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Formylatable methionine transfer RNA from Mycoplasma: purification and comparison of partial nucleotide sequences with those of other prokaryotic initiator tRNAs

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ABSTRACT

The major species of the formylatable methionine tRNA from Mycoplasma mycoides var capri has been purified. The 5'- and 3'-terminal sequences of the purified tRNA are pC-G- and C-A-A-C-C-A_{OH}, respectively. Thus, this tRNA also contains the unique structural feature found in two other prokaryotic initiator tRNAs in that the first nucleotide at the 5'-end cannot form a Watson-Crick type of base-pair to the fifth nucleotide from the 3'-end. The Mycoplasma tRNA does not contain ribothymidine; however, a specific uridine residue in the sequence G-U-ψ-C-G- can be enzymatically methylated by E. coli extracts to yield G-T-ψ-C-G. Since ribothymidine is absent in crude tRNA from this strain of Mycoplasma, the absence of T is probably due to the lack of a U→T modifying enzyme.

INTRODUCTION

Following the nucleotide sequence analysis of yeast cytoplasmic initiator tRNA,[1] the sequence of three mammalian cytoplasmic initiator tRNAs[2,3] and the partial sequence of wheat germ[4] and salmon testes (Gillum, Urquhart, Smith and RajBhandary, unpublished) cytoplasmic intiator tRNAs are now known. A unique structural feature common to these eukaryotic cytoplasmic initiator tRNAs is that they lack the sequence G-T-ψ-C- found so far in all tRNAs that are active in protein biosynthesis, including the E. coli initiator tRNA; instead, they contain the sequence G-A-U-C- or G-A-U*-C-.[5-8] This finding has prompted us to examine whether prokaryotic initiator tRNAs as a class also possess their own unique structural feature.

The nucleotide sequence of E. coli initiator tRNA was determined by Dube et al.[9] A distinguishing feature of the E. coli initiator tRNA is that it lacks the Watson-Crick base-pair between the first nucleotide of the 5'-end and the fifth nucleotide from the 3'-end found so far in all tRNAs. This unique feature

has recently been found also in the initiator tRNA from the prokaryotic alga Anacystis nidulans.[10] This paper describes the purification and partial nucleotide sequence of a formylatable methionine tRNA from Mycoplasma mycoides var capri. It is shown that this tRNA also contains the same unique structural feature in the acceptor stem common to the other prokaryotic initiator tRNAs. This finding suggests that the absence of a Watson-Crick "base-pair" between the first nucleotide at the 5'-end and the fifth nucleotide from the 3'-end is a structural feature common to all prokaryotic initiator tRNAs.

MATERIALS AND METHODS

General. Sources of tRNA, enzymes, radioactive materials, chemicals and techniques were all as described previously.[5,8,11] All column adsorbents were thoroughly equilibrated with starting buffer before use. Crude E. coli aminoacyl-tRNA synthetases, which also served as a source of methionyl-tRNA formylase, tRNA pyrophosphorylase and tRNA methylase, were prepared from E. coli B62 according to Muench and Berg[12] as described previously.[13] The oligonucleotides C-G- and C-A-A-C-C-A_{OH} were isolated by DEAE-cellulose chromatography of T_1-RNase digests of yeast[13] and E. coli $tRNA_f^{Met}$.[11] They were phosphorylated with [^{32}P] at the 5'-end as before.[8] The specific activity of (1) [^{35}S] methionine used in the formylation studies was 8.3 Ci/mmol, (2) [^{3}H] methionine used for amino acid acceptance assays was 6.3 Ci/mmol, (3) [^{14}C]-methyl-S-adenosylmethionine and [^{3}H]-methyl-S-adenosylmethionine used for methylation studies were 60 mCi/mmol and 8.3 Ci/mmol, respectively, (4) [^{32}P]ATP used for the 5'-labeling of oligonucleotides was about 50 Ci/mmol and (5) [^{3}H]ATP used for the repair of the C-C-A- terminus was 33 Ci/mmol.

M. mycoides var capri PG3 was obtained in 1961 from D.G. ff. Edward (Wellcome Research Laboratories) and has subsequently been checked for identity and purity by R.M. Leach (Central Public Health Laboratories).

