MOLECULAR AND CELLULAR BIOLOGY, Jan. 2000, p. 468–477 Vol. 20, No. 2
0270-7306/00/$04.00+0

Eukaryotic Translation Initiation Factor 4E (eIF4E) Binding Site and the Middle One-Third of eIF4GI Constitute the Core Domain for Cap-Dependent Translation, and the C-Terminal One-Third Functions as a Modulatory Region

SHIGENOBU MORINO,[1] HIROAKI IMATAKA,[1] YURI V. SVITKIN,[1] TATYANA V. PESTOVA,[2] AND NAHUM SONENBERG[1]*

Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Quebec H3G 1Y6, Canada,[1] and Department of Microbiology and Immunology, State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203[2]

Received 30 August 1999/Accepted 6 October 1999

The mammalian eukaryotic initiation factor 4GI (eIF4GI) may be divided into three roughly equal regions; an amino-terminal one-third (amino acids [aa] 1 to 634), which contains the poly(A) binding protein (PABP) and eIF4E binding sites; a middle third (aa 635 to 1039), which binds eIF4A and eIF3; and a carboxy-terminal third (aa 1040 to 1560), which harbors a second eIF4A binding site and a docking sequence for the Ser/Thr kinase Mnk1. Previous reports demonstrated that the middle one-third of eIF4GI is sufficient for cap-independent translation. To delineate the eIF4GI core sequence required for cap-dependent translation, various truncated versions of eIF4GI were examined in an in vitro ribosome binding assay with β-globin mRNA. A sequence of 540 aa encompassing aa 550 to 1090, which contains the eIF4E binding site and the middle region of eIF4GI, is the minimal sequence required for cap-dependent translation. In agreement with this, a point mutation in eIF4GI which abolished eIF4A binding in the middle region completely inhibited ribosomal binding. However, the eIF4GI C-terminal third region, which does not have a counterpart in yeast, modulates the activity of the core sequence. When the eIF4A binding site in the C-terminal region of eIF4GI was mutated, ribosome binding was decreased three- to fourfold. These data indicate that the interaction of eIF4A with the middle region of eIF4GI is necessary for translation, whereas the interaction of eIF4A with the C-terminal region plays a modulatory role.

All eukaryotic cellular (except organellar) mRNAs possess a cap structure (m^7GpppN, where N is any nucleotide) at the 5′ end. The cap structure is bound by translation initiation factor 4F (eIF4F) as the first step of cap-dependent translation. eIF4F consists of three subunits, eIF4E, eIF4A, and eIF4G. eIF4E binds the cap structure directly and consequently is required for cap-dependent translation. eIF4A exhibits RNA-dependent ATPase activity and ATP-dependent RNA helicase activity. The helicase activity is though to be required for the melting of mRNA 5′ untranslated region secondary structure to facilitate ribosome binding (for reviews, see references 5, 19, and 28). eIF4G is an adapter protein with a modular structure. It bridges the ribosome to the mRNA via eIF3 (for reviews, see references 20, 22, and 27). The human eIF4G may be divided into three distinct functional domains. The N-terminal one-third (amino acids [aa] 1 to 634) contains the eIF4E binding site (14, 18) and a recently described poly(A) binding protein (PABP) binding site (13). The middle third (aa 635 to 1039) possesses eIF3 and eIF4A binding sites (12) as well as an RNA binding site (6, 24). The C-terminal third (aa 1040 to 1560) contains a second eIF4A binding site (12, 14) and a Mnk1 binding site (26, 30). The middle third of eIF4G is sufficient for cap-independent binding of ribosomes to the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) (24) and for cap-independent but 5′-end-dependent translation (2). The function of the C-terminal region of human eIF4G is unclear.

The eIF4G-related mammalian protein p97/NAT1/DAP-5 (11, 16, 31), which is homologous to the carboxyl two-thirds of eIF4G, does not contain an eIF4A binding site in its C-terminal region (12). While the middle region is phylogenetically conserved, the C-terminal one-third is not. The wheat eIF4G homolog possesses a much shorter C terminus (1), and the *Saccharomyces cerevisiae* eIF4G homolog does not possess a sequence with homology to the mammalian C-terminal third (6).

In this study, using a toeprinting technique with the β-globin mRNA as a template (25), we define the region beginning at the eIF4E binding site and encompassing the middle third of eIF4G as the minimum sequence required for cap-dependent 40S ribosome binding. Our data provide evidence that the C-terminal region plays a modulatory role.

MATERIALS AND METHODS

Construction of plasmids. Plasmids pcDNA3-HA (hemagglutinin), pcDNA3-FLAG, and pcDNA3-GST (glutathione *S*-transferase) plasmids (13) were used as vectors for expression in HeLa cells. pBlueBacHis (Invitrogen) was used for generating recombinant baculoviruses expressing His-tagged eIF4GI and mutants in Sf9 cells. To construct pBlueBacFLAG, the His tag sequence in pBlueBacHis was replaced with the FLAG sequence. Deletion and point mutants of eIF4GI were generated using PCR with primers containing *Eco*RI (5′ primer) and *Xho*I (3′ primer) restriction enzyme sequences. All constructs were sequenced in their entirety.

Antibodies. Anti-hPrt1 (human Prt1), anti-eIF4GI(1–329), and anti-GST antisera were obtained by immunizing rabbits with GST-hPrt1(147–209), GST-eIF4GI(1–329), and GST, respectively. Anti-HA and anti-His_6 monoclonal antibodies were obtained from BAbCo. Anti-Xpress and anti-FLAG monoclonal

* Corresponding author. Mailing address: Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Quebec H3G 1Y6, Canada. Phone: (514) 398-7274. Fax: (514) 398-1287. E-mail: nsonen@med.mcgill.ca.

antibodies were obtained from Invitrogen and Sigma, respectively. Monoclonal anti-eIF4A was a kind gift from H. Trachsel.

Purification of recombinant proteins. His-eIF1, His-eIF1A, His-eIF4A, and His-eIF4E were expressed in *Escherichia coli* BL21(DE3) and purified by Ni-agarose (Qiagen) chromatography as described previously (23, 25). Baculoviruses expressing FLAG-eIF4B or His- or FLAG-eIF4GI proteins were generated by using the pBlueBac baculovirus expression system (Invitrogen). Log-phase Sf9 cells (200 ml of 2×10^6 cells/ml) were infected with a recombinant virus at multiplicity of infection of 5 and cultured for 40 h at 27°C. Expressed proteins were purified by Ni-agarose (Qiagen) or anti-FLAG-agarose (Sigma) chromatography. Concentrations of recombinant proteins were determined by comparison with standard bovine serum albumin (Ambion) following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and staining with Coomassie blue. Sf9 cell proteins copurified with His-tagged proteins were not considered to affect the functional analyses, because nickel resin-bound proteins purified from uninfected Sf9 cells did not inhibit or enhance ribosome binding activity or in vitro translation (data not shown).

40S ribosomal subunits, eIF2, eIF3, and eIF4F were purified from ribosomal pellets of Krebs cell extracts or rabbit reticulocyte lysate as described previously (23).

Assembly and toeprint analysis of 48S complexes. Reaction mixtures (40 μl) containing native α- and β-globin mRNA (Life Technologies) (0.3 μg), His-eIF1 (0.5 μg), His-eIF1A (0.5 μg), eIF2 (3 μg), eIF3 (7 μg), FLAG-eIF4B (1 μg), Met-tRNA$_i^{Met}$ (4 pmol), 40S ribosomal subunits (4 pmol), His- or FLAG-eIF4GI (2 μg, unless indicated), His-eIF4E (0.3 μg), and His-eIF4A (3 μg) were incubated in buffer (2 mM dithiothreitol, 100 mM KCl, 20 mM Tris-HCl [pH 7.6], 2.5 mM magnesium acetate, 100 U of RNasin [Promega], 1 mM ATP, 0.4 mM guanylyl imidodiphosphate [GMP-PNP], 250 μM spermidine) for 5 min at 30°C. Following the addition of 4 pmol of oligonucleotide 5′-GCATTTGCAGAGGA CAGG-3′ (complementary to β-globin positions 177 to 194), incubation was continued for 3 min at 30°C, and the mixture was then placed on ice. For reverse transcriptase reactions, the mixture was incubated for 40 min at 30°C after addition of 1 μl of magnesium acetate (320 mM), 4 μl of deoxynucleoside triphosphate mix solution (5 mM dCTP, dGTP, and dTTP; 1 mM dATP), 1 μl of [α-^{32}P]dATP (6000 Ci/mmol; DuPont, NEN), and 15 U of avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech Inc.). cDNA products were extracted with phenol-chloroform (1:1) and precipitated with ethanol (25). The same primer was used for sequencing of plasmid pBS$^-$(β-globin), harboring β-globin cDNA (10). The products of primer extension and sequence products were resolved side by side on a sequencing gel. In the relevant figures, the full-length cDNA product and the toeprint product are marked "E" and "Complex II," respectively. The intensity of E and complex II was quantitated by BAS-2000 phosphorimager (Fuji). The relative efficiency of complex II formation was calculated as complex II/complex II + E. Values represent the means and standard errors of three independent experiments.

