eIF4G Functionally Differs from eIFiso4G in Promoting Internal Initiation, Cap-independent Translation, and Translation of Structured mRNAs*

Received for publication, April 30, 2001, and in revised form, July 17, 2001
Published, JBC Papers in Press, August 1, 2001, DOI 10.1074/jbc.M103869200

Daniel R. Gallie‡§ and Karen S. Browning¶

From the ‡Department of Biochemistry, University of California, Riverside, California 92521-0129 and the ¶Department of Chemistry and Biochemistry and the Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78712-1096

Eukaryotic initiation factor (eIF) 4G plays an important role in assembling the initiation complex required for ribosome binding to an mRNA. Plants, animals, and yeast each express two eIF4G homologs, which share only 30, 46, and 53% identity, respectively. We have examined the functional differences between plant eIF4G proteins, referred to as eIF4GI and eIFiso4G, with present as subunits of eIF4F and eIFiso4F, respectively. The degree to which a 5′-cap stimulated translation was inversely correlated with the concentration of eIF4F or eIFiso4F and required the poly(A)-binding protein for optimal function. Although eIF4F and eIFiso4F directed translation of unstructured mRNAs, eIF4F supported translation of an mRNA containing 5′-proximal secondary structure substantially better than did eIFiso4F. Moreover, eIF4F stimulated translation from uncapped monocistronic or dicistronic mRNAs to a greater extent than did eIFiso4F. These data suggest that at least some functions of plant eIFiso4F and eIF4F have diverged in that eIFiso4F promotes translation preferentially from unstructured mRNAs, whereas eIF4F can promote translation also from mRNAs that contain a structured 5′-leader and that are uncapped or contain multiple cistrons. This ability may also enable eIF4F to promote translation from standard mRNAs under cellular conditions in which cap-dependent translation is inhibited.

Protein synthesis requires the participation of numerous eukaryotic initiation factors (eIFs)† that assist the binding of 40 S ribosomal subunits to an mRNA and the assembly of the 80 S ribosome at the correct initiation codon. The 5′-cap structure (m7GpppN, where N represents any nucleotide) serves as the binding site for the cap-binding protein eIF4E, the small subunit of eIF4F. eIF4G, the large subunit of eIF4F, interacts with several proteins in addition to eIF4E, including eIF4A (which is required to remove secondary structure within the 5′-leader sequence that would otherwise inhibit scanning of the 40 S ribosomal subunit), eIF3 (which promotes 40 S ribosomal subunit binding to the mRNA), and the poly(A)-binding protein (PABP; which stabilizes eIF4F binding to the 5′-cap) (1–5). The N-terminal domain of eIF4G is responsible for binding eIF4E and PABP; the middle domain binds eIF3 and eIF4A; and in mammalian eIF4G, the C-terminal domain binds a second molecule of eIF4A as well as Mnk1, a MAPK-activated protein kinase responsible for phosphorylating eIF4E (6–8). Consequently, eIF4G functions as a scaffold protein that recruits many of the factors involved in stimulating 40 S ribosomal subunit binding to an mRNA.

Two related but highly distinct eIF4G proteins were first identified in plants (9). The two plant eIF4G proteins, referred to as eIF4G and eIFiso4G, differ in size (165 and 86 kDa, respectively). Two forms of eIF4G are also observed in yeast and mammals (10, 11), but do not differ substantially in molecular mass and are more conserved. Mammalian eIF4GI and eIF4GII are 46% identical (11), and yeast eIF4G1 and eIF4G2 are 53% identical (10), in contrast to plant eIF4G and eIFiso4G, which are only 30% identical.² Mammalian eIF4GII functionally complements eIF4GI to a significant extent (11), and yeast can tolerate the deletion of either gene encoding eIF4G, although at least one gene is required for viability (10). Although these studies suggest that both eIF4G proteins in eukaryotic species are largely functionally similar, differences also have been reported. For example, deletion of the gene encoding yeast eIF4G1 leads to a synthetic lethal interaction with cdc33-1, an eIF4E temperature-sensitive mutant, whereas deletion of the gene encoding eIF4G2 does not (12). Moreover, yeast eIF4G2 supports translation of uncapped polyadenylated mRNA to a greater extent than does eIF4G1 (12). Whether this is due to functional differences or to the in vivo levels of each eIF4G is unknown. Cleavage of mammalian eIF4G occurs following infection with poliovirus or human rhinovirus, resulting in the inhibition of protein synthesis (13, 14). For both viruses, cleavage of eIF4GI occurs prior to that of eIF4GII, and the cleavage of the latter correlates with the loss of protein synthesis following viral infection (13–15). In contrast, the cleavage of eIF4G1 and eIF4GII that occurs during apoptosis is temporally similar (16). Although wheat eIF4F and eIFiso4F support translation in vitro and exhibit RNA-dependent ATP hydrolysis activity and ATP-dependent RNA unwinding activity (17–20), the affinity of eIF4F for hypermethylated cap structures is lower than that of eIFiso4F (21), and the ATPase activity of eIF4F is greater than that of eIFiso4F when mRNA is used to stimulate the RNA-dependent activity (20). Moreover, binding studies with oligonucleotides suggest that eIF4F binding is

* This work was supported by United States Department of Agriculture Grants NRICGP 99-35301-7866 and 00-35301-9086 (to D. R. G.) and by National Science Foundation Grant MCB 980573, Department of Energy Grant DE-FG03-97ER20283, and Welch Foundation Grant F-1339 (to K. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 909-787-7298; Fax: 909-787-3590; E-mail: drgallie@citrus.ucr.edu.
‡ To whom correspondence should be addressed. Tel.: 909-787-7298; Fax: 909-787-3590; E-mail: drgallie@citrus.ucr.edu.
§ The abbreviations used are: eIFs, eukaryotic initiation factors; PABP, poly(A)-binding protein; MAPK, mitogen-activated protein kinase; eEF, eukaryotic elongation factor.
¶ © 2001 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

This paper is available on line at http://www.jbc.org
sensitive to the presence of secondary structure and that eIFiso4F exhibits a binding preference for linear structures (22).

