Replacement of Pseudouridine in Transfer RNA by 5-Fluorouridine Does Not Affect the Ability to Stimulate the Synthesis of Guanosine 5′-Triphosphate 3′-Diphosphate†

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ABSTRACT: The requirement for ribothymidine and pseudouridine in the TΨCG loop of tRNA for its activity in the ribosome and tRNA-stimulated synthesis of guanosine 5′-triphosphate 3′-diphosphate (pppGpp) by stringent factor has been tested by the use of a purified $tRNA^{Phe}$ (883 pmol of phenylalanine incorporated/A_{260} unit) in which 92% of the pseudouridine, 98% of the ribothymidine, 98% of the dihydrouridine, and 88% of the uridines were substituted by 5-fluorouridine. This tRNA was quantitatively as active as control tRNA in inducing pppGpp synthesis. With loose-couple ribosomes, the concentration of tRNA needed to give half-maximal reaction was 0.07 μM for both normal and fluorouridine-substituted tRNA, with vacant tight-couple ribosomes it was 0.05 μM, and with tight couples carrying poly(Phe)-tRNA at the P site the value was 0.02 μM. These results show that at the level of intact tRNA there is no special requirement for modified bases in the TΨCG loop of tRNA in the synthesis of pppGpp.

The mRNA-dependent binding of unacylated tRNA to the A site of the ribosome–mRNA–stringent factor complex of *Escherichia coli* induces stringent factor to synthesize the guanine nucleotide, pppGpp,[1] by pyrophosphoryl transfer from ATP to the 3′-hydroxyl of GTP (Pedersen et al., 1973; Haseltine and Block, 1973; Sy et al., 1973; reviewed in Cashel, 1975). This nucleotide plus its hydrolysis product, ppGpp (Weyer et al., 1976; Fiil et al., 1976; Kari et al., 1977) designated here together as (p)ppGpp, are the primary means by which the processes of RNA synthesis and protein synthesis are interconnected, since, on the one hand, the lack of ability to acylate tRNA is the usual reason for a block in protein synthesis, and, on the other hand, increased levels of (p)ppGpp inhibit the synthesis of stable RNA species (Debenham and Travers, 1977; Van Ooyen et al., 1976) as well as certain mRNAs (Lindahl et al., 1976; Chu et al., 1976a,b).

Stringent factor can catalyze the formation of (p)ppGpp in the absence of ribosomes and tRNA, which can thus be visualized as positive allosteric effectors, but the maximum rate is only 3% that of the ribosomal system and 20% methanol is needed to achieve even this rate (Pedersen and Kjeldgaard, 1977). Certain ribosomal proteins in the absence of tRNA can also function as weak allosteric effectors (Christiansen and Nierhaus, 1976), and it has been reported that the entire tRNA molecule is not required, since the fragment TΨCG and larger oligonucleotides containing this fragment are weakly active in the presence of ribosomes and any mRNA-like polynucleotide (Richter et al., 1974; Erdmann et al., 1976). The unmodified version of TΨCG, UUCG, was inactive in this system, leading Erdmann and his colleagues to conclude that these two modifications serve as a specific recognition signal in tRNA for activation of stringent factor.

This finding represents the first demonstration of any specific function for the ubiquitous TΨCG sequence, common to all elongator tRNAs as well as prokaryotic initiator tRNA (reviewed by Ofengand, 1977). No difference could be detected between control tRNA and tRNA in which TΨCG was replaced by FFCG when tested in each of the partial reactions of ribosomal protein synthesis (Ofengand et al., 1974a), and the inhibition of ribosomal A site binding of AA-tRNA by TΨCG (Ofengand and Henes, 1969; Richter et al., 1973) has now been shown to be equally well performed by UUCG (Sprinzl et al., 1976). In view of the importance of the observation of Erdmann et al. (1976) for an understanding of the functional role of pseudouridine in tRNA, we sought to verify their results by an alternative method which used a modified intact tRNA in order to alleviate the difficulty of working with small tRNA fragments and their attendant very low activity. For this purpose, we elected to test FU-substituted tRNA, since our previous studies (Horowitz et al., 1974) had shown that it was possible to obtain a purified tRNA species in which more than 90% of all the uridine and uridine-derived nucleotides, including pseudouridine, were replaced by FU.

