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Competitive and Noncompetitive Binding of eIF4B, eIF4A, and the Poly(A) Binding Protein to Wheat Translation Initiation Factor eIFiso4G[†]

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ABSTRACT: Eukaryotic translation initiation factor 4G (eIF4G) functions to organize the assembly of initiation factors required for the recruitment of a 40S ribosomal subunit to an mRNA and for interacting with the poly(A) binding protein (PABP). Many eukaryotes express two highly similar eIF4G isoforms. eIFiso4G, one of two isoforms in plants, is highly divergent and unusually small in size. Unlike animal and yeast eIF4G, the domain organization of plant eIF4G proteins is largely unknown. Consequently, little is known about the conservation of plant eIF4G with those in other eukaryotes. In this study, we show that eIFiso4G is similar to other eIF4G proteins in that there are interaction domains for eIF4A and PABP and we identify, for the first time, the interaction domain for eIF4B. In contrast to previous reports, two eIF4A binding domains in eIFiso4G were identified, similar in number and organization to those of animal eIF4G. The eIFiso4G domain organization does differ, however, in that the N-terminal eIF4A binding domain overlaps with the eIF4B and PABP binding domains. Moreover, the eIF4B and PABP binding domains overlap. PABP and eIF4B compete with eIF4A for binding eIFiso4G in the absence of the C-terminal eIF4A binding domain but not when both eIF4A binding domains are present, suggesting that the C-terminal eIF4A interaction domain functions to stabilize the association of eIF4A with eIFiso4G in the presence of eIF4B or PABP. Competitive binding to eIFiso4G was also observed between eIF4B and PABP. These observations reveal an important function of the C-terminal eIF4A binding domain in maintaining the interaction of multiple partner proteins with eIFiso4G despite the substantial divergence in its size and domain organization.

While translation initiation in prokaryotes involves the recruitment of the small ribosomal subunit to an mRNA through RNA−RNA interactions between the 3′-end of its 16 S rRNA and the Shine-Dalgarno sequence 5′-proximal to an initiation codon, recruitment of a 40S ribosomal subunit to a eukaryotic mRNA is facilitated by the action of translation initiation factors that also mediate recognition of the initiation codon and assembly of the 80S ribosome (*1*−*3*). The eIF4 group of initiation factors is particularly important in the recruitment of the 40S subunit. eIF4E, which binds to the 5′-cap structure, eIF4A, an RNA helicase, and eIF4G comprise eIF4F. eIF4G is a modular scaffolding protein that interacts with eIF4E, eIF4A, eIF3 (required for 40S binding to an mRNA), and the poly(A) binding protein (PABP)[1] (*4*). The interaction of eIF4G with eIF4E that is bound to the 5′-cap and PABP bound to the poly(A) tail results in the circularization of an mRNA and stimulates translation by promoting 40S subunit recruitment (*5*, *6*). The interaction between eIF4G and PABP is conserved in animals, yeast, and plants, and the biological function of the interaction has been well characterized (*1*−*8*). In addition to eIF4F, plants contain an isoform, called eIFiso4F, that is composed of eIFiso4E, eIF4A, and eIFiso4G (*9*). In its function as a DEAD/H helicase, eIF4A uses energy from ATP hydrolysis to unwind secondary structure present in the 5′-leader of an mRNA that might otherwise inhibit 40S subunit scanning (*2*). eIF4B enhances the helicase activity of eIF4A (*10*, *11*) and also binds PABP (*8*, *12*, *13*), an interaction that increases the affinity of PABP for poly(A) RNA (*8*, *13*). The interaction of PABP with eIF4B and eIFiso4F increases the ATPase and RNA helicase activities associated with eIFiso4F (*14*). The interaction between PABP and eIF4B is conserved in animals and plants. A single interaction domain for eIF4B is present within a 32-amino acid region representing the C-terminal end of RNA recognition motif (RRM) 1 of PABP (*15*). eIFiso4G interacts with PABP at a domain that overlaps with the eIF4B interaction domain, and the overlapping nature of their binding sites results in their mutually exclusive binding to PABP (*15*). eIFiso4G also interacts weakly with PABP at a second domain encompassing RRM3 and RRM4 (*15*).

In addition to its interaction with PABP, eIF4B interacts with eIFiso4G, eIF4A, eIF3, and RNA (*8*, *12*, *13*). Thus, eIF4B helps to organize the assembly of the translation initiation machinery. Mammalian eIF4B is a dimer for which the central DRYG-rich domain is required (*16*). Although wheat eIF4B is also a dimer, its dimerization domain overlaps its C-terminal RNA-binding domain, and dimerization of the protein is dependent on a physiological concentration of zinc (*17*). Zinc also preferentially promotes the interaction between eIF4B and PABP such that eIFiso4G is unable to compete with eIF4B in binding PABP (*17*).

Several domains required for partner protein interactions have been identified for mammalian eIF4G (*18*−*21*). The interaction

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[1]Abbreviations: *β*-ME, *β*-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; eIF, eukaryotic initiation factor; GST, glutathione *S*-transferase; IRES, internal ribosomal entry site; PABP, poly(A) binding protein; RRM, RNA recognition motif; SDS, sodium dodecyl sulfate.

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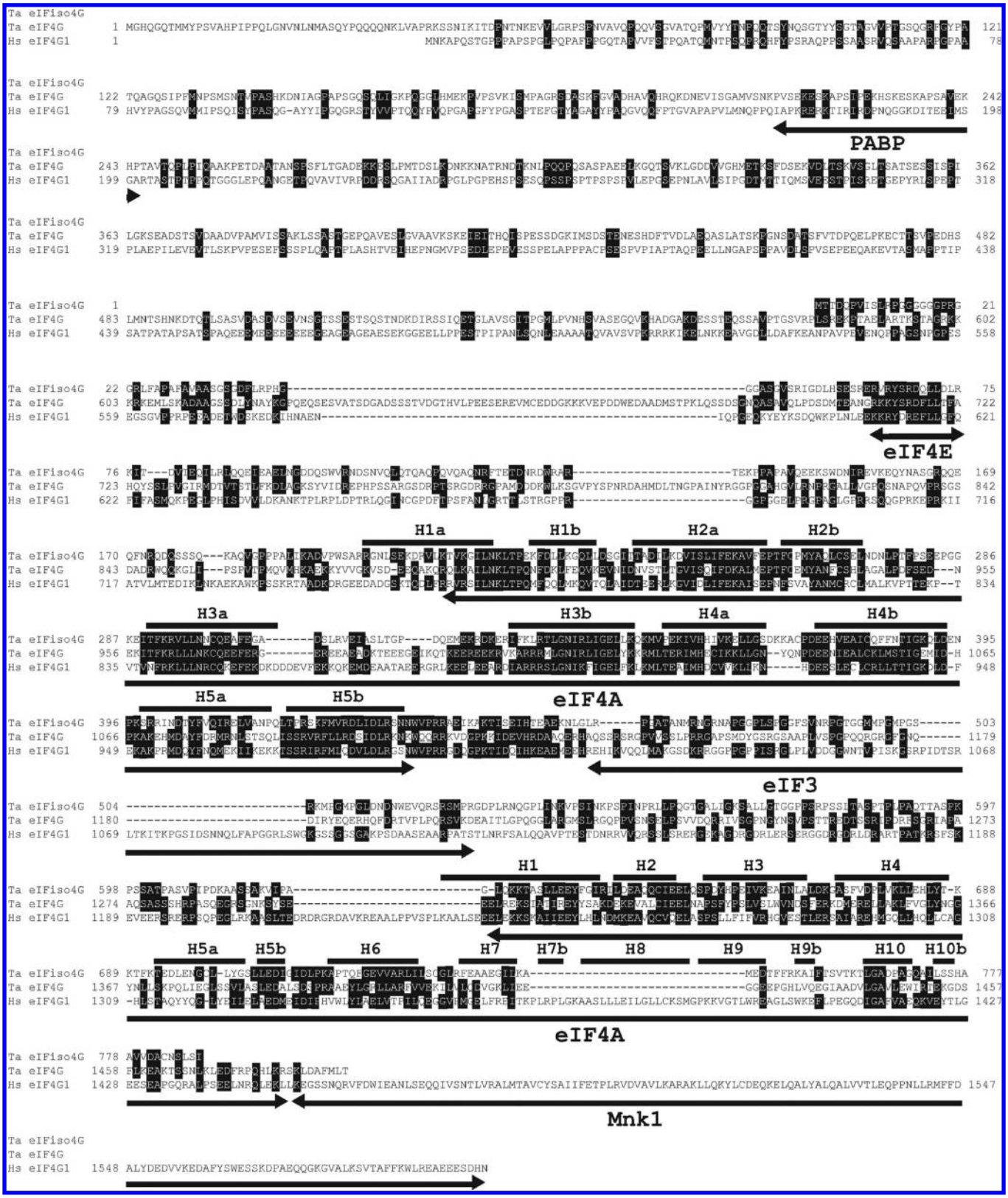

FIGURE 1: Sequence conservation between wheat eIFiso4G and mammalian eIF4G. The sequence of wheat eIFiso4G (Ta eIFiso4G; GenBank accession number M95747) was aligned with wheat eIF4G (Ta eIF4G; GenBank accession number ABO15893) and human eIF4G (Hs eIF4G; GenBank accession number NM_198241). Conservation of identical or similar residues relative to that of wheat eIFiso4G is indicated by shading. Binding sites for select partner proteins of the human eIF4G are indicated below the sequence as reported previously (*3*). The regions corresponding to the helices (H) of the human eIF4GII HEAT-1 domain and the eIF4GI HEAT-2 domain are indicated above the sequence as reported previously (*22*, *23*).

domain for PABP lies N-proximal to that for eIF4E, both of which are present in the N-terminal region of the protein (Figure 1). Mammalian eIF4G contains three HEAT repeat domains, characterized by the presence of antiparallel α-helical hairpins known as HEAT repeats (*22*, *23*). eIF4A binds the first two HEAT domains as well as the linker between these two

domains (*20*, *21*, *24*, *25*). HEAT-1/MIF4G, present in the middle domain of eIF4G, contains five antiparallel α-helical hairpins in a helical stack that are conserved in the plant and yeast homologues (*22*). HEAT-2/MA3, also composed of five HEAT repeats, is largely conserved in the plant homologues (Figure 1) but is absent from yeast eIF4G proteins (*23*). HEAT-1 promotes the helicase activity of eIF4A, whereas HEAT-2 serves a modulatory role (*24*). HEAT-3, composed of three and one-half HEAT repeats in mammalian eIF4G that binds Mnk kinases, is absent from the plant and yeast homologues (Figure 1) (*23*).

Domain mapping of eIFiso4G by yeast two-hybrid analysis suggested that the protein contains just a single eIF4A binding domain (*26*). Deletion of the 52 N-terminal amino acids from eIFiso4G did not abolish eIF4A binding, but deletion of the 90 N-terminal amino acids did, which was interpreted to mean that the eIF4A binding domain lies between residues 52 and 90 (*26*). This region of eIFiso4G, however, contains the eIF4E binding domain (*26*) and shares little similarity with mammalian eIF4G or even wheat eIF4G outside of the eIF4E binding domain (Figure 1). As the two-hybrid assay can yield false negative results, the precise location and number of eIF4A interaction domains in eIFiso4G remain unknown.

eIFiso4G is smaller than animal or yeast eIF4G and shares only limited similarity with other eIF4G proteins, principally within the HEAT-1 domain (Figure 1). The two eIF4G proteins present in plants (i.e., eIF4G and eIFiso4G) are substantially more divergent from one another than are other eIF4G proteins in other species, suggesting a greater functional specialization. However, the extent to which eIFiso4G serves as a scaffolding protein that interacts with multiple factors involved in promoting translation initiation remains unknown. In this study, the interaction of eIFiso4G with proteins and RNA has been examined. In addition to containing the conserved eIF4E and eIF3 binding domains, eIFiso4G contains interaction domains for eIF4B, eIF4A, and PABP. Two domains for eIF4A binding were identified in eIFiso4G as reported for mammalian eIF4G, demonstrating that eIFiso4G is more similar to animal eIF4G than it is to yeast eIF4G. eIFiso4G does differ from animal and yeast eIF4G in that the HEAT-1/eIF4A binding domain overlaps with the eIF4B and PABP binding domains and the eIF4B and PABP binding domains overlap with one another. In the absence of the HEAT-2/eIF4A binding domain, PABP and eIF4B compete with eIF4A for binding eIFiso4G. However, the presence of both HEAT/eIF4A binding domains alleviates the competition, suggesting that the HEAT-2/eIF4A binding domain functions to stabilize the association of eIF4A with eIFiso4G in the presence of eIF4B or PABP. Competitive binding to eIFiso4G was also observed between eIF4B and PABP, indicating that their binding is mutually exclusive. Thus, eIFiso4G has maintained its interaction with multiple partner proteins despite the substantial divergence in its size and domain organization.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression. Constructs for the expression of full-length or truncated eIFiso4G, eIF4B, eIF4A, and PABP were described previously (*12*). All constructs were confirmed by sequencing.

