RECOGNITION BY T FACTOR OF A $tRNA^{phe}_{yeast}$ MOLECULE RECOMBINED FROM 3′ AND 5′ HALVES; AND ITS NON MESSENGER-DEPENDENT BINDING TO RIBOSOMES

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1. Introduction

In bacterial systems, T factors [1] have been shown to be essential for the entrance of aminoacyl-tRNAs into ribosomes through an intermediate ternary complex: Tu–GTP–aminoacyl-tRNA [2–6]. The formation of this complex seems to be a highly specific control step for the translation process, since no formation takes place with many unfunctional forms of tRNA [5–9], such as: deacylated tRNAs, aminoacyl-tRNAs with the α-NH_2 group blocked or modified, or with the terminal ribose altered. Moreover, initiator tRNA from *E. coli*, Met-$tRNA_f$, cannot form the ternary complex [10]; this exception seems to enforce the idea that factor T is specific and can avoid one kind of translation ambiguity.

We are investigating whether tRNAs with the phosphate-carbone backbone interrupted at one or several points, but still acylable by the corresponding aminoacyl-tRNA-synthetase (see [11]), can or can not be attached to the ribosome at the correct site. The present communication deals with the interaction of a recombined molecule made of 3′- and 5′-halves of yeast $tRNA^{phe}$ [12] ($tRNA^{phe}_{(3'+5')}$), with factor T and the poly U-ribosome system.

2. Materials and methods

The origin of the products used is as follows: purified yeast $tRNA^{phe}$: Boehringer, Germany; poly U: Miles, USA; Nitrocellulose membranes: Millipore, USA; (^{3}H) or (^{14}C) phenylalanine, (^{14}C) or α-(^{32}P) GTP: CEA, Saclay, France; tetracycline chloride: Roussel UCLAF, France.

The 3′- and 5′-halves of $tRNA^{phe}$, prepared by the method of Philippsen et al. [12] were obtained as previously described [13]; peaks 1 and 3 were used.

Yeast phenylalanyl-synthetase, prepared according to Fasiolo et al. [14], was a gift from Dr. Ebel's laboratory.

(^{12}C) or (^{14}C)phe-tRNA was obtained by incubation in: Tris (pH 8.1), 50 mM; $MgCl_2$, 15 mM, ATP, 10.5 mM; (^{14}C) phenylalanine (specific activity: 235 mCi/mmole), 42 μM; $tRNA^{phe}$, 10 A_{260} units/ml; phenylalanyl-tRNA-synthetase, 3.3 μg/ml. The reaction was stopped after 20 min incubation at 25° by acidification to pH with acetic acid, and immediately frozen. The amino acid acceptor activity ranged from 1200 to 1400 pmole per 1 A_{260} unit.

(^{14}C)phe-$tRNA_{(3'+5')}$ was prepared in the same manner, except for $MgCl_2$ concentration which was 20 mM, and tRNA which was 20 A_{260} units/ml.

N-acetyl-(^{3}H)phe-tRNA was prepared according to Haenni and Chapeville [15].

T factor was prepared according to Ravel et al. [7], up to the DEAE-Sephadex chromatography step, included. No separation of Tu and Ts was attempted for this investigation.

Ribosomes were prepared from *E. coli* MRE 600, as described previously [16], except that the ribosomes were washed three times and not purified on DEAE-cellulose.

Formation of the Tu–GTP complex was assayed by its retention on nitrocellulose membrane [17], and that of the ternary complex, phe-tRNA–Tu–GTP, by the disappearance of the binary complex from the filter [6]. Binding of phe-tRNA to ribosomes was also assayed by the Millipore filtration technique [18].

3. Results

3.1. *Formation of phe-tRNA–T–GTP complex*

Formation of the ternary complex was followed by the filtration technique on nitrocellulose membranes. The T–GTP binary complex bound on the filter disappeared when increasing amounts of phe-$tRNA_{(3'+5')}$ were added to the reaction mixture (fig. 1). The use of identical concentrations of intact phe-tRNA or of recombined phe-tRNA resulted in similar decay of the GTP–T complex on the filter. With uncharged $tRNA^{phe}_{(3'+5')}$ or intact $tRNA^{phe}$, no formation of the ternary complex occurred, and a slight enhancement of T–GTP formation could even be observed at rather high concentrations, as compared to phe-tRNA. In the absence of T, no GTP was bound under these conditions.

The ability of the recombined phe-$tRNA_{(3'+5')}$ molecule to form the ternary complex indicates that factor T has a limited selectivity since it can choose an abnormal aminoacyl-tRNA. This immediately raises the question as to how the protein synthesis machinery excludes such an aminoacyl-tRNA in which the anticodon loop has been split off. Experiments on the interaction of such a ternary complex with the poly U-ribosome system tend to elucidate this point.

