured in all these cell lines. For comparison, an additional c-myc target gene, ODC, has also been examined in a selection of the lines. The activity of eIF4F in the cell lines has been studied by examining a number of factors which have the potential to control this complex, including the phosphorylation state of eIF4E, the association of eIF4G with eIF4E and expression of both eIF4G and the eIF4E binding proteins.

3.2 Results

3.2.1 Expression of c-myc Target Genes

The protein levels of c-Myc, eIF4E and eIF2α in the nine B-cell lines were determined by western blot analysis, as detailed in section 2.3. Developed blots were exposed to X-ray film for a period of 15 seconds to 30 minutes and the data quantitated by laser densitometric scanning of the film. Expression of c-myc, eIF4E and eIF2α was calculated relative to the loading control, α-tubulin.

As expected, elevated expression of c-myc was observed in the cell lines GM2132, Raji and BL29 (Figure 3.1). However, the protein levels of eIF4E and eIF2α do not display any clear correlation with those of c-myc. Levels of eIF4E and eIF2α measured in GM2132 and Raji are comparable to those in the cell lines with lower c-myc expression (Figure 3.1). The variations in eIF4E and eIF2α expression observed in the LCLs do not correlate with the differences in c-myc levels in these cells. Elevated expression of eIF4E and eIF2α is observed in BL29 cells; compared to the LCLs they have approximately 2- to 4-fold more eIF4E and 5- to 12-fold greater eIF2α levels (Figure 3.1B).
Figure 3.1: Protein levels of c-myc, eIF2α and eIF4E in the panel of B-cell lines. A: Two gels were run in parallel; 7.5% polyacrylamide loaded with 10⁶ cells per lane which after transfer to nitrocellulose was probed for c-myc and 10% polyacrylamide loaded with 0.5x10⁶ cells per lane which, after transfer, was probed for eIF2α, then stripped and re-probed for eIF4E and α-tubulin. B: Quantitation of the data using laser densitometric scanning, normalised to the loading control, α-tubulin.
Since regulation by c-myc occurs at the level of transcription, a northern blot analysis of eIF4E and eIF2α mRNA in these cell lines was performed to discount the possible effects of post-transcriptional alterations of their expression. Northern blots of total cellular mRNA were probed with labelled DNA fragments specific for eIF4E, eIF2α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as detailed in section 2.5. Visualisation and quantitation of the data were performed with a phosphorimager and mRNA expression, relative to the loading control GAPDH, was calculated.

In agreement with the western blot analysis, elevated expression of eIF4E and eIF2α mRNA were observed only in BL29 cells, an increase of 3- to 4-fold and 4- to 5-fold respectively (Figure 3.2B). GM2132 and Raji cells do not express significantly different levels of eIF4E or eIF2α than any of the lines with lower c-myc expression (Figure 3.2). Interestingly, these data also indicate that in BL29 cells there is some dissociation between the mRNA and protein levels of eIF2α (Figures 3.1 and 3.2). Little is known about post-translational control of eIF2α expression; there is some evidence that eIF2α mRNA is more efficiently translated than many other cellular mRNAs (Ernst et al., 1987; Chiorini et al., 1993), which could be consistent with this observation.

Overall, whilst the eIF4E and eIF2α genes may be transcriptional targets of c-myc in growth stimulated cells, these results show that their expression does not correlate with that of c-myc in these transformed B-cell lines. This indicates that, with the exception of the BL29 cell line, these initiation factors do not contribute to the malignant status of the MM and BL cell lines.

In the light of these data, the activity and mRNA levels of ODC were determined in a selection of the B-cell lines, to examine whether a well characterised transcriptional target of c-myc is regulated in these cell types. Samples of the HT29 cell line were used as a control. This line is derived from a human colon carcinoma and has high levels of ODC activity (Garnet et al., 1991). ODC enzyme activity in cell extracts was assayed by monitoring production of 14CO₂, from reactions containing 14C-L-ornithine hydrochloride. The activity of this enzyme was at the lower limits of detection in all the B-cell lines assayed (Figure 3.3A). There has been a previous report that ODC activity in resting murine B lymphocytes is likewise at the lower limits of detection; even when these cells were stimulated with lipopolysaccharides and ODC activity increased several-fold, activity was still in the lower range of detection (Molitor et al., 1994). However, since ODC mRNA is readily detectable in the cells assayed by Molitor et al., expression of the ODC transcript was determined in the B-cell lines which had been assayed for ODC activity.
Figure 3.2: mRNA expression of eIF4E and eIF2α in the panel of B-cell lines. A: Northern blot of 10μg total cellular RNA per lane, probed for eIF4E (transcript size 1.9 Kb), allowed to decay and then probed for eIF2α (transcript size 1.6 Kb, a small amount of the 4.2 Kb transcript was also detected in each cell line (data not shown)), then stripped and re-probed for GAPDH (transcript size 1.6 Kb). B: Quantitation of mRNA expression, normalised to GAPDH, from phosphorimager data.
A northern blot of total cellular RNA, probed for ODC and GAPDH, demonstrated low expression of ODC mRNA in the MM cell line GM1311, the LCLs GM1953 and GM03201 and the BL cell line, Raji, in comparison to the HT29 cells (Figure 3.3B and C). In contrast, significant amounts of ODC mRNA were observed in GM2132 cells, suggesting that c-myc may indeed be increasing expression of this gene in this MM cell line. However, the lack of ODC activity in GM2132 cell extracts points to post-transcriptional repression of ODC in these cells, a common phenomenon which is often mediated translationally (as discussed in section 1.2.5).

3.2.2 Regulation of eIF4F

To further characterise the translational environment of the B-cell tumour lines, three factors which reflect the activity of the eIF4F complex were examined; the phosphorylation state of eIF4E, association of eIF4G with eIF4E and expression of the eIF4E binding proteins, 4E-BP1 and 4E-BP2.

The phosphorylation state of eIF4E was measured by subjecting cell extracts to isoelectric focusing (IEF), over a pH range of 3.9 to 9.4. Focused eIF4E was detected using a western blot procedure as described in sections 2.3.11 and 2.3.12 and the percentage phosphorylation of eIF4E in each sample was calculated (Figure 3.4). The data averaged from three separate experiments show that eIF4E phosphorylation values lie between 40 and 60% in all the cell lines examined, except GM2132 and BL29. The variation in percentage phosphorylation of eIF4E measured in GM1311, GM06923, GM1500C, GM1953, GM03201, GM0892A and Raji cell lines does not correlate with the tumorigenic status of these cell types.

GM2132 and BL29, in contrast, displayed lower levels of phosphorylated eIF4E (Figure 3.4). Only 13% of the eIF4E in BL29 cells is phosphorylated, however, these cells express higher levels of eIF4E than the control cells (Figure 3.1), implying that the total amount of phosphorylated eIF4E in BL29 cells is comparable to that in the LCLs. Since increased expression of eIF4E is not observed in GM2132 cells, the 15% phosphorylation of this protein indicates that these cells have much less of the phosphorylated isoform than any of the other B-cell lines in this study.

The association of eIF4G with eIF4E is an indicator of the formation of the eIF4F complex in a cell. This factor was measured in the B-cell lines by isolating eIF4E and its associated proteins using m7GTP-sepharose affinity chromatography. The proteins eluted from the affinity resin by m7GTP supplemented buffer were analysed by SDS-PAGE followed by western blotting and quantitation. The ratio of eIF4G to eIF4E is similar in all the cell lines (Figure 3.5) irrespective of the phosphorylation state or expression level of eIF4E (Figures 3.4 and 3.1). These data might indicate that
**Figure 3.4:** Phosphorylation state of eIF4E in the B-cell tumour lines. Isoelectric focusing of $0.5 \times 10^6$ cells per lane was followed by western blot analysis; the hyper- and hypo-phosphorylated isoforms are indicated. Percentage phosphorylation was calculated from laser densitometric scanning of blots from three separate experiments and the average value is given.
Figure 3.5: Association of eIF4G with eIF4E in the panel of B-cell lines.
A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on an 8% polyacrylamide gel; following electroblotting the upper section of the blot was probed for eIF4G and the lower section for eIF4E. B: Ratio of eIF4G to eIF4E, calculated from laser densitometric scanning.
factors governing the association of eIF4G with eIF4E are also similar in these cell lines. However, this is clearly not the case, as expression and phosphorylation of eIF4E are not identical in these cell lines. Alternatively, it may be possible that such similar ratios have arisen from different combinations of eIF4F regulatory processes. For example, decreased eIF4E phosphorylation could be compensated for by reduced association with the 4E-BPs or increased eIF4E expression.

To determine whether differences in the eIF4E binding proteins could be contributing to this effect, the protein levels of 4E-BP1 and 4E-BP2 and the phosphorylation state of 4E-BP1 were examined by western blot analysis. Two polyclonal antibodies were available for this determination, one of which was raised against a peptide present in both of the 4E-BPs (anti-BP1/BP2), the other being specific to 4E-BP2 (anti-BP2) (see section 2.3.2). After probing with anti-BP1/BP2, the blot was stripped and probed with anti-BP2 to confirm the identity of the proteins which were detected in the first instance. This showed that, as postulated, expression of these proteins differs in the various cell lines (Figure 3.6). Similar expression of both 4E-BP1 and 4E-BP2 is observed in the control LCLs and in the MM cell lines GM2132, GM06923 and the BL cell line BL29. However, less of both binding proteins is detected in Raji cells than in the aforementioned cell lines and significantly lower expression of 4E-BP1 and 4E-BP2 is observed in the MM cell lines GM1311 and GM1500C (Figure 3.6).

The western blot analysis also reveals the three separate phospho-isoforms of 4E-BP1. Increased phosphorylation of 4E-BP1 results in retarded migration during SDS-PAGE, as a result the upper band represents the most highly phosphorylated 4E-BP1. Quantitation of the three isoforms reveals further differences between the three cell types studied (Figure 3.6B). 98% or more of the 4E-BP1 in the BL cell lines is in the de-phosphorylated form; similarly, <12% of the 4E-BP1 in the MM cell lines GM2132 and GM1500C is phosphorylated. In contrast, a significant proportion of the 4E-BP1 detected in the LCLs is in the hyper-phosphorylated state, up to 70% in the case of GM0892A cells (Figure 3.6B). Of the remaining MM cell lines, quantitation of the GM1311 4E-BP1 must be considered less accurate, due to the low expression of the protein, however, the percentage phosphorylation appears to be similar to that of the 4E-BP1 in GM06923 cells and the LCL with least highly phosphorylated 4E-BP1, GM03201.

In summary, all the B-cell lines express both 4E-BPs to some extent. Two of the MM cell lines, GM1311 and GM1500C display low levels of both 4E-BP1 and 4E-BP2, which would imply that little eIF4E is sequestered by association with these binding proteins in these cells. In contrast, higher levels of both binding proteins are detected in the BL cell lines, Raji and BL29, and the MM cell line GM2132; the 4E-BP1 in these
Figure 3.6: Western blot analysis of the 4E-BPs. A: Western blot of a 15% SDS-polyacrylamide gel loaded with 1x10^6 cells per lane; the upper section of the blot was probed for α-tubulin as a loading control, the lower section of the blot was probed with anti-BP1/BP2, then stripped and re-probed with anti-BP2. B: Quantitation of the three 4E-BP1 phospho-isoforms from laser densitometric scanning.
cells is almost completely in the de-phosphorylated form, i.e., in the state in which it is able to bind eIF4E. Whilst the remaining MM cell line, GM06923, has comparable expression of 4E-BP1 and 4E-BP2 to GM2132 and the BL cell lines, only 41% of the 4E-BP1 in these cells is in the eIF4E binding form. Finally, the LCLs have extremely similar levels of both 4E-BP1 and 4E-BP2 and 55% or less of their 4E-BP1 is in the de-phosphorylated, eIF4E-binding, isoform.

