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Synthesis of Stable RNA in Stringent *Escherichia coli* Cells in the Absence of Charged Transfer RNA

(RNA synthesis/guanosine tetraphosphate/tetracycline)

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ABSTRACT **It has been possible to demonstrate the complete absence of either charged $tRNA^{Glu}$ or charged $tRNA^{Val}$ at 42° by the use of two stringent strains of *E. coli*, one temperature-sensitive for glutamyl-tRNA synthetase and the other temperature-sensitive for valyl-tRNA synthetase. In both strains, stable RNA synthesis ceases, and guanosine tetraphosphate accumulates upon incubation at the nonpermissive temperature. Unique among a series of antibiotics tested, only tetracycline was able to stimulate stable RNA synthesis and to cause disappearance of the guanosine nucleotide. In this regard tetracycline and the "relaxed" gene product appear to be analogous.**

The synthesis of stable RNA is greatly diminished when *Escherichia coli* is unable to synthesize protein as the result of either a nutritional or mutational block. This linkage between protein and RNA synthesis has been termed the stringent response, and is directly or indirectly dependent upon a functional rel^+ allele. Mutants of the rel^+ locus have been isolated (1, 2) and are referred to as "relaxed." Phenotypically, a mutation in the *rel* locus uncouples the stringent response; i.e., it permits RNA synthesis to continue in the absence of protein synthesis. Similarly, when antibiotics that are known to block protein synthesis at the level of the ribosome are added to a stringent strain, RNA synthesis is uncoupled from protein synthesis. It has been suggested that this latter response is dependent upon the accumulation of charged tRNA whose use in protein synthesis has been spared by the presence of the antibiotic (3). Consequently, it was suggested that for RNA synthesis to occur in a stringent strain, a full complement of charged tRNAs and *N*-formylmethionyl-tRNA (4, 5) are required.

More recently, Cashel and Gallant (6–8) identified two unusual guanosine nucleotides, guanosine tetraphosphate and guanosine pentaphosphate (MSI and MSII, respectively), that accumulate during the inhibition of protein synthesis in a stringent strain, but not in a relaxed strain. It was suggested (8) that accumulated ppGpp and/or ppGppp inhibit(s) stable RNA synthesis. Recently, it was reported that ppGpp inhibits the initiation of rRNA synthesis *in vitro* (9).

The question then remains: is it possible to separate the antiobiotic-induced accumulation of charged tRNA from the synthesis of stable RNA *in vivo*? If such a distinction can be made, it will be possible to determine the relationship between the relaxed phenotype and the antibiotic-induced accumulation of stable RNA.

In this communication, we report that tetracycline is unique among several antibiotics tested in that it can stimulate stable RNA synthesis in a stringent strain of *E. coli* in the complete absence of either charged valyl- or glutamyl-tRNA; thus, tetracycline presumably can stimulate RNA synthesis in the absence of any charged tRNA species. Thus, tetracycline seems to have a basic similarity to the *rel* gene product with respect to its mode of action in the absence of charged tRNA. We also show that in the presence of tetracycline, ppGpp and ppGppp are not accumulated in a stringent strain. We conclude that both the acceptor (A) and peptidyl (P) sites on the ribosome must be occupied for stable RNA synthesis to proceed in the absence of protein synthesis.

MATERIALS AND METHODS

Chemicals. Chloramphenicol was obtained from Parke, Davis and Co., Detroit, Mich. Oxytetracycline was obtained from Chas. Pfizer and Co., New York. ^{14}C-Labeled and ^{3}H-labeled uracil were obtained from New England Nuclear Corp., Boston, Mass., and $^{32}PO_4$ was from Amersham/Searle Corp., Arlington Heights, Ill. All chemicals were of reagent grade.

Bacterial Strains, Media, and Growth Conditions. Two temperature-sensitive mutants were used in this study (5F2 and 10B6). They were isolated from strain D2, a K12 strain of *E. coli thy str* F^- and stringent for RNA regulation (rel^+) (10). Strain 5F2 is F^- *thy* $argA^+$ rel^+ and temperature-sensitive for glutamyl-tRNA synthetase (*gluS*).

