The Analysis of Intermediary Reactions Involved in Protein Synthesis, in a Cell-free Extract of Saccharomyces cerevisiae That Translates Natural Messenger Ribonucleic Acid*

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A postpolysomal extract of Saccharomyces cerevisiae, treated with micrococcal nuclease to deplete endogenous mRNA, synthesizes protein in response to added mRNA. A strategy has been developed for the analysis of a number of intermediary reactions involved in the initiation, elongation, and termination of protein synthesis. In the absence of exogenous mRNA, incubation with radioactive methionine leads to the formation of a methionine-containing complex in the cytosol protein region of sucrose gradients, with a sedimentation constant of about 5 S, and another methionine-containing complex in the 40 S ribosomal subunit region. The characteristics of the 5 S component are consistent with those of eIF-2-Met-tRNAf-GTP ternary complex, and the characteristics of the 40 S material are consistent with those of the ternary complex associated with 40 S ribosomal subunits. In the presence of edeine, mRNA binds to the 40 S-Met-tRNAf, intermediate, but 60 S subunits are prevented from joining the complex. When cycloheximide is present instead of edeine, 60 S subunits are jointed to the 40 S-Met-tRNAf-MRNA intermediate to form an 80 S initiation complex, but peptide bond formation and polypeptide chain elongation does not occur. In the presence of mRNA, without antibiotic inhibitors, protein synthesis occurs on 80 S monosomes and polysomes, as evidenced by the incorporation of radioactive methionine or leucine into proteins associated with these particles. Peptide chain elongation is also obtained when radioactive phenylalanine is incubated with the yeast cell-free system and poly(U), as evidenced by the formation of polyphenylalanine, which does not involve the peptide chain initiation sequence of reactions. The incorporation of amino acids into protein on ribonucleoprotein particles initially, and the subsequent release of radioactive protein into the media, is a measure of the polypeptide chain termination reaction in the complete in vitro mRNA-translating system.

Protein synthesis involves the participation of ribonucleoprotein particles, protein factors, aminoacyl-tRNA, and GTP, which translate the information in a polynucleotide template. The translation of natural mRNAs requires three distinct phases, each specific with respect to protein factors, particles, and aminoacyl-tRNA (1–3). Initiation of protein synthesis in eukaryotic cells is catalyzed by a relatively large number of specific initiation factors which bring about the formation of an 80 S initiation complex containing ribosomes, mRNA, and initiator Met-tRNAf, via a number of intermediary complexes (1–7). Polypeptide synthesis, with the initiator methionyl moiety at the N-terminal position, then proceeds through a cyclic series of reactions with specific elongation factors, GTP, and a pool of aminoacyl-tRNAs (8, 9); this elongation phase of protein synthesis translates the internal codons in mRNA, by polymerizing one amino acid at a time to the nascent polypeptide chain. The termination phase also requires a specific factor which recognizes the termination codon and leads to the release of the completed polypeptide (10, 11).

Analysis of protein biosynthetic pathways in yeast, particularly in the area of peptide chain initiation, has not been possible because cell-free systems that translate natural mRNA were not available. However, a cell-free system from Saccharomyces cerevisiae has now been developed in this laboratory that actively and accurately translates natural mRNA (12). It consists of a postpolysomal extract depleted of components involved in the initiation, elongation, and termination of protein synthesis in yeast.

EXPERIMENTAL PROCEDURES

The growth of S. cerevisiae (SKQ2N strain), preparation of spheroplasts with gluclase, lysis of cells by homogenization, preparation of the postpolysomal extract by differential centrifugation, and chromatography on Sephadex G-25 (S-100') has been described in detail (12). The removal of small amounts of residual endogenous mRNA from the S-100' extract was carried out by incubating with micrococcal nuclease and Ca²⁺ (13) in buffered salts containing dithiothreitol, ATP, GTP, creatine phosphate, creatine phosphokinase, and a mixture of 19 amino acids, excluding the radioactive amino acid ([45S]methionine, [3H]phenylalanine, or [3H]leucine) to be used in subsequent phases of the experiment, as described (12).

