Proc. Nat. Acad. Sci. USA
Vol. 70, No. 5, pp. 1564–1568, May 1973

Synthesis of Guanosine Tetra- and Pentaphosphate Requires the Presence of a Codon-Specific, Uncharged Transfer Ribonucleic Acid in the Acceptor Site of Ribosomes

(stringent control/ppGpp (MSI) and pppGpp (MSII)/protein synthesis/*Escherichia coli*)

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Communicated by James D. Watson, February 8, 1973

ABSTRACT **The nucleotides ppGpp and pppGpp accumulate when wild-type, but not *rel*$^-$, strains of *Escherichia coli* are starved for required amino acids. These compounds are synthesized on ribosomes, in the presence of the product of the *rel* gene, from GDP and GTP; ATP is used as the phosphate donor. The signal for making these compounds is the presence of an uncharged tRNA in the ribosomal acceptor site. These compounds are not accumulated if the ribosomes are actively engaged in protein synthesis.**

In many strains of *Escherichia coli* accumulation of stable RNA species is sharply curtailed upon starvation for an essential amino acid (1). This phenomenon is called the stringent response, and is governed by a single genetic locus, the *rel* locus (2). Mutants in this gene continue to accumulate RNA after the onset of amino-acid deprivation. Neidhardt (3) and others (4) demonstrated that the trigger for the stringent response is depletion of one or more of the aminoacyl-tRNA pools, rather than the intracellular concentrations of the amino acids themselves, for inactivation of any of the aminoacyl-tRNA synthetases elicits the stringent response, even in the presence of a full complement of amino acids.

Cashel and Gallant (5) found that amino-acid starvation induced a rapid accumulation of two unusual guanosine phosphates, ppGpp (MSI), and pppGpp (MSII), in stringent, but not in relaxed, strains of bacteria. They postulated that the high intracellular concentration of ppGpp leads to a cessation of ribosomal RNA accumulation and many of the other characteristics of the stringent response. In an earlier communication, we demonstrated that ppGpp and pppGpp are synthesized *in vitro* as a product of an idling step in protein biosynthesis.

MATERIALS AND METHODS

Strains. CP78 was used in all cases. It is from the collection of N. Fiil and is W3110F$^-$ *leu*$^-$ *arg*$^-$ *thi*$^-$ *thr*$^-$ *his*$^-$.

Preparation of Ribosomal Subunits. Cells were grown to a density of 5×10^8 cells per ml at 34° in a medium containing per liter: 5.6 g of KH_2PO_4 (anhydrous), 28.9 g of K_2HPO_4 (anhydrous), 10 g of yeast extract, 10 mg of thiamine, and 10 g of glucose. Where indicated, 500 mg of rifampicin (Schwarz/Mann) was added to the culture, which was incubated for an additional 1.5 hr at 34°. Ribosomes were washed (6) in a buffer of low-salt concentration.

The ribosomes were dissolved in buffer A made 0.1 mM in puromycin and pelleted again. The pellet was redissolved in buffer A and the concentration of ribosomes adjusted to 500 A_{260} units/ml. The ribosomes were then dialyzed for 10 hr in a buffer containing 10 mM Tris-acetate (pH 7.8)–1 mM dithiothreitol–0.1 mM $Mg(OAc)_2$. The dialyzed ribosomes were layered on a 5–20% sucrose gradient in the same buffer, and were spun for 9.5 hr in a Beckman SW 27 rotor at 27,000 rpm at 4°. The fractions containing the 30S and 50S ribosomal subunits were pooled separately, the concentration of $Mg(AOc)_2$ was raised to 20 mM, and the subunits were pelleted by spinning at 60,000 rpm for 4 hr in a Beckman angle 65 rotor. The ribosomal subunits were resuspended in buffer A made 20 mM in $Mg(OAc)_2$, at a concentration of 250 A_{260} units/ml, and incubated at 37° for 20 min. The 30S ribosomal subunits were heated at 37° for 60 sec immediately before they were used.

Purification of the Stringent Factor will be described elsewhere. Unless otherwise indicated, the stringent factor used was free of EF-Tu and EF-Ts, but it contained significant amounts of EF-G as judged by *in vitro* protein synthesis assays. It was also free of tRNA. The preparation of stringent factor used in Table 6 was free of EF-G as judged by *in vitro* synthesis and GTPase assays.

