The poly(A)-binding protein (PABP), a protein that contains four conserved RNA recognition motifs (RRM1–4) and a C-terminal domain, is expressed throughout the eukaryotic kingdom and promotes translation through physical and functional interactions with eukaryotic initiation factor (eIF) 4G and eIF4B. Two highly divergent isoforms of eIF4G, known as eIF4G and eIFiso4G, are expressed in plants. As little is known about how PABP can interact with RNA and three distinct translation interaction factors in plants, the RNA binding specificity and organization of the protein interaction domains in wheat PABP was investigated. Wheat PABP differs from animal PABP in that its RRM1 does not bind RNA as an individual domain and that RRM 2, 3, and 4 exhibit different RNA binding specificities to non-poly(A) sequences. The PABP interaction domains for eIF4G and eIFiso4G were distinct despite the functional similarity between the eIF4G proteins. A single interaction domain for eIF4G is present in the RRM1 of PABP, whereas eIFiso4G interacts with two sites, i.e. one within RRM1–2 and the second within RRM3–4. The eIFiso4G binding site in RRM1–2 mapped to a 36-amino acid region encompassing the C-terminal end of RRM1, the linker region, and the N-terminal end of RRM2, whereas the second site in RRM3–4 was more complex. A single interaction domain for eIF4B is present within a 32-amino acid region representing the C-terminal end of RRM1 of PABP that overlaps with the N-proximal eIFiso4G interaction domain. eIF4B and eIFiso4G exhibited competitive binding to PABP, supporting the overlapping nature of their interaction domains. These results support the notion that eIF4G, eIFiso4G, and eIF4B interact with distinct molecules of PABP to increase the stability of the interaction between the termini of an mRNA.

The initiation of translation in eukaryotes typically requires the involvement of at least 11 initiation factors (eIFs) that facilitate the binding of the 40 S ribosomal subunit to an mRNA and assemble the 80 S ribosome at the correct initiation codon.

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The poly(A)-binding protein (PABP), a protein that contains four conserved RNA recognition motifs (RRM1–4) and a C-terminal domain, is expressed throughout the eukaryotic kingdom and promotes translation through physical and functional interactions with eukaryotic initiation factor (eIF) 4G and eIF4B. Two highly divergent isoforms of eIF4G, known as eIF4G and eIFiso4G, are expressed in plants. As little is known about how PABP can interact with RNA and three distinct translation initiation factors in plants, the RNA binding specificity and organization of the protein interaction domains in wheat PABP was investigated. Wheat PABP differs from animal PABP in that its RRM1 does not bind RNA as an individual domain and that RRM 2, 3, and 4 exhibit different RNA binding specificities to non-poly(A) sequences. The PABP interaction domains for eIF4G and eIFiso4G were distinct despite the functional similarity between the eIF4G proteins. A single interaction domain for eIF4G is present in the RRM1 of PABP, whereas eIFiso4G interacts with two sites, i.e. one within RRM1–2 and the second within RRM3–4. The eIFiso4G binding site in RRM1–2 mapped to a 36-amino acid region encompassing the C-terminal end of RRM1, the linker region, and the N-terminal end of RRM2, whereas the second site in RRM3–4 was more complex. A single interaction domain for eIF4B is present within a 32-amino acid region representing the C-terminal end of RRM1 of PABP that overlaps with the N-proximal eIFiso4G interaction domain. eIF4B and eIFiso4G exhibited competitive binding to PABP, supporting the overlapping nature of their interaction domains. These results support the notion that eIF4G, eIFiso4G, and eIF4B interact with distinct molecules of PABP to increase the stability of the interaction between the termini of an mRNA.

The initiation of translation in eukaryotes typically requires the involvement of at least 11 initiation factors (eIFs) that facilitate the binding of the 40 S ribosomal subunit to an mRNA and assemble the 80 S ribosome at the correct initiation codon. The 5′-cap structure of an mRNA serves as the binding site for eIF4E, the small subunit of eIF4F, which also contains eIF4G and eIF4A. In addition to eIF4E, eIF4F interacts with eIF4A, an RNA helicase, and eIF3, which recruits the 40 S subunit. Two related but highly distinct eIF4G isoforms, referred to as eIF4G and eIFiso4G, are expressed in plants (1) and are present as the large subunit in eIF4F and eIFiso4F, respectively. eIF4G and eIFiso4G differ in size (165 and 86 kDa, respectively) and are only 30% identical (1, 2), whereas the two forms of eIF4G present in yeast and mammals are considerably more conserved (3, 4).

The 5′-cap and the poly(A) tail synergistically stimulate translation (5). The poly(A)-binding protein (PABP) promotes the recruitment of the 40 S subunit (6) and interacts with eIF4G (7–11). The PABP-eIF4G interaction results in the circularization of an mRNA (12–15) and serves as a means test to confirm the integrity of an mRNA prior to the recruitment of the 40 S subunit (16). PABP also interacts with eIF4B (7), eIF4A (19–22) and is involved in organizing the assembly of PABP, eIF4A, eIFiso4G, and RNA (23).

The interaction of eIF4G, eIFiso4G, or eIF4B with PABP increases the binding affinity of the latter for poly(A) RNA (7). The PABP-eIFiso4G interaction also increases eIFiso4G binding to the 5′-cap by 40-fold and stimulates the ATPase and RNA helicase activity of the eIF4F-eIF4A-eIF4B complex (24, 25). Thus, PABP promotes the stable recruitment of eIF4F to an mRNA by accelerating its binding to, and reducing its dissociation from, the 5′-cap (26, 27).

PABP contains four N-terminal RNA recognition motifs (RRMs) and requires a minimum of 12 adenosine residues to bind RNA (28, 29). The C-terminal half of PABP is required for its self-association (30). RRM1 and RRM2 of mammalian and yeast PABP are required to interact with eIF4G (9, 31, 32), whereas the C-terminal domain of mammalian PABP has been suggested to contain the interaction domain for eIF4B (18).

