Purification and Properties of Ribosome-Releasing Factor†

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ABSTRACT: Release of ribosomes from mRNA is dependent on two heat-stable factors, namely, elongation factor G (EFG) and ribosome-releasing factor (RR). RR factor has been purified 500-fold by ammonium sulfate fractionations, DEAE-cellulose, CM-Sephadex, CM-cellulose column chromatography, and Sephadex G-50 gel filtration. The purified material was electrophoretically homogeneous. Molecular weight of RR was determined to be approximately 18,000 by a Sephadex G-100 gel filtration method. When ribosomes were released from ^{14}C-labeled mRNA *in vitro* with purified EFG, purified RR, GTP, and puromycin, no change in the size of mRNA was observed, indicating that these two factors do not hydrolyze mRNA during the release of ribosomes. Polyphenylalanine formation catalyzed by polysomes was stimulated at least 4-fold by pretreatment of polysomes with RR, EFG, puromycin, and GTP. These results indicate that ribosomes released by RR and EFG were active in participating in polyphenylalanine synthesis, and that the presence of these factors is necessary for the release of ribosomes from naturally occurring mRNA. Approximately equimolar amounts of EFG and RR participate in the release of ribosomes.

In preceding communications (Hirashima and Kaji, 1972), it has been reported that for the release of ribosomes from mRNA (run-off of ribosomes), two heat-stable factors, GTP and puromycin, are required. One of these factors was identified as EFG[1] and the other was named RR. The ribosome-bound tRNAs were also released during the release of ribosomes from mRNA and the released ribosomes are active in promoting protein synthesis progammed by MS2-phage RNA. Furthermore, the ribosomes released in this fashion undergo dissociation into their subunits in the presence of dissociation factor (Subramanian and Davis, 1970) or initiation factor IF3 (Sabol *et al.*, 1970). The release of ribosomes from mRNA by these factors is dependent on the presence of puromycin, indicating that ribosomes with nascent polypeptidyl-tRNA are not released from mRNA. With the use of inhibitors, it has been shown that these two factors have to be present simultaneously for the release of ribosomes from mRNA. These results suggest that these factors may release ribosomes at the ends of cistrons to enable the ribosomes to participate in a new round of protein synthesis. In this communication we report purification of RR factor of about 500-fold. With the use of purified RR and EFG factor, it has been shown that the size of mRNA remains the same during the release of ribosomes from mRNA. Approximately equimolar amounts of RR and EFG participate in the release of ribosomes from mRNA.

Materials and Methods

Preparation of Polysomes and EFG. Polysomes were obtained from growing *Escherichia coli* Q13 in an identical fashion to that described in the preceding paper (Hirashima and Kaji, 1972). Although the polysomes had originally been prepared in the presence of tetracycline, subsequent experiments indicated that omission of this antibiotic did not influence the results. Isolated polysomes were stored in liquid nitrogen until used. The activity of polysomes did not decrease for at least 3 months under these conditions.

Preparation of Electrophoretically Pure EFG from E. coli. Partially purified EFG from *E. coli*, prepared according to the method of Lucas-Lenard and Lipmann (1966), was used as a starting material. Purification of EFG was performed according to the methods developed by Parmeggianni (1968), Kaziro and Inoue (1968), and Leder *et al.* (1969). From partially purified EFG the proteins which precipitated between 40 and 60% saturation of $(NH_4)_2SO_4$ were collected. The precipitates were suspended to a final concentration of 4.1 mg/ml in 8 ml of 10 mM potassium phosphate buffer (pH 7.0) containing a mM dithiothreitol (buffer A). The solutions were centrifuged at 2500*g* for 15 min to remove the denatured proteins. Supernatant fluids were dilayzed for 6 hr *vs.* 880 ml of buffer A. The dialyzed solution (10 ml, containing 31.4 mg of protein) was placed on a hydroxylapatite column (1.4 × 16 cm) which had been equilibrated with buffer A. The column was washed with 100 ml of buffer A, and proteins were eluted with a linear gradient of 10–100 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The gradient was established with the use of 150 ml of a solution containing 100 mM potassium phosphate (pH 7.0) as a reservoir and a mixing chamber containing 150 ml of 10 mM potassium phosphate buffer (pH 7.0). The elution was carried out at a flow rate of 9.4 ml/hr and fractions (4 ml) were collected. Fractions containing the peak of EFG activity (fractions 50–54) were pooled, and the pooled fraction was precipitated with $(NH_4)_2SO_4$ (70% saturation). The precipitate was suspended in 3.5 ml of a solution which was 50% saturated with $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.1) and 2 mM dithiothreitol (buffer B). The final pH of the solution was adjusted to neutrality by the addition of NH_4OH. The protein precipitate which did not dissolve during the above treatment was further subjected to extraction with a solution which was 40% saturated with $(NH_4)_2SO_4$ in buffer B. The proteins extracted by the solution containing 40% saturation of $(NH_4)_2SO_4$ were dialyzed overnight against 1 l. of 10 mM Tris-HCl (pH 7.8) and 1 mM dithiothreitol. Denatured proteins were removed by centrifugation at 2500*g* for

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[1] Abbreviations used are: EFG, elongation factor G; RR, ribosome-releasing factor.

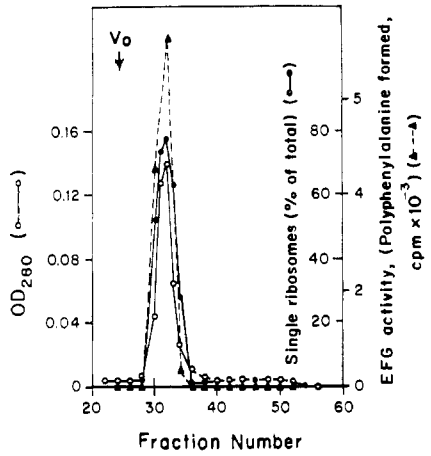

FIGURE 1: Sephadex G-100 column chromatography of purified EFG. The experimental conditions for the Sephadex G-100 column chromatography are described in the text. The reaction mixture (50 μl) for the assay of EFG in the polyphenylalanine formation system is described in the text, except that it contained 88 μg of well-washed ribosomes, 6 μg of EFT, and 2 μl of each fraction. The [^{14}C]polyphenylalanine formed after 20-min incubation at 30° (▲) was plotted against the fraction number. The ability of EFG to release ribosomes from mRNA (●) in the presence of RR factor (0.18 μg) was assayed as described in the text, except that each fraction (15 μl) was used instead of EFG. The amount of single ribosomes expressed as the percentage of total ribosomes was plotted against the fraction number. The absorbancy at 280 mμ (○) was measured directly and plotted against the fraction number. V_0 indicates the void volume of the column.

