THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 8, Issue of March 15, pp. 5566-5573, 1993
Printed in U.S.A.

The p46 Subunit of Eukaryotic Initiation Factor (eIF)-4F Exchanges with eIF-4A*

(Received for publication, August 12, 1992)

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The p46 subunit of eukaryotic initiation factor (eIF)-4F purified from rabbit reticulocyte lysate has previously been found to be composed of eIF-4AI and eIF-4AII in a 4:1 ratio, respectively, whereas the free form of rabbit eIF-4A is composed solely of eIF-4AI. Using sucrose gradient centrifugation and an m^{7}GTP-Sepharose 4B assay, it was shown that eIF-4A exchanges with the p46 subunit of eIF-4F. Incubation of [^{14}C]eIF-4A and eIF-4F resulted in the incorporation of [^{14}C] eIF-4A into the eIF-4F complex. Conversely, the [^{14}C] p46 subunit of [^{14}C]eIF-4F was shown to dissociate from the [^{14}C]eIF-4F complex in the presence of eIF-4A, presumably due to the incorporation of unlabeled eIF-4A. Similar experiments were conducted in which ^{14}C-labeled initiation factors were incubated with rabbit reticulocyte lysate. When [^{14}C]eIF-4A was incubated with lysate, [^{14}C]eIF-4A became incorporated into the eIF-4F complex present in the lysate. Additionally, when [^{14}C]eIF-4F was incubated with lysate, the [^{14}C]p46 subunit of [^{14}C]eIF-4F dissociated from the [^{14}C]eIF-4F complex, most likely due to the exchange of unlabeled eIF-4A (present in the lysate) with the [^{14}C]p46 subunit. The exchange of mouse eIF-4AI and eIF-4AII expressed in *Escherichia coli* was also investigated in the presence of eIF-4F and rabbit reticulocyte lysate. Both the sucrose gradient experiments and m^{7}GTP-Sepharose 4B assays demonstrated that the [^{14}C]p46 subunit of [^{14}C]eIF-4F was displaced in the presence of eIF-4AI or eIF-4AII and that mouse [^{14}C]eIF-4AI or [^{14}C]eIF-4AII became incorporated into the eIF-4F complex in the same manner as rabbit reticulocyte eIF-4A.

Three eukaryotic initiation factors (eIFs),[1] eIF-4A, eIF-4B, and eIF-4F, are required for the binding of the 43 S preinitiation complex to mRNA (1–4). eIF-4F is a protein composed of three subunits, p220, p46, and p24, which recognizes the m^{7}G cap structure of eukaryotic mRNAs in an ATP-independent manner. eIF-4B appears to be a dimer of identical 80-kDa subunits that stimulates the ATPase and mRNA binding activities of eIF-4A and eIF-4F (5, 6) as well as the recycling of the p24 subunit of eIF-4F (7). eIF-4A is a single polypeptide of 46 kDa characterized by an ATP-dependent mRNA binding activity (8) and an RNA-dependent ATPase activity (6). eIF-4A also has the ability to unwind mRNA secondary structure in the presence of ATP as shown by a nuclease sensitivity assay (9) and a bidirectional helicase assay in which eIF-4B is present (10). In addition, two functional genes for eIF-4A, eIF-4AI and eIF-4AII, have been identified, and their amino acid sequences have been found to be 91% identical (11). In rabbit reticulocytes the free form of eIF-4A is believed to be composed of only eIF-4AI, whereas the p46 subunit of eIF-4F is composed of eIF-4AI and eIF-4AII in a 4:1 ratio, respectively (12). The following similarities between the p46 subunit of eIF-4F and eIF-4A have been observed; both proteins co-migrate on polyacrylamide and two-dimensional gels (13, 14), cross-react with the same antibody (15), cross-link to [α-^{32}P]ATP (16), and unwind mRNA secondary structure (9, 10). However, the p46 subunit (when associated with the eIF-4F complex) does cross-link more efficiently to ATP and is a more proficient helicase than free eIF-4A (9, 10, 16).

The ratio of the mRNA levels encoding eIF-4AI and eIF-4AII have been found to vary in different tissues. Levels of mouse eIF-4AII/eIF-4AI mRNA range from 1.4 in the kidney to 0.03 in the thymus (11). These mRNA levels may reflect differences in factor concentration, which could influence the initiation process. In addition, different levels of eIF-4AI and eIF-4AII mRNA may also reflect differences in the ratio of eIF-4A to eIF-4F or even differences in the composition of eIF-4F itself. Thus the manner in which these two forms of eIF-4A interact with eIF-4F is of interest. This study focuses on determining whether free eIF-4A exchanges with the p46 subunit of eIF-4F. eIF-4A purified from rabbit reticulocyte lysate, believed to be approximately 95–100% eIF-4AI as determined by sequence analysis, will be referred to as eIF-4A. In addition, the two forms of mouse eIF-4A (expressed in *Escherichia coli*) were tested for their ability to exchange with the p46 subunit of eIF-4F. The two forms of eIF-4A expressed in *E. coli* will be designated eIF-4AI and eIF-4AII.

Using sucrose gradient centrifugation and a novel m^{7}GTP-Sepharose 4B assay, it has been determined that the p46 subunit of eIF-4F exchanges with free eIF-4A. This exchange phenomenon was demonstrated in two different ways. First, it was shown that [^{14}C]eIF-4A becomes incorporated into the eIF-4F complex, and, second, it was shown that eIF-4A can displace the [^{14}C]p46 subunit of [^{14}C]eIF-4F. Furthermore, both mouse eIF-4AI and eIF-4AII expressed in *E. coli* were found to exchange with the p46 subunit of eIF-4F in the same manner as rabbit reticulocyte eIF-4A.

* This research was supported in part by National Institutes of Health Grant GM-26796 (to W. C. M.) and a grant from the Medical Research Council of Canada (to N. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a studentship from the Deutscher Akademischer Austauschdienst and a graduate fellowship from McGill University.

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[1] The abbreviations used are: eIF, eukaryotic initiation factor; PAGE, polyacrylamide gel electrophoresis; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine.

MATERIALS AND METHODS

Purification of Initiation Factors—Purification of eIF-4A, eIF-4B, eIF-4F, and eIF-3 from rabbit reticulocyte lysate (Green Hectares) to greater than 90% homogeneity was performed as previously described (8, 14). All factors were assayed for activity by globin synthesis or ATP hydrolysis as previously described (6, 14). The two forms of mouse eIF-4A were expressed in *E. coli* and purified as previously described (17).

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in the presence of SDS was performed as previously described (18), except that the polyacrylamide concentration was 12.5% with an acrylamide to bisacrylamide ratio of 58.5 to 1.0. Gels were stained with Coomassie Blue. Kodak X-Omat AR film or phosphoimaging plates were used for autoradiography. A Sci Scan 5000 densitometer (United States Biochemical Corp.) and phosphoimager (Molecular Dynamics) were used to quantitate protein (as Coomassie Blue bands) and radioactivity. Both of these instruments were also used to process the data.

Reductive Methylation—In order to generate *in vitro* radiolabeled proteins, lysine residues were reductively methylated with [^{14}C]formaldehyde (Du Pont-New England Nuclear, specific activity = 40–60 mCi/mmol) and sodium cyanoborohydride as previously described (19). The final labeling solution contained 10 mM $NaCNBH_3$, 1 mM [^{14}C]formaldehyde, and 1 mg/ml protein. The reaction mixture was incubated at room temperature, and reactions were terminated by the addition of NH_4Cl (100 mM final concentration). Incubation times were determined prior to labeling using pilot assays in order to attain 3–5% protein modification. The labeled protein was then dialyzed exhaustively against 20 mM Hepes-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl, and 10% glycerol in order to remove unincorporated label.

