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Stringent Regulation of the Synthesis of a Transfer Ribonucleic Acid Biosynthetic Enzyme: Transfer Ribonucleic Acid(m^5U)methyltransferase from *Escherichia coli*

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This paper describes the regulation of a transfer ribonucleic acid (tRNA) biosynthetic enzyme, the tRNA(m^5U)methyltransferase (EC 2.1.1.35). This enzyme catalyzes the formation of 5-methyluridine (m^5U, ribothymidine) in all tRNA chains of *Escherichia coli*. Partial deprivation of charged $tRNA^{Val}$ can be imposed by shifting strains carrying a temperature-sensitive valyl-tRNA ligase from a permissive to a semipermissive temperature. By using two such strains differing only in the allelic state of the *relA* gene, it was possible to show the tRNA(m^5U)methyltransferase to be stringently regulated. Upon partial deprivation of charged $tRNA^{Val}$, the differential rate of tRNA (m^5U)methyltransferase synthesis was found to decrease in a strain with stringent RNA control (*relA*$^+$), whereas it increased in the strain carrying the *relA* allele. This increase of accumulation of tRNA(m^5U)methyltransferase activity required protein synthesis. Thus, when tRNA is partially uncharged in the cell, the *relA* gene product influences the expression of tRNA(m^5U)methyltransferase gene.

When a required amino acid is removed from cells of *Escherichia coli*, accumulation of ribonucleic acid (RNA) and protein cease coordinately. This so-called stringent response contrasts to the behavior of an *E. coli* strain in which the *relA* gene is mutated. Such a strain continues to make RNA, whereas protein synthesis ceases upon complete deprivation of a required amino acid (25). It is now known that a wild-type strain, but not a strain carrying the *relA* allele, accumulates guanosine tetraphosphate (ppGpp) during amino acid limitation (6). It was therefore suggested that this metabolite was involved in the inhibition of RNA synthesis (5). The synthesis of ppGpp in vitro is known to require uncharged transfer RNA (tRNA), ribosomes, messenger RNA (mRNA), and a functional stringent factor, the product of the *relA* gene (13, 19). The stringent response affects the synthesis of both rRNA and tRNA (9, 21). It was recently shown also to influence the synthesis of ribosomal proteins and elongation factors G and Tu, as well as some other unidentified proteins (7, 11, 12). These macromolecules increase during partial amino acid deprivation in strains carrying an *relA* allele. However, some proteins decrease or are unaffected upon amino acid deprivation in strains carrying the *relA* allele and increase in wild-type strains under the same condition (12). Thus, upon deprivation of aminoacylated tRNA, a mutation in the *relA* gene causes pleiotropic effects (6).

The primary transcription product from tRNA genes are mono- or polycistronic tRNA precursors of a length that exceeds the mature tRNA (24). By the action of specific endo- and exonucleases, modifying enzymes, and, in a few cases, the tRNA nucleotidyltransferase, a mature tRNA is made. In this process the tRNA modifying enzymes participate at different stages. In all tRNA molecules of *E. coli*, 5-methyluridine (m^5U, ribothymidine) is present in the common tetranucleotide sequence m^5U-Ψ-C-G (loop IV) (27). The enzyme catalyzing the formation of m^5U in tRNA, tRNA(m^5U)methyltransferase, uses *S*-adenosyl-L-methionine as methyl donor and polycistronic as well as monocistronic tRNA precursor as substrate (2, 22, 23). Thus, the enzyme tRNA methyltransferase belongs to the tRNA biosynthetic enzymes. This group of enzymes has not been investigated at all thus far with respect to regulation of its members, although knowledge in this field will be important to fully understand the biosynthesis of tRNA. To learn about the regulation of this group of enzymes, we focused on the question of whether or not the tRNA(m^5U)methyltransferase from *E. coli* strain B is under stringent control. We imposed partial deprivation of charged $tRNA^{Val}$ by shifting a strain with a temperature-sensitive valyl-tRNA ligase from a permissive to a semiper-

missive temperature. By using two such strains differing only in the allelic state of the *relA* gene, it was possible to show that the tRNA(m^{5}U)methyltransferase was stringently regulated, i.e., under these conditions, changes in rate of enzyme synthesis parallelled changes in rate of stable RNA synthesis.

