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Random-splitting of tRNA transcripts as an approach for studying tRNA–protein interactions

Ruslan Aphasizhev, Sergey Beresten, Vladimir Pugachev and Lev Kisselev

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 117984, Russian Federation

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Location of phosphodiester bonds essential for aminoacylation of bovine $tRNA^{Trp}$ was identified using a randomly cleaved transcript synthesized in vitro. It was found that cleavage of phosphodiester bonds after nucleotides in positions 21, 22, 36–38, 57–59, 62 and 64 were critical for aminoacylation capacity of $tRNA^{Trp}$-transcript. These cleavage sites were located in the regions of tRNA molecule protected by the cognate synthetase against chemical modification and in the regions presumably outside the contact area as well. These results indicate that for maintenance of aminoacylation ability the intactness of the certain regions of the tRNA backbone structure is necessary. Random splitting of non-modified RNA with alkali followed by separation of active and inactive molecules and identification of cleavage sites developed in this work may become a general approach for studying the role of RNA covalent structure in its interaction with proteins.

RNA-transcripts, Beef; Tryptophanyl-tRNA synthetase; Aminoacylation

1. INTRODUCTION

Highly specific interaction of tRNAs and cognate aminoacyl-tRNA synthetases (EC 6.1.1) is essential for the exact reproduction of genetically encoded protein primary structure (see [1]). In studying this process much attention was paid to the identification of nucleotides responsible for recognition of the cognate tRNA (identity elements) by an aminoacyl-tRNA synthetase and for discrimination from the other tRNAs (see [2]). X-ray analysis was applied to study the three-dimensional structure of the enzyme–tRNA complexes (see [3]). It was also shown that conformational changes occur in tRNA when it forms the complex with the aminoacyl-tRNA synthetase [4,5]. It may indicate that conformational tension is common for tRNA in the complex probably being important for the productive enzyme–substrate interaction.

We suggest that the tRNA transition into the 'stressed' state is directly related to the intactness of the covalent backbone structure of the molecule. In general, much effort was focused on the specific role of various bases in structure and function of tRNA whereas the role of the sugar–phosphate backbone in tRNA–protein interactions was not considered systematically.

The aim of this work is to identify in the tRNA molecule the phosphodiester bonds essential for aminoacylation ability. We have used tRNA transcripts lacking posttranscriptional modifications because after partial alkaline hydrolysis of non-modified tRNA it is possible to generate a statistically reliable set of cleavages of phosphodiester bonds (ladder). In the case of mature RNA it is difficult due to the protection of some phosphodiester bonds by minor nucleosides and by modification of ribose residues. As a model we have used the b-$tRNA^{Trp}$-transcript having kinetic parameters of aminoacylation reaction similar to those of the mature $tRNA^{Trp}$ (not shown).

Correspondence address: R. Aphasizhev, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 117984, Russian Federation. Fax: (7) (095) 135 1405.

Abbreviations: b (prefix), bovine; QRS, glutaminyl-tRNA synthetase; WRS, tryptophanyl-tRNA synthetase.

2. MATERIALS AND METHODS

2.1. *General*

WRS (specific activity 17,000 units/mg) was isolated from beef pancreas as described earlier [6]. T7 RNA polymerase was prepared from *E coli* strain TG1 containing plasmid pAR 1219 as described [7]. Oligonucleotides were synthesized by solid-phase phosphoramidite method using a Beckman system I plus DNA synthesizer. Calf intestinal phosphatase, T4 polynucleotide kinase, RNAse T1 and [γ-^{32}P] ATP (specific activity 3,000 Ci/mmol) were obtained commercially.

2.2. *tRNA gene construction*

Three oligonucleotides were synthesized: (i) the 28-mer oligo corresponding to the promoter sequence for T7 RNA polymerase containing *Bam*HI site at the 3′ end; (ii) the 90-mer oligo including the b-$tRNA^{Trp}$ sequence and *Bst*NI site at the 5′ end; (iii) the 44-mer oligo complementary to 22 nucleotides of the oligonucleotides (i) and (ii). After the filling-in of the obtained duplex using Klenow fragment it was cloned in pUC19 plasmid.

2.3. *In vitro transcription*

Unmodified $tRNA^{Trp}$ transcripts were synthesized with T7 RNA polymerase from synthetically constructed gene. They were purified by electrophoresis on 14% polyacrylamide gel and eluted [8]. Although

the transcripts contain 5′-triphosphate they are aminoacylated to specific activity of 1,125 pmol/A_{260}.

2.4. *End labelling of tRNA*

Transfer RNA was dephosphorylated with phosphatase and rephosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase [9] The labelled transcript was purified on a 14% polyacrylamide/8 M urea gel to single nucleotide resolution. tRNA of the correct length (75 nucleotides) was excised and eluted with 50 mM Tris (pH 7.0).

