RIBOSOME SPECIFICITY FOR THE FORMATION OF GUANOSINE POLYPHOSPHATES

Jose Sy, Nam-Hai Chua, Yasuo Ogawa, and Fritz Lipmann

The Rockefeller University
New York, N.Y. 10021

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SUMMARY: Ribosomes obtained from Bacillus brevis (ATCC 8185) were slightly active in synthesizing guanosine polyphosphates, which activity was greatly stimulated by addition of Escherichia coli stringent factor. Chlamydomonas reinhardtii chloroplast ribosomes did not produce guanosine polyphosphates on incubation but responded with abundant synthesis to addition of the stringent factor from E. coli. In contrast, cytoplasmic ribosomes from the same organism did not respond. Interchange experiments between either subunit from chloroplasts with the E. coli counterparts showed good activity. When the small subunit of cytoplasmic Chlamydomonas ribosomes was combined with the large subunit of E. coli or of chloroplasts, a small but definite response was obtained.

Haseltine et al. (1), by washing with high salt solutions, isolated from ribosomes of stringent E. coli, a factor (stringent factor) which, when complementing depleted ribosomes, would promote the synthesis of ppGpp and pppGpp* with ATP as a pyrophosphoryl donor to GTP or GDP. It had already been observed by these authors that the responding ribosome did not need to be from a stringent organism, a feature which we could confirm early in our studies on the mechanism of the enzymatic pyrophosphorylation of GTP and GDP (2). We have also shown that stringent factor alone can synthesize guanosine polyphosphates (3). Nevertheless, it was quite clear that the ribosome is able to greatly modify, positively as well as negatively, the activity of the enzyme.

The experiments to be reported here were undertaken to test for the kind of ribosomes that could modify the enzymatic response. As mentioned, under certain conditions, for example in the presence of 20% methanol (3), there is a sizeable enzymatic activity in the nonribosomal system. However, the ribosome-

* The abbreviations are: ppGpp, guanosine 5'-diphosphate-3'-diphosphate; pppGpp, guanosine 5'-triphosphate-3'-diphosphate.

wash enzyme in the absence of ribosomes is quite unstable and has to be assayed at relatively high concentrations and at a temperature not above 30°; at 37°, generally used for ribosomal systems, it is very rapidly denatured. At concentrations where a very good response is seen with the ribosome, during the time needed for assay there is frequently only negligible response in the absence of ribosome; the blanks are essentially zero. With regard to the particulars of the ribosomal effect, the work of Kjeldgaard and his colleagues (4) and also of Haseltine and Block (5) show it to be dependent on the presence of a mRNA together with a corresponding uncharged tRNA. Using either a natural system, or an artifical messenger such as poly-U or poly-A, one needs uncharged $tRNA^{Phe}$ or $tRNA^{Lys}$ to get a response, which is abolished when the tRNAs are charged and polypeptide synthesis takes place.

We were particularly interested to see if the general similarity of organelle ribosomes, e.g. in chloroplasts, and bacterial systems might also apply to these ribosomes with regard to their response, even though they were derived from eukaryote organisms. In addition, we tested for the formation of guanosine polyphosphates by ribosomes of a B. brevis which we had worked with extensively in this laboratory (6). Our results indicate chloroplast ribosomes to be competent, in contrast to cytoplasmic ribosomes of the same organism. The ribosomes of our B. brevis showed strong activity with addition of E. coli enzyme, and slight but definite activity for guanosine polyphosphate synthesis without it.

MATERIALS AND METHODS

E. coli K-19 (stringent) ribosomes and stringent factor were obtained as described (3). B. brevis (ATCC 8185) was a gift from Dr. S. G. Lee.

Preparation of B. brevis ribosomes. B. brevis cells were incubated at 4° for 5 hr in 20 mM triethanolamine-HCl, pH 7.7, 0.5 mM EDTA, 10 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 250 μg of lysozyme, 3 μg/ml DNase. The lysate was then spun at 15,00 rpm for 15 min. The supernatant was further spun at 50,000 rpm for 3 hr to obtain a crude ribosomal pellet. Crude ribosomes, resuspended in 40 mM

Table 1. Guanosine polyphosphate synthesis by B. brevis ribosomes.

Additions	% GTP converted to ppGpp + pppGpp
Ribosomes - 40 μg	3
102 μg	5
204 μg	12
Ribosomes (40 μg) + stringent factor	62
Stringent factor	0

The preparation of B. brevis ribosomes and the assay for guanosine polyphosphates were as described in Methods. Stringent factor was that of fraction II previously described (3).

Table 2. Response of chloroplast ribosomes in guanosine polyphosphate synthesis

Additions	% GTP converted to ppGpp + pppGpp
1. Chloroplast ribosomes (33S + 50S)	68
2. Cytoplasmic ribosomes (37S + 57S)	2
3. E. coli ribosomes (30S + 50S)	40
4. E. coli ribosomes + 2 μM thiostrepton	1
5. Chloroplast ribosomes + 2 μM thiostrepton	3

Assays were performed as described in Methods. The following amounts (A_{260}) units of ribosomal subunits were used: E. coli 30S, 0.35; E. coli 50S, 0.58; chloroplast 33S, 0.29; chloroplast 50S, 0.57; cytoplasmic 37S, 0.37; and cytoplasmic 57S, 0.58. In C. reinhardtii, the large and small subunits of chloroplast ribosomes have sedimentation coefficients of 50S and 33S respectively, whereas the large and small subunits of cytoplasmic ribosomes have sedimentation coefficients of 57S and 37S respectively (11). In all cases, the same stringent factor as in Table 1 was added.

