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The Translation Initiation Factor eIF-4B Contains an RNA-Binding Region That Is Distinct and Independent from Its Ribonucleoprotein Consensus Sequence

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eIF-4B is a eukaryotic translation initiation factor that is required for the binding of ribosomes to mRNAs and the stimulation of the helicase activity of eIF-4A. 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 eIF-4B to bind a random RNA, to cooperate with eIF-4A in RNA binding, and to enhance the helicase activity of eIF-4A. We report here that the RNP-CS/RRM alone is not sufficient for eIF-4B 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 eIF-4B to cooperate with eIF-4A in RNA binding and the ability to stimulate the helicase activity of eIF-4A. Point mutations in the RNP-CS/RRM had no effect on the ability of eIF-4B to cooperate with eIF-4A in RNA binding but significantly reduced the stimulation of eIF-4A helicase activity. Our results indicate that the carboxy-terminal RNA-binding region of eIF-4B is essential for eIF-4B function and is distinct from the RNP-CS/RRM.

Initiation of protein synthesis in eukaryotes is a complex multistep process leading ultimately to the binding of the small ribosomal subunit to the mRNA and its proper positioning on the initiator AUG (for a recent review, see reference 37). This event, which is considered rate limiting in translation (25), 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 references 37, 49, and 57]), a process which is dependent on ATP hydrolysis and requires the participation of at least three initiation factors: eIF-4F, eIF-4A, and eIF-4B (reviewed in reference 48). eIF-4F is a heterotrimer composed of eIF-4E, a 24-kDa polypeptide which specifically interacts with the mRNA 5′ cap structure (15, 58); eIF-4A, 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 eIF-4B (19, 42, 47, 50); and p220, whose function is unknown but which is essential for the activity of eIF-4F (16).

The activities of the components of eIF-4F are consistent with models in which eIF-4F binds first to the cap and aligns eIF-4A in close proximity with the mRNA, where it can initiate the melting of the mRNA secondary structure in an ATP-dependent fashion (for reviews, see references 37 and 49). This feature of translation is important, since the amount of secondary structure in the 5′ UTR of a mRNA influences its efficiency of translation (29, 30, 43). It has also been shown that overexpression of eIF-4E in NIH 3T3 cells results in malignant transformation, perhaps by relief of translational repression of certain proto-oncogene mRNAs (32).

The role of eIF-4B in translation initiation is ill-defined. This factor has been characterized from mammalian sources as a phosphoprotein of 80 kDa (6, 60) whose state of phosphorylation positively correlates with cellular translation rates (13, 14, 40). Although no unique functions have been assigned to it, eIF-4B is thought to play a coordinating role during translation initiation (37). It is absolutely required for mRNA binding to ribosomes (6, 60) and considerably stimulates the helicase activity of eIF-4A and eIF-4F (31, 50). Recently, eIF-4B has been shown to possess a ribosome-dependent ATPase activity (24). Another possible function of eIF-4B is the recycling of the eIF-4E component of eIF-4F (46).

The human eIF-4B cDNA encodes a protein of 611 amino acids with a predicted molecular mass of 70 kDa (39), with a ribonucleoprotein consensus sequence (RNP-CS)/RNA recognition motif (RRM) (for a review, see references 5, 23, 27, and 36) near the amino terminus. Accordingly, RNA binding has been demonstrated for rabbit eIF-4B (20), with some preference for AUG triplets, leading to the suggestion that eIF-4B contributes to initiation codon recognition (18).

Purification of eIF-4B to homogeneity from mammalian sources has proven difficult. eIF-4B interacts strongly with eIF-4F, and preparations are often contaminated with the latter (21, 40). Furthermore, in most of the assays performed with eIF-4B, large amounts of protein were used (19, 20, 31, 46, 47, 50), causing concern as to whether the effects observed could be attributed to contaminants in the eIF-4B preparations.

To better understand the function of eIF-4B in translation initiation, we have expressed and purified recombinant human eIF-4B 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 eIF-4B, and evidence is provided for the presence of a potent RNA-binding region in the carboxy-terminal half of eIF-4B, between amino acids 367 and 423.

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MATERIALS AND METHODS

Plasmid construction. To introduce the eIF-4B cDNA into the *Bam*HI site of the bacterial GST fusion protein expression vector pGEX-3X (56), a three-fragment ligation was performed. Fragment 1 consisted of a PCR fragment of the 5′ end of the eIF-4B cDNA (1 to 189 [39]) in which a *Bam*HI site was introduced at position 10 from the 5′ end. After amplification, the PCR product was digested with *Bam*HI and *Bst*XI, yielding a fragment spanning nucleotides 10 to 127. Fragment 2 consisted of the remaining portion of the eIF-4B cDNA (128 to 2013), which was obtained by digesting pET3b-4B (42) with *Bst*XI and *Bam*HI. Fragment 3 consisted of pGEX-3X digested with *Bam*HI. The resulting vector, pGEX-4B, contains the eIF-4B cDNA insert (nucleotides 10 to 2013) flanked by two *Bam*HI sites and in frame with the coding sequence of GST. The expression product is a GST–eIF-4B fusion protein with an expected molecular mass of 106 kDa. The pGEX-4B *Bam*HI fragment was subcloned into pGEM3 (Promega) to form pGEM3-4B in order to generate some of the deletion mutants.

