THE MECHANISM OF TRANSLATIONAL REPRESSION BY IRON REGULATORY PROTEIN

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Abstract

The regulation of the intracellular iron storage protein ferritin is a cytoplasmic mechanism of gene regulation. Translation of ferritin mRNA is regulated by iron via mRNA-protein interactions between iron-responsive elements (IREs) and iron regulatory protein (IRP). In iron-depleted cells, IRP binds to single IREs located in the 5' untranslated region of ferritin mRNA and represses translation. The aim of this thesis was to investigate the mechanism of IRP-mediated translational control of IRE-containing mRNAs. To this end, cell-free translation systems were established in wheat germ extract and rabbit reticulocyte lysate which reconstitute IRE/IRP-mediated control. In these, cellular ferritin mRNA was specifically repressed in the presence of IRP, the IRE element was shown to be necessary and sufficient to mediate this repression, and an IRE with a single nucleotide deletion was not functional. However, the IRE element was shown not to efficiently repress translation when located in a cap-distal position. IRE/IRP-mediated regulation was determined to occur at the level of translation and not as a consequence of changes in mRNA stability. IRE/IRP-mediated regulation *in vitro* was found not to require a polyadenylated mRNA, suggesting that regulation in cells is not mediated by changes in poly (A) tail length. Recombinant IRP was found to be functionally equivalent to placental or reticulocyte IRP, and IRP was determined to be the only mammalian cell-specific protein required for regulation. The molecular mechanism underlying this translational repression was investigated in the cell-free systems by sucrose gradient analysis of complexes between IRE-containing mRNAs and ribosomal subunits and the IRE/IRP complex was shown to prevent the association of the 43S pre-initiation complex (including the small ribosomal subunit) with the mRNA. In addition, the formation of IRE/IRP complexes on the mRNA was found not to be sufficient in itself to block 43S access, but required the IRE to be located close to the cap structure, providing an

explanation for the previously observed "position effect". Evaluation of other newly identified mRNAs containing putative IREs using these cell-free systems provided evidence that erythroid 5-aminolevulinate synthase (eALAS) and mitochondrial aconitase mRNA are also regulated by the same IRP-mediated mechanism. Furthermore, an unrelated mRNA/protein complex formed between the spliceosomal U1A protein and an indicator mRNA containing the U1A binding site, which had previously been implicated in steric repression of translation, was also shown to inhibit 43S association when located in a cap-proximal position. Based on the ability of cap-proximal U1A mRNA/U1A and IRE/IRP complexes to block the binding of the 43S complex to their respective mRNAs, it is suggested that both RNA/protein complexes inhibit translation by steric interference with the binding of the 43S pre-initiation complex. This work thus reveals that the cap-proximal region of eukaryotic mRNAs represents a sensitive target for translational repressor proteins to block the initiation of translation and raises the possibility that this may represent a more general mechanism of translational control.

I would like to dedicate this thesis to the memory of my grandfather James W. M. Gray who died in May 1993 during the course of this work.

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ABBREVIATIONS

A	adenosine
acon	mitochondrial aconitase
ALAS	5-aminolaevulinate synthase
arg	arginine
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	cytidine
ΔC	deletion of the most 5' C in the loop of the IRE (see figure 2)
CAT	chloramphenicol acetyl transferase
cdc	cell division cycle
Ci	Curie
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element-binding protein
cpm	counts per minute
cDNA	complementary DNA
cys	cysteine
CTP	cytosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
eALAS	erythroid-specific isoform of ALAS
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factor
EGTA	ethylene-bis(oxyethylene-nitrilo) tetraacetic acid
eIF	eukaryotic initiation factor
ES	extended spacer
FRP	ferritin repressor protein
G	guanosine
m^7GpppG	7-methyl guanosine triphosphate
GCD	general control constitutively de-repressed
GCN	general control nonde-repressible
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GMP-PNP	guanylylimidodiphosphate
GRP 78	78 kDa glucose regulated protein
GTP	guanosine triphosphate

H	heavy
HCR	Haemin controlled repressor
HDS	HEPES, DTT, spermidine solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HMBA	N,N'- hexamethylene-bis-acetamide
HSE	heparin Sepharose elution
IFN	interferon
IPM	isopropylmalate
IRE	iron-responsive element
IRE-BP	iron responsive element binding protein
IRES	internal ribosomal entry site
IRF	iron regulatory factor
IRP	iron regulatory protein
IRP_B	iron regulatory protein B
kb	kilobase pair(s)
kd	dissociation constant
kDa	kilodalton
L	light
La	Lupus erythematosus antigen
LAP	liver-enriched activating protein
LIP	liver-enriched inhibitory protein
LOX	lipoxygenase
LOX-BP	lipoxygenase binding protein
LPS	lipopolysaccharide
M	Molar
Met	methionine
mRNA	messenger RNA
mRNP	messenger RNP
mut	mutant
NADH	reduced form of nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
NTA-resin	Nitrilotriacetic acid immobilised on crosslinked agarose
NO	nitric oxide
NOP 1	Nucleolar protein 1
NOS	nitric oxide synthase
nt(s)	nucleotide(s)
ODC	ornithine decarboxylase
ORF	open reading frame
P-90	90 kDa IRE-binding protein
PAB	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis

PDGF 2	platelet-derived growth factor 2
PEG	polyethyleneglycol
PBS	phosphate-buffered saline solution
PKC	protein kinase C
PKR	double-stranded RNA-activated kinase
PMSF	phenylmethylsulphonyl fluoride
poly (A)	polyadenosine
pre-mRNA	pre-messenger RNA
RLP	ribosome landing pad
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein particle
rpm	revolutions per minute
RRL	rabbit reticulocyte lysate
RSL	random stem-loop
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
ser	serine
snRNA	small nuclear RNA
snRNP	small nuclear RNP
SSL	suppressor of stem-loop
T	Thymidine
34T	variant IRE at position +34 (see figure 24)
TAE	tris-acetate-EDTA solution
TBE	tris-borate-EDTA solution
TCA	trichloroacetic acid
TCE	translational control element
TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA
TEMED	N,N,N,N,-tetramethylethylene diamine
Tf	transcription factor
TfR	transferrin receptor
T_m	melting temperature
tPA	tissue plasminogen activator
Tris	tris(hydroxymethyl)aminometane
tRNA	transfer ribonucleic acid
$tRNA_i$	initiator tRNA
TS	thymidylate synthase
uORF	upstream open reading frame
U	uridine
U1A	U1 snRNP specific protein A
US	unstructured spacer (see figure 24)

UTP	uridine triphosphate
UTR	untranslated region
UV	ultra violet
WGE	wheat germ extract
wt	wild type
w/v	weight for volume

Chapter 1: Literature Review and aims of the thesis.

1.1 Iron metabolism.

With few exceptions, all organisms require the redox metal iron to support life (Neilands, 1974; Stubbe, 1990). Iron containing proteins are required for a variety of metabolic functions including electron transfer, oxygen transfer, nitrogen fixation and DNA synthesis. However, iron which is not appropriately stored or utilised can react with oxygen to produce hydroxyl radicals, which are harmful to cells by inducing lipid peroxidiation and DNA damage (Gutteridge, 1989; Zamenhof et al., 1954). Therefore, intracellular iron homeostasis must be tightly controlled. Iron is delivered to mammalian cells bound to transferrin, a serum protein with two binding sites for Fe^{3+} ions (Brittenham, 1994). Iron enters cells by the docking of iron-containing transferrin molecules to the transferrin receptor (TfR), a transmembrane protein consisting of two identical subunits of 90 kDa, each of which is thought to bind a single transferrin molecule (Baker and Morgan, 1994). The number of transferrin receptors located on the cell surface is normally rate limiting for the uptake of iron into the cell (Baker and Morgan, 1994). It is generally accepted that transferrin bound to the transferrin receptor is internalised by receptor-mediated endocytosis, and the iron is released from transferrin within the endosomes where the acidic pH leads to its dissociation (Baker and Morgan, 1994). The release of iron within the endosomes is probably accompanied by a reduction of the iron from the ferric to the soluble ferrous state, but the mechanism by which iron then crosses the endosomal membrane is not yet well understood (Halliday et al., 1994). The released iron forms part of an ill defined low molecular weight " intracellular transit iron pool"; from which it is either, (i) incorporated directly into iron-containing proteins involved in cellular metabolism, (ii) may be released e.g. in hepatocytes and macrophages, or, (iii) incorporated into ferritin an iron storage protein which maintains iron

in a soluble form (Halliday et al., 1994). This protein consists of 24 subunits which surround a core of up to 4500 iron atoms in a hydrous oxide ferric-oxyphosphate complex (Theil, 1987). Two distinct types of ferritin subunits exist in mammalian cells, a heavy chain of ~21 kDa and a light chain of ~19 kDa (Theil, 1987). The ratios of these subunits in ferritin vary in different cells and are proposed to reflect the varying roles of ferritin, which can either store intracellular iron for the cells own use, for use by other cells or for detoxification when iron overload exists (Theil, 1987). H-rich ferritins may be primarily involved in detoxification while the main role of L-rich ferritins appears to be the long term storage of iron (Aisen, 1994; Halliday et al., 1994). In bullfrog tadpoles a third ferritin chain known as the M (middle)-chain has been identified (Dickey et al., 1987).

The regulation of iron uptake and storage in mammalian cells by transferrin receptors and ferritin has been shown to be co-ordinately regulated at the post-transcriptional level, and the question of whether haem synthesis, which accounts for 80% of iron utilisation is also regulated in co-ordinate manner has also been raised (Dierks, 1990). This makes an attractive proposition as it would allow the co-ordinate regulation of iron uptake, storage and utilisation.

1.2 The regulation of gene expression.

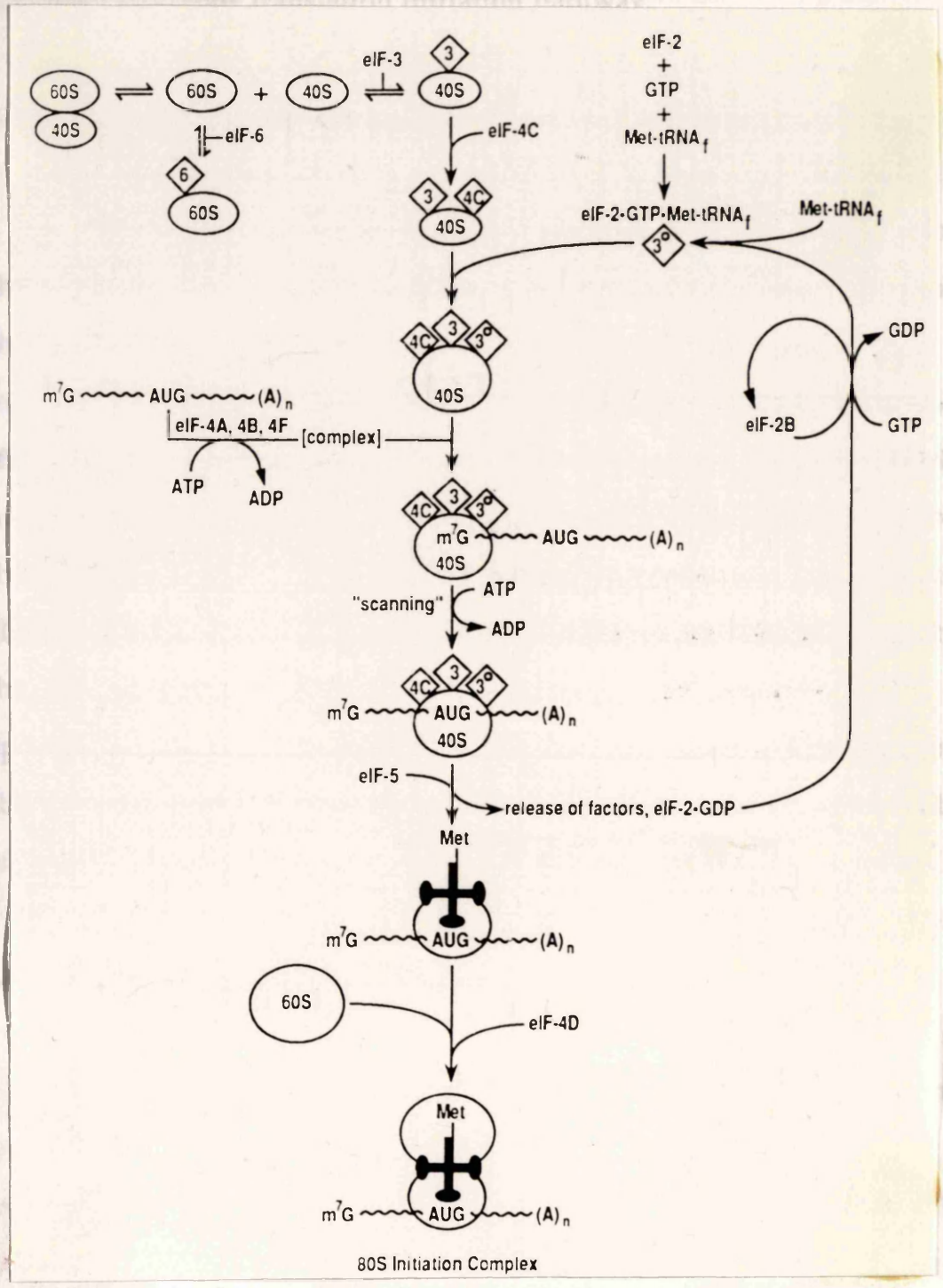
As can be inferred from the example of iron metabolism, the tight and yet flexible regulation of gene expression is essential to all organisms. In eukaryotes, gene regulation is essential in the establishment of specialised cellular functions during developmental differentiation, and conveys responses to environmental stimuli such as hormones, growth factors and nutrients, of which iron is an example of a nutrient which is both necessary and toxic. The specific co-ordinated regulation of ferritin and transferrin receptor synthesis at the post-transcriptional level in response to known cellular stimuli provides a good experimental model to study post-

transcriptional mechanisms of gene regulation. The translational control of ferritin mRNA will be the focus of this thesis.

1.3 Translation of Eukaryotic mRNAs.

Translation of a protein from an mRNA can be divided into three distinct steps: initiation, elongation and termination. Translational initiation is a multi-step process (summarised in figure 1) which results in the formation of an 80S ribosome competent to begin translation at the initiator codon. A large number of factors are involved in this process to mediate interactions between the mRNA, tRNA, and the 40S and 60S ribosomal subunits. Briefly, initiation can be sub-divided as follows; dissociation of 80S ribosomes into the 40S and 60S ribosomal subunits; formation of a 43S pre-initiation complex (Met-tRNA.GTP.eIF-2 and the 40S ribosomal subunit); binding of the 43S complex to the mRNA and joining of the 60S subunit to form an 80S ribosome (Voorma et al., 1994). Once the first peptide bond is formed, elongation continues with the addition of amino-acid residues to the polypeptide chain. This is promoted by four protein translation elongation factors (EF-1α, EF-1$\beta\gamma$, EF-2 and EF-3) and includes three steps; binding of the aminoacyl tRNA to the "A" site of the ribosome together accompanied by GTP hydrolysis, peptide bond formation, and translocation to the "P" site resulting in the hydrolysis of a second GTP molecule (Proud, 1994). Termination occurs when the ribosome encounters one of the three stop codons (a fourth nucleotide following this triplet may also play a role [see 1.5.1.6 and 1.5.3.1]) and a protein release factor induces the cleavage of the synthesised polypeptide from the peptidyl tRNA (Tuite and Stansfield, 1994). Release factor may also influence the number of 40S subunits which remain attached to the mRNA and thus affect re-initiation (see 1.5.1.6 and 1.5.3.1) (Merrick, 1992). Translational regulation occurs predominantly during initiation, but regulation also occurs during

Figure 1 Schematic diagram of the eukaryotic translation initiation pathway.



This figure is reproduced from Merrick (1992) and provides an overview of the initiation pathway and is explained in the text.

elongation and termination. These later two phenomena will not be discussed further as they lie outside the scope of this thesis.

1.4 The Eukaryotic translation initiation pathway.

1.4.1 Dissociation of ribosomes into 40S and 60S ribosomal subunits.

Since the 40S ribosomal subunit interacts with the mRNA prior to the 60S ribosomal subunit, the initial step in translation initiation involves the generation of free ribosomal subunits, as 40S and 60S subunits not involved in translation have a tendency to aggregate in inactive complexes (figure 1). The pool of free 40 and 60S ribosomal subunits is maintained by a number of initiation factors which associate with the subunits and prevent their interaction with one another. The 25 kDa protein eIF-6 (also known as eIF-3A), which binds to the 60S ribosomal subunit, provides the majority of the cellular anti-association activity (Raychaudhuri et al., 1984; Russel and Spremulli, 1979) and is assisted by eIF-3, a multiple polypeptide complex of about 600-650 kDa and eIF-4C (also known as eIF-1A), a 18 kDa protein both of which interact with the 40S ribosomal subunit (Goumans et al., 1980).

1.4.2 Formation of the 43S pre-initiation complex.

The 40S ribosomal subunit is thought to associate with a ternary complex consisting of eIF-2, a heterotrimeric 125 kDa protein, GTP and the methionine-charged initiator tRNA (Met-tRNA$_i$) (Benne et al., 1979; Konieczny and Safer, 1983) prior to its interaction with mRNA (figure 1). The hypothesis that the small ribosomal subunit interacts with the ternary complex prior to its association with the mRNA is based on sucrose gradient analyses which allowed the isolation of complexes between the 40S ribosomal subunit and the ternary complex (43S pre-initiation complexes) and not of complexes between mRNA and 40S ribosomal subunit unless

Met-tRNA$_i$ was present (Benne and Hershey, 1978; Trachsel et al., 1977). The formation of the 43S complex does not require GTP hydrolysis as non-hydrolyzable GTP analogues can functionally substitute for GTP in the formation of this complex (Hershey, 1991). The formation of the 43S complex is stimulated by eIF-3 which is thought to remain bound to the 40S ribosomal subunit (Peterson et al., 1979). However in the absence of kinetic data, the possibility that unstable intermediate 40S.mRNA complexes form in the absence of Met-tRNA$_i$ cannot be ruled out.

1.4.3 Association of mRNA with the 43S pre-initiation complex.

The eIF-4 group of initiation factors facilitates the binding of the 43S complex to the mRNA in an ATP-dependent process (figure 1). The roles and interactions between these factors are not yet fully defined. A 24 kDa protein, eIF-4E (also known as eIF-4α) is the only translation initiation factor capable of direct interaction with the cap structure and it appears as if this protein mediates the initial contact with the mRNA (as reviewed in Rhoads, 1988) and (Sonenberg, 1988). It has been proposed that eIF-4E recognises the cap as part of the eIF-4F complex which in addition to eIF-4E contains two other proteins, a 45 kDa protein eIF-4A (also known as eIF-4β) and p220 (also known as eIF-4γ) (Grifo et al., 1983). Several lines of evidence suggest that eIF-4A may cycle in and out of complexes with eIF-4E and p220 (Buckley and Ehrenfeld, 1987; Etchison and Milburn, 1986; Pause et al., 1994; Ray et al., 1985). Following the interaction of eIF-4F with the cap, an 80 kDa protein eIF-4B (Benne and Hershey, 1978; Trachsel et al., 1977), which may function as a dimer (Abramson et al., 1988), associates with eIF-4F if they have not already associated by the time eIF-4F binds to the m^7G cap (Grifo et al., 1983). At this point unwinding of secondary structure in the vicinity of the cap occurs, mediated either by eIF-4F or by eIF-4A which has an ATPase-dependent RNA helicase activity and is a member of the DEAD box family of proteins (Grifo et al., 1984; Lawson et al., 1989; Pause and Sonenberg, 1992;

Ray et al., 1985; Rozen et al., 1990). The abilities of eIF-4F and eIF-4A to unwind secondary structure is greatly facilitated by the presence of eIF-4B (Lawson et al., 1989; Ray et al., 1985; Rozen et al., 1990). With some or all of these factors associated and at least some unwinding of mRNA, binding of the 43S complex occurs (forming a 48S pre-initiation complex). Since eukaryotes appear to lack specific ribosome binding sequences, it is inferred that the binding of the 43S complex is mediated by mRNA-associated protein factors. eIF-4F and eIF-4B may represent the factors which facilitate binding as they are capable of interacting with eIF-3, which is part of the 43S complex (Hershey, 1991). The eIF-4F mediated interaction possibly occurs through p220 which interacts with the 40S ribosomal subunit as well as with eIF-3 (Joshi et al., 1994). Since the eIF-4F complex is thought to be located at the cap, the 43S complex probably enters the mRNA at or near the 5' end of the mRNA. It has been proposed that the 43S complex then repositions itself to the initiator AUG in a process known as scanning (Kozak, 1978). This process is ATP-dependent and it is thought that the 43S complex migrates along the 5'UTR in a 5'->3' direction until it encounters an AUG codon where protein synthesis is initiated (Kozak, 1980). This hypothesis implies that the first AUG encountered will be the predominant site where protein synthesis is initiated (see 1.5.1.2). The majority of current experimental evidence supports the scanning model of initiation for the majority of cellular mRNAs (alternative models are discussed below in 1.4.6). In summary; the vast majority of mRNAs (90%) conform to the first AUG rule (Kozak, 1987a) and introduction of an upstream in frame AUG in a favourable context (see 1.5.1.2) prevents initiation from the original AUG (Kozak, 1989b; Kozak, 1983); the use of circular mRNAs revealed that the ribosome cannot bind directly to the AUG implying that ribosome binding requires a free mRNA terminus (Kozak, 1979); in support of this, 43S pre-initiation complexes were found to be associated with the 5' end of the mRNA in the absence of ATP (Kozak, 1980), whereas in the presence of ATP and edeine (an antibiotic which is proposed to interfere with AUG

recognition thus allowing ribosomes to ignore the AUG codon) ribosomes were distributed along the entire length of the mRNA, indicating the ability of ribosomes to migrate along the mRNA (Kozak and Shatkin, 1978); and finally, insertion of stem-loop structures into the 5'UTR stall migrating 43S complexes within the 5'UTR, preventing translation (Kozak, 1989a). The requirement for ATP for scanning may be related to the ATP dependence of secondary structure unwinding within the mRNA; however, the cellular helicase activities responsible for the unwinding of secondary structures within the 5'UTR are not yet fully characterised. It is not clear whether the 43S pre-initiation complex itself possesses helicase activity, in which case eIF-4A and eIF-4B may be dispensable once the 43S complex has accessed the mRNA. However, if eIF-4A and eIF-4B are required for the unwinding of more distal structures then the association of additional molecules of eIF-4A and eIF-4B may be required.

1.4.4 Recognition of the initiator codon.

The initiation of protein synthesis normally commences at the first AUG codon in the mRNA, but it is not entirely clear how the 43S complex recognises the initiator codon. However, genetic evidence from *Saccharomyces cerevisiae* clearly implies a role of eIF-2 in AUG recognition. These studies identified mutants in the α and β subunits of eIF-2 which suppress a mutation of the AUG initiator codon to AUU (Cigan et al., 1989; Donahue et al., 1988). Once the 43S complex is correctly positioned at the initiator codon, the anti-codon of Met-tRNA$_i$ directly interacts with the AUG codon (Cigan et al., 1988). The sequences surrounding the AUG codon affect the efficiency with which it is recognised (see 1.5.1.2), but it is not clear how these sequences influence the translational machinery. It has been suggested that the optimal sequence is that which allows the greatest interaction of eIF-2 (Thomas et al., 1992).

1.4.5 Joining of the 60S ribosomal subunit.

Once the initiator tRNA is correctly positioned at the AUG initiator codon, eIF-5 triggers the hydrolysis of the GTP bound to eIF-2 (as reviewed in Merrick, 1992). Some speculation exists as to whether eIF-4C also participates in this event, as it appears complexed with eIF-5 (Schreier et al., 1977; Trachsel et al., 1977). The hydrolysis of GTP causes the release of initiation factors from the small ribosomal subunit (Chakrabarti and Maitra, 1992; Paterson et al., 1979). This hydrolysis occurs more rapidly in the presence of the 60S subunit, which may indicate that the 40S and 60S subunits are already in contact (Merrick, 1979; Merrick et al., 1975). eIF-6 is also thought to be released from the 60S subunit at this point and subunit joining occurs (figure 1). The formation of the first peptide bond seems to be stimulated by eIF-4D (also known as eIF-5A); however the absolute requirement for this factor in general translation initiation has recently been challenged (Kang and Hershey, 1994). eIF-2 is released from the 40S subunit with GDP bound to it. In order to regenerate eIF-2 for subsequent rounds of initiation, it must first be recycled by the activity of eIF-2B (also known as GEF), with which it interacts causing the dissociation of GDP (as reviewed in Clemens, 1994). The affinity of GDP for eIF-2 is far higher than that of GTP (Panniers et al., 1988). eIF-2B appears to remain bound to eIF-2 either until a new eIF-2.GTP complex is formed or prior to ternary complex association with the small ribosomal subunit (Proud, 1986).

1.4.6 Alternative mechanisms for the initiation of translation.

1.4.6.1 Re-initiation.

Re-initiation is a rare mechanism for the initiation of protein synthesis. By definition re-initiation can only occur on mRNAs which contain more than one open reading frame i.e. are di- or polycistronic.

Truly di- or polycistronic mRNAs have not yet been identified in eukaryotes, but several eukaryotic mRNAs contain short open reading frames (uORFs) upstream of the main open reading frame (Kozak, 1987a). After translation of the first ORF is complete, it is postulated that the 60S ribosome dissociates from the mRNA while the 40S ribosomal subunit may stay associated and resume scanning (see 1.5.3.1) (Kozak, 1984; Kozak, 1987c; Liu et al., 1984). It is not clear which (if any) of the components of the translation initiation machinery remain associated with the 40S after translation of short ORFs, but at least a new ternary complex has to be acquired in order to allow re-initiation to occur at downstream sites.

1.4.6.2 Cap-independent initiation.

The 5'UTRs of picornaviral RNAs are unusually long, ranging between 600-1200 nucleotides in length, and contain multiple AUG codons, extensive secondary structure, and no cap structure (Jackson et al., 1994). These features suggest that picornaviral mRNAs would be very poorly translated by a conventional mechanism of initiation. Artificial dicistronic mRNAs in which the 5'UTRs of these viruses were inserted between the first and second ORF were used to show that the 5'UTRs of these viruses promote cap-independent internal initiation of translation (Jang et al., 1988; Pelletier and Sonenberg, 1988). The translation of the downstream ORF was not a result of efficient re-initiation being promoted by these viral sequences, as translation of the second ORF was observed under several conditions where translation of the first ORF did not occur. In addition, these mRNAs were shown to be truly dicistronic and not cleaved by nucleases (OH and Sarnow, 1993). Deletional analysis of the 5'UTRs of these viral RNAs showed that a large region [known as IRES (internal ribosome entry site) or RLP (ribosome landing pad)] was required to promote internal initiation (Jackson et al., 1994). The large size of these IRES elements lead to the notion that they form complex structures. The ribosome is thought to

enter just 3' of this structure in an area which contains two additional features important for internal initiation, a pyrimidine tract and an AUG codon located 20-25 nucleotides downstream (Meerovitch et al., 1991; Pilipenko et al., 1992). In a subset of picornaviruses (cardioviruses and aphthoviruses) this AUG codon is the site of initiation (Kaminski et al., 1990). In others (entero and rhinoviruses) it is not the site of initiation; however it is essential and may serve as an entry point for ribosomes (Meerovitch et al., 1991; Pelletier et al., 1988) which appear to scan and subsequently initiate at the next AUG. The distance between this AUG codon and the pyrimidine tract and perhaps other as yet undefined elements of the IRES are critical, as insertions and deletions which alter this spacing are deleterious (Kaminski et al., 1994; Pilipenko et al., 1992).

It was proposed that cellular factors play a role in internal initiation, since cap-independent initiation can be observed in cells in the absence of viral infection (Trono et al., 1988). The initiation factor eIF-2, which amongst its many functions promotes AUG recognition has been implicated in internal initiation (Del Angel et al., 1989) and eIF-4F has also surprisingly been shown to be required for this type of initiation (Pause et al., 1994). Interestingly, eIF-4F in which the p220 subunit has been cleaved during poliovirus infection still promotes internal initiation, albeit less efficiently (Buckley and Ehrenfeld, 1987). Several other proteins including the auto-antigen La, poly-pyrimidine tract binding protein and a protein of 97 kDa have been suggested to play a role in internal initiation; however a clarification of the roles of these proteins is required (Jackson et al., 1994).

In addition to picornaviruses, IRESes have been identified in an increasing number of viruses including hepatitis C virus (Tsukiyama-Kohara et al., 1992) and infectious bronchitis virus where it is located within the coding sequences of a tricistronic mRNA and is thought to mediate translation of the third cistron (Liu and Inglis, 1992).

Two monocistronic cellular mRNAs, GRP78 (Macejak and Sarnow, 1991) and *Antennapedia* (OH et al., 1992) have also been shown to contain

5'UTRs which can support internal initiation and the IRES in *Antennapedia* mRNA is the shortest functional IRES identified to date, being only 252 nucleotides in length (OH et al., 1992). 55 nucleotides from this *Antennapedia* IRES are highly conserved amongst *Drosophila* species and when placed in a dicistronic mRNA are sufficient to support internal initiation in cultured *Drosophila* cells (OH and Sarnow, 1993).

1.5 Translational Regulation.

1.5.1 Determinants of translational efficiency.

1.5.1.1 The cap structure.

The majority of eukaryotic cellular mRNAs have an inverted monomethyl-guanosine (cap) which is post-transcriptionally attached via a 5'-5' triphosphate linkage to the 5' terminus of the primary transcript (Banerjee, 1980). Although, the cap structure has a role in mRNA processing (Izaurralde et al., 1994), stability (Banerjee, 1980) and possibly transport (Hamm and Mattaj, 1990; Jarmolowski et al., 1994) its primary cytoplasmic role appears to be in increasing the efficiency of mRNA translation (Banerjee, 1980; Shatkin, 1976). The methyl group located on the guanosine ring appears to be necessary for translation as a non-methylated cap is unable to stimulate translation (Horikami et al., 1984). Conditions which impair the interaction of cap binding proteins with the cap, such as poliovirus infection (see 1.4.6.2) result in a severe inhibition of cellular mRNA translation. In cell-free systems, mRNA translation appears to be more cap dependent in wheat germ extract than in rabbit reticulocyte lysate (Sonenberg, 1988).

1.5.1.2 Context of the initiator codon.

The most frequently used initiator codon in eukaryotic translation is AUG, although some viral and cellular mRNAs also initiate at non-AUG codons such as GUG or ACG. However, the sequences which surround the initiator codon influence the frequency with which a particular initiator codon is utilised. From a survey of 699 vertebrate mRNAs GCCGCC^{A}/$_{G}$CCAUGG emerged as a statistically conserved consensus sequence (where the initiator AUG is underlined) for the initiation of translation (Kozak, 1987a). The relevance of this consensus sequence was confirmed by COS cell transfections. A purine at position -3, was found to be the most important upstream determinant for efficient translation (Kozak, 1986b) and interestingly, a purine in this position (usually an A) is the most highly conserved nucleotide after the AUG itself in both vertebrate and plant mRNAs (Heidecker and Messing, 1986; Kozak, 1987a). Likewise, a G in position +4 was shown to be favourable for efficient translation (Grünert and Jackson, 1994; Kozak, 1986b). The remaining nucleotides in the consensus appear to play a less important role in promoting efficient translation, with their influence only becoming apparent when the positions -3 and +4 are occupied by unfavourable pyrimidines (Kozak, 1986b; Kozak, 1987b). Similar comparisons in *Drosophila melanogaster*, yeast, protozoa and plants (Cavener and Ray, 1991) show a variation in the preferred consensus sequences, but a purine at position -3 is preferred in all groups. Recently, it has been shown that positions +5 and +6 also play a role in determining the efficiency of translational initiation. The presence of an A at position +5 (where a C is also stimulatory) appears to be more important than the nucleotide at +6, where the presence of a U has a mildly stimulatory effect (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994). However, the effects mediated by both these positions is most obvious when a weak initiator codon such as a non-AUG codon is present.

When the most 5' AUG in the mRNA lies in a poor context, it is often ignored by the scanning complex which initiates at another AUG further downstream, in a process called "leaky scanning" (Kozak, 1986b). The translation of some cellular mRNAs relies on leaky scanning, as exemplified by the liver transcription activator LAP and repressor LIP. These proteins are encoded by the same mRNA which contains three in-frame initiator codons. The first is located in a poor context and is normally ignored by the scanning 43S complex, with the translation of the longer LAP occuring mainly from the second AUG. Since the context of the second AUG is also not optimal, a proportion of 43S complexes scan to the third AUG where translation of LIP is initiated (Descombes and Schibler, 1991).

1.5.1.3 Leader length.

The majority of eukaryotic mRNAs have 5'UTRs of about 20-100 nucleotides in length (Kozak, 1987a). The effect of leader length has mainly been determined by the use of experimentally generated indicator constructs, as comparisons of naturally occuring mRNAs with short and long 5'UTRs are often hampered by other parameters such as secondary structure and upstream AUGs (Kozak, 1991a; Kozak, 1991b). Very few naturally occuring mRNAs have 5'UTRs shorter than 20 nucleotides and shortening the 5'UTR of artificial constructs to less than 12 nucleotides impairs the efficiency of translation from the AUG at its end (Kozak, 1991b). This is consistent with similar studies in yeast where decreasing the length of the 5'UTR of phosphoglycerate kinase mRNA from 45 to 21 or 7 nucleotides decreased the translational efficiency by 40-50% (van den Heuvel et al., 1989). The efficiency of translation from AUGs preceded by a short 5'UTR can be increased by the introduction of secondary structures downstream of the initiator codon which are proposed to "pause" the 43S pre-initiation complex at or near the AUG, allowing more time for recognition of the initiator codon (Kozak, 1991b). The 80S ribosome

assembled at the AUG then "melts" out the stem-loop allowing translation (see 1.5.1.4). Interestingly, the optimal placement for a stem-loop structure to increase the efficiency of translation from an initiator codon which is inefficiently utilised due to a short leader sequence or a poor context (see 1.5.1.5) is 14 nucleotides downstream of the AUG. This corresponds to the distance the leading edge of the ribosome "sitting" on the initiator AUG codon reaches into the ORF (Kozak, 1990a). Increasing the length of the 5'UTR by insertion of unstructured spacers results in an increase in the efficiency of translation. Sucrose gradient analysis revealed that this was due to the loading of additional 43S pre-initiation complexes on to the 5'UTR, a phenomenon described as "pre-loading" (Kozak, 1991a). However, many cellular mRNAs which have unusually long 5'UTRs are poorly translated due to the presence of upstream AUGs, small ORFs (see 1.5.1.5) and/or strong secondary structures (1.5.1.4) (Kozak, 1987a). This appears to be especially common in mRNAs encoding proto-oncogenes, transcription factors, or growth factors and their receptors suggesting that they may have been designed by nature to be poorly translated.

1.5.1.4 RNA secondary structure.

Transfection studies in mammalian cells and *in vitro* analyses suggested that the translational consequences of introducing secondary structures into the 5'UTR of indicator mRNAs were related to both the stability and position of the introduced structures (Kozak, 1986a; Kozak, 1989a; Pelletier and Sonenberg, 1985a). Glycerol gradient analysis revealed that a moderately stable stem loop structure in a cap-proximal position blocks access of the 43S pre-initiation complex to the mRNA (Kozak, 1989a). In addition, cross-linking studies showed that the interaction of eIF-4A either alone or as part of eIF-4F and eIF-4B with the mRNA was also severely inhibited (Lawson et al., 1986; Pelletier and Sonenberg, 1985b). In contrast, when the distance between the m^7G cap and the structure was

sufficient to allow 43S entry, the progression of the 43S pre-initiation complex and associated factors appeared to disrupt the secondary structure allowing translation to occur (Kozak, 1989a). Transfection of cells with an mRNA containing an AUG codon within this hairpin showed that this AUG was utilised, demonstrating that the scanning 43S ribosome "melted" the stem-loop rather than "hopping" or "shunting" over it (Kozak, 1986a).

In contrast, the introduction of a more stable stem-loop structure (- 61 kcal/mol) blocked translation *in vitro* even when located downstream of the cap. RNAse protection experiments identified a "stalled" 43S pre-initiation complex 5' to the inhibitory structure, suggesting that it was too stable to be disrupted by the 43S pre-initiation complex (Kozak, 1989a). Therefore, the inhibitory effects of introducing secondary structures into the 5'UTR depend on their stability and position, and can be elicited by at least two mechanisms.

Interestingly, mRNAs which are normally poorly translated because of stable secondary structure within their 5'UTRs are translated more efficiently in NIH 3T3 cells overexpressing eIF-4E (Koromilas et al., 1992). Overexpression of eIF-4E also induces transformation of cells to form foci on soft agar and causes tumours in nude mice (Lazaris-Karatzas et al., 1990) and this factor is now considered to be a potent proto-oncogene (Smith et al., 1990). Cellular levels of eIF-4E are thought to limit translation initiation by imposing competition between mRNAs to be translated (Duncan et al., 1987; Hiremath et al., 1985). Overexpression of eIF-4E may thus cause an increase in the pool of eIF-4F available to initiate translation and overcome selection against mRNAs with structure-burdened 5'UTRs. Thus the oncogenic potential of eIF-4E has been attributed to its ability to increase the translation of otherwise poorly translated mRNAs, such as those coding for growth factors whose 5'UTRs tend to contain extensive secondary structure (see below). The translation of at least two "growth related" mRNAs, cyclin D1 and ornithine decarboxylase, have been shown to be increased in response to eIF-4E overexpression (Rosenwald et al., 1993; Shantz and Pegg,

1994). The possibility remains that currently unrecognised biological activities of eIF-4E contribute to its oncogenicity.

Genetic studies in *S. cerevisiae* identified two gene products which overcome the inhibitory effects on translation imposed by cap-proximal secondary structures (Gulyas and Donahue, 1992; Yoon et al., 1992). Both these proteins (SSL1 and SSL2) have homology to nucleic acid binding proteins and SSL2 also shares homology with ATP-dependent helicases. However, neither of these proteins appears to be the yeast homologue of known mammalian initiation factors and their role in translation has recently been questioned, as they have been reported to be physically associated with the basal transcription factor TfIIH (Bootsma and Hoeijmakers, 1993).

Cellular mRNAs which contain extensive secondary structure in their 5'UTR include the human platelet-derived growth factor 2 (PDGF 2) mRNA which has a 1022 nucleotide GC-rich 5'UTR which confers a 30-40 fold reduction of CAT mRNA translation in transfected COS cells when placed into a CAT reporter mRNA (Rao et al., 1988). A GC-rich region of 140 nucleotides immediately preceding the AUG was found to inhibit CAT translation nearly as effectively as the entire 5'UTR, indicating that this region is responsible for the poor synthesis of PDGF 2 (Rao et al., 1988). Removal of the 5'UTR of PDGF 2 mRNA considerably increased translatability, which correlated with increased transforming potential of NIH 3T3 cells by PDGF 2 (Rao et al., 1988).

Ornithine decarboxylase (ODC), an enzyme involved in the synthesis of polyamines required for cell proliferation, is regulated at several levels. ODC mRNA is poorly translated, but its translation can be stimulated by growth factors and mitogens. Transfection experiments showed that translation of ODC mRNA is impeded by a 140 nucleotide GC-rich region close to the m^7G cap, which is predicted to form a very stable stem loop structure (Grens and Scheffler, 1990; Manzella and Blackshear, 1990). Mutations which reduce the stability of the predicted structure increase the

translational efficiency of ODC mRNA (Johannes and Berger, 1992). Translation of ODC mRNA was also poor when the orientation of the inhibitory region was inverted, indicating that the structure is inhibitory in itself rather than serving as a binding site for a repressor protein. It has been proposed that the growth related stimulation of ODC mRNA translation results from increased activity of translation initiation factors that allow the translation machinery to negotiate the previously impenetrable secondary structure (Manzella and Blackshear, 1990). Indeed, insulin stimulation of ODC mRNA translation correlates with increased phosphorylation of eIF-4B and eIF-4E (Manzella et al., 1991). Polyamines may affect mRNA structure, so it is possible that changes in intracellular polyamine concentration (reported to alter ODC mRNA translation), could influence the stability of the secondary structure in ODC (and perhaps other) mRNAs, and thus regulate their own production (Ito et al., 1990).

In the 3'UTR, secondary structure in itself has not yet been ascribed a role in regulating cellular mRNA translation. However, long range interactions between the 5'UTR and the 3'UTR have been implicated in regulating the translation of zein mRNA in *Zea mays* (Spena et al., 1985).

1.5.1.5 Upstream AUGs and upstream open reading frames.

The presence of AUG codons or short ORFs upstream from the main open reading frame generally reduces the efficiency of translation (Kozak, 1991c), due to the low efficiency of re-initiation by eukaryotic ribosomes. The effect of upstream AUGs and ORFs is dependent on the context surrounding the AUG and translational inhibition by upstream ORFs can be modulated by mutations which convert them into less efficient translational start sites (Kozak, 1981; Kozak 1984; Kozak, 1986b). The inhibitory effects of upstream ORFs can also be modulated by changing the sequence of the last codon and nucleotides 3' of it, which affect the ability of the ORF to mediate the resumption of scanning rather than dissociation of

the ribosome (Miller and Hinnebusch, 1989). In addition, the distance between the uORF and the main initiator codon influences the degree of inhibition, so that increasing the distance between the uORF and the protein coding frame increases the efficiency of translation possibly by increasing the available time for the initiation complex to reassemble (Kozak, 1987c). As discussed in section 1.5.1.3 the frequency of initiation from an upstream AUG codon which lies in a poor context, or even a non AUG codon (GUG or UUG) can be increased by the introduction of a moderately stable stem-loop structure downstream of it.

1.5.1.6 The poly (A) tail.

Most mammalian mRNAs are polyadenylated soon after transcription in the nucleus and carry 200-250 adenylate residues at their 3' terminus as they are transported into the cytoplasm (Hershey, 1991). The signals controlling polyadenylation are located within the 3' UTR and consist of a well conserved AAUAAA motif and a less conserved second motif downstream (Sachs and Wahle, 1993). In general the length of the poly (A) tail is decreased to about 50-70 residues in the cytoplasm, however the poly (A) tail can also be lengthened by cytoplasmic enzymes (Hershey, 1991). The deadenylation of many mRNAs which occurs during development is generally associated with a reduction in translation, however, the translation of some mRNAs like *Xenopus laevis* histone mRNAs and mammalian protamine mRNAs correlates with poly (A) tail shortening (Jackson and Standart, 1990; Ruderman et al., 1979; Woodland, 1980; Kleene, 1989). For many mRNAs it is unclear whether differences in polyadenylation leads to changes in translational efficiency or whether changes in the poly (A) tail length are the result of translational induction. However, in the case of the tissue plasminogen activator (tPA) mRNA in mouse oocytes, maturation-dependent polyadenylation was demonstrated to be the cause of translational activation (Huarte et al., 1987; Strickland et

al., 1988; Vassalli et al., 1989). The presence of a poly (A) tail of sufficient length is sufficient for translational activation of tPA mRNA (Vassalli et al., 1989), however other mRNAs such as G10 from *Xenopus* require *in vivo* polyadenylation for translational activation (McGrew et al., 1989). This suggests that for some mRNAs the process of polyadenylation rather than the length of the poly (A) tail itself determines the translational fate of the mRNA.

In yeast, the stimulatory effect of the poly (A) tail appears to be dependent on the poly (A) tail binding protein (PAB) (Sachs and Davis, 1989). Disruption of the PAB gene in yeast is lethal and can be partially rescued by suppressor mutants in the L46 protein of the 60S ribosomal subunit. Interestingly, five other suppressors were also assumed to be associated with the 60S ribosomal subunit due to their hypersensitivity to cycloheximide (Sachs and Davis, 1989). Based on these findings and the demonstration that poly (A) tails promote subunit joining *in vitro* (Munroe and Jacobson, 1990), a model was proposed whereby PAB bound to the poly (A) tail facilitates the recycling of the 60S subunit for renewed initiation (Sachs and Deardorff, 1992). Interestingly, some reports have suggested that the poly (A) tail and the m^7G cap along with its associated initiation factors act in concert to enhance translation (Gallie, 1991; Gallie and Tanguay, 1994; Munroe and Jacobson, 1990).

1.5.2 Global regulation of translation by phosphorylation.

Numerous components of the translational machinery are phosphoproteins including at least 13 initiation factors, three elongation factor subunits, three ribosomal proteins and a number of amino-acyl tRNA synthases (Hershey, 1991). The phosphorylation status of some of these, including several initiation factors changes in response to stress such as nutrient deprivation, extreme temperature and serum deprivation. eIF-2, is the only initiation factor whose phosphorylation correlates with an

inhibition of translation and this is considered to be the central control point in the initiation pathway (Clemens, 1994; Hershey, 1991). The α subunit of eIF-2 can be phosphorylated by a double stranded RNA-activated kinase (PKR) in response to double stranded RNA or interferon, or by a haemin-controlled repressor kinase (HCR) in response to haem deficiency in reticulocytes (as reviewed in Clemens, 1994), or by GCN2 kinase in yeast in response to uncharged tRNA (see 1.5.3.1). Phosphorylation of serine 51 (Colthurst et al., 1987), and perhaps also serine 48 (Kramer, 1990; Kudlicki et al., 1987; Kaufman et al., 1989; Choi et al., 1992) in the α-subunit does not inhibit the activity of eIF-2 *per se*, but inhibits its recycling (see 1.4.5) (Clemens, 1994; Pain, 1986). The binding of phosphorylated eIF-2 to eIF-2B forms a stable complex in which the bound GDP cannot be exchanged for GTP (Pain 1986; Dholakia and Wahba, 1989; Rowlands et al., 1988). As eIF-2 is normally in excess of eIF-2B, this essentially sequesters the cellular eIF-2B activity, and phosphorylation of a relatively small proportion of eIF-2 leads to a general inhibition of translation (Hershey, 1991). Some evidence suggests that eIF-2.GDP is normally bound to the 60S ribosomal subunit and that the phosphorylated form of eIF-2 prevents stable subunit joining (Gross et al., 1985; Gross et al., 1987; Thomas et al., 1985; Matts et al., 1988; Ramaiah et al., 1992). In addition, phosphorylation of eIF-2B has been shown to increase its activity by five fold (Dholakia and Wahba, 1988), which may under some conditions suppress the effects of phosphorylated eIF-2.

