Identification and functional analysis of internal ribosome entry segments in Apaf-1 and BAG-1 mRNAs

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Abstract

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The apoptotic protease activating factor (Apaf-1) plays a central role in apoptosis: interaction of this protein with procaspase-9 leads to cleavage and activation of this initiator caspase. In common with other mRNAs whose protein products have a major regulatory function, the 5' untranslated region (UTR) of Apaf-1 is long, G-C rich and has the potential to form secondary structure. An internal ribosome entry segment (IRES) was identified in the 5' UTR of Apaf-1, located in a 233 nucleotide region towards the 3' end of the leader. The Apaf-1 IRES is active in several cell lines, although to differing degrees, which suggests an important requirement for non-canonical trans-acting factors in internal ribosome entry.

BAG-1 (also known as RAP46/HAP46) was originally identified as a 46 kDa protein that bound to and enhanced the anti-apoptotic properties of Bcl-2. BAG-1 exists as three major isoforms (designated p50, p46 and p36 or BAG-1L, BAG-1M and BAG-1S respectively) that are translated from a common transcript. The 5' untranslated region upstream of the p36 open reading frame (ORF) is also long and G-C rich and analysis of this region indicated that the translation initiation of the most highly expressed isoform (p36/BAG-1S) can occur by both internal ribosome entry and by cap-dependent scanning. The BAG-1 IRES also exhibits different activity in several cell lines, but this does not correlate with the changes in BAG-1 isoform expression observed in transformed cells.

Functional analysis of the Apaf-1 and BAG-1 IRESs show that they are active during the early stages of apoptosis induced by TNF-related apoptosis inducing ligand (TRAIL), which has also been observed with other IRESs. The IRESs do not appear to function during mitosis but following heat shock, when there is a downregulation of cap-dependent translation, the expression of the p36 isoform of BAG-1 is maintained by internal ribosome entry.
Acknowledgments

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Thanks also to Anne for finding me a project that has kept me occupied after a couple of false starts (which didn’t even make “good thesis data”!). Your enthusiasm always keeps us going, although the lab meeting a couple of weeks after giving birth to Edward perhaps showed us your dedication to work can be taken too far.

In addition I would like to thank my friends and family for their support, especially to Nana, who would have been extremely proud of me for getting this far. A special thanks goes to Nic for putting up with my slow writing pace, and for checking me grammar and speling even if you don’t know one end of a ribosome from the other.

Finally, here’s a little comment I found that I hope will inspire you with confidence...

“Science is always wrong. It never solves a problem without creating ten more.”

George Bernard Shaw (1856-1950)
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Abbreviations

4E-BP  eIF4E-binding protein
AMV   Avian myeloblastosis virus
Apaf-1 Apoptotic protease activating factor 1
ATP   adenosine 5'-triphosphate
ATPase adenosine triphosphatase
BAG-1 Bcl-2 associated athanogene 1
BiP   immunoglobulin heavy chain binding protein
bp    base pairs
BSA   bovine serum albumin
CaMV  Cauliflower Mosaic virus
CARD  caspase recruitment domain
CAT   chloramphenicol acetyltransferase
cDNA  complementary DNA
ced   cell death abnormal
CIAP  calf intestinal alkaline phosphatase
CMV   cytomegalovirus
cpm   counts per minute
CTP   cytidine 5'-triphosphate
DAP5  Death-associated protein 5
dATP  deoxyadenosine 5'-triphosphate
dCTP  deoxycytidine 5'-triphosphate
ddNTP dideoxynucleotide 5'-triphosphate
DEAE-Dextran  Diethylaminoethyl-Dextran
DED   Death effector domain
dGTP  deoxyguanosine 5'-triphosphate
DISC  Death-inducing signalling complex
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  Dimethyl Sulfoxide
DNA   deoxyribonucleic acid
DNase deoxyribonuclease
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<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine 5′-triphosphate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminoethanetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methanesulfonate</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electromobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAG</td>
<td>Fragment of apoptotic cleavage of eIF4G</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>FLASH</td>
<td>FLICE-associated huge protein</td>
</tr>
<tr>
<td>FMDV</td>
<td>foot and mouth disease virus</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>Grp78</td>
<td>glucose-regulated protein 78</td>
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<tr>
<td>GTP</td>
<td>guanosine 5′-triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanidine triphosphatase</td>
</tr>
<tr>
<td>Hap46</td>
<td>hsp70- and hsc70-associating protein</td>
</tr>
<tr>
<td>HAV</td>
<td>hepatitis A virus</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HL60</td>
<td>human leukemic cell line 60</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heteronuclear ribonuclear protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Hop</td>
<td>hsp70-hsp90 organising protein</td>
</tr>
<tr>
<td>HRI</td>
<td>Hemin-regulated inhibitor kinase</td>
</tr>
<tr>
<td>HRV</td>
<td>human rhinovirus</td>
</tr>
<tr>
<td>Hsc</td>
<td>heat shock cognate</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IGFII</td>
<td>insulin-like growth factor II</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin class G</td>
</tr>
<tr>
<td>IL-3</td>
<td>interleukin-3</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry segment</td>
</tr>
<tr>
<td>ITAF</td>
<td>IRES-specific cellular <em>trans</em>-acting factor</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kcal</td>
<td>kilocalorie</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>met-tRNA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>initiator methionyl tRNA</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanesulfonate</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>Mnk1</td>
<td>MAP kinase interacting kinase 1/ MAP kinase signal integrating kinase 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear factor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-Nitrophenyl β-D-Galactopyranoside</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PABP</td>
<td>poly(A) binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCBP2</td>
<td>poly(rC) binding protein 2</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF2</td>
<td>platelet derived growth factor 2</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-endoplasmic reticulum-related kinase</td>
</tr>
<tr>
<td><em>Pfu</em></td>
<td><em>Pyrococcus furiosus</em></td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N, N'-bis[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>PKR</td>
<td>RNA-activated protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTB</td>
<td>polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>PV</td>
<td>poliovirus</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAP46</td>
<td>Receptor associated protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonucleic acid hydrolyase</td>
</tr>
<tr>
<td>RNasin</td>
<td>ribonucleic acid hydrolase inhibitor</td>
</tr>
<tr>
<td>RRL</td>
<td>rabbit reticulocyte lysate</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous Sarcoma virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SELEX</td>
<td>Selective evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td><em>Taq</em></td>
<td><em>Thermophilus aquaticus</em></td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TLCK</td>
<td>Naα-p-tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>uORF</td>
<td>upstream open reading frame</td>
</tr>
<tr>
<td>unr</td>
<td>upstream of n-ras</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5’-triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
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Chapter One

Introduction

1.1 Eukaryotic protein synthesis

Translational control plays a considerable role in overall gene expression in both prokaryotes and eukaryotes. Control of translation can be directed at either overall protein synthesis in the cell (global control) or the translation of subsets of messenger RNAs (mRNAs) or even, in some cases, a single mRNA (selective control).

Eukaryotic protein synthesis comprises three stages: initiation, elongation, and termination. It is at the level of initiation that most cases of translational control occur and it is possible that up to 10% of eukaryotic genes could have their expression regulated by a translational mechanism (Willis, 1999).

The initiation phase refers to the binding of the ribosomal subunits to the mRNA and positioning of the subunits at the first codon of the open reading frame (ORF) of a mRNA. The rate of initiation determines the rate of protein synthesis of most mRNAs and thus is thought to be the rate-limiting step of this process (Pain, 1996; Mathews et al., 2000).

Once the translation machinery is in place at the start codon, the elongation phase begins. The message is decoded and a polypeptide assembled from the correct tRNA-bound amino acids by repeated cycles of tRNA binding, peptide bond formation, ribosomal translocation and release of the spent tRNA. During termination, a stop codon is recognised and the final peptidyl-tRNA bond is hydrolysed, the nascent polypeptide chain is released, and the ribosomal subunits dissociate from the mRNA.
1.2 Eukaryotic initiation factors (eIFs)

The events of the initiation pathway can be divided into four basic steps. Following a termination event, the 80S ribosome dissociates into 40S and 60S subunits. The 40S ribosomal subunit is then bound by the initiator methionyl-tRNA complex (met-tRNA$_i$) to create the 43S preinitiation complex. This complex binds to the mRNA and migrates to the initiation codon. Once in position at the initiation codon, the 40S subunit associates with the 60S subunit to create the 80S ribosome, which is then competent to translate the open reading frame (ORF) of the message.

Each step of initiation requires the presence of several eukaryotic initiation factors (eIFs). The properties of the canonical eIFs, many of which are protein complexes, are summarised in Table 1.1. The activities of many of the eIFs, and hence the control of global and selective protein synthesis, can be regulated by phosphorylation in response to many intracellular signalling pathways and also by modification by specific cleavage events.

1.3 Cap-dependent translation initiation

1.3.1 Dissociation of ribosomal subunits and formation of the 43S preinitiation complex

The majority of ribosomal subunits in the cell are found as associated 80S ribosomes and the subunits must be dissociated before initiation can occur. The smaller 40S subunit is maintained in a dissociated form by the binding of eIF1A and eIF3, while binding of the eIF6 protein to the larger 60S subunit prevents association with the smaller 40S subunit (Figure 1.1A).

The formation of the ternary complex and binding of the unique initiator methionyl tRNA (Met-tRNA$_i$) to the 40S ribosome is thought to be a rate-limiting step and the initiation
<table>
<thead>
<tr>
<th>Name</th>
<th>Role</th>
</tr>
</thead>
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<tr>
<td>elF1</td>
<td>AUG recognition</td>
</tr>
<tr>
<td>elF1A</td>
<td>Met-tRNA, binding to 40S subunit, 40S dissociation</td>
</tr>
<tr>
<td>elF2</td>
<td>Three subunits: α binds elF2B, β binds elF2B and elF5, γ binds GTP and Met-tRNA, and has GTPase activity</td>
</tr>
<tr>
<td>elF2B</td>
<td>Guanine nucleotide exchange factor (GEF) for elF2</td>
</tr>
<tr>
<td>elF3</td>
<td>Eleven subunits, known functions include binding to RNA, elF1, elF4B, elF4G, elF5 and 40S subunits</td>
</tr>
<tr>
<td>elF4A</td>
<td>ATP-dependent RNA helicase</td>
</tr>
<tr>
<td>elF4AI</td>
<td>ATP-dependent RNA helicase</td>
</tr>
<tr>
<td>elF4B</td>
<td>Binds RNA, stimulates elF4A helicases</td>
</tr>
<tr>
<td>elF4E</td>
<td>Binds 5' terminal m'GTP cap structure</td>
</tr>
<tr>
<td>elF4GI</td>
<td>Binds elF4E, 4A, 3, PABP and RNA</td>
</tr>
<tr>
<td>elF4GII</td>
<td>Binds elF4E, 4A, 3, PABP and RNA</td>
</tr>
<tr>
<td>elF5</td>
<td>Stimulates GTPase activity of elF2 (GAP)</td>
</tr>
<tr>
<td>elF5B</td>
<td>Joining of 60S subunit at initiation codon</td>
</tr>
<tr>
<td>elF6</td>
<td>Binding and dissociation of 60S ribosomal subunit</td>
</tr>
</tbody>
</table>

**Table 1.1** Properties of the eukaryotic initiation factors (elFs)
Figure 1.1 Assembly of the 43S preinitiation complex

(A) Dissociation of ribosomal subunits by the binding of eukaryotic initiation factors eIF1A, eIF3 and eIF6.

(B) The inert eIF2-GDP complex is recycled by the guanine nucleotide exchange factor (GEF) eIF2B. The active eIF2-GTP binary complex joins to the initiator methionyl tRNA (Met-tRNA) to form the ternary complex.

(C) The ternary complex binds to the dissociated 40S subunit to form the 43S preinitiation complex.
factor eIF2 plays a crucial role in the regulation of initiation (Hershey and Merrick, 2000). During release of the initiation factors from the previous round of initiation, GTP bound to eIF2 is hydrolysed to GDP. The binary complex of eIF2-GDP cannot bind Met-tRNA, so eIF2B binds to eIF2-GDP and catalyses nucleotide exchange, recycling eIF2 into the active GTP bound form (Figure 1.1B). The eIF2-GTP complex then joins with the Met-tRNA, and this ternary complex binds the eIF1A- and eIF3-complexed 40S ribosomal subunit to form the 43S preinitiation complex (Figure 1.1C).

Ternary complex formation is inhibited by the phosphorylation of eIF2 at Ser51 of the α subunit by four kinases, HRI, PKR, PERK and GCN2, which are activated in response to cellular stress. The phosphorylation tightens the binding of eIF2 to eIF2B and prevents the recycling of GDP to GTP, therefore maintaining eIF2 in an inert form.

1.3.2 Initiation factor recruitment to the mRNA

Following transcription, mRNA synthesised in the nucleus is processed. All cellular eukaryotic RNAs are capped with an m⁷GTP structure, spliced and polyadenylated before being exported from the nucleus as part of a messenger ribonucleoprotein complex (mRNP). The cap structure is important for pre-mRNA splicing and nucleocytoplasmic transport and also increases the efficiency of translation. Polyadenylation of the messages also helps stimulate translation as well as stabilising the message (Sachs, 2000b).

To allow binding of the preinitiation complex the cap-binding complex eIF4F assembles at the 5′ end of the mRNA. The cap structure is bound by one of the components of the eIF4F complex, eIF4E, which resembles a cupped hand (Marcotrigiano et al., 1997; reviewed in Hershey and Merrick, 2000). The cap is bound by the concave surface of the protein and the convex surface of eIF4E interacts with eIF4G, which is in turn bound to
eIF4A, the final subunit of the heterotrimeric eIF4F complex. eIF4E can bind the m$^7$GTP cap structure alone but when it is part of the eIF4F complex, cap binding is greatly enhanced.

The abundance and phosphorylation state of eIF4E limits the rate of initiation in the cell. A family of binding proteins, the 4E-BPs, can reduce the amount of eIF4E available to form eIF4F complexes by competing for eIF4E binding with eIF4G. Hyperphosphorylation of the 4E-BPs in response to many types of extracellular stimuli, including hormones, growth factors and mitogens, greatly reduces their affinity for eIF4E resulting in an increase in the rate of cap-dependent translation (Figure 1.2A). Phosphorylation of eIF4E by the MAP kinase interacting kinase/MAP kinase signal integrating kinase (Mnk1) in response to the ERK and p38 MAP kinase pathways also increases the affinity of cap binding (Raught et al., 2000).

More than 90% of mRNAs are considered “strong” mRNAs that can be translated efficiently. However, certain mRNAs that encode growth regulatory proteins have long, highly structured 5' UTRs that make them a poor substrate for eIF4F binding. When more eIF4E is available to bind eIF4G, this repression is relieved. It is not surprising, therefore, that the overexpression of eIF4E induces malignant transformation of cultured cells by activating the translation of many of these genes such as c-myc, cyclin D1 and ODC (reviewed in De Benedetti and Harris, 1999). Examination of eIF4E levels in many types of naturally occurring tumour cells found that the protein was overexpressed in a broad range of tumours. There is an especially large difference in eIF4E levels between normal and breast cancer cells (reviewed in Hershey and Miyamoto, 2000).

The largest component of the eIF4F complex, eIF4G, exists as two isoforms in the cell (eIF4GI and eIF4GII) that share 46% identity at the amino acid level. Both proteins can act as a "multipurpose ribosome adapter" (Hentze, 1997) as eIF4G acts as a scaffold on which many other initiation factors can bind (Gingras et al., 1999). Along with the interaction with eIF4E, eIF4G also binds RNA, eIF3, the eIF4E kinase Mnk1 and the poly(A)-binding protein (PABP)
Figure 1.2 48S preinitiation complex formation
(A) The cap-binding protein eIF4E is sequestered by a family of binding proteins, the 4E-BPs. Phosphorylation of 4E-BPs in response to positive growth signals releases eIF4E, allowing eIF4F formation.
(B) Phosphorylation of eIF4E by the kinase Mnk1 increases the affinity for cap-binding.
(C) The 43S preinitiation complex assembles onto the mRNA via interactions between eIF3 and eIF4G, the major component of the eIF4F complex.
(Figure 1.2B). The binding of PABP to eIF4G allows interactions between the 5' and 3' ends of a message and may play a role in the efficient recycling of ribosomes between rounds of translation (Hershey and Merrick, 2000). A third isoform of eIF4G, known variously as p97, NAT1 and DAP5 (Imataka et al., 1997; Yamanaka et al., 1997; Levy-Strumpf et al., 1997) lacks the amino terminal third of eIF4G and may inhibit cap-dependent translation by forming initiation complexes that lack eIF4E and PABP (Imataka et al., 1997). A potential role for DAP5 in cap-independent translation will be discussed later (Section 1.6.2).

1.3.3 mRNA binding and scanning of the 48S preinitiation complex

Interaction between eIF3 and eIF4G allows the 43S preinitiation complex to bind to the mRNA (Figure 1.3A). The complex is hypothesised to migrate or “scan” through the 5' untranslated region (UTR, sometimes referred to as the non-coding region or NCR) of the mRNA to the initiation codon. Regions of secondary structure that occur by intramolecular hydrogen bonding in the mRNA are unwound by the ATP-dependent helicase activity of eIF4A, which is enhanced by eIF4B (Figure 1.3B) (Hershey and Merrick, 2000).

1.3.4 Selection of the initiation codon, release of initiation factors and elongation

The 5' untranslated regions of most eukaryotic mRNAs are short (typically 20-100 nt) and, in the scanning model, the first AUG codon is used to initiate translation. Investigation of 699 eukaryotic mRNAs identified a consensus sequence of GCC(A/G)CCAUGG surrounding the initiation codon (Kozak, 1987). The nucleotides of particular importance in this sequence are the G at +4 (where the A of the AUG is designated +1) and the purine at -3. Other codons (CUG, ACG and GUG) can also initiate translation but again this is dependent on the context of the surrounding sequence (Kozak, 1989).
Figure 1.3 Ribosome migration to the initiation codon

(A) The 48S preinitiation complex begins to scan through the 5’ untranslated region (UTR). Unwinding of regions of secondary structure is facilitated by the ATP-dependent helicase activity of eIF4A, in concert with eIF4B (not shown).

(B) The ribosome continues to scan until an initiation codon in good context (GCC(A/G)CCAUGG) is recognised. This recognition is aided by eIF1 and eIF5. The initiation factors are then released while the GTPase activating protein (GAP) eIF5 catalyses the hydrolysis of the GTP bound to eIF2.

(C) The dissociated 60S ribosomal subunit is recruited, forming the 80S complex. This junction reaction requires the presence of eIF5B and hydrolysis of GTP.
The initiation factor eIF1 may play a part in AUG recognition and an initiation codon in good context is postulated to “stall” the migration of the preinitiation complex long enough for the hydrolysis of GTP bound to the α subunit of eIF2 to occur. This turnover is promoted by the GTPase activating protein (GAP) activity of eIF5 (reviewed in Hershey and Merrick, 2000; Pestova et al., 2000). The eIF2-GDP binary complex has less affinity for the 40S subunit, causing the ejection of the initiation factors with the concurrent recruitment of the 60S subunit (Figure 1.3C). The junction reaction, requiring the presence of eIF5B and GTP, catalyses the formation of 80S complexes that are then able to begin the elongation phase of peptide synthesis (Pestova et al., 2000).

1.4 Control of translation initiation by 5’ untranslated regions

According to the scanning model of translation initiation, the consensus sequence surrounding the initiation codon would appear to be the only determinant of efficient translation. Although most eukaryotic mRNAs have 5’ UTRs of less than 200 nt (83% in a study of 954 mRNAs by Suzuki et al., 2000), many genes whose protein products are associated with the control of cell growth and cell death have a 5’ UTR of more than 200 nt. These atypical 5’ UTRs can contribute to the expression of the message by either inhibiting scanning, preventing initiation at the authentic initiation codon or by encouraging ribosome binding to internal regions of the 5’ UTR by mechanisms that either require the presence of the 5’ cap structure or operate independently of the cap.

1.4.1 Stable secondary structure

Binding of the heterotrimeric eIF4F complex to the 5’ end of the mRNA can be inhibited by stable secondary structures. In vivo, many messages with 5’ UTRs containing
stable secondary structures are translationally repressed (Section 1.3.2) and Gray and Hentze, (1994) showed that artificial hairpins were able to block scanning in vitro. Hairpins with a free energy of -30 kcal mol\(^{-1}\) can inhibit translation of a downstream reporter when inserted in close proximity to the extreme 5' end of the mRNA. These structures may mask the cap structure and prevent the binding of eIF4E (Figure 1.4A). Other artificial hairpins with a free energy of -50 kcal mol\(^{-1}\) can impede scanning when present further into the 5' UTR. These secondary structures may inhibit the ATP-dependent helicase activity of eIF4A. This factor, in concert with eIF4B, is able to unwind small areas of secondary structure that occur via intramolecular hydrogen bonding. However, larger secondary structures formed by G-C rich nucleotide sequences may be too stable for the helicase to melt the base-pairings. This would impair the migration of the preinitiation complex to the initiation codon, inhibiting translation of the message (Figure 1.4A).

1.4.2 Upstream open reading frames (uORFs) and leaky scanning

The scanning model of translational initiation assumes that the first AUG codon encountered in the UTR is the initiation codon. Upstream AUG codons are found in the 5' UTRs of many mRNAs and some of these are involved in controlling the expression of the physiological ORF.

In the simplest model, if the scanning ribosome encounters an AUG codon in good context then an initiation event will occur. If the first initiation codon is inefficiently recognised, other preinitiation complexes will scan further into the message and initiate translation at another downstream AUG codon in a process known as leaky scanning (Figure 1.4B). This can create isoforms of the same protein with different amino termini if the two initiation codons are in frame. If the initiation codons are out of frame then two or more distinct protein products may be translated from the same message.
Figure 1.4 Control of translation initiation by 5' untranslated regions

(A) G-C rich sequences can form stable secondary structures that either mask the cap-binding structure, preventing eIF4E binding, or inhibit the helicase activity of eIF4A.

(B) Upstream initiation codons may be used for initiation in preference to the physiological initiation codon. This can either inhibit translation of the physiological open reading frame (ORF) or create isoforms of the protein with N-terminal extensions.

(C) In ribosome shunting the ribosome binds via the cap, but bypasses areas of secondary structure before initiating translation at a downstream initiation codon.
In certain cases the upstream ORFs (uORFs) may be completely distinct from, or overlap out of frame with, the physiological ORF. After termination of translation of a complete upstream ORF, a reinitiation event must take place to enable translation of the appropriate ORF. The ribosome must remain associated with the mRNA long enough for another initiation complex to form at the initiation codon, which would require at least another eIF2-GTP-Met-tRNA$_i$ ternary complex (Geballe and Morris, 1994; Geballe and Sachs, 2000).

This mechanism is used to control expression of GCN4, a transcriptional activator of many genes that are involved in amino acid metabolism. In favourable growth conditions, amino acids are abundant and four short upstream ORFs are translated in preference to the GCN4 ORF. During starvation conditions, eIF2α is phosphorylated and the upstream AUG codons are inefficiently recognised leading to translation of the correct GCN4 ORF, the product of which can counter the cellular conditions (Hinnebusch, 1996).

1.4.3 Ribosomal shunting

Structures in the 5' UTRs of viral and cellular RNAs may not just be inhibitory to scanning and, in the poorly understood “shunting” model, the preinitiation complex begins scanning from the 5' end of the message but then bypasses the structure before initiating at a downstream AUG codon (Figure 1.4C). This model, also known as jumping, discontinuous scanning or hopping, remains poorly studied and the actual mechanism by which the 40S ribosome or scanning complex can bypass large regions of secondary structure is still a mystery.

This method of translation was first proposed for the 35S mRNA of cauliflower mosaic virus (CaMV) (Futterer et al., 1993) and more recently translation of adenovirus and hsp70 mRNAs was proposed to be by this mechanism (Yueh and Schneider, 1996; Yueh and
The 5' UTRs of late viral mRNAs contain a tripartite leader that consists of an unstructured 5' end followed by a region of complex secondary structure. Although messages that can be translated by a shunting mechanism can usually also be translated by cap-dependent scanning, both methods of initiating translation cannot take place on the same message at the same time. However, during conditions when the amount of eIF4F complex in the cells is limiting, these messages are translated purely by shunting. It appears that conserved and repeated sequences in the 5' UTR of these mRNAs that are complementary to the 18S ribosomal RNA are important for this process (Yueh and Schneider, 2000).

1.5. Cap-independent translation and internal ribosome entry segments.

A further mechanism for the initiation of translation has been postulated in recent years. As well as inhibiting the normal scanning mechanism of translation initiation, a region of stable secondary structure in the 5' UTR of an mRNA can direct translation initiation in a cap-independent manner. In this case, a complex structural element forms in the 5' untranslated region of the mRNA that allows recruitment of ribosomes directly to an initiation codon. Structures of this type were originally termed ribosome landing pads, but are now known as internal ribosome entry segments (IRESs). The initiation codon of a message translated via internal ribosome entry may be a considerable distance (e.g. more than 600 nt) from the 5' end of the primary mRNA sequence (For recent reviews see Jackson, 2000; Belsham and Jackson, 2000).

Dicistronic assays are commonly used to identify IRES elements and these tests are covered in more detail in Chapters 3 and 4. Insertion of a sequence that can direct internal ribosome entry between the two cistrons of a dicistronic mRNA will result in increased translation of the downstream open reading frame. Various tests must be performed to ensure
that the increase in expression of the downstream cistron is independent of cap-dependent scanning to the first cistron and is also not due to the presence of functional monocistronic mRNAs (reviewed in Carter et al., 2000).

1.5.1 The picornaviral paradigm

Internal ribosome entry was first observed in the picornaviruses, a family of positive strand RNA viruses. The viral genomes have many features that first indicated that they would be poor substrates for the cap-dependent scanning mechanism:

The 5' UTRs are long (typically 600 to 1400 nt) and G-C rich. Therefore they are probably highly structured, which can inhibit both ribosome binding and migration through the 5'UTR of a mRNA (Section 1.4.1). Viral transcripts are bound at the 5' end by a virally encoded protein (VPg), which is cleaved off in the infected cell, meaning transcripts are translated from an uncapped mRNA. The 5' UTRs contain many AUG codons that, although poorly conserved, may prevent scanning to the authentic initiation codon (Section 1.4.2).

The picornaviruses were the first viral group that were proposed to initiate translation by internal ribosome entry. Based on the homologies of IRES sequences, the picornaviruses can be classified into two large groups and one minor group (Jackson and Kaminski, 1995). The first major family includes the human rhinoviruses (HRV) and the enterovirus group (which includes the polioviruses). The cardioviruses, such as Encephalomyocarditis virus (EMCV) and Theiler's murine encephalomyelitis virus (TMEV), and the aphthoviruses, e.g. foot and mouth disease virus (FMDV), make up the second class. The third minor class contains the hepatoviruses such as Hepatitis A virus (HAV).

In most cases, one of the proteases encoded by the viral genome (2A in entero- and rhinoviruses, L in FMDV), as well as functioning in processing of the viral polyprotein, also cleaves the central component of the eIF4F complex, eIF4G. This causes a downregulation of
cap-dependent protein synthesis as it bifurcates the eIF4E and PABP binding portions of eIF4G from the eIF3·40S ribosome binding components of the molecule (Figure 1.5A). However, the uncapped viral transcripts can still be efficiently translated under these conditions, thus conferring a selective advantage to the virus.

1.5.2 Ribosome recruitment to picornaviral IRESs

Picornavirus IRESs have been classified on the basis of their sequences, secondary structures and mechanisms of ribosome recruitment into two major and one minor class (Section 1.5.1 and Jackson and Kaminski, 1995). In all cases ribosome binding is not just directed by cis-acting motifs in the IRES, but is also dependent on the presence of other trans-acting factors (Figure 1.5B, for more detail see sections 1.5.4 and 1.5.5). It is possible that sequences within the IRES may be functionally similar to the Shine-Dalgarno sequences in prokaryotic mRNAs, which bind ribosomes upstream of an initiation codon in a polycistronic mRNA.

The only common feature of all the classes of picornavirus IRESs is a 25 nt segment situated at the 3' end of the IRES. This sequence begins with around ten pyrimidine nucleotides (termed the oligopyrimidine tract), which usually contains a conserved UUUC sequence, followed by a G-poor sequence before ending with an AUG triplet (Figure 1.5).

In Class I IRESs, which include the entero- and rhinoviruses, the AUG at the 3' end of the segment is "silent", being rarely used for initiation. Instead it is thought to be used to aid recruitment of the 40S ribosomal subunit, which then initiates translation from the next AUG downstream of the ribosome entry site. This AUG can be a considerable distance downstream of the entry site (up to 120 nt) and it is unclear whether the ribosome scans or shunts to the initiation codon. IRESs from Class II, which comprises the cardio and aphthovirus IRESs,
Figure 1.5 Internal ribosome entry

(A) Picornaviral proteases cleave the central component of the eIF4F complex, eIF4G, into two fragments. This cleavage bifurcates the eIF4E-binding portion of the molecule from the eIF3-40S subunit complex causing a reduction in cap-dependent translation.

(B) Picornaviral messages are uncapped and recruit ribosomes directly to the initiation codon via an internal ribosome entry segment (IRES). This binding is facilitated by primary sequences such as the oligopyrimidine tract, secondary structural elements in the IRES such as the GNRA tetraloop and C-rich bulge and tertiary interactions. Host encoded factors may also stabilise secondary structures or facilitate ribosome binding (Adapted from Belsham and Jackson, 2000).
conform to the precise placement model where the AUG at the 3' end of the conserved sequence is used as the initiation codon.

1.5.3 Cellular IRESs

The first clue that indicated that cellular mRNAs could also be translated by internal ribosome entry was the observation that the immunoglobulin heavy-chain-binding protein (BiP/Grp78) mRNA was still translated during the early stages of poliovirus infection when cap-dependent translation of the host cell was shut down (Sarnow, 1989). Later work showed that the 5' UTR of BiP contained an internal ribosome entry segment, and this was the first example of a cellular mRNA that could be translated by this mechanism (Macejak and Sarnow, 1991).

In the past few years the number of cellular mRNAs that contain 5' UTRs that can initiate internal ribosome entry in the dicistronic assay has increased massively and it would be impossible to cover the literature devoted to each gene in detail, although an attempt to summarise the work is made in Table 1.2. The products of these genes are involved in several cellular processes, most often the control of cell growth or programmed cell death (apoptosis). Many have been found to encode transcription factors and there is also evidence that the translation initiation factors eIF4G and DAP5 can be translated by an IRES.

In some cases, an IRES is present in the 5' UTR of an mRNA that is also under the influence of complex transcriptional and posttranscriptional controls (e.g. c-myc and VEGF) providing many alternative ways of regulating expression of the gene. Messages that are translated by internal ribosome entry can also be scanned in the usual manner of cap-dependent translation (Stoneley et al., 2000b). In addition, three of the cellular IRESs identified so far (c-myc, FGF-2 and Pim-1) can also be translated from alternative upstream CUG codons in a manner analogous to leaky scanning (Willis, 1999). The amount of
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1/RUNX1</td>
<td>One of a family of transcription factors with a critical role in hematopoesis (Pozner et al., 2000).</td>
</tr>
<tr>
<td>Antennapedia</td>
<td>A homeotic gene involved in early developmental organisation in <em>Drosophila melanogaster</em> (Oh et al., 1992; Ye et al., 1997).</td>
</tr>
<tr>
<td>c-jun</td>
<td>A sequence specific DNA binding protein (Sehgal et al., 2000).</td>
</tr>
<tr>
<td>c-myc</td>
<td>A transcription factor overexpressed in various cancers with roles in growth, differentiation and apoptosis (Nanbru et al., 1997; Stoneley et al., 1998).</td>
</tr>
<tr>
<td>Connexin 43</td>
<td>The major gap junction protein expressed in the ventricle of the heart (Schiavi et al., 1999).</td>
</tr>
<tr>
<td>Cyr61</td>
<td>A serum-induced secreted protein that induces tumour growth and angiogenesis in vivo and induces movement of vascular endothelial cells in culture (Johannes et al., 1999).</td>
</tr>
<tr>
<td>DAP5/NAT1/p97</td>
<td>A member of the eIF4G family that lacks the eIF4E binding site (Henis-Korenblit et al., 2000).</td>
</tr>
<tr>
<td>eIF4GI</td>
<td>Central component of the eIF4F complex (Gan and Rhoads, 1996; Gan et al., 1998 Johannes and Sarnow, 1998).</td>
</tr>
<tr>
<td>ER1α</td>
<td>Estrogen receptor 1α. A 45kDa truncated form can be translated via an IRES (Barraille et al., 1999).</td>
</tr>
<tr>
<td>FGF2/bFGF</td>
<td>Human fibroblast growth factor 2 (Vagner et al., 1995).</td>
</tr>
<tr>
<td>Gtx</td>
<td>A murine homeodomain protein that acts as a transcriptional repressor (Chappell et al., 2000).</td>
</tr>
<tr>
<td>IGFII</td>
<td>Human insulin-like growth factor II (Teerink et al., 1995).</td>
</tr>
<tr>
<td>Kv1.4</td>
<td>A cardiac voltage-gated potassium channel (Negulescu et al., 1998).</td>
</tr>
<tr>
<td>MYT2</td>
<td>A secreted DNA binding protein expressed in the CNS (Kim et al., 1998).</td>
</tr>
</tbody>
</table>
Notch2 | A negative regulator of neuronal differentiation in early brain development contains an IRES within the protein coding region of the mRNA that directs translation of a shorter isoform (Lauring and Overbaugh, 2000).
---|---
NRF | NF-κB repressing factor, a transcriptional repressor (Oumard et al., 2000).
ODC | Ornithine decarboxylase, an enzyme that performs the first step in polyamine biosynthesis (Pyronnet et al., 2000).
PDGF2/c-sis | Platelet-derived growth factor 2 (Bernstein et al., 1997).
Pim1 | A serine-threonine protein kinase that cooperates with c-myc to induce cellular transformation (Johannes et al., 1999).
p58<sup>PITSLRE</sup> | A protein kinase with a role in cell cycle progression. The shorter p58 form is translated from an IRES within the coding region of the longer p110 isoform (Cornelis et al., 2000).
Ultrabithorax | A homeotic gene from D. melanogaster (Ye et al., 1997).
VEGF | Vascular endothelial growth factor (Stein et al., 1998; Miller et al., 1998; Huez et al., 1998; Akiri et al., 1998).
XIAP | The X-linked inhibitor of apoptosis (Holcik et al., 1999).

**Table 1.2** Cellular genes that can be translated by internal ribosome entry
literature demonstrating the use of the IRES element in maintaining translation of messages under conditions when cap-dependent translation is reduced is still relatively sparse, but is covered in more detail in section 1.6.

1.5.4 Cis-acting elements in IRESs

As IRESs are found in 5' UTRs that are long and G-C rich, the contribution of secondary and tertiary structures to IRES activity is obviously a significant one. Deletion analysis of many viral and cellular IRESs shows that the most important sequences for internal ribosome entry are concentrated at the 3' end of the 5' UTR. These sequences may be secondary structures or small regions of primary sequence which cause the internal binding of ribosomes either directly or via other trans-acting factors (Section 1.5.5).

There is a great degree of primary sequence divergence in picornaviral serotypes but this genetic drift still permits conservation of predicted secondary structure, which allows particularly important secondary structures to be identified. Two structural motifs that are common to both major classes of picornaviruses are a GNRA tetraloop and a C-rich bulge, which are both found at the end of longer structures (Figure 1.5).

The secondary structures of cellular IRESs are more difficult to predict, as there is usually not enough sequence data from a large enough number of species to allow identification of covariant bases. Most predictions of secondary structure in cellular IRES sequences instead are based entirely on structural modelling, which cannot be relied upon without the confirmation of experimental data. One such example of the use of computer modelling was the attempt to identify common motifs in the secondary structure of three cellular IRES sequences (Le and Maizel Jr, 1997). This work proposed that a conserved secondary structure motif (consisting of a Y-shaped double hairpin followed by a smaller single hairpin) existed in the BiP and Antennapedia IRESs. Unfortunately, although this
motif has never been determined by structural probing, or even proven to have any effect on IRES function, it has since been fortuitously “found” in the IRESs identified in FGF-2, VEGF, PDGF2, c-jun and NRF by purely theoretical secondary structural modelling (Huez et al., 1998; Sella et al., 1999; Sehgal et al., 2000; Oumard et al., 2000).

So far, the only secondary structure of a cellular IRES to be thoroughly modelled has been the c-myc IRES (Figure 1.6 and Le Quesne et al., submitted for publication). This approach used a mixture of phylogenetic analysis, to identify conserved nucleotides of probable importance, and chemical modification of the bases in the RNA sequence to identify the paired and unpaired bases in the sequence. The ribosome entry window was also determined by introduction of out of frame AUG initiation codons which would impair translation of a downstream reporter gene if ribosome entry occurred upstream of the modified sequence.

Sequence alignment and deletion studies of many viral and cellular IRESs shows that the most important sequences for IRES activity reside towards the 3’ end of the 5’ UTRs. However, most IRESs need the full length 5’ UTR for maximal function of the IRES. Small deletions, insertions or base substitutions can also have large effects on IRES activity and in the case of the c-myc IRES, a single C-U mutation found in a high proportion of multiple myeloma patients alters the repertoire of binding partners and possibly alters the secondary structure of domain 2 (Figure 1.6 and Paulin et al., 1998; Chappell et al., 2000b).

The conserved UUUC and oligopyrimidine tract that is conserved in all picornaviruses (Section 1.5.2), is also present in the 5’ UTR of the ODC mRNA from several species and a similar motif is also found in the 5’ UTRs of PDGF2/c-sis, XIAP and the Kv1.4 cardiac voltage-gated potassium channel (Pyronnet et al., 2000; Bernstein et al., 1997; Holcik et al., 1999; Negulescu et al., 1998).
Figure 1.6 Secondary structural model of the c-myc IRES adapted from Le Quesne et al., submitted for publication.
Other primary sequence motifs may act in a functionally analogous manner to the RNA-RNA contacts between the 16S rRNA and the Shine-Dalgarno (SD) sequence upstream of prokaryotic translation initiation codons. Despite the fact that the exact CCUCC sequence in prokaryotic 16S rRNA that pairs with the SD sequence to promote ribosome binding is deleted in eukaryotic 18S rRNAs, there are several sequences upstream of the initiation codons of IRESs that could act in a similar manner. One such sequence, CCGGC GGGU, is found in the 5' UTR of the homeodomain gene, Gtx, and is completely complementary to the 18S rRNA. This single 9 nt sequence represses translation when placed upstream of a monocistronic reporter gene but can act as an IRES in the dicistronic assay (Hu et al., 1999; Chappell et al., 2000a). When present in multiple copies, the 9 nt segments can act synergistically in the dicistronic assay to create a powerful artificial IRES. Similar sequences to the 9 nt IRES are found in many of the other cellular IRESs although they may be surrounded by other short sequences that can act to repress or encourage IRES activity (Vince Mauro, personal communication).

1.5.5 Trans-acting Factor requirements for internal ribosome entry

Dicistronic reporter vectors, which have no viral coding regions present, show an obvious requirement for host encoded rather than virally encoded trans-acting factors for internal ribosome entry. Again, studies on the factor requirements of viral IRESs have yielded the most information although the factors that bind to cellular IRESs are beginning to be revealed. Most of the work carried out to date has used in vitro systems with the various factors being added, removed or modified to show their requirement in internal ribosome entry.

The model in vitro translation rabbit reticulocyte lysate (RRL) system contains all the canonical initiation factors and is commonly used to translate unstructured RNAs via cap-
dependent scanning. Translation of most viral IRESs in RRL is inefficient, although this repression is relieved when extracts from HeLa cells are added. This demonstrates a requirement for non-canonical factors in the initiation of translation from an IRES. However, many of the factors that have been identified as IRES binding proteins by affinity purification have not always been correlated with an increase in IRES activity. It is extremely likely that a ribonucleoprotein complex must form on the IRES to allow ribosome binding rather than the presence of a single factor. One recently proposed name for this group of factors is the IRES-specific cellular trans-acting factors or ITAFs (Pilipenko et al., 2000).

