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Ribosomal protein L7/L12 is required for optimal translation

(protein synthesis/ionic conditions/missense errors)

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ABSTRACT **We have tested the performance *in vitro* of *Escherichia coli* ribosomes containing or lacking the protein L7/L12. When the experiments are performed in an optimized mixture of ions (polymix), L7/L12 is required for maximal rate of synthesis as well as for minimal missense error frequency. The results in conventional Tris/Mg^{2+}/NH_4Cl buffers are different; in these buffers, only the rate of synthesis is strongly dependent on the presence of L7/L12. In addition, we show that there is a large difference between the optimal Mg^{2+} concentration required for speed of translation and that for accuracy of translation in conventional buffer. These optima are very close in polymix. Finally, we show that the contribution of L7/L12 to the speed of translation is obscured in translation systems that are limited by substrates. We conclude that it is not possible to analyze details of the mechanism of translation in conventional buffers.**

The L7/L12 protein of the *Escherichia coli* 50S ribosome owes its double name to the fact that it can be isolated either as an NH_2-terminally acetylated species, L7, or as a species with a free NH_2 terminus, L12 (1). It is unusual also because the sum of both species on the ribosome is between three and four copies per particle (2–4). In solution it is most stable as a dimer (5, 6), which suggests that a ribosome may contain two such dimers. Finally, it is distinguished by being the only ribosomal protein for which high-resolution crystallographic information is available (7).

It seems likely that the functions of this protein in translation are important ones, because it is required *in vitro* for the expression of maximal activity by initiation, elongation, and termination factors [see review by Möller (8)]. Indeed, the removal of L7/L12 was found (9, 10) to inhibit markedly protein synthesis by the deficient ribosomes. On the other hand, Koteliansky *et al.* (11) as well as Glick (12) have reported other experiments in which the removal of L7/L12 from ribosomes had at most a marginal effect on their ability to synthesize polypeptides. Because L7/L12 is involved in the factor-dependent hydrolysis of GTP on ribosomes (9, 13–21), these conflicting results confuse assessments of the role of GTP hydrolysis in protein synthesis.

We have reinvestigated the influence of L7/L12 on the activities of ribosomes, using a recently developed system that supports translation *in vitro* with a missense error frequency approaching that of living bacteria (22). Our results show that the expression of both the maximal rate of polypeptide synthesis and the minimal missense error rate are strongly dependent on the presence of L7/L12 in ribosomes. In addition, systematic studies of the assay conditions show that rate-limiting substrate concentrations as well as the absence of certain polyelectrolytes obscure the functional contributions of this protein.

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MATERIALS AND METHODS

Purifications. Frozen *E. coli* MRE 600 cells were used as starting material unless otherwise stated. All preparations were finally dialyzed against the phosphate/polyamine buffer, "polymix," used in the translation assays (see below). The 70S ribosomes were prepared by the method of Jelenc (23), which uses passage through a Sephacryl S200 column in polymix with 1 M ammonium chloride instead of high-salt wash and pelleting by ultracentrifugation. The ribosomes were treated four times with ammonium chloride/ethanol for the preparation of L7/L12-depleted 70S core particles (9). L7/L12 was purified as described (24). The soluble fraction II of Wood and Berg (25) was prepared from *E. coli* K-12 P99C grown as described in ref. 26.

Chemicals. *E. coli* tRNA from strain MRE 600 was purchased from Boehringer Mannheim. Preparations of poly(U), ATP, GTP, phospho*enol*pyruvate, pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), putrescine, and spermidine were obtained from Sigma. Radioactive amino acids [^{14}C]phenylalanine, [^{3}H]leucine, [^{3}H]valine, and [^{3}H]-serine were purchased from the Radiochemical Centre (Amersham, England). Sephacryl S200 was obtained from Pharmacia.

***In Vitro* Translation Assays.** The poly(U)-directed incorporation assays are done in either polymix buffer (22) or a conventional Tris buffer (27). Polymix buffer is composed of 5 mM magnesium acetate, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, 95 mM potassium chloride, 5 mM ammonium chloride, and 1 mM dithiothreitol titrated to pH 7.5. The conventional buffer contains 20 mM Tris·HCl, 10 mM magnesium acetate, 100 mM ammonium chloride, and 1 mM dithiothreitol, adjusted to pH 7.5.

Each 100-μl reaction mixture contained in addition 1 mM ATP, 1 mM GTP, 6 mM phospho*enol*pyruvate, 1 μg of pyruvate kinase, 10 μg of poly(U), 200 μg of total *E. coli* tRNA, 10 μl of soluble fraction II, 5000 pmol of [^{14}C]phenylalanine, 1500 pmol of [^{3}H]leucine, and 10–20 pmol of 70S ribosomes, 70S ribosomal core particles, or reconstituted 70S ribosomes. The standard incubation time was 30 min at 37°C. Incubation, quenching, filtration, and calculations were done as described (22). This system made up in polymix buffer is our standard *in vitro* system referred to in the text and in the figure legends unless otherwise stated. Modifications are described in the appropriate figure legend.