The phosphorylation of several initiation factors including eIF-4F, eIF-4B and eIF-2B (see above) correlates with an increase in translational activity (Morley, 1994). Phosphorylation of the α subunit of eIF-4F, eIF-4E at serine 53, has been reported to be absolutely required for its activity and mutants in which this serine is replaced by an alanine are incapable in participating in the formation of 48S pre-initiation complexes (Joshi-Barve et al., 1990). Recently, the phosphorylated form of eIF-4E was shown to have a greater affinity for the cap than the non-phosphorylated form (Minich et al., 1994). Interestingly, the same serine to alanine mutant of eIF-4E does not

cause malignant transformation when over-expressed (Lazaris-Karatzas et al., 1990). However, currently unpublished data from the same group (reported at a recent meeting) called into question the identity of this serine as a phosphorylation site. Phosphorylation of eIF-4B, eIF-3 and p220, which have multiple phosphorylation sites, occurs under the same conditions known to increase phosphorylation of eIF-4E (as reviewed in Morley, 1994).

1.5.3 Gene specific regulation of translation.

1.5.3.1 GCN4.

The mRNA for GCN4 which encodes a transcriptional activator of amino acid biosynthesis genes in *S. cerevisiae* is translationally controlled in response to amino acid starvation (Hinnebusch, 1984; Thireos et al., 1984) and represents the only mRNA for which the mechanism of translational control is currently well defined. The 5'UTR of GCN4 mRNA is 600 nucleotides in length and a ~240 nucleotide internal segment of this 5'UTR containing four uORFs can confer GCN4 regulation to a heterologous reporter mRNA (Mueller et al., 1987). Each of these uORFs in isolation causes repression of the main open reading frame independently of amino acid concentration (see 1.5.1.5) (Mueller and Hinnebusch, 1986). The repression exerted by each isolated uORF varies according to their role in GCN4 regulation and is determined in part by their position within the 5'UTR, but mainly by the sequences surrounding their termination codons, with uORF4 (counting from the 5' end) being the most inhibitory (Miller and Hinnebusch, 1989; Mueller and Hinnebusch, 1986; Williams et al., 1988). Point mutations and deletions of uORF 2 and 3 revealed that uORF 1 and 4 play the dominant role in GCN4 regulation (Mueller and Hinnebusch, 1986; Williams et al., 1988), where uORF 4 is required for efficient repression of GCN4 and uORF 1 acts as a positive element which derepresses GCN4 translation under conditions of amino acid starvation

(Mueller et al., 1987; Mueller and Hinnebusch, 1986). The termination sequences at uORF1 were shown to promote the efficient resumption of scanning allowing ribosomes to re-initiate either at GCN4 or under repressing conditions at uORF2, 3 or particularly uORF4 (Abastado et al., 1991a). Since increasing the distance between uORF1 and uORF4 resulted in an increased translation from uORF4 under derepressing conditions (Abastado et al., 1991a; Abastado et al., 1991b), it was proposed that ribosomes require additional time to regain their capacity to re-initiate under derepressing conditions. This results in failure of ribosomes to re-initiate at uORF 2, 3 or 4 under derepressing conditions; instead they initiate at the GCN4 main open reading frame as the additional time required to scan to this initiation site allows them to become competent for re-initiation (Abastado et al., 1991a; Abastado et al., 1991b).

Several cellular factors are known to play a role in GCN4 regulation and it was proposed that these factors alter the capacity of ribosomes to re-initiate in response to growth conditions (Williams et al., 1988). These factors are divided into two groups, the positive (GCN) factors required for the derepression of GCN4 expression and the negative GCD factors which are required for repression of GCN4. Since the GCN factors are not required for derepression in gcd strains (where the constitutive derepression of GCN4 mRNAs mimics GCN4 mRNA in which the AUG codons of all four uORFs have been mutated) it was suggested that the role of the GCN factors was to antagonise the GCD factors (Mueller and Hinnebusch, 1986). Genetic and biochemical analysis have shown that GCD1, 2, 6 7 and GCN3 are subunits of the yeast homologue of eIF-2B (Bushman et al., 1993a; Bushman et al., 1993b; Cigan et al., 1991; Foiani et al., 1991) and that GCD11 is the γ subunit of eIF-2 (Hannig et al., 1993). Interestingly mutants sui2 and sui3 in the α and β subunits of eIF-2 respectively have a gcd like phenotype (Williams et al., 1989). Cloning of GCN2, a ribosome associated kinase revealed that adjacent to its kinase domain is a histidyl-tRNA synthase like domain which is required for GCN2 function *in vivo* (Ramirez et al., 1991;

Wek et al., 1989; Wek et al., 1990). Aminoacyl-tRNA synthases distinguish between charged and uncharged forms of tRNA, and it was suggested that this domain acts as the sensor of uncharged tRNA for GCN4 activation (Wek et al., 1989). Activation of the synthase domain by uncharged tRNA would in turn lead to an activation of the GCN2 kinase domain. GCN2 has homology to HCR and PKR (see 1.5.2) (Ramirez et al., 1991), and was shown by genetic and biochemical means to phosphorylate eIF-2α (Dever et al., 1992). Thus in analogy to the regulation of global protein synthesis this leads to a reduction in the amount of active eIF-2 available for recruitment into initiation complexes (see 1.5.2). Therefore, additional scanning time is required for ribosomes to regain their ability to re-initiate, resulting in initiation competent complexes being reformed between uORF4 and the main GCN4 ORF in amino acid deprived conditions.

1.5.3.2 Regulation of translation by proteins binding to RNA motifs within the 5'UTR.

The binding of repressor proteins to sites within the 5'UTR facilitates the rapid regulation of subsets of specific genes/mRNAs in response to cellular or extracellular stimuli. The most intensively studied example of this type of translational regulation is that of ferritin mRNA which is discussed in section 1.6. However, several mRNAs have been recently identified which appear to control their translation by the binding of repressor proteins to RNA motifs within their 5'UTR. The following examples are amongst the best characterised.

Synthesis of ribosomal proteins in *S. cerevisiae, Xenopus laevis* and mammalian cell lines is known to be translationally regulated. This group of mRNAs typically contains short 5'UTRs of about 35-50 nucleotides with a polypyrimidine tract of about 8-14 nucleotides in a cap-proximal position. The 5'UTRs of *Xenopus* S19, murine S16 and murine L30 mRNAs are sufficient to confer regulation on reporter mRNAs in micro-injection and

transfection experiments (Amaldi et al., 1989; Levy et al., 1991). Deletion or mutation of the polypyrimidine tract in murine L32 and S16 mRNAs abolished translational control (Kaspar et al., 1992; Levy et al., 1991). However, the interpretation of these results is complicated by the involvement of these sequences in determining transcription start sites, which alter the position of the polypyrimidine sequence with respect to the m^7G cap. As with ferritin (see 1.6.5), regulation of murine S16 mRNA depends on the position of the polypyrimidine tract within the 5'UTR (Hammond et al., 1991). Although the polypyrimidine tract is necessary for regulation, it has not yet been demonstrated to be in itself sufficient. A 56 kDa protein binds to the polypyrimidine tract of the murine L32 5'UTR (Kaspar et al., 1992), whereas at least four proteins appear to interact with the 5'UTR of *Xenopus* L1 mRNA (Cardinali et al., 1993). Two of these proteins bind to the polypyrimidine tract, and one of them has a molecular weight of 57 kDa. A 57 kDa protein also interacts with the polypyrimidine tract of *Xenopus* L14 and S1 mRNAs (Cardinali et al., 1993). Binding of this protein to L1 mRNA is abolished when the polypyrimidine tract is relocated further downstream within the 5'UTR. However, binding of the 56/57 kDa proteins to the polypyrimidine tracts of L32 and L1 mRNAs does not correlate with the rate of synthesis of ribosomal proteins, and whether or not they are part of a regulatory complex remains to be determined.

Mst87F and a family of related genes involved in *Drosophila* spermatogenesis are translationally controlled via a conserved 12 nucleotide sequence within their 5'UTRs (Kuhn et al., 1991). Mutation of this element results in a loss of translational control and constitutive mRNA translation (Schäfer et al., 1990). The cap-proximal position of this element (+28), known as the translational control element or TCE, is conserved in all members of this family (Kuhn et al., 1991) and is functionally relevant as relocating it further from the cap structure impairs its ability to regulate translation (Kempe et al., 1993). A single protein was shown to cross-link to this element, though gel retardation analysis

suggested that this protein is part of a complex of proteins associated with this element and that sequences surrounding the TCE may influence the composition of this protein complex (Kempe et al., 1993).

The human thymidylate synthase (TS) mRNA whose product is involved in DNA synthesis, has been shown to undergo translational autoregulation *in vitro* (Chu et al., 1991). A 29 nucleotide stem-loop motif including the initiator codon of TS mRNA was shown to specifically interact with the TS protein (Chu et al., 1993). In contrast to ferritin (see 1.6.5), the stem-loop structure in TS mRNA is located 81 nucleotides downstream from the cap at the initiator codon, a situation which more closely resembles prokaryotic translational regulation. It is not yet clear whether this 29 nucleotide element is sufficient to direct translational control, particularly since a second binding site for TS has been identified in the ORF of the mRNA.

1.5.3.3 Regulation of translation by proteins binding to the 3'UTR.

Interestingly, mRNA translation can also be regulated by elements in the 3'UTR which serve as protein binding sites. Translational regulation by the 3'UTR is often associated with changes in poly(A) tail length (Jackson, 1993). The alterations in the length of the poly(A) tail may be a cause or a secondary consequence of translational regulation, depending on the system under investigation.

The best defined example of regulation where changes in polyadenylation are not required is that of erythroid 15-lipoxygenase (LOX) mRNA. Reticulocyte LOX has the unusual ability to attack intact phospholipid molecules and participates in the breakdown of internal cellular membranes during the late stages of reticulocyte maturation. Since LOX mRNA is expressed in erythroblasts, it must be translationally silenced in immature erythroid cells. The 576 nucleotide 3'UTR of rabbit LOX mRNA contains ten repeats of a slightly varied 19 nucleotide pyrimidine-

rich element, which starts 50 nucleotides downstream from the translation termination codon (Fleming et al., 1989). In human reticulocyte LOX mRNA, four such repeats seem to suffice for regulation. These repeats specifically interact with a 48 kDa protein, termed LOX-BP (Ostareck-Lederer et al., 1994) which can repress translation of LOX mRNA in rabbit reticulocyte cell-free translations. The 5' UTR and coding region of the LOX mRNA are not required for LOX-BP-mediated repression, as the LOX 3'UTR is sufficient to confer translational repression to a CAT reporter mRNA *in vitro* in the presence of LOX-BP (Ostareck-Lederer et al., 1994). There is currently little information on the mechanism by which LOX-BP blocks translation.

In contrast to LOX mRNA, the translational control of maternal mRNAs, during the early stages in development appears to involve changes in polyadenylation (Richter, 1993). One class of maternal mRNAs are translationally silenced during oogenesis and become polyadenylated and translated during maturation (Richter, 1993). This "unmasking" of these mRNAs requires two types of signal both of which are located in the 3' UTR, the nuclear polyadenylation signal and a U-rich sequence called the cytoplasmic polyadenylation element (CPE) in *Xenopus* and adenylation control element (ACE) in mouse (Fox et al., 1989; Huarte et al., 1992; McGrew et al., 1989). The sequence of the CPE and its position with reference to the nuclear polyadenylation signal controls the timing and extent of polyadenylation (Paris and Richter, 1990; Simon et al., 1992).

Several proteins which interact with CPEs in different mRNAs have been identified, including CPEB which binds to B4 mRNA and an 82 kDa protein which binds to G10 mRNA (Mc Grew and Richter, 1990; Paris et al., 1991). CPEB interacts with the CPE element of B4 mRNA in extracts from oocytes and eggs, however the 3' UTR of B4 mRNA only becomes polyadenylated in egg extracts (Paris et al., 1991). Addition of $p34^{cdc2}$ kinase to oocyte extracts which induces phosphorylation of CPEB coincides with CPE-dependent polyadenylation of B4 mRNA (Paris et al., 1991), suggesting

that polyadenylation occurs as a result of phosphorylation of CPEB. In contrast however, the interaction of the 82 kDa protein with G10 mRNA can only be detected in egg extracts suggesting that these proteins mediate control in different manners (Mc Grew and Richter, 1990). The function of these proteins in cytoplasmic polyadenylation and translation control requires further characterisation.

1.6 Regulation of ferritin.

1.6.1 Organisation of ferritin genes.

The organisation of both heavy and light chain mammalian ferritin genes is similar, with both genes spanning several kilobases of DNA and containing 3 introns (Costanzo et al., 1985; Hentze et al., 1986; Leibold and Munro, 1987; Santoro et al., 1986). The placement of these introns is conserved in H and L chains and between species but their size and sequences vary (Leibold and Munro, 1987). Analysis of H and L-chain mRNAs showed them to be closely related in structure, containing unusually long 5'UTR's of between 160-200 nucleotides, with coding regions of nearly identical length and 3'UTR's of around 150 nucleotides (Munro and Eisenstein, 1989).

1.6.2 Translational regulation of ferritin synthesis by iron.

The expression of ferritin was found to be regulated by iron almost 50 years ago (Granick, 1946). The failure of actinomycin D, an inhibitor of transcription, to inhibit the stimulatory effect of iron on ferritin synthesis implies that regulation was likely to occur post-transcriptionally and lead to the suggestion that ferritin mRNA is translationally regulated (Rogers and Munro, 1987; Zähringer et al., 1976). Sucrose gradient analyses revealed that cytoplasmic ferritin mRNA preferentially becomes associated with

translationally active polysomes upon iron induction, consistent with the idea that ferritin synthesis was translationally regulated (Aziz and Munro, 1986; Rogers and Munro, 1987). Further support for the translational control of ferritin came from the demonstration that ferritin mRNA levels remain constant irrespective of the degree of ferritin induction by iron (Aziz and Munro, 1986; Rogers and Munro, 1987; Shull and Theil, 1982; Shull and Theil, 1983).

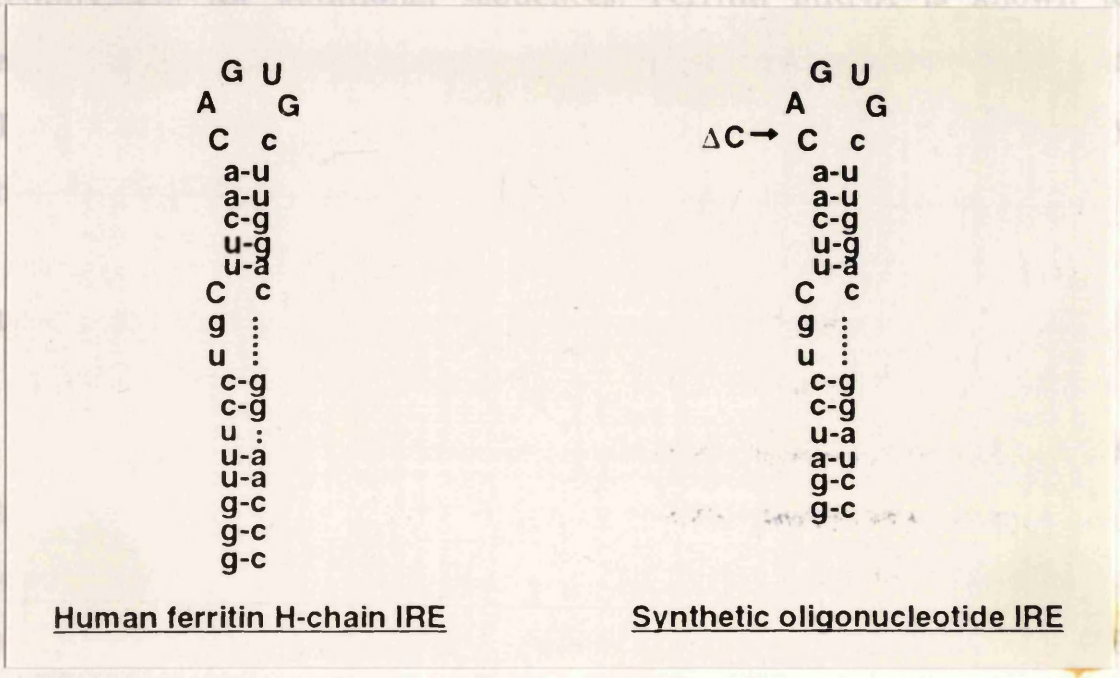
1.6.3 Cis-acting ferritin sequences required for regulation in response to iron.

The unusually long 5'UTR of ferritin was hypothesised to play a role in regulation based on studies of other translationally controlled mRNAs, which had implicated the involvement of sequences within their 5'UTRs in regulation (McGarry and Lindquist, 1985; Mueller and Hinnebusch, 1986). Transfection studies which linked the ferritin 5'UTR to heterologous indicator mRNAs revealed that the 5'UTR of ferritin H-chain mRNA contained all the sequences required to confer iron mediated regulation (Hentze et al., 1987a). Furthermore, deletional analysis identified a ~35 nucleotide region of the 5'UTR which was required for regulation (Hentze et al., 1987a; Hentze et al., 1987b). This region contains a 28 nucleotide sequence which is conserved in both H and L chain mRNAs and was predicted to form a moderately stable stem-loop structure (~ -5.0 kcal/mol) (Aziz and Munro, 1987; Hentze et al., 1987b; Leibold and Munro, 1987; Leibold and Munro, 1988). To assess the ability of this stem-loop structure to mediate iron regulation, synthetic oligonucleotides resembling this putative stem-loop structure were introduced into the 5'UTR of heterologous reporter mRNAs. Transient transfection showed that this stem-loop structure was sufficient to transfer regulation to either cytoplasmic or secreted proteins, implying that this stem loop functions as a regulatory element both on free and endoplasmic reticulum-associated

mRNAs (Hentze et al., 1988; Hentze et al., 1987b). Ferritin mRNA translation is known to mainly occur on free ribosomes but about 15% of ferritin mRNA translation occurs on endoplasmic reticulum associated ribosomes (Halliday et al., 1994). The requirement for this stem-loop structure was further demonstrated by the deletion of two separate cytosine nucleotides, one in the loop and the other in the upper stem (see figure 2) both of which abolished iron regulation (Hentze et al., 1988; Hentze et al., 1987b). The corresponding stem-loop identified in the L-chain ferritin was also shown to function in translational control (Aziz and Munro, 1987; Leibold and Munro, 1988). Thus the control of both H and L-chain ferritin is mediated by a conserved stem-loop structure known as the iron responsive element or IRE (Aziz and Munro, 1987; Hentze et al., 1987b).

Although the IRE is generally accepted to be sufficient for iron regulation, the degree of regulation of reporter mRNAs containing the ferritin 5'UTR or IRE alone was not as pronounced as the changes observed in ferritin synthesis (Aziz and Munro, 1987; Hentze et al., 1987a; Hentze et al., 1987b). This observation and reports suggesting that areas 5' and 3' to the IRE, the so called "flanking sequences", and a region of the 3'UTR were also important for regulation (Dickey et al., 1988; Harrell et al., 1991; Wang et al., 1990), stimulated a closer examination of these differences. However, this confirmed that substitution of the ferritin promoter and the coding region did not affect regulation showing that neither were required for translational regulation, in transfected cells (Aziz and Munro, 1987; Caughmann et al., 1988) or *in vitro* (Brown et al., 1989). Similarly, transfection of ferritin mRNAs containing an intact 5'UTR or a 5'UTR containing a deletion of the 3' flanking sequences, or removal of all the non IRE sequences showed that these mRNAs were indistinguishably regulated (Caughmann et al., 1988; Goossen and Hentze, 1992). Likewise, deletion of sequences in the 3'UTR had no effect on the range of iron regulation in transfected cells (Caughmann et al., 1988) or *in vitro* (Brown et al., 1989). These results strongly suggest that the IRE is the only ferritin sequence

Figure 2 The human ferritin H-chain IRE and a synthetic IRE.



The sequence of the human H-chain ferritin IRE and the synthetic IRE used in Hentze et al., 1987. The non-functional deletion derivative of the IRE used in Henzte et al., 1987 and this thesis is indicated by the ΔC, which indicates the deletion of the first C in the conserved loop of the IRE.

required to mediate iron responsive changes in ferritin translation. The exact range of regulation is dependent on cell type (Goossen and Hentze, 1992), and may be influenced by culture conditions, and although the iron regulation of reporter mRNAs is often noted to be smaller than that of endogenous ferritin (Coulson and Cleveland, 1993; Goossen and Hentze, 1992) the above comparisons suggest that this is a result of the very efficient translation of non-repressed ferritin mRNA and does not reflect a requirement for additional sequences. Ferritin mRNA is known to efficiently compete with globin mRNA for translation factors (Shull and Theil, 1982), which has been long recognised as a one of the most efficiently translated mRNAs.

1.6.4 Structure of the IRE.

Comparisons of IRE elements from ferritin heavy and light chains from different species and of transferrin receptor IREs (see section 1.6.14) resulted in the definition of a consensus IRE shown in figure 2 (Casey et al., 1988; Hentze et al., 1988). The phylogenetically conserved features of the IRE included i) a six membered loop with the sequence 5' CAGUGN 3' where N stands for any nucleotide except G; ii) an "upper stem" of four or five paired nucleotides; iii) an unpaired C residue separated by five nucleotides from the loop; iv) a "lower stem" of somewhat variable length (Hentze et al., 1988). Nuclease and chemical probing of the 5' end of ferritin mRNA supported the prediction that the IRE was a stem-loop structure with a six nucleotide loop and several bulges in the stem (Bettany et al., 1992; Wang et al., 1990). However, it has recently been suggested that base pairing may occur between positions 1 and 5 in the loop i.e. a C-G interaction (Henderson et al., 1994). The ferritin IRE bulge containing the conserved unpaired C was found to contain three nucleotides 5' UGC 3' (Bettany et al., 1992). Such bulges are known to both destabilize and introduce kinks into stem areas and may explain why some nucleotides in the upper stem were

found to be sensitive to reagents known to cleave single stranded regions of RNA (Wang et al., 1990; Bettany 1992). In addition, at least for bullfrog H-chain mRNA, the stem was found to extend beyond the IRE into the flanking sequences (Wang et al., 1990).

1.6.5 The importance of the IRE position for iron regulation.

The conserved cap-proximal position of the IRE within the 5' UTR of different ferritin mRNAs was shown to be functionally relevant, because an IRE only poorly mediated translational regulation when moved >60 nucleotides downstream from the m^7G cap in transfected HeLa and B6 cells (Goossen et al., 1990; Goossen and Hentze, 1992). This effect was observed independently of whether or not the sequences introduced 5' to the IRE contained secondary structure (Goossen and Hentze, 1992). It had been proposed that the role of the flanking sequences was to ensure that IRE elements which vary slightly in their positions within different ferritin mRNAs are located a constant distance of 8-12 nucleotides from the cap (Harrell et al., 1991). This was based on the observation that an inverse relationship exists between the distance of the IRE from the cap and the length of the flanking sequence in different ferritin mRNAs, however as discussed in section 1.6.3 these sequences appear to play no role. The results of Goossen and co-workers (Goossen et al., 1990; Goossen and Hentze, 1992) suggest that the correct positioning of the IRE within the 5'UTR is required for efficient regulation and raises the possibility that varying transcriptional start sites could modulate IRE-mediated translational repression. Heterologous transcriptional start sites have been noted for ferritin mRNA isolated from the reticulocytes of bull frog tadpoles and this heterogeneity was suggested to play a role in the developmental control of ferritin, however this remains to be determined (Didsbury et al., 1986).

1.6.6 Interaction of a cytosolic protein with the IRE.

The interaction of a cytosolic protein with the IRE was initially demonstrated by gel retardation analysis of extracts from rat tissues and cell lines which revealed that specific RNA/protein complexes were formed with IRE-containing mRNAs (Leibold and Munro, 1988). RNA/protein complex formation was found to be diminished when cells were pre-treated with iron, concurrent with an increase in ferritin synthesis. These observations lead to the proposal that this protein bound to and repressed ferritin translation when intracellular iron levels were low (Rouault et al., 1988). Scatchard analysis revealed that this protein has both a high (Kd = 10-30 pM) and low affinity (Kd = 2-7 nM) interaction with IRE, and addition of heparin as a competitor of nucleic acid-protein interactions resulted in a loss of the majority of the lower affinity interactions (Haile et al., 1989). Treatment of cells with the iron chelator desferrioxamine resulted in an increase in the number of high affinity complexes (Haile et al., 1989; Rouault et al., 1988). Deletion of the first cytosine in the loop prevented the formation of high affinity complexes and abolished translational regulation confirming that high affinity complex formation is required for regulation (Haile et al., 1989; Hentze et al., 1988; Rouault et al., 1988). UV crosslinking of labelled-RNA-protein complexes and subsequent RNAse digestion (label transfer) showed that a protein of approximately 87 kDa interacted with the IRE (Leibold and Munro, 1988). An apparently similar 90 kDa inhibitor of ferritin translation was identified in rabbit reticulocyte lysates and subsequently purified biochemically to apparent homogeneity from rabbit liver (Walden et al., 1989). The protein repressed translation of ferritin mRNA in wheat germ extract, which had previously been observed to occur freely (Shull and Theil, 1982), thus confirming the identity of this protein as repressor of ferritin synthesis (Walden et al., 1989). An analogous protein of 90-100 kDa was purified by IRE affinity chromatography from human liver and placenta, and its identity demonstrated by modulation of its IRE-

binding activity in response to agents known to affect the binding activity of the cytosolic regulatory protein (see 1.6.10) (Neupert et al., 1990; Rouault et al., 1989).

1.6.7 Structural features of the IRE required for its interaction with the cytosolic regulatory protein.

The structural requirements for the interaction between an IRE and this repressor protein known as the iron regulatory protein (IRP) [previously called the IRE-BP (Rouault et al., 1988), IRF (Neupert et al., 1990), FRP (Walden et al., 1989) and P90 (Harrell et al., 1991)] have been extensively studied. Foot-printing and toe-printing revealed that IRP protects the entire IRE sequence but not those sequences flanking the IRE, suggesting that IRP has the potential to make multiple contacts with various regions of the IRE (Harrell et al., 1991). Substitutions of nucleotides in the conserved loop showed that the requirement for a particular nucleotide at any of these positions varies, with the most 3' nucleotide of the loop, which is the least conserved, being most tolerant to change (Barton et al., 1990; Jaffrey et al., 1993; Leibold et al., 1990). Deletions or insertion of nucleotides in the loop were more detrimental than substitutions suggesting that the size of the loop is important for function (Hentze et al., 1987b; Rouault et al., 1988; Jaffrey et al., 1993). It has recently been suggested that positions 2-4 in the loop are primarily responsible for contacting IRP as nucleotides 1 and 5 have been proposed to be base paired to one another (Henderson et al., 1994). Substitutions of the conserved C in the invariable bulge of the IRE were better tolerated than deletion of this nucleotide, indicating that the presence of a bulge itself is more important than the presence of any particular nucleotide (Barton et al., 1990; Jaffrey et al., 1993; Leibold et al., 1990). Interestingly, a three nucleotide bulge (as is present in the ferritin H-chain IRE) was found to mediate more efficient IRP interactions than a single nucleotide bulge (as found in transferrin receptor

IREs see 1.6.14) (Henderson et al., 1994). The stem regions of ferritin and transferrin receptor IREs (see 1.6.14), both of which can mediate translational repression when located in the 5'UTR (Casey et al., 1988), are not conserved, suggesting that these regions provide the required secondary structure or, alternatively, that interactions of this region with the IRP are mediated via the sugar-phosphate backbone. Mutants which disrupt the complementarity of the upper stem severely diminish IRP-binding, while complementary mutations in the other side of the stem restore the majority but not all of the binding activity (Barton et al., 1990; Bettany et al., 1992; Leibold et al., 1990; Rouault et al., 1988). This indicates that there may be some contribution of primary sequences in the stem to the IRE/IRP interaction. Mutations which increase or decrease the length of the upper stem are detrimental, which may be a consequence of the necessity for a correct spatial arrangement between the bulged C and the loop, or reflect a requirement for a helical twist of 5 base pairs (Barton et al., 1990; Bettany et al., 1992; Jaffrey et al., 1993). Likewise, shortening of the lower stem, which reduces the stability of the IRE, severely affects IRE/IRP interactions (Barton et al., 1990).

The above data suggest that the overall three-dimensional structure of the IRE appears to be of greatest importance in order to provide the correct spatial arrangement of the primary recognition features. The conservation of sequences in loop and the bulged C suggest that the primary recognition features may be restricted to these regions, although sequences in the stem, especially the upper stem, may also be important. This is supported by analysis of cross-linked IRE/IRP complexes, which implicated a region from just 5' of the bulge to the fifth nucleotide in the loop to be in close contact with IRP (Basilion et al., 1994). This correlates well with previous observations that substitution of the last nucleotide in the loop has a less profound effect on IRE/IRP interactions than substitution of other loop nucleotides (Jaffrey et al., 1993). Interestingly, no base substitution in the IRE has yet been identified which increases the affinity of the IRE for IRP.

1.6.8 Cloning of the IRP.

IRP cDNAs have been cloned from human (Hirling et al., 1992; Rouault et al., 1990), mouse (Philpott et al., 1991), rat (Yu et al., 1992) and rabbit (Patino and Walden, 1992). The main open reading frame of the cDNAs encodes a protein of 889 amino acids with predicted molecular weights of 98.3 kDa, 98.1 kDa, 97.9 kDa and 98.5 kDa, respectively. Human IRP is estimated to have a pI of 6.2, close to the experimentally determined pI of 6.4-6.5 (Hirling et al., 1992). Comparisons of the predicted amino acid sequences showed that there was a high degree of homology between species, with the rabbit IRP being 92.6% identical to the mouse, 94.2% to the human and 92.8% identical to the rat IRP (Patino and Walden, 1992). The majority of non-identical amino acids are conservative changes resulting in an even higher similarity between IRPs.

The corresponding mRNAs of the cloned IRPs are similar in size with human being the longest (4 kb) and mouse (3.4 kb) the shortest, while the IRP mRNAs of rat and rabbit are both 3.6 kb in length. The cloned IRPs have 5'UTRs of between 71 and 236 nucleotides in length which contain no IRE like sequences or regions of significant homology between species. The 5'UTR of rat IRP which was cloned by primer extension is highly unusual in that it contains an inverted repeat, two direct repeats, and three uORFs, however this remains to be confirmed by genomic sequence (Yu et al., 1992). The length of the 3' UTR varies between species from 660 to 850 nucleotides and the 3' UTRs of rabbit, human and rat share considerable sequence homology, that between the rat and the shorter human 3' UTR being 68%. The potential functional relevance of this homology or of the unusual features of the rat 5'UTR for regulation of IRP synthesis remain to be determined.

1.6.9 Homology of IRP with aconitase and other proteins.

Comparison of human IRP with sequences in the data banks revealed a surprising homology with the Krebs cycle enzyme mitochondrial aconitase, an iron-sulphur protein which catalyses the isomerisation of citrate to iso-citrate (Hentze and Argos, 1991; Rouault et al., 1991). IRP is 30-33% identical and 53-56% homologous to the pig and yeast aconitases, respectively, and 53% identical to *E.coli* aconitase (Prodromou et al., 1992). Overlaying the sequence of IRP on to crystal structure of mitochondrial aconitase (Robbins and Stout, 1989a; Robbins and Stout, 1989b), showed that residues which form the active site of aconitase are conserved in IRP, including 3 cysteines which co-ordinate 3 of the 4 iron atoms involved in its 4S-4Fe cluster. Surface residues which are not so critical to aconitase function were not conserved in IRP (Rouault et al., 1991). This lead to the proposal that IRP may also be an iron-sulphur protein, and indeed may be identical with cytosolic aconitase which although previously described had not been cloned. The sensitivity of both cytosolic aconitase and IRP activity to redox agents (see 1.6.10.1), the similar size of cytosolic aconitase and IRP and the localisation of both their genes on chromosome 9 lent support to this idea (Hentze and Argos, 1991; Rouault et al., 1991). A comparison of sequenced peptides between cytoplasmic aconitase purified from beef liver and the predicted amino acid sequence of human IRP confirmed that human IRP and bovine cytosolic aconitase are essentially identical (Kennedy et al., 1992). In addition, IRP and aconitase were shown to have homology to another isomerase, isopropylmalate (IPM) isomerase from *Mucor circinelloides*. IPM isomerase which also contains a 4Fe-4S cluster, catalyses the second step in the leucine biosynthesis pathway and is 22% identical and 47% homologous to IRP (Hentze and Argos, 1991). The implications of the homology between IRP and aconitase will be discussed with respect to the regulation of IRP activity (see 1.6.10.4-5). Comparisons with sequences in the data bank revealed that human IRP also contains a

nucleotide binding consensus sequence which most closely resembles the NADH binding site of *E.coli* oxidoreductase and a metal binding site. However no sequences which resemble known RNA binding motifs were identified (Rouault et al., 1991).

1.6.10 Regulation of IRP activity.

1.6.10.1 Redox sensitivity of IRP.

The activity of IRP is sensitive to treatment with redox reagents whereby exposure of IRP to oxidising agents like diamide diminished its IRE-binding activity, however this activity is fully restored by treatment with reducing agents such as 2-mercaptoethanol or DTT. The redox sensitivity of IRP suggested that it may require free sulphydryl groups to bind IRE, oxidation of which resulted in disulphide bridge formation, thus making them unavailable for such interactions (Hentze et al., 1989a). The irreversible inactivation of IRP by N-ethylmaleimide (NEM), an alkylating agent which blocks free sulphydryl groups supported this hypothesis. Treatment of cell lysates from iron starved or untreated cells with 2-mercaptoethanol revealed that the degree of activation of IRE-binding activity was much greater with untreated cells. It was therefore suggested that the redox perturbations of IRP activity *in vitro* reflected changes in the activity of IRP in cells in response to different intracellular iron concentrations, which affected the amount of IRP in the active (reduced) and inactive (oxidised) state. The high and low affinity binding forms were proposed to result from conformational changes in IRP mediated by at least one sulphydryl pair, resulting in low IRE-binding activity in oxidised IRP and a 100 fold increase in IRE-binding activity in reduced IRP (Haile et al., 1989) and active IRP in cells was subsequently shown to be in the reduced form (Müllner et al., 1992). However, although both redox agents and iron were found to regulate the activity of IRP, treatment of cells with either

diamide or iron salts revealed that the effects of redox and iron perturbations on IRP were qualitatively different (Müllner et al., 1992). The relationship between the sensitivity of IRP to redox agents and iron will be discussed in the sections (1.6.10.2 and 1.6.10.4-5).

1.6.10.2 The role of haem and non-haem iron in IRP regulation.

Despite intensive study, controversy still exists as to whether IRP activity is altered by intracellular levels of iron itself (chelatable iron) or iron in the form of haem. Investigators who support haem as being the regulator of IRP activity argue that IRP regulation does not require the release of iron from haem by haem oxygenase based on the inability of the iron chelator deferrioxamine, which cannot chelate iron from haem, to block the effects on haem addition (Ward et al., 1984). In this model for IRP regulation, addition of iron to cells results in an increase in haem synthesis and excess haem modulates the activity of IRP, and a study which used inhibitors of the haem biosynthetic pathway lent credence to this idea (Ward et al., 1984). However, others found that addition of deferrioxamine to cells did interfere with the decrease in IRE-binding and changes in ferritin and transferrin receptor synthesis otherwise observed upon haem addition (Haile et al., 1990; Rogers and Munro, 1987; Rouault et al., 1985). In contrast to the findings of Ward et al. (1984), a study which utilised inhibitors of haem biosynthesis and haem degradation in conjunction with iron chelators indicated that chelatable iron was the inducer of ferritin synthesis (Eisenstein et al., 1991). In addition, a separate study which investigated the effects of protoporphoryrin IX (the immediate precursor of haem which contains no iron) addition to cells also supported the role of chelatable iron in regulating IRP activity (Müllner et al., 1992).

1.6.10.3 The model for haem regulation of IRP activity.

The effects of haemin on ferritin translation have been studied in vitro and translation of ferritin mRNA was found to be partially de-repressed by pre-treatment of IRP with haemin, but not with iron salts, prior to addition to wheat germ extract. This effect was not mediated by free radicals (Lin et al., 1990) and haemin did not induce crosslinking or degradation of IRP (Lin et al., 1990). However, haemin stably associated with IRP (Lin et al., 1991) and this interaction was localised to a specific 17 kDa proteolytic fragment of IRP (Lin et al., 1991). The affinity of IRP for haem was found to be intermediate between that of the haem binding protein apohemopexin and BSA (Lin et al., 1991). The interaction of haemin with IRP was increased by the presence of reducing agents and inhibited by the addition of NEM suggesting that cysteine residues may be involved (Lin et al., 1991).

It was proposed that iron regulation of ferritin synthesis by haem resulted from the destruction rather than modification of IRP, based on the observation that haem appeared to stimulate the degradation of IRP in cells (Goessling et al., 1992). This degradation was suggested to have two phases, an initial reversible phase where IRP exists in 200 kDa complexes, and a second irreversible phase which coincided with ferritin induction where the 200 kDa IRP complex was lost and smaller peptides were generated (Goessling et al., 1992; Goessling et al., 1994). Consistent with this, re-repression of ferritin translation was found to require protein synthesis presumably to regenerate IRP (Goessling et al., 1994)..

However, a number of findings suggested that the *in vitro* effects of haem on IRP were not fully specific; firstly haemin inhibited the binding of other RNA binding proteins and the activity of a variety of enzymes (Haile et al., 1990), secondly, Scatchard analysis revealed that haem equally affected both low and high affinity interactions of IRP (Haile et al., 1990); and thirdly that other protoporphryrins especially cobalt protoporphryrin also inhibited

IRE/IRP interactions (Haile et al., 1990) and finally, unlike changes in IRP activity induced by iron, those mediated by haemin were not resistant to dilution or chromatography (Haile et al., 1990). In addition, several lines of evidence strongly suggest that IRP is not regulated by degradation in cells. Firstly full IRP-binding activity can be recovered from cell lysates of cultured cells or live rats which have been treated with either chelatable iron or haemin by treatment with 2-mercaptoethanol, indicating that IRP has not been degraded but post-translationally modified in a reversible manner (Haile et al., 1989; Hentze et al., 1989a; Rothenberger et al., 1990; Yu et al., 1992). Secondly, the changes in IRP activity when cells are moved from high to low iron-containing media were not found to be affected by the inhibitors of protein synthesis puromycin or cycloheximide (Hentze et al., 1989a; Tang et al., 1992). Thirdly, the activity of human recombinant IRP could be modulated in stably transfected cells without changes in the rate of its synthesis or degradation (Emery-Goodman et al., 1993; Tang et al., 1992).

1.6.10.4 The model for IRP regulation by chelatable iron.

Concerns over the conflicting data obtained for haem as an IRP regulator in conjunction with the observation that IRP shares extensive homology with the iron sulphur protein mitochondrial aconitase (see 1.6.8) prompted a further investigation of the role of chelatable iron in regulating IRP activity. A model for the regulation of IRP by chelatable iron has been proposed, based on two characteristics of mitochondrial aconitase. Firstly, the active site cleft of mitochondrial aconitase is located between three tightly packed domains and a fourth domain which is attached by a flexible hinge (Rouault et al., 1991). Secondly, the fourth iron in the cluster of mitochondrial aconitase, which is liganded to its substrate citrate is labile and lost upon purification. Purified mitochondrial aconitase is therefore inactive and contains a 4S-3Fe cluster which can be reconstituted to a 4S-4Fe cluster *in vitro* by iron loading procedures resulting in active aconitase.

Although it is not clear whether such changes can occur in the cluster of mitochondrial aconitase in cells, it has been proposed that they could provide a working model for regulation of IRP by iron (Hentze and Argos, 1991; Rouault et al., 1991). When intracellular iron concentrations are low, removal of iron from the cluster would result in the opening of the active site cleft presumably by pivoting of the fourth domain on the flexible hinge, thus allowing IRE-binding and repression of ferritin translation. Moreover, the RNA-binding activity of IRP was reduced when it was subjected to iron loading procedures used to reconstitute the mitochondrial aconitase cluster (Constable et al., 1992). This effect was not due to the formation of free radicals and was specific for iron rather than other metals (Constable et al., 1992). Furthermore, these procedures did not affect the interaction between U1A another RNA binding protein (which does not contain an iron-sulphur cluster) and its binding site (Constable et al., 1992). However addition of iron-treated IRP to wheat germ extract reconstituted its IRE-binding activity (Constable et al., 1992), due to the presence of reducing agents in the extract. This result may explain why iron salts had previously failed to influence ferritin translation in wheat germ extract (Lin et al., 1990). This reactivation provided evidence that the *in vitro* inactivation of IRP by iron salts is reversible and does not result from degradation of IRP as was also shown by SDS-PAGE analysis (Constable et al., 1992). Iron loading of IRP in lysates derived from deferrioxamine treated cells gave similar results (Haile et al., 1992a). Activation of IRE-binding activity by reducing agents at alkaline pH in lysates from haemin-treated cells could be blocked by the addition of citrate (Haile et al., 1992a), which is known to stabilise the labile fourth iron in aconitase. Therefore conditions which stabilise the aconitase iron-sulphur cluster also interfere with the activation of IRE-binding activity consistent with the proposal that changes in IRE-binding are mediated by modification of the iron-sulphur cluster (Haile et al., 1992a). Interestingly, IRP from haemin-treated cells was found to possess significant aconitase activity, comparable to that of mitochondrial aconitase, whereas

iron-loading of IRP from deferrioxamine-treated cells or of purified recombinant IRP resulted in the appearance of aconitase activity (Emery-Goodman et al., 1993; Gray et al., 1993; Haile et al., 1992a). IRP can therefore possess either IRE-binding or aconitase activity with iron manipulations either in cells or *in vitro* resulting in a reciprocal change in these activities. The available evidence therefore strongly favours a reversible post-translational mechanism for IRP regulation with iron as the major regulator of IRP activity but does not rule out the possibility that haem regulates IRP under certain conditions. Furthermore, it seems that regulation of IRP2 (see 1.6.12) requires synthesis of new IRP2 (Pantopolous et al, manuscript in preparation), raising the possibility that this IRE-binding protein is regulated by a degradation mechanism. It is therefore possible that the cell type used in the studies of Thach and co-workers contained a relatively high proportion of IRP2 and that the loss of IRP observed in this study represented loss of IRP2 and not IRP.

1.6.10.5 Cluster status and co-ordination of IRP with IRE-binding (or aconitase) activity.

The fact that IRP from haemin treated cells has aconitase activity implies that the non-IRE-binding form of IRP contains a 4S-4Fe cluster. However these experiments did not clarify the cluster composition of IRP with IRE-binding activity. Indirect evidence had previously suggested that the IRE-binding form of IRP might be the apo-form (Hirling et al., 1992; Walden et al., 1989). To characterise the various forms of IRP, IRP was subjected to treatment with ferricyanide which causes disassembly of the cluster by oxidative destruction. Treatment of IRP from haemin treated cells with a low concentration of ferricyanide was sufficient to abrogate aconitase activity but not to activate IRE-binding in the presence of low concentrations of reducing agents. However, at higher ferricyanide concentrations the IRE-binding activity of IRP from haemin treated cells

was induced by low concentrations of reducing agents and was not antagonised by the presence of substrate (Haile et al., 1992b). Previous characterisation of the IRE-binding activity of defined forms of cytoplasmic aconitase had shown that these characteristics were shared by the 4S-3Fe and apo-forms of cytoplasmic aconitase respectively (Haile et al., 1992b). Therefore these results strongly suggest that IRP active in IRE-binding in iron starved cells is the apo-form of the protein.

Mutation of any of the cysteine (cys) residues (437, 503 and 506) predicted to co-ordinate the iron-sulphur cluster to serine (ser) abolished aconitase activity consistent with these cysteines having a role in cluster formation (Hirling et al., 1994; Philpott et al., 1993). However, all these mutants possess IRE-binding activity which could not be iron-regulated in transfected cells, thus directly demonstrating that the cluster is required for iron regulation (Hirling et al., 1994; Philpott et al., 1993). Treatment of wild type apo-IRP, and single and double ser mutants with diamide and NEM revealed that oxidation of IRP involves cys 437 and either cys 503 or 506 (see 1.6.10.1), and moreover that cys 437 was the site of NEM inactivation (see 1.6.10.1). However, alkylating agents such as iodoacetamide which transfer a smaller moiety than NEM do not block IRE-binding activity of wild type IRP and the ser 437 mutant has IRE-binding activity both of which show that an area in the vicinity of this cysteine rather than cysteine 437 itself is involved in RNA binding. It is unclear how high concentrations of reducing agents activate IRE-binding *in vitro* seemingly overriding the presence of an iron-sulphur cluster in IRP. It has been proposed that reduction of 4S-4Fe IRP may cause cysteine 437 to separate from the cluster, which remains intact bound only to cysteines 503 and 506. IRE-binding activity is lost when the reducing agent is removed (Haile et al., 1992a) as cysteine 437 is quickly re-integrated into the cluster. It is unlikely however, that the conditions within the cell could induce this form of IRP, and IRP probably cannot not interact with RNA in cells when the cluster is assembled but requires conversion to the apo-form, through reduction of the intra-cellular iron

concentration. The harsh conditions required for disassembly of the cluster *in vitro* suggest that this may be an enzymatic process in cells. However, it is clear that the enzymatic activity of IRP is not required for this process (Philpott et al., 1994) and such a putative enzyme activity remains to be identified.

1.6.10.6 Regulation of IRP by nitric oxide.

It is known that nitric oxide (NO) from macrophages inactivates mitochondrial aconitase (Drapier et al., 1991). NO is a free radical with a high chemical reactivity towards iron, and the loss of aconitase activity in targeted cells coincides with the formation of nitrosyl-iron complexes, suggesting that NO targets the iron-sulphur cluster in aconitase (Drapier et al., 1991). This raised the possibility that NO may also affect the activity of IRP. Consistent with this idea, exposure of IRP to NO gas *in vitro* resulted in an increase in IRE-binding activity (Drapier et al., 1993). Stimulation of macrophages with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) which results in the induction of nitric oxide synthase (NOS) and the production of NO (Weisz et al., 1994) caused a decrease in the cytoplasmic aconitase activity and an increase in IRE-binding activity (Drapier et al., 1993; Weiss et al., 1993). The increase in IRE-binding activity stimulated by NO was equal to that observed for deferrioxamine treatment of cells. Inhibitors of NOS activity such as N^G-methyl-L-arginine prevented the induction of IRE-binding activity showing that this effect was due to the production of NO and not due to some other effect of induction by LPS or IFN-γ (Drapier et al., 1993; Weiss et al., 1993). NO is also synthesised by other mammalian cells such as vascular cells, hepatocytes and neurons (Snyder and Bredt, 1992) and the effect on IRP could also be observed in non macrophage cell lines (Drapier et al., 1993; Weiss et al., 1993) suggesting that this mechanism of IRP-mediated regulation may not be restricted to macrophages. Stimulation of NO production also induced translational repression of ferritin mRNA

and reporter mRNAs containing a functional IRE (Weiss et al., 1993). Whether the effect of NO is mediated through an interaction with the iron cluster that causes a conformational change in IRP which allows RNA binding or by destruction of the cluster, is not known. The ability of NO to form iron-nitrosyl complexes also raises the possibility that NO may exert its effect in a manner similar to deferrioxamine by chelation of the intracellular iron pool.