Polyribonucleotides were purchased from Miles Research Biochemicals, and are random copolymers when they contain two or more different bases. The molar ratios of the bases are: poly(A,G) 1.0:1.0, poly(U,A) 1.3:1.0, poly(U,C) 1.1:1.0, poly(C,G,U) 1.1:2.3:1.4, poly(A,C,U) 1.1:1.0:1.4, poly-(A,C,G) 1.2:1.0:1.2, poly(A,G,U) 1.4:1.4:1.1.

Transfer RNA. Purified tRNAs for N-formylmethionine, phenylalanine, valine, and glutamic acid were purchased from Boerhringer–Mannheim. Purified $tRNA_I^{Ala}$ and $tRNA_{II}^{Ala}$ were a gift of B. Roe and B. Dudock, $tRNA_{II}^{Tyr}$ was a gift of U. Rajbahandary, $tRNA_{II}^{Ser}$ was a gift of N. Nishimura and R. Roberts, and $tRNA^{Ser}$ was purified by the method of Gillam *et al.* (7) from bulk *E. coli* K-12 tRNA purchased from Sigma Biochemicals.

Preparation of the Charged tRNA. The charging reactions were 0.15 M Hepes (pH 7.5), 20 mM $Mg(OAc)_2$, 4 mM ATP, 1 mM dithiothreitol, 100 μl/ml of charging enzymes—prepared by passage of an 100,000 × *g* supernatant fraction over a DEAE-sephadex column equilibrated with 10 mM Hepes (pH 7.5)–10 mM $Mg(OAc)_2$–1 mM dithiothreitol–0.30 M

Abbreviations: Buffer A, 10 mM Tris-acetate(pH 7.8)–14 mM $Mg(OAc)_2$–60 mM $K(OAc)_2$–1 mM dithiothreitol. ppGpp is guanosine 3′,5′-bis(diphosphate), pppGpp is guanosine 3′-diphosphate, 5′-triphosphate.

TABLE 1. *The requirement for 30S and 50S ribosomal subunits, messenger RNA, and uncharged tRNA*

Reaction mixture	nmol of ppGpp and pppGpp synthesized	
	Rifampicin treated	No rifampicin
Complete	5.2	5.6
−30S	<0.1	<0.1
−50S	<0.1	<0.1
−poly(A,C,U,G)	0.5	4.0
−tRNA	<0.1	<0.1
−Stringent factor	<0.1	<0.1

The reaction conditions were described (6). Reactions contain, where indicated, 30 μg of 50S subunits, 20 μg of 30S subunits, 25 μg of poly(A,C,U,G) 20 μg of uncharged bulk tRNA from *E. coli* K-12, and 10 μg of stringent factor. The reactions were incubated at 37° for 30 min.

NH_4Cl, and 100 μg/ml of uncharged purified tRNA. *N*-Acetyl-Phe-$tRNA^{Phe}$ was prepared by reaction of Phe-$tRNA^{Phe}$ with *N*-acetyl-succinimide (a gift of Charles Kantor and Maria Pellegrini), followed by elution from a benzoylated DEAE-cellulose column (7).

Binding and Release Assays were done as described by Nirenberg and Leder (20).

R17 RNA Was Prepared from R17 phage (a kind gift of Gilbert Jay) by phenol extraction and ethanol precipitation. The precipitate was suspended in distilled water to give a final concentration of RNA of 10 mg/ml.

RESULTS

Synthesis of ppGpp and pppGpp Requires Messenger RNA and Uncharged tRNA. Initially we observed that reaction mixtures containing ribosomes repeatedly washed with high salt, the 0.5 M-NH_4Cl wash of ribosomes from stringent strains, ATP, GPT, salts, and buffer synthesized ppGpp and pppGpp (6). We noted no requirement for either uncharged tRNA or mRNA. Further purification of the components of the reaction reveals that the synthesis of ppGpp and pppGpp requires both the 30S and 50S ribosomal subunits, an RNA template to serve as a message, and an uncharged tRNA capable of recognizing the codons carried by the message. These requirements have also been demonstrated by Pedersen, Lund, and Kjeldgaard (*Nature*, in press).

