Transfer RNA^{Phe} isoacceptors possess non-identical set of identity elements at high and low Mg^{2+} concentration

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Abstract Primary structures of phage T5- and *Escherichia coli*-encoded tRNA^{Phe} are distinct at four out of 11 positions known as identity elements for *E. coli* phenylalanyl-tRNA synthetase (FRS). In order to reveal structural requirements for FRS recognition, aminoacylation of wild-type phage T5 tRNA^{Phe} gene transcript and mutants containing substitutions of the identity elements at positions 20, 34, 35 and 36 was compared with *E. coli* tRNA^{Phe} gene transcript. The wild-type phage T5 transcript can be aminoacylated with the same catalytic efficiency as the *E. coli* counterpart. However, the maximal aminoacylation rate for T5 and *E. coli* transcripts was reached at different Mg^{2+} concentrations: 4 and 15 mM, respectively. Aminoacylation assays with tRNA^{Phe} mutants revealed that (i) phage transcripts with the substituted anticodon bases at positions 35 and 36 were efficient substrates for aminoacylation at 15 mM Mg^{2+} but not at optimal 4 mM Mg^{2+}; (ii) any change of G34 in phage transcripts dramatically decreased the aminoacylation efficiency at both 4 and 15 mM Mg^{2+} whereas G34A mutation in the *E. coli* transcript exhibits virtually no influence on aminoacylation rate at 15 mM Mg^{2+}; (iii) substitution of A20 with U in the phage transcript caused no significant change in the aminoacylation rate at both Mg^{2+} concentrations; (iv) phage transcripts with double substitutions A20U+A35C and A20U+A36C were very poor substrates for FRS. Collectively, the results indicate the non-identical mode of tRNA^{Phe} recognition by *E. coli* FRS at low and high Mg^{2+} concentrations. Probably, along with identity elements, the local tRNA conformation is essential for recognition by FRS.

Key words: Protein–nucleic acid recognition; Aminoacyl-tRNA synthetase; tRNA gene transcripts; tRNA identity elements, bacteriophage T5; tRNA conformation

1. Introduction

The correct selection and aminoacylation of a tRNA by its cognate aminoacyl-tRNA synthetase (aaRS) is crucial for the accuracy of protein synthesis. It is known that aaRS are able to recognize a small set of tRNA nucleotides defined as 'identity elements'. This concept is based on the results of numerous studies mainly using two approaches. The first strategy evaluates efficiency and specificity of nonsense suppressor tRNAs and their mutant forms (in vivo). The second strategy is based on the measurements of aminoacylation kinetics of tRNAs and tRNA gene transcripts and their mutants with purified aaRS (in vitro). By applying these two approaches the tRNA structural elements required for specific recognition by cognate aaRS mostly from *Escherichia coli* and yeast were identified (reviewed in [1,2]).

In bacteriophage T5 genome tRNAs specific for 20 amino acids are encoded and some even exhibit isoacceptors [3]. Nucleotide sequences of phage tRNA genes are known, most of which significantly deviate from the canonical cloverleaf folding and show low level of homology with their *E. coli* counterparts [4]. Since both phage T5 and *E. coli* tRNAs are parts of the same translation machinery, it is reasonable to consider them as isoacceptors. Consequently, phage T5 tRNAs could serve as unique models for a better understanding of the structure and functioning of tRNAs. In particular, some phage-encoded tRNAs contain certain nucleotides which differ from those identified earlier as identity elements for *E. coli* aaRS. The tRNA^{Phe} *E. coli*–phage pair (Fig. 1) seem to be among the most interesting in this respect.

The identity elements for the *E. coli* FRS in the *E. coli* tRNA^{Phe} encounter 11 and 10 nucleotides revealed by in vitro [6] and in vivo [7] strategies, respectively. The importance for aminoacylation of at least U20, G34, A35, A36 and U59 was confirmed by applying in vitro selection from randomized libraries [8]. Apart from dissimilarity of certain identity elements, phage T5 tRNA^{Phe} also has some other features in the primary structure (Fig. 1, compare A and B): e.g. unpaired bases in anticodon stem U28–U42 and U29–U41, changing of the conservative for *E. coli* tRNA nucleotides G21 by U, C48 by G and base pair R52–Y62 by C52–G62 [9].

Here, we present the data on in vitro aminoacylation of the phage T5 tRNA^{Phe} wild-type and mutant transcripts with purified *E. coli* FRS in comparison with aminoacylation of corresponding *E. coli* tRNA^{Phe} transcripts.

