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A Comparative Study of the 50S Ribosomal Subunit and Several 50S Subparticles in EF-T- and EF-G-Dependent Activities†

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ABSTRACT: A series of ribosomal subparticles derived from the 50S subunit has been studied and compared in EF-T- and EF-G-dependent reactions. Three different 50S cores were prepared by CsCl isopycnic centrifugation and one by NH_4Cl-ethanol extraction. The 50S CsCl core a had lost proteins L1, L7, L8, L10, L12, L16, L25, L33, and some L6 and L11. The 50S CsCl core b additionally lacked protein L6, and 50S CsCl core c also lacked proteins L5, L15, L18, L27, L28, L30, and most of L9, L14, L19, and L21. The 50S NH_4Cl-ethanol core had lost up to 90% of proteins L7,L12 and 30–60% of proteins L8, L10, and L29. The 50S CsCl core a had much reduced activity in EF-G and none in EF-T GTPase reactions while 50S CsCl cores b and c were inactive. Addition of proteins L7,L12 restored the activity for both the EF-T- and EF-G-dependent GTPase with all of the three 50S CsCl cores, increasing stepwise from core c to core a. The 50S NH_4Cl-ethanol core was partially active in the EF-G GTPase over the 2–30 m*M* Mg^{2+} range tested, while EF-T only showed some activity in the upper portion of this range. EF-T GTPase activity with 50S CsCl cores was dependent on aminoacyl-tRNA. Stimulation of EF-G GTPase activity by poly(U) plus $tRNA^{Phe}$ at low Mg^{2+} concentration was present with all of the 50S cores. Guanine nucleotide · EF-G · ribosome complex forming ability closely followed GTPase activity with all 50S cores. The decrease in activity of the 50S CsCl and NH_4Cl-ethanol cores was caused neither by removal of 5S RNA nor by inability to associate with the 30S subunit, although association with 50S CsCl cores b and c required a somewhat higher Mg^{2+} concentration. The results show that down to the 50S CsCl core c the 50S sites of interaction with EF-T and EF-G are still closely related, and provide additional support for a common ribosomal center where the elongation factor dependent GTP hydrolysis is triggered. The activity of this center is strongly dependent on the presence of proteins L7,L12, and less of L6, but these proteins are not absolutely essential. The other split proteins appear to be less significant. It does not seem, therefore, that proteins L5, L18, and L25, which are missing from 50S CsCl core c and have been reported to form a GTPase- and ATPase-active complex with 5S RNA (Horne, J. R., Erdmann, V. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2870), are required for elongation factor dependent GTP hydrolysis.

Several proteins of the 50S ribosomal subunit have been reported to function in elongation factor dependent activities (for a review, see Haselkorn and Rothman-Denes, 1973; Pongs et al., 1974). However, the characterization of the ribosomal components involved in these reactions is still far from complete, and also comparison between the results of different authors presents difficulties due to the large number of parameters inherent in a system of great complexity. Frequently, the isolated subparticles and the extracted proteins have not been sufficiently characterized. To examine more closely the proposed 50S ribosomal center for both EF-G and EF-T[1] activities (Haselkorn and Rothman-Denes, 1973) we have undertaken a systematic study of the effect of several parameters on the elongation factor dependent activities of a series of well-defined ribosomal subparticles. Particular attention was paid to the role of Mg^{2+} and ribosomal association. Methanol, which along with other organic solvents has been shown to increase affinity among components of biological systems (Monro and

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[1] Abbreviations used are: GMPPCP, 5′-guanylylmethylene diphosphonate; EF-T, the complex formed by elongation factors EF-Tu and EF-Ts; EF-G, elongation factor G; $tRNA^{Phe}$, phenylalanine-specific transfer ribonucleic acid.

Marcker, 1967; Tompkins et al., 1970; Spirin and Lishnevskaya, 1971; Sy et al., 1973) and to affect a great number of reactions of the elongation process (Ballesta and Vazquez, 1972a; Hamel and Nakamoto, 1972a; Voigt and Parmeggiani, 1973), was used as an auxiliary tool for determining the activities of the individual ribosomal proteins or groups of proteins removed from the 50S subparticles.

Experimental Procedure

Materials. Poly(U) and GTP (Li^+ salt) were obtained from Boehringer (Mannheim, West Germany) and the latter passed through a column of AG 50 W-X2 cation exchange resin (H^+ form, Bio-Rad Laboratories, Richmond, Calif.). Transfer RNA was purchased from Schwarz (Orangeburg, N.Y.). L-[^{14}C]Phenylalanine (513 Ci/mol), carrier-free $^{32}P_i$, [^{3}H]GTP, and [^{3}H]GMPPCP were purchased from The Radiochemical Centre (Amersham, U.K.). The purity of the radioactive nucleotide was controlled by thin-layer chromatography on PEI cellulose in 0.75 *M* phosphate buffer (pH 3.5). [γ-^{32}P]GTP was prepared as described (Sander et al., 1972). Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were from Serva (Heidelberg, West Germany). Sucrose (Merck, für biochemische und mikrobiologische Zwecke) was treated with diethyl pyrocarbonate to inactivate contaminating ribonuclease (Solymosy et al., 1968). Tris(hydroxymethyl)aminomethane (Trizma base) was obtained from Sigma (St. Louis, Mo.). 2,5-Diphenyloxazole was from Packard (Downers Grove, Ill.). All other reagents were analytical grade.