Isolation of tRNA. M. mycoides var capri PG3 was grown in 2 l flasks containing 1 l of dilute medium[14] at 37° and for 48 h without aeration. The inoculum used was 0.5% of an 18 h culture grown at 37° in the 'normal' medium.[14] Cells from 30l of medium

were collected by centrifugation of the medium at 0°C at 23,000 x g. The cells were then immediately added to a mixture of equal volumes of phenol and 6% p-aminosalicylate solution and were gently shaken at 0° for 18 h. The nucleic acids were isolated and fractionated as before,[14] except that the neutral polysaccharide was removed first, the DNA was then removed by the fractional precipitation of its cetyltrimethylammonium salt and the ribosomal RNA was separated from the tRNA by precipitation with 1 M NaCl. Each fraction obtained was recycled separately through the entire fractionation procedure. Typical yields from the cells from 30l of medium were: tRNA, 80 mg; rRNA, 500 mg; DNA, 90 mg. The high A + T content of the DNA and hybridization studies using this DNA have been reported before.[15,16]

Purification of M. capri $tRNA_f^{Met}$. This involved chromatography of the tRNA on BD-cellulose, DEAE-Sephadex A-50 processed according to the method of Nishimura et al[17] and, when necessary, RPC-5 at pH 4.4.[18] Assay for amino acid acceptance was as described previously[11] and the enzyme used was from E. coli and not from the homologous system.

Chromatography on BD-Cellulose. Crude M. capri tRNA (800 O.D. units) was applied to a column (1.5 x 100 cm) of BD-cellulose and the column was eluted with a linear gradient (0.375 to 0.6 M; total volume of gradient, 3 l) of NaCl containing 10 mM $MgCl_2$. Preliminary studies showed that all the methionine tRNAs present were eluted off the column by 0.6 M NaCl. Consequently, at the end of this gradient (fraction 440), the remaining tRNAs were eluted together with 1 M NaCl containing 20% ethanol and 10 mM $MgCl_2$. Fractions (5 ml) were collected every 20 min. Fig. 1 shows the fractionation profile and the pattern of methionine acceptor activity. As shown below, both peaks of methionine tRNAs could be formylated by E. coli methionyl-tRNA formylase and are hence designated $tRNA_{f\ I}^{Met}$ and $tRNA_{f\ II}^{Met}$. Fractions 298-325 were pooled for $tRNA_{f\ I}^{Met}$. Fractions 345-390 were pooled for $tRNA_{f\ II}^{Met}$ and yielded 125 O.D. units of approximately 15% pure methionine tRNA.

Chromatography on DEAE-Sephadex. $tRNA_{f\ II}^{Met}$ (125 O.D. units) from the BD-cellulose column was applied to a column of DEAE-Sephadex (1.5 x 70 cm) and the column was eluted with a linear

gradient (0.35 to 0.45 M; total volume of gradient, 1.5 l) of NaCl containing 10 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.5). Fractions of 3 ml were collected every 20 min. Fractions 310-318 (Fig. 2) were pooled for $tRNA_{f\ II}^{Met}$ and gave 4 O.D. units of tRNA which was at least 90% pure. The material present in the edges of the peak could be further purified by chromatography on RPC-5 column.

Chromatography on RPC-5. tRNA (20 O.D. units) from two or more DEAE-Sephadex columns (see above) was applied to a column (0.3 x 60 cm) of RPC-5. The column was eluted with a linear gradient (0.35 to 0.65 M; total volume of gradient, 200 ml) of NaCl containing 10 mM ammonium acetate pH 4.4 and 10 mM $MgCl_2$, and was run at 300-400 psi. Fractions of 0.8 ml were collected every min. Fractions 135-150 were pooled for $tRNA_{f\ II}^{Met}$ and yielded 4 O.D. units of 95% pure methionine tRNA.

Assay for the extent of formylation of M. capri methionine tRNAs. This was carried out as before.[11,13] The tRNAs were aminoacylated in the presence of [^{35}S] methionine and N^{10}-formyltetrahydrofolate using E. coli aminoacyl-tRNA synthetases. The [^{35}S] aminoacyl-tRNAs were isolated and 40,000-60,000 cpm were treated with alkali. [^{35}S] met and [^{35}S] fmet produced were separated by paper electrophoresis at either pH 7.5 (.03 M phosphate buffer) or at pH 3.5 (.05 M pyridinium acetate buffer). The radioactive products were located by scanning and/or autoradiography and quantitated by liquid scintillation counting. Both of the M. capri methionine tRNA peaks I and II from the BD-cellulose column (Fig. 1) were found to be essentially quantitatively formylated (>95%).

