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# 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

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**Eukaryotic translation is initiated following binding of ribosomes either to the capped 5′ end of an mRNA or to an internal ribosomal entry site (IRES) within its 5′ nontranslated region. These processes are both mediated by eukaryotic initiation factor 4F (eIF4F), which consists of eIF4A (helicase), eIF4E (cap-binding protein), and eIF4G subunits. Here we present a functional analysis of eIF4F which defines the subunits and subunit domains necessary for its function in initiation mediated by the prototypical IRES element of encephalomyocarditis virus. In an initiation reaction reconstituted in vitro from purified translation components and lacking eIF4A and -4F, IRES-mediated initiation did not require the cap-binding protein eIF4E but was absolutely dependent on eIF4A and the central third of eIF4G. This central domain of eIF4G bound strongly and specifically to a structural element within the encephalomyocarditis virus IRES upstream of the initiation codon in an ATP-independent manner and with the same specificity as eIF4F. The carboxy-terminal third of eIF4G did not bind to the IRES. The central domain of eIF4G was itself UV cross-linked to the IRES and strongly stimulated UV cross-linking of eIF4A to the IRES in conjunction with either eIF4B or with the carboxy-terminal third of eIF4G.**

The first step in initiation of eukaryotic protein synthesis is formation of a 43S complex that consists of eukaryotic initiation factor 3 (eIF3) and a ternary complex (Met-tRNA$_i^{Met}$, eIF2, and GTP) bound to the 40S small ribosomal subunit (32). The second, rate-limiting step is binding of the 43S complex to mRNA. Translation of most mRNAs is cap dependent and follows binding of 43S complexes to the 5′ m$^7$G cap-proximal region. A smaller number of mRNAs contain an internal ribosomal entry site (IRES) within the 5′ nontranslated region (5′ NTR) that promotes binding of the 43S complex to a position in the mRNA that can be far downstream of the 5′ terminus. Cap-dependent binding of ribosomes to eukaryotic mRNAs involves the initiation factors eIF4A, eIF4B, and eIF4F and ATP (48). We have recently found that the initiation mediated by the IRES of encephalomyocarditis virus (EMCV) has the same requirements (45).

eIF4A exhibits RNA-dependent ATPase activity and, in conjunction with eIF4B, RNA helicase activity (29, 49). The RNA-binding activity of eIF4A is enhanced by eIF4B in an ATP-dependent manner (1). eIF4B is a 70-kDa phosphoprotein that contains an RNA recognition motif (RRM) near its amino terminus that can bind to 18S rRNA (34, 36) and a second centrally located domain that binds RNA in a sequence nonspecific manner (33, 38). eIF4F consists of eIF4E (cap-binding protein), eIF4A, and eIF4G subunits. eIF4G is a 154-kDa polypeptide that coordinates the activity of eIF4F by interacting specifically with its eIF4A and eIF4E subunits, as well as with eIF3 (27, 31) and probably also with RNA (10). eIF4E binds to amino acids 409 to 457 of eIF4G (31). eIF4F also exhibits an RNA helicase activity that is stimulated by eIF4B and that is 20-fold more potent than the corresponding activity of eIF4A (20, 47, 49). Free eIF4A exchanges with the eIF4A subunit of eIF4F, and it has been suggested that it may gain access to mRNA only as a subunit of this factor (40, 54).

A model to explain the role of these mRNA-binding eIFs and ATP hydrolysis in ribosome binding to capped mRNAs suggests that eIF4F first binds to the 5′ end of an mRNA through interaction of its eIF4E subunit with the m$^7$G cap. Stimulation of eIF4F's helicase activity by eIF4B then melts RNA secondary structure in the vicinity of the cap to generate a single-stranded RNA region that can enter the mRNA binding site of the 40S subunit. eIF4B's interaction with 18S rRNA may guide the 40S subunit to this single-stranded region of RNA. The only major difference between this model and a model to explain the role of these factors in IRES-mediated ribosomal binding to mRNA is that the latter does not involve an interaction between eIF4F and the m$^7$G cap. We have proposed that instead, specific, cap-independent binding of eIF4F to sequences in the EMCV IRES that include the J-K domain some 50 nucleotides (nt) upstream of the initiation codon directs ribosomal binding to a defined internal binding site and is a prerequisite for initiation (45).

The functions provided by eIF4F in directing ribosomal binding to the capped 5′ ends of mRNAs are disrupted in cells infected by poliovirus following cleavage of eIF4G by the poliovirus-encoded 2A proteinase (2A$^{pro}$) (7), and cap-dependent initiation is shut off as a result. The primary 2A$^{pro}$ cleavage site in rabbit eIF4G is Arg$^{486}$-Gly$^{487}$ (28). Translation of IRES-containing mRNAs such as EMCV is not impaired under shut-off conditions (18, 21) even though eIF4F is required for initiation mediated by the EMCV IRES (45). These observations

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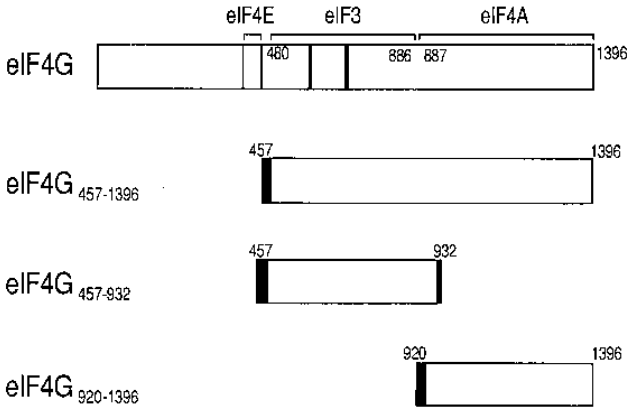


FIG. 1. Schematic representation of the structure of human eIF4G and mutant derivatives. eIF4E interacts with amino acids 409 to 457 of eIF4G (27); this region and regions of eIF4G with which eIF3 and -4A interact (31) are indicated. The positions of the proposed RNP-1 and RNP-2 motifs (10) are represented by black vertical bars. The black bars at the amino termini of $eIF4G_{457-1396}$, $eIF4G_{457-932}$, and $eIF4G_{920-1396}$ and at the carboxy terminus of $eIF4G_{457-932}$ represent vector-encoded amino acid residues. Amino acid residues of eIF4G are numbered according to the published sequence (53).

suggest that one or more functions provided by eIF4F in cap-dependent initiation may be dispensable for IRES-mediated initiation. Proteolytic cleavage separates the domain of eIF4G that interacts with eIF4E from eIF3, eIF4A, and putative RNA-binding domains (Fig. 1) and as a result uncouples cap recognition from ribosome-binding and helicase functions (27, 39).

