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DISCRIMINATION BETWEEN PURINE AND PYRIMIDINE BASE AT THE 3' TERMINUS OF THE tRNA MOLECULE BY THE STRINGENT FACTOR SYSTEM FROM ESCHERICHIA COLI

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SUMMARY: Crude stringent factor, prepared from a mutant strain with low levels of tRNA nucleotidyl transferase, synthesizes little or no (p)ppGpp in the presence of $tRNA^{Phe}$-CpC; addition of yeast tRNA nucleotidyl transferase, however, fully restores (p)ppGpp formation, indicating that the complete CCA terminus of the tRNA molecule is a prerequisite in the (p)ppGpp synthesizing reaction. When the terminal purine is replaced by a pyrimidine base as in the case of $tRNA^{Phe}$-CpCpC; or when the latter is extended by addition of AMP yielding $tRNA^{Phe}$-CpCpCpA, both modified tRNAs are low in stimulating the (p)ppGpp synthesizing reaction. Hence activation of the stringent factor by tRNA requires (i) the terminal purine base and (ii) the precise fitting of the CCA terminus to the acceptor site of the ribosome.

Introduction

Guanosine polyphosphates, ppGpp and pppGpp[1], are known to play a key role in regulating protein and RNA synthesis in stringent strains of E. coli (for review see ref. 1). Lack of an essential amino acid leads to cessation of protein and RNA synthesis while (p)ppGpp is accumulated. It is generally accepted that (p)ppGpp specifically blocks stable RNA synthesis (2, 3), and that (p)ppGpp is synthesized on ribosomes by the stringent factor, an enzyme that transfers pyrophosphate from ATP to the 3'-position of the ribose ring of either GDP or GTP, yielding ppGpp and pppGpp, respectively (4, 5).

It is also accepted that the in vitro reaction is initiated when uncharged tRNA is codon-specifically bound to the A-site of the ribosome (6, 7), indicating that uncharged tRNA is the trigger for (p)ppGpp production. Recently it has

[1]Abbreviations: ppGpp, guanosine 5'-diphosphate, 3'-diphosphate; pppGpp, guanosine 5'-triphosphate, 3'-diphosphate; $tRNA^{Phe}$-CpC, $tRNA^{Phe}$ missing the terminal AMP residue; $tRNA^{Phe}$-C, $tRNA^{Phe}$ missing the terminal AMP and the penultimate CMP residue.

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been shown that the intact ribose residue in the terminal adenosine of the tRNA molecule is essential in this reaction (8). Formation of (p)ppGpp was not induced by tRNA modified in the 3'-position of the terminal adenosine. This high degree in specificity seemed to be at variance with the finding that tRNA lacking the terminal adenosine, $tRNA^{Phe}$-CpC, is also active in this reaction (8); most likely tRNA-CpC is repaired by a tRNA nucleotidyl transferase (EC.2.7.7.25) present in our stringent factor preparations as a contaminant. The tRNA nucleotidyl transferase is known to repair the CCA end of the tRNA molecule in vitro, whereas its in vivo function is less well established. Deutscher et al. (9) studied the effect of the mutation of the tRNA nucleotidyl transferase gene upon the rel A gene and found that in vivo tRNA-CpC induces a small but significant response in cca mutants of rel A^+ cells. This result is not in agreement with our findings that tRNA-CpC does not trigger synthesis of ppGpp on ribosomes until it is repaired by the tRNA nucleotidyl transferase (8). Therefore it seemed to be essential to study the effect of tRNA-CpC in vitro using stringent factor from the cca mutant strain. The data reported here will show that the stringent factor from such a mutant is not activated by tRNA-CpC indicating that the unmodified CCA terminus is essential. Extending these studies to other tRNA molecules modified at the CCA end, it will be shown that tRNA-CpCpC and tRNA-CpCpCpA have little or no activity in triggering guanosine polyphosphate synthesis.

MATERIALS AND METHODS

E. coli strain CGSC 2834/a or CP78 (rel A^+, cca^-, his^-, leu^-, arg^-, thr^-, BI^-; kindly provided by Dr. M.P. Deutscher, Farmington, Conn. USA) were grown as reported (9, 10). 50S and 30S ribosomal subunits were prepared according to published procedures (10). Nucleotides were obtained from Pharma-Waldhof, Germany; uncharged $tRNA^{Phe}_{yeast}$ and poly(U) came from Boehringer, Mannheim; polyethyleneimine thin-layer sheets (PEI) from Macherey-Nagel, Düren, Germany; (α-^{32}P) GTP was purchased from New England Nuclear. tRNA-CpCpC and tRNA-CpCpCpA were prepared from tRNA-CpC (8) by incubation with yeast nucleotidyl transferase and (^{14}C)CTP (11, 12). The tRNA-CpCp(^{14}C)C was purified on Sephadex G-25 as described (12). For preparation of tRNA-CpCpCpA, tRNA-CpCp(^{14}C)C was incubated with ATP and nucleotidyl transferase (12). Analysis of the two products by alkaline hydrolysis indicated that about 90% of the tRNA molecules had the terminal end CpCpC or CpCpCpA which is in good agreement with previous findings (11, 12).

