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The role of the CCA sequence of tRNA in the peptidyl transfer reaction

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Abstract Peptidyl transfer is a key step in the process of protein biosynthesis. To examine the role of the universal CCA terminal sequence of tRNA in the process of peptidyl transfer, various mutant transcripts of *Escherichia coli* valine tRNA were constructed. Peptidyl transferase activity, monitored by the 'fragment reaction' with a slight modification, was decreased by mutation at any one base of CCA. The effect of mutation was moderate in the UCA, CUA and CCG mutants. Replacement of A76 by a pyrimidine nucleotide, or replacement of either C74 or C75 by a purine nucleotide caused a marked decrease in the activity. These findings suggested that the universal CCA terminus of tRNA makes a functional interaction with ribosomal RNA by base-pairing.

Key words: tRNA; CCA sequence; Peptidyl transfer; Fragment reaction; RNA–RNA interaction

1. Introduction

In the translational processes, tRNAs play crucial roles. Each tRNA interacts with cognate aminoacyl-tRNA synthetase, which ensures a high degree of accuracy of aminoacylation. Extensive studies have revealed the recognition elements of many tRNAs by their cognate synthetases [1–3]. In addition to the accurate aminoacylation, proper interactions of tRNAs with mRNA, several protein factors, ribosome and so on are necessary to ensure translational fidelity.

All tRNA molecules possess a CCA sequence at their 3'-end [4]. In spite of these quite clear facts, the question why CCA is universal remains unanswered. In the aminoacylation step, recognition by each aminoacyl-tRNA synthetase would prefer characteristic structures rather than common structures such as the CCA in order to distinguish the amino acid specific tRNAs. On the contrary, the ribosomal processes are likely to require common features of tRNA because of the absence of the selection step of amino acid specificity. In this context, the functions of the CCA sequence in these processes have to be investigated more extensively.

In protein biosynthesis, peptidyl transferase, the ribosomal activity responsible for catalysis of peptide bond formation, plays a central role [5]. Chemical footprinting studies have shown that tRNA is in close contact with some bases in the region of 23 S rRNA, several of which correspond to the sites of protection by antibiotics [6]. These studies suggest that intermolecular RNA–RNA interactions are involved in this process.

The peptidyl transferase activity can be monitored with a simplified assay known as the 'fragment reaction' [7]. This reaction does not require mRNA, protein factors or GTP. In my study, this method was applied to the base substituted mutants of CCA sequence of valyl-tRNA^{Val} and the contribution of the CCA sequence to the peptidyl transfer activity was determined.

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Abbreviations: ARS, aminoacyl-tRNA synthetase; ValRS, valyl-tRNA synthetase.

2. Materials and methods

2.1. Preparation of template DNAs and in vitro transcripts

Synthetic deoxynucleotide oligomers carrying the T7 promoter and tRNA genes were ligated into pUC19 and transformed into *Escherichia coli* strain JM109 [8,9]. The template DNA sequences were confirmed by dideoxy sequencing [10]. Transcripts of the tRNA genes were prepared in a reaction mixture containing 40 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 2 mM spermidine, 10 mM $MgCl_2$, bovine serum albumin (50 μg/ml), 2.0 mM each NTP, 20 mM 5' GMP, *Scr*FI or *Eco*T22I-digested template DNA (0.2 mg/ml), 2 units of inorganic pyrophosphatase (Sigma) and pure T7 RNA polymerase (50 μg/ml) [8,11,12]. The transcripts were purified by 15% polyacrylamide gel electrophoresis.

2.2. 3' end analysis of transcript

[^{32}P]pCp ligated transcripts were purified by 15% polyacrylamide gel electrophoresis. After digestion with RNase T2 (Sankyo), ^{32}P-labeled nucleotides were subjected to two-dimensional thin-layer chromatography (Avicel SF) [13], and detected by autoradiography.

2.3. Purification of ValRS

ValRS was partially purified from *Escherichia coli* strain Q13 according to Lagerkvist and Waldenström [14] except that CM-TOYOPEARL 650M (TOSOH) was used instead of Amberlite IRP-64.

2.4. Aminoacylation

Aminoacylation was performed at 37°C in 100 μl of a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 2.5 mM ATP, 10 μM transcript RNA, various concentrations of partially purified *Escherichia coli* Q13 ValRS, and 100 μM L-[U-^{14}C]valine (9.62 GBq/mmol; Amersham).