T_2-RNase digestion of tRNA and analysis of the products by two-dimensional thin layer chromatography. 1-2 O.D. units of tRNA were digested with T_2-RNase (1-2 units) in 50 µl of 20 mM ammonium acetate (pH 4.5) at 37° for 5 h. The solution was then repeatedly evaporated under vacuum to dryness on parafilm. The residue was dissolved in H_2O (2 µl) and applied to a thin-layer cellulose plate (10 x 10 cm). Chromatography in the first dimension was in isobutyric acid-concentrated ammonia-water, 557:38:385 v/v; and after drying in t-butanol-concentrated HCl-water, 70:15:15 v/v in the second dimension. The nucleotidic material

was visualized under a Mineralight uv lamp. For the quantitative analysis of radioactive spots containing [^{3}H], the spots corresponding to the various nucleotides were scraped off the plate and mixed with 10 ml of a toluene based scintillation medium containing 33% Triton X-100 for counting.

Polyacrylamide gel electrophoresis and elution of ^{32}P-labeled tRNA. Electrophoresis was performed at room temperature on 15% polyacrylamide-gel slabs (2 mm x 20 cm) containing .75% N,N'-methylene-bis-acrylamide and 6 M urea at 200 V for 20 h. Running buffer contained 90 mM Tris-base-90 mM boric acid-5 mM EDTA at pH 8.2.[8] [^{32}P] labeled material was detected by autoradiography (Fig. 4I) and nucleotidic material by staining with methylene blue (Fig. 4II-IV). After autoradiography, the gel containing [^{32}P]-labeled tRNA was homogenized in a Dounce homogenizer in 0.3 M NaCl containing 0.03 M trisodium citrate and crude yeast tRNA (1 O.D. unit) as carrier. The gel was removed by centrifugation, re-extracted and the supernatants were combined, diluted 5-fold with water and added to a column (0.7 x 5 cm) of DEAE-cellulose equilibrated with 50 mM TEAB pH 8.0. After washing with 50 mM TEAB to remove the salt, the tRNA was eluted with 2 M TEAB pH 8.0. The eluate was evaporated to dryness several times and the tRNA was dissolved in water and stored at -20°.

5'-end group labeling of M. capri $tRNA_f^{Met}{}_{II}$. First, the 5'-terminal phosphate was removed as follows. $tRNA_f^{Met}{}_{II}$ (0.18 O.D. unit, 300 pmoles) was treated with E. coli alkaline phosphatase (0.01 unit) in 50 mM Tris-HCl (pH 8.0, 10 µl) at 55° for 60 min. Phosphatase was inactivated by the addition of nitrilotriacetic acid (to 5 mM) as before.[5]

For the labeling of 5'-end group with [^{32}P], the incubation mixture (15 µl) contained 60 pmoles of tRNA treated with phosphatase; Tris-HCl (pH 8.0), 50 mM; $MgCl_2$, 10 mM; 2-mercaptoethanol, 15 mM; [^{32}P]ATP (240 pmoles) and T_4 polynucleotide kinase, 12 units. The kinetics of 5'-labeling was followed by removing aliquots (2 µl) at timed intervals during the incubation at 37° and measuring the amount of acid insoluble radioactivity. It was found that over 50% of the 5'-terminal phosphate was replaced by [^{32}P] in 20 min. The remaining 240 pmoles of tRNA were then phosphorylated under the same conditions for 20 min. The products

were subjected to polyacrylamide-gel electrophoresis (Fig. 4) and located by autoradiography and the bands corresponding to tRNA were eluted as described above.

5'-end group labeling of fragments produced by the action of T_1-RNase on M. capri $tRNA_{f\ II}^{Met}$. This was carried out as described previously,[5] and the [^{32}P]-labeled fragments were separated by two-dimensional electrophoresis (Fig. 7).[19,20]

Determination of the size and the 5'-terminal nucleotides of [^{32}P]-labeled fragments. After autoradiography, the spots on the fingerprint were eluted and the 5'-termini identified as previously described.[5] The size of each fragment could be estimated as follows: An amount of each oligonucleotide (corresponding to about 2000 Cerenkov cpm) was taken to dryness in parafilm and dissolved in a marker solution (2 µl) which contained 0.06 O.D. unit each of nucleotides of chain length 1-6 (isolated from T_1-RNase digests of tRNA). The solution was then applied to a thin-layer cellulose plate and then chromatographed in n-propanol-concentrated ammonia-water, 55:10:35 v/v, which separates oligonucleotides of chain lengths 1-6 (and probably up to 9 or 10) according to size provided that they all contain only one G residue. In most cases, the [^{32}P]-labeled oligonucleotides of a given size move slightly slower than the uv marker because the latter have 3'-phosphates; the former have 5'-phosphates and this solvent system is capable of distinguishing between oligonucleotides with phosphate at the 5' or 3' ends (Fig. 8).

