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Purification and characterization of two initiation factors required for maximal activity of a highly fractionated globin mRNA translation system

(protein synthesis/rabbit reticulocyte)

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ABSTRACT **Two additional initiation factors (IF-M4 and IF-M5) have been purified and characterized both physically and biologically. IF-M4 is active as a single polypeptide chain with a molecular weight of 48,000. In contrast, IF-M5 is active as a complex with a molecular weight of about 500,000 and consists of seven major and several minor polypeptide components. Analysis of IF-M5 in two polyacrylamide gel electrophoresis systems indicated that one of the major polypeptide chains of IF-M5 was the 35,000 dalton subunit of IF-MP. This analysis also revealed that IF-M2A, IF-M3, and elongation factor 2 were present as minor components. Both IF-M4 and IF-M5 are required to achieve maximal activity in an assay system dependent on exogenous globin mRNA, but neither factor has been observed to stimulate model reactions that utilize artificial templates [poly(U) or AUG].**

Fractionation of the 0.5 M KCl wash of reticulocyte ribosomes and the postribosomal supernatant has yielded seven homogeneous protein factors required for polypeptide synthesis on artificial polyribonucleotide templates (1–6). With purified but nonhomogeneous preparations of these factors, an additional factor, initiation factor (IF)-M3, was found to be required for translation of globin mRNA (7). Utilization of homogeneous initiation and elongation factors to complement highly purified IF-M3 preparations, however, resulted in significant loss of ability to translate globin mRNA. This has allowed the purification of two additional factors, IF-M4 and IF-M5, which restore activity to the initial level obtained with the unfractionated 0.5 M KCl ribosomal wash.

IF-M4, active as a single 48,000 dalton polypeptide chain, is required for translation of globin mRNA. Based on its physical properties and chromatographic behavior during purification, IF-M4 appears to be identical to IF-E_4 described by Staehelin *et al.* (8) and to IF_{EMC} described by Wigle and Smith (9, 10), although it does not appear to be specific for viral mRNA.

In contrast, IF-M5, a high-molecular-weight complex, is shown by sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis to consist of seven major polypeptide chains. One major component of the IF-M5 complex has been identified as the 35,000 dalton subunit of IF-MP by both $NaDodSO_4$ and acid urea polyacrylamide gel electrophoresis. In addition to these subunits, eight minor polypeptides copurify with IF-M5 under conditions that would insure no contamination by other known initiation factors. Three of these minor species, however, have been identified as IF-M2A, IF-M3, and elongation factor (EF)-2, indicating a stable association with the IF-M5 complex. Based on a sedimentation value of 15 S for the complex and on the molecular weights of its polypeptide components, IF-M5 appears to be related to the IF-E_3 complex of Staehelin *et al.* (8), the TC complex of Freienstein and Blobel (11), and IF-M3 of Strycharz *et al.* (12), which have been recently reported.

Abbreviations: mRNPs, messenger ribonucleoprotein particles; $NaDodSO_4$, sodium dodecyl sulfate; IF, initiation factor; EF, elongation factor; IF-M2B, an unfractionated mixture of IF-$M2B_\alpha$ and IF-$M2B_\beta$.

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MATERIALS AND METHODS

Purification of Initiation and Elongation Factors. Highly purified or homogeneous initiation and elongation factors were prepared as described: IF-M1 (1); IF-M2A (2); IF-M2B($\alpha + \beta$) (4); IF-M3 (7); IF-MP (3); EF-1 (5); and EF-2 (6). The purity of the nonhomogeneous factors used in some assays is indicated by the step in the published purification procedure or by the fold purification. Except for the crude preparations used in Table 1, each preparation of an initiation factor was free of contamination by other known initiation or elongation factors.

IF-M4 and IF-M5 were purified by chromatography of 550 mg of the crude step 3 IF-M2A on a 5 × 100 cm Sepharose 6B column equilibrated with 20 mM Tris·HCl, pH 7.5–1 mM dithiothreitol–0.1 mM EDTA–200 mM KCl. IF-M4 (45 mg) eluted just ahead of IF-M2B. IF-M4 was resolved from IF-M2B by chromatography on a 5 × 50 cm Sephadex G-75 column equilibrated with 20 mM Tris·HCl, pH 7.5–1 mM dithiothreitol–0.1 mM EDTA–100 mM KCl. The IF-M4 eluting in the void volume (25 mg) was applied directly to a 1.6 × 10 cm phosphocellulose column (Whatman P-11) equilibrated with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes), pH 6.5–1 mM dithiothreitol–0.1 mM EDTA–100 mM KCl. IF-M4 activity (8 mg) eluted at 100 mM KCl and was subsequently applied to a 1.6 × 10 cm DEAE-cellulose (Whatman DE-52) column in the same buffer. IF-M4 (3.4 mg) eluted from the column at 210 mM KCl.

