Higher-order structure of bovine mitochondrial tRNA^{Phe} lacking the 'conserved' GG and TΨCG sequences as inferred by enzymatic and chemical probing

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ABSTRACT

Bovine mitochondrial (mt) phenylalanine tRNA (tRNA^{Phe}), which lacks the 'conserved' GG and TΨCG sequences, was efficiently purified by the selective hybridization method using a solid phase DNA probe. The entire nucleotide sequence of the tRNA, including modified nucleotides, was determined and its higher-order structure was investigated using RNaseT₂ and chemical reagents as structural probes. The D and T loop regions as well as the anticodon loop region were accessible to RNaseT₂, and the N-3 positions of cytidines present in the D and T loops were easily modified under the native conditions in the presence of 10mM Mg²⁺. On the other hand, the nucleotides present in the extra loop were protected from the chemical modification under the native conditions. From the results of these probing analyses and a comparison of the sequences of mitochondrial tRNA^{Phe} genes from various organisms, it was inferred that bovine mt tRNA^{Phe} lacks the D loop/T loop tertiary interactions, but does have the canonical extra loop/D stem interactions, which seem to be the main factor for bovine mt tRNA^{Phe} to preserve its L-shaped higher-order structure.

INTRODUCTION

The higher-order structure of tRNA is thought to be closely related to its function. A normal tRNA has a set of tertiary interactions in its molecule to hold its higher-order structure. For example, interactions between the 'conserved' GG sequence in the D loop and the TΨCR sequence in the T loop are important to maintain the L-shaped tertiary structure (1). On the other hand, mitochondrial (mt) DNA sequence analyses have revealed that many animal mt tRNA genes lack some invariant and semi-

invariant nucleotides which are necessary for forming tertiary interactions (2). However, there have been only a few investigations of the higher-order structures of mt tRNAs having unusual characteristics. de Bruijn and Klug (3) have shown that the tertiary structures of bovine and human mt tRNA^{Ser(GCU)} [tRNA^{Ser} having the anticodon GCU and corresponding to the codon AGY; formerly it was called tRNA^{Ser(AGY)} (3–6)] lacking a D arm are very similar to that of yeast cytosolic (cyt) tRNA^{Phe}. Yokogawa *et al.* (4,5) found that bovine mt tRNA^{Ser(UGA)} [tRNA^{Ser} having the anticodon UGA and corresponding to the codon UCN; formerly it was called tRNA^{Ser(UCN)} (4,5)] seems to possess a unique cloverleaf structure, although the D loop/T loop interaction is preserved. And Ueda *et al.* (6) reported that the tertiary interactions present in bovine mt tRNA^{Ser(GCU)} are important for recognition by homologous seryl-tRNA synthetase. Although Gebhardt-Singh and Sprinzl (7) showed that bovine mt serine tRNAs could bind bacterial elongation factor Tu, translational capability of the mt tRNAs^{Ser} has not been fully demonstrated *in vitro*.

The bovine mt tRNA^{Phe} gene also lacks some invariant nucleotides, such as the GG sequence in the D loop region and the TTCRA sequence in the T loop and thus tRNA^{Phe} should have a loose tertiary structure. The experimental evidence for this structural lability was given by Kumazawa *et al.* (8) who first purified the bovine mt tRNA^{Phe} and measured its melting profiles. They also showed that the mt tRNA^{Phe} could be efficiently aminoacylated by its homologous phenylalanyl-tRNA synthetase but not at all by cytosolic or bacterial synthetase (8). Kumazawa *et al.* (9) also established a homologous *in vitro* poly(U)-directed translation system using bovine mt ribosomes, elongation factors and tRNA^{Phe}, with which they proved that bovine mt tRNA^{Phe} with a loose tertiary structure actually functions in the mt translation process. Therefore, a detailed structural analysis of bovine mt tRNA^{Phe} would be the first step

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for understanding the influence of the unusual characteristics of mt tRNAs on mt protein synthesis. However, difficulty in the purification of this tRNA species [in the conventional column chromatography, several peaks corresponding to mt tRNA^{Phe} sometimes appear (8)] has prevented us from further developing this kind of experiment. To solve this problem, we have devised a selective hybridization method using a solid phase DNA probe to obtain a single species of mt tRNA^{Phe} in sufficient quantity for structural analyses. Here, we report on the primary structure of the tRNA^{Phe}, including modified nucleotides, and its higher-order structure as inferred from enzymatic and chemical probing experiments.

