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

NATHALIE MÉTHOT,[1] GAVIN PICKETT,[2] JACK D. KEENE,[2] and NAHUM SONENBERG[1]

[1] Department of Biochemistry and McGill Cancer Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada
[2] Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710, USA

ABSTRACT

Translation initiation factor eIF-4B is an RNA-binding protein that 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 eIF-4A. In addition to an RNA recognition motif (RRM) located near its amino-terminus, eIF-4B contains an RNA-binding region in its carboxy-terminal half. The eIF-4A helicase stimulatory activity resides in the carboxy-terminal half of eIF-4B, and the RRM has little impact on this function. To better understand the role of the eIF-4B 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 eIF-4B. These experiments were designed to test the RNA recognition specificity of the two eIF-4B regions implicated in RNA binding and to assess the influence of eIF-4A 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 eIF-4A, influenced the structure of the high-affinity RNA ligands selected by eIF-4B, and eIF-4A by itself did not select any specific RNA target. Previous studies have demonstrated an interaction of eIF-4B with ribosomes, and it was suggested that this association is mediated through binding to ribosomal RNA. We show that the RRM of eIF-4B interacts directly with 18S rRNA and this interaction is inhibited by an excess of the eIF-4B in vitro-selected RNA. eIF-4B could bind simultaneously to two different RNA molecules, supporting a model whereby eIF-4B promotes ribosome binding to the 5′ untranslated region of a mRNA by bridging it to 18S rRNA.

Keywords: ribosome binding; RNA binding protein; 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 (eIF-4A, eIF-4B, and eIF-4F) and the hydrolysis of ATP (for reviews, see Hershey, 1991; Merrick, 1992). eIF-4F is comprised of three subunits (eIF-4E, eIF-4A, 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, eIF-4E. The eIF-4A subunit, an RNA-dependent ATPase that cycles through the eIF-4F complex (Yoder-Hill et al., 1993; Pause et al., 1994) is the prototype member of the DEAD box family of RNA helicases (reviewed by Schmid & Linder, 1992; Gorbalenya & Koonin, 1993; Pause & Sonenberg, 1993). A model has been proposed (Sonenberg, 1988) whereby eIF-4A, as part of the eIF-4F complex and together with eIF-4B, unwinds secondary structure present in the 5′ untranslated region (UTR) of an 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 & 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 & Shatkin, 1980; Sonenberg, 1981; Gehrke et al., 1983). In addition, components of the eIF-4F helicase machinery can suppress

Reprint requests to: Nahum Sonenberg, McGill University, Department of Biochemistry and McGill Cancer Centre, 3655 Drummond Street, Rm 807, Montréal, Quebec H3G 1Y6, Canada; e-mail: Sonenberg@medcor.mcgill.ca.

the inhibition of translation caused by secondary structure in the mRNA 5′ UTR. This was demonstrated for eIF-4E using NIH 3T3 cells (Koromilas et al., 1992) and for eIF-4B 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 (eIF-4A and eIF-4E), 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 & 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.

eIF-4B is an 80-kDa phosphoprotein that is required for binding of the mRNA to the 40S subunit (Trachsel et al., 1977; Benne & Hershey, 1978). eIF-4B stimulates the ATPase and RNA helicase activities of eIF-4A and eIF-4F (Rozen et al., 1990; Pause & Sonenberg, 1992). It contains an RNA recognition motif (RRM; or RNA-binding domain, RBD; for reviews see Kenan et al., 1991; Mattaj, 1993; Burd & 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). Although 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 eIF-4A helicase stimulatory activity of eIF-4B maps to the carboxy-terminal half of the protein, with the RRM playing only a minor role (Méthot et al., 1994). eIF-4B was also shown to possess RNA annealing activity (Altmann et al., 1995). Finally, eIF-4B associates with eIF-4F, as evidenced by their copurification (Grifo et al., 1983).

To characterize further the role of eIF-4B 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 eIF-4B preferentially interacts. We demonstrate by RNA-binding studies using deletion and point mutants of eIF-4B that the RRM is responsible for binding the selected RNAs, and confirm the nonspecific RNA-binding role of the carboxy-terminal RNA-binding region. Mutagenesis of the selected RNAs indicates that eIF-4B recognizes individual nucleotides in the context of a higher order RNA structural organization. UV crosslinking experiments with radiolabeled 18S rRNA and eIF-4B demonstrate a specific interaction. Finally, eIF-4B 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 eIF-4B participates in mRNA binding to the 40S ribosomal subunit by virtue of its ability to associate with 18S rRNA.

RESULTS

eIF-4B recognizes a specific RNA secondary structure

Five RNA selection experiments were performed using RNA pools of either 25 or 40 random nucleotide positions (Table 1). In Experiment A, RNA selection was carried out using full-length eIF-4B (Fig. 1A). Two additional selection experiments were performed to test whether the presence of eIF-4A can alter the RNA-binding specificity of eIF-4B (Experiment B), or whether eIF-4A 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 eIF-4B (Fig. 1A). Experiments A, B, and C contained ATP in the binding buffer, eIF-4A binding to RNA is ATP-dependent (Grifo et al., 1982; Pause et al., 1993), and cooperation between eIF-4A and eIF-4B in RNA binding requires ATP hydrolysis (Abramson et al., 1988; Méthot et al., 1994).

