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Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes

(protein synthesis/multi-protein complex/40S ribosomal binding/reductive alkylation)

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ABSTRACT **Initiation factor IF-E3 from rabbit reticulocytes was isolated from a high salt extract of ribosomes prepared according to the procedure of Schreier and Staehelin (*J. Mol. Biol.* 73, 329–349, 1973). The factor was highly purified from the crude extract by ammonium sulfate fractionation, sucrose gradient centrifugation, salt gradient elution from DEAE-cellulose and phosphocellulose columns, and glycerol gradient centrifugation. IF-E3 stimulated cell-free protein synthesis dependent on an exogenous globin mRNA fraction 4- to 5-fold. The factor under nondenaturing conditions behaved as a large multipolypeptide complex, but was separated into 11 major protein components by two-dimensional polyacrylamide gel electrophoresis with urea and sodium dodecyl sulfate. The stoichiometry and molecular weights (range: 28,000–140,000) of the IF-E3 proteins were determined. None of the components corresponded to ribosomal proteins found in high salt-washed ribosomes. $^{14}CH_3$-IF-E3 was prepared by reductive alkylation without detectable loss of its initiation factor activity, and bound stoichiometrically to 40S ribosomal subunits, but not to 60S or 80S ribosomes. $^{14}CH_3$-IF-E3 isolated from the 40S complex contained only nine of the 11 original protein components.**

Initiation factors for protein synthesis have been isolated from a variety of types of eukaryotic cells (1–11), those obtained from rabbit reticulocytes in the laboratories of Staehelin (1–4) and Anderson (5–7) having been best characterized. Among the numerous initiation factors isolated from high salt extracts of ribosomes, a very large multiprotein complex has been found (1, 6) which may contain 10 different polypeptides (4, 12). This factor is required for initiation of globin synthesis, and is implicated in the binding of mRNA to 40S ribosomal subunits (3, 6). Further elucidation of the role played by the multiprotein complex depends on a more thorough characterization of the factor. We report here the isolation from rabbit reticulocytes of the factor, called IF-E3*, and its purification and physical characterization.

METHODS

Crude Initiation Factors. Initiation factors were isolated from rabbit reticulocyte lysates as described by Schreier and Staehelin (2). Three crude fractions were obtained by treating the high salt ribosomal extract with ammonium sulfate (3): fraction A, 0–40% saturation, fraction B, 40–50% saturation, and fraction C, 50–70% saturation. IF-E3 was purified from fraction A as described in *Results*.

Abbreviations: NaDodSO$_4$/electrophoresis, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; DTT, dithiothreitol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; buffer A, buffer containing 20 mM Tris·HCl at pH 7.6, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 5% glycerol; buffer B, buffer containing 20 mM potassium phosphate at pH 7.2, 0.2 mM EDTA, 7 mM 2-mercaptoethanol and 5% glycerol.

* The factor nomenclature used here is based on that of Schreier and Staehelin (3).

Assay for IF-E3 Activity. Initiation factor activity was determined in a system for protein synthesis according to the procedures of Schreier and Staehelin (2). The assay utilized ribosomal subunits and pH 5 enzyme fractions prepared from the livers of Sprague-Dawley rats (14). Globin mRNA was purified from high salt-washed polysomes by dissolving the polysomes (about 7000 $A_{260\,nm}$ units) in 20 ml of 5 mM Tris·HCl at pH 7.6, 2% sucrose, and 1.4% lithium dodecyl sulfate, and centrifuging the mixture through a sucrose gradient in the Beckman 15 zonal rotor. In addition to the fractions of IF-E3 to be assayed, the system contained 5 μg of fraction B and 10 μg of fraction C described above, and 3 μg of protein containing IF-E6 (4), partially purified from the top fractions of the sucrose gradient described in *Results* below. The assay measured the incorporation of [^{3}H]leucine (specific activity 500 Ci/mol) into trichloroacetic acid-precipitable protein.

