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Translational activation of uncapped mRNAs by the central part of human eIF4G is 5′ end-dependent

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ABSTRACT

Translation initiation factor (eIF) 4G represents a critical link between mRNAs and 40S ribosomal subunits during translation initiation. It interacts directly with the cap-binding protein eIF4E through its N-terminal part, and binds eIF3 and eIF4A through the central and C-terminal region. We expressed and purified recombinant variants of human eIF4G lacking the N-terminal domain as GST-fusion proteins, and studied their function in cell-free translation reactions. Both eIF4G lacking its N-terminal part (aa 486–1404) and the central part alone (aa 486–935) exert a dominant negative effect on the translation of capped mRNAs. Furthermore, these polypeptides potently stimulate the translation of uncapped mRNAs. Although this stimulation is cap-independent, it is shown to be dependent on the accessibility of the mRNA 5′ end. These results reveal two unexpected features of eIF4G-mediated translation. First, the C-terminal eIF4A binding site is dispensable for activation of uncapped mRNA translation. Second, translation of uncapped mRNA still requires 5′ end-dependent ribosome binding. These new findings are incorporated into existing models of mammalian translation initiation.

Keywords: translation initiation factors; cap-independent translation; 2′O-Allyl oligoribonucleotides; IRP1; cap-analogue

INTRODUCTION

Initiation of protein synthesis in eukaryotes involves the sequential binding of 40S and 60S ribosomal subunits to a messenger RNA molecule, leading to the assembly of a complete 80S ribosome at the initiator codon for subsequent polypeptide formation. A crucial step is the binding of the 43S preinitiation complex, comprising the 40S ribosomal subunit, methionine-initiator tRNA, GTP, and the initiation factors eIF2 and eIF3, to the mRNA 5′ end. This recruitment of the 43S complex is considered to constitute the rate-limiting step in translation, and generally requires the presence of the m^7GpppN cap structure at the 5′ end of the mRNA (Merrick & Hershey, 1996). The cap structure is recognized by the cap-binding protein eIF4E, a process that is greatly stimulated by interaction with the N-terminal part of eIF4G (Haghighat & Sonenberg, 1997), to form the core of the eIF4F complex. Recent work on mammalian eIF4G has revealed that it interacts also with eIF4A, an RNA-dependent ATPase, through its C terminal, and with eIF3, a multiprotein complex, through its central third (Lamphear et al., 1995). Additionally, the central part of eIF4G also harbors a second site for eIF4A interaction (Imataka & Sonenberg, 1997) (see also Fig. 1). This modular nature of eIF4G supports models according to which small ribosomal subunits are recruited to capped mRNAs via the simultaneous association of eIF4G with both eIF3 and eIF4E (Hentze, 1997; Morley et al., 1997). It is thought that this cap-dependent binding near the 5′ end is followed by linear migration of the preinitiation complex along the mRNA, ensuring faithful recognition of the authentic initiator codon (Jackson, 1996). There, the complex reorganizes and is joined by the 60S subunit.

There are indications for an even more versatile nature of eIF4G (Hentze, 1997). In yeast, biochemical evidence exists for a direct interaction between eIF4G and the poly(A) binding protein (Pab1p) as a basis for poly(A) tail-promoted translation (Tarun & Sachs, 1996; Sachs et al., 1997; Tarun et al., 1997). Furthermore, eIF4G also functions during internal initiation of translation on some viral and cellular mRNAs. This type of translation depends on the presence of specialized RNA structures within the 5′ untranslated region (UTR) of these mRNAs

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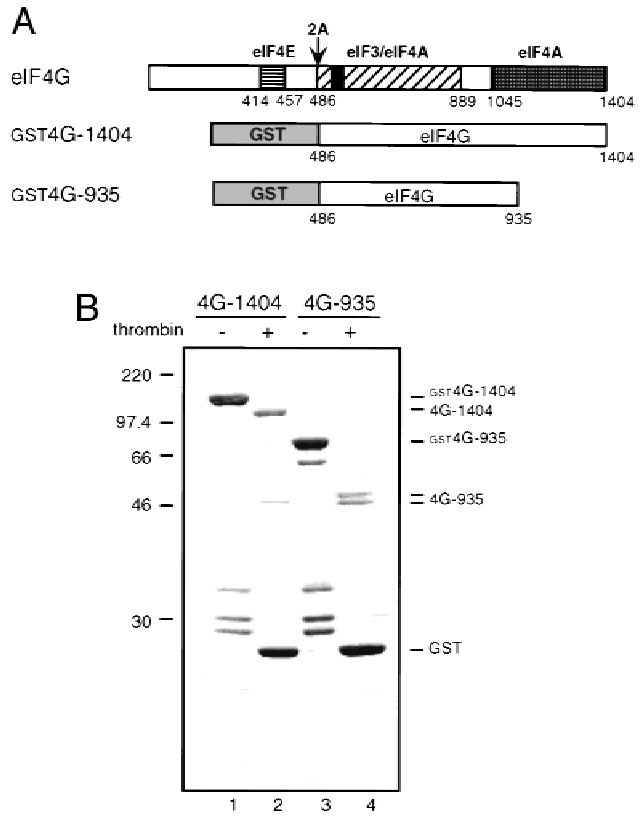

FIGURE 1. Expression of recombinant eIF4G variants. **A:** Schematic representation of the functional domains of human eIF4G and the recombinant GST-fusion proteins used in this study. The regions for interaction with eIF4E, eIF3, and eIF4A (Lamphear et al., 1995; Imataka & Sonenberg, 1997) are boxed, and the 2A protease cleavage site is indicated by an arrow. The black box in eIF4G represents the region of homology with the putative RNA-binding domain of the yeast eIF4Gs (Goyer et al., 1993). **B:** Two micrograms of purified recombinant proteins GST4G-1404 and GST4G-935, before (lanes 1 and 3) and after (lanes 2 and 4) thrombin cleavage, were subjected to SDS-PAGE and stained with Coomassie Blue. Molecular mass markers (in kDa) are indicated on the left. Positions of recombinant proteins and the GST fragment are indicated on the right.

