FORMATION OF GUANOSINE TETRAPHOSPHATE (MAGIC SPOT I) IN HOMOLOGOUS AND HETEROLOGOUS SYSTEMS*

Dietmar RICHTER

Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Berlin-Dahlem, W. Germany

Received 4 June 1973

1. Introduction

The *in vitro* synthesis of the magic spot compounds, the guanosine tetra (ppGpp) or pentaphosphate (pppGpp), are catalyzed by ribosomes from *E. coli* and a stringent factor [1]; the latter present in the ribosomal NH_4Cl wash of stringent *E. coli* transfers pyrophosphate from ATP into the 3′-position of guanosine-5′-diphosphate [2]. Earlier reports [1] indicated that synthesis of the two magic spots was stimulated by elongation factor G (EFG); however, further purification of the ribosomes revealed that messenger RNA and codon-specific uncharged tRNA were essential rather than EF-G (Haseltine and Block, personal communication [3]).

The present paper contributes to the specificity of the formation of guanosine-5′-diphosphate-3′-diphosphate (magic spot I). The results show that this reaction requires 30 S as well as 50 S ribosomal subunits. However, when 70 S ribosomes were split into P-I-protein fraction and P-I-ribosomes by treatment with ethanol and NH_4Cl [4], only P-I-ribosomes were essential for MS I formation. Experiments with ribosomes from various sources indicated that synthesis of MS I was restricted to bacterial ribosomes. Slight stimulation was obtained with mitochondrial ribosomes from yeast. Hybrid ribosomes formed by brain and *E. coli* ribosomal subunits were inactive to catalyze MS I formation.

In addition, our data show that neither cAMP nor the ATP analogue adenylylimido-diphosphate (AMPPNP) could replace ATP as phosphate donor; however, AMPPNP inhibited MS I formation when ATP was present. No inhibition occurred with cAMP or ouabain, an inhibitor of *E. coli* ATP phosphohydrolase [5].

2. Methods

E. coli strain and method of isolation of NH_4Cl washed ribosomes and stringent factor were the same as described by Haseltine et al. [1]. The reaction mixture (50 μl) for the synthesis of MS I contained 20 mM Tris–HCl, pH 8.0, 20 mM Mg-acetate, 2 mM DTT, 40 mM NH_4Cl, 5 μg uncharged $tRNA^{phe}$, 5 μg poly U, 10 μg 0.5 M NH_4Cl ribosomal wash (used as source of the stringent factor), between 0.2 and 2 mM α-[^{32}P]GTP (specific activity 10 mCi/mmol), 4 mM ATP, and ribosomes as specified in the legends of fig. 1. and the tables. After incubation at 37°C for 4 hr the reaction was stopped by addition of 1 μl 88% formic acid; 1 μl aliquots were applied to PEI-cellulose sheets and chromatographed in 1.5 M KH_2PO_4 (pH 3.4) as described [6]. The sheets were autoradiographed for 12 hr and spots corresponding to MS I, GTP and GDP were cut out and counted. Under the conditions described here little MS II was formed which was due to endogenous GTPase activity present in the NH_4Cl ribosomal wash. Ribosomal subunits from *E. coli* were prepared by the method of Takeda and Lipmann [7]; 30 S ribosomes [8], 40 S and 60 S subunits from brain [9], ribosomes from reticulocyte [10], from yeast cytoplasm and mitochondria [11] were prepared as described. P-I-ribosomes and P-I-protein from 70 S ribosomes (*E. coli*) were obtained by

*Paper No. 57 on 'Ribosomal Proteins'. Preceeding paper is by A.P. Czernilofsky, E.E. Collatz, G. Stöffler and E. Kuechler, Proc. Natl. Acad. Sci., submitted.