PABP is differentially phosphorylated in plants, yeast, and sea urchin (33, 34) and its phosphorylation state determines its affinity and type of binding to poly(A) RNA (17). Phosphorylated PABP binds poly(A) RNA cooperatively but with a 10-fold lower affinity than hypophosphorylated PABP, which binds non-cooperatively (17). The highest degree of cooperative binding was observed between phosphorylated and hypophosphorylated PABP (17), indicating that an interaction between PABP of differing phosphorylation states is favored. This sug-
PABP Interaction with eIF4G and eIF4B

gests that the assembly of PABP molecules on a poly(A) tail is likely to be heterogenous in its phosphorylation state.

The phosphorylation state of PABP also determines the strength and specificity of its interaction with eIF4B, eIF4G, and eIFiso4G (17). eIF4G promotes cooperative RNA binding of hypophosphorylated PABP but not phosphorylated PABP (17), suggesting that eIF4G specifically interacts with hypophosphorylated PABP. In contrast, eIFiso4G promotes RNA binding of hypophosphorylated and phosphorylated PABP (17). eIF4B increases RNA binding of phosphorylated PABP to a greater extent than hypophosphorylated PABP (17).

The observation that PABP can interact with three distinct initiation factors raises the question of whether each factor interacts at the same or different sites within PABP. If all three factors share the same interaction site in PABP, the cooperative binding exhibited between PABP molecules of differing phosphorylation states, together with the specificity of initiation factor interaction provided by its phosphorylation state, may enable eIF4G (or eIFiso4G) and eIF4B to avoid competition by interacting with distinct molecules of PABP bound to a poly(A) tail. Alternatively, eIF4G (or eIFiso4G) and eIF4B may interact at independent sites within PABP.

In this study, we show that the interaction domains for eIF4G and eIFiso4G in plant PABP differ in number and location. Whereas the eIF4G and eIF4B interaction domains are located within the RRM1 of PABP, eIFiso4G interacts at two sites, i.e. one within RRM1–2 that overlaps the eIF4B binding site and a second within RRM3–4. Competition between eIFiso4G and eIF4B to bind PABP supported the notion that their binding sites in PABP overlap and that these factors are required to interact with separate molecules of PABP.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression—pET3d-eIFiso4G, pET3d-eIF4B, and pET3d-eIF4G were generous gifts from Karen Browning (University of Texas, Austin). pET19b-eIF4B, pET19b-PABP-(1–651), and pET19b-PABP-(1–393) were described previously (23). Following PCR amplification of the appropriate region of the PABP coding region, the amplified fragments were introduced into the EcoRI and BamHI sites of pGEX-2TK or into the NdeI/BamHI or XhoI/BamHI sites of PET19b (Novagen), and the PABP polypeptides expressed as GST or His10-tagged fusions, respectively. To generate pGEX-2TK-eIF4G(1–498), the corresponding region of eIF4G was amplified and introduced into the BamHI/EcoRI sites of pGEX-2TK. All constructs were confirmed by sequencing.

Protein expression was performed in Escherichia coli BL21 cells following induction with 1 mm isopropyl-β-D-galactopyranoside. Pelleted cells were sonicated in Buffer B-100 (20 mM HEPES, 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT) and following centrifugation to remove cell debris, crude cell extracts were used for analysis or the His10-tagged or GST-tagged fusion proteins were purified first using TALON metal affinity resin (Clontech) or glutathione-Sepharose 4B resin (Amersham Biosciences), respectively. For far Western analysis, PABP polypeptides without the GST fusion were recovered following thrombin (Amersham Biosciences) digestion on resin.

RNA Binding Analysis—Poly(A)-Sepharose 4B and poly(C)-, poly(G)-, or poly(U)-agarose (Sigma) were washed three times with Buffer B-100 (supplied with 5 mM DTT, 0.1% Triton X-100). PABP polypeptides were incubated with the resin at 4 °C for 1 h. The resin was washed four times with Buffer B-100 (with 5 mM DTT, 0.1% Triton X-100), re-suspended in SDS sample buffer, and heated. The supernatant was analyzed by SDS-PAGE and the gel stained with Coomassie.

Protein Pull-down Assay—GST fusion protein was added to glutathione-Sepharose 4B resin (Amersham Biosciences) washed three times with pre-cooled Buffer B-100 (supplied with 1 mM DTT, 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. Prey protein 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 Na2HPO4, 1.4 mM KH2PO4) 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, micrococcal nuclease was added to the pull-down assay. The nuclease was added to the resin containing the bait protein and incubated in Buffer B-100 (without EDTA) and supplied with calcium at 37 °C for 30 min. Prey protein was then added and the reaction incubated at 4 °C for an additional 2 h. Identical conditions were used for control reactions in which the nuclease was lacking.

Far Western Analysis—Bait protein was transferred to 0.22-mm polyvinylidene difluoride membrane by electroblootting. The membrane was blocked by 5% milk in Buffer B-100 for 1 h. The prey protein was radiolabeled using either purified, recombinant maize casein kinase II or protein kinase A (New England Biolabs) in a standard kinase assay reaction. The prey protein was used to probe the membrane by incubating in 1% milk in Buffer B-100 for 1 h at 4 °C. The membrane then was washed in Buffer B-100 (supplied with 0.1% Triton X-100) extensively. Protein interaction was detected using autoradiography.

Western Analysis—Protein was transferred to 0.22-mm polyvinylidene difluoride membrane by electroblotting. The membranes were blocked in 5% milk, 1% NaN3 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 1:10,000 for 1 h. The blots were washed twice with TPBS and the signal detected typically between 1 and 15 min using chemiluminescence (Pierce). eIFiso4F antiserum (a generous gift of K. Browning) was used at 1:2000 dilution.