termed internal ribosome entry sites (IRES) (Pelletier & Sonenberg, 1988; Ehrenfeld, 1996; Pestova et al., 1996; Sachs et al., 1997). During infection by picornaviruses of the enterovirus, rhinovirus, and aphtovirus groups (Belsham & Sonenberg, 1996), eIF4G is cleaved by viral proteases to yield an N-terminal third containing the binding site for eIF4E and a larger C-terminal part containing the binding sites for eIF3 and eIF4A (Lamphear et al., 1993, 1995). This cleavage coincides with inhibition of cellular cap-dependent translation and activation of the viral IRES-driven translation (Belsham & Sonenberg, 1996). The C-terminal fragment resulting from eIF4G cleavage has been directly implicated in the activation of IRES-driven translation (Lamphear et al., 1995; Pestova et al., 1996). In support of this, experiments in rabbit reticulocyte lysate (RRL) have shown that cleavage of eIF4G by viral proteases inhibits translation of capped mRNAs, but enhances that of IRES-containing mRNAs and, curiously, also of uncapped mRNAs (Ohlmann et al., 1995, 1996; Borman et al., 1997). These effects are still detectable after depleting eIF4E and the N-terminal fragment of eIF4G (Ohlmann et al., 1996). Activation of the translation of uncapped mRNAs was also seen when purified rabbit eIF4G C-terminal fragment is added to RRL (Borman et al., 1997; Ohlmann et al., 1997).

The two eIF4Gs from *Saccharomyces cerevisiae* were reported to possess an RNA recognition motif (RRM) in the region corresponding to the central domain of the mammalian homologue (Goyer et al., 1993). Although this central region appeared to be poorly matched by the originally published human eIF4G amino acid sequence (Yan et al., 1992), the correction of frameshifting sequencing errors (Imataka & Sonenberg, 1997 and this study) now reveals substantial homology. Correlating with this, recombinant human eIF4G fragments were shown to bind directly to the IRES of EMCV virus (Pestova et al., 1996). Direct mRNA binding by eIF4G might, therefore, substitute for binding to eIF4E during IRES-mediated initiation of translation.

In this study, we generated recombinant eIF4G polypeptides corresponding to the C-terminal viral cleavage product (aa 486–1404) or the central portion of human eIF4G (aa 486–935) in *Escherichia coli* (Fig. 1A) to characterize the effects on translation of capped and uncapped mRNAs in RRL. Using the translational repressor protein IRP-1 or antisense 2′ O-allyl oligoribonucleotides as additional experimental tools, we revealed unexpected functional properties of the central region of eIF4G.

RESULTS

Expression of recombinant variants of human eIF4G

Sequences from the human eIF4G cDNA were inserted into pGEX-2T for bacterial expression as N-terminal GST-fusion proteins. All constructs start from codon 486 of human eIF4G, corresponding to the cleavage site of viral 2A protease (Lamphear et al., 1993). GST4G-1404 contains both the central and C-terminal third, whereas GST4G-935 represents only the central region of eIF4G. The domain structure of eIF4G and previously mapped binding regions for eIF4E, eIF3, and eIF4A (Lamphear et al., 1995; Mader et al., 1995; Imataka & Sonenberg, 1997) are indicated in Figure 1A. After purification, GST4G-1404 and GST4G-935 were digested with thrombin to remove the GST domain from the recombinant proteins (see Materials and Methods), yielding the 4G-1404 and 4G-935 preparations. In Figure 1B are shown recombinant proteins before (lanes 1 and 3) and after thrombin cleavage (lanes 2 and 4). Both GST fusion proteins (Fig. 1B, lanes 1 and 3) contain smaller peptides, which likely are products of C-ter-

minal degradation of eIF4G because they are also cleaved by thrombin. 4G-935 appears as a doublet, possibly resulting from thrombin cleavage at aa 514, where another Pro-Arg-Lys motif is present in a suitable context.

The central domain of human eIF4G acts as a dominant negative inhibitor of capped and as an activator of uncapped mRNA translation in vitro

The dependence of translation on the cap structure in RRL can be manipulated experimentally (see Jackson et al., 1995 for review). Suboptimal K^+ concentrations (Chu & Rhoads, 1978) and high mRNA concentrations (Chu & Rhoads, 1980) reduce the inhibitory effect of cap-analogue (m^7GpppG) on translation. More recently, it has been shown that the efficiency of translation of uncapped versus capped mRNA increases at high mRNA concentration (0–32 ng/μL) (Svitkin et al., 1996). We tested this effect for two different mRNAs, encoding chloramphenicol acetyltransferase (CAT) or the yeast nucleolar protein NopI, and obtained qualitatively similar results to those reported previously (Svitkin et al., 1996), although at lower mRNA concentrations (0–4.2 ng/μL, data not shown). In the following translation assays, the amounts of capped and uncapped mRNAs were adjusted to yield comparable signal intensities for CAT and Nop I proteins.

The effects of GST4G-1404 and GST4G-935, before and after thrombin cleavage, on the translation of capped (c-) Nop I mRNA and uncapped (u-) CAT mRNA are shown in Figure 2A. Cap-dependent translation is inhibited by both recombinant proteins (compare the translation of c-NopI mRNA in Fig. 2A, lanes 4, 5, 7, and 8 with lane 3), albeit to different extents. The effect is more pronounced when GST4G-1404 is used instead of GST4G-935, and before removal of the GST domain. By contrast, uncapped mRNA translation is activated when GST4G-1404 and GST4G-935 are added (compare the translation of u-CAT mRNA in Fig. 2A, lanes 4, 5, 7, and 8 with lane 3). No effects on translation were observed with either the untreated (Fig. 2A, lane 6) or the thrombin-cleaved GST domain (Fig. 2A, lane 9), as controls. The activation is higher in the case of GST4G-935 and after thrombin cleavage (Fig. 2A, lanes 7 and 8), indicating that the N-terminal GST domain of the recombinant fusion proteins partially interferes with their function. Interestingly, this correlates with approximately 10-fold increases in RNA binding by 4G-1404 and 4G-935 after cleavage by thrombin, when the 5′-UTR of CAT mRNA is used as a probe in gel shift experiments (data not shown).