In this report, we describe our results with unfractionated tRNA and $tRNA^{Phe}$, both highly substituted with FU. With either loose or tight ribosomal couples, with or without peptidyl-tRNA at the P site, the ability of FU-substituted tRNA to induce (p)ppGpp synthesis was quantitatively as great as control tRNA. We conclude that at the level of intact tRNA there is no strong specific requirement for the TΨCG sequence. Part of this work has been reported in preliminary form (Chinali et al., 1976).

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[1] Abbreviations used are: pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; FU, F, or f^5Urd, 5-fluorouridine; f^5Cyd, 5-fluorocytidine; nbt^3Urd, 3-(3-amino-3-carboxypropyl)uridine; BD-cellulose, benzoylated DEAE-cellulose; DEAE, diethylaminoethyl; EF-Tu, elongation factor Tu from *E. coli;* EF-G, elongation factor G from *E. coli;* poly(Phe)-tRNA, polyphenylalanyl-tRNA; Brij 58, polyoxyethylene(20)cetyl ether; PEI, polyethylenimine; RPC, reverse-phase chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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TABLE I: Base Composition of Fluorouracil-Substituted $tRNA^{Phe}$ as Determined by Chemical ^3H Incorporation.

Nucleoside	Residues per 76 total: Expd[a]	Obsd	Ratio of obsd/expd
Urd	8 + 1[b]	1.04	0.12
f^5Urd	(16)	14.9	0.93
Ψrd	3	0.24	0.08
hUrd	2	0.04	0.02
m^5Urd	1	0.016	0.02
nbt^3Urd	1	Nd	Nd
Guo	23	22.8	0.99
Cyd	21	21.3	1.01
Ado	14	14.1	1.01
m^7Guo	1	0.78[c]	0.78
ms^2i^6Ado	1	Nd	Nd
f^5Cyd	0	0.51	

[a] Yaniv and Barrell (1969). [b] 4-Thiouridine is recovered as uridine in the ^3H-labeling procedure. [c] Corrected for 64% recovery. Nucleoside analysis was performed in duplicate by the Randerath labeling procedure as previously described (Horowitz et al., 1974). Nd, analysis not done.

Experimental Procedures

Materials

FU-substituted $tRNA^{Phe}$ was purified from FU-substituted mixed tRNA (Horowitz et al., 1974) by two cycles of BD-cellulose chromatography, first in the unacylated and then in the aminoacylated form. Crude FU-tRNA (9100 A_{260} units; 26 pmol of Phe acceptance/A_{260} unit) was chromatographed on a 2.6 × 30 cm column of BD-cellulose at 4 °C in 50 mM NaOAc (pH 5), 10 mM $Mg(OAc)_2$, 10 mM mercaptoethanol, and a linear gradient of NaCl. Although normal $tRNA^{Phe}$ is eluted at 0.8 M NaCl under these conditions, FU-$tRNA^{Phe}$ remained bound to the resin and did not elute, even at 1.5 M NaCl. A gradient of ethanol in 1.5 M NaCl plus the above buffer was required for elution. The main $tRNA^{Phe}$ peak (1000 A_{260} units; 180 pmol/A_{260} unit) was pooled, extracted with phenol, and recovered by ethanol precipitation. After aminoacylation under scaled-up standard assay conditions (Ofengand et al., 1974b) with a purified Phe-tRNA synthetase, the Phe-tRNA was chromatographed on a 2.6 × 24 cm column of BD-cellulose as described above. The presence of the phenylalanyl group retarded the elution, resulting in a further purification. The late-eluting portion of the peak (91 A_{260} units; 466 pmol/A_{260} unit; 32% of input) was rechromatographed again and the Phe-tRNA peak divided into two parts. The Phe-tRNA was chemically deacylated after treatment with Macaloid and desalted by gel filtration. The final yield (52% of input) was 21.8 A_{260} units at 883 pmol/A_{260} unit and 6.0 A_{260} units at 460 pmol/A_{260} unit. The former fraction was used in all of the experiments. It was essentially free of uridine and uridine-derived residues according to the nucleoside composition given in Table I.