All of the canonical initiation factors that are required for the scanning mechanism are required for efficient IRES activity with the exception of the cap-binding protein eIF4E (Pestova et al., 1996a). As always, there is an exception to this broad rule and the IRESs from hepatitis C virus (HCV) and some pestiviruses can directly recruit 40S ribosomal subunits in vitro without the need for the canonical initiation factors (Pestova et al., 1998).

As mentioned previously, cleavage of eIF4G by virally encoded proteases causes a shut off of host cell protein synthesis resulting in the selective translation of viral transcripts. The C-terminal fragment produced after cleavage by viral proteases maintains the binding sites for eIF3, eIF4A and Mnkl and is able to support internal ribosome entry (Section 1.6.1).

During apoptosis, eIF4G is also targeted for cleavage by caspase-3, a member of a family of proteases that are activated in response to apoptotic signals such as growth factor withdrawal or cellular stress (Section 1.6.2). This cleavage separates eIF4G into three fragments and the central fragment may be used to assemble initiation complexes during apoptosis, when cap-dependent translation is downregulated by the modification of many of the initiation factors (Section 1.6.2).

The DAP5/p97/NAT1 protein, which is homologous to the C-terminus of eIF4G binds to and enhances the function of its own IRES, suggesting a positive regulatory feedback that
could control the expression of the DAP5 protein. During apoptosis, DAP5 is cleaved into two fragments and the p86 apoptotic cleavage product is even more effective at driving translation of the DAP5 IRES (Henis-Korenblit et al., 2000).

Most of the non-canonical factors that have been described as being essential for IRES activity had been previously identified as being involved in the splicing of pre-mRNAs, although others are novel and solely involved in regulating IRES activity. The first non-canonical factor to be ascribed a role in internal ribosome entry was the La autoantigen. La was shown to stimulate translation from the poliovirus (PV) IRES when added to RRL, although the amounts of La supplemented into the reaction to stimulate translation was much greater than that seen to have a physiological effect (Svitkin et al., 1994). La also binds to the IRESs from HCV and EMCV (Ali and Siddiqui, 1997; Kim and Jang, 1999) and other cellular proteins found to bind these IRESs and others include the Poly(rC)-binding protein 2 (PCBP2) and heterogeneous nuclear ribonucleoprotein C (hnRNPC) (reviewed in Carter et al., 2000; Belsham and Jackson, 2000).

The polypyrimidine tract binding protein (PTB), which exists as three isoforms in cells and is also known as heterogeneous nuclear ribonucleoprotein I (hnRNP I), also binds to several viral IRESs. PTB had been originally identified as a protein involved in spliceosome assembly and the regulation of alternative splicing that bound to pyrimidine rich sequences in introns (Garcia-Blanco et al., 1989; Gil et al., 1991; Patton et al., 1991).

PTB contains four RNA recognition motifs (RRMs) and forms dimers in solution possibly allowing the PTB dimer to contact several points on the IRES at once (Perez et al., 1997). As the name of the protein suggests, various pyrimidine rich sequences in the viral IRESs have been identified as the regions of PTB binding with one of the most important sites, unsurprisingly, being the oligopyrimidine tract located upstream of the AUG triplet used.
in ribosome entry or initiation (Sections 1.5.2 and 1.5.4 reviewed in Belsham and Jackson, 2000).

The role of PTB in picornaviral IRES translation was first shown with the EMCV IRES (Jang and Wimmer, 1990) and further work has suggested the presence of PTB is also required for the activities of the FMDV, poliovirus, HCV and HRV2 IRESs (Luz and Beck, 1991; Ali and Siddiqui, 1995; Kaminski et al., 1995; Hunt and Jackson, 1999). PTB cannot act alone in encouraging ribosome binding to the initiation codon and other factors are required for efficient internal ribosome entry.

Another factor purified from HeLa cell extracts that could activate the HRV2 IRES in reticulocyte lysates was found to be \textit{unr} (Hunt et al., 1999) an ubiquitously expressed protein of no previously assigned function that was first found as a transcriptional unit located upstream of \textit{N-ras} (Jeffers et al., 1990). In the same study, the \textit{unr} protein was found to be associated with a novel protein designated \textit{unrip} (\textit{unr-interacting protein}), which is homologous to one of the eIF3 subunits (Hunt et al., 1999).

The \textit{unr} protein contains five cold shock domains, which are found in nucleic acid binding proteins such as the prokaryotic cold shock proteins and eukaryotic Y box factors. It was found to bind strongly to single stranded RNA and DNA, but not double stranded DNA (Jacquemin-Sablon et al., 1994). The sequences to which \textit{unr} binds were determined by in \textit{vitro} selection (Selective evolution of ligands by exponential enrichment, SELEX) and consisted of a stretch of purine nucleotides followed by a conserved core of AAGUA/G or AACG (Triqueneaux et al., 1999) although neither of these motifs are present in the HRV2 IRES.

While the non-canonical IRES \textit{trans}-acting factors that facilitate translation of the viral IRESs have been examined in great depth, the corresponding role of cellular IRES-binding proteins is much less researched. An interaction between PTB and the VEGF IRES
has been demonstrated (Huez et al., 1998), and the La autoantigen and hnRNPC form a ribonucleoprotein complex on the XIAP IRES, along with two other novel factors (Holcik and Korneluk, 2000 and Martin Holcik, personal communication).

Again, as is the case with both eIF4GI and DAP5, there has recently emerged the possibility that some of the non-canonical factors required for internal ribosome entry may themselves be translated via an IRES. A recent study by Carter and Sarnow, (2000) suggests that this is the case for the La autoantigen and it appears that unr may be also translated by an IRES (Hélène Jacquemin-Sablon, personal communication). These results, as well as the discovery of an IRES that can control translation of DAP5 (Henis-Korenblit et al., 2000), suggest that this may be a common mechanism in ensuring maintenance of expression of IRES trans-acting factors to ensure translation of other IRES driven messages under conditions when cap-dependent translation is compromised.

1.6 Potential roles for internal ribosome entry segments

1.6.1 Picornaviral infection

Shortly after picornaviral infection, there is a reduction in general cellular translation with an associated increase in translation of viral messages (Section 1.5.1). Cleavage of eIF4G by virally encoded L and 2A proteases stops eIF4G acting as a bridge between the m7GTP cap structure and the 40S ribosome as the eIF3 and eIF4E binding sites become separated, inhibiting cap-dependent translation (Figure 1.5). This provides a rationale for the preferential translation of viral mRNAs due to their mechanism of internal ribosome entry (Lamphear et al., 1995; Ohlmann et al., 1996; Pestova et al., 1996b).

It has also been shown that the eukaryotic IRESs from c-myc, BiP and eIF4G remain functional following picornaviral infection (Johannes and Sarnow, 1998). Recently, a cDNA
microarray showed that of 7000 messages studied on the chip, over 200 remained associated with polysomes after viral infection (Johannes et al., 1999). Further studies of the 5’ UTRs of some of the most strongly associated messages (e.g. Pim1 and Cyr 61) revealed that they could be translated by internal ribosome entry.

During a variety of other cellular conditions cap-dependent protein synthesis rates are reduced, usually as a result of modifications of the initiation factors. Recent work has demonstrated that a possible function for IRESs is to maintain protein synthesis of certain mRNAs when a reduction in translation occurs.

1.6.2 Translation inhibition during apoptosis

Programmed cell death or apoptosis is a process that is important in numerous biological systems including tissue development and immune system maintenance (Jacobson et al., 1997). The disruption of apoptosis can result in the progression of cancer and the onset of degenerative disorders.

In the nematode Caenorhabditis elegans, morphogenetic apoptosis during development requires the products of three essential genes, ced-9, ced-4 and ced-3 (Horvitz et al., 1994). The product of the ced-9 gene acts as an inhibitor of the activities of the ced-3 and ced-4 gene products, which promote apoptosis. The genes of the Bcl-2 family are the mammalian homologues of ced-9 and the many proteins in this family can be either anti-apoptotic (e.g. Bcl-2, Bcl-XL, Bcl-w, Diva/Boo) or pro-apoptotic (e.g. Bax, Bad, Bid) (Adams and Cory, 1998; Reed, 1998; Reed et al., 1998). The mammalian ced-3 homologues comprise a group of related cysteine proteases, termed caspases, since they cleave proteins after a specific aspartate residue. They all exist as zymogens and are cleaved and activated in response to apoptotic signals (Reviewed in Cohen, 1997; Cryns and Yuan, 1998; Nunez et al., 1998).
The discovery of the human homologue of CED-4, the apoptotic protease-activating factor (Apaf-1) (Zou et al., 1997) by in vitro reconstitution of apoptotic extracts confirmed the conservation of the suicide programme between nematodes and humans.

Apaf-1 plays a role in activating the initiator caspase, caspase-9 which then goes on to activate caspases-3 and -7. These effector caspases are important in both the activation of other caspases and also in cleavage of many of the other intracellular components which lead to the morphological and intracellular changes associated with apoptosis (Section 1.7.1).

Another distinct caspase cascade exists in cells which is induced by cell-surface receptors. This involves the formation of a death inducing signalling complex (DISC) by oligomerisation of receptors which causes the autoactivation of caspases-8 and -10. The intracellular or mitochondrial caspase cascade induced by Apaf-1, as well as inducing apoptosis in its own right, may also act as an amplification step after the induction of the receptor-mediated apoptotic pathway (Sun et al., 1999).

During programmed cell death induced by a number of stimuli in many different cell lines, a reduction in the rate of overall protein synthesis is observed. The addition of active caspases to in vitro translation systems has the same effect. It has only been in recent years that the underlying changes in translation factors have begun to be clarified (reviewed in Clemens et al., 2000). The first component that was studied was eIF4G, which was of interest because the cleavage of this molecule during picornaviral infection by virally encoded proteases also leads to the inhibition of protein synthesis in the cell (Clemens et al., 1998; Marissen and Lloyd, 1998; Morley et al., 1998).

Both eIF4GI and eIF4GII are cleaved by the effector caspase, caspase-3, into three distinct fragments, designated fragments of apoptotic cleavage of eIF4G (FAGs) (Bushell et al., 2000a). Caspase-3 cleaves eIF4GI at two sites, the first between residues 492 and 493 downstream of a DLLLD sequence and the second between 1136 and 1137 downstream of a
DRLD sequence. Initial cleavage events at either of these two sites generate intermediate fragments of 120 and 150 kDa respectively. The three fragments resulting from the second cleavage event are designated N-FAG, M-FAG and C-FAG. Other possible caspase-3 recognition sites exist within the molecule, but these may be inaccessible to the protease.

The N-terminal fragment, N-FAG contains the PABP binding site while C-FAG contains one of the eIF4A binding sites and also the binding site for the eIF4E-kinase Mnk1. The 76 kDa M-FAG is still able to bind eIF4E, eIF4A and eIF3 unlike the C-terminal eIF4G cleavage fragment generated by viral proteases. M-FAG may be able to maintain cap-dependent translation in a modified eIF4F complex, albeit with a reduced efficiency.

The other components of the eIF4F complex, eIF4A and eIF4E are not cleaved, although eIF4E becomes dephosphorylated probably as a result of the separation from the eIF4E kinase, Mnk1 (Bushell et al., 2000b). Dephosphorylated eIF4E has a reduced affinity for cap binding and is instead bound by 4E-BP1, which is also cleaved during apoptosis (Tee and Proud, 2000). However, the cleavage of 4E-BP1 occurs at a later stage of apoptosis than that of eIF4G (four hours compared to two hours after the induction of apoptosis) and appears to be cell-line specific.

eIF4B, which stimulates the helicase and ATPase activities of eIF4A and aids binding of the ribosome to the mRNA, is cleaved between residues 45 and 46 after a DETD sequence to produce a 60 kDa cleavage product. This species remains associated with the eIF4F complex and still contains a DRYG sequence, which is essential for self-association of eIF4B molecules and interaction with the p170 subunit of eIF3.

Of the large, multi-subunit eIF3, only the p35 subunit is targeted for caspase-mediated cleavage. This subunit has no known function, but has been shown to readily dissociate from the eIF3 complex, where it is more susceptible to caspase cleavage than when in the associated state. p35 eIF3 is cleaved between residues 242 and 243 after a DLAD motif and
the final two amino acids of the molecule are also lost. The cleavage product is still able to associate with the eIF4F complex, by binding to M-FAG.

As previously described, eIF2 plays a central role in the formation of the 43S preinitiation complex and therefore the overall control of protein synthesis (Section 1.3.1) and the caspase-3 mediated cleavage of the α subunit of eIF2 has also been described recently (Bushell et al., 2000b). PKR, one of the kinases that can phosphorylate Ser51 of eIF2α, has been implicated in the induction of apoptosis under conditions of cell stress and the downregulation of translation in response to eIF2α phosphorylation may even be a trigger of apoptosis (Srivastava et al., 1998).

Despite the overall reduction in protein synthesis during apoptosis, certain messages remain translated. The altered eIF4F complex containing M-FAG may maintain a limited amount of cap-dependent initiation, however, the role of IRES-initiated translation in apoptotic cells is of particular interest. Both DAP5 and the La autoantigen are cleaved during apoptosis (Henis-Korenblit et al., 2000; Ayukawa et al., 2000) and these modifications may be advantageous for IRES-driven translation. In the case of DAP5, the cleavage product exhibits a greater affinity for the IRES in the DAP5 5’ UTR, and the cleavage of La changes the localisation of the protein. A C-terminal nuclear localisation signal is removed by caspase cleavage, which allows the N-terminal RNA binding portion to localise to the cytoplasm.

Work from our laboratory has recently shown that the c-myc IRES is utilised during apoptosis (Stoneley et al., 2000a) and the IRESs in the 5’ UTRs of XIAP and DAP5 are also used to maintain translation initiation during apoptosis (Holcik et al., 1999; Holcik et al., 2000b; Henis-Korenblit et al., 2000).
1.6.3 Translation during the cell cycle and cell growth

The importance of phosphorylation in controlling the activities of the eukaryotic initiation factors has been described previously and phosphorylation of eIF2B, eIF4B, eIF4E and eIF4G increases rates of translation and cell proliferation. Conversely, phosphorylation of the eIF2α subunit leads to an inhibition of translation and cell growth by sequestering eIF2B (Section 1.3.1). The cap-binding protein eIF4E is phosphorylated in response to growth factors and mitogens and in this case the increase in translation rate is due to the increased association of eIF4E with eIF4G (Section 1.3.2).

During mitosis, protein synthesis rates are reduced to 25% of that in interphase cells and this is due to changes in the rate of initiation (Fan and Penman, 1970). One of the few alterations in the initiation factor machinery during mitosis that has been described so far is the dephosphorylation of eIF4E (Bonneau and Sonenberg, 1987). However, despite the reduction in cap-dependent translation, some mRNAs are still actively translated during mitosis.

One such message encodes ornithine decarboxylase (ODC), an enzyme involved in polyamine synthesis. During the cell cycle there are two rapid and transient peaks of ODC expression. The first peak is during transition from the first gap phase (G1) to the DNA synthesis phase (S). During this stage general protein synthesis rates increase and the increase in ODC is dependent on the levels of eIF4E. However, the second peak of ODC expression occurs during the transition from the second gap phase (G2) into mitosis (M) where there is a strong inhibition of protein synthesis due to the dephosphorylation of eIF4E (Pyronnet et al., 2000).

This observation led to the hypothesis that translation of ODC was mediated by a cap-independent mechanism during mitosis. The ODC 5' UTR was found to direct internal ribosome entry in a dicistronic assay, albeit relatively weakly compared to many viral and
cellular IRESs (Pyronnet et al., 2000). However, the activity of the ODC IRES increased by around 2.4 fold in cells arrested at the G2/M boundary. In the same report, Pyronnet and collaborators also showed evidence for the continuing translation of c-myc during mitosis by an IRES dependent mechanism (Pyronnet et al., 2000) and the IRES from HCV maintains translation of a downstream reporter gene in a dicistronic assay during mitosis (Honda et al., 2000).

Another intriguing IRES was discovered that can drive expression of the PITSLRE protein kinase during mitosis (Cornelis et al., 2000). This cyclin-dependent kinase exists as two isoforms, a p110 form and a shorter p58 isoform that is exclusively expressed during mitosis. The interesting observation is that both isoforms are translated from the same mRNA, meaning the IRES drives translation of the shortened protein isoform from within the mRNA that encodes a longer, cap-dependently translated isoform. This is unusual, but not unique as an IRES within the coding region was proposed to account for the translation of alternative isoforms of Estrogen Receptor 1α (Barraille et al., 1999) and recent work has found an IRES within the coding region of Notch2 (Lauring and Overbaugh, 2000).

In short, it appears that IRESs may be used to direct translation of genes during mitosis, although there again appears to be a role for trans-acting factors in this regulation that are specifically expressed during the cell cycle (Sachs, 2000a).

Internal ribosome entry may also be important in the regulation of cell proliferation, especially as a number of IRESs have been identified in growth factors and other proto-oncogenes (Table 1.2). In particular, angiogenesis, the formation of new capillaries from existing blood vessels which has implications in the development of secondary cancers, may be controlled by internal ribosome entry. The cellular messages of four genes that encode proteins involved in angiogenesis (VEGF, FGF2, PDGF and Cyr61) have all been proposed to
initiate translation via an IRES (reviewed in Carter *et al.*, 2000) with the PDGF IRES required during cell differentiation (Bernstein *et al.*, 1997).

### 1.6.4 Translational control during heat shock

When cells are incubated between five and ten degrees above their optimal growth temperature, within minutes a general repression of protein synthesis is observed with a concurrent preferential translation of heat shock protein (Hsp) mRNAs. This heat shock response (which can also be induced in response to heavy metals, hypoxia and glucose deprivation) prevents the accumulation of misfolded proteins that could be harmful to the cell. This response to elevated temperature or "acquired thermotolerance" sees a reduction in translation in mammalian cells to 80% of normal levels after incubation at 41°C.

The Hsps are a family of molecular chaperones of varying sizes that assist in folding and transport of proteins under normal cellular conditions. When the cell is exposed to stress (either heat or those mentioned above) the Hsps bind to proteins to protect them from unfolding, or in cases when proteins become severely damaged, target them for degradation by the 26S ubiquitin related proteasome pathway.

A role for the Hsps in the inhibition of apoptosis has been proposed recently with recent reports demonstrating that Hsp70 and Hsp90 prevent activation of the caspase cascade of apoptosis by binding to Apaf-1, the central component of the apoptosome (Section 1.7.2 and Beere *et al.*, 2000; Saleh *et al.*, 2000; Pandey *et al.*, 2000b). Another heat shock protein, Hsp27, binds to cytochrome c, which binds to Apaf-1 and is essential for apoptosome formation (Pandey *et al.*, 2000a). One hypothesis to explain this interaction may be that under conditions when the Hsps are able to maintain cellular proteins in a favourable state, Apaf-1 and cytochrome c remain bound by excess heat shock chaperones. Under severe stress when
the survival of the cell is threatened, Apaf-1 and cytochrome c may be released, allowing formation of the apoptosome and initiation of the intracellular caspase cascade.

Upregulation of Hsp expression counters the repression of protein synthesis during heat shock and the Hsps are also able to rapidly restore protein synthesis during recovery from heat shock as the cellular mRNAs remain stable during heat shock, being neither degraded nor modified. Protein synthesis rates can also recover as an adaptive response during a prolonged period of heat shock in mammalian cells. This adaptive response requires new transcription of hsp70 mRNA.

Most of the reduction in protein synthesis in heat shocked cells occurs as a result of changes at the level of initiation, although elongation rates also change (reviewed by Rhoads and Lamphear, 1995; Duncan, 1996). Firstly, there is a reduction in 43S preinitiation complex formation. eIF2α becomes phosphorylated, hindering eIF2B mediated guanine nucleotide exchange, which is essential for recycling of eIF2α between cycles of initiation. Ribosomal protein S6, eIF4B and eIF4E are all dephosphorylated, the latter causing a reduction in the cap-binding affinity of eIF4E and the dissociation of eIF4F complexes. Recent work has demonstrated that Hsp27 specifically binds eIF4G, which also dissociates initiation complexes (Cuesta et al., 2000). All these changes only take place during moderate or severe heat shock and the molecular mechanisms that occur during mild heat shock are still uncharacterised.

Although synthesis of all heat shock proteins continues during heat shock, the mechanism directing translation of only the mRNA encoding human Hsp70 has been established. The hsp mRNAs from Drosophila have long (200-250 nt) 5' UTRs, which contain conserved sequences at similar distances from the 5' m7GTP cap. They have a high (45-50%) adenosine content with a low potential for the formation of secondary structure. It is possible that Hsp mRNA translation occurs by internal ribosome entry especially as the first
The cellular IRES to be found was in the 5' UTR of BiP/Grp78, a variant of Hsp70 that localises to the endoplasmic reticulum. However, the hsp70 mRNA is instead translated by ribosome shunting (Section 1.4.3), which relies on sequences within the 5' UTR that are complementary to 18S rRNA (Yueh and Schneider, 2000).

Translation rates are also reduced during hypoxia, another environmental stress that causes the induction of the heat shock proteins. The 5' UTR of one of several genes induced in hypoxic conditions, vascular endothelial growth factor (VEGF), contains an IRES. The IRES is used to maintain translation of the protein during exposure of cultured cells to 1% oxygen (Stein et al., 1998).

1.6.5 Genotoxic stress

Several cellular responses to DNA damage (genotoxic stress) caused by exposure to chemical or environmental mutagens exist to prevent harmful mutations being maintained in the next generation. Two well-studied checkpoints in the cell cycle at the G1/S and G2/M boundaries exist to prevent progression into the next phase of the cell cycle. One example is the accumulation of the transcription factor p53 in response to DNA damage, which regulates the progression into the DNA replication stage of the cell cycle. This allows various mechanisms in the cell time to repair the lesions in the DNA. However, in cases of extreme stress, an apoptotic programme is induced to remove the damaged cell from the population.

There is also a downregulation in translation rates in response to DNA damage, which prevents the accumulation of mutated proteins. Again, modulation of the activities of the eIF4F complex and eIF2 play a significant role in this shut down of cap-dependent scanning (reviewed in Sheikh and Fornace, 1999). The changes in the initiation factor machinery in response to genotoxic stress are less severe than those observed in apoptosis, and may reflect a reversible, pre-apoptotic state from which the cell can still survive.
The central component in the ternary complex, eIF2, is phosphorylated at Ser51 of the α subunit by the kinases HRI, PKR and GCN2 in response to stress. As previously described, this prevents the recycling of eIF2 between successive rounds of initiation and inhibits ternary complex formation. Tee and Proud (2000) found that treatment of cells with various DNA damaging agents resulted in a dephosphorylation of p70 S6 kinase and 4E-BP1. Dephosphorylated 4E-BP1 shows increased affinity for eIF4E and therefore prevents the association of eIF4E with eIF4G in the cap-binding complex.

The transcription of eIF5 and eIF1 are induced by UV-irradiation and exposure to the alkylating agent MMS. These respectively cause errors in replication by forming thymidine dimers or by attaching alkyl groups to bases. eIF5 and eIF1 are important factors in controlling the fidelity of initiation and this induction may prevent the translation of mutant mRNAs (Sheikh and Fornace, 1999).

Recent work in our laboratory has investigated the role of the c-myc IRES during DNA damage. After treatment of cells with EMS, another alkylating agent, there is an inhibition of cap-dependent translation associated with dephosphorylation of eIF4E, p70 S6 kinase and 4E-BP1. However, the c-myc IRES is used to maintain translation of c-Myc under these conditions (Subkhankulova et al., in preparation).

1.7 The apoptotic protease activating factor Apaf-1

1.7.1 Identification of Apaf-1

When the human homologue of CED-4 was identified by the in vitro reconstitution of apoptotic extracts and designated the apoptotic protease-activating factor (Apaf-1) (Zou et al., 1997), other apoptosis-activating factors were identified as Apaf-2 and Apaf-3 but further studies recognised these as procaspase-9, one of the initiator caspases, and cytochrome c, a
component of the respiratory electron transport chain, normally found exclusively in the mitochondria.

The Apaf-1 molecule contains three domains which allow the protein to play a central role in the initiation of the cellular suicide programme by forming the physical core of the apoptotic machinery, the apoptosome. The N-terminal caspase recruitment domain (CARD) interacts with another CARD on the procaspase-9 molecule. This is followed by a domain which shares 22% identity and 48% conservation at the amino acid level with CED-4. This domain binds to and hydrolyses dATP, another essential component for caspase activation. The C-terminal portion of Apaf-1 is made up of a series of WD-40 repeats, so called because they are rich in tryptophan and aspartate residues (Hu et al., 1998b).

The first models of caspase activation by Apaf-1 involved the binding of Bcl-XL with this repression relieved during an apoptotic stimulus (e.g. Pan et al., 1998; Hu et al., 1998a). The discovery that Apaf-1 does not associate with Bcl-2-like proteins and is also localised to a distinct compartment of the cell has led to a revision of the model of apoptosome formation (Moriishi et al., 1999; Conus et al., 2000; Hausmann et al., 2000; Figure 1.7).

Knockout of Apaf-1 expression in developing mouse embryos revealed that Apaf-1 is essential for three types of developmental apoptosis: morphogenetic (the sculpture of embryonic structures), histogenetic (the control of appropriate cell number) and phylogenetic apoptosis (the deletion of unnecessary structures) (Cecconi et al., 1998; Yoshida et al., 1998; reviewed by Cecconi, 1999). Homozygous Apaf-1 knockout mice die early in embryogenesis and show reduced apoptosis leading to many morphological abnormalities. The interdigital webs persist and the lenses do not form properly, the retinas and brain overgrow and the palate does not close.

In murine Apaf-1 knockout cell lines, overexpression of c-Myc can promote tumorigenesis in a manner analogous to cell lines lacking p53 (Soengas et al., 1999).
Figure 1.7 Activation of the caspase cascade by Apaf-1
(A) Cytochrome c is released from the mitochondria in response to an apoptotic stimulus.
(B) Binding of cytochrome c and dATP causes the oligomerisation of Apaf-1 and binding of procaspase-9.
(C) Activated caspase-9 is released from the apoptosome after undergoing autoproteolysis. This goes on to cleave pro-caspase-3.
Conversely, overexpression of Apaf-1 increases the sensitivity of cells to stimulators of apoptosis such as etoposide and paclitaxel (Perkins et al., 1998).

1.7.2 The role of Apaf-1 in apoptosome formation

The current model of apoptosome formation is illustrated in Figure 1.7. Apaf-1 monomers reside in the cytoplasm (Hausmann et al., 2000) and are maintained in an inert form by self-association of the C-terminal WD-40 repeats with the N-terminal CARD (Hu et al., 1998b). Following an apoptotic stimulus, cytochrome c that has been released from the mitochondria binds to Apaf-1 (Li et al., 1997), possibly via the WD-40 repeat domain (Purring-Koch and McLendon, 2000). dATP binds to the ATPase domain of Apaf-1 at a P loop found in Walker box A in the ced-4 homology region (Cardozo and Abagyan, 1998). Two alternative models exist for the next stage in caspase activation, either binding of cytochrome c causes a conformational change in the Apaf-1 molecule which is required for efficient binding of dATP (Jiang and Wang, 2000) or hydrolysis of dATP bound to the N-terminal ATPase domain alters the conformation of the molecule to allow cytochrome c binding (Benedict et al., 2000). Recent evidence favours the former sequence of events (Purring-Koch and McLendon, 2000). Either way, cytochrome c binding and hydrolysis of the dATP causes oligomerisation of Apaf-1 monomers into an "apoptosome" consisting of up to eight Apaf-1 molecules (Cain et al., 1999; Zou et al., 1999; Saleh et al., 1999).

In this induced proximity model, the oligomerisation permits binding of procaspase-9 via the N-terminal caspase recruitment domains (CARDs). This encourages interactions between procaspase-9 molecules, thus allowing intermolecular cleavage and autoactivation to occur. This initiator caspase can then cleave procaspase-3, one of the effector caspases essential for cleavage of a number of key substrates during the execution phase of apoptosis.
1.7.3 Regulation of Apaf-1 activity by binding partners

Apaf-1 was first implied to exist in a trimeric complex with procaspase-9 and one of the antiapoptotic members of the Bcl-2 family, Bcl-X\textsubscript{i} (Hu \textit{et al.}, 1998a). Another anti-apoptotic member of the Bcl-2 family, known as Boo or Diva, was also identified as an Apaf-1 binding partner (Inohara \textit{et al.}, 1998; Song \textit{et al.}, 1999). A model was proposed that involved the sequestration of Apaf-1 by binding of Bcl-2 family proteins until an appropriate apoptotic stimulus allowed release of Apaf-1 and activation of caspase-9. However, binding of Apaf-1 to these proteins was usually shown by co-immunoprecipitation of whole cell extracts or yeast two hybrid binding experiments. Experiments with endogenous Apaf-1 failed to identify an interaction with either anti- or pro-apoptotic Bcl-2 proteins (Moriishi \textit{et al.}, 1999; Conus \textit{et al.}, 2000). The demonstration of a dispersed cytoplasmic localisation of Apaf-1 compared to the localisation of Bcl-2 and Bcl-X\textsubscript{i} to organelle membranes also appears to suggest an indirect role of Bcl-2 proteins in inhibiting Apaf-1 activity (Hausmann \textit{et al.}, 2000).

Two forms of procaspase-9 can also inhibit the downstream activation of the caspase cascade. Procaspase-9 can be phosphorylated at Ser196 by the Akt kinase and the phosphorylated form can still bind Apaf-1 but cannot be processed in response to cytochrome \textit{c} (Cardone \textit{et al.}, 1998). Alternative splicing of procaspase-9 transcripts produces a C-terminally truncated form termed casp9b or casp9s that cannot be processed on binding to Apaf-1 via a CARD-CARD interaction (Seol and Billiar, 1999).

As previously discussed, the heat shock chaperone family can also regulate the formation of the apoptosome. Hsp90 binds to Apaf-1 and prevents binding of cytochrome \textit{c} (Pandey \textit{et al.}, 2000b), which is also sequestered by Hsp27 (Pandey \textit{et al.}, 2000a). Hsp70 binds to Apaf-1 and prevents ATP hydrolysis, possibly via interaction with the CARD domain.
Another recent addition to the repertoire of Apaf-1 binding proteins is Aven, which also interacts with Bcl-X\textsubscript{L} (Chau \textit{et al.}, 2000).

### 1.7.4 Splice isoforms of Apaf-1 and other CED-4-like proteins

The discovery of several splice variants of Apaf-1 showed that the prototype Apaf-1 isoform identified by Zou \textit{et al.}, (1997), although found in certain cells, is not the predominant form. The original Apaf-1 form, containing 12 WD-40 repeats has now been designated as Apaf-1S (Figure 1.8A). The Apaf-1 homologues identified from mice (Cecconi \textit{et al.}, 1998; Yoshida \textit{et al.}, 1998) and Drosophila (Rodriguez \textit{et al.}, 1999; Zhou \textit{et al.}, 1999; Kanuka \textit{et al.}, 1999) also exist as at least two alternatively spliced isoforms.

The most ubiquitously expressed isoform, termed Apaf-1XL (Figure 1.8B), contains an extra 11 amino acids between the CARD and CED-4-like domains and an extra 43 amino acids inserted into the C-terminal end of the protein, which create a thirteenth WD-40 repeat. This extra WD-40 repeat is essential for cytochrome \textit{c} binding and Apaf-1 oligomerisation (Benedict \textit{et al.}, 2000). Another highly expressed isoform, Apaf-1LN, contains the same 12 WD-40 repeats as the originally published sequence but also has the 11 amino acid insert. Despite not containing the essential 13 WD-40 repeats, it is not thought to function as an inhibitor of apoptosome formation or procaspase-9 formation.

Two possible splice variants have also been identified by RT-PCR although there is a lack of evidence for the expression of the related proteins. Apaf-1LC contains an extra WD40 repeat but lacks the 11 amino acid insert and another isoform, designated Apaf-1XS, was only found in peripheral blood lymphocytes (PBL) (Hahn \textit{et al.}, 1999). This sequence has part of the WD-40 region deleted and is also missing the 33 nt sequence between the CARD and CED-4 domains.
Figure 1.8 Apaf-1 and other proteins with homology to CED-4
(A) The Apaf-1 molecule as originally described by Zou et al., (1997). The N-terminus of the molecule contains a caspase recruitment domain (CARD) and this is followed by a domain with homology to CED-4. This contains a P-loop and consensus Walker A and B boxes, which are typical of ATPase domains. The C-terminus contains a number of WD-40 repeats rich in tryptophan and aspartate residues.
(B) Apaf-1 splice isoforms as determined by RT-PCR. Apaf-1 XL is the most abundantly expressed form of Apaf-1 and contains 13 WD-40 repeats.
(C) Nod1/CARD4 and FLASH (FLICE-associated huge protein) are more recently identified mammalian proteins with homology to CED-4.
Other mammalian proteins with homology to CED-4 have recently been identified (Figure 1.8C). Nod1/CARD4 has a similar domain organisation to Apaf-1, but contains a leucine-rich domain at the C-terminus of the protein instead of the tryptophan and aspartate rich WD-40 repeats (Bertin et al., 1999; Inohara et al., 1999). Nod1 acts in a similar way to Apaf-1 by binding to RICK, a kinase with a CARD domain. RICK molecules in close proximity are able to undergo autophosphorylation, activating RICK, which in turn activates nuclear factor kappa B (NF-κB) (Inohara et al., 2000). Another protein that contains a CED-4 like domain, the FLICE-associated huge protein (FLASH), interacts with caspase-8 in the death-inducing signalling complex (DISC) (Imai et al., 1999).

1.7.5 Apaf-1 expression

Apaf-1 is expressed in almost all tissues studied with the exception of skeletal muscle. In these cells very little Apaf-1 mRNA can be detected by RT-PCR or Northern blot analysis and no Apaf-1 protein is observed by Western blotting (Zou et al., 1997; Burgess et al., 1999). This may be an important control point as after strenuous activity mitochondria in skeletal muscles have been shown to swell and/or degenerate (Zamora et al., 1995), which could lead to accidental triggering of the apoptotic pathway as a consequence of cytochrome c release.

Apaf-1 is efficiently expressed despite containing a 5' untranslated region (UTR) that would be expected to inhibit translation of the mRNA. The 5' UTRs of both the human and mouse versions of Apaf-1 have an overall homology of 56% and are long (578 and 585 nucleotides respectively) and G-C rich (67 and 66% respectively). Such sequences would be expected to form stable secondary structures that would inhibit the migration of ribosomes to the initiation codon.

The Apaf-1 5' UTRs also contain upstream AUG codons (two in the human, one in the murine version). The single upstream AUG codon in the murine 5' UTR is conserved in
the human version and lies in a region of 70% homology approximately 40 nucleotides upstream of the physiological start site (Figure 1.9). These initiation codons may initiate translation of upstream open reading frames in preference to the physiological ORF.

1.8 The Bcl-2-associated athanogene BAG-1

1.8.1 Identification of BAG-1 protein isoforms

When attempting to isolate Bcl-2 binding partners from a mouse embryo cDNA library, Takayama and colleagues identified a protein that enhanced cytoprotection of cells exposed to different apoptotic stimuli. This protein was accordingly named the Bcl-2-associated athanogene-1 (BAG-1), athanos being the Greek for “against death” (Takayama et al., 1995). The human homologue of BAG-1 was cloned in a later study (Takayama et al., 1996) and was also cloned independently as RAP46/HAP46, a protein that interacted with nuclear hormone receptors (Zeiner and Gehring, 1995).

Subsequent studies revealed that human BAG-1 could be expressed as up to four protein products, while only two isoforms of BAG-1 are observed in murine cells. All these isoforms are generated by alternative translation initiation from a single transcript (Figure 1.10A; Packham et al., 1997; Takayama et al., 1998; Yang et al., 1998).

The BAG-1 proteins differ at their N-termini and this determines both their subcellular localisation and the role they play in the cell (Figure 1.10B). The 50 kDa form of BAG-1 (BAG-1L) is translated from a non-canonical CUG codon and contains an SV40-like nuclear localisation signal. The other forms, BAG-1M/p46, BAG-1S/p36 (which is the most common form) and a rare p29 isoform, are primarily cytoplasmic and initiate at alternative AUG codons (Packham et al., 1997; Takayama et al., 1998). All BAG-1 isoforms contain different numbers of an acidic repeat motif (TRSEEX) of unknown function, an ubiquitin-like domain.
Figure 1.9 Alignment of 5' untranslated regions from human and murine Apaf-1 indicating conserved nucleotides. The sequences share 56% identity and are 67 and 66% G-C rich. The physiological initiation codons are capitalised and in bold. Putative initiation and termination codons of upstream open reading frames (uORFs) are in bold and italicised. All initiation codons are aligned with the eukaryotic initiation codon consensus sequence determined by Kozak.
Figure 1.10 Structure of BAG-1 mRNA and protein isoforms

(A) BAG-1 mRNA from humans and mice showing the positions of the initiation codons of the major isoforms.

(B) The three major protein isoforms of human BAG-1. The p50/BAG-1L protein contains a nuclear localisation signal that is not present in the other isoforms (p46/BAG-1M and p36/BAG-1S). All isoforms contain differing numbers of an acidic repeat motif, an ubiquitin-like domain and a C-terminal BAG domain. The rare p29 isoform is not shown.
and a C-terminal BAG domain, which is present in four other mammalian BAG family members (Takayama et al., 1999). The two murine forms of BAG-1 correspond to the human p50 and p36 isoforms but migrate slightly differently during SDS-PAGE analysis.

As well as the SV40-like nuclear localisation signal (NLS) present in the p50 isoform, a putative bipartite NLS can also be found between the ubiquitin-like and BAG domains of all the human BAG-1 isoforms although this does not appear to function. The trafficking of BAG-1 isoforms between the cytoplasm and the nucleus may instead be regulated by the translocation of BAG-1 binding partners such as the growth factor and steroid hormone receptors.

1.8.2 The role of BAG-1 in modulating chaperone activity

BAG-1 isoforms have been implicated in many diverse cellular processes but unfortunately many of the early studies only focused on the expression of a single isoform. It is only recently, with the realisation that BAG-1 exists as several isoforms, that work has started to determine whether the isoforms function differently.

BAG-1 was first cloned as a Bcl-2 binding partner but was also identified in other screens attempting to identify binding partners for the glucocorticoid receptor and the hepatocyte growth factor (HGF) receptor (Zeiner and Gehring, 1995; Bardelli et al., 1996). As there was no obvious homology between these BAG-1 binding partners and others such as Raf-1 (Wang et al., 1996) and other receptors, it was thought that the presence of another protein might mediate these diverse interactions. As the Bcl-2 interaction was ATP-dependent, a possible interaction of BAG-1 with the heat stress induced molecular chaperone Hsp70 or its constitutively expressed form Hsc70 was investigated. The binding of BAG-1 to Hsp70 and Hsc70 was demonstrated and it is possible that this interaction with the chaperone
machinery is one of the reasons why so many binding partners of BAG-1 have been identified (Takayama et al., 1997; Zeiner et al., 1997). 

Hsp70 and Hsc70 contain three domains, an N-terminal ATPase domain which is important for substrate turnover, a substrate binding domain and a C-terminal domain of unknown function (reviewed in Bukau and Horwich, 1998). ATP hydrolysis is the rate-limiting step in the turnover of substrates, and BAG-1 isoforms bind to the ATPase binding domain of Hsp70 and Hsc70 and stimulate nucleotide turnover (Bimston et al., 1998). The C-terminal BAG domain shared by the isoforms and other BAG family members mediates this interaction, and is predicted to form several amphipatic helices (Stuart et al., 1998).

Most studies suggest that BAG-1 isoforms have a negative effect on chaperone activity (Takayama et al., 1997; Zeiner et al., 1997; Nollen et al., 2000). However, one recent study showed that the BAG-1 protein isoforms could have opposite effects on the heat shock response. Transfection of p46 BAG-1 into cells causes an inhibition of refolding of a heat- or chemically denatured substrate whereas the p36 isoform stimulates refolding by the Hsc70 chaperone (Luders et al., 2000b).

