Regions of tRNA Important for Binding to the Ribosomal A and P Sites†

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ABSTRACT: Studies on the enzymatic inhibition of phenylalanyl-tRNA^{Phe} and formylmethionyl-tRNA$_F^{Met}$ binding to the ribosomes by defined tRNA fragments indicate that beside the anticodon the following regions of tRNA are important for ribosomal A-site interaction: the TpΨpCp sequence, the CpCpA end, and hU loop. In contrast, binding to the ribosomal P site is not inhibited by the fragments of uncharged yeast tRNA^{Phe} containing the hU or the TΨC loop of the molecule. Comparative studies on the inhibitory effect of the oligonucleotides TpΨpCpGp and UpUpCpGp indicate that the presence of the minor bases in TΨC loop is not an essential prerequisite for the binding of tRNA to the ribosomal A site. Furthermore, evidence is presented that shows that the binding of the TpΨpCpGp oligonucleotide to the ribosomes influences the ribosomal P site and increases there the efficiency of the codon–anticodon interaction. It is suggested that the TpΨpCpGp binds to the ribosomal A site and competes there with the TΨC loop of the aminoacyl-tRNA for the same binding site. A model for the interaction between tRNA and the ribosomal A site is proposed that involves partial unfolding of hU and TΨC loops of the tRNA and, therefore, suggests the dynamic involvement of tRNA in protein synthesis.

The comparative study on the primary structure of tRNAs (Barrel and Clark, 1974; Sigler, 1975) reveals that a great majority of these molecules contain a GpTpΨpCpPup sequence. This invariable tRNA region is always separated from the other invariable tRNA sequence, the 3′-terminal CpCpA end, by a constant number of nucleotides (Figure 1). This finding suggests that the GpTpΨpCp sequence may serve an important, but so far unknown, functional role. Most likely, this function will be localized in an event in which all tRNAs are involved, such as the elongation process during protein synthesis (Leder, 1973).

After the first sequential analysis of ribosomal 5S RNA was completed it was suggested that a sequence GpApApCp, which is present in this ribosomal RNA and is complementary to GpTpΨpCp, is a binding site for tRNA (Brownlee and Sanger, 1967; Forget and Weissman, 1967). Subsequently, this hypothesis was strengthened by the observation that the nonenzymatic binding of aminoacyl-tRNA to the ribosome can be inhibited by the oligonucleotide TpΨpCpGp (Ofengand and Henes, 1969; Shimizu et al., 1970). More direct evidence that this interaction really takes place was obtained by equilibrium dialysis of a radioactive labeled TpΨpCpGp and a specific 5S RNA–protein complex (Erdmann et al., 1973). In these experiments it could be shown that TpΨpCpGp interacts specifically with 5S RNA and that this interaction can be prohibited by a specific chemical modification of two adenines in 5S RNA. Since all these experiments strongly indicated that there is a specific TpΨpCpGp–5S RNA interaction on the ribosome, it was of interest to investigate this interaction in more detail, that is, to localize it on the ribosomal A and/or P site. In our previous communication (Richter et al., 1973) we showed that TpΨpCpGp fragment is able to inhibit the EF-Tu dependent binding of Phe-tRNA^{Phe} to ribosomes. Now we present the results of a more detailed study in which other tRNA fragments were analyzed for possible ribosome interaction and their effect upon the enzymatic binding of tRNA to ribosomes.

Materials and Methods

Poly(uridylic acid) (poly(U)),[1] tRNA from *Escherichia coli* MRE 600, *E. coli* tRNA$_F^{Met}$, and tRNA from yeast were obtained from Boehringer (Mannheim, Germany). tRNA^{Phe} from yeast was purified from bulk yeast tRNA by the method of Schneider et al. (1972). [^{32}P]tRNA from *E. coli* A19 cells was a gift of Dr. J. Zimmermann (Max-Planck-Institut für molekulare Genetik, Berlin). Pancreatic RNase (EC 3.1.4.22) 2 mg/ml, polynucleotide phosphorylase (EC 2.7.7.8) 30 units/mg, alkaline phosphatase (EC 3.1.3.1) 1 mg/ml, and snake venom phosphodiesterase (EC 3.1.4.1) 1 mg/ml were products from Boehringer (Mannheim). T_1 RNase (EC 3.1.4.8) (50 000 U/ml) was obtained from Sankyo Inc. (Tokyo). Uridylyl-(3′–5′)uridine (UpU), guanosine 5′-diphosphate (GDP), guanosine 5′-triphosphate (GTP), and cytidine 5′-diphosphate (CDP) were from Boehringer. ApUpG was obtained from Miles Laboratories. ApUp[^{14}C]G with a specific activity of 200 mCi/mmol was a gift of Dr. O. Pongs (Max-Planck-Institut für molekulare Genetik, Berlin). [^{14}C]Phenylalanine, specific activity 370 mCi/mmol, and L-[^{3}H]methionine, specific activity 7.5 Ci/mmol, were from New England Nuclear (Boston, Mass.). Puromycin was from Serva (Heidelberg). Analytical grade urea obtained from Baker Chemicals (Deventer, Holland) was used. Urea con-

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[1] Abbreviations used are: A_{260} unit, the quantity of the material contained in 1 ml of a solution that has an absorbance of 1 at 260 nm when measured in a 1-cm pathlength cell; 1 A_{260} unit of 70S ribosomes was assumed to be 25 pmol; 1 A_{260} unit 30S ribosomal subunit 67 pmol; 1 A_{260} unit 50S ribosomal subunit 39 pmol; 1 A_{260}, unit of UpUpCpGp or TpΨpCpGp 25 nmol; 1 A_{260} unit ApUpG 33 nmol; tRNA$_F^{Met}$, methionine specific initiator transfer RNA from *E. coli;* fMet-tRNA$_F^{Met}$, formylmethionyl-tRNA$_F^{Met}$; tRNA^{Phe}, phenylalanine transfer RNA; Phe-tRNA^{Phe}, phenylalanyl-tRNA^{Phe}; Ac-Phe-tRNA^{Phe}, *N*-acetylphenylalanyl-tRNA^{Phe}; EF-T, a mixture of elongation factors Tu and Ts; EF-G, elongation factor G; poly(U), poly(uridylic acid); UpU, uridylyl-(3′–5′)uridine; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; IF, initiation factors.

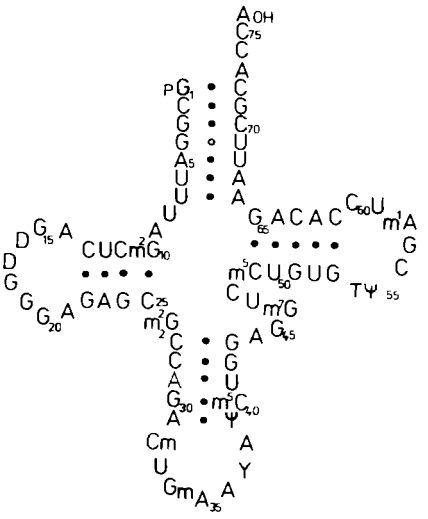

FIGURE 1: Nucleotide sequence of yeast $tRNA^{Phe}$ (RajBhandary et al., 1967).

