Translation Initiation Factors eIF-iso4G and eIF-4B Interact with the Poly(A)-binding Protein and Increase Its RNA Binding Activity*

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Hanh Le‡, Robert L. Tanguay‡, M. Luisa Balasta§, Chin-Chuan Wei§, Karen S. Browning§, Anneke M. Metz‡, Dixie J. Goss§, and Daniel R. Gallie‡

From the ¶Department of Biochemistry, University of California, Riverside, California 92521-0129, the §Department of Chemistry, Hunter College, New York, New York 10021, and the ¶Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712-1096

The 5′-cap and the poly(A) tail act synergistically to increase the translational efficiency of eukaryotic mRNAs, which suggests that these two mRNA elements communicate during translation. We report here that the cap-associated eukaryotic initiation factors (eIFs), i.e. the two isoforms of the cap-binding complex (eIF-4F and eIF-iso4F) and eIF-4B, bind to the poly(A)-binding protein (PABP) both in the presence and absence of poly(A) RNA. The interactions between PABP and eIF-4F, eIF-iso4F, and eIF-4B were measured in the absence of poly(A) RNA using far Western analysis and confirmed by direct fluorescence titration studies. The functional consequence of the interaction between these initiation factors and PABP was examined using RNA binding assays and RNA mobility shift analysis. eIF-4F, eIF-iso4F, and eIF-4B promoted PABP activity through a shift in its equilibrium affinity for poly(A). eIF-iso4G, the large subunit of eIF-iso4F, was the subunit responsible for the interaction between eIF-iso4F and PABP and was the subunit that promoted PABP RNA binding activity. Truncation analysis of eIF-iso4G indicated that a domain close to its N-terminal end appeared to be involved in binding PABP. These results suggest that the interaction between PABP and eIF-4B and eIF-iso4G may be involved in mediating the functional co-dependence observed between the cap and the poly(A) tail during translation.

Most eukaryotic mRNAs contain a cap (m7GpppN) and a poly(A) tail, which are functionally co-dependent regulators of translation (1). The cap serves as the binding site for eukaryotic initiation factor (eIF)1 4E (a subunit of eIF-4F), and the poly(A) tail binds the poly(A)-binding protein (PABP). Plants also contain an isoform of eIF-4F, called eIF-iso4F (2). The translation initiation factors eIF-4F, eIF-4B, and eIF-4A are thought to participate in the ATP-dependent binding of mRNA to the 40 S ribosomal subunit (reviewed in Refs. 3 and 4). Specifically, these factors likely catalyze the ATP-dependent unwinding of any secondary structure of the mRNA. eIF-4G, the large subunit of eIF-4F, also binds eIF-3, an initiation factor thought to be necessary for 40 S subunit recruitment to an mRNA (5).

Several studies have suggested that PABP may play a role during translation initiation: PABP has been shown to be necessary for 40 S ribosomal subunit binding to an mRNA and for formation of the 48 S initiation complex (6, 7). Moreover, the functional co-dependence observed between the cap and the poly(A) tail (1, 8) suggests that there might be an interaction between PABP and one or more of those initiation factors associated with the cap. eIF-4F and eIF-4B were implicated as playing a role in mediating the interaction between the cap and the poly(A) tail in that exogenous poly(A) added to wheat germ lysate inhibited translation, but its effect was reversed following the addition of eIF-4F and eIF-4B (9). The poly(A)-mediated sequestration of eIF-4F and eIF-4B may have been a result of their binding to the PABP-poly(A) complex or their binding directly to the poly(A) RNA itself. These studies suggest that the 3′-end of an mRNA plays a role during initiation and that PABP may interact either directly or indirectly with those initiation factors associated with the 5′-cap. In this report, we used far Western analysis, fluorescence titration, and RNA mobility shift assays to demonstrate that PABP does bind eIF-4F, eIF-iso4F, and eIF-4B either in the presence or absence of poly(A) and that their interaction increases PABP RNA binding activity.

MATERIALS AND METHODS

Plasmid Constructs and In Vitro RNA Synthesis—The T7-based construct containing the (A)30 tract has been described previously (1). The DNA concentration was quantitated spectrophotometrically following linearization and brought to 0.5 mg/ml. In vitro transcription was carried out as described previously (10) using 40 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 100 μg/ml BSA, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM GTP, 10 mM DTT, 0.3 unit/ml RNasin (Promega), and 0.5 unit/ml T7 RNA polymerase. Radiolabeled probes were made as uncap RNAs using [α-32P]ATP. The full-length transcripts were resolved and electroeluted from 4% polyacrylamide gels, and RNA yields were quantitated spectrophotometrically.

