RNA and protein interactions by eIF4B during translation initiation

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A Monique et Gilles, qui m'ont donné de nombreux encouragements, et qui ont toujours su rétablir, au besoin, l'équilibre si nécéssaire à l'accomplissement d'un travail de cette envergure, et à mon frère Michel, dont le courage et les succès ont été et sont toujours une source d'inspiration.

Abstract

One of the most enduring questions pertaining to eukaryotic translation initiation is how the 40S ribosomal subunit recognizes and binds at or near the cap-structure of mRNAs. Eukaryotic initiation factor 4B (eIF4B) is one of the factors that are required for this step of protein synthesis. eIF4B stimulates the RNA helicase activity of eIF4A and eIF4F, melting RNA secondary structure in the 5' untranslated region (UTR), and is thus believed to contribute to ribosome binding by creating an area of single-stranded RNA accessible to the 40S ribosomal subunit. We studied the mechanism of action of eIF4B by initiating a structure-function analysis of this factor. An RNA binding site, located in the carboxy-terminal end between amino acids 367 and 423, was found essential for non-specific RNA binding and eIF4A helicase stimulation. This region is distinct and independent from the canonical RNA Recognition Motif (RRM) located near the amino-terminus. The latter plays no role in non-specific RNA binding and has little impact on the eIF4A helicase stimulation. A self-association region located between residues 213-312 was identified. This segment is rich in aspartic acid, arginine, tyrosine and glycine (DRYG) residues, and can self-associate independently from other regions of eIF4B. The DRYG domain also interacts directly with the p170 subunit of eIF3. Finally, iterative *in vitro* RNA selection demonstrated that the eIF4B RRM is functional and binds specifically to RNA stem-loop structures. The RRM also associates with 18S rRNA. eIF4B possesses two independent RNA binding sites and associates with two different RNA molecules simultaneously. We conclude that eIF4B is organized into three distinct domains: the carboxy-terminal eIF4A RNA helicase stimulatory domain, the DRYG dimerization and eIF3 p170 interaction domain, and the RRM. Furthermore, two additional mechanisms by which eIF4B could stimulate ribosome binding to the mRNA are now apparent. eIF4B may target the 40S ribosomal subunit to the 5' UTR of a mRNA by binding simultaneously to 18S rRNA and the mRNA, and by interacting directly with eIF3.

Résumé

Le mécanisme par lequel la sous-unité ribosomale 40S se lie à la structure coiffe en 5' d'un ARNm demeure une question épineuse du domaine de la traduction chez les eukaryotes. Le facteur eukaryotique d'initiation de traduction eIF4B est l'une des protéines essentielles à ce processus. eIF4B contribue à la liaison du ribosome à l'ARNm en stimulant l'activité ARN-hélicase du facteur de traduction eIF4A. Le déroulement de structures secondaires d'ARN par le tandem eIF4A/eIF4B crée une aire d'ARN simple-brin dans la partie 5' non-codante de l'ARNm, accessible au ribosome. Nous avons étudié le mode d'action d'eIF4B par le biais d'une analyse structurelle et fonctionelle de ce facteur. L'extrémité carboxy d'eIF4B possède un site d'adhésion non-spécifique à l'ARN, qui est absolument nécéssaire à la stimulation de l'activité hélicase d'eIF4A. Le motif de reconnaissance d'ARN (RRM) d'eIF4B ne joue pas ou peu de rôle à cet effet. Une région médiant l'homodimérisation d'eIF4B, située entre les acides-aminés 213 et 312 de la protéine, a été identifiée. Ce segment promouvoie la dimérisation indépendamment des autres régions d'eIF4B et est capable d'interagir directement avec la sous-unité p170 d'eIF3. Enfin, le RRM d'eIF4B se lie spécifiquement à des ARNs possédant une tige, une boucle et une adénosine non-conforme dans la tige et s'associe à l'ARNr 18S. eIF4B est capable de se lier simultanément à deux molécules d'ARN différentes. Ainsi, eIF4B possède trois domaines distincts et indépendants: l'extrémité carboxy, qui stimule l'activité hélicase d'eIF4A, le domaine DRYG, qui est responsable de l'homodimérisation et de l'interaction avec la sous-unité p170 d'eIF3, et le RRM, qui s'associe a l'ARNr 18S. Deux mécanismes supplémentaires par lesquels eIF4B stimule la liaison de la sous-unité ribosomale 40S à l'ARNm peuvent être envisagés. eIF4B se lierait simultanément à l'ARNr 18S et à l'ARNm, et eIF4B interagirait directement avec eIF3, déja présent sur le ribosome. Ainsi, eIF4B établit un pont entre l'ARNm et le ribosome.

Foreword

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Preface

The results presented in Chapters 2, 3 and 4 of this thesis have been published or submitted in the following journals:

Chapter 2: Méthot, N., Pause, A., Hershey, J.W.B. and Sonenberg, N. (1994) Translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. Mol. Cell. Biol. **14**, 2307-2316.

I have performed all the technical aspects of this work. Arnim Pause supplied some purified recombinant eIF4A. John W.B. Hershey provided the eIF4B cDNA and polyclonal antisera directed against eIF4B.

Chapter 3: Méthot, N., Pickett, G., Keene, J.D., Sonenberg, N. (1996) In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: The role of the RNA recognition motif. RNA **2**, 38-50.

Dr. Gavin Pickett contributed to this work by performing the selection/amplification experiments against the N250 and NΔ253 fragments of eIF4B. Dr. Pickett also folded several of his selected RNA, as well as many of mine, using the MFOLD program from the GCG package. Therefore, Dr. Pickett responsible for part of Figure 3.1, as well as Fig. 3.2. Dr. Jack Keene is the supervisor of Dr. Pickett.

Chapter 4: Méthot, N., Song, M.-S., Sonenberg, N. (1996). A region rich in aspartic acid, arginine, tyrosine and glycine mediates eIF4B self association and interaction with eIF3. Submitted.

Min Soo Song performed the far western experiment shown in Figure 6.5 A and B. He also performed some of the heart muscle kinase labeling reactions.

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List of Abbreviations

AMV-4	alfalfa mosaic virus 4
ARM	arginine rich motif
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	basic local alignment search tool
cDNA	cloned deoxyribonucleic acid
DNA	deoxyribonucleic acid
DTT	1,4-dithiotreiol
ΔG^o	free Gibb's energy
EDTA	ethylenediamine tetraacetic acid
E. coli	Escherichia coli
EF	elongation factor
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
4E-BP	eukaryotic initiation factor 4E binding protein
FMDV	foot and mouth disease virus
GDP	guanosine diphosphate
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HCR	heme contolled repressor
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMK	heart muscle kinase
hnRNP	heteronuclear ribonuclear protein
HIV	human immunodeficiency virus
IF	initiation factor
IPTG	isopropyl-β-D-thiogalactopyranoside
IRE	iron responsive element
IRF	iron regulatory factor
IRES	internal ribosome entry site
Kd	dissociation constant
kDa	kilodalton
KH	hnRNP K protein homology

met_tRNA_i	initiator methionine transfer RNA
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
NMR	nuclear magnetic resonance
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein kinase RNA-activated
PMSF	phenylmethylsulfonyl fluoride
poly(A)	polyadenylate
RF	release factor
RNA	ribonucleic acid
RNP-CS	ribonucleoprotein consensus sequence
RRM	RNA recognition motif
rRNA	ribosomal ribonucleic acid
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
SELEX	selection of ligands by exponential enrichment
snRNP	small nuclear ribonucleoprotein
TOP	tract of pyrimidines
tRNA	transfer ribonucleic acid
uORF	upstream open reading frame
UTR	untranslated region
UV	ultraviolet

Acknowledgments

Becoming a researcher involves more than the acquisition of technical and cognitive skills. The way to approach a problem, the "philosophy of science" if you will, is a very important aspect and is taught by a few very special people that are met throughout life. I would like to take these lines to thank these special people as well as may others that contributed to the success of my Ph.D. thesis.

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Chapter I

General Introduction

Chapter I: General Introduction

1.1 Perspective

The assembly of large macromolecular complexes on nucleic acids is a common theme for events that control gene expression, such as transcription, RNA cleavage and polyadenylation, RNA splicing and protein synthesis. In eukaryotes, the translation of a mRNA requires the participation of over 200 macromolecules. These include tRNAs, rRNAs, ribosomal proteins, initiation factors, elongation factors and release factors.

Many of the details behind the three major phases of translation - initiation, elongation, and termination - are known today. Interest in protein synthesis has been rejuvenated since it was found that translation can actively contribute to the control of gene expression, cell growth and differentiation. An intimate knowledge of each step of translation and each factor which promotes these steps, must be acquired in order to fully understand translational control. Indeed, the combination of classical biochemical techniques and yeast genetics have already reaped numerous rewards. In parallel, recent advancement in the field of RNA and RNA binding proteins are providing fresh insights into how translation might work.

This thesis aims at examining the close relationship between one translation factor in particular, eIF4B, and other macromolecules, including mRNA, rRNA and proteins. Because of the complexity of protein synthesis, a more detailed summary of translation and a description of some of the key translation factors will be given. It will be followed by a view of translation from the RNA perspective, and an introduction to RNA binding proteins.

1.2 Protein synthesis: a brief overview

The first phase of protein synthesis is referred to as initiation, and consists of the binding of the 40S ribosomal subunit at or near the cap structure (m7GpppN, where N is any nucleotide) that is present at the 5' end of all cytoplasmic mRNAs. According to the

Kozak model (Kozak, 1978), the ribosome thereafter scans vectorially the mRNA until the initiator AUG is found. Once the 40S ribosomal subunit is in place on the initiator codon, it is joined by the 60S subunit to form the 80S initiation complex. The ribosome is now capable of entering the peptidyl transfer and translocation cycles which characterize the elongation phase of protein synthesis.

Our understanding of elongation and termination mainly originates from prokaryotic studies (for reviews, see Moldave, 1985; Slobin, 1990). However, since several components of the protein synthesis machinery are highly homologous between prokaryotes and eukaryotes (i.e. EF-Tu and eEF1A; EF-G and eEF2; 16S rRNA and 18S rRNA; 23S rRNA and 28S rRNA), elongation and termination are believed to proceed in a very similar fashion in both systems.

The elongation phase of protein synthesis requires eukaryotic elongation factor (eEF) 1A, complexed to GTP and an aminoacyl-tRNA, to bind to the ribosome in a codon-dependent manner. The correct match between the codon and the tRNA anticodon triggers the hydrolysis of GTP, the ejection of eEF1A-GDP and the placement of the aminoacyl-tRNA in the A site of the ribosome. Peptide bond formation results from a nucleophilic attack by the α amino group of the aminoacyl-tRNA in the A site, on the carbonyl of the aminoacyl-tRNA (or peptidyl-tRNA) in the P site. This reaction is most likely catalyzed by both rRNA and ribosomal proteins in the peptidyl transferase center of the 60S ribosomal subunit (Noller et al., 1992). Following the reaction, the peptidyl-tRNA is now in the A site, while the P site contains a stripped tRNA.

The GDP moiety bound to eEF1A must be exchanged for GTP in order for eEF1A to promote another round of aminoacyl-tRNA binding. The exchange is catalyzed by eEF1B. Translocation, which results in the movement of the mRNA by three nucleotides, is facilitated by eEF2 and GTP. This results in the placement of the stripped tRNA in the E site, from which it will be ejected. The peptidyl-tRNA is relocated to the P site, while the A site is vacant and ready to accept a new aminoacyl-tRNA.

Protein synthesis is completed when eRF1 bound to GTP recognizes one of the three termination codons exposed in the A site. eRF induces the hydrolysis of the aminoacyl linkage, and the newly synthesized protein, as well as eRF-GDP, are released.

1.3 Initiation of translation

Prokaryotic and eukaryotic elongation, as well as termination, bear many similarities. Initiation of translation, on the other hand, proceeds quite differently in eukaryotes, especially at the mRNA-ribosome binding step (reviewed by Kozak, 1983; Voorma, 1996).

Prokaryotic initiation of translation requires three initiation factors (IFs). IF2 binds to fmettRNA$_i$ and GTP, and takes position in the P site of the 30S ribosomal subunit. IF3 binds to the 30S ribosomal subunit where it acts as an anti-association factor. In addition, IF3 ensures that the correct AUG codon is used as the initiation site by destabilizing interactions between the anticodon loop of fmettRNA$_i$ and non-AUG codons (Gualerzi & Pon, 1990). The function of IF1 is unclear. None of the three prokaryotic initiation factors are required for the actual binding of the mRNA, which is uncapped, to the 30S ribosomal subunit. mRNA-30S ribosome association is largely mediated by base-pairing between the Shine-Dalgarno (SD) sequence present in the 5' UTR of most prokaryotic mRNAs, and the anti-Shine-Dalgarno sequence (ASD) located at the 3' end of the 16S rRNA (Shine & Dalgarno, 1975). Ribosomal protein S1 has also been speculated to help the recognition process by binding a polypyrimidine-rich region that is often found upstream of the SD sequence in *E. coli* mRNAs (Gribskov, 1992). Since the SD sequence is the primary determinant for ribosome binding, prokaryotic ribosomes can bind mRNAs internally. This is in stark contrast with eukaryotic ribosomes which, except under the circumstances described in section 1.6.4, bind at or near the 5' cap structure of mRNAs. Although 5' UTR secondary structure is inhibitory to prokaryotic protein synthesis, no RNA unwinding system has evolved since translation starts before transcription has ended. Thus, secondary structure masking the SD sequence is less likely to form.

In contrast, the binding of eukaryotic mRNAs on the 40S ribosomal subunit cannot occur in the absence of accessory proteins, and requires the participation of at least 11 eukaryotic initiation factors (eIFs). It is primarily through biochemical methods - fractionation of the protein synthesis machinery and reconstitution with purified components - that the step-wise assembly of the initiation complex and the function of various factors were elucidated (Safer et al., 1976; Trachsel et al., 1977; Benne & Hershey, 1978). A list of eukaryotic translation initiation factors and their activities is given in Table 1.1.

Events leading to initiation of translation occur on two parallel pathways which converge when the 40S ribosomal subunit binds to the mRNA (For reviews, see Hershey, 1991; Merrick, 1992; Merrick & Hershey, 1996). The ribosome must be rendered competent for interaction with the mRNA, and the mRNA must be made accessible for ribosome binding. The steps that lead to the assembly of an initiation complex on a mRNA are depicted in Figure 1.1.

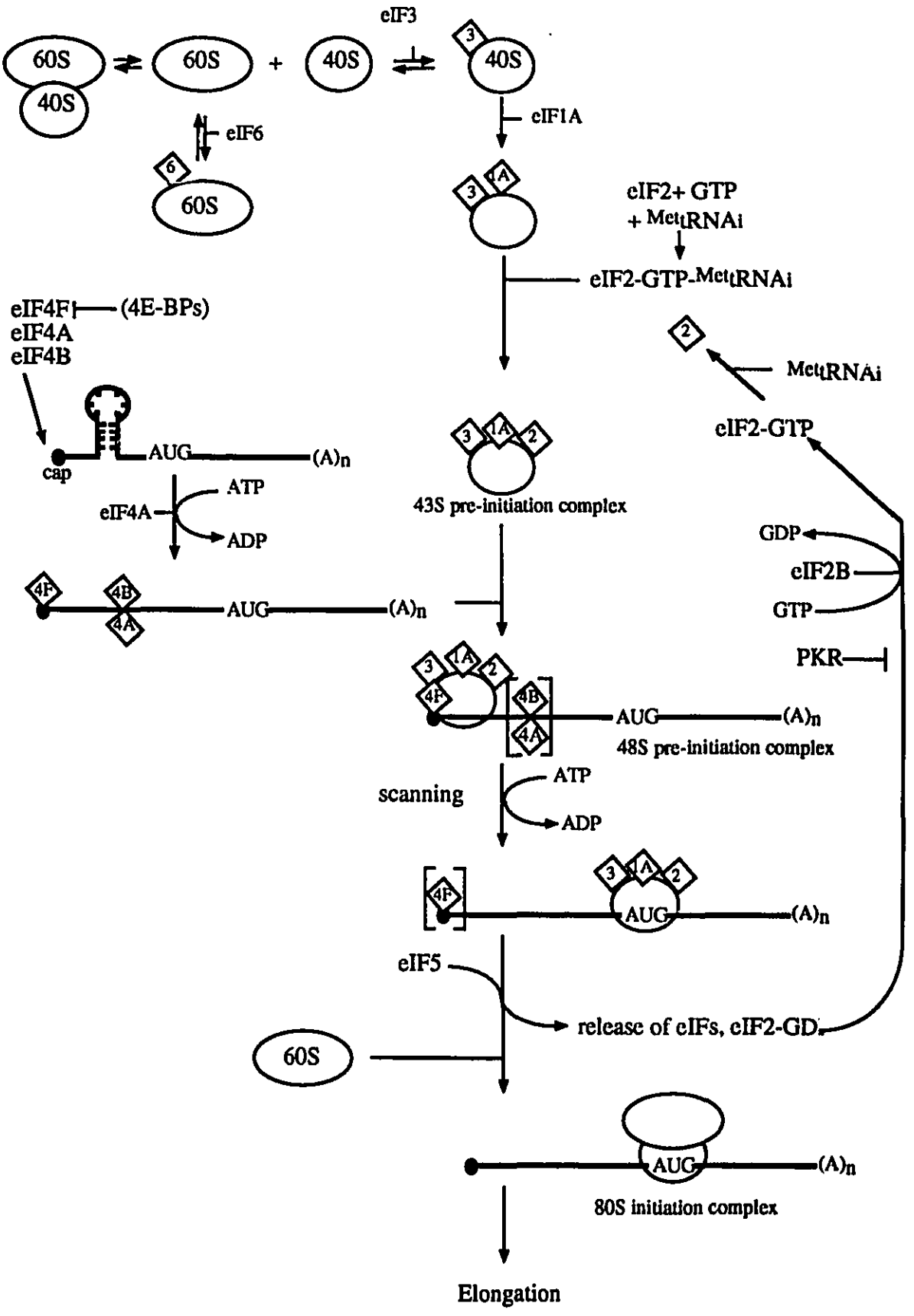
1.3.1 Subunit dissociation

The first step of translation initiation involves the generation of a sufficient pool of free 40S and 60S ribosomal subunits. The conditions that govern the dissociation of an 80S ribosome into its components are poorly defined. On the other hand, it has been shown that several initiation factors help to maintain 40S and 60S subunits apart once they have separated. eIF1A is a small (17-22 kDa) protein whose amino acid sequence is well conserved between mammals, plants and yeast (Dever et al., 1994; Wei et al., 1995). eIF1A binds the 40S ribosomal subunit and prevents its association with the 60S subunit (Goumans et al., 1980). This function is shared by eIF3 (Goumans et al., 1980) a

Table 1.1 Mammalian Translation Initiation Factors (adapted from Merrick & Hershey, 1996)

Name	Subunits	Mass (kDa)	Function
eIF1		15	enhances initiation complex formation
eIF1A		17	promotes ribosomal subunit dissociation and $Met tRNA_i$ binding
eIF2	3		binds $Met tRNA_i$ and GTP
	α	36	site of phosphorylation on Ser-51, major point of translational control
	β	37	binds $Met tRNA_i$
	γ	52	binds $Met tRNA_i$ and GTP
eIF2B	5		nucleotide exchange factor for eIF2
eIF2C		94	stabilizes ternary complex on 40S ribosome in the presence of RNA
eIF3	9		anti-association factor. Promotes ternary complex and mRNA binding to the 40S ribosomal subunit
	p35	35	
	p36	36	
	p40	40	
	p44	44	
	p47	47	
	p66	66	binds RNA
	p110	110	
	p115	115	
	p170	170	
eIF4A		46	ATPase and DEAD box RNA helicase
eIF4B		80	binds RNA and stimulates eIF4A RNA helicase activity
eIF4F	3		binds to the cap-structure. RNA helicase activity
	eIF4E	24	mRNA cap-structure binding
	eIF4A	46	ATPase and DEAD box RNA helicase
	eIF4G	220	binds eIF4A, eIF4E and eIF3. Cleaved during poliovirus infection
eIF5		58	promotes GTPase with eIF2 and ejection of eIFs
eIF6		25	binds to 60S ribosomal subunit. Anti-subunit joining activity

Fig. 1.1 Model for eukaryotic initiation of translation (Adapted from Merrick 1992). The major steps of translation initiation and the role played by some of the eukaryotic initiation factors (eIFs) are depicted. Free 40S and 60S ribosomal subunits are generated, with eIF6 acting as an anti-subunit association factor. The 43S pre-initiation complex is formed by binding of eIF3, eIF1A and eIF2-GTP-MettRNA$_i$. Secondary structure in the 5' untranslated region of the mRNA is removed by eIF4F, eIF4B and eIF4A. The 43S pre-initiation complex binds at or near the cap-structure of the mRNA to form the 48S pre-initiation complex. It is not known whether eIF4A and eIF4B [brackets] remain on the 48S complex and if they participate in the next step of initiation, mRNA scanning. Once the initiator AUG has been identified, eIF5 induces the hydrolysis of GTP and the release of eIFs, and the 60S ribosomal subunit joins to form the 80S initiation complex. The GDP moiety on eIF2 is exchanged for GTP by eIF2B. Also shown are negative regulators of translation initiation. PKR phosphorylates eIF2 and prevents the GTP exchange reaction (section 1.4.1). 4E-BPs block translation by binding to the cap-binding protein eIF4E (section 1.4.2).


eIF3
60S
40S
60S
+
40S
40S
eIF6
6
60S
eIF1A
3
1A
eIF2+ GTP
+ MettRNAi
eIF2-GTP-MettRNAi
eIF4F (4E-BPs)
eIF4A
eIF4B
2
MettRNAi
eIF2-GTP
cap
AUG
(A)n
ATP
eIF4A
ADP
3
1A
2
43S pre-initiation complex
GDP
eIF2B
GTP
4F
4B
4A
AUG
(A)n
PKR
48S pre-initiation complex
scanning
ATP
ADP
eIF5
release of eIFs, eIF2-GDP
60S
80S initiation complex
Elongation

multisubunit complex of at least 9 polypeptide chains in mammals, and with an aggregate mass of 600 000 kDa (Benne & Hershey, 1976; Safer et al., 1976; Schreir et al., 1977). eIF3 binds to the 40S ribosome where, in addition to its ability to block subunit association, (Benne & Hershey, 1978; Trachsel et al., 1977), it stimulates the mRNA binding step (Trachsel et al., 1977). eIF6 is the third anti-association factor, and binds exclusively to the 60S ribosomal subunit (Russell & Spremulli, 1979; Raychaudhuri et al., 1984).

1.3.2 Ternary complex formation

As opposed to prokaryotic initiation of translation, in eukaryotes the initiator methionine tRNA (mettRNA$_i$) appears to be charged on the 40S ribosome prior to the mRNA binding step (Schreier et al., 1973; Darnbrough et al., 1973). mettRNA$_i$ gains access to the P site of the 40S ribosomal subunit in the form of a ternary complex with eIF2 and GTP (Safer et al., 1976; Chen et al., 1972). When present at low concentration, the ternary complex is stabilized by eIF3 and eIF2C (Gupta et al., 1990). Association of eIF2-mettRNA$_i$-GTP with the 40S ribosomal subunit is enhanced by eIF1A and eIF3 (Benne & Hershey, 1978; Peterson et al., 1979; Goumans et al., 1980; Garcia-Barrio et al., 1995). At this point, the small ribosomal subunit, to which eIF1A, eIF3 and eIF2·^{met}tRNA$_i$·GTP are associated, is capable of binding an mRNA and is referred to as the 43S pre-initiation complex. The fact that it can be detected in cell lysates indicates that the 43S pre-initiation complex is an intermediate of translation (Darnbrough et al., 1973; Smith & Henshaw, 1975).

1.3.3 mRNA binding

The mRNA must also undergo preparatory steps before it can bind to the 43S pre-initiation complex. There is a wealth of evidence that secondary structure in the 5' untranslated region (UTR) of a mRNA is inhibitory to translation (Pelletier and Sonenberg, 1985; Kozak, 1986; reviewed by Kozak, 1989b). Pelletier and Sonenberg (1985b) have shown that increased secondary structure in the vicinity of the cap (m^7GpppN) renders this

structure less accessible to a number of initiation factors involved in the mRNA binding step. The effect of secondary structure on translation is more deleterious if a stable stem-loop is located near the cap structure (Kozak, 1989b). Furthermore, there is a positive correlation between the amount of secondary structure in the 5' UTR, and a requirement for a cap during translation. Inosine substitution in reovirus RNA, which reduces the stability of RNA secondary structures, resulted in a decreased requirement for the cap structure and ATP hydrolysis in translation initiation (Morgan & Shatkin, 1980; Kozak, 1980). Alfalfa mosaic virus RNA and heat shock proteins mRNAs, all of which have long, unstructured 5' UTRs, were efficiently translated in cells containing inactivated eIF4F, a translation initiation factor required for cap-dependent translation (reviewed by Sonenberg, 1988). These observations led to a model which argues that secondary structure in the 5' UTR must be removed prior to 43S pre-initiation complex binding (Sonenberg, 1988). Removal is believed to be accomplished by a group of initiation factors which bind to the cap of the mRNA, either directly or indirectly. According to this model, eIF4F is the first factor to associate with the cap structure. Mammalian eIF4F is a heterotrimer (Tahara et al., 1981; Edery et al., 1983; Grifo et al., 1983) comprised of: i) eIF4E, a 24 kDa protein which recognizes specifically the cap structure (Sonenberg et al., 1978); ii) eIF4A, an RNA-dependent ATPase (Grifo et al., 1984) and bidirectional RNA helicase (Ray et al., 1985; Rozen et al., 1990); and iii) eIF4G (previously known as p220), a factor essential for cap-dependent translation (Etchison et al., 1984) which may act as a scaffold linking eIF4E, eIF4A and eIF3 (Mader et al., 1995; Lamphear et al., 1995). The binding of eIF4F to the cap positions eIF4A in proximity to its substrate, RNA secondary structure. In conjunction with eIF4B, an RNA-binding protein which stimulates the ATPase and helicase activities of eIF4A, eIF4A is believed to unwind stem-loop structures and creates an area of single-stranded RNA accessible for ribosome binding (Grifo et al., 1982; Ray et al., 1985; Rozen et al., 1990). The 43S pre-initiation complex binds the mRNA at or near the cap structure, and scans the 5' UTR in a vectorial fashion until the initiator AUG is found (scanning

model; Kozak, 1978). In the majority of cases, the first AUG codon encountered is used to initiate protein synthesis (Kozak, 1987). There are, however, exceptions to this rule. The scanning ribosome may overlook an AUG codon which is located less than 10 nucleotides from the cap (Kozak, 1991), or if the AUG codon is found in an unfavorable context (i.e. surrounding sequences; Kozak, 1987). In some rare instances, non-AUG codons such as CUG, ACG and GUG will serve as initiation sites (see Grünert & Jackson, 1994).

1.3.4 AUG codon recognition

The factors which contribute to AUG recognition are ill-defined. Statistical and experimental evidence (Kozak, 1986b; Kozak, 1987; Kozak, 1987b; Grünert & Jackson, 1994) strongly suggest that nucleotide sequences surrounding an AUG codon influence initiator codon choice. The optimal sequence around an AUG is GCCA/GCCAUGG, and is known as the Kozak consensus sequence (reviewed by Kozak, 1991). It is unlikely that rRNA-mRNA interactions play a role in the selection of the initiator codon, although it has been noted that a region in 18S rRNA offers some degree of complementarity to the Kozak consensus (Lagúnes-Otero, 1993). Recognition of the initiator AUG *per se* requires the ternary complex eIF2.$_{met}$tRNAi·GTP. A codon-anticodon interaction between the AUG and $_{met}$tRNA$_i$ is critical (Cigan et al., 1988). Furthermore, in a genetic reversion analysis, it was shown that mutations in the β subunit of eIF2 alter the ribosomal start site selection during the scanning process (Donahue et al., 1988). A similar search for factors contributing to initiator AUG recognition permitted the identification of the yeast protein encoded by the SUI1 gene (Yoon & Donahue, 1992). Sui1 protein is homologous to eIF1 (Kasperaitis et al., 1995). eIF4B has also been suggested as a candidate for AUG codon recognition, based on its ability to bind AUG triplets better than any other variety of triplets tested (Goss et al., 1987). Wheat germ eIF4B, on the other hand, failed to demonstrate this property (Sha et al., 1994). Thus, the involvement of eIF4B in AUG recognition remains unclear.

1.3.5 Subunit joining

AUG codon recognition by the scanning ribosome is followed by joining of the 60S ribosomal subunit to form the 80S initiation complex. It has been proposed, for prokaryotic ribosomes, that complementary sequences in the 16S and 23S rRNA contribute to subunit joining by direct base pairing (Herr & Noller, 1979). Subunit joining is catalyzed by eIF5, which binds to the 40S ribosomal subunit and induces the hydrolysis of the GTP molecule carried by eIF2 (Trachsel et al., 1977; Benne & Hershey, 1978). Some genetic evidence links the poly(A) binding protein (PABP) with an increased efficiency of 60S subunit joining (Sachs & Davis, 1989). Recently however, it was shown that PABP more likely functions at an earlier step of translation initiation, by helping the recruitment of 40S ribosomal subunits to the mRNA (Tarun & Sachs, 1995).

GTP hydrolysis induces the release or eIF2 as well as the other translation initiation factors bound to the 48S pre-initiation complex (Peterson et al., 1979). In order for eIF2 to re-enter another round of initiation, the GDP moiety must be exchanged for GTP. This reaction is catalyzed by eIF2B, an enzymatic complex comprised of 5 subunits (reviewed by Price & Proud, 1994).

1.4 Regulation of translation initiation

In most instances, the rate-limiting step of protein synthesis is initiation. This conclusion is based on several observations (reviewed by Mathews et al., 1996). For example, the rate of initiation for ovalbumin mRNA is one event every 6.5 seconds (Palmiter, 1975). Since an elongating ribosome requires two seconds to vacate the initiation site, it is clear that the rate of initiation of ovalbumin mRNA is less than the maximal theoretical rate. In addition, toeprinting experiments suggest that a ribosome occupies 35 nucleotides on a mRNA. Yet, the spacing between elongating ribosomes in a polysome is 80 to 100 nucleotides. (Reviewed by Mathews et al., 1996). Finally, because the majority

of mRNAs in a cell are resistant to low concentrations of cyclohexamide, an elongation inhibitor, it is believed that the rate of translation of most mRNA is limited by the efficiency of initiation (Lodish and Jacobsen, 1972). Since 48S initiation complexes are less abundant than 43S pre-initiation complexes (Darnbrough et al., 1973), the precise step that appears limiting in protein synthesis is binding of the ribosome to the mRNA or scanning.

Protein synthesis rates can be altered for all mRNAs, or for a specific subset of mRNAs. Modification of initiation factor activity is one of the means by which a cell may regulate translation. Formation of the ternary complex and changes in the activities of the cap binding proteins (namely eIF4E), both of which affect the binding of the 43S pre-initiation complex to the mRNA, are two of the mechanisms by which protein synthesis is regulated.

1.4.1 Ternary complex formation

AUG codon recognition and subunit joining results in the ejection of eIF2-GDP. The latter is incapable of binding $met tRNA_i$ (Walton & Gill, 1975). Furthermore, eIF2 exhibits a 100-fold greater affinity for GDP than GTP (Proud, 1992). Exchange of the GDP moiety for GTP requires eIF2B (reviewed by Price & Proud, 1994). Phosphorylation of eIF2 on serine 51 of the α subunit causes a 150-fold increase in the affinity of eIF2B for eIF2-GDP. eIF2-GDP phosphorylated on S51 therefore acts as a competitive inhibitor and blocks the exchange reaction (Rowlands et al., 1988). This results in a depletion of eIF2 charged with GTP and $met tRNA_i$, and an overall reduction of protein synthesis rates. Three eIF2α–specific kinases have been identified so far (reviewed by Clemens, 1996). PKR (protein kinase RNA-activated) is a double-stranded RNA-activated ser/thr kinase which plays a pivotal role in the interferon-mediated shut-down of protein synthesis during viral infection (Hovanessian, 1989). HCR (heme controlled repressor) phosphorylates eIF2α in the absence of hemin (Chen et al., 1991). GCN2 is a yeast kinase that phosphorylates eIF2 during amino acid deprivation (Wek et al., 1989).

The importance of eIF2α phosphorylation on translation initiation rates, and by extension, on cell growth, is illustrated by the possible tumor-suppressor role of PKR (Koromilas et al, 1992b; Meurs et al., 1993; Barber et al., 1995), and by the oncogenic potential of an eIF2α mutant in which serine-51 has been replace by alanine (Donzé et al., 1995). The mechanism of transformation for mutants of PKR and eIF2α S51A is unknown, and may not necessarily be the same (Barber et al., 1995b).

1.4.2 Cap binding proteins and melting of RNA secondary structure

The activities of factors involved in the melting of RNA secondary structure [eIF4F (eIF4E, eIF4A, eIF4G) and eIF4B] contribute to the regulation of translation rates and cell growth. The RNA unwinding activity associated with translation may be limiting under normal growth condition, since eIF4E (and consequently eIF4F), present at 0.01 to 0.2 molecules per ribosome, is the least abundant of translation initiation factors (Hiremath et al., 1985; Duncan et al., 1987). Other initiation factors such as eIF4B and eIF4G are found at 0.5 molecules per ribosome (Duncan & Hershey, 1983; Duncan et al., 1987), while eIF4A is present at 3 molecules per ribosome (Duncan & Hershey, 1983). Even though eIF4A is the most abundant initiation factor, the eIF4A-related RNA helicase activity is conditional on the amount of eIF4F. The latter is 20-times more effective as a helicase than free eIF4A (Rozen et al., 1990) and there is evidence that eIF4A functions through eIF4F via an exchange mechanism (Yoder-Hill et al., 1993; Pause et al., 1994).

Several experiments demonstrate the critical role of eIF4E in the control of cell growth. Overexpression of eIF4E in NIH 3T3 and Rat-2 cells induced malignant transformation (Lazaris-Karatsas et al., 1990), while in HeLa cells, it deregulated cell growth (de Benedetti & Rhoads, 1990). Microinjection of purified eIF4E and eIF4F into quiescent NIH 3T3 cells altered their morphology (Smith et al., 1990). In contrast, a decrease in the level of eIF4E with anti-sense RNA resulted in lengthened cell division time in HeLa cells (de Benedetti et al., 1991) or a phenotype reversion of ras-transformed cells (Rinker-Schaeffer et al.,

1993). The effects of eIF4E on cell growth are abolished by a mutation of Ser-53 to Ala (Lazaris-Karatzas et al., 1990; De Benedetti & Rhoads, 1990; Smith et al., 1990).

The mechanism by which eIF4E transforms cells is unclear. It has been suggested that elevated eIF4E levels cause an increase in the level of eIF4F helicase activity. This in turn removes a translational block imposed on mRNAs with high amounts of secondary structure in their 5' UTR (Lazaris-Karatzas et al., 1990). Significantly, several proto-oncogene mRNAs possess exceptionally long and G-C burdened 5' UTRs (see Kozak, 1991). Preferential translation of specific mRNAs in eIF4E-transformed cells has been demonstrated for ornithine decarboxylase mRNA (Rousseau et al., 1996). Microinjection of eIF4E mRNA in *Xenopus* eggs also led to the selective translation of activin mRNA without stimulating total protein synthesis (Klein & Melton, 1994).

Alternative mechanisms for eIF4E-induced transformation cannot be ruled out. A portion (20%) of eIF4E localizes to the nucleus (Lejbkowicz et al., 1992), and eIF4E could be involved in nucleocytoplasmic transport of mRNAs. Indeed, a greater fraction of cyclin D1 mRNA localized to the cytoplasm in eIF4E-transformed cells than in control NIH 3T3 cells (Rosenwald et al., 1995; Rousseau et al., 1996).

