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Preparation of biologically active *Ascaris suum* mitochondrial tRNA^{Met} with a TV-replacement loop by ligation of chemically synthesized RNA fragments

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ABSTRACT

***Ascaris suum* mitochondrial tRNA^{Met} lacking the entire T stem was prepared by enzymatic ligation of two chemically synthesized RNA fragments. The synthetic tRNA could be charged with methionine by *A.suum* mitochondrial extract, although the charging activity was considerably low compared with that of the native tRNA, probably due to lack of modification. Enzymatic probing of the synthetic tRNA showed a very similar digestion pattern to that of the native tRNA^{Met}, which has already been concluded to take an L-shape-like structure [Watanabe *et al.* (1994) *J. Biol. Chem.*, 269, 22902–22906]. These results suggest that the synthetic tRNA possesses almost the same conformation as the native one, irrespective of the presence or absence of modified residues. The method of preparing the bizarre tRNA used here will provide a useful tool for elucidating the tertiary structure of such tRNAs, because they can be obtained without too much difficulty in the amounts necessary for physicochemical studies such as NMR spectroscopy.**

INTRODUCTION

All the prokaryotic and eukaryotic cytoplasmic tRNAs (referred to hereafter as 'usual tRNAs') have a cloverleaf secondary structure possessing three looped stems (arms), whereas most of the animal mitochondrial (mt) tRNAs have secondary structures quite different from that of usual tRNAs (1). In particular, most nematode mt tRNAs have a TV-replacement loop, which replaces the T arm and the variable loop in usual tRNAs with a less-structured loop consisting of 4–12 nucleotides (2–5). The tertiary interactions in these tRNAs have been inferred mainly from their gene sequences by referring to the tertiary structure of yeast tRNA^{Phe}, the crystal form of which has been determined unequivocally by X-ray analysis (6), supported by NMR analysis for its solution form (7).

Although three sets of binary combinations with regard to the T loop (4) common to usual tRNAs (G18-Ψ55, G19-C56 and T54-A58) are not present in nematode tRNA^{Met}, the other five commonly existing sets of binary and ternary combinations [U8-A14-A21, A9-A12-U23, A15-U (L4), A22-G (L3) and G10-U25-G (L2)] are conserved, leading to the speculation that even such tRNAs lacking the entire T stem can be folded into an L-shape-like structure roughly similar to that of usual tRNAs (8). This assumption has been supported by the enzymatic and chemical probing of mt tRNAs of several nematode species (9).

To date, there have been a number of reports concerning synthetic tRNAs obtained chemically and/or enzymatically. *Escherichia coli* tRNA^{fMet} (10,11) and yeast tRNA^{Ala} (12) have been prepared by enzymatic ligation of chemically synthesized oligoribonucleotides, and *E.coli* tRNA^{fMet} (13), yeast tRNA$_i^{Met}$ (14) and *E.coli* tRNA^{Ala} (15) have been fully synthesized chemically. As for the aminoacylation activity of these tRNAs, those synthesized so as to include modified bases (12,15) had 42–90% activity compared with that of the native tRNAs, whereas tRNAs synthesized only with unmodified nucleotides showed only 3–28% the activity. The low aminoacylation activity observed for the unmodified tRNAs is thus assumed to be due to a lack of modified residues which are involved in recognition by the cognate aminoacyl-tRNA synthetases. However, these experiments have been performed only with usual tRNAs possessing the normal cloverleaf structure; unusual tRNAs with incomplete cloverleaf structures have yet to be investigated.