1.8.3 The role of BAG-1 in apoptosis

The original identification of BAG-1 as a Bcl-2 binding partner has led to interest in the contribution BAG-1 makes to apoptotic pathways and how the expression of BAG-1 proteins may change as a result of malignant transformation. BAG-1 can enhance protection from apoptosis induced by several mechanisms such as exposure to anti-Fas antibody or staurosporine and overexpression of p53, but cannot protect against apoptosis induced by apoptin, a protein expressed by the chicken anaemia virus (Takayama et al., 1995; Danen-van Oorschot et al., 1997). The C-terminus of BAG-1 was shown to bind to the catalytic domain of the kinase Raf-1, causing an activation of Raf-1 kinase activity and the suppression of
apoptosis (Wang et al., 1996). This work suggested that the activation of Raf-1 in response to BAG-1 binding might target Raf-1 to cellular substrates that are different to those that are phosphorylated by Raf-1 as part of the MAP kinase signalling cascade.

BAG-1 has been found to interact with many intracellular receptors, but again most of the studies have only focused on a single BAG-1 isoform. For example, the hepatocyte and platelet-derived growth factors can suppress apoptosis and murine p33 BAG-1 binds to the receptors of both these ligands (Bardelli et al., 1996). Again, this interaction was mediated via the C-terminal domain of the protein and BAG-1 was able to inhibit apoptosis induced by staurosporine treatment. The p36 isoform binds to the retinoic acid receptor (RAR), and in this case inhibits the ability of the RAR ligand, trans-RA, to induce apoptosis (Liu et al., 1998).

The role of the different BAG-1 isoforms in receptor binding is beginning to be determined. Binding and transactivation of the androgen and vitamin D3 receptors can only be accomplished by the p50 isoform with the shorter proteins having no effect (Froesch et al., 1998; Guzey et al., 2000), and similarly transactivation of the glucocorticoid receptor was only affected by the p50 and p46 isoforms (Kullman et al., 1998; Kanelakis et al., 1999; Schneikert et al., 1999). Folding of the glucocorticoid receptor (GR) into a form that is competent for steroid binding requires the presence of five proteins from the chaperone system (Hsp90, Hsp70, Hop/p60, Hsp40 and p23). BAG-1 can compete with Hop for binding to Hsp70 (Gebauer et al., 1998) and at low levels causes the release of Hop from the GR folding complex, with no inhibition of folding. However, when BAG-1 is overexpressed by transfection, complex assembly and GR folding is inhibited (Kanelakis et al., 1999). This may reflect another problem with the approaches taken to show BAG-1 functions as most involve expression of BAG-1 cDNAs at levels that do not reflect the physiological levels.
However, the overexpression of BAG-1 in these experiments may reflect the status of BAG-1 in transformed cells.

1.8.4 BAG-1 and cancer

Not surprisingly for a protein involved in the prevention of cell death, overexpression of BAG-1 has been observed in many tumours. However, the observed increase in BAG-1 mRNA does not account for the alteration of BAG-1 isoform expression observed in these cells. In many tumours, while the expression of all BAG-1 isoforms increases, there is a larger relative increase in the expression of p50/BAG-1L (Takayama et al., 1998; Tang et al., 1999; Yang et al., 1999a).

Most approaches look at changes in BAG-1 immunostaining in tissue samples and the expression of BAG-1 in breast cancer has been studied in the most detail. Western blotting of BAG-1 isoforms from breast cancer-derived cell lines and tissue samples showed a correlation with the estrogen receptor status of the cell (Brimmell et al., 1999), and BAG-1 had previously been found to interact with this receptor in vitro (Zeiner and Gehring, 1995). However, the expression and localisation of BAG-1 in these cells is mediated by an estrogen-independent mechanism and a separate study failed to identify this correlation (Tang et al., 1999). The overall survival (OS) of patients with increased nuclear staining of BAG-1 (which would be due to an increase in p50/BAG-1L expression) was also different in two separate studies. Investigation of 140 invasive breast carcinomas by Tang et al., (1999) postulated a shorter OS for patients with increased nuclear staining of BAG-1, whereas Krajewski et al., (1999) found the opposite, albeit with a much smaller sample of patients.

An extensive study of BAG-1 expression in normal and cancer cells identified the particular overexpression of p50/BAG-1L in tumours compared to normal tissues (Takayama et al., 1998). Overexpression of all the BAG-1 isoforms is observed in many cervical cancers
(Yang et al., 1999a), and the p36 isoform of BAG-1 is commonly expressed in the cells of patients with B-cell chronic lymphocytic leukaemia (B-CLL). In this cancer, there is a possible association between high levels of BAG-1 expression and a worse clinical response of the patient to chemotherapy (Kitada et al., 1998). The p36 isoform was also expressed in a high percentage of human glioma cell lines and primary tumour specimens (Roth et al., 2000). BAG-1 colocalises with the cytoskeletal proteins cytokeratin and actin and overexpression of murine p33 BAG-1 enhances tumour cell migration and metastasis (Naishiro et al., 1999)

1.8.5 Other BAG-1 functions

The function of the ubiquitin-like domain found in all BAG-1 isoforms is beginning to be determined. BAG-1 appears to act as a link between the Hsp70 chaperone and the ubiquitin proteasome by aiding transfer of non-native polypeptides to the proteasome (Luders et al., 2000a). In this way, BAG-1 helps to organise a network between the folding and degradation of protein substrates.

Overexpression of p46 BAG-1 causes an increase in overall transcription in heat-shocked cells (Zeiner et al., 1999). This increased transcription correlates with a redistribution of p46 BAG-1 from the cytoplasm to the nucleus. The p46 isoform can bind to long fragments of DNA by virtue of a positively charged decapeptide sequence at the extreme N-terminus of the protein (Zeiner et al., 1999). Deletion or mutation of this N-terminal lysine and arginine-rich region abrogates DNA binding, suggesting the p36 isoform cannot act as a transcription factor. However, the murine p33 isoform, which does not contain this motif, is able to bind nuclear factor (NF) 1-like sequences in the promoter of the human polyomavirus JCV and activate its transcription (Devireddy et al., 2000). A NF-1 motif is also present in the promoter of human BAG-1 which suggests that BAG-1 may be able to regulate its own transcription (Yang et al., 1999b).
1.8.6 Translational Control of BAG-1

All of the initiation codons for BAG-1 isoforms are in poor context when compared to the consensus sequence surrounding eukaryotic initiation codons determined by Kozak, (1987), and in vitro experiments have suggested that the four isoforms of BAG-1 are translated by “leaky scanning” (Yang et al., 1998). However, expression of BAG-1 in vivo does not support this hypothesis especially as the p36 isoform is the predominant form in all cell types (Packham et al., 1997; Takayama et al., 1998). To translate this isoform, a scanning ribosome would have to disregard eight CUG and two AUG codons, some in frame and in reasonable Kozak consensus, before reaching the p36 open reading frame. This would be an extremely rare event according to the scanning model of translation initiation.

It is therefore important to study how BAG-1 isoform expression is regulated at the level of translation and interestingly, the sequence upstream of the human p36 open reading frame of BAG-1 is long (410 nucleotides), highly conserved in the murine 5′ UTR (76%) and GC rich (73%) (Figure 1.11). This would suggest that this region might form secondary structures that inhibit scanning, or potentially form a complex structural element that can direct ribosomes directly to the initiation codon of the p36 ORF. This could be achieved by either internal ribosome entry or ribosome shunting and both phenomena have been observed in other messages that encode proteins involved with the heat shock response, i.e. the ER homologue of Hsp70, BiP/Grp78, contains an IRES (Macejak and Sarnow, 1991) and the hsp70 mRNA can be translated by ribosome shunting (Yueh and Schneider, 2000).

1.9 Project background and aims

Previous work in our laboratory had identified an IRES in the 5′ UTR of the proto-oncogene c-myc mRNA (Stoneley et al., 1998) and, in an attempt to identify further cellular
Figure 1.11 Alignment of 5' untranslated regions of human and murine BAG-1 indicating conserved nucleotides. The sequences share 75.7% identity and are 73 and 68% G-C rich. Initiation codons for each BAG-1 open reading frame (ORF) are shown in bold.
genes that could be translated by internal ribosome entry, the 5’ UTRs of Apaf-1 and BAG-1 were examined. Both 5’ UTRs are long and G-C rich and share a significant degree of homology between the human and murine sequences. This would suggest that these regions have the potential to form secondary structures and could form a complex structural element e.g. an IRES.

The work in this thesis explains how internal ribosome entry segments were identified to contribute to the translation of both of these genes, and details attempts made to determine a functional role for the IRESs under conditions when cap-dependent translation rates are reduced.
Chapter 2

Materials and methods

2.1. General Reagents

2.1.1 Reagent and equipment suppliers

Unless otherwise stated all chemical reagents were of analytical grade and were obtained from BDH laboratory supplies (Lutterworth, Leicestershire, UK), Fisher Scientific (Loughborough, Leicestershire, UK), ICN Flow Ltd (Thame, Oxfordshire, UK) or Sigma Chemical company Ltd (Poole, Dorset, UK).

Products for molecular biological techniques were routinely purchased from Calbiochem (c/o CN Biosciences UK, Beeston, Nottingham), Gibco-BRL (Paisley, Scotland), New England Biolabs (NEB) (c/o CP Labs, Bishops Stortford, Hertfordshire, UK), MBI Fermentas (c/o Helena Biosciences, Sunderland, Tyne and Wear), Pharmacia Biotech (Milton Keynes, Buckinghamshire, UK), Promega (Southampton, UK), Roche UK Ltd (Lewes, East Sussex, UK) and Stratagene Ltd (Cambridge, UK). Radiolabelled chemicals were obtained from Amersham International Plc (Little Chalfont, Buckinghamshire, UK) and NEN Dupont (Hounslow, UK).

Reagents for bacterial cell culture were obtained from Oxoid (Unipath, Basingstoke, Hampshire, UK). Foetal calf serum (FCS) for mammalian tissue culture was obtained from Gibco-BRL or Helena Biosciences.

All tissue culture plastic was supplied by TPP (c/o Helena Biosciences, Sunderland, Tyne and Wear). Zeta-probe nitrocellulose membrane for Northern analysis of mRNA was obtained from Bio-Rad laboratories (Hemel Hempstead, Hertfordshire, UK) and Protran nitrocellulose membrane for immunoblotting of protein lysates was from Schleicher & Schuell (Dassel, Germany).
2.1.2 Antibodies

BAG-1 was detected using the mouse-monoclonal antibody mix (3.9F1E11 + 3.10G3E2) (Neomarkers c/o Stratech Scientific, Luton, Bedfordshire) at 1:1000 dilution. Firefly luciferase was detected with a rabbit polyclonal antibody (Sigma) at 1:1000 dilution. Actin was detected with a 1:2000 diluted mouse monoclonal antibody (Sigma). The FLAG epitope was detected with a mouse monoclonal antibody conjugated to horseradish peroxidase (Sigma) diluted 1:500. Dr Simon Morley, University of Sussex, kindly provided rabbit antibodies to eIF4E and eIF4G which were both used at a dilution of 1:2000. Secondary antibodies raised against mouse and rabbit IgG and conjugated to horseradish peroxidase were obtained from Sigma and routinely diluted 1:2000.

2.2 Tissue Culture Techniques

2.2.1 Tissue culture solutions

Phosphate Buffered Saline (PBS): (4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4).

2 × HEPES Buffered Saline: (50 mM HEPES, pH 7.05, 1.5 mM Na₂HPO₄, 280 mM NaCl)

2.2.2 Cell Lines

All cell lines were supplied by the American Type Culture Collection (ATCC), with the exception of the CAL51 and GI-101 cell lines, which were a kind gift from Dr. Graham Packham (CRC Wessex Medical Oncology Unit, Southampton General Hospital). Details of the origin of cell lines are given in Table 2.1.
<table>
<thead>
<tr>
<th>Name</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa S3</td>
<td>Human cervical epitheloid carcinoma</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human hepatocyte carcinoma</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell line immortalised with adenovirus DNA</td>
</tr>
<tr>
<td>Balb/c-3T3</td>
<td>Murine embryonic fibroblast cell line</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human breast carcinoma</td>
</tr>
<tr>
<td>Cos-7</td>
<td>Monkey epithelial cell line (CV-1) immortalised with SV40 DNA</td>
</tr>
<tr>
<td>MRC-5</td>
<td>Lung</td>
</tr>
<tr>
<td>CAMA1</td>
<td>Human breast cancer</td>
</tr>
<tr>
<td>CAL51</td>
<td>Human breast carcinoma</td>
</tr>
<tr>
<td>SY 5Y</td>
<td>Neuronal</td>
</tr>
<tr>
<td>MDA-MB</td>
<td>Human breast carcinoma</td>
</tr>
<tr>
<td>GI-101</td>
<td>Human breast carcinoma</td>
</tr>
<tr>
<td>CHO-T</td>
<td>Chinese hamster ovary-T cells</td>
</tr>
<tr>
<td>GM637</td>
<td>A fibroblast line derived from a healthy individual</td>
</tr>
</tbody>
</table>

**Table 2.1:** Cell lines utilised with details of their origin.

### 2.2.3 Maintenance of cell lines

All of the cell lines were cultured in gamma sterilised plasticware in Dulbecco's modified eagle medium, (DMEM) without sodium pyruvate (Gibco-BRL), supplemented with 10% FCS, with the exception of SY5Y cells which were grown in a 50:50 mixture of DMEM and HAMS F12 media (Gibco-BRL), supplemented with 10% FCS. The adherent cells were grown to confluence in 10 cm petri dishes, washed with phosphate buffered saline (PBS) and treated with 1× trypsin-0.5 mM EDTA solution (Gibco-BRL). Approximately 1×10⁶ cells were diluted into fresh medium and reseeded into a 75 cm² flask. Cells grown in suspension were maintained at concentrations between 5×10⁵-1×10⁶ cells/ml. All cells were routinely grown at 37°C in a humidified atmosphere containing 5% CO₂.
2.2.4 Transfection of cell lines

2.2.4.1 Calcium phosphate-mediated DNA transfection

Calcium phosphate-mediated DNA transfection of mammalian cells was performed essentially as described by Jordan et al. with minor modifications (Jordan et al., 1996).

Approximately 20 hours before transfection, \(1 \times 10^5\) cells were seeded in each well of a 6-well plate in 2 ml of complete medium.

A solution of 10 \(\mu\)l of 2.5 M \(\text{CaCl}_2\) and 2.2 \(\mu\)g of plasmid DNA (2 \(\mu\)g of luciferase plasmid and 0.2 \(\mu\)g of pcDNA3.1/HisB/lacZ or pJ7lacZ) was diluted with sterile de-ionised water to a final volume of 100 \(\mu\)l. This 2\(\times\) \(\text{Ca}^{2+}\)/DNA solution was added in a dropwise manner to an equal volume of 2\(\times\) HEPES buffered saline whilst bubbling air through the mixture. The calcium phosphate-DNA co-precipitate was allowed to form for 10 min and was then added slowly to the 2 ml of medium covering the cells. After exposing the cells to the precipitate for 15-20 hours at 37°C in 5% \(\text{CO}_2\), the medium was removed and the cells were washed twice with PBS. Subsequently, fresh medium was added and the cells were grown for a further 24 hours before harvesting.

2.2.4.2 FuGENE 6 mediated DNA transfection

Approximately 20 hours before transfection, \(1 \times 10^5\) cells were seeded in each well of a 6-well plate in 2 ml of complete medium. For each well, 3 \(\mu\)l of FuGENE 6 transfection reagent was diluted in 97 \(\mu\)l of serum-free DMEM and incubated at room temperature for 5 min. The diluted FuGENE 6 was added to a solution containing 1 \(\mu\)g of plasmid DNA at a concentration between 0.1 and 1.0 \(\mu\)g/\(\mu\)l and incubated at room temperature for a further 15 min before being added to the cells. No washing of the transfection medium was required and cells were harvested approximately 40 hours after transfection.
2.2.4.3 DEAE-Dextran mediated DNA transfection

Approximately 20 hours prior to transfection, $1 \times 10^6$ COS7 cells diluted in 10 ml of fresh medium (DMEM + 10% FCS) were seeded in a 10 cm petri dish. Just prior to transfection, the culture medium was removed and replaced with 5 ml of serum free DMEM while transfection mixes were prepared: 5 ml of filter sterile 0.25 M Tris pH 7.5 was added to 20 ml of serum free DMEM. For each transfection, 0.5 ml of 10 mg/ml DEAE-Dextran and 2 µg of plasmid DNA was added to 4 ml of the DMEM/Tris solution. The serum free medium was removed from the cells and replaced with the DNA mix. After 20 minutes pre-incubation at 37°C, chloroquine was added to the medium to a final concentration of 40 µg/ml and the cells were returned to 37°C for 2-3 hours. The transfection solution was removed and the cells washed twice with PBS before 10 ml of fresh medium was added to each petri dish.

Total cellular RNA was harvested from the cells 48 hours after transfection using the TRI Reagent method (Chomczynski, 1993; Section 2.4.22).

2.2.5 Induction of apoptosis

2.2.5.1 TRAIL induced apoptosis of HeLa cells

The TNF-related apoptosis inducing ligand (TRAIL) induces apoptosis by oligomerisation of death receptors. HeLa cells in 6 well plates were transfected by the calcium phosphate method and 40 hours after transfection the medium was supplemented with His-tagged TRAIL to a final concentration of 0.25 µg/ml. This was purified from BL21 cells transformed with the plasmid pET28b (a kind gift from Dr. Marion MacFarlane, University of Leicester). Cells were harvested at times of 0, 1, 2, 4, 6 and 8 hours after addition of TRAIL.
2.2.5.2 Induction of apoptosis in HeLa cells with EMS

High concentrations of the DNA damaging agent, Ethyl methanesulfonate (EMS) induce apoptosis in cultured cells. 40 hours after transfection of HeLa cells by the calcium phosphate method, the medium was supplemented with EMS to a final concentration of 50 mM. Cells were harvested at the same time points.

2.2.6 Cell cycle synchronization of cell populations

Overnight treatment with nocodazole (Methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate, dissolved to 2mg/ml in DMSO and diluted 1:1000) arrests cells undergoing mitosis by inhibiting spindle pole formation. Cells arrested in mitosis are found in suspension and so were harvested by centrifuging the medium at $1500 \times g$ and washing the pellet twice in phosphate buffered saline. Half of the cells were lysed to measure luciferase activities in M phase, while the other half were resuspended in fresh media. These cells, released from the nocodazole block, were grown for a further 2 hours and then harvested when in G1.

Treatment with aphidicoline (from Nigrospora sphaerica, dissolved in DMSO to a final concentration of 5mg/ml and diluted 1:1000) overnight prevents exit from S-phase by inhibiting eukaryotic nuclear DNA synthesis. Again, some cells were harvested when arrested in S phase while others were released from the aphidicoline block by washing the cells three times with phosphate buffered saline before adding fresh media. These cells were then grown for a further 6-8 hours to allow progression into G2, where they were harvested.
2.3 Bacterial Methods

2.3.1 Bacterial media and supplements

**LB medium:** 10 g Bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl dissolved in 1 l of deionised water.

**LB agar plates:** 10 g Bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl dissolved in 1 l of deionised water and supplemented with 15 g of agar.

**Ampicillin:** a stock solution of 50 mg/ml of the sodium salt of ampicillin was prepared using sterile deionised water and used at a final concentration of 50 μg/ml.

2.3.2 Bacterial strains

The *Escherichia coli* strain JM109 was used in most bacterial manipulations

JM109: e14*(mrcA)recA1, endA1, gyr A96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB), F', traD36, proAB, lacZΔM15

The XL1-Blue strain of Epicurian coli® supercompetent cells (Stratagene) was used during PCR-directed mutagenesis protocols

XL1-Blue: recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1 lac [F', proAB, lacZΔM15, Tn10 (Tet)]

The BL21 strain of *E.coli* was used for the production of recombinant TRAIL

BL21(DE3): F', ompT, hsdSB, (rB',mB'), dcm, gal

2.3.3 Preparation and transformation of competent cells

A single colony from an LB plate was inoculated into 2.5 ml of LB medium and incubated overnight at 37°C with shaking. The entire overnight culture was inoculated into 250 ml of LB medium supplemented with 20 mM MgSO₄ and incubated at 37°C until the A₆₀₀
reached 0.4-0.6. Cells were pelleted by centrifugation at 4,500 × g for 5 min at 4°C using a GSA rotor (Sorvall). Following centrifugation, the pellet was gently resuspended in 100 ml of ice-cold filter sterile TFB1 (30 mM KAc, 10 mM CaCl$_2$, 50 mM MnCl$_2$, 100 mM RbCl, 15% (v/v) glycerol, adjusted to pH 5.8 with 1 M glacial acetic acid). After incubating on ice for 5 min, the cells were centrifuged at 4,500 × g for 5 min at 4°C. The pellet was resuspended in 10 ml of ice-cold filter sterile TFB2 (1 mM MOPS, pH 6.5, 75 mM CaCl$_2$, 10 mM RbCl, 15% (v/v) glycerol, adjusted to pH 6.5 with 1 M KOH) and the cells were incubated on ice for 1 hour. Finally, 200 μl aliquots of the cells were rapidly frozen in an isopropanol/dry ice bath and stored at −80°C.

Competent cells were thawed on ice and ligation products or plasmid DNA (10 ng) were added to 50 μl of competent cells and incubated on ice for 20 min. After heating the mixture at 42°C for 2 min, 150 μl of antibiotic free LB medium was added. Subsequently, the cells were incubated with gentle shaking at 37°C for 45 minutes. Finally, the sample was spread onto a pre-warmed LB agar plate containing ampicillin and then incubated at 37°C for 16-20 hours.

2.4 Molecular Biology Techniques

2.4.1 Buffers and solutions

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

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

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

5× TBE loading buffer: 50% v/v glycerol, 200 mM Tris, 200 mM acetic acid, 5mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol
DNA sequencing formamide loading dyes: 100% deionised formamide, 0.1%(w/v) Xylene cyanol FF, 0.1%(w/v) Bromophenol blue, 1mM EDTA

RNA formamide loading buffer: 80% deionised formamide, 10 mM EDTA, 0.1% SDS, 0.1% Xylene cyanol FF, 0.1% Bromophenol blue

Oligonucleotide labelling buffer (OLB): Mix solutions A, B, and C in the ratio 2:5:3

A) 1.2 M Tris-HCl, pH 8.0, 0.12 M MgCl₂, 1.75% (v/v) β-mercaptoethanol, and 0.5 mM of dATP, dGTP and dTTP

B) 2M HEPES-NaOH, pH 6.6

C) 1.6 mg/ml Hexadeoxyribonucleotides in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7

2.4.2 Plasmids

pGL3

pRF (previously designated pGL3R2)

pRMF (previously pGL3R2utr)

phpRMF (previously pGL3R2utrH)

pRCAT

pSKL

pRHRVF

pREMCVF

Synthesis of all the above plasmids is detailed in (Stoneley, 1998; Stoneley et al., 1998; or Stoneley et al., 2000b).

pBluescript II SK (+) (Stratagene)

pcDNA3.1/HisB/lacZ (Invitrogen)

pJ7/lacZ (a kind gift from Dr. David Heery, University of Leicester)
2.4.3 Ethanol precipitation of DNA and RNA

Nucleic acids were precipitated by the addition of 0.1 volume of 3 M NaAc, pH 5.2 and 2 volumes of absolute ethanol, or 0.1 volume of 7.5 M ammonium acetate, pH 7.6 and an equal volume of isopropanol. The sample was incubated on ice or at −20°C for 15-30 minutes following which the DNA was pelleted by centrifugation at 12,000 × g for 10 minutes. Excess salt was removed from the pellet by washing with 70% ethanol, and then the DNA was dried briefly and resuspended in either TE (Section 2.4.1) or sterile deionised water.

2.4.4 Determination of nucleic acid concentrations

DNA and RNA concentrations were determined spectrophotometrically by monitoring the absorbance at 260nm. 50 µg/ml of dsDNA, 40 µg/ml of RNA and 20 µg/ml of oligonucleotide were taken to be equivalent to 1 OD unit.

2.4.5 Phenol/chloroform extraction

Solutions of nucleic acid were separated from contaminating proteins by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After vigorous mixing, the phases were separated by centrifugation at 12,000 × g for 5 minutes. The upper aqueous phase was removed to a separate tube, to which an equal volume of chloroform:isoamyl alcohol was added. Following extraction and separation of the phases, the aqueous layer was transferred to a new tube and the nucleic acid was precipitated.

2.4.6 Agarose gel electrophoresis

Fragments of DNA or RNA were fractionated according to their molecular weight by electrophoresis through agarose gels. For most purposes, agarose was melted in 1× TBE buffer (Section 2.4.1) and cooled. If the DNA in the gel was to be eluted, the agarose solution
was prepared with 1× TAE buffer instead (Section 2.4.1). 2 μl of 10mg/ml ethidium bromide was added to the agarose solution and the gel was cast in a suitable support. The set gel was submerged in 1× TBE or 1× TAE buffer in a horizontal electrophoresis tank. Samples were mixed with 0.2 volumes of 5× TBE loading buffer (Section 2.4.1) and separated in the gel at up to 8 V/cm. After electrophoresis, the DNA was visualised on a UV transilluminator. DNA fragments to be eluted were visualised using a low intensity UV lamp. Agarose containing the required fragment was excised from the gel using a clean scalpel blade.

2.4.7 Purification of DNA from agarose gels or solution using glassmilk and Qiagen minicolumns

Glassmilk (BIO101) and Qiaquick spin columns (Qiagen) were used to purify DNA fragments amplified by PCR, to isolate DNA when a change of reaction buffer was required or to extract DNA from melted agarose gel slices.

If the DNA was to be isolated by the glassmilk method, DNA in solution was incubated with 3 volumes of 6 M NaI and 5 μl of glassmilk on ice. If the DNA was present in an agarose gel slice, the mass of the slice was measured and the gel slice was firstly melted at 55°C in 3 volumes of 6 M NaI before addition of the glassmilk solution. The glassmilk was pelleted by centrifugation and washed twice with 0.5 ml of NEW wash solution (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 52.6% v/v Ethanol). After the glassmilk was resuspended in 10 μl of sterile deionised water, it was incubated at 45-55°C for 5 min to elute the DNA. Following centrifugation, the supernatant was removed to a fresh tube and the elution process was repeated.

For the Qiaquick spin method, 3 volumes of buffer QG were added to the solution or gel slice that contained the DNA to be purified. Gel slices were subsequently melted at 55°C. The DNA was bound to the silica matrix in the Qiaquick spin column by centrifugation of the
DNA solution for 1 minute at 12,000 × g. Flowthrough was discarded and the DNA was washed by addition of buffer PE followed by centrifugation of the column for 1 minute. After removal of the flowthrough, a further 1 minute centrifugation ensured complete removal of the wash buffer from the column. DNA was eluted into a fresh microcentrifuge tube by the addition of 30 μl of sterile deionised water to the column, followed by a 1 minute incubation at room temperature before centrifugation at 12,000 × g for 1 minute.

2.4.8 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 394 machine (Protein and Nucleic Acid Chemistry Laboratory, Leicester University) at a 0.2 μM scale. Oligonucleotides were purified by ethanol precipitation with 0.1 volume of 3M NaAc, pH 5.2 and 3 volumes of absolute ethanol. Samples were incubated at –20°C for 30 min and the precipitate was pelleted by centrifugation at 12,000 × g for 20 min. After washing with 70% ethanol the pellet was dried and then resuspended in 100 μl of TE. The concentration of the oligonucleotide was determined by measuring the absorbance at 260 nm (Section 2.4.4).

2.4.9 Oligonucleotides

Details of the oligonucleotides employed are given in table 2.2
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
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<tbody>
<tr>
<td>ApU 5'</td>
<td>TTGAATTCAGAAGAGGTAGCGAGTGG</td>
</tr>
<tr>
<td>ApU 3'</td>
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<td>APMUT1</td>
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<td>APMUT1R</td>
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<td>APMUT2R</td>
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<tr>
<td>A470 5'</td>
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<tr>
<td>A470 3'</td>
<td>CGCCATGGAAGGGAAACGGAACAGGA</td>
</tr>
<tr>
<td>A523 3'</td>
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<td>A345 5'</td>
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<td>MmApF</td>
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<td>MmApR</td>
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<tr>
<td>BRNase F</td>
<td>GTGGATCCGCAAGAAGATGACACCTGATG</td>
</tr>
<tr>
<td>Luc 3'</td>
<td>GCGTATCTCTCTCATAGCCTT</td>
</tr>
<tr>
<td>H3 luc 3'</td>
<td>ATAAGCTTTGCGTATCTCTTCTAGGCTCT</td>
</tr>
<tr>
<td>SV40 5'</td>
<td>GAGCTATTCCAGAAGTATAGGTA</td>
</tr>
<tr>
<td>SV40 en R</td>
<td>CGGTCGACGGATCCGCTGTGGA</td>
</tr>
<tr>
<td>Hpin reverse</td>
<td>ACTGCGGACCAGTTATCATCC</td>
</tr>
<tr>
<td>FLAG F</td>
<td>ATCCATGGACTCAAAAGACGATGACGACAAAGGCCATGGGC</td>
</tr>
<tr>
<td>FLAG R</td>
<td>GGCACATGGGCCTTGCTCGTACGTCTCTTGTAGTCCATGGAT</td>
</tr>
<tr>
<td>FLAG PCR</td>
<td>CCATGGACTCAAAAGACGATGACGAC</td>
</tr>
<tr>
<td>Hpin F</td>
<td>TATCTAGAAAGCTTGGCATTCCGGT</td>
</tr>
<tr>
<td>Hpin R</td>
<td>CGACTAGTGGGGCTAGAGCTTGAT</td>
</tr>
</tbody>
</table>

**Table 2.2:** Oligonucleotides used in molecular biology manipulations.
2.4.10 Standard PCR reaction

Additives such as dimethyl sulfoxide (DMSO) and Betaine prevent formation of secondary structures in the polymerase chain reaction (PCR) and so were routinely used in the amplification of G-C rich templates (Varadaraj and Skinner, 1994; Henke et al., 1997; Rees et al., 1993). To ensure accuracy of amplified products the high fidelity DNA polymerase from *Pyrococcus furiosus*, *PfuTurbo™* (Stratagene), was used.

Standard PCR reactions were performed in a final volume of 50 μl containing 1× Cloned *Pfu* Buffer (20 mM Tris-HCl pH 8.8, 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1 mg/ml nuclease free BSA), 1 M Betaine, 2% DMSO, 100 ng of template DNA, 125 ng of both the upstream and downstream primers, 0.2 mM of each dNTP and 2.5 units of *PfuTurbo™* DNA polymerase. Reactions were performed in a Techne Genius Thermal Cycler. DNA was initially denatured by heating at 94°C for 3 min after which the samples were heated at 94°C for 30 s (denaturation), 57-65°C for 30 s (annealing) and 72°C for 60 s (extension), respectively, for 25-35 cycles. A final extension at 72°C for 10 minutes was performed to optimize the yield of specific amplification products.

2.4.11 RT-PCR

As the 5' UTR of the murine form of Apaf-1 is G-C rich, secondary structures in RNA can inhibit the reverse transcriptase activity of AMV reverse transcriptase at 42°C. To compensate for this the Roche Molecular Biochemicals' *C. therm.* Polymerase One-Step RT-PCR System was used to amplify the murine Apaf-1 5' UTR. The Klenow fragment of DNA polymerase from *Carboxydothermus hydrogenoformans* possesses an intrinsic magnesium dependent reverse transcriptase activity and so can be used for high fidelity reverse transcription as well as amplification.
The reaction was carried out according to the manufacturer’s protocols with some minor modifications. Briefly, two master mixes were made up, the first master mix containing 0.4 mM dNTPs, 7% DMSO, 1 M Betaine, 5 mM DTT, 20 units of RNAsin ribonuclease inhibitor (Promega), 0.3 μM upstream primer (MmApF), 0.3 μM downstream primer (MmApR) and 100 pg of poly(A)+ RNA from Balb-c cells (Section 2.4.23) in a total volume of 25 μl. This was combined with the second master mix which contained 1× RT-PCR buffer (containing 2% DMSO and 2.5 mM MgCl₂) 2 μl of C. therm polymerase mixture (a mixture of C. therm. polymerase and Taq DNA polymerase) in a total volume of 25 μl. The two master mixes were combined and the reverse transcription performed in a Techne Genius thermal cycler for 30 min at 65°C followed by a 2 min denaturation step at 94°C followed by 35 cycles 30 s at 94°C, 30 s at 58°C and 1 min at 72°C. After cycling, the reaction was incubated at 72°C for 7 min. A 5 μl sample of the reaction was analysed by agarose gel electrophoresis to check the amplification products, after which the remainder of the reaction was purified (Section 2.4.7) before subcloning.

2.4.12 PCR mutagenesis

In vitro site-directed mutagenesis was used to make point mutations and introduce restriction enzyme cleavage sites in various reporter constructs. The QuikChange™ Site-Directed Mutagenesis method (Stratagene) was used. 250 ng of the wild type plasmid was combined with 125 ng of each mutagenic oligonucleotide primer, 1× Pfu polymerase reaction buffer, 200 μM dNTPs and 2.5 units of PfuTurbo™ DNA polymerase in a total volume of 50 μl. After 18 cycles of amplification (Denaturation at 95°C for 30s, annealing at primer Tm – 5°C for 2 min and extension of 1 minute per kb template at 68°C), the reaction was cooled on ice for 2 min. A 5 μl sample was removed and used to check the integrity of the reaction products by agarose gel electrophoresis. The rest of the reaction was then treated with 10
units of the methylase specific restriction endonuclease \textit{DpnI} for 1 hour at 37°C, which selectively digested the parental template DNA. 4 μl of the reaction products were transformed into Epicurian Coli® XL1-Blue supercompetent cells (Stratagene) as described in section 2.3.3. DNA extracted from transformed colonies was sequenced to confirm the presence of the mutation (Section 2.4.21).

\textbf{2.4.13 Restriction enzyme digestion}

DNA was digested with restriction enzymes in a total volume of 10-50 μl under the conditions recommended by the suppliers. Reactions were incubated at the appropriate temperature for 1-2 hours. If sequential digestions required a change of buffer conditions, DNA was purified as described in Section 2.4.7.

\textbf{2.4.14 Preparation of DNA for subcloning}

\textbf{2.4.14.1 Filling in of recessed 3’ ends and removal of overhanging 3’ ends}

The large (Klenow) fragment of \textit{E. coli} DNA polymerase I was used to fill in the recessed 3’ ends of DNA fragments and remove the overhanging 3’ ends. The reaction was performed in a final volume of 25 μl, containing 80 μM of each dNTP, a maximum of 2 μg of digested DNA and 1× Fill-in buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgCl₂, 0.1 mM DTT, 100 μg/ml BSA) or 1× Restriction enzyme buffer supplemented with 100 μg/ml of BSA. 1-5 units of Klenow DNA polymerase were added and the reaction was incubated at 30°C for 15 min. The reaction was stopped by heating at 75°C for 10 min, after which the DNA was purified.
2.4.14.2 Alkaline phosphatase treatment of DNA

Linearised plasmids were treated with calf intestinal alkaline phosphatase (CIAP) to remove a phosphate group from the 5' ends and prevent self-ligation. Following restriction digestion, heating the reaction at 65°C for 15 min inactivated the restriction enzyme. Dephosphorylation was performed in a final volume of 50 µl in 1× Restriction enzyme buffer. For DNA fragments with overhanging 5' ends the reaction was incubated for 30 min at 37°C using 1 unit of CIAP, after which another unit of enzyme was added and the incubation was repeated. For DNA fragments with blunt ends, the reaction was incubated at 37°C for 15 min followed by 56°C for 15 min using 1 unit of CIAP, and these incubations were repeated after the addition of another unit of CIAP. Heating at 75°C for 10 min terminated the reaction and the DNA was purified (Section 2.4.7). For those restriction enzymes that are resistant to heat-inactivation, the DNA was first purified, then resuspended in 50 µl of 1× CIAP reaction buffer (50 mM Tris-HCl, pH 9.3, 1 mM MgCl₂, 0.1 mM ZnCl₂). The reactions were then performed as described above.

2.4.15 Annealing complementary oligonucleotides

The complementary oligonucleotides FLAG F and FLAG R were annealed to create an oligonucleotide cassette that was subsequently used in a ligation reaction. Equimolar amounts of oligonucleotide (5 µg of each) were combined and heated at 75°C for 10 min. The oligonucleotides were then annealed by incubating the mixture at 37°C for 10 min followed by incubation on ice for 10 min. The restriction endonuclease NcoI was used to digest the nucleotide cassette. Finally, the concentration of the oligonucleotide cassette was adjusted to approximately 40 nM.
2.4.16 Ligations

Ligations were performed in a total volume of 10 μl. Vector DNA (50 ng) was mixed in a 1:3 molar ratio with insert DNA in a reaction containing 1× T4 DNA ligase buffer (40 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP) and 2.5 units of T4 DNA ligase. For ligations involving fragments with overhanging termini, the reaction was incubated at 16°C for 2-16 hours. 4%(w/v) polyethylene glycol 8000 was included in reactions in which all DNA termini were blunt. The efficiency of these blunt ended ligations was further improved by incubating the reaction for at least 16 hours at 16°C. After the appropriate incubation, 5 μl of the ligation reaction was heat inactivated for 10 min at 65°C, before transformation into competent E. coli (Section 2.3.3).

2.4.17 Radiolabelled DNA markers

1 μg of pBR322 DNA was digested with 5 units of HpaI for 20 min in a volume of 10 μl and the reaction was stopped by heating at 90°C for 2 min. The DNA fragments were radiolabelled using the Klenow fragment of E. coli DNA polymerase I in a reaction volume of 15 μl containing 1× restriction enzyme buffer, 100 μg/ml of BSA, 1 mM dCTP, 10 μCi of [α-32P]dCTP (800 Ci/mmol) and 5 units of Klenow DNA polymerase. The reaction was incubated at 30°C for 15 min and stopped by the addition of RNA formamide loading buffer (Section 2.4.1).

2.4.18 Small scale preparation of plasmid DNA

A single colony of E. coli was inoculated into 5 ml of LB media containing ampicillin and incubated overnight at 37°C in a shaking incubator. Approximately 1.5 ml of the culture was decanted into a labelled tube and the bacteria were pelleted by centrifugation. The pellet was resuspended in 100 μl of ice-cold solution I (25 mM Tris-HCl, 10 mM EDTA, 50 mM
glucose, pH 8.0). After a 5 minute incubation at room temperature, 200 μl of solution II (1% (w/v) SDS, 0.2 M NaOH) was added and the solutions were mixed gently. The sample was incubated on ice for 5 min, following which 150 μl of 7.5 M NH₄Ac, pH 7.6 was added. After briefly mixing the solutions using a vortex, the sample was incubated on ice for a further 5 min. The precipitated matter was pelleted by centrifugation at 12,000 × g for 5 min and the supernatant was removed to a fresh tube. Plasmid DNA was precipitated with ethanol from this solution as described in section 2.4.3. Finally, the washed and dried pellet was resuspended in 30 μl of TE or sterile deionised water. Diagnostic restriction digests were performed using 5 μl of this solution.

2.4.19 Large scale preparation of plasmid DNA

The ammonium acetate method described by Saporito-Irwin et al., (1997) was used to prepare milligram quantities of plasmid DNA suitable for most manipulations and as a template for in vitro transcription. An overnight culture of E. coli containing the plasmid was inoculated into 250 ml of LB media supplemented with ampicillin. The culture was grown for 12-16 hours in a 37°C shaking incubator. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C. The pellet was resuspended in 3 ml of ice-cold solution I and incubated at room temperature for 5 min. Following which, 6 ml of solution II was added and the sample was incubated on ice for 10 min. This solution was neutralised with 4.5 ml of 7.5 M NH₄Ac, pH 7.6 and incubated for a further 10 min on ice. The precipitated matter was pelleted by centrifugation at 10,000 × g for 10 min at 4°C and the supernatant was removed to a fresh tube. Isopropanol (0.6 volumes) was added and the solution was incubated at room temperature for 10 min. The insoluble material was pelleted by centrifugation (10,000 × g) for 10 min at room temperature. The plasmid DNA in the pellet was resuspended thoroughly in 2 M NH₄Ac, pH 7.4. The insoluble matter was pelleted as before and the supernatant
removed to a fresh tube. After the addition of 1 volume of isopropanol, the solution was incubated at room temperature for 10 min and the plasmid DNA was pelleted by centrifugation. Following resuspension of the pellet in 1 ml of sterile deionised water, contaminating RNA was removed by adding 100 µg of RNase A and incubating the solution at 37°C for 15 min. Proteins were then precipitated by the addition of 0.5 volume of 7.5 M \( \text{NH}_4\text{Ac}, \) pH 7.6 and incubation at room temperature for 5 min. The precipitated proteins were pelleted by centrifugation and the supernatant was removed to a fresh tube. Finally, the plasmid DNA was precipitated using an equal volume of isopropanol, pelleted by centrifugation and washed with 70% ethanol. The resulting pellet was resuspended in a volume of 0.5-1 ml of TE or filter sterile water.