In order to demonstrate the requirement for the message and tRNA, it is necessary first to free the ribosomes of both endogenous message and tRNA. The ribosomes used in the experiments described below were freed of message by incubating cells for 1.5 hr in the presence of 500 μg/ml of rifampicin. This treatment prevents initiation of new RNA chains and allows the ribosomes to run-off the existing mRNA molecules. Subunits were prepared from the run-off ribosomes by dialyzing them against 0.1 mM $Mg(OAc)_2$ and separating the subunits on a sucrose gradient. (The dialysis against the low concentration of magnesium releases bound tRNA.) Table 1 demonstrates the requirement for both ribosomal subunits, a mRNA [in this case, the random copolymer poly(A,C,U,G)], a mixture of the uncharged tRNAs, and the stringent factor. The stringent factor used in these experiments was eluted at 0.25 M NH_4Cl from a DEAE-Sephadex column and contains less than 1% nucleic acids. Table 1 also shows that the rifampicin treatment is necessary to free the subunits of endogenous message.

TABLE 2. *The requirement for specific codon recognition by uncharged tRNA*

Message	nmol of ppGpp and pppGpp synthesized with various tRNAs					
	$tRNA^{Phe}$	N-Acetyl-Phe-$tRNA^{Phe}$	$tRNA^{Met}$	$tRNA^{Val}$	$tRNA^{Glu}$	$tRNA_{II}^{Tyr}$
Poly(A,U,G)	—	—	2.4	3.2	1.8	2.2
Poly(A,C,G)	—	—	0.2	<0.1	0.4	0.1
Poly(A,C,U)	—	—	0.2	0.2	0.1	4.2
Poly(G,C,U)	—	—	0.3	0.1	0.1	0.1
Poly(U)	6.4	<0.1	<0.1	<0.1	<0.1	<0.1
Poly(U,C)	4.8	—	—	—	—	—
Poly(U,A)	6.2	—	—	—	—	—
Poly(A,G)	<0.1	—	—	—	—	—
Poly(A)	<0.1	—	—	—	—	—
Poly(C)	<0.1	—	—	—	—	—

Reaction mixtures as in Table 1, except 4 μg of the polyribonucleotide indicated, 5 μg of the purified uncharged tRNA indicated or *N*-Acetyl'Phe-$tRNA^{Phe}$ and 5 μg of the stringent factor were used. The low level of synthesis with $tRNA^{Val}$ and $tRNA_f^{Met}$ and poly(G,C,U) may be due to the relatively high proportion of G residues in this copolymer.

The synthesis of ppGpp and pppGpp requires specific recognition by the uncharged tRNA of the codons of the message. Purified tRNA species only stimulate ppGpp and pppGpp synthesis if the RNA serving as a message contains codons they can recognize (see Table 2). For example, purified $tRNA^{Phe}$, which recognizes the codons UUU and UUC, stimulates the synthesis of ppGpp and pppGpp if the reactions contain poly(U), poly(U,C), or poly(U,A), but not if they contain poly(A), poly(C), or poly(A,G). Purified $tRNA_f^{Met}$ recognizes the codon AUG. Of the four possibly random copolymers containing three of the four nucleotides, only poly(A,U,G) serves as an effective messenger RNA for $tRNA_f^{Met}$. Purified $tRNA_{II}^{Tyr}$ recognizes the codons UAU and UAC. Both poly(U,A,C) and poly(A,U,G) can serve as message for $tRNA_{II}^{Tyr}$, but poly(G,C,U) and poly(C,G,A) cannot.

The stimulation of the reaction by $tRNA^{Phe}$ with poly(U) as a template is completely inhibited by the antibiotic tetracycline at a concentration of 0.1 mM (data not shown). Levin (9) demonstrated that at this concentration tetracycline inhibits the poly(U)-directed binding of uncharged $tRNA^{Phe}$ to ribosomes. Tetracycline also inhibits the binding of the aminoacyl-tRNA–EF-Tu–GTP complex to the acceptor site of the ribosomes (10). Purified $tRNA^{Phe}$ that has been subjected to periodate oxidation no longer stimulates the synthesis of ppGpp and pppGpp (data not shown). *N*-Acetyl-Phe-$tRNA^{Phe}$ purified to remove both uncharged $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ does not promote the synthesis of the ppGpp and pppGpp.