RNA Binding Assay. GST-tagged eIFiso4G polypeptides were expressed in *Escherichia coli* BL21 cells following induction with 1 mM IPTG and a crude extract made in buffer B-100 [200 mM HEPES (pH 7.2), 100 mM KCl, 10% glycerol, and 2 mM DTT] without EDTA but with protease inhibitor cocktail (Sigma). Poly(A) Sepharose 4B or poly(G) agarose (Sigma) was equilibrated in washing buffer (buffer B-100 without EDTA but with 0.1% Triton X-100). The crude extract was incubated with the resin at 4 °C for 20 min while being shaken. The resin was washed extensively with washing buffer and the bound protein resuspended in SDS sample buffer. The supernatant was analyzed by SDS−PAGE and the gel stained with Coomassie Brilliant Blue.

Protein Interaction Assay. Protein interactions were analyzed as described previously (*12*) with some modification. GST fusion protein was added to glutathione Sepharose 4B resin (Amersham) washed three times with precooled buffer B-100 (supplied with 0.1% Triton X-100). Following incubation with shaking at 4 °C for 1 h, the resin was collected by centrifugation and the supernatant removed. Recombinant prey protein from crude extract was added to the resin and incubated for 2 h. The resin was washed four times with Tween phosphate-buffered saline (TPBS) (0.1% Tween 20, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) supplemented with 1 mM DTT. Bound protein was released following the addition of SDS sample buffer to the resin. Following heating and centrifugation, the protein was resolved by SDS−PAGE and the gel stained or used for Western analysis.

To eliminate apparent protein interaction resulting from RNA tethering, RNase A was added to the binding reaction assay. RNase A was added to the resin containing the bait protein and incubated at 37 °C for 20 min. The prey protein was treated similarly. The prey protein was then added and the reaction mixture incubated at 4 °C for an additional 2 h. After extensive washing, bound protein was released following the addition of SDS sample buffer to the resin. The protein was resolved by SDS−PAGE, transferred to a membrane, and used for Western analysis.

Western Analysis. Protein was transferred to a 0.22 mm polyvinylidene difluoride membrane by electroblotting. The membranes were blocked in 5% milk and 1% NaN_3 in TPBS followed by incubation with antiserum in TPBS with 1% milk for 1.5 h. Membranes were washed twice with TPBS and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL) diluted to 1:10000 for 1 h. The blots were washed twice with TPBS, and the signal was detected typically between 1 and 15 min using chemiluminescence (Pierce). Anti-His antiserum was purchased from Santa Cruz Biochemical Inc. eIF4A and eIFiso4G antisera (a generous gift of K. Browning) were used at a 1:1000 dilution. Anti-eIF4B antiserum was generated using recombinant eIF4B.

RESULTS

Several Regions within eIFiso4G Contribute to RNA Binding. Mammalian eIF4G contains at least two RNA binding domains (*27*, *28*). A nucleotide binding domain in wheat eIFiso4G between residues 170 and 443 bound to oligo(dT) cellulose but bound to poly(A) RNA poorly despite the ability of full-length eIFiso4G to do so (*29*, *30*). To identify the RNA binding domains in eIFiso4G, we introduced the cDNA encoding eIFiso4G into pGEX-2TK to express a GST-tagged fusion protein (i.e., GST-eIFiso4G$_{1-788}$) (Figure 2A, lane 2). GST-eIFiso4G$_{1-788}$ was incubated with poly(A) Sepharose 4B resin, the resin washed extensively, and bound protein analyzed by SDS−PAGE.

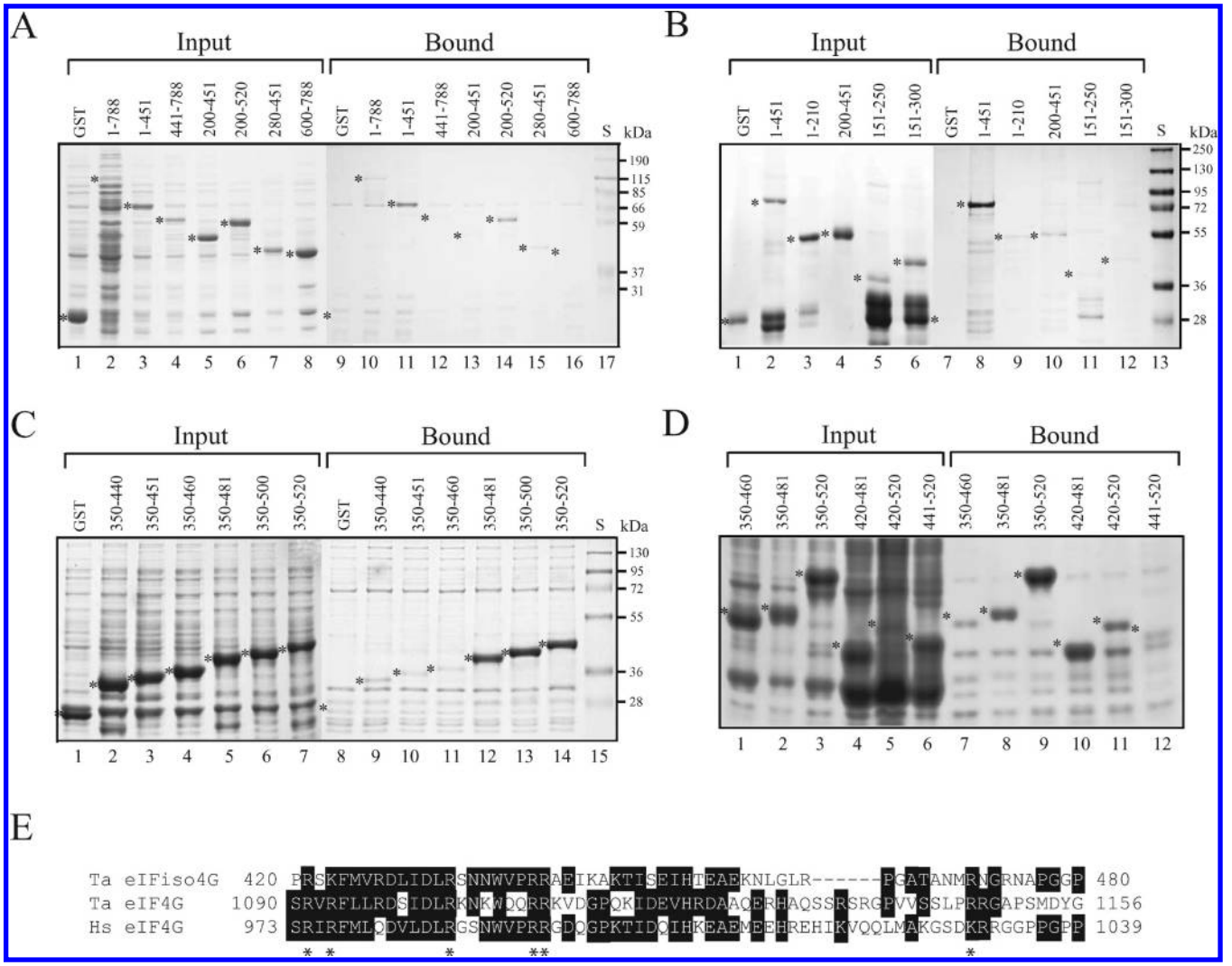

FIGURE 2: Identification of poly(A) RNA binding domains in wheat eIFiso4G. (A−D) eIFiso4G polypeptides were expressed as GST fusion proteins in *E. coli* (Input), and RNA binding activity was determined by adding crude extract to poly(A) Sepharose 4B resin (Bound). Bound proteins were analyzed by SDS−PAGE and detected using Coomassie staining. GST was employed as a negative control. The region of eIFiso4G included in each polypeptide is indicated numerically by the residues included. S denotes molecular weight standards. (E) Comparison of the RNA binding domain between residues 430 and 480 of wheat eIFiso4G (Ta eIFiso4G) with the corresponding region of wheat eIF4G (Ta eIF4G) and human eIF4G (Hs eIF4G). Conservation of identical or similar residues relative to those of wheat eIFiso4G is indicated by shading. Basic residues conserved among plant eIFiso4G, eIF4G, and human eIF4G are denoted with asterisks. The portion of eIFiso4G or eIF4G illustrated in each case is indicated with residue numbers before and after each sequence.

Relative to the amount of input protein, GST-eIFiso4G$_{1-788}$ bound to poly(A) RNA whereas GST did not (in Figure 2A, compare lane 10 to lane 9), in good agreement with our earlier observations using the native protein (*30*). GST-eIFiso4G$_{1-451}$ bound poly(A) efficiently, whereas GST-eIFiso4G$_{441-788}$ did not (Figure 2A, lanes 11 and 12, respectively). Although GST-eIFiso4G$_{200-451}$ and GST-eIFiso4G$_{280-451}$ bound poly(A) poorly (Figure 2A, lanes 13 and 15, respectively), GST-eIFiso4G$_{200-520}$ did bind (Figure 2A, lane 14), suggesting that a second region, involving residues 451−520, was also involved in RNA binding. The C-terminal region between residues 600 and 788 did not bind poly(A) (Figure 2A, lane 16).

To map the N-terminal RNA binding domain more precisely, we tested additional polypeptides within the region of 451 N-terminal residues. In contrast to eIFiso4G$_{1-451}$, which bound poly(A) Sepharose resin strongly (Figure 2B, lane 8), GST-eIFiso4G$_{1-210}$ and GST-eIFiso4G$_{200-451}$ exhibited substantially weaker binding activity (Figure 2B, lanes 9 and 10, respectively), indicating that dividing the N-terminal 451-amino acid domain in half resulted in a considerable loss of poly(A) binding activity. Polypeptides spanning the site of division, e.g., GST-eIFiso4G$_{151-250}$ and GST-eIFiso4G$_{151-300}$, also exhibited poor binding to poly(A) RNA (Figure 2B, lanes 11 and 12, respectively). These data suggest that the N-terminal RNA binding domain requires much of the region of 451 N-terminal residues for full binding to poly(A) RNA, similar to the position of the second RNA binding domain in mammalian eIF4G (*28*).