Protein Purification—For all steps in its purification (11), PABP activity was followed using binding to poly(A) RNA as determined by complex formation in a mobility shift assay. Briefly, the 25–60% saturated ammonium sulfate fraction of wheat germ extract was applied to Affi-Gel blue (Bio-Rad) equilibrated in Buffer A (100 mM KAc, 1 mM CaCl2, 1 mM MgAc, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT). The column was washed with Buffer A containing 4 mM NaCl. PABP was eluted with Buffer A containing 2 mM guanidine HCl. The eluted protein was dialyzed overnight against several changes of Buffer A containing 50 mM KAc. The dialyzed sample was applied to a Mono Q column equilibrated in Buffer A containing 50 mM KAc. The effluent from the Mono Q column was collected and applied to a poly(A)-Sepharose 4B affinity column equilibrated in Buffer A. PABP was eluted with Buffer A containing 1 mM urea and 2 mM LiCl and dialyzed extensively.

Wheat germ eIF-4F and eIF-iso4F (12), eIF-4B (13), and eIF-2 and
eIF-3 (14) were purified as described. Recombinant eIF-iso4G and eIF-iso4E were purified as described (15).

**Far Western Assay**—200 ng of BSA, PABP, or initiation factors in 25 mM Tris (pH 7.5), 1 mM MgAc, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, and 1 mM DTT was applied to a nitrocellulose membrane using a slot-blot apparatus. The membrane was blocked with 10 mg/ml nonfat milk protein in buffer at room temperature for 40 min. The membrane was then probed for 90 min with 3 μg/ml purified PABP or with a fraction derived from either wheat embryos or wheat leaves that was enriched for PABP. The membrane was washed three times in 10 mg/ml BSA, dried, and blocked in 5% nonfat dry milk for 30 min. The membrane was incubated with anti-wheat PABP antibodies, rinsed, and incubated with goat anti-rabbit antibodies (for the detection of PABP) or anti-mouse antibodies (for the detection of eIF-iso4E) conjugated with horseradish peroxidase. The signal was visualized using chemiluminescence detection.

**Fluorescence Studies**—All solutions for fluorescence were prepared in 25 mM Tris (pH 7.5) containing 100 mM KAc, 1 mM DTT, 1 mM CaCl₂, 1 mM MgCl₂, and 10% glycerol. The formation of the binary protein-protein complexes was studied by direct fluorescence titration. A solution of purified PABP (0.6–0.8 μM) was titrated with increasing amounts of one of the other proteins, eIF-4F, eIF-iso4F, eIF-4B, or eIF-4A. In some experiments, T2 RNase was present (5 units). All fluorescence measurements were performed with a Spex 11 spectrophluorometer. The excitation wavelength was 280 nm; emission was monitored at 310 or 320 nm; and the slit widths were 1.5 mm for both excitation and emission. The normalized fluorescence difference (ΔF/ΔF₀) between the protein-protein complex and the sum of the individual fluorescence spectra was used to determine the equilibrium association constant (Kᵣ). The details of the data fitting are described elsewhere (16).

**Mobility Shift Assay**—For all gel shift assays, radiolabeled (Δν) RNA was synthesized in vitro and gel-purified to remove the DNA template, unincorporated nucleotides, and less than full-length RNA. 1 ng of radiolabeled RNA and the indicated amount of protein were used for the binding reactions in a 15-μl volume containing 25 mM Tris (pH 7.5), 1 mM MgAc, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, 1 mM DTT, 0.5 mM MgCl₂, and 2 μl of 2.5 mg/ml heparin were added, and the reaction was incubated for an additional 10 min at 0 °C. The RNA-protein complexes were resolved on a native 4% polyacrylamide gel with a 10% acrylamide stacking gel. The gel-purified complexes were then probed for the presence of bound PABP (0.6–0.8 μM) with crude wheat germ extract or with a recombinant eIF-iso4G. In some experiments, T2 RNase was present (5 units). All fluorescence measurements were performed with a Spex 11 spectrophluorometer. The excitation wavelength was 280 nm; emission was monitored at 310 or 320 nm; and the slit widths were 1.5 mm for both excitation and emission. The normalized fluorescence difference (ΔF/ΔF₀) between the protein-protein complex and the sum of the individual fluorescence spectra was used to determine the equilibrium association constant (Kᵣ). The details of the data fitting are described elsewhere (16).

**RESULTS**

PABP Binds to eIF-4F, eIF-iso4F, and eIF-4B—To investigate whether the cap-associated initiation factors (eIF-4F, eIF-iso4F, eIF-4B, and eIF-4F) interact with PABP, we used far Western analysis, which assays for protein/protein interactions. eIF-4F and eIF-iso4F can be purified from wheat germ as a two-subunit (no eIF-4A) or three-subunit (containing eIF-4A) complex depending on the purification scheme employed (2, 17). eIF-iso4E that was present in an extract has undergone SDS-PAGE. The membrane was then incubated with crude wheat germ extract or with a solution of 10 mg/ml BSA. Following incubation with the extract, the membranes were washed and probed with anti-eIF-iso4E antibodies, and the signal was determined using chemiluminescence. A positive signal for eIF-iso4E indicates that eIF-iso4E that was present in an extract has bound to the immobilized eIF-iso4G. Following incubation of the membrane with wheat germ extract, eIF-iso4E was detected bound to eIF-iso4G, but not to the eIF-4A or BSA control (Fig. 1A, top panel). Similar results were obtained when wheat leaf extract was used instead of wheat germ extract (Fig. 1A, middle panel). eIF-iso4E was not detected when the membrane was incubated with BSA (Fig. 1A, bottom panel), demonstrating that the eIF-iso4E signal observed in the top and middle panels is not due to cross-reaction between the anti-eIF-iso4E antibodies and recombinant eIF-iso4G.

