Anticodon Loop Structure of *Torulopsis utilis* tRNA^{Val} and Valine Acceptance

トルラ酵母 tRNA^{Val} のアンチコドンループの構造とバリン受容能

Kazuko OGAWA, Shosuke TAKEMURA and Kazuya NISHIKAWA¹

Institute of Molecular Biology, School of Science, Nagoya University,
Furou-chou, Chikusa-ku, Nagoya 464-01, JAPAN

¹Present address: Department of Biological Sciences, Faculty of Bioscience and Biotechnology,
Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 227, JAPAN
TEL.: 81-45-922-1111, ext. 2470, FAX.: 81-45-923-1136

(Received 25, May 1993 Accepted 1, July 1993)

ABSTRACT

A variant of *Torulopsis utilis* tRNA^{Val} containing base substitution (C to U) at the third letter of the anticodon has been prepared by several enzymatic reaction steps *in vitro*. The valine-accepting activity of this variant, tRNA^{Val}(IAU), was found to be decreased to a great extent. This result suggests that the third letter (C) of the anticodon of yeast tRNA^{Val} is strictly recognized by the Valyl-tRNA synthetase (ValRS). Combinations of 5'-half and 3'-half fragments were used to assess the importance of the anticodon loop conformation on aminoacylation. The result of this experiment indicated that the base-stacking interactions in the 3'-side region of the anticodon loop have a crucial role in the recognition of this tRNA by its cognate ValRS. In summary, both of the nucleotide sequence and the conformation of the anticodon loop are significant elements for *T. utilis* tRNA^{Val}-ValRS recognition.

Key words: Anticodon Loop, tRNA Identity, Aminoacylation, Base Substitution

1. INTRODUCTION

The nucleotides in the anticodon loop of many tRNAs are reported to be involved in recognition by their cognate aminoacyl-tRNA synthetases.¹ In the cases of aminoacylation by *Escherichia coli* formylmethionyl-tRNA synthetase and glutamyl-tRNA synthetase, the anticodon sequences of their cognate tRNAs play critical roles in determining the specificity.^{2,3} In the case of yeast valine tRNA, Mirzabekov *et al.* reported that the defect of A35-C36p led to the loss of aminoacylation activity.⁴ Chambers *et al.* suggested from chemical modification studies on tRNA^{Val}, that the change of nucleotide at position 36 from C to U destroyed its normal

acceptor activity.⁵ These data suggest that the anticodon region of tRNA^{Val} forms part of the recognition site for its cognate Valyl-tRNA synthetase (ValRS).

Recently, an enzymatic method has been developed in our laboratory, which allows the replacement of nucleotide sequences in tRNA with any arbitrary oligonucleotides, and various tRNA^{Tyr} and tRNA^{Phe} variants were prepared by this method.⁶⁻¹¹ One of the variant tRNA^{Tyr} whose anticodon sequence was changed from G-Ψ-A to G-A-A (anticodon for phenylalanine) acquired specificity for phenylalanine acceptance.⁷ In this paper, a tRNA^{Val} variant containing the anticodon I-A-U corresponding to that for isoleucine was

prepared by the similar enzymatic method and assayed for aminoacylation activities. Several kinds of complexes between 5'- and 3'-half molecules were also tested for aminoacylation without re-joining with RNA ligase (fragment reactions). The results of these experiment suggest both of the nucleotide sequence and the conformation of the anticodon loop are required for recognition of *T. utilis* tRNA^{Val} by its cognate ValRS.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

