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Modification at position 9 with 1-methyladenosine is crucial for structure and function of nematode mitochondrial tRNAs lacking the entire T-arm

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ABSTRACT

The mitochondria of the nematode *Ascaris suum* have tRNAs with unusual secondary structures that lack either the T-arm or D-arm found in most other organisms. Of the twenty-two tRNA species present in the mitochondria of *A.suum*, twenty lack the entire T-arm and two serine tRNAs lack the D-arm. To understand how such unusual tRNAs work in the nematode mitochondrial translation system, we analyzed post-transcriptional modifications of 11 mitochondrial tRNA species purified from *A.suum*, 10 of which lacked a T-arm and one of which lacked a D-arm. The most characteristic feature of nematode mitochondrial tRNAs lacking a T-arm was the presence of 1-methyladenosine at position 9 (m^1A_9). Synthesis of T-armless tRNAs with or without the modified nucleoside showed that T-armless tRNAs without the modification had much lower aminoacylation and EF-Tu-binding activities than native tRNAs. The addition of a single methyl group to A9 of these tRNAs was sufficient to restore nearly native levels of aminoacylation and EF-Tu-binding activity as well as tertiary structure, suggesting that m^1A_9 is a key residue for the activity of T-armless tRNAs. Thus, m^1A_9 is indispensable for the structure and function of T-armless tRNAs of nematode mitochondrial origin.

INTRODUCTION

Almost all known tRNAs have a common cloverleaf secondary structure with conserved tertiary interactions (1). However, some exceptions have been known for metazoan mitochondrial (mt) tRNAs (1). In particular, nematode mitochondria possess extremely unusual tRNAs that lack either the T-arm or the D-arm (2–5). For these two types of truncated tRNAs, nematode mt translation provides two unique elongation factors, EF-Tu1 for the tRNAs lacking the T-arm (6) and EF-Tu2 for two serine tRNAs lacking the D-arm (7). We previously reported that chemically synthesized nematode mt tRNAs without any modified nucleoside were aminoacylated much less efficiently than the native tRNAs (8), suggesting that post-transcriptional modifications in nematode mt tRNAs have crucial roles in their function. To characterize these unique tRNAs in the nematode mt translation system, it is necessary to analyze their post-transcriptional modifications.

In this work, we characterized these tRNAs by isolating and determining the RNA sequences of mt tRNAs from nematode *Ascaris suum*, including a search for modified nucleosides. We report that all the T-armless tRNAs possess 1-methyladenosine at position 9 (m^1A_9) and that this modification is structurally and functionally important for tRNAs in *A.suum* mitochondria as demonstrated by the aminoacylation assays, EF-Tu-binding analyses and enzymatic probing of these tRNAs with or without the m^1A_9 modification. It was reported that m^1A_9 in human mt $tRNA^{Lys}$ sustains the cloverleaf structure by preventing Watson–Crick base-pairing of A_9 with U_{64} in the T-stem (9). This study shows that m^1A_9 contributes to the structures of tRNAs from *A.suum* mitochondria in a different way.

MATERIALS AND METHODS

Purification of individual mt tRNAs from *Ascaris suum*

A.suum was kindly provided by Drs K. Kita and Y. Watanabe (University of Tokyo). The crude RNA fraction was extracted

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from 200 g of *A.suum* as described (10). About 600 A_{260} units of total tRNA was obtained from the crude RNA fraction by purification on a DEAE Sepharose Fast Flow column (Amersham Biosciences) according to the literature (11). Individual mt tRNAs were purified by chaplet column chromatography (11), which was invented for tRNA isolation based on a hybrid selection method using solid-phase DNA probes (12).

The probes for each tRNA have the following sequences:

$tRNA^{Leu}_{UAA}$, 5′-AGTTGTCCCATATCTTTTACGCTTAAAACA-3′;
$tRNA^{Trp}$, 5′-TGAAAACCAAG-AGTTTAAACTTAACTTAAA-3′;
$tRNA^{Gln}$, 5′-CTATACTACAACCCTTTTACACCAAAAATA-3′;
$tRNA^{Lys}$, 5′-AAAAATCTAACACTTTAAACTTAAGTTAAC-3′;
$tRNA^{Glu}$, 5′-CGAAAAAGAAATATACAAAAAATTTATACT-3′;
$tRNA^{Tyr}$, 5′-CAAAAGAAAAAATTCTTTAGATTTACAATC-3′;
$tRNA^{His}$, 5′-AAGCTCCAATTTCACTCATATCACAAACAA-3′;
$tRNA^{Asp}$, 5′-CGACAATAAGGTATAATAAAAAATTATACT-3′;
$tRNA^{Asn}$, 5′-CTAAGAGAATCACCCATTGATCAACAGTCA-3′;
$tRNA^{Cys}$, 5′-AAAGCCCACAAATCCACCTAATCTGCAATT-3′;
$tRNA^{Ser}_{UGA}$, 5′-TTTGTGTTTTCAAAACACAAAACAACTAGTTC-3′.

Each probe is complementary to certain 30 nucleosides near the 5′ or 3′ end of the individual tRNA genes (2,13), whose 3′ end was biotinylated. We isolated about one A_{260} unit for each mt tRNA, whose purity was determined to be 50–90%, using the denaturing PAGE.