Preparation of Elongation Factors and Ribosomes. EF-T and EF-G were purified to electrophoretic homogeneity from *Escherichia coli* BT2^r or A19 as described (Parmeggiani, 1968; Parmeggiani et al., 1971) except that Sephadex G-200 filtration was replaced by chromatography on DEAE-Sephadex A-50. They were stored in 50 m*M* Tris-HCl (pH 7.8, 20°)–2 m*M* dithiothreitol containing 50% glycerol at −25°. One microgram of EF-G and EF-T corresponds to 12 and 15 pmol, respectively (Parmeggiani and Gottschalk, 1969a; Arai et al., 1973). In the absence of ribosomes or Phe-tRNA, EF-T catalyzed hydrolysis of 0.0003 to 0.002 mol of GTP per mol of factor in 1 min at 30° (Gordon, 1969). Crude ribosomes obtained from the pellets of the clarified cell extract were purified by washing twice with 0.5 *M* NH_4Cl–20 m*M* Tris-HCl (pH 7.8)–10 m*M* $MgCl_2$. Ribosomes (0.5–1 g) in 23 ml of washing solution were each time pelleted at 55,000 rpm for 6 hr through 12 ml of 18% sucrose in washing solution using a Spinco 60 Ti rotor. Ribosomal subunits were prepared by centrifugation of about 0.5 g of washed ribosomes in a Spinco 15 Ti zonal rotor through a 5–25% sucrose gradient in 20 m*M* Tris-HCl (pH 7.8), 30 m*M* KCl, 30 m*M* NH_4Cl and 0.5 m*M* $MgCl_2$. The sucrose gradient was prepared according to Leifer and Kreuzer (1971) and was linear after correction for radial dilution. All ribosome preparations were stored at −25° in 20 m*M* Tris-HCl (pH 7.8)–10 m*M* $MgCl_2$–30 m*M* KCl–30 m*M* NH_4Cl containing 50% glycerol. Ribosomal subunit preparations were checked for purity by analytical sucrose gradient centrifugation. The 50S preparations containing less than 5% visible contamination and 30S preparations apparently free from 50S but frequently containing 5–10% of 42S material were used. These 50S preparations showed with EF-T at 40 m*M* NH_4^+ 1–5% of the GTPase activity observed in the presence of 30S subunits, and with EF-G at 80 m*M* NH_4^+, they showed 5–10% of the activity. One A_{260} unit was taken to represent 25 pmol of 70S, 39 pmol of 50S, or 67 pmol of 30S particles, respectively (Tissières et al., 1959; Hill et al., 1970). Protein determinations were carried out according to Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Preparation of Ribosomal Cores and Ribosomal Proteins. The 50S CsCl core a was prepared using a method similar to that of Meselson et al. (1964) except that instead of 50 m*M* Mg^{2+}, 20 m*M* $MgCl_2$ was used. According to Maglott and Staehelin (1971) 2 m*M* NaEDTA was also present. The 50S subunits (200–400 A_{260} units) in 0.5 ml of 20 m*M* Tris-HCl (pH 7.8)–30 m*M* KCl–30 m*M* NH_4Cl–20 m*M* $MgCl_2$ were mixed with 12 ml of 5.25 *M* CsCl containing 20 m*M* Tris-HCl–20 m*M* $MgCl_2$–3.5 m*M* 2-mercaptoethanol and 2 m*M* NaEDTA (final pH 7.4; method I). After centrifugation for 40 hr at 40,000 rpm in a Spinco 50 Ti rotor, 25–30 fractions per tube were collected and the absorption at 260 nm measured. The 50S CsCl cores b and c were prepared by a modification of this method in which the 12 ml of 5.25 *M* CsCl contained 100 m*M* potassium acetate (pH 6.0)–10 m*M* $MgCl_2$–1 m*M* NaEDTA (method II). NH_4Cl–ethanol extracted 50S particles were prepared as described (Sander et al., 1972) with 1 *M* NH_4Cl as final concentration. All subparticles were dialyzed against 10 m*M* $MgCl_2$–20 m*M* Tris-HCl (pH 7.8)–30 m*M* NH_4Cl–30 m*M* KCl containing 50% glycerol and stored at −25°. Proteins L7 and L12, used as a mixture, came from a 0.25 *M* NH_4Cl–50% ethanol extraction of 70S ribosomes which yielded pure L7,L12. We used this method to obtain large amounts of these proteins for reconstitution. Ribosomal protein fractions were stored at 5° in 1 *M* NH_4Cl–50 m*M* Tris-HCl (pH 7.8)–1 m*M* dithiothreitol.

(Phe-)tRNA^{Phe}. $tRNA^{Phe}$ was obtained 30–40% pure by chromatography of total *E. coli* tRNA (Gillam et al., 1967) and was charged as described (Chinali and Parmeggiani, 1973). One A_{260} unit of partially purified $tRNA^{Phe}$ was assumed to contain 1600 pmol of tRNA.

Preparation of 5S RNA. Ribosomes from *E. coli* MRE600 were washed and 5S RNA prepared from the washed 70S ribosomes according to Brownlee and Sanger (1967). All glassware and stock solutions were treated with diethyl pyrocarbonate before use (Solymosy et al., 1968). RNA sedimenting as 16S and 23S material as well as 5S RNA free from tRNA were obtained in the expected proportions: 21, 40 and 2% of the input of A_{260} units, respectively.

GTPase Assays. [γ-^{32}P]GTP hydrolysis was measured as liberation of inorganic phosphate. With EF-T, reaction mixtures contained, in a final volume of 75 μl, 20 m*M* Tris-HCl (pH 7.8)–40 m*M* NH_4Cl and 200–1000 pmol of [γ-^{32}P]GTP (500–1500 Ci/mol); 3 μg of poly(U), 100 pmol of [^{14}C]Phe-tRNA, $MgCl_2$, EF-T, ribosomal particles, and 20% of methanol (v/v) were added as indicated. Poly(U) was included in the assays with methanol although it was found to be slightly inhibitory in this case. Controls omitting Phe-tRNA were often done. Activity of the 50S cores in the presence of proteins L7,L12 was assayed after incubation (10 min at 30°) of 5 pmol of particles with 70 pmol (0.9 μg) of L7,L12 and varying amounts of 30S subunits in a total volume of 15 μl containing 20 m*M* Tris-HCl (pH 7.8)–200 m*M* NH_4Cl–8 m*M* $MgCl_2$. The presence of 30S subunits in the reconstitution mixture greatly enhanced the efficiency of reactivation. The other components were then

added to yield the desired concentrations in a final volume of 75 μl. Two-minute incubations at 30° were used throughout the experiments because the reaction was found to be linear in all conditions during this period of time. After incubation, 80 μl of 1 *M* $HClO_4$ containing 3 m*M* KH_2PO_4 was added to each tube followed by centrifugation for 5 min at 500*g;* to 100 μl of the supernatant were added 300 μl of cold 20 m*M* sodium molybdate and 400 μl of cold isopropyl acetate. After vigorous mixing for 30 sec and a 30-sec centrifugation at 500*g*, 150 μl of the upper phase was placed on Whatman No. 3MM filter disks (23 mm diameter) and dried and the radioactivity measured using a Tri-Carb liquid scintillation spectrometer Model 3380. The scintillation fluid contained 6 g of 2,5-diphenyloxazole in 1 l. of toluene.

With EF-G, reaction mixtures contained, in a final volume of 75 μl, 20 m*M* Tris-HCl (pH 7.8) and 20–30 nmol of [γ-^{32}P]GTP (5–15 Ci/mol); $MgCl_2$, EF-G, ribosomes, 3 μg of poly(U) plus 100 pmol of $tRNA^{Phe}$, and 20% (v/v) methanol were added as indicated in the figure legends. Preincubation with L7,L12 was done as described for EF-T. Most experiments were performed at both 40 and 80 m*M* NH_4Cl. However, all figures show only the activities obtained at 40 m*M* NH_4Cl. The liberated inorganic phosphate was measured as above after a 10-min incubation at 30°.

Assay of EF-G Dependent Binding of GTP and GMPPCP. The reaction mixtures contained 48 pmol of EF-G and 5 pmol of 70S or 50S particles, plus or minus 35 pmol of 30S subunits, in 75 μl of 50 m*M* Tris-HCl (pH 7.8)–14 m*M* $MgCl_2$–10 m*M* NH_4Cl–2 m*M* KCl–14 m*M* 2-mercaptoethanol–3% glycerol and either 2 μ*M* [^{3}H]GTP (1000 Ci/mol) plus 1.5 m*M* fusidic acid or 2.5 μ*M* [^{3}H]GMPPCP (1000 Ci/mol). Methanol was added to 20% (v/v) where indicated. After 10 min of incubation at 30° and again at 120 min, 30-μl samples were withdrawn and pipetted under 5 ml of cold wash buffer onto the surface of a nitrocellulose filter (Millipore HAWP). Suction was then immediately applied so that the filtration process lasted only 2–3 sec. The wash buffer was 50 m*M* Tris-HCl (pH 7.8)–13 m*M* $MgCl_2$–10 m*M* NH_4Cl–14 m*M* 2-mercaptoethanol with 0.1 m*M* fusidic acid and/or 20% methanol if present in the original reaction mixture. The filters were dried and the radioactivity measured as above.