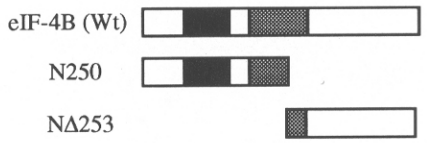
Sequences of several of the selected RNAs are shown in Figure 1B. Examination of the RNA sequences selected by full-length eIF-4B (Experiment A) revealed the presence of a GGAA/C motif in 19 of 20 clones. The three positions immediately upstream of the GGAA/C motif were less well conserved, but appeared to be preferentially occupied by the sequence GUU (Fig. 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. 2). The RNAs selected by eIF-4B (Experiment A) contain a stem-loop structure, with the terminal loop comprised of 3–5 nt. The base of the loop is formed by a G-C or C-G base

TABLE 1. Summary of selection experiments performed.

Experiment	Target	Length of randomized region	Rounds of selection
A	eIF-4B[a]	40	8
B	eIF-4B[a] + eIF-4A	40	8
C	eIF-4A	40	8
D	N250[a]	25	5
E	NΔ253[a]	25	5

[a] Purified as GST-fusion proteins.

A



B

	clone	selected sequence	frequency
Exp. 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
Exp. 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
Exp. D	D1	U**GGAA**AUUGUAAGAAUUAUCGTGGC	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 $\frac{A}{C}$

FIGURE 1. Representative RNA sequences selected from randomized RNA libraries using eIF-4B (Experiment A), eIF-4B + eIF-4A (Experiment B), and N250 (Experiment D). **A:** Schematic representation of eIF-4B mutants used as targets in selection/amplification experiments. Black box, RNA recognition motif (amino acids 97–175); stippled box, DRYG-rich region (amino acids 214–327). **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 eIF-4A 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.

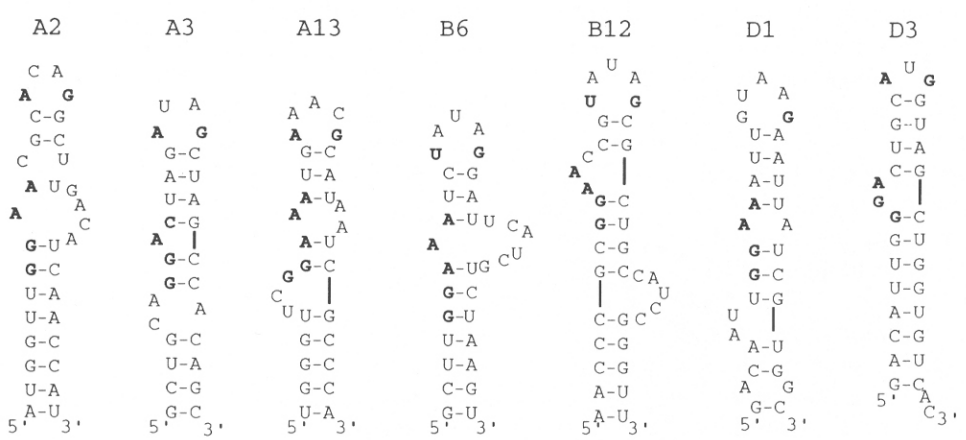


FIGURE 2. Predicted secondary structures of certain ligands selected by eIF-4B (clones A2, A3, and A13), eIF-4B + eIF-4A (B6 and B12), and N250 (D1 and D3). The conserved GGAA/C motif is highlighted in bold type as well as the conserved nucleotides at the 5′ and 3′ position of the loop.

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 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 1–4 nt.

To determine whether eIF-4A affects the structure or nucleotide sequence requirements for specific RNA binding by eIF-4B, a combination of eIF-4A and eIF-4B 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 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. 2). Thus, the presence of eIF-4A did not appear to modify the RNA-binding preference of eIF-4B. 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 conferred a slight selective advantage. The possible significance of this observation will be addressed below.

The selection using eIF-4A 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 eIF-4A is a weak nonspecific RNA-binding protein, at least under these conditions (Pause et al., 1993).