Here, we present an analysis of the role of eIF4F in IRES-mediated initiation. Purified recombinant eIF4A and specific domains of eIF4G were used in assays to identify the subunits of eIF4F that are required for EMCV IRES-mediated initiation and to dissect their functions. Formation of 48S preinitiation complexes in a reconstituted in vitro initiation assay was found to be absolutely dependent on eIF4A and on the central third of eIF4G. This process did not require the cap-binding protein eIF4E. This central domain of eIF4G bound strongly and specifically to the J-K domain of EMCV IRES with the same specificity as eIF4F. The same domain of eIF4G strongly stimulated binding of eIF4A to the EMCV IRES in conjunction with either eIF4B or with the carboxy-terminal third of eIF4G. The implications of these observations for the mechanism and regulation of IRES-mediated initiation are discussed.

## MATERIALS AND METHODS

**Plasmids.** pTE1 contains EMCV nt 315 to 1155 (8) downstream of the T7 promoter in vector pTZ18R (Pharmacia, Piscataway, N.J.). pTE10 is identical to pTE1 except that EMCV nt 702 to 762 inclusive have been replaced by the nucleotides GGGAAUUCCC (8).

pET15($His_6$-eIF4G) was constructed by inserting an *Eco*RV-*Eco*RV fragment derived from pSK-HFC1 (23) that contains the complete human eIF4G coding and 3′ NTR sequences (53) into the *Sma*I site of $pT7H_61$ (45). pET28($His_6$-$eIF4G_{457-1396}$) was constructed by cloning a *Bam*HI-*Hin*dIII restriction fragment derived from pSK-HFC1 between the *Bam*HI and *Hin*dIII sites of pET28b(+) (Novagen, Madison, Wis.). pET28($His_6$-$eIF4G_{457-932}$) was constructed by deleting a *Kpn-Kpn*I fragment from pET15($H_6$-eIF4G), religating the larger restriction fragment, and cloning a *Bam*HI-*Hin*dIII restriction fragment derived from it between the *Bam*HI and *Hin*dIII sites of pET28b(+). pET28($His_6$-$eIF4G_{920-1396}$) was constructed by cloning an *Spe*I-*Hin*dIII fragment derived from pSK-HFC1 between the *Nhe*I and *Hin*dIII sites of pET28b(+).

**Purification of initiation factors and 40S ribosomal subunits.** 40S ribosomal subunits and the initiation factors eIF2, eIF3, and eIF4F were prepared from rabbit reticulocyte lysate (Green Hectares, Oregon, Wis.) as described previously (45). Recombinant eIF4A, eIF4B, and pyrimidine tract-binding protein 1 (PTB-1) were expressed in *Escherichia coli* BL21(DE3) and were purified as described previously (14, 45). The recombinant polypeptides $eIF4G_{457-1396}$, $eIF4G_{457-932}$, and $eIF4G_{920-1396}$ were expressed in *E. coli* BL21(DE3) or B834 (DE3). The level of expression of $eIF4G_{920-1396}$ was much higher than that of either $eIF4G_{457-1396}$ or $eIF4G_{457-932}$. These polypeptides were purified chromatographically by elution with 100 mM imidazole from a $Ni^{2+}$-nitrilotriacetic acid matrix (Qiagen Inc., Chatsworth, Calif.). $eIF4G_{457-1396}$ and $eIF4G_{457-932}$ were then applied to a heparin-Sepharose (Pharmacia) matrix in buffer (20 mM Tris-HCl [pH 7.5], 2 mM dithiothreitol 0.5 mM EDTA) with 100 mM KCl and eluted by using the same buffer with 300 mM KCl.

**Western blot (immunoblot) analysis.** Polypeptides were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were incubated at 25°C for 60 min in Tris-buffered saline containing 0.20% Tween 20 (TBS-T) and 5% dry milk and then for 60 min with a primary antibody. We used an anti-eIF2 polyclonal antibody (1:500; a kind gift from R. Jagus), antibodies directed against each of the subunits of eIF2 (1:1,000; a kind gift from U. Bommer), anti-eIF2/2B polyclonal antibodies (1:100; a kind gift from R. Matts), an anti-eIF3 (p170 subunit) monoclonal antibody (1:20,000; a kind gift from D. Etchison), an anti-eIF4A monoclonal antibody (1:10; a kind gift from H. Trachsel), a goat anti-eIF4B polyclonal antibody (1:1,000; a kind gift from J. Hershey), an anti-eIF4G monoclonal antibody (1:5,000; a kind gift from D. Etchison), an anti-PTB polyclonal antibody (1:1,000; a kind gift from P. Sharp), and a T7-Tag monoclonal antibody (1:5,000; Novagen) which recognizes the vector-encoded amino acid sequence MASMTGGQQMG at the amino termini of $eIF4G_{457-1396}$ and $eIF4G_{457-932}$. After being washed with TBS-T, membranes were treated with peroxidase-linked immunoglobulin G in combination with the Amersham ECL system and exposed to X-ray film.

**Preparation of EMCV RNA and [$^{35}$S]methionyl initiator tRNA.** Plasmids pTE1 and pTE10 were linearized with *Pst*I and transcribed in vitro either in the presence or in the absence of [$^{32}$P]UTP (~3,000 Ci/mmol; Amersham, Arlington Heights, Ill.) with T7 RNA polymerase mutant DEL172-3, which terminates efficiently at termini with 3′ overhanging ends (30). RNA transcripts derived from these plasmids are termed wild type and mutant D1. They were purified as described previously (44). The specific activities of mRNA preparations used in assembly of 48S complexes for analysis in sucrose density gradients were in the range of 300,000 to 500,000 cpm/μg of RNA. [$^{35}$S]Met-$tRNA_i^{Met}$ was prepared as described previously (45).

**Assembly and sucrose density gradient centrifugation of 48S complexes.** 48S complexes were assembled by incubating 1 μg of EMCV RNA for 6 min at 30°C in a 100-μl reaction volume that contained buffer A (2 mM dithiothreitol, 100 mM potassium acetate, 20 mM Tris [pH 7.6]) with 2.5 mM magnesium acetate, 100 U of RNasin (Promega), 1 mM ATP, 0.4 mM guanylimidodiphosphate (GMP-PNP), 250 μM spermidine, 6 pmol of [$^{35}$S]Met-$tRNA_i^{Met}$, 6 pmol of 40S subunits, and combinations of PTB (1.5 μg) and the initiation factors eIF2 (4 μg), eIF3 (10 μg), eIF4A (4 μg), eIF4B (2 μg), eIF4F (2 μg), $eIF4G_{457-1396}$ (2 μg), $eIF4G_{457-932}$ (1 μg), and $eIF4G_{920-1396}$ (1 μg) as indicated in the text. 48S and ribonucleoprotein (RNP) complexes were resolved by centrifugation through a 10 to 30% sucrose gradient in buffer A with 6 mM magnesium acetate for 16 h at 4°C and 24,000 rpm, using a Beckman SW41 rotor. The radioactivity of gradient fractions was determined by Cerenkov counting.