Analytical Sucrose Gradient Centrifugation. The 7–25% linear sucrose gradients contained 20 m*M* Tris-HCl (pH 7.8)–40 m*M* NH_4Cl, 20% methanol and $MgCl_2$ as indicated. After incubation for 10 min at 30°, 100- to 200-μl samples containing 2 to 3 A_{260} units of particles were centrifuged in a Spinco SW 40 rotor for 3.5 hr at 38,000 rpm and analyzed with an ISCO density gradient fractionator Model 640.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The method of Kaltschmidt and Wittmann (1970a) was used except that, after drying, the samples were dissolved in 6 *M* urea–50 m*M* Tris-HCl (pH 7.8)–7 m*M* 2-mercaptoethanol and dialyzed for 5–10 hr against a sample gel solution that lacked riboflavine and $(NH_4)_2S_2O_8$.

Determination of 5S RNA. The 5S RNA content of the various particles was estimated by polyacrylamide gel electrophoresis (Richards et al., 1965) in sodium dodecyl sulfate using the ionic conditions and sample preparation of van Diggelen and Bosch (1973). Four A_{260} units of 50S particles or 0.4 A_{260} unit of commercial tRNA as the standard was applied to 6 × 70 mm 10% gels overlayered with 10 mm of 5% spacer gel. After running for 9 hr at 1 mA/gel (~25 V), the RNA was fixed with 20% formaldehyde for 8–10 hr. The gels were washed (8 hr with H_2O) and stained for another 8 hr with 0.025% Acridine Orange in 20 m*M* Tris-HCl (pH 8.0). After destaining gels were scanned at 490 nm.

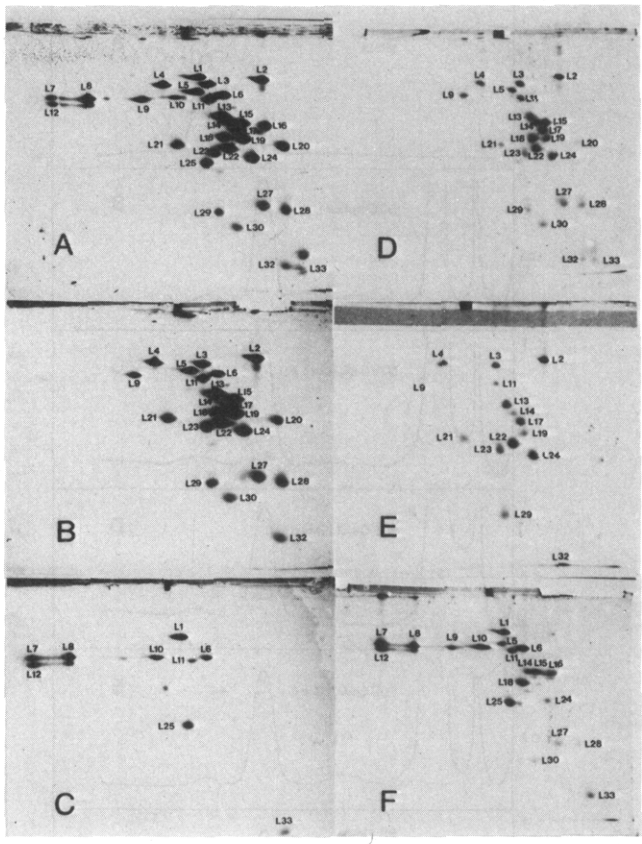

FIGURE 1: Two-dimensional polyacrylamide gel electrophoresis of the proteins from 50S subunits (panel A), the proteins present in 50S CsCl cores a, b, and c (panels B, D, and E, respectively), and the split proteins from 50S CsCl core a (panel C) and from 50S CsCl cores b plus c (panel F).

Results

Characterization of 50S Particles. The electrophoretic pattern of total 50S proteins (Figure 1A) was the same as that reported by Kaltschmidt and Wittmann (1970b), except that slightly above protein L33 an additional spot was frequently observed. In line with observations of other authors (Kaltschmidt and Wittmann, 1970b; Nierhaus and Montejo, 1973), proteins L26 and L31 did not appear in the two-dimensional gels of our 50S ribosome preparations.

Using method I to prepare 50S CsCl cores as described in the Experimental Procedure section, only one major absorbance peak was observed in the CsCl density gradients (50S CsCl core a). With method II, two main absorbance peaks were found, a lighter fraction (50S CsCl core b) and a denser one (50S CsCl core c). Occasionally a very light fraction was observed floating above cores b and c that was not tested. The 50S CsCl cores from *E. coli* BT2^r and A19 had identical protein composition. Figures 1B and 1C show the electrophoretic patterns of 50S CsCl core a proteins and the corresponding split proteins, respectively. Proteins L1, L7, L8, L10, L12, L16, L25, and L33 were quantitatively removed, and L6 and L11 were partially extracted. In this case protein L16 was not recovered, but in other experi-

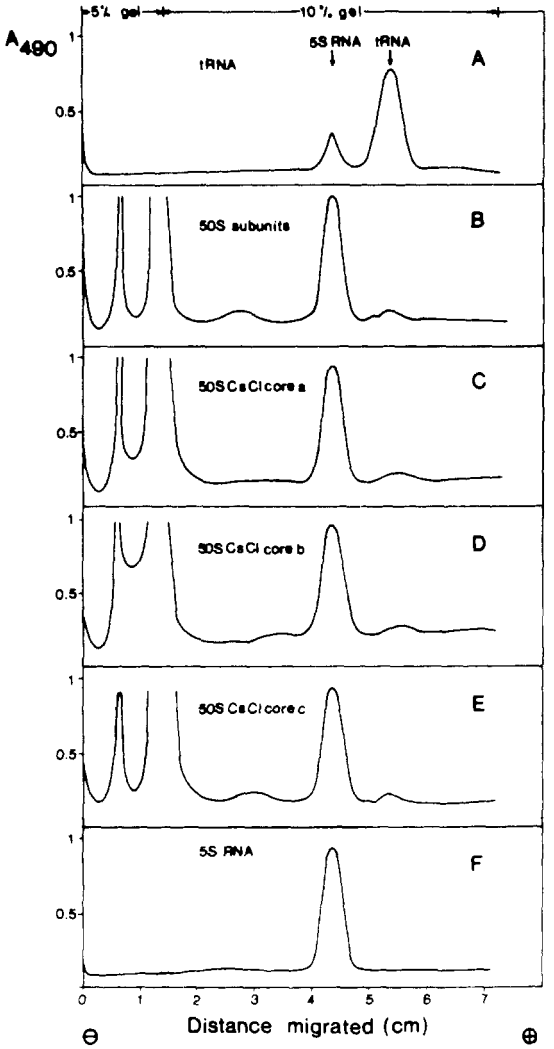

FIGURE 2: The 5S RNA content of the three 50S CsCl cores determined by polyacrylamide gel electrophoresis. The samples consisted of: (A) 0.4 A_{260} unit of control tRNA; (B-E) 4 A_{260} units each of 50S subunits and 50S CsCl cores a, b, and c, respectively; (F) 0.4 A_{260} unit of purified 5S RNA. The 50S NH_4Cl-ethanol core gave the same pattern as 50S CsCl core a.

ments it appeared in the split protein fraction. Figures 1D and 1E show the protein patterns of 50S CsCl cores b and c, respectively, and Figure 1F shows that of the corresponding split proteins. The 50S CsCl core b differed from core a only in its complete lack of protein L6. The 50S CsCl core c had additionally lost proteins L5, L15, L18, L27, L28, L30, most of L9, L14, L19, and L21. When protein L20 was recovered, it was a part of 50S CsCl cores b and c. A certain degree of variability with regard to the relative amounts of L9 and in some cases also L2 could not be entirely eliminated. Some 50S CsCl core c preparations contained traces of protein L18, but in most cases it was completely absent. However, the general composition of the 50S CsCl cores was well reproducible with different ribosomal preparations. With 1 *M* NH_4Cl-50% ethanol up to 90% of proteins L7,L12 were extracted; 30-60% of proteins L8, L10, and L29 were also removed.