Selected RNAs are bound through the RRM

eIF-4B 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 eIF-4B containing either the RRM (fragment N250; Experiment D) or the carboxy-terminal RNA-binding region (fragment NΔ253; Experiment E; Fig. 1A). The primary and secondary structure of the N250-selected RNAs is strikingly similar to pool A and pool B sequences (Figs. 1B, 2). The GGAA/C motif was present in 20 of the 22 sequenced clones, with a predicted secondary structure consisting of a bulged stem-loop. These data strongly suggest that the region of eIF-4B 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 eIF-4B. This is consistent with earlier data that characterized the carboxy-terminus of eIF-4B as a nonspecific RNA-binding site (Méthot et al., 1994). Indeed, examination of the sequences of 20 independent clones, using the eIF-4B 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 eIF-4B, the apparent dissociation constant of recombinant eIF-4B (non-GST fusion) for some selected RNAs (A2, A3, and A13) was assessed by a nitrocellulose retention assay (Fig. 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 eIF-4A selected RNA, C2, was 200 nM. This confirms that eIF-4B 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 of the 23 sequenced clones), was also estimated at 12.5 nM (Fig. 3A), thus showing that eIF-4A does not modify the RNA-binding specificity or affinity of eIF-4B for the selected RNAs. The experiment shown in Figure 3A was conducted in the presence of 30 ng of competitor polyA RNA. In the absence of this competitor, eIF-4B could not distinguish pool-A RNAs from pool-C RNAs, presumably because these RNAs bound nonspecifically to the carboxy-terminal RNA-binding site (data not shown).

Selection/amplifications with full-length eIF-4B 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 eIF-4B, the affinity constants of wild-type eIF-4B and the N250 fragment for A3 RNA were compared. N250 bound A3 RNA with a dissociation constant of 12.5 nM (Fig. 3B), a value identical to the K_d of wild-type eIF-4B for A3 RNA (Fig. 3A). N250 bound extremely weakly to the nonspecific control RNA ligands C2 and N40 (Fig. 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 eIF-4B, which comprises the RRM, is sufficient for specific RNA-binding equivalent to that of wild-type eIF-4B.

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

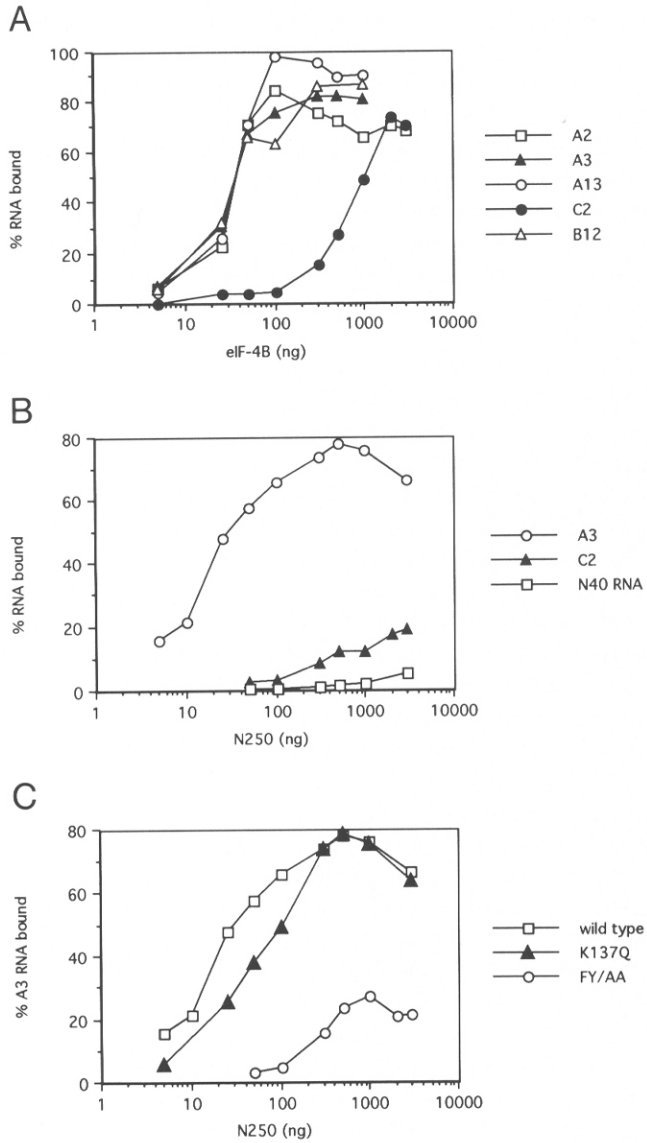


FIGURE 3. RNA-binding specificity of eIF-4B and mutants of eIF-4B. **A:** Binding of eIF-4B (non GST fusion) to selected RNAs from Experiment A (clones A2, A3, and A13), Experiment B (clone B12), and Experiment C (clone C2). **B:** Comparative affinities of the N250 mutant for the eIF-4B-selected RNA clone A3, non-eIF-4B-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 the Materials and methods.