10 min. The solution (1.06 ml), containing 2.3 mg of protein, was diluted with 2 ml of the buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 1 mM $MgSO_4$, and 1 mM dithiothreitol (buffer C), placed on a DEAE-cellulose column (1.5 × 14.5 cm) which had been equilibrated with buffer C. The column was washed with 47 ml of buffer C with a flow rate of 12.6 ml/hr. After the unadsorbed protein was washed away, the EFG was eluted with a linear gradient of KCl (50–300 mM) in 10 mM Tris-HCl (pH 7.8), 1 mM $MgSO_4$, and 1 mM dithiothreitol (buffer D). The gradient was established with the use of 70 ml of 300 mM KCl in buffer D as a reservoir, and 70 ml of 50 mM KCl in the same buffer in the mixing chamber. The proteins were eluted with a flow rate of 15.9 ml/hr and fractions (1.1 ml) were collected. The peak of EFG activity (fractions no. 94–110) was pooled. The pooled fraction was concentrated and dialyzed against 1 l. of the buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 1 mM $MgSO_4$, and 1 mM dithiothreitol (buffer E) overnight. Denatured proteins were removed by centrifugation at 2500g for 10 min. The solution (1 ml) containing 390 μg of protein/ml was placed on a Sephadex G-100 column (superfine grade) (1.4 × 31 cm) which had been equilibrated with buffer E. The proteins were eluted with buffer E with a flow rate of 3.8 ml/hr and 0.72 ml/tube was collected. The peak of EFG activity (fractions 30–33) was pooled and concentrated by exposing to dry Sephadex G-200. The solution was then dialyzed overnight against 500 ml of buffer A which contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, 50 mM NH_4Cl, and 6 mM β-mercaptoethanol. Denatured protein was removed by centrifugation and the solution (180 μg of protein/ml) was stored in liquid nitrogen. For the assessment of homogeneity of EFG, polyacrylamide gel electrophoresis was carried out on a 7.5% acrylamide gel in 0.025 M Tris–0.192 M glycine buffer (pH 8.4). After electrophoresis with a current of 4 mA/tube for 100 min, the protein was stained. The EFG preparation was electrophoretically homogeneous. Further evidence for the homogeneity of this EFG was obtained from the observation that it was eluted as a single symmetrical peak from a Sephadex G-100 (superfine grade) gel filtration column as shown in Figure 1. As noted in this figure, the ultraviolet (uv) absorption profile coincided with EFG activity measured by its ability to promote polyphenylalanine formation in the presence of EFT (elongation factor T). The uv absorption also coincided with the ability to release ribosomes from mRNA in the presence of RR factor.

Ribosomes. Ribosomes washed three times with 1 M NH_4Cl were prepared from *E. coli* as described previously (Igarashi and Kaji, 1967).

Preparation of [^{14}C]Uracil Pulse-Labeled Polysomes and Extraction of mRNA from Polysomes. [^{14}C]Uracil-labeled polysomes were prepared as described previously (Hirashima and Kaji, 1972), except that [^{14}C]uracil was given for 75 sec at 37° to the culture of *E. coli* Q 13. The solution (2.18 ml) containing 62,580 cpm (14,550 cpm/OD_{260} unit) of [^{14}C]-uracil pulse-labeled polysomes/ml was mixed with 0.44 ml of 60 mM sodium acetate (pH 5.1), 60 mM EDTA, and 1.2% sodium dodecyl sulfate. To 2.6 ml of this solution was added 2.6 ml of water-saturated phenol. The mixture was shaken for 5 min, and the upper aqueous phase was subjected to a second extraction with phenol. The aqueous layer, containing RNA, was dialyzed against 500 ml of 1 mM potassium acetate buffer (pH 5.6) for 1.5 hr. The dialyzed solution was concentrated to 0.8 ml with dry Sephadex (G-200) and again dialyzed for 3 hr against 1.5 l. of 1 mM potassium acetate buffer (pH 5.6). The solution thus obtained had 92,500 cpm of [^{14}C]RNA/ml and was stored in liquid nitrogen until used. A similar procedure was used for extracting mRNA from polysomes after the release of ribosomes.

Preparation of rRNA. For the preparation of rRNA, a solution (2 ml) containing 200 mg of ribosomes, which had been washed once with 1 M NH_4Cl as described previously (Igarashi and Kaji, 1967), was mixed with 2 ml of 10 mM sodium acetate buffer (pH 5.1) containing 0.1 M NaCl, 0.005 M EDTA, and 0.2% of sodium dodecyl sulfate. The suspension was mixed with an equal volume of water-saturated phenol, and the aqueous phase, containing RNA, was withdrawn and extracted again with an equal volume of phenol. The aqueous phase was mixed with two volumes of absolute ethanol and potassium acetate was added to this mixture to a final concentration of 1%. The mixture was kept standing at −20° for 2 hr. The RNA fraction precipitated by ethanol was washed again with ethanol and potassium acetate, and suspended in 20 mM sodium acetate buffer (pH 5.1) containing 0.1 M NaCl. The insoluble material was removed by centrifugation. The RNA solution thus obtained was dialyzed against 1 l. of the same acetate buffer for 6 hr. For further purification of rRNA, 0.4 ml of the RNA solution (256 OD_{260}/ml) was placed on a 27-ml linear sucrose gradient (2.5–15%) in the sodium acetate buffer (pH 5.1) as described above, and centrifuged at 24,000 rpm for 15 hr at 4° in a Beckman SW 25.1 rotor. Peaks of 16S and 23S RNA were pooled, precipitated with alcohol, suspended in distilled water, and dialyzed against distilled water overnight.

