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The Role of G Factor in Protein Synthesis. Studies on a Temperature-Sensitive *Escherichia coli* Mutant with an Altered G Factor*

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ABSTRACT: A temperature-sensitive *Escherichia coli* mutant with a specific defect in a supernatant factor required for protein synthesis has been studied. The altered factor has been identified as G by DEAE-Sephadex column chromatography: in a polyuridylic acid-directed cell-free system the addition of wild-type G restores the ability of the mutant supernatant to synthesize polyphenylalanine. When NH_4Cl-washed ribosomes and purified T factor from the mutant are incubated in the presence of polyuridylic acid, guanosine triphosphate, and [^{14}C]phenylalanine transfer ribonucleic acid, the binding of aminoacyl transfer ribonucleic acid to ribosomes proceeds normally. Paper chromatography of the products of the binding reaction shows the presence of a dipeptide peak with trace amount of tripeptides. The addition of wild-type G to the binding mixture results in a decrease of the dipeptide peak with parallel increase of tripeptides and longer polypeptide chains. Experiments with puromycin indicate that ribosomes from the mutant are not impaired in their ability to form a peptide bond with the antibiotic. In conclusion washed ribosomes from the mutant are able to synthesize a single peptide bond. Chain elongation depends upon the addition of G factor prepared from wild type.

In bacterial extracts distinct factors for initiation (Stanley *et al.*, 1966; Eisenstadt and Brawerman, 1966; Revel and Gros, 1966; Clark and Marcker, 1965; Brown and Doty, 1968), elongation (Nakamoto *et al.*, 1963; Lucas-Lenard and Lipmann, 1966), and termination (Capecchi, 1967; Ganoza and Nakamoto, 1966; Takanami and Yan, 1965; Bretscher *et al.*, 1965) have been described. Our knowledge of the number of protein factors required for polypeptide synthesis has been growing very fast but, so far, the genetic approach to the problem has been neglected.

Several laboratories have isolated temperature-sensitive mutants of *Escherichia coli* (Kohiyama *et al.*, 1966; S. Brenner, personal communication) and strains with altered aminoacyl-

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tRNA synthetases (Neidhardt, 1966) and ribosomes (Apirion, 1966) have also been described. In a previous paper (Tocchini-Valentini and Mattoccia, 1968), we reported the isolation of a temperature-sensitive *E. coli* mutant with a specific defect in a supernatant factor required for protein synthesis. This altered factor is necessary for phenylalanine polymerization in a poly U directed cell-free system starting from aminoacyl-tRNA. Its heat stability and property of binding tightly to the ribosomes are reminiscent of factor G[1] (Lucas-Lenard and Lipmann, 1966).

Kinoshita *et al.* (1968) found that a fusidic acid resistant mutant of *E. coli* possesses an altered G factor and similar results have been independently obtained in our laboratory (G. M. Rinaldi, unpublished results).

In the present paper we provide chromatographic evidence that the altered factor in the temperature-sensitive mutant extract coincides with G. The alteration affects both polymerizing and ribosome-linked GTPase activities of the factor. To investigate the role of G factor in the polymerization process, we used a cell-free system derived from the mutant, where a negligible amount (if any) of active G is bound to the ribosomes.

Materials and Methods

The origin of the wild-type and mutant strains, the growth medium, and the preparation of S-30 were described in a previous paper (Tocchini-Valentini and Mattoccia, 1968). *E. coli* stripped tRNA (General Biochemicals) was charged with [^{14}C]Phe (Schwarz BioResearch, 350 mCi/mmole) as reported by Conway (1964). GTP-γ-^{32}P at a specific activity of 2 Ci/mmole was supplied by International Chemical Nuclear Corp. (the contaminating $^{32}P_i$ was reduced from 1 to 0.3% by stepwise DEAE-cellulose column chromatography (Moffat, 1964)).

Preparation of S-150 and Ammonium Sulfate Fraction. The S-30 was centrifuged at 105,000*g* for 3 hr. The upper two-thirds of the supernatant was aspirated and recentrifuged at 150,000*g* for 4 hr. The upper layer resulting from the second centrifugation constitutes the S-150 fraction.

The fraction of S-150 precipitating between 40 and 70% saturation with ammonium sulfate was dissolved in 0.01 M Tris-HCl (pH 7.4) containing 0.001 dithiothreitol and dialyzed against the same buffer overnight.

Ammonium Chloride Washed Ribosomes. Ribosomes pelletted from S-30 were washed with NH_4Cl as described by Nishizuka and Lipmann (1966a).