The Signal is the Presence of Uncharged tRNA in the Acceptor Site. The synthesis of ppGpp and pppGpp requires the presence of an uncharged tRNA molecule in the acceptor site of the ribosome. To establish this, we have studied specific known initiation sequences on a natural message using purified tRNA species. Steitz (11) has demonstrated that ribosomes can be correctly positioned at the natural start sequences of the phage R17 RNA by forming the initiation complex. Both the protein and RNA sequences at these starts are known: the amino-acid sequence for the coat pro-

TABLE 3. *Specific stimulation at the initiation complex by uncharged tRNA^{Ala} and tRNA^{Ser}*

Additions	Reaction number										
	1	2	3	4	5	6	7	8	9	10	11
R17 RNA	+	−	+	+	+	+	+	+	+	+	+
fMet-tRNA$_f^{Met}$	+	+	−	−	+	+	+	+	+	+	+
Initiation factors	+	+	+	+	−	+	+	+	+	+	+
Uncharged tRNA^{Ala}	+	+	+	−	+	−	−	−	−	−	−
Uncharged tRNA^{Ser}	−	−	−	+	−	−	+	−	−	−	−
Uncharged tRNA^{Val}	−	−	−	−	−	−	−	−	+	−	−
Uncharged tRNA^{Glu}	−	−	−	−	−	−	−	−	−	+	−
Uncharged tRNA^{Phe}	−	−	−	−	−	−	−	−	−	−	+
nmol of ppGpp + pppGpp	3.2	<0.1	<0.1	<0.1	0.5	<0.1	2.1	<0.1	<0.1	<0.1	<0.1

All reactions contain 20 μg and 15 μg of 50S and 30S ribosomal subunits, respectively, and 5 μg of stringent factor. They also contain 20 μg of R17 RNA, 0.5 μg of IF-1, 0.5 μg of IF-2, and 0.5 μg of IF-3, 5 μg of fMet-tRNA$_f$, and 5 μg of purified uncharged tRNA species where indicated.

tein begins fMet-Ala-Ser, for the synthetase, fMet-Ser-Lys, and for the A protein fMet-Arg-Ala (11, 12). The relative frequency of initiation at the different starts depends strongly on the integrity of the RNA template. The more intact the template, the higher the proportion of starts at the coat protein sequence (12).

Table 3, column *6* shows that the initiation complex itself does not synthesize ppGpp and pppGpp in the presence of the stringent factor. The initiation complex was formed by incubating reactions containing the purified initiation factors IF-1, IF-2, and IF-3 (kindly supplied by Dr. John Hershey), fMet-tRNA$_f$, R17 RNA, and 30S and 50S ribosomal subunits. In this case the donor site is occupied by the fMet-tRNA$_f^{Met}$ and the acceptor site is empty, because the reaction contains no tRNA capable of recognizing the second codon of any of the starting sequences.

Formation of the initiation complex was verified (see Table 5) by direct binding experiments. Almost half of the ribosomes bind fMet-tRNA$_f^{Met}$ in these experiments, two-thirds of the fMet-tRNA$_f^{Met}$ bound is in the donor site as judged by puromycin release, and more than two-thirds of the total binding is dependent upon the inclusion of the initiation factors. All the reaction mixtures described in this paper are 11 mM in $Mg(OAc)_2$, because the rate of ppGpp and pppGpp synthesis falls sharply at concentrations below this level.

The ppGpp and pppGpp nucleotides are synthesized if an uncharged tRNA species capable of recognizing the codon in the acceptor site of the ribosome is added to the initiation complex. As Table 3 shows, purified uncharged tRNA^{Ala} and tRNA^{Ser} (columns *1* and *7*) both stimulated the synthesis of ppGpp and pppGpp when added to the initiation complex. This result probably reflects starts at both the coat protein and synthetase genes. Steitz (12) has shown that the ratio of fMet-Ala to fMet-Ser dipeptide synthesis can be approximately one if the RNA is somewhat fragmented. In order to promote the synthesis of ppGpp and pppGpp, the uncharged tRNA must be specific for the codon adjacent to the fMet start. Purified tRNA^{Val}, tRNA^{Glu}, and tRNA^{Phe} (columns *9* and *10*) cannot replace tRNA^{Ala} or tRNA^{Ser}. The purified tRNA^{Ala} and tRNA^{Ser} must be binding at the acceptor site, for they recognize codons adjacent to the initiation codon of the coat and synthetase genes, respectively. If the ribosomes are not positioned at the start sequences by formation of the initiation complex (columns *3* and *4*), neither tRNA^{Ala} nor tRNA^{Ser} stimulates the reaction. In the absence of fMet-tRNA$_f$ uncharged tRNA^{Ala} and tRNA^{Ser} do not stimulate ppGpp and pppGpp synthesis. Furthermore, the inclusion of the initiation factors in a reaction that contains both fMet-tRNA$_f$ and uncharged tRNA^{Ala} (column *1* compared to *5*) stimulates the synthesis of ppGpp and pppGpp 5-fold.

