Escherichia coli Stringent Factor Binds to Ribosomes at a Site Different from that of Elongation Factor Tu or G†

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ABSTRACT: The binding of *Escherichia coli* stringent factor to ribosomes has been studied; the reaction depends on 50S and 30S ribosomal subunits and poly(U) as messenger RNA. The ribosome-stringent factor complex is formed at 5–10 m*M* magnesium acetate; NH_4 ions are inhibitory. Binding of the stringent factor to the 70S–mRNA complex is not stimulated by uncharged tRNA. The ribosomal binding site(s) for the stringent factor does not overlap with the one known for elongation factor Tu (EF-Tu) or G (EF-G). Ribosomes carrying either EF-Tu or EF-G are active in binding the stringent factor; however, they are inactive in synthesizing guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp). The latter result is due to the blockage of the ribosomal acceptor site by the aminoacyl-tRNA and/or elongation factors. That stringent and elongation factors do not compete for identical ribosomal region(s) is supported by: (1) the reverse experiments where ribosomes charged with the stringent factor are fully active in EF-Tu or EF-G dependent functions; (2) ribosomes that lack the two ribosomal proteins L7 and L12 known to be essential for EF-Tu and EF-G functions bind the stringent factor and are active in synthesizing pppGpp and ppGpp.

The in vitro synthesis of ppGpp[1] and pppGpp is recognized as a transfer of pyrophosphate from ATP onto the 3′-OH group of GDP or GTP; the reaction is catalyzed by a stringent factor closely associated with 70S ribosomes from stringent strains of *Escherichia coli* (Haseltine et al., 1972; Sy and Lipmann, 1973). In the presence of an excess of 5′-AMP and pppGpp this reaction can be reversed (Sy, 1974a,b). The stringent factor promotes synthesis of guanosine polyphosphates only when activated by a ribosome–mRNA complex carrying uncharged, codon-specific tRNA in the acceptor site (Pedersen et al., 1973; Haseltine and Block, 1973).

So far little is known of the function of the stringent factor at the ribosomal level. This protein factor from *E. coli* does not seem to be species specific and responds to ribosomes from *Bacillus stearothermophilus* and *Bacillus subtilis* as well as to mitochondrial or chloroplast ribosomes, but not to 80S ribosomes from the cytosol of higher organisms (Richter, 1973a; Sy et al., 1974). In stringent strains of *E. coli* and *B. stearothermophilus* the factor is associated with 70S and native 50S but not with native 30S ribosomal subunits (Ramagopal and Davis, 1974; Richter and Isono, 1974). That the stringent factor is only loosely attached to the ribosomes is indicated by its rapid release into the supernatant fraction when ribosomes are washed with NH_4Cl (Haseltine et al., 1972) or when converted into 50S and 30S subunits (Sy et al., 1973; Richter et al., 1974).

However, the binding of the stringent factor to ribosomes has not yet been reported. Formation of a ribosome–stringent factor complex would allow a study not only of the ribosome–stringent factor interaction but also of the relationship between ribosomal regions involved in stringent and elongation factor functions. The latter point is of particular interest since it is not unreasonable to expect that stringent and elongation factors interact with very closely related or even identical ribosomal regions. This would parallel recent findings where the two elongation factors, EF-Tu and EF-G, have been found to share sites on the ribosome which at least partially overlap (Cabrer et al., 1972; Miller, 1972; Richman and Bodley, 1972; Richter, 1972). On the other hand, the finding that ribosomes depleted of the two ribosomal proteins, L7 and L12, are active in guanosine polyphosphate synthesis (Richter, 1973a; Lund et al., 1973) but not in elongation factor dependent functions (Hamel et al., 1972) seems to imply that stringent and elongation factors are bound to different ribosomal regions. The results to be reported (1) describe conditions for binding stringent factor to ribosomes and (2) present direct evidence that stringent and elongation factors recognize nonidentical sites on the ribosome.

Experimental Section

Materials. Elongation factors Tu, Ts, and G were isolated from *Escherichia coli* strain CGSC 2834/a and purified by the method of Arai et al. (1972). EF-Tu and EF-G were contaminated by less than 2% with other proteins as estimated by sodium dodecyl sulfate gel electrophoreses (Weber and Osborn, 1969). Ribosomal subunits (50S and 30S) were prepared by zonal centrifugation (Eikenberry et al., 1970). [^{14}C]Phe-tRNA was prepared with a specific activity of 450 mCi/mg of tRNA. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard protein. GMPPCP[1] and poly(U) came from Miles Laboratories; AMPPNP[1] and $tRNA_{yeast}^{Phe}$

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[1] Abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; EF-Tu, elongation factor ; EF-G, elongation factor G; GMPPCP, β,γ-methyleneguanosine 5′-triphosphate; AMPPNP, β,γ-imidoadenosine 5′-triphosphate.