Preparation of Ribosomes Derived from Polysomes. For the preparation of run-off ribosomes derived from polysomes, a solution (1.25 ml) containing 11.26 OD_{260} units of polysomes, 10 mM Tris-HCl (pH 7.6), 8.2 mM $MgSO_4$, 80 mM NH_4Cl, 6 mM β-mercaptoethanol, 3.2 mM phosphoenolpyruvate, 0.16 mM GTP, 0.84 mM puromycin, 39 μg of

pyruvate kinase, 2.95 μg of RR factor, and 7 μg of EFG was incubated for 20 min at 30°. The reaction mixture was then placed on a Sephadex G-200 column (0.9 × 27 cm) which had been equilibrated with buffer F containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, 50 mM NH_4Cl, and 6 mM β-mercaptoethanol. Elution was carried out with the same buffer at a flow rate of 4.3–5.4 ml/hr, and fractions (0.6 ml/tube) were collected. The fractions containing ribosomes were detected by measuring the OD_{260} absorbancy of eluates. The fractions containing ribosomes (tubes 8–9) were pooled and were used for polyphenylalanine formation programmed by poly(U). In some cases RR or EFG was omitted from the reaction mixture. In these cases, polysomes, instead of run-off ribosomes, were present in the preparation.

Reaction Mixture for Polyphenylalanine Formation. The reaction mixture (0.55 ml) for formation of polyphenylalanine under the direction of poly(U) contained 50 mM Tris-HCl (pH 7.8), 13.4 mM $MgSO_4$, 60 mM NH_4Cl, 1 mM dithiothreitol, 0.2 mM GTP, 3.6 mM phosphoenolpyruvate, 13 μg of pyruvate kinase, 11,400 cpm of phenylalanyl-tRNA (250 cpm/μg of tRNA), 40 μg of poly(U) (ammonium salt), 4 μg of EFG, 34.5 μg of EFT prepared according to Lucas-Lenard and Lipmann (1966), and various amounts of ribosomes. The mixture was incubated for 10 min at 30° and the [^{14}C]polyphenylalanine formed was measured by counting the radioactivity insoluble in hot (95°) trichloroacetic acid.

Assay of RR. The reaction mixture (0.25 ml) for the assay of RR factor contained 10 mM Tris-HCl (pH 7.4), 8.2 mM $MgSO_4$, 80 mM NH_4^+, 6 mM β-mercaptoethanol, 3.2 mM phosphoenolpyruvate, 0.16 mM GTP, 0.05 mM puromycin, 7.8 μg of pyruvate kinase, 1.9 OD_{260} units of polysomes, 19 μg of partially purified EFG, and various amounts of RR factor. The reaction mixture was incubated for 15 min at 30°. After the incubation, 0.22 ml of the reaction mixture was placed on a 5-ml linear sucrose density gradient (15–30%) in buffer A and centrifuged at 38,000 rpm for 55 min in a Beckman SW 50.1 rotor at 4°. One unit of RR factor activity was defined as the amount of enzyme which converts 2 OD_{260} units of polysomes to single ribosomes in 15 min at 30° under these conditions.

Gel Electrophoresis. For assessment of homogeneity of RR factor disc electrophoresis was carried out in the absence of urea, according to Reisfeld *et al.* (1962) and Davis (1964). After the sample was applied, electrophoresis was carried out with a current of 4 mA/tube for 3 hr. After electrophoresis proteins in the gel were stained with 1% Amido Black 10B in 7.5% acetic acid for 1.5 hr, and then destained in a 7.5% acetic acid solution. For the detection of acidic proteins, gel electrophoresis was carried out in the absence of urea on a 7.5% acrylamide gel (Tris-HCl, pH 8.7) according to the method of Davis (1964).

Results

Purification of RR Factor. STEP I. PREPARATION OF SOLUBLE CRUDE EXTRACT FROM *E. coli*. Commericial *E. coli* Q 13 cells (450 g wet weight) were ground in a mortar with 900 g of alumina and extracted with 900 ml of the buffer B containing 10 mM Tris-HCl (pH 7.8), 10 mM magnesium acetate, 60 mM KCl, and 6 mM β-mercaptoethanol. To this extract was added deoxyribonuclease I (0.5 μg/ml). After incubation at 0° for 15 min, the extract was centrifuged at 30,000*g* for 20 min, and the supernatant was saved. The precipitate was reextracted with 100 ml of buffer B, and the extract (100 ml) was combined with the supernatant. The combined extract was incubated for 30 min at 35° in a solution containing 55 mM Tris-HCl (pH 7.8), 11 mM magnesium acetate, 60 mM KCl, 0.92 mM ATP, 0.092 mM phosphoenolpyruvate, 312 μg of pyruvate kinase, 11 mM β-mercaptoethanol, and 29 μM each of 20 amino acids. The mixture was centrifuged at 30,000*g* for 30 min and the supernatant fluid was further centrifuged at 150,000*g* for 3.5 hr to remove ribosomes. The upper 80% of the supernatant was withdrawn. A small portion of the supernatant fluid was dialyzed against buffer F and used for the assay of RR activity. The major portion of this supernatant was subjected to further purificaion as described in step II.

STEP II. AMMONIUM SULFATE FRACTIONATION. For further purification proteins which were precipitated from the supernatant obtained in step I at 45–70% saturation of $(NH_4)_2SO_4$ were collected and dialyzed against buffer G (10 mM Tris-HCl (pH 7.8), 20 mM NH_4Cl, 1 mM $MgSO_4$, and 6 mM β-mercaptoethanol). For the assay of the activity of this fraction, a portion of this fraction was dialyzed against buffer F.