2.5. Reaction of N-acetyl-[^{14}C]valyl-tRNA^{Val} with puromycin

N-Acetyl-[^{14}C]valyl-tRNA^{Val} was prepared according to Robertson and Wintermeyer [15]. However, a dihydroxyboryl-substituted polyacrylamide column was used instead of a BD-cellulose column [16]. Ribosomes were prepared according to a published procedure [17]. The reaction mixture contained (prior to methanol addition) 50 mM Tris-HCl (pH 7.5), 0.4 M KCl, 20 mM $Mg(OAc)_2$, 3.0 A_{260} units of ribosomes, 1 mM puromycin, and *N*-acetyl-[^{14}C]valyl-tRNA^{Val} variants. After preincubation of 100 μl portions at 0°C and the reaction was initiated by the addition of 50 μl of methanol. Each time, 45 μl was withdrawn from the reaction mixture and the reaction was terminated by the addition of 100 μl of 0.1 M $BeCl_2$ (neutralized) and 0.3 M NaOAc (pH 5.5) saturated with $MgSO_4$. Ethyl acetate (0.5 ml) was added. Then the mixture was agitated for 5 s at room temperature, and centrifuged. The upper layer (0.45 ml of ethyl acetate) was mixed with 2.5 ml of scintillation fluid (prepared by dissolving 20 g BBOT in 1 l of toluene

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plus 250 ml of 2-methoxyethanol), and counted by a scintillation counter [18].

3. Results

The peptidyl transferase activities were investigated in a variety of the base substitution mutants of valyl-tRNA^{Val} (N74N75N76) by use of a 'fragment reaction' (Fig. 1). In these experiments, complete molecules of *N*-acetyl-[^{14}C]valyl-tRNA^{Val} mutants were used instead of so-called fragments of tRNAs [7]. Peptidyl transferase activity was estimated from the measurements of the formation of *N*-acetyl-[^{14}C]valyl-puromycin in the reaction of *N*-acetyl-[^{14}C]valyl-tRNA^{Val} and puromycin in the presence of 33% methanol.

Fig. 2 shows the effects of the mutation of A76 to other nucleotides. Substitutions of A76 to C76 or U76 caused a marked decrease in the formation of *N*-acetyl- [^{14}C]valyl-puromycin. Substitution to G76 also decreased the activity, although to a lesser extent.

Figs. 3 and 4 show the effects of substitutions C75 and C74, respectively. Both of these base substitutions affected the formation of *N*-acetyl-[^{14}C]valyl-puromycin. Substitution of C74 or C75 to U caused a relatively mild decrease in activity, although other mutations resulted in a critical damage of activity.

4. Discussion

tRNA is involved in every step of protein biosynthesis. In order to translate the genetic information with high fidelity, accurate aminoacylation, and proper interactions of tRNAs with several protein factors and ribosome are indispensable.

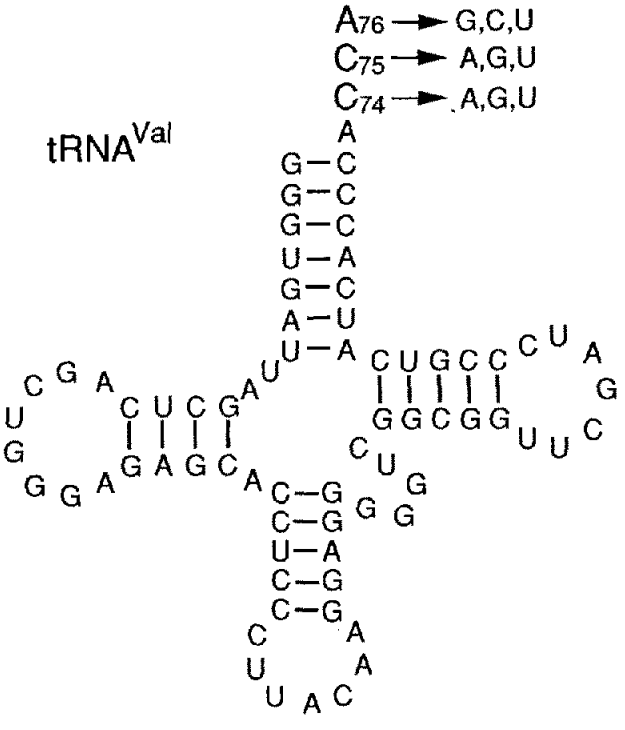

Fig. 1. The cloverleaf representation of the *Escherichia coli* tRNA^{Val} transcripts. The mutations in this study are indicated by arrows. Base numbering is according to Sprinzl et al. [4].