DEAE-Sephadex A-50 Column Chromatography. A DEAE-Sephadex column (1.25 × 25 cm) was equilibrated with 0.010 M Tris-HCl (pH 7.4), 0.15 M KCl, and 0.001 M dithiothreitol. The dialyzed 40–70% ammonium sulfate fraction (100–150 mg) was layered on the top and eluted with a linear KCl gradient (0.15–0.35 M). The mixing chamber contained 300 ml of 0.15 M KCl and the reservoir 300 ml of 0.35 M KCl, both in 0.010 M Tris-HCl (pH 7.4) and 0.001 M dithiothreitol. The flow rate was about 0.3 ml/min.

Assay for Phenylalanine Polymerization. The standard mixture contained in a total volume of 0.25 ml: 12.5 μmoles of Tris-HCl (pH 7.4), 40 μmoles of NH_4Cl, 2.5 μmoles of magnesium acetate, 3 μmoles of dithiothreitol, 10 μg of Poly U, 50 mμmoles of GTP, 50 μg of [^{14}C]Phe-tRNA (16 $\mu\mu$moles of [^{14}C]Phe), 200 μg of NH_4Cl-washed ribosomes, and supernatant factors as indicated. The mixture was incubated at 30° for 15 min and the reaction was stopped with 5% cold trichloroacetic acid. The tubes were heated at 100° for 10 min, and the precipitate was collected on Millipore filters and counted in a Packard TriCarb scintillation spectrometer using Bray's solution (Bray, 1960).

Binding Assay. The binding mixture was the same as for the polymerization assay. After incubation at 30°, the reaction was stopped with 3 ml of a cold buffer containing 0.01 M Tris-HCl (pH 7.4), 0.010 M magnesium acetate, and 0.16 M NH_4Cl. The diluted mixture was immediately filtered through Millipores, as described by Nirenberg and Leder (1964). The filters were dissolved in Bray's solution and counted as above.

Assay for GTPase Activity. The procedure was similar to that described by Nishizuka and Lipmann (1966a). The reaction was started with the addition of GTP-γ-^{32}P and incubation was carried out at 30° for 15 min.

Analysis of the Products of the Binding Reaction. After incubation, 1 ml of binding mixture was quickly chilled in ice, layered over 1 ml of 10% sucrose containing 0.010 M Tris-HCl (pH 7.4), 0.010 M magnesium acetate, and 0.16 M NH_4Cl, and centrifuged at 150,000*g* for 4 hr. Hydrolysis of ribosomal pellets was carried out with 0.3 N KOH at room temperature for 15 hr. The samples were neutralized with HCl, spotted on Whatman No. 3MM paper with appropriate standards, and subjected to descending chromatography in 1-butanol saturated with 2 M ammonium hydroxide. After a run of 10–12 hr, the strips were dried, cut into pieces (1 × 3.5 cm), and counted in Bray's solution in a Packard TriCarb.

Reaction with Puromycin of Polyphenylalanine-Charged Ribosomes. Ribosomes from S-30 were charged with [^{14}C]-Phe, as described by Maden *et al.* (1968), and washed five times by repeated resuspension and recentrifugation in 0.01 M Tris-HCl (pH 7.4), 0.010 M magnesium acetate, and 0.5 M NH_4Cl. Polyphenylalanine-charged ribosomes were incubated with puromycin in 0.05 M Tris-acetate (pH 7.2), 0.01 M magnesium acetate, and 0.1 M NH_4-acetate at 30° for 10 min. The extent of reaction was estimated by the *m*-cresol assay (Maden *et al.*, 1968).

Results

Our first attempt was to find suitable conditions for reducing the residual polymerizing activity of the mutant extract. Preincubation of the mutant supernatant at either 30 or 37° and storage at −20° were effective in reducing the ability of the mutant to synthesize polyphenylalanine but the same treatments had no effect on the supernatant derived from the wild type. The time course of phenylalanine polymerization by using S-150 from the wild type and the mutant shows that, at shorter times of incubation, the amount of hot trichloroacetic acid precipitable material produced by the mutant is negligible compared with the wild-type control (Figure 1). In this experiment S-150 from the mutant was preincubated at 30° for 60 min.

Identification of the Altered Factor as G by DEAE-Sephadex Column Chromatography. A 40–70% ammonium sulfate preparation obtained from wild-type S-150 was fractionated on a DEAE-Sephadex column by using a 0.15–0.35 M KCl gradient (Figure 2). A good resolution of T and G factors was achieved

[1] Tu, Ts, and G are the supernatant factors described by Lucas-Lenard and Lipmann (1966).