**Primer extension analysis of 48S complexes.** 48S complexes were assembled on unlabeled EMCV RNA essentially as described above by incubating a 40-μl reaction mix for 6 min at 30°C. Incubation was continued for 3 min at 30°C following addition of 4 pmol of the oligonucleotide 5′-GTCAATAACTCCTCT GG-3′ (complementary to EMCV nt 957 to 974). The reaction mix was then placed on ice. The reaction mix was incubated for 45 min at 30°C after addition of 1 μl of magnesium acetate (320 mM), 4 μl of deoxynucleoside triphosphates (5 mM dCTP, dGTP, and dTTP; 1 mM dATP), 1 μl of [α-$^{32}$P]dATP (~6,000 Ci/mmol; Amersham), and 15 U of avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega, Madison, Wis.) and was then extracted with phenol-chloroform (1:1). cDNA products were compared with a dideoxynucleotide sequence ladder obtained by using the same primer and pTE1 plasmid DNA after electrophoresis through 6% polyacrylamide sequencing gels.

**UV cross-linking of EMCV RNA.** The UV cross-linking reaction was done by using a UV-Stratalinker (Stratagene, La Jolla, Calif.) essentially as described previously (44). Purified EMCV RNA ($10^5$ to $10^6$ cpm) that had been labeled with [$^{32}$P]UTP during transcription with T7 polymerase was incubated for 10 min at 30°C in a 20-μl reaction volume containing buffer A with 2.5 mM magnesium acetate, 20 U of RNasin (Promega), 0.25 mM spermidine, and combinations of the factors eIF4A (3 μg), eIF4B (1.5 μg), and eIF4F (2 μg) and the eIF4G fragments $eIF4G_{457-1396}$ (2 μg), $eIF4G_{457-932}$ (0.5 μg), and $eIF4G_{920-1396}$ (0.5 μg) either with or without 1 mM ATP, as indicated in the text.

## RESULTS

**Expression and purification of eIF4G domains.** Polypeptides that correspond to amino acid residues 457 to 1396, 457 to 932, and 920 to 1396 of human eIF4G were expressed in *E. coli* as fusion proteins that contain six His residues at their N termini (Fig. 1), allowing a simple purification by metal chelate affinity chromatography (15). $eIF4G_{457-1396}$ and $eIF4G_{457-932}$

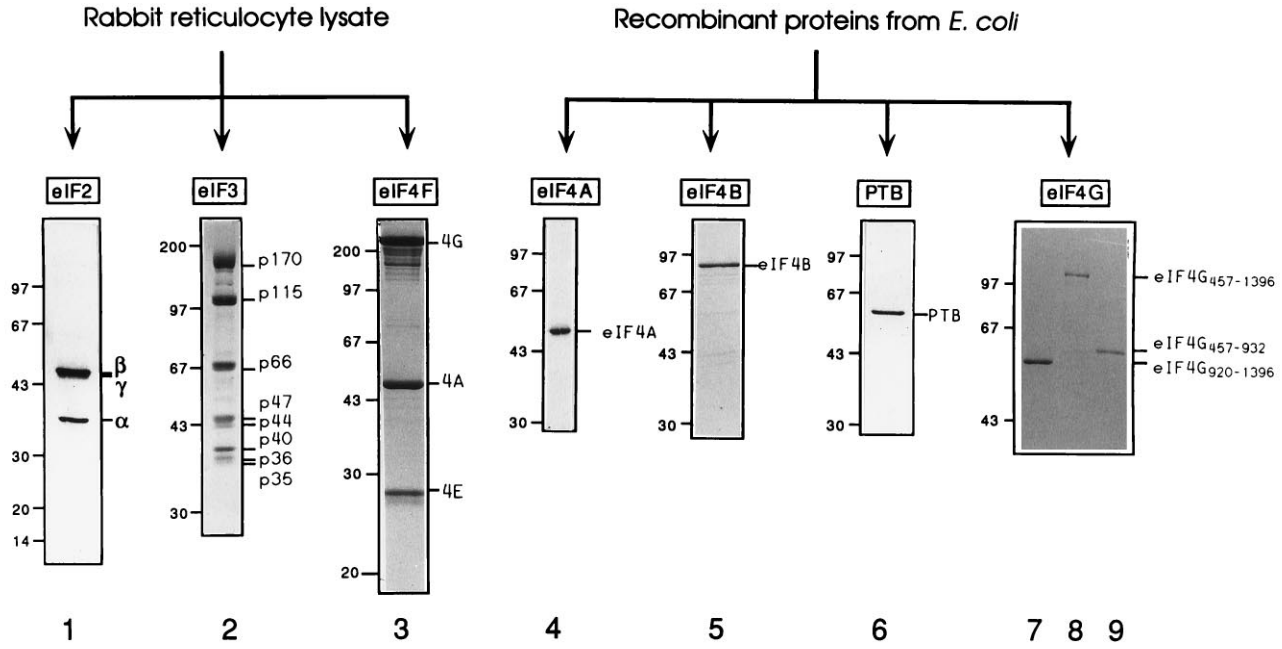


FIG. 2. Overview of the factors used in assaying EMCV IRES-mediated formation of 48S complexes. Purified proteins were analyzed by electrophoresis on SDS–5 to 20% gradient polyacrylamide gels and by Coomassie blue staining. The positions of molecular weight marker proteins are indicated in kilodaltons to the left of each panel. Subunits of eIF2, eIF3, and eIF4F and recombinant eIF4A, eIF4B, PTB, $eIF4G_{457-1396}$, $eIF4G_{457-932}$, and $eIF4G_{920-1396}$ are indicated to the right of appropriate panels. Lane 1, 4 μg of eIF2; lane 2, 5 μg of eIF3; lane 3, 4 μg of eIF4F; lane 4, 2 μg of recombinant human eIF4A; lane 5, 1.5 μg of recombinant human eIF4B; lane 6, 2 μg of recombinant human PTB; lane 7, 1.5 μg of recombinant human $eIF4G_{920-1396}$; lane 8, 1.5 μg of recombinant human $eIF4G_{457-1396}$; lane 9, 1 μg of recombinant human $eIF4G_{457-932}$.

were purified further by chromatography using a heparin-Sepharose matrix. The SDS-PAGE analysis of purified recombinant eIF4G polypeptides is shown in Fig. 2 (lanes 7 to 9). The largest of these polypeptides ($eIF4G_{457-1396}$) corresponds closely to the larger, C-terminal fragment released from eIF4G by picornavirus proteinase cleavage (27, 28).

**Stable interaction of domains of eIF4G with the EMCV IRES.** We have previously found that eIF4F binds specifically to the J-K domain of the EMCV IRES (Fig. 3A) and that this interaction correlates with the ability of the IRES to mediate internal ribosomal entry (45). This domain is sufficiently structured to inhibit primer extension weakly at $C_{786}$ on naked RNA (e.g., Fig. 3B, lane 1). It is further stabilized by interaction with eIF4F, resulting in strong inhibition of primer extension at this position (45). To characterize this interaction in greater detail, primer extension analysis was done on EMCV RNA in the presence of the recombinant polypeptides $eIF4G_{457-1396}$, $eIF4G_{457-932}$, and $eIF4G_{920-1396}$. A prominent cDNA product that terminated at $C_{786}$ was detected in the presence of $eIF4G_{457-1396}$ and $eIF4G_{457-932}$ (Fig. 3B, lanes 2 and 3) and corresponded precisely in length to the cDNA product detected in the presence of eIF4F. The stable interactions of $eIF4G_{457-1396}$ and $eIF4G_{457-932}$ with the IRES were ATP independent (data not shown). Arrest of primer extension at $C_{786}$ was not detected in the presence of $eIF4G_{920-1396}$ (Fig. 3B, lane 4), and the majority of cDNA products corresponded to full-length copies of the RNA template. These observations indicate that the central third of eIF4G (amino acid residues 457 to 932) was responsible for the specific interaction of eIF4F with the J-K domain of the EMCV IRES.