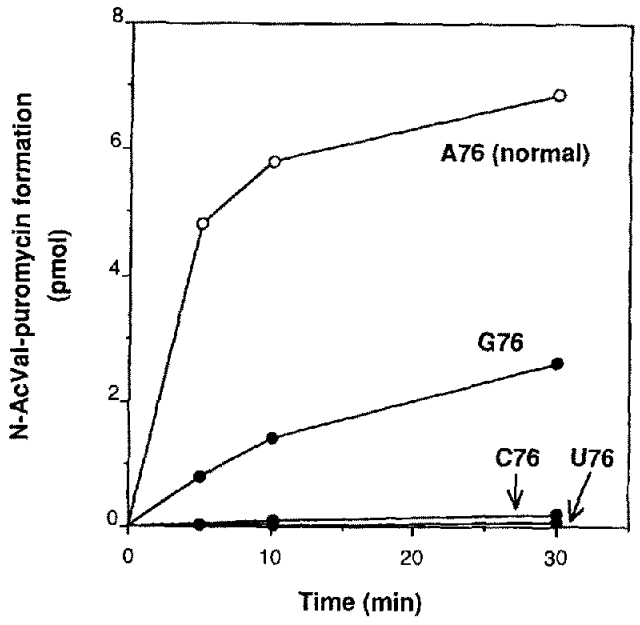

Fig. 2. Time course of the formation of *N*-acetyl-[^{14}C]valyl-puromycin in the reaction of *N*-acetyl-[^{14}C]valyl-tRNA^{Val} (N76 variants) and puromycin.

All tRNA molecules terminate with the CCA sequence at their 3′ ends without exception [4]. However, the reason why CCA is universal has yet to be clarified. In the aminoacylation step, ARS must selectively recognize both amino acids and tRNAs. The tRNA recognition also requires the CCA sequence, but the requirement is sometimes small [12], probably because ARS prefers more characteristic features. Whenever a similar shaped but noncognate amino acid is misactivated by ARS, the cognate tRNA can eliminate it by hydrolysis [19–21]. In this step, the universal CCA sequence, especially A76, plays an important role in tRNA^{Val} to prevent the formation of threonyl-tRNA^{Val} [12], but this proofreading mechanism is not found in all tRNAs and ARSs systems (K. Tamura, unpublished results).

The ribosomal processes are more general steps for tRNAs. Therefore, these processes are likely to be more deeply concerned in the universal CCA sequence. In particular, the peptidyl transfer step is the most likely because of the physical proximity to the functional position. The enzymatic activity responsible for catalysis of peptide bond formation on the ribosome is called peptidyl transferase [5]. This activity can be monitored by a simplified assay known as the 'fragment reaction', which originally used to measure the transfer of *N*-formyl-methionine from a short fragment of tRNA to the amino group of puromycin to form a model peptide bond [7]. The fragment reaction requires only a large ribosomal subunit, appropriate ionic conditions, and 33% methanol or ethanol, in addition to the *f*-methionyl-oligonucleotide and puromycin substrates, with no requirement of the small subunit, mRNA, protein factors or GTP. The validity of the model reaction has been supported by the stereochemical specificity of the substrates and highly specific inhibition of the reaction by antibiotics that are known as peptidyl transferase inhibitors [22]. In the present study, *N*-acetyl-valyl-tRNA^{Val} was used instead of *f*-methionyl-oligoribonucleotide. *N*-Acetyl-aminoacyl-tRNA

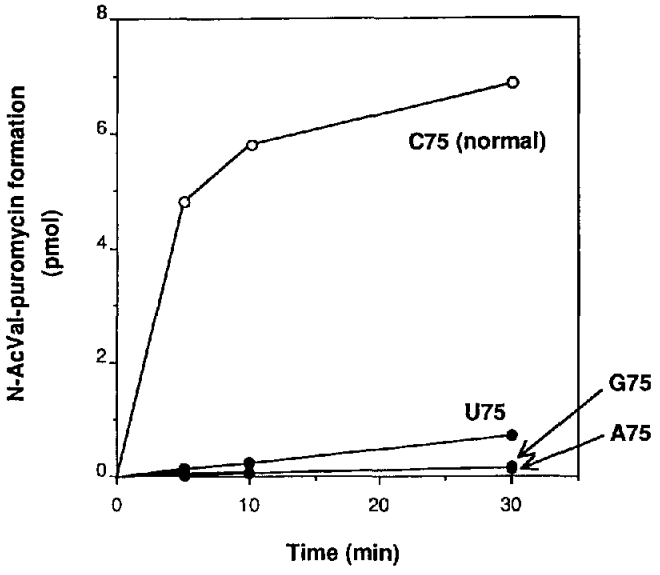

Fig. 3. Time course of the formation of *N*-acetyl-[^{14}C]valyl-puromycin in the reaction of *N*-acetyl-[^{14}C]valyl-tRNA^{Val} (N75 variants) and puromycin.