MATERIALS AND METHODS

Abbreviations. Abbreviations used in this paper are those recommended by the Commission on Biochemical Nomenclature (CBN-1970): m^{5}U, 5-methyluridine (ribothymidine); the tRNA(m^{5}U)methyltransferase producing m^{5}U is called tRNA(m^{5}U)-methyltransferase (EC 2.1.1.35), and ppGpp, guanosine 5'-diphosphate (2'- or 3'-diphosphate).

Bacterial strains. Bacterial strains used in this study were NF314 (*leu valS$^+$ relA$^+$*); NF536 [*leu valS*(Ts) *relA$^+$*]; and NF537 [*leu valS*(Ts) *relA*]. These strains are isogenic derivatives from *Escherichia coli* B strain AS19 and were kindly provided by N. Fiil, Department of Microbiology, University of Copenhagen, Copenhagen, Denmark. *E. coli* K-12 strain GB1-5-39 is *trmA5*, i.e., it completely lacks m^{5}U in its tRNA (4). tRNA from this strain is a specific substrate for the tRNA(m^{5}U)methyltransferase. (3)

Medium and cultivation. Bacteria were grown in potassium morpholinopropane sulfonate medium (17) supplemented with 0.4% glucose, 100 μg of L-leucine per ml, 4 mM K_2HPO_4, and [^{3}H]uracil. All cultures were grown aerobically on a rotary shaker in Erlenmeyer flasks with a minimum 1:5 ratio of culture volume to flask volume. Before the experiments, the cultures were pregrown at the permissive temperature (29.5°C) in the same medium for 6 to 10 generations to assure that the cells were in balanced growth. The optical density at 420 nm (OD_{420}) was always kept below 1.0 (150 μg [dry weight] per ml). The growth rate was determined by the rate of increase in cell mass between 0.1 and 1.0 at OD_{420} and is expressed as the specific growth rate constant k, calculated by the relation k = ln 2/mass doubling time (h). Growth was followed by an OD_{420} as measured with a Zeiss PMQII spectrophotometer with a path length of 1 cm.

Measurement of protein and RNA. RNA was measured as the incorporation of [5-^{3}H]uracil, and protein concentration was determined colorometrically. This was performed in the following way. Cells were grown in the presence of [5-^{3}H]uracil (0.01 μCi/μg; 25 μg/ml). Samples of 5 ml from the culture were made 5% in ice-cold trichloroacetic acid. After standing overnight at 4°C, the precipitates were suspended, and 250 μl was taken into 5% trichloroacetic acid containing 40 μg of uracil per ml. This sample was transferred to a Whatman GF/C glass-fiber filter, washed three times with 5 ml of ice-cold 5% trichloroacetic acid containing 40 μg uracil per ml and once with 20 ml of acetone, and then dried, and the radioactivity was measured in a scintillation counter. The rest of the suspended precipitate was washed once with 3 ml of 5% trichloroacetic acid and then suspended in 1 M NaOH and incubated at 37°C until lysis (10). After a ten-times dilution, the protein concentration was determined by the method of Lowry et al. (16).

Preparation of enzyme extracts. At different times, 40 to 80 ml of the culture was rapidly chilled, and the cells were collected by centrifugation at 12,100 × g for 10 min at 4°C. The cells were washed once in 5 ml of 6 mM potassium phosphate buffer (pH 7.3) containing 6 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid, and 10% ethylene glycol, and suspended in 2 ml of the same buffer. After sonic disruption with a Branson Sonifier B12 for 10 × 20 s at a power setting of 2.0, cell debris was removed by centrifugation at 27,100 × g for 15 min. The extract was then dialyzed overnight in the same buffer at 4°C and assayed for tRNA(m^{5}U)methyltransferase activity the next day. Enzyme extracts retained 90% of the activity after 7 days of storage at 4°C.

Preparation of tRNA. The tRNA preparations used for assaying the tRNA(m^{5}U)methyltransferase were prepared from strain GB1-5-39 (*trmA5*) according to the method of Avital and Elson (1). The cells were grown in a rich medium, harvested in late logarithmic phase, and washed with 0.9% NaCl. The method involves phenol extraction in the presence of chloroform, stripping of the amino acids {2 h at 25°C in 1.8 M tris(hydroxymethyl)aminomethane [Tris]-acetate, pH 8.2}), and fractionation of rRNA and tRNA with 2 M LiCl in 0.1 M potassium acetate (pH 5.0). The tRNA was dialyzed for 22 h against 6 liters of 10^{-6} M Mg^{2+}. A solution of 1 mg of tRNA per ml gives an adsorption at 260 nm of 25 (1-cm path length).