Partial digestion of 5'-[^{32}P]-labeled fragments with snake venom phosphodiesterase. The incubation mixture (30 µl) contained 20 mM Tris-HCl and 5 mM potassium phosphate (pH 8.5); 40-80,000 Cerenkov cpm of 5'-[^{32}P]-labeled oligonucleotide and snake venom phosphodiesterase (Worthington) 0.02 µg/pmol of oligonucleotide. Incubation was at room temperature. Aliquots (5 µl) were removed at intervals over 4 h and applied to DEAE-cellulose paper, the final aliquot being incubated for a further 15 min at 37°. The paper was subjected to electrophoresis at pH 3.5 in pyridinium acetate buffer at 7 V/cm for 15 h. The identity of some oligonucleotides was tentatively identified from their M values,[5,19] and the identity of others was unequivocally established by comparison with markers.

Isolation of 3'-terminal fragment present in T_1-RNase digest of $tRNA_{f\ II}^{Met}$. In the procedure used for labeling the fragments present in T_1-RNase digests, the 3'-terminal fragment would be present in the form $[^{32}P](X\text{-}Y\text{-}Z\text{-----})C\text{-}C\text{-}A_{OH}$ and would be the only fragment not containing a G residue. None of the $[^{32}P]$-labeled fragments (Fig. 7) corresponded to a possible 3'-terminal fragment. However, spot 2 (Fig. 7), when chromatographed in n-propanol-ammonia-water (Fig. 8), contained two compounds, one longer than the main compound which was characterized as $[^{32}P]C\text{-}C\text{-}G$. This longer oligonucleotide was isolated and purified to a state suitable for partial digestion with snake venom phosphodiesterase and sequence analysis in either of the following ways: (a) The slower moving oligonucleotide (Fig. 8, spot 2b) was eluted from a thin-layer cellulose plate, was rerun in the same solvent and then freed from impurities co-eluted from the cellulose by electrophoresis on DEAE-cellulose paper in 7% formic acid. (b) Alternatively, the 3'-terminal fragment was separated by electrophoresis of spot 2 of Fig. 7 on DEAE-cellulose paper in 0.03 M pyridinium acetate (pH 3.5) at 7 V/cm for 15 h, eluted after washing the paper with ethanol to remove pyridinium acetate, and then subjected to electrophoresis on DEAE-cellulose paper in 7% formic acid at 7 V/cm for 15 h.

Methylation of M. capri tRNA.

(a) Crude M. capri tRNA. The incubation mixture (1.5 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM 2 mercaptoethanol, 125 nmoles $[^{14}C]$S-adenosylmethionine (previously neutralized with Tris-HCl pH 8.0), 3 nmoles crude M. capri tRNA and crude E. coli aminoacyl-tRNA synthetases (2 mg). Incubation was at 37° for 90 min. The incubation mixture was deproteinized with phenol and then centrifuged. The methylated tRNA was recovered[11] from the aqueous layer by ethanol precipitation. The ethanol precipitation was repeated once more, the tRNA was then dialyzed against 1 M NaCl in 10 mM Tris-HCl (pH 7.5) and finally against 10 mM Tris-HCl (pH 7.5).

For the analysis of the products of methylation, the methylated tRNA (1 nmol, approximately 20,000 cpm) was mixed with 2 nmol of crude E. coli tRNA and was then digested with T_2-RNase. The products were separated by two-dimensional thin-layer chro-

matography (see above), nucleotidic material was located under a uv lamp and [^{14}C]-containing products were located by autoradiography.

(b) $tRNA_f^{Met}{}_{II}$. The incubation mixture (0.72 ml) contained 80 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 30 nmol [^{3}H]S-adenosylmethionine (previously neutralized with 1 M Tris-HCl pH 8.0), 1 nmol M capri $tRNA_f^{Met}{}_{II}$ and crude E. coli aminoacyl-tRNA synthetases (2 mg). Incubation was at 37° for 90 min and the methylated tRNA was worked up as described above.