All mutant eIF-4B 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 *Bam*HI and one of the following enzymes: *Bgl*II, *Hin*cII, *Xho*I, *Sac*I, *Cla*I, or *Bcl*I, respectively. The fragments corresponding to the expected sizes were gel purified and religated into pGEX-3X that had been digested with *Bam*HI. The N464 deletion mutant was obtained by digesting pGEM3-4B with *Bcl*I and *Xho*I, isolating the 338-bp eIF-4B cDNA fragment and ligating it to pGEX-4B that had been digested with *Xho*I and *Sma*I. The N423 deletion was obtained by linearizing pGEX-4B with *Sma*I and digesting with exonuclease III for various amounts of time. Following mung bean nuclease treatment, the DNA was digested with *Bam*HI and blunt ended with T4 DNA polymerase. The truncated fragments were gel purified and ligated into *Sma*I-treated pGEX-3X. The N-terminal deletion (NΔ253) was obtained by linearizing pGEM3-4B with *Hin*dIII and then by digestion with exonuclease III. The DNA was treated as described above, and truncated fragments were ligated into *Sma*I-treated pGEX-3X. Deletion mutants were sequenced at the junction of eIF-4B cDNA and vector DNA to confirm the position of the truncation. All mutant fusion proteins contain 7 to 12 non-eIF-4B 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 by PCR (51). Amplified fragments were sequenced and subcloned into pGEX-4B.

Protein expression and purification. *Escherichia coli* BL21 was transformed with pGEX-4B DNA that encodes the wild-type (wt) form or mutant forms of eIF-4B. Overnight cultures (20 ml) were diluted into 1 liter in fresh Luria broth containing 50 μg of ampicillin per ml and grown at 37°C until an optical density at 600 nm of 1.0 was reached. Isopropyl-β-D-thiogalactopyranoside (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 (phosphate-buffered saline [PBS], 0.2 mM EDTA, 2 mM dithiothreitol [DTT]). Cells were lysed on ice by six sonication cycles of 20 s each. Immediately before sonication, a protease inhibitor mixture consisting of phenylmethylsulfonyl fluoride (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 centrifugation at 40,000 rpm for 30 min in a Ti-60 rotor (Beckman), and the supernatant was incubated for 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 three times with 15 volumes of wash buffer. Protein was eluted off the beads by three washes with 1 ml of 10 mM reduced glutathione (GIBCO) in 50 mM Tris-Cl (pH 8.5) and once 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 eIF-4B. To remove the latter, mutants that retained the first 367 amino acids were further purified on a heparin EconoPak column (Bio-Rad) in at fast-protein liquid chromatography 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 with centriprep-30 concentrators (Amicon), and dialyzed 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/liter of culture for wt GST–eIF-4B to 10 mg/liter for N250 and N171. Recombinant eIF-4A was purified by the method of Pause and Sonenberg (42).

RNA synthesis. The RNA substrate used in the RNA-binding and the helicase assays was generated with pGEM3 (Promega) and pGEM MO1/2 vectors (52). pGEM3 was linearized with *Bam*HI and transcribed with SP6 polymerase, yielding a 41-nucleotide transcript. pGEM MO1/2 was linearized with *Hin*cII and transcribed with T7 polymerase, yielding a 68-nucleotide transcript. The nucleotide sequences of the two strands are as follows: 41-nucleotide strand, 5′GAAUA CAAGCU UGCAUGCCUGCAGGUC<u>GACUCUAGAGGA UC</u>3′; 68-nucleotide strand, 5′GGGAGACCGGAAUUCCC CAUGGCUGACUAAUUUUUUUUAUUUAUGCAGAG GGGG<u>GAUCCUCUAGAGUC</u>3′ (duplex region is underlined). Transcriptions were carried out as recommended by the supplier (Promega) with [α-^{32}P]GTP (50 μCi; 3,000 Ci/mmol) for the 41-nucleotide 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. 3A). The transcripts were separated on an 8% denaturing (8 M urea) polyacrylamide gel, and the bands were visualized by autoradiography or UV shadowing, excised, and eluted for 12 h at 4°C in 0.4 ml of a solution of 0.5 M ammonium acetate (NH_4OAc), 1 mM EDTA and 0.1% sodium dodecyl sulfate (SDS). RNAs were phenol-chloroform extracted and ethanol precipitated. The annealing of the two strands was performed at a twofold molar excess of the unlabeled over the labeled transcript in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.2)–250 mM NaCl–1 mM EDTA. The RNA mixture was heated at 95°C for 5 min and slowly cooled to 37°C for 2 h to allow for hybridization. The duplex was purified on a 7.5% nondenaturing polyacrylamide gel, visualized by autoradiography, excised, and eluted as described above.

GST–eIF-4B binding to RNA. GST–eIF-4B binding to RNA was measured in a nitrocellulose filter binding assay. GST–eIF-4B (1 to 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 magnesium acetate [$Mg(OAc)_2$], 75 mM KCl), containing 0.1 mg of bovine serum albumin (BSA) per

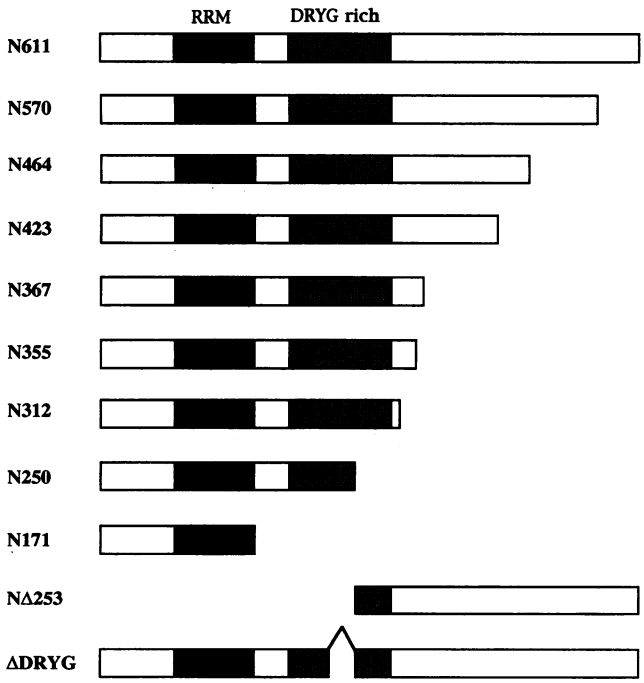

FIG. 1. Schematic representation of GST–eIF-4B deletion mutants. wt eIF-4B contains 611 amino acids. The RRM is located between amino acids 97 and 175, and a DRYG-rich region is located between residues 214 and 327.