2. Materials and methods

2.1. Construction of plasmids harboring *tRNA*^{Phe} genes

Plasmid DNAs with the *E. coli* tRNA^{Phe} gene under control of the phage T7 promoter, both wild-type and containing substitutions in positions 34, 36 and 20 [6], were kindly provided by Prof. O. Uhlenbeck (University of Colorado, USA). Plasmid pT5F0 containing the phage T5 tRNA^{Phe} gene under T7 promoter was described earlier [10]. Nucleotide substitutions in the phage T5 tRNA^{Phe} gene were introduced by PCR-mediated mutagenesis as described [11] and confirmed by sequencing. In order to enhance the yield of transcription products

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Abbreviations: aaRS, aminoacyl-tRNA synthetase(s), E.C. 6.1.1; FRS, phenylalanyl-tRNA synthetase; Phe-tRNA^{Phe}, phenylalanyl-tRNA^{Phe}; EF–Tu, elongation factor Tu; PAGE, polyacrylamide gel electrophoresis

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with T7 RNA polymerase, additional substitutions (G3 for A and C70 for T) were introduced into all variants of the T5 $tRNA^{Phe}$ gene. Similar changes were made in the *E. coli* $tRNA^{Phe}$ gene, and it was shown that these substitutions did not influence the aminoacylation [6]. For reasons of simplicity, the G3–C70 $tRNA^{Phe}$ transcripts are referred here as 'wild-type'.

2.2. Synthesis and purification of tRNA gene transcripts

In vitro transcription of *Bst*NI-digested plasmid DNA with T7 RNA polymerase was performed as described [10]. Transcripts were purified by a two-step procedure. At the first purification step denaturing 15% PAGE was used [10]. To remove transcripts with additional non-template-directed nucleotides at the 3′ terminus, they were further purified by interaction with *Thermus thermophilus* $EF\text{–}Tu_{His6}$ as described [12]. After purification by PAGE, tRNA was aminoacylated by *E. coli* FRS in standard conditions (see below) and ethanol-precipitated from 2.5 M ammonium acetate. Binding of 2–4 μM Phe-$tRNA^{Phe}$ transcript to EF–Tu 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 a 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 30 min at 37°C and desalted on Biogel P6 column. When transcripts were poorly aminoacylated, purification was performed by non-denaturing 15% PAGE.

2.3. FRS and aminoacylation kinetics

E. coli FRS was purified as described [10]. The final enzyme preparation had a specific activity of 200 U/mg. Aminoacylation was carried out at 37°C in the buffer containing 30 mM HEPES, pH 7.45, 25 mM KCl, 2–15 mM $MgCl_2$, 4 mM dithiothreitol, 2 mM ATP, 10 μM L-[^{3}H]phenylalanine (126 Ci/mmol; Amersham), 0.1–2.0 μM transcripts and 0.5–1.0 nM *E. coli* FRS. For aminoacylation assay, transcripts were heated to 90°C in water, immediately placed in ice and then kept at 37°C for 2 min in the reaction buffer prior to FRS addition. Both *E. coli* and phage T5 transcripts could be aminoacylated by *E. coli* FRS to >1500 pmol phenylalanine/A_{260} unit of tRNA. Phenylalanine incorporation was measured as described [8]. Initial rates for at least five tRNA concentrations were plotted using Eadie-Hofstee analysis. The kinetic parameters represent the average of at least two separate determinations, and the values of k_{cat}/K_M can be considered to be within 10–15% of the indicated values.

Table 1
Kinetic parameters of aminoacylation with *E. coli* FRS for the *E. coli*- and phage T5-encoded $tRNA^{Phe}$ gene transcripts and their mutants at 4 and 15 mM Mg^{2+} concentrations

$tRNA^{Phe}$ (transcript)	$MgCl_2$ (mM)	K_M (μM)	k_{cat} (min^{-1})	Relative k_{cat}/K_M
E. coli				
Wild-type	4	0.855	140	0.27
	15	0.815	485	(1.00)
G34A[a]	15	0.850	340	0.67
U20A[a]	15	1.025	210	0.33
Phage T5				
Wild-type	4	0.950	550	0.97
	15	1.940	205	0.18
A20U	4	0.940	370	0.65
	15	0.795	225	0.47
A36U[a]	15	0.605	215	0.60
A36G[a]	15	0.685	210	0.52
A36C[a]	15	1.210	235	0.32
A35U[a]	15	0.690	140	0.33
A35C[a]	15	0.700	190	0.45

[a]Values at 4 mM $MgCl_2$ are not presented due to poor aminoacylation ability.