Next, u- or c-CAT mRNAs were incubated with different amounts of 4G-1404 (Fig. 2B). The results were quantified and plotted in Figure 2C. The activation of u-CAT translation is dose-dependent and detectable at

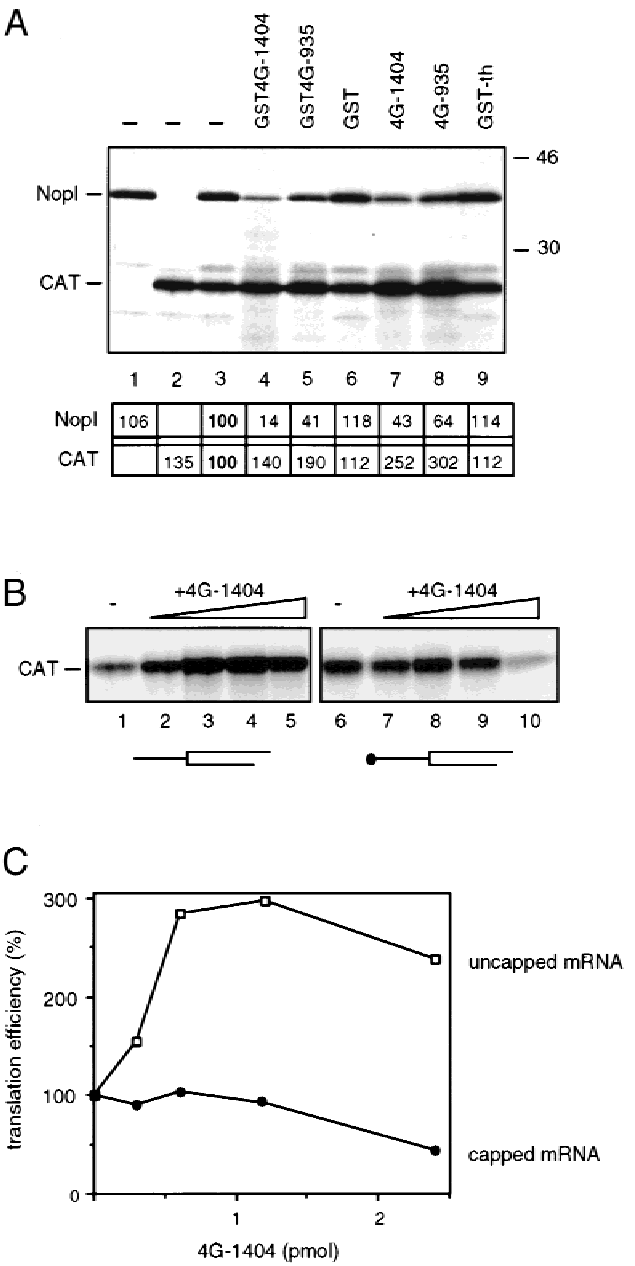

FIGURE 2. Effect of recombinant eIF4G fragments on translation in RRL. **A:** Five nanograms of c-NopI mRNA (lane 1) or 12.5 ng of u-CAT mRNA (lane 2) or both (lanes 3–9) were incubated with buffer (lanes 1, 2, 3); 2.4 pmol of GST4G-1404 (lane 4), GST4G-935 (lane 5), or GST (lane 6); or 2.4 pmol of 4G-1404 (lane 7), 4G-935 (lane 8), and GST-th (lane 9). The table below gives the efficiency of c-NopI and u-CAT translation in the different conditions compared to the controls in lane 3 (set to 100%). Molecular size markers (in kDa) are indicated on the right. **B:** Dose-dependent activation of uncapped mRNA translation (left panel) and inhibition of cap-dependent translation (right panel) by 4G-1404. u-CAT (12.5 ng) or c-CAT (5 ng) mRNAs were incubated in the absence (lanes 1, 6) or presence of 0.3 (lanes 2, 7), 0.6 (lanes 3, 8), 1.2 (lanes 4, 9), or 2.5 pmol (lanes 5, 10) of 4G-1404. **C:** Quantitative analysis of the experiment shown in B. Translation in the absence of recombinant proteins was set to 100% for both u-CAT and c-CAT mRNAs. The same dose-response pattern for activation of uncapped mRNA translation was found in three independent experiments. The maximal level of activation by 4G-1404 (or by 4G-935, compare Figs. 3 and 6) was found to vary from approximately three- to sixfold, depending on the batch of RRL used.

low amounts of added 4G-1404 (0.3 pmol, Fig. 2B, lane 2), whereas the inhibition of c-CAT mRNA translation requires higher amounts (2.4 pmol, Fig. 2B, lane 10). We expect this range of addition to be roughly comparable to the amount of endogenous eIF4G in our assay, based on previous determinations of eIF4A concentration in RRL (Pause et al., 1994) and the molar ratio of eIF4A to eIF4G in eukaryotic cells (Duncan et al., 1987). Because no other mRNA was added to the reaction, we can exclude that this activation results from a competition between different reporter mRNAs. Furthermore, the effects of 4G-1404 and 4G-935 on translation are mRNA sequence-independent as they are equally observable using the c- or u-version of either NopI or CAT mRNAs (Fig. 3).

Translational activation of uncapped mRNAs by 4G-935 requires a free 5′ end

Translation of uncapped mRNAs at high concentrations in RRL has been suggested to involve ribosome binding to sites within the body of the message (Dasso & Jackson, 1989; Svitkin et al., 1996). In contrast to these results, recently it has been reported that translation of uncapped, nonadenylated mRNAs in vivo involves scanning and depends on the presence of a free 5′ end (Gunnery et al., 1997). We decided to address this issue in our assay by blocking the 5′ end of uncapped mRNA in two different ways. Antisense 2′ O-allyl oligoribonucleotides bound to the mRNA 5′ end had been shown previously in RRL to inhibit the translation of capped mRNAs while not affecting internal translation initiation driven by an IRES (Johansson et al., 1994). Furthermore, they had been instrumental in revealing 5′ end-independent translation initiation mediated by the poly(A) tail in yeast extract (Preiss & Hentze, 1998). Therefore, we first hybridized antisense 2′ O-allyl oligoribonucleotides to the 5′ end (oligo 1) or to a downstream position within the open reading frame of the CAT mRNA (oligo 169) as a negative control (Fig. 4A), and translated the hybrids in RRL (Fig. 4B). Oligo 1 efficiently represses translation of a capped mRNA (Fig. 4B, lane 3), whereas oligo 169 has only a minor effect (Fig. 4B, lane 4). The noncomplementary oligo X serves as an additional negative control (Fig. 4B, lane 2). The specific repression by oligo 1 is equally manifest when an uncapped mRNA is examined (Fig. 4B, lanes 5–8), indicating that, at least at these low mRNA concentrations, translation of uncapped mRNA is 5′ end-dependent. This finding is consistent with the lack of detectable smaller polypeptides that would be expected to be generated if internal ribosome binding had occurred (Preiss & Hentze, 1998). Interestingly, when translation of u-CAT mRNA is activated by 4G-935 (Fig. 4B, compare lane 9 with lane 5), identical responses to the presence of the different oligos are observed (Fig. 4B, lanes 10–12). This result suggests that 4G-935-promoted initiation on an uncapped mRNA is 5′ end-dependent. The residual translation of capped or uncapped CAT mRNA in the presence of oligo 1 (Fig. 4B, lanes 3 and 7) most likely reflects incomplete hybrid formation rather than a low level of 5′- and cap-independent initiation. Contrary to the experimental observation, such

