

Canonical Eukaryotic Initiation Factors Determine Initiation of Translation by Internal Ribosomal Entry

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Received 26 June 1996/Returned for modification 26 August 1996/Accepted 10 September 1996

Translation of picornavirus RNA is initiated after ribosomal binding to an internal ribosomal entry site (IRES) within the 5' untranslated region. We have reconstituted IRES-mediated initiation on encephalomyocarditis virus RNA from purified components and used primer extension analysis to confirm the fidelity of 48S preinitiation complex formation. Eukaryotic initiation factor 2 (eIF2), eIF3, and eIF4F were required for initiation; eIF4B and to a lesser extent the pyrimidine tract-binding protein stimulated this process. We show that eIF4F binds to the IRES in a novel cap-independent manner and suggest that cap- and IRES-dependent initiation mechanisms utilize different modes of interaction with this factor to promote ribosomal attachment to mRNA.

Initiation of eukaryotic protein synthesis is the process of assembly of an 80S initiation complex containing initiator tRNA^{Met}, 40S, and 60S ribosomal subunits at the initiation codon of an mRNA (42). The first step in this process is formation of a 43S complex that consists of eukaryotic initiation factor 2 (eIF2), eIF3, and initiator Met-tRNA^{Met} bound to the 40S subunit. The second, rate-limiting step is the binding of mRNA to the 43S complex to form a 48S preinitiation complex. This occurs in one of two ways.

The first mechanism is characteristic of most mRNAs. It involves recognition of the m⁷GpppX cap at the free 5' end of mRNA by the 4E subunit of eIF4F, followed by binding of the 43S complex at or close to the cap. Binding and 5'-3' scanning by this complex to the first AUG codon require ATP hydrolysis and unwinding of secondary structure in the 5' untranslated region (UTR) by eIF4F or eIF4A in cooperation with eIF4B. This mechanism of ribosomal entry imposes several restrictions on the structure of mRNAs that use it: they are capped, have relatively short, unstructured 5' UTRs, and are monocistronic because initiation is limited to the 5'-most AUG codon (34).

A second, cap-independent mechanism of ribosome binding is used by mRNAs that contain an internal ribosomal entry site (IRES). IRES-mediated initiation is used by a number of cellular mRNAs, including those that encode the transcription factors TFIID (TATA-binding protein) and *HAP4* (25), the growth factors fibroblast growth factor 2 and insulin-like growth factor 2 (63, 67), the homeotic genes *Antennapedia* and *Ultrabithorax* (45), the translation initiation factor eIF4G (14), and the immunoglobulin heavy-chain binding protein (39). This mechanism of translation initiation has been usurped by a number of viruses (e.g., references 49 and 65) and is exemplified by encephalomyocarditis virus (EMCV) (21, 26). The EMCV IRES is about 450 nucleotides (nt) long, is highly structured, and lies immediately upstream of AUG₈₃₄, which is the 11th AUG triplet in the 5' UTR and the initiation codon for synthesis of the viral polyprotein (11, 27, 52, 66). The 5' UTRs of IRES-containing mRNAs differ from those of con-

ventional mRNAs in many respects: they contain multiple AUG triplets and extensive secondary structure, they may be uncapped and exceptionally long, and they can by definition promote end-independent translation of a downstream cistron in bicistronic mRNAs. The secondary and/or tertiary structures of IRES elements are critical for their ability to promote initiation, in clear distinction from cap-dependent mRNAs, whose 5' UTRs do not contain structural features that enhance translation (35). These unusual properties suggest that IRES- and cap-dependent initiation differ mechanistically.

IRES-mediated initiation may involve novel activities to direct binding of ribosomes to internal sites that are not required for cap-dependent translation. One such novel factor is the pyrimidine tract-binding protein (PTB), which binds specifically to the EMCV and other IRES elements (6, 7, 22, 27, 37, 50). The requirements for canonical eIFs in IRES- and cap-dependent initiation may also differ quantitatively and qualitatively. The effects of several eIFs on EMCV translation have been investigated. Stimulation of IRES-mediated translation by one or more of the initiation factors eIF4A, eIF4B, eIF4E, and eIF4F and specific binding of eIF2/2B and eIF4B to the EMCV IRES have been reported (2, 4, 15, 48, 53, 55, 56, 61, 68), although some of these findings have been disputed (24, 59, 60). Moreover, involvement of these factors has been inferred from studies using unfractionated or semifractionated translation systems which are not adequate for unequivocal identification of the factors that are necessary and sufficient for IRES-mediated initiation.

We report here that we have reconstituted IRES-mediated initiation *in vitro* up to the stage of 48S complex formation, using purified components (tRNA^{Met}, EMCV RNA, 40S subunits, and initiation factors) in order to define which factors are required for this process and to begin to characterize their functions during it. A similar approach has been very productive for studying cap-dependent initiation (e.g., references 5, 57, and 64). Primer extension analysis was used to verify that 48S complexes had assembled at the EMCV initiation codon AUG₈₃₄. Accurate and efficient assembly of 48S complexes on the EMCV IRES *in vitro* was found to require only eIF2, -3, and -4F and was stimulated by eIF4B and to a lesser extent by PTB. IRES-mediated initiation is therefore determined by exactly the same set of canonical eIFs as standard cap-dependent initiation.

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Primer extension analysis also revealed a stable, cap-independent interaction between eIF4F and the J-K domain of the EMCV IRES, about 50 nt upstream of the initiation codon. Mutations that impaired this interaction also impaired IRES-mediated 48S complex formation. This observation suggests that cap- and IRES-dependent modes of initiation utilize different modes of interaction with the same factor (eIF4F) to promote ribosomal attachment to mRNA.

MATERIALS AND METHODS

Plasmids. pTE1 contains EMCV nt 315 to 1155 downstream of the T7 promoter in vector pTZ18R (Pharmacia, Piscataway, N.J.); this sequence corresponds to the complete IRES and nt 834 to 1155 of the coding region. Plasmids pTE10 and pTP1 are identical to pTE1 except that in pTE10, EMCV nt 702 to 762 inclusive have been replaced by the nucleotides GGGAAUUGCC (13) and in pTP1, EMCV nt 769 to 774 have been replaced by the dinucleotide AU.

The oligonucleotides 5'-GATCCGGGGTACCTAGGCGGCCG-3' and 5'-GATCGCGGCCCTAGGTACCCCGG-3' were inserted into the *Bam*HI site of pET-15b (Novagen, Madison, Wis.) to construct pT7H₆-1.

pET(His₆-eIF4A) was constructed by inserting cDNA (nt 1 to 307 of the eIF4A I coding region) that had been amplified by PCR and digested with *Bam*HI and *Stu*I and a *Stu*I-*Not*I restriction fragment that contains the remainder of eIF4A (32) between *Bam*HI and *Not*I sites of pT7H₆-1.

pET(His₆-eIF4B) was constructed by inserting a PCR fragment (nt 1 to 382 of the eIF4B coding sequence [43]) that had been digested with *Bam*HI and *Pml*I and a *Pml*I-*Avr*II restriction fragment that contains the remainder of eIF4B between the *Bam*HI and *Avr*II sites of pT7H₆-1.

Purification of factors and 40S ribosomal subunits. 40S ribosomal subunits were prepared from rabbit reticulocyte lysate (RRL; Green Hectares, Oregon, Wis.). Ribosomes were precipitated by centrifugation for 4 h at 4°C and 30,000 rpm in a Beckman 50.2Ti rotor and resuspended in buffer A (20 mM Tris-HCl [pH 7.6], 2 mM dithiothreitol [DTT], 6 mM MgCl₂) with 0.25 M sucrose and 150 mM KCl to a concentration of 50 to 150 A₂₆₀ U/ml. This suspension was incubated with 1 mM puromycin for 10 min at 0°C and then for 10 min at 37°C before addition of KCl to 0.5 M. Ribosomal subunits were resolved by centrifugation of 0.7-ml aliquots of this suspension through a 10 to 30% sucrose gradient in buffer A with 0.5 M KCl for 17 h at 4°C and 22,000 rpm, using a Beckman SW27 rotor. 40S subunits were precipitated from 1-ml gradient fractions by centrifugation for 18 h at 4°C and 50,000 rpm in an SW55 rotor. Pellets were resuspended in buffer B (20 mM Tris-HCl [pH 7.6], 0.2 mM EDTA, 10 mM KCl, 1 mM DTT, 1 mM MgCl₂, 0.25 M sucrose) to a concentration of 60 to 80 A₂₆₀ U/ml.