The observation that GST-eIFiso4G$_{200-451}$ exhibited only weak binding to poly(A) but GST-eIFiso4G$_{200-520}$ bound poly(A) more strongly suggested the possibility that the sequence following residue 451 contributed to RNA binding. GST-eIFiso4G$_{350-520}$, GST-eIFiso4G$_{350-500}$, and GST-eIFiso4G$_{350-481}$ bound poly(A) (Figure 2C, lanes 14, 13, and 12, respectively), but GST-eIFiso4G$_{350-460}$, GST-eIFiso4G$_{350-451}$, and GST-eIFiso4G$_{350-440}$ bound poorly (Figure 2C, lanes 11, 10, and 9, respectively), suggesting that the C-terminal border lies between residues 460 and 481. GST-eIFiso4G$_{420-520}$ retained binding

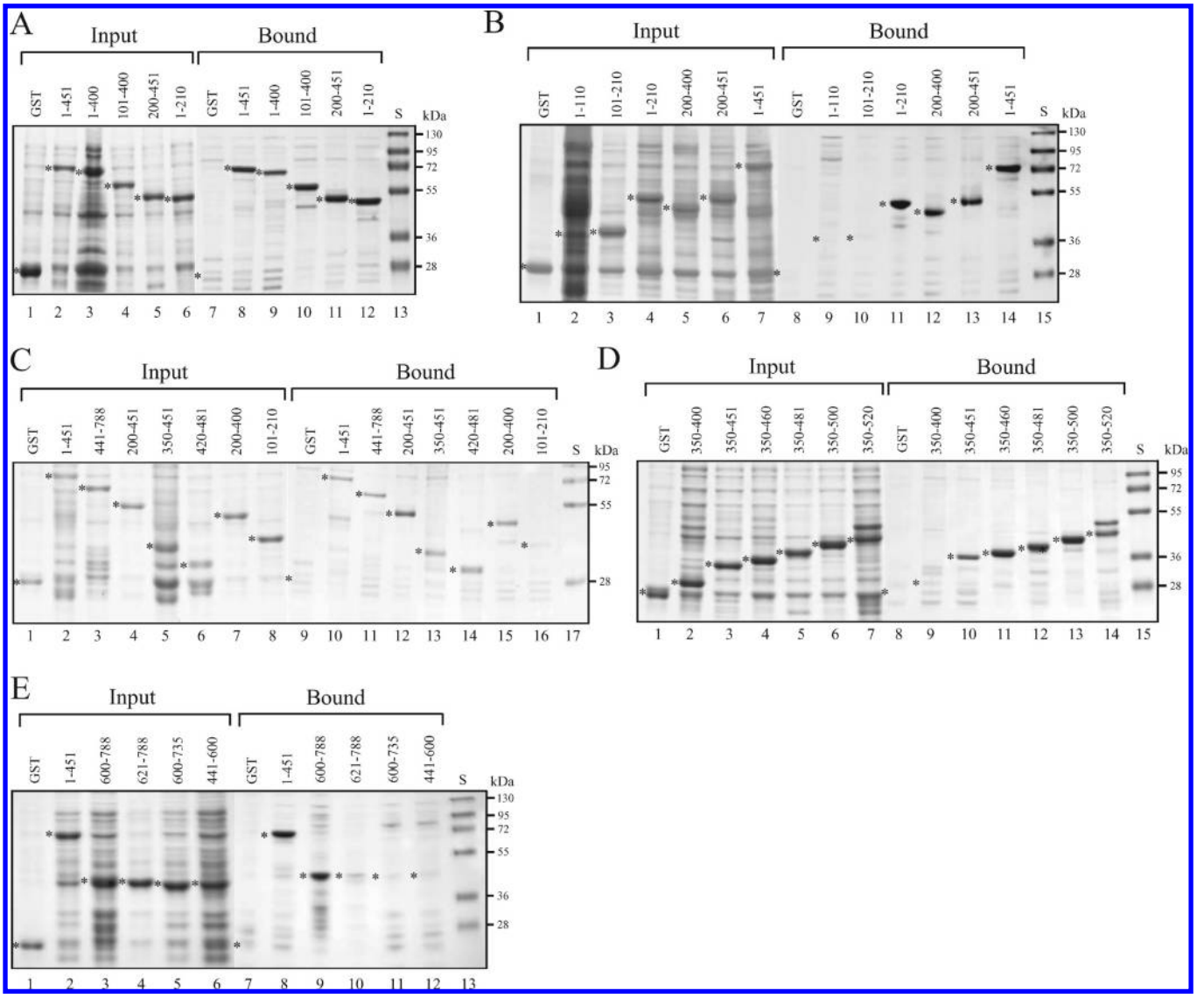

FIGURE 3: Identification of poly(G) RNA binding domains in wheat eIFiso4G. (A−E) eIFiso4G polypeptides were expressed as GST fusion proteins in *E. coli* (Input), and RNA binding activity was determined by adding crude extract to poly(G) agarose resin (Bound). Bound proteins were analyzed by SDS−PAGE and detected using Coomassie staining. GST was employed as a negative control. The region of eIFiso4G included in each polypeptide is indicated numerically by the residues included. S denotes molecular weight standards.

activity to poly(A), but GST-eIFiso4G$_{441-520}$ did not (Figure 2D, lanes 11 and 12, respectively), suggesting that the N-terminal border lies between residues 420 and 441. GST-eIFiso4G$_{420-481}$ was sufficient for binding poly(A) (Figure 2D, lane 10). This region shares sequence similarity with the corresponding region in wheat and human eIF4G, including several arginine residues conserved among the proteins (Figure 2E).

The previously identified nucleotide binding domain between residues 170 and 443 in eIFiso4G was reported to bind poly(G) strongly, poly(A) weakly, and poly(U) or poly(C) not at all (*29*). We next investigated whether those regions in eIFiso4G that bind poly(A) can also bind poly(G) RNA. As observed with poly(A), GST-eIFiso4G$_{1-451}$ bound poly(G) agarose resin efficiently as did GST-eIFiso4G$_{1-400}$, GST-eIFiso4G$_{101-400}$, GST-eIFiso4G$_{200-451}$, and GST-eIFiso4G$_{1-210}$ (Figure 3A, lanes 8−12, respectively). Although GST-eIFiso4G$_{1-210}$ bound poly(G) (Figure 3B, lane 11), GST-eIFiso4G$_{1-110}$ and GST-eIFiso4G$_{101-210}$ did not (Figure 3B, lanes 9 and 10, respectively), suggesting that both of these regions contribute to poly(G) binding. GST-eIFiso4G$_{200-400}$ bound poly(G) to an extent similar to that of GST-eIFiso4G$_{200-451}$ (Figure 3B, lanes 12 and 13, respectively). As GST-eIFiso4G$_{1-210}$ and GST-eIFiso4G$_{200-451}$ bound poly(G) strongly but bound poly(A) weakly at best, the data suggest that several regions within the region of 451 N-terminal residues contribute to RNA binding and that this region binds to poly(G) RNA more strongly than it does to poly(A) RNA.

GST-eIFiso4G$_{350-451}$ bound poly(G) to an extent similar to that of GST-eIFiso4G$_{200-451}$ (Figure 3C, lanes 13 and 12, respectively). GST-eIFiso4G$_{420-481}$ bound to poly(G) (Figure 3C, lane 14), demonstrating that this domain can bind poly(G) or poly(A). GST-eIFiso4G$_{350-400}$ did not bind poly(G), but GST-eIFiso4G$_{350-451}$, GST-eIFiso4G$_{350-460}$, GST-eIFiso4G$_{350-481}$, GST-eIFiso4G$_{350-500}$, and GST-eIFiso4G$_{350-520}$ did bind (Figure 3D, lanes 9−14, respectively), suggesting that the C-terminal border lies between residues 400 and 451, preceding the C-terminal border between residues 460 and 481 necessary for poly(A) binding.

Although the region between residues 600 and 788 did not bind poly(A) (Figure 2A, lane 16), GST-eIFiso4G$_{441-788}$ did bind poly(G) (Figure 3C, lane 11). GST-eIFiso4G$_{600-788}$ bound

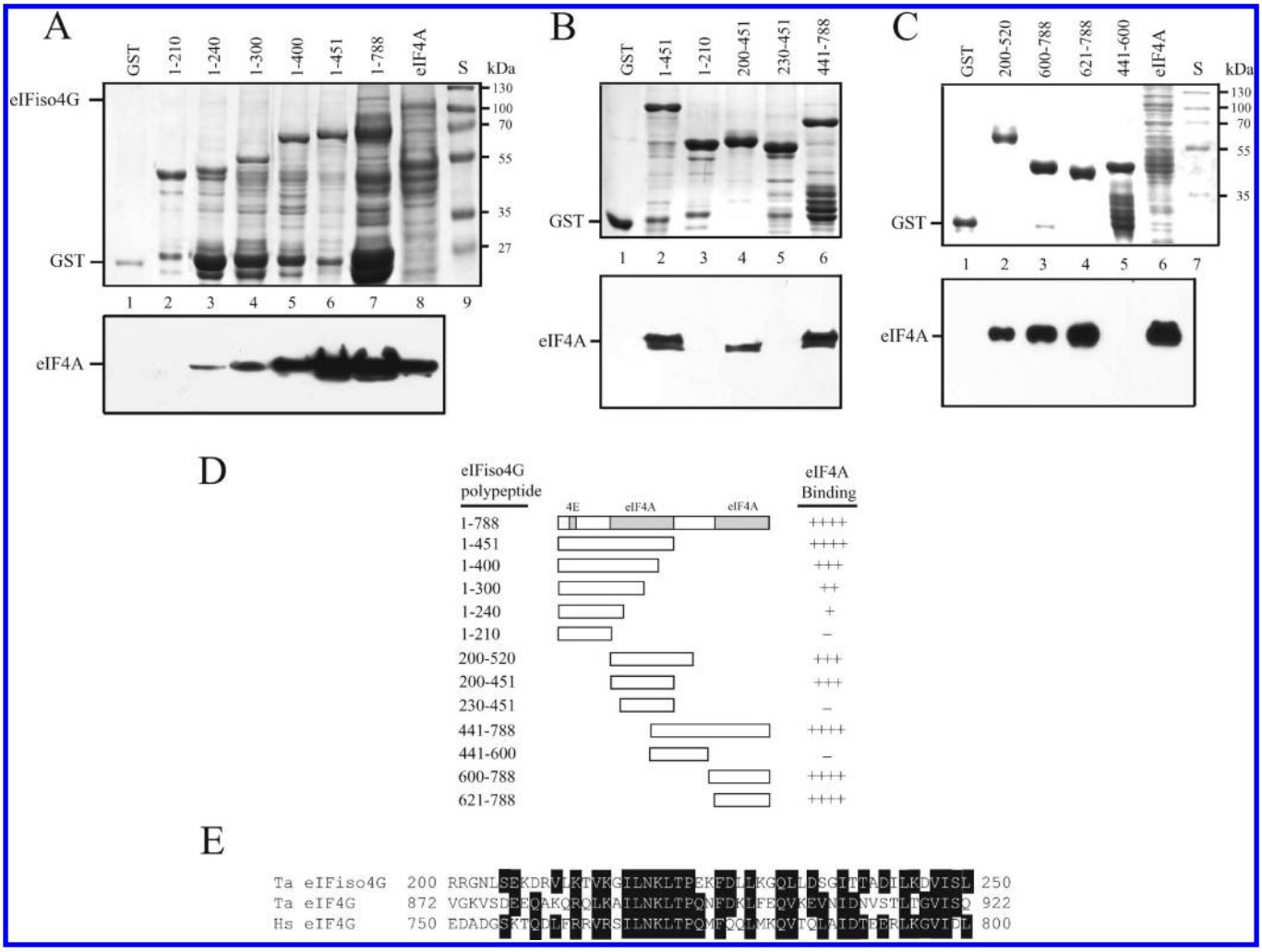

FIGURE 4: eIF4A binds the HEAT-1 and HEAT-2 domains or eIFiso4G. (A−C) eIFiso4G peptides were expressed as GST fusion proteins in *E. coli* (top panels). The region of eIFiso4G included in each peptide is indicated numerically by the residues included. Binding of full-length eIF4A to the indicated eIFiso4G peptides was assessed following binding of the GST fusion proteins to glutathione Sepharose resin (bottom panels). Bound eIF4A was resolved by SDS−PAGE and detected using Western analysis. GST was employed as a negative control. (D) Summary of the eIF4A binding activity of eIFiso4G peptides. The strength of eIF4A binding is indicated by the number of pluses. Hyphens denote a lack of eIF4A binding. (E) Comparison of the sequence between residues 200 and 250 containing the essential region for binding eIF4A within the HEAT-1 domain in wheat eIFiso4G (Ta eIFiso4G) with the corresponding region of wheat eIF4G (Ta eIF4G) and human eIF4G (Hs eIF4G). Conservation of identical or similar residues in wheat eIFiso4G relative to the other sequences is indicated by shading.

poly(G) as strongly as GST-eIFiso4G$_{1-451}$ did (Figure 3E, lanes 9 and 8, respectively), whereas GST-eIFiso4G$_{621-788}$ and GST-eIFiso4G$_{600-735}$ did not bind (Figure 3E, lanes 10 and 11, respectively), suggesting that the RNA binding activity exhibited by GST-eIFiso4G$_{600-788}$ was specific and required most of the C-terminal region. No poly(G) binding activity was exhibited by GST-eIFiso4G$_{441-600}$ (Figure 3E, lane 12), indicating that the region between the central and C-terminal RNA binding domains does not contribute to RNA binding.

eIFiso4G Contains Two eIF4A Binding Domains. To examine whether eIF4A binds the eIFiso4G HEAT-1 and HEAT-2 domains, which exhibit conservation with the HEAT-1/eIF4A and HEAT-2/eIF4A binding domains of mammalian eIF4G (Figure 1), we performed pull-down assays with eIF4A and eIFiso4G. GST-eIFiso4G$_{1-788}$ bound His-tagged, full-length eIF4A as did GST-eIFiso4G$_{1-451}$ (Figure 4A, bottom panel, lanes 7 and 6, respectively). The binding of eIF4A to GST-eIFiso4G$_{1-400}$, GST-eIFiso4G$_{1-300}$, and GST-eIFiso4G$_{1-240}$ was progressively weaker, with no binding to GST-eIFiso4G$_{1-210}$ detected (Figure 4A, bottom panel, lanes 5, 4, 3, and 2, respectively), suggesting that the region up to residue 451 is required for full binding of eIF4A (Figure 4D). In mapping the N-terminal border, we observed that eIF4A bound GST-eIFiso4G$_{200-451}$, which represents the HEAT-1 domain, but not GST-eIFiso4G$_{230-451}$ (Figure 4B, bottom panel, lanes 4 and 5, respectively), suggesting that the 200 N-terminal residues of eIFiso4G are not involved in binding eIF4A and that the N-terminal border of this eIF4A interaction domain lies between residues 200 and 230 (Figure 4D).