Tris-OAc, pH 7.8, 10 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 20 mM KCl, were then spun at 45,000 rpm for 4 hr over a 40% sucrose layer. The pelleted ribosome was again resuspended in buffer.

Preparation of ribosomal subunits. E. coli ribosomes were dissociated into subunits by the high-salt puromycin procedure of Blobel and Sabatini (7). Ribosomes were incubated with 1 mM puromycin (adjusted to pH 7.5 with KOH) in the presence of a high-salt buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM

KCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol. The mixture was incubated at 37° for 10 min and an aliquot containing approximately 30 A_{260} units was layered on to a 5-20% sucrose gradient containing the high-salt buffer. The gradients were centrifuged at 39,000 rpm for 3 hr at 18° in the SB283 rotor of an International centrifuge. Fractions containing the 30S and 50S subunits were pooled separately and the ribosomal subunits were pelleted by centrifugation at 40,000 rpm for 16 hr in a Spinco 40 rotor. Ribosomal subunits were further purified by two additional cycles of high-salt sucrose gradient centrifugation as described above. Both the 30S and 50S subunits prepared by this procedure contained no cross-contamination, and were completely free of tRNA and mRNA.

Subunits of both cytoplasmic and chloroplast ribosomes of C. reinhardtii were prepared by three cycles of high-salt sucrose density gradient centrifugation according to the method described (8).

Assay for guanosine polyphosphate synthesis. The synthesis of ppGpp and of pppGpp was assayed essentially as described (2). Ribosomal subunits were incubated either together or separately at 30° for 60 min in a 50-μl reaction mixture containing 40 mM Tris-OAc, pH 8.0, 20 mM $Mg(OAc)_2$, 4 mM dithiothreitol, 4 mM ATP, 0.4 mM [α-^{32}P]GTP (20-30 Ci/mole), 10 μg of poly-A,U,G, 27 μg of tRNA, and 3 μg of a fraction II preparation of stringent factor (2). The reactions were terminated by the addition of 1 μl of formic acid and the mixture was centrifuged to remove the precipitated materials. Of the resulting supernatant, 2 μl were chromatographed on polyethyleneimine cellulose thin layer sheets and spots corresponding to pppGpp, ppGpp, GTP, and GDP were cut out and counted. The results were expressed as the per cent of GTP that had been converted into pppGpp and ppGpp.

RESULTS

Reaction with B. brevis ribosomes. Since the B. brevis ribosomes used were obtained from the late log growth phase, they may not be the best possible preparation. Nevertheless, as shown in Table 1, a very good response was

Table 3. Subunit interchange in ribosome-dependent guanosine polyphosphate synthesis.

Additions	% GTP converted to ppGpp + pppGpp
1. E. coli 30S + E. coli 50S	40
2. E. coli 30S + chloroplast 50S	51
3. Chloroplast 33S + E. coli 50S	34
4. Cytoplasmic 37S + E. coli 50S	7
5. Cytoplasmic 37S + chloroplast 50S	9
6. Chloroplast 33S + cytoplasmic 57S	2
7. E. coli 30S + cytoplasmic 57S	1

Assay conditions were as described in Table 2. The subunits, when assayed alone, gave less than 2% conversion of GTP to ppGpp + pppGpp. Stringent factor was added to all assays.

obtained on addition of the E. coli enzyme, and a definite synthetic activity, particularly with high concentrations of ribosomes, without it.

Reaction with ribosomes of C. reinhardtii. Chloroplast and cytoplasmic ribosomes do not synthesize guanosine polyphosphates without the addition of E. coli enzyme. However, on addition of enzyme, the response from chloroplast ribosomes is quite remarkable and, indeed, somewhat better than that from E. coli (Table 2). In contrast, ribosomes from Chlamydomonas cytoplasm were without activity, even on addition of enzyme. Both the responses of E. coli and of chloroplast ribosomes are inhibited by thiostrepton. Thus one may conclude that the response of the chloroplast ribosome, like that of E. coli, is abolished by blocking the acceptor site which is generally assumed to be one of the causes of inhibition of protein synthesis by thiostrepton (9).

Since it had been shown that the combination of larger and smaller subunits of the E. coli ribosome is essential for a response (4,5), we tried to see 1) if an interchange between E. coli and chloroplast subunit was possible, and 2) if cytoplasmic ribosomal subunits would give response when combined with the active counterpart of E. coli or chloroplast. The data of Table 3 indicate that the small subunit of E. coli combined with the large one of the chloroplast

gives a somewhat better response than both of the E. coli subunits. However, in reverse, the small chloroplast unit together with the large E. coli subunit gives a slightly lower response than the combination of the two E. coli subunits. In the second part of Table 3, the combination of cytoplasmic and chloroplast as well as E. coli subunits was tested, and the results here are that the large cytoplasmic with either the small chloroplast of E. coli does not show any response, while the reverse combination, the large subunit of the active ribosomes together with the small subunit of the cytoplasmic shows a small but definite response. This response may be related to the ability of an analogous combination between small eukaryotic and large bacterial subunits that showed some ability for peptide bond formation in experiments reported in the literature (10).

It might be mentioned in conclusion that Richter has reported (11) that mitochondrial ribosomes were found to be much less active for ppGpp synthesis than reported here for the chloroplast; however, it is well known that it is much easier to obtain active chloroplast ribosomes from chloroplasts than from mitochondria.

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