A 0 to 70% ammonium sulfate (AS) saturation fraction was prepared from the 0.5 M KCl RRL ribosomal salt wash (RSW) fraction. Ribosomes in buffer C (5 mM Tris HCl [pH 7.6], 0.1 mM EDTA, 1 mM DTT, 0.25 M sucrose) were stirred on ice with 0.5 M KCl for 30 min. The supernatant recovered after centrifugation for 4 h at 45,000 rpm in an SW55 rotor was made 40 mM in Tris-HCl (pH 7.6), and powdered AS was added slowly with constant stirring on ice to 70% saturation. Stirring was continued on ice for 30 min, and a 0 to 70% AS saturation precipitate was collected by centrifugation for 20 min at 15,000 rpm in a Sorvall SS-34 rotor, dissolved in buffer D (1 mM DTT, 0.2 mM EDTA, 10% [vol/vol] glycerol, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.6]) with 100 mM KCl, and dialyzed overnight at 4°C against the same buffer.

Zero to 40%, 40 to 50%, and 50 to 70% AS saturation fractions (termed F1, F2, and F3, respectively) were prepared from the 0.5 M KCl RSW fraction from 3 liters of RRL. PTB and the factors eIF3, -4B, and -4F were purified from F1, eIF2 was purified from F2, and eIF4A was purified from F3, using established procedures (12, 20, 41). eIF4A was purified further by using a HiTrap Blue matrix as described previously (48).

Recombinant eIF4A and -4B were expressed in *Escherichia coli* BL21(DE3) and were purified by chromatography using Ni²⁺-nitrilotriacetic acid, heparin-Sepharose, poly(U)-Sepharose, and HiTrap Blue matrices (48). Recombinant PTB-1 was expressed and purified as described elsewhere (22).

In vitro transcription. Plasmids pTE1, pTE10, and pTP1 were linearized with *Pst*I and transcribed in vitro in the presence of [³²P]UTP (~3,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) with T7 RNA polymerase mutant DEL172-3 (which terminates efficiently and precisely at termini with 3' overhanging ends [38]). Synthetic transcripts derived from these three plasmids are termed wild type (*wt*), D1, and D2, respectively, and are, including non-EMCV residues, 888, 837, and 884 nt long, respectively. They were purified using by Nuc-trap columns as described previously (50). The specific activities of mRNA preparations were in the range of 300,000 to 500,000 cpm/μg of RNA.

Preparation of [³⁵S]methionyl initiator tRNA. Labeled Met-tRNA^{Met} was prepared by the method of Stanley (62), using aminoacyl-tRNA synthetase purified from *E. coli* MRE600 to charge rabbit tRNA (2 mg/ml) with 1 mCi of [³⁵S]methionine (~1,250 Ci/mmol) per ml and 0.1 mM unlabeled methionine for 30 min at 37°C. The reaction mix was phenol extracted, filtered by using a Nuc-trap column, and ethanol precipitated.

Assembly and sucrose density gradient centrifugation of 48S complexes. 48S complexes were assembled by incubating 1 μg of EMCV RNA for 10 min at 30°C in a 100-μl reaction volume that contained buffer E (2 mM DTT, 100 mM potassium acetate, 20 mM Tris [pH 7.6]) with 2.5 mM magnesium acetate, 100 U of RNasin (Promega), 1 mM ATP, 0.1 mM guanylimidodiphosphate (GMP-PNP), 0.25 mM spermidine, 6 pmol of [³⁵S]Met-tRNA^{Met}, and 6 pmol of 40S subunits and combinations of fractions F1 (30 μg), F2 (15 μg), and F3 (15 μg) and PTB (1.5 μg) and initiation factors eIF2 (4 μg), eIF2B (2.5 μg), eIF3 (10 μg), eIF4A (2 μg), eIF4B (2 μg), and eIF4F (1 or 4 μg) as indicated in the text. 48S and ribonucleoprotein (RNP) complexes were resolved by centrifugation through a 10 to 30% sucrose gradient in buffer E 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.

Western blot (immunoblot) analysis. Polypeptides were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis 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 anti-eIF2 polyclonal antibodies (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 (1:1,000; 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), and an anti-PTB polyclonal antibody (1:1,000; a kind gift from P. Sharp). 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.

Primer extension analysis. 48S complexes were assembled on unlabeled EMCV RNA as described above by incubating a 40-μl reaction mix for 10 min at 30°C. Incubation was continued for 3 min at 30°C following addition of 4 pM oligonucleotide 5'-GTCAATAACTCTCTGG-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-³²P]dATP (~6,000 Ci/mmol; ICN Radiochemicals), 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 ethanol precipitated, resuspended, and analyzed by electrophoresis through 6% polyacrylamide sequencing gels. cDNA products were compared with a dideoxynucleotide sequence ladder obtained by using the same primer and pTE1 plasmid DNA.

RESULTS

Fractionation of RSW and reconstitution of 48S complex formation. IRES-mediated 48S complex formation was analyzed by incubating EMCV RNA in RRL under normal translation conditions in the presence of GMP-PNP and then resolving RNP and ribosomal complexes by sucrose density gradient centrifugation. GMP-PNP is a nonhydrolyzable GTP analog that causes 48S complexes to accumulate. EMCV RNA was used as a model IRES because it is well characterized and very active and because the ribosomal binding site is very close to the initiator codon AUG₈₃₄ so that initiation does not involve ribosomal scanning. 48S complexes formed under these conditions contained up to 30% of the input [³²P]UTP-labeled RNA (Fig. 1A).

To identify the initiation factors that are required for IRES-mediated initiation, a 0 to 70% AS precipitation fraction was first prepared from the 0.5 M KCl RSW. This RSW fraction contained all activities necessary for the formation of 48S complexes from 40S subunits and Met-tRNA^{Met} on the EMCV IRES (Fig. 1A). In this assay, the RSW fraction was at least half as active as RRL in promoting incorporation of [³²P]UTP-labeled RNA into 48S complexes. 48S preinitiation complexes did not form under identical conditions (Fig. 1B) on a corresponding EMCV RNA template (mutant D1) that contains an internal deletion (nt 702 to 762) and is inactive in translation (13). This deletion is distant from both the 5' terminus and the authentic initiation codon (AUG₈₃₄) and by definition cannot affect canonical 5'-end-dependent initiation of translation (34). 48S preinitiation complexes had therefore assembled on *wt* EMCV RNA as a result of internal ribosomal entry.

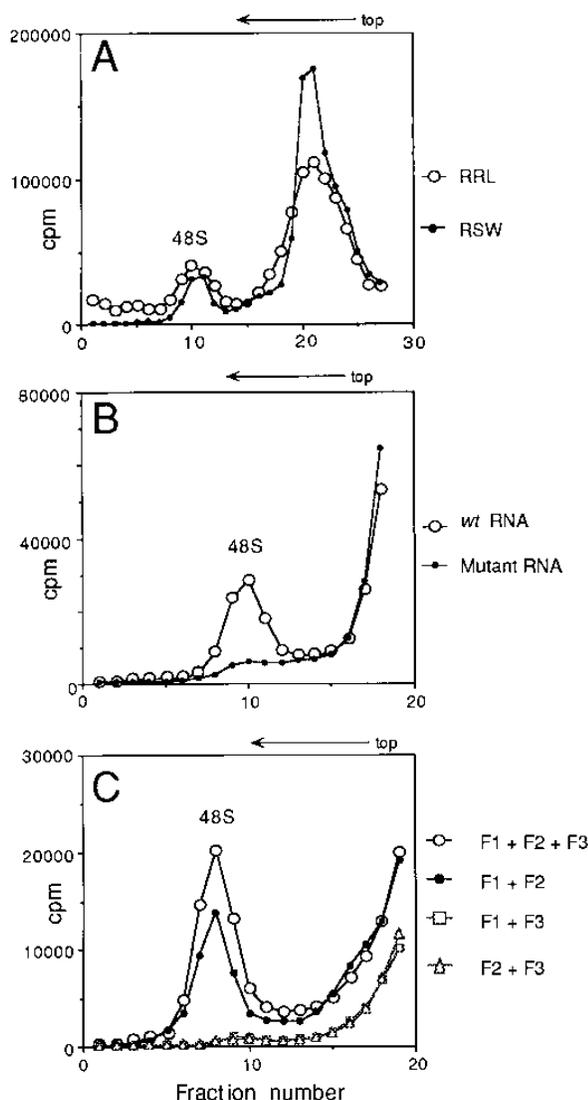


FIG. 1. 48S preinitiation complex formation on EMCV RNA. Assays were carried out with *wt* EMCV RNA and either RRL or a 0 to 70% AS RSW fraction and other components as described in Materials and Methods (A), the 0 to 70% AS RSW fraction and other components with either *wt* or D1 mutant RNA (B), and RSW fractions F1, F2, and F3 and other components with *wt* RNA (C). 48S complexes were resolved on sucrose gradients. Sedimentation was from right to left. Fractions from the upper part of the sucrose gradient have been omitted from graphs in panels B and C for greater clarity.