Identification of modified nucleotides of mt tRNAs

5′ or 3′-[^{32}P]-labeled *A.suum* mt tRNAs were further purified by gel electrophoresis and sequenced by the method of Donis-Keller (14) using RNases T_1, U_2, PhyM and CL_3. In addition, the nucleotide sequences of $tRNA^{Leu}_{UAA}$, $tRNA^{Trp}$, $tRNA^{Gln}$, $tRNA^{Lys}$ and $tRNA^{Glu}$, including modified nucleotides, were analyzed by two-dimensional thin layer chromatography (2D-TLC) as previously described (15).

Construction of *A.suum* mt tRNAs containing m^1A9 as a sole modified base ($tRNA(m^1A9+)$)

The unmodified *A.suum* mt $tRNA^{Phe}$ and $tRNA^{Met}$ were synthesized as described in (8). The $tRNA^{Phe}(m^1A_9+)$ and $tRNA^{Met}(m^1A_9+)$ were constructed according to the scheme shown in Figure 1 using RNA fragments, F1: ACUCUGUU, F2: GUUUAUGUUUUAAAAUAUGACUUUG(U), F3: AAGAAGUUGGGAAAUGUU- AGGAGUGCCA(G), M1: AAUAAGAU, M2: GGAUAAGUUGAGUCUGUGAGGUUCA(C) and M3: UACCCUCUUGGUGUUUUU-CUCUUAUUGCCA(C), which were chemically synthesized using ABI 391 DNA synthesizer. After the synthesis of these fragments, the nucleosides in the parentheses were deprived by $NaIO_4$ treatment as below to generate the fragment bearing 3′-phosphate ends.

3′-,5′-diphosphorylation of m^1A (SIGMA) was performed basically as described (16). Molecular mass of the purified nucleotide was confirmed by mass spectrometry. Preparation of RNA fragments, 5′ end phosphorylation using T4 polynucleotide kinase (Toyobo), 3′ end-nucleoside deprivation

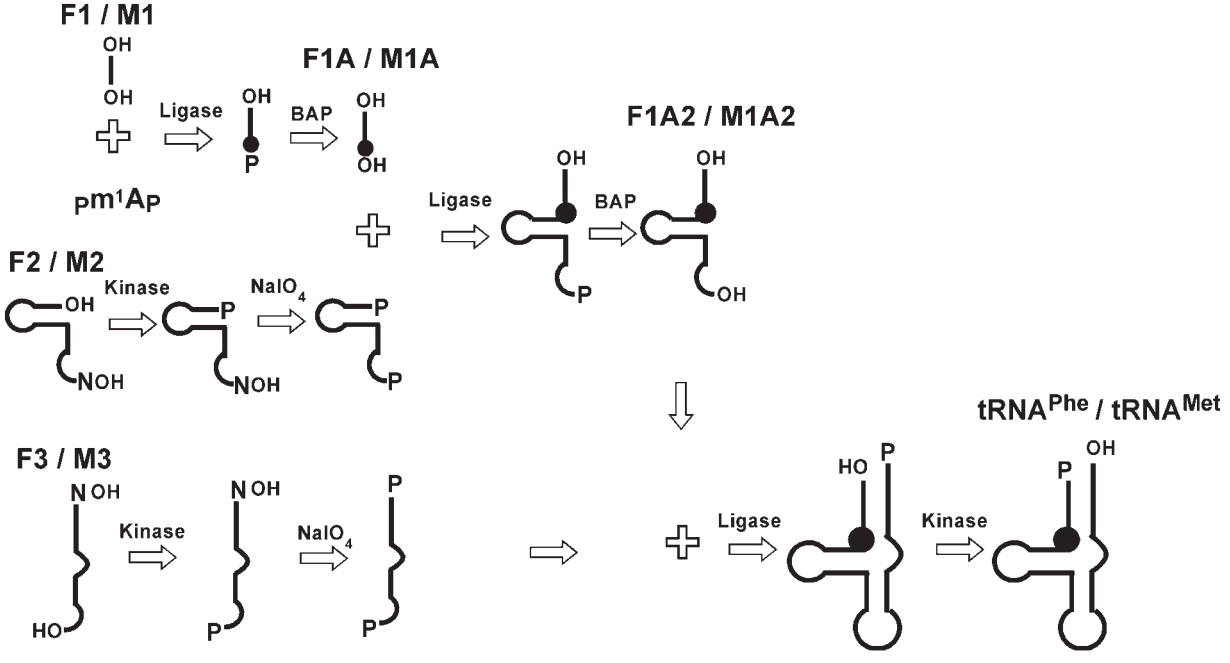


Figure 1. Diagrams showing how *A.suum* mt $tRNAs(m^1A_9+)$ were constructed. The sequences of RNA fragments [F1/M1, F2/M2 and F3/M3] are described in Materials and Methods. The m^1A_9 is described as the filled circle. The chemically synthesized tRNAs containing m^1A_9 were further converted to molecules bearing 5′-phosphates and 3′-OH ends.