2.5. *Preparative aminoacylation of the tRNA^{Trp} transcript*

The labelled transcript (10^6 cpm) was partially hydrolyzed with 25 mM NaOH at 65°C for 30 s and ethanol precipitated. tRNA samples were dissolved in 10 μl of 15 mM $MgCl_2$, heated to 60°C and slowly cooled to 25°C prior to addition of the reaction mixture. Aminoacylation reaction was performed in a 100 μl mixture containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 2.5 mM ATP, 0.4 mM L-Trp, 1 nM WRS and 0.2 mM of 5′-labelled tRNA^{Trp}-transcript at 25°C for 5 min.

2 6. *Separation of aminoacylated and uncharged tRNAs*

Tryptophanyl-tRNA and uncharged tRNA were separated by microcolumn chromatography on benzoylated DEAE-cellulose according to [10] and analyzed on 14% sequencing gel. After autoradiography, the position of nucleotides was determined by comparison of the alkaline cleavage pattern with that derived from tRNA digestion with RNAse T1.

2.7. *Binding studies using gel retardation analysis*

Assays for partially hydrolysed tRNA. WRS binding as performed in 50 mM Tris (pH 7.6), 15 mM $MgCl_2$, 25 mM KCl, 5 mM DTT, 10 μM radiolabelled tRNA-transcripts (3–6 10^5 cpm) and 10 μM WRS After incubation at 4°C for 10 min, glycerol was added to a final concentration of 20% and then the reaction mixture was loaded onto a pre-electrophoresed 8% non-denaturing polyacrylamide gel Electrophoresis was run at 10 mA for 12 h at 4°C. The RNA band was excised and eluted as described [8] Then it was analysed on a 14% sequencing gel or by radioactivity measurement (Cerencov counting)

3. RESULTS AND DISCUSSION

As seen from Fig. 1 (lanes 1 and 2) all phosphodiester bonds in the tRNA-transcript are hydrolysable at almost equal rates except for more labile YpA sequences (positions 13, 20, 35, 40 and 61) as was reported earlier for some tRNAs [11]. The partially cleaved tRNA^{Trp} transcripts were aminoacylated and fractionated on BD-cellulose. Inactive (non-acylated) tRNA was eluted first and tryptophanyl-tRNA was recovered in the second peak (not shown). We found that numerous cleavages of backbone structure do not interfere with the fractionation procedure developed earlier [10] for intact tRNA molecules. For tryptophanyl-tRNA no cleavages were observed in positions 21, 22, 36, 37, 38, 57–59, 62 and 64: the degree of splitting is relatively weak for positions 41–46, 67–71 and relatively strong for positions 27, 63 and 65 (Fig. 1, lane 6). In Fig. 2 these positions are marked in secondary (A) and tertiary structures (B). No meaningful difference was recorded for the other positions.

Identity elements are partially known for tRNA^{Trp} of higher eukaryotes from structural mapping with RNases and chemical modifications [10,12]. Our results indicate that the cleavages preventing aminoacylation are located not only in the region protected by WRS (anticodon loop and acceptor stem) [13], but also in the regions presumably not interacting directly with the enzyme (Fig. 2).

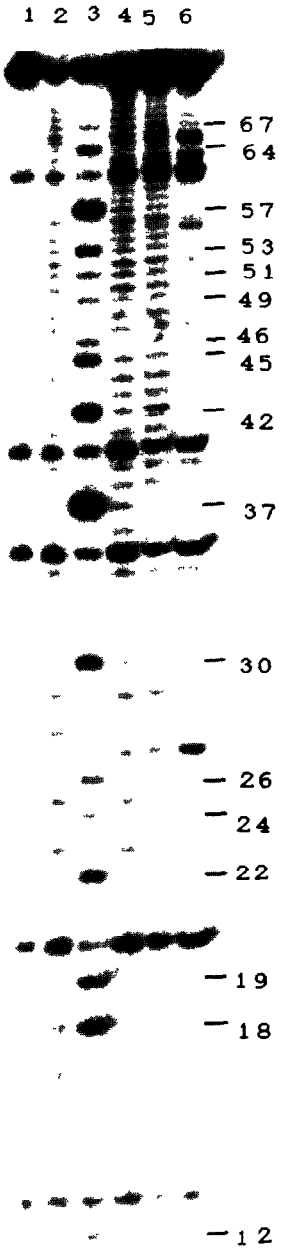

Fig. 1. Autoradiography of 14% polyacrylamide gel in 7 M urea after electrophoresis of 5′-^{32}P-labelled cleaved tRNA^{Trp} and tryptophanyl-tRNA transcripts. Lane 1, tRNA^{Trp}-transcript (control), lane 2, the same after alkaline treatment (ladder); lane 3, tRNA^{Trp}-transcript after T1 RNAse hydrolysis (ladder); lane 4, tRNA^{Trp}-transcript, treated with alkali and then incubated with bWRS, lanes 5 and 6, tRNA^{Trp}-transcripts treated with alkali, aminoacylated with tryptophan and then separated on BD-cellulose. (5) tRNA fraction eluted with 1 5 M NaCl (non aminoacylated), (6) tRNA fraction eluted with 1 5 M NaCl + 20% EtOH (tryptophanylated). Numbers (right side) = nucleotide positions in T1 ladder.