Gel Shift Assay—Radiolabeled poly(A)30 RNA was synthesized in vitro and gel-purified to remove the DNA template, unincorporated nucleotides, and less than full-length RNA. Radiolabeled RNA and proteins were added to a 15-µl binding reaction containing 25 mM HEPES, pH 7.5, 1 mM MgAc, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, 1 mM DTT, 0.5 mg/ml yeast RNA, and 1 mM Mg-ATP for 15 min at 4 °C. Following incuba-
tion, 2 μl of 80% glycerol and 2 μl of 0.25 mg/ml heparin was added and the reaction incubated for an additional 10 min at 4 °C. The RNA-protein complexes were resolved on a native 5% polyacrylamide gel, dried, and analyzed by autoradiography.

RESULTS

The RRMs of PABP Differ in RNA Binding Ability and Nucleotide Specificity—RRMs 1 and 2 of Xenopus PABP are capable of binding RNA as individual domains and exhibit a preference for the purine-rich sequence (35). RRM3 and RRM4 do not bind RNA as individual domains but together, RRM3–4 binds RNA and exhibits a preference for poly(G) RNA (35). To determine whether plant PABP was similar in its RNA binding requirements, the cDNA encoding PABP was introduced into pGEX-2TK and the full-length GST-PABP fusion protein (i.e. PABP-(1–651)) was overexpressed in E. coli (Fig. 1A, lane 1). PABP-(1–651)-containing crude extract was incubated with poly(A)-Sepharose or poly(U)-, poly(C)-, or poly(G)-agarose, the resin washed extensively, and the bound protein analyzed by SDS-PAGE. PABP-(1–651) bound to poly(A) and poly(G) RNA, whereas its binding to poly(U) and poly(C) RNA was just detectable (Fig. 1A, lanes 2–5).

To examine the binding specificity of the individual RRM domains, PABP polypeptides representing one or more RRMs (Fig. 1B) were tested. PABP RRM1 (i.e. PABP-(28–112)) failed to bind poly(A), poly(G), or poly(U) RNA (Fig. 1, C–E, lanes 1, respectively) but RRM1–2 (i.e. PABP-(28–200)), RRM1–3 (i.e. PABP-(28–290)), or RRM1–4 (i.e. PABP-(28–393)) were able to bind poly(A) and poly(G) RNA but not poly(U) RNA (Fig. 1, C–E, lanes 2–4, respectively). PABP RRM2 (i.e. PABP-(104–200)) was able to bind poly(A) and poly(G) RNA but not poly(U) RNA (Fig. 1, C–E, lanes 5, respectively) as did RRM2–3 (i.e. PABP-(104–290)) and RRM2–4 (i.e. PABP-(104–393)) (Fig. 1, C–E, lanes 6 and 7, respectively). In contrast, PABP RRM3 (i.e. PABP-(200–290)) or RRM4 (i.e. PABP-(306–393)) failed to bind RNA (Fig. 1, C–E, lanes 8 and 10, respectively) but together, RRM3–4 bound poly(A) and poly(U) RNA but not poly(G) RNA (Fig. 1, C, E, and D, lane 9, respectively). No RRM or combination of RRMs was able to bind poly(C) (data not shown). These results suggest that wheat PABP is similar in some respects to animal PABP in that its RRM2 is competent to bind RNA alone and that RRM3 and RRM4 do not bind RNA as individual domains but together are competent to bind RNA. Wheat PABP differs from animal PABP in that RRM2-containing polypeptides bind poly(G) as well as poly(A) and that RRM3–4 binds to poly(A) and poly(U) instead of poly(G).

**eIF4B, eIF4G, and eIFiso4G Bind PABP within the RRM Domains**—The eIF4B binding site within human PABP was suggested to reside within its C-terminal domain (18). To determine whether eIF4B binds to the same domain in wheat PABP that it does in human PABP, GST-wheat eIF4B was used.
**PABP Interaction with eIF4G and eIF4B**

**FIGURE 2. eIF4B, eIFiso4G, and eIF4G require RRM1 of PABP for binding.** GST-eIF4B-(1–651), GST-eIFiso4G-(1–450), or GST-eIF4G-(1–498) were used to pull down the indicated His-tagged PABP polypeptides (top panels). Bound PABP was detected using anti-His tag antiserum. The amount of GST-eIF4B, GST-eIFiso4G, or GST-eIF4G used in the pull-down assay is shown in the Coomassie-stained gel (bottom panels).

in pull-down assays with full-length wheat PABP (i.e. PABP-(1–651)), the N-terminal, RRM-containing region of PABP (i.e. PABP-(1–393)), the C-terminal region of PABP (i.e. PABP-(391–651)), and PABP in which RRM1–2 was deleted (i.e. PABP-(200–651)). eIF4B bound full-length PABP (Fig. 2, lane 1), in good agreement with our previous observations (23). eIF4B also bound the N-terminal, RRM-containing region of PABP (Fig. 2, lane 2). Little or no binding was observed to the C-terminal region of PABP or to PABP in which RRM1–2 was deleted (Fig. 2, lanes 3–4, respectively). These data suggest that wheat eIF4B may bind in or around RRM1–2 of wheat PABP, a significant difference from that reported for the interaction between the human orthologs.

In yeast and animals, the RRM1 and RRM2 of PABP are required to interact with eIF4G (9, 31, 32). To determine whether wheat eIF4G (or eIFiso4G) binds to the same domains in wheat PABP, GST-eIFiso4G-(1–450) or GST-eIF4G-(1–498) were used to pull down the same PABP polypeptides tested for interaction with eIF4B above. eIFiso4G bound full-length PABP (Fig. 2, lane 5), in good agreement with our previous observations (7) and bound the N-terminal, RRM-containing region of PABP (Fig. 2, lane 6). No binding was observed for the C-terminal region of PABP or PABP in which RRM1–2 was deleted (Fig. 2, lanes 7 and 8). Identical results were observed when GST-eIF4G was used to pull down these same PABP polypeptides (Fig. 2, lanes 9–12). These data suggest that, like eIF4B, eIF4G and eIFiso4G may bind in or around RRM1–2 of PABP.