3. Results

Since Mg^{2+} concentration in the reaction mixture significantly affected the rate of aminoacylation [13], the optimal magnesium ion conditions for aminoacylation of phage T5 and *E. coli* $tRNA^{Phe}$ (transcripts) by *E. coli* FRS were determined. As shown in Fig. 2, the maximal aminoacylation rate for phage T5 and *E. coli* transcripts was reached at 4 and 15 mM Mg^{2+}, respectively. At the ionic conditions optimal for each transcript the catalytic efficiency of aminoacylation (k_{cat}/K_M) was similar (Table 1). Under the non-optimal ionic conditions (4 or 15 mM Mg^{2+}) these values were 4–5 times lower; in the case of *E. coli* transcript at the expense of the k_{cat} drop, while for phage transcript both K_M and k_{cat} were altered (Table 1).

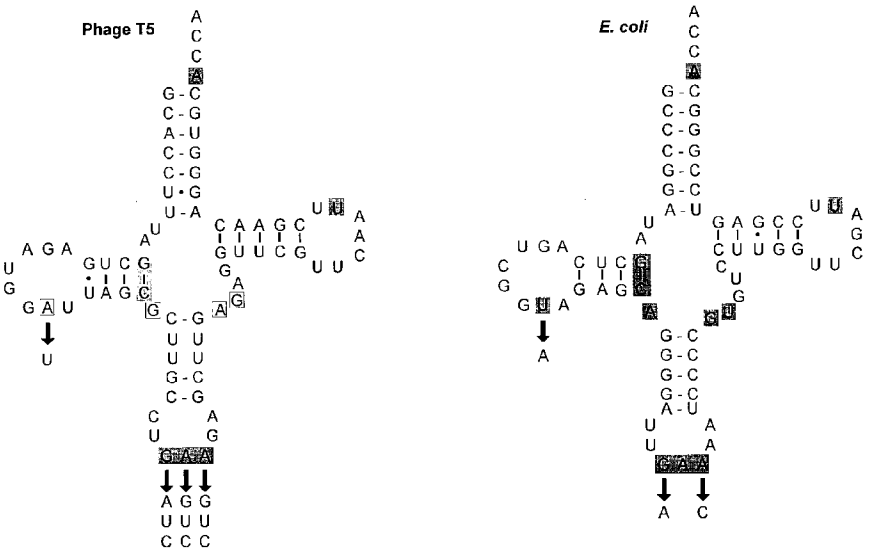

Fig. 1. Cloverleaf presentation of bacteriophage T5 [4] and *E. coli* [5] $tRNA^{Phe}$ (base modifications are not shown). The nucleotides identified as identity elements [6] are shaded in the *E. coli* tRNA structure. In phage tRNA, nucleotides are shaded if they coincide with identity elements and framed if they do not coincide with them. Arrows indicate the nucleotide substitutions performed in this work.

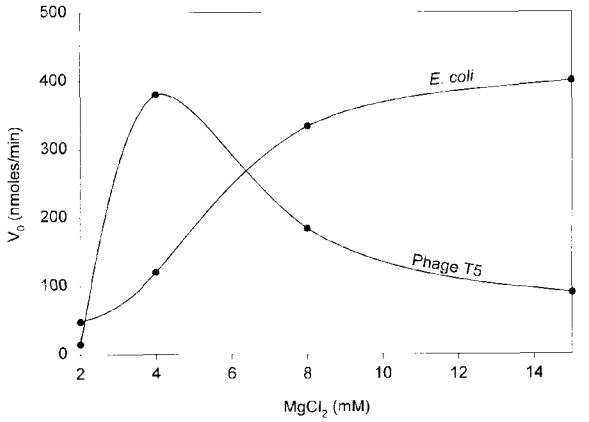

Fig. 2. Mg^{2+} dependence of initial rates of aminoacylation for the *E. coli* and phage T5 $tRNA^{Phe}$ gene transcripts.