came from Boehringer Mannheim Corp. [α-^{32}P]GTP (specific activity 5–13 Ci/mmol) was obtained from New England Nuclear. Hydroxylapatite was purchased from Clarkson Chemical Co.; polyethyleneimine thin-layer plates (Cel 300 PEI) came from Macherey-Nagel Co., Düren, Germany; X-ray films (RP/R-54) were from Kodak, Rochester, N.Y. Sodium [^{3}H]borohydride (sp act. 6–40 Ci/mmol) was purchased from Amersham, Braunschweig, Germany. A_{260} units were calculated by measuring absorbance at 260 nm with a 1-cm cuvette against 10 m*M* Tris-HCl buffer (pH 7.4); 1 mg of ribosomes corresponds to 16 A_{260} units. Ribosome concentration was calculated by determining the ab-absorbance at 260 nm: 1 A_{260} unit of 70S ribosomes $\simeq$ 25 pmol, 1 A_{260} unit of 50S $\simeq$ 39 pmol, 1 A_{260} unit of 30S $\simeq$ 67 pmol.

Preparation and Purification of the Stringent Factor. Growth Conditions. The cells were cultured in a tryptone–glucose–yeast extract medium (Richter et al., 1974). In order to find out whether stringent factor activity depends on growth conditions, 1 g of cells obtained from the early, mid, or late logarithmic phase was homogenized in an Omnimix Microequipment (Sorvall) with 4 g of glass beads (diameter, 0.17–0.18 mm) and 1 ml of buffer 1 containing 0.2 μg/ml of DNase (Haseltine et al., 1972; 10 m*M* Tris-HCl (pH 7.8), 14 m*M* magnesium acetate, 60 m*M* KCl, and 1 m*M* dithiothreitol) for 3 × 30 sec at 4°. The homogenate was extracted with 8 ml of buffer 1, stirred for 10 min at 4°, and centrifuged at 30,000*g* for 20 min. The supernatant fraction was collected and spun at 170,000*g* for 3 hr. The ribosomal pellet was dissolved in buffer 1 and recentrifuged as above. The ribosomes were suspended in 200 μl of buffer 1 and assayed for stringent factor activity. Ribosomes obtained from cells grown to a density of 20 × 10^8 cells/ml (0.5 A_{600} unit/ml = 5 × 10^8 cells/ml in a 1-cm cuvette) showed the highest activity in guanosine polyphosphate production. The specific activities of the stringent factor prepared from cells harvested in early, mid, and late logarithmic phases were 4.9, 5.8, and 10.7 nmol/min per mg, respectively. For large scale preparations the cells were cultured in 100-l. carboys to a density of 20 to 30 × 10^8 cells/ml. The cell suspension was cooled by adding 50 l. of crushed ice and passed through a Sharples continuous flow centrifuge. The cell paste was washed twice with buffer 1. Three–four grams of cells (wet weight; centrifuged at 10,000*g* for 30 min) was obtained per liter of medium, and stored in liquid nitrogen until used.

Isolation of Crude Ribosomes. The stringent factor was released from the ribosomes by conversion of 70S into 30S and 50S subunits (Richter et al., 1974). This technique minimizes contamination by ribosomal proteins and yields stringent factor preparations more readily soluble in low salt solutions; at the same time, 50S and 30S subunits free of stringent and elongation factors can be obtained.

E. coli cells (800 g wet weight) were suspended in 2000 ml of buffer 1 with 0.2 μg/ml of DNase and homogenized in a laboratory pressure cell model 15 M (Gaulin Corp., Everett, Mass.) at 6000 psi. The homogenate was stirred at 4° for 10 min and spun at 30,000*g* for 25 min. The cell debris was reextracted with 400 ml of buffer 1 with 0.2 μg/ml of DNase and centrifuged as above. Ribosomes were pelleted from the supernatant fractions by a spin at 170,000*g* for 3 hr (Beckman rotor Ti60); each tube was rinsed three times with 8 ml of buffer 2 (10 m*M* Tris-HCl (pH 7.8), 0.1 m*M* magnesium acetate, and 6 m*M* mercaptoethanol). The ribosomal pellets were suspended in the same buffer to a concentration of 1068 A_{260} units/ml. The ribosomes were dialyzed against 4 × 4 l. of buffer 2 and either immediately processed or stored in liquid nitrogen. The yield was 25 to 30 g of crude ribosomes per 800 g of cells.

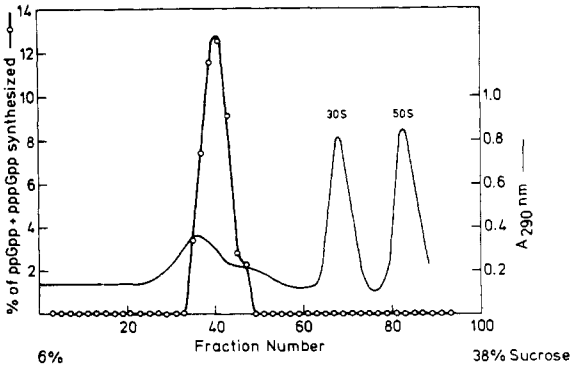

FIGURE 1: Zonal centrifugation pattern of stringent factor and ribosomal subunits.

