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Abstract

Structural, functional and mechanistic analysis of the Bag-1 internal ribosome entry site

Bag-1 is an anti-apoptotic protein involved in the regulation of a number of cellular processes, notably as a co-chaperone for the 70kDa heat shock proteins. At least four protein products of Bag-1 have been isolated, p50, p46, p36 and a minor isoform, p29. The 5' UTR of the p36 isoform of Bag-1 has been shown to contain an internal ribosome entry segment (IRES). The internal ribosome entry mediated mechanism of translation has been shown to maintain Bag-1 expression when cap-dependent translation is compromised during heat shock.

Many IRESes require *trans*-acting protein factors for optimal IRES activity. Bag-1 IRES activity is cell-type specific and is inefficient in cell lines with low endogenous levels of the *trans*-acting factors poly (rC) binding protein 1 (PCBP1) and polypyrimidine tract binding protein 1 (PTB1). Activity of the Bag-1 IRES can be stimulated *in vitro* and *in vivo* by overexpression of PTB and PCBP1.

PTB and PCBP1 bind specifically to the minimal active Bag-1 IRES element. A secondary structural model of the minimal Bag-1 IRES was obtained by chemical and enzymatic probing of IRES RNA *in vitro*. Addition of PTB and PCBP1 modulates the secondary structure of the Bag-1 IRES in the ribosome-landing region.

Overexpression of Bag-1 proteins in cells subjected to genotoxic stress has been shown to protect cells from stress-induced growth inhibition and cell death. The Bag-1 IRES is functional in heat-shocked cells and cells treated with chemotherapeutic agents and this correlates with a redistribution of PTB and PCBP1 from the nucleus of the cell to the cytoplasm. A model for the mechanism of action of the Bag-1 IRES and the influence of PTB and PCBP1 is proposed.

Acknowledgements

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Abbreviations

4E-BP	eIF4E-binding protein
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
CIAP	calf intestinal alkaline phosphatase
Cpm	counts per minute
CTP	cytidine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DAP5	death-associated protein 5
dGTP	deoxyguanosine 5'-triphosphate
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DMS	Dimethyl sulfate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide
DTT	dithiothreitol

dTTP	deoxythymidine 5'-triphosphate
E.coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EGF	epidermal growth factor
eIF	eukaryotic initiation factor
EMCV	encephalomyocarditis virus
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FCS	fetal calf serum
FGF-2	fibroblast growth factor
FMDV	foot-and-mouth disease virus
GDP	guanosine diphosphate
GST	glutathione-S-transferase
GR	glucocorticoid receptor
GTP	guanosine 5'-triphosphate
GTPase	guanidine triphosphatase
Hap46	hsc70/hsp70 associating protein
HAV	hepatitis A virus
HCV	Hepatitis C virus
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HGF	hepatocyte growth factor
HnRNP	heteronuclear ribonuclearprotein
Hip	hsp70-interacting protein
Hop	hsp70/hsp90-organising protein
HRV	human rhinovirus

Hsc	heat shock cognate
Hsp	heat shock protein
IGF-II	insulin-like growth factor II
IRES	internal ribosome entry segment/site
ITAF	IRES-specific cellular *trans*-acting factor
Kb	kilobases
Kcal	kilocalorie
KDa	kilodalton
KH	hnRNPK homology domain
LB	Luria-Bertani broth
MAPK	Mitogen-activated protein kinase
MCS	multiple cloning site
Met	methionine
Met-tRNA$_i$	initiator methionyl tRNA
Mnk1	MAP kinase interacting kinase 1/ MAP kinase signal integrating kinase 1
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
nt	nucleotide
ODC	orthinine decarboxylase
ORF	open reading frame
PABP	poly (A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCBP1/2	poly(rC) binding protein 1/2
PCR	polymerase chain reaction

PDGF2	platelet derived growth factor 2
PKA	
PKR	RNA-activated protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PTB	polypyrimidine tract binding protein
PV	poliovirus
RAP46	receptor associated protein
RNA	ribonucleic acid
RNase	ribonucleic acid hydrolyase
RNasin	ribonucleic acid hydrolyase inhibitor
RRL	rabbit reticulocyte lysate
RRM	RNA recognition domain
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
tRNA	transfer ribonucleic acid
uORF	upstream open reading frame
UTR	untranslated region
UTP	uridine 5'-triphosphate

UV	ultraviolet
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

Chapter 1

Introduction

1.1. Eukaryotic protein synthesis

Eukaryotic protein synthesis or translation is a three-step process comprising initiation, elongation and termination. The initiation phase describes the formation of an 80S ribosome at the appropriate translational start site. During elongation, tRNAs charged with amino acids recognize the protein sequence by codon: anticodon recognition and peptide bond formation occurs to add the appropriate amino acid to the growing peptide chain. This is followed by translocation of the ribosome along the mRNA to the next codon where the process is repeated. The termination phase occurs when a termination codon is reached, there is no tRNA complex to recognize a termination codon and consequently the eukaryotic release factor eRF1 binds and hydrolyses the peptidyl-tRNA to release the complete polypeptide.

1.2. Initiation of protein synthesis

Initiation is believed to be the rate-limiting step and as such is subject to a great deal of regulation (Pain, 1996). Regulation at the level of translation initiation is very important in generating a more rapid response to stimuli in the cell than regulation at the transcriptional level, and also allows specific genes or more general subsets of genes to be targeted for regulation. Two major mechanisms for initiation of eukaryotic protein synthesis have been described, cap-dependent initiation and cap-independent or internal initiation.

1.3. Cap-dependent translation initiation

The Kozak model of cap-dependent scanning is used to explain conventional translation initiation in eukaryotes (Kozak, 1987). This mechanism requires recognition of the 5' cap structure, a methylated guanine and is globally dependent upon a number of eukaryotic initiation factors (eIFs). The main functions of the initiation factors required are summarized in **table 1.1**.

1.3.1. Assembly of the 43S pre-initiation complex

After a round of translation has taken place, the two ribosomal subunits, 40S and 60S are maintained in a dissociated state. The 40S subunit is bound by eIF1A and eIF3 and the 60S subunit by eIF6 (**Figure 1.1A**). This prevents the subunits from interacting and forming a complete 80S ribosome before they have been assembled at the appropriate initiation site.

The first event in a new round of protein synthesis is the formation of the 43S pre-initiation complex, which comprises the 40S small ribosomal subunit, the initiator Met-tRNA$_i$, energy in the form of GTP and the initiation factors eIF2, eIF3 and eIF1A. EIF2 can only bind the Met-tRNA$_i$ in its GTP bound form. During release of eIF2 from the initiation complex, the GTP bound to it is hydrolysed to GDP. In order for eIF2 to participate in a new round of protein synthesis, it must be bound by a guanine nucleotide exchange factor, eIF2B, which recycles eIF2•GDP to eIF2•GTP (**Figure 1.1B**).

The availability of eIF2 for translation initiation is regulated by phosphorylation. Upon cellular stress, a number of kinases including PKR and PERK phosphorylate eIF2. Phosphorylated eIF2 binds tightly to eIF2B and prevents nucleotide exchange from taking place, inhibiting translation initiation. However, the GTP-bound form of eIF2 can bind the Met-tRNA$_i$, generating a ternary complex

Name	Role
eIF1	AUG recognition
eIF1A	Met-tRNA$_i$ binding to 40S subunit, 40S dissociation
eIF2	3 subunits: α binds eIF2B, β binds eIF2B and eIF5, γ binds GTP and Met-tRNA$_i$, GTPase activity
eIF2B	5 subunits Guanine nucleotide exchange factor (GEF) for eIF2
eIF3	11 subunits, binds to RNA, eIF1, eIF4B, eIF4G, eIF5 and 40S subunits
eIF4AI	ATP-dependent RNA helicase
eIF4AII	ATP-dependent RNA helicase
eIF4B	Binds RNA, stimulates eIF4A helicases
eIF4E	Binds 5' terminal m^{7}GTP cap structure
eIF4GI	Binds eIF4E, 4A, 3, PABP and RNA
eIF4GII	Binds eIF4E, 4A, 3, PABP and RNA
eIF5	Stimulates GTPase activity of eIF2 (GAP)
eIF5B	Joining of 60S subunit at initiation codon, GTPase
eIF6	Binding and dissociation of 60S ribosomal subunit

Table 1.1. Properties of the eukaryotic initiation factors (eIFs)

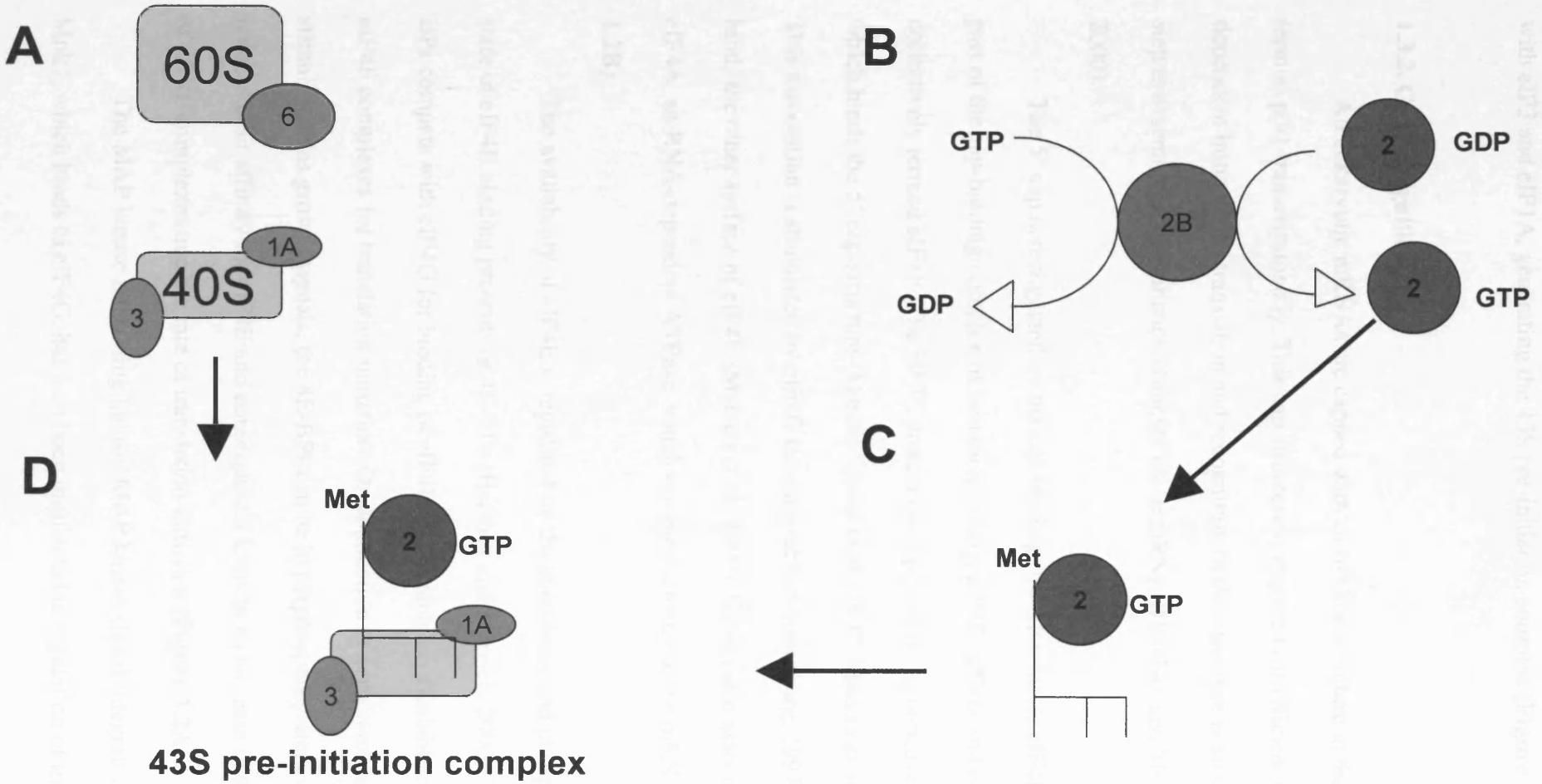


Figure 1.1. Assembly of the 43S pre-initiation complex.

A. The 60 and 40S ribosomal subunits are maintained in a dissociated state between rounds of protein syntheis by eIF6, eIF1A and eIF3

B. Inactive eIF2·GDP is recycled into the active eIF2·GTP form by the guanine nucleotide exchange factor, eIF2B.

C. eIF2·GTP binds to the initiator methionyl tRNA ($tRNA_i$), to form the ternary complex.

D. The ternary complex binds the 40S ribosomal subunit to form the 43S preinitiation complex.

(**figure 1.1C**), which is competent to bind the 40S ribosomal subunit, complexed with eIF3 and eIF1A, generating the 43S pre-initiation complex (**Figure 1.1D).**

1.3.2. Cap-recognition

All eukaryotic mRNAs are capped with an m^7GTP structure at their amino-termini post-transcriptionally. This cap structure is required for efficient cap-dependent initiation of translation and recognition of this structure is an important step in assembly of an initiation complex on an mRNA (Hershey and Merrick, 2000).

The 5' cap is recognized by the cap-binding initiation factor eIF4E, which is part of the cap-binding complex of initiation factors, eIF4E, eIF4G and eIF4A, collectively termed eIF4F. The eIF4E protein has a pocket on its concave side, which binds the 5' cap-structure (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). This association is stimulated by eIF4G (Haghighat and Sonenberg, 1997), which binds the other surface of eIF4E (Matsuo *et al.*, 1997). EIF4G also associates with eIF4A, an RNA-dependent ATPase, which can bind directly to the mRNA (**Figure 1.2B**).

The availability of eIF4E is regulated by the abundance and phosphorylation state of eIF4E binding proteins or 4E-BPs (Hershey and Merrick, 2000). The 4E-BPs compete with eIF4G for binding of eIF4E, decreasing the available complete eIF4F complexes for translation initiation. On application of certain extracellular stimuli such as growth signals, the 4E-BPs can be hyperphosphorylated, which reduces their affinity for eIF4E and consequently leads to an increase in the number of eIF4F complexes and the rate of translation initiation (**Figure 1.2A**).

The MAP kinase interacting kinase/ MAP kinase signal integrating kinase Mnk1, which binds to eIF4G, has also been implicated in regulation of translational

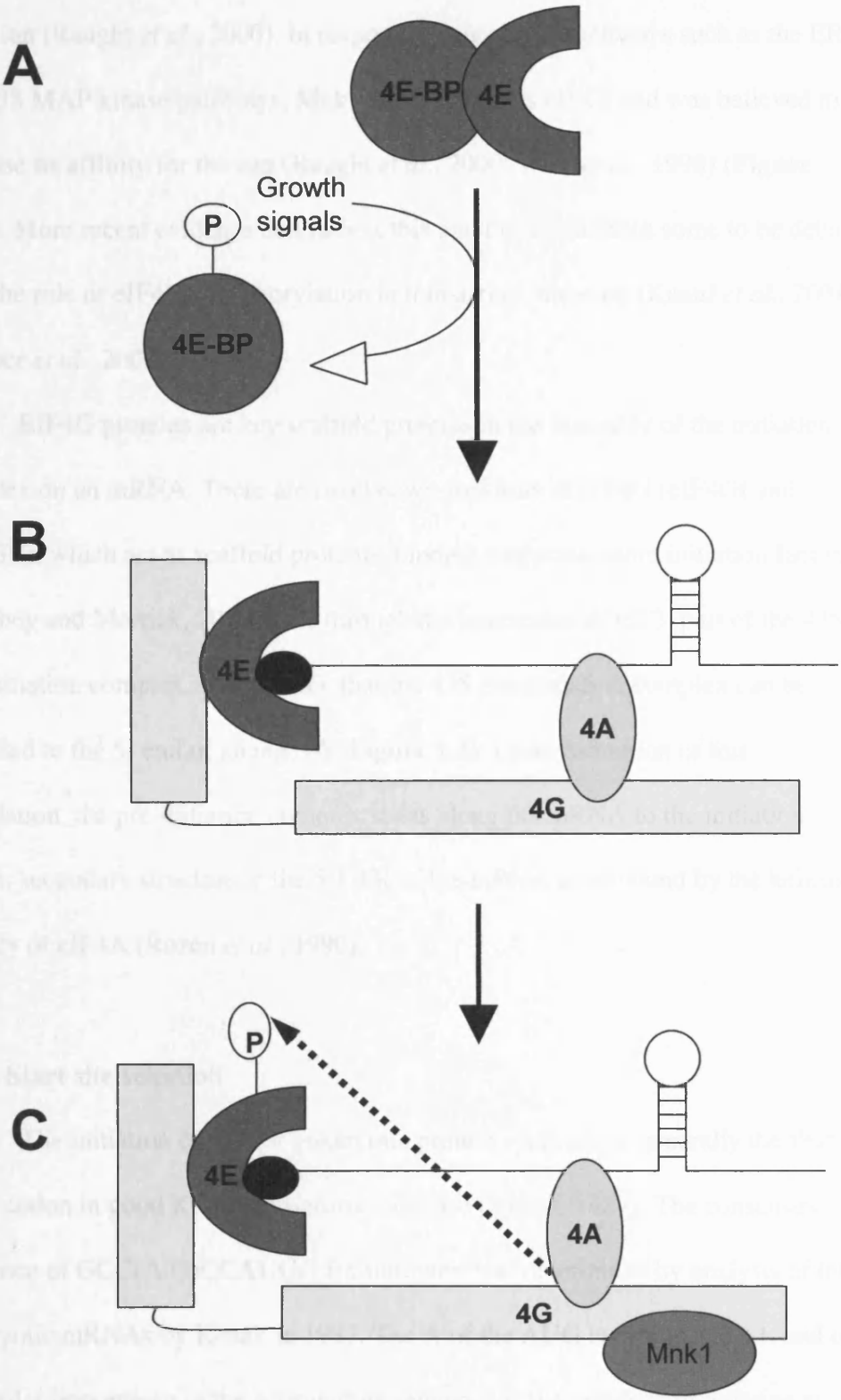


Figure 1.2. Formation of the eIF4F complex.

A. The cap-binding protein eIF4E is sequested by 4E-BPs. Growth signals cause hyperphosphorylation of 4E-BPs, releasing eIF4E.

B. eIF4E binds eIF4G, which is bound to eIF4A, to form the eIF4F complex, which is recruited to the cap of the mRNA.

C. eIF4E can be phosphorylated by Mnk1, which increases the affinity of eIF4E for the cap.

initiation (Raught *et al.*, 2000). In response to signalling pathways such as the ERK and p38 MAP kinase pathways, Mnk1 phosphorylates eIF4E and was believed to increase its affinity for the cap (Raught *et al.*, 2000; West *et al.*, 1998) (**Figure 1.2C**). More recent evidence contradicts this and there continues some to be debate over the role of eIF4E phosphorylation in translation initiation (Knauf *et al.*, 2001; Scheper *et al.*, 2002)

EIF4G proteins are key scaffold proteins in the assembly of the initiation complex on an mRNA. There are two known isoforms of eIF4G (eIF4GI and eIF4GII), which act as scaffold proteins, binding numerous other initiation factors (Hershey and Merrick, 2000). It is through the interaction of eIF3, part of the 43S pre-initiation complex, with eIF4G, that the 43S pre-initiation complex can be recruited to the 5' end of an mRNA (**Figure 1.3**). Upon formation of this association, the pre-initiation complex scans along the mRNA to the initiation codon; secondary structure in the 5'UTR of the mRNA is unwound by the helicase activity of eIF4A (Rozen *et al.*, 1990).

1.3.3. Start site selection

The initiation codon for eukaryotic protein synthesis is generally the first AUG codon in good Kozak consensus sequence (Kozak, 1987). The consensus sequence of GCC(A/G)CC**AUG**G for initiation was determined by analysis of 699 eukaryotic mRNAs by Kozak in 1987. The A of the AUG is designated +1, and of particular importance in the surrounding sequence is the presence of a purine at –3 and a G at +4. There is some debate over the importance of the nucleotides following the G at +4 but no definitive consensus for this sequence has been determined (Kozak, 1997; Grünert and Jackson, 1994). In addition, alternative initiation codons such as CUG, GUG and ACG can sometimes be used to initiate

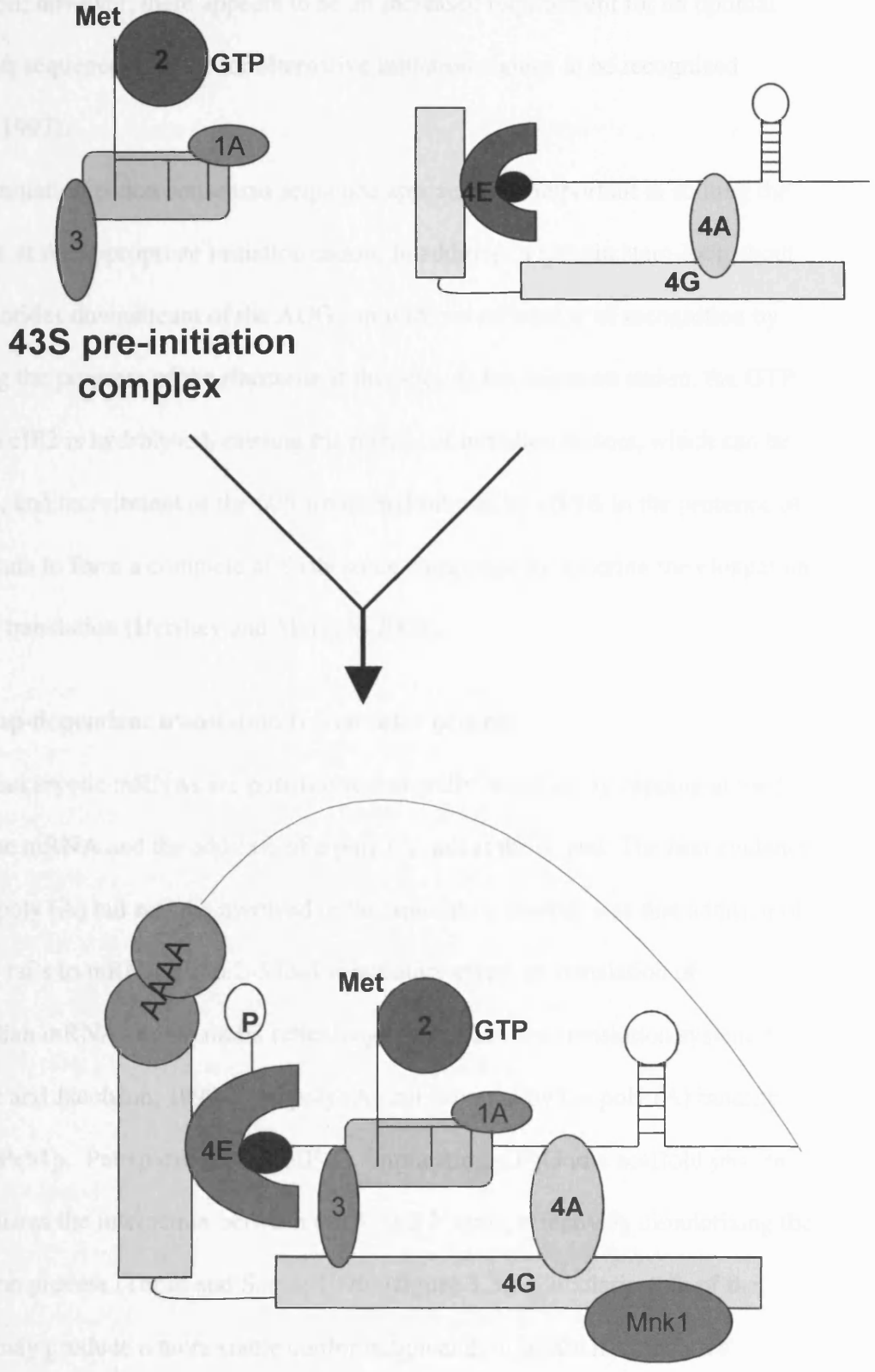


Figure 1.3. Assembly of the 48S pre-initiation complex.
The 43S pre-initiation complex assembles on the mRNA through interactions between eIF3 and eIF4G

translation; however, there appears to be an increased requirement for an optimal consensus sequence in order for alternative initiation codons to be recognized (Kozak, 1997).

Initiation codon consensus sequence appears to be important in stalling the ribosome at the appropriate initiation codon. In addition, a hairpin stem-loop about 18 nucleotides downstream of the AUG can improve efficiency of recognition by impeding the progress of the ribosome at this site. At the initiation codon, the GTP bound to eIF2 is hydrolysed, causing the release of initiation factors, which can be recycled, and recruitment of the 60S ribosomal subunit by eIF5B in the presence of GTP occurs to form a complete 80S ribosome competent for entering the elongation phase of translation (Hershey and Merrick, 2000).

1.3.4. Cap-dependent translation is a circular process

Eukaryotic mRNAs are post-transcriptionally modified by capping at the 5' end of the mRNA and the addition of a poly (A) tail at the 3' end. The first evidence that the poly (A) tail may be involved in the translation process was that addition of poly (A) tails to mRNA had a 2-3 fold stimulatory effect on translation of mammalian mRNAs in the rabbit reticulocyte lysate *in vitro* translation system (Munroe and Jacobson, 1990). The poly (A) tail is bound by the poly (A) binding protein, Pab1p. Pab1p can bind to eIF4G, implicating eIF4G as a scaffold protein that mediates the interaction between the 5' and 3' ends, effectively circularising the translation process (Tarun and Sachs, 1996) (**figure 1.3.).** Circularisation of the mRNA may produce a more stable conformation and, in addition, stimulates translation, possibly by allowing greater efficiency of ribosome recycling due to the proximity of the 5' and 3' ends (reviewed in Sachs, 2000).

1.4. Regulation of cap-dependent initiation

1.4.1. Regulation of initiation factor availability

Regulation of translation occurs at the level of initiation via a large number of mechanisms. The role of phosphorylation of initiation factors in response to extracellular stimuli and cell stress has been discussed (**sections 1.3.1. and 1.3.2.**). Dephosphorylation of eIF4E, 4E-BPs and phosphorylation of eIF2 represent mechanisms for down-regulating protein synthesis during cell stress (Mathews *et al.*, 2000). There are, however, other mechanisms acting at the initiation factor level to down-regulate protein synthesis, for example through cleavage or sequestration of initiation factors.

Cleavage of eIF4G is a control mechanism utilized in a variety of cellular circumstances; for example, eIF4G is cleaved by caspase 3 during apoptosis (Clemens *et al.*, 1998; Morley *et al.*, 1998), and by virally encoded proteases during picornaviral infection (Sarnow, 1989). During heat shock, eIF4G is sequestered into insoluble granules by hsp27. This serves two purposes, firstly, it inhibits protein synthesis to prevent accumulation of misfolded protein aggregates and secondly, it allows rapid release of eIF4G after heat shock and hence restores protein synthesis levels without the requirement for *de novo* synthesis of eIF4G (Cuesta *et al.*, 2000).

1.4.2. Regulation by the 5'UTRs of mRNAs

The 5'-untranslated region (5'UTR) is increasingly understood to be a major site of regulation of translation. The vast majority (around 90%) of eukaryotic mRNAs have 5'-untranslated regions that are between 10 and 200 bases long (Kozak, 1987). Of the remaining 10%, two-thirds encode proto-oncogenes or genes with protein products implicated in cell growth or cell death (Willis, 1999).

Translation of mRNAs encoding genes involved in cell proliferation is generally tightly regulated as misregulation of translation of these proteins has implications for uncontrolled cell growth and oncogenesis. Translational control is particularly important in regulating the amount of protein made in such cases as it acts as an additional defence should the gene be transcriptionally deregulated. The presence of a long 5'UTR would therefore imply that there are some features present in the 5'UTR that are important for regulation of these genes. There are indeed a number of mechanisms whereby the 5'UTR is involved in translational control. These are summarized in **figure 1.4**.

1.4.2.1. Upstream open reading frames and leaky scanning

Upstream open reading frames (uORFs) are found in approximately 10% of mRNAs, often those with long 5'UTRs. The majority of mRNAs containing uORFs are involved in cell proliferation (Kozak, 1991). Two-thirds of proto-oncogenes have uORFs (Geballe *et al.*, 1994) and they are also found in yeast genes (Hinnebusch et *al.*, 1996), and viral genes (Moustakas *et al.*, 1993). The general role of uORFs appears to be to inhibit translation of the downstream product, over-expression of which is likely to be deleterious.

The cap-dependent scanning model dictates that the first AUG codon in good context is likely to be used as an initiation codon (Jackson, 2000). However, this is an over-simplification, there are other factors which influence whether an AUG is likely to be recognized. Firstly, considering context, if the AUG is not in good Kozak consensus sequence then it may be inefficiently recognized, in which case the ribosome may scan further and initiate at a downstream initiation codon (**figure 1.4A**). This process is termed leaky scanning and can be used to produce multiple protein products with alternative amino termini from a single transcript,

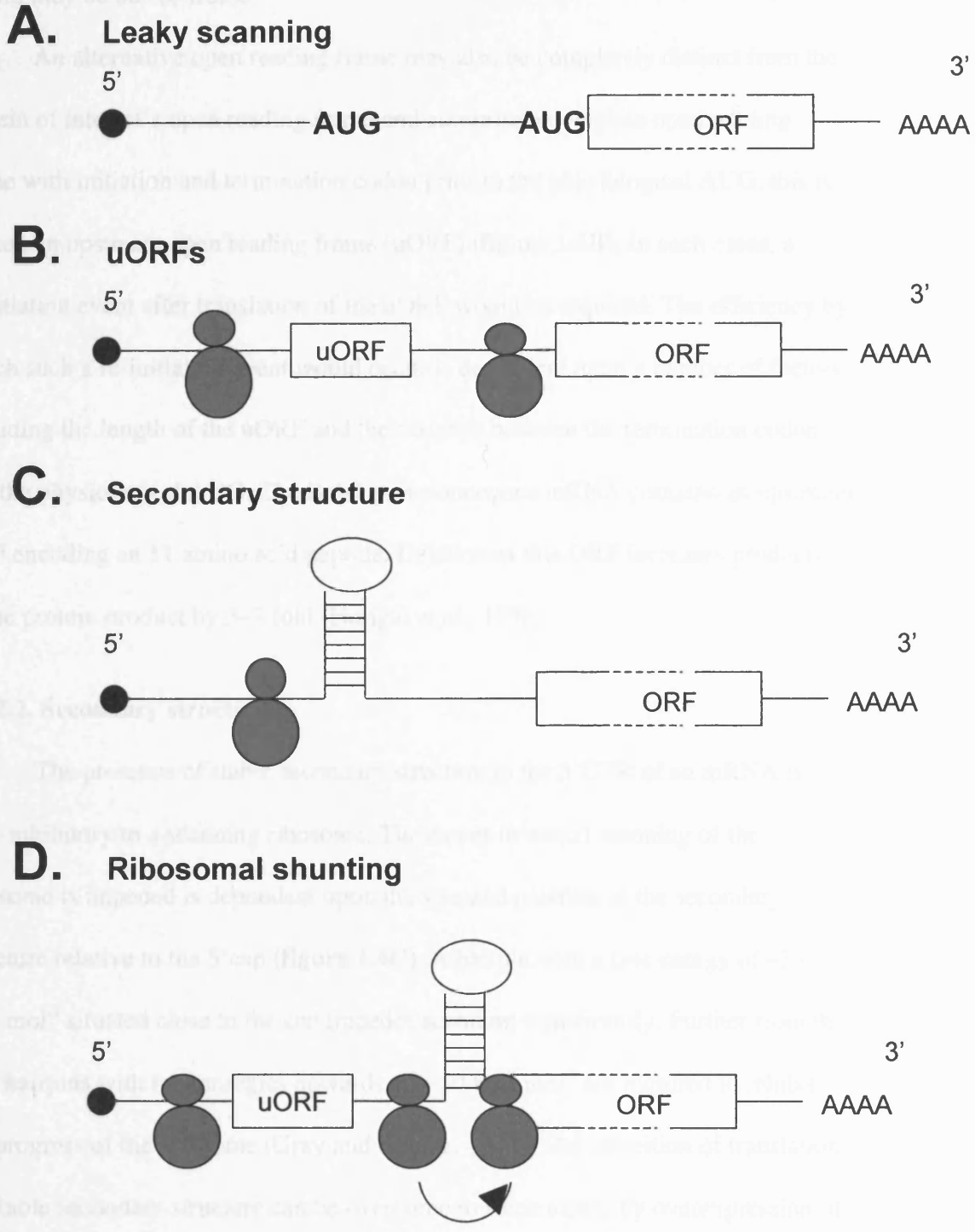


Figure 1.4. Mechanisms of translational regulation through 5' untranslated regions.
Ribosomes are indicated in blue.

A. Leaky scanning
B. Upstream open reading frames
C. Stable secondary structure
D. Ribosomal shunting

should the alternative initiation codons be in-frame, and distinct protein products should they be out-of-frame.

An alternative open reading frame may also be completely distinct from the protein of interest's open reading frame and comprise a complete open reading frame with initiation and termination codon prior to the physiological AUG, this is termed an upstream open reading frame (uORF) (**figure 1.4B).** In such cases, a reinitiation event after translation of the uORF would be required. The efficiency by which such a re-initiation event would occur is dependent upon a number of factors including the length of the uORF and the distance between the termination codon and the physiological AUG. The Bcl-2 proto-oncogene mRNA contains an upstream ORF encoding an 11 amino acid peptide. Deletion of this ORF increases production of the protein product by 5–7 fold (Harigai *et al.*, 1996).

1.4.2.2. Secondary structure

The presence of stable secondary structure in the 5'UTR of an mRNA is very inhibitory to a scanning ribosome. The extent to which scanning of the ribosome is impeded is dependent upon the size and position of the secondary structure relative to the 5'cap (**figure 1.4C**). A hairpin with a free energy of –30 kcal mol^{-1} situated close to the cap impedes scanning significantly. Further from the cap, hairpins with free energies upwards of –50 kcal mol^{-1} are required to inhibit the progress of the ribosome (Gray and Hentze, 1994). The inhibition of translation by stable secondary structure can be overcome to some extent by overexpression of eIF4E, the limiting component of eIF4F complexes. Another component of eIF4F, eIF4A, acts as an RNA helicase, unwinding secondary structure, facilitated by eIF4B (Rozen *et al.*, 1990). Presumably overexpression of eIF4E generates more eIF4F complexes and hence more eIF4A to unwind secondary structure.

1.4.2.3. Ribosomal shunting

A mechanism whereby large areas of stable secondary structure or uORFs can be bypassed and as such do not inhibit scanning is described as ribosomal shunting (**figure 1.4D**). Relatively few examples of this mechanism have been described and in some cases, these can be attributed to internal ribosome entry as opposed to shunting. Nonetheless, shunting was first proposed as a mechanism of translation for the 35S RNA of the cauliflower mosaic virus (CaMV) (Futterer *et al.*, 1993). This mechanism allows the ribosome to bypass 7 uORFs. The mechanism itself is poorly understood, however there are implications for *trans*-acting factors, a spacer of defined length and interactions with the 18S ribosomal subunit (Huang and Schneider, 1991; Yueh and Schneider, 2000).

1.5. Internal ribosome entry

Work on the translation process in picornaviruses in the late 1980s revealed an alternative method of initiation in viral messages, termed cap-independent initiation, internal initiation or internal ribosome entry (Pelletier and Sonenberg, 1988). Typically, this mechanism of translation is dependent upon a complex structural element in the 5'UTR termed an internal ribosome entry site or segment (IRES). This element is used to recruit the ribosome at an internal site, independent of the 5' cap.

1.5.1. The picornavirus paradigm

The picornaviruses are a family of at least 6 genera of positive strand RNA viruses, all of which are translated exclusively by internal ribosome entry (Belsham and Jackson, 2000). The picornaviruses have a number of common features that would make them very poor substrates for cap-dependent translation. Significantly,

RNAs produced from picornaviral genomes were found to lack a 5' cap structure, instead their 5' termini are protected from degradation by the binding of a virally encoded polypeptide (VPg) to the 5' end within virions, which is believed to be cleaved off after infection leaving an uncapped terminus in the cytoplasm (Belsham and Jackson, 2000). In addition, the 5' UTRs are long and GC-rich giving the propensity for the formation of complex secondary structure, which would undoubtedly impede a scanning ribosome. Another important feature of picornaviral RNAs that would prevent canonical cap-dependent translation taking place is the presence of numerous upstream initiation codons, some in strong context but which produce no product (Herman *et al.*, 1989).

During picornaviral infection, cap-dependent translation is compromised by the cleavage of eIF4G by virally encoded proteases (Borman *et al.*, 1997, 1995; Roberts *et al.*, 1998). The C-terminal fragment of eIF4G produced by this cleavage lacks the eIF4E-binding site and has been found to be sufficient for translation of picornaviral RNA genomes (Ohlmann *et al.*, 1996; Pestova *et al.*, 1996). An alternative mechanism of translation initiation therefore gives viral messages a selective advantage for translation upon infection of eukaryotic cells.

Internal ribosome entry was first discovered in the poliovirus (Pelletier and Sonenberg, 1988). Site directed mutagenesis of 5 of the AUGs in poliovirus did not alter the translational efficiency of the downstream ORF, suggesting reinitiation is not responsible for production of the polioviral proteins as mutagenesis would increase the ribosomes initiating at the physiological AUG should this be the case (Herman *et al.*, 1989). This realization left two likely options, ribosomal shunting or recruitment of the ribosome to an internal site. The test classically used to confirm a functional IRES is present in a 5'UTR to recruit ribosomes internally is the dicistronic reporter assay. Briefly, the first cistron will be translated by the

canonical cap-dependent scanning mechanism. The putative IRES is cloned into the intercistronic region and if this can indeed recruit ribosomes internally then initiation at the second cistron will also be observed.

Since the discovery of internal ribosome entry in picornaviruses, it has been found that this mechanism is not restricted to picornaviruses but has also been identified in flaviviruses such as HCV (Rijnbrand *et al.*, 2001), retroviruses (Waysbort *et al.*, 2001) and a growing number of others. The picornaviral IRESes are, however, the best studied and have a number of common features. A picornaviral IRES tends to be around 450 nucleotides (nt) long, located within a 5'UTR of typically 600 to 1400nt (Roberts *et al*, 1998). Based on a number of features, not least of which is the primary sequence and predicted secondary structure of the picornaviral IRESes, they have been broadly classified into two major (Class I and II) and one minor group, which includes the hepatitis A virus IRES (Jackson and Kaminski, 1995). These groups also encompass distinct ribosome-landing sites, and different degrees of dependence on *trans*-acting factors.

1.5.2. Secondary structure of picornaviral IRESes

The secondary structural composition of viral IRESes is critical for IRES activity. The helical segments are relatively susceptible to point mutations and as such base covariation in helices and variation in loops tends to occur between picornaviral species, permitting sequence variation that does not disrupt the secondary structure of the IRES. The secondary structural elements present in IRES sequences with less than 95% identity are probably a result of stabilizing selection and not a consequence of a high degree of sequence conservation (Witwer *et al.*, 2001).

In many cases, IRESes have been shown to be composed of structural domains. Deletion analysis of poliovirus and EMCV 5'UTRs showed that particular segments have positive or negative effects on translation (Herman *et al.*, 1989). The secondary structures are believed to function primarily by presenting individual primary sequence motifs to the translational apparatus allowing a modular structure to the IRES, whereby each motif may contribute to IRES activity and as such amputation of this motif may diminish but not abolish IRES activity. Some IRESes seem to depend on the strength, number and spacing of various individual IRES modules.

The 3'end itself tends to be important for viral IRES activity. This was shown by the fact that hybridisation of a complementary DNA fragment to the 5' end of certain capped/uncapped picornavirus mRNAs had little or no effect on initiation. Hybridisation to the 3' part blocks translation, suggesting the 3' part of the 5'UTR is important for IRES-driven translation (Herman *et al.*, 1989).

The class I picornaviruses include the rhinoviruses and enteroviruses, for example the human rhinovirus (HRV) and the poliovirus (PV). Class II comprise the cardioviruses and apathoviruses for example the foot-and-mouth disease virus (FMDV) and the encephalomyocarditis virus (EMCV). Based on base covariance of aligned viral sequences and subsequent secondary structural predictions of the IRESes, general secondary structures for class I (**figure 1.5A**) and class II (**figure 1.5B**) picornaviruses have been produced.

The two major classes of picornaviral IRESes are also distinct in their mechanism of ribosome landing. Class II picornaviruses conform to the precise recruitment model where the ribosome is recruited directly to the initiation codon (Kaminski *et al.*, 1990, 1994). In contrast, class I picornaviruses conform to the 'land-and-scan' model of ribosome landing, the AUG to which the ribosome binds

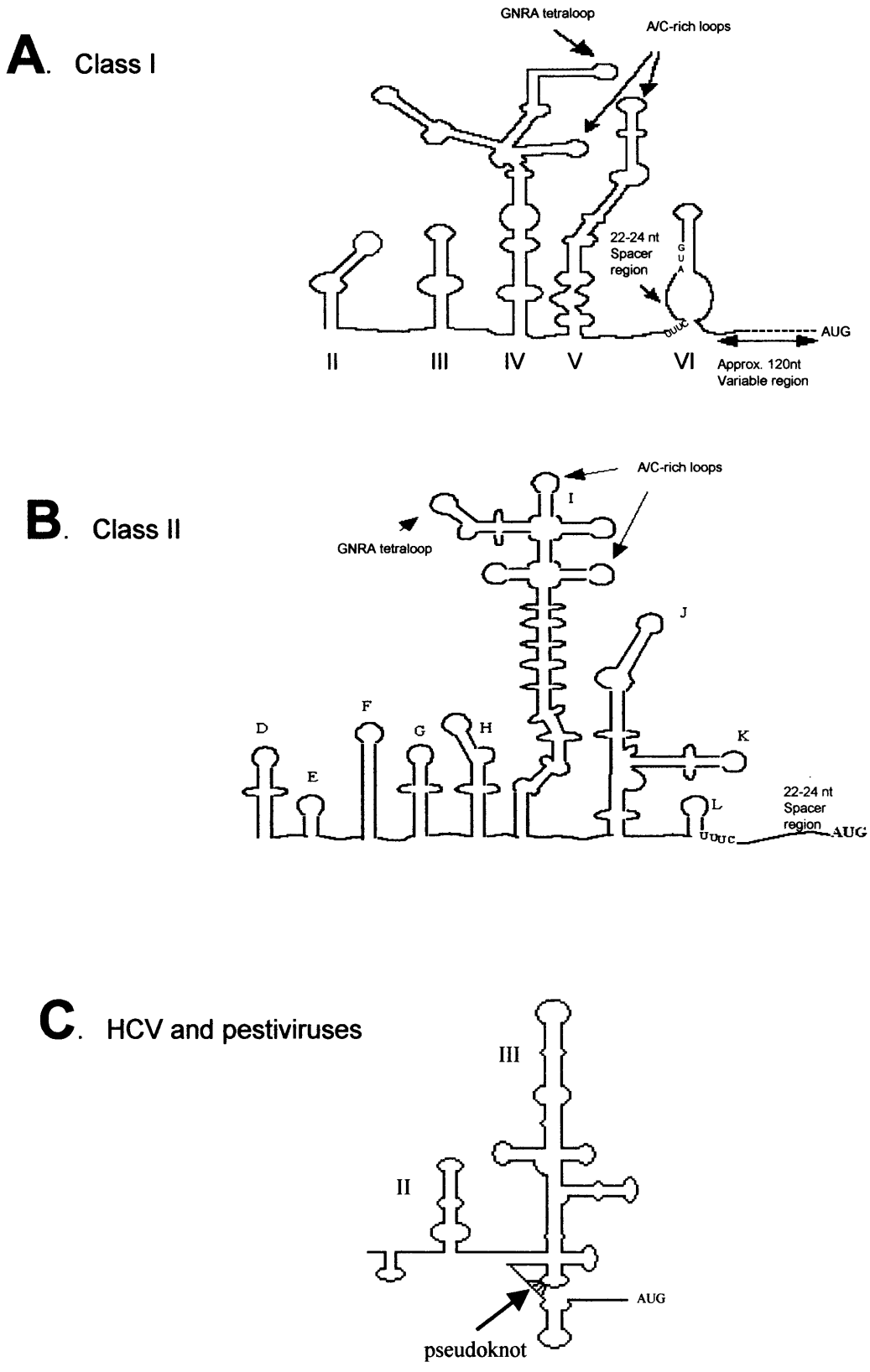


Figure 1.5. Predicted secondary structures of the three major classes of viral IRESes.

A. Class I picornaviruses, including entero- and rhinoviruses.

B. Class II picornaviruses, including cardio- and aphthoviruses.

C. Hepatitis C and pestiviruses.

initially is silent and the ribosome subsequently scans along the RNA for approximately 150nt and initiates translation at the next initiation codon (Pestova *et al.*, 1994; Ohlmann and Jackson, 1999).

The only other group of IRESes that have been studied in much detail with regards to secondary structure are those of the pestiviruses, including the hepatitis C virus IRES and pestiviral IRESes such as the classical swine fever virus IRES. These consist of a simpler structural core including a conserved pseudoknot upstream of the initiation codon, which is an absolute requirement for internal ribosome entry (Fletcher and Jackson, 2002) (**Figure 1.5C**).

In general, although comparisons may be drawn among secondary structural models for IRESes there remain broad differences even within one family of viruses, such as the *picornaviridae*. Attempts have been made to propose essential structural elements common to all viral IRESes; for example, it has been proposed that a common structural core exists including a key pseudoknot at the 3' end of the IRES (Le and Maizel, 1998). More recent evidence suggests this is not the case as there is no entirely conserved pseudoknot and even when a pseudoknot has been identified, this does not dictate that such a structure is an absolute requirement for IRES function (Ohlmann and Jackson, 1999). The advent of the use of structure probing and subsequent mutational analysis to support structural predictions based on phylogenetic and computer modelling analysis permits a higher degree of certainty in the generation of secondary structural models and supports the idea of several major groups of IRES structures, which function by similar mechanisms within a group. Given that the major groups of viral IRESes appear to function by different mechanisms, with different efficiencies, *trans*-acting factor and canonical initiation factor requirements, it is unlikely that a common structural core can be identified to unify the groups.

1.5.3. Sequence analysis of picornaviral IRESes

Picornaviral IRESes are broadly grouped, as discussed above, on the basis of secondary structural similarity; however, there is very little primary sequence similarity. Class I entero- and rhinovirus IRES sequences vary by as much as 36% (Rivera *et al.,* 1988). Interestingly, the majority of the variation observed between structurally related IRESes is within base-paired segments of the IRES structure and does not perturb the structural arrangement itself, being essentially silent. This is consistent with the proposal that the secondary structural arrangement of the IRES RNA serves to present unpaired primary sequence motifs in an appropriate conformation for recognition by the translational machinery.

There is one sequence motif at the 3' end of the IRES that is absolutely conserved between all picornaviruses. This consists of an oligopyrimidine tract of approximately 10 nucleotides including a conserved UUUC sequence, followed by a G-poor sequence and finally, the AUG codon (Jackson, 2000). There is some speculation that sequences in this region may be responsible for interactions with the 18S ribosomal subunit. An appealing alternative explanation is that the presence of a G-poor sequence could provide an unstructured region for ribosome landing. Consistent with this, the sequence but not the length of the G-poor region has been shown to vary. In addition, sequences in this region could be conserved as binding sites for *trans*-acting factors.

There are other primary sequence motifs that are frequently found within exposed loops of IRESes, such as A-rich bulges. Cardiovirus and apathovirus IRESes have a conserved A-rich bulge, however it has been found that mutations have little effect on IRES activity itself but cause class II IRESes to have increased dependence on a *trans*-acting factor, polypyrimidine-tract binding protein (PTB), for activity (Fletcher *et al.*, 2002). A-rich bulges are also found in group I introns

where they are essential for activity through forming tertiary interactions dependent on adenosines (Fletcher *et al.*, 2002).

Other common primary sequence motifs in exposed loops are polyloops of the G(n)NRA variety. Ribosomal RNAs, self-splicing introns and other highly structured RNA molecules often have helical stems capped by one of 3 classes of tetraloop, CUYG, UNCG and G(n)NRA (Abramovitz and Pyle, 1997). While CUYG and UNCG tetraloops are thought to stabilize RNAs, G(n)NRA tetraloops are only marginally more stable than unstructured loops and are proposed to be primarily important for tertiary interactions. Picornaviral class I and II IRESes both contain conserved GNRA tetraloops, in domains IV and I respectively (**figure 1.5**), although the appropriate receptor sequences have not been identified if these are indeed involved in tertiary interactions (deQuinto and Martínez-Salas, 1997; Robertson *et al.*, 1999).

1.6. Cellular IRESes

The first indication that cellular mRNAs can be translated by a cap-independent mechanism was that the human heavy-chain immunoglobulin protein Bip mRNA could be translated in poliovirus infected cells, where eIF4G was cleaved and hence, cap-dependent translation was inhibited (Sarnow, 1989). It was then shown through use of the dicistronic reporter assay that the Bip 5'UTR can direct translation of the second cistron, cap-independently (Macejak and Sarnow, 1991).

IRESes have since been identified in a rapidly growing number of cellular messages, including the proto-oncogene c-*myc*, apoptotic protease activating factor (Apaf-1) and several growth factors such as vascular endothelial growth factor

(VEGF, Akiri *et al.*, 1998) and the fibroblast growth factor 2 (FGF2, Vagner *et al.*, 1995) among others (summarized in **table 1.2**).

1.6.1. Conditions where cap-dependent translation is compromised

The cellular IRESes identified so far tend to be in key regulatory genes where the protein products are required during cellular processes where cap-dependent translation is reduced or abolished (Stoneley *et al.*, 2000; Willis, 1999). During apoptosis, cap-dependent translation is inhibited by the cleavage of initiation factors such as eIF4G by caspases (Clemens *et al.*, 1998; Morley *et al.*, 1998). IRESes producing pro-apoptotic products, such as c-myc, Apaf-1 and death-associated protein 5 (DAP5) have been shown to function during apoptosis (Stoneley *et al.*, 2000; Mitchell *et al.*, 2001 and Henis-Korenblit *et al.*, 2000). Similarly, IRESes producing anti-apoptotic proteins such as the X-linked inhibitor of apoptosis (XIAP) have been found to function (Holcik *et al.*, 2000).

Protein synthesis rates are reduced during other cellular circumstances such as hypoxia. The VEGF IRES functions during hypoxia to maintain high levels of VEGF to rescue tissue from ischemic injury (Stein *et al.*, 1998). A decrease in cap-dependent translation is also observed during amino acid starvation due to decreased phosphorylation of eIF4E (Fernandez *et al.*, 2001). Under such circumstances, translation from the cationic amino acid transporter (cat-1) IRES is up-regulated, as is transcription of this mRNA, leading to an accumulation of cat-1 protein which facilitates uptake of amino acids (Fernandez *et al.*, 2001). Where known, cellular conditions inhibitory to cap-dependent translation but where IRES mediated translation is maintained is described for each IRES in **table 1.3**.

In contrast to viral translation, most cellular transcripts containing IRESes can also be translated by conventional cap-dependent initiation. All of the mRNAs

Gene Type	Name
Transcription factors	*Antennapedia*, *Ultrabithorax*, C-*myc*, N-*myc*, MYT2, AMLI/RUNXI, Gtx, c-Jun, Mnt, Nkx6.1, NRF, YAP1, Smad5, HIP-1alpha, Hairless.
Translation and RNA processing factors	La, eIF4GI, TIF4631, DAP5/p97/NAT1
Stress response factors	XIAP, APC, Apaf-1, Bag-1, Bip/GRP78
Growth factors and growth receptors	FGF2, PDGF2/c-sis, VEGF-A, IGF-II, estrogen receptor alpha, IGF-1 receptor, Notch2
Cytoskeletal proteins	ARC, MAP2
Kinases and related	Pim1, p58/PITSLRE, alpha Cam kinase II, CDK inhibitor p27, PKCδ
Channels/transporters	KV-14, BetaF1-ATPase, Cat-1
Other	Bip, Connexin-43, Connexin-32, Cyr61, ODC, Dendrin, Neurogranin/RC3, NBS1, FMR1, Rbm3, NDST

Table 1.2. Representative list of cellular IRESes identified to date and their cellular functions.
Data from the IRES database at:
http://ifr31w3.toulouse.inserm.fr/IRESdatabase/

Cellular circumstance	IRESes regulated
Development	Antennapedia, Ultrabithorax, C-myc
Apoptosis	C-myc, DAP5, XIAP, PKCδ
Genotoxic stress	C-myc
Hypoxia	C-myc, HIF-1alpha, VEGFA
Cell cycle	Hairless, p58/PITSLRE, PKCδ, ODC
Differentiation	AML1/RUNX1, PDGF2/c-sis
Heat/cold shock	Bip, Bag-1, Rbm3
Amino acid starvation	Cat-1

Table 1.3. Cellular conditions where IRES-mediated translation is regulated

Table to show cellular circumstances where cap-dependent translation is compromised but IRES-driven translation maintains production of the proteins listed. Data from the IRES database at: http://ifr31w3.toulouse.inserm.fr/IRESdatabase/

identified to contain IRESes so far are growth-related and as such are subject to stringent regulation (Willis, 1999). IRESes appear to act as an additional level of regulation to ensure proteins essential for cell survival are synthesized during certain cellular circumstances. The nature of genes capable of being translated by internal ribosome entry has, however, implicated misregulation of IRESes to be important in oncogenesis.

1.6.2. Secondary and primary structure of cellular IRESes

In contrast to picornaviruses, very little structural similarity has been proposed for cellular IRESes. All picornaviral IRESes have been proposed to have a common structural core motif consisting of a Y shaped (branched) stem-loop and a pseudoknot at the 3' end of the IRES (Le and Maizel, 1997). This has also been suggested for a number of cellular IRESes although it must be stressed that this is a prediction based on computer modelling and not on true mechanistic data. Such a motif has been proposed to exist in IRESes including the human immunoglobulin heavy chain binding protein (Bip) and human fibroblast growth factor receptor (FGFR-2) among others. Internal initiation has been not correlated with the presence of the Y-motif, 5' and 3' deletions of Bip still have IRES activity even when the proposed Y motif is lost (Yang *et al.*, 1997). The VEGF, c-*myc* and Apaf-1 IRESes all lack a Y motif and consequently this certainly cannot be a defining feature for cellular IRES function (Chappell *et al.*, 2000; Mitchell *et al.*, 2003).

Some studies suggest more modular arrangements for some cellular IRESes for example, the Gtx homeodomain protein IRES has been found to have a 9-nucleotide motif with 100% complementarity to the 18S ribosomal subunit. This motif has full IRES activity and intriguingly can be linked to produce much higher levels of IRES activity than observed naturally (Chappell *et al.*, 2000). Other

IRESes are beginning to be elucidated which contain sequences homologous to the 18S ribosomal subunit. The VEGF IRES contains a sequence homologous to human 18S rRNA 14nt upstream of the initiator AUG (Akiri *et al.*, 1998). In addition, sequence motifs observed in viral IRESes have also been identified in cellular IRESes; for example, the VEGF IRES also contains an unpaired GNRA motif, common in picornaviral IRESes (Huez *et al.*, 1998). This evidence suggests that some IRESes have short segments with full IRES activity and others consist of structural motifs that contribute to IRES activity.

To date, relatively few secondary structural models have been proposed for cellular IRESes, however the structural models defined give valuable insights into the mechanism of action of the IRESes. Structural remodelling appears to be critical to the function of the cat-1 and Apaf-1 IRESes, whereby the *trans*-acting factors PTB and unr perturb the secondary structure of the Apaf-1 IRES to produce an unstructured window, which facilitates ribosome binding. Translation by the cat-1 IRES is regulated by translation of an upstream open reading frame, which causes remodelling of the IRES into an active conformation, regulated by amino acid availability (Yaman *et al.*, 2003). Clearly, secondary structure determination is an important factor in elucidating the mechanisms by which individual IRESes function.

No significant comparisons can be drawn between the cellular IRESes described so far. It is likely that cellular IRESes have common structural motifs to be recognized by *trans*-acting factors rather than significant primary sequence similarity (Stein *et al.*, 1998).

1.7. Protein factor requirements for IRES activity

In common with the cap-dependent mechanism of translation initiation, IRES-mediated translation generally requires protein factors in order to recruit the ribosome to the appropriate initiation site. There are exceptions to this rule; the cricket paralysis virus can recruit the ribosome directly to a non-canonical initiation codon with no requirement for initiation factors or other proteins (Jan *et al.*, 2002). Nonetheless, the majority of IRESes require at least some of the canonical initiation factors required in cap-dependent translation and, in addition, most have been found to require additional *trans*-acting factors or IRES-interacting factors (ITAFs) that are not required for the cap-dependent mechanism.

1.7.1. Canonical initiation factor requirements

The canonical initiation factor requirements for IRES activity of viral and cellular IRESes varies considerably. In general, it is considered that the minimum requirements for picornaviral recruitment of 40S ribosomes consists of the initiation factor eIF4A, eIF4B and the central region of eIF4G, in addition to ATP (Pestova *et al.*, 1996a; Pestova *et al.*, 1996b). There are exceptions to this; for example, Hepatitis A virus (HAV) translation requires intact eIF4G (Borman and Kean, 1997).

HCV and the pestiviruses do not follow the picornavirus precedent. These can assemble 48S initiation complexes with eIF3 and ternary complex eIF2-GTP-Met-tRNA$_i$ only (Sizova *et al.*, 1998; Pestova *et al.*, 1998). The recruitment of the 40S ribosomal subunit to these IRESes consequently occurs in a manner analogous to the prokaryotic Shine-Dalgarno sequence, with the complex IRES structure in place of the Shine-Dalgarno sequence. This implies that none of the initiation factors generally required for ribosomal recruitment are necessary, with the

exception of the ternary complex, eIF2-GTP-Met-tRNA$_i$, that is eIF4A, eIF4B, eIF4E and eIF4G are all dispensable. In addition, there is no requirement for ATP (Pestova *et al.*, 1998).

Cellular IRESes have been little studied in this respect; however, it is generally assumed that they require all of the canonical initiation factors required for cap-dependent translation, with the possible exception of eIF4E and the amino-terminus of eIF4G. Some cellular IRESes function efficiently in circumstances where eIF4G is cleaved for example, the Bip IRES functions during polioviral infection (Sarnow, 1989; Macejak and Sarnow, 1991). The orthinine decarboxylase (ODC) IRES has also been shown to function independently of eIF4E but it is unknown whether these requirements apply to other cellular IRESes (Pyronnet *et al.*, 2000).

1.7.2. *Trans*-acting factor requirements

The function of both viral and cellular IRESes is further complicated by their requirement for non-canonical *trans*-acting protein factors. In addition to at least some of the canonical initiation factors required for cap-dependent scanning, a number of *trans*-acting protein factors have been demonstrated to be essential for, or to enhance the activity of viral IRESes. Some of the corresponding factors involved in cellular IRES activity have recently begun to be elucidated. A number of roles for *trans*-acting factors in the IRES-mediated mode of translation initiation have been proposed. Some *trans*-acting factors function by interacting with ribosomes to facilitate ribosome recruitment. They may stabilize secondary or tertiary structural elements to allow direct recruitment of the ribosome, or may be involved in the recruitment of other cellular factors through protein-protein interactions or stabilizing IRES structures required for binding other proteins (Walter *et al.*, 1999).

Trans-acting factors have recently been shown to modulate the secondary structure of the Apaf-1 IRES, opening up an unstructured region to facilitate ribosome binding (Mitchell *et al.*, 2003).

The two major classes of picornaviral IRESes differ in their requirement for *trans*-acting factors. Class II IRESes, the apathoviruses and cardioviruses, function efficiently in a cell-free translation system, the rabbit reticulocyte lysate *in vitro* translation system, and can also direct translation in translating extracts derived from HeLa cells. Class I IRESes, the enteroviruses and rhinoviruses, in contrast function inefficiently in the rabbit reticulocyte lysate system but translation can be restored by addition of HeLa cell extract (Meerovitz *et al.*, 1993). Hepatoviruses function inefficiently in both systems. This evidence gave an indication that Class I IRESes require factors deficient in the rabbit reticulocyte lysate system but that are present in sufficient quantities in HeLa cells. None of the cellular IRESes identified so far can direct translation in the rabbit reticulocyte translation system; however, addition of specific *trans*-acting factors or cell lysates can enhance translation in this system and *in vivo*.

The amount and availability of *trans*-acting factors varies considerably according to cell line, leading to cell type specificity of many IRESes. The HAV IRES requires factors enriched in the liver as the virus is translated most efficiently in liver cells (Glass and Summers, 1993). The Apaf-1 and N-*myc* IRESes function efficiently in neuronal cell lines and consequently are probably stimulated by the presence of neuronal specific *trans*-acting factors (Mitchell *et al.*, 2003; Jopling and Willis, 2001). Apaf-1 IRES-driven translation has been shown to be enhanced by addition of PTB but can be enhanced further by addition of neuronal PTB (n-PTB, Mitchell *et al.*, 2003).

Another key feature of some IRESes is that they require a 'nuclear event' in order to direct translation efficiently (Stoneley *et al.*, 2000). That is, transcripts introduced directly into the cytoplasm of HeLa cells are not capable of directing translation; however, if plasmids are introduced into the nucleus, the same IRES can direct translation efficiently. This evidence suggests that the RNA must passage through the nucleus prior to being capable of internal initiation. For this reason, many of the *trans*-acting factors identified so far are predominantly localized in the nucleus as attempts have been made to replicate the nuclear event.

Expression of certain *trans*-acting factors can be enhanced during specific cellular circumstances. Enhanced levels of *trans*-acting factors may contribute to the increased IRES activity observed during cellular circumstances where cap-dependent translation is inhibited. During hypoxia, expression of a known IRES *trans*-acting factor, PCBP1, is enhanced and could consequently stimulate IRES-driven translation under such circumstances (Zhu *et al.*, 2002). Many *trans*-acting factors are modulated during cellular stress conditions, by cleavage or phosphorylation, which leads to a redistribution from the nucleus to the cytoplasm of the cell. PTB is cleaved during polioviral infection and during apoptosis. The carboxyl-terminal fragment generated relocalises to the cytoplasm (Back *et al*, 2002). PTB can also be phosphorylated at its nuclear localization signal (NLS) by PKA, again resulting in redistribution to the cytoplasm, where translation takes place (Xie *et al.*, 2003). Availability of *trans*-acting factors could therefore present another level of regulation of IRES-driven translation and knowledge of the specific set of *trans*-acting factors required for certain IRESes gives valuable insights into their mechanism of action. Translation from all of the IRESes studied so far requires a different set of *trans*-acting factors, although many of the proteins identified are capable of influencing translation from a number of different IRESes. It is tempting

to speculate that elucidation of further factors will reveal distinct subsets of factors required for groups of IRESes that function by similar mechanisms. *Trans*-acting factors identified so far and the IRESes they influence are summarized in **table 1.4**. The most commonly identified *trans*-acting factors and their influence on IRES-driven translation are detailed below.

1.7.2.1. La

The first *trans*-acting factor identified was La, the Lupus Antigen. This protein is required for Hepatitis C virus (Isoyama *et al.*, 1999; Ali *et al.*, 2000), poliovirus (Meerovitch *et al.*, 1993) and XIAP (Holcik and Korneluk, 2000) cap-independent translation amongst others. It is cleaved during apoptosis and is redistributed to the cytoplasm, previously being mainly localized to the nucleus (Ali *et al.*, 2000; Ramos *et al.*, 2000). La is also cleaved by picornaviral proteases, again resulting in redistribution to the cytoplasm (Meerovitch *et al.*, 1993). Cleavage during apoptosis occurs at a different cleavage site to that used by poliovirus but produces a similar truncated protein (Ramos *et al.*, 2000). Cleaved, cytoplasmic La is capable of stimulating translation although the low level of La present in the cytoplasm anyway is sufficient for translation of some IRESes, including HCV (Isoyama *et al.*, 1999). Cell stress and viral infection consequently increase the cytoplasmic levels of La, generating greater availability of this protein for enhancement of IRES activity during inhibition of cap-dependent translation.

1.7.2.2. PTB

One of the most commonly identified *trans*-acting factors for viral IRES function is the Polypyrimidine-Tract binding Protein (PTB), also known as hnRNP1 (heterogeneous nuclear ribonucleoprotein). PTB is involved in the splicing process,

ITAF/*trans*-acting factor	**IRESes interacts with**
PTB (hnRNP1)	EMCV, FMDV, TMEV, PV1, HRV, HCV, HAV, Human T-lymphotrophic virus type 1, Apaf-1, IGF-IR
N-PTB (neuronal PTB)	TMEV
La	EMCV, HCV, PV1, Human T-lymphotrophic virus type 1, HIV-1 (gag RNA), XIAP, Bip/Grp78, Coxsackievirus B3
Unr	HRV, Apaf-1
ITAF45	FMDV
HnRNPE2 (PCBP2)	PV1, HRV, Coxsackievirus B3, c-*myc*
HnRNPE1 (PCBP1)	PV1, c-*myc*
HnRNPC1/C2	PDGF2/c-sis, XIAP
HnRNPL	HCV
HnRNPK	c-*myc*
DAP5	DAP5, c-*myc*, Apaf-1, XIAP
GAPDH	HAV
Nucleolin	HRV, PV1
ELAV/Hu	p27
Ribosomal protein S9	HCV, CSFV
Ribosomal protein S5	HCV

Table 1.4. Table of ITAFs/ *trans*-acting protein factors and the corresponding IRESes they have been identified to interact with. Data from the IRES database at: http://ifr31w3.toulouse.inserm.fr/IRESdatabase/

it binds the polypyrimidine tract that precedes the 3' splice site in eukaryotic introns and regulates alternative splicing (Romanelli *et al.*, 2000). PTB functions as a dimeric RNA binding protein with four RNA recognition motifs (RRMs) for RNA-binding (Wollerton *et al.*, 2001). It binds to UC rich polypyrimidine tracts, normally five or more bases long and has been shown to specifically bind to many IRESes, including those of class I and class II picornaviruses (Yuan *et al.*, 2002).

Picornaviral IRESes have a polypyrimidine tract preceding the AUG codon, which presents a binding site for PTB and consequently PTB appears to bind to the majority of picornaviral IRESes. Class I IRESes including HRV and PV have a requirement for PTB (Hunt *et al.*, 1999; Hellen *et al.*, 1994). PTB has also been shown to moderately enhance the IRES activity of FMDV (Pilipenko *et al.*, 2000) and EMCV (Witherall *et al.*, 1993; Pilipenko *et al.*, 2000). Interestingly, the dependence of some strains of class II picornaviruses on PTB may be due to mutations in a conserved A-rich bulge of class II IRESes. Such mutations have little effect on IRES activity itself but cause class II IRESes to have increased dependence on PTB for activity (Kaminski and Jackson, 1998; Fletcher *et al.*, 2002).

More recently, PTB has been seen to interact with and enhance activity of cellular IRESes, notably the VEGF and Apaf-1 IRESes (Huez et *al.*, 1998; Mitchell *et al.*, 2001). There is evidence, however, that PTB can also act in an inhibitory manner. PTB binds to the Bip IRES and inhibits its activity (Kim *et al.*, 2000). In some cases, low levels of PTB enhance IRES activity but high levels suppress it, for example on the EMCV IRES (Kim *et al.*, 1999). In this case, addition of another *trans*-acting factor, La, relieves the suppression (Kim *et al.*, 1999). This evidence suggests there is complex interplay between *trans*-acting protein factors that determine whether IRES activity is enhanced or suppressed. PTB can either enhance

or inhibit IRES-dependent translation depending on the mRNA and the level of PTB available (Kim *et al.*, 2000).

1.7.2.3. Unr and unrip

Unr (upstream of N-ras) has been demonstrated to interact with a range of IRESes and in some cases acts synergistically with PTB. Unr is an RNA binding protein with 5 cold shock domains, which each act as an RNA-binding motif. The recognition sequence for unr is a stretch of purine nucleotides followed by a conserved core of AAGUA/G or AACG (Triqueneaux *et al.*, 1999). Unr and PTB have a synergistic effect on the HRV (Hunt *et al.*, 1999) and Apaf-1 IRESes (Mitchell *et al.*, 2001). A binding partner for unr was described, in association with HRV, termed unr-interacting protein (unrip, Hunt *et al.*, 1999). Unrip is a member of the GH-WD repeat family with 6 WD-40 repeats. Proteins containing such repeats are often associated with other proteins and appear to act as bridging proteins for protein complexes. No enhancement in IRES activity was, however, observed on adding unr and unrip in conjunction, over that of unr alone (Hunt *et al.*, 1999).

1.7.2.4. The Poly-C binding proteins 1 and 2

PCBP1 and PCBP2 are closely related proteins of the heterogeneous ribonucleoprotein (hnRNP) family of RNA binding proteins. PCBP1 and 2 have 90% sequence similarity (Blyn *et al.*, 1996) where PCBP1 is thought to have been generated by a retrotransposition event of a fully processed PCBP2 RNA (Leffers *et al.*, 1995). They also belong to the KH domain superfamily of nucleic acid binding proteins (hnRNP K Homology domain). RNA-binding proteins associated with nuclear mRNAs (hnRNPs) play a primary role in control of post-transcriptional

events (Adinolfi *et al.*, 1999) and the PCBPs have been shown to be important in binding and stabilizing mRNAs.

In capped mRNAs, PCBP1 and 2 bind 3'UTRs, as these are the only sites where ribonucleoprotein (RNP) complexes can remain undisturbed during active translation. Their role in binding 3'UTRs is not as general translation factors as they have no effect on a range of capped mRNAs (Blyn *et al.*, 1996). The PCBPs are important in the formation of a sequence specific alpha-globin RNP complex associated with alpha-globin mRNA stability (Leffers *et al.*, 1995) Alpha-globin is only expressed in erythroids and accumulates during erythroid differentiation. This process is dependent on a very long half-life of the mRNA, which is achieved through formation of a stabilizing RNP complex including PCBP1 or PCBP2 (Wang *et al.*, 1995). The erythropoietin 3' UTR also contains putative stabilizing and destability elements. The amount of erythropoietin is regulated at the level of mRNA stability during hypoxia by formation of a ribonucleoprotein complex associated with a hypoxia-inducible protein binding site (HIPBS), including PCBP1 and PCBP2 (Czyzyk-krzeska *et al.,* 1999). Interestingly, there is hypoxic up-regulation of PCBP1 but not PCBP2 mRNA and protein mediated by p38 mitogen-activated protein kinase (Zhu *et al.*, 2002).

A role for the PCBPs in stabilizing IRES elements has recently been proposed. Both proteins bind the 5' terminal cloverleaf structure of the poliovirus IRES. PCBP2 also binds stem-loop IV, which is a large, central motif in the poliovirus, required for viral translation and viability (Blyn *et al.*, 1996; Gamarnik *et al.*, 2000). The PCBP- RNA cloverleaf interaction is not directly required for efficient translation initiation, but for stabilization of the RNA (Murray *et al.*, 2001), mutations in the PCBP C-rich binding sites in stem-loop IV or the cloverleaf structure destabilize the IRES structure (Blyn *et al.,* 1995; 1996). Both PCBP1 and

PCBP2 are also required for HAV translation (Graff *et al.*, 1998), and translation from other class I picornaviral IRES elements (Walter *et al.*, 1999). PCBP2 can interact with type II elements but does not appear to promote translation, this interaction could, however, be involved in viral replication (Blyn *et al.*, 1996). It would be interesting to determine whether PCBP1 can bind to cellular IRESes whose translation is maintained during hypoxia, for example the VEGF IRES, as expression of PCBP1 can be induced by hypoxia (Zhu *et al.*, 2002).

1.7.2.5. Initiation factors

Death Associated Protein 5 (DAP5) or p97 is a homologue of eIF4G, lacking the eIF4E binding site and consequently is thought of as analogous to the central fragment of eIF4G. Translation of DAP5 itself can be mediated by internal ribosome entry and IRES-driven translation can be promoted by the presence of DAP5 protein (Sella *et al.,* 1999). A cleaved fragment of DAP5 that is generated during apoptosis, termed p86, has also been shown to stimulate translation of cellular IRESes that drive translation of genes involved in apoptosis (Henis-Korenblit *et al.*, 2000). Overexpression of the caspase-cleaved p86 fragment of DAP5 or the central region of eIF4G, known as M-FAG/p76 can stimulate IRES activity of IRES elements found in some pro-apoptotic mRNAs such as Apaf-1 and DAP5 (Nevins *et al.*, 2003). Thus, it appears that the presence of cleaved fragments of this initiation factor family during apoptosis may accelerate the apoptotic process (Nevins e*t al.*, 2003). Interestingly, there was a reduction in IRES-driven translation of the anti-apoptotic protein XIAP during etoposide-induced apoptosis (Nevins *et al.*, 2003). This suggests that cleaved eIF4G and DAP5 may enhance IRES driven translation of a subset of IRESes during apoptosis.

1.8. The p36 isoform of Bag-1 can be translated by internal ribosome entry.

The murine Bag-1 protein was identified as a Bcl-2 binding partner in a screen of a mouse embryonic cDNA library with Bcl-2, an anti-apoptotic protein, and was the only positive clone identified (Takayama *et al.*, 1995). Bag-1 was accordingly designated 'Bcl-2 associated athanogene 1' (Takayama *et al.*, 1995). The Bag-1 gene shares no homology with the Bcl-2 family but was shown to enhance the cytoprotective properties of Bcl-2 in cells exposed to a range of apoptotic stimuli (Takayama *et al.*, 1995). The murine Bag-1 gene was found to encode a single transcript and two protein isoforms generated by alternative initiation of translation from two in-frame initiation codons (**figure 1.6A**).

The human homologue of Bag-1 has an N-terminal extension compared to the murine sequence (Takayama *et al.*, 1996); and has since been shown to encode at least 3 major and one minor isoform of human Bag-1, all generated from a single transcript (**figure 1.6**). The human homologue was also cloned independently as RAP46 (46kDa Receptor associated protein) in association with the glucocorticoid receptor and subsequently, in association with other nuclear hormone receptors (Zeiner and Gehring, 1995). The nomenclature for this protein was again altered when it was cloned in association with the 70kDa heat shock proteins, as this was believed to represent the major interaction of Bag-1. Through this interaction, Bag-1 can mediate interactions with a vast array of other proteins and consequently influences diverse cellular processes (Zeiner *et al.*, 1997). Bag-1 is therefore also known as hsp70/hsc70-associated protein (HAP46). For simplicity, the nomenclature Bag-1 will be used throughout this study.

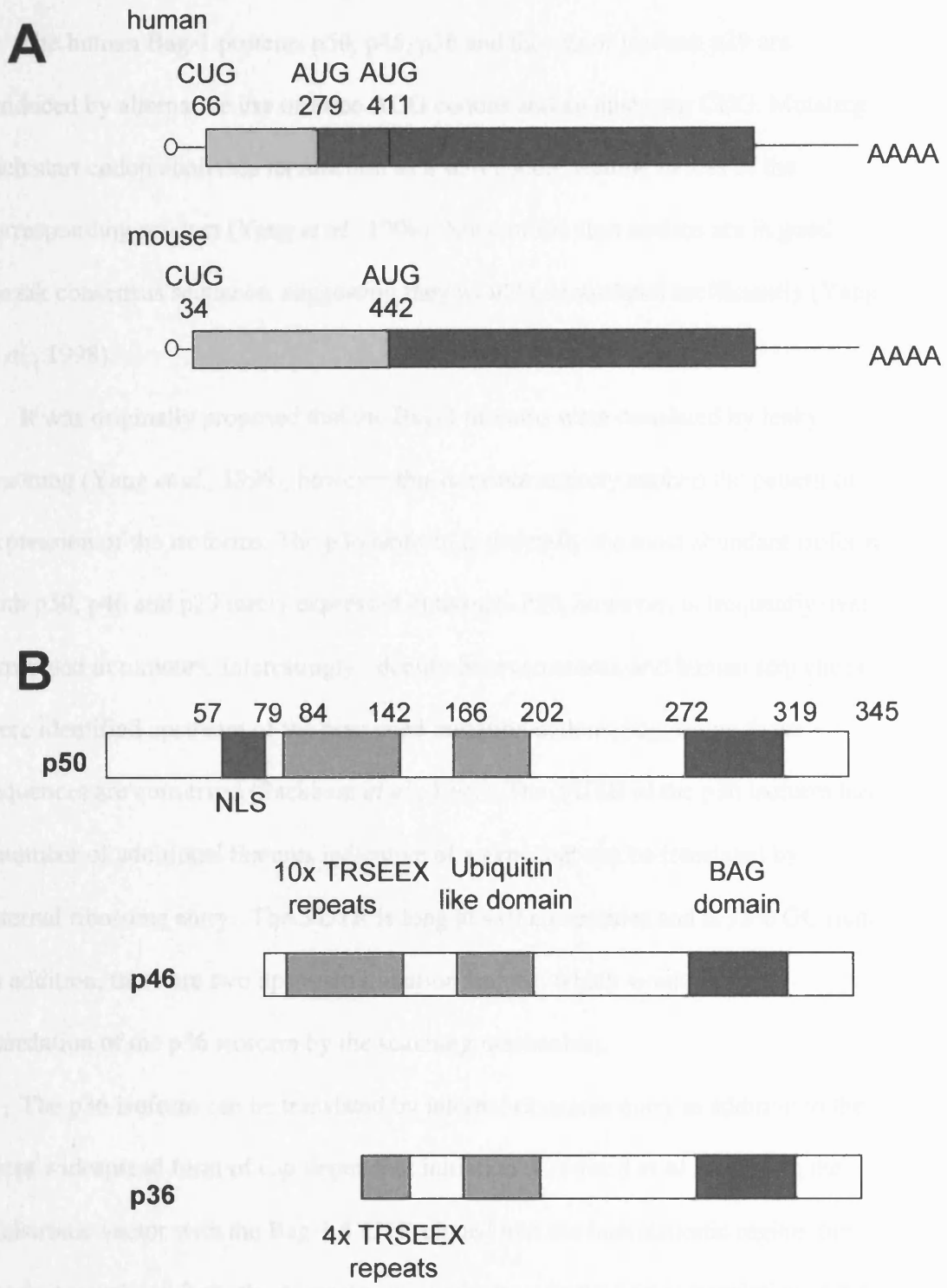


Figure 1.6. Human and mouse Bag-1 proteins are translated from a single transcript.