3.2.3 Expression of eIF4G

An additional factor which could influence eIF4F formation is the level of eIF4G present in a cell. eIF4G protein levels were assessed by western blot analysis, using cell extracts which were prepared in the presence of protease inhibitors and used immediately. This revealed two points of interest. eIF4G protein expression is similar in the LCLs and the BL cell lines, however, reduced levels of eIF4G are observed in the MM cell lines GM1311 and GM1500C and very little eIF4G is detected in GM2132 cells (Figure 3.7). Additionally, a band was observed in the GM2132 track that is of a similar size to that produced by proteolytic cleavage of eIF4G by picornaviral proteases (Figure 3.7A). The blot was originally probed with anti-4G(C), antiserum raised against a peptide from the C-terminal region of eIF4G (see section 2.3.2), for comparison, the blot was stripped and re-probed with anti-4G(N). This revealed the presence of four bands which closely resemble those that have been identified as the N-terminal cleavage products of eIF4G (Figure 3.7B) (Lamphear et al., 1995; Lamphear & Rhoads, 1996).

The presence of these apparent N- and C-terminal fragments of eIF4G, coupled with the extremely low amount of intact eIF4G observed in these GM2132 cell extracts, implies that specific proteolytic cleavage of eIF4G could be occurring. To eliminate the possibility that an alteration in the size of the eIF4G mRNA is involved, a northern blot was performed. Approximately 2 μg of poly(A)+ RNA, isolated from the GM2132 MM cell line and the control GM03201 cells, was used for this analysis. The blot was hybridised with a radio-labelled DNA probe made from a fragment of eIF4G cDNA and a single band of the expected size, 5.3 kb, was detected in both lanes (Figure 3.8). The blot was subsequently hybridised with a GAPDH specific probe which demonstrates that similar amounts of eIF4G mRNA are present in the two cell lines (Figure 3.8).

3.2.4 Cleavage of eIF4G by an Activity in GM2132 Extracts

To further investigate this apparent cleavage of eIF4G in GM2132 cell lines, a crude assay for a specific proteolytic activity in GM2132 extracts was developed. GM2132 and GM03201 cells were harvested as normal, then resuspended in a fixed volume of PBS; varying proportions of the two cell lines were added together and extracts
Figure 3.7: Expression of eIF4G protein in the B-cell lines. Western blot of an 8% SDS-polyacrylamide gel loaded with 1.5x10⁶ cells per lane; the position of molecular weight markers (kDa) is indicated. A: The blot was initially probed with anti-4G (C). B: The blot was stripped and re-probed with anti-4G(N). C: The blot was finally probed for α-tubulin as a loading control.
Figure 3.8: eIF4G mRNA in the MM line GM2132 and the control line GM03201. Northern blot of approximately 2 μg poly(A)+ mRNA per lane, probed for eIF4G then stripped and re-probed for GAPDH as a loading control.
Figure 3.8: eIF4G mRNA in the MM line GM2132 and the control line GM03201. Northern blot of approximately 2 μg poly(A)+ mRNA per lane, probed for eIF4G then stripped and re-probed for GAPDH as a loading control.
prepared in the same way as for Figure 3.7 (see section 2.3.5). Thus, extracts were
made from equal total numbers of cells (1.5\times10^6) but with a different ratio of GM2132
to GM03201 cells in each. The extracts were subjected to SDS-PAGE immediately,
followed by western blot analysis; the blots were initially probed with anti-4G(N),
then stripped and re-probed with anti-4G(C).

The amount of intact eIF4G which could be expected in each extract (i.e. in the
absence of any cleavage activity) was predicted, using the eIF4G values obtained
from lanes 1 and 7 (Figure 3.9A). These values were compared to those measured
from the mixed extracts. This demonstrated that there was less intact eIF4G in the
mixed extracts than was predicted (Figure 3.9B). For example, in the extract where
equal amounts of GM2132 and GM03201 were mixed, 11-fold less eIF4G was
observed than would be predicted (Figure 3.9A, lane 4 and 3.9B, 50% GM2132).
Conversely, greater amounts of the N- and C- terminal fragments were also observed
in the mixed extracts than was calculated from GM2132 and GM03201 cells alone
(Figure 3.9A and C). A similar result was obtained in a separate experiment.

These results indicate that there is an activity in GM2132 cells which results in
cleavage of exogenous eIF4G. It is worth noting that these extracts were prepared in
the presence of inhibitors specific for serine and thiol proteases, which prevent non­
specific degradation of eIF4G; in the absence of such inhibitors the protein is barely
detectable in any of the cell lines. Clearly these inhibitors do not prevent this specific
cleavage of eIF4G into discrete N- and C-terminal products.

3.3 Summary

The data presented in this chapter demonstrate a lack of correlation between the
expression of c-myc and its two proposed target genes, eIF4E and eIF2α. This
suggests that c-Myc does not act as a transcription factor for these genes in either the
control or the tumour derived B-cell lines. The exception to this appears to be the BL
cell line BL29, in which elevated levels of both eIF4E and eIF2α mRNA and protein
were observed. However, in the absence of a similar observation in any of the other
cell lines, it is possible that the high levels of these two proteins in BL29 cells could
simply be a reflection of the transformed status of these cells, rather than a direct
consequence of elevated c-myc. Increased mRNA expression of the well
characterised c-myc target gene, ODC, was detected in the MM cell line GM2132 but
not in the BL cell line Raji. Clearly it would be interesting to determine if this is also
the case in BL29 cells, however, difficulties in culturing the BL29 cell line meant that
only a limited supply of these cells was ever available for this study, which
prevented determination of the ODC activity and mRNA expression in this cell line.
Thus, whilst c-Myc may be regulating ODC transcription in GM2132 cells, it is
Figure 3.9: Cleavage of eIF4G from GM03201 cells by an activity in GM2132 extracts. A: Western blot of an 8% SDS-polyacrylamide gel loaded with an equivalent of $1.5 \times 10^6$ cells per lane; the upper panel shows the blot probed with an N-terminal eIF4G antibody; the lower panel shows the same blot stripped and re-probed with a C-terminal eIF4G antibody. The position of molecular weight markers (KDa) is indicated. Lane 1: 100% GM2132; lane 2: 83% GM2132, 17% GM03201; lane 3: 67% GM2132, 33% GM03201; lane 4: 50% GM2132, 50% GM03201; lane 5: 33% GM2132, 67% GM03201; lane 6: 17% GM2132, 83% GM03201; lane 7: 100% GM03201. B and C: Quantitation of the data by laser densitometric scanning; predicted levels were calculated from the 100% GM2132 and GM03201 values according to the percentage of cells used in each extract.
**A**

Relative units

**B**

Intact eIF4G

N-term. Fragments

% GM2132 in extract

Observed eIF4G

Predicted eIF4G

**C**

Observed C-term.

Predicted C-term.

% GM2132 in extract

Observed N-term.

Predicted N-term.
difficult to draw a general conclusion about the regulation of ODC by c-Myc in all the B-cell lines studied.

Further investigation of the translational environment in these cell lines, in particular focusing on eIF4F, revealed a number of significant points. All the cell lines studied appear to have similar ratios of eIF4G bound to eIF4E. This is difficult to reconcile with the other factors which could affect this association - expression and phosphorylation of both eIF4E and 4E-BP1 and the protein levels of 4E-BP2 and eIF4G - which are far less constant in these cell lines. However, in each cell line, the combination of these different factors may compensate for each other, producing similar effects. For example, greater expression of eIF4E is measured in the BL cell line, BL29, than in any of the other lines, but the phosphorylation of eIF4E in this cell line is low and 100% of the 4E-BP1 expressed by these cells is in the de-phosphorylated isoform. Comparable expression of eIF4E and eIF4G is observed in the three LCLs, phosphorylation of eIF4E is similar in these cells, although the GM0892A cell line has the most highly phosphorylated eIF4E, and there are no major differences in the eIF4E binding proteins between these cell lines.

The data from the MM cell lines are more difficult to reconcile, particularly those for GM2132 cells. Expression of eIF4E does not vary between the four cell lines. Phosphorylation values for eIF4E measured in GM1311, GM06923 and GM1500C cells fall within 20% of each other. Expression of the 4E-BPs is not high in GM1311 and GM1500C cells and whilst greater expression of the 4E-BPs is observed in GM06923 cells, only 53% of their 4E-BP1 is in the de-phosphorylated isoform. Hence, the differences in available, active eIF4E between these three lines may be small. There is some variability in the expression of eIF4G in the three MM cell lines and the least eIF4G is detected in GM1500C cells. However, whether this has an affect on the association of eIF4G with eIF4E depends upon which of the factors is limiting in this system; it is commonly asserted that eIF4E is the limiting component of eIF4F, although this has only been shown in HeLa cells (Duncan et al., 1987) and has been disputed in reticulocyte lysate (Rau et al., 1996). In contrast, a low percentage of the eIF4E in GM2132 cells is phosphorylated, and a significant proportion of the 4E-BP1 detected in these cells is de-phosphorylated. This suggests that only low amounts of eIF4E are available to form active eIF4F complexes in the GM2132 cell line. Since the data presented in figure 3.5 are only indicative of the ratio of eIF4G associated with eIF4E and not a quantitative representation of this complex, low availability of eIF4E coupled with the reduced amount of intact eIF4G could be postulated to produce the result observed. Alternatively, the eIF4G bound to eIF4E could be less susceptible to proteolytic attack than the free protein and the eIF4G isolated by m7GTP-sepharose affinity isolation might therefore represent the majority of the intact protein in these cells.
The most striking observation in the GM2132 cell line is that eIF4G appears to be specifically cleaved into two products, which are extremely similar to the N- and C-terminal fragments produced by the picornaviral proteases (Lamphear et al., 1995; Lamphear & Rhoads, 1996). Furthermore, there is an activity in GM2132 cell extracts that is capable of causing the same cleavage of exogenous eIF4G. It has been shown that the C-terminal product of picornaviral proteolysis is sufficient to support cap-independent internal initiation of translation of viral RNAs (Ohlmann et al., 1996; Pestova et al., 1996; Borman et al., 1997). This finding therefore suggests that there may be a translational advantage for IRES bearing mRNAs in these cells. Indeed, in this MM cell line, the translational overexpression of c-myc (which contains an IRES), could be hypothesised to be mediated, at least in part, by the eIF4G cleavage.
CHAPTER 4

GRANULOCYTIC DIFFERENTIATION OF HL60 CELLS: REGULATION OF TRANSLATION

4.1 Introduction

A complex series of changes in the pattern of gene expression are central to the process of differentiation. Whilst much is known about alterations in gene expression which occur at the level of transcription in differentiating cells, little is known about translational control of gene expression during this process. Globally, protein synthesis rates would be expected to be greatly reduced in terminally differentiated cells compared to a proliferating cell type. However, earlier in the process of differentiation, alterations in the expression or activity of translation initiation factors could occur that affect the translation of individual mRNAs, or a particular sub-population of mRNAs, rather than having a large effect on global protein synthesis. Study of the differentiation of PC12 cells in response to NGF treatment suggests that this may indeed be the case (Frederickson et al., 1992; Kleijn et al., 1995).