Reductive Alkylation of IF-E3. IF-E3 (1 mg), purified by glycerol gradient centrifugation (see *Results*), was treated with 4 μmol of [^{14}C]formaldehyde (New England Nuclear, 44 Ci/mol) and 2 μmol of sodium borohydride according to the procedure of Ottesen and Svensson (15). The reaction was carried out in 1 ml of buffer containing 100 mM potassium borate at pH 9.0, 0.18 mM EDTA, 90 mM KCl, and 4.5% glycerol at 0°; the sodium borohydride was added in 10 portions of 0.2 μmol each at 3 min intervals. Typical preparations yielded about 0.8 mg $^{14}CH_3$-IF-E3 with a specific activity of about 6000 cpm/μg of protein.

RESULTS

Purification of IF-E3. The A fraction described in *Methods* was dissolved in 11 ml of buffer A (20 mM Tris·HCl at pH 7.6, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 5% glycerol) containing 100 mM KCl. Aliquots of 2 ml were layered over 15–30% (wt/vol) sucrose gradients in buffer A and 100 mM KCl and were centrifuged for 24 hr at 25,000 rpm in a Beckman SW 27 rotor. The contents were fractionated and assayed for IF-E3 activity as described in *Methods*. Active fractions were found in the middle portion of the gradient as shown in Fig. 1, panel A, and were pooled. Analysis of the protein components by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO4/electrophoresis) is shown in Fig. 2, gel B. Fractions at the top of the gradient contained material which stimulated protein synthesis in the presence of ammonium sulfate fractions B and C and purified IF-E3. This activity likely corresponds to IF-E6 reported by Staehelin *et al.* (4), and was partially purified by chromatography on DEAE-cellulose and phosphocellulose for use in the assays for IF-E3 activity. A detailed description of the purification and properties of IF-E6 will be presented elsewhere.

The fractions containing IF-E3 were further purified by ion-exchange column chromatography. The material from the

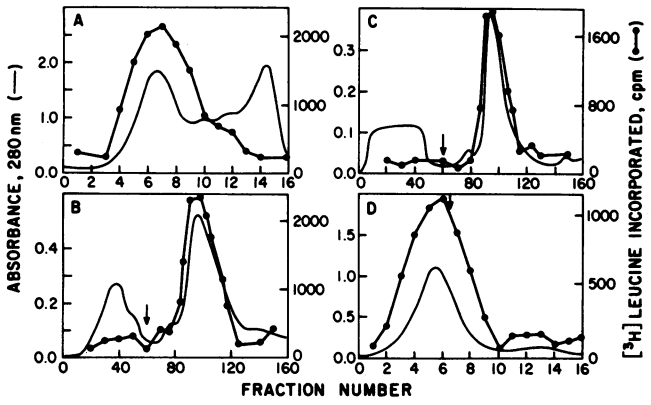

FIG. 1. Purification of IF-E3. Procedures for the four steps are described in the *text*. IF-E3 (10 μl aliquots) was assayed as described in *Methods*. Absorbancy was measured in a Gilford 2400 spectrophotometer; a 0.2 cm flow cell was used for the determinations shown in panels A and D. Panel A: Sucrose gradient centrifugation. Fractions of about 2 ml were collected; fraction 16 represents the top. Panel B: chromatography on DEAE-cellulose. The salt gradient was begun at fraction 60 (see arrow) and 3 ml fractions were collected. Panel C: chromatography on phosphocellulose. The salt gradient was begun at fraction 60 and 2 ml fractions were collected. Panel D: glycerol gradient centrifugation. Fractions of about 0.75 ml were collected; fraction 16 represents the top.