IF-M5 (114 mg), which eluted in the void volume of Sepharose 6B, was further purified by phosphocellulose chromatography on a 5 × 10 cm column equilibrated with 20 mM Tris·HCl, pH 7.9–1 mM dithiothreitol–0.1 mM EDTA–200 mM KCl. Elution was performed with a 200–1000 mM KCl gradient. IF-M5 activity (37 mg) was eluted at 400 mM KCl.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ polyacrylamide gel electrophoresis of IF-M4 and IF-M5, was performed as described using a modification (3) of the Weber and Osborn procedure (13). Electrophoresis of samples in acidic 6.5 M urea was performed as described by Panyim and Chalkley (14).

Poly(U)-Directed Polyphenylalanine Synthesis. The poly(U)-directed synthesis of polyphenylalanine at 4 mM $MgCl_2$ (2) was used to analyze IF-M2A and IF-M2B activity present in fractions from Sepharose 6B chromatography.

Table 1. Globin mRNA translation in a fractionated protein synthesis system

Initiation factor components	pmol of [^{14}C]valine incorporated	
	−mRNA	+mRNA
A. Unfractionated 0.5 M KCl ribosomal wash	5	42
B. Partially purified M1, M2A, M2B, M3	4	30
C. Highly purified M1, M3; homogeneous M2A, M2B$_\alpha$, M2B$_\beta$, MP	4	6
D. Highly purified M1, M3; homogeneous M2A, M2B$_\alpha$, M2B$_\beta$, MP; crude DEAE-M2	4	34
E. Highly purified M1, M3, M4, M5; homogeneous M2A, M2B$_\alpha$, M2B$_\beta$, MP	3	31

Exogenous globin mRNA (0.02 A_{260} unit) was translated by 0.4 A_{260} unit of salt-washed RNase-treated ribosomes using unfractionated reticulocyte tRNA (0.3 A_{260} unit), 2 μg of EF-1, 1 μg of EF-2, and 80 μg of crude reticulocyte aminoacyl-tRNA synthetase (15). In addition, the following initiation factor levels were used: A, 120 μg of DEAE-treated ribosomal wash fraction (15); B, 40 μg of step 3 IF-M1 (1), 20 μg of step 4 IF-M2A (2), 15 μg of step 4 IF-M2B (2), and 15 μg of step 3 IF-M3 (7); C, 15 μg of step 5 IF-M1 (1), 5 μg of step 5 IF-M3 (7), 0.2 μg of IF-M2A, 0.3 μg of IF-M2B$_\alpha$, 0.6 μg of IF-M2B$_\beta$, and 1.7 μg of IF-MP; D, in addition to the factors listed in (C), the reaction mixture contained 33 μg of crude DEAE-M2; E, in addition to the factors listed in (C), the reaction mixture contained 4 μg of IF-M4 and 15 μg of IF-M5.

Translation of Exogenous Globin mRNA. Globin mRNA was translated at 37° in 100-μl reaction mixtures which contained: 85 mM KCl; 3 mM $MgCl_2$; 50 μM unlabeled amino acids except for [^{14}C]valine (specific activity 256 Ci/mol); 1 mM ATP; 0.2 mM GTP; 6 mM phospho*enol*pyruvate; 0.2 international unit of pyruvate kinase; 1 mM dithiothreitol; and 20 mM Tris·HCl, pH 7.5 (15). Specific details are presented in the legends of Fig. 1 and Table 1. mRNA and mRNA-dependent ribosomes from rabbit reticulocyte polysomes were prepared as described by Krystosek *et al.* (16).