1.6.10.7 Phosphorylation of IRP.

Phosphorylation of IRP by protein kinase C (PKC) has been observed *in vitro* and two peptides containing putative PKC phosphorylation sites from human IRP were efficiently phosphorylated, one containing serine 138 and the other containing serine 711 which was the preferred target of PKC. Both these serines are predicted to be accessible to kinases and ser 711 is thought to lie close to the outer edge of the active cleft near to an arginine residue implicated in citrate binding (Eisenstein et al., 1993). Stimulation of PKC activity in cells resulted in phosphorylation of IRP and correlated with an increase in the amount of IRP present in the high affinity binding form (Eisenstein et al., 1993). PKC plays a role in a number of signal transduction pathways and might allow extracellular agents such as hormones and growth factors to override or alter the effects of intracellular factors such as iron. This may be the case in T-cell proliferation where ferritin and transferrin synthesis are altered, concomitant with an increase in IRE-binding activity increasing the availability of iron for incorporation into iron-containing proteins required for proliferation (Teixeira and Kühn, 1991).

1.6.11 Areas of IRP implicated in RNA binding

Chemical modification of the critical cysteine residues in IRP has suggested that they are not directly involved in IRE-binding (see 1.6.5.10). UV crosslinking data have implicated a region from residues 121-130, which is predicted to lie deep inside the active site cleft to interact with the IRE (Basilion et al., 1994). Two of the residues in this region correspond to active site residues in mitochondrial aconitase while others are thought to comprise a region of IRP not present in mitochondrial aconitase. However, mutational analysis has also implicated several arginine residues (residues 536, 541 and 780), which are known to mediate RNA/protein interactions in some cases, to be involved in IRE-binding (Philpott et al., 1994). One of these, Arg 780 is located in the fourth domain consistent with the finding that deletion of 132 nucleotides at the C-terminal of IRP inactivated IRE-binding (Hirling et al., 1992). Taken together, these results suggest that residues in domains 1, 3 and 4 some of which may be arginines contact IRP. In addition, these results support the model that a conformational change associated with the aconitase and IRE-binding forms of IRP may involve movement of domain four away from domains 1-3 by pivoting of the hinge linker domain. This movement around the hinge linker exposes residues deep within the active site cleft for binding to the IRE.

1.6.12 Additional IRPs.

Gel retardation assays of extracts prepared from rodent cell lines revealed the presence of a second iron-responsive IRE-binding protein known as IRP2 (Leibold and Munro, 1988; Müllner et al., 1989; Rothenberger et al., 1990) estimated by label transfer to be ~105 kDa, slightly larger than IRP (Henderson et al., 1993). Cross-linking of IRE/IRP and IRE/IRP2 complexes and subsequent protease digestion, showed that some of the peptides generated co-migrated, indicating that IRP and IRP2 are distinct but

structurally related proteins (Henderson et al., 1993). Antibodies raised against IRP did not recognise IRP2 consistent with it being a distinct protein (Henderson et al., 1993). IRP and IRP2 have equal affinities for a ferritin IRE, but a variant IRE-containing the loop sequence GAGAGU (rather than CAGUGC) is only recognised by IRP (Hirling et al., 1994). The activity of IRP2 appears to be cell-type specific, being most active in tissues associated with iron transport across cell layers (Henderson et al., 1993).

Purified human IRP migrates as a doublet (Neupert et al., 1990; Tang et al., 1992) suggesting that a second IRP may also exist in human cells. However protease digestion revealed that the heterogeneity between the two species was restricted to the N-terminal peptide (Hirling et al., 1992). This is consistent with the doublet representing allelic forms of IRP, or different forms of IRP arising from alternative splicing or post-translational modification. Hybridisation of genomic DNA from human cells with the human IRP cDNA identified a single IRP gene covering 40 kb of chromosome 9 (Hentze et al., 1989b; Hirling et al., 1992; Rouault et al., 1990). However screening with a degenerate oligonucleotide generated from a peptide sequence of IRP resulted in the isolation of a cDNA which was similar to but distinct from IRP and was localised to chromosome 15 (Rouault et al., 1990). This second human IRP clone awaits further characterisation.

1.6.13 Other mechanisms of ferritin regulation.

In addition to the well defined translational control of ferritin mRNA, iron and iron compounds have been shown to affect the transcription, transport and stability of ferritin mRNA (Harford et al., 1994). However, although these effects may contribute to ferritin regulation, translation accounts for a 30-50 fold range in ferritin regulation whereas transcription, for example, only accounts for a 2-5 fold difference in the range of regulation in response to iron. Ferritin transcription is also altered

by a number of other agents and circumstances including differentiation, cytokines (which may also affect ferritin mRNA translation), adenovirus E1A and transcription factor NF-κB (Harford et al., 1994).

1.6.14 Regulation of transferrin receptor (TfR) mRNA

Synthesis of the TfR is also controlled by IRP in response to changes in intracellular iron levels (Casey et al., 1989). Five IRE elements are located in the 3'UTR of TfR mRNA however, they are not sufficient for its iron regulation. A rapid turnover element is also required, with the entire regulatory region spanning ~680 nucleotides of the 3'UTR (Casey et al., 1988; Casey et al., 1989; Müllner and Kühn, 1988; Owen and Kühn, 1987). However iron regulation can be reconstituted with as little as 250 nucleotides of the 3' UTR containing the rapid turnover determinant flanked by three IRE elements B, C and D (where IRE A is the most 5' IRE) (Casey et al., 1989). Deletion of this area renders TfR mRNA stable, while deletion of the most 5' cytosine in the loop of each of these IREs, which renders them incapable of high affinity interactions with IRP, results in TfR mRNA being constitutively degraded (Casey et al., 1989; Koeller et al., 1991; Rouault et al., 1988). TfR mRNA is initially cleaved in a site-specific manner, and the cleavage site has been mapped by primer extension of the 3' cleavage product to a single stranded region just 3' of IRE C (Binder et al., 1994). The endonuclease responsible for this degradation appears to be short lived and remains to be further characterised (Koeller et al., 1991). Temperature-dependent elution of full-length TfR mRNA and the 3' cleavage product from poly (U) Sephadex showed that cleavage is not preceded by poly (A) tail shortening (Binder et al., 1994). When intracellular iron concentrations are low the binding of IRP to TfR mRNA is thought to protect it from endonuclease attack, thereby increasing synthesis of transferrin receptors and consequently iron uptake into the cell.

1. 7 Aims of the thesis.

The aim of the initial part of this work was to develop cell-free systems to study IRP-mediated translational control of IRE-containing mRNAs *in vitro*. These were characterised for their potential to reproduce the known characteristics of the IRE/IRP system defined from studies in cells. These systems were then utilised to elucidate the mechanism of IRE/IRP repression, using sucrose gradient analysis of initiation complexes formed in these cell-free systems to study the interaction of ribosomal subunits with IRP-bound mRNAs. The association of ribosomal subunits with mRNAs in which the IRE was located in cap-distal positions was also examined, in order to search for a molecular explanation for the previous observation that in cells the position of the IRE within the 5'UTR is critical for its function. Finally, these systems were used to characterise newly identified IRE-containing mRNAs whose translation may be regulated by a similar mechanism.

Chapter 2: Experimental Materials and Methods.

2.1 Materials.

2.1.1 Enzymes.

Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, FRG). RNase-free DNase I and Klenow fragment of *E.coli* DNA polymerase I were from Serva (Heidelberg, Germany). T7 RNA polymerase was supplied by Stratagene (Heidelberg, Germany) or United States Biochemicals (USB; OH, USA), and Inhibit Ace was purchased from 5Prime-3Prime (PA, USA). T4 DNA ligase was from Gibco-BRL (Eggenstein, Germany). T4 polynucleotide kinase was from Pharmacia (Uppsala, Sweden).

2.1.2 Purified Proteins.

BSA was from Sigma (Diesenhofen, Germany). Purified human recombinant U1A protein from *E.coli* (Stripecke and Hentze, 1992) was a gift from Dr. R. Stripecke (EMBL) and recombinant human IRP from vaccinia virus infected HeLa cells (Gray et al., 1993) was kindly provided by Sabine Quick (EMBL).

2.1.3 Specialised reagents.

Wheat germ for the preparation of wheat germ extract was supplied by General Mills Inc. (CA, USA) and rabbit reticulocyte lysate was from Serva (Heidelberg, Germany). Oligo (dT) cellulose, rRNA and polyclonal anti-ferritin antiserum were purchased from Boehringer Mannheim. Polyclonal anti-U1A antiserum was from Nina Dathan (EMBL) and anti-eALAS antiserum was from B. Goossen (EMBL). CL4B protein A Sepharose,

heparin Sepharose CL6B, poly (U) Sepharose and the oligolabelling kit were from Pharmacia. Protein and nucleic acid molecular weight markers and vanadyl ribonucleoside complex were supplied by Gibco-BRL. Bacterial media were purchased from Gibco-BRL, and tissue culture media were supplied by Seromed (Berlin, FRG). m^7GpppG cap analogue was supplied by New England Biolabs (NEB; MA, USA). Agarose was supplied by BRL and acrylamide, bis-acrylamide and dimethyldichlorosilane solution were purchased from BDH (Poole, UK). SDS was from Serva, guanidine thiocyanate was purchased from Fluka Chemie (Neu-Ulm, Germany). HEPES was obtained from Gibco-BRL. Imidazole and heparin were from Sigma and Ni^{2+}-NTA agarose beads and Qiagen tip 20 and 500 plasmid purification columns were from Qiagen (Hilden, Germany). GMP-PNP was from Boehringer Mannheim and edeine was a gift from Dr. R. Jackson (Cambridge, UK). Ribonucleotides and cycloheximide were from Sigma. The Mermaid system for the purification of short DNA fragments was from Bio 101 Inc (CA, USA) and the Sequenase kit for sequencing was from USB. Entensifying solutions, Entesify A and B were provided by Dupont (Bad Homburg, Germany) and Kodak X-OMAT AR films were obtained from Sigma. 3MM chromatography paper and 3MM circular cellulose paper filters were from Whatman (UK) and NY13 Nytran filters and nitro-cellulose filters were purchased from Schleicher and Schuell (Dassel, Germany). BCA protein assay system was supplied by Pierce (Illinois, USA) and ready-safe liquid scintillation cocktail was from Beckman (CA, USA). Bacterial strains RR1, XL1-Blue and BL21(DE3) were from B. Goossen (EMBL). Other chemicals not listed here were obtained from Sigma or Merck (Darmstadt, Germany).

2.1.4 Isotopes.

[α-^{35}S]dATP (1000 Ci/mmol), [α-^{32}P]UTP (800 Ci/mmol), [α-^{32}P] dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (> 5000 Ci/mmol) and [^{35}S] methionine (>1000 Ci/mmol) were obtained from Amersham International, (Amersham, UK).

2.1.5 Synthetic oligodeoxyribonucleotides.

Oligodeoxyribonucleotides were synthesised by the phosphoramidate method (Sinha et al. 1984, Matteucci and Caruthers 1981) on an Applied Biosystems synthesizer. All oligodeoxyribonucleotides used in this work were made in the laboratory of Dr. B. Sproat at EMBL by Susie Weston, Samantha O'Loughlin and Viviane Adam.

2.2 Methods.

2.2.1 Microbiological Techniques.

2.2.1.1 Bacterial strains used.

The *E.coli* strains RR1 (genotype: $F^-\Delta$[gpt-proA]62, leu, supE44, ara14, galK2, lacY1, Δ[mcrC-mrr], rpsL20 [str^r], xyl-5, mtl-1, $RecA^+$) and XL1-Blue (genotype: endA1, hsdR17 [r_k-, m_k+], supE44,thi-1, λ-, recA1, gyrA96, relA1, [lac-], [F', proAB, $lacI^QZ\Delta M15$, Tn10, {tet^r}]) were used for propagation of pGEM-3zf plasmids. BL21(DE3) (genotype: F^- ompT $r_B^-m_B^-$[DE3]) was used for propagation of pT7-His-hIRF.

2.2.1.2 Maintenance and media.

RR1, XL1-Blue and BL21(DE3) transformed with plasmids carrying the ampicillin resistance gene were grown in Luria Bertani (LB) medium or on LB agar plates and 100 µg/ml of ampicillin at 37°C or in the case of BL21(DE3) at 25°C (see 2.2.10.2) as described (Sambrook et al., 1989).

2.2.1.3 Preparation of competent cells.

Overnight cultures of *E. coli* cells (2 ml) were diluted 1 in 100 in LB-medium containing 0.1 % glucose, grown until OD_{600}=0.5 and placed on ice. Cells were pelleted at 3,000 rpm for 5 min at 0°C, resuspended in 100 ml ice-cold 100 mM $MgCl_2$, pelleted again at 0°C and resuspended in 100 ml ice-cold 100 mM $CaCl_2$. After 20 min on ice, cells were pelleted for a final time at 0°C before being resuspended in 10 ml 15% glycerol containing 100 mM $CaCl_2$ and frozen in liquid nitrogen and stored at -80°C.

2.2.1.4 Transformation of competent cells.

Cells (50 µl) were thawed on ice, prior to addition of 1-2 ng of ligated plasmid DNA (see 2.2.3.5). Cells remained on ice for a further 30 min, were heat shocked at 42°C for 45 sec, and then replaced on ice for a further 2 min. Cells were incubated at 37°C for 60 min in 450 µl of LB medium, prior to plating on LB agar plates containing 100 µg/ml ampicillin.

2.2.2 Quantitation of nucleic acids.

Nucleic acids were quantified by measuring the A_{260} in a LKB Ultrospec 2 spectrophotometer. 1-5 µl of DNA, RNA or oligodeoxyribonucleotides were diluted with 600 µl water in quartz cuvettes and the quantities were calculated as follows: DNA 50 µg/ml for 1 absorption unit at OD_{260}, RNA 40 µg/ml for 1 absorption unit at OD_{260} and oligodeoxyribonucleotides 50 µg/ml for 1 absorption unit at OD_{260}.

2.2.3 Recombinant DNA techniques.

2.2.3.1 Restriction enzyme digests.

Digests of DNA were performed according to the manufacturers' instructions, using the buffers supplied.

2.2.3.2 Purification of DNA fragments.

DNA fragments (~60 nucleotides) were gel purified using Mermaid, a kit designed for purifying fragments of 10-220 nucleotides, according to the manufacturers' instructions.

2.2.3.3 Phosphorylation of oligodeoxyribonucleotides.

Pairs of complementary oligodeoxyribonucleotides (1.25 µg) were annealed in a final volume of 14 µl by incubation at 95°C for 5 min, then for 10 min at 56°C and finally at room temperature for 20 min. The annealed oligodeoxyribonucleotides were phosphorylated in a 20 µl reaction containing 2 µl 10x kinase buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine and 1 mM EDTA), 2 µl 10 mM ATP and 2 µl T4 polynucleotide kinase (10 units/µl) for 25 min at 37°C. After phosphorylation 25 µl of 1 M NaCl and 205 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) were added to the annealed, phosphorylated oligodeoxyribonucleotides prior to ligation.

2.2.3.4 Conversion of fragments with protruding 5' ends to blunt ends.

2 µg of plasmid DNA linearised by digestion with restriction enzymes was incubated at 37°C for 20 min in a 50 µl reaction containing 2 µl of a 2 mM dNTP solution (dATP, dCTP, dGTP, dTTP), 5 µl 10 x nick translation buffer (0.5 M Tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM DTT and 500 µg/ml BSA) and 6 units of Klenow fragment of DNA polymerase I. The Klenow polymerase was heat-inactivated at 68°C for 5 min and the DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with 2 volumes of ethanol and 1/10th volume of 3 M sodium acetate pH 5.2 prior to subsequent digestion by other restriction enzymes.

2.2.3.5 Ligations.

Ligations involving non-cohesive or cohesive ends used a 10-fold molar excess or a 3 fold molar excess of insert respectively over plasmid

vector, and were performed overnight at 14°C using 1 unit T4 DNA ligase (for 50-200 ng of vector DNA) in the accompanying BRL buffer.

2.2.3.6 Colony Hybridisation.

^{32}P-labelled oligodeoxyribonucleotide probes were generated by phosphorylating oligodeoxyribonucleotides in a 30 µl reaction containing 50-100 ng of oligodeoxyribonucleotide, 10 units T4 polynucleotide kinase, 3 µl 10 x kinase buffer (see 2.2.3.3) and 3 µl [γ-^{32}P]ATP. Bacterial colonies were grown overnight on agar plates and lifted on to nitro-cellulose filters. Filters were soaked in 1% SDS for 3 min, 0.5 M NaOH, 1.5 M NaCl for 5 min and 0.5 M Tris pH 8.0, 1.5 M NaCl for 5 min. These were then baked at 80°C in a vacuum oven for 1.5 hours and pre-hybridised for 2 hours in 25 ml Church buffer (0.5 M Na_2HPO_4/ NaH_2PO_4 pH 7.2, 1 mM EDTA pH 8.0, 7% SDS). Hybridisation in 25 ml Church buffer was performed overnight at an appropriate temperature (5°C lower than the predicted Tm of the oligodeoxyribonucleotide/DNA hybrid as predicted by the G-C content of the hybrid) and filters were washed for 3 x 20 min in 1% SDS, 50 mM Na_2HPO_4/ NaH_2PO_4 pH 7.2 at the same temperature as the hybridisation. Filters were exposed to Kodak X-OMAT AR film at -80°C.

2.2.3.7 Plasmid preparations.

Mini and maxi plasmid preparations were prepared on Qiagen tip 20 and 500 respectively, using reagents provided by the manufacturer according to their instructions except that plasmids were extracted with an equal volume of phenol/chloroform/isoamylalcohol (25: 24: 1) after precipitation with iso-propanol, re-precipitated in 2 volumes of ethanol, washed twice with 70% ethanol, dried and resuspended in 200 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

2.2.3.8 DNA sequencing reactions.

Sequencing of double stranded plasmid DNA was performed by a modified version of the chain termination method using the Sequenase kit, which contains a recombinant T7 DNA polymerase. For primer annealing, 6 µl of DNA template (3 µg) was mixed with 1 µl (3 ng) of the -40 primer provided and 1 µl of 1 M NaOH, and incubated at 68°C for 10 min. 2 µl of TES buffer (560 mM TES [N-tris {hydroxymethyl}-methyl-2 aminoethanosulphonic acid], 240 mM HCl, 100mM $MgCl_2$) was added and incubated for 10 min at room temperature. 0.1 M DTT (1 µl), 2 µl labelling mix (diluted 1 in 10 in water), 0.25 µl [^{35}S] dATP and 2 µl of Sequenase polymerase (diluted 1 in 8 in water) were mixed and 5.5 µl of this mixture was added to the annealed DNA for 5 min at room temperature. The reactions were terminated by addition of 3.5 µl of the resulting labelling reaction to microtiter wells containing 2.5 µl of the dideoxy G, A, T, C termination mixes which were prewarmed to 37°C. After thorough mixing the reactions were incubated at 37°C for 5 min and were stopped by addition of 4 µl of the stop solution. Samples were heated to 95°C for 2 min and immediately loaded on to 8% denaturing polyacrylamide gels.

2.2.4 Electrophoresis

2.2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed essentially as described (Sambrook et al., 1989). 0.7- 1.5% agarose gels were prepared in 0.5 x TBE (45 mM Tris-HCl pH 8.3, 45 mM boric acid, 1 mM EDTA pH 8.0) by boiling the agarose for several minutes. Ethidium bromide was added to a concentration of 0.5 µg/ml before pouring. Before loading DNA samples, 1/6th volume of agarose sample buffer (30% (w/v) glycerol, 0.25%

bromophenol blue, 0.25% xylene cyanol FF), was added. 3 volumes of formamide containing 0.25% bromophenol blue was added to RNA samples which were denatured at 95°C for 2 min prior to loading. Gels (8 cm in length) were run in 0.5 x TBE at 80-100 volts and nucleic acids were visualised under UV irradiation.

2.2.4.2 DNA sequencing gels.

Sequencing reactions were resolved on 0.2 mm thick, 8% acrylamide (24:1 acrylamide: bis-acrylamide), 7 M urea, 1 x TBE, pH 8.8, gels. The eared plate was treated with dimethyldichlorosilane solution, and the back plate with technical ethanol before polishing. To polymerise 20 ml of gel, 100 µl of 10 % ammonium persulphate and 40 µl TEMED were added. Gels were pre-run at 50 watts for 20-30 min, and a metal plate was used to distribute the heat over the gel evenly. Prior to loading, samples were heated at 95°C for 1 min and gels were electrophoresed in 1 x TBE at 50W for between 1-2.5 hours. Gels were soaked in fixative (10% methanol and 10% acetic acid) for 10 min, transferred to 3 MM chromatography paper, dried and exposed to Kodak X-OMAT AR film at -80°C.

2.2.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed essentially as described by Laemmli (1970). SDS-PAGE for the separation of ^{35}S-Met labelled proteins were 1 mm thick. Separating gels were 13.5% polyacrylamide (30% acrylamide, 0.8% bis-acrylamide), 375 mM Tris-HCl pH 8.7, 0.1% (w/v) SDS with a 4% polyacrylamide (30% acrylamide, 0.8% bis-acrylamide), 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS stacking gel. The separating gel (10 ml) was polymerised with 100 µl 10% ammonium persulphate and 10 µl TEMED, the stacking gel (3 ml) with 30 µl 10% ammonium persulphate and 5 µl TEMED. Gels used to resolve unlabelled proteins were 1.5 mm thick, 10% polyacrylamide (30%

acrylamide, 0.8% bis-acrylamide), 375 mM Tris-HCl pH 8.7, 0.1% (w/v) SDS separating gels with 5% polyacrylamide (30% acrylamide, 0.8% bis-acrylamide), 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS stacking gel. The separating gel (40 ml) was polymerised with 400 µl 10% ammonium persulphate and 16 µl TEMED, the stacking gel (8 ml) with 80 µl 10% ammonium persulphate and 8 µl TEMED. Before loading, samples were mixed with an equal volume of 2 x sample buffer (10% 2-mercaptoethanol, 4% (w/v) SDS, 20% glycerol, 160 mM Tris-HCl pH 6.8 and 0.25% bromophenol blue). Gels were electrophoresed in 1 x Tris glycine buffer (50 mM Tris, 0.4 M glycine, 0.1% SDS, pH 8.8) at 150 volts for 2 hours or 8 hours for resolving ^{35}S-Met labelled and unlabelled proteins respectively. Gels used to resolve ^{35}S-Met labelled proteins were soaked in fixative (10% methanol and 10% acetic acid), and entensifying solutions, Entensify A and Entensify B for 20 min each before being transferred to 3 MM chromatography paper, dried and exposed to Kodak X-OMAT AR film at -80°C. Gels for resolving unlabelled proteins were stained with 0.05 % Coomassie blue, 50 % methanol and 10 % acetic acid for 1 hour. Destaining was effected with 30 % methanol, 10 % acetic acid and soaking for an appropriate length of time. If required, Coomassie stained gels were soaked in water overnight and then silver stained. Gels were soaked in a 1% silver nitrate solution for 30 min, and in developer (3% sodium carbonate, 5% formaldehyde) for up to 10 min and developing was stopped in 5% acetic acid.

2.2.4.4 6M urea polyacrylamide gels.

Denaturing gels for the purification of oligodeoxyribonucleotides or short RNA transcripts were 1.5 mm thick, 15% polyacrylamide (20%:1% acrylamide, bis-acrylamide), 6 M urea, 1 x TBE, pH 8.0. Gels (70 ml) were polymerised by addition of 500 µl of 10% ammonium persulphate and 120 µl TEMED. Samples were loaded after denaturation at 95°C for 2 min in

formamide containing 0.25 % bromophenol blue and gels were electrophoresed in 1 x TBE at 30 Watts for 30 min (using a metal plate to distribute heat) or 10 Watts for 90 min.

2.2.4.5 8M urea polyacrylamide gels.

Denaturing gels for the analysis of ^{32}P-labelled mRNAs were 0.4 mm thick, 4% acrylamide, 8 M urea, 1 x TBE gels. Gels (15 ml) were polymerised with 100 µl 10% ammonium persulphate and 10 µl TEMED. Samples were loaded in formamide containing 0.25% bromophenol blue after denaturation at 95°C for 2 min. Electrophoresis in 1 x TBE was at 50 Watts using a metal plate (for even distribution of heat) for 30-45 min, and gels were then exposed wet at -80°C to a Kodak X-OMAT AR film.

2.2.4.6 Non-denaturing polyacrylamide gels.

Native gels for the resolution of RNA/protein complexes were 1.5 mm thick 4% polyacrylamide (30%: 0.5% acrylamide: bis-acrylamide), 1 x TBE, pH 8.0. Gels (40 ml) were polymerised with the addition of 300 µl 10% ammonium persulphate and 100 µl TEMED. Samples were loaded in 50% glycerol containing 0.25% bromophenol blue. Electrophoresis in 1 x TBE was at 150 volts for 45-90 min, after which gels were soaked in fixative (40% methanol, 10% acetic acid) for 10 min before being transferred to 3 MM chromatography paper, dried and exposed to Kodak X- OMAT AR film at -80°C.

2.2.5 Recombinant DNA constructs.

2.2.5.1 Ferritin IRE-containing plasmids.

IRE-wt and IRE-mut (referred to in earlier publications as I-12.CAT and I-19.CAT respectively) were created from L5(+26mer)CAT and +IRE ΔC-165, (Hentze et al., 1988) respectively by Dr. M. Hentze (EMBL), by insertion of a *Bam* HI-*Pst* I fragment containing the IRE or ΔC IRE and the CAT ORF from these plasmids into pGEM 3zf(-) digested with *Bam* HI-*Pst* I. 34T.CAT was created by insertion of a *Bam* HI-*Xba* I fragment containing the IRE from F64 (Goossen and Hentze, 1992) into IRE-wt digested with *Bam* HI-*Xba* I.

US.CAT was created from IRE-wt digested with *Bam* HI, the *Bam* HI cohesive ends were filled in using Klenow fragment of *E.coli* DNA polymerase I, prior to a further digestion with *Xba* I. A gel purified *Stu* I-*Xba* I fragment from F64 containing the IRE and sequences 5' of the IRE was then introduced into this *Bam* HI blunt-*Xba* I digested IRE-wt. ES.CAT was created from US.CAT by insertion of annealed phosphorylated oligodeoxyribonucleotides into US.CAT digested with *Xho I*. The sequence of the sense strand oligodeoxyribonucleotide was 5' TCGAGATTTA ACCTCTTCCA ACCCAAAGGC CTCT 3'.

2.2.5.2 eALAS and aconitase IRE-containing plasmids.

ALAS.CAT (Melefors et al., 1993) was a gift from Dr. R. Stripecke (EMBL). Acon.IRE was created from pGA (Zheng et al., 1990), a pGEM-3Z vector containing the porcine mitochondrial aconitase cDNA lacking 10 nucleotides of the 5'UTR which was a gift from Dr L. Zheng (Indiana, USA). Annealed phosphorylated oligodeoxyribonucleotides were inserted into pGA which had been digested with *Bst* EII and partially digested with *Eco* RI, to recreate the 5' end of the aconitase cDNA which contains the IRE. The

sequence of the sense strand oligodeoxyribonucleotide was 5' AATTGACCTC ATCTTTGTCA GTGCACAAAA TGGCGCCTTA CAGCCTACTG 3'. The oligodeoxyribonucleotides used to create acon.ΔC differed from those used to create acon.IRE at one nucleotide (the underlined C) to create a delta C derivative of the aconitase IRE.

2.2.5.3 Non IRE-containing plasmids for *in vitro* transcription/translation.

Plasmids U1A-wt, U1A-mut and US-U1A (Stripecke and Hentze, 1992) were a gift from Dr. R. Stripecke (EMBL), and plasmids hUIA (Scherly 1989) and NOP1 (Schimmang et al., 1989) for the transcription of internal control mRNAs were kindly provided by Dr D. Scherly (EMBL) and Dr. U. Nehrbass (EMBL), respectively.

2.2.5.4 IRP expression and purification plasmids.

Plasmids pSPT-TR38 (Neupert et al., 1990) for the generation of the IRE affinity column and pSPTACAT (Constable et al., 1992) for the generation of "non-IRE" RNA for a control column in the affinity purification of placental IRP were kindly provided by Dr. L. Kühn (ISREC, Epalinges, Switzerland) and Dr A. Constable (EMBL) respectively.

Plasmid pT7-His-hIRF (Gray et al., 1993) for the expression of recombinant human IRP in *E.coli* was a gift from B. Goossen (EMBL).

2.2.6 Mammalian cell culture conditions

Mammalian cell lines B6 and HeLa were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum and 1 unit/ml penicillin/streptomycin, at 37°C, 5% CO_2 in 175 cm^2 flasks. Fresh media was added every two days and cells were passaged about

every seven days. Cells were passaged by aspiration of old medium, two washes with trypsin-EDTA (2 ml), incubation of cells in 2 ml trypsin-EDTA at 37°C for 5 min and addition of 8 ml of fresh media, the detached cells were then taken up by gentle pipetting. An appropriate volume of this cell suspension was used to inoculate fresh dishes. Prior to harvesting, cells were grown on 155 mm dishes.

2.2.7 Preparation of total RNA from mammalian cell lines.

Medium was aspirated and cells were washed three times with 1 x PBS (4 mM KCl, 4 mM KH_2PO_4, 17.5 mM Na_2HPO_4, 125 mM NaCl, pH 7.4 [NaCl/Pi]) (4°C) before the cells were harvested by scraping into ice cold 1 x PBS. Cells (30-200 x 10^6) were lysed in a buffer containing 4 M guanidinium thiocyanate, 0.5% SDS, 25 mM sodium citrate and 0.7% 2-mercaptoethanol, pH 7.0, and the DNA was sheared by passing the lysate through a 20G needle. The RNA was pelleted by centrifugation of the lysate through a 5.7 M CsCl gradient containing 0.25 M EDTA pH 8.0 at 32,000 rpm for 16-18 hrs at 20°C in a Beckman SW40 rotor. RNA was dissolved in 500 µl water, precipitated in 2.5 volumes of ethanol and a 1/20th volume 3M sodium acetate pH 5.2, washed in 80% ethanol, dried, redissolved in 100 µl of water and frozen at -80 °C.

2.2.8 Isolation of polyadenylated mRNA from cells.

Poly (A)-enriched RNA was isolated from 1 mg of total mRNA using 0.2 g of oligo (dT) cellulose which had been rehydrated for 30 min and washed 4 times for 10 min in 1 x poly A buffer (20 mM Tris pH 7.4, 0.75 M NaCl, 2 mM EDTA pH 8.0 and 0.2% (w/v) SDS) at 37°C. RNA was added to the beads in an equal volume of 2 x poly A buffer, incubated at room temperature for 20 min, spun at 4,000 g for 3 min and the supernatant was

removed. The RNA bound to the beads was washed 5 times in 1 x poly A buffer prior to being eluted with 0.1 % SDS. The eluted RNA was extracted with equal volumes of phenol, then phenol/chloroform/isoamylalcohol (25:24:1) and finally with chloroform/isoamylalcohol (24:1). The RNA was then precipitated in 2.5 volumes of ethanol and 1/20th volume of 3M sodium acetate pH 5.2, washed twice in 80% ethanol, dried and redissolved in water and stored at -80°C. Poly $(A)^+$ RNA from MEL cells induced to differentiate with HMBA was a gift from Dr Ö. Melefors (EMBL).

2.2.9 Generation of RNA transcripts *in vitro*

2.2.9.1 Generation of short RNA transcripts from single stranded DNA templates.

[^{32}P]-Labelled RNA probes for gel retardation assays were transcribed after annealing of an 18 nucleotide T7 primer to the transcription template by heating to 95°C for 1 min and cooling slowly to room temperature (Milligan et al., 1987). Transcription reactions contained 1 x transcription buffer (40 mM Tris pH 8.8, 1 mM spermidine, 5 mM DTT, 50µg/ml BSA, 0.01% Triton X-100, 80 ng/ml PEG-8000), 4 mM ATP, 4 mm CTP, 4 mm GTP, 1.3 mM UTP, 3 µM [α-^{32}P] UTP, 7 mM $MgCl_2$, 1 unit InhibitAce and 140 units T7 RNA polymerase. The sequence of the DNA template for the transcription of the ferritin IRE was 5' GGGATCCGTC CAAGCACTGT TGAAGCAGGA TCCCTATAGT GAGTCGTATT A 3'. Unlabelled competitor RNAs for the ferritin IRE, eALAS IRE, ΔC IRE, RSL and *toll* were transcribed by the same method except that the UTP concentration was raised to 4 mM and no [α-^{32}P]UTP was added. The sequence for the ΔC IRE template varies from the sequence above only at the underlined G (which is deleted). The sequence of the eALAS template is 5' GTCCTGTTGC CCTGCACTGA GGACGAACGA ATGACCCTAT AGTGAGTCGT ATT 3', the template for the RSL transcript: 5' GGGTACGACC AAGTTCGTGA

CAACTTCTCT ACCCTATAGT GAGTCGTATT A 3' and the template for the *toll* transcript 5'CGAACAAACA CGCACTGGCA CTGTTGTTTA CCCTATAGTG AGTCGTATTA 3'. Transcription templates and *in vitro* transcripts were gel purified, eluted in a buffer containing 0.1% SDS, 0.5 mM ammonium acetate, 10 mM magnesium acetate and 1 mM EDTA. Eluted RNA was extracted with equal volumes of phenol, then phenol/ chloroform/isoamylalcohol (25:24:1) and subsequently with chloroform/ isoamylalcohol (24:1), precipitated with 2.5 volumes of ethanol and 1/20th volume of 3M sodium acetate, pH 5.2, washed twice in 80% ethanol, dried and redissolved in water and stored at -80°C.

2.2.9.2 Generation of RNA transcripts from linearised plasmids.

The CAT, U1A, NOP1, TR38 and UCAT mRNAs were transcribed from *Hind* III (full-length), *Pvu* II (short) or *Bam* HI (TR38, UCAT and NOP1) linearised plasmids in standard reactions containing 1 mM ATP, CTP, UTP, 30 mM DTT, 0.2 units Inhibit Ace, 7 mM m^7GpppG, 1 x Stratagene transcription buffer and 4 units T7 RNA polymerase. After 5 min at 37°C, 1 mM GTP (final concentration) was added and the reaction was continued for an additional 60 min before treatment with 1 unit DNase I/ 10 µl (20 min at 37°C) and extracted with equal volumes of phenol, and phenol/ chloroform/isoamylalcohol (25: 24: 1). To remove excess m^7GpppG, mRNAs were applied to two successive 1 ml Sephadex G-50 columns as described in Maniatis *et al.*, (1982) except that columns were equilibrated with sterile water rather than STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). Finally, the mRNA was precipitated with 2.5 volumes of ethanol and 1/20th volume of 3 M sodium acetate, pH 5.2, washed twice in 80% ethanol, dried, redissolved in sterile water and stored at -80°C. [^{32}P]-Labelled mRNAs were transcribed as above from either *Hind* III or *Pvu* II linearised plasmids with the exception of the final UTP concentration which was reduced to 0.5 mM, and the inclusion of 2.5 µM [α-

^{32}P] UTP for full-length (~8 x 10^6 cpm/µg RNA) or 5 µM [α-^{32}P] UTP for shortened transcripts (~1.6 x 10^7 cpm/µg RNA). [α-^{32}P] labelled RNAs for gel retardation assay probes were transcribed from *Xba* I linearised IRE-wt in similar reactions containing 0.1 mM UTP and 2.5 µM [α-32P] UTP (~2.9 x 10^7 cpm/µg RNA).

2.2.10 Purification of IRP.

2.2.10.1 Purification of IRP from human placenta.

IRP was purified from human full term placenta (provided by Professor Heep, Heidelberg) by a modified version of the method of Neupert et al. (1990). Fresh placenta was dissected free of connective tissue and washed thoroughly in PBS (see 2.2.7). The tissue was suspended in an equal volume of extraction buffer (0.1 M [3-{N-Morpholino}propane-sulphonic acid] pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 3 mM sodium azide and 1 mM PMSF) and homogenised in a Polytron blender at speed 4 for 5 min and subsequently in a Dounce glass chamber, five strokes at speed 4. The placental extract was cleared of debris by successive centrifugation for 10 min at 3,000 rpm, 15 min at 8,500 rpm and 60 min at 25,500 rpm. The placental extract was concentrated by precipitation with 70% ammonium sulphate and dialysed against buffer A (10 mM Hepes pH 7.5, 40 mM KCl, 3 mM $MgCl_2$ and 5% glycerol) to remove the ammonium sulphate. The dialysed lysate was then subjected to heparin Sepharose chromatography to pre-purify nucleic acid binding proteins which have an affinity for heparin. Heparin Sepharose CL-6B (10 g) was equilibrated overnight in buffer A. The dialysed lysate was added to the heparin Sepharose beads and mixed gently for 3 hours, the beads were washed three times with buffer A and the bound proteins were then eluted with buffer A containing 250 mM KCl. The heparin Sepharose elutions (HSE) containing IRP activity were pooled and subjected to affinity chromatography. Poly (U) Sepharose beads were

equilibrated with RNA binding buffer (100 mM KCl, 25 mM Hepes pH 7.5) with 15 µl of InhibitACE. Polyadenylated transcripts TR38 and UCAT, transcribed from pST-TR38 (300 µg) and pSPTACAT (400 µg) respectively were applied to separate batches of poly (U) Sepharose beads to create a specific IRE and a non specific RNA column.

The HSE was incubated with 1/20th volume of 200 mM vanadyl ribonucleoside complex, an RNAse inhibitor, 35 µg/ml rRNA and 5 mg/ml heparin for 30 min at room temperature. 2-mercaptoethanol (2%) was added and the HSE was centrifuged at 10,000 rpm for 10 min to remove aggregates formed by the presence of vanadyl ribonucleoside complex, diluted 1 in 3 in buffer A and applied to the non specific RNA column. The flow through was then applied to the IRE affinity column, which was washed with 10 ml of buffer A containing 5 mg/ml heparin followed by buffer A alone. The bound material was then eluted with 10 ml 1 M KCl. The eluted material was diluted 1 in 10 with buffer A minus KCl before applying to a second IRE affinity column. The bound material was again eluted in 1 M KCl and the purity of the IRP as assessed on silver-stained polyacrylamide gels.

The authenticity of the purified protein was confirmed by a) comparison of the eluate from the IRE affinity column with the eluate from the control column, b) function as a specific IRE-binding protein, c) function as a specific translational repressor *in vitro*. The protein was quantified by BCA protein assay (Pierce) and was normally stored in small aliquots as a 0.25 mg/ml stock solution in buffer N (24 mM Hepes, pH 7.6, 150 mM potassium acetate, 1.5 mM $MgCl_2$, 5% glycerol) at -80°C. The first preparation of IRP was stored in 1 M KCl.

2.2.10.2 Expression and purification of recombinant hIRP.

pT7-His-hIRF was used to transform *E.coli* BL21(DE3). Optimal expression of soluble IRP was obtained from dense overnight cultures grown in LB medium at 25°C in the absence of IPTG (isopropyl ß-D-thiogalactopyranoside). Growth at 37°C and/or induction with IPTG resulted in the expressed IRP being located in inclusion bodies. Cells were lysed in 250 mM NaCl, 0.5 % NP40, 20 mM Tris HCl pH 8.0, by one cycle of freeze/thawing followed by sonification on ice (30 pulses with a Branson Cell Disruptor B15). Cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall RT6000B centrifuge. Supernatants were adjusted to 0.4 M KCl, applied to Ni^{2+}-NTA (nitrilotriacetic acid)-agarose and washed once with one column volume of buffer N (see 2.2.10.1) containing 0.4 M KCl, once with buffer N alone, followed by two washes with buffer N containing 5 mM imidazole. Recombinant IRP was then eluted with buffer N containing 50 mM imidazole and was stored frozen at -80°C. Quantitation was achieved by comparison with IRP standards of known concentration on silver stained SDS-polyacrylamide gels. A dense 1 litre culture of *E.coli* yields 0.8-3 mg of purified hIRP. IRP expressed in vaccinia virus infected HeLa cells was a gift from Sabine Quick (EMBL) and was of the purity shown in Gray et al. (1993).

2.2.11 Preparation of wheat germ extract.

Wheat germ (stored desiccated at 4°C) was homogenised in a buffer containing 40 mM HEPES pH 7.6, 90 mM KCl, 1 mM magnesium acetate, 2 mM $CaCl_2$ and centrifuged twice at 14,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor. The supernatant was removed taking care to avoid the lipid layer and was applied to a 1.5 x 40 cm Sephadex G-25 column, eluted with 20 mM Hepes pH 7.6, 50 mM KCl, 1 mM $Mg(Ac)_2$ and 0.1% 2-mercaptoethanol and subjected to a final centrifugation at 14,000 rpm at 4°C. The most turbid

supernatant fractions were pooled, and frozen in aliquots in liquid N_2 and stored at -80°C.

2.2.12 Cell-free translation.

Cell-free translations were performed essentially as described by Clemens (1984) in the presence of [^{35}S] methionine (10 µCi/µl) programmed with poly (A)-enriched RNA (20-25 ng/µl) or 1-5 ng of capped *in vitro* transcribed U1A, NOP1, aconitase or CAT mRNAs. Reactions in RRL (12 µl) contained either 0.68 mM Mg^{2+} or were adjusted to 2.1 mM Mg^{2+} using $MgCl_2$ where stated, the K^+ concentration was adjusted to 63 mM K^+ using potassium acetate. Reactions in wheat germ extract (15 µl) were adjusted to 117 mM K^+ and 1.86 mM Mg^{2+} using potassium acetate and $MgCl_2$. IRP stored in 1 M KCl was diluted in HDS (12 mM Hepes pH 7.6, 1 mM DTT and 4 µM spermidine) whereas IRP stored in buffer N (see 2.2.10.1) was diluted in buffer N and U1A protein (Stripecke and Hentze, 1992) was diluted into 20 mM HEPES pH 7.6 and added to mRNAs on ice either immediately (IRP) or 15 min (U1A) prior to the addition of the translation extract. Translations which did not receive either IRP or U1A protein were compensated with the appropriate buffer. Short competitor transcripts were added to mRNAs on ice prior to addition of translation extract. Inhibitors of translation (GMP-PNP, cycloheximide, anisomycin, m^7GpppG cap analogue and edeine) were assayed by addition and incubation at 25°C (WGE) or 30°C (RRL), 3 min prior to the addition of mRNA. Translation reactions were incubated at 30°C (RRL) or 25°C (WGE) for 1 h.

2.2.13 Trichloroacetic acid (TCA) precipitation.

^{35}S-Met labelled translation products (2 µl) were spotted on to circular cellulose filter papers and dried. ^{35}S-Met labelled proteins were then precipitated with ice cold 10% (w/v) TCA for 30 min, boiled twice in

5% (w/v) TCA for 5 min, washed briefly in room temperature 5% (w/v) TCA and dehydrated twice with 99% ethanol and dried. TCA precipitable material was measured by analysis of filters in 6 ml of liquid scintillation cocktail in a Beckman LS 8100 or LS6000SC scintillation counter.

2.2.14 Immunoprecipitation of ^{35}S-Met labelled protein products.

Translation of ferritin, eALAS and U1A mRNA was assessed by immunoprecipitation of ^{35}S-Met polypeptides using polyclonal antiserum as follows: the volume of the translation reaction was increased from 30-45 µl to 500 µl with buffer D (0.3 M NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4), 5 µl of polyclonal anti-ferritin, 1 µl of anti-eALAS or 9 µl of anti-U1A antiserum was added and incubated at 4°C for 60 min with mild agitation before the addition of 30 µl of protein A Sepharose CL4B beads. The incubation was continued at 4°C with mild agitation for an additional 60 min. The beads were subsequently washed in 3 x 500 µl of buffer D and 1 x 500 µl of 10 mM Tris-HCl, pH 7.4, before elution of immunoprecipitates in 20 µl of SDS-PAGE sample buffer (see 2.2.4.3) by heating to 95°C for 10 min.

2.2.15 Gel retardation assays.

Where stated samples were activated with a final concentration of 1.5% 2-mercapatoethanol for 10 min at room temperature (Hentze *et al.*, 1989). Aliquots were incubated with either 12,000 cpm ferritin IRE probe synthesised by the method of Milligan et. al. (1987) (~3.2 x 10^6 cpm/µg RNA) or by *in vitro* transcription from a linearised plasmid (~2.9 x 10^7 cpm/ µg RNA) for 20 min at room temperature and 5-15 mg/ml of heparin was added for an additional 10 min. Unlabelled competitor mRNAs were added 5 min prior to the addition of ferritin IRE probe. RNA/protein complexes were resolved on 4% non-denaturing polyacrylamide gels (see 2.2.4.6).

2.2.16 Northern blotting.

RNA was extracted before and after translation using equal volumes of phenol, then phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/ isoamylalcohol (24:1). The RNA was precipitated using 2.5 volumes of ethanol and 1/20th 3 M sodium acetate pH 5.2 and washed once in 80% ethanol, dried and redissolved in formamide containing 0.25% bromophenol blue. Gels (see 2.2.4.1) were soaked in 0.3 x TAE (13.3 mM Tris, 6.6 mM sodium acetate, 0.33 mM EDTA pH 8.0, pH 7.2) for 2 x 30 min prior to transfer of nucleic acids onto a NY 13 N Nytran filter. The gel was sandwiched with the Nytran filter (pre-soaked in 0.3 x TAE for 5 min) between several sheets of 3 MM Whatman chromatography paper and Scotch Britts soaked in 0.3 x TAE. Transfer was performed at 4°C either overnight at 10 volts or for 6 hours at 30 volts in 0.3 x TAE. The RNA was cross-linked on to the filter in a Stratalinker 2400 (Stratagene, Heidelberg, Germany). Filters were pre-hybrised at 65°C for 1-2 hours in Church buffer (see 2.2.3.6) before addition of the probe in fresh Church buffer. Hybridisation was overnight at 65°C and filters were washed at 65°C for 3 x 20 min in 1% SDS, 50 mM Na_2HPO_4/ NaH_2PO_4 pH 7.2. Filters were exposed to Kodak X-OMAT AR film at -80°C. Randomly labelled probes were prepared from *Hind* III-linearised U1A and IRE-mut plasmids using the Pharmacia Oligolabelling kit according to the manufacturers instructions.