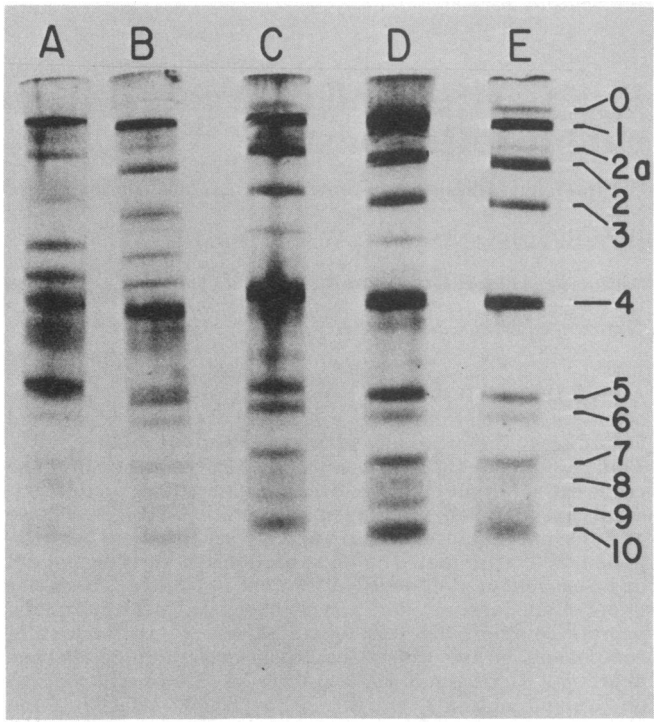

FIG. 2. NaDodSO$_4$/electrophoresis analysis of IF-E3 fractions. Disc gels containing 10% acrylamide and 0.27% *N,N*-methylene-bis-acrylamide were prepared and used according to the procedures of Weber and Osborn (16). Protein was stained with Coomassie brilliant blue. Protein samples (approximately 50 μg each) were applied as follows: A, fraction A; B, after sucrose gradient centrifugation; C, after DEAE-cellulose chromatograph; D, after phosphocellulose chromatography; E, after glycerol gradient centrifugation.

sucrose gradients (135 $A_{260\ nm}$ units) was applied to a column of DEAE-cellulose (Whatman DE-32; 2 × 25 cm) equilibrated with buffer A containing 100 mM KCl. The adsorbed protein was eluted with a 300 ml linear gradient of KCl, 100–300 mM, in buffer A. The IF-E3 activity eluted at about 150 mM KCl as shown in Fig. 1, panel B, and active fractions were combined and dialyzed against 100 mM KCl in buffer B (20 mM potassium phosphate at pH 7.2, 0.2 mM EDTA, 7 mM 2-mercaptoethanol, and 5% glycerol). The solution containing about 52 mg of protein was applied to a column of phosphocellulose (Whatman P11; 0.9 × 25 cm) equilibrated in the same buffer, and the adsorbed protein was eluted with a 200 ml linear gradient of KCl, 100–500 mM in buffer B. As shown in Fig. 1, panel C, the IF-E3 activity eluted at 300 mM KCl as a sharp peak and active fractions were combined. Analysis by NaDodSO$_4$/electrophoresis of the IF-E3 fractions obtained from the two columns is shown in Fig. 2, gels C and D.

The IF-E3 was concentrated to 12 ml by ultrafiltration (Amicon PM 10 filter) and further purified by centrifugation in 15–30% glycerol gradients in buffer A containing 100 mM KCl. Aliquots containing about 2 mg of protein in 1 ml were layered over the gradients, which were centrifuged for 16 hr at 40,000 rpm in a Beckman SW 41 rotor, and then fractionated. The IF-E3 activity corresponded closely with the bulk of the protein which sedimented somewhat more rapidly than β-galactosidase (see arrow in Fig. 1, panel D). The active fractions were pooled, concentrated by ultrafiltration to 1 mg/ml, and stored at −70°. Analysis of the preparation by NaDodSO$_4$/electrophoresis (Fig. 2, gel E) indicated that IF-E3 is a multipolypeptide complex.

