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Requirement of Proteins S5 and S9 from 30S Subunits for the Ribosome-Dependent GTPase Activity of Elongation Factor G

(CsCl 30S core/ribosomal split proteins/30S reconstitution/30S–50S association)

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ABSTRACT **During CsCl isopycnic centrifugation at 20 mM Mg^{++}, *Escherichia coli* 30S ribosomal subunits specifically lose proteins S1, S2, S3, S5, S9, S10, and S14. The resultant 30S core is unable to stimulate the GTPase activity of EF-G in the presence of 50S subunits. Activity could be restored to a small extent by adding back S2, S5, or S9. However, when S5 and S9 were added together, they cooperatively produced 30S particles 1.5 times more active than the original native 30S subunits. The small amount of activity restored by S2 was simply additive to that restored by S5 or S9. None of the other split proteins showed any restoring capability. Ability of the various protein-deficient 30S particles to couple with 50S subunits corresponded closely to their activity in the EF-G GTPase reaction. It is concluded that S5 and S9 together enable the 30S subunit to participate in the formation of a GTPase-active 30S–50S–EF-G complex.**

Elongation factor G(EF-G) is essential for ribosomal polypeptide synthesis. Its presence is required for expression of a GTPase activity involved in translocation of the peptidyl-tRNA from the acceptor to the donor site of a ribosome. The ribosome–EF-G GTPase activity can be manifested also in the absence of elongation or of amino-acyl-tRNA and mRNA (1). In 1966, Nishizuka and Lipmann (2) found that both ribosomal subunits participate in this GTPase reaction. Since then, most work has pointed out the central role of the 50S ribosomal subunit in the interaction of ribosomes with EF-G (3–5). As will be shown in this communication and in more detail elsewhere, the level of GTPase activity is, however, dependent upon the additional presence of the 30S subunit. Recently, proteins L7 and L12 from the 50S subunit were found to be required for the EF-G GTPase reaction (6, 7). We now report our studies on the 30S subunit designed to determine its active components in this GTPase reaction.

Hosokawa *et al.* (8) and Staehelin and Meselson (9) have shown that CsCl isopycnic centrifugation of 30S subunits produces protein-deficient cores and a fraction of seven specific split proteins—S1, S2, S3, S5, S9, S10, and S14 (10–13)—neither of which can substitute for the 30S subunit in protein synthesis. Recombined, however, they form fully functional 30S subunits. The 30S cores specifically lack the ability to bind mRNA and, in turn, cannot bind tRNA (10, 14). We have now observed that this 30S core is also inactive in promoting the ribosome-dependent GTPase activity of EF-G, apparently due to a failure to couple with 50S subunits. A test of the purified split proteins for their ability to restore this activity has shown S5 and S9 to be the necessary components for restoration.

MATERIALS AND METHODS

EF-G and Ribosomal Particles. Pure EF-G and 70S ribosomes were isolated from *Escherichia coli* B/2 as described (15). The NH_4Cl-washed ribosomes were separated into 30S and 50S subunits by sucrose density gradient centrifugation at 0.5 mM Mg^{++} with a Spinco 15 Ti rotor, and further purified by another zonal centrifugation in a Spinco 14 Ti rotor. The 30S subunits were 99% pure and the 50S subunits were 96–97% pure, as measured by sedimentation in linear 7–30% sucrose density gradients. The 30S cores were prepared by CsCl density gradient centrifugation of 30S subunits in the presence of 20 mM Tris·HCl (pH 7.8)–20 mM $MgCl_2$–7 mM 2-mercaptoethanol (16). The CsCl was optical grade from the Harshaw Chemical Co. All ribosomal particles were stored at −35° in 10 mM Tris·HCl (pH 7.8)–10 mM $MgCl_2$–15 mM KCl–15 mM NH_4Cl–50% glycerol. One A_{260} unit was taken to represent 25 pmol of 70S, 39 pmol of 50S, or 67 pmol of 30S particles (7).