2.4.20 Large scale preparation of plasmid DNA by Qiagen column

The Qiagen plasmid purification protocol was used to produce microgram quantities of plasmid DNA suitable for transfection. An overnight culture of \( E. \text{coli} \) containing the plasmid was inoculated into 100 ml of LB media supplemented with ampicillin. The culture was grown for 12-16 hours in a 37°C shaking incubator. Cells were harvested by centrifugation at 5,000 \( \times \) g for 10 min at 4°C. The pellet was resuspended in 4 ml of Buffer P1 (50 mM Tris-HCl pH8.0, 10 mM EDTA, 100 µg/ml RNase A). Cells were lysed by the addition of 4 ml of Buffer P2 (200 mM NaOH, 1% SDS) followed by five minutes incubation at room temperature. 4 ml of Buffer P3 (3.0 M KAc, pH5.5) was added to neutralize the sample. The sample was mixed by inversion and incubated on ice for 15 min. The sample was centrifuged at 20,000 \( \times \) g for 30 min at 4°C and the supernatant was recentrifuged at 20,000 \( \times \) g for 15 min at 4°C to ensure no suspended material was applied to the Qiagen resin. The supernatant was added to a Qiagen-tip 100 that had been equilibrated by the addition of 4 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% v/v isopropanol). The Qiagen-tip 100 was
washed twice by the addition of 10 ml of Buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 
15% v/v isopropanol) and the DNA was then eluted by the addition of 5 ml of Buffer QF 
(1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% v/v isopropanol). The DNA was precipitated by 
the addition of 0.7 volumes of isopropanol followed by centrifugation at 15,000 × g for 30 
min at 4°C. The resulting pellet was washed with 70% ethanol and redisolved in 0.1-0.5 ml 
of TE or 10 mM Tris-HCl pH 8.5.

2.4.21 Double stranded DNA sequencing

Plasmid DNA was isolated using the small scale method (Section 2.4.18) and 
contaminating RNA was digested with 1 μg of RNase A at 37°C for 30 min. After RNase 
treatment, the DNA was ethanol precipitated and resuspended in 10 μl of sterile deionised 
water. The plasmid DNA was denatured by incubating this solution with 0.1 volumes of 2 
mM NaOH, 2 mM EDTA, pH 8.0 at 37°C for 15 min. After which, the solution was 
neutralised with 0.1 volumes of 7.5 M NH₄Ac, pH 7.6 and 1 volume of isopropanol was 
added. Following incubation at -80°C for at least 30 min, the single stranded DNA was 
pelleted by centrifugation at 12,000 × g for 10 min and the pellet was dried. The pellet was 
resuspended in 10 μl of a 2.5 ng/μl solution of sequencing primer and 2 μl of annealing buffer 
(280 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 350 mM NaCl). The plasmid DNA/primer 
solution was heated at 65°C for 2 min, and then incubated at room temperature for 10 min, 
followed by 5 min on ice to achieve primer annealing. Samples were labelled at 20°C for 5 
min, in a reaction containing 0.4 μl [α-³⁵S]dATP (12.5 mCi/ml), 3 μl of labelling mix A (2 
μM dGTP, 2 μM dCTP, 2 μM dTTP), and 1 unit of T7 DNA polymerase. Labelling was 
terminated by the addition of 4 μl of each termination mix (150 μM of each dNTP, 10 mM 
MgCl₂, 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15 μM ddNTP G, A, T, or C) and incubated 
at 42°C for 5 min. Finally, the reaction was stopped by adding 4 μl of formamide loading
dyes (Section 2.4.1). The labelled DNA fragments were fractionated on a 6%
polyacrylamide/7M urea gel following which the gel was dried under a vacuum for 1 hour at
80°C and exposed to Fuji X-ray film for 16-48 hours.

2.4.22 Isolation of total cellular RNA

Total cellular RNA was isolated using TRI Reagent (Chomczynski, 1993), a
development of the guanidium isothiocyanate method described by (Chomczynski and Sacchi,
1987). Adherent cells were lysed by addition of 1 ml of TRI Reagent (Sigma) and removed
with a cell scraper. Once the lysate was transferred to a fresh tube, 200 μl of chloroform was
added and the mixture was vigorously mixed using a vortex for at least 30 s. The aqueous and
organic phases were separated by centrifugation at 12,000 × g for 15 min at 4°C and the upper
aqueous phase was transferred to a fresh tube. An equal volume of isopropanol was added to
this solution and the sample was incubated at room temperature for 10 min. The precipitate
was pelleted by centrifugation at 12,000 × g for 15 min and washed with 75% ethanol. After
briefly drying the pellet, it was resuspended in 30 μl of filter sterile deionised water. A 1 μl
sample was subjected to agarose gel electrophoresis to ensure that degradation had not
occurred. Finally, prior to storage at −80°C, the concentration of the RNA was determined by
measuring its absorbance at 260 nm (section 2.4.4).

2.4.23 Purification of poly(A)^+ mRNA from total cellular RNA

Poly(A)^+ mRNA was purified from total cellular RNA using oligo[dT]_25 magnetic
DynaBeads (Dynal Inc.) according to the manufacturers instructions. Initially, 50 μl of
oligo[dT]_25 beads in 1× Storage buffer (250 mM Tris-HCl, pH 7.0, 20 mM EDTA, 0.1%
Tween 20, 0.02% sodium azide) were washed in 100 μl of 2× Binding buffer (20 mM Tris-
HCl pH 7.5, 1 M LiCl, 2 mM EDTA) and then resuspended in a further 100 μl of 2× Binding
buffer. Filter sterile de-ionised water was used to increase the volume of 50 μg of total cellular RNA to 100 μl. After heating the RNA solution at 65°C for 2 min, it was combined with the bead suspension. The RNA was bound to the beads by continuously mixing the solution at room temperature for 5 min. Following which, the beads were pelleted using the magnet provided and the supernatant was removed. Unbound material was removed by washing the beads twice with 200 μl of wash solution (10 mM Tris-HCl pH 7.5, 150 mM LiCl, 1 mM EDTA). Finally, 10 μl of elution solution (10 mM Tris-HCl, pH 7.5) was added to the beads and the poly(A)+ mRNA was eluted by heating at 65°C for 2 min.

Oligo[dT]_{25} beads were reconditioned by resuspension in 200 μl of Reconditioning solution (0.1 M NaOH) followed by heating to 65°C for 2 min. The beads were then washed at room temperature in Reconditioning solution six times and resuspended in 1 x storage buffer before storage at 4°C.

2.4.24 *In vitro* run-off transcription

10 μg of vector DNA was linearised by restriction digestion using a site downstream of the sequence of interest. Subsequently, the protein was removed by phenol/chloroform extraction and following ethanol precipitation, the DNA was resuspended in 10 μl of filter sterile deionised water. Capped transcripts were synthesised in a reaction containing 1 x Transcription buffer, (80 mM HEPES-KOH, pH 7.5, 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT), 20 units of RNasin, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.25 mM GTP, 1 μM m⁷G(5')ppp(5')G, 1 μg of DNA template and 20 units of T7 RNA polymerase in a final volume of 50 μl. After incubation of the reaction for 1 hour at 37°C, an extra 10 units of T7 RNA polymerase were added and the reaction incubated for a further 30 min.

The reaction was stopped by heating at 70°C for 5-10 min and the DNA template was digested with 10 units of RNase-free DNase I for 15 min at 37°C. Immediately following
digestion, the RNA was phenol/chloroform extracted and unincorporated nucleotides were removed by passing the solution through a Sephadex G-50 column. The RNA was precipitated by the addition of 0.5 volumes of 7.5 M NH₄Ac and 2.5 volumes of ethanol. After incubation at −70°C for 30 min, the RNA was pelleted by centrifugation and washed with 75% ethanol. The pellet was resuspended in 30 µl of filter sterile 0.1× TE and the concentration was determined using the absorbance at 260 nm (section 2.4.4). In addition, 0.5 µl of the RNA was subjected to agarose gel electrophoresis to ensure the product was not degraded.

Radiolabelled transcripts were synthesised in a reaction containing 1× Transcription buffer (40 mM TrisHCl pH 7.9, 6 mM MgCl₂, 10 mM DTT, 10 mM NaCl, 2 mM spermidine), 6 mM KOH, 20 units of RNasin 50 µCi of [α-³²P]UTP (800Ci/mmol), 1 mM of ATP, CTP and GTP, 100 µM of UTP, 1 µg of template DNA and 20 units of T7 RNA polymerase in a final volume of 10 µl. After incubation for 1 hour at 37°C, the template DNA was removed as described previously. RNA was resuspended in 10 µl of loading dyes (80% deionised formamide, 10 mM EDTA, 0.1% SDS, 0.1% Xylene cyanol FF, 0.1% Bromophenol blue). After heating at 85°C for 5 min, transcripts were fractionated on a 4% polyacrylamide/7M urea gel and detected by exposure to Fuji X-ray film for 60 s. A slice of polyacrylamide containing only full length transcripts was excised from the gel, and RNA was extracted by incubation with 0.5 ml of extraction buffer (0.5 M NH₄Ac, 1 mM EDTA, 0.2% SDS) for 16 hours at 4°C. Radiolabelled RNA was precipitated from the supernatant using 0.1 volume of NH₄Ac and 2.5 volumes of ethanol, pelleted by centrifugation, washed with 75% ethanol and resuspended in 50 µl of filter sterile deionised water. Transcript concentrations were then determined by Cerenkov scintillation counting.
2.4.25 RNase protection

The RNA samples were combined with $2.5 \times 10^5$ cpm of radiolabelled riboprobe and precipitated with 0.1 volume of 3 M NaAc pH 4.6 and 2.5 volumes of ethanol for 30 min at -20°C. The RNA was pelleted by centrifugation, washed with 75% ethanol, and briefly dried, after which it was resuspended in 30 µl of hybridisation buffer (80% deionised formamide, 40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA). After heating at 85°C for 5 min, the samples were incubated at 45°C for 16 hours to allow annealing to occur. Subsequently, 300 µl of RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate) was added and single stranded RNA was digested for 90 min at 37°C with RNase ONE™ (Promega) at a concentration of 1 unit/µg of RNA. The reaction was terminated by the addition of 10 µl of 20%(w/v) SDS and proteins were digested with 2.5 µl of 20 mg/ml proteinase K at 37°C for 15 min. The sample was extracted once with phenol/chloroform and the aqueous phase was removed to a separate tube containing 10 µg of carrier tRNA. RNA was precipitated by incubating the sample with 825 µl of 100% ethanol at -20°C for 30 min. After pelleting by centrifugation and washing with 75% ethanol the sample was dried and resuspended in 5 µl of formamide RNA loading dye. RNA was denatured by heating at 85°C for 5 min and fractionated by electrophoresis through a 4% polyacrylamide/7M urea gel. Finally, radiolabelled RNA fragments were detected by analysis of the dried gel using a Molecular Dynamics phosphorimager.

2.4.26 Denaturing RNA agarose gel electrophoresis and Northern blotting

Samples of RNA were denatured by incubation in 1× Gel buffer (20 mM MOPS, pH 7.0, 5 mM NaAc, and 1 mM EDTA), 6.5% formaldehyde, and 50% deionised formamide at 55°C for 15 min in a final volume of 20 µl. 2 µl of RNase free loading buffer (50% glycerol, 1 mM EDTA, 0.4% Bromophenol blue and 0.4% Xylene Cyanol) was added to each sample.
Denatured RNA was fractionated by electrophoresis through a 1% agarose gel containing 1× Gel buffer and 6% formaldehyde. The gel was submerged in 1× Gel buffer and run at 100V for 3-4 hours. After electrophoresis was complete, the portion of gel containing the RNA markers (Gibco-BRL) was removed, stained with ethidium bromide (5 μg/ml) for 10 min and destained for 10 min using 1× Gel buffer. The markers were visualised using a UV transilluminator and photographed for later reference. The remainder of the gel was washed in filter sterile deionised water before being soaked in 0.05 M NaOH for 20 min to remove any formaldehyde and aid transfer of RNA. After rinsing with filter sterile deionised water, the gel was incubated in 20× SSC (3 M NaCl, 0.3 M tri-sodium citrate) for 20 min. The RNA was transferred from the gel to Zetaprobe nitrocellulose membrane (Biorad) using capillary blotting. After transferring for 16 hours, the RNA was fixed to the membrane by baking at 80°C for 2 hours. The filter was then ready for hybridisation with a random-primed radiolabelled probe.

2.4.27 Synthesis of a radiolabelled DNA probe and hybridisation to immobilised RNA

The plasmid pGL3 was digested with Ncol and AvaI and the 1058 bp luciferase-encoding fragment was isolated as described previously (Section 2.4.7). To prepare a random primed radiolabelled DNA probe 20 ng of the DNA fragment was heated at 95°C for 5 min in 10 μl of sterile deionised water. After the DNA was denatured, 3 μl of OLB (Section 2.4.1), 0.5 μl of 10 mM BSA, 1 μl of [α-32P]dCTP (800 Ci/mmol) and 5 units of Klenow DNA polymerase were added and the reaction was incubated at 37°C for 1-2 hours. Unincorporated nucleotides were then removed by passing the probe through a Sephadex G-50 column.

The RNA bound to the nitrocellulose filter (Section 2.4.26) was pre-hybridised with 10 ml of Church-Gilbert buffer (180 mM Na2HPO4, 70 mM NaH2PO4, 7% SDS) supplemented with 0.2 mg/ml of denatured Salmon sperm DNA and 50 μg/ml of bakers yeast.
tRNA (Sigma) for 1 hour at 65°C. The random-primed radiolabelled DNA probe was
denatured by heating at 95°C for 5 min and added directly to the hybridisation buffer.
Hybridisation was then performed at 65°C for 16-24 hours after which the filter was washed
once for 20 min at 65°C with Church-Gilbert buffer followed by 2-4 washes for 20 minutes
with Church Wash buffer 1 (14.4 mM Na$_2$HPO$_4$, 5.6 mM NaH$_2$PO$_4$, 1 mM EDTA, 5% SDS).
Further washes using Church Wash buffer 2 (14.4 mM Na$_2$HPO$_4$, 5.6 mM NaH$_2$PO$_4$, 1 mM
EDTA, 1% SDS) were performed if the background counts on the filter remained high after
the initial washes. Excess moisture was then removed from the filter and radiolabelled probe
was detected by phosphorimager analysis.

2.5 Biochemical Techniques

2.5.1 *In vitro* translation reactions

*In vitro* translation reactions were performed using the Flexi® rabbit reticulocyte lysate
system (Promega) with minor modifications to the manufacturer’s recommendations. Each
reaction contained 8.25 µl of reticulocyte lysate, 0.6 mM MgOAc, 20 units of RNasin, 2 µl of
1 mM complete amino acid mixture and between 20 and 100 ng of RNA substrate in a final
volume of 12.5 µl.

2.5.2 Preparation of cell lysates from transfected cells

After transfection, the medium was aspirated and the adherent cells were washed twice
with phosphate buffered saline (PBS). Cells were lysed by the addition of 200 µl of either 1×
Reporter lysis buffer (Promega) or 1× Passive lysis buffer (Promega) and wells were scraped
with a cell scraper. The lysate was transferred to a tube and subjected to one freeze-thaw
cycle at \(-20^\circ\text{C}\). The insoluble matter was pelleted by centrifugation and the supernatant was removed to a fresh tube and enzyme activity was assayed.

2.5.3 Luciferase assays

The activity of firefly luciferase in lysates prepared from cells transfected with monocistronic reporter vectors were measured using a luciferase reporter assay system (Promega). Lysates were prepared using \(1 \times\) Reporter lysis buffer as described in section 2.5.2. 5 \(\mu\)l of lysate was added to 25 \(\mu\)l of luciferase assay reagent and light emission was measured over 10 seconds using an Optocomp I luminometer (MGM Instruments).

The activity of both firefly and \textit{Renilla} luciferase in cell lysates transfected with dicistronic luciferase plasmids was measured using the Dual-luciferase reporter assay system (Promega) which allows the intensity of the separate bioluminescent signals to be measured in a single luminometer assay. After an initial firefly luciferase-dependent reaction, the “Stop and Glow” substrate quenches firefly luciferase activity while instantaneously activating \textit{Renilla} luciferase activity.

Cell lysates were prepared using \(1 \times\) Passive lysis buffer and 5 \(\mu\)l of lysate was used for each assay. Assays were performed according to the manufacturers recommendations except that only 25 \(\mu\)l of each reagent was used. Light emission was measured in the same manner as described previously.

2.5.4 \(\beta\)-Galactosidase assays

The activity of \(\beta\)-galactosidase in lysates prepared from cells transfected with pcDNA3.1/HisB/lacZ or pJ7lacZ was measured using the Galacto-Light Plus assay system (Tropix). This chemiluminescent assay is more sensitive than the commonly employed ONPG colourimetric assay. 5 \(\mu\)l of cell lysate was added to 25 \(\mu\)l of Galacton Plus reaction
buffer (Galacton-Plus substrate diluted 1:100 with Reaction Buffer diluent [100 mM NaPO₄ pH 8.0, 1 mM MgCl₂]) and incubated at room temperature for 1 hour. After which, 37.5 µl of Light Emission Accelerator II was added and enzyme activity was determined by immediately measuring the light emission from the reaction in a luminometer, as previously described.

2.5.5 Chloramphenicol acetyltransferase (CAT) assays

Lysates were prepared from cells transfected with pRCAT, pRBCAT, or pRMCAT using 1× Passive lysis buffer and heated at 60°C for 10 min to inactivate endogenous deacetylase activity. Reactions were set up containing 100 µl of cell lysate, 0.15 µCi of [¹⁴C]chloramphenicol (0.05 mCi/ml), and 5 µl of n-Butyryl Coenzyme A (5 mg/ml) in a final volume of 125 µl. After incubation at 37°C for 20 hours, the reaction was terminated by the addition of 300 µl of mixed xylenes (Sigma). The samples were mixed thoroughly using a vortex and the organic phase, containing butyrylated [¹⁴C]chloramphenicol, was transferred to a fresh tube. Unmodified [¹⁴C]chloramphenicol remains in the aqueous phase of the reaction. Any contaminating [¹⁴C]chloramphenicol was removed from the solvent by extracting twice with 100 µl of 0.25 M Tris-HCl, pH 8.0. The amount of butyrylated [¹⁴C]chloramphenicol in the xylene phase was measured by scintillation counting using 4 ml scintillant in a Packard Tri-Carb 2000 CA liquid scintillation analyser.

2.5.6 Analysis of reporter gene expression

Each experiment was performed in triplicate on three independent occasions. The expression of firefly and *Renilla* luciferases or chloramphenicol acetyl transferase were adjusted to that of the transfection control (β-galactosidase or firefly luciferase). This value was then expressed relative to that of the control vector. Errors were calculated as the
standard deviation of the reporter gene activities of the three experiments and then expressed as a percentage of the mean activity.

2.6 Protein Techniques

2.6.1 Stock solutions/buffers

1× SDS sample buffer: 50 mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.1% bromophenol blue, 10% β-mercaptoethanol, 1 mM EDTA

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

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

1× SDS running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS pH 8.3

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

Coomassie staining solution: 0.1% Coomassie brilliant blue R-250 dissolved in 5:1:5 methanol:acetic acid:water

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

2.6.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli, 1970. Briefly, gels were cast and run in a Bio-Rad Protean II system and were prepared as detailed in Table 2.3 using a 30%:0.8% acrylamide:bisacrylamide stock solution. Gels were polymerised by the addition of ammonium persulphate (APS) solution.
Table 2.3. Preparation of polyacrylamide gels (* denotes stacking buffer).

<table>
<thead>
<tr>
<th></th>
<th>Resolving Gel</th>
<th>Stacking Gel</th>
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<tbody>
<tr>
<td>%</td>
<td>7.5%</td>
<td>10.0%</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>17.5 ml</td>
<td>14.6 ml</td>
</tr>
<tr>
<td>Resolving buffer</td>
<td>8.75 ml</td>
<td>8.75 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>8.75 ml</td>
<td>11.65 ml</td>
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<tr>
<td>25% APS</td>
<td>135 μl</td>
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<tr>
<td>Total</td>
<td>35 ml</td>
<td>35 ml</td>
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Protein extracts were denatured by the addition of 1× SDS sample buffer containing protease inhibitors (19 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1 μg/ml TLCK, 20 μg/ml PMSF) and heated at 95°C for 5 min prior to loading. Gels were then run in SDS running buffer for 6-20 hours at between 8-40 mA depending on the resolution required. Typically, vertical gels were run at a constant voltage of 150V (minigels) or 40mA (large gels) until the Bromophenol blue dye front reached the bottom of the gel.

2.6.3 Transfer of proteins on to nitrocellulose membranes

Cell extracts were separated by SDS-PAGE and then transferred on to nitrocellulose (Schleicher and Schuell) by semi-dry blotting in transfer buffer (50 mM Tris, 192 mM glycine, 20% methanol) for between 30 and 90 min at 10 V. Protein transfer was visualised temporarily by staining with Ponceau-S solution (0.5% w/v in 5% w/v trichloroacetic acid [TCA]).
2.6.4 Western blotting/immunodetection

Proteins immobilized on to nitrocellulose after SDS-PAGE (2.6.3) were detected immunologically using antibodies to firefly luciferase, actin, BAG-1, eIF4E, eIF4G or the FLAG peptide. Non-specific binding sites were first blocked by incubating the nitrocellulose membranes in a 5% dried milk solution in TBST for 1-4 hours at room temperature. Membranes were then incubated in 5-10 ml of appropriately diluted primary antibody (Section 2.1.2) in 5% milk TBST overnight at 5°C with constant agitation. Excess antibody was removed by rinsing for three 10 min periods in TBST solution. Horseradish peroxidase conjugated secondary antibodies to mouse IgG (BAG-1, actin) or rabbit IgG (firefly luciferase, eIF4E and eIF4G), diluted in 5% milk TBST, were applied to the membranes for a period of 30-45 min at room temperature, again with constant agitation. Membranes were then thoroughly rinsed for at least three 10 min periods in TBST solution. Protein-antibody complexes were detected using an enhanced chemiluminescence (ECL) technique according to the manufacturer's instructions (Amersham) or according to a recipe kindly provided by Professor Ken Siddle (University of Cambridge). For this, 1 ml Luminol solution (50 mg Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in 0.1 M Tris-HCl pH 8.6), 10 μl Enhancer (11 mg para-coumaric acid in 10 ml DMSO) and 3.1 μl 3% hydrogen peroxide were mixed and incubated on the membrane for 60 s. Chemiluminescence was visualised by exposing the membrane to Fuji RX X-ray film for periods of between 10 s and 30 min.

2.6.5 Stripping and re-probing of western blots

Nitrocellulose membranes were stripped of existing protein-antibody interactions by incubation in a solution of 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7 for 10 min at 50°C. Membranes were then washed in TBST and re-probed with a different antibody as described above (Section 2.6.4).
2.6.6 Immunoprecipitation of cell lysates

Immunoprecipitation was performed to purify $[^{35}\text{S}]$methionine labelled FLAG-tagged luciferase from HeLa cells.

$2 \times 10^6$ cells were labelled with 250 µCi of $[^{35}\text{S}]$methionine in 1 ml of methionine free medium (supplemented with 10% FCS dialyzed against PBS) for 30 min. Cells were solubilised in antibody buffer (10 mM Tris-HCl pH7.5, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.5% SDS, 10 mM iodoacetamide) supplemented with protease inhibitors (Section 2.6.2) and disrupted by passage through a 21-gauge needle. Lysates were precleared by incubation with 10 µg of mouse immunoglobulin G (in 150 mM NaCl) and protein A/G + agarose at 4°C for 1 hour with constant agitation. After centrifugation at 4°C at 4,000 $\times$ g for 5 min, the supernatant was removed to a fresh tube. The FLAG-tagged firefly luciferase was immunoprecipitated by incubation with the M5 FLAG antibody conjugated to agarose (Sigma) at 4°C overnight with agitation.

Immunoprecipitated proteins were spun down in a microcentrifuge at 4°C for 5 min at 4,000 $\times$ g, then washed three times with 1 ml of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton, 0.5% Na deoxycholate, 0.1% SDS). The immunoprecipitate was resuspended in 30 µl of SDS sample buffer (Section 2.6.1), heated at 95°C for 5 minutes and centrifuged at 13,000 $\times$ g to remove the agarose beads. The supernatant was then subjected to SDS-PAGE and the gel was stained in Coomassie solution before being destained, dried at 80°C and then exposed to a phosphorimager screen.
Chapter 3

Initiation of translation of Apaf-1 by internal ribosome entry

3.1 Introduction

The role of translation initiation in controlling expression of Apaf-1 has not been determined, despite the fact that the 5' untranslated regions (UTRs) of both the human and mouse versions of Apaf-1 have many of the features of a translationally regulated gene.

The human and murine 5' UTRs have an overall homology of 56%, although the region approximately 40 nt upstream of the physiological start site is even more highly conserved (around 70%). Both sequences are long (578 and 585 nucleotides respectively) and G-C rich (67 and 66% respectively) so have the potential to form complex secondary structures. These may be inhibitory to scanning translation initiation complexes, or may be able to direct internal ribosome entry.

Both versions of the 5' UTR also contain upstream AUG codons (two in the human, one in the mouse). The single upstream AUG codon in the murine 5' UTR is conserved in the human version and lies in the region of 70% homology upstream of the physiological start site. These upstream AUG codons may repress translation initiation at the physiological AUG start codon in a manner analogous to leaky scanning.

As no other work had investigated the possibility that a translational mechanism could play a role in influencing expression of Apaf-1, in the first instance the effect the Apaf-1 5' UTR had on the translation of a heterologous reporter gene was determined.
3.2 The effect of the Apaf-1 5' UTR on translation of monocistronic reporter vectors

The majority of the work in this thesis uses a series of vectors described in Stoneley (1998) based on the vector pGL3-control (Promega). This vector contains a modified version of the luciferase gene from the North American firefly Photinus pyralis. The 60.7 kDa monomeric luciferase enzyme catalyses the ATP-dependent conversion of D-luciferin and oxygen, in the presence of Mg\(^{2+}\), to oxyluciferin and CO\(_2\) with the accompanying emission of light. The level of light emission produced in the reaction is proportional to the amount of luciferase present.

The modified version of the luciferase gene (\(\text{luc}^+\)) in pGL3-control has been altered to remove type II restriction endonuclease recognition sites, remove internal transcription factor binding sites and prevent peroxisome targeting of the protein.

To aid cloning in the pGL3-control vector, a multiple cloning site (MCS) was introduced between the SV40 promoter and initiation codon of the firefly luciferase open reading frame. An oligonucleotide cassette containing restriction endonuclease sites for EcoRV, SpeI, PvuII and EcoRI was inserted between the HindIII and NcoI sites of the plasmid. This vector was designated pGL3 (Figure 3.1A) and was used for all subsequent cloning steps.

To create a monocistronic luciferase expression cassette that could be transcribed \textit{in vitro}, the MCS and \(\text{luc}^+\) gene of pGL3 were removed by digestion with HindIII to XbaI. The 1718 bp product was isolated by agarose gel electrophoresis and ligated between the HindIII and XbaI sites of pBluescript II SK (+) to make pSKL (Figure 3.1B). In this vector, the luciferase ORF is downstream of a T7 RNA polymerase promoter from which transcripts produced \textit{in vitro} can be translated in \textit{in vitro} translation extracts such as the rabbit reticulocyte lysate (RRL) system.
Figure 3.1 Monocistronic Luciferase vectors

(A) The Promega vector pGL3-control was modified by the addition of a further multiple cloning site to create the vector pGL3.

(B) The luciferase expression cassette from pGL3 was ligated into pBluescript II SK (+) (Stratagene) to create the vector pSKL, suitable for in vitro transcription.
3.2.1. Construction of pSKAL

The 5' UTR of human Apaf-1 was amplified from Apaf-1 cDNA (a kind gift from Dr Katharine Ayres) by polymerase chain reaction (PCR) with the oligonucleotide primers ApU 5' and ApU 3'. Restriction enzyme sites were engineered into the oligonucleotide primers (EcoRI and Ncol in the forward and reverse primers respectively). The restriction endonuclease Ncol recognises and cuts at the sequence CCATGG, therefore the presence of the Ncol site at the 3’ end of the PCR product ensured that the physiological AUG start codon of the Apaf-1 mRNA was maintained as the AUG of the luciferase open reading frame. The resultant 590 bp product was digested with EcoRI and Ncol and ligated between the EcoRI and Ncol sites of the vector pSKL (Stoneley, 1998) creating the vector pSKAL (Figure 3.2). The integrity of the insert was confirmed by sequencing of pSKAL in both forward and reverse directions with the oligonucleotide primers T7 and luc3’.

Therefore, the plasmid pSKAL contains the Apaf-1 5’ UTR upstream of the firefly luciferase ORF under the control of a T7 promoter that can be used in an in vitro transcription reaction.

3.2.2. The 5’ untranslated region of Apaf-1 inhibits the translation of a downstream reporter gene in vitro

The vectors pSKL and pSKAL were linearised with Xbal and the digestion products were purified and used to generate capped mRNAs with T7 RNA polymerase in an in vitro transcription reaction. Integrity and concentration of these mRNAs were determined by agarose gel electrophoresis and by measuring the absorbance of the samples at 260 nm. These mRNAs were used to prime rabbit reticulocyte lysates and the luciferase generated after 1 hour was measured using the Promega Luciferase Assay System (Figure 3.3).
Figure 3.2 Construction of pSKAL

(A) The vector pSKL was digested with the restriction enzymes EcoRI and Ncol.

(B) The Apaf-1 5' UTR was amplified from an Apaf-1 cDNA with the oligonucleotide primers ApU 5' and ApU 3' which contain EcoRI and Ncol restriction sites.

(C) The digested Apaf-1 5' UTR cDNA was ligated into the cut pSKL vector to create the plasmid pSKAL.
Figure 3.3 Inhibition of in vitro translation initiation of firefly luciferase by the presence of the Apaf-1 5' UTR

(A) mRNAs from pSKL and pSKAL were transcribed in vitro with T7 RNA polymerase.

(B) Rabbit reticulocyte lysates were primed with capped RNA from either the control (pSKL) or Apaf-1 containing vectors. After one hour luciferase activities were measured.
The presence of the Apaf-1 5' UTR was found to potently inhibit the translation of the downstream luciferase gene, despite the presence of a cap structure, which can alleviate translational repression of moderately structured mRNAs (Pause et al., 1994). For example, when the lysates contained 20 ng of pSKAL RNA, 100-fold less luciferase was produced compared to lysates containing 20 ng of pSKL RNA (Figure 3.3).

These data suggested either that a region of stable secondary structure was obstructing the scanning translation initiation complex or that non-canonical factors, which were not present in the rabbit reticulocyte lysate, were required for translation initiation of Apaf-1.

3.2.3. Construction of the vector pGAL

To test whether the repression of luciferase expression could be relieved in vivo, as is the case with the c-myc 5' UTR (Stoneley et al., 2000b), the Apaf-1 5' UTR was inserted into the pGL3 vector (Figure 3.1b). The monocistronic luciferase reporter vector pGL3 contains the luc+ gene under the control of the SV40 promoter, which is constitutively active in many cell lines. An SV40 late poly(A) signal downstream of the luc+ gene ensures efficient termination and polyadenylation of the luciferase message. A synthetic poly(A) signal and transcriptional pause site upstream of the SV40 promoter terminates spurious transcription initiating within the vector backbone.

The Apaf-1 5' UTR from the vector pSKAL was excised by digestion with the restriction enzymes EcoRI and Ncol. The resulting 580 bp fragment was ligated into the monocistronic vector pGL3 (Stoneley et al., 1998) that had been digested with the same restriction enzymes to create the vector pGAL (Figure 3.4). Again the sequence of the insert was verified by sequencing pGAL with the oligonucleotide primers SV40 5' and luc 3' in the forward and reverse orientations.
Figure 3.4 Construction of the vector pGAL

The EcoRI- NcoI fragment from pSKAL containing the Apaf-1 5' UTR was ligated into the equivalent sites of the vector pGL3 to create the plasmid pGAL.
3.2.4. The Apaf-1 5' UTR causes a reduction in the translation of a luciferase reporter enzyme in vivo

The vectors pGL3 and pGAL were transiently transfected by the calcium phosphate method into HeLa cells, and luciferase activity was measured 40 hours after transfection. The monocistronic plasmids were cotransfected with a plasmid containing the lacZ gene from E. coli, under the control of a CMV promoter (either pJ7lacZ or pcDNA3.1/HisB/lacZ). The tetrameric β-galactosidase enzyme expressed from these plasmids catalyses the hydrolysis of β-galactoside sugars and can be measured using the Galactolight Plus system (Tropix). Firefly luciferase activity was normalised to the β-galactosidase transfection control and then expressed relative to the activity of the control monocistronic vector pGL3 (Figure 3.5).

The presence of the Apaf-1 5' UTR upstream of the luciferase reporter again resulted in a significant decrease in the amount of luciferase produced compared to the control. However, the effect was much less dramatic than that observed in vitro and in this case approximately four-fold less luciferase was produced (Figure 3.5). This would suggest that the presence of stable secondary structure in the Apaf-1 5' UTR was inhibitory to scanning or that the initiation of Apaf-1 translation is mediated by an alternative mechanism that is less efficient than scanning.

To distinguish between these two possibilities, plasmid constructs were generated containing a hairpin which would inhibit scanning of the downstream reporter gene and which would test for the presence of an IRES in the Apaf-1 5' UTR (Figure 3.6).

3.2.5. Construction of phpL and phpAL

A stable hairpin has been successfully used to inhibit translation of a downstream cistron in the vector phpRMF (previously pGL3RutrH; Stoneley et al., 1998). This vector contains a 66 bp palindromic sequence that forms an RNA hairpin when transcribed. Using
Figure 3.5 Apaf-1 5' UTR reduces translation initiation efficiency of firefly luciferase in vivo

(A) mRNAs produced from the vectors pGL3 and pGAL

(B) HeLa cells were transfected with pGL3 or pGAL. The luciferase activities were measured 40 hours after transfection and normalised to a transfection control of β-galactosidase
Figure 3.6 Determining whether the Apaf-1 5’ UTR can direct internal ribosome entry
(A) The RNA hairpin from the vector phpRMF with a predicted free energy of -80 kcal/mol has been previously used to inhibit ribosome scanning (Stoneley et al., 1998)
(B) The hairpin should inhibit translation of firefly luciferase. If the Apaf-1 5’ UTR also acts as an inhibitor of scanning, an inhibition of luciferase expression will also be seen. If the Apaf-1 5’ UTR contains an IRES, the expression of firefly luciferase will be maintained.
the RNA folding program Mfold described by Zuker et al., (1999) the palindromic sequence was predicted to fold into a single stem loop structure with a calculated free energy of −80 kcal/mol (Figure 3.6).

The palindromic sequence was amplified from the vector phpRMF with the oligonucleotide primers HpinF and HpinR. The 127 bp PCR product contained XbaI restriction sites at each end and so was digested with XbaI before being ligated into the SpeI site of the plasmids pGL3 and pGAL. This sited the hairpin sequence upstream of the luc+ gene and the Apaf-1 5’ UTR to create the vectors phpL and phpAL respectively (Figure 3.7). Due to repetition of sequences in the inserted product the predicted hairpin in these vectors is predicted to fold into a more extended structure with a free energy of -94 kcal/mol (Figure 3.8)

3.2.6. A stable hairpin potently inhibits translation of a downstream reporter, but translation is maintained in the equivalent vector containing the Apaf-1 5’ UTR

The presence of the hairpin reduced the luciferase produced from phpL by 200-fold when compared to the control vector pGL3 (Figure 3.9). In contrast, the presence of the hairpin had only a small effect (1.5 fold) on the amount of luciferase produced from phpAL. This reduction in activity may suggest that a certain amount of Apaf-1 translation initiation is due to a scanning mechanism or may have been due to the hairpin destabilising structural elements in the Apaf-1 5’ UTR. More importantly, these data suggest that Apaf-1 translation can indeed be initiated by an alternative mechanism when cap-dependent translation is inhibited e.g. internal ribosome entry.
**Figure 3.7 Construction of phpL and phpAL**

(A) The palindromic hairpin sequence from the vector phpRMF was amplified with the primers HpinF and HpinR, which contained XbaI restriction sites.

(B) The cDNA containing the hairpin sequence was ligated into the Spel sites of the vectors pGL3 and pGAL to create the vectors phpL and phpAL.
Figure 3.8 The extended hairpin present in the monocistronic vectors phpL and phpAL. The structure has a predicted free energy of -94 kcal/mol.
Figure 3.9 The Apaf-1 5' UTR maintains translation of firefly luciferase in the presence of a stable hairpin

(A) mRNAs produced from the monocistronic luciferase reporter vectors pGL3, phpL, pGAL and phpAL.

(B) HeLa cells were transfected with either pGL3, phpL, pGAL or phpAL. The luciferase activities were measured and normalised to a transfection control of β-galactosidase.
3.3 The effect of the Apaf-1 5′ UTR in a dicistronic assay system

The classical test to determine whether a 5′ UTR contains an IRES is to introduce the sequence between the cistrons of a dicistronic message. An IRES will cause an increase in translation of the second cistron, even under conditions when cap-dependent translation is compromised.

One such dicistronic vector is pRF (Figure 3.10A; previously designated pGL3R; Stoneley et al., 1998), which incorporates two luciferase reporter genes with separate chemistries which can be assayed in a single tube. The monomeric 36 kDa luciferase from the coelenterate sea pansy *Renilla reniformis* catalyses the oxidation of coelenterazine to coelenteramide with the accompanying production of CO₂ and light. This gene is the first cistron of the dicistronic cassette of pRF and is translated by a cap-dependent mechanism, whereas expression of the second cistron, firefly luciferase, will be used to measure IRES driven translation.

The *Renilla* luciferase gene was subcloned from pRL-CMV (Promega) and ligated upstream of the *luc*+ gene of pGL3. A chimeric intron also had to be introduced upstream of the *Renilla* ORF in a second cloning step as it is essential for efficient expression of the *Renilla* luciferase gene. The intron is a composite of the 5′ donor site of human β -globin intron 1 and the branch and 3′ acceptor of the variable heavy chain region of an immunoglobulin gene, both altered to match the splicing consensus. The intron sequence also includes a T7 promoter, allowing *in vitro* transcription of the dicistronic message, along with the SV40 promoter-mediated transcription in transiently transfected cells (Figure 3.10A).

When the control dicistronic message in pRF is transcribed, only notable translation of *Renilla* luciferase should be observed (Figure 3.10B). The firefly luciferase cistron may be translated at low levels due to some ribosomes remaining bound to the message after
Figure 3.10 The dicistronic luciferase assay system

(A) The dicistronic luciferase vector pRF, formerly called pGL3R2 (Stoneley et al., 1998).
(B) In the control vector Renilla luciferase is efficiently expressed while firefly luciferase is only translated by a small number of ribosomes by readthrough reinitiation.
(C) If an IRES is inserted between the cistrons, an increase in firefly luciferase is observed.
termination at the stop codon of the *Renilla* gene and reinitiating at the AUG of the firefly luciferase ORF. If an IRES is inserted in the MCS proximal to the firefly gene, a marked up-regulation in firefly luciferase activity is observed (Figure 3.10C).