2.2.17 Sucrose gradient analysis.

Extracts competent for translation were prepared as described in 2.2.12 except unlabelled methionine replaced the ^{35}S-Met. These extracts were incubated at 25°C (WGE) or 30°C (RRL) for 3 min with a final concentration of 0.5 mM cycloheximide prior to addition of mRNA. Analogues and other inhibitors of translation where incubated alongside cycloheximide at final

concentrations of 1-2 mM GMP-PNP, 0.5 mM m^7GpppG cap analogue or 2-4 µM edeine. Reactions in WGE (45 µl) were subsequently programmed with 1 ng of U1A mRNA and 3 ng of full-length or 0.75 ng of short CAT mRNAs. Reactions in RRL (48 µl) were programmed with 2 ng of U1A or NOP1 and 1.5 ng of short CAT mRNAs. 45 ng of IRE competitor transcripts (RRL) or 125 ng of IRP was added to mRNA prior to addition to lysate and reactions which did not receive IRP were compensated with buffer N (see 2.2.10.1). U1A protein (700 ng) or its storage buffer (1 mM DTT and 20 mM Hepes pH 7.2) was mixed with mRNA 15 min prior to addition to the lysate. Initiation assays, at 25°C (WGE) or 30°C (RRL) were stopped 5 min (unless otherwise stated) after the addition of mRNA by addition of dilution buffer (150 mM potassium acetate, 20 mM Hepes pH 7.6, 5 mM $MgCl_2$, 1 mM DTT) and 0.2% glutaraldehyde (WGE) up to 100 µl, and placed on ice for 5 min. Translation initiation complexes were resolved on either 30-10% or 25-5% 5 ml linear sucrose gradients containing 150 mM potassium acetate, 20 mM HEPES pH 7.6, 5 mM $MgCl_2$ and 1 mM DTT. Gradients were centrifuged in a Beckman SW50.1 rotor at 40,000 rpm for 90 min (WGE) or 30,000 rpm for 180 min (RRL) at 4°C using maximum acceleration and no brake. 24 fractions of 210 µl were collected from the bottom of the gradient using a Pharmacia P-1 peristaltic pump and a Pharmacia RediFrac fraction collector. Absorption traces were recorded using a Pharmacia UV HR-10 flow cell with a A_{254} filter. Every second fraction was counted by the Cerenkov method in a Beckman LS6000SC scintillation counter. This procedure is summarised in figure 13.

2.2.18 RNA extraction from sucrose gradient fractions.

RNA from 100 µl of gradient fractions was phenol/ chloroform/isoamylalcohol (25:24:1) extracted twice, ethanol precipitated, dissolved in formamide containing 0.25% bromophenol blue and visualised on a 4% denaturing acrylamide gel (see 2.2.4.5).

2.2.19 Quantitative analysis of ^{35}S-Met incorporation by phosphor imaging.

^{35}S-Met incorporation into proteins was quantified using a Compaq phosphor imager and Molcular Dynamics Image Quant software version 5.6. Each experimental point was evaluated twice.

Chapter 3: Establishment of cell-free translation systems to study IRE/IRP-mediated regulation.

3.1 Introduction.

Iron regulation of ferritin synthesis in mammalian cells is achieved at the translational level via an RNA/protein interaction. The RNA component is a stem loop structure referred to as an iron responsive element (IRE), which is located in the 5'UTR of ferritin mRNA (Aziz and Munro, 1987; Hentze et al., 1987) and acts as a binding site for the iron regulatory protein (IRP). IRP binds to IREs when intracellular iron levels are low and represses ferritin synthesis (Leibold and Munro, 1988; Rouault et al., 1988). The mechanism by which RNA/protein complexes regulate translation has not been established. In order to investigate this mechanism biochemically, cell-free systems which accurately reflect the known characteristics of ferritin regulation in mammalian cells must first be established. In contrast to mammalian cells, plant cells are known to regulate ferritin expression transcriptionally (Lescure et al., 1991), therefore translation competent extracts prepared from a mammalian (rabbit reticulocyte lysate) and a plant source (wheat germ extract) would be expected to differ in their translation of mammalian ferritin mRNA, thus making them suitable and complementary systems to study IRE-mediated translational control *in vitro*. Ferritin mRNA translation was found to be inhibited in rabbit reticulocyte lysate but not in wheat germ extract (Walden et al., 1988), suggesting that rabbit reticulocyte lysate contains a specific inhibitor of ferritin translation. Addition of fractions of rabbit reticulocyte lysate induced repression of ferritin mRNA translation in wheat germ extract, confirming the existence of an inhibitor of ferritin synthesis in the mammalian extract (Walden et al., 1988). Subsequently, a 90 kDa protein required for this repression was purified biochemically to apparent homogeneity from rabbit liver (Walden et al., 1989). These results clearly

supported the hypothesis that such *in vitro* systems are suitable for studying translational repression by IRE/IRP complexes and led us to examine them more closely.

3.2 Results.

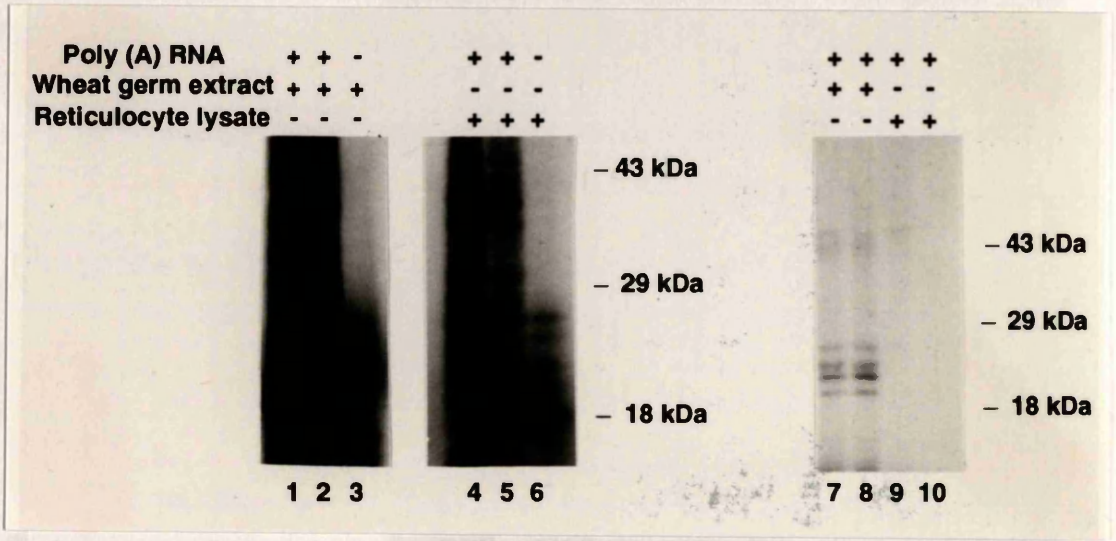
3.2.1 Translation of ferritin mRNA in cell-free translation systems.

3.2.1.1 Correlation between ferritin mRNA translation in cell-free systems and endogenous IRP activity.

To compare the behaviour of ferritin mRNA in the cell-free translation extracts described above, poly (A)-enriched RNA from murine Tk^- fibroblasts (B6 cells) was translated in both systems (figure 3A). Although total protein synthesis was comparable in the two systems (as measured by TCA precipitation of ^{35}S-labelled protein and comparison by SDS PAGE gels) (lanes 1-6), immunoprecipitation using an anti-ferritin antiserum showed that ferritin mRNA translation was lower in rabbit reticulocyte lysate than in wheat germ extract (compare lanes 7-8 with 9-10). To investigate whether wheat germ extract contains an endogenous IRP activity which is capable of IRE-binding but cannot mediate translational repression of mammalian ferritin mRNA, rabbit reticulocyte lysate and wheat germ extract were probed with a radiolabelled IRE, and RNA/protein complexes were assessed by native gel electrophoresis (figure 3B). In order to maximise potential IRP activity, samples of rabbit reticulocyte lysate and wheat germ extract were pre-treated with 2-mercaptoethanol (Hentze et al., 1989a). As shown in figure 3B, rabbit reticulocyte lysate (lanes 2-5) contains IRP whereas wheat germ extract does not (lanes 6-9), providing further evidence that the lack of ferritin mRNA repression in wheat germ extract results from the lack of an endogenous IRP homologue.

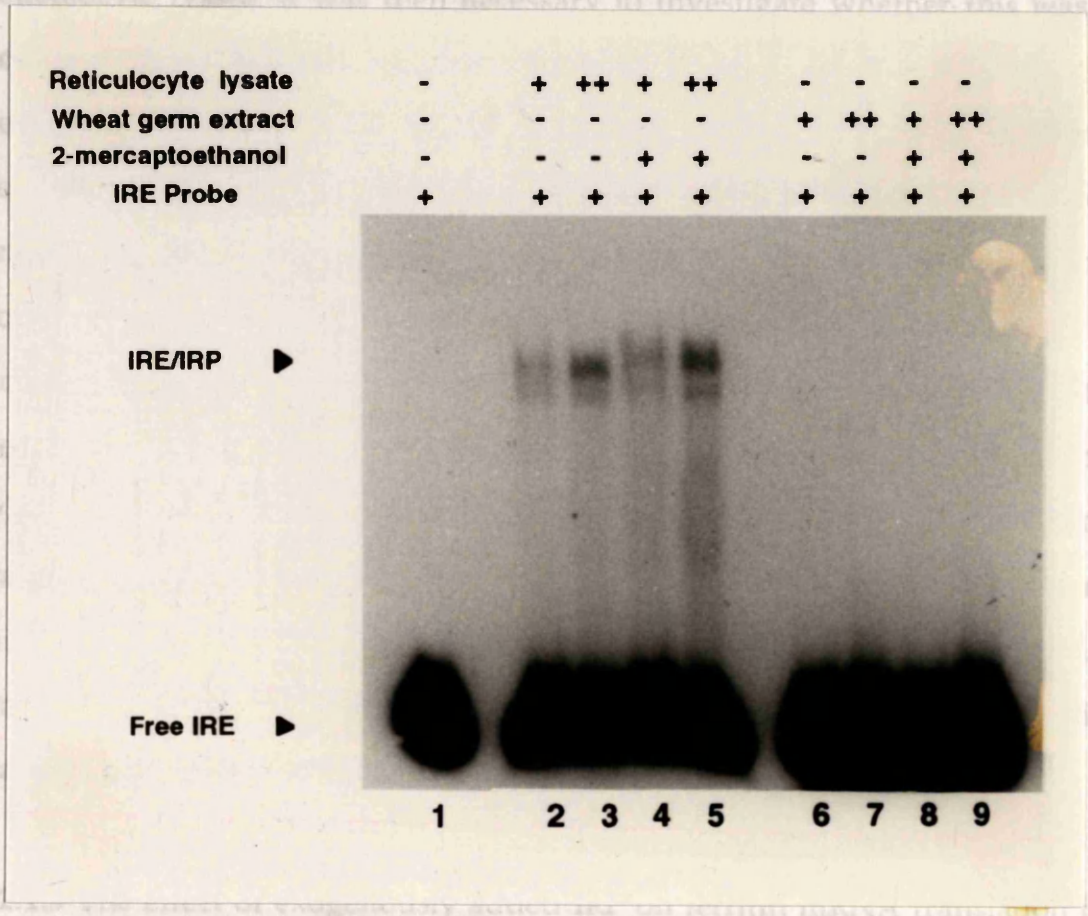
Figure 3. Correlation between ferritin mRNA translation in cell-free systems and IRP activity.

(A) Ferritin mRNA translation in cell-free systems.



Poly (A)-enriched RNA from B6 cells was translated in reticulocyte lysate (4-6 and 9-10) and wheat germ extract (lanes 1-3 and 7-8). 336 ng RNA (lanes 1, 4, 7 and 9), 84 ng RNA (lanes 2, 5, 8 and 10), or no RNA (lanes 3 and 6) was added to the *in vitro* translations. ^{35}S-Met labelled products were analysed directly by SDS-PAGE (lanes 1-6) or immunoprecipitated with anti-ferritin antiserum prior to SDS-PAGE analysis (lanes 7-10) and fluorography. Molecular weight markers are shown on the right.

Figure 3 (B) IRP activity in cell-free translation systems.



4 µl (+) or 8 µl (++) of rabbit reticulocyte lysate (lanes 2-5) or wheat germ extract (lanes 6-9) were analysed by gel retardation assay with 12,000 cpm of ferritin IRE probe (synthesised by the method of Milligan et. al., 1987) for the presence of IRP. Where indicated (+), 2-mercaptoethanol (1.5% final concentration) was added prior to addition of probe. The positions of excess 'free' labelled RNA probes and IRE/IRP complexes are marked.

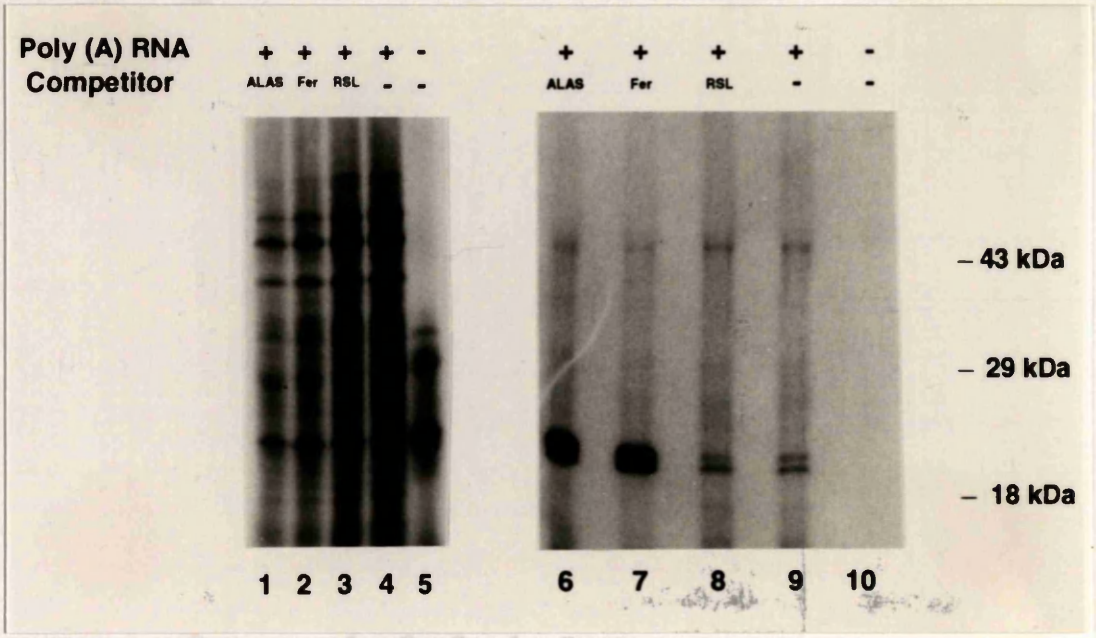
3.2.1.2 De-repression of ferritin translation in rabbit reticulocyte lysate.

Having established that ferritin translation is repressed in rabbit reticulocyte lysate, it was then necessary to investigate whether this was a consequence of IRE/IRP complex formation. If so, it should be possible to sequester the IRP with an excess of IRE competitor RNA, thus relieving repression. Three different types of short (~30 nucleotide) competitor transcripts were prepared; one representing a ferritin IRE, a second containing an eALAS IRE (see chapter 6) and a third which consisted of a non-IRE stem loop (RSL). These competitors were added together with poly(A)-enriched RNA from HeLa cells (figure 4). Immunoprecipitation with anti-ferritin antiserum revealed that only the two IRE competitor transcripts were able to relieve the repression by endogenous IRP (compare lanes 6-7 with 8-9). The competitor transcripts did not affect global mRNA translation (lanes 1-5), suggesting that the specific repression of ferritin translation is due to IRE/IRP interactions.

3.2.1.3 The effect of exogenously added IRP on ferritin mRNA translation in wheat germ extract.

Conversely, it was of interest to determine whether the addition of affinity-purified IRP to wheat germ extract could induce specific repression of ferritin mRNA. IRP was purified from human placenta to greater than 95% purity (see figure 5A). Addition of this highly purified IRP to wheat germ extract induced specific repression of ferritin mRNA translation (figure 5B, compare lanes 2 and 3). This repression was not observed if the protein buffer alone was added (lane 5) and inclusion of a high molar excess of IRE competitor alongside IRP relieved repression (compare lanes 3 and 4), supporting the notion that ferritin mRNA translation was specifically repressed by IRP.

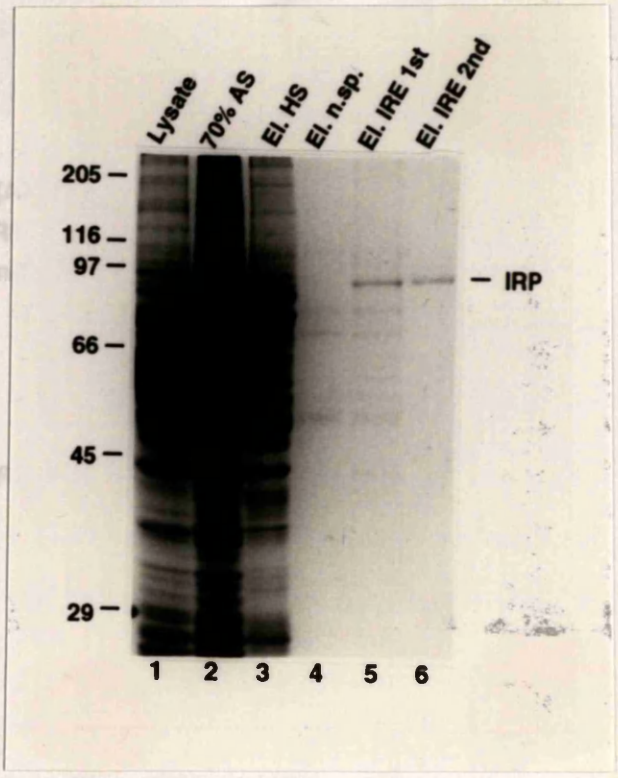
Figure 4. De-repression of ferritin mRNA translation in reticulocyte lysate by IRE competitor transcripts.



Where indicated (+), poly (A)-enriched RNA from HeLa cells was translated in rabbit reticulocyte lysate. 100 ng/30 µl reaction of eALAS (ALAS) IRE (36 nucleotides), ferritin (Fer) IRE (34 nucleotides) or a 34 nucleotide non-IRE stem-loop (RSL) competitor were added to the mRNA prior to addition of the extract. ^{35}S-Met labelled products were subjected to immunoprecipitation with anti-ferritin antiserum (lanes 6-10) or directly analysed by SDS-PAGE (lanes 1-5) and fluorography. Molecular weight markers are indicated on the right.

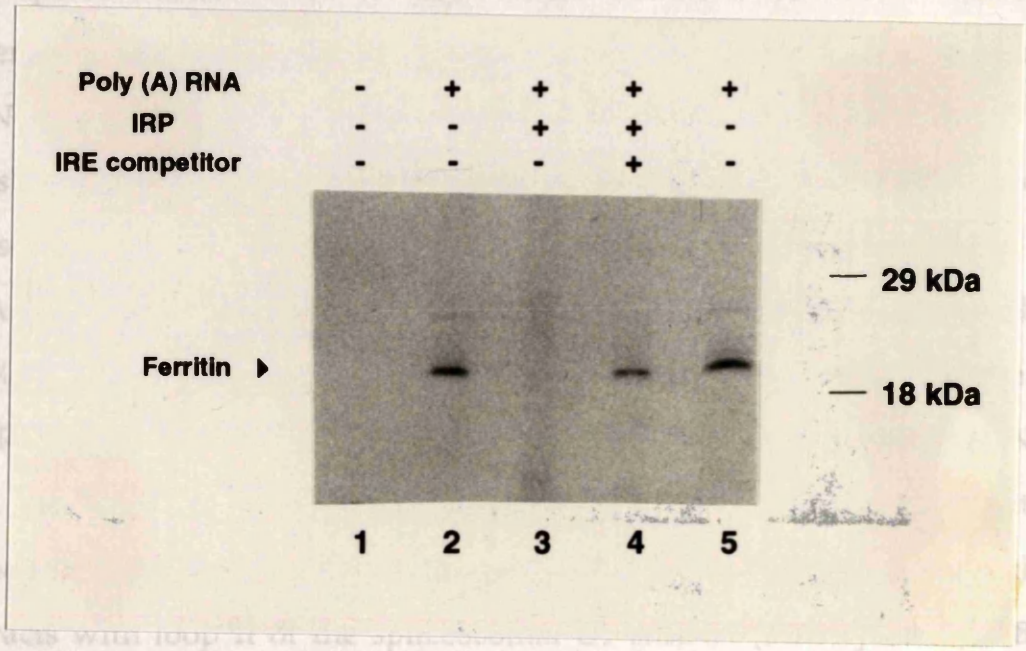
Figure 5. Effect of affinity-purified IRP from human placenta on ferritin translation

(A) Affinity purification of IRP from human placenta.



Aliquots from each purification step were analysed by SDS-PAGE and Coomassie blue staining. Crude placental extract (lane 1) was precipitated with 70% ammonium sulphate (lane 2). The precipitate was subjected to heparin Sepharose chromatography, eluted at 250 mM KCl (lane 3) and subsequently applied to a non specific CAT RNA column (lane 4); the flow through fraction was applied to a specific IRE column (lane 5). The eluate from the specific IRE column was applied to a second IRE affinity column and eluted in 1 M KCl to achieve purification to apparent homogeneity (lane 6).

Figure 5 (B) Repression and de-repression of ferritin mRNA using purified IRP.



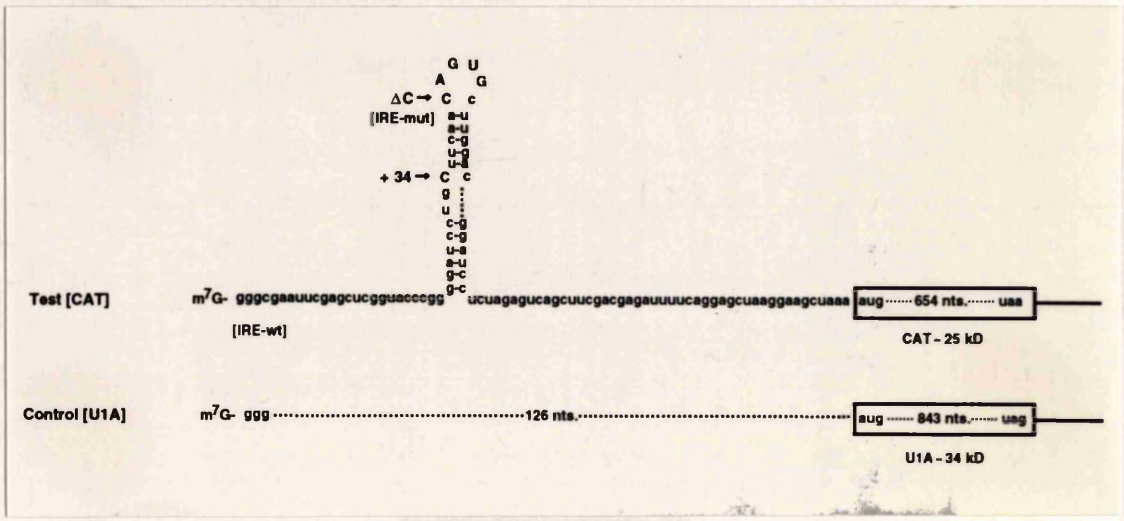
Poly (A)-enriched RNA from HeLa cells was translated in wheat germ extract. In the absence of IRP (-), the buffer used to elute IRP from the IRE affinity column (lane 5) or water (lane 2) was added. Where indicated, 200 ng IRP (+) was added to the translation reactions. A high molar excess of ferritin IRE competitor transcripts over IRP was added alongside IRP where indicated (+). ^{35}S-Met labelled products were subjected to immunoprecipitation prior to analysis by SDS-PAGE and fluorography. Molecular weight markers are indicated on the right.

3.2.2 The cis-acting ferritin sequences required for translational repression by IRP in cell-free translation systems.

3.2.2.1 The cis-acting sequences required for IRE-mediated repression in rabbit reticulocyte lysate.

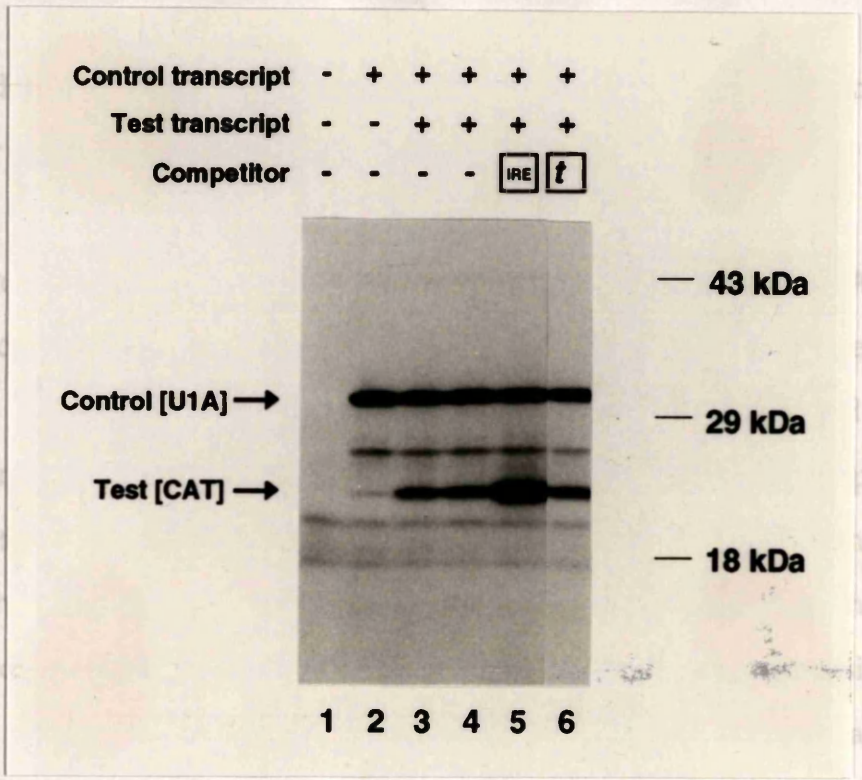
Having established that affinity-purified or endogenous reticulocyte IRP can repress translation of cellular ferritin mRNA in cell-free translation systems, it was of interest to investigate whether an *in vitro* transcribed mRNA containing only the IRE element from the ferritin mRNA would be translationally repressed by IRP *in vitro*. To this end, two mRNAs were transcribed *in vitro* in the presence of m^7GpppG cap analogue using T7 RNA polymerase under conditions previously optimised to ensure that >95% of transcripts were capped (Dr R. Stripecke- PhD thesis). IRE-wt bears an IRE at position +34 (figure 6) in the 5'UTR of a CAT reporter mRNA. U1A was used an internal control containing no ferritin or IRE related sequences, and encodes the U1A protein, an RNA-binding protein that interacts with loop II of the spliceosomal U1 snRNA (Scherly et al., 1989). The use of *in vitro* transcribed mRNAs, bypasses the need for immunoprecipitation of CAT or the U1A protein as both translation products are easily distinguished from the low background of proteins synthesised from endogenous reticulocyte or wheat germ mRNAs (figure 7 lanes 3-6 and figure 8A lanes 2-3). Translation of IRE-wt and U1A mRNA in rabbit reticulocyte lysate (figure 7) resulted in two main polypeptides, the larger ~34 kDa product corresponding to the U1A protein and the smaller ~25 kDa product representing CAT (lane 3 and 4). Although U1A translation occurs efficiently, translation of IRE-wt appears to be repressed (lanes 3-4) suggesting that the IRE represents the only sequences required from ferritin mRNA to confer repression by IRP. To investigate whether translation of IRE-wt is indeed repressed, IRE competitor transcripts were

Figure 6. Schematic representation of the *in vitro* transcribed capped mRNAs.



The sequence of the 5'UTR of test transcript IRE-wt, and the structure of the IRE are indicated. IRE-mut is identical except that the first C in the loop of the IRE has been deleted (ΔC). The IRE is located 34 nucleotides downstream from the cap, with reference to the distance between the conserved unpaired C in the IRE (+34) and the cap structure (Goossen and Hentze, 1992). U1A is a control transcript and contains no ferritin or IRE like sequences. The size of the CAT and U1A mRNA open reading frames (open boxes) and their protein products are given.

Figure 7. Cis-acting sequences required from ferritin mRNA to confer repression in rabbit reticulocyte lysate.



Where indicated (+), 1 ng of U1A and 2.5 ng of IRE-wt mRNA were translated in the absence (-) or presence of 5 ng of IRE (lane 5) or 45 ng of *toll* (lane 6) competitor transcript. Lanes 3-4 represent duplicate controls to assess experimental variation. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. The positions of the U1A and CAT proteins are indicated on the left and molecular weight markers are shown on the right.

added to the translation reactions. The addition of IRE competitor transcripts increased IRE-wt translation by sequestration of IRP (lane 5), without affecting the translation of U1A. Thus, the repressed expression of IRE-wt was due to IRE/IRP complex formation, indicating that the presence of an IRE in position +34 of the 5'UTR of an mRNA is sufficient to mediate repression by IRE/IRP in rabbit reticulocyte lysate.

3.2.2.2 Ferritin sequences required to confer IRP-mediated repression to reporter mRNAs in wheat germ extract.

In contrast to the repressed translation of IRE-wt mRNA in rabbit reticulocyte lysate, IRE-wt was efficiently translated in wheat germ extract (figure 8A). The addition of 250 ng of affinity-purified IRP (an approximately 150-fold molar excess) resulted in a complete repression of IRE-wt translation without affecting the translation of U1A (compare lanes 2-3 with 4-5). Titration of IRP against IRE-wt showed that this specific repression is dose dependent (figure 8B). Furthermore, repression can be relieved by the addition of IRE competitor RNA (figure 10; compare lanes 3-4 with 10-12).

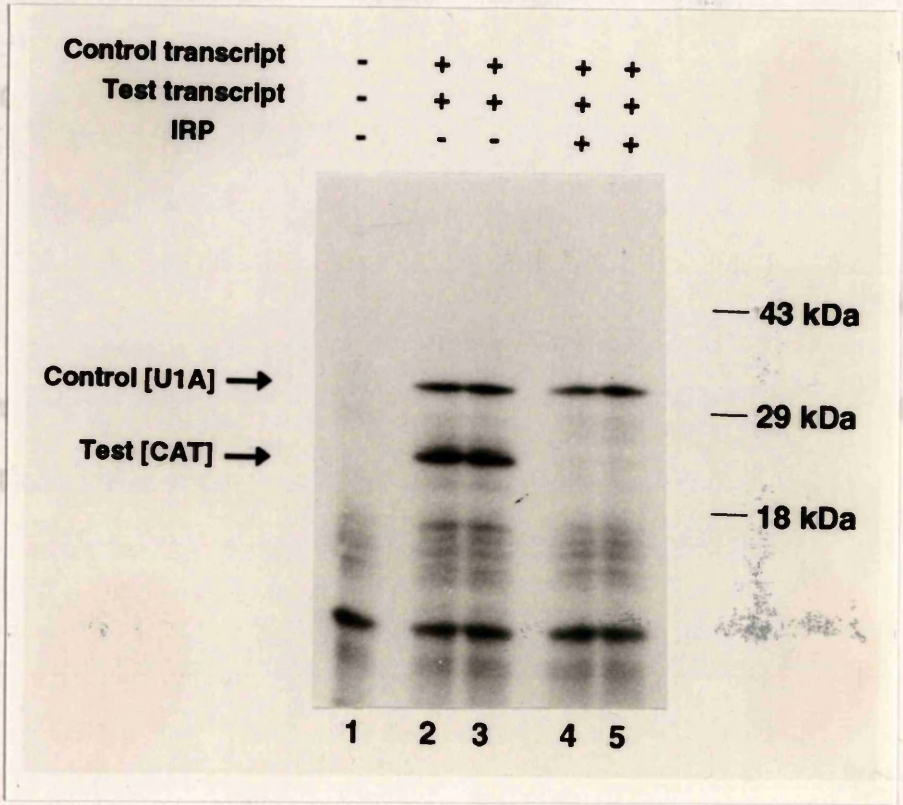
3.2.3 IRP-responsiveness of a ΔC IRE-containing CAT mRNA in cell-free translation systems.

3.2.3.1 IRP-responsiveness of ΔC IRE-containing mRNA in rabbit reticulocyte lysate.

To further characterise these cell-free translation systems, a third *in vitro* transcribed CAT mRNA, IRE-mut, was evaluated. IRE-mut is identical to IRE-wt, except at a single position in the loop of the IRE where the 5' C residue has been deleted (see figure 6). The reduction in the size of the loop

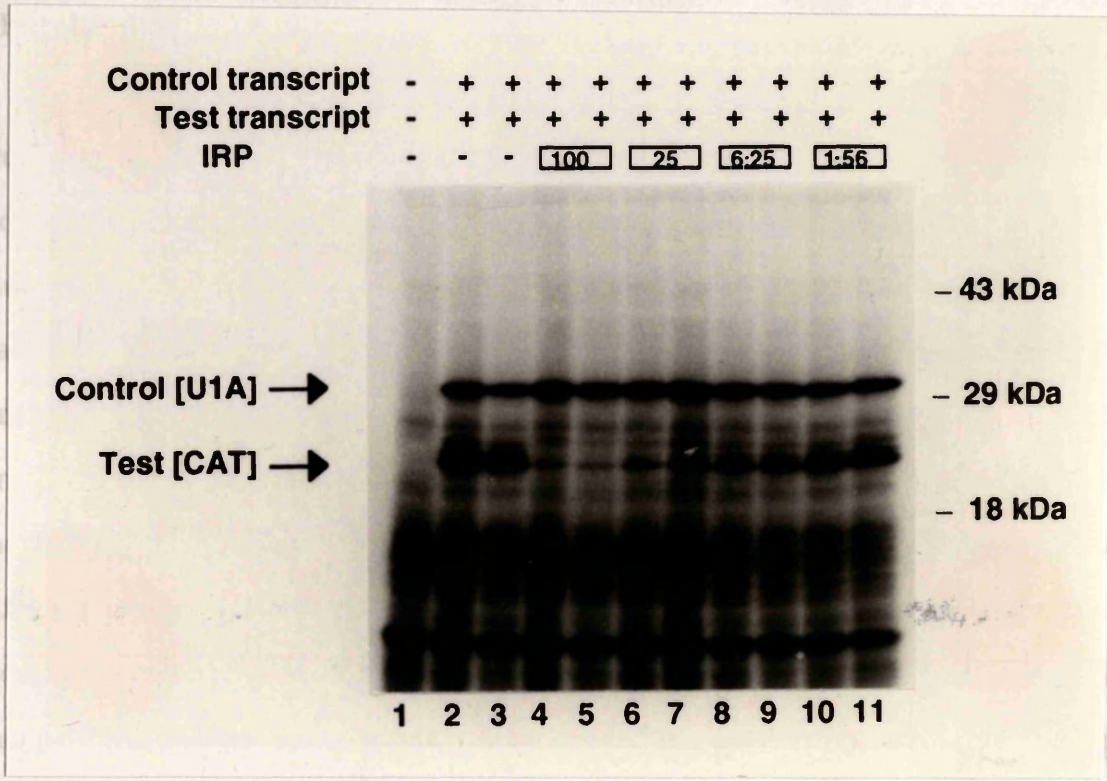
Figure 8. Ferritin sequences required for IRP-mediated repression in wheat germ extract

(A) Translation of *in vitro* transcribed CAT mRNAs containing an IRE in the presence of affinity-purified IRP in wheat germ extract.



Where indicated (+), 2.5 ng of U1A and 5 ng of CAT mRNA were translated in the absence (lanes 2-3) or presence of 250 ng of IRP (lanes 4-5). ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. Molecular weight markers are shown on the right and the positions of the U1A and CAT proteins on the left .

Figure 8 (B). The translation of IRE-wt mRNA in the presence of varying IRP concentrations.

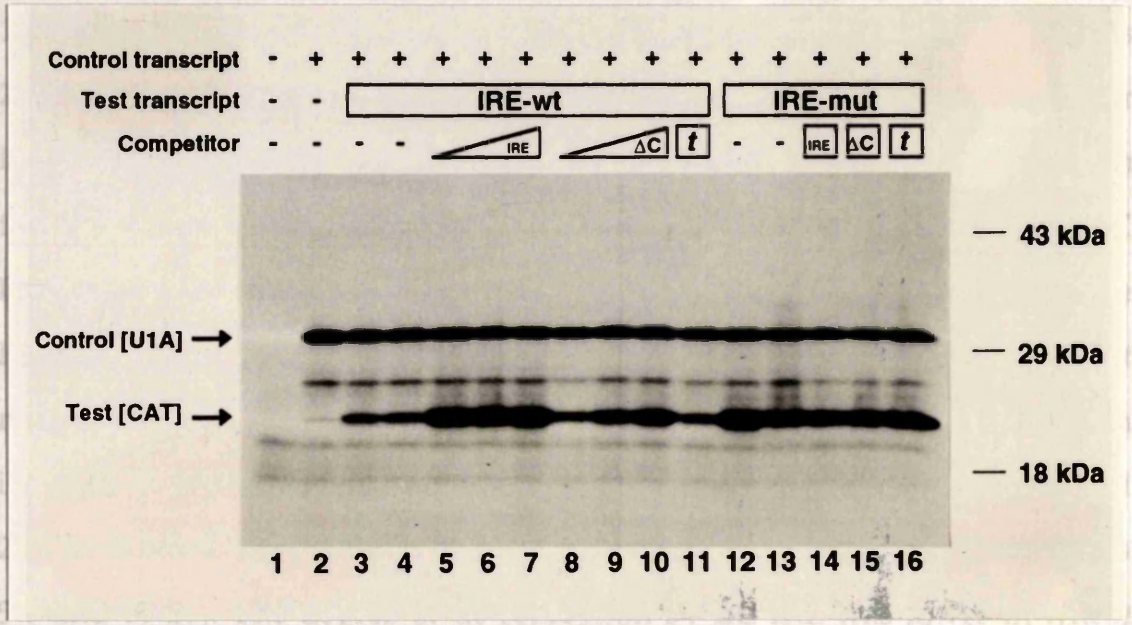


Repression of IRE-wt translation is dose-dependant. Where indicated (+), 2.5 ng of U1A and 5 ng of CAT mRNA were translated in wheat germ extract in the presence of 100 ng (lanes 4-5), 25 ng (lanes 6-7), 6.25 ng (lanes 8-9) or 1.56 ng (lanes 10-11) of affinity-purified IRP. No IRP was added to the translations in lanes 2-3. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography, molecular weight markers are shown on the right and the positions of the U1A and CAT proteins on the left .

from 6 to 5 nucleotides is not predicted to adversely affect stem loop formation, but the ΔC IRE exhibits a severly reduced affinity for IRP, so that the resulting complex can be disrupted by heparin (Haile et al., 1989; Rouault et al., 1988). Cell transfection experiments have shown that such a ΔC IRE is not capable of conferring iron regulation to a reporter gene in cells, predicting that IRE-mut should not be repressed *in vitro* by IRP (Hentze et al., 1988; Hentze et al., 1987b).

Translation in rabbit reticulocyte lysate (figure 9) revealed that, in contrast to IRE-wt, translation of IRE-mut appeared not to be repressed (compare lanes 3-4 with 12-13). Furthermore, IRE-mut translation was not augmented by a high concentration of IRE competitor transcripts (compare lanes 5-7 with 14). A second competitor, which contains the mutated ΔC IRE from IRE-mut was added to translations containing either IRE-wt or IRE-mut, relieving repression of IRE-wt (lanes 8-10) at high concentrations, but not affecting IRE-mut translation (lane 15). This effect is due to the residual affinity of the ΔC IRE competitor for IRP (Goossen et al., 1990), such that sufficient excess of ΔC IRE allows it to sequester the endogenous IRP. The non IRP-binding competitor transcript, *toll*, which forms a stem loop structure closely resembling an IRE (figure 33), had no effect on the translation of either IRE-wt (lane 11) or IRE-mut (lane 16), showing that the presence of small RNA transcripts containing stem-loop structures *per se* does not relieve repression of IRE-wt. It is clear from these results that reticulocyte IRP distinguishes between two mRNAs varying at only one position in the IRE, consistent with previous observations that a ΔC IRE-containing mRNA was not regulated in transfected cells (Hentze et al., 1988; Hentze et al., 1987b).

Figure 9. Analysis of IRE-mut mRNA translation in rabbit reticulocyte lysate.

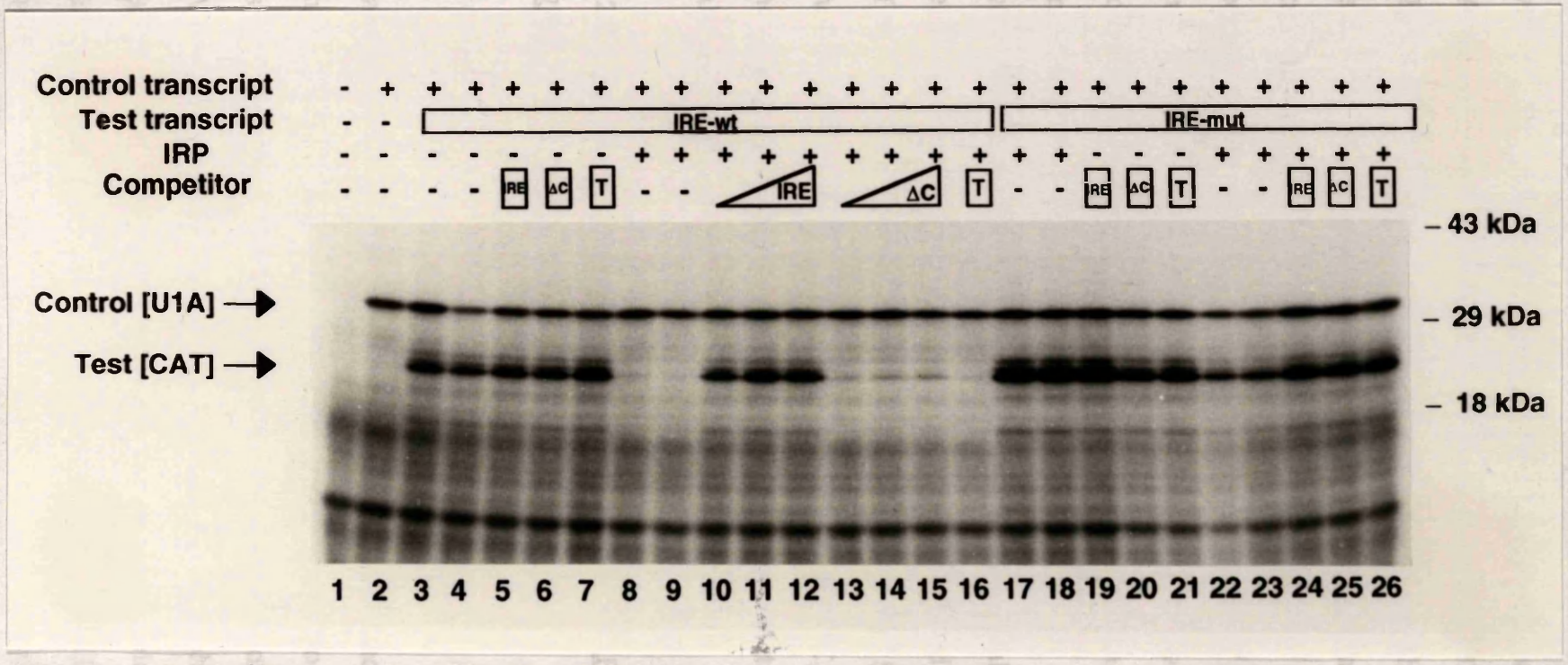


Where indicated (+), 1 ng of U1A and 2.5 ng of IRE-wt (containing a structurally intact IRE) or IRE-mut (containing a ΔC IRE) mRNA were translated in the absence (-) or presence of 5, 15 or 45 ng of IRE (lanes 5-7 respectively and 14), ΔC (lanes 8-10 respectively and 15) or 45 ng of *toll* (t; lanes 11 and 16) competitor transcripts. Lanes 3-4 and 12-13 represent duplicate controls to assess experimental variation. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. The positions of the U1A and CAT proteins are indicated on the left and molecular weight markers are shown on the right.

3.2.3.2 The responsiveness of ΔC IRE-containing mRNAs to purified IRP added to wheat germ extract.

To evaluate whether affinity-purified IRP added to wheat germ extract represses ΔC IRE-containing mRNAs, the IRE-wt, IRE-mut and U1A mRNAs were utilised (figure 10). In the absence of IRP, IRE-wt (lanes 3-4) and IRE-mut (lanes 17-18) were translated equally efficiently. Addition of 150 ng of IRP lead to repression of IRE-wt (lanes 8-9) but not IRE-mut (lanes 22-23). The slight loss of translation of IRE-mut was paralleled by a similar loss of U1A translation and is therefore non-specific (lanes 22-23). Although the IRE competitor relieved repression of IRE-wt (lane 10-12) by IRP, the ΔC IRE competitor did not substantially affect repression of IRE-wt even at the highest concentration (lanes 13-15). Addition of *toll* competitor did not relieve repression of IRE-wt (lanes 16). None of the competitors caused an increase in translation of IRE-mut in the presence of IRP (lanes 24, 25 and 26), or affected translation of IRE-wt or IRE-mut in the absence of IRP (lanes 5-7 and 19-21). The degree of de-repression by ΔC IRE was lower in wheat germ extract than in rabbit reticulocyte lysate suggesting a differential recognition of the IRE element in these systems. However, it must be remembered that the quantity of endogenous IRP in rabbit reticulocyte lysate is considerably less than the quantities of IRP added to wheat germ extract, as shown by gel retardation analysis (compare figures 3B and 25A on page 131) and the lower repression of IRE-wt mRNA in rabbit reticulocyte lysate compared to wheat germ extract containing 150 ng of IRP (compare figures 7 and 8). Thus, the data obtained using wheat germ extract clearly demonstrate that affinity-purified IRP accurately reflects the failure of ΔC IRE-containing mRNAs to be regulated in reticulocyte lysate and in transfected cells.

Figure 10. Affinity purified IRP added to wheat germ extract represses translation of IRE-wt, but not IRE-mut mRNA.



In lanes 3-26, 2.5 ng of U1A and 5 ng of either IRE-wt or IRE-mut mRNA were translated in wheat germ extract. Where indicated (+), 150ng of IRP and/or 5, 15 or 45 ng of IRE, ΔC IRE and 45 ng of *toil* (t) competitors were added to translation reactions. The translation reactions in lanes 1 and 2 received either no mRNA or U1A mRNA, respectively. Lanes 3-4, 8-9, 17-18 and 22-23 represent duplicates to assess experimental variation. ^{35}S-Met labelled products were analyzed by SDS-PAGE and fluorography. Molecular weight markers are indicated on the right and the positions of the U1A and CAT proteins are shown on the left.

