Template-independent synthesis of guanosine tetra- and pentaphosphates on ribosomes

Nadezhda V. Belitsina*, Elena V. Klyachko and Rustem S. Shakulov

*A.N. Bakh Institute of Biochemistry, Academy of Sciences of the USSR, Moscow and All Union Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, USSR

Received 26 July 1983

It is shown that *Escherichia coli* ribosomes carrying poly(Lys)-tRNA can form (p)ppGpp in the presence of stringent factor in the absence of the poly(A) template. Template-independent synthesis of (p)ppGpp is suppressed by tetracycline and partially decreases if deacylated tRNA is omitted.

Guanosine-3',5'-polyphosphate *Stringent factor* *Ribosome* *Tetracycline*

1. INTRODUCTION

For (p)ppGpp synthesis on *Escherichia coli* ribosomes the following components are necessary: pyrophosphoryltransferase (a *relA* gene product or the stringent factor, SF), a template polyribonucleotide, codon-specific deacylated tRNA, ATP as a donor and GTP or GDP as an acceptor of pyrophosphate [1–3]. The binding of SF with the ribosome is strongly dependent on the template [4] and that of the deacylated tRNA is determined by the appropriate codon in the A-site [1,2].

Escherichia coli ribosomes are capable of synthesizing polylysine without poly(A) template using Lys-tRNA as substrate [5,6]. This template-free system of polylysine synthesis seems to be a convenient tool to study the role of mRNA in (p)ppGpp formation.

Here we show that *E. coli* ribosomes carrying poly(Lys)-tRNA without poly(A) can form (p)ppGpp. Thus, the template polyribonucleotide is not requisite for SF-promoted ribosomal synthesis of tetra- and pentaphosphates.

2. MATERIALS AND METHODS

Escherichia coli MRE-600 ribosomes washed 4-times with a mixture of 1 M NH_4Cl and 10 mM $MgCl_2$ [7,8] were used. The purified ribosomes were stored in the frozen state at −70°C in the buffer containing 20 mM Tris–HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol ($pH_{37°C}$ 7.6).

The ribosomes carrying [^{14}C]poly(Lys)-tRNA were isolated from the template-free system of poly(Lys) synthesis [5]. The reaction mixture was prepared in a standard buffer with 20 mM Tris–HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT ($pH_{37°C}$ 7.6); 2.5 ml of this buffer contained 1 nmol ribosomes, 8.5 nmol EF-Tu, 1.8 nmol EF-G, 7.4 nmol [^{14}C]Lys-tRNA (5 mg total tRNA), 0.4 μmol GTP (Fluka), 5 μmol phosphoenolpyruvate (Fluka), 50 μg phosphoenolpyruvate kinase (Boehringer-Mannheim). Incubation was at 37°C for 30 min. The reaction was stopped by cooling and diluting of the incubation mixture up to 13 ml with the standard buffer. The ribosomes from the incubation mixture were pelleted by centrifugation at 49000 rev./min in the Spinco 50 Ti rotor for 2.5 h. The ribosomal pellet was suspended in the standard buffer, and this suspension was clarified by low speed centrifugation; glycerol was added up to 10% and the aliquots of the charged ribosomes were stored at −70°C. The final ribosomal

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00145793/83/$3.00

preparations contained from 1–3 × 10^5 dpm of [^{14}C]poly(Lys)-tRNA/mg ribosomes.

The stringent factor (SF) was prepared from *E. coli* strain NF952 (*thiA, pyrE, argE, his, proA, thr, leu*), the single lysogenic for λc1857S7d (*pyrG*$^+$, *relA*$^+$) [9]. SF was purified as in [10] without the final DEAE-cellulose chromatography procedure. The samples of SF were stored in the buffer containing 5 mM Tris-acetate, 0.5 mM EDTA, 50 mM KCl, 2.5 mM DTT and 50% glycerol ($pH_{25°C}$ 8.5) at −20°C. The total electrophoretic purity of the SF preparation was ⩾70%.