STEP III. DEAE-CELLULOSE COLUMN CHROMATOGRAPHY AND HEATING. The ammonium sulfate fraction prepared above (150 ml containing 3.9 g of protein) was placed on a DEAE-cellulose column (2.5 × 65 cm) which had been equilibrated with buffer G. The column was washed with buffer G with a flow rate of 15 ml/hr until uv-absorbing materials were washed away. During the washing procedure, fractions of 10 ml were collected in each tube. The RR activity of the eluate (wash) was assayed, and fractions having the peak of RR activity (fractions 21–50) were pooled, and concentrated to 40 ml with a Diaflo PM10 filter (Amicon Co.). The solution, containing 5 mg of protein/ml, was heated to 60° for 5 min. Denatured protein was then removed by centrifugation and the solution was concentrated to 20 ml. This solution was then dialyzed against 1.5 l. of buffer H containing 10 mM Tris-HCl (pH 8.0), 20 mM NH_4Cl, 1 mM $MgSO_4$, 0.5 mM dithiothreitol, and 5% glycerol.

STEP IV. CM-SEPHADEX COLUMN CHROMATOGRAPHY. The solution (20 ml containing 118 mg of protein) obtained in step III was placed on a CM-Sephadex C-50 column (2.5 × 60 cm) which had been equilibrated with buffer H. Fractions of 5 ml were collected at a flow rate of 24 ml/hr. The RR activity was assayed and fractions containing peak activity of RR were pooled (fractions 80–110). Pooled fractions were concentrated with dry Sephadex powder to 100 ml (containing 11.2 mg of protein). The solution was dialyzed overnight against 4 l. of buffer I (10 mM potassium acetate buffer (pH 5.0), 20 mM NH_4Cl, 0.5 mM dithiothreitol, and 5% glycerol).

STEP V. CM-CELLULOSE COLUMN CHROMATOGRAPHY. The dialyzed solution obtained in step IV (47.5 ml containing 11.25 mg of protein) was placed on a CM-cellulose column (0.9 × 27 cm) which had been equilibrated with buffer I. The column was washed with 90 ml of the same buffer at a flow rate of 20 ml/hr. Fractions of 2.5 ml/tube were collected. Proteins were eluted with a linear gradient of NH_4Cl (0.02–0.4 M) in buffer containing 10 mM potassium acetate (pH 5.0), 0.5 mM dithiothreitol, and 5% glycerol. The gradient was established by the use of 80 ml of buffer I in the mixing chamber and 80 ml of buffer I containing 0.4 M NH_4Cl in the reservoir. The elution was carried out at a flow rate of 12 ml/hr, and fractions (2 ml/tube) were collected. The uv absorption profile of the eluted proteins, as well as the distribution of RR activity, are shown in Figure 2. Of the two major protein peaks eluted by the gradient, only one peak, eluting at approximately 0.24 M NH_4Cl, contained RR activity. There was no

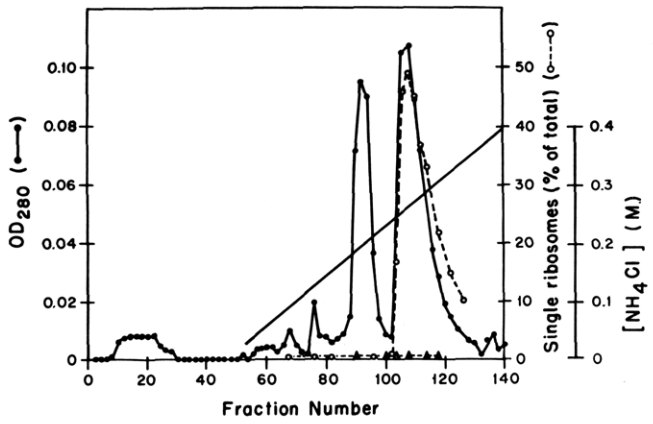

FIGURE 2: CM-cellulose column chromatography of RR factor. The experimental procedures are given in the text. Absorbancy at 280 mμ (●) and RR activity (○) are plotted against the fraction number. For the assay of RR activity, 2 μl of each fraction was used in the standard reaction mixture (0.25 ml). (▲) RR activity in the absence of EFG; —, concentration of NH_4Cl.

releasing activity in the absence of EFG, as shown in the figure. Fractions containing RR activity (104–120) were pooled and concentrated with dry Sephadex powder. The concentrated material was dialyzed overnight against buffer J (10 mM Tris-HCl (pH 7.6), 50 mM NH_4Cl, 10 mM $MgSO_4$, and 0.5 mM dithiothreitol).

STEP VI. SEPHADEX G-50 GEL FILTRATION. The solution containing RR factor (1.25 ml containing 1.17 mg of protein) was placed on a Sephadex G-50 (fine grade) column (1.3 × 45 cm) which had been equilibrated with buffer J. The protein was eluted with buffer J at the flow rate of 10 ml/hr and 0.6-ml fractions were collected. The elution pattern of protein, as well as RR activity, are shown in Figure 3.

A summary of the purification procedure is shown in Table I. It is noted that approximately 500-fold purification of the specific activity was achieved with a yield of 1.5%. Efficient purification was achieved in essentially two steps, namely, DEAE-cellulose and CM-Sephadex column chromatography.

Polyacrylamide Gel Electrophoresis of Purified RR Factor. To examine the purity of RR factor obtained in the last step of purification, the material was subjected to gel electrophoresis at pH 4.5. As shown in Figure 4, only one protein band was observed under these conditions. To test for possible contamination by an acidic protein in the preparation, a similar electrophoresis was carried out at pH 8.7 in Tris–glycine buffer in the absence of urea. Although the data are not shown, no stainable protein was observed in the gel under these conditions. One can conclude from these results that after the purification procedures described above, RR factor was electrophoretically homogeneous.

Molecular Weight of Partially Purified RR Factor. In the experiment illustrated in Figure 5, an approximate molecular weight of RR factor was determined by the use of gel filtration techniques (Whitaker, 1963). A preparation of RR factor

TABLE I: Purification of RR Factor.