### 3.3.1. Construction of pRAF and pRMmAF

The Apaf-1 5' UTR in pSKAL was removed by digestion with *EcoRI* and *NcoI* and the 580 bp fragment was ligated into the dicistronic construct pRF digested with the same enzymes to give pRAF (Figure 3.11). To determine whether the murine version of the Apaf-1 5' UTR sequence determined by Cecconi *et al.*, (1998) and Yoshida *et al.*, (1998) (which is 56% homologous to the human version) could also act as an IRES, the sequence was also introduced into the dicistronic vector system. The sequence was subcloned by reverse transcription of poly(A)* selected RNA from Balb-c cells followed by PCR-mediated amplification with the primers MmAF and MmAR. The resulting 598 bp cDNA was digested with *EcoRI* and *NcoI* and ligated into pRF to make pRMmAF (Figure 3.11). The inserts in both vectors were sequenced in both orientations using the primers RNaseF and luc 3'.

### 3.3.2. The Apaf-1 5' UTR directs internal ribosome entry in dicistronic reporter vectors

HeLa cells were transiently transfected with either pRF, pRAF or pRMmAF by the calcium phosphate method, in conjunction with a vector encoding β-galactosidase as a transfection control. Approximately 40 hours later, expression of *Renilla* and firefly luciferases were assayed using the Dual-luciferase assay kit with β-galactosidase activity measured using the Galactolight Plus system. Measuring the β-galactosidase activity of a sample to normalise the luciferase activities of the dicistronic vectors gives a clearer idea of IRES activity than expressing the luciferase activities as a ratio of one to the other. This is because *Renilla* expression can be altered by the presence of sequences between the cistrons.
Figure 3.11 Construction of the vectors pRAF and pRMmAF.

(A) The expression cassette of the control dicistronic vector pRF.

(B) The human Apaf-1 5' UTR was excised from the vector pSKAL by digestion with EcoRI and Ncol. The murine version was amplified by RT-PCR from Balb-c mRNA and the cDNA digested with EcoRI and Ncol.

(C) The Apaf-1 5' UTRs were ligated into pRF that had been digested with EcoRI and Ncol to create the plasmids pRAF and pRMmAF.
The presence of both human and murine Apaf-1 5' UTR sequences can direct internal ribosome entry in the dicistronic luciferase assay (Figure 3.12). The amount of firefly luciferase produced from the human and murine Apaf-1-containing constructs was 10 and 15-fold over readthrough. These values are slightly higher than those obtained from cells transfected with the HRV-IRES-containing plasmid, pRHRVF but less than those obtained from cells transfected with the plasmid construct which harbours the c-myc IRES, pRMF (Figure 3.12).

The expression of Renilla luciferase is also inhibited by approximately 40% in both pRAF and pRMmAF when compared with that seen in pRF. This may reflect competition between cap-dependent and IRES driven translation on the same message or may also be a consequence of having highly structured RNA sequences between the cistrons. The latter phenomenon has been observed when a stable hairpin is inserted between the cistrons in the vector pRhpF (Mark Stoneley and John Le Quesne, personal communication).

3.3.3. Prevention of cap-dependent scanning of the dicistronic transcript

The result from the dicistronic assay alone does not prove the existence of an IRES in a 5' UTR since the small amount of ribosomal readthrough and reinitiation in the control pRF dicistronic vector could be enhanced by the presence of the 5' UTR in pRAF. The mechanism of ribosomal readthrough and reinitiation is poorly defined but it is postulated that, following termination, the 60S subunits dissociate from the mRNA, while the 40S subunits remain associated and continue to migrate along the mRNA. This continued association may be advantageous to the recycling of the ribosome between rounds of initiation on the same message due to the synergy between the poly(A) tail and cap structure mediated by the PABP-eIF4G-eIF4E complex. Considering the initiation factor eIF3 binds to dissociated 40S subunits, it is also possible that this factor could interact with the 40S subunit and encourage
Figure 3.12 The Apaf-1 5' UTR directs internal ribosome entry.  
(A) The dicistronic mRNAs from the control plasmid pRF and those containing the Apaf-1 5' UTRs or known IRES sequences.  
(B) The dicistronic plasmids containing Renilla and firefly luciferase genes and either human or mouse Apaf-1 5' UTR, the c-myc-IRES or the HRV IRES inserted between them, were transfected into HeLa cells and the activities of both luciferases were measured and normalised to the transfection control.
the binding of a new ternary complex. Thus when the distance between the cistrons of a
dicistronic vector is increased, there is a consequential improvement in the translation of the
downstream cistron (Kozak, 1987).

To eliminate the possibility that the increased intercistronic distance in the vector
pRAF is stimulating the increase in firefly luciferase expression, a vector that prevents
expression of the upstream Renilla luciferase cistron by inhibiting cap-dependent scanning
was used. Insertion of a stable hairpin upstream of the Renilla ORF should inhibit translation
of that cistron, meaning any increase in activity of the downstream cistron cannot be due to
enhanced readthrough occurring on the dicistronic transcript (Figure 3.13A).

A palindromic oligonucleotide cassette was blunt end ligated into the EcoRV
restriction site upstream of the Renilla luciferase cistron in the dicistronic vector pRMF to
create phpRMF (Figure 3.13B). This hairpin, with a calculated free energy of -80 kcal/mol,
could inhibit cap-dependent translation of the upstream cistron while c-myc IRES dependent
translation of the firefly luciferase cistron was sustained (Stoneley et al., 1998).

3.3.4. Construction of phpRAF and phpRMmAF

The vector phpRMF was digested with SpeI and NcoI, the 411 bp fragment containing
the c-myc IRES was removed and the vector backbone purified. The human and murine
Apaf-1 5’ UTRs were removed from pRAF and pRMmAF by digestion with the same
enzymes and the respective 592 and 600 bp products were ligated into the cut phpRMF to
create the equivalent plasmids phpRAF and phpRMmAF (Figure 3.14).

3.3.5. IRES activity is not due to enhanced ribosomal readthrough

The presence of the hairpin was sufficient to impede ribosomal scanning to the first
cistron of the transcript, and Renilla luciferase activity produced from these two constructs
Figure 3.13 Testing for enhanced ribosomal readthrough

(A) The presence of a sequence between the cistrons of the dicistronic vector may be causing an increase in firefly luciferase translation by enhancing ribosomal readthrough and reinitiation. The insertion of a hairpin upstream of the first cistron will inhibit translation of Renilla luciferase and also any reinitiation. Firefly luciferase expression will be maintained if the sequence between the cistrons contains an IRES.

(B) The dicistronic luciferase vector phpRMF (formerly pGL3R2utrH) contains a palindromic sequence upstream of the Renilla cistron which inhibits ribosomal scanning.
**Figure 3.14** Construction of the vectors phpRAF and phpRMmAF

(A) The dicistronic vector phpRMF, containing a palindromic hairpin sequence upstream of the Renilla luciferase cistron.

(B) The human and murine Apaf-1 5' UTRs were excised from the vectors pRAF and pRMmAF by digestion with SpeI and NcoI and ligated into phpRMF digested with the same enzymes to create the vectors phpRAF and phpRMmAF.
was reduced to 15-20% of that in the control pRF vector, while firefly luciferase activity
directed by the Apaf-1 5' UTRs was maintained. This demonstrates that increased
readthrough and reinitiation cannot account for the increased translation of firefly luciferase
(Figure 3.15).

3.3.6. Analysis of dicistronic transcripts

The increase in firefly luciferase expression in cells transfected with the dicistronic
vector may also be due to the production of functional monocistronic firefly luciferase
mRNAs. These may be generated by cryptic promoter signals in the UTR, aberrant splicing
occurring via a cryptic 3' splice acceptor site within the 5' UTR, or even ribonuclease cleavage
(Figure 3.16). Northern analysis will identify any cryptic RNA species in transiently
transfected cells that could account for the increased expression of the downstream cistron.

3.3.7. IRES activity is not due to the production of functional monocistronic luciferase
transcripts

To show that only full length mRNA was being produced from the dicistronic
constructs, COS7 cells were transfected with pGL3, pGAL, pRF and pRAF by the DEAE-
Dextran method. Poly(A)+ selected RNA from these cells was subjected to formaldehyde gel
electrophoresis and then transferred onto nitrocellulose by capillary blotting. The blot was
then probed with radiolabelled luciferase cDNA fragments that were generated using random
prime labelling with the Klenow fragment of DNA polymerase I from *E. coli* in the presence
of [α-32P]dCTP.

Transcripts corresponding to full-length pGL3, pGAL, pRF and pRAF were observed
(Figure 3.17). In all the transfected cells, a transcript of approximately 1.3 knt in length was
also detected (*) which probably represents an aberrantly processed luciferase transcript that
Figure 3.15 The Apaf-1 5' UTR is still able to direct internal ribosome entry in the presence of a stable hairpin

(A) The mRNAs transcribed from dicistronic vectors with or without palindromic hairpins upstream of the Renilla luciferase open reading frame.

(B) HeLa cells were transfected with these constructs and luciferase activities determined. The hairpin sequence inhibits the translation of Renilla luciferase in the vectors phpRAF and phpRMmAF while translation of firefly luciferase is maintained.
Figure 3.16 The presence of the Apaf-1 5' UTR may be causing the production of functional monocistronic firefly luciferase mRNAs which account for the increased expression of firefly luciferase:

(A) A cryptic promoter may be present in the Apaf-1 5' UTR.
(B) A cryptic splice acceptor site may be present in the Apaf-1 5' UTR.
(C) Cleavage by a ribonuclease may be generating monocistronic firefly luciferase mRNAs.
**Figure 3.17** Northern blot analysis of luciferase reporter vectors

Poly(A)$^+$ mRNAs derived from cells alone (Lane 1), or cells transfected with pGL3 (Lane 2), pGAL (Lane 3), pRF (Lane 4) or pRAF (Lane 5) were electrophoresed in the presence of formaldehyde, transferred to nitro-cellulose and then probed with a radiolabelled single stranded DNA specific for firefly luciferase. The non-coding luciferase fragment is indicated (*).
cannot encode full length luciferase. This fragment cannot account for the IRES activity observed in the vector pRAF as densitometry analysis of this luciferase fragment showed that in the vectors pRF and pRAF these transcripts are present in approximately equal amounts (0.8:1 respectively). However, ten times more firefly luciferase is observed in cells transfected with pRAF compared to pRF (Figure 3.11). This transcript is also present in cells that have been transfected with a dicistronic vector containing the c-myc IRES (Stoneley, 1998).

3.4 The role of trans-acting factors in Apaf-1 IRES driven translation

To investigate how widely the Apaf-1-IRES is utilised, a panel of cell lines derived from different tissues, including Human cervical carcinoma (HeLa); Human liver carcinoma (HepG2); Human breast carcinoma (MCF7); Human embryonic kidney (HK293); African Green Monkey kidney (COS7); Human lung (MRC5); Human neuronal cells (SY5Y); Mouse fibroblasts (Balb/c) and Chinese Hamster ovary T (CHO-T) cells were co-transfected using either FuGene 6 or the calcium phosphate method with either pRF, pRAF or, in some cases, pRMmAF (Figure 3.11) and pcDNA3.1/HisB/lacZ.

The expression from both Renilla and firefly luciferase cistrons was assayed and normalised to the transfection control, β-galactosidase and expressed relative to the luciferase activities of the control vector in each line (Figure 3.18). The human Apaf-1 IRES is most active in HeLa and HepG2 cells, and is not used in SY5Y neuronal cells or Balb/c mouse cells suggesting that these cell types lack non-canonical protein factors that are required for internal ribosome entry via the Apaf-1 IRES.

The dicistronic vector pRMmAF, which contains the murine version of the Apaf-1 5' UTR was also tested in the rodent cell lines Balb/c and CHO-T. Interestingly, the murine
Figure 3.18. The Apaf-1 IRES is active in a range of cell lines. The human and mouse Apaf-1 IRESs were transfected into the cell lines shown by either the calcium phosphate method or by using FuGene 6. Luciferase activities were measured and normalised to the β-galactosidase transfection control.
version of Apaf-1 was able to direct IRES activity in the CHO-T cells (8 times more firefly luciferase activity than the control vector) and again not in the Balb/c cells (Figure 3.18).

3.5 Mapping cis-acting elements in the Apaf-1 IRES

3.5.1. Construction of a deletion series

To define the boundaries of the Apaf-1 IRES a series of plasmid constructs was generated containing decreasing lengths of the sequence coding for the 5' UTR. These were based on a restriction map of the Apaf-1 5' UTR which identified six dispersed fragments that were designated a to f (Figure 3.19A).

The simplest truncations were performed by religating pRAF plasmids that had been digested with restriction enzymes that recognised unique restriction sites in pRAF. The Klenow fragment of *E. coli* DNA polymerase I was used to generate blunt ends from the 3' and 5' overhanging ends formed during restriction digestion. The larger fragments were isolated after separation of the two reaction products by agarose gel electrophoresis and allowed to reigate. The deletions of the 3' end of the Apaf-1 IRES in the vectors pRabF (which contains UTR sequence from nucleotides -578 to -330) and pRacF (-578/-233) were created by digesting pRAF with *NcoI* and *SacII* or *PstI* (Figure 3.19C). The 5' deletions in the plasmids pRcfF (-330/1) and pRdfF (-233/1) were made in a similar fashion by digesting pRAF with *EcoRI* and either *SacII* or *PstI* (Figure 3.19B).

Two more truncations were produced by linearising pRAF with *AccIII* or *MaeI* after which overhanging ends were blunted. The vectors were then cut with *NcoI* to yield fragments with one blunt end and one *NcoI* overhanging end. The respective 464 and 60 bp fragments were isolated by agarose gel electrophoresis and ligated into the plasmid pRF that
Figure 3.19 Construction of a deletion series of the Apaf-1 IRES

(A) Restriction map of the Apaf-1 5' UTR as it appears in the dicistronic vector pRAF.

(B) and (C) Deletions of the 5' and 3' portions of the IRES were made by restriction digestion or PCR-mediated amplification to create the vector series, each containing the region of the Apaf-1 IRES indicated.
had been digested with *Pvu*II and *Nco*I. The resulting plasmids pRbfF and pRfF contain sequences from -464 to 1 and -60 to 1 respectively (Figure 3.19B).

The only vector containing a blunt-ended fragment of the Apaf-1 IRES was pRaF (-578/-464), which was made by digesting pRAF with *EcoRI* and *AccIII*. Overhanging ends were filled in and the 114 bp blunt ended product was isolated and ligated into the *Pvu*II restriction site of pRF (Figure 3.19C).

Finally, three truncated versions of the 5′ UTR which had proved difficult to make by restriction digestion were constructed by amplifying sequences from pRAF with the oligonucleotide primer pairs ApU-5′ and A470-3′, ApU-5′ and A523-3′, and A470-5′ and ApU-3′ respectively. The 470, 523 and 115 bp PCR products were digested with *EcoRI* and *NcoI* and ligated into the MCS of pRF to yield pRadF (-578/-115), pRaeF (-578/-60) and pRefF (-115/1).

### 3.5.2. Mapping the Apaf-1 IRES

The ability of these truncated sequences to promote internal ribosome entry on a dicistronic mRNA was compared to the full length 5′ UTR. Although the full length IRES is required for maximal luciferase activity, the data suggest that elements which are vital to internal ribosome entry lie at the 3′ end of the IRES (Figure 3.20).

The most important element appears to reside between nucleotides -233 and 1, as deletions from the 5′ end maintain a large proportion of IRES activity (75% in the case of the df fragment). Once the d fragment is removed, however, there is a considerable decrease in IRES activity to 20% of that measured from the full length IRES in the plasmid containing the ef fragment (Figure 3.20, compare df to ef).

Removing fragments from the 3′ end of the IRES has a more drastic effect on IRES activity. Deletion of fragment f has little effect (the ae fragment maintains 80% of IRES activity).
Figure 3.20 Deletion mapping of the Apaf-1 5' UTR.

(A) Deletions of the Apaf-1 5' UTR were created by restriction enzyme digestion or PCR-mediated amplification and introduced into the dicistronic vector pRF.

(B) Dicistronic vectors containing deletions of the Apaf-1 5' UTR were transfected into HeLa cells and assayed for luciferase activity. The values obtained were then expressed relative to the full length Apaf-1 5' UTR.
activity), but deletion of the next fragment causes another large reduction in IRES activity (to 35%). This reduced IRES activity is maintained, even in the pRaF plasmid, which may suggest that other parts of the IRES may be able to direct some internal ribosome entry, while the whole 5' UTR is essential for maximal activity.

3.5.3. Construction of pRdF

As removal of the d fragment resulted in a significant decrease in IRES activity, this portion of the Apaf-1 5' UTR was tested to determine whether it could drive IRES driven translation alone. The plasmid pRadF was digested with SpeI and PstI, the large reaction product was isolated, the overhanging ends filled in and the vector religated. The resulting vector, pRdF contains the 5' UTR sequence from –233 to –115 (Figure 3.21A).

Further deletion of the Apaf-1 IRES sequence to the portion from –233 to -115 does not reveal a single essential element in this fragment. The plasmid pRdF only has 30% of the activity of the full length 5' UTR, which is similar to the activity of the pRadF truncation. This suggests that the sequences within fragment ef are essential for activity, but require another element in fragment d for efficient IRES activity. (Figure 3.21B)

3.6 A putative ribosome landing site

The truncated def fragment of the Apaf-1 IRES is still able to direct efficient internal ribosome entry in the dicistronic assay, whereas the ef fragment has reduced activity. However, the activity of the d fragment alone is also poor, which suggests an important role for the sequences of ef which cannot act alone to recruit ribosomes.

The def fragment contains several features that suggest that internal ribosome entry directed by the Apaf-1 5' UTR might be similar to that seen in the Class I IRESs. These
Figure 3.21 Further deletion of the Apaf-1 IRES

(A) The vector pRdF was created by removal of the a, b and c fragments of the Apaf-1 IRES by digestion of pRadF with Spel and PstI, followed by religation of the vector backbone.

(B) The relative IRES activities of each deletion was measured and expressed relative to the IRES activity of the full length IRES.
IRESs, which include the entero- and rhinoviruses, recruit ribosomes to an AUG codon downstream of an oligopyrimidine tract. The ribosome then migrates to the next downstream AUG where it initiates translation.

The sequence of the d fragment from the human 5’ UTR is 65% G-C rich, with the equivalent mouse fragment being 68% G-C, whereas the ef fragments are only 60 and 56% G-C rich respectively. Although the d fragment is only 57% conserved, compared to 66% conservation between the ef fragments, this does not rule out the possibility of conserved secondary structures existing in this portion of the Apaf-1 IRES. Predictions of the secondary structure of the human and mouse d fragments made using the Mfold algorithm reveal many possible stable secondary structures that may play a role in ribosome recruitment, either directly or via trans-acting factors (data not shown).

Of more interest is the ef fragment (Figure 3.22), which contains the highest degree of conservation and also contains an oligopyrimidine tract, although this sequence does not contain the conserved UUUC motif found in all picornaviruses. Downstream of the oligopyrimidine tract is a conserved AUG triplet and it is possible that this AUG is involved in ribosome entry.

To determine whether the conserved AUG triplet is important in ribosome entry, as would be expected if the Apaf-1 IRES could be considered a Class I IRES, mutations of the Apaf-1 5’ UTR were made.

3.6.1. Creation of the plasmids pRAM1F, pRAM2F and pRAM1+2F

Mutant forms of the Apaf-1 IRES were synthesised using the Stratagene QuikChange™ protocol (Figure 3.23) using pSKAL as the template. The oligonucleotide primers AM1F and AM1R were used to mutate the first AUG at nucleotides 399-401 of the Apaf-1 5’ UTR to an AGG codon. Similarly, the primers AM2F and AM2R mutated the
Fragment d

Hs |agagauccagggagcgccacugugagggccggaaaccuc 385
Mm |ugauccaggagagcggaccuc 366

AUG1

Hs cgggcagagggauaugcgacacgacaccucaccuauucgggaggaaacag 425
Mm -ggggcgccggucgccggaagccaggcgggagccccgccgcuuucuggcaaucua 422

Fragment e

Hs cguuugcc-guuucccuccac|cgccuggagucucccc-agucu-----uguucccgccag 497
Mm gucuuauagucuuccccc-c|ugggcugcuuuccuuucgaauauacuagccuaccuc 481

Oligopyrimidine tract Fragment f

Hs ugcc-goccuocaccuuaagacc|uagggcgcaagggcgg-
Mm ccggcuggaagggcgccacuuguuggcaucguucuuugccuc 541

AUG2

Hs ---cucaguugcagacucacagagagaaagauucugaggaagAUGg 581
Mm uagcccagccacagccgagagagaaacaccucugagcacaAUGg 589

Figure 3.22 Alignment of the 3’ ends of the human and murine Apaf-1 IRESs showing regions of interest. The physiological initiation codons are capitalised and in bold. Putative initiation codons of upstream open reading frames (uORFs) are in bold and italicised. An oligopyrimidine tract is shown in bold.
Target site for mutation identified in plasmid

Oligonucleotide primers containing the desired mutation are annealed to the denatured plasmid

Mutagenic primers extended with *Pfu Turbo™* DNA polymerase, resulting in nicked circular strands of mutated DNA

Parental DNA template digested with *DpnI* which specifically recognises GmGATC

Circular nicked dsDNA transformed into XL1-Blue cells which repair the nicks in the mutated plasmid

**Figure 3.23** Overview of QuikChange™ site directed mutagenesis method (Stratagene) illustrating the mutation of a potential ATG initiation codon to an AGG triplet.
second, conserved AUG at positions 540-542 to AGG. A double mutant form of the UTR was also created. After confirming the presence of each ATG-AGG mutation by sequencing with the primers T7 and luc3', the three Apaf-1 5' UTRs were excised from the respective host vectors and ligated into pRF, creating the vectors pRAM1F, pRAM2F and pRAM1+2F (Figure 3.24).

3.6.2. Mutation of upstream AUG codons in the Apaf-1 5' UTR increases IRES activity

The mutant versions of the Apaf-1 IRES were transfected into HeLa cells alongside the wild type vector, pRAF, and luciferase activities were measured. Mutation of the first AUG causes a small increase in IRES activity, but mutation of AUG2 causes a bigger increase, which is also present in the double mutant (Figure 3.25). These data suggest that ribosomes may enter upstream of this AUG, with half initiating translation at the upstream, out of frame AUG codon, with the other half initiating at the physiological AUG.

3.7 Summary

3.7.1 Identification of an IRES in the Apaf-1 5' UTR

Experiments performed in vitro and in vivo with monocistronic reporter vectors showed that the presence of the long G-C rich 5' UTR of Apaf-1 upstream of firefly luciferase resulted in an inhibition of translation. However, the effect was much less severe in transfected cells, suggesting that Apaf-1 translation may occur by an alternative mechanism.

Further experiments showed that translation initiation of Apaf-1 occurs by internal ribosome entry and this provides evidence for an IRES in an mRNA whose protein product is associated with the initiator of a caspase cascade during apoptosis. Experiments performed with monocistronic plasmid constructs that contain a stable hairpin imply that initiation of
Figure 3.24 Creation of mutant forms of the Apaf-1 IRES

(A) The Apaf-1 5' UTR in the vector pSKAL was mutated by the QuikChange site-directed mutagenesis method with the mutagenic primers shown.

Construction of pRAM1F

(B) The first ATG triplet in the Apaf-1 IRES was mutated to AGG with the primers AM1 and AM1R, then the mutant sequence was excised by digestion with EcoRI and NcoI.

(C) The mutant IRES was ligated into the vector pRF to create pRAM1F. The plasmids pRAM2F and pRAM1+2F were created in the same way.
Figure 3.25 Mutation of upstream AUG codons causes an activation of Apaf-1 IRES activity

(A) Wild-type and mutant forms of the Apaf-1 IRES were introduced into the dicistronic assay system and transfected into HeLa cells.

(B) The activity of each mutant IRES was expressed relative to the activity of the wild-type sequence.
translation of Apaf-1 mRNA may only occur by internal ribosome entry. Moreover, the
reduction in luciferase activity obtained when the Apaf-1 5' UTR was placed in front of the
luciferase reporter vector suggests that internal ribosome entry initiated by the Apaf-1 IRES is
approximately 70% less efficient than the scanning mechanism of translation. In this way, the
Apaf-1 IRES differs significantly from those identified in the c-myc and FGF-2 mRNAs, since
it has been proposed that initiation of translation of these mRNAs can occur by cap-dependent
mechanisms as well as internal ribosome entry (De Benedetti and Rhoads, 1990; De Benedetti
et al., 1994; Kevil et al., 1995; West et al., 1998).

3.7.2. The role of cell-specific factors in translation of the Apaf-1 IRES

The Apaf-1 IRES was active, although to differing degrees in all human cell types
tested, with the exception of the neuronal line SY5Y. The differences in Apaf-1 IRES-
mediated initiation of translation between the cell lines examined may be due to the levels of
expression of proteins that are required for internal ribosome entry in these cells.
Interestingly, the IRES is most active in cell lines of tumour origin.

The contribution that two IRES specific trans-acting factors make to translation of the
Apaf-1 IRES is currently under investigation (Section 6.2).

3.7.3. Identification of the minimal IRES

Most of the ability of the Apaf-1 5' UTR to initiate internal ribosome entry resides in a
233 nt fragment at the 3' end of the IRES. Although this is a rather small IRES when
compared to those of viral origin, it is a size that is consistent with those that have been
observed in eukaryotic mRNAs e.g. the BiP and FGF-2 IRESs are 220 and 165 nucleotides in
length respectively (Yang and Sarnow, 1997; Vagner et al., 1995).
Two regions of particular interest within this region are an AUG triplet that is conserved in the murine Apaf-1 5' UTR and an oligopyrimidine tract upstream of this, and both of these may act in a similar way to the Class I picornaviral IRESs in ribosome recruitment. Mutation of this conserved AUG increases the activity of the Apaf-1 IRES, which indicates that the AUG may act as an initiation codon in some cases and prevent translation of the physiological open reading frame.

3.7.4. A hypothetical model for the Apaf-1 IRES

This model for ribosome recruitment to the Apaf-1 IRES has two functional sections (Figure 3.26). The 5' end of the UTR is highly inhibitory to scanning, and could play a minor role in recruiting ribosomal subunits. The second half of the UTR is the major ribosomal recruitment region.

The minimal fragment of Apaf-1, def, that maintains 75% of the IRES activity of the full length UTR still contains both upstream AUG codons while the d fragment only contains the first unconserved one. The IRES activity of the d truncation is only as efficient as the ad fragment suggesting there is a synergistic effect of the d and ef fragments on IRES activity.

It is possible that a secondary structural motif in fragment d positions the ribosome at the second conserved upstream AUG (AUG2), present in the ef fragment, which is downstream of a stretch of pyrimidines. The ribosome may initiate some translation at this AUG (like cardio- and some aphthoviruses), but others will scan to the physiological AUG codon in a manner analogous to the entero- and rhino- and the majority of the aphthoviruses. Assuming that the AUG2 mutation, which causes an increase in IRES activity, does not cause a large change in the secondary structure of the IRES, it is possible that AUG2 may help repress the IRES by initiating some translation at this upstream AUG, therefore preventing initiation at the physiological AUG codon. This hypothesis may explain the high IRES
Figure 3.26 A hypothetical model for ribosome recruitment to the Apaf-1 IRES.

The first half of the 5' UTR is highly structured and inhibits ribosome scanning. The second half of the IRES contains the major ribosomal recruitment domain, which consists of a region of secondary structure followed by an oligopyrimidine tract. The ribosome is recruited to a conserved AUG codon, where some ribosomes initiate translation. Other ribosomes continue to migrate to the AUG initiation codon of the physiological open reading frame, where initiation takes place.
activity of the ae fragment which lacks AUG2 but still contains the pyrimidine stretch. In this truncation, the physiological AUG will be the sole initiation codon used.

The putative ribosome landing pad of the Apaf-1 IRES is much closer to the initiation codon than the one that has been experimentally determined by introducing out of frame AUG mutations into the c-myc IRES (Figure 1.6 and Le Quesne et al., submitted for publication). Whether this is due to the c-myc message being efficiently scanned as well as initiating translation via an IRES, whereas the Apaf-1 message is inefficiently scanned and more reliant on IRES driven translation remains to be seen.
Chapter 4

Both cap-dependent scanning and internal ribosome entry mediate translation of BAG-1 isoforms

4.1 Introduction

When human BAG-1 transcripts are translated in vitro, the different isoforms are translated by “leaky scanning” (Yang et al., 1998), and so it would appear that the only determinant of BAG-1 translation is the context of the different initiation codons. However, in vivo the p36 isoform is always expressed at levels greater than or equal to the other isoforms, which suggests that an alternative form of translational control may play an important role in the expression of the BAG-1 isoforms (Packham et al., 1997; Takayama et al., 1998).

Previous work in our laboratory identified an internal ribosome entry segment in the 5' untranslated region of c-myc (Stoneley et al., 1998). C-Myc can also be translated as two protein isoforms generated by initiation from a canonical AUG codon and an upstream in-frame non-canonical CUG triplet. However, these alternate initiation codons are only 36 nt apart and are both downstream of the site of ribosome entry (Figure 1.6 and Le Quesne et al., submitted for publication). The initiation codons of human BAG-1 are much more dispersed along the message but the 5' UTR upstream of the p36 ORF is 411 nt long and 73% G-C rich. This suggests that this region has the potential to form an IRES, which could account for the preferential translation of the p36 isoform. It is because of these features of the BAG-1 mRNA that investigations were carried out to determine whether BAG-1 could be translated by internal ribosome entry.
4.2 The effect of the 5' untranslated region of p36 BAG-1 in dicistronic reporter assays

4.2.1 Subcloning of the p36 BAG-1 5' UTR into pRF

To determine whether the p36 BAG-1 5' UTR could act as an internal ribosome entry segment, a region corresponding to nucleotides 43 to 411 of the BAG-1 p36 5' UTR was amplified by PCR from the plasmid CI-3 (a kind gift from Dr. G. Packham, CRC Wessex Medical Oncology Unit, Southampton General Hospital) using the oligonucleotide primers BAGF and BAG36R. The primers contained restriction endonuclease recognition sites and thus the 381 bp PCR product was digested with EcoRI and NcoI and ligated into the equivalent sites between the cistrons of the dicistronic reporter vector pRF (Stoneley et al., 1998) to create the vector pRBF (Figure 4.1).

4.2.2 The BAG-1 p36 5' UTR directs internal ribosome entry in the dicistronic luciferase vector system

The vectors pRF and pRBF were transfected into HeLa cells by the calcium phosphate method in parallel with dicistronic vectors containing IRESs that had been previously identified. Forty hours after transfection, expression of Renilla and firefly luciferases were assayed and these values were normalised to a transfection control of β-galactosidase and then expressed with respect to the luciferase activities of the control vector pRF (Figure 4.2).

In transient transfections in HeLa cells, the presence of the BAG-1 p36 5' UTR can direct internal ribosome entry in the dicistronic assay. This results in an increase in the expression of the downstream firefly luciferase of 17-fold over that attributable to readthrough and reinitiation of ribosomes on the control transcript produced from pRF (Figure 4.2). This compares favourably with the IRES activity of the cellular IRESs found in Apaf-1 and c-myc (9 and 50 times respectively) and the viral IRESs found in HRV and EMCV (8 and 14 times).
Figure 4.1 Construction of the dicistronic vector pRBF

(A) The BAG-1 5' UTR was amplified from the plasmid CI-3 with the primers BAGF and BAG36R, which contained restriction sites for EcoRI and Ncol respectively.

(B) The digested BAG-1 5' UTR was ligated into the vector pRF to create the plasmid pRBF.
Figure 4.2 The BAG-1 p36 5' UTR directs internal ribosome entry analogous to that seen in other IRES containing vectors

(A) The p36 BAG-1 5' UTR is present in the dicistronic vector pRBF. The cellular IRESs from Apaf-1 and c-myc and the viral IRESs from HRV and EMCV are present in the respective vectors pRAF, pRMF, pRHRVF and pREMCVF.

(B) The luciferase activities of the dicistronic vectors were measured following transient transfection into HeLa cells, normalised to a transfection control of β-galactosidase and expressed relative to the luciferase activities of the control vector pRF.
when present in the same assay system. There is a slight downregulation of expression of the upstream *Renilla* luciferase cistron in the vector pRBF (to about 70% of the control), and this has been observed in other IRES-containing dicistronic transcripts and in some cases may reflect a competition between cap-dependent and IRES-dependent translation on the same transcript.

This repression of the upstream cistron expression can be shown more clearly if the data is plotted as the relative light emission from each plasmid (Figure 4.3A). This shows that in the control dicistronic vector pRF, the expression of the downstream cistron is much less than that of the upstream. In fact, the light emission of firefly luciferase is around 40-fold less than that of *Renilla* luciferase.

Another alternative way of showing data from dicistronic experiments is to plot the data as a ratio of downstream cistron activity to upstream cistron activity (Figure 4.3B). Unfortunately, although commonly employed in much of the literature, this can be misleading as weaker IRESs may be enhanced by their effect on the upstream cistron. As the presence of both the Apaf-1 and BAG-1 5' UTRs cause a downregulation of upstream cistron expression, this consequently has a large effect on the relative luciferase ratios and suggests that the Apaf-1 and BAG-1 IRESs are far more efficient at directing internal ribosome entry than the HRV and EMCV IRESs (Figure 4.3B). More importantly, when the data is shown in this form, the use of a hairpin upstream of the first cistron of the dicistronic reporter would appear to have a further "activating" effect on the activity of a putative IRES sequence.

### 4.2.3 An alternative dicistronic reporter vector

It has been suggested that in some viral IRESs, the presence of the first portion of the viral coding sequence is essential for IRES activity (reviewed in Jackson, 2000). When these sequences were aligned, although they were A-rich, there was no discernable conserved motif.
Figure 4.3 Alternative interpretations of data from the dicistronic luciferase assay

(A) The data are plotted relative to the light emission of firefly luciferase in the dicistronic control vector pRF.

(B) The data are plotted as a ratio of the downstream firefly luciferase cistron expression (which is IRES-dependent) to the upstream Renilla luciferase cistron, which is translated by cap-dependent scanning.
In prokaryotes it is possible that A-rich regions downstream of the initiation codon may increase the efficiency of translation initiation (Dreyfus, 1988). The region downstream of the firefly luciferase initiation codon is also A-rich, so may be contributing to the efficiency of our dual luciferase dicistronic vector system.

Therefore a second dicistronic assay system was used to test whether the BAG-1 p36 5' UTR could also enhance the expression of an alternative downstream reporter gene. In the pRCAT vector (Figure 4.4A) the upstream *Renilla* luciferase cistron is maintained but the downstream firefly luciferase gene is replaced with the chloramphenicol acetyltransferase (CAT) gene from *E. coli*. pRCAT was created by swapping a 2164 bp *NcoI-BamHI* fragment from pRF, containing the *luc*+ gene, SV40 poly(A) and enhancer, with the equivalent 1170 bp *NcoI-BamHI* fragment from pCAT3 (Promega) which contains the CAT ORF, SV40 poly(A) and enhancer from pCAT3 (Stoneley, 1998).

CAT activity is assayed by measuring the transfer of the acetyl group from n-butyryl Coenzyme A onto $[^{14}\text{C}]$chloramphenicol over a defined time period. The butyrylated $[^{14}\text{C}]$chloramphenicol is extracted and measured by liquid scintillation counting (LSC).

4.2.4 **Construction of pRBCAT and analysis of upstream and downstream cistron expression from the vector**

The p36 BAG-1 5' UTR was removed from the vector pRBF by digestion with *EcoRI* and *NcoI* and the 371 bp fragment was introduced into pRCAT, which had been digested with the same restriction enzymes to create the vector pRBCAT (Figure 4.4B).

The LSC assay for measuring CAT activity is much less sensitive than the luciferase assay system and there is also a high level of background CAT activity in untransfected HeLa cells. Coupled with this is the fact that the level of expression of dicistronic messages is always much lower than that from monocistronic messages, and the SV40 promoter upstream
Figure 4.4 Construction of the alternative dicistronic vector pRBCAT
(A) The alternative dicistronic vector pRCAT contains a dicistronic cassette with the chloramphenicol acetyltransferase (CAT) gene as the downstream cistron.
(B) The BAG-1 5' UTR from the vector pRBF was removed by digestion with EcoRI and Ncol and ligated into the vector pRCAT to make the plasmid pRBCAT.
of the dicistronic cassette is not as strong as that from CMV thus any increase in CAT activity is difficult to detect. An equivalent vector pRACAT, which contains the Apaf-1 IRES shows only a slight increase in translation of the downstream cistron (data not shown).

To attempt to compensate for the poor expression of CAT in the vectors pRCAT, pRBCAT and the positive control pRMCAT (which contains the c-myc 5' UTR), the plasmids were transiently transfected into HeLa cells with FuGene 6. This method is far more efficient than the calcium phosphate or DEAE-Dextran method used in previous experiments, and an increase in expression of the downstream CAT reporter driven by the BAG-1 p36 5' UTR was observed. Therefore the BAG-1 p36 5' UTR is also able to direct internal ribosome entry to the second cistron in this alternative dicistronic vector system, albeit at a reduced efficiency compared to the dicistronic luciferase vectors (Figure 4.5).

### 4.2.5 IRES activity is not due to enhanced ribosomal readthrough and reinitiation

As detailed in section 3.3.3 the result from the dicistronic assay alone does not prove the existence of an IRES in the p36 BAG-1 5' UTR. Ribosomal readthrough and reinitiation at the AUG codon of the firefly luciferase could be enhanced by the increased intercistronic length in the pRBF vector compared to the pRF control. To investigate this possibility a dicistronic vector that hinders ribosomal scanning to the upstream Renilla luciferase cistron was also created.

A 411 bp fragment containing the c-myc 5' UTR was removed from the vector phpRMF (Stoneley et al., 1998) by digestion with SpeI and NcoI and replaced with a 383 bp fragment containing the p36 BAG-1 5' UTR, which was subcloned from the vector pRBF by digestion with the same restriction enzymes. The palindromic sequence in this vector, named phpRBF, forms a hairpin with a free energy of -80 kcal mol$^{-1}$ when transcribed (Figure 4.6). The hairpin in the vector phpRBF was sufficient to impede ribosomal scanning, and Renilla
Figure 4.5 An alternative dicistronic reporter assay

(A) The vector pRBCAT was transfected into HeLa cells, alongside negative and positive controls of pRCAT and pRMCAT, respectively.

(B) The Renilla luciferase and CAT activities of each vector were measured and normalised to a transfection control of firefly luciferase. The BAG-1 p36 5' UTR again directs internal ribosome entry in a manner analogous to the c-myc IRES.
Figure 4.6 Construction of the vector phpRBF

The c-myc IRES from the dicistronic vector phpRMF was removed by digestion with SpeI and NcoI. This was replaced by the BAG-1 5' UTR, which had been removed from the vector pRBF by digestion with the same enzymes. The resulting vector, phpRBF, contains a palindromic hairpin sequence upstream of the Renilla luciferase open reading frame.
activity dropped to 30% of that observed in the control vector pRF (Figure 4.7a), while firefly luciferase activity directed by the BAG-1 5' UTR was maintained (Figure 4.7b).

4.2.6 Analysis of the mRNAs expressed from dicistronic luciferase vectors

As discussed in Section 3.3.6, functional monocistronic mRNAs produced from a cryptic promoter, cryptic splice site or ribonuclease recognition site in the BAG-1 p36 5' UTR could be responsible for the enhanced firefly luciferase activity observed in the cells transfected with pRBF. Northern analysis of mRNAs from cells transfected with pRF and pRBF, along with the monocistronic luciferase reporter vectors pGL3 and pGBL (Section 4.3.1) was carried out as detailed in Section 3.3.7.

The cDNA probe which contains an approximately 1 kb portion of the luciferase coding region hybridises to species that correspond to full length dicistronic transcripts in the cells transfected with pRF and pRBF (Figure 4.8). Again the 1.3 kb mRNA observed in Figure 3.17 is observed in all lanes and this is thought to represent an aberrantly processed luciferase transcript with no potential to produce a functional firefly luciferase enzyme. However, more importantly, the probe also hybridised to a transcript of approximately 2.0 kb in size which appears to be the same size as the monocistronic luciferase mRNA produced from pGL3. This fragment could potentially be responsible for the increase in firefly luciferase expression observed in cells transfected with pRBF. To determine whether this transcript can encode the entire firefly luciferase open reading frame, RNase protection analysis was performed.