A summary of the purification is given in Table 1, which shows the amounts of protein and the specific activities obtained. The procedure yielded about 20 mg of IF-E3 which was purified 11-fold from the ammonium sulfate A fraction. It is clear by inspection of the gels shown in Fig. 2 that very little additional purification is obtained by chromatography on phosphocellulose and by the glycerol gradient step. This is consistent with the specific activities reported in Table 1. Thus, the last two steps of purification are primarily useful in showing that the IF-E3 behaves as a complex and is not further fractionated by the procedures used, although a few contaminating proteins are reduced or removed. An IF-E3 solution of 1 mg/ml [determined by the method of Lowry *et al.* (17) with bovine serum albumin as standard] had an absorbancy at 280 nm of 1.7. As shown in Table 2, purified IF-E3 stimulated globin synthesis 4- to 5-fold. Even at about 3-fold excess of IF-E3 added, the omission of the IF-E6 or fractions A and B caused a greater than 3-fold decrease in protein synthesis (data not shown). This is in agreement with the more elegant experiments of Staehelin, where all six factors have been highly purified (4, 12) and all are required for protein synthesis.

Table 1. Purification of IF-E3

Purification step	Protein ($A_{280\ nm}$ units)	Activity (units)	Specific activity
Crude wash	750	—	—
A fraction	310	40	0.13
Sucrose gradient	135	68	0.50
DEAE cellulose	52.1	75	1.44
Phosphocellulose	42.8	65	1.52
Glycerol gradient	39.5	53	1.34

The values cited are derived from a typical preparation obtained from the blood of 90 anemic rabbits. Protein was measured by absorbancy at 280 nm, and 1 unit is defined as the amount of protein in 1 ml of solution which has an $A_{280\ nm}$ of 1 in a 1 cm cell. Activity was measured as described in *Methods*; 1 activity unit refers to the ability to stimulate the incorporation of 1 nmol of [^{3}H]leucine into protein. Specific activity is defined as activity units divided by $A_{280\ nm}$ units.

Table 2. Protein synthesis stimulated by IF-E3

Factor added (μg of protein)	Activity (pmol of [^{3}H]leucine incorporated)	
	IF-E3	$^{14}CH_3$-IF-E3
0	2.1	2.1
1	4.0	5.0
2	6.6	7.6
3	8.5	9.0
4	9.3	9.8

The assay was performed as described in *Methods*.

IF-E3 Is a Large Multipolypeptide Complex. The sedimentation behavior of IF-E3 on glycerol gradients (Fig. 1, panel D) implies that the complex is very large. Analysis by NaDodSO$_4$/electrophoresis indicates that the complex contains at least 10 polypeptides (Fig. 2, gel E). Molecular weights and stoichiometry of the protein components of IF-E3 were determined from densitometric tracings of NaDodSO$_4$ gels as described in Table 3. The results indicate that protein bands 1, 2, 5, 7, and 9 occur in equimolar amounts; protein bands 4 and 10 occur in two copies in relation to those mentioned above, while protein bands 3, 6, and 8 are present in less than one copy. The stoichiometries obtained for preparations from both the fast and the slow sedimenting regions of the glycerol gradient are similar for nearly all the proteins. The proteins in bands 0 and 2a, on the other hand, are present in considerably less than one copy, and the relative amounts are not the same in the two gels shown. In other preparations of IF-E3, band 0 was entirely absent and the protein in band 8 was present in even lower amounts than reported in Table 3. It is therefore possible that proteins 0, 2a, and 8 are contaminants of the complex.