To examine whether eIF-4B, eIF-4F, or eIF-iso4F could selectively bind PABP when presented with a complex protein solution from wheat germ, 200 ng of each purified factor was immobilized onto a nitrocellulose membrane and incubated with a PABP-containing fraction from wheat germ. As before, eIF-4A and BSA were used as negative controls. In another control, a membrane to which a purified factor was bound was incubated with milk protein instead of wheat germ extract. Both the test and control membranes were then probed with anti-wheat PABP antibodies, and the signal was revealed using chemiluminescence. PABP was detected bound to eIF-4B (Fig. 1B, top panel), eIF-iso4F (middle panel), and eIF-iso4G (bottom panel), but not to the eIF-4A or BSA control. No PABP was detected when eIF-4B, eIF-4F, or eIF-iso4F was incubated with milk protein, demonstrating that these purified factors are free of PABP and that the anti-PABP antibodies do not cross-react with the factors.

![Fig. 1. Far Western analysis of eIF-iso4E and eIF-iso4G binding (A) and the interaction between PABP and eIF-4B, eIF-4F, and eIF-iso4F (B). 200 ng of each protein indicated was immobilized on nitrocellulose membranes using slot blotting. In each case, the membranes were then incubated with crude wheat germ extract (top panel), wheat leaf extract (middle panel, or BSA (bottom panel). The membranes were probed for bound PABP using Western analysis with mouse anti-eIF-iso4E antibodies. In B, the membranes were incubated with a wheat germ fraction enriched for PABP. Each incubation factor present in the last lane of each panel was incubated with milk protein instead of wheat germ extract. The membranes were then probed for the presence of bound PABP using anti-PABP antibodies. The Western signal was detected by chemiluminescence.](image-url)
Interaction between eIF-iso4G and eIF-4B and PABP

200 ng of each protein indicated was immobilized onto nitrocellulose membranes using slot blotting. The membranes were incubated with 3 μg/ml purified PABP (A), 3 μg/ml purified PABP with 0.3 μg/ml poly(A) RNA (B), or 3 μg/ml purified PABP and 40 units/ml T2 RNase (C). The membranes were then probed for the presence of bound PABP using Western analysis with anti-PABP antibodies. The Western signal was detected by chemiluminescence.

could not be addressed when crude wheat germ extract was used as the source of PABP. To examine the requirement for poly(A) in the interaction between PABP and the initiation factors, PABP was purified from wheat germ to near homogeneity (11) and used in the far Western analysis. As the purification procedure for PABP involved the denaturation of PABP following Affi-Gel blue chromatography and the removal of poly(A) RNA on subsequent purification using a Mono Q column, the PABP used in these assays should be free of poly(A). However, whether residual RNA remained in the purified PABP was determined directly by subjecting the PABP preparation either to 3'-end labeling using [32P]pCp and RNA ligase or to 5'-end labeling following phosphatase treatment. No nucleic acid was detected in 2 μg of PABP (detection limit of 50 pg) following the resolution of either end-labeled reaction on a sequencing gel (data not shown). Similar results were obtained with eIF-4B, eIF-4F, and eIF-iso4F, indicating that nucleic acids are not present in these preparations.

To examine whether purified poly(A)-free PABP could bind to the cap-associated initiation factors, 200 ng of purified eIF-4B, eIF-4F, eIF-iso4F, eIF-iso4G, and eIF-iso4E was immobilized onto a nitrocellulose membrane. eIF-4A and BSA were again included as negative controls, and purified PABP was included as a positive control for the Western analysis. The membrane was then incubated with 3 μg/ml purified PABP. Following probing with the anti-wheat PABP antibodies, PABP was detected bound to eIF-4B, eIF-4F, eIF-iso4F, and recombinant eIF-iso4G, but not to eIF-iso4E or eIF-4A (Fig. 2A). PABP appeared to bind more strongly to eIF-4B, eIF-4F, and eIF-iso4F than to recombinant eIF-iso4G; however, the far Western assay is, like Western assays in general, only semiquantitative, and differences in the extent of binding must be viewed cautiously. These results with purified PABP confirm the results obtained with crude wheat extract and suggest that PABP interacts with the cap-associated factors even in the absence of poly(A). These results also indicate that it is the eIF-iso4G subunit of eIF-iso4F that interacts with PABP. However, to more rigorously establish the requirements for poly(A) in the interaction between PABP and the cap-associated factors, far Western assays were performed in the presence of poly(A) RNA or in the presence of T2 RNase (Fig. 2, B and C, respectively).