This active RSW fraction was divided into 0 to 40, 40 to 50, and 50 to 70% AS fractions termed F1, F2, and F3, respectively. These fractions were incubated in different combinations with Met-tRNA^{Met}, 40S subunits, and [³²P]UTP-labeled EMCV RNA. F1, F2, and F3 gave the same level of 48S complex formation as that obtained with the 0 to 70% AS fraction. F1 and F2 were essential for 48S complex formation, whereas a significant amount of 48S complex was formed in the absence of F3 (Fig. 1C).

Use of purified eIFs in place of fractions F1, F2, and F3. F1, F2, and F3 contain PTB and all canonical eIFs (16, 20, 41, 57). These three fractions were used as sources for purification of PTB and eIF2, eIF3, and eIF4F (Fig. 2) and eIF2B, eIF4A, and eIF4B (data not shown). Initiation factors of high quality and purity were used, and no cross-contamination of individual

eIFs was detected by Western blotting using antibodies specific for each one of these factors (data not shown). The abilities of these factors to replace the activity of the F1, F2, and F3 fractions in initiation were assayed in order to identify the factors that mediate IRES-dependent 48S complex formation. PTB and the initiation factors eIF2, eIF2B, eIF3, eIF4A, eIF4B, and eIF4F were added to *in vitro* reactions in comparable picomolar quantities. The limiting factor in these reactions was the amount of added EMCV RNA template.

F2 contains eIF2 and eIF2B (a guanine nucleotide exchange factor for eIF2). Purified eIF2 and eIF2B in combination with F1 and F3 resulted in a level of 48S complex formation similar to that obtained with F1, F2, and F3, showing that F2 could be replaced by these two factors (data not shown). eIF4A is present in F3. Purified eIF4A in combination with F1, eIF2, and eIF2B resulted in a level of 48S complex formation similar to that obtained with F1, F2, and F3 (Fig. 3A). F3 could therefore be replaced by eIF4A. Fraction F1 contains PTB and eIF3, eIF4B, and eIF4F. Purified eIF3, eIF4B, eIF4F, and PTB in combination with eIF2, eIF2B, and eIF4A resulted in a level of 48S complex formation that is slightly greater than that obtained with F1, F2, and F3 (Fig. 3B and C), showing that F1 could be replaced by these four factors.

The single-subunit factors used in assembling 48S complexes (PTB, eIF4A, and eIF4B) were overexpressed in *E. coli* and purified by chromatography (Fig. 2). All three recombinant factors were as active as the corresponding native proteins in mediating assembly of 48S complexes (e.g., Fig. 3D and E). These recombinant factors are readily and inexpensively purified in high yield and without risk of contamination by other initiation factors. They were therefore used in all subsequent experiments.

48S complex formation on the EMCV IRES could therefore be reconstituted using Met-tRNA^{Met}, 40S ribosomal subunits, and seven purified proteins. 48S complexes formed from these fractionated components contained up to one-fifth of the input [³²P]UTP-labeled RNA. The efficiency of this reconstituted process is therefore comparable to that in RRL. This result strongly suggests that all essential factors involved in EMCV IRES-mediated initiation up to the stage of 48S complex formation have been identified. 48S complexes assembled in the reconstituted reaction are competent to form 80S initiation complexes in the presence of GTP (in place of GMP-PNP), eIF5, and 60S subunits (data not shown).

Factor and nucleotide requirements for 48S complex formation on EMCV RNA. Each of the purified factors used in assembling 48S complexes was individually omitted from the reaction to determine whether it was required for IRES-mediated initiation. 48S complex formation was absolutely dependent on eIF2 and on eIF3 (Fig. 4A), whereas omission of eIF2B had no effect (data not shown). Omission of eIF4A, eIF4B, or eIF4F reduced 48S complex formation 3-fold, 4-fold, and 8- to 10-fold, respectively (Fig. 4B to D). It is important to note that this analysis does not assess the contribution of any factor to the kinetics of initiation and therefore probably underestimates the importance of these factors in internal ribosomal entry *in vivo*. 48S complex formation in the absence of free eIF4A is consistent with the dispensability of fraction F3 from which it was derived (Fig. 1C). eIF4A was present in these reactions as a subunit of eIF4F, and these experiments have therefore not enabled us to establish whether eIF4A is absolutely required for IRES-mediated initiation. A requirement for eIF4A as a subunit of eIF4F has previously been suggested (48). PTB's effect on 48S complex formation was significant but less pronounced; omission reduced 48S complex formation 1.5- to 2-fold (Fig. 4E). Inclusion of additional

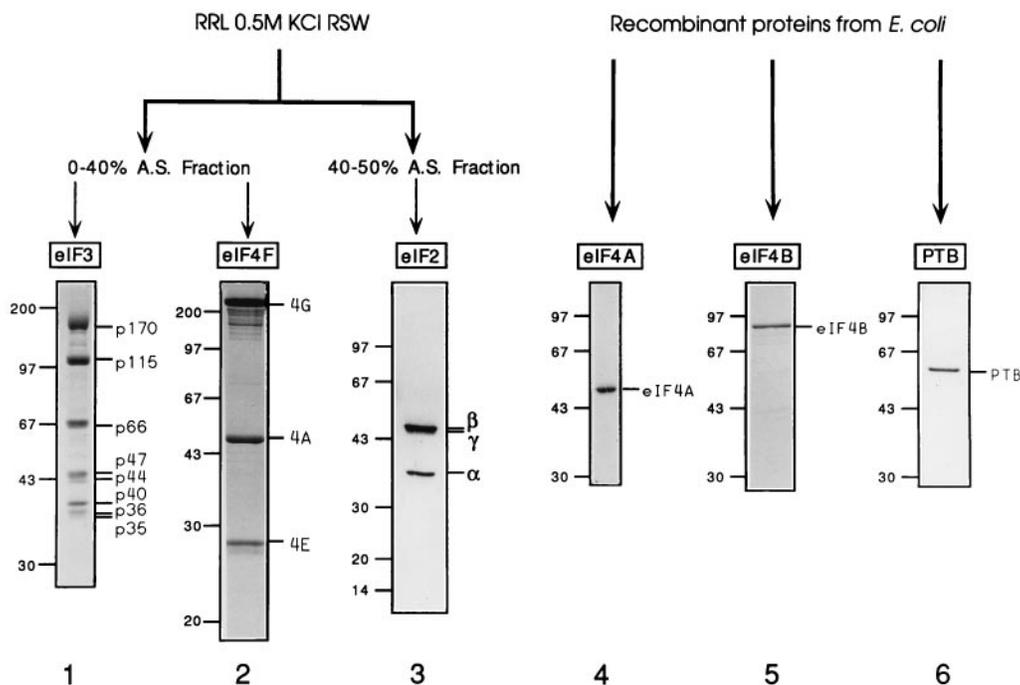


FIG. 2. Overview of the factors used in reconstitution of internal ribosomal entry. SDS-5 to 20% gradient polyacrylamide gels were stained with Coomassie blue. The positions of molecular weight marker proteins are indicated in kilodaltons to the left of each panel. Subunits of eIF2, eIF3, and eIF4F and recombinant eIF4A, eIF4B, and PTB are indicated to the right of appropriate panels. Lane 1, 5 μ g of eIF3; lane 2, 4 μ g of eIF4F; lane 3, 4 μ g of eIF2; lane 4, 2 μ g of recombinant human eIF4A; lane 5, 1.5 μ g of recombinant human eIF4B, lane 6, 2 μ g of recombinant human PTB.

eIF4F in a reaction lacking eIF4A restored the level of 48S complex formation (Fig. 4F). Conversely, inclusion of 5 μ g of additional eIF4A in a reaction lacking eIF4F had no such effect (data not shown). This observation is consistent with suggestions that eIF4A can gain access to mRNA only as a subunit of eIF4F (48, 69) and suggests that another subunit of this factor is required for IRES-mediated initiation. The canonical initiation factors eIF2, eIF3, and eIF4F are therefore essential for EMCV IRES-dependent 48S complex formation *in vitro*.