Sucrose Gradient Centrifugation—Combinations of ^{14}C-labeled and unlabeled initiation factors (eIF-4A, eIF-4F, and eIF-3) were added to 100-μl reactions containing 20 mM Hepes-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl, and 1 mM $Mg(CH_3CO_2)_2$. Reactions were incubated at 4 °C for 90 min and subjected to sucrose gradient centrifugation using a Beckman SW 60 rotor. Linear sucrose gradients (5–18%) containing 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 1 mM $Mg(CH_3CO_2)_2$ were centrifuged for 18.5 h at 32,000 rpm at 4 °C. Gradients were fractionated into 20 samples (200 μl each), 20-μl aliquots of each sample were counted via liquid scintillation spectrometry, and graphs of radioactivity *versus* sucrose gradient fraction number were then plotted. Alternatively, gradients were fractionated into six samples (~600 μl each) and precipitated with 100% trichloroacetic acid (final concentration 10%). Soybean trypsin inhibitor (6 μg) was also added to act as a carrier protein. The solution was left on ice for 60 min and subjected to centrifugation in a microcentrifuge for 15 min at 15,000 rpm to pellet the precipitated protein. The supernatant was carefully removed, and the pellet was washed in 1 ml of 100% acetone and centrifuged for 15 min at 15,000 rpm. The acetone was removed, the washing procedure repeated, and the pellets left to dry overnight. The pellets were then solvated in 20 μl of sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% bromphenol blue), heated for 10 min at 90 °C, and analyzed by SDS-PAGE followed by autoradiography. Graphs of radioactivity *versus* sucrose gradient fraction number were then plotted.

m^{7}GTP-Sepharose 4B Assay—Factor combinations of ^{14}C-labeled and unlabeled initiation factors (eIF-4A, eIF-4B, eIF-4F, and eIF-3) were added to 500-μl reactions containing 20 mM Hepes-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, and 1 mM $Mg(CH_3CO_2)_2$, or 500 μl reactions containing rabbit reticulocyte lysate and 1 mM $Mg(CH_3CO_2)_2$. Reaction mixtures were combined with 100 μl of 7-methyl GTP-Sepharose 4B (Pharmacia) in Eppendorf tubes and rotated for 90 min at 4 °C. The m^{7}GTP-Sepharose 4B column matrix was then rinsed 9 times with 1 ml of 20 mM Hepes-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM KCl, 10% glycerol, and 1 mM $Mg(CH_3CO_2)_2$ in order to remove any unbound protein. The rinsing procedure involved gently mixing the rinsing buffer with the column matrix by inverting the Eppendorf tubes several times followed by a 5-s centrifugation in a microcentrifuge in order to pellet the 7-methyl GTP-Sepharose 4B column matrix. Following the last rinse, the supernatant was removed with a pipette and protein was eluted from the columns with the addition of 1 ml of 200 μM m^{7}GTP (Pharmacia) and 1 mM $Mg(CH_3CO_2)_2$ followed by a 45-min rotation. The column matrix was then pelleted by centrifugation in a microcentrifuge, and the supernatant containing the eluted protein was collected. Two hundred microliters of the supernatant was analyzed using liquid scintillation spectrometry. Protein in the remaining 800 μl was precipitated with trichloroacetic acid as previously described in the sucrose gradient centrifugation methods. After each experiment, the m^{7}GTP column was washed with Hepes-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 1000 mM KCl, 10% glycerol, and 1 mM $Mg(CH_3CO_2)_2$.

RESULTS

Additional characterization of the p46 subunit of eIF-4F and eIF-4A will increase our understanding of how these proteins interact with each other and with other initiation factors. In order to investigate whether an exchange event between eIF-4A and the p46 subunit of eIF-4F occurs, sucrose gradient centrifugation was employed. By incubating labeled eIF-4A with unlabeled eIF-4F (bound to eIF-3) and subjecting these proteins to sucrose gradient centrifugation, an exchange reaction between eIF-4A and the p46 subunit was shown to occur. A polyacrylamide gel of the purified initiation factors used in the following experiments is shown in Fig. 1*A*. Purified initiation factors (eIF-4A, eIF-4F, and eIF-3) were labeled with [^{14}C]formaldehyde by reductive methylation and subjected to sucrose gradient centrifugation. eIF-4A was found in fractions 15–18, eIF-4F in fractions 13–17, and eIF-3 in fractions 5–8 (Fig. 1*B*). As is evident, eIF-4A and eIF-4F are not well resolved from one another. It was therefore necessary to resolve eIF-4A from eIF-4F so that an exchange event between labeled eIF-4A and unlabeled eIF-4F could be detected. Efforts to improve separation of these proteins by increasing the speed or time of centrifugation resulted in destabilization of the eIF-4F complex. Therefore, in order to further resolve eIF-4A from eIF-4F, eIF-4F was incubated with the high molecular weight protein eIF-3. Previous studies have shown that eIF-3 can form a rather stable complex with eIF-4F (20). In addition, the stability of the eIF-4F·eIF-3 complex is reflected in the high salt concentration (500 mM KCl) required for their dissolution during purification (14). Radiolabeled eIF-4F is normally detected in fraction numbers 13–17, but incubation with eIF-3 now results in the shift of approximately one third of [^{14}C]eIF-4F to fractions 5–8 in conjunction with eIF-3, thereby demonstrating that [^{14}C]eIF-4F associates and comigrates with eIF-3 (Fig. 1*C*).

With the separation of eIF-4A and eIF-4F achieved, experiments designed to investigate the possible exchange of eIF-4A with the p46 subunit of eIF-4F were performed. As seen in Fig. 2*A*, [^{14}C]eIF-4A was incubated with eIF-3 (*lanes 1–6*) or with the eIF-4F·eIF-3 complex (*lanes 7–12*). Analysis by SDS-PAGE shows that the majority of the eIF-4A migrates at the top of the gradient (Fig. 2*A*, *lanes 5*, *6*, and *10–12*) while the eIF-3 migrates at the bottom of the gradient (Fig. 2*A*, *lanes 1*, *2*, *7*, and *8*). The addition of [^{14}C]eIF-4A to eIF-4F and eIF-3 results in the association of [^{14}C]eIF-4A with eIF-4F as seen in the autoradiogram (Fig. 2*B*, *lanes 7–9*). The position of [^{14}C]eIF-4A in the gradient when incubated with eIF-3 is not altered, demonstrating that no interaction between eIF-3 and [^{14}C]eIF-4A has occurred (Fig. 2*B*, *lanes 1–3*). These data suggest that [^{14}C]eIF-4A has become incorporated into the eIF-4F complex, while eIF-4F is associated with eIF-3.