Protein immunoprecipitation. HeLa R19 cells (6-cm-diameter dish) were infected with vaccina virus vTF7-3 (3) for 1 h and then transfected with plasmids by using Lipofectin (Gibco BRL). Sixteen hours later, cells were lysed in 400 μl of buffer A (20 mM HEPES-KOH [pH 7.6], 100 mM KCl, 0.5 mM EDTA, 20% glycerol) containing 0.5% Triton X-100, 50 μg of RNase A per ml, and protease inhibitor cocktail (Boehringer Mannheim). After centrifugation, the supernatant was mixed with anti-HA or anti-FLAG antibody (2 μg) immobilized on protein G-Sepharose (10 μl) and incubated for 4 h at 4°C. After being washed with buffer A (400 μl, three times), bound proteins were dissolved in Laemmli buffer. The samples were resolved by SDS-PAGE and analyzed by Western blotting. Protein bands were visualized with an enhanced chemiluminescence detection system (Boehringer Mannheim).

To coprecipitate FLAG-Mnk1 with GST fusion proteins, cell extracts expressing FLAG-Mnk1 and a GST fusion protein were incubated with glutathione-Sepharose beads (15 μl; Amersham Pharmacia Biotech Inc.) for 4 h at 4°C. After being washed with buffer A containing 0.1% Triton X-100 (400 μl, three times), bound proteins were eluted with a buffer (40 μl) containing 20 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0), and 100 mM KCl.

In vitro translation. Rabbit reticulocyte lysate (Promega) was treated with rhinovirus 2A^{pro} (40 μg/ml) for 5 min at 30°C (7), followed by incubation for 10 min on ice with 0.8 mM elastatinal (Sigma). Aliquots (12.5 μl) were supplemented with eIF4E (0.2 μg) and/or wild-type or mutant eIF4GI (1 μg, unless indicated) and programmed for translation with 0.1 μg of capped bicistronic pGEMCAT/EMC/LUC mRNA (7) in the presence of [^{35}S]methionine. Translation reaction mixtures were incubated at 30°C for 60 min and analyzed by SDS-PAGE (12.5% gel). Gels were fixed with 40% methanol–7% acetic acid, treated with En^{3}Hance (Dupont, NEN) and processed for autoradiography. The intensity of the bands was determined with a BAS-2000 phosphorimager.

In vitro protein binding assay. A His-tagged protein (5 μg) was incubated with FLAG-eIF4A (4 μg or 8 μg) immobilized on anti-FLAG agarose resin in buffer A (50 μl) containing 0.1% Triton X-100 for 10 min on ice. The resin was washed with buffer A containing 0.1% Triton X-100 (400 μl, three times) and dissolved in Laemmli buffer. The sample was resolved by SDS-PAGE followed by Western blotting.

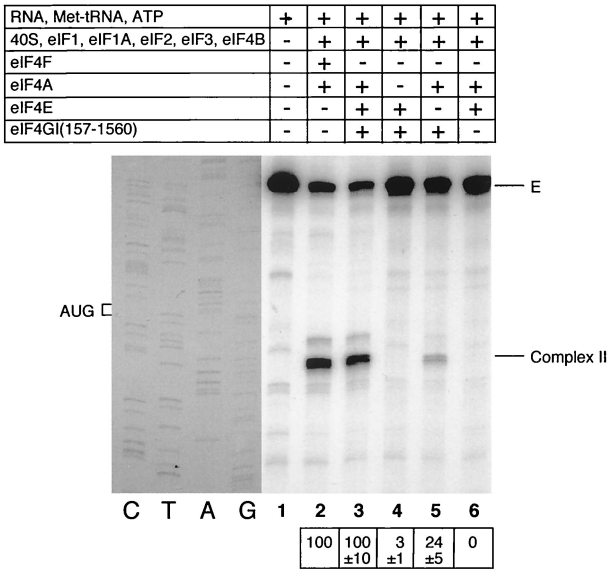


FIG. 1. Toeprint analysis of 48S ribosomal complex formation on β-globin mRNA with recombinant eIF4GI. The components of each reaction mixture are indicated above the lanes. Formation of complex II was quantified as described in Materials and Methods, and the value for eIF4F (lane 2) was set at 100.

RESULTS

The eIF4E binding site and middle third of eIF4G are necessary and sufficient for cap-dependent translation. The toeprinting analysis has proven extremely useful for the study of translation initiation (23, 25). In the presence of β-globin mRNA (a typical cap-dependent mRNA), Met-tRNA, ATP, 40S ribosomal subunits, eIF1, eIF1A, eIF2, eIF3, eIF4B, eIF4A, and eIF4F, a 48S ribosomal complex is formed on the initiation codon of the mRNA. No signal was detected in the presence of mRNA, Met-tRNA, and ATP alone (Fig. 1, lane 1). The ribosomal complex is detected by primer extension as a toeprint 15 to 17 nucleotides downstream from the initiation codon (25) (lane 2). This toeprint is termed complex II (25). To study the function of eIF4GI in cap-dependent translation, eIF4F was replaced by a combination of recombinant eIF4GI(157–1560), eIF4A, and eIF4E. Complex II was formed with these three recombinant proteins as efficiently as with eIF4F (compare lane 3 to lane 2). Control experiments in which eIF4A or eIF4E were omitted were performed. Complex II was not detectable in the absence of eIF4A (lane 4), confirming the importance of eIF4A for 48S ribosomal complex formation. However, the 48S complex was formed at the correct position in the absence of eIF4E (lane 5), albeit with much lower efficiency (compare lane 5 to lane 3). Complex II was not formed, however, when eIF4GI(157–1560) was omitted (lane 6). These results confirm that eIF4G is essential for 48S ribosomal complex formation, and they validate the use of recombinant eIF4G in this assay system.

Recently, the eIF4GI coding sequence was extended by 156 amino acids at the N terminus, to a total of 1560 amino acids (13). Because full-length eIF4GI(1–1560) is difficult to express in cells (13), a truncated protein (32), eIF4GI(157–1560), was the longest form used in this study and is referred to as full-length eIF4GI throughout this report. To delineate the minimal sequence of eIF4GI required for cap-dependent translation, several different fragments of eIF4GI were generated (Fig. 2A), and tested for 48S ribosomal complex formation by the toeprint assay. eIF4GI(550–1560), which lacks a large part