Table 1. Modified nucleotides detected in the individual tRNAs from the nematode, *A.suum*

TRNA	Lacking arm	9	26	34	37	Other
Leu(UAA)	T	m^1A		U*[a]	m^1G	Ψ (2, 27, 28, 31, 32, 40)
Trp	T	m^1A		U*[a]	ms^2i^6A	Ψ (27, 28, 31, 32, 70)
Gln	T	m^1A		U*[a]	m^1G	Ψ (27, 31, 32, 38, 40, 71)
Lys	T	m^1A	$m^2{}_2G$	U*[a]	t^6A	Ψ (25, 27, 28, 39, 69)
Glu	T	m^1A		U*[a]	m^1G	Ψ (25, 27, 29, 32, 71)
Tyr	T	m^1A		Q	ms^2i^6A	Ψ*[b]
His	T	m^1A		Q		Ψ*[b]
Asp	T	m^1A		Q	m^1G	Ψ*[b]
Asn	T	m^1A		Q	m^1G	Ψ*[b]
Cys	T	m^1A			m^1G	Ψ*[b]
Arg*[c]	T	m^1A			m^1G	m^1A8, Ψ (25, 29, 32, 38, 40, 71)
Met*[d]	T	m^1A	m^2G	f^5C		Ψ (3, 27, 71)
Phe*[d]	T	m^1A			m^1G	Ψ (25, 27, 31, 32)
Ser(UGA)	D				ms^2i^6A	Ψ*[b]
Ser(UCU)*[d]	D				t^6A	Ψ (26, 40, 67)

The numbers in the parentheses indicate the positions of modified nucleosides. Each residue is numbered according to (1,2). Abbreviations; $m^2{}_2G$, N^2,N^2-dimethylguanosine; m^2G, N^2-methylguanosine; Q, queuosine; f^5C, 5-formylcytidine; m^1G, 1-methylguanosine; ms^2i^6A, N^6-(3-methyl-2-butenyl)-2-methylthioadenosine; t^6A, N^6-threonylcarbamoyladenosine; Ψ, pseudouridine.

*[a]Modified uridines. Details will be described in other manuscript (Sakurai *et al*., in preparation)

*[b]Positions of modified nucleotides in tRNAs for Tyr, His, Asp, Asn, Cys and Ser(UGA) were investigated by LC/MS analysis of RNase T_1-digested tRNA (data not shown). The location of each Ψ could not be determined by this analysis because its molecular mass is same as uridine.

[c][d]These results were already described in Watanabe *et al*. (20) and (10), respectively.

using $NaIO_4$, and dephosphorylation using *E.coli* alkaline phosphatase (BAP) (Takara Shuzo) were performed as described (17). The ligation reaction was performed basically as described (17) at 11°C for 15–20 h in a buffer consisting of 50 mM Tris–HCl (pH 7.6), 15 mM $MgCl_2$, 3.5 mM DTT, 15 μg/ml BSA, 5% PEG, 0–10% DMSO, 210 μM ATP and 500 U/ml T4 RNA ligase. The RNA fragments were included in the ligation reaction mixture at the concentration of 210 μM for pm^1Ap, 90 μM for F1 and M1, and 30–70 μM for the other fragments.

The ligation products F1A and M1A was purified by monoQ HR 5/5 column (Amersham Pharmacia Biotech) chromatography followed by desalting using Sep-Pak plus C18 Cartridges (Water). The presence of the m^1A at the 3′ end of F1A and M1A was confirmed by two-dimensional thin layer chromatography (15). F1A2, M1A2 and tRNAs thus obtained were purified by 10% denaturing (with 7 M urea) PAGE.

Aminoacylation of tRNAs

To obtain tRNAs bearing 5′-phosphate and 3′-OH ends required for aminoacylation reaction, $tRNA^{Phe}$ and $tRNA^{Met}$ were treated by T4 polynucleotide kinase. *A.suum* mt extract was prepared as described previously (10). Aminoacylation reaction was carried out basically as described in (8), at 37°C in 30 μl of the 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 #6000, 0.01 A_{260} unit of the tRNA, 27 μM [^{14}C] phenylalanine (17.4 Bq/pmol) or 25 μM [^{35}S]-methionine (670 Bq/pmol) and *A.suum* mt extract of 0.8 mg/ml protein. At appropriate times, 9 μl aliquots were withdrawn from the reaction mixture and placed onto dry Whatman 3MM filters. The filters were washed three times with 5% (w/v) trichloroacetic acid (TCA) and once with ethanol, and then dried and measured by a liquid scintillation counter (ALOKA).

Deacylation protection assay

Caenorhabditis elegans mt EF-Tu1/Ts complexes were prepared according to the literature (18). [^{35}S]Met-$tRNA^{Met}$ and [^{14}C]Phe-$tRNA^{Phe}$ were prepared as follows: the tRNAs were aminoacylated as described above except for the reaction scale (100 μl), and purified by phenol extraction and 2-propanol precipitation. The deacylation-protection assay was performed basically as described (6,19) using 5 μM EF-Tu1/Ts complex, and 50 nM [^{35}S]Met-$tRNA^{Met}$ or [^{14}C]Phe-$tRNA^{Phe}$ at 30°C for the deacylation reaction.

Enzymatic probing of tRNAs

Enzymatic probing of tRNAs was performed as described (10). 5′ or 3′ end-[^{32}P]-labeled tRNAs were digested with RNase T_2 (0.00006 or 0.00002 U) or $RNaseV_1$ (0.045 or 0.015 U) in 5 μl of 50 mM sodium acetate (pH 6.0), 20 mM $MgCl_2$. The digestion was performed at 37°C for 7 min.