In order to ascertain if the same assumption can also hold for nematode mt tRNAs lacking the entire T stem, an attempt was made to synthesize mt tRNA^{Met} from *Ascaris suum* (a parasite worm living in pig intestine) by enzymatic ligation of chemically synthesized RNA fragments. The aminoacylation activity and the enzymatic probing of the synthetic tRNA both provide good evidence that the synthetic tRNA possesses the same tertiary structure as that of the native *A.suum* mt tRNA^{Met}, both of which

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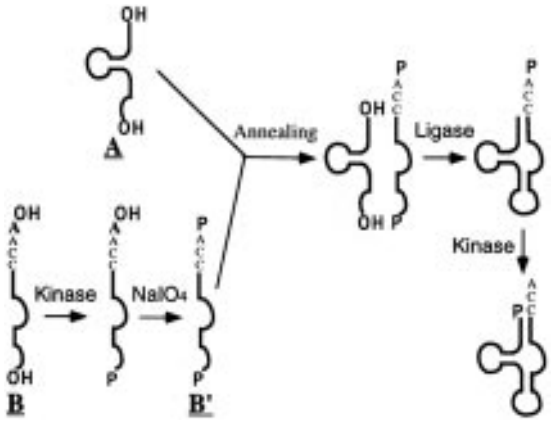

Figure 1. Process of synthesizing *A.suum* mt $tRNA^{Met}$ by a combination of chemical synthesis and ligation.

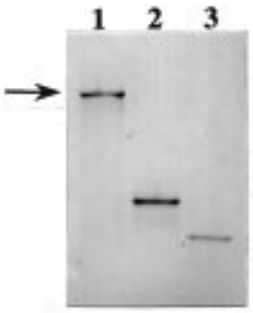

Figure 2. Polyacrylamide gel electrophoresis of the ligation product (lane 1), Fragment A (lane 2) and Fragment B′ (lane 3). The arrow shows the ligation product.

seem to be folded into an L-shape-like structure basically similar to the normal L-shape structure of usual tRNAs.

MATERIALS AND METHODS

Chemicals and enzymes

Fully-protected ribonucleoside β-cyanoehyl-phosphoramidites and CPG-packed columns (CPG supports bound with 1 µmol of blocked adenosine) were purchased from Perceptive Biosystems. T4 polynucleotide kinase was obtained from Toyobo, T4 RNA ligase from Takara Shuzo, $RNaseT_1$ and $RNaseT_2$ from Sankyo, RNasePhyM and $RNaseV_1$ from Pharmacia, $RNaseU_2$ from Sigma, $RNaseCL_3$ and *Neurospora crassa* endonuclease from Boehringer Mannheim, *Bst*NI from New England Biolabs, and Sequenase Ver.2/7-deaza-dGTP sequencing kit from USB. [5′-^{32}P]pCp (105 GBq/mmol), [γ-^{32}P]ATP (105 GBq/mmol) and [L-^{35}S]methionine (>35 GBq/mmol) were purchased from Amersham. Solid phase DNA probe was obtained from Sci-Media.

Chemical synthesis and purification of RNA fragments

Two RNA fragments (Fig. 1A and B) were synthesized by an Applied Biosystems 381A DNA synthesizer at a scale of 1 µmol. Deprotection was performed as described (16). Purification was achieved in two steps. First, fully deprotected RNA fragments were separated from partially deprotected RNAs using a YMC-pack C4-AP HPLC column as described previously (17). In the second step, a full-length RNA fragment was separated from other RNA fragments with shorter chain lengths by 10% denaturing (7 M urea) polyacrylamide gel electrophoresis.

5′-phosphorylation and 3′-dephosphorylation of Fragment B and the synthetic tRNA

Fragment B or the synthetic tRNA (19 nmol) was incubated in 200 µl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 nmol ATP, and 44 U T4 polynucleotide kinase containing 3′-phosphatase activity at 37°C for 1 h. Then, the reaction mixture was loaded on a SuperQ Toyopearl column (Tosoh). After the column was washed with 400 mM ammonium acetate to elute ATP and T4 polynucleotide kinase, the product was eluted with 2 M triethylammonium bicarbonate (pH 7.8) and the resulting solution was dried up.