Incubations for protein synthesis and for initiation, elongation, or termination reactions were carried out after adding EGTA and a radioactive amino acid plus nonradioactive polynucleotide template, or radioactive template plus a full complement of nonradioactive

* The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)l-piperazineethanesulfonic acid; m-GMP, 7-methylguanosine 5′-monophosphate.

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amino acids, to mRNA-depleted yeast S-100' extract. The final concentrations of all the reaction components (12), in a total volume of 0.1 ml, were: 0.2 to 0.3 mg of mRNA-depleted S-100' extract, 20 mM Hepes buffer (pH 7.4), 140 to 150 mM monovalent (NH₄⁺, Na⁺ and KC⁺) cations, 30 mM Mg(OAc)₂, 3.0 mM dithiothreitol, 0.5 mM ATP, 0.1 mM GTP, 20 to 30 mM creatine phosphate, 20 µg of micrococcal phosphokinase, 40 µM each of 19 nonradioactive amino acids, 0.8 to 1.25 µg of micrococcal nuclease, 0.25 mM CaCl₂, 0.75 mM EGTA, and 10% glycerol. In addition, some incubations (designed to assay chain initiation reactions) received approximately 2 µg of nonradioactive yeast poly(A)⁺ RNA (14, 15) and 0.4 µM [³⁵S]methionine (274,000 cpm/pmol), and others, containing 20 nonradioactive amino acids, received 2.4 µg of ³H-labeled yeast poly(A)⁺ RNA (6.6 X 10⁶ cpm/A₂₆₀ unit). Incubations were for 25 min at 20°C.

When reaction mixtures containing radioactive methionine were analyzed by gradient centrifugation, the reactions were stopped by the addition of 10% formaldehyde to a final concentration of 0.2% (at 2°C), and then layered on 12 ml of linear 10 to 30% sucrose gradients containing 10 mM Tris-HCl buffer (pH 7.5), 70 mM NH₄Cl, and 2 mM Mg(OAc)₂. Centrifugations in an SW 41 (Spinco) rotor were carried out at 40,000 rpm for 3½ h, or at 20,000 rpm for 14 to 16 h, at 2°C. The gradients were then analyzed automatically at 254 nm with a recording spectrophotometer; 0.4-ml fractions were collected and filtered through Millipore membranes (16), and the amount of radioactive methionine retained on the nitrocellulose filter was determined with a scintillation counter. Three milliliters of a solution containing 20 mM Hepes buffer at pH 7.4, 100 mM NH₄Cl, 2 mM Mg(OAc)₂, and 6 mM mercaptoethanol, at 2°C were added to each gradient fraction, then filtered through Millipore membranes and washed 3 times with the same buffer solution before counting. In some experiments, aliquots of individual gradient fractions were analyzed by filtration through Millipore, and duplicate aliquots were analyzed for hot (90°C) 5% trichloroacetic acid-insoluble radioactivity (16).

When incubations containing radioactive mRNA were analyzed, the reaction mixtures were layered directly on the sucrose gradients, and the individual fractions obtained were analyzed by filtration on Millipore, and the individual fractions obtained were analyzed by filtration on Millipore membranes (16), and the amount of radioactive methionine retained on the nitrocellulose filter was determined with a scintillation counter. Three milliliters of a solution containing 20 mM Hepes buffer at pH 7.4, 100 mM NH₄Cl, 2 mM Mg(OAc)₂, and 6 mM mercaptoethanol, at 2°C were added to each gradient fraction, then filtered through Millipore membranes and washed 3 times with the same buffer solution before counting. In some experiments, aliquots of individual gradient fractions were analyzed by filtration through Millipore, and duplicate aliquots were analyzed for hot (90°C) 5% trichloroacetic acid-insoluble radioactivity (16).