As with eIF2α, some activities of eIF4E appear to be regulated by phosphorylation. Phosphorylation of eIF4E increases the affinity of this factor for the cap structure (Minich et al, 1994), stimulates the incorporation of eIF4E into the eIF4F complex (Morley et al., 1993; Bu et al., 1993), results in a larger amount of eIF4E associated with the 48S initiation complex (Joshi-Barve et al., 1990), and stimulates protein synthesis *in vitro* (Morley et al., 1990). These observations are consistent with an increased capacity of eIF4E to promote mRNA-ribosome binding when phosphorylated. eIF4E was originally believed to be phosphorylated at serine-53 (Rychlik et al., 1987). Recently, the phosphorylation site has been re-assigned to S-209 (Joshi et al., 1995; Flynn & Proud, 1995; Makkinje et al., 1995; Whalen, Gingras et al., in press). Protein kinase C (Joshi et al., 1995; Whalen, Gingras et al., in press), and protamine kinase (Makkinje et al., 1995) phosphorylate eIF4E at S209

and T210 *in vitro*. Other translation initiation factors such as eIF4G, eIF4B and eIF3 are phosphorylated, and as with eIF4E, an increase in their phosphorylation positively correlates with the overall translational and proliferation status of the cell (Duncan & Hershey, 1984, 1985; Huang & Schneider, 1984; Bonneau & Sonenberg, 1987; Duncan et al., 1987). How phosphorylation affects the activities of eIF4B, eIF4G and eIF3 is unknown at present.

Recently, the identification of eIF4E-binding proteins (4E-BPs) gave new insight to the mechanism of regulation of eIF4E activity (Pause et al., 1994b; Lin et al., 1994). 4E-BPs are small heat and acid-stable proteins which associate with eIF4E and interfere with cap-dependent translation (Pause et al., 1994b). Repression of translation by 4E-BPs is the result of competition between 4E-BPs and eIF4G for binding to eIF4E (Haghighat et al., 1995). The association of eIF4E and 4E-BP1 is affected by the phosphorylation status of 4E-BP1. 4E-BP1 will bind to eIF4E when dephosphorylated on Ser64 (Pause et al., 1994b; Lin et al., 1994).

1.5 Initiation factors required for mRNA unwinding

eIF4E binds to the cap structure of a mRNA and positions eIF4G and eIF4A near potentially inhibitory RNA stem-loops. eIF4G may serve as a scaffold to which eIF4E and eIF4A bind, and may link the 40S ribosomal subunit with the mRNA via a direct interaction with eIF3 (Mader et al., 1995; Lamphear et al., 1995). eIF4A, in conjunction with eIF4B, participates actively in the melting of secondary structure in the 5' UTR.

1.5.1 eIF4A

eIF4A is a 46 kDa protein that is present both as a free form and as part of eIF4F (Edery et al., 1983; Conroy et al., 1990). The use of reconstituted translation extracts demonstrated that eIF4A is required for the binding of the mRNA to the 43S pre-initiation complex (Trachsel et al., 1977; Benne & Hershey, 1978). eIF4A binds ATP (Sarkar et al., 1985;

Rozen et al., 1989), cross-links to oxidized cap structures in an ATP and eIF4B-dependent manner (Grifo et al., 1982; Edery et al., 1983), and hydrolyses ATP in the presence of RNA (Grifo et al., 1984). RNA-dependent ATP hydrolysis is stimulated by eIF4B (Grifo et al., 1984). There appears to be some specificity as to the type of RNA which will induce the ATPase activity of eIF4A, as RNAs lacking secondary structure are better stimulators (Abramson et al., 1987). It is thought that eIF4A is responsible for the ATP requirement during eukaryotic protein synthesis initiation.

Consistent with its role in the ribosome binding step, eIF4A (and eIF4F) possesses bidirectional RNA helicase activity (Ray et al., 1985; Rozen et al., 1990), a property which is also stimulated by eIF4B. eIF4F is 20-times more active than free eIF4A as a helicase (Rozen et al., 1990). The incorporation of eIF4A into eIF4F is due to a direct interaction with the eIF4G component of the cap-binding complex (Lamphear et al., 1995). This association is not strong, as free eIF4A has been shown to exchange with the p46 subunit of eIF4F (Yoder-Hill et al., 1993). In addition, trans-dominant negative studies using mutants of eIF4A that are deficient for RNA binding suggest that free eIF4A does not directly participate in translation , but is rather channeled through eIF4F to melt RNA (Pause et al., 1994). The eIF4A mutants inhibited translation of RNAs devoid of secondary structure, implicating this factor in functions other than unwinding the 5' UTR. Perhaps eIF4A (or eIF4F) alters the secondary/tertiary structure of rRNA during translation initiation.

eIF4A is the prototypical member of the DEAD box family of putative RNA helicases (reviewed by Gorbalenya & Koonin, 1993; Pause & Sonenberg, 1993). These proteins share 9 highly conserved amino acid motifs which include the characteristic asp-glu-ala-asp sequence (DEAD in single letter amino acid code; Linder et al., 1989). DEAD box family members are involved in a wide array of biological processes, such as translation initiation, RNA splicing, ribosome biogenesis, spermatogenesis, oogenesis, cell growth and division (Wasserman & Steitz, 1991). Many of these proteins possess RNA-dependent ATPase

activity, but few have actually been shown to unwind RNA. Among these are p68, a nuclear protein involved in cell growth (Hirling et al., 1989), vasa (Liang et al., 1994), An3, a *Xenopus* oocyte protein (Gururajan et al., 1994), pestivirus NS3 protein (Warrener & Collett, 1995), RNA helicase A (Lee & Hurwitz, 1993), potyvirus CI protein (Lain et al., 1990) and Vaccinia virus NPH-II (Gross & Shuman, 1996). Extensive mutational analysis of eIF4A revealed which amino acids are implicated in ATP binding, ATP hydrolysis, RNA binding and RNA unwinding (Pause & Sonenberg, 1992; Pause et al., 1993). RNA helicase motif I (Ax_4GKT) mediates ATP binding (Rozen et al., 1989; Pause et al., 1992), while motif II (DEAD) is important for ATP hydrolysis (Pause et al., 1992). Motif III (SAT) couples the ATPase with the helicase activity (Pause et al., 1992) while motif VI (HRIGRxxR) is involved in ATP-dependent RNA binding (Pause et al., 1993).

1.5.2 eIF4B

eIF4B is a translation initiation factor which stimulates the ATPase, RNA binding and RNA helicase activities of eIF4A and eIF4F (Grifo et al., 1982; Grifo et al., 1984; Abramson et al., 1988; Ray et al., 1985; Lawson et al., 1989; Rozen et al., 1990). eIF4B has also been reported to favor the binding and release of eIF4E to the cap structure (Ray et al., 1986). These properties help to explain why eIF4B is absolutely required for binding of the mRNA to the ribosome (Trachsel et al., 1977). Finally, eIF4B co-purifies with eIF4F and eIF3 (Etchison & Smith, 1990) and exhibits moderate interaction with purified ribosomes in the presence of eIF4A (Hughes et al., 1993).

The mechanism by which eIF4B stimulates the eIF4A ATPase and helicase activities is unknown. eIF4B binds directly to RNA in an ATP-independent fashion (Grifo et al., 1982) and cross-links to cap-labeled RNAs in the presence of ATP and eIF4F (Edery et al., 1983). Cross-linking of eIF4B to the cap is stimulated by eIF2 (van Heugten et al., 1991). A fluorescence study with a variety of nucleotide triplets indicated some preference of rabbit eIF4B for AUG triplets (Goss et al., 1987). Wheat germ eIF4B showed no such

preference, and its binding to oligoribonucleotides was insensitive to the presence of secondary structure (Sha et al., 1994). Poly(A) was shown to be a good substrate for eIF4B binding (Gallie & Tanguay, 1994), and indeed, inhibition of translation associated with the addition of exogenous poly(A) was reversed by a combination of eIF4A, eIF4B and eIF4F (Gallie & Tanguay, 1994). eIF4B has also been reported to bind 18S rRNA (Brown-Luedi et al., 1978).

The cDNAs for human (Milburn et al., 1990) and yeast (Coppolecchia et al., 1993; Altmann et al., 1993) eIF4B have been cloned. The proteins have a predicted molecular mass of 69 and 49 kDa, respectively, and both contain an RNA Recognition Motif (RRM; see section 1.6.5.5 for details) near the amino-terminus. Human eIF4B has short repeats of aspartic acid, arginine, tyrosine, and glycine residues (D-R-Y-G in single letter amino acid code), and a highly charged carboxy-terminus. Yeast eIF4B, also termed TIF3 or STM1, does not contain a DRYG region but rather possesses a 7-fold repeat of 26 amino acids rich in basic and acidic residues. Disruption of yeast eIF4B is not lethal but causes a slow growth and cold-sensitive phenotype (Altmann et al., 1993; Coppolecchia et al., 1993). The level of identity between human and yeast eIF4B is one of the lowest (17 to 26%, depending on the alignment program) among cloned translation initiation factors (Merrick & Hershey, 1996). The claim that TIF3 is the yeast homologue of mammalian eIF4B is supported by the fact that TIF3 is a high copy suppressor of a temperature-sensitive mutation in yeast eIF4A (Coppolecchia et al., 1993). The RRM of TIF3 is required for the suppressor effect (Coppolecchia et al., 1993). Further, a yeast strain in which TIF3 has been deleted poorly translates mRNAs with secondary structure in their 5' leader sequence (Altmann et al., 1993). Recently, RNA annealing activity for TIF3 and eIF4B has been reported (Altmann et al., 1995), re-opening the debate on the possible involvement of eIF4B in scanning.

eIF4B is a phosphoprotein that is phosphorylated at multiple serine and threonine residues. The level of phosphorylation positively correlates with the translational status of

the cell. eIF4B is less phosphorylated during heat shock (Duncan & Hershey, 1984) and serum starvation (Duncan & Hershey, 1985). In vitro, eIF4B is phosphorylated by at least 5 different kinases (S6 kinase, PKC, PKA, CKI and CKII; Tuazon et al., 1989). eIF4B phosphorylation increases in response to insulin, phorbol ester treatment (Morley & Traugh, 1990; Morley & Traugh, 1993) and epidermal growth factor (Wolthuis et al., 1993). It is not known whether phosphorylation affects the activity of eIF4B.

1.6 RNA and RNA binding proteins during translation initiation

Not all mRNAs are translated with the same efficiency, with a 100-fold range among cellular mRNA (Rhoads et al., 1994). Features both in the 5' and the 3' untranslated region influence the ability of a mRNA to be translated.

1.6.1 The 5' untranslated region

1.6.1.1 The cap structure

All cytoplasmic mRNAs are capped. Apart from its role in mRNA splicing (Edery & Sonenberg, 1985), nucleocytoplasmic transport (Hamm & Mattaj, 1990) and RNA stability (Furuichi et al., 1977), the cap structure increases the efficiency of translation *in vitro* (Shatkin, 1976; reviewed by Sonenberg, 1988) and *in vivo* (Gallie, 1991). Capped mRNAs are translated more efficiently than their uncapped counterparts in cell-free translation extracts (Both et al., 1975) or when electroporated into cells (Gallie, 1991). Capped mRNAs bind more efficiently to ribosomes (Both et al., 1976).

Picornaviruses produce uncapped mRNAs with long, highly structured and AUG-burdened 5' UTRs. The initiation of translation of picornavirus mRNA occurs via a cap-independent mechanism by internal entry of the 40S ribosomal subunit on the mRNA (Pelletier & Sonenberg, 1988; Jang et al., 1988; reviewed by Jackson & Kaminski, 1995). The translation of mRNAs with long unstructured 5' UTRs shows a reduced requirement for the cap (Gehrke et al., 1983).

1.6.1.2 The initiation codon

In 90-95% of cases, the first AUG codon encountered by a scanning ribosome is used as the initiation site (Kozak, 1989). However, the position and the context surrounding the initiator codon influence the efficiency of translation initiation at the correct site. As mentioned in section 1.3.3, data from statistical and systematic mutagenesis studies suggest that in vertebrates, the sequence GCCA/GCCAUGG is optimal for translation initiation (initiation codon underlined; Kozak, 1986, 1987). Recently, it has been shown that the immediate downstream codon influences the efficiency of utilization of the initiator codon, particularly in the case of non-AUG initiators (Grünert & Jackson, 1994). The strongest determinants of recognition efficiency are at position -3 and +4, (numbered relative to the adenine residue, which occupies position +1) which ideally should be occupied by purines (Kozak, 1987b; Kozak, 1986). These rules do not apply to yeast, as AUG recognition is not dependent on context (Cigan et al., 1988b).

Poor context may be responsible for a phenomenon termed "leaky scanning", whereby a 40S ribosomal subunit bypasses the first AUG and initiates at the next one. Several viral mRNAs (reviewed by Kozak, 1986c) are capable of producing two, sometimes three overlapping proteins by overlooking at a certain frequency the first weak AUG codon. Cellular mRNAs may also produce two proteins from a single mRNA species, presumably by leaky scanning. Such is the case for LAP, a transcriptional activator, and LIP, a transcriptional repressor. Both proteins are identical except that LIP lacks part of the amino-terminus (Descombes & Schibler, 1991). In other examples, N-terminally extended pim-1 and int-1 proteins are produced by alternative initiation of translation at CUG and AUG codons (Acland et al., 1990; Saris et al., 1991).

The position of the initiator codon relative to the cap-structure and to other AUG codons in the 5' UTR also influence the fidelity and efficiency of translation initiation. For instance, if the initiator AUG is less that 10 nucleotides downstream from the cap, the

ribosome bypasses it at a high frequency to initiate at the next downstream site (Kozak, 1991).

The presence of in frame AUG codons between the cap structure and the initiator AUG, creates short upstream open reading frames (uORFs) which reduce the efficiency of translation of an mRNA. However, synthesis of proteins having short uORFs in their mRNA 5' UTR may take place by re-initiation. During this process, translation-termination of the first uORF results in 60S subunit dissociation. The 40S ribosomal subunit remains bound to the mRNA and resumes scanning until the next AUG is reached. Alternatively, the 80S ribosomal subunit may remain on mRNA and scan. Re-initiation is usually rare, and the principles that govern its efficiency are not understood. In the case of yeast, re-initiation is used as a means to control the expression of GCN4. The latter is a transcription factor that directs the expression of genes required for amino acid biosynthesis (reviewed by Hinnebusch, 1996). Under normal growth conditions, translation of GCN4 mRNA is repressed by four uORFs. During amino acid starvation translation of GCN4 mRNA is upregulated via a mechanism which involves the phosphorylation of the α subunit of eIF2 by the GCN2 kinase. Phosphorylation of eIF2 ultimately leads to a reduction in the pool of eIF2·mettRNA·GTP complex. Thus, binding of a ternary complex to a 40S ribosomal subunit would occur less often, allowing the scanning ribosome to bypass uORF 2, 3 and 4. Acquisition of a ternary complex while the 40S ribosomal subunit transits between uORF 4 and the authentic start sites allows re-initiation and the synthesis of GCN4..

For certain mRNAs (five reported so far), the peptide encoded by the short uORF plays an active role in the inhibition of translation (see Geballe, 1996).

1.6.1.3 Secondary structure

The presence of stem-loop structures in the 5' UTR is inhibitory to translation (Pelletier & Sonenberg, 1985). Both the stability and the position of the hairpin influence the extent of inhibition. A moderately stable hairpin (ΔG= -30 kcal/mol) impairs translation if positioned near the cap, but has no effect if located 52 nucleotides downstream (Kozak, 1989). On the other hand, stable hairpins (ΔG= -61 kcal/mol) are deleterious to translation, irrespective of their position in the 5' UTR (Kozak, 1989). Secondary structure near the cap decreases 40 S ribosomal subunit binding (Pelletier & Sonenberg, 1985; Kozak, 1986), an effect which correlates with a reduction in initiation factor binding to the cap structure (Lee et al., 1983; Pelletier & Sonenberg, 1985b). The interpretation of these results is that ribosomes require single-stranded RNA near the cap structure to enter on the mRNA. Once bound, the scanning ribosome unwinds moderately stable secondary structure, but stalls when it encounters stable hairpins (Kozak, 1986, 1989). Surprisingly, hairpins located immediately downstream of the initiator AUG stimulate translation, especially if the nucleotide context around the AUG is unfavorable (Kozak, 1990).

Secondary structure (as well as primary sequence elements) may serve as binding sites for specific RNA-binding proteins. When bound to the 5' UTR, these proteins inhibit translation initiation. A well documented case is ferritin mRNA, whose translation is regulated by ceilular iron content (reviewed by Klausner et al., 1993; Rouault et al., 1996). The 5' UTR of ferritin mRNA contains a small stem-loop structure, the iron regulatory element (IRE), which serves as a specific binding site for a 100 kDa protein, the iron regulatory factor (IRF). Under iron-depleted conditions, IRF binds to the IRE and blocks translation of ferritin mRNA. The repression is dependent on the position of the IRE, which must be within 40 nucleotides from the cap-structure (Goosen & Hentze, 1992). The translational block is not dependent on the IRE/IRF relationship, as other RNA-binding proteins, such as U1A snRNP and bacteriophage MS2 coat protein, will inhibit translation, provided their high affinity RNA binding site is located near the cap (Stripecke & Hentze,

1992). The mechanism of translational inhibition involves a reduction in the amount of 43S complex associated with the mRNA cap (Gray & Hentze, 1994). It is not known yet if binding of the IRF to IRE impedes the accessibility of the cap to eIF4F and eIF4B.

Similarly, the translation of a group of spermatocyte RNA appears to be controlled temporally by an RNA sequence in the 5' UTR. The regulatory effect is dependent on the position of this motif, which must be near the cap (Shäfer et al., 1990). Proteins that bind to the regulatory element have been identified by UV crosslinking (Kempe et al., 1993).

Secondary structure may act as a regulatory element during oogenesis and early development. When injected into *Xenopus* oocytes, the translation of a CAT reporter mRNA bearing secondary structure in its 5' UTR was repressed. At fertilization, however, its translation was considerable stimulated without apparent changes in mRNA stability. An increase in RNA helicase activity at fertilization was postulated as a mechanism to explain this phenomenon (Fu et al., 1991). In another study, insulin stimulated the translation of a reporter mRNA bearing the GC-rich 5' UTR of ornithine decarboxylase mRNA. It was suggested that insulin treatment promotes the preferential translation of mRNAs with structured 5' UTRs by activating initiation factors involved in RNA unwinding (Manzella et al., 1991). In light of the fact that insulin promotes the phosphorylation (and hence the inactivation) of 4E-BP1 (Pause et al., 1994; Lin et al., 1994), this mechanism seems plausible.

All vertebrate ribosomal protein mRNAs described to date, as well as eEF1A and eEF2 mRNAs, contain a polypyrimidine tract (TOP; tract of pyrimidines) immediately downstream of the cap structure. This motif is implicated in translational regulation (Levy et al., 1991). The mechanism of control appears to involve phosphorylation of ribosomal protein S6. Following mitogenic stimulation, translation of eEF1A mRNA is selectively up-regulated, as shown from the re-distribution of transcripts from mono-disomes into polysomes (Jefferies et al., 1994). Blockage of S6 phosphorylation with the drug rapamycin results in a selective down-regulation of translation of eEF1α mRNA, and other

transcripts containing the TOP motif. This effect is dependent on the presence of the polypyrimidine tract immediately downstream of the cap (Jefferies et al., 1994b). Based on these data, it has been suggested that S6 phosphorylation leads to the preferential translation of the TOP class of mRNAs (Jefferies et al., 1994b).

There are a few examples of autoregulatory feedback mechanism, whereby binding of a protein to sequences in the 5' UTR and the coding region of its own mRNA blocks translation. Such is the case for thymidylate synthase (Chu et al., 1991), dihydrofolate reductase, (Chu et al., 1993)(Chu, 1993) poly(A) binding protein (de Melo Neto et al., 1995) and p53 (Mosner et al., 1995).

If extensive 5' UTR secondary structure can be employed as a means to reduce the translational efficiency of certain oncogenes (Kozak, 1991c), mutations that either increase or decrease secondary structure may have significant consequences on cell function. For instance, several breast cancer cell lines express a novel transforming growth factor β transcript which lacks much of the long 5' UTR. The shortened leader supports translation that is 7-fold more efficient relative to the wild type sequence (Arrick et al., 1994). In another example, the expansion of a CGG repeat in the 5' UTR of the FMR1 gene of a mildly affected fragile X syndrome patient considerably reduces FMR1 protein levels without affecting FMR1 mRNA stability (Feng et al., 1995).

1.6.2 The 3' untranslated region

The 3' UTR plays a crucial role in mRNA metabolism since it contains signals that affect mRNA stability, localization, polyadenylation and translation efficiency (reviewed by Jackson, 1993). Meiotic maturation, development and differentiation are particularly rich in translational control events mediated by the 3' UTR.

There are numerous examples which demonstrate that a specific protein/RNA interaction in the 3' UTR of a mRNA regulates initiation of translation in a fashion that is independent from changes in mRNA stability (reviewed by Standart & Jackson, 1994).

LOX (erythroid 15-lipoxygenase) mRNA is synthesized and stored as an untranslated mRNA particle until reticulocytes mature into erythrocytes. The LOX mRNA 3' UTR contains 10 tandem repeats of a motif which serves as a specific binding site for a 48 kDa protein. Association of this protein with the 3' UTR inhibits the translation of LOX mRNA at the initiation step (Ostareck-Lederer et al., 1994). A similar temporally regulated inhibition of translation, which is dependent on the specific binding of a protein to RNA sequences in the 3' UTR is seen with protamine mRNA (Kwon & Hecht, 1991). Regulation of translation by sequences in the 3' UTR contributes greatly to the establishment of asymmetry in *Drosophila* and *C. elegans* embryos. Nanos and glp-1 mRNA translation occurs only in specific areas of the embryo, an effect which is mediated by the 3' UTR (Gavis & Lehman, 1995; Evans et al., 1994). Nanos itself, which is a posterior patterning determinant in *Drosophila*, acts as a translational repressor for hunchback (*hb*) mRNA through nanos-response-elements (NRE) located in the 3' UTR of *hb* mRNA (Wharton & Struhl, 1989). Nanos does not bind directly to NRE, but rather exerts its effect via another protein, pumilio (Murata & Wharton, 1995). Recently, the homeodomain protein *bicoid* was shown to repress the cap-dependent translation of *caudal* by binding specifically to sequences in the 3' UTR of *caudal* mRNA (Dubnau & Struhl, 1996; Rivera-Pomar et al., 1996).

How is translation initiation blocked from the 3' UTR? It is possible that a protein binding to the 3' UTR reduces access to the cap-structure by either interacting directly with cap-binding proteins, or indirectly through serial protein-protein interactions. Alternatively, this protein may serve as a nucleation site for the formation of an inactive mRNP particle.

Translation initiation is strongly affected by the polyadenylation status of the mRNA as changes in poly(A) tail length often result in changes in translation initiation efficiency. Such is the case for the activation of maternal mRNAs (for review, see Standart & Jackson, 1994). Signals which modulate polyadenylation lie in the 3' UTR. Again, how the poly(A)

tail enhances translation is unknown, but there appears to be a requirement for the poly(A) binding protein (Tarun & Sachs, 1995).

1.6.4 Alternative modes of translation initiation

The inhibitory effects of secondary structure and/or short uORFs can be avoided by the use of two alternative modes of initiation of translation.

In internal initiation, the ribosome binds to the mRNA independently of the cap, at an internal site within the 5' UTR. The internal entry of the ribosome is dependent on RNA sequence elements and on proteins that bind to these elements (reviewed by Jackson & Kaminski, 1995). The translation of picornavirus RNAs, which are uncapped and which possess long, highly structured 5' UTRs proceeds by internal initiation. Conserved nucleotides and secondary structure within the internal ribosome entry site (IRES) are critical. Non-canonical translation factors such as the La autoantigen and the polypirimidine tract binding protein (PTB) also participate in internal initiation. Both factors can cross-link to picornavirus IRES (Meerovitch et al., 1993; Kaminski et al., 1995). Depletion of PTB from cell-free translation extracts abrogated the translation of IRES-dependent mRNAs without affecting translation of other mRNAs (Kaminski et al., 1995). Addition of La to rabbit reticulocyte lysates increased the accuracy of translation initiation of poliovirus RNA (Svitkin et al., 1994). Internal initiation for cellular proteins such as BiP (Macejak & Sarnow, 1991), *Drosophila* Antennapedia (OH et al., 1992), basic fibroblast growth factor (Vagner et al., 1995) and eIF4G (Gan & Rhoads, 1996) has been reported.

The second alternative mechanism is termed "ribosome shunt". Introduction of a very stable stem-loop in the 5' UTR of the cauliflower mosaic virus RNA had no inhibitory effect on translation, even though initiation required the presence of the cap. The results suggested that the ribosome binds to the mRNA at the cap but bypasses the inhibitory stem-loop during scanning (Fütterer et al., 1993).

1.6.5 RNA binding proteins

RNA binding proteins are involved in a wide variety of cellular functions, such as antitermination (examplified by HIV Tat and lambda phage N protein), mRNA cleavage and polyadenylation (Cleavage and Polyadenylation Specificity Factor or CPSF, Cleavage stimulation Factor, or CStF, poly(A) polymerase), RNA stability (Iron Regulatory Factor, poly(A) binding protein or PABP), RNA nucleocytoplasmic transport (hnRNPs shuttling proteins, HIV rev), RNA localization (staufen) and translation (eIF4B, eIF4G, PABP). The preceding sections gave numerous examples on the requirement for RNA binding proteins in development. In other processes, the role played by RNA-binding proteins is not always evident. For instance, in Fragile X syndrome, one of the most prevalent genetic forms of mental retardation, the protein product of the fragile X gene FMR1 is an RNA binding protein of unknown function (Siomi et al., 1993; Ashley et al., 1993; Siomi et al., 1994). RNA binding proteins have been shown to associate with src, vav and rasGAP, and may thus participate in signal transduction (Taylor & Shalloway, 1994; Bustelo et al., 1995).

RNA can adopt complex secondary and tertiary structures, and consequently, proteins that bind RNA come in a variety of forms. Certain motifs however, are frequently found in RNA binding proteins and in many cases have been shown to contact RNA (for review, see Mattaj, 1993; Burd & Dreyfuss, 1994b).

1.6.5.1 The double stranded RNA binding motif (DSRM)

As its name implies, the double-stranded RNA binding motif is found in a number of proteins that bind double-stranded RNA. Family members include PKR (Green & Mathews, 1992), staufen (St. Johnston et al., 1991), the double-stranded RNA specific adenosine deaminase (O'Connell et al., 1995), TAR RNA binding protein (Gatignol et al., 1993), *E. coli* RNAse III (Kharrat et al., 1995) and RNA helicase A (Gibson & Thompson, 1994). The DSRM is approximately 70 amino acids long. NMR structure of this motif

reveal an αββββα topology (Bycroft et al., 1995; Kharrat et al., 1995). None of the known members of this protein family exhibit sequence specificity.

1.6.5.2 The Arginine-Rich Motif (ARM)

Found in some viral and ribosomal proteins, the Arginine-rich motif is characterized only by an abundance of arginine residues within a short (10-20 amino acids) sequence (Lazinski et al., 1989). HIV Tat and rev are two well-studied ARM-containing proteins which bind specifically and with high affinity to their RNA targets. The structure of the ARM motif of these two proteins are different (Tan et al., 1993) and it is probable that most ARM proteins do not share common structures.

1.6.5.3 The RGG box

Defined as closely spaced arg-gly-gly repeats interspersed between other, often aromatic amino acids, the RGG box usually occurs in proteins that contain other types of RNA binding motifs. Examples of RGG box-containing proteins are hnRNPA1, hnRNPU, FMR1, and nucleolin. The RGG box appears to facilitate RNA binding. Peptides with sequences resembling the RGG box can be modified by methyltransferases to yield dimethylarginine (Najbauer et al., 1993). hnRNP proteins have a high content of dimethylarginine, and it has been suggested that this modification may modulate the RNA binding properties of these proteins (Liu & Dreyfuss, 1995).

1.6.5.4 The KH domain

The prototype member of this RNA binding protein family is hnRNP K, thus the name hnRNP K-homology domain (Siomi et al., 1993). The family now includes ribosomal protein S3, fragile X syndrome protein FMR1 (Siomi et al., 1993), FXR1, a homologue of FMR1 (Siomi et al., 1995) and bicaudal-C (Mahone et al., 1995). The KH domain is

approximately 30 amino acids long and is characterized by conserved hydrophobic residues. A single point mutation at one of these amino acids in the FMR gene of a severely affected fragile X syndrome patient impairs the RNA binding activity of the FMR1 protein (Siomi et al., 1994).

1.6.5.5 The RNA Recognition Motif (RRM)

The RNA Recognition Motif (RRM; also known as RNP motif or RNP-consensus sequence) is the most widely found and best characterized sequence involved in RNA binding (reviewed by Mattaj, 1993; Birney et al., 1993; Burd & Dreyfuss, 1994). Identified in over 200 different proteins to date, the RRM is defined as a loosely conserved region of 80 to 90 amino acids within which an octapeptide, termed RNP1, and an hexapeptide, termed RNP2, form the most distinguishable features. A number of hydrophobic residues interspersed throughout the motif ensure correct folding. The RRM of certain proteins can bind RNA independently of other sequences, while in others, amino acids outside the RRM, or several contiguous RRMs, are needed for wild type RNA binding.

Position 1 of RNP1 is occupied by a basic amino acid (arginine of lysine), while positions 3 and 5 are occupied by aromatic residues (phenylalanine or tyrosine). Mutagenesis of these amino acids in several proteins indicate a critical role for RNA binding or overall function of the protein. For example, substitution of aromatic residues in RNP2 and/or RNP1 reduced or abolished RNA binding for U1A snRNP protein (Scherly et al., 1989; Jessen et al., 1991; Boelens et al., 1991), U2B" snRNP protein (Bentley & Keene, 1991), bacteriophage ø29 connector (Donate et al., 1993), *E. coli* Rho protein (Brennan & Platt, 1991), poly(A) polymerase (Raabe et al., 1994) and hnRNP A1 (Mayeda et al., 1994). Mutations in the RRM can also abolish the activity of the protein, as demonstrated for the yeast MUD2 protein (a putative splicing factor; Abovich et al., 1994), Poly(A) polymerase (Raabe et al., 1994), hnRNPA1 (Mayeda et al., 1994), and splicing factors SF2/ASF and tra-2 (Cáceres & Krainer, 1993; Amrein et al., 1994). However, in the latter

two examples, mutations at the RNP1 aromatic residues did not alter RNA binding activity and it is possible that other regions of the protein contribute to RNA binding.

A critical role for aromatic residues within RNP1 and RNP2 is evident from UV cross-linking studies (Merril et al., 1988; Stump & Hall, 1995). It was suggested that RNA binding is achieved at least in part through ring-stacking interactions between RNA bases and the aromatic amino acids (Kenan et al., 1991). Nuclear magnetic resonance (Hoffman et al., 1991; Görlach et al., 1992; Garrett et al., 1994), crystallographic (Nagai et al., 1990) and co-crystallographic (Oubridge et al., 1994) studies are consistent with this hypothesis. All the RRMs examined at the structural level adopt a $\beta_1\alpha\beta_2\beta_3\alpha\beta_4$ fold, with RNP2 and RNP1 localized within β sheets 1 and 3, respectively. In U1A snRNP, the conserved aromatic amino acids are solvent-exposed (Nagai et al., 1990) and make ring-stacking interactions with some bases of the U1A snRNA (Oubridge et al., 1994). Many of the base-specific contacts, on the other hand, are mediated by a loop between β_2 and β_3 (Oubridge et al., 1994). Indeed, phylogenetic analysis demonstrated that this loop is the most variable region of the RRM (Birney et al., 1993). In domain-swapping experiments, a short amino acid segment between β_2 and β_3 of the U2B" snRNP protein conferred U2A RNA recognition when substituted into the corresponding position in the U1A snRNP protein (Bentley & Keene, 1991; Scherly et al., 1990). These data, combined with the co-crystal structure information, suggest that RNP1 and RNP2 constitute a general RNA binding platform, and amino acids in the variable region of the RRM and at its C-terminus provide specificity determinants (Burd & Dreyfuss, 1994).

Many RRM-containing proteins exhibit RNA binding preferences. The U1A snRNP protein binds specifically to stem-loop II of U1 snRNA and the 3' end of its own mRNA (Scherly et al., 1989; Lutz-Freyermuth et al., 1990; van Gelder et al., 1993), while the 70K snRNP protein binds to stem-loop I of U1 snRNA (Query et al., 1989b). U2B" snRNP protein binds to U2 snRNA, an association which is enhanced in affinity and specificity by U2A' snRNP protein (Scherly et al., 1990). Splicing factors such as U2AF and sxl bind to

polypyrimidine tracts (Zamore et al., 1992; Singh et al., 1995; Samuels et al., 1994; Kanaar et al., 1995). Other putative spicing factors such as hnRNP C and PTB exhibit a similar RNA binding preference (Garcia-Blanco et al., 1989; Görlach et al., 1994). prp 24, hnRNP A1, tra, tra-2, ASF/SF2, poly(A) binding protein, SC35, CStF are all RRM-containing proteins for which a preferential binding site or sequence has been identified (Ghetti et al., 1995; Burd & Dreyfuss, 1994; Hedley & Maniatis, 1991; Tacke & Manley, 1995; Matunis et al., 1993, Takagaki et al., 1992). Thus, it is clear that the RRM is involved in sequence-specific RNA binding.

1.7 Rationale for experimentation

Translation initiation is a highly complex, highly regulated process. The mechanism by which a ribosome recognizes and binds to a mRNA 5' UTR is not well understood, yet, this step is crucial in most cases of translational regulation. Many of the eukaryotic initiation factors, such as eIF4E, eIF4A and eIF2, have been extensively characterized. On the other hand, little information is available on factors such as eIF4B, eIF4G and eIF3.

The absolute requirement for eIF4B during the ribosome binding step, and the ability of eIF4B to bind RNA and stimulate the eIF4A helicase, make this factor a choice study target. Understanding eIF4B may prove key to understanding mRNA binding to the 40S ribosomal subunit. Many questions arise concerning eIF4B: can this factor serve as a link between the ribosome and the mRNA cap structure? Does eIF4B contribute to the scanning process? Can it discriminate between mRNAs and favor the translation of some over others? These questions can not be answered without an understanding of the relationships between eIF4B, RNA and other components of the translational apparatus.

Chapter 2

The translation intiation factor eIF4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence

Abstract

eIF4B is a eukaryotic translation initiation factor that is required for the binding of ribosomes to mRNAs and the stimulation of the helicase activity of eIF4A. It is an RNA binding protein that contains a ribonucleoprotein consensus sequence (RNP-CS)/RNA recognition motif (RRM). We examined the effects of deletions and point mutations on the ability of eIF4B to bind a random RNA, to cooperate with eIF4A in RNA binding and to enhance the helicase activity of eIF4A. We report here that the RNP-CS/RRM alone is not sufficient for eIF4B binding to RNA, and that an RNA binding region, located between amino acids 367 and 423, is the major contributor to RNA binding. Deletions which remove this region abolish the ability of eIF4B to cooperate with eIF4A in RNA binding and the ability to stimulate the helicase activity of eIF4A. Point mutations in the RNP-CS/RRM had no effect on the ability of eIF4B to cooperate with eIF4A in RNA binding, but significantly reduced the stimulation of eIF4A helicase activity. Our results indicate that the carboxy-terminal RNA binding region of eIF4B is essential for eIF4B function, and is distinct from the RNP-CS/RRM.