Two derivatives of 5F2 were constructed by transduction; these are 5F2-rel^+ and 5F2-*rel*. The *argA* thy^+ markers were cotransduced into strain 5F2 from strain NF58 (rel^+ thy^+ *argA met*). A relaxed derivative of strain 5F2 was prepared by cotransduction of *rel* thy^+ *argA* from strain NF59 (*rel* thy^+ *argA met*) into 5F2 and selection for thy^+ *argA*. Transductants were tested for *rel*, and one isolate was selected for study. The two derived 5F2 strains will be referred to as 5F2 rel^+ and 5F2 *rel*. 10B6, is *thy* F^- and temperature-sensitive for valyl-tRNA synthetase (*valS*). Another temperature-sensitive mutant used in this study was isolated by and obtained from F. Neidhardt (11). This strain (NP-29) is rel^+ *thi valS*.

Bacterial strains were routinely grown at 30° in medium (3) supplemented with all L-amino acids (100 μg/ml) except

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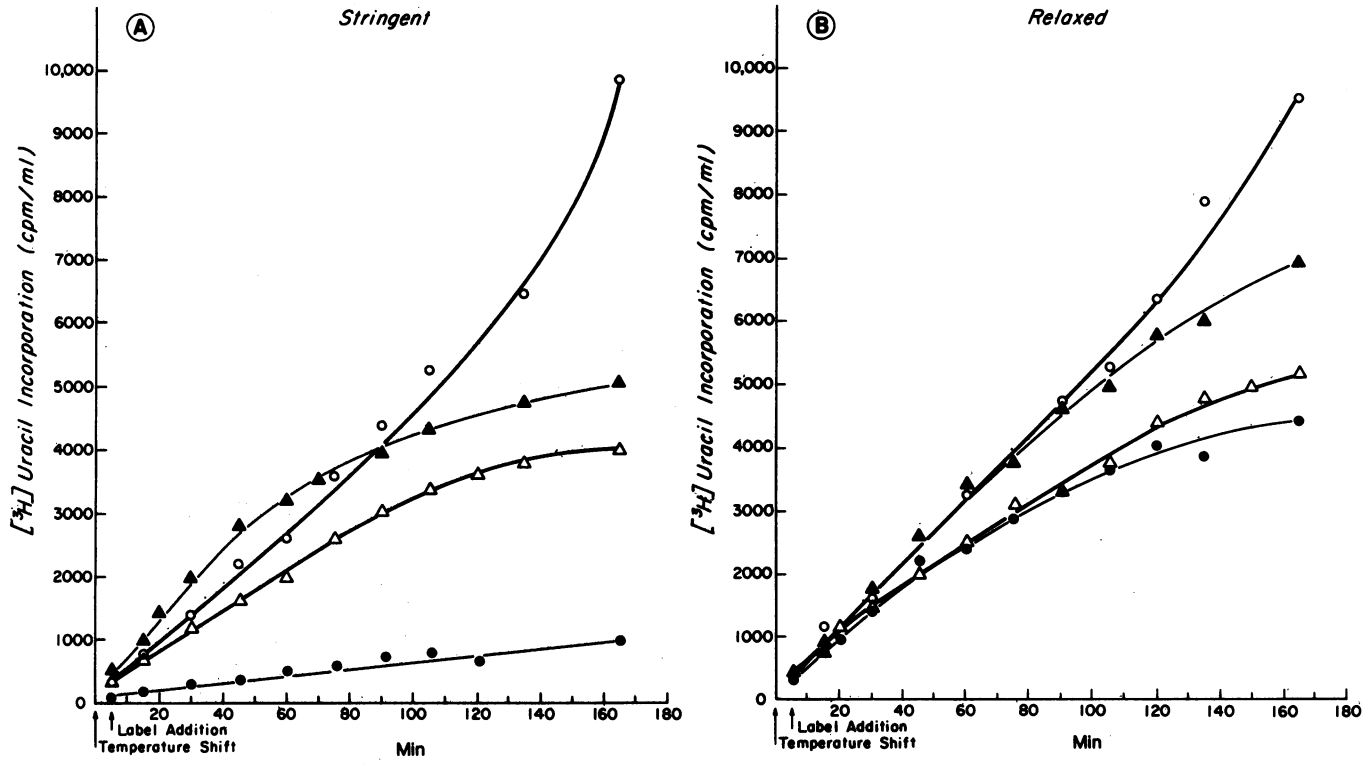

FIG. 1. To an exponential culture of either 5F2-*rel*$^+$(*A*) or 5F2-*rel* (*B*), growing in medium containing 15 μg/ml of uracil, was added 0.1 μCi/ml of [^{3}H]uracil (9.8 Ci/mol). Label and tetracycline were added 5 min after the temperature shift. O——O, 30°; ●——●, 42°; ▲——▲, 42° + tetracycline; △——△, 30° + tetracycline.