Split Proteins. Individual split proteins were purified from total 30S protein extracted with LiCl–urea (17) by a scheme inspired by methods that have been used by Nomura, Traut, and Wittmann (11, 18, 19). A correlation of the different 30S protein nomenclatures used by these groups is given in ref. 13. All chromatography was performed at 4° in a 10 mM Na-phosphate buffer containing 3 mM 2-mercaptoethanol and 6 M urea (reagent grade, Merck), which had been freshly deionized. The pH was adjusted with NaOH to the desired value. Proteins S1 and S2 were separated from the other 30S proteins by chromatography on DEAE-cellulose (Whatman DE 52) at pH 8.0 with a linear 0–200 mM LiCl gradient. S1 and S2 were then individually purified from minor contaminants by elution from CM-cellulose (Whatman CM 52) at pH 5.6 with a linear 0–200 mM LiCl gradient. The 30S proteins that did not absorb to the DEAE-cellulose at pH 8.0 were applied to a column of carboxymethyl (CM)-cellulose at pH 5.6 and eluted with a linear 80–425 mM LiCl gradient. So chromatographed, S3 and S14 were essentially pure, S9 was still somewhat contaminated, and S5 and S10 were mixed with one another. S9 was purified by CM-cellulose chromatography at pH 6.6 with a linear gradient of 150–250 mM LiCl; S5 was separated from S10 by CM-cellulose chromatography at pH 3.8 with a linear 75–200 mM LiCl gradient. After being concentrated to about 1 mg/ml with Aquacide II (Calbiochem), the proteins were stored at 4° in 50 mM Tris·HCl (pH 7.8)–2 M NH_4Cl–3 mM 2-mercaptoethanol. Purity of the proteins was monitored at all steps by polyacrylamide gel electrophoresis at pH 4.5 in 8 M urea (20). So judged, the isolated split proteins were more than 95% pure and contained no cross-contamination. Protein concentrations were measured by the method of Lowry *et al.* (21) with crystalline bovine-serum albumin as standard. Molecular weights of S1, S2, S3, S5, S9, S10, and S14 were