Deprivation of 3′-terminal nucleoside by $NaIO_4$ treatment

Fragment B (19 nmol) was incubated in 100 µl 10 mM $NaIO_4$ at 0°C in the dark for 30 min. To the solution, 2 µl 1 M rhamnose was added and it was incubated at 0°C in the dark for 30 min. After 100 µl 2 M lysine–HCl (pH 8.5) was added to the solution, it was warmed at 45°C for 90 min. Then, 200 µl water and 1 ml ethanol were added and the product was collected by ethanol precipitation.

Ligation of Fragments A and B′

Fragments A and B′ (19 nmol each) were both heated at 65°C for 5 min in a buffer (400 µl) consisting of 100 mM Tris–HCl (pH 7.5) and 15 mM $MgCl_2$ and then annealed; 40 µl of an 11x buffer [50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 38.5 mM dithiothreitol, 165 µg/ml bovine serum albumin and 1.76 mM ATP], and 150 U T4 RNA ligase were then added. After overnight incubation at 11°C, T4 RNA ligase was removed by phenol treatment and the ligated product (the synthetic $tRNA^{Met}$) was separated from the substrate fragments using a monoQ HR HPLC column (Pharmacia). Elution was performed by a linear gradient with 0.3–0.55 M NaCl in 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 7 M urea at a flow rate of 1 ml/min.

Construction of *A.suum* mt $tRNA^{Met}$ by a combination of chemical synthesis and enzymatic ligation

Ascaris suum mt $tRNA^{Met}$ was synthesized by the scheme shown in Figure 1. The 5′-fragment [34 mer (A)], and the 3′-fragment [31 mer (B)] containing one additional Ap at the 3′-end, were chemically synthesized at a scale of 1 µmol using CPG supports bound with 1 µmol of blocked adenosine as a starting substrate. After purification, 8 A_{260} units of each fragment were recovered.

For the 3′-fragment to serve as a donor for the ligation reaction with the 5′-fragment as well as to prevent its self-ligation, both the 5′- and 3′-ends must be phosphorylated. For this purpose, Fragment B was first phosphorylated at the 5′-end with T4 polynucleotide kinase and ATP, and then the 3′-end was treated with $NaIO_4$ followed by amine treatment so as to remove the 3′-terminal adenosine, leading to exposure of the 3′-phosphate. The resulting Fragment B′ and Fragment A were annealed and

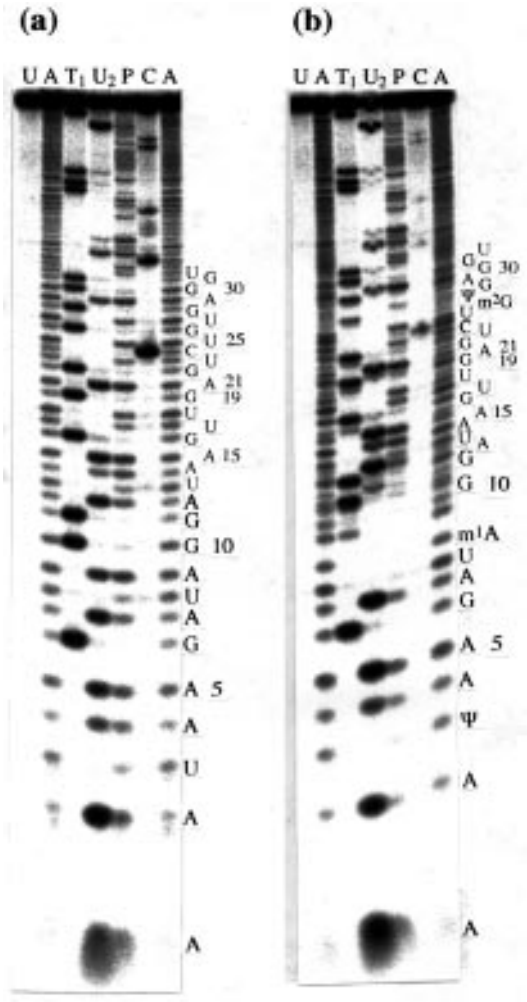

Figure 3. Sequencing gel patterns of the 5′-labeled synthetic *A.suum* mt tRNA^{Met} **(a)** and native *A.suum* mt tRNA^{Met} **(b)**. U, untreated tRNA; A, alkaline ladder; T_1, U_2, P, and C, ladders resulting from digestion with RNaseT_1, RNaseU_2, RNasePhyM and RNaseCL_3 respectively.

ligated with T4 RNA ligase giving a ligated product (Fig. 2) with ~80–90% yield.

