In Vitro Synthesis of Human Protein Synthesis Initiation Factor 4γ and Its Localization on 43 and 48 S Initiation Complexes*

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The rate of protein synthesis is controlled in a large number of physiological situations at the stage of 48 S initiation complex formation, a phase that involves the recruitment of mRNA to the 40 S ribosomal subunit. This process is mediated by the eukaryotic initiation factor-4 (eIF-4) group of translation initiation factors consisting of eIF-4E, eIF-4A, eIF-4B, and eIF-4γ. In order to develop a new tool to study this process, we have produced radiolabeled eIF-4γ by in vitro transcription and translation. Despite the fact that eIF-4γ is predicted from the cDNA sequence to be 205 kDa, no products were found to co-migrate with the slowest forms of authentic eIF-4γ (210-220 kDa), suggesting that these forms derive from extensive modification of the initial polypeptide. The in vitro product also formed a complex with eIF-4E, as judged by its ability to bind to m7GTP-Sepharose. Sucrose gradient sedimentation studies demonstrated that eIF-4γ was present on both 43 and 48 S initiation complexes but not 80 S complexes. This supports a model in which free eIF-4E binds to mRNA followed by binding of the eIF-4E mRNA complex to a 43 S initiation complex already containing eIF-4γ.

Eukaryotic protein synthesis is largely dependent on the presence of an m7GTP-containing cap at the 5' end of messenger RNA (Schatkin, 1976). Recognition of the cap and subsequent unwinding of mRNA secondary structure are catalyzed by the group 4 eukaryotic initiation factors (eIF-4γ) (Hershey, 1991; Thach, 1992; Merrick, 1992). The eIF-4 factors are an interacting group of polypeptides consisting of eIF-4E, a 25-kDa cap-binding protein; eIF-4A, a 48-kDa ATP-dependent RNA helicase; eIF-4B, a 69-kDa RNA-binding protein that stimulates the helicase activity of eIF-4A; and eIF-4γ (also known as p220), a 154-kDa polypeptide that forms complexes with the other factors but whose function is not yet delineated. Various complexes of the eIF-4 polypeptides have been isolated, the best studied of which is termed eIF-4 (Safer, 1989) or eIF-4F (Grifo et al., 1983) and consists of eIF-4A, eIF-4E, and eIF-4γ.

The eIF-4 factors catalyze the binding of mRNA to the 43 S initiation complex, resulting in the 48 S initiation complex, a step which is rate-limiting for protein synthesis under normal conditions (reviewed by Rhoads, 1991). The 43 S initiation complex consists of the 40 S ribosomal subunit, eIF-3, eIF-2, GTP, and tRNA", whereas the 48 S complex contains all components of the 43 S complex plus mRNA and eIF-4E (Hiremath et al., 1989; Joshi-Barve et al., 1990; Joshi et al., 1991). It has not yet been determined whether other eIF-4 factors are present on 43 or 48 S initiation complexes.

Regulation of the levels and activities of eIF-4 polypeptides plays a major role in the overall control of protein synthesis rate (reviewed by Rhoads, 1993a). Phosphorylation of eIF-4E at Ser-53 is critical for both its binding to the 43 S initiation complex in vitro and its stimulation of protein synthesis in vivo. Phosphorylation of eIF-4γ, although not as well studied, likewise stimulates protein synthesis in vitro and correlates with the stimulation of protein synthesis in vivo. Translation of capped mRNAs is also dependent on the intracellular levels of eIF-4 polypeptides. Elevation of eIF-4 levels by either expression from a vector or microinjection causes accelerated cell growth, loss of contact inhibition, formation of tumors, and preferential synthesis of proteins encoded by translationally repressed mRNAs, whereas lowering of eIF-4 levels by antisense RNA causes cells to slow or stop growth. Infection of mammalian cells by certain picornaviruses such as poliovirus, foot-and-mouth disease virus, and rhinovirus leads to the specific cleavage of eIF-4γ into a set of polypeptides ranging from 100 to 130 kDa. This coincides with the inhibition of host protein synthesis and concomitant onset of viral protein synthesis. eIF-4γ levels are also decreased in HeLa cells expressing antisense RNA against eIF-4E mRNA. In such cells, protein synthesis is profoundly impaired, but addition of the eIF-4 complex restores activity. The principal mRNAs translated in cells depleted of eIF-4E and eIF-4γ encode the heat shock proteins.