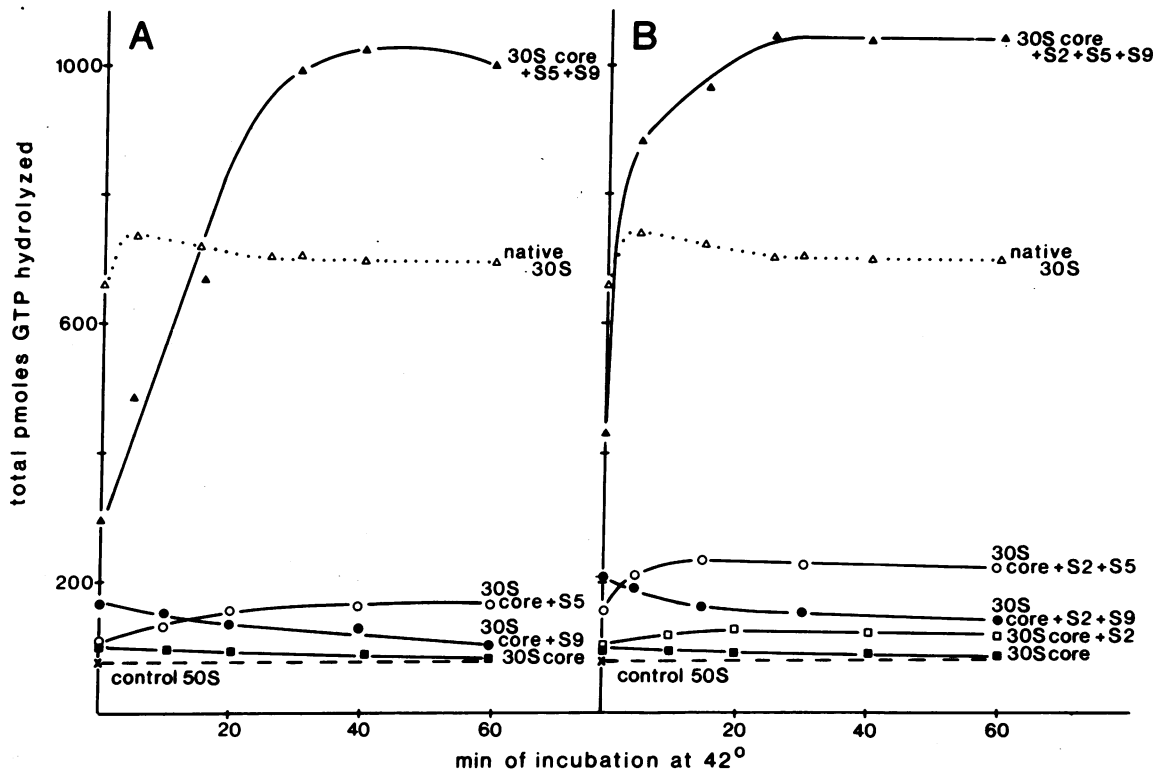

FIG. 1. Ability of S2, S5, and S9—individually and in all possible combinations—to restore activity in the ribosome–EF-G GTPase reaction to CsCl-prepared 30S cores as a function of the time of reconstitution at 42°. Each reconstitution mixture contained 8 pmol of 30S cores and a 1.2-molar excess of each of the split proteins, added as indicated in the figure. To assay GTPase activity after reconstitution 10 pmol of native 50S subunits was added to each reaction mixture. As controls, the 50S subunits were assayed alone (×) and in the presence of either (■) 30S core or (△) native 30S particles.

assumed to be 65,000, 30,000, 32,000, 24,000, 21,000, 16,000, and 15,600, respectively (22).

Reconstitution and GTPase Assay. For reconstitution of 30S particles, the 20-μl reaction mixture contained 0.12 A_{260} units of CsCl 30S cores (8 pmol) and various concentrations and combinations of the individual split proteins in 10 mM Tris·HCl (pH 7.8)–25 mM $MgCl_2$–300 mM NH_4Cl–5 mM KCl–1 mM mercaptoethanol–12% glycerol. After incubation at 42°, 0.25 A_{260} units of 50S subunits (10 pmol), 5.0 μg of EF-G (60 pmol), and 5.0 nmol of [γ-^{32}P]GTP (43–63 Ci/mol, prepared as in ref. 7) were added to the reaction mixture, which was then brought to a final volume of 75 μl containing the following concentrations: 100 mM Tris·HCl (pH 7.8)–20 mM $MgCl_2$–80 mM NH_4Cl–2 mM KCl–15 mM mercaptoethanol–3% glycerol. GTPase activity was measured as the amount of $^{32}P_i$ liberated during a 15-min incubation at 30° (7).

30S–50S Reassociation. Ability of the various 30S particles to couple with 50S subunits was measured by sedimentation in linear 7–30% sucrose gradients in 20 mM Tris·HCl (pH 7.8)–20 mM $MgCl_2$–30 mM NH_4Cl–30 mM KCl. For this procedure, the 20-μl reconstitution mixture described above was doubled in volume and constituents and incubated 20 min at 42°. After addition of 0.50 A_{260} units of 50S subunits along with 250 μl of 25 mM Tris·HCl (pH 7.8)–20 mM $MgCl_2$–50 mM NH_4Cl and incubation for 10 min at 30°, the mixtures were layered on the sucrose gradients. The gradients were spun for 3 hr at 38,000 rpm at 4° in a Spinco SW40 rotor, and the resultant absorption patterns at 254 nm were measured with an ISCO fractionator connected to an external Servogor recorder.

RESULTS

Role of 30S subunits in the EF-G-dependent GTPase reaction

As shown in Table 1 and Figs. 1 and 2, preparations of 50S ribosomal subunits that were contaminated 3–4% with measurable 30S subunits possessed a small residual capability to promote the EF-G-dependent hydrolysis of GTP. Upon addition of native 30S subunits at a 0.8 molar ratio to the 50S subunits, the GTPase activity increased 10-fold or more. Results to be published elsewhere show that the GTPase activity can be further increased when more 30S subunits are added, a plateau in activity being reached only after 30S subunits are present at a several-fold excess over the 50S.

Activity of protein-deficient 30S particles in the GTPase reaction

Under our EF-G GTPase assay conditions, designed to measure the full stimulatory activity of the average 30S subunit by including a slight—but sufficient—excess of 50S subunits, little or no activity was observed for CsCl-prepared protein-deficient 30S cores (Table 1). This variation in activity depended upon the individual 30S core preparation; the best preparations were made at 10–20 mM Mg^{++}, as opposed to 40 mM Mg^{++}. Residual activity, when present, could be

lowered or eliminated by a second centrifugation in CsCl. Addition of all purified split proteins, each at a 1.2-molar excess over core, produced 30S particles more active in the GTPase reaction than the original 30S subunits. Without the 30S core, the split proteins showed no ability to stimulate the GTPase reaction.