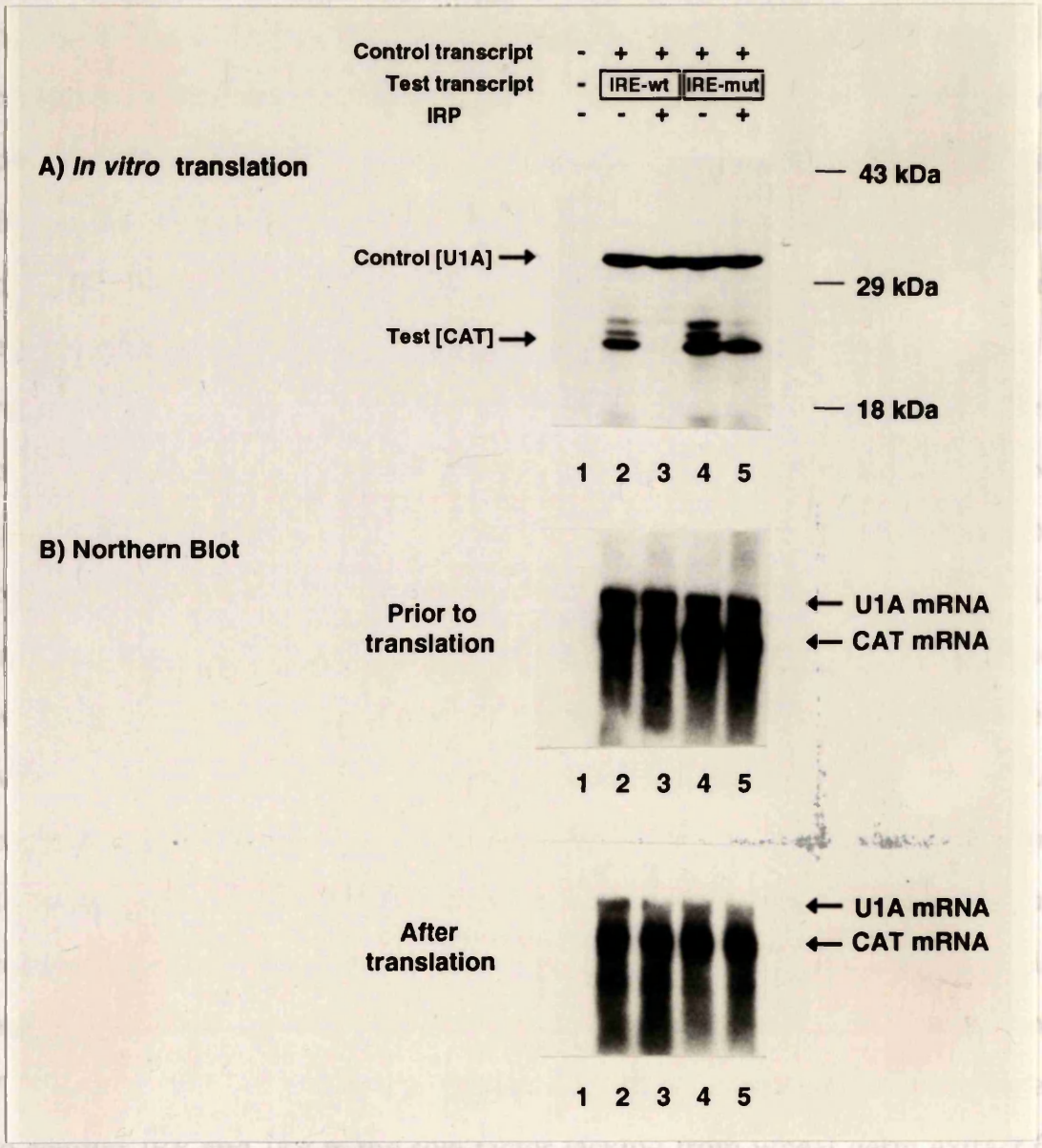
3.2.4 The effect of IRP on mRNA stability *in vitro*.

Iron regulation of ferritin expression can occur in mammalian cells without changes in the steady state levels of cytoplasmic ferritin mRNAs (Aziz and Munro, 1986; Rogers and Munro, 1987; Shull and Theil, 1982; Shull and Theil, 1983). To exclude the possibility that the IRE/IRP-mediated reduction of IRE-wt mRNA expression *in vitro* was a result of IRP-induced transcript degradation, IRE-wt and IRE-mut were translated in wheat germ extract in the presence or absence of purified IRP (figure 11A). Aliquots of the reactions taken before (figure 11B, top panel) and after (figure 11B, bottom panel) translation were phenol/chloroform extracted, and RNA was analysed by Northern blotting. Using U1A as an internal control for RNA recovery, it is apparent that all mRNAs remain stable during the translation reactions (compare the top and bottom panels) and that neither the integrity of IRE-wt (lanes 2 and 3) nor IRE-mut (lanes 4 and 5) mRNAs are affected by the presence of IRP. Therefore the reduction of CAT synthesis induced by the presence of IRP *in vitro* is a result of translational repression and not mRNA degradation.

3.2.5 Functional substitution of affinity-purified IRP by recombinant human IRP in wheat germ extract.

As shown earlier in this chapter, IRP is absolutely required for translational repression of IRE-bearing mRNAs and was assumed to be the only factor necessary, although its function had so far only been evaluated using purified fractions obtained by conventional biochemical means (Walden et al., 1989) or IRE affinity chromatography (figures 5B, 8 and 10). Furthermore, evidence in favour of the possible existence of multiple biochemically and genetically distinct forms of IRP (Hirling et al., 1992; Leibold and Munro, 1988; Rouault et al., 1990) prompted an investigation of

Figure 11. The effect of IRP on mRNA stability *in vitro*.

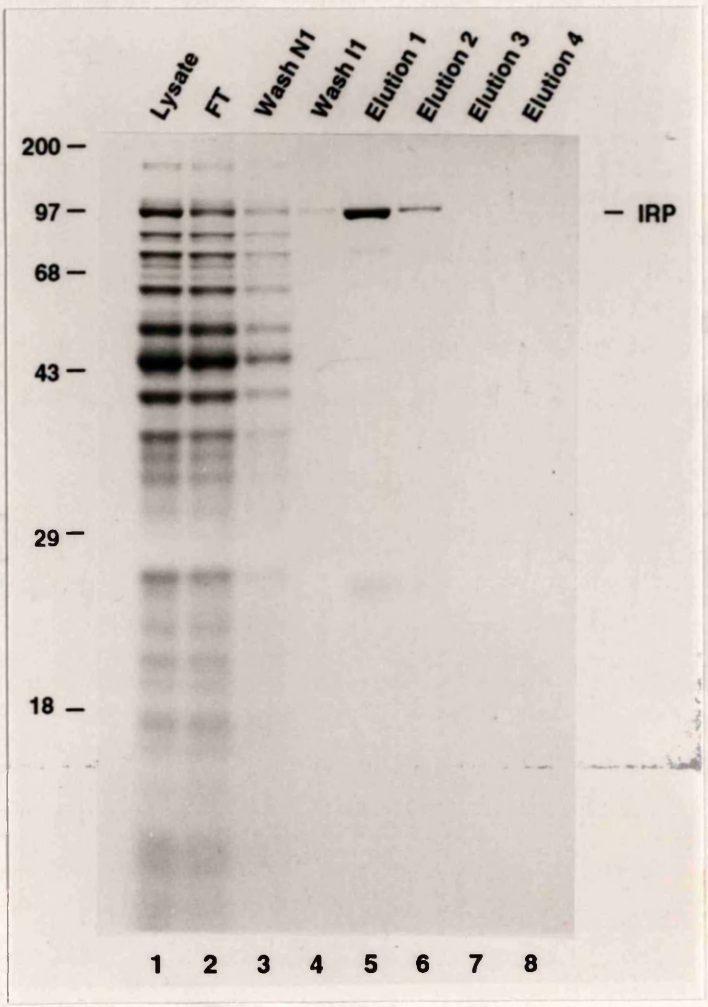


(A). Where indicated, 15 ng of U1A and IRE-wt or IRE-mut mRNAs were translated in WGE in the presence (+) or absence (-) of 450 ng IRP, and the products were analysed by SDS-PAGE analysis and fluorography.
(B). RNA was extracted from aliquots of the translation reaction, before and after translation, Northern blotted and probed simultaneously for U1A and CAT mRNAs.

the activity of recombinant human IRP to repress IRE-wt translation in wheat germ extract. To this end, IRP carrying an N-terminal $(His)_6$ tag was expressed in vaccinia virus-infected HeLa cells and in *E. coli*, and purified by nickel NTA-agarose chromatography (figure 12A). The repressor activity of recombinant IRP from either vaccinia virus-infected HeLa cells or *E.coli* was directly compared to placental IRP by addition of equal amounts of IRP from these sources to wheat germ extract translation reactions containing CAT mRNAs (figure 12B). When IRE-wt mRNA was translated in wheat germ extract, addition of 100 ng of affinity-purified human placental IRP (lane 3), recombinant human IRP purified from either vaccinia virus-infected HeLa cells (lane 8), or *E. coli* (lane 14) all specifically repressed CAT mRNA translation; the buffer used to elute IRP from the nickel Ni^{2+}NTA-agarose column (lane 6 and 12), or column eluates from wild type vaccinia virus-infected HeLa cells (lane 7), or eluates originating from *E. coli* transformed with the host plasmid for IRP expression (lane 13) did not exhibit this effect. Moreover, the recombinant IRPs only repressed the translation of mRNAs containing structurally intact and not mutated IREs (compare lanes 3, 8 and 14 with lanes 5, 11 and 17). When the concentration of placental and recombinant IRPs were titrated against IRE-wt mRNA, the three proteins exhibited similar specific activities (figure 12C, compare lanes 3-6 with lanes 7-10 and 11-14). Therefore, the recombinant IRPs are functionally equivalent to cellular IRP and IRP is the sole factor lacking from wheat germ extract for translational repression *via* IRE/IRP.

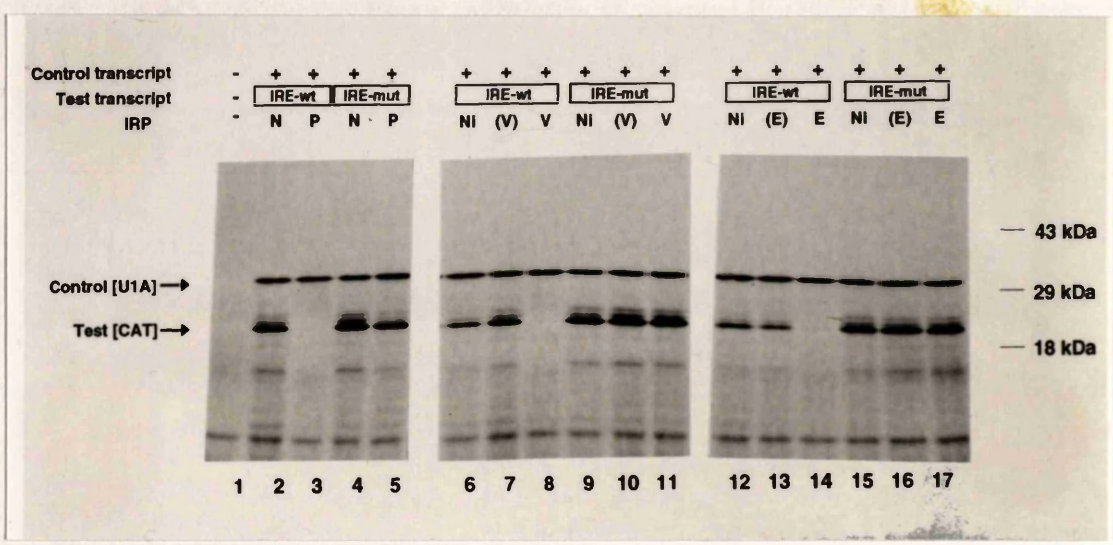
Figure 12. Comparison of recombinant human IRP isolated from vaccinia virus infected HeLa cells or *E.coli* with cellular IRP purified from human placenta.

(A). Purification of recombinant human IRP from *E.coli*.



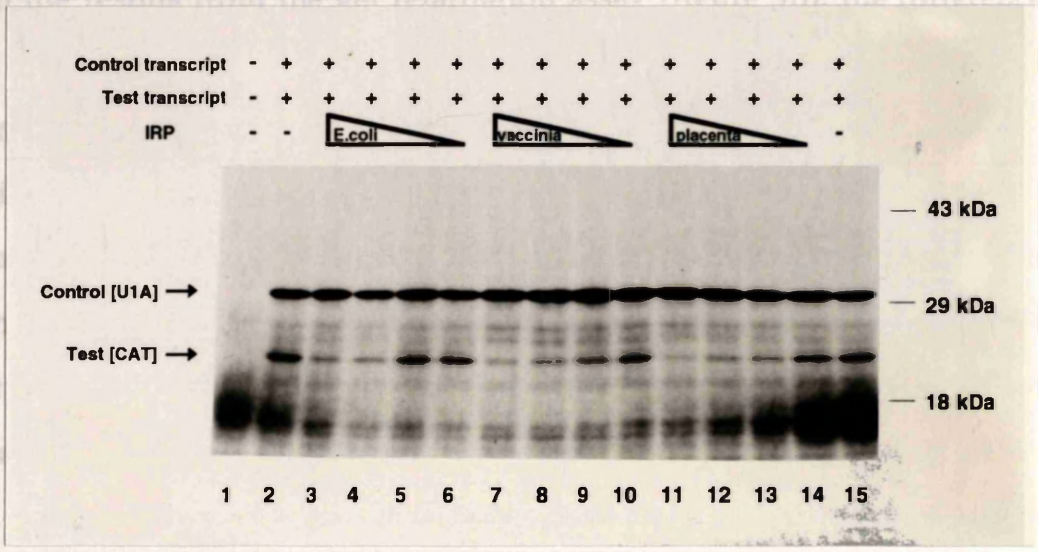
Lysates (lane 1) were applied to nickel NTA-agarose. The majority of proteins in the lysate were not retained by the nickel NTA agarose beads (lane 2). Proteins retained on the nickel NTA agarose were washed once with buffer N containing 0.4 KCl (lane 3), once with buffer N, and twice with buffer N containing low concentrations (5 mM) of imidazole (lane 4) prior to elution (lanes 5-8). Molecular weight markers are shown on the right .

Figure 12 (B). Repression of IRE-wt and IRE-mut mRNAs by recombinant human IRP and placental human IRP.



U1A (2.5 ng) and 5 ng IRE-wt (lanes 2-3, 6-8 and 12-14) or IRE-mut mRNAs (lanes 4-5, 9-11 and 15-17) were translated in WGE in the absence or presence of 100 ng of IRP-purified from human placenta [pIRP] or recombinant IRP from either vaccinia virus infected cells [vIRP] or *E.coli* [eIRP]. In the absence of IRP either buffer N [N], buffer N containing 50 mM imidazole [Ni] or protein preparations from wild type vaccinia virus infected cells [(V)] or *E.coli* transformed with the expression vector alone [(E)] were added. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography.

Figure 12 (C). Comparison of the repressor activities of recombinant human IRP and human placental IRP.



U1A (2.5 ng) and IRE-wt (5 ng) mRNAs were translated in WGE in the absence or presence of 100, 50, 25 or 12.5 ng of recombinant *E.coli* IRP (lanes 3-6), recombinant vaccinia virus expressed IRP (lanes 7-10) or placental IRP (lanes 11-14). ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography.

3.3 Discussion.

The de-repression of ferritin translation in rabbit reticulocyte lysate by short IRE competitor transcripts confirmed that IRE/IRP interactions were responsible for the poor translation of ferritin mRNA in this system. Conversely, addition of IRP purified by RNA affinity chromatography to wheat germ extract containing poly (A) mRNA showed that it exerts activity as a specific repressor of ferritin mRNA translation *in vitro*. Taken together with the results from the gel retardation assay (figure 3B), the difference in the translation of ferritin mRNAs in the two cell-free systems is a consequence of the presence or absence of active IRP, and cannot be attributed to other differences in the translation initiation machinery in animal and plant cells (Merrick, 1992). Therefore, this series of experiments is in agreement with the findings of Walden et al. (1988; 1989), and confirm that both cell-free translation systems reproduce the repression of poly-adenylated ferritin mRNA observed in cells in the presence of active IRP.

The only sequence required from ferritin mRNA to mediate repression by IRP was the IRE element itself, while an IRE with a single nucleotide deletion was not functional, showing that an intact IRE is necessary for repression. No IRP-induced changes in mRNA stability were observed *in vitro*, confirming that the IRP-mediated changes in protein synthesis were achieved at the level of translation. Since the *in vitro* transcribed mRNAs used here were not polyadenylated, it is concluded that IRP-mediated translational repression (at least *in vitro*) does not require the mRNA to be polyadenylated. The advent of recombinant IRP revealed that IRP itself is the only mammalian-specific protein required for IRE-mediated repression, as purification of recombinant IRP from *E. coli* excludes the possibility of inadvertent co-purification of other eukaryotic factors that might otherwise contribute to translational repression by IRP. Consequently, IRP is the only protein absent from wheat germ extract that is required for translational repression. Furthermore, the function of IRP in

an extract derived from plant cells, which do not regulate ferritin mRNA translation indicates that the function of IRP in mammalian cells is not likely to require protein/protein interactions with mammalian-specific co-factors.

In conclusion, the data presented here argue that the *in vitro* systems described are suitable for examining the mechanism of IRE/IRP-mediated translational repression. In addition, the availability of a one step purification of IRP which yields large amounts of protein should greatly facilitate these studies.

Chapter 4: The mechanism of IRE/IRP-mediated regulation.

4.1 Introduction.

While much has been learnt about IRP, the mRNAs to which it binds and the control of IRP activity, surprisingly little is known about the mechanism of how IRE/IRP complexes affect translation. Changes in the length of ferritin mRNA poly (A) tails following iron regulation have not been observed and IRP-mediated translational repression *in vitro* does not require a polyadenylated mRNA (Chapter 3 and Swenson et al., 1991), suggesting that changes in ferritin synthesis are unlikely to be a consequence of changes in the poly (A) tail length. The conserved position of the IRE within the cap-proximal 40 nucleotides of ferritin mRNAs from different species is functionally important. An IRE which is moved further than 60 nucleotides downstream from the cap structure only poorly mediates translational regulation (Goossen et al., 1990; Goossen and Hentze, 1992), indicating that events at the extreme 5' end of the mRNA are most likely to represent the target of IRE/IRP-mediated regulation.

Translational repression by IRE/IRP complexes was suggested not to require specific interactions of IRP with initiation factors, based on experiments in which the IRE was substituted with binding sites for proteins which play no physiological role in eukaryotic translation (such as the spliceosomal protein U1A and the bacteriophage MS2 coat protein). These proteins, which are unlikely to repress translation by specific interactions with translation initiation factors, nevertheless mediate IRE-like translational repression, strongly suggesting that a purely physical (steric) block of the translational machinery is sufficient to repress translation (Stripecke and Hentze, 1992; Stripecke et al., 1994). The cell-free systems described in chapter 3, and sucrose gradient analysis were employed to investigate how IRP and the U1A protein, as an example of a steric inhibitor of translation initiation, affect ribosome access to the mRNA.

4.2 Results.

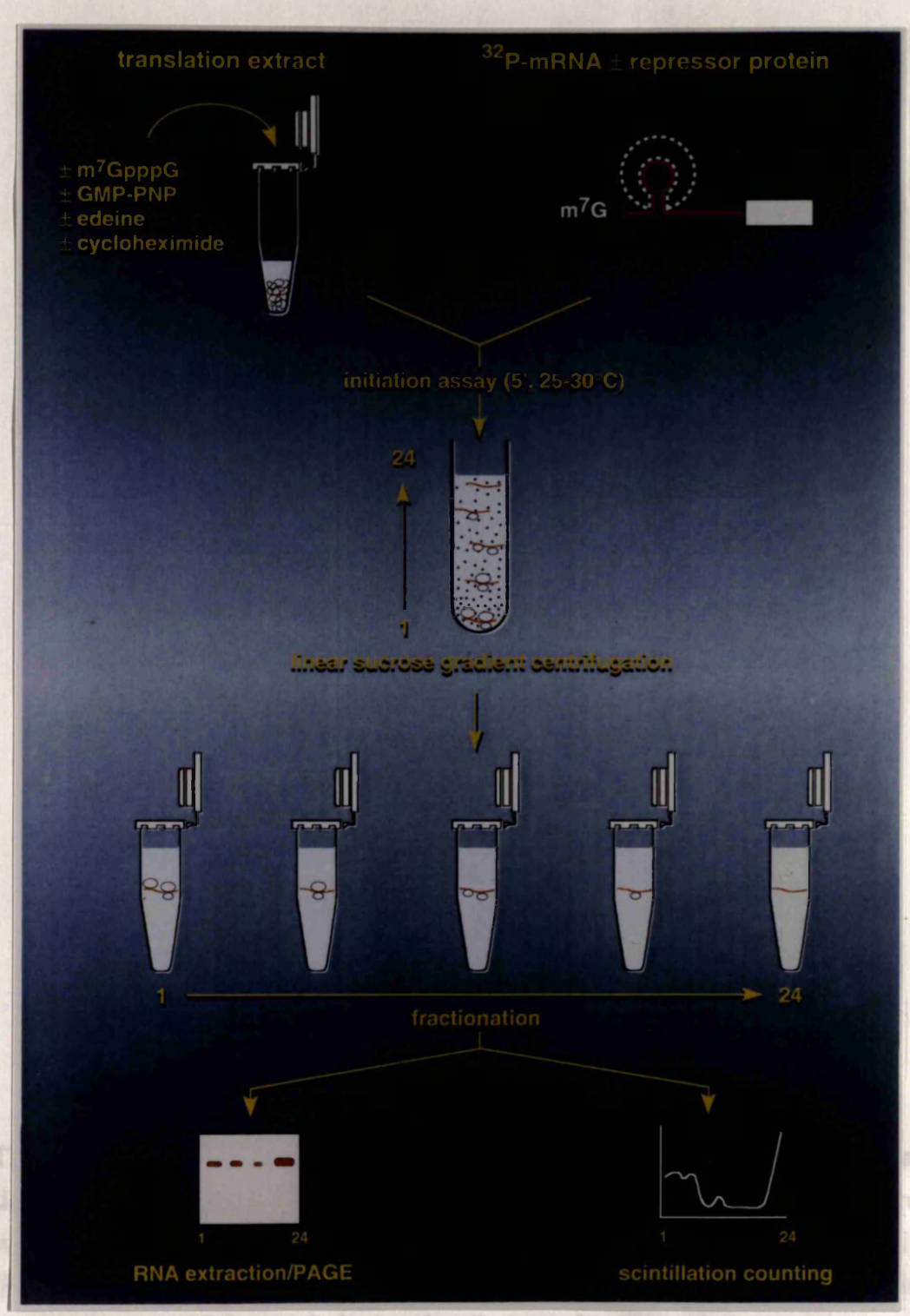
4.2.1 The effect of IRP-binding on the association of ribosomes with IRE-containing mRNAs.

4.2.1.1 The effect of IRP-binding on 80S ribosome assembly.

To investigate which components of the translational machinery associate with an IRE-regulated mRNA in the presence of IRP, translation initiation assays were performed using ^{32}P-labelled mRNAs and translation-competent extracts from wheat germ or rabbit reticulocytes. The resulting initiation complexes were resolved by centrifugation through linear sucrose gradients. Following fractionation, the distribution of mRNA was assessed by RNA extraction and analysis on denaturing polyacrylamide gels or by scintillation counting of the radioactivity associated with each fraction. As shown in figure 20, the latter quantitatively reflected non-degraded full-length mRNAs. The assays were routinely performed in the presence of 0.5 mM cycloheximide, an antibiotic which interferes with the peptidyl transfer reaction (Obrig et al., 1971). Thus, 80S ribosomes which assemble on the mRNA at the AUG initiator codon are trapped and run off is prevented. The procedure for the formation and resolution of initiation complexes is described in materials and methods and depicted in Figure 13.

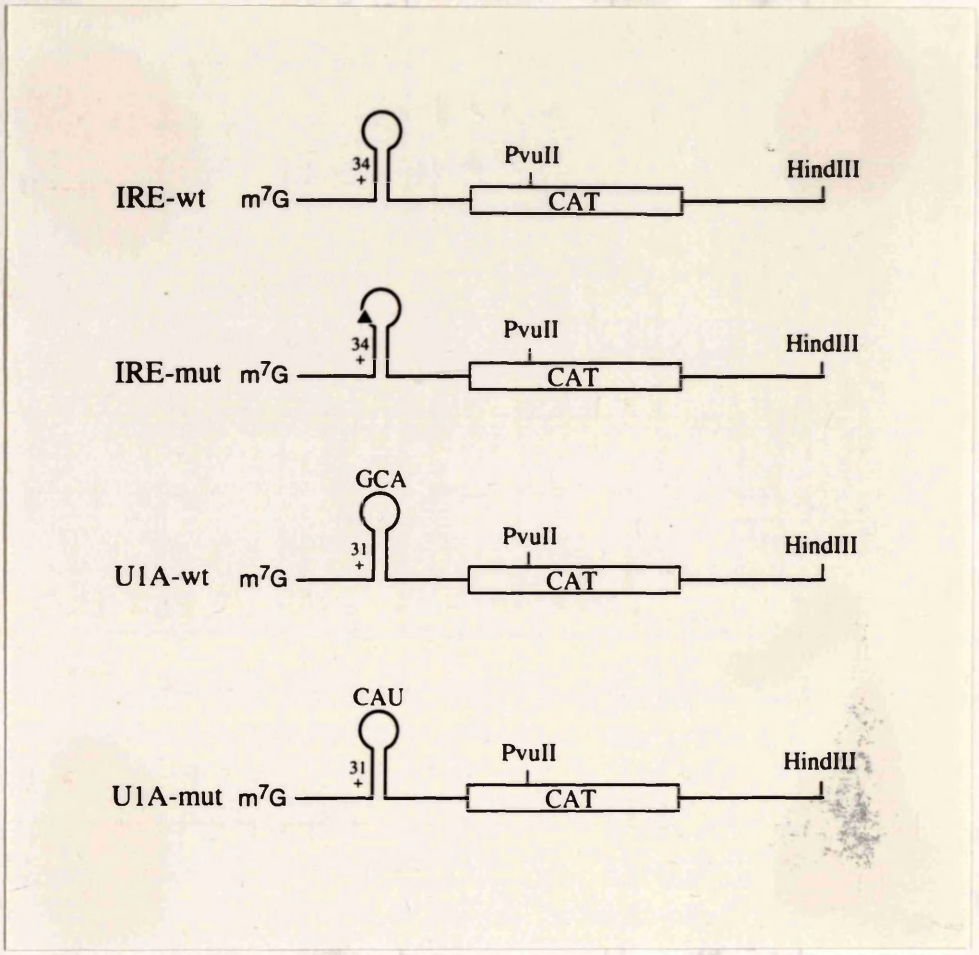
Initially, three mRNAs, IRE-wt, IRE-mut and U1A (figures 6 and 14) whose response to IRP was previously characterised (see chapter 3), were compared in assays in wheat germ extract using human recombinant IRP purified from *E.coli* as a source of IRP activity. When initiation assays were performed with the three different mRNAs, their sedimentation patterns through linear gradients of 10-30% sucrose were roughly similar (figure 15, dotted squares). Small amounts of mRNA (5 fmol) were utilised as this

Figure 13. Schematic representation of initiation assays and resolution of the resulting initiation complexes.



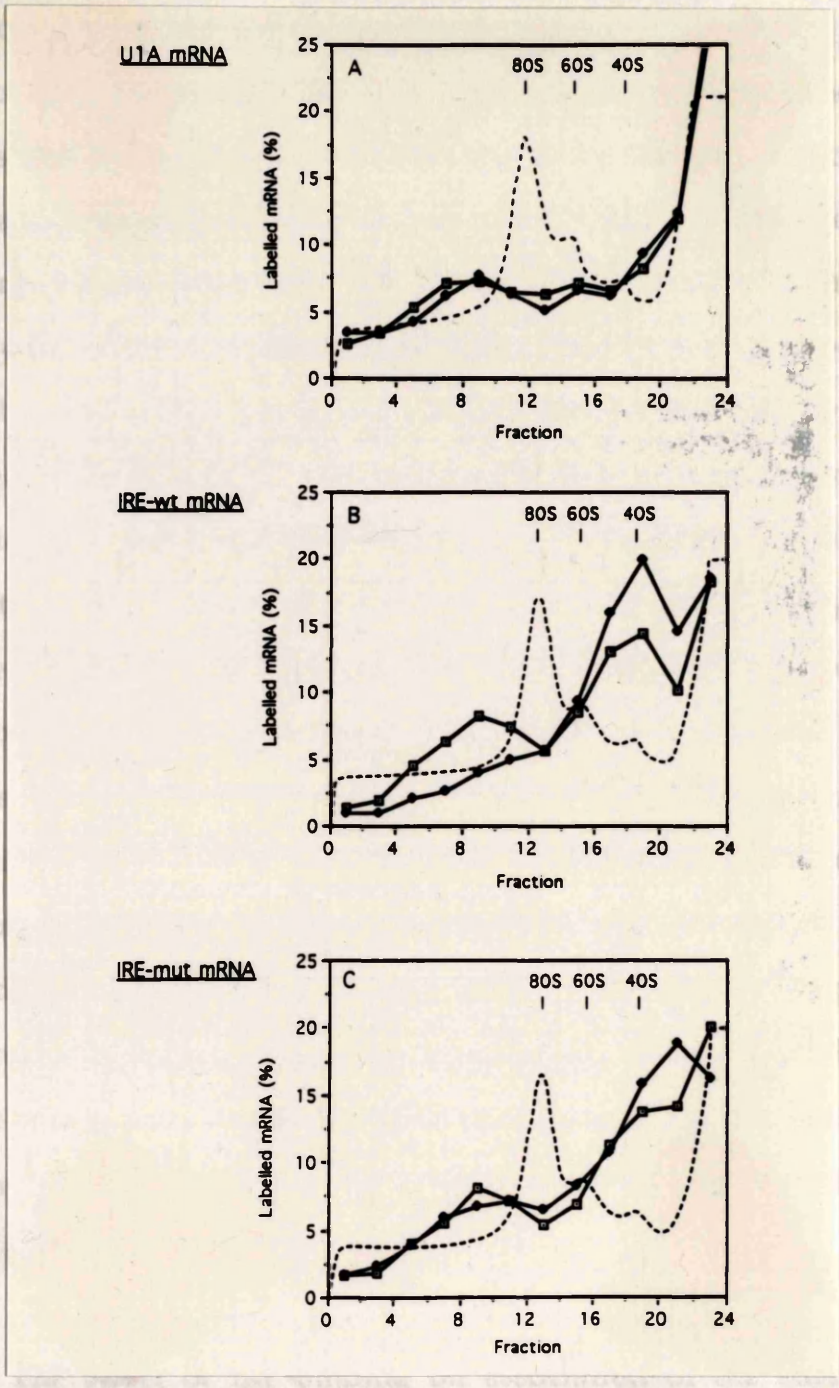
Translation extracts were pre-treated with inhibitors of translation initiation prior to the addition of ^{32}P-mRNA or -mRNA/protein complexes. Initiation complexes were allowed to assemble and were resolved by sucrose gradient centrifugation. Gradients were fractionated, and the radioactivity in the fractions determined by scintillation counting or by RNA extraction and analysis on denaturing polyacrylamide gels.

Figure 14. Diagram of the CAT reporter mRNAs used in this study.



The open reading frame encoding chloramphenicol acetyltransferase is denoted by an open box. The position of the IRE in the 5' UTR is quoted with reference to the distance of the conserved unpaired C residue in the IRE stem (not indicated here) from the cap structure. The black triangle symbolises an IRE which lacks the first nucleotide of the conserved loop. The position of the U1A binding site is quoted with reference to the distance of the insertion site from the cap structure. The RNA sequences indicated above the loop of U1A-wt and U1A-mut represent the only differences between the two transcripts. The AUG initiator codon is located at +103 and +102 in IRE-wt and IRE-mut, respectively, and at +111 in U1A-wt and U1A-mut. The restriction sites used to linearise the plasmids prior to transcription are indicated.

Figure 15. The effect of IRP on ribosome association with IRE-containing mRNAs in wheat germ extract.



Full-length mRNA transcripts A) U1A, B) IRE-wt and C) IRE-mut were assayed in the absence or presence of IRP. Initiation complexes were analysed on 10%-30% linear sucrose gradients and fractionated. The labelled mRNA in the fractions is expressed as a percentage of total counts recovered. The percentage of mRNA is plotted against the fraction number (dotted squares = - IRP; filled diamonds = + IRP). The dashed line denotes the A254 absorption profile, which was identical for gradients -/+ IRP. The positions of 40S, 60S and 80S ribosomal particles are indicated.

resulted in an increase in the fraction of ribosome-associated mRNAs from ~25% to ~40%, which sedimented in fractions 1-13 (table 1). Even in the presence of 0.5 mM cycloheximide, the majority of ribosome-associated mRNAs sedimented faster than 80S, the expected size of monosomes (a significant proportion of mRNAs assayed in rabbit reticulocyte lysate also sedimented faster than 80S - see 4.2.3). The same pattern emerged with anisomycin, another elongation inhibitor, and ^{35}S-Met incorporation was found by SDS-PAGE analysis of translation products to be completely inhibited by as little as 0.1 mM cycloheximide or 0.1 mM anisomycin (table 2), indicating that incomplete inhibition by cycloheximide is unlikely to account for the faster sedimentation rate. It is more likely that these particles represent 80S monosomes with additional 43S pre-initiation complexes associated with the 5' UTR, which are known to form when mRNAs have a sufficiently long 5' UTR (Kozak, 1991a). Addition of recombinant IRP to the translation initiation assays had little effect on the sedimentation pattern of U1A and IRE-mut mRNAs (figure 15A and C, black diamonds), whereas IRE-wt mRNA was largely displaced from fractions 1-13 and appeared to co-sediment with the small ribosomal subunit in fractions 17-21 (figure 15B, black diamonds). This result suggested that the translationally repressed IRE-wt mRNA was associated with a stalled pre-initiation complex.

4.2.1.2 The effect of IRP-binding on association of the small ribosomal subunit with IRE-containing mRNAs.

To further examine this apparent association of repressed IRE-wt mRNA with 43S pre-initiation complexes, inhibitors of translation which induce accumulation of defined initiation intermediates were included in the assays. The m^{7}GpppG cap analogue sequesters initiation factors with affinity for the cap structure of the mRNA. Sequestration of these factors,

Table 1. Distribution of mRNA into 80S or heavier complexes at different mRNA concentrations

	Percentage of mRNA in complexes of 80S or heavier	
mRNA concentration	15 ng/45 µl	3 ng/45 µl
Expt 1	22	33
Expt 2	23	33

Table 2. Inhibition of ^{35}S-Met incorporation by cycloheximide and anisomycin

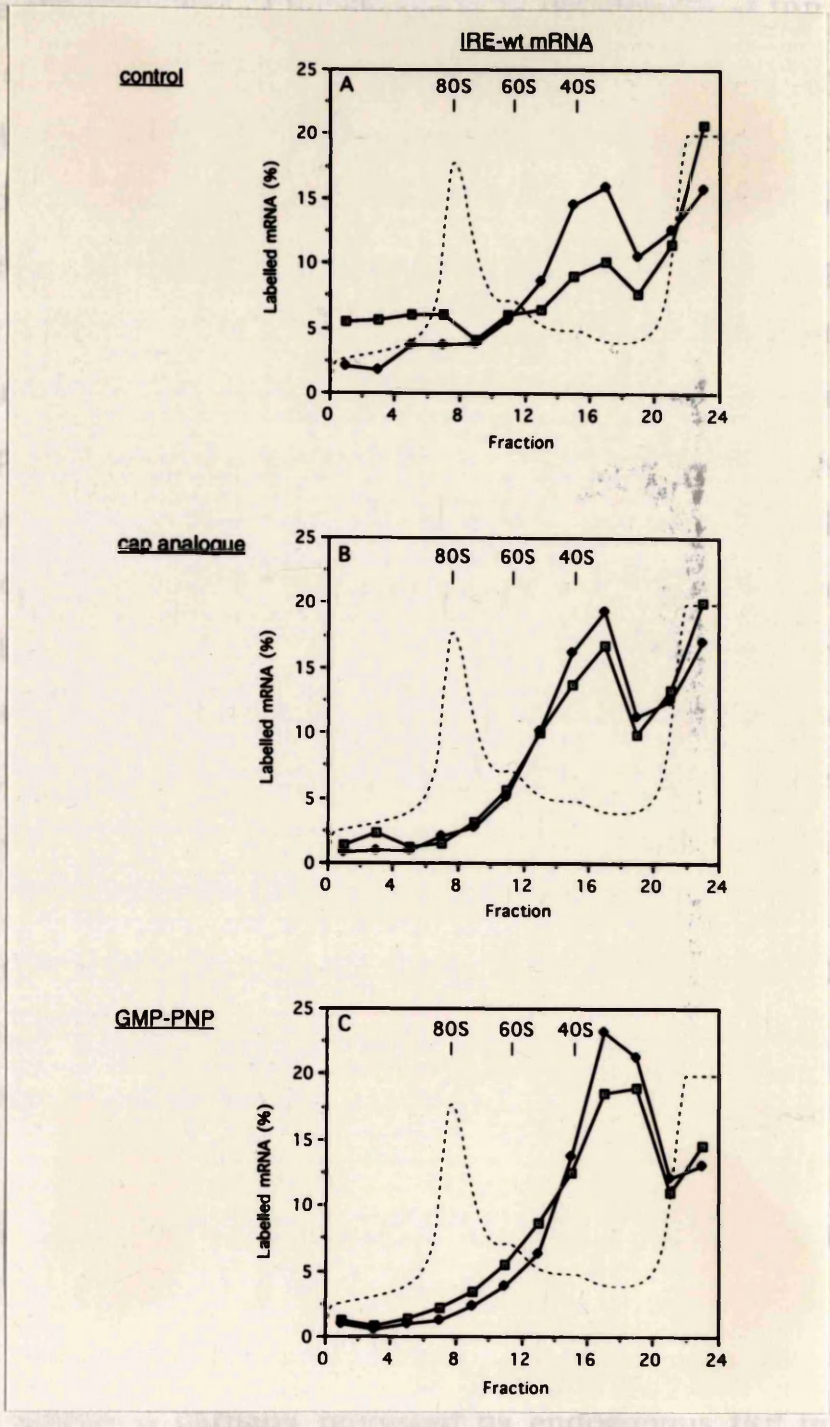
	Percentage inhibition of ^{35}S-Met incorporation	
Inhibitor concentration	Cycloheximide	Anisomycin
0.05 mM	85	92
0.1 mM	93	96
0.2 mM	95	98

which facilitate binding of the 43S complex to the mRNA, leads to the accumulation of mRNA in messenger ribonucleoprotein particles (mRNPs) in the top fractions of the gradient (Antony and Merrick, 1992). The non-hydrolyzable GTP analogue GMP-PNP induces the accumulation of 43S pre-initiation complexes stalled at the initiation codon, because GTP hydrolysis is required prior to the joining of the 60S ribosomal subunit (Anthony and Merrick, 1992; Hershey and Monro, 1966). GMP-PNP and m^7GpppG can hence be used to localise mRNAs engaged with 43S pre-initiation complexes (known as 48S complexes) or no ribosomal subunits, respectively. The sucrose concentration of the gradients was adjusted from 10%-30% to 5-25% to improve the resolution between complexes which sediment slower than 80S. Paradoxically, IRP-bound IRE-wt mRNA displayed similar sedimentation rates (approx. at 40S) both in the presence or absence of m^7GpppG, or GMP-PNP (figure 16). The similar migration profiles of mRNAs under conditions where ribosomes, the small ribosomal subunit or no ribosomal subunits can associate with the mRNA, implies that the sedimentation rate of IRP-bound IRE-wt mRNA at around 40S should not be taken as evidence for the association of IRP-repressed mRNAs with the small ribosomal subunit.

4.2.2 Higher resolution sucrose gradient analysis of mRNA particles inhibited by IRP.

It seemed reasonable to assume that the sedimentation of IRE-wt mRNA could have been influenced by proteins in the extract which bound to the 860 nucleotide long mRNA. Since the elements for IRE/IRP regulation are confined to the 5' UTR, truncated mRNAs (218 nucleotides in length) were transcribed from templates linearised at the *Pvu* II site 115 nucleotides into the CAT-ORF (figure 14). These shortened mRNAs should form initiation complexes, and may allow the resolution of *bona fide* 48S

Figure 16. Association of ribosomal subunits with IRE-wt mRNA in wheat germ extract.



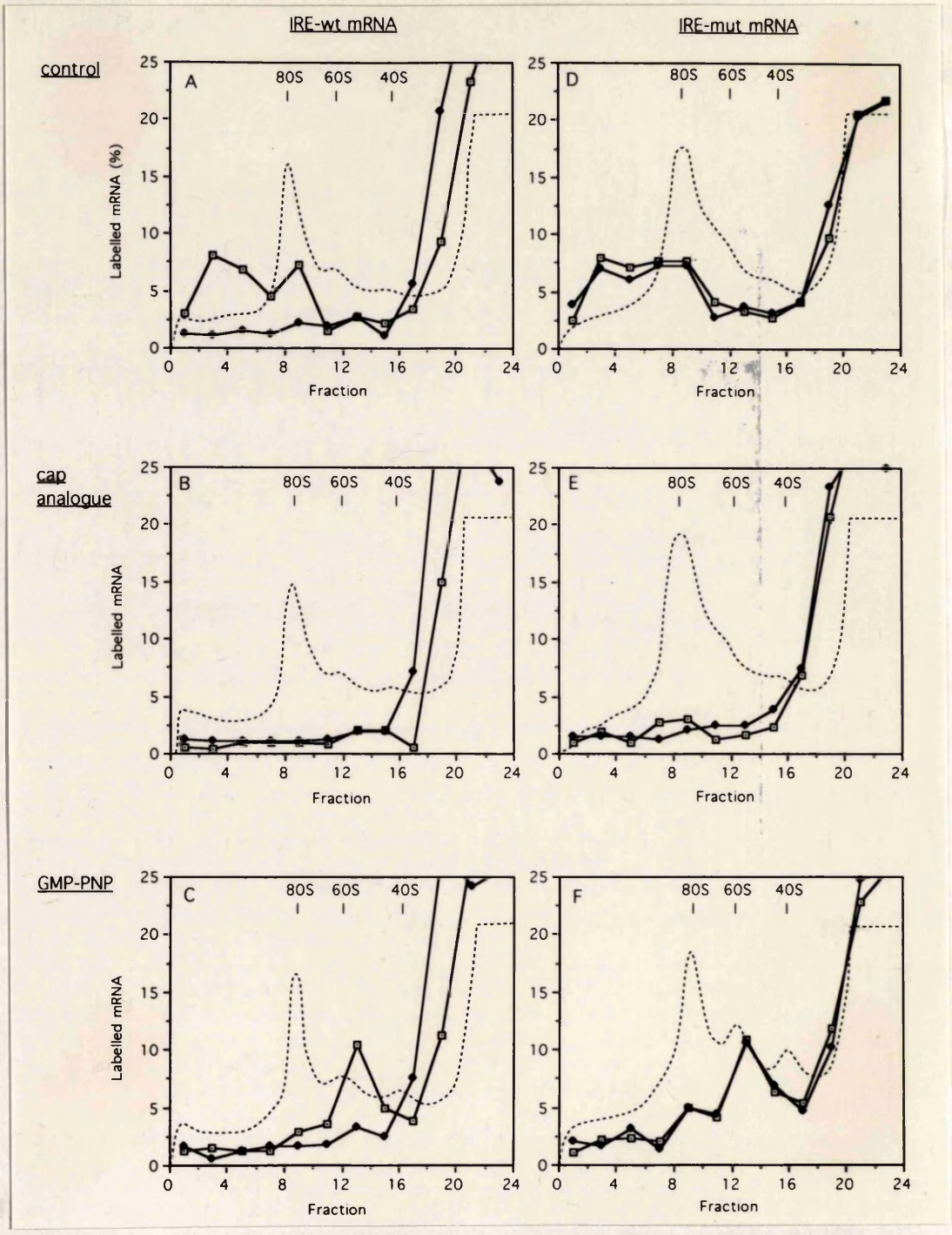
Full-length IRE-wt mRNA was assayed (dotted squares = - IRP; filled diamonds = + IRP) and analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (A), m^7GpppG cap analogue (B), or GMP-PNP (C). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients -/+ IRP. The positions of 40S, 60S and 80S ribosomal particles are indicated.

pre-initiation complexes from mRNPs. As expected, the shortened IRE-wt mRNAs were efficiently bound by ribosomes in the absence of IRP (figure 17A, dotted squares). In contrast to their longer counterparts, they were predominantly found in the top fractions of the gradient when incubated with m^7GpppG cap analogue (figure 17B), and distinctly heavier complexes were observed when GMP-PNP was included (figure 17C, dotted squares), marking the position of 48S complexes. IRP reduced the fraction of ribosome-bound mRNA to background levels and appeared to redistribute it into the top fractions of the gradient (figure 17A, black diamonds). When m^7GpppG cap analogue was included, the mRNA sedimented similarly in the presence of IRP or in its absence, indicating that IRP itself did not affect the sedimentation rate of the mRNA (figure 17B). While a significant fraction of the mRNA had sedimented as 48S complexes with the inclusion of GMP-PNP, IRP caused displacement of this mRNA into the top fractions of the gradients (figure 17C). These results strongly suggest that IRP prevents the mRNA from engaging with 43S pre-initiation complexes. When IRE-mut mRNA was analysed in the same manner (figure 17D-F), this mRNA was unaffected by IRP under all conditions, confirming the specificity of the above results.