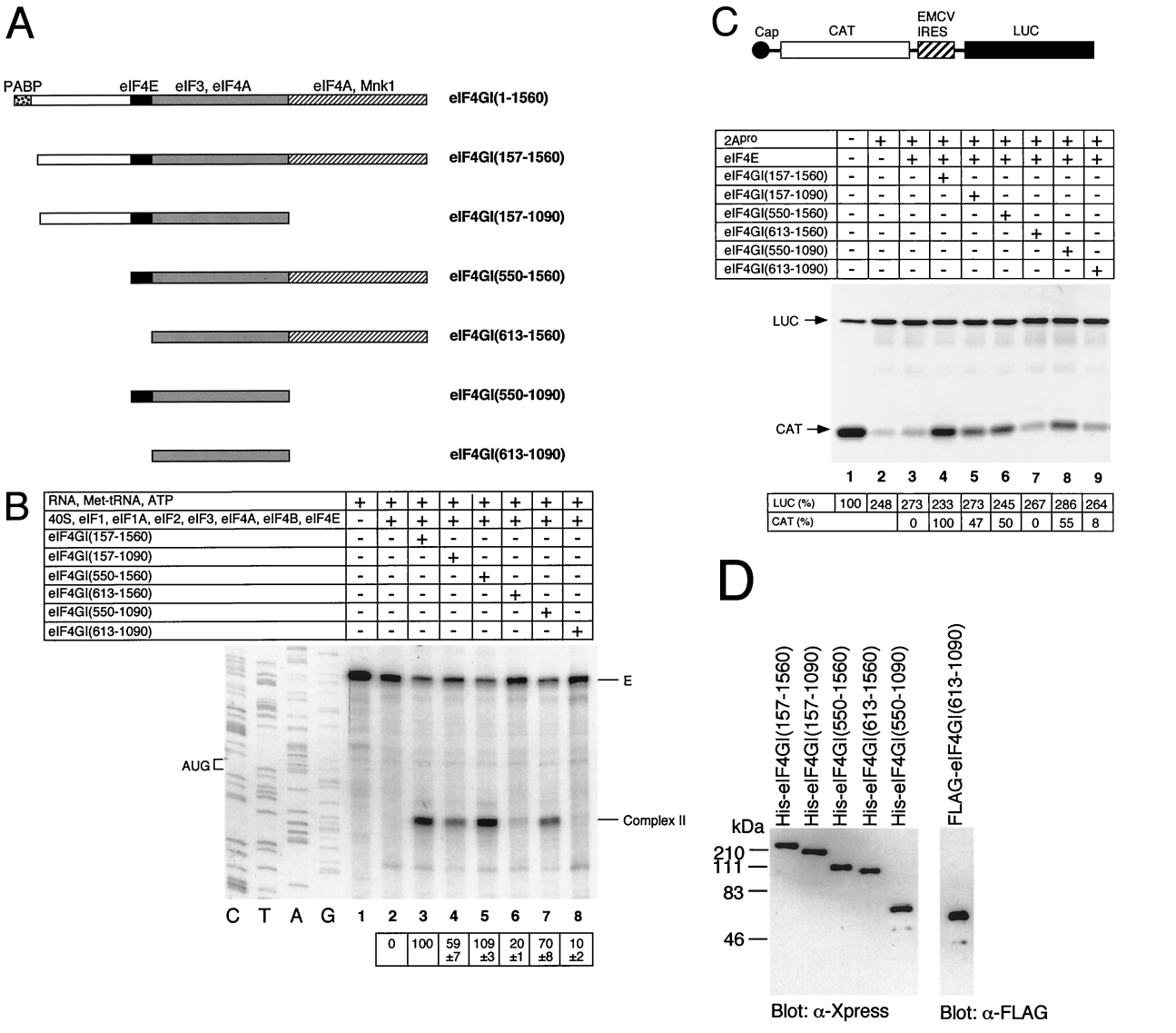


FIG. 2. Functional analysis of eIF4GI deletion mutants. (A) Schematic representation of eIF4GI deletion mutants. PABP, eIF4E, eIF4A, eIF3, and Mnk1 binding sites are indicated. (B) Toeprint analysis of 48S ribosomal complex formation on β-globin mRNA with eIF4GI deletion mutants. The reaction components are indicated above the lanes. The value for eIF4GI(157–1560) (lane 3) was set at 100. (C) Analysis of eIF4GI deletion mutants in a reticulocyte lysate translation system. Translation was performed as described in Materials and Methods. A rabbit reticulocyte lysate treated with rhinovirus $2A^{pro}$ was supplemented with recombinant proteins as indicated and programmed for translation with the capped bicistronic mRNA CAT/EMCV IRES/LUC. For quantitation of luciferase (LUC) synthesis, the value obtained for translation in untreated lysate in the absence of additional proteins (lane 1) was set at 100. For the quantitation of CAT synthesis, the value obtained for translation in the treated lysate in the presence of eIF4E alone was subtracted as background, and then the value for treated lysate translated in the presence of eIF4E and eIF4GI(157–1560) (lane 4) was set at 100. (D) Western blotting of eIF4G deletion mutants. Recombinant protein preparations (~1 μg) containing the same amount of eIF4GI according to Coomassie blue staining were subjected to SDS-PAGE (10% gel) and analyzed by Western blotting with anti-Xpress antibody (to detect the epitope located between the His tag and eIF4G coding sequence) or with anti-FLAG antibody.

of the N-terminal region but retains the eIF4E binding site, functioned as efficiently as control eIF4GI(157–1560) (Fig. 2B, compare lanes 3 and 5). However, deletion of the eIF4E binding site markedly (80%) reduced cap-dependent 40S ribosomal binding (compare lanes 5 and 6). The residual activity (20%) is consistent with the background level of binding observed for control eIF4GI in the absence of eIF4E (Fig. 1). The reasons for the residual activity will be addressed in Discussion. eIF4GI(157–1090), which lacks the C-terminal third, retained ~60% of the activity of control eIF4GI (compare lanes 3 and 4). Strikingly, efficient binding of 40S ribosomes (70% of control) was achieved by an eIF4GI protein possessing only the eIF4E binding site and the middle third (aa 550 to 1090), which contains binding sites for eIF3 and eIF4A (lane 7). In contrast to the results obtained for EMCV IRES RNA (24), the middle domain alone failed to support 40S ribosome binding to β-globin mRNA (lane 8). These results were reproducible with a wide concentration range (0.5 to 4 μg) of eIF4GI and its mutants (data not shown).

To further substantiate these conclusions, we extended the experiments to a rabbit reticulocyte lysate in vitro translation system. The lysate was pretreated with rhinovirus $2A^{pro}$ to cleave the endogenous eIF4G. This treatment results in inhibition of cap-dependent translation and stimulation of IRES-

dependent translation (7, 9). The $2A^{pro}$-treated lysate was programmed with a bicistronic mRNA in which translation of the first cistron (chloramphenicol acetyltransferase [CAT]) is cap dependent but translation of the second cistron (luciferase), which is preceded by the EMCV IRES, is cap independent (7). Treatment with $2A^{pro}$ dramatically (85%) reduced cap-dependent translation, as expected (Fig. 2C, compare lane 2 to lane 1). While addition of eIF4E alone failed to enhance cap-dependent translation of the CAT cistron (lane 3), addition of eIF4E plus eIF4GI(157–1560) restored cap-dependent translation to 65% of the untreated control level (compare lane 4 to lane 1). The eIF4GI C-terminal third fragment is not critical for cap-dependent translation, because eIF4GI(157–1090) retained approximately half of the activity of control eIF4GI (compare lanes 4 and 5). In contrast, the eIF4E binding site is important for cap-dependent translation, because eIF4GI (613–1560) and eIF4GI(613–1090) were extremely feeble in stimulating cap-dependent translation (lanes 7 and 9). These results demonstrate that the minimal region required for cap-dependent translation is the eIF4GI(550–1090) fragment, which contains the eIF4E, eIF3, and one (the middle) eIF4A binding site. Thus, the eIF4A binding site in the C-terminal region (aa 1090 to 1560) of eIF4GI (12, 14) does not play a critical role in cap-dependent translation. In this regard, yeast eIF4Gs, which do not possess a region corresponding to the C-terminal third of human eIF4G, bind eIF4A (21). eIF4GI and deletion mutant proteins used in the above experiments were analyzed by SDS-PAGE followed by Western blotting (Fig. 2D) to indicate that similar amounts of the different proteins were utilized.

Point mutations which abolish eIF4A binding activity of the middle or C-terminal domain. To explore the possible function of the C-terminal eIF4A-binding site and to contrast it with that of the middle domain, we further delimited the middle and C-terminal regions of eIF4GI for eIF4A binding and generated point mutants (Fig. 3 shows amino acid sequences). First, N-terminally truncated versions of the middle domain of eIF4GI were expressed as C-terminally FLAG-tagged proteins in HeLa cells and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting with anti-FLAG, anti-hPrt1 (a subunit of eIF3), and anti-eIF4A antibodies (Fig. 4A). The boundary of eIF4A and eIF3 binding region in eIF4GI resides between aa 672 and 702, because eIF4GI(672–1090) bound both proteins but eIF4GI(702–1090) bound neither (lanes 4 and 5). C-terminally truncated forms of the middle region of eIF4GI were expressed as N-terminally HA-tagged proteins for similar binding assays (Fig. 4B). The C-terminal boundary of eIF4A binding lies between aa 947 and 970, because eIF4GI(642–970) bound eIF4A but eIF4GI(642–947) did not (lanes 6 and 7). Interestingly, binding of eIF3 requires an extended C-terminal sequence compared to eIF4A binding: the C-terminal boundary of eIF3 binding resides between aa 1055 and 1065 (lanes 4 and 5). To show that eIF4GI(672–970) and eIF4GI(672–1065) are the minimal fragments that bind eIF4A or eIF3, HA-tagged fragments of eIF4GI were expressed for immunoprecipitation with anti-HA antibody followed by Western blotting (Fig. 4C). eIF4GI(672–970) bound eIF4A, while eIF4GI(702–970) did not (lanes 3 and 4). eIF4GI(672–1065) bound eIF3, while eIF4GI(672–970) did not (lanes 2 and 3). Thus, the minimal fragments of the eIF4GI middle region which are required for eIF4A and eIF3 binding comprise aa 672 to 970 and 672 to 1065, respectively.

Next, several amino acids in eIF4GI(672–970) which are conserved among eIF4GI, eIF4GII and p97 were mutated to alanines (Fig. 3). HA-tagged eIF4GI-middle third (aa 613 to 1090) fragments harboring the mutations were expressed in HeLa cells and immunoprecipitated with anti-HA antibody for Western blotting with anti-HA, anti-hPrt1, and anti-eIF4A antibodies (Fig. 4D). Mutations of tyrosine 776, phenylalanine 862, or phenylalanine 938 to alanines abolished eIF4A binding, yet the proteins retained eIF3 binding activity (lanes 4, 6 and 8). Other mutations, including phenylalanine 737, phenylalanine 812, or phenylalanine 920 to alanines, affected neither eIF3 nor eIF4A binding (lanes 3, 5 and 7). Thus, eIF3 binds to the middle domain of eIF4GI independently of eIF4A. These results also exclude the possibility that the mutations caused unfolding and denaturation of the proteins. An in vitro binding assay was carried out with the mutant Y776A, which retained 70% of the wild-type eIF3 binding, as determined by laser densitometry (Fig. 4D). Equal amounts of His-eIF4GI(157–1090) or His-eIF4GI(157–1090) Y776A were mixed with FLAG-eIF4A immobilized on anti-FLAG resin. After washing, proteins were eluted and subjected to SDS-PAGE, followed by Western blotting with anti-FLAG or with anti-His-tag. eIF4GI(157–1090) was bound to eIF4A (Fig. 4E, lane 1), while eIF4GI(157–1090) Y776A was not (lane 2), confirming that mutation Y776A abolishes eIF4A binding to the middle third fragment of eIF4GI.