Step	Fraction	Protein (mg)	Total Unit (× 10^2)	Sp Act. (Unit/mg of Protein)	Yield (%)
1	Supernatant	7776	1165	15	100.0
2	$(NH_4)_2SO_4$ fraction	3965	793	20	68.1
3	DEAE-cellulose chromatography	210	618	294	53.0
4	CM-Sephadex chromatography	11.2	220	2000	18.9
5	CM-cellulose	2.47	75	3036	6.4
6	Sephadex G-50	0.21	17	8100	1.5

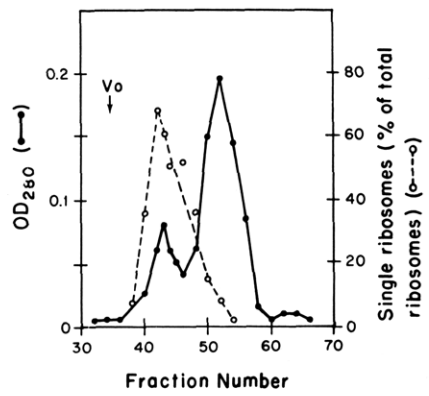

FIGURE 3: Sephadex G-50 column chromatography of RR factor. The experimental conditions are described in the text. Absorbancy at 280 mμ (●) and RR activity (○) are plotted against the fraction number. V_0 indicates the void volume of the column. For the assay of RR activity the contents of the standard reaction mixture (0.25 ml) are described as in the text, except that 2 μl of each fraction obtained from this column were used as a source of RR factor.

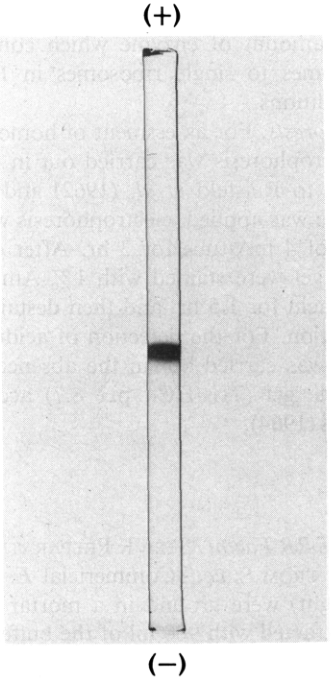

FIGURE 4: Polyacrylamide gel electrophoresis of RR factor. Purified RR factor (after step VI, 10 μg of protein in 0.15 ml of buffer F containing 0.5 M sucrose) was layered between the electrophoresis buffer (0.07 M β-alanine, pH 4.5) and top of the gel (8-cm 10% acrylamide in KOH–acetic acid buffer, pH 4.5). Electrophoresis was carried out with a current of 4 mA/tube for 3 hr. The movement of the protein was from the anode (top) to the cathode (bottom).

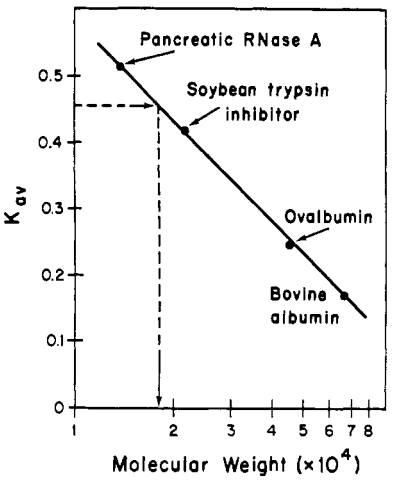

FIGURE 5: Molecular weight determination of RR factor by Sephadex G-100 gel filtration. Gel filtration was carried out according to the method of Whitaker (1963). Partially purified RR factor (after step V) (1.0 mg in 1.0 ml of the buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, and 6 mM β-mercaptoethanol) was placed on a Sephadex G-100 (superfine grade) column (1.3 × 36 cm). Elution of the protein was carried out at 4° with the same buffer at the flow rate of 3.7–4 ml/hr, and fractions (0.6 ml/tube) were collected. As a standard for the molecular weight determination of RR factor, 1 mg each of pancreatic ribonuclease A, soybean trypsin inhibitor (from Worthington Biochemical), ovalbumin (from Matheson Coleman) and bovine serum albumin (Armour Laboratory) were placed on the column, and were eluted in identical fashion. $K_{av} = (V_e - V_0)/(V_x - V_0)$, where V_e is elution volume of the sample, V_x is column volume, and V_0 is void volume of the column. The K_{av}'s of the samples were plotted against the log of the molecular weight of the proteins used as standard. (----→) K_{av} of RR and the corresponding molecular weight of RR.

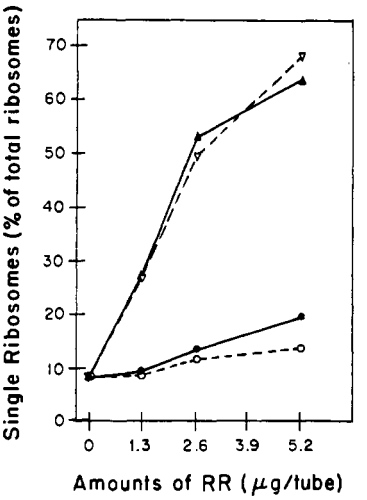

FIGURE 6: The effect of trypsin on RR factor. (—) Dose response curve. For the treatment of RR factor with trypsin, 15 μg of RR factor in 50 μl if buffer F were mixed with 1 μl of a solution containing 1.5 μg of trypsin, and incubated at 30° for 45 min. To ensure that trypsin digested the RR protein in the mixture, an additional 1.5 μg of trypsin in 1 μl was then added to the mixture and incubation was continued at 30° for 4.5 hr, and then at 25° for 13 hr. After the incubation period, 30 μg of soybean trypsin inhibitor was added and the RR activity of the mixture was assayed (●). In some cases, trypsin was omitted during the treatment, namely, only trypsin inhibitor was added (△), or both trypsin and trypsin inhibitor were omitted (▲). For the assay of treated and nontreated RR factor, the reaction mixture (0.25 ml) was as described in the text, except that it contained 11 μg of partially purified EFG and various amounts of RR factor as indicated in the figure. The amount of single ribosomes, expressed as the percentage of total ribosomes after 15-min incubation of the reaction mixture, is plotted against the amount of added RR factor. (○) RR activity of the mixture of trypsin (3 μg) and trypsin inhibitor (30 μg).

after step V was subjected to a Sephadex G-100 (superfine grade) gel filtration and the elution volume of the RR factor was compared with those of proteins of known molecular weight (Andrews, 1964). The elution volumes of bovine albumin, ovalbumin, soybean trypsin inhibitor, and pancreatic ribonuclease A are indicated. From the elution volume of RR factor, the molecular weight was estimated to be approximately 18,000.

