Higher-order Structure of Yeast tRNA^{Tyr} and Its Chimeric Mutants in Aqueous Solution As Studied by Hydroxyl Radical "Footprinting"

ヒドロキシラジカル・フットプリント法による酵母 tRNA^{Tyr}
およびそのキメラ変異体の溶液内高次構造の研究

Ichirou MOTOKI, Takashi YOKOGAWA, Tsunemi HASEGAWA¹, Kimitsuna WATANABE²
and Kazuya NISHIKAWA

Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 227, ¹Institute of Space and Astronautical Science, 3-1-1 Yoshinodai, Sagami-hara, Kanagawa 229, and ²Department of Industrial Chemistry, Faculty of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.
TEL:81-45-922-1111, ext.2470, FAX:81-45-923-1136

(Received 1, October 1993 Accepted 10, November 1993)

ABSTRACT

Hydroxyl radical, generated by reduction of hydrogen peroxide by Fe(II)-EDTA, was used to investigate the higher-order structure of yeast tRNA^{Tyr} and its chimeric mutants in aqueous solution. Exposure of free tRNA^{Tyr} to this reagent gave cleavage patterns consistent with the three-dimensional structure of yeast tRNA^{Phe} established by X-ray crystallography. This probing method was applied to detect the base-mismatches in the anticodon stem region of a chimeric tRNA^{Tyr} mutant, which was constructed by ligating the 5'-half fragment of *T.utilis* tRNA^{Tyr} and the 3'-half fragment of baker's yeast tRNA^{Tyr} with T4 RNA ligase. The result of this analysis clearly showed that this probing method is sensitive enough to detect even a minute variation of the secondary structure of RNA molecules.

Key words: Transfer RNA Conformation, Tertiary Structure, Hydroxyl Radical, Chemical Modification, Chimeric tRNA Mutant, RNA ligase

1. INTRODUCTION

A precise knowledge of RNA structure has become increasingly important in many areas of molecular biology. For example, such knowledge is essential for a detailed understanding of the mechanism of RNA processing such as splicing and editing, and the mechanism involved in protein biosynthesis. Among many other ways to investigate the three-dimensional RNA structures, one promising approach would be the use of chemical reagents for probing the accessible sites in RNA molecule. The principal advantage of this approach is that much less amount of sample RNA is required as compared with other methods, such as NMR

or X-ray crystallography. Recently, Tullius and coworkers have developed a powerful method for probing DNA structure and making footprints of proteins bound to DNA.^{1,2} The hydroxyl radical, generated upon reduction of hydrogen peroxide by Fe(II)-EDTA complex, was the reagent used to modify and cleave DNA at positions that are accessible to solvent. This reagent has following merits for using as a probe for DNA structure, and hopefully for RNA also.^{3,4} 1) Since the active species is very small and uncharged, it is expected to penetrate into the interior areas of the RNA molecule where nucleases or other chemical probes would not be able to access. 2) It is believed to

attack the exposed C1' and C4' atoms of ribose and therefore sequence-independent probing is possible. 3) The cleavage reaction is compatible with most of the physiological conditions (buffer components, concentration, pH, and temperature).

We have developed an efficient method for altering nucleotide sequences in tRNA molecules *in vitro* and constructed several tRNA^{Tyr} mutants.⁵⁻⁸ Here we describe the results of hydroxyl radical footprinting analyses of yeast tRNA^{Tyr} and its chimeric mutants which have base-mismatches in the anticodon stem region.

2. EXPERIMENTAL

MATERIALS. Tyrosine tRNAs were purified from crude mixtures of *Torulopsis utilis* (Torula yeast) tRNA (Jujo Paper Co.) or baker's yeast tRNA (Boehringer Mannheim) by successive column chromatographies on DEAE-Sephadex A-50, DEAE-Sephadex A-25, and BD-cellulose, essentially as described previously.⁹ The final tRNA^{Tyr} preparations accepted 1.6-1.8 nmol tyrosine /A₂₆₀ unit. The sources of enzymes used are as described previously¹⁰ unless otherwise noted. EDTA and 3-(N-morpholino) propanesulfonic acid (MOPS) were obtained from Dojindo Laboratories. Ammonium iron(II) sulfate hexahydrate, sodium L-ascorbate and hydrogen peroxide were from Wako Pure Chemical Industries. 5'-[³²P]pCp (1.1 x 10¹⁴ Bq/mmol) was purchased from Amersham.