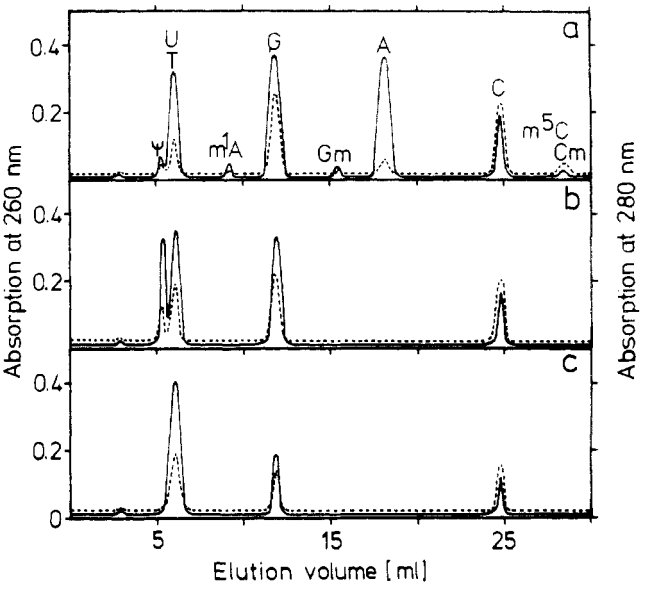

FIGURE 2: Analysis of the nucleoside composition of $tRNA^{Phe}$ (a), TpΨpCpGp (b), and UpUpCpGp (c). Preparation of the samples and the apparatus used is described under Materials and Methods. (—) Absorption at 260 nm, (- - - -) absorption at 280 nm.

taining solutions for chromatography of oligonucleotides was prepared by dissolving the appropriate amount of NaCl and urea in double-distilled water and adjusting the pH with concentrated HCl. Solutions were freshly prepared for each chromatography. DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals Ltd. (England), Sephadex A-25 was from Pharmacia, Uppsala (Sweden). Both column materials were precycled and equilibrated according to the producer's instructions.

Analysis of the nucleoside composition of tRNA fragments and of the oligonucleotides was performed on a nucleoside analyzer described by Uziel et al. (1968). This apparatus was equipped with a Beckman M71 ion-exchange column (0.6 × 40 cm). Samples of maximally 50 μl were applied by an injection syringe (jacketed column and the column head was supplied by LKB-Biocal, München, Germany) and the column was eluted with 0.4 M ammonium formate buffer, pH 4.75, at 50 °C and a flow rate of 0.3 ml/min. Absorbance of the effluent was recorded at 260 and 280 nm simultaneously. The peaks were integrated numerically with a precision of ±2% at 0.1 A_{260} unit of material/peak. For analysis of the nucleoside composition, the oligonucleotide (1 A_{260} unit) was incubated in a 50 μl solution containing 100 mM ammonium acetate buffer, pH 8.8 and 10 mM $MgCl_2$ with 5 μg of snake venom phosphodiesterase and 5 μg of alkaline phosphatase for 4 h at 45 °C. The reaction mixture was directly applied onto a column of the analyzer. For determination of the 3'-end nucleoside the oligonucleotide (2 A_{260} units) was incubated in 30 μl of 100 mM ammonium acetate, pH 8.8, with 5 μg of alkaline phosphatase for 3 h at 37 °C. Then 10 μl of 1 M NaOH was added and incubated for 30 min at 80 °C. After neutralisation with 10 μl of 1 N HCl, the sample was analyzed. The 5'-end nucleoside was determined by analysis following the incubation of 2 A_{260} units of oligonucleotide in 20 μl of 0.1 M ammonium acetate, pH 6.5, with 5 μg of snake venom phosphodiesterase for 30 min at 37 °C.

Polyacrylamide gel gradient microelectrophoresis was performed according to Wolfrum et al. (1974). Ten-microliter capillaries were used and the concentration of gradients was 1–50% monomer at 2% cross-linking. As a gel buffer, 350 mM Tris-H_2SO_4, pH 8.4, and as electrophoresis buffer, 50 mM Tris–glycine, pH 8.4, were used. Samples were applied onto the gel in quantities of 1–5 × 10^{-3} A_{260} units. Electrophoresis was performed at 100–160 V for 20 min. Toluidine blue (0.5% in water) was used for staining. Yeast $tRNA^{Phe}$ and the dodecanucleotide from the anticodon of yeast $tRNA^{Phe}$ (Maelicke et al., 1973) served as markers. The gels were scanned using the Joyce-Loebl double-beam microdensitometer.

The preparation of TpΨpCpGp was performed essentially according to Shimizu et al. (1970). Yeast tRNA (0.810 g) was incubated in 100 ml of 100 mM potassium phosphate buffer, pH 7.0, with 2500 units of T_1 RNase for 10 h at 37 °C. The reaction mixture was diluted with 100 ml of water and solid urea was added up to a concentration of 7 M. The solution was then applied onto a DEAE-cellulose column (2 × 100 cm) equilibrated with 0.05 M NaCl in 7 M urea at pH 7.0. After washing the column with 500 ml of starting buffer, a 2 × 2000-ml gradient of 0.05–0.3 M NaCl in 7.0 M urea, pH 7.0, was applied. The large peak of TpΨpCpGp appearing in the second third of the elution volume was identified by nucleoside analyses (Figure 2b) where the presence of pseudouridine is typical, or by the uv spectrum (shift of the absorption maximum in alkaline conditions due to the pseudouridine (Ofengand and Henes, 1969)). For the removal of urea, fractions containing the TpΨpCpGp were diluted with an equal volume of water and passed through a Sephadex A-25 column (2 × 20 cm) equilibrated with 50 mM NaCl in 20 mM sodium acetate, pH 5.2. After an initial wash with water (until urea was eluted), the oligonucleotide material was removed by washing with 1 M NaCl. The sample was then desalted by Bio-Gel P-2 filtration and concentrated to 3 ml volume by flash evaporation. The second chromatography was performed on a DEAE-cellulose column (1 × 100 cm) equilibrated with 20 mM NaCl and 7 M urea at pH 3.5. TpΨpCpGp fraction (670 A_{260} units) obtained from previous chromatography was applied onto the column and the column was washed with 200 ml of starting buffer before a linear gradient of 2 × 1000 ml of 20–200 mM NaCl and 7 M urea at pH 3.5 was applied. The major peak (at about 1000 ml of the elution volume) contained 340 A_{260} units of TpΨpCpGp. After desalting by gel filtration (Bio-Gel P-2), it was analyzed as shown in Figure 2. Found ratio for Ψ:T:G:C was 1:0.97:0.93:0.99. In some preparations a further purification by chromatography on a DEAE-cellulose column at pH 5.5 (conditions given in the legend to Figure 6)

was necessary. Only preparations that did not contain any adenosine and gave a correct ratio of Ψ:T:G:C were used for inhibition studies.