Table 3. Molecular weights and stoichiometry of IF-E3 components

Protein no.	Stoichiometry		Molecular weights × 10^{-3}	
	Fr 4	Fr 7	Disc gel	Two-dimensional gel
0	0.10	0.30	165	—
1	1.00	1.00	140	140
2a	0.22	0.14	130	—
2	0.88	0.90	120	125
3	0.75	0.74	110	110
4	1.71	1.68	69	72
5	1.09	1.14	47	50
5a	—	—	—	48
6	0.74	0.84	45	46
7	1.08	1.12	37	39
8	0.57	0.75	35	35
9	1.03	1.12	31	33
10	2.01	2.14	28	31

Molecular weights were calculated from both the disc gels (Fig. 2) and the two-dimensional gel (Fig. 4) with the following protein standards: β-galactosidase (135,000), bovine serum albumin (67,500), and ovalbumin (45,000). Stoichiometric determinations were made as follows: two IF-E3 fractions (number 4 and 7) from the glycerol gradient shown in Fig. 1, panel D, were selected which represented material from the slow-sedimenting and the fast-sedimenting side of the peak; the fractions were analyzed by NaDodSO$_4$/electrophoresis as described in Fig. 2, and densitometric tracings of the gels were made; the areas under the peaks corresponding to the numbered bands shown in Fig. 2, gel E, were measured; the values obtained were divided by the corresponding molecular weights and were normalized to protein 1.

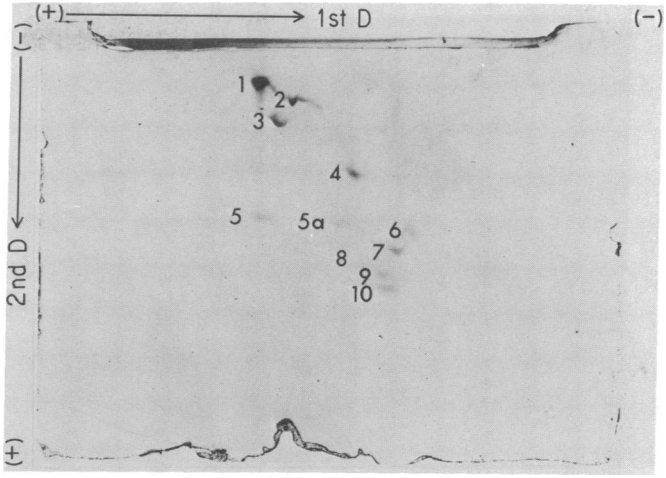

FIG. 3. Urea/NaDodSO$_4$ two-dimensional electrophoresis of IF-E3. The gel system described by Mets and Bogorad (19) was used except that the urea gel was run at pH 4.5 instead of pH 5.0. Further modifications were introduced to prevent irreversible precipitation of IF-E3 proteins during the first dimension: the urea gels were allowed to polymerize overnight and were prerun for 6 hr at 2 mA per gel; IF-E3 (50 μg) was dialyzed extensively against the urea sample buffer and treated with 20 mM iodoacetamide for 30 min at 37° before electrophoresis. After electrophoresis for 16 hr at 0.75 mA per gel, the urea gels were dialyzed against buffer containing 70 mM Bis-Tris-HCl-pH 6.75, 0.1% NaDodSO$_4$, and 0.1% 2-mercaptoethanol and were incorporated into the second dimension and subjected to electrophoresis for 8 hr at 25 mA.

IF-E3 also behaved as a complex when analyzed by gels in a nondenaturing buffer (18). Most of the protein migrated as a complex with a R_m (relative mobility to bromphenol blue) of about 0.2, although about 20% appeared as a broad band of greater mobility. When the gel was analyzed by NaDodSO$_4$/electrophoresis in a second dimension, all 10 protein bands were detectable in the region of the major complex (data not shown).