The anticodon nucleotides G34, A35 and A36 and nucleotide U20 were identified as the main identity elements for *E. coli* FRS [6,8]. Therefore, we have constructed mutant phage T5 $tRNA^{Phe}$ genes with substitutions in each anticodon position by all three nucleotides from 'complementary set' (e.g., G34 with A, C and T) except for A35G substitution. In T5 $tRNA^{Phe}$, the position 20 is occupied by A (Fig. 1A). In the mutant gene we have substituted it by T present in the *E. coli* $tRNA^{Phe}$ gene (Fig. 1B). Since phage T5 and *E. coli* wild-type transcripts differed in Mg^{2+} optimum for aminoacylation (Fig. 2), we compared the catalytic efficiency of the mutant transcripts also at two Mg^{2+} concentrations, corresponding to aminoacylation optimum for both types of transcripts (Fig. 3).

As anticipated, all mutant phage transcripts with substitutions in the anticodon appeared to be very poor substrates for FRS at 4 mM Mg^{2+} (Fig. 3A, data were presented only for one mutant in each position). Surprisingly, substitution of A35 and A36 with all nucleotides from 'complementary set' enhanced efficiency of aminoacylation at 15 mM Mg^{2+} (Fig. 3B and Table 1). Values of k_{cat}/K_M differed insignificantly for various mutants ranging from 1.8 to 3.3 relative to the wild-type transcript in the same reaction conditions. In contrast to mutations localized in the positions 35 and 36, substitution of G34 by A, U and C dramatically decreased aminoacylation at 15 mM Mg^{2+}.

A20U substitution in the phage T5 transcript led to 1.5-fold reduction of catalytic efficiency at 4 mM Mg^{2+} and to its 3.6-fold increase at 15 mM Mg^{2+} (Fig. 3A,B and Table 1). This mutant appeared to be the only one among mutant phage transcripts, which had the same optimum of aminoacylation as the wild-type transcript.

Aminoacylation of the *E. coli* $tRNA^{Phe}$ anticodon and U20A mutants was also measured at different Mg^{2+} concentrations. G34A transcript was aminoacylated with nearly the same efficiency as the wild-type transcript at 15 mM Mg^{2+}, and was a poor substrate at 4 mM Mg^{2+}. Substitution of A36