The difference in cleavage pattern between uncharged

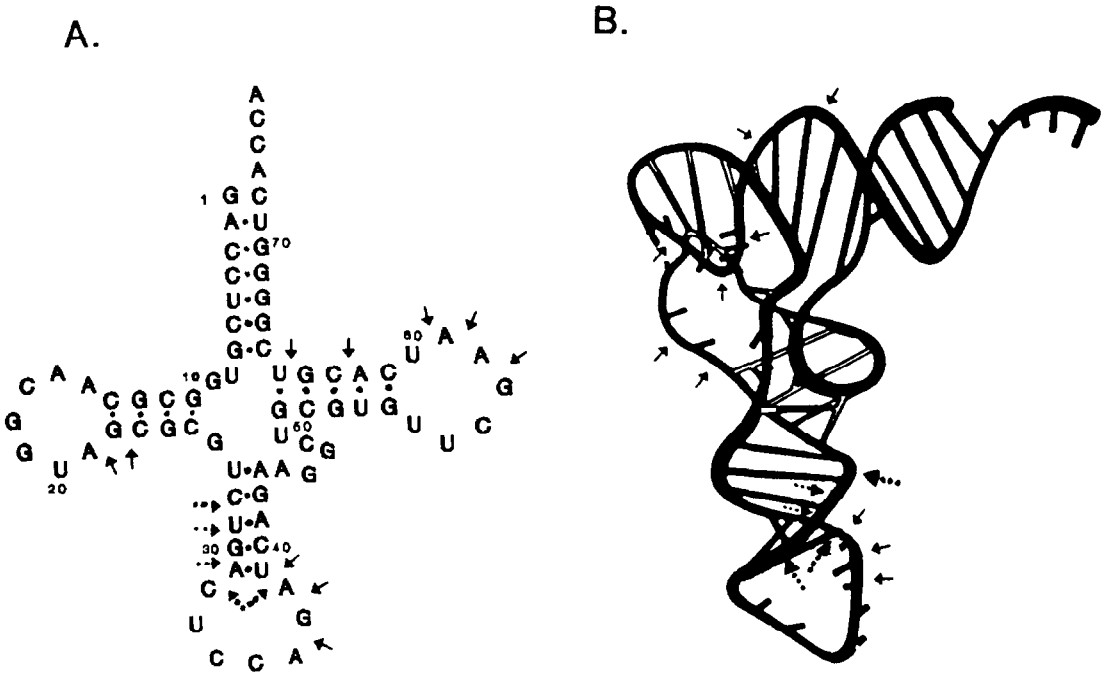

Fig. 2. Cloverleaf representation (A) and tertiary structure (B) of bovine $tRNA^{Trp}$-transcript. Phosphodiester bonds protected against nitrosoethylurea action in the presence of bWRS [13] are shown by dotted arrows. Arrows show the positions of phosphodiester bonds essential for aminoacylation ability The tertiary structure is that of the yeast $tRNA^{Phe}$ [15].

and aminoacylated tRNA-transcripts (compare lanes 5 and 6 on Fig. 1) proves that the random-splitting approach suggested in this work allows to discriminate between phosphodiester bonds exhibiting a different role in the biological function of tRNA. Furthermore, it demonstrates that in tRNA structure some phosphodiester bonds are critical for the acceptor activity while the others are not essential.

Two possible explanations for the cleavage effect on the charging reaction could be proposed. First, when phosphodiester bonds are cut in certain positions, tRNA becomes non-recognizable by the cognate enzyme. Second, the enzyme molecule and the tRNA molecule with the structure relaxed due to the cleavages cannot undergo mutual conformational changes leading to productive complex formation. The results of gel retardation test indicate that partially hydrolyzed tRNA bound to the enzyme (Fig. 3B, lane 1) and the unbound tRNA (Fig. 3B, lane 2) exhibit no differences in cleavage pattern (Fig. 3A, lanes 4 and 5, respectively). When $tRNA^{Trp}$ and $tRNA^{Gln}$ from *E. coli* were applied as controls, the level of non-specific binding was estimated to be about 5–10% (not shown). In other words, partial splitting of different phosphodiester bonds, although being important for aminoacylation, is not critical for enzyme–substrate complex formation. These data are in favour of the second hypothesis which is also in agreement with the results of earlier observations on the mutual conformational changes of $tRNA^{Trp}$ and WRS [14].

