Wheat germ S100 supernatant was resolved into 10 components, all of which are required for tobacco mosaic virus RNA-directed incorporation of amino acid into protein. Two of the components, C2b and C2a, are, respectively, elongation factors 1 and 2, having molecular weights of 51,000 and 72,000. A third factor (C2e) binds Met-tRNA in a GTP-requiring reaction and is absolutely required for the formation of both 40S ribosome-Met-tRNA and 80S ribosome-Met-tRNA complexes. This factor is considered to be eucaryotic initiation factor 2. Formation of 80S ribosome Met-tRNA complexes is augmented 2 to 3-fold by the simultaneous addition of an mRNA, factor C1, and fraction D2(a + b). Factor C1 also reverses the inhibition of translation by an increased concentration of monovalent cations and promotes the translation of a "competitively inhibited" mRNA. These observations suggest that factor C1 and either factor D2a or D2b (or both) function in mRNA attachment reactions. Factor C1 has a molecular weight of 115,000 and appears to be made up of two or three subunits. Factor D2b has been purified to essential homogeneity and has a molecular weight of 55,000. Its interaction with mRNA and its molecular weight suggest that this factor is the wheat germ equivalent of reticulocyte eucaryotic initiation factor 4A.

Eucaryotic protein synthesis is a complex process involving the interaction of a substantial number of initiation and elongation factors (1, 2). As part of a detailed study of the initiation process, we have fractionated wheat germ lysate into a number of components based on their function in the primary reaction of protein synthesis, namely amino acid polymerization. This report describes the resolution of the system and a partial characterization of seven of these components. An additional three factors are dealt with in the accompanying report (3).

MATERIALS AND METHODS

Preparation of S100 Supernatant and Ribosomes. 120 g of wheat germ were homogenized at 20,000 x g for 5 min in 600 ml of 10 mM KCl, 10 mM KHCO3, 1 mM MgAc2, and then homogenized in a Waring Blender at low speed for 60 sec (4). This work was supported by Grants GM-15122, CA-06927, and RR-05539 from the National Institutes of Health, by Grant 8100166 from the United States Department of Agriculture, and by an appropriation from the Commonwealth of Pennsylvania. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Portions of this paper (including "Materials and Methods" and Figs. 1-8) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass.

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### Factors C1, C2, C8, C9, and C10

**Factor C1**
- 100 mg of the 80-90% (M_W) precipitate of Fraction C was dialyzed against 3 ml of a 0.05 M ECl buffer, and the buffer was then diluted to 30 ml. The solution was dialyzed against 2 ml of a 0.05 M ECl buffer. A column of Sephadex G-25 was equilibrated with 2 ml of the buffer. The column was washed with the same buffer, followed by 2 ml of 0.05 M ECl buffer. A column of Sephadex G-25 was equilibrated with 2 ml of the buffer. The column was washed with the same buffer, followed by 2 ml of 0.05 M ECl buffer.

**Factors C2, C8, C9, and C10**
- 100 mg of the 80-90% (M_W) precipitate of Fraction C was dialyzed against 10 ml of a 0.05 M ECl buffer, at which point the solution was dialyzed against 2 ml of 0.05 M ECl buffer. A column of Sephadex G-25 was equilibrated with 2 ml of the buffer. The column was washed with the same buffer, followed by 2 ml of 0.05 M ECl buffer.

### Preparation of 14-aminobutyric acid

1. **14-Aminobutyric acid**
   - 14-Aminobutyric acid was obtained from Sigma Chemical Co. (St. Louis, Missouri). It was used without further purification.

2. **Synthesis of 14-Aminobutyric acid**
   - 14-Aminobutyric acid was synthesized by the method described by Sanger and collaborators. The yield was 90%.

### Assays of Soybean Acid Hydrolase

1. **Tissue Extract**
   - The tissue extract was prepared by homogenizing 10 g of fresh tissue in 100 ml of 0.05 M NaCl buffer, followed by centrifugation at 10,000 g for 10 minutes.

2. **Assay Conditions**
   - The assay was performed at 37°C for 10 minutes. The reaction was terminated by the addition of 5 ml of 0.05 M NaCl buffer, followed by centrifugation at 10,000 g for 10 minutes. The resulting supernatant was used for the assay.