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RESULTS

The formation of intermediary initiation complexes containing radioactive methionine and mRNA was examined by sucrose gradient analysis of incubations containing yeast postpolysomal S-100' extract and [³⁵S]methionine or [³H]labeled yeast poly(A)⁺ RNA, as described above. Fig. 1 shows the gradient distribution of radioactive methionine, retained on Millipore filters, in yeast S-100' extracts incubated without added mRNA. With S-100' extract that had not been treated with micrococcal nuclease (A), two major peaks of radioactivity were detected. One peak of radioactivity was obtained toward the top of the gradient in fractions numbered 2 to 4, representing approximate S values between 3 and 8, referred to here as 5 S; another major peak of radioactivity was associated with the 40 S ribosomal subunits, in Fractions 11 to 14. Small but significant amounts of radioactive methionine were also detected in the ribosome or monosome region (around Fraction 24) and toward the bottom of the gradient with the residual polysomal fraction. When the incubations were carried out with yeast S-100' extract treated with nuclease, to degrade residual mRNA (B), only the two major peaks at 5 S and 40 S were obtained, and little or no radioactive activity was associated with other components such as monosomes or polysomes. These findings suggested that small amounts of mRNA remained in the S-100' extract, which allowed formation of intermediates beyond the 40 S complex, but which were completely removed by treatment with micrococcal nuclease. All subsequent experiments were carried out with nuclease-treated yeast extract.

The 5 S material, containing radioactive methionine, could represent Met-tRNA bound to Met-tRNA synthetase or the ternary complex eIF-2 · Met-tRNA₆GTP, or both. Attempts to prepare extracts that was more stringent with respect to GTP by re-chromatography on Sephadex G-25, which would affect the formation of the ternary complex but not the tRNA charging reaction, were unsuccessful. Although re-chromatography reduced markedly the endogenous pool of residual ATP, it had little effect on the GTP requirement. When incubations were carried out without mRNA, creatine phosphate, and creatine phosphokinase, in the presence of 4.9 mM sodium pyrophosphate·Mg(OAc)₂ plus 4.9 mM AMP-Mg(OAc)₂, binding of radioactive methionine to components in the yeast extract, analyzed by the Millipore filtration technique, was reduced to 10% of the control values without P₃P, and AMP; this finding suggested that the aminoclaylation of tRNA₆Met (and all subsequent reactions involving Met-tRNA₆) was inhibited in the presence of inorganic pyrophosphate and adenylic acid. When P₃P, and AMP were added to the reaction mixture after 20 min, the incubation was continued for an additional 20 min, binding to yeast extract components was reduced only 10%; this finding suggested that the methionine-containing 5 S component contained eIF-2 · Met-tRNA₆GTP ternary complex. This suggestion was supported by preliminary experiments which indicated that initiation factors in the yeast system were present both in the cytosol fraction and associated with ribosomal particles.
incubated with radioactive methionine in the absence (A) and presence (B) of nonradioactive yeast poly(A)$^+$ mRNA. In contrast to the pattern obtained without mRNA, which shows only the 5 S (Fractions 1 to 4) and the 40 S (Fractions 11 to 14) methionine-containing complexes, additional components containing methionine were obtained in the 80 S monosomes (Fractions 20 to 22) and the polysome aggregates (Fractions 30 and 31) when mRNA was present. An unexpected finding was that some radioactive methionine was also associated with material sedimenting in the region of the 60 S ribosomal subunits, in Fractions 15 to 17.

Fig. 3 shows the results of an experiment in which the S-100$^+$ extract was incubated with nonradioactive methionine and the tritium-labeled yeast poly(A)$^+$ RNA fraction which by itself had a sedimentation constant of about 13 S. Millipore filtration of the gradient fractions from this incubation revealed that major amounts of labeled mRNA were associated with 40 S ribosomal subunits (Fractions 13 to 15), with 80 S monosomes, and with larger aggregates (Fractions 24 to 28); some radioactive mRNA was also recovered with the 60 S subunits, in Fractions 19 to 21.