The product was purified by a monoQ HR HPLC column and then treated with T4 polynucleotide kinase to add a phosphate group at the 5′-end and to remove a phosphate group from the 3′-end. Finally, 7 A_{260}units of the synthetic tRNA^{Met} were obtained, the nucleotide sequence of which was confirmed by the Donis-Keller's method (18) using RNases T_1, U_2, PhyM and CL_3 (19,20) to be the same as that of the native tRNA^{Met} except for the modified residues (Fig. 3).

Purification of *A.suum* mt tRNA^{Met}

Ascaris suum total tRNAs were prepared from the body wall muscle of *A.suum* as described (9). *Ascaris suum* mt tRNA^{Met} was isolated from total tRNAs in two steps by the selective hybridization method [according to ref. 21, except that Streptavidin agarose (Gibco-BRL) was used instead of the magnetic resin] using a solid-phase DNA probe possessing a sequence complementary to the residue number U8-C38 of the tRNA and further

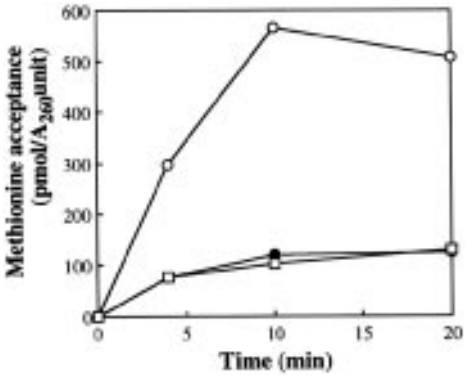

Figure 4. Time course of aminoacylation reaction of native and synthesized *A.suum* mt tRNAs^{Met} with *A.suum* mt extract. Open circles, native tRNA; filled circles, synthetic tRNA; open squares, tRNA transcript.

purification by 10% denaturing polyacrylamide gel electrophoresis. The yield of mt tRNA^{Met} was 0.2 A_{260} unit from 300 A_{260} units of *A.suum* total tRNA. The nucleotide sequence of the tRNA was confirmed by Donis-Keller's method (18).

Preparation of T7 transcripts having the sequence of *A.suum* mt tRNA^{Met}

Various oligodeoxyribonucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer for the construction of tRNA genes. The oligonucleotides were ligated with one another and the resultant DNA was inserted into the multicloning sites of pUC19 by the method of Sampson and Uhlenbeck (22). The nucleotide sequence of the plasmid thus constructed was checked by the dideoxy-termination method (23) using Sequenase Ver.2. The template DNA was prepared from *E.coli* JM109 cells cultivated on a large scale and completely digested by *Bst*NI. The transcription reaction was performed in 4 ml of a reaction mixture containing 800 µg template DNA as described (24) and the products were purified by 10% denaturing polyacrylamide gel electrophoresis. From the gel, a 0.25 A_{260} unit transcript one base longer than the native tRNA^{Met} and one of 0.05 A_{260} unit the same length as tRNA^{Met} were recovered. The transcript having the additional base at the 3′-end was treated with $NaIO_4$ to remove the base and then dephosphorylated at the 3′-end with T4 polynucleotide kinase. After confirming that the product was the same length as tRNA^{Met} by polyacrylamide gel electrophoresis, it was combined with the other transcript having the same original length as tRNA^{Met}. The total yield of the two transcripts thus obtained was 0.26 A_{260} unit.