The oligonucleotide UpUpCpGp was prepared in two steps (Figure 4). First step: the incubation mixture contained 150 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 0.4 mM EDTA, 150 mM NaCl, 17.6 mM UpU, and 10 mM CDP. A 1.0-ml reaction mixture was incubated with 500 μg (15 units) of polynucleotide phosphorylase at 37 °C for 71 h. Then 5 μg of alkaline phosphatase was added and further incubated at 37 °C for 1 h. The reaction mixture was diluted with water to a volume of 15 ml and applied to a Sephadex A-25 column. Fractions containing the product (Figure 5a) were pooled, evaporated, reevaporated with water (3 times), and analyzed. The yield of UpUpC was 190 A_{260} units, $uv_{pH7.0}$: 280/260 = 0.536. Second step: a mixture (0.5 ml) containing 150 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 0.4 mM EDTA, 150 mM NaCl, 12 mM UpUpC, 24 mM GDP (0.5 mg), 15 units of polynucleotide phosphorylase and 250 units of T_1 ribonuclease was incubated at 37 °C for 2 h, diluted to 3 ml with water, and applied onto a Sephadex A-25 column (Figure 5b). Fractions of UpUpCpGp were pooled, evaporated, and reevaporated several times with water. The residue was dissolved in water and passed through a Bio-Gel P-2 column. Yield 160 A_{260} units, $uv_{pH7.0}$: 280/260 = 0.556. The product was analyzed for its nucleoside composition (Figure 2c). The ratio of U:G:C was found to be 2:0.96:0.96.

Preparation of $[^{32}P]TpΨpCpGp$. Twelve A_{260} units of unlabeled TpΨpCpGp and 96 A_{260} units of [^{32}P]labeled tRNA from *E. coli* A 19 cells (2 mCi) were incubated in 2 ml of 100 mM potassium phosphate buffer with 1200 units of T_1 ribonuclease for 12 h at 37 °C. The reaction mixture was diluted with 2 ml of water and applied onto a DEAE-cellulose column. The chromatographic purification of [^{32}P]TpΨpCpGp is described in the legend to Figure 6. Fractions from the third DEAE-cellulose column (pH 5.5) containing the product were pooled and passed through a Bio-Gel P2 column, which had previously been equilibrated with water. The desalted aqueous solution of [^{32}P]TpΨpCpGp was evaporated to dryness by flash evaporation. The sample did not contain any residual salt or urea. Yield 8.2 A_{260} units; 9.92×10^6 cpm (about 40% recovery).

Preparations of $tRNA^{Phe}$ *Fragments.* Yeast $tRNA^{Phe}$ was treated with dilute HCl according to Thiebe and Zachau (1971) in order to excise the base Y. For excision of m^7G the method described by Simsek et al. (1973) was used. The cleavage of the phosphodiester bond at the site where the modified base is missing was achieved by incubation of the $tRNA^{Phe}$(-Y) or $tRNA^{Phe}$(-m^7G), respectively (500 A_{260} units), in a 30-ml solution containing 25 mM sodium acetate, pH 4.5, and 150 mM aniline hydrochloride at 37 °C for 4 h. The reaction mixture was dialyzed against water (3 × 500 ml), made 7 M with respect to urea, and applied onto a Sephadex A-25 column (chloride form; 1 × 100 cm). Chromatography was performed using a linear gradient of 2 × 1000 ml of 100–500 mM NaCl in 7 M urea, pH 3.0, as an eluant. Fractions containing the appropriate peaks (Simsek et al., 1973; Thiebe and Zachau, 1971) were pooled and the urea was removed by dialysis against water. Desalted fractions were concentrated by evaporation dissolved in 10 mM phosphate buffer, pH 7.5, containing 100 mM NaCl and applied onto a Sephadex G-100 column (3 × 100 cm), which was equilibrated and run at 50 °C with the same buffer (Schmidt et al., 1970). Fractions were analyzed by polyacrylamide gradient microelectrophoresis (Wolfrum et al., 1974) and the ones containing a uniform oligonucleotide were pooled, concentrated to one-tenth of the volume by evaporation, and desalted on Bio-Gel P-2 column. Oligonucleotide 47–75 missing the 3′-end adenosine was prepared by the same procedure, starting from $tRNA^{Phe}$pCpC (Maelicke et al., 1974). The fragment 48–59 was prepared by partial digestion of $tRNA^{Phe}$ with pancreatic RNase according to Chang and RajBhandary (1968). $tRNA^{Phe}$ (468 A_{260} units) was incubated with 100 μg of pancreatic RNase in 4 ml of solution containing 100 mM Tris-HCl, pH 7.65, 10 mM magnesium acetate, and 100 mM potassium chloride, at 0 °C for 60 min. The reaction mixture was then extracted with Macaloid adsorbent (National Lead Company, Houston, Texas) three times (0.5 mg of adsorbent/mg of digest). The fragments were isolated by chromatography on DEAE-cellulose columns using buffers containing 7 M urea and by Sephadex G-100 gel filtration at 50 °C (Schmidt et al., 1970). Characterization was performed by polyacrylamide gradient microelectrophoresis. All $tRNA^{Phe}$ fragments were further characterized by determination of their nucleoside composition, identification of the minor bases, and determination of their 3′-end nucleoside.

Ribosomes, IF, EF-Tu. 70S ribosomes and their 50S and 30S subunits were isolated from midlog phase grown *E. coli* A19 cells previously described (Cronenberger and Erdmann, 1975). The isolated particles were stored in TMA I buffer (10 mM Tris-HCl, pH 7.6, at 25 °C, 30 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol) at −80 °C. Crude initiation factors (IF) were prepared essentially according to Dubnoff and Maitra (1971). Elongation factor EF-T was isolated as previously described (Arai et al., 1972) and was free of EF-G activity.

tRNA Binding Assays. Phe-tRNA preparation and binding studies were done essentially according to Ravel and Shorey (1971). In the *E. coli* tRNA bulk, 7% of $tRNA^{Phe}$ was charged with a specific activity of 1.36 mCi/mmol of tRNA. The binding assay was carried out as follows: 20 μl of salt mixture (50 mM Tris-HCl, pH 7.5, 25 mM magnesium acetate, 600 mM NH_4Cl, 5 mM dithiothreitol), 10 μl of poly(U) (4 mg/ml), 0.6–1.5 A_{260} units of 70S ribosomes (or corresponding amounts of 50S and 30S subunits), oligonucleotides or $tRNA^{Phe}$ fragments as indicated, and 30 μl of water were mixed on ice and incubated at 30 °C for 10 min. The reaction mixture was then cooled to 4 °C and supplemented with 5 μl of GTP (0.01 M), 10 μl of Phe-$tRNA^{Phe}$ (2 A_{260} units), and 20 μl of EF-Tu. After a second incubation at 30 °C for 7 min, the reaction mixture was placed on ice, diluted with 1 ml of reaction buffer, and filtered through Millipore membranes. Each filter was washed with 10 ml of buffer (4 °C), dried, placed in toluene liquid scintillation fluid, and counted in a Packard Liquid Scintillation spectrophotometer.