HL60 cells have been widely used in the study of alterations in, and the control of, gene expression in haemopoietic differentiation. HL60 cells are a promyelocytic cell type which can be induced to differentiate into either monocytic or granulocytic cells (Collins, 1987). Granulocytic differentiation of HL60 cells, induced by DMSO treatment, has been used to investigate alterations in protein synthesis in the differentiating cells and the contribution of translational control to the differentiation process. Since changes in eIF4F have the potential to regulate the translation of specific subsets of mRNAs as well as to affect global protein synthesis rates, factors which contribute to the control of eIF4F have been examined.

During differentiation of HL60 cells, as with all differentiating cell types, there is a reduction in the expression of c-myc (Grosso & Pitot, 1984; Filmus & Buick, 1985; Mitchell et al., 1992); this is particularly pronounced in HL60 cells since they overexpress this oncogene (Collins & Groudine, 1982; Dalla-Favera et al., 1982b). This system has therefore also been used for further investigation of the regulation of eIF4E and eIF2α expression by c-Myc.
4.2 Results

4.2.1 Differentiation and c-myc Target Genes

Differentiation was induced in HL60 cells that had been recently diluted to 0.25x10^6 cells/ml with fresh medium (supplemented with 10% FCS), by addition of 1.25% DMSO. In parallel, control experiments were performed; cells were diluted in the same way with media but no inducing agent added. Cell samples were taken for analysis up to 96 hours after addition of DMSO, after which time no increase in the proportion of HL60 cells which assume the characteristics of the terminally differentiated granulocytic cell type is expected (Collins et al., 1978, 1979).

Three characteristics of the differentiating HL60 cells were routinely checked, in order to monitor the differentiation process and to ensure that a number of difficulties which were experienced in culturing the HL60 cell line did not reduce reproducibility of the experiments. The cell count was monitored; cell division continues for the first 48 hours after addition of DMSO at which point growth arrest occurs and no further increase in cell count is observed. Viability of the differentiating cells was also monitored using trypan blue exclusion and was >90% in both differentiated and undifferentiated cells. The morphological characteristics of the HL60 cells at 0 and 96 hours were evaluated; cytospin preparations were giemsa stained (see section 2.2.6), demonstrating development of the following characteristics in differentiated cells: smaller size, decreased nuclear : cytoplasmic ratio, reduced staining of cytoplasmic granules and marked indentation, convolution and segmentation of the nuclei (Figure 4.1A and B).

Finally, HL60 cells were assayed for their ability to produce the superoxide anion, a procedure which is routinely used to identify peripheral blood granulocytes (Segal, 1974; Collins et al., 1979). The cells were treated with TPA, to induce superoxide anion production, and the percentage of cells which reduced nitroblue tetrazolium (NBT) to form blue-black formazan deposits was determined from a cytospin preparation. Typically, < 1% of undifferentiated HL60 cells reduced NBT, whereas approximately 40 to 50% of cells reduced NBT 96 hours after DMSO treatment (Figure 4.1C). The percentage of cells capable of reducing NBT was determined on every occasion that differentiation was induced and if this was < 35% the experiment was discarded.

A further characteristic of differentiating HL60 cells is a reduction in c-myc expression; mRNA levels fall within 30 minutes of DMSO treatment (Mitchell et al., 1992) and, as in other differentiating cell types, c-Myc protein levels would be expected to follow this closely. In order to examine the effect this reduction in c-Myc has on the expression of the two proposed c-Myc target genes, eIF4E and eIF2α, the
Figure 4.1: Morphological characteristics of untreated and differentiated HL60 cells. Photographs taken at 400x magnification. A: Giemsa stained cytospin preparation of untreated HL60 cells. B: Giemsa stained cytospin preparation of differentiated HL60 cells (96 hours after addition of DMSO). C: Cytospin preparation of differentiated HL60 cells that have been assayed with NBT (as detailed in section 2.2.7).
levels of these proteins were determined. Samples of equal numbers of cells, taken at
the indicated time points from 0 to 96 hours, were subjected to western blot analysis;
the data were quantitated by laser densitometric scanning and the protein levels of c-
Myc, eIF4E and eIF2α calculated relative to the loading control, α-tubulin.

As expected, levels of c-Myc fall to 54% of resting levels within 1 hour of DMSO
treatment and are barely detectable by 2 hours (Figure 4.2). The level of eIF4E
protein does not decrease below that in untreated cells until 48 hours after
differentiation is induced, after which time there is a gradual decrease to 45% at 96
hours (Figure 4.2). In contrast, expression of eIF2α decreases within 10 hours of
DMSO addition to 48% of that in untreated cells. However, eIF2α levels rise by 24
hours to 87% and then gradually decrease in a similar manner to that of eIF4E over
the remaining 72 hours (Figure 4.2).

These data show that eIF4E expression does not appear to respond to the changes in
c-Myc protein levels in differentiating HL60 cells. In contrast, there is a correlation
between the decrease in c-Myc protein levels and expression of eIF2α. However, the
reduction in eIF2α is reversed to a large extent by 24 hours and this is a reproducible
phenomenon. This indicates eIF2α expression is initially modulated by c-Myc, but a
different regulator must take effect after the first 10 hours of differentiation.

4.2.2 Protein Synthesis Rates

Two separate methods were used to determine the changes in protein synthesis
which occur during granulocytic differentiation of HL60 cells. In the first instance,
the rate of incorporation of 35S-labelled amino acids into protein, in differentiating
and control cells, was monitored over the initial 10 hours of DMSO treatment.
Duplicate samples were harvested and the material precipitated by TCA was
subjected to scintillation counting, as described in section 2.4.2. No difference
between the protein synthesis rate in the treated and untreated cells was detected
using this technique (Figure 4.3A). This result was completely reproducible;
although this technique does produce significant variation between experiments,
there was no appreciable difference between the rates measured in differentiating
and control cells in any case.

To complement the data presented above, protein synthesis rates were measured for
the entire time course of differentiation, by pulsing cells with 35S-labelled amino
acids for a 1 hour period, at the indicated times, then harvesting duplicate samples
and measuring incorporation as before (see section 2.4.3). The rate of protein
synthesis measured at 0, 2, 4 and 10 hours was similar in differentiating and control
cells (Figure 4.3B). By 24 hours these rates have diverged; the rates measured in
Figure 4.2: Protein levels of c-Myc, eIF2α and eIF4E in differentiating HL60 cells. A: Two gels were run in parallel; 7.5% polyacrylamide loaded with $10^6$ cells per lane which after transfer to nitrocellulose was probed for c-Myc and 10% polyacrylamide loaded with $0.5 \times 10^6$ cells per lane which, after transfer, was probed for eIF2α, then stripped and re-probed for eIF4E and α-tubulin. B: Quantitation of the data using laser densitometric scanning, normalised to the loading control, α-tubulin.
Figure 4.3: Protein synthesis rates in differentiating and control HL60 cells. A: Incorporation of $^{35}$S labeled amino acids (a.a.) over a 10 hour period; the values plotted are the averages obtained from duplicate samples. B: Protein synthesis rate measured in duplicate samples at the indicated time points; cells were pulsed with $^{35}$S labeled amino acids for one hour.
untreated cells increase slightly and are then maintained at this level, as would be expected for normally proliferating cells. In contrast, the rate of protein synthesis measured in differentiating HL60 cells at 24 hours is reduced to that measured at 0 hours and decreases further over the following 72 hours, consistent with the growth arrest observed in these cells.

These data demonstrate that, during the first 10 hours, there is an increase in protein synthesis rate in HL60 cells which have been diluted to a density of 0.25x10⁶ cells/ml with fresh media, irrespective of addition of the differentiation agent. After the first 10 hours, the protein synthesis rates measured in the control and differentiating cells are as would be predicted for the two cell states. In the untreated, proliferating cells the protein synthesis rate rises over the 96 hour period. In the DMSO treated, differentiating cells the protein synthesis rate falls from 10 to 48 hours and remains at a low rate thereafter.

Dilution of HL60 cells with media containing 10% FCS prior to addition of the differentiation agent is essential in obtaining reproducible results and maintaining cell viability for the following 96 hours. However, it is possible that the increase in protein synthesis rate measured during the first 10 hours in response to the addition of fresh media could mask a change in protein synthesis rate induced by the addition of DMSO. To discount this possibility, these experiments were repeated in HL60 cells that had not been given fresh media for at least 48 hours. Under these conditions, the protein synthesis rates measured using both techniques were the same in control and differentiating cells during the first 10 hours (Figure 4.4) and remained so for the following 14 hours when measured over a longer period (Figure 4.4B). This demonstrates that even in the absence of the increase in protein synthesis rate stimulated by addition of media plus FCS, DMSO treatment does not result in an alteration in the protein synthesis rate, when compared to control cells, during the first 24 hour period.

4.2.3 Regulation of eIF4E

Two mechanisms which regulate eIF4E have been studied during granulocytic differentiation in HL60 cells. The phosphorylation state of eIF4E has been measured and its association with the eIF4E binding proteins, 4E-BP1 and 4E-BP2, has been assessed.

Isoelectric focusing followed by western blot analysis was used to measure the phosphorylation state of eIF4E, in samples taken from differentiating and control HL60 cells throughout the 96 hour time course. Laser densitometry was used to quantitate the data and the percentage phosphorylation calculated. Phosphorylation
Figure 4.4: Protein synthesis rates in differentiating and control HL60 cells not initially diluted with fresh media. A: Incorporation of $^{35}$S labeled amino acids (a.a.) over a 10 hour period; the values plotted are the averages obtained from duplicate samples. B: Protein synthesis rate measured in duplicate samples at the indicated time points; cells were pulsed with $^{35}$S labeled amino acids for one hour.
of eIF4E ranges from approximately 20 to 30% in growing HL60 cells and does not vary significantly on the addition of serum (Figure 4.5B). When HL60 cells are treated with DMSO, an approximately 2-fold increase in eIF4E phosphorylation is observed, peaking at 4 to 6 hours (Figure 4.5A and C). Whilst the percentage phosphorylation of eIF4E falls gradually from 10 to 96 hours it remains at a higher level than at 0 hours throughout this period.

These data show that a significant change in eIF4E phosphorylation occurs during granulocytic differentiation of HL60 cells. The initial increase in eIF4E phosphorylation is observed in the absence of any concurrent changes in the protein synthesis rate, when compared to control cells. Although this alteration in eIF4E phosphorylation does not affect global protein synthesis, it is possible that it has an effect on the translation of messages which are proposed to compete particularly poorly for eIF4F, i.e. those with long, GC rich 5' UTRs.

The expression of both 4E-BP1 and 4E-BP2 was determined during the time course of differentiation by western blot analysis, to examine the role the 4E-BPs have in the differentiation of HL60 cells. As in section 3.2.2, the blot was initially probed with anti-BP1/BP2, then stripped and re-probed with anti-BP2. It is apparent that both binding proteins are expressed in HL60 cells and that levels of these proteins do not vary significantly throughout differentiation (Figure 4.6). This experiment also demonstrates that the majority of the 4E-BP1 detected exists as the middle phospho-isoform of the three. The de-phosphorylated isoform of 4E-BP1 (which is least retarded by SDS-PAGE) is the only form of the protein to associate with eIF4E (Lin et al., 1994), therefore, little 4E-BP1 would be expected to be associated with eIF4E in these cells.