Cap-dependent initiation of translation in eukaryotes requires both GTP (as part of a ternary complex containing Met-tRNA^{Met} and eIF2) and ATP (for the RNA helicase activity of eIF4A and eIF4F). These nucleotides were individually omitted or replaced by nonhydrolyzable analogs to determine the requirement for them in IRES-dependent initiation. GTP was routinely replaced by GMP-PNP without impairing 48S complex formation, consistent with previous observations (42). ATP was essential for 48S complex formation and could not be replaced by adenylylimidodiphosphate (AMP-PNP) (Fig. 4G).

Assembly of 48S complexes from purified components is IRES dependent. D1 mutant EMCV RNA was used to confirm that assembly of 48S complexes from purified initiation factors was IRES mediated. As noted above, this RNA contains an internal deletion (nt 702 to 762) that by definition (34) cannot affect canonical 5'-end-dependent initiation of translation but which abrogates its activity in IRES-mediated initiation (8, 13). 48S complexes were not assembled on D1 mutant RNA following incubation with purified translation components (Fig. 4H). This result is identical to that obtained in assays using the same mutant RNA and the 0 to 70% AS fraction from which these factors were derived (Fig. 1B). Assembly of 48S complexes on *wt* EMCV RNA mediated by purified factors was therefore the result of internal ribosomal entry. The integrity of the *wt* and D1 mutant RNAs on which 48S and RNP com-

plexes had formed was compared with that of input RNA. No significant degradation of either RNA occurred as a result of incubation with initiation factors, sucrose density gradient centrifugation, and extraction from peak 48S and RNP fractions (Fig. 5). The first result confirms that formation of 48S complexes from purified components is dependent on the integrity of the IRES and could not have resulted from ribosomal entry at the 5' end followed by scanning to the first AUG codon. The second result eliminates the possibility that 48S complexes had assembled by a conventional end-dependent mechanism on fragmented EMCV RNA.

Determination of the site of 48S complex assembly by primer extension inhibition. The initiation site for synthesis of the EMCV polyprotein is AUG₈₃₄ (66). Most ribosomes initiate translation at this codon, but a low level of initiation occurs at AUG₈₂₆ (29, 30). We have used primer extension inhibition (19) to determine whether the 48S complexes described above had assembled at the correct location on the EMCV IRES.

Primer extension done in the presence of 40S subunits, fractions F1 and F3, and eIF2 and eIF2B yielded prominent cDNA products that terminated 15 to 17 nt 3' to the A in AUG₈₃₄ and less prominent products that terminated at C₇₈₆ and 15 to 17 nt 3' to AUG₈₂₆ (Fig. 6A, lane 2). A similar set of cDNAs was detected after probing 48S complexes assembled from 40S subunits, PTB, eIF2, eIF3, eIF4A, eIF4B, and eIF4F (Fig. 6B, lane 1). Eukaryotic 48S complexes inhibit primer extension at positions 15, 16, and 17 nt 3' to the A of the initiation codon on which they have assembled (3). Detection of stop sites 15 to 17 nt 3' to AUG₈₃₄ and AUG₈₂₆ therefore indicates that 48S complexes had assembled precisely at these two codons.

Primer extension was done on D1 mutant RNA (see Fig. 8A) in the presence of translation components to confirm the validity of this method as an assay of IRES-mediated initiation. D1 RNA is inactive in translation and in 48S complex forma-

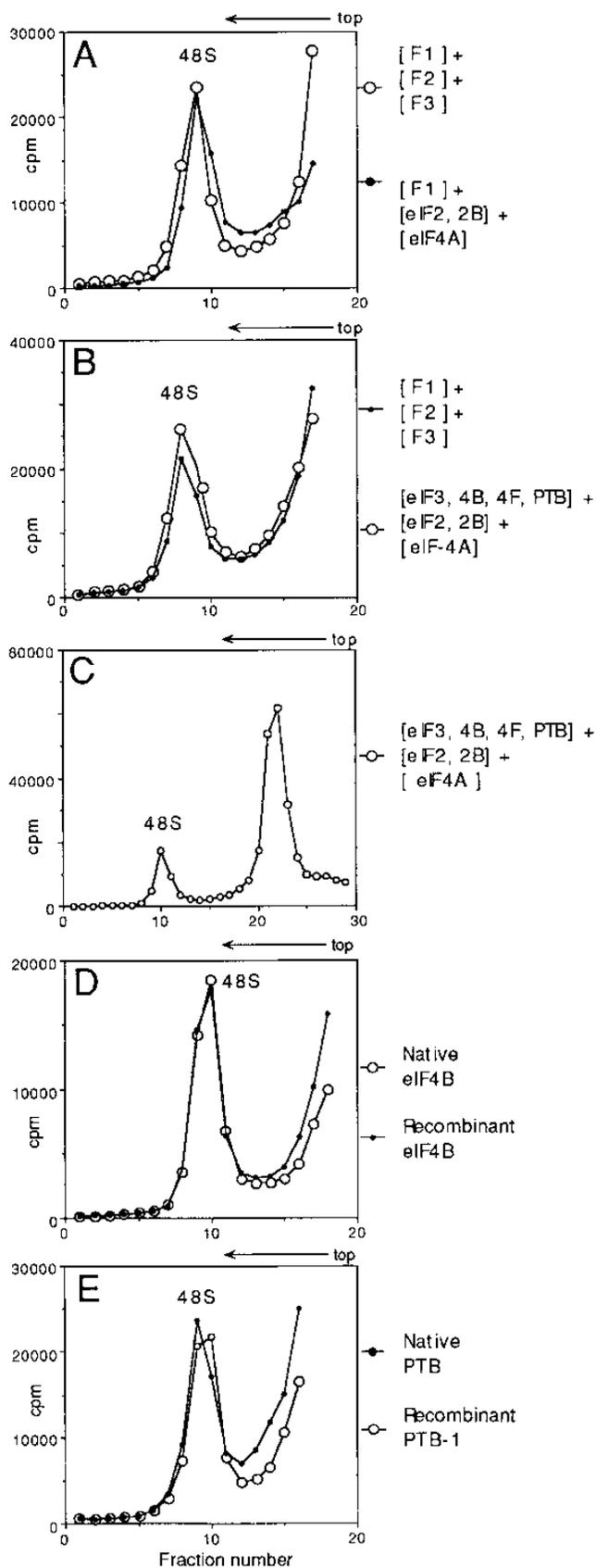


FIG. 3. Replacement of RSW fractions F1, F2, and F3 by purified initiation factors. Assays were carried out under standard conditions using fractions F1, F2,

tion (9, 13) (Fig. 1B and 4H). The results of primer extension were consistent with these properties. Stop sites due to assembly of 48S complexes at AUG₈₂₆ and AUG₈₃₄ were not detected on D1 RNA (data not shown).