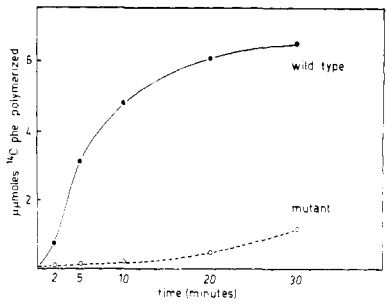

FIGURE 1: Time course of polyphenylalanine synthesis in the presence of S-150 from wild type and mutant, 1 ml of standard mixture (see assay for phenylalanine polymerization), containing 0.8 mg of NH_4Cl-washed ribosomes from wild type and 0.4 mg of S-150 from either wild type or mutant, was incubated at 30°. Samples (0.2 ml) were taken at different times for measuring radioactivity incorporated into hot trichloroacetic acid insoluble material. The lower curve represents polymerizing activity of mutant S-150 after being preincubated at 30° for 60 min.

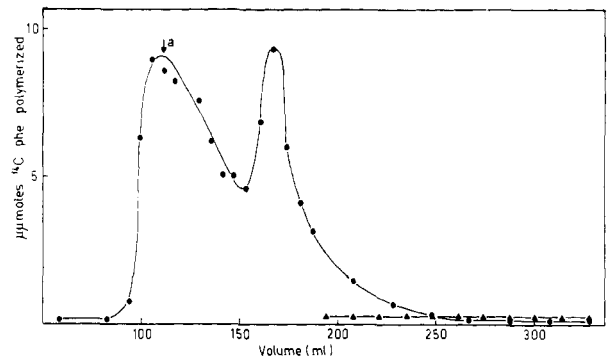

FIGURE 3: DEAE-Sephadex column chromatography of 40–70% ammonium sulfate fraction from mutant. Column fractions (0.020 ml) were tested for their ability to polymerize phenylalanine, as described in Methods. Polymerizing activity of fractions: (●—●—●) in the presence of 4 μg of b from wild type and (▲—▲—▲) in the presence of 12 μg of a from mutant.

as demonstrated by the small peak of residual polymerizing activity eluted between 150 and 200 ml. The T factor was eluted first as two peaks containing a mixture of Ts and Tu; the G factor was eluted later as a single broader peak. The S-150 from the mutant was complemented only by the fractions contained in the G area (Figure 2). On the other hand, when a 40–70% ammonium sulfate preparation from the mutant S-150 was fractionated on a DEAE-Sephadex column (Figure 3), only the peak corresponding to T factor could be detected and no fraction able to complement T was found in the G area.

The addition of wild-type G to NH_4Cl-washed ribosomes resulted in a twofold stimulation of GTPase activity (Table I), but the corresponding fraction prepared from the mutant gave no stimulation at all.

Binding of Phenylalanyl-tRNA to the Ribosomes. When NH_4Cl-washed ribosomes and S-150 from the mutant were incubated with poly U, GTP, and [^{14}C]Phe-tRNA, the binding of aminoacyl-tRNA to ribosomes proceeded normally to saturation levels (Figure 4, upper curve). In the same conditions negligible amounts of hot trichloroacetic acid insoluble material were formed (Figure 4, lower curve). The addition of G factor from the wild type resulted in a rapid onset of phenylalanine polymerization. The mutant supernatant, therefore, contains the protein factors required for the binding of aminoacyl-tRNA to ribosomes (Tu and Ts): G is not involved in the binding step but in the polymerization process.

Analysis of the Products of the Binding Reaction. In these experiments we decided to substitute S-150 with purified T in order to reduce the extent of Phe-tRNA hydrolysis. A clear stimulation of Phe-tRNA binding (data not shown) was observed by adding increasing amounts of purified T. A three- to fourfold stimulation was constantly found at saturation levels. Paper chromatography of the products of the binding reaction was run after alkaline hydrolysis of the ribosomes.

Figure 5 shows the radioactivity profile when either wild-type or mutant ribosomes were incubated with purified T from the wild type. A dipeptide peak and trace amounts of tri- and tetrapeptides were always found, irrespective of the source of ribosomes (Figure 5, clear area).