Aminoacylation of tRNAs

Ascaris suum mt extract was prepared as described (9). Aminoacylation of tRNAs was carried out at 37°C in 20 µl of a reaction mixture containing 100 mM Tris–HCl (pH 8.7), 15 mM $MgCl_2$, 4 mM ATP, 20 mM KCl, 0.8 mM spermidine, 0.5 mM spermine, 5% polyethylene glycol (PEG 6000), 25 µM [^{35}S]methionine (500 Bq/pmol), and *A.suum* mt extract. To stop the reaction, 6 µl aliquots of the reaction mixture were mixed with 7 µl of a cold dye solution containing 0.02% bromphenol blue, 0.02% xylene cyanol, 0.1 M sodium acetate (pH 5.0) and 8 M urea. The mixture was loaded onto 10% polyacrylamide gel containing 0.1 M sodium

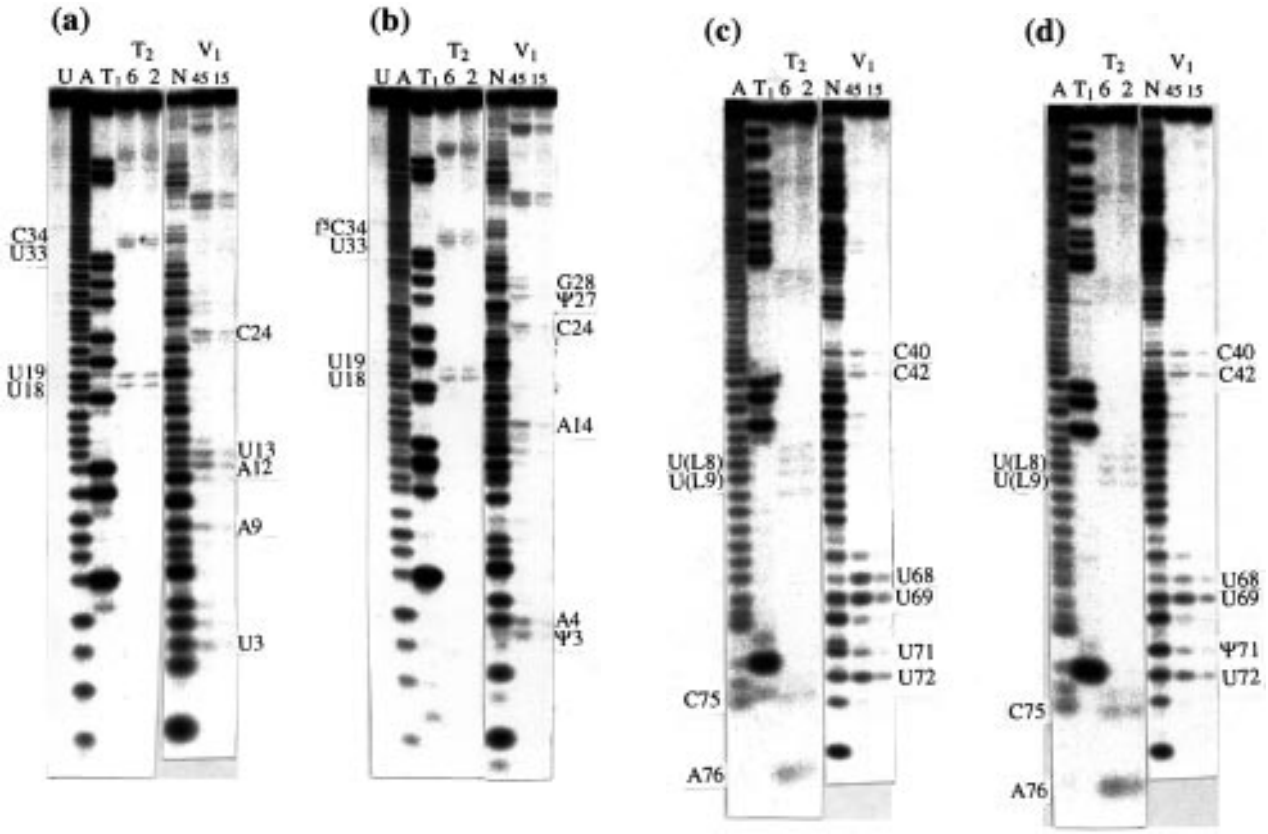

Figure 5. Enzymatic probing of the 5′-labeled synthetic *A.suum* mt tRNA^{Met} **(a)** and native *A.suum* mt tRNA^{Met} **(b)**, as well as 3′-labeled synthetic *A.suum* mt tRNA^{Met} **(c)** and native *A.suum* mt tRNA^{Met} **(d)**. Labeled tRNA was reacted with 0.00006 (6) or 0.00002 (2) U of RNaseT_2, or 0.045 (45) or 0.015 (15) U of RNaseV_1. U, untreated tRNA; A, alkaline ladder; T_1, RNaseT_1 ladder; N, ladder of digestion with *N.crassa* endonuclease serving as a size marker of RNaseV_1 which digests at the 5′-side of phosphodiester bonds. All the data are summarized in Figure 6.

acetate (pH 5.0), and 8 M urea (25). After electrophoresis, the gel was stained with toluidine blue and dried. The radioactivity corresponding to each tRNA band was estimated by an imaging analyzer (BAS 1000; Fuji Photo).

For the analysis of [^{35}S]methionine acceptance, the reaction mixture contained 0.01 A_{260} unit tRNA and mt extract of 0.8 mg/ml protein. To estimate the kinetic parameters for aminoacylation, the reaction mixture contained tRNA (8.5–170 nM of the native tRNA^{Met} and 0.85–3.4 μM of the synthetic or transcribed tRNA^{Met}) and mt extract of 0.4 mg/ml protein.