Whether the two types of eIF4G present in eukaryotic cells exhibit specialization in determining which mRNAs are translated or whether they differ in the efficiency in which they support translation has not been investigated for any species. In this study, the functional differences of plant eIF4G and eIFiso4G were investigated during the translation of capped or uncapped mRNAs, mRNAs containing a structured 5'-leader, or dicistronic mRNAs. The addition of eIF4F or eIFiso4F (in which eIF4G and eIFiso4G are present as subunits, respectively) to lysates depleted of eIF4F and eIFiso4F supported the translation of an unstructured mRNA; however, only eIF4F significantly supported translation from an mRNA with a structured 5'-leader. eIF4F increased the translation of an uncapped mRNA and stimulated the translation from the second cistron of a dicistronic mRNA to a greater extent than did eIFiso4F, suggesting that eIF4F has evolved to promote translation from nonstandard mRNAs, i.e. those that lack a cap, contain a structured 5'-leader, or contain multiple cistrons, or has evolved to promote translation from standard mRNAs under cellular conditions in which cap-dependent translation is inhibited, whereas eIFiso4F may be largely limited to facilitating translation from standard mRNAs. Additionally, the concentration of eIF4F, eIFiso4F, and PABP determined the extent to which the cap stimulated translation: PABP was required for the cap to stimulate translation efficiently; however, increased levels of PABP, eIF4F, or eIFiso4F substantially reduced the competitive advantage that a cap conferred to an mRNA. These observations suggest that eIF4F and eIFiso4F have undergone functional specialization that allows them to discriminate between mRNAs. Moreover, these observations suggest that developmental changes in the cellular concentration of eIF4F, eIFiso4F, or PABP may influence the extent of cap-dependent translation.

MATERIALS AND METHODS

**Plasmid Constructs and in Vitro RNA Synthesis—**The T7-based monocistronic and dicistronic luciferase constructs have been described previously (23). DNA concentration was quantitated spectrophotometrically following linearization and brought to 0.5 mg/ml. In vitro transcription was carried out as described previously (24) using 40 mM Tris- HCl (pH 7.5), 6 mM MgCl2, 100 μg/ml bovine serum albumin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM GTP, 10 mM dithiothreitol, 0.3 units/μl RNasin (Promega), and 0.5 units/μl T7 RNA polymerase. The constructs used terminated in a A 50 tail. Capped RNAs were synthesized using 5 μg of template in the same reaction mixture as described above, except that GTP was used at 160 μM, and 1 mM m7GpppG was included. Under these conditions, >85% of the mRNA is capped. The free energy of secondary structures used in this study was calculated at a temperature of 37 °C using MFOLD of GCG Software Package Version 10, which is based on the Zuker algorithm for determining multiple optimal and suboptimal secondary structures (25) with the folding parameters as described (26).

**Protein Purification and Western Analysis—**Wheat PABP (27), eIF4F and eIFiso4F (9), eIF4B (28), and recombinant eIF4G and eIFiso4E (30) were purified as described. The purification of eIF4F and eIF4E will be described elsewhere.

Proteins from control and depleted wheat germ lysates were resolved on a standard SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to 0.22 μm nitrocellulose membrane by electroblotting. Following transfer, the nitrocellulose membranes were blocked in 5% milk and 0.01% thimerosal in TPBS (0.1% Tween 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na2HPO4, and 0.14 mM KH2PO4), followed by incubation with primary antibodies diluted typically 1:1000 to 1:2000 in TPBS with 1% milk for 1.5 h. The blots were then washed twice with TPBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Southern Biotechnology Associates, Inc.) diluted to 1:10,000 for 1 h. The blots were washed twice with TPBS, and the signal was detected typically between 1 and 15 min using chemiluminescence (Amersham Pharmacia Biotech).

**In Vitro Translation Assays—**200 μl of wheat germ extract (Promega) was loaded to 300 μl of m7GTP-Sepharose (Amersham Pharmacia Biotech) or 100 μl of poly(A)-agarose (Sigma) and incubated with rotation at 4 °C for 30 min. The lysate was collected by centrifugation (800 x g for 1 min) through a spin column (Promega) and used immediately. The extent of depletion of eIF4F, eIF4E, eIFiso4G, eIFiso4E, eIF4A, eIF4B, eIF3, eEF2, PABP, or Hsp101 was determined by Western analysis following resolution of the extract by SDS-polyacrylamide gel electrophoresis. mRNA constructs were translated using complete or depleted wheat germ lysates as described by the manufacturer, except that all amino acids were unlabeled. The lysates were supplemented with recombinant initiation factors or factors purified from wheat germ extract as indicated. In wheat germ lysate, eIF4A is present in a >30-fold molar excess relative to eIF4G (31). Consequently, a similar ratio was used when lysates were supplemented with eIF4A. The ratio of eIF4B to eIF4F has not been measured; and therefore, the ratio used for supplementation was determined empirically. The reactions were incubated for 3 h, and 2-μl aliquots were assayed in a Monolight 2010 luminometer for luciferase activity. Each mRNA construct was translated in triplicate, and the mean ± S.D. for each construct is reported.