tRNA(m^{5}U)methyltransferase assays. tRNA from strain GB1-5-39 (*trmA5*), which completely lacks m^{5}U, was used as a specific substrate for the tRNA(m^{5}U)methyltransferase (3, 4). The assay was performed in a reaction mixture of 100 μl with the following composition: 0.1 M Tris-hydrochloride (pH 8.0), 2 mM glutathione, 0.1 mM ethylenediaminetetraacetic acid, 10 mM $MgSO_4$, 20 mM NH_4Cl, 60 nmol of *S*-[*methyl*-^{14}C]adenosyl-L-methionine per ml (specific activity, about 25 mCi/mmol), 800 μg of tRNA per ml from strain GB1-5-39, and varying amounts of enzyme. Two enzyme concentrations were routinely used, and the amount of enzyme was always such that the rate of methylation was proportional to the amount of enzyme used. The concentration of *S*-adenosyl-L-methionine and tRNA was high enough to ensure maximal velocity of the enzymatic reaction. The reaction was carried out at 37°C for 40 min when the reaction was stopped by adding 3 ml of ice-cold 0.01 M $La(NO_3)_3$ in 0.5 M $HClO_4$. The precipitates were washed three times directly on a glass-fiber filter (Whatman GF/C) with 5 ml of 5% trichloroacetic acid and 20 ml of acetone. The filters were dried, and the radioactivity was measured in a scintillation counter. Specific activities are given in units per milligram of protein. One enzymatic unit transfers 1 μmol of methyl group to tRNA per min at 37°C.

Materials. Potassium morpholinopropane sulfonate and *N*-tris(hydroxymethyl)-methylglycine was from Sigma Chemical Co., and *S*-[*methyl*-^{14}C]-adenosyl-L-methionine and [5-^{3}H]uracil were from The Radiochemical Centre, Amersham, Buckinghamshire, England.

RESULTS

Three isogenic strains of *E. coli* B were used in this study: strain NF314 (*relA*$^{+}$ *valS*$^{+}$), strain NF536 [*relA*$^{+}$ *valS*(Ts)] and strain NF537 [*relA valS*(Ts)]. In the *valS*(Ts) strains, the level of charged tRNA^{Val} (i.e. valyl-tRNA^{Val}) diminishes at a growth temperature above 32°C. It is known that deprivation of aminoacylated tRNA, i.e., accumulation of uncharged tRNA, will allow the accumulation of stable RNA in the strain carrying the *relA* allele, but not in the strain carrying the *relA*$^{+}$ allele. We used these strains to study how the synthesis of tRNA(m^{5}U)methyltransferase will respond to the *relA* mutation upon restriction of charged tRNA.

RNA and protein synthesis upon partial deprivation of charged tRNA. Cultures were grown at the permissive temperature (29.5°C), and synthesis of protein and stable RNA was monitored. At an approximate OD_{420} of 0.8, part of the culture was diluted into prewarmed medium at the semipermissive temperature 35.5°C (Fig. 1). In the parental strain an immediate increase (40%) in the rate of accumulation of both RNA and protein was observed after the temperature shift. In the strain carrying the *relA*$^{+}$ *valS*(Ts) alleles there was a 25% decrease of RNA accumulation, whereas synthesis of protein continued at about the preshift rate. In the strain carrying the *relA valS*(Ts) alleles, there was, as expected, a 60% increase of RNA accumulation after the temperature shift, whereas protein synthesis continued at about the same rate as before the temperature shift. Measurements of the rate of RNA and protein synthesis when shifted to 37°C showed the same qualitative response (Table 1).

Synthesis of tRNA(m^{5}U)methyltransferase. Figure 2 shows the differential rate of tRNA(m^{5}U)methyltransferase synthesis [i.e., tRNA(m^{5}U)methyltransferase synthesis per total protein synthesis] in the three strains at 29.5°C and when shifted to the semipermissive temperature 35.5°C. After the temperature shift, the rate of tRNA(m^{5}U)methyltransferase synthesis was unaffected in the parental strain (*relA*$^{+}$ *valS*$^{+}$) and decreased slightly (from 0.7 to 0.5) in strain NF536 [*relA*$^{+}$ *valS*(Ts)]. In strain NF537 [*relA valS*(Ts)], there was an immediate increase (from 0.7 to 1.2; about 70%) of the differential rate of tRNA(m^{5}U)methyltransferase synthesis. Qualitatively, the same results were obtained when a temperature