Although it is clear that eIF-4γ is essential for capped mRNA translation, neither its structure nor function are well understood. eIF-4γ migrates as if it were 220 kDa on SDS-PAGE (Etchison et al., 1982), but its cDNA sequence predicts a polypeptide of only 154 kDa (Yan et al., 1992). Furthermore, eIF-4γ migrates as a cluster of polypeptides with mobilities corresponding to 200-220 kDa (Etchison et al., 1982; Duncan et al., 1987; Lamphere and Pannier, 1990), raising the possibilities of multiple genes, alternative splicing, partial proteolysis, or post-translational modification. Finally, and fundamental to an understanding of the mechanism of eukaryotic protein synthesis, it is not known how or when during the initiation process eIF-4γ interacts with other initiation factors or subribosomal initiation complexes. Among the various models proposed for the recruitment of mRNA to polysomes (Abramson et al., 1988;
Rhoads, 1988; Sonenberg, 1990; Thach, 1992), there are differences as to whether mRNA initially binds to free eIF-4E or to the initiation complex. Whether or not eIF-4E is bound to the 40 S ribosomal subunit or is assembled stepwise on it.

In the present study, we have developed a new tool to study the structure and function of eIF-4y, which consists of a radioactive form of eIF-4y produced by in vitro transcription and translation. This has shed new light on the heterogeneity of eIF-4y, its interaction with eIF-4E, and its location in subribosomal complexes prior to and after mRNA binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—mGTP was purchased from Sigma. Pancreatic RNAase inhibitor (RNAGuard) and mGTP-Septapeptide were purchased from Pharmacia LKB Biotechnology Inc. The plucripts SK II(+) vector, ribonucleotides, T3 RNA polymerase, mGpppG, and DNase I were purchased from Stratagene (La Jolla, CA). Restriction enzymes were purchased from Promega (Madison, WI). [3S]Met (1000 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA). Edeine was purchased from Calbiochem (San Diego, CA), and calf liver tRNA was from Boehringer Mannheim. Reticulocyte lysate was prepared from New Zealand white rabbits by the method of Clemens (1985). Immobilon-P paper was purchased from Millipore (Bedford, MA).

**In Vitro Transcription**—The eDNA insert of AHFCl (nucleotides 323-5014; Yan et al., 1992) corresponds to the entire open reading frame of the initiation transcript, which could then be purified and used as a transcription template, pSK-HFC1 as described above and then immediately added to new translation mixtures (200 pl). The reaction mixtures contained the same components as described above for in vitro transcription except that (i) the lysate was not treated with nuclease; (ii) dithiothreitol, tRNA, and tritiated mRNA were omitted; (iii) 2.5 mM MgCl2 was included. The mixtures were incubated at 37 °C for 18 min prior to the determination of translation by the addition of 1 volume of ice-cold Buffer B (20 mM triethanolamine-HCl, pH 7.6, 100 mM KCl, 2 mM Mg(OAc)2). Where indicated, reactions were made either 400 μM in m7GTP and Mg(OAc)2 or 10 μM in edeine after the first 10 min of incubation. Reaction mixtures were allowed to stand on ice for 20 min and then loaded onto sucrose gradients which consisted of 10.5 ml of 20-40% (w/v) sucrose in Buffer C (as B but with 25 mM KCl) with an 800-pl overlay of 15% sucrose in Buffer B. Samples were centrifuged for 15 h at 29,000 x g and 4 °C in a Beckman Instruments SW41 Ti rotor. The gradients were then analyzed by upward displacement and continuous monitoring at 254 nm. Fractions of 400 μl were collected, and 100-μl aliquots were diluted with 300 μl of a solution containing 0.6 M NaOH, 2% H2O2, and 50 μg/ml bovine serum albumin and incubated at 37 °C for 10 min. Radiolabeled protein was then precipitated with the addition of 0.5 ml of ice-cold 50% (v/v) trichloroacetic acid solution containing 2.5 mM Met. The precipitates were collected on 2.5-cm diameter GF/C filters (Whatman, Hillsboro, OR), washed with 15 ml of 5% trichloroacetic acid, dried, and counted in 5 ml of Aquasol (Amer sham Corp.) scintillant. Using this protocol, [35S]-labeled proteins, translated using total reticulocyte lysate mRNA, do not bind to the ribosomal complexes (Joshi, 1992).