to alanines (FY/AA). Previous mutagenesis (Merril et al., 1988; Brennan & Platt, 1991; Cáceres & 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. 3C). Taken together, the data clearly establish the RRM of eIF-4B 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 eIF-4B RRM, we mutated the GGAA/C stretch. Modifications were introduced by: (1) deleting the bulged A residue (ΔA mutant); (2) pairing the bulged A residue to a U residue (A = U mutant); (3) mutating the two G residues to Cs, while introducing compensatory changes to keep the stem intact (GG → CC mutant). Mutations (1) and (2) were intended to test whether the bulged A functioned in a defined structural context. Mutation (3) 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. 4). 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 eIF-4B 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 mutants, but was still severely deficient compared to wild-type oligoA3 RNA (Fig. 4). Thus, the loop sequence, in addition to the GGAA/C motif, appears to be required for efficient RNA recognition by the N250 fragment of eIF-4B.

eIF-4B binds A3 RNA in HeLa cytoplasmic extracts

The association of eIF-4B with the selected RNAs was characterized using recombinant protein. It was therefore important to test whether cellular eIF-4B, containing posttranslational modifications such as phos-

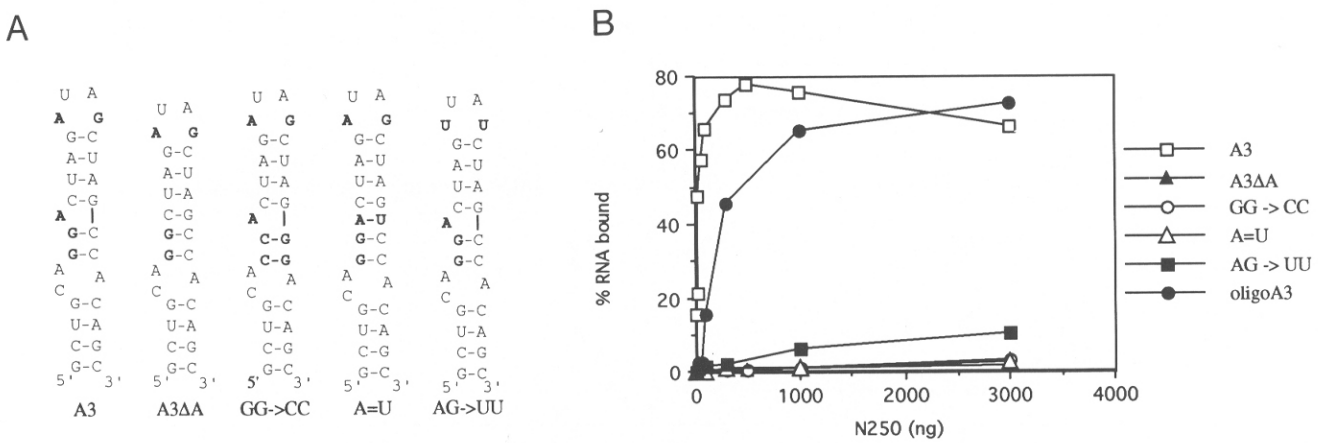


FIGURE 4. Mutagenesis of conserved nucleotides in the A3 RNA ligand selected from the randomized RNA library: Effect on binding by the N250 fragment of eIF-4B. The predicted secondary structures for wild-type A3 RNA and mutants of A3 RNA are illustrated (**A**). Nitrocellulose filter-binding conditions are described in the Materials and methods.

phorylation (Duncan & Hershey, 1984, 1985), could bind to A3 RNA and whether eIF-4B 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 crosslinked to oligoA3 RNA in HeLa cell extracts. One protein migrated at a similar molecular mass (~85 kDa) as eIF-4B (Fig. 5, lane 1). When the labeled mutant A3ΔA RNA was used, the 85-kDa polypeptide failed to crosslink (lane 2), consistent with the idea that this band corresponds to eIF-4B. 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 crosslinked to oligoA3 (lane 3), but not to A3ΔA RNA (lane 4). To clearly identify the 85-kDa band, UV crosslinking of oligoA3 RNA in HeLa cell extracts was followed by immunoprecipitation with either a polyclonal antibody directed against eIF-4B or with pre-immune serum. The 85-kDa protein was immunoprecipitated by the eIF-4B antibody (lane 5), but not by the pre-immune serum (lane 6), thus demonstrating that HeLa eIF-4B can specifically bind to A3 RNA. Similarly, in the rabbit reticulocyte lysate, this protein was immunoprecipitated by the eIF-4B antibody (lane 7), but not by pre-immune serum (lane 8). Thus, native eIF-4B from human and rabbit lysates recognizes A3 RNA with the same specificity as recombinant eIF-4B.

eIF-4B binds 18S rRNA through its RRM

eIF-4B 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 crosslinking and competition experiments. PolyA RNA (5 μg) was added in each assay to block nonspecific binding by the carboxy-terminal RNA-binding region. Recombinant eIF-4B crosslinked efficiently to rat 18S rRNA (nt 1–1678; Fig. 6A, lane 2), as measured by label transfer. The lower molecular weight bands seen in all lanes are eIF-4B degradation products, because they are rec-