Release of the Stringent Factor by Zonal Centrifugation. The crude ribosome suspension was adjusted with buffer 2 to 50–70 A_{260} units/ml. This solution (50 ml) was applied to a linear sucrose gradient (6–38% sucrose, w/v, in buffer 2) in a 1.8-l. zonal rotor (Spinco B XV zonal rotor) and centrifuged at 26,000 rpm for 17 hr. Fractions (18 ml) were collected from the gradient, and the absorbance at 290 nm was recorded. Aliquots (5 μl) of the fractions were assayed for stringent factor activity. A typical elution and activity profile, depicted in Figure 1, shows that the stringent factor activity is clearly separated from the ribosomal subunits and emerges as a single peak with a small shoulder ahead of the main activity peak. Fractions 35 to 47 were combined; the yield was 2150 mg of crude stringent factor protein from 800 g of cells. 50S and 30S ribosomal subunits free of stringent factor activity were collected by centrifugation at 200,000*g* for 18 hr.

Zonal centrifugation can also be carried out with higher quantities of crude ribosomes (up to 50,000 A_{260} units per gradient), but in this case, although the stringent factor is almost quantitatively released from the ribosomes, there is no separation into 50S and 30S subunits.

Ammonium Sulfate Precipitation. Ammonium sulfate (43 g) was added to each 100 ml of the crude stringent factor preparation from the zonal gradient, the solution was stirred for 20 min at 4°, and the precipitated protein centrifuged and dissolved in 4 ml of buffer (20 m*M* Tris-HCl (pH 7.8), 40 m*M* KCl, 1 m*M* dithiothreitol, and 5% glycerol, w/v). After dialysis against 2 × 2 l. of the same buffer the stringent factor was kept in liquid nitrogen in small aliquots. At this step, the yield of protein was 1750 mg from 800 g of cells.

Hydroxylapatite Chromatography. Protein (580 mg) from the ammonium sulfate fractionation was applied to a hydroxylapatite column (size 2 × 15 cm) equilibrated with 5 m*M* phosphate buffer (pH 7.8), containing 14 m*M* magnesium acetate, 60 m*M* potassium acetate, 1 m*M* dithiothreitol, and 5% glycerol (w/v). The flow rate was adjusted to 20 ml/hr and 6-ml fractions were collected. Protein was eluted stepwise with 15, 30, 60, 100, and 200 m*M* phosphate buffer containing the same components as used for equilibration. Stringent factor activity was eluted with 60 m*M* phosphate buffer; a peak with minor activity was obtained at a concentration of 100 m*M* phosphate. Fractions containing stringent factor activity were collected and

Table I: Purification Steps of the Stringent Factor.

Purification Steps	Total Protein (mg)	Specific Act. (nmol/min per mg)	Total Enzyme Units (nmol/min)
Zonal centrifugation	1810	8.5	15,385
Ammonium sulfate	1590	9.2	14,628
Hydroxylapatite	91.5	110.0	10,065
DEAE-Sephadex A-50	2.5	2520	6,300
Glycerol gradient	0.4	3910	1,564

concentrated by ammonium sulfate precipitation as described above. The yield was 30.5 mg of protein per hydroxylapatite column.

DEAE-Sephadex A-50 Chromatography. The DEAE-Sephadex column (size 1.5 × 30 cm) was equilibrated with buffer (20 m*M* Tris-HCl (pH 7.8), 2 m*M* dithiothreitol, 5 m*M* magnesium acetate, and 5% glycerol, w/v) containing 100 m*M* KCl. The stringent factor preparation from the hydroxylapatite chromatography (90 mg of protein) was applied to the column. Protein was eluted with a linear gradient of KCl from 100 to 500 m*M* (300 × 300 ml) in buffer (see above). The fractions with stringent factor activity (eluted at 200 to 300 m*M* KCl) were combined, concentrated, and kept in liquid nitrogen as described for the other purification steps. The yield was 2.5 mg of stringent factor protein.

Glycerol Gradient Centrifugation. Stringent factor protein (100 μl) containing 1 mg/ml of protein was layered on top of a 5.4-ml linear glycerol gradient (10–30%, w/v, in 20 m*M* Tris-HCl (pH 7.8), 1 m*M* dithiothreitol, and 40 m*M* KCl) and centrifuged in an SW 65 rotor at 55,000 rpm for 16 hr. Fractions of 180 μl were collected from the bottom of the tube using an Isco gradient fractionator. The main protein peak coincided with the stringent factor activity. The molecular weight was calculated to be 70,000 ± 5000. Based on sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969) the stringent factor from the glycerol gradient step was about 80% pure. The yield from 800 g of cells was 300 to 400 μg of protein. The progress of purification is described in Table I. The activity for the stringent factor was determined by the conversion of [α-^{32}P]GTP into ppGpp and pppGpp and was derived from experiments with a linear dependence on the protein factor. Table I shows that about 10% of the stringent factor activity originally present in the zonal centrifugation step was recovered in the glycerol gradient step. The main losses were obtained during chromatography. Similar low yields of purified stringent factor were reported by Cochran and Byrne (1974).