4.2.3 Mechanism of translational repression by IRP in rabbit reticulocyte lysate.

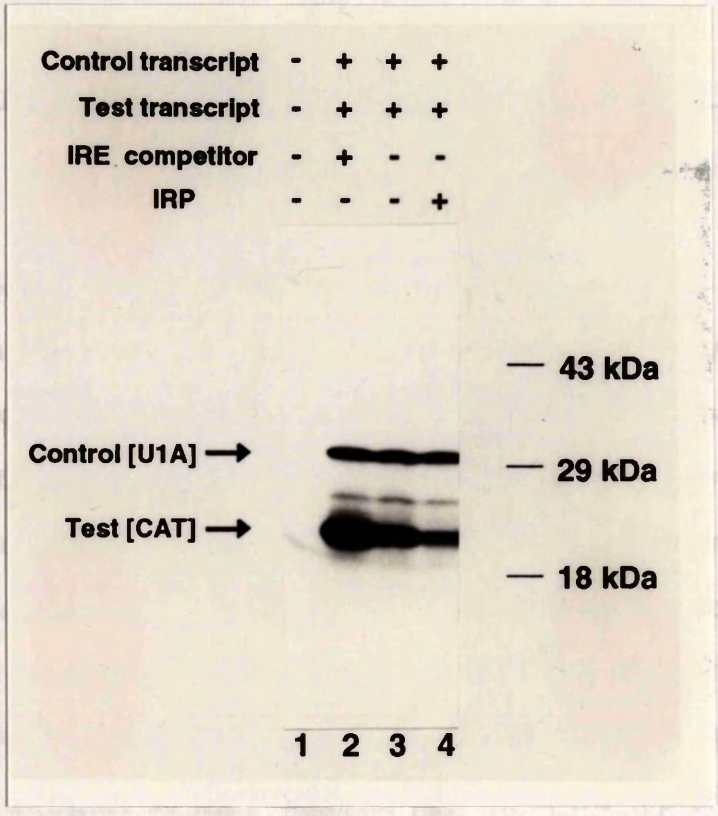
IRE-wt mRNA is partially repressed by endogenous IRP in rabbit reticulocyte lysates (figure 7 lanes 3-4 and 18 lane 3). To augment the translational repression of IRE-wt mRNA, the endogenous IRP was supplemented with recombinant human IRP (figure 18, lane 4); conversely, translation was completely de-repressed when the endogenous IRP was competitively sequestered with IRE-containing oligoribonucleotides (figure 18, lane 2). Employing this approach, the mechanism of translational

Figure 17. Sucrose gradient profiles of translation initiation assays performed in wheat germ extract with shortened mRNAs.



IRE-wt (A-C) and IRE-mut (D-F) mRNAs were assayed (dotted squares = - IRP; filled diamonds = + IRP) and analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (A and D), m^7GpppG cap analogue (B and E), or GMP-PNP (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients -/+ IRP. The positions of 40S, 60S and 80S ribosomal particles are indicated.

Figure 18. Translation of IRE-wt mRNA under repressing and de-repressing conditions in rabbit reticulocyte lysate.



U1A (1 ng) and 2.5 ng of full-length IRE-wt mRNAs were co-translated in the presence of either 15 ng IRE competitor RNA (lane 2) or 125 ng recombinant human IRP (lane 4). ^{35}S-labelled translation products were analysed by SDS-PAGE and fluorography. Molecular weight markers are indicated on the right and the positions of the U1A and CAT proteins are shown on the left.

repression by IRP was investigated in this mammalian translation system. As shown in figure 19, sucrose gradient analysis of short IRE-wt and IRE-mut mRNAs in the presence and absence of IRP yielded similar results to the wheat germ system. The effect of IRP on IRE-wt mRNA was even more pronounced, strengthening the argument that IRP affects 43S pre-initiation complex binding.

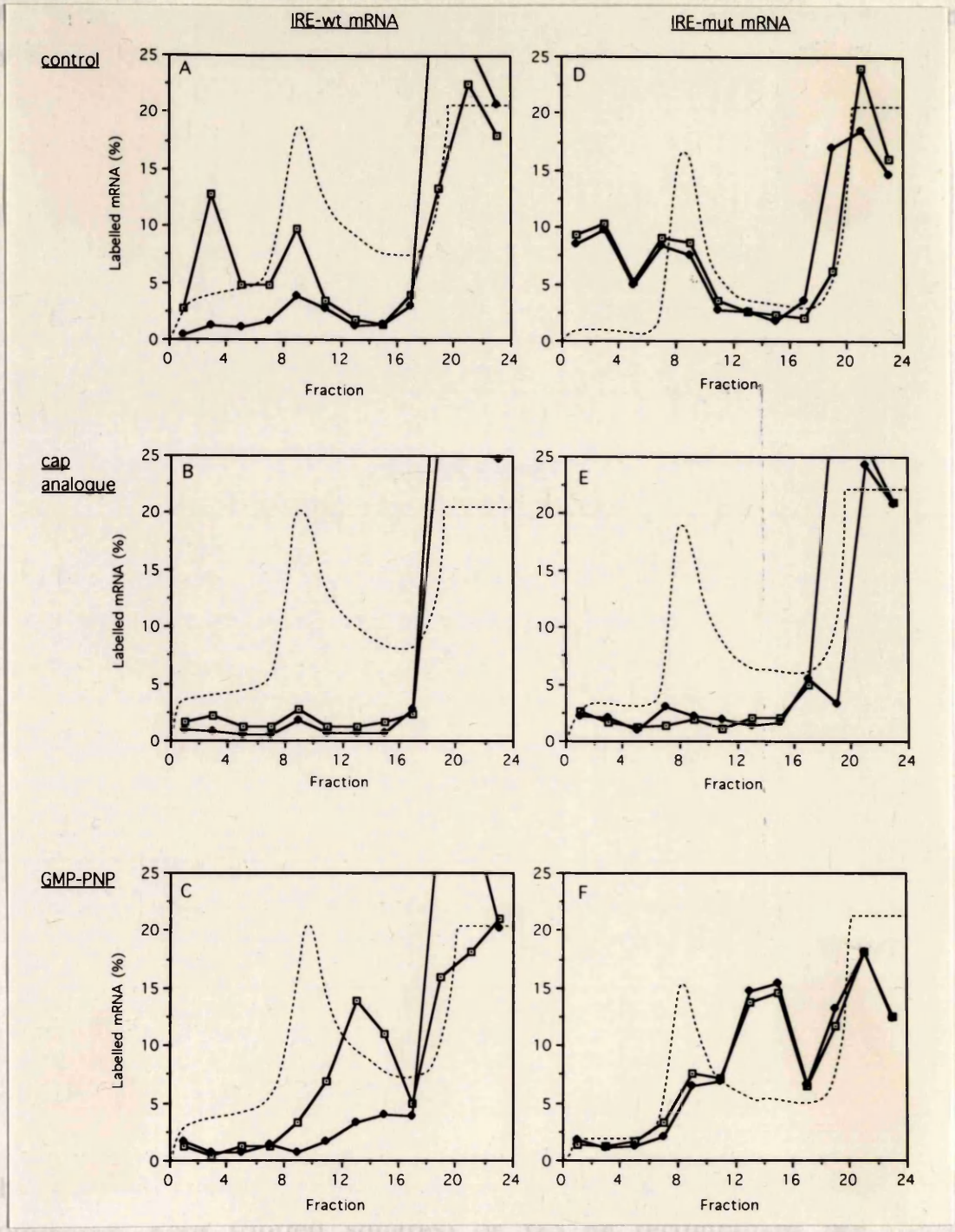
4.2.4 The influence of IRP on mRNA stability during the analytical procedure.

It is possible that the increase of radiolabel in the top fractions of the gradients in the presence of IRP (figures 17 and 19) may represent free nucleotides caused by mRNA degradation, although the absence of changes in mRNA stability induced by IRP in cell-free translations (see figure 11) make this unlikely. To ensure that IRP did not induce degradation of IRE-wt mRNA in this assay system, IRE-wt was assayed in rabbit reticulocyte lysate in the presence of IRE competitors or IRP and RNA was subsequently extracted from fractions and analysed on denaturing polyacrylamide gels. As can be seen in figure 20 the radiolabel present at the top of the gradient in the absence or presence of IRP represents full-length mRNAs in mRNP complexes.

4.2.5 Influence of IRP on the association of 43S translation pre-initiation complexes with mRNA.

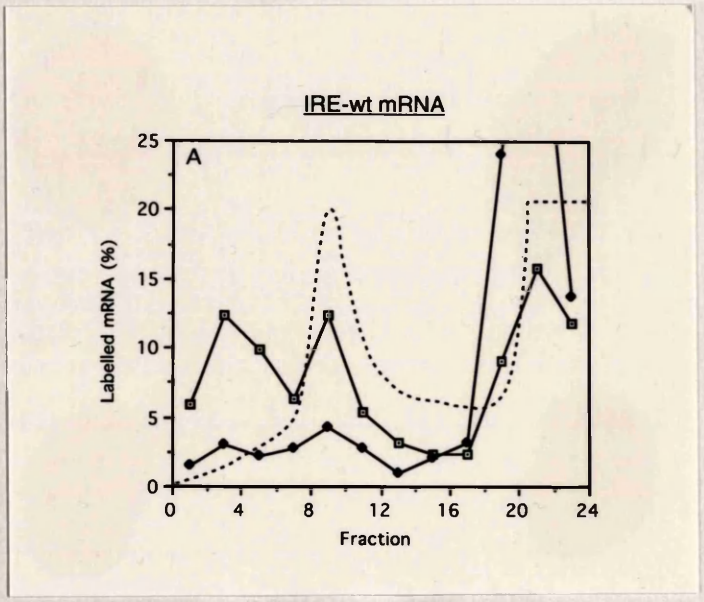
The sedimentation pattern of IRE-wt mRNA in the presence of IRP suggests that the small ribosomal subunit is prevented from binding to the mRNA. The question then arises as to whether this reflects the mechanism by which IRP represses translation or whether the lack of binding could be explained by dissociation of the 43S complex during the analytical

Figure 19. Sucrose gradient profiles of translation initiation assays performed in rabbit reticulocyte lysate.



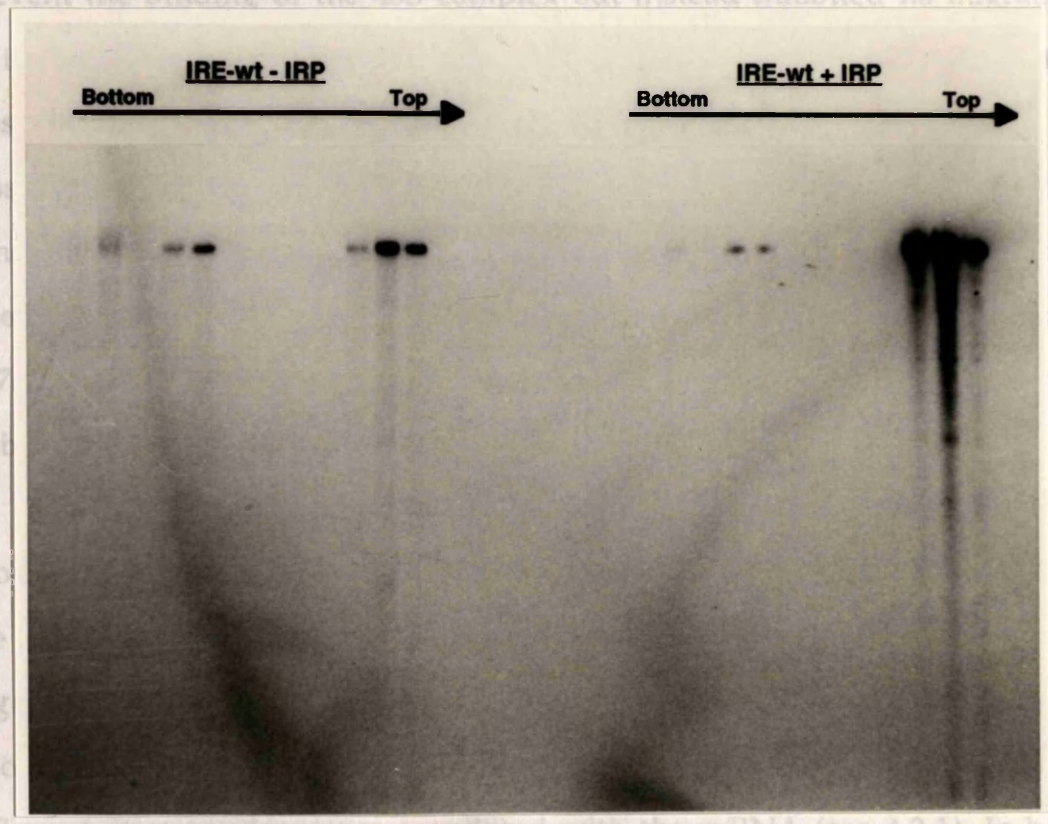
Shortened mRNA transcripts of IRE-wt (A-C) and IRE-mut (D-F) were assayed in the presence of either 45 ng IRE competitor RNA (dotted squares) or 125 ng recombinant IRP (filled diamonds) and analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (A and D), m^7GpppG cap analogue (B and E), or GMP-PNP (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients with added IRE competitor or IRP.

Figure 20. The stability of IRE-wt mRNA during sucrose gradient analysis in the presence of IRP.
(A). Sucrose gradient profiles of translation initiation assays performed in rabbit reticulocyte lysate.



Shortened IRE-wt mRNA was assayed in the presence of either 45 ng IRE competitor RNA (dotted squares) or 125 ng recombinant IRP (filled diamonds) and analysed on 5%-25% linear sucrose gradients. The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients with added IRE competitor or IRP.

Figure 20 (B). The integrity of IRP-bound IRE-wt mRNA recovered from sucrose gradient fractions.

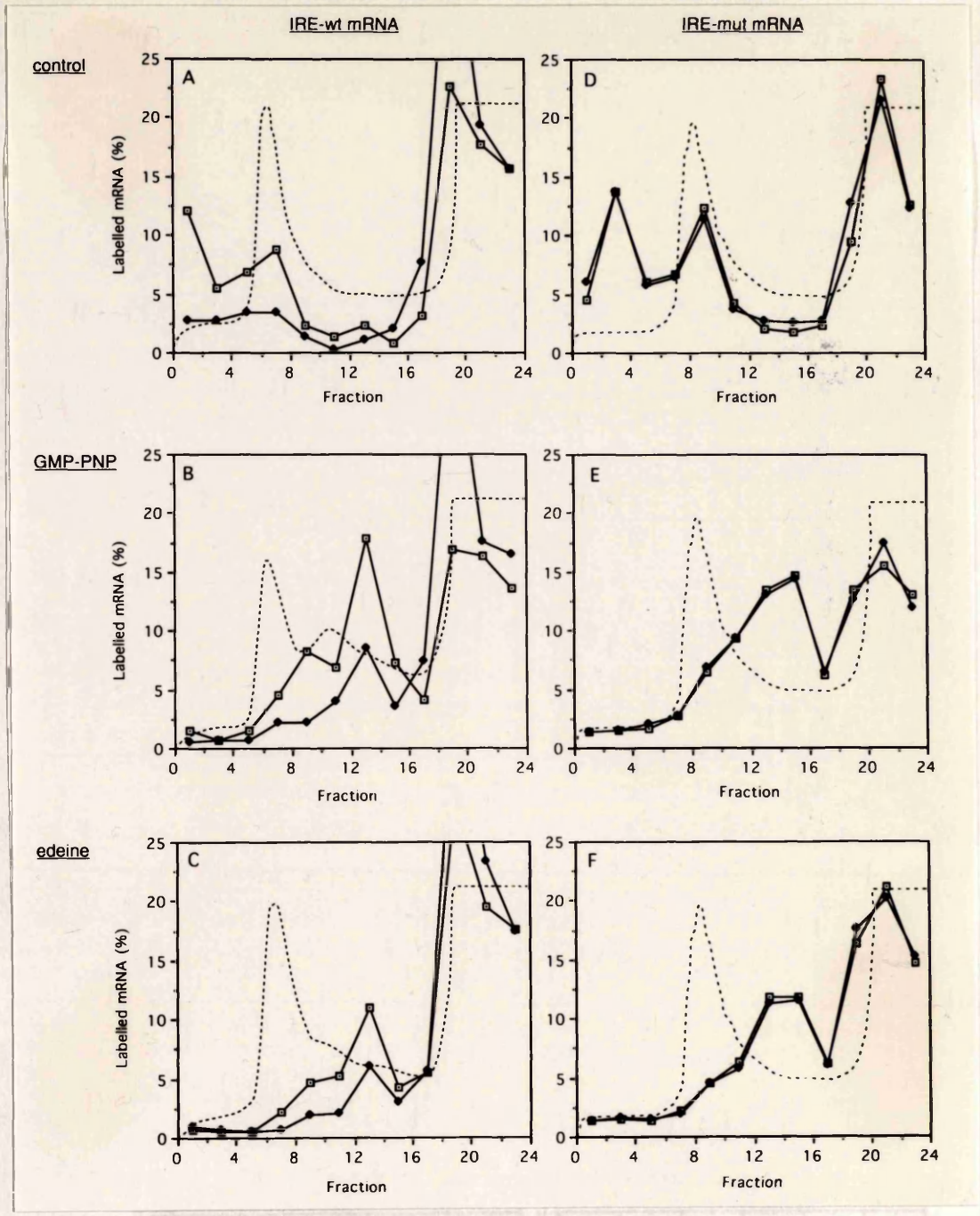


RNA was extracted from 100 µl of the fractions in 20A (lanes 1-12 = + IRE and lanes 13-24 = +IRP) by phenol/chloroform extraction, precipitated and analysed on a 4% denaturing polyacrylamide gel.

procedure. Since GMP-PNP stalls the 43S pre-initiation complex at the AUG initiator codon and prevents joining of the large ribosomal subunit, interactions between the initiator tRNA and the AUG codon might stabilise a 48S complex that would otherwise dissociate. Assuming that IRP did not prevent the binding of the 43S complex but instead inhibited its migration to the AUG codon, this could result in a 43S complex which is associated less strongly with the mRNA and which may therefore be lost by dissociation during the experimental procedure. To directly address this concern, translation assays were performed with edeine, an antibiotic which interferes with AUG recognition by the 43S complex (Kozak and Shatkin, 1978) and thus prevents possible AUG/initiator tRNA-mediated stabilisation of 48S complexes.

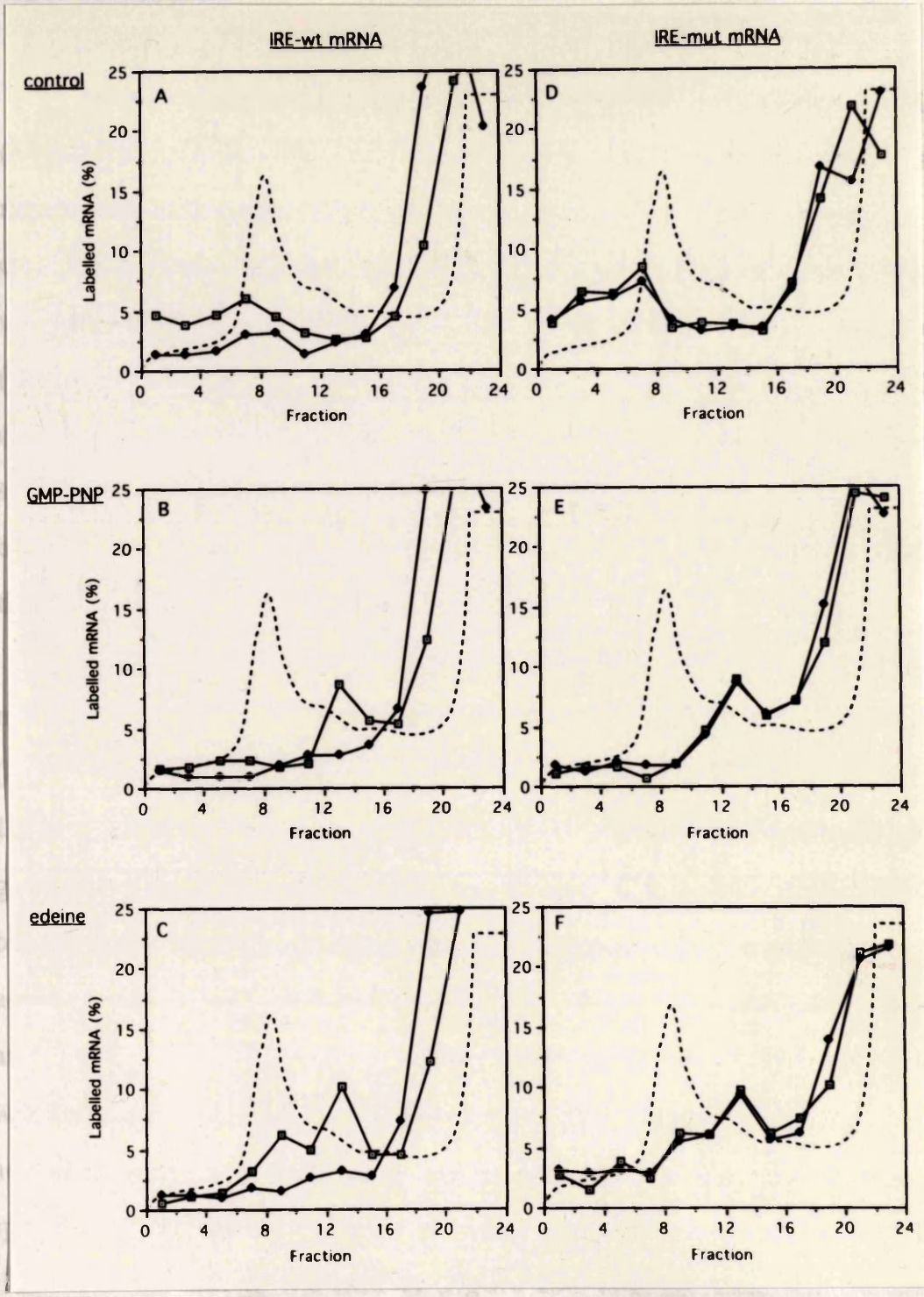
When rabbit reticulocyte IRP was sequestered by competitor IREs, the shortened IRE-wt mRNA sedimented in 48S complexes when assayed in the presence of either GMP-PNP (figure 21B, dotted squares) or edeine (figure 21C, dotted squares). Complexes sedimenting faster than 48S were also observed (see also figure 19C and E); these are indicative of multiple 43S pre-initiation complexes associated with the mRNA (see 4.2.1). In both cases, recombinant IRP caused the loss of 43S complexes from the mRNA (figure 21B-C, black diamonds). IRP had no effect on the sedimentation of short IRE-mut mRNA incubated with GMP-PNP or edeine (figure 21D-F). Furthermore, similar initiation assays with IRE-wt and IRE-mut mRNAs in wheat germ extract confirmed the results described above (figure 22). Thus, IRP prevents the binding of the 43S pre-initiation complex to the mRNA rather than inhibiting its progression to the initiation codon.

Figure 21. Association of 43S pre-initiation complexes during sucrose gradient analysis of rabbit reticulocyte lysate treated with edeine.



Shortened IRE-wt mRNA (A-C) and IRE-mt (lanes D-F) were assayed in the presence of 45 ng IRE competitor RNA (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained either no further additions (A and D), GMP-PNP (B and E) or edeine (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients with added IRE or IRP.

Figure 22. 43S pre-initiation complex association during sucrose gradient analysis of edeine treated wheat germ extract.



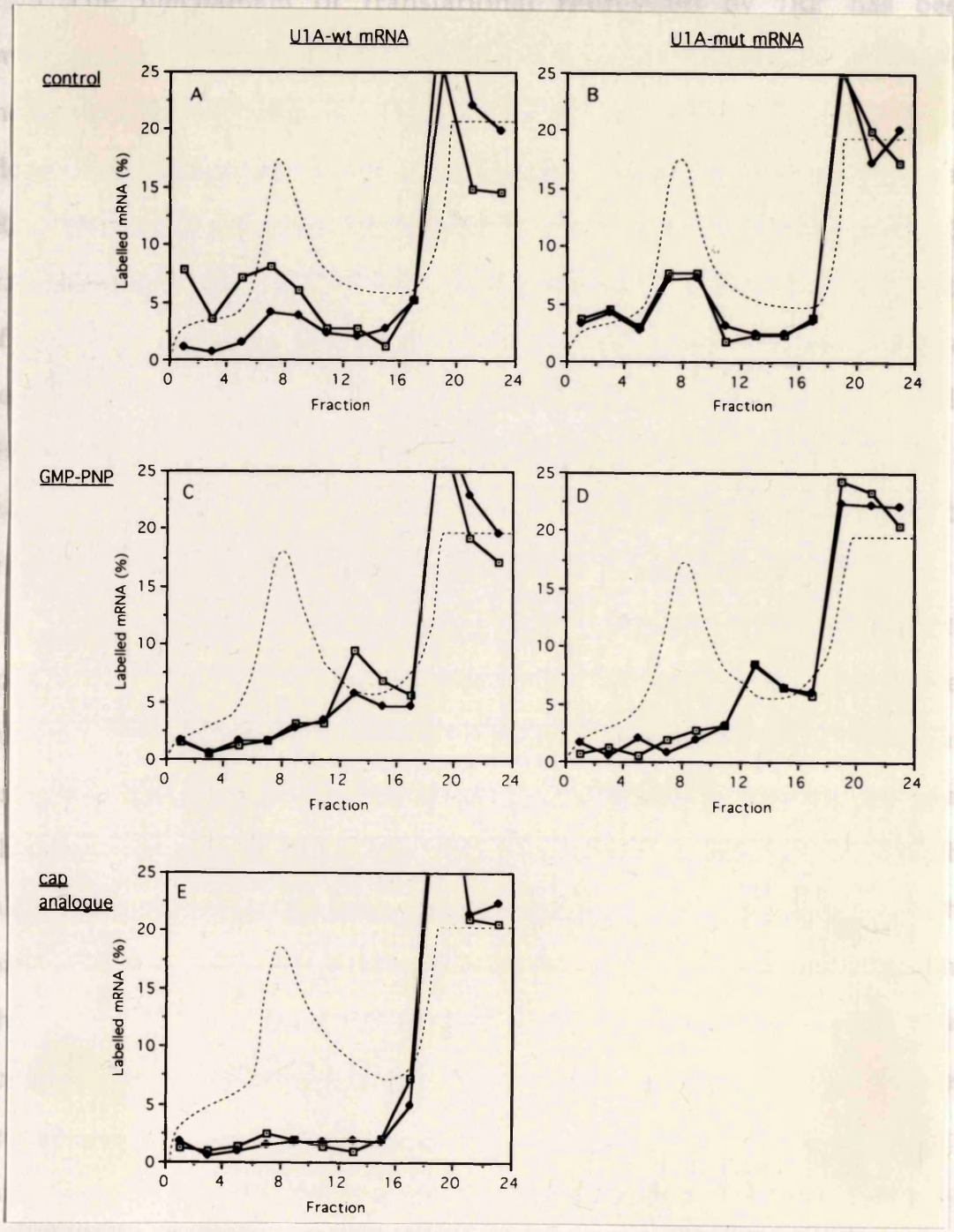
Shortened IRE-wt mRNA (A-C) and IRE-mt (lanes D-F) were assayed in the absence (dotted squares) or presence of 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained either no further additions (A and D), GMP-PNP (B and E) or edeine (C and F). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients independent of the inclusion of IRP.

4.2.6 Steric inhibition of translation by U1/U1A: comparison with ferritin regulation by IRP.

It has been suggested that IRP mediates translation sterically (Stripecke and Hentze, 1992). Thus, the mechanism by which steric repressors that bind to cap-proximal RNA sites affect translation was examined. Two examples of steric regulators have been described, the spliceosomal U1A protein and the bacteriophage MS2 coat protein (Stripecke and Hentze, 1992; Stripecke et al., 1994). CAT reporter mRNAs, one with a high affinity wild type (U1A-wt) and one with a mutant binding site (U1A-mut), were used to examine how the U1A protein inhibits translation initiation. The binding sites were located in a position similar to that of the IRE in IRE-wt mRNA (figure 14).

In the absence of U1A protein, both short U1A-wt and U1A-mut mRNAs bound to ribosomes when assayed in rabbit reticulocyte lysate (figure 23A and B, dotted squares). The U1A protein differentially removed U1A-wt, but not U1A-mut mRNA, from these lower fractions of the gradient (figure 23A and B, black diamonds). The quantitative differences between IRP and U1A as repressors of 80S ribosome formation are in good accord with *in vitro translation* studies (see figures 7 and 32 and Stripecke and Hentze, 1992). In analogy to the approach used for IRP, definition of whether repressed U1A-wt mRNA was associated with 43S complexes was achieved using m^7GpppG and GMP-PNP. As with IRP, the U1A protein specifically blocked translation initiation by preventing access of the 43S ribosomal subunit to the mRNA (figure 23C and E). Thus the manner by which the U1A mRNA/U1A complex blocks translation initiation resembles that of IRE/IRP, supporting the hypothesis that IRP is a steric repressor of translation.

Figure 23. The effect of U1A as a steric repressor of translation on 43S complex association.



Shortened transcripts of U1A-wt (A, C, E) and U1A-mut (B and D) were assayed in the absence (dotted squares) or presence (filled diamonds) of 700 ng recombinant U1A protein, and analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (A and B), GMP-PNP (C and D) or m^7GpppG cap analogue (E). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients -/+ U1A protein.

4.3 Discussion.

The mechanism of translational repression by IRP has been investigated biochemically using *in vitro* translation initiation assays, an indicator mRNA that bears a human ferritin H-chain IRE in a position identical to that of ferritin IREs, and highly purified recombinant human IRP. Collectively, the data demonstrate that IRP prevents binding of the 43S translation pre-initiation complex to the mRNA. The previous evaluation of the CAT reporter mRNAs, which showed that an intact IRE was necessary and sufficient to mediate translational control in transfected cells (Hentze et al., 1988; Hentze et al., 1987b) and in cell-free systems (Chapter 3) indicates that the results reported here reflect the cellular mechanism by which IRP represses the translation of ferritin in iron-starved cells.

It is important to consider the technical validity of the experimental approach used here, since the results with the full-length mRNAs suggested initially that the repressed mRNA co-sedimented with the small ribosomal subunit (figure 15 and 16). This result appeared to indicate that the small ribosomal subunit (as part of the 43S pre-initiation complex) binds to the mRNA and that the IRE/IRP complex blocks its movement towards the initiator codon. This is believed not to be the case for the following reasons. The translation initiation inhibitors m^7GpppG, GMP-PNP and edeine allow accurate identification of different pre-initiation complexes based on their sedimentation through sucrose gradients. The data obtained in translation initiation extracts from both a plant and a mammalian cell source show that IRP displaces mRNAs into fractions that co-sediment with mRNAs not bound by ribosomal subunits, and that IRP prevents the accumulation of 43S pre-initiation complexes otherwise seen in the presence of GMP-PNP. Moreover, several lines of evidence suggest that a 43S pre-initiation complex, once associated with the mRNA, will remain bound to it throughout the analytical procedure. First, glutaraldehyde fixation of translation complexes prior to centrifugation through sucrose gradients was

employed for the experiments using wheat germ extract, but was omitted from samples analysed in rabbit reticulocyte lysate (see 2.2.17). Both approaches yielded similar results. Second, a proportion of mRNAs sediments faster than 48S in the presence of GMP-PNP (figures 19C and F, 21B and E; figure 17C and F). The presence of these complexes suggests that more than one 43S pre-initiation complex is associated with some of the mRNAs. Since the CAT open reading frame begins with the most 5' AUG codon of the mRNA, this result implies that stabilising interactions between the pre-initiation complex and an AUG codon are not required. Third, the formation of 48S complexes in the presence of edeine, which interferes with AUG recognition by the 43S complex also support this interpretation. In addition, hybridisation of an antisense 2'-O-allyl oligoribonucleotide to a region of the 5'UTR including the AUG codon itself permits the binding of the 43S pre-initiation complex but inhibits its progression to the AUG codon. Under these conditions, it remains associated with the mRNA throughout the analytical procedure described here (Dr H. E. Johansson, PhD thesis). The sedimentation of full-length IRE-wt mRNA at around 40S most probably reflects the association of proteins in the extract binding non-specifically to the mRNA, a situation which has also been encountered by others (Sonenberg et al., 1981). Based on the results and the above considerations it is concluded that the IRE/IRP complex prevents translation initiation by blocking access of the 43S pre-initiation complex to the mRNA.

Having obtained information on the mechanism of ferritin regulation, it was of interest to examine whether steric repressors inhibit translation in a similar manner. The data obtained with the U1A protein show that it, too, prevents access of the 43S pre-initiation complex to U1A-wt mRNA. The data reveal that the cap-proximal region of eukaryotic mRNAs represents a sensitive target for translational repressor proteins to sterically block 43S pre-initiation complex binding to an mRNA and raises

the possibility that this may represent a more general mechanism of translational control (see chapter 7).

Chapter 5: The effect of the position of the IRE/IRP complex on the association of ribosomal subunits.

5.1 Introduction.

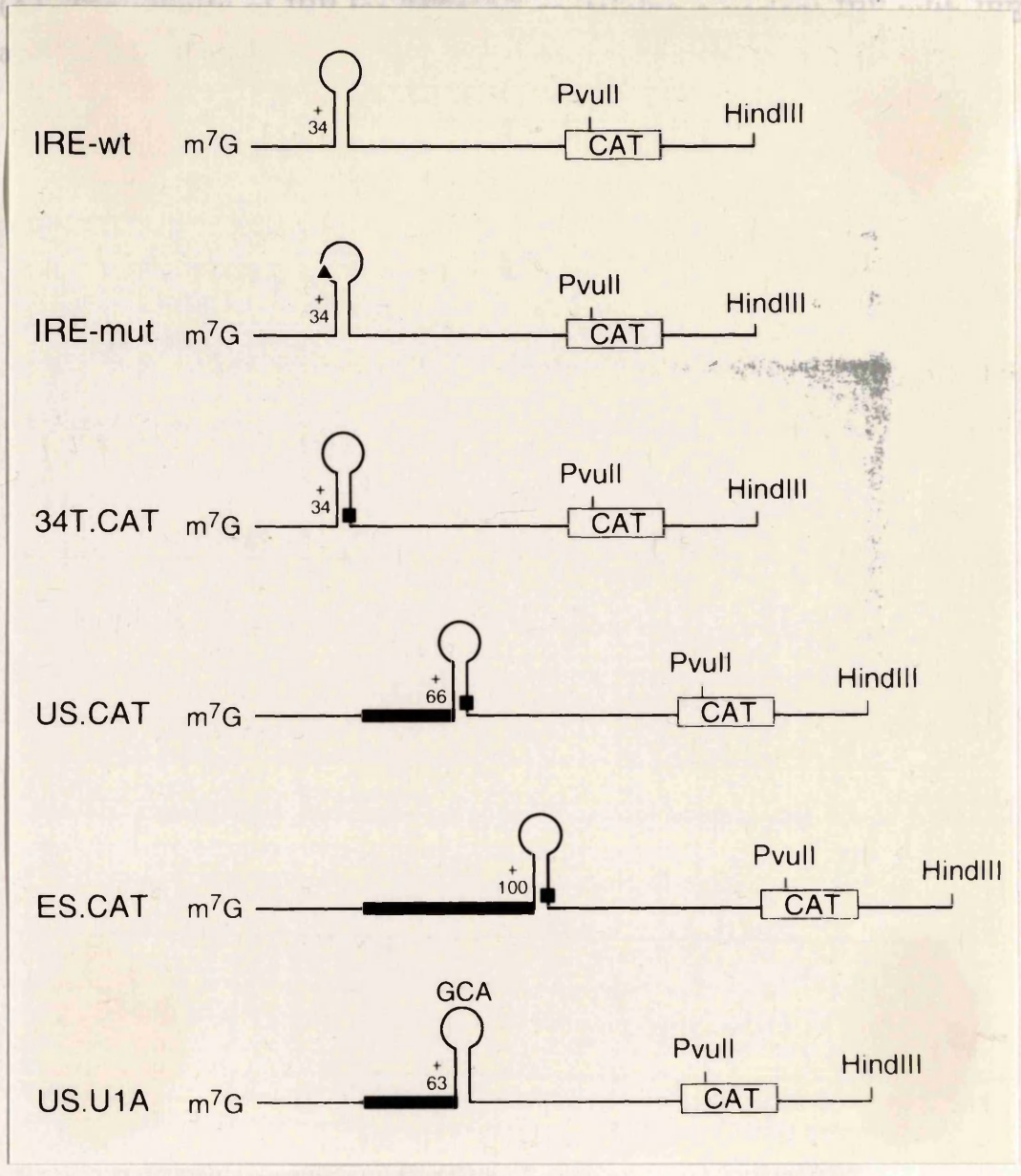
The conservation of the position of the IRE within the 5'UTR of ferritin (and eALAS see chapter 6) mRNAs suggests that the position of the IRE may be of functional relevance. Transfection studies using reporter mRNAs which harboured IRE elements at varying positions within the 5'UTR have shown that the regulation of IRE-containing mRNAs depends on the proximity of the IRE to the m^7G cap (Goossen et al., 1990; Goossen and Hentze, 1992). Repositioning of the IRE further from the m^7G cap resulted in a decreasing range of iron regulation, the strength of this "position effect" varying between cell types. Introduction of either structured or unstructured spacers 5' to the IRE similarly decreased iron regulation (Goossen et al., 1990; Goossen and Hentze, 1992). This effect has also been described for the U1A system in cell-free translation extracts (Stripecke and Hentze, 1992). The assays previously employed for analysing ribosomal subunit association with mRNAs on which RNA/protein complexes were assembled should be suitable for analysis of mRNAs containing RNA binding sites at various locations within the 5'UTR in order to elucidate the mechanistic basis of this position effect. From the results presented in chapter 4, it would seem reasonable to predict that increasing the distance between the cap structure and the RNA/protein complex might allow improved access of the 43S pre-initiation complex to the mRNA.

5.2 Results.

5.2.1 The affinity of mRNAs with differentially located IREs for IRP.

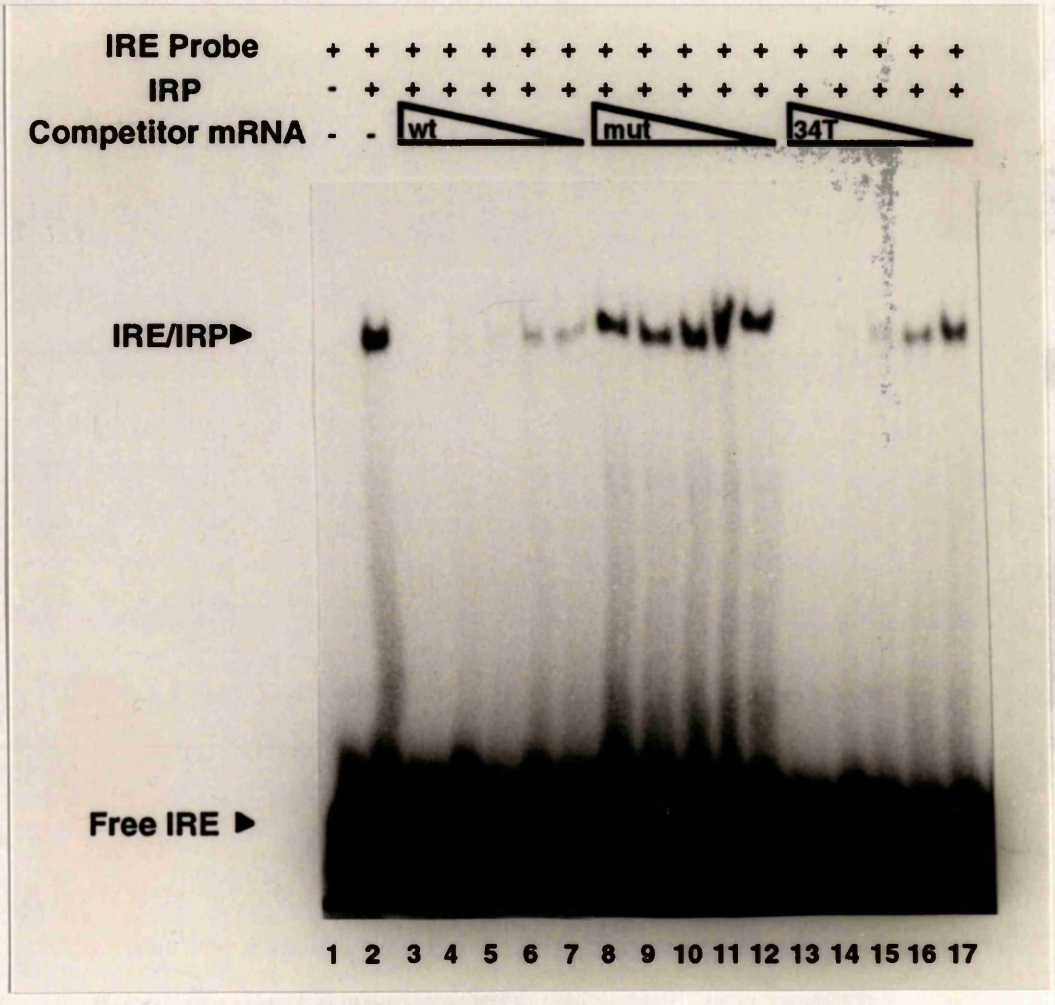
To assess the relative abilities of IRE/IRP complexes located at varying positions within the 5'UTR to inhibit translation initiation in cell-free systems, a series of CAT reporter mRNAs were constructed. These CAT reporter mRNAs contain an IRE element identical to the IRE element previously utilised in the transfection experiments to examine the position effect (Goossen and Hentze, 1992), which varies from the IRE in IRE-wt at one position (figure 24). In 34T.CAT, this IRE is located in an identical position to the IRE in IRE-wt (+34) and serves as a positive control for this IRE variant. Inserts of 32 and 66 nucleotides were introduced 5' to the IRE in US.CAT and ES.CAT, so that the IRE is located at +66 and +100 nucleotides downstream of the cap respectively (figure 24). The sequences of these inserts were predicted neither to form extensive secondary structure nor to disrupt the structure of the IRE itself. To determine whether these mRNAs have similar affinities for IRP, their interaction with IRP was examined by gel retardation analysis. This analysis is critical to allow a clear distinction between the effects of altering the position of the IRE and inadvertently altering its affinity for IRP by introduction of the inserts. To assess the affinity of 34T.CAT mRNA (containing the variant IRE) for IRP, non-radiolabelled full-length 34T.CAT, IRE-wt and IRE-mut mRNAs were used as competitors of complex formation between IRP and a radiolabelled IRE in a gel retardation assay. As seen in figure 25A, 34T.CAT competed for IRP with similar efficiency as IRE-wt, indicating that mRNA containing this variant IRE is efficiently recognised by IRP. Similarly, the introduction of the unstructured spacers into US.CAT and ES.CAT did not cause differences in their IRP-binding characteristics (figure 25B).

Figure 24. Schematic representation of the CAT mRNAs used.



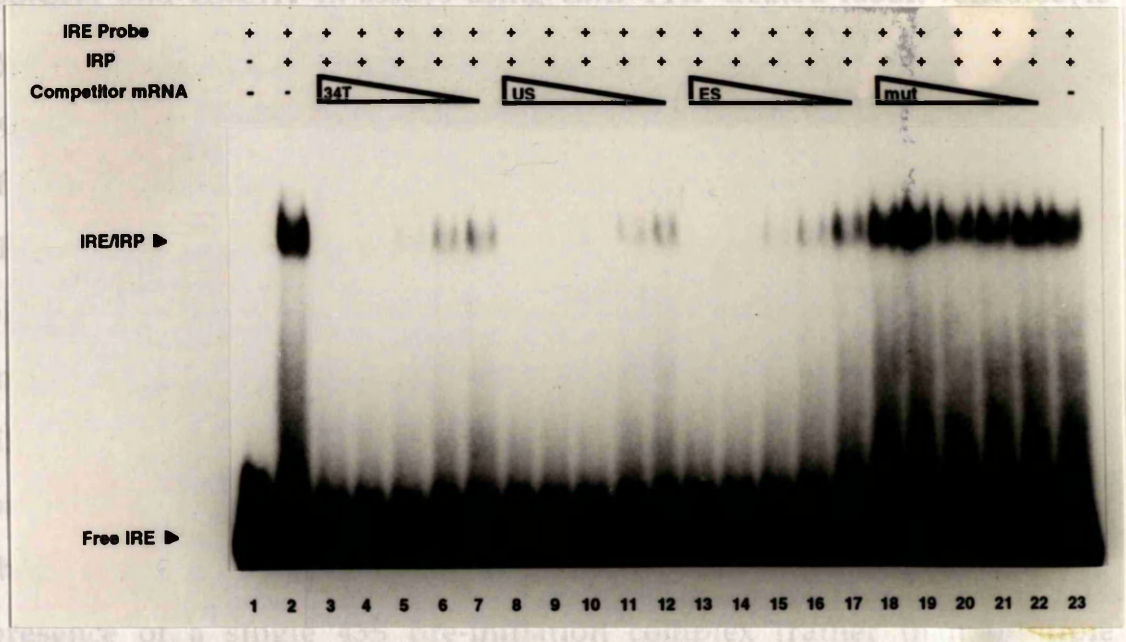
The open reading frame encoding chloramphenicol acetyltransferase is denoted by an open box. The position of the IRE in the 5' UTR is quoted with reference to the distance of the conserved unpaired C residue in the IRE (not indicated here) from the cap structure. The IRE with the black triangle lacks the first nucleotide of the conserved loop and the black squares symbolises those IREs which contain a C to T substitution (+57 in 34T. CAT). The position of the U1A binding site is quoted with reference to the distance of the insertion site from the cap structure. The thick line highlights the introduced spacers. In all mRNAs, the number of nucleotides between the protein binding site and the initiator AUG remains constant.

Figure 25. The affinities of IRE-wt, IRE-mut, 34T.CAT, US.CAT and ES.CAT for IRP as determined by gel retardation analysis.
(A). The affinity of IRP for 34T.CAT containing a variant IRE with IRE-wt and IRE-mut mRNA.



Complex formation between placental IRP (75 ng) and a 34 nucleotide ^{32}P-labelled transcript containing an IRE (lane 2) was competed by addition of 500, 250, 125, 62.5 and 31.25 ng of cold full-length IRE-wt (lanes 3-7), IRE-mut (lanes 8-12) or 34T.CAT (lanes 13-17) mRNA. The positions of IRE/IRP complexes and free IRE probe are marked.

Figure 25 (B). Comparison of the affinity of mRNAs containing a spacer element and 34T.CAT for IRP.



The complex (lanes 2 and 23) between recombinant IRP (75 ng) from *E. coli* to a 34 nucleotide labelled transcript containing an IRE was competed by 500, 250, 125, 62.5 and 31.25 ng of cold full-length 34T.CAT (lanes 3-7), US.CAT (lanes 8-12), ES.CAT (lanes 13-17) or IRE-mut (lanes 18-22) mRNA. The positions of IRE/IRP complexes and free IRE probe are marked.