Loose Couple tRNA-Free Ribosomes. The crude ribosome pellets, obtained by centrifugation at 200 000*g* of a 30S supernatant from a French-press extract of midlog *E. coli* B cells (Brot et al., 1970), were resuspended in 0.5 M NH_4Cl, 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ at a concentration of about 20 mg/mL and washed twice by layering the ribosome suspension (24 mL) on top of an 8-mL cushion of 18% sucrose in the above buffer and centrifugation for 6 h at 55 000 rpm (Spinco 60Ti rotor). Twice-washed ribosomes contained per A_{260} unit less than 10^{-4} unit of stringent factor.[2] NH_4Cl-washed ribosomes were then centrifuged through a two-step sucrose gradient under subunit-dissociating conditions (Chinali and Parmeggiani, 1973) to remove traces of tRNA. The ribosomes were suspended in 20 mL of 20 mM Tris (pH 7.8), 60 mM NH_4Cl, 0.5 mM $MgCl_2$ and layered over 5 mL of 12% sucrose in the above buffer which in turn was layered over 1.5 mL of 20% sucrose in 20 mM Tris (pH 7.8), 60 mM NH_4Cl, 10 mM $MgCl_2$. After a 4-h centrifugation at 45 000 rpm in the Spinco 50Ti rotor, the pellet of the ribosomes was resuspended in 20 mM Tris (pH 7.8), 60 mM NH_4Cl, 10 mM $MgCl_2$, 45% glycerol and stored at −25 °C.

Tight couple ribosomes were prepared according to the procedures described by Noll et al. (1973) and were kindly donated by Dr. L. Hsu. Tight couples carrying poly(Phe)-tRNA in the P site were prepared by incubation in a phenylalanine-limited protein synthesis reaction mixture. Two mixtures (50-μL each) were prepared containing 50 mM Tris (pH 7.8), 80 mM NH_4Cl, 7 mM $MgCl_2$, and 2 mM dithiothreitol. Mixture A contained in addition 0.5 mg/mL poly(U) and 156 A_{260} units/mL tight couples. Mixture B contained in addition 2.4 mM ATP, 0.8 mM GTP, 24 μM [^{14}C]phenylalanine (970 cpm/pmol), 10 μM EF-Tu, 4.8 μM EFG, excess phenylalanyl-tRNA synthetase, and 5.82 A_{260} units/mL $tRNA^{Phe}$. Each mixture was incubated separately for 5 min at 30 °C, chilled to 0 °C, mixed, kept 10 min at 0 °C to maximize A site binding, and then transferred to 30 °C for polypeptide synthesis. The progress of synthesis was followed by treatment of aliquots with 0.5 M NaOH for 20 min at 23 °C, followed by Cl_3AcOH precipitation and millipore filtration. Synthesis was maximal by 10 min, but the reaction was allowed to continue for 15 min. Since only 22% of the ribosomes were active (as determined by A-site binding assays), the average chain length was calculated to be 23. The incubation mixture was placed on a 0.6 × 8 cm column of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM Tris (pH 7.8), 80 mM NH_4Cl, 5 mM $MgCl_2$, 2 mM dithiothreitol and eluted at 4 °C with the same buffer. The pooled peak fractions contained 70% of the A_{260} units and 90% of the radioactivity. The preparation was made fresh for each experiment. For the experiment of Figure 4, a control tight couple preparation was made in the same way but without the addition of mixture B. It was 22% active in EF-Tu-dependent A-site binding.

Stringent Factor. The crude ribosome pellet from 50 g of *E. coli* B, prepared as described above for loose couples, was resuspended overnight at 4 °C in 165 mL of 10 mM Tris-HCl (pH 7.8), 60 mM KCl, 14 mM $MgCl_2$, 0.5 mM EDTA, and 5 mM dithiothreitol, treated with DNase [4 μg/mL] for 30 min at 4 °C, and centrifuged (40 000*g*, 20 min). The pellet was reextracted with the above solution. Subsequent ammonium sulfate fractionation was adapted from Pederson and Kjeldgaard (1977). The combined supernatants (240 mL) were precipitated at 33% saturated ammonium sulfate (pH 7.5), and the supernatant, essentially free of stringent factor activity, was discarded. The precipitate was extracted three times with 80 mL of 50 mM Tris-HCl, 25 mM EDTA, 2 mM dithiothreitol, 0.05% Brij 58 (ICI United States, Inc., Specialty Chemicals Division, Wilmington, Del. 19897), 0.73 M ammonium sulfate, pH 8.5. The pooled extracts were centrifuged (14 h, 25 000 rpm, Spinco 35Ti rotor) to remove residual ribosomes, precipitated at 52% saturated ammonium sulfate, and extracted three times with 10 mL of 33% saturated ammonium sulfate in 20 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol. The residual precipitate, containing most of the stringent factor activity, was resuspended and dialyzed against 20 mM Tris-HCl (pH 7.4), 100 mM NH_4Cl, 2 mM di-