However, the converse experiment needed to be performed in order to conclude that the [^{14}C]eIF-4A is not simply binding a form of eIF-4F that was deficient in eIF-4A and thereby reconstituting the eIF-4F complex. Authentic incorporation of eIF-4A must be accompanied by the loss of the p46 subunit of eIF-4F in order to demonstrate that these two proteins are exchanging with one another. Therefore, [^{14}C]eIF-4F was incubated with eIF-3 (Fig. 3, *lanes 1–6*) or with eIF-4A and eIF-3 (Fig. 3, *lanes 7–12*). As previously shown, analysis by

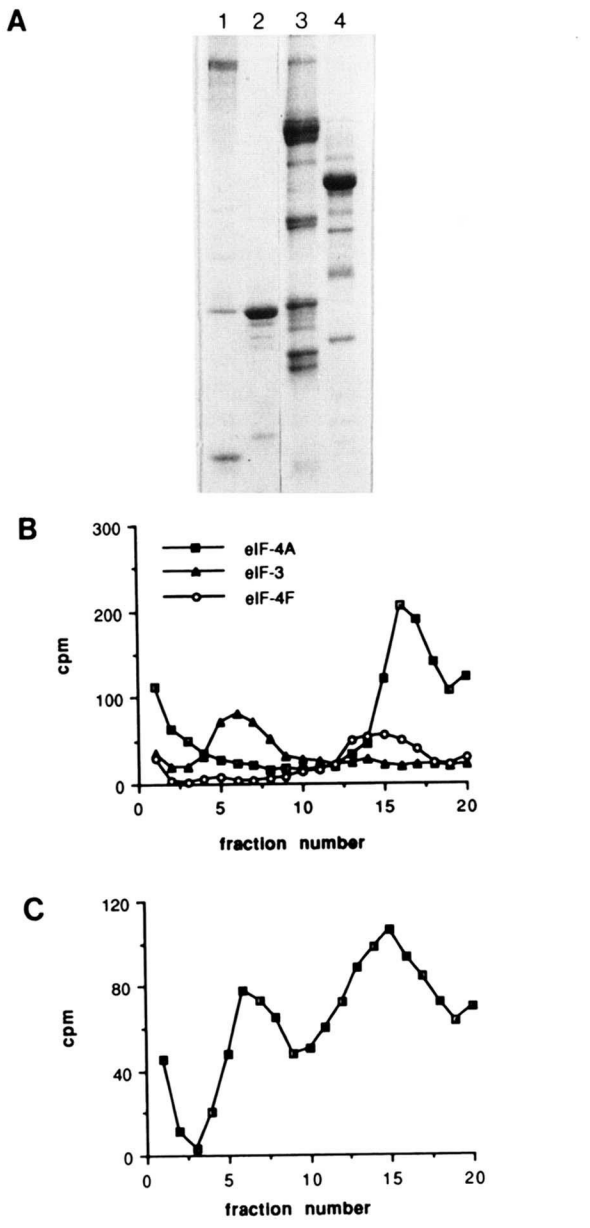

FIG. 1. **Polyacrylamide gel analysis of purified initiation factors and sucrose gradient profile of ^{14}C-labeled initiation factors.** *A*, Proteins were fractionated on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate. *Lane 1*, 2.1 μg of eIF-4F; *lane 2*, 1.2 μg of eIF-4A; *lane 3*, 5 μg of eIF-3; *lane 4*, 1.5 μg of eIF-4B. *B*, approximately 4 μg (5000 cpm/μg) of [^{14}C]eIF-4A, [^{14}C]eIF-4F, and [^{14}C]eIF-3 were subjected to sucrose gradient centrifugation. Gradients were fractionated into 20 samples (200 μl each), and 20-μl aliquots of each sample were analyzed via liquid scintillation spectometry. Represented in this figure is a graph of radioactivity *versus* sucrose gradient fraction number. *C*, [^{14}C]eIF-4F (4 μg) and eIF-3 (40 μg) were incubated for 90 min at 4 °C and subjected to sucrose gradient centrifugation. Gradients were fractionated into 20 samples (200 μl each), and 20-μl aliquots of each sample were analyzed via liquid scintillation spectometry. The graph represents radioactivity *versus* sucrose gradient fraction number.

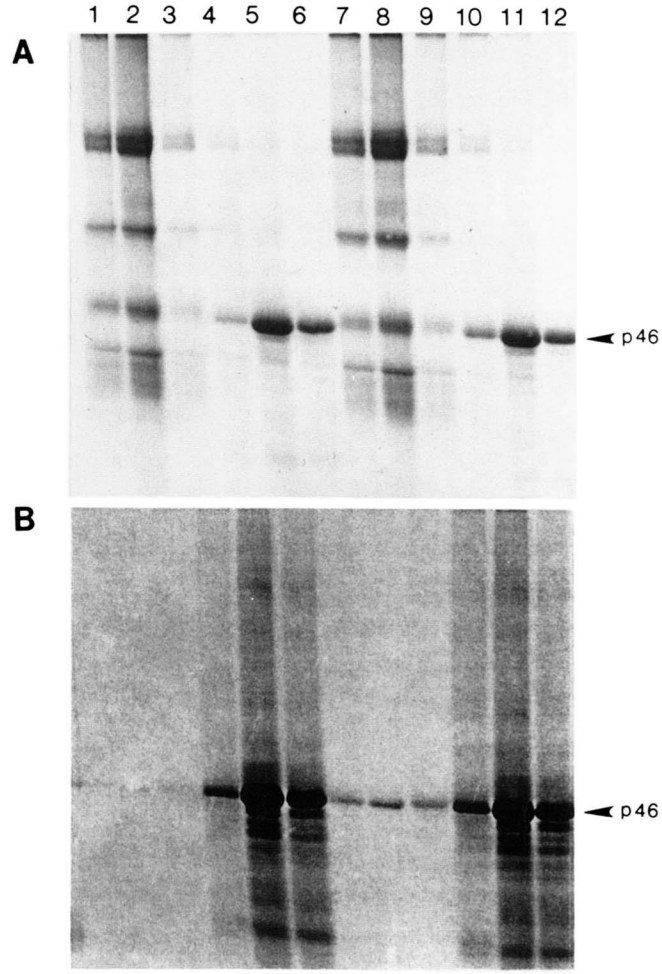

FIG. 2. **The exchange of [^{14}C]eIF-4A with the p46 subunit of eIF-4F as analyzed by sucrose gradient centrifugation.** [^{14}C]eIF-4A and eIF-3 (*lanes 1–6*) and [^{14}C]eIF-4A, eIF-3, and eIF-4F (*lanes 7–12*) were incubated for 90 min at 4 °C and subjected to sucrose gradient centrifugation. Gradients were fractionated into six samples (600 μl each), precipitated with a final concentration of 10% trichloroacetic acid, and analyzed by SDS-PAGE and autoradiography. *Panel A* shows the Coomassie Blue-stained polyacrylamide gel; *panel B* shows the autoradiogram after exposure on phosphoimaging plates. Amounts of initiation factor used are as follows: eIF-3 (40 μg), eIF-4F (5 μg), [^{14}C]eIF-4A (7 μg).