Assay for (p)ppGpp Synthesis. The assay mixture (50 µl) was 20 mM Tris-HCl, pH 7.8, 20 mM magnesium acetate, 40 mM NH_4Cl, 2 mM dithiothreitol, 2 mM ATP, 0.4 mM (α-^{32}P)GTP (spec. act. 27 Ci/mol), 5 pmol of ribosome-stringent factor complex (10), 2.5 µg poly(U) and $tRNA^{Phe}_{yeast}$ as indicated in the legends to the figures. After 1 hr of incubation at 37°C the reaction was stopped with 1 µl of cold 88% formic acid. (p)ppGpp analysis was carried out by polyethyleneimine thin layer chromatography in 1.5 M KH_2PO_4 (pH 3.4). The conversion of radioactive GTP into (p)ppGpp is given as a percentage.

Preparation of Crude and Purified Stringent Factor. Either E. coli strain CGSC 2834/a or CP78 was used for isolating stringent factor and 50S and 30S ribosomal subunits (10). Crude stringent factor was prepared by dissociation of 70S ribosomes at low ionic conditions (10); the released stringent factor was concentrated by ammonium sulfate precipitation (10); this preparation is termed as crude stringent factor. For further purification it was chromatographed on hydroxyl apatite, DEAE-Sephadex A-50 and DEAE-cellulose (De 52, Whatman) columns (8, 10). The latter step removes residual amounts of tRNA nucleotidyl transferase.

Results and Discussion

The first suggestion in our hands that stringent factor preparations contained residual amounts of tRNA nucleotidyl transferase came from experiments where $tRNA^{Phe}$-C incubated with CTP and ATP in the (p)ppGpp synthesizing assay system, was found to be active in triggering synthesis of (p)ppGpp (8). No activity was observed when CTP was omitted. It was assumed that $tRNA^{Phe}$-C was repaired by the correct sequential addition of CMP and AMP due to a contaminating tRNA nucleotidyl transferase. Based on these data it was concluded that only tRNA-CpCpA but not tRNA-CpC or tRNA-C is co-substrate in the stringency reaction. These findings do not agree with those by Deutscher et al. (9) described above. This discrepancy may be due to different sensitivity of the stringent factor system towards tRNA-CpC. Alternatively the effect of tRNA-CpC to bring about an increase in the ppGpp/GTP ratio in vivo may not be a direct result of the stringent response. In an attempt to find out whether $tRNA^{Phe}$-C can activate the cell-free stringent factor system, ribosomal subunits and stringent factor were prepared from wild-type and cca mutant strain of E. coli. When assayed for (p)ppGpp formation crude stringent factor from wild-type but not from cca mutant strain is activated by $tRNA^{Phe}$-CpC (Figure 1). As expected similarly to the wild strain, $tRNA^{Phe}$-CpC cannot serve as co-substrate in the stringency reaction of the cca mutant, eliminating the possibility that the stringent factor system of the cca mutant has an altered sensitivity for tRNA-CpC.

In order to find out whether the lack in activity with $tRNA^{Phe}$-CpC was due to the low levels of tRNA nucleotidyl transferase in the cca mutant the following experiments were carried out. Firstly formation of (p)ppGpp was studied as a function of increasing concentrations of stringent factor assuming that at higher protein concentrations sufficient amounts of tRNA nucleotidyl transferase are present to repair $tRNA^{Phe}$-CpC. This in turn should consequently trigger (p)ppGpp synthesis. Indeed a small but significant amount of (p)ppGpp is formed at high stringent factor concentrations (Figure 2) supporting the

