Eukaryotic initiation factor (eIF) 4G is an integral member of the translation initiation machinery. The molecule serves as a scaffold for several other initiation factors, including eIF4E, eIF4AI, the eIF3 complex, and poly(A)-binding protein (PABP). Previous work indicates that complexes between these proteins exhibit enhanced mRNA cap-binding and RNA helicase activities relative to the respective individual proteins, eIF4E and eIF4A. The eIF4G-PABP interaction has been implicated in enhancing the formation of 48 S and 80 S initiation complexes and ribosome recycling through mRNA circularization. The eIF3-eIF4GI interaction is believed to forge the link between the 40 S subunit and the mRNA. Here we have investigated the behavior in vitro and in intact cells of eIF4GIf molecules lacking either the PABP-binding site, the eIF3-binding site, the middle domain eIF4A-binding site, or the C-terminal segment that includes the second eIF4A-binding site. Although in some cases the mutant forms were recruited more slowly, all of these eIF4G variants could form complexes with eIF4E, enter 48 S complexes and polyosomes in vivo and in vitro, and partially rescue translation in cells targeted with eIF4GI short interfering RNA. In the reticulocyte lysate, eIF4G unable to interact directly with PABP showed little impairment in its ability to support translation, whereas loss of either of the eIF4A-binding sites or the eIF3-binding site resulted in a marked decrease in activity. We conclude that there is considerable redundancy in the mechanisms forming initiation complexes in mammalian cells, such that many individual interactions have regulatory rather than essential roles.

Eukaryotic initiation factor (eIF) 3 4GI has an important role in translation initiation. It serves as a scaffold protein, allowing multiple other initiation factors to assemble at the 5′ cap of an mRNA molecule and promote its recruitment by the small ribosomal subunit. Its binding partners include the cap-binding protein eIF4E, the RNA helicase eIF4A, poly(A)-binding protein (PABP), the eIF4E kinase, Mnk, and eIF3, which in turn interacts with the 40 S ribosomal subunit (for review see Ref. 1). These cooperative interactions enhance the affinity of eIF4E for the mRNA cap (2–4) and increase the helicase and ATPase activities of eIF4A (5–7). Five isoforms of eIF4GI that differ in their N termini have been identified, arising by alternative translation initiation at different AUG codons (8, 9). It has been shown that the two longest isoforms (designated eIF4GI If and eIF4GIe, according to Ref. 8) are the most abundant in cells. The shortest isoform (designated eIF4Gia, also the first cloned (10)) lacks the sequence near the N terminus of eIF4GI that interacts with PABP (11, 12).

The mammalian eukaryotic initiation factor 3 complex, consisting of 12 protein subunits, also has a complex role in translation initiation (13, 14), and its interaction with both the 40 S ribosomal subunit and the central domain of eIF4G is thought to play an important role in forging the link between the mRNA and the ribosome during initiation (15). However, recent work has suggested the possibility of an interaction between eIF3 and cap-bound eIF4E that is independent of eIF4G (16). Two binding sites for eIF4AI have been identified on mammalian eIF4GI, one in the middle domain and a second in the C-terminal domain (11, 15). Each of these binding sites has been identified to have a distinct role in translation. The middle domain is thought to be primarily responsible for the enhancement of the helicase activity of eIF4AI, whereas the C-terminal domain (which is lacking in the yeast homologs) appears to have a more regulatory role (17, 18). Although PABP is not considered a core member of the eIF4 complex, the PABP-eIF4GI interaction has been reported to show a significant stimulatory effect on translation. This effect is thought to be due to the circularization of the mRNA by linking the m7Gppp cap (via eIF4E) with the poly(A) tail via PABP (19). However, the PABP-eIF4GI interaction is not essential for cap-dependent translation, as eIF4G variants or fragments lacking the PABP-binding site are able to support this process, albeit with lower efficiency (17, 20). A synergistic stimulation of mRNA translation occurs when...
mRNA is both capped and polyadenylated (21), although recent work suggests that the poly(A) tail is not always required for optimal mRNA translation in vivo (22).

In this study, we sought to elucidate the importance of the PABP-eIF4GII, eIF4AI-eIF4GII, and eIF3-eIF4GII interactions in translation initiation by removing the ability of the eIF4GII to bind to each of these proteins. Our data show that the loss of the PABP-binding site in eIF4GII had no substantial effect on a number of its characterized properties. These included a lack of effect on the ability of eIF4GII to promote mRNA translation, to interact with eIF4E, to incorporate into 48 S complexes, or to be associated with polysomes in vitro or in cultured cells. Similarly, mutation of the middle domain eIF4AI-binding site had no effect on any of these interactions in vivo. In contrast, however, the loss of either of the two eIF4AI-binding sites resulted in a decreased ability of eIF4GII to support the initiation of translation in the reticulocyte lysate. Interestingly, the eIF3-binding site mutation reduced the ability of the molecule to bind to eIF4E, despite being present in polysome fractions. Taken together, our results suggest a functional redundancy between the interactions between the initiation factors responsible for the recruitment of mRNA for translation.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Plasmids encoding the different variants of eIF4G (Fig. 1) were constructed using standard molecular biology methods, with slight modifications. pcDNA3.1-myc4GIf-4A1 and pcDNA3.1-myc4GIf-3 were constructed by site-directed PCR mutagenesis of pcDNA3.1-myc4GIf (23), pcDNA3.1-myc4GIf-PABP was constructed by complete digestion of pT7.4Gmut (24) and pcDNA3.1-myc4GIf with BamHI and ligation with T4 DNA ligase (Fermentas, UK). pcDNA3.1-myc4GIf-PABP-4A and pcDNA3.1-myc4GIf-PABP-4A-3 were constructed from pcDNA3.1-myc4GIf-PABP by site-directed PCR mutagenesis. In all cases, mutations were confirmed by DNA sequencing. Enzymes were from New England Biolabs unless otherwise specified. pcDNA3.1-mycNMf-WT was as described previously (23).

Cell Culture—NIH 3T3 and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Labtech International) at 37 °C in a humidified atmosphere containing 5% CO2.