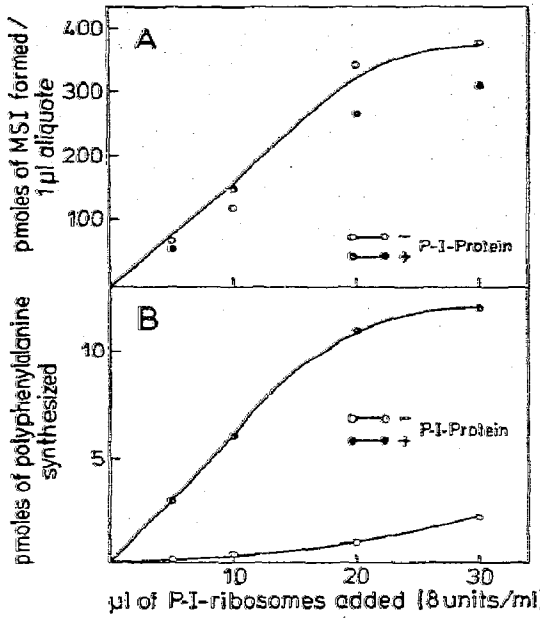

Fig. 1. Formation of MS I and synthesis of polyphenylalanine in the presence of P-I-ribosomes and P-I-protein. Formation of MS I and polypeptide synthesis were carried out in a total reaction volume of 50 µl; where indicated 10 µl of P-I-protein (3.2 mg/ml) were added. P-I-ribosomes contained 8 A_{260} units/ml. The conditions for MS I synthesis were the same as described in table I and in the Methods section. For polyphenylalanine synthesis see ref. [11]. MS I formation with P-I protein fraction and stringent factor alone was 25 pmoles above background and was substracted in fig. 1A, closed circles. P-I-protein alone had no effect in polyphenylalanine synthesis.

the method of Hamel et al. [4]. α-[^{32}P]GTP came from New England Nuclear, AMPPNP from Boehringer, Mannheim. Ouabain was a gift from Dr. V.A. Erdmann.

3. Results and discussion

With a purified system *in vitro* synthesis of guanosine tetraphosphate (MS I) required a stringent factor, 30 S, 50 S, poly U, and $tRNA^{phe}$ (table 1, line 1). No MS I was formed with $tRNA^{met}$ and poly U (table 1, line 2) suggesting that this reaction was dependent on codon-specific interaction of tRNA and mRNA [3]. Omission of either 30 S or 50 S subunits resulted in the complete loss of activity (table 1, line 3 and 4). However, when P-I-ribosomes and P-I-protein were prepared from 70 S ribosomes by the method of Hamel et al. [4] optimal MS I formation occurred

Table 1
Dependence of MS I formation on 30 S, 50 S, poly U and $tRNA^{phe}$.

Experimental conditions	Formation of MS I in pmoles/ µl aliquot
Complete System	450
$-tRNA^{phe}$, $+tRNA^{met}$	21
−30 S	11
−50 S	15
−Poly U	10
−Stringent factor	0

The complete system (50 µl) contained 0.9 A_{260} units of 50 S, 0.6 units of 30 S, 5 µg of $tRNA^{phe}$; for other conditions see Methods section. Where indicated 5 µg of $tRNA^{met}$ was used per assay; $tRNA^{phe}$ and $tRNA^{met}$ were purified according to the methods described elsewhere [12–14].

with P-I-ribosomes alone (fig. 1 A), whereas both, P-I-ribosomes and P-I-protein were essential for polyphenylalanine synthesis (fig. 1 B). Proteins L7 and L12 which are present in the P-I-fraction are required for the function of EF-Tu and EF-G during peptide chain elongation cycle [4, 15–19]. Thus, in contrast to the peptide chain elongation reaction, formation of MS I functioned independently of L7 and L12.

In order to find out whether synthesis of MS I occurred with ribosomes other than bacteria, ribosomes from yeast cytoplasm and mitochondria, from brain and from reticulocyte were complemented with the

Table 2
Complementation of the stringent factor (*E. coli*) with ribosomes from various sources.