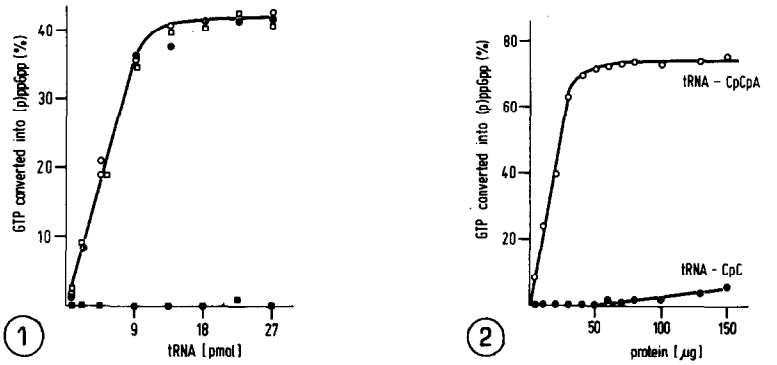

Fig. 1. The Effect of $tRNA^{Phe}$-CpCpA and $tRNA^{Phe}$-CpC upon Synthesis of (p)ppGpp. For (p)ppGpp assay conditions see Method section. Crude stringent factor and ribosomal subunits were prepared either from cca mutant or wild-type strain according to published procedures (10). Formation of (p)ppGpp in the presence of stringent factor and ribosomes from wild-type strain: o——o, with $tRNA^{Phe}$-CpCpA; ●——●, with $tRNA^{Phe}$-CpC; in the presence of stringent factor and ribosomes from cca mutant strain: □——□, with $tRNA^{Phe}$-CpCpA; ■——■, with $tRNA^{Phe}$-CpC.

Fig. 2. Formation of (p)ppGpp with Increasing Concentrations of Stringent Factor in the Presence of $tRNA^{Phe}$-CpC. For assay conditions see Method section and ref. 10. 15 pmoles of $tRNA^{Phe}$-CpC or $tRNA^{Phe}$-CpCpA were present per assay. Crude stringent factor was prepared from cca mutant strain CP78 and assayed at the protein concentrations indicated. Synthesis of (p)ppGpp: o——o, with $tRNA^{Phe}$-CpCpA; ●——●, with $tRNA^{Phe}$-CpC. It should be noted here that the observed activity at high stringent factor concentrations is not detected when $tRNA^{Phe}$-CpC is omitted indicating that it is not a ribosome-independent way of (p)ppGpp formation.

idea that repair of $tRNA^{Phe}$-CpC is a prerequisite for activation of stringent factor. Secondly to show more directly that tRNA nucleotidyl transferase is involved, it was isolated from yeast (11, 12) and added to the (p)ppGpp synthesizing assay system derived from the cca mutant strain. As shown in Figure 3, when present yeast tRNA nucleotidyl transferase completely restores (p)ppGpp formation. Similarly, highly purified stringent factor from wild-type strain, no longer containing residual amounts of tRNA nucleotidyl transferase and hence inactive in the presence of $tRNA^{Phe}$-CpC, is activated when yeast tRNA nucleotidyl transferase is added. That this activity was due to the addition of AMP to $tRNA^{Phe}$-CpC is also supported by the data shown in Table 1 where $tRNA^{Phe}$-CpC was pre-incubated with various nucleotides and yeast tRNA nucleotidyl transferase; after incubation the latter was heat-inactivated and the tRNA dialyzed against buffer to remove nucleotides. Only pre-incubation with ATP and tRNA nucleotidyl transferase leads to the complete restoration of (p)ppGpp synthesis.

Table 1 Effect of tRNA Nucleotidyl Transferase on the Formation of of (p)ppGpp Catalyzed by the Stringent Factor.

Conditions for preincubation of $tRNA^{Phe}$-CpC with various nucleotides	Per cent of GTP converted into (p)ppGpp	
	Stringent factor from	
	cca mutant strain	wild-type strain
+ ATP	35.9	53.8
+ ADP	0	0
+ UTP	0	0
+ ITP	0	0
+ GTP	0	0
+ CTP	2.8	4.3
control with $tRNA^{Phe}$CpCpA	37.8	59.8

Pre-incubation was carried out in a 500-µl reaction volume containing 20 mM Tris-HCl, pH 8.2, 5 mM Mg-acetate, 90 pmoles of $tRNA^{Phe}$-CpC, 40 µg yeast tRNA nucleotidyl transferase and where indicated 2 mM of the nucleotides listed above. Incubation was carried out at 37°C for 1 hr, followed by a step at 60°C for 10 min to inactivate tRNA nucleotidyl transferase. To remove the nucleotides the 500-µl assay mixture was dialyzed against 2 l of 20 mM Tris-HCl buffer, pH 8.2, 5 mM Mg-acetate; 40 µl aliquots of the dialyzed material were assayed in the (p)ppGpp synthesizing system containing 5 µg of stringent factor from cca mutants or wild-type strain. In the control experiment 8 pmoles of $tRNA^{Phe}$-CpCpA were assayed. Stringent factor from wild-type strain was purified according to published procedures (8, 10) and was free of tRNA nucleotidyl transferase.