The far Western analysis was carried out exactly as described for Fig. 2A, except that 3 μg/ml poly(A) RNA was added to the PABP incubation solution of the membrane in Fig. 2B and 40 units/ml T2 RNase was added to the PABP incubation solution of the membrane in Fig. 2C. The presence of T2 RNase did not affect the binding of PABP to eIF-4B, eIF-4F, eIF-iso4F, or recombinant eIF-iso4G (Fig. 2C), confirming that PABP binding to the factors occurs in the absence of poly(A) and that when a stoichiometric amount of poly(A) was included in the incubation reaction, PABP binding to the factors was not significantly affected (Fig. 2B).

**Determination of the Kd for PABP/eIF-4B, PABP/eIF-4F, and PABP/eIF-iso4G by Direct Fluorescence Titration**—To establish the equilibrium binding constant (expressed as Kd) for PABP and either eIF-4B, eIF-4F, eIF-iso4F, or eIF-4A, direct fluorescence titration studies were performed using purified PABP and initiation factors. The natural fluorescence of PABP is altered following the binding of a tightly associated protein. A solution of PABP can therefore be titrated with increasing amounts of a PABP-binding protein, and the shift in fluorescence can be used to calculate the Kd. Such a fluorescence spectrum resulting from the presence of PABP and eIF-4B in the same cuvette is shown in Fig. 3 and is compared with the sum of the fluorescence spectra of separate samples of PABP and eIF-4B at the same concentration as that used in the mixed sample (Fig. 3A). The spectrum of the PABP-eIF-4B complex not only shows an increase in fluorescence intensity, but also shows a shift in emission maximum of ~8 nm, which demonstrates that an interaction is occurring between the two proteins. Similar spectra were obtained for PABP-eIF-4F and PABP-eIF-iso4F. Following the titration of PABP with eIF-4B, eIF-4F, or eIF-iso4F, the difference between the fluorescence obtained from the combination of PABP and each factor and the sum of their individual fluoroscences can be plotted as a function of the factor used for the titration. An example of this is shown in Fig. 3B for PABP and eIF-4F and in Fig. 3C for PABP and eIF-iso4F, which are plots of the normalized difference between the fluorescence of PABP/eIF-4F or PABP/eIF-iso4F and the sum of the individual fluoroscences of PABP and eIF-4F or PABP and eIF-iso4F. From such analyses, the equilibrium binding constant can be calculated (16) for the interactions between PABP and eIF-4B, PABP and eIF-4F, and PABP and eIF-iso4F (Table 1). All three of these initiation factors showed a strong interaction with PABP in the absence of poly(A). No interaction was detected between PABP and eIF-4A. Direct fluorescence titrations were also performed in the presence of T2 RNase to ensure that no intact RNA was present. The binding affinity between PABP and each of these initiation factors in the presence of T2 RNase was identical to that observed in its absence. The equilibrium binding constant calculated for each of the initiation factors with PABP is about an order of magnitude stronger than the Kd determined for the interaction between eIF-4A and eIF-iso4F in the presence of ATP or 2 orders of magnitude stronger than the eIF-4A/eIF-iso4F interaction in the absence of ATP (20). In addition, the PABP/eIF interactions are approximately an order of magnitude stronger than the interaction between eIF-4F and eIF-4B.2 These data confirm the results obtained from the far Western analyses, and the calculated equilibrium binding constants indicate that the interaction between PABP and eIF-4B, eIF-4F, or eIF-iso4F is quite strong and likely to be physiologically relevant.

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2 M. L. Balasta and D. J. Goss, unpublished data.
consequently, the binding complexes, designated complexes A and B, were observed when PABP bound (A)50 (Fig. 4, lane 2) and are characteristic of the PABP/(A)50 interaction. Complex B was seen only upon PABP binding to poly(A) and was not observed for eIF-4B, eIF-4F, or eIF-iso4F (Fig. 4, lanes 3–6 and lanes 12–16). As the packing density for PABP is one molecule bound for every 25 adenosine residues, the (A)50 RNA used in these studies should be long enough to accommodate two molecules of PABP. Complex B may represent a single bound PABP, and complex A may represent two PABPs bound to (A)50.

