EFFECT OF THE PRESENCE OF A pCpCpCpA 3′-TERMINUS IN Phe-tRNA$^{Phe}_{yeast}$ ON THE INTERACTION WITH ELONGATION FACTORS AND WITH THE POLY U-RIBOSOME SYSTEM

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1. Introduction

Previous studies [1] have shown that breaking the phosphate backbone in the vicinity of the anticodon on Phe-tRNA$^{Phe}_{yeast}$ does not modify the reactivity of such a recombined molecule with elongation factor EFT, in the presence of GTP. Although such a ternary complex was able to bind to ribosomes, this binding was not functional since it did not depend upon the presence of synthetic messenger, poly U; furthermore, no polymerization of phenylalanine occurs (unpublished).

Our second tRNA model for detecting the selectivity of EFT_u was a Phe-tRNA$^{Phe}_{(pCpCpCpA)}$ with the 3′–OH terminal sequence lengthened by one cytosine moiety as compared to the universal pCpCpA ending of all known tRNA's. Such a species of tRNA was made available as follows [2]: tRNA nucleotidyl transferase isolated from yeast is able, in the sole presence of CTP, to complete the terminal sequence of tRNA$^{Phe}_{pX}$, tRNA$^{Phe}_{pXpC}$, and of tRNA$^{Phe}_{pXpCpC}$ to three units of CMP, giving tRNA$^{Phe}_{pXpCpCpC}$; the third CMP replacing the normal terminal AMP. The resulting tRNA^{Phe}, with a terminal pCpCpC sequence could accept a terminal AMP when incubated in the presence of ATP and of the enzyme, resulting in a new species of tRNA, fully active towards phenylalanyl-tRNA-ligase.

Obviously, such a molecule presents enormous advantages for the studies of interaction and recognition requirements between an aminoacyl-tRNA, EFT, and ribosomes. First this is a 'modified', active tRNA without any damage either to the integrity of the native tRNA chain (as opposed to the case of the recombined molecule mentioned above, for instance) nor to the chemical nature of any of the nucleotides. Secondly, the lengthening of the non-helical 3′–OH extremity by one nucleotide unit offers a powerful tool to investigate the function of the universal sequence of a charged tRNA, this region being ultimately close to the peptide link formation site during the protein synthesis process. Thirdly, use of such a tRNA in combination with the normal one permits the delineation of the flexibility of the aminoacyl-tRNA (or peptidyl-tRNA) relatively to the ribosome, and the distance restrictions between the amino acid (or nascent peptide) on the donor site and the incoming amino acid on the acceptor site. Preliminary results have already been presented [3].

2. Materials and methods

The origin of the following products: purified yeast tRNA^{Phe}; poly U; [^{3}H] or [^{14}C] phenylalanine; [^{14}C] or [^{32}P] GTP; nitrocellulose membranes; yeast phenylalanyl-tRNA ligase, is the same as previously indicated [1]. Preparation of Phe-tRNA; Phe-tRNA$_{(3'+5')}$; *N*-Ac-Phe-tRNA; factor EFT, as well as the techniques used for the assays of EFT–GTP–aminoacyl-tRNA complex formation, and binding of such complexes to the ribosome, have also been already described [1]. Filtration on Sephadex column [4] has also been used in this study to isolate EFT–GTP–Phe-tRNA ternary complexes. It should be emphasized that all the charged tRNA used during this

Table 1
Ternary complex formation assayed by nitrocellulose membrane filtration.

Additions	[γ-^{32}P] GTP retained on filter (binary EFT–GTP complex) (pmoles)	Ternary complex (pmoles)
No tRNA	7	–
+16.8 pmoles Phe-tRNA$_{(CCCA)}$	7	0
+33.6 pmoles Phe-tRNA$_{(CCCA)}$	6.1	< 1
+10.2 pmoles Phe-tRNA$_{(3'+5')}$	1.1	5.9

The incubation mixture (100 μl) contained: Buffer A (Tris, pH 7.5, 50 mM; KCl, 80 mM; NH_4Cl, 80 mM; $MgCl_2$, 10 mM); dithiothreitol, 5 mM; factor EFT, 25 μg; [γ-^{32}P] GTP, 390 pmoles. Incubation 10 min at 10° in the absence or in the presence of Phe-tRNA$_{(CCCA)}$ at indicated concentrations. Phe-tRNA$_{(3'+5')}$ was used as control. The mixtures were then filtered on nitrocellulose membranes, washed with cold buffer A, dried, and counted in a liquid scintillator.

study has been purified on a DEAE-cellulose column after acylation in order to completely eliminate ATP, phenylalanine, and enzymes.