For kinetic measurements of (p)ppGpp synthesis the reaction mixtures were prepared in the buffer consisting of 20 mM Tris–HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT ($pH_{37°C}$ 7.6). 50 μl aliquots contained 2 pmol ribosomes, or 2–4 pmol [^{14}C]poly(Lys)-tRNA carrying ribosomes, 10 μg total *E. coli* deacylated tRNA (Boehringer-Mannheim), 1 μg SF, 8 nmol GTP (Fluka), 60 nmol ATP (Fluka), 0.1 μCi [^{14}C]GTP (460 mCi/mmol, Amersham); 5 μg poly(A) (Calbiochem), 44 pmol [^{14}C]Lys-tRNA (30 μg total *E. coli* tRNA) and 140 pmol EF-Tu are added to the aliquots where indicated.

Tetracycline (final concentration 0.3 mM) was used to measure the inhibition of (p)ppGpp synthesis.

Incubation was at 37°C. The reaction was stopped by addition of 3 μl 88% HCOOH. After 10 min incubation at 4°C the samples were centrifuged for 5 min at 3000 rev./min; 10 μl aliquots of the supernatants were applied to PEI-cellulose sheets (Polygram CEL 300 PEI, Serva) and chromatographed in 1.5 M KH_2PO_4 (pH 3.4) as in [11]. The sheets were cut out and their radioactivities were measured in the standard toluene–PPO–POPOP mixture using a scintillation spectrometer. The amounts of (p)ppGpp synthesized were calculated from the ratio of [^{14}C]GTP converted to (p)pp[^{14}C]Gpp.

3. RESULTS AND DISCUSSION

Fig.1 presents the kinetics of (p)pp[^{14}C]Gpp synthesis in a classical ribosomal system in the presence of the poly(A) template polynucleotide. The results suggest that (p)ppGpp formation essentially depends on the presence of ribosomes and the stringent factor. The omission of the deacylated tRNA or poly(A) from the system significantly inhibited (p)ppGpp formation.

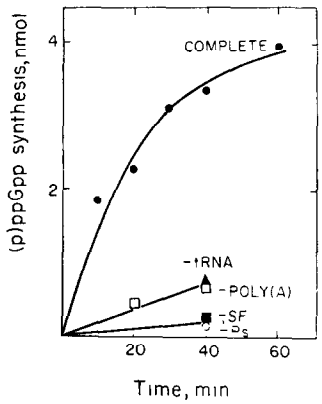

Fig.1. Kinetics of (p)ppGpp synthesis on the ribosomes in the presence of the poly(A) template: (●) complete system (70 S ribosomes, poly(A), deacylated tRNA, SF, ATP, [^{14}C]GTP); (□) the system without poly(A); (▲) the system without deacylated tRNA; (■) the system without SF; (○) the system without ribosomes.

From fig.2 it is seen that (p)ppGpp can be also synthesized by the template-free ribosomes carrying poly(Lys)-tRNA. This synthesis strictly depends on the stringent factor. However, the absence of the deacylated tRNA in the incubation

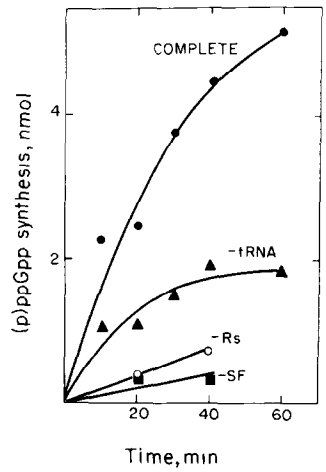

Fig.2. Kinetics of (p)ppGpp synthesis on the ribosomes carrying poly(Lys)-tRNA without the poly(A) template: (●) complete system; (▲) the system without deacylated tRNA; (■) the system without SF; (○) the system without ribosomes.