The protein components of the IF-E3 complex were characterized by two-dimensional polyacrylamide gel electrophoresis performed by using denaturing conditions in both dimensions (Fig. 3). The first dimension utilized a large pore gel in urea buffer at pH 4.5; thus, separation was achieved primarily on the basis of electrostatic charge at acidic pH. The second dimension used an NaDodSO$_4$-containing buffer in order to separate components on the basis of size. Detailed procedures are given in the legend to Fig. 3. The system separates the IF-E3 complex into 11 distinct protein spots. The spots were numbered in the same way as the bands in the one-dimensional NaDodSO$_4$ gel shown in Fig. 2, by using molecular weights from both systems (see Table 3) as the basis for identification of the proteins. This procedure leads to an unambiguous assignment of proteins 1 through 4, but there is no strict correspondence between the proteins numbered 5 through 10 in the two gel systems. Further experimentation is needed to make firm number assignments for these proteins. It appears that protein bands 5 and 6 are separated into three spots, 5, 5a, and 6. Because the two-dimensional urea/NaDodSO$_4$/electrophoresis system separates the IF-E3 components better than the one-dimensional NaDodSO$_4$ gels, it is a more adequate system for the characterization of IF-E3 preparations and can

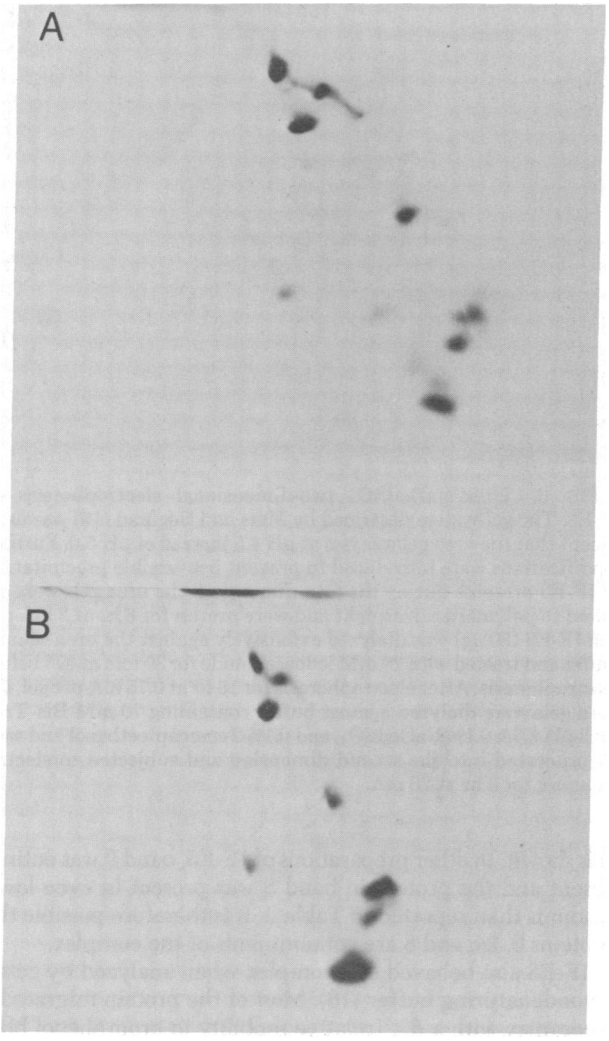

FIG. 4. Autoradiograms of $^{14}CH_3$-IF-E3 on urea/NaDodSO$_4$ two-dimensional gels. The gel system is the same as described in Fig. 3. The gels were dried under vacuum after soaking for 4 hr in 22% 2,5-diphenyloxazole in dimethyl sulfoxide as described by Bonner and Laskey (20). The gels were then exposed to Kodak RP Royal X-omat film for 10 days (panel A) or 21 days (panel B). Panel A: the sample contained 5.5 μg of $^{14}CH_3$-IF-E3 (33,000 cpm) and about 50 μg of unlabeled IF-E3. Panel B: the preparation of a $^{14}CH_3$-IF-E3:40S complex was essentially the same as described in the legend to Fig. 5 except that the amounts of IF-E3 and 40S ribosomal subunits were increased 10-fold. Ten microliter aliquots of the fractions, collected after centrifugation, were assayed in order to determine the position of the $^{14}CH_3$-IF-E3 complex. The peak fractions were pooled and the proteins extracted as described by Hardy *et al.* (21). The extracted proteins (containing 50% of the original radioactivity) were extensively dialyzed against 8 M urea, 10 mM DTT, and 10 mM Bis-Tris acetate at pH 4.0, and concentrated by ultrafiltration (Amicon PM 10 filter). Approximately 15,000 cpm of $^{14}CH_3$-IF-E3 were applied to the gel.

be used to compare IF-E3 proteins with other protein fractions isolated from cells, as described below.