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

Under standard conditions for binding tRNA–T–GTP to ribosomes, the binding of phe-$tRNA_{(3'+5')}$

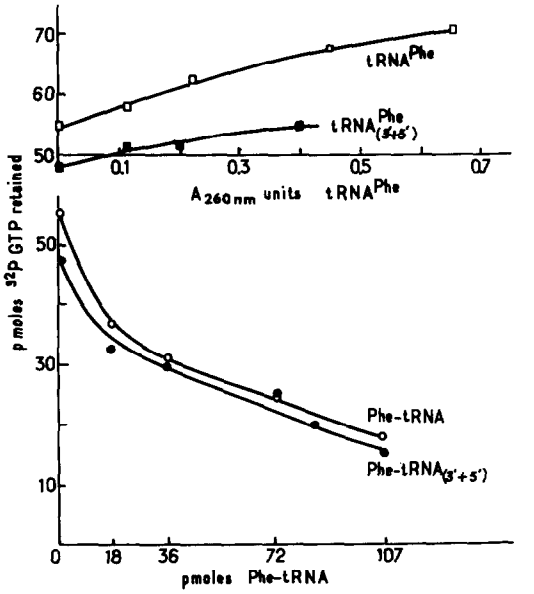

Fig. 1. Formation of phe-$tRNA_{(3'+5')}$–T–GTP complex. The incubation mixture (100 μl) contains; buffer A (Tris, pH 7.5, 50 mM; $MgCl_2$, 10 mM; KCl, 80 mM; NH_4Cl, 80 mM); dithiothreitol, 5 mM; (^{32}P)GTP, 5.25 μM; T factors, 18.5 μg protein; Phe-tRNA (or $tRNA^{phe}$), as indicated. The mixture was incubated 10 min at 10°, then immediately diluted by addition of 1 ml of buffer A, and filtrated on a pre-cooled nitrocellulose membrane. The filter was washed several times with cold buffer A, dried, and counted in a liquid scintillator system. Formation of the T–GTP binary complex had been checked and was proportional to the concentration of T factor.

Table 1
T factor dependency for the binding of recombined phe-$tRNA^{phe}_{(3'+5')}$.

Mg^{2+} (mM)	(^{14}C) phe-$tRNA^{phe}_{(3'+5')}$ bound (pmoles)			
	–T		+T	
	–poly U	+poly U	–poly U	+poly U
5	1.24 (< 1.0)*	1.11 (2.85)	5.46 (< 1.0)	6.13 (10.27)
10	1.33 (< 1.0)	1.81 (5.55)	5.01 (< 1.0)	5.88 (10.39)

Binding of phe-$tRNA_{(3'+5')}$ to ribosomes was assayed by the Millipore filter technique. The reaction mixture (50 μl) contained: Buffer B (Tris, pH 7.6, 50 mM; KCl, 80 mM; NH_4Cl, 80 mM); GTP, 1 mM; dithiothreitol, 10 mM; Mg acetate, as indicated; poly U, 0.4 A_{260} units, ribosomes, 3.6 A_{260} units; factor T, 8.1 μg protein; (^{14}C)phe-$tRNA_{(3'+5')}$, 10 pmoles (or intact (^{14}C)phe-tRNA, 10.4 pmoles). The mixture was incubated 15 min at 25°, then diluted rapidly with 2 ml of cold buffer B containing 10 mM Mg acetate, and filtrated on nitrocellulose membranes. The filter was washed twice with 5 ml of the same cold buffer, dried, and counted in a liquid scintillator.

* Figures in parentheses correspond to intact phe-tRNA under the same conditions.

Table 2
Influence of N-Ac-phe-tRNA and of phe-tRNA on the binding of phe-tRNA$_{(3'+5')}$.

Additions	(^{14}C)phe-tRNA$_{(3'+5')}$ bound (pmoles)
none	4.39
N-Ac-phe-tRNA	7.33
(^{12}C)phe-tRNA (M/M)	5.12
(^{12}C)phe-tRNA (M/2M)	6.09

Same conditions as for table 1. Mg^{2+}, 10 mM; N-Ac-phe-tRNA, 6 pmoles; (^{12}C)phe-tRNA, 10 and 20 pmoles.

to ribosomes was 4–5-fold enhanced in the presence of factor T (table 1); stimulation is the same whether poly U is present or not. Moreover, the stimulation is also the same whether in the presence of 5 or 10 mM Mg^{2+}. These two characteristics:independence from poly U, and non Mg^{2+} dependent binding, allow to differentiate the binding to ribosomes of an intact phe-tRNA from that of a phe-tRNA in which the anticodon loop has been split off.