The central domain of human eIF4G has large blocks of significant sequence homology with the p82 subunit of wheat eIF(iso)4F, and over a conserved 300-amino-acid region, these two polypeptides are 35% identical and 58% similar (2, 10). This conserved region contains sequences that are characteristic of the RRM domains that occur in numerous RNA-binding proteins (4) (Fig. 1). The ability of wheat eIF4F and wheat eIF(iso)4F to bind specifically to the EMCV IRES was assessed using the toeprinting assay. Neither of these two wheat factors arrested primer extension at $C_{786}$ or at any other position on the IRES, and instead only prominent full-length cDNAs were detected (Fig. 4, lanes 3 and 4). As in previous experiments, binding of $eIF4G_{457-1396}$ to the EMCV IRES caused strong and specific arrest of primer extension at $C_{786}$ (Fig. 4, lane 2). These observations provide an attractive explanation for the inability of EMCV RNA to be translated in wheat germ extracts (22) and additionally suggest potential specificity determinants within the IRES-binding domain of eIF4G.

**Activity of domains of eIF4G in IRES-mediated initiation.** We have reconstituted IRES-mediated initiation in vitro on EMCV RNA from purified components in order to define which factors are required and to characterize their functions during this process (45). The reconstituted initiation process is comparable in efficiency and fidelity to the initiation process in rabbit reticulocyte lysate. To investigate the dependence of IRES-mediated initiation on subunits and domains of eIF4F, we used 40S ribosomal subunits, $Met\text{-}tRNA_i^{Met}$, ATP, GMP-PNP, and the initiation factors shown in Fig. 2. GMP-PNP is a nonhydrolyzable GTP analog that causes 48S complexes to accumulate. Assembly of 48S complexes was first assayed by using sucrose density gradient centrifugation to resolve 48S complexes from RNP complexes and free RNA. The same proportion of input RNA was incorporated into 48S complexes in the complete assembly reaction when eIF4F was used as when eIF4A and either $eIF4G_{457-1396}$ or $eIF4G_{457-932}$ were included in the reaction in place of eIF4F (Fig. 5A and B). Assembly of 48S complexes was not detected when eIF4F was

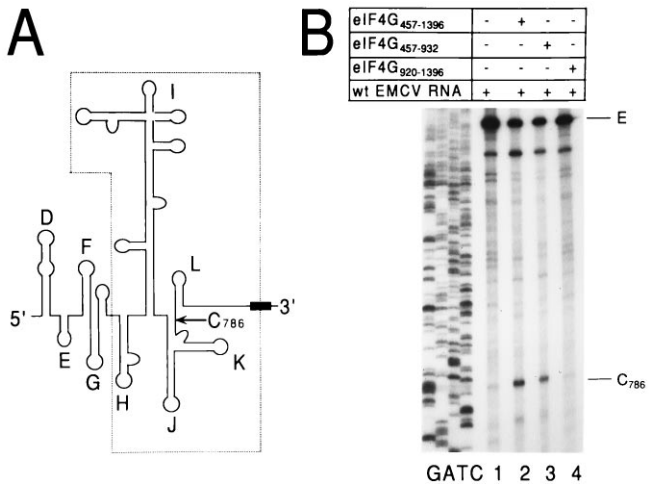


FIG. 3. Specificity of interaction between domains of eIF4G and the EMCV IRES. (A) Consensus secondary structure of nt 260 to 840 of the EMCV IRES based on proposals in references 6 and 46. Domains D to L are named alphabetically as proposed in reference 6. The boundary of the IRES is indicated by a dotted line, and the initiation codon of the viral polyprotein ($AUG_{834}$) is indicated by a black rectangle. The position of the stop site ($C_{786}$) detected by primer extension inhibition in the presence of eIF4F is indicated by an arrow. (B) Primer extension analysis done on EMCV nt 315-1155 RNA after incubation under standard conditions either alone (lane 1) or with 2 μg of $eIF4G_{457-1396}$ (lane 2), 1.5 μg of $eIF4G_{457-932}$ (lane 3), or 1.5 μg of $eIF4G_{920-1396}$ (lane 4). A primer was annealed to nt 957 to 974 within the EMCV coding sequence and was extended with AMV-RT. The cDNA product labeled $C_{786}$ terminated at this nucleotide. The full-length extension product is marked E. Reference lanes G, A, T, and C depict the negative-strand EMCV sequence derived by using the same primer and pTE1 DNA. wt, wild type.

replaced by eIF4A and $eIF4G_{920-1396}$ in a reaction that otherwise included the factors eIF2, eIF3, eIF4B, and PTB (Fig. 5B). The levels of 48S complex formation in reactions that contained eIF4F were all higher in the presence of PTB and eIF4B than in their absence (45). Similar results were obtained when eIF4F was replaced by eIF4A and either $eIF4G_{457-1396}$ or $eIF4G_{457-932}$ (data not shown). It is important to note that this analysis does not assess the contribution of any factor to the kinetics of 48S complex formation.

Most ribosomes initiate translation of EMCV RNA at $AUG_{834}$, but a low level of initiation occurs at $AUG_{826}$ (24, 25). We have found that primer extension analysis is a sensitive and reliable method to assay IRES-mediated assembly of 48S complexes (45). We therefore used this method to confirm that the fidelity of the reconstituted initiation process was comparable to that of initiation in vivo and in cell-free lysates. 48S complexes arrest primer extension 15 to 17 nt 3′ to the A of the initiation codon (3). The results of primer extension analysis of 48S complexes assembled in the complete reconstituted initiation reaction were consistent with the normal pattern of initiation: a prominent set of cDNA products was detected 15 to 17 nt 3′ to the A in $AUG_{826}$ (Fig. 6B, lane 2). These two primer extension stop sites were not detected on analysis of EMCV RNA that had been incubated with 40S subunits and all other constituents of the assembly reaction (including PTB and eIF2, -3, -4A, and -4B) except for eIF4F (Fig. 6B, lane 1). This result was consistent with our previous observations (45), and it indicated that EMCV IRES-mediated 48S complex formation was absolutely dependent on eIF4F. Inclusion of eIF4A and either $eIF4G_{457-1396}$ or $eIF4G_{457-932}$ in place of eIF4F in the complete assembly reaction resulted in formation of 48S complexes at $AUG_{834}$ and to a lesser extent at $AUG_{826}$ (Fig. 6A, lanes 1 and 3). However, 48S complexes did not assemble when eIF4F was replaced by eIF4A and $eIF4G_{920-1396}$ (Fig. 6A, lane 2). Instead, a prominent full-length cDNA product was detected in this assay. The results of primer extension analysis were thus wholly consistent with the results of sucrose density gradient centrifugation assays.