Antibodies—Anti-Myc4A6 mouse monoclonal antibody was obtained from Upstate Biologicals; total eIF2α antibody was obtained from Cell Signaling; M2 anti-FLAG antibody was obtained from Sigma, and rabbit polyclonal antiserum recognizing eIF4GI, eIF4A, PABP, and eIF4E were made in-house using the peptide sequences described previously (23, 25, 26). Rabbit anti-eIF3e antibodies were from Dr. P. Jalinot (Lyon, France) and Professor C. Norbury (Oxford, UK).

m7GTP-Sepharose Affinity Isolation of eIF4E and Associated Factors—For the isolation of eIF4E and associated proteins, cell extracts of equal protein concentration were subjected to m7GTP-Sepharose chromatography (Amersham Biosciences), and the resin was washed twice in buffer (20 mM MOPS-KOH, pH 7.4, 25 mM KCl, 2 mM MgCl2, 2 mM benzamidine, 2 mM 2-mercaptoethanol, 0.1 mM GTP, and 0.25% (v/v) Igepal), with recovered protein eluted directly into sample buffer (27).

Baculovirus Expression and Protein Purification—To create FLAG-tagged fusion proteins, pcDNA3.1-myc4GIf, pcDNA3.1-myc4GIf-4A1, pcDNA3.1-myc4GIf-PABP, and pcDNA3.1-myc4GIf-3 were digested with HindIII and XhoI, and the inserts were ligated into similarly digested pcDNA3.1 containing an N-terminal FLAG epitope (DYKDDDDK). These constructs were then subjected to PCR amplification to insert a NotI site for cloning into the pFASTBAC HTc vector (Invitrogen). The PCR products were then digested with NotI and XbaI and ligated into similarly digested pFASTBAC HTc vector. Recombinant baculoviruses allowing expression of the eIF4GII variants were then produced as per the manufacturer’s instructions (Invitrogen). Sf9 cells were maintained in SF900 II serum-free media (Invitrogen) at 27 °C. [35S]Methionine was obtained from Amersham Biosciences. To produce purified [35S]methionine-labeled proteins, Sf9 cells were infected with the baculoviruses expressing the FLAG-tagged eIF4GI proteins. The cells were grown for 48 h, and the culture medium was then substituted with methionine-free SF900 media, in the presence of [35S]methionine (10 μCi per 10-cm plate) for 24 h. Cells were harvested as described (13), and expressed proteins were purified by affinity chromatography using M2 anti-FLAG-agarose, as per the manufacturer’s instructions. Unlabeled proteins were isolated from cells infected for the same total time but in the absence of added radioactive methionine.

Association of Expressed eIF4G Variants with Isolated Initiation Factors in Vitro—Sf9 cells were infected with the recombinant baculoviruses for 72 h, and cells were harvested, and the expressed proteins were captured with M2 anti-FLAG-agarose, as described above. Beads associated with ~5 μg of tagged eIF4GI variants were washed and then blocked with 10 mg/ml cytochrome c for 30 min on ice. Purified baculovirus-expressed eIF4A or PABP (4 μg) or 10 μg of cell-purified eIF3 complex were added to the beads and incubated for 30 min on ice, and the beads were then washed with lysis buffer. Bound proteins were eluted using SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

Leader Protease Treatment of Rabbit Reticulocyte Lysate (RRL)—Briefly, nuclease-treated RRL (Promega) was treated with ~0.03 μg/ml recombinant foot-and-mouth disease virus (FMDV) Leader (L) protease for 20 min at 30 °C, and the L protease was subsequently inactivated by addition of 200 μM elastatin. Preliminary titration studies were conducted to optimize the enzyme concentration. Samples were analyzed by Western blotting to confirm the complete cleavage of eIF4GI, and the lysate was frozen in aliquots in liquid N2.

Reconstitution of L Protease-treated RRL—eIF4GII proteins expressed and purified from the baculovirus-infected insect cell system were added to the L protease-treated RRL under conditions indicated in the individual figure legends. Translation assays were performed under the conditions and using the reagents supplied by the manufacturer. Translation of exogenous luciferase mRNAs was assayed over 90 min, unless otherwise specified. Capped/poly(A)+ luciferase mRNAs were added to a 100 μM elution buffer (20 mM MOPS-KOH, pH 7.4, 25 mM KCl, 2 mM MgCl2, 2 mM benzamidine, 2 mM 2-mercaptoethanol, 0.1 mM GTP, and 0.25% (v/v) Igepal) with recovered protein eluted directly into sample buffer (27).
Interaction of Recombinant elf4GIf Proteins with Other Initiation Factors—Specified concentrations of either unlabeled or [35S]methionine-labeled recombinant, purified elf4GIf variants were added to 20 μl of RRL under full translational conditions for the times indicated. elf4E and associated proteins were isolated by m7GTP-Sepharose chromatography as described previously (27), and recovered proteins were visualized by immunoblotting using the antisera indicated.

Incorporation of Baculovirus-expressed elf4GIf Variants into 48 S Initiation Complexes in RRL—Specified concentrations of the purified, [35S]-labeled elf4GIf proteins were added to RRL in the presence of the normal mixture of reagents required for translation (see above). To sequester endogenous globin mRNA in pre-existing polysomes, the elongation inhibitor emetine was added to a final concentration of 1 × 10−5 M. In addition the nonhydrolyzable GTP analog, GMPPNP (3 mM), was specially designed to ensure that the untranslated 5’-site small ORF-3 (BSEF, also known as WT, see Ref. 29)), which encoded a small FLAG-tagged peptide, was specially designed to ensure that the untranslated mRNPs formed in cell extracts sedimented more slowly than 40 S ribosomal subunits (29); the plasmid encoding this mRNA was kindly supplied by Drs. M. Grskovic and M. Hentze (EMBL, Heidelberg, Germany). Reactions were layered onto an equal volume of ice-cold Gradient Buffer (20 mM MOPS-KOH, pH 7.2, 25 mM KCl, 2 mM magnesium acetate) containing 1% (v/v) formaldehyde and incubated for a further 15 min on ice. The formaldehyde was then quenched by the addition of 1 M Tris-HCl, pH 7.5, and reactions were layered onto 15–45% sucrose gradients and centrifuged for 1 h, 25 min at 167,000 × gav at 4 °C in a Beckman SW55Ti rotor. Samples were then processed as described above.

 elf4GIf-binding Site Mutants Can Enter Initiation Complexes

RESULTS

Characterization of Proteins Used in This Study—The aim of this work was to examine the effect on the function of elf4GIf of inactivating individual binding sites for some of its key interacting proteins. Two binding sites have been identified in elf4GIf for elf4AI; one is located in the middle domain and the second is in the C-terminal domain (Fig. 1). Several mutations have been identified previously in elf4GIf fragments and in the elf4Gla isoform that disrupt the interaction of elf4GIf with elf4AI (17). One of these mutations, an F978A substitution that disrupted the binding of elf4AI, but not that of elf3, was chosen for this study. A pcDNA3.1 plasmid containing the full-length elf4GIf cDNA with a 5’-Myc tag (23), here referred to as elf4GIf, was subjected to site-directed PCR mutagenesis to