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acetate, 50 mM EDTA, and 62.5 mM EIC was incubated for 3 min at 29°C. The reaction mixture was brought to 70 mM Hepes-KOH pH 7.5, 0.15 mM dithiothreitol, 20 mM glutamate, 2.5 mM Mg acetate, 20 mM EDTA, 50 mM EIC and supplemented with 50 mM ATP, 1.5 mM phosphoenolpyruvate, and 1.7 mg pyruvate kinase. 2.1 μg factor Cbs (eIF4; 2.1 μg fraction D2(c+d), and 1 to 2 pg of D2(a;Tris-mercaptoethanol) were added to a final vol of 100 μl. After incubation for 2 min, 8 μl of 100 mM EIC, 30 mM Tris acetate pH 7.5, 5 mM MgAc2 were added and the mixture was filtered through a nitrocellulose filter, washed twice with the same buffer and counted for radioactivity in a counter. When the reaction mixtures were analyzed on sucrose gradients, a 200 pg incubation was carried out with all components at the same concentration as in the 100 pg direct filtration assay. In some experiments the nucleoside supplement was 100 μM GTP and 950 μM ATP, 1.8 μg AKP-NEA 6 μg factor C1, and 13 μg of fraction D2(b+c) were added as indicated.

**Sucrose Gradient Analysis of Ribosome Complexes.** Incubation mixtures were layered over 5 ml of a linear gradient between 12% and 25% sucrose in 55 mM EIC, 20 mM EDTA, 32 mM Tris acetate pH 7.5, 3.5 mM Mg acetate, 0.05 M Tris/acetate, pH 7.8, and centrifuged for 75 to 85 min at 87,000 rpm in a Spinco SW 50.1 rotor. The gradients were monitored at 254 nm in an ultracentrifuge and 0.05 ml fractions were collected. Two ml of 100 μM EIC, 30 mM Tris acetate pH 7.5, 5 mM Mg acetate were added and the fractions were filtered on nitrocellulose filters and counted in a counter.

**Polyacrylamide Gel Electrophoresis.** SDS-polyacrylamide electrophoresis was done in slab gels (12 cm x 12 cm x 0.1 cm) with the separating gels containing either 10% or 12.5% acrylamide, 0.3% Bis-acrylamide, 0.5 M Tris HC1 pH 8.8, 0.15 M SDS, and the stacking gel containing 7.5% acrylamide, 0.15% Bis-acrylamide, 0.125 M Tris HC1 pH 6.1, 0.18 M SDS. Protein samples were brought to a final concentration of 0.025 M Tris-HCl, pH 6.8, 7.5% (v/v) mercaptoethanol, 20% (v/v) SDS, and 5% (v/v) glycerol, baled for 3 min, and loaded on the gels. The electrophoresis buffer consisted of 0.192 M glycine, 0.025 M Tris base, and 0.18 M SDS. Electrophoresis was run at 25 mA at room temperature until the bromphenol blue tracking dye reached the end of the gel. The gel was then stained with Coomassie blue and destained in a solution containing 30% methanol, 12% acetic acid. For analysis of the products of the competitive translation, a 17% acrylamide gel was used (7). TMV virus-RNA, glutathione, and AKP-NEA were prepared as described (7).

Wheat germ was obtained from General Mills, Vallejo, Calif., and Dextran Sulfate from Bethesda Research Laboratories. DEAE-cellulose (DE 23, DE 57), and phosphorcellulose (P11) were products of Whatman Biochemicals. Heparin-Sepharose was prepared according to Goldblatt and Jaffe (8). Acrylamide gel (6%) was purchased from LKB, bovine serum albumin (BSA), ovalbumin, and chymotrypsigen from Sigma Chemical Co., and Sephadex G-150 from Pharmacia. Protein concentrations of the different factor preparations were determined according to Bradford (11) with the reagents obtained from Boehringer.

**RESULTS**

**Resolution of the Soluble Factors—**The initial step in the resolution of the soluble factors resolves the S100 supernatant into two fractions, C and D, each of which can be used as an assay reagent allowing the purification of the factors in the other fraction (Fig. 1). In practice, fraction C was first resolved into the components indicated, i.e. C1 and C2a-e, and then, using a partially resolved system of the C components (C1, C2b+c, C2e), the D components were fractionated. The requirement of the system for the TMV-RNA2-directed incorporation of amino acids into protein is presented in Table I. Where appropriate, the different components have been designated according to the standardized system (12) to allow a comparison to the fractionated reticulocyte system. The data of Experiment 1 demonstrate the requirement for components C1 and C2b (EF-1) and a strong stimulation by components C2c and C2e (EF-2). The data of Experiment 2 demonstrate a strong requirement for components C2a (EF-1), D2a (eIF-4B), D2b (eIF-4A), D1 (eIF-3), and D2c (EF-2). Fraction D2(c+d) can be further resolved into two components (3, 13).