Previous studies demonstrated that eIF-4F, eIF-iso4F, and eIF-4B bind poly(A) specifically (9), and the preference of eIF-4B for poly(A) was confirmed recently with the mammalian homolog (21). Although PABP/(A)50 complex formation can be detected when as little as 1–2 ng of PABP is used in the binding reaction, higher levels of eIF-4F, eIF-iso4F, and eIF-4B are needed to detect their binding to poly(A). The $K_{d(app)}$ (i.e. apparent $K_d$ as determined by gel shift analysis) for wheat PABP is $10 \text{ nM}$ (11), which is similar to the values of 5 nM determined for yeast PABP (22) and 7 nM measured for human PABP (23). Not surprisingly, PABP exhibits a greater affinity for poly(A) than does eIF-4B ($K_{d(app)} = 55 \text{ nM}$), eIF-4F ($K_{d(app)} = 400 \text{ nM}$), or eIF-iso4F ($K_{d(app)} > 400 \text{ nM}$) (9). To maintain the gel shifts in the linear range of the assay, it is necessary that no more than 50% of the poly(A) RNA in any binding reaction be complexed with protein. Consequently, the binding complexes formed between 1 pmol of eIF-4F or eIF-iso4F, which is 10-fold less than that used in our previous study (9), and (A)50 are not quite visible in the exposure in Fig. 4 (lanes 3 and 13, respectively), but eIF-4B binding to (A)50 can be seen as a faint complex (lanes 4 and 14). Complex formation occurred to a greater extent when both eIF-4F and eIF-4B were present in the binding reaction (Fig. 4, lane 5) relative to the complexes formed by each factor individually (lanes 3 and 4, respectively), eIF-4A did not bind poly(A) to a detectable level either here (Fig. 4, lane 1) or in previous studies (9, 24). Although eIF-4A exhibits weak RNA binding activity as detected by direct fluorescence titration (20), its affinity for RNA in that analysis was...
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FIG. 4. eIF-4B, eIF-4F, and eIF-iso4F increase PABP binding activity. Purified factors were tested individually and in combination in binding reactions containing 0.035 pmol of PABP protein and 1 ng of (A)\(_{\text{poly}}\) and tested in the PABP/poly(A) mobility shift assay. BSA was added to the binding reactions to maintain an equivalent amount of protein. 1 pmol of eIF-4F or eIF-iso4F, 6.8 pmol of eIF-4A, and 0.1 pmol of eIF-4B were used in the binding reaction as indicated. Two complexes (designated complexes A and B) are characteristic of the PABP protein/poly(A) binding reaction, whereas one complex (complex A) is characteristic of the pattern resulting from the binding of the cap-associated factors to poly(A). PhosphorImage analysis of the complexes was used to determine the extent of complex formation.

An order of magnitude less than that measured for eIF-iso4F. The addition of eIF-4A to eIF-4F/eIF-4B in the binding reaction only slightly increased complex formation relative to the combination of eIF-4F and eIF-4B (Fig. 4, compare lane 6 with lane 5). The eIF-4F/poly(A), eIF-iso4F-poly(A), and eIF-4B/poly(A) complexes migrated at approximately the same position on these native gels, in agreement with our previous study (9). The migration of proteins on a native polyacrylamide gel is determined by both their size and charge. eIF-4B and eIF-iso4F are similar in molecular mass and are highly basic proteins, whereas eIF-4F is larger, but is also less basic (25), which may explain the similarity in the positions of the complexes. The greatest extent of complex formation with poly(A) was observed when the cap-associated initiation factors were added to a binding reaction containing PABP (Fig. 4, lane 7). The addition of the cap-associated factors resulted in the appearance of a shifted band of lower mobility above the position where the PABP-poly(A) complex migrated (Fig. 4, compare the bands of complex A in lanes 7–10 with those in lane 2), which may represent the PABP-initiation factor complex. Omission of eIF-4A (Fig. 4, lane 8), eIF-4F (lane 9), or eIF-4B (lane 10) resulted in a decrease in complex formation, particularly of the upper band in complex A that forms when the initiation factors are present in the PABP/poly(A) binding reaction. Similar results were obtained when eIF-iso4F was substituted for eIF-4F in the binding reactions (Fig. 4, lanes 11–20), although the stimulation of binding afforded by eIF-iso4F was less than that observed in the experiments using eIF-4F. No substantial stimulation of PABP/poly(A) complex formation was observed when purified eIF-2, eIF-3, or elongation factor 1a or 2 was added to PABP/poly(A) binding reactions containing the cap-associated initiation factors, and no difference in binding was observed when (A)\(_{\text{poly}}\) RNA containing a cap at the 5’-terminus was used instead of uncapped (A)\(_{\text{poly}}\) RNA in the mobility shift assay (data not shown). These data suggest that the combination of eIF-4F (or eIF-iso4F) and eIF-4B has a greater stimulatory effect on PABP/poly(A) complex formation than do these factors when present individually with PABP.

As a combination of the initiation factors promoted complex formation between PABP and poly(A), eIF-4F, eIF-iso4F, eIF-4B, or eIF-4A was tested in the PABP/poly(A) binding reactions to determine their individual effect on PABP RNA binding activity. eIF-4A did not bind directly to poly(A) (Fig. 5, lane 1) and did not have a significant effect on PABP binding to poly(A) (compare lanes 2 and 3). When both eIF-4B and PABP were present in the binding reaction, the amount of poly(A) bound was greater than the sum of their individual binding affinities (Fig. 5, compare lane 4 with lanes 3 and 5). The presence of both eIF-4F and PABP in the binding reaction also resulted in a substantially greater amount of poly(A) being bound than the sum of their individual binding affinities (Fig. 5, compare lane 7 with lanes 6 and 8). Similar results were observed when eIF-iso4F was substituted for eIF-4F (Fig. 5, compare lane 9 with lanes 8 and 10), although as observed above, eIF-iso4F stimulated PABP binding to poly(A) to a lesser extent than did eIF-4F. These results indicate that eIF-4B, eIF-4F, and eIF-iso4F, but not eIF-4A, interact individually with PABP to increase its RNA binding activity.