The approach described here seems to be useful for the analysis of any specific RNA–protein interactions in cases where it is possible to separate the reaction products into active and inactive fractions.

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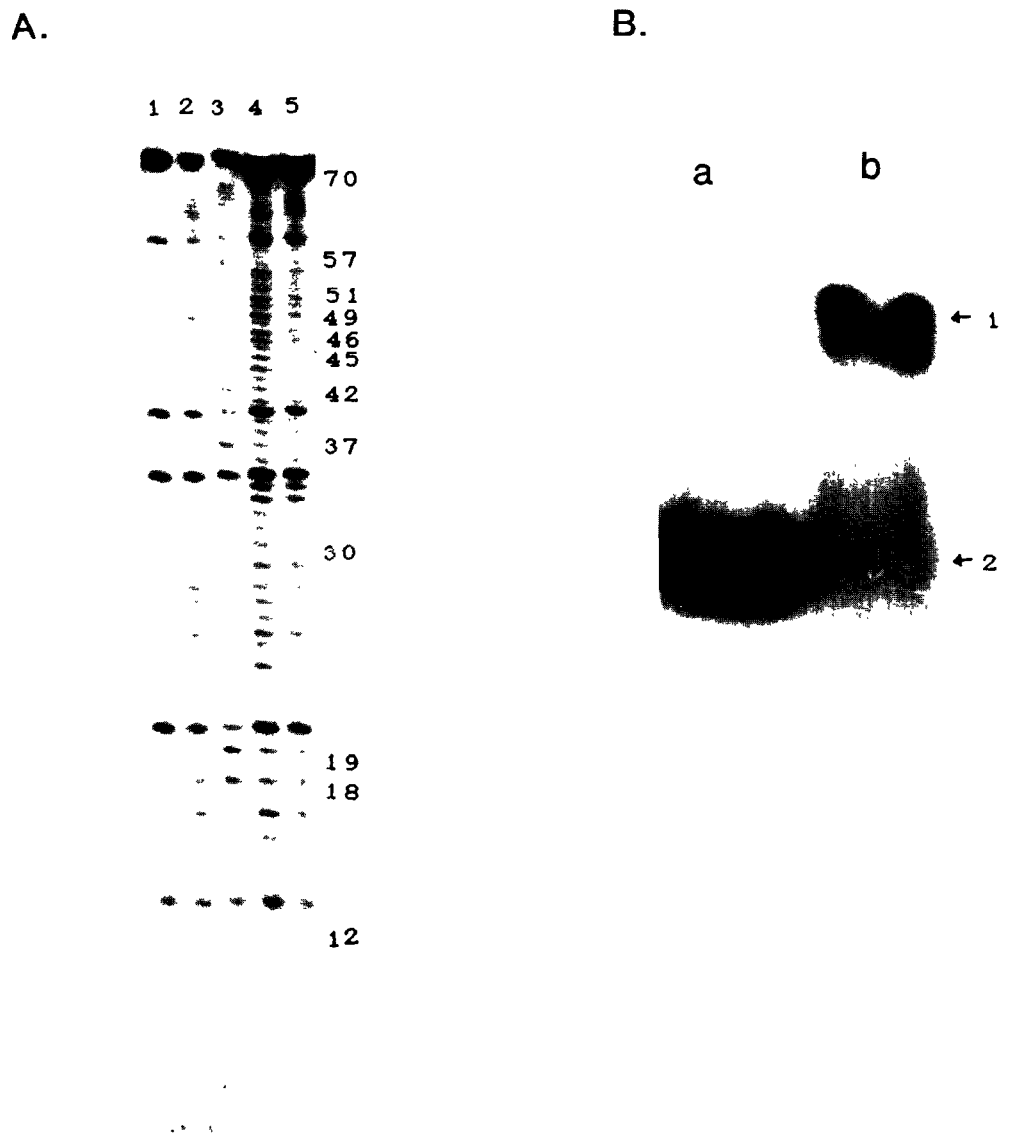

Fig. 3. Structural analysis of the randomly split $tRNA^{Trp}$-transcripts complexed and non-complexed with bWRS. (A) Autoradiogram of 'shifted' and free tRNA separated in a 14% polyacrylamide-urea gel. Lane 1, $tRNA^{Trp}$-transcript (control); lane 2, alkaline ladder; lane 3, T1-ladder; lane 4, tRNA from the complex with WRS; lane 5, free tRNA. (B) Gel retardation analysis of tRNA-transcripts. WRS complex. (a) tRNA after alkaline digestion (control); (b) tRNA incubated with WRS (see section 2); 1, 'shifted' tRNA; 2, free tRNA.

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