Unfractionated *E.coli* tRNA was obtained from General Biochemicals (USA). This total tRNA was charged with phenylalanine by DEAE-cellulose fractions of *E.coli* extracts [5]. Charged tRNA was also purified by absorption on DEAE columns at pH 5; ATP, phenylalanine, and enzymes were eliminated by washing with 0.4 M NaCl in acetate buffer pH 5; Phe-tRNA was eluted with 1.5 M NaCl in the same buffer.

Purified tRNA$^{Phe}_{(CCCA)}$ was obtained as follows: The terminal AMP of a normal, purified tRNA^{Phe} was removed by periodate treatment followed by alkaline phosphatase action [6]. The pCpCpC sequence was then synthesized by incubation with nucleotidyl-tRNA-transferase, in the sole presence of CTP [2]; then the terminal AMP was added back by incubation with ATP. The pCpCpC and pCpCpCpA sequences thus obtained were identified by fingerprint analysis, according to Gangloff et al. [7]. Within experimental errors, the material appeared to be pure.

This tRNA$^{Phe}_{(CCCA)}$ was acylated with phenylalanine by purified yeast phenylalanyl-tRNA-ligase under the same conditions as those described above for normal tRNA^{Phe}.

Ribosomes were from freshly grown *E.coli* MRE 600, and prepared as described elsewhere [8], except that the three ribosome washings were performed with Tris buffer, pH 7.5, 10 mM containing 10 mM Mg acetate and 7 mM β-mercaptoethanol, and, successively, with: 60 mM NH_4 Cl, 1.5 M NH_4 Cl, and 50 mM NH_4 Cl. The DEAE-cellulose step was eliminated. The ribosomes were kept at –90° in the last washing buffer

Phenylalanine polymerization was performed by measuring radioactive material insoluble in TCA after incubation of a mixture containing: Tris buffer, purified radioactive acylated tRNA, washed ribosomes, EFT, and EFG. Detailed conditions are given under the experiments described in Results. When *N*-Ac-Phe-tRNA and/or factor IF were present, the ribosomes were preincubated with the initiation system before addition of EFT, Phe-tRNA, and EFG, as described by Lucas-Lenard and Lipmann [9].

Factors EFT and EFG were prepared at the same time, according to Ravel et al. [10]. Fractions of both factors from DEAE-cellulose column were pooled; this way very little contamination from one to the other can be observed.

3. Results

3.1. *Formation of the EFT–GTP–Phe-tRNA$_{(CCCA)}$ ternary complex*

Ternary complex formation was first assayed by the usual nitrocellulose membrane filtration technique. As shown in table 1, no disappearance of the binary complex EFT–[^{32}P] GTP, retained on the membrane, could be detected when Phe-tRNA$_{(CCCA)}$ was added to the incubation mixture in a concentration double that of the binary complex. Even with five times more Phe-tRNA$_{(CCCA)}$ than the EFT–GTP complex, there was scarcely more than 1 pmole of ternary