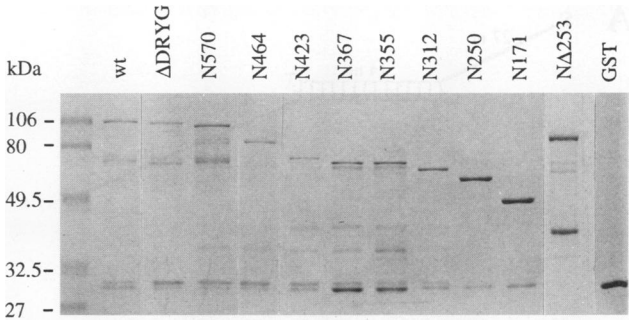

FIG. 2. Polyacrylamide gel analysis of GST–eIF-4B deletion mutants. GST–eIF-4B wt and GST–eIF-4B deletion mutants (approximately 2 μg) were resolved on an SDS–11% polyacrylamide gel and stained with Coomassie blue.

ml and 0.18 pmol of ^{32}P-labeled duplex RNA. The mixture was filtered through a prewetted nitrocellulose membrane (0.45 μm pore size, 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 eIF-4A and GST–eIF-4B in RNA binding was assayed by the nitrocellulose filter binding procedure with minor modifications: GST–eIF-4B mutants (2.5 pmol) and recombinant eIF-4A (22 pmol) were mixed in 40 μl of binding buffer containing 0.1 mg of BSA per ml, 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 eIF-4B, which typically represented less than 1.5% of the total RNA input.

RESULTS

Expression and purification of GST–eIF-4B mutants. eIF-4B was expressed as a GST fusion protein, allowing for a single-step affinity purification with 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–eIF-4B fusion protein for all assays. The GST–eIF-4B fusion protein exhibited wt activity for stimulation of eIF-4A and eIF-4F helicase activity (see below). A series of C-terminal deletions was made by using either conveniently placed restriction enzyme sites or exonuclease III digestion, and one N-terminal deletion mutant (NΔ253) was produced by exonuclease III digestion. Figure 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 (39). 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 five amino acids, while the N-terminal deletion removes the RRM and a portion of the DRYG region.

Purification of GST–eIF-4B with glutathione-Sepharose yielded full-length protein as well as smaller polypeptides. These are presumably degradation products of GST–eIF-4B, as they are immunoreactive towards polyclonal antiserum raised against eIF-4B. 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 with a heparin 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 with poly(U)-Sepharose. A Coomassie blue stain of an SDS-polyacrylamide gel of the eIF-4B deletion mutants which were used in all assays is shown in Fig. 2. wt GST–eIF-4B migrates at a molecular mass of 106 kDa, as expected from the combined mass of GST and eIF-4B (Fig. 2). 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–eIF-4B preparations and homogeneously pure recombinant eIF-4B (42), containing the same amount of full-length protein (as determined by Western blotting [immunoblotting]), were comparable (data not shown). The doublet migrating at 30 kDa is GST as it comigrates with the GST marker (lane GST) and does not react with the anti-eIF-4B antibody (data not shown). Because of the presence of degradation products in the eIF-4B preparations, equimolar amounts of full-length mutant proteins, as determined by Western blotting analysis, were used in all assays.

GST–eIF-4B binding to RNA. The affinity of eIF-4B mutant proteins for RNA was measured by a nitrocellulose filter binding assay. The RNA substrate consisted of a 14-bp duplex region flanked by 5′ single-stranded overhangs (Fig. 3A). wt GST–eIF-4B bound 40% of the RNA. A deletion of 40 amino acids from the C terminus of eIF-4B (mutant N570) had no deleterious effect on RNA binding. Further deletions from the