SDS PAGE in Fig. 3*A* indicates that the majority of the eIF-3 migrates at the bottom of the gradient (*lanes 2* and *8*), while eIF-4A migrates at the top of the gradient (*lane 11*). The autoradiogram (Fig. 3*B*) reveals that the [^{14}C]p46 and [^{14}C]p24 subunits of [^{14}C]eIF-4F bind to eIF-3 (*lanes 2–4*). There is also some [^{14}C]p24 seen in *lanes 5* and *6* that did not bind to eIF-3. However, the addition of eIF-4A to the [^{14}C]eIF-4F·eIF-3 complex (*lanes 7–12*) results in a substantial loss of the [^{14}C]p46 subunit of [^{14}C]eIF-4F and the majority of [^{14}C]p46 subunit now migrates at the top of the gradient (*lane 11*) as free [^{14}C]p46. This experiment demonstrates that the [^{14}C]p46 subunit is displaced, presumably due to the incorporation of unlabeled eIF-4A. The p220 subunit of eIF-4F can be seen most prominently in Fig. 3*B* (*lanes 8–10*). The recovery of the p220 subunit varied somewhat between experiments and did not always coincide with the presence of eIF-4A. The variability of the p220 subunit recovery and the presence of the p24 subunit in every fraction is probably caused by the destabilization of the eIF-4F complex due to sucrose gradient centrifugation. The ratio of [^{14}C]p46/[^{14}C]p24 from the autoradiogram in Fig. 3*B* is quantitated in Fig. 3*C* in order to more clearly illustrate the change in the [^{14}C]p46/[^{14}C]p24 ratio after the addition of eIF-4A. When [^{14}C]eIF-4F and eIF-3 are incubated, the ratio of [^{14}C]p46/[^{14}C]p24 is greatest in *lanes 1–3*. The addition of unlabeled eIF-4A, however, causes the [^{14}C]p46/[^{14}C]p24 ratio to shift so that the majority of the [^{14}C]p46 now migrates in *lanes 11* and *12* as free [^{14}C]p46.

The experiments presented in Figs. 2 and 3 indicate that under conditions in which eIF-4A is the only factor examined

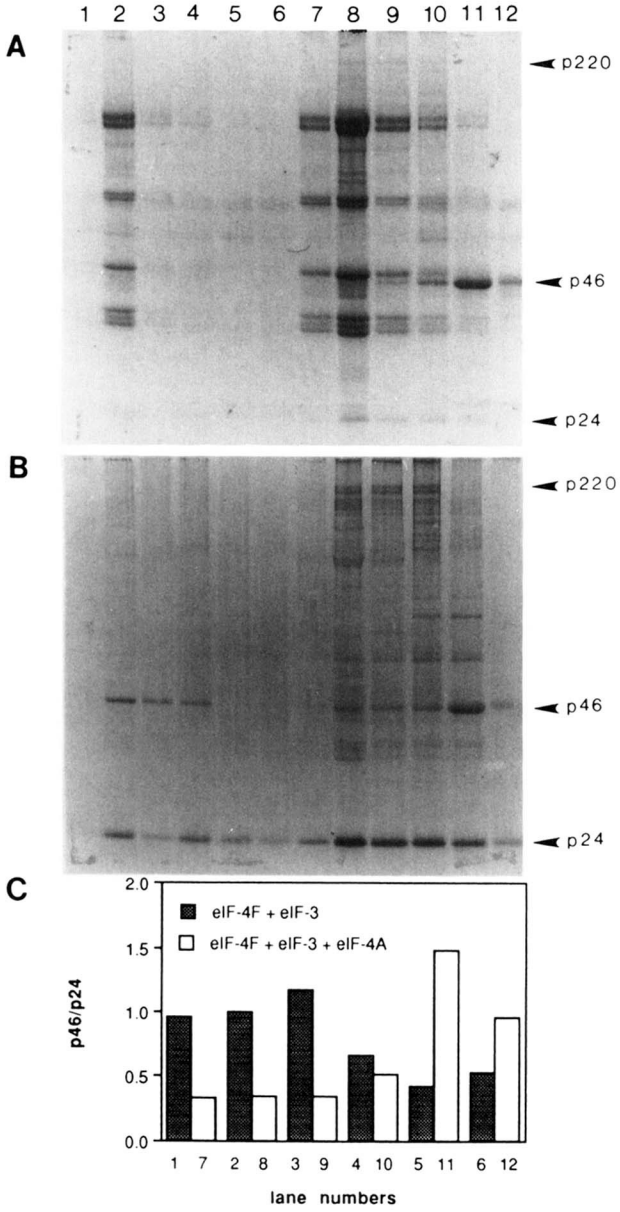

FIG. 3. **The addition of eIF-4A results in the displacement of the [^{14}C]p46 subunit of [^{14}C]eIF-4F as analyzed by sucrose gradient centrifugation.** [^{14}C]eIF-4F and eIF-3 (*lanes 1–6*) and [^{14}C]eIF-4F, eIF-3, and eIF-4A (*lanes 7–12*) were incubated for 90 min at 4 °C and subjected to sucrose gradient centrifugation. Gradients were fractionated into six samples (600 μl each), precipitated with a final concentration of 10% trichloroacetic acid, and analyzed by SDS-PAGE and autoradiography. *Panel A* shows the Coomassie Blue-stained polyacrylamide gel; *panel B* shows the autoradiogram; *panel C* shows a graph of the ratio of the [^{14}C]p46/[^{14}C]p24 subunits of [^{14}C]eIF-4F (obtained by densitometry from the autoradiogram) *versus* sucrose gradient fraction number. Amounts of initiation factor used are as follows: eIF-3 (40 μg), [^{14}C]eIF-4F (4 μg), eIF-4A (7 μg).

(in the presence of eIF-4F·eIF-3), an exchange between the p46 subunit of eIF-4F and eIF-4A is observed. In order to study the exchange reaction more rapidly and examine the effect that additional initiation factors may have on the exchange, a second technique using an m^{7}GTP-Sepharose 4B column matrix was developed. This assay provided a more rapid technique to isolate and examine the composition of the eIF-4F complex after incubations with reticulocyte lysate (which contains all of the protein synthesis components) or individual initiation factors.

First, rabbit reticulocyte cell lysate and [^{14}C]eIF-4A were incubated with m^{7}GTP-Sepharose 4B column matrix for increasing lengths of time in order to determine a sufficient incubation period needed for eIF-4A incorporation. The columns were washed with buffer, and bound eIF-4F was eluted with m^{7}GTP, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE and autoradiography. Analysis by SDS-PAGE shows an increase in eIF-4F binding to the m^{7}GTP-Sepharose 4B column as a function of increased incubation time (Fig. 4*A*) and the autoradiogram reveals that maximal [^{14}C]eIF-4A incorporation into the eIF-4F complex occurred within 90 min at 4 °C (Fig. 4*B*, *lane 4*). Preincubations of [^{14}C]eIF-4A and lysate at 37 °C for 15 and 45 min, respectively, followed by a 90-min incubation at 4 °C with m^{7}GTP-Sepharose 4B did not increase [^{14}C]eIF-4A incorporation (Fig. 4*B*, *lanes 5* and *6*). Therefore, sufficient eIF-4A incorporation could be achieved using a 90-min incubation with m^{7}GTP-Sepharose 4B at 4 °C.