Formation of the Ribosome–mRNA Complex. The reaction was carried out in 250 μl volume, containing 1200 pmol/ml of 50S and of 30S subunits, 200 μg/ml of poly(U), 40 m*M* Tris-HCl (pH 7.8), 10 m*M* magnesium acetate, and 4 m*M* dithiothreitol. The mixture was incubated at 30° for 10 min and cooled to 4° (mixture I).

Formation and Isolation of the Ribosome–Stringent Factor Complex. Mixture I was diluted with 250 μl of mixture II; the latter contained 40 μg/ml of stringent factor (stabilized with 1 mg/ml of bovine serum albumin). The final concentrations of the 500 μl reaction mixture was 20 m*M* Tris-HCl (pH 7.8), 5 m*M* magnesium acetate, and 2 m*M* dithiothreitol. This 500-μl reaction mixture was kept at 4° for 30 min, then layered on top of 1.6 ml of a 5% sucrose solution (containing 20 m*M* Tris-HCl (pH 7.8), 5 m*M* magnesium acetate, and 2 m*M* dithiothreitol) and centrifuged at 160,000*g* for 3.5 hr (Beckman rotor Ti50). Binding of the stringent factor to the ribosome occurred at 4° as well as at 37°. In experiments where only 50S or 30S ribosomal subunits were assayed the centrifugation time was 6.5 hr. The ribosomal pellet was carefully rinsed with buffer (5 m*M* Tris-HCl (pH 7.8), 15 m*M* magnesium acetate, and 2 m*M* dithiothreitol) and finally dissolved in 50 μl of the same buffer. Aliquots were assayed for A_{260}/A_{280} absorbance and stringent factor activity. The assay for stringent factor activity was carried out in 50-μl reaction volume that contained if not otherwise indicated 100 pmol/ml of the ribosome-stringent factor complex, poly(U), tRNA^{Phe}, and salt conditions as described in the assay for ppGpp + pppGpp production (see below).

When ribosomes complexed with crude stringent factor were isolated by centrifugation and analyzed for pppGpp and ppGpp production, it was found that the guanosine pentaphosphate was preferentially synthesized; little or no tetraphosphate was made. In addition, with the isolated ribosome–stringent factor complex the time curve of pppGpp synthesis no longer showed any lag phase.

Preparation of Tritiated EF-Tu or EF-G. Both factors were radioactively labeled by the reductive methylation method (Means and Feeney, 1968) using formaldehyde and [^{3}H]borohydride (Kleinert and Richter, 1975). [^{3}H]EF-Tu or [^{3}H]EF-G (specific activity 1–10 Ci/mmol; mol wt of EF-Tu, 45,000; mol wt of EF-G, 80,000) prepared by this method were active in the polyphenylalanine synthesizing reaction as well as in EF-Tu or EF-G specific functions (Kleinert and Richter, 1975).

Binding of [^{3}H]EF-Tu or [^{3}H]EF-G to Ribosomes. In principle ribosomes were charged with EF-G or EF-Tu as reported (Richter, 1972). The ribosome–poly(U) complex (mixture I) was prepared as outlined above and complemented with [^{3}H]EF-Tu, GMPPCP, and [^{14}C]Phe-tRNA or with [^{3}H]EF-G, [^{14}C]GDP, and fusidic acid (Richter, 1972). Reaction mixture I (1 ml) was complemented with 1 ml of mixture III; the latter contained 40 m*M* Tris-HCl (pH 7.8), 4 m*M* dithiothreitol, 100 m*M* NH_4Cl, 1 m*M* GMPPCP, 2000 pmol/ml of [^{3}H]EF-Tu (sp act. 0.4 Ci/mmol), and 800 pmol/ml of [^{14}C]Phe-tRNA; the final concentrations were 5 m*M* magnesium acetate, 50 m*M* NH_4Cl, 40 m*M* Tris-HCl (pH 7.8), 0.5 m*M* GMPPCP, and 4 m*M* dithiothreitol. The combined mixtures were kept at 30° for 10 min and applied to a 12–30% linear sucrose density gradient (w/v; 18.6 × 18.6 ml in 20 m*M* Tris-HCl (pH 7.8), 2 m*M* dithiothreitol, 50 m*M* NH_4Cl, and 15 m*M* magnesium acetate) and centrifuged in an SW 27 rotor at 20,000 rpm for 14 hr. The gradient was fractionated using an Isco gradient fractionator collecting 46 × 0.8 ml fractions. Aliquots were counted for ^{3}H and ^{14}C radioactivity and were measured at A_{260}/A_{280} nm. In parallel experiments the ribosomes were precharged with 1500 pmol/ml of [^{3}H]EF-G (sp. act. 1.5 Ci/mmol), 2000 pmol/ml [^{14}C]GDP, and 3 m*M* fusidic acid (Richter, 1972). Between 0.3 and 0.45 mol of [^{3}H]EF-Tu or [^{3}H]EF-G were bound per mol of ribosome (Kleinert and Richter, 1975). The isolated ribosome–[^{3}H]EF-Tu–GMPPCP–[^{14}C]Phe-tRNA complex was stable under the conditions used for the stringent factor assay (see below) and was recovered almost quantitatively when recentrifuged.