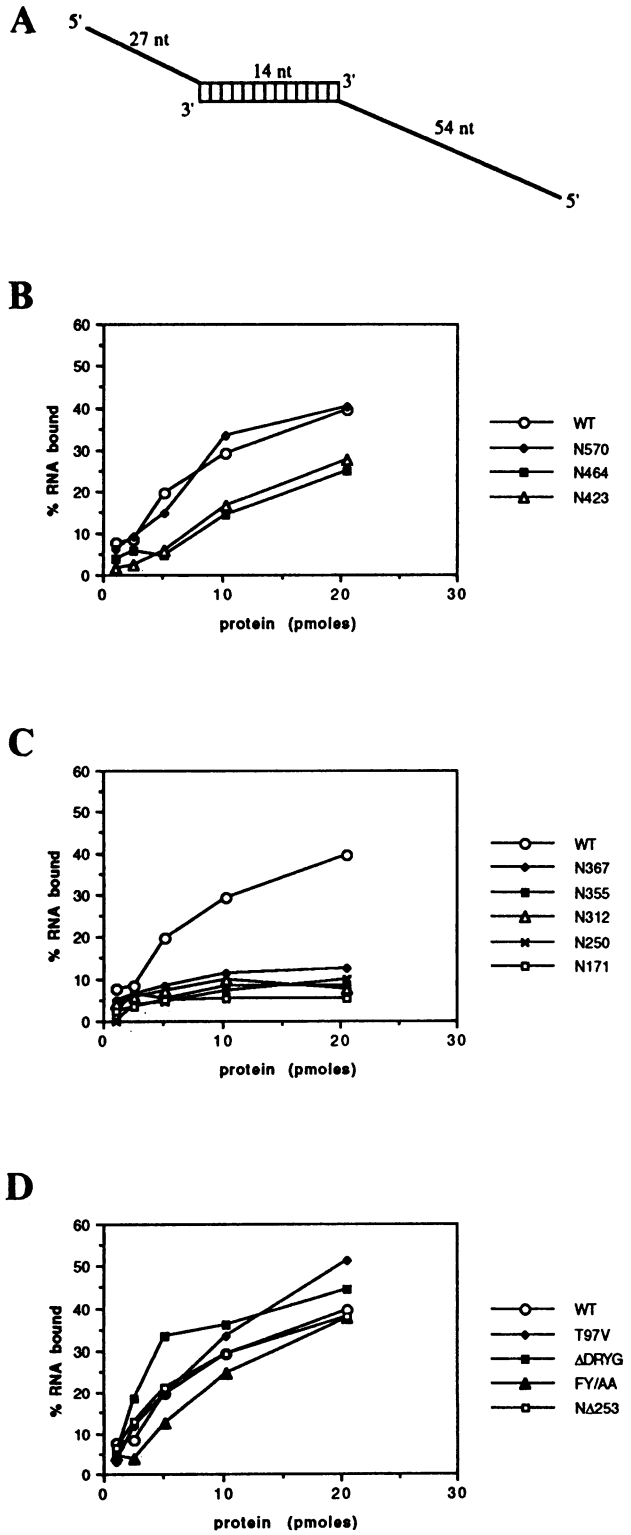

FIG. 3. RNA-binding properties of GST–eIF-4B mutants. (A) Schematic representation of the RNA substrate used for the nitrocellulose filter binding assays and the eIF-4A–eIF-4B-directed helicase assay. The substrate was prepared as described in Materials and Methods and contains a double-stranded region 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–eIF-4B mutants. RNA (0.18 pmol) was incubated with increasing amounts of protein. Each point represents the average of at least two independent determinations.

		RNP-2								RNP-1							
	97								137								
eIF-4B	T	A	F	L	G	N	L			K	G	F	G	Y	A	E	F
U1A snRNP	T	I	Y	I	N	N	L			R	G	Q	A	F	V	I	F
U2B" snRNP	T	I	Y	I	N	N	L			R	G	Q	A	F	V	I	F
hnRNP A1 (1)	K	L	F	I	G	G	L			R	G	F	G	F	V	T	Y
U1 70K	T	L	F	V	A	R	V			R	G	Y	A	F	I	E	Y
La RNP	S	V	Y	I	K	G	F			K	G	S	I	F	V	V	F
eIF-4B mutation																	
T97V	V																
FY/AA														A		A	

FIG. 4. Alignment of eIF-4B RNP-1 and RNP-2 with homologous segments of other characterized RRM-containing proteins. Sequence alignments were obtained elsewhere (28). Positions are numbered according to the eIF-4B sequence. RNP-1 and RNP-2 residues are boxed. Mutated eIF-4B residues are indicated at the bottom. hnRNP, heterogeneous nuclear RNP.

C terminus (mutants N464 and N423) led to a small reduction (40%) in RNA binding (Fig. 3B). However, RNA-binding activity was significantly reduced for mutants N367, N355, N312, and N250 (four- to sixfold at maximal binding [Fig. 3C]). These data indicate that the RRM alone (located between amino acids 97 and 175) is not sufficient to account for most of the RNA-binding activity of eIF-4B. Mutant N171, in which the extreme C-terminal portion of the RRM has been removed, showed the least RNA-binding activity (eightfold reduction at maximal binding [Fig. 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 on the basis of 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. 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). T-97 is located at the edge of RNP-2 (Fig. 4). Data derived from X-ray diffraction of the crystal structure of the small nuclear RNP (snRNP) U1A suggest that the corresponding threonine residue (threonine 11) may form hydrogen bonds with the RNA (26). Mutagenesis of T-11 of U1A to valine abolished RNA binding (26). Phenylalanine 139 and tyrosine 141, which occupy positions 3 and 5 of RNP-1, are also highly conserved throughout the RRM family (see Fig. 4). It has been suggested that they participate in ring-stacking interactions with nucleotide bases of the RNA (28). This is based on the finding that the phenylalanine at position 5 of RNP-1 in heterogeneous nuclear RNP A1 cross-links to RNA upon UV irradiation (38). Also, mutations of phenylalanines into alanines at positions 3 and 5 of the Rho protein RNP-1 resulted in weakened RNA binding (8). These mutations would be expected to strongly affect RNA binding by eIF-4B, were the RRM solely responsible for eIF-4B binding to the RNA.

The T97V and the FY/AA mutants bound RNA to approximately wt levels (Fig. 3D). Moreover, the NΔ253 mutant, in which the RRM was completely removed, bound RNA with similar affinity to wt GST–eIF-4B (Fig. 3D). To demonstrate