eIF-4F and eIF-iso4F are composed of a small subunit (eIF-4E and eIF-iso4E, respectively) and a large subunit (eIF-4G and eIF-iso4G, respectively). To determine which subunit of eIF-iso4F is responsible for the increase in PABP binding to poly(A), eIF-iso4E and eIF-iso4G were overexpressed and purified from E. coli and tested separately in PABP/poly(A) RNA binding reactions (the complete cDNA for eIF-4G has not been isolated, and therefore, recombinant eIF-4G could not be tested). The addition of eIF-iso4G stimulated PABP binding to poly(A) in a dose-dependent fashion (Fig. 6, top panel, lanes 3–6). No increase in PABP binding to poly(A) was observed when BSA was substituted for eIF-iso4G, and no direct binding of eIF-iso4G to poly(A) was observed (Fig. 6, bottom panel, lanes 5–8). The lack of detectable poly(A) binding by recombinant eIF-iso4G may be due to the absence of the post-translational modifications that it normally undergoes when expressed in wheat (25). eIF-iso4E alone did not bind directly to (A)\(_{\text{poly}}\) (Fig. 6, bottom panel, lanes 1–4), and it did not stimulate PABP binding to poly(A) over the wide range of eIF-iso4E concentrations tested (middle panel, lanes 3–6). eIF-4E also failed to stimulate PABP binding to poly(A) (data not shown). These data suggest that eIF-iso4G, and not eIF-iso4E, specifically enhances PABP binding activity and are in agreement with the specific binding of PABP to eIF-iso4G in the far Western assay (see Fig. 2).

The molar range over which eIF-4F, eIF-iso4F, and eIF-4B enhance PABP RNA binding activity was examined next. In the data presented in Fig. 4, the molar ratio of eIF-4F/eIF-iso4F to PABP in the binding reaction was 28:1, whereas the ratio of eIF-4B to PABP was 2:8:1. This is assuming that only one molecule of PABP is bound to the poly(A) RNA. If two molecules of PABP are bound, the ratio drops to 14:1 for eIF-4F/eIF-iso4F:
PABP and 1.4:1 for eIF-4B:PABP. This molar ratio resulted in a substantial increase in PABP/poly(A) binding relative to the reaction containing PABP alone (Fig. 7, compare the third lane with the second lane). As the molar ratio of the initiation factors decreased in 2-fold increments with respect to PABP, there was a commensurate decrease in the stimulation of PABP binding afforded by these factors. However, substantial increases in poly(A) RNA binding activity were observed even when in the range of 0.35:1 for eIF-4B:PABP and 3.5:1 for eIF-4F/eIF-iso4F:PABP (Fig. 7, sixth lane), assuming only one molecule of PABP bound to the (A)50 RNA, and stimulation was observed at even lower ratios of the initiation factors to PABP.

To examine whether eIF-iso4G and eIF-4B enhanced PABP RNA binding activity over a wide range of PABP concentrations, a series of binding reactions was performed in which the level of the initiation factor was held constant and the amount of PABP present was varied from 0 up to 30 ng in the reaction. In the absence of PABP, no detectable binding was observed between (A)50 RNA and the level of eIF-iso4G (Fig. 8A, lane 1) or eIF-4B (Fig. 8C, lane 1) used in the reaction. The presence of eIF-iso4G (Fig. 8B, lane 2) or eIF-4B (Fig. 8C, lane 2) enhanced the binding of 0.9 ng of PABP to poly(A) relative to the same amount of PABP in the absence of either initiation factor (Fig. 8A, lane 2). Similar enhancement afforded by eIF-iso4G and eIF-4B was observed throughout the range of PABP concentrations tested until >50% of the poly(A) RNA had been bound and the linear range of the assay exceeded. These data suggest that eIF-iso4G and eIF-4B enhance PABP RNA binding activity over a wide molar range.

eIF-4F, eIF-iso4F, and eIF-4B Increase PABP Equilibrium Binding by Decreasing the Rate of PABP Dissociation from Poly(A)—To determine the mechanism by which eIF-4F, eIF-iso4F, and eIF-4B increase PABP RNA binding activity, we followed the effect of these factors on the binding kinetics of PABP to (A)50. For the dissociation experiments, binding reactions containing PABP and (A)50 in the presence or absence of each initiation factor were allowed to reach equilibrium, at which point, the reactions were challenged with an excess of...
unlabeled poly(A) RNA. Aliquots were then removed at time points and resolved on a native polyacrylamide gel. In the presence of recombinant eIF-iso4G, not only was the extent of complex formation enhanced (Fig. 9B), as observed in Fig. 6, but binding reached equilibrium within 20 s, whereas up to 10 min was required for PABP(poly(A) complex formation to reach equilibrium in the absence of eIF-iso4G as determined by mobility shift analysis (data not shown). In the dissociation reactions, the presence of eIF-iso4G decreased the rate of PABP dissociation from (A)50 (Fig. 9, compare B and A). Similar results were obtained for eIF-4B, and the kinetics of PABP dissociation from labeled poly(A) within 1 min following the addition of unlabeled poly(A), whereas a reduction in the rate of dissociation was observed when either eIF-iso4G or eIF-4B, but not eIF-4A, was present in the binding reaction.