[2] One unit of stringent factor activity is defined below.

thiothreitol, 7% glycerol (buffer I) and applied to a 2.5 × 12 cm column of phosphocellulose (Whatman P11) equilibrated with buffer I (Cochran and Byrne, 1974). The column was washed with 150 mL of buffer I and then eluted with 400 mM NH_4Cl in buffer I. Fractions containing stringent factor activity were pooled, precipitated with 50% ammonium sulfate, resuspended and dialyzed against buffer I, and applied to a second phosphocellulose P11 column (0.8 × 25 cm) which was eluted with a linear NH_4Cl gradient from 100 to 400 mM in buffer I. Active fractions were pooled, precipitated with 50% ammonium sulfate, resuspended in 25 mM Tris-HCl (pH 7.8), 80 mM NH_4Cl, 2 mM dithiothreitol, 7% glycerol (buffer II), dialyzed against this buffer for 6 h, and then applied to an 0.8 × 20 cm DEAE-cellulose column (Whatman DE-52) equilibrated with buffer II. Elution was with a 120-mL linear gradient from 80 to 260 mM NH_4Cl in buffer II. Fractions containing stringent factor activity were precipitated by addition of ammonium sulfate to 50%, resuspended in 50% glycerol, 0.5 M NH_4Cl, 25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, and stored at −20 °C.

One unit of stringent factor is defined as the amount of enzyme which catalyzes the conversion of 1 nmol of GTP to pppGpp in 1 min at 30 °C in the standard stringent factor assay.

Other Materials. Unmodified *E. coli* $tRNA^{Phe}$ (11–1200 pmol/A_{260} unit), mixed *E. coli* tRNA, and poly(U) were obtained from Boehringer, and poly(A,C_2) was synthesized from ADP and CDP with polynucleotide phosphorylase. The actual base composition, determined spectrophotometrically at 260 and 285 nm after alkaline hydrolysis, was 1A:1.8C. [α-^{32}P]-GTP was purchased from New England Nuclear. Macaloid (beneficiated Hector Clay) was obtained from the Baroid Division of the National Lead Co., Houston, Texas. It was washed by blending 10 g with 500 mL of water for 5 min three times with recovery of the Macaloid by centrifugation. The precipitate was finally suspended in 500 mL of boiling water to make a 2% solution. EF-Tu and EF-G were prepared by standard procedures (Miller and Weissbach, 1974; Parmeggiani et al., 1971). Phenylalanyl-tRNA synthetase (approximately 50% pure) was prepared by DEAE-cellulose chromatography of the mixed synthetase preparation of Muench and Berg (1966).

Methods

(p)ppGpp Synthesis Assay. The standard assay mixture (25 μL) contained 50 mM Tris-HCl (pH 7.8), NH_4Cl as indicated, 20 mM $MgCl_2$, 2 mM dithiothreitol, 2.5 μg of poly(U), 4 mM ATP, 0.8 mM [α-^{32}P]GTP (5–15 Ci/mol), ribosomes, tRNA, and stringent factor as indicated. Incubation was in polystyrene tubes at 30 °C for the times indicated for each experiment. Synthesis was proportional to time over this range. Reaction was stopped by addition of 3 μL of 50% HCOOH, 10 mM GTP, 5 mM GDP. After 10 min at 0 °C, the samples were centrifuged for 5 min at 6000*g* and 2 μL of the supernatant was spotted on 10-cm-long PEI cellulose TLC sheets (Macherey-Nagel Co., Duren, West Germany). The sheets were chromatographed in saturated KH_2PO_4 adjusted to pH 3.4 with HCOOH (Cashel and Kalbacher, 1970) until the solvent front had traveled 8.5 cm. In this system GDP, GTP, ppGpp, and pppGpp had R_f values of 0.60, 0.45, 0.26, and 0.12, respectively. After drying and locating the GTP and ATP spots under UV light, the spots containing the pppGpp and ppGpp were cut out together, by taking the area from 3 mm below the GTP spot to 3 mm above the application line. The remaining GTP and contaminating GDP (this stringent factor preparation is free of GTPase) was cut out as a zone from 3 mm below the GTP spot to just above the blue secondary solvent front line which occurs at R_f 0.8. The pieces of TLC sheet were counted under 10 mL of a toluene-based scintillation fluid. The fraction of [α-^{32}P]GTP recovered as pppGpp and ppGpp was determined as the ratio of radioactivity in (p)ppGpp to that in (p)ppGpp + GTP + GDP, and converted to nanomoles synthesized by multiplication by the amount of GTP in the reaction, 20 nmol.