GST-eIFiso4G$_{441-788}$ bound eIF4A (Figure 4B, bottom panel, lane 6), whereas GST-eIFiso4G$_{441-600}$ did not (Figure 4C, bottom panel, lane 5), suggesting the presence of a second eIF4A interaction domain in the C-terminal region of eIFiso4G. GST-eIFiso4G$_{600-788}$ bound eIF4A to a extent similar to that of GST-eIFiso4G$_{200-520}$ (Figure 4C, bottom panel, lanes 3 and 2, respectively). eIF4A also bound GST-eIFiso4G$_{621-788}$ (Figure 4C, bottom panel, lane 4), which represents the HEAT-2 domain.

These data suggest that eIFiso4G contains two eIF4A binding domains corresponding to the HEAT-1 and HEAT-2 domains of animal eIF4G but differs from yeast eIF4G, which contains only the HEAT-1/eIF4A binding domain (*31*).

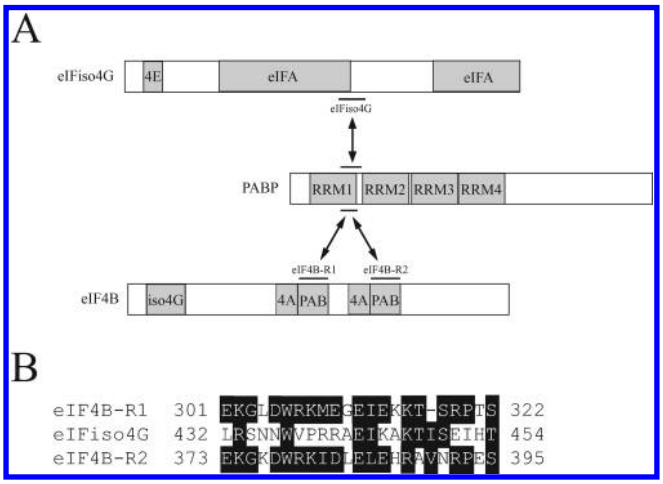

FIGURE 5: Conservation of PABP interaction domains in eIFiso4G and eIF4B. (A) Domain organization of eIFiso4G, PABP, and eIF4B. The eIFiso4G and eIF4B interaction domains present in the RRM1 of PABP are indicated as are the two PABP interaction domains present in eIF4B and the region of eIFiso4G containing a similar sequence. (B) Comparison of the two PABP interaction domains present in eIF4B with the region of eIFiso4G containing a similar sequence element. Conservation of identical or similar residues in wheat eIFiso4G relative to the two PABP interaction domains present in eIF4B is indicated by shading.

The PABP Binding Region Overlaps the HEAT-1/eIF4A Binding Domain in eIFiso4G. In contrast to mammalian eIF4G and PABP that interact at a single site (*18*, *19*), wheat PABP contains two well-separated interaction domains for eIFiso4G (*15*). eIFiso4G binds PABP strongly at a site that encompasses the C-terminal end of the first RRM and the linker region between RRM1 and RRM2 (*15*). eIFiso4G also binds PABP weakly at a second site that requires the RRM3 and RRM4 domains (*15*). The lack of sequence conservation between the two eIFiso4G interaction sites suggests that two independent regions of PABP contact eIFiso4G. In addition, both eIFiso4G and eIF4B bind to overlapping regions at the C-terminal end of PABP RRM1 (Figure 5A) and eIFiso4G and eIF4B compete for binding to this site (*15*, *17*). This suggests that the PABP interaction domain in eIFiso4G may share homology with the two PABP interaction domains in eIF4B that are significantly similar in sequence with one another (*12*). Using the PABP interaction domains in eIF4B to search for a similar sequence in eIFiso4G revealed a region between residues 432 and 453 that exhibited similarity (Figure 5B).

We then identified the regions in eIFiso4G that contributed to PABP binding. Relative to the input amount, full-length PABP efficiently bound GST-eIFiso4G$_{1-788}$ and GST-eIFiso4G$_{1-451}$ (Figure 6A, bottom panel, lanes 2 and 3, respectively). GST-eIFiso4G$_{441-788}$ and GST-eIFiso4G$_{600-788}$ failed to interact with

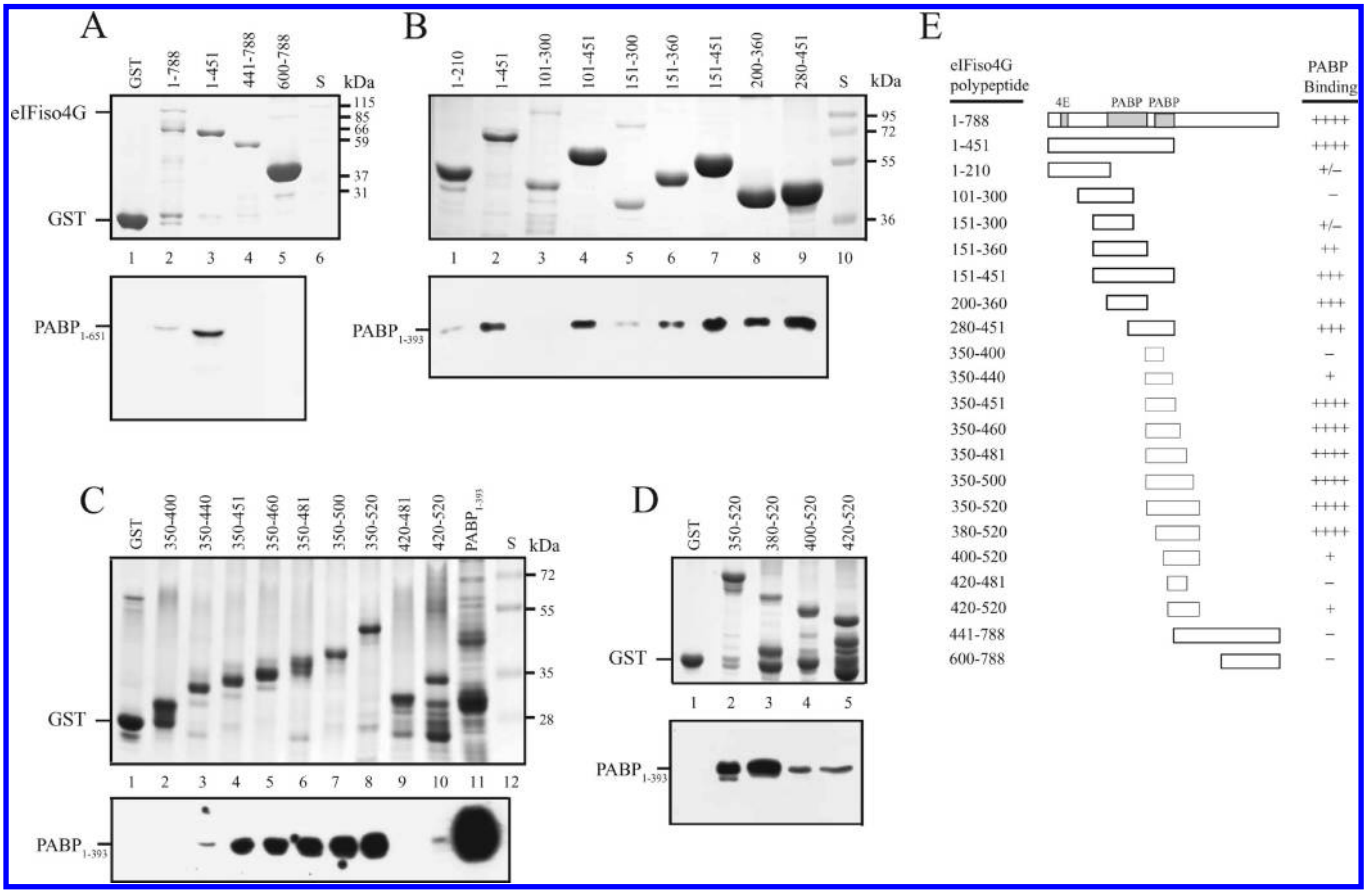

FIGURE 6: Regions within eIFiso4G contributing to PABP binding. (A–D) eIFiso4G peptides were expressed as GST fusion proteins in *E. coli* (top panels). The region of eIFiso4G included in each peptide is indicated numerically by the residues included. Binding of full-length PABP (i.e., 1–651) (panel A) or the PABP N-terminal region containing the four RRMs (i.e., 1–393) (panels B–D) to the indicated eIFiso4G peptides was assessed following binding of the GST fusion proteins to glutathione Sepharose resin (bottom panels). Bound PABP was resolved by SDS–PAGE and detected using Western analysis. GST was employed as a negative control. S denotes molecular weight standards. (E) Summary of the PABP binding activity of eIFiso4G peptides. The strength of PABP binding is indicated by the number of pluses. Hyphens denote a lack of PABP binding.

PABP (Figure 6A, bottom panel, lanes 4 and 5, respectively), suggesting that the N-terminal region of eIFiso4G is responsible for binding PABP. GST-eIFiso4G$_{101-451}$ and GST-eIFiso4G$_{1-451}$ bound PABP (Figure 6B, bottom panel, lanes 4 and 2, respectively), but GST-eIFiso4G$_{101-300}$ did not (Figure 6B, bottom panel, lane 3). Binding of PABP to GST-eIFiso4G$_{151-300}$ or GST-eIFiso4G$_{1-210}$ was weak and variable (Figure 6B, bottom panel, lane 5 or 1, respectively). GST-eIFiso4G$_{151-360}$ and GST-eIFiso4G$_{200-360}$ bound PABP (Figure 6B, bottom panel, lanes 6 and 8, respectively), but relative to the input amount, the binding was weak. These data suggest that the region between residues 200 and 360 contributes, albeit weakly, to binding PABP (Figure 6E).

Stronger binding to GST-eIFiso4G$_{151-451}$ and GST-eIFiso4G$_{280-451}$ (Figure 6B, bottom panel, lanes 7 and 9, respectively) may be due to the higher level of bait protein used and/or the presence of additional sequence between residues 360 and 451 contributing to PABP binding. To examine this, we observed that GST-eIFiso4G$_{350-520}$ bound PABP (Figure 6C, bottom panel, lane 8) as did GST-eIFiso4G$_{350-500}$, eIFiso4G$_{350-481}$, eIFiso4G$_{350-460}$, and eIFiso4G$_{350-451}$ (Figure 6C, bottom panel, lanes 7, 6, 5, and 4, respectively). PABP exhibited only weak binding to eIFiso4G$_{350-440}$ and failed to bind eIFiso4G$_{350-400}$ at all (Figure 6C, bottom panel, lanes 3 and 2, respectively). PABP bound eIFiso4G$_{420-520}$ only weakly and did not bind eIFiso4G$_{420-481}$ (Figure 6C, bottom panel, lanes 10 and 9, respectively).