Translocation by one codon down the coat protein sequence eliminates stimulation of ppGpp and pppGpp synthesis by uncharged tRNA^{Ala}. This translocation brings a different codon into the acceptor site (14). The movement is accomplished by adding to the reaction charged tRNA^{Ala} and the elongation factors EF-Tu, EF-Ts, and EF-G (a gift

TABLE 4. *Uncharged tRNA must recognize the codon at the acceptor site*

Additions	1	2	3	4	5	6	7	8	9	10	11
Uncharged tRNA^{Ala}	+	+	+	+	−	−	−	−	+	+	+
Charged tRNA^{Ala}	−	+	+	+	−	−	−	−	−	−	+
Uncharged tRNA^{Ser}	−	−	−	−	+	+	+	+	−	−	+
Charged tRNA^{Ser}	−	−	−	−	−	+	+	+	+	−	−
Charged tRNA^{Phe}	−	−	−	−	−	−	−	−	−	+	−
EF-Tu + EF-Ts	−	−	+	+	−	−	+	+	+	+	+
EF-G	−	−	−	+	−	−	−	+	+	+	+
nmol of ppGpp + pppGpp	3.5	2.4	<0.1	<0.1	2.0	1.4	<0.1	<0.1	2.8	2.4	1.8

Reaction mixtures as in Table 3, except 4 μg of fMet-tRNA. Where shown, the reaction contains 1.5 μg of EF-Tu, 1 μg of EF-Ts, and 0.2 μg of EF-G.

of H. Weissbach and D. I. Miller). As is shown in Table 4, (column *3*) the reactions that contain the elongation factors and charged $tRNA^{Ala}$ do not accumulate ppGpp and pppGpp in the absence of the elongation factors EF-Tu and EF-Ts. Lines *1* to *4* of Table 4 probably reflect initiations at the coat protein sequence, and lines *5* to *8* reflect initiations at the synthetase sequence.

As Table 5 shows, measurement of the binding of charged $tRNA^{Ala}$ to the ribosomes demonstrates that at least one-third of the ribosomes are correctly positioned at the start of the coat protein cistron. Binding of charged tRNA is dependent upon the formation of the initiation complex. Translocation of the complex down the coat protein sequence in the presence of the elongation factors is confirmed by the puromycin release of 75% of the charged $tRNA^{Ala}$ bound. Furthermore, as seen in Table 4, only charged $tRNA^{Ala}$ inhibits the stimulation of the reaction by uncharged $tRNA^{Ala}$. Charged $tRNA^{Ser}$ and charged $tRNA^{Phe}$ show no inhibition. Similarly, stimulation by uncharged $tRNA^{Ser}$ is inhibited by charged $tRNA^{Ser}$ but not by charged $tRNA^{Phe}$ in the presence of the elongation factors.

Confirmation of the Idling Ribosome Hypothesis. ppGpp and pppGpp are not synthesized *in vitro* if the ribosomes are actively engaged in protein synthesis. This fact is illustrated in the experiment pictured in Fig. 1*a*, in which poly(U) is used to direct polyphenylalanine synthesis. The ppGpp and pppGpp nucleotides are not made until the supply of charged amino acids is exhausted, at which point the synthesis of ppGpp and pppGpp begins. Charged $tRNA^{Phe}$ in the absence of the elongation factors EF-Tu and EF-Ts does not inhibit the reaction. Other charged tRNA species, such as Ala-$tRNA^{Ala}$, Val-$tRNA^{Val}$, and *N*-acetyl-Phe-$tRNA^{Phe}$, do not inhibit uncharged $tRNA^{Phe}$-promoted synthesis of ppGpp and pppGpp, even in the presence of the elongation factors.