tRNA^{Val} was purified from a crude mixture of *T. utilis* tRNA (Jujo Paper Co.) by chromatography on columns of DEAE-Sephadex A-50 and DEAE-Sephadex A-25 essentially as described previously.¹²⁻¹⁴ The final tRNA^{Val} preparation accepted 1.6 nmol valine/A₂₆₀ unit. Oligonucleotides, pC-Ap, pU-Ap, and pC-C-Ap were prepared as follows. *T. utilis* 5S rRNA was digested completely with RNase T1 (Sankyo), and fragments C-A-Gp, U-A-Gp, and A-C-C-A-U-A-C-Gp were purified by column chromatographic procedures.¹⁵ These fragments were digested with RNase U2 (Sankyo), and resulting C-Ap, U-Ap, and C-C-Ap were then 5'-phosphorylated with T4 polynucleotide kinase (3'-phosphatase free, Boehringer Mannheim). The sources of enzymes used are as described previously^{9,16} except that *E. coli* alkaline phosphatase (BAP) was obtained from Takara shuzo Co. and ribonuclease CL3 from Pharmacia, respectively. L-[¹⁴C]-valine (275 mCi/mmol), L-[³H]-valine (47 Ci/mmol), L-[¹⁴C]-isoleucine (342 mCi/mmol) and [γ -³²P]-ATP were purchased from Amersham. Valyl-tRNA synthetase (ValRS) was purified from freshly harvested *T. utilis* cells essentially according to the method of Lagerkvist and Waldenstrom¹⁷ with a slight modification.

2.2. Preparation of Half Molecules of tRNA^{Val}

(A) *T. utilis* tRNA^{Val} dissolved in 100 mM sodium acetate (pH 4.5)-5 mM MgCl₂ (67 A₂₆₀ units/ml) was subjected to limited digestion

with RNase U2 (final concentration; 25 units/ml, incubated at 0°C for 90 min). The reaction was stopped by adding an equal volume of phenol saturated with 3 mM EDTA and shaking vigorously. After back-extraction of the phenol layer with H₂O, the combined aqueous layer was acidified to pH 1 with 2 N HCl and kept at 0°C overnight to cleave the 2',3'-cyclic phosphodiesterbond. After neutralization and dilution with 7 M urea-10 mM Tris-HCl(pH 7.6), the sample was charged on a column of DEAE-Sephacel. The elution was carried out with a linear gradient of NaCl in 7 M urea-10 mM Tris-HCl(pH 7.6).¹⁸ The fractions containing 5'-half or 3'-half fragment were desalted by adsorption to and desorption with 0.8 M NaCl from small columns of DEAE-Sephacel, followed by ethanol precipitation. The half fragments (65 A₂₆₀ units /ml) were dephosphorylated in 50 mM Tris-HCl(pH 7.6)-10 mM MgCl₂ by incubation with 9 units/ml of BAP at 37°C for 30 min, followed by the phenol treatment as described above. Finally, samples were applied onto 10 % polyacrylamide slab gels containing 7 M urea and fragments HO-G1—A35OH (U2-5') and HO-C38—A73OH (U2-3') were purified.

(B) *T. utilis* tRNA^{Val} dissolved in 10 mM Tris-HCl(pH 7.6)-10 mM MgCl₂ (15 A₂₆₀ units/ml) was subjected to limited digestion with RNase T1 (final concentration; 3600 units/ml, incubated at 0°C for 60 min). The reaction was stopped by the phenol treatment, followed by the HCl treatment and dephosphorylation with BAP as described above. The sample was applied onto 10 % polyacrylamide slab gels containing 7 M urea and fragments HO-G1—I34OH (T1-5') and HO-A35—A76OH (T1-3') were purified.

2.3. Preparation of Variant 5'-Half Fragments

Fragment HO-G1—A35OH (U2-5') was used as an "acceptor" in ligation reactions to produce variant 5'-half fragments. As "donors", pU-Ap, or pC-Ap were separately ligated to the 3'-end of the U2-5' fragment. Ligation reaction was carried out in 50 mM

Tris-HCl(pH 7.6)-15 mM MgCl₂-10 µg/ml BSA-600 µM ATP-3.5 mM DTT with 100 units /ml T4 RNA ligase (LIG, obtained from Pharmacia) at 6°C overnight. The concentrations of the "acceptors" and the "donors" were 35-45 µM and 200 µM, respectively. The reaction was stopped by heating the mixture at 70°C for 5 min. After precipitation with ethanol three times and treatment with BAP as described above, the sample was applied to 15 % polyacrylamide/urea gel to purify the ligated product. Fragment HO_G1—A35-U-AOH or HO_G1—A35-C-AOH was prepared by using pU-Ap or pC-Ap as a "donor", respectively.