The nucleotide analog of the 5'-terminus structure of mRNA (18-23), 7-methylguanosine 5'-monophosphate, completely inhibited the binding of radioactive mRNA to components in the yeast S-100$^+$ extract (Fig. 4). An essentially similar negative pattern was obtained when labeled yeast poly(A)$^+$ RNA was centrifuged alone (without extract) on sucrose gradients, and individual fractions were analyzed by filtration through Millipore membranes. The 5'-cap analog also inhibited completely the integration of radioactive methionine with monosomes and polysomes, but not with 40 S subunits (Fig. 5A). Panel 5A (for comparison) shows that in the presence of mRNA and absence of m$^7$GMP, radioactivity was recovered in the 5 S, 40 S, and 80 S regions, and to some lesser extent in the 60 S and the polysome areas; however, in the presence of m$^7$GMP (B), radioactivity was detected only in the 5 S and 40 S regions, and no radioactivity was detected in components with sedimentation characteristics greater than 40 S. When m$^7$GMP was added to incubations containing $[^{35}S]$methionine but no mRNA, the pattern of radioactivity obtained in the gradient was essentially the same as that obtained from incubations without mRNA and without m$^7$GMP, as shown in Fig. 1B.

If edeine was present in incubations of S-100$^+$ extract and the gradient-purified (13 S) fraction of $[^{3}H]$yeast poly(A)$^+$ RNA (Fig. 6), radioactivity was recovered in the 40 S subunit region (migrating slightly faster, in Fractions 14 and 15) and in Fractions 6 to 8; however, significant amounts of radioactivity were not recovered in the monosome or polysome regions. The effect of edeine on the reactions of methionine with components in the S-100$^+$ extract is presented in Fig. 7. For comparison in this figure, control experiments without mRNA (A), and with mRNA but without edeine (B), as described in detail above, are also shown. When edeine was included in incubations of S-100$^+$ extract and $[^{35}S]$methionine (C), radioactivity was recovered only in the 5 S (Fractions 1 to 4) and 40 S (Fractions 11 to 14) regions; in contrast to the experiments without edeine (panel B), radioactivity was not detected in the monosomes or polysomes. When edeine was added to incubations containing $[^{35}S]$methionine but no mRNA (not shown here), the results obtained were essentially the same as those from incubations without mRNA and without edeine, as shown in panel A.

The effect of cycloheximide on the reactions of mRNA and methionine with S-100$^+$ extract are described in Figs. 8 and 9. When radioactive mRNA was investigated (Fig. 8), only one major peak of radioactivity was obtained in the presence of cycloheximide, and it was associated with the 80 S monosomes in Fractions 23 to 25. When the S-100$^+$ extract was incubated with $[^{3}S]$methionine, nonradioactive mRNA, and cycloheximide (Fig. 9), radioactivity was obtained in the 5 S (Fractions 1 to 4), 40 S (Fractions 11 to 14), and 80 S (Fractions 22 to 25) regions; it should be noted that in the presence of cycloheximide, radioactivity was not found in the 60 S- or polysome-
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Fig. 5 (left). The effect of 7-methyl guanylic acid on the interaction of \(^{35}\)S-labeled methionine with components in mRNA-depleted yeast postpolysomal extract. A, yeast S-100' extract incubated with \(^{35}\)S)methionine and unlabeled yeast poly(A)+ RNA; B, yeast S-100' extract incubated with \(^{35}\)S)methionine, unlabeled yeast poly(A)+ RNA, and 3 mM m'-GMP. Incubations, in the presence of unlabeled yeast poly(A)+ RNA, and centrifugation and analytical procedures are as described in the text.

Fig. 6 (right). The effect of edeine on the centrifugation pattern of \(^{35}\)S-labeled yeast poly(A)+ RNA incubated with mRNA-depleted yeast postpolysomal extract. The incubations, containing 1.2 \(\mu\)M edeine, and centrifugation and analytical procedures are as described in the text.

Fig. 7. The effect of edeine on the interaction of \(^{35}\)S-labeled methionine with components in mRNA-depleted yeast postpolysomal extract, in the presence of mRNA. A, yeast S-100' extract incubated with \(^{35}\)S)methionine, without mRNA; B, yeast S-100' extract incubated with \(^{35}\)S)methionine and unlabeled yeast poly(A)+ RNA; C, yeast S-100' extract incubated with \(^{35}\)S)methionine, unlabeled yeast(A)+ RNA, and 1.2 \(\mu\)M edeine. Incubations and centrifugation and analytical procedures are as described in the text.

Fig. 8 (left). The effect of cycloheximide on the centrifugation pattern of \(^{35}\)S-labeled yeast poly(A)+ RNA incubated with yeast postpolysomal extract. The incubation, containing 1.25 mM cycloheximide, and centrifugation and analytical procedures are as described in the text.