Association of the 4E-BPs with eIF4E during HL60 cell differentiation was determined by m7GTP-sepharose affinity chromatography, followed by SDS-PAGE and western blot analysis, using anti-BP1/BP2 and anti-BP2. 4E-BP1 was not routinely detectable by this method, but 4E-BP2 is clearly shown to associate with eIF4E (Figure 4.7A). It is also apparent that there is some variation in the ratio of 4E-BP2 associated with eIF4E; the ratio of 4E-B2 to eIF4E decreases by approximately 35% after 1 hour of DMSO treatment and then increases to a similar ratio to that in untreated cells at 6 to 10 hours. Another reduction in the ratio is observed at 24 hours, followed by a return to a ratio greater than 60% of that in untreated cells for the remaining 72 hours of the experiment (Figure 4.7B). These data are difficult to interpret given that no corresponding changes occur in the association of eIF4G with eIF4E (data to be presented in section 5.2).
Figure 4.5: Phosphorylation state of eIF4E in differentiating HL60 cells. A: Western blot of IEF gel; samples (1x10^6 cells) were taken from DMSO treated HL60 cells at the indicated time points. B: Western blot of IEF gel; samples (1x10^6 cells) were taken from untreated HL60 cells; percentage phosphorylation was calculated from data from laser densitometric scanning. C: Plot of average percentage phosphorylation values from three separate experiments, calculated from laser densitometry data. The error bars represent +/- one standard deviation, where no bar is plotted this value is less than 2.
**Figure 4.6:** Western blot of SDS-PAGE, showing the eIF4E binding proteins in differentiating HL60 cells. Samples (1×10^6 cells) were taken at the indicated times and analysed on a 15% SDS-polyacrylamide gel, then transferred to nitrocellulose; the upper section of the blot was probed for α-tubulin as a loading control (upper panel), the lower section of the blot was probed with anti-BP1/BP2 (middle panel), then stripped and re-probed with anti-BP2 (lower panel).
Figure 4.6: Western blot of SDS-PAGE, showing the eIF4E binding proteins in differentiating HL60 cells. Samples (1x10⁶ cells) were taken at the indicated times and analysed on a 15% SDS-polyacrylamide gel, then transferred to nitrocellulose; the upper section of the blot was probed for α-tubulin as a loading control (upper panel), the lower section of the blot was probed with anti-BP1/BP2 (middle panel), then stripped and re-probed with anti-BP2 (lower panel).
Figure 4.7: Association of 4E-BP2 with eIF4E in differentiating HL60 cells. A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on a 15% polyacrylamide gel; following transfer to nitrocellulose the upper section of the blot was probed for eIF4E and the lower section with anti-BP1/BP2. B: Ratio of 4E-BP2 to eIF4E, calculated from laser densitometric scanning.
Two types of signalling pathway are known to be involved in controlling initiation of translation via eIF4E (see section 1.2.4). 4E-BP1 is regulated by the FRAP/mTOR pathway, which activates p70S6 kinase and the unidentified 4E-BP1 kinase. Two types of MAP kinase pathways can regulate eIF4E phosphorylation; the growth activated MAP kinase pathways involving the Erk kinases and the stress activated MAP kinase pathway which includes the p38 kinase. To determine whether signalling via these pathways might be involved in the translational responses in differentiating HL60 cells, two approaches were employed.

The activation of the FRAP/mTOR pathway was investigated by harvesting samples of DMSO treated cells were over a 10 hour period and subjecting them to western blot analysis. The blot was probed with an antibody which detects the p70 and p85 S6 kinases, which when activated by phosphorylation display decreased mobility on SDS-PAGE. No change in the phosphorylation state of p70S6 kinase is observed during the first 10 hours of differentiation (Figure 4.8), which implies that there is no change in the activity of the FRAP/mTOR pathway. Comparison with a sample of HL60 cells treated with rapamycin, a FRAP/mTOR pathway inhibitor, reveals that a proportion of the p70S6 kinase is in the active, phosphorylated form.

The effects of the inhibitors rapamycin and PD98059 (which is specific to the Erk MAP kinase pathway) on the increase in eIF4E phosphorylation were examined. HL60 cells were treated with either rapamycin or PD98059 for 1 hour, in order to determine the effect these inhibitors have on undifferentiated HL60 cells. The treated cells were then subjected to IEF followed by western blot analysis. This experiment shows that rapamycin has no affect on the phosphorylation state of eIF4E; the percentage phosphorylation measured was 24%, comparable to that in untreated cells (Figure 4.9A). In contrast, treatment with PD98059 reduced the phosphorylation of eIF4E to less than 2% (Figure 4.9A), suggesting that the Erk MAP kinase pathway is responsible for maintaining basal levels of eIF4E phosphorylation in growing HL60 cells.

Having established the effect of these inhibitors on undifferentiated HL60 cells, cells were treated with either rapamycin or PD98059 for 30 minutes before DMSO addition to determine their effect during differentiation. Samples were harvested throughout the first 10 hours of differentiation and the phosphorylation state of eIF4E was determined. The induction of eIF4E phosphorylation observed during differentiation is not inhibited by rapamycin; this observation is consistent with the evidence that suggests regulation of eIF4E phosphorylation occurs via a pathway other than the FRAP/mTOR pathway (discussed in section 1.2.4) (Figure 4.9B). The
Figure 4.8: The S6 kinases in differentiating HL60 cells. Western blot of a 7.5% polyacrylamide gel loaded with $1 \times 10^6$ cells per lane; the blot was probed with an antiserum specific for the p70 and p85 S6 kinases. Lanes 1-7: samples were taken from HL60 cells treated with DMSO at the indicated time points, lane 8 (R): HL60 cells treated with rapamycin for 30 minutes.
Figure 4.9: Effect of the inhibitors Rapamycin and PD98059 on the phosphorylation state of eIF4E in differentiating HL60 cells. A: Western blot of an IEF gel, showing the effect of a 1 hour treatment with the inhibitors on resting cells. B: Western blot of an IEF gel; HL60 cells were pre-treated with the indicated inhibitor, or left untreated, for 30 minutes, then stimulated to differentiate by addition of DMSO; samples were taken at the indicated times. C: The fold increase in eIF4E percentage phosphorylation from 0 to 6 hours was calculated from laser densitometric scanning of the blots. Data calculated from the experiments shown in Figure 4.5 is also included.
induction in eIF4E phosphorylation also occurs in the presence of PD98059, in spite of the reduced eIF4E phosphorylation initially caused by this inhibitor (Figure 4.9B). This implies that the growth factor regulated MAP kinase pathway, mediated by Erk1 and Erk2, is not required for the induction of eIF4E phosphorylation that occurs when HL60 cells are stimulated to undergo granulocytic differentiation with DMSO. It is interesting to note that the percentage phosphorylation of eIF4E at 6 hours in the PD98059 treated differentiating HL60 cells is similar to that in control differentiating cells (Figure 4.9B). As a result, the increase in eIF4E phosphorylation in PD98059 treated differentiating cells is almost 4 times that which occurs in control differentiating HL60 cells (Figure 4.9C).

4.2.5 Reverse Transcription and PCR of Immunoprecipitation Products

As proposed in section 4.2.3, the increase in eIF4E phosphorylation observed in differentiating HL60 cells, which occurs in the absence of a corresponding change in the rate of protein synthesis, could be involved in increasing the translation of a specific subset of mRNAs. Testing this proposal requires a method which allows the identification of such messages. Therefore, a scheme was devised for isolating and identifying the mRNAs bound by eIF4E. Quantitative analyses of such messages before and after a change in the phosphorylation state of eIF4E, taking into account any alterations in their expression, could allow the identification of mRNAs which are specifically translationally up-regulated in a cap-dependent manner.

The method employed for this analysis is outlined in Figure 4.10; immunoprecipitation of eIF4E from cell lysates allows isolation of the mRNA bound by eIF4E, i.e. that which is being actively translated in a cap-dependent manner. This mRNA can be purified by phenol extraction followed by ethanol precipitation and then subjected to reverse transcription. The result is a population of cDNA which can be further analysed by PCR. The basis for this technique is not without precedent; a similar method has been used to isolate and characterise the thymidylate synthase protein in complex with its own mRNA (Chu et al., 1994).

To develop this method, more eIF4E antiserum was required and was produced by immunising a rabbit with a peptide conjugated to keyhole limpet haemacyanin. The peptide corresponded to 21 amino acids from the N-terminal region of the protein and was identical to that used to raise the original eIF4E antiserum (see section 2.3.3). Once the rabbit serum had been harvested, the antibody was affinity purified, using peptide conjugated to an agarose support matrix (see section 2.3.3). This antibody was used for a series of test immunoprecipitation reactions, using cell extract from 10^7 HL60 cells per reaction; the products of these were subjected to western blot
Figure 4.10: Scheme outlining experimental approach for analysing mRNA bound by eIF4E. See section 4.2.5.
analysis and show that 20 μl of the purified antibody specifically isolates detectable amounts of eIF4E (Figure 4.11A).

To isolate RNA using this antiserum, immunoprecipitations were performed in the presence of vanadyl ribonucleoside complexes (VRC) and nucleic acid purified from the products by phenol extraction. The isolated material was treated with DNase and then subjected to reverse transcription. The cDNA produced in this manner was used as the template for PCR reactions using two sets of primers; specific for a 221 bp fragment in exon 2 of c-myc and a 248 bp fragment in the GAPDH sequence. It was determined that mRNA could be specifically isolated using this method, provided the HL60 cell extracts were pre-cleared with normal rabbit serum plus protein A sepharose and the immunoprecipitation was carried out in the presence of a blocking agent, yeast tRNA. Figures 4.11B and C show examples of the products from such experiments.

However, PCR reactions of this nature do not provide quantitative data about the amount of template initially present in each reaction. This information is required if changes in the mRNA bound by eIF4E are to be assessed. For this purpose, competitive PCR was performed; competitor DNA for both the c-myc and GAPDH reactions was produced from a 415 bp fragment, of unrelated sequence, as detailed in section 2.5.12. Sequences complementary to the c-myc or GAPDH gene specific primers are were added to the competitor fragment using two rounds of PCR as outlined in Figure 2.1. The first PCR reaction employed composite primers, consisting of the last 10 bp of the gene specific primer and the first 10 bp of the competitor fragment; the products of this reaction were purified and used for a second round of PCR with the gene specific primers. The products of the second reaction were also purified and used as competitor DNA in the competitive PCR reactions.

Initial attempts at the competitive PCR technique were unsuccessful. Time restrictions did not allow this work to be pursued further; however, the initial data presented in Figure 4.11 suggest that the method outlined could be developed for the analysis of eIF4E-bound mRNA.