Assay for Synthesis of Guanosine Polyphosphates. This reaction was carried out in 25 or 50 μl reaction volume (Richter and Isono, 1974; Richter et al., 1974) containing

Table II: Stringent Factor Binding to Ribosome Functions Independently of Cofactors.[a]

Exptl Conditions	Synthesis of pppGpp + ppGpp (%)
Control	48 (45)
+ ATP	49 (54)
+ GTP	52 (55)
+ AMPPNP	47 (48)
+ GMPPCP	49 (53)

[a] Binding of the stringent factor to the 70S–mRNA complex was carried out in the absence or presence of the nucleotides listed in the left column. The stringent factor–ribosome complex was isolated by centrifugation (see Experimental Section); 5 pmol of isolated 70S particles was assayed in a 50 μl reaction volume for pppGpp + ppGpp synthesis. Where indicated the various nucleotides were present at a concentration of 0.4 m*M*. The numbers in parentheses are from experiments where binding of stringent factor to ribosomes was done in the presence of 50 μg/ml of uncharged $tRNA^{Phe}$ and the nucleotides listed in the left column of the table.

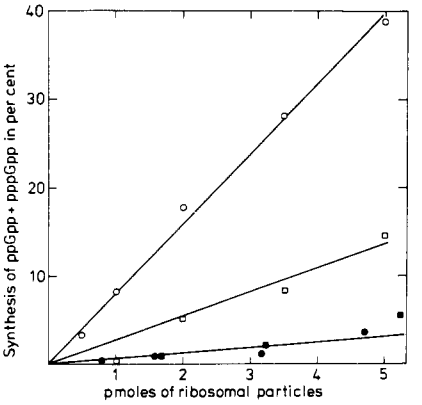

FIGURE 2: Formation of the ribosome-stringent factor complex. For detailed conditions see the Experimental Section. Stringent factor binding to ribosomes was carried out either in the presence of 50S, 30S, and poly(U) (○ — ○); of 50S and 30S (□ —□); of 50S (● — ●); or of 30S (■ — ■). The various ribosome-stringent factor complexes were isolated by centrifugation and analyzed for guanosine polyphosphate production. The abscissa gives the picomoles of ribosomal particles tested in 50 μl reaction volume.

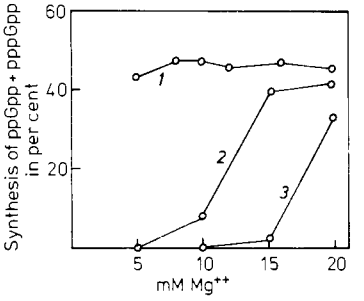

FIGURE 3: Binding of stringent factor in the presence of various ions. Formation of the ribosome-stringent factor complex was carried out at the Mg concentrations indicated. Where listed the complex was formed in the presence of 40 m*M* (2) or 200 m*M* NH_4Cl (3); in the upper curve (1) binding was performed in the absence of NH_4^+. The complexed ribosomes were isolated by centrifugation through a 5% sucrose solution (see Experimental Section) that contained the same Mg and NH_4 concentrations as used for the complex formation. In a 50-μl reaction mixture containing 20 m*M* Tris-HCl (pH 7.8), 20 m*M* magnesium acetate, 40 m*M* NH_4Cl, and 2 m*M* dithiothreitol the complexed ribosomes (100 pmol/ml reaction volume) were complemented with 20 μg/ml of $tRNA^{Phe}$, 4 m*M* ATP, and 0.177 m*M* [α-^{32}P]GTP. The mixture was incubated and analyzed for ppGpp + pppGpp production as outlined in the Experimental Section.

165 pmol/ml of 50S subunits, 180 pmol/ml of 30S subunits, 20 μg of $tRNA_{yeast}^{Phe}$/ml, 50 μg of poly(U)/ml, 4 m*M* ATP, 20 m*M* Tris-HCl (pH 7.8), 20 m*M* magnesium acetate, 0.177 m*M* [α-^{32}P]GTP, 40 m*M* NH_4Cl, 2 m*M* dithiothreitol, and if not otherwise indicated 8–10 μg/ml of purified stringent factor (glycerol or DEAE-Sephadex step). The reaction mixture was incubated at 37° for 1 or 2 hr. pppGpp and ppGpp were analyzed by PEI thin-layer chromatography; the plates were exposed to X-ray films (Cashel and Kalbacher, 1970); spots corresponding to labeled compounds were cut out and counted in Bray's solution. The conversion of radioactive GTP into pppGpp and ppGPP is given as a percentage.

Preparation of Ribosomes that Lack L7 and L12. To prepare ribosomal particles free of L7 and L12, 50S subunits were treated with ethanol-NH_4Cl (Hamel et al., 1972) and analyzed by two-dimensional gel electrophoresis according to Kaltschmidt and Wittmann (1970); the particles were free of L7 and L12 and termed 50S[−(L7/L12)]. The latter were incubated with 30S ribosomes to form 70S particles (see conditions for preparing reaction mixture I), termed 70S[−(L7/L12)], and were isolated by linear sucrose density centrifugation.