A. Schematic diagram of the human and mouse Bag-1 transcripts

B. Schematic diagram of the 3 major Bag-1 isoforms in humans, indicating key features.

1.8.1. Translational control of Bag-1

The human Bag-1 proteins p50, p46, p36 and the minor isoform p29 are produced by alternative use of three AUG codons and an upstream CUG. Mutating each start codon abolishes its function as a start codon, leading to loss of the corresponding product (Yang *et al.,* 1998). None of the start codons are in good Kozak consensus sequence, suggesting they would be translated inefficiently (Yang *et al.*, 1998).

It was originally proposed that the Bag-1 proteins were translated by leaky scanning (Yang *et al.,* 1998), however this does not entirely explain the pattern of expression of the isoforms. The p36 isoform is generally the most abundant isoform, with p50, p46 and p29 rarely expressed in tissues. P50, however, is frequently over-expressed in tumours. Interestingly, identity between mouse and human sequences were identified upstream of the presumed initiation codons, suggesting those sequences are conserved (Packham *et al.*, 1997). The 5'UTR of the p36 isoform has a number of additional features indicative of a gene that can be translated by internal ribosome entry. The 5'UTR is long at 410 nucleotides and is 73% GC rich. In addition, there are two upstream initiation codons, which would inhibit translation of the p36 isoform by the scanning mechanism.

The p36 isoform can be translated by internal ribosome entry in addition to the more widespread form of cap-dependent initiation (Coldwell *et al.*, 2001). In the dicistronic vector with the Bag-1 5'UTR cloned into the intercistronic region, the product translated from the downstream cistron was derived from translation at the p36 initiation codon. There was no significant translation from the upstream initiation codons, indicating that only the p36 isoform can be translated by internal ribosome entry (Coldwell *et al.*, 2001). Both the cap-dependent and cap-independent mechanisms of translation contribute to expression of the p36 isoform

of Bag-1, although IRES-driven translation of Bag-1 operates at around one-third of the efficiency of cap-dependent translation in HeLa cells (Coldwell, 2001).

1.9. Roles of the Bag-1 proteins

The Bag-1 proteins have in common a central ubiquitin-like domain and a carboxyl terminal BAG domain, which is conserved amongst other, recently identified, members of the Bag protein family (Takayama *et al.*, 1999) (**figure 1.6B**). The three major isoforms differ at the amino terminus, which has important implications for both their protein binding specificities and subcellular location. Amino-terminal sequences present in human p50 Bag-1 RNA but not p36 are sufficient to confer nuclear localization upon green fluorescent protein (Packham *et al.*, 1997). The p50 isoform contains an SV40-like nuclear localization signal (NLS) at its amino-terminus, which is thought to be responsible for the mainly nuclear distribution of this isoform. P46 and p36 lack this NLS and as such are predominantly localized cytoplasmically (Yang *et al.*, 1998; Packham *et al.*, 1997). Interactions of the Bag-1 proteins and the cellular processes they influence are summarized in **figure 1.7**.

The amino-terminal extensions lead to a different number of repeats of an N-terminal hexapeptide motif with p50 and p46 containing ten repeats and p36, four. These differences are thought to bestow different protein binding specificities on the three isoforms as the repeats allow formation of extended amphipathic alpha helices. In addition, there are six positively charged residues located in a cluster on top of the helix at the amino-terminus of the p46 and p50 isoforms. P36 lacks this protein-binding motif and so the three isoforms could conceivably have different protein binding specificities.

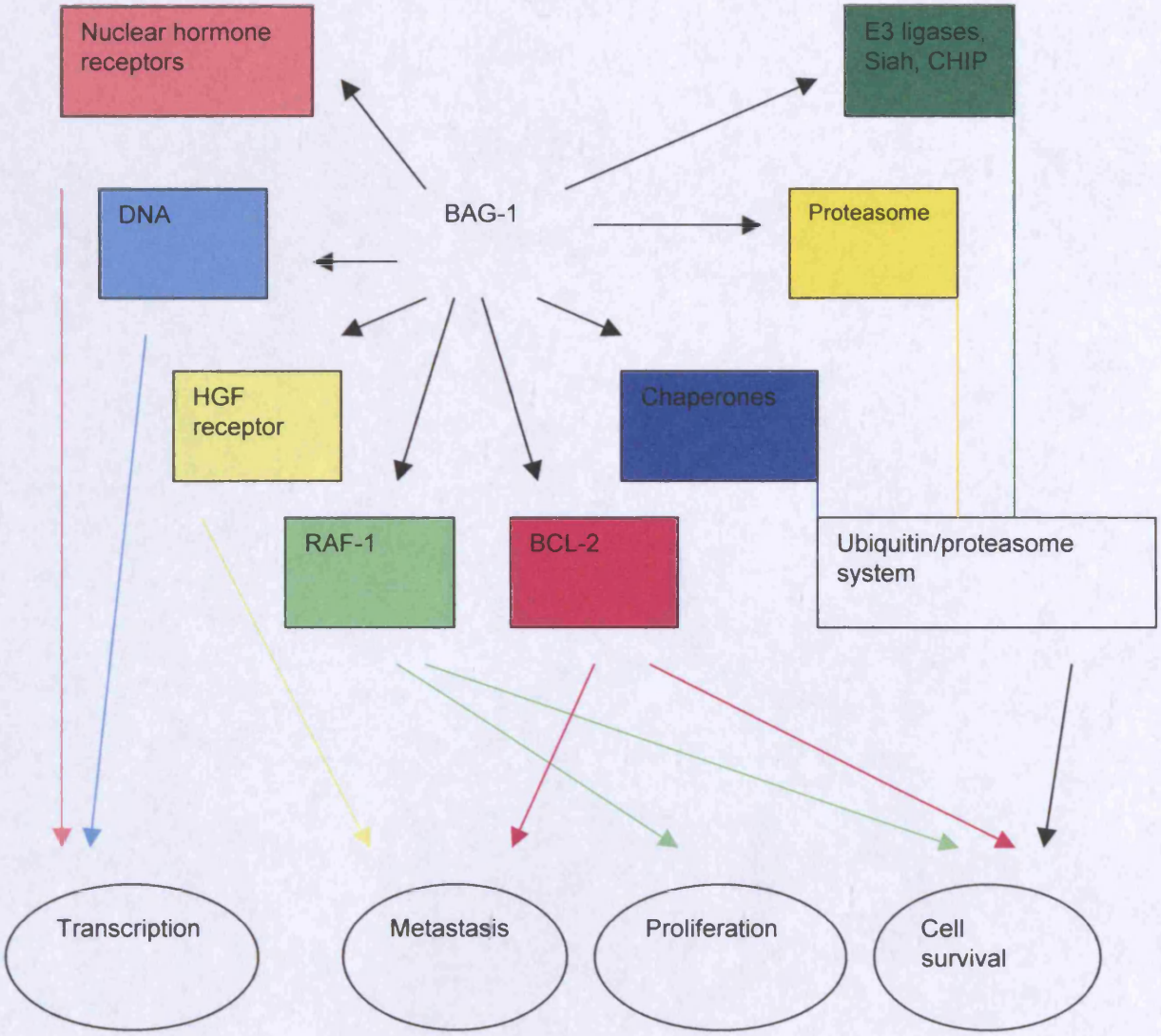


Figure 1.7. Interactions of the Bag-1 proteins.
Schematic diagram to show major interactions of the Bag-1 proteins and the cellular processes they influence. Adapted from Cutress *et al*, 2002.

1.9.1. Bag-1 can bind to DNA and stimulate transcription

The p46 and p50 isoforms of Bag-1 have been shown to bind to DNA and activate transcription when localized in the nucleus (Zeiner *et al.*, 1999; Niyaz *et al.*, 2001). The p46 isoform is predominantly cytoplasmic at 37°C and in the cytoplasm its primary role is probably regulation of hsp70 activity. The p46 protein is redistributed to the nucleus after heat shock, possibly in conjunction with hsp70 and can then activate transcription (Niyaz *et al.*, 2001). P46 and p50 can bind to DNA from various different origins and therefore act as general DNA binding proteins. (Zeiner *et al.*, 1999). P46 can also bind and enhance the transcriptional activity of the Cytomegalovirus (CMV) early gene promoter under unstressed conditions (Takahashi *et al.*, 2001); and can bind the JCV (human JC virus) promoter and activate viral transcription (Yang *et al.*, 1998).

Deletion analysis showed that DNA binding was mediated through the amino-terminus (Zeiner *et al.*, 1999). The p50 and p46 isoforms of Bag-1 are generally negatively charged proteins with a high proportion of acidic amino acids, however the amino-termini contains six positively charged amino-acids arranged in clusters of 3 basic residues each. Both clusters are involved in binding DNA (Niyaz *et al.*, 2001; Schmidt *et al,* 2003). The p36 isoform lacks the positively charged amino-terminus and as such is believed to be predominantly involved in hsp70-mediated protein refolding pathways (Niyaz *et al.*, 2001). Increased expression of the p46 and p50 isoforms of Bag-1 makes cells more resistant to heat stress by enhancing expression of molecular chaperones like hsp70 and compensating for general shutdown of transcriptional activity (Zeiner *et al.*, 1999; Niyaz *et al.*, 2001). Stressful conditions may lead to the selection of subpopulations with an increased level of Bag-1 proteins, this may provide one explanation for the overexpression of

p50 in cancer cells as it makes them better able to cope with stress (Niyaz *et al.*, 2001).

1.9.2. The role of Bag-1 in the heat shock protein chaperone system.

All three major Bag-1 isoforms have been shown to interact directly with the ATPase domain of the hsp70 family of heat shock proteins via their conserved C-terminal BAG domain (Luders *et al.*, 2000). Heat shock proteins act as chaperones and mediate the correct assembly of other proteins. Their roles include generation of the correct folding of steroid receptors for hormone binding and refolding of denatured or misfolded proteins (Schneikert *et al.*, 2000). The interaction of the Bag-1 isoforms with the 70kDa heat shock proteins is an appealing explanation for the interaction of Bag-1 proteins with a diverse range of proteins, with the 70kDa heat shock proteins acting as an intermediary.

1.9.2.1. The role of hsp70/hsc70 in steroid hormone binding

Some steroid receptors, for example the glucocorticoid receptor (GR), must bind to hsp90 in order to attain the correct conformation for ligand binding. A complex including the hsp90 chaperone directs ATP-dependent partial unfolding of GR ligand to open a hydrophobic steroid binding cleft, which can then be accessed by steroid. Initially, hsp70 binds the GR through its carboxyl terminus and is converted to a state that binds hsp90; this process is ATP-dependent and requires hsp40, a protein that accelerates ATP hydrolysis. In the second step, hsp90 is converted from its ADP to ATP-bound form and conversion of the GR to its steroid-binding form generally occurs using hsp90, Hop (hsp organizer protein) and p23, although hsp90, hsp70 and ATP alone are sufficient for refolding the GR (Rajapandi

et al., 2000). This system can be reconstituted efficiently from the 5 proteins, hsp90, hsp70, Hop, p23 and hsp40, which form a heterocomplex.

Bag-1 has a regulatory role as a co-chaperone in the hsp90-based chaperone system (Gebauer *et al.*, 1997; Takayama *et al.*, 1999). Another co-chaperone for this system is the hsc70-interacting protein (Hip) (Hohfëld *et al.*, 1995). The carboxyl-terminal BAG domain of Bag-1 binds to the amino-terminal ATPase domain of hsp70 and accelerates nucleotide exchange (Zeiner *et al.*, 1997). The reported effect of Bag-1 on the hsp90-chaperone system varies according to the system used. In the majority of cases, Bag-1 has been shown to inhibit the protein-refolding pathway, however a study comparing p46 and p36 suggests these two isoforms may have opposing effects. Both isoforms stimulate the ATPase activity of hsp70, in association with hsp40 (Luders *et al.*, 2000). P46 has been found to inhibit the hsp70 chaperone system by locking misfolded proteins into a state that does not allow refolding (Zeiner *et al.*, 1997). P36, in contrast, was proposed to promote the folding activity of hsc70 in a concentration dependent manner (Luders *et al.*, 2000). It was proposed that transient interactions made by the different amino termini of the Bag-1 proteins with the substrate could be responsible for their opposing effects (Luders *et al*, 2000).

An additional study indicated that the effect of the p36 isoform of Bag-1 on the hsp90-chaperone system is concentration dependent. At the levels of Bag-1 present in rabbit reticulocyte lysate, which are very low compared to Hip and the components of the heterocomplex, there is no influence of Bag-1 on folding of the GR receptor into its steroid hormone binding state (Kanelakis *et al.*, 1999). At low concentrations of Bag-1, however, the release of Hop is promoted, which may cause disassembly of the hsp90 heterocomplex and promote the formation of new hsp90-Hop-hsp70 complexes with an increase in the rate of the hsp90-based chaperone

cycle (Kanelakis *et al.*, 1999). At higher concentrations approaching a molar ratio of 0.4 Bag-1: hsp70, there is inhibition of GR folding. Interestingly, Hop or Bag-1 added independently inhibit luciferase refolding but when added in conjunction, compensate for the inhibition (Gebauer *et al.*, 1998).

Hip also binds to the ATPase domain of hsp70 and stabilizes the ADP-bound state of hsp70, which binds substrate tightly. Hip and Bag-1 have been shown to compete with one another for binding to the ATPase domain of hsp70 (Gebauer *et al.*, 1997). In the case of the p46 isoform of Bag-1, Hip has been shown to oppose the inhibition of GR folding by p46, thus the two proteins have a regulatory role whereby the outcome is dependent on their relative concentrations (Kanelakis *et al.*, 2000). Hip blocks the effect of the p46 isoform of Bag-1 at a 5:1 molar ratio. There is a 10:1 molar ratio present in the rabbit reticulocyte lysate *in vitro* translation system, which would counteract inhibition by Bag-1. In transformed cells, where there is frequently overexpression of the longer isoforms, the p46 isoform of Bag-1 could inhibit receptor unfolding (Kanelakis *et al.*, 2000).

1.9.2.2. Translational inhibition by heat shock

Cells may be subjected to stress by a heat-shock elevating their temperature 5-10 degrees above their optimal temperature (Schneider *et al.*, 2000). The heat shock response serves to protect cells against death induced by heat stress and to allow rapid recovery. The heat shock response itself is not limited to heat stimuli but also operates during other stress conditions such as cold and oxygen deprivation (hypoxia). As a rapid response is required to a heat stress stimulus, there is generally control at the translational level and mRNA levels or stability are not normally altered (Schneider *et al.*, 2000). Consequently, there is a large reduction in global protein synthesis within minutes as a result of heat shock to a cell. Many

proteins are denatured (partially unfolded) by heat stress and inhibition of protein synthesis therefore prevents accumulation of denatured or misfolded proteins (Joshi-Barve *et al.,* 1992). There is a concurrent preferential translation of heat shock proteins.

The mechanisms by which cap-dependent translation is inhibited occur primarily at the level of initiation, by phosphorylation and sequestration of initiation factors. The initiation factor eIF4G is sequestered into insoluble granules by hsp27, this serves two purposes; firstly, inhibiting cap-dependent translation by limiting the amount of available eIF4G and secondly, as eIF4G is still functional, it may be released in the recovery from heat shock in order to rapidly restore protein synthesis to normal (Cuesta *et al.*, 2000). In addition, the availability of eIF2 may be limited in some cell types by phosphorylation of eIF2α (Duncan and Hershey, 1984; Joshi-Barve *et al.*, 1992). Formation of the cap-binding complex is further inhibited by dephosphorylation and possible inactivation of the cap-binding protein eIF4E (Duncan and Hershey, 1984; Duncan *et al.*, 1987). There is also hypophosphorylation of the 4E-BPs, which sequester eIF4E, reducing the available eIF4F complexes. Impaired activity of eIF3 and eIF4B may also contribute to the reduction in protein synthesis (Joshi-Barve *et al.*, 1992). All of these changes are reversible, allowing rapid restoration of the normal rate of protein synthesis after heat shock.

The other key change in protein synthesis during heat shock is the continued and elevated production of the heat shock proteins. The heat shock proteins hsp70 and hsp27 are primarily responsible for thermotolerance and protection of the protein synthesis apparatus in cells. The inducible forms of hsp70 are under the transcriptional control of the heat shock factor (HSF) as well as a number of physiological processes such as cell cycle control, proliferation and differentiation

(Morimoto *et al.*, 1998). Hsp70 and hsp90 are among a group of mRNAs resistant to eIF4E depletion and hsp90 and hsp70 shift from light to heavy polysomes in cells heat-shocked to 43°C, generating a massive increase in hsp70 synthesis (Joshi-Barve *et al.*, 1992). In addition, large deletions in the 5'UTR of heat shock proteins abolish their translation at high temperatures (Joshi-Barve *et al.*, 1992). This evidence suggests a translational mechanism is at least partly responsible for upregulation of heat shock proteins as part of the heat shock response. It was originally suggested that hsp70 may be translated by ribosomal shunting, reliant on sequences within the 5'UTR that are complementary to the 18S ribosomal subunit (Yueh and Schneider, 2000). Recent evidence, however, indicates that the sequences complementary to the 18S ribosome may be part of a much larger recognition site for the ribosome, in that an IRES is present in the 5'UTR of the hsp70 mRNA (Rubtsova *et al.*, 2003). There is not yet a general consensus as to whether hsp70 mRNA is translated by ribosomal shunting or IRES-driven translation.

The heat shock proteins act as molecular chaperones. During recovery from heat shock, the ATP-dependent chaperones hsp40, hsp60, hsp70 and the hsp100 family participate in refolding protein intermediates (Cuesta *et al.*, 2000). Misfolded/ aggregated proteins may be degraded by the ubiquitin proteasome pathway, which is linked to the heat shock chaperone network (Cuesta *et al.*, 2000). The heat shock chaperones limit protein denaturation and enhance the thermoresistance of the cell.

During normal cellular circumstances, hsp70 recognises and binds to extended hydrophobic segments in substrates through the carboxyl-terminal peptide domain while the N-terminal ATPase domain regulates the binding process (Schneikert *et al.*, 1999). Substrates interact transiently with the ATP bound form of

hsp70 but this binding is stabilised when ATP is hydrolysed (Schneikert *et al.*, 1999), a process which is regulated by the various co-chaperones, including Bag-1. The heat shock proteins chaperone new/distorted proteins folding into shape, shuttle proteins from one compartment to another and transport old proteins to be degraded (Adachi *et al.*, 1996).

It has recently been shown that the IRES identified in the p36 isoform of Bag-1 can maintain synthesis of p36 during heat shock (Coldwell *et al.*, 2001). This suggests that expression of Bag-1 is required during heat shock, in addition to continued expression of hsp70, for the cell's heat shock response.

1.9.2.3. Bag-1 and the proteasome

Molecular chaperones such as the heat shock proteins recognize hydrophobic regions exposed on unfolded proteins and stabilize non-native conformations. This prevents formation of insoluble protein aggregates and promotes folding of the proteins to their native states. Energy dependent proteases such as the 26S proteasome degrade irreversibly degraded proteins that fail to be folded properly, preventing accumulation of misfolded proteins. In the process of ubiquitylation, polyubiquitin chains are attached to a protein substrate. Polyubiquitin chains are normally sufficient to target a protein to the proteasome where deubiquitylation, unfolding and degradation occur (Alberti *et al.*, 2002). CHIP is an hsp70/90 associating ubiquitin ligase that labels chaperone-presented proteins that are to be degraded with ubiquitin (Alberti *et al.*, 2002).

Bag-1 acts as a coupling factor between the molecular chaperones and the proteasomal complex. Bag-1 accepts substrates from hsp70 and presents them to CHIP (Demand *et al.*, 2001). Bag-1 can stimulate release of chaperone substrates from hsp/hsc70 due to its nucleotide exchange activity, which could stimulate the

release of substrate to the proteasome. Bag-1 and CHIP co-operate to switch the activity of hsc/hsp70 from protein folding to protein degradation (Demand *et al.*, 2001).

In addition to their role in the protein folding response and degradation pathways, the interaction of Bag-1 proteins with the 70kDa heat shock proteins has significance for its interaction with many other proteins and cellular processes. Bag-1 proteins readily form ternary complexes with the hsp70 heat shock proteins and other proteins such as steroid hormone receptors (Stuart *et al.*, 1998; Gebauer *et al.*, 1997).

1.9.3. The role of Bag-1 in apoptosis

Apoptosis or programmed cell death is a vital cellular process, disruption of which has implications for uncontrolled cell proliferation and progression of cancer. Bag-1 interacts with multiple cellular targets and suppresses apoptosis induced by a wide range of stimuli. Bag-1 was originally identified by virtue of its interaction with Bcl-2, where it was found to promote the anti-apoptotic properties of this protein, blocking a step in the apoptotic pathway (Takayama *et al.*, 1996).

1.9.3.1. Apoptosis

The Bcl-2 family proteins are key regulatory proteins in the process of apoptosis. Bcl-2 family members can be pro-apoptotic (e.g. Bax, Bad, Bid), or anti-apoptotic (Bcl-2, BCl-XL, BCl-w, Boo) and the ratio of these proteins is a key determinant of the progression of apoptosis. These proteins act on the apoptotic cascade at the level of caspase activation (Hengartner *et al.*, 2000; Cryns and Yuan, 1998).

Caspases are cysteine-dependent aspartate-specific proteases, which exist in the cell as inactive pro-forms or zymogens and require cleavage in order to be activated. Initiator caspases are activated at induction of apoptosis (reviewed in Chen and Wang, 2002) and activate effector caspases by cleavage. Effector caspases mediate the effects of apoptosis such as cleavage of cellular substrates like cytoskeletal structural proteins, nuclear proteins and translation initiation factors or activation of degradative enzymes such as DNases. Caspases degrade key targets required for cell and nuclear integrity including lamin A, hnRNPs, poly (ADP-ribose) polymerase and DNA-dependent protein kinase (reviewed in Cryns and Yuan, 1998).

There are two distinct caspase cascades, which are activated according to the apoptotic stimuli applied (Sun *et al.*, 1999). The mitochondrial pathway occurs in response to a variety of cell stresses such as heat shock, irradiation, osmotic shock, DNA damage or treatment with chemotherapeutic drugs amongst others. The extent of cell stress is a major determinant of whether apoptosis will be induced. Pro-apoptotic Bcl-2 family proteins in the cytosol relocate to the mitochondrial surface upon cell stress and interact with anti-apoptotic proteins. Dependent on the ratio of pro- to anti-apoptotic proteins, the function of anti-apoptotic proteins may be disrupted. This leads to the generation of pores in mitochondrial membranes and subsequent release of cytochrome c (reviewed in Gottelib, 2000). Cytochrome c released from the mitochondria complexes with Apoptotic Protease activating factor 1 (Apaf-1) and pro-caspase-9. In an ATP-dependent reaction, caspase-9 is activated by cleavage Activated caspase-9 can cleave and activate effector caspases such as caspase-3, which triggers the apoptotic cascade (Cain *et al.*, 1999; Saleh *et al.*, 1999)(**figure 1.8.**).

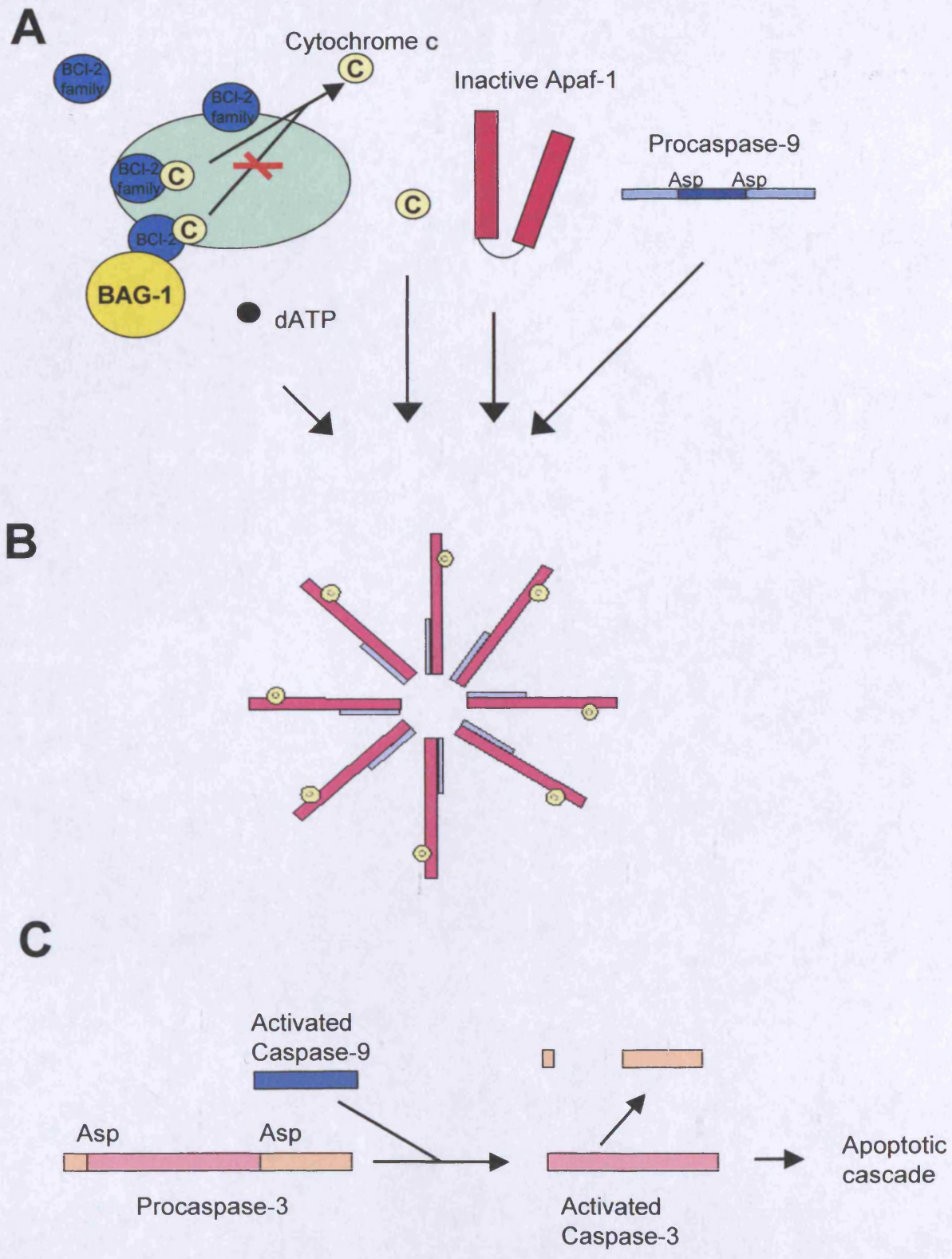


Figure 1.8. Activation of the caspase cascade by the mitochondrial pathway

A. Cytochrome c is released from the mitochondria in response to an apoptotic stimulus. This step can be blocked by anti-apoptotic BCl-2 family members (red cross). Bag-1 can increase the anti-apoptotic properties of BCl-2.

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 the prodomain from procaspase-3.

The death-receptor mediated pathway occurs in response to signals such as binding of death-inducing ligand to cell surface receptors such as Fas or the tumour necrosis factor (TNF) receptor, or by cytotoxic T-lymphocytes, which induce apoptosis of damaged or virally infected cells (reviewed in Hengartner *et al.*, 2000; Cryns and Yuan, 1998). In receptor-induced apoptosis, transmembrane cell surface receptors such as Fas, TNF-receptor 1 (TNFR1) or TRAIL receptors such as Death receptors 4 and 5 (DR4/5) are bound by their respective ligand, for example, the TNF-related apoptosis-inducing ligand (TRAIL). Induction of apoptosis after ligand binding is very rapid. The death receptors have death domains (DDs) on their intracellular domains, DDs aggregate on ligand binding and adaptor molecules such as Fas-associated death domain protein (FADD) bind. This complex can recruit other proteins, generating a death inducing signalling complex (DISC). The DISC can recruit the initiator pro-caspase-8 and activate it by cleavage (Hengartner *et al.*, 2000; Cryns and Yuan, 1998). Caspase-8 can activate effector caspases and can also cleave Bid, this releases the carboxyl-terminal domain which can translocate to the nucleus where it interacts with Bcl-2 family proteins and can cause the release of cytochrome c from the mitochondria, resulting in an amplification of apoptosis (Hengartner *et al.*, 2000).

1.9.3.2. Translational inhibition by apoptosis

Upon application of an apoptotic trigger to cells one of the most critical events is a rapid and significant decrease in protein synthesis. This inhibition of translation can be attributed to cleavage and alterations in the phosphorylation state of a number of initiation factors. Caspases cleave eIF4G (Clemens *et al.*, 1998; Morley *et al.*, 1998), one subunit of eIF3 (Bushell *et al.*, 2000), and eIF4B (Bushell *et al.*, 2000), inhibiting protein synthesis. In addition, increased phosphorylation of

eIF2 (Bushell *et al.*, 2000) and dephosphorylation of eIF4E (Bushell *et al.*, 2000) contribute to the global inhibition of protein synthesis.

A number of IRESes have been identified which are functional during apoptosis; these include pro-apoptotic proteins such as Apaf-1 (Mitchell *et al.*, 2001), DAP5 (Henis-Korenblit *et al.*, 2000) and c-*myc* (Stoneley *et al.*, 2000); and anti-apoptotic proteins such as XIAP (Holcik *et al.*, 1999; Holcik *et al.*, 2000). These IRESes maintain production of key proteins required for the progression or inhibition of apoptosis.

1.9.3.3. The roles of Bag-1 in apoptosis

Bag-1 has been shown to increase the anti-apoptotic properties of Bcl-2 under a range of apoptotic stimuli (Takayama *et al.*, 1995). Bcl-2 can inhibit the release of cytochrome c from mitochondria by localising to mitochondrial membranes and, together with other Bcl-2 family members, is believed to influence the formation of ion channels in membranes (reviewed in Gottleib, 2000). This could consequently influence the permeability of intracellular membranes resulting in prevention of the release of cytochrome c and also inhibits the release of calcium from the endoplasmic reticulum (ER). Bcl-2 can also act later in apoptosis, by binding to Apaf-1 and inhibiting the formation of the Apaf-1/procaspase-9 complex and also inhibits progression through the cell cycle (Schorr *et al.*, 1999). Bag-1 can overcome Bcl-2 mediated enhancement of exit from the cell cycle and interacts with Bcl-2 to co-operatively interfere with the apoptotic cascade at the level of caspase activation (Takayama *et al.*, 1995). Bcl-2 and Bag-1 act just before the release of cytochrome c step (**figure 1.8**).

Bag-1 can also inhibit apoptosis through interaction with growth factors and steroid receptors. Bag-1 forms complexes with a number of hormones and growth

factor receptors and modulates their ability in apoptosis, generally enhancing growth factor mediated resistance to apoptosis (Yang *et al.*, 1999). The C-terminus of Bag-1 is required to bind growth receptors but the whole of Bag-1 is necessary to protect against apoptosis. Perhaps the best-studied example of the prevention of apoptosis through binding of Bag-1 to steroid hormone receptors is in conjunction with the retinoic acid receptor (RAR). Retinoids are used as anti-cancer drugs due to their roles in inhibition of cell proliferation, induction of cell differentiation and promotion of apoptosis (Liu *et al.*, 1998). Retinoic-acid and retinoic-X receptors heterodimerise and bind retinoic-acid response elements to regulate transactivation (Liu *et al.*, 1998).

Bag-1 interacts with the RAR but not the RXR and prevents heterodimerisation, therefore suppressing retinoic-acid induced transactivation of RARs on RAREs (Liu *et al.*, 1998).

Bag-1 interacts with a range of growth factor receptors such as the hepatocyte (HGF) heparin-binding EGF-like growth factor receptor and PDGF receptors and enhances their anti-apoptotic function (Bardelli *et al.*, 1996; Stuart *et al.*, 1998; Lin *et al.*, 2001). EGF gene expression is upregulated in response to cell stress, consistent with its cytoprotective function (Lin *et al.*, 2001). The Bag-1/EGF interaction isn't favoured when cells undergo programmed cell death as Bag-1/ EGF transfected cells demonstrate increased resistance to apoptosis induced by etoposide, a topoisomerase inhibitor, in comparison to cells expressing Bag-1 or EGF (Lin *et al.*, 2001).

The role of Bag-1 in the prevention of apoptosis in a variety of systems suggests Bag-1 may function as an adaptor to mediate the interaction between survival factors and apoptotic machinery.

1.9. Bag-1 provides protection against cell death

The ERK (extracellular signal-related kinases) survival pathway over-rides apoptotic signals by upregulating expression of anti-apoptotic members of the Bcl-2 family and has been shown to upregulate expression of Bag-1 (Perkins *et al.*, 2003). Interestingly, Bag-1 itself binds to Raf-1, a protein kinase in the ERK pathway (Wang *et al.*, 1996). Bag-1 has been shown to activate the kinase activity of Raf-1, therefore providing a potential feedback loop for the ERK survival pathway (Perkins *et al.*, 2003). Bag-1 interacts with Raf-1 and hsp70 at partially overlapping sites therefore their binding to Bag-1 is competitive (Song *et al.*, 2001). When levels of hsp70 are elevated after heat shock, Raf-1 is displaced by hsp70 for binding to Bag-1 and DNA synthesis is arrested (Song *et al.*, 2001). Bag-1 may therefore also function in the heat shock response to co-ordinate cell growth signals and mitogenesis (Song *et al.*, 2001).

Overexpression of Bag-1 has been shown to protect cells against apoptosis and growth-inhibition induced by a variety of cellular stresses, this may be mediated by its effects on the ERK survival pathway and, in addition, the role of Bag-1 in the heat shock response. Overexpression of Bag-1 protected both breast and cervical cancer cell lines, MCF7 and C33A respectively, against apoptosis induced by genotoxic stress resulting from treatment with chemotherapeutic agents (Chen *et al.*, 2002; Townsend *et al.*, 2003). Bag-1 overexpression was also found to protect cells against stress induced by hypoxia, heat shock and radiation treatment (Townsend *et al.*, 2003). Protection induced by Bag-1 was found to require the ubiquitin-like domain, which is important in association of Bag-1 with the proteasome. In addition, the interaction of Bag-1 with the 70kDa heat shock proteins is important in maintaining the protective properties of Bag-1 (Townsend *et al.*, 2003). The Bag-1 IRES is functional during heat shock and could consequently contribute to the cell

survival pathways during heat shock by maintaining production of Bag-1 protein. It would consequently be of interest to determine whether the Bag-1 IRES is functional during other cellular stresses to maintain production of the pro-survival protein, Bag-1.

1.11. Overexpression of Bag-1 in cancers

The role of Bag-1 in apoptosis, its interactions with various growth factors, activities as a transcription factor and protective properties against cell stress lead to important implications for oncogenesis and radio/chemotherapy resistance. Bag-1 is overexpressed in many human tumours including breast, cervical, colorectal carcinomas, thyroid neoplasms, lung and gastric cancers tumours (Yang *et al.*, 1998; Yang *et al.*, 1999; Kikuchi *et al.*, 2002; Ito *et al.*, 2003; Yao *et al.*, 2002; Yawata *et al.*, 1999). Overexpression of Bag-1 suppresses apoptosis, promotes cell migration and modulates hormone-dependent transcription (Townsend *et al.*, 2002).

Overexpression of Bag-1 may lead to inhibition of apoptosis induced by cancer therapies. Bag-1 has been shown to protect cells against a variety of anti-cancer drugs (Chen *et al.*, 2002; Townsend *et al.*, 2003). Bag-1 can also inhibit apoptosis induced by anti-cancer drugs through its ability to modulate steroid hormone receptors. Retinoids are used as a cancer treatment as a result of their ability to induce apoptosis in cancer cells and to inhibit cell proliferation (Liu *et al*, 1998). Bag-1 increases resistance to retinoic-acid induced apoptosis by binding to the retinoic acid receptor and consequently preventing transactivation from this receptor (Liu *et al*, 1998). The oestrogen receptor (ER) is a target for some cancer therapies such as tamoxifen. There is a correlation between overexpression of Bag-1 and the oestrogen receptor in breast cancers (Brimmel *et al.*, 1999). Bag-1 potentiates the activity of ER, which mediates proliferative and survival responses

to oestrogen in hormone dependent breast cancers (Cutress *et al.*, 2002). The Androgen receptor (AR) is important in prostate cancer and Bag-1 increases sensitivity of AR expressing cells to androgens and decreases sensitivity to cypoterone acetate, an anti-androgen used clinically in the treatment of prostate cancer (Froesch *et al.*, 1998). Thus, the presence or absence of Bag-1 could be a major influence in determining the type of therapy most appropriate for certain cancers.

1.12. Project aims

The knowledge that the p36 isoform of Bag-1 can be translated by internal ribosome entry has important implications for many cellular circumstances. It has already been shown that the Bag-1 IRES is functional during heat shock but it is likely that the Bag-1 IRES can maintain production of Bag-1 under other cellular circumstances, given its importance in apoptosis and its protective properties against a range of cell stresses. One aim of my project is to investigate other cellular circumstances where cap-dependent translation is inhibited to identify whether the Bag-1 IRES is functional and to determine how potential *trans*-acting factors may influence IRES activity under such circumstances.

The mechanism, by which the Bag-1 IRES functions, as with other cellular IRESes, is poorly understood. It is of interest to begin to elucidate the mechanisms of action of cellular IRESes, particularly given that the cellular IRESes identified so far allow translation of products that are frequently aberrantly regulated in cancers. Knowledge of the mechanisms by which individual IRESes or groups of IRESes function will allow targeting of specific mRNAs in certain cell types without affecting expression of the protein in other cell types. Interference with IRES activity is already a growing field of drug design, particularly by interfering with

viral IRES translation such as that from HIV and HCV (Ekblom, 2001). The main aims of my project are to study the mechanism of action of the Bag-1 IRES with regards to its secondary structure and *trans*-acting factor requirements, in addition to assess the effect of putative *trans*-acting factors on the secondary structure of the Bag-1 IRES.

Chapter 2

Materials and Methods

2.1. General reagents

2.1.1. Reagents and equipment suppliers

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

2.1.2. Antibodies

Bag-1 was detected using a mouse monoclonal antibody at a dilution of 1:1000 (Neomarkers). Actin was detected with a mouse monoclonal antibody at a dilution of

1:2000 (Sigma). Rabbit antibodies to PCBP1 were kindly provided by Dr. R. Andino. Polyclonal rabbit antibodies against PTB and unr were raised in the Jackson laboratory. Rabbit antibodies to eIF4E, eIF4G and eIF4A were kindly provided by Dr. Simon Morley of the University of Sussex. Secondary antibodies raised against mouse and rabbit IgG and conjugated to horseradish peroxidase were obtained from Sigma and used at a dilution of 1:5000.

2.2. Tissue Culture Techniques

2.2.1. Tissue culture solutions

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

2.2.2. Cell Lines

Cell lines CAL51 and GI-101 were kind gifts from Dr. Graham Packham (CRC Wessex Medical Oncology Unit, Southampton General Hospital). All others were purchased from the American Type Culture Collection (ATCC). Details of the cell lines used can be found in **table 2.1.**

Name	Cell Type
HeLa S3	Human cervical epithelioid carcinoma
HEK293	Human embryonic kidney cell line immortalised with adenovirus DNA
MCF7	Human breast carcinoma
Cos-7	Monkey epithelial cell line (CV-1) immortalised with SV40 DNA
Cama-1	Human breast cancer
CAL51	Human breast carcinoma
GI-101	Human breast carcinoma
CALU1	Human lung cancer

Table 2.1. Descriptions of cell lines used.

2.2.3. Maintenance of cell lines

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

2.2.4. Transfection of cell lines

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

2.2.4.1. Fugene-6 mediated DNA transfection

Cells were transfected using FuGene 6 (Roche) as specified by the manufacturer. Approximately 20 hours prior to transfection, 1 X 10^5 cells were seeded into each well of a 6-well plate in 1ml of complete media. 3µl of Fugene-6 was added to 97µl of serum-free DMEM media per well and incubated at room temperature for five minutes, following which the solution was added to 1µg of plasmid DNA, and 0.2µg pcDNA3.1/*lacZ* transfection control. The DNA/Fugene-6 reaction mixture was incubated for a further fifteen minutes at room temperature and added drop wise to the cells. Cells were harvested 40 hours after transfection. Unless otherwise indicated, all

transfections were performed in duplicate. Error bars represent standard deviations of three independent experiments. Where observed differences between data sets were small, a students *t*-test was performed to ascertain the significance of the difference. The probability of such differences being observed by chance are indicated in brackets where $P < 0.05$ is taken to represent a significant difference.

2.2.4.2. Lipofectin mediated RNA transfection

Cationic liposome-mediated RNA transfection of mammalian cells was carried out essentially as described by Dwarki *et al.*, 1993. Cells were seeded as described in section 2.2.4.1. The medium was aspirated after 20 hours, the cells washed with PBS and the medium replaced with 1 ml of Opti-MEM I reduced serum medium (GIBCO-BRL) supplemented with 10mM glutamine. The cells were incubated at 37°C while the transfection mix was assembled. 12.5µg of Lipofectin was added to 1ml of Opti-MEM medium containing 2mM L-glutamine, and the mixture incubated at room temperature for 20 minutes. 5µg of capped, polyadenylated RNA was added to the transfection mixture and incubated for a further 10 minutes at room temperature. The medium was aspirated from the cells and replaced with the transfection mix. Cells were returned to 37°C and harvested 8 hours after transfection. Transfections were performed in duplicate. Error bars represent standard deviations as determined from three independent experiments.

2.2.5. Induction of apoptosis and cell stress

2.2.5.1. TRAIL induced apoptosis

The TNF-related apoptosis inducing ligand TRAIL induces apoptosis via the death receptor-mediated pathway of apoptosis. HeLa cells were plated into 6-well plates, as described previously, and transfected by the Fugene-6 method. 40 hours post-transfection, 1μg/ml of His-tagged TRAIL was added to the medium. TRAIL protein was purified from BL21 cells transformed with the plasmid pET28b (A kind gift from Marion MacFarlane, University of Leicester). Cells were harvested at set time-points after TRAIL treatment.

2.2.5.2. Induction of cell stress by chemotherapeutic agents

HeLa cells were plated into 6-well plates, as described previously, and transfected by the Fugene-6 method. 24 hours post-transfection, 1, 2 or 4nM of vincristine was applied to cells. 24 hours later, cells were lysed and assayed for luciferase activity. Alternatively, 24 hours post-transfection, 1, 2, 4, 10 or 20nM cis-platinum(II)diamine dichloride(cisplatin) were applied to cells for 24 hours prior to harvesting.

2.2.5.3. Serum starvation

To induce growth inhibition by serum starvation, cells were transfected as before. 24 hours post-transfection, the medium was aspirated and cells washed extensively with PBS to remove serum. The medium was replaced with fresh medium

containing 10%, 0.5% or no serum. Cells were grown for a further 24 hours prior to harvesting.

2.3. Bacterial Methods

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

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

2.3.1. Preparation of competent cells

A single bacterial colony was inoculated into 2.5ml of LB medium and incubated overnight at 37°C in a shaking incubator. The entire overnight culture was used to inoculate 250ml of LB medium supplemented with 20mM $MgSO_4$. The culture was incubated at 37°C in a shaking incubator until A_{595} = 0.4-0.6. Cells were pelleted by centrifugation at 4500x *g* for 5 min at 4°C. The pellet was resuspended in 100ml ice-cold filter sterile TFB1 (30mM Kac, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% (v/v) glycerol, adjusted to pH 5.8 with 1M acetic acid). The solution was incubated on ice for 5 min. Cells were pelleted as before and resuspended in 10 ml of ice-cold filter-sterile TFB2 solution (1mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% (v/v) glycerol, adjusted to pH 6.5 with 1M KOH), incubated on ice for 1 hour. 200µl aliquots of cells were snap frozen in liquid nitrogen or dry ice/isopropanol and stored at -80°C.

2.3.2. Transformation of competent cells

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

2.4. Molecular Biology Techniques

2.4.1. Buffers and solutions

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

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

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

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

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

2.4.2. Plasmids used

- pRF (previously designated pGL3R2), pRHRVF, pRMF, pGL3, pSp64RLPolyA. pSp64RHRVLPolyA, pRHpF (all described in Stoneley *et al.*, 2000)
- pRBF, pGBL, pRHpBF, pHpL and pHpBL (all described in Coldwell, 2001).
- pSKL (Stoneley, 1998)

- pSKGAP:E/H (Paulin, 1997)
- pJ7*lacZ* (A gift of Dr. David Heery, University of Leicester)
- pcDNA3.1/p36, pcDNA3.1/p46, pcDNA3.1/p50 (a kind gift from Paul Townsend, CRC Wessex Medical Oncology Unit, Southampton General Hospital)

For expression of His-tagged recombinant proteins used, cDNAs were cloned into PET28a vectors by Joanne Evans, enabling expression of protein in *Escherichia coli* and subsequent purification of the protein. For expression in tissue culture cells, the cDNAs were subcloned into pCDNA3.1 by Joanne Evans and for expression in insect cells (for purification of protein) subcloned into pBlueBac4 (Invitrogen) by Sally Mitchell (All described in Evans, 2003).

2.4.3. Purification of nucleic acids

2.4.3.1. Determination of nucleic acid concentrations

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

2.4.3.2. Phenol-chloroform extraction

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

mixture was centrifuged at 12,000x *g* for 3 minutes and the aqueous layer removed to a new tube prior to precipitation of the nucleic acid.

2.4.3.3. Ethanol precipitation of nucleic acids

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

2.4.4. Agarose gel electrophoresis

Electrophoresis through agarose gels was carried out to separate nucleic acids according to molecular weight. For the majority of manipulations, agarose was melted in 1 X TBE buffer, unless DNA fragments were to be eluted, in which case 1 X TAE gels were prepared. 2µl of 10mg/ml ethidium bromide was added to the melted agarose gel solution and the gel was cast in a gel tray. Samples were mixed with 5 X TBE loading buffer and loaded into the gel. Gels were run submerged in 1 X TBE or 1 X TAE buffer in a horizontal electrophoresis tank, typically at 100V, and visualised on a UV transilluminator.

2.4.5. Purification of DNA from agarose gels.

DNA fragments were excised from agarose gels and purified using a Qiaquick spin column (Qiagen). 3 volumes of buffer QG were added to the gel slice and the solution melted at 55°C. The gel solution was added to a Qiaquick spin column and

bound by centrifugation at 12,000x *g* for 1 minute. DNA fragments were washed with buffer PE by centrifugation and last traces of buffer removed by an additional centrifugation as before. DNA was eluted in 30µl of sterile deionised water by incubation on the column for one minute prior to centrifugation.

2.4.6. Oligonucleotide synthesis and purification

Oligonucleotides (**table 2.2**) were synthesised by GIBCO-BRL or PNACL (Protein and Nucleic Acid Sequencing Laboratory, Leicester University) at a scale of 0.2µM. GIBCO-BRL oligonucleotides were diluted by addition of 100µl TE (10mM Tris-HCl pH 8.0, 1mM EDTA) and concentration determined by absorbance at 260nm. PNACL oligonucleotides were ethanol precipitated (**section 2.4.3.3**). Oligonucleotides were then resuspended in 100µl TE. Concentration was determined as before (**section 2.4.3.1**).

2.4.7. Polymerase chain reaction (PCR)

PCR reactions were performed using 100ng of template in a reaction containing 1 X cloned *Pfu* buffer (Stratagene), 125ng of each primer, 0.2mM dNTPs, 1M Betaine, 2% (v/v) DMSO and 2.5 units PfuTurbo^{TM} DNA polymerase (Stratagene). Reactions were performed in a Techne Genius Thermal cycler. DNA was denatured at 94°C for three minutes and then subjected to cycling by denaturing at 94°C for 1 minute, annealing at 5° below the oligonucleotide melting temperature for 2 minutes and extending at 72°C for 1 minute. The reaction was cycled for 35 cycles and finally extended for 10 minutes at 72°C.

Oligonucleotides

Name	Sequence
GENERAL OLIGONUCLEOTIDES	
Luc3'	5' - GCGTATCTCTTCATAGCCTT - 3'
RnaseF	5' - GCAAGAAGATGCACCTGATG - 3'
BAGF	5' - TCGAATTCTGGGCGGTCAACAAGTGCGG - 3'
BAG36R	5' - ATCCATGGCTTCGCCCTGGGTCGCC - 3'
BAGSpeF	5' – GTCGACCTCGAATCACTAGTCAGC – 3'
PRIMERS FOR DELETIONS OF THE BAG-1 5' UTR	
BAG225F	5' - GCGGGAATTCCCGACCCACCAGGG - 3'
BAG281F	5' - CCGGAGCGAGGAATTCACCCTGAG - 3'
BAG358R	5' - GCCTCTTCACTCCATGGCGCTTCC - 3'
BAG411R	5' - TCTTCCATGGCTTCGCCCTGGGTC - 3'
BAG258F	5' - CAGGGGCGAATTCGGCGCTCG - 3'
BAG274F	5' - GCGCTCGAATTCCGCGGATGAAGA - 3'
BAG292F	5' - GAAGAAGAATTCCCGGCGCCGCTC - 3'
BAG312R	5' - CTCCGGGTCAACTCCATGGTCCGG - 3'
BAG290R	5' - GGCGCCATGGTTTCTTCTTCATCC - 3'
PRIMERS TO INTRODUCE OUT-OF-FRAME AUGs	
AUG1F	5' - GCCCCCGGCCCAGCATGGTCCGCCTCCCTC - 3'
AUG1R	5' - GAGGGAGGCGGACCATGCTGGGCCGGGGGC - 3'
AUG 2	5' - CGACCCAATGGGGGCGCCGCCGCCGGCGCT - 3'
AUG 2R	5' - AGCGCCGGCGGCGGCGCCCCCATTGGGTCG - 3'
AUG 3	5' - GCTCGATGGCCGCGGATGAAGAAGAAAACC - 3'
AUG 3R	5' - GGTTTTCTTCTTCATCCGCGGCCATCGAGC - 3'
AUG 4	5' - GGATGAAGAAAACATGGCGCCGCTCGACCC – 3'
AUG 4R	5' – GGGTCGAGCGGCGCCATGTTTTCTTCATCC – 3'
AUG 5	5' – AAAACCCGGCGGCGATGGACCCGGAGCGAG –3'
AUG 5R	5' – CTCGCTCCGGGTCCATCGCCGCCGGGTTTT – 3'
AUG 6	5' - GGCGCCGCTCGACATGGAGCGAGGAGTTGA - 3'
AUG 6R	5' - TCAAGTCCTCGCTCCATGTCGAGCGGCGCC - 3'
AUG 7	5' - CGAGGAGTTGACATGGAGCGAGGAGTTGAC - 3'
AUG 7R	5' - GTCAACTCCTCGCTCCATGTCAACTCCTCG - 3'
AUG 8	5' – AGGAGTTGACCCGGAATGGGGAAGCGACCT –3'
AUG 8R	5' – AGGTCGCTTCCCCATTCCGGGTCAACTCCA – 3'
AUG 9	5' - AGCGACATGGAGTGAAGAGGCGACCCAGAG - 3'
AUG 9R	5' - CTCTGGGTCGCCATTTCACTCCATGTCGCT - 3'
PRIMERS TO MUTATE THE p46 INITIATION CODON	
P46AGGF	5' - GGCCGCGGAGGAAGAAGAAA - 3'
P46AGGB	5' - TTTCTTCTTCCTCCGCGGCC - 3'
P46STOPF	5' - GGCCGCGGTAGAAGAAGAAA - 3'

P46STOPB	5' - TTTCTTCTTCTACCGCGGCC - 3'
PRIMERS TO MUTATE THE A-RICH LOOP	
Amut2F	5' – GGATGAAGAAGGGGGCCCGGCGC – 3'
Amut2B	5' – GCGCCGGGCCCCCTTCTTCATCC – 3'
PRIMERS FOR PRIMER EXTENSION	
Primer 1	5' – ATCCGCGGCCTGCGAGCGCCGGCG – 3'
Primer 2:	5' – TCAGGGTCAACTCCTCGCTCCGGG – 3'
Primer 3:	5' – GCCTCTTCACTCCAGGTCGCTTCC – 3'
Primer 4:	5' – TCGCCTCCTCACTCTGGG – 3'
Primer 5:	5' – GCGGATAGAATGGCGCCGGG – 3'

Table 2.2. Table of oligonucleotides used.

2.4.8. PCR mutagenesis

Mutagenic PCRs were carried out by performing two half-reactions using the standard PCR procedure (**section 2.3.6**). The regions 5' and 3' of the mutation to be introduced into the Bag-1 5'UTR were amplified using the primer pairs BAGF/ mutant reverse primer and BAG36R/ mutant forward primer. PCR products were gel isolated and 1µl of a 1/500 dilution of each product was used as the template for a PCR reaction using the primer pair BAGF/ BAG36R. The mutant Bag-1 5'UTR produced was digested with *Eco*RI and *Nco*I and ligated into the vector pRF.

2.4.9. Restriction enzyme digestion

DNA was digested with restriction enzymes using buffers and quantities recommended by the manufacturers. The reaction volume was typically 10-50µl and the reaction mixture was incubated at the recommended temperature for 1-2 hours. For digests with restriction sites occurring close to one another or requiring different buffers, the DNA was incubated with one enzyme, the enzyme deactivated by heat inactivation at 65°C for 10 min and the DNA extracted by phenol-chloroform extraction, ethanol precipitated and incubated with the second enzyme. Following the digestion period, the restriction enzymes were deactivated as before.

2.4.10. Alkaline phosphatase treatment of DNA for subcloning

Prior to ligation, linearised plasmids were treated with calf intestinal alkaline phosphatase (CIAP) to remove the 5' phosphate in order to prevent re-annealing of the plasmid vector. Dephosphorylation was performed in a final reaction volume of 50µl

with 1 unit of CIAP and 1 X CIAP buffer (MBI Fermentas). The reaction mixture was incubated at 37°C for 15 min followed by a 15 min incubation at 56°C. The reaction was terminated by heat inactivation at 75°C for 10 min.

2.4.11. Ligations

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

2.4.12. Preparation of plasmid DNA

2.4.12.1. Mini-preparations

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

washed in 70% ethanol, air-dried and re-suspended in 30µl sterile deionised water. 5µl of prepared plasmid DNA was digested with restriction enzymes to identify successful clones.

2.4.12.2. Maxi-preparations

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

centrifugation as before. The pellet was washed with 70% ethanol, air-dried and resuspended in 0.5ml sterile deionised water.

2.4.12.3. Preparations by Qiagen/Wizard columns

To produce plasmid DNA suitable for transfection, the Wizard plasmid purification kit (Promega) was used to produce up to 30μg of DNA. Alternatively, the Qiagen midi/mega kit was used to produce larger quantities of plasmid DNA according to the manufacturer's instructions.

2.4.13. Double-stranded DNA sequencing

Plasmid DNA was denatured by incubation with 0.1 volumes of 2mM NaOH, 2mM EDTA, pH 8.0 at 37°C for 15 mins. The solution was then neutralised with 0.1 volumes of 7.5M NH_4Ac, pH 7.4, and 1 volume of isopropanol was added. Following incubation at room temperature for 10 minutes, the single stranded DNA was pelleted by centrifugation at 12,000x *g* for 10 minutes and air-dried. The pellet was resuspended in 10μl of a 2.5ng/μl solution of sequencing primer and 2μl of annealing buffer (280mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 350mM NaCl). The plasmid DNA/primer solution was heated at 65°C for 10 minutes, and then incubated at 37°C for 10 minutes, followed by 5 minutes on ice to achieve primer annealing. Samples were labelled at 20°C for 5 minutes, in a reaction containing 0.4μl [α-^{35}S] dATP (12.5mCi/ml), 3μl of labelling mix A (2μM dGTP, 2μM dCTP, 2μM dTTP), and 1 unit of T7 DNA polymerase. Labelling was terminated by the addition of 2.5μl of each termination mix (150μM of each dNTP, 10mM $MgCl_2$, 40mM Tris-HCl, pH 7.5, 50mM NaCl, 15μM ddNTP G, A, T, or C) and incubated at 42°C for 5 minutes.

Finally, the reaction was stopped by adding 4μl of formamide loading dyes. The labelled DNA fragments were fractionated on a 6% polyacrylamide/7M urea gel following which the gel was dried under vacuum for 1 hour at 80°C and exposed to a phospor screen (Molecular Dynamics) overnight or for several days. Alternatively, DNA sequencing was carried out using a sequencing kit (USB), according to the manufacturer's instructions.

2.4.14. 5' end-labelling of oligonucleotides

Oligonucleotides were 5' end-labelled by treatment with T4 polynucleotide kinase (PNK, MBI Fermentas). Reactions were performed in a final reaction volume of 50μl containing 5pmol oligonucleotide, 1 X T4 PNK buffer, 10μl [^{32}P]-γ-ATP (0.37MBq/μl) and 10 units of T4 PNK. The kinase reaction was incubated at 37°C for 30 min and terminated by heat inactivation of the enzyme at 75°C for 10 min. Unincorporated nucleotide was removed by passage through a Sephadex G-50 column. Concentration of the radiolabelled oligonucleotides was determined by Cerenkov schintillation counting.

2.5. RNA methods

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

2.5.1. *In vitro* run-off transcription

Template DNA was linearised from the appropriate plasmid by restriction digestion using a site downstream of the region of interest (*Hpa*I for dicistronic

constructs, *Nco*I for monocistronic). Capped transcripts were synthesised in a reaction mixture containing 1 X transcription buffer (MBI Fermentas), 40 U RNAguard or RNasin, 1mM ATP, 1mM UTP, 1mM CTP, 0.5 mM GTP, 1μM m^7G(5')ppp(5')G, 1 μg DNA template and 20 U T7 or T3 RNA polymerase to a final volume of 50μl. Uncapped transcripts were synthesised using the same reaction mixture with the exception of adding a total of 1mM GTP and no m7G(5')ppp(5')G. The reaction mixture was incubated at 37°C for 1 hour, following which a further 10 units of RNA polymerase were added and the incubation continued for 30 minutes. The DNA template was digested with 10 units of RNase-free DNase I for 15 min at 37°C. The RNA was purified by phenol/chloroform extraction and passaged through a Sephadex G-50 column (Amersham) to remove unincorporated nucleotide. The RNA was ethanol precipitated and resuspended in 50μl of sterile deionised water. 5μl of the RNA was subjected to agarose gel electrophoresis to ensure the integrity of the product.

For radiolabelled RNAs, CTP was replaced with 50μCi [α-^{32}P]CTP. For electrophoretic mobility shift assays, with the exception of the inclusion of radiolabelled nucleotide and a decrease of unlabelled CTP to a final concentration of 0.5mM, the reaction mixture was identical to that described for unlabelled transcripts. For the purposes of UV-crosslinking assays, the reaction was performed in a 20μl volume and contained 1 X transcription buffer, 1mM ATP and GTP, 0.75mM UTP, 0.25mM 4-thioUTP, 50μCi of [α-^{32}P] CTP (400Ci/mmol), 40 units of RNA guard or RNasin, 1μg of template DNA and 20 units of T7 or T3 RNA polymerase. The RNA was synthesised and isolated in the same manner as unlabelled RNA. The

concentration of radiolabelled transcripts was determined by Cerenkov schintillation counting.

2.5.2. Electrophoretic Mobility Shift Assays

Approximately 25,000cpm of labelled transcript was incubated with protein (as indicated) in buffer mix containing 40U RNAguard, 2µl of 5 X transcription buffer (200mM Tris-HCL [pH 8.0], 40mM $MgCl_2$, 10mM spermidine, 250mM NaCl, 50mM DTT, 15µg tRNA), and 2µl of 10mM ATP in a reaction volume of 15µl for ten minutes at room temperature. 5 X DNA loading dye was added and samples loaded onto 0.7 X TBE agarose gels. Samples were electrophoresed at 100V for approximately 3 hours in 1 X TBE loading buffer. All buffers and loading dyes were filter-sterilized. Gels were dried under vacuum at 60°C for two hours and exposed to a phosphor screen (Molecular Dynamics).

2.5.3. Folding RNA

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

2.5.4. Binding proteins to RNA

Transcripts were incubated with protein in buffer mix containing 40U RNAguard, 2µl of 5 X transcription buffer (200mM Tris-HCL [pH 8.0], 40mM

$MgCl_2$, 10mM spermidine, 250mM NaCl, 50mM DTT, 15μg tRNA), and 2μl of 10mM ATP in a reaction volume of 15μl for ten minutes at room temperature.

2.5.5. Modification of RNA using DMS

Chemical modification was carried out by addition of 10μl of a 1 in 12 dilution of DMS (dimethyl sulfate) in ethanol and subsequent incubation at 0°C for one hour. 50 μg of carrier tRNA were added before the reaction was halted by ethanol precipitation.

2.5.6. Modification of RNA using RNase VI

2μl RNA were incubated with 2μl V1 buffer (Roche) and 2μg tRNA in a reaction volume of 10μl and the mixture was split into 2 tubes. To the first tube, 1.25μl RNase V1 and 3.75μl water were added. To the second, 5μl of sterile water was added as a control. The tubes were incubated for 15 minutes at room temperature and 20μl of Stop buffer (Roche) added. The mixtures were incubated at -20° C for 30 minutes or overnight. RNA was pelleted by centrifugation and washed with 70% ethanol. RNA was resuspended in 2.5 μl sterile water.

2.5.7. Primer extension

The procedure for primer extension was adapted from Stern *et al.*, 1998. 1μl of 5' end-labelled primer (2pmol/μl) was combined with 1μl hybridization buffer (250 mM K-HEPES pH 7.0, 500mM KCl) and 2.5μl RNA (i.e. in molar excess relative to the primer). The mixture was incubated at 85°C for 1 minute and allowed to cool at room temperature for 10-15 minutes. 3μl of extension mix was added to the cooled

hybrid, consisting of 0.5µl *C.therm* reverse transcriptase (Roche), 1µl *C.therm* reaction buffer (Roche), 0.33µl dNTP stock (110µM each dGTP, dCTP, dTTP, dATP), 0.66µl extension buffer (1.3M Tris-HCl pH 7.4, 100mM $MgCl_2$, 100mM DTT), and 0.5µl sterile deionised water. The reaction mixture was incubated at 37°C for 30 minutes, at which time 1µl of chase mix (1mM each dGTP, dCTP, dTTP, dATP) was added and incubation continued for a further 15 minutes. The reaction was stopped by the addition of 3µl 3M NaAc pH 5.4 and 90µl ethanol. The mixture was vortexed, incubated at 0°C for 1 hour and spun in a microcentrifuge at high speed (12,000x *g*) for 15 minutes. The supernatant was carefully drawn off, the pellet air-dried and resuspended into 10µl of gel loading buffer (7M urea, 0.03% (w/v) xylene cyanol and bromophenol blue dyes). The products of the reaction were then heated to 100°C for 2.5 minutes, chilled briefly on ice and 2-5µl were quickly loaded onto a 7M urea 6% polyacrylamide sequencing gel.

2.6. Biochemical techniques

2.6.1. Buffers and solutions

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

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

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

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

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

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

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

2.6.2. *In vitro* translation

5ng/μl RNA was used to prime the Promega rabbit reticulocyte Flexi®-lysate *in vitro* translation system according to the manufacturer's instructions. Each reaction contained 8.25μl of reticulocyte lysate, 0.6mM MgOAc, 20 units of Rnasin, 2μl of complete amino acid mixture and 100ng of RNA substrate. The reactions were performed in a final volume of 12.5μl. 0.1μg of each protein was added where appropriate. All experiments were performed in duplicate. Error bars represent standard deviations from three independent experiments. Where observed differences between data sets were small, a students *t*-test was performed to ascertain the significance of the difference. The probability of such differences being observed by chance are indicated in brackets where $P < 0.05$ is taken to represent a significant difference.

2.6.3. Preparation of translating extracts

Cells were grown to confluence in 150cm^2 flasks and the media aspirated. 14ml of dissociation buffer (GIBCO-BRL) were added and the cell suspension transferred to a 50ml tube. Each flask was washed with 7ml PBS twice and the wash

solution added to the cell suspension. Cells were pelleted by centrifugation at 5,000 x *g* for 10 minutes and the supernatant discarded. Cells were resuspended in 10ml of PBS and pelleted as before to wash. The supernatant was discarded and cells resuspended in 1ml buffer (0.5µl 1M DTT, 8.75µl 25mg/ml creatine kinase, 9.4µl 10mg/ml tRNA) per ml of cells. Cell lysates were prepared by passaging the mixture through a 22G needle five times and a 25G needle 30 times. The cell lysates were transferred into 1.5ml aliquots and cell debris pelleted by centrifugation at 12,000x *g* for 10 minutes at 4°C. The supernatants were aliquoted and frozen at -80°C.

2.6.4. Preparation of extract from placenta

Placenta was weighed then homogenised in chilled buffer comprising 1ml hypotonic buffer (10mM HEPES pH 7.4, 1.5mM MgOAc, 1.5mM KCl), 0.5µl 1M DTT, 8.57µl 25mg/ml creatine kinase and 9.5µl 10mg/ml yeast tRNA per gram of placenta. The resultant extract was sonicated and passaged through a 22G then a 25G needle. Aliquots were centrifuged at 12,000x *g* at 4°C for ten minutes and the supernatant removed to fresh tubes and stored at -80°C.

2.6.5. Dialysis of placental extract.

Placental extract was syringed into a slide-a-lyzer dialysis cassette (Perbio) and dialysed against buffer containing 1.5mM MgOAc, 10mM HEPES pH 7.4, 0.5mM DTT at 4°C for 24 hours with one buffer change.

2.6.6. *In vitro* translation assays with translating extract

5ng/μl RNA was used to prime the *in vitro* translation system containing 1 X salts and spermidine (10mM KCl, 1.25M MgOAc, 2mM $NaCl_2$ and 0.1M sperimidine), 1 X translation buffer (175mM HEPES pH7.4, 5mM ATP, 1mM GTP, 5mM DTT, 125 mM creatine phosphate, 2mg/ml creatine phosphokinase), 0.25μl 1mM complete amino acid mix, 40U RNAguard and 5μl translating extract. The reaction was performed in a final volume of 12.5μl. 0.1μg of each protein was added where appropriate. Luciferase activities were assayed as described in **section 2.6.8**, and the firefly and *Renilla* values expressed relative to the control plasmid pRF which was assigned a value of 1.

2.6.7. Harvesting lysates from transfected cells

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

2.6.8. Luciferase assays

5µl of lysate from transfected cells was used to assay enzyme activity. In the case of monocistronic constructs, the luciferase reporter assay system (Promega) was used. 25µl of luciferase assay reagent was added to the lysate and light emission measured over 10s using an Optocomp I luminometer (MGM Instruments).

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

2.6.9. β-galactosidase assays

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

2.6.10. SDS-polyacrylamide gel electrophoresis

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

	Resolving gel		Stacking gel
%	10%	12.5%	6%
distilled water	2.1ml	1.7ml	1.8ml
resolving/stacking buffer	1.25ml	1.25ml	2.5ml
30%:0.8% acrylamide:bisacylamide	1.67ml	2.1ml	0.67ml
25% ammonium persulphate	50µl	50µl	50µl

Table 2.3. Recipes for SDS-polyacrylamide gels

25% ammonium persulphate was added last to polymerise the gel. Samples were denatured prior to loading by boiling at 100°C for two minutes. Gels were run in SDS running buffer at a constant voltage of 150V for approximately 1-2 hours.

To analyse endogenous protein levels, cell lysates were prepared in 1 x SDS buffer supplemented with protease inhibitors and solubilised by passage through a 25G needle. Protein extracts were heated to 95°C for 2 min prior to loading and analysed by SDS-polyacrylamide gel electrophoresis (PAGE) as described above. Gels were typically run at a constant voltage of 150V in SDS running buffer until the bromophenol blue dye front reached the bottom of the gel, typically between 1-2 hours.

2.6.11. Transfer of proteins onto nitrocellulose membranes.

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

2.6.12. Western blotting/immunodetection

Proteins immobilized on nitrocellulose membranes (**Section 2.6.11**) were detected immunologically using antibodies to PTB, unr, PCBP1, eIF4G, eIF4E, eIF4A, actin or Bag-1. Binding at non-specific sites was first blocked by incubation of the nitrocellulose membranes in a 5% dried milk in TBST solution for approximately 1 hour at room temperature, with agitation. The blocking solution was aspirated and replaced with 10-20ml of appropriately diluted primary antibody (**Section 2.1.2**) in 5% milk TBST solution and incubated overnight at 4°C with agitation. Excess antibody was removed by washing for 10 minutes with TBST, repeated 3 times. Membranes were incubated with peroxidase-conjugated secondary antibodies raised against mouse immunoglobulins (Dako A/S) or rabbit immunoglobulins (Sigma) diluted 1:2000 and 1: 10,000 respctively, for 1 hour at room temperature with agitation. Excess secondary antibody was removed by washing for 10 minutes with TBST, repeated 3 times. Protein-antibody complexes were detected by enhanced chemiluminescence (ECL) according to a recipe kindly provided by Professor Ken Siddle (University of Cambridge). 1ml of luminol solution (50mg luminol [5-amino-2,3-dihydro-1,4-phyhalazinedione] in 0.1M Tris-HCl pH 8.6), 10µl enhancer (11mg para-coumaric acid in 10ml DMSO) and 3.1µl 3% hydrogen peroxide were mixed and applied to the membrane for 60s with agitation. Chemiluminescence was visualised by exposure of the membrane to Fuji RX X-ray film for between 10s and 15min.

2.6.13. Stripping and re-probing of western blots

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

2.6.14. Measuring protein synthesis rates by incorporation of ^{35}S Methionine

Approximately 1 X 10^5 cells were seeded into each well of a 6-well plate and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The media was aspirated and the cells washed 3 X with PBS before adding 1ml of methionine-free media supplemented with 10% (v/v) dialysed fetal calf serum. 10μCi of ^{35}S-Methionine was added to each well, in addition to the appropriate concentration of vincristine or cisplatin, as denoted in the text, as required. For concentration-dependence experiments, cells were incubated for a further 24 hours. Cells were harvested by washing 3 X with PBS, scraped using a cell scraper and resuspended in 1ml of 10% TCA. Cell suspensions were stored at -80°C. To assay methionine incorporation, cell suspensions were filtered onto Whatman glass fibre filter paper circles, washed thoroughly with ice-cold 10% TCA and air-dried. Dried papers were placed in schintillation vials with 5ml schintillation fluid and methionine incorporation measured by counting for 1 minute using a schintillation counter.

2.6.15. UV-crosslinking

Radiolabelled transcript was generated from pSKBL linearised with *Nco*I and approximately 2.5pmol (5 X 10^5cpm) per reaction was incubated with 0.25μg of protein in 1 X UV-crosslinking buffer (10mM HEPES [pH 7.4], 3mM $MgCl_2$, 100mM KCl, 5mM creatine phosphate, 1 mM DTT, 1mM ATP, 6% (v/v) glycerol, 0.1 μg/μl tRNA). For competition assays, unlabelled competitor RNAs were added with labelled RNA. Reaction mixtures were incubated for 15 minutes at room temperature. Heparin (0.05μg/μl) was added to the samples and they were incubated for a further 15 minutes at room temperature. The reaction mixtures were UV irradiated using a 312nm UV light source (UVP) at a distance of 3cm for 30 minutes on ice. 0.2mg per ml of RNase A (Sigma) and RNase V1 (Ambion) was added to the mixture to degrade any unprotected RNA by incubation at 37°C for 30 minutes. An equal volume of 2 X SDS sample buffer was added and the samples separated on a 10% polyacrylamide gel by SDS-PAGE. Gels were fixed in destaining solution and dried at 80°C under vacuum. RNA-protein complexes were visualised on a Molecular Dynamics Phosphorimager.

2.6.16. Indirect immunofluorescence

Approximately 1 X 10^5 cells were seeded in each well of a 4-well chamber slide and grown for 24 hours. Cells were heat-shocked to 44°C by addition of pre-warmed media and subsequent incubation for 30 minutes in a hybaid oven. Alternatively, cells were left at 37°C in a 5% CO_2 humidified incubator. The media was aspirated and cells washed with PBS and incubated at -20°C with pre-chilled 50% acetone/ 50% methanol solution for five minutes to fix. The fix solution was removed and cells air-dried for 15 minutes. Cells were washed three times with PBS and

blocked for 10 minutes with 3% BSA in PBS before incubation with the primary antibody for 15-30 minutes. Primary antibodies utilised were as follows; mouse monoclonal α-Bag-1 or α-PTB at 1:10 dilution in 3% BSA in PBS, rabbit polyclonal α-PCBP1 at 1:25 or rabbit polyclonal α-eIF4G at 1: 300. Following incubation, cells were washed three times in PBS and incubated with either FITC-conjugated α-mouse IgG antibody at 1:20 or TRITC-conjugated α-rabbit IgG antibody at 1:40 for fifteen minutes. Cells were washed as before and the chambers removed. A drop of mounting buffer (90% (v/v) glycerol, 10% (v/v) Tris, pH 9.0) was added to each well and coverslips placed over the cells. Sides of the coverslips were fixed with clear nail varnish. Cells were visualised by fluorescence microscopy immediately or stored at 4°C in the dark.

Chapter 3

Determination of the secondary structure of the Bag-1 IRES

3.1. Introduction

One of the key criteria in the identification of mRNAs that contain putative IRESes is the presence of a long, GC-rich 5'UTR capable of folding into a stable secondary structure. Formation of stable secondary structure in the 5'UTR of an mRNA is a requirement for IRES function in the majority of cases. The secondary structure of viral IRESes has been characterised much more extensively than cellular IRESes. Viral IRESes can be broadly classified into two major groups and one minor group based on secondary structure predictions. In contrast, cellular IRES structure is much less defined, however there appears to be much more variation in secondary structure predictions of cellular IRESes, even those within a gene family such as the *myc* gene family (Keith Spriggs and Sally Mitchell, personal communication). In addition, there is evidence that short primary sequence motifs as opposed to secondary structure are vital for IRES activity, for example the 9nt IRES of the Gtx homeodomain gene (Chappell *et al.*, 2000).

A secondary structural model provides an invaluable starting point for analysis of the mechanism of IRES function, allowing identification of key motifs through subsequent mutagenesis and thus providing scope for up or down-regulating IRES activity. The effects and mechanism of action of *trans*-acting factors can also be deduced based on secondary structural perturbations caused by protein binding

(Mitchell *et al.*, 2003). In addition, a secondary structural model provides an excellent basis for subsequent tertiary structural analysis by NMR and X-ray crystallography.

3.2. The 225-411 fragment of the Bag-1 IRES retains 100% IRES activity

Deletion constructs, created by restriction digestion and re-ligation into the dicistronic vector pRF, have previously been generated, corresponding to the 5' and 3' segments of the 5'UTR. These constructs were denoted pRBagF5' and pRBagF3' respectively (Coldwell *et al.*, 2001). Assaying IRES activity resulting from transfection of these constructs into HeLa cells revealed that the construct pRBagF5' had virtually no IRES activity. In contrast, the construct pRBagF3' retained 75% of the IRES activity observed with the full-length 5'UTR in the same system. These results implied that the most important structures for IRES activity are located in the 3' region of the 5'UTR. Such findings encouraged the generation of a finer deletion series of the Bag-1 5'UTR to determine, in more detail, the location and sequence of important regions for IRES activity.

The 5'UTR of Bag-1 contains an IRES, which is capable of directing translation of the p36 isoform but also comprises sequences that are part of the coding region for the longer Bag-1 protein isoforms, p50 and p46. As the longer isoforms appear to be translated entirely by cap-dependent translation (Coldwell *et al.*, 2001), it is possible that the Bag-1 IRES consists of short primary sequence motifs as opposed to complex secondary structure. Primary sequence motifs would not inhibit translation of the longer isoforms by the cap-dependent scanning mechanism which may occur should the 5'UTR be highly structured.

Oligonucleotide primers were designed to amplify segments of the 5'UTR by polymerase chain reaction. The resultant fragments were cloned into the dicistronic vector pRF to create a deletion series (**figure 3.1**). The region of the pRBagF3' construct corresponded to the segment between nucleotides 279 and 411 in the 5'UTR where 279 is the location of the initiation site for the p46 isoform and 411 is the initiation site for the IRES-driven p36 isoform. The deletion series was consequently concentrated in the region of 225-411 nucleotides (**figure 3.2a**). The resultant deletion constructs were transfected into HeLa cells and luciferase activities assayed (**figure 3.2b**).

