Aminoacylation of tRNA gene transcripts is strongly affected by 3′-extended and dimeric substrate RNAs

Natalya Kholod[1,a], Natalya Pan'kova[a], Vladimir Ksenzenko[a], Lev Kisselev[b,*]

[a]*Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russia*
[b]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 117984, Russia*

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Abstract Kinetic parameters of aminoacylation by *E. coli* phenylalanyl-tRNA synthetase vary for phage T5 tRNA^{Phe} gene transcript from 0.950 to 2.545 μM for K_m and from 550 to 400 min^{-1} for k_{cat}. To reveal the source of this variability for various RNA preparations, homogeneity of the transcripts has been examined. Presence of 3′ extensions and dimer formation in transcript preparations reduced the catalytic efficiency k_{cat}/K_m several-fold. We have shown that the proportion of dimers and 3′-extended transcripts in tRNA preparations is sensitive to single-base substitutions in tRNA. While wild-type phage T5 tRNA^{Phe} gene transcript contains about half of dimeric molecules, for some mutants this value increases up to 90% or drops to 0%. Phage T5 tRNA^{Phe} gene with anticodon stem nucleotide substitutions used as a template in run-off transcription produces 5 times less 3′-extended molecules than the wild-type gene. In view of all these results kinetic parameters of aminoacylation reaction for many wild-type and mutant tRNA gene transcripts should be reevaluated.

Key words: tRNA gene transcript; tRNA dimer; Aminoacylation kinetics; Phage T5 tRNA^{Phe}

1. Introduction

A common approach to identify tRNA identity elements in vitro is based on measuring the aminoacylation rates of various tRNA derivatives by purified aminoacyl-tRNA synthetase (aaRS) (for reviews see [1–3]). The relative specificity of an aaRS for various tRNA substrates is compared using k_{cat}/K_m ratio. The tRNA gene transcripts being efficient substrates for most of the aaRS allow quantitative evaluation of the effects of base substitutions on tRNA recognition by aaRS. Decreased catalytic efficiency may indicate that an identity element has been changed by the given nucleotide substitution. However, impurities of tRNA transcripts and conditions for aminoacylation reaction may significantly alter the estimated values for the kinetic constants.

For aminoacylation assays, tRNA gene transcripts are usually activated in various buffers (see, for example [4–6]). Conditions for activation are often not indicated being considered unimportant for aminoacylation kinetics. However, activation of the transcripts may cause formation of dimers [7]. It is known that the current tRNA gene transcript purification utilizes denaturing 10–20% PAGE (see, for example [8,9]). However, presence of 3′-extended transcripts in the preparations obtained by this technique was also reported [7,10,11]. By this way inactive transcripts may not only lack the acceptor activity, but also compete with the normal-size transcripts for the aaRS in aminoacylation assay.

In view of the above considerations it seems appropriate to examine carefully a contribution of non-correct-size (3′-extended and dimeric) substrates to estimation of kinetic parameters of the aminoacylation reaction. Here, we have analyzed the influence of mutations in phage T5 encoded tRNA gene transcript on the yield of 3′ extensions and dimer formation after activation of transcripts and in parallel on the kinetic parameters of phenylalanylation reaction.

2. Materials and methods

2.1. Plasmids and in vitro transcription of tRNA genes

Construction of the plasmid pT5F0 containing phage T5 tRNA^{Phe} gene under the control of phage T7 promoter and introduction of nucleotide substitutions were described earlier [12]. Plasmid DNA for transcription was isolated using FlexiPrep kit (Pharmacia). In vitro transcription of *Bst*OI-digested plasmid DNA with T7 RNA polymerase was performed as described [12]. In some experiments 1 μCi of [α-^{32}P]ATP (2000 Ci/mmol, Obninsk, Russia) was added to a reaction mixture. T7 RNA polymerase was isolated as described [13]. Transcripts were analyzed by electrophoresis in denaturing PAG containing 8 M urea, in non-denaturing PAG (without urea) or in acidic PAG. Denaturing PAGE was carried out according to standard protocols using TBE running buffer [14]. Non-denaturing PAGE was performed at room temperature in Tris-borate buffer without EDTA. Typically, gels were 0.75 mm thick, and voltage was 10 V/cm that allowed to keep the temperature of the gel below 40°C. Electrophoresis in acidic PAG was performed as described for non-denaturing PAGE except that 50 mM sodium acetate, pH 5.48 was used as running buffer, and the voltage was 6 V/cm.