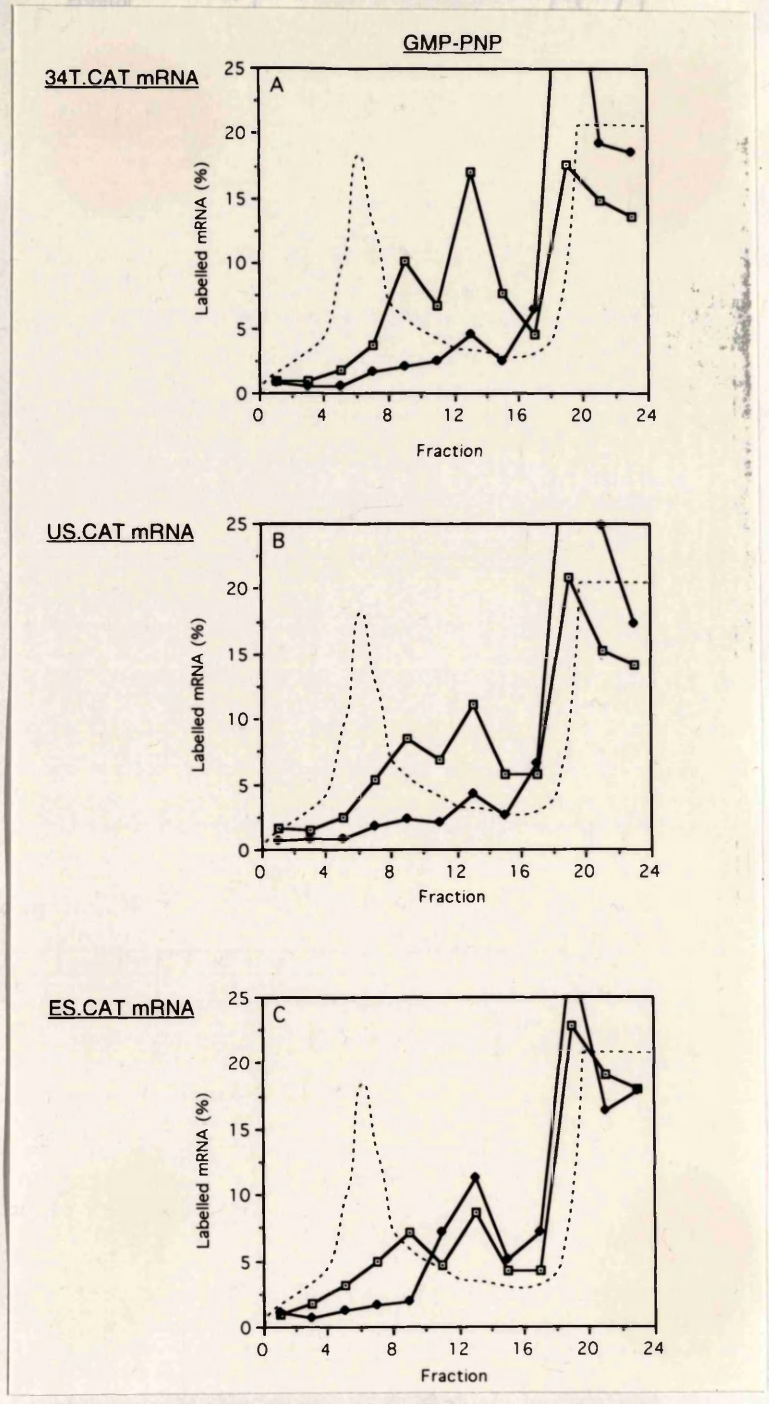
5.2.2 The binding of ribosomal subunits to mRNAs with cap-proximal and cap-distal IRE/IRP complexes.

Having determined that IRP has similar affinities for 34T.CAT, US.CAT and ES.CAT, the ability of IRE/IRP complexes in US.CAT and ES.CAT to block access of the 43S pre-initiation complex was examined using the initiation assays described in chapter 4. Analysis of 34T.CAT, US.CAT and ES.CAT in assays using GMP-PNP-treated rabbit reticulocyte lysate, containing either IRE competitor transcripts or recombinant IRP revealed that all three transcripts were affected by IRP (figure 26). 34T.CAT (figure 26A) and US.CAT (figure 26B) behaved in a fashion similar to that of IRE-wt (compare to figure 19) indicating that 43S pre-initiation complex binding was being prevented by the IRE/IRP complexes present on these mRNAs. Unlike previously characterised mRNAs containing an intact IRE, ES.CAT did not redistribute into the mRNP area of the gradient in the presence of IRP, but sedimented at the 48S area of the gradient indicating that a single 43S complex is assembled on ES.CAT mRNA (figure 26C). The presence of a single 43S pre-initiation complex (rather than multiple complexes) on ES.CAT could indicate that the IRE/IRP complex prohibits scanning along the 5'UTR, or causes the 43S complex to pause temporarily before continuing towards the AUG. Additionally, the results could reflect kinetic differences in 43S pre-initiation complex binding to the mRNA.

5.2.3 The effect of IRP on 80S complex assembly on ES.CAT.

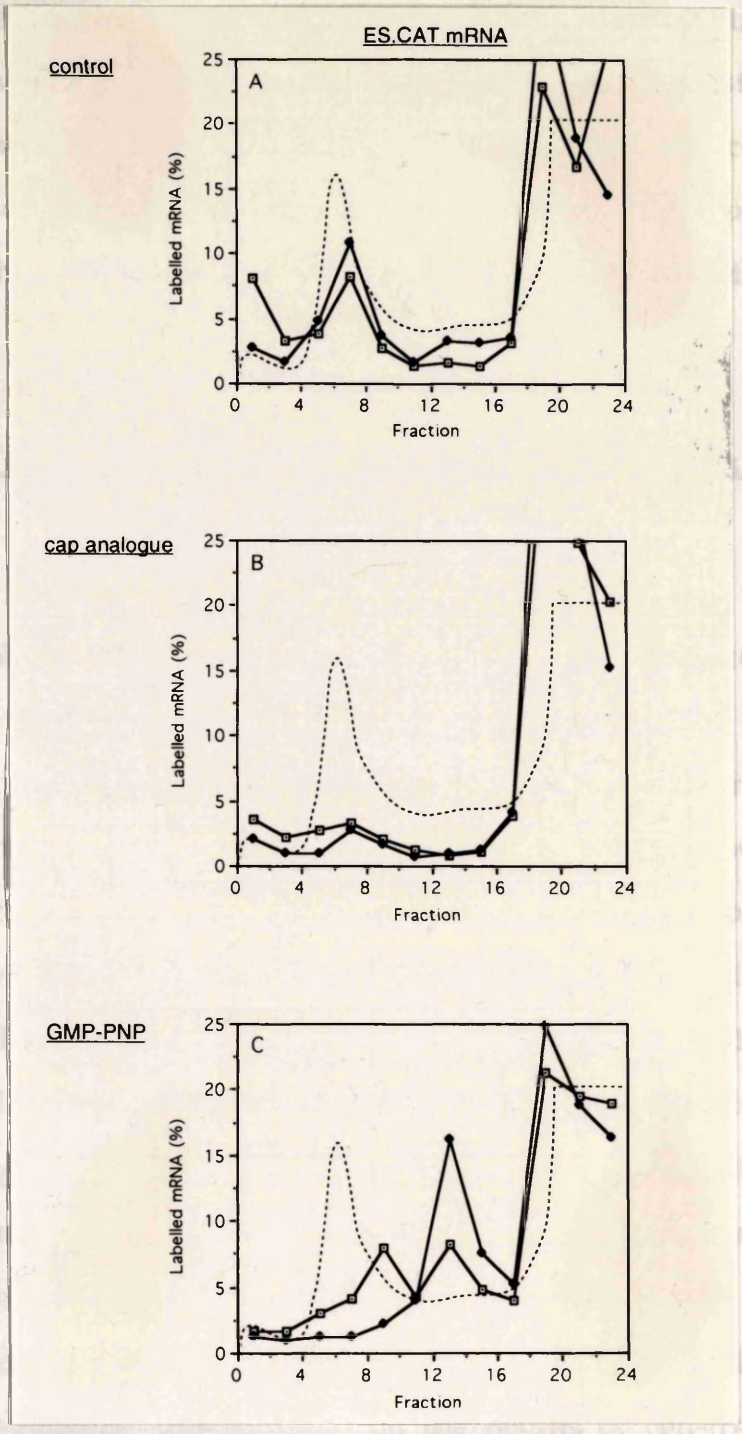
To further investigate the effect of IRE/IRP complexes assembled on ES.CAT, ES.CAT was assayed in the presence of IRE competitors or IRP with either no analogue, m^7GpppG cap analogue or GMP-PNP (figure 27). As seen previously, only a single 43S pre-initiation complex assembles on ES.CAT in the presence of IRP and GMP.PNP (figure 27C), while no

Figure 26. The association of 43S complexes with 34T.CAT, US.CAT and ES.CAT in rabbit reticulocyte lysate.



Shortened 34T.CAT (A) and US.CAT (B) and ES.CAT (C) mRNAs were assayed in the presence of 45 ng of IRE (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. All assays contained GMP-PNP. The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients independent of the inclusion of IRP.

Figure 27 Assembly of 80S complexes on ES.CAT in the presence of IRP.



Shortened ES.CAT mRNAs were assayed in the presence of 45 ng of IRE (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (A), m^7GpppG cap analogue (B) or GMP-PNP (C). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients independent of the inclusion of IRP.

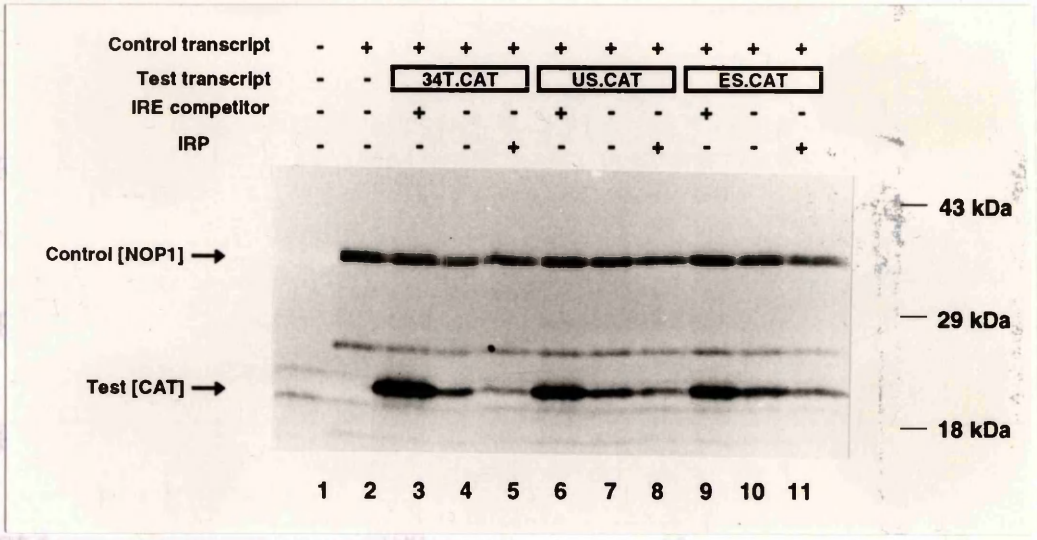
complexes assemble in the presence of IRP and cap analogue (figure 27B). In the absence of analogues, complexes sedimenting at around 80S were observed when IRP was included (figure 27A), suggesting that 43S complexes which gain access to the 5'UTR can form 80S complexes. No complexes sedimenting faster than 80S were observed in the presence of IRP indicating that loading of multiple ribosomal particles on to the mRNA did not occur.

5.2.4 CAT synthesis directed by mRNAs containing IREs in cap-distal positions.

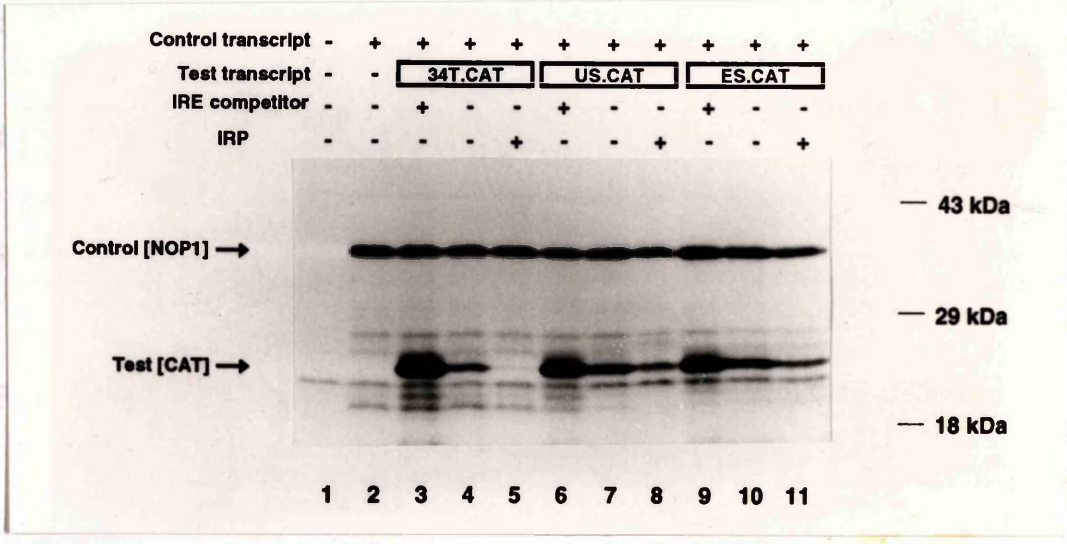
The above analyses revealed that altering the distance between the cap and the IRE influenced the access of ribosomal subunits to the mRNA. To determine if this would be reflected in changes in the rate of protein synthesis, the translation of 34T.CAT, US.CAT and ES.CAT were examined in rabbit reticulocyte lysate in the presence of IRE competitor transcript or exogenously added recombinant IRP (figure 28). For this evaluation, NOP1, which encodes a 38 kDa nucleolar protein (Schimmang et al., 1989) was used as a control mRNA rather than U1A, as an early termination product of U1A co-migrates with the CAT protein, impeding exact quantitative analysis of the results. The translation of 34T.CAT, US.CAT and ES.CAT in the presence and absence of IRP was examined both at low (0.7 mM) and high magnesium concentrations (2.1 mM), since other workers found an effect of magnesium concentration on the results of cell-free translations (Dasso and Jackson, 1989; Kozak, 1990b). Translations performed at either magnesium concentration yielded similar results (figure 28A and B), with 34T.CAT being most and ES.CAT least efficiently repressed by IRP. Much of the variation seen in table 3 appears to be due to variations in the quantitation procedure itself. For instance, successive quantitations of experiment 1 at 0.7 mM magnesium gave a decrease in the repressional

Figure 28. Translation of 34T.CAT, US.CAT and ES.CAT in rabbit reticulocyte lysate.

A



B



The lysates used in (A) were unsupplemented (basal level: 0.7 mM Mg^{2+}) and (B) were supplemented with $MgCl_2$ to a final concentration of 2.1 mM Mg^{2+}. NOP1 (2 ng) and 2.5 ng of 34T.CAT, US.CAT or ES.CAT mRNA were co-translated in the presence of either 15 ng of IRE competitor transcript (lanes 3, 6 and 9) or 150 ng recombinant IRP (lanes 5, 8 and 11). ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. Molecular size markers are indicated on the right and the positions of the NOP1 and CAT proteins are shown on the left. The exposure time of gel B was twice that of gel A.

Table 3. IRP-mediated repression of CAT synthesis directed by 34T.CAT, US.CAT and ES.CAT

	Percentage repression of CAT synthesis *		
mRNA	34T.CAT	US.CAT	ES.CAT
0.7 mM $MgCl_2$			
Expt 1	100	37	25
	100	62	28
Expt 2	100	42	25
	100	75	44
Expt 3	100	28	15
	100	21	14
2.1 mM $MgCl_2$			
Expt 1	100	37	23
	100	35	29
Expt 2	100	37	29
	100	42	28
Expt 3	100	55	37
	100	40	30

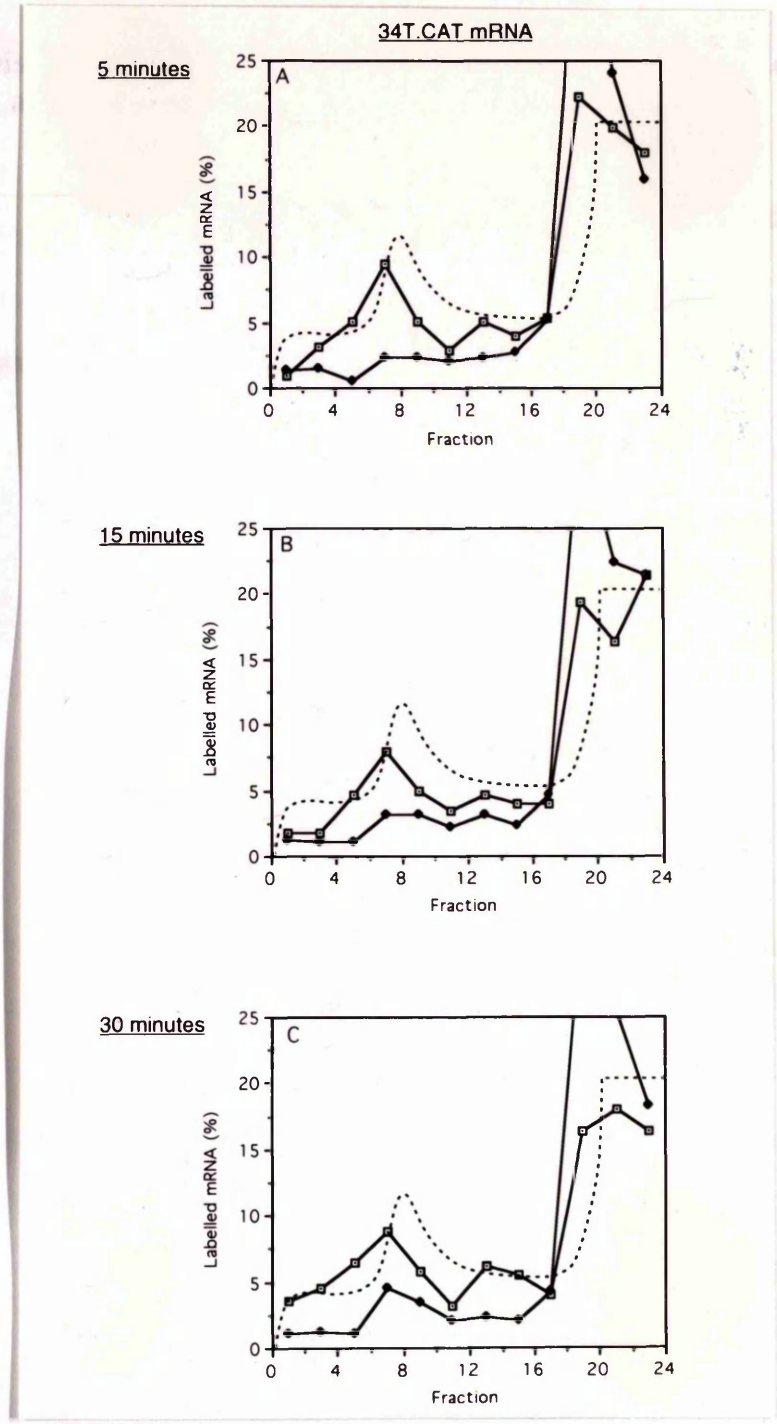
* Figures are expressed as a percentage repression compared to 34T.CAT which is taken as 100%. Each experimental point was quantified twice to allow for experimental variation.

efficiency of between 37% and 62% for US.CAT. These results indicate that the degree of repression mediated by an IRE progressively diminishes as it is positioned further from the m^7G cap structure and therefore, that these mRNAs display the "position effect "*in vitro*.

5.2.5 Kinetic analysis of 80S complex formation on 34T.CAT, US.CAT and ES.CAT.

The translation data suggest that 80S ribosomes competent to translate must assemble on a proportion of US.CAT and ES.CAT mRNAs. However, in the previous initiation assays, no significant accumulation of ribosomal complexes on US.CAT mRNA was observed. To address this apparent discrepancy, initiation assays were allowed to proceed for 5, 15 and 30 min since standard *in vitro* translation assays are incubated for 1 hour while the standard initiation assays incubate for only 5 min. When, 34T.CAT, US.CAT and ES.CAT were assayed at 2.1 mM magnesium, mRNAs located in complexes of 80S or heavier after a 5 min initiation assay were reduced compared to the amount observed at the lower magnesium concentration (figures 29-31 and Table 4). This correlates with the lower amounts of CAT and NOP1 synthesis observed at this magnesium concentration (figure 28). Analysis by Antony and Merrick (1992) showed that the formation of 80S complexes under these conditions is slower than observed here at the low magnesium concentration, with many mRNAs being located in 43S complexes after 2 or even 5 min of initiation, requiring 10 min for maximal ribosome association of mRNAs in 80S complexes. 34T.CAT mRNA was predominately found in mRNP complexes when IRP was present after 5, 15 or 30 min of initiation (figure 29). This indicates that the translational block imposed by a cap-proximal IRE/IRP complex is not overcome by increasing the length of assay. The small increase in the number of 80S or heavier complexes assembled on

Figure 29 Assembly of 80S complexes on 34T.CAT mRNA during a 30 min initiation assay in rabbit reticulocyte lysate containing 2.1 mM magnesium.



Shortened 34T.CAT mRNA was assayed in the presence of 45 ng of IRE (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained no analogue and were incubated for either 5 (A), 15 (B) or 30 min (C). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients independent of the inclusion of IRP.

Table 4 Distribution of mRNA into 80S or heavier complexes during a 5 min assay at different magnesium concentrations

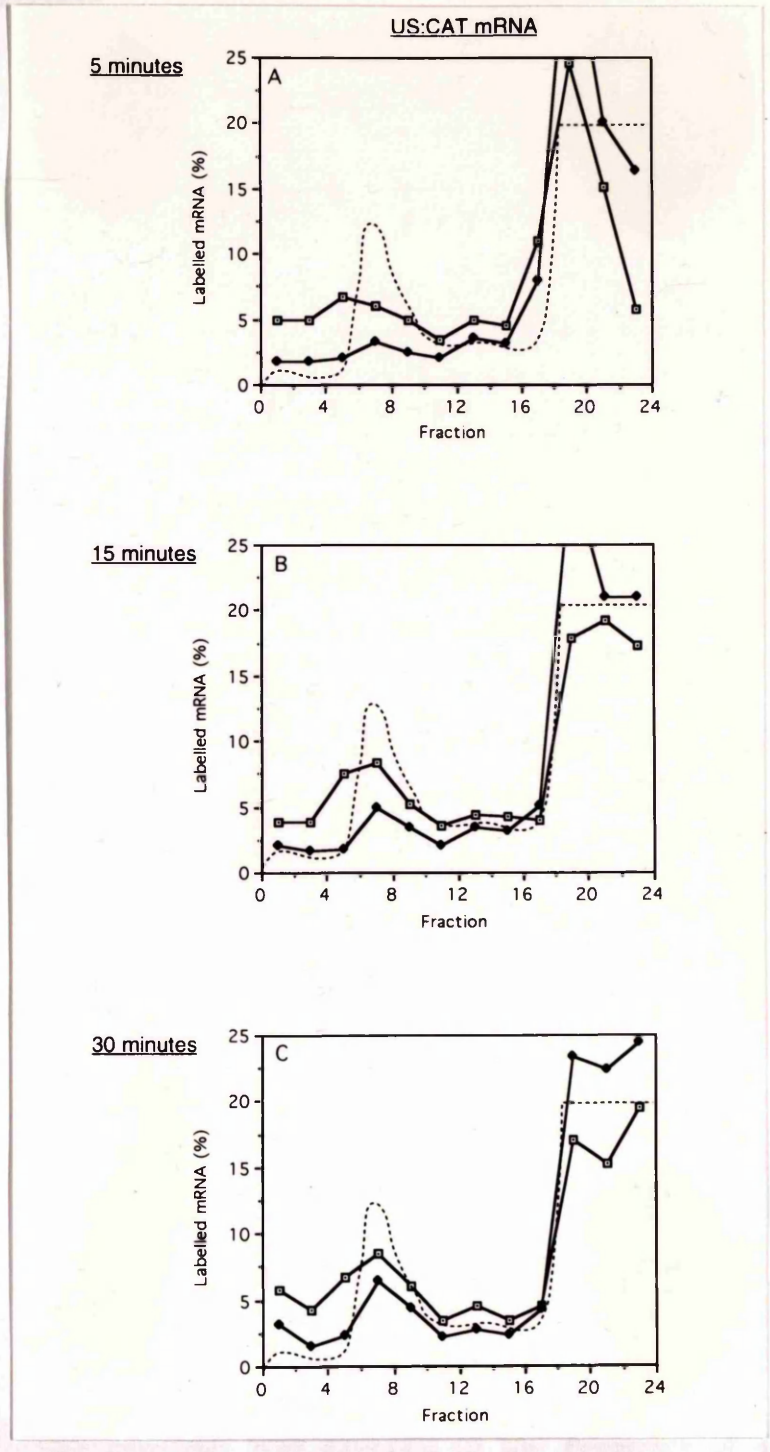
Magnesium concentration	Percentage of mRNA in complexes of 80S or heavier
0.7 mM	32
1.4 mM	28
2.1 mM	23

34T.CAT in the presence of IRP after 30 min is in good accord with the extensive but incomplete repression of 34T.CAT translation by IRP observed in the translation assays (figure 28).

After a 5 min initiation assay in the presence of IRP, US.CAT was predominantly located in mRNP complexes (figure 30A), where it was located after a 5 min assay at the low magnesium concentration (figure 26). When the initiation assay was allowed to proceed for 15 min, a proportion of the US.CAT mRNA moved into ribosome-associated complexes of 80S or heavier, with a significant amount of US.CAT mRNA being located there after 30 min, accounting for the increased CAT synthesis directed by US.CAT as compared to 34T.CAT in the presence of IRP (figure 30A and B).

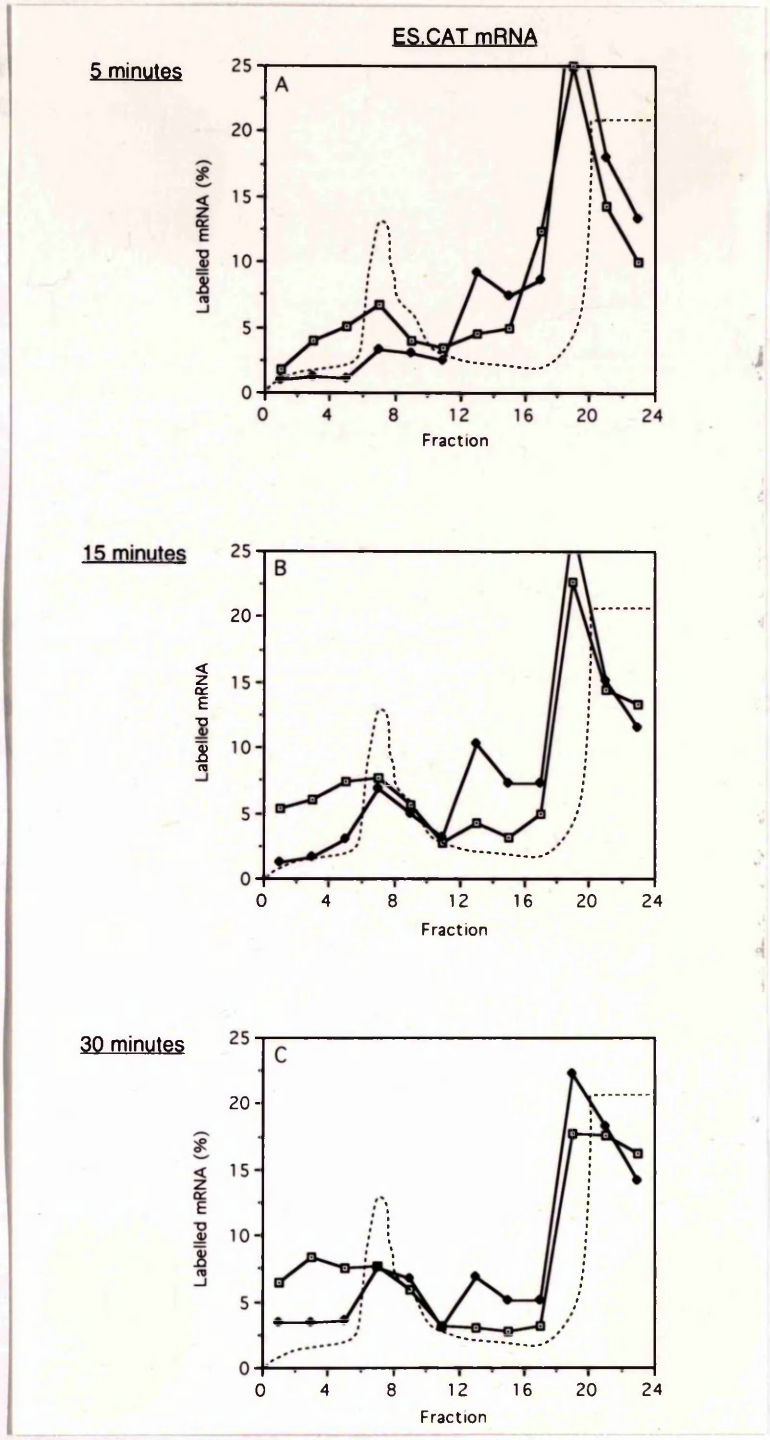
Analysis of ES.CAT in the presence of IRP showed that it was predominately located in 48S complexes after 5 min (figure 31A), which is in contrast to previous analysis at the low magnesium concentration (figure 27), where ES.CAT is located in 80S complexes. This difference is probably the result of the previously observed differences in the kinetics of 80S formation at low and high magnesium concentrations, which result in 80S complexes being assembled on ES.CAT during a 5 min assay containing a low magnesium concentration (see 5.3). After 15 minutes of assay in the presence of IRP, a shift of ES.CAT mRNA from 48S complexes to 80S complexes was observed (figure 31B) and after 30 min a greater proportion of ES.CAT mRNA was located in 80S complexes than in 48S complexes (figure 31C). Note that the percentage of ES.CAT accumulated in 80S or heavier complexes after 30 min is greater than US.CAT, which correlates well with the relative amounts of CAT synthesis from these mRNAs in the presence of IRP (Table 5).

Figure 30 Ribosome assembly on US.CAT mRNA in rabbit reticulocyte lysate containing 2.1 mM magnesium during a 30 min assay.



Shortened US.CAT mRNA was assayed in the presence of 45 ng of IRE (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained no analogue and were incubated for either 5 (A), 15 (B) or 30 min (C). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients independent of the inclusion of IRP.

Figure 31 Assembly of 43S complexes and 80S complexes on ES.CAT mRNA during a 30 min initiation assay in rabbit reticulocyte lysate containing 2.1 mM magnesium.



Shortened ES.CAT mRNA was assayed in the presence of 45 ng of IRE (dotted squares) or 125 ng recombinant IRP (filled diamonds), and analysed on 5%-25% linear sucrose gradients. The assays contained no analogue and were incubated for either 5 (A), 15 (B) or 30 min (C). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile, which was identical for gradients independent of the inclusion of IRP.

Table 5 IRP-mediated reduction of ribosome assembly on 34T.CAT, US.CAT and ES.CAT during a 30 min assay

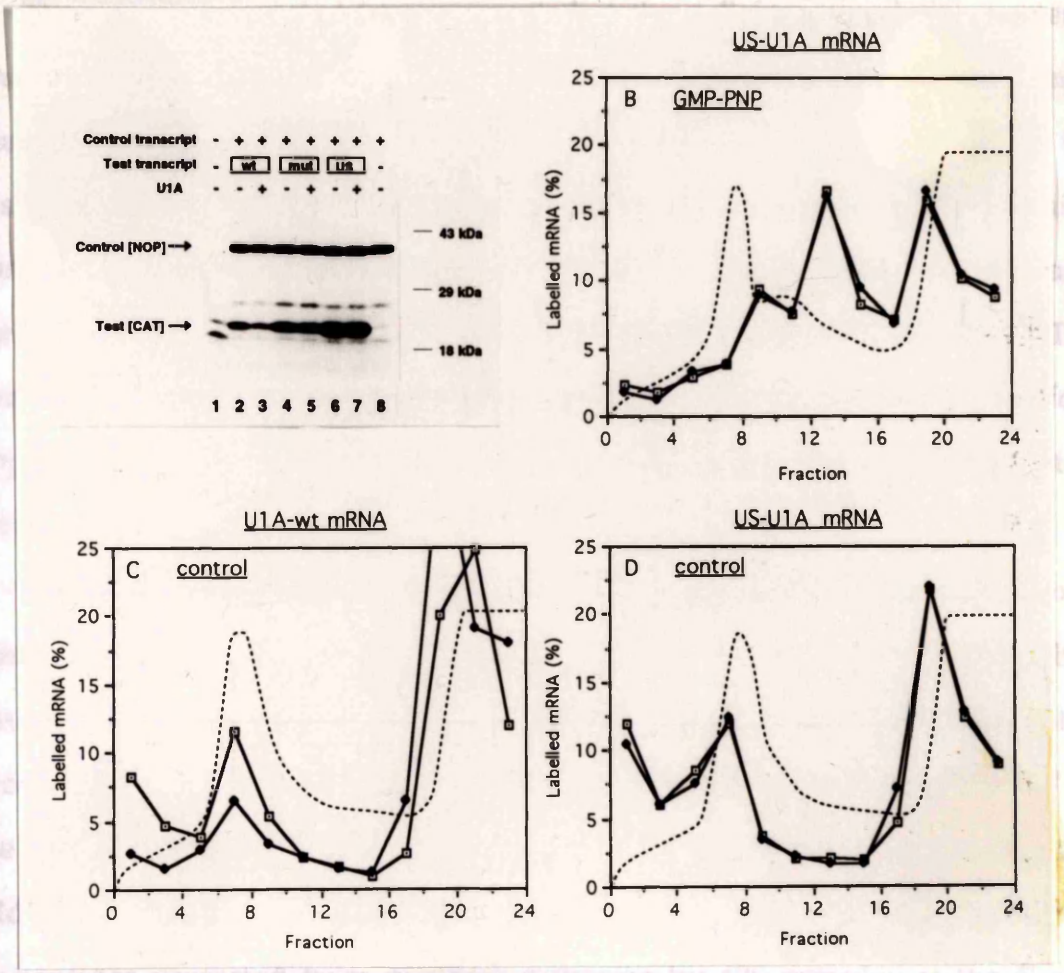
mRNA	Percentage of IRP-mediated reduction of 80S or heavier complex assembly*
34T.CAT	100
US.CAT	64
ES.CAT	27

* Figures are expressed as a percentage reduction of 80S or heavier complex assembly compared to 34T.CAT which is taken as 100%.

5.2.6 The effect of cap-proximal mRNA/U1A complexes on binding of the small ribosomal subunit.

Since cap-proximal U1A mRNA/U1A complexes also block access of the 43S pre-initiation complex to mRNAs (chapter 4) and the position of the U1A binding site is also important for U1A mediated translational repression, it was of interest to analyse the position dependence of this system. US-U1A mRNA bears an identical U1A binding site to the U1A-wt mRNA examined in chapter 4, located 32 nucleotides further downstream of the cap structure at +63 (figure 24). As shown by cell-free translation (figure 32A), full-length US-U1A mRNA is similarly unresponsive to U1A protein as is U1A-mut mRNA (lanes 4-7), demonstrating the effect of binding site position on translational repression. Shortened versions of these transcripts (249 nucleotides) were subjected to initiation assays and sucrose gradient analysis. In contrast to U1A-wt, US-U1A formed 48S and 66S complexes in the presence of GMP-PNP irrespective of the presence of the U1A protein (Figure 32B, dotted squares and black diamonds). Thus, 43S complexes can associate with the mRNA even in the presence of U1A protein when the binding site was positioned further downstream. Consequently, complexes of 80S and heavier assemble irrespective of the presence of the repressor protein (Figure 32D). U1A-wt mRNA, included as a control for the activity of the U1A protein in this assay (figure 32C), was translationally repressed as seen before (Figure 32A). Therefore, in contrast to the IRE-containing mRNAs, US-U1A, which contains a protein binding site at a position similar to the IRE in US.CAT, allows access of multiple 43S complexes to the 5'UTR in the presence of its binding protein. The possible reasons for this difference in the sedimentation patterns of these mRNA/protein complexes are discussed below.

Figure 32. The effect of cap-distal U1A mRNA/U1A complexes on the assembly of ribosomal subunits on US-U1A.



A). Translation of U1A-wt, U1A-mut and US-U1A in reticulocyte lysate containing U1A protein. NOP1 (2 ng) and 1 ng of full-length U1A-wt, U1A-mut or US-U1A mRNAs were translated in rabbit reticulocyte lysate in the absence or presence of 120 ng U1A protein. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. Molecular weight markers are indicated on the right and the positions of the NOP1 and CAT proteins are shown on the left. B-D) Sucrose gradient profiles of translation initiation assays performed in rabbit reticulocyte lysate. Shortened US-U1A (B and D) or U1A-wt (C) mRNAs were assayed without (dotted squares) or with (filled diamonds) 700 ng U1A protein and were analysed on 5%-25% linear sucrose gradients. The assays contained either no analogue (C and D) or GMP-PNP (B). The labelled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A254 absorption profile, which was identical for gradients -/+ U1A protein.

5.3 Discussion.

The results presented here suggest that the cell-free translation systems and initiation assays accurately reflect the position effect previously observed in transfected cells. This is supported by the similar range of the loss of repression from US.CAT and iron regulation from F64 (which contain the identical spacer and IRE) in transfected B6 cells (Goossen and Hentze, 1992). Although ES.CAT was less repressed by IRP than US.CAT, a correlate of this mRNA has not been tested in transfected cells. It therefore appears to be valid to examine the mechanistic basis of the position effect *in vitro*.

The available data strongly suggest that 43S pre-initiation complexes bind more efficiently to US.CAT than 34T.CAT in the presence of IRP, but that the IRE/IRP complex in US.CAT still proves a formidable barrier to the access of 43S complexes. ES.CAT, contains an additional 34 nucleotides 5' to the IRE compared to US.CAT, the presence of which appears to provide the additional space required for the entry of a 43S complex. The number of nucleotides protected from nuclease digestion by 43S complex at the 5' end of the mRNA has been found to vary from 35-80 nucleotides (Kozak, 1989a; Kozak and Shatkin, 1977). The ribosomal complexes formed on ES.CAT after a 5 min assay varied, most probably as a result of the slower kinetics of initiation at higher magnesium concentrations. The slower kinetics of initiation under this condition may result from the progress of the 43S complex through the 5'UTR being affected by an increase in the stability of secondary structures or be a direct effect on the joining of 60S ribosomal subunits. The slowing in initiation kinetics in combination with the presence of the IRE/IRP complex, which may also be stabilised by the higher magnesium concentration (Wang et al., 1990), therefore allows observation of the 43S initiation intermediate in the absence of GMP-PNP, which is probably more transient in nature at the lower magnesium concentration (Antony and Merrick, 1992 and data not shown). However, single 43S pre-

initiation complexes appear to access ES.CAT efficiently at both magnesium concentrations suggesting the observed reduction of CAT synthesis from ES.CAT is the result of not loading multiple 43S complexes on the mRNA. In the absence of IRP a second 43S pre-initiation complex is normally "pre-loaded" (see 1.5.1.3). The absence of multiple loaded 43S complexes on ES.CAT may indicate that 43S complexes which have assembled on ES.CAT are temporarily stalled blocking the association of additional complexes. This is supported by the analysis at higher magnesium concentrations which revealed that 43S complexes associated with IRP-bound ES.CAT form 80S complexes more slowly. The analysis of ES.CAT therefore implies that IRE/IRP complexes located in this position temporarily interfere with scanning. Bearing this in mind, the lack of accumulation of 48S pre-initiation complexes on US.CAT (figure 30B and C) may indicate that complexes which have accessed this mRNA are not stalled implying that IRP is not firmly associated with US.CAT at the time of 43S entry. Alternatively, this may indicate that the kinetics of 43S binding to this mRNA are slow compared to the kinetics of overcoming the scanning impediment.

The rapid accumulation of 43S complexes on US-U1A suggests that the U1A mRNA/U1A complex does not pose as strong a barrier to 43S complex entry as IRE/IRP when located at a position similar to the IRE in US.CAT. This may be due to the weaker affinity of the U1A protein (Kd = 0.02-30 nM) for its binding site compared to IRP (Kd= 0.01-0.09 nM for active IRP) (Stripecke et al., 1992) allowing improved access of ribosomes to the mRNA. Alternatively, the smaller size of the U1A protein (34 as opposed to 100 kDa) may increase the effective "loading" area on the mRNA that is exposed for entry of 43S complexes. Currently, it is not known whether steric repression by RNA/protein complexes is mediated through the stabilisation of weak RNA secondary structures or whether the protein itself causes the steric block. These models are not mutually exclusive and the easier access of 43S complexes to US-U1A may be a combination of both the smaller size of the

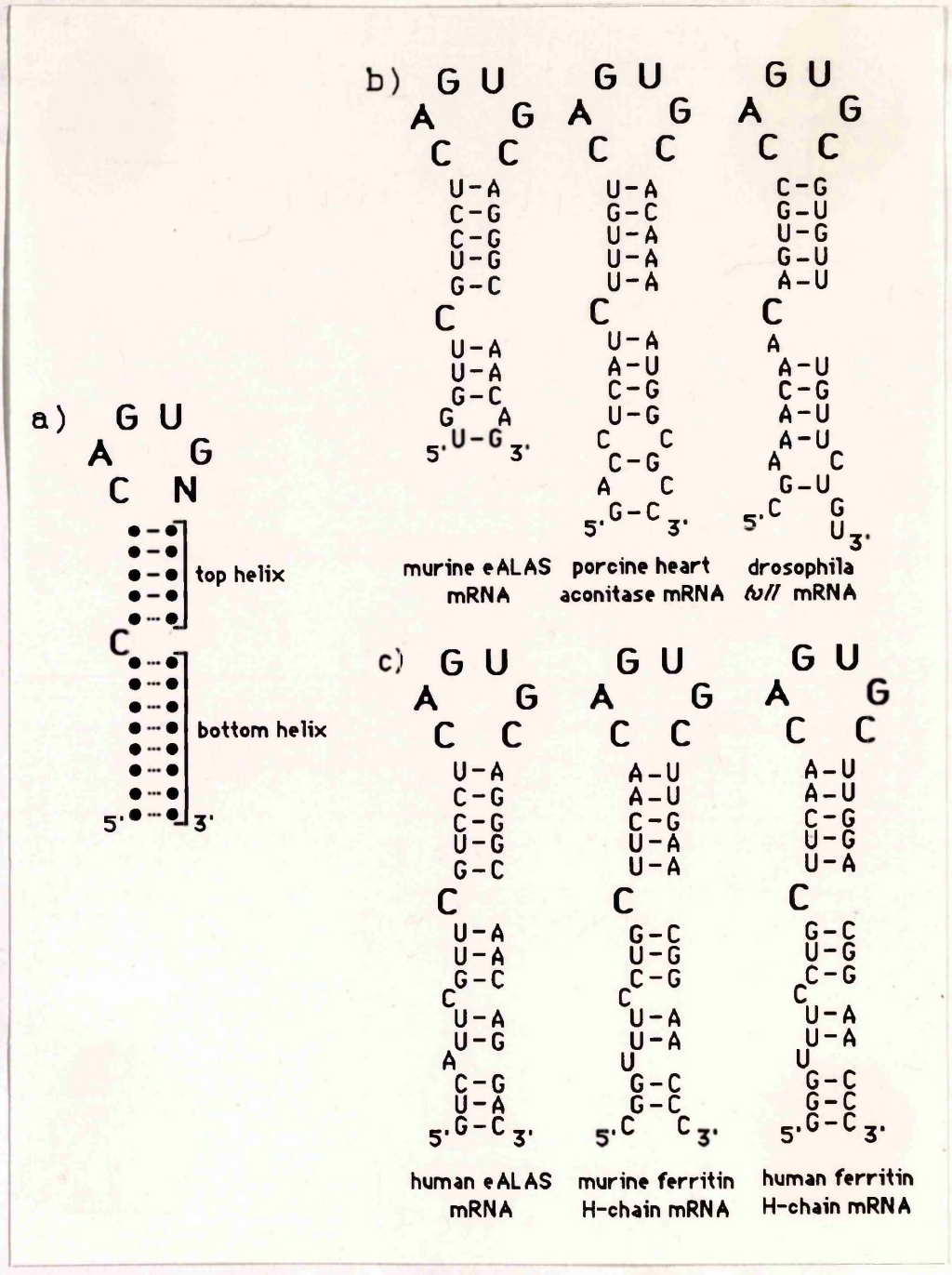
protein and its weaker interaction with its binding site resulting in a less stabilised secondary structure.

6.1 Introduction.

Bearing in mind that the abundance of IRP in cells far exceeds the requirement for the repression of ferritin and transferrin receptor mRNAs (Rouault et al., 1987), it was of interest to determine whether genes involved in other aspects of iron metabolism or inter-related metabolic pathways may also be controlled by IRP. The presence of IREs in such mRNAs could possibly allow the co-ordinated regulation of these genes. A computer-aided search of the EMBL data bank identified 64 putative IREs located in the untranslated and coding regions of mRNAs. Since all IRE elements known to mediate translational regulation are located in the 5'UTR of mRNAs, only IREs which were identified in the 5'UTR of their mRNAs were considered further. These putative IREs were found in the murine erythroid δ-aminolaevulinic acid synthase (eALAS) mRNA, the porcine mitochondrial aconitase mRNA and the *Drosophila melanogaster toll* mRNA (figure 33). In addition, comparison of the murine eALAS mRNA with the recently cloned human eALAS mRNA (Dr. B. May) revealed that it also contained a putative IRE element in its 5'UTR (figure 33). Gel retardation analysis showed that the putative IRE motifs from eALAS and mitochondrial aconitase mRNAs specifically interact with IRP, whilst the *toll* IRE motif did not (Dandekar et al., 1991; Zheng et al., 1992).

eALAS is the first and perhaps rate limiting enzyme in erythroid haem biosynthesis (Bothwell et al., 1983), a pathway responsible for 80% of the body's iron turnover. IRP-mediated regulation of this enzyme could therefore provide a link between the major utilisation pathway of iron and its uptake and storage. The role of mitochondrial aconitase, a Krebs cycle enzyme,in iron metabolism is at first less obvious, but aconitase is an iron-

Figure 33. The ferritin H-chain IRE, putative IREs identified by a computer assisted search of the data banks and the consensus IRE-motif.



(a) The phylogenetically derived IRE consensus motif. N represents any ribonucleotide except G, black dots connected with solid bars indicate Watson-Crick base pairing (allowing G-U or U-G interactions), black dots with broken bars symbolise optional hydrogen bonding. (b and c) Comparison between ferritin H-chain IREs and IREs identified using a computer assisted approach as described in Dandekar *et al.* (1991).

sulphur protein and its cytoplasmic counterpart has be shown to be identical to IRP (Kennedy et al., 1992). However, it is currently not known if and how these mRNAs are translationally regulated. The cell-free translation systems described in chapter 3 should provide a reliable method of assessing the ability of such IREs to direct translational repression.