**RESULTS**

**eIF4F Targets Translation from an mRNA with a Structured 5'-Leader to a Greater Extent than Does eIFiso4F—**An alignment of eIF4G from wheat, human, and Saccharomyces cerevisiae revealed that plant eIF4G and eIFiso4G are most conserved with eIF4G of other eukaryotes in the region responsible for interaction with eIF4A and eIF3 (Fig. 1) (1). A second conserved region is the eIF4E-binding domain (1, 32). The Mnk1-binding domain and a second eIF4A-binding site have been mapped to the C-terminal region of human eIF4G (6, 8), but are not present in yeast and plant eIF4F proteins. However, plant eIF4G and eIFiso4G do contain a domain near their C terminus that shares limited conservation with the human
eIF4G and eIFiso4G Functionally Differ

3 K. S. Browning, unpublished data.

Fig. 2. Reduction in eIF4F and eIFiso4F from wheat germ lysate. 200 µl of wheat germ lysate was incubated with 200 µl of m7GTP-Sepharose for 30 min, and Western analysis was performed to confirm the reduction in the levels of eIF4E and eIF4G (i.e. subunits of eIF4F) and eIFiso4E and eIFiso4G (i.e. subunits of eIFiso4G). An equal amount of protein (60 µg) of each lysate was examined.

eIF4G proteins and is absent from the yeast orthologs. Plant eIFiso4G differs most from eIF4G in that it lacks an ~700-amino acid-long N-terminal region present in eIF4G. In this respect, plant eIF4G is more similar to human eIF4G than is eIFiso4G. The yeast eIF4G proteins also contain an N-terminal region, although it is shorter than that present in plant eIF4G or in either mammalian eIF4G protein. Although the domain responsible for interaction with PABP is not conserved among eIF4G proteins, it is located within this N-terminal region of human and yeast eIF4G proteins. The PABP interaction domain within plant eIF4G has not been identified precisely, but the putative site in eIFiso4G has been mapped to its N-terminal region (33).

To examine the function of eIF4G or eIFiso4G in vitro, it was necessary to generate an eIF4G- and eIFiso4G-dependent lysate. This was accomplished by depleting wheat germs lysate of eIF4F (composed of eIF4A and eIF4E) and eIFiso4F (composed of eIFiso4G and eIFiso4E) through their binding to m7GTP-Sepharose. Western analysis confirmed that the level of eIF4E and eIFiso4E was reduced by 90–95%, as was that of eIF4G and eIFiso4G (Fig. 2). To examine whether the depleted lysate was eIF4F- or eIFiso4F-dependent, capped luc-A50 mRNA was translated in a fractionated lysate supplemented with increasing amounts of purified eIF4F or eIFiso4F. The extent to which the reporter mRNA was translated was determined by measuring luciferase activity. A reduction in the level of eIF4F and eIFiso4F reduced translation by >95% (compare translation in complete and fractionated lysates) (Fig. 3), as would be expected following a reduction in those initiation factors that are normally required for efficient translation. Residual translational activity of the fractionated lysate may be the result of the low level of either eIF4G and eIFiso4G remaining in the lysate (Fig. 2) or a result of PABP (see below). Supplementation with 16 nM eIF4F increased translation from the reporter mRNA nearly 10-fold in the fractionated lysate, but did not affect translation of the same mRNA in the unfragmented lysate (Fig. 3). Translation in the fractionated lysate was also dependent on eIFiso4F, whereas supplementation of the unfragmented lysate with eIFiso4F did not affect translation (Fig. 3). A comparison of their relative effects on translation reveals that eIF4F was more stimulatory than was eIFiso4F. Native eIF4F and recombinant eIF4F are equally active in supporting translation in vitro, as are native eIFiso4F and recombinant eIFiso4F (30), as native eIFiso4F and recombinant eIFiso4F (30). Therefore, there is no significant difference in the fraction of each purified factor that is active. The stimulation afforded by eIFiso4F was more nonlinear at the highest concentration used for this factor than that observed for eIF4F, suggesting that even higher concentrations of eIFiso4F would not yield a level of translation comparable to that observed for eIF4F. The nonlinearity of the activity of these factors has been reported previously (34). The greater nonlinearity of eIF4F at lower concentrations may indicate its higher affinity for RNA compared with eIFiso4F, a possibility that is supported by the observation that the eIFiso4F dose-response curve is slightly sigmoidal (Fig. 3; see Fig. 5C). These data suggest that the endogenous level of eIF4F and eIFiso4F in the unfragmented lysate is necessary for maximum translational activity and that their removal from the lysate renders the fractionated lysate dependent upon the addition of either eIF4F or eIFiso4F.

To examine whether eIF4F and eIFiso4F differ in the extent to which they support translation of an mRNA with a structured 5'-leader, it was first necessary to determine the translational characteristics of mRNAs with or without secondary structure in the eIF4F/eIFiso4F-dependent lysate. A stable stem-loop structure containing a 24-base pair stem of m7GTP was introduced 4 nucleotides downstream of the 5' terminus of the luc reporter mRNA (referred to as SL24-luc-A50). In addition, deletions were made within the 24-base pair stem-loop to generate less stable structures with 19-, 13-, and 7-base pair stems of ΔG = −31.8, −21.3, and −4.5 kcal/mol, respectively (referred to as SL19-luc-A50, SL13-luc-A50, and SL7-luc-A50, respectively). The presence of these structures was shown previously to inhibit translation in unfragmented lysate as a function of their stability (35). To determine the effect of the stem-loop on translation, each construct was synthesized in vitro as a capped mRNA containing a 3'-A50 tail and translated at three different concentrations in a lysate reduced in eIF4F and eIFiso4F. When the eIF4F/eIFiso4F-dependent lysate was programmed with a 9.6 ng/µl concentration of each factor, the expression of the luc reporter mRNA was reduced by 95% (compare translation in the left and right panels) of each lysate was examined. The nonlinearity of the activity of the reduced amount of luciferase produced (compare the absolute levels of expression in Fig. 4 and note the difference in scale on the x axis). However, the presence of the same stem-loop structures was progressively less inhibitory as the concentration of the input mRNA decreased (see the relative values to the right of each histogram in Fig. 4, which are relative to the expression from the control luc-A50 mRNA), and translation from the SL7-luc-A50 and SL13-luc-A50 mRNAs was actually higher than...
that from the control mRNA at the lowest RNA concentration tested. Consequently, secondary structure of lower stability (e.g. SL17-tuc-A50 and SL13-tuc-A50 mRNAs) was inhibitory only at a high RNA concentration, whereas more stable secondary structure (e.g. SL19-tuc-A50 and SL24-tuc-A50 mRNAs) was inhibitory at all RNA concentrations tested, albeit less inhibitory at low RNA concentrations. These data suggest that the translational machinery, such as eIF4A, which functions as an RNA helicase and as a subunit of eIF4F (or eIFiso4F), is required during translation in direct proportion to the degree of secondary structure present in the 5'-leader of an mRNA (36), is sufficient to unwind moderately stable secondary structure when an mRNA is present at a low concentration. However, higher concentrations of mRNA may act to titrate the RNA helicase activity such that the structure cannot be removed from all of the input RNA, resulting in the inhibition of translation by secondary structure of even moderate stability.