N-Terminal deletions were also tested. PABP bound eIFiso4G$_{380-520}$ as strongly as it did eIFiso4G$_{350-520}$ (Figure 6D, bottom panel, lanes 3 and 2, respectively). Weak binding, however, was observed for eIFiso4G$_{400-520}$ and eIFiso4G$_{420-520}$ (Figure 6D, bottom panel, lanes 4 and 5, respectively). These data suggest that the region between residues 380 and 451, particularly the sequence between residues 420 and 451, contributes to binding PABP (Figure 6E). Within this region lies the sequence from residue 432 and 453 that shares similarity with the two PABP interaction domains of eIF4B (Figure 5B). Deletion of most of this conserved sequence, e.g., in eIFiso4G$_{350-440}$, resulted in the virtual loss of interaction with PABP (Figure 6C, bottom panel, lane 3).

Together, these data indicate that the regions between residues 200 and 360 and residues 380 and 451 of eIFiso4G contribute to binding PABP. Because two regions within PABP are known to contact eIFiso4G (*15*), the binding of each region of PABP to eIFiso4G was tested separately. PABP$_{200-393}$, encompassing RRM3 and RRM4, bound eIFiso4G$_{200-360}$ (Figure 7A, bottom panel, lane 2), which lacks the eIFiso4G region spanning residues 380 and 451. PABP$_{200-393}$ also bound eIFiso4G$_{200-400}$ and eIFiso4G$_{200-451}$ (Figure 7A, bottom panel, lanes 3 and 4, respectively) but did not bind eIFiso4G$_{400-520}$ (Figure 7A, bottom panel, lane 7), which represents the second region capable of interacting with PABP. These data demonstrate that the eIFiso4G region spanning residues 200 and 360 binds PABP RRM3 and RRM4 specifically. PABP$_{18-200}$, which contains the eIFiso4G binding site within the C-terminal end of RRM1 and the linker between RRM1 and RRM2 (*15*), bound eIFiso4G$_{200-360}$, eIFiso4G$_{200-400}$, eIFiso4G$_{200-451}$, eIFiso4G$_{400-520}$, and eIFiso4G$_{350-520}$ (Figure 7B, bottom panel, lanes 2−6, respectively), suggesting that this region of PABP contacts both regions (identified above) of eIFiso4G (i.e., between residues 200 and 360 and between residues 380 and 451) that contribute to binding PABP.

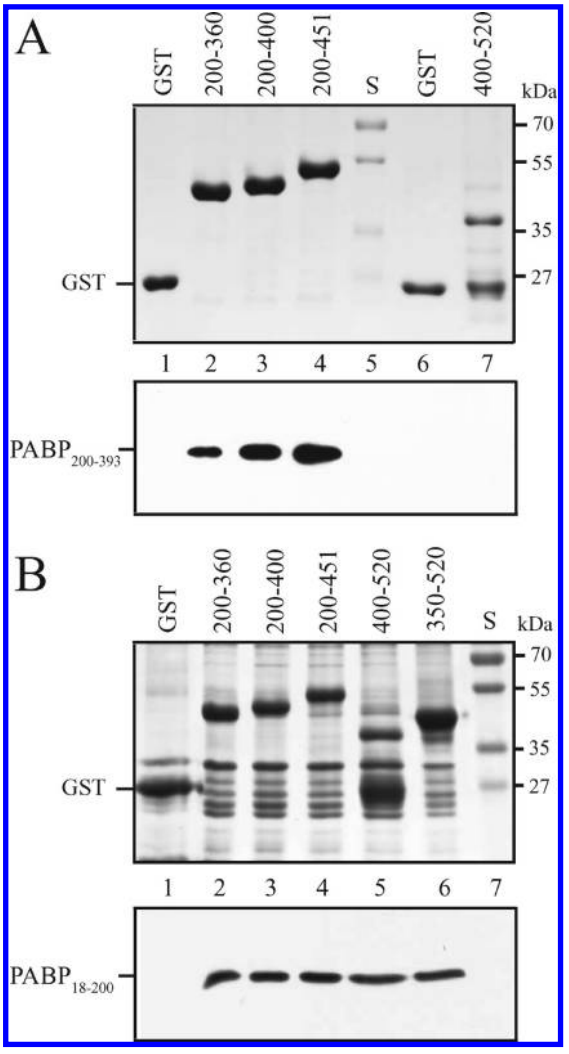

FIGURE 7: Specificity of PABP binding to eIFiso4G. eIFiso4G peptides were expressed as GST fusion proteins in *E. coli* (top panels). The region of eIFiso4G included in each peptide is indicated numerically by the residues included. Binding of His-tagged (A) PABP$_{200-393}$ or (B) PABP$_{18-200}$ to the indicated eIFiso4G peptides was assessed following binding of the GST fusion proteins to glutathione Sepharose resin (bottom panels). Bound PABP was resolved by SDS−PAGE and detected using Western analysis. GST was employed as a negative control. S denotes molecular weight standards.

The eIF4B and PABP Binding Domains Overlap in eIFiso4G. eIFiso4G interacts with eIF4B at a single N-proximal site (*12*), suggesting a possible single eIF4B binding site in eIFiso4G. eIF4B bound GST-eIFiso4G$_{1-451}$ and GST-eIFiso4G$_{441-788}$ (Figure 8A, bottom panel, lanes 3 and 4, respectively) but did not bind GST-eIFiso4G$_{1-400}$ (Figure 8A, bottom panel, lane 2), suggesting that the eIF4B interaction domain may overlap a region present within the two halves of the protein tested. Within the N-terminal half of eIFiso4G, eIF4B failed to bind GST-eIFiso4G$_{1-210}$ or GST-eIFiso4G$_{200-400}$ (Figure 8B, bottom panel, lane 2 or 3, respectively), consistent with its failure to bind GST-eIFiso4G$_{1-400}$. Weak binding to GST-eIFiso4G$_{200-451}$ was observed, but binding to GST-eIFiso4G$_{200-520}$ was as strong as it was to GST-eIFiso4G$_{1-788}$ (Figure 8B, bottom panel, lanes 4, 5, and 9, respectively). These data indicate that the region between residues 451 and 520 contributes significantly to the binding of eIF4B but that sequence N-proximal to residue 451 may also contribute to its binding. eIF4B bound GST-eIFiso4G$_{420-788}$ and,

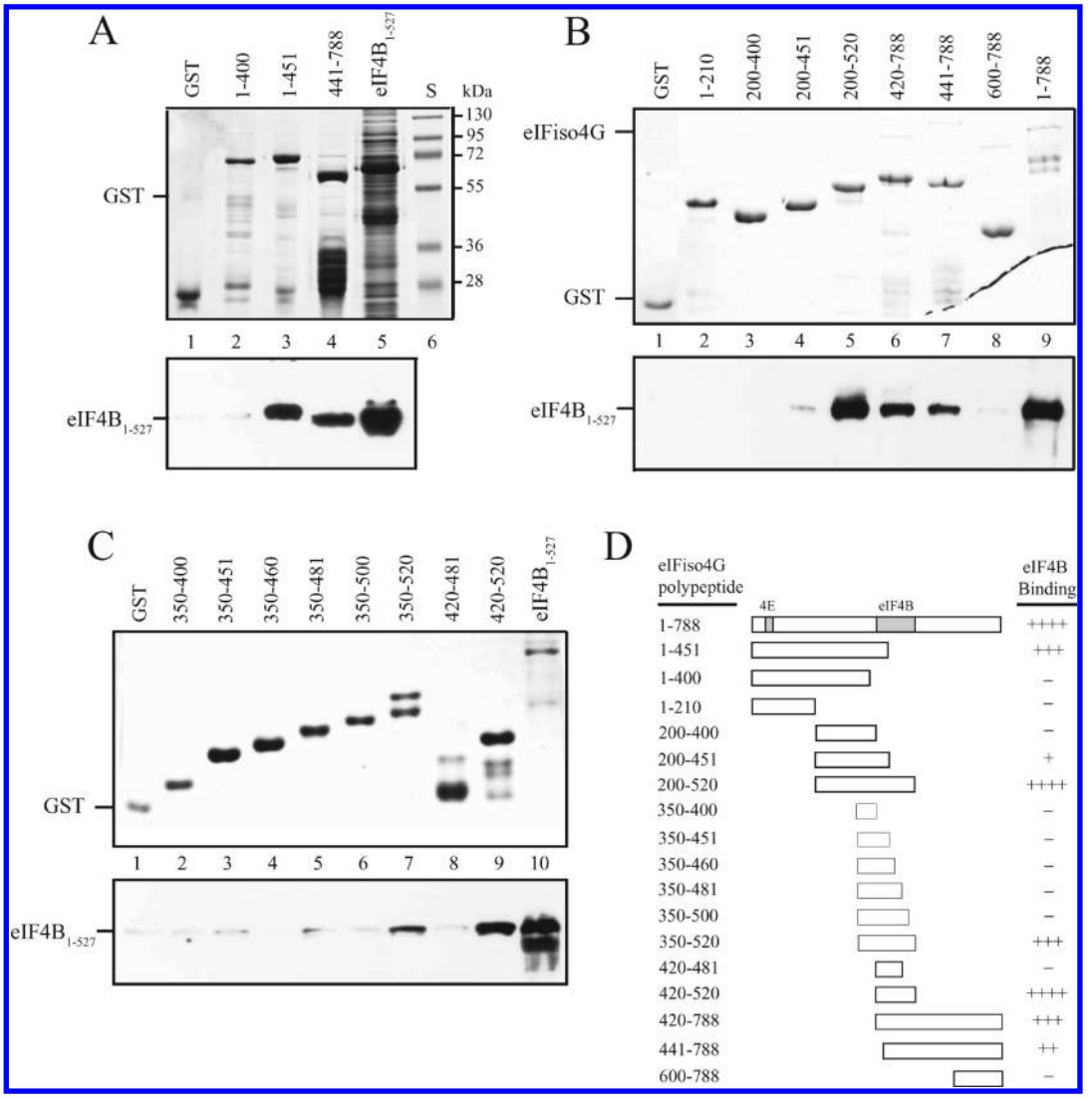

FIGURE 8: eIF4B binds eIFiso4G at a single site. (A−D) eIFiso4G peptides were expressed as GST fusion proteins in *E. coli* (top panels). The region of eIFiso4G included in each peptide is indicated numerically by the residues included. Binding of full-length eIF4B (i.e., residues 1−527) to the indicated eIFiso4G peptides was assessed following binding of the GST fusion proteins to glutathione Sepharose resin (bottom panels). Bound eIF4B was resolved by SDS−PAGE and detected using Western analysis. GST was employed as a negative control. (D) Summary of the eIF4B binding activity of eIFiso4G peptides. The strength of eIF4B binding is indicated by the number of pluses. Hyphens denote a lack of eIF4B binding.

more weakly, GST-eIFiso4G$_{441-788}$, but did not bind GST-eIFiso4G$_{600-788}$ (Figure 8B, bottom panel, lanes 6−8, respectively).