With an optimal incubation time for eIF-4A incorporation established, the following m^{7}GTP-Sepharose 4B experiments were conducted. First, a series of experiments were performed in which eIF-4A and eIF-4F were incubated together in order to demonstrate that eIF-4A exchanges in and out of the eIF-4F complex. To control for any nonspecific binding of eIF-4A to the m^{7}GTP-Sepharose 4B matrix, [^{14}C]eIF-4A was incubated with the column. As seen in Fig. 5 (*lane 1*), [^{14}C]eIF-4A does not bind to the column matrix. Second, the incubation of [^{14}C]eIF-4A with eIF-4F results in the incorporation of [^{14}C]eIF-4A into the eIF-4F complex (*lane 2*). Third, the incubation of [^{14}C]eIF-4F with the column results in the recovery of all three subunits (*lane 3*). However, the ratio of the three subunits of eIF-4F is altered after eIF-4F is eluted from the column. The recovery of the p24 subunit is favored due to the specific binding of p24 subunit for the m^{7}G cap structure and the recovery of the p46 and p220 subunits is reduced. The poor recovery of the p46 and p220 subunits may

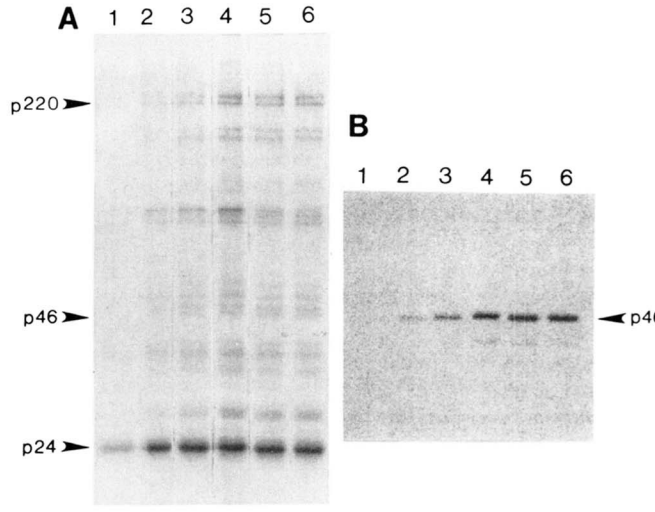

FIG. 4. **Time curve of [^{14}C]eIF-4A incorporation into eIF-4F (present in rabbit reticulocyte lysate).** An m^{7}GTP-Sepharose 4B assay was performed as described under "Materials and Methods." Proteins were precipitated with a final concentration of 10% trichloroacetic acid, fractionated on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate, and analyzed by autoradiography. *Panel A* shows the Coomassie Blue-stained polyacrylamide gel; *panel B* shows the autoradiogram. *Lanes 1–4* represent 5-, 20-, 45-, and 90-min incubations of [^{14}C]eIF-4A and rabbit reticulocyte lysate (500 μl) with m^{7}GTP-Sepharose 4B columns at 4 °C. In *lanes 5* and *6*, [^{14}C]eIF-4A and rabbit reticulocyte lysate (500 μl) were preincubated at 37 °C for 15 (*lane 5*) and 45 min (*lane 6*) and subsequently incubated with m^{7}GTP-Sepharose 4B columns for 90 min at 4 °C. Amount of [^{14}C]eIF-4A used was 7 μg (~5000 cpm/μg).

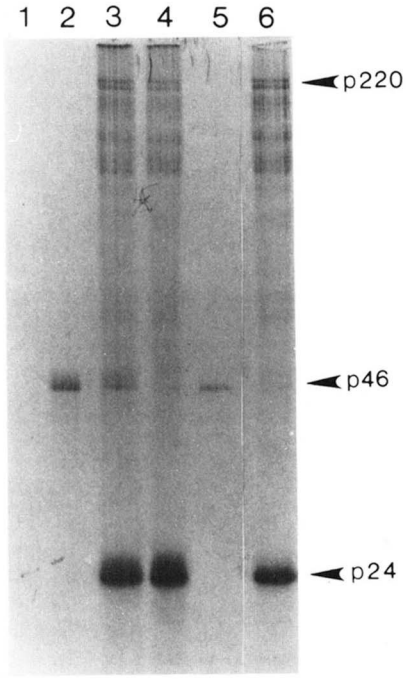

FIG. 5. **The exchange of eIF-4A with the p46 subunit of eIF-4F as analyzed by m^{7}GTP-Sepharose 4B assays.** An m^{7}GTP-Sepharose 4B assay was performed as described under "Materials and Methods." Proteins were precipitated with a final concentration of 10% trichloroacetic acid and fractionated on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate and analyzed by autoradiography. *Lane 1*, [^{14}C]eIF-4A; *lane 2*, [^{14}C]eIF-4A and eIF-4F; *lane 3*, [^{14}C]eIF-4F; *lane 4*, [^{14}C]eIF-4F and eIF-4A; *lane 5*, [^{14}C]eIF-4A and lysate (500 μl); *lane 6*, [^{14}C]eIF-4F and lysate (500 μl). The amounts of initiation factors used are as follows: eIF-4A (12 μg), [^{14}C]eIF-4A (7 μg; ~5000 cpm/μg), eIF-4F (5 μg), [^{14}C]eIF-4F (4 μg; ~7000 cpm/μg).

be due to the inability of the these subunits to remain in a stable complex throughout the experimental procedure. Finally, the incubation of [^{14}C]eIF-4F with eIF-4A results in the loss of the [^{14}C]p46 subunit of eIF-4F, most likely due to the incorporation of unlabeled eIF-4A (*lane 4*). Next, the exchange of eIF-4A with p46 was further investigated in the reticulocyte lysate system. The exchange between eIF-4A and the p46 subunit under these *in vitro* conditions provides convincing evidence that these subunits naturally exchange with one another in the cell. The results obtained in the reticulocyte lysate are the same as those seen when eIF-4F and eIF-4A are incubated alone. The incubation of [^{14}C]eIF-4A with lysate results in the incorporation of [^{14}C]eIF-4A into the eIF-4F complex present in the lysate, as previously described (*lane 5*). Furthermore, the incubation of [^{14}C]eIF-4F with lysate results in the loss of the [^{14}C]p46 subunit, most likely due to the exchange of unlabeled eIF-4A (present in the lysate) into the [^{14}C]eIF-4F complex (*lane 6*).

Having established conditions for the m^{7}GTP-Sepharose 4B assay, it was of interest to test the different forms of eIF-4A, eIF-4AI and eIF-4AII, for their ability to exchange with eIF-4F. Both eIF-4AI and eIF-4AII have also been cloned and sequenced in mouse, and eIF-4AI has been cloned and sequenced in rabbit. Although rabbit eIF-4AII has only been partially sequenced, the similarity between mouse and rabbit eIF-4AI (100% identical at the amino acid level and 90% identical at the nucleotide level within the protein coding region) suggests that the mouse and rabbit eIF-4AII sequences are also very similar. In the rabbit reticulocyte lysate system, it has been shown that the p46 subunit contains eIF-4AI and eIF-4AII (ratio of approximately 4:1, respectively), whereas there appears to be little or no eIF-4AII in purified rabbit eIF-4A (12). Thus, since eIF-4AII appears to be preferentially associated with eIF-4F, it was thought that eIF-4AII might exchange into the eIF-4F complex more efficiently than eIF-4AI. The cloned cDNAs for mouse eIF-4AI and eIF-4AII have also been expressed and the proteins isolated from *E. coli*. The mouse eIF-4AI and eIF-4AII proteins were tested for their ability to exchange with the p46 subunit of eIF-4F in the sucrose gradient experiments and the m^{7}GTP-Sepharose 4B assay. The purified proteins were tested for activity in a reconstituted hemoglobin synthesis system and a bidirectional RNA helicase assay, and both forms of mouse eIF-4A exhibited approximately the same specific activity as eIF-4A prepared from rabbit reticulocyte lysate (data not shown). In the following experiments, the two different forms of mouse eIF-4A were analyzed and compared with eIF-4A purified from rabbit reticulocyte lysate. When the three forms of eIF-4A were incubated with [^{14}C]eIF-4F as seen in Fig. 6*A*, each was capable of displacing the [^{14}C]p46 subunit of [^{14}C]eIF-4F (*lanes 2–4*). The incubation of [^{14}C]eIF-4F with the column matrix resulted in the recovery of all three subunits of eIF-4F as expected (Fig. 6*A*, *lane 1*). In addition, the three types of [^{14}C]eIF-4A (the two different forms of mouse eIF-4A and rabbit eIF-4A) exchanged with the p46 subunit to approximately the same extent when incubated with eIF-4F (Fig. 6*B*, *lanes 1–3*) or when incubated with lysate (Fig. 6*B*, *lanes 4–6*). The amount of incorporation of the three different types of