**eIF4B Interacts within the C-terminal Portion of RRM1—** Wheat PABP binds to eIF4B at each of two conserved 41-amino acid repeat domains on either side of the C-terminal RNA binding domain of eIF4B (23), whereas human PABP has been reported to bind close to the N terminus of human eIF4B (18). The observation that the two PABP binding sites present in wheat eIF4B exhibit a high degree of similarity suggests that PABP may contain a single eIF4B-interaction domain. To examine this possibility, the PABP polypeptides used for the RNA binding analysis in Fig. 1 were resolved on a SDS-PAGE gel, transferred to membrane, and used for far Western analysis using purified, full-length eIF4B as the probe. The PABP polypeptides were expressed as GST fusion proteins, however, the GST domain was removed by digestion of each purified fusion protein with thrombin prior to resolution by SDS-PAGE. Following renaturation of the PABP polypeptides, eIF4B bound to RRM1 (Fig. 3A, bottom panel, lane 1), demonstrating that this domain alone was sufficient to bind eIF4B. Binding was also observed for RRM1–2, RRM1–3, and RRM1–4 (Fig. 3A, bottom panel, lanes 2–4). The strength of the signal was reduced as the molecular mass of the polypeptide increased. As a similar amount of each PABP polypeptide was used for the analysis (Fig. 3A, top panel), the decrease in the signal largely correlated with a decrease in the molar amount of RRM1. Little to no binding of eIF4B was observed for any other region of PABP (Fig. 3A, bottom panel, lanes 5–10), suggesting that a single interaction domain for eIF4B is present in PABP and is contained within RRM1.

To more precisely determine the region within the PABP RRM1 that is responsible for binding eIF4B, portions of RRM1 were tested for their ability to bind eIF4B in the far Western assay. As before, the PABP polypeptides were expressed as GST fusion proteins and the GST domain removed by thrombin digestion prior to resolution by SDS-PAGE. Because truncation of RRM1 alone would result in polypeptides that would be extremely small that may result in poor protein folding, truncations of RRM1 were performed using the RRM1–2 construct. eIF4B does not bind RRM2 (Fig. 3A, bottom panel, lane 5), therefore, the presence of RRM2 would not complicate the analysis.

N-terminal truncation of PABP to residue 81 (i.e. PABP-(81–200)) reduced but did not abolish the ability of eIF4B to bind (Fig. 3B, bottom panel, lane 2). Truncation to residue 94 (i.e. PABP-(94–200)) did not further affect binding of eIF4B (Fig. 3B, bottom panel, lane 3). Truncation to residue 104 (i.e. PABP-(104–200)), however, abolished eIF4B binding (Fig. 3B, bottom panel, lane 4) as observed in Fig. 3A (bottom panel, lane 5). Because eIF4B binds RRM1 alone (residues 28–112), these data suggest that eIF4B binds PABP between residues 94 and 112 and does not require sequences present in the linker region between RRM1 and RRM2 or sequences within RRM2. The presence of a single eIF4B binding site in PABP is consistent with the repeat nature of the two PABP binding sites in eIF4B (23). The reduction in binding observed for PABP-(81–200) and PABP-(94–200) may indicate that additional sequences within RRM1 contributes to eIF4B binding either directly or indirectly by assisting in protein folding. The region between residues 94 and 112 contains the forth β-sheet (i.e. S4) of RRM1 and the loop between the second helix (H2) and S4 (Fig. 4). Binding of eIF4B to the RRM1 of wheat PABP is significantly
different from the reported eIF4B binding site in human PABP (18). This is not surprising as the PABP binding site within wheat eIF4B (23) also differs in number and location from that reported for human eIF4B (18).

**PABP RRM1 Interacts with eIF4G**

To determine whether eIF4G binds to the same domain in plant PABP as it does in the human and yeast orthologs, the PABP polypeptides used for the eIF4B binding analysis were used for far Western analysis using purified, recombinant eIF4G as the probe. As before, PABP polypeptides in which the GST domain was removed were used. eIF4G bound to the RRM1 (Fig. 5A, **bottom panel, lane 1**), demonstrating that, as with eIF4B, this domain is sufficient to bind eIF4G. Binding was also observed to RRM1–2, RRM1–3, and RRM1–4 although the strength of the signal decreased as additional domains were included (Fig. 5A, **bottom panel, lanes 2–4**). Longer polypeptides may not renature as efficiently following their transfer to the membrane. Little to no binding of eIF4G was observed for any other region of PABP (Fig. 5A, **bottom panel, lane 5–10**), suggesting that RRM1 is solely responsible for binding eIF4G (Fig. 5B). This observation sug-

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**FIGURE 3. Mapping the eIF4B interaction domain in wheat PABP.** In A, PABP polypeptides were expressed as GST fusion proteins in E. coli, the GST domain removed by thrombin digestion, the polypeptides resolved by SDS-PAGE and detected using Coomassie staining (**top panel**). Note that incomplete digestion was achieved in **lane 10** so that the large molecular weight band represents uncut fusion protein. Binding of full-length eIF4B to the indicated PABP polypeptides was determined by far Western analysis in which [32P]-labeled eIF4B was used as the probe (**bottom panel**). Bound eIF4B was analyzed by autoradiography. MW, molecular weight standards. In B, PABP polypeptides in which the GST was removed (**top panel**) were used for far Western analysis with [32P]-labeled eIF4B as the probe (**bottom panel**). In C, summary of eIF4B binding to PABP polypeptides. Strength of eIF4B binding is indicated by the number of pluses. —, no detectable binding.

**FIGURE 4. Comparison of eIFiso4G and eIF4B interaction domains of wheat PABP (Ta PAB) with the corresponding sequence of tobacco PAB3 (Nt PAB3), Arabidopsis PAB2 (At PAB2), human PAB (Hs PAB), and Saccharomyces cerevisiae PAB (Sc PAB).** Conservation of identical residues relative to wheat PABP is indicated by shading. The RNP1 and RNP2 motifs conserved among PAB proteins are indicated by asterisks. Helices (H) and β-sheets (S) as determined for human PAB (42) are indicated. RRM1 and RRM2 domains are indicated by brackets and the eIFiso4G and eIF4B interaction domains indicated by lines. The portion of PABP illustrated in each case is indicated by **residue numbers before and after each sequence.** Residues in wheat PABP discussed in the text are indicated **above the sequence.**
gests that wheat eIF4G differs significantly from yeast and animal eIF4G in that only RRM1 of wheat PABP is required for binding wheat eIF4G, whereas RRM1 and RRM2 of yeast and animal PABP are required for binding yeast and animal eIF4G (9, 31, 32).