To examine the effect of eIF4F or eIFiso4F on the translation of a structured mRNA, the SL13-tuc-A50 mRNA construct was selected as an mRNA with moderately stable secondary structure. This mRNA also exhibited the greatest potential range in expression as exemplified by the degree to which it was translated at high or low RNA concentrations (Fig. 4). Supplementation of the eIF4F/eIFiso4F-dependent lysate programmed with eIF4F increased translation from SL13-tuc-A50 mRNA up to 14.7-fold (Fig. 5B), whereas supplementation with eIFiso4F did not increase translation from the same mRNA (Fig. 5D). In contrast, translation from the control (i.e. unstructured) mRNA increased when the lysate was supplemented with either eIF4F (Fig. 5A) or eIFiso4F (Fig. 5C). For the structured mRNA (Fig. 5B), eIF4F was less stimulatory at high concentrations than at lower concentrations. This effect is also observed for unstructured mRNAs if eIF4F is added to the lysate to a level higher than was used for these studies (data not shown). An excess of eIF4F (i.e. a level of eIF4F that is in excess of the binding capacity of the input mRNA) may compete with the bound eIF4F for other factors needed for translation. The secondary structure present in the construct of Fig. 5B may reduce the amount of eIF4F that can bind the mRNA, which would result in a condition of excess eIF4F at a lower concentration than normally observed for an unstructured mRNA. Once in excess, free eIF4F may compete with the bound eIF4F for other necessary factors, resulting in an apparent lower level of stimulation. As eIF4F is considered to be present in limiting amounts in vivo in eukaryotes, such in vitro conditions of excess eIF4F are unlikely to be present in vivo. These data suggest that eIF4F and eIFiso4F differ considerably in their ability to promote translation from an mRNA containing a structured 5'-leader.

The introduction of each stem-loop increases the length of the 5'-leader relative to the construct lacking the secondary structure, which could have an effect on translation efficiency. For example, introduction of SL24, SL19, SL13, or SL7 results in a leader length of 64, 53, 41, or 29 nucleotides, respectively. To determine whether the length of the 5'-leader influences the degree to which eIF4F or eIFiso4F increases translation, capped luc-A50 mRNAs containing a 17-, 72-, or 144-nucleotide unstructured 5'-leader were translated in the eIF4F/eIFiso4F-dependent lysate and supplemented with eIF4F or eIFiso4F. Expression was affected only to a small extent as a function of the 5'-leader length, with a 2-fold increase in translation as a function of the length of the leader when increased from 17 to 72 nucleotides (Table I). The addition of eIF4F increased translation 4.4–6-fold, whereas an ~2-fold increase was observed for eIFiso4F. The length of the leader did not substantially alter the stimulatory effect of either factor, suggesting that neither eIF4F nor eIFiso4F discriminates between mRNAs based on their 5'-leader length.

eIF4F Promotes Cap-independent Translation to a Greater Extent than Does eIFiso4F—mRNAs that initiate protein synthesis using an internal ribosome entry site, such as those of the picornaviral family, often have long structured leader sequences. The observation that secondary structure proximal to the 5'-cap inhibits eIFiso4F function to a greater extent compared with eIF4F suggests that eIF4F may be able to promote cap-independent translation to a greater extent compared with eIFiso4F. To examine this possibility, capped and uncapped luc-A50 mRNAs were translated in the eIF4F/eIFiso4F-dependent lysate supplemented with increasing amounts of either factor. The addition of eIF4F at a concentration of just 2 nM increased translation from uncapped mRNA by 4.8-fold and from capped mRNA by 6.8-fold (Table II). Higher concentrations of eIF4F increased translation up to 11-fold. In contrast, eIFiso4F at a concentration of 5 nM increased translation from uncapped mRNA by only 1.4-fold, and no increase was observed for capped mRNA. Higher concentrations of eIFiso4F resulted in up to a 3.7-fold stimulation of translation from uncapped mRNA and 2.8-fold from capped mRNA. These data suggest that both eIF4F and eIFiso4F can promote cap-independent translation; however, eIF4F facilitates cap-independent translation to a greater extent than does eIFiso4F, consistent with its greater ability to promote translation from mRNAs that contain secondary structure proximal to the 5'-cap. Interestingly, despite the reduction in the level of eIF4F and eIFiso4F, there was little alteration in the extent to which translation was stimulated by the presence of a 5'-cap (Table II).