RESULTS

Identification of Two Initiation Factors Required for Translation of Globin mRNA. To assess the function of individual protein factors in the translation process, five initiation factors (M1, M2A, M2B$_\alpha$, M2B$_\beta$, and MP) and two elongation factors (EF-1 and EF-2) have been purified to homogeneity (1–6). While these factors were able to totally reconstitute the activity present in unfractionated 0.5 M KCl ribosomal wash in assays that are dependent on artificial templates (unpublished observations), translation of globin mRNA required an additional partially purified factor, IF-M3 (7). With more highly purified preparations of factors, however, severe impairment of globin mRNA translation was observed (Table 1, compare A and B with C). To determine whether other factors required for efficient translation of globin mRNA might have been lost during purification of IF-M3, we tested the three crude DEAE fractions of 0.5 M KCl ribosomal wash protein for ability to restore activity in this assay. These fractions are the material not adsorbed to DEAE-cellulose at 100 mM KCl (DEAE-M1), the protein subsequently eluted with 210 mM KCl (DEAE-M3), or the sample obtained with the final 400 mM KCl column wash

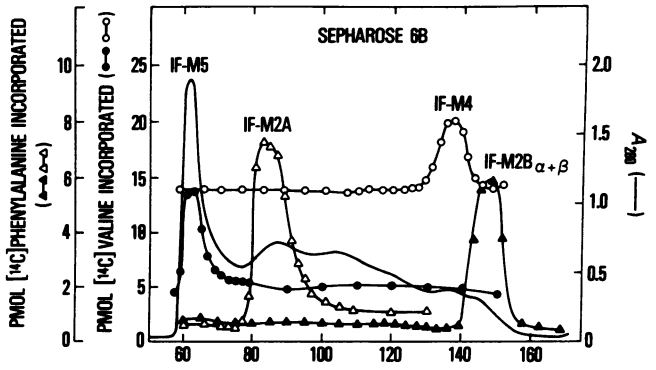

FIG. 1. Sepharose 6B chromatography of crude DEAE M2. Crude DEAE M2 (550 mg) was chromatographed on a Sepharose 6B column (5 × 100 cm) equilibrated with 200 mM KCl–20 mM Tris·HCl, pH 7.5–1 mM dithiothreitol–0.1 mM EDTA. IF-M4 and IF-M5 were assayed by translation of 0.02 A_{260} unit of exogenous globin mRNA using 0.4 A_{260} unit of salt-washed RNase-treated ribosomes (16). The translation system also contained 0.3 A_{260} unit of unfractionated rabbit reticulocyte tRNA and 80 μg of crude rabbit reticulocyte aminoacyl-tRNA synthetases per 100-μl reaction mixture (15). The amounts of initiation factors used were as follows. (*a*) Globin mRNA translation assay for IF-M5 using homogeneous or highly purified factors (●): 20 μg of IF-M1, 0.2 μg of IF-M2A, 0.3 μg of IF-M2B$_\alpha$, 0.6 μg of IF-M2B$_\beta$, 5 μg of IF-M3, 1.7 μg of IF-MP, 2 μg of EF-1, and 1 μg of EF-2. (*b*) Globin mRNA translation re-assay across the column for IF-M4 (○): in addition to the factor amounts listed in (*a*), 5-μl aliquots of the peak fraction of IF-M5 activity were used. IF-M2A (△) and IF-M2B (▲) were assayed by poly(U)-directed polyphenylalanine synthesis as described (2, 4).

(DEAE-M2). Although crude DEAE M3 was partially effective, crude DEAE M2 was found to completely restore the activity (Table 1D); this fraction was subsequently chromatographed on Sepharose 6B to identify the missing factors (Fig. 1). The column fractions were assayed for stimulation of globin synthesis using two types of initiation factor preparations: (*a*) preparations of IF-M1, IF-M2A, IF-M2B, and IF-M3 that are relatively crude but mutually exclusive, and (*b*) homogeneous IF-M2A, IF-M2B$_\alpha$, IF-M2B$_\beta$, and IF-MP, with highly purified but nonhomogeneous IF-M1 and IF-M3. With the partially purified preparations of initiation factors, the total activity was 70% of that obtained with DEAE-treated 0.5 M KCl ribosomal wash, and little stimulation was detected by any of the column fractions. In contrast, with homogeneous or highly purified preparations of initiation factors the activity supported by highly purified IF-M3 was only 5% of the activity obtained with the ribosomal wash; this was increased to 20% by factor(s) eluting in the void volume of Sepharose 6B (IF-M5). Re-assay of the column using the peak fraction of the void volume activity permitted detection of a second stimulatory factor (IF-M4) that eluted just before IF-M2B; the addition of these two factors restored activity in the globin synthesis assay to 50% of the level obtained with 0.5 M KCl ribosomal wash. As can be seen in Fig. 1, neither of these activities corresponds to IF-M2A or IF-M2B (α or β), which were assayed by poly(U)-directed polyphenylalanine synthesis.