Two control experiments confirmed that formation of 48S preinitiation complexes was IRES mediated. First, assembly of 48S complexes on mutant D1 EMCV RNA from the complete set of purified translation components was not detected by sucrose density gradient centrifugation (Fig. 5A). This mutant RNA lacks nt 702 to 762 of the 5′ NTR and is known to be inactive in IRES-mediated initiation of translation (8, 45). Initiation could therefore not have occurred by ribosomal scanning from the 5′ end of intact EMCV mRNA. Second, as in our earlier experiments (45), we compared input RNA with RNAs on which 48S and RNP complexes had assembled and found that no significant degradation had occurred (data not shown). 48S complexes could therefore not have assembled by an end-dependent mechanism on fragmented EMCV RNA.

These results confirm that eIF4F is essential for internal initiation mediated by the EMCV IRES and show that this factor can be replaced by eIF4A and either $eIF4G_{457-1396}$ or $eIF4G_{457-932}$ without loss of activity. The role played by eIF4F in this process therefore does not require its eIF4E subunit, the amino-terminal third of the eIF4G subunit (to which eIF4E binds [31]), or the carboxy-terminal third of eIF4G (to which eIF4A binds [27]).

**Requirement for eIF4A in EMCV IRES-mediated initiation.** The reconstituted initiation reaction and the two assays described above were next used to determine the requirement for eIF4A in EMCV IRES-mediated initiation. Assembly of 48S complexes in the reconstituted initiation reaction was not detected by sucrose density gradient centrifugation when eIF4F was replaced by either $eIF4G_{457-1396}$ (Fig. 5C) or $eIF4G_{457-932}$ (Fig. 5D) in the absence of eIF4A. Similarly, primer extension analysis indicated that 48S complexes were

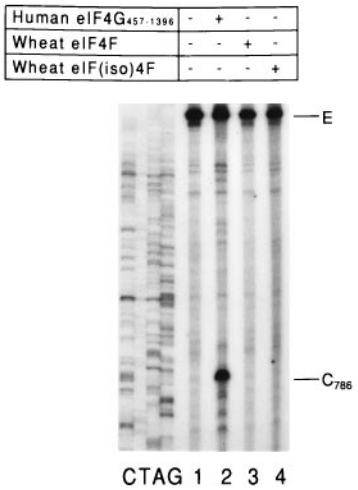


FIG. 4. Specificity of interaction between eIF4F and the EMCV IRES. Primer extension analysis was done on EMCV nt 315-1155 RNA after incubation under standard conditions either alone (lane 1) or with 1.5 μg of recombinant human $eIF4G_{457-1396}$ (lane 2), 1.5 μg of wheat eIF4F (lane 3), or 1 μg wheat eIF(iso)4F (lane 4). A primer was annealed to nt 957 to 974 within the EMCV coding sequence and was extended with AMV-RT. The position of the stop site ($C_{786}$) detected by primer extension inhibition in the presence of $eIF4G_{457-1396}$ is indicated. The full-length extension product is marked E. Reference lanes G, A, T, and C depict the negative-strand EMCV sequence derived by using the same primer and pTE1 DNA.

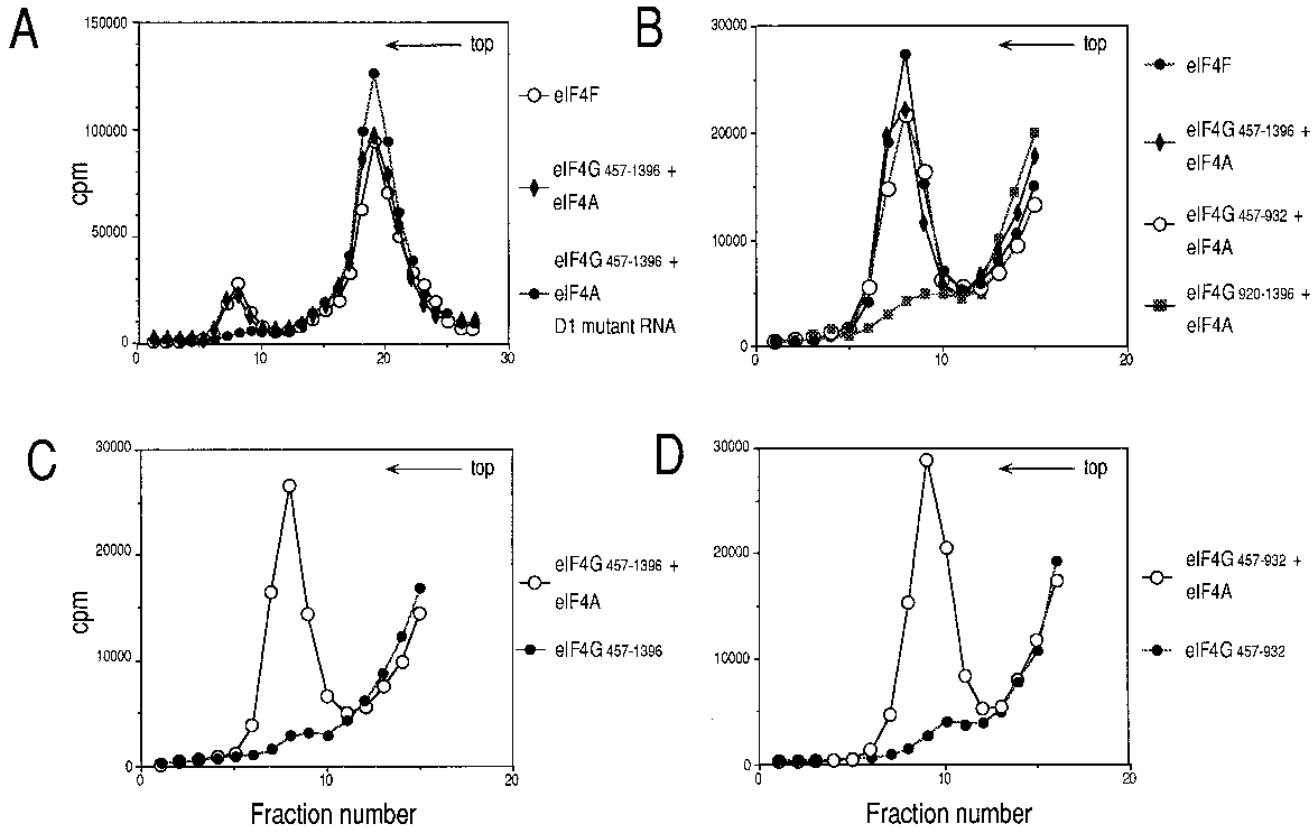


FIG. 5. Dependence of EMCV IRES-mediated 48S preinitiation complex formation on individual initiation factors. Assays in panels A and B were done with eIF2, -3, -4A, and -4B and PTB with eIF4F or $eIF4G_{457-1396}$ (A) and eIF4F, $eIF4G_{920-1396}$, $eIF4G_{457-932}$, or $eIF4G_{920-1396}$ (B), as indicated, and other components as described in Materials and Methods. Assays in panels C and D were done with eIF2, -3, and -4B and PTB with $eIF4G_{457-1396}$ and -4A or $eIF4G_{457-1396}$ alone (C) and $eIF4G_{457-932}$ and -4A or $eIFG_{457-932}$ alone (D), as indicated, and other components as described in Materials and Methods. All assays were done with wild-type EMCV transcripts except where indicated in panel A. 48S complexes were resolved on sucrose density gradients. Sedimentation was from right to left. Fractions from upper parts of the sucrose gradient have been omitted from graphs in panels B to D for greater clarity.