Mapping the PABP Interaction Domain within eIF-iso4G—The domains within eIF-iso4G responsible for binding eIF-iso4E and eIF-4A and the domains required for ATP hydrolysis and polypeptide synthesis have been identified using recombinant truncated eIF-iso4G proteins purified from E. coli (19). These same eIF-iso4G truncation mutations were overexpressed and purified to near homogeneity from E. coli (19). The truncated eIF-iso4G proteins were then added to PABP/poly(A) binding reactions to determine which eIF-iso4G deletion mutants were affected in their ability to interact and promote PABP RNA binding activity (Fig. 11A). As seen in Figs. 7–9, the addition of purified recombinant eIF-iso4G increased PABP binding to poly(A) (Fig. 11A, compare lane 3 with lane 2). Deletion of the N-terminal 52 amino acids from eIF-iso4G had a small effect on its ability to increase PABP activity, and the deletion of the N-terminal 90 amino acid residues significantly reduced its ability to promote PABP RNA binding activity. Some residual activity remained in the N90, N136, and N186 deletion mutants, which might suggest that the N-terminal domain may be necessary but not sufficient to promote PABP binding activity. It is also possible that changes in protein structure caused by these truncations may indirectly contribute to the extent to which eIF-iso4G binds to PABP. Deletions from the C-terminal end to positions 462, 489, and 511 (eIF-iso4G is 787 amino acids in length) did not reduce its ability to increase PABP binding activity using mobility gel shift assays. B, each truncated eIF-iso4G protein was tested for its ability to bind directly to poly(A) RNA.

Several recent studies have suggested that an interaction between the 5’- and 3’-termini of an mRNA is important for binding activity. In this study, we investigate the role of the C-terminal domain of eIF-iso4G in promoting PABP binding activity using mobility gel shift assays. A, each truncated eIF-iso4G protein was tested for its ability to enhance PABP binding activity using mobility gel shift assays. B, each truncated eIF-iso4G protein was tested for its ability to bind directly to poly(A) RNA.

**DISCUSSION**

Several recent studies have suggested that an interaction between the 5’- and 3’-termini of an mRNA is important for...
efficient translation initiation (1, 6–9). Such an interaction could be indirect or could involve the direct interaction between those proteins bound to the cap and the poly(A) tail. In this study, we have presented evidence from three independent approaches that suggests that the cap-associated factors, i.e. eIF-4F, eIF-iso4F, and eIF-4B, can interact with PABP. Although it remains to be determined to what extent this interaction occurs in vivo, the data suggest that these interactions might form the basis for the functional dependence between the cap and the poly(A) tail during translation.

We observed PABP binding to eIF-4F, eIF-iso4F, or eIF-4B using a far Western assay and direct fluorescence measurements. In contrast, no binding was detected between PABP and eIF-4A or between PABP and BSA, suggesting that the interactions between the cap-associated factors and PABP are specific. Moreover, binding between the cap-associated initiation factors and PABP was stronger than the protein/protein interactions between the initiation factors themselves (e.g. between eIF-4F and eIF-4A or between eIF-4F and eIF-4B). The equilibrium binding constant calculated for PABP and either eIF-4B, eIF-4F, or eIF-iso4F was stronger than that determined for eIF-4A and eIF-iso4F by 2 orders of magnitude (20) and was stronger than the interaction between eIF-4F and eIF-4B by an order of magnitude. Although the fluorescence analysis was carried out in the absence of poly(A), far Western analysis detected little difference in the interaction between PABP and the initiation factors when poly(A) was present or not. Mobility shift analysis suggested that a functional consequence of the interaction between PABP and eIF-4B, eIF-4F, or eIF-iso4F is a decrease in the rate of PABP dissociation from poly(A). Although these factors also appeared to increase the rate of PABP/poly(A) association, in a result of a decrease in the rate of PABP/poly(A) dissociation. eIF-iso4G was the subunit of eIF-iso4F responsible for interaction and promotion of PABP RNA binding activity. Using deletion analysis, the domain within eIF-iso4G that was required for this interaction with PABP mapped to the N-terminal region. This region also contains the eIF-iso4E-binding domain and may interact with eIF-4A (19), observations that suggest that this region of eIF-iso4G may be primarily involved in protein/protein interactions. As deletions within a protein may also affect protein/protein interactions through changes in protein folding, more detailed mutational analysis of the N-terminal region will be necessary to precisely delineate the interaction domain(s).