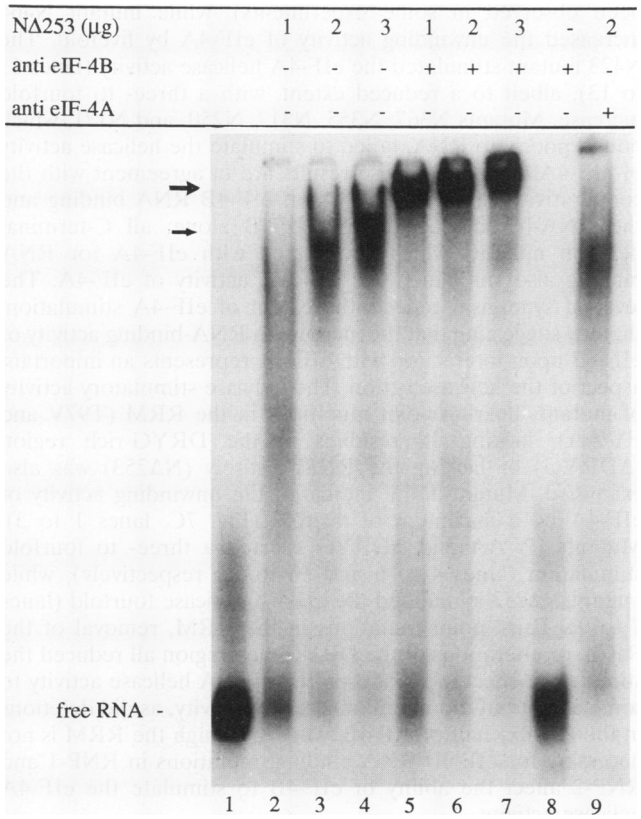

FIG. 5. Mobility shift assay analysis of mutant NΔ253. NΔ253 [prior to poly(U) purification] was incubated in buffer containing 20 mM HEPES-KOH (pH 7.2), 2 mM DTT, 0.5 mM $Mg(OAc)_2$, 5% glycerol, and 75 mM KCl in a final volume of 20 μl. Affinity-purified monoclonal antibody against eIF-4B (1.3 μg [38b]) was added as indicated. Mixtures with and without antibody were preincubated 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-bisacrylamide) containing 5% glycerol in 0.5× Tris-borate-EDTA which had been preelectrophoresed 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.

that the RNA-binding activity in this preparation is attributed to the eIF-4B mutant and not to an *E. coli* contaminating protein, a gel retardation assay (supershift) in the absence and presence of a monoclonal antibody against eIF-4B was performed. Addition of mutant NΔ253 to the RNA resulted in the formation of a gel-retarded RNA-protein complex (Fig. 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 wt eIF-4B (data not shown). In the presence of a monoclonal antibody raised against eIF-4B, the mobility of the complex is further reduced, indicating that the protein component of the complex is indeed eIF-4B (lanes 5 to 7). The antibody itself did not bind to the RNA (lane 8), and a control polyclonal antibody raised against eIF-4A failed to supershift the eIF-4B–RNA complex (lane 9). These results demonstrate the presence of an RNA-binding site at the C-terminal half of eIF-4B, 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 eIF-4B activity. The ΔDRYG mutant bound RNA at wt levels (Fig. 4C), indicating that the integrity of this region is not required for RNA-binding activity of eIF-4B. These results, together with those of C-terminal deletion analyses, indicate that the critical region in eIF-4B for RNA binding is located between amino acids 367 and 423.

Cooperation between GST–eIF-4B mutants and eIF-4A in RNA binding. The affinity of eIF-4B for RNA is increased in the presence of eIF-4A and ATP. The amount of RNA bound by the combination of eIF-4A and eIF-4B is greater than the sum of RNA bound by the individual components, thus indicating a synergistic effect (2, 20). UV cross-linking studies have shown that the majority of the RNA is bound to eIF-4B and not to eIF-4A under these conditions (data not shown). We have used the filter binding assay to examine the effects of mutations on the ability of eIF-4B to cooperate with eIF-4A in binding to RNA. The data are summarized in Fig. 6. All assays were performed in the presence of ATP. As previously shown (1, 41), eIF-4A alone has a very low affinity for RNA, as only 4% of the substrate is bound by eIF-4A. wt GST–eIF-4B, 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 eIF-4B to bind RNA. We used low amounts of eIF-4B (2.5 pmol) in this assay to observe the cooperative effect with eIF-4A. When eIF-4A was included with wt GST–eIF-4B, mutant N570, and mutant N464, the amount of bound RNA was increased by fivefold, thus showing a clear synergistic effect (Fig. 5A). Mutant N423 could also cooperate with eIF-4A, but with a twofold reduction in the total amount of RNA bound compared with that by the wt (Fig. 6A). RNA-binding activity of mutants N367, N355, N312, N250, and N171 on the other hand was not significantly increased by eIF-4A (Fig. 6B). The point mutants T97V and FY/AA exhibited synergism at 100 and 70% of wt levels, respectively (Fig. 6C). Strikingly, RNA binding of the NΔ253 mutant was also stimulated by eIF-4A (60% of wt [Fig. 6C]). These results are in agreement with the presence of an RNA-binding site at the C-terminal half of eIF-4B. 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 eIF-4A at 80% of wt levels (Fig. 6C). Deletion of 244 amino acids from the carboxy end results in the complete loss of cooperativity. Thus, the carboxy 188 amino acids of eIF-4B are dispensable for cooperativity with eIF-4A. 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 eIF-4A. This suggests that the RRM is not essential for the apparent change of affinity of eIF-4B for RNA that is engendered by eIF-4A and ATP hydrolysis.

Helicase stimulatory activity of GST–eIF-4B mutants. Since eIF-4B stimulates the helicase activity of eIF-4A, it was pertinent to test the effect of eIF-4B mutations in a helicase assay. The RNA substrate was the same as that described in the two previous assays (Fig. 3A). eIF-4A alone exhibited some unwinding activity, as 4% of the duplex RNA was converted into the monomeric form (Fig. 7A, lane 1). Addition of GST–eIF-4B stimulated the unwinding activity of eIF-4A in a dose-dependent manner (lanes 2 to 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 to 7 and 8 to 10, respectively). Mutant N570 showed a ninefold stimulatory effect over eIF-4A helicase (wt levels of stimulation have