not formed at either $AUG_{826}$ or $AUG_{834}$ when eIF4F was replaced by $eIF4G_{457-1396}$ in the absence of eIF4A (Fig. 6B, lane 5). Instead, a prominent full-length cDNA product was detected in this assay. These results showed that EMCV IRES-mediated initiation is absolutely dependent on eIF4A.

The helicase activity of eIF4A requires ATP hydrolysis, but this requirement can also be met by dATP (49; see also a discussion in reference 19). IRES-mediated initiation is absolutely dependent on eIF4A (see above) and on ATP (45), and it was therefore of interest to determine whether dATP could substitute for ATP in IRES-mediated initiation. 48S preinitiation complex formation in a complete assembly reaction in which eIF4A and $eIF4G_{457-1396}$ were included in place of eIF4F was assayed by using sucrose density gradient centrifugation (Fig. 7). The level of 48S complex formation was reduced slightly by inclusion of dATP in place of ATP in the reconstituted initiation reaction. 48S complex formation was not detected when ATP was omitted from the assembly reaction, consistent with previous observations (45). This result suggests that the requirement for ATP in IRES-mediated initiation is accounted for by the requirement for this cofactor during unwinding or restructuring of the IRES by eIF4A.

**UV cross-linking of initiation factors to the EMCV IRES.** eIF4G associates with eIF3, -4A, -4E, and probably -4B (27, 31). The observation that $eIF4G_{457-932}$ and $eIF4G_{457-1396}$ also bind specifically to the J-K domain of the EMCV IRES independently of other initiation factors (Fig. 3B, lanes 2 and 3) suggests that a function of eIF4G in internal initiation may be to promote the interaction of other initiation factors with the IRES. We used UV cross-linking to analyze the interactions of the initiation factors eIF4A, eIF4B, eIF4F, and eIF4G with the EMCV IRES.

UV cross-linking of eIF4A to [$^{32}$P]UTP-labeled EMCV IRES-specific RNA was barely detectable and was not significantly enhanced by inclusion of ATP in the reaction mixture (Fig. 8A, lane 3, and data not shown). This result is consistent with previous observations (33, 41). However, the eIF4A, eIF4E, and eIF4G subunits of eIF4F all became strongly labeled when this factor was cross-linked to EMCV RNA (Fig. 8A, lanes 1 and 2). The eIF4G subunit of eIF4F was degraded slightly either during this reaction or during subsequent RNase treatment of cross-linked RNP complexes.

To dissect the interaction between eIF4F and the EMCV IRES, recombinant eIF4A and fragments of eIF4G were UV cross-linked to the EMCV IRES either alone or in combination. Reactions were done under normal translation buffer (20 mM Tris-HCl [pH 7.6], 2.5 mM magnesium acetate, 100 mM potassium acetate, 1 mM ATP) and temperature (30°C) conditions. $eIF4G_{457-1396}$ and particularly $eIF4G_{457-932}$ became labeled after UV cross-linking to the EMCV IRES, whereas UV cross-linking of $eIF4G_{920-1396}$ to [$^{32}$P]UTP-labeled EMCV RNA was not detected (Fig. 8B, lanes 2, 4, and 6). The interaction of these polypeptides with the EMCV IRES was not affected by omission of ATP from the reaction mixture (data

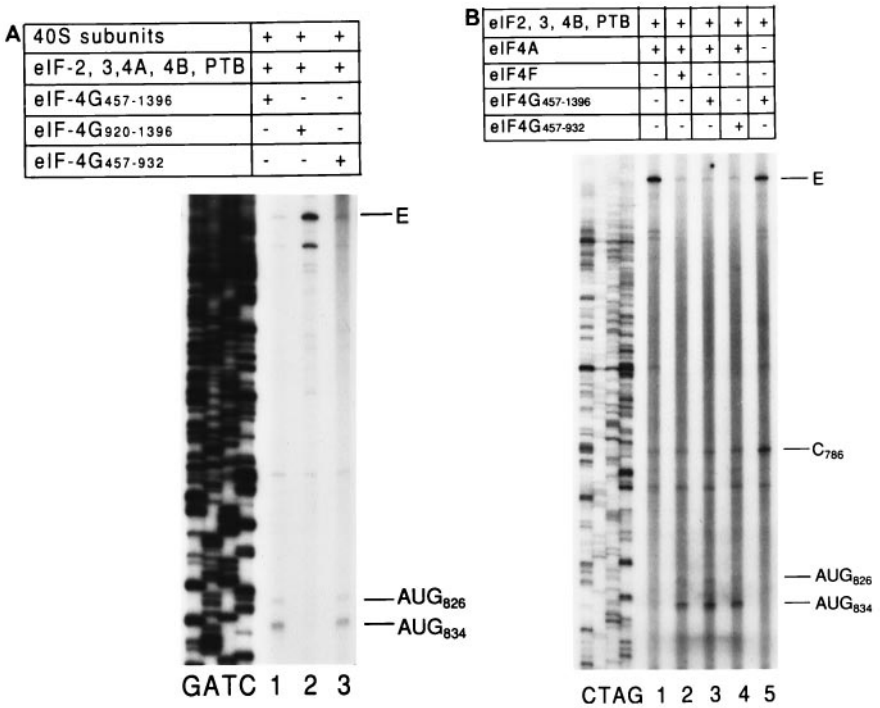


FIG. 6. Primer extension analysis of 48S preinitiation complexes. EMCV RNA transcripts were incubated under standard reaction conditions for assembly of 48S complexes as follows: (A) with 40S ribosomal subunits, PTB, eIF2, eIF3, eIF4A, and eIF4B and either $eIF4G_{457-1396}$ (lane 1), $eIF4G_{920-1396}$ (lane 2), or $eIF4G_{457-932}$ (lane 3); (B) with 40S ribosomal subunits, PTB, eIF2, eIF3, and eIF4B and either eIF4A (lane 1), eIF4A and eIF4F (lane 2), eIF4A and $eIF4G_{457-1396}$ (lane 3), eIF4A and $eIF4G_{457-932}$ (lane 4), or $eIF4G_{457-1396}$ (lane 5). A primer was annealed to nt 957 to 974 within the EMCV coding sequence and was extended with AMV-RT. cDNA products labeled $AUG_{834}$ and $AUG_{826}$ terminated at stop sites 15 to 17 nt upstream of the stated initiation codon. The full-length extension product is marked E. Reference lanes G, A, T, and C depict the negative-strand EMCV sequence derived by using the same primer and pTE1 DNA.