Wheat germ lysate is highly message-dependent because of a low concentration of endogenous transcript and a high level of unengaged translational machinery. As a consequence, those features that increase the competitiveness of an mRNA, such a 5’-cap, would not be expected to confer a translational advantage under the noncompetitive conditions that prevail in nor-

---

**Fig. 4.** 5'-Proximal secondary structure is inhibitory to translation in the eIF4F/eIFiso4F-reduced lysate. A stem-loop (SL) with a 7-, 13-, 19-, or 24-base pair stem was introduced 4 nucleotides downstream of the 5' terminus of luc-A50 mRNA, resulting in SL7-luc-A50, SL13-luc-A50, SL19-luc-A50, and SL24-luc-A50, respectively. The free energy (ΔG) of the control leader (i.e. present in luc-A50) and each stem-loop construct is indicated. The luc mRNA constructs were synthesized in vitro as capped polyadenylated mRNAs and translated in eIF4F/eIFiso4F-dependent wheat germ lysate at three concentrations: 9.6 ng/μl (left panel), 2.4 ng/μl (middle panel), and 0.5 ng/μl (right panel). Each mRNA construct was translated in triplicate, and the mean ± S.D. of the absolute level of expression from each construct is reported as a histogram. Luciferase expression is also indicated as a percentage (indicated to the right of each histogram) of the SL24-luc-A50 mRNA.
mal lysate. Accordingly, competitive translation might be achieved by increasing the concentration of mRNA to a level that exceeds the translational capacity of the lysate or by removing the excess capacity of those factors most important in determining competitive translation. To test the former prediction, complete and eIF4F/eIFiso4F-dependent lysates were programmed with increasing amounts of capped and uncapped luc-A50 mRNAs, and the cap dependence was determined by the ratio of protein synthesis from capped to uncapped mRNAs (Table III). The presence of a 5′-cap did not confer a translational advantage when the mRNA was present at a low concentration in either the complete or eIF4F/eIFiso4F-dependent lysate, results consistent with those in Table II. Note that the lower translation from capped mRNA relative to uncapped mRNA reflects a lower yield of in vitro transcribed mRNA when made in the capped versus uncapped form. As mRNA concentration was increased in the lysate, the presence of the 5′-cap became increasingly important in stimulating translation. These results suggest that even following a reduction in the level of eIF4F and eIFiso4F, translation remains largely less competitive than that observed in vivo and indicates another factor, such as PABP, that may be important in influencing cap function remains in excess in the fractionated lysate.

Because PABP interacts with the eIF4G and eIFiso4G subunits of eIF4F and eIFiso4F, respectively (33, 37), recruits eIF4G to an mRNA in the absence of a cap or functional eIF4E

**Fig. 5. eIF4F (but not eIFiso4F) supports the translation of an mRNA with 5′-proximal secondary structure.** The eIF4F/eIFiso4F-dependent lysate was programmed with 7.5 ng/μl luc-A50 (A and C) or SL-luc-A50 (B and D) mRNA and supplemented with the indicated amounts of eIF4F (A and B) or eIFiso4F (C and D). eIF4A was included at a 1:70 ratio of eIF4A to eIF4F or at a 1:30 ratio of eIF4A to eIFiso4F. Each mRNA construct was translated in triplicate, and the mean ± S.D. for each construct is reported. The 1-fold stimulation provided by eIF4F or eIFiso4F is indicated above each histogram. **Luc**, luciferase.

**TABLE I**

eIF4G and eIFiso4G functionally differ

Capped luc-A50 mRNAs containing a 17-, 72-, or 144-nucleotide 5′-leader were translated in the eIF4F/eIFiso4F-dependent lysate. The lysate was supplemented with the indicated amounts of eIF4F or eIFiso4F, and the amount of luciferase synthesized was measured in a luminometer. eIF4A was included at a ratio of 1:70 (for eIF4F assays) or 1:30 (for eIFiso4F assays). Luciferase expression is indicated as light units (LU) from 2 μl of each reaction. -Fold increase is relative to translation in depleted lysate without the addition of any factor. nt, nucleotides.

| 5-Leader length (nt) | No factor added | eIF4F (4 nM) | Increase | eIFiso4F (10 nM) | Increase |
|----------------------|----------------|-------------|-----------|----------------|---------|
| 17                   | 862,000 ± 14,700 | 5,210,000 ± 139,000 | 6.0 | 1,680,000 ± 30,200 | 1.9 |
| 72                   | 1,410,000 ± 19,700 | 6,230,000 ± 324,000 | 4.4 | 2,840,000 ± 122,000 | 2.0 |
| 144                  | 1,020,000 ± 10,200 | 4,840,000 ± 199,000 | 4.7 | 2,460,000 ± 118,000 | 2.4 |
**Table II**

The degree of cap-dependent translation increases with RNA concentration.

| RNA (ng/μl lysate) | Luciferase expression | Stimulation by a cap |
|-------------------|-----------------------|----------------------|
|                   | Uncapped mRNA | Capped mRNA | LU | fold | LU | fold |
|-------------------|---------------|-------------|-----|------|-----|------|
| 0                 | 130,000 ± 5600 | 118,000 ± 6490 | 1   | 0.9  |
| 2                 | 626,000 ± 21,300 | 803,000 ± 8830 | 4.8 | 1.3  |
| 4                 | 641,000 ± 23,100 | 1,080,000 ± 33,600 | 9.5 | 1.7  |
| 8                 | 1,230,000 ± 22,100 | 1,570,000 ± 3300 | 10  | 1.3  |
| 12                | 1,300,000 ± 44,300 | 1,310,000 ± 24,900 | 11  | 1.0  |
| 16                | 1,470,000 ± 19,100 | 1,180,000 ± 36,700 | 10  | 0.8  |

**Table III**

The control wheat germ lysate or the eIF4F/eIFiso4F-dependent lysate was programmed with increasing amounts of capped or uncapped luc-A50 mRNAs. The amount of luciferase synthesized was measured in a luminometer. Luciferase expression is indicated as light units (LU) from 2 μl of each reaction.