The 5S RNA content of 50S NH_4Cl-ethanol and CsCl cores was compared with that of 50S subunits using polyacrylamide gel electrophoresis (Figure 2). The 5S RNA was identified by the following parameters: (a) sedimentation coefficient; (b) electrophoretic behavior as compared with reports of other authors (Geroch et al., 1968; van Diggelen and Bosch, 1973); (c) 5S RNA purified according to Brownlee and Sanger (1967) moved as a single sharp band in the gels to the exact position of the 5S RNA band from 50S subunits and cores; (d) the amount of 5S RNA found was independent of the degree of fragmentation of 23S RNA although breakdown of 23S RNA into smaller fragments became evident upon standing of the samples before treatment with sodium dodecyl sulfate.

The 5S RNA was fully present in the NH_4Cl-ethanol core, 50S CsCl cores a and b, and in most cases also in core c (Figure 2), in agreement with Staehelin et al. (1969). Furthermore, 5S RNA did not dissociate from any of the cores upon passage of the latter through a Sepharose 4B column (not illustrated). No difference was observed in the 5S RNA content of 50S CsCl cores from *E. coli* $BT2^r$ or A19.

Activity of 50S CsCl Cores in the Ribosome-EF-T GTPase Reaction. To reduce the possibility that activity of any of the 50S CsCl cores in the EF-T-dependent GTPase reaction would be overlooked by choosing too narrowly defined reaction conditions, we have tested them at three Mg^{2+} concentrations and with increasing amounts of 30S subunits. Saturation with 30S subunits allows a more valid comparison of the activities of the different 50S particles since it eliminates possible artefacts due to the presence of functionally different populations in the purified 30S subunits (Kurland et al., 1969). The amount of 50S particles was kept constant because at least for EF-G the ribosomal center that allows the expression of this GTPase activity is situated on the 50S subunit (Bodley et al., 1970; Brot et al., 1971; Voigt et al., 1974). The ability of methanol to induce or stimulate GTPase activity was also tested. As already shown by other authors (Ballesta and Vazquez, 1972a; Hamel and Nakamoto, 1972a), methanol stimulates the EF-T-dependent GTPase with 30S plus 50S subunits by an order of magnitude. The 40 m*M* NH_4^+ present in the assays is near optimal with 30S and 50S subunits both with and without methanol (Voigt et al., 1974).

The effect of addition of proteins L7,L12 on activity was also tested since they have been found to play a primary role in the ribosome- EF-T GTPase reaction (Haselkorn and Rothman-Denes, 1973). In this way, we wanted to characterize the action of other ribosomal proteins. As a control, 50S subunits supplemented with a twofold excess of 30S subunits and saturating amounts of proteins L7,L12 were used. The activity of this control was equivalent to that of 70S ribosomes supplemented with L7,L12.

As Figure 3 shows, neither 50S CsCl core a, b, nor c was active in the EF-T-dependent GTPase reaction at any of the Mg^{2+} or 30S subunit concentrations used unless proteins L7,L12 or methanol was present. With the addition of only L7,L12 (panels D-F), activity of all three 50S CsCl cores rose progressively with Mg^{2+} concentration and reached 80, 50, and 20% of the control, respectively, at 30 m*M* Mg^{2+} and a ca. twofold excess of 30S subunits.

With methanol, but without proteins L7,L12 (panels G-I), 50S CsCl core a showed some activity even at 5 m*M* Mg^{2+} and CsCl core b at 14 and 30 m*M* Mg^{2+}. The 50S CsCl core c was for all practical purposes inactive. The need to test activity at increasing amounts of 30S subunits is particularly evident in all of these cases.

With both methanol and proteins L7,L12 (panels J-L), the highest activities were observed. The 50S CsCl core a

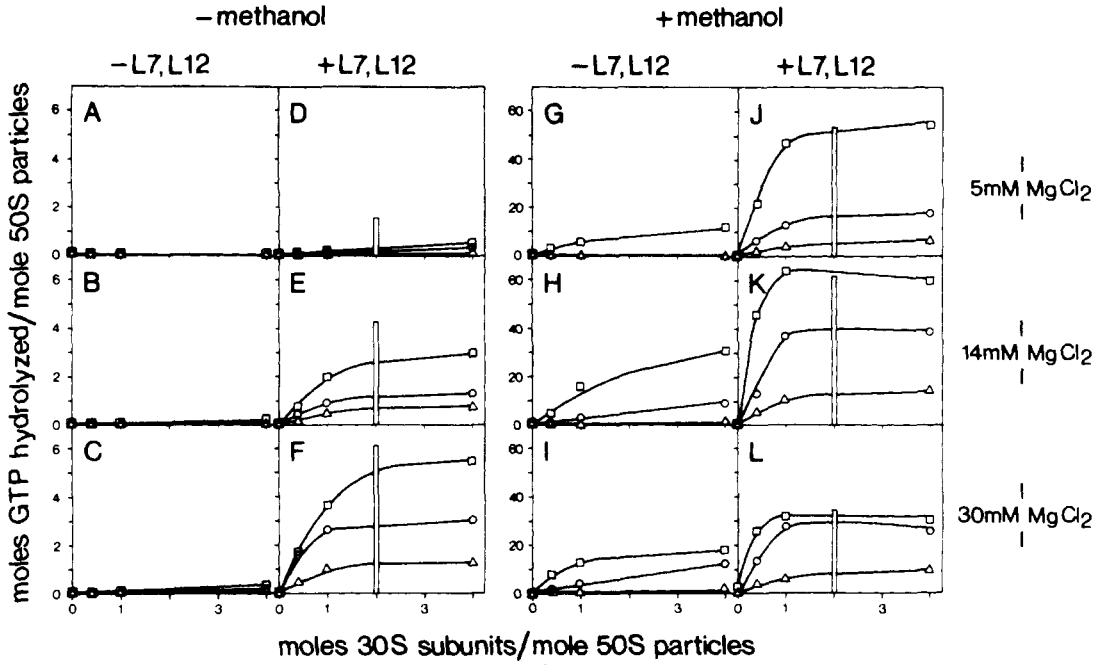

FIGURE 3: Activity of 50S CsCl cores a, b, and c in the ribosome-EF-T GTP hydrolysis. Activity was tested at different Mg^{2+} concentrations with 50 pmol of EF-T and 5 pmol of 50S CsCl core, preincubated with or without proteins L7,L12 and increasing amounts of 30S subunits, in the absence (A-F) or presence (G-L) of 20% methanol. One-hundred picomoles of Phe-tRNA and 3 μg of poly(U) were present in all experiments: (□) 50S CsCl core a; (○) 50S CsCl core b; (△) 50S CsCl core c. Columns represent the controls with 5 pmol of 50S subunits plus 10 pmol 30S subunits preincubated with proteins L7,L12; incubation time, 2 min at 30°.

was as active as the 50S subunit control at all Mg^{2+} concentrations; 50S CsCl core b increased progressively in activity, almost reaching the control level at 30 m*M* Mg^{2+}, and 50S CsCl core c showed 30% of the control level at 30 m*M* Mg^{2+}.