2.2. Purification of tRNA gene transcripts

tRNA gene transcript was purified either by denaturing 15% PAGE [13] or by affinity chromatography. Transcription mixture after completion of the reaction was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and ethanol-precipitated. A pellet was dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl and loaded on 0.1–0.5 ml DEAE-cellulose column (DE52, Whatman). Transcripts were eluted with the same buffer containing 1 M NaCl, ethanol-precipitated, dissolved, aminoacylated by *E. coli* FRS (see Section 2.3) and ethanol-precipitated from 2.5 M ammonium acetate. Binding of 2–4 μM Phe-tRNA^{Phe} transcript to *T. thermophilus* EF-Tu$_{His6}$ [15] was carried out in solution containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM NH_4Cl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM GTP, and ~0.75 mg/ml of EF-Tu*GTP for 5 min in ice. The ternary complex was loaded on 0.5 ml Ni^{2+}-NTA column (Qiagen) in the same buffer. Elution of Phe-tRNA^{Phe} was performed with the buffer containing 1 M NaCl and 100 μM GDP instead of GTP. Transcripts were ethanol-precipitated, deacylated in the buffer containing 100 mM Tris-HCl, pH 8.7, 5 mM $MgCl_2$ for

*Corresponding author. Fax: +7 (095) 135 1405.
E-mail: kissel@imb.imb.ac.ru

[1]Present address: Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 117984, Russia.

Abbreviations: aaRS, aminoacyl-tRNA synthetase(s), EC 6.1.1; FRS, phenylalanyl-tRNA synthetase; EF-Tu, elongation factor Tu; PAG(E), polyacrylamide gel (electrophoresis)

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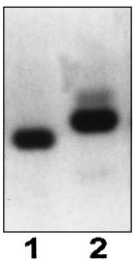

Fig. 1. Transcription of phage T5 tRNA^{Phe} (lane 2) and mutant tRNA^{Phe} (T28G, T29G, T41C, T42C) (lane 1) genes with T7 RNA polymerase. Autoradiograph of 6% denaturing PAG.

30 min at 37°C and desalted on Biogel P6 column (Bio-Rad). tRNA transcripts were stored frozen in water.

2.3. Aminoacylation kinetics

To obtain fully active transcripts heating at 90°C in water followed by immediate cooling on ice was used. Dimerization of transcripts was achieved by heating at 90°C in buffer A (30 mM HEPES-KOH, pH 7.45, 50 mM KCl and 0.3 mM $MgCl_2$) with slow cooling to 37°C. Homogeneous *E. coli* phenylalanyl-tRNA synthetase (FRS) was purified as described [12]. Aminoacylation reaction was run at 37°C in the buffer containing 30 mM HEPES, pH 7.45, 25 mM KCl, 4 mM $MgCl_2$, 4 mM dithiothreitol, 2 mM ATP, 10 μM L-[^{3}H]phenylalanine (126 Ci/mmol, Amersham) or L-[^{14}C]phenylalanine (225 mCi/mmol, Amersham), 0.1–2.0 μM transcripts and 0.5–1.0 nM *E. coli* FRS. Prior to FRS addition, the mixture was kept at 37°C for 2 min. Phenylalanine incorporation was measured as described earlier [12]. Initial rates for at least five tRNA concentrations were plotted using Eadie-Hofstee analysis. The kinetic parameters were calculated as an average of at least two independent determinations, k_{cat}/K_m were estimated with 10% error.