Introduction

Initiation of protein synthesis in eukaryotes is a complex multistep process leading ultimately to the binding of the small ribosomal subunit to the messenger RNA, and its proper positioning on the initiator AUG (for a recent review, see Merrick, 1992). This event, which is considered rate limiting in translation (Jagus et al., 1981), requires the participation of at least 12 initiation factors. Although it has been studied extensively *in vitro* with purified components, the mechanism by which initiation of translation occurs and is regulated is not well understood. One of the prerequisites for ribosome binding to mRNA is believed to be the melting of secondary structure in the 5' untranslated region (UTR; for reviews see Merrick, 1992; Rhoads, 1991), a process which is dependent on ATP hydrolysis and requires the participation of at least three initiation factors: eIF4F, eIF4A and eIF4B (reviewed in Rhoads, 1988). eIF4F is a heterotrimer composed of eIF4E, a 24 kDa polypeptide which specifically interacts with the mRNA 5' cap structure (Sonenberg et al., 1978), eIF4A, a 50 kDa polypeptide, which is the prototype of the DEAD box family and exhibits RNA-dependent ATPase and bidirectional RNA helicase activity in combination with eIF4B (Grifo et al., 1983; Ray et al., 1987; Rozen et al., 1990; Pause et al., 1993), and p220, whose function is unknown but is essential for the activity of eIF4F (Etchison et al., 1984).

The activities of the components of eIF4F are consistent with models in which eIF4F binds first to the cap and aligns eIF4A in close proximity with the mRNA, where it can initiate the melting of the mRNA secondary structure in an ATP-dependent fashion (for review: Merrick, 1992; Rhoads, 1991). This feature of translation is important since the amount of secondary structure in the 5' UTR of a mRNA influences its efficiency of translation (Koromilas et al., 1992; Kozak, 1986; Pelletier & Sonenberg, 1985). It has also been shown that overexpression of eIF4E in NIH 3T3 results in malignant transformation,

perhaps by relief of translational repression of certain proto-oncogene mRNAs (Lazaris-Karatsas et al., 1990).

The role of eIF4B in translation initiation is ill-defined. This factor has been characterized from mammalian sources as a phosphoprotein of 80 kDa (Benne & Hershey, 1978; Trachsel et al., 1977) whose state of phosphorylation positively correlates with cellular translation rates (Duncan & Hershey, 1984; Duncan & Hershey, 1985; Morley & Traugh, 1990). Although no unique functions have been assigned to it, eIF4B is thought to play a coordinating role during translation initiation (Merrick, 1992). It is absolutely required for mRNA binding to ribosomes (Benne & Hershey, 1978; Trachsel et al., 1977) and considerably stimulates the helicase activity of eIF4A and eIF4F (Lawson et al., 1989; Rozen et al., 1990). Recently, eIF4B has been shown to posses a ribosome-dependent ATPase activity (Hughes et al., 1993). Another possible function of eIF4B is recycling of the eIF4E component of eIF4F (Ray et al., 1986).

The human eIF4B cDNA encodes a protein of 611 amino acids and a predicted molecular mass of 70 kDa (Milburn et al., 1990), with a ribonucleoprotein consensus sequence (RNP-CS)/RNA recognition motif (RRM; for review, see Bandziulis et al., 1989; Haynes, 1992; Kenan et al., 1991; Mattaj, 1993) near the amino terminus. Accordingly, RNA binding has been demonstrated for rabbit eIF4B (Grifo et al., 1984), with some preference for AUG triplets, leading to the suggestion that eIF4B may contribute to initiation codon recognition (Goss et al., 1987).

Purification of eIF4B to homogeneity from mammalian sources has proven difficult. eIF-4B interacts strongly with eIF4F, and preparations are often contaminated with the latter (Grifo et al., 1982;Morley & Traugh, 1990). Furthermore, in most of the assays performed with eIF4B, large amounts of protein were used (Grifo et al., 1983; Grifo et al.,

1984; Lawson et al., 1989; Ray et al., 1985), causing concern as to whether the effects observed could be attributed to contaminants in the eIF4B preparations.

To better understand the function of eIF4B in translation initiation, we have expressed and purified recombinant human eIF4B as a fusion protein with glutathione-*S*-transferase (GST). The effects of deletions and point mutations on RNA binding and helicase stimulatory activities of eIF-4B were studied. Here, we show that the RRM does not account for most of the RNA binding activity of eIF4B, and evidence is provided for the presence of a potent RNA binding region at the carboxy terminal half of eIF4B, between amino acids 367 and 423.

Materials and Methods

Plasmid construction

To introduce the eIF4B cDNA into the BamHI site of the bacterial GST fusion protein expression vector pGEX-3X (Smith & Johnson, 1988), a three-fragment ligation was performed. Fragment 1 consisted of a PCR fragment of the 5' end of the eIF4B cDNA (1-189; Milburn et al., 1990) in which a BamHI site was introduced at position 10 from the 5' end. After amplification, the PCR product was digested with BamHI and BstXI, yielding a fragment spanning nucleotides 10 to 127. Fragment 2 consisted of the remaining portion of the eIF4B cDNA (128-2013), which was obtained by digesting pET3b-4B (Pause et al., 1993) with BstXI and BamHI. Fragment 3 consisted of pGEX-3X digested with BamHI. The resulting vector, pGEX-4B contains the eIF4B cDNA insert (nucleotide 10-2013) flanked by two BamHI sites and in frame with the coding sequence of GST. The expression product is a GST-eIF4B fusion protein of expected molecular mass of 106 kDa. The pGEX-4B BamHI fragment was subcloned into pGEM3 (Promega) to form pGEM3-4B in order to generate some of the deletion mutants.

All mutant eIF4B proteins were expressed as GST-fusion proteins. C-terminal deletion mutants N570, N367, N355, N312, N250 and N171 were obtained by digesting pGEM3-4B with BamHI and one of the following enzymes: BglII, HincII, XhoI, SacI, ClaI or BclI, respectively. The fragments corresponding to the expected sizes were gel-purified and religated into pGEX-3X that had been digested with BamHI. The N464 deletion mutant was obtained by digesting pGEM3-4B with BclI and XhoI, isolating the 338 bp eIF-4B cDNA fragment and ligating it to pGEX-4B that had been digested with XhoI and SmaI. The N423 deletion was obtained by linearizing pGEX-4B with SmaI and digesting with exonuclease III for varying amounts of time. Following Mung Bean nuclease treatment, the DNA was digested with BamHI and blunt ended with T4 DNA polymerase. The truncated fragments were gel-purified and ligated into SmaI-treated pGEX-3X. The N-

terminal deletion (NΔ253) was obtained by linearizing pGEM3-4B with HindIII followed by digestion with Exonuclease III. The DNA was treated as described above and truncated fragments were ligated into SmaI-treated pGEX-3X. Deletion mutants were sequenced at the junction of eIF4B cDNA and vector DNA to confirm the position of the truncation. All mutant fusion-proteins contain 7 to 12 non-eIF4B amino acid residues at the carboxy-terminus, depending on which of the termination codons was provided by the pGEX-3X vector. Site-directed mutagenesis was performed using PCR (Saiki et al., 1988). Amplified fragments were sequenced and subcloned into pGEX-3X.

Protein expression and purification

E.coli BL21 was transformed with pGEX-4B DNA that encodes the wild type form or mutant forms of eIF4B. Overnight cultures (20 ml) were diluted into 1 L in fresh LB containing 50 μg/ml of ampicillin, and grown at 37°C until an OD_{600} of 1.0 was reached. IPTG was added to 0.1 mM and the cells were grown for an additional 90 min, harvested and resuspended in 10 ml of lysis buffer (PBS, 0.2 mM EDTA, 2 mM DTT). Cells were lysed on ice by 6 sonication cycles of 20 sec each. Immediately before sonication, a protease inhibitor mixture consisting of PMSF (1 mM), leupeptin (20 μg/ml), benzamidine (1 mM), aprotinin (50 μg/ml), pepstatin A (10 μg/ml) and soybean trypsin inhibitor (50 μg/ml) was added to the cell suspension. Cellular debris was removed by centrifuging at 40,000 rpm for 30 min in a Ti-60 rotor (Beckman), and the supernatant was incubated 15 min on ice with 1 ml of a 50% glutathione-agarose bead suspension (Pharmacia) equilibrated in wash buffer (PBS, 0.2 mM EDTA, 2 mM DTT, 1% Triton X-100). The beads were pelleted and washed 3X with 15 volumes of wash buffer. Protein was eluted off the beads by washing 3X with 1 ml of 10 mM reduced glutathione (Gibco) in 50 mM Tris-Cl, pH 8.5, and 1X with 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.5, 500 mM KCl. We found that the 500 mM KCl fraction consistently contained a higher ratio of full length to truncated eIF4B. To remove the latter, mutants that retained the first 367

amino acids were further purified on a heparin EconoPak column (Bio-Rad) using an FPLC system (Pharmacia). Elution was performed in buffer A (20 mM Tris-Cl pH 7.3, 2 mM DTT, 0.1 mM EDTA, 10 % glycerol). The truncated forms eluted at 150 mM KCl, while the full length protein eluted between 200 and 300 mM KCl. Mutants N171, N250, N312 and N355 did not contain as much of the degradation products and were not purified further. Mutant NΔ253 was purified on poly(U) Sepharose (Pharmacia) and eluted with Buffer A in 500 mM KCl. Fractions were pooled, concentrated using centriprep-30 concentrators (Amicon) and dialysed in buffer A containing 75 mM KCl. Aliquots were stored at -70°C. Yields varied considerably from mutant to mutant, ranging from 0.5 mg per liter of culture for wt GST-eIF4B, to 10 mg per liter for N250 and N171. Recombinant eIF4A was purified according to Pause and Sonenberg (1992).

RNA synthesis

The RNA substrate used in the RNA binding and the helicase assays was generated using pGEM 3 (Promega) and pGEM MO1/2 vectors (Scheffner et al., 1989). pGEM 3 was linearized with BamHI and transcribed with SP6 polymerase, yielding a 41 nucleotide transcript. pGEM MO1/2 was linearized with HincII and transcribed with T7 polymerase, yielding a 68 nucleotide transcript. The nucleotide sequences of the two strands are as follows: 41 nt strand:
$^{5'}$GAAUACAAGCUUGCAUGCCUGCAGGUCGACUCUAGAGGAUC$^{3'}$, 68 nt strand;
$^{5'}$GGGAGACCGGAAUUCCCCAUGGCUGACUAAUUUUUUUUAUUUA
UGCAGAGGGGGGAUCCUCUAGAGUC$^{3'}$ (duplex region is underlined).
Transcriptions were carried out as recommended by the supplier (Promega) using α^{32}P-GTP (50 μCi; 3000 Ci/mmol) for the 41 nt strand (specific activity 10^5 cpm/pmol). The transcription of the 68 nucleotide strand was performed with unlabeled nucleotides. The two transcripts share a region of complementarity of 14 nucleotides which is flanked by 5' overhangs (see Fig. 2.3A). The transcripts were separated on a 8% denaturing (8 M urea)

polyacrylamide gel and the bands were visualized by autoradiography or U.V. shadowing, excised and eluted for 12 h at 4 °C in 0.4 ml of 0.5 M NH_4OAc, 1 mM EDTA and 0.1% SDS. RNAs were phenol/chloroform extracted and ethanol precipitated. The annealing of the two strands was performed at a 2-fold molar excess of the unlabeled over the labelled transcript in 20 mM Hepes-KOH, pH 7.2, 250 mM NaCl and 1 mM EDTA. The RNA mixture was heated at 95°C for 5 min and slow cooled to 37°C for 2 h to allow for hybridization. The duplex was purified on a 7.5% non-denaturing polyacrylamide gel, visualized by autoradiography, excised and eluted as described above.

GST-eIF4B binding to RNA

GST-eIF4B binding to RNA was measured in a nitrocellulose filter binding assay. GST-eIF4B (1-20 pmol) was incubated for 2 min at 37°C in 40 µl of binding buffer (17 mM Hepes-KOH, pH 7.2, 2 mM DTT, 5% glycerol, 0.5 mM $Mg(OAc)_2$, 75 mM KCl), containing 0.1 mg/ml BSA and 0.18 pmol of ^{32}P-labelled duplex RNA. The mixture was filtered through a pre-wetted nitrocellulose membrane (0.45 µm type HA, Millipore) and the filter was washed with 1 ml of ice-cold binding buffer, air dried for 30 min and counted in a scintillation counter.

Cooperativity between eIF4A and GST-eIF4B in RNA binding was assayed by the nitrocellulose filter binding procedure with minor modifications: GST-eIF4B mutants (2.5 pmol) and recombinant eIF4A (22 pmol) were mixed in 40 µl of binding buffer containing 0.1 mg/ml BSA, 0.5 mM ATP and 0.18 pmol of RNA duplex. The mixture was incubated for 1 min and filtered as described above. All assays were corrected for the fraction of RNA bound in the absence of eIF4B, which typically represented less than 1.5% of the total RNA input.

Results

Expression and purification of GST-eIF4B mutants

eIF4B was expressed as a GST-fusion protein, allowing for a single-step affinity purification using a glutathione-Sepharose column and providing a standard purification scheme for most of the mutants. We were unable to remove the GST portion of the protein with factor Xa, and consequently used the GST-eIF4B fusion protein for all assays. The GST-eIF4B fusion protein exhibited wild type activity for stimulation of eIF4A and eIF4F helicase activity (see below). A series of C-terminal deletions were made by either using conveniently placed restriction enzyme sites or exonuclease III digestion, and one N-terminal deletion mutant (NΔ253) was produced by exo III digestion. Figure 2.1 illustrates the mutants which were purified. The RRM is confined between amino acids 97 and 175, and a region rich in aspartic acid, arginine, tyrosine and glycine (DRYG) spans amino acids 214 to 327 (Milburn et al., 1990). C-terminal deletion mutants are designated according to the C-terminal amino acid retained, while the N-terminal deletion mutant is named according to the last N-terminal amino acid removed. Point mutations in the RRM and an 18 amino acid deletion (amino acids 230 to 247) in the DRYG rich region were also made. All C-terminal deletions retain the entire RRM, except for mutant N171 which lacks the last 5 amino acids, while the N-terminal deletion removes the RRM and a portion of the DRYG region.

Purification of GST-eIF4B using glutathione-Sepharose yielded full length protein as well as smaller polypeptides. These are presumably degradation products of GST-eIF4B as they are immunoreactive towards polyclonal antiserum raised against eIF4B. We have attempted by using various growth conditions to reduce the extent of the degradation, but without success. To enrich for the intact fusion protein, all point mutants and C-terminal deletion mutants that retained the first 367 amino acids were further purified using a heparin

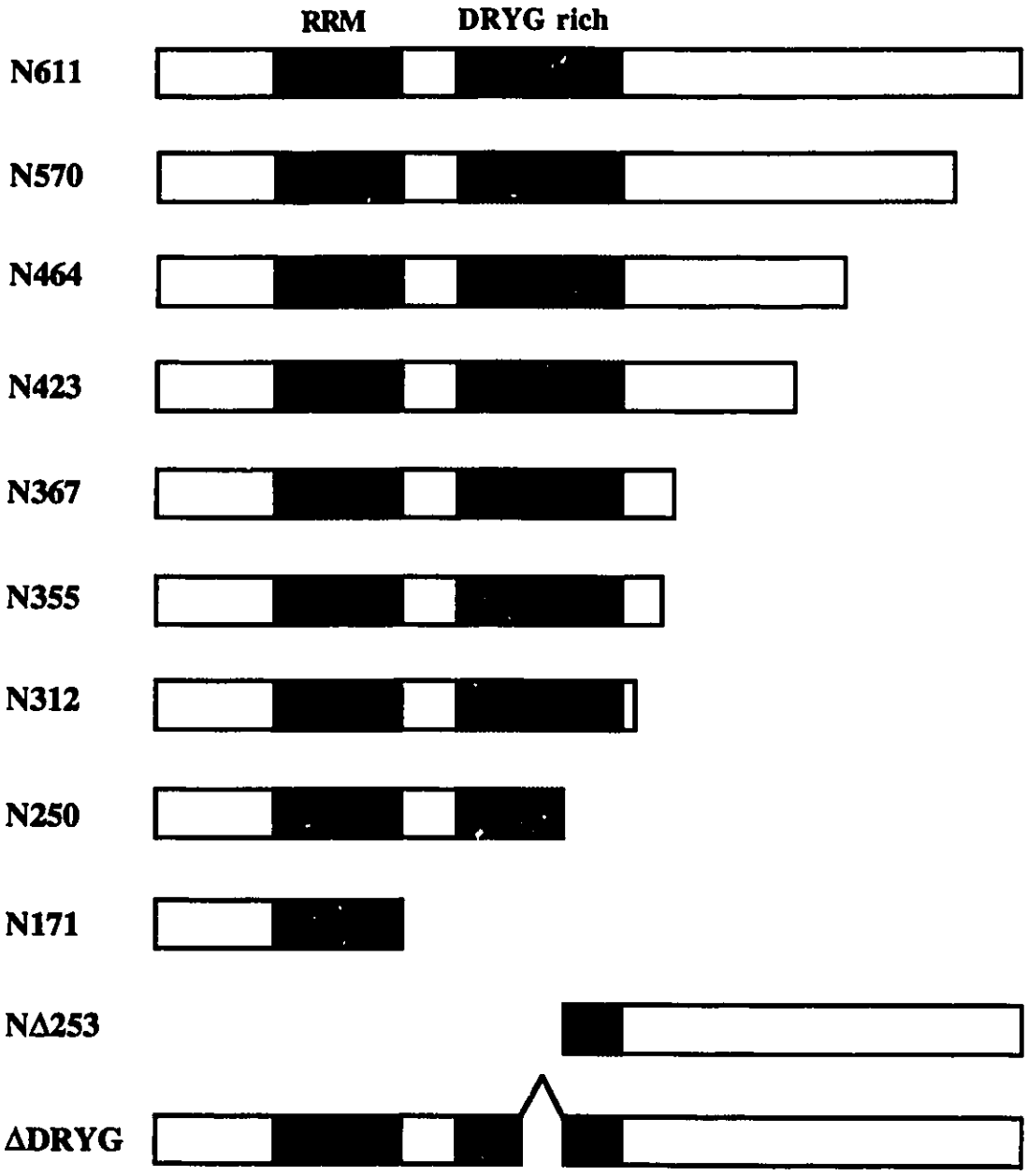


Fig. 2.1. Schematic representation of GST-eIF4B deletion mutants. Wt eIF4B contains 611 amino acids. The RRM is located between amino acids 97 and 175, and a DRYG-rich region is located between residues 214 and 327.

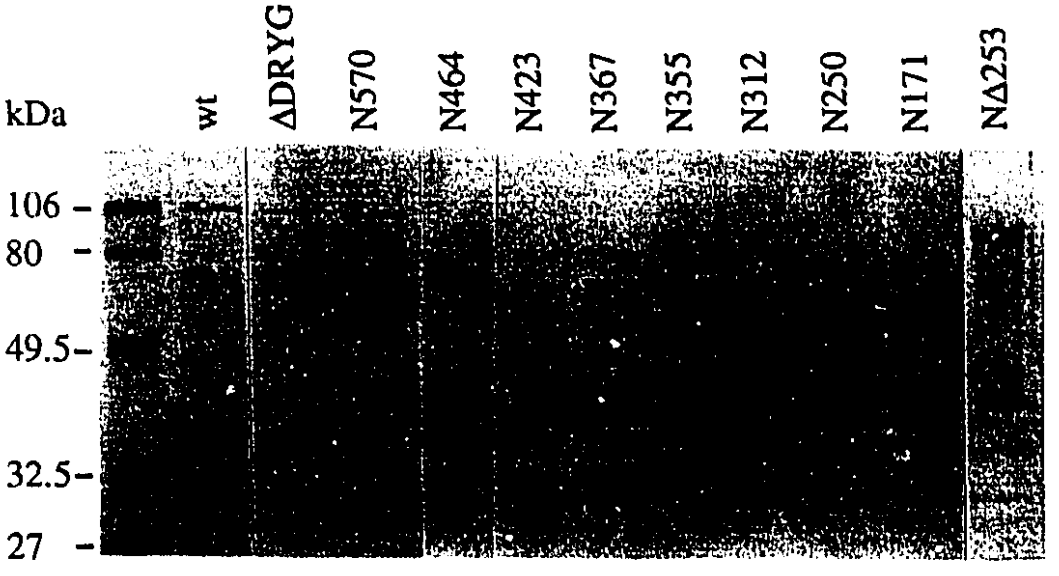


Fig. 2. 2. Polyacrylamide gel analysis of GST-eIF4B deletion mutants. GST-eIF4B wt and GST-eIF4B deletion mutants (approx. 2 µg) were resolved on an 11% SDS-polyacrylamide gel and stained with Coomassie blue.

column. Mutants N171, N250, N312 and N355 did not contain appreciable amounts of degradation products and were not further purified. The N-terminal deletion mutant (NΔ253) did not bind to the column and was further purified using poly-U Sepharose. A Coomassie blue stain of an SDS polyacrylamide gel of the eIF4B deletion mutants which were used in all assays is shown in Fig. 2.2. Wild-type GST-eIF4B migrates at a molecular mass of 106 kDa, as expected from the combined mass of GST and eIF4B (Fig. 2.2, wt). Note that the purified preparations still contain degradation products (mutant NΔ253, in particular, contains a significant amount of a 40 kDa degradation product). However, these appear unlikely to affect the results, as the helicase stimulatory activity of wt GST-eIF4B preparations and homogeneously pure recombinant eIF4B (Pause et al., 1993), containing the same amount of full length protein (as determined by Western blotting), were comparable (data not shown). The doublet migrating at 30 kDa is GST as it co-migrates with purified GST and does not react with the anti-eIF-4B antibody (data not shown). Because of the presence of degradation products in the eIF4B preparations, equimolar amounts of full length mutant proteins, as determined by Western blotting analysis, were used in all assays.

GST-eIF4B binding to RNA

The affinity of eIF4B mutant proteins for RNA was measured by a nitrocellulose filter binding assay. The RNA substrate consisted of a 14 base pair duplex region flanked by 5' single strand overhangs (Fig. 2.3A). Wild type GST-eIF4B bound 40% of the RNA. A deletion of 40 amino acids from the C-terminus of eIF4B (mutant N570) had no deleterious effect on RNA binding. Further deletions from the C-terminus (mutants N464 and N423) led to a small reduction (40%) in RNA binding (Fig. 2.3B). However, RNA binding activity was significantly reduced for mutants N367, N355, N312 and N250 (4 to 6-fold at maximal binding; Fig. 2.3C). This data indicates that the RRM alone (located between amino acids 97 and 175) is not sufficient to account for most of the RNA binding activity

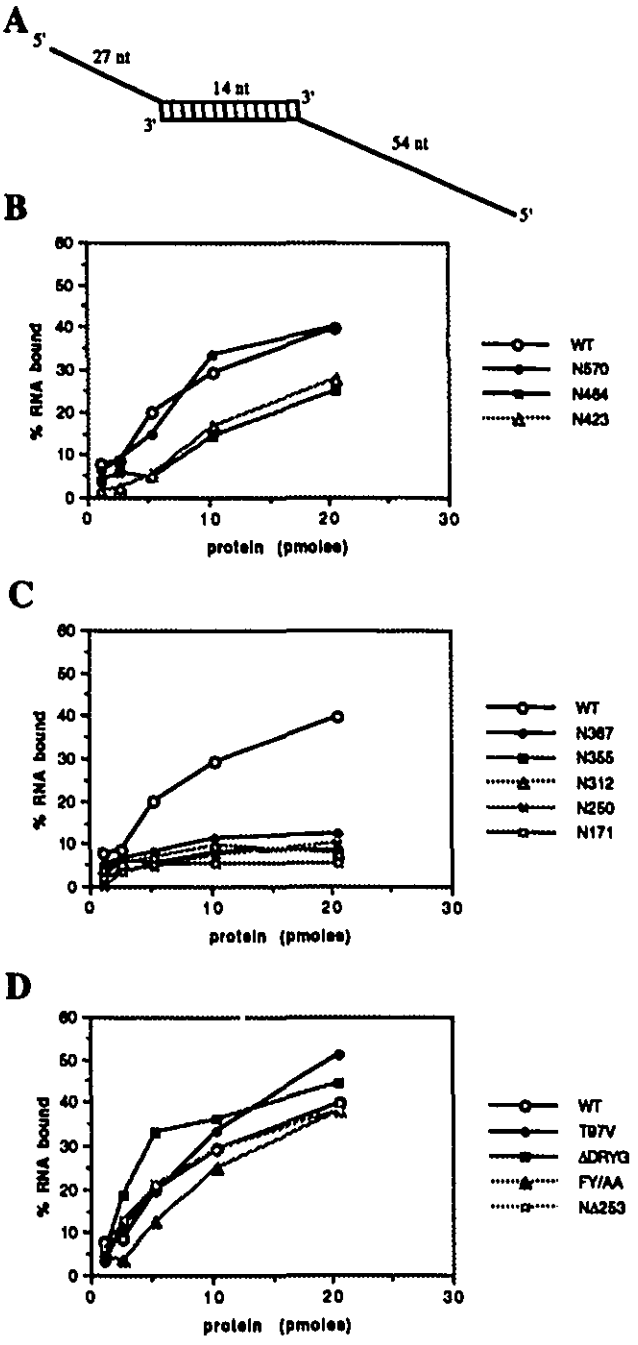


Fig. 2.3. RNA binding properties of GST-eIF4B mutants. A. Schematic representation of the RNA substrate used for the nitrocellulose filter binding assays and the eIF4A/eIF4B-directed helicase assay. The substrate was prepared as described in Materials and Methods and contains a double-stranded region of 14 bp with 5' terminal extensions of single-stranded tails of 27 and 54 nucleotides. B and C. Nitrocellulose filter binding assay with GST-eIF4B mutants. RNA (0.18 pmol) was incubated with increasing amounts of protein. Each point represents the average of at least 2 independent determinations.

of eIF4B. Mutant N171, in which the extreme C-terminal portion of the RRM has been removed, showed the least RNA binding activity (8-fold reduction at maximal binding; Fig. 2.3C), suggesting that some binding affinity is provided by the RRM. GST alone did not bind RNA (data not shown). The abrupt decrease in RNA binding observed with mutant N367 suggests a potential RNA binding site localized between amino acids 367 and 423. However, a loss of RRM-dependent RNA binding activity due to a conformational change cannot be ruled out based on these results.

To distinguish between these possibilities, we generated point mutations at highly conserved residues within the RRM which have been shown to be important for RNA binding in other proteins containing this motif (Fig. 2.4). In addition, the entire RRM was deleted. Threonine 97 was mutated to valine (mutant T97V), and a double mutant, in which phenylalanine 139 and tyrosine 141 were replaced by alanines, was produced (mutant FY/AA). T97 is located at the edge of RNP-2 (see Fig. 2.4). Data derived from X-ray diffraction of the crystal structure of the snRNP protein U1A suggests that the corresponding threonine residue (threonine 11) may form hydrogen bonds with the RNA (Nagai et al., 1990). Mutagenesis of T11 of U1A to valine abolished RNA binding (Jessen et al., 1991). Phenylalanine 139 and tyrosine 141, which occupy positions 3 and 5 of RNP-1, are also highly conserved throughout the RRM family (see Fig. 2.4). It has been suggested that they participate in ring-stacking interactions with nucleotide bases of the RNA (Keene & Query, 1991). This is based on the finding that the phenylalanine at position 5 of RNP-1 in hnRNP A1 cross-links to RNA upon U.V. irradiation (Merril et al., 1988). Also, mutations of phenylalanines into alanines at positions 3 and 5 of the Rho protein RNP-1 resulted in weakened RNA binding (Brennan & Platt, 1990). These mutations would be expected to strongly affect RNA binding by eIF4B, were the RRM solely responsible for eIF4B binding to the RNA.

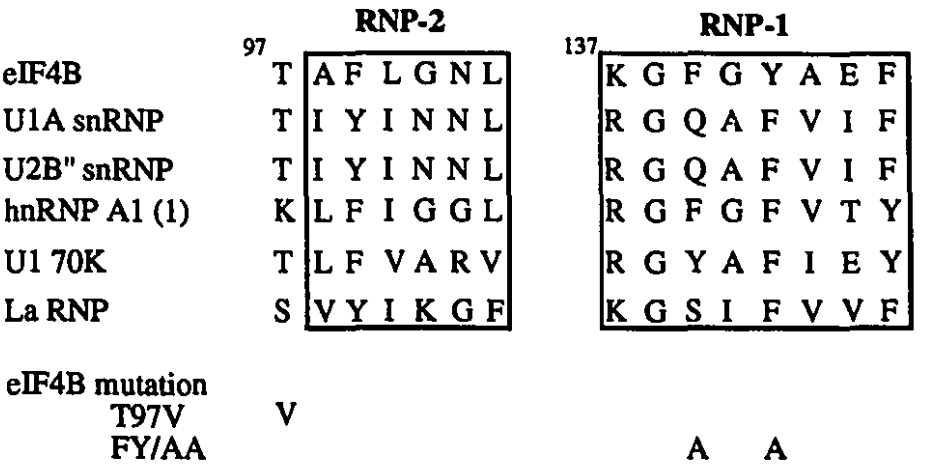


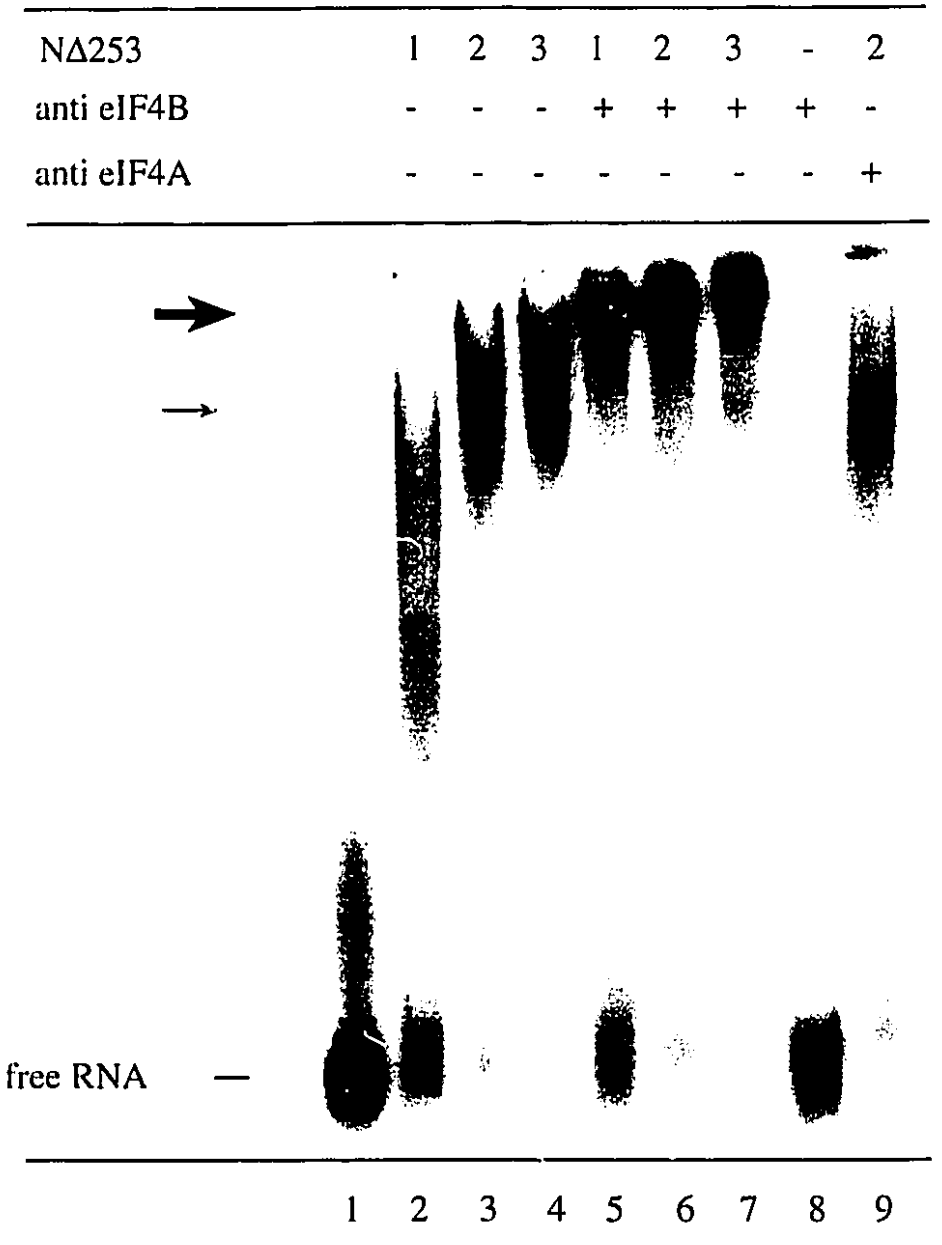
Fig. 2.4. Alignment of eIF4B RNP-1 and RNP-2 with homologous segments of other characterized RRM-containing proteins. Sequence alignments were obtained from Keene & Query, 1991. Positions are numbered according to the eIF4B sequence. RNP-1 and RNP-2 residues are boxed. Mutated eIF4B residues are indicated at the bottom of the figure.

The T97V and the FY/AA mutants bound RNA to approximately wild type levels (Fig. 2.3D). Moreover, the NΔ253 mutant, in which the RRM was completely removed, bound RNA with similar affinity to wt GST-eIF4B (Fig. 2.3D). To demonstrate that the RNA binding activity in this preparation is attributed to the eIF4B mutant, and not to an *E. coli* contaminating protein, a gel retardation assay (super shift) in the absence and presence of a monoclonal antibody against eIF4B was performed. Addition of mutant NΔ253 to the RNA resulted in the formation of a gel retarded RNA-protein complex (Fig. 2.5). The mobility of this complex decreased with increasing amounts of protein (lanes 2 to 4), presumably resulting from several proteins binding to a single RNA molecule. This was also noted with wild type eIF4B (data not shown). In the presence of a monoclonal antibody raised against eIF4B, the mobility of the complex is further reduced, indicating that the protein component of the complex is indeed eIF4B (lanes 5-7). The antibody itself did not bind to the RNA (lane 8), and a control polyclonal antibody raised against eIF4A failed to supershift the eIF4B-RNA complex (lane 9). These results demonstrate the presence of an RNA binding site at the C-terminal half of eIF4B, downstream of the RRM. We also made an 18 amino acid deletion in the DRYG rich region (mutant ΔDRYG; deletion of amino acids 230 to 247) to assess its role in eIF4B activity. The ΔDRYG mutant bound RNA at wild type levels (Fig. 2.4C), indicating that the integrity of this region is not required for RNA binding activity of eIF4B. These results, together with C-terminal deletion analyses, indicate that the critical region in eIF4B for RNA binding is located between amino acids 367 and 423.

Cooperation between GST-eIF4B mutants and eIF4A in RNA binding

The affinity of eIF4B for RNA is increased in the presence of eIF4A and ATP. The amount of RNA bound by the combination of eIF4A and eIF4B is greater than the sum of RNA bound by the individual components, thus indicating a synergistic effect (Abramson et al., 1988; Grifo et al., 1984). U.V. cross-linking studies have shown that the majority

Fig.2.5. Mobility shift assay analysis of mutant NΔ253. NΔ253 (prior to polyU purification) was incubated in buffer containing 20 mM Hepes-KOH, pH 7.2, 2 mM DTT, 0.5 mM $Mg(OAc)_2$, 5% glycerol and 75 mM KCl in a final volume of 20 µl. Affinity-purified monoclonal antibody against eIF4B (1.3 µg; N. Méthot and A. Darveau: to be published later) was added as indicated. Mixtures with and without antibody were pre-incubated for 10 min at 37°C. RNA (0.18 pmol) was added for a further incubation of 5 min at 37°C. Reactions were stopped by the addition of 5 µl of a solution containing 50% glycerol and 20 mM EDTA. Complexes were resolved on a 0.75 mm thick 7.5% native polyacrylamide gel (50:1 acrylamide to bisacrylamide) containing 5% glycerol in 0.5 X Tris-Borate-EDTA (TBE) which had been pre-electrophoresed for 30 min at 22 mA at 4°C. Electrophoresis was carried out at a constant current of 22 mA for 2 h at 4°C. Gels were dried and exposed with intensifying screens at -70°C. Lane 1; RNA alone. Small arrow: RNA-NΔ253 complex. Large arrow: supershifted complex.