MATERIALS AND METHODS

Chemicals, enzymes and tRNAs

Dimethylsulphate (DMS) was obtained from Aldrich, diethylpyrocarbonate (DEPC) from Sigma, hydrazine from Pierce and sodium borohydride, aniline and other chemicals from Wako Pure Chemicals. [5'-³²P]pCp (3000 Ci/mmol) and [¹⁴C] L-phenylalanine (485.7 mCi/mmol) were purchased from NEN Research Products, [γ -³²P]ATP (3000 Ci/mmol) from Amersham. T4 polynucleotide kinase was obtained from Nippon Gene, T4 RNA ligase and RNasePhyM from Pharmacia and *E. coli* A19 alkaline phosphatase from Takara Shuzo, RNaseT₁ and RNaseT₂ from Sankyo, RNaseU₂ from Sigma and RNaseCL₃ from Boehringer Mannheim. Yeast tRNA^{Phe} was purchased from Boehringer Mannheim. Unfractionated mt tRNAs (tRNA^{Mix}) were extracted from the mitoplast fraction of bovine liver as described previously (4).

Procedure for the selective hybridization method using a solid phase DNA probe

A biotinylated DNA probe, 5'-TGTTTATGGAGTTGGGAG-ACTCATCTAGGC-Biotin-3' which is complementary to the 30 nucleotides of tRNA^{Phe} gene (10) extending from the anticodon stem (G40) to the discriminator base (A73), was chemically synthesized using an Applied Biosystems model 391 DNA synthesizer with the use of 3'-Biotin-ONTM CPG (CLONTECH). The 3'-biotinylated DNA probe was purified by electrophoresis on a 15% polyacrylamide gel in the presence of 7 M urea. The purified 3'-biotinylated DNA probe (10 μ g) dissolved in 500 μ l of 10 mM Tris-HCl (pH 7.5) was mixed with 1 mg of magnetic resin [Dynabeads M-280 Streptavidin (Dyna)] which had been pre-washed with 10 mM Tris-HCl (pH 7.5) and incubated for 30 min at room temperature. Thus, the 3'-biotinylated DNA probe was bound to the magnetic resin through the Biotin-Avidin interaction. After incubation, the resin was washed three times with 10 mM Tris-HCl (pH 7.5). This solid phase DNA resin was stored at 4°C.

Mitochondrial tRNA^{Mix} was dissolved in 900 mM NaCl, 90 mM sodium citrate (pH 7.0) [6 $\times$ SSC] to a final concentration of 100 A₂₆₀ units (5 mg)/ml. The tRNA solution (100 μ l) was mixed with 1 mg of the solid phase DNA resin which had been pre-washed with 6 $\times$ SSC several times. The mixed solution was incubated for 30 min at room temperature. After incubation, the hybridized resin was separated from the tRNA solution containing tRNAs other than tRNA^{Phe} by using a magnetic particle concentrator (Dyna). The hybridized resin was washed with 3 $\times$ SSC until the absorbance at 260 nm of the wash solution was almost zero. Then the hybridized resin was suspended in 0.1 $\times$ SSC and incubated at 60°C for 2 min. Mitochondrial

Figure 1. RPC-5 column chromatography of a bovine liver mt tRNA^{Phe} fraction purified by the selective hybridization method. About 2 A₂₆₀ units of the tRNA^{Phe} fraction were applied onto an RPC-5 column (5 mm $\times$ 6 cm). A linear gradient of NaCl from 0.4 M (20ml) to 1.0 M (20ml) in a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM Mg(OAc)₂ was used for elution. The thick, solid line indicates the absorbance monitored at 260 nm, the thin solid line with closed circles [¹⁴C] phenylalanine acceptor activity, and the dashed line with open circles hybridization activity measured by Cherenkov counting.