**Functional Characterization of Factors C2b (EF-1) and C2c—**Fraction C2b (c + c) (fraction C2 purified through the heparin-Sepharose step) has sufficient EF-1 activity such that 2 μg provide a saturating level of activity in the poly(U)-catalyzed polymerization of phenylalanine. When chromatographed on a column of AAc 44, its activity in the protein synthesis assay was resolved into two components (Fig. 2).
The heavier component eluted at a position corresponding to a molecular weight of 51,000 (Fig. 3), and its activity was coincident with the EF-1 activity. Analysis by SDS-acrylamide gel electrophoresis showed a single band with molecular weight of 56,000 (Fig. 4, lane a). The lighter component in the AcA fractionation (factor C2c) eluted at a position corresponding to a molecular weight of 24,000 (Fig. 3). It had no EF-1 activity, and SDS-acrylamide gel electrophoresis showed several bands ranging in molecular weight from 15 to 20,000 (Fig. 4, lane f).

Functional Characterization of Factor C2e (eIF-2)—The binding of Met-tRNA<sub>Met</sub> to ribosomes as determined by sucrose gradient analysis was completely dependent on factor C2e (see legend to Fig. 7). When the reaction was assayed by retention of radioactivity on nitrocellulose filters in the presence of 5 mM Mg<sup>2+</sup>, a similar strong requirement for this factor was obtained (Table II). The filter assay for ribosome-dependent binding of Met-tRNA<sub>Met</sub> could also be used to monitor two other components, factor D2(c + d) and fraction D1. Factor D1 was required to almost the same extent as factor C2e, while factor D2(c + d) stimulated the ribosome-dependent reaction 2.5-fold. At a low Mg<sup>2+</sup> concentration (0.2 mM), factor C2e bound Met-tRNA<sub>Met</sub> in a GTP-dependent reaction.

| Component omitted | +Ribosome | -Ribosome | Δ  |
|-------------------|-----------|-----------|---|
| Met-tRNA<sub>Met</sub> bound | pmol | pmol |  |
| C2e | 0.40 | 0.09 | 0.31 |
| D1 | 0.03 | 0.12 | 0.09 | 0.03 |
| D2(c + d) | 0.22 | 0.09 | 0.13 |
| C2d in place of C2e | 0.54 | 0.15 | 0.39 |

TABLE II

Formation of Met-tRNA<sub>Met</sub> factor complexes as assayed by retention of radioactivity on nitrocellulose filters

The conditions were those of the standard assay for the formation of ribosome-Met-tRNA<sub>Met</sub> complexes as described under "Materials and Methods." Where indicated, 4 μg of factor C2d were added in place of C2e.

| Factor added | µg | Met-tRNA<sub>Met</sub> bound | GTP | GDP | pmol |
|--------------|----|-----------------------------|-----|-----|------|
| C2e | 2.9 | 0.13 | 0.04 |
| C1 | 5.8 | 0.41 | 0.08 |
| C2b | 5.0 | 0.01 | 0.01 |
| D1 | 7.8 | 0.01 | 0.01 |
| D2(a + b) | 7.6 | 0.00 | 0.00 |
| D2(c + d) | 1.9 | 0.06 | 0.31 |
| C2d | 6.0 | 0.32 | 0.31 |

TABLE III

The reaction with this component, however, was independent of GTP and, under the 5 mM Mg<sup>2+</sup> condition of the ribosome binding assay (Table I), this fraction could not replace C2e or C2d in functioning as an eIF-2-like component. On SDS-gel electrophoresis, factor C2e showed a considerable number of bands, with the predominant ones having molecular weights of 36,000 and 50,000 (data not shown).

Functional Characterization of Factor C2a (EF-2)—The requirement for this component was realized when the resolved system was assayed with ribosomes washed with 0.6 M KCl (K<sub>M</sub><sup>700</sup> ribosomes). The purification steps on DEAE and hydroxylapatite were initially developed to provide EF-2 from an otherwise discarded fraction. Having observed that this fraction restored the reaction with the K<sub>M</sub><sup>700</sup> ribosomes, it was chromatographed on a column of AcA 44. The results presented in Fig. 5 show that the TMV-RNA-catalyzed amino acid polymerizing activity co-elutes with the EF-2 (poly(U)-dependent Phe-tRNA polymerizing) activity. Based on these observations, we have designated C2e as eIF-2, the factor that binds Met-tRNA<sub>Met</sub> in a ternary complex with GTP (1, 2). The data of Tables II and III also show that factor C2e can be replaced in the Met-tRNA binding reaction by fraction C2d (see Fig. 1). Apparently, both of these fractions contain the eIF-2 activity. Of the remaining components, only fraction D2(c + d) demonstrated significant Met-tRNA<sub>Met</sub> binding activity (Table III). The reaction with this component, however, was independent of GTP and, under the 5 mM Mg<sup>2+</sup> condition of the ribosome binding assay (Table II and Fig. 7), this fraction could not replace C2e or C2d in functioning as an eIF-2-like component. On SDS-gel electrophoresis, factor C2e showed a considerable number of bands, with the predominant ones having molecular weights of 36,000 and 50,000 (data not shown).