Primer extension inhibition was next used to examine the requirements of IRES-mediated initiation for individual factors. Analysis of 48S complexes assembled from 40S subunits and eIF2, -3, -4A, -4B, and -4F yielded extension products (Fig. 6B, lane 2) that were similar to those detected on analysis of complexes formed from these components and PTB (Fig. 6B, lane 1). The prominence of the set of bands 3' to AUG₈₃₄ increased about twofold and that of the bands 3' to AUG₈₂₆ was slightly reduced as a result of PTB's inclusion in the assembly reaction. Stop sites 15 to 17 nt upstream of AUG₈₃₄ and AUG₈₂₆ were detected when 40S subunits were incubated with eIF2, -3, -4B, and -4F (Fig. 6C, lane 3) or with PTB and eIF2, -3, -4A, and -4F (Fig. 6C, lane 5). These two stop sites were not detected when 40S subunits were incubated with PTB and either eIF2, -3, and -4B (Fig. 6B, lane 3) or eIF2, -3, -4A, and -4B (Fig. 6C, lane 4). Instead, a prominent full-length cDNA and smaller amounts of shorter cDNAs were detected in each of these assays. These results indicate that 40S subunits and either crude RSW fractions or purified initiation factors assembled into 48S complexes at two locations on EMCV RNA. The majority of 48S complexes assembled at AUG₈₃₄, and a smaller proportion assembled at AUG₈₂₆. eIF2, eIF3, and eIF4F were essential for 48S complex formation; PTB, eIF4A, and eIF4B enhanced this process. These results are wholly consistent with the results of experiments in which the dependence of IRES-mediated 48S complex formation on individual factors was assayed by sucrose density gradient centrifugation.

A prominent cDNA that terminated at C₇₈₆ and a lesser amount of full-length cDNA were detected after probing the RNP complex formed from EMCV RNA, PTB, and eIF2, -3, -4A, -4B, and -4F in the absence of 40S subunits (Fig. 6B, lane 4). Primer extension done in the absence of 40S subunits and factors yielded a large proportion of full-length cDNA and a set of shorter, less prominent products (Fig. 6A, lane 1; Fig. 6B, lane 5). None of these shorter cDNAs correspond to the prominent stop sites detected 15 to 17 nt upstream of AUG₈₂₆ and AUG₈₃₄ in the presence of 40S subunits and eIFs.

Stable binding of eIF4F to the EMCV IRES. The stop site detected by primer extension at C₇₈₆ is within the J₁ helix at the base of the J-K domain (see Fig. 8A). Primer extension is inhibited weakly at this position on naked RNA (Fig. 6B, lane 5) and significantly more strongly in the presence of initiation factors. This observation suggests that binding of one or more factors to the IRES stabilizes the J-K domain. PTB had previously been found not to influence inhibition of primer extension at C₇₈₆ (Fig. 6B, lanes 1 and 2). To identify the protein(s) that arrests primer extension at this residue, primer extension analysis was done using *wt* EMCV RNA and different combinations of the initiation factors (eIF2, eIF3, eIF4A, eIF4B, and eIF4F) that are involved in IRES-mediated 48S complex formation. A prominent cDNA product that terminated at C₇₈₆

and F3 or fraction F1 and eIF2, -2B, and -4A (A), fractions F1, F2, and F3 or native eIF2, -2B, -3, -4A, -4B, and -4F and PTB (B), native eIF2, -3, -4A, -4B, and -4F and PTB (C), native eIF2, -2B, -3, -4A, -4B, and -4F and native or recombinant eIF4B (D), and native eIF2, -3, -4A, and -4F and PTB and PTB or recombinant PTB-1 with other reaction components (E) as described in Materials and Methods. Sedimentation was from right to left, and the positions of 48S complexes are indicated. Fractions from the upper part of the sucrose gradient have been omitted from graphs in panels A, B, D, and E for greater clarity.

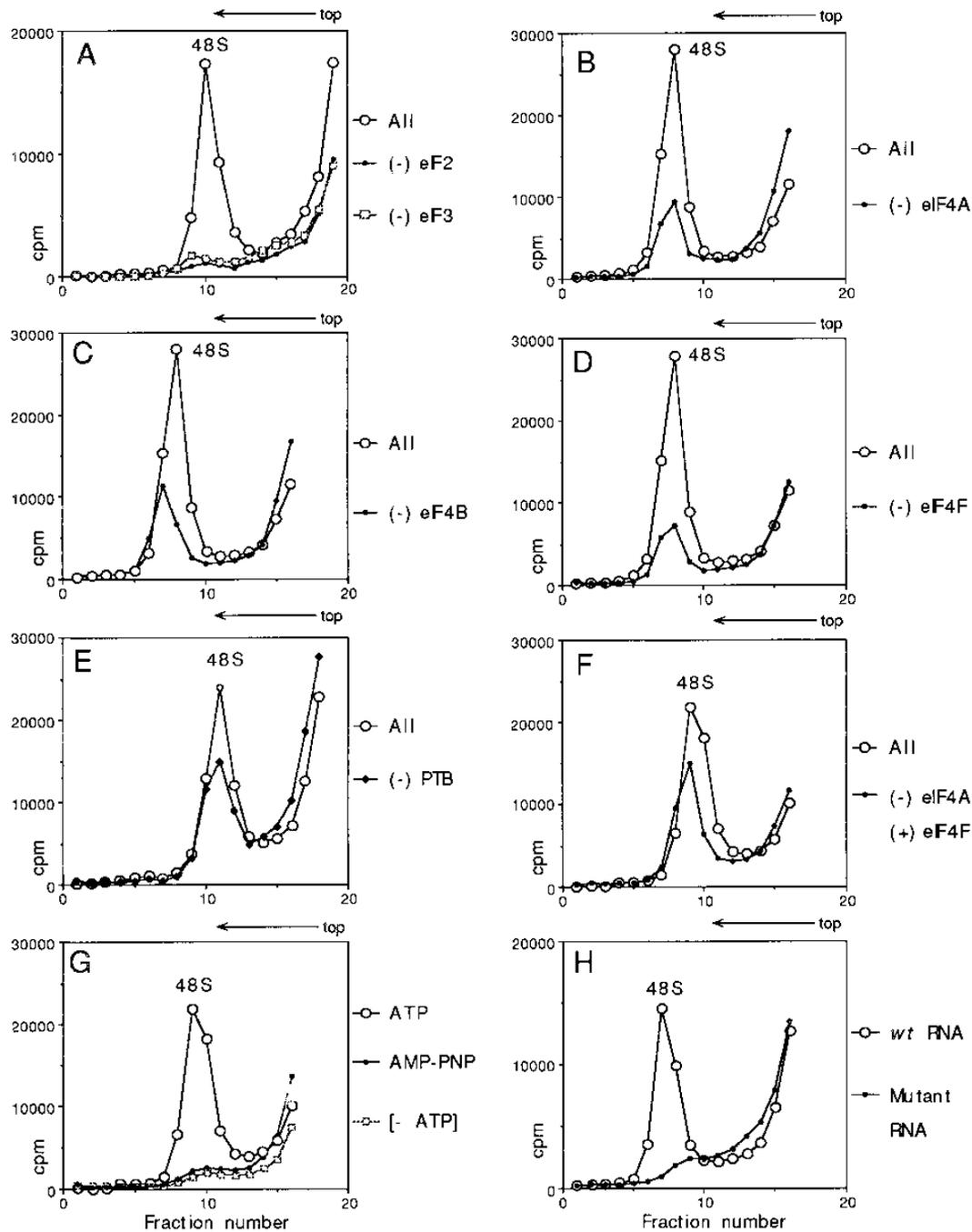


FIG. 4. Dependence of internal ribosomal entry in vitro on individual purified components. Assays were carried out as follows: (A) using the complete set of initiation factors (eIF2, -2B, -3, -4A, -4B, and -4F and PTB) or the complete set lacking either eIF2 or eIF3; (B to H) using the complete set of factors with or without eIF4A (B), with or without eIF4B (C), with or without eIF4F (D), with or without PTB (E), either with eIF4A or without eIF4A but with 3 μ g of additional eIF4F (F), without ATP or with 1 mM ATP or 1 mM AMP-PNP (G), and with *wt* or Δ nt 701-763 mutant EMCV RNA (H). Sedimentation was from right to left. Fractions from the upper part of the sucrose gradient have been omitted from graphs in panels A to H for greater clarity. The complete assay shown in panels B to D is the same because these panels represent a single experiment run in the same rotor.

was detected in the presence of eIF4A, eIF4B, and eIF4F and either eIF2 or eIF3 (Fig. 7A, lanes 1 to 3). The same product was detected in the presence of eIF4F alone, and its intensity was not altered by inclusion of eIF4A or -4B (Fig. 7A, lanes 6 to 8). Extension inhibition at C_{786} was not detected in the presence of eIF4A and eIF4B together (Fig. 7A, lane 9) or in the presence of eIF2, eIF3, eIF4A, or eIF4B individually (Fig.