Results

Conditions for Ribosome–Stringent Factor Complex Formation. The experiments shown in Figures 2 and 3 were designed to study the conditions for binding stringent factor to ribosomes. In principle ribosomes incubated with stringent factor under various conditions were isolated by centrifugation and analyzed for ppGpp and pppGpp production. Figure 2 (upper curve) shows that optimal binding of the stringent factor to ribosomes occurred with the 70S–mRNA complex. When 50S or 30S ribosomal subunits alone were incubated with the factor and isolated by centrifugation, the stringent factor did not cosediment with the subunits (Figure 2, closed circles and closed squares). The stimulation of the formation of the ribosome–stringent factor complex by poly(U) is probably due to a stabilizing effect on the 70S -ribosomes. When analyzed on a linear sucrose gradient, stringent factor binding occurred only to the 70S and not to the 50S or 30S subunits (see also Figure 5). Based on the binding of radioactively labeled stringent factor to ribosomes it was calculated that about 0.2 to 0.3 mol of stringent factor (assuming a mol wt of 70,000) was bound per mol of 70S ribosomes; in comparison, 0.3 to 0.45 mol of EF-Tu or EF-G was bound per mol of 70S ribosomes (Kleinert and Richter, 1975).

Figure 3 shows that the ribosome–stringent factor complex is formed at rather low Mg concentrations (5 mM); in the presence of NH_4 ions little or no stringent factor was bound to the ribosome. The sensitivity of the ribosome–stringent factor complex to NH_4 ions decreased with increasing Mg concentrations. The binding of the stringent factor to ribosomes was not enhanced when ATP, GTP, AMPPNP, or GMPPCP and/or uncharged tRNA were added prior to the centrifugation step (Table II). This is in contrast to ribosomal complexes formed with elongation factors known to be dependent on cofactors.

Interaction of the Stringent Factor, EF-Tu, and EFG with Ribosomes. Several groups have reported that EF-Tu and EF-G recognize a common region on the ribosome which suggested that both factors function alternately on

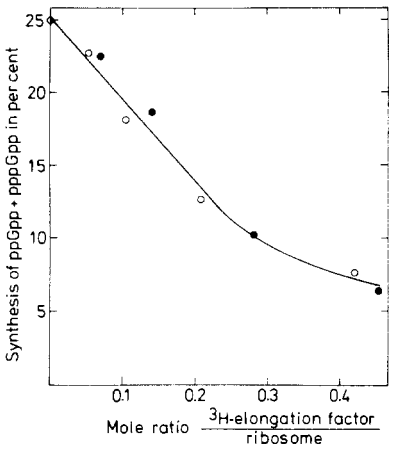

FIGURE 4: Inhibition of the stringent factor activity by ribosomal bound elongation factors. Ribosomes carrying either [^{3}H]EF-Tu, GMPPCP, and [^{14}C]Phe-tRNA or [^{3}H]EF-G-[^{14}C]GDP-fusidic acid were complemented with stringent factor and assayed for pppGpp + ppGpp production (see Experimental Section). The various mole ratios of ^{3}H-labeled elongation factor-ribosome were obtained by the addition of 70S ribosomes: (○ — ○) ribosomes carrying [^{3}H]EF-Tu, GMPPCP, [^{14}C]Phe-tRNA; (● — ●) ribosomes carrying [^{3}H]EF-G, [^{14}C]GDP, and fusidic acid.

the ribosome (Richter, 1972; Cabrer et al., 1972; Richman and Bodley, 1972; Miller, 1972). The existence of such a common ribosomal region has been demonstrated by competition experiments where ribosomes precharged with one elongation factor no longer will function with the complementary factor indicating that both factors compete for the same or overlapping site on the ribosome. In order to find out whether stringent and elongation factors make use of closely related or even identical site(s) on the ribosome, the latter, precharged with the stringent factor, was complemented with [^{3}H]EF-Tu or [^{3}H]EF-G. Table III shows that the ribosome–stringent factor complex not only was fully active in the peptide chain elongation cycle but also in binding of either of the two elongation factors. These data seemed to indicate that the stringent and elongation factor interact with different ribosomal regions. The possibility can be excluded that the stringent factor is released from the ribosome during incubation with the elongation factors. Under the experimental conditions used the ribosome–stringent factor complex was still intact after incubation (data not shown). Also, the stoichiometry of the stringent factor binding to the ribosome (see above) should be sufficiently high to cause significant inhibition of the elongation factor function. The assumption that stringent and elongation factors function on different ribosomes is controverted by experiments shown below where ribosomes carrying EF-Tu or EF-G were inactive in ppGpp and pppGpp production but active in binding the stringent factor.