**RESULTS**

**In Vitro Transcription of eIF-4y**—In order to arrive at a correct mechanism for the action of the eIF-4 factors in catalyzing the rate-limiting binding of mRNA to the 40 S ribosomal subunit, it is necessary to know which factors are present in each of the successive complexes that are formed in the course of initiation. Previously we presented evidence that eIF-4E is present in the 48 S but not 43 S complex (Hiremath et al., 1989; Joshi-Barve et al., 1990; Joshi et al., 1991). This was demonstrated using radioactive eIF-4E produced by in vitro transcription of eIF-4E cDNA and translation of the RNA product. Earlier attempts to localize eIF-4E immunologically had been inconclusive (Hiremath et al., 1985). In preliminary experiments, we similarly attempted to localize eIF-4y on initiation complexes by immunological techniques (Cole et al., 1988). A monoclonal antibody against eIF-4y was used for detection of the protein in translation mixtures separated by ultracentrifugation. In that study, eIF-4y could not be detected on initiation complexes unless samples were fixed with formaldehyde prior to centrifugation, raising the possibility of functional associatation.

To develop a more sensitive and quantitative methodology, we took advantage of the recent cloning of eIF-4y cDNA (Yan et al., 1992) to produce an in vitro transcript, which could then be used for in vitro translation. The mRNA sequence of eIF-4y, however, contains four upstream open reading frames (uORFs). Because of the possibility that these uORFs could reduce the
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**FIG. 1. Diagram of the plasmid pSK-HFC1 and in vitro transcription of eIF-4γ.** The insert of AHFCl was excised by digestion with EcoRI and inserted into pBluescript II(-) at EcoRI sites. The HFC1 cDNA contains a complete coding region as indicated by the location of initiator and stop codons. The plasmid DNA was linearized by SalI digestion. A T3 promoter was used for in vitro transcription. The thin arrow inside the circle indicates the orientation of the eIF-4γ open reading frame. The thick arrow indicates the orientation of vector gene lacZ. Inset, linearized pSK-HFC1 was transcribed in a capping tran-scription system as described under "Experimental Procedures." In vitro transcription products were assayed by electrophoresis in an 8% SDS-polyacrylamide gel and analyzed the proteins using a 5% SDS-polyacrylamide gel and analyzed the proteins by a combination of immunoblotting and autoradiography (Fig. 3). Rather surprisingly, this assay revealed that the major translation product migrated approximately the same (205 kDa) as the fastest isoform of authentic eIF-4γ (206 kDa), as detected by both immunoblotting (+mRNA, lane I; note the doublet at 205--206 kDa) and autoradiography (+mRNA, lane A). The data in Fig. 3 also show that the in vitro products were reactive with anti-eIF-4γ antibodies. We do not understand why the synthetic eIF-4γ does not co-migrate with the more slowly migrating isoforms of endogenous eIF-4γ. However, preliminary experiments involving the addition of canine microsomal membranes for co-translational processing did not cause a shift to the slower forms, suggesting that eIF-4γ is not glycosylated (data not shown).

**FIG. 2. In vitro translation of eIF-4γ mRNA.** In vitro translation was performed as described under "Experimental Procedures." Aliquots (2 μl) were removed at the indicated times and analyzed by SDS-PAGE on an 8% polyacrylamide gel followed by autoradiography. -mRNA indicates a translation reaction incubated without the eIF-4γ transcript for 90 min. The mobilities of standard proteins are indicated on the right.