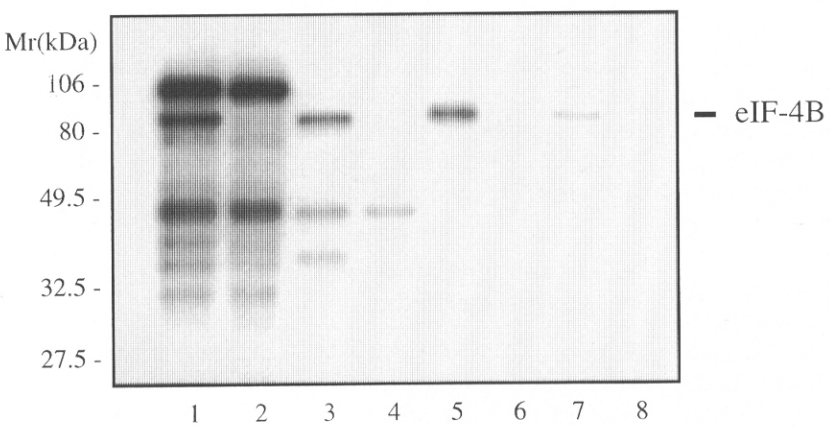


FIGURE 5. Native eIF-4B binds to A3 RNA. ^{32}P-labeled A3 RNA or A3ΔA RNA were UV crosslinked with HeLa or rabbit reticulocytes extracts. Following RNAse A treatment, the proteins marked by label-transfer were resolved on SDS-PAGE. A3 RNA, lanes 1, 3, 5–8; A3ΔA RNA, lanes 2 and 4; HeLa extracts, lanes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