Analysis of [^{3}H] methyl labeled oligonucleotide obtained by digestion of [^{3}H] methyl-M. capri $tRNA_f^{Met}{}_{II}$ with T_1-RNase. The [^{3}H] methyl labeled M. capri $tRNA_f^{Met}{}_{II}$ (0.25 O.D. unit, approximately 500,000 cpm) was digested with T_1-RNase (0.25 unit) in 20 mM Tris-HCl (pH 7.5) for 5 h at 37°. A similar digest of E. coli $tRNA_f^{Met}$ (10 O.D. units) was carried out to obtain a marker of the oligonucleotide T-ψ-C-A-A-A-U-C-C-G-. The two digests were mixed and to this was added T-ψ-C-G- (2.5 O.D. units). The mixture was fractionated on a column (0.3 x 60 cm) of RPC-5 run at 37° and at 300 psi pressure, using a linear gradient (0.5 to 3 M; total volume of gradient, 250 ml) of ammonium acetate.[21] Fractions (0.9 ml) were collected every 2 min. Aliquots (25 µl) were combined with 5 ml of scintillation medium which contained 33% Triton X-100[22] and were counted for [^{3}H] radioactivity.

RESULTS

Purification of M. capri $tRNA_f^{Met}{}_{II}$. Chromatography of M. capri tRNA on BD-cellulose yielded two peaks of methionine acceptor activity as assayed by using E. coli aminoacyl-tRNA synthetases (Fig. 1). Both of these peaks of methionine accepting tRNAs could be converted to fMet-$tRNA_f^{Met}$ by E. coli extracts in the presence of N^{10}-formyl tetrahydrofolate[11,13]. Consequently, these tRNAs are designated $tRNA_f^{Met}{}_I$ and $tRNA_f^{Met}{}_{II}$. The major peak of $tRNA_f^{Met}{}_{II}$ was purified essentially to homogeneity by further chromatography on DEAE-Sephadex (Fig. 2) and the material from the edges of this peak was further purified on RPC-5 (Fig. 3). All the work described herein was carried out on the material isolated after RPC-5 chromatography which was about 95% pure as judged by its methionine acceptor activity.

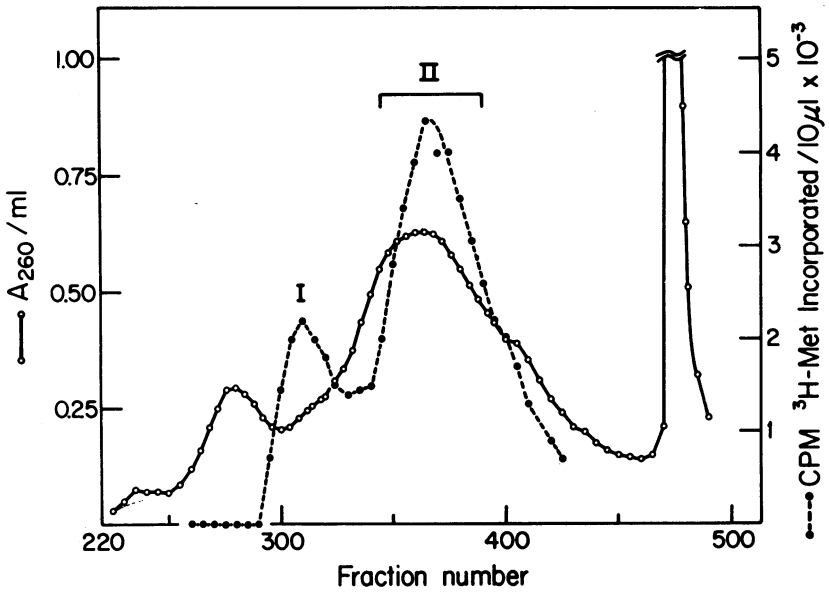

Fig. 1: Chromatography of crude M. capri tRNA on BD-cellulose.

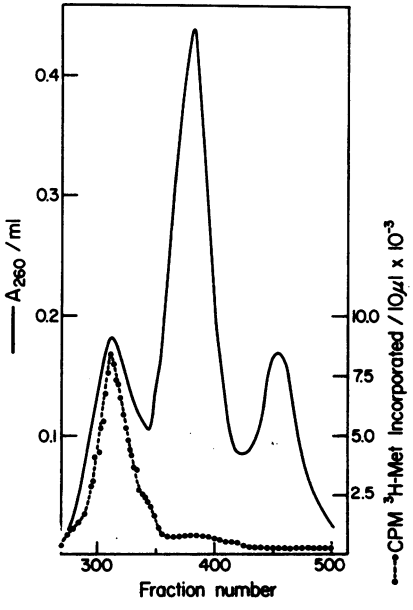

Fig. 2: Chromatography of partially purified M. capri $tRNA_{f}^{Met}$ II (fractions 350-390 of Fig. 1) on DEAE-Sephadex A-50.