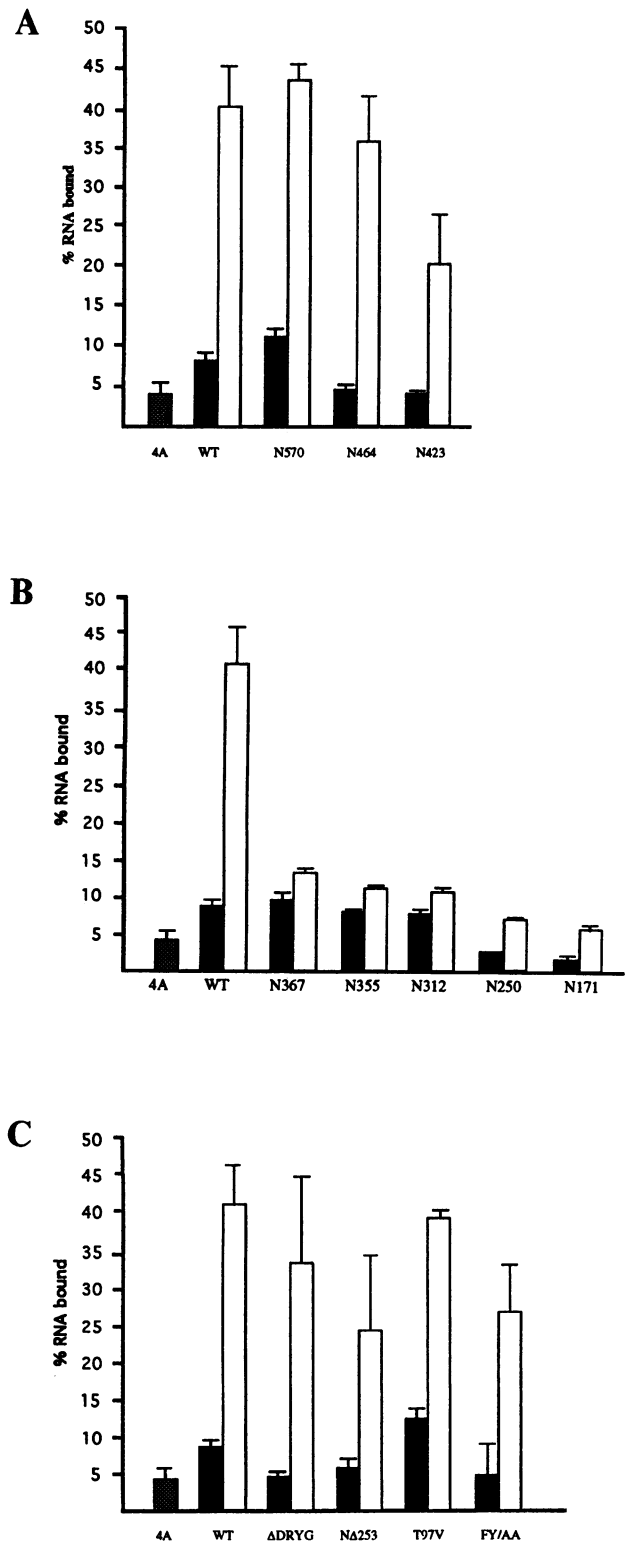

FIG. 6. Cooperation between eIF-4A and eIF-4B in RNA binding. The RNA substrate shown in Fig. 3A (0.18 pmol) was incubated with either eIF-4A alone (22 pmol, stippled box), GST–eIF-4B alone (2.5 pmol, black box), or GST–eIF-4B and eIF-4A (2.5 and 22 pmol, respectively; white box) as described in Materials and Methods. Each point represents the average of at least two independent determinations, and the standard deviations are indicated.

been observed in some experiments), while mutant N464 increased the unwinding activity of eIF-4A by fivefold. The N423 mutant stimulated the eIF-4A helicase activity (lanes 11 to 13), albeit to a reduced extent, with a three- to fourfold increase. Mutants N367, N355, N312, N250, and N171, which bound poorly to RNA, failed to stimulate the helicase activity of eIF-4A (Fig. 7B). These results are in agreement with the cooperativity effects of eIF-4A on eIF-4B RNA binding and the RNA-binding activity of eIF-4B alone; all C-terminal deletion mutants which cooperated with eIF-4A for RNA binding also stimulated the helicase activity of eIF-4A. The level of synergism reflects the extent of eIF-4A stimulation, thereby suggesting that the increase in RNA-binding activity of eIF-4B upon interaction with eIF-4A 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 eIF-4A by a maximum of fivefold (Fig. 7C, lanes 1 to 3). Mutants FY/AA and ΔDRYG exerted a three- to fourfold stimulation (lanes 4 to 6 and 10 to 12, respectively), while mutant NΔ253 stimulated the eIF-4A helicase fourfold (lanes 7 to 9). Thus, point mutations in the RRM, removal of the RRM, or alterations of the DRYG-rich region all reduced the stimulatory effect of eIF-4B on the eIF-4A helicase activity to some extent but did not eliminate the activity, as did deletions in the carboxy half of eIF-4B. Thus, although the RRM is not necessary for eIF-4B RNA binding, mutations in RNP-1 and RNP-2 affect the ability of eIF-4B to stimulate the eIF-4A helicase activity.