RESULTS

Identification of modified nucleosides of nematode mt tRNAs

We isolated mt tRNAs from nematode, *A.suum* as described in Materials and Methods, whose sequences were confirmed by the methods of Donis-Keller (14) (data not shown). As for the five tRNAs for Leu(UAA), Trp, Gln, Lys and Glu, modified nucleosides were analyzed by the method of Kuchino *et al*. (15). All 11 tRNAs purified in this work were analyzed by LC/MS (Figure S1 showed the results for six tRNAs other than above five tRNAs). Table 1 summarized modifications of nematode mt tRNAs analyzed in this and previous works (10,20). Various modified nucleosides, such as m^1A_9, $m^2{}_2G_{26}$, Q_{34}, m^1G_{37}, $ms^2i^6A_{37}$ and t^6A_{37}, were found in

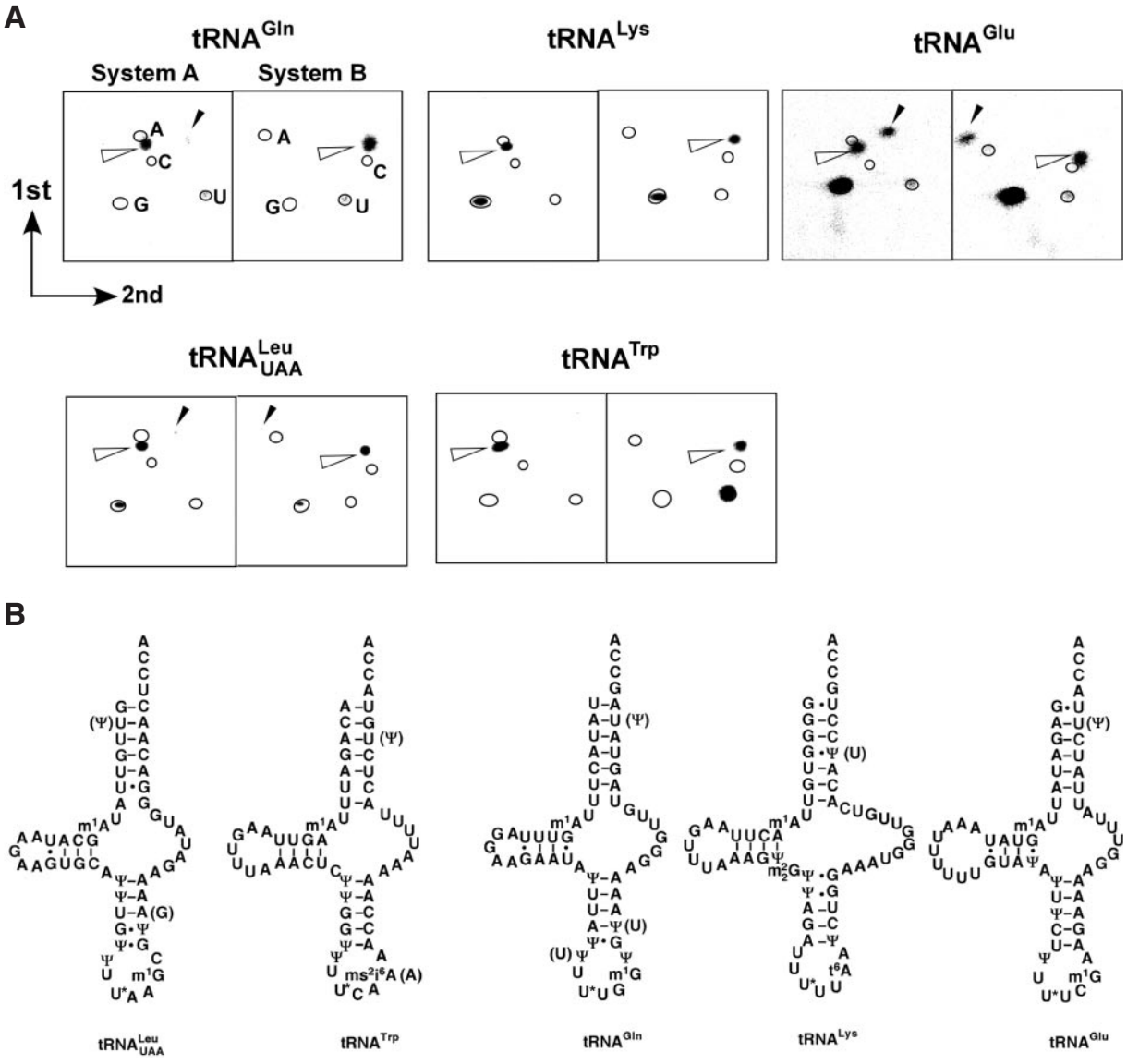


Figure 2. (**A**) Two-dimensional thin layer chromatography of modified nucleosides at position 9 of mitochondrial tRNAs. Open circles indicate spots of nucleotides (A, U, G and C bearing 5′-phosphate) detected by UV shadowing. Open and filled triangles indicate m^1A and m^6A [which is considered to be converted form of m^1A (21)], respectively. Nucleotide analysis was performed as follows. Each 3′ fragment generated by alkaline digestion of the tRNA was labeled at its 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled RNAs were loaded onto 10% denaturing polyacrylamide gel and each 5′-labeled 3′-fragment was excised from the gel. The fragments were then digested with P_1 nuclease and the resulting 5′-labeled mononucleotides were analyzed by TLC. 2D-TLC analyses were performed using the solvents; isobutyric acid/concentrated ammonia/water (66:1:33, v/v/v) for the first dimension in both systems, 2-propanol/HCl/water (70:15:15, v/v/v) for the second dimension in system A, and ammonium sulfate/0.1 M sodium phosphate, pH 6.8/1-propanol (60 g:100 ml:2 ml) for the second dimension in system B. 5′-nucleotides of tRNAs were also detected by TLC because each 5′-fragment generated by alkaline digestion of the tRNA was also labeled at its 5′-terminus by the phosphorylation reaction described above (although the 5′-fragment had 5′-phosphate even before the reaction, the 5′-phosphate may have exchanged with labeled phosphate of [γ-^{32}P]ATP), and the 5′-labeled 5′-fragments migrated together with the 5′-labeled 3′-fragments on the gel. (**B**) Nucleotide sequences of *A.suum* mt tRNAs. Abbreviations are the same as in Table 1. Asterisks (*) show modified uridines, whose details will be described in another manuscript (Sakurai *et al*., in preparation).

nematode mt tRNAs. Among them, only m^1A_9 was common in T-armless tRNAs but not in D-armless tRNAs (Table 1).