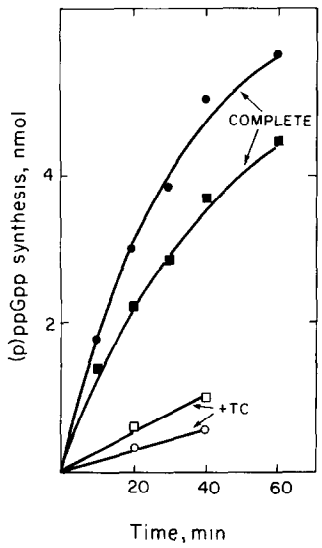

Fig.3. Kinetics of (p)ppGpp synthesis in the presence of tetracycline (TC): (●) complete system with the ribosomes in the presence of poly(A); (○) the same as (●) plus 0.3 mM TC; (■) complete system with ribosomes carrying poly(Lys)-tRNA without poly(A); (□) the same as (■) plus 0.3 mM TC.

mixture does not completely block this synthesis. Thus, the ribosomes carrying poly(Lys)-tRNA without the poly(A) template are capable of binding deacylated tRNA and synthesizing (p)ppGpp.

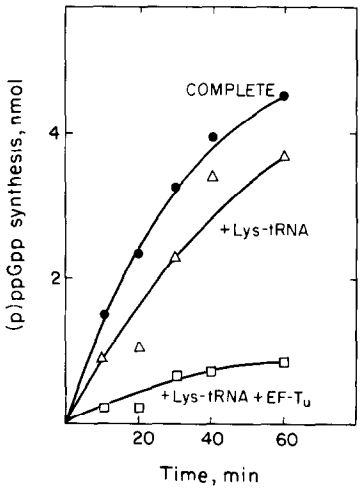

Fig.4. Kinetics of (p)ppGpp synthesis on the ribosomes in the presence of poly(A): (●) complete system; (△) complete system plus Lys-tRNA; (□) complete system plus Lys-tRNA and EF-Tu.

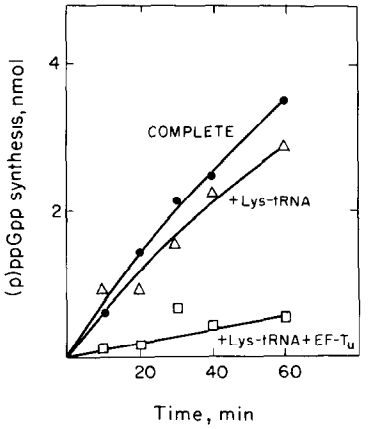

Fig.5. Kinetics of (p)ppGpp synthesis on the ribosomes carrying poly(Lys)-tRNA without the poly(A) template: (●) complete system; (△) complete system plus Lys-tRNA; (□) complete system plus Lys-tRNA and EF-Tu.

Fig.3 demonstrates that tetracycline (at 0.3 mM) inhibits the tetra- and pentaphosphate formation not only in classical ribosomal system with poly(A), but also at the system with ribosomes carrying poly(Lys)-tRNA without poly(A).

EF-Tu-dependent binding of Lys-tRNA to the ribosomal A site blocks (p)ppGpp formation (fig.4,5). However, Lys-tRNA without EF-Tu added to the incubation mixture does not decrease essentially the rate of (p)ppGpp synthesis. In this case Lys-tRNA itself does not compete apparently with SF-dependent deacylated tRNA binding to the ribosome.

The results obtained demonstrate that the template polynucleotide is not requisite for SF-promoted ribosomal synthesis of (p)ppGpp. It is seen that the ribosomes carrying poly(Lys)-tRNA in the P-site can synthesize (p)ppGpp in the absence of the template and that this proceeds at almost the same rate as the template charged ribosomes.

ACKNOWLEDGEMENTS

We thank Professors A.S. Spirin and A.B. Chetverin for their interest in this work and helpful discussion, and Mrs E.A. Arutunijan for excellent technical assistance.

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