The deletion 225-411, 54 nucleotides longer than pRBagF3', retained 100% IRES activity, suggesting that the 1-224 nucleotide section is dispensable for IRES activity. IRES activity of the other deletions was considerably lower, indicating that the 225-411 section comprises the minimal complete IRES element. The 225-358 section, which is the 5' section of the minimal element, retains 50% IRES activity. This region comprises approximately half of the minimal element, indicating that the structures necessary for IRES activity may be spread throughout the 225-411 region, and not localised to a smaller segment. Any further deletions decrease IRES activity significantly.

The wild-type IRES activity of the 225-411 segment cannot be attributed to a decrease in *Renilla* luciferase activity and a consequent increase in the ratio of firefly to *Renilla* luciferase. Normalising the luciferase activities to a transfection control of β-galactosidase determined that firefly and *Renilla* luciferase levels are equivalent in the 225-411 and full-length 5'UTR constructs (**figure 3.3**). The dicistronic vector

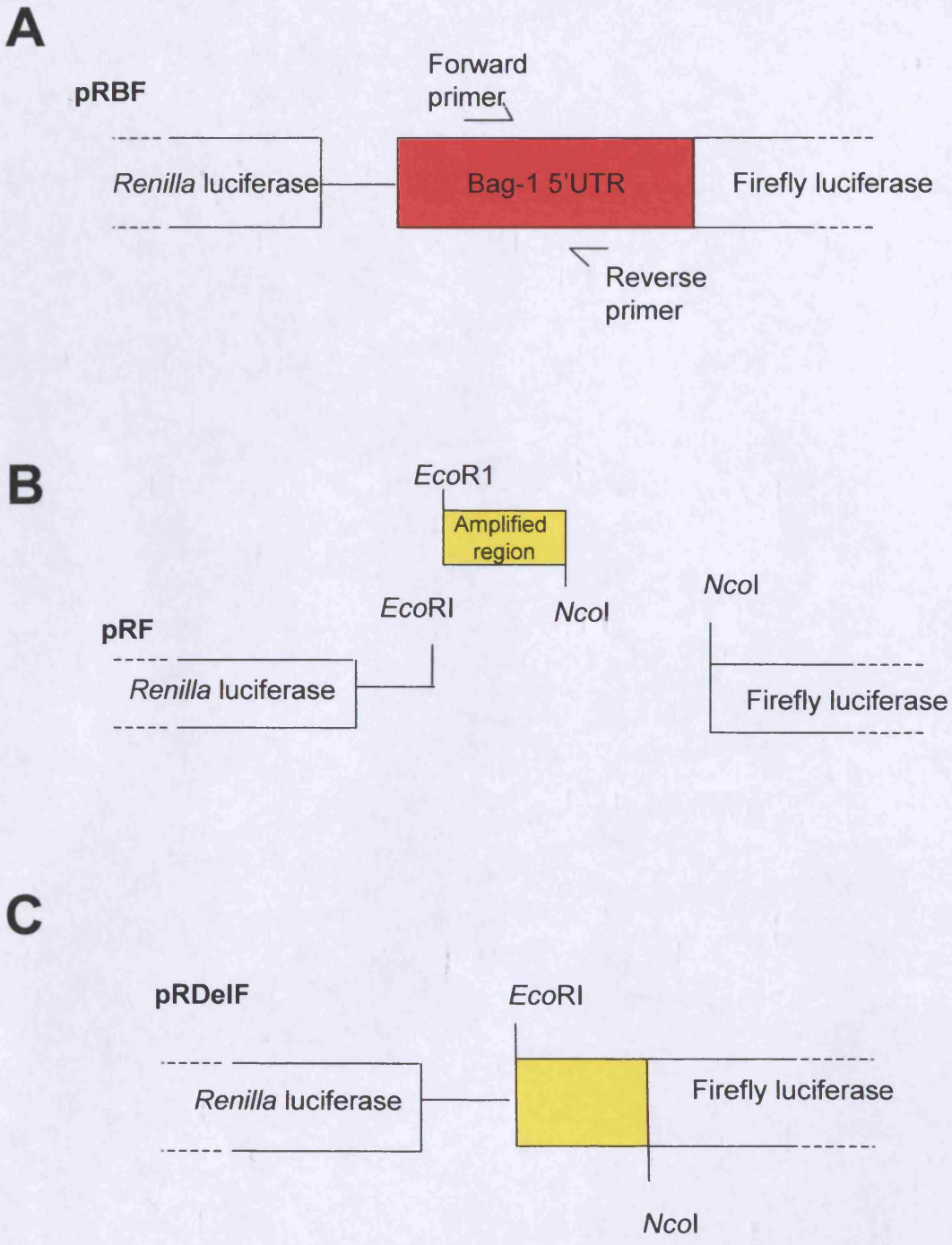


Figure 3.1. Construction of deletions in the 5' UTR of Bag-1

A. Specific regions of the Bag-1 5'UTR were amplified with primers that introduced an *Eco*R1 site (forward primer) and an *Nco*I site (reverse primer) either side of the region of interest.

B. The amplified segment was digested and ligated into the vector pRF which had been digested with *Eco*RI and *Nco*I and dephosphorylated.

C. This created the deletion constructs, pRDelF.

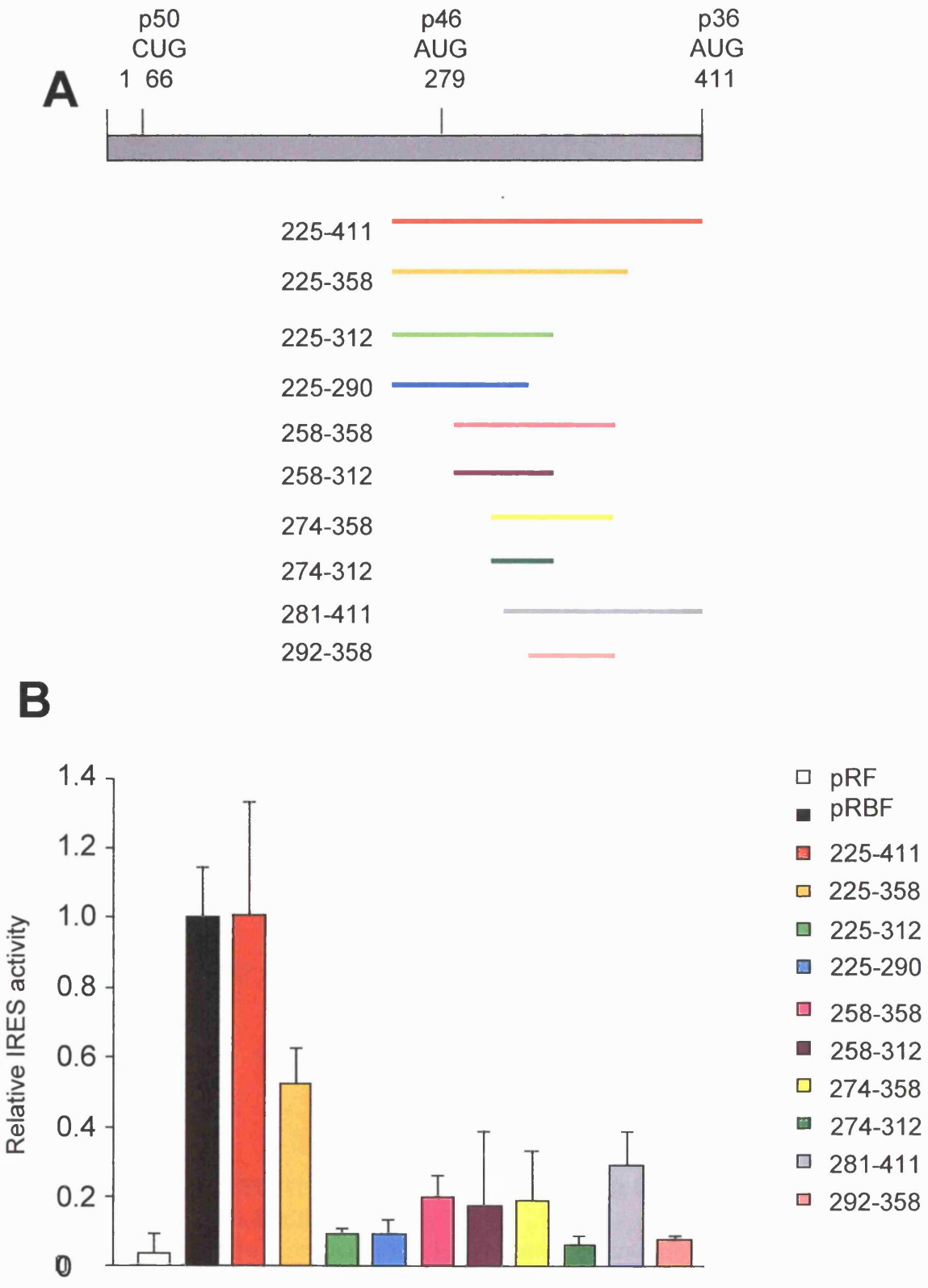


Figure 3.2. The 225-411 fragment of the Bag-1 5'UTR is the minimal element with 100% IRES activity.

A. Schematic diagram showing the relative positions of deletion fragments amplified from the Bag-1 5'UTR

B. Deletion constructs were transfected into HeLa cells and IRES activity expressed as a ratio of firefly/*Renilla* luciferase relative to full-length pRBF, which was assigned a value of 1.

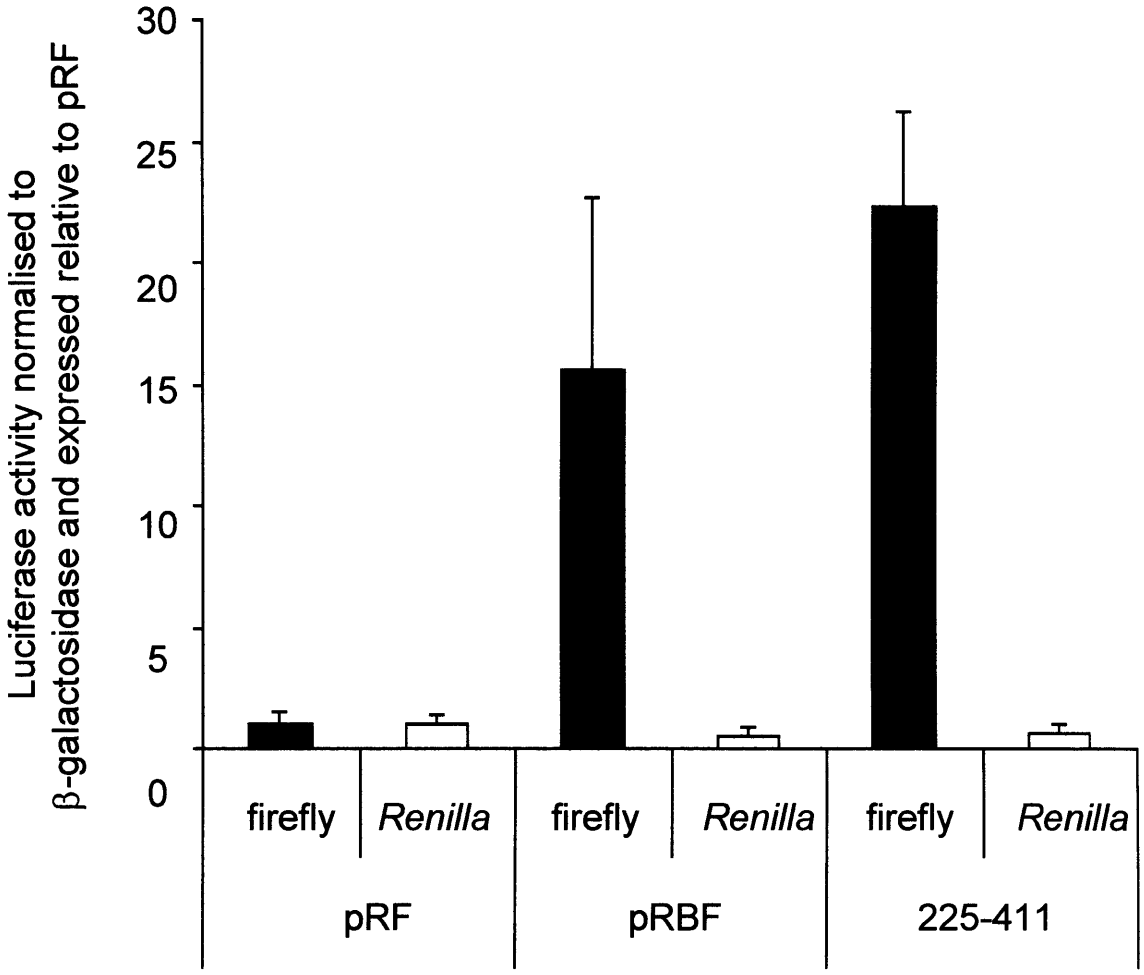


Figure 3.3. The 225-411 fragment of the Bag-1 5'UTR is the minimal element with 100% IRES activity.

The deletion construct 225-411 was transfected into HeLa cells. Firefly and *Renilla* luciferase activities were normalised to a transfection control plasmid expressing β-galactosidase and expressed relative to pRF, which was assigned a value of 1. The plasmid pRBF was also transfected as a positive control. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

containing the 225-411 segment of the Bag-1 5'UTR will henceforth be known as pRBminF.

3.3. Activity of the minimal element is not due to alterations in the efficiency of ribosomal readthrough

It is possible that alteration in the length of the intercistronic region of the dicistronic vector could contribute to the firefly luciferase activity observed with the minimal IRES element as this could increase the efficiency of ribosomal readthrough. Alternatively, inhibitory structural elements could be deleted in the construction of pRBminF. To address this possibility, constructs were generated with a stable hairpin loop structure cloned into the intercistronic region, before the *EcoR*I cloning site. The full-length Bag-1 5'UTR and the minimal IRES element were cloned into the vector pRHpF to generate the constructs pRHpBF and pRHpBminF respectively (**figure 3.4**). The constructs were transiently transfected into HeLa cells and luciferase activities assayed (**figure 3.5**). The dicistronic vectors pRBF and pRBminF were also transfected into HeLa cells as controls to compare with the firefly luciferase activities of the hairpin constructs. With each type of vector, the full-length 5'UTR and the minimal IRES element exhibited comparable levels of IRES activity, indicating that the minimal IRES element does comprise the entire boundaries of the Bag-1 IRES and the 5' section, 1-224, is dispensable for IRES activity. This region may be required solely for translation of the longer isoforms.

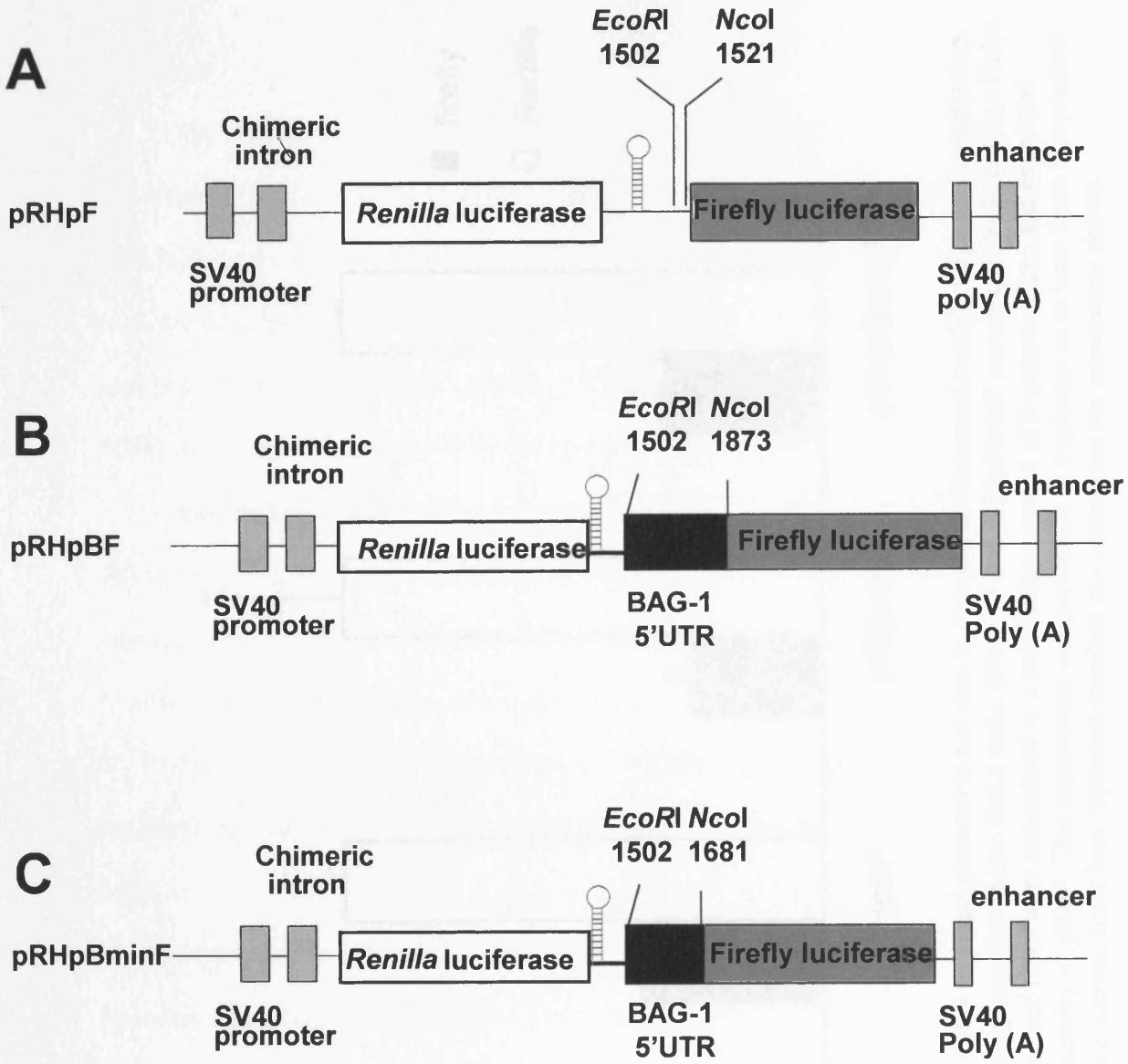


Figure 3.4. Construction of the vectors pRHpBF and pRHpBminF.

A. The vector pRHpF was digested with *EcoR*I and *Nco*I.

B. The Bag-1 5'UTR was removed from the vector pRBF by digestion with *EcoR*I and *Nco*I and ligated into pRHpF to create pRHpBF.

C. The Bag-1 IRES element, 225-411 of the 5'UTR, was removed from the vector pRBminF by digestion with *EcoR*I and *Nco*I and ligated into pRHpF to create pRHpBminF

All of the resultant constructs contain a palindromic hairpin sequence 5' of the *EcoR*I site.

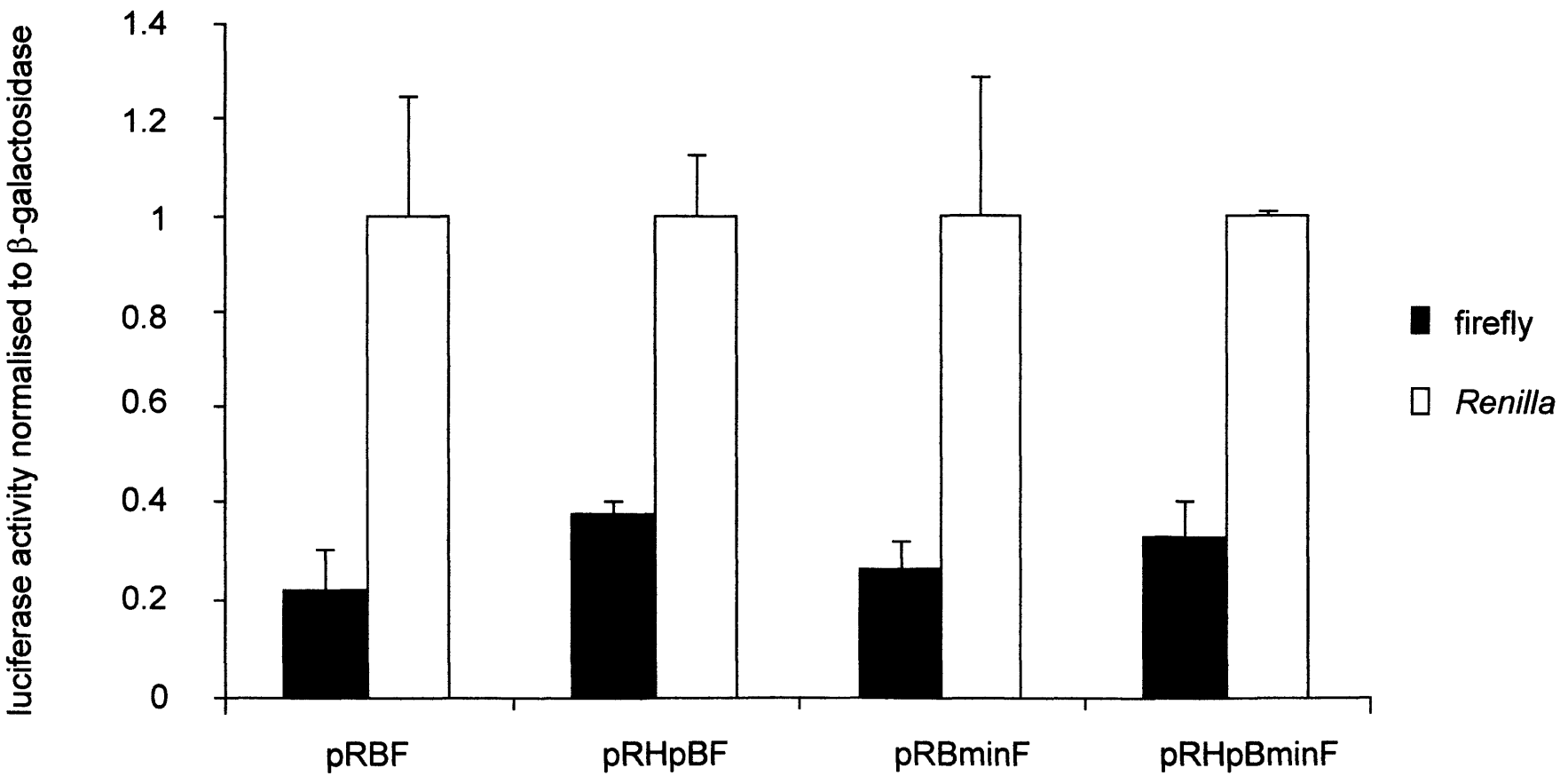


Figure 3.5. IRES activity of the Bag-1 IRES minimal element is not due to enhanced ribosomal readthrough and reinitiation. pRBF and pRBminF were transiently transfected into HeLa cells. pRHpBF and pRHpBminF were also transfected into HeLa cells. Firefly and *Renilla* luciferase activities were normalised to a transfection control of β-galactosidase and expressed relative to the *Renilla* luciferase activity of pRBF. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation from the experiment shown.

Discussion

The indication that a large area of the Bag-1 5'UTR, comprising up to 186 nucleotides is required for Bag-1 IRES activity suggests that function of the Bag-1 IRES is dependent on secondary structural motifs as opposed to short primary sequence elements. The size of the IRES compares well with other cellular IRESes such as the XIAP IRES of 152 nucleotides (Holcik *et al.*, 2000), the 165nt IRES of FGF-2 (Le *et al*, 1997) and the 175nt IRES of VEGF (Miller *et al.*, 1998).

Interestingly, sequence alignments and deletion studies of a number of cellular IRESes including the Apaf-1, FGF-2, XIAP and VEGF IRESes show that the most important sequences for IRES activity reside towards the 3' end of the 5'UTRs (Coldwell *et al.*, 2000; Le *et al.*, 1997; Holcik *et al.*, 2000; Huez e*t al.,* 1998; Miller *et al.*, 1998). It is possible in the case of the Bag-1 IRES that the 5' sequences are only required for coding of the longer isoforms and as such are dispensable for IRES function.

With both the full-length Bag-1 5'UTR and the minimal IRES element, there may be a slight increase in firefly luciferase activity relative to *Renilla* luciferase activity in the hairpin constructs compared to the dicistronic vectors (**figure 3.5**). It is possible that ribosomal readthrough in the dicistronic vectors may reduce the efficiency of IRES driven translation, possibly through unwinding of the secondary structure of the IRES in order to allow scanning to take place. Consequently, inhibiting ribosomal readthrough by introducing a hairpin before the IRES element may allow greater efficiency of IRES-driven translation.

3.4. Identifying the ribosome-landing region

3.4.1. The ribosome is not recruited to the Bag-1 IRES through the p46 initiation codon

Two major mechanisms have been described for ribosome landing in IRES elements; firstly the land-and-scan model, where the ribosome is recruited to an upstream site, frequently an upstream AUG codon, and then scans to the initiation codon. An alternative mechanism is the direct recruitment model where the ribosome is recruited directly to the initiation codon. This mechanism occurs on class II picornaviral IRESes. Class I picornaviral and cellular IRESes, in contrast, appear to conform to the land-and-scan model of ribosome recruitment. The scanning distance is typically around 150 nucleotides (Pelletier *et al.*, 1998).

There are two upstream initiation codons in the Bag-1 5'UTR, the CUG encoding the p50 isoform, which is located at position 66 and the AUG initiation codon at position 279, encoding the p46 isoform. The p46 initiation codon is a good candidate for recruiting the ribosome as it is located 132bp upstream of the IRES initiating AUG. Primers were designed to mutate the AUG codon into a different amino acid, AGG encoding arginine or alternatively to a stop codon, UGA (**figure 3.6 and figure 3.7a**). Neither mutation had any significant influence on the amount of active luciferase generated, suggesting that the ribosome is not recruited by the p46 initiation codon (**figure 3.7b**).

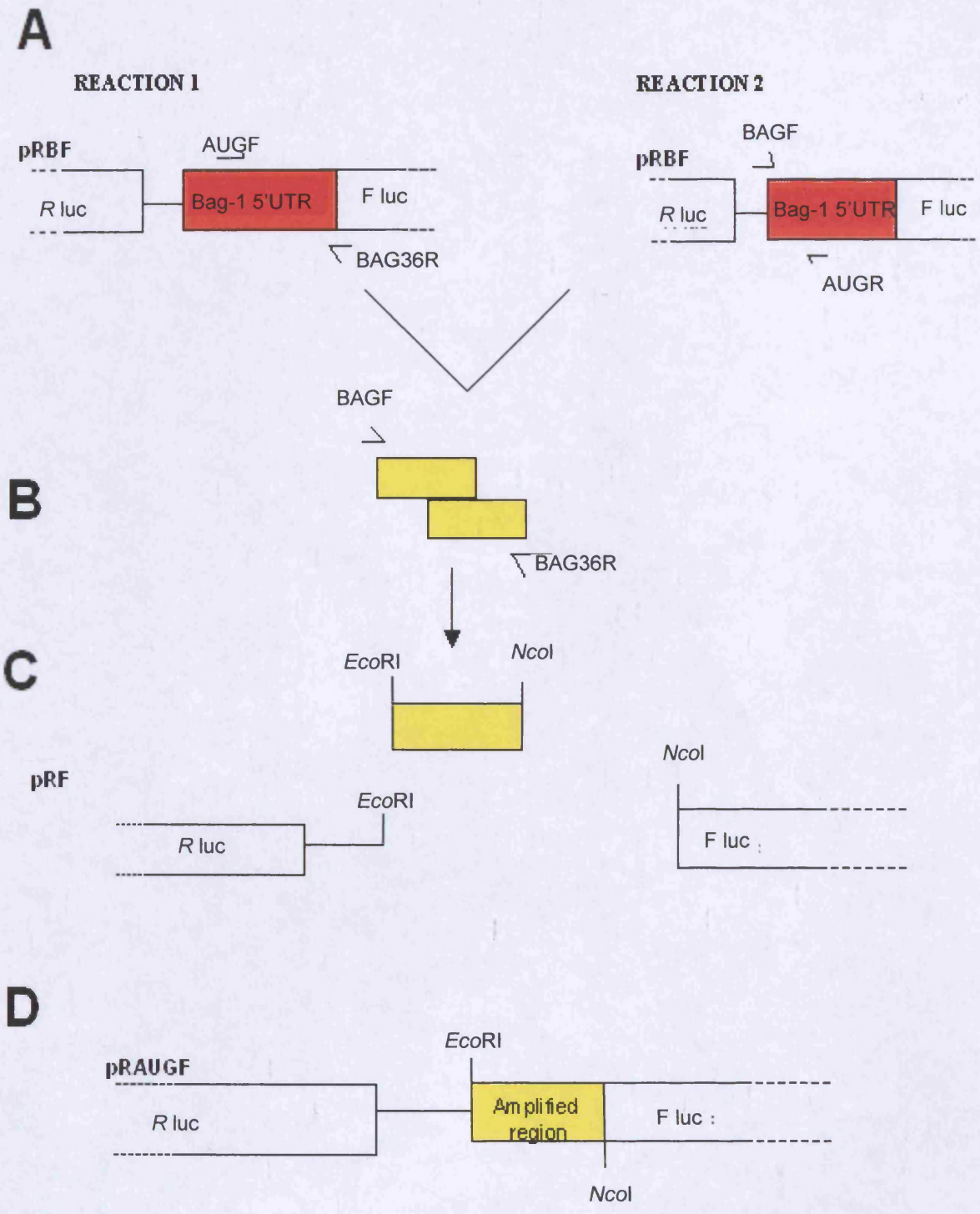


Figure 3.6. Construction of out-of-frame AUG mutants in the 5' UTR of Bag-1

A. The mutation was introduced by performing two PCR reactions, Reaction 1 with a forward primer bearing the mutated sequence, AUGF, and BAG36R. Reaction 2 with the BAGF forward primer and a reverse primer containing the AUG mutation. R luc represents *Renilla* luciferase and F luc represents firefly luciferase in all cases

B. The amplified segments were used as templates for another PCR reaction using BAGF and BAG36R to generate a full-length 5'UTR bearing the mutation.

C. The fragment was digested with *EcoR*I and *Nco*I and ligated into the vector pRF which had been digested with *EcoR*I and *Nco*I and dephosphorylated.

D. This created the out-of-frame AUG constructs, pRAUGF.

A

1 -gaattctggg cggtcaacaa gtgcgggcct ggctcagcgc ggggggcgc
51 -ggagaccgcg aggcgaccgg gagcggctgg gttcccggct gcgcgccctt
101-cggccaggcg ggagccgcgc cagtcggagc ccccggccca gcgtggtccg
151-cctccctctc ggcgtccacc tgcccggaga ctgccagcg ggcatgaccg

p46 STOP: tag
201-acccaccagg ggcgccgccg ccggcgctcg caggccgcgg atgaagaaga
p46 AGG: agg

251-aaacccggcg ccgctcgacc cggagcgagg agttgacccg gagcgaggag
301-ttgaccctga gtgaggaagc gacctggagt gaagaggcga cccagagtga
351-ggaggcgacc cagggcgaag ccatgg

B.

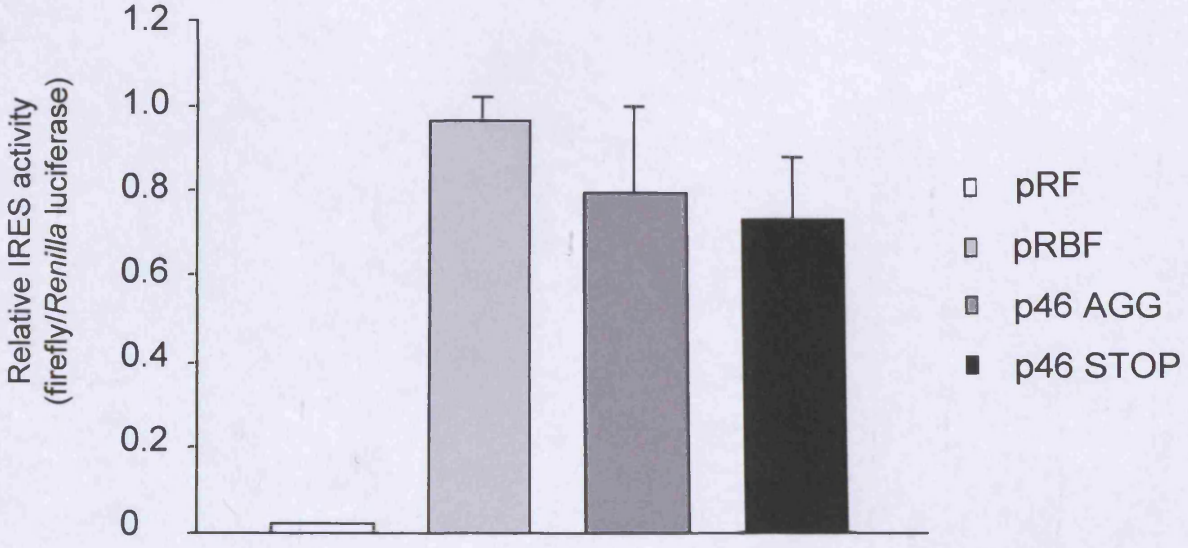


Figure 3.7. Mutating the p46 initiation codon has no influence on internal ribosome entry.

A. Schematic to show the mutations introduced into the Bag-1 5'UTR. Mutated sequence is shown in blue. The Bag-1 initiation codons are shown in red. The numbers represent the position of the nucleotide within the 5'UTR.

B. pRBF constructs with mutations at the p46 initiation site were transfected into HeLa cells in parallel with pRBF or the empty vector pRF. IRES activity is expressed as firefly/*Renilla* luciferase relative to pRBF, which is assigned a value of 1.

3.4.2. The Bag-1 IRES conforms to the land-and-scan model of ribosome landing

In order to analyse the ribosome-landing site of the p36 isoform in the Bag-1 5'UTR, out-of-frame AUG codons were introduced. If an out-of-frame AUG codon is introduced upstream of the ribosome landing site, active luciferase should be generated. If an AUG codon is introduced downstream of the ribosome-landing site, this will be the first initiation codon encountered by the ribosome and as such translation will commence here and inactive luciferase will result. Primers were designed to introduce several out-of-frame AUG codons at reasonably spaced intervals into the Bag-1 5'UTR (**figure 3.8 and figure 3.9a**). PCR mutagenesis was undertaken using these primers and the gel-purified fragments were cloned between the *Renilla* and firefly luciferase cistrons of the vector pRF. The resultant constructs were transiently transfected into cells and the cell lysates assayed for luciferase activity (**figure 3.9b**). A comparison of the relative activities of the mutated constructs shows that the ribosome makes important contacts between AUG6 and AUG7, between 97 and 115 nucleotides upstream of the initiator AUG.

Discussion

This data indicates that the Bag-1 IRES functions via the land-and-scan model of ribosome landing but is not recruited by the p46 initiation codon. It is interesting to note that in the murine 5'UTR there is a GUG instead of an AUG at this position and indeed only two murine Bag-1 isoforms have been identified (Takayama *et al.,* 1995). The lack of this initiation codon in the murine 5'UTR suggests that it is unlikely to be

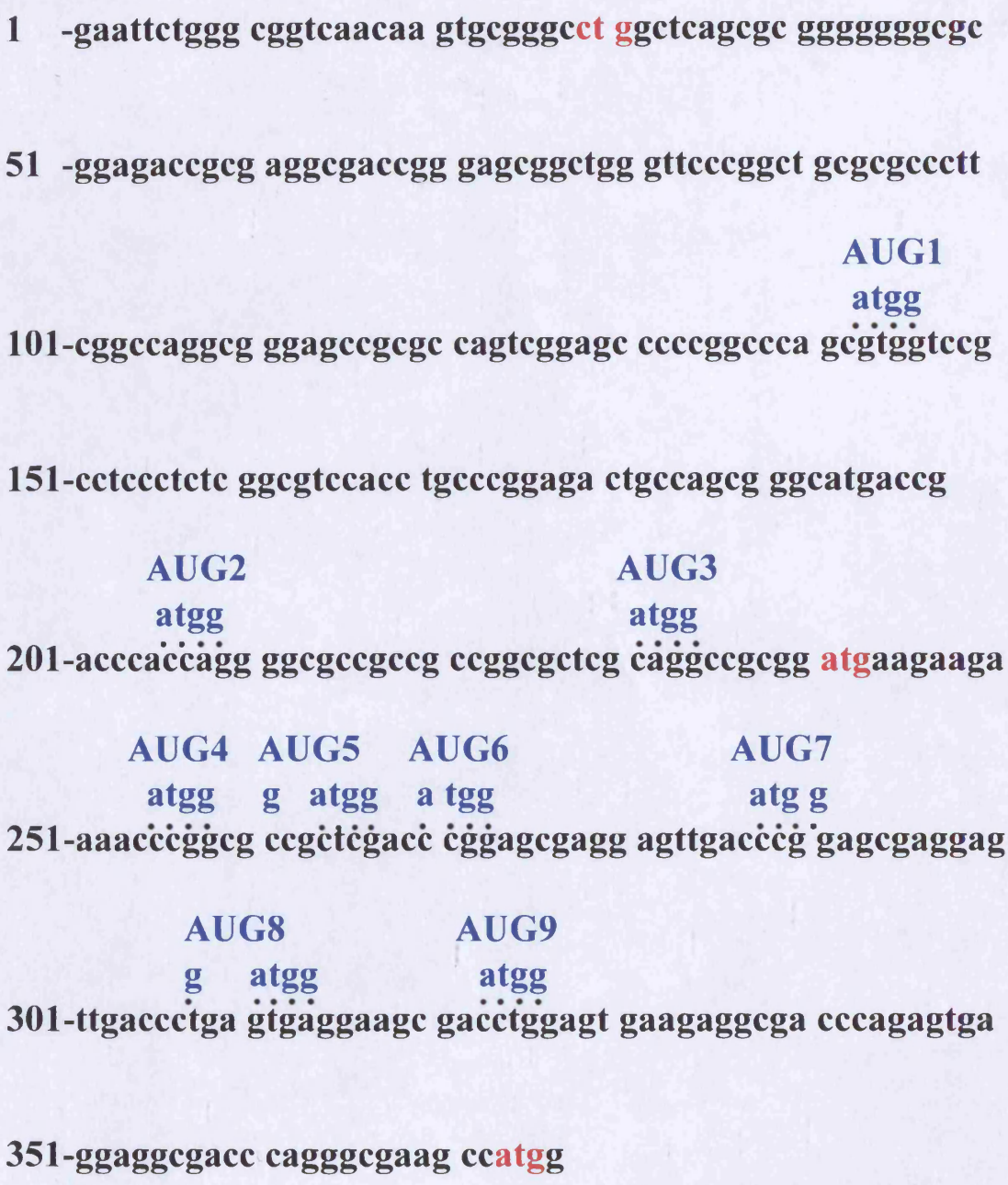


Figure 3.8. Position of AUG mutants in the Bag-1 5'UTR
Wild-type initiation codons are indicated in red, in line with the sequence. Introduced AUG codons and additional mutations to introduce the initiation codons in good Kozak consensus sequence are indicated in blue.

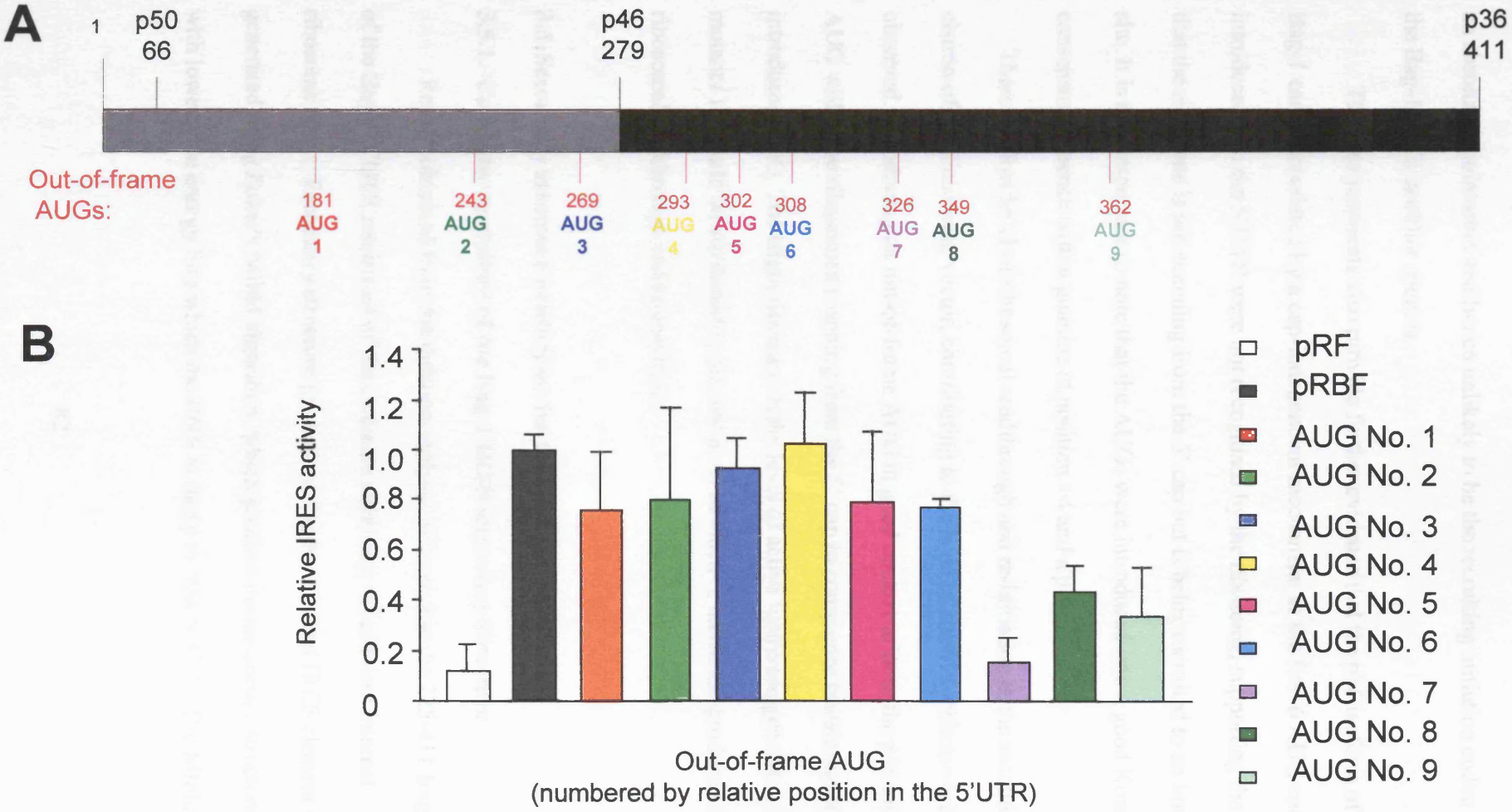


Figure 3.9. The ribosome-landing region is located between nucleotides 308 and 326.

A. Schematic showing the position of out-of-frame AUG codons introduced into the Bag-1 5'UTR.

B. Relative activity of out-of-frame AUG mutants. Mutated pRBF constructs were transfected into HeLa cells in parallel with pRBF as a control, and IRES activity was expressed as the ratio of firefly to *Renilla* luciferase, relative to pRBF, which was assigned a value of 1.

an absolute requirement and hence unlikely to be the recruiting initiation codon for the Bag-1 IRES in either species.

These experiments also provide further evidence that the p36 isoform of Bag-1 can be translated by a cap-independent mechanism as the first 6 AUG codons introduced into the 5'UTR were not recognised by the ribosome, supporting the idea that the ribosome is not scanning from the 5' cap but is being recruited to an internal site. It is also important to note that the AUGs were introduced into a good Kozak consensus sequence with a guanine at position +4 and a purine at –3.

There is a low level of ribosomal readthrough and re-initiation at the second cistron of the dicistronic vector, contributing to the level of firefly luciferase activity observed. Introducing an out-of-frame AUG in good context prior to the p36 initiator AUG will cause ribosomes scanning from the 5' cap to commence translation at the introduced AUG. The slight decrease in the level of active luciferase generated in mutants 1-6 could be attributed to the low level of inactive luciferase produced by ribosomal readthrough and re-initiation.

3.5. Secondary structure predictions for the Bag-1 IRES

3.5.1. Computer modelling of the Bag-1 IRES secondary structure

Results obtained from the deletion analysis indicate that the 225-411 fragment of the Bag-1 5'UTR retains all of the elements necessary for efficient internal ribosome entry. Secondary structure predictions for the minimal IRES element were generated using Zuker's Mfold algorithm, which predicts the secondary structures with lowest free energy into which the RNA is likely to fold at 37°C. The Mfold

algorithm produced 7 potential structures, with significant similarities. A dot plot was produced (**figure 3.10**) which shows the base pairs most likely to form as they have minimum free energy. The lower triangle shows the optimal base pairings. In addition, a plot depicting the frequency of which a base appeared single-stranded in the 7 putative structures was generated (**figure 3.11a**). 50% of bases are exclusively single or double-stranded in all of the structures generated. From this plot, potential structural motifs may be predicted (**figure 3.11b**).

All of the structures generated included a prominent single-stranded region comprising the p46 initiation codon and at least ten bases following it. It was notable that this region was very A-rich, when the 5'UTR as a whole is GC-rich. The exposed loop could represent a protein-binding motif for a protein that binds to A-rich regions or may perhaps act as an unstructured spacer. There is no complementary motif to this string of adenines and it is therefore very likely to be a single-stranded motif, as depicted on the single-stranded frequency plot. Another notable feature is that the first and last twenty bases appear to show a complementary pattern of single and double stranded bases. Indeed, all of the structures generated show base pairing between the 3' and 5' ends of the IRES to form a stem. There are a number of probable foldings for the central region of the IRES and so it is not possible to define a complete model for the Bag-1 IRES on the basis of free energy minimization alone. It should be noted that the first 12 and last 3 bases of the 225-411 region are not included in the structural models as these were subject to mutations in order to introduce restriction sites. In light of this, only the 237-408 region of the 5'UTR is likely to be required for Bag-1 IRES activity.

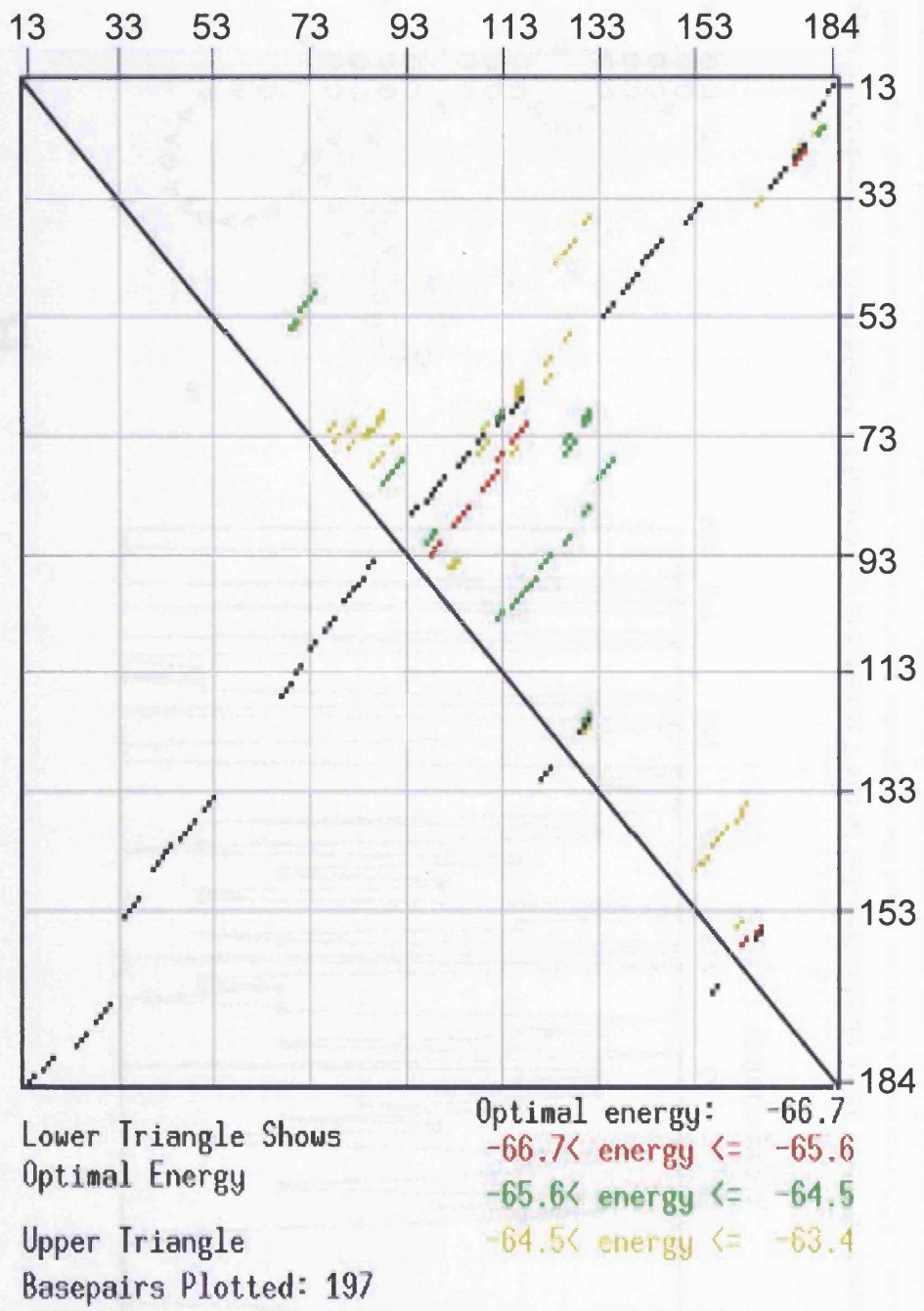


Figure 3.10. Energy dotplot of minimal IRES element

Dots in the lower triangle represent base-pairings with the lowest free energy, the most likely to form. The coloured dots in the upper triangle represent base pairs within 5% of the optimal free energy. Numbers indicate the position of the base in the 5'UTR where the first base of the IRES is taken as 1.

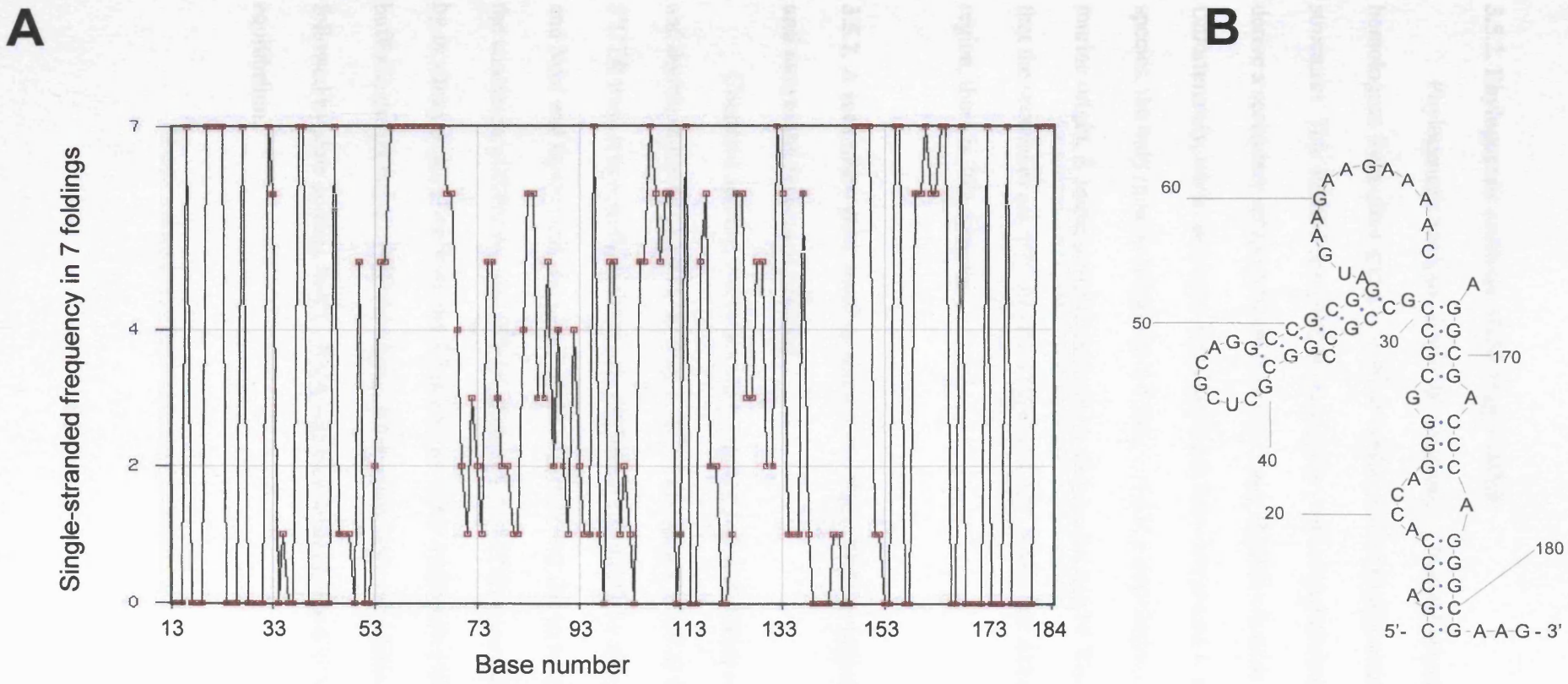


Figure 3.11. Plot to show frequency of bases being single-stranded in the 7 structures generated by Mfold from un-constrained sequence

A. The first base of the minimal IRES element is taken as 1 and the remainder numbered accordingly. Red squares indicate individual bases. Bases with a frequency of 7 are single-stranded in all structures generated. Bases with a frequency of 0 are double-stranded in all structures generated.

B. Conserved motifs in the 7 structures generated

3.5.2. Phylogenetic analysis of the Bag-1 IRES

Phylogenetic analysis, where the sequence of interest is compared to homologues from other species, is often useful in determining conserved bases and structures. This method was used in conjunction with chemical structure probing to derive a secondary structural model for the c-*myc* IRES (LeQuesne *et al.*, 2001). Unfortunately, while the c-*myc* homologue has been sequenced in a variety of species, the only reliable sequences for Bag-1 and it's homologues are of human and murine origin. A sequence alignment of the human and murine Bag-1 5'UTRs shows that the sequences are 66% identical (**figure 3.12**). Within the minimal element region, there is 70% identity.

3.5.2. A secondary structural model for the Bag-1 IRES constrained by chemical and enzymatic structure probing

Chemical and enzymatic structure probing was undertaken to identify single and double-stranded bases in the Bag-1 IRES. The Bag-1 IRES or the entire Bag-1 5'UTR were removed from the vector pRBminF and pRBF by digestion with *EcoR*I and *Nco*I and ligated into the vector pSKL digested with the same enzymes to create the constructs pSKBminL and pSKBL (**figure 3.13**). RNA transcripts were generated by *in vitro* transcription from the T7 promoter. RNA was incubated with probing buffer in the presence of 10mM MgAc. RNA transcripts were folded by rapid heating followed by slow cooling to 4°C. RNA was then incubated at 0°C to allow structural equilibrium.

Figure 3.12. Sequence Alignment of the 5' untranslated regions of human and murine BAG-1.

Conserved nucleotide highlighted in red. Initiation codons for each BAG-1 open reading frame (ORF) are shown in bold. The human Bag-1 IRES is located in the region 237-411 of this sequence.

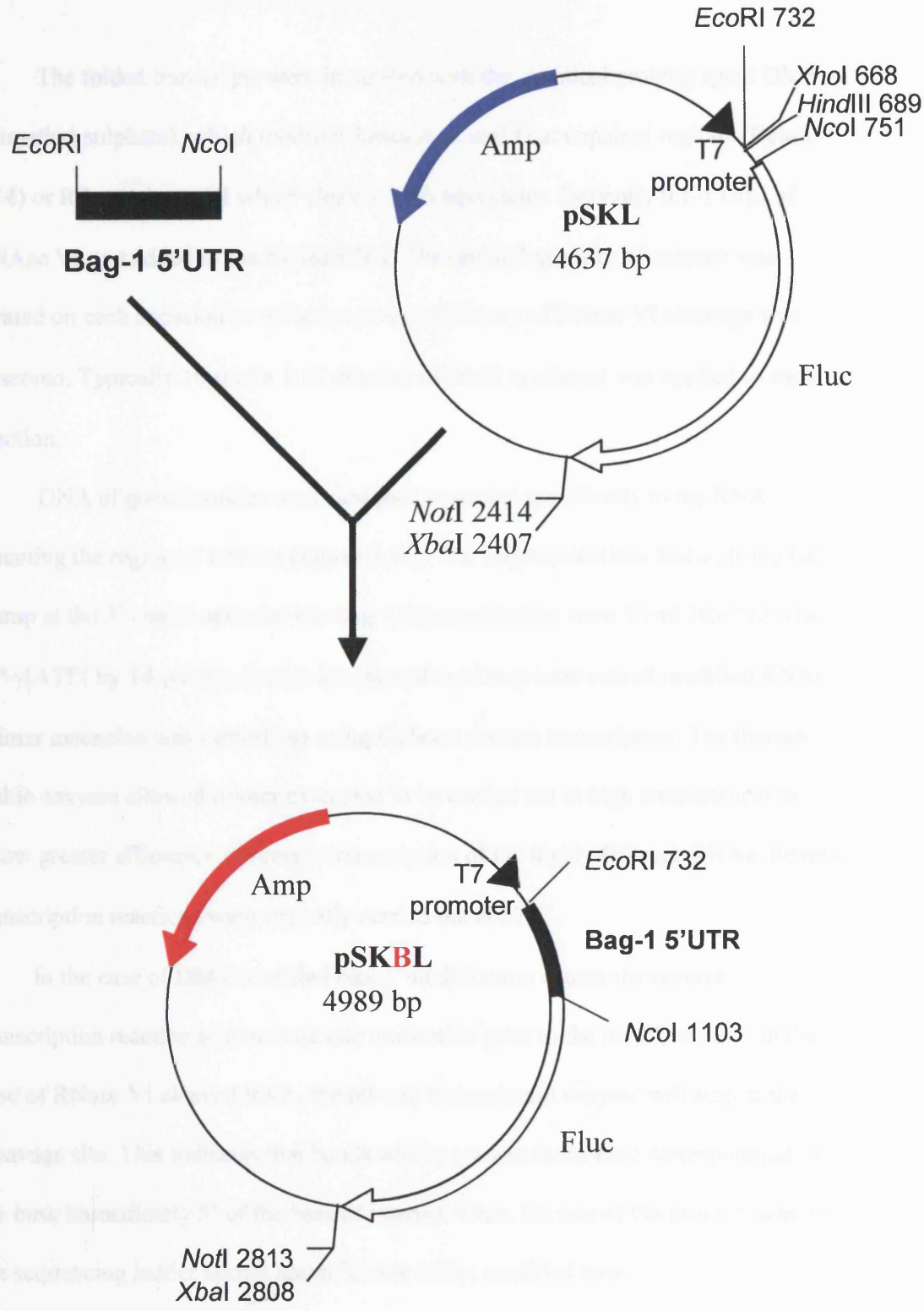


Figure 3.13. Construction of pSKBL.

The Bag-1 5'UTR was digested from pRBF with *Eco*RI and *Nco*I. The fragment was ligated into the vector pSKL, which was digested with *Eco*RI and *Nco*I and dephosphorylated. The resultant plasmid was pSKBL

The folded transcripts were incubated with the chemical probing agent DMS (Dimethyl sulphate), which modifies bases A, C and G at unpaired regions (**figure 3.14**) or Ribonuclease VI which cleaves 5' to base-pairs. Typically 0.1-1 U/μl of RNAse VI was added to the folded RNA. The optimal quantity of enzyme was titrated on each occasion as variation in the efficiency of RNase VI cleavage was observed. Typically 10μl of a 1:12 dilution of DMS in ethanol was applied to each reaction.

DNA oligonucleotides were designed to anneal specifically to the RNA spanning the region of interest (**figure 3.15**). The oligonucleotides had a strong GC clamp at the 3' end to optimise binding. Oligonucleotides were 5'end-labelled with ^{32}P-γ[ATP] by T4 polynucleotide kinase and used to primer extend modified RNAs. Primer extension was carried out using *C.therm* reverse transcriptase. The thermo stable enzyme allowed primer extension to be carried out at high temperatures to allow greater efficiency of reverse transcription of the highly GC-rich RNAs. Reverse transcription reactions were typically carried out at 57°C.

In the case of DMS-modified bases, modification causes the reverse transcription reaction to terminate one nucleotide prior to the modified base. In the case of RNase VI cleaved RNA, the reverse transcriptase enzyme will stop at the cleavage site. This indicates that bands will be present at the base corresponding to the base immediately 5' of the base of interest. Thus, the size of the product against the sequencing ladder allows identification of the modified base.

Primer extension was carried out with each primer on RNAs modified with DMS and/or RNase VI, in addition to unmodified RNAs treated in parallel as a control.

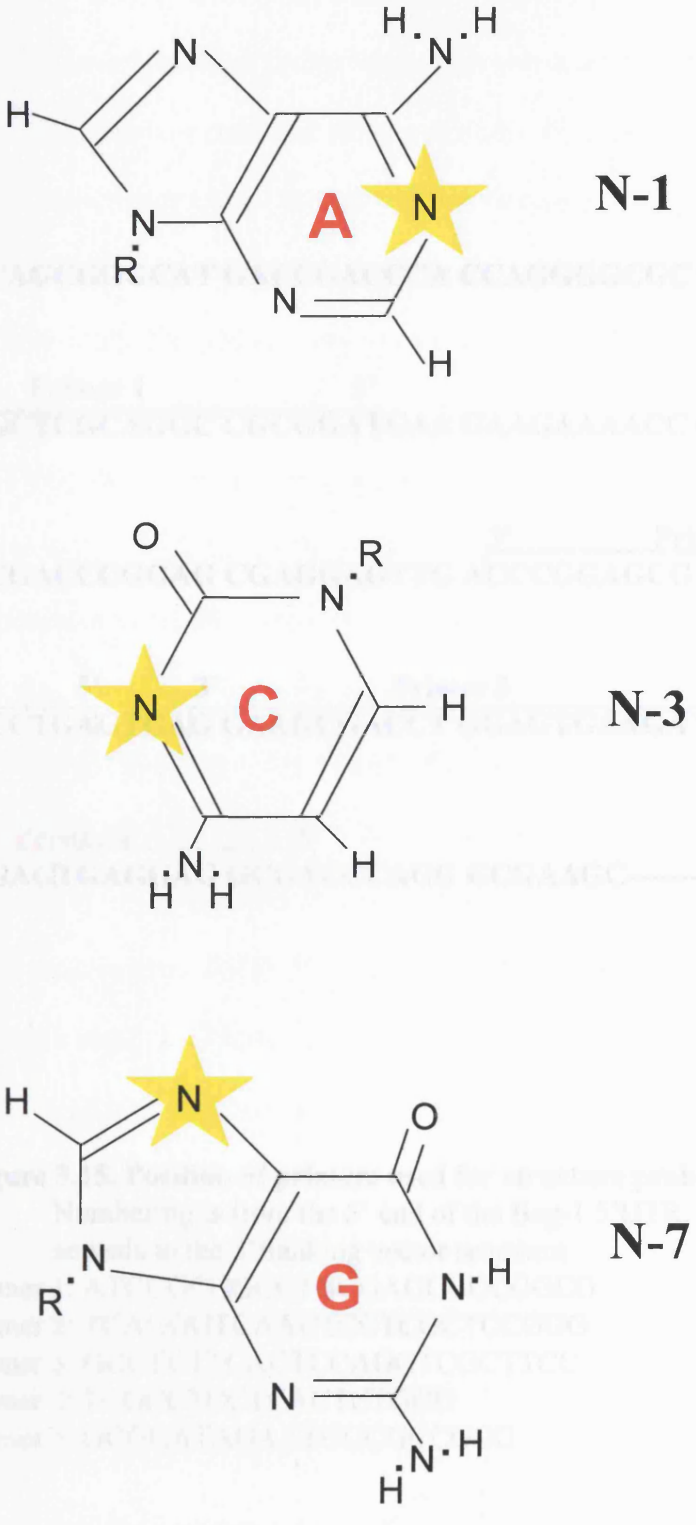


Figure 3.14. Schematic showing positions of chemical modification by DMS.
DMS attaches a methyl group at the positions represented by a star.

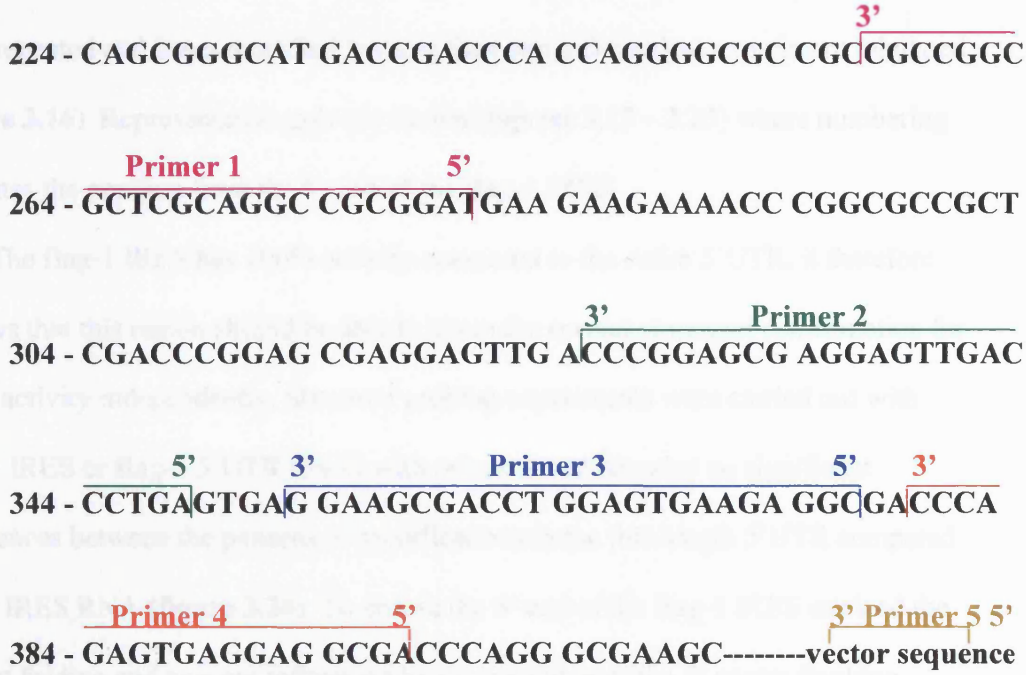


Figure 3.15. Position of primers used for structure probing.
Numbering is from the 5' end of the Bag-1 5'UTR. Primer 5 anneals to the 3'flanking vector sequence
Primer 1: ATCCGCGGCCTGCGAGCGCCGGCG
Primer 2: TCAGGGTCAACTCCTCGCTCCGGG
Primer 3: GCCTCTTCACTCCAGGTCGCTTCC
Primer 4: TCGCCTCCTCACTCTGGG
Primer 5: GCGGATAGAATGGCGCCGGG

Positions where the reverse transcriptase enzyme terminates prematurely in unmodified RNAs were denoted as background. Such events are generally attributed to robust structural features or particular structural motifs. As a consequence some regions of the IRES structure cannot be well defined. Structure probing experiments were repeated and bases modified in more than one independent experiment tabulated (**figure 3.16**). Representative gels are shown (**figures 3.17 – 3.23**) where numbering indicates the position from the 5' end of the Bag-1 IRES.

The Bag-1 IRES has 100% activity compared to the entire 5'UTR, it therefore follows that this region should be able to attain the correct structural conformation for IRES activity independently. Structure probing experiments were carried out with Bag-1 IRES or Bag-1 5'UTR RNAs with primer 4 and revealed no significant differences between the patterns of modifications in the full-length 5'UTR compared to the IRES RNA (**figure 3.24**). To ensure the 5' end of the Bag-1 IRES attained the correct folding and was not influenced by juxtaposition of the 5' vector flanking sequence to the position immediately 5' of the IRES, all reactions were carried out with the full-length Bag-1 5'UTR. In addition, to analyse bases at the extreme 3' end of the IRES, it was necessary to include 3' flanking sequence to provide a position for primer annealing. It is possible that the presence of 5' and 3' vector sequence could generate alternative foldings for the IRES, involving interactions with these vector sequences, however this possibility is unlikely given the ability of the Bag-1 IRES to function in a large range of vectors with different 5' and 3' flanking sequences.

Using the strongly modified positions from the tabulated data, constraints were placed on Zuker's Mfold algorithm to force bases to be double or single stranded at

Position	Base	Background	RNase VI	DMS
1	C		++	
2	G		++	
4	C	++		
5	C		++	
6	C	+	++	
7	A	++	+	
9	C	++		
10	A	++		
11	G		++	
13	G	+		
14	G	++		
15	C	++		
16	G	++		
26	G	++		++
27	C	++		
29	C			++
32	G			++
33	C			++
34	A	++		
35	G			++
36	G	++		
37	C	++		
38	C	+	++	
39	G	+		+
43	A			++
44	T	++		
47	A	+		++
48	G	+		+
49	A	+		+
50	A	+		+
51	G	+		+
52	A	+		+
53	A	+		+
54	A	+		++
55	A	++		
56	C			+
59	G			++
60	G			++
61	C	++		
62	G	++		
63	C			++
64	C			++
65	G			+
66	C			+
68	C			++
70	A	++		++
72	C	++		
73	C	++		++
74	G			++
76	A			++
77	G			++
78	C			+
79	G		++	++
80	A			++
81	G		++	++
83	A			++
85	T		++	
86	T	++		
88	A	++		++

Position	Base	Background	RNase VI	DMS
91	C		++	
92	G		++	
94	A			++
95	G			++
96	C		++	
97	G		++	
98	A		++	++
99	G		+	++
100	G		++	++
101	A			+
102	G			++
103	T	+	++	
104	T		++	
105	G	++	++	++
107	C	+	+	++
108	C	++		
109	C		++	
110	T		++	
111	G		++	
112	A			++
113	G			++
114	T		++	
115	G	++		
117	G			++
118	G			++
119	A			+
120	A	++		
121	G	++		++
122	C	++		++
123	G	++		
124	A	++		
125	C	++		
126	C	++		
127	T	++		
128	G	++		
141	G		+	
145	C	++		
146	A		++	
147	G		+	
148	A			++
150	T		+	
151	G	+		
152	A	++		
153	G	++		
154	G	++		
155	A			++
156	G	+		
158	C	++		
159	G		++	
160	A	++		
161	C	++		
162	C	++		
163	C		++	
164	A			+
166	G	++		
168	C	++	++	
169	G		++	
171	A			++
172	G			++

Figure 3.16. Table of modified bases.
Positions modified predominantly by RNase VI are coloured red, positions modified predominantly by DMS are coloured green. A single + depicts a weak modification, a double ++ indicates a strong modification, as determined by eye. Positions where pairing cannot be defined due to background are coloured blue. Only bases where modifications or background were observed are tabulated. All modifications were observed in at least two independent experiments.

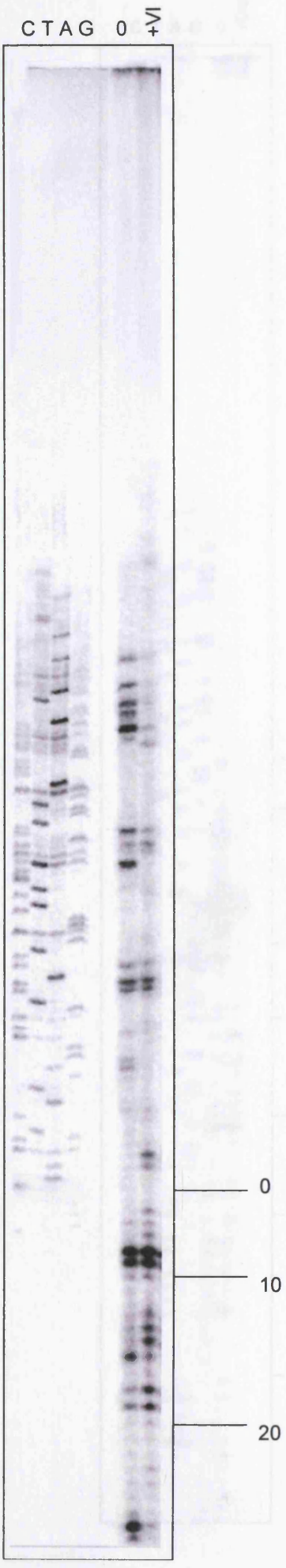


Figure 3.17. Double-stranded bases in the 1-25 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with RNaseVI and reverse transcribed with Primer 1. The 0 control lane shows unmodified RNA treated in parallel.

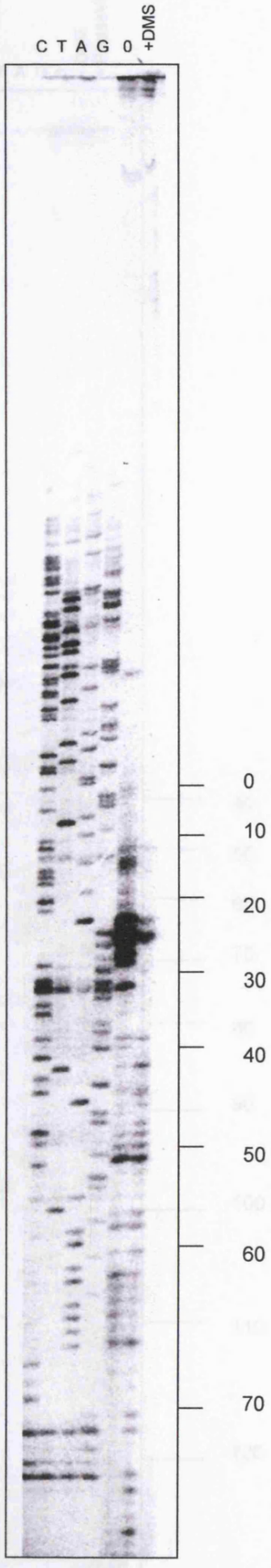


Figure 3.18. Single-stranded bases in the 1-80 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with DMS and reverse transcribed with Primer 2. The 0 control lane shows unmodified RNA treated in parallel.

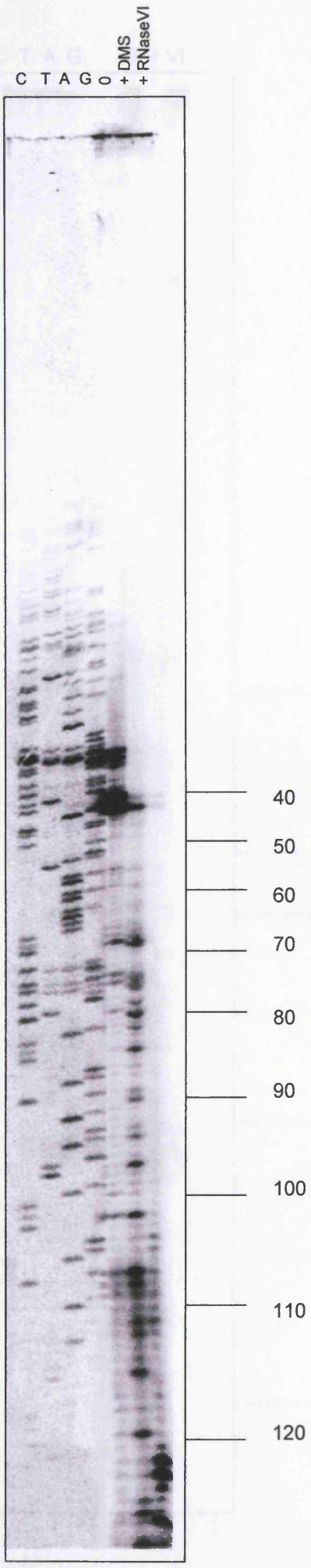


Figure 3.19. Single and double-stranded bases in the 40-130 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with DMS or RNase VI and reverse transcribed with Primer 3. The 0 control lane shows unmodified RNA treated in parallel.