CONSTRUCTION OF CHIMERIC tRNA.

Chimeric tRNA^{Tyr} mutants which have base-mismatches in the anticodon stem were constructed by following procedures. First, *T. utilis* or baker's yeast tRNA^{Tyr} was cleaved at G36 (arrowheads in Fig. 1) into two half molecules (pC1-G36p and HOΨ37-A78OH) by limited digestion with RNase T1. tRNA^{Tyr} dissolved in 20 mM Tris-HCl(pH 7.6)-100 mM MgCl₂ (15 A₂₆₀ units /ml) was subjected to limited digestion with RNase T1 (3,600 units /ml) at 0°C for 30 min. The reaction was stopped by phenol treatment. Aqueous layer was acidified to pH 1 with 2 N HCl and kept at 0°C overnight to cleave the 2',3'-cyclic

phosphodiester bond. After neutralization, the sample was loaded onto 10 % polyacrylamide / 7 M urea gels and the fragments pC1-G36p (5'-half) and HOΨ37-A78OH (3'-half) were separated and eluted from the gel blocks. Second, equimolar amounts of baker's yeast 5'-half (B5) and *T. utilis* 3'-half (T3) were mixed and reannealed by heating at 65°C for 5 min and cooling slowly to room temperature over 2-3 hr. The annealed half fragments (16 μM each) were dephosphorylated at the 3'-end of the 5'-half and phosphorylated at the 5'-end of the 3'-half by incubation with T4 polynucleotide kinase (80 units /ml, from Takara shuzo) in 50mM Tris-HCl(pH 7.6)-15mM MgCl₂-15 μg/ml BSA-160 μM ATP-3.5 mM DTT at 37°C for 30 min, then ligated by addition of T4 RNA ligase (33 units /ml, from Pharmacia) and incubation for further 30 min. The ligated product was purified by electrophoresis on a 10 % polyacrylamide / 7 M urea gel. This chimeric mutant, named tRNA^{Tyr} (B5T3), has two base-mismatches (C×A and A×G) in the anticodon stem (Fig. 1). Similarly, another chimeric mutant, tRNA^{Tyr} (T5B3), can be constructed from *T. utilis* 5'-half (T5) and baker's yeast 3'-half (B3), and has one base-mismatch C×U in the anticodon region.

HYDROXYL RADICAL FOOTPRINTING.

Native and Chimeric tRNAs were 3'-end-labeled with [³²P]pCp by using T4 RNA ligase and purified by electrophoresis on 10% polyacrylamide / 7 M urea gels. Prior to hydroxyl radical footprinting, the labeled tRNAs were renatured by annealing in the presence of 10 mM MgCl₂ as described above. Probing reaction was performed in the buffer containing 4.5 mM MOPS-NaOH (pH7.6), 12 mM KCl, 0 ~ 5 mM MgCl₂, and hydroxyl radical generating system (basic system; 0.05 mM ammonium iron(II) sulfate, 0.1 mM EDTA, 5 mM ascorbate and 0.15 % hydrogen peroxide or multiples thereof) at 30 or 45°C for 2 min. The reaction was stopped by adding 10 % glycerol which is an efficient scavenger of hydroxyl radical.² Sequencing markers were generated by partial digestion of tRNAs with RNase T1 or partial alkaline hydrolysis as described by Donis-Keller.¹¹ Samples were

Fig. 1. Construction of chimeric tRNA^{Tyr} variants from native tRNA^{Tyr}s.

Nucleotides which are different between *T. utilis* and baker's yeast tRNA^{Tyr}s are circled. Open arrowheads indicate the sites of limited cleavage with RNase T1. Each of the 5'- and 3'-half fragments were separated by polyacrylamide gel electrophoresis, then rejoined with the aid of RNA ligase to form tRNA^{Tyr}(B5T3) and tRNA^{Tyr}(T5B3).