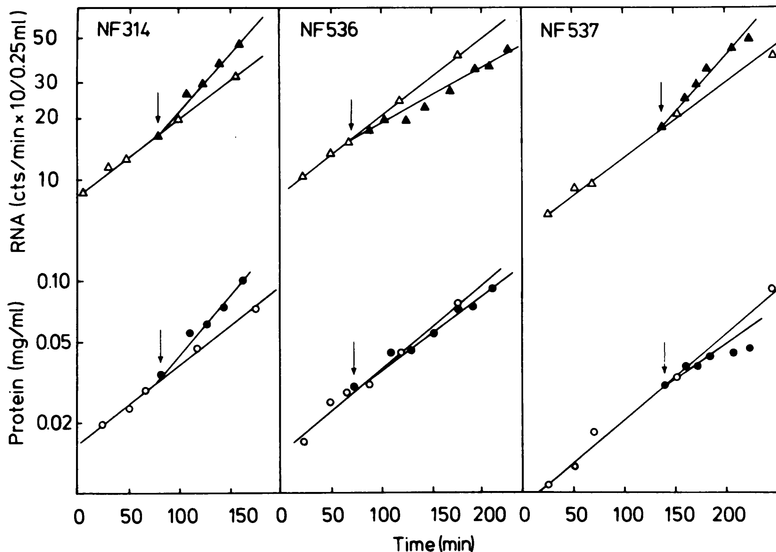

FIG. 1. *Accumulation of RNA and protein as function of time in strains NF314 (relA*$^{+}$ *valS*$^{+}$*),* NF536 [*relA*$^{+}$ *valS*(Ts)] and NF537 [*relA valS*(Ts)]. *Cells were grown at 29.5°C (○,△) to an OD_{420} of 0.8 when a portion of the culture was diluted four times in prewarmed media at 35.5°C (●, ▲). Correction has been made for this dilution for the values given in the figure. Total RNA (triangles) was measured as [5-^{3}H]uracil incorporation into trichloroacetic acid-precipitable material. Protein (circles) was measured as described by Lowry et al. (16).*

TABLE 1. *Synthesis of RNA, protein, and tRNA(m⁵U)methyltransferase in strains NF314, NF536, and NF537 after a shift in growth temperature from 29.5 to 35.5 or 37°C*

Bacterial strain and temp (°C)	Differential rate of tRNA (m⁵U)methyltransferase synthesis[a]	Relative rate of synthesis[b]		
		Protein	RNA	tRNA(m⁵U)methyltransferase
NF314 (*relA*⁺ *valS*⁺)				
29.5	0.7	1.00	1.00	1.0
35.5	0.7	1.47	1.38	1.3
37.0	0.7	1.50	1.50	1.6
NF536 [*relA*⁺ *valS*(Ts)]				
29.5	0.7	1.00	1.00	1.0
35.5	0.5	0.89	0.76	0.7
37.0	0.4	0.28	0.11	0.2
NF537 [*relA* *valS*(Ts)]				
29.5	0.7	1.00	1.00	1.0
35.5	1.2	0.94	1.59	1.6
37.0	1.0	0.25	1.92	0.5

[a] Differential rate of synthesis calculated as the slope obtained from plots as shown in Fig. 2.

[b] Relative rate of synthesis was calculated as the product of the diferential rate of synthesis and the initial rate of protein synthesis (*k* for protein) and normalized to the value obtained at 29.5°C.

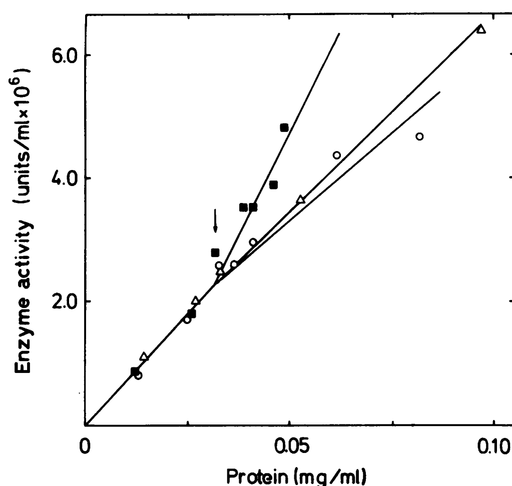

FIG. 2. *Differential rate of tRNA(m⁵U)methyltransferase synthesis in strain NF314 (relA⁺ valS⁺) (△); NF536 [relA⁺ valS(Ts)] (○); and NF537 [relA valS(Ts)] (■). The arrow indicates the shift in growth temperature from 29.5 to 35.5°C.*

shift from 29.5 to 37°C was employed (Table 1). Thus, the differential rate of tRNA(m⁵U)-methyltransferase synthesis was markedly affected by the availability of charged tRNA^{Val}, and the effect was influenced by the *relA* gene product.