The inhibition of the reaction by charged tRNA in the presence of the elongation factors EF-Tu and EF-Ts probably reflects a competition between the Phe-$tRNA^{Phe}$–EF-Tu–GTP complex and uncharged $tRNA^{Phe}$ for the acceptor site. The ppGpp and pppGpp nucleotides are not synthesized in reactions that contain Phe-$tRNA^{Phe}$, uncharged $tRNA^{Phe}$, EF-Tu, EF-Ts, and stringent factor purified free of the elongation factor EF-G. No protein synthesis occurs in this reaction. Columns *1* and *5* of Table 6 show that EF-G is not required for the synthesis of ppGpp from GDP or pppGpp from GTP by purified stringent factor. The accumulation of ppGpp in the reaction containing GTP in EF-G (Table 6, column *3*) is due both to the hydrolysis of GTP to GDP by EF-G and to the observed hydrolysis of pppGpp to ppGpp by EF-G in a

TABLE 5. *tRNA binding to ribosomes*

	pmol fMet-$tRNA^{Met}$ bound		pmol Ala-$tRNA^{Ala}$ bound	
	−Puromycin	+Puromycin	−Puromycin	+Puromycin
1. fMet-$tRNA_f^{Met}$	1.5	1.0	—	—
2. fMet-$tRNA_f^{Met}$ + initiation factors	7.4	3.0	—	—
3. fMet-$tRNA_f^{Met}$ + initiation factors + Ala-$tRNA^{Ala}$ + EF-Tu:EF-Ts:EF-G	—	—	8.0	3.3
4. Ala-$tRNA^{Ala}$ + initiation factors + EF-Tu:EF-Ts:EF-G	—	—	2.4	2.2

Binding reactions are those described in *Methods*. The 50-μl reaction mixtures contain 30 μg of 50S subunits, 20 μg of IF-1, 0.5 μg of IF-2, 0.5 μg of IF-3, 5 μg of fMet-$tRNA_f^{Met}$, 5 μg of $tRNA^{Ala}$, 0.2 μg of EF-G, 1.5 μg of EF-Tu, and 1 μg of EF-Ts (where indicated). In lines *1* and *2*, fMet-$tRNA_f^{Met}$ carried [^{3}H]Met (15 Ci/mmol). In lines *3* and *4*, Ala-$tRNA^{Ala}$ carried [^{3}H]Ala (20 Ci/mmol).