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generate the F978A mutation, and is referred to as eIF4GIf-4A1 (Fig. 1). To test the effectiveness of this point mutation in inactivating the binding of eIF4A to the middle domain, it was necessary to eliminate the second binding domain in the C-terminus. For this purpose we used a truncated fragment of eIF4GI- (amino acids 1–1176) that corresponds to a cleavage site destroyed by caspase 3 during apoptosis (26). This fragment, here termed NMf, was inserted into pcDNA3myc to produce NMf-WT (23) (Fig. 1). The chosen middle domain eIF4AI-binding site mutation was also inserted into this fragment to produce NMf-4A (Fig. 1). A mutation in eIF4GI disrupting the PABP-eIF4GI interaction has also been characterized previously (24, 33), although the location of the mutation for the eIF3-binding site was identified by N. Sonenberg (see Footnote 5). As indicated, the NMf-WT truncation results in removal of the C-terminal eIF4A-binding site.

Table 1. Schematic diagram showing eIF4GIf mutations inactivating the PABP, central domain eIF4A, and eIF3-binding sites. Site-directed mutagenesis was used to introduce the indicated mutations into the eIF4GIf nucleotide sequence in the pcDNA3.1myc plasmid backbone (23). The mutations used to inactivate the central domain eIF4A- and PABP-binding sites have been described previously (24, 33), although the location of the mutation for the eIF3-binding site was identified by N. Sonenberg (see Footnote 5). As indicated, the NMf-WT truncation results in removal of the C-terminal eIF4A-binding site.

![Diagram](image)

To examine binding of eIF3 to eIF4GIf, we employed an antiserum recognizing the eIF3e subunit of the complex. Although a degree of binding to the resin occurred in the absence of eIF4GIf (Fig. 2C, lane 2 versus lane 3), binding of eIF3 to the mutant eIF4GIf protein was reduced to levels less than those obtained with the wild-type protein (lane 4 versus lane 3). In our experience, background binding of eIF3 to FLAG affinity beads has been a particular problem for interaction studies and affects determinations using antisera recognizing the whole eIF3 complex or raised against individual subunits. As seen in Fig. 2, A and B, the mutation in the eIF3-binding site had no effect on the ability of eIF4GIf to bind either PABP (lane 5 versus lanes 2 and 4) or eIF4A (lane 5 versus lanes 2–4), respectively.

**Loss of Either of the eIF4A-binding Sites or the eIF3-binding Site Decreases the Ability of eIF4GIf to Support Initiation of Translation, although Loss of the PABP-binding Site Has a Minimal Effect**—Treatment of nuclease-treated RRL with picornavirus 2A or L protease is known to inhibit translation in this system through cleavage of endogenous eIF4GIf (1) (Fig. 3A). The L protease can then be inactivated by addition of the cysteine protease inhibitor, elastatin. This allows the subsequent addition of recombinant eIF4G variants, which remain resistant to cleavage even after 2 h of incubation in the RRL (Fig. 3A) and are able to restore cap-dependent translation (17, 35, 36). We have used this well characterized, albeit limited, system to address the ability of the variant forms of eIF4GIf to influence translation of a reporter mRNA (28) following cleavage of the endogenous protein. These results (Fig. 3B) confirm that rela-
**elf4GIf-binding Site Mutants Can Enter Initiation Complexes**

The mutation of the PABP-binding site appeared to reduce the ability of elf4GIf to recover translation when added at low concentrations, but at higher concentrations the activity of this variant approached that of the wild-type protein (Fig. 3B). Although not proven directly, we think the most likely explanation for this dose effect of the −PABP mutant is that the interaction with PABP may allow elf4GIf to act more efficiently at catalytic concentrations. However, elf4GIf-4A1, NMf-WT, and elf4GIf-3 were substantially less effective in facilitating translation, although low levels of activity were possible at higher concentrations used in these assays (100 nM; Fig. 3B). In agreement with published data (17), loss of the C-terminal elf4A-binding site, although detrimental, was less severe than loss of the middle domain binding site. However, the published work focused exclusively on the activity of variants of the shorter elf4GIfa form, which lacks the PABP-binding site. Our data show that the presence of the PABP-binding site in elf4GIf does not modify the effect of the elf4A mutations, indicating that elf4A and PABP enhance translation independently. elf4AI is involved in unwinding secondary structure in the mRNA molecule, and previous work has demonstrated that mutations in the elf4A protein that compromise its activity particularly affect the translation of mRNAs with structured 5′-untranslated regions (37). To address the question of how translation is affected by compromising the elf4GIf-elf4A interaction and to provide a more stringent test of the helicase activity of elf4F complexes, we employed a luciferase mRNA containing a small stem loop (∆G = 4.5 kcal/mol) 10 nucleotides from the 5′ cap, shown previously to decrease translation initiation compared with unstructured mRNA (28). Fig. 3C shows that although this mRNA was translated in all cases, there appeared to be no selective effect of the elf4AI-binding site mutation or any other mutation tested on the ability of elf4GIf to support translation of mRNA containing a 5′ stem loop. Detailed comparison of the restoration activity of wild-type elf4GIf activity between individual experiments showed some variation between protein preparations purified from different batches of baculovirus-infected cells. Nevertheless, the comparisons between wild-type and mutant forms of elf4GIf presented here are representative of experiments repeated several times with different protein preparations.