It was important to establish that PABP was not a contaminant in the preparations of the initiation factors used in this study. Several lines of evidence show that the eIF-4B, eIF-4F, and eIF-iso4F used in this study were free of PABP. Anti-wheat PABP antibodies did not detect PABP in the purified eIF-4B, eIF-iso4F, and eIF-4B used for far Western analysis (Fig. 1) or in enzyme-linked immunosorbent assays (data not shown). The binding complex formed by eIF-4B, eIF-4F, or eIF-iso4F with poly(A) did not result in the formation of any complex B, which is characteristic of the presence of PABP protein. Finally, recombinant eIF-iso4G overexpressed and purified from E. coli rules out the possibility of any copurification with PABP. It was also necessary to establish that the protein preparations did not contain RNA. RNA was not detected in the protein preparations following either 5' or 3'-end labeling. If poly(A) were required for the interaction between PABP and the initiation factors detected by fluorescence analysis, nearly stoichiometric amounts would need to be present, which would have been easily detected by the RNA end labeling and which would have been sufficient to prevent PABP binding to the radiolabeled (A)50 RNA used in the mobility shift assays. Moreover, the inclusion of T2 nuclease did not alter the interaction between the initiation factors and PABP as determined by fluorescence titration and far Western analysis.

The effect of eIF-4B, eIF-4F, and eIF-iso4G on PABP was specific as no interaction was observed between PABP and eIF-4A, eIF-4E, or eIF-iso4E. Although it is possible that eIF-4F, eIF-iso4F, or eIF-4B binds directly to poly(A) in the PABP/poly(A) complex, the affinity of eIF-4B, eIF-4F, or eIF-iso4F for poly(A) is considerably less than that of PABP. Therefore, the interaction between eIF-4B, eIF-4F, and eIF-iso4F and PABP is more likely to result in an increase in PABP RNA binding activity. This conclusion is supported by the observation that purified recombinant eIF-iso4G, which itself does not exhibit detectable binding to poly(A) under the conditions used in the mobility shift assay, increases PABP binding to poly(A).

The PABP/A50 complex was quantitatively shifted to a lower mobility (the upper band of complex A in Fig. 4) when eIF-4F, eIF-iso4F, eIF-iso4G, or eIF-4B was present. This was most clearly seen when a combination of the initiation factors was present in the binding reaction. PABP was detected in both bands, whereas the presence of eIF-4B was detected only in the band of lower mobility (the upper band of complex A) when the shifted complexes were transferred to membrane and probed with anti-PABP or anti-eIF-4B antibodies, respectively (data not shown). However, Western analysis did not detect eIF-4F and eIF-iso4F in the PABP-containing complexes, suggesting that these factors dissociate from the complex during electrophoresis. Similar conclusions have been reached with several DNA-binding proteins involved in the assembly of transcriptional regulatory complexes at their target DNA. The paired-like homeobox protein (Phox1) enhances the binding of the serum response factor to the serum response element through protein/protein interactions, but does not result in an alteration in the mobility of the serum response factor-serum response element complex during PAGE and is thought to dissociate from the complex during electrophoresis (27). The Tax protein of human T-cell lymphotropic virus type 1 activates expression of the human T-cell lymphotropic virus type 1 long terminal repeat through protein/protein interactions with cellular CREB (cAMP-regulated enhancer-binding protein) to promote CREB binding to its target DNA (cAMP response element). As with the Phox1/serum response factor interaction, Tax increases CREB-cAMP response element complex formation, but does not alter the mobility of the complex as it also dissociates from the complex during PAGE (28–31). Although eIF-4B remains associated with the PABP/poly(A) complex, eIF-4F and eIF-iso4F appear to dissociate from the PABP/poly(A) complex during electrophoresis. Nevertheless, the fluorescence titration studies and far Western analysis confirm that eIF-4F and eIF-iso4F do bind PABP in solution.

How then may eIF-4B, eIF-4F, and eIF-iso4F increase PABP RNA binding activity? They may induce a conformational change in PABP or promote its multimerization, which results in a decrease in the rate of dissociation of PABP from poly(A), as has been shown for the effect of Tax on CREB binding activity (29, 31) or for the effect of Phox1 on serum response
factor binding activity (27). Previous studies have suggested that PABP remains as a monomer in the absence of poly(A), but multimerizes on binding to poly(A), and requires the PABP C-terminal domain to do so (32). Any conformational change that would favor PABP binding to poly(A) or increase PABP multimerization could explain the initiation factor-mediated increase in the equilibrium binding of PABP to poly(A).

What purpose does the interaction between the cap-associated initiation factors and PABP serve during translation initiation? One possibility is that their interaction stabilizes PABP/initiation factor binding to an mRNA as a means to promote efficient translation. The initiation factor-mediated increase in PABP/poly(A) equilibrium binding supports this possibility. Another consequence of such an interaction might be to confer a translational advantage to those mRNAs containing both a cap and a poly(A) tail and would serve as a selection mechanism that screens for only intact mRNAs. A third possibility may be to promote re-initiation through the physical proximity of the termini. Recent studies on mRNA degradation have also suggested that an interaction between PABP and the 5′-terminus is important to maintain the integrity of an mRNA. For example, during the turnover of MFA2 mRNA in yeast, deadenylation is followed by decapping (33), and the presence of poly(A)-bound PABP prevents decapping of the mRNA (34) by DCP1, the decapping enzyme (35). These observations suggest that, as in translation initiation, mRNA turnover may depend on the interaction between the termini of an mRNA. The interaction between PABP and the cap-associated initiation factors may therefore play a role in both translation initiation and mRNA turnover regulation.

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