The nonenzymatic binding of Ac-Phe-$tRNA^{Phe}$ was done similarly to Ravel and Shorey (1971). The experimental conditions were those as described above for Phe-$tRNA^{Phe}$ except that in the reaction mixture the $MgCl_2$ concentration was raised to 10 mM and that no EF-Tu was added. The specific activity for Ac-[^{3}H]Phe-$tRNA^{Phe}$ was 5 mCi/mmol of tRNA.

Initiation factor and ApUpG dependent binding of [^{3}H]-F-Met-$tRNA_F^{Met}$ to ribosomes was carried out according to Dubnoff and Maitra (1971). Purified *E. coli* $tRNA_F^{Met}$ was aminoacylated to 60% with a specific activity of 1 Ci/mmol. In the standard reaction mixture (80 μl) were: 50 mM Tris-HCl, pH 7.8, 8 mM $MgCl_2$, 85 mM NH_4Cl, 2 mM dithiothreitol, 0.6–1.5 A_{260} units of 70S ribosomes (or corresponding amounts of 50S and 30S subunits), 0.3 A_{260} units of ApUpG, 2.5 mM GTP, and, if indicated, oligonucleotides or

TABLE I: Inhibitory Effects of Different Oligonucleotides upon tRNA Binding to Ribosomes.

Oligonucleotide	Binding of tRNA (%)		
	Phe-tRNA^{Phe}	Ac-Phe-tRNA^{Phe}	fMet-tRNA$_F^{Met}$
None	100	100	100
TpΨpCpGp	22	83	117
UpUpCpGp	24	–[b]	–[b]
GpApUpU	95	–[b]	–[b]

[a] Binding assays were done as described under Materials and Methods. 70S ribosomes were preincubated with a 1000-fold molar excess of oligonucleotide. One-hundred percent of Phe-tRNA^{Phe}, Ac-Phe-tRNA^{Phe}, and F-Met-tRNA$_F^{Met}$ binding corresponds to 0.34, 0.10, and 0.87 mol of tRNA bound per mol of ribosome. [b] Experiments were not done.

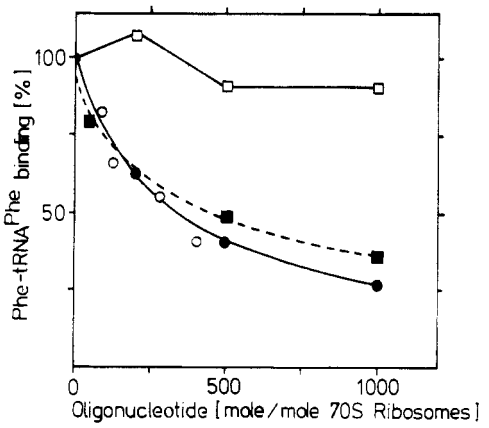

FIGURE 3: Inhibition of EF-Tu dependent Phe-tRNA^{Phe} binding to 70S ribosomes by different oligonucleotides. One-hundred percent binding corresponds to 4.8 pmol of Phe-tRNA^{Phe} bound per 23.4 pmol of 70S particles. Inhibitory effects of GpApApU (□-□-□), UpUpCpGp (■-■-■), TpΨpCpGp (●-●-●), and [^{32}P]TpΨpCpGp (○-○-○). Experimental details as described under Materials and Methods.

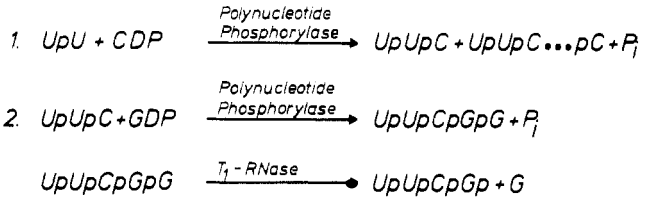

FIGURE 4: Reaction scheme for the preparation of UpUpCpGp.

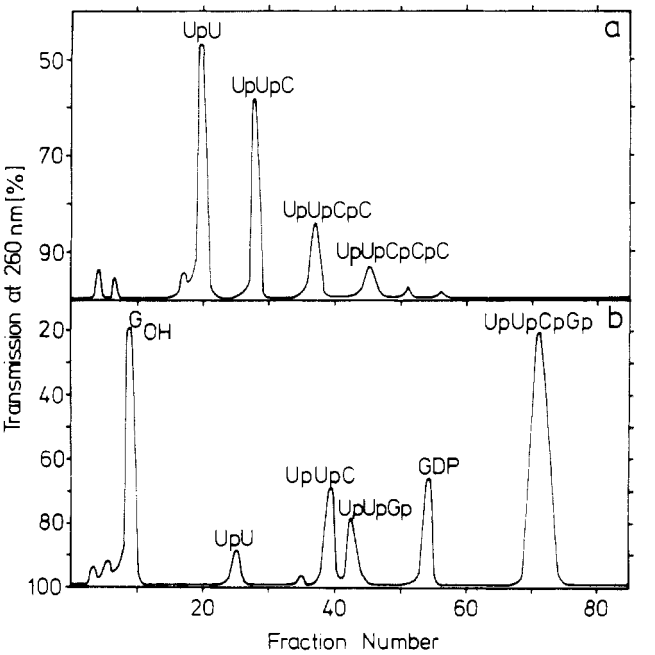

FIGURE 5: Isolation of UpUpCpGp. (a) Chromatography of the reaction mixture obtained after first step of the synthesis (Figure 4). A Sephadex A-25 column (carbonate form, 1 × 40 cm) equilibrated with 10 mM triethylammonium bicarbonate (TBC), pH 7.5, was used. Reaction mixture (see Materials and Methods) was eluted with a linear gradient consisting of 2 × 500 ml of 10–500 mM TBC, pH 7.5. Fractions of 12 ml were collected. (b) Chromatography of the reaction mixture obtained after the second step (Figure 4) on a Sephadex A-25 column (carbonate form, 2 × 40 cm). Elution was made by a linear gradient of 2 × 500 ml of 10–800 mM TBC, pH 7.5. Again, 12-ml fractions were collected.

tRNA^{Phe} fragments. After a 20-min preincubation at 30 °C, the samples were placed on ice and fMet-tRNA$_F^{Met}$ was added in a 3:1 ratio to ribosomes. The samples were again incubated at 30 °C for 10 min, cooled, passed through a Millipore filter, and counted.

Results

A method is described that allows a large-scale preparation of the oligonucleotide TpΨpCpGp by T_1 RNase digestion of unfractionated tRNA from yeast, its isolation by repeated ion-exchange chromatography, and characterization by analysis of the nucleoside composition (Figure 2). In order to show that the tRNA binds to ribosomes via its TpΨpGp sequence, the 70S ribosomes were preincubated with TpΨpCpGp oligonucleotide and, subsequently, the enzymatic binding of Phe-tRNA^{Phe} and fMet-tRNA$_F^{Met}$ and the nonenzymatic binding of Ac-Phe-tRNA^{Phe} were measured. From the enzymatic binding of fMet-tRNA$_F^{Met}$ and the nonenzymatic binding of Ac-Phe-tRNA^{Phe} to the ribosome it is known that these two tRNA species would bind to the ribosomal P-site, while the Phe-tRNA^{Phe} binding is only taking place at the ribosomal A site. As can be seen from Table I, the ribosomes that have been preincubated with TpΨpCpGp have lost their ability to bind enzymatically Phe-tRNA^{Phe}. In contrast, the binding of tRNAs to the ribosomal P site was not inhibited but in the case of fMet-tRNA$_F^{Met}$ a slight stimulation was observed. The inhibition of Phe-tRNA^{Phe} binding to the A site of the ribosome by TpΨpCpGp is a maximal inhibition that was achieved by using a 1000-fold excess of this oligonucleotide over 70S ribosomes. The dependence of the extent of inhibition upon the concentration of TpΨpCpGp is shown in Figure 3.