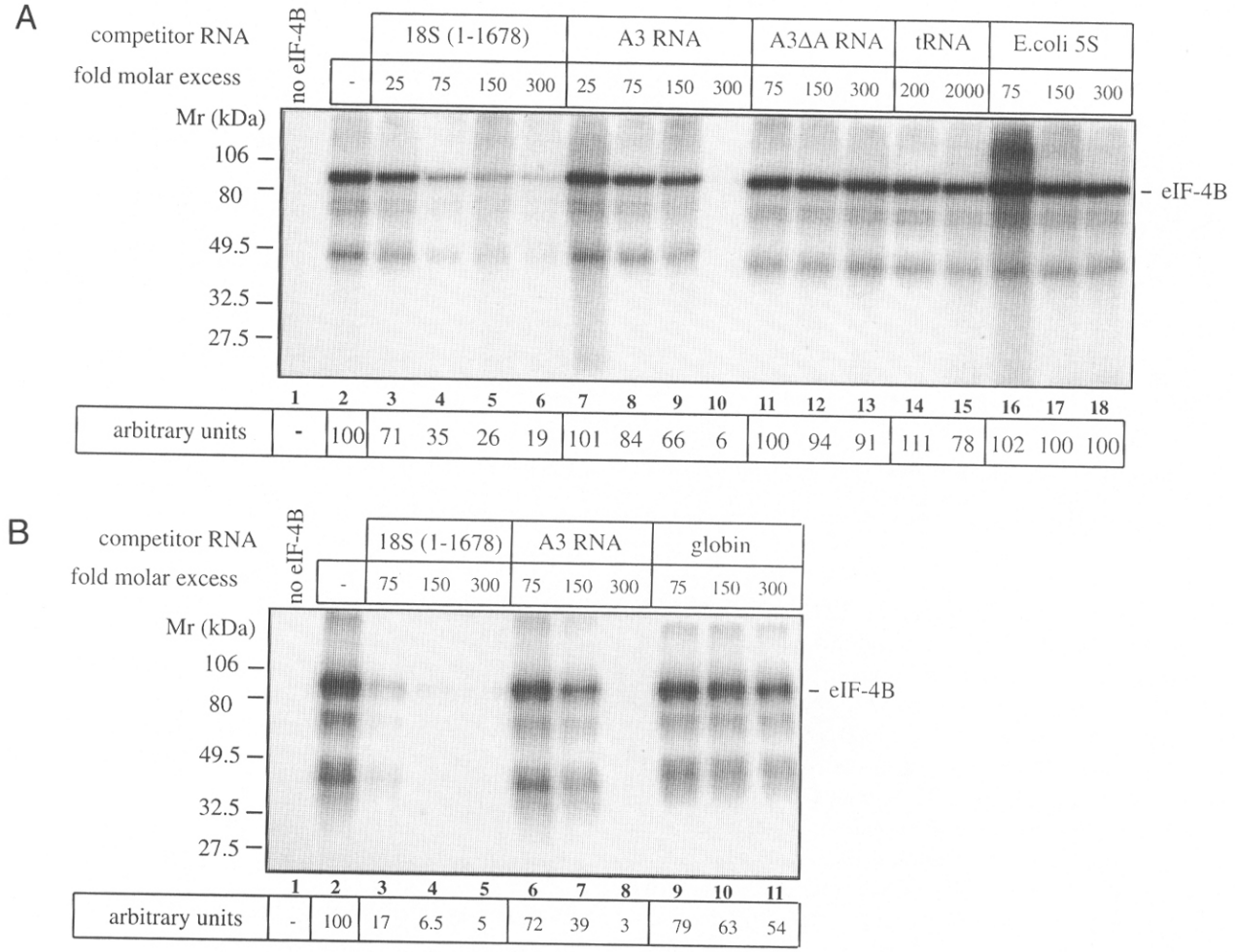


FIGURE 6. eIF-4B specifically photocrosslinks to 18S rRNA. Recombinant eIF-4B (0.5 μg) was incubated with labeled 18S rRNA (1–1678) and 5 μg of polyA RNA in the presence of a molar excess of unlabeled RNA competitors. Samples were then UV-irradiated, digested with RNAse A, and analyzed for label transfer on SDS-PAGE as described in the Materials and methods. **A:** Lane 1, no eIF-4B; lane 2, no competitor; lanes 3–6, increasing molar excess of unlabeled 18S (1–1678) rRNA; lanes 7–10, increasing molar excess of A3 RNA; lanes 11–13, increasing molar excess of A3ΔA RNA; lanes 14 and 15, tRNA; lanes 16–18, increasing molar excess of *E. coli* 5S RNA. **B:** Lane 1, no eIF-4B; lane 2, no competitor; lanes 3–5, unlabeled 18S (1–1678) rRNA; lanes 6–8, A3 RNA; lanes 9–11, globin RNA.

ognized by an antibody directed against eIF-4B (data not shown). Crosslinking was inhibited by up to fivefold 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 crosslinking of 18S rRNA to eIF-4B, 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 6B, lanes 6–8. A3ΔA, on the other hand, failed to compete, even at a high molar excess (Fig. 6A, lanes 11–13). Neither were nonspecific RNAs such as tRNA and *Escherichia 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. 6B, lanes 3–5 and 6–8, respectively; the difference in competition relative to lanes 4–6 of Fig. 6A may be due to different RNA preparations used). Globin RNA was a weak competitor, with only a twofold 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 eIF-4B to 18S rRNA is mediated through the RRM.

eIF-4B can bind simultaneously to two RNA molecules

One of the mechanisms by which eIF-4B could promote binding of the mRNA to the 40S ribosomal subunit is by bridging the mRNA and the ribosome. Such

a model posits that eIF-4B 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 eIF-4B could simultaneously bind a specific and a nonspecific RNA substrate, eIF-4B, N250, or NΔ253 were each incubated with polyA-Sepharose beads and radiolabeled A3 RNA. The amount of A3 RNA bound to polyA-Sepharose via an eIF-4B bridge was assessed (Fig. 7). No A3 RNA was bound to polyA-Sepharose in the absence of eIF-4B (lane 2). In the presence of eIF-4B, approximately 10% of input A3 RNA was recovered on polyA-Sepharose (lane 3). Addition of an excess of unlabeled A3 reduced by ~fourfold the amount of labeled A3 RNA bound to polyA-Sepharose (lane 4). Predictably, N250 (lane 5) and NΔ253 (lane 6) were incapable of bridging significant amounts of A3 RNA to polyA-Sepharose. These results demonstrate that eIF-4B can simultaneously bind two RNA molecules and the binding requires both the RRM and the carboxy-terminal RNA-binding region for this function.

DISCUSSION

In this study, in vitro RNA selections were performed to determine the RNA-binding specificity of the eIF-4B RRM. RNAs with conserved primary sequence and secondary structure were generated and exhibited high affinity for the eIF-4B 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 eIF-4B RRM binds a specific RNA and defines primary and secondary elements required for specific RNA binding.

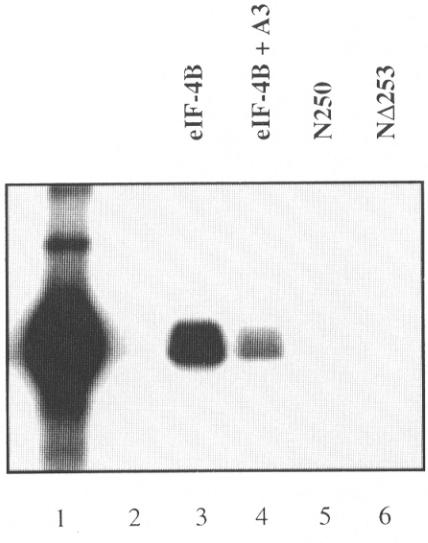


FIGURE 7. eIF-4B can bind two RNA molecules simultaneously. eIF-4B, N250, or NΔ253 (7×10^{-12} mol) were incubated 5 min at 37 °C in a final volume of 50 μL in buffer A containing 15 μL (packed volume) of polyA-Sepharose beads (Pharmacia). A3 RNA (50,000 cpm or 20 pmol) was added for a further 5 min at 37 °C. Beads were washed three times with 500 μL of buffer A, and radioactivity retained was analyzed on an SDS-15% polyacrylamide gel and visualized by autoradiography. Lane 1, A3 RNA load; lane 2, poly-A RNA Sepharose beads alone; lane 3, eIF-4B; lane 4, eIF-4B and unlabeled A3 RNA; lane 5, N250; lane 6, NΔ253.