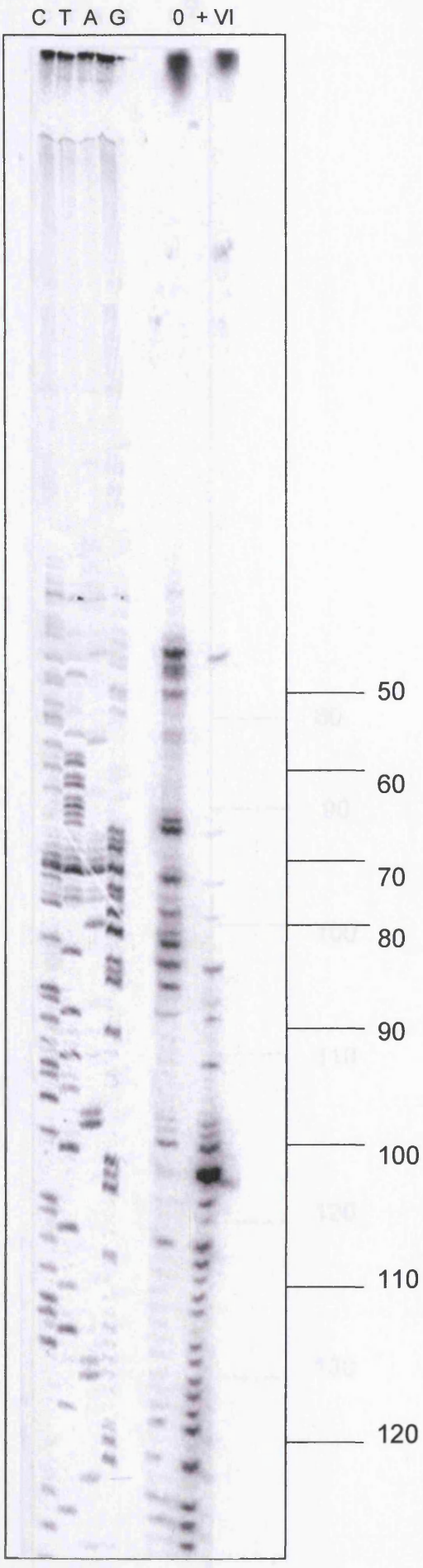


Figure 3.20. Double-stranded bases in the 50-130 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with RNAseVI and reverse transcribed with Primer 4. The 0 control lane shows unmodified RNA treated in parallel.

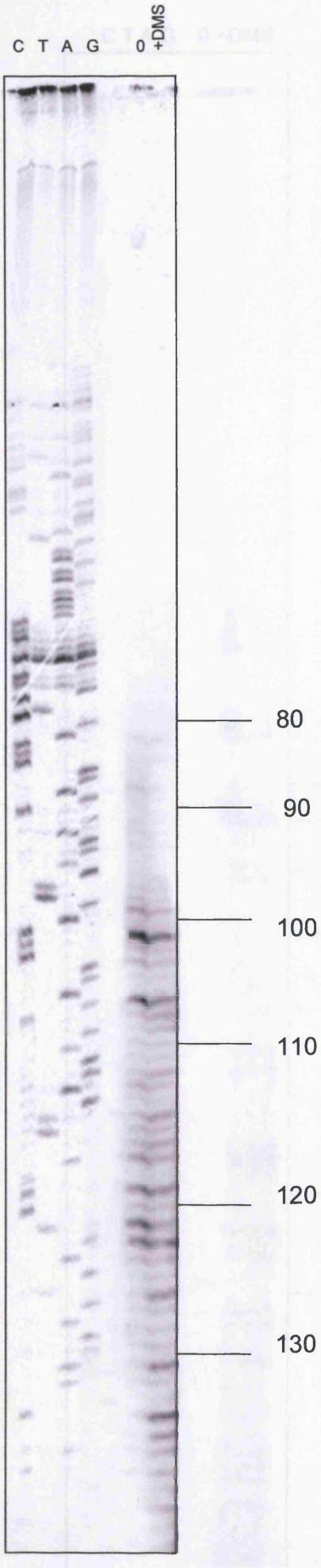


Figure 3.21. Single-stranded bases in the 80-140 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with DMS and reverse transcribed with Primer 4. The 0 control lane shows unmodified RNA treated in parallel.

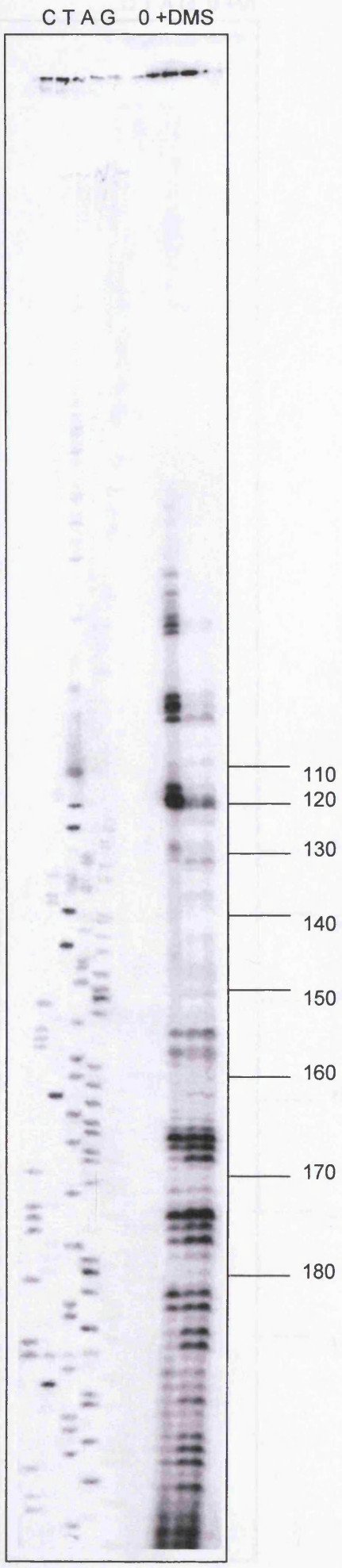


Figure 3.22. Single-stranded bases in the 110-184 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with DMS and reverse transcribed with Primer 5. The 0 control lane shows unmodified RNA treated in parallel.

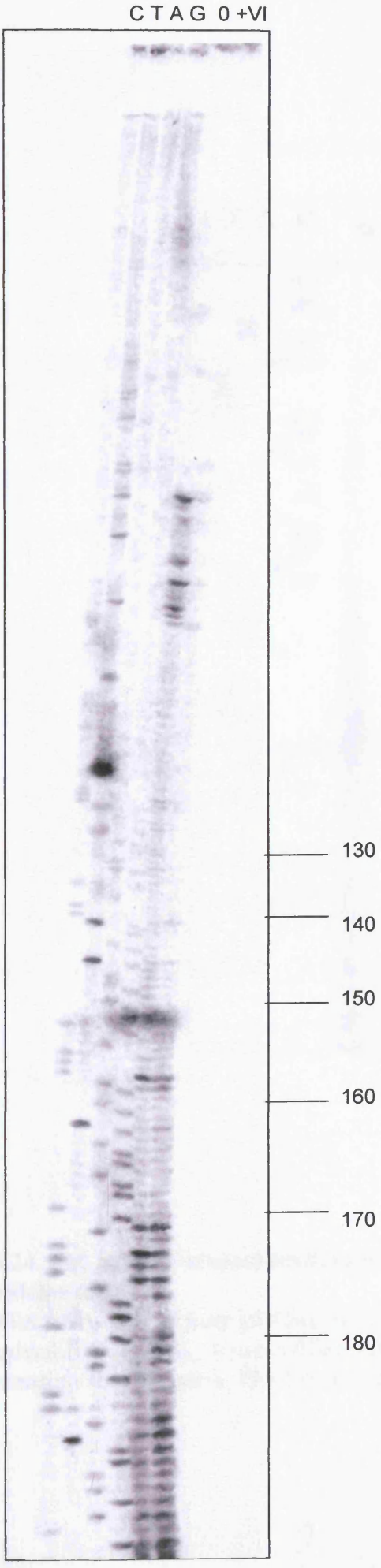


Figure 3.23. Double-stranded bases in the 130-184 nucleotide segment of the Bag-1 IRES.
Bag-1 IRES RNA was incubated with RNAseVI and reverse transcribed with Primer 5. The 0 control lane shows unmodified RNA treated in parallel.

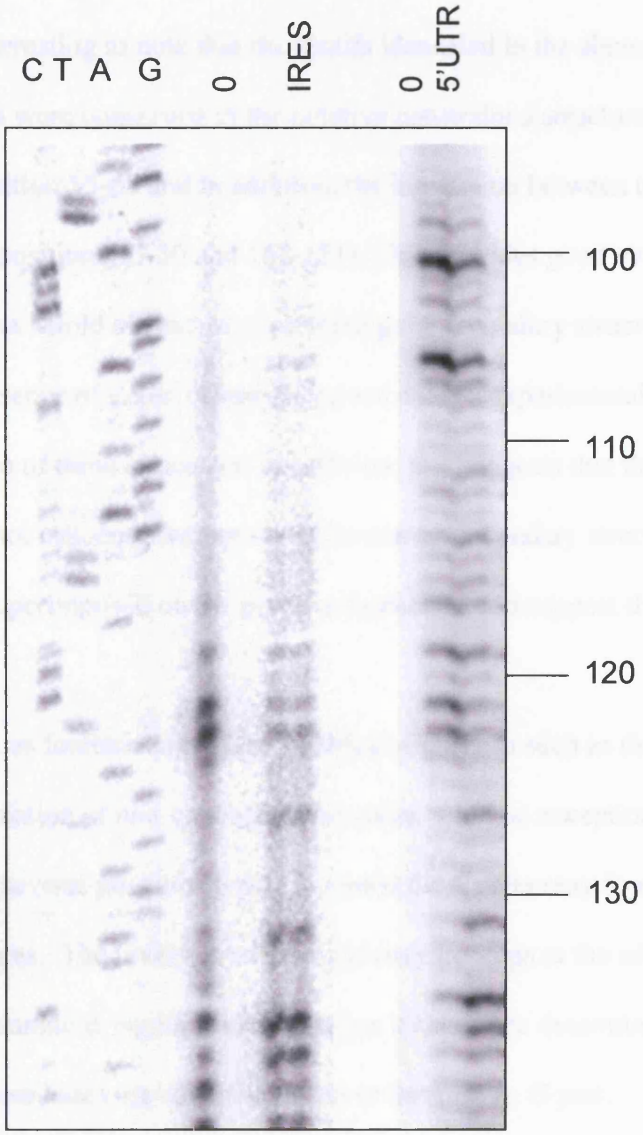


Figure 3.24. The pattern of modifications is the same for RNA derived from pSKBL and pSKBminL.

IRES RNA derived from pSKBminL or RNA representing the entire Bag-1 5'UTR, derived from pSKBL, was modified with DMS and single-stranded bases identified by extension with primer 4. The 0 control lanes show unmodified RNA treated in parallel.

the positions which had been strongly modified and a single structure was produced (**figure 3.25**). It is interesting to note that the motifs identified in the absence of structure probing data were conserved in the putative constrained structures including the A-rich loop at position 55-68 and in addition, the interaction between the 5' and 3' ends of the IRES (positions 13-30 and 168-181). This provides good evidence for the effectiveness of the Mfold algorithm at predicting the secondary structure of the Bag-1 IRES in the absence of experimentally derived data as experimental data supports the formation of these structures. In addition, this suggests that the 5' and 3' vector sequences do not influence formation of the correct secondary structure. Weak modifications were superimposed on the predicted structure and support the predicted structure generated.

There are numerous limitations to Zuker's Mfold algorithm such as the prevention of the formation of non-canonical base pairs, with the exception of G: U base-pairs. There are several positions where potential G: A pairs may form, these are indicated by dotted lines. The reverse transcriptase enzyme stops at the adenine at position 26 and the guanine at position 172 therefore it cannot be determined from these data whether these bases are single-stranded or form an A: G pair.

The secondary structure of the Bag-1 IRES is superimposed on the human: mouse sequence alignment in **figure 3.26**. Although there is some degree of sequence variation in the mouse sequence, the corresponding sequences appear to support formation of the same helices. Where insertions are present in the mouse sequence, these occur in single-stranded regions, potentially increasing the size of the single-stranded regions, which may not perturb the secondary structure.

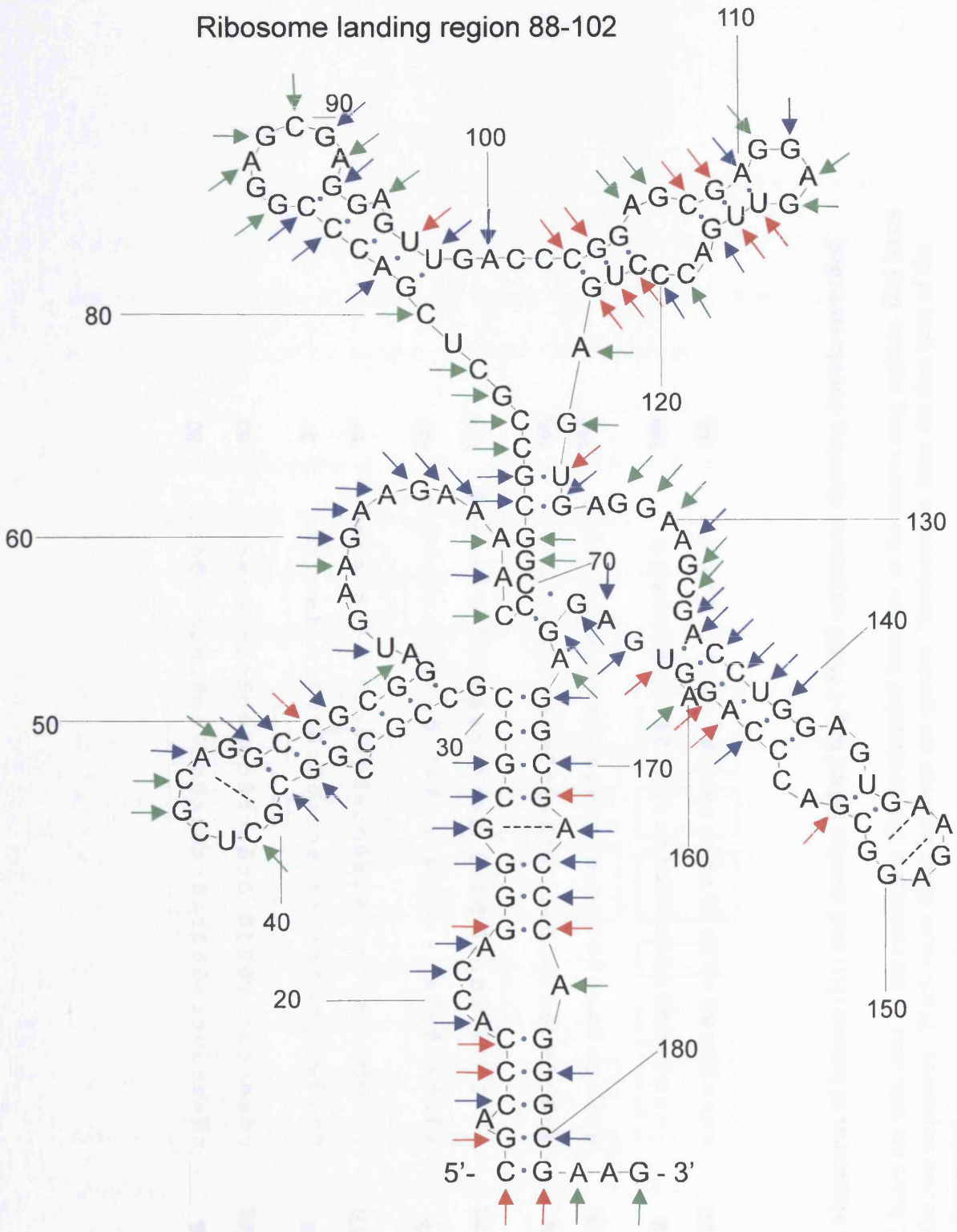


Figure 3.25. Predicted secondary structural model for the Bag-1 IRES. Secondary structural model with strong and weak modifications superimposed. Red arrows indicate double-stranded bases, green arrows indicate single-stranded bases and blue arrows indicate positions where background obscures other data. Blue dots indicate base-pairing and dotted lines indicate potential base pairing. Numbering is from the 5' end of the minimal Bag-1 IRES (225-411) with the first base of the IRES designated 1.

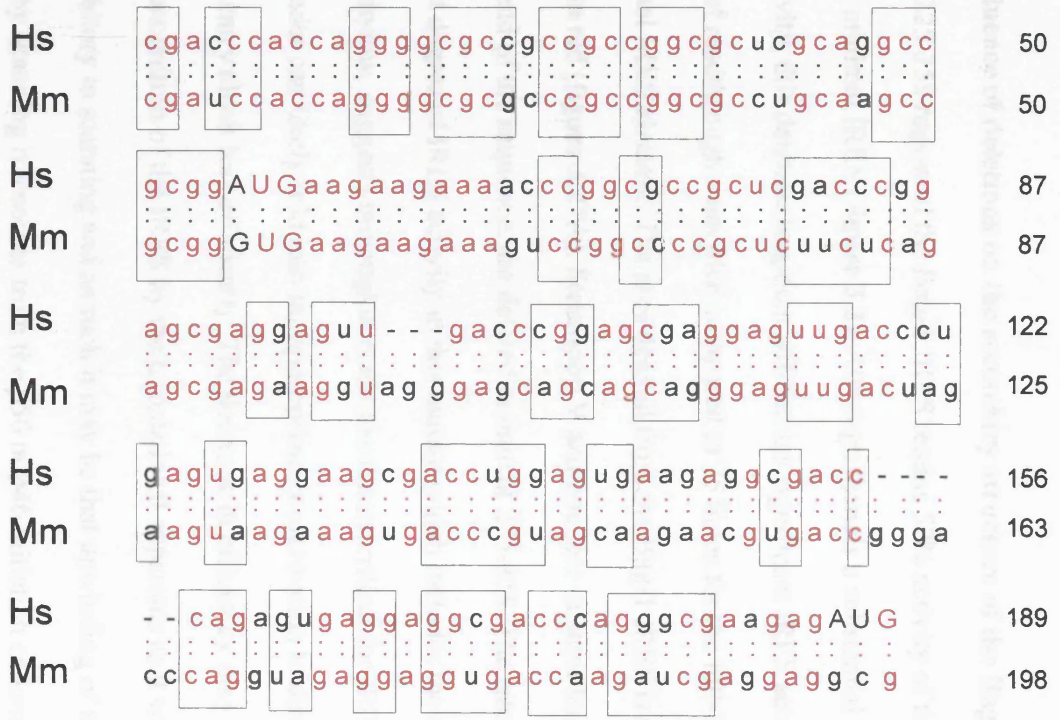


Figure 3.26. Sequence alignment of human (Hs) and murine (Mm) Bag-1 IRES sequences showing double-stranded bases.

Double stranded bases are indicated by the rectangles. Single-stranded bases are in the intervening regions. Red bases are identical in the two sequences. Black bases differ between the species. Numbering is from the first base of the IRES, which is designated 1.

3.5. Mutational analysis of the Bag-1 IRES

The production of a secondary structural model for the Bag-1 IRES allows the design of mutants to determine which motifs in the IRES structure are critical for Bag-1 IRES activity and, in addition, interpretation of deletion and ribosome-landing mutant activities in light of secondary structural perturbations they may cause.

3.6.1. Influence of deletions on the secondary structure of the Bag-1 IRES

The 225-358 region of the Bag-1 IRES retains 52% activity of the full-length 5'UTR or minimal IRES (**figure 3.2**). Although there is a substantial reduction in IRES activity, this deletion fragment still retains significant IRES activity, 13 times the level of readthrough from pRF compared to 25 times for the full-length 5'UTR or the minimal IRES element. The area deleted from the Bag-1 IRES is shown on the structure in red (**figure 3.27A**). Stem-loop V and one side of stem-loop I, consisting of the 3' end of the sequence, are deleted in mutant 225-358. The retention of a significant degree of IRES activity in this mutant which includes the ribosome landing window, suggests this region is not absolutely critical for IRES activity and the remainder can likely fold into the appropriate conformation to direct internal ribosome entry albeit less efficiently. The decrease in efficiency may be due to reduced recognition of the IRES by the translational apparatus as it would no longer be as inhibitory to scanning and as such it may be that unwinding of the IRES structure by scanning ribosomes from the p50 or p46 initiation codons could contribute to decreased IRES efficiency. Alternatively, stem-loop V contains an AG-rich polyloop, which is potentially closed by a sheared G:A pair, a feature often

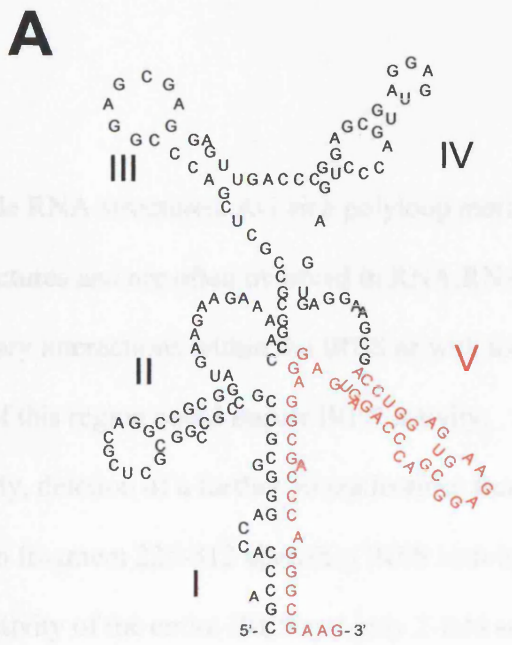


Figure 3.27. Effect of deletion mutants on the secondary structure of the Bag-1 IRES.

Deleted regions of the IRES are highlighted in red.

A. The 225-358 fragment, which has 52% activity of the full-length Bag-1 5'UTR (figure 3.2.).

B. The 225-312 fragment, which has 9% activity of the full-length Bag-1 5'UTR (figure 3.2.).

observed in stable RNA structures. AG-rich polyloop motifs are frequently found in stable RNA structures and are often involved in RNA:RNA interactions, implicating this loop in tertiary interactions within the IRES or with the translational apparatus, hence deletion of this region could impair IRES activity.

Interestingly, deletion of a further 36 nucleotides from the 3' end of the IRES to form the deletion fragment 225-312 abolishes IRES activity. The resultant construct has 9% of the activity of the entire IRES and only 2-fold activity over that of pRF suggesting this fragment cannot act as a functional IRES (**figure 3.2**). This deletion removes stem-loop IV in addition to V and the 3' half of I. It is likely that this mutation would disrupt the secondary structure of the IRES such that a functional IRES cannot be generated and suggests integrity of the central region may be essential for IRES activity. This region encompasses the 3' end of the ribosome-landing region and as such its deletion probably prevents ribosome recruitment.

3.6.2. The ribosome-landing region

The ribosome-landing region is marked on the predicted secondary structure in **figure 3.25**. The ribosome appears to make important contacts in the central region of the structure, which is critical for IRES function. The out-of-frame AUG codons are marked on the structure with base changes that alter the sequence highlighted in blue and bases that form part of the new initiation codon but which were not mutated highlighted in green (**figure 3.28**). Analysis of alterations in the structure indicates that these changes should, in most cases, retain the structural integrity of the IRES. The mutations to construct AUGs 2, 3 and 5 are isolated in loops and are not

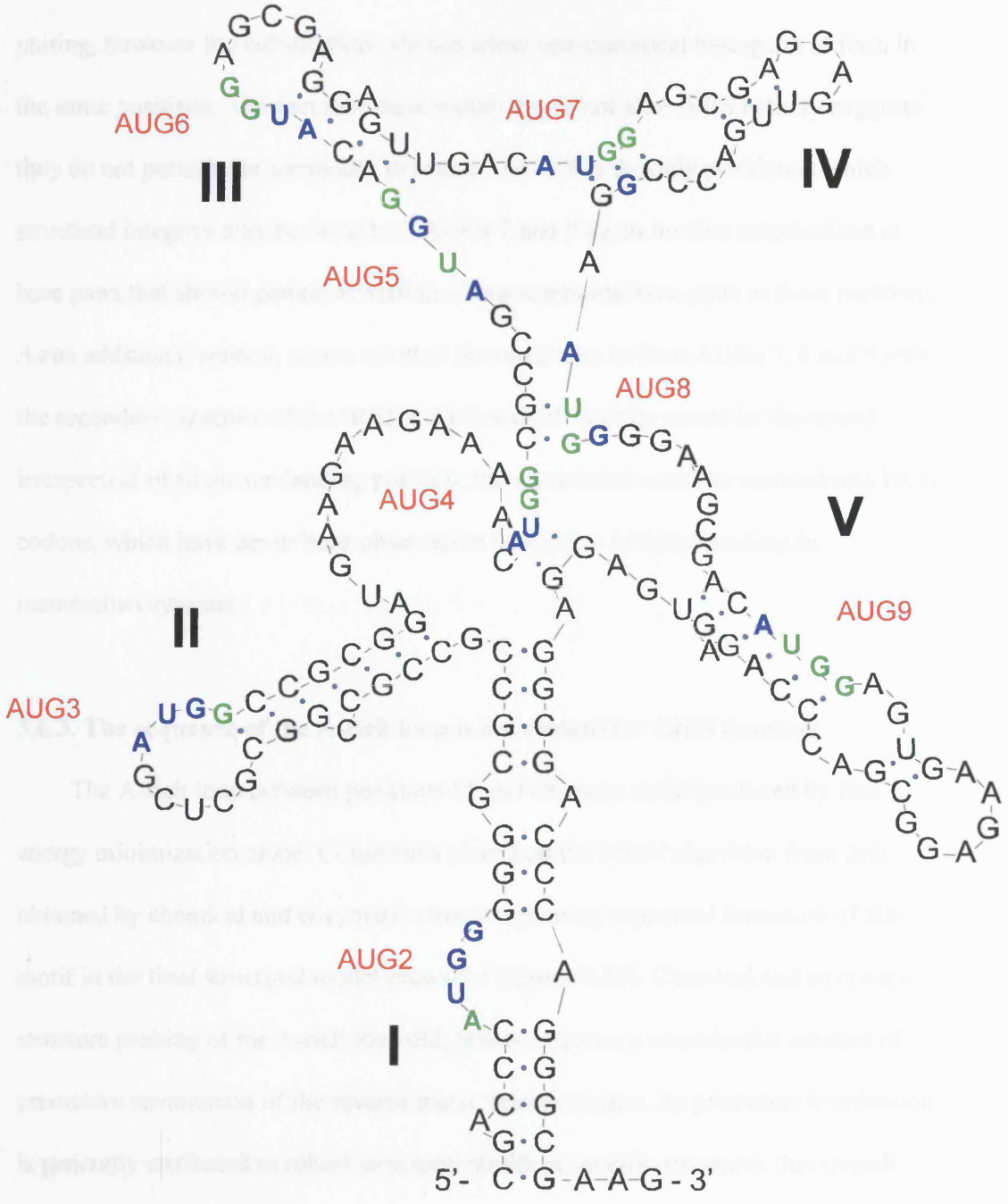


Figure 3.28. Positions of out-of-frame AUG mutants on the secondary structure of the Bag-1 IRES

Mutants are labelled in red. Mutations that alter the sequence are highlighted in blue and those that do not alter the sequence in green.

predicted to alter the structure. AUGs 4 and 6 substitute nucleotides involved in base pairing, however the substitutions should allow non-canonical base-pairs to form in the same positions. The fact that these mutations do not alter IRES activity suggests they do not perturb the secondary structure. AUG 8 is the only position at which structural integrity may be disturbed. AUGs 7 and 9 again involve substitutions at base pairs that should permit formation of non-canonical base pairs at these positions. As an additional control, to test whether the mutations to form AUGs 7, 8 and 9 alter the secondary structure of the IRES such that IRES activity would be decreased irrespective of ribosome landing position, the same bases could be mutated into UUG codons, which have never been observed to be used as initiation codons in mammalian systems.

3.6.3. The sequence of the A-rich loop is not critical for IRES function

The A-rich loop between positions 55 and 68 was a motif predicted by free energy minimization alone. Constraints placed on the Mfold algorithm from data obtained by chemical and enzymatic structure probing supported formation of this motif in the final structural model presented (**figure 3.25**). Chemical and enzymatic structure probing of the A-rich loop did, however, cause a considerable amount of premature termination of the reverse transcriptase enzyme. As premature termination is generally attributed to robust structural motifs or specific structures that impede progress of the enzyme, it is possible that this loop may be critical in tertiary interactions. The A-rich loop is conserved in all of the potential structures generated and the central 11 (of 13) bases conserved in the murine 5'UTR; therefore the

importance of the sequence of this loop was investigated by mutagenesis. A mutant was constructed by PCR mutagenesis to substitute A (position 64) to A (position 67) to guanines (**figure 3.29A**). The mutant construct was transfected into HeLa cells and assayed for IRES activity. There is no significant decrease in IRES activity on mutating this region, suggesting sequence at least may be unimportant for IRES activity (**figure 3.29B**). Many viral IRESes contain AU rich spacer elements in their IRES secondary structures and this may be a potential role for the A rich loop; however this does not explain the presence of a significant amount of pausing of the reverse transcriptase enzyme in this region. It will be interesting to determine whether length of this sequence is important for IRES activity and also to determine the influence of amputation of this loop on IRES activity.

Discussion

The data presented here indicates that the Bag-1 IRES is contained within the 225-411 region of the Bag-1 5'UTR. This region forms a complex secondary structural motif with several loops, which may be accessible for protein binding, act as unstructured spacers or be important for tertiary interactions. The central region, including the ribosome-landing site is subject to numerous pauses by the reverse transcriptase enzyme making it difficult to assign a clear structure. It is possible that this region is not structurally rigid but can attain a number of structural motifs. Alternatively, tertiary interactions between elements in this region may restrict access by both modification agents and also, the reverse transcriptase enzyme.

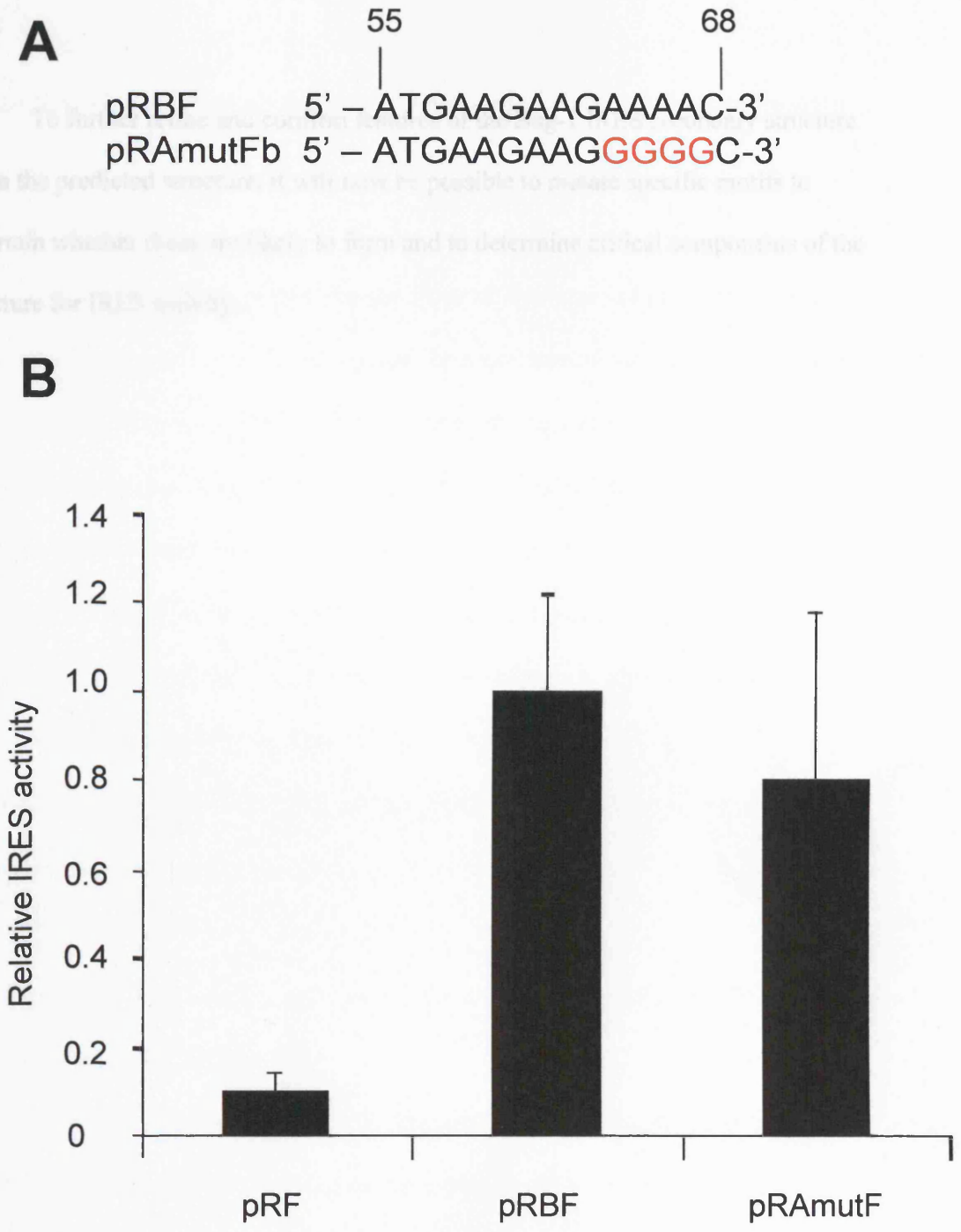


Figure 3.29. Effect of A-rich loop mutations on Bag-1 IRES activity.

A. Nucleotides coloured red are mutated in the construct pRAmutF

B. IRES activity (firefly/*Renilla* luciferase) of the dicistronic constructs pRF, pRBF and pRAmutF after transfection into HeLa cells. IRES activity is expressed relative to pRBF which is assigned a value of 1.

To further refine and confirm features of the Bag-1 IRES secondary structure given the predicted structure, it will now be possible to mutate specific motifs to ascertain whether these are likely to form and to determine critical components of the structure for IRES activity.

Chapter 4

Analysis of the *trans*-acting factor requirements for Bag-1 internal initiation

4.1. Introduction

A key feature of the cellular and viral IRESes studied so far is that they show cell-type specificity. This suggests there are determinants in cell lines which influence the efficiency of IRES-driven translation and recently this role has been attributed to *trans*-acting protein factors. Cellular IRESes and class I picornaviral IRESes drive translation inefficiently in the rabbit reticulocyte lysate *in vitro* translation system, however in the majority of cases significant translation can be restored by supplementing the *in vitro* translation system with cell extracts or specific *trans*-acting factors. Thus, it was of interest to determine whether the Bag-1 IRES functions with cell-type specificity and consequently to attempt to elucidate the *trans*-acting factor requirements of this IRES.

4.2.The Bag-1 IRES is inactive when transcripts are introduced directly into the cytoplasm of HeLa cells.

A nuclear event appears to be an important factor in the activation of many IRESes, particularly cellular IRESes, for example, the immunoglobulin heavy chain binding protein, Bip (Yang and Sarnow, 1997), the c-*myc* IRES (Stoneley *et al.*, 2000), and the Apaf-1 IRES (Coldwell, 2001) require a nuclear event for efficient translation initiation. Constructs introduced directly into the cytoplasm of cells are not capable of directing translation of the downstream cistron, whereas plasmids transfected into the nucleus of the same cell lines can be translated by internal ribosome entry and an upregulation in translation of the downstream

cistron is observed. The nuclear event has been proposed to comprise nuclear protein factors or a conformational change to the mRNA structure that is induced in the nucleus in order for the IRES to attain an active conformation (Stoneley *et al.*, 2000).

To determine whether the Bag-1 IRES requires a nuclear event, the Bag-1 5' UTR was cloned between the *Renilla* and firefly luciferase cistrons of the plasmid pSP64RLpolyA. The plasmid pSp64RBagLpolyA was generated by amplification of the Bag-1 5'UTR from the vector pRBF using the primers BAGSpeF and BAG36R (**table 2.2.**). The PCR product was digested with *Spe*I and *Nco*I and ligated into pSp64RLpolyA vector, which had been digested with *Xba*I and *Nco*I (**figure 4.1**). Capped and polyadenylated dicistronic transcripts were generated by *in vitro* transcription from the T7 polymerase promoter in the presence of cap analogue (**figure 4.2a**). RNA transfections of the transcripts into HeLa cells using the carrier lipofectin were performed. Cells were harvested after 8 hours and assayed for luciferase activity (**figure 4.2b**).

A positive control with HRV in place of the Bag-1 5' UTR was transfected simultaneously. The HRV IRES is acknowledged as being capable of initiating the translation of transcripts introduced directly into the cytoplasm (Stoneley *et al.*, 2000). As a negative control, RNA derived from pSp64RLpolyA without the IRES cloned into the intercistronic region was also transfected into HeLa cells. The Bag-1 IRES has previously been shown to be more active than the HRV IRES in HeLa cells in a dicistronic context when plasmids are transfected into the cell nucleus. The vectors pRBF and pRHRVF stimulate translation of the downstream cistron 17 and 8 fold over the empty vector respectively (Coldwell, 2001). This would suggest that the Bag-1 IRES should direct translation at least as efficiently

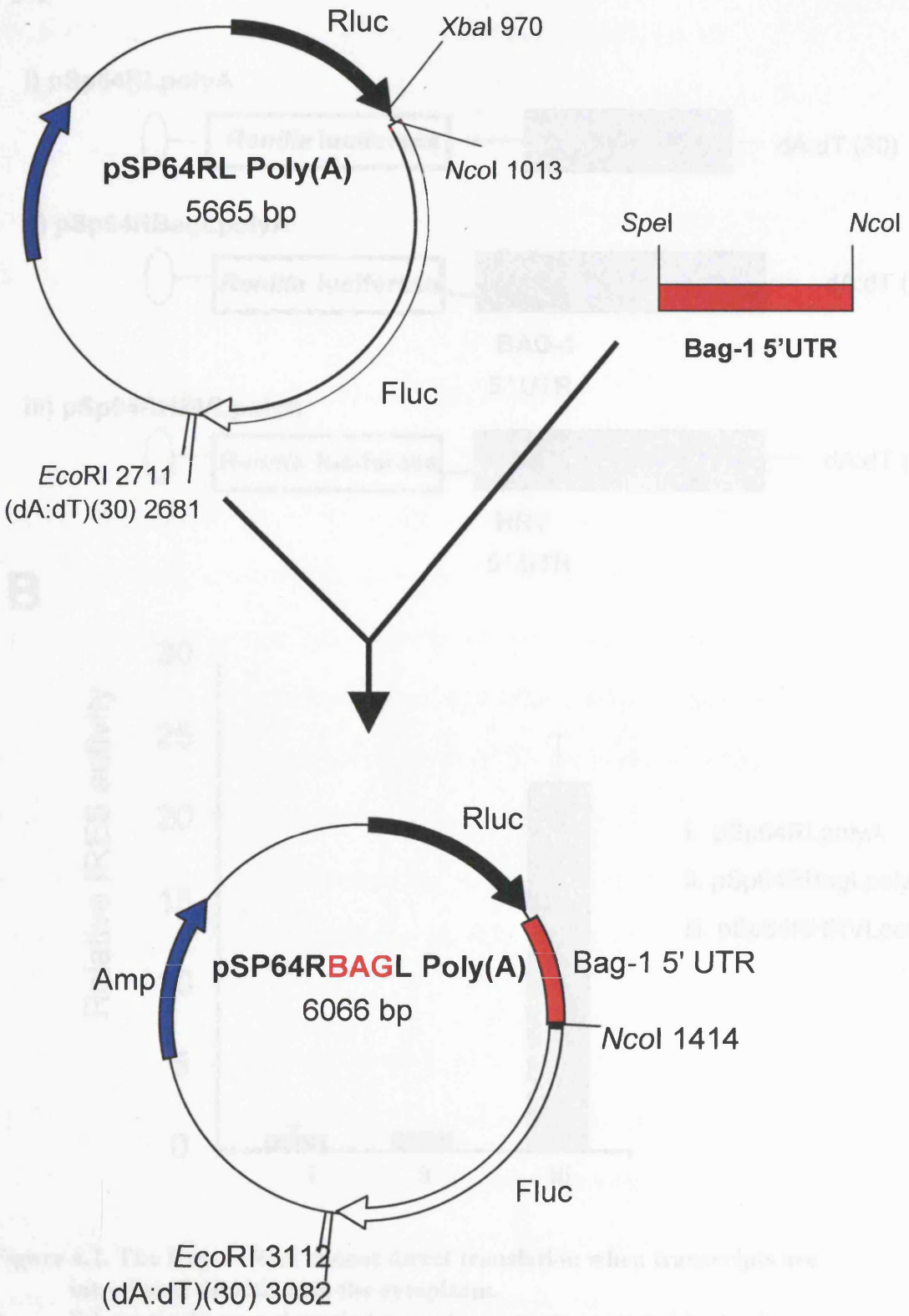


Figure 4.1. Construction of pSp64BLpolyA.
The Bag-1 5' UTR was amplified from pRBF by PCR using the primers BAGSpeF and BAG36R, to introduce a *Spe*I restriction site. The PCR product was digested with *Spe*I and *Nco*I and the fragment ligated into the vector pSp64RLpolyA which had been digested with *Xba*I and *Nco*I and dephosphorylated.

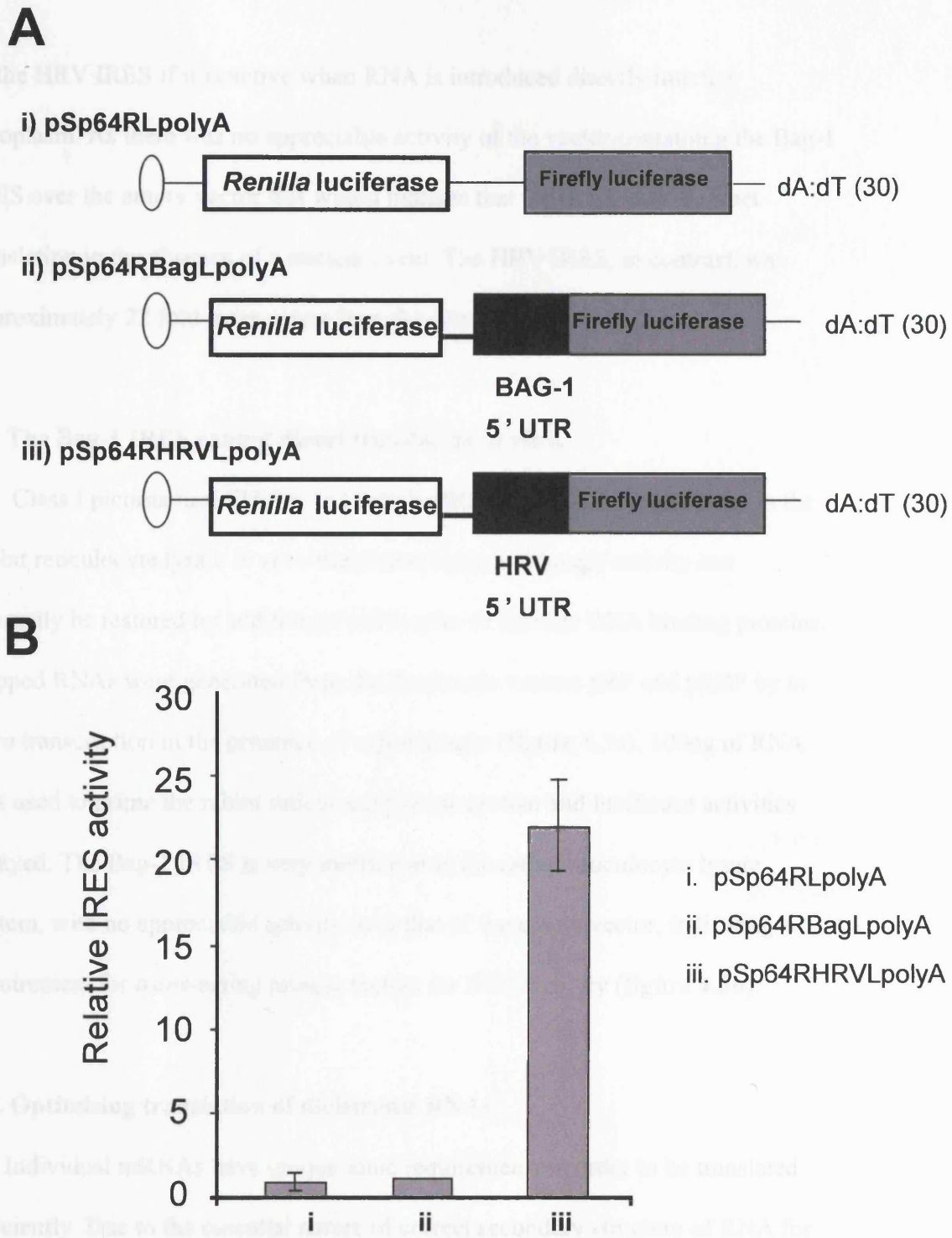


Figure 4.2. The Bag-1 IRES cannot direct translation when transcripts are introduced directly into the cytoplasm.

A. Schematic diagram depicting capped transcripts generated by *in vitro* transcription, i) psp64RLpolyA, ii) pSp64RLpolyA with the Bag-1 5'UTR cloned into the intercistronic region, iii) pSp64RLpolyA with the HRV 5'UTR cloned into the intercistronic region.

B. Relative IRES activity, represented by the ratio of firefly/*Renilla* luciferase of each construct after transfection into the cytoplasm of HeLa cells. IRES activity is expressed relative to that of pSp64RLpolyA, which is assigned a value of 1. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

as the HRV IRES if it is active when RNA is introduced directly into the cytoplasm. As there was no appreciable activity of the vector containing the Bag-1 IRES over the empty vector this would indicate that the IRES cannot direct translation in the absence of a nuclear event. The HRV IRES, in contrast, was approximately 22 fold more active than the empty vector.

4.3. The Bag-1 IRES cannot direct translation *in vitro*.

Class I picornaviral IRESes and cellular IRESes function inefficiently in the rabbit reticulocyte lysate *in vitro* translation system although activity can generally be restored by addition of cell lysates or specific RNA binding proteins. Capped RNAs were generated from the dicistronic vectors pRF and pRBF by *in vitro* transcription in the presence of cap analogue (**figure 4.3a**). 100ng of RNA was used to prime the rabbit reticulocyte lysate system and luciferase activities assayed. The Bag-1 IRES is very inefficient in the rabbit reticulocyte lysate system, with no appreciable activity over that of the empty vector, indicating a requirement for *trans*-acting protein factors for IRES activity (**figure 4.3b**).

4.4. Optimising translation of dicistronic RNAs

Individual mRNAs have unique ionic requirements in order to be translated efficiently. Due to the essential nature of correct secondary structure of RNA for IRES function, the ionic concentration may be critical. Thus, in order to assess the optimum ionic requirements for translation of the *Renilla* and firefly luciferase cistrons in the vectors pRF and pRBF, the salt concentrations present in the rabbit reticulocyte lysate system were titrated. The Mg^{2+} concentration present in each translation reaction was constant in the translation buffer with a final

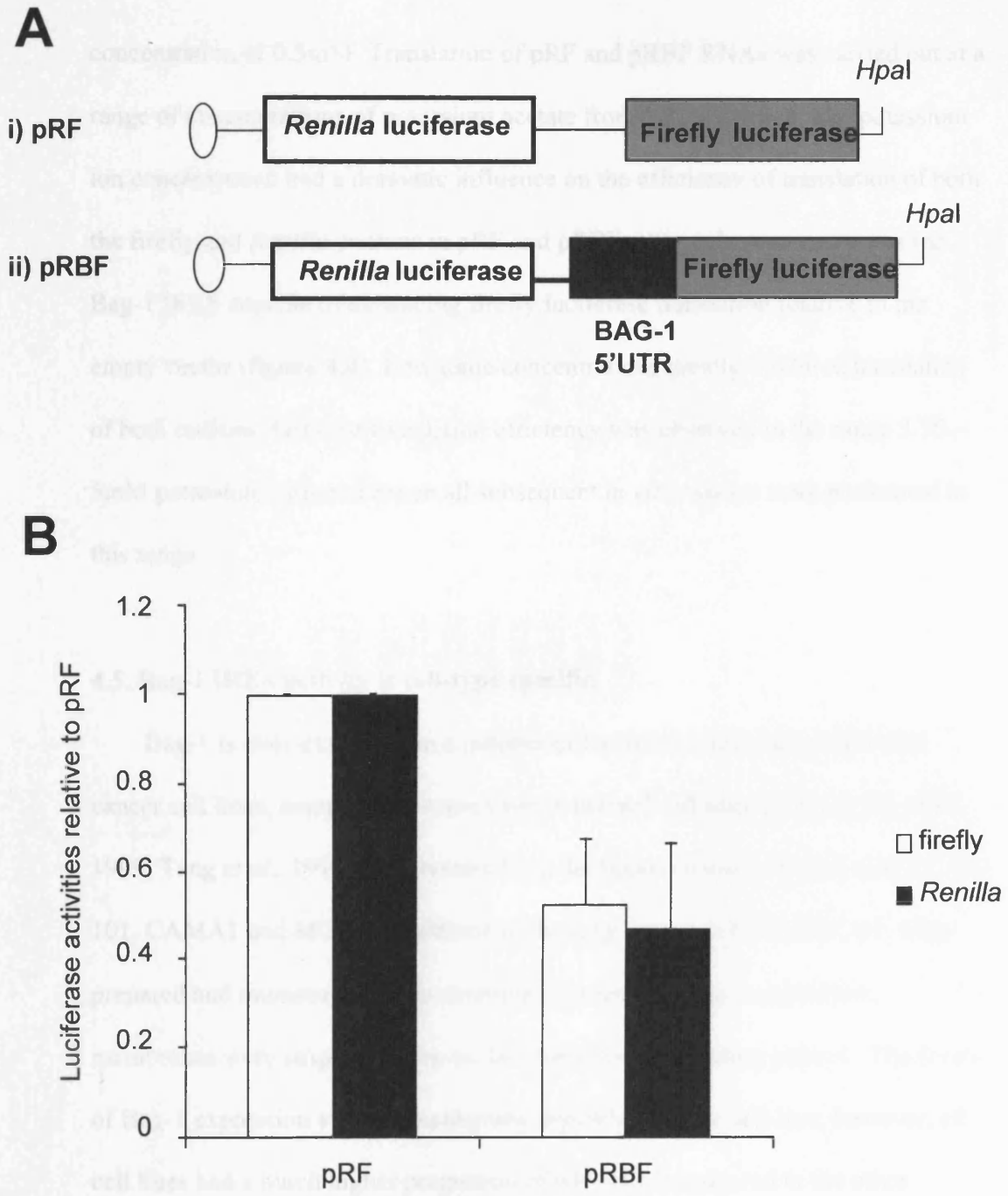


Figure 4.3. The Bag-1 IRES cannot direct translation in the rabbit reticulocyte lysate *in vitro* translation system

A. Schematic diagram depicting dicistronic RNAs used to prime the rabbit reticulocyte lysate system, i) pRF and ii) pRBF, with the Bag-1 5'UTR cloned into the intercistronic region.

B. Luciferase activity measured as relative light units and expressed relative to *Renilla* or firefly luciferase of pRF which are assigned values of 1. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

concentration of 0.5mM. Translation of pRF and pRBF RNAs was carried out at a range of concentrations of potassium acetate from 1.25 – 7.5mM. The potassium ion concentration had a dramatic influence on the efficiency of translation of both the firefly and *Renilla* cistrons in pRF and pRBF, although in no cases was the Bag-1 IRES capable of enhancing firefly luciferase translation relative to the empty vector (**figure 4.4**). Low ionic concentrations greatly inhibited translation of both cistrons. Greatest translation efficiency was observed in the range 3.75 – 5mM potassium ions and hence all subsequent *in vitro* assays were performed in this range.

4.5. Bag-1 IRES activity is cell-type specific.

Bag-1 is over-expressed in a number of human cervical, lung and breast cancer cell lines, compared to expression in normal cell lines (Yang *et al.*, 1998, 1999; Tang *et al.*, 1999). Cell lysates from the breast cancer cell lines CAL51, G1-101, CAMA1 and MCF7, in addition to the lung cancer cell line CALU1, were prepared and immunoblotted to determine the level of Bag-1 expression, membranes were stripped and re-probed for actin as a loading control. The levels of Bag-1 expression varied considerably dependent on the cell line; however, all cell lines had a much higher proportion of p36 when compared to the other isoforms (**figure 4.5**). pRF and pRBF were also transiently transfected into the cell lines and relative IRES activity expressed as a ratio of firefly to *Renilla* luciferase (**figure 4.6b**). IRES activity varies considerably according to cell line, however no significant correlation between IRES activity and the expression pattern of the isoforms can be identified. It is possible that differing endogenous levels of *trans*-acting protein factors could contribute to IRES activity *in vivo*.

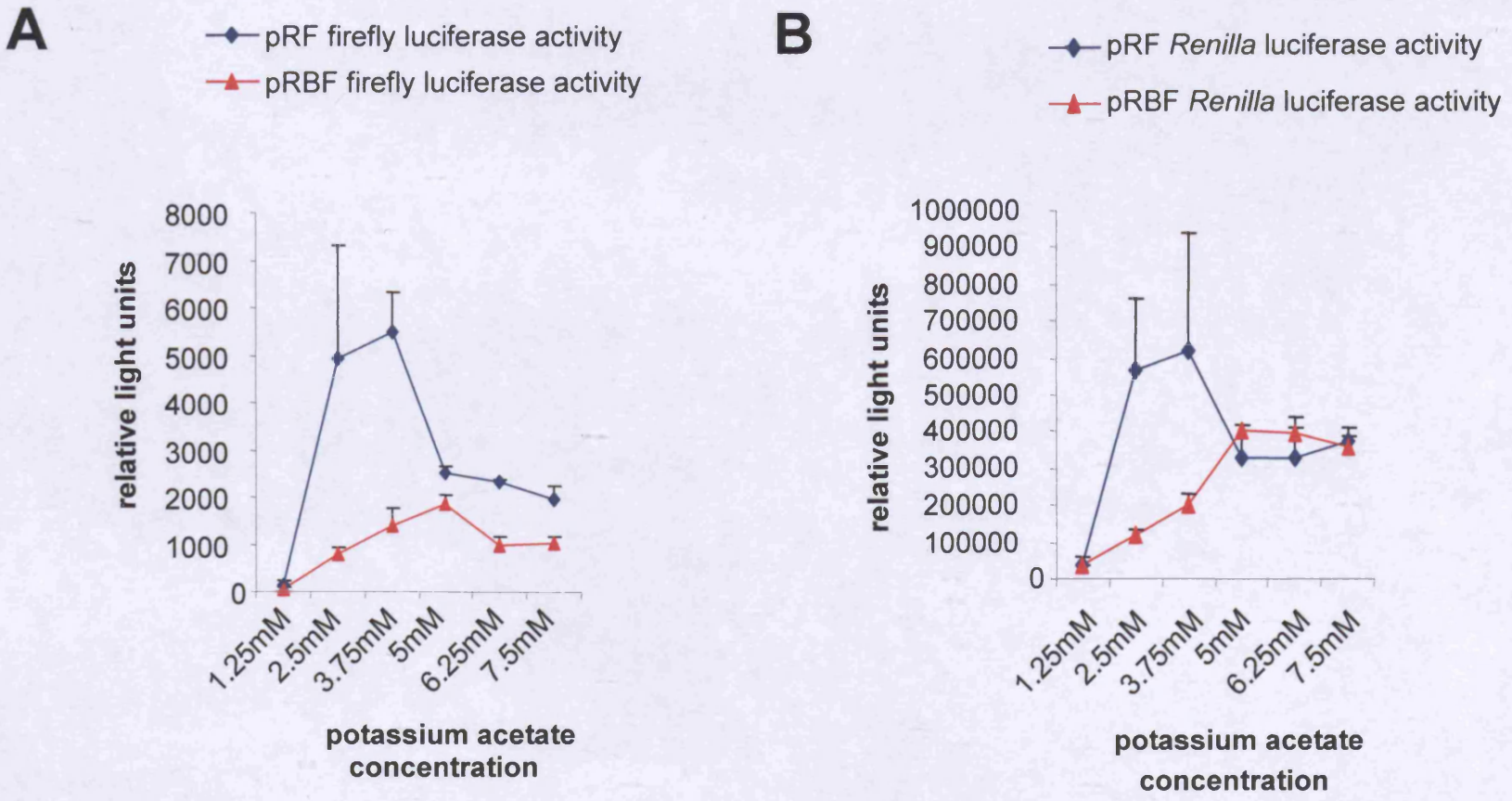


Figure 4.4. K^+ ion titration with dicistronic RNAs to optimise the rabbit reticulocyte lysate system

A. Firefly luciferase activity of pRF and pRBF on addition of increasing concentrations of potassium acetate.

B. *Renilla* luciferase activity of pRF and pRBF on addition of increasing concentrations of potassium acetate.

Error bars represent standard deviations from the mean luciferase activity of duplicate samples.

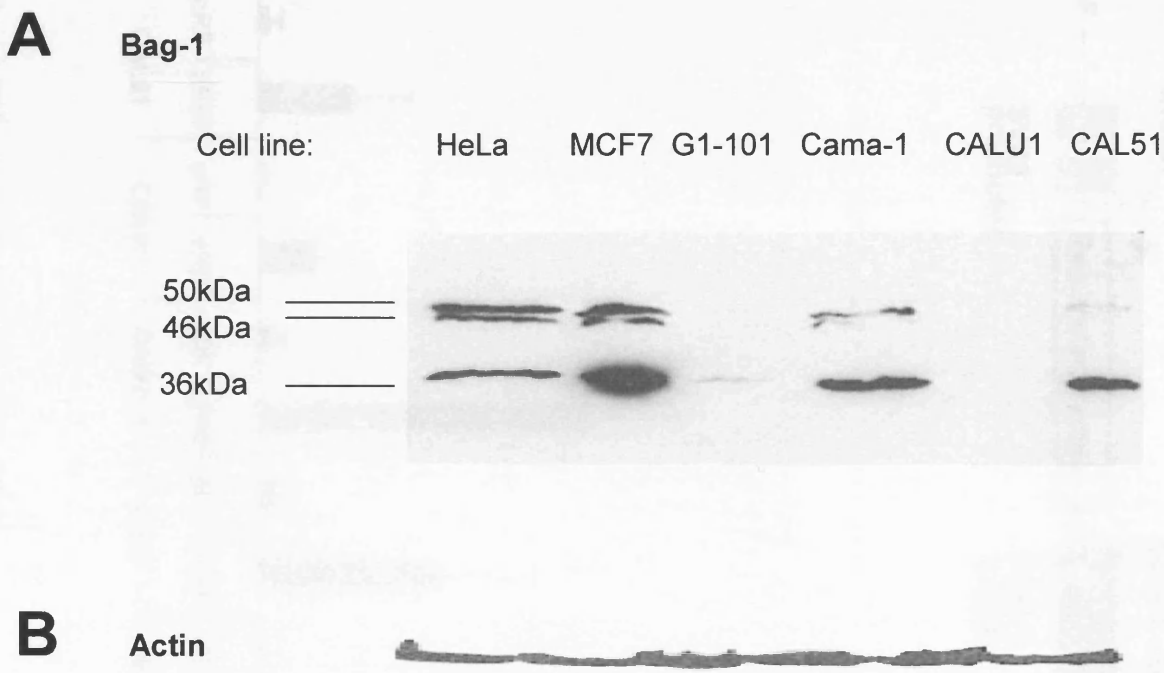


Figure 4.5. Western blot of Bag-1 in cell lines

A. Autoradiograph of a western blot of the cell lysates indicated with anti-Bag-1 monoclonal antibody. The molecular weight of the Bag-1 protein isoforms are marked in kDa.

B. The western blot was stripped and re-probed with anti-actin monoclonal antibody as a loading control.

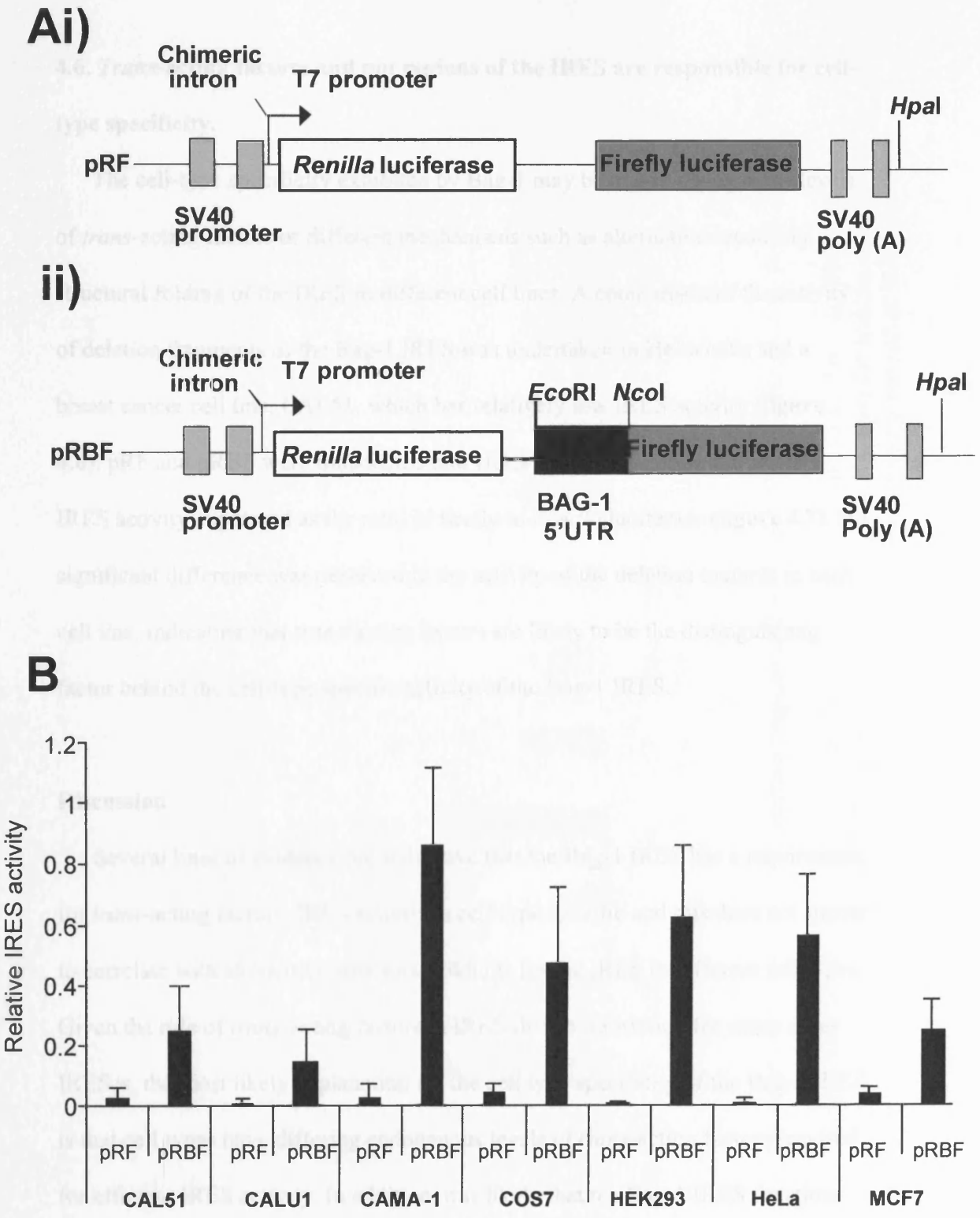


Figure 4.6. Bag-1 IRES activity is cell-type specific.

A. Schematic diagram depicting plasmid constructs transfected into the cell lines indicated, i) pRF and ii) pRBF

B. Plasmids were transfected into the cell lines indicated and relative IRES activity is expressed as firefly/*Renilla* luciferase. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars indicate standard deviation.

4.6. *Trans*-acting factors and not regions of the IRES are responsible for cell-type specificity.

The cell-type specificity exhibited by Bag-1 may be due to endogenous levels of *trans*-acting factors or different mechanisms such as alternative secondary structural folding of the IRES in different cell lines. A comparison of the activity of deletion fragments of the Bag-1 IRES was undertaken in HeLa cells and a breast cancer cell line, CAL51, which has relatively low IRES activity (**figure 4.6**). pRF and pRBF were transfected into HeLa and CAL51 cells and relative IRES activity expressed as the ratio of firefly to *Renilla* luciferase (**figure 4.7**). No significant difference was observed in the activity of the deletion mutants in each cell line, indicating that *trans*-acting factors are likely to be the distinguishing factor behind the cell-type specific activity of the Bag-1 IRES.

Discussion

Several lines of evidence are indicative that the Bag-1 IRES has a requirement for *trans*-acting factors. IRES activity is cell-type specific and this does not appear to correlate with alternative structural foldings for the IRES in different cell types. Given the role of *trans*-acting factors in IRES-driven translation for many other IRESes, the most likely explanation for the cell type specificity of the Bag-1 IRES is that cell types have differing endogenous levels of *trans*-acting factors required for efficient IRES activity. In addition, it is likely that the Bag-1 IRES functions inefficiently in the rabbit reticulocyte lysate *in vitro* translation system due to low levels of the requisite *trans*-acting factors.

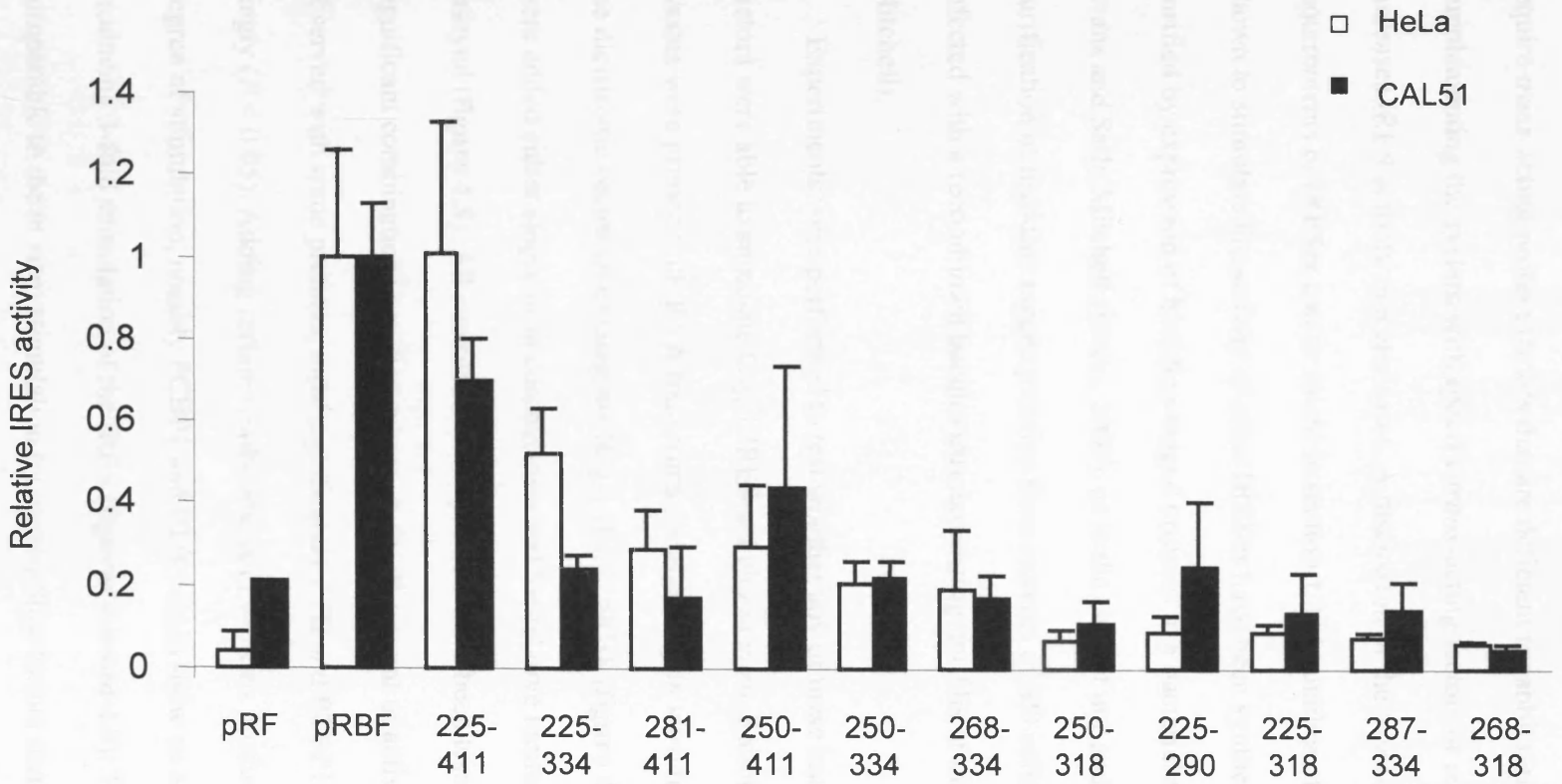


Figure 4.7. A similar pattern of IRES activity of deletion fragments of the Bag-1 5'UTR is observed in HeLa and CAL51 cells

Deletion constructs were transfected into CAL51 cells (black bars) and HeLa cells (white bars) and IRES activity expressed as firefly/*Renilla* luciferase, as a percentage of full-length pRBF IRES activity for comparison. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

4.7. PTB and PCBP1 can enhance Bag-1 IRES activity in a dicistronic vector.

Class I picornaviral IRESes and cellular IRESes, which function inefficiently in the rabbit reticulocyte lysate *in vitro* translation system, have been shown to require *trans*-acting protein factors that are deficient in rabbit reticulocyte lysate. Supplementing the system with specific *trans*-acting factors or cell lysates increases IRES activity in many cases. A discussion of the *trans*-acting factor requirements of IRESes can be found in section 1.3. A number of protein factors shown to stimulate the activity of other IRESes have been synthesised and purified by expression of histidine-tagged proteins in a bacterial system by Joanne Evans and Sally Mitchell (Evans, 2003), or in the case of unr and unrip, through purification of histidine tagged proteins from cultures of Sf9 cells that had been infected with a recombinant baculovirus expressing unr-His or unrip-His (Sally Mitchell).

Experiments were performed to test whether any of these known *trans*-acting factors were able to stimulate Bag-1 IRES activity *in vitro*. Rabbit reticulocyte lysates were primed with RNA transcripts generated by *in vitro* transcription from the dicistronic vector containing the Bag-1 IRES, pRBF (**figure 4.3a**). Proteins were added either singly or in combination and the relative luciferase activities assayed (**figure 4.8**). All combinations of proteins have been tested but only significant combinations are shown here. A small amount of activation can be observed with some proteins, most significantly PTB and PCBP1, when added singly ($P < 0.05$). Adding certain combinations of proteins produced a significant degree of stimulation, notably PCBP1 and PTB, which show an additive effect, producing 3-fold stimulation of the IRES (**figures 4.8 and 4.9**). This is comparable to the *in vitro* stimulation by *trans*-acting factors seen with other

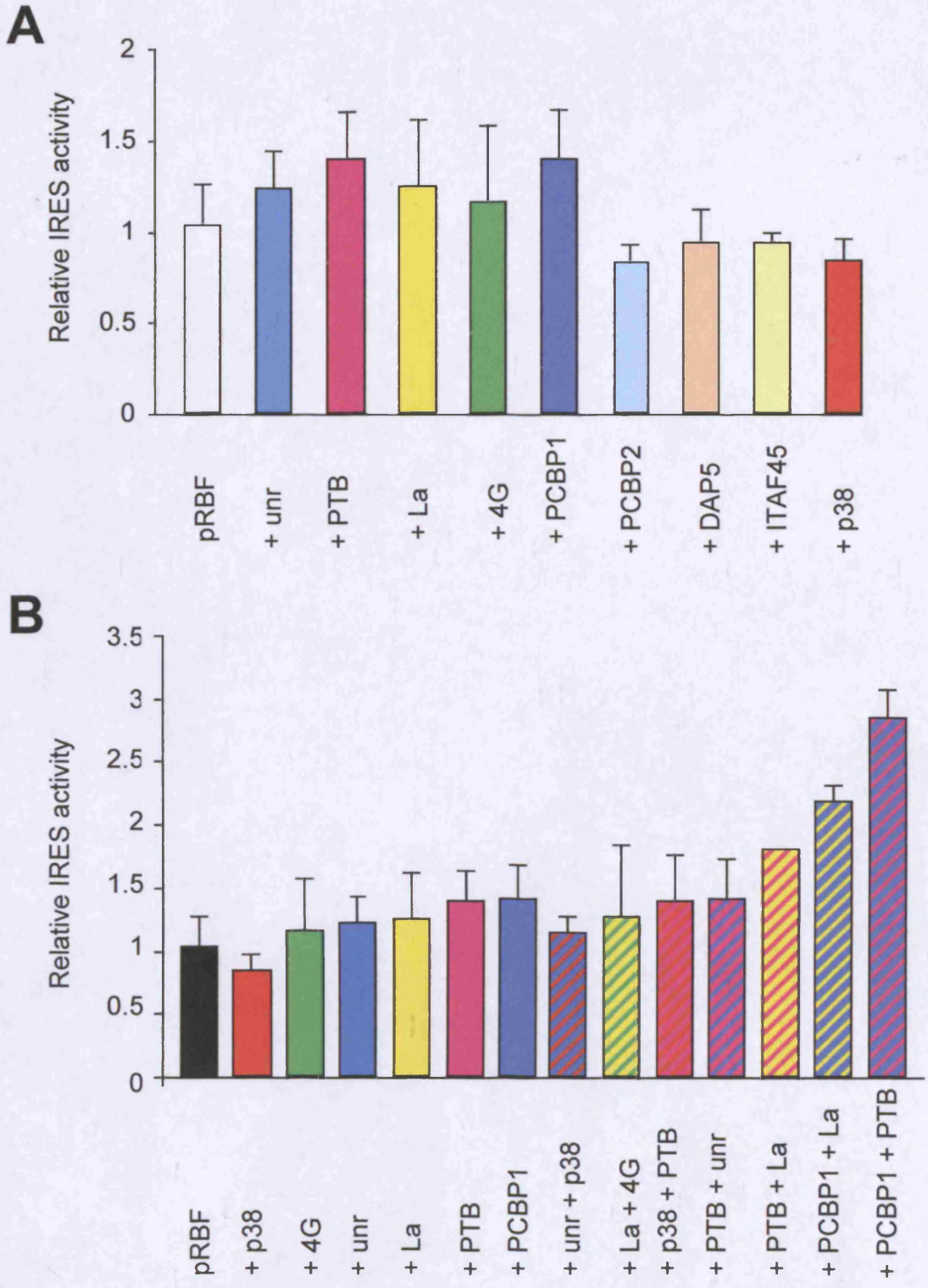


Figure 4.8. Bag-1 IRES activity can be enhanced by supplementing the rabbit reticulocyte lysate system with potential *trans*-acting factors

A. IRES activity expressed as firefly/*Renilla* luciferase, relative to pRBF (which is assigned a value of 1) on addition of 100ng each protein.

B. IRES activity, expressed as firefly/*Renilla* luciferase, relative to pRBF (which is assigned a value of 1) on addition of 100ng each protein either singly or in combinations shown. Each colour in the striped bars represents one of the two proteins added in combination.

Where indicated in the text, a students *t*-test was performed to ascertain the probability (*P*) of the stated difference being observed by chance. A *P* of <0.05 is taken to represent a significant difference.

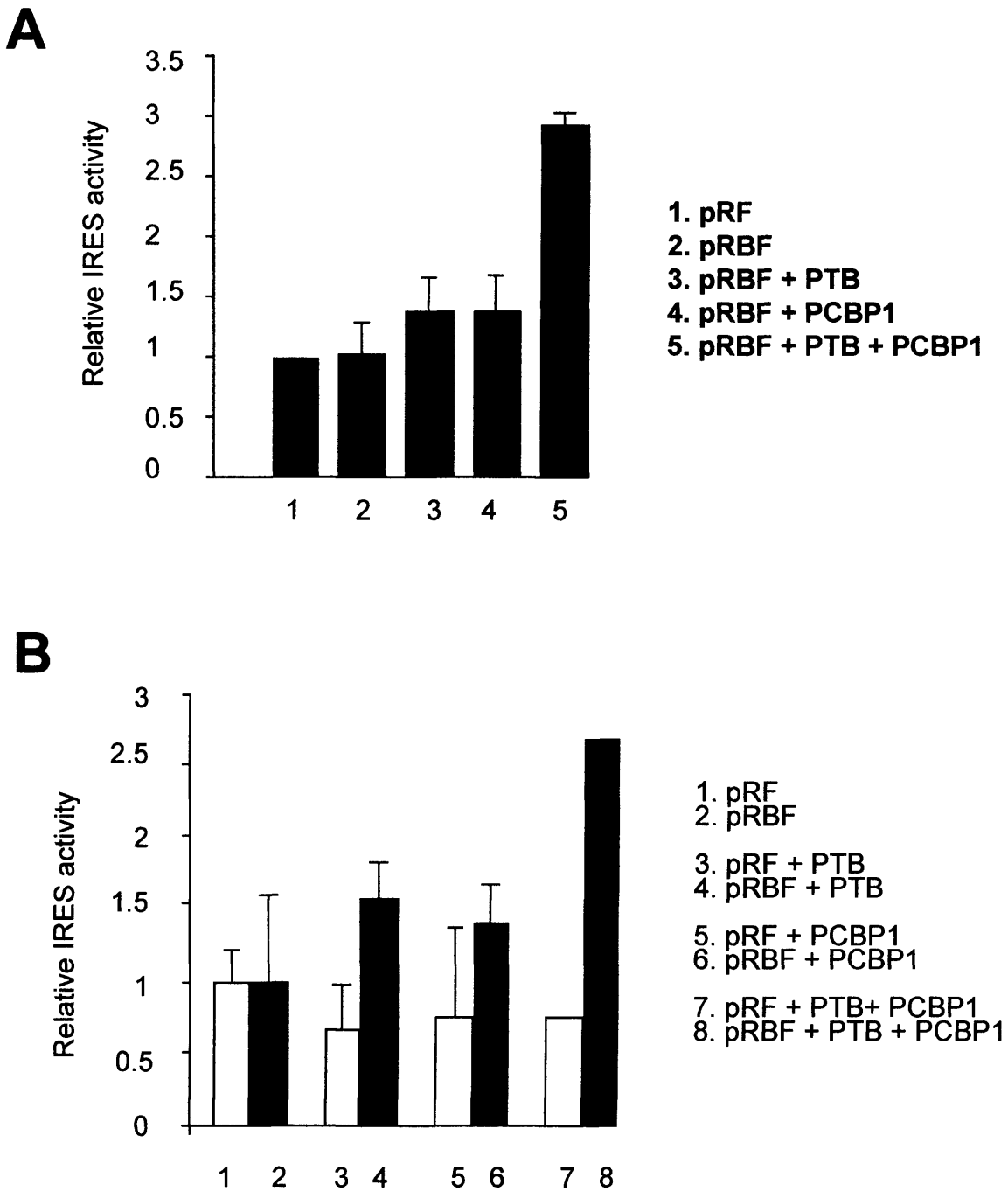


Figure 4.9. PTB and PCBP1 enhance Bag-1 IRES activity *in vitro*

A. IRES activity expressed as firefly/*Renilla* luciferase relative to pRBF alone, on addition of 100ng of each protein to the rabbit reticulocyte lysate system. IRES activity is expressed relative to pRF, which is assigned a value of 1.