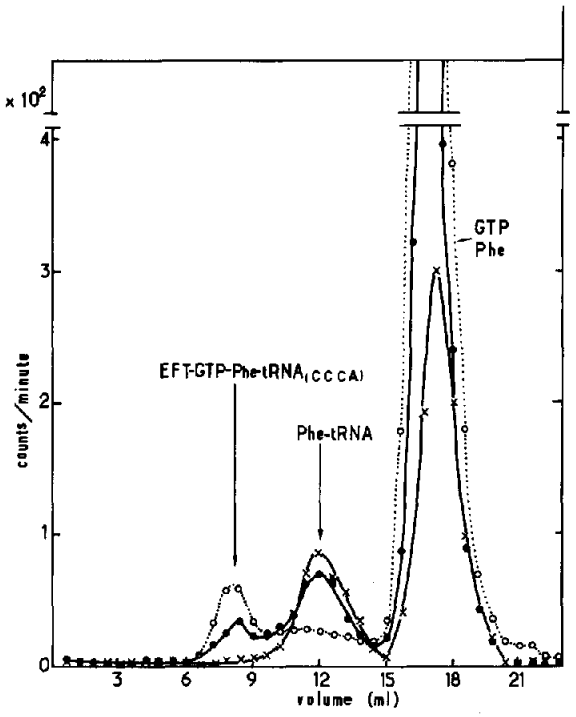

Fig. 1. Isolation of (T–GTP–Phe-tRNA$_{(CCCA)}$) ternary complex on a Sephadex G-100 column. The incubation medium (50 μl) contained: Tris, pH 7.5, 50 mM; KCl, 80 mM; NH_4Cl, 80 mM; $MgCl_2$, 10 mM; dithiothreitol, 5 mM; [γ-^{32}P] GTP, 45 pmoles; Phe-tRNA$^{Phe}_{(CCCA)}$ from yeast, 12 pmoles; factor EFT, 45 μg. The mixture was incubated at 30° for 15 min and cooled in ice. The ternary complex was separated from free Phe-tRNA and GTP by filtration on a Sephadex G-100 column equilibrated in a cacodylate buffer containing: cacodylate, pH 6.8, 10 mM; NH_4Cl, 160 mM; $MgCl_2$, 10 mM; dithiothreitol, 2 mM, and EDTA, 1 mM. Elution of the complex was performed by the cacodylate buffer at 4° with a flow rate of 1 ml/10 min. Fractions of 0.3 ml were collected. Aliquots were dried on glass filters and counted in a liquid scintillator for ^{32}P and ^{14}C. The ternary complex was localized by both [^{32}P] GTP (o···o) and [^{14}C] Phe-RNA$_{(CCCA)}$ (•–•–•) countings. Free Phe-tRNA$_{(CCCA)}$ location (x–x–x) was identified by control filtrations without either GTP or EFT.

complex formed. Under similar conditions, Phe-tRNA$_{(3'+5')}$, used as reference, in roughly equimolar concentration to that of the EFT–GTP complex, displaced most of the latter from the nitrocellulose membrane.

A double control of the ternary complex formation with Phe-tRNA$_{(CCCA)}$ was performed by the Sephadex column technique. Both Sephadex G-50 and Sephadex G-100 were used. As illustrated in fig.1 the ternary complex, EFT–GTP–Phe-tRNA$_{(CCCA)}$ could be detected, in significant amounts, albeit at a rather low efficiency.

These results tend to ascribe a structural change to the artificial Phe-tRNA carrying an additional C in its –CCA 3′-OH region, and this change is evidenced by the weak formation of the EFT–GTP–Phe-tRNA$_{(CCCA)}$ complex.

3.2. *Binding of tRNA–EFT–GTP to ribosomes*

Despite the low efficiency of the ternary complex formation, it was possible to obtain a good binding of the Phe-tRNA$_{(CCCA)}$ to the poly U-ribosome system. This binding is dependent upon poly U, EFT_u and GTP. As shown in fig.2a there is an absolute requirement for EFT at low Mg^{2+} concentrations. Nevertheless, the optimum Mg^{2+} concentration for enzymatic binding was 10 mM instead of 5 mM as for natural Phe-tRNA (fig.2b). On the other hand, the binding efficiency of the artificial Phe-tRNA is somewhat lower (around 55%); this efficiency, however, varied with the batch of tRNA used and decreased with the storage time of the charged tRNA$_{(CCCA)}$ much faster than deacylation of Phe-tRNA$_{(CCCA)}$ during the same length of time.

This observed lower efficiency was not due to an alteration, nor to the destruction of some components in the binding system by a contaminant present in the Phe-tRNA$_{(CCCA)}$ preparation. In fact, excess of any one of the components did not change the binding efficiency. The experiment was as follows: after 30 min incubation at 30° under standard conditions, duplicate samples were incubated another 30 min with the addition of either: poly U, ribosomes, poly U + + ribosomes, or EFT. The amount of Phe-tRNA$_{(CCCA)}$ bound was each time the same.