In all conditions Phe-tRNA and poly(U) remained a stringent requirement with all of the cores used (not illustrated).

Activity of 50S CsCl Cores in the Ribosome-EF-G GTPase Reaction. Unlike EF-T, EF-G is capable of supporting the ribosome-dependent GTPase in the absence of poly(U) and tRNA. However, depending on the Mg^{2+} concentration, its activity can be stimulated up to 3-4 times by these components of ribosomal polypeptide synthesis (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966; Parmeggiani et al., 1974). We have tested the 50S CsCl cores in the EF-G-dependent GTPase reaction in both of these conditions. As a control, 50S subunits supplemented with proteins L7,L12 and a sixfold excess of 30S subunits were used to achieve maximal activity.

Activities of the 50S CsCl cores in the absence of poly(U) and $tRNA^{Phe}$ but otherwise in the same conditions as the EF-T-dependent GTPase assays are shown in Figure 4. Without proteins L7,L12 and methanol (panels A-C), only CsCl core a was partially active. This is at variance to its lack of activity with EF-T. Upon addition of proteins L7,L12 (panels D-F), all three CsCl cores showed activity, as in the EF-T-dependent GTPase reaction except at 5 m*M* Mg^{2+} where only 50S CsCl core a was active. The increase in their activity as a function of Mg^{2+} concentration leveled off at or before 14 m*M* Mg^{2+}. At this point with sixfold excess of 30S subunits, 50S CsCl cores a, b, and c showed 67, 26, and 10% of the control activity, respectively.

With methanol but without proteins L7,L12 (panels G-I), only 50S CsCl core a at 5 and 14 m*M* Mg^{2+} and 50S CsCl core b at 14 m*M* $MgCl_2$ showed low activity. In the additional presence of L7,L12 (panels J-L) the highest CsCl core activities were observed, just as with EF-T. CsCl core a was as active as the control at all three Mg^{2+} concentrations, CsCl core b was as active as the control at 14 and 30 m*M* Mg^{2+}, and CsCl core c activity increased from 50% of the control level at 5 m*M* Mg^{2+} to 90% at 30 m*M* Mg^{2+}.

Figures 4E, J, and K especially show that 50S CsCl core a retained the known ability of 50S subunits (Voigt et al., 1974) to support the EF-G GTPase at 40 m*M* NH_4^+ in the absence of 30S subunits. This ability was lost by CsCl cores b and c.

The molar ratio of elongation factor to 50S particles had no major influence on the GTPase activities. A certain difference was found in 50S CsCl core c that at a ratio of 1:1 displayed no activity with EF-G vs. a 10% activity at a ratio of 10:1; with EF-T at both ratios approximately 25% of the control activity was observed (data with 1:1 ratio not shown).

Table I summarizes the effects of poly(U) and $tRNA^{Phe}$ on the EF-G-dependent GTPase activities of the 50S subunit and the three 50S CsCl cores in the presence of 30S subunits. At 5 m*M* Mg^{2+}, the presence of the $tRNA^{Phe}$ and poly(U) stimulated GTP hydrolysis of the control 50S plus 30S subunits threefold; at 14 m*M* Mg^{2+}, they inhibited slightly while at 30 m*M* a 70% inhibition was observed. Methanol virtually abolished stimulation and strongly accentuated inhibition which reached 90% of the control at 30 m*M* Mg^{2+}. Replacement of the 50S subunit with any of the 50S CsCl cores hardly affected the stimulation at 5 m*M* Mg^{2+}, but reduced the inhibition at 30 m*M* Mg^{2+}. Methanol prevented both stimulation and inhibition by $tRNA^{Phe}$ and poly(U), except that in the presence of 50S CsCl core a a considerable inhibition at 14 and 30 m*M* Mg^{2+} was found. As expected, the 30S subunit was in all cases an absolute requirement for the action of $tRNA^{Phe}$ plus poly(U) on the EF-G-dependent GTPase activity (data not shown).

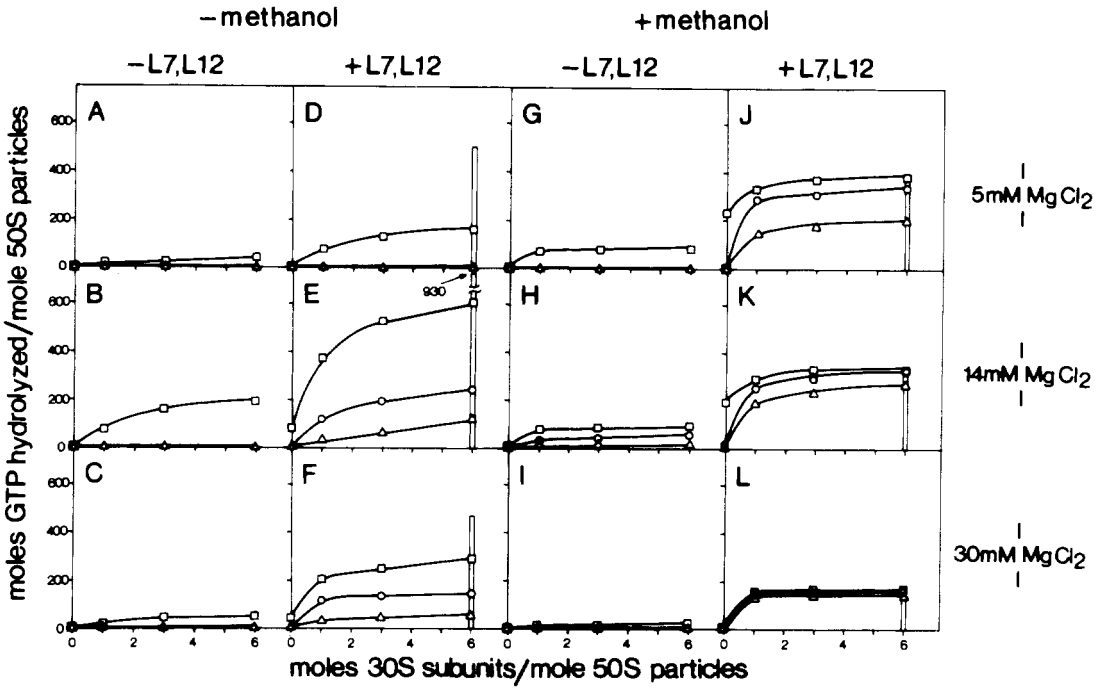

FIGURE 4: Activity of 50S CsCl cores a, b, and c in the ribosome-EF-G GTPase uncoupled from polypeptide synthesis. Activity was tested at different Mg^{2+} concentrations with 50 pmol of EF-G and 5 pmol of 50S CsCl core, preincubated with or without proteins L7,L12 and increasing amounts of 30S subunits, in the absence (A–F) or presence (G–L) of 20% methanol: (□) 50S CsCl core a; (○) 50S CsCl core b; (△) 50S CsCl core c. Columns represent the controls with 5 pmol of 50S subunits plus 30 pmol of 30S subunits preincubated with proteins L7,L12; incubation time, 10 min at 30°.