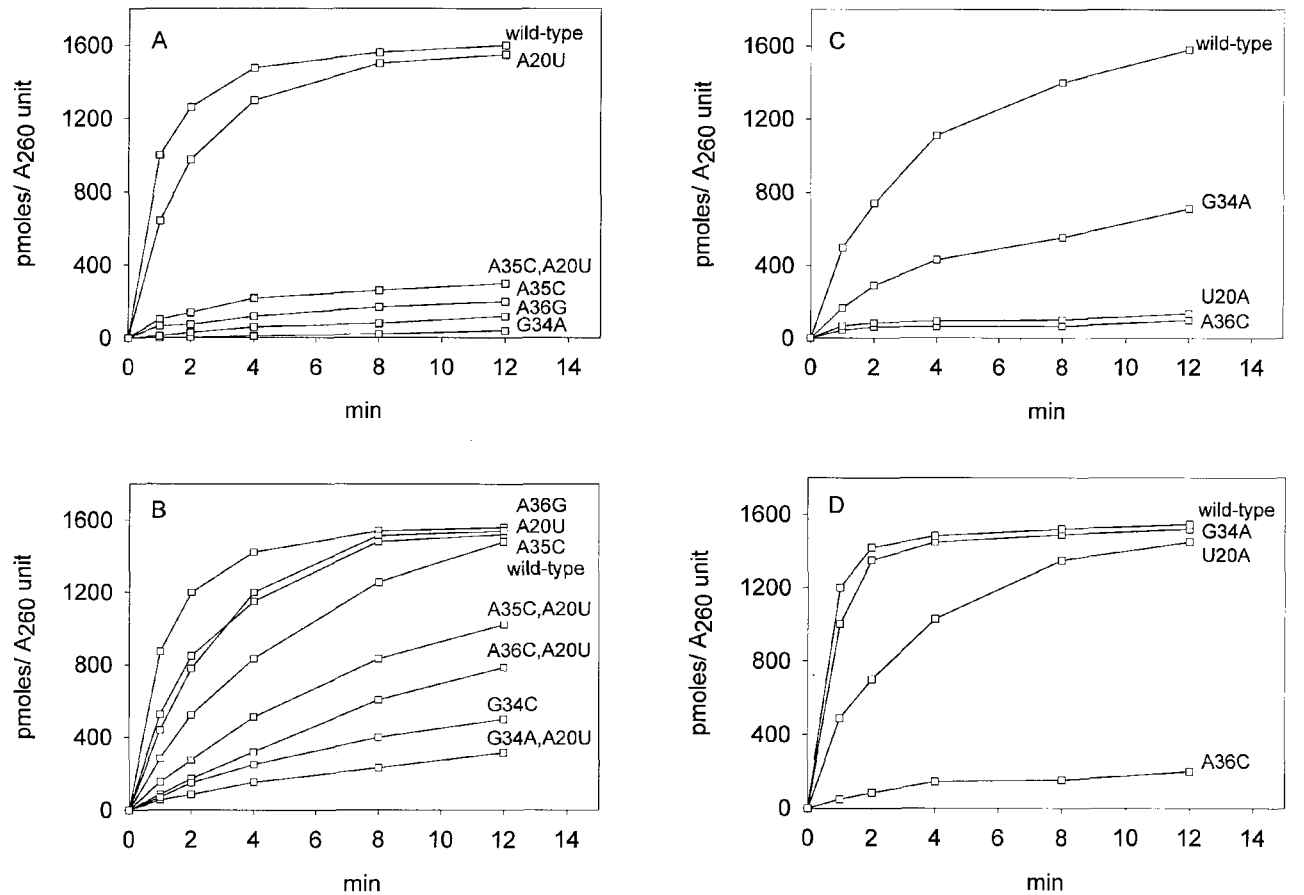

Fig. 3. Aminoacylation rates for the wild-type and mutant phage T5 transcripts at 4 mM Mg^{2+} (A) and 15 mM Mg^{2+} (B), and for *E. coli* transcripts at 4 mM Mg^{2+} (C) and 15 mM Mg^{2+} (D). FRS and tRNA concentrations were 0.6 nM and 0.44 μM, respectively.

with C led to loss of phenylalanylation by FRS at both Mg^{2+} concentrations. As for U20A transcript, at 15 mM Mg^{2+} its catalytic efficiency decreased only 3 times whereas at 4 mM Mg^{2+} it was completely inactive (Fig. 3C,D and Table 1).

In order to follow the effect of anticodon mutations in phage transcripts in comparison with the *E. coli* anticodon mutants, double mutations were introduced into T5 $tRNA^{Phe}$ gene (G34A and A20T; A35C and A20T, and A36C and A20T). All these transcripts were shown to be poor substrates for aminoacylation both at 4 and 15 mM Mg^{2+} (Fig. 3A,B).

4. Discussion

It was always assumed (though not thoroughly examined) that tRNA isoacceptors from one source exhibit the same set of identity elements. Comparison of primary structures of isoacceptors was considered as one of the direct approaches to narrow the set of identity elements for the given aaRS [14,15]. Identity elements for *E. coli* FRS were determined when sequence information for the only one isoacceptor from the *E. coli* $tRNA^{Phe}$-FRS system was available [6]. Later, sequence examination of phage T5-encoded $tRNA^{Phe}$, which can be considered as a natural isoacceptor of its *E. coli* counterpart, have revealed nucleotide substitutions in 4 out of 11 positions known as identity elements (Fig. 1). The data presented here demonstrated the ability of T5 $tRNA^{Phe}$ gene transcript to be efficiently phenylalanylated by *E. coli* FRS, provoking the suggestion that isoacceptors can possess different set of identity elements. Moreover, the set of identity elements for *E. coli* and phage T5 transcripts looks surprisingly different, depending on the ionic conditions in the reaction mixture. In this way, these two $tRNA^{Phe}$ isoacceptors with the same aminoacylation parameters have significantly different Mg^{2+} optimum (Fig. 2).

Our data on aminoacylation of phage T5 $tRNA^{Phe}$ gene transcripts containing substitutions in the positions 35 and 36 indicated that all these mutant transcripts can be phenylalanylated with *E. coli* FRS at 15 mM Mg^{2+} with higher catalytic efficiency than the wild-type transcript (Table 1). For *E. coli* $tRNA^{Phe}$ gene transcript, the G34A mutation did not significantly decrease the k_{cat}/K_M ratio at 15 mM Mg^{2+}. Therefore, if the testing of aminoacylation efficiency would be done only at 15 mM Mg^{2+} concentration one may arrive to the conclusion that anticodon bases are not essential as identity elements for the given aaRS. However, all three anticodon nucleotides were shown to be identity elements for cognate tRNA recognition by FRS from *E. coli* [6–8], *Thermus thermophilus* [16], human [17], yeast cytoplasm [18] and mitochondria [19], and it seems reasonable to consider the anticodon as an identity element for FRS conserved in evolution. Indeed, measurements of the catalytic constants at 4 mM Mg^{2+} for both *E. coli* and phage T5 transcripts containing substitutions of the anticodon nucleotides proved the importance of all three positions, 34, 35 and 36, for the specific recognition by cognate FRS.