To map the C-terminal border of the eIF4B binding domain, C-terminal residues were deleted from GST-eIFiso4G$_{350-520}$, to which eIF4B bound (Figure 8C, bottom panel, lane 7). In contrast to GST-eIFiso4G$_{350-520}$, eIF4B failed to bind GST-eIFiso4G$_{350-500}$, GST-eIFiso4G$_{350-481}$, GST-eIFiso4G$_{350-460}$, GST-eIFiso4G$_{350-451}$, or GST-eIFiso4G$_{350-400}$ to an extent greater than it did GST (Figure 8C, bottom panel, lane 6, 5, 4, 3, 2, or 1, respectively). This suggests that the C-terminal border of the eIF4B binding domain lies between residues 500 and 520. eIF4B also bound GST-eIFiso4G$_{420-520}$ but failed to bind GST-eIFiso4G$_{420-481}$ (Figure 8C, bottom panel, lanes 9 and 8, respectively), indicating that the eIF4B interaction domain lies between residues 420 and 520 that overlap with the PABP binding region between residues 380 and 451 (Figure 8D).

eIF4B and PABP Compete for Binding to eIFiso4G. The overlap between the PABP and eIF4B binding domains in eIFiso4G suggested the possibility of competitive binding to eIFiso4G. To examine this, GST-eIFiso4G$_{350-520}$, which contains the region for interaction with eIF4B and PABP, was used in pull-down assays with eIF4B and PABP. His-eIF4B$_{45-280}$, which contains the interaction domain for eIFiso4G but not for PABP (*12*), was present in molar amounts equal to that of GST-eIFiso4G$_{350-520}$, whereas PABP$_{1-393}$, which contains the eIFiso4G and eIF4B interaction domains, was added in increasing molar amounts. The level of binding of His-eIF4B$_{45-280}$ to GST-eIFiso4G$_{350-520}$ in the absence of PABP was substantially reduced by PABP when present at just a 1:0.1 molar ratio, with further reductions at higher molar ratios (Figure 9, top panel, lanes 2−6). As His-eIF4B$_{45-280}$ lacks the interaction domain for PABP (*12*), the ability of PABP to weaken the interaction between eIF4B and eIFiso4G was a result of competition with eIF4B for binding to eIFiso4G.

eIF4B dimerization and the interaction between eIF4B and PABP are promoted by Zn^{2+} at physiological concentrations (*17*). To examine whether Zn^{2+} affects the competition between PABP and eIF4B in binding eIFiso4G, the same binding reactions were repeated in the presence of 0.2 mM Zn^{2+}. His-eIF4B$_{45-280}$ lacks the eIF4B dimerization domain and lacks the

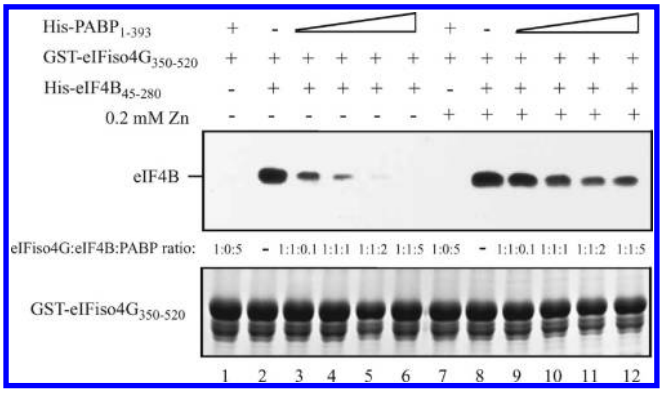

FIGURE 9: eIF4B and PABP compete for binding to eIFiso4G. The amount of GST-eIFiso4G$_{350-520}$, which contains the overlapping interaction domains for eIF4B and PABP, used in each pull-down assay is shown in the Coomassie-stained gel (bottom panels). His-eIF4B$_{45-280}$, which contains the interaction domain for eIFiso4G but lacks the interaction domains for PABP, was added to each reaction mixture in a constant amount. His-PABP$_{1-393}$, which contains the interaction domains for eIFiso4G and eIF4B, was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{350-520}$ and His-eIF4B$_{45-280}$ in the absence or presence of zinc. Following binding of GST-eIFiso4G$_{350-520}$ to glutathione Sepharose resin, the amount of His-eIF4B$_{45-280}$ bound to GST-eIFiso4G$_{350-520}$ was detected by Western analysis using anti-eIF4B antiserum (top panels).

two PABP binding domains (*17*), which might otherwise complicate the analysis of the competition between eIF4B and PABP in binding eIFiso4G. Higher levels of PABP were required to compete with eIF4B in binding eIFiso4G in the presence of Zn^{2+} (Figure 9, top panel, lanes 8−12), suggesting that Zn^{2+} may stabilize the interaction between eIF4B and eIFiso4G, particularly in the absence of the ability of eIF4B to dimerize.

The HEAT-2/eIF4A Binding Domain Functions To Stabilize Binding of eIF4A to eIFiso4G in the Presence of eIF4B. The eIF4B binding domain in eIFiso4G overlaps partially with the HEAT-1/eIF4A binding domain. To examine whether eIF4A affects the binding of eIF4B to eIFiso4G, GST-eIFiso4G$_{200-788}$, which contains both HEAT/eIF4A binding domains and the eIF4B binding domain, was used in pull-down assays with full-length eIF4B and eIF4A. His-eIF4B$_{1-527}$ and GST-eIFiso4G$_{200-788}$ were used at equal molar amounts, and eIF4A was added in increasing amounts. An equal molar ratio of eIF4A only slightly weakened binding of eIF4B to GST-eIFiso4G$_{200-788}$, with similar results observed when Mg^{2+} was included or, to lesser extent, when both Mg^{2+} and Zn^{2+} were included (Figure 10A, top panel).

As there are two eIF4A binding domains in eIFiso4G, we examined whether eIF4B affected binding of eIF4A to the HEAT-1 domain of eIFiso4G when the HEAT-2/eIF4A binding domain was absent, i.e., in GST-eIFiso4G$_{200-520}$, which contains the eIF4B and HEAT-1/eIF4A binding domains but lacks the HEAT-2/eIF4A binding domain. Full-length eIF4A and GST-eIFiso4G$_{200-520}$ were present in equal molar amounts, whereas His-eIF4B$_{45-280}$, which contains the eIFiso4G interaction domain but lacks both eIF4A interaction domains (*12*), was added in increasing amounts. The presence of eIF4B weakened binding of eIF4A to GST-eIFiso4G$_{200-520}$ regardless of whether Mg^{2+} or Zn^{2+} was present (Figure 10B, top panel). When the binding reactions were repeated using an eIFiso4G polypeptide that contained the HEAT-2/eIF4A binding domain as well as the HEAT-1/eIF4A binding domain, i.e., GST-eIFiso4G$_{200-788}$, eIF4B was unable to reduce the level of binding of eIF4A to eIFiso4G (Figure 10C, top panel). These results indicate that the HEAT-2/eIF4A binding domain functions to stabilize the binding of eIF4A to eIFiso4G in the presence of eIF4B.

The HEAT-2/eIF4A Binding Domain Functions To Stabilize Binding of eIF4A to eIFiso4G in the Presence of PABP. Because the HEAT-1/eIF4A binding domain in eIFiso4G overlaps with the region required for PABP binding, we investigated whether full-length eIF4A and PABP compete for binding to eIFiso4G. His-PABP$_{1-651}$ and GST-eIFiso4G$_{200-788}$, which contains both HEAT/eIF4A binding domains and the PABP binding region, were used in equal molar amounts, and eIF4A was added in increasing amounts. eIF4A had little to no effect on binding of PABP to GST-eIFiso4G$_{200-788}$ regardless of whether Mg^{2+} or Zn^{2+} was present (Figure 11A, top panel).

We then examined whether PABP, like eIF4B, could compete with eIF4A for binding GST-eIFiso4G$_{200-520}$, which contains the PABP and HEAT-1/eIF4A binding domains but lacks the HEAT-2/eIF4A binding domain. Full-length eIF4A and GST-eIFiso4G$_{200-520}$ were present in equal molar amounts, whereas PABP was added in increasing amounts. PABP did reduce the level of binding of eIF4A to eIFiso4G in the absence of the HEAT-2/eIF4A binding domain regardless of whether Mg^{2+} or Zn^{2+} was present (Figure 11B, top panel). When the binding reactions were repeated using GST-eIFiso4G$_{200-788}$, which contains both HEAT/eIF4A interaction domains, PABP was unable to reduce the level of binding of eIF4A to eIFiso4G (Figure 11C, top panel). These results indicate that the HEAT-2/eIF4A binding domain functions to stabilize the binding of eIF4A to eIFiso4G in the presence of PABP as it does when eIF4B is present.

DISCUSSION

As a scaffolding protein, the interactions of eIF4G with eIF4E, eIF4A, and PABP are highly conserved in eukaryotes and are required to promote the assembly of the translational machinery with an mRNA (*1*−*6*, *8*). In plants, the interactions between eIFiso4G and eIF4E, eIF4A, eIF4B, and PABP have been thoroughly demonstrated (*8*, *12*, *13*, *15*, *17*). Functionally, the interaction between eIFiso4G and PABP strengthens the binding of eIFiso4G to the 5′-cap (*32*), increases the affinity of PABP for poly(A) RNA (*8*, *13*), and increases the ATPase and RNA helicase activities of eIFiso4F (*14*). Although the eIFiso4G binding domains in eIF4B and PABP have been identified (*12*, *15*) and the functional consequences of the interactions between eIFiso4G and its partner proteins have been extensively characterized (*8*, *11*, *14*, *32*), the domain structure of eIFiso4G had remained largely unknown. The compact nature of eIFiso4G, however, suggested potential significant differences in the organization of its interaction domains that could provide insight into the evolution of this important scaffolding protein.

eIF4G from animals and yeast differs significantly in that animal eIF4G contains two HEAT/eIF4A binding domains whereas yeast eIF4G contains just one (*20*, *31*). In human eIF4GI, a 40-amino acid RNA binding domain between residues 682 and 720 that is N-proximal to the HEAT-1 domain was required for ribosomal scanning (Figure 12) (*28*). A second RNA binding domain between residues 774 and 1116 that includes the HEAT-1 domain was required for binding of β-globin mRNA, whereas the region between residues 761 and 988 was the minimum domain required for binding the internal ribosomal