NΔ253 1 2 3 1 2 3 - 2
anti eIF4B - - - + + + + -
anti eIF4A - - - - - - - +
free RNA
1 2 3 4 5 6 7 8 9

of the RNA is bound to eIF4B and not to eIF4A under these conditions (data not shown). We have used the filter binding assay to examine the effects of mutations on the ability of eIF4B to cooperate with eIF4A in binding to RNA. The data is summarized in Figure 2.6. All assays were performed in the presence of ATP. As previously shown (Abramson et al.,1987), eIF4A alone has very low affinity for RNA, as only 4% of the substrate is bound by eIF4A. Wild type GST-eIF4B, mutant N570 and mutant N464 bound 8, 11 and 5% of the RNA, respectively. These results are not substantially different from the values observed in the absence of ATP, indicating that ATP does not affect the ability of eIF4B to bind RNA. We used low amounts of eIF4B (2.5 pmol) in this assay to observe the cooperative effect with eIF4A. When eIF4A was included with wt GST-eIF4B, mutant N570 and mutant N464, the amount of bound RNA was increased by 5-fold, thus showing a clear synergistic effect (Fig. 2.6A). Mutant N423 could also cooperate with eIF4A, but with a 2-fold reduction in the total amount of RNA bound as compared to wild type (Fig. 2.6A). RNA binding activity of mutants N367, N355, N312, N250 and N171 on the other hand was not significantly increased by eIF-4A (Fig. 2.6B). The point mutants T97V and FY/AA exhibited synergism at 100% and 70% of wt levels, respectively (Fig. 2.6C). Strikingly, RNA binding of the NΔ253 mutant was also stimulated by eIF4A (60% of wt; Fig. 2.6C). These results are in agreement with the presence of an RNA binding site at the C-terminal half of eIF4B. Furthermore, they indicate that this RNA binding site, and not the RRM, is responsible for the cooperative effect. The ΔDRYG mutant could also cooperate with eIF4A at 80% of WT levels (Fig. 2.6C). Deletion of 244 amino acids from the carboxy end results in the complete loss of cooperativity. Thus, the carboxy 188 amino acids of eIF4B are dispensable for cooperativity with eIF4A. The integrity of the RRM , however, is not required for the synergistic effect, as point mutants in the RRM and NΔ253 could still cooperate with eIF4A. This suggests that the RRM is

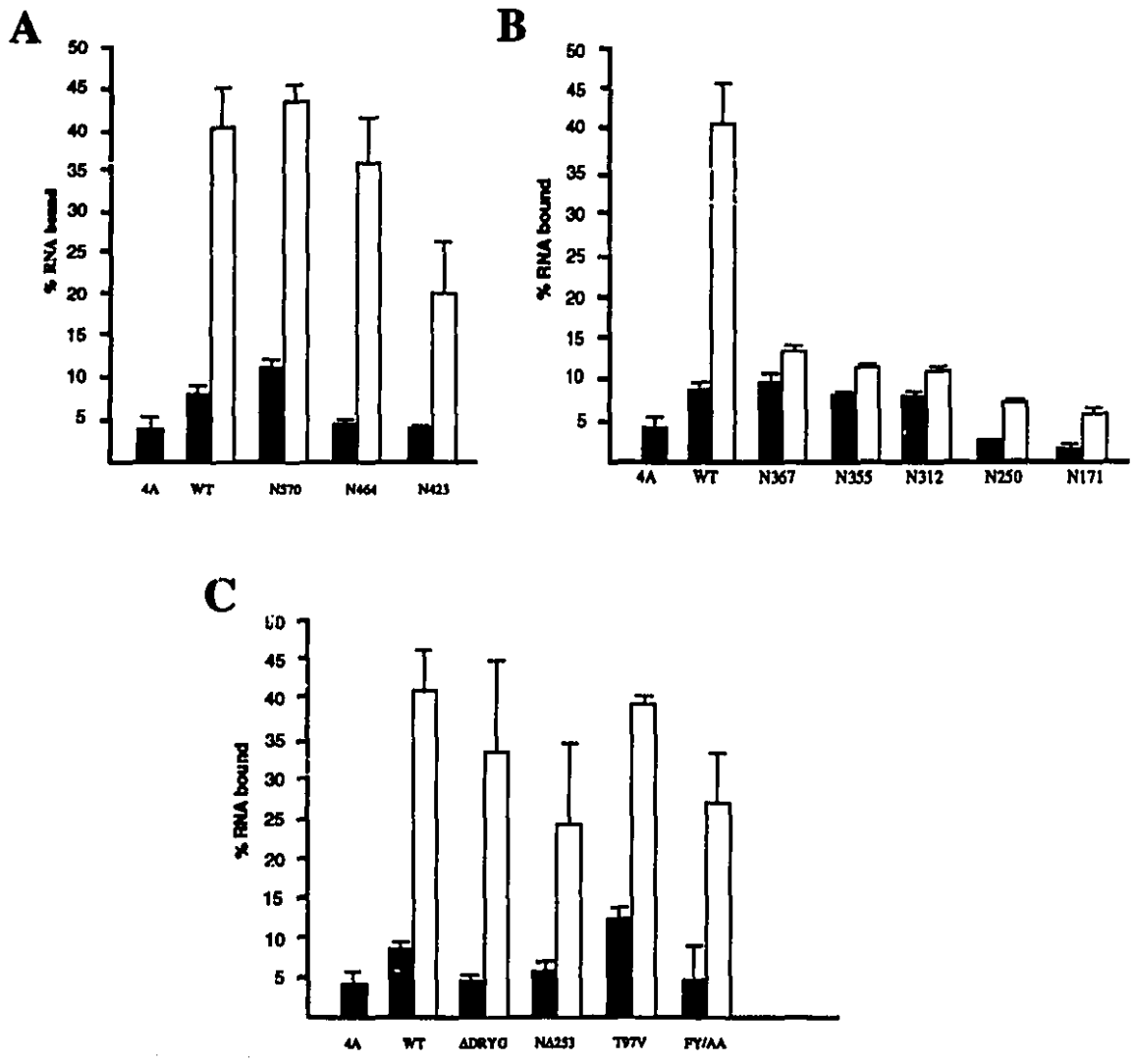


Fig. 2.6. Cooperation between eIF4A and eIF4B in RNA binding. The RNA substrate shown in Fig. 2.3A (0.18 pmol) was incubated with either eIF4A alone (22 pmol; stippled box), GST-eIF4B alone (2.5 pmol; black box) or GST-eIF4B and eIF4A (2.5 pmol and 22 pmol, respectively; white box), as described in Materials and Methods. Each point represents the average of at least 2 independent determinations and the standard deviations are indicated.

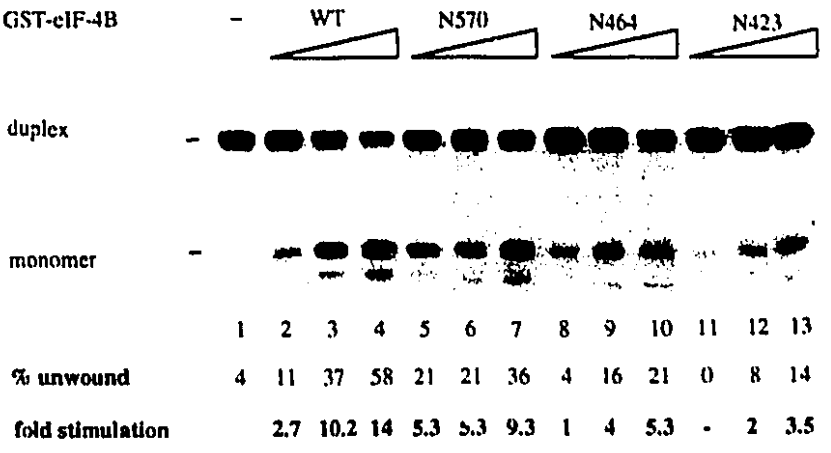
not essential for the apparent change of affinity of eIF4B for RNA that is engendered by eIF4A and ATP hydrolysis.

Helicase stimulatory activity of GST-eIF4B mutants

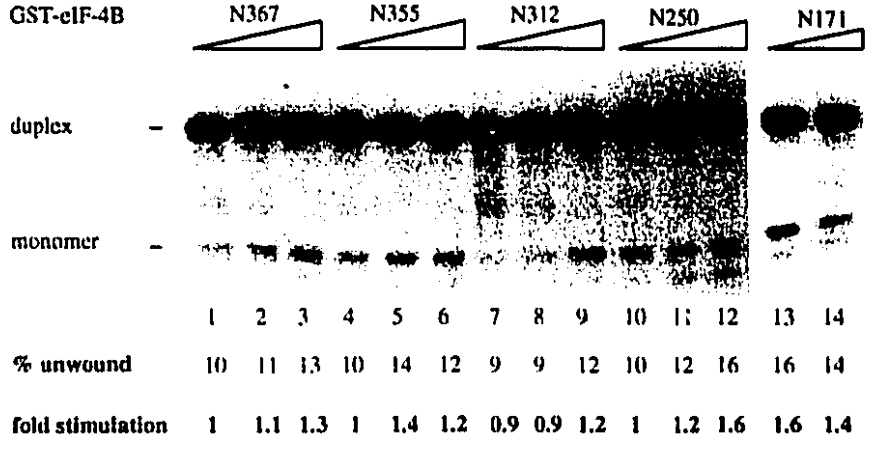
Since eIF4B stimulates the helicase activity of eIF4A, it was pertinent to test the effect of eIF4B mutations in a helicase assay. The RNA substrate was the same as that described in the two previous assays (see Fig. 2.3A). eIF4A alone exhibited some unwinding activity, as 4% of the duplex RNA was converted into the monomeric form (Fig. 2.7A, lane 1). Addition of GST-eIF4B stimulated the unwinding activity of eIF4A in a dose-dependent manner (lanes 2-4), with a 14-fold maximal increase. A dose-dependent stimulation was also observed with mutants N570 and N464, but to lower levels (lanes 5-7 and 8-10, respectively). Mutant N570 showed a 9-fold stimulatory effect over eIF4A helicase (wt levels of stimulation have been observed in some experiments), while mutant N464 increased the unwinding activity of eIF-4A by 5-fold. The N423 mutant stimulated the eIF4A helicase activity (lanes 11-13), albeit to a reduced extent, with a 3 to 4-fold increase. Mutants N367, N355, N312, N250 and N171, which poorly bound to RNA, failed to stimulate the helicase activity of eIF4A (Fig. 2.7B). These results are in agreement with the cooperativity effects of eIF4A on eIF4B RNA binding and the RNA binding activity of eIF4B alone; all C-terminal deletion mutants which cooperated with eIF4A for RNA binding also stimulated the helicase activity of eIF4A. The level of synergism reflects the extent of eIF4A stimulation, thereby suggesting that the increase in RNA binding activity of eIF4B upon interaction with eIF4A represents an important aspect of the helicase action. The helicase stimulatory activity of mutants bearing point mutations in the RRM (T97V and FY/AA), lacking 18 residues in the DRYG rich region (ΔDRYG) or lacking the RRM entirely (NΔ253) was also examined. Mutant T97V increased the unwinding activity of eIF4A by a maximum of 5-fold (Fig. 2.7C, lanes 1-3). Mutants FY/AA and ΔDRYG exerted a 3 to 4-fold stimulation (lanes 4-6 and 10-12, respectively), while mutant NΔ253

Fig. 2.7. eIF4A helicase stimulatory activity of GST-eIF4B mutants. GST-eIF4B mutant protein (1, 5 and 10 pmol) was incubated with 45 pmol of eIF4A and 20-40 fmol of labelled RNA duplex in 20 µl of unwinding buffer (17 mM Hepes-KOH, pH 7.2, 2 mM DTT, 5% glycerol, 0.5 mM $Mg(OAc)_2$, 0.5 mM ATP, 75 mM KCl, 20 U RNAsin) for 20 min at 37°C. The reaction was stopped by the addition of 5 µl of 50% glycerol, 2% SDS, 20 mM EDTA. Duplex and monomer RNA species were resolved on a 15% SDS polyacrylamide gel and visualized by autoradiography. The amounts of duplex and monomer RNA were quantitated on a Fujix Bas 2000 Phosphoimager. The level of monomeric RNA in the absence of eIF4A (~ 5% of total RNA) was subtracted from all values. The percentage of unwinding for was calculated as the amount of monomer RNA divided by the sum of duplex and monomer RNA. Background eIF4A unwinding was 4% in A, 10% in B and 5% in C.

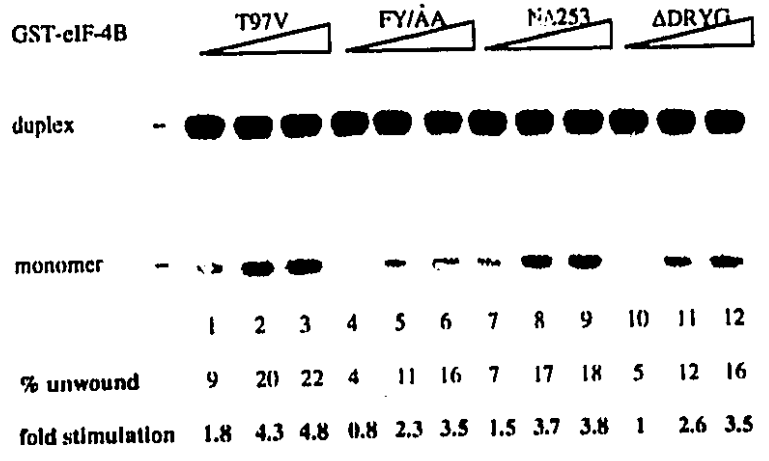
A


GST-eIF-4B
–
WT
N570
N464
N423
duplex
–
monomer
–
1 2 3 4 5 6 7 8 9 10 11 12 13
% unwound
4 11 37 58 21 21 36 4 16 21 0 8 14
fold stimulation
2.7 10.2 14 5.3 5.3 9.3 1 4 5.3 - 2 3.5

B


GST-eIF-4B
N367
N355
N312
N250
N171
duplex
–
monomer
–
1 2 3 4 5 6 7 8 9 10 11 12 13 14
% unwound
10 11 13 10 14 12 9 9 12 10 12 16 16 14
fold stimulation
1 1.1 1.3 1 1.4 1.2 0.9 0.9 1.2 1 1.2 1.6 1.6 1.4

C


GST-eIF-4B
T97V
FY/AA
ΔDRYG
duplex
–
monomer
–
1 2 3 4 5 6 7 8 9 10 11 12
% unwound
9 20 22 4 11 16 7 17 18 5 12 16
fold stimulation
1.8 4.3 4.8 0.8 2.3 3.5 1.5 3.7 3.8 1 2.6 3.5

stimulated the eIF4A helicase 4-fold (lanes 7-9). Thus, point mutations in the RRM, removal of the RRM or alterations of the DRYG-rich region all reduced the stimulatory effect of eIF4B on the eIF4A helicase activity to some extent, but did not eliminate the activity as did deletions in the carboxy half of eIF4B.

Discussion

A summary of the effects of deletions and mutations in eIF4B on RNA binding, cooperation with eIF4A in RNA binding and helicase stimulatory activity is shown in figure 2.8. The major conclusion of this study is that eIF4B contains a region required for RNA binding that is separate and independent of the RNP-CS/RRM. This region is located between amino acids 367 and 423, and is critical for the RNA binding of eIF4B. This conclusion is based principally on the finding that an eIF4B truncated protein lacking the RRM binds RNA with similar affinity to the intact protein. Consistent with this conclusion is the finding that most of the RNA binding activity is lost upon deletion of 244 amino acids from the carboxy terminus, which leaves the RRM intact. Furthermore, point mutations at highly conserved residues in the RRM failed to reduce RNA binding activity of eIF4B, again dissociating the RRM from the major RNA binding function.

We have determined that the RRM is not absolutely required for the ATP-dependent cooperation between eIF4A and eIF4B for RNA binding, and that regions involved in this process localize to the C-terminal of eIF4B. Moreover, the synergistic activity of the eIF4B deletion mutants correlate with their capacity to stimulate the helicase activity of eIF4A: mutant N570 behaves like wild type eIF4B in both assays. Mutant N464, which cooperates with eIF4A to a slightly lower level than the wild type, also stimulates the eIF4A helicase activity to a lesser extent. Mutant N423 is similar to N464. In contrast, mutants N367, N355, N312, N250 and N171 do not cooperate with eIF4A in RNA binding or stimulate the helicase activity of eIF4A. The RRM, on the other hand, seems to stimulate helicase activity. Deletion of the RRM reduced (4-fold) the helicase stimulatory activity of eIF4B, even though the ability to cooperate with eIF4A in RNA binding is only

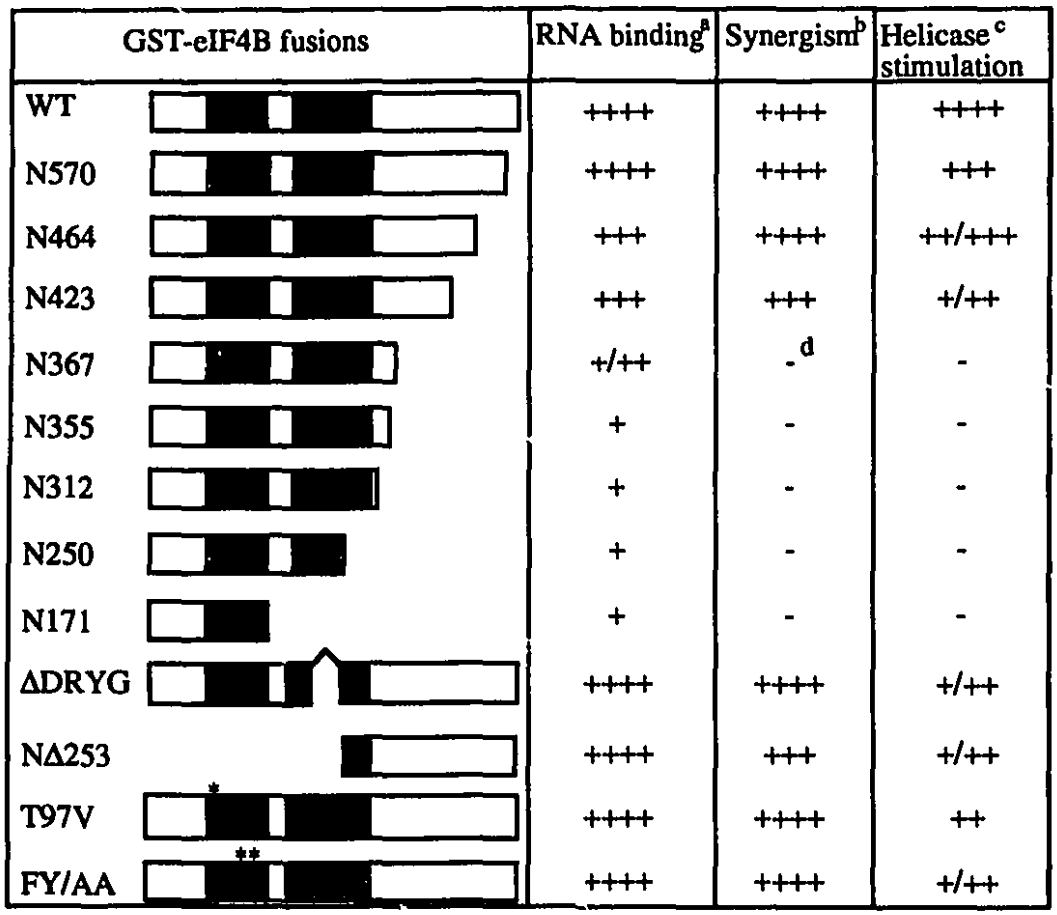


Fig. 2.8. Summary of deletion and point mutation constructs and their ability to bind RNA, cooperate with eIF4A at RNA binding and stimulate the eIF4A helicase activity. Black boxes represent the RRM. Stippled boxes represent the DRYG-rich region. Asterixes (*) represent point mutations. +:10-25% of wild type activity; ++:25-40; +++: 40-65; ++++:65-100.

a Values were derived from Fig. 2.3 B and C

b Values were derived from Fig. 2.6

c Values were derived from Fig. 2.7

d No activity detected.

marginally decreased. Point mutations at conserved RRM residues reduced (4-fold) the ability of eIF4B to enhance the helicase activity of eIF4A. In these assays, we cannot exclude the possibility that the reduction is due to a conformation change caused by these mutations.

Because mutant N423 cooperates with eIF4A in RNA binding, whereas mutant N367 is completely inactive, and similarly mutant N423 binds RNA at 50% of wt efficiency, whereas mutant N367 is much less efficient, we propose that the RNA binding site is located between amino acid positions 367 and 423. One cluster of basic amino acids is found between residues 367 and 423: KLERRPRERH (amino acids 395 to 404) which might be critical for RNA binding. A second cluster of basic amino acids (RNARRRESEK) is present 30 residues downstream. Five basic amino acid stretches (RP/GPRREREE/K) have also been identified in yeast eIF4B in the corresponding region (Altmann et al., 1993; Coppolecchia et al., 1993). Furthermore, homology searches (BLAST program; Altschul et al., 1990) have revealed that residues 386 to 445 of eIF4B share similarities with known RNA binding proteins such as snRNP U1 70K (Query et al., 1989) and U2AF (Lee et al., 1993, Valcárcel et al., 1993; data not shown). An arginine rich motif (ARM) is found in a number of prokaryotic and eukaryotic regulators of viral gene expression (Lazinski et al., 1989), and is required for the RNA binding activity of proteins such as Tat and Rev of HIV (Calnan et al., 1991; Delling et al., 1991) and the hepatitis delta antigen (Lee et al., 1993b). The latter is particularly of relevance since 2 ARMs separated by 29 amino acids (an arrangement similar to eIF4B), are essential for RNA binding. Mutagenesis in the arginine rich region of eIF4B will be required to directly test its role in RNA binding.

What is the role of the RRM in the eIF4B function? The RRM is a loosely conserved domain of 80 to 90 amino acids found in proteins that participate in diverse reactions involving RNA, such as polyadenylation, general and alternative splicing, RNA transport

and translation (Bandziulis et al., 1989; Keene & Query, 1991; Dreyfuss et al., 1993). The hallmark of the RRM is the presence of two conserved sequences termed RNP1 and RNP2. Deletion analyses have shown that for several proteins (U1A snRNP, U2B" snRNP, 70K snRNP), the RRM is sufficient for binding to RNA (Query et al., 1989; Scherly et al., 1990, 1990b). On the other hand, in the case of the La autoantigen and Ro60, extensive amino acid sequences flanking the RRM are needed for RNA binding (Pruijn et al., 1991), indicating that residues outside the RRM are also required for RNA binding. The eIF4B RRM seems to bind RNA at basal levels as mutants N367, N355, N312 and N250 could bind RNA, albeit very poorly, while mutant N171, in which 5 amino acids at the carboxy-terminal end of the RRM were removed, bound RNA near background levels.

An intriguing hypothesis is that the RRM may have specificity for a certain type of RNA, such as ribosomal RNA. An eIF4A-dependent association between ribosomes and eIF4B has been reported (Hughes et al., 1993). Fluorescence studies suggested that eIF4B binds preferentially to AUG triplets, as this trinucleotide competed efficiently with poly(A) for binding to eIF4B (Goss et al., 1987). The RRM is not required for eIF4B RNA binding, and is not essential for cooperativity with eIF4A. However, it could be involved in protein-protein interactions, as in the case of U2B" and U2A' snRNP (Scherly et al., 1990). The C-terminal RNA binding region, on the other hand, may serve as a non-specific RNA binding site, as would be expected to be found in a general translation factor.

What is the nature of the functional interaction between eIF4A and eIF4B in RNA binding and helicase activity? To date, there is no evidence for direct physical interaction between eIF4A and eIF4B. We have attempted to detect such an interaction by a co-immunoprecipitation approach, the yeast two-hybrid system (Fields & Song, 1989), far western (Blanar & Rutter, 1992) and gel shift assay, but obtained negative results (N. Méthot, unpublished results). It is possible that eIF4A alters the secondary structure of the

RNA and makes it a better target for eIF4B binding. The RNA binding properties of eIF4B suggest that it binds to RNA before eIF4A. This is supported by the lower Kd of eIF-4B (5×10^{-7} M) compared to eIF4A (> 10^{-6} M), and by the finding that in a mixture containing eIF4A, eIF4B, ATP and RNA (with an 8-fold molar excess of eIF4A to eIF4B), only eIF4B detectably cross-links to RNA (N. Méthot, unpublished observations). eIF4A may interact transiently with eIF4B and change its conformation via ATP hydrolysis. Several polypeptides are known to change their affinity or specificity for DNA or RNA upon interaction with other proteins. One of the best characterized examples is U2B" snRNP, an RRM-type RNA binding protein. Alone, U2B" can bind to both U1 and U2 RNA. In the presence of U2A', U2B" will bind only to U2 RNA with higher affinity (Scherly et al., 1990). Other examples are cleavage-stimulation factor (CstF) and cleavage -polyadenylation specificity factor (CPSF), which are required for correct cleavage and polyadenylation of mRNA. CstF, which contains an RRM, can UV cross-link to RNA in a non AAUAAA dependent manner. However, when both CstF and CPSF are present, only mRNA bearing the polyadenylation signal AAUAAA will be efficiently cross-linked by CstF. It has thus been suggested that protein-protein interactions between CstF and CPSF modulate the affinity of CstF for AAUAAA containing RNA (Takagaki et al., 1992).

Recently, SSL2, a 95 kDa protein with motifs similar to the DEXH box family of helicases has been cloned in yeast (Gulyas & Donahue, 1992). Indirect evidence suggests that this polypeptide may interact with SSL1, a zinc finger-containing protein (Yoon et al., 1992). Both factors, which have been cloned by a genetic screen which selected for suppressors of a translational block of the HIS4 mRNA due to the presence of a stable stem loop structure in the 5' UTR, were suggested to be novel yeast translation initiation factors. The functional relationship between SSL1 and SSL2 is reminiscent of the interaction between eIF4A and eIF4B, and it is possible that they exert their effects in an analogous manner.

Yeast eIF4B gene has been cloned recently by two groups (Altmann et al., 1993; Coppolecchia et al., 1993). Although it shares only limited homology with its human counterpart, yeast eIF4B retains some of the characteristic features of mammalian eIF4B: an RRM near the amino-terminus, and repetitive sequences of charged amino acids in the middle (human eIF4B) or at the carboxy-terminus (yeast eIF4B). As it is composed of 436 amino acids, yeast eIF4B is much smaller than human eIF4B (611 residues), and lacks the entire serine-rich carboxy-terminal portion of human eIF4B. Significantly, the last 188 amino acids of human eIF4B, that are not present in yeast, are dispensable for RNA binding, cooperation with eIF4A, and to a certain extent, stimulation of eIF4A helicase.

The identification of a novel RNA binding site in eIF4B provides a means to identify target RNAs by using the SELEX method (Tuerk & Gold, 1990). Knowledge of the mechanism of action of eIF4B is critical for the understanding of how ribosomes bind to eukaryotic mRNAs, and how this process is regulated.

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Chapter 3

In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA recognition motif

Abstract

Translation initiation factor eIF4B is an RNA binding protein which promotes the association of the mRNA to the 40S ribosomal subunit. One of its better characterized features is the ability to stimulate the activity of the DEAD box RNA helicase eIF4A. In addition to an RNA Recognition Motif (RRM) located near its amino-terminus, eIF4B contains an RNA binding region in its carboxy-terminal half. The eIF4A helicase stimulatory activity resides in the carboxy-terminal half of eIF4B, while the RRM has little impact on this function. To better understand the role of the eIF4B RRM, it was of interest to identify its specific RNA target sequence. To this end, *in vitro* RNA selection/amplifications were performed using various portions of eIF4B. These experiments were designed to test the RNA recognition specificity of the two eIF4B regions implicated in RNA binding and to assess the influence of eIF4A on the RNA binding specificity. The RRM was shown to bind with high affinity to an RNA stem-loop structure with conserved primary sequence elements. Discrete point mutations in an *in vitro*-selected RNA identified residues critical for RNA binding. Neither the carboxy-terminal RNA interaction region, nor eIF4A, influenced the structure of the high affinity RNA ligands selected by eIF4B, and eIF4A by itself did not select any specific RNA target. Previous studies have demonstrated an interaction of eIF4B with ribosomes, and it was suggested that this association is mediated through binding to ribosomal RNA. We show that the RRM of eIF4B interacts directly with 18S rRNA and this interaction is inhibited by an excess of the eIF4B *in vitro*-selected RNA. eIF4B could bind simultaneously to two different RNA molecules, supporting a model whereby eIF4B promotes ribosome binding to the 5' untranslated region of a mRNA by bridging it to 18S rRNA.

Introduction

In eukaryotes, the association of mRNA with the small ribosomal subunit is a highly regulated event. This process requires the participation of at least three initiation factors (eIF4A, eIF4B and eIF4F) and the hydrolysis of ATP (for reviews, see Merrick, 1992; Hershey, 1991). eIF4F is comprised of three subunits (eIF4E, eIF4A and p220), and binds to the cap structure that is present at the 5' end of all cellular mRNAs via the cap-binding protein subunit, eIF4E. The eIF4A subunit, an RNA-dependent ATPase which cycles through the eIF4F complex (Yoder-Hill et al., 1993; Pause et al., 1994) is the prototype member of the DEAD box family of RNA helicases (reviewed by Pause and Sonenberg, 1993; Gorbalenya and Koonin, 1993; Schmid and Linder, 1992). A model has been proposed (Sonenberg, 1988) whereby eIF4A as part of the eIF4F complex and together with eIF4B, unwinds secondary structure present in the 5' untranslated region (UTR) of a mRNA (Ray et al., 1985; Rozen et al., 1990), rendering the mRNA accessible for attachment to the 40S ribosomal subunit. The unwinding model is consistent with numerous reports that extensive secondary structure in the mRNA 5' UTR inhibits ribosome binding (e.g. Pelletier and Sonenberg, 1985; Kozak, 1986), and that the requirement for the cap correlates with the presence of secondary structure. mRNAs with low amounts of secondary structure are less dependent on the presence of the cap for their initiation of translation (Morgan and Shatkin, 1980; Sonenberg, 1981; Gehrke et al., 1983). In addition, components of the eIF4F helicase machinery can suppress the inhibition of translation caused by secondary structure in the mRNA 5' UTR. This was demonstrated for eIF4E using NIH 3T3 cells (Koromilas et al., 1992) and for eIF4B using yeast (Altmann et al., 1993).

Despite the supporting evidence for the mRNA unwinding model and the extensive biochemical characterization of some of the translation initiation factors implicated in unwinding (eIF4A and eIF4E), it is not clear how the 40S ribosomal subunit recognizes

and binds to the mRNA. Unlike prokaryotic initiation of translation, where association of the mRNA with the 30S subunit is mediated by base pairing interactions between the 16S rRNA and the mRNA Shine-Dalgarno sequence (Shine and Dalgarno, 1975; Jacob et al., 1987), eukaryotic ribosomes are not believed to position themselves on the mRNA through Shine-Dalgarno-like interactions. The attachment of the 40S subunit to the mRNA is more likely mediated by interactions between ribosomal proteins or rRNA and initiation factors.

eIF4B is an 80 kDa phosphoprotein which is required for binding of the mRNA to the 40S subunit (Trachsel et al., 1977; Benne and Hershey, 1978). eIF4B stimulates the ATPase and RNA helicase activities of eIF4A and eIF4F (Rozen et al., 1990; Pause and Sonenberg, 1992). It contains an RNA recognition motif (RRM; or RNA binding domain, RBD; for reviews see Kenan et al., 1991; Mattaj, 1993; Burd and Dreyfuss, 1994b) near the N-terminus (Milburn et al., 1990), and a second RNA binding region in the carboxy-terminal half of the protein (Méthot et al., 1994; Naranda et al, 1994). While the carboxy-terminal region binds random RNAs with high affinity, the RRM binds such RNAs inefficiently (Méthot et al., 1994; Naranda et al., 1994). The eIF4A helicase stimulatory activity of eIF4B maps to the carboxy-terminal half of the protein, with the RRM playing only a minor role (Méthot et al., 1994). eIF4B was also shown to possess RNA annealing activity (Altmann et al., 1995). Finally, eIF4B associates with eIF4F, as evidenced by their copurification (Grifo et al., 1983).

To characterize further the role of eIF4B in translation initiation, we investigated whether the RRM recognizes a specific RNA sequence, as it has been shown for this motif in many other proteins. Here, using iterative *in vitro* genetic selection, we have identified RNA molecules with which eIF4B preferentially interacts. We demonstrate by RNA binding studies using deletion and point mutants of eIF4B that the RRM is responsible for binding the selected RNAs, and confirm the non-specific RNA binding role of the carboxy-terminal

RNA binding region. Mutagenesis of the selected RNAs indicates that eIF4B recognizes individual nucleotides in the context of a higher order RNA structural organization. UV cross-linking experiments with radiolabeled 18S rRNA and eIF4B demonstrate a specific interaction. Finally, eIF4B is shown to interact with two different RNA molecules simultaneously, which is consistent with an RNA-RNA bridging model. In total, these data suggest that eIF4B participates in mRNA binding to the 40S ribosomal subunit by virtue of its ability to associate with 18S rRNA.

Materials and Methods

Vectors, protein expression and purification

The N250 FY/AA mutation was generated by digesting pGEX-4B FY/AA (Méthot et al., 1994) with ClaI, creating blunt ends on the vector with the Klenow fragment of *E. coli* DNA polymerase and religating. N250 K137Q was obtained by first introducing the point mutation in full-length eIF4B by two-step PCR mutagenesis. The resulting vector, pGEX-4B K137Q, was linearized with ClaI, blunt-ended and religated. The presence of the point mutations and the integrity of the PCR-amplified DNA sequence were verified by sequencing. N250 FY/AA, N250 K137Q, N250, NΔ253 and GST-eIF4B were expressed in *E. coli* BL-21 and purified as described (Méthot et al., 1994). eIF4A and eIF4B were purified from *E. coli* K38 as described by Pause and Sonenberg (1992).