4.3 Summary

Study of granulocytic differentiation in HL60 cells has provided additional information about the regulation of eIF4E and eIF2α by c-Myc. When c-Myc levels decrease during the differentiation process, eIF4E expression is unaffected; eIF4E is maintained at initial levels for the first 24 hours of differentiation. This implies that eIF4E is not regulated by c-Myc in HL60 cells. In contrast, eIF2α expression
Figure 4.11: eIF4E immunoprecipitation (IP) and RT PCR. A: Western blot analysis of eIF4E immunoprecipitated from HL60 cell extracts with the indicated amount of eIF4E antiserum; CE: cell extract from $1 \times 10^6$ HL60 cells. B and C: Ethidium bromide stained agarose gels showing products of PCR reactions using the indicated templates. +ve: reverse transcription (RT) of HL60 total RNA, +Ab: RT of RNA isolated by eIF4E IP, -Ab: RT of RNA isolated from negative control IP, -ve: no template. The sizes, in bp, of a DNA ladder are given and the arrows indicate the amplified products. B: PCR with primers specific for a 221 bp c-myc fragment. C: PCR with primers specific for a 248 bp GAPDH fragment.
correlates with that of c-myc during the first 10 hours of the differentiation process, although the reduction in eIF2α protein levels (to 48% of initial levels at 10 hours) is not as great as the decrease in c-Myc (to 24% of initial levels at 10 hours). This suggests that c-Myc is indeed responsible for regulating expression of eIF2α, to some extent, during these first 10 hours. However, by 24 hours after addition of DMSO there is a clear divergence between levels of c-Myc and eIF2α protein; eIF2α expression increases to 87% of that at the beginning of the experiment. Thus, whilst c-Myc may be regulating eIF2α expression in the initial stages of granulocytic differentiation of HL60 cells, other factors can also control eIF2α expression and these dominate from 24 hours onwards.

Further investigation of eIF4E during the differentiation of HL60 cells demonstrated that this factor is regulated in the early stages of granulocytic differentiation. Examination of the eIF4E binding proteins, 4E-BP1 and 4E-BP2, showed that the majority of the 4E-BP1 in HL60 cells is in a phospho-isoform that cannot bind eIF4E and that this does not change throughout the differentiation process. In contrast, 4E-BP2 does associate with eIF4E and the ratio of these two proteins, when they are isolated by m7GTP-sepharose affinity chromatography, varies. However, the data to be presented in chapter 5 suggest that the variation in association of eIF4E with 4E-BP2 does not influence the association of eIF4G with eIF4E and it is difficult to conclude how this variation could be affecting the initiation of translation.

An increase in the phosphorylation of eIF4E in HL60 cells occurs within 4 to 6 hours of DMSO treatment which is not observed in control HL60 cells. Phosphorylation increases from an average of 21% at 0 hours to 39% at 6 hours. Investigation of the signalling pathways which might be involved in such regulation revealed no changes in the FRAP/mTOR pathway. Correspondingly, the inhibitor rapamycin, which blocks the FRAP/mTOR pathway, does not influence the increase in eIF4E phosphorylation. This is consistent with the evidence that eIF4E is regulated by a MAP kinase pathway and not via FRAP/mTOR (Flynn & Proud, 1996b; Morley, 1997; Morley & McKendrick, 1997). Use of the inhibitor PD98059 demonstrates that the growth factor regulated (Erk) MAP kinase pathway is not involved in the increased phosphorylation of eIF4E that occurs during differentiation of HL60 cells. However, this pathway does appear to be responsible for maintaining basal levels of eIF4E phosphorylation in this system, since treatment of normally growing HL60 cells causes a decrease in phosphorylation from 24% to less than 2%.

These data show that neither the FRAP/mTOR nor the Erk MAP kinase pathways are involved in signalling an increase in eIF4E phosphorylation when HL60 cells are treated with DMSO. The only other pathway that has been identified which regulates eIF4E phosphorylation is the stress activated p38 MAP kinase pathway.
(Fukunaga & Hunter, 1997; Morley, 1997; Morley & McKendrick, 1997; Waskiewicz et al., 1997). It may be possible that whilst the growth activated MAP kinase pathway maintains eIF4E phosphorylation in growing HL60 cells, it is the p38 pathway that controls the change in eIF4E phosphorylation that occurs when these cells are stimulated to undergo granulocytic differentiation.

Interestingly, when the increase in eIF4E phosphorylation is observed in differentiating HL60 cells, no corresponding change in protein synthesis occurs. During the first 10 hours there is an increase in protein synthesis rate in HL60 cells diluted with media containing 10% FCS (which is part of the method used to stimulate differentiation), but this increase is independent of the addition of DMSO. Protein synthesis was also monitored in HL60 cells that were undiluted, to ensure that the increase in rate was not masking some other effect stimulated by DMSO addition. This also shows that protein synthesis rates do not differ between DMSO treated and untreated cells, during the first 24 hours in this case. Thus, the 2-fold increase in eIF4E phosphorylation seen in differentiating HL60 cells has no discernible effect on the rate of protein synthesis in these cells. However, whilst the change in eIF4E phosphorylation does not alter global protein synthesis rates, it is possible that it could have a specific effect on the translation of certain mRNAs.

In order to investigate this hypothesis, an attempt was made to establish a method for examining changes in the mRNAs bound by eIF4E. Whilst time constraints did not allow this technique to be developed to the point where it could be used to obtain data from differentiating HL60 cells, the initial work carried out to this end shows that the process of immunoprecipitation, followed by reverse transcription of the isolated RNA, can be used to produce a population of cDNA amenable to analysis by PCR.
CHAPTER 5

GRANULOCYTIC DIFFERENTIATION OF HL60 CELLS: THE ROLE OF eIF4G

5.1 Introduction

Regulation of initiation of translation through alterations in the availability, or the activity, of the eIF4F complex has been shown to occur via a number of different control mechanisms. Two separate but interconnected effects appear to be involved in this process; increased formation of eIF4F from its constituent proteins and increased ability of the complex to bind the mRNA cap structure. Phosphorylation of eIF4E and eIF4G both appear to have an affect on these aspects of eIF4F function (Morley et al., 1991; Bu et al., 1993; Minich et al., 1994; Morley & Pain, 1995a, b; Haghighat & Sonenberg, 1997). Additionally, regulation of eIF4E availability by the 4E-BPs can alter the association of eIF4E with eIF4G (Haghighat et al., 1995; Mader et al., 1995).

Whilst eIF4E has long been considered the limiting component of eIF4F, there is only a small amount of evidence to support this. Although eIF4E has been shown to be limiting in HeLa cells (Duncan et al., 1987), in reticulocyte lysates there is a functional excess of the protein (Rau et al., 1996). It would therefore seem possible that the expression or availability of other components of eIF4F could have an impact on formation of this complex. In support of this premise, it has been recently shown that overexpression of eIF4G can transform cultured cells (Fukuchi-Shimogori et al., 1997); which suggests that alterations in this component of eIF4F alone can influence translation.

The granulocytic differentiation of HL60 cells has been used in this study to investigate the contribution translational control makes to the differentiation process. In addition to the work presented in section 4.2.3, further investigation of the eIF4F complex has been carried out in this system, focusing on the role of eIF4G. The expression of eIF4G has been examined and alterations in the formation of eIF4F have been measured by determining the association of eIF4G with eIF4E in DMSO treated HL60 cells. These factors have also been investigated in HL60 cells induced to undergo granulocytic differentiation by treatment with retinoic acid and in HL60 cells subjected to oxidative stress.
5.2 Results

5.2.1 eIF4G expression and association with eIF4E during granulocytic differentiation of HL60 cells

To measure the association of eIF4G with eIF4E, samples of differentiating HL60 cells were analysed using m7GTP affinity chromatography. As before, HL60 cells were diluted to 0.25x10^6 cells/ml and treated with 1.25% DMSO. Samples of 7x10^6 cells were harvested at the indicated points throughout the 96 hour period, subjected to m7GTP-sepharose affinity chromatography and the proteins eluted with m7GTP supplemented buffer were used for a western blot analysis. The ratio of eIF4G associated with eIF4E was calculated from quantitation of the blots by laser densitometry. An increase in the ratio of eIF4G to eIF4E was observed within 30 minutes. The increase in ratio continued until 24 hours and the ratio then decreased at 48 hours (Figure 5.1). At 72 and 96 hours after induction, eIF4G was barely detectable in the column eluant (Figure 5.1A). This indicates that an increase in the formation of eIF4F complexes has occurred in HL60 cells during the course of the experiment.

To determine the protein levels of eIF4G in differentiating HL60 cells, cell samples were taken in parallel to those used for m7GTP-sepharose affinity chromatography. Western blot analysis of these samples showed an increase in the eIF4G protein level which corresponds with the observed increase in association of eIF4G with eIF4E (Figure 5.2A). Comparison of these two data sets demonstrated that, up to 10 hours after DMSO addition, the changes in these two factors occur in parallel (Figure 5.2B). At 24 and 48 hours a divergence between the protein levels of eIF4G and the association of eIF4G with eIF4E can be observed. eIF4G protein levels decrease while the ratio of eIF4G to eIF4E rises before falling at 48 hours. At 72 and 96 hours, eIF4G protein was not detectable, which correlates with the reduced association of eIF4G with eIF4E observed at these points (Figures 5.1 and 5.2).

These data suggest that the increase in association of eIF4G with eIF4E that occurs in DMSO treated HL60 cells, during the first 10 hours, may be caused by the enhanced expression of eIF4G. From 24 to 48 hours, eIF4G protein levels decrease more rapidly than the ratio of eIF4G to eIF4E, indicating that other factors may be involved in regulating the association during this time. By 72 hours, eIF4G becomes undetectable and as a result little of the protein is recovered with eIF4E (Figures 5.1 and 5.2).

A similar effect was observed in a number of experiments and eIF4G protein levels always correlated with the initial increase in its association with eIF4E. However, there was significant variation in when the peak in association occurred and the
Figure 5.1: Association of eIF4G with eIF4E in DMSO treated HL60 cells (before adaptation). A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on an 8% polyacrylamide gel; following transfer to nitrocellulose the upper section of the blot was probed for eIF4G and the lower section for eIF4E. B: Ratio of eIF4G to eIF4E, calculated from laser densitometric scanning.
Figure 5.2: Expression of eIF4G in DMSO treated HL60 cells (before adaptation). A: Western blot of 1.5x10^6 cells loaded on an 8% polyacrylamide gel, probed for eIF4G. B: Plot of fold change in eIF4G expression, calculated from laser densitometry of the blot, and fold change in the ratio of eIF4G/eIF4E, taken from Figure 5.1.
magnitude of the response (data not shown). Finally, after the HL60 cells had been in continuous culture for over 2 months, the phenomenon was no longer detectable, and the small variations in the association of eIF4G with eIF4E that were observed (all less than 2-fold) were not accompanied by corresponding changes in eIF4G expression (Figure 5.3). Yet, according to the criteria defined in section 4.2.1, the HL60 cells were differentiating normally. This implies that the induction in eIF4G expression and association of eIF4G with eIF4E is not a necessary part of the process of granulocytic differentiation in HL60 cells. It is interesting to note that when the induction is not observed, neither is the large decrease in eIF4G protein, nor the concomitant reduction in its association with eIF4E, that occurs from 72 hours (Figure 5.3).

The culture of HL60 cells used for these experiments was obtained from Dr C. Bunce (Department of Medicine, Birmingham University) who cultures HL60 cells for the purpose of studying differentiation. A new culture of HL60 cells was obtained from this source and a sequence of events similar to those described above occurred. An initial induction in eIF4G expression and association with eIF4E was observed, but this response was variable and eventually was not detectable (data not shown). These observations suggest that when the HL60 cell culture was first acquired, some change in the culture conditions (e.g. use of different FCS) could have stimulated the response of the cells to addition of fresh media and DMSO. It is possible that, as the cells were continuously cultured over time, they ‘adapted’ to whatever the new factor was and as a result the response was no longer stimulated.

In order to exclude any other aspects of this phenomenon from the data obtained using differentiating HL60 cells, the experiments detailed in chapter 4 were performed with ‘adapted’ cells. The HL60 cells used for all procedures described in chapter 4 had been grown in continuous culture for at least two months and no significant alterations in eIF4G expression, or in its association with eIF4E, were detectable in those cells when differentiation was induced (as in Figure 5.3).