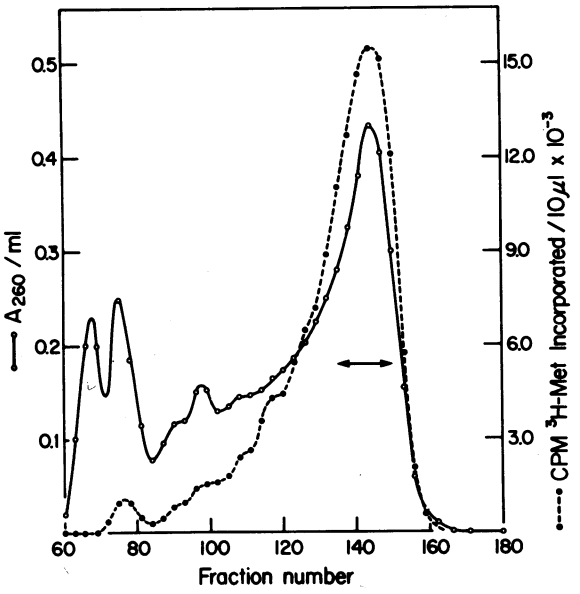

Fig. 3: Chromatography of partially purified M. capri $tRNA_{f\ II}^{Met}$ (fractions 302-309, 319-330 of Fig. 2) on RPC-5.

Polyacrylamide gel electrophoresis of purified $tRNA_{f\ II}^{Met}$ yielded mainly two closely running bands in an approximate ratio of 4:1 (Fig 4, III). These correspond in mobility to markers of E. coli[9] (Fig. 4, IV) and sheep mammary gland[3] (Fig. 4, II) initiator tRNAs and are probably 77 and 75-76 nucleotides long, respectively. On the basis of the following evidence, we assume that the M. capri $tRNA_{f\ II}^{Met}$ purified as above consists of two tRNA chains and that the only difference between them is the absence of 3'-terminal A in one of these tRNAs. Thus, (i) both of these tRNAs contain pCpGp at the 5'-end and (ii) incubation of M. capri $tRNA_{f\ II}^{Met}$ with E. coli extracts in the presence of [^{3}H]ATP[11] results in the incorporation of [^{3}H]A at the 3'-end of tRNA to the extent of 10-15% of total tRNA present (data not shown).

5'-Terminal Sequence of M. capri $tRNA_{f\ II}^{Met}$ is pC-G-. The M. capri $tRNA_{f\ II}^{Met}$ was labeled with [^{32}P] at the 5'-end and the 5'-[^{32}P] labeled tRNA was subjected to gel electrophoresis. Fig. 4, I shows that most of the radioactivity is present in two closely spaced bands (1 and 2). A comparison of this with the gel electrophoretic pattern (Fig. 4) obtained from unlabeled $tRNA_{f\ II}^{Met}$ and sheep mammary gland[3] and E. coli initiator tRNAs[9] indicate

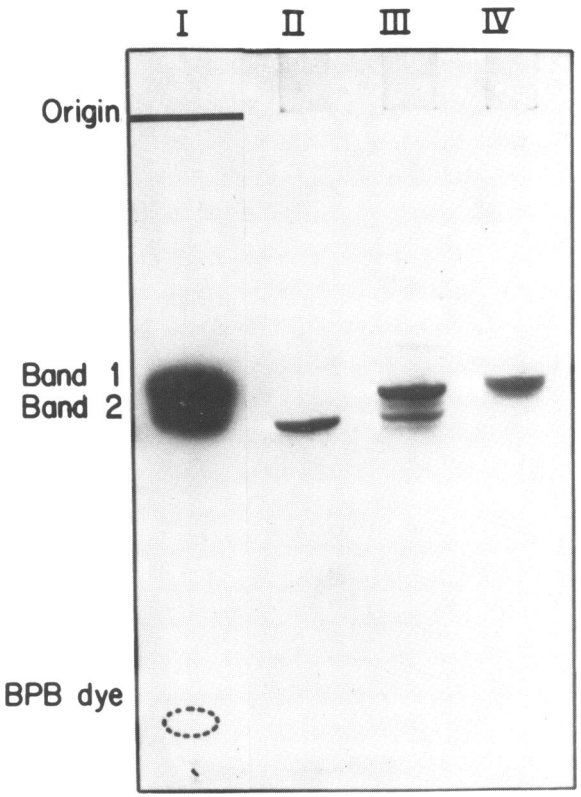

Fig. 4: Electrophoresis on polyacrylamide gel of 5'-[^{32}P] labeled M. capri $tRNA_{f\ II}^{Met}$ (I) and sheep mammary gland initiator tRNA (II), unlabeled M. capri $tRNA_{f\ II}^{Met}$ (III) and E. coli initiator tRNA (IV). I, [^{32}P] labeled material detected by autoradiography; II-IV, nucleotidic material detected by staining.