7A, lanes 4 and 10; Fig. 7B, lanes 1 and 3). Extension inhibition at C_{786} in the presence of eIF4F was not affected by omission of ATP, replacement of ATP by AMP-PNP, or inclusion of GTP (Fig. 7C, lanes 1 to 4). This result is consistent with the RNA-binding properties of eIF4F (1, 28). These observations indicate that eIF4F interacts strongly with EMCV mRNA in a novel manner that does not involve binding of eIF4E to a

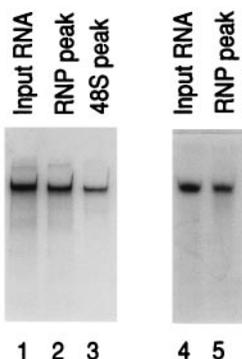


FIG. 5. Integrity of EMCV RNA in 48S and RNP complexes. [³²P]UTP-labeled *wt* (lanes 1 to 3) or Δ nt 701-763 mutant EMCV RNAs (lanes 4 and 5) extracted from peak 48S (lane 3) or RNP fractions (lanes 2 and 5) and corresponding input RNAs (lanes 1 and 4) were analyzed by electrophoresis in 5% polyacrylamide gels and exposed to X-ray film. *wt* and mutant RNAs were about 890 and 840 nt long, respectively.

5'-terminal m⁷G cap. This conclusion is strongly supported by our finding that a recombinant polypeptide that corresponds to the central third of the eIF4G subunit of eIF4F arrests primer extension at C₇₈₆ on the EMCV IRES and can be UV cross-linked to EMCV IRES-containing RNA (51).

Complexes formed between eIFs and capped eukaryotic mRNAs are not sufficiently stable to arrest AMV-RT (3), and so the inhibition of primer extension caused by interaction of eIF4F and the EMCV IRES suggests that this complex is exceptionally stable and/or long-lived. To investigate the specificity of this interaction, primer extension was done on *wt* EMCV RNA or on D1 or D2 mutant EMCV RNA in the presence eIF4F. The D1 mutant RNA lacks the apical two-thirds of the J-K domain, but the results of enzymatic probing indicate that the integrity of the basal J₁ and J₂ helices is maintained (13). This RNA is inactive in initiation (Fig. 1B and 4H) (9) and translation (13). In the D2 mutant RNA, residues UAAAAA₇₆₉₋₇₇₄ have been replaced by the sequence AU (Fig. 8A). This mutant RNA was designed to resemble (but is not identical to) the previously described EMCV mutant 768 Δ 4, which is inactive in translation *in vivo* and which has lost the

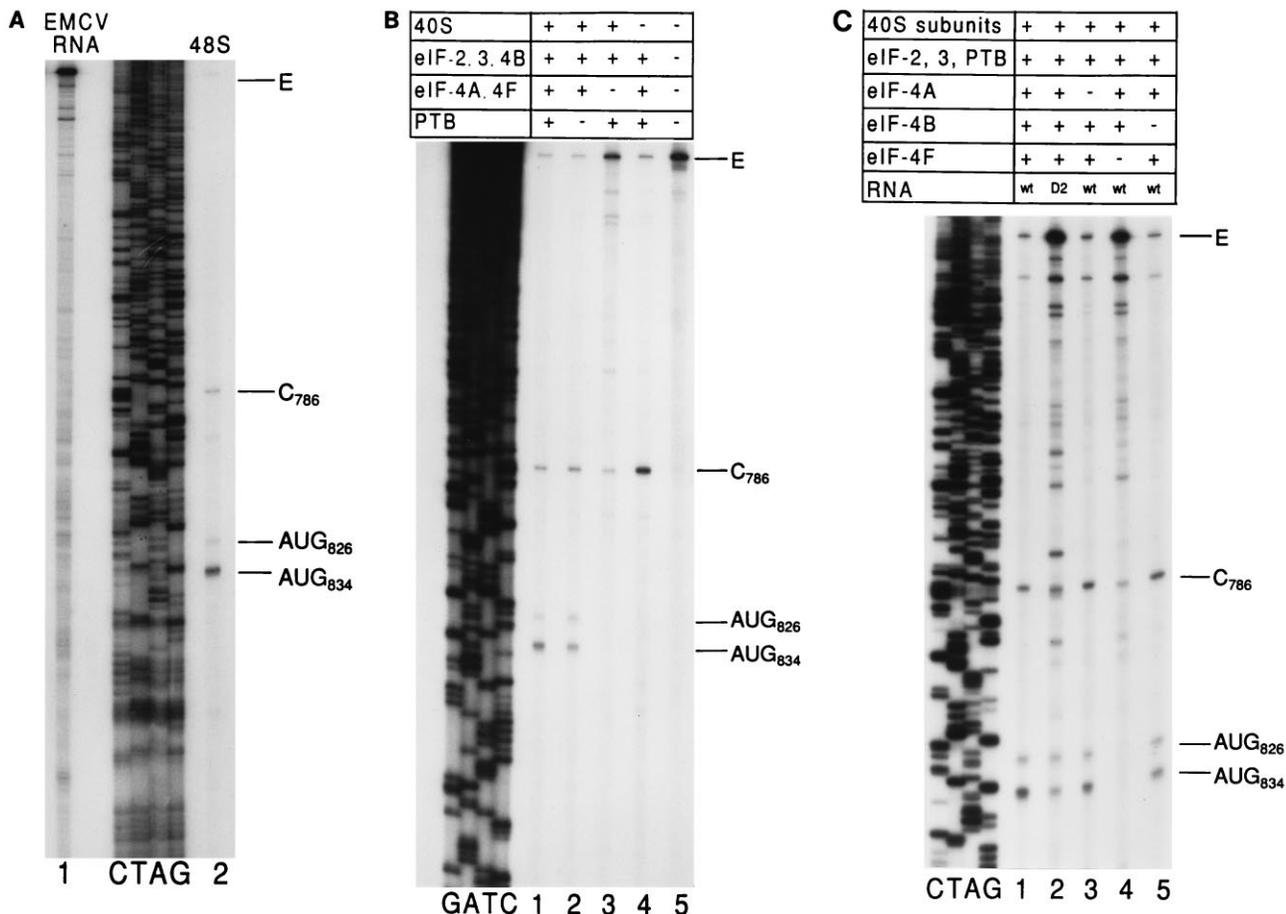


FIG. 6. Primer extension analysis of 48S preinitiation complexes. EMCV RNA transcripts were incubated under standard reaction conditions for assembly of 48S complexes as follows: (A) with 40S subunits, fractions F1 and F3, and eIF2 and eIF2B (lane 2) or without these components (lane 1); (B) with (lanes 1 to 3) or without (lanes 4 and 5) 40S subunits and without initiation factors (lane 5) or with eIF2, -3, -4A, -4B, and -4F and PTB (lanes 1 and 4), with this set of factors except for PTB (lane 2), or with this set except for eIF4A and eIF4F (lane 3); (C) with 40S subunits and eIF2, eIF3, and PTB (lanes 1 to 5), with eIF4A (lanes 1, 2, 4, and 5), with eIF4B (lanes 1 to 4), and with eIF4F (lanes 1 to 3 and 5). *wt* nt 315-1155 EMCV RNA was used in all reactions except for that shown in lane 2 of panel C, in which D2 mutant RNA was used. A primer (5'-GTCAATAACTCCTCTGG-3') was annealed to EMCV nt 957 to 974 (within the coding sequence) and was extended with AMV-RT. cDNA products labeled AUG₈₃₄ and AUG₈₂₆ terminated at stop sites 15 to 17 nt from the stated AUG codon. The cDNA product labeled C₇₈₆ 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 using the same primer and pTE1 DNA.