In order to investigate the role of the modified bases ribothymidine and pseudouridine in the tRNA binding to the ribosomes, inhibition experiments with unmodified UpUpCpGp were performed (Table I, Figure 3). This oligonucleotide was prepared enzymatically in two steps using polynucleotide phosphorylase (Figure 4) and purified by ion-exchange chromatography (Figure 5). In a similar experiment, as described for TpΨpCpGp, it was observed that also UpUpCpGp was able to inhibit the binding of Phe-tRNA^{Phe} to the ribosomal A site (Table I, Figure 3).

In order to show that the inhibition by TpΨpCpGp is specific and involves the binding of this oligonucleotide to a specific tRNA binding site on the ribosome, it was necessary to determine the stoichiometry of its binding. For this reason, [^{32}P]TpΨpCpGp was isolated from *E. coli* [^{32}P]tRNA (Figure 6) and incubated with 70S ribosomes. As is shown in Table II,

TABLE II: Stoichiometry of TpΨpCpGp and Phe-tRNA^{Phe} Binding to 70S Ribosomes.

		TΨpCpGp Binding		Phe-tRNA^{Phe} Binding	
Experiment	70S ribosomes (mol)	mol	mol/mol of 70S	mol	mol/mol of 70S
A	3.4×10^{-11}	0.0	0.0	6.8×10^{-12}	0.200
B	3.4×10^{-11}	3.3×10^{-12}	0.097	3.2×10^{-12}	0.094

[a] Experiment A shows the maximal enzymatic binding of Phe-tRNA^{Phe} to 70S ribosomes. Experiment B is similar to A except that the 70S ribosomes had previously been incubated with a 200-fold molar excess of [^{32}P]TpΨpCpGp. That the radioactive [^{32}P]TpΨpCpGp preparation inhibits equally as well aminoacyl-tRNA as other preparations of TpΨpCpGp or UpUpCpGp is illustrated in Figure 3. For other experimental details see Materials and Methods.

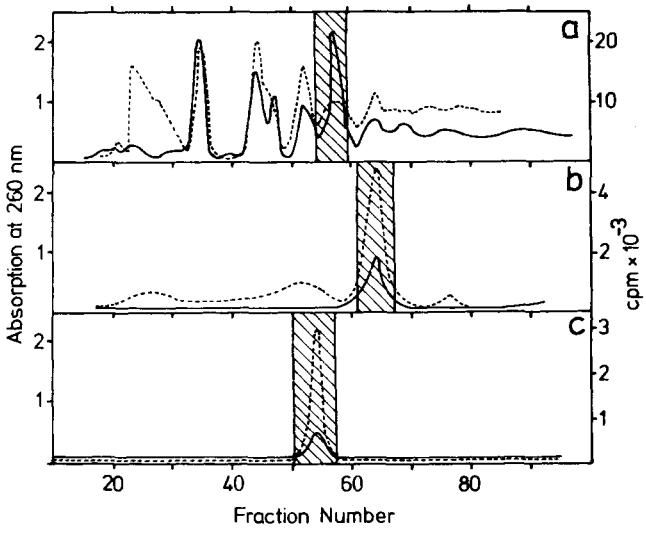

FIGURE 6: Isolation of [^{32}P]TpΨpCpGp from *E. coli* tRNA. T_1-ribonuclease digest of [^{32}P]tRNA (see Methods) was applied onto a DEAE-cellulose column (50 × 1 cm) equilibrated with 0.01 M NaCl in 7 M urea at pH 7.0. The column was washed with 50 ml of starting buffer and then with a linear gradient (2 × 450 ml) of 0.01–0.3 M NaCl in 7 M urea at pH 7.0. Fractions of 10 ml were collected. Radioactivity was determined in 2-μl aliquots (a). Fractions 54–59 (hatch marks) were pooled, diluted with 20 ml of 7 M urea, pH adjusted to 3.5 by HCl and the solution was applied onto a column of DEAE-cellulose (50 × 1 cm) equilibrated with 0.01 M NaCl in 7.0 M urea at pH 3.5. The column was washed with 50 ml of starting buffer, then with 2 × 500 ml of NaCl gradient 0.01–0.2 M in 7 M urea, pH 3.5. Fractions of 10 ml were collected and the radioactivity estimated in 5-μl aliquots (b). Fractions 61–67 (hatching) were pooled and applied onto a third column of DEAE-cellulose (30 × 1 cm) equilibrated with 0.01 M NaCl in 7 M urea at pH 5.5. This column was run with a 2 × 400 ml gradient of NaCl, 0.01–0.3 M in 7 M urea at pH 5.5. Fractions of 8 ml were collected and radioactivity was determined in 10-μl aliquots (c). Fractions 50–57 were pooled and worked up as described under Materials and Methods. (—) Uv absorption at 260 nm, (- - - -) radioactivity in cpm/aliquot.

this preparation of 70S ribosomes was able to bind only about 20% of Phe-tRNA^{Phe}. If the same 70S ribosomes were preincubated with about 200-fold excess of [^{32}P]TpΨpCpGp, 10% of 70S ribosomes bind this oligonucleotide. Subsequent enzymatic binding of Phe-tRNA^{Phe} showed that only 50% of the tRNA can bind to these 70S particles, compared to ribosomes that were not preincubated with TpΨpCpGp. Therefore, 50% inhibition of Phe-tRNA^{Phe} binding was achieved under these conditions. This experiment further shows that each molecule of TpΨpCpGp bound to ribosomes caused a reduction of Phe-tRNA^{Phe} binding by a corresponding stoichiometric amount.

In the experiments shown in Figure 3, the efficiency of the inhibition by three oligonucleotides, TpΨpCpGp, [^{32}P]-

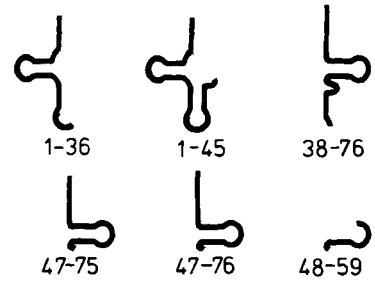

FIGURE 7: Fragments of tRNA^{Phe} from yeast used in the inhibition experiments.