Other Methods. Aminoacylation assays were performed as previously described (Ofengand et al., 1974b). Treatment of FU-$tRNA^{Phe}$ with Macaloid was done by first washing the Macaloid two times with 10 mM KOAc (pH 5). The tRNA solutions (7–27 A_{260} units/mL) were then made 0.4% in Macaloid and 25 mM in Bicine (pH 7.5). After 15 min of stirring at room temperature, the Macaloid was removed by centrifugation at 15 000 rpm for 15 min. Chemical deacylation of Phe-$tRNA^{Phe}$ was done in 0.8 M Tris (pH 9), 0.4 M KCl at 37 °C (Strickland and Jacobson, 1972). The $T_{1/2}$ was 4.8 min. After 25 min of incubation (97.4% stripped), the pH was adjusted to 5.6 with glacial HOAc and the tRNA precipitated with 2 volumes of ethanol and recovered. The concentration of ribosomes and poly(U) was measured by UV adsorption. One A_{260} unit of ribosomes in 10 mM Mg^{2+} and of poly(U) in water was taken to be 25 pmol and 33 μg, respectively.

Results

(p)ppGpp Formation with FU-Substituted Mixed tRNA. In initial studies to ascertain the effect of replacement of Ψrd (and all other uridine-derived residues) by f^5Urd in tRNA, we tested the activity of highly substituted (90–95% of all uridine-derived residues replaced by f^5Urd) unfractionated tRNA under conditions such that the amount of (p)ppGpp synthesized was proportional to the amount of tRNA added. When poly(U) was used as a mRNA, there was no detectable difference between FU-substituted and control tRNA in stimulating the synthesis of (p)ppGpp. Similar results were obtained when poly(A,C_2) was used in order to obtain a selection of codons lacking uridine, although in this case only 65% of the activity of the control tRNA was reached. However, even 65% activity is 6–12 times the 5–10% remaining Ψrd in the tRNA after FU substitution. Nevertheless, because of the possible ambiguities in any system using an undefined tRNA mixture, a purified $tRNA^{Phe}$ species lacking Ψrd was prepared. $tRNA^{Phe}$ was selected for further study because preliminary experiments using unsubstituted purified $tRNA^{Val}$ with poly(U_2,G) or poly(A,U,G), or mixed tRNA with poly(A,U,G), poly(C), or poly(G,C) did not stimulate synthesis sufficiently in our hands, despite literature reports to the contrary (Pedersen et al., 1973; Haseltine and Block, 1973).

Preparation of a Purified FU-Substituted $tRNA^{Phe}$. Essentially standard methods were used to prepare the purified $tRNA^{Phe}$ species. The only unusual aspect was the marked shift in elution position on BD-cellulose of the FU-substituted species. Our previous studies with $tRNA^{Val}_1$ (Horowitz et al., 1974) did not reveal any such large alteration in chromatographic behavior. We ascribe this to the nbt^{3}U residue present in normal *E. coli* $tRNA^{Phe}$ and probably replaced by f^5Urd in FU-substituted tRNA. The absence of this zwitterionic residue which is located in a highly exposed region of the molecule by analogy to U_{47} of yeast $tRNA^{Phe}$ (Kim, 1976) would certainly make the tRNA more hydrophobic as was observed.

Despite this shift into a relatively unpopulated region of the chromatogram, the $tRNA^{Phe}$ species proved very difficult to purify. RPC-5 chromatography (Kelmers et al., 1974) at pH 5 or 7 was not helpful, and best results were obtained by ami-