B. IRES activity of pRF or pRBF in the rabbit reticulocyte lysate system supplemented with 100ng of PTB, PCBP1 or both proteins. IRES activity is expressed as firefly/*Renilla* luciferase relative to pRF, which is assigned a value of 1.

cellular IRESes, for example the Apaf-1 IRES activity is stimulated around 3 fold *in vitro* on addition of unr and PTB (Mitchell *et al.*, 2001). No stimulation of firefly luciferase activity from the empty vector pRF is observed (**figure 4.9b**), confirming that the enhancement in firefly luciferase activity is due to stimulation of IRES-driven translation and not ribosomal readthrough. The additive effect observed suggests that a complement of proteins may be necessary for full IRES activity.

4.8. The Bag-1 IRES is active in reticulocyte lysate when cap-dependent scanning is impeded

The constructs pRHpF and pRHpBF were used to generate capped dicistronic RNAs in which the second cistron can only be translated by internal ribosome entry as a stable hairpin structure is present before the firefly cistron, cloned 5' to the IRES in the case of pRHpBF (**figure 4.10a**). The pRHpBF RNAs can maintain production of firefly luciferase in the rabbit reticulocyte lysate system whereas the hairpin reduces any ribosomal readthrough into the firefly luciferase cistron to background in the control vector. In both cases, *Renilla* luciferase is translated by cap-dependent translation at approximately the same efficiency (**figure 4.10b**).

The use of dicistronic RNAs generated from the hairpin constructs shows that the IRES is functional in translating the firefly luciferase cistron in this system when cap-dependent translation by ribosomal readthrough and re-initiation at the second cistron is impeded. Supplementing the rabbit reticulocyte lysate system with PTB and PCBP1 also stimulated IRES-driven translation in this context (**figure 4.11a**). The other candidate *trans*-acting factors were also tested

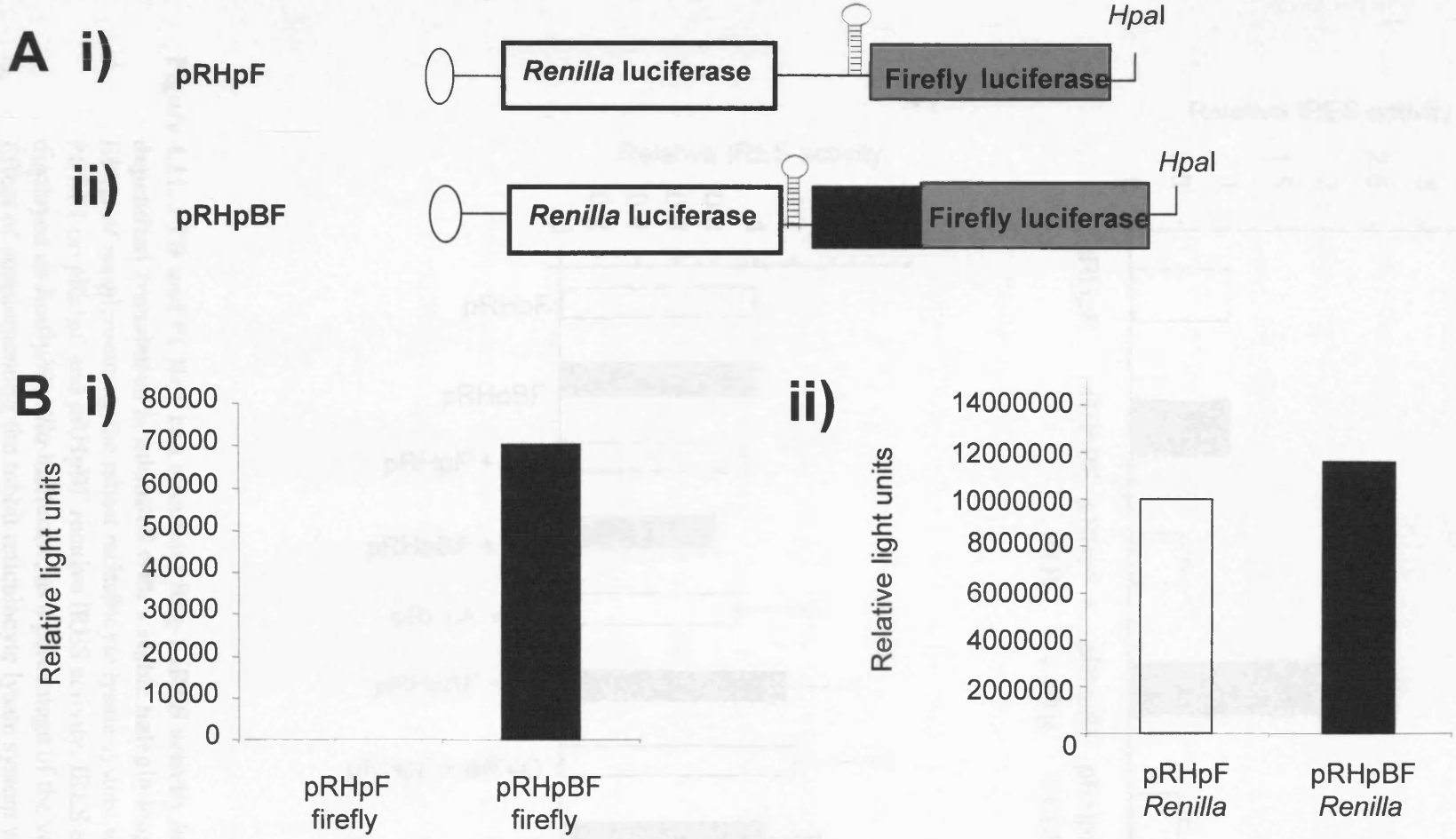


Figure 4.10. The Bag-1 IRES is active in the rabbit reticulocyte lysate system when cap-dependent scanning is inhibited by a stable hairpin structure.

A. Schematic diagram depicting RNAs generated from i) pRHpF and ii) pRHpBF

B. Graph of i) firefly and ii) *Renilla* luciferase activity of pRHpF and pRHpBF RNAs translated in the rabbit reticulocyte lysate system. This experiment was performed on at least three independent occasions and representative data is shown.

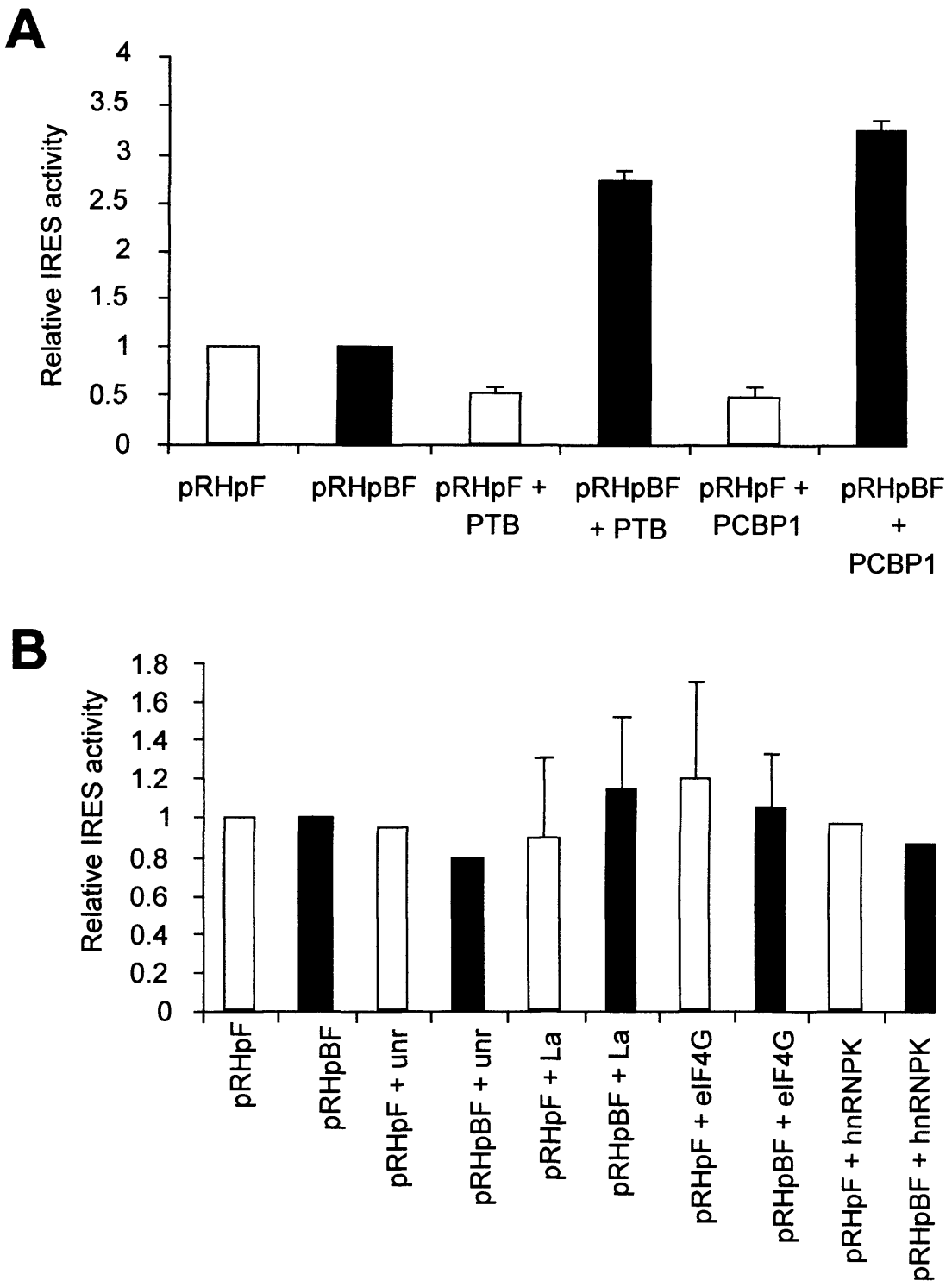


Figure 4.11. PTB and PCBP1 can stimulate Bag-1 IRES activity *in vitro* when cap-dependent translation is inhibited with a stable hairpin loop.

A. Effect of supplementing the rabbit reticulocyte lysate system with PTB and PCBP1 on pRHpF and pRHpBF relative IRES activity. IRES activity is displayed as firefly/*Renilla* luciferase, as a percentage of the vectors alone.

B. Effect of supplementing the rabbit reticulocyte lysate system with other potential *trans*-acting factors on relative IRES activity of pRHpF and pRHpBF. IRES activity is displayed as firefly/*Renilla* luciferase as a percentage of the vectors alone.

with no obvious stimulation of IRES activity with the possible exception of La (**figure 4.11b**). A small stimulation of IRES activity of around 1.2 fold over the levels of pRHpBF firefly luciferase activity alone and over the levels of the empty vector pRHpF was observed. A stimulation of the same extent was also observed with the dicistronic construct pRBF, and a more significant effect in combination with PCBP1, to around 2-fold over the levels of pRBF alone (**figure 4.8**). Thus, La cannot be ruled out as a candidate *trans*-acting factor for the Bag-1 IRES.

4.9. The Bag-1 5'UTR inhibits translation in a monocistronic vector

Capped monocistronic RNAs were generated from the vectors pSKL and pSKBL (**figure 3.6**) and used to prime the rabbit reticulocyte lysate system. The Bag-1 5'UTR inhibited translation of the firefly luciferase reporter by around 6 fold, indicating that the Bag-1 5'UTR is very inhibitory to cap-dependent translation and thus is likely to be highly structured (**figure 4.12**). PTB and PCBP1 both enhanced Bag-1 IRES activity and hence translation of firefly luciferase from pSKBL by around 1.4 fold (**figure 4.13a**). The proteins unr and unrip inhibited cap-dependent translation of pSKL, however these proteins did not inhibit translation of pSKBL and stimulated IRES activity slightly (**figure 4.13b**).

Discussion

Bag-1 IRES activity can be specifically stimulated in the rabbit reticulocyte lysate *in vitro* translation system in a range of vectors by PTB and PCBP1. The stimulation of IRES activity observed with PTB or PCBP1 individually was relatively small but reproducible. When both proteins were added together, a 3-

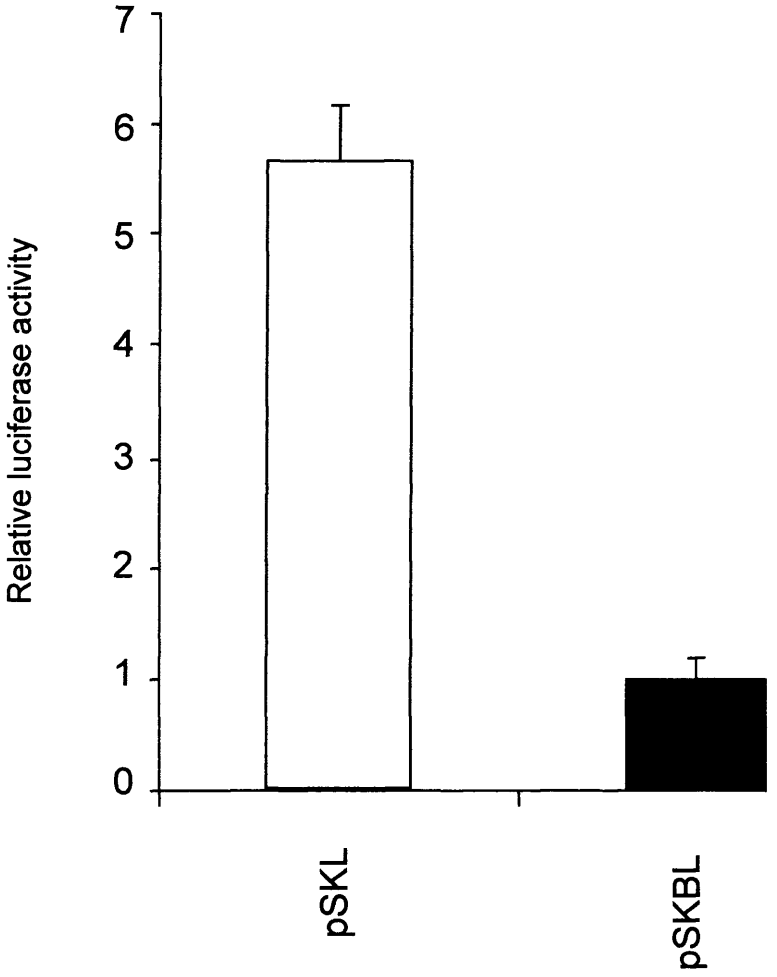


Figure 4.12. The Bag-1 5'UTR inhibits translation by cap-dependent scanning.

Relative luciferase activity (firefly luciferase) of monocistronic RNAs pSKL and pSKBL in the rabbit reticulocyte lysate system, relative to pSKBL, where pSKBL is assigned a value of 1. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

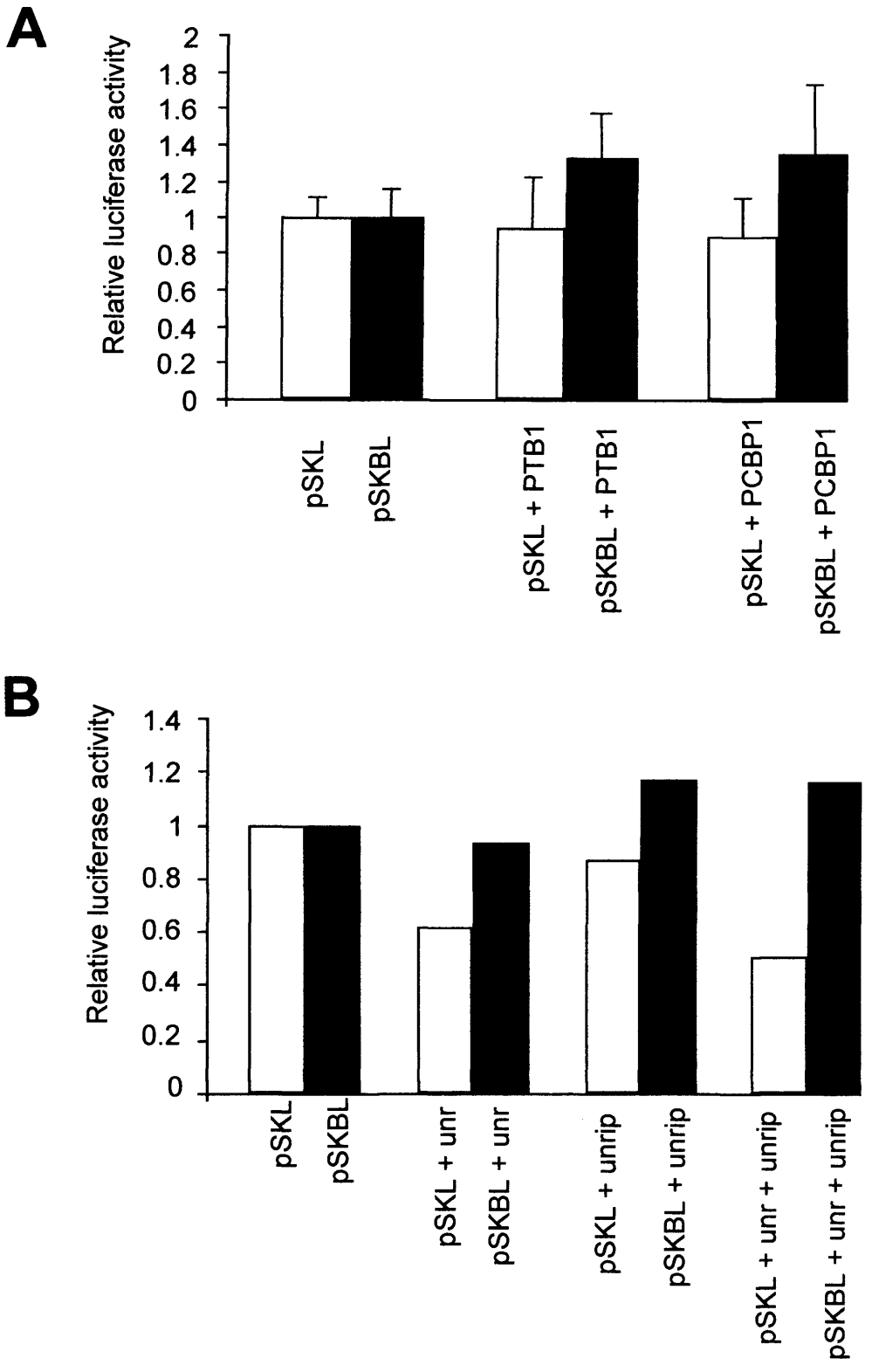


Figure 4.13. Activity of the Bag-1 IRES in the monocistronic vector pSKL in rabbit reticulocyte lysate supplemented with potential *trans*-acting factors.

A. luciferase activity (firefly luciferase) on addition of PCBP1 and/or PTB, relative to pSKL or pSKBL alone (which are assigned values of 1)

B. luciferase activity (firefly luciferase) on addition of unr and/or unrip, relative to pSKL or pSKBL alone (which are assigned values of 1)

fold stimulation of IRES activity was observed, suggesting that these proteins may act in combination as Bag-1 IRES *trans*-acting factors.

4.10. PTB and PCBP1 enhance IRES activity *in vivo*

To test whether PTB, PCBP1 or any of the other candidate *trans*-acting factors could stimulate the function of the Bag-1 IRES *in vivo,* co-transfections were carried out using pRBF with either plasmids expressing PCBP1 or PTB, singly or in combination into the cell lines that showed low Bag-1 IRES activity. The proteins of interest have been cloned into the vector pCDNA3 and transfection of these plasmids into cell lines has previously been shown to increase the level of the proteins in the cell, assessed by immunoblotting (Evans, 2003). The concentrations of PTB and PCBP1 were titrated in each cell line in order to assess the optimum concentration for maximal stimulation of Bag-1 IRES activity. A titration in the cell line CAL51 is shown (**figure 4.14**).

Transfection of CAL51 and CALU1 cell lines with constructs expressing either PTB, PCBP1 or both constructs simultaneously stimulated IRES activity significantly ($P<0.01$) (**figure 4.15a and b**). In contrast, transfection of MCF7s with PTB and/or PCBP1 did not significantly stimulate Bag-1 IRES activity ($P>0.05$) (**figure 4.15c**). This cell line shows slightly higher IRES activity than CAL51 or CALU1 cells but the differences in stimulation by PTB and PCBP1 may be attributed to the endogenous protein levels in the cell. Overexpression of these proteins in cell lines that already contain sufficient levels to support Bag-1 IRES activity would have limited effect. Thus, cell lysates were immunoblotted for PTB, PCBP1 or actin as a loading control (**figure 4.15d**). A correlation can be drawn between endogenous protein levels and activation of the IRES by these

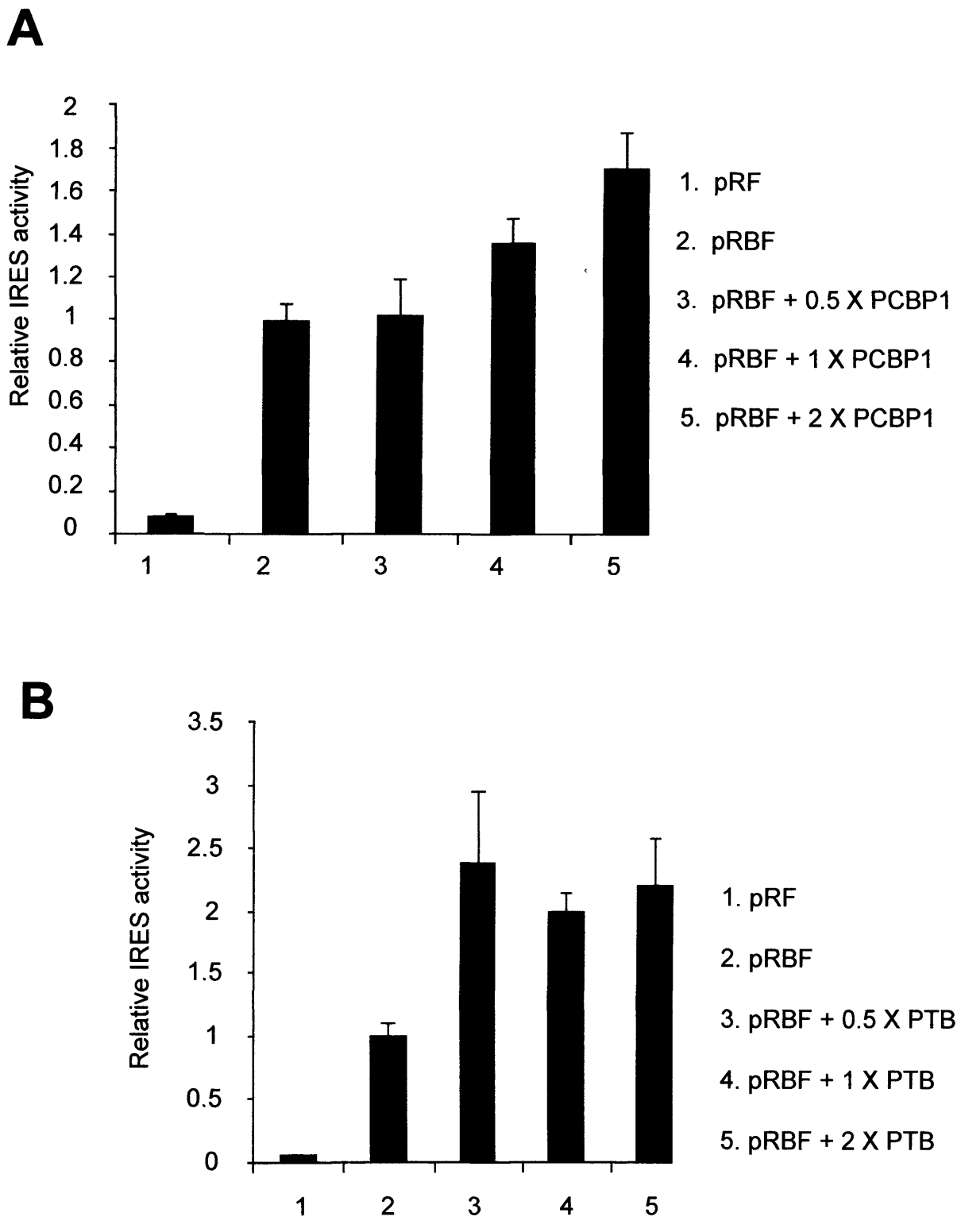


Figure 4.14. Titration of PCBP1 and PTB in CAL51 cells.

A. Titration with co-transfection of pRBF and increasing ratios of PCBP1. Relative IRES activity is expressed as firefly/*Renilla* luciferase relative to pRBF alone, which is assigned a value of 1.

B. Titration with co-transfection of pRBF and increasing ratios of PTB. Relative IRES activity is expressed as firefly/*Renilla* luciferase relative to pRBF alone, which is assigned a value of 1.

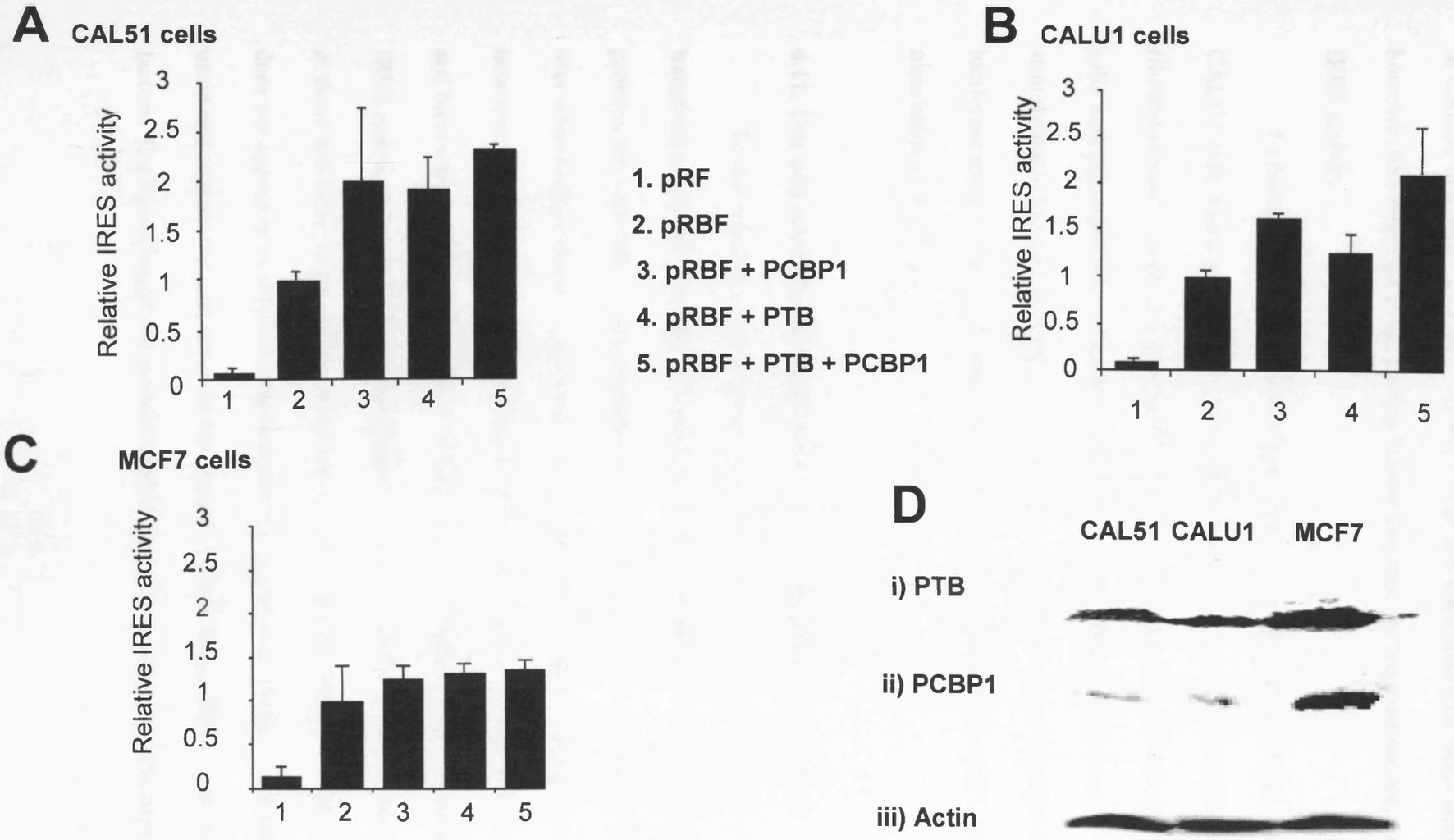


Figure 4.15. PTB and PCBP1 stimulate IRES activity in cell lines with low endogenous PTB and PCBP1 levels

IRES activity (firefly/*Renilla* luciferase) of co-transfections into A) CAL51, B) CALU1 and C) MCF7 cells, relative to pRBF, which was assigned a value of 1. Where indicated in the text, a students *t*-test was performed to ascertain the probability (*P*) of the stated difference # being observed by chance. $P < 0.05$ was taken to indicate a significant difference.

D) Autoradiograph of western blots of i) PTB, ii) PCBP1 and iii) actin as a loading control in lysates of the cell lines indicated.

proteins as MCF7s have comparatively high levels of PTB and PCBP1 compared to CALU1 or CAL51 cell lines. As MCF7s do not show maximal IRES activity, however, this indicates other protein factors may also be required for optimal IRES activity.

To ensure the increase in firefly/*Renilla* ratio observed in CAL51 and CALU1 cells was due to an increase in IRES activity and not an increase in ribosomal readthrough, the vector pRF was co-transfected into CAL51 or CALU1 cells with plasmids expressing the proteins of interest (**figure 4.16**). No stimulation of the downstream cistron is observed and the ratio of firefly/*Renilla* luciferase remains the same, suggesting the enhancement of downstream cistron translation is IRES-specific.

4.11. Unr and unrip do not significantly enhance IRES activity *in vivo*

To test whether unr and unrip act as Bag-1 *trans*-acting factors *in vivo*, co-transfections of pRF or pRBF in combination with plasmids overexpressing these proteins were carried out into a range of cell lines. The only stimulation observed was when both proteins were co-transfected with pRBF into the CALU1 cell line however, the results obtained with this combination of proteins were very variable and have a high degree of error and as such may not represent a true stimulation of IRES activity (**figure 4.17a**). To examine the endogenous levels of unr and unrip in these cell lines, immunoblots were carried out as before (**figure 4.17b**). There does not appear to be a correlation between the endogenous levels of unr and unrip and as such unr and unrip do not appear to act as Bag-1 IRES *trans*-acting factors. The recombinant unr protein used in the *in vitro* assays and the expression

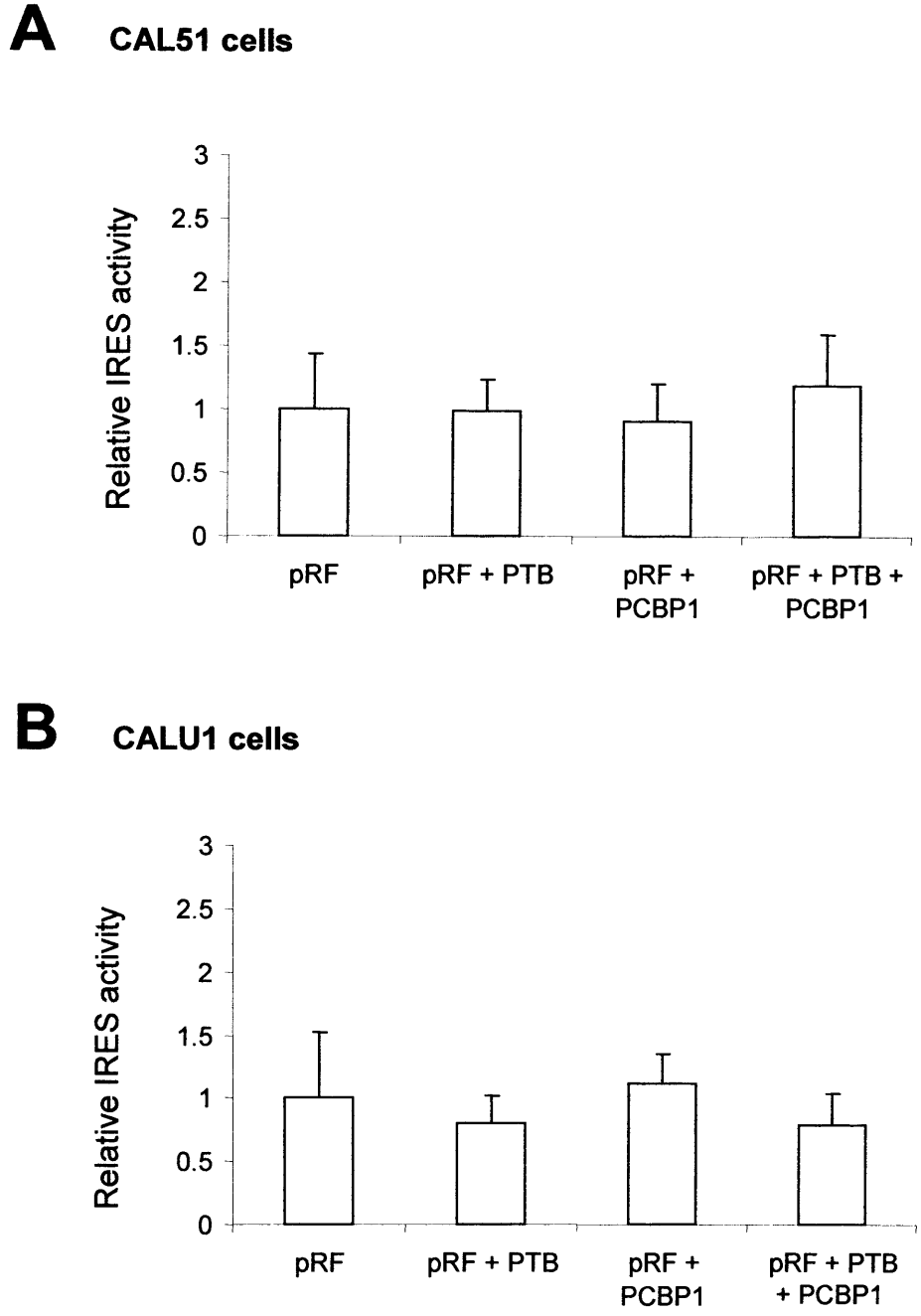


Figure 4.16. Activity of the downstream cistron of pRF is not enhanced by co-transfection of PTB and/orPCBP1

Relative IRES activity (firefly/*Renilla* luciferase) of co-transfections into

A) CAL51 cells relative to pRF alone, which is assigned a value of 1.

B) CALU1 cells relative to pRF alone, which is assigned a value of 1.

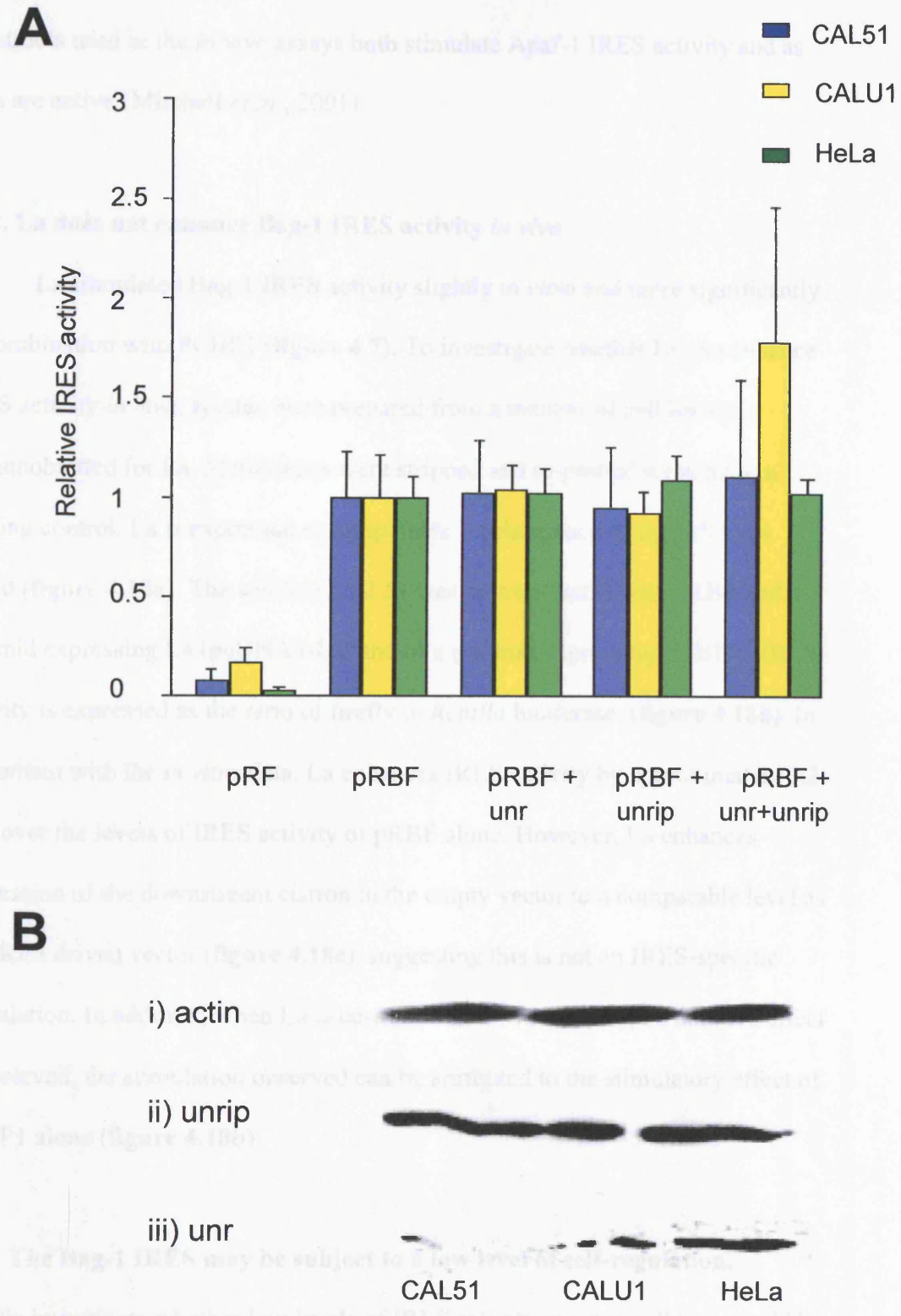


Figure 4.17. The effect of overexpression of unr and unrip on Bag-1 IRES activity

A. Plasmids expressing unr and/or unrip (PCDNA3) were co-transfected with pRBF into CAL51 (blue bars), CALU1 (yellow bars) or HeLa (green bars) cell lines. IRES activity is expressed as firefly/*Renilla* luciferase relative to pRBF, which is assigned a value of 1.

B. Autoradiograph of western blots of CAL51, CALU1 or HeLa cell lysates probed with i) anti-actin as a loading control, ii) anti-unrip or iii) anti-unr. Molecular weights of each protein are indicated in kDa.

constructs used in the *in vivo* assays both stimulate Apaf-1 IRES activity and as such are active (Mitchell *et al.*, 2001).

4.12. La does not enhance Bag-1 IRES activity *in vivo*

La stimulated Bag-1 IRES activity slightly *in vitro* and more significantly in combination with PCBP1 (**figure 4.7**). To investigate whether La can enhance IRES activity *in vivo*, lysates were prepared from a number of cell lines and immunoblotted for La. Membranes were stripped and re-probed for actin as a loading control. La is expressed at comparable levels in each of the cell lines tested (**figure 4.18a**). The cell line CAL51 was co-transfected with pRBF and a plasmid expressing La (pcDNA3-La) and/or a plasmid expressing PCBP1. IRES activity is expressed as the ratio of firefly to *Renilla* luciferase. (**figure 4.18b**). In agreement with the *in vitro* data, La enhances IRES activity by approximately 1.2 fold over the levels of IRES activity of pRBF alone. However, La enhances expression of the downstream cistron in the empty vector to a comparable level as the IRES driven vector (**figure 4.18c**), suggesting this is not an IRES-specific stimulation. In addition, when La is co-transfected with PCBP1, no additive effect is observed, the stimulation observed can be attributed to the stimulatory effect of PCBP1 alone (**figure 4.18b**).

4.13. The Bag-1 IRES may be subject to a low level of self-regulation.

To investigate whether low levels of IRES activity in some cell lines could be attributed to the low level of Bag-1 p36 protein in cell lines, potentially impeding self-regulation of IRES activity, co-transfections with a vector expressing Bag-1 p36 protein were performed. The vector pCDNA3, containing p36, was kindly

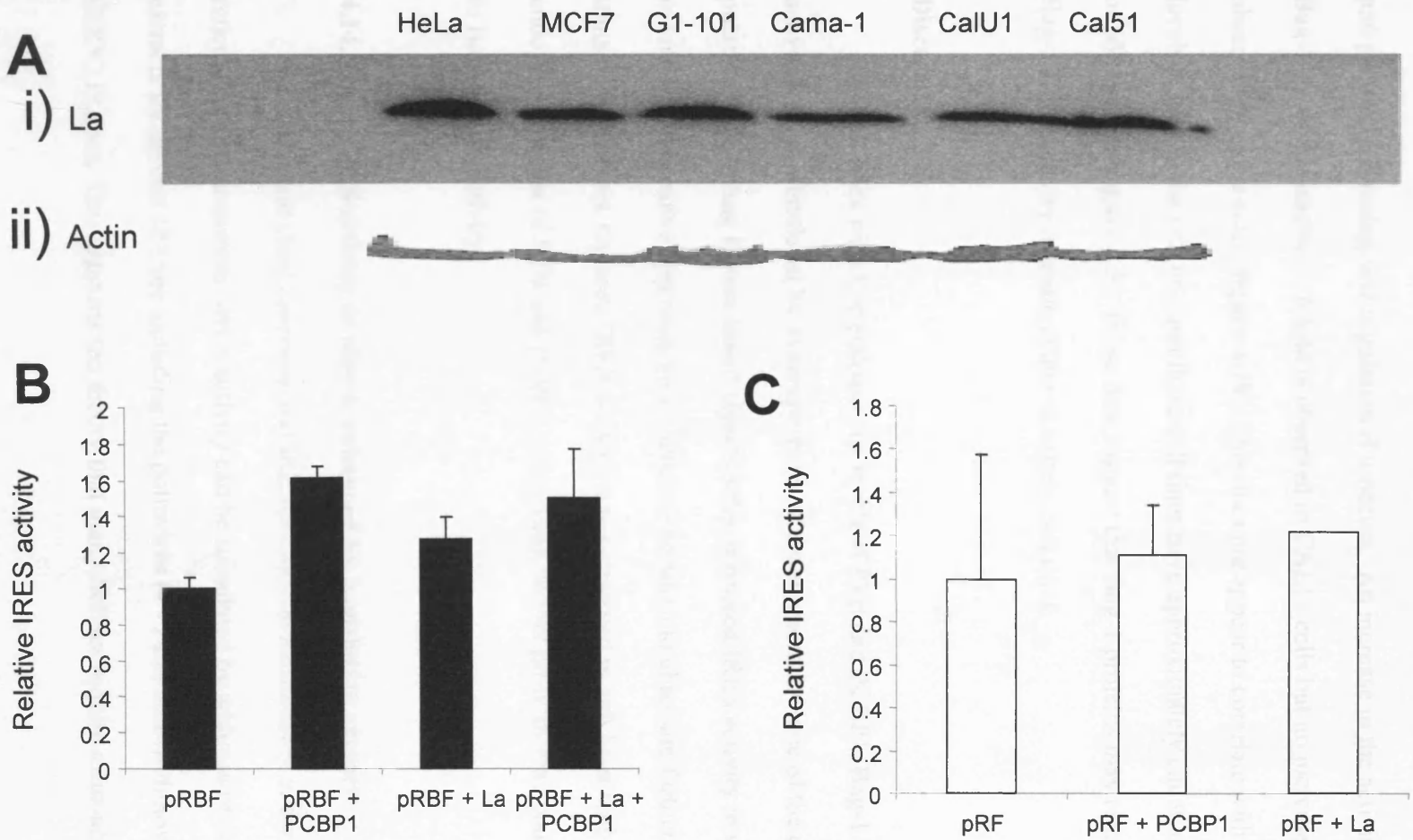


Figure 4.18. La does not enhance Bag-1 IRES activity *in vivo*

A. Autoradiograph of a western blot of the cell lysates indicated with i) anti-La and ii) anti-actin.

B. Relative IRES activity (Firefly/*Renilla* luciferase) of pRBF with co-transfection of PCBP1, La or both proteins, expressed relative to pRBF alone, which is assigned a value of 1

C. Firefly/*Renilla* luciferase activity of the empty vector pRF with co-transfection of PCBP1 or La, expressed relative to pRF alone, which is assigned a value of 1.

supplied by Paul Townsend (CRC Wessex Medical Oncology Unit, Southampton General Hospital). This vector allows the Bag-1 p36 protein to be over-expressed in cells. Co-transfection with dicistronic Bag-1 (pRBF) will increase the level of p36 protein, enhancing self-regulation if it occurs. An increase in the activity of Bag-1 by approximately 1.6 fold is observed in CAL51 cells but no increase is observed in HeLa cells (**figure 4.19**). This does not appear to correlate with the levels of p36 in the cell line and these cell lines have approximately equal levels of p36 protein (**figure 4.5**). These data suggest that Bag-1 proteins may regulate Bag-1 IRES activity to some extent in certain cell lines.

Discussion

In cell lines with low endogenous levels of PTB and PCBP1, Bag-1 IRES activity can be stimulated by overexpression of these proteins. None of the other putative *trans*-acting factors tested significantly enhanced IRES activity *in vitro* or *in vivo*. An alternative approach must therefore be taken to elucidate further Bag-1 trans-acting factors. Optimal IRES activity is not observed in cell lines with high endogenous levels of PTB and PCBP1, suggesting further proteins may contribute to Bag-1 IRES activity.

4.14. Bag-1 IRES activity *in vitro* is enhanced by translation extracts.

Cellular and class I picornaviral IRESes function inefficiently in the rabbit reticulocyte lysate system. IRES activity can be stimulated by addition of cell extracts for several IRESes including the poliovirus (PV) and human rhinovirus (HRV) IRESes. This supports the theory that many IRESes require *trans*-acting

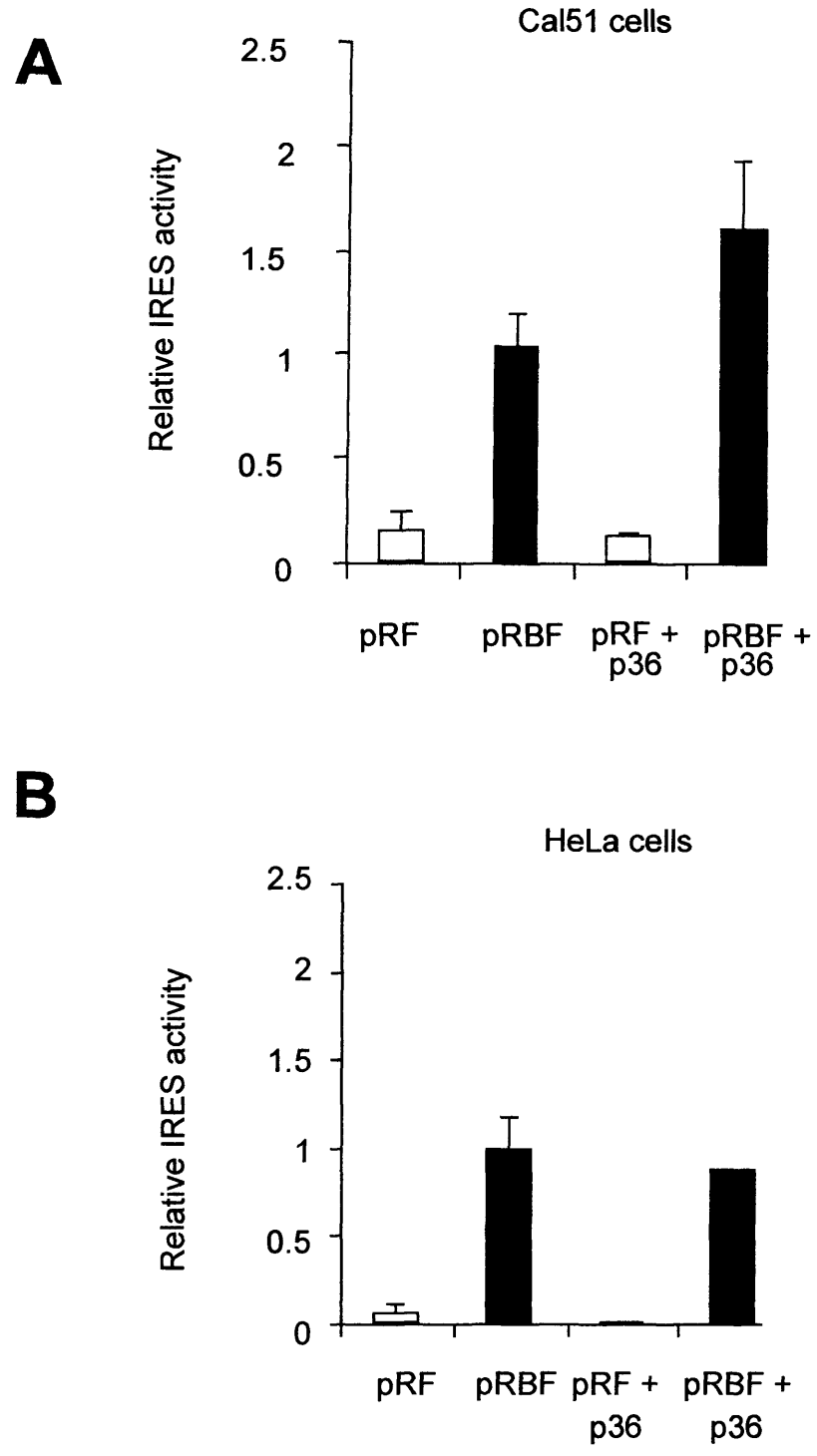


Figure 4.19. Effect of overexpression of Bag-1 isoforms on activity of the Bag-1 IRES

A. Relative IRES activity represented as firefly/*Renilla* luciferase (where pRBF is assigned a value of 1) on transfection of Cal51 cells with pRF or pRBF alone or co-transfection with p36 protein expression plasmids.

B. Relative IRES activity represented as firefly/*Renilla* luciferase (where pRBF is assigned a value of 1) on transfection of HeLa cells with pRF or pRBF alone or co-transfection with p36 protein expression plasmids.

factors that are deficient in the rabbit reticulocyte lysate system (Graff *et al.*, 1998).

Placenta is a rich source of RNA binding proteins and hence an ideal candidate material for identification of *trans*-acting factors. Whole cell extract was prepared from placenta. Lysates were dialysed against H100 buffer (1.5mM MgOAc, 10mM HEPES pH 7.4, 0.5mM DTT) to remove excess salt and were used to supplement the rabbit reticulocyte lysate system. Dicistronic RNAs from pRBF were used to prime the rabbit reticulocyte lysate system and IRES activity is expressed as a ratio of firefly to *Renilla* luciferase (**figure 4.20**). Placental extract was shown to stimulate Bag-1 IRES activity on at least three independent occasions however the extent of stimulation varied considerably from between 4 and 60 fold over the levels of pRBF or pRF alone. This variation may be attributed to the sensitivity of this system to ion concentrations. Optimising the system for placental extract will be useful for the future identification of Bag-1 *trans*-acting factors as this extract stimulates IRES activity significantly, in some cases restoring IRES activity to *in vivo* levels.

4.15. A number of proteins from translating extracts can bind directly to the Bag-1 IRES.

To determine whether specific protein factors from placental extract can bind to the Bag-1 IRES, electrophoretic mobility shift assays (EMSAs) were carried out with placental and HeLa cell extracts. ^{32}P-[rCTP] labelled Bag-1 IRES RNA was generated by *in vitro* transcription from pSKBL linearised with *Nco*I in the presence of [α-^{32}P]-CTP (**figure3.7a**). 25,000cpm (0.46pmol.) of radiolabelled RNA was incubated alone or with increasing amounts of cell extract for 10-15

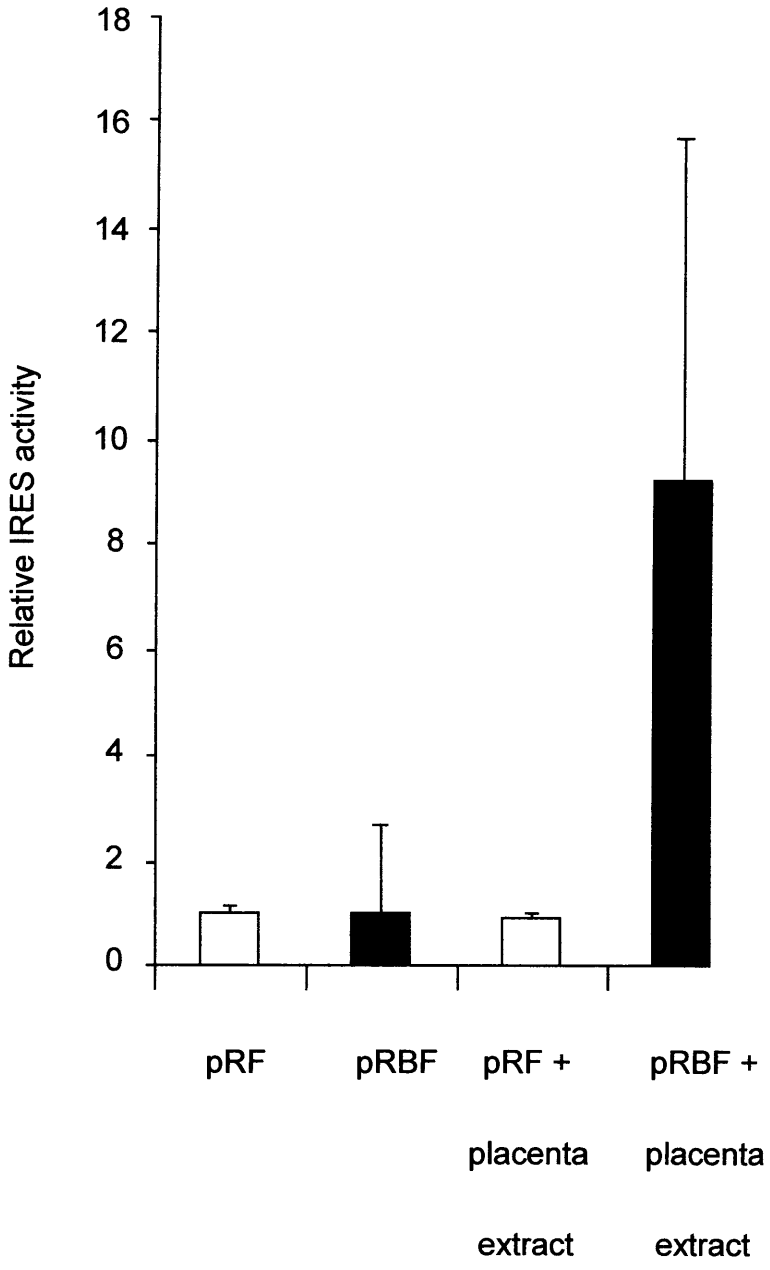


Figure 4.20. Placental extract can enhance Bag-1 IRES activity *in vitro*
IRES activity (firefly/*Renilla* luciferase) of pRF and pRBF on addition of placental extract into the rabbit reticulocyte lysate system. IRES activity is expressed relative to pRF, which is assigned a value of 1.

minutes at room temperature. The protein-RNA complexes were immediately separated on 0.6% agarose/1 x TBE gels and the products visualised using a phosphorimager. Proteins binding to the radiolabelled RNA generate an increase in the molecular weight of the complex and hence, a shift in the position of the complex on the gel.

On addition of increasing amounts of cell extract, there is an increase in the shift observed in the size of the complex suggesting a number of proteins from both extracts bind to the RNA (**figure 4.21**). The proteins may bind with different affinities or be present at differing concentrations in the extracts so that addition of more extract allows multiple copies of one protein to bind, in the case of proteins that may have multiple binding sites. Interestingly, there is a difference in the pattern of protein binding with the different extracts, supporting the proposal that *trans*-acting factors contribute to cell-type specificity.

To elucidate the sizes and number of proteins from placental cell extract binding to the Bag-1 IRES, UV-crosslinking analysis was carried out. ^{32}P-[rCTP] labelled Bag-1 IRES RNA was generated as before (**figure3.7a**). 500,000 cpm (9.25pmol.) of RNA in addition to an increasing molar excess of unlabelled specific or non-specific competitor RNAs, was incubated with placental cell extract. Non-specific RNA was generated by *in vitro* transcription from pSKGAP:E/H, linearised with *Hind*III (Paulin, 1997) and specific competitor RNA comprised unlabelled Bag-1 IRES RNA. The protein-RNA complexes were cross-linked using a 312nm UV light source for 30 minutes at 0°C. Unbound RNA was digested with an RNase cocktail of RNases T1 and A. The protein-RNA complexes were separated on 10% polyacrylamide gels by SDS-PAGE. Products were detected using a phosphorimager (**figure 4.22**).

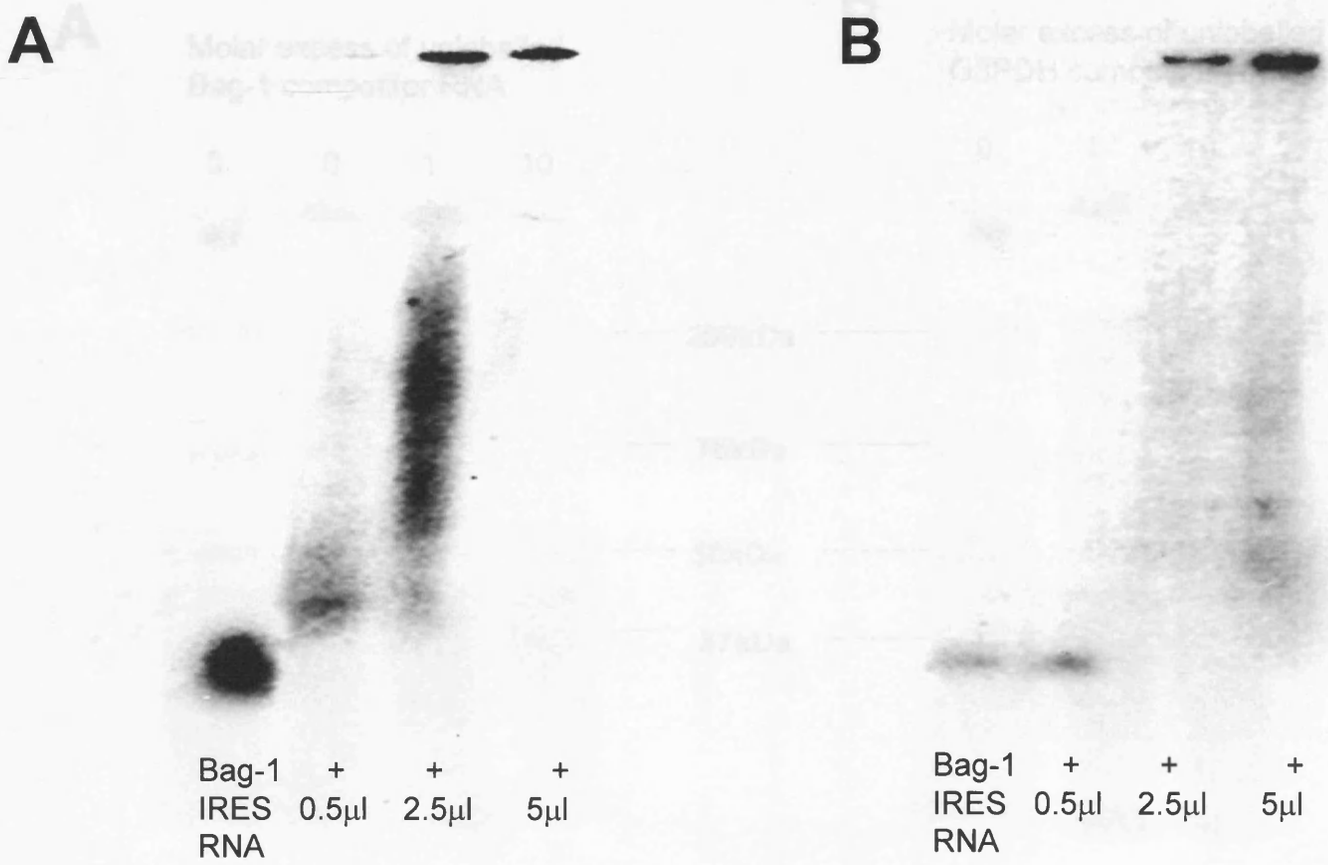


Figure 4.21. A complex of proteins from translating extracts can bind to the Bag-1 IRES

A. Autoradiograph of a 0.7X TBE agarose gel. EMSAs were performed with 25,000cpm ^{32}P[CTP]-labelled Bag-1 IRES RNA and increasing amounts of placental cell extract (indicated in µl) or

B. HeLa cell extract (indicated in µl). Gel retardations are observed on addition of cell extract.

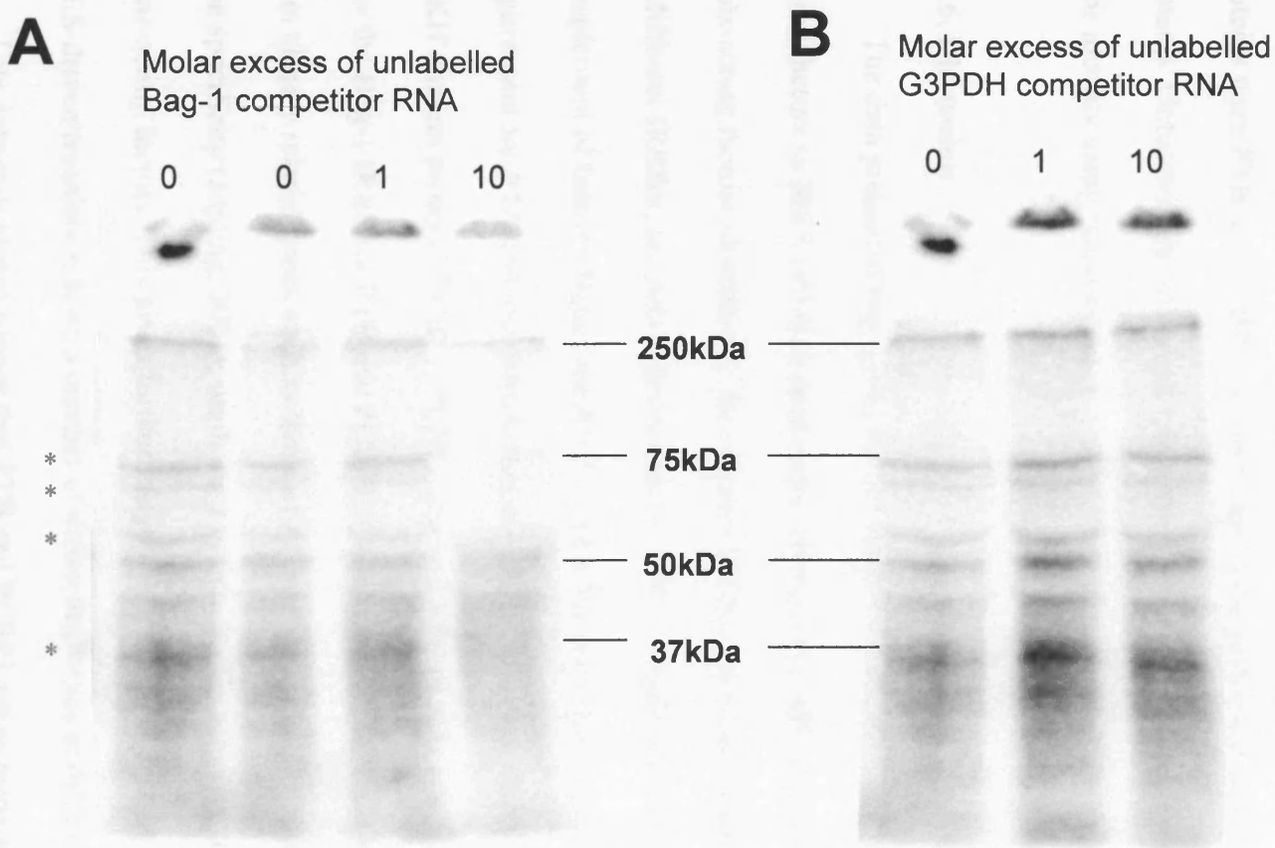


Figure 4.22. Several proteins from placental cell extract bind specifically to the Bag-1 IRES

A. Autoradiograoh of UV cross-linked products separated on a 10% SDS-PAGE gel. 500,000cpm ^{32}P[CTP]-labelled Bag-1 IRES RNA and an increasing molar excess of unlabelled Bag-1 RNA was UV cross-linked to 2µl placental cell extract.

B. Autoradiograoh of UV cross-linked products separated on a 10% SDS-PAGE gel. 500,000cpm ^{32}P[CTP]-labelled Bag-1 IRES RNA and an increasing molar excess of unlabelled G3PDH RNA was UV cross-linked to 2µl placental cell extract.

The molecular weight of size markers are indicated in kDa and proteins binding specifically to Bag-1 IRES RNA are indicated by red astericks.

Proteins of approximate sizes of 75, 70, 55 and 37kDa remain bound on addition of an excess of unlabelled non-specific competitor RNA (G3PDH) but are competed off by a 10-fold excess of unlabelled specific Bag-1 competitor RNA. These proteins bind specifically to the Bag-1 IRES and therefore represent potential *trans*-acting factors. The 55 and 37kDa proteins are the approximate sizes of PTB and PCBP1 respectively. To confirm whether the 55 and 37kDa proteins were PTB and PCBP1, western blots were performed on the cross-linked proteins. Unfortunately, the levels of proteins present were insufficient to confirm their identity using this method.

4.16. Discussion

The data presented here gives further support for the vital role of *trans*-acting factors in IRES cell-type specificity. Intriguingly, although a number of *trans*-acting factors identified so far enhance IRES-driven translation of a variety of different IRESes, the data indicate that each IRES requires a distinct complement of factors. Hence the Apaf-1 IRES has been shown to have a requirement for PTB and unr (Mitchell *et al.*, 2001), the c-*myc* IRES for a number of KH domain proteins, PCBP1, PCBP2 and hnRNPK (Evans *et al.*, 2003) and now the Bag-1 IRES for PTB and PCBP1 (Pickering *et al.*, 2003). Even IRESes from closely related genes such as those of the *myc* gene family differ in their cell-type specificity (Jopling, 2001), implying a system highly sensitive to the levels of *trans*-acting factors. This gives further insights into the difference in efficiency of IRES-driven translation from a number of different IRESes in different cell lines.

This data provides evidence that PTB and PCBP1 act as *trans*-acting factors for Bag-1 IRES activity but suggests that further proteins remain to be elucidated.

There are several factors which point to this conclusion, firstly, MCF7 cells do not have maximal levels of IRES activity but cannot be stimulated further by addition of PTB or PCBP1. This cell line has high endogenous levels of these proteins, indicating there must be additional contributing factors to achieve optimal IRES activity. Secondly, PTB and PCBP1 stimulate IRES activity to a maximum of 3-fold in an *in vitro* translation system, far below the *in vivo* levels of IRES activity observed in a number of cell lines. In addition, placental cell lysate has been shown to stimulate IRES activity significantly, perhaps to *in vivo* levels. Further optimisation of the *in vitro* translation system with placental extract will be required to produce a reliable guide to the extent to which IRES activity can be enhanced by addition of cell extract. A number of proteins can be isolated from placental cell extract which bind specifically to the Bag-1 IRES and it will be interesting to identify these proteins and further elucidate the Bag-1 IRES *trans*-acting factor requirements, giving insight into its mechanism of action.

Chapter 5.

Interaction of *trans*-acting factors with the Bag-1 IRES

5.1. Introduction

The majority of viral and cellular IRESes identified require *trans*-acting protein factors in addition to at least some of the canonical initiation factors used for the cap-dependent scanning model of translation. A number of roles have been proposed for *trans*-acting protein factors; they may interact with ribosomes to facilitate ribosome recruitment, stabilise or modify secondary or tertiary structures for direct ribosome recruitment or may assist in recruitment of other protein factors by protein: protein interactions (Walter *et al.*, 1999).

Notably, several *trans*-acting factors have been shown to interact with other factors and enhance IRES activity synergistically. The best studied example being PTB and unr, which act synergistically in enhancing IRES activity of cellular IRESes such as the Apaf-1 IRES (Mitchell *et al.*, 2001) and viral IRESes such as the HRV IRES (Hunt *et al.*, 1999). There is also evidence for the stabilisation of IRES structures by their specific *trans*-acting factors, for example PCBP1 and PCBP2 stabilise the 5' cloverleaf structure that is conserved in class I picornaviral IRESes (Murray *et al.*, 2001). In addition, PTB and unr have recently been shown to modulate the secondary structure of the Apaf-1 IRES to attain efficient ribosome entry (Mitchell *et al.*, 2003).

Chapter 4 showed that PTB and PCBP1 enhance Bag-1 IRES activity both *in vitro* and *in vivo*. In cell lines with low endogenous levels of PTB and PCBP1, Bag-1 IRES activity can be stimulated by overexpression of these proteins, suggesting they act as Bag-1 *trans*-acting factors and contribute to the cell-type

specific activity of the Bag-1 IRES. As such, it was of interest to determine whether PTB and PCBP1, can bind to the Bag-1 IRES and subsequently, to ascertain the influence of these proteins on the secondary structure of the Bag-1 IRES.

5.2. PTB and PCBP1 interact directly and specifically with the Bag-1 IRES

Radiolabelled Bag-1 5'UTR RNA was generated from *in vitro* transcription reactions primed with DNA derived from the monocistronic construct pSKBL (**figure 5.1A**), the resulting RNA was incubated with protein and the products separated on 0.5% TBE agarose gels. When BAG-1 5'UTR RNA was incubated with PTB or PCBP1, which both enhance IRES activity *in vitro* and *in vivo*; a decrease in the mobility of the RNA was observed (**figure 5.1B and C**), suggesting both of these proteins bind the 5'UTR directly. In addition, when both proteins were incubated with radiolabelled Bag-1 IRES RNA, an increased gel retardation is observed suggesting these proteins can bind to the Bag-1 5'UTR simultaneously and do not compete for the same binding site (**figure 5.2A**). To test the specificity of this interaction, the proteins were also incubated with a non-specific RNA segment from glyceraldehyde-3-phosphate dehydrogenase (G3PDH) of approximately the same size. No alterations in mobility of this non-specific RNA were observed with any of the proteins tested (**figure 5.2B**).

To confirm the interaction of PTB and PCBP1 with the Bag-1 IRES, UV-cross linking analysis was performed. Thus, radiolabelled Bag-1 IRES RNA was generated as before and incubated with PCBP1 and/or PTB, samples were exposed to UV light, any RNA not bound to protein digested with RNAses and the products separated by PAGE. Both PTB and PCBP1 either singly or in combination interacted with Bag-1

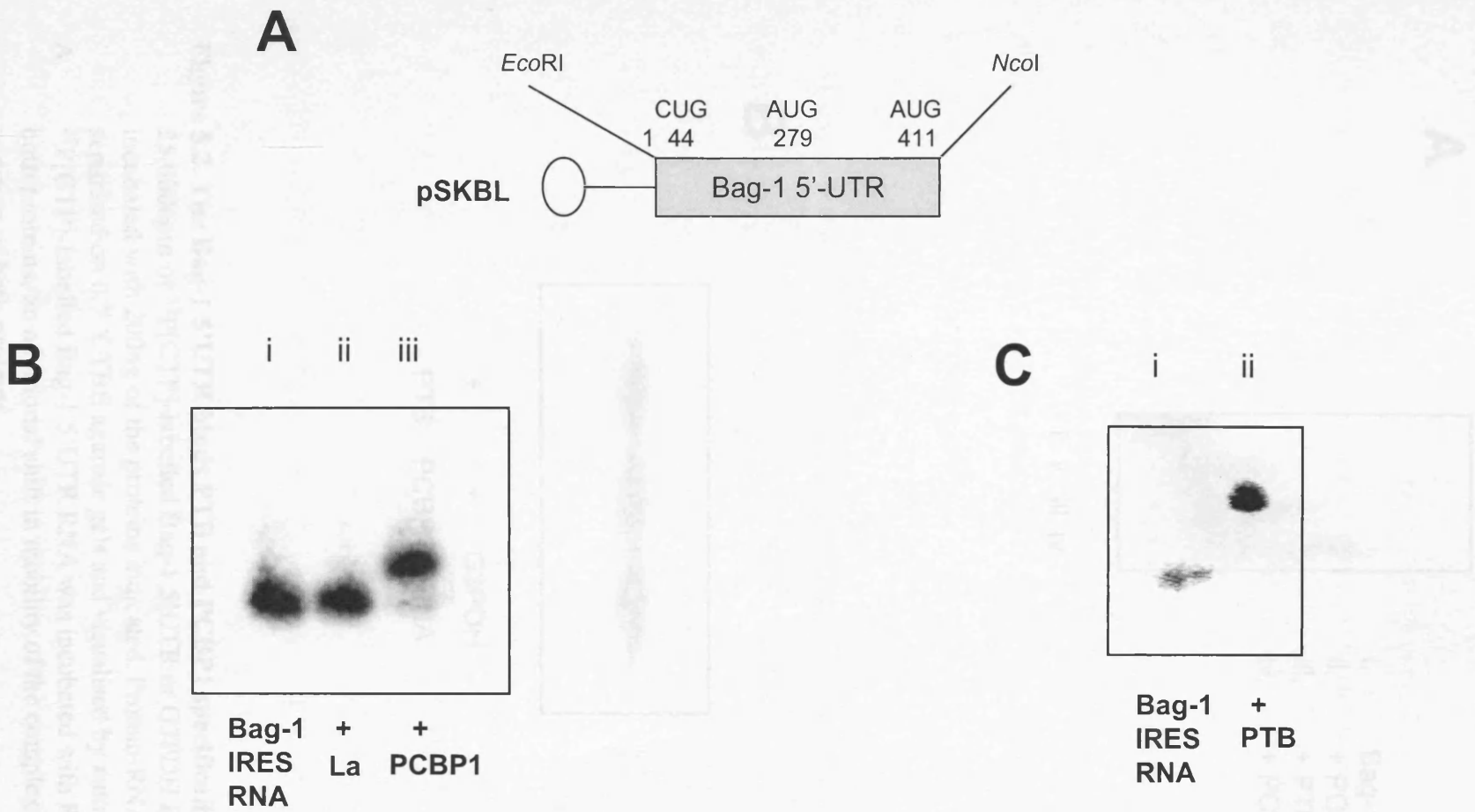


Figure 5.1. The Bag-1 5'UTR binds PTB and PCBP1 specifically in EMSAs

A Schematic diagram depicting transcripts generated from pSKBL.

B 25,000cpm of ^{32}P[CTP]-labelled Bag-1 5'UTR RNA was incubated with 200ng of the proteins indicated. Protein-RNA complexes were separated on 0.7 X TBE agarose gels and visualised by autoradiography. Lane i) radiolabelled Bag-1 IRES RNA, ii) plus La, iii) plus PCBP1. A decrease in the mobility of the RNA was observed with PCBP1, but not La.