IF-E3 May Be Radioactively Labeled by Reductive Alkylation Without Loss of Biological Activity. A purified preparation of IF-E3 was treated with [^{14}C]formaldehyde and sodium borohydride as described in *Methods*. $^{14}CH_3$-IF-E3 was obtained with a specific activity of 6000 cpm/μg of protein, which corresponds to about 54 methyl groups per IF-E3 complex. Analysis by urea/NaDodSO$_4$ two-dimensional electrophoresis, followed by autoradiography (Fig. 4, panel A), indicated that each of the protein components of IF-E3 became

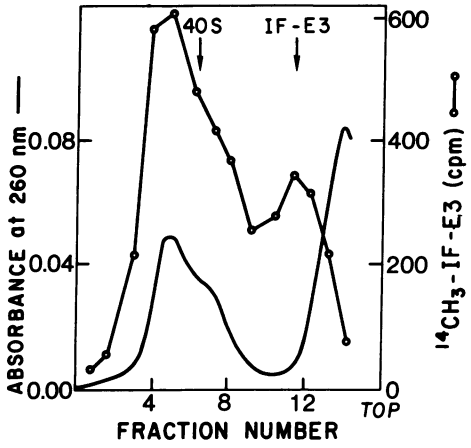

FIG. 5. Binding of $^{14}CH_3$-IF-E3 to 40S ribosomal subunits. A reaction of 50 μl (4 mM magnesium acetate, 70 mM KCl, 20 mM Tris·HCl at pH 7.6, 7 mM 2-mercaptoethanol) containing 5 μg of $^{14}CH_3$-IF-E3 (6000 cpm/μg) and 15 μg of 40S ribosomal subunits (prepared from rat liver as described in *Methods*) was incubated for 15 min at 37°. The reaction mixture was layered on a 15–30% sucrose gradient in the same buffer and centrifuged for 2 hr at 50,000 rpm in a Beckman SW56 rotor. Fractions of six drops were collected and counted in 5 ml of Triton-Toluene scintillation liquid. The arrows indicate the peak positions of free 40S ribosomal subunits and free IF-E3 sedimented in separate gradients.

labeled. The positions of radioactivity corresponded exactly to the positions of the stained spots of carrier, unlabeled IF-E3. The initiation factor activity of the $^{14}CH_3$-IF-E3 preparation was compared with that of the untreated factor (Table 3); no change in specific activity due to reductive alkylation was found.

The Protein Components of IF-E3 Do Not Correspond to Any Ribosomal Proteins. Since IF-E3 is large, it is plausible that it represents a complex of ribosomal proteins and/or initiation factors which have been separated and characterized by other means. The former possibility has been tested directly by coelectrophoresis of $^{14}CH_3$-IF-E3 and a total protein extract of either 40S or 60S ribosomal subunits prepared from rabbit reticulocyte ribosomes washed in high salt buffers (22). The urea/NaDodSO$_4$ two-dimensional electrophoresis system was used under conditions where ribosomal proteins were in large excess over $^{14}CH_3$-IF-E3. The positions of ribosomal proteins were determined by staining with Coomassie brilliant blue, while IF-E3 was determined by autoradiography. None of the radioactive spots coincided with any of the ribosomal proteins (data not shown). We conclude that no part of the IF-E3 complex is composed of ribosomal proteins. The possibility that other initiation factors may be present in IF-E3 is under investigation.