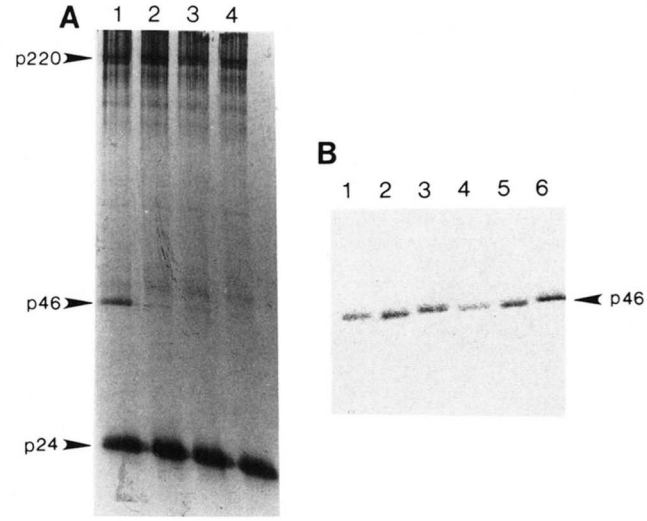

FIG. 6. **Mouse eIF-4AI and eIF-4AII purified from *E. coli* exchange with the p46 subunit of eIF-4F in the same manner as eIF-4A purified from rabbit reticulocyte lysate.** An m^{7}GTP-Sepharose 4B assay was performed as described under "Materials and Methods." Proteins were precipitated with a final concentration of 10% trichloroacetic acid, fractionated on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate, and analyzed by autoradiography. *A*, exchange of rabbit eIF-4A and mouse eIF-4AI and eIF-4AII with the [^{14}C]p46 subunit of [^{14}C]eIF-4F. *Lane 1*, [^{14}C]eIF-4F; *lane 2*, [^{14}C]eIF-4F and rabbit eIF-4A; *lane 3*, [^{14}C]eIF-4F and mouse eIF-4AI; *lane 4*, [^{14}C]eIF-4F and mouse eIF-4AII. *B*, exchange of rabbit [^{14}C]eIF-4A and mouse [^{14}C]eIF-4AI and [^{14}C]eIF-4AII with the p46 subunit of eIF-4F. *Lane 1*, eIF-4F and rabbit [^{14}C]eIF-4A; *lane 2*, eIF-4F and mouse [^{14}C]eIF-4AI; *lane 3*, eIF-4F and mouse [^{14}C]eIF-4AII; *lane 4*, lysate (500 μl) and rabbit [^{14}C]eIF-4A; *lane 5*, lysate (500 μl) and mouse [^{14}C]eIF-4AI; *lane 6*, lysate (500 μl) and mouse [^{14}C]eIF-4AII. Amounts of initiation factors purified from rabbit reticulocyte lysate used are as follows: eIF-4A, 12 μg; [^{14}C]eIF-4A, 7 μg (~5000 cpm/μg); eIF-4F, 5 μg; [^{14}C]eIF-4F, 4 μg (~7000 cpm/μg). Amounts of mouse eIF-4AI and eIF-4AII expressed in *E. coli* used are as follows: eIF-4AI (12 μg), [^{14}C]eIF-4AI (7 μg; ~5000 cpm/μg), eIF-4AII (12 μg), [^{14}C]eIF-4AII (7 μg; ~5000 cpm/μg).

[^{14}C]eIF-4A into the eIF-4F complex varied between experiments (compare *lane 4* and *lane 6*). The error observed in these experiments may be attributed to the procedure used to rinse the individual m^{7}GTP-Sepharose 4B columns that is required to eliminate unbound protein. Since each column is individually rinsed (see "Materials and Methods" for details), it is likely that the amount of protein (eIF-4F) that remains bound to each individual column or the amount of eIF-4A that remains associated with the eIF-4F complex differs between experiments. This assay therefore serves to answer the basic question of whether the different forms of eIF-4A exchange with the p46 subunit of eIF-4F; in all cases, *E. coli*-expressed mouse eIF-4AI and eIF-4AII and rabbit eIF-4A were found to exchange with the p46 subunit of eIF-4F.

The ability of [^{14}C]eIF-4AI and [^{14}C]eIF-4AII to exchange with the p46 subunit of eIF-4F was also examined using sucrose gradient centrifugation as previously described. Both mouse [^{14}C]eIF-4AI and [^{14}C]eIF-4AII became incorporated into the eIF-4F complex (while associated with eIF-3) to the same extent as rabbit [^{14}C]eIF-4A (data not shown). In addition, the [^{14}C]p46 subunit of [^{14}C]eIF-4F was displaced in the presence of mouse eIF-4AI or eIF-4AII (data not shown). Thus, these results are consistent with those obtained from the m^{7}GTP-Sepharose 4B assays.

Finally, experiments were conducted in order to determine if the addition of other initiation factors could enhance the exchange of [^{14}C]eIF-4A with the p46 subunit of eIF-4F. The effect of the addition of eIF-3 on eIF-4A exchange was examined first. eIF-3 has been shown to be required for the proteolytic cleavage of the p220 subunit of eIF-4F in polio infected cells (21). Therefore, it has been postulated that eIF-3 binds to eIF-4F inducing a conformational change in eIF-4F, which then allows the polio protease, 2A^{pro}, to cleave the p220 subunit. In addition, the initiation factors, eIF-4F, eIF-3, and eIF-4B form a stable complex as seen from protein purification. A 500 mM KCl sucrose gradient is required to separate eIF-3 from eIF-4F and eIF-4*B*, again demonstrating the close association of eIF-3 with eIF-4F (14). It has also been shown in the previous sucrose gradient experiments presented in this paper that eIF-4F is capable of binding to eIF-3. Therefore, because of the interaction between eIF-3 and eIF-4F, it was of interest to examine the effect that eIF-3 may have on the exchange of the p46 subunit of eIF-4F with eIF-4A. The addition of eIF-3 to [^{14}C]eIF-4F resulted in an increase in [^{14}C]eIF-4F recovery from the m^{7}GTP-Sepharose 4B column. The binding of eIF-3 to eIF-4F probably resulted in the stabilization of the eIF-4F complex, thereby increasing the amount of eIF-4F recovered. However, the addition of eIF-3 to [^{14}C]eIF-4A and eIF-4F did not increase the amount of [^{14}C]eIF-4A that became incorporated into the eIF-4F complex.