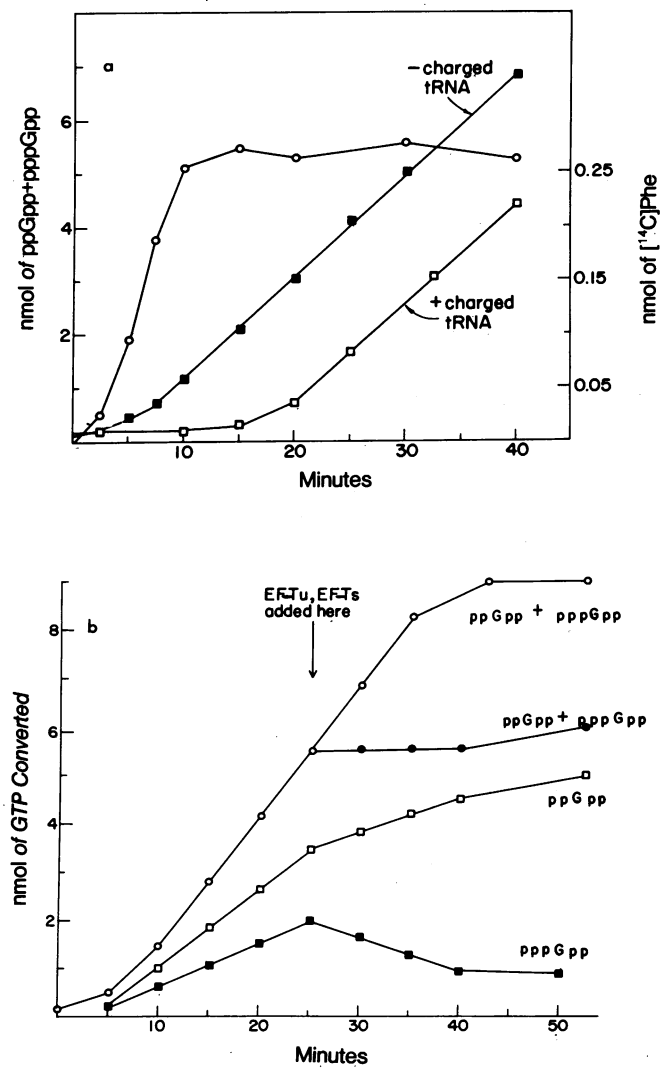

FIG. 1. (*a*) ppGpp and pppGpp are not synthesized when ribosomes are actively engaged in protein synthesis. Reaction conditions are those described in Table 6. *Open circles* represent nmol of [^{14}C]Phe incorporated into acid-insoluble counts. *Squares* represent nmol of ppGpp and pppGpp synthesized.

(*b*) Action of EF-Tu and EF-Ts on ppGpp and pppGpp synthesis. Reaction conditions are those described in Table 6. All reactions contain Phe-$tRNA^{Phe}$. *Filled symbols* indicate reactions containing EF-Tu and EF-Ts.

TABLE 6. *ppGpp and pppGpp synthesis by purified stringent factor*

	GTP substrate				GDP substrate	
	1	2	3	4	5	6
EF-Tu:						
EF-Ts	−	+	−	+	−	−
EF-G	−	−	+	+	−	+
nmol ppGpp	0.1	<0.1	1.9	<0.1	3.0	3.2
nmol pppGpp	3.4	<0.1	1.6	<0.1	<0.1	<0.1
nmol [^{14}C]-Phe incorporated	<0.05	<0.05	<0.05	0.28	−	−

Each reaction contains, per 50 μl: 30 μg of 50S and 20 μg of 30S ribosomal subunits, 5 μg of poly(U), 5 μg of charged [^{14}C]Phe-tRNA^{Phe} (50 Ci/mol), 5 μg of uncharged tRNA^{Phe}, and 1 μg of stringent factor purified free of EF-Tu, EF-Ts, and EF-G. Where indicated, mixtures also contain 1.5 μg of EF-Tu, 1.0 μg of EF-Ts, and 0.2 μg of EF-G.

ribosome-dependent reaction (Fig. 1*b*, and unpublished observations).

DISCUSSION

Cashel and Gallant (5) originally proposed that a reaction normally involved in protein synthesis idles during the stringent response and produces ppGpp and pppGpp. Others (15, 16) have suggested on the basis of drug inhibition experiments that ribosomes may be directly involved. Lund and Kjeldgaard (17) and others (18) have concluded that the presence, but not the synthesis, of messenger RNA is required for the synthesis of ppGpp. Earlier work by Neidhardt and coworkers (3, 4) has highlighted the key role of uncharged tRNA as the trigger for the stringent response.

The *in vitro* data on the synthesis of ppGpp neatly ties together these earlier experiments. The direct involvement of the translation machinery in the synthesis of ppGpp and pppGpp demonstrated that this is the site of synthesis. The signal that triggers the idling reaction is the presence of uncharged tRNA in the acceptor site of a ribosome bound to a mRNA. After a translocation event, the acceptor site should be able to receive an uncharged tRNA if the appropriate aminoacyl-tRNA species is not available, and the pool of the cognate uncharged tRNA is large. This should be the case after the stimuli which induce the stringent response, such as starvation for a required amino acid or inactivation of an aminoacyl-tRNA synthetase.

This is the first report of a biologically significant functional binding of uncharged tRNA to ribosomes. Levin (9, 19) has demonstrated that uncharged tRNA does bind to the ribosome in a codon-specific way. If ribosomes are actively engaged in protein synthesis, ppGpp [illegible] pppGpp do not accumulate either *in vivo* or in the *in vitro* reaction, implying that either they are not made at all or that they are made and immediately degraded. If the former is the case, cessation of the reaction may be due to the exclusion of uncharged tRNA from the acceptor site by the aminoacyl-tRNA–EF-Tu–GTP complex. If ppGpp and pppGpp are made all the time, but are immediately degraded during protein synthesis, they may then be intermediates in protein synthesis, rather than the products of a side reaction.

We thank Walter Gilbert for advice and discussions, and F. S. Pedersen, E. Lund, and N. O. Kjeldgaard for communicating their results before their publication. This work was supported by NIH Grant GM 09541.

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