To determine which of the split proteins were essential for restoring activity, a series of reconstitutions and assays was performed; a different split protein was omitted from each reconstitution mixture. As Table 1 shows, S5 and S9 proved to be the necessary components, the absence of S5 having a somewhat more dramatic effect than that of S9. Only minor variations from the activity of the fully reconstituted 30S particles were observed upon omission of any one of the other five split proteins. The requirement for S5 and S9 was independent of Mg^{++} concentration over the range (10–20 mM) tested.

Restoring ability of individual split proteins

The lack of restored 30S activity in the EF-G GTPase reaction in the absence of proteins S5 and S9 naturally leads to the question: can they alone impart activity to the cores? Fig. 1*A* shows that only about 13% of the native 30S activity could be restored by either S5 or S9 alone at the 1.2-molar excess of protein over core used. Combined, however, the action of S5 and S9 proved to be extremely cooperative; particles 1.5-times more active than the native 30S control were produced. The reason for this greater activity is unclear since one copy of both S5 and S9 appears to be present in the native 30S subunit (23).

Although formation of fully active 30S particles from cores and proteins did not stringently require a 42° incubation, such an incubation was necessary to restore maximal activity in a reasonable period of time. The reactivation time could also be decreased by use of concentrations of S5 and S9 proteins higher than the 1.2-molar excess over 30S core used for the experiment illustrated in Fig. 1*A*. However, no further increase in activity of the final GTPase was observed.

A further examination of the individual split proteins revealed that the 30S restoring ability of S5 or S9 depended upon their concentration in the reaction mixture. As Fig. 2 shows, S5 could maximally restore 38% of the native 30S activity when present at more than a 5-fold excess over 30S core. With S9, a maximum restoration of 19% of native activity was reached at a 2-fold excess. Surprisingly, S2 also showed activity, restoring 20% of the native 30S activity when present at a 13.5-molar excess over core, the highest ratio tested. The presence of S2 failed, however, to have any striking effect on the restoring ability of either S5 or S9, the restoration of 30S activity in both cases being simply additive (Fig. 1*B*). Moreover, S2, S5, and S9 together showed no significant increase in final 30S activity restored over the combination of S5 and S9 alone. However, with S2 present, the rate at which S5 and S9 were able to restore 30S activity in the GTPase reaction was markedly increased (Fig. 1*A* and *B*).

50S coupling ability of protein-deficient 30S particles

As a step toward elucidation of the mechanism by which S5 and S9 restore activity to 30S cores in the ribosome–EF-G GTPase reaction, the various protein-deficient 30S particles were tested for their ability to associate with 50S subunits, as judged by the production of particles sedimenting faster than

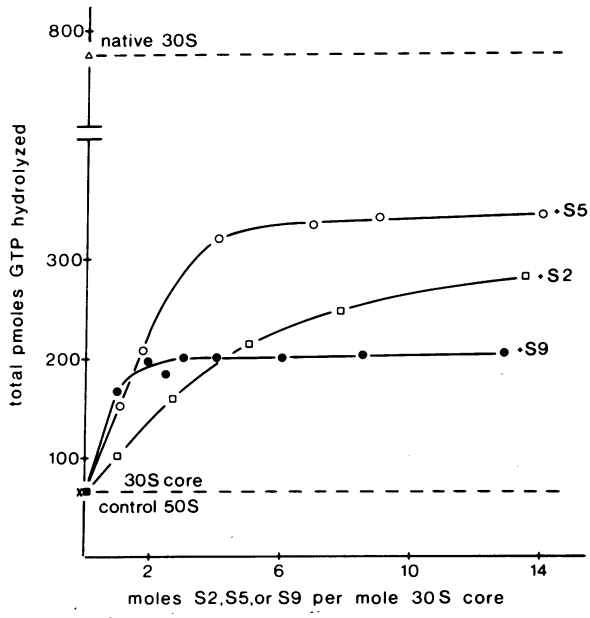

FIG. 2. The individual ability of proteins (□) S2, (○) S5, and (●) S9 to restore activity in the ribosome–EF-G GTPase reaction to 30S cores, as a function of their relative concentrations in the reconstitution mixture. All GTPase assays were performed in the presence of 10 pmol of 50S subunits. Each *point* represents the total activity observed with 8 pmol of 30S particles. These particles had been reconstituted in the presence of increasing concentrations of the given split proteins under the following observed optimal conditions: a 60-min incubation at 42° with S2 and S5, or without a 42° incubation with S9. As controls, 50S particles were assayed alone (×) and in the presence of either (■) 30S core or (△) native 30S subunits.