Enzymatic probing of tRNAs

Enzymatic probing of tRNAs was performed as described (26) with the following modification: 5′- or 3′-end-labeled tRNAs were digested with RNaseT_2 (0.00006 or 0.00002 U) or RNaseV_1 (0.045 or 0.015 U) in 50 mM sodium acetate (pH 6.0), 20 mM $MgCl_2$. The digestion was performed at 37°C for 7 min.

RESULTS

Aminoacylation activity of the synthetic tRNA

The synthetic tRNA^{Met} was shown to have methionine-accepting activity as catalyzed by the *A.suum* mt extract (see Fig. 4). The activity was ~1/5 that of the native tRNA^{Met}. To ascertain whether the low activity of the synthetic tRNA^{Met} was due to some form of side reaction during the chemical synthesis (incomplete deprotection, etc.) or to a lack of the modified residues, the tRNA^{Met} transcript synthesized with T7 RNA polymerase was used as a reference. The tRNA transcript had the same curve in the [^{35}S]-labeled methionine accepting reaction (Fig. 4) and showed similar K_m and V_{max} values to those of the synthetic tRNA^{Met} (Table 1). The K_m and V_{max} values of both the synthetic and transcribed tRNAs^{Met} were almost 40-fold larger and 40–50% lower respectively, than the corresponding values of the native tRNA^{Met}, resulting in a V_{max}/K_m value almost 1/70 that of the native tRNA^{Met} (Table 1). These results indicate that the low charging activity of the synthetic tRNAs is due to a lack of modified residues.

Table 1. Kinetic parameters for aminoacylation of *A.suum* mt tRNAs^{Met} by *A.suum* mt extract

tRNA^{Met}	K_m(μM)	V_{max} (relative)	V_{max}/K_m (relative)
Native	0.12	1	1
Synthetic	4.9	0.59	0.014
Transcript	5.6	0.72	0.015

K_m and V_{max} were determined from Lineweaver–Burk plots. Relative V_{max} and V_{max}/K_m values are based on the values of native tRNA.