Reconstitution of L7/L12-Depleted Core Particles. Purified L7/L12 was either added to the core particles before these had been introduced in the assay mixture or added into a complete assay mixture already containing the core particles. Both methods gave the same results, so in each assay the technically most convenient order of addition was used.

Table 1. Translational activity of 70S ribosomes and L7/L12-depleted 70S core particles

Incorporation	70S ribosomes	70S cores	70S cores/ 70S ribosomes
Sense, pmol phenylalanine	1200	30	0.025
Missense, relative to phenylalanine			
Leucine	4.7×10^{-4}	4.0×10^{-3}	8.5
Valine	1.4×10^{-4}	6.0×10^{-4}	4.3
Serine	6.5×10^{-5}	5.4×10^{-4}	8.3

Assay conditions were as described in *Materials and Methods* with leucine, valine, or serine added as the noncognate amino acid. The missense incorporation is calculated as the ratio (noncognate)/(noncognate + cognate) amino acid in the hot trichloroacetic acid-precipitable product.

RESULTS

Dependence on L7/L12. The activities of 70S ribosomes and 70S cores—i.e., L7/L12-depleted ribosomes prepared as described by Hamel *et al.* (9)—were compared in a high-fidelity *in vitro* system primed with poly(U) (22). The data summarized in Table 1 show that under these conditions the removal of L7/L12 leads to a reduction of phenylalanine incorporation to 2.5% of the control as well as to a 4- to 8-fold increase of the misincorporation of leucine, valine, and serine.

The relatively poor performance of 70S cores might be due at least in part to damage done during core preparation rather than to the absence of the L7/L12 *per se*. In order to distinguish between these alternatives, the effects of readdition of L7/L12 to 70S cores was studied.

The data summarized in Fig. 1 show that readdition of L7/L12 to 70S cores stimulates the phenylalanine incorporation to within one third of the activity of the starting 70S ribosomes. Therefore, while there has been significant inactivation of the 70S cores, the readdition of L7/L12 leads to more than a 10-fold stimulation of peptide synthetic activity. Most significant is the finding that readdition of L7/L12 reduces the missense error rate with leucine to a level close to that of the starting 70S ribosomes (Fig. 1). In conclusion, the reduction of ribosome activity attending removal of L7/L12 is only marginally due to damage of the cores and is primarily due to the absence of L7/L12.

The L7/L12 concentration dependence of the activity of ribosomes is similar to that reported by others (9, 18, 28). Thus, the data in Fig. 1 are consistent with a return of maximal activity to a core particle after the addition of an average of one dimer rather than the expected two dimers of L7/L12. Unfortunately, our data would not permit us to detect a requirement for two dimers per active ribosome within the background of a majority of functionally inactive cores that would bind only one dimer.

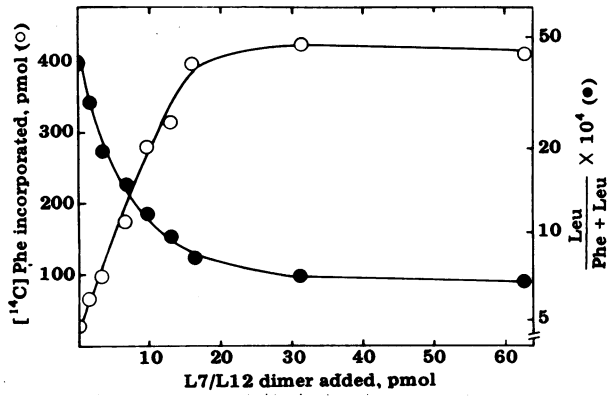

FIG. 1. Activation of 70S core particles by addition of purified L7/L12. The missense incorporation is plotted on a logarithmic scale.

Effect of Ionic Conditions. The results of Koteliansky *et al.* (11) as well as those of Glick (12) have suggested, in contrast to the data described above, that the presence or absence of L7/L12 has little or no effect on the ability of the ribosomes to translate poly(U). However, their experiments were done in a conventional Tris/Mg^{2+}/NH_4Cl system, while ours are done in polymix (22). In order to determine the extent to which the different results depend on the assay conditions, we have compared the activities of 70S ribosomes to those of 70S cores in conventional buffer and in polymix.