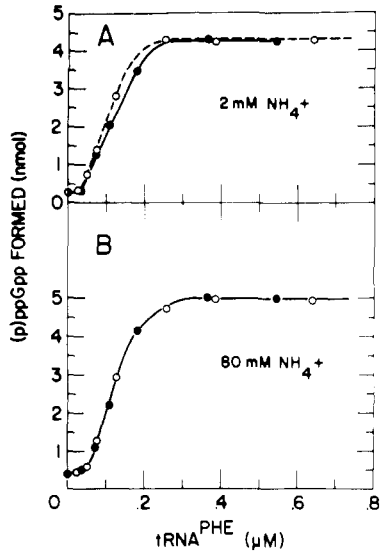

FIGURE 1: Ability of normal and FU-substituted purified $tRNA^{Phe}$ to stimulate (p)ppGpp synthesis with loose couples. The standard assay was used with 0.34 A_{260} unit of loose couples, 0.15 (A) or 0.30 (B) unit of stringent factor, and either 2 or 80 mM NH_4Cl, as indicated, in a 30-min incubation. The $tRNA^{Phe}$ concentration was determined by aminoacylation assays: (○-○) normal $tRNA^{Phe}$ (1100 pmol/A_{260} unit); (●-●) FU-$tRNA^{Phe}$ (883 pmol/A_{260} unit).

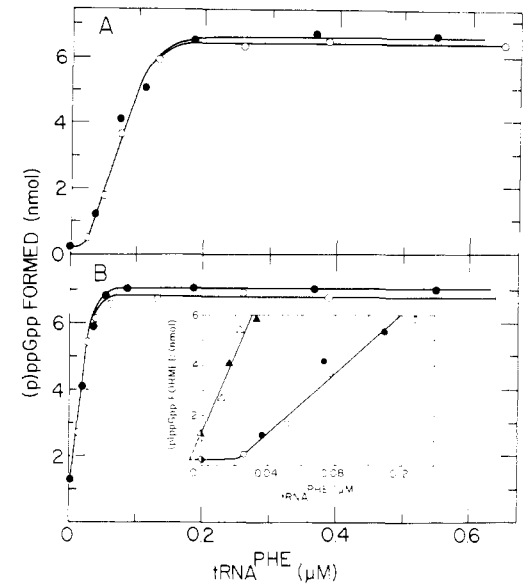

FIGURE 2: Ability of normal and FU-substituted purified $tRNA^{Phe}$ to stimulate (p)ppGpp synthesis with tight couples. The standard assay was used with 0.1 A_{260} unit of either tight couples or tight couples carrying poly(Phe)-tRNA at the P site, 1.2 μg of poly(U), 80 mM NH_4Cl, and 0.5 unit of stringent factor for 30 min. The $tRNA^{Phe}$ concentration was determined as in the legend to Figure 1: (○-○) control $tRNA^{Phe}$; (●-●) FU-$tRNA^{Phe}$. (A) Tight couples. (B) Tight couples carrying poly(Phe)-tRNA at the P site. Inset: expanded scale of a part of Figure 2. (△-△) Control $tRNA^{Phe}$ from part B; (▲-▲) FU-$tRNA^{Phe}$ from part B; (○-○) control $tRNA^{Phe}$ from part A; (●-●) FU-$tRNA^{Phe}$ from part A.

noacylation with phenylalanine. The added hydrophobic character of this amino acid shifted the elution position sufficiently to give a reasonably purified tRNA, although at low yield. Despite the relatively low aminoacylation capacity of 883 pmol/A_{260} unit, the nucleoside composition (Table I) was quite close to that expected for $tRNA^{Phe}$ and showed in addition the expected high degree of substitution of all the uridine-derived residues. In particular, 92% of the Ψrd was replaced by f^5Urd. Even if all of the Ψrd remaining were uniquely localized to the TΨCG sequence, 76% of the tRNA preparation would still be completely free of any Ψrd. This preparation was used in all of the following experiments.

(p)ppGpp Formation with Purified FU-Substituted $tRNA^{Phe}$. The tRNA dependence of (p)ppGpp synthesis using high salt-washed ribosomes ("loose couples") is shown in Figure 1. After an initial lag, which we interpret (see below) as the filling of the ribosomal P site, there was a linear increase in the rate of the reaction which saturated at approximately 0.2 μM. FU-substituted $tRNA^{Phe}$ behaved identically to control $tRNA^{Phe}$ at 80 mM NH_4Cl (Figure 1B) and was only 20% less active at 2 mM NH_4Cl (Figure 1A). The lower limit of 2 mM NH_4Cl was determined by the amount of salt in the stringent factor and ribosome preparations while the 80 mM value was chosen as the concentration which allowed optimum discrimination among modified tRNA species (Chinali et al., 1978) without too severely decreasing the rate of (p)ppGpp synthesis (Chinali et al., 1978; Sy et al., 1973). Clearly there is essentially no difference in activity between the two tRNA species at either salt concentration.