valine, leucine, isoleucine, and proline. Cultures used for incorporation studies were incubated at 30° in 20-ml flasks and shaken in a New Brunswick Gyrotory Shaker. At about 5×10^8 cells per ml, the cultures were shifted to 42° (see Fig. 1). 1-ml Samples were periodically removed for radioactivity measurement into tubes containing 0.10 ml (1 mg) of bovine-serum albumin, and an equal volume of cold 10% trichloroacetic acid was added. Samples were then filtered and washed on 25-mm glass filters (Reeve Angel 934AH). The amount of radioactivity was determined by counting in a toluene-based scintillation fluid with a Packard model 3320 spectrometer. Antiobiotics were used at a final concentration of 250 μg/ml.

Level of Charged tRNA and ppGpp Measurement. The level of charged glutamyl- and valyl-tRNA was determined in 150-ml cultures of strains 5F2, 10B6, and NP-29 (12). Cultures were either grown at 30° or placed at 42° for 30 min before harvest and determination (12) of the level of charged tRNA. The tRNA determination involves the use of cold CCl_3COOH for stopping growth and aminoacyl-tRNA synthetase activity. The isolated tRNA is divided into two portions, one treated with periodate and the other not. Each portion is "stripped" of amino acids, and the charging levels are determined from the ratio of the amino-acid acceptance of the periodate-treated aliquot to the acceptor activity of the untreated sample. When antibiotics were added at 42°, the culture was incubated for an additional 30 min before the level of charged tRNA was estimated.

The accumulation of ppGpp and GTP were measured by the method of Cashel (13), with minor variations. 1 ml of exponentially growing cells (4 to 5×10^8/ml) in medium (3) containing 0.2 mM KH_2PO_4 was combined with 50 μCi of carrier-free [^{32}P]orthophosphate, and 100-μl samples were removed into an equal volume of 2 N formic acid at 0, 7, and 20 min after temperature shift. The remainder of the procedure was exactly as described (13).

RESULTS

RNA synthesis in aminoacyl-tRNA synthetase mutants

Strains 5F2, 10B6, and NP-29 show an abrupt cessation of protein and RNA synthesis at 42°, but grow well at 30° (refs. 11, 14, and unpublished data). The data presented in Fig. 1*A* show RNA synthesis in the stringent strain, 5F2 *rel*$^+$ (temperature-sensitive for glutamyl-tRNA synthetase), during incubation at either 30° or 42°. At 30°, RNA synthesis continues uninterrupted; at 42°, however, RNA synthesis proceeds at a level about 5% of that observed at 30°. On the other hand, in the isogenic relaxed strain (Fig. 1*B*), RNA synthesis continues at a high rate at both 30° and 42° for as long as 180 min.

The effect of several antibiotics on RNA synthesis in these strains was investigated. Tetracycline (or oxytetracycline) stimulated RNA synthesis at 30° and 42° in strain 5F2 *rel*$^+$ (Fig. 1*A*) to about the same extent as did the presence of the *rel* allele in the absence of tetracycline (Fig. 1*B*). Furthermore, the addition of tetracycline to 5F2 *rel* results in a 35% greater rate of RNA synthesis at 42° than in its absence. These experiments were repeated with strain 10B6, which possesses a severely blocked valyl-tRNA synthetase. Once again, RNA synthesis was greatly stimulated at 30° and 42° by tetracycline (data not shown). Several other drugs (fusidic acid, 1

mg/ml; streptomycin, chloramphenicol, and puromycin, 250 μg/ml) were tested for their effect upon RNA synthesis in strains 5F2-*rel*$^+$ and 10B6 at 42° and were without effect. Of the antibiotics tested, only tetracycline stimulates RNA synthesis at 42° in both these strains. On the other hand, stimulation of RNA synthesis at 42° in strain NP-29 can be observed in the presence of chloramphenicol (ref. 15; unpublished data). Thus, in strain NP-29, also temperature-sensitive for valyl-tRNA synthetase, the stimulation of RNA synthesis by an antibiotic is not unique to tetracycline.