Physical Properties of IF-M4 and IF-M5. IF-M4 purified to 95% homogeneity has an apparent molecular weight of 48,000, estimated by $NaDodSO_4$ polyacrylamide gel electrophoresis (Fig. 2). Native IF-M5 migrates as a single complex of polypeptides during alkaline disc polyacrylamide gel electrophoresis (data not shown) and by R_F analysis (3) has a molecular weight of approximately 700,000. Native IF-M5 also sediments as a symmetrical boundary with a sedimentation coefficient of about 15 S (data not shown). $NaDodSO_4$ poly-

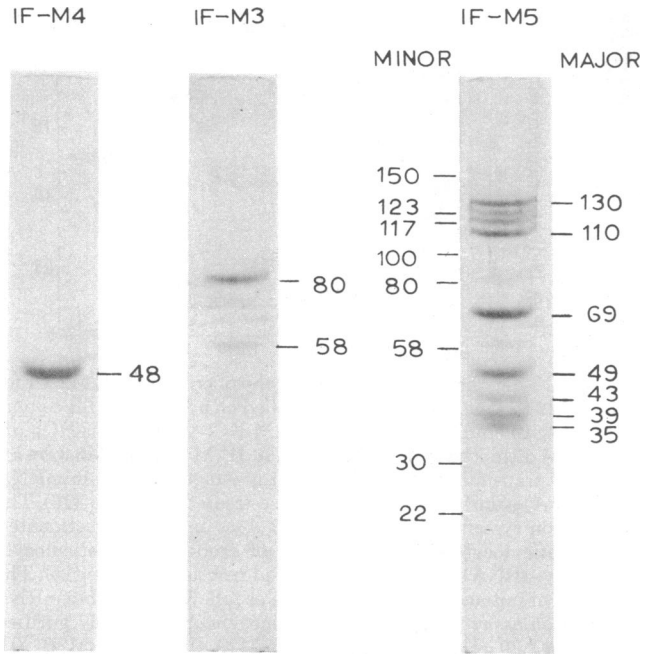

FIG. 2. Analysis of IF-M3, IF-M4, and IF-M5 by $NaDodSO_4$ polyacrylamide gel electrophoresis. Numbers indicate the apparent molecular weights $\times 10^{-3}$ of the polypeptide bands. The major and minor components of IF-M5 are indicated. The amounts of IF-M3, IF-M4, and IF-M5 applied per gel were 5, 4, and 15.4 μg, respectively.

acrylamide gel electrophoresis indicates, however, that IF-M5 is a complex composed of seven major and eight minor polypeptide chains (Fig. 2). Assuming the IF-M5 complex is composed of one copy of each major polypeptide chain and binds variable amounts of the minor polypeptide components, an apparent molecular weight of $\geq$475,000 is obtained. Comparison of IF-M5 with IF-M3, which is also required for translation of globin mRNA (7), shows a markedly different polypeptide composition (Fig. 2).

Relationship of IF-M4 and IF-M5 to Other Initiation and Elongation Factors. By analysis with both acid urea and $NaDodSO_4$ polyacrylamide gel electrophoresis, IF-M4 is unambiguously resolved from all other known homogeneous initiation and elongation factors. Because of the large number of polypeptide components of IF-M5, this large complex was examined for known initiation and elongation factors by the addition of single factors to IF-M5 and analysis by both $NaDodSO_4$ (Fig. 3) and acid urea (Fig. 4) polyacrylamide gel electrophoresis. This permitted identification both by size and charge-to-mass ratio; resolution in these two systems is sufficiently good to determine if exact superimposition of polypeptide bands occurs. The presence of known initiation or elongation factors in IF-M5 is indicated by increased density of an IF-M5 polypeptide band in both gel systems without generation of a new polypeptide band. By these criteria, IF-M2A, IF-M3, and EF-2 are present as minor components in IF-M5; one of the three subunits of IF-MP is present as a major component. Although analysis by $NaDodSO_4$ polyacrylamide gel electrophoresis indicates the possible presence of IF-M4, acid urea polyacrylamide gel electrophoresis does not confirm this. Thus, most of the polypeptides present in IF-M5 do not correspond to the other initiation or elongation factors.