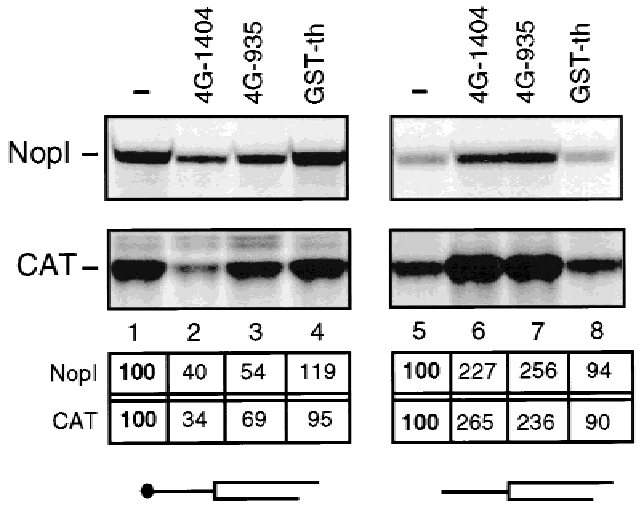

FIGURE 3. Effects of the eIF4G variants are not restricted to specific mRNAs. Five nanograms of c- (left panels) or 12.5 ng of u- (right panels) NopI and CAT mRNAs were incubated in the absence of recombinant proteins (lanes 1, 5), in the presence of 2.4 pmol of 4G-1404 (lane 2), 4G-935 (lane 3), and GST-th (lane 4), or in the presence of 0.6 pmol of 4G-1404 (lane 6), 4G-935 (lane 7), and GST-th (lane 8). The table below gives the efficiency of translation relative to the translation in the absence of recombinant proteins (lanes 1 and 5, set to 100%).

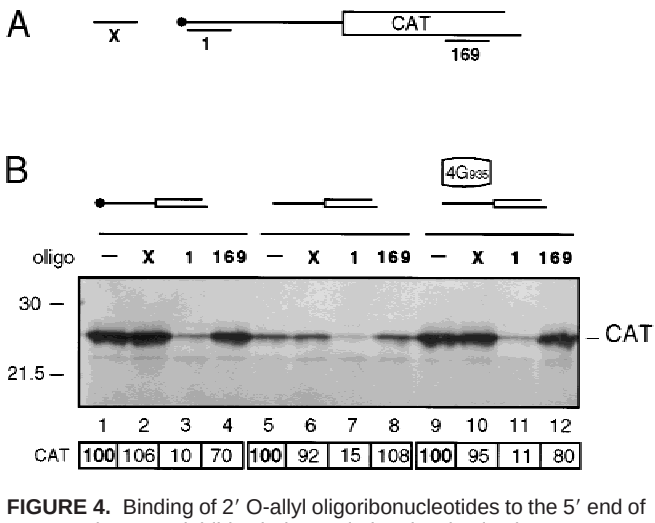

FIGURE 4. Binding of 2′ O-allyl oligoribonucleotides to the 5′ end of uncapped mRNAs inhibits their translational activation by 4G-935. **A:** Schematic representation of the OT.CAT mRNA 5′ UTR. Target positions of the oligonucleotides are indicated. **B:** Pre-annealed mixtures of the oligonucleotides indicated above the lanes and 2.5 ng of c-OT.CAT (lanes 1–4) or 12.5 ng of u-OT.CAT mRNA (lanes 5–12) were translated in the absence of recombinant proteins (lanes 1–8) or in the presence of 1.2 pmol of 4G-935 (lanes 9–12). The table shows the efficiency of translation relative to the translation in the absence of oligonucleotides (lanes 1, 5, and 9, set to 100%).

internal initiation events would be expected to yield similar absolute levels of translation under both conditions (Fig. 4B, compare lanes 3 and 7).

This finding is independently confirmed using an mRNA with an iron-responsive element (IRE) close to the 5′ end (IRE-CAT) (Fig. 5A). The specific binding of IRP-1 to this IRE represses translation by preventing the association of the small ribosomal subunit with capped mRNAs (Gray & Hentze, 1994). In Figure 5B, u-IRE-CAT mRNA was incubated with increasing amounts of 4G-935 protein and an excess of IRE competitor RNA (lanes 1–5) or recombinant IRP-1 (lanes 6–10). The IRE competitor is used to sequester the endogenous IRP activity present in RRL (Gray & Hentze, 1994). The binding of IRP-1 reduces translation of u-IRE-CAT to 7% in the absence of 4G-935 (Fig. 5B, lanes 1 and 6), confirming that uncapped mRNA translation is still 5′ end-dependent. Moreover, at every concentration of 4G-935 protein tested, IRP-1 reduces translation to an equivalent extent (6–8%) (Fig. 5B, lanes 2–5 and 7–10).

Translational enhancement of uncapped mRNAs by 4G-935 and cap-analogue occurs by independent mechanisms

The inhibitory effect of cap-analogue on translation of capped mRNA in vitro is a well-documented phenomenon (Chu & Rhoads, 1978, 1980) that results from competition between the cap structure and the cap-analogue for the same cellular factors. More recently, it was also shown that cap-analogue can activate translation of uncapped mRNA in RRL (Anthony & Merrick, 1991), yeast (Iizuka et al., 1994; Tarun & Sachs, 1995), and wheat germ (Gallie & Tanguay, 1994) translation extracts at concentrations ranging from 0.3 to 0.5 mM. The intriguing similarities between the effects of cap-analogue and the 4G variants demanded further investigation. c-Nop I mRNA and u-CAT mRNA translation was analyzed in the presence of increasing concentrations of the cap-analogue, m^7GpppG, confirming the inhibitory effect on capped and the stimulation of uncapped mRNA translation (Fig. 6A, lanes 1–5). Equivalent results were obtained at a range of added Mg^{2+} concentrations (0.17–0.66 mM), arguing against the possibility that chelation of Mg^{2+} by the added cap-analogue is responsible for these effects on translation (data not shown). Next, the cap-analogue titration was repeated in the presence of 0.6 pmol of 4G-935 or thrombin-cleaved GST (GST-th) as control. As summarized in Figure 6B, u-CAT mRNA translation is stimulated to a similar extent by 4G-935 addition in the absence or presence of cap analogue at all concentrations tested. Because both effects are approximately additive even at saturating concentrations of cap analogue (0.15–1.2 mM), we suggest that 4G-935 and cap analogue