Selection/amplification

Selection/amplification was performed as described (Tuerk and Gold,1990; Tsai et al.,1991). The synthetic DNA oligonucleotides used were : T7 Univ, Rev Univ, Linear N25 and Linear N40. A description of the first three oligonucleotides is provided in Tsai et al., 1991. Linear N40 was designed similarly to linear N25 except that it contained a random region of 40 bases instead of 25. Selection experiments A, B and C were performed in buffer A (20 mM HEPES-KOH, pH 7.3, 2 mM DTT, 0.5 mM $Mg(OAc)_2$, 5% glycerol, 75 mM KCl) in the presence of 40 U RNAsin (Promega) and 1 mM ATP. For the initial round of selection, a pool of 10^{14} different RNA molecules was used and subsequent rounds were performed with 40 pmoles of RNA. For each round of experiments A and B, 2 µg of purified GST-4B (2×10^{-11} moles) served as target, while in experiments B and C, 2 µg of purified eIF4A (4×10^{-11} moles) were used. RNA-protein complexes were isolated by precipitation with either glutathione-sepharose (Pharmacia; Experiments A and B) or protein G-sepharose coupled to a monoclonal antibody directed against eIF4A (Experiment C). To remove non-specific RNAs binding to glutathione-

sepharose or protein G-sepharose or anti-eIF4A, the RNA pool prior to each round of selection was incubated 5 min at 25°C with 100 µl of a 50% slurry of either glutathione-sepharose or protein G-sepharose coupled to anti eIF4A, and equilibrated in buffer A containing 1 mM ATP. RNA in the flow-through was ethanol-precipitated and resuspended in 100 µl of buffer A containing 1 mM ATP and 40 units RNAsin. Proteins were added in amounts indicated above and mixed with the RNA for 5 min at 25°C. Subsequently, 100 µl of a 50% slurry of beads equilibrated in buffer A and 1 mM ATP were added to the reaction and gently mixed for 5 min at room temperature. The reaction was transferred to a BioRad poly prep column, and the beads were washed 5 times with 500 µl of buffer A, resuspended in 400 µl of water and phenol-chloroform extracted. The RNA was ethanol-precipitated and reverse transcribed for 1 h at 42°C in 20 µl of 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 50 µg/ml BSA, and 1 mM of each deoxynucleotide, to which 100 ng of UnivRev primer and 5 units of AMV reverse transcriptase (Gibco-BRL) were added. An aliquot (10 µl) of this reaction served as template for a PCR amplification (15 rounds) performed with UnivRev and T7 Univ primers. The resulting DNA was phenol-chloroform extracted, ethanol precipitated and used for T7 RNA polymerase-directed in vitro transcriptions under conditions described below. Following the 8th round, the amplified DNA was treated with T4 polynucleotide kinase (Pharmacia), agarose gel-purified and cloned into pBluescript KS linearized with SmaI. Clones bearing an insert were sequenced by the dideoxy method. Experiments D and E were performed under identical conditions except for the omission of ATP in the incubation buffer. Selection/amplification was repeated 5 times using 2 µg of either N250 (experiment D) or NΔ253 (experiment E) as targets. Following the 5th round of amplification, the DNA was digested with BamHI and cloned into pGEM3Z(+) linearized with BamHI. Clones were sequenced by the dideoxy method (Pharmacia).

In Vitro Transcriptions

The first round of selection was performed on an RNA pool consisting of 10^{14} molecules, which was generated with T7 RNA polymerase (Promega) in a final volume of 400 µl, under conditions recommended by the supplier. All other transcriptions were carried out in 100 µl volumes under identical conditions. A tracer amount of α^{32}P GTP (20 µCi) was added to each reaction to follow the synthesis and the efficiency of the selection. Following transcription , DNA was removed with 10 U of RNAse-free DNAse I (Boehringer Mannheim) and the RNA was purified on a denaturing 8% polyacrylamide gel (Experiments A, B and C). Quantitation was done by Cherenkov counting and spectrophotometric measurement.

All RNA transcripts used in filter binding analysis and UV-cross linking assays were synthesized to a specific activity of 2 x 10^{17} cpm/mole RNA, and purified by denaturing-polyacrylamide gel electrophoresis, except for 18S rRNA (1-1678). N40, A3 and mutants of A3 RNA were generated by T7 RNA polymerase-directed in vitro transcription from DNA templates of either vector (pBluescript KS clones linearized with BamH1) or oligonucleotide (wild type and mutant A3 sequences) origin. Reaction conditions were as recommended by the supplier. Unlabeled A3 and A3ΔA RNAs were generated under the standard Promega protocol, and purified by denaturing gel electrophoresis. A3 and A3ΔA RNA were further purified on a 7.5% non-denaturing polyacrylamide gel to remove all double-stranded RNA activated protein kinase (PKR)-stimulating activities. Rat 18S rRNA (1-1678) was generated from transcription with T7 RNA polymerase. The template used in the transcription reactions was obtained by PCR amplification of the appropriate sections of pGEM2-18S (gift from I. Wool) using a 5' primer bearing a T7 promoter. The primer pairs for rat 18S rRNA (1-1678) were:
AATACCTAATACGACTCACTATAGGGCGATACCTGGTTGATCCTGCC and
AACGCAAGCTTATGACCCGCACTTACTG; Transcription with SP6 RNA polymerase

using pSP64-globin linearized with BamHI yielded globin RNA. *E.coli* 5S rRNA was purchased from Boehringer Mannheim.

Filter binding analysis

Nitrocellulose retention assays were performed essentially as described (Méthot et al, 1994). Briefly, indicated amounts of proteins were incubated in 40 µl of buffer A containing 0.1 mg/ml BSA, 30 ng of polyA RNA and approximately 0.18 pmoles of ^{32}P-labeled RNA substrate. The mixture was incubated for 2 min at 37°C and filtered through a pre-wetted nitrocellulose membrane (0.45 µm pore size; type HA; Millipore). The filter was washed with 1 ml of ice cold buffer A, air-dried for 30 min and retained radioactivity was quantitated by scintillation counting. Each point represents the average of at least 2 independent binding reactions and is corrected for the amount of RNA retained in the absence of protein, which typically represents less than 1.5 % of the RNA input.

UV cross-linking

HeLa cytoplasmic extract was prepared by growing HeLa S3 cells to 80% confluency and lysing the cells in 20 mM HEPES-KOH, pH 7.3, 75 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and 1% Triton X-100. Nuclei and cell debris were removed by centrifugation. Rabbit reticulocyte lysate was purchased from Promega. OligoA3 or A3ΔA RNA (10^5 cpm or 0.5 pmoles) were mixed with 45 µg of HeLa extract or 5 µl of rabbit reticulocyte lysate, along with 1 ug of poly(A) RNA, 1 µg of poly(U) RNA and 1 µg of tRNA in 20 µl of FBB 75. Following 5 min incubation at 37°C, the extract was UV-irradiated on ice for 5 min with a 15W General Electric G15T8 germicidal lamp from a distance of 1.5 cm. Excess RNA was digested for 30 min at 37°C with 20 µg of RNAse A. Where indicated, eIF4B was immunoprecipitated with an anti-eIF4B polyclonal antibody. Samples were subjected to SDS-PAGE and visualized by autoradiography. For 18S rRNA cross-linking studies, ^{32}P-labeled 18S rRNA [(1-1678); 1.4×10^{-14} moles] and competitor

RNA as indicated were mixed simultaneously in 20 µl of buffer A containing 5 µg of poly(A) RNA and 0.5 µg recombinant eIF-4B. Incubation and irradiation conditions were identical to those described above. Following RNAse A digestion, the samples were subjected to SDS-PAGE and visualized by autoradiography. Cross-linked products were quantified using a Fujix Bas 2000 Phosphorimager.

Results

eIF4B recognizes a specific RNA secondary structure

Five RNA selection experiments were performed using RNA pools of either 25 or 40 random nucleotide positions (Table 3.1). In experiment A, RNA selection was carried out using full length eIF4B (Fig. 3.1A). Two additional selection experiments were performed to test whether the presence of eIF4A can alter the RNA binding specificity of eIF4B (experiment B), or whether eIF4A by itself binds to a specific RNA sequence (experiment C). Selections using deletion mutants that contain the RRM (fragment N250; experiment D) or the C-terminal RNA binding sequence (fragment NΔ253; experiment E) were performed to discern the RNA binding specificities of the two RNA binding regions of eIF4B (Fig. 3.1A). Experiments A, B and C contained ATP in the binding buffer, as eIF4A binding to RNA is ATP-dependent (Grifo et al., 1982; Pause et al., 1993), and cooperation between eIF4A and eIF4B in RNA binding requires ATP hydrolysis (Abramson et al. 1988, Méthot et al., 1994).

Sequences of several of the selected RNAs are shown in Figure 3.1B. Examination of the RNA sequences selected by full length eIF4B (Exp. A) revealed the presence of a GGAA/C motif in 19 out of 20 clones. The three positions immediately upstream of the GGAA/C motif were less well conserved but appeared to be preferentially occupied by the sequence GUU (Fig. 3.1B). Stretches of C and A were also prominent. The *in vitro*-selected RNAs were folded using the MFOLD program (Devereux et al., 1984) to yield a common RNA secondary structure (Fig. 3.2). The RNAs selected by eIF4B (Exp. A) contain a stem-loop structure, with the terminal loop comprised of three to five nucleotides. The base of the loop is formed by a G-C or C-G base pair, and the 5' position of the loop is an A in 73% of the clones, or a U in the remaining clones. The 3' position of the loop is occupied by G in the vast majority of the clones (93%). The conserved primary sequence

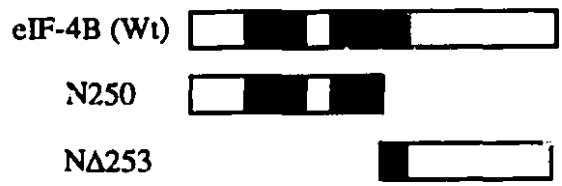
Table 3.1 Summary of selection experiments performed

experiment	target	length of randomized region	rounds of selection
A	eIF4B [1]	40	8
B	eIF4B [1] + eIF4A	40	8
C	eIF4A	40	8
D	N250 [1]	25	5
E	NΔ253 [1]	25	5

[1] Purified as GST-fusion proteins.

Fig. 3.1. Representative RNA sequences selected from randomized RNA libraries using eIF4B (experiment A), eIF4B + eIF4A (experiment B) and N250 (experiment D). A) Schematic representation of eIF4B mutants used as targets in selection/amplification experiments. Black box; RNA Recognition Motif (amino acids 97 to 175). Stippled box; DRYG-rich region (amino acids 214 to 327). B) RNA sequences of individual clones selected in experiments A, B and D. The conserved GGAA/C motif present in A, B and D pool clones is highlighted in bold type. Sequences from experiments C and E, using eIF4A and NΔ253 as targets, respectively, are not shown. The consensus sequence derived from experiments A and B is indicated. A total of 20 clones for experiment A, 23 for experiment B, 28 for experiment C, 22 for experiment D and 20 for experiment E were isolated after 8 rounds (A, B and C) or 5 rounds (D and E) of selection/amplification.

A



B

	clone	selected sequence	frequency
A	A1	CGGGCCACCAACGACAUUACAACCA**GGAC**UGGAUUGCCAGCUA	X1
	A2	AAUGGUU**GGAA**CGCACAGGCUUGACAUCAACCAUCAAUCC	X3
	A3	CCAGCUGCA**GGAC**UAGAUAGCUAGCUACAGCACACCGUGA	X1
	A4	CGCGUGUC**GGAA**UAGAGCGUUAAGCUCAUCCCCUGACCAAACC	X1
	A5	AAGGUU**GGAA**CGCACAGGCUUGACAUCGACCAUCAAUCC	X1
	A6	UACAGUU**GGAC**CGGUCGCCGGCAGCUGUGACACAUAAUUC	X1
	A8	ACCAUAGGCUU**GGAA**AUCUAUAGGAUCAUCGUCUAAGUAA	X1
	A13	CUUGGGUUC**GGAA**AUGAAACGCAUAAUCGCCCAUCUAUGA	X3
	A17	CUUCCAACGCACA**GGCU**UGACAUCAACCACAAUCCG	X1
B	B5	AAAAGAACACAACACC**GGAA**CGCAGAAGGCCUGAAUCAGU	X3
	B6	ACCAUAGGCUU**GGAA**AUCUAUAGGAUCAUCGUCUAAGUAA	X3
	B11	CACCACAUAACAUAGUC**GGAA**AGAACACGUCUAAUCCUAU	X2
	B12	AGAAUAAAAAACCCGC**GGAA**CCGUAUAGCGCUGCAUCCGG	X11
	B20	CUUAGCACCACAACAACGCUU**GGAC**UGGCCGCCAGCAAGU	X1
	B23	GAUAGU**GGAA**CACACAAGGUAUGCAUCCUAUCCCAAUGAC	X1
	B24	AACAGUU**GGAC**GGUCGCCGGCAGCUGUGACACAUAAUUC	X1
	B32	CCAGCUGCA**GGAC**UAGAUAGCUAGCUACAGCACACCGUGA	X3
D	D1	UG**GAAA**UUGUAAGAAUUAUCGTGGC	X13
	D3	G**GGAC**UGCAUGGCAGCUGGUGUCAC	X1
	D6	**GGAC**UAGAUGCUAGCAAUGAACUUC	X1
	D8	U**GGAC**UAGUUGCUAGCAAAUGACCC	X1
	D9	**AGAC**UGCAGAACAUCUGAUACAUGC	X1
	D11	CCGTAG**AGAG**CAAGCUCCGGGUUCG	X1
	D15	CACGCA**GGAC**UUGAAGCAAGCUAC	X1

Consensus for experiments A and B $\frac{G}{C}$ U $\frac{U}{C}$ G G A $\frac{A}{C}$

GGAA/C also lies in a defined structural context. In all cases, at least one of the A residues is bulged and at least one of the G residues is paired at the base of the bulge. The most prevalent arrangement (50% of independent clones) consists of both G residues paired at the base of the bulge, and the 5' most A residue unpaired. The size of the bulge is variable, ranging from one to four nucleotides.

To determine whether eIF4A affects the structure or nucleotide sequence requirements for specific RNA binding by eIF4B, a combination of eIF4A and eIF4B was used to generate high affinity RNA ligands by iterative selection *in vitro* (experiment B). Pool B sequences contained the common GGAA/C motif found in experiment A , as well as an abundance of C and A residues (Fig 3.1B; B-clones). Significantly, all but one of the clones from experiment B are different from the clones obtained in experiment A, demonstrating that the presence of the GGAA/C motif in pool B does not result from contamination with pool A clones. Folding of the pool B RNA sequences produced a hairpin secondary structure similar to that of experiment A (Fig. 3.2). Thus, the presence of eIF4A did not appear to modify the RNA binding preference of eIF4B. A feature of the pool B clones, however, is that 14 of the 23 clones sequenced contained a stretch of A residues at their 5' end. Of these 14 sequences, 11 were identical, suggesting that the A-richness may have confered a slight selective advantage. The possible significance of this observation will be addressed below.

The selection using eIF4A as a target (experiment C) did not result in the amplification of RNA bearing recognizable common sequences or secondary structures (data not shown). This supports the notion that eIF4A is a weak non-specific RNA binding protein, at least under these conditions (Pause et al., 1993).

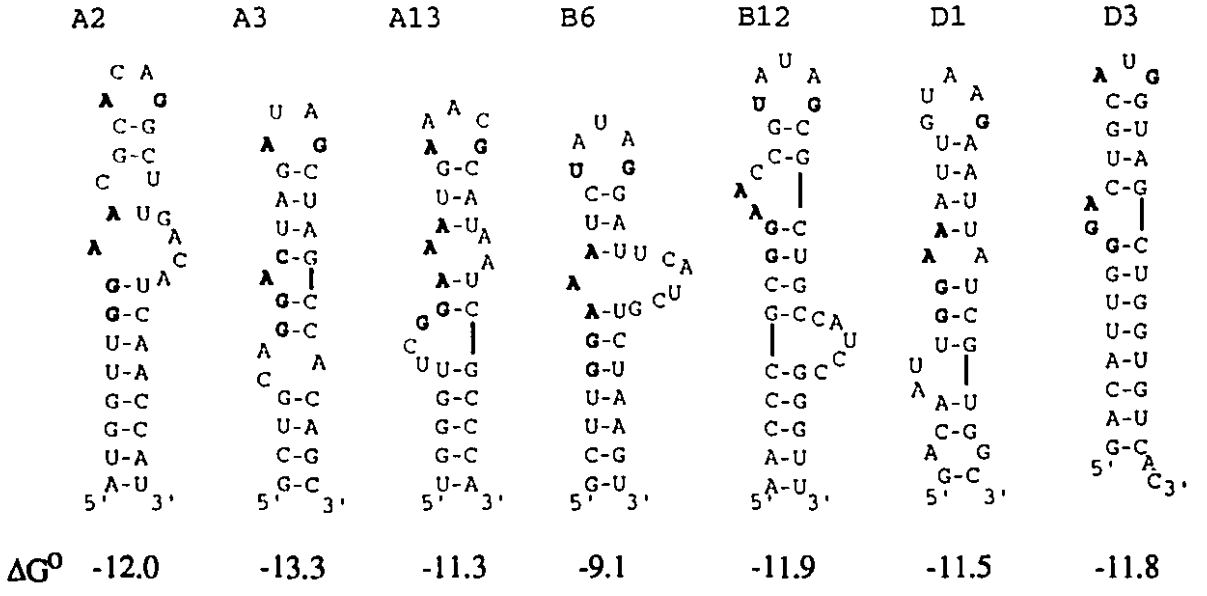


Fig. 3.2. Predicted secondary structures of certain ligands selected by eIF4B (clones A2, A3 and A13), eIF4B + eIF4A (B6 and B12) and N250 (D1 and D3). The conserved GGAA/C motif is highlighted in bold type as well as the conserved nucleotides at the 5' and 3' position of the loop.

The selected RNAs are bound through the RRM

eIF4B may bind the selected RNAs either through its RRM, or its carboxy-terminal RNA binding site or a combination of both regions. To identify the specific RNA-binding site, *in vitro* iterative selection-amplification was performed with portions of eIF4B containing either the RRM (fragment N250; experiment D) or the carboxy-terminal RNA binding region (fragment NΔ253; experiment E; Fig. 3.1A). The primary and secondary structure of the N250-selected RNAs is strikingly similar to pool A and pool B sequences (Figs. 3.1B and 3.2). The GGAA/C motif was present in 20 out of the 22 sequenced clones, with a predicted secondary structure consisting of a bulged stem-loop. These data strongly suggest that the region of eIF4B responsible for binding the selected RNAs in experiments A, B and D is the RRM. The presence of the carboxy-terminal RNA binding site did not affect the RNA binding preference of eIF4B. This is consistent with earlier data which characterized the carboxy-terminus of eIF4B as a non-specific RNA binding site (Méthot et al., 1994). Indeed, examination of the sequences of 20 independent clones, using the eIF4B fragment NΔ253 for selection (experiment E), did not reveal any common sequence (data not shown).

To confirm that the *in vitro*-selected RNAs contained high affinity binding sites for eIF4B, the apparent dissociation constant of recombinant eIF4B (non-GST fusion) for some selected RNAs (A2, A3 and A13) was assessed by a nitrocellulose retention assay (Fig. 3.3A). The dissociation constants (K_d) were identical for all three pool A RNAs tested, at 12.5 nM. For comparison, the K_d for an eIF4A selected RNA, C2, was 200 nM. This confirms that eIF4B can bind specifically and with high affinity to RNAs derived from pool A. The dissociation constant for pool B RNA clone B12 (which was selected 11 times out of the 23 sequenced clones), was also estimated at 12.5 nM (Fig. 3.3A), thus showing that eIF4A does not modify the RNA binding specificity or affinity of eIF4B for the selected RNAs. The experiment shown in Fig. 3.3A was conducted in the presence of 30

ng of competitor poly(A) RNA. In the absence of this competitor, eIF4B could not distinguish pool A RNAs from pool C RNAs, presumably because these RNAs bound non-specifically to the carboxy-terminal RNA binding site (data not shown).

Selection/amplifications with full length eIF4B and the N250 fragment yielded high affinity RNA ligands that have the potential to fold into similar secondary structures. To evaluate the contribution of the C-terminal RNA binding region to the overall RNA binding strength of eIF4B, the affinity constants of wild type eIF4B and the N250 fragment for A3 RNA were compared. N250 bound A3 RNA with a dissociation constant of 12.5 nM (Fig. 3.3B), a value identical to the K_d of wild type eIF4B for A3 RNA (Fig. 3.3A). N250 bound extremely weakly to the non-specific control RNA ligands C2 and N40 (Fig. 3.3B). From this experiment, it is clear that the carboxy-terminal RNA binding site neither changes the specificity of RNA binding, nor influences its affinity for specific RNAs. The N250 segment of eIF4B, which comprises the RRM, is sufficient for specific RNA binding equivalent to that of wild type eIF4B.

To show that the RRM is directly involved in RNA binding, studies using single amino acid mutants of the ribonucleoprotein consensus sequence-1 (RNP1) in the N250 fragment were conducted. The lysine residue at position 1 of RNP1 was changed to a glutamine (K137Q) , and the phenylalanine and tyrosine residues at position 3 and 5 of RNP1, respectively, were both mutated to alanines (FY/AA). Previous mutagenesis (Merril et al., 1988; Brennan and Platt, 1991; Cáceres and Krainer, 1993; Mayeda et al., 1994; Raabe et al., 1994), NMR (Hoffman et al., 1991; Görlach et al., 1992) and crystallographic studies (Nagai et al., 1990; Oubridge et al., 1994) pointed to these residues as key determinants of RRM-mediated RNA binding in several RNA binding proteins (reviewed in Kenan et al., 1991). The K137Q and the FY/AA mutations increased the dissociation constant for A3 RNA by 3-fold and 360-fold, respectively (Fig. 3.3C). Taken together, the data clearly

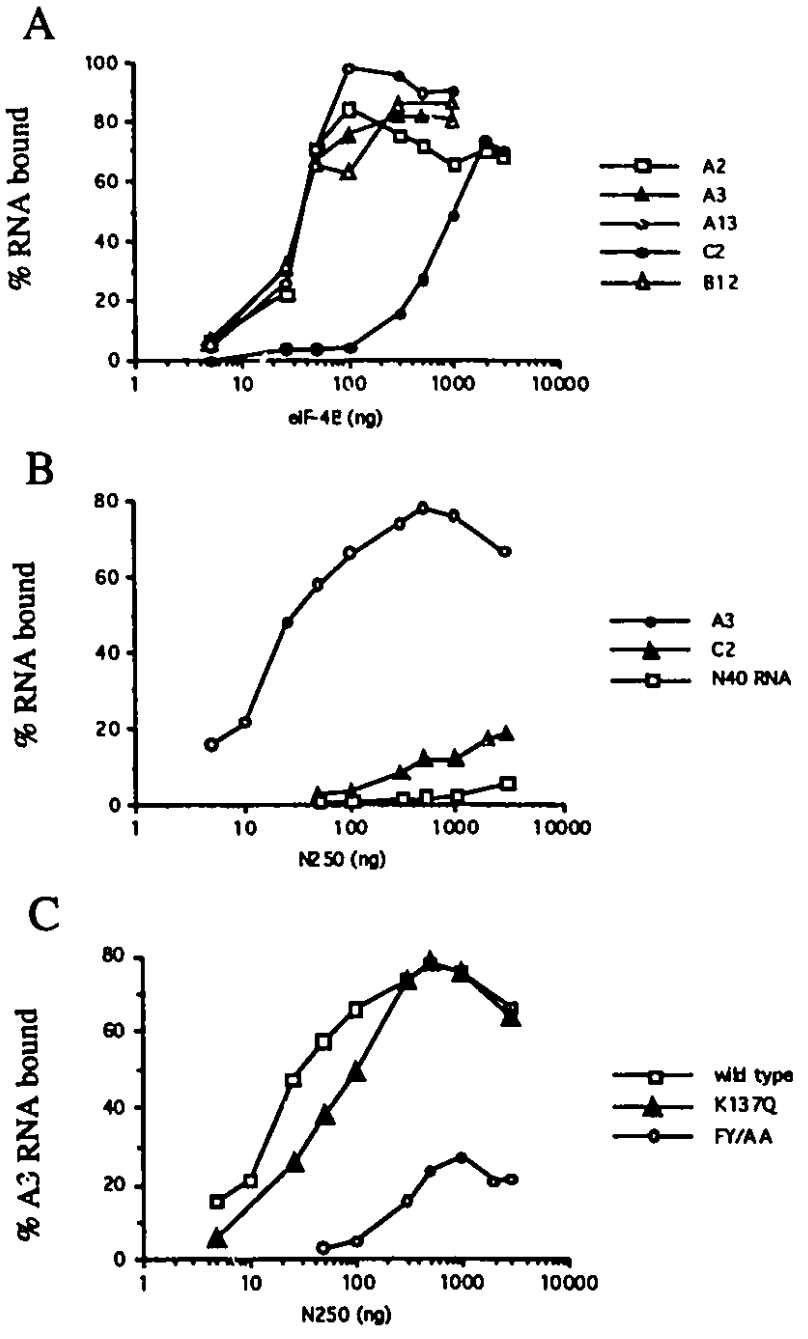


Fig. 3.3. RNA binding specificity of eIF4B and mutants of eIF4B. A) Binding of eIF4B (non GST fusion) to selected RNAs from experiments A (clones A2, A3 and A13), experiment B (clone B12) and experiment C (clone C2). B) Comparative affinities of the N250 mutant for the eIF4B-selected RNA clone A3, non eIF4B-selected clone C2, and random RNA pool N40. C) Effect of point mutations in the ribonucleoprotein-1 consensus sequence (RNP-1 CS) on binding of N250 to A3 RNA. Nitrocellulose filter binding conditions are described in Materials and Methods.

establish the RRM of eIF4B as part of the domain responsible for specific binding to the selected RNA sequences.

Nucleotide sequence and structure requirements for specific RNA binding

The probability that any tetranucleotide will have the sequence GGAA/C is approximately 0.03. To assess the functional significance of this motif for recognition by the eIF4B RRM, we mutated the GGAA/C stretch. Modifications were introduced by i) deleting the bulged A residue (ΔA mutant); ii) pairing the bulged A residue to a U residue (A=U mutant); iii) mutating the two G residues to Cs, while introducing compensatory changes to keep the stem intact (GG->CC mutant). Mutations (i) and (ii) were intended to test whether the bulged A functioned in a defined structural context. Mutation (iii) tested the primary sequence requirements by changing the conserved G residues without altering the overall secondary structure of A3 RNA. Wild type A3 RNA synthesized from an oligonucleotide-directed transcription system similar to that used to generate mutants of A3 RNA was used as a positive control (referred to as oligoA3). N250 bound to oligoA3 RNA efficiently, but with a 10-fold increase in K_d compared to plasmid-derived A3 RNA (data not shown). The reason for this discrepancy is unclear, but could be due to the five extra nucleotides present at the 3' end of oligoA3 RNA affecting its folding. All mutants of the GGAA/C motif of oligoA3 RNA were completely deficient in their ability to bind N250 (Fig. 3.4B). Clearly, any modification of the GGAA/C motif prevented the association of N250 to the RNA. The need for strict structure requirements is evident from the observation that the A residue must be bulged in order for eIF4B to recognize A3 RNA. The GG->CC mutant did not bind to N250 even though the predicted secondary structure of A3 RNA was unaffected by these changes. This demonstrates the importance of the RNA primary sequence. The contribution of the loop sequence to RNA recognition was examined by mutagenesis of the A and G residues in the 5' and 3' positions of the loop (AG->UU mutant). This mutant bound to N250 somewhat better than the GGAA/C motif

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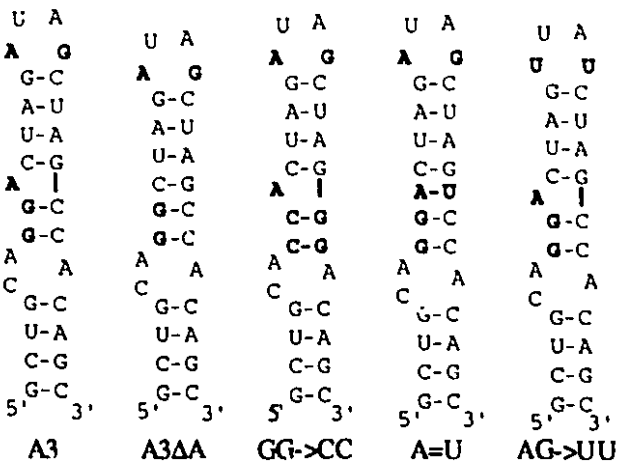

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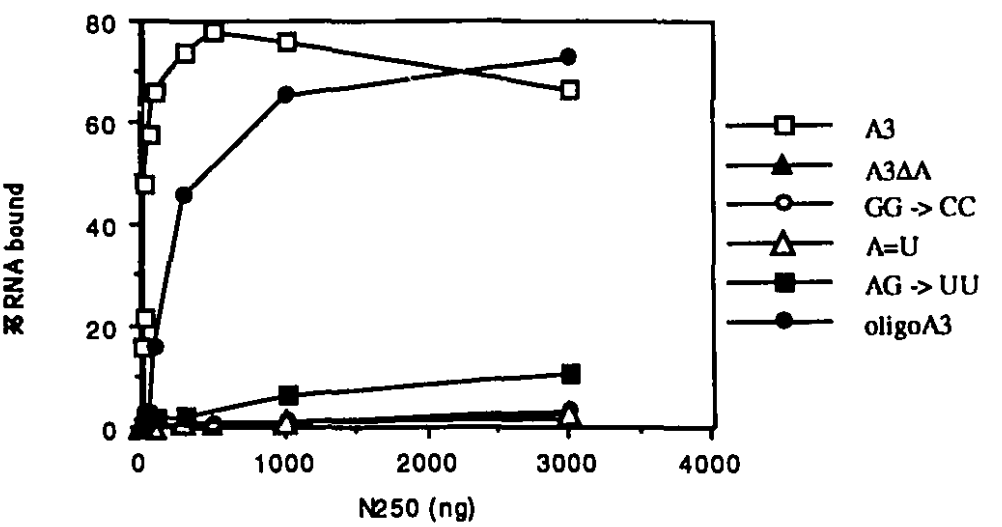

Fig. 3.4. Mutagenesis of conserved nucleotides in the A3 RNA ligand selected from the randomized RNA library: effect on binding by the N250 fragment of eIF4B. The predicted secondary structures for wild type A3 RNA and mutants of A3 RNA are illustrated (A). Nitrocellulose filter binding conditions are described in Materials and Methods.

mutants, but was still severely deficient compared to wild type oligoA3 RNA (Fig. 3.4B). Thus, the loop sequence, in addition to the GGAA/C motif, appears to be required for efficient RNA recognition by the N250 fragment of eIF4B.

eIF4B binds A3 RNA in HeLa cytoplasmic extracts

The association of eIF4B with the selected RNAs was characterized using recombinant protein. It was therefore important to test whether cellular eIF4B, containing postranslational modifications such as phosphorylation (Duncan and Hershey, 1984; Duncan and Hershey, 1985), could bind to A3 RNA and whether eIF4B can efficiently compete with other cellular proteins for binding to A3 RNA. To this end, UV-irradiation of ^{32}P-labeled oligoA3 RNA and A3ΔA RNA in HeLa S10 cell extracts was performed. Three major proteins were cross-linked to oligoA3 RNA in HeLa cell extracts. One protein migrated at a similar molecular mass (~85 kDa) as eIF4B (Fig. 3.5, lane 1). When the labeled mutant A3ΔA RNA was used, the 85 kDa polypeptide failed to cross-link (lane 2), consistent with the idea that this band corresponds to eIF4B. A similar experiment was performed using a rabbit reticulocyte lysate to examine whether the specific recognition of A3 RNA is conserved in another animal species. An 85 kDa protein cross-linked to oligoA3 (lane 3), but not to A3ΔA RNA (lane 4). To clearly identify the 85 kDa band, UV cross-linking of oligoA3 RNA in HeLa cell extracts was followed by immunoprecipitation with either a polyclonal antibody directed against eIF4B or with pre-immune serum. The 85 kDa protein was immunoprecipitated by the eIF4B antibody (lane 5), but not by the pre-immune serum (lane 6), thus demonstrating that HeLa eIF4B can specifically bind to A3 RNA. Similarly, in the rabbit reticulocyte lysate, this protein was immunoprecipitated by the eIF4B antibody (lane 7), but not by pre-immune serum (lane 8). Thus, native eIF4B from human and rabbit lysates recognizes A3 RNA with the same specificity as recombinant eIF4B.

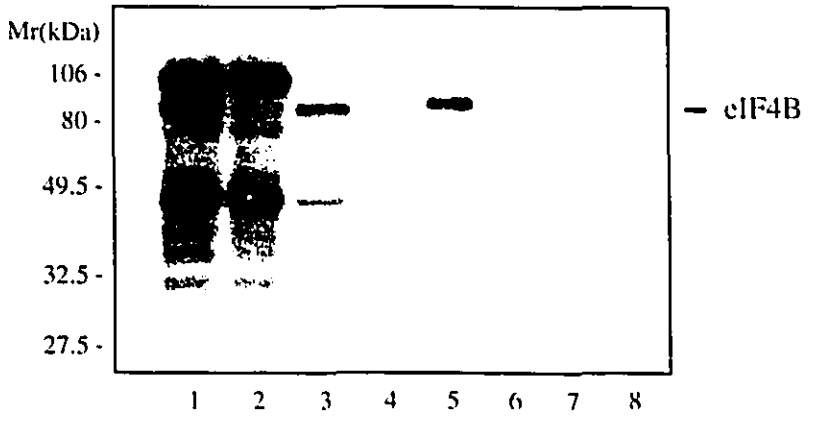

Fig. 3.5. Native eIF4B binds to A3 RNA. ^{32}P-labeled A3 RNA or A3ΔA RNA were UV cross-linked with HeLa or rabbit reticulocytes extracts. Following RNAse A treatment, the proteins marked by label-transfer were resolved on SDS-PAGE. A3 RNA: lanes 1,3, 5-8. A3ΔA RNA: lanes 2 and 4. HeLa extracts: lanes 1,2, 5 and 6. Rabbit reticulocyte lysate: lanes 3,4,7 and 8. The position of eIF4B is marked by an arrowhead. The identity of eIF4B was confirmed by immunoprecipitation with a polyclonal antibody directed against eIF4B (lanes 5 and 7) or with pre-immune serum (lanes 6 and 8).

eIF4B binds 18S rRNA through its RRM

eIF4B binds to ribosomes and it was suggested that this binding is mediated by an interaction with 18S rRNA (Hughes et al., 1993; Naranda et al., 1994). The possibility that the recognition of 18S rRNA occurs specifically through the RRM was examined by UV cross-linking and competition experiments. Poly(A) RNA (5 μg) was added in each assay to block non-specific binding by the carboxy-terminal RNA binding region. Recombinant eIF4B cross-linked efficiently to rat 18S rRNA (nucleotide 1-1678; Fig.3.6A lane 2), as measured by label transfer. The lower molecular weight bands seen in all lanes are eIF4B degradation products, since they are recognized by an antibody directed against eIF-4B (data not shown). Cross-linking was inhibited by up to 5-fold upon addition of excess unlabeled 18S rRNA (lanes 3-6). To determine whether the RRM contributed to the specificity of the binding, competition experiments using unlabeled A3 RNA and A3ΔA RNA against 18S (1-1678) rRNA binding were performed. A3 RNA reduced the cross-linking of 18S rRNA to eIF4B, although less efficiently at lower molar excess ratios, than unlabeled 18S rRNA (lanes 7-9). However, with 300-fold molar excess, A3 RNA showed a marked increase in its ability to compete against 18S RNA, suggesting a possible cooperative effect (lane 10). This result was highly reproducible, as shown in Figure 3.6B, lanes 6-8. A3ΔA, on the other hand, failed to compete, even at a high molar excess (Fig. 4.6A; lanes 11-13). Neither were non-specific RNAs such as tRNA and E. coli 5S RNA able to compete (lanes 14-15 and 16-18, respectively). The ability of globin mRNA to compete for 18S (1-1678) rRNA binding was also tested. Again, 18S (1-1678) and A3 RNA were efficient competitors (Fig. 3.6B; lanes 3-5 and 6-8, respectively; the difference in competition relative to lanes 4-6 of Fig. 4.6A may be due to different RNA preparations used). Globin RNA was a weak competitor, with only a 2-fold reduction in binding at a 300-fold molar excess over labeled 18S RNA (lanes 9-11). These results support the argument that specific binding of eIF4B to 18S rRNA is mediated through the RRM.

Fig. 3.6. eIF4B specifically photocross-links to 18S rRNA. Recombinant eIF4B (0.5 μg) was incubated with labeled 18S rRNA (1-1678) and 5 μg of poly(A) RNA in the presence of a molar excess of unlabeled RNA competitors. The samples were then UV-irradiated, digested with RNAse A and analysed for label transfer on SDS-PAGE as described in Materials and Methods. (A) Lanes: 1, no eIF-4B; 2, no competitor; 3-6, increasing molar excess of unlabeled 18S (1-1678) rRNA; 7-10, increasing molar excess of A3 RNA; 11-13, increasing molar excess of A3ΔA RNA; 14 and 15, tRNA; 16-18, increasing molar excess of *E. coli* 5S RNA. (B) Lanes: 1, no eIF4B; 2, no competitor; 3-5, unlabeled 18S (1-1678) rRNA; 6-8, A3 RNA; 9-11, globin RNA.