3. Results

3.1. Effect of nucleotide substitutions in the tRNA^{Phe} gene on synthesis of 3′-extended transcripts and dimer formation

One–two nucleotide extension was observed in the course of run-off transcription of various tRNA genes [7,10,11]. Quantification of the synthesized transcripts after their separation by denaturing PAGE revealed varying yields of extended products [7]. Here we found that certain nucleotide substitutions in the tRNA gene caused significant decrease of the ratio between the correct-size and one–two nucleotide 3′-extended transcripts. Reconstitution of the pairing in the anticodon stem of phage T5 tRNA^{Phe} mutant led to 5-fold decrease of the yield for the 3′-extended molecules (Fig. 1).

Dimerization of tRNAs and tRNA gene transcripts was often observed earlier (see [7,16] and references therein). An ability to form dimers was studied for various phage T5 tRNA^{Phe} mutant transcripts (Fig. 2). Dimer formation depended on nucleotide substitutions in the tRNA gene transcripts (Table 1). The wild-type tRNA^{Phe} gene transcript formed under the given conditions yielded about 50% of dimer molecules, whereas substitution of A20 with U resulted

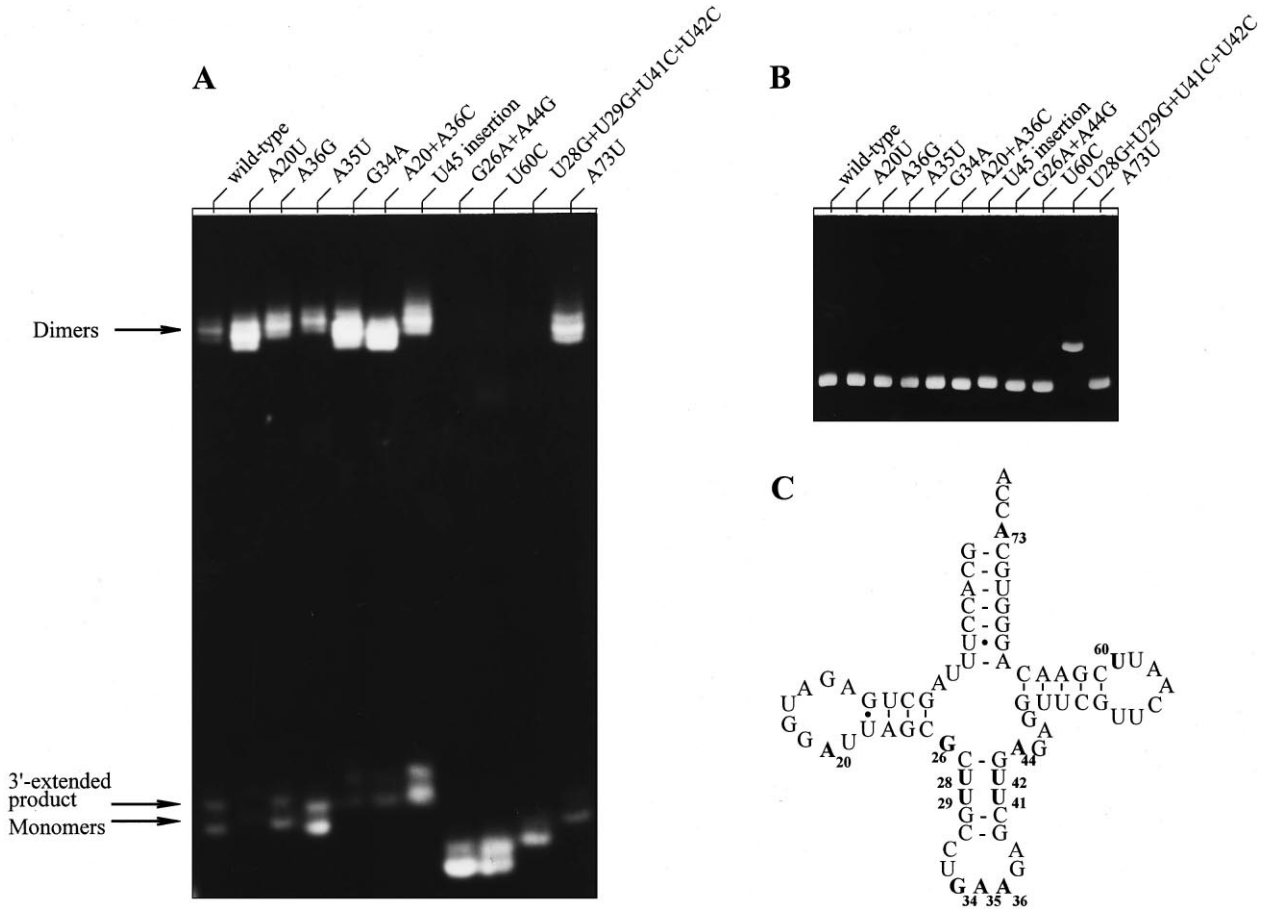

Fig. 2. Effect of nucleotide substitutions in phage T5 tRNA^{Phe} gene transcripts on dimer formation. Wild-type and mutant transcripts heated in buffer A were separated by 15% non-denaturing (A) and 15% denaturing PAGE (B). Cloverleaf presentation of bacteriophage T5 tRNA^{Phe} gene transcript (C) taken from [12].

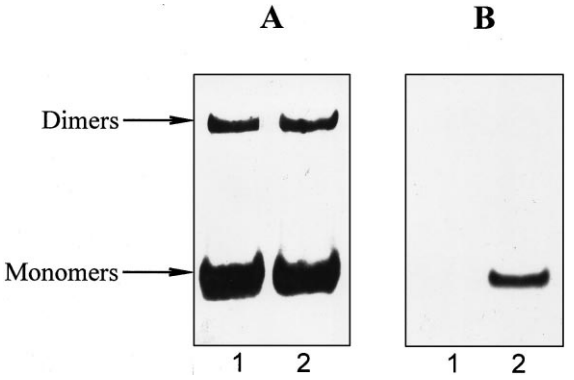

Fig. 3. Aminoacylation of phage T5 tRNA^{Phe} gene transcripts in conditions favoring dimer formation. A: The transcripts heated in buffer A (see Section 2) were aminoacylated with [^{14}C]phenylalanine by FRS and separated on 15% acidic PAG (lane 2). Separation of non-aminoacylated transcripts (lane 1). B: Autoradiograph of the polyacrylamide gel shown in A.