Stringent Factor and EF-Tu Bind to Different Sites on the Ribosome. In order to study the reverse reaction with ribosomes carrying the elongation factor and also to show more directly the binding of the stringent factor to a ribosomal site different from that of EF-Tu or EF-G, 70S ribosomes charged with EF-Tu or EF-G were complemented with the stringent factor. As shown in Figure 4 synthesis of ppGpp and pppGpp decreased with increasing amounts of [^{3}H]EF-Tu or [^{3}H]EF-G bound per mole of ribosomes. These data seem to be in conflict with the assumption made above that stringent and elongation factor bind to different ribosomal regions. The observed inhibition of ppGpp and

Table III: Ribosomal Bound Stringent Factor Does Not Inhibit the Functions of [^{3}H]EF-Tu or [^{3}H]EF-G.[a]

	70S–Poly-(U) Complex	70S–Poly(U)–Stringent Factor Complex
1. EF-T + EF-G dependent synthesis of polyphenylalanine in pmol	4.5	4.6
2. pmol of [^{3}H]EF-Tu bound per pmol of ribosomes	0.42	0.40
3. pmol of [^{3}H]EF-G bound per pmol of ribosomes	0.38	0.38

[a] Binding of the stringent factor to ribosomes was performed as described in the Experimental Section. The isolated ribosomal complexes were analyzed either for polyphenylalanine synthesis or for binding of [^{3}H]EF-Tu or [^{3}H]EF-G (Kleinert and Richter, 1975).

pppGpp production, however, could also be due to a blocked acceptor site; several groups have reported that ribosomal bound EF-G interferes with the binding of aminoacyl-tRNA to the acceptor site (Bàliga and Munro, 1971; Richter, 1973b; Modolell and Vazquez, 1973; Modolell et al., 1973; Nombela and Ochoa, 1973). As shown by Haseltine and Block (1973) the ppGppp and pppGpp production is only triggered when uncharged tRNA can enter the vacant acceptor site on the ribosome.

In order to demonstrate that ribosomes complexed with EF-Tu were capable of binding the stringent factor, the isolated 70S–EF-Tu–GMPPCP–Phe-tRNA complex was complemented with the stringent factor and reisolated by centrifugation (70S–EF-Tu–stringent factor complex). The complexed ribosomes were dialyzed against low Mg concentrations (0.1 m*M*) and applied to a linear sucrose density gradient.

Under the ionic conditions used ribosomes carrying the stringent factor should release the latter into the top fractions of the gradient. Indeed, as depicted in Figure 5A (closed circles), the stringent factor appears in the first fractions of the gradient supporting the finding reported above that ribosomes carrying EF-Tu, GMPPCP, and Phe-tRNA do bind the stringent factor. No stringent factor activity was observed when the 70S–EF-Tu–stringent factor complex was stabilized by high Mg concentrations (Figure 5A, open circles). This indicated that the stringent factor was still bound to the 70S–EF-Tu–GMPPCP–Phe-tRNA complex and that as long as the ternary complex EF-Tu–GMPPCP–Phe-tRNA occupied the acceptor site of the ribosome no uncharged tRNA could be bound and consequently no pyrophosphate transfer could occur. In contrast, when ribosomes free of EF-Tu but charged with the stringent factor were analyzed by gradient centrifugation at high Mg concentrations the stringent factor activity coincided with the 70S peak (Figure 5B, open circles), whereas under low ionic conditions (0.1 m*M* magnesium acetate) the stringent factor was released into the top fractions of the gradient (Figure 5B, closed circles).

The experiments cited above are supported by data obtained with active tritiated stringent factor, labeled by the reductive methylation method. The radioactive factor was bound to ribosomes regardless of whether the ribosomes carried EF-Tu or EF-G (data not shown). Although the stringent factor was not free of contaminating proteins, the

Table IV: Stringent Factor Binding to Ribosomes That Lack L7 and L12.[a]

Experimental Conditions	% of pppGpp + ppGpp Synthesized	pmol of [^{3}H]EF-Tu Bound per pmol of Ribosome
70S	33.5	0.38
70S[−(L7/L12)]	33.3	0.08
70S[−(L7/L12)] + L7/L12	34.8	0.38
L7/L12	1.1	0

[a] The stringent factor or [^{3}H]EF-Tu was bound to 70S ribosomes or to 70S particles depleted of L7 and L12. The ribosome–factor complex was isolated by centrifugation through a 5% sucrose solution and then either analyzed for guanosine polyphosphate production or for [^{3}H]EF-Tu binding (for details see the Experimental Section).