To characterize further these variants of elf4GIf, we also monitored their effect on the rate of translation by assaying for luciferase activity at early times. In agreement with the data presented in Fig. 3, Fig. 4A shows that when present at lower concentrations, the variants unable to interact with PABP or elf4A were in general less effective than the wild-type protein at driving luciferase synthesis. However, at higher concentrations, there was no difference in the rate of luciferase production between incubations receiving elf4GIf and elf4GIf-PABP, although the elf4GIf-4A1 variant remained less competent at driving translation. Similar effects were seen when the reporter mRNA contained a stem loop close to the cap structure (Fig. 4B). The most straightforward explanation of these data is that the variant proteins are, to differing extents, defective in their ability to support efficient de novo initiation. However, an alternative interpretation of these data is that this behavior may also reflect the relative inefficiency in displacing the L protease-
**eIF4GIf-binding Site Mutants Can Enter Initiation Complexes**

**A**

![Diagram](image)

**B**

![Diagram](image)

**C**

![Diagram](image)

**FIGURE 3.** eIF4GIf mutants rescue translation in messenger-dependent lysate pre-treated with L protease. **A,** upper, to cleave endogenous eIF4G, nuclease-treated reticulocyte lysate was preincubated with FMDV L protease, as described under "Experimental Procedures." Elastatinal was then added to the depleted lysates, and translation assays were then performed with and without supplementation with the baculovirus-expressed, purified variants of the eIF4GIf proteins indicated. After 2 h incubation at 30 °C, eIF4GIf was visualized by SDS-PAGE and immunoblotting. **Lower,** diagrammatic representation of the luciferase reporter construct used in this work. The eIF4GIf variants (or buffer) were added to the L protease-treated lysate at a final concentration of 10, 20, 50, or 100 nM, as indicated on the inset key, together with 20 μg/ml reporter luciferase mRNA. Luciferase activity was determined after 90 min incubation at 30 °C. The results from the two panels represent assays carried out with different purified eIF4GIf preparations. These data are expressed as a percentage of luciferase activity obtained with eIF4GIf-WT at 100 nM (set at 100%). **C,** upper, diagrammatic representation of the luciferase reporter with a stem loop introduced near the 5′ terminus (28) used in this work. The eIF4GIf variants were added to the L protease-treated lysate at the concentrations shown, together with 20 μg/ml of stem-loop luciferase mRNA. Luciferase activity was determined after 90 min incubation at 30 °C. Similar results were obtained with assays carried out with at least two different preparations of purified eIF4GIf. These data are expressed as a percentage of luciferase activity obtained with eIF4GIf-WT at 100 nM (set at 100%).

The in Vitro Interaction of eIF4E with eIF4GIf Is Unaffected by the Loss of the PABP-, eIF4AI-, or eIF3-binding Sites—The core eIF4G complex required for recruitment of the 40 S preinitiation complex to the mRNA consists of eIF4E, eIF4G, and eIF4A, with eIF4E responsible for binding the mRNA cap structure and bringing the eIF4G complex onto the mRNA. Although none of the mutations specifically targeted the eIF4E-binding site, we wished to determine whether their lower efficiency in supporting translation was because of a decreased ability to assemble into eIF4F. For this experiment we used a non-nuclease-treated reticulocyte lysate (Promega), which we pretreated with L protease. Translation reactions were supplemented with 50 nM recombinant, purified eIF4GIf together with 20 μg/ml luciferase mRNA and incubated for 2 min prior to recovery of eIF4E and associated proteins by m7GTP-Sepharose chromatography. Input material (Fig. 5, A and top panel of C) or proteins recovered from the affinity resin (Fig. 5, B and bottom panel of C) were eluted with sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting. Exogenous eIF4GIf proteins were detected either by eIF4GIf-specific antibodies or by antisera recognizing the FLAG tag. The results in Fig. 5B indicate that all of the eIF4GIf variants tested were rapidly incorporated into complexes with eIF4E. As expected, no full-length endogenous eIF4GIf was recovered in the L protease-treated sample (Fig. 5B, 1st lane), and little eIF4A was recovered in eIF4F unless exogenous eIF4G was restored (2nd to 5th lanes). However, both eIF4GIf-4AI and NMIf-WT were as effective as wild-type eIF4GIf in restoring recovery of eIF4A into eIF4F (Fig. 5C). These data suggest that the presence of either the middle or the C-terminal eIF4A-binding site is sufficient to allow eIF4G to recruit eIF4A into stable eIF4F complexes and confirm the data shown in Fig. 2B. It was not possible in these experiments to analyze the recruitment of PABP by the different eIF4GIf variants in this system, as complexes containing PABP bound to the N-terminal fragment of endogenous eIF4GIf produced by the L protease cleavage would also be recovered on the beads through interaction of this fragment with eIF4E. Therefore, in a separate series of experiments (Fig. 5D), we compared the protein binding behavior of FLAG-eIF4GIf with that of FLAG-eIF4GIfa (which naturally lacks the N-terminal PABP-binding site (24, 33)) when incubated in the presence and absence of poly(A) m7GTP-mRNA in a postribosomal supernatant derived from untreated reticulocyte lysate. The tagged eIF4G and associated proteins were recovered using anti-FLAG-agarose. Fig. 5D shows that although eIF4E was recovered to a similar extent under all conditions, PABP was only recovered in association with eIF4GIf in the absence of added mRNA (lane 3 versus lane 1). However, in the presence of poly(A) m7GTP-mRNA, co-isolation of PABP with eIF4GIf increased markedly (lane 4 versus lane 3), and some PABP was also recovered with eIF4GIf (lane 2 versus lane 1). The additional PABP is most likely
recruited by binding to the poly(A) tail of the added mRNA, which is in turn associated via its cap with the eIF4E component of eIF4F. Consistent with this, the additional PABP was not recruited by poly(A) mRNA nor under conditions where the eIF4E-eIF4G interaction was prevented by the addition of the negative regulator protein 4E-BP1 (data not shown).