C lane i) radiolabelled Bag-1 IRES RNA, ii) plus PTB. A decrease in the mobility of the RNA was observed with PTB

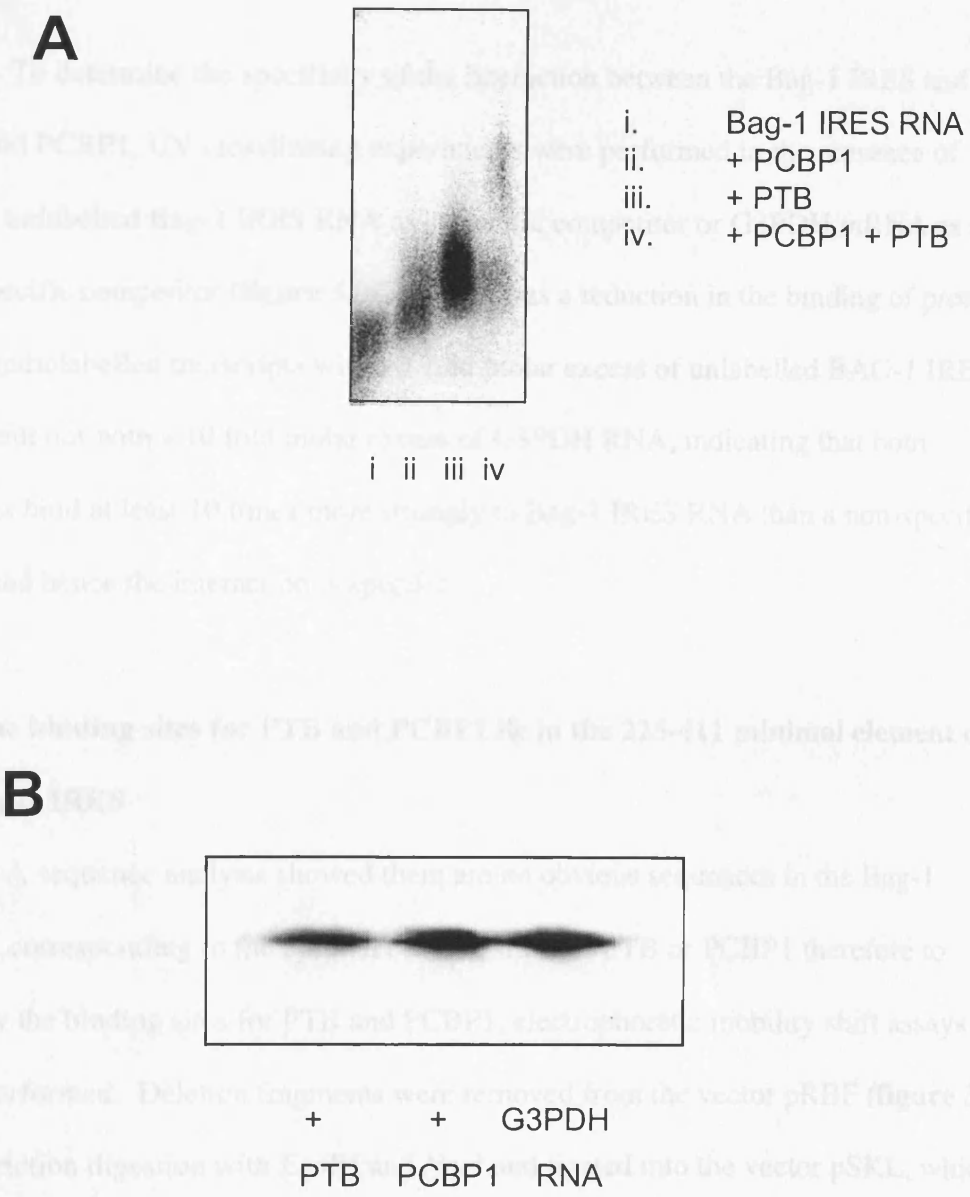


Figure 5.2. The Bag-1 5'UTR binds PTB and PCBP1 specifically in EMSAs
25,000cpm of ^{32}P[CTP]-labelled Bag-1 5'UTR or G3PDH RNA was incubated with 200ng of the proteins indicated. Protein-RNA complexes were separated on 0.7 X TBE agarose gels and visualised by autoradiography.

A. ^{32}P[CTP]-labelled Bag-1 5'UTR RNA was incubated with PCBP1, PTB or both proteins, an additional shift in mobility of the complex is observed on addition of both proteins.

B. No alterations in mobility were observed on incubation of the proteins with a non-specific ^{32}P[CTP]-labelled RNA of approximately the same length generated from G3PDH.

IRES RNA, producing a protected band of the size of the protein of interest (**figure 5.3A**).

To determine the specificity of the interaction between the Bag-1 IRES and PTB and PCBP1, UV crosslinking experiments were performed in the presence of excess unlabelled Bag-1 IRES RNA as a specific competitor or G3PDH mRNA as a non-specific competitor (**figure 5.3C**). There was a reduction in the binding of protein to the radiolabelled transcripts with a 1 fold molar excess of unlabelled BAG-1 IRES RNA, but not with a 10 fold molar excess of G3PDH RNA, indicating that both proteins bind at least 10 times more strongly to Bag-1 IRES RNA than a non-specific RNA and hence the interaction is specific.

5.3. The binding sites for PTB and PCBP1 lie in the 225-411 minimal element of the Bag-1 IRES

A sequence analysis showed there are no obvious sequences in the Bag-1 5'UTR corresponding to the optimal binding sites for PTB or PCBP1 therefore to identify the binding sites for PTB and PCBP1, electrophoretic mobility shift assays were performed. Deletion fragments were removed from the vector pRBF (**figure 3.2**) by restriction digestion with *Eco*RI and *Nco*I and ligated into the vector pSKL, which had been pre-digested with the same enzymes and dephosphorylated (**figure 5.4A**). Radiolabelled RNAs were generated from these plasmids by *in vitro* transcription reactions and these were incubated with PCBP1 (**figure 5.4B**).

As expected, incubating radiolabelled Bag-1 IRES RNA corresponding to the minimal IRES element, which comprises the region 225-411, with increasing amounts of PCBP1 showed that PCBP1 bound directly to this region of the IRES. To further refine the binding site for PCBP1, the protein was incubated with other deletion

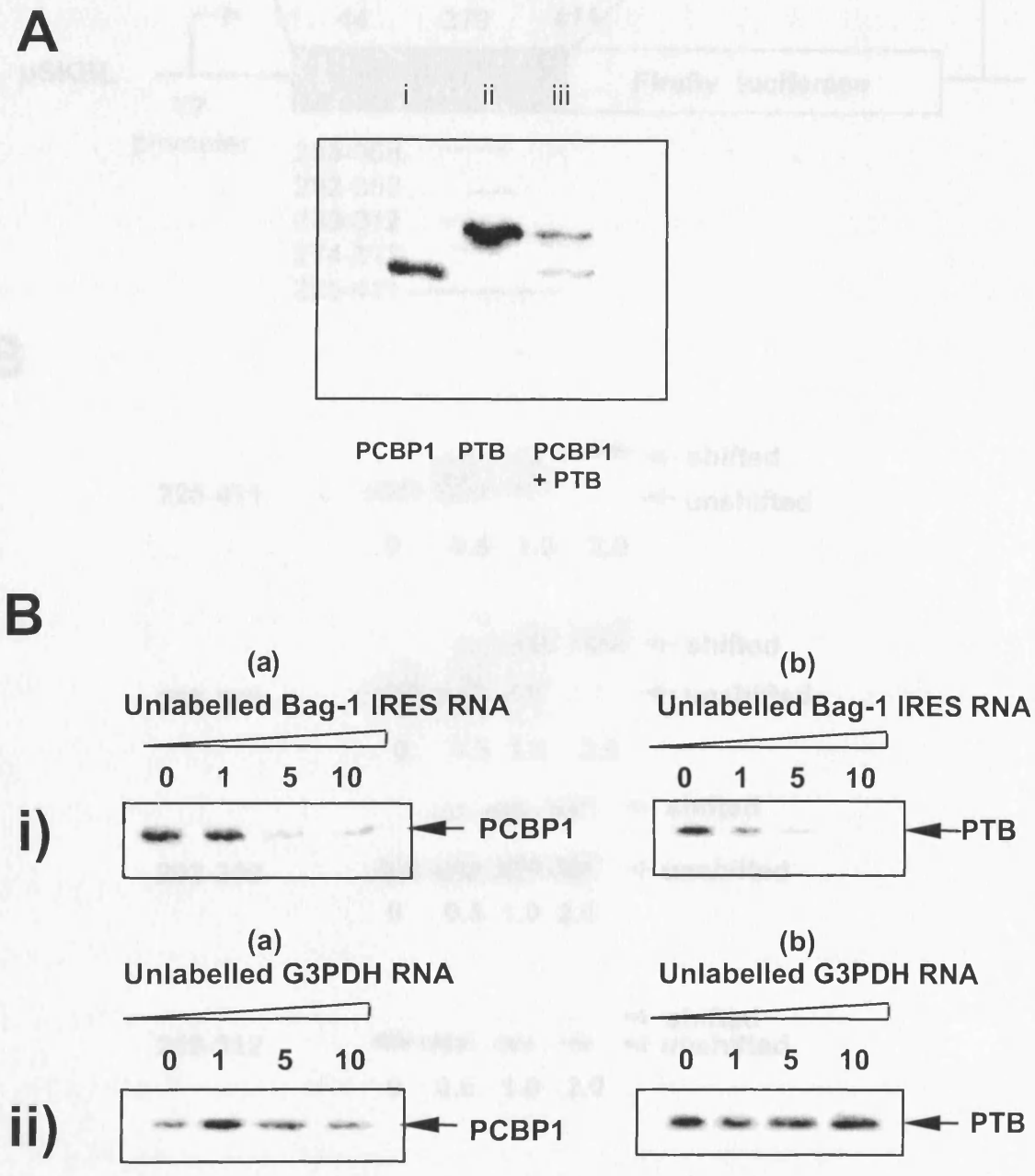


Figure 5.3. The Bag-1 5'UTR specifically binds PTB and PCBP1

500,000cpm of ^{32}P[CTP]-labelled Bag-1 IRES RNA was UV cross-linked to 200ng of each protein indicated. Unbound RNA digested with RNases, the products separated on 10% SDS-PAGE gels and visualised by autoradiography.

A. UV-crosslinking analysis of ^{32}P[CTP]-labelled Bag-1 IRES RNA in combination with (i) PCBP1, (ii) PTB or (iii) PTB and PCBP1

B. UV-crosslinking competition analysis. ^{32}P[CTP]-labelled Bag-1 5'UTR RNA incubated with (a) PCBP1 or (b) PTB and increasing molar excess of i) a specific competitor, unlabelled Bag-1 5'UTR RNA or ii) a non-specific competitor RNA generated from G3PDH.

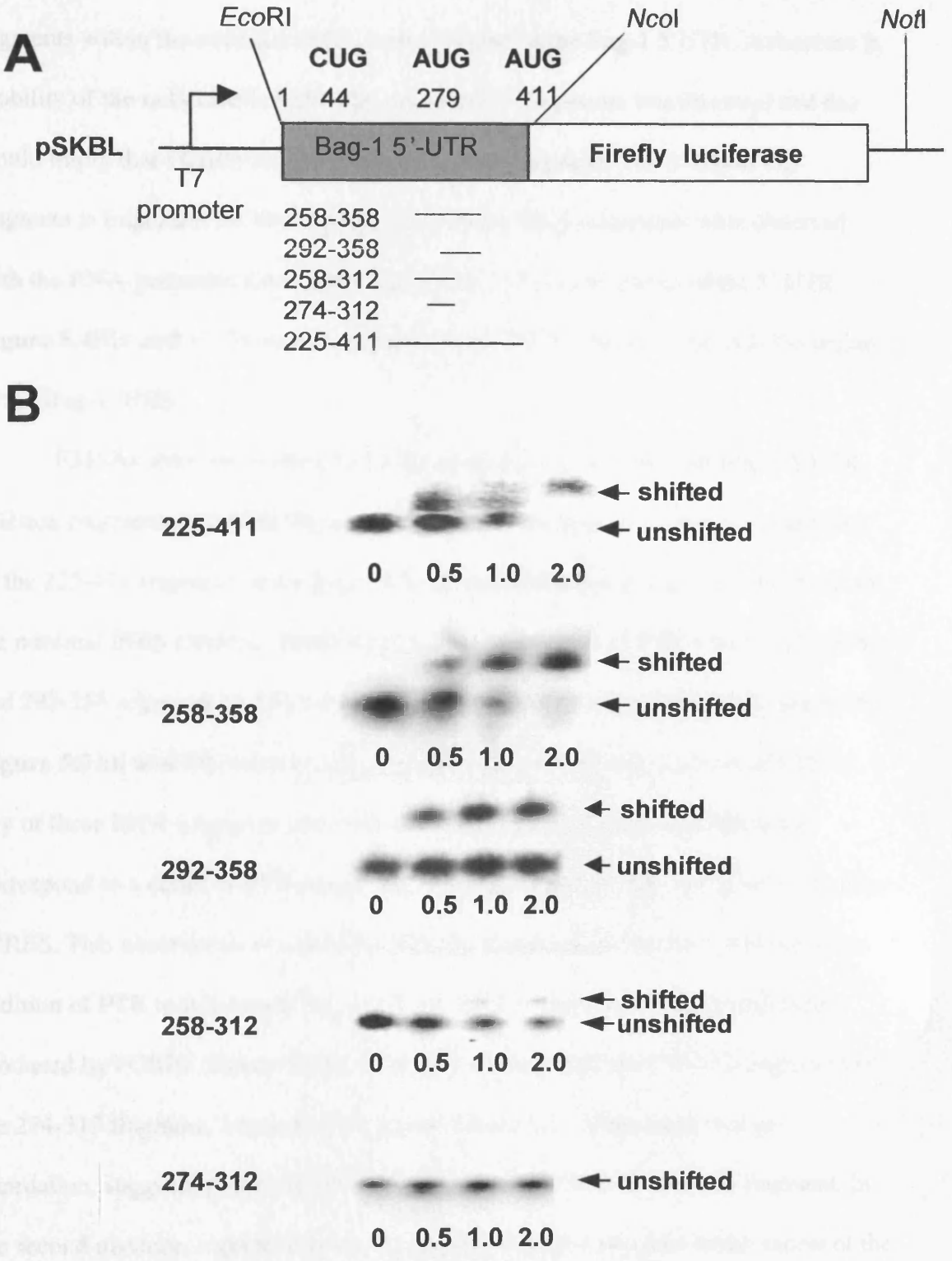


Figure 5.4. The binding site for PCBP1 on the Bag-1 IRES was mapped by EMSAs with deletion fragments of the 5'UTR..

A. Schematic diagram showing position of deletion fragments generated from the monocistronic vector pSKBL by subcloning of deletions generated by PCR into pSKBL.

B. 25,000cpm of ^{32}P[CTP]-labelled RNAs corresponding to deletion fragments of the Bag-1 5'UTR were incubated with the molar ratios of PCBP1 indicated. Products were separated on 0.7 X TBE agarose gels and visualised by autoradiography.

segments within the minimal IRES element region of the Bag-1 5'UTR. A decrease in mobility of the radiolabelled 258-358 and 292-358 fragments was observed and this would imply that PCBP1 binds to a 66-nucleotide fragment. The 3' end of this fragment is important for binding since no protein-RNA complexes were observed with the RNA generated from either 258-312 or 274-312 segments of the 5' UTR (**figure 5.4Biv and v**). These results indicate that PCBP1 binds to the 312-358 region of the Bag-1 IRES.

EMSAs were also carried out with incubation of radiolabelled Bag-1 5'UTR deletion fragments and PTB (**figure 5.5A**). PTB was found to cause a gel retardation of the 225-411 fragment of the Bag-1 5'UTR indicating that this protein also binds to the minimal IRES element. Incubating increasing amounts of PTB with the 258-358 and 292-358 segments of RNA shows that this protein bound to these RNA segments (**figure 5.5Aii and iii**). Interestingly, addition of a two fold molar excess of PTB to any of these RNA fragments gave rise to a shifted band of a position that would correspond to a dimer of PTB suggesting multiple copies of PTB may bind to the Bag-1 IRES. This observation is consistent with the increased gel retardation observed on addition of PTB to full-length Bag-1 5'UTR RNA compared to the gel retardation produced by PCBP1 (**figure 5.2A**). PTB also interacts with the 258-312 fragment and the 274-312 fragment, but in the first instance there is no clear additional gel retardation, suggesting only one PTB monomer may be able to bind this fragment. In the second instance, a gel retardation is only observed at a two fold molar excess of the protein and may be non-specific. Consequently, PTB appears to bind at multiple sites along the 258-358 region.

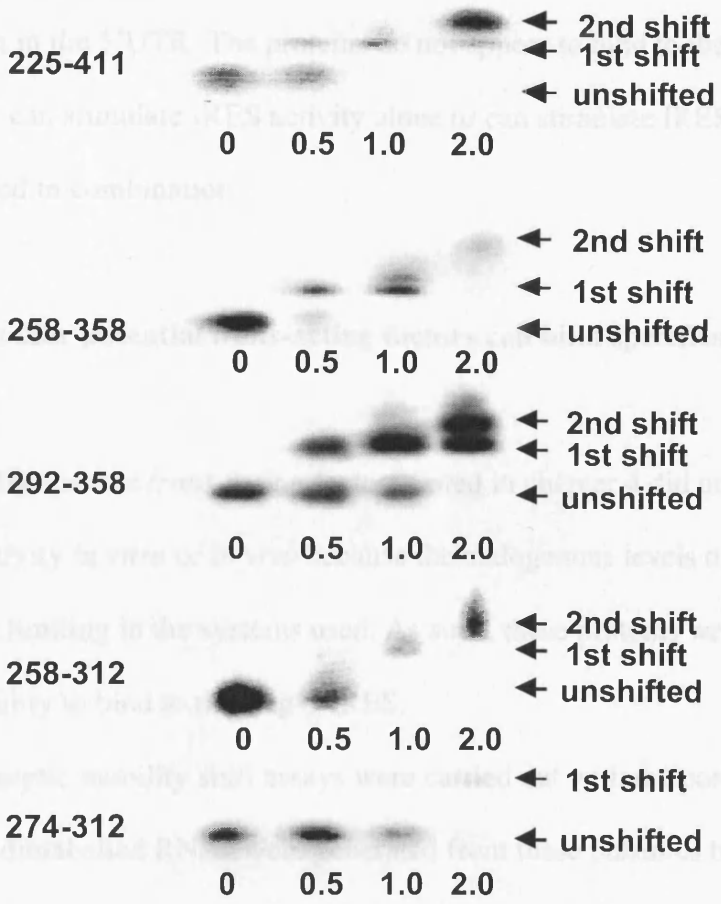


Figure 5.5. The binding sites for PTB on the Bag-1 IRES were mapped by EMSAs with deletion fragments of the 5'UTR. 25,000cpm of ^{32}P[CTP]-labelled RNAs corresponding to deletion fragments of the Bag-1 5'UTR were incubated with the molar ratios of PTB indicated. Products were separated on 0.7 X TBE agarose gels and visualised by autoradiography.

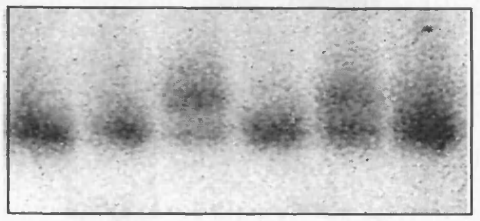
Discussion

Of the potential *trans*-acting factors tested for stimulation of Bag-1 IRES activity in chapter 4, only PTB and PCBP1 appeared to show a significant degree of stimulation of Bag-1 IRES activity. PTB and PCBP1 also bind directly and specifically to a region within the boundaries of the IRES element itself (deletion fragment 225-411), suggesting they are binding to the IRES structure and not an upstream position in the 5'UTR. The proteins do not appear to bind to the same site and therefore can stimulate IRES activity alone or can stimulate IRES activity further when added in combination.

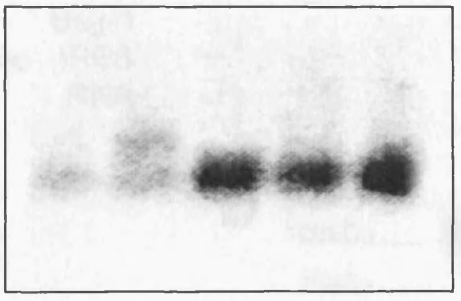
5.4. None of the other potential *trans*-acting factors can bind specifically to the Bag-1 IRES

It is possible that the *trans*-acting factors tested in chapter 4 did not enhance IRES activity *in vitro* or *in vivo* because the endogenous levels of these proteins were not limiting in the systems used. As such, these proteins were also tested for their ability to bind to the Bag-1 IRES.

Electrophoretic mobility shift assays were carried out with the potential *trans*-acting factors. Radiolabelled RNAs were generated from these plasmids by *in vitro* transcription reactions as before and these were incubated with the proteins of interest. Protein: RNA complexes were separated on 0.7% TBE agarose gels. No difference in mobility of Bag-1 IRES RNA was observed with the other proteins tested, suggesting they do not bind the Bag-1 IRES (**figure 5.6**). EMSAs were also carried out with unr and unrip, both alone and in combination as unrip is known to interact with unr. A clear gel retardation can be observed with the addition of unr suggesting unr is binding to the Bag-1 IRES (**figure 5.7A**). Unrip was not capable of binding to the Bag-1 IRES



Bag-1 IRES RNA	+	+	+	+	+
	La	PCBP1	eIF4G	La + PCBP1	La + eIF4G



Bag-1 IRES RNA	+	+	+	+
	PCBP1	PCBP2	DAP5	ITAF45

Figure 5.6. The Bag-1 5'UTR does not bind other proteins tested. 25,000cpm of ^{32}P[CTP]-labelled RNAs corresponding to deletion fragments of the Bag-1 5'UTR were incubated with 200ng of the proteins indicated. Products were separated on 0.7 X TBE agarose gels and visualised by autoradiography. Mobility shifts are only observed in the presence of PCBP1.

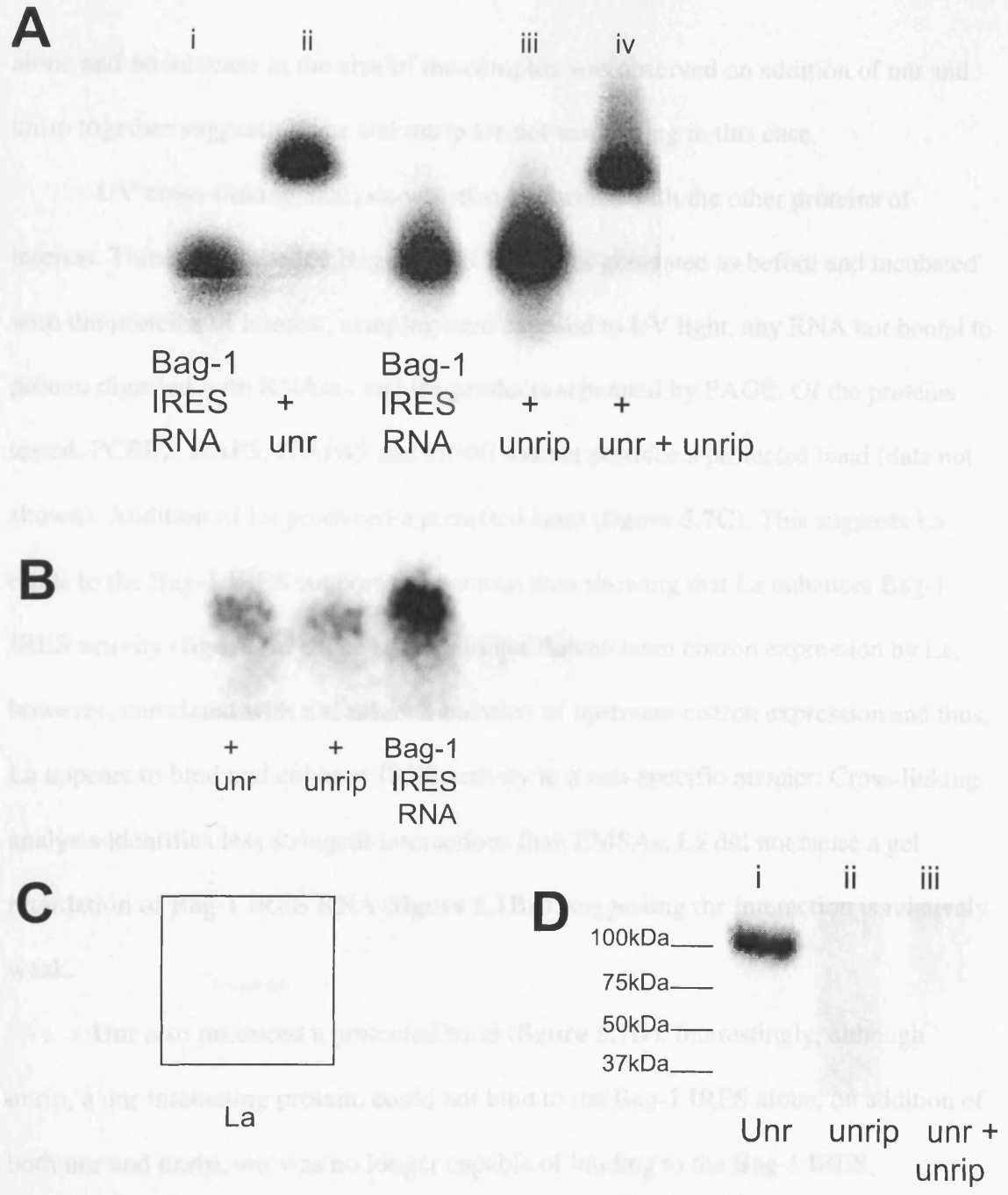


Figure 5.7. Unr binds to the Bag-1 IRES but unrip cannot bind, even in conjunction with unr.

EMSAs were performed using 25,000cpm ^{32}P[CTP]-labelled Bag-1 IRES RNA plus 200ng of each protein indicated. Products were separated on 0.7% TBE agarose gels. UV cross-linking analyses were performed using 500,000cpm ^{32}P[CTP]-labelled Bag-1 IRES RNA plus 200ng of each protein indicated. Products were separated on 10% SDS-PAGE gels. All products were visualised by autoradiography.

A. EMSA of ^{32}P[CTP]-labelled Bag-1 5'UTR RNA i) alone, or with ii) unr, iii) unrip or iv) both proteins.

B. EMSA of ^{32}P[CTP]-labelled non-specific G3PDH RNA with i) unr or ii) unrip.

C. UV crosslinking analysis of ^{32}P[CTP]-labelled Bag-1 5'UTR RNA with La.

D. UV crosslinking analysis of ^{32}P[CTP]-labelled Bag-1 5'UTR RNA with i) unr, ii) unrip or iii) unr + unrip. The molecular weights of the size markers used are indicated in kDa. The molecular weights of unr and unrip are approximately 97kDa and 38kDa respectively.

alone and no increase in the size of the complex was observed on addition of unr and unrip together suggesting unr and unrip are not associating in this case.

UV cross-linking analysis was also performed with the other proteins of interest. Thus, radiolabelled Bag-1 IRES RNA was generated as before and incubated with the proteins of interest, samples were exposed to UV light, any RNA not bound to protein digested with RNAses and the products separated by PAGE. Of the proteins tested, PCBP2, DAP5, ITAF45 and eIF4G did not produce a protected band (data not shown). Addition of La produced a protected band (**figure 5.7C**). This suggests La binds to the Bag-1 IRES supporting previous data showing that La enhances Bag-1 IRES activity (**figure 4.7**). The stimulation of downstream cistron expression by La, however, correlated with a similar stimulation of upstream cistron expression and thus, La appears to bind and enhance IRES activity in a non-specific manner. Cross-linking analysis identifies less stringent interactions than EMSAs; La did not cause a gel retardation of Bag-1 IRES RNA (**figure 5.1Bii**), suggesting the interaction is relatively weak.

Unr also produced a protected band (**figure 5.7D**). Interestingly, although unrip, a unr interacting protein, could not bind to the Bag-1 IRES alone, on addition of both unr and unrip, unr was no longer capable of binding to the Bag-1 IRES, suggesting that the interaction of unr with unrip may prevent unr binding to the Bag-1 IRES in this system (**figure 5.7Dii and iii**).

The same quantities of each protein were used in both the EMSA and crosslinking assays. It is possible that unr can make transient interactions with the Bag-1 IRES in the presence of unrip which can be detected by EMSA but are not stable enough to protect the Bag-1 IRES RNA from RNase degradation in the crosslinking assay.

5.5. Unr binds to the Bag-1 IRES minimal element

To determine where the binding site or sites for unr in the Bag-1 IRES are located, EMSAs were carried out with radiolabelled RNAs derived from deletion fragments of the Bag-1 IRES as before (**figure 5.8**). In common with the binding sites of PTB and PCBP1, unr bound to the minimal IRES and also to the 258-358 and 292-358 fragments of the IRES but binding to the smaller fragments was greatly reduced, suggesting unr may be binding at multiple sites in the IRES RNA.

To further investigate the specificity of the interaction of unr with the Bag-1 IRES, UV-crosslinking analysis was carried out with the addition of specific or non-specific unlabelled competitor RNAs. Addition of a 10 fold molar excess of unlabelled BAG-1 IRES RNA, or a 10 fold molar excess of G3PDH RNA both decreased unr binding to the radiolabelled Bag-1 IRES RNA (**figure 5.9**). This suggests unr has comparative affinity for both the Bag-1 IRES and the non-specific competitor and as such the interaction is unlikely to be specific.

Discussion

While La and unr both appear to bind to the Bag-1 IRES, these interactions seem to be non-specific. The interaction of La with the Bag-1 IRES is relatively weak as no interaction can be detected in EMSA experiments although La is capable of binding to the Bag-1 IRES in the less stringent cross-linking assays. As unr can bind to the Bag-1 IRES in EMSAs, it could be determined that unr binds to the minimal IRES element; however, unr can bind to smaller fragments but with less efficiency suggesting unr may be binding multiple sites in the Bag-1 IRES. These non-specific interactions, however, suggest these proteins do not act as Bag-1 *trans*-acting factors.

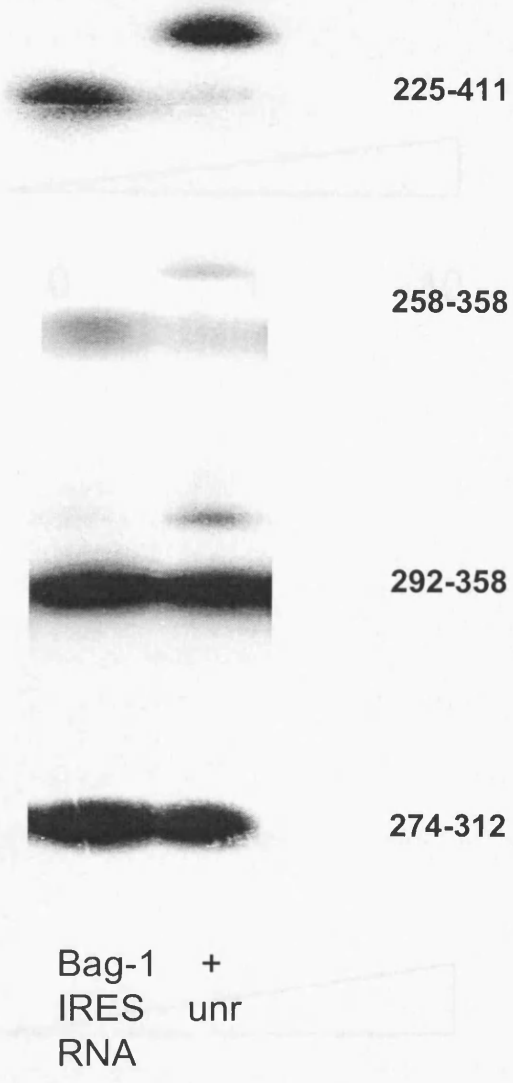
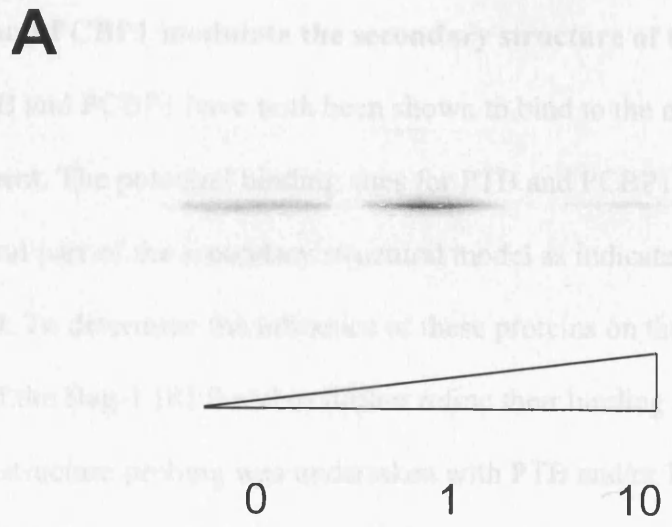


Figure 5.8. Unr also binds to the Bag-1 IRES minimal element in EMSAs

25,000cpm of ^{32}P[CTP]-labelled Bag-1 5'UTR transcripts were incubated alone or with 200ng unr. Protein-RNA complexes were separated on 0.7% TBE agarose gels and products visualised by autoradiography.



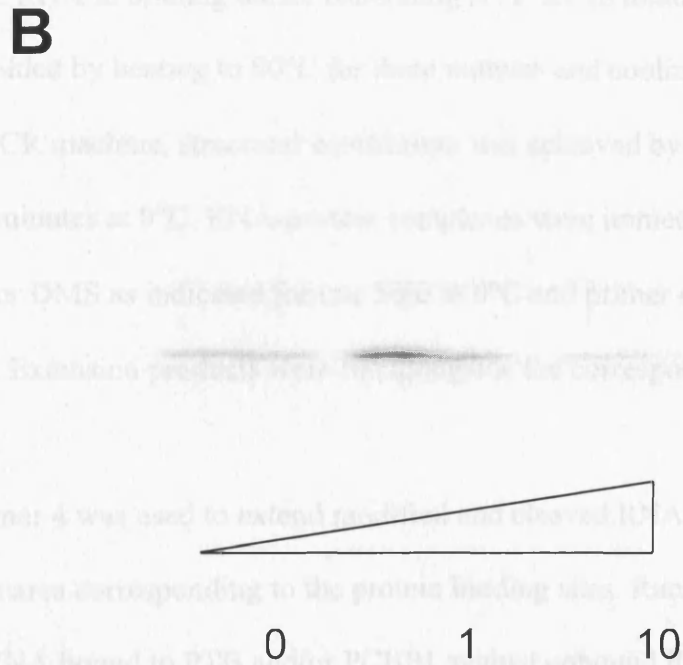


Figure 5.9. Unr binds non-specifically to the Bag-1 IRES
Autoradiographs of 10% SDS-PAGE gels.

A. UV-crosslinking of 500,000cpm of ^{32}P[CTP]-labelled Bag-1 IRES RNA with 200ng unr in the presence of increasing molar quantities of unlabelled Bag-1 IRES competitor RNA.

B. UV-crosslinking of 500,000cpm of ^{32}P[CTP]-labelled Bag-1 IRES RNA with 200ng unr in the presence of increasing molar quantities of unlabelled G3PDH non-specific competitor RNA.

5.6. PTB and PCBP1 modulate the secondary structure of the Bag-1 IRE

PTB and PCBP1 have both been shown to bind to the minimal Bag-1 IRES element. The potential binding sites for PTB and PCBP1 span a large region in the central part of the secondary structural model as indicated on the model in **figure 5.10**. To determine the influence of these proteins on the secondary structure of the Bag-1 IRES and to further refine their binding sites, chemical and enzymatic structure probing was undertaken with PTB and/or PCBP1 bound to Bag-1 IRES RNA.

PTB and PCBP1 were incubated with Bag-1 IRES RNA generated from pSKBminL RNA in binding buffer containing ATP for 10 minutes. The RNA had been pre-folded by heating to 80°C for three minutes and cooling to 4°C over 1 hour in a PCR machine, structural equilibrium was achieved by incubating for a further 10 minutes at 0°C. RNA-protein complexes were immediately treated with RNase VI or DMS as indicated for one hour at 0°C and primer extension performed. Extension products were run alongside the corresponding sequencing ladder.

Primer 4 was used to extend modified and cleaved RNAs as this primer extends the area corresponding to the protein binding sites. Running DMS modified RNA bound to PTB and/or PCBP1 against unbound RNA highlights a number of bases in this region that appear to become single-stranded on addition of protein (**figure 5.11**). The bases with an altered pattern of modification are highlighted in the green section on the chart of modifications in **figure 5.12**. The corresponding bases are also indicated by green arrows on the structural model of the Bag-1 IRES in **figure 5.13**.

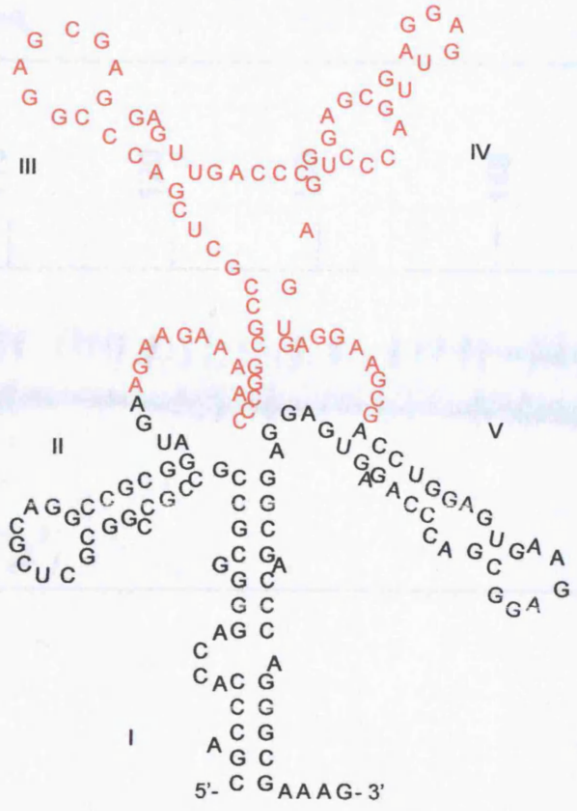


Figure 5.10. The binding sites for PTB and PCBP1 are located in the central region of the Bag-1 IRES.

The region of protein binding is shown in red on the secondary structural model of the Bag-1 IRES

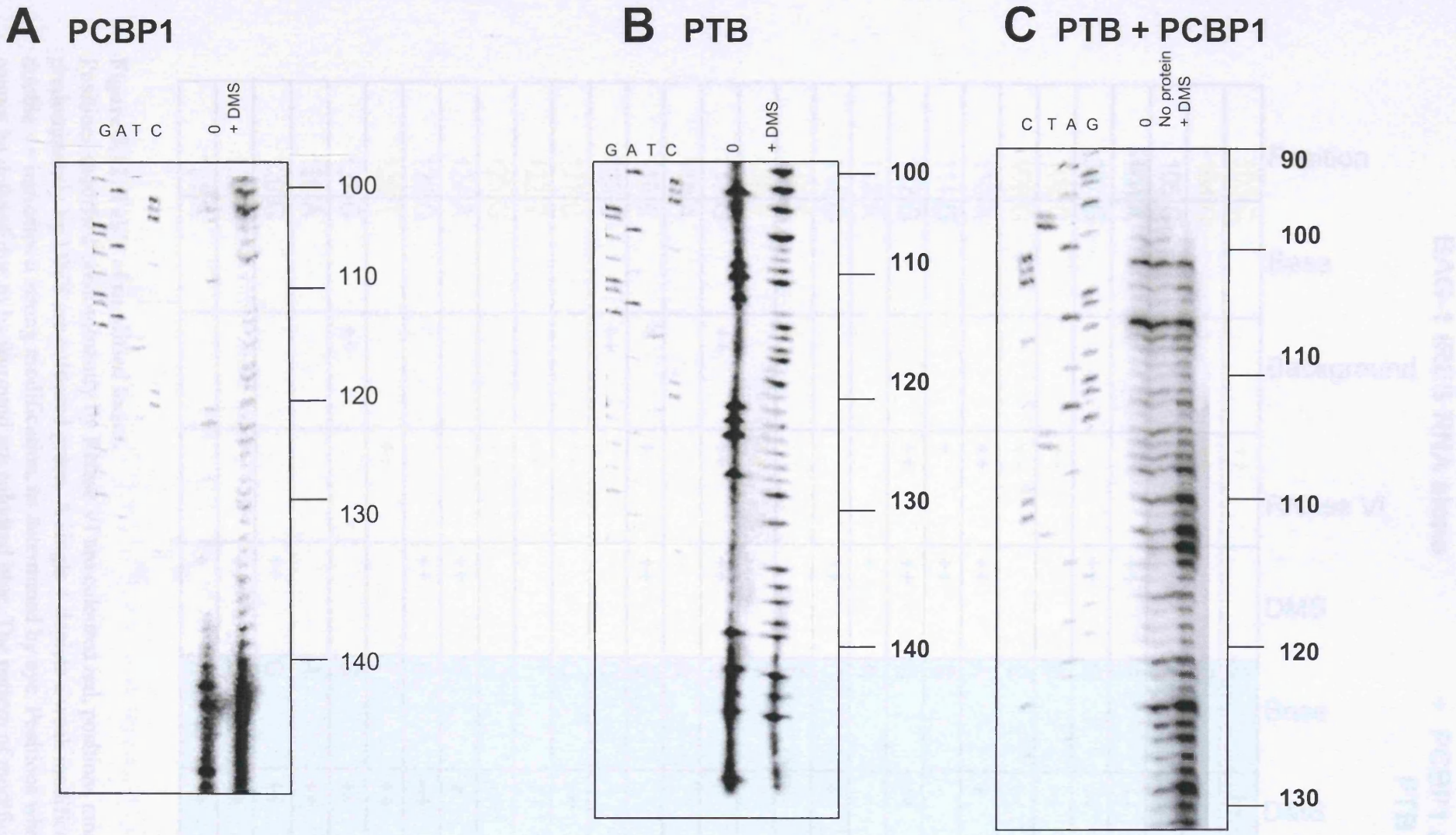


Figure 5.11. Single-stranded bases in the 90-150 nucleotide segment of the Bag-1 IRES on addition of PTB and/or PCBP1.
Single-stranded bases identified by modification of Bag-1 IRES RNA with DMS and reverse transcription with ^{32}P [γATP] end-labelled Primer 4. Products were separated on 7% sequencing gels against the corresponding sequencing ladder. The 0 control lane contains unmodified RNA treated in parallel.

	BAG-1 IRES RNA alone				+ PCBP1 and PTB	
Position	Base	Background	RNase VI	DMS	Base	DMS
103	C		++		C	++
104	G		++		G	
105	G				G	
106	A			++	A	++
107	G			++	G	++
108	C		++		C	++
109	G		++		G	+
110	A		++	++	A	+
111	G		+	++	G	
112	G		++	++	G	++
113	A			+	A	
114	G			++	G	++
115	T	+	++		T	
116	T		++		T	
117	G	++	++	++	G	
118	A				A	
119	C	+	+	++	C	
120	C	++			C	
121	C		++		C	++
122	T		++		T	++
123	G		++		G	++
124	A			++	A	+
125	G			++	G	++
126	T		++		T	++
127	G	++			G	++
128	A				A	++
129	G			++	G	++
130	G			++	G	++
131	A			+	A	++

Figure 5.12. Table of modified bases.
Positions modified predominantly by RNase VI are coloured red, positions modified predominantly by DMS are coloured green. A single + depicts a weak modification, a double ++ indicates a strong modification, as determined by eye. Positions where pairing cannot be defined due to background are coloured blue. The pattern of modification on addition of protein is highlighted in green. All modifications were observed in at least two independent experiments.

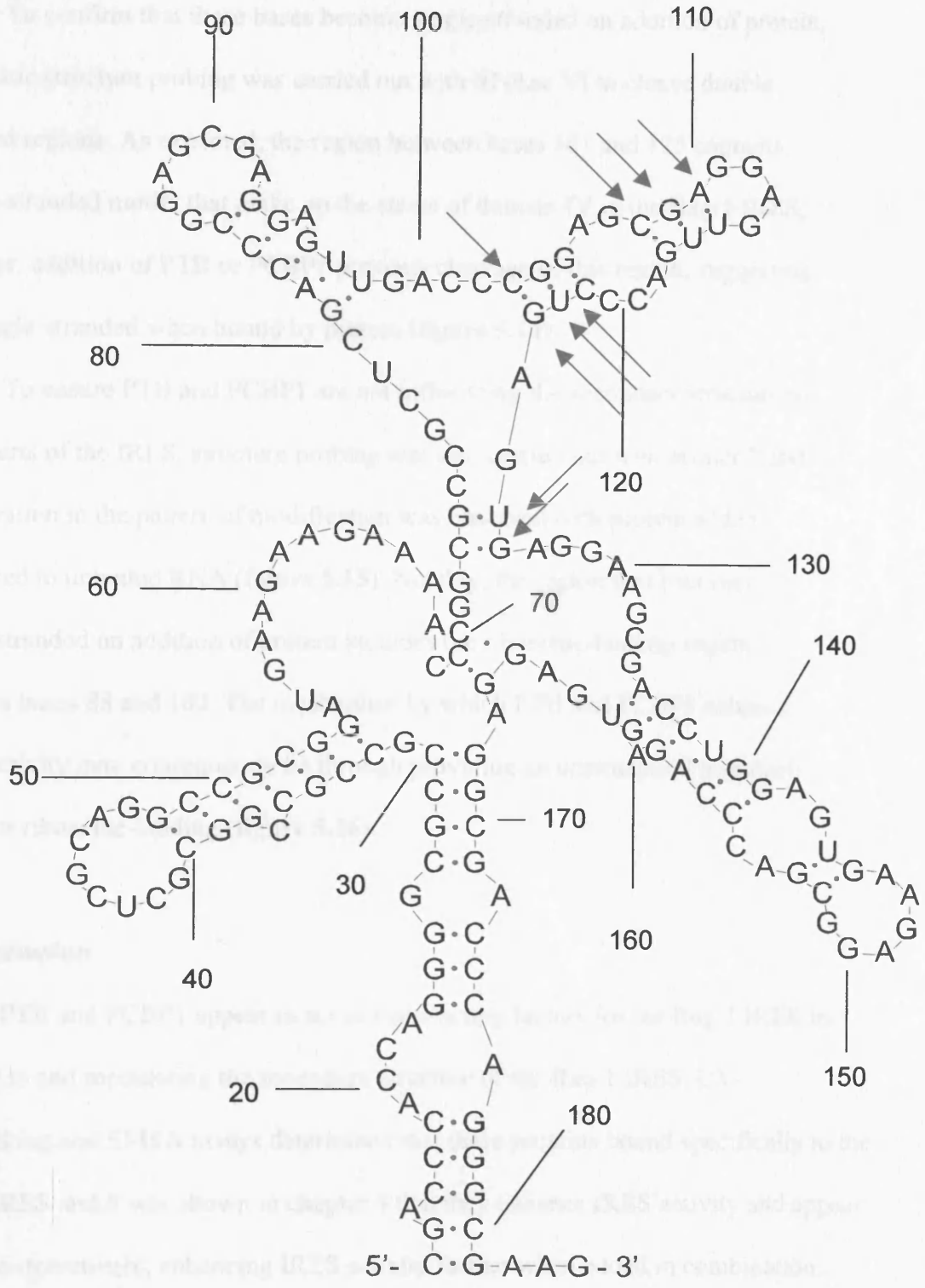


Figure 5.13. Positions at which PTB and/or PCBP1 binding alter Bag-1 IRES structure.

Bases which show a different pattern of DMS modification (become single stranded) on addition of PTB and/or PCBP1 are marked with green arrows.

To confirm that these bases become single-stranded on addition of protein, enzymatic structure probing was carried out with RNAse VI to cleave double stranded regions. As expected, the region between bases 101 and 125 contains double-stranded motifs that make up the stems of domain IV in the Bag-1 IRES; however, addition of PTB or PCBP1 prevents cleavage of this region, suggesting it is single-stranded when bound by protein (**figure 5.14**).

To ensure PTB and PCBP1 are not influencing the secondary structure in other parts of the IRES, structure probing was also carried out with primer 2 and no alteration in the pattern of modification was observed with protein added compared to unbound RNA (**figure 5.15**). Notably, the region that becomes single-stranded on addition of protein includes the ribosome-landing region between bases 88 and 102. The mechanism by which PTB and PCBP1 enhance IRES activity may consequently be through providing an unstructured region of RNA for ribosome-landing (**figure 5.16**).

5.7. Discussion

PTB and PCBP1 appear to act as *trans*-acting factors for the Bag-1 IRES by binding to and modulating the secondary structure of the Bag-1 IRES. UV-crosslinking and EMSA assays determined that these proteins bound specifically to the Bag-1 IRES and it was shown in chapter 4 that they enhance IRES activity and appear to act co-operatively, enhancing IRES activity further when added in combination. Both proteins appear to open up stem-loop IV of the Bag-1 IRES but when added in combination, the modifications were generally stronger suggesting that maintenance of the 'open' structure may be more stable on addition of both proteins.

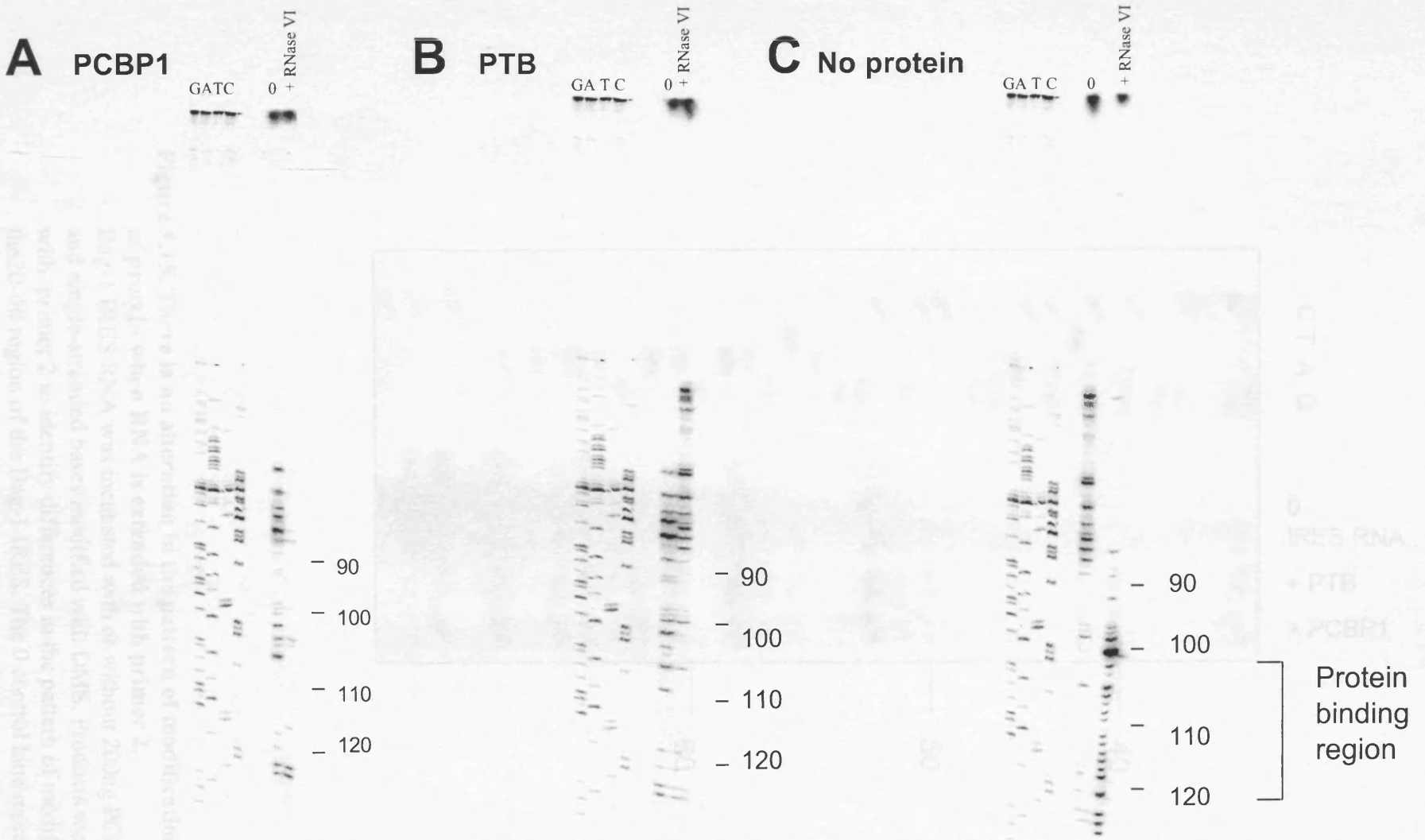


Figure 5.14. Cleavage of stem-loop IV by RNase VI is prevented by addition of PTB or PCBP1.

A. Bag-1 IRES RNA incubated with PCBP1 and RNase VI.

B. Bag-1 IRES RNA incubated with PTB and RNase VI.

C. Bag-1 IRES RNA incubated with RNase VI in the absence of protein.

RNA fragments were extended with ^{32}P-end-labelled primer 4 and run against the corresponding sequencing ladder on 7% sequencing gels. The 0 control lane contains unmodified RNA treated in parallel.

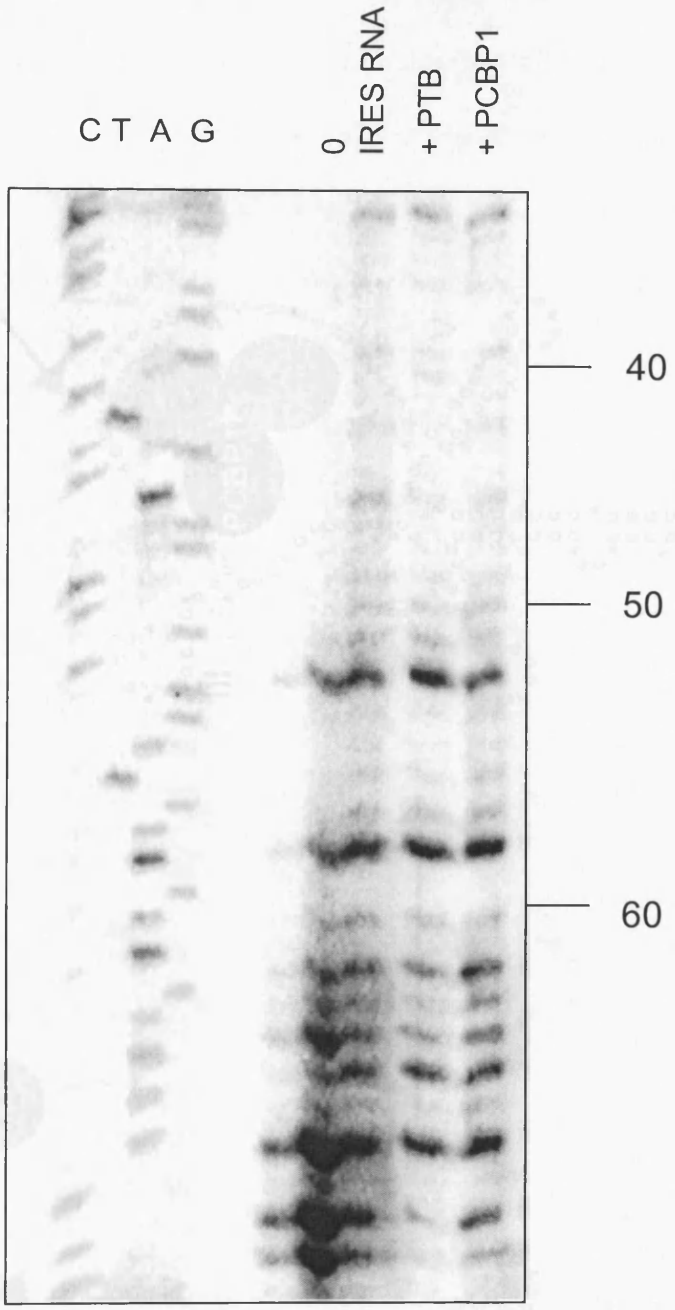


Figure 5.15. There is no alteration in the pattern of modification on addition of protein when RNA is extended with primer 2.
Bag-1 IRES RNA was incubated with or without 200ng PCBP1 or PTB and single-stranded bases modified with DMS. Products were extended with primer 2 to identify differences in the pattern of modification in the20-60 region of the Bag-1 IRES. The 0 control lane represents unmodified RNA treated in parallel.

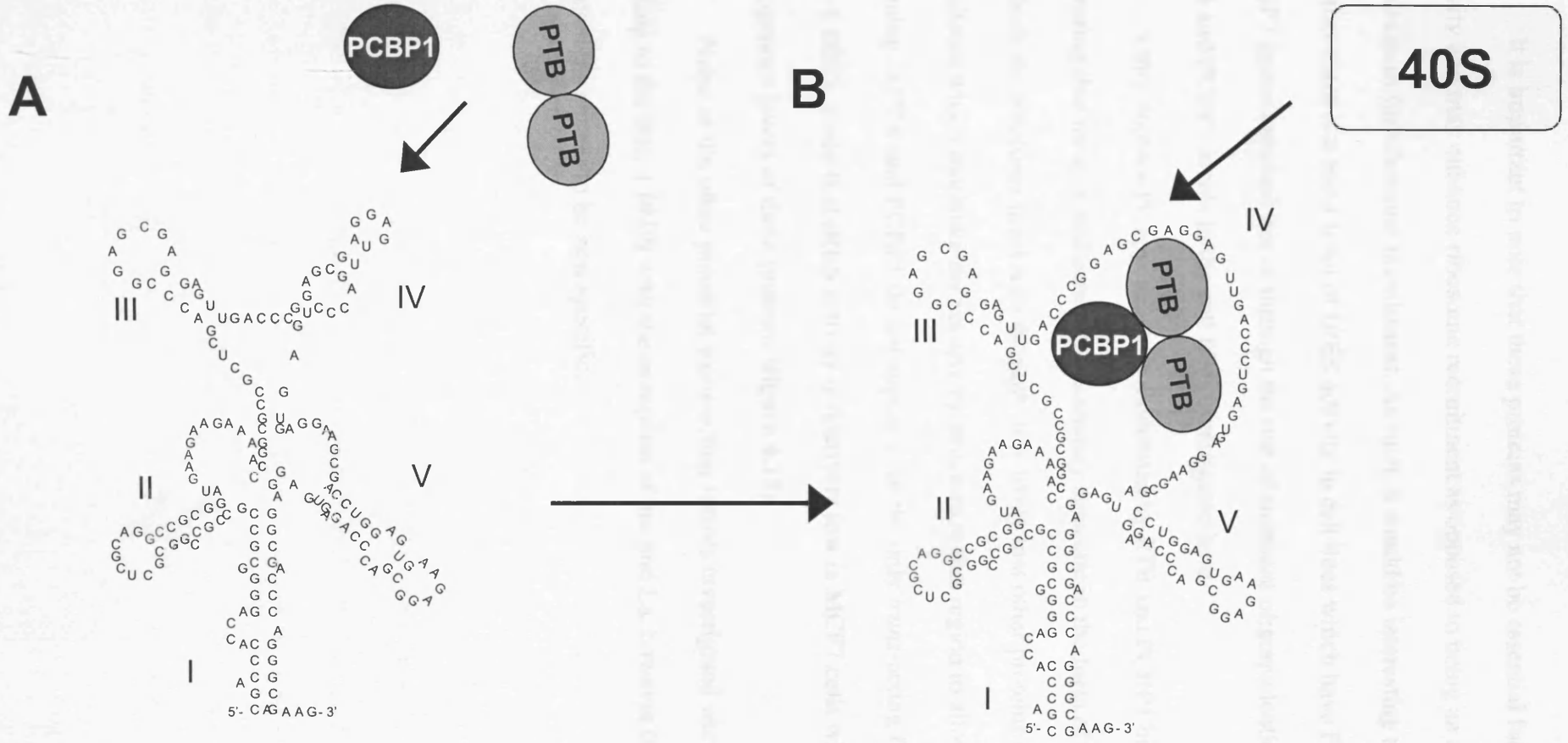


Figure 5.16. Proposed model for the mechanism of PTB and PCBP1 enhancement of IRES activity.

A. PTB and PCBP1 bind to the Bag-1 IRES

B. Binding of proteins alters the structural conformation of the Bag-1 IRES to open domain IV into a loop.

C. The ribosome can bind to the single-stranded region of the protein-bound conformation of the Bag-1 IRES.

It is important to note that these proteins may not be essential for Bag-1 IRES activity and may enhance ribosome recruitment as opposed to being an absolute requirement for ribosome recruitment. As such, it would be interesting to observe whether there is a basal level of IRES activity in cell lines which have PTB and/or PCBP1 genes knocked out or through the use of antisense oligonucleotides to decrease PTB and PCBP1 levels in the cell lines investigated here.

Only domain IV appears to be modulated by PTB and PCBP1 binding, indicating that there is still extensive secondary structure in the form of domains V and I, which the ribosome must scan through. It is likely that other proteins will be elucidated which modulate the secondary structure in this region to allow ribosomal scanning as PTB and PCBP1 do not appear to be the only *trans*-acting factors for the Bag-1 IRES, given that IRES activity is relatively low in MCF7 cells which have high endogenous levels of these proteins (**figure 4.15**).

None of the other potential *trans*-acting factors investigated were capable of binding to the Bag-1 IRES with the exception of unr and La, however these interactions appear to be non-specific.

Chapter 6.

Functional roles for the Bag-1 IRES

6.1. Introduction

The role of cellular IRESes is generally assumed to involve maintenance of translation under circumstances where cap-dependent translation is compromised, presumably maintaining production of proteins required under such circumstances. Examples include the c-*myc* and XIAP IRESes, which maintain production of their protein products during apoptosis (Stoneley *et al.*, 2000, Holcik *et al.*, 2000), the VEGF IRES, which functions during hypoxia (Stein *et al.*, 1998, Akiri *et al.*, 1998) and the FGF IRES, which remains active during cell growth, when available eIF4E is limiting (Vagner *et al.*, 1995).

The Bag-1 IRES has been shown to function during heat shock (Coldwell *et al.*, 2001). The Bag-1 protein is a key co-chaperone for the 70kDa heat-shock proteins (hsp70/hsc70) indicating that production of this protein during heat shock and recovery is required. In addition, overexpression of Bag-1 proteins has been shown to protect cells against cell death induced by heat shock (Townsend *et al.*, 2003). The Bag-1 proteins have a diverse range of cellular functions due to interactions with many different proteins involved in cellular processes ranging from apoptosis and regulation of steroid hormone binding to transcriptional activation (Takayama *et al.*, 1995, Zeiner *et al*, 1995, Niyaz *et al.*, 2001). Interestingly, overexpression of Bag-1 proteins also protects cells against cell death or growth inhibition induced by treatment with chemotherapeutic agents (Chen *et al.*, 2002; Townsend *et al.*, 2003).

Thus, it was of interest to address the identification of other cell stress conditions in which the Bag-1 IRES functions to maintain production of the p36 isoform of Bag-1.

6.2. Bag-1 and cancer

There is increasing evidence that Bag-1 proteins are overexpressed in a diverse range of cancers (**section 1.11.**). Work has focused particularly on overexpression of Bag-1 in breast cancers where increased levels of Bag-1 are observed in approximately 92% of cases studied (Townsend *et al.*, 2002). Interestingly the p50 isoform is rarely observed in normal cells however it is frequently found to be overexpressed in cancers (Takayama *et al.*, 1998, Yang *et al.*, 1998, Yang *et al.*, 1999), indicating that increased IRES activity under these circumstances cannot be entirely responsible for the increased Bag-1 expression observed in these cells. This suggests a cap-dependent mechanism may be implicated in overexpression of these proteins.

The Bag-1 5'UTR is very GC-rich, highly structured (**figure 3.23.**) and has an inhibitory effect on cap-dependent translation in the rabbit reticulocyte lysate system (**figure 4.12**). These features suggest p50 and p46 would be translated very inefficiently and indeed in normal cells are rarely expressed. The long, structured 5'UTR would make the Bag-1 mRNA a poor substrate for recognition by the eIF4F complex, however it has been shown that overexpression of limiting components of this complex, after cellular transformation for example, leads to increased recognition of mRNAs with inhibitory 5'UTRs, consequently leading to increased cap-dependent translation of these mRNAs.

Western analysis was performed to determine the relative expression levels of the initiation factors eIF4E and eIF4G in cell lines with differing levels of Bag-1 expression. Cell lysates were prepared from a range of human breast, lung and cervical cancer cell lines (**described in table 2.1**.). The levels of eIF4E are roughly equivalent in each cell line, therefore there does not appear to be a direct correlation between eIF4E and overexpression of Bag-1 (**figure 6.1Aii**). In contrast, levels of eIF4G do show a broad correlation with eIF4G highly expressed in cell lines with high levels of Bag-1 such as HeLa and MCF7 cells, and with significantly lower expression in cell lines with low levels of Bag-1 such as CAL51 and CALU1 cells (**figure 6.1Ai**).

Discussion

The data presented here indicate that there is a broad correlation between expression levels of eIF4G and the three major Bag-1 isoforms. This observation may reflect a general inhibition of protein synthesis due to deficient eIF4G in some cell lines as a similar pattern of expression is observed with the *trans*-acting factors PTB and PCBP1. Both proteins are expressed at significantly higher levels in MCF7 cells compared to CAL51 and CALU1 cell lines (**figure 4.15**).

It would be of interest to immunoblot cell lysates for the initiation factor eIF4A, which is a helicase and unwinds secondary structure in the 5'UTR during the initiation process. The Bag-1 IRES must be able to be unwound as otherwise it would not be possible for p50 or p46 to be expressed. The hairpin construct pRHpBF is capable of directing translation of the downstream cistron in the rabbit reticulocyte

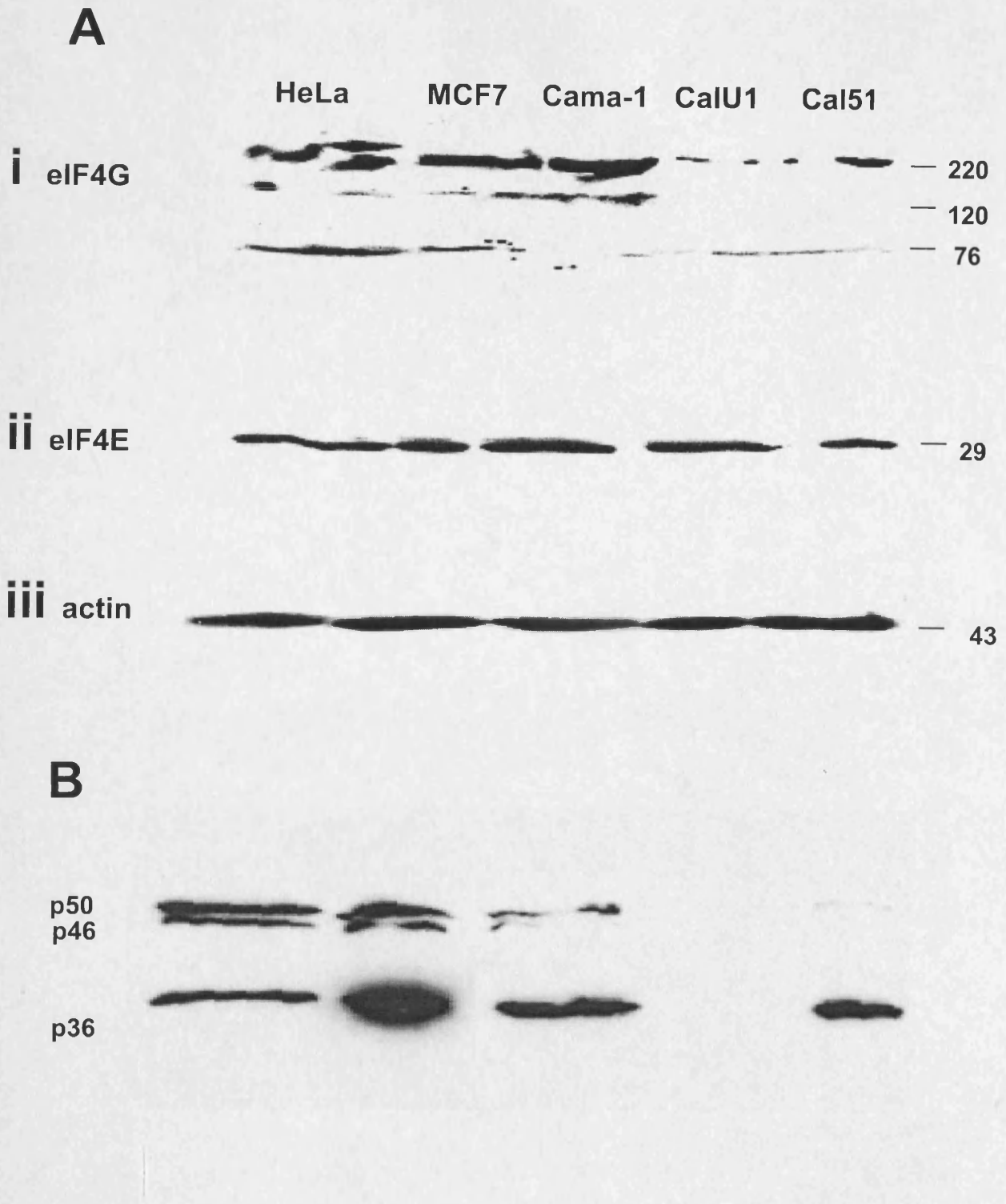


Figure 6.1. eIF4G tends to be highly expressed in cell lines with relatively high levels of Bag-1 protein.

A. Autoradiographs of western blots of i) eIF4G, ii) eIF4E and iii) actin in HeLa, MCF7, Cama-1, CalU1 and Cal51 cell lysates. The approximate molecular weights of the proteins indicated are marked in kDa.

B. Autoradiograph of a western blot of Bag-1 in the cell lysates indicated with the positions of the p36, p46 and p50 isoforms marked. Note that this blot was shown in **figure 4.5** and is reproduced here for ease of comparison with the levels of eIF4G and eIF4E in the corresponding cell lysates.

lysate system whereas the dicistronic vector pRBF cannot. This may be attributed to ribosomal readthrough from the upstream cistron and reinitiation at the p36 initiation codon due to unwinding of the IRES structure. Overexpression of this initiation factor could conceivably lead to increased unwinding of the secondary structure comprising the IRES and consequently greater efficiency of translation of the longer isoforms p50 and p46 by the cap-dependent scanning mechanism.

6.3. The Bag-1 IRES functions during cell stress

6.3.1. TRAIL induced apoptosis

One of the key events in apoptosis is inhibition of protein synthesis, which occurs by caspase-mediated cleavage of initiation factors such as eIF4G and eIF4A and alterations in the phosphorylation state of initiation factors such as eIF4E, eIF4E-BP1 and eIF2α (Clemens *et al.*, 2000). A number of the cellular IRESes identified so far have key roles in the apoptotic process; both anti and pro-apoptotic proteins have been identified. Of these, several have been shown to function during apoptosis and hence to maintain production of key proteins presumably required for progression or inhibition of apoptosis. Pro-apoptotic proteins include c-*myc* and DAP5 (Stoneley *et al.*, 2000, Henis-Korenblit *et al.*, 2000) whilst anti-apoptotic proteins include XIAP (Holcik *et al.*, 1999).

Bag-1 has been shown to have multiple anti-apoptotic roles. Some of the best defined include association with BCl-2, enhancing the anti-apoptotic properties of this protein (Takayama *et al.*, 1995). Bag-1 also associates with the retinoic-acid receptor where it inhibits retinoic acid induced apoptosis (Liu *et al.*, 1998). As such, it

was of interest to determine whether the Bag-1 IRES functions during apoptosis. Previous experiments by Mark Coldwell investigating Bag-1 IRES activity during TRAIL-induced apoptosis indicated that the Bag-1 IRES might be functional during the early stages of apoptosis (Coldwell, 2001). These experiments were performed using the dicistronic vectors pRF and pRBF and the monocistronic vector pGL3. Luciferase activity from the monocistronic vector decreased to 59% of untreated cells after two hours, however the presence of the Bag-1 IRES in the dicistronic vector allowed translation of the downstream cistron with approximately 82% of the efficiency of IRES activity in untreated cells. This rapidly decreases at later stages of the time course to the same level as the control, however the data has a high degree of error and as such merits further investigation.

The TNF-related apoptosis inducing ligand (TRAIL) was used in these experiments to induce apoptosis in HeLa cells. TRAIL binds to TRAIL receptors on the cell surface and induces apoptosis via the death receptor-mediated mechanism of apoptosis. Addition of TRAIL causes a general decrease in protein synthesis within two hours, correlating with cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP) and condensation of cell nuclei (Stoneley *et al.*, 2000).

HeLa cells were transfected with the monocistronic vector pGL3 which expresses firefly luciferase or a monocistronic vector containing the Bag-1 IRES with a hairpin introduced 5' to the IRES to prevent translation of firefly luciferase by cap-dependent scanning, pHpBL (**figure 6.2A**). These constructs represent more physiologically relevant products than the dicistronic constructs as they produce monocistronic mRNAs. The use of monocistronic constructs also prevents any

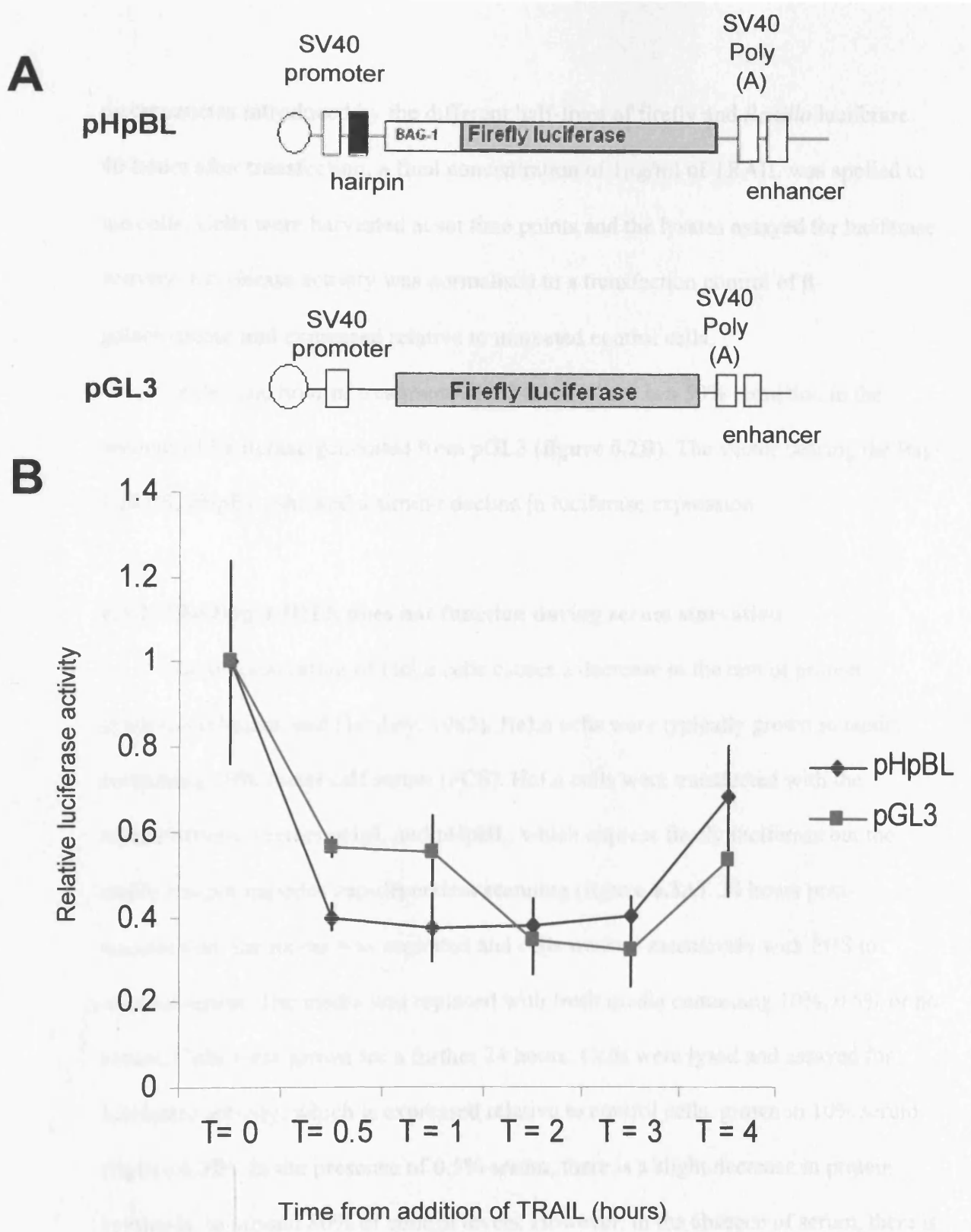


Figure 6.2. The Bag-1 IRES does not maintain translation of Bag-1 during TRAIL induced apoptosis.

A. Schematic diagram of the monocistronic hairpin construct pHpBL and the monocistronic control vector pGL3.

B. Apoptosis was induced in HeLa cells transfected with pHpBL or pGL3 by addition of TNF-related apoptosis-inducing ligand (TRAIL). Luciferase activity is normalised to a transfection control of β-galactosidase and expressed relative to untreated cells. This experiment was performed in duplicate on three independent occasions and cumulative data is shown. Error bars represent standard deviation.

discrepancies introduced by the different half-lives of firefly and *Renilla* luciferase. 40 hours after transfection, a final concentration of 1μg/ml of TRAIL was applied to the cells. Cells were harvested at set time points and the lysates assayed for luciferase activity. Luciferase activity was normalised to a transfection control of β-galactosidase and expressed relative to untreated control cells.