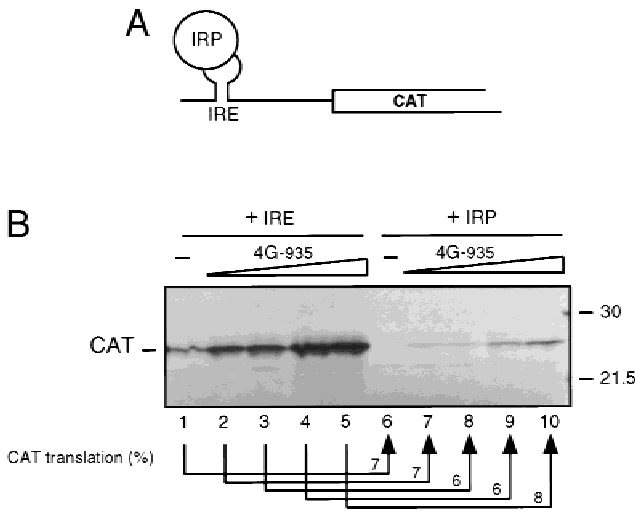

FIGURE 5. Binding of IRP-1 near the 5′ end of uncapped mRNAs inhibits their translational activation by 4G-935. **A:** Schematic representation of the IRE-CAT mRNA 5′ UTR. **B:** Translation of 12.5 ng of u-IRE-CAT mRNA was performed with 0 (lanes 1, 6), 0.3 (lanes 2, 7), 0.6 (lanes 3, 8), 1.2 (lanes 4, 9), or 2.5 (lanes 5, 10) pmol of 4G-935. Reactions shown in the left half of the panel (lanes 1–5) received a 20-fold molar excess of competitor IRE relative to the reporter mRNA. Reactions on the right were instead supplemented with 2 pmol of recombinant IRP-1 (lanes 6–10). Numbers at the arrows below the figure specify the translation in the presence of IRP-1 relative to the corresponding control lane in %.

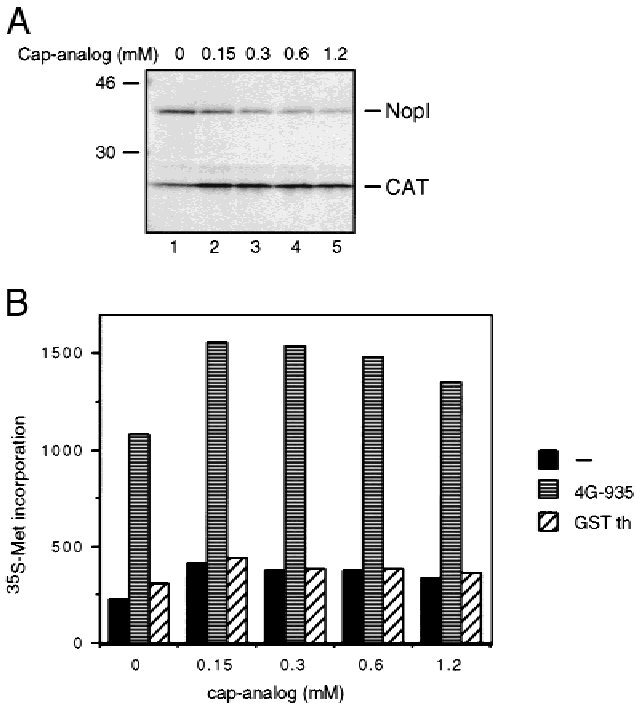

FIGURE 6. Cap-analogue and 4G-935 independently activate translation of uncapped mRNAs. **A:** Five nanograms of c-NopI and 12.5 ng of u-CAT mRNAs were translated in the presence of increasing concentrations of the cap-analogue m^7GpppG (as stated above the lanes). **B:** Graph summarizing the effect of cap analogue on u-CAT mRNA translation in the absence of recombinant protein and in the presence of 0.6 pmol of 4G-935 or GST-th. ^{35}S-Met incorporation was determined by PhosphorImager analysis and expressed in arbitrary light units.

act through, at least partly, independent mechanisms (see Discussion).

DISCUSSION

In this paper, we show that a bacterially expressed recombinant protein corresponding to the C-terminal viral cleavage product of human eIF4G (4G-1404) activates translation of uncapped mRNAs in RRL. Similar observations were made previously using partially purified preparations of the C-terminal fragment of rabbit eIF4G after L protease cleavage (Ohlmann et al., 1997). Our data confirm these observations and now also exclude any involvement of eIF4G-associated, co-purifying factors. As a new and unexpected finding, we report that the central domain of human eIF4G (4G-935) preserves the activating effect on the translation of uncapped mRNAs seen with the whole C-terminal cleavage product in our assays. Transfection studies suggest different requirements for EMCV IRES-driven translation, because the central third of human eIF4G failed to have a stimulatory effect (Imataka & Sonenberg, 1997). On the other hand, the central third of human eIF4G—in conjunction with eIF4A—was shown to promote 40S binding to the EMCV IRES in a reconstituted cell-free system (Pestova et al., 1996). Interestingly, a translational repressor (p97/ DAP-5/NAT1) with homology to the central and C-terminal thirds of eIF4G was identified recently (Imataka et al., 1997; Levi-Strumpf et al., 1997; Yamanaka et al., 1997). Transfection studies showed that p97/NAT1 inhibits both cap-dependent and EMCV IRES-driven translation (Imataka et al., 1997; Yamanaka et al., 1997).

The C-terminal eIF4A binding site of eIF4G (Lamphear et al., 1995; Imataka & Sonenberg, 1997) is shown here to be dispensable for the activation of uncapped mRNA translation in RRL. The interaction of eIF4A with the central region of eIF4G could possibly suffice to recruit eIF4A into the preinitiation complex, or eIF4A binding to the preinitiation complex could be stabilized by further interactions in the complex. That the central domain of eIF4G enhances binding of eIF4A to the EMCV IRES only in conjunction with eIF4B, whereas the whole C-terminal domain does so on its own (Pestova et al., 1996) may be seen in support of the latter. On the other hand, the known eIF4Gs of fungal origin lack homology to the mammalian C-terminal eIF4A binding region (Morley et al., 1997), perhaps arguing for a certain redundancy of binding sites.