TpΨpCpGp, and UpUpCpGp, each prepared by a different way, is demonstrated. There is no difference in the inhibitory effect between the oligonucleotides isolated from tRNA and containing modified bases and the oligonucleotide that was prepared by a synthetic method. Inhibition of the binding of Phe-tRNA^{Phe} to the A site by [^{32}P]TpΨpCpGp is the same as in the case of the two nonradioactive materials. This shows that it was justified to use this oligonucleotide prepared in a different way for the determination of the stoichiometry of the binding (Table II). Since in this case only a limited amount of radioactively labeled oligonucleotide was available, the maximal inhibition could not be achieved. An arbitrarily chosen tetranucleotide, GpApUpU, served as a control for these experiments and showed, as expected, no inhibition of the tRNA binding.

The experiments described here have shown that the TpΨpCpGp fragment of the tRNA binds to the ribosomes. Further, it was of interest to scan other regions of tRNA for their possible interaction with either the ribosomal A or P site. Therefore, different oligonucleotide fragments were isolated from yeast tRNA^{Phe}. As outlined in Figure 7, these fragments included such which contained the TpΨpCpGp as part of a larger sequence, as well as fragments originating from other parts of the tRNA molecule. In order to test the function of the terminal adenosine, which is common for all tRNA species, for the binding to the ribosomal sites, fragments were prepared that differ only in the presence or absence of the 3′-terminal A_{76}. The large-scale preparation of the fragments was performed according to known methods (Thiebe and Zachau, 1971; Simsek et al., 1973). Cleavage of the phosphodiester bond was performed either chemically at the sites occupied by minor bases Y, or 7-methylguanosine, or by a limited pancreatic ribonuclease digestion. All fragments used in this study were uniform with respect to their chain length, as judged by gel gradient microelectrophoreses (Wolfrum et al., 1974). This method allows the separation of tRNA^{Phe} species that differ only by one nucleotide in chain length (Sprinzl et al., 1975) and

TABLE III: Inhibitory Effect of Different Yeast $tRNA^{Phe}$ Fragments upon the Enzymatic Binding of Phe-$tRNA^{Phe}$ and fMet-$tRNA_F^{Met}$ to Ribosomes.

$tRNA^{Phe}$ Fragment	fMet-$tRNA_F^{Met}$ Binding (%)	Phe-$tRNA^{Phe}$ Binding (%)
1-36	85	35
1-45	81	48
38-76	79	28
47-76	70	30
47-75	71	84
48-59	93	32

[a] Prior to the tRNA binding experiments the ribosomes were incubated with a 170-fold molar excess of tRNA fragment. Other experimental conditions as described under Materials and Methods.

TABLE IV: Stoichiometry of ApUpG and fMet-$tRNA_F^{Met}$ Binding to 70S Ribosomes in the Absence and Presence of TpΨpCpGp.

Experiment	Assay Supplemented with: TpΨpCpGp	fMet-$tRNA_F^{Met}$	Puromycin	Component Bound [mol/mol 70S]: ApUpG	fMet-$tRNA_F^{Met}$
A	−	−	−	0.15	−
B	−	+	−	2.98	0.90
C	+	+	−	0.86	1.00
D	+	+	+	0.53	0.40

[a] Experiments A-D contained 0.3 A_{260} unit of [^{14}C]ApUpG/assay. In experiments C and D 70S ribosomes were incubated with an 800-fold molar excess of TpΨpCpGp prior to fMet-$tRNA_F^{Met}$ binding. The release of fMet-$tRNA_F^{Met}$ bound to the ribosomal P site was measured by puromycin release, as illustrated in experiment D, which is otherwise identical with experiment C. Puromycin concentration in experiment D was 0.5 mM and the $MgCl_2$ concentration was 5 mM. For other experimental details see Materials and Methods.

is therefore a powerful method for the characterization of RNA fragments.

In the inhibition experiments these fragments were preincubated with 70S ribosomes and then the enzymatic binding of fMet-$tRNA_F^{Met}$ or the binding of Phe-$tRNA^{Phe}$ was measured (Table III). As expected, the tRNA fragments containing the TpΨpCpGp part of the molecule were inhibiting very efficiently the binding of Phe-$tRNA^{Phe}$ but not the binding of fMet-$tRNA_F^{Met}$. Surprisingly, the fragment 47-75 containing the TΨC loop (loop IV) but missing the 3′-terminal adenosine is not very effectively inhibiting the EF-Tu dependent Phe-$tRNA^{Phe}$ binding to the ribosomal A site. It was also surprising that the fragments 1-36 and 1-45, where the TpΨpCpGp sequence is absent but which contain the whole D loop (loop I) of the tRNA, are inhibiting the Phe-$tRNA^{Phe}$ binding. Similar to TpΨpCpGp or UpUpCpGp, the D-loop containing fragments do not inhibit the F-Met-$tRNA_F^{Met}$ binding to the ribosomal P site. As a matter of fact, we could not find any fragment of nonaminoacylated $tRNA^{Phe}$ that would influence the enzymatic binding of the initiator tRNA to the ribosome.

It is known that nonaminoacylated tRNAs are unable to inhibit the formation of a ternary complex between EF-Tu and GTP and aminoacyl-tRNA (Jerez et al., 1969). In agreement with this observation the fragments listed in Table III, as well as the oligonucleotides TpΨpCpGp and UpUpCpGp, did not inhibit the formation of a ternary complex between the Phe-$tRNA^{Phe}$ and EF-Tu and GTP (data not shown), and, therefore, it can be postulated that the inhibition of the Phe-$tRNA^{Phe}$ binding takes place on the ribosomal level, i.e., the fragments are bound directly to the 70S ribosomes.

Next the possibility was considered whether or not the binding of TpΨpCpGp to the ribosome influences the events occurring on the ribosomal P site. As is shown in Tables I and IV, the interaction of TpΨpCpGp with 70S ribosomes does stimulate significantly the binding of F-Met-$tRNA_F^{Met}$ to the ribosomal P site. In addition, several points are interesting to note concerning the ApUpG (initiator triplet) binding to the 70S ribosome. As can be seen in experiment A (Table IV), there is only small amount of binding of ApUpG to the ribosome in the absence of fMet-$tRNA_F^{Met}$, while the addition of fMet-$tRNA_F^{Met}$ leads to the binding of nearly 3 mol of ApUpG/70S ribosome. Although the addition of TpΨpCpGp to the 70S ribosome does not inhibit fMet-$tRNA_F^{Met}$ binding, it reduces significantly the ApUpG binding. Experiment C illustrates that in the presence of TpΨpCpGp only 1 mol of ApUpG is bound per 70S ribosome. Thus, 2 mol of ApUpG/ribosome were initially bound most likely unspecifically. To localize the remaining ApUpG on the 70S ribosome the 70S ribosome-TpΨpCpGp-fMet-$tRNA_F^{Met}$-ApUpG complex was treated with puromycin and the release of ApUpG and fMet-$tRNA_F^{Met}$ was followed by radioactivity. Experiment D in Table IV clearly shows that with the puromycin induced release of fMet-$tRNA_F^{Met}$ a proportional amount of ApUpG triplet is released, which strongly suggests that an ApUpG-fMet-$tRNA_F^{Met}$ interaction is taking place at the ribosomal P site. The puromycin reaction was carried out in the presence of 5 mM magnesium, a condition that is optimal for enzymatic binding of fMet-$tRNA_F^{Met}$ but not for the puromycin reaction, which requires 15-20 mM magnesium, and it explains why only 50% of fMet-$tRNA_F^{Met}$ was released by puromycin. It is also important to point out that the binding of puromycin to 70S or 50S ribosomes is not influenced by prebound TpΨpCpGp to the ribosomes (equilibrium dialysis experiments, data not shown).