TABLE 1. *Activity of various split protein-deficient 30S particles in the ribosome–EF-G GTPase reaction*

30S particles	mol of GTP hydrolyzed per mol of 30S particles
Native 30S	82.2
30S core	4.2
30S core + (S1,S2,S3,S5,S9,S10,S14)	92.4
30S core + (—,S2,S3,S5,S9,S10,S14)	79.7
30S core + (S1,—,S3,S5,S9,S10,S14)	83.1
30S core + (S1,S2,—,S5,S9,S10,S14)	85.0
30S core + (S1,S2,S3,—,S9,S10,S14)	15.2
30S core + (S1,S2,S3,S5,—,S10,S14)	29.8
30S core + (S1,S2,S3,S5,S9, —, S14)	81.5
30S core + (S1,S2,S3,S5,S9,S10, —)	75.0

Protein-deficient 30S cores prepared by CsCl isopycnic centrifugation of native 30S subunits were incubated with a 1.2-molar excess of each of the given split proteins and tested in the presence of a 1.25-molar excess of native 50S subunits for activity in the EF-G-dependent GTPase reaction. When the 50S subunits were assayed alone, 5.4 mol of GTP were hydrolyzed per mol of 50S subunits; this background activity has been subtracted from the values given in the table. When all split proteins were tested only in the presence of 50S subunits and the background was subtracted, 0.3 mol of GTP was hydrolyzed per mol of 50S particles.