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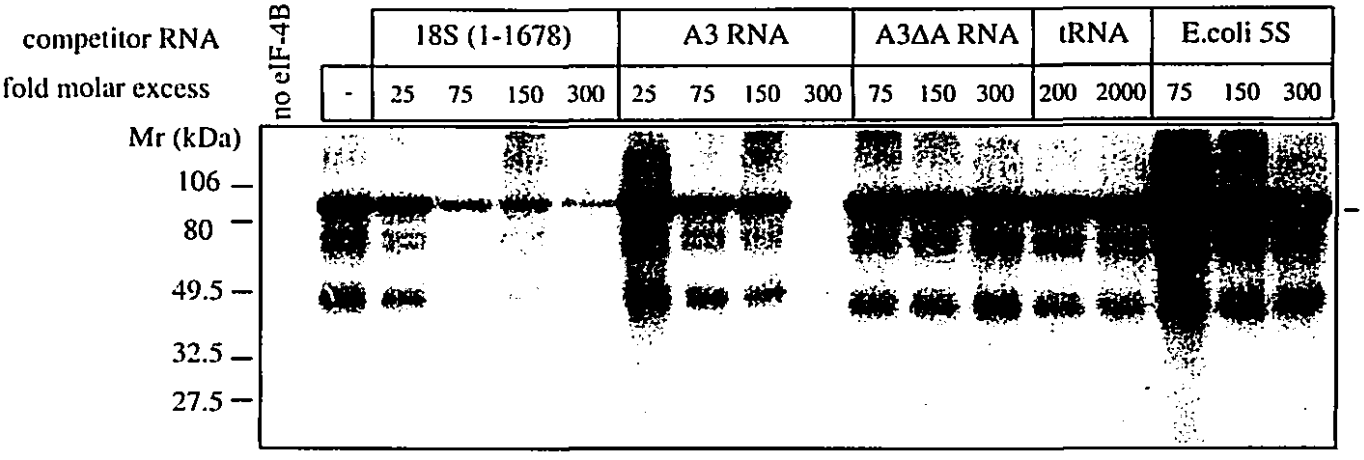

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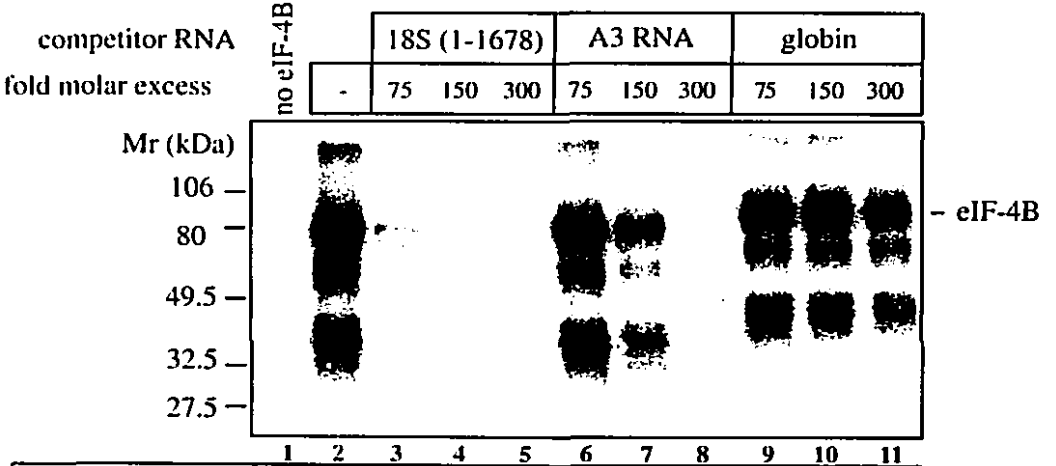
arbitrary units	-	100	71	35	26	19	101	84	66	6	100	94	91	111	78	102	100	100

B



	1	2	3	4	5	6	7	8	9	10	11
arbitrary units	-	100	17	6.5	5	72	39	3	79	63	54

eIF4B can bind simultaneously to two RNA molecules

One of the mechanisms by which eIF4B could promote binding of the mRNA to the 40S ribosomal subunit is by bridging the mRNA and the ribosome. Such a model posits that eIF4B can interact simultaneously with two RNA molecules, namely the 18S rRNA bound to the RRM, and mRNA bound to the carboxy-terminal RNA binding region. To examine directly whether eIF4B could simultaneously bind a specific and a non-specific RNA substrate, eIF4B, N250 or NΔ253 were each incubated with polyA-Sepharose beads and radiolabeled A3 RNA. The amount of A3 RNA bound to poly(A)-Sepharose via an eIF4B bridge was assessed (Fig. 3.7). No A3 RNA was bound to poly(A)-Sepharose in the absence of eIF4B (lane 2). In the presence of eIF4B, approximately 10% of input A3 RNA was recovered on poly(A)-Sepharose (lane 3). Addition of an excess of unlabeled A3 reduced by ~4-fold the amount of labeled A3 RNA bound to poly(A)-Sepharose (lane 4). Predictably, N250 (lane 5) and NΔ253 (lane 6) were incapable of bridging significant amounts of A3 RNA to poly(A)-Sepharose. These results demonstrate that eIF4B can simultaneously bind two RNA molecules and the binding requires both the RRM and the carboxy-terminal RNA binding region for this function.

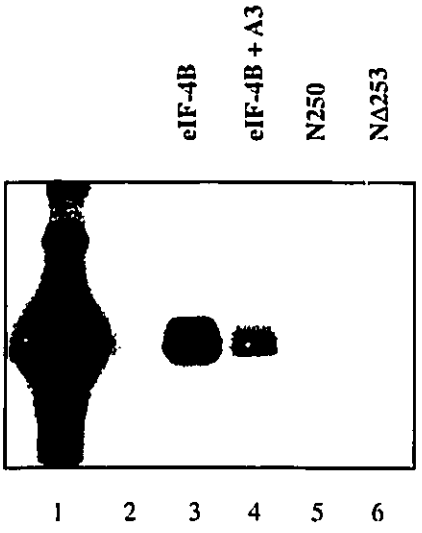


Fig. 3.7. eIF4B can bind two RNA molecules simultaneously. eIF4B, N250 or NΔ253 (7 x 10^{-12} moles) were incubated 5 min at 37°C in a final volume of 50 µl in buffer A containing 15 µl (packed volume) of polyA-Sepharose beads (Pharmacia). A3 RNA (50 000 cpm or 20 pmoles) was added for a further 5 min at 37°C. The beads were washed 3 times with 500 µl of buffer A, and the radioactivity retained was analysed on a SDS-15% polyacrylamide gel and visualized by autoradiography. Lanes: 1, A3 RNA load; 2, poly-A RNA Sepharose beads alone; 3, eIF4B; 4, eIF4B and unlabeled A3 RNA; 5 N250; 6, NΔ253.

Discussion

In this study, *in vitro* RNA selections were performed to determine the RNA binding specificity of the eIF4B RRM. RNAs with conserved primary sequence and secondary structure were generated and exhibited high affinity for the eIF4B RRM. Mutational studies with one of the selected RNAs, A3 RNA, indicate a strict requirement for the conserved nucleotides in a structural context consisting of a bulged stem-loop. This context appears to be flexible, as shown by the variety of the size and nucleotide sequence of the bulge and the loop. These results demonstrate for the first time that the eIF4B RRM binds a specific RNA and defines primary and secondary elements required for specific RNA binding.

The effect of the RNA helicase eIF4A on eIF4B RNA binding was also investigated (Experiment B). eIF4A increases the affinity of eIF4B for RNA by approximately 5-fold, and the increase in activity has been ascribed to the carboxy-terminal RNA binding region (Méthot et al., 1994). The results of experiment B demonstrate that eIF4A does not modify the type of RNA ligands that are preferentially bound by eIF4B. However, an increase in the content of adenosine residues present in pool B RNAs compared to those of pool A RNAs was noticed (Fig.3.1). This change could reflect an activation of the carboxy-terminal RNA binding region, as eIF4A cooperates with eIF4B in RNA binding (Abramson et al. 1988, Méthot et al., 1994). Although in experiment E, iterative RNA selection with the NΔ253 mutant did not yield specific sequences, filter binding studies using unlabeled RNA homopolymers as competitors indicated that the carboxy-terminal RNA binding region interacted preferentially with poly(A) RNA (unpublished results). Furthermore, a preferential interaction between poly(A) and wheat germ eIF4B has been reported (Gallie and Tanguay, 1994). Thus, in the absence of eIF4A, the eIF4B RRM is the dominant RNA binding domain. In the presence of eIF4A and ATP, the carboxy-terminal RNA binding region may contribute to the overall RNA binding affinity.

A role for the specific interaction between eIF4B and its RNA ligand was addressed by searching databases for sequences predicted to adopt a secondary structure similar to that of A3 RNA, using the RNAmot program (Laferriere et al., 1994). A few matches were obtained, but the sequences corresponded to promoter or intron regions. The absence of functionally relevant matches could be attributed to the variability of size and sequence tolerated in the internal and terminal loops of the RNA targets and to limitations of the RNAmot program. For instance, even though A2, A3, A13 and B12 RNA possess different internal and terminal loops, they bound to eIF4B with the same affinity. It is also possible that the selected RNAs do not reflect exactly the natural RNA ligand(s). *In vitro* selections/amplifications are performed under conditions in which the type of interactions that can modulate RNA-protein interactions *in vivo* are absent, and the size of the randomized region of the initial RNA target (i.e. the complexity of tertiary structures) is limited. Selected RNAs may represent optimal sequences for binding under *in vitro* conditions and may not necessarily have natural counterparts with the same affinity for the protein target. The selection/amplification experiment against hnRNPA1 (Burd and Dreyfuss, 1994) provides an example. High affinity RNA ligands to hnRNPA1 contain sequences resembling 5' and 3' splice sites, and bind hnRNPA1 with a dissociation constant of 1 nM. Such RNA ligands are consistent with the role of hnRNPA1 in alternative splicing (Mayeda et al., 1992). When the affinity of this protein for the authentic 3' or 5' splice site of the human ß-globin gene was measured, a K_d of 70 nM was found. This value is lower than the K_d measured for the intron of ß-globin (300 nM) but it is still considerably higher than the optimal selected sequence. Thus, iterative *in vitro* selection can generate RNAs that can bind to their target with a higher affinity than to natural ligands. Selection/amplification did succeed in recreating the natural RNA binding sites of proteins whose targets were already known, such as Bacteriophage T4 DNA polymerase, U1A snRNP, HIV-1 Rev and IRF (Tuerk and Gold, 1990; Tsai et al., 1991; Bartel et al., 1991;

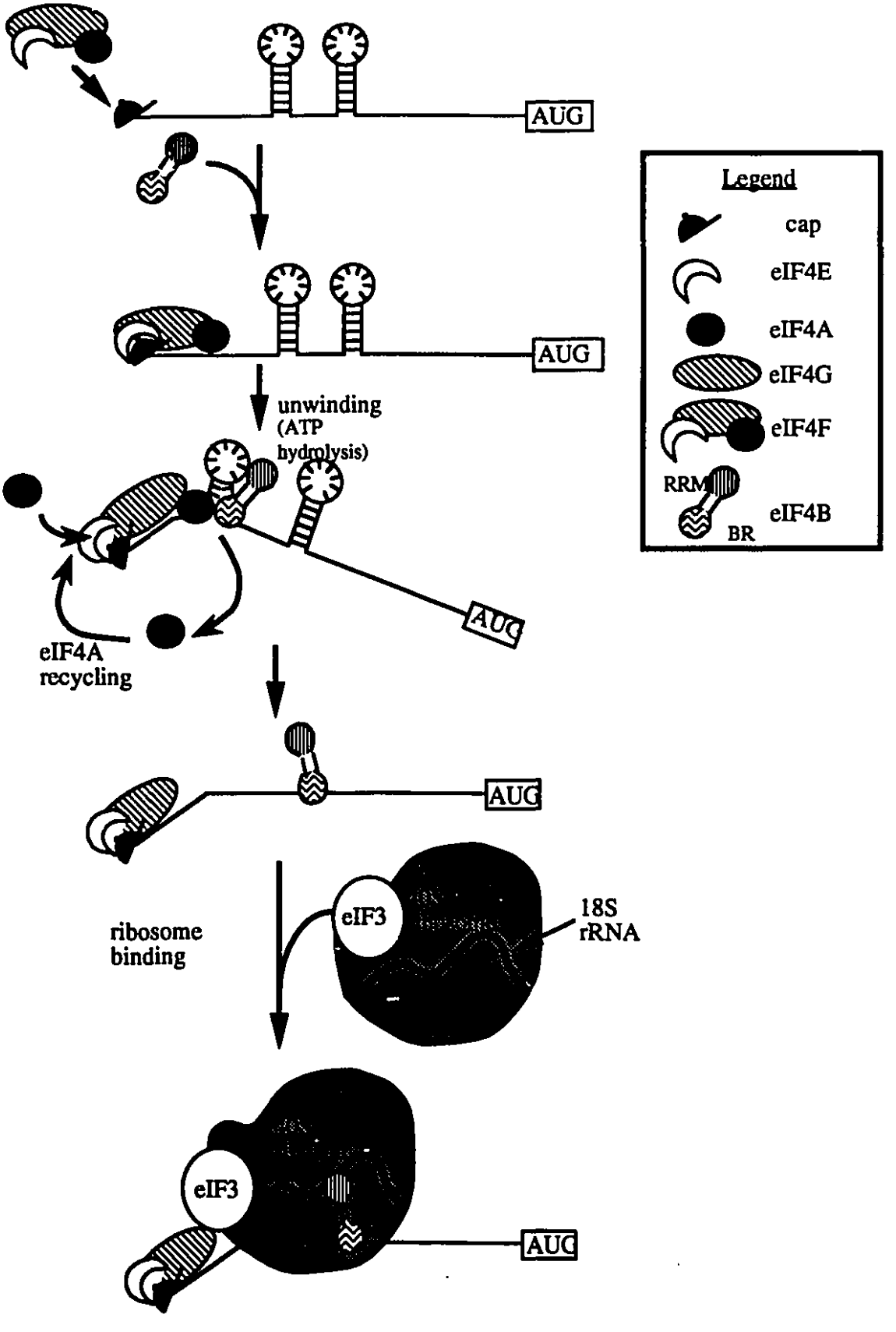
Henderson et al., 1994). In the case of T4 DNA polymerase and IRF, however, two groups of high affinity RNA ligands were obtained, showing that more than one valid solution can be found for a single problem (Tuerk and Gold, 1990; Henderson et al., 1994).

Given the characteristics of eIF4B and its ability to bind ribosomes (Hughes et al., 1993) the 18S rRNA seemed a logical ligand for this factor. Specific association of eIF4B with 18S rRNA was shown by UV cross-linking and competition studies (Fig. 3.6). On a molar basis, 18S rRNA was the best competitor relative to other RNAs such as globin, *E. coli* 5S rRNA, and tRNA. The fact that A3 RNA, but not A3ΔA, was able to compete against 18S rRNA (albeit to a lesser extent at lower molar ratios than 18S rRNA itself) suggests that the RRM is responsible for mediating RNA binding. We identified a region in the rat 18S rRNA (nucleotides 465 to 645; Chan et al., 1984) that contains the conserved GGAC sequence in the consensus folding. However, deletion of the GGAC sequence from an *in vitro* synthesized 18S rRNA containing nucleotides 465 to 645 did not reduce RNA binding by the N250 fragment (data not shown). It is possible that other parts of the 18S rRNA, or other protein factors, may contribute to the binding, or that the 465-645 region of rRNA does not fold properly when isolated.

The two RNA binding regions of eIF4B appear to provide a bridge between two different RNA molecules. Full length eIF4B was able to bridge A3 RNA and poly(A) RNA. N250, which binds A3 RNA with high affinity but lacks the carboxy-terminal RNA binding region, was unable to provide a significant bridge. Similarly, NΔ253, which lacks the RRM but still contains the carboxy-terminal RNA binding region, could not interact with A3 RNA and poly(A)-Sepharose simultaneously.

The data presented here suggest a model for the role of eIF4B during translation initiation (Fig. 3.8). According to this model, eIF4F binds to the cap structure of the mRNA and properly positions the initiation factors eIF4A and eIF4B on the 5' UTR. These proteins are thought to unwind the secondary structure in the 5' UTR to create a ribosome binding site. One of the features which directs the 40S subunit to the 5' UTR, and not to other unstructured areas of the mRNA, could be a direct interaction between the eIF4B RRM and sequences in 18S rRNA . Our data are compatible with a model proposed by Altmann et al. (1995), who have shown that eIF4B possesses RNA annealing activity, and suggested that eIF4B promotes rRNA-mRNA base pairing. Thus, the simultaneous interaction of eIF4B with the mRNA 5' UTR and with rRNA provides a means by which the ribosome is targeted to the proper region of the mRNA, upstream of the initiation codon.

Fig. 3.8. Model for the role of eIF4B during translation initiation. eIF4B contributes to the binding of the 40S ribosome by bridging the 18S rRNA and the mRNA. The steps leading to ribosome binding are the following: eIF4F binds to the cap-structure of the mRNA, and is later joined by eIF4B. These factors unwind the proximal mRNA 5' secondary structure in an ATP-dependent manner, creating an area of single stranded RNA accessible for ribosome binding. This process also requires the cycling of eIF-4A through the eIF-4F complex (Pause et al., 1994). eIF4B remains bound to the mRNA via its carboxy-terminal RNA binding region. The 40S ribosomal subunit binds to the mRNA via a specific interaction between the eIF4B RNA Recognition Motif (RRM) and the 18S rRNA. Another contributing factor for ribosome binding is eIF3 via its interaction with eIF4G (Lamphear et al., 1995). BR; basic region. cap; ^{m7}Gppp.


AUG
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Legend
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eIF4E
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RRM
BR
eIF4B

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Chapter 4

A region rich in aspartic acid, arginine, tyrosine and glycine (DRYG) mediates eIF4B self association and interaction with eIF3

Abstract

The binding of mRNA to the ribosome is mediated by eukaryotic initiation factors eIF4F, eIF4B, eIF4A, and eIF3. eIF4F binds to the mRNA cap structure and, in combination with eIF4B, is believed to unwind the secondary structure in the 5' untranslated region (UTR) to facilitate ribosome binding. eIF3 associates with the 40S ribosomal subunit prior to mRNA binding. eIF4B copurifies with eIF3 and eIF4F through several purification steps, suggesting the involvement of a multisubunit complex during translation initiation. To understand the mechanism by which eIF4B promotes 40S ribosome binding to the mRNA, we studied its interactions with partner proteins using the filter overlay assay ("Far Western") and the two hybrid system. Here, we show that eIF4B self associates and also interacts directly with the p170 subunit of eIF3. A region rich in aspartic acid, arginine, tyrosine and glycine, termed DRYG domain, is sufficient for self association of eIF4B, both *in vitro* and *in vivo*, and for interaction with the p170 subunit of eIF3. These experiments suggest that eIF4B participates in mRNA-ribosome binding by acting as an intermediary between the mRNA and eIF3, via a direct interaction with the p170 subunit of eIF3.

Introduction

Initiation of translation in eukaryotes is thought to occur by binding of the 40S ribosomal subunit at or near the cap structure of a mRNA, followed by ribosome scanning of the 5' untranslated region until the initiator AUG is encountered (Mathews et al., 1996). Initiation of translation requires the assembly of macromolecular complexes, both on the 40S ribosome and on the mRNA. Eukaryotic initiation factors (eIFs) 1A and 3 associate with the 40S ribosomal subunit prior to the mRNA binding step. eIF1A and eIF3 inhibit joining of the 40S and 60S ribosomal subunits in the absence of mRNA (Nakaya et al., 1973; Benne & Hershey, 1976; Goumans et al., 1980). The next event in the assembly of translation-competent 40S ribosomal subunits is the binding of a ternary complex composed of eIF2, GTP and met-tRNA$_i$, an association which is stabilized by eIF3 (Benne & Hershey, 1978; Peterson et al., 1979; Goumans et al., 1980). This complex is referred to as the 43S pre-initiation complex.

A pre-requisite for binding of the mRNA to the 43S pre-initiation complex is the interaction of several translation initiation factors with the mRNA. The first translation factor to make contact with the mRNA is eIF4F. This factor is a heterotrimer comprised of i) eIF4E, a 24 kDa protein which binds the cap structure present at the 5' end of all cellular mRNAs (Sonenberg et al, 1978). ii) eIF4A, a bidirectional RNA helicase (Ray et al., 1986; Rozen et al., 1990), and iii) eIF4G, a 220 kDa polypeptide to which both eIF4E and eIF4A bind directly (Mader et al., 1995; Lamphear et al., 1995). eIF3 has also been shown to interact with the carboxy-terminus of eIF4G, and has thus been postulated to serve as a link between the mRNA and the 40S ribosome (Lamphear et al., 1995).

eIF4B facilitates the binding of the mRNA to the 43S pre-initiation complex, as demonstrated by reconstitution studies of an *in vitro* translation system (Trachsel et al., 1977). eIF4B is an RNA-binding protein with a canonical RNA Recognition Motif (RRM; for reviews see Mattaj et al., 1993; Burd and Dreyfuss, 1994b; Nagai et al., 1995) near its amino-terminus, and an arginine-rich RNA binding region in the carboxy-terminus (Méthot

et al., 1994; Naranda et al., 1994). The arginine-rich region binds RNA non-specifically, while the RRM binds specifically to 18S rRNA (Méthot et al., 1996). eIF4B can associate with a specific RNA target and a non-specific RNA simultaneously, suggesting that it may serve to facilitate the binding of the 40S ribosomal subunit to the mRNA by acting as a bridge between the mRNA and the 18S rRNA (Méthot et al., 1996). Besides its RNA binding activity, eIF4B stimulates the ATPase (Grifo et al., 1984; Abramson et al., 1987) and RNA helicase activities of eIF4A (Ray et al., 1985; Rozen et al., 1990). A functional interaction between eIF4A and eIF4B *in vivo* is also evident since yeast eIF4B, expressed from a multicopy plasmid, is able to complement a temperature-sensitive mutant of eIF4A (Coppolecchia et al., 1993). More recently, mammalian and yeast eIF4B were shown to possess RNA annealing activity (Altmann et al., 1995). It is thought that eIF4F, properly positioned on the mRNA near the cap structure via the eIF4E subunit, unwinds RNA secondary structure in the 5' untranslated region of the mRNA, in conjunction with eIF4B, to create a site accessible for ribosome binding (Sonenberg, 1988). This model is consistent with the observation that secondary structure in the 5' UTR of a mRNA is inhibitory to translation (Pelletier et al, 1985; Baim et al., 1985). Furthermore, a mRNA with extensive 5' UTR secondary structure is better translated in cells that overexpress eIF4E (Koromilas et al., 1992), but translates poorly in a yeast strain that has been disrupted for the eIF4B gene, TIF3 (Altmann et al., 1993).

To gain further understanding into how eIF4B promotes binding of mRNA to the 40S ribosomal subunit, we examined protein-protein interactions mediated by eIF4B. We demonstrate that recombinant eIF4B self associates *in vitro* and *in vivo*. We mapped the homotypic interaction site of eIF4B to a 99 amino acid region rich in aspartic acid, arginine, tyrosine and glycine (DRYG-rich region; Milburn et al., 1990). Filter overlay assays ("Far Western" ; Blanar & Rutter, 1992) on cell extracts reveal that eIF4B interacts with the p170 subunit of eIF3, and that this interaction is also carried out by the DRYG domain.

Materials and Methods

Vectors, proteins:

The construction of pGEX4B, N367, N355, N312, N250, N171, NΔ253 and Δ230-247 has been previously described (Méthot et al., 1994). pGEXN213 was obtained by digesting pGEX4B with EcoNI and SmaI. The vector-containing fragment was blunt-ended with the Klenow fragment of *E. coli* DNA polymerase and religated. pGEXNΔ180 and NΔ352 were generated by exonuclease III/Mung Bean nuclease digestion, as described in Méthot et al., 1994. pGEX 180-312 was created by digesting pGEXNΔ180 with SacI and SmaI. The vector-containing fragment was blunt-ended and religated. pGEX2THMK 213-312 was obtained by digesting pGEX-4B with EcoNI and SacI. The 300 bp fragment was blunt-ended and ligated into pGEX2T[128/129] (Blanar & Rutter, 1992) that had been linearized with EcoRI and blunt-ended. pGEX2THMK-4B was constructed by excising the BamHI fragment from pGEX-4B, blunt-ending it and ligating into pGEX2T[128/129] that had been linearized with EcoRI and blunt-ended. Due to the leakiness of the tac promoter and the extensive degradation of the resulting GST-eIF4B fusion protein, the GST-FLAG-HMK-eIF4B fusion was put under the control of the T7 promoter. An NdeI site was created at the initiator ATG codon of the GST cDNA, by polymerase chain reaction (PCR), using the primers AACAGTACATATGTCCCCTATACTAG and CTGTGCCAAGTGGTCG, and pGEX-4B as template. The PCR product was digested with NdeI and BstXI, and ligated to the BstXI-PstI fragment excised from pGEX4B and to pET3b (Studier et al., 1990) that had been cut with NdeI and PstI. The resulting vector, pET2THMK-4B, generates a GST-FLAG-HMK fusion protein with eIF4B which is under the control of the T7 promoter. The GSTHMK-eIF4B fusion protein was overexpressed in *E. coli* K38 cells, and induction was performed by heat shock at 42°C for 30 min. All GSTHMK-eIF4B fragments were purified on glutathione-Sepharose (Pharmacia). GSTHMK-eIF4B wt was further purified on an Heparin EconoPak column (BioRad), as described previously for GST-eIF4B (Méthot et al., 1994). For the yeast two hybrid

system expression vectors, pGBT9 and pGADGH, which carry the GAL4 DNA binding domain (GAL4-DB) and the GAL4 transactivator domain (GAL4-TA), respectively, were used (gifts from P. Bartel, State University of New York, Stony Brook and G. Hannon, Cold Spring Harbor Laboratory). pGBT9-4B was constructed by cutting pGEX-4B with BamHI. The excised eIF4B DNA was ligated in pGBT9 linearized with BamHI. For pGADGH-4B, the BamHI fragment from pGEX-4B was blunt-ended with the Klenow fragment of *E. coli* DNA polymerase, and ligated into pGADGH digested with SmaI. To generate N312 GAL4-DB fusions, pGBT9-4B was digested with SacI or EcoNI, respectively, and PstI. Similarly, N312 GAL4-TA fusions were generated by cutting pGADGH-4B with SacI or EcoNI, respectively, and SalI. Following blunt-ending with T4 DNA polymerase, the vector-containing fragments were religated. Recombinant eIF4B was purified as described (Pause et al., 1992).

Far Western Analysis

Purified GST-HMK-eIF4B fusions (1-3 μg) were ^{32}P-labeled using heart muscle kinase (Sigma) as described (Blanar & Rutter, 1992). Cellular extracts and *E. coli* extracts expressing various fragments of eIF4B were resolved by SDS-polyacrylamide gel electrophoresis and blotted on PVDF membranes (Millipore) or nitrocellulose. Immobilized proteins were denatured by incubating the membranes with 6M urea in HBB buffer (25 mM Hepes-KOH pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT). The proteins were renatured *in situ* by by a series of 10 minute incubations in 3M urea/HBB, 1.5M urea/HBB, 0.75M urea/HBB, 0.38M urea/HBB and 0.18M urea/HBB. Although not necessary, we found that these denaturation-renaturation steps improved the sharpness of the signal. The membranes were blocked with 5% milk in HBB, and incubated overnight in hybridization buffer (20 mM Hepes-KOH pH 7.5, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, 1% milk) containing the ^{32}P-labeled probe at 250 000 cpm/ml and

unlabeled purified GST at 1 μg/ml. The membranes were washed 3 times with hybridization buffer and processed for autoradiography.

Two-Hybrid System Analysis

Yeast strain Y526 (Bartel et al., 1993) was transformed with various combinations of the yeast expression constructs by the lithium acetate method (Gietz et al., 1992). Identification of colonies with a reconstituted GAL4 activity was performed by an X-Gal colony filter assay. Colonies were replica-plated onto a nitrocellulose filter (Hybond-N, Amersham) and permeabilized by immersion in liquid nitrogen. The filters were placed on Whatman 3MM paper which had been soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) containing 1 mM X-Gal, and incubated overnight at 37°C to allow for color development. For quantitative β-galactosidase assays, randomly picked colonies were grown in liquid selection medium before the cells were harvested and lysed with glass beads. β-galactosidase was quantitated as described (Rose & Botstein, 1983). Expression of GAL4-DB and GAL4-TA fusion proteins was monitored by Western blotting. Cells (3×10^7) were lysed with glass beads in Laemmli buffer, and loaded on an SDS-12% polyacrylamide gel and transferred to a PVDF membrane. Fusion proteins were detected using an antibody raised against GAL4 which can detect both the DNA binding domain and the transactivator domain of GAL4 (a generous gift from James Hopper, Pennsylvania State University).

Immunoprecipitations

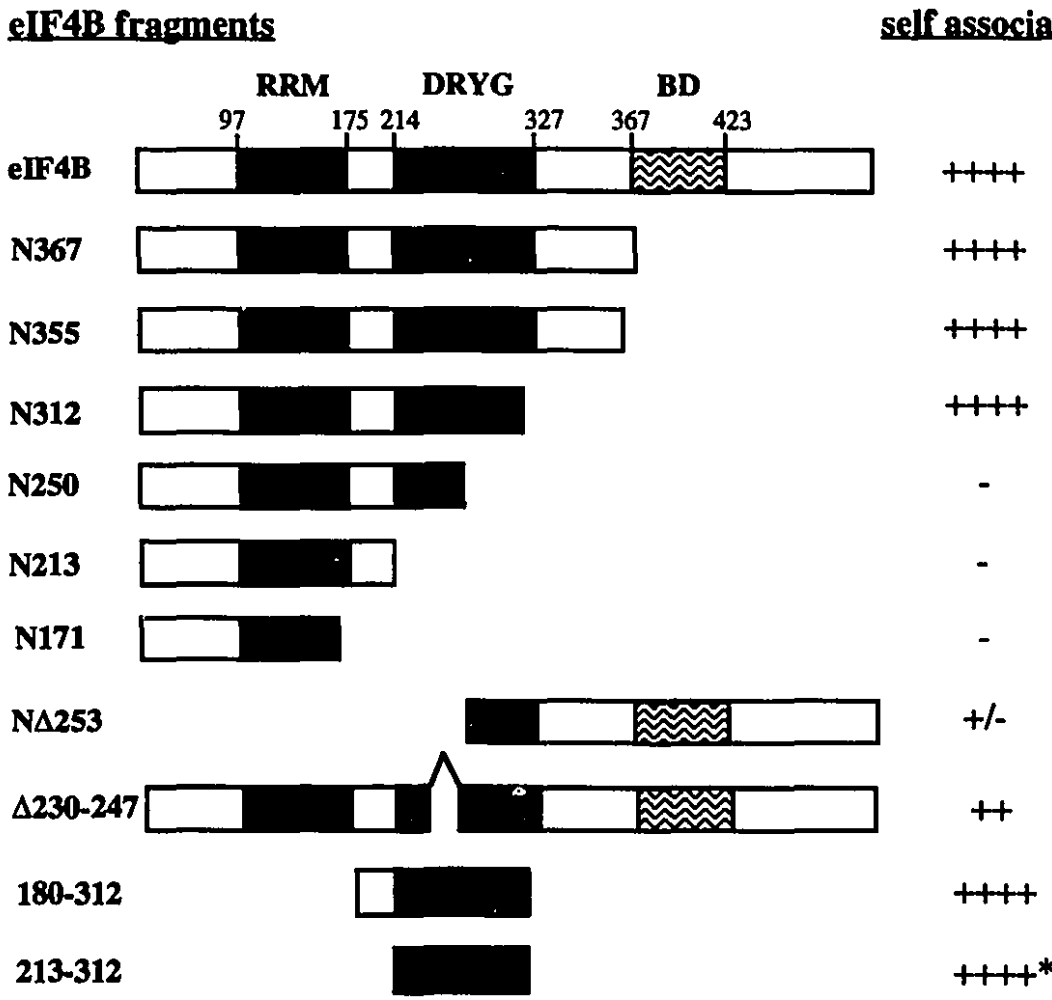
HeLa R19 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100). Nuclei and cellular debris were removed by centrifugation. Antibodies (α-eIF4B polyclonal 706, pre-immune 706 and α-eIF4B monoclonal 9E11) were added to 200 μg of cell extract and incubated on ice for 30 min. A 50% suspension (50 μl) of protein A-sepharose (Repligen) that had been

washed in lysis buffer and pre-incubated with BSA, was added to the mixture, and incubated end-over-end at 4°C for 90 min. The beads were washed 6 times with 1 ml of lysis buffer and boiled in Laemmli buffer to which 100 mM of β-mercaptoethanol had been freshly added. Proteins were resolved on SDS- 8% or 11% polyacrylamide gels, and transferred onto PVDF membranes. Immunoblotting was performed with α-human eIF4B (monoclonal 9E11), goat α-rabbit eIF3 (generous gift from J. Hershey), and α-actin (ICN), and immunoreactive proteins were visualized by chemiluminescence (ECL, Amersham).