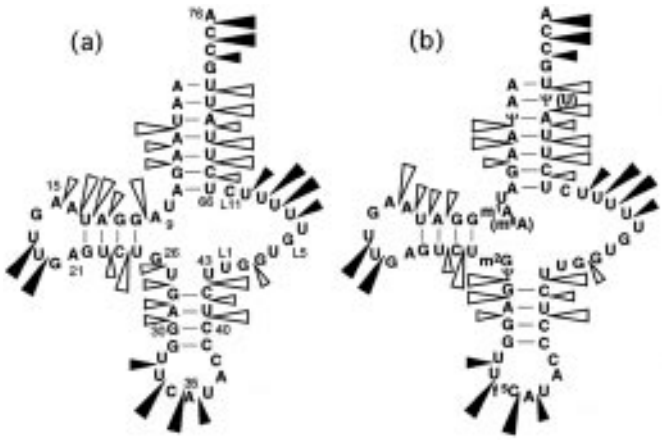

Figure 6. Summary of enzymatic probing of the synthetic *A.suum* $tRNA^{Met}$ **(a)** and native *A.suum* $tRNA^{Met}$ **(b)**. Filled and open triangles indicate the cleavage sites with $RNaseT_2$ and $RNaseV_1$ respectively (the cleavage strengths are shown by the triangle sizes) as deduced from the results in Figure 5. The base numbering conforms to that of Wolstenholme *et al.* (1). Abbreviations for modified nucleosides are as follows; Ψ, pseudouridine; m^1A, 1-metyladenosine; m^2G, 2-metylguanosine; f^5C, 5-formylcytidine.

Enzymatic probing of the synthetic $tRNA^{Met}$

To compare the tertiary structures of the synthetic and native $tRNAs^{Met}$, enzymatic probing was performed using single strand-specific $RNaseT_2$ and double strand-specific $RNaseV_1$. The limited digestion patterns obtained by polyacrylamide gel electrophoresis for both tRNAs are shown in Figure 5 and the summarized results are illustrated in Figure 6. The patterns of $RNaseT_2$ digestion were exactly the same for the two species, while those of $RNaseV_1$ digestion were almost the same except that sites 9 and 26 were both cleaved in the synthetic $tRNA^{Met}$, but not in the native one. These results clearly demonstrate that the secondary as well as the tertiary structures of the synthetic and native $tRNA^{Met}$ are almost the same.

DISCUSSION

Recently, methods for the artificial preparation of RNA molecules have made great progress. The *in vitro* transcription method which utilizes T7 RNA polymerase to transcribe any RNA gene with the T7 promoter sequence upstream of it on a plasmid (22) is very easily utilized, and any RNA with a relatively long chain length can be synthesized. One of the main drawbacks of this method, however, is that the transcription yield is dependent on the sequence of the RNA to be synthesized; in particular, the enzyme prefers G at the start point of the transcription and it appears that the yield may be related to the structural stability of the RNA product. Another disadvantage is that modified residues cannot be introduced directly into the transcript.

On the other hand, the merits of the chemical synthesis method which has recently become available are that any RNA can be synthesized independently of the nucleotide sequence and that modified residues can be introduced into the RNA. However the yield seems to be inversely dependent on the length of the RNA; for example, the yield of Fragment A (34mer) is 8 A_{260} units using a 1-µmol CPG column, while that of the full-length tRNA (64mer) is 0.3 A_{260} unit. Thus, we attempted to combine the chemical synthesis of short RNA oligomers and their enzymatic ligation. Although such a strategy has already been reported with usual tRNAs possessing the normal cloverleaf structure (10–12), it has not been adapted to the preparation of mt tRNAs with unusual secondary structures.