2.4. Repair of the 3'-Half Fragment

The 3'-terminal sequence of the U2-3' fragment (HO_C38—A73OH) was repaired by ligating pC-C-Ap, followed by BAP treatment under the similar conditions described above. The repaired U2-3' fragment (HO_C38—A73-C-C-AOH, 30 µM) was phosphorylated at the 5'-end in 50 mM Tris-HCl(pH 7.6)-10 mM MgCl₂-2 mM ATP-5 mM DTT with 80 units /ml T4 polynucleotide kinase at 37°C for 30 min. The reaction was stopped by phenol-treatment and the reaction product, pC38—A73-C-C-AOH, was freed from ATP and ADP by chromatography on a small column of DEAE-Sephacel, followed by ethanol-precipitation.

2.5. Reconstruction of tRNA^{Val} Variants

Equimolar amounts of fragments HO_G1—A35-U-AOH and pC38—A73-C-C-AOH were mixed in 50 mM Tris-HCl(pH 7.6)-15 mM MgCl₂ and annealed by heating at 65°C for 10 min and cooling slowly to room temperature over 2-3 hr. The annealed half fragments (16 µM each) were ligated by incubation in 50 mM Tris-HCl(pH 7.6)-15 mM MgCl₂-15 µg/ml BSA-160 µM ATP-3.5 mM DTT with 33 units /ml T4 RNA ligase at 37°C for 30 min, followed by purification by electrophoresis on a polyacrylamide/urea gel. The product of this reaction was termed tRNA^{Val}(IAU). As a control, tRNA^{Val}(IAC), whose nucleotide sequence is just the same as that of intact tRNA^{Val} from *T. utilis*, was also reconstructed by using HO_G1—A35-C-AOH instead of HO_G1

—A35-U-AOH as the 5'-half fragment.

2.6. Aminoacylation Assay

Combinations of 5'-half and 3'-half fragments were annealed in 20 mM Tris-HCl(pH 7.6)-10 mM MgCl₂, prior to aminoacylation assays. Reaction mixtures contained 100 mM Tris-HCl(pH 7.6)-10 mM MgCl₂-40 mM KCl-4 mM ATP-25 µM L-[¹⁴C]-valine or isoleucine (100 µCi/µmol), and tRNAs or complexes of the 5'- and 3'-half fragments at concentrations as described for each assay. Samples were preincubated at 30°C for 5 min and reactions initiated by addition of ValRS. Aliquots(25 µl each) were withdrawn at pre-determined time intervals and spotted on small pieces of Whatman 3MM filters, and the filters were washed twice with 0.2 N HCl, once with ethanol, dried, and the radioactivities on the filters were counted in a liquid scintillation counter. Initial rate of aminoacylation was determined with L-[³H]-valine (47 Ci/mmol). Concentrations of tRNAs used ranged from 0.7 µM to 2.1 µM for intact tRNA^{Val} and tRNA^{Val}(IAC), and from 4.5 µM to 9.0 µM for tRNA^{Val}(IAU). The complex of fragments HO_G1—I34OH and pC35—A76OH was assayed at the concentrations ranging from 0.7 µM to 3.5 µM, and the complex of fragments HO_G1—A35-C-AOH and pC38—A76OH from 3.0 µM to 15.0 µM.

3. RESULTS

3.1. Construction of tRNA^{Val} Variants with the Substitution at the Third Position of the Anticodon

Figure 1 shows the scheme for the construction of tRNA^{Val}(IAU) which has a replacement (C36to U36) at the third position of the anticodon. As a control, tRNA^{Val}(IAC), whose nucleotide sequence is just the same as that of intact tRNA^{Val} was also prepared by the same scheme except that pC-Ap was used instead of pU-Ap at the first ligation step. The final products in the scheme were designed so as not to contain the 5'-terminal phosphate. This was necessary to ensure reasonably high yield of the desired product. The absence of phosphate group at the 5'-end of the 5'-half fragment prevented the formation of a useless

Fig. 1. Scheme for the construction of tRNA^{Val} variant containing substitution (from C36 to U36) at the third letter of the anticodon.

by-product (circularized tRNA) at the final ligation step.