Source of ribosomes	Formation of MS I in pmoles/ µl aliquot
E. coli	755
Yeast cytoplasm	0
Reticulocyte	0
Calf brain	0
Yeast mitochondria	45

The incubation mixture contained: 1.0 A_{260} units of 70 S ribosomes from *E. coli*, or 1.6 units of yeast ribosomes, or 1.8 units of reticulocyte or calf brain ribosomes, or 2.5 units of yeast mitochondrial ribosomes; since those ribosomal preparations contained still endogenous mRNA poly U was omitted and $tRNA^{phe}$ replaced by an unpurified tRNA mixture (40 µg/assay). The incubation was carried out at 23°C for 10 hr; a blank (10 pmoles/µl aliquot) caused by the stringent factor alone was substracted.

Table 3
The function of hybrid ribosomes.

Ribosomal subunits	Formation of MS I in pmoles/μl aliquot	Binding of [^{14}C]phe-tRNA in pmoles
30 S	20	1.2
50 S	45	0.4
30 S + 50 S	648	5.7
40 S	0	0.9
60 S	0	0.4
40 S + 60 S	0	3.8
40 S + 50 S	49	2.3
30 S + 60 S	31	1.5
30 S + 50 S + 40 S + 60 S	605	n.d.

Formation of MS I was carried out in 50 μl vol containing 0.9 A_{260} units of 50 S, 0.6 units of 30 S (both from *E. coli*), 1.1 units of brain 60 S, and 0.6 units of brain 40 S; other conditions were the same as described in the Methods section. Nonenzymatic binding of [^{14}C]phe-tRNA was done in a total vol of 100 μl as described elsewhere [23]; the ribosomal concentrations were: 0.47 A_{260} units of 50 S, 0.35 units of 30 S subunits (both from *E. coli*), 0.52 units of 60 S, and 0.3 units of 40 S subunits (both from calf brain).

Table 4
Effect of AMPPNP and of cAMP on the synthesis of MS I.

Conditions	Formation of MS I in pmoles/μl aliquot
Complete	510
−ATP, + AMPPNP (4 mM)	0
−ATP, + cAMP (4 mM)	0
Complete + AMPPNP (2.5 mM)	520
Complete + AMPPNP (5.0 mM)	320
Complete + AMPPNP (10 mM)	15
Complete + ouabain (2 mM)	495

The complete reaction mixture (50 μl) contained 0.9 A_{260} units of 50 S and 0.6 units of 30 S *E. coli* ribosomal subunits; where indicated 3′-5′-cAMP was used. Other conditions see Methods section.

stringent factor from *E. coli*. Table 2 shows that 80 S ribosomes from eukaryotic organisms were inactive to complement the bacterial stringent factor. With ribosomes from yeast mitochondria, however, MS I was formed. The low activity of those ribosomes was probably due to a contamination by endogenous ATPases or RNAases; more crude preparations of mitochondrial ribosomes not only were inactive but caused a strong inhibition of MS I formation when combined with bacterial ribosomes (not shown).

Only recently, it has been shown that ribosomal subunits from prokaryotes and eukaryotes were functionally interchangeable [20–22]. The hybrid ribosomes obtained from 50 S (*E. coli*) and 40 S subunits (brime shrimp) showed limited function by synthesizing fmet-puromycin or *N*-acetylphe–phe; no activity was observed with 60 S and 30 S [20]. In table 3 ribosomal subunits from *E. coli* and brain were interchanged and assayed for MS I formation and non enzymic binding of [^{14}C]phe-tRNA. The results indicated that the stringent factor from *E. coli* was ribosome-specific and did not form MS I with hybrid ribosomes; we confirmed the findings by Klein and Ochoa [20] that [^{14}C]phe-tRNA was nonenzymatically bound by hybrid ribosomes consisting of 50 S and 40 S but not of 30 S and 60 S (table 3). It should be noted that hybrid ribosomes obtained from 50 S and 40 S did not yield a distinct '70 S' ribosomal peak when analyzed on a sucrose gradient (not shown); the lack of this peak might be explained by the instability of the hybrid ribosomes. That brain ribosomal subunits contained unspecific inhibitors was unlikely since 70 S-dependent synthesis of MS I was not blocked by the 40 S and 60 S particle (table 3).