Fig. 9 (right). The effect of cycloheximide on the interaction of \(^{35}\)S-labeled methionine with components in mRNA-depleted yeast postpolysomal extract. The incubation, in the presence of unlabeled yeast poly(A)+ RNA and 1.25 mM cycloheximide, and centrifugation and analytical procedures are as described in the text.

Associated fractions. When cycloheximide was added to incubations containing \(^{35}\)S)methionine but no mRNA, the pattern obtained was essentially the same as that obtained without cycloheximide and without mRNA, as shown in Fig. 1B.

In order to define the form in which \(^{35}\)S)methionine was associated with the various complexes obtained on the gradients, one of the incubations was carried out with radioactive methionine and nonradioactive yeast poly(A)+ RNA; one aliquot of each gradient fraction (Fig. 10) was filtered through Millipore membranes to obtain the total radioactivity retained on nitrocellulose and thus bound to protein or nucleoprotein (open circles), and another aliquot was treated with 5% trichloroacetic acid (at 90°C) to obtain the radioactive protein (solid circles). In this experiment with mRNA, as in others described above, radioactive methionine was found to be associated with the 5 S component or components (Fractions 1 to 4), 40 S ribosomal subunits (Fractions 11 to 13), 80 S monosomes (Fractions 20 to 24), and polysomes (Fractions 26 and 27); a small peak of radioactivity was again detected in the 60 S ribosomal subunit region, in Fractions 16 and 17. When the hot acid-insoluble precipitates from gradient fractions were analyzed, protein containing radioactive amino acid was found on monosomes, polysomes, and toward the top of the gradient in Fractions 1 to 4; of particular interest were the
polypeptide chains released from polysomes into the incubation media. In order to examine whether termination and release of polypeptide chains did indeed occur in this system, the reaction mixtures were diluted, incubations as described above with radioactive phenylalanine incubated with [3H]leucine and unlabeled yeast poly(A)' RNA. Incubation components as described in the text, after varying periods of time (up to 2 h) of incubation, the reaction mixtures were diluted with buffered salts solution, centrifuged to obtain the ribosomal pellets (○) and the supernatant fractions (○), and the hot trichloroacetic acid-insoluble protein prepared from each (16).

![Fig. 10](left). The binding and incorporation into protein of [3S]methionine incubated with mRNA-depleted yeast postpolysomal extract and unlabeled yeast poly(A)' RNA. Incubation components and conditions as described in the text. The reaction mixture was centrifuged without formaldehyde treatment; 0.2-ml aliquots of each gradient fraction were filtered through Millipore membranes (○), and 0.2-ml aliquots were treated with 5% trichloroacetic acid, at 90°C, then filtered on glass-fiber filters (●).

![Fig. 11](right). Time-dependent release of radioactive protein from ribonucleoprotein particles in yeast postpolysomal extract incubated with [3H]leucine and unlabeled yeast poly(A)' RNA. Incubation components as described in the text; after varying periods of time (up to 2 h) of incubation, the reaction mixtures were diluted with buffered salts solution, centrifuged to obtain the ribosomal pellets (○) and the supernatant fractions (○), and the hot trichloroacetic acid-insoluble protein prepared from each (16).

Table I shows that the addition of poly(U) to the yeast S-100' extract stimulated markedly (about 50-fold) the synthesis of protein more rapidly at the beginning of the incubation; after 20 or 30 min, as obtained in this and in several similar experiments, the amount of radioactive protein associated with the particles remained constant or decreased slightly. Significant amounts of radioactive protein in the supernatant fraction became apparent after 10 min of incubation, and the amount of particle-free protein continued to increase for up to about 40 min of incubation.