Limited digestion of intact tRNA^{Val} with RNase U2 was used to generate half fragments HO-G¹-A³⁵-OH (U2-5') and HO-C³⁸-A⁷³-OH (U2-3'). In order to ensure good separation of the 5'- and 3'-half fragments, dephosphorylation by BAP treatment prior to purification

by polyacrylamide gel electrophoresis was required. Since the C74-C-A76 end was deleted from the 3'-half fragment, it had to be repaired by joining pC-C-A_p with RNA ligase, followed by removal of 3'-phosphate with polynucleotide kinase. The 5'-half fragment (U2-5') was ligated to pU-A_p or pC-A_p at the 3'-end and treated with BAP to produce 5'-fragments HO-G¹-A³⁵-U(or C)³⁶-A³⁷-OH. Joining these 5'-fragments and the repaired 3'-fragment with RNA ligase gave tRNA^{Val}(IAU) or tRNA^{Val}(IAC). The nucleotide sequences of the constructed tRNAs were confirmed by sequence analyses of the 3'-[³²P-pCp] labeled tRNAs according to the method of Donis-Keller *et al.*¹⁹(data not shown).

3.2. Valine-Accepting Activity of Variant tRNAs

The time courses of valine acceptance of variant tRNAs were measured with purified ValRS. As shown in Fig. 2a, the reconstructed tRNA^{Val}(IAC) could be fully aminoacylated as natural tRNA^{Val}. This result indicates that the construction was performed without any damage to the valine-accepting activity. tRNA^{Val}(IAU) was found to be able to accept valine, but the extent was only 5 % of the acceptance of native tRNA^{Val} or tRNA^{Val}(IAC). Even with the use of more than 160 fold concentration of ValRS, the valine acceptance of tRNA^{Val}(IAU) was only about 30 % of that of native tRNA^{Val} or tRNA^{Val}(IAC)(Fig. 2b). These results indicated that the base-substitution at the third position in the anticodon (from C to U) had deteriorative effect on the valine-accepting activity of tRNA^{Val}.

The kinetic parameters for aminoacylation were determined under the conditions described in 2.6. and by Lineweaver-Burk analyses. The K_m for native tRNA^{Val} or reconstructed tRNA^{Val}(IAC) was 1.3 μM or 1.2 μM, respectively. The variant tRNA(IAU) had much higher K_m, 15 μM (11 fold increase as compared with native tRNA^{Val}). The values of V_{max} were given as relative rate to tRNA^{Val}; tRNA^{Val}=100, tRNA^{Val}(IAC)=160, and tRNA^{Val}(IAU)=14 (7 fold decrease).

Therefore, value of V_{max}/K_m for $tRNA^{Val}(IAU)$ is about 80 fold lower than that of native $tRNA^{Val}$. These data strongly suggest that in the case of *T. utilis* $tRNA^{Val}$ the third letter of anticodon (36th position from the 5'-end) is strictly recognized by ValRS and has strong influence on the aminoacylation activity of $tRNA^{Val}$.

Fig. 2. Valine acceptance of intact $tRNA^{Val}$ (◇), $tRNA^{Val}(IAC)$ (●), and $tRNA^{Val}(IAU)$ (▲). Reaction mixtures contained 100 mM Tris-HCl(pH 7.6)-10 mM $MgCl_2$ -40 mM KCl-4 mM ATP-25 μM L-[^{14}C]-Valine (100 $\mu Ci/\mu mol$), 0.8 μM tRNA, and ValRS. Incubation was at 30°C for the times indicated. (a)0.34 unit/ml ValRS was used for each assay. (b)56 units/ml ValRS was used for each assay.

3.3. Aminoacylation of the 5'-Half and 3'-Half Fragment Combinations

The results above indicated that ValRS recognizes a specific nucleotide in the anticodon. In order to assess the importance of steric characters of the anticodon loop, fragment combination assays were designed as follows. If a nick is introduced into the anticodon loop, it will destroy or at least modify the native conformation of the loop.

Fig. 3 Combinations of 5'-half and 3'-half fragments assayed for valine acceptance after annealing.