**FIG. 3. In vitro translated eIF-4γ bound to m7GTP-Sepharose.** The foregoing results indicate that in vitro produced eIF-4γ resembles at least one form of the in vivo protein in electrophoretic mobility but say nothing about whether the eIF-4γ is functionally active. Unfortunately, no biochemical activity has yet been determined for eIF-4γ, unlike the other eIF-4 polypeptides. However, eIF-4γ is isolated from both mammalian (Grifo et al., 1983; Edery et al., 1983) and plant (Lax et al., 1985; Browning et al., 1987) sources in a complex with eIF-4E. If the in vitro translated eIF-4γ is fully functional, it should similarly form a complex with eIF-4E. To test this, translation mixtures were subjected to m7GTP-Sepharose affinity chromatography, since eIF-4E is the only initiation factor to bind specifically to m7GTP (reviewed by Rhoads, 1991). (eIF-4A and eIF-4B can be cross-linked to periodate-oxidized mRNA caps but only in the presence of the eIF-4 complex, which contains eIF-4E.) Fig. 4 shows the results of m7GTP-Sepharose chromatography of the translation mixture. Although a large percentage of the 35S-labeled eIF-4γ appeared in the unbound fraction (UB), about 20--30% of the synthetic products remained bound and could be specifically eluted with m7GTP (B). Since data from Hela cell extracts suggest that the ratio of eIF-4γ to eIF-4E is approximately 2.5:1 (Duncan et al., 1987; the relative ratios in rabbit reticulocyte lysates have not been reported) and that the major-
ity of eIF-4E exists in a "free" form, the amount of eIF-4y retained by the matrix does not seem unreasonable.

**Localization of eIF-4y on Translation Initiation Complexes—**
Models proposed by various investigators for the formation of 48 S initiation complexes (see Introduction) can be divided into four categories. (i) The eIF-4 complex binds to free mRNA, unwinds mRNA secondary structure, and then dissociates from the mRNA as the 43 S initiation complex joins. (ii) Binding occurs as in (i), except that eIF-4 is transferred to the 43 S initiation complex along with mRNA. (iii) Free eIF-4E binds first to mRNA and then to the 43 S initiation complex which already contains eIF-4y. (iv) mRNA binds directly to the 43 S initiation complex, which already contains both eIF-4E and eIF-4y. These four models predict four different situations with respect to the presence of eIF-4E and eIF-4y on 43 and 48 S initiation complexes. (i) Neither complex contains either protein. (ii) The 43 S complex contains neither protein, but the 48 S complex contains both. (iii) The 43 S complex contains eIF-4y but not eIF-4E, whereas the 48 S complex contains both. (iv) Both complexes contain both proteins. Since it was now possible to synthesize both radiolabeled eIF-4E (Hiremath et al., 1989; Joshi-Barve et al., 1990; Joshi et al., 1991) and radiolabeled eIF-4y (as described above), the presence of these two initiation factors on subribosomal complexes could be tested to provide some insight into the true mechanism of 48 S complex formation.

We separated initiation complexes containing either radiolabeled eIF-4E or eIF-4y by ultracentrifugal sedimentation (Fig. 5). Under the conditions used here, 35S-labeled eIF-4E was found to have some affinity for 43 S initiation complexes (Fig. 4, panel A, fractions 15–19). When lysates were incubated in the presence of the cap analog m7GTP, an inhibitor of 48 S complex formation, this eIF-4E was not removed from the complexes (panel B), indicating that it did not represent low levels of 48 S initiation complexes. However, incubation of the lysate in the presence of euride, which causes an accumulation of 48 S initiation complexes (Safer et al., 1978), resulted in an increase in the amount of eIF-4E bound to ribosomal complexes (panel A versus panel C) and a shift of sedimentation to that representative of 48 S complexes (panel C, fractions 18–21). The second peak at 66 S (panel C, fractions 23–26) represents dimers of 48 S complexes, due to joining of a second ribosomal subunit to the mRNA (Safer et al., 1978). These results agree with those obtained previously (Hiremath et al., 1989; Joshi-Barve et al. 1990; Joshi et al., 1991) except that in the first two of these studies, the low level of eIF-4E in the 43 S region was not detected. Similar results were previously obtained when lysates were incubated with GMPPNP, which also leads to a build-up of the 48 S complex, except no peak at 66 S was observed (Hiremath et al., 1989; Joshi-Barve et al., 1990). Overall, these data confirm the idea that eIF-4E is a component of the 48 S initiation complex. The amount of eIF-4E associated with the 43 S complex is considerably less and possibly non-specific (see "Discussion").