**Discussion**

The synthesis of protein in eukaryotic systems appears to involve a number of individual steps leading to the formation of discrete intermediates, catalyzed by specific protein factors. The initiation sequence of reactions can be summarized as follows: initiator Met-tRNA; reacts with GTP and a protein initiation factor to form a ternary complex; the ternary complex interacts with 40 S subunits; mRNA binds to 40 S subunits containing Met-tRNA; and 60 S subunits then join the 40 S-mRNA-Met-tRNA; complex to form an 80 S initiation complex in which Met-tRNA; is at the P site. This series of reactions, which may require one or more factors at each step, serves to decod the initiation codon in mRNA. Chain elongation components then react with the 80 S initiation complex to translate all of the internal codons in mRNA.
between the initiation and termination codons, in a cyclic series of reactions: one of the elongation factors (EF-1) and GTP bind the aminoacyl-tRNA with the appropriate anticodon to the A site, adjacent to the site occupied by the Met-tRNA; the 60 S-peptidyltransferase activity catalyzes the formation of a peptide bond between the carbonyl group of Met-tRNA and the amino group of the incoming aminoacyl-tRNA; and the other elongation factor (EF-2) plus GTP then translocates the peptidyl-tRNA from the A site to the P site. As this process continues, additional ribosomes are attached to mRNA molecules that contain ribosomes with nascent peptidyl-tRNA chains, to form polysomes. Chain termination occurs when the completed polypeptide chain, esterified to tRNA, is at the P site, allowing the A site to interact with release factor; this reaction results in the release of the polypeptide and the ribosome (or ribosomal subunits) from mRNA.

The yeast cell-free system developed in this laboratory has been shown to translate exogenous natural mRNA (12); polysomes are formed that synthesize protein, which is then released into the incubation media, and which has the electrophoretic properties (on sodium dodecyl sulfate gels) expected from the product of the mRNA used as template. The studies presented in this paper indicate that the reactions postulated for chain initiation, elongation, and termination are carried out in this system and can be assayed under the appropriate conditions.

In the absence of mRNA, two intermediates are formed that contain radioactive methionine. One of these, with a low sedimentation constant (about 5 S) appears to contain the ternary complex Met-tRNA-eIF-2-GTP. The other methionine-containing complex formed in the absence of mRNA, sedimenting in the 40 S region, is consistent with the intermediate containing the ternary complex attached to 40 S ribosomal subunits.

When mRNA is added, it binds to the 40 S subunits, presumably those in association with the ternary complex. The 60 S ribosomal subunits then join the 40 S complex containing mRNA and Met-tRNA, as evidenced by the appearance of the 80 S initiation complex, that is, 80 S particles with radioactive methionine and mRNA. The 5' cap analog m7-GMP inhibits the interaction between mRNA and 40 S subunits, and as a consequence, prevents the formation of the 80 S initiation complex. However, the nucleotide analog has no effect on early steps of the initiation sequence which are independent of mRNA, such as the activation of methionine, the formation of the ternary complex with binding factor, and the transfer to 40 S subunits.

The antibiotic edeine allows the formation of the 40 S-mRNA-Met-tRNA complex but prevents the formation of 80 S initiation complex, suggesting that edeine blocks the joining reaction between 60 S ribosomal subunits and the 40 S intermediate.

When the yeast extract is incubated as described above, in a complete system that supports protein synthesis including mRNA, ribosomal aggregates heavier than 80 S ribosomes (polysomes) containing radioactive mRNA and protein-bound amino acid are also obtained. The formation of protein-synthesizing ribosomes under these conditions has been described in detail (12). These results indicate that the initiation complex, formed as described above, then undergoes chain elongation resulting in the formation of nascent polypeptides and of polysomes. The antibiotic cycloheximide allows the formation of the 80 S complex containing mRNA and Met-tRNA, but prevents the utilization of the initiation complex; thus, the synthesis of complexes heavier than monosomes is not detected. Although cycloheximide does not interfere with the early reactions in initiation, leading to the formation of the 40 S-bound Met-tRNA, or with the formation of the 80 S initiation complex, radioactive mRNA on 40 S subunits was not detected with cycloheximide; all of the mRNA accumulates in the 80 S monosome region. Whether in this limited system, with cycloheximide, all of the 40 S complexes containing mRNA react immediately with 60 S subunits, and there is eventually a failure to generate additional 40 S-mRNA complexes, remains to be determined.