not shown). The presence of $eIF4G_{457-1396}$ in the reaction mixture stimulated UV cross-linking of eIF4A to the EMCV IRES very strongly (Fig. 8B, lanes 1 and 5). The strong UV cross-linking of eIF4A to the EMCV IRES in the presence of $eIF4G_{457-1396}$ was not enhanced further by eIF4B (data not shown). Inclusion of $eIF4G_{457-932}$ in the reaction had a slight stimulatory effect on UV cross-linking of eIF4A to the EMCV IRES (compare lanes 1 and 7 in Fig. 8B), whereas inclusion of $eIF4G_{920-1396}$ did not affect the labeling of eIF4A in this assay (compare lanes 1 and 3 in Fig. 8B). These observations indicate that a fragment of eIF4G that consists of the central domain (which interacts with the EMCV IRES [see above]) and the carboxy-terminal domain (which interacts with eIF4A [27]) promoted the interaction of eIF4A with the EMCV IRES. Individually these two domains did not have similar activities.

The observation that the domain of eIF4G to which eIF4A binds and which promotes eIF4A's interaction with the IRES was not required for internal ribosomal entry suggests that either this stimulatory function is not essential for IRES-mediated initiation or that other factors can also promote eIF4A's interaction with the IRES. eIF4B is known to cooperate with eIF4A and eIF4F in binding to RNA (20, 33), and we therefore investigated its effects on eIF4A's interaction with the IRES. Recombinant eIF4B became weakly labeled after UV cross-linking to [$^{32}$P]UTP-labeled EMCV IRES-specific RNA (data not shown), and labeling of this factor was enhanced by inclusion of eIF4A in the reaction mixture (Fig. 8C, lane 1). The presence of eIF4B slightly enhanced cross-linking of eIF4A to the EMCV IRES (compare Fig. 8A, lane 3, and Fig. 8C, lane 1). Inclusion of $eIF4G_{457-932}$ in a reaction mixture that contained either eIF4A or eIF4B had a slight stimulatory effect on UV cross-linking of these two factors to the EMCV IRES (Fig. 8C, lanes 2 and 3). However, inclusion of $eIF4G_{457-932}$ in a reaction mixture that contained both eIF4A and eIF4B significantly enhanced UV cross-linking of eIF4B to the EMCV IRES and had an even greater stimulatory effect on labeling of eIF4A (Fig. 8C, lane 4).

The results of UV cross-linking therefore show that

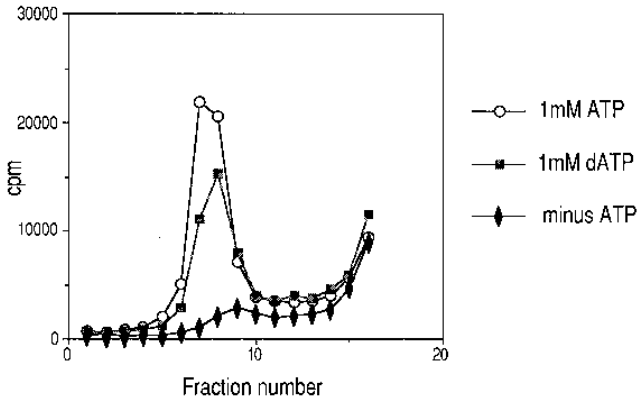


FIG. 7. Dependence of EMCV IRES-mediated 48S preinitiation complex formation on ATP. Assays were done with wild-type EMCV RNA transcripts, PTB, eIF-2, -3, -4A, and -4B, $eIF4G_{457-1396}$, and other components either as described in Materials and Methods or as indicated. 48S complexes were resolved on sucrose density gradients. Sedimentation was from right to left. Fractions from upper parts of the sucrose gradient have been omitted for greater clarity.

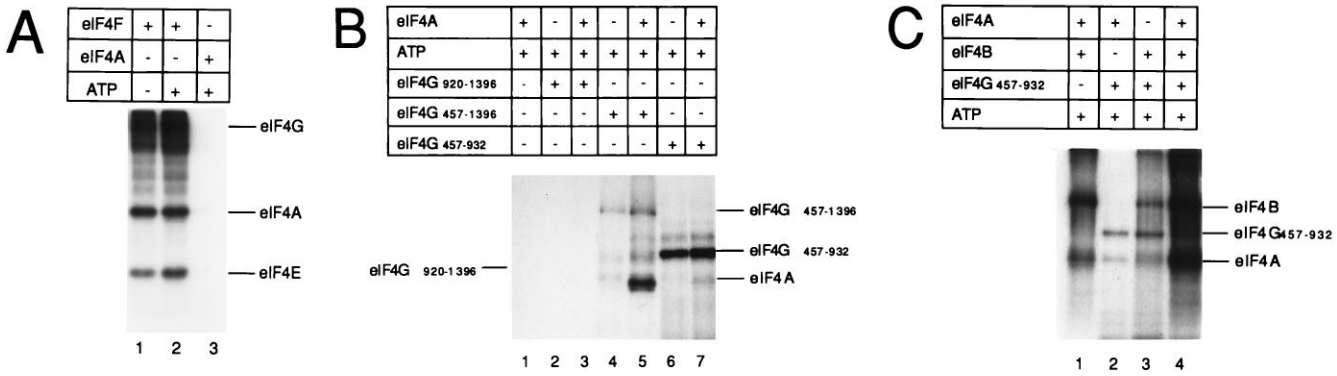


FIG. 8. UV cross-linking of initiation factors to the EMCV IRES. (A) Native eIF4F (lanes 1 and 2) and native eIF4A (lane 3) were UV cross-linked to [$^{32}$P]UTP-labeled EMCV (nt 315 to 1155) RNA transcripts in the presence (lanes 2 and 3) or absence (lane 1) of 1 mM ATP. (B) Recombinant eIF4A (lanes 1, 3, 5, and 7), eIF4G$_{920\text{-}1396}$ (lanes 2 and 3), eIF4G$_{457\text{-}1396}$ (lanes 4 and 5), and eIF4G$_{457\text{-}932}$ (lanes 6 and 7) were UV cross-linked to [$^{32}$P]UTP-labeled EMCV (nt 315 to 1155) RNA transcripts in the presence of 1 mM ATP. (C) Recombinant eIF4A (lanes 1, 2, and 4), eIF4B (lanes 1, 3, and 4), and eIF4G$_{457\text{-}932}$ (lanes 2 to 4) were UV cross-linked to [$^{32}$P]UTP-labeled EMCV (nt 315 to 1155) RNA transcripts in the presence of 1 mM ATP. Samples were digested with cobra venom nuclease and RNases A and $T_1$. Proteins were separated by SDS-PAGE on an SDS–15% polyacrylamide gel.

eIF4G$_{457\text{-}1396}$ and eIF4G$_{457\text{-}932}$ interact specifically with the EMCV IRES whereas eIF4G$_{920\text{-}1396}$ does not. These observations are entirely consistent with the results of primer extension analysis of the interaction between domains of eIF4G and the EMCV IRES. Additionally, these results indicate that eIF4B and eIF4G$_{457\text{-}932}$ together, or eIF4G$_{457\text{-}1396}$ by itself, strongly stimulated binding of eIF4A to the IRES.