4.2.7 Construction of pBAGRNase

The primers BRNaseF and H3luc3' were used to amplify a 597 nucleotide region of the vector pRBF which contained the end of the Renilla gene, the BAG-1 5' UTR and the first
Figure 4.7 BAG-1 IRES activity is not due to enhanced ribosomal readthrough

(A) The vector phpRBF contains a palindromic sequence upstream of the *Renilla* cistron which forms a stable hairpin when transcribed.

(B) The presence of the hairpin results in a significant reduction of *Renilla* activity in the vector phpRBF compared to that seen in the control pRF.

(C) The increase in firefly luciferase activity seen in pRBF is maintained in phpRBF.
Figure 4.8 Northern blot of luciferase reporter vectors

Poly(A)^+ mRNAs derived from cells alone (Lane 1), or cells transfected with pGL3 (Lane 2), pGBL (Lane 3), pRF (Lane 4) or pRBF (Lane 5) were electrophoresed in the presence of formaldehyde, transferred to nitro-cellulose and then probed with a radiolabelled single stranded DNA specific for firefly luciferase. The non-coding luciferase fragment is present in all transfected cells, but a species of approximately 2.0 kb is also observed in lane 5 which may represent a functional monocistronic firefly luciferase transcript.
101 nt of the firefly gene. The PCR product was digested with BamHI and HindIII and ligated into pBluescript II SK (+) (Stratagene) creating the vector pBAGRNase (Figure 4.9).

A representation of the RNase protection analysis is shown in Figure 4.10. The vector pBAGRNase must first be linearised with NotI to create a template for in vitro transcription. An antisense riboprobe produced from this vector by in vitro transcription with T7 RNA polymerase will be 669 nt in length. This riboprobe will hybridise to and protect a 597 nt fragment of the full length dicistronic transcript from digestion with a single stranded RNA-specific nuclease. If the 5' end of the 2.0 kb transcript is located within the BAG-1 5' UTR sequence, then an additional fragment will be protected. This fragment would have to be at least 101 nt in length to contain the entire firefly luciferase open reading frame.

4.2.8 RNase protection analysis of the mRNAs transcribed from pRBF

RNase protection was carried out exactly as described in Stoneley et al., (1998). pBAGRNase was digested with NotI and purified to create a linear template for in vitro transcription. An antisense riboprobe was generated by run off transcription by T7 RNA polymerase in the presence of [α-32P]UTP. Full length (669 nt) radiolabelled transcripts were isolated after separation on a 4% polyacrylamide/7M urea gel by the crush and soak method (Maxam and Gilbert, 1977).

TRI Reagent (Sigma) was used to isolate total RNA from HeLa and COS7 cells either mock transfected or transfected with pRBF using FuGene 6 (HeLa cells) or the DEAE-Dextran method (COS7 cells). Poly(A)+ mRNA was selected from 10µg of the total RNA using Dynabeads Oligo dT(25) from Dynal. The 669 nt antisense radiolabelled riboprobe was annealed to this poly(A)+ mRNA or a control of 10µg of yeast tRNA for 16h at 45°C.

Single stranded RNA, which in the case of the radiolabelled transcript corresponds to vector sequence from pBAGRNase was digested with RNase ONE™ (Promega) and the products were size fractionated by electrophoresis through a 4% polyacrylamide/7M urea gel.
Figure 4.9 Construction of the vector pBAGRNase

(A) A portion of the vector pRBF corresponding to the end of the Renilla luciferase open reading frame, the BAG-1 5' UTR and the first 101 nt of the firefly luciferase gene was amplified using the primers BRNaseF and H3luc3' which contained BamHI and HindIII restriction sites.

(B) The cDNA was digested with BamHI and HindIII and ligated into the pBluescript II SK (+) plasmid (Stratagene) to create the vector pBAGRNase.
A Transcribe antisense riboprobe from pBAGRNase

B Anneal radiolabelled probe to transcript

C Digest single stranded RNA with RNase ONE

**Figure 4.10** RNase protection analysis of dicistronic mRNAs

(A) and (B) The antisense riboprobe is synthesised by run off transcription from the linearised pBAGRNase template in the presence of [α-32P]UTP and annealed to dicistronic mRNAs.

(C) The single stranded radiolabelled RNA corresponding to pBAGRNase vector sequence is digested with a ribonuclease. The protection of a 599 nt fragment indicates that dicistronic transcripts are intact, whereas protection of fragments between this size and 101 nt indicates that functional monocistronic luciferase transcripts in the cell may be the cause of the increased firefly luciferase activity observed in cells transfected with pRBF.
alongside undigested riboprobe. The 597 nucleotide fragment which corresponds to the end of the *Renilla* gene, the BAG-1 5' UTR and the first 101 nt of the firefly luciferase gene should be protected from digestion.

A protected fragment of the expected size was observed by phosphorimager analysis (Figure 4.1IB). Any monocistronic transcripts that could encode full-length luciferase would be at least 101nt in length. As the only fragment approaching this size was observed in all lanes, the increased translation of firefly luciferase observed in cells transfected with pRBF must occur on intact dicistronic mRNAs. In addition a fragment of between 309 and 404 nt in size was protected in the mRNA from HeLa cells (Figure 4.11C). As this fragment was observed in both transfected and untransfected cells, it is likely that it appears as the result of hybridisation of the 368 nt portion of the riboprobe, which is complementary to the p36 BAG-1 5' UTR, with endogenous BAG-1 mRNA. These data strongly suggest that the 5' UTR upstream of the p36 BAG-1 ORF contains an IRES.

### 4.3 Translation of monocistronic BAG-1 reporter constructs

Some cellular mRNAs that can be translated in an IRES-dependent manner can also be translated by the cap-dependent scanning mechanism. For example, the FGF-2 and c-myc messages can be translated by both mechanisms but, at least in the case of c-myc, cap-dependent scanning is a more efficient mechanism at initiating translation than IRES driven translation (Stoneley *et al*., 2000b). Our initial experiments and the work of others suggest that both mechanisms play a role in controlling expression of the human isoforms of BAG-1 (Figure 4.2 and Packham *et al*., 1997; Yang *et al*., 1998). Thus to investigate the contribution the IRES makes to the expression of BAG-1 isoforms the monocistronic vector system based on the vector pGL3 (Stoneley *et al*., 1998) was employed.
Figure 4.11 RNase protection analysis of dicistronic mRNAs

(A) The 669nt antisense riboprobe used to investigate the integrity of dicistronic transcripts. 597nt of the riboprobe will hybridise to the transcript from pRBF.

(B) and (C) RNase protection results of dicistronic RNAs show that IRES activity is not due to the presence of functional monocistronic firefly luciferase transcripts. Panel B shows the analysis of mRNA from COS7 cells while panel C shows the results of analysis of HeLa cell mRNA. The radiolabelled antisense riboprobe was hybridised to a yeast tRNA control (Lane 1), poly(A)+ mRNA from untransfected cells (Lane 2), and poly(A)+ mRNA from cells transfected with pRBF (Lane 3). Lane 4 contains undigested riboprobe.
4.3.1 Construction of pGBL, phpBL and pGBAL

The monocistronic firefly luciferase reporter vectors pGL3 and phpL (described in Sections 3.2.3 and 3.2.5) were digested with *EcoRI* and *NcoI*. The BAG-1 5' UTR from the vector pRBF was excised by digestion with the same enzymes and the 376 bp product was ligated into the digested vectors to create the plasmids pGBL and phpBL respectively (Figure 4.12). The oligonucleotide primers BA36LF and BA36LR were used to mutate the AUG initiation codon of firefly luciferase in the vector pGBL to an AGG codon, using the QuikChange site-directed mutagenesis protocol (Stratagene) giving rise to the vector pGBAL (Figure 4.13).

4.3.2 IRES driven translation of BAG-1 is not as efficient as cap-dependent scanning

The monocistronic constructs were transfected into HeLa cells and 40 hours post-transfection the samples were divided, either being prepared for SDS-PAGE or lysed in passive lysis buffer for measurement of reporter gene activity. As usual, luciferase activity was measured and normalized to a transfection control of β-galactosidase. The luciferase isoforms produced were also separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The isoforms were then visualised by immunoblotting with an anti-luciferase antibody and the enzyme expressed from the pGL3 plasmid was observed as a 60 kDa protein (Lane 2 of Figure 4.14B).

With the BAG-1 5' UTR upstream of the reporter gene in the vector pGBL two additional isoforms of luciferase at 74 and 70 kDa that contained N-terminal extensions were observed (Figure 4.14B, lane 3). These represent the p50 and p46 isoforms that initiate from the upstream CUG and AUG codons respectively. However, the “p36” isoform is present in a much greater amount than either of the larger isoforms, which is contradictory to the suggestion that all the isoforms are purely translated by the leaky scanning mechanism.
Figure 4.12 Construction of the monocistronic reporter vectors pGBL and phpBL

The BAG-1 5' UTR from the vector pRBF was removed by digestion with EcoRI and NcoI and ligated into the vectors pGL3 and phpL to create the vectors pGBL and phpBL.
Figure 4.13 Construction of pGBAL

(A) The vector pGBL was used as a template for QuikChange site directed mutagenesis (Stratagene) with the mutagenic oligonucleotide primers BΔ36LF and BΔ36LR.

(B) The mutagenic primers were extended with *Pfu Turbo* DNA polymerase and the parental template was digested with the enzyme *DpnI*.

(C) The initiation codon of p36 BAG-1/firefly luciferase was mutated to AGG in the vector pGBAL.
Figure 4.14 The translation of p36 BAG-1 by internal ribosome entry is not as efficient as cap-dependent scanning

(A) Illustration of mRNAs produced from the monocistronic reporter vectors pGL3, phpL, pGBL, phpBL and pGBAL

(B) Western blot of firefly luciferase isoforms translated from monocistronic and dicistronic reporter vectors.

(C) Luciferase activities of the monocistronic reporter constructs after transient transfection into HeLa cells, normalized to a transfection control of β-galactosidase.

When the luciferase activity of the pGBL plasmid was measured, the luciferase activity was found to be 46% of that expressed from the control plasmid (Figure 4.14C). This is probably due to the relative inactivity of the longer isoforms, which can be shown when the AUG codon that would initiate the p36 isoform was mutated to an AGG codon in the vector pGBAL. In this case, production of luciferase in the transfected cells was strongly inhibited to 5% of the pGL3 control, yet the proteins representing the p50 and p46 isoforms were still observed by immunoblotting (Figure 4.14B, lane 5). This shows that the fusion of luciferase with the p50 and p46 ORFs produces proteins that cannot efficiently catalyse the turnover of D-luciferin, probably by disrupting the correct folding of the enzyme.

A stable hairpin upstream of the luciferase gene in the vector phpL strongly inhibited translation (to 6% of the control) yet when the same hairpin was inserted upstream of the BAG-1 5' UTR in phpBL, it only reduced expression of luciferase to 15% of that observed in pGL3 (Figure 4.14C). Western analysis of these plasmid constructs showed that the translation of the longer isoforms was inhibited especially the "p50" isoform while the 60kDa native luciferase was still efficiently expressed (Figure 4.14B, Lane 4). These data suggest that both ribosome scanning and internal ribosome entry are used for translation of the p36 isoform of BAG-1. However, the scanning mechanism is more efficient than the IRES-mediated translation since three times more luciferase is expressed from pGBL than phpBL.

When cells were transfected with the dicistronic vector pRBF, nearly all the luciferase present was initiated from the p36 AUG but not the upstream initiation codons (Figure 4.14B, lane 7). These data suggest that only the p36 isoform can be translated by internal ribosome entry. An extremely small amount of the "p46" isoform is expressed in cells transfected with
the dicistronic vector, which suggests that this isoform may also be able to initiate by internal ribosome entry. However, this isoform may be being translated by a readthrough-reinitiation mechanism, which may explain why there is a small change in the IRES activity of the BAG-1 5’ UTR in the vector phpRBF compared to pRBF. A small proportion of ribosomes that are initiating luciferase expression by a readthrough-reinitiation mechanism will be inhibited by the presence of the upstream stable hairpin.

4.4 Deletion analysis of the BAG-1 IRES

To determine whether solely the p36 isoform is translated by an IRES, two truncated versions of the p36 5’ UTR were introduced into the dicistronic vectors. The 5’ UTR up to the p46 ORF was amplified from pRBF by PCR with the primers BAGF and BAG46R. The 236 bp cDNA was digested with EcoRI and Ncol and inserted into pRF to make the vector pRB5’F (Figure 4.15B).

In a second truncation, the 5’ end of the BAG-1 p36 5’ UTR was removed from the vector pRBF by digestion with EcoRI and SacII. The 236 bp restriction fragment was removed, the overhanging and recessed ends polished by the Klenow fragment of E. coli DNA polymerase I and the vector was religated, generating the vector pRB3’F (Figure 4.15A). The AUG initiation codon of firefly luciferase was also mutated to AGG in the vector pRBΔF by the PCR-directed QuikChange site directed mutagenesis method (Stratagene) using the primers BA36LF and BA36LR (Figure 4.15C).
Figure 4.15 Construction of BAG-1 IRES deletions and mutations

(A) The vector pRB3'F was created by removal of the EcoRI-SacII fragment of the vector pRBF.

(B) The sequence up to the p46 ORF was amplified with the primers BAG F and BAG46 R. This cDNA was ligated into the vector pRF to make the vector pRB5'F.

(C) The initiation codon of p36 BAG-1/firefly luciferase was mutated by QuikChange site directed mutagenesis with the primers BA36LF and BA36LR. This created the vector pRBΔF.
4.4.1 Deletion analysis indicates that only the p36 isoform of BAG-1 is translated by internal ribosome entry

As before, the dicistronic vectors were transfected into HeLa cells by the calcium phosphate method, in conjunction with β-galactosidase and harvested 40 hours after transfection. The luciferase activities of each vector were compared to the control vector pRF and the activity of the full length BAG-1 IRES was also measured.

During immunoblotting of the luciferase isoforms produced from monocistronic and dicistronic reporter vectors, some “p46” isoform is still observed which indicates that this isoform may also be translated by internal ribosome entry. However, the vector pRB5’F, which contains the 5’ UTR of the p46 isoform of BAG-1 has no IRES activity in the dicistronic assay suggesting this is not the case (Figure 4.16). It is possible, though, that IRES driven expression of p46 BAG-1 may require downstream sequence elements that are absent in the pRB5’F plasmid as is observed in certain viral IRESs (reviewed in Jackson, 2000).

Mutation of the AUG initiation codon of the p36 ORF to an AGG codon, however, again resulted in a dicistronic vector, pRBΔF, with no IRES activity in HeLa cells (Figure 4.16). This suggests that the 5’ UTR of p46 BAG-1 cannot recruit ribosomes even in the presence of downstream BAG-1 sequences. An increase in luciferase expression would be expected, albeit at a reduced level as the N-terminally extended luciferase that would be produced from this vector would not be folded properly and thus have reduced activity as is observed in the monocistronic vector pGBAL.

In contrast, the vector pRB3’F, which contains only the sequence between the p46 AUG and the p36 AUG, is still able to direct efficient internal ribosome entry (Figure 4.16). This vector maintained around 75% of the IRES activity of the full-length p36 5’ UTR (13 times the activity attributable to readthrough compared to 17 times for the full-length). This
Figure 4.16 Deletion analysis indicates that only the p36 isoform of BAG-1 is translated by internal ribosome entry

(A) Truncated versions of the BAG-1 IRES were introduced into the dicistronic reporter system. The sequence upstream of the p46 ORF is present in the vector pRB5'F and the sequence between the p46 and p36 initiation codons is present in pRB3'F. The AUG of luciferase in pRB is mutated to AGG in the vector pRBΔF.

(B) Relative luciferase activities of the truncated and mutated dicistronic vectors in HeLa cells. Only the vector pRB3'F maintains IRES activity, which is not as efficient as the full length IRES.
suggests the most important sequences for internal ribosome entry reside in this region of the 5' UTR.

Analysis of the sequence does not reveal any of the features that are common to the viral IRESs such as the oligopyrimidine tract, which is observed in the Apaf-1 IRES. Whether this region can be shortened further is currently under investigation. There is also the possibility that other primary sequence motifs with complementarity to the 18S rRNA may be involved in the recruitment of ribosomes to the BAG-1 IRES, as is the case in the Gtx IRES (Chappell et al., 2000a). The motif of CCGGC|GGU from the IRES of the murine homeodomain protein, Gtx, is complementary to the murine 18S rRNA. This sequence is not found anywhere in the murine or human BAG-1 5' UTRs, although other short stretches of either BAG-1 5' UTR may be complementary to 18S rRNA and this possibility is currently being explored.

One of the most interesting conclusions that can be drawn from the deletion experiments is that the BAG-1 IRES is located in a region that can also encode longer isoforms of the BAG-1 protein. This is unusual among IRESs, but not unique. Shorter, IRES-initiated isoforms have also been observed for p110/p58^PITSLRE^ a protein kinase involved in cell cycle regulation, p67/p45 Estrogen Receptor α and Notch2 (Cornelis et al., 2000; Barraille et al., 1999; Lauring and Overbaugh, 2000).

4.5 The BAG-1 IRES and cellular transformation

Increased BAG-1 mRNA expression has been observed in both tumour cell lines and patient samples, but this does not account for the alteration of BAG-1 isoform expression observed in these cells. In many tumours, for example cervical and invasive breast
carcinomas and colon and prostate cancers, expression of p50/BAG-1L is increased (Tang et al., 1999; Yang et al., 1999a).

4.5.1 IRES activity varies between cell lines

Therefore to determine whether the IRES activity correlates with the levels of the isoforms observed, the vectors pRF and pRBF were transfected into a panel of cell lines (Figure 4.17), including five breast cancer derived lines, CAL 51, CAMA1, GI-101, MCF7 and MDA-MB-453. IRES activity was also measured in other cell types, fibroblast (GM637), embryonic kidney (HEK293), cervical carcinoma (HeLa) and lung carcinoma (MRC5). COS7 cells from the African green monkey and Balb-c cells from mice were also used to investigate whether the IRES could be used in other species.

IRES activity varies greatly between these cell lines, suggesting that trans-acting factors, present at different levels in the cells play a role in regulating IRES-driven translation (Figure 4.17). This has also been observed in previous work in our laboratory where we have shown that the activities of the Apaf-1 and c-myc IRESs differ between cell lines (Section 3.4; Coldwell et al., 2000 and Stoneley et al., 2000b). However, the pattern of expression is different to that observed previously with the c-myc and Apaf-1 IRESs suggesting that the BAG-1 IRES may require different trans-acting factors. For example, the BAG-1 IRES shows its greatest level of activity in MCF7 cells whilst the c-myc IRES only has a low level of function in these cells (Figure 4.17, Stoneley et al., 2000b). In contrast the c-myc IRES is very active in MRC5 cells whilst the BAG-1 IRES shows less activity in these cells (Figure 4.17).

The BAG-1 IRES fails to direct internal ribosome entry in the rabbit reticulocyte lysate system (Becky Pickering, personal communication) and this phenomenon has been observed with many viral and cellular IRESs, including the c-myc IRES (Stoneley et al., 2000b) which
Figure 4.17 BAG-1 IRES activity varies between cell lines

The vectors pRF and pRBF were transfected into cell lines of different origin and IRES activity was measured, normalised to a transfection control of β-galactosidase and expressed relative to the luciferase activity of the control vector pRF in each cell line.
again suggests a role for cell-specific trans-acting factors being involved in IRES driven translation. This may also explain the difference between the pattern of expression observed between in vivo expression of BAG-1 isoforms and the in vitro translation results of BAG-1 mRNA obtained by Yang et al., (1998).

The BAG-1 IRES is also active in cells from other species, suggesting that either conserved secondary structures are formed, which are recognized by IRES binding factors or certain conserved primary sequences are being recognized for binding in these cells.

4.5.2 IRES activity does not correlate with BAG-1 isoform expression or expression of the initiation factors eIF4E and eIF4G

An antibody that recognized all the BAG-1 isoforms was used to investigate whether the different IRES activities in some of the cell lines studied correlated with the expression of the isoforms (Figure 4.18A). The level of the protein isoforms varies between cell lines but there is not a correlation with the different IRES activities of the dicistronic vectors in these cells. Our data would suggest that both scanning and cap-independent mechanisms are responsible for the BAG-1 p36 over-expression in these cells. For example, the relative ratios of the p36 isoform to the others are comparable between MCF7 and GI-101 cells (Figure 4.18A, lanes 3 and 4) yet there is a 12-fold difference in the luciferase generated by internal ribosome entry (Figure 4.17).

One possibility that exists is that under normal cellular conditions the BAG-1 message is solely translated by internal ribosome entry, leading to the expression of only the p36 isoform. As the 5' UTR of BAG-1 is G-C rich, it may be considered a “weak” mRNA in its ability to direct cap-dependent translation (Section 1.3.2). During conditions when the cap-binding protein eIF4E is overexpressed, such as after transformation, this repression would be relieved and all of the isoforms would then be translated by leaky scanning. This would
Figure 4.18 Immunoblotting of cell lysates

(A) BAG-1 isoforms were detected in some of the cell lines tested for IRES activity with a mouse monoclonal antibody mix. There is no correlation between variations in IRES activity and changes in isoform expression. Actin was used as a loading control.

(B) The initiation factors eIF4G and eIF4E were also immunoblotted and again no correlation was found between expression of these initiation factors and the changes in BAG-1 isoform expression.
explain why p50 and p46 BAG-1 are rarely observed in normal cells (Takayama et al., 1998) and may mean that, instead of IRES activity correlating with isoform expression, a change in the cap-dependent translation of BAG-1 could be causing this effect.

Therefore, the possibility that increased expression of eIF4E is leading to the altered expression of BAG-1 isoforms in transformed cells is also of interest. Accordingly, the membrane containing the cell samples was stripped of existing antibody complexes and reprobed for eIF4E and the central scaffold of the eIF4F complex, eIF4G (Figure 4.18B). Unfortunately, there was no obvious correlation between the expression of eIF4E and the expression of the BAG-1 isoforms in the various cell lines studied. However, this does not completely rule out the possibility that the overexpression of eIF4E makes an indirect contribution to the altered expression of BAG-1 in cancer.

4.6 Summary

4.6.1. The p36 BAG-1 5' UTR can initiate internal ribosome entry on dicistronic transcripts

These results show that the most ubiquitously expressed p36 isoform of BAG-1, a protein implicated in a wide variety of cellular processes including apoptosis, transcription and heat shock, can be translated by the alternative mechanism of internal ribosome entry. The presence of the BAG-1 5' UTR can direct internal initiation of translation of the downstream cistron in two different dicistronic assay systems. IRES driven translation is initiated on intact dicistronic transcripts and is not due to enhanced ribosomal readthrough.
4.6.2. The IRES driven translation of p36 BAG-1 is not as efficient as cap-dependent scanning

The IRES containing messages of c-myc and FGF-2 can be translated by both cap-dependent and IRES-dependent mechanisms and this is also the case for the p36 isoform of BAG-1. Cap-dependent scanning, which also accounts for the translation of the longer p50 and p46 isoforms, is a more efficient mechanism at initiating translation than IRES driven translation and this has also been shown for the c-myc IRES (Stoneley et al., 2000b).

4.6.3. Only the p36 isoform of BAG-1 is translated by internal ribosome entry

Deletion of the p36 BAG-1 5' UTR shows that the full length UTR is essential for maximal activity but a 132 nt portion maintains 75% of the IRES activity of the full length. This segment is shorter than most viral IRESs but is a similar length to some of the cellular IRESs e.g. the FGF and BiP IRESs where regions of 165, and 220 nucleotides respectively have been shown to exhibit internal ribosome entry (Vagner et al., 1995; Yang and Sarnow, 1997). Deletion of the Apaf-1 IRES showed that a 233 nt region could also maintain a significant proportion of the IRES activity of the full length 5’ UTR (Section 3.5 and Coldwell et al., 2000).

The deletions also indicate that the BAG-1 IRES is present in a region that can also code for longer isoforms of the protein. This phenomenon has also been observed in mRNAs encoding the PITSLRE protein kinase, Estrogen Receptor α and Notch2, in which IRESs present in the protein coding region of the mRNA direct the translation of shorter isoforms of the protein (Cornelis et al., 2000; Barraill et al., 1999; Lauring and Overbaugh, 2000).

Because the BAG-1 IRES is present in a protein coding region, it is possible that only a short region of primary sequence is required for internal ribosome entry, as has been observed in the 9 nt IRES present in the 5’ UTR of mRNA encoding the murine
homeodomain protein Gtx (Chappell et al., 2000a). The murine version of the BAG-1 IRES does not contain the CCGGCGGGGU module which is complementary to the 18S rRNA, nor does the human.

4.6.4. The role of trans-acting factors in controlling expression of the BAG-1 IRES

The BAG-1 IRES is active in all the cell lines tested, although to differing degrees. The differing activities may be due to the differential expression of IRES specific trans-acting factors in these cells and it appears that the BAG-1 IRES requires different binding proteins to the c-myc or Apaf-1 IRESs. It is also active in cells from other species, which suggests that either conserved secondary structures are formed and it is these that are recognized by IRES binding factors or short stretches of conserved primary sequences in the message are sufficient to encourage ribosome entry.

The expression of the different BAG-1 isoforms in these cells was tested by immunoblotting and showed that there was no apparent correlation between the efficiency of the IRES in initiating translation of firefly luciferase in the dicistronic luciferase assay and the pattern of expression of the isoforms in these cells. Immunoblotting of two of the members of the eIF4F complex, eIF4E and eIF4G also failed to link the pattern of BAG-1 isoform expression with the levels of these factors.
Chapter 5

Functional analyses of the Apaf-1 and BAG-1 internal ribosome entry segments

5.1 Possible roles for internal ribosome entry segments

The cap-dependent scanning mechanism of translation is inhibited by a variety of cellular conditions. The downregulation of cap-dependent protein synthesis during picornaviral infection due to cleavage of eIF4G by viral proteases provides a rationale for the preferential translation of viral mRNAs due to their mechanism of internal ribosome entry. It has also been shown that several eukaryotic IRESs such as BiP, c-myc, Cyr61 and Pim1 are functional following Picornaviral infection (Johannes and Samow, 1998; Johannes et al., 1999).

During apoptosis there is a reduction in translation as a result of cleavage of eIF4G and other components of the initiation factor machinery by the intracellular caspase cascade (reviewed in Clemens et al., 2000). The c-myc IRES is used to initiate translation during apoptosis (Stoneley et al., 2000a) as are those found in the 5' UTRs of DAP5 and XIAP (Henis-Korenblit et al., 2000; Holcik et al., 1999; Holcik et al., 2000b).

The de-phosphorylation of eIF4E causes a reduction in cap-dependent translation during mitosis (Bonneau and Sonenberg, 1987) and IRESs are used to direct the translation of p58^{PITSLRE} and ODC during mitosis (Cornelis et al., 2000; Pyronnet et al., 2000).

Following cellular stresses such as DNA damage, heat shock and hypoxia, there is a shut down of scanning by modulation of the activities of the eIF4F complex and/or eIF-2 (Panniers, 1994; Rhoads and Lamphear, 1995; Schneider, 2000). The c-myc IRES remains
active following DNA damage (Subkhankulova et al., in preparation) and the VEGF IRES was found to be active during hypoxia (Stein et al., 1998).

To attempt to determine cellular states when the IRESs of Apaf-1 and BAG-1 are used, three conditions when cap-dependent translation is compromised were studied, namely apoptosis, mitosis and heat shock.

Our data suggest that the IRES in the Apaf-1 message is used as the default pathway for protein synthesis as the message is only poorly translated by cap-dependent scanning. This would also imply that it is important for a cell to be able to maintain a constant, albeit low, cellular level of Apaf-1 which is not all that surprising given the central role that Apaf-1 plays in apoptosis and the consequences of overexpression of Apaf-1.

All isoforms of BAG-1 are translated by the cap-dependent scanning mechanism but only the p36 isoform is translated by internal ribosome entry. The pattern of expression of BAG-1 isoforms observed in untransformed cells suggests that the BAG-1 IRES may also be used as the default initiation pathway for translation of this message and cap-dependent scanning only occurs under certain conditions.

5.2 Investigation of IRES-driven translation during apoptosis

Whilst the caspase-mediated cleavage of initiation factors was being elucidated, efforts were made to determine whether an internal ribosome entry segment in the 5' UTR of a gene could be used to maintain translation of the message when there is a general shut-off of translation in apoptotic cells. As Apaf-1 and BAG-1 are both central components of the apoptotic pathway, although these proteins have opposing effects, there is a potential that the IRESs found in the 5' UTRs of these messages may be used to maintain translation of the
proteins during apoptosis as has been observed for the IRESs in the 5’ UTRs of c-myc, XIAP and DAP-5.

Several different apoptotic stimuli have been used to induce apoptosis in studies of translation and two alternate methods were chosen for this study. The TNF-related apoptosis inducing ligand (TRAIL) binds to several TRAIL receptors, causing an aggregation of the intracellular “death domains” of these receptors (MacFarlane et al., 1997; Goodwin and Smith, 1998). The formation of this death-inducing signalling complex (DISC) recruits and activates procaspase-8, one of the initiator caspases.

Several DNA-damaging agents have also been found to induce apoptosis. At low concentrations, the addition of these agents causes the arrest of the cell cycle at the G1/S boundary. Higher concentrations of these agents cause the induction of an apoptotic programme. One of the commonly used DNA damaging agents used to induce apoptosis is etoposide, a topoisomerase II inhibitor. Alkylating agents such as ethane methylsulphonate (EMS), which damages DNA by ethylating the N-7 position of guanine and the N-3 position of adenine, can also induce apoptosis at high concentrations (Tanya Subkhankulova, personal communication).

5.2.1 The Apaf-1 and BAG-1 IRESs maintain translation of a reporter gene during the early stages of apoptosis induced by TRAIL

Apoptosis of HeLa cells triggered by the addition of TRAIL causes a reduction in overall protein synthesis within two hours, as determined by measuring the incorporation of [35S]methionine (Stoneley et al., 2000a). This reduction in protein synthesis correlates with the caspase-mediated cleavage of eIF4G and the “classic” caspase substrate poly(ADP-ribose) polymerase (PARP). Eight hours after induction of apoptosis, 95% of cells have condensed cell nuclei, which is measured by staining with propidium iodide (Stoneley et al., 2000a).
HeLa cells were transfected with pRF, pRAF or pRBF and, 40 hours after transfection, apoptosis was induced by the addition of TRAIL to a final concentration of 0.25 µg/ml, or EMS to a final concentration of 50 mM. Cells were harvested at several time points and the luciferase activities were measured and normalised to a transfection control of β-galactosidase. This protein has a half-life of approximately 20 hours, so the levels of β-galactosidase expression should remain constant during the 8 hours of apoptosis induction.

As expected, the activity of the upstream Renilla cistron decreases over the time course, which reflects the reduction in cap-dependent translation. For example, in cells treated with TRAIL, this reduction occurs gradually over the 8 hours of the time course to about 35% of the Renilla luciferase activity of untreated cells transfected with pRF, pRAF or pRBF (Figure 5.1). The levels of firefly luciferase expressed from the monocistronic vector pGL3 also decrease over time (Figure 5.2). This vector will be translated by a cap-dependent mechanism, as opposed to the IRES-dependent expression of firefly luciferase from the vectors pRAF and pRBF. The expression of firefly luciferase from pRAF and pRBF also reduces over time, but not as quickly as that expressed from pGL3 (Figure 5.2). Thus two hours after the addition of TRAIL, the luciferase activity of pGL3 is 59% of that of untreated cells, but the luciferase activities of pRAF and pRBF are 73 and 82% respectively (Figure 5.2). However, during the later stages of the time course, the luciferase activities of pRAF and pRBF are approximately equal to those of pGL3, suggesting the IRESs are no longer used for translation initiation at this time. This is in contrast to the use of the IRESs in c-myc and human rhinovirus (HRV), which maintain efficient translation of firefly luciferase through to the later stages of TRAIL-induced apoptosis (Stoneley et al., 2000a).
Figure 5.1 Cap-dependent protein synthesis is inhibited in HeLa cells treated with TRAIL.

TNF-related apoptosis-inducing ligand (TRAIL) was added to HeLa cells to induce apoptosis. Cap-dependent protein synthesis of Renilla luciferase was measured in cells transfected with pRF, pRAF or pRBF at the times indicated.
Figure 5.2 Apaf-1- and BAG-1-IRES dependent protein synthesis is maintained during the early stages of TRAIL-induced apoptosis in HeLa cells. 

TNF-related apoptosis-inducing ligand (TRAIL) was added to HeLa cells to induce apoptosis. IRES-dependent protein synthesis of firefly luciferase was measured in cells transfected with pRAF or pRBF at the times indicated. Cap-dependent protein synthesis of firefly luciferase was measured in cells transfected with the monocistronic vector pGL3.
5.2.2 The Apaf-1 and BAG-1 IRESs do not function during EMS-induced apoptosis

When HeLa cells were treated with EMS, there was a much more rapid reduction in the Renilla luciferase activities of cells transfected with pRF, pRAF and pRBF, albeit with differing severities (Figure 5.3). The activities of firefly luciferase from cells transfected with pGL3, pRAF or pRBF also reduce quickly (Figure 5.4) but this time, the reduction is at the same rate which suggests that the Apaf-1 and BAG-1 IRESs are not active during EMS induced apoptosis. Unfortunately, the concentration of EMS used in this experiment may be inducing a downturn in both cap-dependent and IRES dependent translation too quickly for the maintenance of Apaf-1 or BAG-1 IRES-driven firefly luciferase expression to be measured.

Thus in cells exposed to lower concentrations of EMS, which induces a reduction in cap-dependent protein synthesis without triggering apoptosis, the Apaf-1 IRES is still able to initiate translation of firefly luciferase in the dicistronic assay (Subkhankulova et al., in preparation). The maintenance of Apaf-1 expression from the IRES during genotoxic stress possibly reflects the need for the cell to quickly undergo apoptosis in cases when the cell is unable to repair severe DNA lesions.

5.2.3 Summary

These results suggest that the IRESs of Apaf-1 and BAG-1 maintain translation of these proteins during the early stages of TRAIL-induced apoptosis, but not during the later stages of TRAIL induced apoptosis or any time during apoptosis induced by EMS treatment. The cell line used in this study is one of the most efficient at directing internal ribosome entry via the 5' UTRs of Apaf-1 and BAG-1, and consequently the requirement for new synthesis of either Apaf-1 or the p36 isoform of BAG-1 during apoptosis may not be as great in HeLa cells. The IRES-driven translation of the firefly luciferase reporter may be observed during
Figure 5.3 Cap-dependent protein synthesis is inhibited in HeLa cells treated with EMS
The DNA alkylating agent Ethylmethyl sulfonate (EMS) was added to HeLa cells to induce apoptosis. Cap-dependent protein synthesis of *Renilla* luciferase was measured in cells transfected with pRF, pRAF or pRBF at the times indicated.
The DNA alkylating agent Ethylmethyl sulfonate (EMS) was added to HeLa cells to induce apoptosis. The cap-dependent and IRES-dependent protein synthesis of firefly luciferase was measured in cells transfected with pGL3, pRAF or pRBF at the times indicated.
apoptosis induced in one of the cell lines in which Apaf-1 or BAG-1 IRES expression is lower. It is possible that the IRESs may be induced during the early stages of apoptosis in cells with lower levels of Apaf-1 or p36 BAG-1 to either enable apoptosis to proceed or prevent caspase activation. This effect has also been observed during experimentation with the XIAP IRES. The continued IRES-dependent translation of XIAP depends on both the cell line and also the treatment used to induce apoptosis (Martin Holcik, personal communication).

As Apaf-1 is pro-apoptotic it is possible that the cell does not require new synthesis of the protein once the caspase cascade is underway, which may explain why the Apaf-1 IRES is only used during the early stages of apoptosis induced by TRAIL. Similarly, although BAG-1 is an anti-apoptotic protein, it can only inhibit the early stages of the apoptotic programme. BAG-1 enhances the function of Bcl-2, which is thought to prevent the release of cytochrome c into the cytosol, where it can bind to Apaf-1. This part of the apoptotic pathway is upstream of the site of action of XIAP, which binds to caspases and prevents their activation and is also translated by an IRES.

This may be why the Apaf-1 and BAG-1 IRESs only appear to be used during the initial stages of apoptosis, whereas the c-myc IRES remains functional during the later stages. The c-Myc protein has a half-life of only 20 minutes and so expression of this short-lived protein is maintained by the IRES long after cap-dependent protein synthesis has been shut off. The expression of c-myc may be required for the final stages of cell death such as phagocyte recognition and engulfment when it may be required to activate the transcription of specific genes. Conversely the Apaf-1, BAG-1 and XIAP IRESs may be required to regulate the complex interplay between the pro- and anti-apoptotic pathways during the initial stages of apoptosis. However, once the death of the cell is inevitable, only those IRESs required to maintain expression of short lived proteins during the latter stages of apoptosis remain functional.
5.3 Investigation of IRES-driven translation during the cell cycle

The downregulation of general protein synthesis during mitosis was originally observed by Fan and Penman (1970), but it was many years before one of the underlying reasons for this reduction in protein synthesis was established to be the dephosphorylation of the cap-binding protein eIF4E (Bonneau and Sonenberg, 1987). During this change in cap-dependent translation, the poliovirus mRNA remains translated (Bonneau and Sonenberg, 1987) and the recent discovery that several other IRESs function during mitosis has led to the hypothesis that this may be a general strategy by which certain messages can remain translated during mitosis (Honda et al., 2000; Cornelis et al., 2000; Pyronnet et al., 2000; reviewed in Sachs, 2000a). It is for this reason that the dicistronic assay was again used to determine whether the Apaf-1 and BAG-1 IRESs can be used to maintain translation of firefly luciferase during mitosis.

5.3.1 Synchronisation of cell populations

Five populations of HeLa cells were transiently transfected with pRF, pRAF or pRBF along with a plasmid encoding the lacZ gene as a transfection control. Two populations of the transfected cells were arrested at the G2/M phase border by overnight treatment with nocodazole, which arrests cells undergoing mitosis by preventing tubulin polymerisation thus inhibiting formation of the spindle pole. Another two populations of cells were synchronised at the G1/S phase border by overnight exposure to aphidicoline, which inhibits nuclear DNA replication. The fifth population were maintained in an asynchronous state.

Mitotic cells were harvested or washed in PBS and replated to remove the nocodazole block. The latter cells were harvested 2 hours later after progression into the first gap phase of the cell cycle (G1). Cells synchronised at the G1/S phase border were either harvested or
washed with PBS to release the aphidicoline block. These cells progressed into the second gap phase, \( G_2 \), where they were harvested 6-8 hours later.

### 5.3.2 Expression of Renilla and firefly luciferases from dicistronic reporter vectors is reduced during mitosis

The luciferase activities of the upstream and downstream cistrons were measured at each stage of the cell cycle and normalised to the activity of the transfection control, \( \beta \)-galactosidase. This protein has a half-life of approximately 20 hours and should therefore be expressed at a constant level throughout the cell cycle. These results were then expressed relative to the luciferase activities measured in a population of cells that had been maintained in an asynchronous state (Figure 5.5).

During mitosis, there is a 30-50% decrease in the *Renilla* luciferase activity of the cell lysates compared to that of asynchronous cells, reflecting a down turn in cap-dependent translation. However, there is also a 25-40% reduction in firefly luciferase activity during mitosis in cells transfected with either pRAF or pRBF. These results suggest that neither the Apaf-1 nor BAG-1 5' UTRs can maintain expression of firefly luciferase in our dicistronic reporter vector system during mitosis.

As the levels of both reporter genes return to normal by the \( G_1 \) phase of the cell cycle, it was decided to investigate the two hours between mitosis and the \( G_1 \) phase in detail to determine whether internal ribosome entry is induced at a faster rate than cap-dependent translation. This would be important to return levels of Apaf-1 and BAG-1 to normal as they may have reduced during mitosis. Otherwise, the cell may not be able to produce an appropriate response to apoptotic stimuli.
Figure 5.5 The Apaf-1 and BAG-1 IRESs do not function during mitosis, when cap-dependent protein synthesis rates are reduced.
Luciferase activities of cells transfected with pRF, pRAF or pRBF were measured at each stage of the cell cycle, normalised to a transfection control of β-galactosidase and expressed relative to cells maintained in an asynchronous state.
5.3.3 Cap-dependent and IRES-dependent protein synthesis rates are induced at the same rate following mitosis

Cells transfected with pRF, pRAF and pRBF were arrested at the G2/M border by nocodazole treatment and then harvested at 30 minute intervals after the release of the nocodazole block. The luciferase activities of the upstream and downstream cistrons were measured and normalised to a transfection control of β-galactosidase. Once again the activities at each time were expressed as a proportion of the activities of asynchronous cells (Figure 5.6).