Figure 2. Primary structure of bovine mt tRNA^{Phe} and summary of enzymatic and chemical probing experiments. a) The complete nucleotide sequence determined by the post-labeling methods of Kuchino *et al.* (13) and Donis-Keller (14). The base numbering conforms to the literature (2). Arrows indicate the cleavage sites with RNaseT₂ and the length of the arrow being proportional to the sensitivity to RNaseT₂. Circled residues were reactive to the chemical reagents under the native conditions. Boxed residues correspond to residues which were protected under the native conditions but reactive under the semi-denaturing and the denaturing conditions. Triangles correspond to the residues protected under the native and the semi-denaturing conditions. b) Two dimensional thin layer chromatograms of the nucleotide at position 39 of tRNA^{Phe} II and III developed by solvent system 2 (see Materials and Methods).

tRNA^{Phe} can be eluted from the resin by this procedure. The elution procedure was repeated twice. Finally, mt tRNA^{Phe} was recovered by ethanol precipitation in the presence of glycogen (Boehringer Mannheim) (final concentration 20 μ g/ml).

Figure 3. Enzymatic probing using RNaseT₂. Limited digestion patterns of 3'-labeled yeast cyt tRNA^{Phe} (a) and bovine mt tRNA^{Phe} (b); lane 1, untreated tRNA; lane 2, RNaseT₁ ladder; lane 3, alkaline ladder; lane 4, digestion with 0.01 unit of RNaseT₂; lane 5, digestion with 0.002 unit of RNaseT₂.

Detection of mt tRNA^{Phe}

Mitochondrial tRNA^{Phe} was detected by dot hybridization as reported previously (4) and conventional aminoacylation assay (8). An oligodeoxyribonucleotide, 5'-TGCCTTGCTTTGGGT-TA-3' which is complementary to the 17 nucleotides of tRNA^{Phe} extending from the D stem (T13) to the anticodon stem (A31), was synthesized using an Applied Biosystems model 391 DNA synthesizer, and labeled at the 5'-end with T4 polynucleotide kinase and [γ -³²P]ATP. This labeled oligodeoxynucleotide was used as the hybridization probe. The dot hybridization was carried out at 43°C.

End-labeling of tRNA

Prior to 5'-end labeling, the tRNA sample was dephosphorylated with *E. coli* alkaline phosphatase and labeled with T4 polynucleotide kinase and [γ -³²P]ATP as described by Silberklang *et al.* (11). For 3'-end labeling, tRNA was labeled with T4 RNA ligase and [5'-³²P]pCp (12). The labeled tRNA was purified by electrophoresis on a 15% polyacrylamide/7 M urea gel, eluted from the gel and precipitated with ethanol.

RNA sequencing

The nucleotide sequence of mt tRNA^{Phe} was determined by the method of Kuchino *et al.* (13) with a slight modification, and Donis-Keller's method (14) using the RNases T₁, U₂, PhyM and CL₃ (15, 16). Modified nucleotides were identified by two-dimensional thin layer chromatography (TLC) using the following

Figure 4. Chemical probing of mt tRNA^{Phe} as developed on 15% polyacrylamide/7 M urea gels in the absence (a) or the presence (b) of 10% glycerol. [A] (left 4 lanes), probing with DEPC at N-7 of adenosine; [C] (middle 4 lanes), probing with DMS at N-3 of cytidine; [G] (right 4 lanes), probing with DMS at N-7 of guanosine. [-], control without chemical modification but the chain scission step was performed; [N], modified under the native conditions (10mM MgCl₂, 37°C); [S], modified under the semi-denaturing conditions (1 mM EDTA, 37°C); [D], modified under the denaturing conditions (1 mM EDTA, 90°C).

two solvent systems. System 1: isobutyric acid/25% ammonia/water (66 : 1 : 33, by vol.) for the first dimension, and isopropanol/36% HCl/water (70 : 15 : 15, by vol.) for the second dimension. System 2: isobutyric acid/25% ammonia/water (66 : 1 : 33, by vol.) for the first dimension, and 100ml of 0.1 M sodium phosphate (pH 6.8), 60 g of ammonium sulfate and 2 ml of n-propanol for the second dimension (13).