Loss of RR Activity upon Trypsin Treatment. The behavior of RR factor during the purification procedure described above strongly suggested that the active principle was protein. However, a possibility existed that nonprotein material might be bound strongly to a protein and this material was responsible for RR activity. In order to eliminate such a possibility, the experiment indicated in Figure 6 was carried out. In this experiment, RR factor was treated with trypsin overnight and the activity was compared with the control RR factor. It is clear from this figure that trypsin treatment almost completely abolished the activity. A control experiment indicated that a mixture of trypsin and trypsin inhibitor did not have any effect on the formation of run-off ribosomes from polysomes. One can therefore conclude that the active principle of RR factor requires protein, or is itself a protein.

Lack of Ribonuclease Activity of RR Factor. In the preceding communication (Hirashima and Kaji, 1972), it has been shown that during the release of ribosomes from mRNA, RR factor and EFG did not convert mRNA to acid soluble material, suggesting that the possible ribonuclease activity of these factors is not responsible for the conversion of polysomes into single ribosomes. Since our assay method for the release of ribosomes from mRNA was dependent on the conversion of polysomes to single ribosomes, it appears important for us to establish that even the size distribution of mRNA does not change during this conversion of polysomes into single ribosomes. This would establish unequivocally that the conversion is due to the release of ribosomes from mRNA and not to the partial breakdown of mRNA to a smaller size. In the experiment shown in Figure 7, naturally occurring mRNA labeled with [^{14}C]uracil was isolated and the effect of EFG and RR factor on the size distribution of the mRNA was studied. As shown in this figure (A,B) no appreciable change in the size distribution of isolated mRNA due to RR and EFG was observed. We can therefore conclude that these two factors together with GTP do not have any ribonuclease activity. In other experiments shown in this figure (C,D), the labeled mRNA isolated was mixed with nonlabeled polysomes in the presence and absence of the two factors. The size distribution of labeled mRNA was then examined. It is clear from this figure that the size of mRNA was not influenced by the addition of EFG and RR factor. However, it was noticed in this experiment that incubation of labeled mRNA with nonlabeled polysomes shifted the distribution of mRNA toward lighter positions, suggesting that ribonuclease activity of polyribosomes may make the mRNA somewhat smaller (compare A and C, and B and D). In order to determine if the slight shift in the size distribution of mRNA was responsible for the conversion of polysomes into single ribosomes, the experiment indicated in Figure 8 was performed. In this experiment, polysomes were pulse labeled with [^{14}C]uracil and the size distribution of mRNA

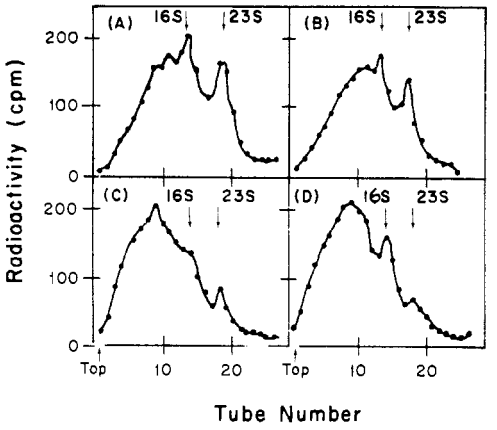

FIGURE 7: Lack of ribonuclease activity of RR factor and EFG in the presence of polysomes. The complete reaction mixture (0.25 ml) for the assay of ribonuclease activity of RR factor and EFG was identical with that for the assay of RR activity, except that it contained 1.9 OD_{260} units of unlabeled polysomes, 0.23 μg of purified RR factor, 1 μg of purified EFG, and 8000 cpm of [^{14}C]-uracil-labeled mRNA isolated as described in the text. The reaction mixture was incubated at 30° for 15 min. After the incubation, the solution was mixed with 50 μl of buffer containing 60 mM sodium acetate (pH 5.1), 60 mM EDTA, and 1.2% sodium dodecyl sulfate. From this mixture, RNA was isolated by the phenol extraction method. The RNA was dialyzed against 1 l. of 10 mM sodium acetate buffer (pH 5.1) containing 50 mM NaCl and 5 mM EDTA for 2.5 hr. The dialysate containing labeled RNA was placed on a 5-ml linear sucrose density gradient (5–20%) in the same buffer that was used for the dialysis. The tubes were centrifuged at 50,000 rpm for 210 min at 4° in a Beckman SW 50.1 rotor. After the fractions were collected from the top of the tube, the acid-insoluble radioactivity of the fractions was measured and plotted against the tube number. (A) Unlabeled polysomes, RR factor, and EFG were omitted from the complete system; (B) unlabeled polysomes were omitted; (C) RR and EFG were omitted; (D) complete reaction mixture. Sedimentation was from left to right.