After one hour of treatment with TRAIL, there is a 50% reduction in the amount of luciferase generated from pGL3 (**figure 6.2B**). The vector bearing the Bag-1 IRES, pHpBL, showed a similar decline in luciferase expression.

6.3.2. The Bag-1 IRES does not function during serum starvation

Serum starvation of HeLa cells causes a decrease in the rate of protein synthesis (Duncan and Hershey, 1985). HeLa cells were typically grown in media containing 10% foetal calf serum (FCS). HeLa cells were transfected with the monocistronic vectors pHpL and pHpBL, which express firefly luciferase but the stable hairpin impedes cap-dependent scanning (**figure 6.3A**). 24 hours post-transfection, the media was aspirated and cells washed extensively with PBS to remove serum. The media was replaced with fresh media containing 10%, 0.5% or no serum. Cells were grown for a further 24 hours. Cells were lysed and assayed for luciferase activity, which is expressed relative to control cells, grown in 10% serum (**figure 6.3B**). In the presence of 0.5% serum, there is a slight decrease in protein synthesis, to around 80% of control levels. However, in the absence of serum, there is a reduction in protein synthesis to around 40-50% of the control cells, indicating that protein synthesis is significantly impeded. Interestingly, there remains some

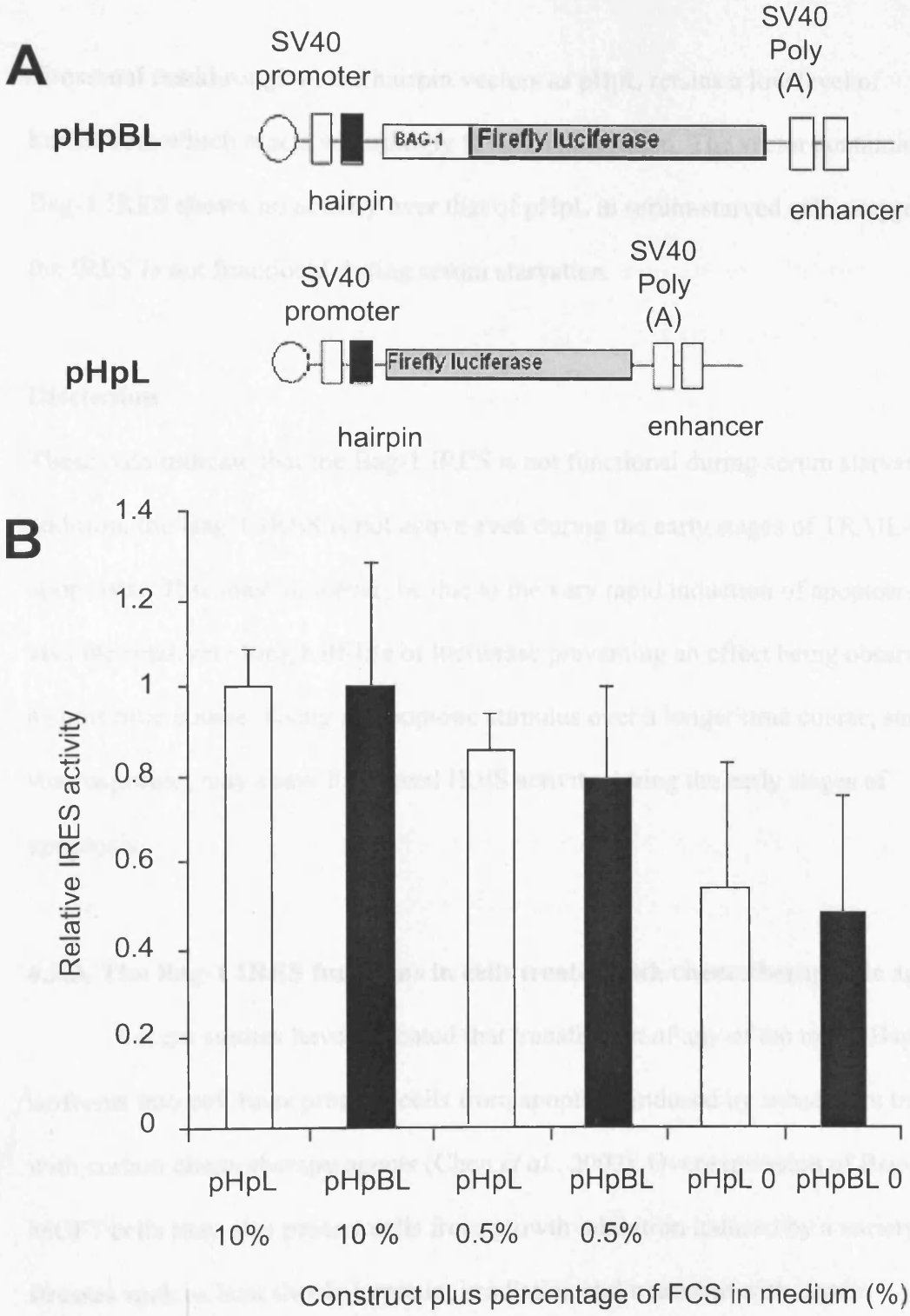


Figure 6.3. The Bag-1 IRES does not maintain production of Bag-1 protein in serum-starved cells

A. Schematic diagram of the monocistronic hairpin vectors pHpBL and pHpL.

B. Serum starvation was induced in HeLa cells transfected with pHpBL or pHpL by replacement of media with media containing 10, 0.5 or 0% FCS for 24 hours post-transfection. Luciferase activity is normalised to a transfection control of β-galactosidase and expressed relative to the luciferase activity of the same constructs transfected into cells maintained with 10% FCS, which were assigned a value of 1. This experiment was performed in duplicate on three independent occasions and cumulative data is shown. Error bars represent standard deviation.

ribosomal readthrough in the hairpin vectors as pHpL retains a low level of translation, which reacts accordingly to serum starvation. The vector containing the Bag-1 IRES shows no activity over that of pHpL in serum-starved cells, suggesting the IRES is not functional during serum starvation.

Discussion

These data indicate that the Bag-1 IRES is not functional during serum starvation. In addition, the Bag-1 IRES is not active even during the early stages of TRAIL-induced apoptosis. This may, however, be due to the very rapid induction of apoptosis and also the relatively long half-life of luciferase preventing an effect being observed over a short time course. Using an apoptotic stimulus over a longer time course, such as staurosporine, may show functional IRES activity during the early stages of apoptosis.

6.3.3. The Bag-1 IRES functions in cells treated with chemotherapeutic agents

Recent studies have indicated that transfection of any of the major Bag-1 isoforms into cell lines protects cells from apoptosis induced by subsequent treatment with certain chemotherapy agents (Chen *et al.*, 2002). Overexpression of Bag-1S in MCF7 cells may also protect cells from growth inhibition induced by a variety of cell stresses such as heat shock, hypoxia, irradiation and treatment with certain chemotherapeutic drugs (Townsend *et al.*, 2003). There is therefore compelling evidence for the role of Bag-1 in the response to a vast variety of cellular stress conditions and as such it is of considerable interest to detemine what role, if any, the

Bag-1 IRES has in maintaining production of the p36 isoform of Bag-1 during these stresses.

Vincristine is a chemotherapeutic agent with multiple effects on cells. Work by Townsend *et al.* (2003) suggested that overexpression of Bag-1 protects cells from apoptosis induced by this chemotherapeutic agent. To confirm that a reduction of total protein synthesis occurs on addition of vincristine, new protein synthesis after vincristine treatment was assessed by incorporation of ^{35}S-methionine. The rate of protein synthesis was measured by ^{35}S-methionine incorporation after a 12 or 24-hour treatment with 4nM vincristine (**figure 6.4A**). No decrease in total protein synthesis was observed after a 12-hour treatment, however a 24-hour treatment decreased total protein synthesis to approximately 55% of the level of untreated cells. This duration of treatment was used for all subsequent vincristine experiments. In addition, the rate of protein synthesis was assessed after a 24-hour treatment with various concentrations of vincristine (**figure 6.4B**). Increasing the concentration of vincristine to a maximum of 4nM final concentration caused a progressive decrease in the level of protein synthesis to around 60% with concentrations ranging from 2-4nM of vincristine. No decrease in protein synthesis was observed with 1nM of vincristine.

To determine whether the Bag-1 IRES is functional in vincristine-treated cells, HeLa cells were transfected with the monocistronic hairpin vector pHpBL or control vectors pHpL or pRL (**figure 6.5A**). 24 hours post-transfection, 1, 2 or 4nM of vincristine was applied to cells. 24 hours later, cells were lysed and assayed for luciferase activity. Cell lysates were also assayed for β-galactosidase, which had been co-transfected as a transfection control, and results are shown normalised to β-

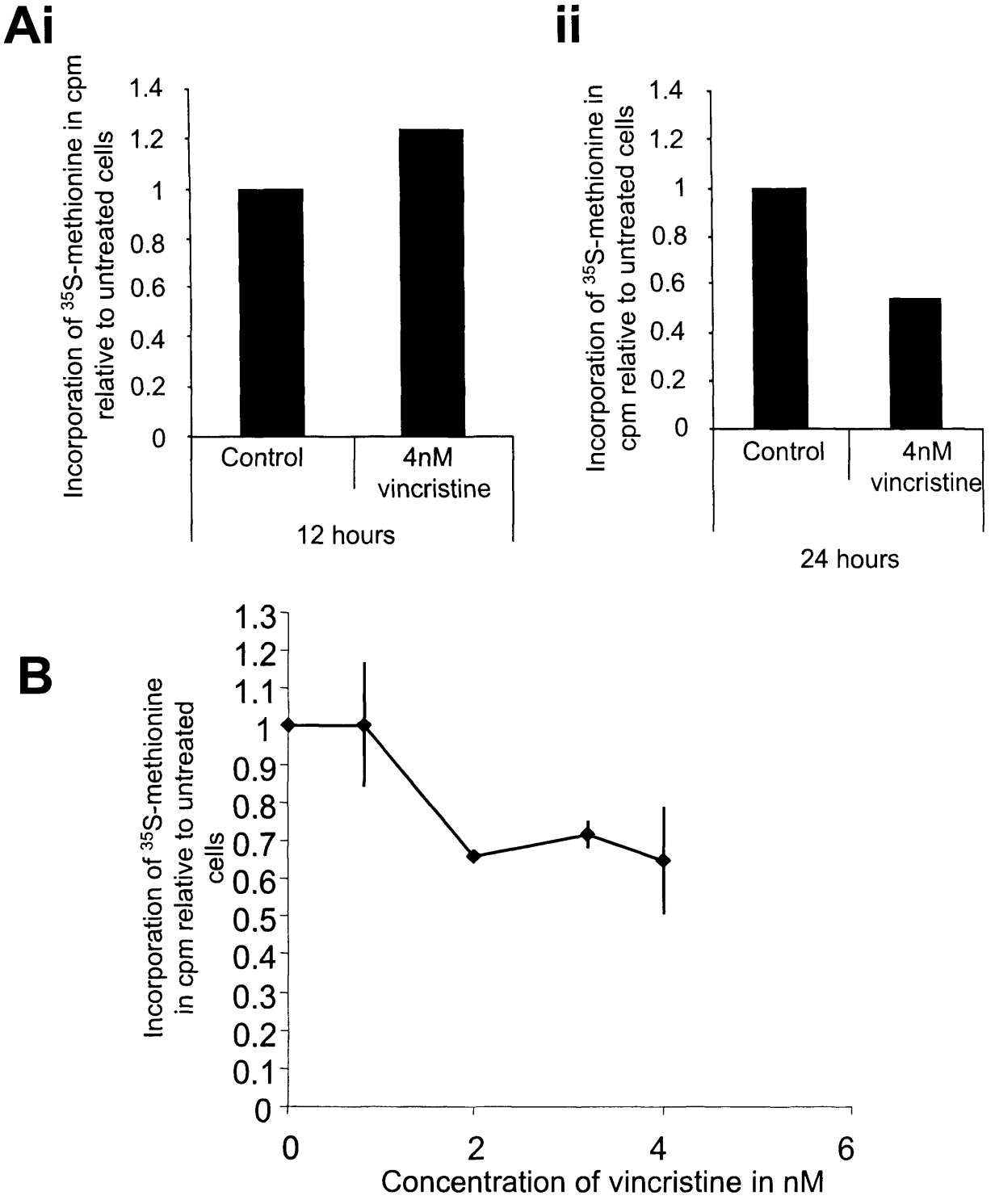


Figure 6.4. The global rate of protein synthesis in HeLa cells decreases with vincristine treatment.

A. The rate of protein synthesis measured by incorporation of ^{35}S-Methionine during a i) 12 or ii) 24 hour treatment of HeLa cells with 4nM of vincristine. The level of protein synthesis was measured in cpm and expressed relative to untreated cells, which were assigned a value of 1.

B. The rate of protein synthesis measured by incorporation of ^{35}S-Methionine during a 24 hour treatment of HeLa cells with varying concentrations of vincristine. The level of protein synthesis was measured in cpm and expressed relative to untreated cells, which were assigned a value of 1

These experiments were performed in duplicate and error bars represent standard deviation.

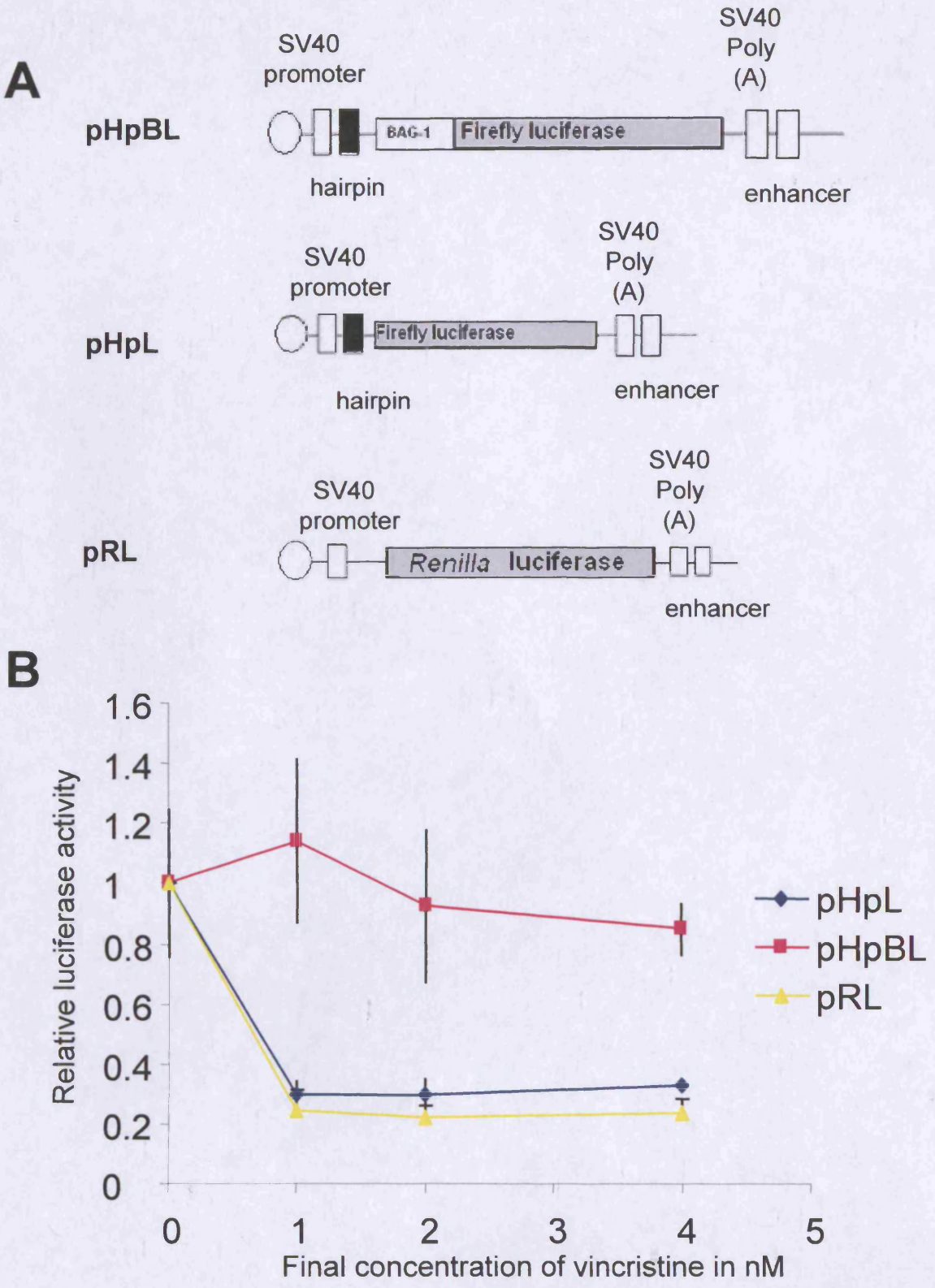


Figure 6.5. The Bag-1 IRES is functional in vincristine-treated cells, when cap-dependent translation is compromised.

A. Schematic diagrams of plasmids transfected, pHpBL, pHpL and pRL

B. HeLa cells transfected with pHpL, pRL or pHpBL were treated with 0, 1, 2 or 4nM of vincristine and cell lysates assayed for luciferase activity. Luciferase activity is normalised to a transfection control of β-galactosidase and expressed relative to the luciferase activity of the same constructs transfected into untreated cells, which are all assigned values of 1. This experiment was performed in duplicate on at least three independent occasions and representative data is shown. Error bars represent standard deviation.

galactosidase (**figure 6.5B**). Luciferase activity from pHpL or pRL decreased dramatically at all concentrations of vincristine applied, to around 20-30% of the level of luciferase observed in untreated cells. This indicates that some ribosomes are able to scan through the hairpin structure and direct translation in the pHpL and pHpBL vectors however, IRES driven translation will be the major mechanism of translation occurring for pHpBL. As such, a slight decrease in IRES activity is observed in cells treated with 2 or 4nM of vincristine but as this constitutes a maximum of 10% decrease in luciferase generated, this could be attributed to the inhibition of cap-dependent scanning. Treatment of cells transfected with pRL with vincristine showed a similar decline of luciferase activity as observed with pHpL, confirming pHpL is being translated by cap-dependent scanning.

Another chemotherapy agent that has been studied in conjunction with Bag-1 is cisplatin (cis-platinum(II)diamine dichloride). Chen *et al.*, 2002 found that all three Bag-1 isoforms protect cells from apoptosis induced by cisplatin in a cervical cancer cell line with low levels of Bag-1, C33A. In contrast, Townsend *et al.*, 2003 found no protection of cisplatin-treated MCF7 cells on overexpression of Bag-1. To test whether the Bag-1 IRES is functional in cisplatin treated cells, HeLa cells were transfected with pHpL or pHpBL, and after 24 hours subjected to a 24-hour treatment with 1,2,4, 10 or 20nM cisplatin **(figure 6.6A)**. A decrease in luciferase activity can be observed from both plasmids and the plasmid containing the Bag-1 IRES does not appear to be translated any more efficiently than the empty vector.

It is possible that there is sufficient Bag-1 protein available in HeLa cells so that the pathways leading to Bag-1 IRES activation are not turned on. Chen *et al.*

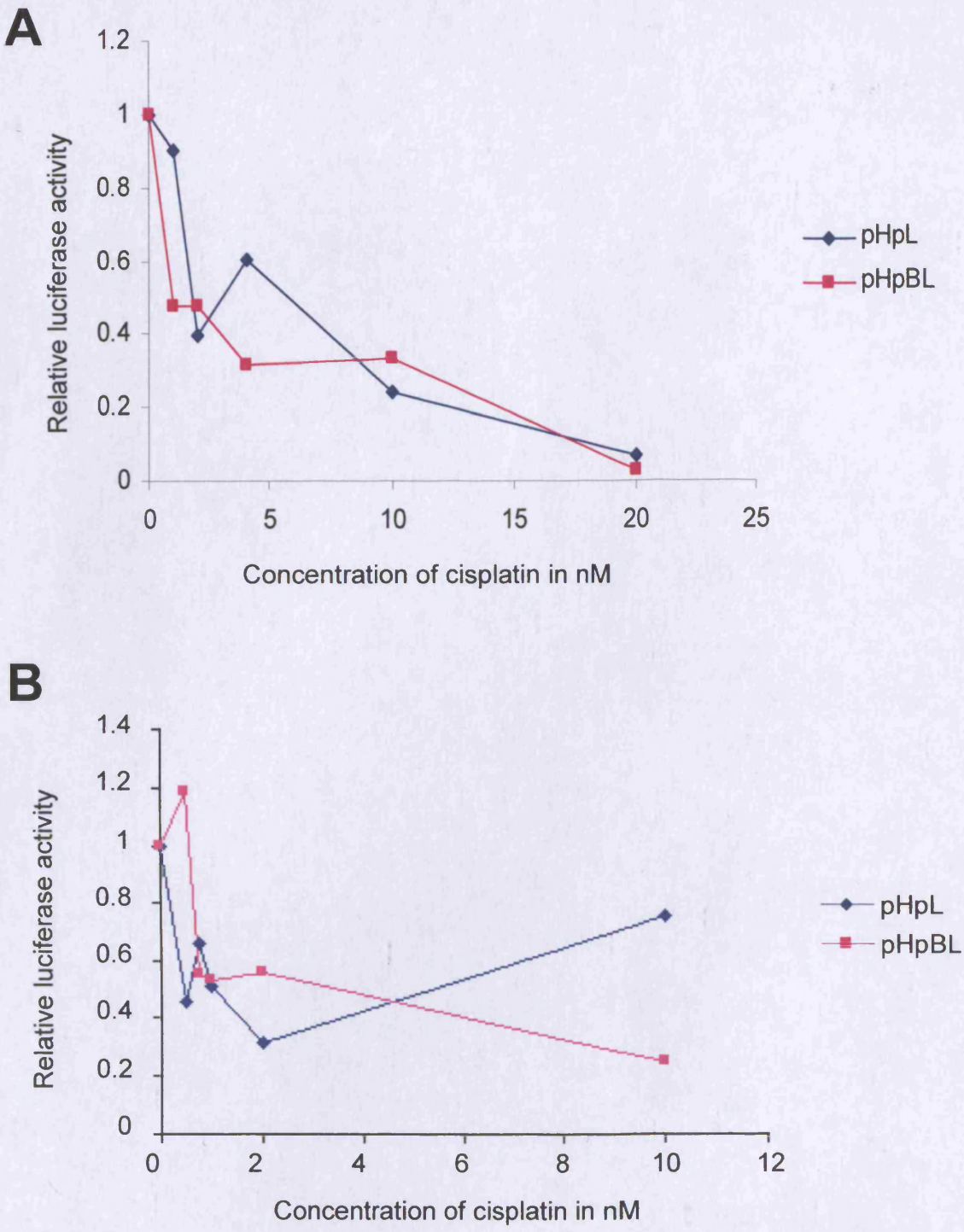


Figure 6.6. The Bag-1 IRES is not functional in cisplatin-treated cells, when cap-dependent translation is compromised.

A. HeLa cells transfected with pHpL or pHpBL were treated with 0, 1, 2, 4, 10 or 20nM of cisplatin and cell lysates assayed for luciferase activity. Luciferase activity is expressed relative to the luciferase activities of the same constructs transfected into untreated cells, which are assigned a value of 1.

B. CAL51 cells were treated with 0.5, 0.75, 1, 2 or 10nM of cisplatin or left untreated and cell lysates assayed for luciferase activity. Luciferase activity is expressed relative to the luciferase activities of the same constructs transfected into untreated cells, which are assigned a value of 1.

(2002) used the cervical cancer cell line C33A, which has low endogenous levels of Bag-1. To exclude this possibility, the experiment was repeated in a cell line with a lower endogenous level of Bag-1. CAL51 cells were transfected with pHpL or pHpBL and 24 hours later subjected to a 24-hour treatment with 0.5, 0.75, 1, 2 or 10nM of cisplatin (**figure 6.6B**). In excess of 0.5nM of cisplatin there is again a comparable decrease in luciferase generated from both pHpL and pHpBL. However, there is no reduction in luciferase generated from pHpBL in the presence of 0.5nM of cisplatin although luciferase generated from pHpL is reduced to approximately 45%. These results suggest the Bag-1 IRES may be functional in the presence of very low concentrations of cisplatin in CAL51 cells, which contain a relatively low level of endogenous Bag-1 protein.

Discussion

It will be useful to repeat these experiments in different cell lines with low endogenous levels of Bag-1 and with a number of other chemotherapeutic agents, for example etoposide, palitaxel and doxorubicin, to try and establish the mechanism by which IRES activity may be maintained. It would be particularly interesting to investigate whether the IRES is functional during palitaxel and doxorubicine treatment as the p36 isoform was not found to protect cells from apoptosis under the conditions used by Chen *et al.*, 2002.

The chemotherapeutic drugs used have multiple functions in the cells and as such it cannot be certain whether the Bag-1 IRES is functioning to prevent apoptosis or whether the IRES is functional due to cells being arrested at specific stages of the

cell cycle when treated with specific chemotherapeutic drugs. Further experiments will be necessary and with other chemotherapeutic agents and inhibitors of various signalling pathways in order to elucidate the signalling mechanisms leading to IRES activity during cell stress conditions and to try and establish which of the many effects of these drugs are influencing pathways leading to Bag-1 IRES activity.

An investigation into the activity of the Bag-1 IRES during the cell cycle suggested the Bag-1 IRES is not functional during mitosis, when cap-dependent translation is reduced (Coldwell, 2001). When HeLa cells are arrested at the G_2/M phase or G_1/S phase, both firefly and *Renilla* luciferase activity of the dicistronic reporter constructs pRF and pRBF were reduced by a comparable level. Further investigation into the two hours between mitosis and the G_1 phase to determine whether the Bag-1 IRES was used to induce expression of the downstream cistron at a faster rate than the upstream cistron again indicated that protein synthesis via the IRES is induced at comparable levels as cap-dependent translation during this phase of the cell cycle. These data indicate that the Bag-1 IRES does not function during mitosis and as such, suggests the IRES may be functional due to apoptotic stimuli induced by vincristine treatment as opposed to cell cycle arrest. However, further experiments will be necessary to determine the mechanisms of Bag-1 IRES induction during cell stress.

6.4. Subcellular localisation of Bag-1 and Bag-1 *trans*-acting factors, PTB and PCBP1, after cell stress

It is possible that IRES elements function efficiently during cell stress despite a reduction in the generally required translation factors as relocalisation of *trans*-acting protein factors may occur. Many of the IRES *trans*-acting factors (ITAFs) identified shuttled between the nucleus and cytoplasm. It is possible that a relocalisation of these proteins to the cytoplasm may be required for activation of IRESes (Michael *et al.*, 1997, Makeyev and Liebhaber 2002). Many proteins have been shown to relocalise to different subcellular compartments during cell stress, for example the p46 isoform of Bag-1 has been shown to move into the nuclear compartment during cell stress (Zeiner *et al.*, 1999, Niyaz *et al.*, 2001)

Utilising monoclonal Bag-1 and PTB antibodies and polyclonal PCBP1 antibodies, indirect immunofluorescence was carried out on control cells and cells that had been treated with chemotherapeutic agents in order to assess the localisation of Bag-1 and Bag-1 *trans*-acting factors during chemotoxic stress. Approximately 1 X 10^5 HeLa cells were plated into chamber slides and treated with 4nM of vincristine, 4nM of cisplatin or maintained under control conditions for 24 hours. Cells were fixed using 50:50 acetone: methanol and proteins bound by addition of primary antibody. The localisation of the proteins was determined using fluorescently labelled secondary antibodies and fluorescence microscopy.

6.4.1. Localisation of Bag-1 proteins after chemotoxic stress

In control cells, Bag-1 proteins are predominantly localised to the cytoplasm with a significant amount of staining in the nucleus (**figure 6.7A**). This follows as Bag-1S is predominantly cytoplasmically localised, Bag-1L, nuclear and Bag-1M shuttles between the compartments (Packham *et al.*, 1997; Schneikert *et al.*, 1999, Zeiner *et al.*, 1999). The monoclonal antibody used recognises all three isoforms of Bag-1. Upon vincristine treatment there is a significant redistribution of Bag-1 proteins predominantly into the nucleus, as can be observed from co-localisation with DAPI staining of the DNA (**figure 6.7B**). Again, on addition of the chemotoxic agent cisplatin there is a significant redistribution of Bag-1 protein to the nucleus (**figure 6.8**).

6.4.2. Localisation of Bag-1 *trans*-acting factors PTB and PCBP1 after chemotoxic stress

PTB is a nuclear protein which is thought to be involved in splicing and as such is almost exclusively nuclearly localised during normal cellular circumstances (Kamath *et al.*, 2001)(**figure 6.9Aii**), however a very significant cytoplasmic redistribution is observed on treatment of the cells with vincristine (**figure 6.9Bii**). This would localise PTB to the appropriate compartment for chaperoning translation of Bag-1, perhaps allowing greater efficiency of IRES-driven translation under these circumstances.

In contrast to PTB, PCBP1 has a more even distribution between the cytoplasm and nucleus in control cells, supporting the theory that PCBP1 shuttles

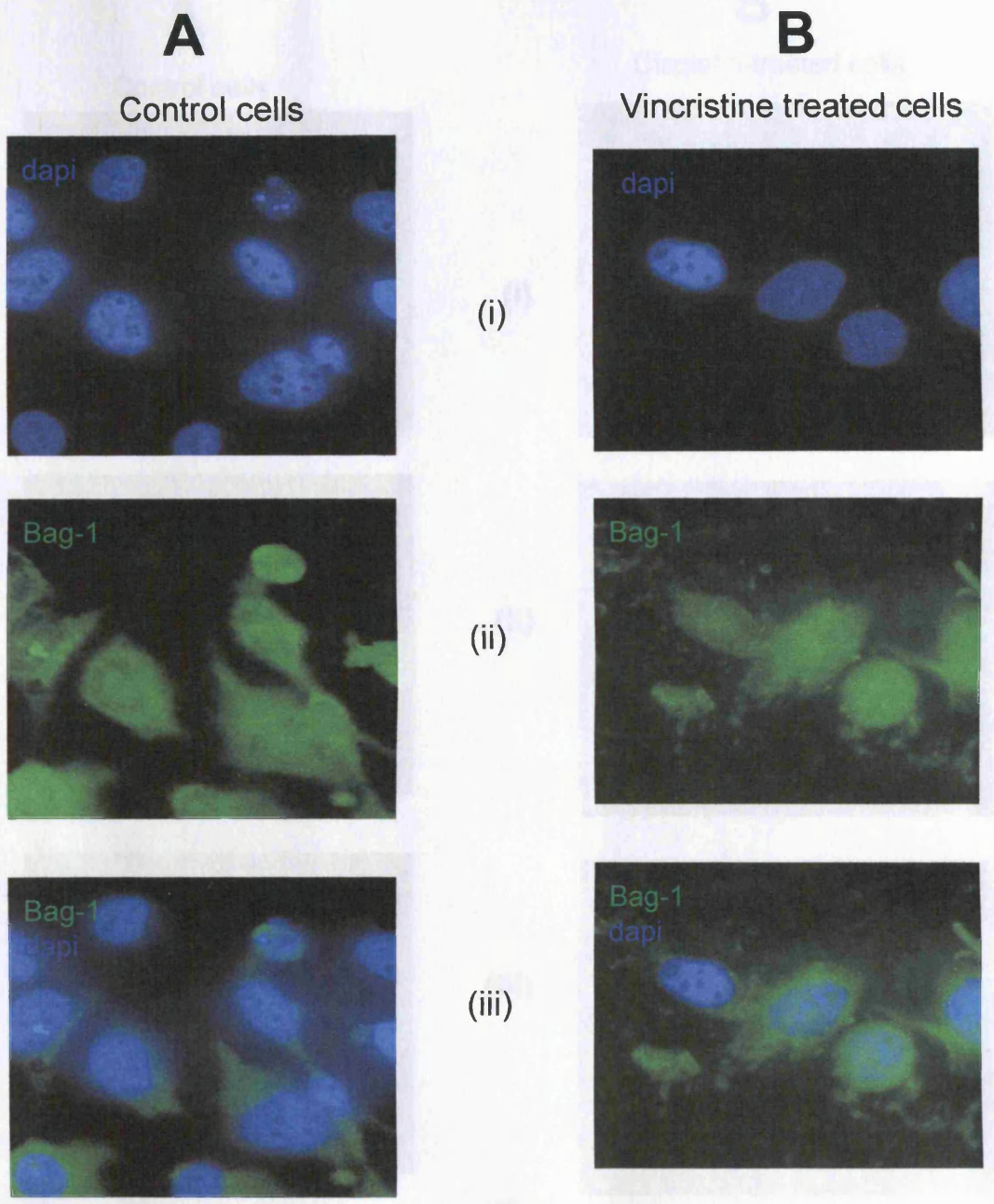


Figure 6.7. There is an increase in nuclear localisation of Bag-1 on vincristine treatment.

HeLa cells were treated with 4nM vincristine for 24 hours or left untreated. After treatment, cells were subjected to indirect immunofluorescence with anti-Bag-1 antibody and a FITC-conjugated secondary antibody. DNA in the nuclei is visualised with Dapi.

A. Control cells. i) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1.

B. Vincristine-treated cells. I) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1

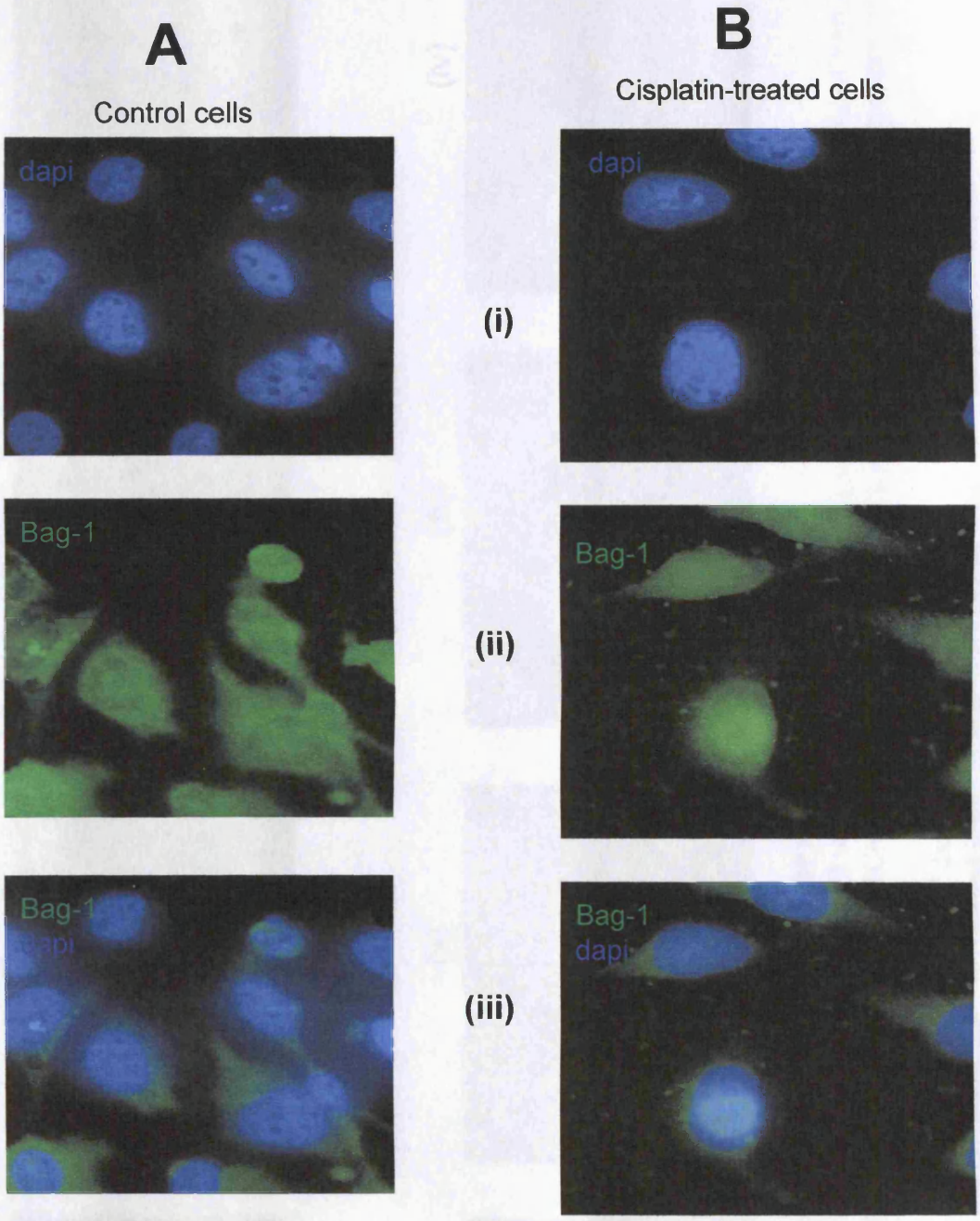


Figure 6.8. There is an increase in nuclear localisation of Bag-1 on cisplatin treatment.

HeLa cells were treated with 4nM cisplatin for 24 hours or left untreated. After treatment, cells were subjected to indirect immunofluorescence with anti-Bag-1 antibody and a FITC-conjugated secondary antibody. DNA in the nuclei is visualised with Dapi.

A. Control cells. i) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1. The control cells panels are reproduced from **figure 6.7** as these treatments were performed in parallel.

B. Vincristine-treated cells. I) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1.

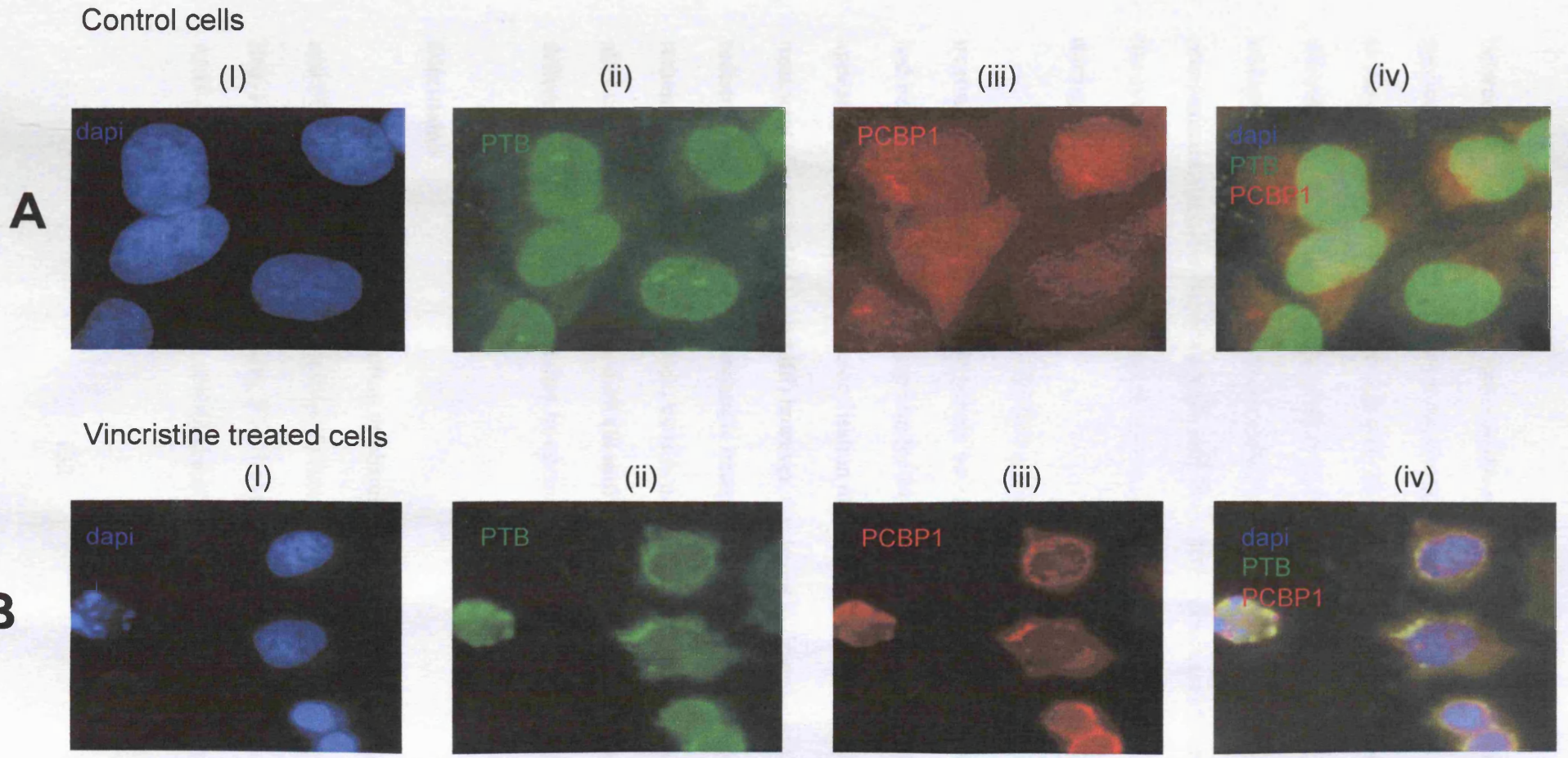


Figure 6.9. There is an increase in co-localisation of PTB and PCBP1 on vincristine treatment.

HeLa cells were treated with 4nM vincristine for 24 hours or left untreated. After treatment, cells were subjected to indirect immunofluorescence with anti-PTB and anti-PCBP1 antibodies and FITC- and TRITC-conjugated secondary antibodies respectively. Nuclei were visualised by staining with dapi.

A. Control cells. i) dapi, ii) PTB, iii) PCBP1, iv) merged image of dapi, PTB and PCBP1

B. Vincristine-treated cells. i) dapi, ii) PTB, iii) PCBP1, iv) merged image of dapi, PTB and PCBP1

between the cytoplasm and nucleus (Leffers *et al.,* 1995)(**figure 6.9Aiii**). Again, on application of a 24-hour vincristine treatment, PCBP1 shows a dramatic redistribution to the cytoplasm (**figure 6.9Biii**). Merging the images of PTB and PCBP1 after vincristine treatment shows apparently complete co-localisation of the two proteins, whereas there is some degree of co-localisation in control cells but this is predominantly nuclear (**figure 6.9Aiv and Biv**). Both proteins therefore co-localise in the cytoplasmic compartment where they may enhance Bag-1 IRES-driven translation during cell stress conditions.

To test whether PTB and PCBP1 also relocalise as a result of cisplatin treatment, indirect immunofluorescence was carried out on control cells and cells that had been treated with 4nM of cisplatin for 24 hours prior to fixing of the cells. There appears to be some degree of relocalisation of PTB to the cytoplasm after cisplatin treatment (**figure 6.10Aii and Bii**) however, this is not as dramatic as the redistribution observed after vincristine treatment and a large proportion of PTB remains in the nucleus. In addition, there is no significant relocalisation of PCBP1 after cisplatin treatment (**figure 6.10Aiii and Biii**). These results suggest that a different signalling mechanism may be operating as a result of cisplatin treatment.

Discussion

Elucidation of the signalling mechanisms that lead to redistribution of PTB-1 and PCBP1 would give valuable insights into the pathways that lead to regulation of Bag-1 IRES activity. Interestingly, PTB-1 can be phosphorylated by the 3', 5'-cyclic AMP-dependent protein kinase (PKA) at a site close to the nuclear localisation signal;

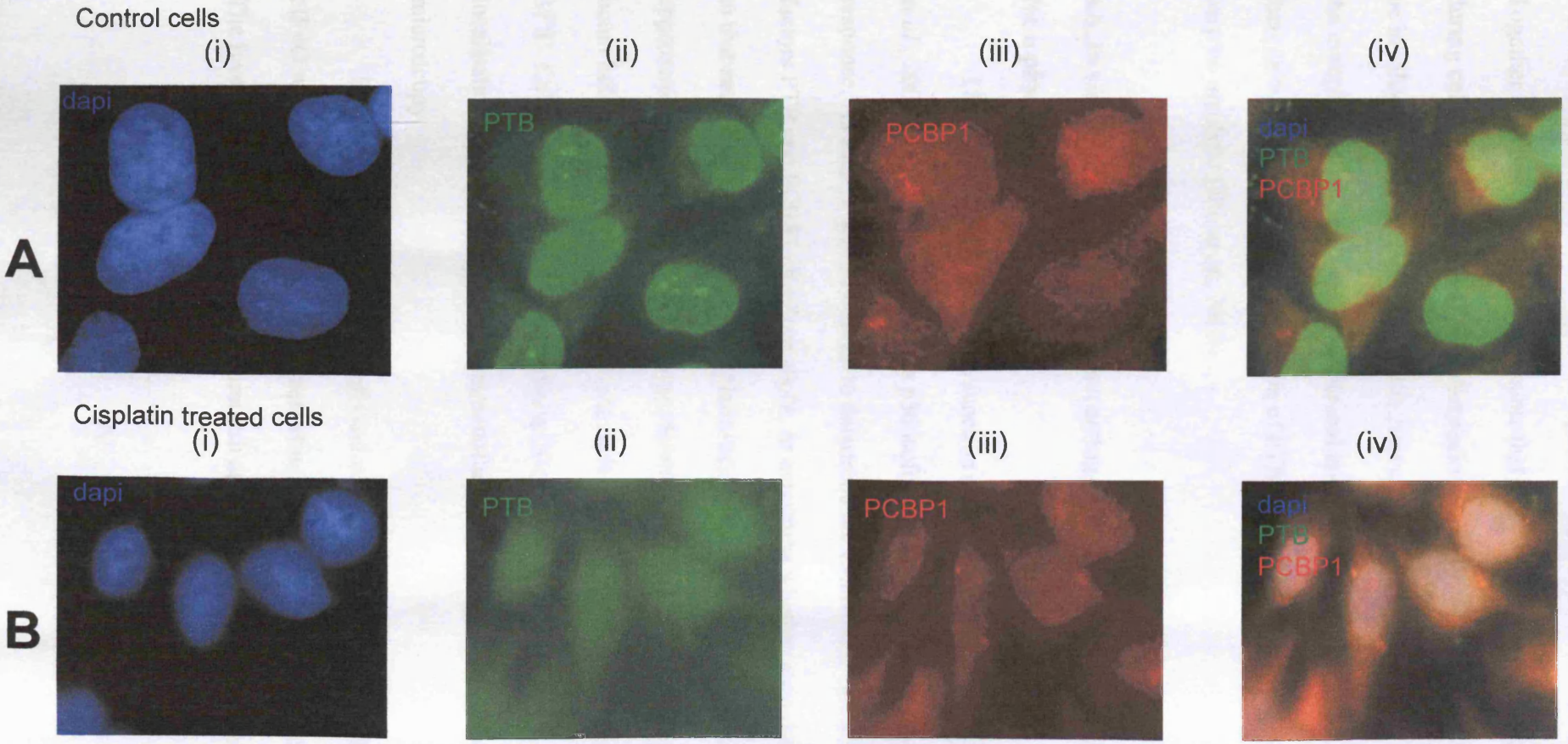


Figure 6.10. There is an increase in co-localisation of PTB and PCBP1 on cisplatin treatment.

HeLa cells were treated with 4nM cisplatin for 24 hours or left untreated. After treatment, cells were subjected to indirect immunofluorescence with anti-PTB and anti-PCBP1 antibodies and FITC- and TRITC-conjugated secondary antibodies respectively. Nuclei were visualised by staining with dapi..

A. Control cells. i) dapi, ii) PTB, iii) PCBP1, iv) merged image of dapi, PTB and PCBP1. Note that control cell panels are reproduced from **figure 6.9** as these treatments were carried out in parallel.

B. Cisplatin-treated cells. i) dapi, ii) PTB, iii) PCBP1, iv) merged image of dapi, PTB and PCBP1

this causes a relocalisation of the PTB-1 protein to the cytoplasm (Xie *et al.*, 2003). Together with this evidence, it is possible that PTB-1 relocalises to the cytoplasm during cell stress as a result of phosphorylation. An alternative mechanism that may be involved in re-distribution of PTB is cleavage of the protein. PTB re-distributes to the cytoplasm after polioviral infection and apoptosis (Back *et al.*, 2002), however there is no increase in phosphorylation of PTB suggesting cleavage by viral proteases may be involved (Xie *et al.*, 2003)

6.5. *In vivo* localisation of IRES-interacting proteins after heat shock – evidence for a physiological role.

The Bag-1 IRES is known to function efficiently during heat shock (Coldwell *et al.*, 2001), indicating a role for the p36 isoform of Bag-1 in the heat shock response. As such, it was of interest to determine the localisation of the *trans*-acting factors PTB and PCBP1 after heat shock, to determine whether a similar mechanism to that seen in vincristine but not cisplatin-treated cells is operating. Thus, approximately 1 X 10^5 HeLa cells were plated into chamber slides and grown for 24 hours before being subjected to a 30-minute heat shock at 44°C or maintained at 37°C. Cells were immediately fixed using 50:50 acetone: methanol and protein localisation determined by indirect immunofluorescence and fluorescence microscopy.

In order to confirm that the cells had undergone heat shock, the localisation of eIF4G was determined. eIF4G is sequested into granules by hsp27 during heat shock. The levels of eIF4G protein do not diminish as it is subsequently released during

recovery to allow rapid recovery from heat shock. Thus, in cells maintained at 37°C eIF4G shows a predominantly cytoplasmic distribution with some nuclear staining (**figure 6.11A**). After a 30 minute heat shock, there is decreased nuclear staining and the cytoplasmic staining has a granulated appearance (**figure 6.11B**). This suggests that the treatment applied is sufficient to cause cell stress.

Bag-1 proteins show an increase in nuclear localisation after heat shock (**figure 6.12**), as observed with vincristine treatment, supporting the theory that Bag-1 proteins are taken into the nucleus after cell stress. PCBP1 and PTB again show a dramatic redistribution to the cytoplasm (**figures 6.13 and 6.14 respectively**), indicating that a signalling mechanism is operating during cell stress to redistribute these proteins into the cytoplasm. There is again a dramatic increase in co-localisation of these proteins, allowing them to function additively in stimulation of the Bag-1 IRES (**figure 6.15**). It is not yet known whether these proteins interact and act co-operatively in enhancing IRES activity but this would be a good area for future study.

6.6. Discussion

Conditions of cell stress are very rapidly accompanied by a down-regulation of protein synthesis, generally through cleavage, sequestration or alterations in the phosphorylation state of translation initiation factors. This is a vital reaction for the cell as it serves to prevent accumulation of misfolded proteins and allows rapid recovery from cell stress.

mRNAs that can be translated by internal ribosome entry have been found in a large number of key regulatory genes and their products have subsequently been

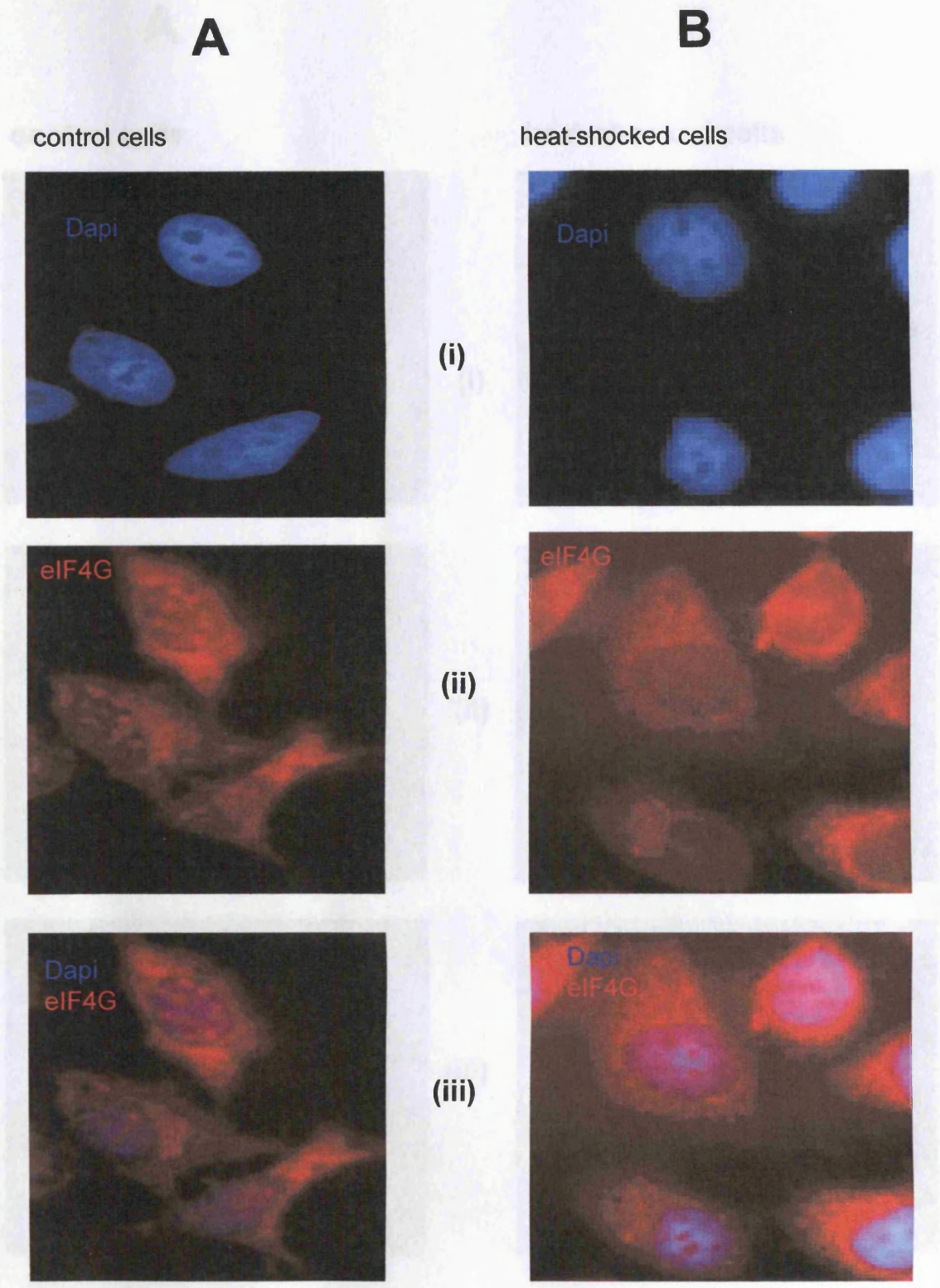


Figure 6.11. eIF4G is sequested into granules by hsp27 during heat shock

HeLa cells were heat-shocked treated to 44°C for 30 minutes prior to fixing or were maintained at 37°C before being fixed and subjected to indirect immunofluorescence with an anti-eIF4G antibody and a TRITC-conjugated secondary antibody. The nuclei were visualised by staining with dapi.

A. Control cells, i) dapi, ii) eIF4G, iii) merged image of dapi and eIF4G.

B. Heat-shocked cells, i) dapi, ii) eIF4G, iii) merged image of dapi and eIF4G

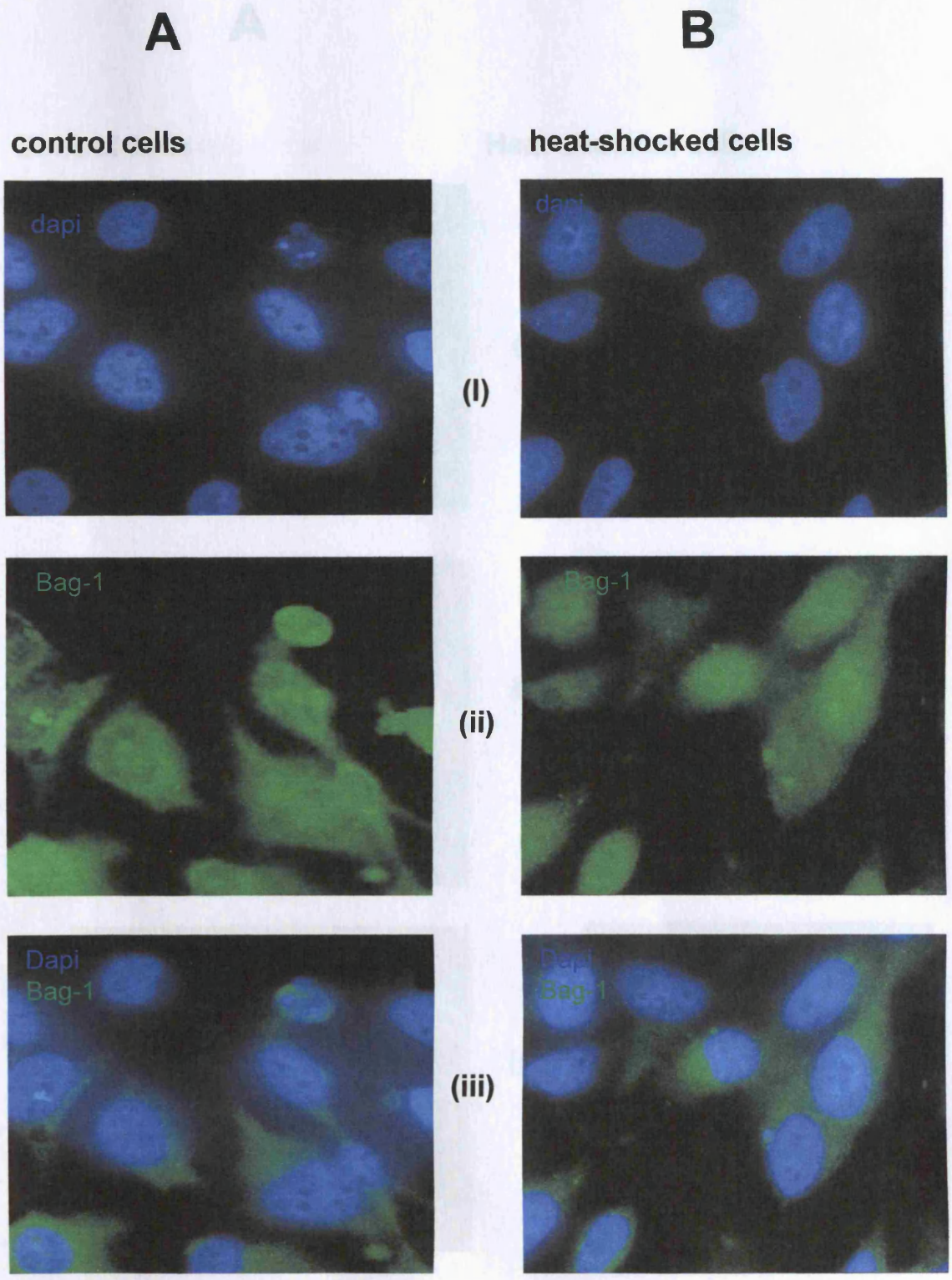


Figure 6.12. Bag-1 shows increased nuclear localisation on heat-shock treatment

HeLa cells were heat-shocked treated to 44°C for 30 minutes prior to fixing or were maintained at 37°C before being fixed and subjected to indirect immunofluorescence. The nuclei were visualised by staining with Dapi.

A. Control cells. i) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1. Note that the control cell panels are reproduced from **figure 6.7** as these treatments were performed in parallel.

B. Heat-shocked cells. i) dapi, ii) Bag-1, iii) merged image of dapi and Bag-1.

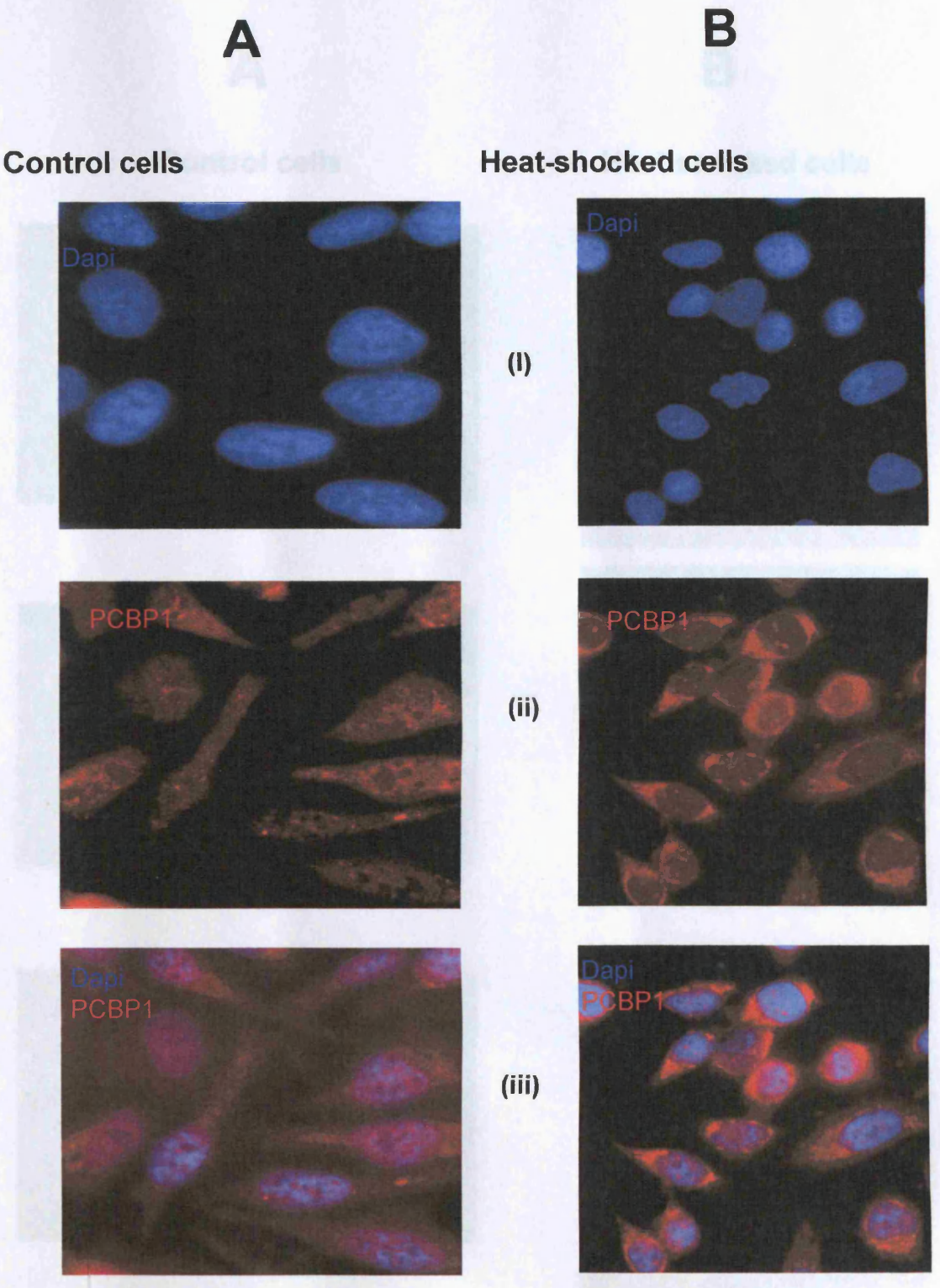


Figure 6.13. PCBP1 is re-distributed to the cytoplasm on heat-shock treatment

HeLa cells were heat-shocked treated to 44°C for 30 minutes prior to fixing or were maintained at 37°C before being fixed and subjected to indirect immunofluorescence with an anti-PCBP1 antibody and TRITC-conjugated secondary antibody. The nuclei were visualised by staining with Dapi.

A. Control cells. i) dapi, ii) PCBP1, iii) merged image of dapi and PCBP1.

B. Heat-shocked cells. i) dapi, ii) PCBP1, iii) merged image of dapi and PCBP1

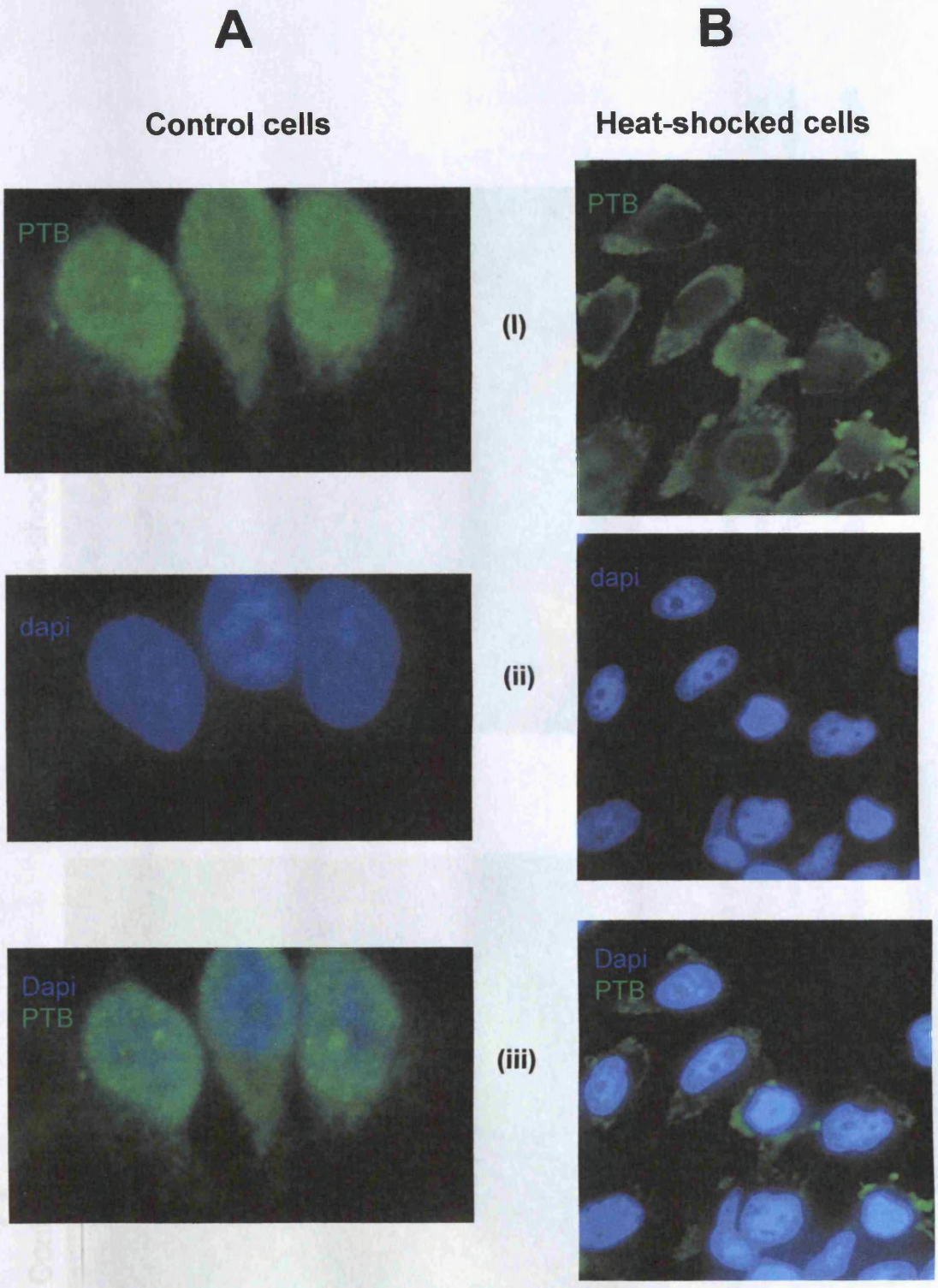


Figure 6.14. PTB is re-distributed to the cytoplasm on heat-shock treatment

HeLa cells were heat-shocked treated to 44°C for 30 minutes prior to fixing or were maintained at 37°C before being fixed and subjected to indirect immunofluorescence with an anti-PTB antibody and FITC-conjugated secondary antibody. The nuclei were visualised by staining with Dapi.

A. Control cells. i) dapi, ii) PTB, iii) merged image of dapi and PTB.

B. Heat-shocked cells. i) dapi, ii) PTB, iii) merged image of dapi and PTB

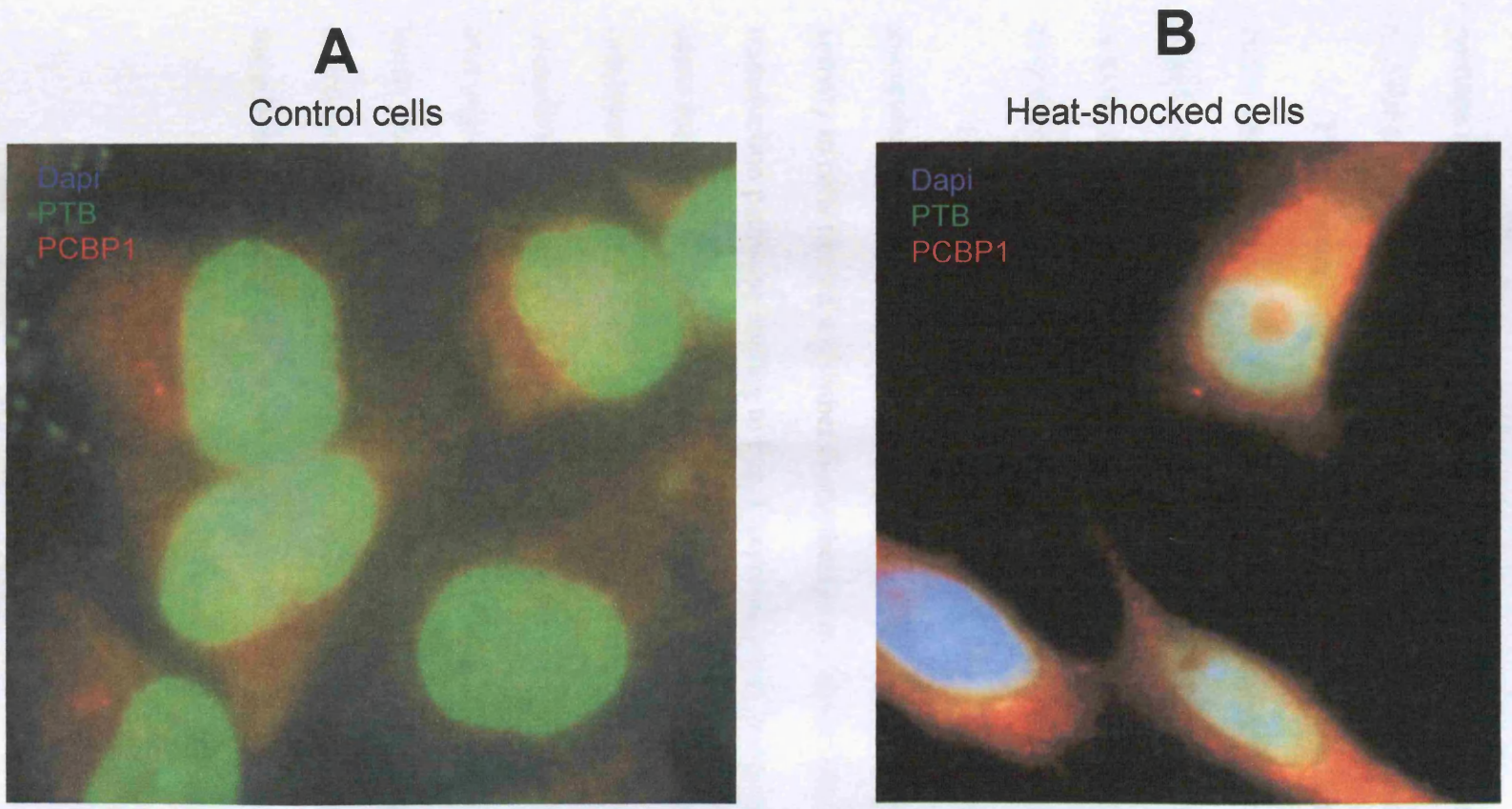


Figure 6.15. There is increased co-localisation of PTB and PCBP1 after heat-shock treatment

HeLa cells were heat-shocked treated to 44°C for 30 minutes prior to fixing or were maintained at 37°C before being fixed and subjected to indirect immunofluorescence with anti-PTB and anti-PCBP1 antibodies and FITC- and TRITC-conjugated secondary antibodies respectively. Nuclei were visualised by staining with dapi..

A. Control cells, merged image of dapi, PCBP1 and PTB. Note that control cell panels are reproduced from **figure 6.9** as these treatments were carried out in parallel.

B. Heat-shocked cells, merged image of dapi, PCBP1 and PTB

found to be translated during conditions of cell stress that require such proteins. The data presented here indicates that the Bag-1 IRES may also be used to maintain translation of Bag-1 under conditions of cell stress in addition to heat shock. This is perhaps not surprising given the multifunctional role of Bag-1 in a large number of cellular processes where it appears to be a key regulatory protein.

The discovery that PTB and PCBP1 are re-distributed into the cytoplasm under conditions of cell stress provides some rationale for the ability of these proteins to activate the Bag-1 IRES, perhaps contributing to greater efficiency of IRES activity under conditions where IRES-driven translation is operating exclusively and may alternatively be impeded by the reduction in canonical initiation factors.

There are numerous other cellular stress conditions under which it would be interesting to investigate the role of the Bag-1 IRES such as investigation of IRES activity in cells treated with other chemotherapeutic agents; elucidation of the signal transduction pathways leading to Bag-1 expression and the specific events which cause the IRES to remain active is of particular interest. In addition, cell stress conditions such as hypoxia may potentially identify other roles for Bag-1. There is some evidence that Bag-1 may be involved in protecting cells against hypoxia in that overexpression of Bag-1S appears to protect cells from apoptosing under hypoxic stress (Townsend *et al.,* 2003). With that in mind, it is interesting to note that expression of PCBP1 is induced under hypoxic stress; this induction is mediated by the p38 mitogen-activated kinase pathway (Zhu *et al.*, 2002).

Chapter 7

Discussion

7.1. Mechanism of action of cellular IRESes

Viral IRESes, particularly those of the picornaviridae have been extensively studied in recent years. This has allowed subdivision into classes within which similarity of protein factor requirements and secondary structure is observed. As yet, studies of cellular IRESes are preliminary and no structural comparisons can be drawn. All appear to require *trans*-acting factors but the proteins required vary for each IRES.

The knowledge that a large number of cellular IRESes studied so far appear to function during specific conditions when cap-dependent translation is compromised is indicative that common mechanisms of IRES activity may be elucidated for IRESes that function during the same cellular circumstances, perhaps with structural features and *trans*-acting factor requirements in common. This study has elucidated the mechanism of action of the Bag-1 IRES and indicates that this IRES may function during cellular circumstances where cap-dependent translation is inhibited, in addition to heat shock.

7.2. A secondary structural model of the Bag-1 IRES.

Relatively little work has been undertaken so far towards the elucidation of the secondary structure of cellular IRESes. Studies of viral IRESes are greatly simplified by the availability of numerous strains of any particular species, allowing sequence conservation and covariation to contribute towards the derivation of a secondary structural model. For some cellular genes, sequence of

the 5'UTR is available for numerous species allowing sequence alignments to be produced which were used in the derivation of the secondary structural model of the c-*myc* IRES (Le Quesne, 2000), however it has not been proven that all species contain a conserved IRES. In the case of the Bag-1 IRES, full sequences for the 5'UTR are only available for murine and human species, both of which contain a functional IRES (Coldwell, 2001), consequently sequence alignments are of relatively little use in constructing a secondary structural model.

Deletion analysis refined the boundaries of the Bag-1 IRES to a relatively short region of the 5'UTR, 225-411. Failure to reduce this region further is indicative that structural motifs are required for Bag-1 IRES-driven translation. It was originally proposed that short primary sequence motifs may be important for Bag-1 IRES driven translation, given that complex secondary structures may impede translation of the longer isoforms by the cap-dependent scanning mechanism. This is not the case, and indeed the majority of cellular IRESes also appear to be translated by the cap-dependent scanning mechanism, for example Apaf-1 and c-*myc*, despite the presence of IRESes made up of complex secondary structural motifs (Le Quesne *et al.,* 2001; Mitchell *et al.,* 2003).This suggests the secondary structural motifs involved, while inhibitory to scanning, do not preclude it.

The Bag-1 IRES comprises a central domain, including the ribosome-landing site, which may have complex structure including tertiary structures given the inhibitory nature of this region to progression of the reverse transcriptase enzyme. Given the preliminary model for this region, it will now be possible to perform mutational analysis and amputation of particular domains in order to assess their importance for IRES-driven translation. Review of the activity of

deletion mutants in light of the secondary structural model goes some way towards this analysis, showing the importance of the central region for IRES-driven translation. Mutations in this region effectively abolish IRES-driven translation, although some of these mutations encroach on the ribosome-landing site. Care must be taken with the design of future mutations, with the introduction of compensatory mutations to assess whether the structure or sequence of motifs in this region are important for IRES activity.

7.3. Protein factor requirements of the Bag-1 IRES

The Bag-1 IRES has a number of mechanistic features in common with other cellular IRESes; it fails to function in the rabbit reticulocyte lysate *in vitro* translation system, has cell type specificity according to the cell line transfected and requires a nuclear event prior to IRES activity being observed. All of these features are indicative of a definitive requirement for the appropriate cellular environment for IRES activity.