The effect of the RNA helicase eIF-4A on eIF-4B RNA binding was also investigated (Experiment B). eIF-4A increases the affinity of eIF-4B for RNA by approximately fivefold, 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 eIF-4A does not modify the type of RNA ligands that are preferentially bound by eIF-4B. However, an increase in the content of adenosine residues present in pool-B RNAs compared to those of pool-A RNAs was noticed (Fig. 1). This change could reflect an activation of the carboxy-terminal RNA-binding region, as eIF-4A cooperates with eIF-4B 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 polyA RNA (unpubl. results). Furthermore, a preferential interaction between polyA and wheat germ eIF-4B has been reported (Gallie & Tanguay, 1994). Thus, in the absence of eIF-4A, the eIF-4B RRM is the dominant RNA-binding domain. In the presence of eIF-4A and ATP, the carboxy-terminal RNA-binding region may contribute to the overall RNA-binding affinity.

A role for the specific interaction between eIF-4B 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 eIF-4B 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 & Dreyfuss, 1994a) 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 & Krainer, 1992). When the affinity of this protein for the authentic 3′ or 5′ splice site of the human β-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 β-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 & Gold, 1990; Bartel et al., 1991; Tsai 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 & Gold, 1990; Henderson et al., 1994).

Given the characteristics of eIF-4B and its ability to bind ribosomes (Hughes et al., 1993) the 18S rRNA seemed a logical ligand for this factor. Specific association of eIF-4B with 18S rRNA was shown by UV cross-linking and competition studies (Fig. 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 (nt 465–645; 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 nt 465–645 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 eIF-4B appear to provide a bridge between two different RNA molecules. Full-length eIF-4B was able to bridge A3 RNA and polyA 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 polyA-Sepharose simultaneously.

The data presented here suggest a model for the role of eIF-4B during translation initiation (Fig. 8). According to this model, eIF-4F binds to the cap structure of the mRNA and properly positions the initiation factors eIF-4A and eIF-4B 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 that directs the 40S subunit to the 5′ UTR, and not to other unstructured areas of the mRNA, could be a direct interaction between the eIF-4B RRM and sequences in 18S rRNA. Our data are compatible with a model proposed by Altmann et al. (1995), who have shown that eIF-4B possesses RNA-annealing activity, and suggested that eIF-4B promotes rRNA–mRNA base pairing. Thus, the simultaneous interaction of eIF-4B 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.

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 *Cla* I, 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 eIF-4B by two-step PCR mutagenesis. The resulting vector, pGEX-4B K137Q, was linearized with *Cla* I, 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-eIF-4B were expressed in *E. coli* BL-21 and purified as described (Méthot et al., 1994). eIF-4A and eIF-4B were purified from *E. coli* K38 as described by Pause and Sonenberg (1992).

Selection/amplification

Selection/amplification was performed as described (Tuerk & 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 pmol of RNA. For each round of Experiments A and B, 2 μg of purified GST-4B (2×10^{-11} mol) served as target, whereas in Experiments B and C, 2 μg of purified eIF-4A (4×10^{-11} mol) were used. RNA–protein complexes were isolated by precipitation with either glutathione-sepharose