Therefore, different combinations of 5'-half and 3'-half fragments were annealed to form tRNA-like complexes which have a nick at different sites in the anticodon loop (Fig. 3). Complex A consisted of the T1-5' and T1-3' fragments derived from limited digestion of $tRNA^{Val}$ with RNase T1. This complex has a nick between the first letter(I34) and second letter(A35) of the anticodon. Complexes B was prepared from the U2-5' fragment ligated to pC-Ap (described in 2.3.) and the repaired U2-3' fragment (2.4.). this complex has a nick between A37 and C38 at the 3'-side of the anticodon. The valine accepting activities of these complexes are shown in Fig. 4. In this assay, various molar ratios of the 5'- and 3'-half fragments were mixed and annealed, while keeping the total amount constant (50 pmoles). Therefore, "100 % 3'-half" in the abscissa means 50 pmoles 3'-half fragment and no 5'-fragment, and "50 % 3'-half" means 25 pmoles each of 5'- and 3'-half fragments, and so on. As can be seen in the figure, maximum valine-acceptance of complex A was observed when equimolar amounts (25 pmoles each) of 5'- and 3'- fragments were annealed, and the complex was fully aminoacylated (25 pmoles of the complex A accepted 25 pmoles valine). Therefore the cleavage between I34 and A35 had no crucial effect on the aminoacylation reaction. It seems likely that the anticodon

loop region of complex A keeps or at least is able to restore the original conformation when it interacts with ValRS. On the other hand, the

Fig. 4 Valine acceptance of complex A (●) and complex B (○) at various molar ratios of the 5'-half and 3'-half fragments. Reaction mixtures (50 μ l each) contained coponents described in Fig. 2 except that total amount of 50 pmoles of the 5'- and 3'-half fragments were used. Incubation was at 30°C for 20 min.

valine accepting activity of complex B was about 10 % of complex A (and native tRNA^{Val}) at most. Since complex B has all the nucleotide elements in tRNA^{Val}, and the ligated product of complex B, namely tRNA^{Val}(IAC), had full activity (Fig. 2), the cleavage between A37 and C38 had caused the decrease in aminoacylation activity. This cleavage might have changed the gross conformation of the 3'-side of anticodon loop, resulting in the disturbance of tRNA-ValRS interaction. The kinetic parameters for aminoacylation of complexes A and B were obtained preliminarily. Complex A had K_m value of 3.2 μ M and V_{max} of 200 (tRNA^{Val}=100). Those of Complex B were 15 μ M and 9, respectively. These data indicate that both of the nucleotide sequence and conformation of the anticodon loop are important for yeast tRNA^{Val} to be recognized by its cognate ValRS.

3.4. Isoleucine Accepting Activity of tRNA^{Val}(IAU)

The variant tRNA^{Val}(IAU) has an anticodon corresponding to isoleucine, IAU. The

homology between the nucleotide sequence of tRNA^{Ile} and tRNA^{Val} is about 60 %. Since the anticodon sequence of *E. coli* tRNA^{Ile} is known to be crucial for isoleucine acceptance, the possibility that tRNA^{Val}(IAU) had acquired the ability to accept isoleucine was examined. However, this was not the case. The variant tRNA^{Val}(IAU) did not accept isoleucine at all under the various conditions tested. This might mean that some other important recognition element(s) for IleRS (for example, t⁶A37) is deficient in tRNA^{Val}(IAU), or tRNA^{Val}(IAU) has some "negative" recognition element(s) for IleRS.

4. DISCUSSION

Enzymatic method was utilized to prepare yeast tRNA^{Val} variants which have base-substitutions in the anticodon loop. Alteration of the cytosine (C36) at the third position of the anticodon to uracil (U36) drastically reduced the aminoacylation activity. This result is in good agreement with that of Chambers *et al.*,⁵ who have shown that conversion of the third letter of yeast tRNA^{Val} from C to U by chemical modification caused a loss of aminoacylation activity. These results indicate that the C36 residue in the anticodon of tRNA^{Val} is one of the critical determinants for recognition by yeast ValRS. Cytosine has an amino group and uracil a keto group at the C(6) position of pyrimidine ring, respectively. As for hydrogen bonding, these two would function oppositely: amino group works as a proton donor and keto group as a proton acceptor. So, U36 would be rejected from the C36-recognition site, if hydrogen bonding is the major factor of recognition.