DISCUSSION

A summary of the effects of deletions and mutations in eIF-4B on RNA binding, cooperation with eIF-4A in RNA binding, and helicase stimulatory activity is shown in Fig. 8. The major conclusion of this study is that eIF-4B 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 activity of eIF-4B. This conclusion is based principally on the finding that an eIF-4B truncated protein lacking the RRM binds RNA with an affinity similar to that of 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 eIF-4B, 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 eIF-4A and eIF-4B for RNA binding and that regions involved in this process localize to the C terminus of eIF-4B. Moreover, the synergistic activity of the eIF-4B deletion mutants correlates with their capacity to stimulate the helicase activity of eIF-4A: mutant N570 behaves like wt eIF-4B in both assays. Mutant N464, which cooperates with eIF-4A to a slightly lower level than the wt, also stimulates the eIF-4A 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 eIF-4A in RNA binding or stimulate the helicase activity of eIF-4A. The RRM, on the other hand, seems to stimulate helicase activity. Deletion of the RRM reduced (fourfold) the helicase-stimulatory activity of eIF-4B, even though the ability

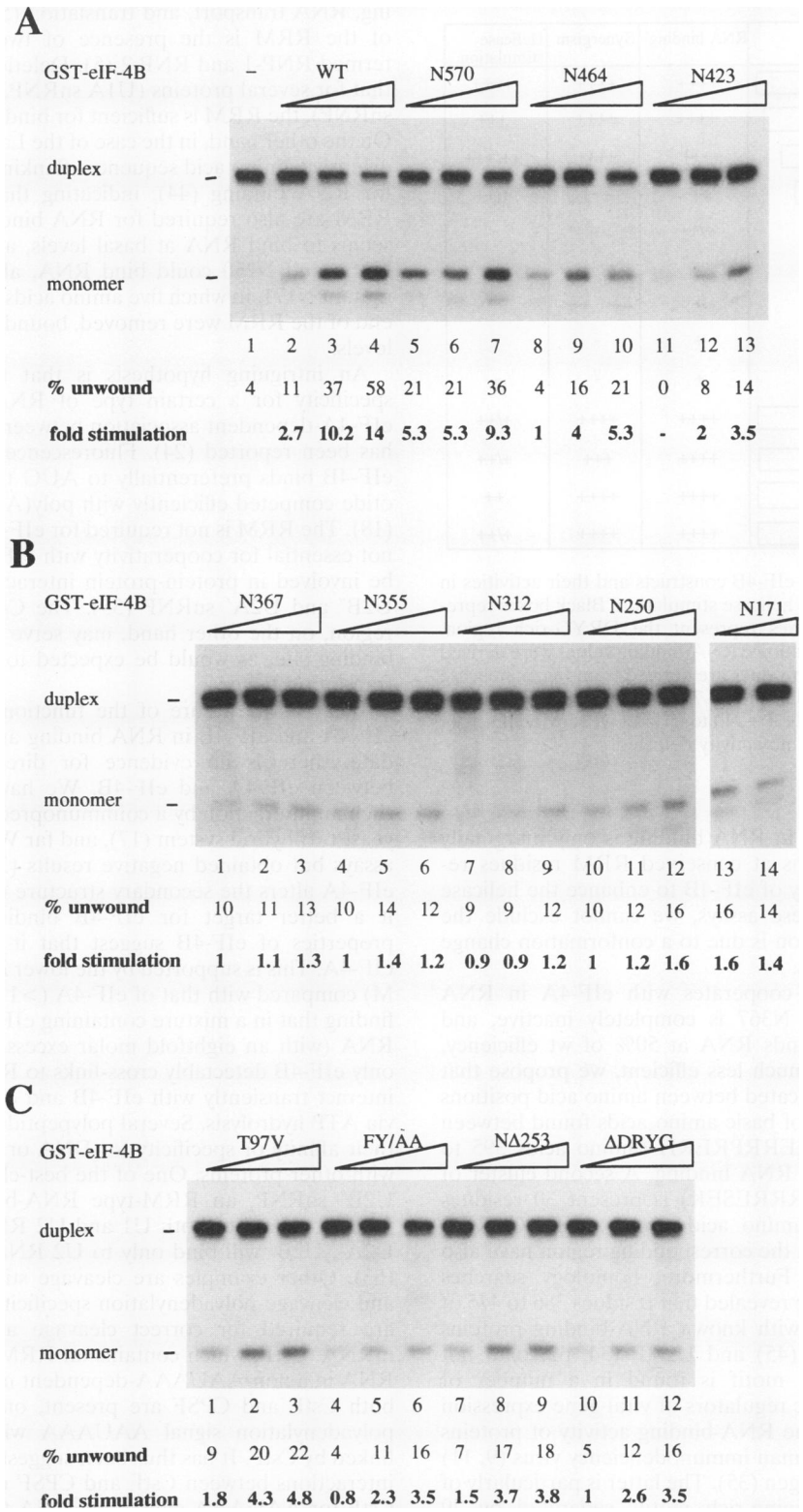

FIG. 7. eIF-4A helicase stimulatory activity of GST–eIF-4B mutants. GST–eIF-4B mutant protein (1, 5, and 10 pmol) was incubated with 45 pmol of eIF-4A and 20 to 40 fmol of labeled RNA duplex in 20 μl of unwinding buffer [17 mM HEPES-KOH (pH 7.2), 2 mM DTT, 5% glycerol, 0.5 mM $Mg(OAc)_2$, 0.5 mM ATP, 75 mM KCl, 20 U of 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 an SDS–15% polyacrylamide gel and visualized by autoradiography. The amounts of duplex and monomer RNA were quantitated on a Fujix BAS2000 phosphoimager. The level of monomeric RNA in the absence of eIF-4A (~5% of total RNA) was subtracted from all values. The percentage of unwinding was calculated as the amount of monomer RNA divided by the sum of duplex and monomer RNA. Background eIF-4A unwinding was 4% in panel A, 10% in panel B, and 5% in panel C.