Two Domains within PABP Interact with eIFiso4G—To determine whether eIFiso4G shares the same interaction domain in PABP as eIF4G, the PABP polypeptides were expressed as GST fusion proteins and used in pull-down assays with full-length eIF4G.

RRM1 of PABP (i.e. GST-PABP-(28–112)) failed to pull down eIF4G (Fig. 6, bottom panel, lane 1). As the same RRM1-containing polypeptide was competent to bind eIF4G and eIF4B, this suggested that the eIFiso4G binding site differed from the other two factors. In contrast to the results with RRM1 alone, RRM1–2, RRM1–3, and RRM1–4 where able to pull down eIF4G (Fig. 6, bottom panel, lanes 2–4). Binding was also observed for RRM2, RRM2–3, and RRM2–4, although the signal was weaker (Fig. 6, bottom panel, lanes 5–7). No binding of eIFiso4G to RRM3 (i.e. PABP-(200–290)) or RRM4 (i.e. PABP-(306–393)) was detected (Fig. 6, bottom panel, lanes 8 and 10, respectively) but together, binding between RRM3–4 and eIFiso4G was just detectable (Fig. 6, bottom panel, lane 9). These data suggest that eIFiso4G binds within RRM1 and RRM2 of PABP, similar to that reported for yeast and animal eIF4G (9, 31, 32) but representing a significant difference from the binding requirements observed for wheat eIF4G (Fig. 5).

Our data indicated that RRM1–2 and RRM3–4 each contained an eIFiso4G binding domain. Because of the sequence and structural conservation among the RRMs, we investigated whether the sequence requirements for each eIFiso4G binding site were similar. Deletions from N-terminal and C-terminal regions of the RRM1–2 or RRM2–3 polypeptides were made and tested for their ability to pull down full-length eIFiso4G. A C-terminal deletion to residue 169 of the RRM1–2 (i.e. PABP-(28–169)) did not impair its ability to bind eIF4G relative to RRM1–2 (i.e. PABP-(28–200)) (Fig. 7A, bottom panel, compare lane 2 to 3). Binding of eIFiso4G to RRM2–3 (i.e. PABP-(104–290)) was just detectable (Fig. 7A, bottom panel, lane 4) as observed in Fig. 6. N-terminal deletion of residue 168 abolished eIFiso4G binding (Fig. 7A, bottom panel, lane 5).

To more precisely determine the C-terminal boundary of the eIFiso4G binding region within RRM1–2, further deletions were made within the RRM1–2 polypeptide. C-terminal deletions of residues 168, 158, 148, or 129 of RRM1–2 (i.e. PABP-(28–168), PABP-(28–158), PABP-(28–148), or PABP-(28–129), respectively) did not impair its ability to bind eIFiso4G relative to RRM1–2 (i.e. PABP-(28–200)) (Fig. 7B, bottom panel, compare lanes 2–6 to 6). Note that more protein of each PABP polypeptide was used in the pull-down assay to increase detection of the signal. This also resulted in some apparent binding of eIFiso4G to PABP-(28–112), which likely represented background binding as it was variable.

FIGURE 5. Mapping the eIF4G interaction domain in wheat PABP. In A, PABP polypeptides were expressed, digested, and resolved (top panel) as described in the legend to Fig. 3. Binding of eIF4G to the indicated PABP polypeptides was determined by far Western analysis in which 32P-labeled eIF4G was used as the probe (bottom panel). Bound eIF4G was analyzed by autoradiography. MW, molecular weight standards. B shows a summary of eIF4G binding to PABP polypeptides.

FIGURE 6. Mapping the eIFiso4G interaction domains in wheat PABP. Purified PABP polypeptides expressed as GST fusion proteins (top panel) were used in pull-down assays to detect binding of eIFiso4G as determined by Western analysis (bottom panel).
N-terminal deletions of RRM1–2 were also tested. Deletion to residues 81 or 94 (i.e. PABP-(81–200) or PABP-(94–200), respectively) did not abolish binding to eIFiso4G (Fig. 7C, bottom panel, lanes 1 and 2), whereas deletion to residue 104 resulted in a substantial reduction in the observed signal that, although weak, was reproducible. Together, these results suggested that the eIFiso4G binding site in RRM1–2 resides between residues 94 and 129. To test this directly, polypeptides representing residues 81–129 (i.e. PABP-(81–129)) or residues 94–129 (i.e. PABP-(94–129)) were tested for their ability to bind eIFiso4G. Binding of eIFiso4G was observed for both polypeptides (Fig. 7D, bottom panel, lanes 2 and 3), suggesting that eIFiso4G binding to RRM1–2 resides within residues 94–129 (Fig. 7E). This represents a region that overlaps yet is not identical with the eIF4B binding site that was mapped between residues 94 and 112 (Fig. 4). Specifically, eIFiso4G binding required sequence between residues 113 and 129 but this region was not required for binding eIF4B. However, sequence between residues 94 and 112 was required for binding eIF4B and eIFiso4G. The region between residues 94 and 129 contains S4, i.e. the forth β-sheet of RRM1, the loop between the second helix (H2) and S4, the RRM1–2 linker region, and S1, i.e. the first β-sheet of RRM2 (Fig. 4).