**40 S Ribosome Recruitment to mRNA Is Not Prevented by Loss of the eIF4AI- or PABP-binding Sites**—The main role of the eIF4F complex is to recruit the 43 S preinitiation complex to the mRNA to form what is commonly known as the 48 S preinitiation complex (40). As eIF4GIf-WT, eIF4GIf-4A, and eIF4GIf-PABP variants are able to associate with eIF4E, we wanted to determine whether the decrease in translation observed in Figs. 3 and 4 was because of a lower ability to recruit the 43 S initiation complex to mRNA. To address this, [35S]methionine-labeled, baculovirus-expressed proteins were added to reticulocyte lysate translation systems (not pre-treated with either nuclease or L protease) under conditions designed to monitor their recruitment into 48 S complexes formed in response to added mRNA. This involved adding the elongation inhibitor, emetine, to trap the endogenous globin mRNA in pre-existing polysomes such that any further formation of initiation complexes was dependent on added mRNA (41). The nonhydrolyzable GTP analog, GMPPNP, was added to block 60 S subunit joining and allow 48 S complexes to accumulate (42, 43). Incorporation of the eIF4GIf variants was then determined following incubation of the systems for 2 min in the absence or presence of mRNA, followed by sucrose gradient centrifugation. The mRNA used for these experiments was a short construct designed to ensure that untranslated mRNP complexes sedimented more slowly on sucrose gradients than 40 S ribosomal complexes (29). In preliminary calibration experiments (not shown), we defined the sedimentation peaks of this mRNA in mRNP (slightly heavier than the main protein peak), and this is referred to as “free RNA” in Fig. 6A, left panel, and in 48 S ribosomal complexes. Fractions corresponding to these peaks were pooled, and the particles present in each pool were recovered by centrifugation. The pellets were dissolved in sample buffer, resolved by SDS-PAGE, and proteins detected by PhosphorImager analysis for [35S]-eIF4G, with the presence of eIF2α used as an indicator for the presence of the 40 S complex (Fig. 6A, right panel). These data show that all three eIF4GIf variants rapidly became incorporated into both the mRNP complex and 48 S preinitiation complexes in an mRNA-dependent manner, suggesting that 40 S ribosome recruitment to mRNA is not prevented by loss of the eIF4AI- or PABP-binding sites. However, eIF4GIf-PABP did appear to show a different distribution relative to the other variants, with the amount in the 48 S region lower relative to that in the mRNP from the same gradient. This result may indicate a lower efficiency of this mutant in supporting the recruitment of mRNA to the 43 S preinitiation complex.

To complement this study, we also examined [35S]-eIF4GIf variant incorporation into 48 S initiation complexes associated with polysomes during the translation of endogenous globin mRNA (Fig. 6B). For this, each eIF4GIf variant was added to translation-competent, untreated RRL in the absence of inhibitors and incubated under translation conditions for 3 min. GMPPNP was then added in order to trap 40 S complexes in the process of initiation onto polysomal mRNAs (“half-mers” (44)). After a further incubation for 3 min, lysates were subjected to sucrose gradient centrifugation, and those fractions containing 40 S ribosomes, 60 S ribosomes, light (P1), or heavy polysomes
Purified baculovirus-expressed eIF4GIf variants were wild-type protein.

**FIGURE 5.** eIF4GIf-binding Site Mutants Can Enter Initiation Complexes

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**A**

| eIF4GIf-WT | eIF4GIf-4A1 | eIF4GIf-PABP | eIF4GIf-3 |
|------------|------------|-------------|-----------|
| L-treated  | L-treated  | L-treated   | Untreated |
| FLAG       | eIF4A      | eIF4E       |           |

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**B**

| eIF4GI    | eIF4A      | eIF4E       |
|-----------|------------|-------------|
| m7GTP-Sepharose |         |             |

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**C**

| eIF4GI    | eIF4A      | eIF4E       |
|-----------|------------|-------------|
| m7GTP-Sepharose |        |             |

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**D**

| eIF4GI    | PABP       | eIF4A      | eIF4E       |
|-----------|------------|------------|-------------|
| +         | -          | -          | -           |
| -         | -          | +          | +           |
| -         | +          | -          | -           |
| +         | +          | -          | -           |
| anti-FLAG-IP |        |             |             |

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The presence of PABP, eIF4E, and eIF2α in the fractions was monitored to indicate the sedimentation of eIF4F and 40 S complexes. The main conclusion from Fig. 6B (right panel) is that under these *in vitro* conditions, all three variants were able to enter the polysomal fractions. Although there is a suggestion that the mutant proteins were more heavily distributed toward the lighter (40 S + 60 S) fractions than the wild type, technical limitations in labeling the eIF4GIf proteins and using low concentrations to maintain translational competence in the system (Figs. 3 and 4) meant that it was not possible to quantify these data precisely enough to compare the kinetic behavior of these proteins. In several repeats of this experiment, we have consistently observed very low signals for the labeled eIF4G in the case of the wild-type protein (Fig. 6B, 1st 3 lanes).

An interesting possibility to explain this difference between the wild type and the mutants could be that the complexes containing the mutant forms turn over less rapidly than those formed with the wild type. However, the technical limitations discussed above preclude detailed analysis of this difference, and the main conclusion from these experiments is that the mutant forms do become incorporated into polysomal complexes.

*eIF4GIf-4A1, eIF4GIf-PABP, and eIF4GIf-3 Are Able to Partially Rescue Translation in eIF4GI siRNA-targeted Cells*—Although the reticulocyte lysate system is the most active mammalian *in vitro* translation system available, it contains relatively high levels of initiation factors and is unlikely to reflect the competitive conditions in most intact cells. We have therefore complemented the above studies by examining the effect of expression of the eIF4GIf variants in cells subjected to knockdown of the endogenous eIF4GI. Plasmids encoding siRNA-resistant versions of the eIF4GIf variants were transfected into HeLa cells that were concomitantly transfected with siRNA targeting endogenous eIF4GI (30). The effectiveness of the knockdown of the endogenous eIF4GI was shown by immunoblotting in two independent experiments (Fig. 7A, left and right panel) were pooled separately, and the protein was isolated by centrifugation as described above. Recovered protein was resolved by SDS-PAGE and detected by PhosphorImager analysis or by probing with the specific antisera indicated.

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*additional content...*
eIF4GIf-binding Site Mutants Can Enter Initiation Complexes

As a control, cells were also transfected with a plasmid encoding an siRNA containing three changed nucleotides, such that it no longer targeted eIF4GI (Fig. 7A, lanes 2 and 9 versus lanes 1 and 8, respectively). Concomitant transfection of siRNA-resistant eIF4GIf variants with the siRNA resulted in expression of total eIF4GIf to levels similar to those observed for the endogenous protein (Fig. 7A, lanes 4–7 and 11–15). eIF4E and associated proteins were also recovered by m'GTP-Sepharose chromatography, and protein was eluted with sample buffer and resolved by SDS-PAGE. The recovery of expressed eIF4GIf variants was analyzed by immunoblotting either with eIF4GIf-specific antibodies or with antisera recognizing the Myc tag. The results shown in Fig. 7A (lanes 4–7 and 11–15) indicate that all of the eIF4GIf variants tested were incorporated into complexes with eIF4E, including novel variants lacking the binding sites for both PABP and eIF4A (lane 12), PABP and eIF3 (lane 13), eIF4A and eIF3 (lane 14), and one lacking the sites for all three ligands, PABP, eIF4A, and eIF3 (lane 15). Unfortunately, problems with nonspecific binding of these ligands to Sepharose resins, commonly observed and illustrated in Fig. 7B, led to inconsistent data on the recovery of these proteins in eIF4F complexes. For example, although lanes 5 and 13 (versus lanes 4 and 11, respectively) of Fig. 7A show reduced recovery of PABP in material recovered following m'GTP-Sepharose chromatography of extracts from cells expressing the eIF4GIf-PABP variants, data in other cases (e.g. lane 12) were less consistent. This problem was considerably magnified under the conditions employed for these experiments analyzing cell extracts, where relatively large protein inputs were required to visualize eIF4GIf variants that are expressed at low levels in vivo. Furthermore, eIF4GII will still be recovered in the eIF4F complex under these conditions and will contribute to some extent to the eIF4A signal observed in the absence of eIF4GII (30). As stated above, and illustrated in Fig. 7B, nonspecific binding of eIF3 to these resins precludes attempts to monitor binding of this protein complex to eIF4G in cell extracts.