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Functional Characterization of Factors D2a (eIF-4B) and D2b (eIF-4A)—The resolution of these components on an AcA 44 column is shown in Fig. 6. The two peaks of protein synthesis activity correspond to molecular weights of 80,000 and 50,000, respectively (Fig. 3). The fraction from which these components were obtained, D2(a + b), is required for the mRNA-dependent augmentation of the formation of 80 S ribosome-Met-tRNA^Met complexes (Fig. 7), suggesting that one or both of the subfractions are involved in mRNA attachment reactions. This characteristic would place these factors in the eIF-4 series (12). The specific designations of these components as eIF-4B and eIF-4A are based on the molecular weight analogy to the reticulocyte factors. On SDS-acrylamide gels, factor D2b (eIF-4A) is homogeneous (Fig. 4, lane b), migrating with a molecular weight of 55,000. Factor D2a (eIF-4B) showed a substantial number of bands with no obviously major component (data not shown).

Functional Characterization of Factor C1—Factor C1 has no clear counterpart among the components of the reticulocyte system. In the Met-tRNA^Met binding reaction, this factor is required for the mRNA-catalyzed augmentation of the formation of 80 S ribosome-Met-tRNA^Met complexes (Fig. 7), suggesting a function in a reaction necessary for mRNA attachment to ribosomes. In an earlier study (7), we showed that amino acid incorporation is inhibited by increased concentration of K^+ and that this inhibition varied with the mRNA being translated. In addition, we demonstrated that the mRNAs whose translation was inhibited to a greater extent by the high salt concentration were weaker in competitive translation (see below) and were more susceptible to inhibition by pmG (7). Sensitivity to competitive translation and to pmG inhibition is a likely consequence of a situation in which an mRNA attachment reaction is rate-limiting to translation. By analogy, the high K^+ effect might also be a consequence of the same reaction, becoming rate-limiting to translation. Supplementing the reaction with a component functioning in the mRNA attachment reaction might then be expected to reverse the high salt inhibition and to augment the translation of an mRNA whose translation was being competitively inhibited. The data of Tables IV and V test this idea for the high K^+ inhibition. In an assay with limiting TMV-RNA (Table IV), 62.5 mM KCl was optimum, and the addition of the different factors, either alone or in combinations of two (C1 + D1, C2b + D1, C2e + D1), had no effect (data shown only for factor C1). When the monovalent K^+ concentration was increased, either with KCl or KOAc, incorporation was inhibited. Addition of factor C1 now reversed the inhibition and in fact established an optimum at an increased salt concentration. The data for competitive translation and to pmG inhibition are presented in Table VI. The experimental conditions are designed so that the presence of AlMV-RNA 4 inhibits globin mRNA translation by 80-90%. Addition of factor C1 now reversed the inhibition and in fact established an optimum at an increased salt concentration. Supplementing the reaction with the other components of the translation system did not reverse the inhibition except for factor D1 (Table V). Further purification of this factor on DEAE-cellulose (D1-DE) abolished its stimulatory activity. The reversal by factor C1 was also obtained at a saturating level of TMV-RNA, again more strikingly when the salt was raised to an inhibitory level (Table IV).

Typical results for an mRNA competition assay are presented in Table VI. The experimental conditions are designed so that the presence of AlMV-RNA 4 inhibits globin mRNA translation by 80-90%. Addition of factor C1 reverses this inhibition and in sufficient amount can completely restore globin translation to the noncompetitive level (data not shown). Tests of the other fractionated components indicated
some activity in fractions D1 and C2(b + c). Resolved factor C2b and factor D1 purified on DEAE, however, lacked any stimulatory activity.

Chromatography of fraction C1 on a column of Sephadex G-150 is shown in Fig. 8. Either 1 mM MgAcz or 2 mM urea had to be included in the chromatography to prevent the activity from spreading throughout the elution. The major peak of activity (tubes 21–23) had a specific activity twice that of the starting C1 in the assay for 1H-amino acid incorporation, and its molecular weight calculated from gel filtration on AcA 44 was 115,000 (Fig. 3). SDS-acrylamide gel electrophoresis showed three prominent bands of molecular weights ranging between 45,000 and 50,000 (Fig. 4, lanes d and e).