We assessed the effect of the anticodon loop conformation on the aminoacylation capacity by the fragment combination assay. Mirzabekov *et al.* reported that yeast tRNA^{Val} dissected between I34 and A35 retained full activity of valine acceptance.²⁰ One of the complexes in our experiment (complex A) had similar propeties. Surprisingly, however, complex B which has a nick between A37 and C38 accepted valine only to the extent of 10 %

of complex A. One possible explanation for this result would be as follows. X-ray analyses of tRNA crystals have revealed that five consecutive nucleotides from position 34 to 38 of anticodon loop have base stacking interactions and the stacking is coaxially continued into the anticodon stem.^{21,22} When the cleavage site in the anticodon loop is between I34 and A35 as in the case of complex A, the base stacking above may be conserved and the steric feature of anticodon loop important for tRNA-ValRS interaction may be maintained. On the other hand, cleavage between A37 and C38, like complex B, would interrupt the base stacking of the anticodon loop, and consequently decrease the tRNA-ValRS interaction.

In summary, this study revealed that both of the nucleotide sequence and the conformation of anticodon loop are significant factors for *T. utilis* tRNA^{Val}-ValRS recognition.

REFERENCES

- ¹ D.H. Gauss, F. Gunter, and M. Sprinzl, *Nucleic. Acids Res.*, 6, r1 (1979)
- ² L.H. Schulman and H. Pelka, *Proc. Natl. Acad. Sci. USA*, 80, 6755 (1983)
- ³ L.H. Schulman and H. Pelka, *Biochemistry*, 24, 7309 (1985)
- ⁴ A.D. Mirzabekov, D. Lastity, E.S. Levina, and A.A. Bayev, *Nature New Biol.*, 229, 21 (1971)
- ⁵ R.W. Chambers, S. Aoyagi, Y. Furukawa, H. Zawadzka, and O.S. Bhanot, *J. Biol. Chem.*, 248, 5549 (1973)
- ⁶ T. Ohyama, K. Nishikawa, and S. Takemura, *Nucleic Acids Symp. Ser.*, 12, 137 (1983)
- ⁷ K. Nishikawa, M. Uritani, M. Miyazaki, and S. Takemura, *Nucleic Acids Symp. Ser.* 15, 125 (1984)
- ⁸ T. Ohyama, K. Nishikawa, and S. Takemura, *Nucleic Acids Symp. Ser.*, 16, 213 (1985)
- ⁹ T. Ohyama, K. Nishikawa, and S. Takemura, *J. Biochem.*, 97, 29 (1985)
- ¹⁰ T. Ohyama, K. Nishikawa, and S. Takemura, *J. Biochem.*, 99, 859 (1986)
- ¹¹ M. Kato, K. Nishikawa, M. Uritani, M. Miyazaki, and S. Takemura, *J. Biochem.*, 107, 242 (1990)
- ¹² K. Ogawa, M. Kawakami, Y. Shimizu, and S. Takemura, *J. Biochem.*, 91, 1241 (1982)
- ¹³ S. Nishimura, F. Harada, U. Narushima, and T. Seno, *Biochim. Biophys. Acta*, 142, 133 (1967)
- ¹⁴ S. Takemura and M. Miyazaki, *J. Biochem.*, 65, 159 (1969)
- ¹⁵ K. Nishikawa and S. Takemura, *J. Biochem.*, 76, 925 (1974)
- ¹⁶ S. Yamashiro-Matsumura and S. Takemura, *J. Biochem.*, 86, 335 (1979)
- ¹⁷ U. Lagerkvist and J. Waldenstrom, *J. Biol. Chem.*, 242, 3021 (1967)
- ¹⁸ T. Mizutani, M. Miyazaki, and S. Takemura, *J. Biochem.*, 64, 839 (1968)
- ¹⁹ H. Donis-Keller, A.M. Maxam, and W. Gilbert, *Nucleic Acids Res.*, 4, 2527 (1977)
- ²⁰ A.D. Mirzabekov, L.Y. Kazarinova, and A.A. Bayev, *Molec. Biolog. U.S.S.R.*, 3, 879 (1969)
- ²¹ S.H. Kim, J.L. Sussman, F.L. Suddath, G.J. Quigley, A. McPherson, A.J.H. Wang, N.C. Seeman, and A. Rich, *Proc. Natl. Acad. Sci. USA*, 71, 4970 (1974)
- ²² N.H. Woo, B.A. Roe, and A. Rich, *Nature*, 286, 346 (1980)