that band 1 corresponds to intact tRNA and is approximately 77 nucleotides long, whereas band 2 probably represents labeled tRNA lacking part of the 3'-terminal CCA and is 75-76 nucleotides long. Both bands 1 and 2 were eluted from the gel; as shown below, both of them contain the sequence [^{32}P]C-G- at the 5'-end.

(i) 5'[^{32}P] labeled $tRNA_{f\ II}^{Met}$ (bands 1 and 2) were separately treated with T_2-RNase and the [^{32}P] nucleoside diphosphates produced were analyzed by thin layer chromatography. Fig. 5 shows that both bands 1 and 2 yield [^{32}P]Cp as the only radioactive product. Thus, C is present at the 5'-end of both bands 1 and 2 of 5'-[^{32}P] labeled $tRNA_{f\ II}^{Met}$.

(ii) 5'-[^{32}P] labeled $tRNA_{f\ II}^{Met}$ (bands 1 and 2) were separately treated with T_1-RNase. In both cases, the radioactive

oligonucleotide behaved identically to an authentic marker of [^{32}P]C-G- obtained by phosphorylation of C-G- with polynucleotide kinase and γ-[^{32}P]ATP in the following systems: (a) thin-layer chromatography in n-propanol-conc. ammonium hydroxide-water, 55:10:35, v/v, which is known to separate oligonucleotides which contain one G residue strictly according to size, (b) electrophoresis at pH 3.5 on cellulose acetate and (c) electrophoresis in 7% formic acid on DEAE-cellulose paper (Fig. 6).

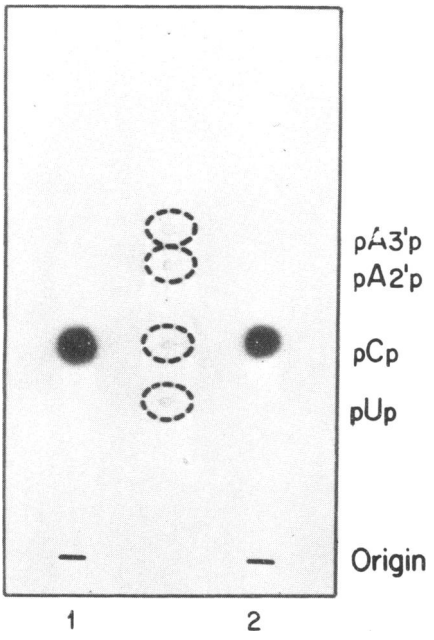

Fig. 5: Autoradiography of 3', 5'-nucleoside diphosphates obtained by digestion of 5'-[^{32}P] labeled *M. capri* $tRNA_{f\ II}^{Met}$ (Bands 1 and 2 of Fig. 4) with T_2-RNase. Thin-layer chromatography was as described previously.[5] Dotted circles indicate the location of ultraviolet absorbing markers of the various nucleoside diphosphates added to the digests before chromatography. The markers used were synthesized chemically and contain isomeric mixtures of 2',5' and 3',5'-diphosphates. The solvent system used separates the two isomeric adenosine diphosphates, but not the C and U derivatives.