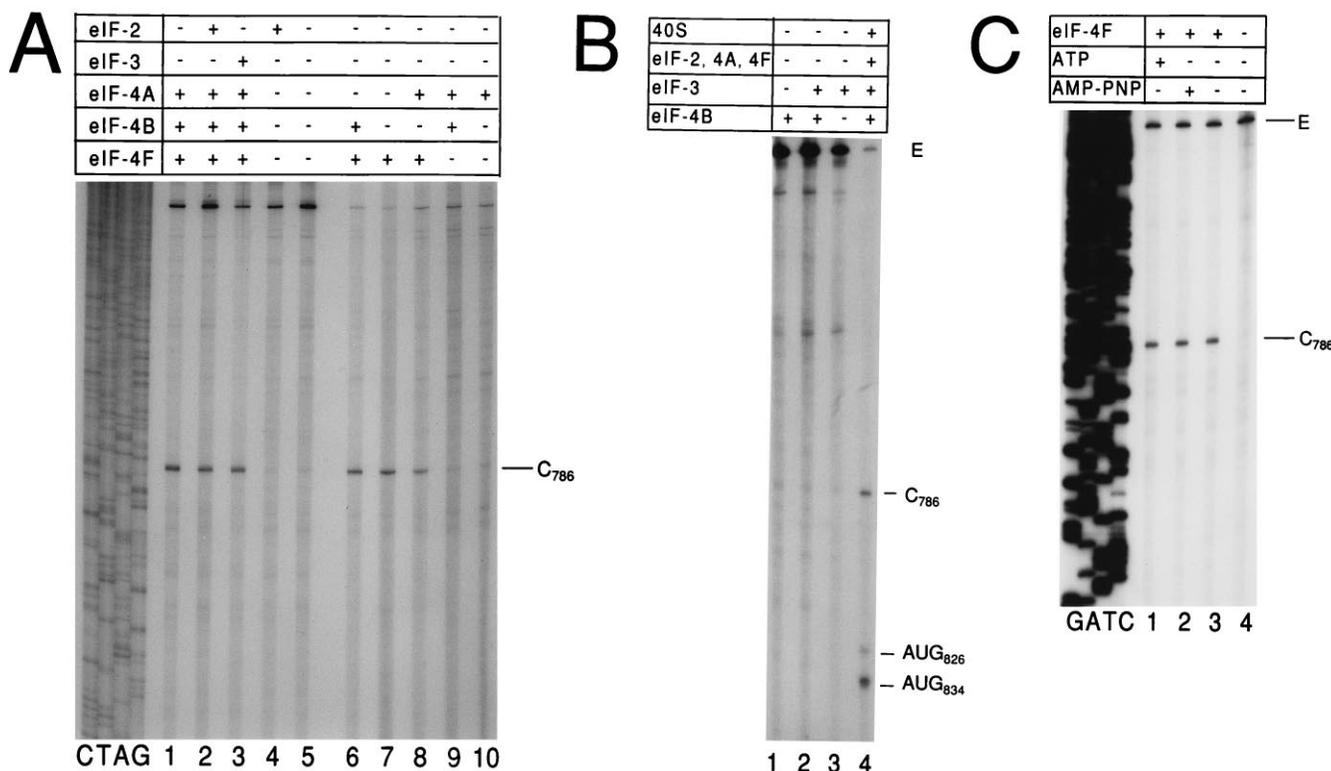


FIG. 7. Primer extension analysis of an RNP complex formed on EMCV RNA. *wt* nt 315-1155 EMCV RNA was incubated under standard conditions for assembly of 48S complexes as follows: (A and B) with 40S subunits, eIF2, -3, -4A, -4B, and -4F as indicated; (C) with eIF4F, 1 mM ATP, and 1 mM AMP-PNP as indicated or with 1 mM ATP and 0.4 mM GTP (lane 4). An oligonucleotide primer was annealed within the EMCV coding sequence and extended with AMV-RT. The cDNA product labeled C₇₈₆ 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.

normal ability of *wt* EMCV IRES to competitively inhibit IRES-dependent and cap-dependent initiation (23). Formation of 48S complexes at AUG₈₃₄ on D2 RNA is strongly impaired (Fig. 6C, lanes 1 and 2). Primer extension inhibition at C₇₈₆ was detected on the *wt* RNA but was only faintly apparent on D2 RNA and was not detected on D1 RNA (Fig. 8B, lanes 1 to 6). These observations indicate that the integrity of the J-K domain is essential both for IRES function and for the stabilizing interaction between eIF4F and the IRES.

DISCUSSION

Initiation of translation by internal ribosomal entry was reconstituted *in vitro* using EMCV IRES-containing RNA transcripts and purified translation components to identify the factors that are required for this process. Assembly of 48S preinitiation complexes at the correct initiation codon (AUG₈₃₄) was verified by primer extension analysis. To ensure that previously uncharacterized initiation factors would be detected, this process was first reconstituted by using RSW fractions from RRL which were then replaced by purified factors. A number of new conclusions can be reached on the basis of this study.

Internal ribosomal entry is mediated by canonical eIFs. The reconstituted initiation process described here is as accurate as initiation in RRL and is only 1.5-fold less efficient. All essential factors involved in EMCV IRES-mediated initiation have therefore been identified. Purified eIF2, eIF3, and eIF4F as well as Met-tRNA_i^{Met}, ATP, GTP (or nonhydrolyzable GTP analogs), and 40S ribosomal subunits were necessary to recon-

stitute IRES-mediated 48S complex formation *in vitro*; eIF4B and to a lesser extent PTB were found to enhance this process. As noted above, the analysis presented here does not provide information about the contribution of individual factors to the kinetics of internal initiation. In addition, the results presented here do not discriminate between a role for eIF4A by itself and as a subunit of the eIF4F holoenzyme.

The observation that PTB enhanced EMCV IRES-mediated initiation but was not essential for this process is consistent with observations that sequestration of PTB in an *in vitro* translation system and deletion of the principal PTB binding site from the EMCV IRES both impaired but did not abrogate initiation (8, 11, 13). The initiation process outlined here occurs on naked RNA, and PTB's enhancement of 48S complex formation is likely to be significant in the cytoplasmic environment of competing cap-dependent mRNAs and RNA-binding proteins. For example, PTB has a higher affinity than the cytoplasmic protein glyceraldehyde 3-phosphate dehydrogenase for domain IIIa of the hepatitis A virus IRES (58). PTB thus has the ability to prevent destabilization of this RNA structural element by glyceraldehyde 3-phosphate dehydrogenase and consequent impairment of IRES-mediated initiation (58).

The results presented here indicate that internal ribosomal entry can be mediated solely by canonical initiation factors and does not require factors whose function is restricted to IRES-mediated initiation. This conclusion implies that it is the IRES itself rather than such IRES-specific factors that determines entry of ribosomes at an internal rather than a 5'-terminal position on an mRNA. The factors involved in IRES-mediated initiation are all RNA-binding proteins but do not mediate