Enzymatic probing using RNaseT₂

The 3'-end labeled bovine mt tRNA^{Phe} and yeast cyt tRNA^{Phe} (10,000 cpm each) containing carrier tRNA (0.05 A₂₆₀unit)

were digested separately with 0.01 unit or 0.002 unit of RNaseT₂ at 37°C for 10 min in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂, essentially according to the method of Vary and Vournakis (17). Alkaline hydrolysis was performed at 90°C for 2 min in 50 mM sodium carbonate (pH 9.0) and 1 mM EDTA. The digestion with RNaseT₁ was performed at 55°C for 10 min with 0.05 unit RNaseT₁ in 30 mM sodium citrate (pH 4.5), 1 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol and 8 M urea. The hydrolyzed samples were immediately loaded on a 15% polyacrylamide gel containing 7 M urea and 10% glycerol.

Chemical modification of tRNAs with DEPC and DMS

Modifications at N-7 of adenine with DEPC and N-3 of cytosine and N-7 of guanine with DMS were performed by the methods already published (18, 19).

The following buffers were used in this experiment. Buffer A: 50 mM sodium cacodylate (pH 7.0), 10 mM MgCl₂. Buffer B: 50 mM sodium cacodylate (pH 7.0), 1 mM EDTA.

Under 'native' conditions, 3'-end labeled tRNA (about 20,000 cpm) containing 10 µg of carrier tRNA was preincubated in buffer A (200 µl) at 37°C for 10 min. For base modification with DEPC, 20 µl of DEPC were added and the mixture was incubated at 37°C for 1 hr. For modification with DMS, 0.5 µl of DMS was added and the mixture was incubated at 37°C for 12 min.

Under 'semi-denaturing' conditions, the base modification was performed by the same procedure as described above except that buffer B was used.

Under 'denaturing' conditions, tRNA was preincubated in buffer B (300 µl) at 0°C for 5 min. For modification with DEPC, 5 µl of DEPC were added and the mixture was incubated at 90°C for 15 min. For modification with DMS, 0.5 µl of DMS was added and the mixture was incubated at 90°C for 2 min.

For probing N-3 of cytosine and N-7 of guanine, the alkylated residues were treated with hydrazine and sodium borohydride, respectively. Modified residues were split with aniline by the method already published (18).

The oligonucleotides following the above reactions were analyzed by electrophoresis on 15% polyacrylamide/7 M urea gels in the presence or absence of 10% glycerol.

RESULTS

Isolation of bovine mt tRNA^{Phe}

Mitochondrial tRNA^{Phe} was isolated from bovine liver mt tRNA^{Mix} by the selective hybridization method using a solid phase DNA probe as described in Materials and Methods. By a single run of this procedure, about 0.1 A₂₆₀unit of tRNA^{Phe} and its derivatives (see below) was obtained from 10 A₂₆₀units of mt tRNA^{Mix} (data not shown). This procedure was repeated 26 times, resulting in about 2 A₂₆₀units of mt tRNA^{Phe} in total. This fraction was further subjected to RPC-5 column chromatography (5). tRNA^{Phe} was detected by hybridization and aminoacylation assays. Fig. 1 shows the elution profile of mt tRNA^{Phe}. Three peaks containing mt tRNA^{Phe} were obtained in fractions 21–22 (named tRNA^{Phe} I), 24–25 (tRNA^{Phe} II) and 27–28 (tRNA^{Phe} III). These fractions were separately pooled, with final recoveries being 0.13 A₂₆₀unit, 0.27 A₂₆₀unit and 0.11 A₂₆₀unit, respectively.

Primary structure of bovine mt tRNA^{Phe}

The nucleotide sequences of mt tRNAs^{Phe} I, II and III were determined by the post-labeling methods of Donis-Keller (14)

and Kuchino *et al.* (13). Fig. 2a shows the nucleotide sequence of tRNA^{Phe} II. The base numbering conforms to the literature (2). If the modified nucleotides are not taken into account, the nucleotide sequence is identical to the already published gene sequence (10). In tRNA^{Phe} II, three modified nucleotides, m¹A, m²G and Ψ, were unambiguously identified and positioned at 9, 10 and 39, respectively. One nucleotide at the 3' position adjacent to the anticodon (position 37) could not be discriminated between ms²i⁶A and i⁶A because both migrated similarly on the two different TLC's used in this study. tRNA^{Phe} I was shown to lack the 3'-terminal adenylylate of tRNA^{Phe} II (data not shown). Ψ at position 39 was replaced by an unmodified U in tRNA^{Phe} III (Fig. 2b).

