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Automated Chemical Synthesis of Biologically Active tRNA Having a Sequence Corresponding to *Ascaris suum* Mitochondrial $tRNA^{Met}$ toward NMR Measurements[1]

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RNA samples corresponding to *Ascaris suum* mitochondrial $tRNA^{Met}$ were chemically and automatically synthesized in amounts sufficient for NMR measurement. Conventional and rapid deprotection methods gave tRNA samples with the same amino acid-accepting activity as those prepared by other method; enzymatic synthesis, and enzymatic ligation of chemically synthesized fragments. The synthetic tRNA showed the same ^{1}H-NMR spectrum in the iminoproton region as the ligated tRNA. This rapid and reliable preparation method thus provides biologically active tRNA for NMR measurement, and further, it is applicable for synthesis of other large synthetic RNAs, by combining the site-specific isotopic labeling method.

Key words: *Ascaris suum*, chemical synthesis, mitochondria, NMR, tRNA.

Recently, NMR work on RNA molecules has been greatly improved and a number of RNA structures have been solved (*1*). This improvement has come mainly from the introduction of the *in vitro* transcription system with T7 RNA polymerase (*2*) and stable isotopic labeling (*3-5*). *In vitro* transcription with labeled NTP allows the production of labeled RNA samples with desired sequences. Using this method, longer RNAs such as tRNA can be prepared efficiently. To further enhance the NMR analyses of RNA, several methods of obtaining site-specifically labeled RNA samples have been devised. We previously demonstrated the possibility of procuring biologically active tRNA molecules in sufficient amount for NMR measurement by enzymatic ligation of chemically synthesized RNA fragments (*6*). By this method, labeled nucleotides or oligonucleotides can be incorporated into RNA sequences. Furthermore, it has recently been shown that segmental isotopic labeling can be achieved by enzymatic ligation of RNaseH-cleaved fragments (*7*).

The most straightforward way to produce site-specifically labeled RNA samples may be automated chemical synthesis. Recently, a number of research groups have produced a variety of labeled nucleotides. The combination of such labeled nucleotides with automated chemical synthesis should extend the possibilities of NMR in the structural analyses of RNAs. The problem with automated synthesis has been its lower efficiency for RNAs with longer sequences. However, recent progress in chemical synthesis of RNA seems to overcome this problem. In this paper, we show that a 64-nt long RNA having a sequence corresponding to mitochondrial methionine tRNA from *Ascaris suum* (a parasite worm living in pig intestine) (mt $tRNA^{Met}$, Fig. 1) can be synthesized with an automated DNA/RNA synthesizer, and that the synthesized sample exhibits the same amino-acid accepting activity and NMR spectrum as samples produced by other methods.

Most animal mitochondrial (mt) tRNAs have secondary structures different from that of usual tRNAs which commonly possess a cloverleaf secondary structure (*8*). In particular, most nematode, including *A. suum*, mt tRNAs have a TV-replacement loop consisting of 4-12 nucleotides, which replaces the T arm and the variable loop in usual tRNAs (*9-12*). The tertiary structure of these unusual tRNAs has been biochemically analysed (*13, 14*). However, the detailed tertiary structure has not been elucidated, since it is quite difficult to prepare such tRNAs from living organisms in amounts sufficient for structural analysis. Such tRNAs thus seem appropriate candidates for chemical and enzymatic synthesis.

We previously synthesized the mt $tRNA^{Met}$ by enzymatic ligation of chemically synthesized RNA fragments. From the amino acid-accepting activity and enzymatic probing of the ligated tRNA, the ligated tRNA was inferred to possess almost the same tertiary structure as that of the native mt $tRNA^{Met}$. This type of tRNA synthesizing method, *i.e.*, enzymatic ligation of chemically synthesized RNA fragments, was used only when the synthesis yield for a large RNA fragment (>40 nt) was very low.

The present work is concerned with improving the synthesis yield for a large RNA fragment. We were able to do this by using several post-synthesis modifications,