Table I: Effect of Poly(U) plus $tRNA^{Phe}$ on EF-G GTPase Activity of Various 50S Particles.[a]

30S Subunits Plus	Poly(U), $tRNA^{Phe}$	Minus Methanol			Plus 20% Methanol		
		5 m*M* Mg^{2+}	14 m*M* Mg^{2+}	30 m*M* Mg^{2+}	5 m*M* Mg^{2+}	14 m*M* Mg^{2+}	30 m*M* Mg^{2+}
50S subunits	−	550	960	824	446	360	184
	+	1643	887	258	488	189	18
50S CsCl core a	−	206	732	375	441	347	187
	+	580	796	303	435	230	132
50S CsCl core b	−	80	204	213	436	340	182
	+	231	224	191	443	344	192
50S CsCl core c	−	49	115	85	233	302	178
	+	178	103	66	220	280	175

[a] Values given as moles of GTP hydrolyzed/mole of 50S particles; 5 pmol of 50S particles and 30 pmol of 30S subunits were preincubated with 70 pmol of L7,L12 (10 min, 30°) and assayed with 50 pmol of EF-G in the presence or absence of 3 μg of poly(U) plus 100 pmol of purified $tRNA^{Phe}$; incubation time, 10 min at 30°.

Activity of NH_4Cl–Ethanol Extracted 50S Particles in the Ribosome-Dependent EF-T and EF-G GTPase Reactions. With EF-T, NH_4Cl–ethanol core showed the same activity as illustrated in Figure 3 for 50S CsCl core a except that at 30 m*M* Mg^{2+} without added proteins L7,L12 and methanol 25% residual activity was present. This was likely due to the unextracted portion of L7,L12 (data not shown).

With EF-G, the NH_4Cl–ethanol core was about 40% as active as the control and activity could be restored by proteins L7,L12 over the whole range of Mg^{2+} concentrations tested (Figure 5). Methanol lowered the effective range of Mg^{2+}, as shown already for the 70S ribosome (Voigt and Parmeggiani, 1973). The relatively high activity of the ethanol core in these experiments as compared with previous ones (cf. Sander et al., 1972) is due to the lower NH_4^+ concentration used (40 vs. 80 m*M*). The effect of $tRNA^{Phe}$ and poly(U) on the EF-G GTPase activity was not affected by substituting the 50S subunit with 50S NH_4Cl–ethanol core.

Activity of 50S Cores in Fusidic Acid · EF-G · GDP · Ribosome and EF-G · GMPPCP · Ribosome Complex Formation. The complex-forming ability of the 50S CsCl cores closely followed their EF-G-dependent GTPase activities (Table II). With GTP and fusidic acid, the 50S CsCl cores showed a greater dependence upon the presence of the 30S than did the 50S subunits. With GMPPCP, 30S subunits were required by both the 50S CsCl cores and subunits. Methanol increased complex formation and enabled 50S subunits but not CsCl cores to bind GMPPCP and EF-G without 30S subunits. Complex formation was practically complete within 10 min at 30° for 50S subunits but with the 50S CsCl cores in some cases could be significantly increased with a longer incubation.

Association Behavior of 50S CsCl Cores. All three 50S CsCl cores efficiently coupled with 30S subunits (Figure 6). The 50S NH_4–ethanol core and 50S CsCl core a were nearly as good as 50S subunits (panels A, B, H, I, O, P); thus, proteins L1, L8, L10, L16, L25, and L33 in addition to L7

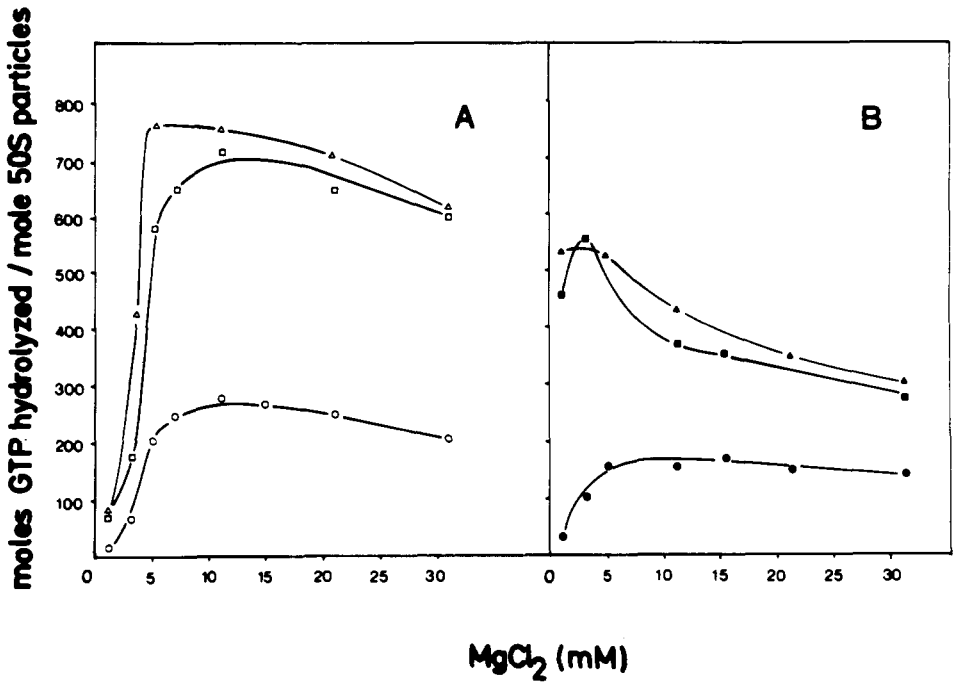

FIGURE 5: Ribosome-EF-G GTPase activity of 50S NH_4Cl-ethanol core as a function of Mg^{2+} concentration; 50 pmol of EF-G, 5 pmol of 50S particles, and 15 pmol of 30S subunits without (A) or with (B) 20% methanol: (△,▲) 50S subunits; (○,●) 50S NH_4Cl-ethanol core without proteins L7,L12; (□,■) 50S NH_4-ethanol core with proteins L7,L12; incubation time, 10 min at 30°.

Table II: EF-G-Dependent Binding of GTP or GMPPCP.[a]

50S	Min at 30°	mol Bound per 100 mol of 50S Particles: GTP + Fusidic Acid, − Methanol, − 30S	GTP + Fusidic Acid, − Methanol, + 30S	GTP + Fusidic Acid, + Methanol, − 30S	GTP + Fusidic Acid, + Methanol, + 30S	GMPPCP, − Methanol, − 30S	GMPPCP, − Methanol, + 30S	GMPPCP, + Methanol, − 30S	GMPPCP, + Methanol, + 30S
50S subunits	10	27	47	55	70	0	45	38	68
	120	31	56	58	85	0	56	41	65
50S CsCl core a	10	0	5	0	14	0	10	0	7
	120	1	4	2	22	0	12	0	16
50S CsCl core b	10	1	0	0	3	0	4	0	0
	120	0	3	0	12	0	5	0	1
50S CsCl core c	10	0	1	0	0	0	1	0	0
	120	0	0	0	0	0	0	0	0
50S NH_4Cl–ethanol core a	10	7	32	15	43	0	20	5	49
	120	5	34	22	68	0	28	8	55

[a] For experimental conditions see Experimental Procedure.

and L12 seem to play no major role in association. Part of the resulting couples sedimented as 62S particles (Infante and Baierlein, 1971), particularly at 10 m*M* Mg^{2+} (panels B, C, I, J, P, Q). The 50S CsCl cores b and c formed only 62S material with 30S at 20 and 30 m*M* Mg^{2+} (panels K, L. R, S), which together with the requirement of higher Mg^{2+} concentration suggests a weakened interaction between these particles and the 30S subunit. The 50S CsCl core c was as active in association as 50S CsCl core b. Thus, the many proteins additionally removed from 50S CsCl core c do not seem to play an essential role in subunit association.