Enzymatic probing of yeast cyt tRNA^{Phe} and bovine mt tRNA^{Phe} with RNaseT₂

The accessibility to RNaseT₂ was compared between yeast cyt tRNA^{Phe} and bovine mt tRNA^{Phe} II. Fig. 3 (a and b) shows the limited digestion patterns of both tRNAs^{Phe} with RNaseT₂. In the presence of 10 mM Mg²⁺, yeast tRNA^{Phe} was cleaved only in the anticodon loop region and at the CCA end. On the other hand, mt tRNA^{Phe} was digested not only in these regions but also in the D loop and T loop regions under the same conditions. The detailed cleavage sites of mt tRNA^{Phe} are shown schematically in Fig. 2a.

Chemical probing of bovine mt tRNA^{Phe} with DEPC and DMS

To obtain further information on the higher-order structure of bovine mt tRNA^{Phe}, chemical probing with DEPC and DMS was performed. Typical examples of such probing experiments are shown in Fig. 4. Similar analyses were also done on yeast cyt tRNA^{Phe} under the same conditions (data not shown). As a whole, bovine mt tRNA^{Phe} was more sensitive to chemical reagents than yeast cyt tRNA^{Phe}. Data obtained from the experiments on yeast cyt tRNA^{Phe} essentially confirmed the results already published (18). The reactivity of A, C and G residues in bovine mt tRNA^{Phe} is described in detail below.

Carbethoxylation of adenosine N-7 with DEPC. An autoradiogram resulting from the modification of N-7 of adenosine is shown in Fig. 4 (left 4 lanes). Under the native conditions, residues A14, A15, A21, A35, A36, i⁶A37 (or ms²i⁶A37), A59 and A62 were reactive. Under the semi-denaturing conditions, all adenosine residues were reactive, except for A31 and A38, which became barely reactive when the tRNA was heated at 90°C. The reactivities of the adenosine residues are summarized on the cloverleaf structure of mt tRNA^{Phe} in Fig. 2a.

Alkylation of cytidine N-3 with DMS. Fig. 2a and Fig. 4 (middle 4 lanes) show the results of modification of cytidine N-3 of mt tRNA^{Phe}. Under the native conditions, residues C16, C17 and C18 in the D loop region, C54, C56, C57 and C58 in the T loop region and C74 and C75 at the CCA end were reactive. Under the semi-denaturing conditions, five more cytidine residues, C25, C63, C65, C66 and C72, became reactive. All the cytidine residues were reactive under the denaturing conditions.

Alkylation of guanosine N-7 with DMS. As shown in Figs. 2a and 4 (right 4 lanes), only guanosine residues G4, G7, G24 and G34 were reactive under the native conditions. Under the semi-denaturing conditions, all guanosine residues were reactive.

used for the aminoacylation assay also contained the CCA-repair enzyme.

The higher-order structure of mt tRNA^{Phe} was inferred from the results of enzymatic and chemical probing experiments. Whereas yeast cyt tRNA^{Phe} having the normal D loop/T loop interactions was cleaved with RNaseT₂ only at the anticodon loop and the CCA terminus in the presence of Mg²⁺ (Fig. 3a), the D loop and T loop regions of bovine mt tRNA^{Phe} were sensitive to RNaseT₂ under the same conditions (Fig. 3b). The higher sensitivity of mt tRNA^{Phe} to RNaseT₂ may be ascribed to the loose tertiary structure due to the lack of the GG and TΨC sequences in the D and T loop, respectively.