The classes of viral IRESes elucidated have distinct requirements for *trans*-acting factors, with class I IRESes functioning inefficiently in the rabbit reticulocyte lysate *in vitro* translation system but class II IRESes capable of maintaining translation in this system. If viral and cellular IRESes operate through similar mechanisms, the *trans*-acting factors required to stimulate viral IRES activity may also stimulate cellular IRESes. In support of this, there is increasing evidence that several *trans*-acting factors regulate both viral and cellular IRESes (**table 1.4**). This does not preclude the possibility that additional factors may contribute to cellular IRES function, distinct from those that regulate viral IRES-driven translation. Considering the Apaf-1 IRES, unr and PTB have been found to

stimulate IRES activity synergistically; however, they cannot completely restore IRES activity to optimal levels when supplied in cell lines deficient in these factors, indicating that further factors may be required.

Two Bag-1 IRES *trans*-acting factors, PTB and PCBP1, were identified from a pool of viral *trans*-acting factors, supporting the theory that viral and cellular IRESes may share a common pool of *trans*-acting factors. None of the other proteins tested were found to activate the Bag-1 IRES specifically although it is possible they may stimulate IRES activity in combination with another, as yet unknown, *trans*-acting factor. PTB and PCBP1 were found to stimulate IRES activity only in cell lines with low endogenous levels of these factors, and as such these factors contribute to the cell type specific activity of the Bag-1 IRES. Interestingly, PTB and PCBP1 enhance Bag-1 IRES activity but are not sufficient for optimal IRES activity to be observed. MCF7 cells have relatively high levels of PTB and PCBP1 and IRES activity in this cell line cannot be significantly enhanced by overexpression of these proteins however, IRES activity is around 2.5 fold lower than that observed in HeLa cells, suggesting other factors must also be contributing to IRES activity.

Attempts to identify further Bag-1 IRES *trans*-acting factors from cell lines have met with some success. IRES activity can be enhanced dramatically in the rabbit reticulocyte lysate *in vitro* translation system, to levels comparable with those observed *in vivo*, by the addition of placental cell extract, a rich source of RNA-binding proteins. Isolation of proteins from this extract that can bind specifically to the Bag-1 IRES has revealed at least four candidate *trans*-acting factors, two of which are comparable in size to PTB and PCBP1. Isolation of these proteins and an assessment of their effect on IRES activity in functional assays

will be necessary to determine whether these proteins act as Bag-1 *trans*-acting factors. Therefore, much work remains to identify the proteins required for Bag-1 IRES activity and also, to elucidate the nature of the 'nuclear event' in order to fully understand the cell-type specific activity and the mechanism by which the Bag-1 IRES functions.

7.4. *Trans*-acting factors modulate the secondary structure of the Bag-1 IRES

Although a large number of *trans*-acting factors have been identified, which influence the activity of numerous IRESes, the mechanisms by which they function to regulate IRES activity are poorly understood. A few examples exist whereby the roles of *trans*-acting factors have been deduced. The poly C binding proteins PCBP1 and PCBP2 appear to function by stabilizing secondary structure. Both proteins have a role in stabilizing mRNAs through binding to 3'UTRs, but have also been shown to stabilise structural elements of the polioviral IRES (Murray *et al.*, 2001). Recently, it was deduced that PTB and unr bind to the Apaf-1 IRES and modulate its structure to allow ribosome binding (Mitchell *et al.*, 2003).

PTB and PCBP1 were both shown to bind specifically to regions within the minimal Bag-1 IRES element, suggesting they are directly influencing the Bag-1 IRES. Analysis of the secondary structure of the Bag-1 IRES in the presence of these proteins showed a clear difference in the structure in the region of protein binding. Both proteins bind in the central region of the IRES where the structure is difficult to define as the reverse transcriptase enzyme pauses at numerous sites, making it impossible to determine whether certain bases are single-or double-stranded. As reverse transcriptases are subject to premature termination in highly

structured regions, it is possible that tertiary interactions in this area may contribute to the inhibition of enzyme progression observed. Alternatively, the structure in this region may be flexible and take up numerous conformations. In which case, a potential role for *trans*-acting factors would be stabilization of the optimal structure. This does not appear to be the case for the Bag-1 IRES.

PTB and PCBP1 modulate the secondary structure of the Bag-1 IRES to open up a single-stranded region including the ribosome-landing region. The ribosome requires a single-stranded region in which to land and thus it appears that PTB and PCBP1 function by facilitating ribosome recruitment.

It may be that the structure in this region is indeed flexible in the absence of PTB and PCBP1 and consequently addition of these proteins stabilises the 'open' conformation, producing a single stranded region. Alternatively, this region may be highly structured and PCBP1 and PTB could consequently enhance ribosome recruitment by producing a single-stranded landing site.

7.5. The Bag-1 IRES maintains production of Bag-1 during certain cell stresses.

Bag-1 transcripts can be translated by two mechanisms in the case of the p36 isoform, cap-dependent scanning and internal initiation, contributing around 70% and 30% to translation of this isoform respectively in HeLa cells under normal cellular circumstances (Coldwell, 2001). Some cellular IRESes are used exclusively to direct translation of their protein product during all cellular circumstances in which cases it appears that the highly structured 5'UTR cannot be traversed by a scanning ribosome. This is the situation for the L-*myc*, which is translated exclusively by internal ribosome entry (Jopling, 2001). The majority of

IRES-containing genes, however, can be translated by both cap-dependent scanning and internal initiation suggesting the IRESes serve an alternative purpose. Many cellular IRESes appear to exist in order to maintain production of essential proteins under cellular circumstances during which cap-dependent translation is compromised.

The Bag-1 IRES has previously been shown to maintain production of Bag-1 during heat shock and subsequent recovery, with the p36 isoform the only isoform produced during heat shock, when cap-dependent translation is inhibited (Coldwell *et al.*, 2001). There was also some indication that the Bag-1 IRES may function during the early stages of apoptosis, in correlation with its role as an anti-apoptotic protein acting at the early stages of apoptosis. There is, however, insufficient evidence to substantiate this as a role for Bag-1 IRES-mediated translation. Further experiments with a range of apoptotic stimuli and treatment durations will be required to address this possibility.

Overexpression of any isoform of Bag-1 has been shown to protect cells from growth inhibition caused by treatment with chemotherapeutic drugs (Chen *et al.*, 2002; Townsend *et al.*, 2003). When cap-dependent translation is inhibited by treatment of HeLa cells with vincristine, the Bag-1 IRES has been shown to be functional, suggesting that maintenance of production of the p36 isoform may have a role in protecting cells against certain chemotherapeutic agents. Analysis of the effects of other chemotherapeutic drugs on Bag-1 IRES activity has so far proved inconclusive. If the Bag-1 IRES is shown to retain activity during treatment with a range of chemotherapeutic drugs, it will be possible to begin to elucidate the signalling pathways leading to maintenance of IRES activity. The ERK survival signalling pathway has been shown to over-ride apoptotic signals by

upregulating anti-apoptotic BCl-2 proteins through transcription dependent mechanisms and has recently been shown to maintain production of Bag-1 mRNA (Perkins *et al.*, 2003). Using specific inhibitors to this pathway and other signalling pathways it will be possible to deduce the mechanism by which Bag-1 IRES-driven translation is maintained. The c-*myc* IRES has been shown to maintain c-*myc* expression following DNA damage induced by chemotherapeutic agents and the proteins responsible were found to lie upstream of the p38 mitogen-activated protein kinase (MAPK) and/or signal-regulated protein kinase (ERK)/MEK (MAPK/ERK kinase) signalling pathways (Subkhankulova *et al.*, 2001).

7.6. Regulation of IRES activity by availability of *trans*-acting factors.

IRES-driven translation can be regulated through the availability of *trans*-acting factors. This is similar to cap-dependent translation in that it is regulated by availability of initiation factors. The availability of *trans*-acting factors may be limited due to the presence of low endogenous levels of the requisite factors but there are several other ways in which the availability of such factors can be limited. Firstly, the *trans*-acting factors identified so far tend to have diverse cellular roles in addition to influencing IRES activity. PTB has a role in regulating alternative splicing. PCBP1 has been shown to bind to a range of mRNAs and enhance their stability during certain cellular circumstances. RNA-binding proteins associated with nuclear mRNAs (hnRNPs) play an important role in the control of post-transcriptional events (Adinolfi *et al.*, 1999). In addition, there may be competition for *trans*-acting factors between a number of cellular IRESes.

PTB, for example, enhances the IRES activity of several cellular IRESes including those of Apaf-1, IGF-IR and Bag-1.

The availability of *trans*-acting factors may also be limited by their localisation. The majority of *trans*-acting factors identified are predominantly nuclear in location; however, they may be redistributed under certain cellular circumstances. PTB can be phosphorylated by the 3', 5'-cyclic AMP-dependent protein kinase (PKA) at a site close to the nuclear localisation signal; causing a relocalisation of PTB to the cytoplasm (Xie *et al.*, 2003). PTB can also be cleaved by virally encoded proteases after polioviral infection and during apoptosis (Back *et al.*, 2002). Both events result in a redistribution of PTB to the cytoplasm.

The carboxyl-terminal cleavage fragments of PTB produced from cleavage by picornaviral proteases or caspases during apoptosis have been shown to inhibit translation from the polioviral IRES, whereas full-length PTB is required for polioviral IRES-driven translation (Back *et al.*, 2002). Modulation of *trans*-acting factors may cause them to differentially regulate IRES-driven translation.

This study shows that PTB and PCBP1 both accumulate in the cytoplasm following heat shock and following treatment with the chemotherapeutic drug vincristine. The mechanism by which these proteins are relocalised to the cytoplasm has not yet been elucidated but it is plausible that cell stress events may cause phosphorylation of these proteins and subsequent relocalisation. Nonetheless, the redistribution of these proteins increases their cytoplasmic concentration, placing them in proximity to assist with Bag-1 IRES-driven translation. This and the evidence presented above, suggests that IRES-mediated translation may be regulated under cell stress conditions, when cap-dependent

translation is inhibited, by increased availability and/or modulation of *trans*-acting factors.

It has also been shown that there is hypoxic upregulation of PCBP1 expression (Zhu *et al.*, 2002). It is therefore possible that some *trans*-acting factors may be upregulated during cell stress conditions, again altering their availability. Overexpression of Bag-1 has been shown to protect cells from cell death induced by hypoxia (Townsend *et al.*, 2003). Given the increased levels of PCBP1 during hypoxia it would be of interest to determine whether the Bag-1 IRES is functional.

It is becoming increasingly evident that availability and modulation of *trans*-acting factors may present a stringent level of regulation of IRES-driven translation under different cellular circumstances and in different cell types. The study of the mechanism of action of *trans*-acting factors in light of secondary structural models of IRES elements will also allow progression towards a better understanding of cellular IRES function. Given the potentially oncogenic nature of many genes regulated by IRES-driven translation, knowledge of the mechanism of IRES-driven translation may provide a crucial first step towards regulating the expression of these genes for the purpose of cancer therapy.

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Polypyrimidine tract binding protein and poly r(C) binding protein 1 interact with the BAG-1 IRES and stimulate its activity *in vitro* and *in vivo*

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ABSTRACT

The 5′-untranslated region of Bag-1 mRNA contains an internal ribosome entry segment (IRES) and the translation of Bag-1 protein can be initiated by both cap-dependent and cap-independent mechanisms. In general, cellular IRESs require non-canonical *trans*-acting factors for their activity, however, very few of the proteins that act on cellular IRESs have been identified. Proteins that interact with viral IRESs have also been shown to stimulate the activity of cellular IRESs and therefore the ability of a range of known viral *trans*-acting factors to stimulate the Bag-1 IRES was tested. Two proteins, poly r(C) binding protein 1 (PCBP1) and polypyrimidine tract binding protein (PTB), were found to increase the activity of the Bag-1 IRES *in vitro* and *in vivo*. The regions of the Bag-1 IRES RNA to which they bind have been determined, and it was shown that PCBP1 binds to a short 66 nt section of RNA, whilst PTB interacts with a number of sites over a larger area. The minimum section of the RNA that still retained activity was determined and both PCBP1 and PTB interacted with this region suggesting that these proteins are essential for Bag-1 IRES function.

INTRODUCTION

The human Bag-1 gene (Bcl-2 associated athanogene) encodes three major isoforms generated from a single transcript, p50, p46 and p36 (BAG-1L, BAG-1M and BAG-1S respectively) that differ at their N-termini. BAG-1L, which initiates from a non-canonical CUG codon, contains an SV40-like nuclear localisation signal (NLS) at its N-terminus, and this is thought to be responsible for the mainly nuclear distribution of this isoform (1). The AUG-initiated isoforms, BAG-1M and BAG-1S, lack this NLS and as such are localised predominantly cytoplasmically (1,2). The variation in the N-termini of the Bag-1 proteins also leads to different protein binding specificities and the isoforms have diverse cellular roles. Bag-1 was originally identified as RAP46 (receptor associated protein) through its interaction with the glucocorticoid receptor (3) and has been found to associate with numerous other members of the steroid hormone receptor superfamily. It was also documented as HAP46 (Hsc70/Hsp70 associated protein) through its interaction with the 70 kDa heat-shock proteins (4,5) where it has a role as a co-chaperone in the protein folding response (6–8). The murine homologue, Bag-1, was identified through its interaction with Bcl-2, an anti-apoptotic gene (9). Bag-1 has been shown to interact with Bcl-2 and to promote the anti-apoptotic properties of this protein, blocking a step in the apoptotic pathway (10).

It was originally suggested that the three main isoforms of Bag-1, in addition to a minor isoform, p29, are translated by leaky scanning (1), however, expression of Bag-1 *in vivo* does not support this hypothesis (1,2,11). Recently we have demonstrated that the p36 isoform of Bag-1 can be translated by internal ribosome entry through use of an internal ribosome entry segment (IRES) in addition to the cap-dependent scanning mechanism (11). IRES elements are used to initiate translation under conditions where cap-dependent scanning is compromised (12) and our data suggest that the IRES of Bag-1 is required to maintain translation of the p36 isoform following heat shock (11).

The internal ribosome entry mechanism of translation was first identified in picornaviruses and these have been widely studied in terms of structure, mechanisms and *trans*-acting factor requirements (13). Viral IRESs vary widely in their dependence on *trans*-acting factors. For example, some viral IRESs such as the encephalomyocarditis virus are able to function well *in vitro* (14,15), in contrast, other viral IRESs such as polio virus or the human rhino virus (HRV) require the addition of extracts derived from HeLa cells to *in vitro* systems before they are active (16–18). In the case of HRV, two proteins that are required have been identified, upstream of N-ras (unr) (19), and polypyrimidine tract binding protein (PTB) (20). A number of additional viral IRES binding/activating proteins have been identified, including the La autoantigen which is used by polio virus IRES (21), and poly r(C) binding protein 2 (PCBP2) which binds to polio virus IRES (22) and has been shown to activate entero/rhino virus IRESs *in vitro* (23). These proteins are thought to act as RNA chaperones to either maintain or aid the RNA to form a structure that is competent for ribosome recruitment.

Recently, a large number of cellular IRESs have been identified but the mechanisms by which they initiate translation are poorly understood (12). The protein factor

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requirements for cellular IRESs are much less well defined although data produced thus far would suggest that each IRES has a requirement for a specific set of *trans*-acting factors. First, cellular IRESs show considerable cell tropism and this would suggest that levels of endogenous *trans*-acting factors vary between cell lines (24–26). Secondly, the proteins that interact with two cellular IRESs studied so far are different, thus the Apaf-1 IRES requires PTB and unr for function *in vitro* and *in vivo* (27), whilst the XIAP IRES has a requirement for La (28).

In this study the protein factor requirements for efficient Bag-1 IRES activity has been investigated. We demonstrate a direct and specific interaction of PTB and poly r(C) binding protein 1 (PCBP1) with the Bag-1 IRES, which stimulates IRES function both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmid constructs

The plasmids pRF and pRBF harbouring deletion segments of Bag-1 are described in Figures 1A, 2A and 4A. The plasmid pSKL is based upon the vector pSK+bluescript (Stratagene); the Bag-1 5′-untranslated region (5′-UTR) or deletions were cloned into this vector in frame with the firefly luciferase gene (Figs 3A and 5A). Deletion fragments were generated by PCR using specific primers to the regions required. For expression of proteins used, the cDNAs were present in PET28a vectors, enabling expression of protein in *Escherichia coli* and subsequent purification of the protein. For expression in tissue culture cells, the cDNAs were subcloned into pCDNA3.1 and for expression in insect cells (for purification of protein) subcloned into pBlueBac4 (Invitrogen).

Protein expression

Proteins were overexpressed in *E.coli* from the pET28a vector by the addition of isopropyl-β-D-thiogalactopyranoside to the growth medium. The proteins that contained a His tag were purified using a nickel affinity column (Qiagen). Alternatively, unr was purified from cultures of Sf9 cells that had been infected with a recombinant baculovirus expressing unr-His (Invitrogen). Cells were harvested and lysed in phosphate-buffered saline containing 0.1% Triton X-100, and the tagged protein purified on a nickel affinity column.

Cell culture and transient transfections

Cells were typically grown in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal calf serum, under humidified atmosphere containing 5% CO_2. The cell lines CALU1, CAL51 and CAMA1 were a kind gift from Dr G. Packham (CRUK Unit, Southampton, UK). MCF7 and HeLa were originally purchased from ATCC. Cells were transfected using FuGene 6 (Roche) as specified by the manufacturer. Alternatively, calcium phosphate-mediated transfections were performed as described, with minor modifications (29). Lysates were prepared from transfected cells using 1× passive lysis buffer. Firefly and *Renilla* luciferase activities were measured using the 'Stop and glo' dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions with the exception that only 25 µl of each reagent was used. Light emission was measured over 10 s using an OPTOCOMP I luminometer. Activity of the β-galactosidase transfection control was measured using a Galactolight Plus assay system (Tropix). All transfections were carried out in triplicate on at least three independent occasions.

In vitro transcription reactions

Vector DNA was linearised by restriction digestion using a site downstream of the region of interest (*HpaI* for dicistronic, *NcoI* for monocistronic); transcripts were synthesised in a reaction mixture containing 1× transcription buffer [40 mM HEPES–KOH (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 10 mM NaCl], 40 U RNAguard or RNasin, 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, 1 µM $m^7G(5')ppp(5')G$, 1 µg of DNA template and 20 U T7 or T3 RNA polymerase to a final volume of 50 µl. For radiolabelled RNAs, CTP was replaced with 50 µCi [α-^{32}P]CTP. The reaction mixture was incubated at 37°C for 1.5 h and the RNA purified.

In vitro translation reactions

The Promega rabbit reticulocyte flexi-lysate *in vitro* translation system was primed with 5 ng/µl RNA and used according to the manufacturer's instructions. The reaction was performed in a final volume of 12.5 µl and 0.1 µg of each protein was added where appropriate. Luciferase activities were assayed as described above, and the firefly and *Renilla* values expressed relative to the control plasmid pRF, which was assigned a value of 1. All experiments were performed in triplicate on at least three independent occasions.

UV-crosslinking analysis

Radiolabelled transcript was generated from pSKBL linearised with *NcoI*. Approximately 2.5 pmol per reaction was incubated with 0.25 µg of protein in 1× UV-crosslinking buffer [10 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100 mM KCl, 5 mM creatine phosphate, 1 mM DTT, 1 mM ATP, 6% glycerol, 0.1 µg/µl tRNA] for 15 min at room temperature. For competition assays, unlabelled competitor RNAs were added with labelled RNA. The reaction mixtures were UV irradiated using a 305 nm UV light source for 30 min on ice. RNase A and RNase V1 (0.2 mg/ml) were added to the mixture to degrade any unprotected RNA by incubation at 37°C for 30 min. Sample buffer was added and the samples separated on a 10% polyacrylamide gel by SDS–PAGE. Gels were dried at 80°C under vacuum for 2 h and analysed on a Molecular Dynamics Phosphorimager.

Electrophoretic mobility shift assays (EMSAs)

Approximately 20 pmol of RNA was incubated with protein as appropriate in a buffer mix containing 40 U RNAguard, 2 µl of 5× transcription buffer [200 mM Tris–HCl (pH 8.0), 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl, 50 mM DTT, 15 µg tRNA], and 2 µl of 10 mM ATP in a reaction volume of 15 µl for 10 min at room temperature. DNA loading dye was added (50% sucrose, 0.1 M EDTA, 0.2% bromophenol blue) and samples loaded onto 0.7× Tris-borate-EDTA (TBE) agarose gels. Samples were electrophoresed at 100 V for ~3 h in 1× TBE loading buffer. All buffers and loading dyes were

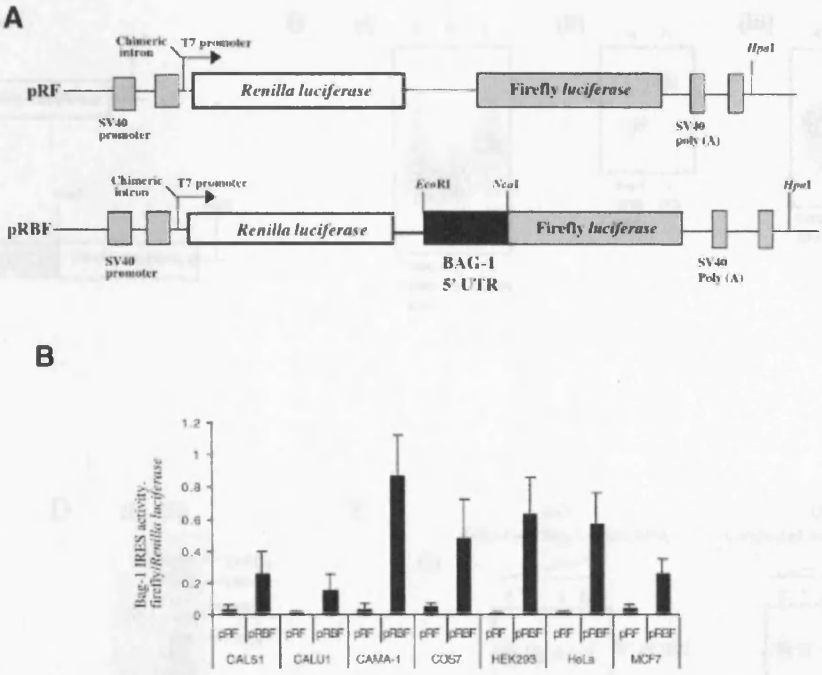

Figure 1. A comparison of the efficiency of Bag-1 IRES-mediated translation in cell lines of different origin. (**A**) Schematic representation of the dicistronic reporter constructs pRF and pRBF where pRBF contains the Bag-1 5′-UTR inserted into the vector pRF and fused in-frame with the firefly luciferase gene. (**B**) The plasmids pRF and pRBF were transfected into the cell lines indicated. CAL51, CAMA-1 and MCF7s are of human breast carcinoma origin; CALU1, human lung cancer; COS7, a monkey epithelial cell line (CV-1) immortalised with SV40 DNA; HEK293, a human embryonic kidney cell line immortalised with adenovirus; and HeLa S3, of human cervical epitheloid carcinoma origin. IRES activity was expressed as the ratio of downstream cistron expression to upstream cistron expression (firefly/*Renilla* luciferase), with any differences in transfection efficiencies corrected for using the β-galactosidase transfection control (PJ7*lacZ*). Error bars indicate standard deviations as determined from at least three independent experiments performed in triplicate.

filter-sterilised. The gels were dried under vacuum at 60°C for 2 h and exposed on a phosphoimager.

RESULTS

Comparison of Bag-1 IRES-mediated internal initiation between cell types

The majority of cellular IRESs studied thus far show considerable cell tropism in that they do not work efficiently in all cell types (24–26). This is presumably because the expression of specific IRES *trans*-acting factors varies between cell lines (24–26). The ability of the Bag-1 IRES to function in a variety of cell lines was tested by transfecting HeLa, COS7, HEK293, MCF7, CAL51, CALU1 and CAMA1 cell lines with the dicistronic construct pRBF or the control construct pRF. The expression from both *Renilla* and firefly luciferase cistrons was assayed and normalised to the transfection control β-galactosidase. The efficiency of the IRES is represented as a ratio of firefly luciferase to *Renilla* luciferase expression from pRBF (Fig. 1A). As expected, the Bag-1 IRES showed a wide range of activities in different cell types and firefly luciferase activity was found to vary considerably according to cell line (Fig. 1B). The Bag-1 IRES was highly active in CAMA-1 and active in HeLa cells, COS7 and HEK293 cells, yet relatively inactive in MCF7, CAL51 and CALU1 cells (Fig. 1B). The expression of the Bag-1 IRES in these cell lines differed from that observed with the c-*myc* IRES (25) and the Apaf-1 IRES (30), which would again suggest that cellular IRESs have different requirements for *trans*-acting factors.

PCBP1 and PTB bind to the Bag-1 IRES

EMSAs were performed to identify putative Bag-1 IRES *trans*-acting factors by using a range of known IRES interacting proteins including PCBP1 and PCBP2 (22), PTB (20), DAP5 (31,32), La (28) and unrip (19). Radiolabelled Bag-1 IRES RNA was generated from *in vitro* transcription reactions primed with DNA derived from the monocistronic constructs (Fig. 2A); the resulting RNA was incubated with protein and the products separated on 0.5% TBE agarose gels. Only when BAG-1 IRES RNA was incubated with PTB or PCBP1 was a decrease in the mobility of the RNA observed (Fig. 2B and B.M.Pickering and A.E.Willis, unpublished data), suggesting both of these proteins bind the IRES directly. No difference in mobility of Bag-1 IRES RNA was observed with the other proteins tested, for example La (Fig. 2B, i, lane b). To test the specificity of this interaction, the proteins were also incubated with a non-specific RNA segment from glyceraldehyde-3-phosphate dehydrogenase (G3PDH) of approximately the same size. No alterations in mobility of this RNA were observed with any of the proteins tested (Fig. 2B, iii). To confirm this interaction, UV-crosslinking analysis was performed. Thus, radiolabelled Bag-1 IRES RNA was incubated with PCBP1 and/or PTB, samples were

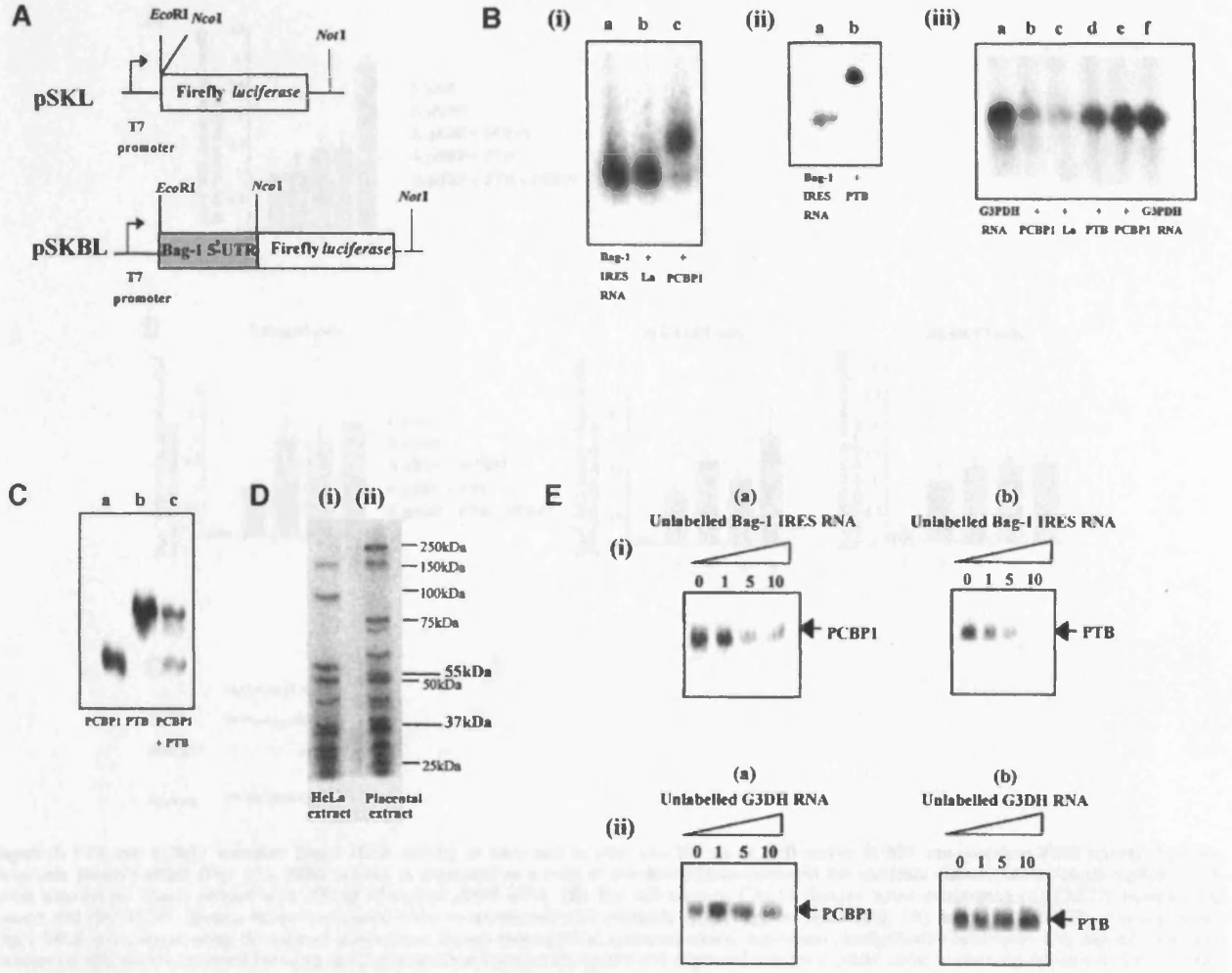

Figure 2. PTB and PCBP1 bind directly to Bag-1 IRES RNA. (**A**) Schematic diagram of the monocistronic plasmids pSKL and pSKBL where pSKBL contains the Bag-1 5′-UTR fused in-frame with the luciferase gene. (**B**) (i and ii) EMSAs of radiolabelled Bag-1 IRES RNA alone (i and ii, lane a) and in combination with 0.2 μg of protein (i, lane b), La (i, lane c PCBP1). A gel retardation is observed with PTB and PCBP1, suggesting complex formation, but not with La. (iii) Control EMSAs were carried out with non-specific radiolabelled RNA of the same size from G3PDH (lanes a and f, G3PDH RNA alone). No gel retardation is observed with 0.2 μg of any protein tested (lanes b–e). (**C**) UV-crosslinking assay of radiolabelled Bag-1 IRES RNA in combination with 0.2 μg of PTB and/or PCBP1, again showing both proteins binding to Bag-1 RNA. (**D**) UV-crosslinking assay of radiolabelled Bag-1 IRES RNA with cell extracts. (Lane i) HeLa extract and (lane ii) placental extract. Bands corresponding in size to PTB (55 kDa) and PCBP1 (37 kDa) can be observed in both lanes, marked in bold type. Sizes and position of markers are indicated in plain type. There is also a marker at 37 kDa. (**E**) (i) UV-crosslinking assay of radiolabelled Bag-1 IRES RNA in combination with (a) 0.2 μg of PCBP1, where binding is competed by the addition of a 5× molar excess of unlabelled Bag-1 IRES RNA and (b) 0.2 μg of PTB, where binding is competed by the addition of an equimolar quantity of unlabelled Bag-1 IRES RNA. (ii) UV-crosslinking assay of radiolabelled Bag-1 RNA in combination with (a) 0.2 μg of PCBP1 or (b) 0.2 μg of PTB, where there is no competition observed with a 10× molar excess of unlabelled G3DH RNA.

exposed to UV light, any RNA not bound to protein digested with RNases and the products separated by PAGE. Both PTB and PCBP1 either singly or in combination interacted with Bag-1 IRES RNA (Fig. 2C). Crosslinking analysis was also undertaken with cell extracts made from HeLa or placenta. Thus, radiolabelled Bag-1 IRES RNA was incubated with HeLa or placental extracts, exposed to UV light and unbound RNA was digested with RNases. The products were separated by PAGE. A number of proteins that interact with the Bag-1 IRES were identified in both extracts with sizes of approximately 150, 55, 45, 37, 30 and 29 kDa in addition to some extract-specific proteins (Fig. 2D). The 55 and 37 kDa proteins are the same size as PTB and PCBP1, respectively. The other proteins remain to be identified.

To determine the specificity of the interaction between the Bag-1 IRES and these proteins, UV-crosslinking experiments were performed in the presence of excess unlabelled Bag-1 IRES RNA or G3PDH mRNA (Fig. 2E). Both proteins bind specifically to the Bag-1 IRES. Hence, there was a reduction in the binding of protein to the radiolabelled transcripts with a 1-fold molar excess of unlabelled BAG-1 IRES RNA, but not with a 10-fold molar excess of G3PDH RNA (Fig. 2E, i and ii).

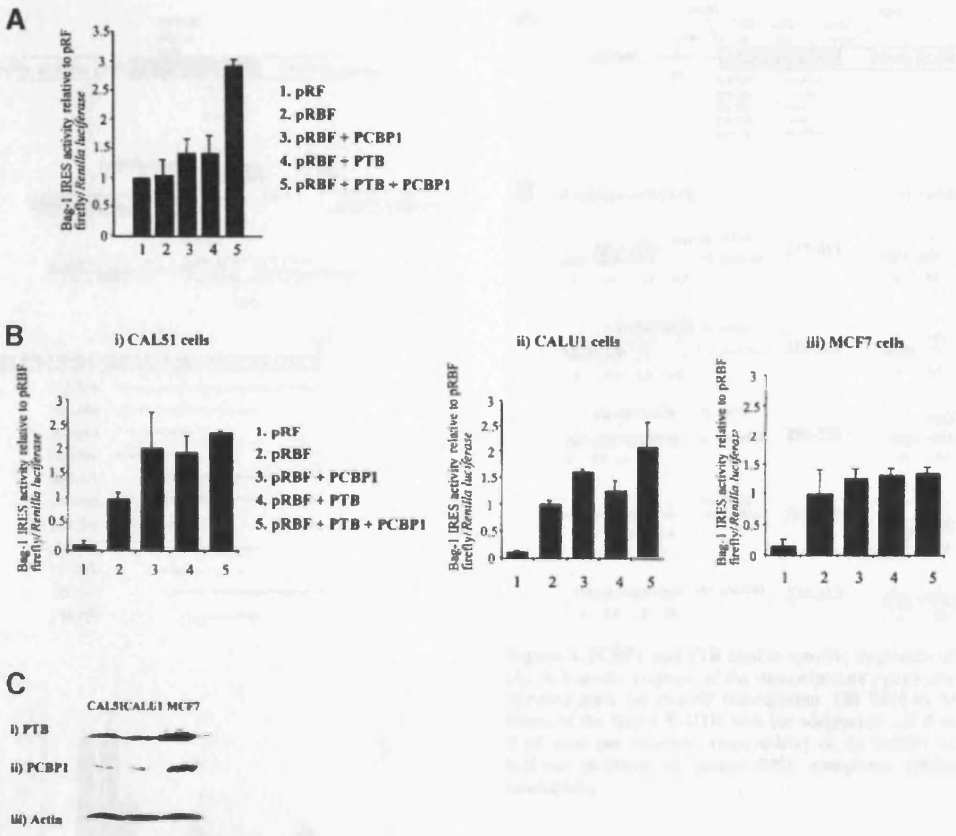

Figure 3. PTB and PCBP1 stimulate Bag-1 IRES activity *in vitro* and *in vivo*. (**A**) 200 ng of PTB and/or PCBP1 can stimulate IRES activity from the dicistronic plasmid pRBF (Fig. 1A). IRES activity is expressed as a ratio of the downstream cistron to the upstream cistron (firefly/*Renilla* luciferase), in rabbit reticulocyte lysates primed with 100 ng of capped pRBF RNA. (**B**) The cell lines (i) CAL51 (human breast carcinoma), (ii) CALU1 (human lung cancer) and (iii) MCF7 (human breast carcinoma) were co-transfected with plasmids containing the pRBF (Fig. 1A) and/or PCBP1/PTB. Activity of the Bag-1 IRES is expressed using the ratio of downstream cistron expression to upstream cistron expression (firefly/*Renilla* luciferase) with any differences in transfection efficiencies corrected for using the β-galactosidase transfection control and expressed relative to pRBF alone to show the increase in IRES activity produced by each *trans*-acting factor. Error bars indicate standard deviations as determined from at least three independent experiments performed in triplicate. (**C**) Western blots of cell lysates for endogenous protein levels with (i) anti-PTB antibody, (ii) anti-PCBP1 antibody and (iii) anti-actin antibody as a loading control show a correlation with the level of activation shown by transfection of each protein in each cell line.

Crosslinking analysis was also performed with La, unrip, DAP5 and PCBP2 but no protected RNA was detected (data not shown).

PCBP1 and PTB stimulate the Bag-1 IRES *in vitro* and *in vivo*

In general, cellular IRESs work very inefficiently (if at all) *in vitro*, but we have shown that it is possible to stimulate certain cellular IRESs by the addition of known viral *trans*-acting factors (27). The activity of the Bag-1 IRES in a dicistronic RNA (generated from pRBF and pRF; Fig. 1A) was tested in the rabbit reticulocyte lysate system with the addition of PCBP1 and PTB. The Bag-1 IRES functioned very inefficiently *in vitro* and no appreciable luciferase activity was detected over that produced from RNA derived from the vector pRF which does not contain an IRES (Fig. 1A). However, activation was observed with PTB and PCBP1, when added singly, each stimulating IRES activity to 1.4-fold (Fig. 3A). Moreover, addition of PCBP1 and PTB had an additive effect in combination producing 3-fold stimulation of the IRES (Fig. 3A).

To test whether these proteins could stimulate the function of the Bag-1 IRES *in vivo*, co-transfections were carried out using pRBF with plasmids expressing PCBP1 or PTB either singly or in combination into the cell lines which showed low Bag-1 IRES activity (Fig. 1). Thus, transfection of CAL51 and CALU1 with either PTB or PCBP1 alone had a stimulatory effect, which was additive when the plasmids containing DNA encoding these proteins were transfected in combination (Fig. 3C, i and ii). In contrast, transfection of MCF7s with PTB and/or PCBP1 did not significantly stimulate Bag-1 IRES activity (Fig. 3C, iii). To test whether there was a correlation between the expression of PTB/PCBP1 and IRES function western analysis was performed and cell lysates were immunoblotted for PTB, PCBP1 or actin as a loading control (Fig. 3D). There is a good correlation between endogenous protein levels and activation of the IRES by these proteins. For example, MCF7s which are not stimulated by co-transfection

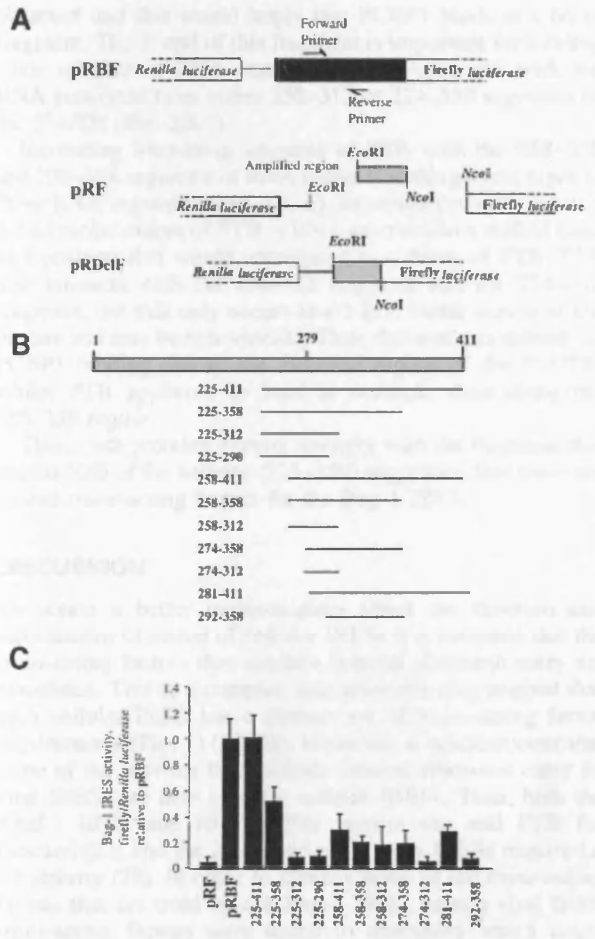

Figure 4. PCBP1 and PTB bind to the minimal active element of the Bag-1 IRES. (**A**) Schematic diagram showing construction of deletion constructs from the discistronic plasmid pRBF (Fig. 1A) by PCR where delF indicates the forward primer, which introduces an *Eco*RI site and delR the reverse primer, which introduces an *Nco*I site, primer sequences are described in Materials and Methods. (**B**) Representation of the sections of the Bag-1 5′-UTR amplified in comparison with the full-length 5′-UTR. (**C**) Relative IRES activity of the deletion constructs in HeLa cells taken as a ratio of firefly/*Renilla* luciferase, normalised to a β-galactosidase transfection control and expressed relative to pRBF, which is assigned a value of 1.

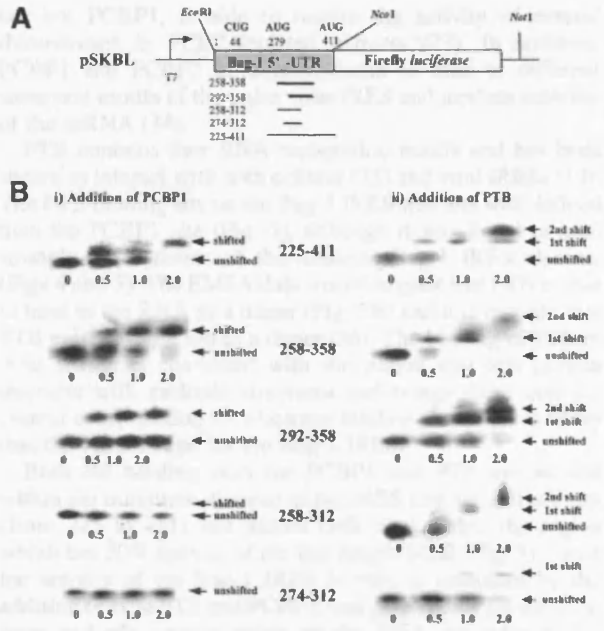

Figure 5. PCBP1 and PTB bind to specific fragments of the Bag-1 5′-UTR. (**A**) Schematic diagram of the monocistronic constructs pSKL and pSKBL showing sites for run-off transcription. (**B**) EMSAs of radiolabelled segments of the Bag-1 5′-UTR with the addition of 2.5, 5 or 10 μg/μl (0.5, 1 or 2 μl total per reaction, respectively) of (i) PCBP1 or (ii) PTB. Arrows indicate positions of protein–RNA complexes (shifted) or RNA alone (unshifted).

with PTB and/or PCBP1 have comparatively high levels of these proteins. In contrast, CALU1 or CAL51 cell lines which are stimulated by these proteins have lower expression of PTB and PCBP1 than MCF7 cells (Fig. 2D).

A comprehensive deletion analysis was then performed to identify the minimal element that harboured IRES activity to determine whether PTB or PCBP1 were essential for Bag-1 IRES function.

The minimum active fragment of the Bag-1 IRES is 186 nt

Regions of the Bag-1 5′-UTR DNA were obtained by PCR and subcloned into the dicistronic reporter vector pRF (Fig. 4A and B). Deletions were transfected into HeLa cells and cell lysates assayed for luciferase activity (Fig. 4C). A region of 218 nt from 225 to 411 was identified as the minimal element since this region of RNA retained 100% IRES activity. The 5′ end of this section of RNA must be critical for function of the IRES since deleting a further 25 nt at the 5′ end reduced the activity of the 258–441 fragment to 25% (Fig. 4C). It is possible to delete an additional 107 nt from the 3′ end and retain 50% of the IRES function with the 225–358 fragment, but the removal of a further 46 nt to generate the 225–312 fragment resulted in an inactive IRES (Fig. 4C). These data would suggest that the first 225 nt are not required for an active IRES, but this section of RNA may bind other, perhaps regulatory, proteins.

PCBP1 and PTB bind to the minimal active fragment

EMSAs were then performed to identify the regions of Bag-1 IRES RNA that could be bound by PCBP1 and PTB. Fragments of the DNA encoding Bag-1 IRES RNA were obtained by PCR and inserted into the vector pSKL (Fig. 5A). Radiolabelled RNAs were generated from these plasmids by *in vitro* transcription reactions and these were incubated with PTB or PCBP1 (Fig. 5B, i). The minimum active fragment 225–411 binds both PTB and PCBP1 as efficiently as the full-length RNA (Figs 5B and 2B). To refine the binding sites further, RNA was generated from additional deletion constructs (Fig. 5A) and these were used in EMSAs. Thus, incubating the 258–358 segment of radiolabelled Bag-1 IRES RNA with increasing amounts of PCBP1 showed that PCBP1 bound directly to this region of the IRES. A decrease in mobility of the radiolabelled 292–358 fragment was also

observed and this would imply that PCBP1 binds to a 66 nt fragment. The 3′ end of this fragment is important for binding since no protein–RNA complexes were observed with the RNA generated from either 258–312 or 274–312 segments of the 5′-UTR (Fig. 5B, i).

Incubating increasing amounts of PTB with the 258–358 and 292–358 segments of RNA shows that this protein binds to these RNA segments (Fig. 5B, ii). Interestingly, addition of a 2-fold molar excess of PTB to RNA gave rise to a shifted band of a position that would correspond to a dimer of PTB. PTB also interacts with the 258–312 fragment and the 274–312 fragment, but this only occurs at a 2-fold molar excess of the protein and may be non-specific. Thus, this analysis refined the PCBP1 binding site to the 292–312 region of the 5′-UTR, whilst PTB appeared to bind at multiple sites along the 258–358 region.

Thus, both proteins interact strongly with the fragment that retains 50% of the activity (225–358) suggesting that these are indeed *trans*-acting factors for the Bag-1 IRES.

DISCUSSION

To obtain a better understanding about the function and mechanisms of action of cellular IRESs it is essential that the *trans*-acting factors that mediate internal ribosome entry are elucidated. This is a complex task since the data suggest that each cellular IRES has a distinct set of *trans*-acting factor requirements (Fig. 1) (24–28). However, it would appear that some of the proteins that mediate internal ribosome entry on viral IRESs are also used by cellular IRESs. Thus, both the Apaf-1 IRES and HRV IRESs require unr and PTB for function (27) and the XIAP and polio virus IRESs require La for activity (28). In order to identify some of the *trans*-acting factors that are used by the Bag-1 IRES, known viral IRES *trans*-acting factors were tested to determine which could interact with the Bag-1 IRES RNA. Of the seven proteins that were tested, only two, PTB and PCBP1, could bind in EMSAs (Fig. 2B). UV-crosslinking studies showed that both PCBP1 and PTB interact directly and specifically with Bag-1 IRES RNA (Fig. 2C and D). Interestingly, these proteins would appear to work in concert since a combination of these two proteins stimulated the Bag-1 IRES 3-fold *in vitro* (Fig. 3B). Moreover, it was possible to increase the activity of the Bag-1 IRES in the cell lines that had low Bag-1 IRES activity by co-transfection of pRBF with plasmids containing cDNAs encoding these proteins (Fig. 3C, i and ii).

PCBP1, which is a member of the KH domain family of single-stranded nucleic acid binding proteins (33), was found to bind strongly to a 66 nt fragment (Fig. 5). The proteins in the KH domain family generally bind to cytidine-rich sequences (33), however, there is not a cytidine-rich stretch in the linear sequence to which PCBP1 binds. It is likely, therefore, that this protein is recognising secondary or tertiary structural motifs in the Bag-1 IRES. Computer predictions have been carried out using the mfold program, although, in the absence of experimentally derived data it is difficult to predict the structure of RNA that is being recognised. It is of interest to note that PCBP2, which has 90% amino acid similarity to PCBP1, does not bind to Bag-1 IRES RNA (data not shown). Therefore, these proteins must recognise distinct structural motifs. In this regard, it has been shown that PCBP2, but not PCBP1, is able to restore the activity of entero/rhinoviruses in PCBP-depleted extracts (23). In addition, PCBP1 and PCBP2 have been found to bind to different structural motifs of the polio virus IRES and mediate stability of the mRNA (34).

PTB contains four RNA recognition motifs and has been shown to interact with both cellular (35) and viral IRESs (13). The PTB binding site on the Bag-1 IRES was less well defined than the PCBP1 site (Fig. 5), although it was found to bind strongly to segments of the minimal Bag-1 IRES element (Figs 4 and 5). The EMSA data would suggest that PTB is able to bind to the RNA as a dimer (Fig. 5B) and it is thought that PTB exists in solution as a dimer (36). The binding of PTB to viral IRESs is consistent with the notion that this protein interacts with multiple structures and brings them into the correct conformation for ribosome binding (13) and it is likely that the same is true for the Bag-1 IRES.

Both the binding sites for PCBP1 and PTB are located within the minimum element of the IRES that has full activity (from 225 to 411) and indeed both bind within the region which has 50% activity of the full-length IRES (Fig. 5). Since the activity of the Bag-1 IRES *in vitro* is enhanced by the addition of both PTB and PCBP1, and given that PTB seems to have multiple contact points on the RNA, we suggest that these proteins act as RNA chaperones and allow the Bag-1 IRES to attain the correct structure that is competent for 40S ribosomal subunit entry. To test this theory, work is being carried out to obtain a secondary structural model for the Bag-1 IRES in the presence of these proteins.

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SHORT REPORTS

The p36 isoform of BAG-1 is translated by internal ribosome entry following heat shock

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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) and one minor isoform (p29), which are translated from a common transcript. The differing amino terminus determines both the intracellular location and the repertoire of binding partners of the isoforms which play different roles in a variety of cellular processes including signal transduction, heat shock, apoptosis and transcription. Although *in vitro* data suggest that the four BAG-1 isoforms are translated by leaky scanning, the patterns of isoform expression *in vivo*, especially in transformed cells, do not support this hypothesis. We have performed *in vivo* analysis of the BAG-1 5′ untranslated region and shown that translation initiation of the most highly expressed isoform (p36/BAG-1S) can occur by both internal ribosome entry and cap-dependent scanning. 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. *Oncogene* (2001) **20,** 4095–4100.

Keywords: BAG-1; IRES; translation; internal ribosome entry; heat shock

Human BAG-1 (Zeiner and Gehring, 1995; Takayama *et al.*, 1996) can be expressed as up to four protein products that are generated by alternative translation initiation from a single transcript (Figure 1a). Translation of the 50 kDa form of BAG-1 (BAG-1L) is initiated at a non-canonical CUG codon and this isoform contains an SV40-like nuclear localization signal. The other forms, p46/BAG-1M, p36/BAG-1S (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 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 (1986). It has been 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 (Packham *et al.*, 1997; Takayama *et al.*, 1998), and moreover the p36 isoform is the predominant form in all cell types.

The BAG-1 isoforms regulate several disparate cellular processes. These include cell survival (Takayama *et al.*, 1995), signal transduction (Wang *et al.*, 1996) and protein refolding (Bimston *et al.*, 1998; Stuart *et al.*, 1998; Luders *et al.*, 2000; Nollen *et al.*, 2000).

The majority of eukaryotic mRNAs are translated by the cap-dependent scanning mechanism of initiation. Some mRNAs are translated by an alternative mechanism where the 5′ UTR forms a complex structural element termed an internal ribosome entry segment (IRES) (reviewed by Gray and Wickens, 1998). IRESs have now been identified in a number of eukaryotic mRNAs and recent work has shown that cellular IRESs are used in situations when the cap-dependent scanning mechanism of translation is compromised; for example the c-*myc*, DAP5 and XIAP-1 IRESs are all used to maintain expression of the corresponding proteins during apoptosis (Stoneley *et al.*, 2000a; Henis-Korenblit *et al.*, 2000; Holcik *et al.*, 2000).

A number of features of the 5′ UTR upstream of the human p36 open reading frame of BAG-1 suggested that it would be involved in the translational regulation of BAG-1. For example, it is long (410 nucleotides) and G-C rich and has alternative translation initiation start codons including an upstream non-canonical CUG. To investigate whether the p36 BAG-1 5′ UTR could contain an internal ribosome entry segment, a dicistronic reporter assay was used. The dicistronic reporter vector pRF (previously designated pGL3R (Stoneley *et al.*, 1998), incorporates both the firefly and sea pansy (*Renilla reniformis*) luciferase reporter genes. A region of cDNA encoding the BAG-1 p36 5′ UTR was cloned between the cistrons of the vector pRF to create the vector pRBF (Figure 2a). In transient

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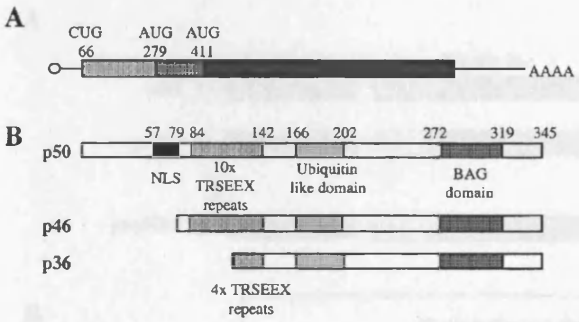

Figure 1 Structure of BAG-1 mRNA and protein isoforms. (**a**) Human BAG-1 mRNA showing the positions of the initiation codons of the three major isoforms. (**b**) The three major protein isoforms of human BAG-1. The p50/BAG-1L protein contains a nuclear localization signal that is not present in the 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

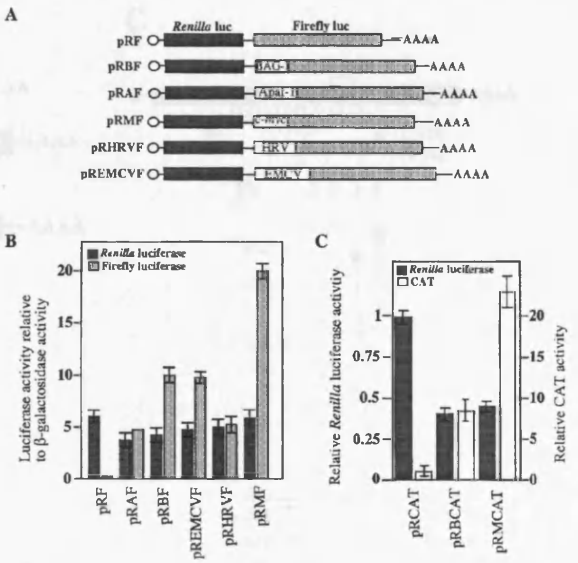

Figure 2 The p36 5′ untranslated region of BAG-1 can direct internal ribosome entry in dicistronic reporter assays. (**a**) The dicistronic luciferase transcript from pRF (formerly known as pGL3R) has been described previously (Stoneley *et al*., 1998). The vectors containing IRESs present in the 5′ UTRs of c-*myc* (pRMF), Apaf-1 (pRAF), HRV (pRHRVF) and EMCV (pREMCVF) have been described elsewhere (Stoneley *et al*., 1998, 2000b; Coldwell *et al*., 2000). A region corresponding to nucleotides 43–411 of the BAG-1 p36 5′ UTR was generated by PCR-mediated amplification from the plasmid CI-3 using the oligonucleotide primers 5′-TCGAATTCTGGGCGGTCAA-CAAGTGCGG-3′ and 5′-ATCCATGGCTTCGCCCTGGGTC-GCC-3′ and introduced between the upstream *Renilla* luciferase cistron and downstream firefly luciferase cistron. This fragment was also introduced into pGL3 (Promega) to create the vector pGBL. In all vectors the position of the physiological AUG initiation codon of the p36 BAG-1 5′ UTR is maintained with that of the firefly or CAT gene within the *Nco*I recognition site. (**b**) HeLa cells were transfected with the constructs shown containing the cellular IRESs from Apaf-1 and c-*myc* and the viral IRESs from HRV and EMCV and the putative BAG-1 IRES by calcium phosphate-mediated DNA transfection (Jordan *et al*., 1996). Luciferase expression was assayed using the Dual luciferase assay kit (Promega) 40 h after transfection and normalized to the transfection control of β-galactosidase (cells were transfected with pcDNA3.1/HisB/*lac*Z from Invitrogen), which was assayed for by the Galactolight plus assay system (Tropix). All light emissions were measured over 10 s using an Optocompl Luminometer (MGM instruments). The BAG-1 p36 5′ UTR directs internal ribosome entry analogous to that seen in other IRES containing vectors. (**c**) In the vector pRCAT, the firefly luciferase cistron is replaced by the CAT gene. CAT activity was measured by liquid scintillation counting using the CAT assay system (Promega) according to the manufacturer's protocol. The BAG-1 p36 5′ UTR can direct translation of an alternative downstream reporter gene in the vector pRBCAT, in a manner related to translation driven by the c-*myc* IRES in the vector pRMCAT

transfections in HeLa cells, the presence of the BAG-1 p36 5′ UTR resulted in an increase in the expression of the downstream firefly luciferase of 17-fold over that attributable to readthrough and re-initiation of ribosomes on the control transcript produced from pRF (Figure 2b). This compares favourably with the IRES activity of the cellular IRESs found in Apaf-1 and c-*myc* (nine and 50 times respectively), and the viral IRESs found in HRV and EMCV (eight and 14 times) when present in the same assay system (Figure 2b). There is a slight downregulation of expression of the upstream *Renilla* cistron in the vector pRBF (to about 70% of the control). This has been observed in other IRES-containing dicistronic transcripts and most likely reflects a competition between cap-dependent and IRES-dependent translation on the same transcript. The BAG-1 p36 5′ UTR can also direct internal ribosome entry in an alternative dicistronic assay system in which the firefly gene is replaced with a gene encoding chloramphenicol acetyl transferase (CAT) (Figure 2c; Stoneley, 1998).

In order to ensure that the apparent internal ribosome entry observed was not due to either enhanced ribosomal readthrough or the presence of cryptic promoters or splice sites in the RNA, two control experiments were performed. In the first instance, a palindromic sequence that forms a hairpin with a free energy approximately equivalent to -68 kcal mol^{-1} was inserted upstream of the *Renilla* ORF in the vector pRBF to create the vector phpRBF (Figure 3a). This hairpin in the vector phpRBF was sufficient to impede ribosomal scanning, and *Renilla* luciferase activity dropped to 7% of that observed in the control vector pRF (Figure 3b), while firefly luciferase activity directed by the BAG-1 5′ UTR was maintained (Figure 3b). Secondly, to ensure that only intact dicistronic messages were being transcribed from the vector pRBF, RNase protection assays were performed. A radiolabelled 669 nt antisense riboprobe (Figure 3c,d, lane 4) was annealed to mRNA from cells that had either been mock transfected (Figure 3d, lane 2) or transfected with pRBF (Figure 3d, lane 3). A protected fragment of the expected size was observed, 597 nt, which contains the 3′ end of the *Renilla* ORF, the BAG-1 5′ UTR and 101 nt of the firefly luciferase

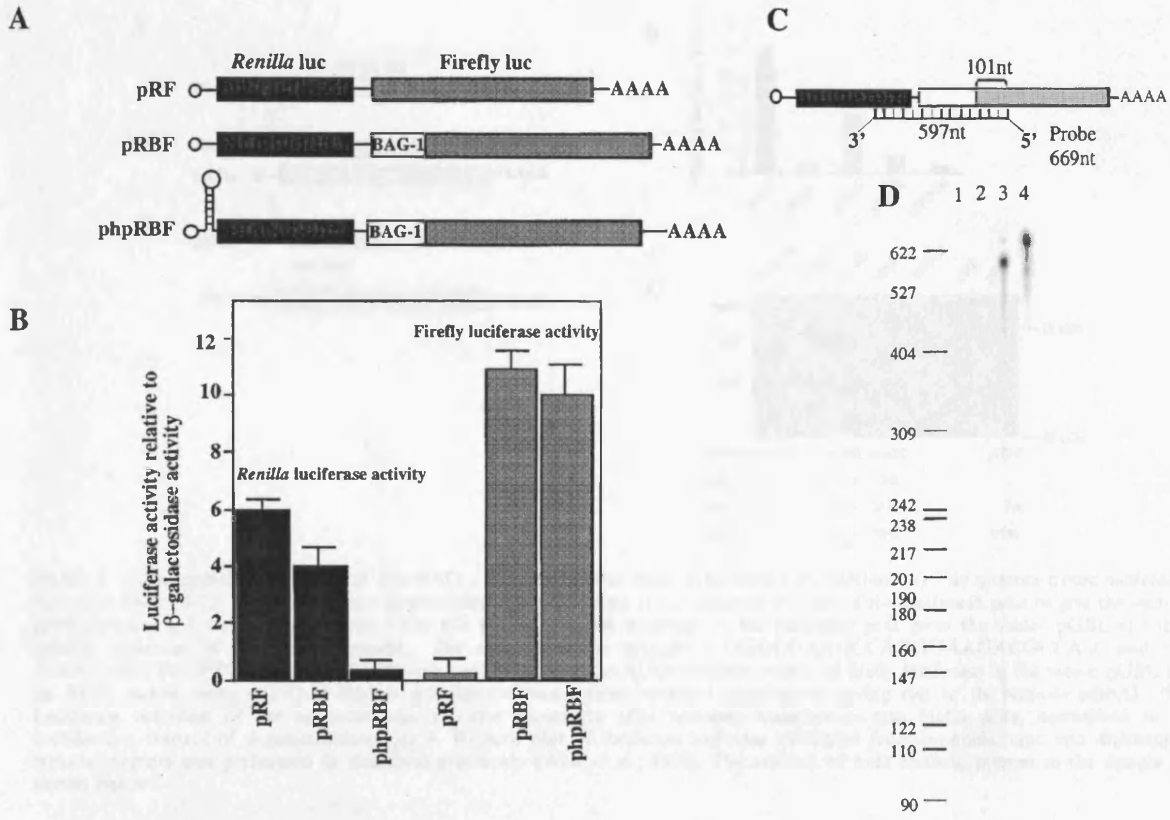

Figure 3 IRES activity is not due to enhanced ribosomal readthrough and re-initiation and internal ribosome entry is initiated on intact dicistronic transcripts. (**a**) The vector phpRBF was created by insertion of a palindromic sequence upstream of the *Renilla* cistron in pRBF which would form a stable hairpin when transcribed. The vector phpRBF was created by ligating the palindromic sequence. 5′-CGCCGCGGGGCGCCGCCGGCCACGTGGGTACCCACGTGGCCGGCGGCGCCCCGCGGCGGCCG-3′ into the *Eco*RV site of pRBF. This would give rise to a hairpin with a free energy of approximately −68 Kcal/mol. (**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 but the increase in firefly luciferase activity seen in pRBF is maintained in phpRBF. (**c**) A 669 nt antisense riboprobe was used to investigate the integrity of dicistronic transcripts. 597 nt of the riboprobe will hybridize to the transcript from pRBF. The vector pBAGRNase used was generated by amplifying a 597 nucleotide region of the vector pRBF using the primers 5′-GTGGATCCGCAAGAAGATGCACCTGATG-3′ and 5′-ATAAGCTTGCGTATCTCTTCATAGCCTT-3′. The PCR product was digested with *Bam*HI and *Hind*III and ligated into pBluescript SK$^+$ (Stratagene). The RNase protection was carried out exactly as described in Stoneley *et al.* (1998). (**d**) RNase protection results of dicistronic RNAs show that IRES activity is not due to the presence of functional monocistronic firefly luciferase transcripts. The radiolabelled antisense riboprobe was hybridized to a yeast tRNA control (lane 1), polyA$^+$ mRNA from untransfected cells (lane 2), and polyA$^+$ mRNA from cells transfected with pRBF (lane 3). Lane 4 contains undigested riboprobe

mRNA (Figure 3d, lane 3). Taken together these data strongly suggest that the 5′ UTR of BAG-1 contains an IRES. The IRES is present in a region that can also code for longer isoforms of the protein and this is unusual among IRESes, but not unique e.g. a shorter IRES-initiated isoform has also been observed for p110/p58^{PITSLRE} (Cornelis *et al.*, 2000).

The translation of certain cellular mRNAs can be initiated by both internal ribosome entry and the cap-dependent scanning mechanism (Stoneley *et al.*, 2000b; Vagner *et al.*, 1996). It was likely that this would also be the case with the p36 isoform of BAG-1 since it has been shown that translational control of the human isoforms of BAG-1 involves the scanning mechanism of translation (Packham *et al.*, 1997; Yang *et al.*, 1998). To investigate the contribution the IRES makes to the expression of BAG-1 isoforms the DNA encoding the BAG-1 5′ UTR was inserted into the monocistronic firefly luciferase vector pGL3 (Figure 4a) to create the vector pGBL. Insertion of the DNA encoding the BAG-1 5′ UTR upstream of the firefly luciferase gene reduced the luciferase activity to 46% of that expressed from the control plasmid (Figure 4b). This is probably due to the relative inactivity of the two additional isoforms at 74 and 70 kDa of luciferase that contained N-terminal extensions that were observed (Figure 4c). These represent the p50 and p46 isoforms that initiate from the upstream CUG and AUG codons respectively (Figure 4c, pGBL lane). A stable hairpin upstream of the luciferase gene in the

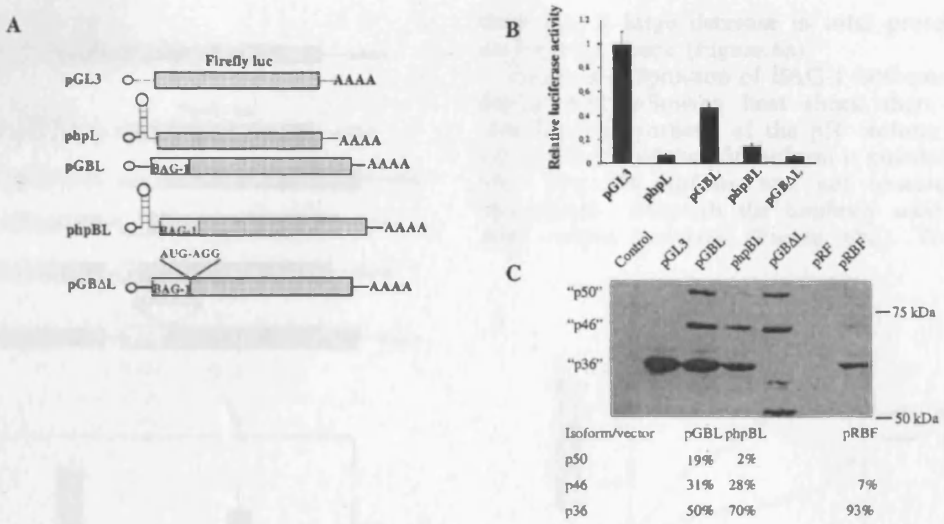

Figure 4 Cap-dependent scanning of p36 BAG-1 is more efficient than IRES driven translation. (**a**) The monocistronic luciferase transcript from pGL3. A palindromic sequence (described in Figure 3) was inserted in front of the luciferase gene to give the vector phpL containing a stable RNA hairpin. The p36 BAG-1 5′ UTR upstream of the luciferase gene gives the vector pGBL and the hairpin upstream of this makes phpBL. The oligonucleotide primers 5′-GGGCGAAGCCAGGGAAGACGCCA-3′ and 5′-TGGCGACTTCCCTGGCTTCGCCC-3′ were used to mutate the AUG initiation codon of firefly luciferase in the vector pGBL to an AGG codon, using the Quik-change site-directed mutagenesis protocol (Stratagene) giving rise to the vectors pGBΔL. (**b**) Luciferase activities of the monocistronic reporter constructs after transient transfection into HeLa cells, normalized to a transfection control of β-galactosidase. (**c**) A Western blot of luciferase isoforms translated from monocistronic and dicistronic reporter vectors was performed as described previously (West *et al*., 1998). The amount of each isoform present in the sample is shown below

vector phpL strongly inhibited translation of luciferase from this construct to 6% of the value obtained with the control construct pGL3 (Figure 4b). The same hairpin, when inserted upstream of the BAG-1 5′ UTR in phpBL, reduced expression of luciferase to 30% when compared to pGBL, however, the level of luciferase produced is still fivefold higher than with phpL (Figure 4b). This would suggest that in the monocistronic construct, pGBL, the p36 isoform BAG-1 is translated primarily by the scanning mechanism and that this is more efficient than internal ribosome entry. However, in the presence of the stable hairpin the BAG-1 IRES is used to maintain expression of the reporter enzyme. These results were confirmed by Western analysis of proteins derived from these plasmid constructs. Thus the translation of the longer isoforms of luciferase was inhibited by the presence of the hairpin, especially the 'p50' isoform, while the 60 kDa luciferase isoform was still efficiently expressed (Figure 4c, pGBL lane). No luciferase was observed in Western analysis performed on samples isolated from cells transfected with phpL (data not shown). When the AUG codon that would initiate the p36 isoform was mutated to an AGG codon in the vector pGBΔL (Figure 4a), production of luciferase in the transfected cells was again strongly inhibited. However, the proteins representing the p50 and p46 isoforms were still observed by immunoblotting (Figure 4c, pGBΔL lane). In contrast, in cells transfected with the dicistronic vector pRBF, nearly all the luciferase present was initiated from the p36 AUG but not the upstream initiation codons (Figure 4c, pRBF lane).

To determine whether only the p36 isoform is translated by internal ribosome entry, two truncated versions of the DNA encoding the p36 5′ UTR were introduced into the dicistronic vectors. The vector pRB5′F that contained the 5′ UTR up to the p46 ORF (Figure 5a) had no IRES activity in the assay after transient transfection in HeLa cells (Figure 5b). The vector pRB3′F that contains only the sequence between the p46 AUG and the p36 AUG had approximately 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 suggests the most important sequences for internal ribosome entry reside in this region of the 5′ UTR. This segment is shorter than most viral IRESs, but is a similar length to some of the cellular IRESs e.g. the FGF-2 (Vagner *et al*., 1995), Apaf-1 (Coldwell *et al*., 2000) and BiP IRESs (Yang and Sarnow, 1997). As a control, mutation of the AUG initiation codon of the p36 ORF to an AGG codon also resulted in a dicistronic vector pRBΔF with no IRES activity.

During heat shock there is a large reduction in global protein synthesis rates due to the changes in the phosphorylation states of many eukaryotic initiation factors (for review see Rhoads and Lamphear, 1995; Schneider, 2000) and sequestration of eIF4G by Hsc70 (Cuesta *et al*., 2000). However, there is still synthesis of certain key proteins that are required during this

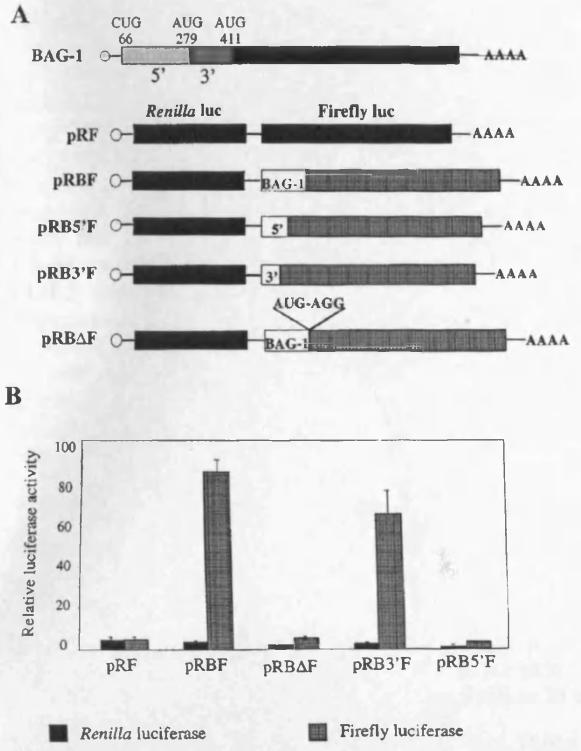

Figure 5 Deletion analysis indicates that only the p36 isoform of BAG-1 is translated by internal ribosome entry. (**a**) Truncated versions of the BAG-1 IRES were introduced into the dicistronic reporter system. The primers 5′-TCGAATTCTGGGCGGTC-AACAAGTGCGG-3′ and CTCCATGGGCGGCCTGCGAGC-GCC-3′ amplified the p46 5′ UTR which was inserted into pRF to make the vector pRB5′F. The vector pRB3′F was produced by religation of pRBF after the removal of a 236 bp restriction fragment generated by digestion with *Eco*RI and *Sac*II such that the sequence between the p46 and p36 initiation codons is present in this vector. In the vector pRBΔF, the AUG initiation codon of luciferase in pRBF is mutated to AGG. (**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

process and it has been shown that the mRNAs that correspond to these proteins are subject to translational regulation. The p36 isoform of BAG-1 protein is involved in the protein refolding response although its precise role following heat shock is not clear. *In vitro* experiments have shown that it can aid the refolding activity of the constitutively expressed Hsc70 (Luders *et al*., 2000), whilst *in vivo* it has been shown to inhibit protein refolding that is dependent on Hsp70, which is only expressed during heat shock (Nollen *et al*., 2000). However, expression of this BAG-1 isoform following heat shock is clearly important thus the ability of the BAG-1 IRES to maintain the expression of the p36 isoform following heat shock was examined. HeLa cells were heated to 44°C by the addition of pre-heated media and after 30 min at this temperature were returned to 37°C by the addition of media. As expected there was a large decrease in total protein synthesis during heat shock (Figure 6a).

Immunoprecipitation of BAG-1 isoforms shows that during and following heat shock there is a large reduction in synthesis of the p50 isoform of BAG-1, yet expression of the p36 isoform is maintained (Figure 6bi). The p46 isoform was not observed in these experiments, although the antibody used detects all three major isoforms (Figure 6bii). To determine

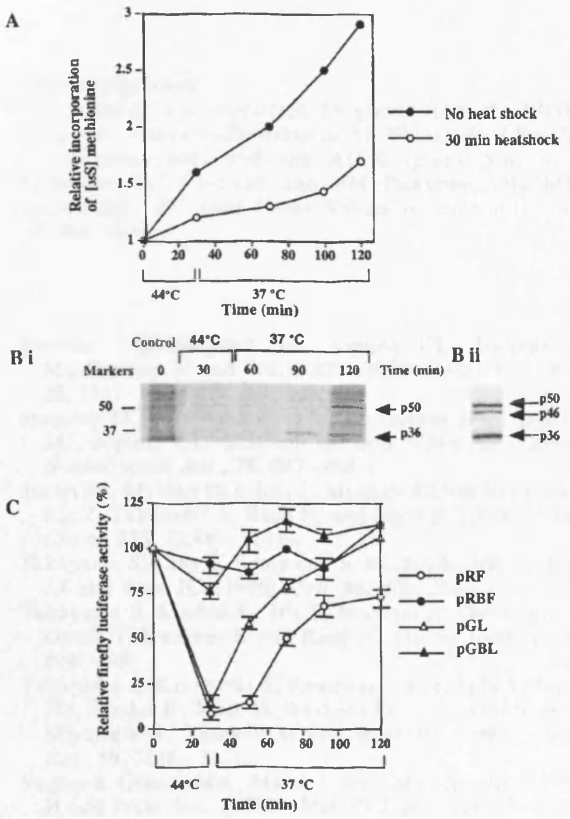

Figure 6 The BAG-1 IRES is used following heat shock. HeLa cells were heated to 44°C by the addition of warm media and maintained at this temperature for 30 min. They were returned to 37°C by the addition of media at this temperature. Samples were taken at the time points indicated. (**a**) Incorporation 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 was performed as described previously (Luscher and Eisenman, 1988; Stoneley *et al*., 2000a) cells were labelled with ^{35}S-methionine for 30 min before harvesting and the BAG-1 isoforms were immunoprecipitated overnight at 4°C using a BAG-1 monoclonal antibody mix (3.9F1E11 + 3.10G3E2, Neomarkers). The data suggest that following heat shock and during the recovery period there is a relative increase in the synthesis of the p36 isoform of BAG-1 when compared to the p50 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. In each case the presence of the BAG-1 IRES causes firefly luciferase to be synthesized under conditions when the cap-dependent scanning mechanism of translation initiation is down-regulated

whether the BAG-1 IRES was utilised to maintain expression of the p36 isoform, cells were transfected with either pGL3, pGBL, pRF or pRBF, and then treated as described above. Cells were harvested at the time points indicated and firefly luciferase levels were assayed and normalised to the transfection control of β-galactosidase. The data show that firefly luciferase was still synthesised from cells that were transfected with the constructs that harbour the BAG-1 IRES whilst in cells transfected with the control plasmids pGL3 and pRF there was a decrease in luciferase expression (Figure 6c). Thus we conclude from these experiments that in contrast to Hsp70 where shunting is used to maintain protein expression following heat shock (Yueh and Schneider, 2000), initiation of the p36 isoform of BAG-1 following heat shock occurs by internal ribosome entry.

In conclusion we have investigated the translation initiation of BAG-1 and have shown that the 5′ UTR of the p36 isoform can be synthesised by internal ribosome entry. Given the putative roles of the BAG-1 p36 isoform in the protein refolding response following heat shock (Luders *et al.*, 2000; Nollen *et al.*, 2000) when the cap-dependent scanning mechanism of translation is reduced (Rhoads and Lamphear, 1995), we investigated whether the IRES was active during this process to sustain expression of this protein. We show that there is synthesis of the p36 isoform of BAG-1 following heat shock and that this is mediated by the IRES.

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