Figure 2 shows the nucleotide at position 9 of each tRNA was m^1A. N^6-methyladenosine (m^6A) was also detected, especially for $tRNA^{Glu}$ (Figure 2), but it is probably generated from m^1A through the Dimroth rearrangement reaction (21) during the purification and analytical procedures. Figure S1 also showed the existence of m^1A in the T-armless tRNAs.

Aminoacylation activities of T-armless tRNAs with or without m^1A_9

Since m^1A_9 was identified as the sole modified nucleoside at position 9 in all of the T-armless tRNAs of nematode mitochondrial origin, it seems likely that this nucleoside has a crucial role to play in the functioning of these tRNAs. To investigate this possibility, unmodified tRNAs and tRNAs

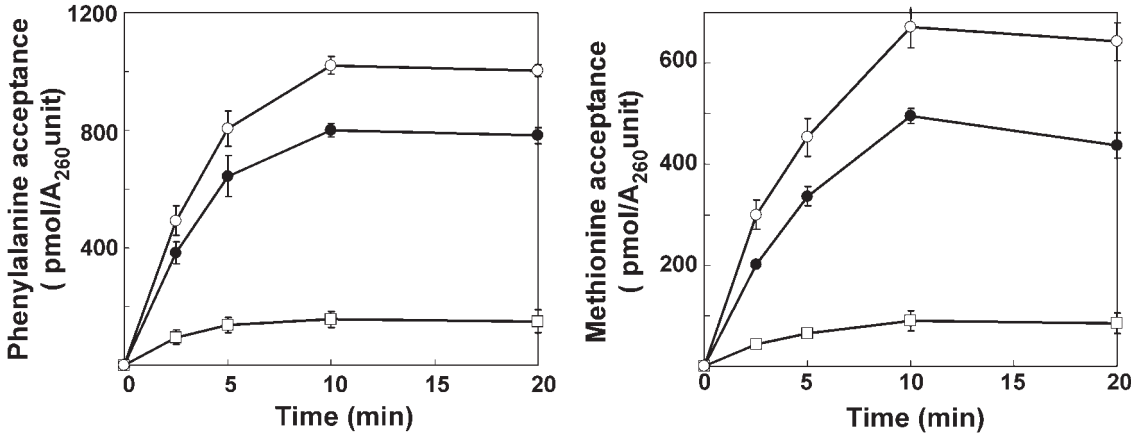


Figure 3. Aminoacylation activities of *A.suum* mt tRNA^{Phe} (left) and tRNA^{Met} (right) with three different forms. Open squares, unmodified tRNAs; open circles, native tRNAs; filled circles, tRNAs(m^1A_9+). Blank value at each time point, which was measured by same procedures using the reaction mixture without tRNA, was subtracted from each raw data.

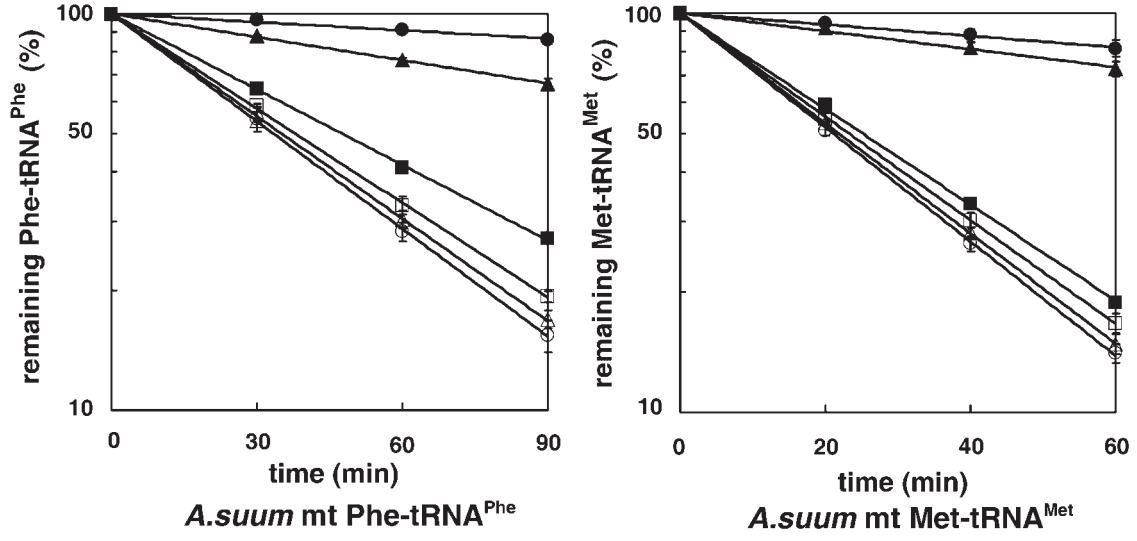


Figure 4. Deacylation-protection assay of the aminoacyl ester bond against hydrolysis (19) of *A.suum* mt Phe-tRNA^{Phe} (initial concentration: 50 nM) (left) and *A.suum* mt Met-tRNA^{Met} (initial concentration: 50 nM) (right) in the presence of 5 μM *C.elegans* mt EF-Tu1/Ts complex. Unmodified tRNAs (squares), tRNAs(m^1A_9+) (triangles) and native tRNAs (circles) were analyzed. Filled and open symbols show the deacylation profile with and without EF-Tu1/Ts, respectively.