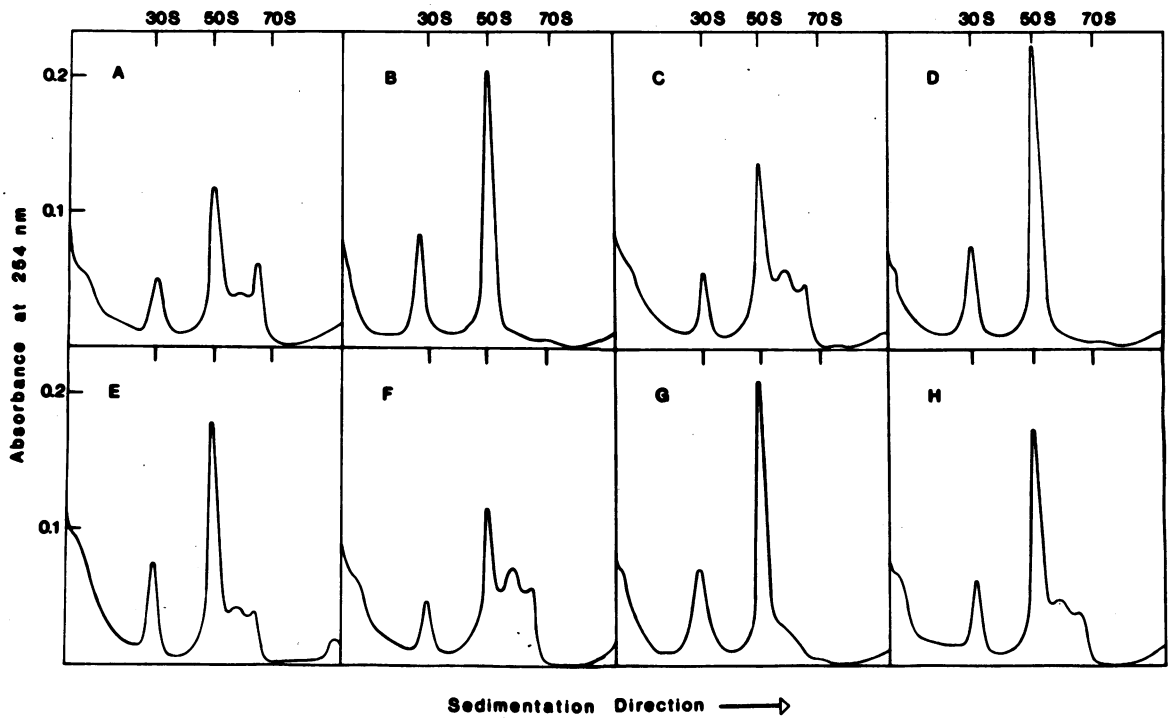

FIG. 3. 50S coupling ability of protein-deficient 30S particles as measured in linear 7–30% sucrose gradients containing 20 mM Mg^{++}. The gradients were layered with mixtures containing native 50S subunits incubated together with either (*A*) native 30S subunits, (*B*) CsCl 30S cores; or with 30S particles reconstituted from 30S cores and (*C*) all seven split proteins, (*D*) all split proteins except S5, (*E*) all split proteins except S9, (*F*) all split proteins except S10, (*G*) S5 alone, (*H*) S5 and S9 together. Each split protein was present at a 1.2-molar excess over 30S core during reconstitution except for S5 in (*G*), which was present at a 3-fold excess. Centrifugation conditions: Spinco SW40 rotor, 38,000 rpm, 3 hr at 4°. Absorption at the top of the gradients was due to the presence of mercaptoethanol in the 30S reconstitution mixture.

50S in sucrose density gradients. In direct correlation with the GTPase results, 30S cores failed to associate with 50S subunits (Fig. 3*B*) but could be restored to full activity upon addition of all seven split proteins (Fig. 3*A* and *C*). Again, this restoration depended primarily upon the presence of protein S5 (Fig. 3*D*) and, to a lesser extent, upon that of S9 (Fig. 3*E*). No difference in restored 50S coupling ability was observed when either S1, S2, S3, or S14 was omitted from the mixture of split proteins; sedimentation patterns identical with Fig. 3*C* were obtained in each case. Upon omission of S10, however, a small but reproducible stimulation in coupling occurred (Fig. 3*F*). Although this apparent inhibition of 30S–50S association by S10 may be physiologically significant, it is more likely due to nonspecific binding of S10, which is known to aggregate readily (18), to the ribosomal subunits.

Alone, S5 at high molar ratios to core could impart a small amount of 50S coupling activity (Fig. 3*G*); but, as with GTPase activity, S9 together with S5 was required to restore essentially full 50S coupling ability to the 30S cores (Fig. 3*H*).

Our failure to observe a sedimentation constant of 70 S for reassociated 30S–50S subunits is probably due to a combination of great hydrostatic pressure during the high-speed sedimentation analysis (24) and to weakened interaction between subunits in the absence of mRNA and tRNA.

DISCUSSION

Our experimental results point out the importance of 30S subunits in the ribosome–EF-G GTPase reaction and show that their activity depends upon the presence of proteins S5 and S9. Proteins S1, S3, S10, and S14 are dispensible—at least in the 10–20 mM Mg^{++} range tested. In general, S2 is also dispensible; however, it does appear capable of inducing a minor amount of 30S activity that becomes apparent in the absence of S5 and S9.

Because the 50S coupling ability of the various protein-deficient particles tested corresponded almost directly to their activity in the EF-G GTPase reaction and because the 50S subunit is known to bind EF-G under several experimental conditions (3, 4), S5 and S9 undoubtedly function by enabling the 30S subunit to participate in the formation of a GTPase-active 30S–50S–EF-G complex. Such a central role for S5 and S9 in protein synthesis is in agreement with the low capability of 30S particles reconstituted in the absence of either S5 or S9 to participate in protein synthesis, as determined by Nomura *et al.* (11).

The tempting possibility exists that S5 and S9 sit at the 30S–50S interface and in so doing directly regulate or help mediate the GTPase reaction. In support of this possibility, Huang and Cantor (25) have recently found S5 and S9 to be among six 30S proteins strongly shielded by the 50S subunit from reaction with fluorescein isothiocyanate. Alternatively, binding of S5 and S9 may simply induce conformational changes that activate other portions of the core responsible for coupling with 50S and stimulation of the GTPase reaction.

The extreme cooperativity of S5 and S9 in activating the 30S core is most exciting in light of the recent finding by Bickle *et al.* (26) that S5 and S9 can be crosslinked by reaction of 30S subunits with bis-(methyl)suberimidate and are, there-

fore, close neighbors. This result suggests that S5 and S9 interact directly, perhaps inducing conformational changes in one another that enable the 30S to couple with the 50S subunit. That the cooperativity shown by S5 and S9 is not due to any interdependency in binding to the 30S subunit during reconstitution has been observed by Mizushima and Nomura (12).

The fact that S5 plays a vital role in the ribosome–EF-G GTPase reaction is consistent with earlier work on the antibiotic spectinomycin. Spectinomycin appears to inhibit protein synthesis by blocking some aspect of ribosomal translocation (27), and S5 has been identified as the component responsible for the sensitivity of ribosomes to this antibiotic (28).