Haseltine et al. [1] showed that a magic spot was formed when GTP was replaced by β-γ-methylene guanosine-5′-triphosphate (GMPPCP). In table 4 ATP was replaced by AMPPNP or cAMP, but no magic spot was formed under those conditions. Thus neither cAMP nor AMPPNP could be used as phosphate donor. However, AMPPNP but not cAMP was a competitor for ATP in the MS I reaction (table 4). Ouabain, an inhibitor of membrane-bound ATPases [5] did not block formation of MS I (table 4).

The results reported here show that synthesis of MS I is restricted to the bacterial type of ribosomes. The experiments with P-I-ribosomes indicated that L7 and L12 were not required for MS formation; a similar observation has been made by Kjeldgaard (personal communication). The stringent factor seems to function on a ribosomal region different of that for EF-Tu or EF-G. However, 50 S subunits deprived of L7 and L12 showed EF-Tu and EF-G dependent GTPase activity when methanol was present [24]; Ballesta and

Vazquez [24] suggested that L7 and L12 were essential for an appropriate ribosomal confirmation. In the light of the data presented here this confirmation might be not required for the function of the stringent factor.

Acknowledgement

The author thanks Drs. H.G. Wittmann and V.A. Erdmann for critical discussion.

References

[1] Haseltine, W.A., Block, R., Gilbert, W. and Weber, K. (1972) Nature 238, 381.
[2] Sy, J. and Lipmann, F. (1973) Proc. Natl. Acad. Sci. U.S. 70, 306.
[3] Pedersen, F.S., Lund, E. and Kjeldgaard, N.O. (1973) Nature New Biol. 243, 13.
[4] Hamel, E., Koka, M. and Nakamoto, T. (1972) J. Biol. Chem. 247, 805.
[5] Hafkenscheid, J.C.M. and Bonting, S.L. (1968) Biochim. Biophys. Acta 151, 204.
[6] Cashel, M. and Kalbacher, B. (1970) J. Biol. Chem. 245, 2309.
[7] Takeda, M. and Lipmann, F. (1966) Proc. Natl. Acad. Sci. U.S. 56, 1875.
[8] Richter, D. (1973) J. Biol. Chem. 248, 2853.
[9] Zomzely-Neurath, C.E. (1972) in: Methods in Molecular Biology (Last, J.A. and Lasikin, A.I., eds), Vol. 2, p. 147, Marcel Dekker, New York.
[10] Krisko, I., Gordon, J. and Lipmann, F. (1969) J. Biol. Chem. 244, 6117.
[11] Richter, D. and Lipmann, F. (1970) Biochemistry 9, 5056.
[12] Gilliam, J., Blew, D., Warrington, R.C., Tigerstrom, M.v. and Tener, G.M. (1968) Biochemistry 7, 3459.
[13] Henes, C., Krauskopf, M. and Ofengand, J. (1969) Biochemistry 8, 3024.
[14] Richter, D. and Lipmann, F. (1970) Nature 227, 1212.
[15] Kischa, K., Möller, W. and Stöffler, G. (1971) Nature New Biol. 233, 62.
[16] Brot, N., Yamasaki, E., Redfield, B. and Weissbach, H. (1972) Arch. Biochem. Biophys. 148, 148.
[17] Sopori, M. and Lengyel, P. (1972) Biochem. Biophys. Res. Commun. 46, 238.
[18] Sander, G., Marsh, R.C. and Parmeggiani, A. (1972) Biochem. Biophys. Res. Commun. 47, 866.
[19] Highland, J.H., Bodley, J.W., Gordon, J., Hasenbank, R. and Stöffler, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 147.
[20] Klein, H.A. and Ochoa, S. (1972) J. Biol. Chem. 247, 8122.
[21] Lee, S.G. and Evans, W.R. (1971) Science 173, 241.
[22] Grivell, L.A. and Walg, H.L., Biochem. Biophys. Res. Commun. 49, 1452.
[23] Richter, D. (1972) Biochem. Biophys. Res. Commun. 46, 1850.
[24] Ballesta, J.P.G. and Vazquez, D. (1972) FEBS Letters 28, 337.