(m^1A_9+) specific for both phenylalanine and methionine were synthesized as described in Materials and Methods. The amino acid-acceptance rates of native tRNA, unmodified tRNA and tRNA(m^1A_9+) were compared. The amino acid-acceptance rates of the unmodified tRNAs were 10–20% of those of native tRNA^{Phe} and tRNA^{Met} (Figure 3). On the other hand, tRNA^{Phe} (m^1A_9+) and tRNA^{Met} (m^1A_9+) exhibited much higher acceptance rates (70–80% of native tRNAs) than the unmodified tRNAs. These results indicate that m^1A_9 is quite important for the recognition of T-armless tRNAs by aminoacyl-tRNA synthetases [Phenylalanyl-tRNA synthetase (PheRS) and Methionyl-tRNA synthetase (MetRS) in this case] of *A.suum* mitochondria.

EF-Tu-binding activity of T-armless tRNAs depends on the m^1A_9 modification

To examine the contribution that m^1A_9 makes to nematode mt tRNA binding to nematode mt EF-Tu1, we performed the deacylation protection assay (6,19). The slow deacylation rates of native Phe-tRNA^{Phe} and Met-tRNA^{Met} in the presence of EF-Tu1 indicate that EF-Tu1 binds to these tRNAs and protects them from deacylation (Figure 4). In contrast, unmodified Phe-tRNA^{Phe} (Figure 4, left) and Met-tRNA^{Met} (Figure 4, right) were scarcely bound by EF-Tu1. The introduction of only a single modification (m^1A_9) into these tRNAs restored their EF-Tu-binding activities to nearly those of native tRNAs (Figure 4). These results suggest that

m^1A at position 9 is essential for the binding of T-armless tRNAs by nematode mt EF-Tu1, probably because the modification permits the adoption of the correct tertiary structure by the tRNA.

The effect of m^1A_9 on the higher-order structure of T-armless tRNA

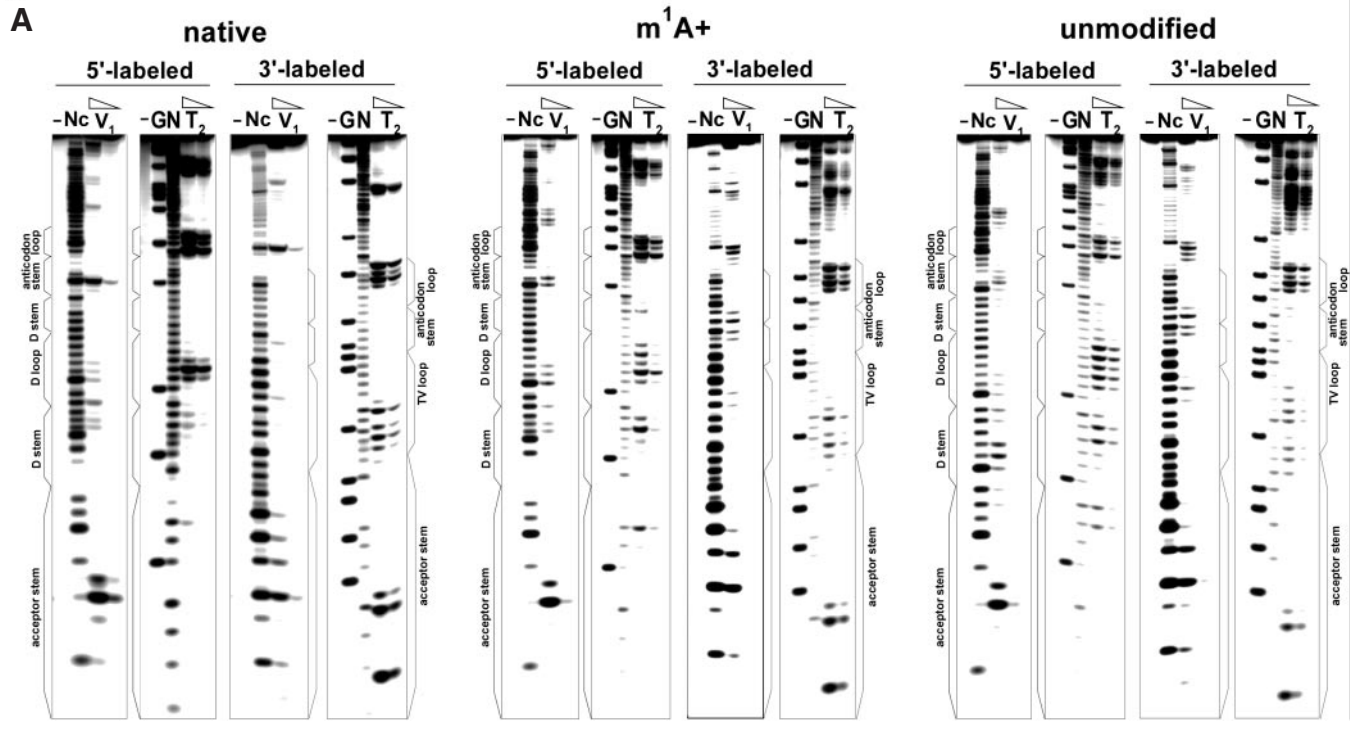
To elucidate the effect of m^1A_9 on the higher-order structures of the T-armless tRNAs, we adapted the enzymatic probing technique using RNase T_2, which is specific for single-stranded RNA regions, and RNase V_1, which is specific for double-stranded RNA regions (10). As shown in Figure 5, the modified tRNAs (m^1A_9+) showed almost the same cleavage pattern as native tRNA, whereas unmodified tRNAs showed a slightly different pattern in their D-arm and TV-replacement loop. We also performed UV-melting analysis to determine the T_m values of these tRNAs. Although no appreciable differences in the T_m values between m^1A_9-containing and unmodified tRNAs were detected for $tRNA^{Phe}$ (49.5°C) and $tRNA^{Met}$ (52°C), subtle differences were apparent in the shape of the melting curves (data not shown). These results indicate that m^1A_9 has no significant effect on tRNA thermostability, but has a pronounced effect on the higher-order structure of these tRNAs, which would be crucial for their recognition by aminoacyl-tRNA synthetases and EF-Tu.