The subribosomal localization of 35S-labeled eIF-4y was also determined (Fig. 5, panels D–F). eIF-4y was found in association with 43 S complexes in both control (panel D) and m7GTP-inhibited (panel E) lysates. When lysates were treated with edeine, eIF-4y shifted to 48 and 66 S complexes and had an identical distribution to that of eIF-4E (cf. panels C and F). Unlike eIF-4E, there was little or no increase in the amount of eIF-4y associated with monomeric (i.e. 43 and 48 S) initiation complexes when edeine was added (panels D and E versus panel F). We repeated the experiment using GMPPNP instead of edeine and measured the presence of 35S-labeled eIF-4y in gradient fractions. The results were the same as with edeine, except no peak at 66 S was observed (data not shown).
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FIG. 5. Subribosomal localization of eIF-4E and eIF-4γ. Translation reactions containing either 35S-labeled eIF-4E (panels A-C) or 35S-labeled eIF-4γ (panels D-F) were prepared as described under "Experimental Procedures." Reactions were incubated for 10 min at 30 °C and then no inhibitor (panels A and D), 400 μM m7GTP (panels B and E), or 10 μM edeine (panels C and F) was added. Incubation was continued for 8 min at 30 °C. Ribosomal complexes were resolved by sucrose gradient centrifugation and the presence of alkali-stable, acid-precipitable radiolabel in collected fractions was measured.
initiation (Kozak, 1987b). Even though the 5'-UTR was segregated from the endogenous cellular mRNA (Kozak, 1987b), and four uORFs, assuming that the 5'-UTR of eIF-4y mRNA was very unusual; it endogenous eIF-4y. The second reason for concern was the finding that one of the upstream AUGs was the true initiation codon. Another concern was that this unusual 5'-

m7GTP...

m7GTP, which, at 1000 CPM/m mole, contributes 1 μm to the Met concentration. The endogenous Met is estimated from isotope dilution experiments to be 3.5 μm, so that the final specific activity of Met is 345 cpmmol, assuming 70% counting efficiency. In a typical reaction, 100,000 cpmmol α-[35S]-labeled eIF-4E were synthesized. As the eIF-4E sequence contains 2 Met residues (Rychlik et al., 1997), this corresponds to 96.6 fmmol of eIF-4E. The translation system contains 4 nm endogenous eIF-4E (Hiremath et al., 1985), so the specific activity of eIF-4E in the primary translation reaction was 100,000/(96.6 fmmol) = 3450 cpmmol fmmol. For the secondary translation reaction (in which initiation complexes were analyzed), 450,000 cpmmol of eIF-4E, or 488 fmmol, were added. The 200-μl reaction contained 800 fmmol of endogenous eIF-4E (see above), to produce a final specific activity for eIF-4E of 450,000/(800 + 960) = 377 cpmmol fmmol.

The specific radioactivity of eIF-4y was calculated as for eIF-4E except that 20,000 cpmmol of eIF-4y were synthesized, eIF-4y contains 24 Met residues, the molar concentration of eIF-4y is 8-fold higher than that of eIF-4E (Duncan et al., 1988), and 191,000 cpmmol of eIF-4y were added to the secondary translation reaction (Fig. 5) to produce a final specific activity for eIF-4y of 90.2 cpmmol fmmol.

From the specific activity of [35S]Met and published estimations for the endogenous concentrations of eIF-4E and eIF-4y, it is possible to calculate the amounts of these two proteins in each of the initiation complexes (Table I). There was a 3-4-fold difference in the molar amounts of eIF-4E and eIF-4y on 43 S complexes, but the amounts of each protein on 48 S complexes were nearly the same, as were the amounts of each protein on 66 S complexes. These data most closely resemble the predictions of model (ii) above, in which the 43 S complex already contains eIF-4y, and the 48 S complex is formed when an (eIF-4E-mRNA) complex binds to the 43 S complex (Fig. 6).