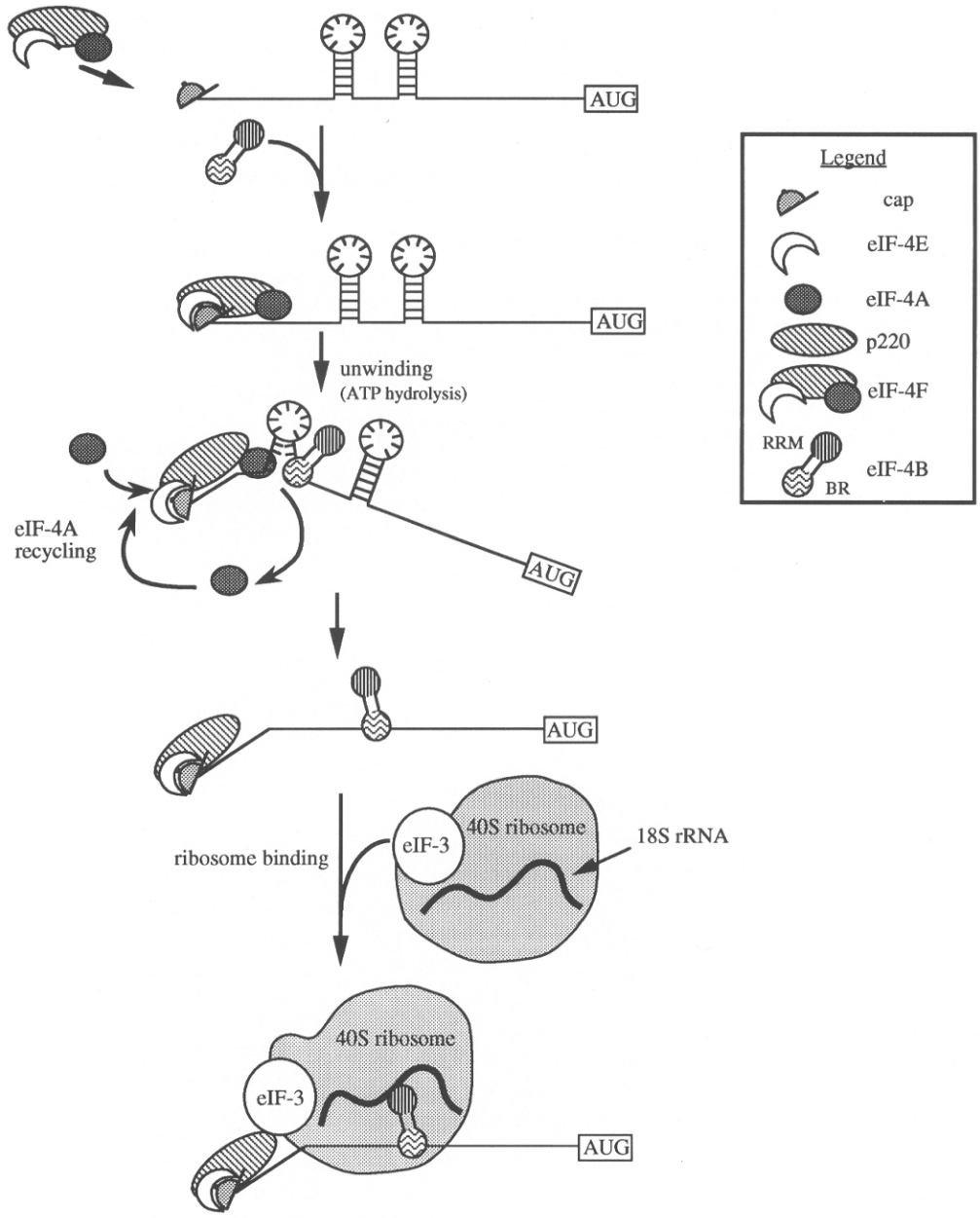


FIGURE 8. Model for the role of eIF-4B during translation initiation. eIF-4B contributes to the binding of the 40S ribosome by bridging the 18S rRNA and the mRNA. The steps leading to ribosome binding are the following: eIF-4F binds to the cap-structure of the mRNA, and is later joined by eIF-4B. These 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). eIF-4B remains bound to the mRNA via its basic carboxy-terminal RNA-binding region (BR; basic region). The 40S ribosomal subunit binds to the mRNA via a specific interaction between the eIF-4B RNA recognition motif (RRM) and the 18S rRNA. Another contributing factor for ribosome binding is eIF-3 via its interaction with p220 (Lamphear et al., 1995).

(Pharmacia; Experiments A and B) or protein G-sepharose coupled to a monoclonal antibody directed against eIF-4A (Experiment C). To remove nonspecific RNAs binding to glutathione-sepharose or protein G-sepharose or anti-eIF-4A, 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 eIF-4A, 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 five 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 eighth round, the amplified DNA was treated with T4 polynucleotide kinase (Pharmacia), agarose gel-purified, and cloned into pBluescript KS linearized with *Sma* I. 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 five times using 2 μg of either N250 (Experiment D) or NΔ253 (Experiment E) as targets. Following the fifth round of amplification, the DNA was digested with *Bam*H I and cloned into pGEM3Z(+) linearized with *Bam*H I. 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 conducted in 100 μL volumes under identical conditions. A tracer amount of ^{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-crosslinking assays were synthesized to a specific activity of 2×10^{17} cpm/mol RNA, and purified by denaturing-PAGE, 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 *Bam*H 1) 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% nondenaturing 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: AATACCTAATACGACTCAC TATAGGGCGATACCTGGTTGATCCTGCC and AACGCA AGCTTATGACCCGCACTTACTG. Transcription with SP6 RNA polymerase using pSP64-globin linearized with *Bam*H I 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 pmol 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 two independent binding reactions and is corrected for the amount of RNA retained in the absence of protein, which was typically less than 1.5% of the RNA input.

UV crosslinking

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 μg 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 15-W 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, eIF-4B was immunoprecipitated with an anti-eIF-4B polyclonal antibody. Samples were subjected to SDS-PAGE and visualized by autoradiography. For 18S rRNA crosslinking studies, ^{32}P-labeled 18S rRNA [(1–1678); 1.4×10^{-14} mol] and competitor RNA as indicated were mixed simultaneously in 20 μL of buffer A containing 5 μg of polyA 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. Crosslinked products were quantified using a Fujix Bas 2000 Phosphorimager.

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