Biological Properties of IF-M4 and IF-M5. IF-M4 and IF-M5 were tested for their ability to stimulate translation of both artificial and natural templates. IF-M4 had no effect on polyphenylalanine synthesis or IF-MP-dependent Met-$tRNA_f$ binding (data not shown). At saturating levels of other initiation and elongation factors (including IF-M5), the translation of globin mRNA was stimulated 9-fold by IF-M4 (Fig. 5A). IF-M5 showed no ability to bind Met-$tRNA_f$, even though it appears to contain the 35,000 dalton subunit of IF-MP, and was not capable of stimulating polyphenylalanine synthesis (data not shown). With saturating levels of all other required initiation and elongation factors (including IF-M4), the translation of globin mRNA was stimulated 10-fold by IF-M5; the total activity of the fractionated system with saturating levels of IF-M4 and IF-M5 approached that obtained with 0.5 M KCl ribosomal wash (Table 1, Fig. 5A and B). With increasing levels of IF-M5, only a partial dependence of the globin assay on IF-M3 was observed (Fig. 5B). However, at nonsaturating levels of IF-M5, the stimulation by IF-M3 was much more apparent.

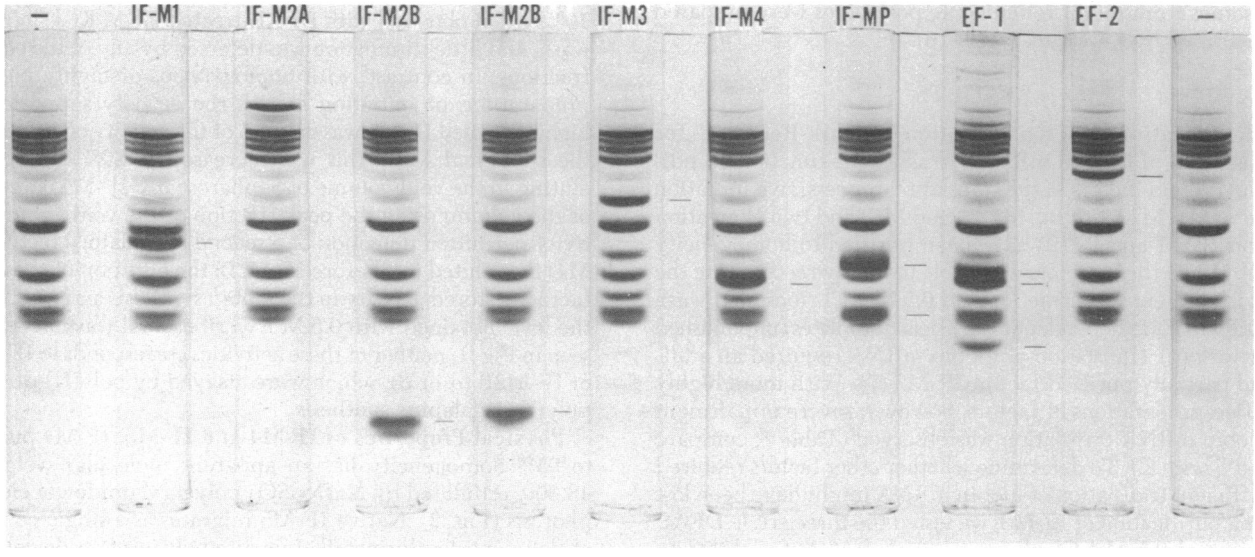

FIG. 3. Analysis of IF-M5 components by $NaDodSO_4$ polyacrylamide gel electrophoresis. IF-M5 (15.4 μg) was mixed individually with 0.5–2 μg of the indicated highly purified initiation and elongation factors and were prepared for electrophoresis as described (5). The presence of IF-M2A, IF-M3, the 35,000 dalton subunit of IF-MP, and EF-2 in or binding to the IF-M5 complex is supported by exact superimposition of the polypeptide bands. The position to which the polypeptide band(s) of the individual factor would migrate is indicated by the line(s) to the right of each gel.