**DISCUSSION**

In a previous study, we cloned the cDNA for eIF-4y (Yan et al., 1992). Evidence that the cDNA truly corresponded to eIF-4y was obtained by synthesizing peptides predicted by the sequence, raising antibodies against them, and demonstrating that they reacted with a polypeptide of 220 kDa which was degraded during poliovirus infection. Such an approach, however, did not provide evidence that the eIF-4y mRNA was fully and accurately represented in the cDNA. This was of particular concern for reasons. First, the cDNA predicted a protein of only 154 kDa, as opposed to the 220 kDa estimated for the in vitro protein from migration on SDS-PAGE. The results presented in the present study (Figs. 2 and 3) argue that the cDNA contains the entire coding region, since the in vitro product migrated at 205 kDa, nearly the same as the fastest isofrom of endogenous eIF-4y. The second reason for concern was the finding that the 5'-UTR of eIF-4y mRNA was very unusual; it contained 386 nucleotides, considerably more than in the average cellular mRNA (Kozak, 1987b), and four UORFs, assuming that the AUG at nucleotide 369 was the initiation codon. In addition, this AUG was not in optimal sequence context for initiation (Kozak, 1987b). Even though the 5'-UTR was sequenced five times, cloning errors conceivably have introduced erroneous termination codons in the 5'-UTR, obscuring the fact that one of the upstream AUGs was the true initiation codon. Another concern was that this unusual 5'-

**TABLE I**

| Treatment | Particle | eIF-4E | eIF-4y |
|-----------|----------|--------|--------|
|           |          | cpmmol | fmmol  | cpmmol | fmmol  |
| Control   | 43 S     | 7789   | 20.7   | 7010   | 77.7   |
| mGTP      | 43 S     | 8902   | 21.5   | 6129   | 67.2   |
| Edcine    | 48 S     | 22,928 | 60.0   | 6277   | 69.6   |
| 66 S      | 18,854   | 50.0   | 4686   | 54.0   |
| 48 S + 66 S | 41,482 | 110.0  | 11,148 | 123.6  |

Derived from Fig. 5.

The specific radioactivity of eIF-4E was calculated as follows. In Method 2 (see "Experimental Procedures"), translation reactions were 1 CPM/ml in [35S]Met, which, at 1000 CPM/mole, contributes 1 μm to the Met concentration. Of these, post-translational processing seems the most likely. The fact that in vitro synthesized eIF-4y migrated like the fastest isofrom of endogenous eIF-4y suggests that an initial polypeptide of 154 kDa is post-translationally modified in the reticulocyte system to form migrating at 205 kDa. However, this form is unable to be further modified to the slower migrating isoforms in this cell-free system. In other studies, we have shown that the same eIF-4y when expressed in HeLa cells produces a cluster of polypeptides identical in mobility to the endogenous eIF-4y. This suggests that the intact HeLa cell is capable of carrying out the full range of post-translational modifications necessary to convert eIF-4y to the slowest migrating forms, whereas the reticulocyte lysate system is capable of only some modifications.

What is the nature of the putative post-translational modifications? The rabbit reticulocyte lysate system possesses some post-translational modification activities, such as acetylation, isoprenylation, and phosphorylation, but not others, such as signal peptide cleavage or core glycosylation (Walter and Blobel, 1983). Of these, phosphorylation seems the most likely for the following reasons. It is known that eIF-4y is a phosphoprotein and can be phosphorylated in vitro by protein kinase A, protein kinase C, and protase-activated kinase 1 (McMullin et al., 1988; Tuazon et al., 1989; Morley et al., 1991). Furthermore, the eIF-4y amino acid sequence contains many consensus phosphorylation sites for these and other kinases (Yan et al., 1992). The phosphopeptide map of eIF-4y isolated from cultured cells metabolically labeled with [32P]Pi is quite complex, suggesting a multitude of phosphorylated sites (Tuazon et al., 1989). Finally, the presence of phosphate residues is known to cause retardation of protein mobility on SDS-PAGE (Chauchereau et al., 1991; Paris et al., 1991, Lichtenberg-Kraag and Mandelkow, 1990; Grasser et al., 1991). The model we propose is that the anomalously slow electrophoretic mobility of the various eIF-4y isoforms in vitro and in vivo results from the incorporation of a large number of phosphate residues, and that the more rapidly migrating bands are various underphosphorylated forms of the protein.