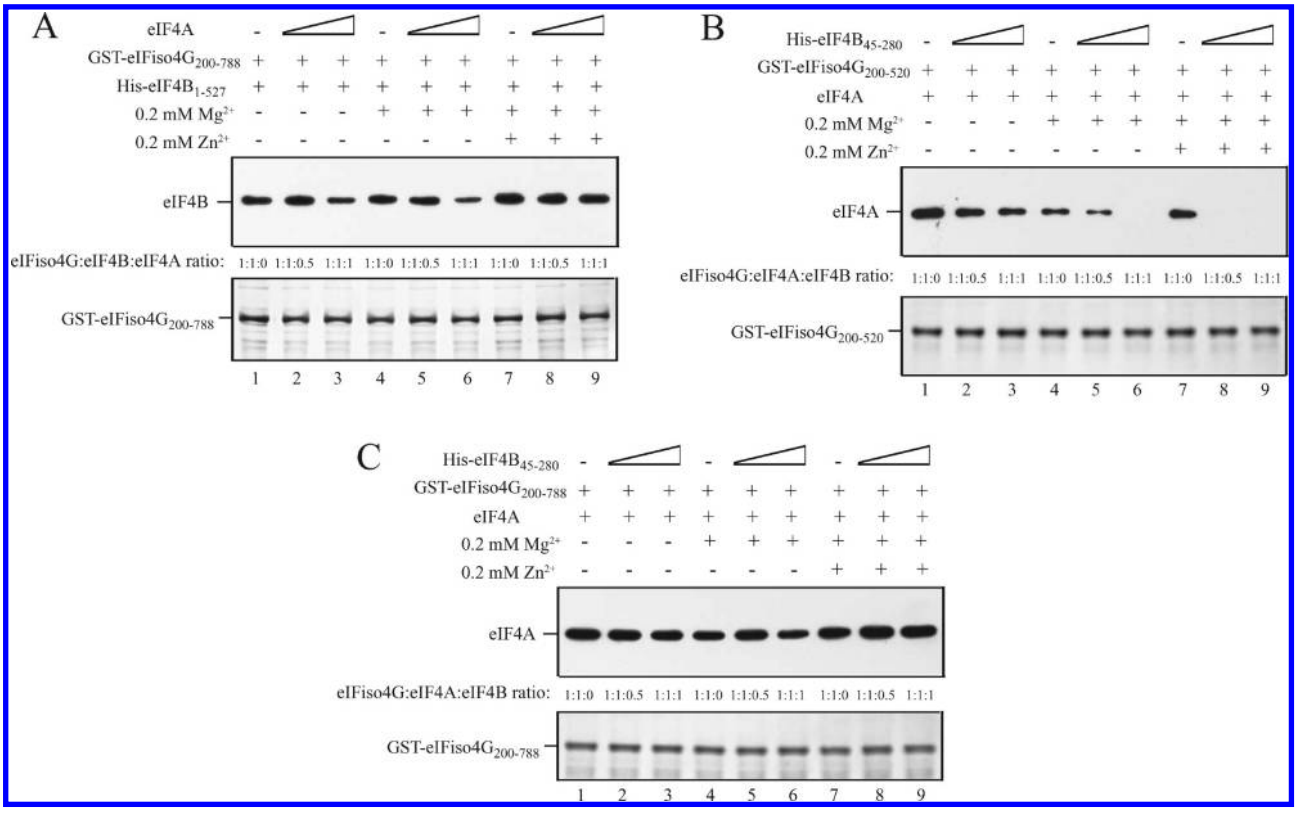

FIGURE 10: Both HEAT/eIF4A binding domains are required to maintain the association of eIF4A with eIFiso4G in the presence of eIF4B. (A) The amount of GST-eIFiso4G$_{200-788}$, which contains the interaction domains for eIF4B and eIF4A, used in each pull-down assay is shown in the Coomassie-stained gel (bottom panel). Full-length His-eIF4B$_{1-527}$ was added to each reaction mixture in a constant amount. Full-length eIF4A was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-788}$ and eIF4B in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-788}$ to glutathione Sepharose resin, the amount of eIF4B bound to GST-eIFiso4G$_{200-788}$ was detected by Western analysis using anti-eIF4B antiserum (top panel). (B) The amount of GST-eIFiso4G$_{200-520}$ used in each pull-down assay, which contains the eIF4B binding domain and the HEAT-1 but not HEAT-2/eIF4A binding domain, is shown in the Coomassie-stained gel (bottom panel). Full-length eIF4A was added to each reaction mixture in a constant amount. His-eIF4B$_{45-280}$, which lacks the eIF4A binding sites, was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-520}$ and eIF4A in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-520}$ to glutathione Sepharose resin, the amount of eIF4A bound to GST-eIFiso4G$_{200-520}$ was detected by Western analysis using anti-eIF4A antiserum (top panel). (C) The amount of GST-eIFiso4G$_{200-788}$ used in each pull-down assay, which contains the eIF4B interaction domain and both HEAT/eIF4A interaction domains, is shown in the Coomassie-stained gel (bottom panel). Full-length eIF4A was added to each reaction mixture in a constant amount. His-eIF4B$_{45-280}$ was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-788}$ and eIF4A in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-788}$ to glutathione Sepharose resin, the amount of eIF4A bound to GST-eIFiso4G$_{200-788}$ was detected by Western analysis using anti-eIF4A antiserum (top panel).

entry site (IRES) of encephalomyocarditis virus (*27*). The RNA binding domains in yeast eIF4G proteins differ in that RNA binding was observed for the 82 N-terminal amino acids as well as the regions flanking either side of the HEAT-1 domain but not for the HEAT-1 domain itself (*33*, *34*).

The RNA binding domain of mammalian eIF4G encompassing the HEAT-1 domain corresponds to the region between residues 134 and 517 in eIFiso4G (Figure 1). eIFiso4G is similar to mammalian eIF4G in that its HEAT-1 domain alone exhibited RNA binding activity when binding to poly(G) was examined. The demonstration that RNA binding activity was observed when the entire HEAT-1 domain was tested suggests that the observed RNA binding activity was not an artifact of a truncated HEAT domain. Additional sequence N-proximal to the HEAT-1 domain, however, was required for binding to poly(A), suggesting that sequence prior to the HEAT-1 domain contributes to RNA binding in a sequence-specific manner (Figure 12). A nucleotide binding site was previously reported to be present in eIFiso4G between residues 170 and 443 that exhibited specificity for poly(G) RNA (*29*), consistent with our observation that the eIFiso4G HEAT-1 domain binds poly(G) RNA.

A second RNA binding domain lies between residues 420 and 481 in eIFiso4G, which largely flanks the C-terminal side of the HEAT-1 domain as observed for yeast eIF4G proteins (*33*, *34*). This second eIFiso4G RNA binding domain corresponds to residues 973−1039 in human eIF4GI, which largely represents the C-terminal end of the domain required for binding β-globin mRNA (*27*). Interestingly, deletion analysis of human eIF4G suggested that the C-terminal end of the C-proximal RNA binding domain contributes more significantly to its general RNA binding activity than does the N-terminal end (*27*). A third RNA binding domain was identified within the HEAT-2 domain of eIFiso4G that bound poly(G) but not poly(A) (Figure 12). No such activity has been reported for the mammalian HEAT-2 domain, suggesting that eIFiso4G may possess an additional RNA binding domain or, because of sequence specificity, has yet to be identified in animal eIF4G. Thus, although multiple RNA binding domains are present in human, yeast, and plant eIF4G, differences in their arrangement for each exist.

eIF4A binds the HEAT-1 and HEAT-2 domains in human eIF4G as well as the linker region between these two domains (*20*−*23*). The HEAT-1 domain stimulates the helicase

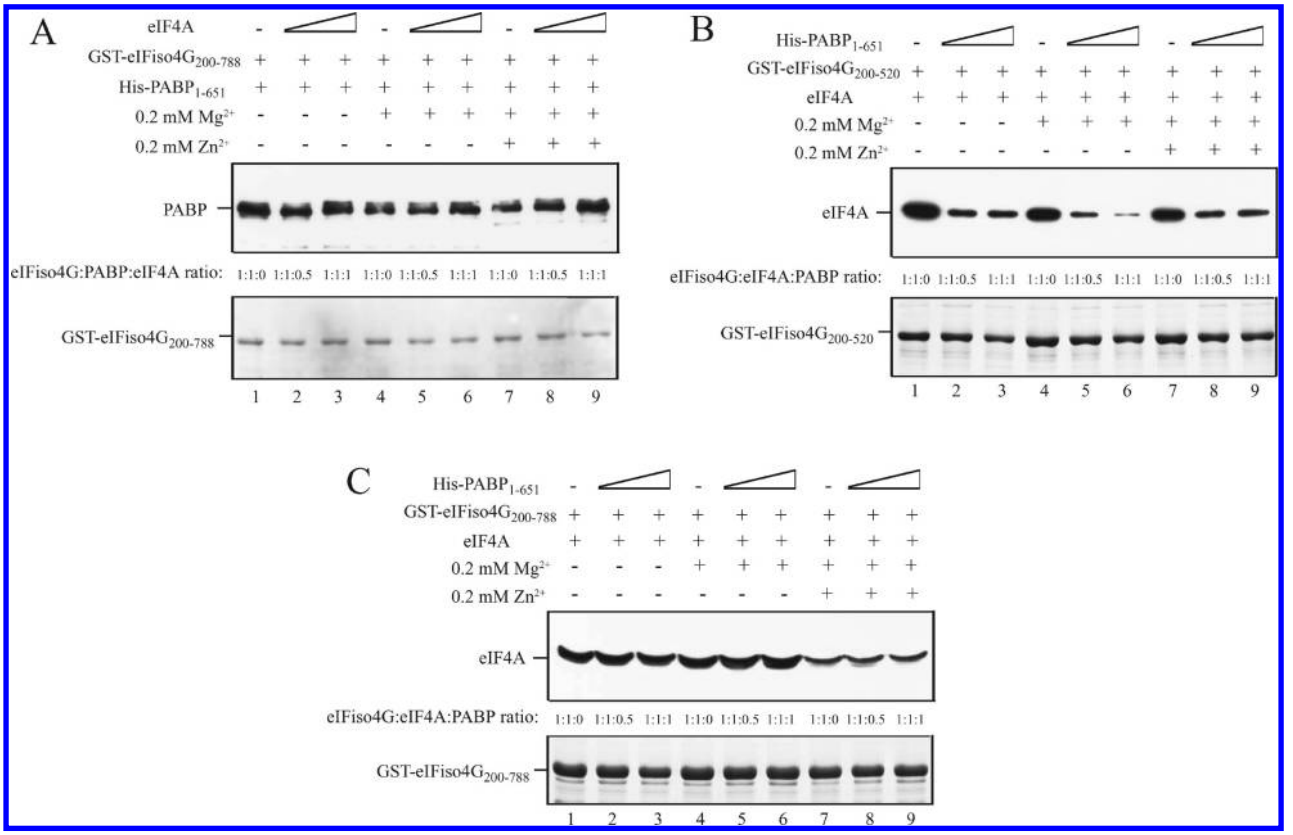

FIGURE 11: Both HEAT/eIF4A binding domains are required to maintain the association of eIF4A with eIFiso4G in the presence of PABP. (A) The amount of GST-eIFiso4G$_{200-788}$, which contains the interaction domains for eIF4A and PABP, used in each pull-down assay is shown in the Coomassie-stained gel (bottom panel). Full-length His-PABP$_{1-651}$ was added to each reaction mixture in a constant amount. Full-length eIF4A was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-788}$ and PABP in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-788}$ to glutathione Sepharose resin, the amount of PABP bound to GST-eIFiso4G$_{200-788}$ was detected by Western analysis using anti-PABP antiserum (top panel). (B) The amount of GST-eIFiso4G$_{200-520}$, which contains both PABP interaction domains and the HEAT-1 but not HEAT-2/eIF4A interaction domain, used in each pull-down assay is shown in the Coomassie-stained gel (bottom panel). Full-length eIF4A was added to each reaction mixture in a constant amount. His-PABP $_{1-651}$ was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-520}$ and eIF4A in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-520}$ to glutathione Sepharose resin, the amount of eIF4A bound to GST-eIFiso4G$_{200-520}$ was detected by Western analysis using anti-eIF4A antiserum (top panel). (C) The amount of GST-eIFiso4G$_{200-788}$, which contains both PABP and HEAT/eIF4A interaction domains, used in each pull-down assay is shown in the Coomassie-stained gel (bottom panel). Full-length eIF4A was added to each reaction mixture in a constant amount. His-PABP$_{1-651}$ was added in increasing amounts to binding reaction mixtures containing GST-eIFiso4G$_{200-788}$ and eIF4A in the absence or presence of 0.2 mM Mg^{2+} or 0.2 mM Zn^{2+}. Following binding of GST-eIFiso4G$_{200-788}$ to glutathione Sepharose resin, the amount of eIF4A bound to GST-eIFiso4G$_{200-788}$ was detected by Western analysis using anti-eIF4A antiserum (top panel).

activity of eIF4A, while the HEAT-2 domain performs a modulatory role (*35*). The HEAT-1 and HEAT-2 domains likely contact separate surfaces of eIF4A and contribute to its interaction with eIF4G (*25*). Yeast eIF4G lacks the region corresponding to the HEAT-2 domain (as well as the HEAT-3 domain); consequently, the recruitment of eIF4A to yeast eIF4G must rely on its interaction with a single binding domain.

eIF4A bound eIFiso4FG between residues 200 and 451, which comprise the HEAT-1 domain. A previous report had suggested the 90 N-terminal amino acids were required for binding eIF4A based on the observation that deletion of this region abolished the interaction between eIFiso4G and eIF4A in a yeast two-hybrid assay (*26*). However, the effect of such a deletion on protein folding or stability in yeast was not determined. Moreover, this same N-terminally truncated eIFiso4G polypeptide retained its ability to promote ATP hydrolysis in the presence of eIF4A (*26*), suggesting that an interaction between eIFiso4G and eIF4A was maintained in solution.