Discussion

The results presented in this communication show that the TpΨpCpGp fragment of the tRNA, which contains an invariable part of the tRNA sequences (Barrell and Clark, 1974), binds with 1:1 stoichiometry to the *E. coli* ribosomes. This interaction affects the EF-Tu dependent binding of aminoacyl-tRNA to the A site, whereas the binding of tRNAs to the P site is not inhibited. It is established that the binding of uncharged tRNA to the ribosomal A site is required for the ribosome-dependent synthesis of pppGpp and ppGpp (Haseltine and Block, 1973; Richter, 1976). We showed previously that the TpΨpCpGp fragment is able to replace uncharged tRNA in this process and by doing so also induces the synthesis of pppGpp and ppGpp. It is therefore likely that the TpΨpCpGp binds to the same place as the tRNA, namely to the A site of the ribosomes. Since we also showed that the TpΨpCpGp fragment interacts with specific 5S RNA-protein complexes (Erdmann et al., 1973; Horne and Erdmann, 1972), it is suggestive that parts of the 5S RNA sequence interact with this oligonucleotide. The 5S RNA sequence most likely responsible for this interaction is the conserved GpApApC sequence (Brownlee and Sanger, 1967; Forget and Weissman,

1967), which should, therefore, be located at the ribosomal A site.

However, working with a very complex macromolecular system we are not able to exclude completely the possibility that the observed inhibitory effects and the observed induction of ppGpp and pppGpp synthesis are not caused by some long-range effects. The binding of the oligonucleotide to some other part of the ribosomes could in principle also influence the events on the ribosomal A site. On the other hand, taking into consideration all information about the specific interactions of the tRNA and its fragments with ribosomes or the ribosomal components (Erdmann et al., 1973), the latter interpretation does not seem plausible.

In our previous work (Erdmann et al., 1973) it could be shown by equilibrium dialysis experiments that not only the oligonucleotide containing the two minor bases ribothymidine and pseudouridine (TpΨpCpGp) but also UpUpCpGp is able to interact with 5S RNA, as long as it is complexed with its specific binding proteins. Therefore, the inhibition of the EF-Tu dependent binding of Phe-tRNA^{Phe} to the ribosome by UpUpCpGp was now investigated and an inhibition that was comparable with the inhibition caused by TpΨpCpGp was observed. From these results we have to conclude that the modification of tRNA leading to T and Ψ is not an absolutely essential prerequisite for ribosomal interaction. This finding is in agreement with the work of other authors (Johnson et al., 1970; Svensson et al., 1971; Marcu et al., 1973) who found that some tRNA species that are less modified in loop IV and contain uridine instead of ribothymidine are able to participate in the ribosomal elongation process. Results of Ofengand et al. (1974) permit a similar conclusion. These authors prepared tRNA^{Val} species from *E. coli* in which both the Ψ and T in loop IV were replaced by 5-fluorouridine. This tRNA could participate in ribosomal protein synthesis. It is therefore clear that the presence or absence of these modifications in loop IV of tRNA is not crucial and does not determine whether or not this tRNA can bind to the ribosomal A site. On the other hand, the limits of sensitivity of the in vitro assays cannot exclude the possibility that this modification increases the efficiency of the binding of the tRNA. This has been suggested in our previous work, which revealed that the binding of TpΨpCpGp to 5S RNA–protein complexes is three times higher than that of UpUpCpGp (Erdmann et al., 1973). More recently, we have obtained experimental evidence that points towards a possible specific function of T and Ψ in loop IV of tRNA, since we have found that only the tRNA fragment TpΨpCpGp and not UpUpCpGp can trigger the ribosome dependent synthesis of ppGpp and pppGpp (Erdmann et al., 1975).

It is also interesting to note, that the tRNA species possessing in loop IV a different sequence than GpTpΨpCp are taking part in specific processes, which do not involve the binding to the ribosomal A site. Such processes include participation of tRNA in eukaryotic protein synthesis initiation (Simsek et al., 1973, 1974; Piper and Clark, 1973, 1974), a tRNA involvement in bacterial cell wall synthesis (Roberts, 1972) and a function as a primer for RNA dependent DNA polymerase (Harada et al., 1975).

The observation described here that TpΨpCpGp inhibits the enzymatic binding of aminoacyl-tRNA to the ribosomal A site is in accordance with previous reports in which the nonenzymatic binding of tRNA to the ribosomal A site was measured (Ofengand and Henes, 1969; Shimizu et al., 1970). Ofengand and Henes (1969) observed that the interaction of TpΨpCpGp with 70S ribosomes is not an equilibrium process, which is in agreement with our experiments. Using a high excess of radioactive oligonucleotide we have never observed any higher binding of TpΨpCpGp as 0.6–0.8 mol/mol of the ribosome. As can be seen from Table II, 1 mol of TpΨpCpGp replaces 1 mol of aminoacyl-tRNA at the ribosomal A site. Thus, the binding is very specific. After the radioactive oligonucleotide is bound it forms a stable complex with the 50S subunit, which can be isolated by gel filtration. It is therefore implied that a conformational change is induced in the 50S ribosomal subunit after binding of this tRNA fragment.

Further, other large fragments of tRNA^{Phe} were investigated for possible interactions with the ribosomal A and/or P site. It was again found that the oligonucleotides that contain the TpΨpCpGp sequence inhibited only the binding of tRNA to the ribosomal A site. In addition, other fragments that did not contain the TpΨpCpGp sequence were also inhibitory (Table III). We have no evidence of exactly which part of the 5′ fragments of tRNA are interacting with the ribosome, although the data suggest strongly that the hU loop or its stem are of primary importance. Also, it cannot yet be concluded where on the 70S ribosome this part of the tRNA interacts. In this context it is of interest to note that recent photoaffinity labeling experiments with Val-tRNA^{Val} indicate that the 4-thiouridine residue of aminoacyl-tRNA is in proximity of 16S RNA when bound to the ribosomal A site (Schwartz et al., 1975).

The concentrations of the larger fragments required for inhibition are considerably lower than the concentration of the TpΨpCpGp or UpUpCpGp tetranucleotides. As was shown by NMR studies, the secondary structure of the TΨC stem in the 3′ fragments is identical with the secondary structure of this part in the intact tRNA (Lightfoot et al., 1973). It can therefore be expected that the conformation of TpΨpCpGp is more similar to the native conformation in the 3′ half-fragments as in the tetranucleotide TpΨpCpGp and, therefore, leads to a more efficient binding to the ribosomes.