| eIF4F (nM)   | Uncapped mRNA | Capped mRNA | Stimulation by a cap |
|-------------|---------------|-------------|----------------------|
| 0           | 130,000 ± 5600 | 118,000 ± 6490 | 1.06 |
| 2           | 626,000 ± 21,300 | 803,000 ± 8830 | 6.8 |
| 4           | 641,000 ± 23,100 | 1,080,000 ± 33,600 | 9.2 |
| 8           | 1,230,000 ± 22,100 | 1,570,000 ± 3300 | 13 |
| 12          | 1,300,000 ± 44,300 | 1,310,000 ± 24,900 | 11 |
| 16          | 1,470,000 ± 19,100 | 1,180,000 ± 36,700 | 10 |

**FIG. 6**

Supplementation of depleted wheat germ lysate with initiation factors and PABP. Wheat germ lysate was incubated with m7GTP-Sepharose (lane 2) or poly(A)-agarose (lane 7) for 30 min, and Western analysis was performed to determine the extent of depletion of eIF4G, eIF4F, PABP, eIF3, eIF4A, and eIF4B. Western analysis of eEF2 and Hsp101 was included as a control. Unfractionated wheat germ lysate is included in the first and last lanes of each panel. An equal amount of protein (60 μg) of each lysate was examined. The amounts of each purified factor used for the supplementation experiments are represented in lanes 3–6 in the top six panels and lanes 3–5 in the bottom two panels. For eIF4G: lane 3, 16 nM; lane 4, 8 nM; lane 5, 4 nM; lane 6, 2 nM. For eIFiso4G: lane 3, 40 nM; lane 4, 20 nM; lane 5, 10 nM; lane 6, 5 nM. For PABP: lane 3, 411 nM; lane 4, 137 nM; lane 5, 69 nM; lane 6, 34 nM. For eIF4A: lane 3, 800 nM; lane 4, 300 nM; lane 5, 150 nM. For eEF2: lane 3, 55 nM; lane 4, 22.5 nM; lane 5, 11 nM. No signal is present in lanes 3–6 for eIF3, eEF2, and Hsp101, as these membranes represent those used for the analysis of eIF4G, eIFiso4G, and PABP that had been stripped and reprobed.
lysate was substantially lower (Table IV) than that observed in the eIF4F/eIFiso4F-dependent and complete lysates (Fig. 3), suggesting that in addition to eIF4F and eIFiso4F, PABP contributes to the overall translational activity of the lysate.

Both PABP and eIF4E function to recruit eIF4G to an mRNA, which is a necessary prerequisite for the recruitment of the 43S complex. Although PABP may recruit eIF4G to an mRNA whether a 5' cap is present or not, the effect of PABP on eIF4F recruitment would be expected to be greater for a capped mRNA, in which eIF4E (which binds to the cap) could contribute more to the recruitment of eIF4G, than for an uncapped mRNA, to which eIF4E would not be able to bind. Consistent with the prediction of a preferential recruitment of eIF4F by PABP to a capped mRNA, the presence of PABP at an appropriate concentration would be expected to increase the extent to which translation is cap-dependent. To test these predictions, capped and uncapped mRNAs were translated in the PABP-depleted lysate in the presence of increasing amounts of PABP (Fig. 7). In the lysate depleted of PABP and reduced in the level of eIF4F and eIFiso4F (Fig. 6), the presence of a 5'-cap stimulated translation by 4.2-fold (Fig. 7). Supplementation with low concentrations of PABP increased the translation of both the capped and uncapped mRNAs, but did so preferentially for the mRNA with a 5'-cap, resulting in an increase in the stimulation afforded by a cap of up to 11.2-fold. As the concentration of PABP was increased further, the competitive advantage conferred by a cap was increasingly lost, resulting in a decline in translation specifically from the capped mRNA and, consequently, a reduction in cap function. The data presented in Table IV and Fig. 7 suggest that the concentration of PABP contributes to the extent to which a cap stimulates translation from an mRNA: the presence of a moderate amount of PABP preferentially stimulates the translation of capped mRNA, presumably through the recruitment of eIF4G as a result of the combined efforts of PABP and eIF4E. However, the competitive advantage provided by a 5'-cap is lost with an increase in the concentration of eIF4F, eIFiso4F, or PABP.

**TABLE IV**

| Compounds | eIF4F (nM) | eIFiso4F (nM) |
|-----------|------------|---------------|
| Uncapped mRNA | Capped mRNA | Increase | Increase |
| LU | -fold | LU | -fold |
| 0 | 657 ± 23 | 1 | 6910 ± 390 | 10 |
| 2 | 15,700 ± 850 | 24 | 103,000 ± 5050 | 6.6 |
| 4 | 67,400 ± 1890 | 100 | 198,000 ± 4750 | 2.9 |
| 8 | 229,000 ± 20,800 | 350 | 377,000 ± 9050 | 5.5 |
| 16 | 522,000 ± 46,900 | 790 | 627,000 ± 32,600 | 1.2 |

**Fig. 7.** PABP is required for optimal cap-dependent translation. The PABP-dependent lysate was programmed with (0.6 ng/μl) capped or uncapped luc-A50 mRNAs. The lysate was supplemented with the indicated amounts of PABP, and the amount of luciferase synthesized was measured in a luminometer. Luciferase expression is indicated as light units from 2 μl of each reaction.