A second factor, eIF-4B, was incubated with eIF-4F and eIF-4A in order to determine if its presence effected the exchange of the p46 subunit of eIF-4F with eIF-4A. The addition of eIF-4B in the presence of ATP has previously been shown to stimulate the ATPase and mRNA binding activities of eIF-4A and eIF-4F (5, 6). Furthermore, eIF-4B has been shown to be required for the recycling of the p24 subunit of eIF-4F (7). It is not known if eIF-4B also stimulates the recycling of the p46 and p220 subunits of eIF-4F. The addition of eIF-4B to [^{14}C]eIF-4A and eIF-4F or [^{14}C]eIF-4A, eIF-4F, and eIF-3 did not enhance the exchange of eIF-4A with the p46 subunit of eIF-4F (data not shown). The experiments described above were also performed in the presence of ATP, and, similarly, no increase in eIF-4A exchange was detected (data not shown). The fact that no detectable stimulation or inhibition of exchange was observed in the presence of eIF-3, eIF-4B, or ATP may reflect the limitations of the m^{7}GTP-Sepharose 4B assay.

DISCUSSION

Using two different techniques, rabbit reticulocyte eIF-4F, which has been previously shown to be composed of eIF-4AI and eIF-4AII in a 4:1 ratio, respectively (12), was found to exchange with free eIF-4A composed solely of eIF-4AI. The two techniques employed were sucrose gradient centrifugation and an m^{7}GTP-Sepharose 4B assay. Examination of the exchange between the p46 subunit of eIF-4F and eIF-4A via sucrose gradient centrifugation required the addition of eIF-3. This high molecular weight initiation factor, previously shown to bind to eIF-4F, was used to enhance the separation of eIF-4A from eIF-4F in the gradient. It was observed that eIF-3 bound to eIF-4F, resulting in the comigration of eIF-4F and eIF-3 at the bottom of the gradient, thereby separating eIF-4F from eIF-4A. With the separation of eIF-4A and eIF-4F achieved, the exchange between the p46 subunit of eIF-4F and eIF-4A was examined. First, [^{14}C]eIF-4A was found to exchange into the eIF-4F·eIF-3 complex, and second, the [^{14}C]p46 subunit of [^{14}C]eIF-4F was found to dissociate from the [^{14}C]eIF-4F·eIF-3 complex in the presence of eIF-4A.

The second method used to examine the exchange between the p46 subunit of eIF-4F and eIF-4A was an m^{7}GTP-Sepharose 4B assay. Initially, [^{14}C]eIF-4A and rabbit reticulocyte lysate were incubated with m^{7}GTP-Sepharose 4B in order to determine the appropriate length of time required for the exchange between eIF-4A and the p46 subunit of eIF-4F to occur. An increase in eIF-4F (present in lysate) binding to the m^{7}GTP-Sepharose 4B matrix was observed as the time of incubation was increased. Maximal [^{14}C]eIF-4A incorporation into the eIF-4F complex was shown to occur within 90 min at 4 °C. It is difficult to determine the actual rate of exchange using this method, since the increase in eIF-4F binding to the m^{7}GTP-Sepharose 4B matrix is also accompanied by an increase in eIF-4A exchange. Future experiments involving the use of fluorescently labeled eIF-4A may be useful in order to determine the actual rate of exchange between eIF-4A and the p46 subunit of eIF-4F. The rate at which fluorescently labeled eIF-4A would exchange into the eIF-4F complex could be more easily monitored.

With an optimal incubation time of 90 min established for the exchange of [^{14}C]eIF-4A with the p46 subunit of eIF-4F, additional experiments were performed. Incubation of [^{14}C]eIF-4A with lysate resulted in the incorporation of [^{14}C]eIF-4A into the eIF-4F complex present in the lysate. Additionally, incubation of [^{14}C]eIF-4F with lysate caused the dissociation of the [^{14}C]p46 subunit of [^{14}C]eIF-4F, presumably due to the incorporation of unlabeled eIF-4A present in the lysate. The same results were observed when only eIF-4A and eIF-4F were incubated together; [^{14}C]eIF-4A exchanged with the p46 subunit of eIF-4F and the [^{14}C]p46 subunit of [^{14}C]eIF-4F dissociated from the [^{14}C]eIF-4F complex in the presence of eIF-4A.

In addition, mouse eIF-4AI and eIF-4AII expressed in *E. coli* were found to exchange with the p46 subunit of eIF-4F to the same extent as eIF-4A purified from rabbit reticulocyte lysate. The incorporation of [^{14}C]eIF-4AI or [^{14}C]eIF-4AII into the eIF-4F complex and the loss of the [^{14}C]p46 subunit of [^{14}C]eIF-4F in the presence of unlabeled eIF-4AI or eIF-4AII was observed. Both [^{14}C]eIF-4AI or [^{14}C]eIF-4AII were also incubated with lysate and again, [^{14}C]eIF-4AI or [^{14}C]eIF-4AII became incorporated into the eIF-4F complex present in the lysate. It might have been expected that eIF-4AII

would exchange into the the eIF-4F complex to a greater extent, since eIF-4AII appears to be preferentially associated with eIF-4F (12); however, this could not be determined. Our inability to distinguish between the differences in the exchange of eIF-4AI and eIF-4AII in the m^{7}GTP-Sepharose 4B assay reflects the limitations of this technique. The rinsing procedure required to remove unbound protein from the m^{7}GTP-Sepharose 4B matrix may be responsible for the differences in the amount of [^{14}C]eIF-4A recovered in these experiments. Since each m^{7}GTP-Sepharose 4B column is individually rinsed nine times, the amount of eIF-4F that is bound to the column or the amount of [^{14}C]eIF-4A that is associated with the eIF-4F complex is likely to differ slightly between experiments. However, the m^{7}GTP-Sepharose 4B assay as well as the sucrose gradient experiments provides important information, which suggests that eIF-4AI and eIF-4AII exchange with the p46 subunit of eIF-4F. Perhaps a more sensitive technique would detect differences in the exchange between these two forms of eIF-4A. It should also be noted that in rabbit reticulocytes, the p46 subunit of eIF-4F is composed of eIF-4AI and eIF-4AII in a 4:1 ratio, respectively (12). However, the ratio of mouse eIF-4AI to eIF-4AII mRNA varies in different tissues (11), and therefore the composition of the p46 subunit of mouse eIF-4F from different tissues may also vary. Thus, the exchange of free eIF-4A with the p46 subunit of eIF-4F may be influenced by different ratios of eIF-4AI and eIF-4AII and/or the composition of the p46 subunit of eIF-4F itself. The fact that the addition of eIF-3, eIF-4B, or ATP did not stimulate the exchange of eIF-4A with the p46 subunit of eIF-4F in the m^{7}GTP-Sepharose 4B assays may also reflect the quantitative limitations of the assay.

The exchange between free eIF-4A and the p46 subunit of eIF-4F demonstrates that eIF-4F is not a highly stable complex. The instability of the eIF-4F complex is also shown by the change in the ratio of the subunits after eIF-4F is recovered from the m^{7}GTP-Sepharose 4B column. In these experiments, the recovery of the p24 subunit is approximately 20 times greater than the recovery of the p46 or p220 subunits of eIF-4F. However, in the sucrose gradient experiments, the recovery of the p24 and p46 subunits of eIF-4F was almost equivalent. Additionally, after purification of eIF-4F, the ratio of the p220, p46, and p24 subunits (as determined from SDS-PAGE), is approximately 1:1:4, respectively. These data may suggest that the stability of the eIF-4F complex may deteriorate after it binds the m^{7}G cap structure. Thus, the binding of eIF-4F to the m^{7}G cap structure may cause the destabilization of the eIF-4F complex, thereby allowing eIF-4A to diffuse down the mRNA and unwind mRNA secondary structure. The destabilization of the eIF-4F complex may also facilitate the exchange of eIF-4A with the p46 subunit of eIF-4F, since eIF-4F is not maintained as a stable complex. However, the exchange of the p46 subunit of eIF-4F with eIF-4A does occur in the absence of m^{7}GTP as seen in the sucrose gradient experiments; thus, the binding of eIF-4F to the mRNA cap structure may only serve to enhance the exchange. It should also be noted that the poor recovery of the p46 and p220 subunits of eIF-4F could be due to the washing conditions performed following incubation of eIF-4F with the m^{7}GTP-Sepharose 4B matrix.