In order to reduce contamination of the system with small amounts of active G, T factor was prepared from the mutant and incubated with ribosomes as before (Figure 6). A clear

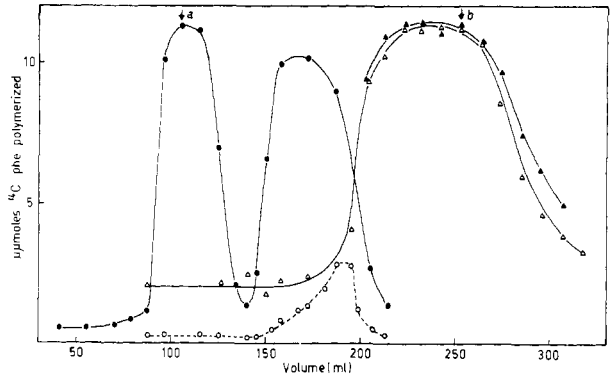

FIGURE 2: DEAE-Sephadex column chromatography of 40–70% ammonium sulfate fraction from wild type. Column fractions (0.020 ml) were tested for their ability to polymerize phenylalanine, as described in Methods. (○- - -○- - -○) Residual polymerizing activity of fractions. Polymerizing activity of fractions: (●—●—●) in the presence of 4 μg of b; (▲—▲—▲) in the presence of 16 μg of a; and (△—△—△) in the presence of 150 μg of mutant S-150.

TABLE I: Ribosome-Linked GTPase Activity of G Factor from Wild Type and Mutant.[a]

	^{32}P Hydrolyzed (mμmoles)
Ribosomes	0.220
Wild-type G	0.081
Wild-type G + ribosomes	0.550
Mutant G	0.020
Mutant G + ribosomes	0.245

[a] The reaction mixture (see GTPase assay in Methods) contained, in a final volume of 0.25 ml, 4 mμmoles of GTP-γ-^{32}P, 100 μg of NH_4Cl-washed ribosomes from wild type, and 5 μg of G factor.

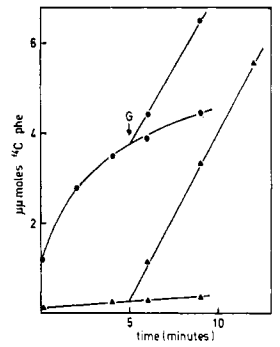

FIGURE 4: Effect of G factor on polymerizing activity of S-150 from mutant; 2 ml of standard mixture (see assay for phenylalanine polymerization), containing 1.6 mg of NH_4Cl-washed ribosomes from wild type and 0.8 mg of S-150 from mutant, were incubated at 30°. Samples of 0.25 ml were taken at different times and the reaction was stopped with 3 ml of cold buffer for binding assay or 3 ml of cold 5% trichloroacetic acid for polymerization assay. (●—●—●) μμmoles of [^{14}C]Phe bound to ribosomes (see binding assay) and (▲—▲—▲) μμmoles of [^{14}C]Phe precipitated with hot trichloroacetic acid (see polymerization assay).

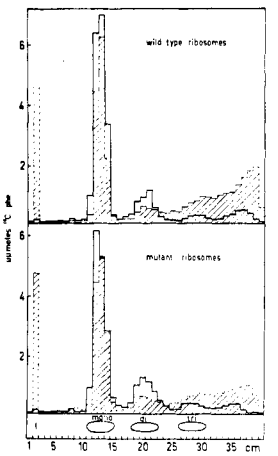

FIGURE 5: Paper chromatography of the products of the binding reaction in the presence of T factor from wild type; 1 ml of scaled-up binding mixture (see binding assay in Methods), containing 0.8 mg of NH_4Cl-washed ribosomes from either wild type or mutant and 130 μg of T factor from wild type, was preincubated at 37° for 5 min. The reaction was started with the addition of [^{14}C]Phe-tRNA and continued for 5 min at 30°. The subsequent steps of the procedure are described in Methods. The clear area represents the radioactivity profile in the absence of G factor, the dotted area in the presence of 1 μg/ml of G factor from wild type.

peak of dipeptides was still observed with both preparations of ribosomes but tri- and tetrapeptides were less evident (Figure 6, clear area).

We can conclude that, in the absence of G, ribosomes from the mutant are able to form a peptide bond between two molecules of Phe-tRNA as well as wild-type ribosomes. The addition of limited amounts of G factor to the binding mixture resulted in a decrease of dipeptide peak with parallel increase of tripeptides, tetrapeptides, and longer polypeptide chains which stuck at the origin (Figures 5 and 6, dotted area).

Reaction of Puromycin with Polyphenylalanine-Charged Ribosomes. Maden *et al.* (1968) have described a rapid method for measuring the puromycin release of polyphenylalanine from tRNA. The technique is based on differential solubilities of substrate and product in *m*-cresol. We prepared [^{14}C]-polyphenylalanine-charged ribosomes from wild type and mutant and studied puromycin release in the absence of supernatant factors (Table II). Both preparations of ribosomes reacted very well with puromycin, and the release values are very close to those obtained by Maden *et al.* (1968) with *E. coli* MRE 600. The results clearly show that ribosomes from the mutant are not impaired in their ability to form a peptide bond with puromycin.