●, Watson-Crick type base-pairs; ○, wobble base pairs; ×, base-mismatches introduced.

loaded onto a 15 % polyacrylamide gel containing 7 M urea, 45 mM Tris-borate (pH8.3), 1 mM EDTA, and 10 % glycerol. After electrophoresis, the cleavage patterns were visualized by autoradiography and, when necessary, quantification was done by densitometry of the autoradiogram with the aid of Gilford Spectrophotometer Response II equipped with gel scanning apparatus.

3. RESULTS AND DISCUSSION

Most of the cytoplasmic tRNAs of known sequence can be folded into the same secondary structure arrangement called cloverleaf model. In this arrangement, each of the four double-helical stem regions has invariant numbers of base pairs. The acceptor stem, anticodon stem and TΨC-stem have 7, 5 and 5 base pairs, respectively. D-stem has 3 or 4 Watson-Crick type base pairs. However, the reason why the stems have constant numbers of base pairs is still unclear. Naturally-occurring single-point mutations in the stem regions of tRNA have resulted in temperature sensitivity or ambiguity of aminoacylation.¹²⁻¹⁴ Many of the mitochondrial tRNAs have been shown to have unusual sizes of loop and stem structures.¹⁵

We have developed a method to construct chimeric mutant of yeast tRNA^{Tyr} molecules which have reduced numbers of base pairs in the anticodon stem region. As shown in Fig. 1, nucleotide sequences of tRNA^{Tyr}s from *T. utilis* and baker's yeast differ from each other only at 8 positions (circled in the figure). Chimeric tRNA^{Tyr} mutants (B5T3 and T5B3) could be easily and efficiently (final yield; higher than 60 %) constructed by switched ligation of the half fragments. Melting profile analyses revealed that both chimeric tRNAs are thermally destabilized, tRNA^{Tyr}(B5T3) being more unstable.⁸ Aminoacylation characteristics of these chimeric mutants will be published elsewhere. The purpose of the present study is to assess the possibility to use the hydroxyl radical footprinting for detecting the base-mismatch(es) embedded in the double-stranded stem region of tRNAs.

As shown in Fig. 2(a), hydroxyl radicals cleaved *T. utilis* tRNA^{Tyr} at most positions of

Fig. 2. Hydroxyl radical footprinting of *T. utilis* tRNA^{Tyr}. (a) Autoradiogram of a 15 % polyacrylamide sequencing gel of 3'-end-labeled *T. utilis* tRNA^{Tyr}. Lanes: L, partial alkaline hydrolysis ; G, partial RNase T1 digest ; 1~3, hydroxyl radical probing of free tRNA^{Tyr} (increasing amounts of hydroxyl radical were used from left to right) ; 4~6, hydroxyl radical probing in the presence of 0.1 mg /ml bovine serum albumin (same). The regions of protection from hydroxyl radical are indicated by brackets on the right. (b) tRNA cleavage data superimposed on the cloverleaf structure of this molecule and (c) the three-dimensional structure of yeast tRNA^{Phe}.¹⁶ Shaded areas are regions of protection from hydroxyl radical.

the RNA backbone except those at the D and TΨC loops. Addition of bovine serum albumin (up to 200μg /ml) in the reaction mixture did not affect the efficiency or patterns of the reaction. This will be an important feature when one is going to study on the interaction between tRNA and proteins (for example, aminoacyl-tRNA synthetase). Although the protection at the D loop region is ambiguous in this autoradiogram, shorter exposure of the gel to X-ray films gave clearer results (Fig. 3(a)). The result is essentially in agreement with the results of similar experiments on yeast tRNA^{Phe} by Lantham and Cech⁴ and consistent with the three-dimensional structure of yeast tRNA^{Phe} which was established by X-ray crystallography.¹⁶

Patterns of hydroxyl radical probing on *T. utilis* tRNA^{Tyr} and chimeric tRNA^{Tyr}(B5T3) are compared in Fig. 3. Overall cleavage patterns are very similar to each other both in the presence and absence of Mg²⁺ ions. However, cleavage at the regions of interest, that is, around the base-mismatches C29×A45 and A30×G44, was obviously more enhanced in the chimeric tRNA^{Tyr} (Fig. 3(a)). This point was made more clear by tracing the appropriate lanes in the autoradiogram by a densitometer (Gilford Spectrophotometer) (Fig. 3(b)). As shown in Fig. 3(c), base-mismatched region in tRNA^{Tyr}(B5T3) could be clearly detected by the hydroxyl radical probing method. Probably, the C1' or C4'