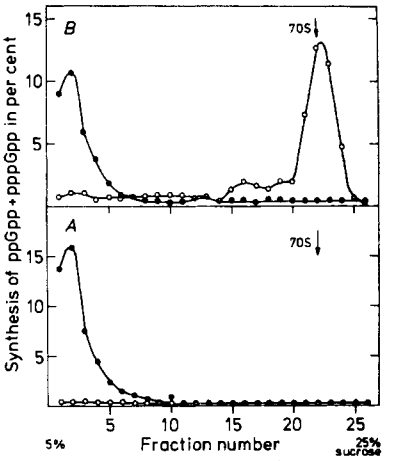

FIGURE 5: Binding of stringent factor to ribosomes precharged with EF-Tu, GMPPCP, and Phe-tRNA. The 70S-poly(U) complex (reaction mixture I) was incubated with (A) or without (B) [^{3}H]EF-Tu, GMPPCP, and [^{14}C]Phe-tRNA and was isolated by sucrose gradient centrifugation. The complexed ribosomes were charged with the stringent factor and re-isolated by centrifugation through a 5% sucrose solution. The ribosomal pellets were dissolved in and dialyzed against either buffer 2 (10 m*M* Tris-HCl (pH 7.8), 0.1 m*M* magnesium acetate, and 6 m*M* mercaptoethanol) or buffer 3 (10 m*M* Tris-HCl (pH 7.8), 15 m*M* magnesium acetate, and 6 m*M* mercaptoethanol). The complexed ribosomal preparations (20 to 50 A_{260} units/gradient) were applied to a linear sucrose density gradient (5–25% sucrose, w/v; 6.5 × 6.5 ml) made with buffer 2 (A and B, closed circles) or buffer 3 (A and B, open circles). The gradient was centrifuged in an SW 40 rotor at 38,000 rpm for 3.5 hr. Fractions of 0.5 ml were collected; 20-μl aliquots were analyzed in a total volume of 50 μl in the absence (○) or presence (●) of 165 pmol/ml of 50S and 180 pmol/ml of 30S (for details see Experimental Section).

results are in good agreement with those obtained with the unlabeled factor.

Binding of Stringent Factor to Ribosomes Depleted of L7 and L12. Recently, it has been shown that ribosomes lacking the two proteins L7 and L12 from the 50S subunit are fully active in the stringency reaction (Richter, 1973a; Lund et al., 1973). Table IV shows that these ribosomal particles are also active in binding stringent factor but are unable to accept radioactively labeled EF-Tu.

Discussion

Our results show that the *E. coli* stringent factor is bound to the 70S ribosome but not to either of the two ribosomal subunits. In contrast to the elongation factors, formation of the ribosome–stringent factor complex does not depend on cofactors. There is neither a stimulation by tRNA nor by GTP nor by ATP, although the latter has a heat-stabilizing effect on the factor (Lück and Richter, 1975). The inhibition of ppGpp + pppGpp production by ribosomal bound EF-Tu or EF-G is evidently due to the presence of Phe-tRNA or of EF-G in the acceptor site of the ribosome. As shown by a number of groups the acceptor site is very closely related or even identical with the ribosomal site for binding EF-G (Baliga and Munro, 1971; Richter, 1973b; Modolell and Vazquez, 1973; Modolell et al., 1973; Nombela and Ochoa, 1973). The importance of a vacant acceptor site for the stringency reaction is evident, since synthesis of ppGpp + pppGpp is only initiated when uncharged tRNA (Haseltine and Block, 1973) or a significant tRNA fragment such as TpψpCpGp (Richter et al., 1974) is bound to the ribosomal acceptor site.

The finding that EF-Tu complexed to the ribosome does not interfere with the ribosomal binding of the stringent factor strongly suggests that both factors bind to different site(s) on the ribosome. These data coincide with the result that ribosomes depleted of two proteins of the large ribosomal subunit, L7 and L12, still bind the stringent but not the elongation factor Tu, again indicating that the two factors recognize and interact with nonidentical ribosomal regions. This result overlaps with recent findings that ribosomes lacking L7 and L12 are fully active in the stringency reaction (Richter, 1973a; Lund et al., 1973). In contrast, L7 and L12 have been found to affect a number of steps of the protein synthesizing cycle that includes (1) initiation factor 2-directed fMet-tRNA binding to ribosomes (Kay et al., 1973; Fakunding et al., 1973); (2) EF-Tu dependent binding of aminoacyl-tRNA as well as EF-Tu linked GTP hydrolysis (Hamel et al., 1972; Brot et al., 1972; Sander et al., 1972); (3) EF-G dependent functions such as translocation, GTP hydrolysis, and binding to ribosomes (Hamel et al., 1972; Brot et al., 1972; Sander et al., 1972; Kischa et al., 1971); and (4) releasing factor 1 and 2 directed functions like formation of a ribosome-releasing factor–UAA complex (Brot et al., 1974). Ribosomes depleted of L7 and L12 are inactive in the reactions described above. So far direct evidence that L7 and L12 are involved in the binding of the protein factors to the ribosome has only been obtained for EF-G (Acharya et al., 1973). The mutual exclusion of EF-Tu and EF-G as well as of releasing factor 1 and 2 (Cabrer et al., 1972; Miller, 1972; Richman and Bodley, 1972; Richter, 1972; Tate et al., 1973) in binding to the 70S ribosome has led to the conclusion that these factors react alternately on the ribosome during the protein synthesizing cycle.

Although it is not clear whether the stringent factor is permanently bound to the ribosome, our results may indicate that the stringent factor is not a true protein factor that alternately binds to and is released from the ribosome as known for the elongation factors. The contention that the stringent factor is attached to a specific site on the ribosome throughout the protein synthesizing cycle (Ramagopal and Davis, 1974) is furthermore supported by our experiments that ribosomal bound stringent factor does not block the functions of the elongation factors. Also, the stringent factor has not yet been detected in a cellular fraction other than the ribosomal one (Cochran and Byrne, 1974).

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