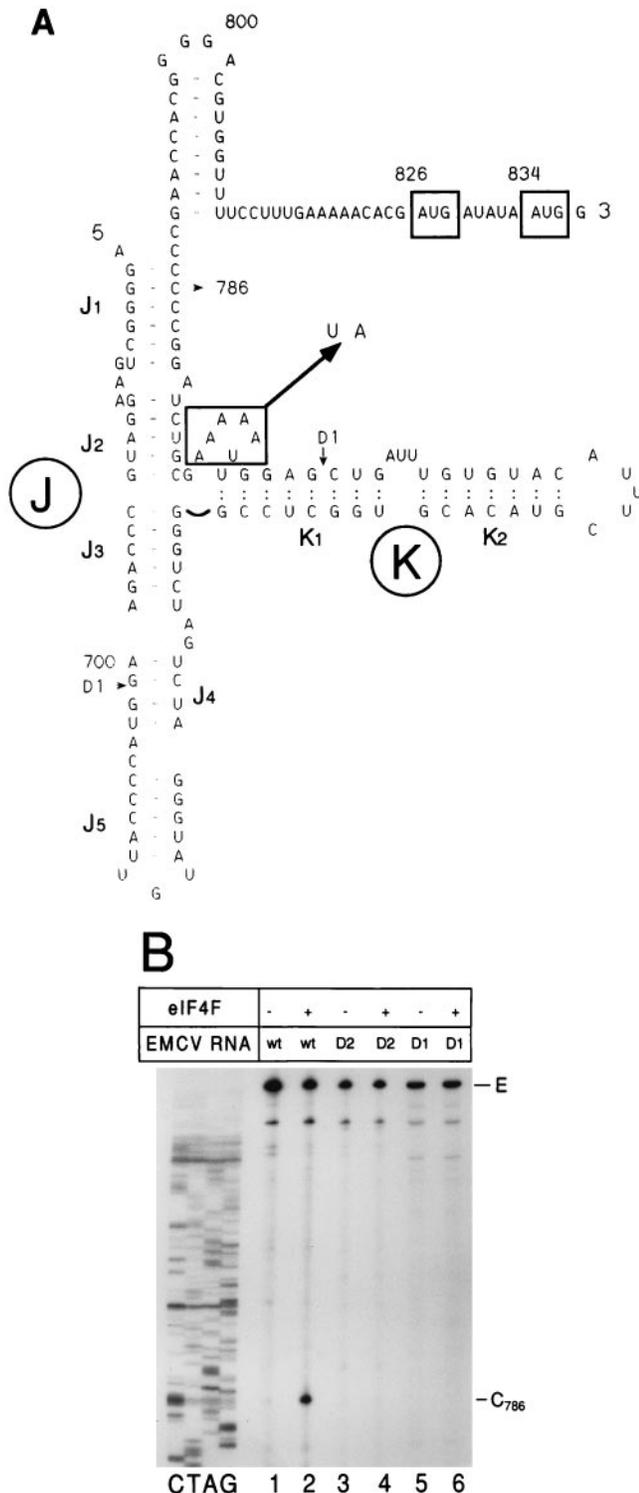


FIG. 8. Specificity of interaction between eIF4F and the EMCV IRES. (A) Structure of the J-K domain of the EMCV IRES as in reference 52 (sequence according to Evstafieva et al. [13]; nomenclature of domains and helices as in reference 11). The initiation codons AUG₈₂₆ and AUG₈₃₄ are boxed. The stop site (C₇₈₆) detected in the presence of eIF4F and the borders of the deletion (nt 702 to 762 inclusive) in mutant D1 are indicated by arrowheads. The residues (nt 769 to 774) substituted in the D2 mutant by the sequence AU are boxed. (B) Primer extension analysis done on wt (lanes 1 and 2), D1 (lanes 3 and 4), or D2 (lanes 5 and 6) EMCV nt 315-1155 RNA after incubation under standard conditions either alone (lanes 1, 3, and 5) or with 2 μ g of eIF4F (lanes 2, 4, and 6). A primer was annealed within the EMCV coding sequence and was extended

efficient binding of 40S subunits to random RNA sequences. The ordered structure of the EMCV IRES may act as a scaffold to which these factors bind in a manner that orients them such that they are able to mediate entry of 40S subunits at a specific internal site.

Initiation mediated by the EMCV IRES was absolutely dependent on ATP and eIF2, -3, and -4F. eIF2 is required to bind Met-tRNA^{Met} to 40S subunits, and eIF3 stabilizes this interaction (42). Both factors are constituents of the resulting 43S complex. A second function of eIF3 is to promote binding of this 43S complex to mRNA. 43S complexes by themselves do not bind mRNA, and so this step probably involves eIF3's interacting with other factors rather than directly with RNA.

Cap-independent interaction of eIF4F with the EMCV IRES. The interaction of eIF3 with the cap-binding complex eIF4F may promote loading of 43S complexes on to mRNA near to the 5'-terminal cap (18, 36). We have detected a novel interaction of eIF4F with internal mRNA sequences that by definition does not involve binding of eIF4E to the m⁷G cap, and we propose that this interaction plays an analogous role in promoting internal ribosomal entry. The interaction of eIF4F with the IRES stabilized the J-K domain sufficiently to inhibit primer extension at C₇₈₆. This interaction may be an important early step in internal initiation. The observation that substitution or deletion of residues upstream of C₇₈₆ abrogated the stabilizing interaction of eIF4F with the J-K domain and impaired IRES activity is consistent with this model.

eIF4F had been considered to have no role in IRES-mediated initiation because cleavage of its eIF4G subunit following poliovirus infection shuts off cap-dependent initiation but does not impair translation of EMCV and other IRES-dependent mRNAs (12, 39, 59, 65). Proteolytic cleavage of eIF4G separates its eIF3, eIF4A, and RNA binding domains from the domain that interacts with the cap-binding protein eIF4E and as a result uncouples cap recognition from ribosome-binding and helicase functions (36, 40, 46, 51). Internal initiation can be stimulated *in vitro* by addition of eIF4F (2, 56), and a recent report suggests that the function of eIF4F in initiation can be attributed to the C-terminal two-thirds of its eIF4G subunit (46). We have found that the central third of eIF4G binds specifically to the J-K domain of the EMCV IRES and that the activity of eIF4F in IRES-mediated initiation can be provided by eIF4A and this portion of eIF4G (51). Taken together, these observations suggest that possible roles of eIF4F in IRES-mediated initiation are to provide a helicase activity after its eIF4G subunit has bound to a specific site within the IRES and to mediate binding of 43S complexes to the IRES.

IRES-mediated initiation is dependent on ATP. ATP may be required for recycling of eIF4F and is required for cap-dependent binding of ribosomes to mRNA and for ribosome scanning to the initiation codon (33, 42). These steps result from ATP-dependent unwinding of the mRNA at the 5' end, initiated by eIF4F and continued in a 5'-3' direction by eIF4A. Initiation of EMCV translation is cap independent and does not involve scanning (30), and so the ATP dependence of EMCV initiation was not anticipated. The observations that 48S complex formation on the EMCV IRES depended strongly on both ATP and eIF4F suggest that this process also involves restructuring or unwinding of RNA. The interaction of

with AMV-RT. The cDNA product labeled C₇₈₆ 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.

eIF4F with the J-K domain positions eIF4A on the EMCV IRES a short distance upstream of the initiation codon. This ATP-independent interaction may be followed by binding and hydrolysis of ATP by eIF4A, thereby unwinding part of the IRES to create a single-stranded region that can enter the mRNA binding cleft of the 40S subunit. This mechanism is consistent with the helicase activity of eIF4F which can unwind an RNA substrate that contains an internal single-stranded region (54).

Implications for the regulation of translation initiation.

Binding of 43S complexes to mRNA is the rate-limiting step in translation and is a frequent target for regulation. eIF4E plays a key role in the regulation of translation (23). It is the target for two regulatory mechanisms which down-regulate the cap-binding activity of eIF4F. The first mechanism involves sequestration of eIF4E by specific 4E-binding proteins, which prevents its binding to eIF4G and thus decreases the translation of capped mRNAs (17, 48). The second mechanism involves dephosphorylation of eIF4E, which reduces its association with eIF4G (10) and its cap-binding activity (44). We have found that eIF4F binds to the EMCV IRES independently of the interaction between eIF4E and a 5'-terminal m⁷G cap. This observation suggests that repression of cap-dependent translation by these two regulatory mechanisms, for example, during heat shock, growth arrest, mitosis, and viral infection, could result in selective translation of mRNAs that contain IRES elements.

ACKNOWLEDGMENTS

We thank R. J. Jackson and W. C. Merrick for helpful comments during the course of this study. We gratefully acknowledge U. Bommer, D. Etchison, J. Hershey, R. Jagus, R. Matts, P. Sharp, and H. Trachsel for providing antibodies. We thank S. Kato, M. Davies, R. Rhoads, and P. Sharp for the human eIF4A, eIF4B, eIF4G, and PTB-1 cDNAs, respectively. We thank C. G. Proud, W. C. Merrick, and U. Maitra for generous gifts of eIF2, eIF2B and eIF4B, and eIF5, respectively, and D. L. Lyakhov for the DEL172-3 mutant T7 RNA polymerase.

This work was supported by grant NAT 000 from the International Science Foundation to I.N.S. and grants HTECH.CRG 941251 from NATO and 75195-544804 from the Howard Hughes Medical Institute to C.U.T.H. and I.N.S.

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