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including 5-ethylthio-1*H*-tetrazole as an activator (*15-20*). The tRNA was synthesized at the 1-μmol scale on an ABI 394 DNA/RNA synthesizer according to the method described (*15*). RNA phosphoramidite monomers and supports used in the synthesis employ 6-*N*-phenoxyacetyl protection for the exocyclic amine of adenosine, 2-*N*-dimethyl(dimetylamino)methylene for guanosine and 4-*N*-isobutyryl for cytidine. The syntheses were carried out (15-min coupling time) using a 0.75 M solution of 5-ethylthio-1*H*-tetrazole (Applied Biosystems) in acetonitrile as an activator (*16-18*). The oligoribonucleotides were cleaved on the instrument and the removal of the exocyclic amine protecting groups was complete within 6 h in a 3:1 mixture of ammonium hydroxide and ethanol at 55°C. After the base-protecting groups were removed, the solution was divided into two portions of 10 (for Sample A) to 1 (for Sample B) by volume, and each sample was evaporated to dryness. The silyl protecting groups of Sample A were removed with a neat solution of triethylamine trihydrofluoride for 24 h at ambient temperature (*15-17*). To Sample B was added 9 μl/A_{260} unit (40 μg) of triethylamine trihydrofluoride followed by 3 μl/A_{260} unit of dimethyl-formamide. The mixture was vortexed and heated to 55°C for 1 h (*19, 20*). To each sample was added 2 μl/A_{260} unit of H_2O and crude RNA was precipitated with 100 μl/A_{260} unit of 1-butanol after chilling at −20°C for 30 min. Two hundred A_{260} units of crude tRNA were obtained from Sample A and 20 from Sample B.

These crude tRNAs were analyzed by anion-exchange HPLC in monitoring the eluates by the absorbance at 260 nm (NucleoPac PA-100 column, Dionex Corp., 250×4 mm) (Fig. 2a), and by 10% denaturing (7 M urea) polyacrylamide gel electrophoresis (Fig. 2b). A Shimadzu column oven (CTO-6A) was used in conjunction with anion-exchange HPLC to analyze these tRNAs. The slowly-desilylated Sample A and the rapidly-desilylated Sample B showed identical patterns in the HPLC and PAGE analyses. These crude tRNAs were then purified by 10% denaturing (7 M urea) polyacrylamide gel electrophoresis. The purified tRNAs were phosphorylated at the 5′-end with T4 polynucleotide kinase. Twenty-six A_{260} units of tRNA were obtained from Sample A, and 1.4 from Sample B. The yield

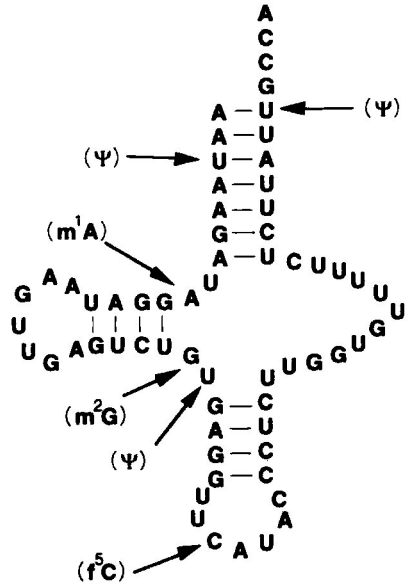


Fig. 1. **Synthetic *A. suum* mt tRNA^{Met}.** Modified bases of the native tRNA are shown in parentheses.

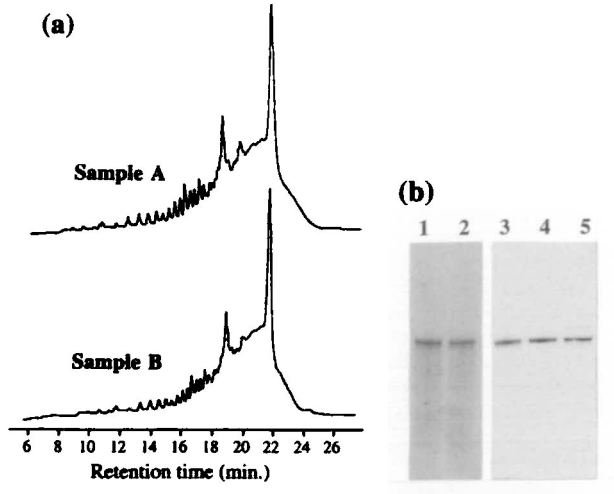
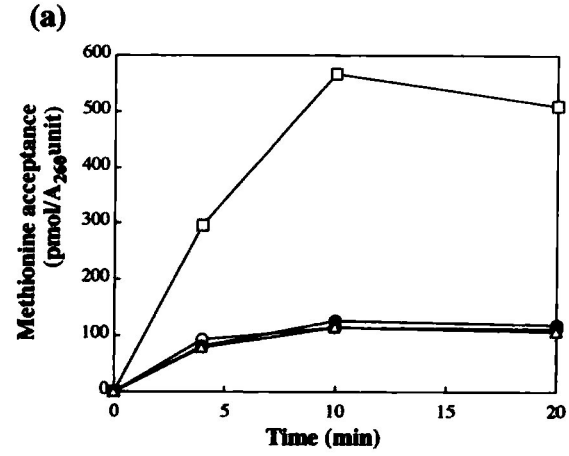