Our results indicate that S2 plays some role in the GTPase reaction and also facilitates the reconstitution of 30S particles by S5 and S9. These activities cannot be explained by contamination. It is interesting to note that S2 has also been indicated to contribute to the aminoacyl-tRNA binding site (29).

The results reported here do not affect the fact that the GTP cleavage center seems to be localized on a 50S–EF-G complex (3–5) but point out the great importance of S5 and S9 from the 30S subunit in the EF-G–ribosome GTPase reaction. Elucidation of the mechanism of the EF-G–ribosome GTPase reaction during elongation can be expected only when the role of the different components has been clarified with respect to the complete system, which alone allows polypeptide synthesis.

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1. Lucas-Lenard, J. & Lipmann, F. (1971) *Annu. Rev. Biochem.* **40**, 409–448.
2. Nishizuka, Y. & Lipmann, F. (1966) *Arch. Biochem. Biophys.* **116**, 344–351.
3. Bodley, J. W. & Lin, L. (1971) *Nature* **227**, 60–61.
4. Brot, N., Spears, C. & Weissbach, H. (1971) *Arch. Biochem. Biophys.* **143**, 286–296.
5. Modolell, J., Vazquez, D. and Monro, R. E. (1971) *Nature New Biol.* **230**, 109–112.
6. Kischa, K., Möller, W. & Stöffler, G. (1971) *Nature New Biol.* **233**, 62–63.
7. Sander, G., Marsh, R. C. & Parmeggiani, A. (1972) *Biochem. Biophys. Res. Commun.* **47**, 866–873.
8. Hosokawa, K., Fujimura, R. K. & Nomura, M. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 198–204.
9. Staehelin, T. & Meselson, M. (1966) *J. Mol. Biol.* **16**, 245–249.
10. Traub, P. & Nomura, M. (1968) *J. Mol. Biol.* **34**, 575–593.
11. Nomura, M., Mizushima, S., Ozaki, M., Traub, P. & Lowry, C. V. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 49–61.
12. Mizushima, S. & Nomura, M. (1970) *Nature* **226**, 1214–1218.
13. Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Randall-Hazelbauer, L., Birge, E. A., Nomura, M., Kaltschmidt, E., Mizushima, S., Traut, R. R. & Bickle, T. A. (1971) *Mol. Gen. Genet.* **111**, 327–333.
14. Raskas, H. & Staehelin, T. (1967) *J. Mol. Biol.* **23**, 89–97.
15. Parmeggiani, A., Singer, C. & Gottschalk, E. M. (1971) in *Methods in Enzymology*, eds. Grossmann, L. & Moldave, K. (Academic Press, New York), Vol. 20, part C, pp. 291–302.
16. Meselson, M., Nomura, M., Brenner, S., Davern, C. & Schlessinger, D. (1964) *J. Mol. Biol.* **9**, 696–711.
17. Spitnik-Elson, P. (1965) *Biochem. Biophys. Res. Commun.* **18**, 557–562.
18. Moore, P. B., Traut, R. R., Noller, H., Pearson, P. & Delius, H. (1968) *J. Mol. Biol.* **31**, 441–461.
19. Hindennach, I., Stöffler, G. & Wittmann, H. G. (1971) *Eur. J. Biochem.* **23**, 7–11.
20. Leboy, P. S., Cox, E. C. & Flaks, J. G. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 1367–1374.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
22. Craven, G. R., Voynow, P., Hardy, S. J. S. & Kurland, C. G. (1969) *Biochemistry* **8**, 2906–2915.
23. Traut, R. R., Delius, H., Ahmad-Zadeh, C., Bickle, T. A., Pearson, P. & Tissières, A. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 25–38.
24. Infante, A. A. & Baierlein, R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1780–1785.
25. Huang, K. & Cantor, C. R. (1972) *J. Mol. Biol.* **67**, 265–275.
26. Bickle, T. A., Hershey, J. W. B. & Traut, R. R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1327–1331.
27. Anderson, P., Davies, J. & Davis, B. D. (1967) *J. Mol. Biol.* **29**, 203–215.
28. Bollen, A., Davies, J., Ozaki, M. & Mizushima, S. (1969) *Science* **165**, 85–86.
29. Randall-Hazelbauer, L. L. & Kurland, C. G. (1972) *Mol. Gen. Genet.* **115**, 234–242.