Knockdown of eIF4GII expression in these studies resulted in a decrease of about 50% in the overall rate of translation, estimated as the incorporation of [35S]methionine into total protein over 1 h (Fig. 7C), an effect not observed with the mutant siRNA. Consistent with the in vitro data shown in Fig. 3, expression of each of the eIF4GIIf variants was able to increase the rate of translation over that in the silenced cells, but not to the control level. However, although all the eIF4GIIf variants consistently promoted this partial restoration of protein synthesis, it was not possible to discern any differences in efficiency between them. Indeed, surprisingly, the triple mutant affecting all three binding sites performed as well as any of the other variants in this assay. The failure to rescue to the control level, even with the wild-type eIF4GII, may be due to not all silenced cells being co-transfected with the siRNA-resistant eIF4GIIf cDNAs. Alternatively, replacement of endogenous eIF4GI with just the full-length version (eIF4GIIf) may not be sufficient for full translation as five N-terminally distinct versions of eIF4GI are present in normal cells (8, 9), and the importance of the simultaneous presence of all of these in normal cells has not been elucidated. However, in a separate study beyond the scope of the work described here (30), we have observed that, relative to eIF4GIIf, the N-terminally truncated eIF4GIIa variant exhibits a significantly lower ability to restore translation under these assay conditions. This is the case even though this variant can be expressed to higher levels. It is not clear at present why eIF4GIIa has less ability to support translation than the eIF4GIIf-PABP mutants, although one cannot exclude the possibility that the N-terminal sequence upstream of the PABP-binding site may also have a previously unidentified function.

To analyze further the behavior of the mutants, we examined the incorporation of each of the expressed siRNA-resistant, single site-mutated eIF4GIIf proteins into complexes associated with polysomes. Fig. 8A shows the polysome profiles of extracts from these cells and demonstrates a decrease in the number of...
polysomes present in the knockdown cells (eIF4GIf siRNA versus mutant siRNA). Fig. 8B (left panel) shows that although expression of the variants was similar in each case (3rd to 6th lanes), levels attained by transfection with myc-eIF4GIf were lower than that observed for the endogenous protein in cells transfected with the mutant siRNA (1st lane). In no case did expression of the siRNA-resistant eIF4GI variants markedly affect the polysome content (Fig. 8A), reflecting the incomplete restoration of overall translation seen in Fig. 7B. After sucrose gradient centrifugation, the indicated fractions were pooled separately, and the polysomes were recovered as described above and resolved by SDS-PAGE, with detection using the specific eIF4GI antiserum (Fig. 8B, right panel). These data show that consistent with a partial rescue of translation, the eIF4GIf-WT was recovered in the polysomal fraction (Fig. 8B, 9th lane) to a level similar to that of the endogenous protein (7th lane). Surprisingly, the eIF3-binding mutant was also associated with the remaining polysomes (Fig. 8B, 12th lane) suggesting that interaction with eIF3 was not required for the association of eIF4GIf with ribosomes. Interestingly, the results for the PABP (Fig. 8B, 10th lane) and the eIF4A-binding site mutants (11th lane) in the HeLa cells show these variants recovered in polysomes, results that were distinctly different in the RRL (Fig. 6). This distinction between the whole cell and in vitro systems may reflect differences in concentration of the eIF4GIf molecules in the two situations or may be due to the presence of competing cleavage fragments of endogenous eIF4G in the reticulocyte lysate assay system.

**In Vivo Analysis of the Ability of eIF4GIf Variants to Promote de Novo Translation Initiation**—Previous studies have demonstrated that depletion of endogenous eIF4G in mammalian cells and Xenopus oocytes had surprisingly little effect on ongoing protein synthesis rates...
elF4GIf-binding Site Mutants Can Enter Initiation Complexes

In current models for the stepwise initiation of protein synthesis (40, 47, 48), elF4G has been identified as the scaffold protein responsible for assembling the elF4F complex onto mRNA via interaction with elF4E, other proteins, and possibly RNA. This assembly leads on to the next step, the recruitment of the 40 S subunit onto the mRNA through the interaction between elF4G and elF3. However, although it is known that

S10 extract. In steady state cells (not treated with NaCl), all variants were found in association with elF4E (Fig. 9B). During recovery from salt shock, the interactions between elF4E and elF4G-WT, elF4GIf-4A1, or elF4GIf-PABP were rapidly reformed (Fig. 9B, middle panel). elF4GIf-3, however, was less well represented in elF4F at 7.5 min. After 15 min of recovery, however, the elF4GIf-3 protein was clearly seen in the elF4F complex (Fig. 9B, right-hand panel). These data suggest that although this mutation did not prevent the association of elF4GIf with elF4E, it did slow down its incorporation under conditions where the mutant protein was in competition with endogenous wild-type elF4GIf.

We then examined the ability of these proteins to be incorporated into complexes associated with translating light (Fig. 9C, P1) and heavy polysomes (Fig. 9C, P2). The polysome profiles of the extracts derived from these cells confirmed the pronounced polysome dissociation resulting from salt shock and the rapid reassociation during recovery (31, 32). Similarly, endogenous elF4GIf was associated with polysomes (Fig. 9C, left panel); the level of elF4GIf in polysomes decreased, as expected, during salt shock (middle panel), and elF4GIf re-entered the polysome fraction during recovery (right panel). We also examined the re-incorporation of the expressed myc-elF4GIf variants after 30 min of recovery. Fig. 9D shows that in actively growing (untreated) cells, elF4GIf-WT, elF4GIf-4A1, and elF4GIf-PABP were detected in both heavier and lighter polysomes, indicating the involvement of all three elF4GIf proteins in 40 S-mRNA complexes. Interestingly, however, we consistently found that comparison of the signals in the polysome fractions relative to the total expression (Fig. 9D, lane S10) showed both the elF4GIf-4A1 and elF4GIf-PABP variants to be relatively under-represented in the polysome fractions. Despite problems with expression levels, in a number of separate experiments we also found that the elF4GIf-3 variant behaved in a similar manner (Fig. 9D). These results confirm results observed with the RRL, in which both elF4GIf-4A1 and elF4GIf-PABP were retained in the 40 S, 60 S, and light polysome fractions (Fig. 6B).