To delimit the eIF4A binding site in the C-terminal region of eIF4GI, GST fusion fragments were expressed together with HA-tagged eIF4A in HeLa cells. Cell extract was immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS-PAGE followed by Western blotting with anti-HA antibody for eIF4A or anti-GST for eIF4GI fragments (Fig. 5A). The N-terminal boundary of eIF4A binding resides between aa 1201 and 1235, because eIF4GI(1201–1445) bound eIF4A but eIF4GI(1235–1445) did not (lanes 4 and 5). The C-terminal boundary resides between aa 1370 and 1411, because eIF4GI(1201–1411) bound eIF4A but eIF4GI(1201–1370) failed to bind (lanes 2 and 3). Thus, based on this analysis, eIF4GI(1201–1411) is the minimal region for eIF4A binding in the C-terminal region.

To create point mutations in the eIF4A binding region of the eIF4GI C-terminal third, three amino acid stretches of eIF4GI were mutated to alanines (FVR1239AAA, KKV1351AAA, and FEQ1377AAA [Fig. 3]). These amino acid stretches are conserved in eIF4GII but not in p97, whose C-terminal region does not bind eIF4A (12). HA-eIF4GI(1040–1560) or mutated fragments were expressed in HeLa cells and immunoprecipitated with anti-HA antibody (Fig. 5B). eIF4GI(1040–1560) FVR1239AAA did not bind eIF4A (lane 3), while the other two mutants, KKV1351AAA and FEQ1377AAA (lanes 4 and 5), bound eIF4A as well as wild-type protein (lane 2). To confirm that eIF4GI(1040–1560) FVR1239AAA was unable to bind eIF4A in vitro, equal amounts of His-tagged eIF4GI (1040–1560) or eIF4GI(1040–1560) FVR1239AAA were mixed with FLAG-eIF4A immobilized on anti-FLAG resin. After washing, proteins were analyzed by Western blotting for eIF4A or for His-tagged proteins (Fig. 5C). eIF4GI(1040–1560) bound eIF4A (lane 1), while eIF4GI(1040–1560) FVR1239AAA did not (lane 2). These binding assays suggest that either one or all of the amino acids FVR (aa 1239 to 1241) of eIF4GI are involved in eIF4A binding.

Distinct roles of the middle and C-terminal domains in eIF4A binding. To examine the physiological significance of eIF4A binding to the two binding sites in eIF4GI, recombinant full-length eIF4GI(157–1560) point mutants which abrogate eIF4A binding to either the middle or the C-terminal region were generated (Fig. 6A; Western blotting shows that equal amounts of protein were used in the assay), and used in the ribosome toeprinting assay (Fig. 6B). Binding of eIF4A to the

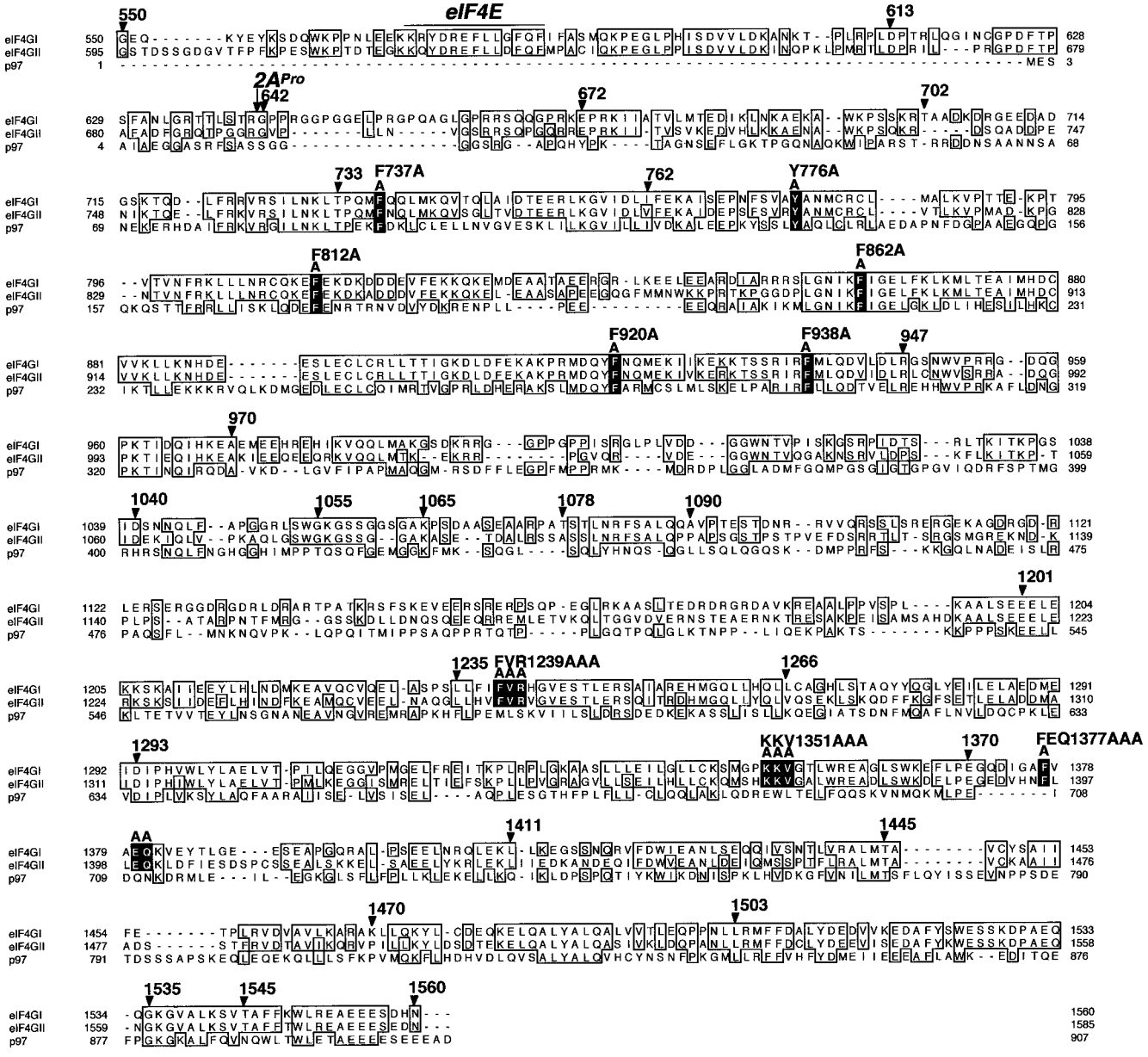


FIG. 3. Protein sequence alignment of human eIF4GI (13, 32), eIF4GII (7), and p97 (11). Conserved amino acids are boxed. Amino acids mutated to alanine are highlighted. The eIF4E binding site (18) and the rhinovirus 2A^{pro} cleavage site (15) are also indicated.

middle third of eIF4GI is essential for the 48S ribosomal complex formation, because no complex II was formed with full-length eIF4GI Y776A (compare lanes 3 and 4). Surprisingly, binding of eIF4A to the C-terminal region is also important for efficient 48S ribosomal complex formation, because ribosome complex II formation was decreased three- to fourfold when wild-type eIF4GI was replaced with eIF4GI FVR1239AAA (compare lanes 3 and 5). To substantiate these results, we performed a titration experiment whereby three different amounts of full-length wild type and mutant (FVR1239AAA) were tested in the toeprinting assay (Fig. 6C). At all concentrations, eIF4GI FVR1239AAA exhibited only 20 to 30% activity of wild-type eIF4GI in the ribosomal complex formation assay.

It could be argued that FVR1239AAA mutation causes partial denaturation of eIF4GI, which affects the functions of the other regions of eIF4GI. To address this possibility, the effects of the mutations in the middle and C-terminal regions in the context of full-length eIF4GI on eIF4A, eIF3, and eIF4E binding were determined. Coimmunoprecipitation was performed with anti-eIF4GI(1–329) antiserum following assembly of translation factors on β-globin mRNA (Fig. 6D). Mutation of either the middle (lane 3) or C-terminal (lane 4) eIF4A binding site dramatically decreased (10-fold, as determined by laser densitometry) the association of eIF4A with eIF4GI. In contrast, binding of eIF4E and eIF3 was not affected by either mutation. Thus, the defect of the mutant proteins in formation of the ribosomal complex is most probably due to their failure in eIF4A binding.