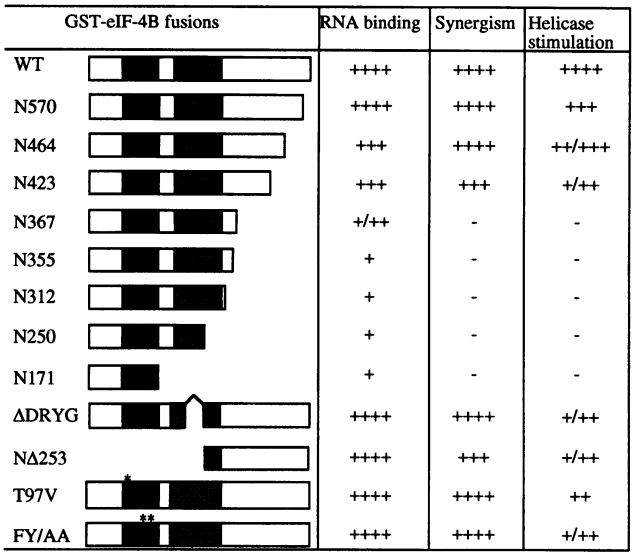

FIG. 8. Summary of GST–eIF-4B constructs and their activities in RNA binding, synergism, and helicase stimulation. Black boxes represent the RRM. Stippled boxes represent the DRYG-rich region. Asterisks represent point mutations. RNA-binding values were derived from Fig. 3B and C; synergism values are derived from Fig. 6; helicase stimulation values are derived from Fig. 7. +, 10 to 25% of wt activity; ++, 25 to 40% of wt activity; +++, 40 to 65% of wt activity; ++++, 65 to 100% of wt activity; −, no activity detected.

to cooperate with eIF-4A in RNA binding is only marginally decreased. Point mutations at conserved RRM residues reduced (fourfold) the ability of eIF-4B to enhance the helicase activity of eIF-4A. 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 eIF-4A 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 found between residues 367 and 423, KLERRPRERH (amino acids 395 to 404), 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/GPRRE REE/K) in yeast eIF-4B in the corresponding region have also been identified (3, 10). Furthermore, homology searches (BLAST program [4]) have revealed that residues 386 to 445 of eIF-4B share similarities with known RNA-binding proteins such as snRNP U1 70K (45) and U2AF (34, 62; data not shown). An arginine-rich motif is found in a number of prokaryotic and eukaryotic regulators of viral gene expression (33) and is required for the RNA-binding activity of proteins such as Tat and Rev of human immunodeficiency virus (9, 11) and the hepatitis delta antigen (35). The latter is particularly of relevance, since two arginine-rich motifs separated by 29 amino acids (an arrangement similar to eIF-4B) are essential for RNA binding. Mutagenesis in the arginine-rich region of eIF-4B will be required to directly test its role in RNA binding.

What is the role of the RRM in the eIF-4B function? The RRM is a loosely conserved region 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 (5, 12, 28). The hallmark of the RRM is the presence of two conserved sequences termed RNP-1 and RNP-2 (5). Deletion analyses have shown that for several proteins (U1A snRNP, U2B″ snRNP, and 70K snRNP), the RRM is sufficient for binding to RNA (45, 53, 54). 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 (44), indicating that residues outside the RRM are also required for RNA binding. The eIF-4B 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 five amino acids at the carboxy-terminal end of the RRM were removed, bound RNA near background levels.

An intriguing hypothesis is that the eIF-4B RRM has specificity for a certain type of RNA, such as rRNA. An eIF-4A-dependent association between ribosomes and eIF-4B has been reported (24). Fluorescence studies suggested that eIF-4B binds preferentially to AUG triplets, as this trinucleotide competed efficiently with poly(A) for binding to eIF-4B (18). The RRM is not required for eIF-4B RNA binding and is not essential for cooperativity with eIF-4A. However, it could be involved in protein-protein interactions, as in the case of U2B″ and U2A′ snRNP (55). The C-terminal RNA-binding region, on the other hand, may serve as a nonspecific RNA-binding site, as would be expected to be found in a general translation factor.

What is the nature of the functional interaction between eIF-4A and eIF-4B in RNA binding and helicase activity? To date, there is no evidence for direct physical interaction between eIF-4A and eIF-4B. We have attempted to detect such an interaction by a coimmunoprecipitation approach, the yeast two-hybrid system (17), and far Western (7) and gel shift assays but obtained negative results (38a). It is possible that eIF-4A alters the secondary structure of the RNA and makes it a better target for eIF-4B binding. The RNA-binding properties of eIF-4B suggest that it binds to RNA before eIF-4A. This is supported by the lower K_d of eIF-4B (5×10^{-7} M) compared with that of eIF-4A ($>1 \times 10^{-6}$ M) and by the finding that in a mixture containing eIF-4A, eIF-4B, ATP, and RNA (with an eightfold molar excess of eIF-4A to eIF-4B), only eIF-4B detectably cross-links to RNA (38a). eIF-4A may interact transiently with eIF-4B 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 example 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 a higher affinity (53). 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 (59).

Recently, SSL2, a 95-kDa protein with motifs similar to the DEXH box family of helicases, has been cloned in *Saccharomyces cerevisiae* (22). Indirect evidence suggests that this polypeptide may interact with SSL1, a zinc finger-containing protein (63). 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 eIF-4A and eIF-4B, and it is possible that they exert their effects in an analogous manner.

The yeast eIF-4B gene has been cloned recently by two groups (3, 10). Although it shares only limited homology with its human counterpart, yeast eIF-4B retains some of the characteristic features of mammalian eIF-4B: an RRM near the amino terminus and repetitive sequences of charged amino acids in the middle (human eIF-4B) or at the carboxy terminus (yeast eIF-4B). As it is composed of 436 amino acids, yeast eIF-4B is much smaller than human eIF-4B (611 residues) and lacks the entire serine-rich carboxy-terminal portion of human eIF-4B. Significantly, the last 188 amino acids of human eIF-4B, which are not present in *S. cerevisiae*, are dispensable for RNA binding, cooperation with eIF-4A, and to a certain extent, stimulation of eIF-4A helicase.

The identification of a novel RNA-binding site in eIF-4B provides a means to identify target RNAs by the SELEX method (61). Knowledge of the mechanism of action of eIF-4B is critical for the understanding of how ribosomes bind to eukaryotic mRNAs and how this process is regulated.

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