The second cistron was translated as an uncapped mRNA in the eIF4F/eIFiso4F-dependent lysate in the presence of increasing amounts of eIF4F or eIFiso4F. Because the level of eIF4A and eIF4B was reduced in the eIF4F/eIFiso4F-dependent lysate, the supplementation of the lysate with eIF4F or eIFiso4F was performed in the presence or absence of additional eIF4A and eIF4B (Fig. 8). Translation from the second cistron was then determined by measuring the resulting level of luciferase synthesized. The addition of 16 nM eIF4F (together with eIF4A and eIF4B) increased translation from the second cistron up to 16-fold (at 4 nM), whereas the addition of 40 mM eIFiso4F (together with eIF4A and eIF4B) increased translation only 3-fold (Fig. 8). When eIF4F or eIFiso4F was added in the absence of additional eIF4A and eIF4B, eIF4F increased translation from the second cistron up to 16-fold (at 4 nM), whereas the addition of eIFiso4F increased translation only 3-fold (at 7.5 nM) (Fig. 8). Interestingly, the maximum increase in translation from the second cistron occurred at 4 nM eIF4F or 7.5 mM eIFiso4F, and higher levels of either factor resulted in lower levels of internal initiation. This is in contrast to the data obtained when eIF4A and eIF4B were present; translation from the second cistron continued to increase as a function of the increase in eIF4F and eIFiso4F (up to 16 and 40 nM, respectively), suggesting that eIF4A and/or eIF4B increased the activity of eIF4F and eIFiso4F over a greater concentration range perhaps because the endogenous level of eIF4A and eIF4B had become limiting.
than does eIFiso4F. mRNA, it does not substantially promote internal initiation to stimulate translation from a 5′/H11032ulatory effect (Fig. 9), suggesting that, in contrast to its ability with PABP at a lower concentration than did recombinant eIF4G or eIFiso4G. The addition of either eIFiso4F-dependent lysate supplemented with increasing concentrations (Fig. 9). The addition of PABP had only a small stimulation of eIF4A and eIF4B was included with eIF4F or eIFiso4F. The degree to which the second cistron of the dicistrionic mRNA was translated in the eIF4F/eIFiso4F-dependent lysate. The lysate was supplemented with the indicated amounts of eIF4F or eIFiso4F in which eIF4A and eIF4B were added at ratios of 1:70 and 1:4, respectively, for eIF4F and eIFiso4F. Right panel, the lysate was supplemented with the indicated amounts of eIF4F or eIFiso4F in the absence of eIF4A and eIF4B. The degree to which the second cistron of the dicistrionic mRNA was translated in each assay was determined by the amount of luciferase synthesized. Luciferase expression is indicated as light units from 2 μl of each reaction.

These data suggest that although eIF4F and eIFiso4F can stimulate internal initiation, they require eIF4A and/or eIF4B for their maximum stimulatory effect.

To determine whether eIF4A or eIF4B was responsible for increasing the ability of eIF4F or eIFiso4F to promote translation from the second cistron, the dicistrionic mRNA was translated in the eIF4F/eIFiso4F-dependent lysate with factors added either separately or in combination (Table V). The addition of eIF4A or eIF4B alone increased translation from the second cistron by 2.6- and 2.4-fold, respectively, whereas the addition of eIF4A and eIF4B had a 2.1-fold effect. The combination of eIF4A and eIF4F or of eIF4A and eIFiso4F had a synergistic effect on increasing second cistron translation, as did the combination of eIF4B and eIF4F or of eIF4B and eIFiso4F, although not as great as when eIF4A was employed. An even greater stimulation of translation was observed when a combination of eIF4A and eIF4B was included with eIF4F or eIFiso4F.

To determine whether it was the eIF4G subunit of eIF4F that was responsible for increasing translation from the second cistron, the dicistrionic mRNA was translated in the eIF4F/eIFiso4F-dependent lysate supplemented with increasing amounts of recombinant eIF4G or eIFiso4G. The addition of eIF4G increased translation from the second cistron up to 20-fold, whereas the addition of eIFiso4G increased translation ~2-fold (Table VI). eIF4A was less effective in stimulating recombinant eIF4G function than it was for native eIF4F, suggesting that the native form may be more competent for interaction with this partner protein. Similar observations were made previously, in which native eIF4F interacted with PABP at a lower concentration than did recombinant eIFiso4G or recombinant eIF4F (33). The addition of either eIF4E or eIFiso4E did not increase translation from the second cistron and even decreased translation slightly at high concentrations (Fig. 9). The addition of PABP had only a small stimulatory effect (Fig. 9), suggesting that, in contrast to its ability to stimulate translation from a 5′-proximal cistron of a capped mRNA, it does not substantially promote internal initiation under the conditions used. Together, these results suggest that it is the eIF4G (or eIFiso4G) subunit that is responsible for promoting internal initiation and that eIF4G promotes internal initiation to a greater extent than does eIFiso4G. Moreover, the relative effect of recombinant eIF4G compared with eIFiso4G (Table VI) was very similar to that obtained for native eIF4F and eIFiso4F (Table V), supporting the conclusion that the observed differences are inherent properties of these proteins and not a result of differences in the active fraction of each factor preparation.

**DISCUSSION**

The presence of two highly divergent eIF4G proteins in plants has suggested the possibility of their functional specialization. The results presented in this study indicate that the greatest difference between these two factors was observed for capped mRNAs containing a structured 5′-leader: eIF4F supported translation from such an mRNA to a greater extent than did eIFiso4F. The presence of secondary structure within a leader blocks efficient scanning of the 40 S ribosomal subunit (40, 41). eIF4F exhibits higher activity than does eIFiso4F when mRNA is used to stimulate the RNA-dependent ATPase activity in the presence of eIF4A (20). This helicase activity is required for the ATP-dependent unwinding of mRNA secondary structure (17–20). The higher ATP hydrolysis activity of eIF4F is consistent with its greater ability to promote translation from structured mRNA. The present observations and the