To further support these results, the mutants used for ribo-

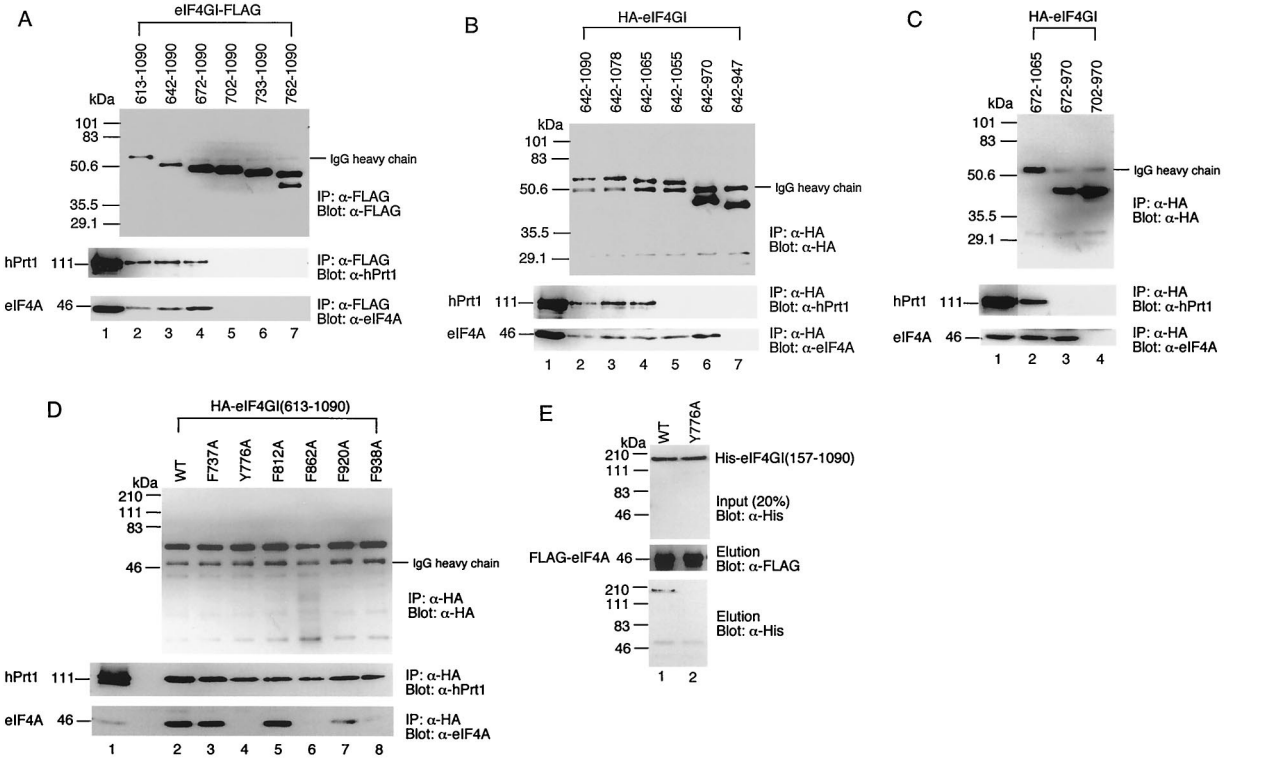


FIG. 4. Minimal essential region for eIF4A and eIF3 binding in the middle fragment of eIF4GI and point mutation for eIF4A binding. (A) N-terminal boundary. N-terminally truncated middle region fragments of eIF4GI were expressed as C-terminally FLAG-tagged proteins in HeLa cells, using the vaccinia virus system as described in Materials and Methods. Cell extract (1 mg) was immunoprecipitated (IP) with anti-FLAG antibody, and immunoprecipitates were resolved by SDS-PAGE (10% gel) for Western blotting with an anti-FLAG (upper panel), anti-hPrt1 (middle panel), or anti-eIF4A (lower panel) antibody. HeLa cell extract (40 μg of protein) was used as a control for the Western blotting in lane 1. IgG, immunoglobulin G. (B) C-terminal boundary. C-terminally truncated middle region fragments of eIF4GI were expressed in HeLa cells as N-terminally HA-tagged proteins. Cell extracts were immunoprecipitated with anti-HA antibody for Western blotting with an anti-HA (upper panel), anti-hPrt1 (middle panel), or anti-eIF4A (lower panel) antibody. HeLa cell extract (40 μg of protein) was used as a control for the Western blotting in lane 1. (C) Minimal essential region for eIF4A and eIF3 binding. HA-eIF4GI(672–1065), HA-eIF4GI(672–970), or HA-eIF4GI(702–970) was expressed in HeLa cells and processed as for panel B. (D) Coimmunoprecipitation of point mutants of the eIF4GI middle region with eIF4A and eIF3. HA-eIF4GI(613–1090) wild type (WT) or HA-eIF4GI(613–1090) point mutants were expressed in HeLa cells and processed as for panel. (E) Recombinant eIF4GI(157–1090) Y776A does not bind eIF4A. Four micrograms of FLAG-eIF4A immobilized on anti-FLAG resin was incubated with 5 μg of His-eIF4GI(157–1090) wild type or His-eIF4GI(157–1090) Y776A on ice for 10 min. After washing, bound proteins were solubilized with SDS sample buffer and subjected to SDS-PAGE followed by Western blotting with an anti-FLAG (middle panel) or anti-His (lower panel) antibody. Twenty percent of the input His-tagged protein was resolved by SDS-PAGE followed by Western blotting with anti-His antibody (upper panel).

some complex formation were examined in the $2A^{pro}$-treated rabbit reticulocyte lysate. $2A^{pro}$ treatment dramatically reduced cap-dependent translation of CAT (Fig. 6E, compare lane 2 to lane 1). Addition of eIF4E by itself could not restore cap-dependent translation (lane 3), as shown above (Fig. 2C). However, addition of eIF4E together with eIF4G restored cap-dependent translation to 58% of the control level (compare lane 4 to lane 1). Addition of eIF4GI Y776A did not restore cap-dependent translation (lane 5), confirming that the binding of eIF4A to the middle domain of eIF4GI is essential for translation. Similarly, the importance of eIF4A binding to the C-terminal domain was corroborated, as eIF4GI FVR1239AAA stimulated translation to a much lesser extent than wild-type eIF4GI (24% of the wild-type level [compare lanes 4 and 6]). To substantiate this conclusion, we performed a titration experiment in which different amounts of full-length wild type and mutant (FVR1239AAA) were tested in in vitro translation (Fig. 6F). The capacity of eIF4GI FVR1239AAA to stimulate translation was four- to sixfold lower than the wild-type level within the range of the protein amounts examined.

The Mnk1 binding site does not overlap with the eIF4A binding site in the C-terminal region of eIF4GI. Recently, the serine/threonine kinase Mnk1 (4, 29) was shown to bind to the C-terminal region of eIF4G and to phosphorylate eIF4E (26, 30). However, it was not determined whether Mnk1 competes with eIF4A for binding to the C-terminal region. To address this question, progressive amino- or carboxy-terminal deletion mutants of the C-terminal region of eIF4GI were expressed as GST fusion proteins together with FLAG-Mnk1 in HeLa cells. Cell extracts were precipitated with glutathione-Sepharose, and bound proteins were eluted with glutathione (Fig. 7A) or immunoprecipitated with anti-FLAG antibody (Fig. 7B). The N-terminal boundary for Mnk1 binding resides between aa 1411 and 1470, because eIF4GI(1411–1560) bound Mnk1 but eIF4GI(1470–1560) did not (Fig. 7A, lanes 3 and 4). The C-terminal boundary resides between aa 1545 and 1560, because eIF4GI(1411–1560) bound Mnk1 but eIF4GI(1235–1545) did not (Fig. 7B, lanes 3 and 4). Thus, the Mnk1 binding site, eIF4GI(1411–1560), does not overlap with the C-terminal eIF4A binding site (Fig. 8, top).

DISCUSSION

In this report we demonstrate that a core sequence of 540 aa, comprising the middle third and eIF4E binding site of eIF4GI, is required and sufficient for cap-dependent transla-