Since it is now clear that high salt-washed ribosomes are not representative of the native ribosomal structure in *E. coli*, we repeated the above experiments at 80 mM NH_4Cl with vacant ribosomal tight couples (Noll et al., 1973) (Figure 2A) and with tight couples carrying peptidyl-tRNA at the P site (Figure 2B) in order to more closely mimic the physiological state of the ribosome during the functioning of the stringent factor-tRNA-ribosome complex. Again, FU-substituted $tRNA^{Phe}$ was indistinguishable from control $tRNA^{Phe}$.

These results are summarized in Table II, where the apparent K_m of the two tRNAs are compared under various conditions. There was essentially no difference between control and FU-substituted tRNA, but clear differences between the ribosome preparations were observed. Vacant tight couples had a slightly increased affinity for tRNA compared to loose couples, while the greatest effect (twofold) occurred when the P site of tight couples was occupied with peptidyl-tRNA instead of with unacylated tRNA. Note that the control vacant tight couple preparation was carried through the same procedure used to prepare the loaded tight couples. In addition, the lag in tRNA concentration disappeared when loaded tight couples were used (Figure 2B), as expected if the lag were due

TABLE II: Apparent K_m of $tRNA^{Phe}$ with Different Ribosome Preparations.[a]

Ribosomes	NH_4Cl (M)	App $K_m \times 10^8$ (M) Control	FU
Loose couples	2	7.6	9.1
Loose couples	80	7.0	7.0
Tight couples	80	5.2	5.4
Tight couples with poly(Phe)-tRNA at P site	80	2.1	2.2

[a] Experimental details are described in the legends to Figures 1 and 2. Apparent K_m was taken to be equivalent to the concentration of tRNA needed to give half-maximal rate of reaction. The half-maximal rate was calculated as (plateau value − 0 tRNA value) divided by 2 plus 0 tRNA value, and the tRNA concentration so obtained was corrected for the tRNA concentration required before any stimulation of (p)ppGpp synthesis occurred. This latter value was obtained by direct extrapolation of the linear parts of the curves in Figures 1 and 2 to the basal value in the absence of tRNA. In Figure 2B, it was assumed that the high basal value was due to residual tRNA, hence the basal value of Figure 2A was used and extrapolation in this case required the addition of 0.7×10^{-8} M tRNA to the observed value (see inset to Figure 2) rather than a subtraction as in the other cases.

to filling of the P site. From the inset to Figure 2B, an estimate of 0.5 pmol of tRNA per 2.5 pmol of tight couples can be calculated for this putative P-site stoichiometry which is close to the estimated active fraction of this ribosome preparation (22%).

Relative Activity of Loose and Tight Couples for (p)ppGpp Synthesis. Comparison of the data of Figures 1 and 2 already shows that tight couples are more active than loose couples for (p)ppGpp synthesis, just as they are for A- and P-site binding. In order to make this comparison in a more quantitative way, the relative ability of tight and loose couples for both EF-Tu-dependent A-site binding of Phe-tRNA and stringent factor dependent interaction of $tRNA^{Phe}$ at the A site were measured under conditions of limiting ribosomes for both assays. EF-Tu-dependent A-site binding of Phe-tRNA was assayed by standard methods (Schwartz et al., 1975), and the ribosome-dependent synthesis of (p)ppGpp is shown in Figure 3. The vacant tight couples were 3.4 times more active than the loose couples in Phe-tRNA binding to the A site (data not shown), and, from Figure 3, 3.1 times more active in (p)ppGpp synthesis. Thus, there does not appear to be any differential inactivation of the A site for the two types of binding of tRNA.

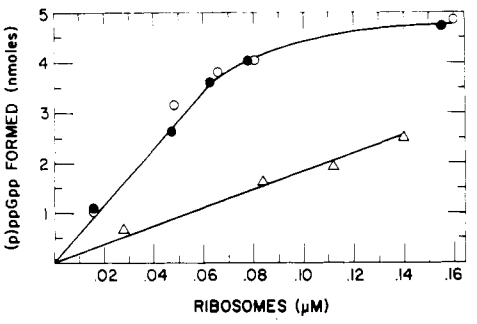

FIGURE 3: Relative activity of loose and tight couples in (p)ppGpp synthesis. The standard incubation for (p)ppGpp synthesis was used with 80 mM NH_4Cl, 0.15 unit of stringent factor, 0.64 μM $tRNA^{Phe}$, and ribosomes as indicated for 30 min at 30 °C. The preparation of loose and tight couples and analysis for (p)ppGpp were as described under Materials and Methods: (○) tight couples; (●) tight couples carrying poly(Phe)-tRNA; (△) loose couples.