The observation that the central third of human eIF4G can bind directly to the viral EMCV-IRES (Pestova et al., 1996) prompted us to test recombinant 4G-1404 and 4G-935 for RNA binding. Both proteins displayed a general RNA-binding capacity in gel shift assays (data not shown). We (see Materials and Methods) and others (Imataka & Sonenberg, 1997) revised the originally published eIF4G cDNA sequence (Yan et al., 1992; Lamphear & Rhoads, 1996). The amino acid sequence revisions between codons 597 and 636 significantly improve the alignment to the yeast eIF4Gs in the region of the RNP-2 motif of the RRM-like domain in which the proteins were assumed previously to diverge (Goyer et al., 1993). Taken together, these findings are consistent with the notion that RNA binding mediated by the central domain of eIF4G may represent a relevant activity also for the case of non-IRES mRNAs. They may also explain the strong enhancement of cap-binding by eIF4E in the presence of complete eIF4G (Haghighat & Sonenberg, 1997).

How does the central region of eIF4G activate uncapped mRNA translation? Information relevant to this question can be extracted from a number of recent studies. Addition of the translational repressor 4E-BP1 to extracts inhibits the translation of capped and uncapped mRNAs as well as the cleavage of eIF4G by L protease (Ohlmann et al., 1995, 1996). This suggests that the interaction between eIF4E and eIF4G, which is disrupted by the 4E-BPs, leads to a conformational change in eIF4G required for access to the L protease cleavage site and for the translation of uncapped mRNAs (Ohlmann et al., 1996). By contrast, a study in yeast recently suggested that the eIF4E/4G interaction negatively affects the translation of uncapped mRNA (Tarun & Sachs, 1997). Although these apparent differences between the two systems remain unresolved at present, it is conceivable that eIF4E-binding to mammalian eIF4G exposes an RNA-binding function in the central region. Deletion of the N-terminal part of eIF4G may mimic this effect. It is interesting that the addition of cap-analogue to translation extracts from rabbit reticulocytes, yeast, and wheat germ also activates the translation of uncapped mRNAs (Anthony & Merrick, 1991; Gallie & Tanguay, 1994; Iizuka et al., 1994; Tarun & Sachs, 1995). We show here that the effects of cap-analogue and the central region of eIF4G are additive and, therefore, appear to function through independent yet possibly related mechanisms. In micrococcal nuclease-treated RRL, short capped fragments of cellular mRNAs likely sequester a substantial fraction of eIF4G together with eIF4E (Jackson et al., 1995). Uncapped mRNAs must attract eIF4G without the aid of the cap/ structure eIF4E interaction. In this context, the additive effect of cap-analogue and 4G-935 can be explained by their additive effect on the availability of free eIF4G. Cap-analogue liberates eIF4E/4G complexes from the capped mRNA fragments, whereas recombinant 4G-1404 or 4G-935 contribute directly to this pool, because they are inefficiently sequestered by the capped mRNA fragments. The inhibitory effect of 4G-1404 and 4G-935 on the translation of capped mRNAs can be explained by their assembly into initiation factor complexes without a bias for capped mRNAs, thus gradually outcompeting the endogenous eIF4G.

A second challenging issue raised is the 5′ end-dependence of the translation of uncapped mRNAs in

RRL, which contrasts with the ability of mammalian ribosomes to initiate internally at IRES elements, even on circular mRNAs (Chen & Sarnow, 1995). This clearly demonstrates that "threading" of the translation machinery onto an mRNA is not obligatory for initiation. The repressive effect of general RNA-binding proteins on uncapped mRNA translation in RRL has been attributed to the prevention of internal initiation events (Dasso & Jackson, 1989; Svitkin et al., 1996), although no direct tests such as shown here (Figs. 4, 5) were employed. Using a yeast cell-free translation system, we found that the poly(A) tail can promote translation initiation at internal sites on uncapped mRNAs with blocked 5′ ends. In yeast, the tethering function of the cap structure appears to be crucial to enforce faithful, 5′ end-directed initiation at the authentic AUG start codon (Preiss & Hentze, 1998). By contrast, the translation even of uncapped mRNAs is strictly 5′ end-dependent in all conditions employed here.

This unexpected property of the mammalian translation apparatus could have several, not mutually exclusive explanations. First, the body of mRNAs may be masked by general mRNP proteins that interfere with internal ribosome binding (Svitkin et al., 1996), unless specifically promoted by an IRES element. Second, initiation complexes mediating 40S subunit binding may engage in a preferred, yet nonobligatory conformation that resembles the eye of a needle. Third, a mammalian translation initiation factor may display an inherent affinity for the 5′ triphosphate terminus of the uncapped mRNAs used in this (and other) studies. The answer to this enigma should yield important information concerning the ribosome binding step in mammalian translation initiation, including the role of the poly(A) tail during this process in mammalian cells.

In summary, this study underscores the critical role of eIF4G in coordinating translation initiation and reveals novel properties of this molecule in determining ribosome recruitment to mRNAs in mammalian translation.

MATERIALS AND METHODS

Plasmids

Plasmid pSK-HFC1 containing the human eIF4G cDNA (Yan et al., 1992) was amplified by PCR using the primers B1 (5′-GAA GGA TCC GGG CCC CCA AGG GGT GGG-3′) and B2 (5′-CAA TCA AGG GAC TCT TCA-3′). The amplification product was digested with *Bam*H I–*Hpa* I and re-inserted into the *Bam*H I–*Hpa* I sites of pSK-HFCI to create plasmid p220Bam. A *Bam*H I–*Eco*R I fragment from p220Bam was inserted into *Bam*H I and *Eco*R I sites of pGEX-2T (Pharmacia) to create plasmid pGEX4G1404. pGEX4G935 is derived from pGEX4G1404 by digestion with *Kpn* I and *Eco*R I and religation after blunting with T4 DNA polymerase. All plasmids were subjected to sequence analysis. The sequence of the PCR-derived region of p220Bam revealed four discrepancies with the published human eIF4G sequence (Yan et al., 1992; Lamphear & Rhoads, 1996): 2068-GCA GGT GG-2075 was found to be 2068-GCA GGG TG-2075; 2152-CCG AGG ACG-2160 was found to be 2152-CCG AGG A**A**C G-2161; 2224-CTA TCC AAC AT-2234 was found to be 2225-CTA T**G**C CAA CAT-2236; 2270-CGG AAA GCC AA-2280 was found to be 2272-CGG AAA **A**GC CAA-2283 (bold: nucleotides that were missing in the original cDNA sequence, EMBL database accession number: AJ001046). The corrected sequence was confirmed by re-sequencing pSK-HFC1 as well as by PCR amplification of the same eIF4G DNA fragment from HeLa cell cDNA. Our corrections agree with the independent findings of other investigators (Imataka & Sonenberg, 1997).