## DISCUSSION

Initiation of EMCV translation epitomizes IRES-mediated internal ribosomal entry (13, 19). This process has been reconstituted in vitro from purified translation components, enabling the requirements for individual factors in this process to be determined and their functions to be characterized (45). In this study, we have dissected the essential role of the initiation factor eIF4F in IRES-mediated initiation of EMCV translation.

First, the requirements for the eIF4A subunit and for domains of the eIF4G subunit of eIF4F in EMCV IRES-mediated initiation were determined directly by replacing eIF4F with combinations of these polypeptides in the reconstituted initiation process. Recombinant eIF4A and eIF4G polypeptides were used in these experiments to eliminate the possibility of contamination by eIF4F. The activity of eIF4F in this process was absolutely dependent on eIF4A and on the central third of eIF4G but did not require the amino- or carboxy-terminal third of eIF4G. Omission of eIF4E did not impair IRES-mediated initiation. Although the assays described here did not assess the kinetics of 48S complex formation, it is nevertheless clear that these results support and significantly extend conclusions reached on the basis of experiments done using semifractionated translation assay systems (39, 50, 52).

The requirement for eIF4A in EMCV IRES-mediated initiation described here, the previously identified requirement for ATP (45), and the finding reported here that ATP could be replaced by dATP all suggest that EMCV IRES-mediated initiation involves unwinding of RNA. mRNA is unwound in cap-dependent initiation to enable ribosomes both to bind to mRNA close to the 5′-terminal cap and to scan to the initiation codon. EMCV initiation does not involve scanning (25), and it is therefore probable that the role of the helicase activity of eIF4F is to unwind a segment of the IRES downstream of the site to which it binds, generating a single-stranded region that can enter the mRNA binding site of the 40S subunit. It has been suggested that eIF4A gains access to mRNAs as a subunit of eIF4F (42, 54), possibly as a consequence of its interaction with sequences in the C-terminal third of eIF4G (27, 39). However, internal initiation and UV cross-linking of eIF4A to the EMCV IRES both occurred in the absence of this domain (albeit at reduced levels), indicating that the carboxy-terminal third of eIF4G is not essential either for EMCV IRES-mediated initiation or to promote eIF4A's interaction with RNA. Both processes were stimulated by eIF4B in reactions containing eIF4A and eIF4G$_{457\text{-}932}$ in place of eIF4F. These observations lead us to suggest that the proposed role of the carboxy-terminal third of eIF4G in promoting eIF4A's interaction could also be mediated by eIF4B and eIF4G$_{457\text{-}932}$ following site-specific binding of the latter to the IRES. Experiments to define the interactions of eIF4B with eIF4G$_{457\text{-}932}$ and eIF4A are in progress. The redundancy of the carboxy-terminal third of eIF4G in promoting eIF4A's interaction with RNA does not preclude this domain having additional functions, such as interacting with eIF2 (26). However, it may be significant that the *Saccharomyces cerevisiae* homologs of eIF4G do not contain sequences equivalent to this carboxy-terminal segment of mammalian eIF4G (10).

eIF4F provides essential functions in IRES-mediated initiation in addition to the helicase activity of its eIF4A subunit (45). We have identified the central, strongly conserved domain of eIF4G as the second component of eIF4F that is essential for EMCV IRES function. This domain has three distinct properties that are relevant to its role in initiation. First, it determines the specific binding of eIF4F to the J-K domain of the IRES, which is an important early step in initiation of EMCV translation (45). The central third of eIF4G contains sequences that are characteristic of RRM domains (Fig. 1), suggesting that this region may fold into an RNA-binding structure of this type (4, 10). Second, the central third of eIF4G cooperates with eIF4B in promoting the interaction of eIF4A with the IRES. Third, this domain binds to eIF3 (27) and may bind to eIF4B.

Taken together, these observations suggest the following model for EMCV IRES-mediated initiation. First, the specific ATP-independent interaction of eIF4G with the IRES promotes assembly of an RNP complex consisting of eIF4A, -4B, -4E, and -4G. eIF4G may already be associated with eIF4A and -4B when it binds to the IRES because eIF4F binds stably

to eIF4B and is in equilibrium with its constituent eIF4A, -4E, and -4G subunits (11, 12, 54). Protein components of this complex almost certainly interact with each other, and UV cross-linking analysis suggests that they all also bind to the IRES (reference 35 and this report). Stable RNA-protein complexes consisting of eIF4B and eIF4F have been described previously, and it has been suggested that they are active helicase complexes (20). The specific interaction of eIF4F with a tertiary structure involving the J-K domain of the EMCV IRES would result in assembly of such a complex at a defined site and promote ATP-dependent unwinding of a specific segment of the IRES by eIF4A. This generates a single-stranded region that can enter the mRNA binding site of the 40S subunit. The interaction of eIF4G with eIF4B and eIF3 (11, 27, 31) may direct binding of 43S preinitiation complexes to a specific location in the IRES. eIF4B binds to 40S subunits (16), and eIF3 is a component of the 43S preinitiation complex. The resulting 48S complex may be positioned more precisely on the IRES and further stabilized by interactions of eIF2 and the p115 and p66 subunits of eIF3 with the IRES (reference 9 and our unpublished observations). Experiments to test predictions of this model and its applicability to other IRES elements are in progress.

The observations that EMCV IRES-mediated initiation does not require eIF4E or the amino-terminal third of eIF4G (to which eIF4E binds) are consistent with the known effects of two mechanisms that regulate the activity of this factor. IRES-mediated initiation is not inhibited by sequestration of eIF4E by specific 4E-binding proteins which prevents its interaction with eIF4G (12, 40) or by the proteolytic cleavage of eIF4G that occurs during infection of cells by poliovirus and some other picornaviruses (7, 21, 39, 43). Cleavage separates the amino-terminal 4E binding domain of eIF4G from other functional domains of the molecule (27, 28, 31). These mechanisms both inhibit cap-dependent initiation of translation. Cap-dependent initiation is also inhibited by dephosphorylation of eIF4E, which reduces its cap-binding activity (37) and its association with eIF4G (5). The results obtained here in analyses using EMCV RNA as a model IRES therefore suggest that it is likely that it and other IRES-containing mRNAs will be translated more efficiently than most cellular mRNAs during conditions such as growth arrest, mitosis, heat shock, or viral infection that result in dephosphorylation of eIF4E.

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