5.2.2 eIF4G expression and association with eIF4E in serum stimulated HL60 cells

To further investigate the phenomenon described above, a third culture of HL60 cells was obtained from Dr C. Bunce. As before, the induction in eIF4G protein levels and an increased association of eIF4G with eIF4E were observed when the cells were diluted with fresh media and treated with DMSO (data not shown). In order to determine whether this response was specific to DMSO treatment, or was occurring as a result of diluting the cells with media at the start of the experiment, HL60 cells were diluted to 0.25x10^6 cells/ml and samples taken over 96 hours as before. The samples were subjected to m7GTP-sepharose affinity chromatography followed by
Figure 5.3: Association of eIF4G with eIF4E and eIF4G expression in DMSO treated HL60 cells (after adaptation). A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on an 8% polyacrylamide gel; following transfer to nitrocellulose the upper section of the blot was probed for eIF4G and the lower section for eIF4E. B: Western blot of 1.5x10⁶ cells loaded on an 8% polyacrylamide gel, probed for eIF4G. C: Plot of fold change in eIF4G expression and fold change in the ratio of eIF4G/eIF4E, calculated from laser densitometric scanning of the blots.
western blot analysis and this showed that serum stimulation causes an increase in the association of eIF4G with eIF4E of a magnitude similar to that observed when HL60 cells were treated with DMSO (Figure 5.4). An increase in eIF4G protein levels was also observed, which continues until 72 hours after induction and then decreases at 96 hours (Figure 5.4). When this experiment was performed in 'adapted' HL60 cells, no increase in eIF4G expression or association with eIF4E was detected (Figure 5.5).

These data suggest that the stimulation of eIF4G expression and association of eIF4G with eIF4E that was observed in DMSO treated HL60 cells, can be attributed to the addition of fresh media containing 10% FCS at the beginning of the experiment. After the initial 10 to 24 hour period, differences between cells treated with DMSO and the untreated cells can be observed (Figures 5.2 and 5.4). This variation could arise from the differences in the growth characteristics of the two cell populations, since differentiating HL60 cells undergo growth arrest 48 hours after addition of DMSO.

5.2.3 eIF4G expression in response to retinoic acid and oxidative stress

In order to ascertain more about the nature of the response of 'un-adapted' HL60 cells to addition of fresh media, two further investigations were carried out. During the first 10 hours, the increases in eIF4G protein levels and its association with eIF4E are similar in diluted HL60 cells in the presence or absence of DMSO. This implies that the granulocytic differentiation process does not have an effect on the eIF4G response until 24 hours after the cells are first exposed to DMSO. Granulocytic differentiation can also be induced in HL60 cells by treatment with 1 µM retinoic acid (RA) (Breitman et al., 1980). To determine whether the induction in eIF4G expression and association with eIF4E occurs during granulocytic differentiation induced in this way, eIF4G protein levels were monitored in RA treated HL60 cells.

In contrast to serum stimulated and DMSO treated HL60 cells, RA treatment caused a 2-fold decrease in eIF4G by 1 hour (Figure 5.6). eIF4G protein levels remain below those at 0 hours until 48 hours after RA treatment. As in granulocytic differentiation induced by DMSO, RA treated cells continue to grow and divide up to 48 hours after treatment with the differentiation agent. After this time, cell counts remain constant until 96 hours. The fact that the induction of eIF4G expression and association with eIF4E occurs in response to addition of fresh media and serum might imply that the phenomenon is a growth related serum response. However, the absence of an increase in eIF4G in growing, RA treated HL60 cells, coupled with the fact that the increase in eIF4G protein levels and association with eIF4E, in response to addition of
Figure 5.4: Expression of eIF4G in serum stimulated HL60 cells (before adaptation). A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on an 8% polyacrylamide gel; following transfer to nitrocellulose the upper section of the blot was probed for eIF4G and the lower section for eIF4E. B: Western blot of 1.5x10⁶ cells loaded on an 8% polyacrylamide gel, probed for eIF4G. C: Plot of fold change in eIF4G expression and fold change in the ratio of eIF4G/eIF4E, calculated from laser densitometric scanning of the blots.
Figure 5.5: Association of eIF4G with eIF4E and expression of eIF4G in serum stimulated HL60 cells (after adaptation). A: Proteins isolated by m7GTP sepharose affinity chromatography were analysed by SDS-PAGE on an 8% polyacrylamide gel; following transfer to nitrocellulose the upper section of the blot was probed for eIF4G and the lower section for eIF4E. The variations in loading are due to the precipitated proteins incompletely re-dissolving. B: Western blot of 1.5x10⁶ cells loaded on an 8% polyacrylamide gel, probed for eIF4G. C: Plot of fold change in eIF4G expression and fold change in the ratio of eIF4G/eIF4E, calculated from laser densitometric scanning of the blots.
Figure 5.6: Expression of eIF4G in retinoic acid treated HL60 cells (before adaptation). A: Western blot of 8% polyacrylamide gel loaded with $1.5 \times 10^6$ cells per lane, probed for eIF4G. B: Quantitation of the data by laser densitometric scanning, expressed as fold change in eIF4G protein levels.
media plus serum, is eventually lost in normally growing HL60 cells, suggests that this is not a growth related response.

An alternative explanation for these observations is that the new agent that HL60 cells are exposed to when a new culture is obtained, is in some way detrimental to the cells and induces a stress related response. To explore this possibility further, H$_2$O$_2$ was used to induce oxidative stress in HL60 cells that had not been exposed to fresh media for at least 48 hours. HL60 cells were treated with 50 μM H$_2$O$_2$ and samples taken from 0 to 10 hours. These samples were subjected to western blot analysis to determine eIF4G protein levels. This experiment demonstrated that no increase in eIF4G protein is induced by this dose of H$_2$O$_2$ (Figure 5.7A).

HL60 cells were also treated with increasing concentrations of H$_2$O$_2$ for 6 hours and then harvesting and analysing the cells as before. This demonstrated that oxidative stress does not induce an increase in eIF4G protein at this time (Figure 5.7B). Indeed, when cells were treated with 100 or 250 μM H$_2$O$_2$ the amount of eIF4G detected decreases. The 250 μM dose of H$_2$O$_2$ resulted in significant cell death and was presumably inducing apoptosis in the HL60 cells. In this context, it is interesting to note the appearance of a band of the expected size for the C-terminal cleavage product of eIF4G (Figure 5.7B). This might suggest that the eIF4G protein is being cleaved when apoptosis is induced.

### 5.3 Summary

Investigation of the association of eIF4G with eIF4E and the protein levels of eIF4G during granulocytic differentiation of HL60 cells revealed that, under the experimental conditions used for the studies presented in chapter 4, no significant alteration in either of these factors occurs. However, it was observed that an increase in the association of eIF4G with eIF4E occurs in newly acquired HL60 cells when they are exposed to media containing 10% FCS. Similar increases, up to 24 hours, were observed in the presence or absence of DMSO. In both cases, an increase in eIF4G protein levels also occurred. A comparison of the changes in the expression of eIF4G and the ratio of eIF4G to eIF4E demonstrated that, during the first 10 hours, the two inductions parallel each other closely. Thus, the increase in association of eIF4G with eIF4E would appear to be mediated by an increase in eIF4G.

The focus of research on regulation of eIF4F complex formation has, on the whole, been on eIF4E, the supposedly limiting component of this complex. The data presented here suggest that alteration in the levels of eIF4G can also regulate formation of eIF4F. This implies that in HL60 cells, eIF4G may be the limiting component of eIF4F, rather than eIF4E.
Figure 5.7: Effect of H$_2$O$_2$ treatment on eIF4G protein expression in HL60 cells (before adaptation). A: Western blot of an 8% polyacrylamide gel, loaded with 1.5x10$^6$ cells per lane, probed for eIF4G. Cells were treated with 50 µM H$_2$O$_2$ and samples harvested at the indicated times. B: Western blot of an 8% polyacrylamide gel, loaded with 1.5x10$^6$ cells per lane, probed with anti-4G(C); the position of molecular weight markers (kDa) is indicated. Cells were treated with the indicated concentration of H$_2$O$_2$ for 6 hours.
However, this phenomenon was difficult to reproduce precisely and gradually reduced in magnitude, as the HL60 cells were continuously cultured over a period of approximately 2 months. This process was observed on each occasion that a new culture of HL60 cells was obtained. These observations suggest that the HL60 cells are responding to some new factor in their culture conditions and that, over time, they are adapting to this change and lose the eIF4G response.

The fact that the induction in eIF4G expression and corresponding changes in the association of eIF4G with eIF4E are no longer observed when HL60 cells have been in culture for some time, demonstrates that this phenomenon is not essential to the normal growth of HL60 cells. In order to allow comparison with DMSO treated cells, the expression of eIF4G was measured in HL60 cells induced to undergo granulocytic differentiation by RA treatment. This showed that when differentiation is induced with RA, the eIF4G response to the initial dilution of the HL60 cells with fresh media is suppressed. However, whichever differentiation agent is used, HL60 cells continue to grow until 48 hours after exposure to the stimulus. Thus, whilst the increase in eIF4G expression and association with eIF4E clearly occurs in response to addition of media containing FCS to HL60 cells, it may not be a growth related response.

It is possible that the increase in eIF4G expression and association with eIF4E observed when HL60 cells are diluted with fresh media, occurs in response to a stress inducing agent. Indeed, there is precedence for cultured mammalian cells adapting to low levels of a stress inducing agent (for example H$_2$O$_2$; Wiese et al., 1995). To test this possibility, HL60 cells were subjected to oxidative stress, induced by treatment with H$_2$O$_2$. eIF4G protein levels were measured in HL60 cells given different doses of H$_2$O$_2$ and over time; no increase in eIF4G was observed in response to these treatments. These data imply that oxidative stress does not reproduce the conditions HL60 cells are exposed to when the increase in eIF4G expression and association with eIF4E is observed.

However, treatment of HL60 cells with H$_2$O$_2$ did produce one very interesting result. At a dose of 250 μM H$_2$O$_2$, apoptosis appeared to be induced in the HL60 cells. Analysis of the eIF4G in these cells demonstrated a decrease in the level of intact eIF4G protein and what appeared to be a C-terminal cleavage product of eIF4G was observed in the same lane. These data suggest that eIF4G may be being proteolytically cleaved in HL60 cells undergoing apoptosis.
CHAPTER 6

DISCUSSION

In this thesis, cultured cells of haemopoietic origin have been used to study eukaryotic initiation factors and their regulation. Cell lines derived from patients with the B-cell tumours Burkitt's lymphoma and Multiple Myeloma were examined for changes in the initiation factors that might correlate with the transformed phenotype. HL60, a differentiation inducible cell line derived from a patient with acute promyelocytic leukaemia, was used to investigate alterations in protein synthesis, and its regulators, during granulocytic differentiation.

The main conclusions of this study are: i) expression of eIF4E does not correlate with c-Myc levels either in B-cell lines or in differentiating HL60 cells; ii) eIF2a expression does not correlate with c-Myc expression in the B-cell lines and is only responsive to c-Myc levels in differentiating HL60 cells during the first 10 hours of differentiation; iii) a cellular protease can cleave eIF4G into specific N- and C-terminal fragments, similar to those observed upon picornaviral infection, in the absence of any such infection; iv) during granulocytic differentiation of HL60 cells, a 2-fold increase in eIF4E phosphorylation occurs in the absence of any alteration in protein synthesis rate, suggesting that it may specifically affect the translation of a sub-set of mRNAs; v) formation of the eIF4F complex, as measured by monitoring changes in the association of eIF4G with eIF4E, can be mediated by alterations in the expression of eIF4G, implying that this component of eIF4F may be limiting in HL60 cells.