In addition to the binding site within RRM1–2, a second, eIFiso4G binding site was observed within RRM3–4 (Fig. 6). eIFiso4G binding to RRM3–4 consistently resulted in a weaker signal than that observed with RRM1–2. As neither RRM3 nor RRM4 alone was sufficient to bind eIFiso4G (Fig. 6), this suggested that the region encompassing the C-terminal portion of RRM3 to the N-terminal portion of RRM4 that corresponded to the binding site within RRM1–2 may be required for eIFiso4G binding. In addition to the structural similarities between these two corresponding regions, there is significant sequence conservation (Fig. 8A). The difference in sequence between the regions might be expected to account for the difference in eIFiso4G binding affinity of RRM1–2 versus RRM3–4.

As shown above, residues 94–112 are critical for eIFiso4G binding to RRM1–2. To determine whether the corresponding region in RRM3–4 (i.e. residues 272–288) (Fig. 8A) was functionally similar to residues 94–112 in supporting eIFiso4G binding, residues 94–110 were replaced with residues 272–288 in PABP-(94–200) to generate PABP-(272–288/111–200) (Fig. 8B). eIFiso4G bound PABP-(272–288/111–200) to a lower detectable level than that observed for PABP-(94–200) (Fig. 9, bottom panel, compare lane 2 to 1) or RRM3–4, i.e. PABP-(200–393) (Fig. 9, bottom panel, compare lane 2 to 5). This suggested that the region encompassing residues 272–288 was not functionally equivalent to residues 94–110 or was not able to function in the context of the RRM1–2. In support of the former possibility, truncation of RRM3–4 to residue 272 (i.e. PABP-(272–393)) resulted in a substantial reduction in detectable eIFiso4G binding relative to intact RRM3–4 (i.e. PABP-(200–393)) (Fig. 9, bottom panel, compare lane 3 to 5), data suggesting that the sequence between residues 200 and 271 is necessary for eIFiso4G binding, whereas deletion of a region encompassing the corresponding region in RRM1–2, i.e. PABP-(94–200), was fully competent to bind eIFiso4G (Fig. 9, bottom panel, lane 1). Residues 200–271 were necessary for eIFiso4G binding but not sufficient as the entire RRM3 (i.e. PABP-(200–290)) was unable to bind eIFiso4G (Fig. 6, bottom panel, lane 8).

eIFiso4G binding to RRM1–2 consistently resulted in a stronger detectable signal than its binding to RRM3–4. Because residues 272–288 did not appear to be functionally equivalent to residues 94–110 when tested for their ability to support eIFiso4G binding to RRM1–2, it is possible that they are responsible for the lower detectable binding of eIFiso4G to RRM3–4. If so, replacing residues 272–288 in PABP-(272–393) with residues 94–112 might be expected to improve eIFiso4G binding. To test this, PABP-(94–110/289–393) was generated in which residues 272–288 in PABP-(272–393) were replaced with residues 94–110 (Fig. 8B). eIFiso4G bound PABP-(94–110/289–393) to a higher detectable level than that observed...
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for PABP-(272–393) (Fig. 9, bottom panel, compare lane 4 to 3) although it did not restore the signal to the level observed for the entire RRM3–4 (i.e. PABP-(200–393)) (Fig. 9, bottom panel, compare lane 4 to 5) or to the even higher level observed for PABP-(94–200) (Fig. 9, bottom panel, compare lane 4 to 1). This suggested that residues 94–110 were not sufficient to restore full binding of eIFiso4G to RRM3–4.

The minimum binding region of RRM1–2 contained within residues 94–129 differs from the corresponding region in RRM3–4 in that the linker region between RRM3 and RRM4 is 25 amino acids long, whereas the linker region between RRM1 and RRM2 is only 9 amino acids long (Fig. 8A). To test if the weaker binding signal of eIFiso4G to RRM3–4 is a consequence of a longer linker region between RRM3 and RRM4, its length was reduced by deleting 16 amino acids to result in a length identical to that between RRM1 and RRM2 (i.e. PABP-(200–393)(Δ290–305/K289L)) (Fig. 8B). The eIFiso4G binding signal of this polypeptide was substantially weaker relative to eIFiso4G binding to RRM3–4 (Fig. 9, bottom panel, compare lane 5 to 6). This observation suggests that the longer length of the linker sequence between RRM3 and RRM4 (compared with the linker between RRM1 and RRM2) was not responsible for the reduced detectable binding of eIFiso4G to RRM3–4. Indeed, the sequence between residues 290 and 305 was necessary for eIFiso4G binding, although it was not sufficient as it is contained within the N-terminal truncated RRM3–4 construct, i.e. PABP-(272–393), which was unable to bind eIFiso4G (Fig. 9, bottom panel, lane 3).

Deletion of the eIFiso4G and eIF4B Binding Sites within PABP Results in the Loss of the Functional Interaction of eIFiso4G and eIF4B with PABP—The above results suggest that eIF4B interacts within PABP RRM1 and eIFiso4G interacts within PABP RRM1–2. We have shown previously that the interaction of eIF4B or eIFiso4G with PABP increases the binding affinity of the latter to poly(A) RNA, principally through an increase in its multimeric binding (17). Thus, one important function of the eIF4B-PABP and eIFiso4G-PABP interactions is to increase the recruitment of PABP to an mRNA. To determine whether the functional interaction of eIF4B or eIFiso4G with PABP requires the eIF4B or eIFiso4G binding sites, the effect of eIF4B or eIFiso4G with PABP increases the binding affinity of the latter to poly(A) RNA, principally through an increase in its multimeric binding (17). Thus, one important function of the eIF4B-PABP and eIFiso4G-PABP interactions is to increase the recruitment of PABP to an mRNA. To determine whether the functional interaction of eIF4B or eIFiso4G with PABP requires the eIF4B or eIFiso4G binding sites, the effect of eIF4B or eIFiso4G on the binding of full-length PABP or PABP lacking RRM1–2 (i.e. PABP-(200–651), which retains RNA binding activity but lacks the binding sites for eIF4B and eIFiso4G) to poly(A)₅₀ RNA was examined. Both full-length PABP and PABP-(200–651) bound poly(A)₅₀ RNA in the gel shift assay (Fig. 10A), consistent with the results when binding to poly(A)-Sepharose was examined (Fig. 1C). A second complex, containing two PABP molecules bound to the poly(A)₅₀ RNA, was observed at PABP concentra-