It should be noted that in the studies on determination of the identity elements for different aaRS including *E. coli* FRS 'standard' incubation mixture containing high Mg^{2+} concentrations (10–20 mM) were most often used. However, the estimates of the free Mg^{2+} concentration in *E. coli* are from 0.3 to 2.0 mM [20,21], and higher Mg^{2+} concentrations are not required for the catalytic action of aaRS [22]. It was shown that Mg^{2+} strongly affected the coupling between two FRS active sites, and Phe, Mg·ATP and $tRNA^{Phe}$ dissociation constants for the different binding sites were significantly altered at high Mg^{2+} concentrations [23]. In addition, yeast FRS is susceptible for misacylation at high Mg^{2+} concentrations [13]. Therefore, it can be assumed that at non-physiological ionic conditions $tRNA^{Phe}$-FRS recognition is significantly changed in a way when tRNA conformation appears to be more important than a set of individual nucleotides.

Application of the in-vitro-transcript approach used in these studies for determination of identity elements presumes a similarity between the conformation of modified (mature) tRNA and corresponding tRNA gene transcript. It was shown that at high Mg^{2+} concentration the yeast $tRNA^{Phe}$ gene transcript folded normally, and the NMR spectral features of the transcript resembled those of mature $tRNA^{Phe}$ whereas at the low Mg^{2+} concentration the transcript did not adopt the native spatial structure [24]. Consequently, in the latter case the mature yeast $tRNA^{Phe}$ was much better substrate for FRS than the transcript [25]. Differences in the local conformation between modified (mature) and unmodified (transcript) *E. coli* $tRNA^{Phe}$ were also noticed [26]. However, at present no information is available on the minor nucleotides present in mature phage T5 $tRNA^{Phe}$. It should be mentioned that phage T5 specific tRNAs exhibit low content of modified nucleotides as follows from the sequence analysis of phage T5 tRNAs specific to Asn, Asp, Gln, Leu and Pro [5] and from the data on nucleotide composition of some other individual T5 tRNAs [27]. For example, phage T5 $tRNA^{Asp}$ and $tRNA^{Gln}$ do not contain modified nucleotides other than T and ψ [28,29]. Therefore, we anticipate a low content of minor nucleotides in the phage T5 $tRNA^{Phe}$ as well. Taking into account these observations along with our data concerning the ability of T5 $tRNA^{Phe}$ transcript to reach maximal rate of aminoacylation at low magnesium concentration, one may anticipate similar conformation of mature T5 $tRNA^{Phe}$ and the corresponding transcript under the physiological conditions in contrast to *E. coli* $tRNA^{Phe}$ which contains as much as 10 modified nucleotides [5]. Therefore, phage T5 $tRNA^{Phe}$ gene transcript can be considered as a proper substrate for determination of identity elements for the *E. coli* FRS.

While mutations in the anticodon of phage and *E. coli* transcripts at 4 mM Mg^{2+} affected similarly the aminoacylation rates, for mutations at the position 20 we met with an apparent discrepancy between the data obtained with two types of tRNA substrates. Substitution of U20 with A in the *E. coli* transcripts led to the vast deceleration of the aminoacylation rate whereas the wild-type phage T5 transcript, which contained A20, could be efficiently aminoacylated with the *E. coli* FRS. In addition, the 'inverse' A20U substitution in the phage T5 transcript decreased insignificantly the aminoacylation ability (Table 1). The nucleotide 20 was not supposed to interact with other nucleotides in tertiary structure, and therefore its substitution was not anticipated to alter the folding. However, replacement of U20 with A in the *E. coli* transcript was shown to reduce the rate of lead cleavage due to alteration of tRNA conformation [6]. Since A20 is present in the phage wild-type transcript, different conformation of phage and *E. coli* transcripts can be assumed. Consequently, not only individual nucleotides themselves but the local tRNA

conformation as well could contribute to the specific recognition of $tRNA^{Phe}$ by FRS in *E. coli*.

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