One of our aims in the molecular cloning of eIF-4y cDNA was the expression of the protein for structural and functional studies. Complex formation of eIF-4y with eIF-4E, as evidenced by retention of eIF-4y on a m7GTP affinity column (Fig. 4), indicates that in vitro translated eIF-4y can be correctly folded in the reticulocyte lysate system. This, then, represents the first in vitro assay for eIF-4y activity, namely binding to eIF-4E. As such, it should be useful for studies of domains in both eIF-4y and eIF-4E that are required for their interaction, as well as the potential role of phosphorylation. Furthermore, it should allow definition of the conditions under which an eIF-4y-eIF-4E
complex can form. For example, we have shown that association of eIF-4E with the 40 S ribosomal subunit requires both ATP and mRNA (Rhoads et al., 1993b). There may be similar requirements for an association of eIF-4E and eIF-4y.

Another new in vitro assay for eIF-4y activity, and one which gives insight into the mechanism of initiation, is the ability of eIF-4y to bind to initiation complexes (Fig. 5). This is additional evidence that in vitro eIF-4y is fully active. A number of models have been proposed for the formation of the 48 S initiation complex (see "Results"). The findings of the present study are useful for distinguishing among these models (Figs. 5 and 6). It is conceivable that eIF-4E and eIF-4y form a complex only on the 40 S ribosome. The complex referred to as eIF-4 (previously eIF-4F) has only been isolated by high salt treatment of ribosomal pellets (which include initiation complexes). The finding that eIF-4E and eIF-4y are differentially distributed among initiation complexes (Fig. 5 and Table I) provides more direct evidence for such a model.

It is not clear how one should interpret the finding that some eIF-4E is associated with the 40–43 S region of the gradient, albeit at only approximately one fourth the molar amount of the 48 S initiation complex (Table I). In previous studies, eIF-4E was found on 48 S but not 43 S initiation complexes (Hiremath et al., 1989; Joshi-Barve et al., 1990). In a later study (Joshi et al., 1991), gradient conditions were modified to stabilize initiation complexes by lowering the salt concentration, and this produced both an increase in yield of eIF-4E on 48 S complexes and a small amount of eIF-4E at 43 S. It is likely that this represents nonspecific binding of eIF-4E to the 40 S ribosome. As evidence of this, formaldehyde fixation and centrifugation in the presence of 100 mM KCl produced eIF-4E on 48 S but not 43 S complexes (Joshi, 1992). Alternatively, this may represent specific but low affinity binding of eIF-4E to eIF-4y or some other component of the 43 S initiation complex. In such a scenario, the affinity of eIF-4E for this component would increase when the eIF-4E becomes bound to mRNA.

In practical terms, the ability to synthesize eIF-4y in vitro should provide a valuable tool for furthering our knowledge of protein synthesis initiation. Radioactive eIF-4y can be used to study the interactions of this protein with other participants in the initiation process to which it is known to bind (eIF-3, eIF-4A, eIF-4B, and the 40 S ribosomal subunit), as well as explore interactions with other components (e.g., mRNA, eIF-2, etc.). Furthermore, the ability to make deletions and substitutions of amino acid residues should allow one to discover domains and binding sites on eIF-4y which may lead to an understanding of its biochemical function.

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FIG. 6. Model for 48 S initiation complex formation. Truncated circles represent the small and large ribosomal subunits. The wavy line is mRNA, in which the solid circle represents the cap. The L-shaped line is tRNA Met. Initiation factors are represented by circles: 2, eIF-2; 3, eIF-3; 4, eIF-4E; γ, eIF-4y. The placement of eIF-2 and eIF-3 on the 43 and 48 S initiation complexes is based on published studies (reviewed by Rhoads, 1991). The absence of eIF-4E from 43 and 80 S complexes and its presence on 48 S complexes is based on the present work, as well as on previous studies (Hiremath et al., 1989; Joshi-Barve et al., 1990; Joshi et al., 1991). The placement of eIF-4y on both 43 and 48 S initiation complexes and its absence from 80 S complexes is based on the present work. The juxtaposition of eIF-3 with eIF-4y and eIF-4y with eIF-4E in the model is based on published studies showing that these factors have affinities for each other. Other factors that are known to participate in 48 S initiation complex formation (eIF-4A and eIF-4B) are not depicted because their presence on initiation complexes has not yet been reported.
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