3.3. *Polymerization of phenylalanine from Phe-tRNA$_{(CCCA)}$*

The function of the Phe-tRNA$_{(CCCA)}$ bound on the ribosome was examined by the polymerization reaction in the presence of factor EFG. At optimum Mg^{2+} concentration (10 mM) for the binding of this tRNA species enhanced by EFT, no polymerization activity could be detected, whereas the presence of initiation factors and of *N*-Ac-Phe-tRNA promoted the poly-

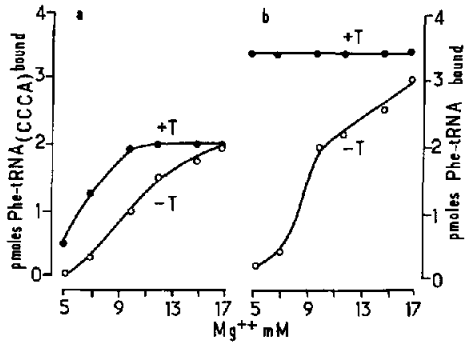

Fig. 2. Factor EFT and Mg^{2+} dependence for the binding of Phe-$tRNA_{(CCCA)}$ to ribosome. The reaction mixture (25 μl) contained: Buffer B (Tris, pH 7.6, 50 mM; KCl, 80 mM; NH_4Cl, 80 mM); GTP, 1 mM; dithiothreitol, 5 mM; Mg acetate, as indicated; poly U, 0.1 A_{260} units; ribosomes, 1 A_{260} units; EFT, 12 μg; [^{14}C] Phe-$tRNA_{(CCCA)}$, 3.5 pmoles (or normal [^{14}C] Phe-tRNA, 4 pmoles). The mixture was incubated at 30° for 30 min, then quickly diluted with 2 ml of cold buffer B containing 10 mM Mg^{2+} and filtrated on nitrocellulose membranes. The filters were washed twice with 5 ml of the same cold buffer, dried, and counted in a liquid scintillator.

merization (table 2). The degree of stimulation of the polymerization by the initiation system was, however, dependent on the Mg^{2+} concentration. Maximum polymerization was obtained around 5 mM and leveled off at 10 mM (fig.3). However, in the absence of initiation factors and of *N*-Ac-Phe-tRNA, polymerization was detectable between 3 to 5 mM Mg^{2+} (fig.3); the stimulation was thus only 2-fold at 5 mM Mg^{2+}. The fact that the optimum Mg^{2+} concentration for phenylalanine polymerization, in the absence of initiation factors and *N*-Ac-Phe-tRNA, is located in a very low range of molarity is not particular to the modified tRNA. It has also been observed with natural Phe-tRNA. This shift in Mg^{2+} concentration, and the efficiency of polymerization in the absence of initiator tRNA reflects the state of the ribosomes used. This, however, is not the purpose of the present communication and will be discussed elsewhere. In addition, the initial rate of polymerization was 3 to 4-fold higher in the presence of initiation factors and *N*-Ac-Phe-tRNA than in their absence, for all cases studied.

3.4. *Crossed system with natural and artificial Phe-tRNA*

Fig.3 shows that polyphenylalanine formation with

Table 2
Polyphenylalanine synthesis with Phe-$tRNA_{(CCCA)}$.

	PolyPhe formed* (pmoles)
+ EFG	< 0.05
+ IF + *N*-Ac-Phe-tRNA	0.92

* 4 pmoles Phe-tRNA input; 1.7 pmoles bound in the absence of EFG.
Identical samples of 25 μl containing the 'binding buffer' (Mg^{2+}, 10 mM) and 4 pmoles [^{14}C] Phe-$tRNA_{(CCCA)}$ were preincubated 20 min at 25°. At that time, 1.7 pmoles Phe-tRNA were bound to the ribosomes as assayed by the Millipore filter technique on a control sample. To one sample, 3.2 μg of factor EFG was added and incubation prolonged for another 10 min at 37°. For the effect of IF and *N*-Ac-Phe-tRNA on the polymerization, the *N*-Ac-Phe-tRNA (5 pmoles) was pre-attached on the ribosome in a medium (25 μl) containing: salt buffer; GTP, 1 mM; ribosomes, 1 A_{260} unit; poly U, 0.1 A_{260} unit; 20 min at 25°. Polymerization was then started by the addition of 4 pmoles Phe-$tRNA_{(CCCA)}$ 9 μg EFT, and 3.2 μg EFG and incubation resumed for 10 min at 37°. The reaction was stopped by addition of 4 ml of 5% trichloroacetic acid. The suspension was heated to 90° for 10 min. The hot TCA precipitate was filtered on nitrocellulose membranes and washed 5 times with 4 ml 1% TCA. The filters were dried, and counted in PPO-POPOP toluene scintillation liquid.