DISCUSSION

Modified nucleosides have been considered to play an important role in the structure and function of tRNA (22,23). However, information on the nucleoside modifications of tRNAs is relatively scarce, as compared to the enormous amount of information on the tRNA gene sequences of various organisms (1). To determine the nucleoside modifications present in nematode mt tRNAs with unusual secondary structures, we purified mt tRNAs and analyzed them by several methods. Although only a small amount of mt tRNAs is included in the eukaryotic cells in comparison with a large amount of cytoplasmic tRNAs, the hybrid selection method using solid-phase DNA probes (11) facilitated efficient isolation of eleven mt tRNAs at same time from only 200 g of *A.suum* worms.

All T-armless tRNAs analyzed so far have m^1A at position 9 (Table 1). Other T-armless tRNAs of nematodes, for which RNA sequence information at DNA level is available, *A.suum* (3), *C.elegans* (3), *Onchocerca volvulus* (4), *Ancylostoma duodenale* (5) and *Necator americanus* (5), are known to also possess A_9 at the DNA level, indicating that this position is most probably modified to m^1A. These facts strongly suggest that m^1A_9 is important in terms of structure and/or function for T-armless tRNAs.

Our previous results showed that the aminoacylation efficiency of unmodified $tRNA^{Met}$ of *A.suum* mitochondria was much lower than that of the native $tRNA^{Met}$ (8). *A.suum* mt $tRNA^{Met}$ lacking the T-arm has six modified nucleosides (10). Thus, one or some of these modified nucleosides should be necessary for the correct structure and efficient aminoacylation of the tRNA. The most plausible candidate for this is m^1A_9 because we here found m^1A_9 was common in the T-armless tRNAs of *A.suum* mitochondria. In this study, we synthesized two T-armless tRNAs possessing m^1A_9 as the sole modified nucleoside ($tRNA^{Phe}$ and $tRNA^{Met}$), to investigate the role of m^1A_9 in maintaining their higher-order structures as well as the aminoacylation and EF-Tu1-binding activities. All of the experimental results clearly demonstrated that m^1A_9 plays a crucial role in the biological activities of the T-armless tRNAs in terms of the