It has been concluded that the rate of RNA synthesis in a stringent bacterial population reflects the intracellular concentration of aminoacyl-tRNAs (3). The simplest interpretation of why RNA synthesis is stimulated with tetracycline at 42° in strains 5F2-*rel*$^+$, 10B6, and NP-29 is that upon addition of an antibiotic the intracellular concentration of the affected aminoacyl-tRNA is increased. This increase could be caused by a slight leakiness of the defective synthetase, combined with an antiobiotic-induced reduction of a requirement for charged-tRNA in protein synthesis.

Estimation of levels of charged tRNA

The level of glutamyl- and valyl-tRNA were examined in strains 5F2-*rel*$^+$, 10B6, and NP-29 at 30° and 42° in the presence and absence of antibiotic (Table 1). The % of charged glutamyl-tRNA in strain 5F2-*rel*$^+$ at 30° is 65–70% in either the presence or absence of tetracycline. However, glutamyl-tRNA is not present at detectable levels at 42° in strain 5F2-*rel*$^+$. When strain 5F2-*rel*$^+$ was incubated at 42° for 30 min, the addition of either tetracycline or chloramphenicol did not result in detectable increase in the level of charged glutamyl-tRNA (Table 1). A similar result was also observed in strain 10B6 for valyl-tRNA charging. On the contrary, when strain NP-29 was incubated at 42°, the level of valyl-tRNA was dramatically increased after the addition of chloramphenicol (Table 1 and F. Neidhardt, personal communication). Consequently, despite the absence at 42° of charged glutamyl- and valyl-tRNA in strains 5F2-*rel*$^+$ and 10B6 *rel*$^+$, respectively, RNA synthesis is observed only in the presence of tetracycline. This result is very similar to the effect produced by the presence of the *rel* allele. In strain NP-29, however, the accumulation of valyl-tRNA occurs at 42° in the presence of chloramphenicol, thus making any distinction between an indirect effect resulting from the accumulation of valyl-tRNA and a direct effect on RNA synthesis impossible.

TABLE 1. *Aminoacyl-tRNA charging levels in E. coli*

Strain	Growth temperature (°C)	Addition to growth culture	% Charged tRNA: Glutamyl-	% Charged tRNA: Valyl-
5F2				
Stringent	30	None	69	—
		Tetracycline	67	—
	42	None	0	—
		Tetracycline	0	—
		Chloramphenicol	0	—
10B6				
Stringent	30	None	—	85
	42	None	—	0
		Tetracycline	—	0
NP-29				
Stringent	30	None	—	40
	42	None	—	0
		Chloramphenicol	—	34

TABLE 2. *Synthesis of ppGpp and GTP*

Strain	Growth temperature (°C)	Additions	cpm/5 × 10^6 cells: ppGpp	cpm/5 × 10^6 cells: GTP
5F2				
Stringent	30	None	320	12,600
		Valine	4,040	7,560
		Valine + tetracycline	700	19,300
	42	None	11,100	22,900
		Tetracycline	870	34,200
		Fusidic acid	12,000	14,400
		Chloramphenicol	15,000	17,450
5F2				
Relaxed	30	None	200	37,000
		Valine	420	12,670
		Valine + tetracycline	1,050	49,850
	42	None	1,160	68,980
		Tetracycline	880	48,300
		Fusidic acid	820	54,000
		Chloramphenicol	960	57,350

Zero-time samples obtained for ppGpp were 250–750 cpm/5 × 10^6 cells, and for GTP were 1000–2000 cpm/5 × 10^6 cells. All antibiotics were present at a concentration of 250 μg/ml. Only the data from the 20-min sample are presented.

Synthesis of ppGpp and GTP

The accumulation of ppGpp is invariably observed in the absence of stable RNA synthesis (6, 7, 15–19). Therefore, ppGpp accumulation should be observed in either strain 5F2-*rel*$^+$ or 10B6 (both *rel*$^+$) at either 42° or 30° when protein synthesis is inhibited. The accumulation of $^{32}P_i$ label in ppGpp (and GTP for comparison) in strain 5F2 "stringent" and in an isogenic 5F2 "relaxed" derivative was examined in the presence and absence of several antibiotics (Table 2). At 42°, ppGpp accumulation is greatly stimulated in strain 5F2-stringent; in strain 5F2-relaxed, however, no significant accumulation of ppGpp is observed. Further, the pattern of ppGpp accumulation is not altered if 250 μg/ml of either chloramphenicol or fusidic acid is present. In contrast, the addition of tetracycline to 5F2-stringent at 42° completely abolishes the accumulation of ppGpp.