The results of the chemical probing analyses yielded more precise information about the higher-order structure of tRNA^{Phe}. All the residues in the stem regions were protected from the modification except for G4, G7, G24 and A62 under the native conditions. The reactivity of G4 can be explained by the G4-A69 mismatch in the acceptor stem. Although A69 was not reactive to DEPC, the difference of reactivity between G4 and A69 may be ascribed to the difference in the molecular size of DMS and DEPC. Susceptibility of G7 to DMS may be explained by the presence of neighboring two pyrimidines, and that of G24 may be due to the distortion of the D stem, as discussed in the literature (19). The mild reactivity of A62 may reflect weak base pairing of U52-A62 and/or weak base stacking due to the structural looseness of the T loop region. Mitochondrial tRNA^{Phe} has many cytidines in both the D and T loops (Fig. 2a). If some of these cytidines were to form hydrogen bonds with other residues, the N-3 of the cytidines would function as a hydrogen acceptor. However, as shown in Figs. 2a and 4, these residues, C16, 17, 18, 54, 56, 57 and 58, were very easily modified with DMS under the native conditions. Therefore, it seems unlikely that mt tRNA^{Phe} has D loop/T loop interactions.

As shown in Fig. 5, the D stem and its neighboring residues, the extra loop and the anticodon loop regions are widely conserved among various animal mt tRNA^{Phe} genes as well as in the yeast nuclear tRNA^{Phe} gene. Surprisingly, they are conserved even in the nematode mt tRNA^{Phe} gene lacking the T arm region. With respect to the sequence similarity of the D stem region among tRNA^{Phe} genes, Roe *et al.* pointed out that in yeast cyt tRNA^{Phe} the D stem and its neighboring residues are important for aminoacylation (20). However, since major recognition sites for yeast phenylalanyl-tRNA synthetase are not located in the D stem (21), the sequence similarity in the D stem region may be necessary to preserve the higher-order structure for recognition by phenylalanyl-tRNA synthetase. It is well known that the residues around the D stem and those in the extra loop are spatially close to each other (22–26). Therefore, it is likely that there are some tertiary interactions between these regions also in bovine mt tRNA^{Phe}. As shown in Fig. 2a, A14, A15 and A21 are classified as reactive under the native conditions. However, A21 is very easily modified with DEPC but A14 and A15 are only slightly modified (Fig. 4a). Moreover, A44, G45 and A46 are protected under the native conditions. The modification patterns of bovine mt tRNA^{Phe} are very similar to those of yeast cyt tRNA^{Asp} which has only 4 nucleotides in the extra loop, lacking the nucleotide at position 47 (19). Interestingly, mt tRNA^{Phe} also has 4 nucleotides in the extra loop and the sequence of these residues is identical to that of yeast cyt tRNA^{Asp}. Consequently, it is very reasonable to assume that the arrangement of these residues is spatially similar in bovine mt tRNA^{Phe} and in yeast cyt tRNA^{Asp} (24). Based on these

considerations, we propose 6 tertiary interactions in bovine mt tRNA^{Phe}, as shown in Fig. 6: (a) U8-A14, (b) m¹A9-U12-A23, (c) m²G10-C25-G45, (d) U13-A22-A46, (e) A15-U48 and (f) A26-A44. Interactions (a), (b) and (c) are found in yeast tRNA^{Phe} (1). If the U8-A14 interaction exists in bovine mt tRNA^{Phe}, the N-7 of A14 should not be reactive under the native conditions. However, A14 was actually slightly reactive. This may be explained by assuming that bovine mt tRNA^{Phe} has a loose tertiary structure at 37°C. In fact, studies on the melting profile of this tRNA revealed that melting began even at 25°C (8). With respect to the other tertiary interactions postulated above, Sampson *et al.* (27) have described a similar possible combination of tertiary interactions for a T7 RNA transcript based on the yeast cyt tRNA^{Phe} sequence. Several mutant yeast cyt tRNA^{Phe} transcripts that have the (d), (e) and (f) combination of interactions proposed for bovine mt tRNA^{Phe} (Fig. 6) were shown to have high activity in aminoacylation reactions (27). Therefore, the predicted tertiary interactions probably exist in bovine mt tRNA^{Phe}. As described above, bovine mt tRNA^{Phe} lacks the D loop/T loop interactions but has the canonical extra loop/D stem interactions, the latter probably maintaining the overall L-shaped tertiary structure by fixing the extra loop and D stem in a proper arrangement. Likewise other mt tRNAs having somewhat unusual primary or secondary structures, could retain the L-shaped tertiary structure by using some of the canonical interactions found in the normal cyt tRNAs.

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