The data summarized in Fig. 2 *Upper* show that the Mg^{2+} concentration optimum for phenylalanine incorporation by preparations of both 70S ribosomes and 70S cores in conventional buffer is close to 10 mM. In contrast, the missense error rate for leucine incorporation under these conditions increases by more than an order of magnitude when the Mg^{2+} concentration is raised from 5 mM to 10 mM, and it continues to rise by another order of magnitude when the Mg^{2+} concentration is further increased to levels that inhibit phenylalanine incorporation. The responses of both 70S cores and 70S ribosomes to changes in the Mg^{2+} concentration are similar for the leucine missense frequency, but there is a 20-fold difference in the phenylalanine incorporation between the two preparations at the Mg^{2+} concentration corresponding to maximal activity. Clearly, the Mg^{2+} dependence of the optimal activity and that for minimum error rate are uncoupled in the conventional system.

The Mg^{2+} concentration dependences of the cores and 70S ribosomes in polymix are significantly different (Fig. 2 *Lower*). Thus, both the optimal phenylalanine incorporation and the minimal missense error with leucine are observed at Mg^{2+} concentrations in the neighborhood of 5 mM. Although the maximal phenylalanine incorporation is slightly higher for 70S cores in conventional buffer compared to that in polymix buffer, that for 70S ribosomes is about twice as high in polymix as in conventional buffer. Significantly, near the optimal Mg^{2+}

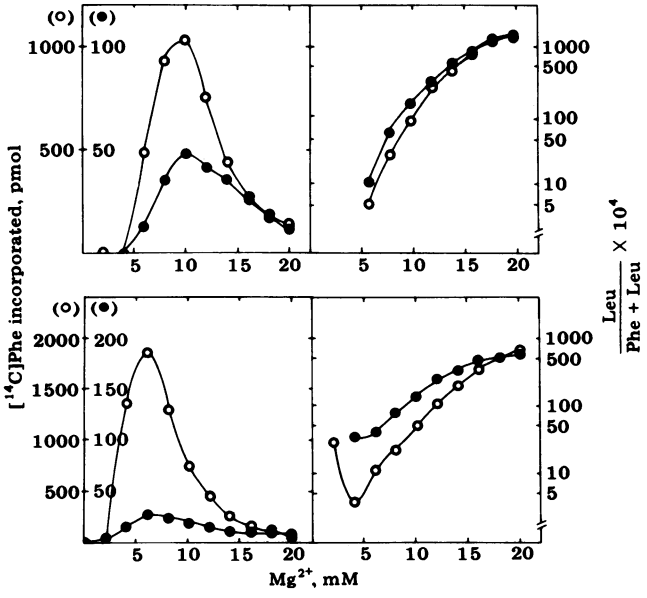

FIG. 2. Mg^{2+} dependence of incorporation in a Tris/NH_4Cl system (*Upper*) and in the polymix system (*Lower*). ○, 70S ribosomes; ●, 70S core particles.

concentration there is a roughly 10-fold difference in the error rates with leucine for 70S cores compared to 70S ribosomes.

Comparison of the activities of 70S cores and 70S ribosomes in the two buffer systems (Fig. 2) shows that the ionic environment certainly influences the outcomes of these experiments. Thus, in conventional buffer, the dependence of the error rate on the presence or absence of L7/L12 is relatively small, at most a factor of 2, at all Mg^{2+} concentrations. On the other hand, polymix enhances the absolute activity of 70S ribosomes and couples the Mg^{2+} dependence of this activity to that of the missense error frequency. Most relevant to the present study is the finding that the dependence of the missense error rate on L7/L12 is quite evident in polymix. Nevertheless, the differences we observe in the two buffer systems will not account for the discrepancies between our results and those of Koteliansky *et al.* (11) and Glick (12).

Effects of Substrate Limitation. An important difference between our experimental design and that of Koteliansky *et al.* (11) and Glick (12) is that we employ aminoacyl-tRNA synthetases as well as a nucleoside triphosphate-regenerating system to maintain substrate levels at high steady-state levels. However, these workers have employed systems in which a fixed amount of substrate is added initially, and the substrate concentrations are allowed to decrease over the course of the incubation period. We therefore explored the possibility that the discrepancy in the results with L7/L12 could be due to differences in the degrees of substrate limitation in the two systems.

The effects of varying the tRNA and nucleoside triphosphate concentrations on the speed as well as the accuracy of translation in both conventional buffer and polymix are described in detail elsewhere (22). We have extended these studies to include effects on core particles. We find that raising the amounts of nucleoside triphosphates, tRNA, or amino acids to levels higher than those used in the above experiments does not enhance the performance of either cores or 70S ribosomes in either conventional buffer or polymix (data not shown). In other words, all the data reported here are for systems in which the rates are limited by the ribosome or core concentrations.

On the other hand, if amino acid or tRNA concentrations or both are lowered significantly, the apparent differences between the activities of cores and 70S ribosomes are reduced because 70S ribosomes exhaust the substrates faster than cores do. One illustration of the effects of substrate limitation is given in Fig. 3.