To further specify the nature of such a binding, competition experiments were done with either *N*-acetyl-phenylalanine-tRNA or phe-tRNA. It is known that at 10 mM Mg^{2+}, under the same conditions used, N-Ac-phe-tRNA attaches to the peptidyl site [19]; a saturation curve of (^{3}H) labelled *N*-Ac-phe-tRNA binding to ribosomes enabled the determination of optimum binding conditions which were then used to perform the competition of (^{14}C)phe-tRNA$_{(3'+5')}$ binding to ribosomes by *N*-Ac-phe-tRNA.

Not only did the presence of *N*-Ac-phe-tRNA show no inhibitory effect whatsoever, but it even resulted in a marked stimulation (table 2). This indicates that phe-tRNA$_{(3'+5')}$ does not enter the peptidyl site. (^{12}C)phe-tRNA did not either inhibit the binding of the split molecule to ribosomes (table 2), and also resulted in a constant stimulation, which in turn indicates that phe-tRNA$_{(3'+5')}$does not enter the aminoacyl site. This last assumption is confirmed by the results obtained with tetracyclin which interacts with the aminoacyl site [20], thus inhibiting phe-tRNA binding. As a matter of fact, at low Mg^{2+} concentration and in the presence of T, tetracyclin, at 10^{-4} M has no effect on the binding of phe-tRNA$_{(3'+5')}$, whereas it inhibits 70% of the binding of intact phe-tRNA (table 3), as previously observed [21]

4. Discussion

The ability of the recombined phe-tRNA$_{(3'+5')}$ molecule to form a ternary complex with T and GTP demonstrates the failure of factor T to discriminate between functional aminoacyl-tRNA and all the non-functional ones during protein synthesis. This is the first direct example giving evidence of the limitation of factor T control upon the entrance of aminoacyl-tRNA into the ribosome. The formation of this ternary complex also suggests that the integrity of the codon-anticodon loop is not essential for the recognition of tRNA^{phe} by factor T, nor does the structural change modulated by the break of a phosphodiester bond in the anticodon region affect this recognition. Consequently, the recombined molecule is still able to assume a tridimensional structure, quite close to that of the original tRNA^{phe}, since it is recognized by three different protein components, i.e. phenylalanyl-tRNA-synthetase [12] polynucleotide phosphorylase [13], and factor T.

Table 3
Effect of tetreacyclin on the binding of phe-tRNA and phe-tRNA$_{(3'+5')}$.

		−T		+T	
		−poly U	+poly U	−poly U	+poly U
(^{14}C)phe-tRNA	−tetra	0.24	1.25	0.74	10.50
	+tetra	0.18	1.13	0.33	3.60
(^{14}C)phe-tRNA$_{(3'+5')}$	−tetra			5.29	6.35
	+tetra			3.46	6.73

Same conditions as for table 1. Mg^{2+}, 5 mM; tetracyclin, 0.4 mM. The values are given in pmoles bound.

The ability of phe-tRNA$_{(3'+5')}$ to bind to ribosomes reveals one aspect of the exclusion mechanism of unwanted aminoacyl-tRNAs from the proper site on the ribosome. The fact that this binding requires the presence of factor T, eliminates the possibility of a completely non-specific interaction between a nucleic acid molecule and the ribosome. Such a kind of interaction seems unlikely, because the non-enzymatic binding (in the absence of T) is very low, even at 10 mM Mg^{2+} (table 1). However, the attachment of phe-tRNA$_{(3'+5')}$ to ribosomes does not change, whether poly U is present or not in the reaction mixture. This indicates that, as might be expected, the codon-anticodon recognition is not involved in the binding of this abnormal phe-tRNA; such a binding is nonsense and proably not operational. Moreover, such an attachment is quite different from that of another modified yeast phe-tRNA in which only the Y base is excised, and which responds to poly UUC, or to poly U in the presence of high Mg^{2+} concentration or of streptomycin [22]. The lack of inhibition by *N*-Ac-phe-tRNA, phe-tRNA, or tetracyclin, suggests that phe-tRNA$_{(3'+5')}$ either does not occupy the peptidyl nor the aminoacyl site, or that it is not correctly adjusted to those sites. It has previously been shown that aminoacyl-tRNA, when misadjusted to its proper ribosomal site, did lose its reactivity towards streptomycin or puromycin [21, 23]. The interesting fact: *N*-Ac-phe-tRNA, and to a lesser degree phe-tRNA, stimulating the binding of this modified molecule to ribosomes, suggests that a structural change occurs in the ribosome when binding of aminoacyl-tRNA takes place.

We thus tentatively conclude that phe-tRNA$_{(3'+5')}$ can form a ternary complex, phe-tRNA$_{(3'+5')}$–T–GTP, which in turn can attach to ribosomes in a region where the binding of *N*–Ac–phe–tRNA and that of phe–tRNA does not interfere. Consequently, when, in the cell, a tRNA has its anticodon split by a nuclease, whether it is first charged and then cut, or vice-versa, it will not be eliminated by factor T; but it will not interfere with protein synthesis because it is not attached on the correct sites for polypeptide elongation.

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