serves as an analog of peptidyl-tRNA [23,24]. *N*-Acetyl-phenylalanyl-tRNA^{Phe} binds preferentially to the P site and not to the E site of ribosome [25]. In the absence of mRNA the A site is not occupied [26–28]. In the present experiments, the transfer of the *N*-acetyl-valyl moiety of *N*-acetyl-valyl-tRNA^{Val} in the P site to the puromycin was measured. The present findings show that the peptidyl transferase activity was still retained when N74 or N75 was U, or N76 was G, although with lower activities. These findings suggest that not only a Watson–Crick type of base pairing but also a wobble base pairing interaction enables the peptidyl transfer reaction.

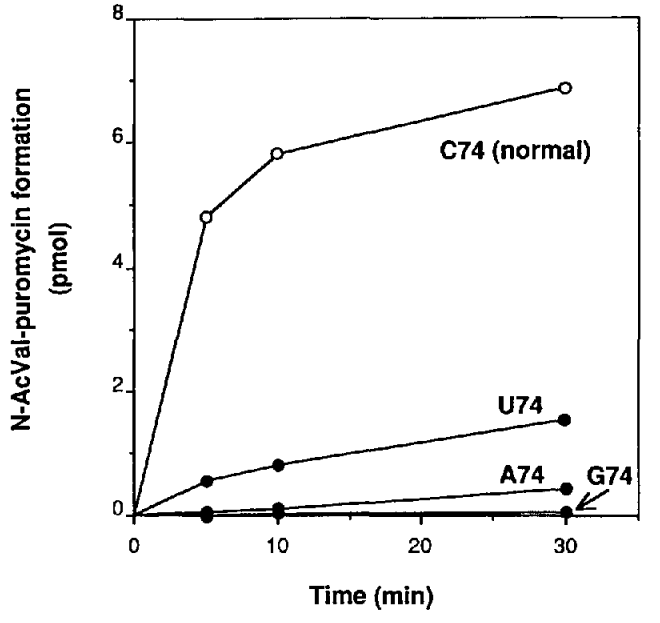

Fig. 4. Time course of the formation of *N*-acetyl-[^{14}C]valyl-puromycin in the reaction of *N*-acetyl-[^{14}C]valyl-tRNA^{Val} (N74 variants) and puromycin.

Chemical footprinting studies have suggested that the peptidyl transferase activity lies in the central loop of domain V of 23 S rRNA. Bases protected by tRNA are found almost exclusively in domain V, and most of them are localized in the central loop or its surroundings [6,29]. Stepwise deletion experiments have suggested that the 3′-terminal CCA-aminoacyl moiety of the tRNA is deeply involved in these protections [6,29]. The mutation experiment in *Escherichia coli* 23 S rRNA by site-directed mutagenesis suggested the importance of conserved nucleotides in peptide bond formation [30]. Whether the peptidyl-transferase activity requires intermolecular RNA–RNA interactions still remains an unanswered fundamental question. A recent exciting experiment has shown that the extensively deproteinized rRNA has peptidyl-transferase activity, suggesting that only the RNA portion of the large ribosomal subunit is sufficient for the activity [31]. The present study strongly suggests that the base pairings, probably Watson–Crick type of base pairings, between all three bases of the terminal CCA and bases on 23 S rRNA are essential for the peptidyl-transferase activity.

RNA–RNA interactions may be more actively engaged in the RNA world. The interactions play a central part in the ribozyme reaction in terms either of the molecular recognition or of the catalytic process [32]. Since the first peptidyl transfer was accomplished when the early ribosome free of protein was established, the essential RNA–RNA interactions between proto tRNA and proto ribosome would still be conserved. A model for the evolution of a peptide-specific proto-ribosome into a modern template-dependent ribosome also suggested these interactions [33]. Therefore, I believe that the 3′ terminus of tRNA was determined at that moment, and the CCA sequence together with its function has been conserved during evolution.

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