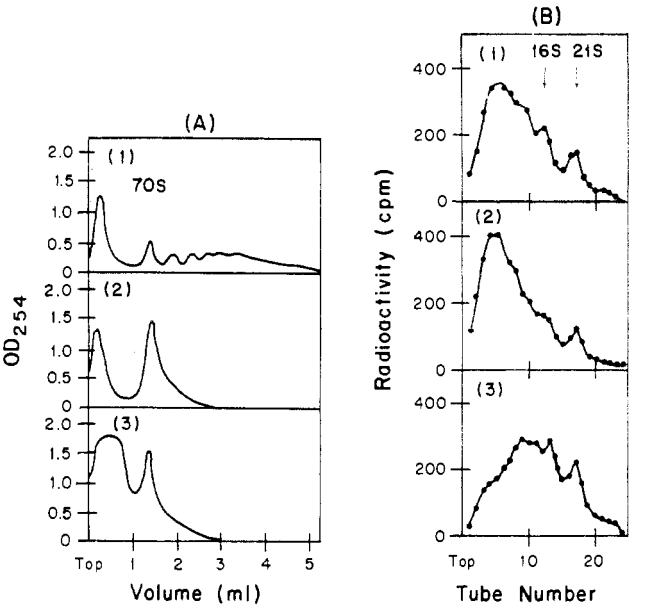

FIGURE 8: Evidence of preservation of mRNA during the release of ribosomes from mRNA by RR factor and EFG. For the conversion of labeled polysomes into single ribosomes, the reaction mixture (0.5 ml) was identical with the standard complete reaction mixture, except that it contained 3.9 OD_{260} units of [^{14}C]uracil pulse-labeled polysomes (containing 16,000 cpm of mRNA). The reaction mixture was incubated at 30° for 15 min and divided into two parts at the end of the reaction. One part was used to detect the conversion of polysomes into single ribosomes as described in the text, the other was used for the extraction of mRNA from the polysomes as described in Figure 7. (1) RR and EFG factors were omitted from the complete reaction mixture; (2) complete reaction mixture; (3) 3.25 OD_{260} units of rRNA added to the complete reaction mixture. (A) Sedimentation behavior of ribosomes after the treatment; (B) sedimentation behavior of mRNA isolated from labeled polysomes used in part A.

from the polysome was examined under the condition where polysomes were either incubated alone or converted into single ribosomes in the presence of EFG and RR factor. It is clear from this figure (B1 and B2) that the size distribution of mRNA after most of the polysomes were converted to single ribosomes was almost identical with size distribution of mRNA from the polysomes which had been incubated in the absence of these factors. These results strongly indicated that the change of size distribution of mRNA is not related to the conversion of polysomes into single ribosomes. To establish this point further, the conversion of polysomes into single ribosomes was carried out in the presence of a large amount of nonlabeled rRNA to inhibit the possible ribonuclease activity of the ribosomes. As shown in Figure 8A (3), the presence of nonlabeled rRNA did not inhibit the release of ribosomes from mRNA at all, but the size distribution of mRNA after the conversion of polysomes (B3) into single ribosomes indicated that during the release of ribosomes from mRNA no endonucleolytic cleavage of the mRNA takes place.

Effect of Pretreatment of Polysomes with RR Factor on Polyphenylalanine Formation. If the notion is correct that RR factor is required for the relase of ribosomes from mRNA, one can expect that this factor is requird or stimulatory for the polyphenylalanine formation catalyzed by the ribosomes that are defived from polysomes. The ribosomes in the polysomes would first have to be released in order to participate in the polyphenylalanine formation programmed by poly(uridylic acid). This is indeed the case, as illustrated in the experiment shown in Figure 9. In this experiment, polysomes were treated with puromycin in the presence or absence of RR factor of EFG. The run-off ribosomes thus made, as well as polysomes, were isolated free from puromycin, and polyphenylalanine formation by these ribosomes were examined under the condition where the amount of polyphenylalanine formed was proportional to that of ribosomes added. It is noted in this figure that where ribosomes are limiting, the pretreatment of the polysomes with both EFG and RR factor resulted in an approximately 4-fold stimulation of polyphenylalanine formation. A possible reason why the polysomes that were not pretreated had approximately one-fourth of the activity of the pretreated ribosomes is that the polysome preparation had already contained some monosomes (approximately 25% of the total ribosome was monosomes). Those monosomes which were already present would function for polyphenylalanine synthesis while those in the polysomes would first have to be released before they participate in polyphenylalanine.

Quantitative Relationship between RR Factor and EFG for the Release of Ribosomes from mRNA. In an attempt to obtain the molar ratio between RR factor and EFG necessary for the release of ribosomes from mRNA under our conditions, various amounts of EFG factor were added to a reaction mixture containing a constant amount of RR factor. As shown in Figure 10, the amount of ribosomes released increased linearly as the purified EFG was increased, reaching a plateau at approximately 1.08 μg of EFG/reaction mixture. Assuming that the molecular weights of RR factor and

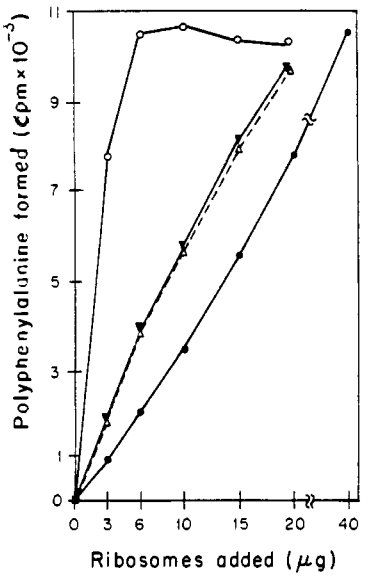

FIGURE 9: Polyphenylalanine formation by ribosomes released from naturally occurring polysomes. Preparation of ribosomes released from polysomes by RR factor and EFG is as described in the text. The reaction mixture for polyphenylalanine formation by these ribosomes is also described in the text. The amount of [^{14}C]polyphenylalanine formed in a 10-min incubation is plotted against the amount of ribosomes used. (1) (○) Ribosomes derived from polysomes by the action of RR factor and EFG; (2) (△) polysome preparation treated as described in (1) except for the omission of RR factor; (3) (▲) polysome preparation treated as described in (1) except for the omission of EFG; (4) (●) ribosomes washed three times with 1 M NH_4Cl as described in the text.

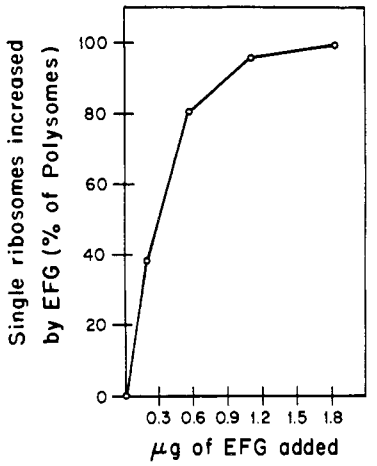

FIGURE 10: The reaction mixture (0.25 ml) for the release of ribosomes from mRNA was as described in the text, except that it contained 1.52 OD_{260} units of polysomes, 0.18 μg of purified RR factor (after the final step of the purification procedure), and various amounts of purified EFG prepared as in Figure 1. The reaction mixture was incubated for 15 min as 30° and the sedimentation behavior of ribosomes was analyzed as described in the text.