FIGURE 8. Construct design to determine the degree of similarity between the eIFiso4G binding sites in RRM1–2 and RRM3–4. In A, alignment of the portion of RRM1–2 containing the eIFiso4G interaction domain with the corresponding sequence of RRM3–4. Conservation of identical residues is indicated by shading. The RNP1 and RNP2 motifs conserved among PAB proteins are indicated by asterisks. Helices (H) and β-sheets (S) as determined for human PAB (42) are indicated. RRM1 and RRM2 domains are indicated by brackets and the eIFiso4G interaction domain is indicated with a line. The portion of PABP illustrated in each case is indicated by the residue numbers before and after each sequence. Residues in wheat PAB discussed in the text are indicated above and below the respective sequence. In B, the construct design to test the function of the effect of swapping residues 94–110 for residues 272–288 or the deletion of residues 290–305. The ability of each resulting polypeptide to bind eIFiso4G is shown in Fig. 9.
tions in which little or no unbound RNA remained (Fig. 10A, see complex 2). To maintain binding in the linear range of the assay, a small amount of each PABP was used, resulting in a single complex with poly(A) RNA (Fig. 10B, lane 3). No binding of eIFiso4G to poly(A) RNA was observed at the maximum amount used (Fig. 10B, lane 2). However, the addition of eIFiso4G to a binding reaction containing full-length PABP promoted the binding of PABP to the RNA, resulting in the formation of a second complex (Fig. 10B, lanes 4–6, see complex 2). This result is in excellent agreement with our previous observation that eIFiso4G promotes PABP binding to poly(A) RNA (17). In contrast, the addition of eIFiso4G to a binding reaction containing PABP-(200–651) had little effect on the binding of PABP-(200–651) to the RNA (Fig. 10B, lanes 8–10), indicating a loss of the functional interaction between these proteins when PABP lacks the major eIFiso4G binding site. These results also indicate that the second eIFiso4G binding site present in the RRM3–4 of PABP-(200–651) is not sufficient for eIFiso4G to promote binding of PABP to poly(A) RNA.

As observed previously (7, 17), eIF4B can bind poly(A) RNA and forms a complex that migrates similarly to a monomer of PABP bound to poly(A) RNA using electrophoretic mobility shift analysis. 8 and C, eIFiso4G and eIF4B, respectively, were tested for their ability to increase the RNA binding activity of full-length PABP or PABP lacking RRM1–2 (i.e. PABP-(200–651)) using electrophoretic mobility shift analysis. To maintain binding in the linear range of the assay, 25 ng of PABP or 20 ng of PABP-(200–651) were used, resulting in a single complex when either alone was bound to poly(A)RNA. In each panel, the faster migrating (i.e. monomeric) complex is referred to as Complex 1 and the slower migrating (i.e. multimeric) complex is referred to as Complex 2.

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**FIGURE 9.** Analysis of the degree of similarity between the eIFiso4G binding sites in RRM1–2 and RRM3–4. Purified GST-PABP constructs (top panel) as illustrated in Fig. 8 were used in pull-down assays to detect binding of eIFiso4G as determined by Western analysis (bottom panel).

**FIGURE 10.** eIFiso4G and eIF4B binding sites in PABP are required for each to promote PABP binding to poly(A) RNA. In A, full-length recombinant PABP and PABP-(200–651) bound poly(A)RNA using electrophoretic mobility shift analysis. 8 and C, eIFiso4G and eIF4B, respectively, were tested for their ability to increase the RNA binding activity of full-length PABP or PABP lacking RRM1–2 (i.e. PABP-(200–651)) using electrophoretic mobility shift analysis. To maintain binding in the linear range of the assay, 25 ng of PABP or 20 ng of PABP-(200–651) were used, resulting in a single complex when either alone was bound to poly(A)RNA. In each panel, the faster migrating (i.e. monomeric) complex is referred to as Complex 1 and the slower migrating (i.e. multimeric) complex is referred to as Complex 2.
of eIFiso4G to compete with eIF4B for binding with PABP was then monitored by the amount of PABP that remained bound to eIF4B. GST-tagged eIF4B was used in the binding reactions to facilitate its isolation on glutathione resin with the associated PABP. To prevent direct binding of eIFiso4G to eIF4B (23), which would obscure the detection of competition between eIFiso4G and eIF4B for PABP, GST-eIF4B-(69–527) was employed in the assay as it lacks the interaction site for eIFiso4G.

In the absence of eIFiso4G in the binding reaction, eIF4B readily bound PABP (Fig. 11, top panel, lane 1). However, the addition of increasing amounts of eIFiso4G resulted in a decrease in the amount of PABP bound to eIF4B (Fig. 11, top panel, lanes 2–6). A significant reduction of PABP bound to eIF4B was observed when the amount of eIFiso4G in the binding reaction approached two-thirds of eIF4B (e.g., Fig. 11, top panel, lane 4). These data indicate that the interaction of eIFiso4G with PABP precludes interaction of eIF4B with PABP, suggesting a competition between eIFiso4G and eIF4B in binding PABP. The observation that eIFiso4G and eIF4B compete for binding PABP is consistent with their overlapping binding sites.

**DISCUSSION**

We have investigated the degree to which the RNA binding specificity of plant PABP and its interaction with initiation factors is conserved with PABP in other eukaryotes. We show that, like its animal ortholog, the RRMs in wheat PABP exhibit different RNA binding specificities. Whereas RRM1 and RRMs of *Xenopus* PABP were capable of binding RNA as individual domains (35), only RRMs of wheat PABP are competent to bind RNA as an isolated domain. RRMs, however, exhibited specificity for purine-rich RNA, similar to RRM1 and RRMs of *Xenopus* PABP (35). In contrast, RRMs of wheat PABP bound poly(A) and poly(U) specifically, whereas RRMs of *Xenopus* PABP bound poly(G) and poly(U) (35).