The addition of valine to K12 strains results in inhibition of protein synthesis by starvation for isoleucine (20). Thus, when valine is added to strain 5F2-stringent at 30°, ppGpp accumulation is observed as expected (Table 2). However the addition of tetracycline and valine to 5F2-*rel*$^+$ at 30° abolishes any significant ppGpp accumulation, consistent with the observation that RNA synthesis is stimulated.

DISCUSSION

Two mutants of *E. coli* defective in different aminoacyl-tRNA synthetases have been isolated (strains 5F2 and 10B6). These strains are *totally* blocked in their ability to charge their

cognate tRNAs at 42° (Table 1). Consequently, charged tRNA of either species cannot be caused to accumulate at the nonpermissive temperature in the presence of antibiotics (Table 1), in strains 5F2-*rel*$^+$ and 10B6. However, in other temperature-sensitive mutants for aminoacyl-tRNA synthetases (ref. 12; Table 1, strain NP-29) specific charged tRNAs do accumulate in the presence of antibiotics. Such an accumulation is probably due to a combination of residual synthetase activity and a sparing effect on the use of the charged tRNA as a result of the antibiotic-induced inhibition of protein synthesis. The addition of tetracycline to 5F2 *rel*$^+$ and 10B6 *rel*$^+$ at 42° has the unusual effect of stimulating RNA synthesis (Fig. 1). Thus, RNA synthesis is induced in a stringent strain of *E. coli* in the complete absence of a specific charged tRNA. This result has not been observed previously in a stringent strain, but is a normal condition in a relaxed strain (21). The stimulation of RNA synthesis by tetracycline in strain 10B6 (14) at 42° has been observed previously, as has stimulation of RNA synthesis by chloramphenicol in strain NP-29 (15). The levels of charged tRNA were not determined in these cited experiments; consequently, no conclusions could be made about whether the effect of these antibiotics on the stimulation of RNA synthesis was direct or indirect (see above). It appears from the data in this paper that tetracycline must induce a relaxed response in a stringent strain by its interaction with the ribosomes.

It has been reported that tetracycline inhibits tRNA binding at the A-site of ribosomes (for a review, see ref. 22), most likely by blocking the interaction of the GTP–EF-Tu–aminoacyl-tRNA complex with the A-site. The addition of tetracycline has little effect on the P-site of ribosomes, except at high concentrations where an apparent conformational change of the ribosome results (22). Consequently, the P-site is normally occupied by a peptidyl-tRNA in the presence of tetracycline.

A stringent response for RNA synthesis also is seen in a stringent strain when the P-site is unoccupied, as suggested from the data of Shih *et al.* (5). Addition of trimethoprim, an inhibitor of dihydrofolate reductase, indirectly inhibits formation of *N*-formylmethionyl-tRNA, and a stringent response is observed. Consequently, the lack of *N*-formylmethionyl-tRNA at the P-site results in inhibition of RNA synthesis. Furthermore, the addition of chloramphenicol to trimethoprim-inhibited stringent cells yields an increased rate of RNA synthesis by blocking protein synthesis and allowing the P-site to become saturated with the small amount of *N*-formylmethionyl-tRNA formed (5). Thus, it would appear that ribosomal RNA synthesis can only continue in a stringent strain if both the A and the P sites on ribosomes are occupied.

The stringent response can be further characterized, as shown by Cashell and Gallant (6, 8), by the accumulation of ppGpp and ppGppp. These investigators have suggested that as protein synthesis is inhibited (idles) ppGpp and ppGppp are produced, and that it is the accumulation of these compounds that may regulate rRNA synthesis. This interpretation is compatible with our conclusions and is supported by the recent observations of Haseltine *et al.* (23), who have been able to demonstrate the *in vitro*, ribosome-dependent accumulation of ppGpp in the presence of supernatant factors. Haseltine *et al.* (22) conclude that the signal that triggers the idling reaction referred to by Cashel and Gallant and the subsequent accumulation of ppGpp is the presence of an unoccupied A-site. Whether or not elongation factor G is required for ppGpp accumulation remains to be determined, since the *in vitro* data of Haseltine *et al.* (23) disagree with published findings of Atherly (24).

Finally, according to our interpretation, the relaxed phenotype either bypasses the necessity for an occupied A- (or) P-site for rRNA synthesis or it results in a ribosomal conformation that mimics occupation of the A- and P- sites.

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