Discussion

Lucas-Lenard and Lipmann (1966) have demonstrated that three soluble protein factors (Tu, Ts, and G) are required for polypeptide synthesis in bacterial systems, but the exact role of these factors in protein synthesis is still unclear. Recent studies have shown that both Tu and Ts react with GTP to form a complex with aminoacyl-tRNA and this complex is the reactive species which binds aminoacyl-tRNA to template-charged ribosomes (Ravel *et al.*, 1967; Ravel, 1967; Gordon, 1967, 1968; Lucas-Lenard and Haenni, 1968; Ertel *et al.*, 1968). Regarding the role of G factor, Nishizuka and Lipmann (1966b) have correlated its ribosome-linked GTPase activity with its stimulation of amino acid polymerization in the presence of Tu and Ts. They suggested that factor G may be involved in the translocation process which occurs during protein biosynthesis. However, when NH_4Cl-washed ribosomes are incubated with Phe-tRNA in the absence of supernatant factors (Pestka, 1968a), more than 50% of [^{14}C]Phe-tRNA can be converted into oligophenylalanine-tRNA. The oligophenylalanine product consists of 73, 16, and 9.7% of di-, tri-, and tetraphenylalanine, respectively (Pestka, 1968a). If the formation of tri- and larger peptides involves translocation processes, the above results clearly show that trace amounts of supernatant factors, G in particular, are still present on the ribosomes even after extensive washing with NH_4Cl. The possibility that a limited amount of G, tightly bound to the ribosomes, may be responsible for peptide-bond

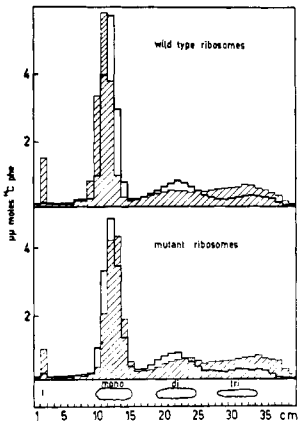

FIGURE 6: Paper chromatography of the products of the binding reaction in the presence of T factor from mutant. Incubation conditions are the same as in Figure 5, except for 120 μg of T factor from mutant instead of wild type. Clear and dotted area as in Figure 5.

TABLE II: Reaction with Puromycin of Polyphenylalanine-Charged Ribosomes from Wild Type and Mutant.[a]

Samples	[^{14}C]Phenylalanine-Charged Ribosomes			
	Wild Type		Mutant	
	$\mu\mu$-moles	% Release	$\mu\mu$-moles	% Release
Control	2.6	0	1.82	0
Puromycin (10^{-3} M)	1.24	48	0.66	64

[a] The incubation mixture contained, in a final volume of 0.1 ml, either 100 μg of polyphenylalanine-charged ribosomes from wild type or 200 μg from mutant. NH_4Cl washing of ribosomes, incubation conditions, and *m*-cresol assay are described in Methods.

formation cannot be ruled out by the experiments with salt-washed ribosomes. The same criticism can be applied to the study of puromycin reaction with 70S ribosomes (Bretscher and Marcker, 1966; Rychlik, 1966; Zamir *et al.*, 1966; Gottesman, 1967) or 50S subunit (Monro, 1967; Monro and Marcker 1967).

The occurrence of a mutant with a specific alteration of G factor provides us with a system where a negligible amount (if any) of active G bound to ribosomes is present. The analysis of the products of the binding reaction indicates that ribosomes from the mutant are able to form the first peptide bond, as well as ribosomes from the wild type. In the absence of G, there is a block of polypeptide synthesis at the level of dipeptides. Many lines of evidence (Monro *et al.*, 1967) indicate that puromycin reaction takes place by the same mechanism as peptide-bond formation in protein synthesis. When puromycin is reacted with polyphenylalanine-charged ribosomes from the mutant, 60% puromycin release occurs in the absence of any supernatant factor. It seems, therefore, that the factor responsible for peptide-bond formation is integrated into the ribosome structure and has different properties from the supernatant G (Monro *et al.*, 1967). Our data are consistent with the idea that G factor is not directly required for peptide-bond synthesis but is necessary for chain elongation (Pestka, 1968b; Erbe and Leder, 1968).

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