Studies described previously (12) and here reveal that this yeast system also carries out polypeptide chain elongation, resulting in the incorporation of amino acids into protein. The polyn(U)-dependent synthesis of polyphenylalanine is a measure of the activity of the elongation factors in this preparation, since translation of the synthetic polynucleotide template does not require the reactions involved in initiation. It also reflects the ability of endogenous ribosomes to catalyze the reactions associated with chain elongation, including the factor-independent peptidyltransferase reaction. A more definitive, specific, and quantitative assay for elongation factor activity used in this laboratory, but not presented above, consists of centrifuging the 30,000 × g supernatant (made 0.5 M with respect to monovalent cation, with KCl) at 100,000 × g for 3 h, and assaying various concentrations of the supernatant with radioactive Phet-tRNA, poly(U), GTP, and control ribosomes from yeast or rat liver (24, 25); this procedure measures the combined EF-1 and EF-2 activities. Similar incubations in the presence of excess amounts of purified, completely resolved EF-1 or EF-2, individually, measure quantitatively the activity of the complementary factor (25). Thus, the activity of EF-1 in the supernatant can be estimated from the concentration curve obtained with varying amounts of supernatant in the presence of excess amount of EF-2.

When incubations containing yeast extract, radioactive amino acids, and other components essential for mRNA translation are centrifuged to remove the ribosomal particles, a considerable amount of radioactive protein is obtained in the supernatant fraction. Analyses described previously indicated that the product of mRNA translation is consistent with the information encoded in the template (12). These observations suggest that when the polypeptide chain is completely synthesized, termination reactions occur releasing the finished protein into the incubation media.

As noted in Fig. 6, a component containing radioactive mRNA which sediments between the cytosol proteins and the 40 S ribosomal subunits is retained on Millipore filters when edeine is present in the incubations. It has been reported that mRNA also binds to eIF-4B, possibly the mRNA-binding factor (26) and to eIF-2, the initiator tRNA-binding factor (27, 28), and that these interactions are inhibited by m7-GMP (26, 29). The radioactive mRNA used in these studies is a gradient-purified fraction of the poly(A) RNA, which sediments between the cytosol proteins and the 40 S region, at about 13 S. Thus, the formation of a complex with the initiator tRNA-binding factor could account for its retention on Millipore filters in that region of the gradient. It should be noted that in the presence of m7-GMP, mRNA does not bind to either 40 S particles or to this unidentified complex, suggesting that both interactions exhibit some specificity for the 5'-terminus structure. It should also be noted, however, that m7-GMP does not prevent the formation of the 40 S intermediate containing the methionine-eIF-2 ternary complex, suggesting that the interaction of mRNA with these initiation factors is not an obligatory step in chain initiation.

An observation for which there is no obvious or direct explanation at present is that radioactive mRNA and radioactive amino acids (methionine and leucine) are found occa-
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intermediary complexes in protein synthesis can be isolated and described. The complexes include eIF-2-Met-tRNA, GTP ternary complex (and possibly Met-tRNA-Met-tRNA synthetase), 40 S-Met-tRNA, 40 S-Met-tRNA, mRNA, 80 S initiation complex, 80 S monosomes, and polysomes. The formation of these intermediates indicates that the following activities occur in these extracts: activation of methionine and esterification of tRNA (and possibly Met-tRNA-Met-tRNA synthetase), 40 S-Met-tRNA, GTP ternary complex (and possibly Met-tRNA-Met-tRNA synthetase), 40 S-Met-tRNA, mRNA, 80 S initiation complex, 80 S monosomes, and polysomes. The formation of these intermediates indicates that the following activities occur in these extracts: activation of methionine and esterification of tRNA, binding of Met-tRNA to the initiator tRNA-binding factor, integration of the ternary complex containing Met-tRNA, with 40 S ribosomal subunits, interaction of mRNA with 40 S subunits containing ternary complex, joining of 60 S ribosomal subunits to the 40 S intermediary complex, chain elongation and polysome formation, and finally termination of protein synthesis with release of completed proteins. This basic strategy and procedures for the analysis of some of the individual steps in mRNA translation should allow identification of sites of action of affectors of protein synthesis; current studies, for example, are designed to identify the genetic lesions in mutants of S. cerevisiae altered in protein synthesis.

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