To compare the rate of tRNA(m⁵U)methyltransferase synthesis to that of RNA and protein at the different temperatures, the following calculation was made. The product of the differential rate of synthesis and the rate of total protein synthesis (i.e., *k* for protein) was determined and normalized to the rate of synthesis at 29.5°C. The results are shown in Table 1. As expected in the wild-type strain *relA*⁺ *valS*⁺, the relative rate of protein, RNA, and tRNA(m⁵U)methyltransferase synthesis changed coordinately. In strain NF536 [*relA*⁺ *valS*(Ts)], this rate decreased for all the macromolecules studied from 1.0 to about 0.7 to 0.9 and further to 0.1 to 0.3 when growth temperature was increased from the permissive temperature to 35.5 and 37°C, respectively. However, in strain NF537 [*relA* *valS*(Ts)] there was a dissociation of the relative rate of protein synthesis from that of both RNA and tRNA(m⁵U)methyltransferase synthesis. While the relative rate of protein synthesis stayed the same at 35.5°C and decreased to 0.25 at 37°C, the relative rate of both RNA and tRNA(m⁵U)methyltransferase synthesis increased at 35.5°C to 1.6. At 37°C, the relative rate of tRNA(m⁵U)methyltransferase synthesis was 0.5 as compared with that of RNA which was 1.9. This was due to the overall reduction of total rate of protein synthesis due to the limited amount of charged tRNA^{Val}. However, the relative rate of tRNA(m⁵U)methyltransferase synthesis was higher than the relative rate of total protein synthesis and, furthermore, the ratio between enzyme synthesis and total protein synthesis at 37°C (0.5:0.25 = 2.0) is higher than that at 35.5°C (1.6:0.94 = 1.7). Thus, the cell favors even more the synthesis of tRNA(m⁵U)methyltransferase at 37°C. It can therefore be concluded that the relative

changes in the rate of tRNA(m^5U)methyltransferase synthesis respond in the same way as stable RNA and not as total protein.

Stringent response causes an abrupt increase in the level of ppGpp (5). However, many unrelated metabolic activities respond to amino acid deprivation in a *relA*$^+$ strain but not in an *relA* strain (6). Since metabolites such as polyamines have been shown to stimulate the tRNA methyltransferases, it was necessary to show that the observed increase and decrease in the relative rate of enzyme synthesis was not due to some metabolic activation and/or inhibition of preexisting enzyme molecules (15). Extracts from NF536 [*relA*$^+$ *valS*(Ts)] grown at 29.5 and 37°C were mixed in different proportions and combinations with extracts from strain NF537 [*relA valS*(Ts)] grown at the same temperatures. Furthermore, a partially purified tRNA(m^5U)methyltransferase was mixed with these extracts. We also mixed extracts from *E. coli* K-12 strain LMUR-5-266 (*relA lys trmA5*) starved for lysine with different extracts from strains NF536 and NF537. Within the experimental errors, no indication of activator and/or inhibitor was observed. Furthermore, Fig. 3 shows that the increase of the tRNA(m^5U)-methyltransferase activity in strain NF537 [*relA valS*(Ts)] when shifted to 35.5°C is not observed in the presence of chloramphenicol, a known inhibitor of protein synthesis.