During the transition from mitosis to the first gap phase of the cell cycle in HeLa cells, the expression of firefly luciferase translated from the Apaf-1 and BAG-1 IRESs increased at the same rate as the cap-dependent synthesis of Renilla luciferase. This indicates that there is no specific induction of IRES-dependent translation over cap-dependent protein synthesis during the progression of the cell cycle following mitosis.

5.3.4 Summary

These results suggest that the Apaf-1 and BAG-1 IRESs are unable to maintain translation during mitosis, when cap-dependent protein synthesis rates are reduced. One possibility for the lack of Apaf-1 or BAG-1 IRES expression may be the fact that these proteins have long half-lives and are maintained at suitable levels during mitosis, whereas both c-myc and ODC are short-lived proteins and new, IRES-driven expression is required.

However, results from the other studies on IRES dependent translation during the cell cycle indicate that there may be a significant difference with the approach taken in the experiments with the Apaf-1 and BAG-1 IRESs. The dicistronic cassette in the pRF family of vectors is expressed from the SV40 promoter and during mitosis, the Renilla activities of the vectors pRF, pRAF and pRBF reduce to 30-40% during mitosis. In the study of HCV IRES-
Figure 5.6 There is no difference in induction of cap-dependent and IRES-dependent protein synthesis during exit from mitosis

HeLa cells were arrested at the G2/M boundary by addition of nocodazole. Luciferase activities of cells transfected with pRF, pRAF or pRBF were measured at the times indicated after release of the nocodazole block and expressed relative to those of cells maintained in an asynchronous state.
driven translation during the cell cycle, the stably transfected dicistronic luciferase cassette is under the control of the stronger CMV promoter (Honda et al., 2000). In these cells, the expression of Renilla luciferase appears to be fairly constant throughout the cell cycle with the expression of firefly luciferase being induced during mitosis. This suggests that the reduction of Renilla luciferase activity in the pRF vectors may be instead reduced by a transcriptional mechanism. If this were the case, the amount of dicistronic RNA available for IRES-dependent translation would also be reduced.

Pyronnet et al., (2000) suggested that the c-myc IRES is still able to initiate translation during mitosis by using a vector described in Nanbru et al., (1997). This expresses a dicistronic cassette with an upstream CAT ORF and a Myc-CAT fusion protein as the downstream ORF, under the control of a CMV promoter. Similar experiments with the pRMF vector, which contains the c-myc IRES, failed to detect any maintenance of firefly luciferase translation during mitosis (Tanya Subkhankulova, personal communication). The monocistronic vector used to show the induction of CAT expression from the ODC IRES during G2/M progression also contained a CMV promoter.

The dicistronic vectors used by Cornelis et al., (2000) contain an SV40 promoter, but express firefly luciferase as the cap-dependent cistron and β-galactosidase as the IRES-dependent ORF. During mitosis, the physiological levels of the p58 isoform of PITSLRE do increase significantly, but it is possible that the “induction” of p58\textsuperscript{PITSLRE} IRES activity shown from the Luc-β gal dicistronic vector is a consequence of the wide variation in the half lives of these two proteins and may therefore be due to a transcriptional mechanism, rather than a translational one.

Firefly luciferase has a half-life of approximately 3 hours (Thompson et al., 1991) but the half-life of the LACZ protein is 20 hours, so the β-galactosidase activity of cell lysates would be constant during mitosis, independent of whether or not it is being translated by the
p58^{PITSLRE} IRES. The induction of IRES activity in this study was calculated as the ratio of β-galactosidase to firefly luciferase in the cells. If one assumes that the expression of dicistronic mRNA was constant throughout the cell cycle, then a reduction in the amount of cap-dependently synthesised firefly luciferase in mitotic cells and the maintenance or induction of β-galactosidase expression by an IRES-dependent mechanism would result in an increase in the ratio of downstream to upstream cistron expression. However, this “induction” would also be seen if the expression of the dicistronic cassette was reduced by a transcriptional mechanism. While the amount of firefly luciferase drops in the cells, the levels of β-galactosidase would drop at a much slower rate or stay constant, and an induction would again be seen.

The half-lives of Renilla and firefly luciferase do vary slightly (5.3 hours compared to 3 hours; Bronstein et al., 1994), although not to the same degree as firefly luciferase to β-galactosidase. If the data from the Apaf-1 and BAG-1 experiments are interpreted in the same way, then a small change in the ratio of Renilla luciferase expression to firefly luciferase is observed in the cells transfected with pRBF, but not those transfected with pRAF (Figure 5.7). However, this change is not as great as that observed by Cornelis et al., (2000) and is probably due to the difference in half-lives of the two proteins expressed from the dicistronic vectors.

One approach to determine whether the Apaf-1, BAG-1 or c-myc IRESs are able to function during the cell cycle in our dual luciferase dicistronic system would be to change the SV40 promoter upstream of the dicistronic cassette to the CMV promoter. Unfortunately, this promoter is far more efficient than the SV40 promoter and thus overexpression of dicistronic mRNAs has been found to limit the amount of IRES-driven translation from a dicistronic vector containing either the c-myc or Apaf-1 IRESs (Stoneley et al., 2000b and Sally Mitchell, personal communication). This is probably a consequence of the factors responsible for
Figure 5.7 A possible induction of BAG-1 IRES activity during mitosis may be due to differences in luciferase half lives. By plotting the ratio of cap-dependently synthesised Renilla luciferase to firefly luciferase synthesised by the Apaf-1 or BAG-1 IRESs in the vectors pRAF or pRBF, a small upregulation of internal ribosome entry mediated by the BAG-1 IRES is observed.
IRES-driven translation being present in limiting amounts in cells and this phenomenon has been observed for the entero- and rhinovirus IRESs, but not those of the cardio- and aphthoviruses (Borman et al., 1997).

**5.4 Investigation of IRES-driven translation during heat shock**

The reduction of cap-dependent protein synthesis during heat shock occurs by the phosphorylation of eIF2α, the sequestration of eIF4E by 4E-BPs and the binding of Hsp27 to eIF4G. So far, the translation of cellular IRESs during heat shock has not been investigated, although the first cellular IRES to be discovered was in the 5' UTR of BiP/Grp78, a variant of Hsp70 that is associated with the endoplasmic reticulum. It appears that adenoviral mRNAs and those encoding human Hsp70 are translated during heat shock by the mechanism of ribosomal shunting (Yueh and Schneider, 2000).

As a co-chaperone of both the heat-induced Hsp70 and the constitutively expressed Hsc70, BAG-1 obviously plays an important role in the heat shock response. Recent work has proposed differential functions for the BAG-1 isoforms in heat shock, with the p36 isoform of BAG-1 aiding the protein refolding capacity of Hsc70 (Luders et al., 2000b). As we have found that this is the only isoform of BAG-1 that can be translated by internal ribosome entry, the obvious question to ask is whether the IRES is used to initiate the sole translation of this isoform during heat shock. Heat shock is also of importance to Apaf-1 as recent work has shown that the heat shock proteins are able to bind to Apaf-1 and prevent caspase activation. As may be the case in DNA damage, the Apaf-1 IRES may be required to maintain expression of the protein in case an apoptotic programme is required following severe heat shock.

Also, the Apaf-1 or BAG-1 IRESs may be used to initiate translation in the early stages of heat shock recovery. Protein synthesis rates take time to recover from an elevated
temperature and the translation of p36 BAG-1 from the IRES may be important in aiding recovery as the preferential expression of this isoform would be beneficial in recovery.

As part of the response to heat shock treatment to 46°C, c-Myc proteins accumulate through an increase in both the stability of the protein and also via an increase in translation of the c-Myc protein (Luscher and Eisenman, 1988). Obviously, the IRES in the 5' UTR of c-myc may be contributing to this maintenance of expression during heat shock and so this temperature was used to determine whether the Apaf-1 or BAG-1 IRESs were able to maintain translation of firefly luciferase in the dicistronic assay.

5.4.1 Heat shock at 46°C is inhibitory to the dual luciferase assay

One of the problems with our dual luciferase dicistronic vector system is that firefly luciferase is a model substrate used to show the refolding kinetics of the heat shock proteins (e.g. Luders et al., 2000b; Nollen et al., 2000) and so, unsurprisingly, denatured luciferase proteins only have negligible activity in the dual luciferase assay after exposure to 46°C for up to two hours (data not shown). To overcome these problems of denaturation, it was decided to attempt to demonstrate the continued synthesis of firefly luciferase by internal ribosome entry during heat shock by immunoprecipitating radiolabelled firefly luciferase. As several species cross-react with the luciferase antibody in Western blotting, it was decided to epitope tag the luciferase to aid in the purification of the protein.

The FLAG epitope is a short peptide sequence that is commonly used for the detection of recombinant proteins (Hopp et al., 1988). If the hydrophilic FLAG sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys is introduced at the N-terminus of luciferase, it will remain accessible for recognition by antibodies and therefore can be used for purification of the FLAG-luciferase fusion protein.
Accordingly, vectors were made that would express a FLAG-firefly luciferase fusion protein from a dicistronic vector containing either the Apaf-1 or BAG-1 IRES. As a control for measuring the decrease in cap-dependent translation, a monocistronic luciferase vector containing the FLAG epitope was also created.

5.4.2 Construction of pRAΦF, pRBΦF, pGΦL

A double stranded oligonucleotide cassette formed by hybridisation of the oligonucleotides FLAGF and FLAGR was digested with Ncol and ligated into the plasmids pGL3, pRAF and pRBF that had been digested with the same enzyme and treated with CIAP to minimise self-ligation (Figure 5.8). Clones that contained the FLAG sequence in the correct orientation were identified by PCR with the oligonucleotides FLAG PCR and SV40enR. This yielded a 1.8 kb amplification product when the FLAG sequence was in the correct orientation. Sequencing of the plasmids confirmed the presence of a single copy of the FLAG sequence, fused to the ORF of firefly luciferase. In each of these plasmids, the FLAG epitope also contains an initiator methionine.

The presence of the FLAG epitope at the N-terminus of the firefly luciferase protein did not appreciably affect its enzyme activity in the dual luciferase assay, unlike the fusions of BAG-1 coding sequence in the vector pGBAδL (Figure 4.14 and data not shown).

5.4.3 Western blotting and immunoprecipitation of FLAG-tagged luciferase

HeLa cells were transfected with the vectors pGΦL, pRAΦF and pRBΦF and 40 hours after transfection, the cells were lysed and prepared for SDS-PAGE. The FLAG epitope could only be efficiently detected by Western blotting cells transfected with the plasmid pGΦL (Figure 5.9A) and this probably reflects the relatively efficient expression of monocistronic vectors compared to dicistronic vectors. Unfortunately, the expression of the
Figure 5.8 Construction of the vectors pGΦL, pRAΦF and pRBΦF

(A) The oligonucleotides FLAG F and FLAG R containing the FLAG epitope sequence (shown in bold) were annealed and digested at the Ncol sites (shown in italics).

(B) The digested cassette was ligated into the cut vectors indicated to create the plasmids pGΦL, pRAΦF and pRBΦF.
Figure 5.9 Expression of FLAG-tagged luciferase is only efficient from the monocistronic reporter vector pGΦL.

(A) FLAG-tagged luciferase expressed in HeLa cells transfected with pGΦL, pRAΦF or pRBΦF was detected with an anti-FLAG antibody (M2) raised in mice and conjugated to horseradish peroxidase (Sigma).

(B) HeLa cells transfected with no vector, pGΦL, pRAΦF or pRBΦF were labelled with [35S]methionine for 30 min. The lysates were pre-cleared with mouse IgG and then incubated with anti-FLAG antibody (M2) conjugated to agarose (Sigma). Immunoprecipitated proteins were separated by SDS-PAGE and the fixed and dried gel was exposed to a phosphorimager screen.
FLAG-tagged firefly luciferase from the dicistronic vectors pRAΦF and pRBΦF could not be detected in an immunoprecipitation from cells growing at 37°C and labelled with $[^{35}\text{S}]$methionine for 30 minutes (Figure 5.9B).

5.4.4 The BAG-1 IRES is used to maintain expression of p36 BAG-1 during heat shock to 44°C and during heat shock recovery

More recent experiments by Anne Willis have concentrated on the expression of BAG-1 during recovery from heat shock at a less severe temperature of 44°C, which also permits measurement of firefly luciferase activity. Protein synthesis rates of normal and heat shocked HeLa cells were assayed by measuring the incorporation of $[^{35}\text{S}]$methionine of cells either maintained at 37°C, or heated to 44°C and then allowed to recover at 37°C for 90 min. The heat shocked cells exhibited a greatly reduced rate of protein synthesis, although this was beginning to recover 90 minutes after the return of the cells to 37°C (Figure 5.10A).

As expression of firefly luciferase from the FLAG-tagged vector pRBΦF was almost undetectable, the state of native BAG-1 in the cells was investigated instead. BAG-1 proteins were isolated from normal and heat shocked cells by immunoprecipitation using the mouse monoclonal antibody mix used in Section 4.

The BAG-1 proteins were labelled by addition of $[^{35}\text{S}]$methionine to cells for 30 minutes either in cells maintained at 37°C, cells heat shocked to 44°C for 30 minutes, or at 30 minute intervals during recovery at 37°C.

At first the synthesis of both p36 and p50 BAG-1 are reduced, but there is a preferential induction of p36 BAG-1 in the HeLa cells during the first hour of recovery from heat shock (Figure 5.10B, lanes 60 and 90 min). By 90 min after heat shock, cap-dependent translation has recovered and the expression of the p50 isoform is observed (Figure 5.10B,
Figure 5.10 The BAG-1 IRES is used following heat shock.

(A) Incorporation of radiolabelled methionine into cells shows that there is a decrease in total protein synthesis during and following heat shock.

(B) Immunoprecipitation of BAG-1 isoforms during heat shock and during recovery shows that there is a higher amount of synthesis of the p36 isoform.

(C) Cells were transfected with either the monocistronic plasmids pGBL and pGL3 or the dicistronic plasmids pRF and pRBF. Firefly luciferase activities were determined during and following heat shock.
lane 120 min). This suggests, before cap-dependent protein synthesis recovers, the expression of p36 BAG-1 is induced by IRES-dependent translation.

This hypothesis is confirmed in cells transfected with pGL3, pGBL, pRF or pRBF. Firefly luciferase activities were measured at various times during and after a 30 minute heat shock to 44°C and, in cells transfected with the control vectors pGL3 and pRF, the expression of firefly luciferase during a heat shock to 44°C is reduced to around 5% of that of cells maintained at 37°C. The levels of firefly luciferase expression then gradually increase during incubation at 37°C, correlating with the recovery of cap-dependent protein synthesis. In contrast, the expression of firefly luciferase is maintained throughout the heat shock and recovery in those cells transfected with vectors that contain the BAG-1 IRES (Figure 5.10C).

5.4.5 Summary

The downregulation of cellular protein synthesis in response to environmental stresses such as heat shock is obviously of great benefit to the cell, preventing the accumulation of aberrantly folded proteins. It should not be surprising, therefore to find that the p36 isoform of BAG-1, which has been previously found to have a stimulatory effect on the protein refolding cycle of Hsp70, remains translated by internal ribosome entry during heat shock. The observation that the c-myc IRES is also able to maintain translation of firefly luciferase during heat shock at 44°C (Catherine Jopling, personal communication), suggests that the preferential translation of IRES-containing mRNAs during heat shock recovery may allow the translation of certain mRNAs while general protein synthesis is inhibited.

Several other cellular mRNAs that contain internal ribosome segments may remain translated during heat shock. The first cellular IRES was discovered in the 5’ UTR of BiP/Grp78, a homologue of Hsp70 and there is an induction of human fibroblast growth factor
Expression of Hsp70 itself during heat shock is also maintained by a translational mechanism, although in this case it is by ribosomal shunting. According to Yueh and Schneider (2000), the 5' UTR of hsp70 is unable to direct internal ribosome entry in a dicistronic assay, but no information is given on how this was tested. For example, the dicistronic vector assay may have been carried out in a cell line or *in vitro* translation system that was lacking *trans*-acting factors that are essential for IRES activity. Also Hsp70 coding region that might have been essential for IRES activity may have been missing from the dicistronic vector or a relatively inefficient dicistronic vector like pRCAT may have been used to determine internal ribosome entry. One line of evidence that suggests the hsp70 mRNA contains an IRES was the observation that a circularised full length hsp70 mRNA could initiate translation in rabbit reticulocyte lysate (RRL) (Carter *et al.*, 1999).
Chapter 6

Discussion

6.1 Identification of internal ribosome entry segments in the 5' UTRs of Apaf-1 and p36 BAG-1

Apaf-1 plays a central role in the regulation of programmed cell death by controlling the activation of members of the caspase family of proteases. Several features of the 5' untranslated region of the Apaf-1 mRNA suggested that translational control may regulate the expression of the Apaf-1 protein. Experiments with monocistronic reporter vectors showed that the 5' untranslated region of Apaf-1 was a potent inhibitor of the translation of a downstream reporter gene both in rabbit reticulocyte lysates and transfected HeLa cells. When inserted between the cistrons of a dicistronic reporter vector, the Apaf-1 5' UTR was found to increase the expression of a downstream reporter gene. This increase in expression was found to be due to internal ribosome binding on intact dicistronic transcripts.

BAG-1 is involved in several cellular processes due to its interaction with the heat shock chaperone family. Several isoforms of the BAG-1 protein exist which are translated from alternative initiation codons. While in vitro experiments suggest that the isoforms are translated by leaky scanning, the expression of BAG-1 isoforms in cells does not correlate with this observation, with the p36 isoform being the most abundant. Consequently, the translation of p36 BAG-1 was found to be initiated by both internal ribosome entry and cap-dependent scanning. However, IRES dependent translation of p36 BAG-1 is not as efficient as cap-dependent scanning.

Attempts were also made to identify a functional role for these novel IRESs during conditions when cap-dependent translation is downregulated as a result of modifications of the initiation factor machinery. The IRESs appear to be used to maintain translation of Apaf-1
and BAG-1 during the early stages of TRAIL-induced apoptosis and the BAG-1 IRES is used to maintain translation of the p36 isoform of the protein during heat shock. This isoform has been found to aid the protein refolding activity of the Hsp70 machinery and so the specific expression of p36 BAG-1 should aid in the recovery of the cell from heat shock.

6.2 The role of trans-acting factors in IRES-dependent translation

The activities of the Apaf-1 and BAG-1 IRESs differ greatly between cell lines, suggesting that the expression of non-canonical initiation factors required for efficient internal ribosome entry varies between cell lines. However, the activity of each IRES also differs in the same cell line which suggests that each IRES may have a specific set of binding partners. For example, while both IRESs exhibit high activity in HeLa cells, the activity of the BAG-1 IRES is much higher than the Apaf-1 IRES in MCF7 cells.

A long term aim of the laboratory is to reconstitute an *in vitro* translation extract capable of maintaining IRES-dependent translation as the rabbit reticulocyte lysate system lacks many of the non-canonical factors required for the translation of most viral and cellular IRESs. The factors unr (upstream of N-ras) and PTB (the polypyrimidine tract binding protein) are required for the activity of the HRV IRES (Hunt and Jackson, 1999; Hunt *et al.*, 1999). The addition of unr and PTB to rabbit reticulocyte lysate was found to increase the production of firefly luciferase from dicistronic mRNAs transcribed *in vitro* from the vector pRAF, which contains the Apaf-1 IRES (Mitchell *et al.*, in press).

Electromobility shift assays (EMSAs) and UV-cross-linking assays found that unr binds directly to the Apaf-1 IRES and PTB only binds if unr is present. Using the deletions of the Apaf-1 IRES described in Section 3.5.2, unr was found to bind to the df fragment (the 233 nt upstream of the initiation codon), which maintains 75% of the IRES activity of the full
length 5’ UTR. This sequence does not contain the optimal unr binding motif of AAGUA/G or AACG determined by SELEX, but neither does the IRES in the 5’ UTR of human rhinovirus.

The observed variation of Apaf-1 IRES activity between cell lines correlates broadly with the content of unr and PTB in these cells. In COS7 and SY5Y cells the expression of these proteins is particularly reduced, but overexpression of unr or PTB cDNAs in these cells caused an increase in Apaf-1 IRES activity. This is the first time that the stimulation of the activity of a cellular IRES by non-canonical factors has been observed in vitro and in vivo.

When dicistronic mRNAs harbouring the HRV IRES are transfected directly into the cytoplasm of cells, IRES activity is still maintained. However, dicistronic mRNAs containing the Apaf-1 IRES failed to generate any firefly luciferase when transfected into the cytoplasm of HeLa cells (Sally Mitchell, personal communication) and this is paralleled in other eukaryotic IRESs such as the one found in the c-myc 5’ UTR (Stoneley et al., 2000b). This appears to suggest a “nuclear event” is essential for IRES activity, which could represent either the modification of the mRNA or the binding of a trans-acting factor that is usually localised to the nucleus. One such factor that may act in this way is the autoantigen La, which interacts with several viral and eukaryotic IRESs. During apoptosis, caspase cleavage causes the bifurcation of the N-terminus of the La protein, which contains the RNA binding moiety and the C-terminal nuclear localisation signal (Ayukawa et al., 2000). This results in the relocalisation of the truncated La protein to the cytoplasm, where it may act to bind IRESs more efficiently, maintaining translation of IRES-containing messages during apoptosis.

As well as determining the role of known IRES-binding proteins in the translation of Apaf-1, attempts are also being made to identify novel trans-acting factors that are essential for internal ribosome entry. One novel approach to identify IRES binding proteins has involved the use of a RNA sequence that was identified by its ability to bind to streptomycin
(Wallace and Schroeder, 1998). The RNA aptamer or “StreptoTag” (Figure 6.1A; described in Bachler et al., 1999) is introduced at the 3’ end of the IRES sequence and this RNA is then incubated with HeLa cell extract or rabbit reticulocyte lysate. After the protein-RNA complex is immobilised to a streptomycin-Sepharose affinity matrix the proteins are eluted with excess antibiotic.

Several binding partners for the Apaf-1 IRES can be isolated by this method (Sally Mitchell, personal communication), and similar experiments with the IRESs in the BAG-1 and c-myc 5’ UTRs also isolated potential binding proteins. Unfortunately, an extremely large amount of starting material is required to isolate enough of these proteins for sequencing and therefore attempts to sequence these novel proteins have so far yielded no information.

An alternative method, which should quickly provide sequence information for proteins that bind to the Apaf-1 and Bag-1 IRESs, is the yeast three hybrid system (SenGupta et al., 1996; illustrated in Figure 6.1B). This system works on a similar principle to the yeast two-hybrid screen used to identify or confirm protein-protein interactions in vivo (Fields and Song, 1989). The LexA DNA binding domain is fused to the MS2 coat protein, which contains an MS2 RNA binding domain. A hybrid RNA containing the MS2 stem loop motif and the IRES sequence of interest is then screened for interactions with a library of prey proteins fused to the B42 transcriptional activation domain. Positive interactions complete the transcriptional unit and allow blue/white colour selection and growth on HIS- media.

This method is currently being used to screen a cDNA library derived from human testes for proteins that bind to the c-myc IRES. Unfortunately the size of the hybrid bait RNA appears to determine the efficiency of the system, with RNAs over 300 nt in length failing to produce positive interactions. For example, when the HRV IRES and unr were used as the RNA bait and prey protein respectively, a positive interaction could not be produced (Jo Evans, personal communication).
Figure 6.1 Methods for detecting RNA-protein interactions

(A) The “StreptoTag” streptomycin-binding RNA aptamer fused to the 3’ end of an IRES sequence (adapted from Bachler et al., 1999).

(B) Interactions between the components of the yeast three hybrid system (adapted from SenGupta et al., 1996). A hybrid RNA containing the sequence of interest is used to screen for interactions with members of a cDNA library.
6.3 Minimal IRES elements and predictions of secondary structure

Deletions of the Apaf-1 and BAG-1 IRESs produced truncations that were still able to function efficiently compared to the full length IRES sequence. The 233 nt before the initiation codon of Apaf-1 could maintain 75% of the IRES activity of the full length 5' UTR. A deletion that included just the sequence from –233 to –115 (fragment d) was still able to direct internal ribosome entry, but not very efficiently. The Apaf-1 IRES may direct internal ribosome entry in a manner similar to the Class I Picornavirus IRESs and the conserved AUG triplet in fragment f may be important for Apaf-1 IRES activity. Mutation of the AUG codon to an AGG triplet appears to activate Apaf-1 IRES activity which suggests that some ribosomes initiate translation at the upstream AUG codon, while others initiate translation at the physiological initiation codon. This hypothesis is backed up by the fact that the ae truncation of the Apaf-1 IRES, which loses the conserved AUG triplet, has a similar activity to the full length IRES.

The oligopyrimidine tract in fragment e may also be important for IRES activity. However, a more recent IRES truncation containing only the d and e fragments does not direct internal ribosome entry as efficiently as the d-f fragment and mutation of the oligopyrimidine tract to an oligopurine tract has only a negligible effect on IRES activity (Sally Mitchell, personal communication). Other oligopyrimidine tracts exist in the Apaf-1 IRES and the effect of mutation of these sequences is also being tested.

Further deletions of the BAG-1 IRES are also currently being made in an attempt to determine a minimal IRES sequence. As the BAG-1 IRES is located in coding sequence it is possible that the essential sequence for ribosome recruitment may be an extremely short module like the 9 nt IRES found in the 5' UTR of the Gtx mRNA. If the BAG-1 IRES is indeed found to be a short primary sequence, then predictions of the secondary structure of the
IRES may be unnecessary. However, the Apaf-1 5' UTR is hypothesised to act like a Class I IRES, and thus it is of interest to be able to model the secondary structure of the Apaf-1 IRES.

Creating secondary structure predictions of either the full length Apaf-1 and BAG-1 IRESs or even the functional deletions (e.g. the def fragment of the Apaf-1 5' UTR or the 3' end of the BAG-1 IRES) with the M-fold program produces many possible secondary structures. To constrain the possible secondary structures, the approach with many viral IRESs relies on analysis of covariation between the sequences of viral serotypes. A similar phylogenetic analysis of several mammalian c-myc 5' UTR sequences allowed the identification of particularly important sequences, and a first structure of the c-myc IRES based on sequence conservation was proposed in Stoneley et al., (1998).

Unfortunately, this kind of phylogenetic analysis of the Apaf-1 and BAG-1 5' UTRs is practically impossible as only the messages from humans and mice have been sequenced. The 5' UTR of the ced-4 mRNA from C. elegans is only 46 nt in length and the available sequence information for the Drosophila and zebrafish homologues of Apaf-1 appears to be incomplete.

Without experimental data to constrain both raw structural data and conserved sequence motifs into a coherent model, no conclusions can be made. The secondary structure of the c-myc IRES from Stoneley et al., (1998) was extensively revised after the identification of paired and unpaired bases by chemical modification and primer extension experiments (Figure 1.6 and Le Quesne et al., submitted for publication).

6.4 The identification of novel IRESs

Since the identification of an internal ribosome entry segment in the 5' UTR of the BiP mRNA, there has been great interest in cellular IRESs and in the last few years the number of cellular mRNAs found to be translated in this way has increased dramatically. As IRES
sequences rarely contain obvious motifs in the primary sequence, the discovery of novel IRES elements has previously relied on observations that certain proteins remain translated during conditions when cap-dependent translation is downregulated or that the 5' UTR of an mRNA possesses several features that make it a candidate for internal ribosome entry.

With the advent of cDNA microarray technology, which allows the differential identification of mRNAs that remain associated with polysomes under conditions when cap-dependent translation is inhibited, many more sequences that contain IRESs will be identified. This technique has been successfully used to identify novel IRESs that direct translation after picornavirus infection (Johannes et al., 1999) and many independent efforts are now being made to use this technique to screen for mRNAs that remain translated under different conditions e.g. apoptosis, mitosis and T lymphocyte activation.

The use of this technology to identify hundreds of species that can initiate translation by internal ribosome entry will possibly lead to a greater understanding of primary sequence motifs that are essential for IRES activity. This will be especially true if more short IRES modules are found within coding sequences that can translate shorter IRES-initiated forms of proteins, as is the case for BAG-1, PITSLRE, ERα and Notch2.

Recent work in our laboratory and others has also shown that IRESs may exist in the 5' UTRs of several members of gene families. The proto-oncogene N-myc shows considerable homology to c-myc and is typically overexpressed in neuroblastomas. The 5' UTR of N-myc is able to direct internal ribosome entry at levels similar to c-myc in non-neuronal cells. However, in neuronal cells, the N-myc IRES is up to seven times more active than the c-myc IRES which suggests that trans-acting factors specific for the N-myc IRES are expressed in these cells (Jopling and Willis, in press). Another member of the Myc/Max/Mad family of transcriptional regulators that possesses a 5' UTR that contains an IRES is mnt/rox
(Stoneley et al., in press) and the 5' UTRs of L-myc and c-myb may also be able to direct internal ribosome entry.

In terms of Apaf-1 like molecules, two other proteins with CED-4 like domains have been identified in mammalian cells, the FLICE-associated huge protein (FLASH) and Nod1/CARD4 (Imai et al., 1999; Bertin et al., 1999; Inohara et al., 1999). The 5' UTR of Nod1/CARD4 is 425 nt long but is only 46% GC rich, which suggests a reduced potential for the formation of secondary structure. Nonetheless, the region of the 5' UTR immediately upstream of the initiation codon contains an upstream AUG triplet along with an oligopyrimidine tract, which suggests that this 5' UTR is an interesting candidate for study. However, when the Nodi 5' UTR is subcloned into the dicistronic vector pRF, it fails to increase the translation of the downstream firefly luciferase reporter gene (Sally Mitchell, personal communication).

The 5' UTR of human FLASH is 197 nt long and 64% G-C rich and contains a single, out of frame upstream AUG triplet, while the murine version is 256 nt in length, 71% GC rich with three out of frame upstream AUG codons. The sequences are not particularly well conserved, although the region immediately upstream of the initiation codon is 71.9% conserved. Thus the FLASH mRNA may also be able to direct translation by internal ribosome entry.

Of the four human BAG-1 homologues identified by Takayama et al., (1999) only the open reading frame of BAG-2 has been defined. Definite start codons for BAG-3 and BAG-5 have not been determined and the 309 nucleotides upstream of the putative AUG initiation codon of BAG-3 contains two in frame CUG triplets, while the 225 nt of the BAG-5 5' UTR contains an in frame AUG as well as an in frame CUG codon. This obviously raises the possibility that these initiation codons may be used to initiate translation of longer isoforms of these proteins, as is observed with BAG-1. Although there is no significant homology
between the 5’ UTRs of any of the BAG family members, this does not rule out the possibility that other members of the BAG family of molecular chaperone regulators may be translated by internal ribosome entry.

6.5 Functional roles for IRESs

The observation that the Apaf-1 and BAG-1 IRESs maintain translation of a reporter gene during the early stages of TRAIL-induced apoptosis complements previous work in our laboratory and by others that certain mRNAs that contain IRESs will be translated during apoptosis after modification of the initiation factor machinery causes a reduction in cap-dependent translation (Holcik et al., 1999; Henis-Korenblit et al., 2000; Stoneley et al., 2000a; reviewed in Holcik et al., 2000a).

The Apaf-1 and BAG-1 IRESs do not appear to function during mitosis, although this may be due to a downregulation of transcription of the dicistronic reporter constructs, rather than a translational mechanism. However, during environmental stresses, both IRESs do function. Low levels of the alkylating agent EMS are able to induce a response to genotoxic stress without provoking apoptosis and in HeLa cells treated with 20 mM EMS, a reduction in cap-dependent protein synthesis is observed. The Apaf-1 IRES is able to maintain the translation of firefly luciferase in the dicistronic vector system under these conditions (Subkhankulova et al., in preparation). The BAG-1 IRES functions during recovery from heat shock to cause the preferential expression of the p36 BAG-1 isoform, which encourages the protein refolding activity of the Hsp70 chaperone system.

One area of particular interest in studying the role of the Apaf-1 and BAG-1 IRESs is during mammalian development. Apaf-1 is essential for apoptosis during murine development (Cecconi et al., 1998; Yoshida et al., 1998) and the small proportion of male
Apaf-1 knockout mice that survive to adulthood are infertile (Honarpour et al., 2000). During murine development, the different isoforms of BAG-1 are expressed in different cell types and at different times (Crocoll et al., 2000).

An approach using a dual luciferase vector to test for the activity of the FGF-2 IRES in transgenic mice (Creancier et al., 2000) showed that IRES activity was especially strong in the heart and brain of mouse embryos, but in the adult only the brain exhibited high FGF-2 IRES activity. We intend to study IRES-driven expression in a developing organism using a vector that will allow the in situ assay of IRES activity. Using the vector pEF-BOS as a base the vector pEF-GFP-IRES-βgal is currently being constructed. This will contain an expression cassette under the control of the powerful EF1α promoter, which is between 1.5 and 50x stronger than the SV40 or RSV promoters (Mizushima and Nagata, 1990). The dicistronic message will express Green Fluorescent Protein (GFP) as the upstream, cap-dependent cistron and lacZ as the downstream IRES-driven cistron. Expression of both of these reporter genes can be easily detected in situ.

6.6 Concluding remarks

The work in this thesis has described the identification and analysis of two novel cellular IRESs and this adds to our understanding of the mechanisms of control of translation initiation in mammalian cells. However, there is still much to be learnt, as a full understanding of cellular internal ribosome entry will not be complete until the non-canonical trans-acting factors required for this process have been identified. This is because: i) regulation of such factors by phosphorylation or protease-mediated cleavage would be key to this process and ii) establishment of an in vitro translation system is vital to allow the dissection of key elements within the IRES.
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Initiation of Apaf-1 translation by internal ribosome entry

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The apoptotic protease activating factor (Apaf-1) plays a central role in apoptosis: interaction of this protein with procaspase-9 leads to cleavage and activation of this initiator caspase. In common with other mRNAs whose protein products have a major regulatory function, the 5' untranslated region (UTR) of Apaf-1 is long, G-C rich and has the potential to form secondary structure. We have shown that the 5' UTR of Apaf-1 contains an internal ribosome entry segment, located in a 233 nucleotide region towards the 3' end of the leader, and that the translation initiation of this mRNA occurs only by internal ribosomal entry. The Apaf-1 IRES is active in almost all human cell types tested, including Human cervical carcinoma (HeLa), Human liver carcinoma (HepG2), Human embryonic kidney (HK293), African Green Monkey kidney (COS7) and Human lung (MRC5). The Apaf-1 IRES initiates translation as efficiently as the HRV 40 repeats (Hu et al., 1998a) and also by self-association of C-terminal WD-40 repeats (Hu et al., 1998b). Following an apoptotic stimulus, cytochrome c that has been released from the mitochondria binds to Apaf-1 (Li et al., 1999). At the same time dATP binds to Apaf-1 at a P loop found in the ced-4 homology region (Cardozo and Abagyan, 1998). Hydrolysis of the dATP causes oligomerization of Apaf-1 monomers into an 'apoptosome' consisting of up to eight Apaf-1 molecules (Zou et al., 1999; Saleh et al., 1999). This oligomerization permits binding of procaspase-9 and encourages interactions between procaspase-9 molecules, thus allowing intermolecular cleavage and activation to occur. This initiator caspase can then cleave procaspase-3, one of the effector caspases essential for cleavage of a number of key substrates during the execution phase of apoptosis.

Apaf-1 is expressed in almost all tissues studied with the exception of skeletal muscle. In these cells very little Apaf-1 mRNA can be detected by RT-PCR or Northern blot analysis and no Apaf-1 protein is observed by Western blotting (Zou et al., 1997; Burgess et al., 1999). This may be an important control point as after strenuous activity mitochondria in skeletal muscles have been shown to swell and/or degenerate (Zamora et al., 1995), which could lead to accidental triggering of the apoptotic pathway as a consequence of cytochrome c release.

Homozygous Apaf-1 knockout mice die early in embryogenesis and show reduced apoptosis leading to many morphological abnormalities (Ceconi et al., 1998; Yoshida et al., 1998). In murine Apaf-1 knockout cell lines, overexpression of c-Myc can promote tumorigenesis in a manner analogous to cell lines lacking p53 (Soengas et al., 1999). Conversely, overexpression of Apaf-1 increases the sensitivity of cells to stimulators of apoptosis such as etoposide and paclitaxel (Perkins et al., 1998).

The 5' untranslated regions (UTRs) of both the human and mouse versions of Apaf-1 have an overall homology of 56% and have many of the features of a translationally regulated gene. They are long (578 and 585 nucleotides respectively), G-C rich (67 and 66% respectively) and contain upstream AUG codons (two in the human, one in the mouse). The single upstream AUG codon in the murine 5' UTR is conserved in the human version and lies in a region of 70% homology.

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approximately 40 nucleotides upstream of the physiological start site.

The high degree of secondary structure in the 5' UTR of an mRNA can contribute to the control of translation in a number of ways. For example, regions of stable secondary structure may be inhibitory to the normal scanning mechanism of translation initiation (Pain, 1996), or they may contain an internal ribosome entry segment (IRES). IRESs were originally identified in the 5' UTRs of picornaviral RNAs and they act to direct ribosome binding straight to the initiation codon, either directly or indirectly via other linking factors (Jackson et al., 1994, 1995; Jackson and Kaminski, 1995). Recently a number of IRESs have been identified in several eukaryotic mRNAs whose protein products are associated with cell growth or cell death including vascular endothelial growth factor (VEGF) (Stein et al., 1998; Miller et al., 1998), fibroblast growth factor-2 (FGF-2) (Vagner et al., 1995), platelet derived growth factor (PDGF) (Bernstein et al., 1997) and c-myc; (Nanbru et al., 1997; Stoneley et al., 1998).

In this study we have investigated the 5' UTR of Apaf-1 and shown that it contains an IRES. This is the first example of an IRES to be identified in a gene whose protein product is intimately associated with the initiation of apoptosis and our data suggest that initiation of translation of Apaf-1 only occurs by internal ribosome entry and not by the more generally used scanning mechanism of translation.

Results

The 5' untranslated region of Apaf-1 inhibits the translation of a downstream reporter gene in vitro

The 5' UTR of Apaf-1 was introduced upstream of the luciferase start codon at the NcoI site in the vector pSKL (Stoneley et al., 1998); this ensured that it was in the correct reading frame (Figure 1a). Capped mRNAs generated from this vector using T7 RNA polymerase were then used to prime rabbit reticulocyte lysates and the amount of luciferase generated was measured (Figure 1b). The presence of the Apaf-1 5' UTR was found to potently inhibit the translation of the downstream luciferase gene. For example, when the lysates contained 20 ng of pSKAL RNA, 100-fold less luciferase was produced (Figure 1b). This suggested either that a region of stable secondary structure was obstructing the scanning translation initiation complex or that non-canonical factors which were not present in rabbit reticulocyte lysates were required for translation initiation of Apaf-1.

The Apaf-1 5' UTR causes a reduction in the translation of a luciferase reporter enzyme in vivo

The Apaf-1 5' UTR was inserted into the monocistronic luciferase reporter vector pGL3 (Figure 2a) to create the vector pGAL. Both constructs were transfected by the calcium phosphate method into HeLa cells, and luciferase activity measured (Figure 2b). In the presence of the Apaf-1 5' UTR upstream of the luciferase reporter there was again a significant decrease in the amount of luciferase produced compared to the control. However, the effect was much less dramatic than that observed in vitro and in this case approximately fourfold less luciferase was produced (Figure 2b). This would suggest either the presence of stable secondary structure in the Apaf-1 UTR which was inhibitory to scanning or that the initiation of Apaf-1 translation is mediated by an alternative mechanism which is less efficient than scanning. To distinguish between these two possibilities, a stable hairpin (Stoneley et al., 1998) was introduced upstream of the control vector and the Apaf-1 5' UTR to create the vectors pPL and pPAl respectively. The presence of the hairpin reduced the luciferase produced by the control vector 200-fold, but had only a small effect (1.5-fold) on the amount of luciferase produced from pPAl. This effect may have been due to the hairpin destabilizing structural elements in the Apaf1 5' UTR. This data suggests that Apaf-1 translation can indeed be initiated by an alternative mechanism e.g. internal ribosome entry. To determine whether Apaf-1 5' UTR contained an IRES it was inserted into the dicistronic construct pRF (formerly known as pGL3R, Stoneley et al., 1998) to give pRAF which incorporates both the firefly and sea pansy (Renilla reniformis) luciferase reporter genes (Figure 3a).