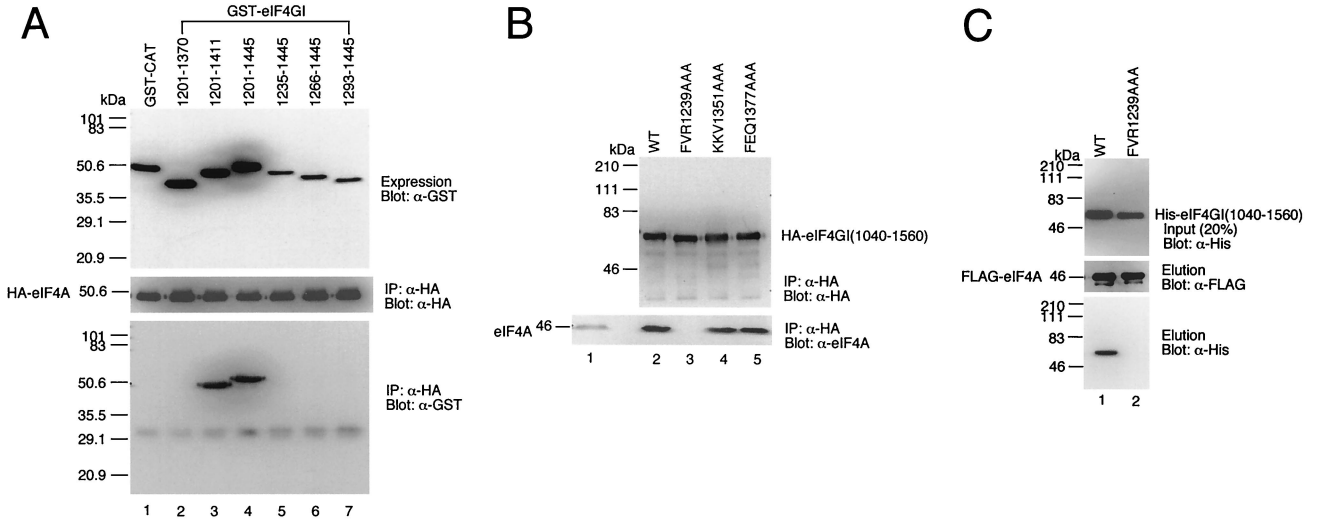


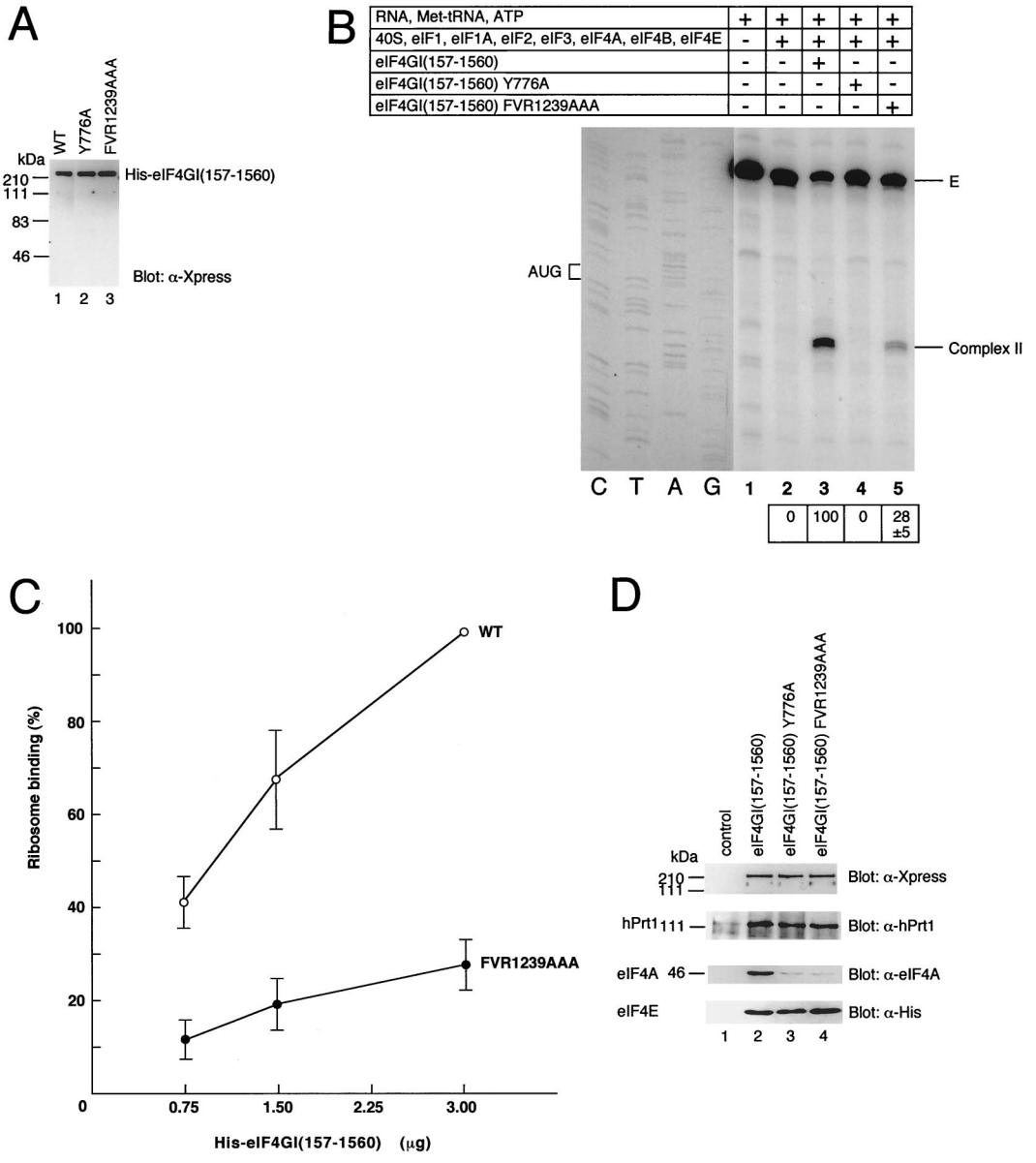
FIG. 5. Demarcation of the C-terminal eIF4A-binding domain of eIF4GI and point mutants. (A) Coimmunoprecipitation of deletion mutants of the eIF4GI C-terminal domain with eIF4A. GST-CAT or GST-eIF4GI deletion mutants were coexpressed with HA-eIF4A in HeLa cells, using the vaccinia virus system as described in Materials and Methods. One-twentieth of the cell extract was used for Western blotting with anti-GST antibody (upper panel). The remaining extract was immunoprecipitated (IP) with anti-HA antibody, and immunoprecipitates were resolved by SDS-PAGE (15% gel) followed by Western blotting with anti-HA (middle panel) or anti-GST (lower panel) antibody. (B) Coimmunoprecipitation of point mutants of the eIF4GI C-terminal domain with eIF4A. HA-eIF4GI(1040–1560) wild type (WT) or HA-eIF4GI(1040–1560) point mutants were expressed in HeLa cells. The cell extract was immunoprecipitated with anti-HA antibody, and immunoprecipitates were resolved by SDS-PAGE (10% gel) followed by Western blotting with an anti-HA (upper panel) or anti-eIF4A (lower panel) antibody. HeLa cell extract (40 μg of protein) was used as a control for Western blotting in lane 1. (C) Recombinant eIF4GI(1040–1560) FVR1239AAA does not bind eIF4A in vitro. Four micrograms of FLAG-eIF4A immobilized on anti-FLAG resin was incubated with 5 μg of His-eIF4GI(1040–1560) or His-eIF4GI(1040–1560) FVR1239AAA on ice for 10 min. After washing, bound proteins were solubilized for SDS-PAGE followed by Western blotting with an anti-FLAG (middle panel) or anti-His (lower panel) antibody. Twenty percent of the input His-tagged protein was subjected to SDS-PAGE followed by Western blotting with anti-His antibody (upper panel).

tion. The middle third of eIF4GI binds two initiation factors, eIF4A and eIF3 (12), and possesses an RNA binding site (24). Owing to these features, this segment can mediate the internal entry of a 43S ribosome preinitiation complex on the EMCV IRES (24) and stimulate translation of uncapped RNAs in a reticulocyte lysate (2). However, all eukaryotic mRNAs possess a cap structure, and most are considered to be translated in a cap-dependent manner. Our present results demonstrate the importance of the middle region of eIF4GI for cap-dependent translation and further underscore the important role of the eIF4E-binding site for cap-dependent translation. We have also delimited the binding sites of eIF4A, eIF3, and Mnk1 on eIF4GI (Fig. 8, top).

Another important feature of eIF4GI characterized here is that binding of eIF4A to the C-terminal third region is required for robust translation, suggesting that the C-terminal region of eIF4GI plays a modulatory role. How does binding of eIF4A to the C-terminal region modulate the function of the core domain of eIF4GI? We consider two models, which are not necessarily mutually exclusive (Fig. 8). In model A, the C-terminal third folds over the middle region to inhibit its function in an autoinhibitory manner. eIF4A is sandwiched between the middle and C-terminal regions of eIF4GI, thus relieving the inhibition by the C-terminal region. Such a model may explain how eIF4AIII, a newly characterized translation modulator, functions (17). eIF4AIII, which exhibits 65% amino acid identity with eIF4AI, possesses RNA-dependent ATPase activity and ATP-dependent helicase activity, but it fails to substitute for eIF4AI in the ribosomal binding assay. In addition, eIF4AIII inhibits translation in a reticulocyte lysate (17). Interestingly, eIF4AIII binds eIFGI only through the middle region of eIF4GI. It is therefore conceivable that eIF4AIII fails to alleviate the inhibitory activity of the C-terminal part of eIF4G and instead forms an inactive eIF4F complex. The mechanism of action of a translation repressor protein, p97, may also be explained by this model. p97 is homologous to the C-terminal two-thirds segment of eIF4G, and it binds to eIF4A and eIF3 but not to eIF4E or PABP. p97 inhibits both cap-dependent and -independent translation, probably by sequestering eIF4A and eIF3 (11). Interestingly, the N-terminal half of p97, which corresponds to the middle region of eIF4G, binds eIF4A, but the C-terminal half, which corresponds to the C-terminal third of eIF4G, fails to bind eIF4A (12). Thus, the defect of p97 in stimulating translation might result from the inability of eIF4A to bind to the C-terminal half of p97, thus failing to counteract its autoinhibitory function.