in more than 90% of dimers. In contrast, when pairing in the anticodon stem was restored by substitution of U28·U42 and U29·U41 pairs with G28·C42 and G29·C41 pairs, respectively, dimerization was completely abolished (Fig. 2, Table 1).

3.2. *Influence of 3′ extension and dimer formation on aminoacylation of tRNA gene transcripts*

It was evident from numerous early works that presence of intact CC-3′ terminal sequence in tRNA molecule was necessary for its aminoacylation [17]. However, preparations of transcripts purified by denaturing PAGE contained significant amounts of one–two nucleotide-extended products [7,10]. Here, we have studied the aminoacylation of phage T5 tRNA^{Phe} gene transcripts purified and activated under various conditions. It was shown that dimers retained their stability in the presence of FRS, but were not aminoacylated (Fig. 3). Consequently, since both incorrect-size 3′-extended products and dimers were not aminoacylated, the presence of these transcripts should significantly affect the kinetics of the aminoacylation reaction. This effect became apparent as 'lowering' of the plateau of aminoacylation (Fig. 4A). The maximal amount of phenylalanyl-tRNA^{Phe} (about 70%) formed at saturating level of the reaction correlated for various samples either with 30% of inactive 3′-extended transcripts or with 30% of dimers (not shown). Catalytic efficiency (k_{cat}/K_m) of

Table 1
Dimer formation by phage T5 tRNA^{Phe} gene transcript and its mutants

Transcripts	Dimers (%)
Wild-type	48
A20U	91
G34A	72
A73U	70
A20U+A36C	70
A36G	57
U45 insertion	51
A35U	41
U60C	12
G26A+A44G	0
U28G+U29G+U41C+U42C	0

The dimer formation was estimated after non-denaturing PAGE by scanning the films using Kodak 1D program. For details of dimerization conditions see Section 2.