DISCUSSION

In current models for the stepwise initiation of protein synthesis (40, 47, 48), elF4G has been identified as the scaffold protein responsible for assembling the elF4F complex onto mRNA via interaction with elF4E, other proteins, and possibly RNA. This assembly leads on to the next step, the recruitment of the 40 S subunit onto the mRNA through the interaction between elF4G and elF3. However, although it is known that

(45, 46). However, in both cases there was a much more profound effect on de novo protein synthesis. Accordingly, we exploited the reversible response of mammalian cells to exposure to hypertonic media (“salt shock”), which strongly inhibits protein synthesis at the level of initiation (45), to examine the behavior of initiation factors during the rapid, de novo recruitment of mRNA during recovery (31, 32). Because this system provides a more stringent test of elF4G function (45), we have used NIH 3T3 cells transfected with each of the four elF4GIf variants, as indicated. After 96 h of incubation, cells were harvested, and the resulting extracts were prepared and analyzed for total (endogenous) elF4GIf (Fig. 9D, middle panel) using the anti-Myc antibody to track specifically the behavior of the expressed proteins. As before, lysates were prepared at the indicated times and analyzed for expression of elF4GIf variants (Fig. 9B, top panels) or elF4GIf co-isolated with elF4E on m7GTP-Sepharose (lower panels). Because levels of expression varied between variants, it is necessary to compare the data from each of the m7GTP-Sepharose analyses with those from the corresponding

\begin{align*}
\text{elF4GIf-binding Site Mutants Can Enter Initiation Complexes} & \\
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\end{align*}
such factors as eIF4A, PABP, and eIF3 bind to eIF4G, the effect of the loss of these interactions on translation initiation in vivo and in vitro has not been fully determined. This study was designed to determine the effect of removing the eIF4A-, eIF3-, or PABP-binding sites on eIF4G function. Interestingly, the results indicate that the loss of any one of these interactions has a minimal effect on the ability of eIF4G to support the initiation of translation in cells but can have greater effects on translation assayed with in vitro RRL systems in which the endogenous eIF4G has been depleted by proteolysis. This may reflect the fact that in the latter system the added proteins are tested more stringently in that they must compete with the proteolytic degradation fragments of endogenous eIF4G (15, 35). Loss of the PABP site did not eliminate the ability of eIF4G to support translation initiation in either the RRL (Figs. 3 and 4) or in HeLa cells with eIF4G targeted by siRNA (Fig. 7), but rather, the variant was less effective than the wild-type eIF4G at lower concentrations. In contrast, the over-expression of eIF4G harboring this same mutation (in the context of eIF4Gl) in Xenopus oocytes was found to inhibit both the translation of co-injected poly(A)⁺ mRNA and progesterone-induced maturation, suggestive of a dominant negative effect (24).

Evidence for the importance of the eIF4G-PABP interaction in mammalian cells comes from a recent study using cell extracts artificially depleted of PABP (48). These extracts exhibited defects in translational activity and in initiation complex formation that were rescued by addition of wild-type PABP but not by the same mutant as used in the present work, which was unable to bind to eIF4G. It is possible that the variations in effects between studies reflect differences between cell types (or in vitro systems derived from them), in levels of eIF4G and PABP relative to each other and to other binding partners that may provide alternative linkages within initiation complexes. For example, PABP can interact indirectly with eIF4A via Paip1 (50) is also able to bind eIF4B (51) and enters eIF4F complexes containing mRNA by associating with the poly(A) tail of the latter (Fig. 5D). Any of these interactions may mediate an alternative means of interacting with eIF4F and could potentially be sufficient to support translation in the absence of the direct association of PABP with eIF4G. In this context it is of interest to note that fragments of PABP that exclude the eIF4G binding domain retain the ability to promote translation of mRNAs to which they are tethered (52).

Disruption of the binding sites for eIF4A in the middle and C-terminal domains of eIF4G has been shown previously to reduce its ability to restore translation in protease 2A-treated reticulocyte lysates (17). Our results described in Fig. 3 support those findings, including the observation that mutation of the eIF4A-binding site in the middle domain of eIF4G is more detrimental to protein function than the loss of the C-terminal domain. Because the earlier study used the eIF4Glα isoform that lacks the PABP-binding site, our work further shows that the presence of the N-terminal extension present in the eIF4Gl isoform, which includes the PABP-binding site, our work further shows that the presence of the N-terminal extension present in the eIF4Gl isoform, which includes the PABP-binding site, does not affect this response. Despite the reduced ability of the middle domain mutant eIF4GI-4A1 to rescue translation, this protein became incorporated into eIF4F complexes (Fig. 5) and associated with both 48 S initiation complexes and polysomes (Fig. 6) in reticulocyte lysates. As mentioned above, it is possible that the greater sensitivity of the translation assays to the mutation may reflect the presence of the L protease cleavage fragment p100 of eIF4G with which the added protein would have to compete for other ligands such as eIF3 and RNA. However, another interpretation is that these in vitro assays