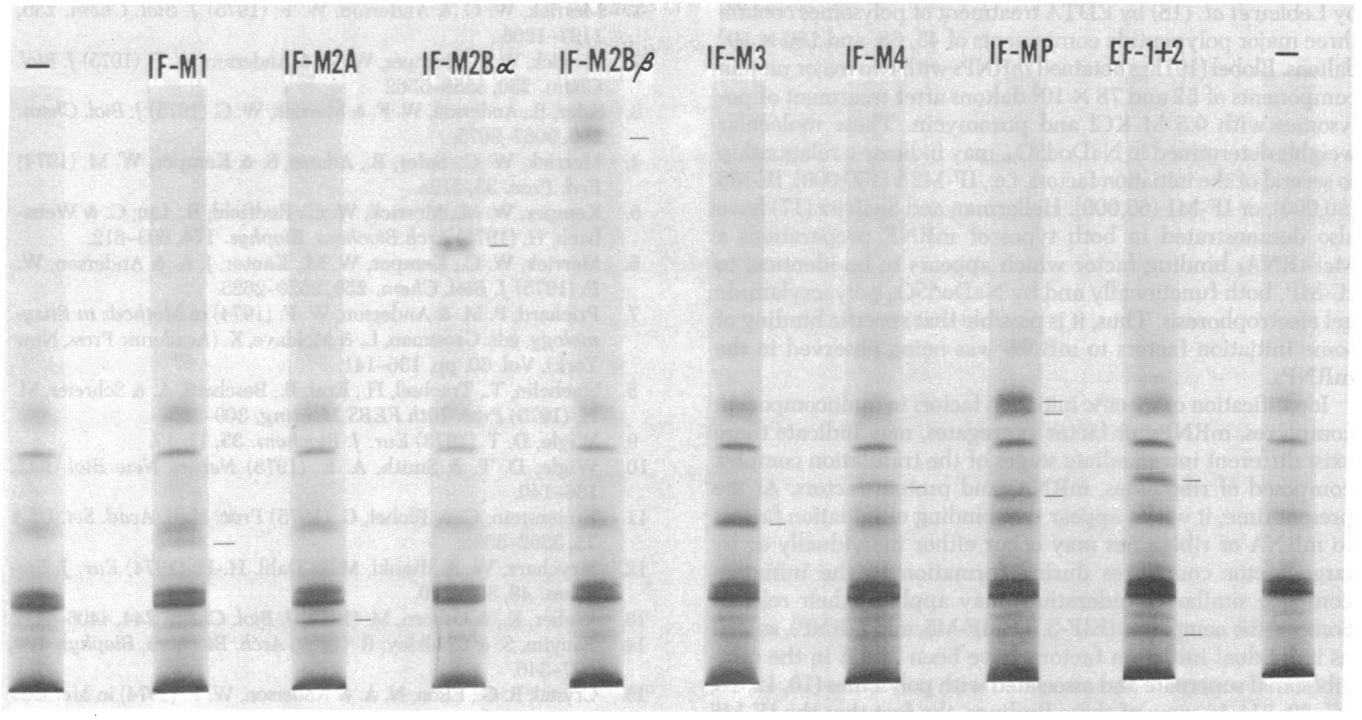

FIG. 4. Analysis of IF-M5 by acid urea polyacrylamide gel electrophoresis. Highly purified initiation and elongation factors (0.5–2 μg) were mixed individually with 10.4 μg of IF-M5. The samples were then precipitated with ethanol and the protein was incubated for 30 min at 37° in 10 M urea–50 mM dithiothreitol. After addition of acetic acid to a final concentration of 5%, electrophoresis was performed as described (14). In agreement with analysis by $NaDodSO_4$ polyacrylamide gel electrophoresis, the presence of IF-M2A, IF-M3, one of the three IF-MP subunits, and EF-2 in the IF-M5 complex preparation is demonstrated.

DISCUSSION

This paper has reported the purification and properties of two initiation factors, M4 and M5. IF-M4, on the basis of size and chromatographic behavior, appears to be similar to IF_{EMC} described by Wigle and Smith (9, 10) and to IF-E_4 described by Staehelin *et al.* (8). Similarly, IF-M3 probably corresponds to IF-E_6 of Staehelin *et al.* (8). Although IF-M4 is required for translation of endogenous or exogenous globin mRNA, no stimulation of any assay utilizing artificial template has been observed; in contrast to IF_{EMC}, IF-M4 does not appear to be specific for viral mRNA, and activity is not lost on storage at the vapor temperature of liquid nitrogen.