Expression of recombinant proteins

The recombinant proteins GST-4G-1404, GST-4G-935 [aa numbering is according to the revised cDNA sequence (Imataka & Sonenberg, 1997)], and GST (see Fig. 1) were expressed in the *E. coli* strain BL21(DE3) transformed with the vectors pGEX4G1404, pGEX4G935, and pGEX2T, respectively. Cells were grown in LB medium at 37 °C to an O.D. (600 nm) of 0.5 and induced with 0.3 mM IPTG for 4 h at 23 °C. Bacterial pellets were resuspended in PBS, including 1 mM DTT, 1 mM PMSF, and 1 mg/mL lysozyme, incubated for 30 min at 4 °C, and sonicated. Triton X-100 (1%) was added to the samples after sonication and cellular debris was removed by centrifugation at 6,000 rpm for 10 min at 4 °C in a Sorvall SS34 rotor. The supernatant was incubated at 4 °C for 1 h with glutathione-agarose beads (Sigma) and then washed with PBS. GST fusion proteins were eluted with 10 mM reduced glutathione (Sigma), 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, yielding GST-4G-1404, GST-4G-935, and GST. Following elution, the proteins 4G-1404, 4G-935, and GST-th were obtained by thrombin cleavage of the corresponding GST fusion proteins in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2,5 mM $CaCl_2$, 5 U/mL thrombin (Novagene). After 3 h at 23 °C, the reactions were stopped with 0.5 mM PMSF and 2.5 mM EGTA, and the products were analyzed by SDS-PAGE followed by Coomassie Blue staining. The concentration of the eluted GST fusion proteins was determined by Bio-Rad protein assay. The molar concentration of active recombinant eIF4G proteins was assumed to be the same before and after thrombin cleavage. The N-terminal sequence of the recombinant proteins 4G-1404 and 4G-935 is: **GS**GPPRGGPG… (aa in bold derive from pGEX2T vector after thrombin cleavage).

Recombinant His-tagged IRP-1 was expressed and purified as previously described (Gray et al., 1993).

In vitro transcription

Transcription reactions with T7 RNA polymerase yielding >95% capping efficiency were performed as described previously (Gray & Hentze, 1994), omitting m^7GpppG for uncapped mRNAs. Accurate determination of mRNA concentration and monitoring of its integrity was achieved by trace labeling with α^{32}P-UTP and agarose gel electrophoresis. The mRNAs were generated using the following templates: CAT mRNA, pI-19.CAT; OT.CAT mRNA, pOT.CAT; IRE.CAT mRNA, pIRE.CAT; all *Hin*d III-linearized; and NopI mRNA, *Bam*H I-linearized

pNOPI (Stripecke & Hentze, 1992). All CAT mRNAs possess identical open reading frames and 3′ UTR sequences, and exhibit related 5′ UTR regions. pOT.CAT was generated from pI-19.CAT (Gray et al., 1993) by introducing four nucleotide changes to suppress initiation at upstream non-AUG codons: A_{28} to G, C_{31} to U, G_{51} to A, and G_{75} to C. pIRE-CAT differs from pI-12.CAT (Gray et al., 1993) by a C to T change at position 57, destroying a *Bam*H I site while preserving IRE function (Goossen & Hentze, 1992).

Cell-free translations

Five nanograms of capped mRNAs or 12.5 ng of uncapped mRNAs were translated as described previously (Gray & Hentze, 1994). Reactions (12 μL) contained 4.8 μL micrococcal nuclease-treated rabbit reticulocyte lysate (RRL, Promega), 0.33 mM added $MgCl_2$, 0.5 U of Prime RNAse Inhibitor (5′ $\rightarrow$3′), 67 μM amino acids (except methionine), 0.33 MBq of ^{35}S-methionine (37TBq/mmol, Amersham), and 1 μL of recombinant eIF-4G proteins or corresponding buffer. Quantification of translation products after SDS-PAGE was done with a PhosphorImager (Molecular Dynamics, ImageQuant software).

Regional probes

2′-O-allyl oligoribonucleotides were annealed to OT.CAT as described previously (Johansson et al., 1994). Annealed mixtures containing 5 ng of c-OT.CAT or 12.5 ng of u-OT.CAT mRNA, 16 pmol oligonucleotide, and 190 ng of *E. coli* rRNA were added to translation reactions.

Sequences of oligonucleotides were: X, CUA CAC GUC UAC CA; 1, AGC UCG AAU UCG CCC; 169, **T**GA CUG AAA UGC CU**C** (bold: deoxyribonucleotide).

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REFERENCES

Anthony DD, Merrick WC. 1991. Eukaryotic initiation factor (eIF)-4F. *J Biol Chem 266*:10218–10226.

Belsham GJ, Sonenberg N. 1996. RNA–protein interactions in regulation of picornavirus RNA translation. *Microbiological Reviews 60*:499–511.

Borman AM, Kirchweger R, Ziegler E, Rhoads RE, Skern T, Kean KM. 1997. eIF4G and its proteolytic cleavage products: Effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. *RNA 3*:186–196.

Chen C, Sarnow P. 1995. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science 268*: 415–517.

Chu LY, Rhoads RE. 1978. Translational recognition of the 5′-terminal 7-methylguanosine of globin messenger RNA as a function of ionic strength. *Biochemistry 17*:2450–2455.

Chu LY, Rhoads RE. 1980. Inhibition of cell-free messenger ribonucleic acid translation by 7-methylguanosine 5′-triphosphate: Effect of messenger ribonucleic acid concentration. *Biochemistry 19*:184–191.

Dasso MC, Jackson RJ. 1989. On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system. *Nucleic Acids Res 17*:3129–3144.

Duncan R, Milburn SC, Hershey JWB. 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggests a role in translational control. *J Biol Chem 262*:380–388.

Ehrenfeld E. 1996. Initiation of translation by picornavirus RNAs. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translational control*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 549–573.

Gallie DR, Tanguay R. 1994. Poly(A) binds to initiation factors and increases cap-dependent translation in vitro. *J Biol Chem 269*:17166–17173.

Goossen B, Hentze MW. 1992. Position is the critical determinant for function of iron-responsive elements as translational regulators. *Mol Cell Biol 12*:1959–1966.