IF-E3 Binds to 40S Subunits. The interaction of $^{14}CH_3$-IF-E3 with 40S subunits was studied by analytical sucrose gradient centrifugation. As shown in Fig. 5, the labeled factor formed a stable complex with 40S ribosomal subunits in the absence of any of the other components involved in initiation of protein synthesis. Details of the binding conditions and gradient analyses are given in the figure legend. The IF-E3:40S complex sedimented faster than 40S subunits to a position in the gradient corresponding to about 50 S. With conditions of a 2-fold excess of ribosomes, greater than 90% of the recovered $^{14}CH_3$-IF-E3 was present in the ribosomal complex. As the amount of factor is increased relative to 40S subunits, the amount bound saturates at a level of 0.8 copies of $^{14}CH_3$-IF-E3 per ribosome, by assuming a mass of 724,000 daltons for IF-F3

(data not shown). No binding interaction was detected with 60S or 80S ribosomes and the binding to 40S subunits was not influenced by the presence or absence of GTP.

Bound IF-E3 Is Composed of Nine Polypeptides. To determine the composition of the IF-E3 bound to the 40S ribosomal subunit, a complex of $^{14}CH_3$-IF-E3 and 40S subunits was isolated and the proteins were extracted and analyzed by urea/NaDodSO$_4$ two-dimensional electrophoresis. As shown in Fig. 4, panel B, bound $^{14}CH_3$-IF-E3 gives rise to a cleaner pattern of radioactivity than is obtained with the factor before it is bound (Fig. 4, panel A). Comparison of the two panels shows that proteins 5a and 8 are entirely absent from the bound factor. Otherwise, the intensities of the remaining nine protein spots are generally comparable. The radioactive pattern was identical when protein was extracted from the ribosomal complex by the method of Mizushima and Nomura (23) rather than by that of Hardy *et al.* (21).

DISCUSSION

IF-E3 has been highly purified from rabbit reticulocytes and is required for protein synthesis in an assay employing exogenous globin mRNA (Table 2). Much of the evidence presented here indicates that IF-E3 is a large multi-protein complex: it was not fractionated by salt gradient elution from two kinds of ion-exchange columns; it sedimented in a glycerol gradient as a symmetrical peak with constant specific activity (Fig. 1, panel D) and protein content (Table 3); during electrophoresis in nondenaturing buffer, the bulk of the protein migrated as a complex which contained all of the major protein components. On analysis by one-dimensional NaDodSO$_4$/electrophoresis, 10 major protein bands were seen (Fig. 2, gel E). The protein band pattern obtained is similar to those reported for IF-E3 by Staehelin *et al.* (4) and for the high-molecular-weight complex eluted from native 40S subunits described by Freienstein and Blobel (13) and by Sundkvist and Staehelin (12). Further analysis of IF-E3 by urea/NaDodSO$_4$ two-dimensional electrophoresis indicated that the factor contained 11 major proteins. None of these proteins corresponded to the proteins present in salt-washed 40S or 60S ribosomal subunits.

Radioactive IF-E3 formed a stable complex with 40S ribosomal subunits in the absence of any of the other components of the initiation process (Fig. 5). Analysis of the bound factor indicated that only nine proteins were present in the ribosomal complex (Fig. 4, panel B). Based on this result and on the stoichiometric determinations (Table 3) we propose that IF-E3 is composed of nine different polypeptides, namely proteins 1, 2, 3, 4, 5, 6, 7, 9, and 10, as numbered in Fig. 3, and that all other proteins are contaminants of the preparation. The mass of such a complex is 724,000 daltons, as calculated from the data in Table 3.

It is not yet clear whether all nine of the proteins identified above are either necessary or sufficient for IF-E3 activity during initiation of protein synthesis. The possibility of heterogeneity in IF-E3 cannot be ruled out. These problems are best answered by separating and purifying each of the protein components, followed by reconstitution of the active factor. It is also of interest to determine whether IF-E3 remains intact during all phases of its involvement in protein synthesis, or whether some proteins separate from the complex at specific stages of initiation.

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