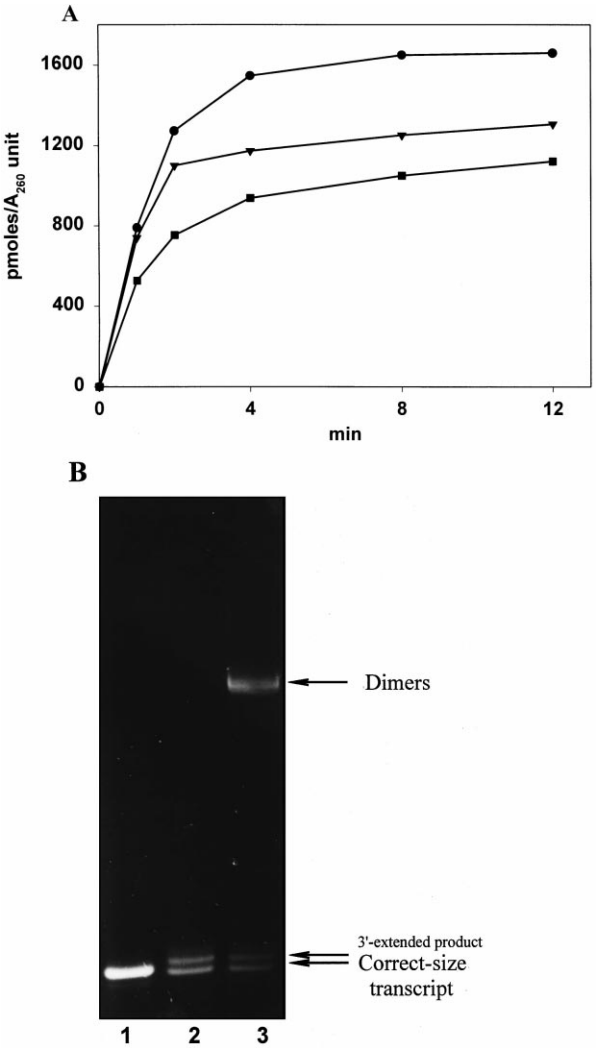

Fig. 4. Aminoacylation of phage T5 tRNA^{Phe} gene transcripts (A) and their subsequent analysis by 15% non-denaturing PAGE (B). The transcript isolated by EF-Tu chromatography and heated in water (circles in A; lane 1 in B), isolated by denaturing PAGE and heated in water (triangles in A; lane 2 in B) or heated in buffer A (squares in A; lane 3 in B).

the aminoacylation for transcripts with 3′ extensions after denaturing PAGE was 82% of the transcripts devoid of the 3′ extensions after purification by EF-Tu affinity chromatography (Table 2). Catalytic efficiency was only 27% for the same preparation activated under the conditions favoring dimer formation. In both cases the decrease of the catalytic efficiency was caused by increase of K_m and decrease of k_{cat}.

4. Discussion

Run-off transcription with T7 or other RNA polymerase using cloned individual tRNA genes proved to be a convenient tool for tRNA studies. The in vitro transcription system seems to be quite efficient in terms of the yield, but the fidelity of the synthesis is far from being optimal. Surprisingly, it was found from quantitative analysis of transcripts synthesized from various tRNA genes, that the amount of 3′-extended molecules (with one or two extra nucleotides) significantly depended on nucleotide substitutions in the DNA template.