Phe-$tRNA_{(CCCA)}$ is enhanced by the presence of an initiator, *N*-Ac-Phe-tRNA; some enhancement was also observed when *N*-Ac-Phe-tRNA was replaced by *N*-Ac-Phe-$tRNA_{(CCCA)}$. On the other hand, the latter could also replace *N*-Ac-Phe-tRNA in the polymerization system with natural Phe-tRNA (table 3). Thus, all four combinations between Phe-tRNA and Phe-$tRNA_{(CCCA)}$, i.e. two homologous and two heterologous combined systems on the P and the A site, are functional. These results suggest that the distance between peptidyl–tRNA and aminoacyl-tRNA in the peptide bond formation site is not necessarily rigid.

4. Discussion

Numerous studies have shown that many unfunctional species of deacylated or acylated tRNA could not, in the presence of GTP, attach to EFT and could therefore not form a ternary complex (for detailed references, cf. [1]). Other modified tRNA's such as

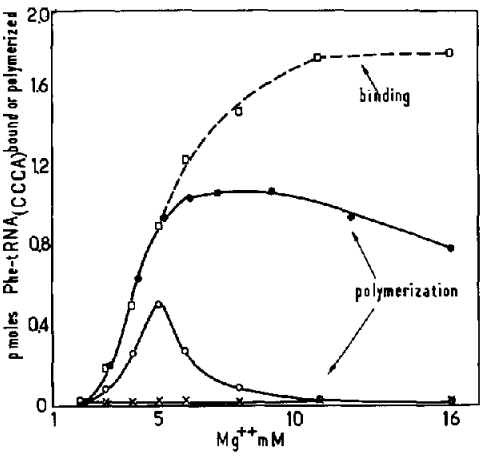

Fig. 3. Effect of Mg^{2+} and initiation factor on the polyphenylalanine synthesis with Phe-$tRNA_{(CCCA)}$. Same conditions as those described in table 2. Mg^{2+} concentrations as indicated.

a Val-tRNA with a crossed covalent linkage between $^4S_{(8)}$ and $C_{(13)}$ [11], or the phenyllactyl-tRNA [12] could, however form this ternary complex. These results were expected since both cross-linked Val-tRNA [13] and phenyllactyl-tRNA [14] were shown to be incorporated into a peptide chain containing the valine, or the deaminated phenylalanine with an ester linkage.

The failure of EFT to discriminate all unfunctional tRNA's was demonstrated by using the Phe-$tRNA_{(3'+5')}$ recombined molecule [1]. Evidence of formation of a ternary complex with a recombined molecule was confirmed by the study with Val-$tRNA_{(3'+5')}$ [11]. These results indicate: i) that formation of a ternary complex is not a sufficient criterion for selecting a functional aminoacyl-tRNA for protein synthesis, since EFT_u–GTP–Phe-$tRNA_{(3'+5')}$ was bound to the ribosomes whether the messenger was present or not [1] but without any polymerization being observed; ii) while it has been proposed [15,16] that a correct site for aminoacyl-tRNA entrance on the ribosome was induced through the interaction with elongation factors or initiation factors, the conformation of the aminoacyl-tRNA carried on the EFT–GTP–aminoacyl-tRNA complex also plays an active role in the positioning of the tRNA on the ribosomal site.

With the modified tRNA species, studied here, the

Table 3
Effect of the replacement of *N*-Ac-Phe-tRNA by *N*-Ac-Phe-$tRNA_{(CCCA)}$ as initiator tRNA for the synthesis of polyphenylalanine with Phe-tRNA.