Figure 1 Inhibition of in vitro translation initiation of firefly luciferase by the presence of the Apaf-1 5' UTR. (a) The expression cassette of the vector pSKL used to measure translation of in vitro transcribed mRNAs. The Apaf-1 5' UTR was inserted at the restriction sites indicated to create the vector pSKAL. (b) Rabbit reticulocyte lysates were primed with capped RNA from either the control (pSKL) or Apaf-1 containing vectors. After 1 h luciferase activities were measured as described under experimental procedures.

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E co R I

\[ 5' \text{UTR} \]

SV40 prom Firefly luc SV40 SV40 polyA enhancer

\( \text{B} \)

\( \text{pGL3} \)
\( \text{phpL} \)
\( \text{pGAL} \)
\( \text{phpAL} \)

\( \text{pGL3} \)
\( \text{phpL} \)
\( \text{pGAL} \)
\( \text{phpAL} \)

\( \text{Renilla luciferase} \)
\( \text{Firefly luciferase} \)

\( \text{SV40 chimeric Renma luc Firefly luc} \)
\( \text{SV40 prom intron Renllla luc} \)
\( \text{SV40 SV40 SV40 polyA enhancer} \)

\( \text{SV40 chimeric renma luc Firefly luc} \)
\( \text{SV40 prom intron Renllla luc} \)
\( \text{SV40 SV40 polyA enhancer} \)

Apaf-1 5' UTR directs internal ribosome entry in dicistronic reporter vectors

HeLa cells were transiently transfected with either pRF, pRAF or pRMmAF (the murine form of Apaf-1 5' UTR which is 56% homologous to the human version) by the calcium phosphate method. Expression of Renilla and firefly luciferase were assayed using the 'Stop & Glo' assay kit (Promega) and we show that both Apaf-1 sequences can direct internal ribosome entry in the dicistronic assay. The amount of firefly luciferase produced from the human and murine Apaf-1 containing constructs was ten and 15-fold over readthrough. These values are slightly higher than those obtained from cells transfected with the HRV-IRES-containing plasmids but less than those obtained from cells transfected with plasmid constructs which harbour the c-myc-IRES (Figure 3b).

IRES activity is not due to enhanced ribosomal readthrough

The result from the dicistronic assay alone does not prove the existence of an IRES since ribosomal readthrough could be enhanced by signals in the UTR. Thus to eliminate this possibility a stable hairpin (−55 kcal/mol) was inserted upstream of the Renilla luciferase to create the plasmid vectors phpRAF and phpRMmAF (Figure 4a). This hairpin was sufficient to impede ribosomal scanning, and Renilla activity produced from these two constructs dropped to 15–20% of wild type, while firefly luciferase activity directed by the Apaf-1 5' UTRs was maintained. This demonstrates that increased readthrough cannot account for the increased translation of firefly luciferase (Figure 4b).

Figure 2 Apaf-1 5' UTR reduces translation initiation efficiency of firefly luciferase in vivo. (a) The monocistronic luciferase reporter vector pGL3. The Apaf-1 5' UTR was inserted at the restriction sites indicated to create the vector pGAL. A stable hairpin (−55 kcal/mol) was also introduced at the restriction site shown to block ribosomal scanning in the vectors phpL and phpAL. (b) HeLa cells were transfected with either pGL3, phpL, pGAL or phpAL. The luciferase activities were measured and normalized to a transfection control of ß-galactosidase.

Figure 3 Apaf-1 5' UTR directs internal ribosome entry. (a) The dicistronic expression cassette of the vector pRF. Putative IRES sequences are subcloned between the different luciferase cistrons. (b) The dicistronic plasmids containing firefly and Renilla luciferase genes and either human or mouse Apaf-1 5' UTR, the c-myc-IRES or the HRV IRES inserted between them, were transfected into HeLa cells and the activities of both luciferases were measured and normalized to the transfection control.

Figure 4 The Apaf-1 5' UTR is still able to direct internal ribosome entry in the presence of a stable hairpin. (a) A palindromic sequence that when transcribed generates a hairpin of free energy −55 kcal/mol was introduced upstream of the Renilla ORF in the vectors pRAF and pRMmAF. (b) HeLa cells were transfected with these constructs and luciferase activities determined.
**Apaf-1 translation is mediated by an IRES**

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**IRES activity is not due to the presence of a cryptic promoter or aberrant splicing**

It is possible that the increase in firefly luciferase activity in the vector pRAF was due to the presence of a cryptic promoter or splicing signals in the 5' UTR, resulting in monocistronic RNAs being produced. To show that only full length mRNA was being produced from the dicistronic constructs, cells were transfected with pGL3, pGAL, pRF and pRAF. Poly(A)' selected RNA was subjected to formaldehyde gel electrophoresis and then transferred onto nitrocellulose. The blot was then probed with 32P-labelled luciferase fragments that were generated using random prime labelling (Figure 5). Transcripts corresponding to full length pGL3, pGAL, pRF and pRAF were observed. In all cell lines a luciferase transcript of approximately 1.3 knt in length was also detected (*) which probably represents an aberrantly processed luciferase transcript. This fragment cannot account for the IRES activity observed in the vector pRAF as densitometry analysis of this luciferase fragment showed that in the vectors pRF and pRAF these transcripts are present in approximately equal amounts (0.8 :1 respectively). However, ten times more firefly luciferase is observed in cells transfected with pRAF compared to pRF (Figure 3b). This transcript is also present in cells that have been transfected with a dicistronic vector containing the c-myc IRES (Stoneley, 1998).

**Mapping the Apaf-1 IRES**

To define the boundaries of the Apaf-1 IRES a series of plasmid constructs was generated containing decreasing lengths of the sequence coding for the 5' UTR (Figure 6). The ability of these truncated sequences to promote internal ribosome entry on a dicistronic mRNA was compared to the full length 5' UTR. Although the full length IRES is required for maximal luciferase activity, the data suggest that elements which are vital to internal ribosome entry lie between nucleotides −233 and 1, since fragments from −115—1 have only 20% of the luciferase activity of the full length 5' UTR, yet fragments from −233—1 maintain 75% of the activity (Figure 6b).

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**The Apaf-1 IRES is used in a wide range of cell lines**

To investigate how widely the Apaf-1 IRES is utilized, a panel of cell lines derived from different tissues, including Human cervical carcinoma (HeLa); Human liver carcinoma (HepG2); Human breast carcinoma (MCF7); Human embryonic kidney (HK293); African Green Monkey kidney (COS7); Human lung (MRC5); Human neuronal cells (SY5Y); Mouse fibroblasts (Balb/c) and Chinese Hamster ovary T (CHO-T) cells were co-transfected using either FuGene 6 or the calcium phosphate method with either pRF or pRAF (Figure 2a) and pcDNA3.1/HisB/lacZ. The expression from both Renilla and firefly luciferase cistrons was assayed and normalized to the transfection control, β-galactosidase and expressed relative to the luciferase activities of the control vector in each line (Figure 7). The human Apaf-1 IRES is most active in HeLa and HepG2 cells, and is not used in SY5Y neuronal cells or Balb/c mouse cells suggesting that these cell types lack non-canonical protein factors that are required for internal ribosome entry via the Apaf-1 IRES.

The dicistronic vector pRMf, which contains the murine version of the Apaf 5' UTR was also tested in the rodent cell lines Balb/c and CHO-T. Interestingly, the murine version of Apaf-1 was able to direct IRES activity in the CHO-T cells (eight times more firefly luciferase activity than the control vector) and again not in the Balb/c cells (Figure 7).

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**Figure 5 Northern blot analysis of Apaf-1.** Poly(A)' mRNAs derived from cells alone (lane 1), pGL3 (lane 2), pGAL (lane 3), pRF (lane 4) and pRAF (lane 5) were electrophoresed in the presence of formaldehyde, transferred to nitrocellulose and then probed with radiolabelled single stranded DNA specific for luciferase.

**Figure 6 Deletion mapping of the Apaf-1 5' UTR.** (a) Deletions of the Apaf-1 5' UTR were created by restriction enzyme digestion and introduced into the dicistronic vector pRF. (b) Dicistronic vectors containing deletions of the Apaf-1 5' UTR were transfected into HeLa cells and assayed for luciferase activity. The values obtained were then expressed relative to the full length Apaf-1 5' UTR.
Apaf-1 translation is mediated by an IRES

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Discussion

Apaf-1 5' UTR contains an IRES

Our data show that translation initiation of Apaf-1 occurs by internal ribosome entry and this provides evidence for an IRES in an mRNA whose protein product is associated with the initiator of a caspase cascade during apoptosis. Experiments performed with monocistronic plasmid constructs that contain a stable hairpin imply that initiation of translation of Apaf-1 mRNA may only occur by internal ribosome entry. Moreover, the reduction in luciferase activity obtained when the Apaf-1 5' UTR was placed in front of the luciferase reporter vector suggests that internal ribosome entry initiated by the Apaf-1 IRES is approximately 70% less efficient than the scanning mechanism of translation. In this way, the Apaf-1 IRES is active, although to differing degrees in all human cell types tested, with the exception of the neuronal line SY5Y. The differences in Apaf-1 IRES-mediated initiation of translation between the cell lines examined may be due to the levels of expression of proteins that are required for internal ribosome entry in these cells. Interestingly, the IRES is most active in cell lines of tumour origin. Most of the ability of the Apaf-1 5' UTR to initiate internal ribosome entry resides in a 233 nt fragment and although this is a rather small IRES when compared to those of viral origin, it is a size that is consistent with those that have been observed in eukaryotic mRNAs e.g. the Bip and FGF-2 IRESs are 220 and 165 nucleotides in length respectively (Yang and Sarnow, 1997; Vagner et al., 1995).

Function of the Apaf-1 IRES

A reduction in the cap-dependent scanning mechanism of translation can be induced by a variety of cellular conditions. A reduction in translation occurs (i) during mitosis due to de-phosphorylation of eIF4E (Bonneau and Sonenberg, 1987); (ii) following cellular stress such as heat shock, osmotic shock and hypoxia which all cause a shut down of scanning by modulation of the activities of the eIF4F complex and/or eIF-2 (Panniers, 1994; Rhoads and Lamphear, 1995); (iii) during picornaviral infection due to cleavage of eIF4G by viral proteases, providing a rationale for the preferential translation of viral mRNAs due to their mechanism of internal ribosome entry (Lamphear et al., 1995; Ohlmann et al., 1996; Pestova et al., 1996) and (iv) during apoptosis where eIF4G is also cleaved, but in this case by caspase-3 (Clemens et al., 1998; Marissen and Lloyd, 1998; Morley et al., 1998). Some of the eukaryotic IRESs identified thus far are active during these situations, hence the VEGF IRES is active during hypoxia (Stein et al., 1998). We have recently shown that the c-myc-IRES is utilized during apoptosis (Stoneley et al., 2000 in press) and it has been shown that many eukaryotic IRESs are functional following Picornaviral infection (Johannes and Sarnow, 1998). Our data would suggest that the Apaf-1 IRES is used continuously, and we propose that internal ribosome entry can be used as a default pathway for protein synthesis initiation. This would also imply that it is important for a cell to be able to maintain a constant...
cellular level of Apaf-1 which is not all that surprising given the central role that Apaf-1 plays in apoptosis. It has recently been shown that X-linked inhibitor of apoptosis (XIAP) contains an IRES and the data suggest that initiation of translation of this mRNA also only occurs by internal ribosome entry (Holcik et al., 1999).

In summary we have shown that Apaf-1 5' UTR contains an IRES and our data demonstrate that translation initiation of Apaf-1 occurs primarily by this mechanism.

Materials and methods

Materials

Media and serum were purchased from Gibco BRL. Luciferase assay kits 'Stop & Glo' and rabbit reticulocyte lysates were purchased from Promega. Galactolight plus lysate and up to 50 ng of RNA as recommended in the manufacturer's instructions.

Cell culture

Cell lines were grown at 37°C in Dulbecco's modified Eagle's Medium supplemented with 10% foetal calf serum (FCS), in a humidified atmosphere containing 5% CO2. SY5Y were purchased from the American Type culture collection. FuGene6 was purchased from Roche Molecular Biochemicals. All other chemicals were purchased from Sigma (Poole, UK).

DNA transfections

Calcium phosphate-mediated DNA transfection of HeLa, HepG2 and Cos-7 cells was performed essentially as described by Jordan et al. (1996). All other cell lines were transfected using FuGene6 according to manufacturer's protocols.

In vitro run-off transcription and in vitro translation

Vector DNA was linearized by restriction digestion using a site downstream of the sequence of interest. Capped transcripts were synthesized in a reaction containing 1 x Transcription buffer (80 mM Hepes-KOH, pH 7.5, 24 mM MgCl2, 2 mM spermidine, 40 mM DTT), 7 mM KOH, 40 units of RNasin, 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, 2 mM mG(5')ppp(5')G, 1 ng of DNA template and 20 units of T7 RNA polymerase in a final volume of 50 µl. After incubation of the reaction for 1 h at 37°C, the RNA was isolated and used to prime a 12.5 µl in vitro translation reaction containing 8.25 µl of Promega rabbit reticulocyte lysate and up to 50 ng of RNA as recommended in the manufacturer's instructions.

Reporter gene analysis

The activity of firefly luciferase in lysates prepared from cells transfected with pGL3 or pGAL was measured using a luciferase reporter assay system (Promega). Light emission was measured over 10 s using an Optocomp-1 Luminometer (MGM instruments). The activity of both firefly and Renilla luciferase in cell lysates transfected with dicistronic luciferase plasmids was measured using the Dual-luciferase reporter assay system (Promega). Assays were performed according to the manufacturer's recommendations except that only 25 µl of each reagent was used. Light emission was measured in the same manner as described previously.

The activity of β-galactosidase in lysates prepared from cells transfected with pcDNA3.1/HisB/lacZ was measured using a Galactolight plus assay system (Tropix). Enzyme activity was then determined by measuring the light emission from the reaction in a luminometer, as previously described.

Northern blot analysis

Total cellular RNA and Poly(A)+ selected mRNA was prepared and analysed by Northern blotting exactly as described previously (West et al., 1995). DNA probes used for the detection of luciferase mRNA species were also as described (Stoneley et al., 1998).

Abbreviations

IRES, internal ribosome entry segment; UTR, untranslated region; Apaf-1, Apoptotic protease activating factor-1; eIF, eukaryotic initiation factor.

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Apaf-1 translation is mediated by an IRES

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Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment

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ABSTRACT

The 5' UTR of c-myc mRNA contains an internal ribosome entry segment (IRES) and consequently, c-myc mRNAs can be translated by the alternative mechanism of internal ribosome entry. However, there is also some evidence suggesting that c-myc mRNA translation can occur via the conventional cap-dependent scanning mechanism. Using both bicistronic and monocistronic mRNAs containing the c-myc 5' UTR, we demonstrate that both mechanisms can contribute to c-myc protein synthesis. A wide range of cell types are capable of initiating translation of c-myc by internal ribosome entry, albeit with different efficiencies. Moreover, our data suggest that the spectrum of efficiencies observed in these cell types is likely to be due to variation in the cellular concentration of non-canonical translation factors. Interestingly, the c-myc IRES is 7-fold more active than the human rhinovirus 2 (HRV2) IRES and 5-fold more active than the encephalomyocarditis virus (EMCV) IRES. However, the protein requirements for the c-myc IRES must differ significantly from these viral IRESs, since an unidentified nuclear event appears to be a pre-requisite for efficient c-myc IRES-driven initiation.

INTRODUCTION

The proto-oncogene c-myc is required for both cell proliferation and programmed cell death (apoptosis), and de-regulated c-myc expression is associated with a wide range of cancers (1,2). It is therefore not surprising that c-myc gene expression is tightly controlled at multiple levels (3). The post-transcriptional regulation of c-myc involves alterations in the stability of both the mRNA and the protein (4–7), and the control of c-myc translation (8–12).

In common with many other genes involved in the regulation of cell growth, the c-myc mRNA has a long and potentially highly structured 5' untranslated region (UTR, located in exon 1). Multiple transcription start sites exist within the gene, giving rise to four transcripts (P0, P1, P2 and P3, with sizes of ~3.1, 2.4, 2.25 and 2.0 kb respectively; 13–15), with the predominant mRNA (P2) having a 5' UTR of ~400 nt. It has been suggested that mRNAs with structured 5' UTRs, such as c-myc, are poorly translated due to their reduced ability to associate with the cap-binding complex, the eukaryotic initiation factor 4F (eIF4F). Indeed, over-expression of the cap-binding protein eIF4E, which is believed to be a limiting component of this complex, causes an increase in the translation of mRNAs with structured 5' UTRs such as c-myc (16–18). Furthermore, in certain circumstances the translational regulation of c-myc is mediated by phosphorylation and inactivation of the eIF4E inhibitor protein 4EBP1 (19).

It has also been shown that the 5' UTR of c-myc contains an internal ribosome entry segment (IRES) (11,12). IRESs were originally identified in the 5' UTRs of picornaviral RNAs and these complex structural elements allow ribosomes to enter at a considerable distance (often >1000 nt) from the 5' end of the mRNA (20–22). Several eukaryotic mRNAs have the potential to initiate translation by an internal ribosome entry mechanism and interestingly many of the mammalian IRESs identified to date have been found in genes whose protein products are associated with the control of cell growth, e.g. c-myc, fibroblast growth factor –2 (FGF-2), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (11,12,23–26).

The region of c-myc mRNA that contains the IRES is located downstream of the P2 promoter (12). Approximately 75–90% of c-myc transcripts are synthesised from this promoter (3). Therefore, the majority of c-myc mRNAs have the potential to initiate translation via internal ribosome entry. The c-myc IRES appears to function under conditions where cap-dependent translation is compromised. Indeed, we have recently shown that the c-myc IRES is utilised during apoptosis when cap-dependent translation is reduced due to cleavage of eIF4G (27). Furthermore, in poliovirus-infected HeLa cells, in which there is a substantial reduction in cap-dependent protein synthesis due to the proteolysis of eIF4G and sequestration of eIF4E, c-myc mRNAs remain associated with heavy polysomes (28). However, since there is some evidence that c-myc mRNA can also be translated by a cap-dependent mechanism, to date it has not been possible to assess the contribution that either mechanism makes to the synthesis of c-Myc polypeptides (12,19).

In this study we present further evidence for the existence of an IRES in the c-myc 5' UTR. In addition our data confirm that...
c-myc mRNAs can also be translated by a cap-dependent mechanism. This has led us to propose that both mechanisms operate in vivo. We demonstrate that the c-myc IRES is active (with one exception) in all cell lines of human origin tested, although there is a wide variation in its efficiency, whereas the IRES is not active in cell lines of murine origin. When compared to IRESs of picomaviral origin, the c-myc IRES is 7- and 5-fold more active than the IRESs derived from HRV and EMCV, respectively. Finally we provide evidence that the c-myc IRES depends on a prior nuclear event for efficient initiation of translation.

**MATERIALS AND METHODS**

**Cell culture**

All cell lines were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, in a humidified atmosphere containing 5% CO₂. The cell lines HeLa (Human cervical epithelial carcinoma), HepG2 (Human hepatocyte carcinoma), HK293 (Human embryonic kidney cell line immortalised with adenovirus DNA), Balb/c-3T3 (Murine embryonic fibroblast cell line), MCF7 (Human breast carcinoma), Cos-7 [Monkey epithelial cell line (CV-1) immortalised with SV 40 DNA] and MEL cells (murine erythroleukaemic cells) were purchased from the American type culture collection. The human SV 40 immortalised fibroblast cell line immortalised fibroblast cell line GM637 was obtained from NIGMS.

**Plasmid constructs**

The plasmids pGL3, pGML (formerly pGL3utr), pRF and pRMF (formerly pGL3r and pGL3Rut) have been described previously (12). cDNA encoding the HRV2 IRES was obtained from the plasmid pXLJ(10-605) (a gift from Dr R. Jackson, University of Cambridge) and inserted into pRF between the 

**In vitro run-off transcription and in vitro translation reactions**

Plasmid constructs were linearised and in vitro transcription reactions were performed using either SP6 (pSP6R(x)L series) or T3 (pSKMyr and pSKMyrΔ1) polymerase as previously described. Capped transcripts were synthesised in a reaction containing 2 mM m<sup>5</sup>ppp(5')G, 0.5 mM GTP and 1 mM of the remaining nucleotides. All RNAs were purified using size exclusion chromatography and quantified using the absorbance at 260 nm. In addition, the integrity of each transcript was verified using agarose gel electrophoresis and ethidium bromide staining.

In vitro translation reactions were performed using rabbit reticulocyte lysate (Promega) according to the manufacturer’s recommendations. The translation products were fractionated by SDS–polyacrylamide gel electrophoresis and visualised using phosphorimager analysis (Molecular Dynamics).

**Cationic liposome-mediated RNA transfection**

Cationic liposome-mediated RNA transfection of mammalian cells was performed as described previously (30). Capped and polyadenylated transcripts were synthesised using in vitro run-off transcription on an EcoRI linearised pSP64R(x)L poly(A) template. Approximately 2 × 10<sup>5</sup> HeLa cells were transfected with 5 μg of RNA previously incubated with 12.5 μg of Lipofectin (Life Technologies Inc.). After 8 h of transfection, cells were harvested and processed for reporter gene analysis.
Reporter gene analysis

The activity of Firefly luciferase in lysates prepared from cells transfected with pGL3, pGML, pHpML and pHpL was measured using a luciferase reporter assay system (Promega). Light emission was measured either over 1 s using a 1253 luminometer (Bio-Orbit) or over 10 s using an Optocomp-1 Luminometer (MGM instruments). The activity of both Firefly and Renilla luciferase in cell lysates with bicistronic luciferase plasmids was measured using the Dual-luciferase reporter assay system (Promega). Assays were performed according to the manufacturer’s recommendations. The activity of β-galactosidase in lysates prepared from cells transfected with pcDNA3.1/HisB/lacZ was measured using a Galactolight plus assay system (Tropix).

RESULTS

c-myc translation initiation can occur by internal ribosome entry and the conventional cap-dependent mechanism

We and others have shown that c-Myc protein synthesis can occur in a cap-dependent manner and by internal ribosome entry (11,12,17,19). To assess the contribution that these two disparate mechanisms make to c-myc expression, a palindromic sequence capable of forming a stable RNA hairpin (−55 kcal/mol) was introduced into the control luciferase reporter construct, (pGL3) and the 5' UTR containing construct (pGML, previously known as pGL3utr) at the SpeI site (Fig. 1A). As a consequence, ribosome scanning from the cap structure of the transcripts produced by the new constructs (pHPL and pHpML) should be severely impeded, whereas ribosomes entering at a site distal to the hairpin will be unaffected. HeLa cells were transfected with pGL3, pGML, pHPL or pHpML and in agreement with our previously published data, the c-myc IRES does not inhibit translation of the downstream Firefly luciferase reporter gene. Moreover, we consistently observe that there is a slight elevation in expression of this enzyme in the presence of the IRES (Fig. 1B). In cells transfected with the construct pHPL there is a 200-fold reduction in the amount of luciferase produced when compared to the control vector pGL3 (Fig. 1B). Hence, as expected the RNA hairpin structure inhibits cap-dependent translation initiation. However, in cells transfected with pHpML, in which the c-myc IRES lies downstream of the RNA hairpin, luciferase expression is stimulated by ~67-fold when compared to pHPL. These data demonstrate that the 5' UTR can promote efficient translation initiation despite the presence of an RNA structure which blocks ribosome scanning from the 5' end and thus provide further support for the presence of an IRES within this leader sequence. Nevertheless, it is notable that the RNA hairpin does reduce luciferase expression from a transcript containing the c-myc 5' UTR by 3-fold. This observation would indicate that mRNAs originating from the P2 promoter must also support a cap-dependent scanning mechanism in addition to internal initiation.

Comparison of c-myc IRES-mediated internal initiation in a range of cell types

We have shown previously that the c-myc IRES is capable of promoting translation of the downstream cistron on a bicistronic mRNA in both HeLa and HepG2 cells. To investigate how widely the IRES is utilised, a range of cell types derived from different tissues, including Cos-7, MCF7, Balb/c-3T3, MEL, MRC5, HK293, GM637, HeLa and HepG2 were co-transfected with either pRF or pRMF and pcDNA3.1/HisB/lacZ (Fig. 2A). The expression from both Renilla and Firefly luciferase cistrons was assayed and normalised to the transfection control β-galactosidase. All experiments were performed on three independent occasions.

Figure 1. A comparison between the efficiency of IRES-mediated translation and scanning. (A) A diagrammatic representation of the monocistronic hairpin containing plasmids pHPL and pHpML. The hairpin was inserted into the SpeI site upstream of the PvuII site. (B) HeLa cells were transfected (in triplicate) with the plasmids shown and Firefly luciferase activity is expressed relative to the transfection control β-galactosidase. All experiments were performed on three independent occasions.
and MEL cells, is that the function of the IRES displays species specificity. However, we have recently shown that this is not the case, since the c-myc IRES isolated from murine cells is active in HeLa cells and yet also relatively inactive in Balb/c-3T3 cells (data not shown).

**c-myc P2 transcripts can be translated by a cap-dependent mechanism in Balb/c-3T3 cells, MCF-7 cells and in reticulocyte lysates**

The relative inactivity of the c-myc IRES in Balb/c-3T3 and MCF7 cells enabled us to analyse the effect of the P2 5' UTR on cap-dependent translation initiation. To this end, these cell lines were transfected with the monocistronic control construct, the 5' UTR-containing constructs, pGL3 and pGML, and the c-myc 5' UTR construct containing the hairpin pHpML respectively. The P2 5' UTR does not inhibit cap-dependent translation initiation, at least in these cell lines (Fig. 3A). However, the additional presence of the hairpin structure was sufficient to prevent scanning demonstrating that the c-myc IRES is relatively inactive in these cell types and consequently c-myc is translated by a cap-dependent mechanism (Fig. 3A).

To further investigate the impact of the P2 5' UTR on cap-dependent translation initiation we turned to reticulocyte lysate. This system cannot support internal ribosome entry on the c-myc leader sequence (our unpublished data; 31), therefore the contribution of the 5' cap structure can be assessed directly. Rabbit reticulocyte lysate was primed with capped or uncapped c-myc transcripts, either bearing the P2 5' UTR sequence (myc) or lacking this element (mycAl) (Fig. 3B). Two species of c-myc protein can arise from the P2 transcripts by use of alternate translation initiation codon (CUG or AUG), which give rise to protein products with apparent molecular weights of 67 and 64 kDa respectively (32). As expected, capping the mycAl RNA stimulated the synthesis of both Myc-1 and 2 polypeptides (Fig. 3B, lanes 1 and 2). This modest effect of 2–2.5-fold is consistent with the previously reported values for relatively unstructured RNAs using this system (33). In the absence of a cap structure, the c-myc 5' UTR reduced the synthesis of both the AUG and CUG-initiated polypeptides by ~90% (Fig. 3B, lanes 3 and 4). It is likely that structural elements within the 5' UTR are responsible for this effect since this element is GC-rich. However, the synthesis of both proteins was enhanced by 14–16-fold on capping of the myc transcript (Fig. 3B, lanes 3 and 4), with the result that the
5' UTR inhibits translation initiation by only 50%. Hence, the P2 5' UTR strongly attenuates the translational efficiency of uncapped c-myc transcripts. Nevertheless, much of this repression is relieved by the presence of a 5' cap. Therefore, translation initiation on the P2 transcript is strongly cap-dependent in the reticulocyte lysate system.

Overexpression of bicistronic mRNAs inhibits the function of the c-myc IRES

Thus far, we have demonstrated that in many cell lines c-myc translation can occur by the alternative mechanism of internal ribosome entry. However, c-myc can also be translated by the conventional cap-dependent mechanism in certain backgrounds. One model that would explain the cell-type specific variation in the efficiency of c-myc IRES-driven translation posits that non-canonical trans-acting factors are required for the recruitment of the 40S ribosome to this element. In this scenario, the activity of one or more of these factors is considerably reduced in the Balb/c-3T3 and MCF7 cell lines. Further evidence in support of this model was provided by experiments in which the bicistronic mRNAs were overexpressed using the powerful cytomegalovirus (CMV) promoter/enhancer region; this transcriptional element has been shown to result in significantly higher levels of expression than the SV40 promoter/enhancer (34). The Renilla luciferase activity measured in cells transfected with a CMV-based control bicistronic plasmid pCRF was significantly greater than that achieved with the analogous plasmid, pRF (-27-fold, Fig. 4A, compare pCRF Renilla luciferase to pRF Renilla luciferase). However, in cells transfected with the 5' UTR-containing construct, pCRMF, there was not a corresponding increase in Firefly luciferase activity when compared to pRMF. Transfection with 4 or 8 μg of pCRMF produced only 4- or 1.25-fold more Firefly luciferase than pRMF, respectively (Fig. 4B). Consequently, using the CMV promoter/enhancer, the apparent activity of the c-myc IRES when calculated relative to readthrough is only 1.5-2-fold compared to 50-fold for the SV40 based constructs (Fig. 4B). These data suggest that a trans-acting factor, which is required for initiation of translation via the c-myc IRES, is present at a limiting concentration. A similar observation has been reported for the entero- and rhinovirus IRESs; the efficiency of translation mediated by these IRESs was considerably reduced when bicistronic mRNAs were expressed at high levels in vivo (35). This phenomenon correlates with a requirement for non-canonical factors, since it was not observed for either cap-dependent translation or translation driven by the cardio- and aphthovirus IRESs (35).

A comparison of the efficiency of the c-myc and viral IRESs

The previous data provided indirect evidence that the function of the c-myc IRES could depend on a non-canonical trans-acting factor. In this respect, it would be analogous to the IRESs of the entero- and rhinoviruses (36). To compare the efficiency of the c-myc, HRV and EMCV IRESs, HeLa cells were transfected with the plasmids pRF, pRMF, pHRVF, pRemcvF. The activities of Renilla and Firefly luciferase were determined and normalised to that of the transfection control, β-galactosidase (Fig. 5). Expression of the upstream cistron, Renilla luciferase, was not greatly affected by the presence of the EMCV, HRV or the c-myc IRES in the intercistronic region (data not shown). A comparison of the downstream cistron activities revealed that all of these elements stimulated Firefly luciferase expression (Fig. 5). However, the extent to which expression from the downstream cistron was enhanced differed widely between these IRESs. In fact, the c-myc IRES elevated Firefly luciferase activity by 70.8-fold, whilst the HRV and EMCV IRESs caused a lesser stimulation of 9.6- and 14-fold, respectively. Thus, these data suggest that both of these IRESs are less efficient in this system at promoting internal ribosome entry than the c-myc IRES.

c-myc IRES-driven translation requires a nuclear event

It has been suggested previously that efficient translation driven by the IRES located in the 5' UTR of the immunoglobulin heavy chain binding protein (Bip) requires a nuclear event (37). Moreover, two specific nuclear protein factors have been identified which interact with the Bip IRES (38). To test whether the c-myc IRES also has such a requirement for
Figure 5. A comparison of the efficiency of HRV, EMCV and c-myc IRES-initiated internal ribosome entry on bicistronic mRNAs transcribed in the nucleus. HeLa cells were transfected in triplicate with either the control plasmid pRF, the c-myc IRES containing plasmid pRFM, the HRV IRES containing plasmid pHRV or the EMCV IRES containing plasmid pEMCV. Upstream cistron (Renilla luciferase) and downstream cistron (Firefly luciferase) activities were determined and normalised to that of the transfection control.

nuclear factors, the plasmid constructs pSP64RL poly(A), pSP64R(c-myc)L poly(A) and pSP64R(hrv)L poly(A) were generated. Bicistronic transcripts containing an m7GpppG cap structure and a polyadenylated tail at the 5' and 3' termini, respectively, were synthesised from each of the plasmids in the pSP64RL(x)Lpoly(A) series by in vitro run-off transcription (Fig. 6A). Cationic liposomes were used to encapsulate equimolar quantities of each transcript and introduce them into the cytoplasm of HeLa cells. After a period of 8 h, the expression from the upstream and downstream cistrons was monitored (Fig. 6B and C). In cells transfected with the control bicistronic transcript, Rluc, the Renilla luciferase cistron was translated efficiently, whilst little expression of the downstream cistron was observed (Fig. 6B and C). Insertion of the HRV IRES between the two cistrons resulted in a 52-fold stimulation of Firefly luciferase activity when compared to the expression due to readthrough-re-initiation (Fig. 6C). In contrast, the expression of the downstream cistron was only enhanced by 1.4-fold on the Rc-mycL transcript (Fig. 6B). Thus, the c-myc IRES cannot stimulate the translation of the downstream cistron on a bicistronic mRNA introduced directly into the cytoplasm. To confirm these data, the plasmids pRF and pRMF were transfected into human TK143 cells previously infected with a recombinant vaccinia virus that expresses the T7 RNA polymerase (vTF7-3) (39). The presence of a T7 RNA polymerase promoter upstream of the Renilla luciferase cistron in pRF and pRMF resulted in the transcription of bicistronic mRNAs in the cytoplasmic compartment. However, the c-myc 5' UTR did not promote internal initiation on mRNAs transcribed in the cytoplasm using the T7/vaccinia system (data not shown). In contrast, the IRESs of the enterovirus and rhinoviruses have been shown to function efficiently using bicistronic mRNAs expressed in this manner (35,40). These data appear to suggest a fundamental difference between the function of the enterovirus and rhinovirus IRESs and that of c-myc. The c-myc IRES is only able to promote internal initiation on transcripts expressed in the nucleus, however the HRV element is capable of performing this task on mRNAs that do not originate in this compartment. Therefore, we propose that a nuclear event is a pre-requisite for efficient c-myc internal initiation.

DISCUSSION

We and others have shown previously that the 5' UTR of c-myc contains an IRES (11,12). We have investigated several features of the c-myc IRES and compared its activity in a range of cell lines and to IRESs of viral origin.

First, using a stable RNA structure to substantially impede ribosome scanning from the 5' cap, we have demonstrated that efficient translation initiation can be restored by positioning the c-myc UTR downstream of this inhibitory element (Fig. 1). This observation provides further evidence that the P2 leader sequence can support internal entry of ribosomes via an IRES. In these experiments, internal initiation directed by the
c-myc IRES is apparently 3-fold less efficient than cap-dependent translation initiation (but see later). However, reporter mRNAs are translated with comparable efficiency whether the 5' UTR is present or not. Thus, we suggest that c-myc mRNAs originating from the P2 promoter are capable of being translated via a cap-dependent mechanism in addition to internal initiation. This hypothesis is strengthened by two observations. First, a reporter mRNA bearing the P2 leader sequence was translated efficiently in cell lines with a significantly reduced capacity to promote 5' UTR-mediated internal initiation (Fig. 3A).

Second, in reticulocyte lysate, a system in which the c-myc IRES is inactive (our unpublished data; 31), c-myc P2 transcripts are translated in a manner that is strongly dependent on the presence of a cap structure (Fig. 3B). In agreement with these data, Carter et al. (31) have recently shown that the considerable repression of translation initiation caused by the P1 5' UTR in rabbit reticulocyte lysate can be relieved by the addition of eIF4F/E (31). Thus, we propose a dual mechanism for c-myc translation initiation. Under conditions where cap-dependent protein synthesis is compromised there is a shift from a cap-dependent to an IRES-directed mechanism of translation initiation. In accord with this hypothesis, we have recently shown that c-myc protein synthesis is maintained during apoptosis by virtue of the IRES, whereas overall cap-dependent translation is significantly inhibited (27).

We have also identified several factors that influence the efficacy of the c-myc IRES. Expression of bicistronic mRNAs containing the c-myc IRES in a panel of cell lines demonstrated that the activity of this element is critically dependent on cellular origin (Fig. 2). Although the IRES stimulated protein synthesis from the downstream cistron in all the cell lines tested, there was a 20-fold disparity between HeLa and MCF7 cells, the lines in which the IRES is most and least active, respectively. This cell-type specific variation in IRES activity implies that the function of this element could be modulated by non-canonical trans-acting factors. In this regard, we have recently demonstrated that ribonuclear protein complexes assembled on the c-myc 5' UTR in vitro using cell extracts from different cell lines vary distinctly in composition (41). Furthermore, overexpression of bicistronic mRNAs using the powerful CMV promoter/enhancer drastically reduced the apparent efficiency of the c-myc IRES (Fig. 4). We speculate that the concentration of a trans-acting factor essential for c-myc IRES-driven translation initiation is limiting under these conditions. The low concentration of this factor could also explain why c-myc internal initiation appears to be 3-fold less efficient than cap-dependent translation (Fig. 1) since transcripts expressed from the monocistronic constructs (pGL3, pGML, pHpL and pHpML) accumulate to a level approximately an order of magnitude higher than those produced from the bicistronic constructs (pRF and pRMF) (our unpublished observations). Significantly, the characteristics described above are not unique to the c-myc IRES. Both cell-type specific variations in IRES activity and saturation of IRES function have also been described for the better defined IRESs of the enteroviruses (35,40). The activity of these elements is known to be dependent on host-specific trans-acting factors suggesting that the c-myc IRES has similar requirements.

A comparison of the c-myc IRES to those of the human rhinovirus (HRV) and encephalomyocarditis virus (EMCV), using bicistronic mRNAs expressed in the nucleus, revealed that it is 7- and 5-fold more active, respectively (Fig. 5). However, the c-myc IRES differs markedly from those of viral origin, in that it is almost completely inactive when present in bicistronic mRNAs introduced directly into the cytoplasmic compartment (Fig. 6 and data not shown). Furthermore, it has also been observed that in contrast to the poliovirus IRES, the c-myc 5' UTR could not promote internal initiation in HeLa cell extracts (42). Taken together, these data strongly suggest that a nuclear experience is an essential pre-requisite for internal initiation mediated by the c-myc IRES. The nature of this nuclear event is currently unknown. However, it is interesting to note that several nuclear factors have been shown to interact with the Bip IRES, the function of which is also dependent on a nuclear origin (37,38). Thus, factors recruited to these IRESs in the nucleus could subsequently promote internal initiation in the cytoplasm (37).

Carter et al. have recently suggested that the c-myc 5' UTR does not contain an IRES (31). However, these experiments were performed in reticulocyte lysate, a specialised translation extract known to contain very limiting amounts of nuclear and cytoplasmic RNA binding proteins (33). We have also found that the c-myc IRES cannot function in reticulocyte lysate (data not shown). In this respect it is similar to the IRESs of the entero- and rhinoviruses, which function inefficiently or not at all in this system. Indeed, reticulocyte lysate must be supplemented with cytoplasmic extracts to support efficient enterovirus internal initiation (36). Most importantly, to our knowledge no eukaryotic cellular IRES has been shown to promote internal initiation in this system. Using bicistronic mRNAs expressed in the nucleus of cell lines, we and others identified an IRES in the c-myc 5' UTR (11,12). This finding has been supported by the observation that c-myc mRNAs are efficiently translated in poliovirus-infected HeLa cells and in cells undergoing apoptosis (27,28). Here we present further evidence that c-myc mRNAs can be translated by internal initiation and we provide additional mechanistic insights. Our data support a model in which both non-canonical trans-acting factors and a nuclear experience participate in c-myc internal ribosome entry. In the light of these results, it is hardly surprising that the c-myc IRES does not function in the reticulocyte lysate system. Finally, we are currently attempting to identify the cytoplasmic and nuclear factors involved in the formation of ribonuclear protein complexes with the c-myc 5' UTR. The effect of these factors on c-myc internal initiation can then be rigorously tested in cell-free extracts.

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