Evidence which supports the exchange of eIF-4A with the p46 subunit of eIF-4F has been demonstrated in previous experiments. Utilization of a fractionated translation assay for cap binding activity demonstrated that eIF-4F or p46-deficient eIF-4F equally stimulate mRNA translation in the presence of eIF-4A (22). These data suggest that the p46-deficient eIF-4F complex may be reconstituted with free eIF-4A and subsequently translate mRNA as efficiently as eIF-4F. Furthermore, the technique of primer extension inhibition adapted to analyze eukaryotic ribosome-mRNA interaction provides evidence for an exchange between eIF-4A and the p46 subunit of eIF-4F. In these experiments, eIF-4A, eIF-4B, and eIF-4F were added to the reaction; however, various factors were pretreated with FSBA. Preincubation of eIF-4B or eIF-4F with FSBA had no effect on ribosome-mRNA complex formation, while preincubation of eIF-4A with FSBA resulted in significant loss of ribosome-mRNA complex formation (23). The observation that the preincubation of eIF-4F with FSBA did not result in the loss of factor-mediated stimulation could be explained by the exchange of free eIF-4A with the FSBA-treated p46 subunit of eIF-4F, thereby forming an active eIF-4F complex.

The p24 subunit of eIF-4F binds to the mRNA cap structure with high affinity and specificity, whereas the possible loss or separation of the p46 subunit of eIF-4F functions to unwind mRNA secondary structure. An analogous situation is observed in bacterial RNA polymerase and its associated protein, σ factor. σ factor is responsible for start site specificity (24, 25). RNA polymerase and σ factor bind to DNA as a complex and after several elongation events, σ is lost from the elongating RNA polymerase/DNA complex. A second comparison of eIF-4F to RNA polymerase is that exposure to

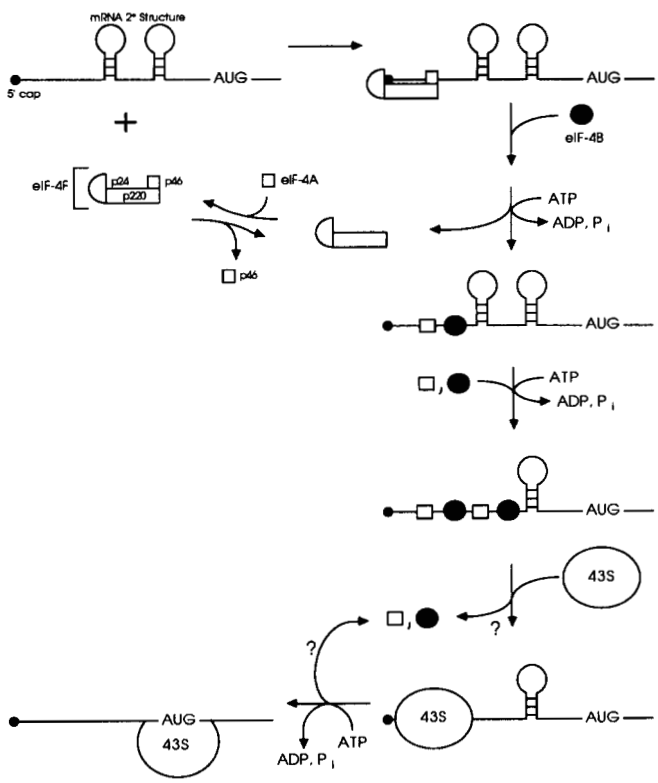

FIG. 7. **Proposed model depicting the interaction of eIF-4A, eIF-4B, and eIF-4F with mRNA and the 43 S preinitiation complex.** After the eIF-4F complex binds the mRNA cap, eIF-4B and the hydrolysis of ATP drives the recycling of the p24 subunit of eIF-4F (and possibly the p220 subunit of eIF-4F). The p46 subunit dissociates from the eIF-4F complex and remains bound to the mRNA. Free eIF-4A exchanges into the p46-deficient eIF-4F complex. Additional eIF-4A and eIF-4B molecules may be required for the unwinding of mRNA secondary structure. After sufficient mRNA secondary structure is unwound, the 43 S preinitiation complex binds the mRNA and scans in an ATP-dependent manner to locate the initiation codon.

phosphocellulose chromatography causes the release of the σ factor (26) or the p46 subunit (9) from the complex.

A proposed scheme describing how eIF-4A, eIF-4B, and the recycling of the p46 subunit of eIF-4F might interact with mRNA and the 43 S preinitiation complex is depicted in Fig. 7. Initially, eIF-4F binds the m^7G cap of the mRNA in an ATP-independent fashion. Subsequently, eIF-4B binds mRNA (if it is not already bound to eIF-4F) and the hydrolysis of ATP drives the recycling of the p24 subunit of eIF-4F and possibly the p220 subunit of eIF-4F. Once the p24 and p220 subunits of eIF-4F are released from the mRNA, free eIF-4A exchanges into eIF-4F, thereby reconstituting the complex. The newly reconstituted eIF-4F complex is now available to participate in a second round of initiation. The p46 subunit of eIF-4F that dissociated from the eIF-4F complex remains bound to the mRNA. The presence of eIF-4A/p46 and eIF-4B combined with the hydrolysis of ATP results in the unwinding of mRNA secondary structure. Additional eIF-4A and eIF-4B molecules may be required for the unwinding of downstream secondary structure. The lack of secondary structure in the 5′ region of the mRNA allows the 43 S preinitiation complex (which contains eIF-4C, eIF-3 bound to the 40 S ribosomal subunit, and the ternary complex composed of the initiator tRNA, GTP, and eIF-2) to bind to the mRNA. The 43 S preinitiation complex scans the mRNA in a 5′ to 3′ direction in an ATP-dependent manner in order to locate the initiating AUG codon. It is not known at which stage eIF-4A and/or eIF-4B are released from the message. These proteins may be released as a function of the 43 S preinitiation complex binding to the mRNA, in which case the ribosome may be responsible for further unwinding of mRNA secondary structure, or these proteins may be released after the 43 S complex has reached the AUG codon, in which case they may be responsible for unwinding all the secondary structure before the initiation codon.

Future experiments will help determine what functional advantage this exchange may provide. Of particular interest is the question of how the exchange between eIF-4A and p46 is affected by RNAs with different degrees of secondary structure. Perhaps RNAs with high degrees of secondary structure require increased exchange rates or increased incorporation of one particular form of eIF-4A in order to unwind the message. Additionally, in order to examine functional differences between eIF-4AI and eIF-4AII, experiments involving mRNA competition or the use of bicistronic mRNA constructs should be helpful. It is possible that one form of eIF-4A may play more of a role in internal initiation or may act more specifically on a particular type of messenger RNA.

Acknowledgments—We thank Don Anthony for many helpful discussions and Diane Hughes for the preparation of Fig. 7.

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