Effect of Methanol on Association Behavior of 50S Cores. As already shown for the 50S subunit by Spirin and Lishnevskaya (1971) and Voigt and Parmeggiani (1973), in the presence of methanol 50S cores could associate with 30S subunits at Mg^{2+} concentrations as low as 1.5 m*M* (Figure 7). Because methanol decreases the apparent sedimentation coefficient (Voigt and Parmeggiani, 1973), fractions of ribosomal particles were rerun on analytical sucrose gradients without methanol at 10 and 0.5 m*M* Mg^{2+} to identify the components (panels E and S). The particles of fraction I were 30S and those of fraction II 50S subunits. Fractions III, IV, and V contained 70S ribosomes. With methanol, some 50S subunits were present as dimers; increasing Mg^{2+} concentration caused increasing dimer formation. This effect became particularly accentuated with 50S CsCl cores. At 20 and 30 m*M* Mg^{2+}, the greatest part of the 30S subunits disappeared as a discrete component and formed higher aggregates (panels R and Y).

Discussion

The work described here was undertaken to investigate and compare the activities of the 50S subunit and of well-

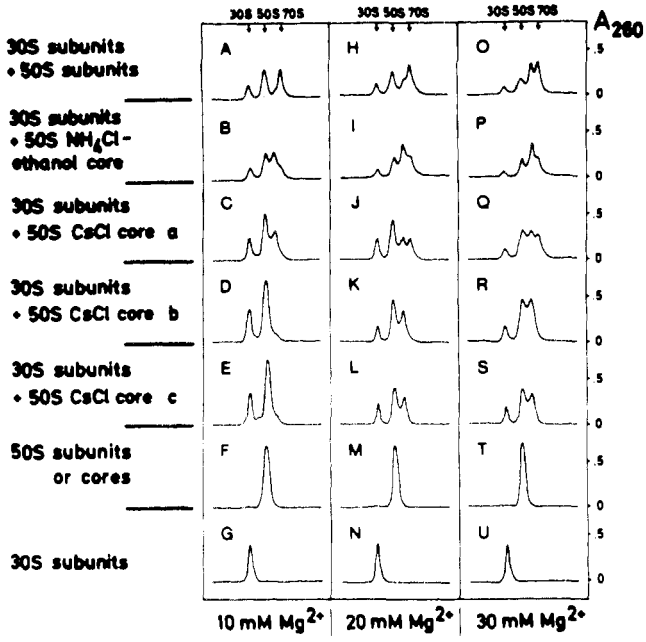

FIGURE 6: Sucrose gradient analysis of the association behavior of 50S NH_4Cl-ethanol core and 50S CsCl cores a, b, and c at 40 mM NH_4^+ as a function of Mg^{2+} concentration. The 30S subunits and 50S core were present in equimolar amounts.

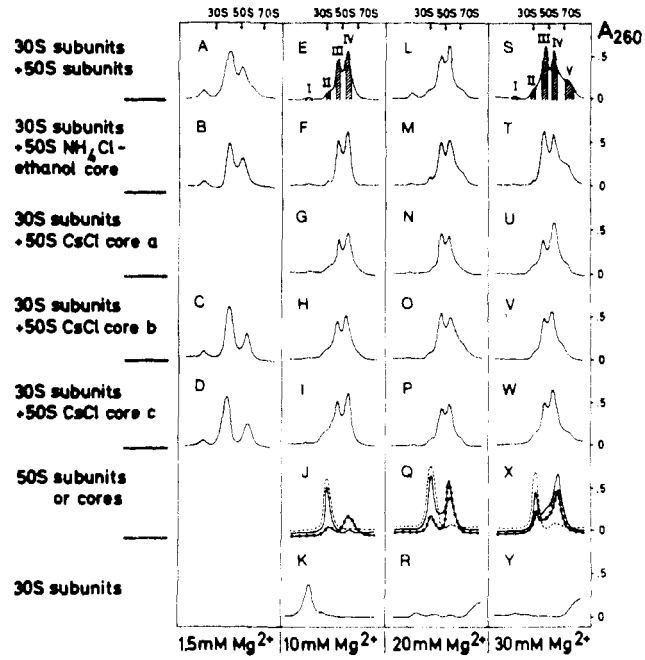

FIGURE 7: Influence of methanol and Mg^{2+} concentration on the association of 30S subunits with 50S NH_4Cl-ethanol core and 50S CsCl cores a, b, and c at 40 mM NH_4^+. In all gradients 20% methanol was present; panels J, Q, and X: (- - -) 50S subunits; (——) 50S CsCl core a or b; (- - - - - -) 50S CsCl core c. The 30S subunits and 50S core were present in equimolar amounts. Shaded areas in E and S indicate fractions pooled to be rerun in gradients without methanol.

characterized 50S subparticles in the EF-T- and EF-G-dependent reactions.

Each of the various 50S cores was able to support EF-T and EF-G GTPase activity to a similar extent. The parallelism between EF-T and EF-G goes along with the results of several authors which indicated that EF-G and EF-T interact with a similar region of the 50S subunit (for a review, see Haselkorn and Rothman-Denes, 1973). The exceptions were: (1) CsCl core a could support a significant amount of activity with EF-G but none with EF-T unless tested in the presence of methanol; (2) 50S CsCl core c was more efficient in supporting EF-G GTPase activity than that of EF-T in the presence of methanol and proteins L7,L12; (3) NH_4Cl–ethanol core was partially active with EF-G at all Mg^{2+} concentrations tested, whereas activity with EF-T was found only at 30 mM Mg^{2+}.

Our results show that L7,L12-free 50S particles can function in both GTPase reactions. However, in line with the observations of several authors (Hamel and Nakamoto, 1972b; Sander et al., 1972; Ballesta and Vazquez, 1972b), these two proteins proved to be the split proteins primarily involved in expression of elongation factor dependent GTPase and binding of EF-G to ribosomes. The importance of proteins L7,L12 for both GTPase reactions is underlined by the finding that 50S CsCl core b, except for some methanol-induced activity at 14 mM Mg^{2+}, required L7,L12 for expression of both GTPase activities. Moreover, proteins L7,L12 were found to restore some activity to 50S CsCl core c.

The 50S CsCl core b which differs from 50S CsCl core a only by the loss of protein L6 offered a good opportunity for studying the action of this protein. Its selective removal greatly reduced EF-G GTPase activity, in line with Schrier et al. (1973). Our results show that this protein plays a similar role also in EF-T-dependent GTP cleavage. However, proteins L7,L12 restored the activity of 50S CsCl core b up to 50% of the control without methanol and up to 95% with methanol. These results go along with the observation of Stöffler et al. (1974), who observed that after blockage of protein L6 the ribosomes were still able to bind proteins L7,L12 to 70% of the control. Additional removal of the proteins L5, L15, L18, L27, L28, L30, and most of L9, L14, L19, and L21, which differentiate 50S CsCl core c from core b, caused a further reduction in activity but still allows considerable EF-T or EF-G activity in the presence of proteins L7,L12. Thus, protein L6 contributes significantly to the elongation factor dependent GTPase activities, but other ribosomal components are involved. With methanol and proteins L7,L12 50S CsCl core c activity with EF-G was restored from 50 to 100% and with EF-T up to 30% of the control depending on the Mg^{2+} concentration.