In the present work, we have focused on the synthesis of *A.suum* mt tRNA. It is especially difficult to isolate the native tRNA because of the difficulty of collecting sufficient numbers of the *A.suum* worm, as well as the very low yield in the preparation of a specific mt tRNA. The yield of the native $tRNA^{Met}$ is only 0.2 A_{260} unit from 300 A_{260} units of unfractionated *A.suum* total tRNA, which itself is isolated from ~200–300 g (wet weight) of *A.suum* body wall muscle (9). *In vitro* transcription using T7 RNA polymerase for synthesizing this $tRNA^{Met}$ species also gave a very low yield; 0.26 A_{260} unit of $tRNA^{Met}$ was recovered from 4-ml of reaction mixture, which is known to yield several A_{260} units of *E.coli* tRNAs in the normal case (22). By a combination of chemical synthesis and enzymatic ligation, 7 A_{260} units of $tRNA^{Met}$ were finally recovered from a 1-µmol-scale column as the starting reaction vessel, indicating the apparent suitability of this method for preparation of tRNAs with unusual secondary structures, whose tertiary structure needs to be elucidated by physicochemical analysis such as NMR spectroscopy.

The K_m value for aminoacylation of the native mt $tRNA^{Met}$ (0.12 µM) is similar to those of other mt tRNAs such as bovine mt $tRNA^{Ser}_{GCU}$ (0.178 µM), $tRNA^{Ser}_{UGA}$ (0.046 µM) (27), $tRNA^{Phe}$ (0.19 µM) (28), and hen mt $tRNA^{Phe}$ (0.15 µM) (29). If this means that the aminoacylation reaction occurs in a similar way in animal mitochondria, it may be said that the incomplete cloverleaf structure of *A.suum* mt $tRNA^{Met}$ is not directly related with the affinity toward the aminoacyl-tRNA synthetase.

The finding that the synthetic $tRNA^{Met}$ and $tRNA^{Met}$ transcripts had similar K_m and V_{max} values provides good evidence that the synthetic tRNA has no artificial modification on the normal residues. The large K_m and low V_{max} values of these tRNAs compared with those of the native $tRNA^{Met}$ strongly suggest that the modified residues in the native tRNA are responsible for recognition with methionyl-tRNA synthetase. By taking it into consideration the fact that the synthetic $tRNA^{Met}$ can be charged with *E.coli* synthetase (preliminary result), whose recognition sites lie mainly in the anticodon (30–32) and the acceptor stem (33,34) regions, it seems that the modified residues of f^5C34, Ψ3 and Ψ71 are involved in recognition with mt methionyl-tRNA synthetase. Site-specific introduction of these modified nucleosides into the synthetic tRNA will clarify this possibility, and this is now under way.

Enzymatic probing of the synthetic and native $tRNAs^{Met}$ gave almost the same patterns in sensitivity toward RNases T_2 and V_1, strongly suggesting that the synthetic tRNA without modified residues has a similar conformation to that of the native tRNA. This provides a rationale for the project to determine the tertiary structure of the bizarre tRNA using chemically synthetic tRNA, in which we are currently involved. In the probing, little difference was observed in the sensitivity—only at positions 9 and 26 (Fig. 6), which may have been arisen from the presence or absence of the modification. The synthetic $tRNA^{Met}$ contained no modified residue, whereas the native $tRNA^{Met}$ contained m^1A9 and m^2G26 at both positions. Since these sites are sensitive toward $RNaseV_1$ in unmodified synthetic $tRNA^{Met}$, the residues A9 and G26 are considered to be involved in base-pairs or in the stacking between

bases. This has been proved to be the case in the other *A.suum* tRNAs (9). Therefore, it is very likely that m^1A9 and m^2G26 are not cleaved by RNases T_2 and V_1, not because they are uninvolved in the binary or ternary combinations of bases but because the modifications themselves are resistant against RNase attack. The results in Figure 3 clearly support this conclusion, showing that both the RNases U_2 and PhyM did not cleave the native $tRNA^{Met}$ at m^1A9 and RNaseT_1 only weakly cleaved it at m^2G26.

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