IF-M5 has been prepared in a high state of purity, as judged by sedimentation velocity analysis and alkaline polyacrylamide gel electrophoresis. However, the presence of several minor polypeptide chains that form a stable association with the IF-M5 complex precludes homogeneity. Freienstein and Blobel (11) and Staehelin *et al.* (8) have also isolated complexes consisting of at least 10 polypeptide chains that have sedimentation values of 15–17 S. IF-M5 prepared by Sepharose 6B chromatography and gradient elution from phosphocellulose is composed of seven major and eight minor components. Analysis by $NaDodSO_4$ and acid urea polyacrylamide gel electrophoresis indicates that the IF-M2A, IF-M3, a component of the multisubunit factor IF-MP, and EF-2 appear to be associated with the IF-M5 complex. Because some of these initiation factors appear to be present in smaller amounts than the seven major protein bands of IF-M5, it is likely that several populations of IF-M5 (defined as a complex of the seven major polypeptide components) exist that bind variable amounts of these factors. A tight association of these factors with the IF-M5 complex is indicated, however, since the chromatographic behavior of the individual initiation factors would preclude copurification with IF-M5.

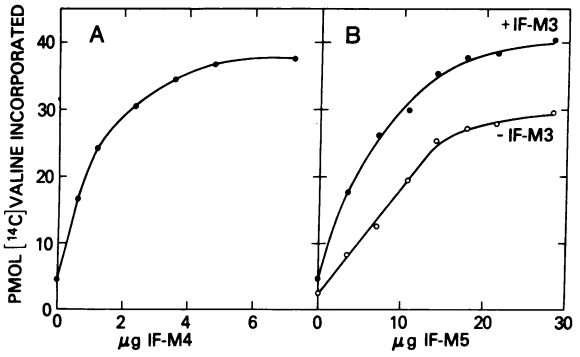

FIG. 5. (A) Dependence of globin mRNA translation on IF-M4. Assay conditions were the same as those of Fig. 1, with the exception that 20 μg of IF-M5 were used per 100-μl reaction mixture. (B) Dependence of globin mRNA translation on IF-M5. Five micrograms of IF-M4 and 5 μg of IF-M3 were used. An incorporation of 40 pmol of [14C]valine was obtained using saturating levels of DEAE-treated ribosomal wash fraction (15).

In spite of the presence of several initiation factors in the IF-M5 complex, only the presence of IF-M3 could be demonstrated by activity. The ability to detect IF-M3 and not IF-M2A is probably due to the 10- to 20-fold greater content of IF-M3 in the IF-M5 complex (as measured by scanning of stained gels at 540 nm). The presence of just one of the three subunits of IF-MP is curious. Since IF-M5 does not bind Met-$tRNA_f$ or mRNA, it would appear that both of the other subunits of IF-MP are required to catalyze these reactions that occur with native IF-MP (17).

A second class of high-molecular-weight complexes containing both protein and mRNA is referred to as messenger ribonucleoprotein particles (mRNPs). The mRNPs prepared

by Lebleu *et al.* (18) by EDTA treatment of polysomes contain three major polypeptide components of 45, 68, and 130×10^3 daltons. Blobel (19) has obtained mRNPs with two major protein components of 52 and 78×10^3 daltons after treatment of polysomes with 0.5 M KCl and puromycin. These molecular weights determined in $NaDodSO_4$, may indicate a relationship to several of the initiation factors, i.e., IF-M2A (150,000), IF-M3 (80,000), or IF-M1 (65,000). Hellerman and Shafritz (17) have also demonstrated in both types of mRNP preparations a Met-$tRNA_f$ binding factor which appears to be identical to IF-MP, both functionally and by $NaDodSO_4$ polyacrylamide gel electrophoresis. Thus, it is possible that specific binding of some initiation factors to mRNA was being observed in the mRNPs.

Identification of specific initiation factors in multicomponent complexes, mRNPs, or factor aggregates, may indicate there exist different intermediate stages of the translation complex composed of ribosomes, mRNA, and protein factors. At the present time, it would appear that binding of initiation factors to mRNA or ribosomes may occur either individually or by large factor complexes during formation of the initiation complex; similar considerations may apply to their release. Some of the complexes (EIF-3, TC, IF-M5, and mRNP), as well as individual initiation factors, have been found in the post-ribosomal supernate and associated with polysomes (10, 12, 16, 17, 20, 21). In view of these findings, the fact that the IF-M5 complex does not contain all known initiation factors, and a lack of direct proof that initiation factors are not present in polysomes, the conclusion recently drawn by Freienstein and Blobel (11) that initiation factors found in ribosome preparations are located exclusively on the native small ribosomal subunit is premature.

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