The 3′ half of the tRNA^{Phe} molecule inhibited the binding to the ribosomal A site efficiently only when the terminal CpCpA sequence was intact. If the terminal adenosine is missing a lower inhibition was observed that is comparable with the inhibitory effect of the tetranucleotides TpΨpCpGp and UpUpCpGp at the same concentration (Figure 3). This may indicate that the ribosomal binding sites of tRNA do not act independently but influence each other. Binding of one region of the tRNA to the ribosome makes another ribosomal region available for interaction. Similar suggestion was made by Schwarz et al. (1974), who postulated that the interaction of the TpΨpCpGp with the ribosome is induced by matching of the codon to the anticodon. Based on this hypothesis, recently a dynamic model for tRNA–ribosome interaction has been proposed (Kurland et al., 1975).

As was shown by x-ray structural analysis (Kim et al., 1974; Robertus et al., 1974), chemical modifications (Rhodes, 1975), and NMR studies (Kan et al., 1974) of yeast tRNA^{Phe}, as well as by equilibrium dialysis of this tRNA with complementary oligonucleotides (Pongs et al., 1973), its TΨC loop (loop IV) is not on the surface of the molecule and is not available for interaction with other partners. Consequently, if the TpΨpCp sequence is required for binding of the tRNA to the ribosomes, a conformational change exposing this part of the molecule must occur. Whether or not this conformational change takes place during aminoacylation of tRNA, EF-Tu interaction, or binding of the aminoacyl-tRNA to the ribosome, is still a matter of discussion (Ofengand and Henes, 1969; Wong et al., 1973; Schwarz et al., 1974). Nevertheless, when the aminoacyl-tRNA is bound to the ribosomal A site it must be partially

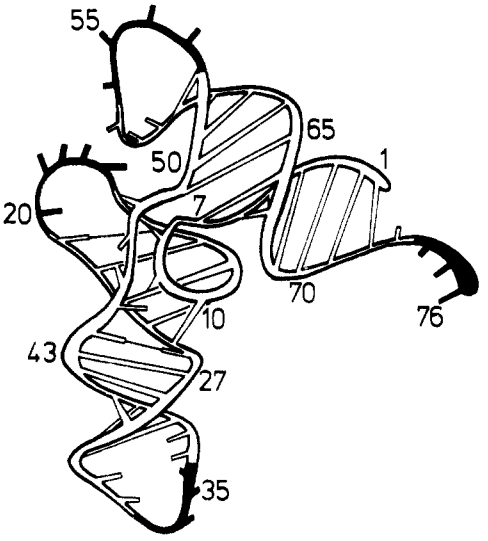

FIGURE 8: Suggested conformation of partially unfolded aminoacyl-tRNA required for binding to the ribosomal A site. This model was suggested to us by Professor F. Cramer. The conformational change involves breaking of the T54-m^1A58, G18-Ψ55, and G19-C56 interactions and unfolding of the TΨC and hU loops. Regions of tRNA which are suggested to interact with the ribosome are indicated by darkened areas in the model. The basic $tRNA^{Phe}$ model has been taken from Kim et al. (1974).

unfolded so that loop I and loop IV can interact with ribosomal components. A schematical model of a partially unfolded tRNA is shown in Figure 8. To achieve this conformational change the tertiary structure interactions G18-Ψ55 and G19-C56 are disrupted. Similarly also the T54-m^1A58 base pair is set free. In this way, interactions of the bases present in loop I and loop IV with its ribosomal partners are made possible. To which extent the other tertiary base pairs are affected during the binding of tRNA to the A site of ribosomes cannot be decided by a simple model study. As was suggested by Sigler (1975), the interactions between G15-C48, C13-G22-m^7G46, and A14-U8 probably persist during the functional cycle of tRNA.

Inhibition of the binding of tRNA to the ribosomal P site by the tRNA fragments studied in this work was not observed. Therefore, it is suggested that at the ribosomal P site only the codon–anticodon interaction (Clark et al., 1968) and the binding of the CpCpA end carrying the peptidyl residue (Monro et al., 1968) are important. On the other hand, the suggested binding of the TpΨpCpGp fragment to the A site affects the specificity of the codon–anticodon interaction at the P site. In the absence of TpΨpCpGp a nonspecific binding of two molecules of the initiator triplet ApUpG was observed. After binding of TpΨpCpGp to the A site, only 1 mol of ApUpG remains bound per mol of the ribosomes and the enzymatic binding of fMet-$tRNA_F^{Met}$ is stimulated. Thus, similarly, as the peptidyl-tRNA bound to the P site stimulates the enzymatic binding of aminoacyl-tRNA to the A site (Baksht and de Groot, 1974), also the binding to the P site may be stimulated by partial or total occupation of the A site.

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Isolation of a Transcriptionally Active Chromosome from Chloroplasts of *Euglena gracilis*†

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ABSTRACT: A transcriptionally active chromosome has been isolated in highly purified form from chloroplasts of *Euglena gracilis.* It contains chloroplast DNA, DNA-dependent RNA polymerase, and other proteins. Transcription occurs at low levels of endogenous DNA, and is indifferent to high levels of exogenous DNA. RNA chain elongation continues for several hours in vitro, and RNA chain initiation, determined by [γ-^{32}P]ATP incorporation, is continuous for at least 1 h in vitro. Maximal rates for RNA synthesis require only a divalent cation and the four ribonucleoside triphosphates. Apparent K_m values for adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, and uridine triphosphate are 4.0, 0.6, 2.5, and 2.3 μM, respectively. As would be expected for a DNA-dependent RNA polymerase, RNA synthesis is inhibited by actinomycin D. However, rifampicin and streptolydigin, inhibitors of procaryotic RNA synthesis, and α-amanitin, an inhibitor of eucaryotic nuclear RNA polymerases II and III, do not inhibit the RNA synthesis reaction. Heparin, which is a potent inhibitor of the initiation of RNA synthesis by a nontemplate bound RNA polymerase, also does not inhibit RNA synthesis. Isolation of transcriptionally active chromosomes should prove to be a useful method to study the mechanism of selective RNA transcription of eucaryotic chromosomes.

An important approach to the study of the regulation of in vivo RNA transcription in eucaryotic cells involves attempts to reconstruct the transcription apparatus in vitro with the minimum appropriate components. This requires solubilization and purification of the RNA polymerases, relevant regulatory molecules, and the DNA template for reconstitution experiments. This approach has led to the discovery of multiple eucaryotic RNA polymerases with separate transcription functions and to the recognition of several distinct transcription systems in the cell nucleus (Roeder and Rutter, 1969; Roeder and Rutter, 1970).

In addition to the nuclear systems, independent genomes are segregated within other cellular organelles such as mitochondria and chloroplasts. DNA-dependent RNA polymerases have been described from mitochondria of *Neurospora* (Küntzel and Schäfer, 1971), *Saccharomyces* (Tsai et al., 1971; Wintersberger, 1970; Scragg, 1971), rat liver (Reid and Parsons, 1971), and *Xenopus* ovaries (Wu and David, 1972), and from chloroplasts of maize (Bottomley et al., 1971; Smith and Bogorad, 1974), wheat leaf (Polya and Jagendorf, 1971a,b) and *Euglena gracilis* (Hallick et al., 1973; Hallick and Rutter, 1973).

These enzymes have been characterized by their ability to transcribe heterologous and homologous DNAs; however,

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[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, ethylenedinitrilotetraacetic acid; ATP, adenosine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.