We compared the activities of cores and 70S ribosomes in polymix with either the normal amount—5000 pmol—of phenylalanine (Fig. 3 *Left*) or with a limiting amount—50 pmol—of phenylalanine (Fig. 3 *Right*). Two relevant effects are evident. First, the maximal initial rate of the phenylalanine incorporation by 70S ribosomes is reduced by more than an order of magnitude at the lower phenylalanine concentration. Second, the incorporation levels off after 10 min in the limiting amino acid mixture, and, as a consequence, the relative activity of 70S cores and 70S ribosomes depends on the time of incubation. In contrast, with a properly titrated incubation mixture (Fig. 3 *Left*) the relative activity of 70S cores and 70S ribosomes is independent of time for at least 30 min. Significantly, the activities of cores relative to 70S ribosomes increases from 2% in excess amino acid to nearly 20% in limiting amino acid after a 30-min incubation.

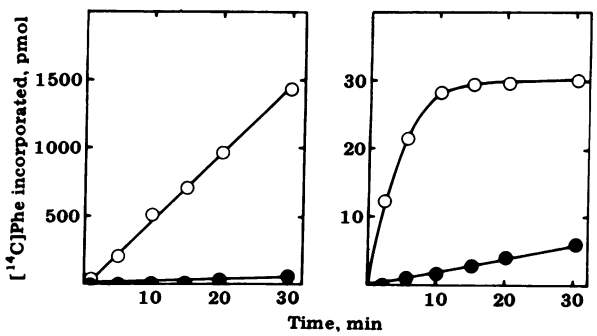

FIG. 3. Time course of incorporation with optimized (*Left*) or limiting (*Right*) amounts of amino acid added. (*Left*) Standard amino acid input, 5000 pmol. (*Right*) Limiting amino acid input, 50 pmol. ○, 70S ribosomes; ●, 70S core particles.

DISCUSSION

Our data show that L7/L12 is required for the maximal rate of polypeptide synthesis at all Mg^{2+} concentrations in both conventional buffer and polymix. Such results confirm previous findings (9, 18). Furthermore, they are consistent with the widely held view that the factor-dependent hydrolysis of GTP, which is mediated by L7/L12, is an important partial reaction in protein synthesis (29, 30).

The contradictory results of others (11, 12), who found little or no dependence on the presence of L7/L12, are also explicable in light of the present results. They have added fixed amounts of aminoacyl-tRNA and GTP to their incubation mixtures. During the course of their incubations a substantial fraction of the added substrates will be consumed by the ribosome-dependent reactions as well as by spontaneous hydrolysis. As a consequence, the rate of incorporation decreases with the time of incubation. Indeed, we can simulate this behavior in our systems by employing limiting amounts of amino acid. Under these substrate-limited conditions, we find that the maximal activity of ribosomes as well as the difference in specific activities of cores and ribosomes are underestimated. Therefore, we suggest that others may have underestimated the influence of L7/L12 on the rate of polypeptide synthesis because they were using assay conditions in which the substrate concentrations were rate limiting.

In general, conventional buffer systems—i.e., those containing Tris buffer with Mg^{2+} as the sole polyvalent cation—support translation *in vitro* less effectively than does the polymix (22). Two reasons for this performance difference are evident in the present data. First, we find in conventional buffer that there is a marked separation between the Mg^{2+} concentration which supports the maximal rate of polypeptide synthesis and that which supports the minimum missense error frequency. In other words, it is impossible to optimize simultaneously the rate and accuracy of translation by varying the Mg^{2+} concentration in a conventional buffer system. In contrast, the two optima are supported by Mg^{2+} concentrations that are almost the same in polymix.

Second, we find in conventional buffer that L7/L12 has a pronounced influence on the rate of polypeptide synthesis at all Mg^{2+} concentrations, but that it has relatively little influence on the missense error frequency under the same conditions. In contrast, the presence or absence of L7/L12 coordinately influences these two performance characteristics of the system in polymix. Such observations suggest that the normal interactions between one or more components of the translation apparatus with the ribosome domain containing L7/L12 are modulated by a number of different polyelectrolytes. These ionic species are absent in conventional buffers, with the consequence that in such buffers the normal coupling of the speed and accuracy functions of L7/L12 is lost.

In conclusion, the present results have several obvious implications for future studies. One is that they show how risky

it is to optimize *in vitro* translation systems with respect to only one performance characteristic such as speed or accuracy; both aspects must be taken into account when *in vitro* assays of translation are under development. Second, it seems likely that some prior results obtained in the conventional buffers and relevant to the influence of ribosomes on the error frequency of translation may require reevaluation. Third, our data show that experiments concerned with the *in vitro* identification of the role played by different components of the translation apparatus are preferably performed under steady-state conditions rather than under those in which substrate limitations are built into the experimental design.

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