Fig. 3. Comparison of hydroxyl radical cleavage patterns between *T. utilis* tRNA^{Tyr} and chimeric tRNA^{Tyr}(B5T3). (a) Autoradiogram of a 15 % polyacrylamide sequencing gel of 3'-end-labeled tRNA^{Tyr}s. Lanes: -, untreated tRNA^{Tyr}s; L, partial alkaline hydrolysis; G, partial RNase T1 digest; EDTA, hydroxyl radical probing in the presence of 5 mM EDTA; [Mg²⁺]1, 3 and 5, hydroxyl radical probing in the presence of 1, 3 and 5 mM Mg²⁺ ion, respectively. (b) Densitometer tracings of the lanes [Mg²⁺] 3 in autoradiogram(a). Solid line, *T. utilis* tRNA^{Tyr}; dotted line, chimeric tRNA^{Tyr}(B5T3). (c) Cloverleaf structure of chimeric tRNA^{Tyr}(B5T3). Solid triangles indicate the sites where accessibility to hydroxyl radical was enhanced in the chimeric tRNA.

atoms of the ribose sugars at this region have become more exposed to solvent by being mismatched with the counterpart residues.

Hydroxyl radical cleavage pattern of chimeric tRNA^{Tyr}(T5B3) was essentially similar to that of chimeric tRNA^{Tyr}(B5T3) above (data not shown). This probably means that the Ψ29•G45 "wobble" pair is not stable under the conditions studied.

Cleavage of RNA by hydroxyl radical has been believed to be independent of the primary and secondary structure of the RNA examined, due to the small size and neutral charge of the reagent.^{3,4} However, the results of this study have shown that this method is sensitive to detect the base-mismatches embedded in the double-stranded stem region. In other words, even a minute variation of the secondary structure can be detected by this probing method if the data are processed quantitatively by appropriate means. This method should be generally useful for structural analyses of low-molecular-weight RNA molecules.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- ¹ T.D. Tullius, *Trends Biochem. Sci.*, **15**, 297 (1987).
- ² T.D. Tullius and B.A. Dombrowski, *Proc. Natl. Acad. Sci. USA*, **83**, 5469 (1986).
- ³ X. Wang and R.A. Padgett, *Proc. Natl. Acad. Sci. USA*, **86**, 7795 (1989).
- ⁴ J.A. Latham and T.R. Cech, *Science*, **245**, 276 (1989).
- ⁵ K. Nishikawa, M. Uritani, M. Miyazaki, and S. Takemura, *Nucleic Acids Symp. Ser.* **15**, 125 (1984).
- ⁶ T. Ohyama, K. Nishikawa, and S. Takemura, *J. Biochem.*, **97**, 29 (1985).
- ⁷ T. Ohyama, K. Nishikawa, and S. Takemura, *J. Biochem.*, **99**, 859 (1986).
- ⁸ K. Nishikawa, *Nucleic Acids Symp. Ser.* **17**, 167 (1986).
- ⁹ S. Hashimoto, M. Kawata, and S. Takemura, *J. Biochem.*, **72**, 1339 (1972).
- ¹⁰ K. Ogawa, S. Takemura, and K. Nishikawa, *J. Adv. Sci.*, **5**, 43 (1993).
- ¹¹ H. Donis-Keller, *Nucleic Acids Res.*, **8**, 3133 (1980).
- ¹² J.D. Smith, L. Barnett, S. Brenner, and R.L. Russell, *J. Mol. Biol.*, **54**, 1 (1970).
- ¹³ J.E. Celis, M.L. Hooper, and J.D. Smith, *Nature New Biol.*, **244**, 261 (1973).
- ¹⁴ F. Tamura, S. Nishimura, and M. Ohki, *EMBO J.*, **3**, 1103 (1984).
- ¹⁵ S. Steinberg, A. Misch, and M. Sprinzl, *Nucleic Acids Res.*, **21**, 3011 (1993).
- ¹⁶ S.H. Kim, in *Transfer RNA, Structure, Properties and Recognition*, eds. P.R. Schimmel, D. Söll, and J.N. Abelson, (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.) pp.83 (1979).