Discussion

Pseudouridine was discovered 20 years ago (Davis and Allen, 1957), its structure determined shortly thereafter (Cohn, 1959, 1960; Scannel et al., 1959; Yu and Allen, 1959), and its localization to a specific region of tRNA, namely, the common TΨCG sequence in loop IV, has been known for 12 years (Zamir et al., 1965). However, no specific functional role has yet been assigned to this modified uridine (reviewed in Ofengand, 1977) despite the complicated intramolecular conversion reaction needed to accomplish its synthesis (Ciampi et al., 1977; reviewed in Schäfer and Söll, 1974). The ribosomal binding function assigned to this sequence (Ofengand and Henes, 1969; Richter et al., 1973) can also be accomplished by the unmodified sequence (Sprinzl et al., 1976). Moreover, a specific attempt to investigate the requirements for pseudouridine in tRNA during protein synthesis by the use of an FU-substituted tRNA lacking pseudouridine showed no effect of this substitution (Ofengand et al., 1974a). The loss of corepressor activity of His-tRNA and Leu-tRNA described by Cortese et al. (1974) is due to the failure to convert U to Ψ in a different region of the tRNA, namely, the anticodon stem region, and requires an enzyme different from the one used to make Ψ in the TΨCG loop. Consequently, the discovery of a biological function of TΨCG that could not be replaced by UUCG (Erdmann et al., 1976) was particularly interesting as the first example of such an effect.

A complication of that work was the very low activity of the fragments. In the experiments of Erdmann et al., it can be estimated that the TΨCG fragment was 400–1000 times less active on a molar basis than intact tRNA. In order to circumvent this situation as well as to test the effect of the absence of pseudouridine in an intact tRNA where the potential for other effector sites might allow an assessment of the relative importance of the TΨCG sequence, we used an approach which had been useful in previous studies, namely, that of examining a tRNA whose uridine and uridine-derived nucleosides were replaced by 5-fluorouridine. Since many residues are replaced, a loss of activity would be difficult to interpret, but retention of activity would show that *none* of the substitutions were important. We obtained the latter result in our previous study (Ofengand et al., 1974a) and the same result was obtained here also. Of course, strictly speaking, we have not replaced pseudouridine and ribothymidine by their unmodified analogues, but by a different modified uridine, 5-fluorouridine. It is highly unlikely that 5-fluorouridine is capable of mimicking the action of T and Ψ in view of the large structural and electronic differences between these residues. These studies confirm and extend our earlier work in the sense that we have now shown that both activities of tRNA at the ribosomal A site, namely, stringent factor catalyzed synthesis of (p)ppGpp, and EF-Tu-dependent binding, are independent of the presence of pseudouridine residues in tRNA.

How then can one best rationalize the results of Erdmann et al. (1976) with our findings? In our opinion, the simplest explanation is that, while the specific modification of UUCG to TΨCG may provide a signal for stringent factor activation, it is likely to be quantitatively an unimportant effect compared to that of other parts of the tRNA molecule, in particular the 3′-terminal region. For example, aminoacyl-tRNA does not induce (p)ppGpp synthesis (Pedersen et al., 1973; Chinali et al., 1978) even though aminoacyl-tRNA obviously contains the TΨCG sequence. A more direct example is presented in the following paper of this issue (Chinali et al., 1978), where it is shown that a variety of 3′-terminally modified tRNAs which obviously still retain their TΨCG sequence are incapable of inducing (p)ppGpp synthesis, although they still retain their capacity to be bound to the ribosome as evidenced by their ability to compete with unmodified control tRNA. If the 3′ end of tRNA plays such a dominant role in determining the allosteric effector activity of tRNA, then it may not be so surprising that FU-substituted tRNA which still retains an intact 3′ end is fully active in (p)ppGpp synthesis.

The subject of 3′-end recognition in the stringent factor reaction is discussed further in the following paper of this issue.

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