An alternative model (Fig. 8, model B) for the function of

FIG. 6. Functional analysis of eIF4GI point mutants. (A) Western blotting of eIF4GI point mutants. One microgram of each recombinant protein preparation was subjected to SDS-PAGE (10% gel) and analyzed by Western blotting with anti-Xpress antibody to detect the epitope located between the His-tag and eIF4GI coding sequence. WT, wild type. (B) toeprinting analysis of 48S ribosomal complex formation on β-globin mRNA with eIF4GI point mutants (2 μg of each). The components of the reaction mixtures are indicated above the lanes. The value for eIF4GI(157–1560) (lane 3) was set at 100. (C) Dose-dependent analysis of eIF4GI FVR1239AAA in a ribosomal binding assay. Increasing amounts of wild-type eIF4GI and eIF4GI FVR1239AAA were used in toeprinting analysis. The value for wild-type eIF4GI (3 μg) was set at 100. (D) Binding of translation factors to eIF4GI point mutants in vitro. The components indicated above the lanes in panel B were mixed and incubated as for toeprinting analysis. The mixture was then immunoprecipitated with anti-eIF4GI(1–329), and immunoprecipitates were analyzed by Western blotting with anti-Xpress for eIF4G, anti-hPrt1 for eIF3, anti-eIF4A, or anti-His for eIF4E. (E) Analysis of eIF4GI point mutants in a rabbit reticulocyte lysate translation system. Translation was performed as described in Materials and Methods. Translation products were analyzed as for Fig. 2C. LUC, luciferase. (F) Dose-response analysis of eIF4GI FVR1239AAA in a rabbit reticulocyte lysate treated with $2A^{pro}$.


A
WT
Y776A
FVR1239AAA
kDa
210
111
83
46
His-eIF4GI(157-1560)
Blot: α-Xpress
1 2 3
B
RNA, Met-tRNA, ATP + + + + +
40S, eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, eIF4E - + + + +
eIF4GI(157-1560) - - + - -
eIF4GI(157-1560) Y776A - - - + -
eIF4GI(157-1560) FVR1239AAA - - - - +
AUG
E
Complex II
C T A G 1 2 3 4 5
0 100 0 28 ±5
C
Ribosome binding (%)
100
80
60
40
20
0
WT
FVR1239AAA
0.75
1.50
2.25
3.00
His-eIF4GI(157-1560) (μg)
D
control
eIF4GI(157-1560)
eIF4GI(157-1560) Y776A
eIF4GI(157-1560) FVR1239AAA
kDa
210
111
Blot: α-Xpress
hPrt1 111
Blot: α-hPrt1
eIF4A 46
Blot: α-eIF4A
eIF4E
Blot: α-His
1 2 3 4
E
2Apro - + + + + +
eIF4E - - + + + +
eIF4GI(157-1560) - - - + - -
eIF4GI(157-1560) Y776A - - - - + -
eIF4GI(157-1560) FVR1239AAA - - - - - +
LUC
CAT
1 2 3 4 5 6
LUC (%) 100 224 236 206 201 209
CAT (%) 0 100 0 24
F
2Apro - + + + + + + + + + +
eIF4E - - + + + + + + + + +
eIF4GI(157-1560) (μg) - - - 0.3 0.6 0.9 1.2 - - - -
eIF4GI(157-1560) FVR1239AAA (μg) - - - - - - - 0.3 0.6 0.9 1.2
LUC
CAT
1 2 3 4 5 6 7 8 9 10 11
LUC (%) 100 148 157 164 157 157 153 165 166 164 145
CAT (%) 0 19 39 77 100 3 10 13 16

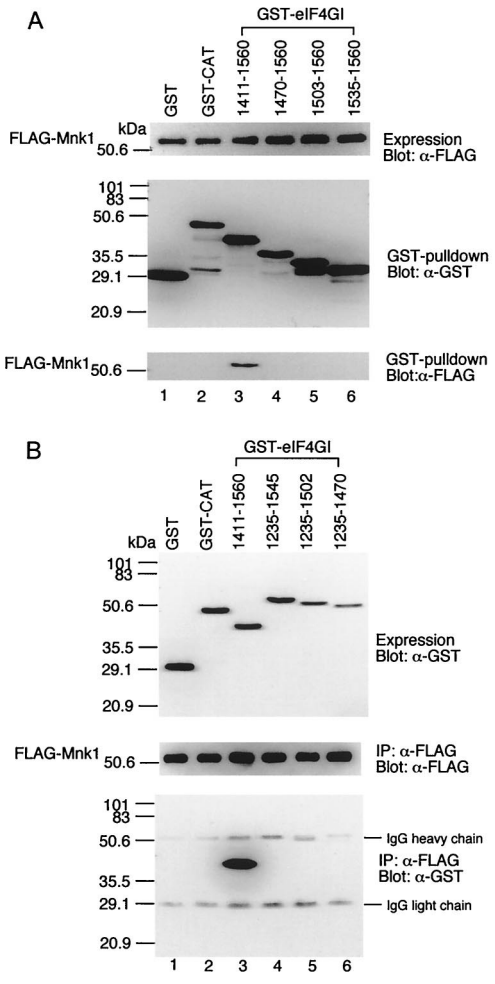


FIG. 7. Demarcation of the Mnk1 binding site in the C-terminal region of eIF4GI. (A) N-terminal boundary. GST, GST-CAT, or GST-eIF4GI deletion mutants were coexpressed with FLAG-Mnk1 in HeLa cells. One-fortieth of the cell extract was subjected to SDS-PAGE for Western blotting with anti-FLAG antibody to confirm the expression of FLAG-Mnk1 (upper panel). The remaining extract was mixed with glutathione-Sepharose beads. Bound proteins eluted with reduced glutathione were subjected to Western blotting with an anti-GST (middle panel) or anti-FLAG (lower panel) antibody. (B) C-terminal boundary. GST, GST-CAT, or GST-eIF4GI deletion mutants were coexpressed with FLAG-Mnk1 in HeLa cells. One-fortieth of the cell extract was subjected to SDS-PAGE for Western blotting with anti-GST antibody to confirm the expression of GST fusion proteins (upper panel). The remaining extract was immunoprecipitated with anti-FLAG antibody, and immunoprecipitates were subjected to Western blotting with anti-FLAG (middle panel) or anti-GST (lower panel) antibody. IgG, immunoglobulin G.

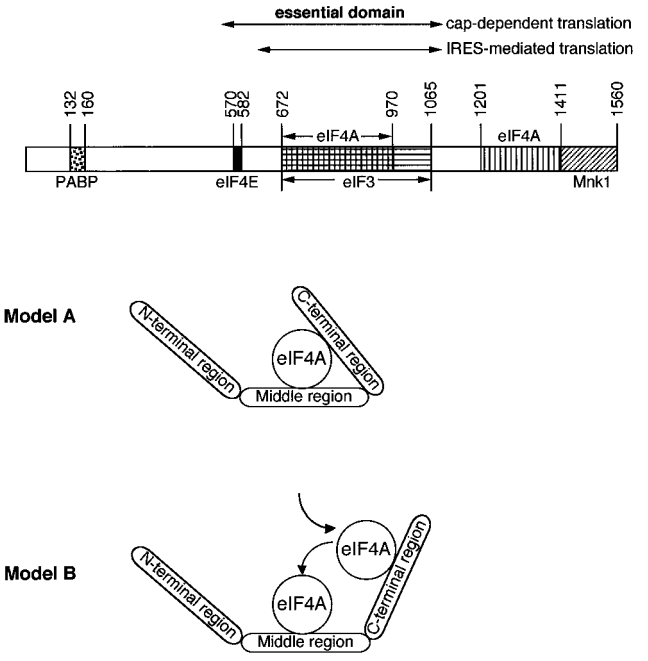


FIG. 8. Model of eIF4GI functional domains. Previous studies have mapped the eIF4E (18) and PABP (13) binding sites to the N-terminal third of eIF4G. The middle third region was shown to bind eIF4A and eIF3 (12), while the C-terminal third region was shown to bind eIF4A (12, 14) and Mnk1 (26). See Discussion section for explanations of models.

the C-terminal region is that the middle region of eIF4GI is sterically hidden from free eIF4A by the C-terminal region. eIF4A binds first to the C-terminal region and is subsequently transferred to the middle region. This idea is consistent with another feature of eIF4AIII: while eIF4AIII strongly binds to the middle third of eIF4GI, it binds the full-length eIF4GI very poorly (17). To distinguish between these models, dissociation constants between eIF4A and each region or full-length of eIF4GI ought to be determined. Also, the number of eIF4A molecules that bind to eIF4GI at a given time will need to be established.

The C-terminal third of eIF4G also plays a role in the phosphorylation of eIF4E. The distal C-terminal region of eIF4GI contains a binding site for the serine/threonine kinase Mnk1 (Fig. 7). It has been shown that the C-terminal third of eIF4G recruits Mnk1 to phosphorylate eIF4E in vivo (26, 30), which is thought to stimulate cap-dependent translation. Phosphorylation of eIF4G itself may also affect translation. Interestingly, several serum-responsive phosphorylation sites are localized in the C-terminal third region of eIF4GI (B. Raught, A.-C. Gingras, S. P. Gygi, H. Imataka, S. Morino, A. Gradi, R. Aebersold, and N. Sonenberg, unpublished data).