Results

The self association site of eIF4B maps to the DRYG-rich region

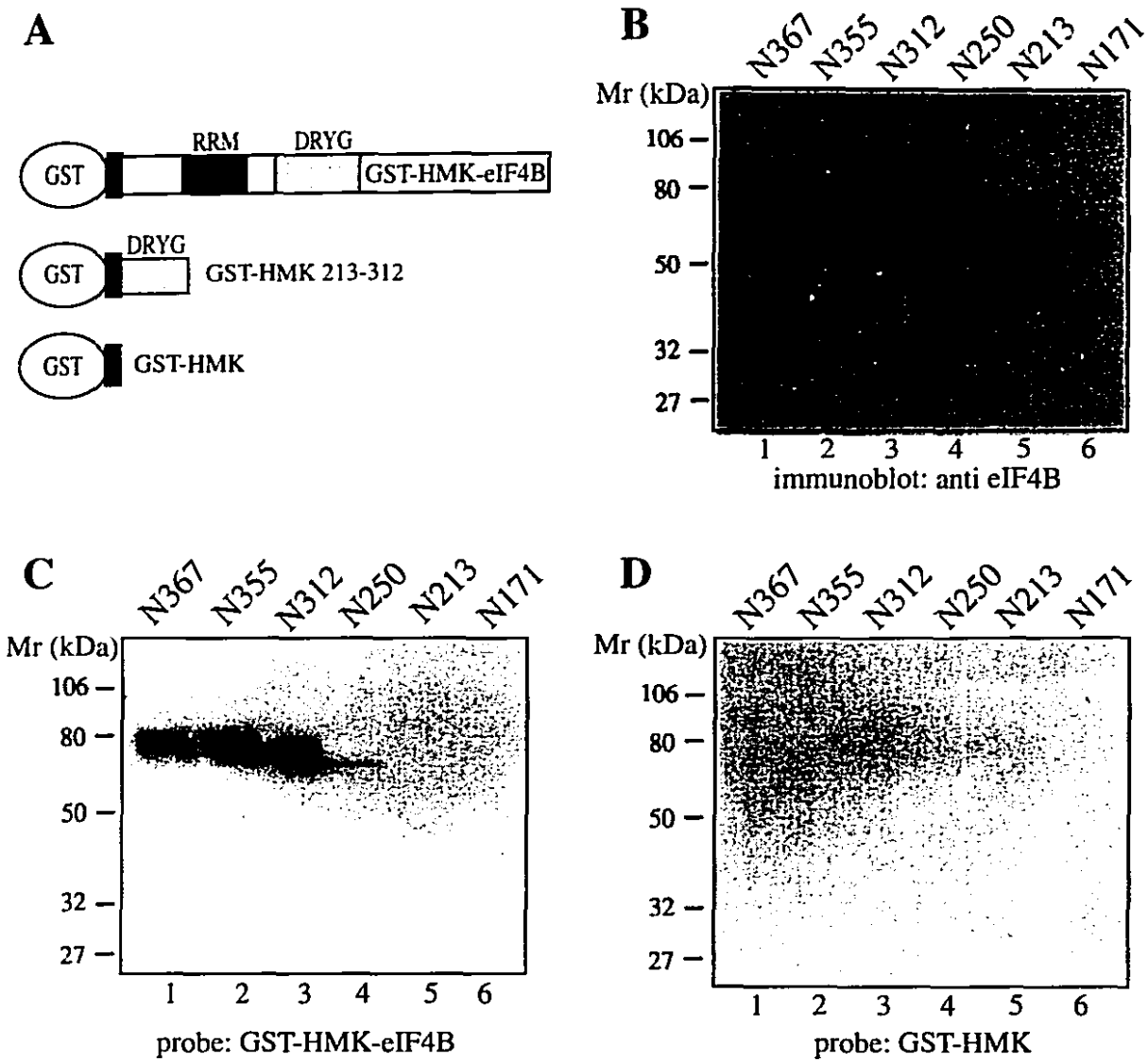
Human eIF4B is a 69 kDa protein that migrates aberrantly on SDS-polyacrylamide gels at 80 kDa (Milburn et al., 1990). The molecular mass of rabbit reticulocyte eIF4B, as determined by gel-filtration chromatography in the presence of 0.5 M KCl, is 220 kDa, which led to the suggestion that eIF4B is an asymmetric dimer (Grifo, 1982). Previous structure/function analyses, using a series of truncated eIF4B proteins, identified regions responsible for sequence non-specific and specific RNA binding and eIF4A helicase stimulation (Méthot et al., 1994; Naranda et al., 1994; Méthot et al., 1996). eIF4B fragments were used here to map the self association region of eIF4B by a filter overlay assay (Far Western; Blanar & Rutter, 1992). Figure 4.1 illustrates the various truncations which were used, and summarizes the results. *E. coli* crude extracts expressing different C-terminal truncations of eIF4B were resolved on SDS-polyacrylamide gels, transferred to PVDF membranes and probed with a ^{32}P-labeled GST-eIF4B fusion protein tagged with the heart muscle kinase (HMK) phosphorylation site (Fig. 4.2A, upper construct). To monitor background dimerization due to GST-GST interactions (Trakshel & Maines, 1988), duplicate filters were probed with HMK-tagged GST (Fig. 4.2D, lower construct). Background was minimized by the addition of a large excess of unlabeled GST protein in the hybridization buffer. The different GST-fusion eIF4B proteins were expressed to similar levels, as determined by Western blotting (Fig. 4.2B). All fragments of eIF4B retaining the amino-terminal 312 amino acids of the protein were capable of interacting with eIF4B (Panel C, lanes 1 to 3; for wt eIF4B, see Fig. 4.3B). Further deletions generated eIF4B fragments that were unable to associate with eIF4B (fragments N250, N213 and N171; lanes 4 to 6. The faint band seen for N250 is artifactual since it does not migrate exactly with the N250 protein ; compare lane 4 from Fig. 4.2B and 4.2C, and is not reproducible). ^{32}P-labeled GST-HMK failed to interact with any of the fragments of eIF4B



* determined from its reactivity as a probe

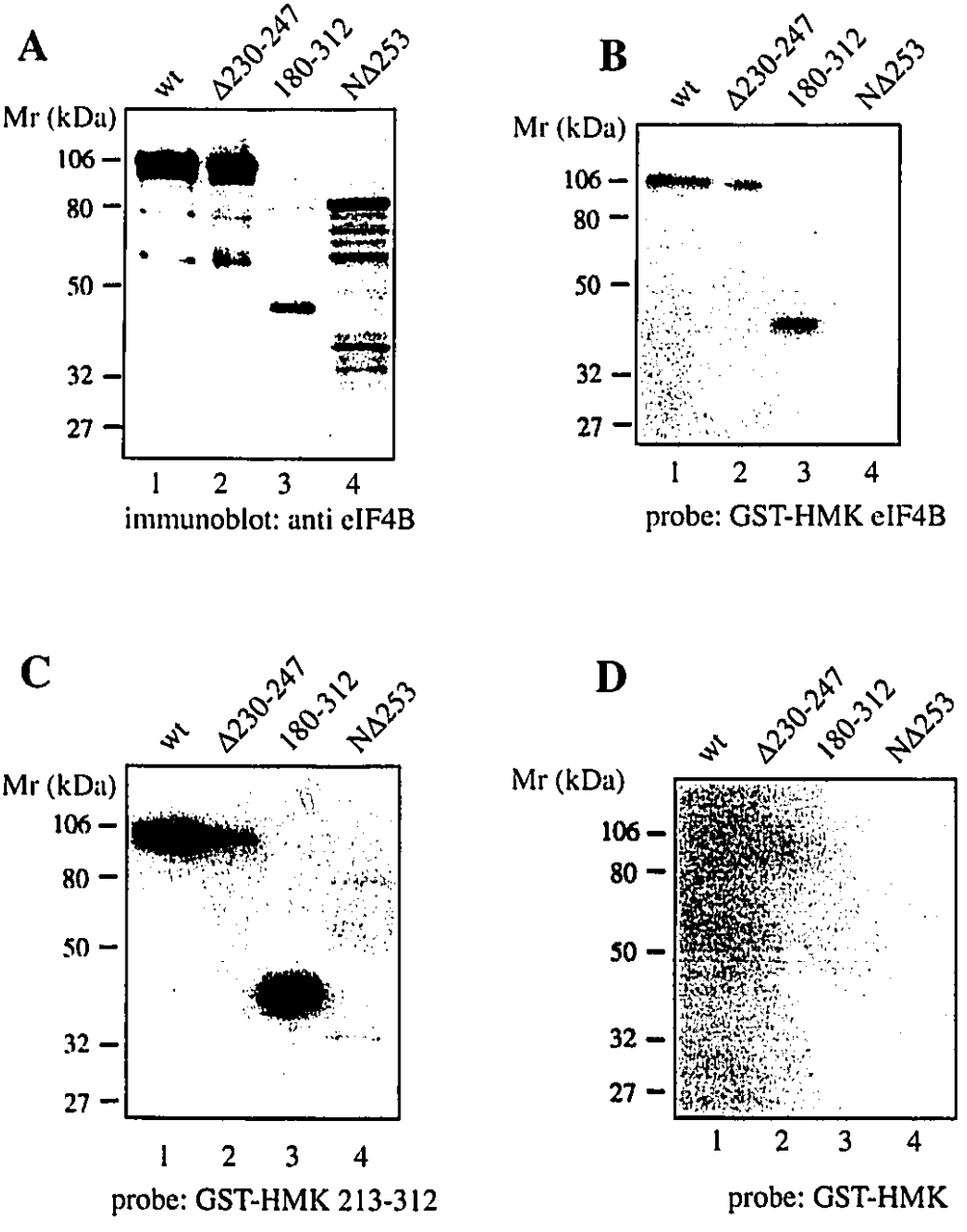
Fig. 4.1. Schematic representation of eIF4B fragments used in this study. The RNA Recognition Motif (RRM), the DRYG-rich region , while the carboxy-terminal RNA binding site, or basic domain (BD) are indicated. Also shown is summary of the self association activity of each of these fragments, as determined by Far Western analysis. ++++ : 100%; ++: 25-50%; +/-: 5% or less.

Fig. 4.2: Mapping of the eIF4B self association site. Panel A. GST-HMK probes used in this study. The heart muscle kinase phosphorylation site is represented by a grey rectangle. Also shown are the RNA Recognition Motif (RRM) and the DRYG-rich region. Panel B. Immunoblot using monoclonal α-eIF4B 12B1 on *E. coli* extracts expressing various fragments of eIF4B. Panel C. Far Western analysis on a similar membrane as shown in panel B, with ^{32}P-labeled GST-HMK-eIF4B as probe. Panel D. GST-GST interaction background. An identical membrane as shown in Panel D was probed with ^{32}P-labeled GST-HMK probe. Under the conditions used, no GST-GST interaction was observed.


A
RRM
DRYG
GST
GST-HMK-eIF4B
DRYG
GST
GST-HMK 213-312
GST
GST-HMK
B
N367
N355
N312
N250
N213
N171
Mr (kDa)
106
80
50
32
27
1 2 3 4 5 6
immunoblot: anti eIF4B
C
N367
N355
N312
N250
N213
N171
Mr (kDa)
106
80
50
32
27
1 2 3 4 5 6
probe: GST-HMK-eIF4B
D
N367
N355
N312
N250
N213
N171
Mr (kDa)
106
80
50
32
27
1 2 3 4 5 6
probe: GST-HMK

(Panel D), excluding the possibility that the signal arise from GST-GST interaction. The results suggest that the self association domain of eIF4B lies between amino acids 213 and 312, with the core domain possibly residing between residues 250 to 312. Amino acids 213 to 312 encompass the DRYG-rich region of eIF4B (Milburn et al, 1990). To determine whether this region can mediate homotypic interactions independently from other regions of eIF4B, four kinds of experiments were performed: i) a fragment encompassing amino acids 180 to 312 was tested for its ability to interact with eIF4B. ii) An amino-terminal deletion fragment (NΔ253) that contains the carboxy-terminal half of the DRYG region was tested for interaction with the GST-HMK-eIF4B to determine whether portion 254 to 312 of the DRYG region is capable of supporting eIF4B self association. iii) a GST-HMK fusion encompassing amino acids 213 to 312 of eIF4B was used as a probe in the filter overlay assay. iv) Amino acids 230 to 247 were removed from the DRYG-rich region to create the Δ230-247 mutant. The relative amounts of GST-eIF4B wild type (wt), Δ230-247, 180-312 and NΔ253 were estimated by Western blotting with a polyclonal antibody against eIF4B (Fig. 4.3, panel A).When an identical blot was probed with ^{32}P-labeled GST-HMK-eIF4B, a 3-fold decrease in the ability of Δ230-247 to interact with the probe, relative to the wt, was observed (Panel B; compare lanes 1 and 2). Fragment 180-312 reacted with the probe as well as wt eIF4B (lane 3), while NΔ253 failed to react (lane 4). Another fragment (NΔ352) was also unable to interact with eIF4B (data not shown). The GST-HMK 213-312 probe (Fig. 4.2A, middle construct) interacted with various eIF4B truncations in a similar fashion as full length eIF4B (Panel C), except for NΔ253, which showed a very weak interaction (lane 4). Taken together with the previous results, these experiments clearly demonstrate that the region between amino acids 213 and 312 is necessary and sufficient to mediate eIF4B homotypic interactions. The inability of NΔ253 to react with the probe at levels comparable to wt suggests that amino acids 254 to 312 alone are not sufficient to mediate an interaction. Also, amino acids 230 to 247 contribute to the self association function.

Fig. 4.3: The DRYG domain is sufficient to mediate self association. Panel A. Immublot using polyclonal α-eIF4B antibody 706 on *E. coli* extracts expressing GST-eIF4B fusions wt, Δ230-247, 180-312 and NΔ253. Panel B. Far Western analysis on a similar membrane as shown in panel B, with ^{32}P-labeled GST-HMK-eIF4B as probe. Panel C. Same as in Panel B, except that ^{32}P-labeled GST-HMK 213-312 was used as a probe. Panel D. Same as in B and C except that ^{32}P-labeled GST-HMK was used as a probe to monitor the GST dimerization background.


A
wt
Δ230-247
180-312
NΔ253
Mr (kDa)
106
80
50
32
27
1 2 3 4
immunoblot: anti eIF4B
B
wt
Δ230-247
180-312
NΔ253
Mr (kDa)
106
80
50
32
27
1 2 3 4
probe: GST-HMK eIF4B
C
wt
Δ230-247
180-312
NΔ253
Mr (kDa)
106
80
50
32
27
1 2 3 4
probe: GST-HMK 213-312
D
wt
Δ230-247
180-312
NΔ253
Mr (kDa)
106
80
50
32
27
1 2 3 4
probe: GST-HMK

eIF4B forms dimers in solution and *in vivo*

The results obtained by the Far Western analysis were further supported by chemical cross-linking assays. Ethylene glycol*bis*(succinimidylsuccinate; EGS) was used as a homobifunctional cross-linking reagent that reacts with primary amines. All reactions, including a non-cross-linked control, were carried out with equal amounts of eIF4B. In the absence of EGS, eIF4B migrated at an estimated molecular mass of 83 kDa on an SDS-PAG (Fig. 4.4, lane 1). Addition of small amounts of EGS (0.1 to 1 mM) resulted in the dose-dependent formation of a high molecular mass complex migrating at 176 kDa , which is consistent with eIF4B dimerization. Cross-linking was resistant to KCl concentrations of 0.5 M, and was not enhanced by the presence of eIF4A or RNA (data not shown). The diffuse mobility of eIF4B monomers in the presence of cross-linking reagents has been observed elsewhere (Parsell et al., 1994), but is not understood. The self association region (amino acids 213 to 312) contains only one lysine residue, perhaps explaining why EGS cross-linking occurred at low efficiency.

Finally, the yeast two hybrid system (Fields & Song, 1989) was used to determine whether eIF4B can self associate *in vivo*. Fragments of eIF4B (N312 and 213-312) were expressed both as GAL4 transactivator domain fusions proteins and GAL4 DNA binding domain fusions proteins, and a series of combinatorial pairs were tested for their ability to reconstitute GAL4 transactivation activity. The level of expression in yeast was determined by Western blotting using a polyclonal antibody directed against GAL4 (Fig. 4.5). Both GAL4 transactivation domain fusions were detected (lanes 2 and 4). On the other hand, none of the DNA binding fusions, nor the GAL4 DNA binding domain generated by the pGBT9 vector, were detected. After a very long exposure, however, very small amounts of DNA binding domain fusion proteins could be seen (data not shown). The reasons for

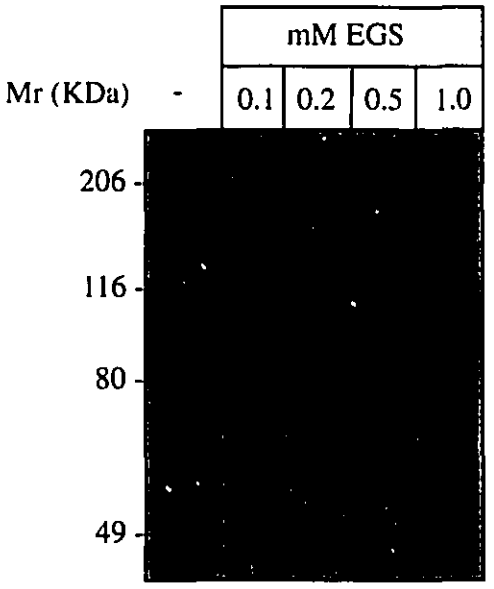


Fig. 4.4: Recombinant eIF4B can form dimers *in vitro*. Purified recombinant eIF4B (1 µg or 14 pmoles) was incubated for 20 min at 25°C with homobifunctional cross-linker EGS [ethylene glycol *bis*(succinimidylsuccinate); Pierce], at the indicated concentration, in a final volume of 20 µl, in 20 mM Hepes-KOH, pH 7.5, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100. The reactions were stopped by the addition of 5 µl of 1M Tris-HCl, pH 8.0, and Laemmli buffer. The samples were boiled and resolved on an SDS-8% polyacrylamide gel, blotted onto a PVDF membrane (Millipore) and probed with monoclonal antibody 9E11 directed against eIF4B. The signal was detected using the Renaissance chemiluminescence system (DuPont).

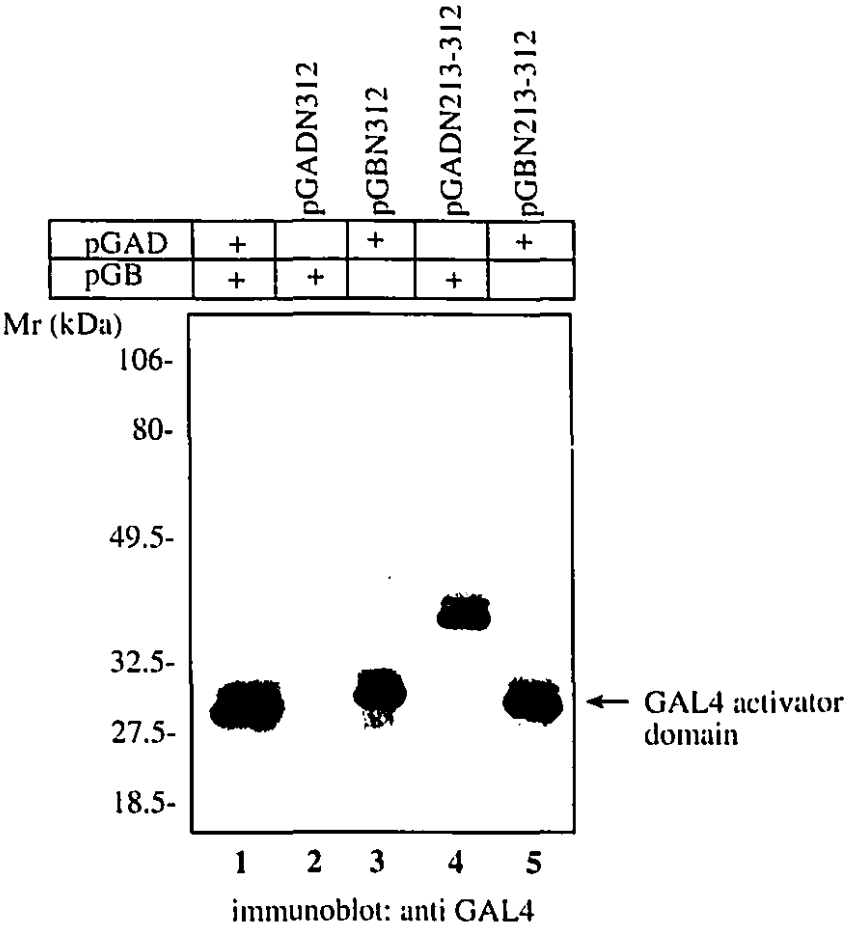


Fig.4.5: Expression of GAL4-eIF4B fusion proteins used in the two-hybrid system analysis. Yeast cells (3 x 10^7) transformed with pGAD-eIF4B and pGBT9 or pGB-eIF4B and pGADGH were lysed with glass beads in Laemmli buffer and blotted onto a nitrocellulose membrane. GAL4 fusion protein levels was determined using an antibody directed against GAL4, which can recognize both the activator domain and the DNA binding domain of the protein.

Table 4.1 two hybrid system

DNA binding (pGBT9)	Transactivation (pGADGH)	colony color	β-galactosidase activity [a] (fold over background)
N312	N312	blue	348
N312	213-312	white	1.0
213-312	N312	light blue	14.7
213-312	213-312	white	1.6

[a] Values represent the level of β-galactosidase activity above background levels, and are averaged from at least three independent assays.

the low expression levels of GAL4 DNA binding domain fusion proteins are unknown, but could be due to the instability of the proteins. Table I summarizes the level of β-galactosidase activity obtained from several experiments. N312 homodimerized and dimerized with 213-312, yielding β-galactosidase activities of 348 and 14.7-fold over background, respectively. These data are asymmetric, as only N312, but not the 213-312 fragment, expressed as a GAL4 transactivator fusion, could dimerize with 213-312. A directionality with results of protein-protein interaction assays has often been observed with the two hybrid system (e.g. Estojak et al., 1995). This experiment shows that the DRYG-mediated protein-protein interaction occurs *in vivo*, and confirms the importance of the DRYG rich region for self association.

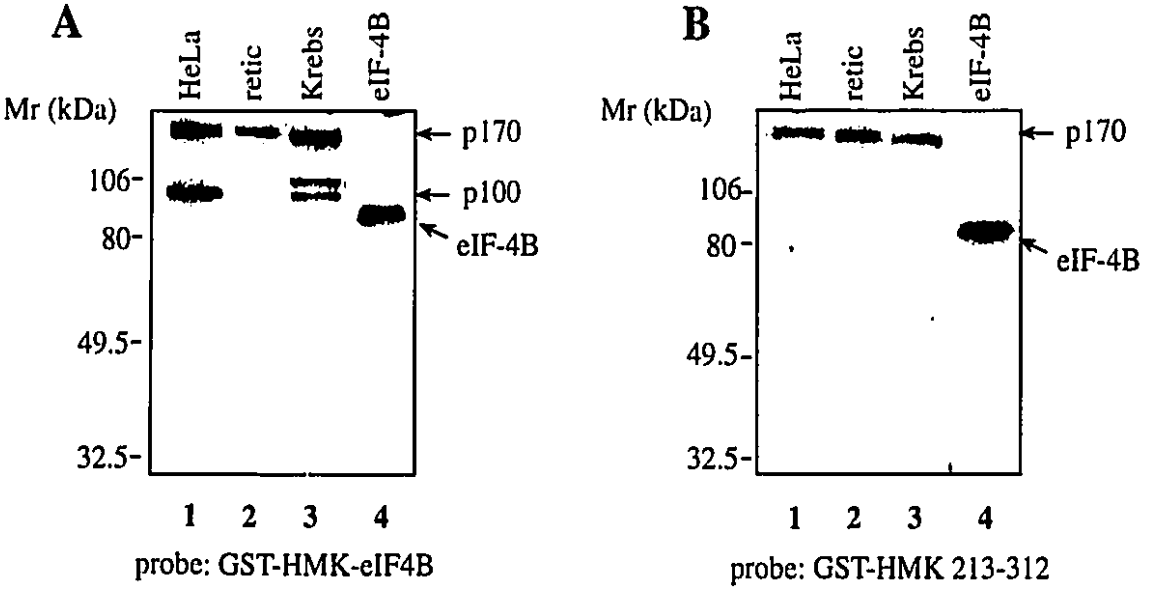
eIF4B interacts directly with eIF3

eIF4B copurifies through many purification steps with eIF3 and eIF4F, and is separated from these factors by high salt (0.5M KCl). This interaction could have biological relevance. To determine if eIF4B associates directly with eIF3 and eIF4F, and to further study these interactions, a filter overlay assay was performed with various cell extracts, using different eIF4B fragments as probes. GST-HMK eIF4B bound to two major proteins of approximately 170 kDa and 100 kDa and also bound weakly to a protein of 80 kDa in a HeLa extract and reticulocyte lysate (Fig. 4.6A, lanes 1 and 2). A filter overlay assay performed with a higher percentage polyacrylamide gel also revealed the presence of a 40 kDa reacting protein (data not shown). The 100 kDa protein is absent from rabbit reticulocyte lysates (lane 2). In Krebs extracts, an additional protein of 110 kDa is present (lane 3). The 80 kDa polypeptide co-migrated with recombinant eIF4B (lane 4), and thus is probably eIF4B. When an identical blot was probed with GST-HMK 213-312, only the 170 kDa and the 80 kDa proteins reacted (Fig. 6B). Thus, the DRYG domain does not interact with the 100 and 110 kDa proteins. A control blot probed with GST-HMK did not yield any signal (data not shown).

We sought to determine the identity of the 170 kDa protein. eIF3 is a multisubunit translation initiation factor which is comprised of 9 polypeptides: p35, p36, p40, p44, p47, p66, p110, p115 and p170 (Meyer et al., 1982; Behlke et al., 1986). Because eIF4B was reported to associate with eIF3 in cell extracts, we examined the possibility that the 170 kDa polypeptide is the high molecular weight subunit of eIF3. To this end, two different preparations of eIF3 (generous gifts from W. Merrick and H. Trachsel), one lacking intact p170 subunit (preparation #1) and one containing intact p170 (preparation #2), were resolved on an SDS-polyacrylamide gel and transferred onto nitrocellulose. An immunoblot using a monoclonal antibody directed against the p170 subunit of eIF3 shows the presence of the intact polypeptide in preparation #2 (Fig. 4.6C). Probing a duplicate membrane with ^{32}P-labeled GST-HMK-213-312 revealed proteins of 170 kDa and 80 kDa in HeLa cell and rabbit reticulocyte extracts (Fig. 4.6D, lanes 1 and 2). eIF3 preparation #1 contained a polypeptide, migrating at approximately 125 kDa, that interacted weakly with the DRYG domain (lane 3). eIF3 preparation #2, on the other hand, contained only one strongly reactive polypeptide migrating at 170 kDa (lane 4). We tested the possibility that the 125 kDa polypeptide corresponded to the p110 and p115 subunits of eIF3 by probing bacterial extracts expressing recombinant p110 and p115. We found no evidence that these eIF3 subunits interact directly with eIF4B (data not shown). Taken together, these results demonstrate that eIF4B associates directly with the p170 subunit of eIF3, and that this interaction is mediated by the DRYG domain.

To further substantiate the conclusion that eIF4B interacts with eIF3, co-immunoprecipitation experiments were performed. eIF4B was immunoprecipitated from HeLa cell extracts using either an eIF4B polyclonal antibody (706), pre-immune serum (706 PI) or a monoclonal antibody that recognizes the carboxy-terminal end (9E11). The immunoprecipitates were blotted onto nitrocellulose and probed with antibodies directed against various proteins. eIF4B was precipitated with both α–eIF4B antibodies (Fig. 7 Panel A, lanes 2 and 4), whereas no protein was precipitated with the pre-immune serum

Fig. 4.6: The DRYG domain of eIF4B interacts with the p170 subunit of eIF3. Panel A. Far Western analysis on cytoplasmic extracts (75 μg) using ^{32}P-labeled GST-HMK-eIF4B as probe. Recombinant eIF4B (0.5 μg) was also included as positive control. The position and molecular mass of interacting proteins are indicated by arrows. Panel B. Same as in A except that ^{32}P-labeled GST-HMK 213-312 was used as a probe. Panel C. Western blot analysis using a monoclonal antibody directed against the p170 subunit of eIF3. Preparation #1 of eIF3 contains a p170 degradation fragment that is immunoreactive towards the antibody. Preparation #2 of eIF3 contains partially degraded p170. Panel D. Far Western analysis on a similar membrane as shown in C. ^{32}P-labeled GST-HMK 213-312 was used as a probe.


A
HeLa
retic
Krebs
eIF-4B
Mr (kDa)
106-
80-
49.5-
32.5-
p170
p100
eIF-4B
1 2 3 4
probe: GST-HMK-eIF4B
B
HeLa
retic
Krebs
eIF-4B
Mr (kDa)
106-
80-
49.5-
32.5-
p170
eIF-4B
1 2 3 4
probe: GST-HMK 213-312
C
HeLa
retic
eIF3 (prep#1)
eIF3 (prep#2)
eIF4B
Mr (kDa)
200-
116-
80-
49-
1 2 3 4 5
immunoblot: anti p170
D
HeLa
retic
eIF3 (prep#1)
eIF3 (prep#2)
eIF4B
Mr (kDa)
200-
116-
80-
49-
1 2 3 4 5
probe: GST-HMK 213-312

(lane 3). Approximately 30% of the total eIF4B was recovered in the immunoprecipitate. eIF-3 co-immunoprecipitated efficiently with eIF4B, as judged from the large amount present in the immune complex relative to the total extract (10% of eIF3 input was recovered; Panel B, compare lanes 1, 2 and 4). This result is consistent with a direct interaction between eIF4B and the p170 subunit of eIF3. As a negative control, the presence in the immune complexes of proteins not known to be related to eIF4B function was examined. Actin (Panel C) and the La autoantigen (data not shown) were not found in the eIF4B immunoprecipitates.

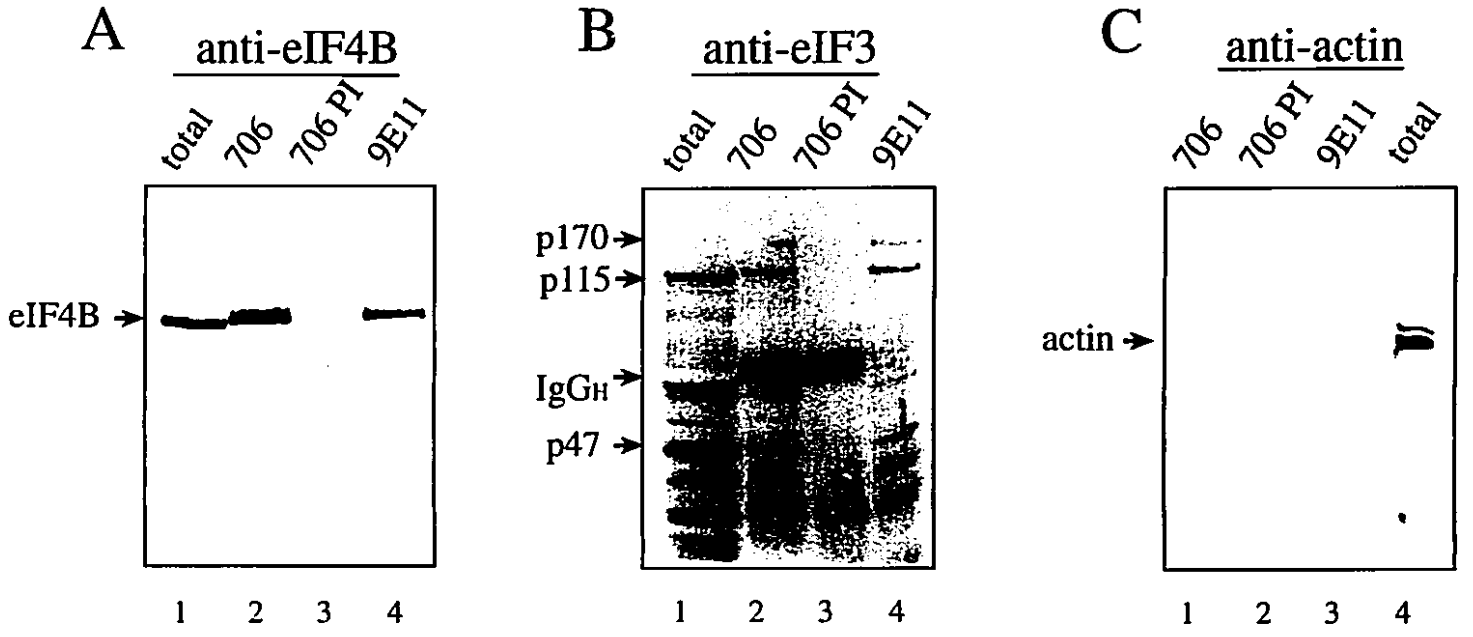


Fig. 4.7: eIF3 co-immunoprecipitates with eIF4B. Western blots on immunoprecipitates. eIF4B from HeLa cell extracts (200 µg) was immunoprecipitated with polyclonal antibody 706 or monoclonal antibody 9E11. Pre-immune 706 serum was used as negative control. Panel A. α-eIF4B western. Panel B. α-eIF3 western. The position of three of the eIF3 subunits is indicated by arrows. Panel C. α-actin western. For each experiment, 30 µg of HeLa extract was included to monitor the efficiency of the immunoprecipitation.

Discussion

The DRYG-rich region

In this study, we have mapped a self association domain in eIF4B to a portion of the protein rich in aspartic acid, arginine, tyrosine and glycine (DRYG-rich region), located between amino acids 214 and 327. Residues 213 to 312 are sufficient for the interaction. Deletion of the carboxy-terminal-most part of the DRYG-rich region (mutant N250, N213 and N171) resulted in a complete loss of self association. Similarly, NΔ253, in which the carboxy-terminal-most part of the DRYG-rich region is left intact, interacted with eIF4B very weakly. Removal of 18 amino acids in the DRYG domain (Δ230-247), led to a 3-fold decrease in self association. These data suggest that amino acids between position 250 and 312 are crucial for the interaction, but not sufficient since NΔ253, which still has amino acids 254 to 312 intact, showed virtually no activity, and the Δ230-247 deletion, which is outside amino acids 250 to 312, exhibited a decrease in self association activity. It is possible that both of these deletions induce a conformational change which prohibits dimerization. Alternatively, a minimal amount of residues outside the 250-312 region, or amino acids 250 to 254, are necessary for dimerization. There is also the possibility that two segments within the DRYG domain mediate self-association. The data do not permit to conclude whether the predominant species of eIF4B is a dimer, trimer or tetramer.

How is self association promoted? Programs such as Chou and Fasman predict a very high content of random coil structure within the DRYG-rich region, probably owing to the abundance of glycine residues. The binding energy may be provided by electrostatic interactions between the alternating positive and negative charges. However, glutaraldehyde cross-linking and gel-filtration studies showed that eIF4B self association was resistant to KCl concentration of at least 500 mM (N. Méthot, unpublished, J. A. Grifo, 1982). Homodimerization could be the result of hydrophobic interactions between the tyrosine residues, where phenyl rings may stack. Alternatively, self association may be mediated by the formation of β-sheets between two eIF4B monomers. The DRYG-rich

region of eIF4B does not show homology to any known proteins except for the SRYG repeats of the azoospermia factor AZF (Ma et al., 1993). This homology may be coincident rather than conservation. However, it may also represent a novel type of protein-protein interaction domain in which tyrosine residues play an important role.

Function of self association

Previous studies on eIF4B have shown that the NΔ253 fragment exhibits non-specific RNA binding and eIF4A helicase stimulation (Méthot et al., 1994). Furthermore, the N250 fragment binds to a specific RNA substrate with an affinity comparable to that of wt eIF4B (Méthot et al., 1996). Both of these fragments are severely deficient for self association. These results suggest that eIF4B self interaction is not required for RNA binding and for eIF4A helicase stimulation. Indeed, the N312 fragment (self association-positive; helicase-stimulation-negative) had no trans-dominant negative effects in a helicase assay and in *in vitro* translation of a reporter mRNA. Note that helicase assays are performed with a high excess of eIF4B over RNA, and it is possible that the need for self association is bypassed, since more than one eIF4B molecule can bind to duplex RNA (Méthot et al., 1994).

The role of self association for eIF4B function is not immediately clear. We used several deletions in the DRYG domain to determine whether the self association region of eIF4B and the p170 interaction domain overlap. All the truncations that abolished eIF4B self interaction also abolished eIF3 binding (N.M., unpublished. observations). Two possibilities may explain these results. i) the self association and p170 interaction sites overlap. This would predict that eIF4B homodimerization prevents p170 interaction. ii) eIF4B must dimerize in order to generate a site for p170 binding. We cannot at the present distinguish between these two possibilities because expression systems for intact p170 are not available. However, it is possible that a large proportion of the GST-HMK 213-312 probe already exists as a dimer (or multimer). This might explain why the eIF4B self association signal in a HeLa extract is weaker than the p170 interaction signal (both eIF4B

and eIF3 are present at approximately 0.5 molecules per ribosome in HeLa cells). Furthermore, the p170 protein does not contain a region homologous to the DRYG domain, which is not consistent with a shared interaction domain between eIF4B and p170. On the other hand, overexpression of eIF4B in COS cells results in a general reduction of translation, a phenomenon that remains unexplained (Milburn et al., 1990, Naranda et al., 1994). Removal of the DRYG domain abolished the ability of eIF4B to inhibit translation (Naranda et al., 1994). This could be consistent with the notion that eIF4B self association prevents interaction with eIF3. However, some deletion mutants that retained the DRYG domain did not inhibit translation when overexpressed, while others that lacked the DRYG domain, were strong translational inhibitors (Naranda et al., 1994). Thus, the translational block induced by overexpression of eIF4B cannot be attributed solely to its ability to self associate or interact with eIF3.

Our findings have implications for the mechanism of ribosome binding to mRNA. We have previously postulated that eIF4B serves as a bridge between the mRNA and the 40S ribosomal subunit by virtue of its ability to bind to the mRNA and the 18S rRNA (Méthot et al., 1996). The direct interaction between eIF3, which is present on the 43S pre-initiation complex prior to mRNA binding, and eIF4B, provides an additional link between the mRNA and the ribosome (see Fig. 4.8). The abundance of protein-protein and protein-RNA interactions during mRNA-ribosome binding ensures that the mRNA is recognized by ribosomes with high fidelity.