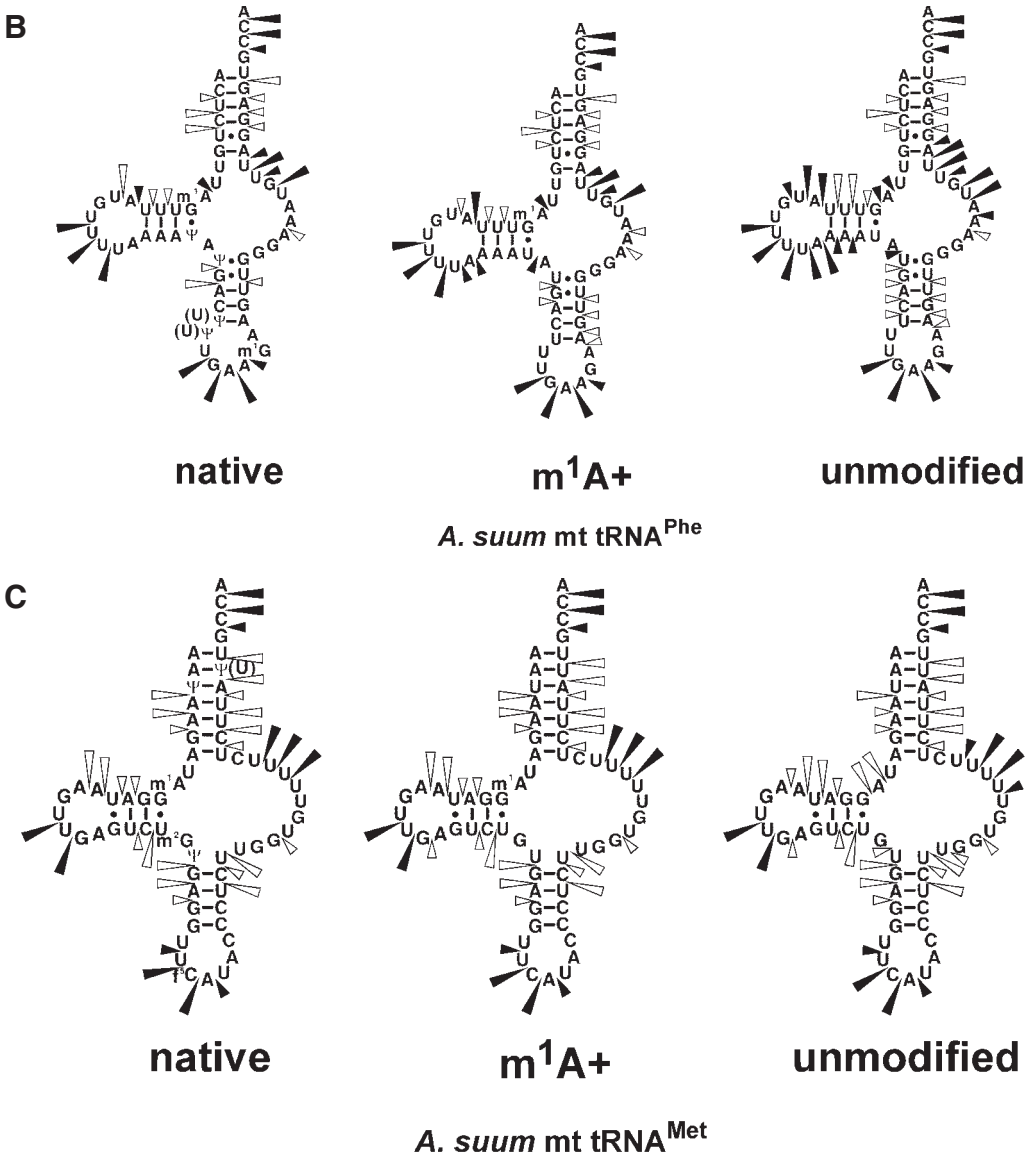


Figure 5. Enzymatic probing of modification variants of 5′- or 3′-labeled *A.suum* mt $tRNA^{Phe}$ and $tRNA^{Met}$. (**A**) Labeled $tRNAs^{Phe}$ were reacted with 0.009 or 0.003 U/μl of RNase V_1 and 0.000012 or 0.000004 U/μl of RNase T_2. Open triangles indicate the amounts of RNases. Symbols: −, untreated tRNA; N, alkaline ladder; G, RNase T_1 ladder; Nc, ladder of digestion with *Neurospora crassa* endonuclease as a size marker of $RNaseV_1$, which digest at the 5′ side of phosphodiester bonds. (**B** and **C**) Summary of enzymatic probing of modification variants of the $tRNA^{Phe}$ and $tRNA^{Met}$. Filled and open triangles indicate the cleavage sites with $RNaseT_2$ and $RNaseV_1$, respectively (the cleavage strengths are shown by the triangle sizes).

aminoacylation and EF-Tu1-binding activities, probably by preserving the higher-order structures of the tRNAs to the native types.

As for how the subtle differences found in the structure probing experiments can account for the large differences observed in the functional studies, a possible explanation is as follows. The digestion pattern of the hinge region of the tRNA for Phe or Met depended on the existence of 1-methyl group at A9 in the RNase probing experiments. Any change in the structure of the tRNA hinge region might affect the distance between the CCA sequence and the anticodon. This distance seems to be important for tRNA recognition by ARSs because bacterial PheRS (24) and MetRS (25) mainly recognize elements around the CCA-end and the anticodon. From the same reason, it seems that *A.suum* PheRS and MetRS did not efficiently aminoacylate tRNAs lacking 1-methyl group at A9. Since *C.elegans* mt EF-Tu1 recognizes position 13 and 14 of T-armless tRNA (M. Sakurai *et al*., manuscript in preparation), the subtle difference in the structure around position 9 would have caused the large differences in EF-Tu1-binding activity.

Structural importance of m^1A_9 was also reported for human mt $tRNA^{Lys}$ (9,26) in which it was revealed that the unmodified $tRNA^{Lys}$ was folded into a non-canonical stem–loop structure, including elongated acceptor stem with base pairs A_8–U_{65}, A_9–U_{64} and G_{10}–C_{63}. It should be noted that native

human mt $tRNA^{Lys}$ contains a normal T-arm and loop (although it lacks the consensus nucleotide sequence) and folds into a cloverleaf structure (26). It is regarded that 1-methyl group at A_9 of the tRNA maintains canonical cloverleaf structure by precluding an undesired Watson–Crick base-pairing of A_9 with U_{64} in the T-stem. Our results showed that the structures of native and unmodified $tRNAs^{Met}$ were slightly different from each other, although they were not so dramatically different in the secondary structural level as the case of human mt $tRNA^{Lys}$ (9,26). It is difficult for unmodified $tRNA^{Phe}$ and $tRNA^{Met}$ to form an alternative structure with a long acceptor stem, such as that formed by unmodified human $tRNA^{Lys}$. This is because the 8th and 10th base-pair positions of the long acceptor stems of $tRNA^{Phe}$ and $tRNA^{Met}$ and the 9th base-pair position in $tRNA^{Phe}$ are not complementary. Thus, in nematode mt T-armless tRNA, the main role of m^1A_9 seems not to be the inhibition of an alternative structure with a long acceptor stem, but the maintenance of the proper structure around m^1A_9 (i.e. the tRNA hinge region). Since m^1A_9 in T-armless tRNAs affected tRNA activities with three different proteins (MetRS, PheRS and EF-Tu1), it presumably contributes not only to the local structure around position 9, but also to the overall structure of the tRNAs. In the RNase probing experiments, the lack of 1-methyl group at position 9 affected mainly the hinge region, including the connector region between acceptor- and D-stems, and the TV-replacement loop (Figure 5B and C). Thus, the lack of methyl group might affect the overall structure, such as the angle between the acceptor and anticodon stems.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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