Goyer C, Altmann M, Lee HS, Blanc A, Deshmukh M, Woolford JJL, Trachsel H, Sonenberg N. 1993. *TIF4631* and *TIF4632*: Two yeast genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an RNA recognition motif-like sequence and carry out an essential function. *Mol Cell Biol 13*:4860–4874.

Gray NK, Hentze MW. 1994. Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J 13*:3882–3891.

Gray NK, Quick S, Goossen B, Constable A, Hirling H, Kühn LC, Hentze MW. 1993. Recombinant iron regulatory factor functions as an iron-responsive element-binding protein, a translational repressor and an aconitase. A functional assay for translational repression and direct demonstration of the iron switch. *Eur J Biochem 218*:657–667.

Gunnery S, Maivali U, Mathews B. 1997. Translation of uncapped mRNA involves scanning. *J Biol Chem 272*:21642–21646.

Haghighat A, Sonenberg N. 1997. eIF4G dramatically enhances the binding of eIF4E to the mRNA 5′-cap structure. *J Biol Chem 272*:21677–21680.

Hentze MW. 1997. eIF4G: A multipurpose ribosome adapter? *Science 275*:500–501.

Iizuka N, Najita L, Franzusoff A, Sarnow P. 1994. Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. *Mol Cell Biol 14*:7322–7330.

Imataka H, Olsen HS, Sonenberg N. 1997. A new translational regulator with homology to eukaryotic translation initiation factor 4G. *EMBO J 16*:817–825.

Imataka H, Sonenberg N. 1997. Human eukaryotic translation initiation factor 4G (eIF4G) possesses two independent binding sites for eIF4A. *Mol Cell Biol 17*:6940–6947.

Jackson RJ. 1996. A comparative view of initiation site selection mechanisms. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translational control*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 71–112.

Jackson RJ, Hunt SL, Reynolds JE, Kaminski A. 1995. Cap-dependent and cap-independent translation: Operational distinctions and mechanistic interpretations. *Curr Top Microbiol Immunol 203*:1–29.

Johansson HE, Belsham GJ, Sproat BS, Hentze MW. 1994. Target-specific arrest of mRNA translation by antisense 2′-O-alkyl-oligoribonucleotides. *Nucleic Acids Res 22*:4591–4598.

Lamphear BJ, Kirchweger R, Skern T, Rhoads RE. 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. *J Biol Chem 270*:21975–21983.

Lamphear BJ, Rhoads RE. 1996. A single amino acid change in protein synthesis initiation factor 4G renders cap-dependent translation resistant to picornaviral 2A protease. *Biochemistry 35*:15726–15733.

Lamphear BJ, Yan R, Yang F, Waters D, Liebig HD, Klump H, Kuechler E, Skern T, Rhoads RE. 1993. Mapping the cleavage site in protein synthesis initiation factor eIF-4γ of the 2A proteases from

human coxsackievirus and rhinovirus. *J Biol Chem 268*:19200–19203.
Levi-Strumpf N, Deiss LP, Berissi H, Kimchi A. 1997. DAP-5, a novel homolog of eukaryotic translation initiation factor 4G isolated as a putative modulator of gamma interferone-induced programmed cell death. *Mol Cell Biol 17*:1615–1625.
Mader S, Lee H, Pause A, Sonenberg N. 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. *Mol Cell Biol 15*:4990–4997.
Merrick WC, Hershey JWB. 1996. The pathway and mechanism of eukaryotic protein synthesis. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translational control*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 31–69.
Morley SJ, Curtis PS, Pain VM. 1997. eIF4G: Translation's mystery factor begins to yield its secrets. *RNA 3*:1085–1104.
Ohlmann T, Pain VM, Wood W, Rau M, Morley SJ. 1997. The proteolytic cleavage of eukaryotic initiation factor (eIF) 4G is prevented by eIF4E binding protein (PHAS-I; 4E-BP) in the reticulocyte lysate. *EMBO J 16*:844–855.
Ohlmann T, Rau M, Morley SJ, Pain VM. 1995. Proteolytic cleavage of initiation factor eIF-4γ in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. *Nucleic Acids Res 23*:334–340.
Ohlmann T, Rau M, Pain VM, Morley SJ. 1996. The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. *EMBO J 15*:1371–1382.
Pause A, Methot N, Svitkin Y, Merrick WC, Sonenberg N. 1994. Dominant negative mutants of mammalian eIF-4A define a critical role for eIF-4F in cap-dependent initiation of translation. *EMBO J 13*:1205–1215.
Pelletier J, Sonenberg N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature 334*:320–325.
Pestova TV, Shatsky IN, Hellen CUT. 1996. Functional dissection of eukaryotic initiation factor 4F: The 4A subunit and the central domain of 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol Cell Biol 16*:6870–6878.
Preiss T, Hentze MW. 1998. Dual function of the messenger RNA cap structure in poly(A)-tail-promoted translation in yeast. *Nature 392*:516–520.
Sachs AB, Sarnow P, Hentze MW. 1997. Starting at the beginning, middle, and end: Translation initiation in eukaryotes. *Cell 89*:831–838.
Stripecke R, Hentze MW. 1992. Bacteriophage and spliceosomal proteins function as position-dependent *cis/trans* repressors of mRNA translation in vitro. *Nucleic Acids Res 20*:5555–5564.
Svitkin YV, Ovchinnikov LP, Dreyfuss G, Sonenberg N. 1996. General RNA binding proteins render translation cap dependent. *EMBO J 15*:7147–7155.
Tarun S, Sachs AB. 1997. Binding of eukaryotic initiation factor 4E (eIF4E) to eIF4G represses translation of uncapped mRNA. *Mol Cell Biol 17*:6876–6886.
Tarun SZ Jr, Sachs AB. 1995. A common function for mRNA 5′ and 3′ ends in translation initiation in yeast. *Genes & Dev 9*:2997–3007.
Tarun SZ Jr, Sachs AB. 1996. Association of the yest poly(A) tail binding protein with translation initiation factor eIF-4G. *EMBO J 15*:7168–7177.
Tarun SZ Jr, Wells SE, Deardorff JA, Sachs AB. 1997. Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. *Proc Natl Acad Sci USA 94*:9046–9051.
Yamanaka S, Poksay KS, Arnold KS, Innerarity TL. 1997. A novel translational repressor mRNA is edited extensively in livers containing tumors caused by the transgene expression of the apoB mRNA-editing enzyme. *Genes & Dev 11*:321–323.
Yan R, Rychlik W, Etchison D, Rhoads RE. 1992. Amino acid sequence of the human protein synthesis initiation factor eIF-4γ. *J Biol Chem 267*:23226–23231.