Many of the data presented in chapters 3, 4 and 5 are measurements of steady state protein levels produced using western blotting. Before discussing the implications of these data, it is worth noting some of the limitations of this technique. Three aspects of the detection method can affect the accuracy and reproducibility of the data: the X-ray film, the enhanced chemiluminesence detection system and the primary antibody. X-ray film imposes limitations on the range of luminescence that can be detected; if the signal is too weak, the resultant band may be too faint to be accurately quantitated, if the signal is too strong, the film may be 'burned-out', i.e. the maximum exposure of the film occurs and any luminescence above this level is not detected. Enhanced chemiluminesence detection systems are based on oxidation of a substrate which subsequently emit light; the reaction is catalysed by hydrogen peroxide and the peroxidase enzyme conjugated to the secondary antibody, amplification of the signal is achieved by performing the oxidation in the presence of a chemical enhancer which increases the light output and extends the time of light emission. As a result, if the substrate or components of the oxidation/amplification
system become limiting (e.g. over time) the accuracy of the signal produced can be
affected. Detection of individual proteins with a specific monoclonal or polyclonal
antibody is dependent upon the dilution at which the antibody is used and the
amount of protein loaded on the SDS-PA gel. Too high a concentration of antibody
can result in reduced specificity of detection and 'overloading' of the protein to be
detected can result in too strong a signal.

In order to minimise some of the effects of these limitations, the antibodies used for
western blotting were characterised in two ways. Initially, the optimum
concentration of antibody, which gave an acceptable signal with minimal
background in under 30 minutes (typically 1 to 10 minutes) was determined.
Additionally, the linear range of chemiluminescence detected by the X-ray film used
was determined for each antibody, as described in section 2.3.12, and experiments
performed so that the signal should fall within this range. However, comparison of
the data produced in this way with western blots quantitated using radio-labelled
secondary antibody or protein A and phosphorimaging could have been beneficial in
gauging what degree of accuracy was possible with this method. It could also have
been useful to support some of the data presented with other techniques. For
example, assays of activity (e.g. of the eIF4F complex), or measurements of synthesis
and degradation rates of individual proteins (e.g. eIF2α and eIF4E) could have been
performed in addition to the measurement of steady state protein levels.

6.1 Regulation of eIF4E and eIF2α Expression by c-Myc

Expression of eIF4E and eIF2α has been shown to correlate with that of c-myc under a
number of conditions, including in growth stimulated fibroblasts, c-myc transformed
fibroblasts and in response to induction of the myc-ER construct (Rosenwald et al.,
1993b). The promoters of both eIF4E and eIF2α have been shown to possess E-box
elements (Rosenwald et al., 1993b; Jones et al., 1996) and in the eIF4E promoter, this
sequence has been shown to bind Myc-Max complexes and to be essential for
transcription (Jones et al., 1996). Hence, eIF4E and eIF2α fulfil a number of the
criteria which define c-myc target genes (Henriksson & Lüscher, 1996 and see section
1.3.3). However, much remains to be determined about the regulation of eIF4E and
eIF2α by c-Myc. To this end, expression of eIF4E, eIF2α and c-myc was examined in
B-cell tumour lines that overexpress c-myc and during the granulocytic
differentiation of HL60 cells.

Protein levels of c-Myc, eIF4E and eIF2α were measured in the panel of B-cell lines
derived from Multiple Myeloma patients, Burkitt's lymphoma patients and normal
healthy donors. As expected, high c-Myc was detected in the MM cell line GM2132
and the BL cell lines Raji and BL29, compared to the control LCLs. However,
increased protein levels of eIF4E and eIF2α were only observed in the BL29 cells. This pattern of expression was confirmed when levels of eIF4E and eIF2α mRNA were measured. Additionally, the small variations in c-Myc that were observed in the LCLs were not accompanied by differences in eIF4E or eIF2α. These data suggest that in these B-cell lines, with the possible exception of BL29, eIF4E and eIF2α expression is not responsive to c-Myc.

Protein levels of eIF4E and eIF2α were also monitored in HL60 cells stimulated to undergo granulocytic differentiation. During this process c-Myc levels fall rapidly, to 30% of the original level within 2 hours. However, eIF4E protein levels do not follow this pattern and remain at a similar level to that observed in untreated cells until 48 hours after induction of differentiation. In contrast, expression of eIF2α does correlate with that of c-Myc, during the first 10 hours of differentiation. By this time, eIF2α protein levels have decreased to 48% of those in undifferentiated cells. However, 24 hours after differentiation is initiated, eIF2α expression increases, returning to 87% of original levels.

In summary, expression of eIF4E does not appear to be regulated by c-Myc in either of the systems studied. Likewise, eIF2α expression does not correlate with that of c-myc in the B-cell lines. The only exception to this is the BL29 cell line which displays high levels of both eIF4E and eIF2α mRNA and protein. However, in the absence of a similar correlation in the other cell lines it is difficult to conclude whether the increased expression of eIF4E and eIF2α is a direct consequence of high c-Myc levels or whether the high levels of these proteins reflect the transformed status of these cells. Expression of eIF2α does appear to be regulated by c-Myc during the early stages of granulocytic differentiation. It is however, also apparent that other factors influence eIF2α protein levels during differentiation, since from 24 hours levels of the two proteins no longer correlate. In order to conclude whether these changes in eIF2α expression are a direct result of the alterations in c-Myc levels it would be necessary to examine transcription of eIF2α mRNA and to determine if any reduction in eIF2α mRNA in response to decreased c-Myc occurred in the absence of protein synthesis. It would also be of interest to measure rates of synthesis and degradation of eIF2α protein to determine how these might contribute to the demonstrated change in steady state protein levels.

For comparison with eIF4E and eIF2α, ODC, a well characterised c-Myc target gene, was also examined in a selection of the B-cell lines. ODC mRNA expression and enzyme activity were both measured in the MM cell lines GM2132 and GM1311, the LCLs GM1953 and GM03201 and the BL cell line Raji. Of these cell lines, high levels of ODC mRNA were only observed in GM2132; in contrast, Raji cells, which have comparable levels of c-Myc to GM2132 cells, did not display high ODC mRNA levels.
Although the high expression of ODC mRNA in GM2132 cells could be attributed to the high level of c-Myc in these cells, ODC enzyme activity was at the lower limits of detection in the GM2132 cell line, implying that ODC expression is post-transcriptionally repressed in GM2132 cells. These observations in GM2132 cells emphasise the point that de-regulation of a gene which has the potential to transform cells requires loss of more than one level of control.

ODC mRNA and enzyme activity were not examined in differentiating HL60 cells because it had been shown previously that ODC mRNA does not decrease until 48 hours after stimulation of granulocytic differentiation (Rius & Aller, 1989). Additionally, another report has shown that ODC activity increases during granulocytic differentiation of HL60 cells, peaking at 24 hours (Luk et al., 1982). Hence, expression of ODC mRNA is not responsive to c-Myc in differentiating HL60 cells and once again, post-transcriptional control of ODC is observed.

The examination of eIF4E and eIF2α expression demonstrates that the relationship between these two initiation factors and c-myc is more complex than has been suggested by previous studies. However, this is not entirely unprecedented since many of the c-Myc target genes characterised to date do not respond to alterations in c-Myc levels in all circumstances. For example, whilst expression of α-prothymosin does respond to the decrease in c-Myc in differentiating HL60 cells (Smith et al., 1993), α-prothymosin is not responsive to constitutive expression of c-myc or to activation of Myc-ER in proliferating cells (Eilers et al., 1991; Mol et al., 1995). In addition, as described above, ODC expression does not correlate with that of c-myc in differentiating HL60 cells.

The evidence that eIF4E and eIF2α can be transcriptionally regulated by c-Myc, as summarised at the beginning of this section, is convincing. However, these determinations have been carried out in a limited number of systems. One reason for examining the relationship between expression of c-myc, eIF4E and eIF2α in cells of haemopoietic origin was to add to the existing evidence for regulation of the two initiation factors by c-Myc. However, the data presented here show that levels of eIF4E and eIF2α proteins do not always correlate with c-myc expression. In particular, eIF4E protein levels do not alter in response to the decrease in c-Myc observed in HL60 cells undergoing granulocytic differentiation, implying either that after the decrease in c-Myc eIF4E protein levels are maintained by some other mechanism (e.g. increased protein stability) or that some other transcription factor can maintain eIF4E mRNA levels in the absence of c-Myc. Interestingly, it has recently been shown that two novel regulatory factors interact with the promoter of eIF4E (Johnston et al. 1998), suggesting that factors other than c-Myc may indeed regulate transcription of eIF4E mRNA.
Although the data from this study do not show a correlation between expression of 
c-myc, eIF4E and eIF2α under most of the conditions examined, they do not 
necessarily disprove the hypothesis that c-Myc transcriptionally regulates the 
expression of these initiation factors. However, they do suggest a modification of 
this hypothesis to one which proposes that c-Myc can transcriptionally regulate 
eIF4E and eIF2α, but is not the only factor which is able to do so and is not always 
the major determinant of their expression.

6.2 Proteolytic Cleavage of eIF4G

Determination of eIF4G protein levels using western blot analyses produced an 
interesting observation in two different situations during the course of this study. In 
HL60 cells treated with 250 μM H₂O₂ and in GM2132 cells, reduced amounts of 
eIF4G protein were observed and an apparent C-terminal cleavage product of eIF4G 
was detected. N-terminal cleavage products of eIF4G were also detected in GM2132 
cells, and it was demonstrated that GM2132 cell extracts could promote cleavage of 
exogenous eIF4G in a control cell line extract. The fragments detected are strikingly 
similar to those produced by picornaviral 2A proteases and the FMDV L protease 
(Lamphear et al., 1995; Lamphear & Rhoads, 1996).

These observations suggest that eIF4G has been specifically cleaved into N- and C-
terminal fragments by a cellular protease. There is a precedent for cleavage of eIF4G 
in this manner by a non-picornaviral protease. Whilst direct cleavage of eIF4G by 2A 
and L proteases has been demonstrated (Lamphear et al., 1995; Haghighat et al., 
1996), recent investigations have suggested that the predominant eIF4G cleavage 
activity in poliovirus infected cells is a cellular factor which is activated by the 2A 
protease (Bovee et al., 1998).

What might be the consequence of eIF4G cleavage in these cells? It has been shown 
that the C-terminal domain of eIF4G is sufficient to support translation mediated by 
an IRES in the absence of intact eIF4G (Ohlmann et al., 1996; Pestova et al., 1996b). 
The presence of an eIF4G C-terminal fragment in GM2132 cells and H₂O₂ treated 
HL60 cells therefore suggests that translation of mRNAs containing an IRES might be 
facilitated in these cells.

In the case of GM2132 cells, it has been shown that c-myc expression is up-regulated 
by a translational mechanism (Paulin et al., 1996). Whilst a disparity between c-myc 
mRNA and protein levels has been demonstrated in all four of the MM cell lines used 
in the present study, GM2123 cells display the greatest increase in c-Myc protein 
levels (Paulin et al., 1996). Given that c-myc mRNA contains an IRES (Nanbru et al.,
1997; Stoneley et al., 1998), it is possible that the translational increase in c-myc expression observed in GM2132 cells could, in part, be mediated by the cleavage of eIF4G.