	Polyphenylalanine formed (pmoles)
[^{14}C] Phe-tRNA + *N*-Ac-Phe-tRNA	1.8
Phe-tRNA + *N*-Ac-Phe-tRNA + IF	2.9
Phe-tRNA + *N*-Ac-Phe-$tRNA_{(CCCA)}$	1.9
Phe-tRNA + *N*-Ac-Phe-$tRNA_{(CCCA)}$ + IF	2.9

Pre-attachment of *N*-Ac-Phe-tRNA and of IF, and synthesis of polyphenylalanine were performed as described under table 2. Combinations of the crossed system between normal and artificial Phe-tRNA are illustrated by the following diagram:

			Phe	AcPhe	Phe	AcPhe	
AcPhe	Phe	AcPhe	A	A	A	A	Phe
A	A	A	C	C	C	C	A
C	C	C	C	C	C	C	C
C	C	C	C	C	C	C	C

Representation of the homologous and heterologous combinations on the ribosomes.

ability of Phe-$tRNA_{(CCCA)}$ to form the EFT–GTP–Phe-tRNA complex, to be bound to ribosomes, and to be polymerized into polyphenylalanine, demonstrates that this artificial tRNA can mimic the natural tRNA in these steps of the protein synthesis. However, this does not mean that the addition of one CMP in the –CCA 3'-OH region does not induce any structural changes, but that the change is not significant enough to be discriminated by the protein factors and the ribosomes necessary for the protein synthesis. In fact, the very weak formation of EFT–GTP–Phe-$tRNA_{(CCCA)}$, though significant, was the first indication of a structural change such that recognition of the tRNA molecule by EFT was disturbed; this results either from a modification of the affinity and therefore the stability, or from conversion of most of the tRNA population into molecules in a conformational state which is not recognized by the factor. The second indication of a structural change comes from the Mg^{2+}-dependence for the binding of the EFT–GTP–Phe-$tRNA_{(CCCA)}$ complex to ribosomes. The shift from 4–5 mM Mg^{2+}, optimum for the binding of natural Phe-tRNA, to 10 mM Mg^{2+}

for that of Phe-tRNA$_{(CCCA)}$ is demonstrative. It should be pointed out here that the amount of Phe-tRNA$_{(CCCA)}$ bound to ribosomes (fig. 2a) was much higher than the amount of ternary complex formed. This suggests that interaction with ribosomes stabilizes the ternary complex, or that ribosomes interaction with Phe-tRNA$_{(CCCA)}$ changed the latter to a conformation more favorable for factor EFT recognition. Quantitatively, it was hardly possible to bind more than 60% of the Phe-tRNA$_{(CCCA)}$ under various conditions, whereas binding of native Phe-tRNA was generally almost complete.

The binding properties of the EFT$_u$–GTP–Phe-tRNA$_{(CCCA)}$ underlines one fundamental aspect of the multiple interaction between EFT, aminoacyl-tRNA's and ribosomes, namely the fact that the aminoacyl-tRNA in the ternary complex plays an active role of its own for its positioning on the ribosome. A slight structural modification introduced at the 3′-OH end by the pCpCpCpA sequence of the artificial tRNA affects its binding ability, in agreement with the study using the non-functional EFT$_u$–GTP–Phe-tRNA$_{(3'+5')}$.

Finally, despite 50% efficiency of binding, formation of polyphenylalanine with Phe-tRNA$_{(CCCA)}$ was worse, even under optimal conditions, i.e. the presence of IF and of *N*-Ac-Phe-tRNA. The initial polymerization rate with Phe-tRNA$_{(CCCA)}$ was also much lower than that with natural Phe-tRNA (not shown). These results suggest that the structural change of the artificial Phe-tRNA, or the mis-adjustment on ribosomes of the altered ternary complex, markedly affects the translocation and/or the peptidyl transferase activity. However, the effect is to reduce, and not to destroy, the activity of peptide formation. This suggests that the additional length of one nucleotide unit in this non-helical region (according to the usual clover-leaf model) bearing the amino acid, does not have a drastic effect on the polyphenylalanine synthesis. Hence, this would indicate a flexibility of the aminoacyl-tRNA on the ribosome and a rather loose distal restriction on the peptide linkage formation sites. This idea is strengthened by experiments using crossed combinations between Phe-tRNA, *N*-Ac-Phe-tRNA, Phe-tRNA$_{(CCCA)}$, and *N*-Ac-Phe-tRNA$_{(CCCA)}$ (table 3 and fig.3). The heterogeneous system worked apparently as well as the homogeneous system.

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