Table 2
Kinetic parameters of aminoacylation with *E. coli* FRS for phage T5 $tRNA^{Phe}$ gene transcript

Sample preparation	Kinetic parameters of aminoacylation		
	K_m (μM)	k_{cat} (min^{-1})	Relative k_{cat}/K_m
EF-Tu purification followed by heating in			
H_2O	0.950	550	(1.00)
0.3 mM $MgCl_2$	*	*	ND
Denaturing PAGE purification followed by heating in			
H_2O	1.000	480	0.82
0.3 mM $MgCl_2$	2.545	400	0.27

*, Difficult to measure (see Section 3).
ND, not determined.

Subsequently, various mutant tRNA transcripts, if not carefully purified, contained varying amounts of inactive molecules (Fig. 1).

Heating and cooling is currently used to activate the tRNA gene transcripts for aminoacylation assays [4–6,18]. However, aggregation of tRNAs and tRNA gene transcripts was often observed during heating and cooling, preparative isolation and storage ([16] and references therein). Nevertheless, in most studies the activated transcripts were not tested for oligomerization. It is known that Mg^{2+} ions play an important role in tRNA gene transcript folding (for example see [19]) and various Mg^{2+} concentrations are often used for activation of the transcripts [4–6]. We observed that addition of Mg^{2+} to the buffer for activation even at low concentration (0.3 mM) enhanced dimerization. Only activation in water did not provoke RNA dimerization [7].

Surprisingly, the dimerization was extremely sensitive to the nucleotide sequence: even single nucleotide substitution in tRNA gene transcript changed the ability to form dimers (Fig. 2; Table 1). Consequently, various preparations of transcripts activated in the conditions favoring dimer formation could contain significant and varying amounts of dimeric molecules. An extreme case was observed for the A20U mutant of phage T5 $tRNA^{Phe}$ gene transcript where nearly 100% dimerization occurred (Fig. 2; Table 1). Obviously, this caused very poor aminoacylation of such preparation (data not shown). Inactive dimers in wild-type and mutant transcripts may cause confusion in identification of identity elements by measuring the aminoacylation capacity of 'activated' transcripts. For example, for phage T5 $tRNA^{Phe}$ the very poor aminoacylation ability for A20U mutant should provoke the conclusion that the nucleotide in position 20 is an identity element for this tRNA. In fact, as shown here, this effect was caused by high level of dimerization of the transcript molecules rather than alteration of the identity element, and when the A20U mutant was activated at the conditions preventing dimerization, it was aminoacylated with almost the same catalytic efficiency as the wild-type transcript [20].

Since tRNA gene transcripts containing the 3′ extensions are enzymatically non-chargeable, their occurrence in the sample should significantly influence the yield of aminoacylation reaction and kinetic parameters. In principle, for the population of homogeneous and active molecules at optical concentrations of all components, virtually all tRNA molecules in the reaction mixture could be aminoacylated. In fact, it was achieved for phage T5 $tRNA^{Phe}$ gene transcript purified using EF-Tu technique (Fig. 4). This result implies that only by applying proper methodology the homogeneous and fully active transcripts could be obtained for further physical or biochemical studies. Furthermore, after EF-Tu purification these transcripts do not require any further activation by denaturation/renaturation which as we have shown here provokes dimerization.

Dimerization of tRNA transcripts also affected the aminoacylation kinetics. It was very difficult to determine K_m and k_{cat} values when dimers of EF-Tu purified tRNA gene transcripts were present in the reaction mixture. For tRNA transcripts purified by denaturing PAGE and containing dimers, kinetic constants were determined (Table 2). Presence of the dimers, mostly 'heterodimers' formed by correct-size and extended transcripts (Fig. 2A) in tRNA sample resulted in significant increase of the K_m value, and the k_{cat} was also changed. Since the amounts of 3′-extended molecules and dimers depend on nucleotide substitutions in tRNA structure, the errors in estimating catalytic efficiencies of wild-type and mutant transcripts may be substantial and different.

In view of our data we suggest to test wild-type and mutant tRNA gene transcripts for homogeneity before aminoacylation assays. For mutant transcripts with reduced aminoacylation levels and lower catalytic efficiency evidence should be presented excluding the interference of dimers and 3′-extended transcripts with the estimated quantitative values.

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