The region between residues 200 and 230 in eIFiso4G was essential for interaction with eIF4A as deletion of this region abolished the interaction with the eIFiso4G HEAT-1 domain. Moreover, an N-terminal eIFiso4G peptide terminating at residue 240 was still able to bind eIF4A, albeit weakly. Deletions from the C-terminal end of the HEAT-1 domain progressively reduced the strength of eIF4A binding, indicating the entire domain was necessary for full binding. The HEAT-1 domain lies within the central domain of eIFiso4G, which is the most highly conserved region among eukaryotic eIF4G proteins (Figure 1). A seven-amino acid sequence, i.e., ILNKLTP, within the essential region between residues 200 and 230, is highly conserved among eukaryotic eIF4G proteins (Figure 4E). Mutation of this sequence in mammalian eIF4G abolished eIF4A binding (*20*). Inclusion of this sequence with the remaining portion of the HEAT-1 domain was required for eIF4A to bind eIF4G (*27*), consistent with our observation that the region between residues 200 and 230 is essential for binding of eIF4A to the eIFiso4G HEAT-1 domain. Analysis of the crystal structure of the eIF4A−eIF4G complex from yeast demonstrated that the asparagine, lysine, and threonine residues of this conserved heptapeptide in eIF4G make direct contacts with specific residues in

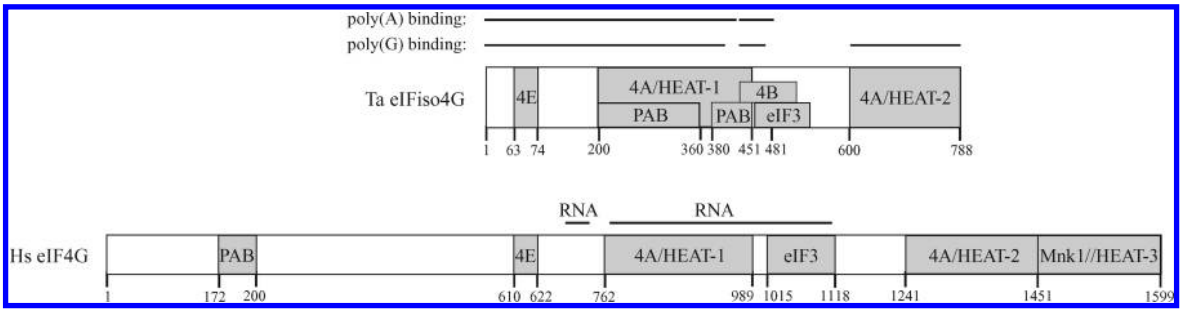

FIGURE 12: Partner protein interactions of eIFiso4G. Comparison of the domain organization of the RNA and protein binding domains in wheat eIFiso4G and human eIF4G. The eIF4E binding domain in eIFiso4G was previously reported (*26*).

eIF4A (*36*). Deletion of helix 5b from the HEAT-1 domain of yeast eIF4G did not affect eIF4A binding, but it was impaired upon enhancement of the ATPase activity of eIF4A (*36*), supporting the notion that the N-terminal portion of the HEAT-1 domain contributes more to the stable association of eIF4A.

Although plant eIF4A binds to eIFiso4G in a region known to make important contacts with eIF4A in animals and yeast, differences also exist. A polypeptide terminating just to the C-terminal end of the conserved ILNKLTP sequence in mammalian p97, a homologue of mammalian eIF4G, was unable to bind eIF4A. Instead, most of its HEAT-1 domain was required for the binding of eIF4A as it is in yeast eIF4G (*20*, *31*, *36*). Mutation of the other highly conserved, HEAT-1 domain elements also abolished or dramatically weakened eIF4A binding, although in at least two instances, these conserved sequences occur in the hydrophobic core of eIF4G that does not interact with eIF4A (*20*, *36*). Although binding of eIF4A to the eIFiso4G HEAT-1 domain required the entire domain for maximum binding, the ability of eIF4A to bind, albeit with lower affinity, to C-terminal truncations of this domain demonstrates that the additional C-terminal sequence that is essential for binding of eIF4A to the mammalian HEAT-1 domain is not essential for eIF4A to bind the eIFiso4G HEAT-1 domain. Because all of the amino acid residues within the HEAT-1 domain of yeast eIF4G identified as contacting eIF4A (*36*) are conserved in eIFiso4G, including several that lie outside the essential region between residues 200 and 230, they likely function to stabilize the binding of eIF4A to the eIFiso4G HEAT-1 domain as they do in yeast eIF4G. Significantly, eIFiso4G (and wheat eIF4G) differs from yeast eIF4G in that it does not contain a tryptophan at residue 184 that corresponds to Trp-579 of yeast eIF4G that contributes to the stable association of yeast eIF4A (Figure 1) (*36*). Moreover, this region was not required for the interaction of eIF4A with eIFiso4G.

A single eIF4A binding domain had been reported for eIFiso4G (*26*). However, the C-terminal region of wheat eIFiso4G (and wheat eIF4G) is highly conserved with the HEAT-2/eIF4A binding domain of mammalian eIF4G (Figure 1). Residues in the mammalian HEAT-2 domain known to contact eIF4A are conserved in the eIFiso4G HEAT-2 domain, such as Asp-1329 and Asp-1333 (Asp-709 and Asp-713 in eIFiso4G, respectively) that contact the N-terminal domain of mammalian eIF4A (*37*). However, Asp-1259, which contacts the C-terminal domain of mammalian eIF4A, is not conserved in eIFiso4G, although it is present in wheat eIF4G. Additionally, eIFiso4G (and wheat eIF4G) lacks the sequence corresponding to α-helices H7b, H8, and H9 of the mammalian HEAT-2 domain (Figure 1), indicating significant divergence from the mammalian homologue. Nevertheless, eIF4A bound the eIFiso4G HEAT-2 domain, demonstrating that eIFiso4G does contain two eIF4A binding domains. Consequently, eIFiso4G is more similar to animal eIF4G, which contains two eIF4A binding domains (*20*), than it is to yeast eIF4G, which contains just a single eIF4A binding domain (*31*).

A single binding site for PABP has been reported in animal and yeast eIF4G that lies on the N-proximal side of the eIF4E binding domain (*18*, *19*, *36*). In turn, animal and yeast eIF4G bind PABP at a single domain (*18*, *38*). In contrast, eIFiso4G binds PABP at two sites, one site encompassing the C-terminal end of RRM1 and the adjacent linker sequence and the second site requiring RRM3 and RRM4 (*15*). The regions between residues 200 and 360 and residues 380 and 451 in eIFiso4G interacted with PABP (Figure 12). The sequence in the second region of eIFiso4G shares similarity with the two PABP binding domains present in eIF4B (*12*), and similar sequences are present in eIF4G from wheat and human in the corresponding location of each protein (Figure 1). This second region of eIFiso4G (i.e., from residue 380 to 451) bound the N-terminal eIFiso4G interaction domain of PABP (Figure 7), encompassing the C-terminal end of RRM1 and the adjacent linker sequence to which eIF4B binds. This observation is consistent with the observation that eIF4B and eIFiso4G bind at overlapping domains within PABP and consequently compete in binding PABP (*15*). The C-terminal eIFiso4G interaction domain in PABP RRM3 and RRM4 bound the N-terminal region of eIFiso4G spanning residues 200 and 360 specifically. The PABP binding region in eIFiso4G lies on the C-proximal side of the eIF4E binding domain within the N-proximal eIF4A binding domain (Figure 12) and therefore differs substantially from animal and yeast eIF4G. The absence of a PABP binding domain N-proximal to the eIF4E binding domain in eIFiso4G may be due to the short length of the N-terminal region of eIFiso4G relative to other eIF4G proteins (Figure 12).

The region in eIFiso4G observed to bind PABP largely comprises the HEAT-1 domain and consequently overlaps with the eIF4A binding site in this same domain. The observed competition between PABP and eIF4A in binding the HEAT-1 domain confirmed the correct identification of the PABP binding region within the HEAT-1 domain and confirmed the overlapping nature of the PABP and eIF4A binding sites. The eIFiso4G peptide used in the competition experiment (Figure 11) represents the entire HEAT-1 domain and the eIF4B binding domain (i.e., eIFiso4G peptide 200−520). Therefore, the ability of PABP to compete with eIF4A in binding this peptide demonstrates that the binding of PABP to the HEAT-1 domain is not an artifact of a truncated HEAT-1 domain. As PABP was able to displace eIF4A from the HEAT-1 domain in the absence of the HEAT-2 domain but not in its presence, this supports the notion that the HEAT-2 domain serves to stabilize the binding of eIF4A to eIFiso4G,

particularly in the presence of PABP. Thus, in the absence of the HEAT-2 domain, binding of eIF4A to the eIFiso4G HEAT-1 domain may be weaker and more likely to be displaced by PABP, whereas the HEAT-2 domain may maintain binding of eIF4A while accommodating the binding of PABP to eIFiso4G.

Although eIF4B has not been reported to bind eIF4G in animals or yeast, eIFiso4G binds eIF4B at a single site adjacent to its N-terminal RNA binding domain (*12*), demonstrating a direct interaction between these two proteins. The eIF4B binding domain in eIFiso4G lies between residues 420 and 520, partially overlapping the eIF4A binding site in the HEAT-1 domain. As observed for PABP, eIF4B competed with eIF4A in binding eIFiso4G in the absence of the HEAT-2 domain but not in its presence, supporting the conclusion that the HEAT-2 domain functions to stabilize the binding of eIF4A to eIFiso4G, particularly in the presence of partner proteins. The observation that full-length wheat eIF4B interacts with eIFiso4G and eIF4A (*12*) may serve as another means of stabilizing the association of eIF4A with eIFiso4G.

The stoichiometry of the interaction between animal eIF4G and eIF4A has been suggested to be either 1:2 or 1:1 (*39*, *40*) but is unknown for eIFiso4G. If the stoichiometry of the eIFiso4G−eIF4A interaction was 1:2 and the binding of eIF4B to eIFiso4G could prevent eIF4A from binding to the HEAT-1 domain without affecting a second molecule from binding to the HEAT-2 domain, the presence of eIF4B would be expected to result in a 50% reduction in the amount of eIF4A associated with eIFiso4G in the competition assay. However, the presence of eIF4B did not significantly weaken the binding of eIF4A to $eIFiso4G_{200-788}$, which contains both HEAT domains. This supports the possibility that both HEAT domains of eIFiso4G participate in binding one molecule of eIF4A as suggested for mammalian eIF4G (*25*) and supports the notion that the HEAT-2 domain serves to stabilize the binding of eIF4A to eIFiso4G. Thus, in the absence of the HEAT-2 domain, binding of eIF4A to eIFiso4G may be weaker and more likely to be displaced by eIF4B, whereas the HEAT-2 domain may maintain binding of eIF4A while accommodating the binding of eIF4B to eIFiso4G.

The eIF4B binding domain between residues 420 and 520 in eIFiso4G overlaps with the region between residues 380 and 451 that contributes to PABP binding, raising the possibility that eIF4B and PABP compete for binding to eIFiso4G. Competition between eIF4B and PABP in binding eIFiso4G was confirmed, suggesting that the binding of eIF4B and PABP to this site within eIFiso4G is mutually exclusive. Physiologically relevant concentrations of zinc promote the homodimerization of eIF4B and the interaction between eIF4B and PABP (*17*). Zinc also stimulates the ATPase activity of the eIF4A−eIF4B−eIFiso4G complex (*17*). Zinc was able to stabilize the interaction between eIF4B and eIFiso4G in the presence of PABP when the domains for PABP interaction and for dimerization in eIF4B were absent. This suggested that the dimerization of eIF4B may contribute to its interaction with eIFiso4G and that zinc stabilizes its interaction in the absence of its dimerization. Despite the stabilizing effect of zinc on the eIF4B−eIFiso4G interaction, competition was still detected between eIF4B and PABP in binding eIFiso4G, albeit at a reduced level. These data demonstrate that eIF4B and PABP compete for binding to eIFiso4G. This is in contrast to the lack of competition between eIF4A and eIF4B or between eIF4A and PABP for binding to eIFiso4G when the HEAT-2 domain is present.

Just as the binding of eIF4B and PABP to eIFiso4G is mutually exclusive, so too is the binding of eIF4B and eIFiso4G to PABP as a consequence of their overlapping interaction domains in PABP (Figure 5A) (*15*). Because multiple molecules of PABP can bind a typical poly(A) tail, the mutually exclusive nature of eIFiso4G and eIF4B binding to PABP would likely result in their interaction with separate molecules of PABP, providing the additional stability of the eIFiso4F−eIF4B−PABP complex through multiple protein interactions. eIF4B also can dimerize, a process stimulated by zinc (*17*). Thus, with respect to interactions between the termini of an mRNA, two scenarios may occur. Separate molecules of PABP on a poly(A) tail may interact directly with eIFiso4G or eIF4B. The observation that the combination of eIFiso4G and eIF4B synergistically promotes the multimeric binding of PABP to poly(A) RNA (*8*) supports the notion that they can bind separate molecules of PABP and increase the stability of the complex. Moreover, wheat PABP exists as differentially phosphorylated species (*41*), and its phosphorylation state affects partner protein selection (*13*), further reducing the potential for competition between eIFiso4G and eIF4B. Alternatively, an eIF4B dimer might interact with eIFiso4G/eIF4A and PABP simultaneously, forming a bridge between these proteins.

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