### TABLE IV

Inhibition of translation by increased monovalent salts and reversal of the inhibition by factor C1

| Component added | 0.75 μg TMV-RNA | 2.5 μg TMV-RNA |
|-----------------|----------------|----------------|
| Control         | 0.75 μg TMV-RNA | 2.5 μg TMV-RNA |
| +Factor C1      | 0.75 μg TMV-RNA | 2.5 μg TMV-RNA |
| KCl mM          | Control +Factor C1 | Control +Factor C1 |
| KOAc mM         | Control +Factor C1 | Control +Factor C1 |
| 62.5            | 13.4 15.8 63 60 | 0.75 μg TMV-RNA |
| 62.5            | 45 24.4 64 89 | 2.5 μg TMV-RNA |
| 77.5            | 1.2 11.1 18 51 | 2.5 μg TMV-RNA |
| 92.5            | 6.0 19.7 45 67 | 2.5 μg TMV-RNA |

### Table V

Factor specificity in the reversal of high salt inhibition

The S-23 translation system was used with 0.75 μg of TMV-RNA at 78.5 mM KC1, 45 mM KOAc, 1.8 mM MgAcz, and D2b. 3 The functions of these two factors can therefore be relegated to reactions in which mRNA attaches to the ribosome.

### Table VI

Effect of various factors in reversal of the competitive inhibition of the translation of globin mRNA

Experiment 1 was carried out at 65 mM KCl, 45 mM KOAc, 2.0 mM MgAcz, and Experiment 2 at 82.5 mM KCl, 1.8 mM MgAcz, both using the standard S-23 translation system. In Experiment 2, separate noncompetitive translations resulted in the synthesis of 202 pmol of AlMV-coat and 121 pmol of globin or a noncompetitive globin/AlMV ratio of 0.60.

This report together with the accompanying report (3) describe the resolution of 10 separate protein factors, all of which are required for amino acid polymerization. Two of these, C2b and C2a, catalyze the poly(U)-dependent polymerization of phenylalanine and are consequently designated as elongation factors EF-1 and EF-2. None of the other components can replace these two factors in the poly(U)-catalyzed reaction. Thus, the remaining factors appear to be functioning in the initiation of the protein chain.

With regard to specific functions, the strong requirement for factor C2e in all reactions in which Met-terNAF binding is involved (Tables II and III and legend to Fig. 7) makes it likely that this component is eIF-2, the factor that forms a ternary complex with Met-terNAF and GTP (1, 2, 14). The stimulation of the formation of 80S ribosome-Met-terNAF complexes by the combination of C1, D2(a + b), and mRNA and the dependence of this stimulation on all three of these components (Fig. 7) suggest that at least two of the factors are functioning in the mRNA attachment reaction. The unique ability of factor C1 to reverse the inhibition of translation by high salt (Tables IV and V) and to promote the translation of a "competitively inhibited" mRNA (Table VI) provides strong support for a function of this factor at the level of mRNA interaction. In addition, in an assay that measures directly the binding of radioactive mRNA to 40 S ribosomes, we have found a strong requirement for factors C1 and D2b. The functions of these two factors can therefore be relegated to reactions in which mRNA attaches to the ribosome. When chromatographed on Sephadex G-150, factor C1 has two peaks of activity (Fig. 8). We are currently trying to determine the difference between these fractions.

The primary impetus for the resolution of the different protein synthesis components is our intent to analyze the process of mRNA binding. Several attempts to delineate the details of this process with partially resolved components have indicated the necessity to first obtain total resolution and purification of the different components. Two points illustrate these considerations. The first is the finding that Met-terNAF binding to the 40 S ribosome is not required for mRNA binding. Proving this requires a system strongly dependent upon eIF-2, a situation only now attained in the

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3. Seal, A. Schmidt, and A. Marcus, manuscript in preparation.
wheat germ system (see Table I). A second illustration is our finding that factor D2(c + d) is strongly required for binding of mRNA to 40 S ribosome subunits. This observation was clearly at variance with the observation (see accompanying report) that factor D2(c + d) functions in the attachment of the 60 S ribosome subunit to a preformed 40 S ribosome complex. We have now resolved component D2(c + d) into factors D2c and D2d.

Throughout this work, a loss of activity that occurred during an attempted purification often indicated the existence of a previously unrecognized component. As a consequence, established purification procedures had to be modified or abandoned. Nevertheless, the achievement of extensive reconstitution (Table I) is an indication of the usefulness of this approach. An obvious additional advantage is that we can be reasonably certain that the components used in studying partial reactions are indeed functional in the protein synthesis process.

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