As indicated in Table 1 pre-incubation of $tRNA^{Phe}$-CpC with ADP, ITP, UTP or GTP had no effect, while a small but significant (p)ppGpp formation was observed with CTP. It is known that tRNA nucleotidyl transferase can introduce an additional CMP residue at the 3' end of tRNA-CpC yielding tRNA-CpCpC 11, 12), which in turn can be extended for one AMP residue. The resulting tRNA-CpCpCpA accepts a variety of amino acids (12), forms with EF-T and GTP a ternary complex but with a rather low efficiency; it shows good binding to ribosomes but a low rate in the polymerization reaction of polyphenyl-

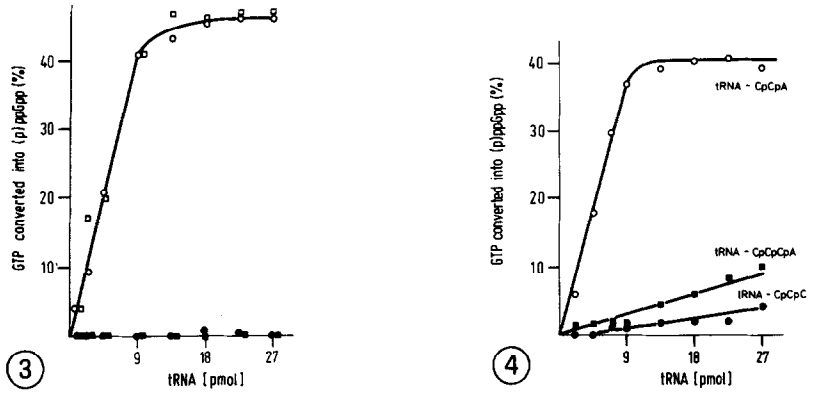

Fig. 3. The Effect of Yeast tRNA Nucleotidyl Transferase on Formation of (p)ppGpp. The assay conditions were as described in Methods section, except that 40 µg of yeast tRNA nucleotidyl transferase were present. Assay was carried out with a crude stringent factor from the cca mutant strain CP78: ■——■, $tRNA^{Phe}$-CpC; □——□, $tRNA^{Phe}$-CpC plus yeast tRNA nucleotidyl transferase; or with purified stringent factor (8, 10) from wild-type strain: ●——●, $tRNA^{Phe}$-CpC; o——o, $tRNA^{Phe}$-CpC plus yeast tRNA nucleotidyl transferase.

Fig. 4. Formation of (p)ppGpp in the Presence of $tRNA^{Phe}$ Modified at the 3' Terminus. Purified stringent factor (8, 10) from the wild-type strain was used in the assay system (see Methods section). Similar results were obtained with stringent factor prepared from the cca mutant.

alanine synthesis (11). These data indicate that aminoacyl-tRNA synthetase as well as the acceptor site at the ribosome are flexible enough to tolerate an extended chain length at the 3' end of the tRNA. It seems to be of interest whether this flexibility also exists in the stringent factor-dependent reaction of (p)ppGpp formation. As illustrated in Figure 4 $tRNA^{Phe}$-CpCpC or $tRNA^{Phe}$-CpCpCpA has only little activity. While at about 10 pmoles of $tRNA^{Phe}$-CpCpA/50 µl assay optimal synthesis of (p)ppGpp was observed, the same concentrations for $tRNA^{Phe}$-CpCpC or $tRNA^{Phe}$-CpCpCpA had less than one-tenth to one-twentieth of the control activity. Further increase of the concentration of $tRNA^{Phe}$-CpCpCpA leads to a proportional increase of (p)ppGpp formation. As regards the significance of (p)ppGpp synthesis with $tRNA^{Phe}$-CpCpCpA or $tRNA^{Phe}$-CpCpC, it cannot be excluded that this increase is due to a contamination of preparations of the modified tRNAs with $tRNA^{Phe}$-CpCpA. As stated in the method section the modified tRNA was about 90% pure. Alternatively at higher concentrations of modified $tRNA^{Phe}$ the ribosome~ mRNA~ tRNA complex could be more stable, thus favouring (p)ppGpp formation. That binding of $tRNA^{Phe}$-CpCpCpA to ribosomes is no longer codon-specific can be

excluded; as with unmodified $tRNA^{Phe}$ it is strictly dependent on poly(U) (not shown).

The results presented here suggest that the mechanism for triggering synthesis of (D)ppGpp by the stringent factor depends on the precise fitting of the 3' terminal of the tRNA molecule to the acceptor site of the ribosome. In this process ribosomes and/or stringent factor not only recognize the intact ribose ring but also discriminate between purine and pyrimidine base at the 3' terminal end of the tRNA molecule. Clearly tRNA-CpC is not a co-substrate in the in vitro stringency reaction. Thus from the in vitro analysis at present there is no good explanation for the in vivo data that tRNA-CpC causes an increase in the ppGpp/GTP ratio. That this increase is related to a reduced turnover rate of ppGpp as it is known for $spoT^-$ strains (13) seems to be unlikely. In vitro experiments with the cca mutant strain indicate a normal degradation of ppGpp (14).

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