**FIGURE 9.** Relative to the wild-type protein, eIF4Gl lacking the eIF3-binding site is incorporated into eIF4F at a slower rate upon recovery of cells from salt shock. A, NIH 3T3 cells were untreated or salt-shocked with 150 mM additional NaCl for 1 h with or without subsequent recovery in normal DMEM supplemented with 10% fetal bovine serum for the time indicated. Cells were then harvested, and the resultant extracts either resolved directly by SDS-PAGE (upper panels) or subjected to m7GTP affinity chromatography. Recovered proteins were resolved by SDS-PAGE and immunoblotting (lower panels). B, NIH 3T3 cells were transfected with the pcDNA Myc constructs encoding the indicated eIF4Gl variants 36 h prior to the experiment. Cells were either untreated or salt-shocked as above and then recovered for various time periods as indicated. Extracts were prepared and either resolved directly (upper panels) or subjected to m7GTP affinity chromatography (lower panels), as described above. Proteins were visualized by SDS-PAGE, and Myc-tagged eIF4G was detected by immunoblotting. C, upper, NIH 3T3 cells were either untreated, salt-shocked, or salt-shocked and then allowed to recover for 30 min. Cell lysates were separated on 15–40% sucrose gradients (upper panel), and light (P1) and heavy polysome fractions (P2) were recovered by sedimentation and analyzed by SDS-PAGE and immunoblotting for associated endogenous eIF4G (lower). D, NIH 3T3 cells were transfected with the pcDNA3.1myc constructs encoding the eIF4Gl variants indicated. Cells were salt-shocked, recovered for 30 min, and extracts prepared and separated on 15–40% sucrose gradients. Polysome fractions were pooled and proteins recovered and analyzed by SDS-PAGE and immunoblotting for the Myc tag, as described above.
represent partial reactions that would not reveal changes in function that affect kinetic behavior or the ability of eIF4Glf to be recycled through successive rounds of initiation. To address this, we investigated the behavior of eIF4Glf variants when expressed in mammalian cells. In HeLa cells we were able to achieve a substantial knockdown of endogenous eIF4Glf levels using an siRNA strategy (30) (Fig. 7A). This induced a 50% decrease in overall protein synthesis rate after 4 days (Fig. 7B). The residual translation may reflect the incomplete knockdown and/or the continued presence of eIF4GII, but these data also recall the persistence of ongoing protein synthesis in mammalian cells (45) or Xenopus oocytes (46) in the absence of intact eIF4G. Expression of siRNA-resistant eIF4Glf variants partially restored the incorporation of [35S]methionine into protein. Although the shortest naturally occurring variant, eIF4Gla, was less efficient in this assay (30), there was no obvious effect of binding site mutations on the activity of eIF4Glf (Fig. 7B). Indeed, and very surprisingly, even a triple mutant form of eIF4Glf with all these sites compromised was just as effective as the wild-type protein. Again there was no detectable effect of mutating either the eIF4A- (middle domain), PABP-, or eIF3-binding sites on the recruitment of expressed eIF4Glf into eIF4F complexes or polysomal initiation complexes (Fig. 8).

Finally we adopted a different strategy, which we have used previously, to examine the behavior of initiation factors during de novo translation during recovery from hypertonic shock (31, 32). Following treatment of cells for 1 h under hypertonic conditions, polysomes were completely disaggregated, and eIF4G was dissociated from eIF4E, but these changes were rapidly reversed when cells were restored to normal medium (Fig. 9, A and C). During the recovery period, the reincorporation of Myc-tagged eIF4Glf variants into eIF4F therefore would, of necessity, occur in competition with endogenous eIF4Glf. As can be seen in Fig. 9B, even under these stringent conditions, both the PABP and eIF4A binding mutants reassociated with eIF4E as efficiently as the wild-type protein. However, relative to the total load (S10), both of these mutants were less efficiently incorporated into polysomal complexes than the wild-type eIF4Glf (Fig. 9C).

Our results with the eIF4Glf variant with reduced ability to interact with eIF3 were initially surprising. Current models of translation initiation depict the interaction of eIF4Glf with eIF3 as being responsible for forging the link between the eIF4F-mRNA complex and the 43 S preinitiation complex (40). Relative to the wild-type protein, we found the eIF4Glf-3 mutant to be inefficient both at rescuing translation in the L protease-treated reticulocyte lysate (Fig. 3) and at becoming incorporated into eIF4F complexes in cells recovering from hypertonic shock (Fig. 9B). However, we could detect no defect in the association of this mutant form of eIF4Glf with polysomes in siRNA-treated HeLa cells (Fig. 8B). These data suggest that the interaction between eIF4Glf and eIF3 is not essential for the association of eIF4F with the 43 S initiation complex in mammalian systems. Interestingly, a stable interaction between eIF4G and eIF3 has never been reported for Saccharomyces cerevisiae, a situation that might be explained by the recent demonstration that the eIF3e subunit of the mammalian factor, which is lacking in budding yeast, plays a key role in the association (53). It now seems likely that the interaction of each of these proteins with mRNA and/or ribosomal RNA could play an important role in the formation of initiation complexes. Two recent papers are pertinent to this discussion. eIF3 has been shown in mammalian cells to remain associated with a cap-binding complex from which eIF4G has largely been evicted by treatment of the cells with rapamycin (16). This suggests that the interaction between these proteins may not be essential for basal translation. However, the demonstration of a rapamycin-sensitive effect of insulin to promote increased association of eIF4Glf with eIF3 (54) suggests the possibility that this interaction may play an important role in the up-regulation of general or specific translation by insulin in mammalian cells.

Overall, the results presented here highlight the inherent redundancy in the mechanism of formation of translational initiation complexes. Multiple interactions of eIF3 with eIF5, eIF1, eIF1A, and eIF2 (14), association of Paip1 with both PABP and eIF4A (50), and suggested interactions of eIF4B with eIF3 (16, 54–56) and PABP (51, 57) all serve to bring the components of initiation complex together. Clearly some of the individual binding activities of initiation factors are not essential for translation or for the formation of initiation complexes to occur. In addition to the report discussed above (16), recent studies have shown the presence in cap-binding complexes of mutant forms of eIF4A that can bind neither the middle nor the C-terminal domains of eIF4G expressed individually (58). Although this could reflect a need for interaction between the domains of eIF4G, it could also indicate the potential for an eIF4G-independent association of eIF4A with these complexes.

In addition to the role of protein-protein interactions in the formation of initiation complexes, it is important to remember that many initiation factors exhibit both general and specific RNA binding activity (59–61). In the case of eIF4G, both S. cerevisiae (62) and mammalian (63) homologs exhibit nonspecific RNA binding activity, with binding kinetics for mammalian eIF4G suggesting of active association at physiological concentrations.6 eIF4G-mRNA interactions could well stabilize 48 S initiation complexes (48). Moreover, general RNA binding activity has been localized to a region toward the N terminus of the central domain of eIF4G (63), which has now been shown to modulate scanning (64). This is of particular interest in the light of the recent observation that the most pronounced effect of deleting S. cerevisiae cells of eIF4G was an accumulation, rather than the expected failure of formation, of 48 S initiation complexes (39). Increasingly, it seems likely that the stepwise model for initiation is an over-simplification and that, in many cases, the multiplicity of interactions provides a basis for a complex pattern of regulatory mechanisms in response to physiological change.

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