When HL60 cells were treated with 250 µM H₂O₂, significant cell death resulted and apoptosis had presumably been induced. Cleavage of eIF4G was not observed when lower doses of H₂O₂ were administered that did not cause cell death. Thus, cleavage of eIF4G appears to be occurring when apoptosis is induced in HL60 cells. Whilst this is an isolated observation and must not be over-interpreted, it does present some interesting possibilities. Apoptosis is an active process which in its early stages often requires both RNA and protein synthesis, it is also characterised by cascades of proteases which specifically degrade a number of proteins (reviewed in Martins & Earnshaw, 1997). It is possible that the observed cleavage of eIF4G is part of this process and contributes to a general shut down of protein synthesis. However, the C-terminal fragment of eIF4G appears to be intact, raising the intriguing possibility that there is a role for internal initiation of translation during apoptosis. It is possible to speculate that whilst cap-dependent protein synthesis is shut off, proteins required for apoptosis are synthesised via internal ribosome entry.

6.3 Translational Control During Granulocytic Differentiation of HL60 Cells

Two aspects of translational control, mediated by eIF4F, were investigated in HL60 cells stimulated to undergo granulocytic differentiation: phosphorylation of eIF4E and formation of the eIF4F complex.

6.3.1 eIF4E Phosphorylation

Measurement of the percentage phosphorylation of eIF4E in HL60 cells induced to differentiate demonstrated that an increase in eIF4E phosphorylation, from an average of 21% to 39%, occurs within 6 hours of DMSO treatment. No alteration in the rate of protein synthesis occurs in differentiating HL60 cells that can be attributed to this increase in eIF4E phosphorylation. From 10 hours, eIF4E phosphorylation decreases, but remains above the level in untreated HL60 cells for the remainder of the experiment. Concomittant with this, protein synthesis decreases, to a rate approximately 50% of that in untreated cells within 48 hours of DMSO addition and decreases further over the final 48 hours. Thus, the alterations in eIF4E phosphorylation do not appear to affect global protein synthesis rates.

Whilst it is conceivable that the observed increase in eIF4E phosphorylation has no effects on translation, it is more likely that such a clearly defined stimulation of eIF4E
phosphorylation, occurring during the early hours of induction of differentiation, does have a purpose. It is possible that the increase in eIF4E phosphorylation has an effect on the translation of mRNAs which are translationally repressed by their 5′ UTRs. Enhanced eIF4E expression has been shown to increase the expression of mRNAs with long, structured 5′ UTRs (Koromilas et al., 1992a) and it is possible that increased eIF4E phosphorylation may have a similar effect. One potential target for such regulation might be the limiting enzyme in the polyamine biosynthesis pathway, ODC. This is suggested by the fact that translation of ODC has been shown to be enhanced in insulin treated cells, when eIF4E phosphorylation increases (Manzella et al., 1991) and that, as previously mentioned, ODC activity increases, independently of any rise in its mRNA level, during granulocytic differentiation of HL60 cells (Luk et al., 1982; Rius & Aller, 1989).

In order to address this possibility and to identify other mRNAs that could be regulated in this way, a method for detecting changes in the mRNA associated with eIF4E was conceived. It was shown that immunoprecipitation of eIF4E allowed specific isolation of RNA which could be purified, reverse transcribed and used for PCR reactions. Time did not allow for this method to be developed to the point where changes in specific mRNAs bound by eIF4E could be quantitated. However, in the future this technique could be developed further to examine changes in the cap-dependent translation of specific mRNAs, or to be used as part of a differential display process for the identification of translationally regulated genes.

The signals which lead to the increase in eIF4E phosphorylation during granulocytic differentiation of HL60 cells were investigated. It was demonstrated that neither the FRAP/TOR signalling pathway nor the Erk mediated MAP kinase pathway is required for the increase in eIF4E phosphorylation to occur. Increases in eIF4E phosphorylation have been shown to be mediated by a number of distinct MAP kinase pathways, depending upon the stimulus used (Flynn & Proud, 1996a, b; Morley, 1997; Morley & McKendrick, 1997; Wang et al., 1998). In addition to the growth factor stimulated (Erk) MAP kinase pathway, eIF4E phosphorylation has been shown to be regulated by the p38, stress induced, MAP kinase pathway; both of these signalling pathways are thought to target Mnk1 which phosphorylates eIF4E (Fukunaga & Hunter, 1997; Waskiewicz et al., 1997; Wang et al., 1998). Thus, it is conceivable that the stimulation of eIF4E phosphorylation observed in differentiating HL60 cells is mediated by the p38 MAP kinase pathway. It would be of interest to test this proposal by determining whether the p38 pathway inhibitor, SB203580, prevents the increase in eIF4E phosphorylation.

Whilst the Erk MAP kinase pathway inhibitor, PD98059, did not inhibit an increase in eIF4E phosphorylation in differentiating HL60 cells, this inhibitor did cause a
reduction in eIF4E phosphorylation, to less than 2%, in proliferating HL60 cells. This observation implies that the growth factor stimulated MAP kinase pathway is required to maintain basal levels of phosphorylation in proliferating HL60 cells. There have been previous reports which demonstrate that one or more phosphatases are involved in regulating eIF4E phosphorylation, in addition to the effects of kinases. In particular, okadaic acid, an inhibitor of the serine/threonine phosphatases 1 and 2A, increases the phosphorylation of eIF4E (Donaldson et al., 1991) or inhibits its de-phosphorylation (Rychlik et al., 1990). Furthermore, it has been shown that an increase in eIF4E phosphorylation in activated porcine peripheral blood lymphocytes is partially due to inhibition of phosphatase activity (Morley & Pain, 1995b). It seems possible therefore that the steady state phosphorylation of eIF4E in proliferating HL60 cells reflects the activity of both phosphatases and the Erk MAP kinase pathway.

It is also interesting to note that the percentage phosphorylation of eIF4E measured after 6 hours, in HL60 cells treated with both PD98059 and DMSO, is similar to that in cells treated with DMSO alone. Hence, the increase in eIF4E phosphorylation in PD98059 treated cells is greater than in control cells. This observation suggests that an increase in eIF4E phosphorylation to this level is of significance in HL60 cells stimulated to undergo granulocytic differentiation.

6.3.2 eIF4F Complex Formation

To assess changes in the formation of the eIF4F complex, the association of eIF4G with eIF4E was determined. When HL60 cells fully 'adapted' to the cell culture conditions used in this study were induced to differentiate, no significant alterations in the association of eIF4G with eIF4E occurred. The variations in the ratio of eIF4G to eIF4E that were observed were all less than 2-fold in magnitude and were not reproducible. Whilst the ratio of eIF4G to eIF4E is not a direct measure of the amount of eIF4F complex in the cells, the absence of any alteration in this ratio suggests that no significant variation in eIF4F is occurring in differentiating HL60 cells.

The eIF4E binding proteins were also examined in the HL60 cell line. Both 4E-BP1 and 4E-BP2 are expressed in HL60 cells and no alteration in the level of either protein occurs during granulocytic differentiation. The different phospho-isoforms of 4E-BP1 are resolved by SDS-PAGE and as a result, western blots also showed that the majority of the 4E-BP1 detected in HL60 cells exists as the middle phospho-isoform of the three. Since it is the least-phosphorylated, fastest migrating, phospho-isoform of 4E-BP1 which binds eIF4E, 4E-BP1 association with eIF4E was not detected at any time during the differentiation of HL60 cells.
In contrast, 4E-BP2 association with eIF4E was detectable. Measurement of the association of 4E-BP2 with eIF4E in differentiating HL60 cells demonstrated alterations in their association. There is a decrease in the ratio of 4E-BP2 associated with eIF4E within 1 hour of DMSO treatment, a return to the initial ratio at 10 hours and another decrease at 24 hours. This implies that at 1 and 24 hours, an increase in the amount of free eIF4E occurs in differentiating HL60 cells. However, this increase in free eIF4E is not reflected by an increase in the amount of eIF4G associated with eIF4E. It is therefore difficult to suggest what role the alteration in association of 4E-BP2 with eIF4E plays in differentiating HL60 cells.

This investigation of the factors which regulate eIF4F complex formation and activity points to one further conclusion. When HL60 cells are stimulated to differentiate with DMSO, it is necessary to dilute them with fresh media at the beginning of the experiment. Measurement of protein synthesis rates in diluted HL60 cells indicated that a serum stimulated increase in protein synthesis rate occurs during the first 10 hours, irrespective of DMSO addition. Given that an increase in eIF4E phosphorylation only occurs in the presence of DMSO, and that no alteration in the association of eIF4G with eIF4E was detected, it must be concluded that another component of the translational machinery is responsible for the observed increase in protein synthesis. Although a decrease in the expression of eIF2α occurs in DMSO treated HL60 cells, it could be speculated that regulation of either eIF2α or eIF2B phosphorylation plays a role in the serum stimulated increase in protein synthesis.

6.4 Regulation of eIF4F Complex Formation by Alterations in eIF4G Expression

In newly acquired HL60 cells an increase in eIF4G protein levels and in association of eIF4G with eIF4E was observed, during the first 24 hours after addition of fresh media plus serum. It became apparent that this phenomenon was variable in magnitude and could not be detected in HL60 cells that had been in culture for over 2 months, which had presumably ‘adapted’ to the new culture conditions. Nonetheless, these observations are interesting in that when the phenomenon was observed, the increase in association of eIF4G with eIF4E always correlated with an increase in eIF4G protein levels. These observations suggest that the increase in eIF4G protein causes the increased association of eIF4G with eIF4E.

It is commonly asserted that eIF4E is the limiting component of eIF4F and the affects of alterations in the availability or activity of this protein attest to its regulatory function. However, measurement of relative levels of initiation factors has only been carried out in two systems. In HeLa cells eIF4E was reported to be the least
abundant initiation factor (Duncan et al., 1987) and whilst a similar result was reported in reticulocyte lysate (Hiremath et al., 1985) a more recent determination suggests that there is a functional excess of eIF4E in reticulocyte lysate (Rau et al., 1996). Overexpression of eIF4G has been shown to transform NIH3T3 cells, in the absence of an increase in eIF4E expression (Fukuchi-Shimogori et al., 1997). This demonstrates that an increase in eIF4G protein can have a profound effect on translation and this does not require greater amounts of eIF4E, indeed, if the affects of eIF4G overexpression are mediated by increased eIF4F formation, then eIF4E cannot be limiting in this system.

Likewise, the fact that alterations in the association of eIF4G with eIF4E appear to be caused by changes in the level of eIF4G protein suggests a similar conclusion. Thus, in HL60 cells, eIF4G may be the limiting component of eIF4F, rather than eIF4E. This proposal also explains why changes in the association of 4E-BP2 with eIF4E are not reflected by altered association of eIF4G with eIF4E; if eIF4E is not limiting, small changes in the availability of this protein would be of no consequence. Increased expression of eIF4E has been reported in a number of transformed cell lines and in breast carcinomas (see section 1.2.6). It is possible that in the HL60 cell line, which was derived from a patient with acute promyelocytic leukaemia, eIF4E is expressed at abnormally high levels. However, given the increasing evidence that eIF4G is involved in translational regulation, it would be of interest to determine which component of eIF4F is least abundant in normal mammalian tissues.
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