6.2 Results.

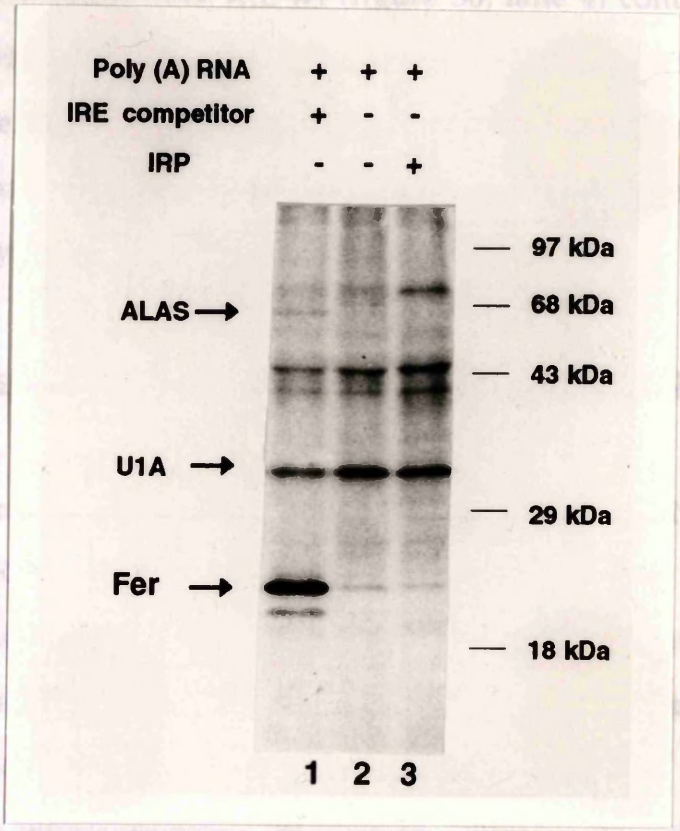
6.2.1 The sensitivity of eALAS mRNA translation to IRP.

The ability of short eALAS competitor transcripts to sequester endogenous reticulocyte IRP and thus relieve repression of ferritin mRNA. (see figure 4 on page 82, lane 6), provided a strong indication that the eALAS IRE was specifically recognised by IRP and therefore that eALAS mRNA may be translationally regulated by IRP. To test this hypothesis, poly (A)-enriched mRNA was prepared from mouse erythroleukaemia (MEL) cells induced with HMBA to differentiate. Non-differentiated MEL cells do not contain significant amounts of eALAS mRNA. Translation of this mRNA in rabbit reticulocyte lysate in the presence of either ferritin IRE competitor or exogenously added recombinant IRP and subsequent immunoprecipitation with anti-eALAS antiserum showed that eALAS mRNA translation is repressed in the presence of either endogenous reticulocyte or recombinant IRP (figure 34, lanes 2 and 3). Co-immunoprecipitation with anti-ferritin and anti-U1A antisera showed that ferritin mRNA translation was also repressed in the absence of short IRE competitor transcripts but the translation of U1A was not, demonstrating the specificity of the observed repression (figure 34, lanes 2 and 3).

6.2.2 The capacity of the eALAS IRE to mediate translational repression by IRP.

Although the above data demonstrate that eALAS mRNA is translationally repressed by IRP, they do not determine whether this sensitivity of eALAS mRNA to IRP is mediated by the IRE alone or involves other areas of the mRNA. To determine whether the IRE identified in eALAS mRNA is alone sufficient for the observed repression,

Figure 34. The translation of eALAS mRNA from induced MEL cells in rabbit reticulocyte lysate.



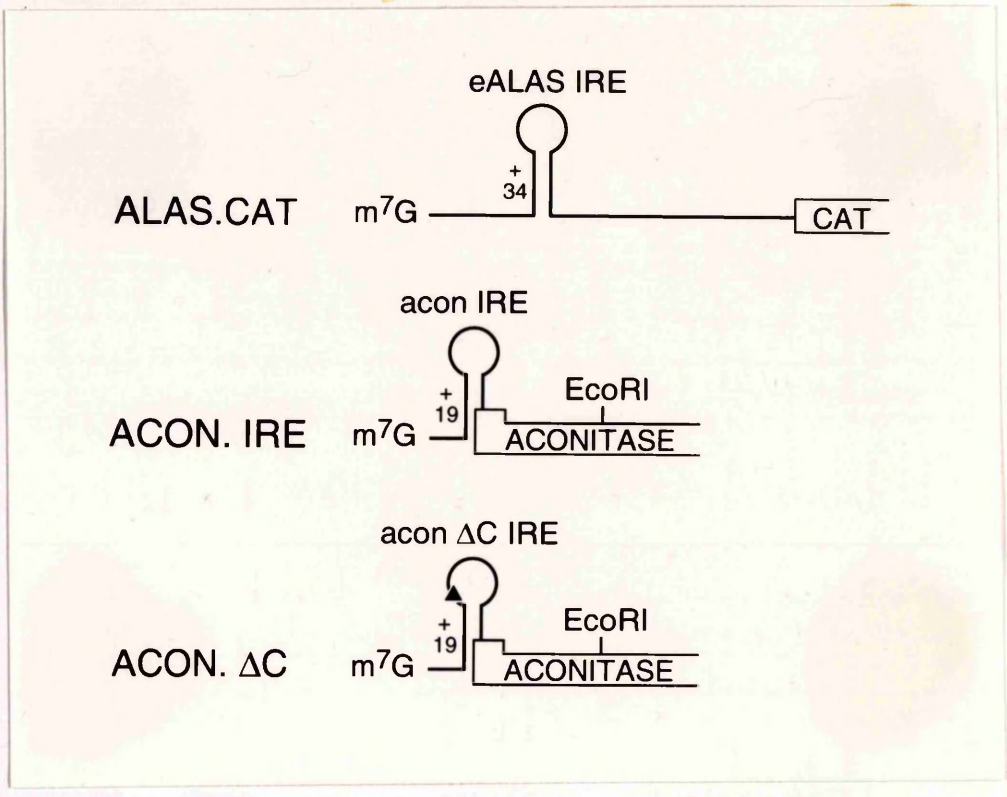
Poly (A)-enriched mRNA was isolated from induced MEL cells and translated in reticulocyte lysate in the presence of 135 ng of ferritin IRE competitor transcript (lane 1) or 750 ng of recombinant IRP from *E.coli* (lane 3). ^{35}S-Met labelled products were immunoprecipitated and then subjected to SDS-PAGE analysis and fluorography. Molecular weight markers are shown on the right and the position of U1A, ferritin and eALAS proteins are indicated on the left.

an eALAS IRE was inserted into the 5'UTR of a CAT reporter mRNA (ALAS.CAT) in an identical position to the ferritin IRE in IRE-wt (figure 35). The translation of ALAS.CAT in wheat germ extract was compared to that of IRE-wt in the absence or presence of 250 ng placental IRP (figure 36). ALAS.CAT translation was specifically repressed by IRP (figure 36, lane 6), although the repression of IRE-wt (figure 36, lane 4) containing the ferritin IRE was consistently stronger than the repression mediated by the eALAS IRE. Thus, eALAS mRNA represents a newly identified IRE-containing mRNA, whose IRE is sufficient to mediate translational repression by IRP in cell-free systems.

6.2.3 The responsiveness of mitochondrial aconitase mRNA to IRP.

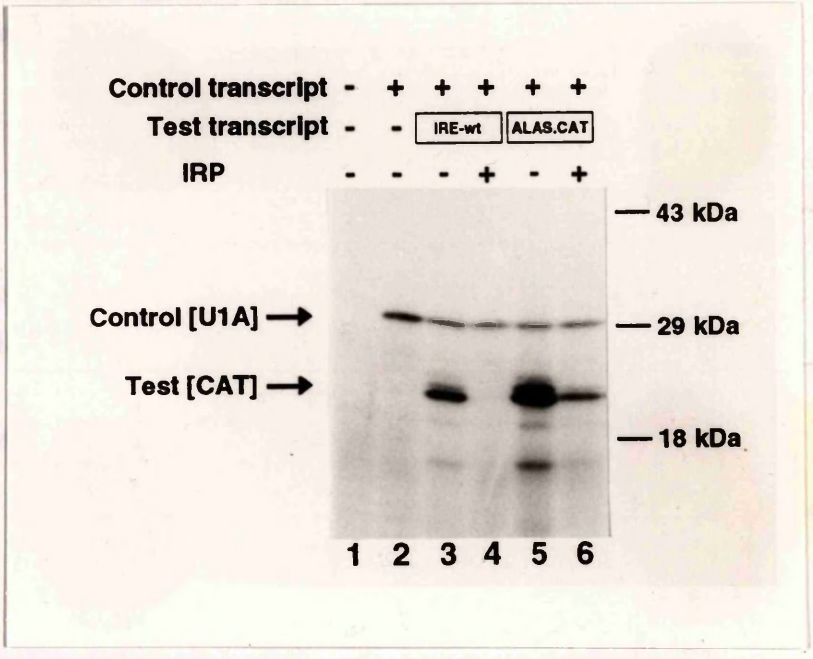
The translation of mitochondrial aconitase mRNA in poly (A)-enriched mRNA could not be directly examined due the lack of high affinity anti-aconitase antiserum required to perform immunoprecipitation assays. Therefore, the translation of *in vitro* transcribed aconitase mRNAs was examined (figure 35). One of these mRNAs, acon.IRE, contains an intact aconitase IRE whilst the other contains an aconitase IRE where the first C in the loop has been deleted (acon.ΔC). Translation of full-length aconitase mRNAs in wheat germ extract did not result in ^{35}S-Met labelled protein products of the predicted size (~83 kDa) (data not shown). It is known that the elongation capacity of wheat germ extract is poor and that production of such large proteins is therefore inefficient (Clemens, 1984). To overcome this technical problem, the aconitase plasmids were linearised at a site within the aconitase coding sequence, prior to transcription. Translation of the truncated aconitase mRNAs in wheat germ extract resulted in heterogeneous protein products of ~35 kDa (figure 37A, lanes 6-9). The heterogeneity of these products is possibly a result of protein proteolysis, as protein products which are not terminated at an authentic termination codon are subject to degradation through identification of the tRNA which

Figure 35. Schematic drawing of the *in vitro* transcribed mRNAs.



The position of the IRE in the 5'UTRs of ALAS.CAT (containing the murine eALAS IRE) and the porcine mitochondrial aconitase mRNAs is quoted with reference to the distance of the conserved unpaired C in the IRE from the cap structure. The delta symbolises an IRE with a deletion of the first nucleotide of the IRE loop. The open box represents the open reading frame. In the aconitase mRNAs, the position of the *Eco* RI site used to linearise plasmids prior to transcription of truncated aconitase mRNAs is shown.

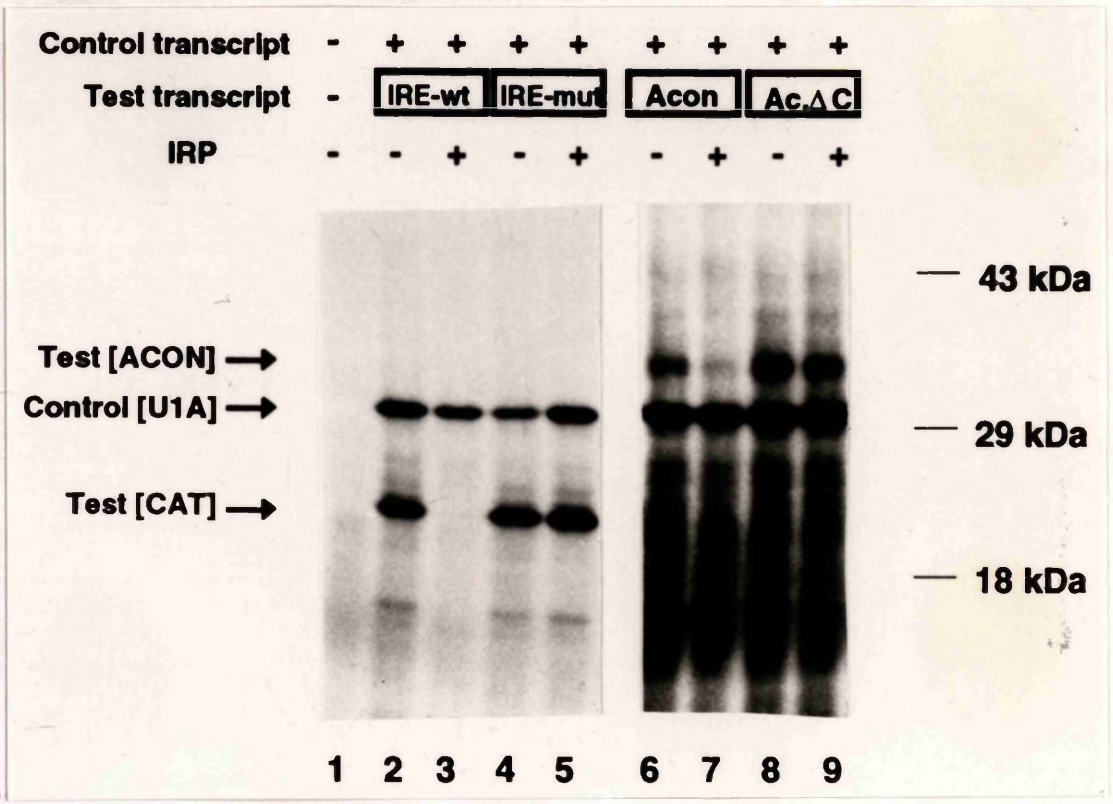
Figure 36. The cis-acting sequences required from eALAS mRNA to direct IRE-mediated regulation in wheat germ extract.



U1A (2.5 ng) and 5 ng of IRE-wt or ALAS.CAT mRNAs were translated in wheat germ extract in the presence (+) or absence (-) of 250 ng of affinity-purified IRP. ^{35}S-Met labelled products were subjected to SDS-PAGE analysis and fluorography. Molecular weight markers are shown on the right and the positions of the U1A and CAT proteins are indicated on the left.

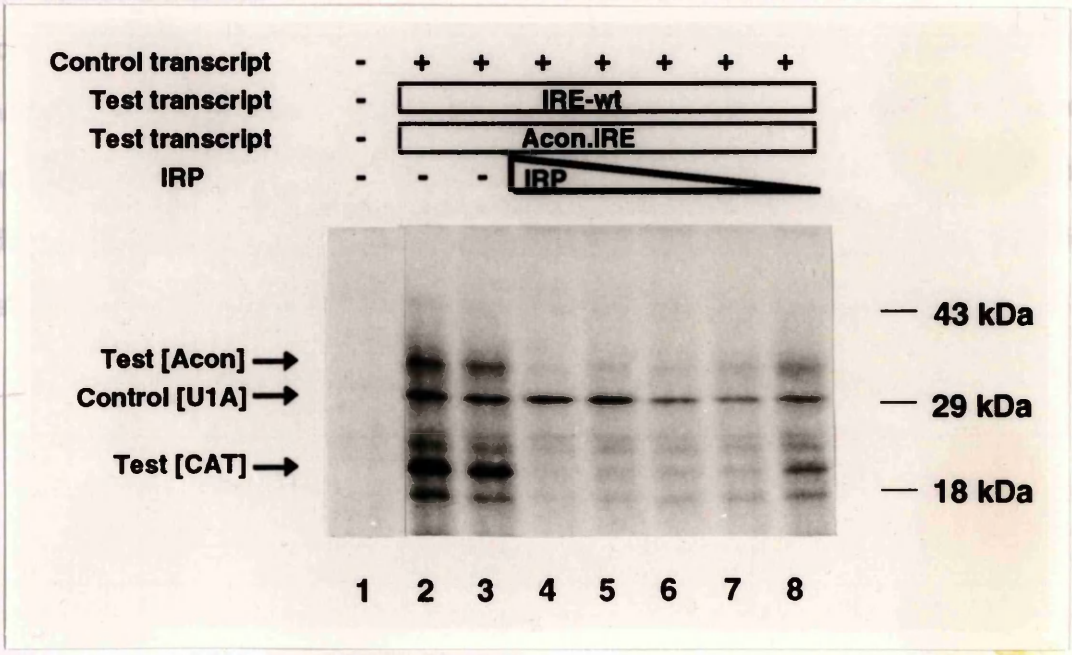
Figure 37. The translation of aconitase mRNAs in the presence of IRP in wheat germ extract

(A). Repression of aconitase mRNA containing an intact IRE by recombinant IRP from *E.coli*.



U1A and equimolar amounts of IRE-wt, IRE-mut, acon.IRE or acon.ΔC mRNAs were translated in the absence or presence of 375 ng of IRP in WGE. ^{35}S-Met labelled products were analysed by SDS-PAGE and fluorography. Molecular weight markers are shown on the right and the positions of the "U1A", CAT and truncated aconitase proteins are indicated on the left.

Figure 37 (B). Titration of IRP against acon.IRE and IRE-wt.



U1A, IRE-wt and acon.IRE mRNAs were translated in WGE in the absence (lanes 2-3) or presence (lanes 4-8) of 500, 250, 125, 62.5 or 31.25 ng of recombinant IRP from E.coli. Translation products were analysed by SDS-PAGE and fluorography. Molecular weight markers are shown on the right and the positions of the "U1A", CAT and truncated aconitase proteins are indicated on the left.

remains bound in the absence of release factor (Minshull and Hunt, 1992). Addition of recombinant IRP from *E.coli* repressed IRE-wt and acon.IRE translation, showing that the aconitase mRNA is responsive to IRP (figure 37A, lanes 3 and 7). However, translation of acon.ΔC. mRNA was not affected by IRP, indicating that an intact IRE is required for IRP-mediated translation repression of aconitase mRNA (figure 37A, lanes 5 and 9). A comparison of the sensitivity of acon.IRE and IRE-wt mRNA to varying concentrations of IRP showed that their sensitivity to the presence of IRP was similar (figure 37B). It is therefore concluded that the translational repression of mitochondrial aconitase mRNA is specific and IRE-mediated. In addition, it is concluded that acon.IRE is repressed with an efficiency similiar to IRE-wt (containing a ferritin IRE).

6.3 Discussion.

The results presented here suggest that the putative IREs previously identified in the eALAS and mitochondrial aconitase mRNAs (Dandekar et al., 1991) are recognised by IRP and mediate translational repression in the presence of active IRP. The regulation of eALAS mRNA in response to intracellular iron levels has subsequently been verified in MEL cells (Melefors et al., 1993). The repression of both mRNAs was specific and in the case of the eALAS mRNA the IRE element was shown to be sufficient for regulation. The translational repression mediated by the eALAS IRE is less efficient than that mediated by the ferritin IRE when placed in the same context, which correlates well with the lower affinity of IRP for eALAS IREs observed in gel retardation assays (A. Constable unpublished data) and the lower range of eALAS mRNA (compared to ferritin mRNA) in MEL cells (Melefors et al., 1993). The lower affinity of the eALAS IRE may be crucial to cellular metabolism, allowing repression of ferritin synthesis at iron concentrations where a basal level of eALAS synthesis, and thus haem production, can still continue.

Short aconitase IRE transcripts appear to compete less efficiently for IRP than the ferritin or eALAS IREs when assayed in gel retardation assays (A. Constable unpublished observations), but aconitase mRNA translation *in vitro* seems to be as efficiently repressed as IRE-wt translation by IRP. This apparent discrepancy may be due to the differing contexts of the surrounding mRNA sequences in IRE-wt and acon.IRE, or due to the increased proximity of the aconitase IRE (+19) to the cap compared to the ferritin IRE in IRE-wt (+34). Since the results presented here do not determine whether sequences in addition to the IRE are required for repression of aconitase mRNA, a contribution of non-IRE sequences to the regulation of aconitase mRNA cannot be excluded. The function of the mitochondrial aconitase IRE in cells has yet to be examined and a possible physiological role of such regulation is discussed in chapter 7.

Finally, the IRE-like motif identified in *Drosophila toll* mRNA, used as an internal control in chapter 3, does not sequester either endogenous reticulocyte (figure 9, lane 11) or purified IRP (figure 10, lane 16) supporting the previous gel retardation analysis and underscoring the likelihood that this sequence does not represent a *bona fide* IRE. Based on the extensive mutational analysis of IREs (see 1.6.7), the variability between the primary sequence of the stem regions of this IRE and functional IREs is not likely to account for its lack of affinity for IRP. However, the predicted stability of the putative *toll* IRE-like motif (-0.4 kcal/mol) is lower than that of other known IREs (-2.1- -6.7 kcal/mol) (Dandekar et al., 1991) raising the possibility that this sequence does not exist in a sufficiently stable stem-loop structure of the correct configuration to be recognised by IRP. Alternatively, it has been suggested that the presence of a 2 nucleotide bulge, rather than a single C or a three nucleotide bulge in the *toll* IRE-like motif is unfavourable for IRE/IRP interactions (Henderson et al., 1994). However, *Drosophila* IRP (Rothenberger et al., 1990) has not been assayed for its ability to recognise the *toll* IRE and it therefore remains possible that this element could function in *Drosophila*.

Chapter 7: General Discussion.

7.1 The requirements for IRE-mediated repression in cell-free systems.

Previous work has shown the IRE element alone to be sufficient to mediate translational regulation in response to intracellular iron levels in transfected cells (Caughmann et al., 1988; Hentze et al., 1987b) and that ferritin or reporter mRNAs containing the majority of the ferritin 5' UTR are repressed in the presence of IRP in cell-free systems (Walden et al., 1988; Walden et al., 1981; Walden et al., 1989). Subsequently, it was shown that a smaller region of the ferritin 5' UTR (92 nucleotides) including the IRE was sufficient to mediate translational repression *in vitro* (Swenson et al., 1991).

The data presented here are fully consistent with previous work and have shown in addition that an intact IRE, in the absence of other ferritin or eALAS sequences including the respective "flanking regions" is sufficient for translational repression in cell-free systems. Consistent with this, a deletion of the 3' flanking region was shown not to affect translational repression in cell-free systems (Dix et al., 1993) or in transfected cells (Goossen and Hentze, 1992). However, a triple mutant in the flanking region which disrupts base pairing between the flanking sequences but does not affect IRP-binding is less efficiently repressed albeit only at low protein concentrations (Dix et al., 1993). This result is difficult to interpret since deletion of the 3' flanking region (Dix et al., 1993; Goossen and Hentze, 1992) or all the flanking sequences has no effect on the range of IRP-mediated regulation (Caughmann et al., 1988). The work presented here also shows that the position of the IRE within the 5'UTR is important for repression *in vitro*, in agreement with other studies both in transfected cells (Goossen et al., 1990; Goossen and Hentze, 1992) and in a cell-free system (Bhasker et al., 1993). The absence of poly (A) tails on the CAT reporter mRNAs used here and in other studies (Swenson et al., 1991) demonstrated that polyadenylation of mRNAs is not required for repression in cell-free

systems. The possibility remains that changes in the length of ferritin poly (A) tails occur *in vivo* in response to changes in intracellular iron levels, but the findings in cell-free systems clearly favour such changes being a secondary consequence rather than a primary cause of repression.

The requirements for trans-acting factors was also examined in these systems and affinity-purified IRP was shown to mediate translational repression in a similar way to the previously described FRP activity (Brown et al., 1989; Walden et al., 1988; Walden et al., 1989). This result is not surprising in the light of the cloning of human IRP and rabbit IRP (FRP) which revealed them to be 94% identical (Hirling et al., 1992; Patino and Walden, 1992; Rouault et al., 1990). The ability of recombinant IRP purified from *E.coli* to repress the translation of IRE-containing mRNAs in wheat germ extract in a quantitatively similar manner to placental IRP revealed that IRP alone was sufficient to mediate repression in the absence of additional mammalian co-factors. Co-expression of recombinant IRP and IRE-containing mRNAs in *S. cerevisiae* has subsequently confirmed IRP to be the only mammalian factor required for repression *in vivo* (Oliveira et al., 1993). The minimal requirements for IRE-mediated repression in cell-free systems were therefore found to be an intact IRE located in a cap-proximal position within the 5'UTR, and the presence of active IRP, which accurately reflects the minimal requirements for IRE-mediated regulation in cells.

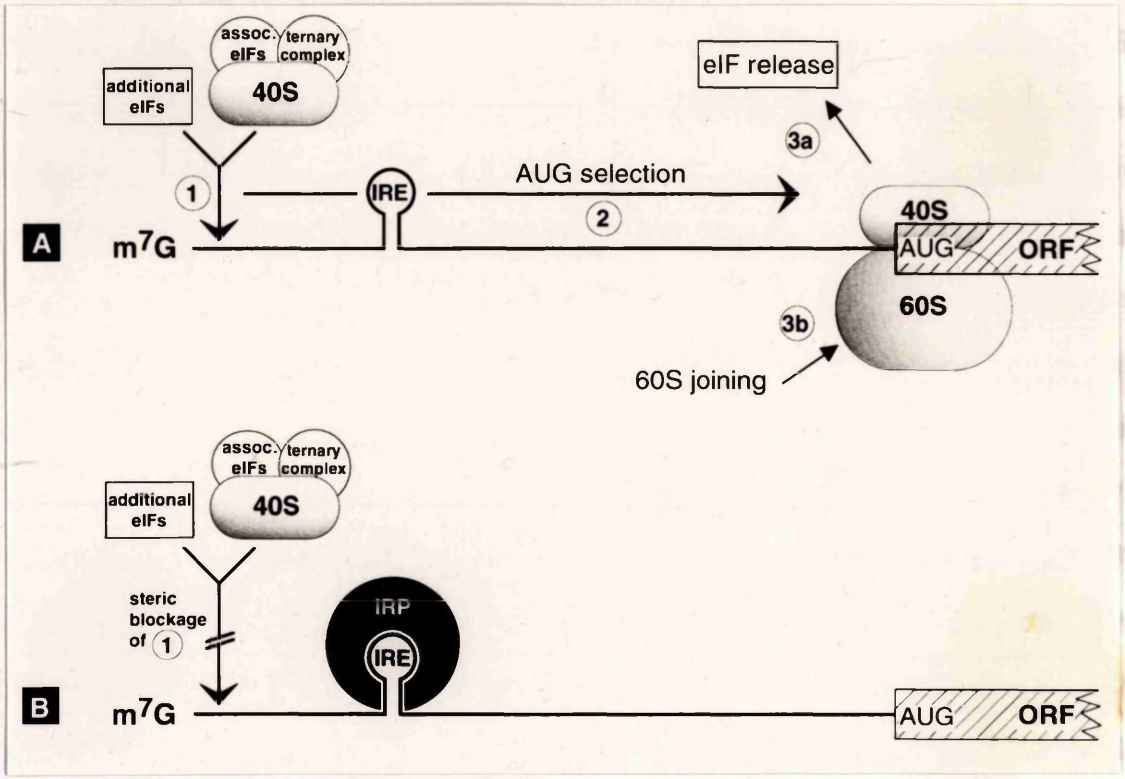
7.2 The mechanism of ferritin, eALAS and mitochondrial aconitase regulation.

The data presented here indicate that the regulation of ferritin, eALAS and most probably mitochondrial aconitase mRNA is achieved by IRP blocking the interaction of the 43S pre-initiation complex with these mRNAs. The hypothesis that these mRNAs are all regulated by a similar mechanism is strengthened by the recent observation that the repression of

eALAS mRNA is position-dependent in rabbit reticulocyte lysate (Bhasker et al., 1993). Furthermore, results obtained by sucrose gradient analyses of ferritin mRNA isolated from rats that had been injected with the iron chelator desferrioxamine, in which repressed ferritin mRNA sedimented in the upper fractions of the gradient, are fully consistent with the proposed mechanism (Aziz and Munro, 1986). The finding that artificial repressors such as the U1A protein inhibit 43S pre-initiation complex access to mRNA when binding to a cap-proximal site, strongly suggests that IRE/IRP-mediated repression is achieved via a steric inhibition of the translation initiation machinery. The ability of IRE/IRP-mediated repression to be re-constituted in *S. cerevisiae* and especially in wheat germ extract where the initiation factors vary substantially from those found in mammalian cells, is also consistent with the argument that IRE/IRP complexes inhibit translation sterically. However, it remains possible that initiation factors in mammals and plants retain sufficient similarity to allow similar specific interactions with IRP to occur in both systems, but it is highly unlikely that the U1A and MS2 proteins contain the necessary sequences to interact with the initiation machinery in a manner similar to IRP. Based on the data presented here, that of Stripecke et. al. (1992; 1994), and the above considerations it is suggested that the IRE/IRP complex sterically blocks the access of the 43S pre-initiation complex to ferritin, eALAS and aconitase mRNAs (figure 38).

It remains to be determined whether the IRE/IRP complex exerts its effect via direct steric hindrance of the 43S complex or whether it interferes with earlier stages in initiation. The proximity of IRE/IRP complexes to the 5' end of the mRNA raises the possibility that they may occlude association of initiation factors which interact with this area of the mRNA prior to the 43S complex and assist in its binding. Stem loop structures located close to the cap structure of mRNAs which prevent access of the 43S pre-initiation complex of mRNAs (Kozak, 1989a) do not seem to affect the interaction of eIF-4E with the mRNA (Lawson et al., 1986; Pelletier and Sonenberg, 1985b).

Figure 38. Model of ferritin regulation by IRP.



A) With IRP not bound to the IRE, (1) binding of the 43S pre-initiation complex to the mRNA is assisted by initiation factors associated with this complex as well as additional eIFs which interact with the mRNA to facilitate 43S association. Subsequently (2), the 43S pre-initiation complex moves along the 5'UTR towards the AUG initiator codon, (3) GTP is hydrolysed, initiation factors are released, and assembly of the 80S ribosome occurs. B) With IRP-bound to the IRE, access of the 43S preinitiation complex to the mRNA is sterically blocked. U1A bound to an mRNA in a position similar to that of the IRE also prevents step 1.

However, stem-loop structures have been shown to prevent the stable association of eIF-4A and eIF-4B with mRNA (Lawson et al., 1986; Pelletier and Sonenberg, 1985b). It is therefore a matter of speculation whether IRE/IRP complexes prevent the assembly of eIF-4A or eIF-4B on to the mRNA or the progression of the eIF-4A/4B helicase activity along the 5'UTR. Inhibition of either the association or progress of these initiation factors and/or the association of the 43S pre-initiation complex may result from direct steric interference by the IRP protein or from stabilisation of the IRE secondary structure by IRP. These models are not mutually exclusive and the observation that binding of IRP to the IRE increases the helical stacking of base pairs surrounding the IRE (Harrell et al., 1991) is consistent with the idea that IRP stabilises the IRE secondary structure. An increase in helical stacking of base pairs close to the 5' end of the mRNA might inhibit the progression of helicase activity, which has been proposed to remove secondary structure from the 5'UTR of mRNAs allowing binding of the 43S pre-initiation complex (see 1.4.3). A number of experimental approaches appear suitable to ascertain whether IRE/IRP complexes affect initiation events prior to the joining of the 43S pre-initiation complex. These include gel retardation analysis of IRP-bound mRNAs purified initiation factors, or isolation and analysis of initiation factors present on IRP-repressed mRNA either by co-immunoprecipitation, cross-linking or *via* streptavidin affinity chromatography using biotinylated 2'O-allyl RNA oligonucleotides directed against downstream regions of IRE-wt mRNA (Dr. H. E. Johansson, PhD thesis). Application of such techniques may provide information about which initiation factors access IRP-bound mRNA and whether the IRE/IRP complex blocks the access of the 43S pre-initiation complex directly.

7.3 Cap-distal RNA/protein complexes.

Two hypotheses have been advanced to explain the "position effect" (Goossen et al., 1990; Goossen and Hentze, 1992). First, the 43S complex may

be excluded from the mRNA when an IRE/IRP complex is located in a cap-proximal but not a cap-distal position. Alternatively, a 43S complex may bind to the mRNA in the presence of IRP and acquire the potential to displace inhibitory RNA/protein complexes during scanning (Goossen et al., 1990; Melefors and Hentze, 1993). The data presented here clearly favour the former hypothesis and exclude the latter. Although the 43S complex appears to bind to US.CAT, US-U1A and ES.CAT more efficiently than to 34T.CAT, only in the case of ES.CAT does this entry appear to occur as efficiently as in the absence of IRP. Implicit in the seemingly unimpeded entry of a 43S complex to IRP-bound ES.CAT is the understanding that IRE/IRP complexes in this position do not interfere with the access of initiation factors to the mRNA. In addition, the results presented here extend the former model as the accumulation of 48S initiation intermediates on ES.CAT in the presence of IRP suggests that IRE/IRP complexes located in a downstream position impede the progression of the 43S complex along the 5'UTR. However, this impediment can clearly be overcome. The impaired progression of the 43S complex along the 5'UTR of ES.CAT could be interpreted to mean that the IRE/IRP complex forms a formidable barrier to helicase activities responsible for unwinding of the mRNA. This activity may either be provided by the 43S complex itself or by 4A/4B complexes which may precede it along the 5'UTR (see 1.4.3). The possibility therefore exists that IRE/IRP complexes both in cap-distal and cap-proximal position impair the progression of the 4A/4B helicase activity (see previous section), with the 4A/4B complex gaining additional potential to overcome IRE/IRP complexes as it traverses the 5'UTR. This speculative hypothesis would provide a unified explanation for the seemingly differing actions of the IRE/IRP complex in various regions of the 5'UTR. However, it remains to be elucidated whether the IRE/IRP complex inhibits any helicase activity and whether IRE/IRP complexes located in different places within the 5'UTR interfere with the translational machinery in the same way.

7.4 How does the translational machinery overcome cap-distal RNA/protein complexes ?

43S complexes appear to co-exist with IRP on ES.CAT and the data suggest that these 43S complexes are temporarily stalled by the IRE/IRP complex. The mechanism by which a bound 43S pre-initiation complex negotiates the impediment posed by a downstream mRNA/protein complex requires further definition. The 43S complex might pass the mRNA/protein complex following either transient dissociation or active displacement of the repressor protein. In the first case, the presence of 43S complexes "waiting" on the mRNA for IRP dissociation would account for the inefficient repression by IRE/IRP complexes in this position. In the second, either a helicase activity associated with the eIF-4A/4B or the 43S complex, or the collision of the 43S complex with the IRE/IRP complex may dislodge the IRP protein. Quantitative comparisons of the release of labelled IRP during incubation in translation extract from 34T.CAT, from which active displacement should not occur, and ES.CAT, may distinguish between these models.

The absence of complexes greater than 80S on IRE-containing mRNAs in the presence of IRP suggests that IRP re-associates with the mRNA after the passage of the 43S complex, preventing the association of multiple 43S complexes with the mRNA. In the case of US-U1A, however, complexes larger than 80S are observed suggesting that binding of the U1A protein is relieved and that the lesser affinity of this RNA/protein complex results in the exclusion of U1A protein from the mRNA.

Although it is highly likely that the IRE/IRP block is overcome by the 43S complex itself, the possibility exists that pausing of this complex in front of the IRE/IRP complex allows 60S joining and that an 80S ribosome overcomes the IRE/IRP complex. If this is the case it should be possible to

detect 80S ribosomes paused in front of the IRE/IRP complex by RNAse footprinting.

Interestingly, the translational machinery has recently been shown to shunt or hop past a region of the 5'UTR of cauliflower mosaic virus (Fütterer et al., 1993). The possibility that the IRE/IRP complex is overcome in such a manner cannot therefore be ruled out. Transfection of reporter mRNAs containing an IRE with a AUG codon in the stem should determine whether shunting or hopping represents the mechanism by which a cap-distant IRE/IRP complex is bypassed in cells. If shunting occurs then the AUG initiator codon in the IRE should not be utilised.

In contrast to rabbit reticulocyte lysate, preliminary experiments not reported in this thesis suggest that ES.CAT is fully repressed in wheat germ extract and that 48S complexes accumulated on the mRNA (N. K. Gray- unpublished data). This suggests that an activity which is required to overcome IRE/IRP complexes in mammalian cells and reticulocyte lysate is absent from wheat germ extract. Exploitation of the differences in these systems may yield additional information about inhibition by cap-distal IRE/IRP complexes and how this inhibition is overcome.

7.5 Multiplicity of mechanisms for translational control by mRNA binding proteins.

Numerous examples of translational control by repressor proteins have been reported from a wide range of eukaryotic organisms. IRP-mediated regulation of ferritin and eALAS mRNA translation is the first for which the interaction with the translational apparatus has been characterised in detail. It is therefore a matter of speculation whether other mRNAs are controlled similarly to ferritin and eALAS. However, the ability of U1A protein to block access of the 43S pre-initiation complex when placed in a cap-proximal position within the 5'UTR of a reporter mRNA suggests that this may be a more general mechanism for translational

repression. The lack of effect of IRP on translation when an IRE is located within the 3' UTR of an mRNA (Casey et al., 1988) appears to exclude translationally-regulated mRNAs like rabbit erythroid 15-lipoxygenase mRNA, where the cis-acting regulatory sequences lie within this region (Ostareck-Lederer et al., 1994). Likewise, regulatory sequences located within the open reading frame are likely to contribute to translational regulation in a different manner (Chu et al., 1993). The position dependence of IRP function might suggest that regulatory sequences in cap-distal positions within the 5' UTR, as in the thymidylate synthase mRNA, regulate translation in a different manner. However, cap-distant IRE elements within the 5'UTR retain some regulatory activity (Goossen and Hentze, 1992) and such a mechanism could be advantageous when only a slight modulation of protein expression is required. Likewise, the differences in the association of 43S complexes with US-U1A and US.CAT, whose binding sites are similarly positioned highlight the possibility that cap-distant complexes may regulate translation more efficiently when the affinity between the RNA binding site and the protein is greater.

Some examples of translational control, however, share obvious similarities with the IRE/IRP system. The regulation of a family of mRNAs involved in spermatogenesis in *Drosophila melanogaster*, including Mst87F, is mediated by a conserved element in the 5'UTR of these mRNAs. As with ferritin, the position of this element with respect to the cap structure is conserved, and movement of this element further downstream severely impedes regulation (Kempe et al., 1993). In addition, the mRNAs encoding different ribosomal proteins are translationally regulated *via* cap-proximal elements in many species, ranging from L32 in *S. cerevisiae* (Dabeva and Warner, 1993) to L30, L32 and S16 in human cells (Levy et al., 1991). Conceivably, differences in the regulatory mechanisms may exist even within this family. A requirement for cap proximity has been demonstrated for the murine S16 mRNA (Hammond et al., 1991). Unfortunately, the putative regulatory proteins that bind to these

translationally-regulated mRNAs have not yet been purified and demonstrated to function *in vitro*, so their functional relationship with IRP remains to be fully established.

In conclusion, it appears as if multiple mechanisms can operate within eukaryotic cells by which proteins regulate the association of an mRNA with the translational apparatus. In principle, the biochemical approach employed here should be applicable to many of these systems.

7.6 The physiological implications of IRE-mediated control on iron metabolism.

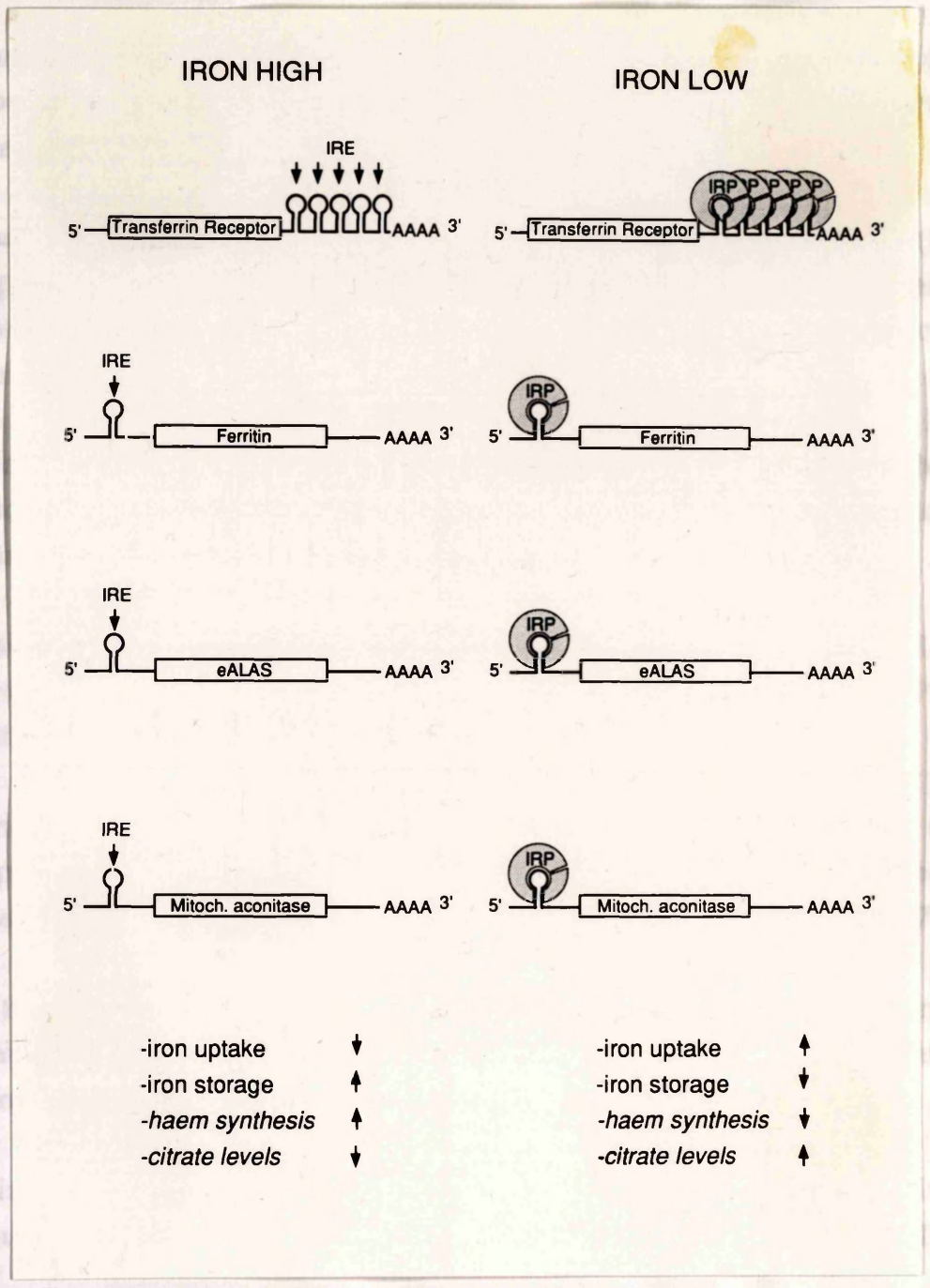
It has long been known that the control of iron uptake and storage into cells is co-ordinately regulated post-transcriptionally via IREs. The results reported here and elsewhere (Bhasker et al., 1993; Dandekar et al., 1991; Melefors et al., 1993) suggest that eALAS mRNA is regulated in a manner qualitatively similar to ferritin. In the human genome, ALAS is encoded by two genes; the "housekeeping" form, whose 5'UTR does not contain an IRE and is ubiquitously expressed, and the erythroid-specific form, which is regulated by IRP, and which accounts for almost all of the ALAS activity in erythroid cells where it appears to be the rate limiting enzyme in haem synthesis (Melefors et al., 1993). Since haem synthesis in erythroid cells accounts for the majority of body iron utilisation it is clear why co-ordinate regulation of intracellular iron levels and haem synthesis is advantageous. Interestingly, the apparent differential affinity of IRP for ferritin and eALAS mRNAs may account for the less stringent regulation of eALAS synthesis by iron, providing these cells with a way to maintain basal haem production under conditions where intra-cellular iron is too scarce to be stored in ferritin. The complete shutdown of ferritin synthesis coupled with the increased production of transferrin receptor synthesis under these conditions ensures that the maximal amount of iron is made available for haem synthesis, the primary function of these cells.

Although it remains to be determined whether mitochondrial aconitase mRNA is regulated by IRP in cells, the possible physiological role of IRE-mediated regulation of mitochondrial aconitase is less clear. However, the observation that IRP has aconitase activity strengthens the notion that the IRE element in mitochondrial aconitase is physiologically relevant. It appears as if the activities of both mitochondrial and cytoplasmic aconitase are regulated by iron so that their activities decrease when iron is scarce. In the case of cytoplasmic aconitase (IRP) this is achieved at the post-translational level by changes in the iron-sulphur cluster, while the data presented here indicate that mitochondrial aconitase is regulated translationally. The cytoplasmic aconitase activity of IRP may represent an evolutionary remnant of a common ancestral aconitase enzyme, or reflect a cellular requirement for an iron-regulated cytoplasmic aconitase activity. Interestingly citrate, the primary substrate of aconitase, is an iron chelator and has been proposed to act as an intracellular carrier of iron (low molecular weight iron pool) (Bacon and Tavill, 1984). The product of the reaction, iso-citrate, has a lower affinity for iron (O'Halloran, 1993), so conversion of citrate into iso-citrate may regulate the amount of citrate available to carry or chelate iron within the cell. Thus, there maybe a physiological reason for the regulation of citrate levels in response to iron availability which is currently not fully understood. In addition to the IRE-binding activity of IRP therefore, the aconitase activity of IRP may influence the intracellular distribution of iron. Cells are known to be restricted in growth when sufficient iron is not present (Klausner et al., 1993), and the presence of an IRE in the mRNA encoding a Krebs cycle enzyme may provide a link between the availability of intra-cellular iron and the growth potential of the cell. Lack of iron may lead to a decrease in mitochondrial aconitase synthesis and activity which may ultimately limit the activity of the Krebs cycle. Interestingly, an IRE-like element has been identified in an mRNA encoding a subunit of succinate dehydrogenase, another Krebs cycle enzyme which is also an iron-containing protein (pers. comm. Dr B.

Ackrell, U.C.S.F., CA, USA). However, it must also be remembered that other iron-containing proteins such as ribonucleotide reductase are known to be required for proliferation (Frausto da Silva and Williams, 1991) .

In conclusion, the IRE/IRP system provides co-ordinated regulation of mRNAs which control intracellular iron homeostasis (figure 39). This system provides flexibility for the differential regulation of mRNAs *via* affinity differences for IRP between the IREs. This may also be achieved *via* the positioning of IREs in the 5'UTRs of their respective mRNAs. As illustrated in this thesis, the IRE/IRP system has also proved to be a suitable model system for studying the underlying mechanisms of translational control by RNA/protein complexes within the 5'UTR of mRNAs.

Figure 39. Biological targets and implications of IRE/IRP regulation.



When iron levels are high, transferrin receptor mRNA is unstable and translation of ferritin, eALAS and mitochondrial aconitase occurs. When iron levels are low, IRP will bind to the IREs in these transcripts. This increases the stability of transferrin receptor mRNA leading to increased numbers of receptors and increased iron uptake. Analogously, the binding of IRP to ferritin, eALAS and mitochondrial aconitase mRNAs leads to lowered iron storage, decreased haem synthesis and increased citrate levels, respectively. Italics indicate that the physiological consequences remain to be directly established *in vivo*. Note also that the translational control of aconitase expression has not yet been demonstrated *in vivo*.

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