Sopori and Lengyel (1972) reported considerable activity of both elongation factors with 50S CsCl β cores prepared according to Staehelin et al. (1969). The activity of 50S CsCl α, β and γ cores has also been studied by Ballesta et al. (1971) and Ballesta and Vazquez (1972b), who described much higher activities with EF-G than we have observed in the case of our 50S CsCl cores. A direct comparison of these results with ours is not possible, because their 50S CsCl cores were prepared with a different method and the protein composition was not sufficiently characterized. A precise characterization of the various ribosomal subparticles is essential since small changes in the Mg^{2+} concentration and pH during core preparation are sufficient to cause variations in their composition. Ballesta and Vazquez have claimed that differences could arise from the use of different bacterial strains, but we have found no variation in protein composition or activity with either *E. coli* $BT2^r$ or A19 cores. Their results with the EF-T GTPase activity of their 50S CsCl cores go along with ours. However, a major difference between our 50S plus 30S subunit-dependent EF-T GTPase activity (Sander et al., 1972; Wolf et al., 1974; cf. Gordon, 1969) and the observations of Vazquez and co-

workers (Ballesta and Vazquez, 1972a, 1973) was the failure to detect in the absence of methanol any activity independent of the presence of Phe-tRNA and poly(U). With methanol, we observed ~10% of the control activity in the absence of aminoacyl-tRNA at 5m*M* Mg^{2+} (G. Sander, unpublished observation).

It is interesting to note that 50S CsCl core c with proteins L7,L12 is still active in EF-T and EF-G GTPase although it lacks proteins L5, L18, and L25, which have been found to form an elongation factor independent GTPase- and ATPase-active complex with 5S RNA (Horne and Erdmann, 1972, 1973). Erdmann et al. (1971) had previously found that 5S RNA is not essential for EF-G-dependent GTP binding to ribosomes, since 50S particles having less than 1% of the normal 5S RNA content conserved 25% of their activity. Thus, it is difficult to see a direct relationship between the elongation factor dependent GTPases and L5 · L18 · L25 · 5S RNA complex dependent GTPase. The recent observation that in the presence of the antibiotic kirromycin EF-T GTPase can function in the absence of ribosomes (Wolf et al., 1974) indicates that the catalytic center of aminoacyl-tRNA and ribosome dependent EF-T GPTase is primarily located on the elongation factor. There are no similar data for EF-G, but because of the similarity of the ribosomal requirements in both GTPase reactions it is tempting to think that also in this case the catalytic center is located on the elongation factor. This possibility is also supported by the existence of a binding site for GTP and GDP on EF-G in procaryotes (R. C. Marsh, G. Chinali, and A. Parmeggiani, unpublished results) and on its counterpart EF-2 in eucaryotes (Chuang and Weissbach, 1972).

The 50S CsCl cores a and b, and in most cases also 50S CsCl core c, contained normal amounts of 5S RNA. Thus, proteins L6, L18, and L25, which have been reported to bind cooperatively 5S RNA with 23S RNA (Gray et al., 1972), are not necessary for a stable association of the two 50S ribosomal RNA species once incorporated into the 50S particle.

With EF-G and our NH_4Cl–ethanol core preparations we obtained in the absence of proteins L7,L12 higher activities (40% vs. 5–10%) than other authors (Hamel and Nakamoto, 1972b; Ballesta and Vazquez, 1972b). This was in part caused by our use of 40 m*M* NH_4^+. At 80 m*M* NH_4^+ only 15% of the control activity was observed.

The inhibition of EF-G GTPase caused by poly(U) and $tRNA^{Phe}$ at 30 m*M* Mg^{2+} disappeared by substituting 50S subunits with any of the three 50S CsCl cores while stimulation at 5 m*M* Mg^{2+} was not affected. It appears, therefore, that inhibition by $tRNA^{Phe}$ and poly(U) (Ballesta and Vazquez, 1973) at unphysiologically high Mg^{2+} concentration is mediated by the 50S subunit and is not directly related with the interaction of mRNA and tRNA with the 30S subunit.

Formation of the fusidic acid · EF-G · GDP · ribosome complex was about doubled in the presence of 30S subunits. This was due to the stabilization of the complex by the 30S subunit which becomes apparent in the presence of a low concentration of GTP as used here. This phenomenon will be described in detail elsewhere. Complex formation with GMPPCP was totally dependent upon the presence of 30S subunits in the absence of methanol. In the latter case, a requirement for the small subunit has been reported using sucrose gradients for isolation of the complex in both bacterial and eucaryotic systems (Parmeggiani and Gottschalk, 1969b; Rao and Moldave, 1969), while Acharya et al. (1973) did not find this requirement. With the nitrocellulose filtration technique Brot et al. (1971) and this group (Sander et al., 1972) reported that 50S subunits alone were able to support formation of EF-G · GMPPCP · ribosome complexes. Since then, we have carefully rechecked this problem and were unable to see any binding of GMPPCP in the absence of the 30S subunit. The state of conformation of the ribosomes from different preparations may offer an explanation for these inconsistencies.

All 50S CsCl cores could associate with the 30S subunit at sufficiently high Mg^{2+} concentration. Therefore, association was not a limiting factor for activity as has been found to be the case for the 30S CsCl core (Marsh and Parmeggiani, 1973; Parmeggiani et al., 1973). This confirms and extends our previous observation on association of NH_4Cl–ethanol core and 50S CsCl core a with 30S subunits (Sander et al., 1972) and agrees with the findings of van Diggelen et al. (1973) for the 50S CsCl γ core. The observation of Morrison et al. (1973) that blockage of protein L6 partially inhibited coupling of the ribosomal subunits agrees with our finding that L6-deficient 50S CsCl core b needed more Mg^{2+} than 50S CsCl core a to couple efficiently with the 30S subunit (Figure 6). On the other hand our data do not support their claim that proteins L27 and L19 are more essential than protein L6 for association. Protein L27 was always absent in our 50S CsCl core c and protein L19 was reduced to approximately one-third of the amount present in 50S CsCl core b, yet no difference in coupling ability was found between these two 50S CsCl cores. Protein L23, which has been found to be the most important 50S protein for association (Morrison et al., 1973), was present in all of our cores.

The data presented in this work provide additional evidence that EF-T and EF-G interact with closely related 50S ribosomal site(s) controlled by many ribosomal proteins among which L7 and L12 play a predominant role. We feel that the ability of the 50S subunits to support in the absence of 30S subunits, under ionic conditions far from the physiological ones, the GTPase activity of EF-G but not of EF-T (Voigt et al., 1974) is mainly dependent on intrinsic differences between the two elongation factors. The 30S subunit is a stringent requirement for the EF-G GTPase associated with the presence of aminoacyl-tRNA and mRNA and becomes determinant also for the uncoupled EF-G GTPase under conditions resembling the physiological ones. Interaction of EF-T and EF-G with a common region on the 50S subunit is reasonable: both EF-T and EF-G are GTPases and act on aminoacyl-tRNA and polypeptidyl-tRNA, respectively, while these are interacting with the ribosomal A site. The complexity of the EF-T- and EF-G-dependent functions in the complete system explains our inability to isolate a single or a limited group of ribosomal protein(s) absolutely essential for their reactions and justifies the need of a large number of ribosomal components controlling their activities.

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