Fig. 2. **HPLC and PAGE analysis of synthetic tRNAs.** (a) Anion-exchange HPLC (at 70°C) of a crude tRNA (upper, Sample A; lower, Sample B) using the gradient described below. Solvent A: 20 mM $LiClO_4$ + 20 mM NaOAc in H_2O : CH_3CN (9 : 1) (pH 6.5). Solvent B: 600 mM $LiClO_4$ + 20 mM NaOAc in H_2O : CH_3CN (9 : 1) (pH 6.5). Flow rate: 1.0 ml/min. (Gradient: 0 to 70% B in 40 min) (b) PAGE analysis of tRNAs. 1, crude tRNA (Sample A); 2, crude tRNA (Sample B); 3, purified tRNA (Sample A); 4, purified tRNA (Sample B); 5, native *A. suum* mt tRNA^{Met}. 0.04 A_{260} unit of crude tRNAs (lanes 1, 2) and 0.01 A_{260} unit of tRNAs (lanes 3, 4, 5) were loaded.



(b)

Symbol	Sample	Km (μM)	Vmax (relative)	Vmax/Km (relative)	Reference
—□—	native tRNA	0.12	1	1	ref. 6
—△—	ligated tRNA	4.9	0.59	0.014	
—○—	sample A	5.6	0.70	0.015	this study
—●—	sample B	4.5	0.60	0.016	

Fig. 3. **The amino acid-accepting activity of tRNAs.** Time course of aminoacylation reaction of native and synthesized *A. suum* mt tRNAs^{Met} with *A. suum* mt extract (a) and their kinetic parameters (b).

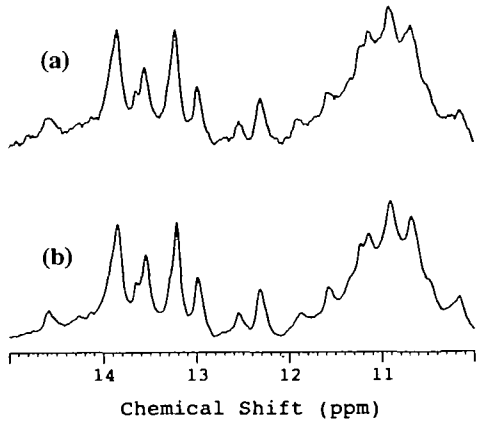


Fig. 4. **^{1}H-NMR spectra of tRNAs.** (a) Synthetic tRNA (Sample A, 25 A_{260} units). (b) Ligated tRNA (100 A_{260} units).

of purified tRNA from Sample B was lower than that from Sample A, probably because the lower amount of Sample B caused larger loss during purification. It is noted, however, that the yields of crude tRNAs were comparable.

The amino acid-accepting activity of these synthetic tRNAs was tested using *A. suum* mt extract prepared as described previously (*14*), whereby [^{35}S]methionine-acceptance and the kinetic parameters for aminoacylation were determined as described (*6*). The synthetic tRNAs were shown to have methionine-accepting activity equivalent to those of the ligated tRNA (Fig. 3a) and the tRNA transcript (*6*), all of which were about 1/5 that of the native $tRNA^{Met}$ (Fig. 3a). The lower activity of the synthetic tRNA compared to the native one is probably due to the absence of modified bases (*6*). The K_m, V_{max} and V_{max}/K_m values of both synthetic tRNAs were respectively similar (Fig. 3b), and were also similar to those of the ligated tRNA and the tRNA transcript.

The synthetic (from Sample A) and ligated tRNA samples were analyzed by NMR. Each of the tRNAs was dissolved in 200 μl of 10 mM sodium cacodylate (pH 6.0)-0.1 mM EDTA-100 mM NaCl with 10 μl of ^2H_2O. The 500 MHz ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer at probe temperature of 283 K. The water signal was suppressed by a Jump-and-Return sequence (*21*). The NMR spectra of these tRNAs in the iminoproton region are shown in Fig. 4. The spectrum of the synthetic tRNA (Fig. 4a) was identical to that of the ligated tRNA (Fig. 4b).

The method described here is useful for providing a sufficient amount of tRNA for NMR analysis. The data of methionine-acceptance activity indicate that the synthetic tRNAs are comparable to the tRNA obtained by the chemical and enzymatic method described previously (*6*). Since the ^{1}H-NMR spectrum of the synthetic tRNA in the iminoproton region is almost identical to that of the ligated tRNA, it can be concluded that both the synthetic and ligated tRNAs can be used for structural study by NMR. Although several tRNAs have been synthesized chemically (*22-25*), this is the first attempt to use wholly chemically synthesized tRNA for NMR analysis. Although the enzymatic ligation method enabled specific labeling, the ligation efficiency depended on the sequence and length of the substrate RNA. By using phosphoramidites of stable-isotopic labeled or chemically modified nucleosides, this method will provide a powerful tool for producing specific labeled or modified RNA samples with long chain lengths like tRNA in amounts sufficient for NMR analysis.

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