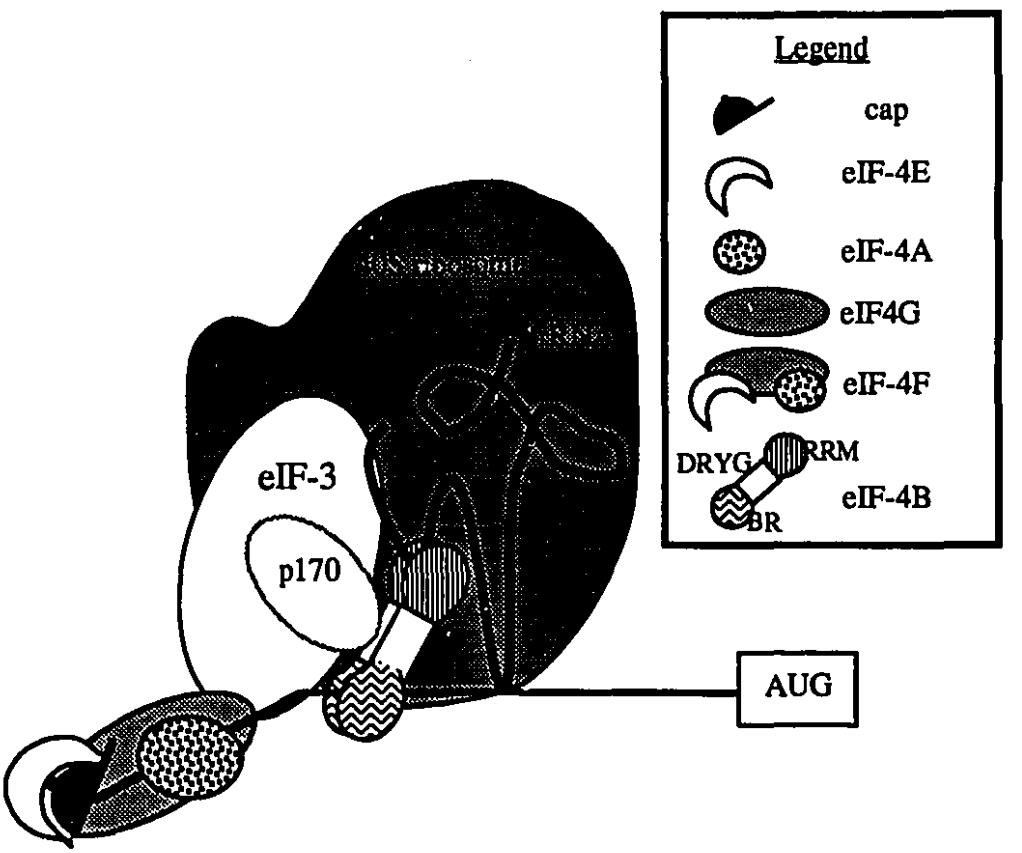


Fig. 4.8: Model for the binding of the 40S ribosomal subunit to a mRNA. Three types of interactions may stabilize the mRNA-ribosome complex. i) eIF3-eIF4G association. ii) eIF4B-eIF3 interaction. iii) eIF4B-18S rRNA interaction.

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Chapter 5

General Discussion

The eukaryotic translation initiation factor 4B was initially identified as a protein absolutely essential for binding of the mRNA to the 40S ribosomal subunit. Early biochemical studies indicated that this factor participates in the ribosome binding step by stimulating the RNA helicase activity of eIF4A and eIF4F. Together, eIF4B and eIF4A or eIF4F melt the secondary structure in the 5' UTR of the mRNA to create an area accessible for ribosome binding. These studies also implicated eIF4B in a number of other processes. Functions for eIF4B during scanning, AUG recognition, eIF4E recycling and poly(A) tail synergism have been proposed.

To clarify the role of eIF4B during protein synthesis, and to understand its mechanism of action during RNA unwinding, we initiated a structure-function analysis of this factor. Our results demonstrate that eIF4B is a highly modular protein with three distinct domains: the RNA Recognition motif (RRM) near the amino-terminus, the dimerization/eIF3 p160 interaction region (DRYG domain) and the non-specific RNA binding and helicase stimulator region in the carboxy-terminus half (Fig. 5.1).

5.1 The carboxy-terminal half of eIF4B

5.1.1 Non-specific RNA binding

The carboxy-terminal half of eIF4B contains the non-specific RNA-binding and the helicase stimulatory modules (Chapter 2). The carboxy-terminal RNA-binding region is located between amino acids 367 and 423. These results are in agreement with those of Naranda et al., (1994) who have detected an RNA binding site between residues 385 and 447. The combined data suggests that the C-terminal RNA binding site is located between amino acids 385 and 423. Two observations demonstrate that this RNA binding site acts independently of the RNA Recognition Motif. Mutations at highly conserved residues within RNP-1 and RNP-2 of the RRM did not significantly affect RNA binding (Fig. 2.3D). Removal of the RRM (mutant NΔ253) also failed to reduce RNA binding activity. Residues 367 to 423 of eIF4B contain two clusters of basic amino acids which could

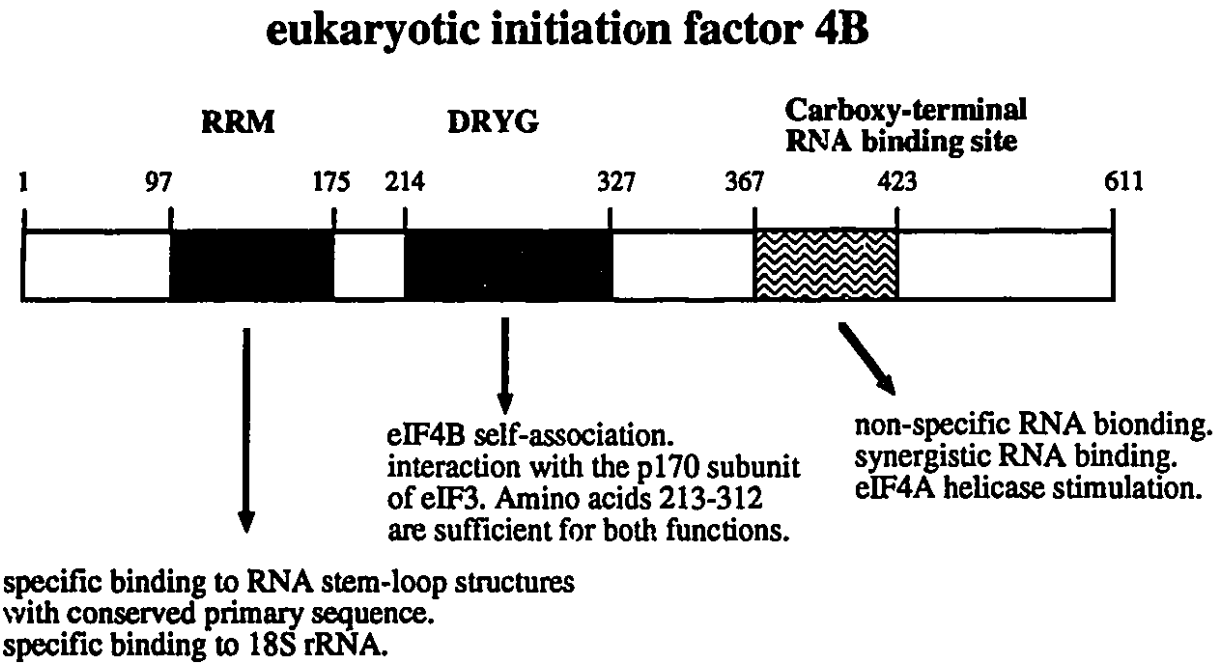


Fig. 5.1 eIF4B is organized into three independent domains. Schematic representation of the domain organization of eIF4B and their functions. eIF4B contains three independent domains: the RNA Recognition Motif (RRM; residues 97-175), the DRYG-rich region (residues 214-327), and the carboxy-terminal RNA binding site (residues 367-611).

potentially contact the negatively charged backbone of RNA. Arginine-rich motifs (ARMs) mediate RNA binding in a number of proteins, such at Human immunodeficiency virus Tat and Rev, and λ-phage N protein (Lazinski et al., 1989). Remarkably, despite the low complexity of interactions between positively charged residues and the RNA backbone, many of the ARM-containing proteins make highly specific contacts with RNA. The C-terminal RNA binding site of eIF4B appears to be non-specific. It bound well to a variety of RNA substrates tested, and it did not select RNAs bearing common sequence or secondary structure elements (Chapter 3).

The eIF4B dissociation constant (K_d) for non-specific duplex RNA was measured at 5 x 10^{-7}M. Binding of this substrate is most likely mediated by the carboxy-terminal RNA binding site. HIV Tat and Rev protein or the Iron Regulatory Factor (IRF), are highly specific RNA-binding proteins and associate with their cognate RNAs with K_ds of 3 x 10^{-9}, 1 x 10^{-9} and 1 x 10^{-11} M, respectively. Proteins such as the Polypyrimidine Tract Binding protein (PTB) and poly(A) binding protein (PABP), which recognize RNAs with low sequence complexity, or eIF4A, which binds RNA non-specifically, exhibit K_ds ranging from 10^{-8} to 10^{-6} M (reviewed by McCarthy & Kollmuss, 1995). The carboxy-terminal RNA-binding site, exhibits a relatively high K_d and low discriminatory ability, and this is consistent with the function of eIF4B as a general translation factor. eIF4B should be capable of stimulating the unwinding of all mRNAs. It cannot be ruled out however, that the C-terminal RNA-binding site binds better to some types of mRNAs and stimulates preferentially their translation. Some experiments support this possibility. When radiolabeled RNA duplex was melted by heat treatment prior to the binding assay, it bound 3-fold less efficiently to eIF4B than intact duplex. This demonstrates a preference by eIF4B for duplex-containing RNAs (N. Méthot, unpublished data). On the other hand, a perfectly duplexed RNA was a poor substrate for eIF4B binding (G. Cosentino, unpubl.). Taken together, these data suggest that eIF4B recognizes junctions between single-stranded and double-stranded areas of RNA. Tighter binding to this particular structure may be important

for the unwinding mechanism. In terms of nucleotide specificity, poly(A) ribohomopolymers were efficient competitors for duplex RNA binding (N. Méthot, unpubl.). A poly(A) RNA binding preference is also evident from selection/amplification experiment B (Chapter 3), where eIF4B, in combination with eIF4A, selected RNAs that were particularly rich in A residues in their 5' end (Fig. 3.1B). In the presence of eIF4A and ATP, the RNA-binding activity of eIF4B is increased 5-fold. RRM point mutants and the NΔ253 fragment of eIF4B were capable of cooperating with eIF4A for RNA binding, thus showing that the C-terminal RNA binding site is the effector of synergistic RNA binding (Fig. 2.6C). An attempt to locate the eIF4B residues involved in non-specific RNA binding was made. Two arginines, at positions 398 and 399, were mutated to asparagines. When tested in the NΔ253 background for duplex RNA binding, these mutants behaved as the wild type NΔ253 (N. Méthot, unpubl.). More extensive mutagenesis studies in the arginine-rich region will be required to identify residues that contact RNA. Alternatively, UV cross-linking of eIF4B (NΔ253 fragment) to a radiolabeled RNA, followed by protease treatment and HPLC purification and microsequencing of labeled fragments could be used.

5.1.2 eIF4A-helicase stimulation

The deletion analysis (Chapter 2) demonstrated that the eIF4A helicase stimulatory activity of eIF4B resides in the carboxy-terminus, between amino acids 253 and 423. This segment is coincident with the non-specific RNA-binding region of eIF4B. Helicase stimulation is conditional on non-specific RNA binding and synergistic RNA binding. All eIF4B fragments that bound duplex RNA were capable of stimulating the eIF4A helicase (compare Fig. 2.3, 2.6 and 2.7). This has implications for the eIF4A/eIF4B RNA melting mechanism.

It is believed that helicase-catalyzed DNA unwinding (and by extension RNA) functions through a protein or a protein complex that possesses at least two RNA-binding sites (Lohman, 1993). This would enable the DNA helicase to bind both single-stranded or

duplex DNA, or two strands of single-stranded DNA simultaneously at the unwinding fork. Many of the known RNA helicases have at least two RNA binding- sites in addition to the Q/HRIGRxxR motif, which has been shown, at least in eIF4A, to mediate RNA binding (Pause et al. 1994). RNA helicase A contains a double-stranded RNA-binding region (see section 1.6.5.1) and an RGG box-like sequences (see section 1.6.5.3). p68 and vasa also contain RGG boxes (Gibson & Thompson, 1994). Mutations in the QRxGRxGRxxxG motif of the vaccinia virus RNA helicase NPH-II abolished ATP hydrolysis without affecting RNA binding (Gross & Shuman, 1996). This argues for the presence of another RNA-binding region in NPH-II, which may mediate stable association with RNA. eIF4A exhibits very poor RNA-binding activity, and except for the HRIGRxxR motif, it does not seem to contain other common RNA-binding sites. eIF4A alone or as part of eIF4F demonstrates poor helicase activity (Rozen et al., 1990; Pause et al., 1994). The stimulatory effect of eIF4B on eIF4A cannot be attributed only to its ability to bind RNA since eIF4F as a complex is an RNA binding protein (Jaramillo et al., 1991).

One possibility is that the HRIGRxxR motif of eIF4A does not permit a stable association of this protein with RNA. Rather, it transiently contacts mRNA to induce ATP hydrolysis. The fact that synergistic RNA binding is essential for the helicase-stimulatory activity of eIF4B suggests that following ATP hydrolysis by eIF4A, a conformational change within eIF4B is induced. This conformational change would increase the affinity of the C-terminal RNA-binding site for mRNA, which ultimately leads to a mechanical disruption of duplex RNA. Thus eIF4B actively participates in the unwinding process. Conformational changes within eIF4A, which take place during ATP and RNA binding (Pause et al., 1994) may contribute to strand separation but are not sufficient.

The functional relationship between eIF4A and the carboxy-terminal half of eIF4B is indicative of a physical interaction. Attempts using the far western approach, the two-hybrid system and RNA gel-shift assays, have failed to demonstrate an interaction. Perhaps an eIF4A/eIF4B association is too transient to allow detection using these methods.

eIF4B is a phosphoprotein with over 10 forms as determined by two-dimentional gel electrophoresis and western blotting (Duncan & Hershey, 1984). Recently, it has become apparent that the covalent derivatives of eIF4B may not all be the result of phosphorylation, and that eIF4B bears other types of post-translational modifications (J.W.B. Hershey, personal communication). Dr. Hershey and his group have found two protein-kinase C phosphorylation sites, and one S6 kinase phosphorylation site. The latter is located in the carboxy-terminal domain at a serine residue near the RNA binding region. It would be interesting to determine the effect of S6 phosphorylation on synergistic RNA binding and helicase stimulation. Introduction of a negative charge by mutating this serine to an aspartic acid may also provide some answers as to the role of phosphorylation on eIF4B activity. Already, it is clear that phosphorylation is not essential for the helicase stimulatory activity since recombinant eIF4B purified from *E. coli* promotes melting of duplex RNA to a similar extent as eIF4B purified from rabbit reticulocyte lysates (A. Pause, unpubl.)

Purification of eIF4B from bacterial lysates proved difficult due to extensive degradation. In particular, a protease-sensitive region is present between amino acids 312 and 355 (Compare the extent of degradation between N312 and N355, Fig. 2.2). This region is located between the DRYG domain and the helicase stimulatory domain. Examination of the hydropathy profile of eIF4B (Milburn et al., 1990) reveals a striking change of topology in this region. These observations support the view that eIF4B is a highly modular protein, with an independent helicase stimulatory domain.

5.2 The DRYG domain

5.2.1 Self association

The next module of eIF4B is the DRYG region, located between amino acids 213 to 312. This segment supports eIF4B self association and interaction with the p170 subunit of eIF3 independently of other regions, and therefore constitutes a domain. As its name

implies, the DRYG domain is rich in aspartic acid, arginine, tyrosine and glycine, and does not bear homology to any known protein except the azoospermia factor AZF (Ma et al., 1993). The latter contains a region rich in S-R-Y-G. It is not known if AZF can dimerize. However, it would be interesting to test the SRYG region for self association activity.

The Chou-Fasman algorithm predicts a random-coil structure for the DRYG domain, owing to the large number of glycine residues. Dimerization, as measured by chemical cross-linking and gel-filtration chromatography, was resistant to 0.5 M KCl (N. Méthot, unpubl.; J. Grifo, 1982). Insensitivity to K^+ ions suggests that self association is not mediated by salt bridges between the aspartic acid and arginine residues of the DRYG domain. An alternative possibility is hydrophobic interactions via the tyrosine residues, possibly by a ring stacking mechanism. Hydrophobic interactions play a pivotal role in protein-protein association, as shown with the "leucine zipper". The latter consists in an α-helical structure where leucine side-chains, which emerge from one side of the helix, interdigitate with leucine side chains from an adjacent helix. Stability of the leucine zipper is achieved primarily through hydrophobic interactions, while specificity, at least for the Fos-Jun and Jun-Jun dimers, is determined by electrostatic interactions between the charged residues at the periphery of the leucine tract (reviewed by Phizicky & Fields, 1995). Perhaps the alternate arginine and aspartic acid residues of the DRYG domain serve a similar specificity function. Alternatively, β–sheet formation between two eIF4B monomers may mediate self association. The DRYG domain appears to be quite selective, since only three proteins, eIF4B and eIF3-p170and a 40kDa polypeptide, associate with it in a far western assay.

The repetitive nature of the DRYG domain and its large size may be indicative of a homotypic interaction taking place over a large surface. Dimerization would require a minimum number of repeats rather than a few essential amino acids, with some of these repeats perhaps contributing more to the stability of the complex than others. For instance, fragment N250 of eIF4B was unable to dimerize (Fig. 4.2). This would suggest that amino

acids 250 to 312 mediate self-association, yet, fragment NΔ253 of eIF4B also failed to dimerize, (Fig. 4.3), as well as fragment 250-312 (N. Méthot, unpubl.) Unless amino acids on either side of residue 250 are absolutely necessary for dimerization, these data support the view that neither N250 nor 250-312 contain by themselves the minimum number of DRYG repeats required for dimerization. Removal of some of these repeats (mutant Δ230-247) reduced but did not eliminate dimerization (Fig. 4.3).

Phosphorylation does not appear to be the only type of post-translational modification found within eIF4B. The DRYG domain could constitute a target for arginine methylation. Monomethyl and dimethylarginine are modifications found in a number of RNA-binding proteins (Najbauer et al., 1993; Liu & Dreyfuss, 1993). Arginines flanked by glycines seem to be the preferred substrate for methyltransferases that modify arginines (Najbauer et al., 1993; Rawal et al., 1995). If the DRYG domain of eIF4B indeed contains dimethylarginine, it would be interesting to determine if this modification affects homodimerization and interaction with the p170 subunit of eIF3.

To date, homodimerization does not seem to be required for any of the known activities of eIF4B. N250 is incapable of dimerizing (Chapter 4), yet binds A3 RNA at wild type level (Chapter 3). NΔ253 is also unable to self-associate, but still binds RNA indistinguishably from wt eIF4B, and stimulates the eIF4A RNA helicase, albeit at lower levels. Since the helicase assay is conducted with a large excess of protein over RNA, it is possible that the need for dimerization is circumvented, as more than one eIF4B molecule can bind per duplex RNA. However, in a cell free translation extract, none of the eIF4B truncations tested, except the 180-312 fragment, could inhibit protein synthesis. The reduction in translation induced by fragment 180-312 varied with different protein preparations and could not be rescued by addition of purified wt eIF4B (N. Méthot, unpubl.). Thus the significance of this reduction is unclear. On the other hand, the experiments shown in Chapter 4 may suggest that dimerization is a pre-requisite for the interaction of eIF4B with the p170 subunit of eIF3. All the mutations that abolished eIF4B

self-association abrogated interaction with eIF3. The predicted amino acid sequence for p170 does not contain a region homologous to the DRYG domain. This is not consistent with a shared domain for eIF4B dimerization and p170 association. Since p170 expression system are lacking at the moment, we are currently unable to test directly whether interaction with p170 is conditional on eIF4B homodimerization.

5.2.2 Interaction with the p170 subunit of eIF3

The DRYG domain of eIF4B interacts with the p170 subunit of eIF3 (Fig. 4.6). In mammals, eIF3 is comprised of at least 9 subunits: p35, p36, p40, p44, p47, p66, p110, p115 and p170 (Meyer et al., 1982; Behlke et al., 1986). The p66 subunit can be cross-linked to 18S rRNA (Nygard & Westermann, 1982) and to mRNA (Westermann & Nygard, 1984; Naranda et al., 1994b). Activities for the other subunits of eIF3 have not been assigned. Nevertheless, it is known that eIF3 stimulates and stabilizes ternary complex association with the 40S ribosomal subunit, and is essential for mRNA binding (Benne & Hershey, 1978; Trachsel & Staehelin, 1979). eIF3 co-purifies with eIF4B and eIF4F (Benne & Hershey, 1976; Schreier et al., 1977). An interaction between the carboxy-terminus half of eIF4G and eIF3 has been demonstrated (Lamphear et al., 1995), but it is not known which of the subunit(s) of eIF3 is implicated. Knowing that a direct interaction takes place between the p170 subunit of eIF3 and the DRYG domain of eIF4B, a clearer picture of the protein-protein and protein-RNA interaction network that is formed during translation initiation has emerged (Fig 5.2). eIF4E binds to the amino terminus of eIF4G while eIF4A and eIF3 associate with the carboxy-terminus of eIF4G. eIF4B contacts eIF3 via the p170 subunit. eIF2, from co-purification data (Shreier et al., 1977), may also interact directly with eIF3. All of the above factors can be co-immunoprecipitated with eIF4B (N. Méthot, unpubl.). It is thus likely that eIF3 serves to link initiation factors that bind mRNA to the 40S ribosome. Indeed, deletion of the entire DRYG domain prevented association of eIF4B with the translational apparatus (Naranda et al., 1994). What remains

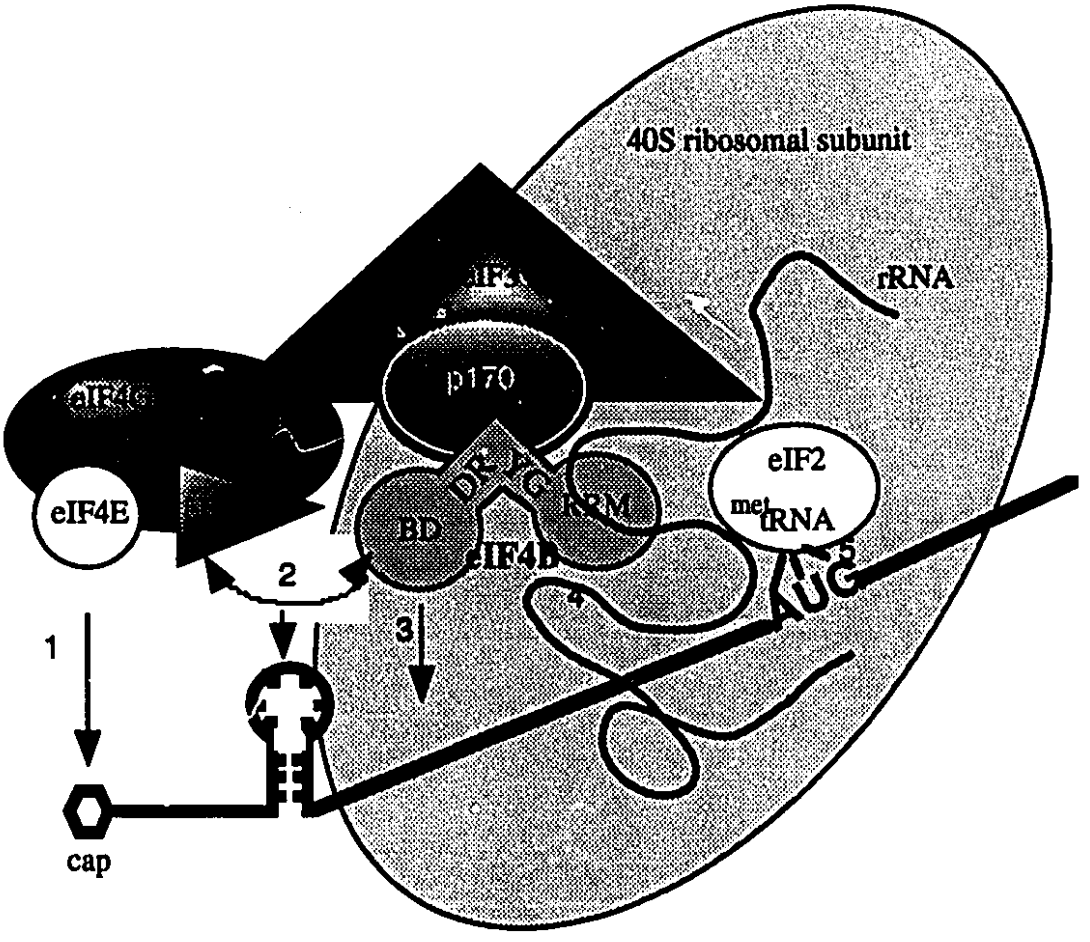
to be solved is whether these interactions take place in solution or on the 40S ribosomal subunit.

5.3 The eIF4B RNA Recognition Motif

The third module of eIF4B is located at the amino terminus and encompasses the first 250 residues of the protein (Chapter 3). This segment recognizes RNA secondary structures with conserved nucleotides at specific positions (Fig. 3.4B). The features of RNA ligands preferentially bound by N250 are identical to those found in RNAs selected with wt eIF4B. Thus, the N250 fragment acts independently of other sequences within eIF4B and constitutes a domain. The amino and carboxy terminal borders of this domain have not been delineated, but point mutagenesis demonstrated a requirement for the RRM to mediate specific RNA binding (Fig 3.3C).

The secondary structure of RNAs specifically recognized by the eIF4B RRM is reminiscent of the structures of RNAs bound by the Iron Regulatory Factor (IRF), HIV Tat and U1A snRNP protein. The Iron Responsive Element (IRE) folds into a stem-loop structure with a six-membered terminal loop and at least one unpaired nucleotide, generally a C, interrupting the stem. The IRE/IRF interaction is dependent on both RNA sequence and secondary structure (Rouault et al., 1996). Similarly, Tat binds to the TAR RNA, a stem-loop structure with a U-rich bulge interrupting the stem. Recognition of TAR by Tat is mainly through the bulge, which distorts the stem (Gait & Karn, 1993). Finally, U1A snRNP protein binds to hairpin I of U1A snRNA by direct contact with the 7 first nucleotides of the 10-mer apical loop (Oubridge et al., 1994). These examples illustrate a common feature of sequence-specific RNA-binding proteins. Specific RNA-protein contacts are mostly made with unpaired RNA bases. Double-stranded RNA, in contrast to DNA, is always found in the A-form, with deep and narrow major grooves that are difficult to access. Minor grooves, which are more accessible, provide less opportunity for hydrogen bonding, and are not as suitable for sequence-specific contacts (reviewed by

Fig. 5.2 Model for protein-protein and protein-RNA interactions that take place during translation initiation. Binding of the 40S ribosomal subunit to the mRNA is aided by translation initiation factors which form a protein-RNA network. eIF4G interacts directly with eIF4E and eIF4A to form the heterotrimer eIF4F. eIF3, which is associated with the 40S ribosomal subunit prior to mRNA binding, also interacts with eIF4G directly. eIF4B functionally interacts with eIF4A to unwind RNA secondary structure in the 5' UTR of the mRNA, and physically associates with the p170 subunit of eIF3 via its DRYG domain, thereby establishing a link between the ribosome and the mRNA. Not shown is the possibility of eIF4B binding as a dimer. eIF2 is bound to the 40S ribosomal subunit and may interact with eIF3. Numbers depict the protein-RNA interactions. (1) eIF4E binds to the cap-structure that is present at the 5' end of the mRNA. (2) eIF4A and eIF4B bind to mRNA stem-loop structures, possibly at single-stranded/double-stranded RNA junctions. (3) The basic domain (BD) of eIF4B also binds to mRNA non-specifically, while (4) the RNA Recognition Motif (RRM) specifically associates with 18S rRNA. (5) The anticodon loop of met_iRNAi base-pairs with AUG for initiation codon recognition. Not shown is an association between the poly(A) tail and the poly(A) binding protein (PABP) which may loop back to interact directly, or indirectly, with initiation factors or the 40S ribosome.


40S ribosomal subunit
rRNA
p170
eIF4E
eIF2
met tRNA
BD
RRM
eIF4b
2
1
3
4
5
AUG
cap

Steitz, 1993). eIF4B recognizes unpaired nucleotides in the A3 RNA terminal loop, but also base-paired nucleotides (GC or GU) at the base of the bulge. The bulge probably distorts the stem to allow access to the GGAA/C motif. It should be interesting to study the interaction between the eIF4B RRM and the A3 RNA by NMR or crystallography. Valuable information on sequence-specificity determinants, applicable to other RNA-binding proteins, may be gained.

In addition to A3 RNA-type stem-loops, the eIF4B RRM specifically recognizes 18S rRNA (Fig. 3.6). Variable domain IV of 18S rRNA (which is conserved among mammalian species; Huysmans & de Wachter, 1986), is predicted to adopt a similar structure as A3 RNA, and contains many of the specific nucleotides required for binding to eIF4B. When tested in isolation, this region bound to the N250 fragment, albeit at only 20% of A3 RNA levels. Deletion of the GGAC motif did not decrease RNA binding. It is therefore probable that eIF4B recognizes a specific tertiary structure in 18S rRNA. The N250 fragment bound efficiently to ribosomes as determined by sucrose gradient co-localization, while the N250 FY/AA mutant bound at 25% of wt levels (N. Méthot, unpubl.). This data lends support to the view that the eIF4B RRM binds to 18S rRNA. Furthermore, a phylogenetic analysis of a large number of RRMs indicates that RRMs of functionally related proteins contain similar sequences (Kim & Baker, 1993; Fukami-Kobayashi et al., 1993). The RRM of eIF4B is most related to that of NSR1, a nucleolar protein involved in ribosome biogenesis (Lee et al., 1992).

Is the eIF4B RRM capable of specifically binding to other types of RNAs? Meyer *et al.*, (1995) have shown an association between eIF4B and the internal ribosome entry site (IRES) of the foot and mouth disease virus (FMDV). Preliminary studies indicated that this interaction is mediated by the RRM (N. Méthot, unpubl.). However, the functional significance of eIF4B binding to the FMDV IRES is unclear (Meyer, 1995). Recently, a cDNA-based in vitro RNA selection technique was developped (Dobbelstein & Shenk, 1995). It would be interesting to apply this method to eIF4B to identify cellular RNAs that

preferentially associate with this factor. The preferential binding of eIF4B to poly(A) RNA also supports the hypothesis that this factor is one of the agents that mediate the synergy between the cap-structure and the poly(A) tail during translation initiation.

5.4 The role of eIF4B in translation initiation

The results presented in chapters 2, 3 and 4 allows conclusions to be drawn on the role of eIF4B during translation initiation. Figure 5.1 depicted eIF4B as part of a large protein-protein and protein-RNA interaction network. eIF4B is required for the binding of the 40S ribosomal subunit for two reasons. First, together with eIF4A, eIF4B actively participates in the melting of secondary structure in the 5' UTR of the mRNA, creating an area accessible for ribosome binding. The carboxy-terminal end of eIF4B by itself is capable of mediating the helicase-stimulatory activity (Chapter 2), and data from synergistic RNA binding and helicase assays favor a model whereby a change in conformation by eIF4B, engendered by eIF4A and ATP hydrolysis, is largely responsible for the disruption of duplex RNA. Secondly, eIF4B stimulates the mRNA-binding step by bridging the 40S ribosomal subunit to the mRNA via protein-protein and protein-RNA interactions. The DRYG domain of eIF4B binds directly to the p170 subunit of eIF3 (Chapter 4), an initiation factor that is found on the 43S pre-initiation complex. eIF3 also directly contacts eIF4G, which is bound to the cap-structure via the cap-binding protein eIF4E (see Fig. 5.1). Furthermore, eIF4B can bind specifically to 18S rRNA (Chapter 3) and can associate with two different RNA molecules simultaneously. This suggests that the 43S ribosome-mRNA complex is stabilized by an mRNA-eIF4B-rRNA link. The RRM of eIF4B is bound to 18S rRNA, while the carboxy-terminal RNA binding region is joined to the mRNA. Multiple protein-protein and protein-RNA interactions ensure the stability and the accuracy of the initiation process. The position of eIF4G at the cap-structure, and eIF4B in the vicinity of the cap, ensures that the 43S pre-initiation complex binds to the mRNA in this area, upstream of the initiator codon.

This work clearly established eIF4B as an agent which links the 43S pre-initiation complex to the mRNA. No evidence for specific AUG codon recognition by eIF4B was found in the selection-amplification experiments (Chapter 3). A participation of eIF4B during scanning cannot be ruled out though. As suggested by Altmann *et al.* (1995), it is possible that eIF4B, due to its RNA annealing and RNA helicase stimulatory activities, aligns the initiator codon with the anticodon loop of the $met tRNA_i$ by annealing the anticodon loop with nucleotide triplets in the 5' UTR and unwinding, together with eIF4A, unproductive matches. eIF2 has been shown to participate in this process through a mechanism that remains to be elucidated (Donahue et al., 1988). Finally, it is not excluded that eIF4B favors the translation of some mRNAs over others by binding preferentially to sequences in the 5' UTR. Preliminary experiments whereby the A3 RNA secondary structure present in the 5' UTR stimulated the translation of an uncapped mRNA, support this possibility (N. Méthot, unpubl.). Much work remains to be done on this factor. Unfortunately, as human eIF4B cannot rescue the temperature-sensitive phenotype of yeast eIF4B, the genetic approach is not a viable option to study the role of human eIF4B during translation initiation. The development of an *in vitro* translation system that is dependent of exogenously added eIF4B would an important tool to gain a better understanding of this factor.

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Original Contributions to Knowledge

1) The carboxy-terminal end of eIF4B contains an RNA-binding site that is responsible for the non-specific RNA-binding activity. This RNA-binding region, which is located between amino acids 367 and 423, mediates synergistic RNA binding with eIF4A and RNA helicase stimulation. Unexpectedly, the RNA Recognition Motif of eIF4B plays no role in non-specific RNA binding and synergistic RNA binding, and contributes little to the RNA helicase stimulatory activity.

2) The eIF4B RNA Recognition Motif (RRM) is functional and binds specifically and with high affinity to RNA molecules that are predicted to fold into a particular stem-loop structure. Secondary structure and individual nucleotides are required for recognition by the RRM. Cellular eIF4B from human and rabbit sources exhibits a similar binding specificity as recombinant eIF4B, indicating a conservation of function among mammalian species. The eIF4B RRM also binds to 18S rRNA. Due to its two RNA binding sites, eIF4B is capable of associating simultaneously to two different RNA molecules. This is the first RNA binding protein directly shown to do so. Taken together, these data suggest an additional mechanism by which eIF4B promotes binding of the mRNA to the 40S ribosomal subunit. eIF4B acts as a bridge by jointly binding mRNA via the carboxy-terminal RNA binding site and 18S rRNA via the RRM.

3) Recombinant eIF4B self associates *in vitro* and *in vivo*. The self association region is located in an portion of eIF4B that is enriched in aspartic acid, arginine, tyrosine and glycine (DRYG), and can act independently of other regions of the protein. In addition to homodimerization, the DRYG domain mediates a specific and direct interaction with the p170 subunit of the translation initiation factor eIF3. These results suggest that eIF4B stimulates binding of the mRNA to the 43S pre-initiation complex by establishing a link

between the mRNA, bound by the carboxy-terminal RNA binding site, and eIF3, already present on the 43S pre-initiation complex.