What is the function of the N-terminal third of eIF4G? This region harbors the eIF4E and PABP binding sites and consequently engages the mRNA via both its 5′ and 3′ ends. While the critical role of the eIF4E-binding site for cap-dependent translation is confirmed in this study, our experiments did not address the importance of the PABP binding site in translation, because the recombinant eIF4GI which we used lacked this site. It should be very interesting, however, to examine how translation is affected when mRNA is circularized through the N terminus of eIF4G in a reconstituted translation system, using full-length eIF4G. However, current models state that the PABP interaction with eIF4G is not required for the first round of translation initiation, but only for subsequent rounds (28). The spacer region between the PABP and eIF4E binding sites (~400 aa) is the least conserved region between eIF4GI and eIF4GII (7). We do not know the function of this region; no protein has been reported to bind this region, and its deletion had no effect on ribosomal binding (Fig. 2B) or on translation in the reticulocyte lysate (Fig. 2C).

Finally, using the in vitro ribosome binding assay, we demonstrated 48S ribosomal complex formation at the correct initiator AUG for the β-globin mRNA in the absence of eIF4E (Fig. 1) or with an eIF4GI mutant lacking the eIF4E binding site (Fig. 2B), albeit with low efficiency (20 to 24% of that of the complete system). The eIF4E-independent ribosome binding does not seem to represent aberrant ribosomes binding,

since the ribosomal complex was not formed at the second AUG of β-globin mRNA (data not shown; the second AUG was out of the photograph in Fig. 1 and 2B). Thus, the binding represents cap-independent but 5′-end-dependent translation, which has been documented in a rabbit reticulocyte lysate (2) and in mammalian cells (8). The first AUG is still predominantly utilized as the translation initiator when the reticulocyte lysate is programmed with uncapped mRNA (2) or when uncapped mRNA is transcribed by RNA polymerase III in mammalian cells (8). These results are consistent with the idea that the cap structure dramatically enhances translation, rather than being absolutely required for translation in eukaryotes.

In summary, we have defined a minimal core sequence of eIF4GI which is required for ribosome binding and translation. The core sequence constitutes only one-third of the entire eIF4GI protein. The C-terminal third region, which does not have a counterpart in yeast, modulates eIF4GI activity. It is therefore of great importance to elucidate the mechanism by which the eIF4G C-terminal region controls translation in metazoan cells.

ACKNOWLEDGMENTS

We thank W. C. Merrick for eIF2, eIF3, and eIF4F proteins used for a preliminary experiment, T. Skern for rhinovirus $2A^{pro}$, A. Gradi for the anti-eIF4GI antibody, and R. Fukunaga for the Mnk1 plasmid. We are indebted to C. Lister for excellent technical assistance. We thank B. Raught and A.-C. Gingras for sharing unpublished data and critically reading the manuscript.

S.M. was supported by research fellowships of the Japan Society for the Promotion of Science for Young Scientists. This work was supported by a grant from the Medical Research Council of Canada to N.S. N.S. is a Distinguished Scientist of the Medical Research Council of Canada and a Howard Hughes Medical Institute International Scholar.

ADDENDUM IN PROOF

Since the submission of this paper, a report by De Gregorio et al. (EMBO J. **18:**4865–4874, 1999) also defined a conserved central domain (aa 642 to 1091) of eIF4G as an autonomous ribosome recruitment core in vivo.

REFERENCES

1. **Allen, M., A. M. Metz, R. T. Timmer, R. E. Rhoads, and K. S. Browning.** 1992. Isolation and sequence of the cDNAs encoding the subunits of the isozyme form of wheat protein synthesis initiation factor 4F. J. Biol. Chem. **267:**23232–23236.
2. **De Gregorio, E., T. Preiss, and M. W. Hentze.** 1998. Translational activation of uncapped mRNAs by the central part of human eIF4G is 5′ end-dependent. RNA **4:**828–836.
3. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
4. **Fukunaga, R., and T. Hunter.** 1997. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. **16:**1921–1933.
5. **Gingras, A.-C., B. Raught, and N. Sonenberg.** 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. **68:**913–963.
6. **Goyer, C., M. Altmann, H. S. Lee, A. Blanc, M. Deshmukh, J. L. Woolford, Jr., H. Trachsel, and N. Sonenberg.** 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.
7. **Gradi, A., H. Imataka, Y. V. Svitkin, E. Rom, B. Raught, S. Morino, and N. Sonenberg.** 1998. A novel functional human eukaryotic translation initiation factor 4G. Mol. Cell. Biol. **18:**334–342.
8. **Gunnery, S., Ü. Mäivali, and M. B. Mathews.** 1997. Translation of an uncapped mRNA involves scanning. J. Biol. Chem. **272:**21642–21646.
9. **Haghighat, A., Y. V. Svitkin, I. Novoa, E. Kuechler, T. Skern, and N. Sonenberg.** 1996. The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. J. Virol. **70:**8445–8450.
10. **Hellen, C. U. T., G. W. Witherell, M. Schmid, S. H. Shin, T. V. Pestova, A. Gil, and E. Wimmer.** 1993. A cytoplasmic 57kDa protein that is required for translation of picornavirus RNA by internal ribosome entry is identical to the nuclear pyrimidine tract-binding protein. Proc. Natl. Acad. Sci. USA **90:**7642–7646.
11. **Imataka, H., H. S. Olsen, and N. Sonenberg.** 1997. A new translational regulator with homology to eukaryotic translation initiation factor 4G. EMBO J. **16:**817–825.
12. **Imataka, H., and N. Sonenberg.** 1997. Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. Mol. Cell. Biol. **17:**6940–6947.
13. **Imataka, H., A. Gradi, and N. Sonenberg.** 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J. **17:**7480–7489.
14. **Lamphear, B. J., R. Kirchweger, T. Skern, and R. E. Rhoads.** 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases: implications for cap-dependent and cap-independent translational initiation. J. Biol. Chem. **270:**21975–21983.
15. **Lamphear, B. J., R. Yan, F. Yang, D. Waters, H. D. Liebig, H. Klump, E. Kuechler, T. Skern, and R. E. Rhoads.** 1993. Mapping of 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.
16. **Levy-Strumpf, N., L. P. Deiss, H. Berissi, and A. Kimchi.** 1997. DAP-5, a novel homolog of eukaryotic translation initiation factor 4G isolated as a putative modulator of gamma interferon-induced programmed cell death. Mol. Cell. Biol. **17:**1615–1625.
17. **Li, Q., H. Imataka, S. Morino, G. W. Rogers, Jr., N. J. Richter-Cook, W. C. Merrick, and N. Sonenberg.** 1999. Eukaryotic translation initiation factor 4AIII (eIF4AIII) is functionally distinct from eIF4AI and eIF4AII. Mol. Cell. Biol. **19:**7336–7346.
18. **Mader, S., H. Lee, A. Pause, and N. Sonenberg.** 1995. The translation initiation factor eIF4E 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.
19. **Merrick, W. C., and J. W. B. Hershey.** 1996. The pathway and mechanism of eukaryotic protein synthesis, p. 31–70. *In* J. Hershey, M. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Morley, S. J., P. S. Curtis, and V. M. Pain.** 1997. eIF4G: translation's mystery factor begins to yield its secrets. RNA **3:**1085–1104.
21. **Neff, C. L., and A. B. Sachs.** 1999. Eukaryotic translation initiation factors 4G and 4A from *Saccharomyces cerevisiae* interact physically and functionally. Mol. Cell. Biol. **19:**5557–5564.
22. **Pain, V. M.** 1996. Initiation of protein synthesis in eukaryotic cells. Eur. J. Biochem. **236:**747–771.
23. **Pestova, T. V., C. U. T. Hellen, and I. N. Shatsky.** 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol. Cell. Biol. **16:**6859–6869.
24. **Pestova, T. V., I. N. Shatsky, and C. U. T. Hellen.** 1996. Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol. Cell. Biol. **16:**6870–6878.
25. **Pestova, T. V., S. I. Borukhov, and C. U. T. Hellen.** 1998. Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature **394:**854–859.
26. **Pyronnet, S., H. Imataka, A.-C. Gingras, R. Fukunaga, T. Hunter, and N. Sonenberg.** 1999. Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. EMBO J. **18:**270–279.
27. **Sachs, A. B., P. Sarnow, and M. W. Hentze.** 1997. Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell **89:**831–838.
28. **Sonenberg, N.** 1996. mRNA 5′ cap-binding protein eIF4E and control of cell growth, p. 271–294. *In* J. Hershey, M. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Waskiewicz, A. J., A. Flynn, C. G. Proud, and J. A. Cooper.** 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. **16:**1909–1920.
30. **Waskiewicz, A. J., J. C. Johnson, B. Penn, M. Mahalingam, S. R. Kimball, and J. A. Cooper.** 1999. Phosphorylation of the cap-binding protein eIF4E by the protein kinase Mnk1 *in vivo*. Mol. Cell. Biol. **19:**1871–1880.
31. **Yamanaka, S., K. S. Poksay, K. S. Arnold, and T. L. Innerarity.** 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:**321–333.
32. **Yan, R., W. Rychlik, D. Etchsion, and R. E. Rhoads.** 1992. Amino acid sequence of the human protein synthesis initiation factor eIF-4γ. J. Biol. Chem. **267:**23226–23231.