Like PABP from animals and yeast, a single interaction domain for eIF4G was identified in wheat PABP (Fig. 12). However, in contrast to animal and yeast PABP where the interaction of eIF4G requires RRM1–2, wheat PABP RRMs was sufficient to interact with eIF4G. The interaction of eIFiso4G with PABP differed substantially from the interaction of eIF4G in that eIFiso4G interacted at two distinct regions (Fig. 12), whereas in mammals and yeast, both eIF4G isoforms interact with PABP at a single site (9, 32). The interaction of eIFiso4G with RRMs of PABP–2 is similar to that reported for eIF4G in animals and yeast, despite the fact that eIFiso4G is substantially smaller than eIF4G.

Residues 94–129 within PAB2 were sufficient to interact with eIFiso4G. Deletion of the loop between H2 and S4 of RRMs or deletion of the RRMs–2 linker region and S1 of RRMs resulted in a substantial loss of eIFiso4G binding, demonstrating that RRMs1 and RRMs2 both contribute to eIFiso4G binding. Like the eIFiso4G binding site in RRMs1–2, binding to RRMs–4 requires both domains, raising the possibility that eIFiso4G binds to the region within RRMs–4 corresponding to the eIFiso4G binding site in RRMs1–2, a possibility supported by the sequence and structural similarities between these two regions. However, replacing residues 94–110 (representing the loop between H2 and S4 as well as S4 of RRMs1), which was required for strong interaction with eIFiso4G, with residues 272–288 (representing the loop between H2 and S4 as well as S4 of RRMs3), substantially reduced detectable eIFiso4G binding. Moreover, replacing residues 272–288 with residues 94–110 resulted in just detectable eIFiso4G binding. These data suggest that eIFiso4G does not bind to the same region in RRMs–4 that it does in RRMs1–2. This conclusion is supported by the observation that deletion of the N-terminal two-thirds of RRMs in a RRMs–4 polypeptide (i.e., PABP-(272–393)) virtually abolished eIFiso4G binding (Fig. 9), whereas similar deletions in a RRMs1–2 polypeptide (i.e., PABP–272–81) or PABP-(94–200) did not (Fig. 7). Reducing the length of the linker region between RRMs3...
and RRM4 to a length identical to that between RRM1 and RRM2 (i.e. PABP-(200–393)(Δ290–305/K289L)) substantially reduced detectable eIFiso4G binding relative to RRM3–4, indicating that binding of eIFiso4G to RRM3–4 is more complex than its binding to RRM1–2. Residues 200–272, 272–288, and 290–305 within RRM2–3 contribute to eIFiso4G binding either directly or perhaps indirectly through protein folding.

Another difference between the eIF4G-PABP (or eIFiso4G-PABP) interaction in wheat versus that in yeast is that poly(A) RNA was not required for the interaction observed in this study, in good agreement with our previous report (7), whereas it is a prerequisite for the eIF4G-PABP interaction in yeast (11). Poly(A) RNA was also not required for the interaction between the human orthologs (9).

The interaction domain for eIF4B in human PABP has been suggested to reside within the PABC domain (18), a highly conserved domain known to interact with eIF3, Paip1, Paip2, ERD15, PC16, PCI243, and CID7 (36–41) that share a PAM2 motif necessary for the interaction with the PABC domain (38, 42, 43). Interestingly, human eIF4B does not contain a PAM2 motif. The interaction domain in wheat PABP for eIF4B lies between residues 94 and 112 in RRM1, which overlaps but is not identical with the eIFiso4G binding site (Fig. 12).

The observation that the interaction domains of eIF4B and eIFiso4G overlap suggested the possibility that these two factors compete for binding PABP. This was supported by the observation that addition of eIFiso4G to a binding reaction containing eIF4B and PABP could prevent the eIF4B/PABP interaction (Fig. 11). Thus, either eIFiso4G or eIF4B can interact with PABP but their simultaneous interaction with the same molecule of PABP at RRM1–2 is precluded. It should be noted that the ability of eIFiso4G to bind PABP within RRM3–4 raises the possibility of the simultaneous interaction of eIFiso4G and eIF4B to the same molecule of PABP but this remains to be demonstrated.

The phosphorylation state of wheat PABP is known to determine the specificity of its interaction with eIF4G, eIFiso4G, and eIF4B (17). eIF4B interacts with phosphorylated PABP more strongly than with hypophosphorylated PABP, whereas eIF4G preferentially interacts with hypophosphorylated PABP (17). eIFiso4G interacts with PABP independent of its phosphorylation state (17). PABP molecules of opposite phosphorylation states bind poly(A) RNA highly cooperatively (17), suggesting that the PABP molecules bound to a poly(A) tail are likely heterogeneous in their phosphorylation state. This would allow eIF4B and eIF4G (or eIF4B and eIFiso4G) to avoid competition in their interaction with PABP.

The role of PABP phosphorylation in determining the specificity of its interaction with eIF4G, eIFiso4G, and eIF4B, in conjunction with the overlapping eIF4G, eIFiso4G, and eIF4B interaction domains in PABP, suggests that two molecules of PABP are required to interact with eIF4G and eIF4B (or eIFiso4G and eIF4B) simultaneously. Such a possibility would involve the interaction of four molecules (i.e. eIF4B, eIF4G, and two molecules of PABP). As PABP self-interacts during its cooperative binding to poly(A) RNA (17, 30) and eIF4B binds eIFiso4G (23), the interaction among four molecules may provide greater stability to the complex than would interactions among three molecules (i.e. eIF4B, eIF4G, and one molecule of PABP). This conclusion is consistent with our observation that the combination of eIF4B and eIF4G (or eIF4B and eIFiso4G) synergistically promotes multimeric binding of PABP to poly(A) RNA (7, 17). Thus, the overlapping nature of the eIF4G, eIFiso4G, and eIF4B interaction domains in PABP may require eIF4B and eIF4G (or eIF4B and eIFiso4G) to interact with distinct molecules of PABP and thereby increase the stability of the interaction between the termini of an mRNA.

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