---

**TABLE V**

| Reaction | eIF4F | eIFiso4F | eIF4A | Luciferase | Increase |
|----------|-------|---------|-------|-----------|----------|
|          | nM    | nM      | μM    | nM        | LU       | -fold    |
| 1        | 0     | 0       | 0     | 0         | 137 ± 2  | 1        |
| 2        | 16    | 0       | 0     | 0         | 2580 ± 28| 19       |
| 3        | 0     | 0       | 1.2   | 0         | 355 ± 3  | 2.6      |
| 4        | 0     | 0       | 21    | 110       | 237 ± 9  | 2.4      |
| 5        | 16    | 0       | 1.2   | 0         | 11,300 ± 588| 83   |
| 6        | 16    | 0       | 0     | 110       | 7320 ± 270| 53      |
| 7        | 16    | 0       | 1.2   | 110       | 13,800 ± 179| 100 |
| 8        | 0     | 40      | 0     | 0         | 291 ± 6  | 2.1      |
| 9        | 0     | 40      | 1.2   | 0         | 1990 ± 46| 15       |
| 10       | 0     | 40      | 1.2   | 110       | 530 ± 14 | 3.9      |
| 11       | 0     | 40      | 1.2   | 110       | 1700 ± 37| 12       |

**TABLE VI**

| Reaction | eIF4G | eIFiso4G | eIF4A | Luciferase | Increase |
|----------|-------|---------|-------|-----------|----------|
|          | nM    | nM      | μM    | nM        | LU       | -fold    |
| 1        | 0     | 0       | 0     | 0         | 2990 ± 36| 1        |
| 2        | 0     | 0       | 1.2   | 0         | 3000 ± 104| 1.0    |
| 3        | 1.8   | 0       | 1.2   | 11,000 ± 779| 3.7   |
| 4        | 3.5   | 0       | 1.2   | 32,800 ± 688| 11      |
| 5        | 7     | 0       | 1.2   | 59,100 ± 1060| 20     |
| 6        | 7     | 0       | 0     | 47,100 ± 1270| 16     |
| 7        | 0     | 7       | 1.2   | 5410 ± 124 | 1.8     |
| 8        | 0     | 14      | 1.2   | 4880 ± 68  | 1.6     |
| 9        | 0     | 28      | 1.2   | 5810 ± 42  | 1.9     |
| 10       | 0     | 28      | 0     | 6960 ± 174 | 2.3     |
The degree to which a 5' cap stimulated translation from an mRNA was partially dependent on the presence of PABP. The addition of a moderate level of PABP to a PABP-depleted lysate preferentially increased translation from capped mRNAs, thereby increasing the cap dependence of the lysate. PABP promotes the recruitment of 40 S ribosomal subunits to an mRNA through its physical contact with eIF4G and increases eIF4F binding to the 5'-cap (4, 7, 33, 50–53), suggesting that the stimulatory effect on cap function observed in Fig. 7 resulted from an increase in eIF4F recruitment to a capped mRNA. Although a moderate level of PABP stimulated cap function, higher concentrations (equivalent to those present in unfraccionated wheat germ lysate) failed to stimulate cap-dependent translation. Similarly, increasing the concentration of eIF4F or eIFiso4F resulted in a decrease in cap dependence. Consequently, the high endogenous level of unengaged eIF4F, eIFiso4F, and PABP in wheat germ lysate appears to be responsible for the low level of cap dependence typically observed in the unfraccionated lysate. These observations suggest that, in addition to eIF4F and eIFiso4F, the concentration of PABP also influences the extent of cap-dependent translation in a cell.

Under what circumstances might the functional differences observed between eIF4F and eIFiso4F be important in plants? mRNAs that contain secondary structure or one or more small open reading frames in the 5'-leader are just two strategies that have evolved to limit the amount of protein synthesized from an mRNA (reviewed in Refs. 54 and 55). Stable secondary structure within a leader can impede the scanning of the 40 S ribosomal subunit in its search for the initiation codon and thereby inhibit translation (40, 41, 56, 57), whereas upstream open reading frames require the involvement of re-initiation or internal initiation mechanisms if the downstream cistron is to be translated (58). Secondary structure within the 5'-leader of an mRNA may serve to regulate translation in response to alterations in the amount or activity of the translational machinery. As eIF4F did not stimulate translation substantially from an mRNA containing a structured leader, eIF4F (or eIF4G) and its cellular concentration may be largely responsible for determining the extent to which structured mRNAs are translated. In plants, the level of eIF4F (but not eIF4E, eIFiso4G, or eIFiso4E) increases substantially during late seed development (59), a developmental stage in which most soluble proteins, including eIFiso4G, undergo proteolysis as the seed prepares to enter a metabolically quiescent stage as part of its maturation program. The developmentally late increase in eIF4G concentration may be required to facilitate the translation of mRNAs under the conditions that prevail during late seed development. The level of eIF4G (but not eIF4E) also increases following a heat shock as a function of the severity of the stress (59). Heat stress results in a loss of cap function and a preferential increase in the translation of uncapped mRNAs to a level equal that of capped mRNAs (60). These in vivo observations are similar to the loss-of-cap function and a preferential increase in the translation of capped mRNAs following an increase in eIF4F concentration (Table IV).

In conclusion, our results suggest that eIF4G (as part of eIF4F) in addition to supporting translation from normal capped mRNAs, may function to facilitate translation from nonstandard mRNAs, i.e. those that contain secondary structure proximal to the 5'-cap, those that lack a cap, and those that contain more than one cistron. In contrast, the function of eIFiso4G (as part of eIFiso4F) may be largely limited to supporting translation from standard mRNAs, i.e. capped mRNAs with an unstructured 5'-leader.
eIF4G Functionally Differs from eIFiso4G in Promoting Internal Initiation, Cap-independent Translation, and Translation of Structured mRNAs
Daniel R. Gallie and Karen S. Browning

J. Biol. Chem. 2001, 276:36951-36960.
doi: 10.1074/jbc.M103869200 originally published online August 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103869200
Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 42 of which can be accessed free at
http://www.jbc.org/content/276/40/36951.full.html#ref-list-1