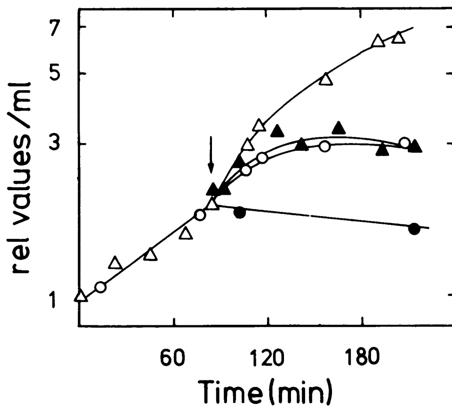

FIG. 3. *Accumulation of RNA (triangles) and tRNA(m^5U)methyltransferase (circles) in the absence (△,○) and presence (▲,●) of 200 μg of chloramphenicol per ml in strain NF537 [relA, valS(Ts)]. The arrow indicates the time when growth temperature was shifted from 29.5 to 37°C, at which time chloramphenicol was added to part of the culture. The amount of RNA and tRNA(m^5U)methyltransferase is given relative to the value at the time of the shift in growth temperature. At this time the culture contained about 2 × 10^{-6} U of enzyme activity per ml and 4,500 cpm of radioactivity per ml.*

DISCUSSION

We have shown that partial deprivation of charged $tRNA^{Val}$ in a strain carrying the *relA*$^+$ *valS*(Ts) alleles results in a more severe inhibition of the production of tRNA(m^5U)methyltransferase than in the synthesis of protein in general (Table 1). On the other hand, in a strain carrying the *relA valS*(Ts) alleles, synthesis of this enzyme is preferentially stimulated up to 70% compared with that of total protein under the same conditions (Table 1). Thus, in the expression of the *trmA* gene, which is responsible for the synthesis of tRNA(m^5U)methyltransferase, it is clear that the *relA* gene product is involved and that the level of charged tRNA is critical.

In our study we measured the kinetics of appearance of the tRNA(m^5U)methyltransferase activity. Several lines of evidence suggest that activation-inhibition of preexisting molecules is not involved. (i) A direct measurement of such activator-inhibitor proved negative. (ii) Upon shift in temperature a new steady state of differential rate of synthesis was obtained (cf. Fig. 2). (iii) The accumulation of tRNA-(m^5U)methyltransferase activity in strain NF537 [*relA valS*(Ts)] at 35.5°C required protein synthesis (Fig. 3). Thus, taken together, our results support a regulatory mechanism operating on the synthesis of tRNA(m^5U)-methyltransferase and should then be similar to the stringent control of the ribosomal proteins (7).

It is well established that biosynthesis of tRNA is under stringent control (14, 18, 20). Several enzymes are involved in the biosynthesis of tRNA but, until now, no studies have been published concerning the regulation of an enzyme involved only in tRNA biosynthesis. Ribosomal RNA and ribosomal proteins are both subjected to stringent control (7). Both these macromolecules constitute part of the same particle, the ribosome, which is part of the protein synthesizing system. The elongation factors EF-G and EF-Tu, which have also been shown to be stringently controlled, are also directly involved in protein synthesis (11). All these proteins are thought to be essential proteins for cell growth. However, mutants lacking tRNA(m^5U)methyltransferase activity in vitro and in vivo grow well under normal laboratory conditions (4). So far this enzyme has only been implicated to be involved in the biosynthesis of tRNA and has no direct function in protein synthesis. Thus, the putative regulatory mechanism(s), which is involved in the regulation of a group of macromolecules showing stringent control, does not discriminate be-

tween essential and nonessential activities and between proteins directly or not directly involved in protein synthesis. However, as a tRNA biosynthetic enzyme, the tRNA(m^5U)-methyltransferase might be considered as part of the translational apparatus and, so far, only such proteins have been shown to be stringently regulated.

Upon deprivation of charged tRNA, wild-type strains immediately accumulate ppGpp, in contrast to a strain carrying the *relA* allele in which the level of ppGpp diminishes or stays the same (5). The levels of ppGpp in the strains used by us (NF314, NF536, and NF537) were analyzed after temperature shift, and the results were as predicted from earlier studies (7; Ny and Björk, unpublished data). It was suggested that the high level of ppGpp in $relA^+$ strain was the signal for repression of rRNA biosynthesis, whereas the synthesis of mRNA was thought not to be stringently regulated (5, 9, 21). However, recently, direct measurement of the mRNA for ribosomal protein showed that mRNA for these proteins was stringently controlled (8). Therefore, it seems clear that stringent regulation is not only influencing stable RNA but might also be influencing some species of mRNA. It has been suggested that ppGpp stimulates the transcription of some genes but inhibits that of others (26). Therefore, it will be important to show the role played by ppGpp on the transcription of tRNA(m^5U)methyltransferase. This enzyme is involved in the biosynthesis of all tRNA chains in *E. coli*, and it will be interesting to learn whether or not other tRNA biosynthetic enzymes are regulated in the same manner as the tRNA(m^5U)methyltransferase.

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