EFG are 18,000 and 84,000, respectively, approximately 12.8 nmoles of EFG would saturate 10 nmoles of RR factor. These calculations indicate that approximately 1 mole of RR factor would require about 1 mole of EFG for its maximum activity and further addition of EFG did not result in the increase of the rate of release.

Discussion

The process of polypeptide synthesis can be divided into three major steps, namely chain initiation, chain elongation and chain termination (Kaji, 1970; Lucas-Lenard and Lipmann, 1971). In an *E. coli* cell-free system for polypeptide synthesis, at least nine soluble factors are known to participate in these steps. There are three initiation factors, IF_1 (A), IF_2 (C), and IF_3 (B) (Iwasaki *et al.*, 1968; Revel *et al.*, 1968) which promotes formation of the initiation complex of formylmethionyl-tRNA, initiation codon and the 30S ribosomal subunits. The chain elongation steps are catalyzed by three elongation factors EFG, EFTu, and EFTs (Lucas-Lenard and Lipmann, 1966). The final step (the termination step) is catalyzed by a codon-specific chain-releasing factor RF1, RF2, and an additional factor RF3 (S) (Caskey *et al.*, 1969; Capecchi and Klein, 1969). Although RR factor described here has not been proven to be distinct from all of these known factors, various biochemical behaviors of RR tend to suggest that RR is different from any of these factors (Hirashima and Kaji, 1972, and in preparation).

After the polypeptide chain is released by RF_1, RF_2, and RF_3, the complex of ribosome and tRNA would remain bound to the mRNA at the end of the cistron. The exact fate of this termination complex remains to be elucidated. It has been suggested that the ribosomes will be released from mRNA as subunits (Mangiarotti and Schlessinger, 1966; Schlessinger *et al.*, 1967; Kaempfer *et al.*, 1968; Philips *et al.*, 1969; Kaempfer, 1971) while others maintain that ribosomes are released from mRNA as 70S ribosomes which would then be dissociated into their subunits by IF_3 (Kohler *et al.*, 1968; Algranati *et al.*, 1969; Subramanian and Davis, 1971). We would like to propose that RR factor described in this paper functions to release ribosomes from the termination complex. The substrate for RR factor and EFG is the complex of tRNA, ribosomes, and mRNA, which is similar to the termination complex. Although data described in this paper were obtained with the "artificial termination complex" which was prepared from polysomes by the action of puromycin, preliminary experiments suggest that RR and EFG do indeed release the ribosomes from the termination complex involving RNA of R17 amber mutant phage. The data presented in this and other communications are consistent with the notion that ribosomes are released by RR factor and EFG as 70S ribosomes but they do not rule out the possibility that they are released as subunits which would then associate rapidly to form 70S ribosomes.

It should be noted that the "termination complex" contains tRNA which corresponds to the COOH end amino acid of the completed polypeptide chain. It was for this reason that a factor called TR (tRNA-releasing factor) was postulated (Ishitsuka and Kaji, 1970). On the other hand, separate studies indicated that tRNA was released simultaneously with the release of ribosomes from mRNA by RR factor and EFG (Hirashima and Kaji, 1972, in preparation). A recent observation that run-off ribosomes did not contain tRNA agrees with this observation (Tai and Davis, 1972). It is therefore no longer necessary to postulate an additional factor such as TR. Furthermore, TR factor, upon purification, was identified as ribonuclease II and polynucleotide phosphorylase indicating that it has no direct bearing on the process of chain termination (Ishitsuka and Kaji, 1972).

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Fractionation of Nucleic Acids by Isoelectric Focusing†

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ABSTRACT: The basis of a fractionation of nucleic acids on gel electrofocusing has been investigated with two model systems: (1) dinucleoside phosphates and trinucleoside diphosphates and (2) isoaccepting species of tRNA. Many of the oligonucleotides and all of the tRNA species examined here (Phe, Arg, fMet, from *Escherichia coli*) formed stable banding patterns in the pH 3–5 range. Most oligonucleotides focused essentially as single bands whose apparent p*I*'s seemed consistent with an isoelectric fractionation arising from protonation of ring nitrogens in A and C. The banding patterns obtained with different tRNA species could not readily be attributed to differences in primary structure. Each of several highly purified isoaccepting species of tRNA gave rise to complex, but rather similar banding patterns. The amino acid accepting activity of components separated from tRNA fMet decreased progressively with decreasing p*I*. Although the possibility of artifact arising from binding to ampholyte could not be excluded, much of the evidence suggests that the multiple forms represent stable conformers, arising from a progressive, but reversible, denaturation of the tRNA during the electrofocusing procedure. Possible explanations for the reversible denaturation and for the apparently anomalous p*I* range of the multiple forms are discussed.

Recent demonstration of viable mRNA preparations capable of programming the synthesis of specific proteins lead to the hope that several eukaryotic mRNA species will soon be isolated and characterized. However, the purification to homogeneity of discrete species of messenger or other trace RNA molecules may prove difficult in view of the limited choice of high-resolution procedures for fractionating nucleic acids of similar size but different primary structure. One of the parameters that may be used to define and to demonstrate charge differences inherent in the primary structure of many biopolymers is the isoelectric point (p*I*) of the molecule. Recently, the procedure of isoelectric focusing has been adapted for fractionating macromolecules according to differences in their p*I* (Kolin, 1955; Svennson, 1961; Vesterberg and Svennson, 1966; Haglund, 1971). In this procedure, amphoteric molecules migrate under an electric field in stable pH gradients where they equilibrate or "focus" at their p*I*. Isoelectric focusing generally offers superior resolution to

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