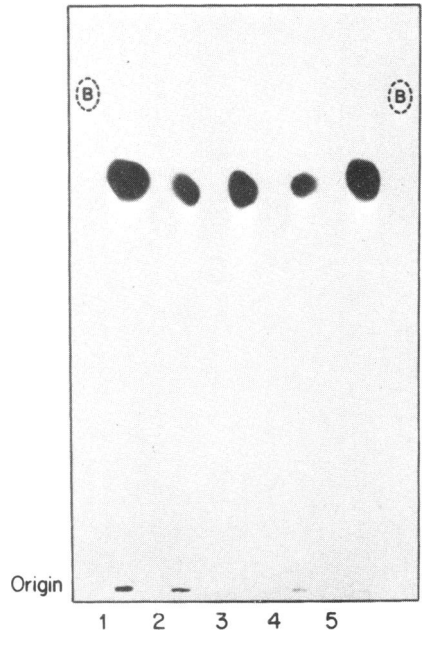

Fig. 6: Autoradiogaph of T_1-RNase digest on 5'-[^{32}P] labeled *M. capri* $tRNA_{f\ II}^{Met}$ (Bands 1 and 2 of Fig. 4). Electrophoresis was on DEAE-paper and in 7% formic acid. B, blue dye marker; 1, T_1-digest on band 1 of Fig. 4; 2, same as 1 but to which [^{32}P]C-G- was added; 3, [^{32}P]C-G- alone; 4, T_1-digest on band 2 of Fig. 4 to which [^{32}P]C-G- was added; and 5, T_1-digest on band 2 alone.

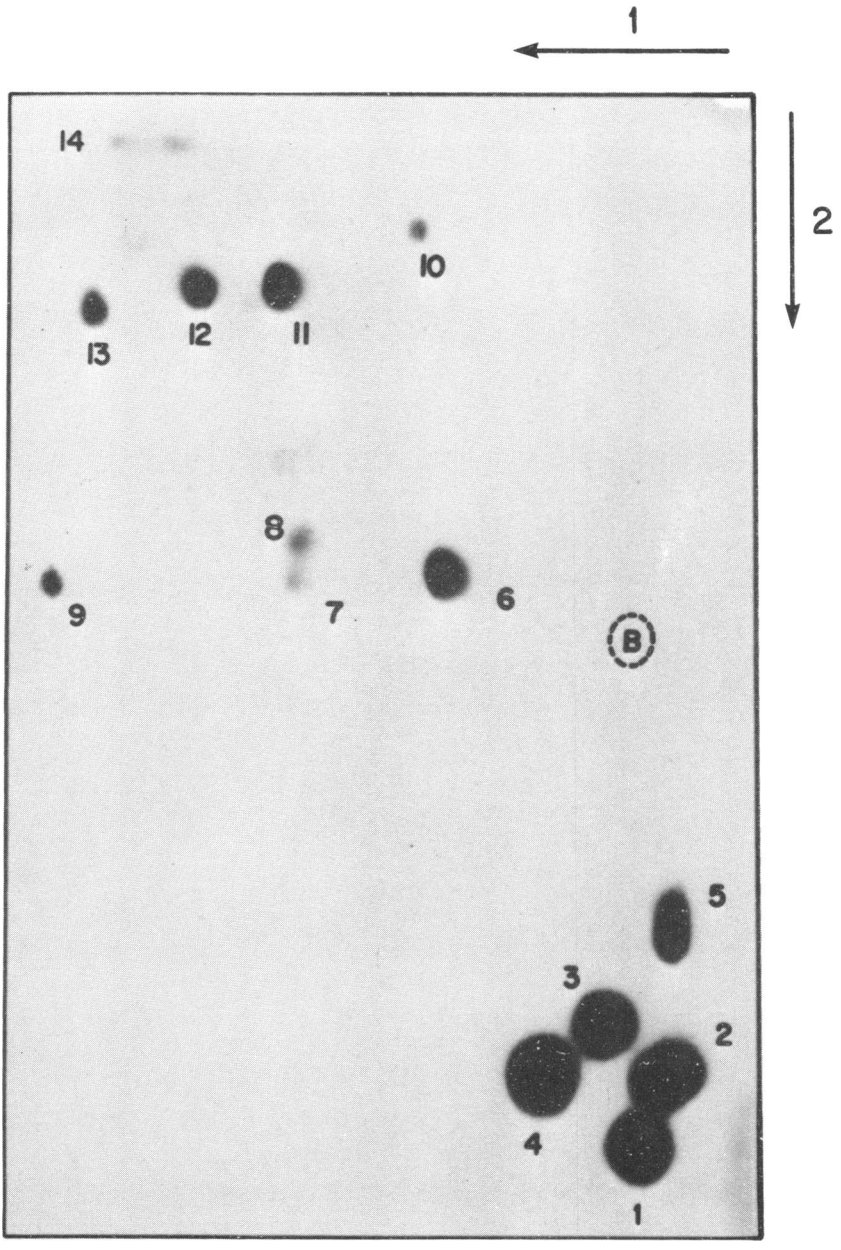

Fig. 7: Autoradiograph of 5'-[^{32}P] labeled oligonucleotides obtained from a T_1-RNase digest on *M. capri* $tRNA_f^{Met}$ II.

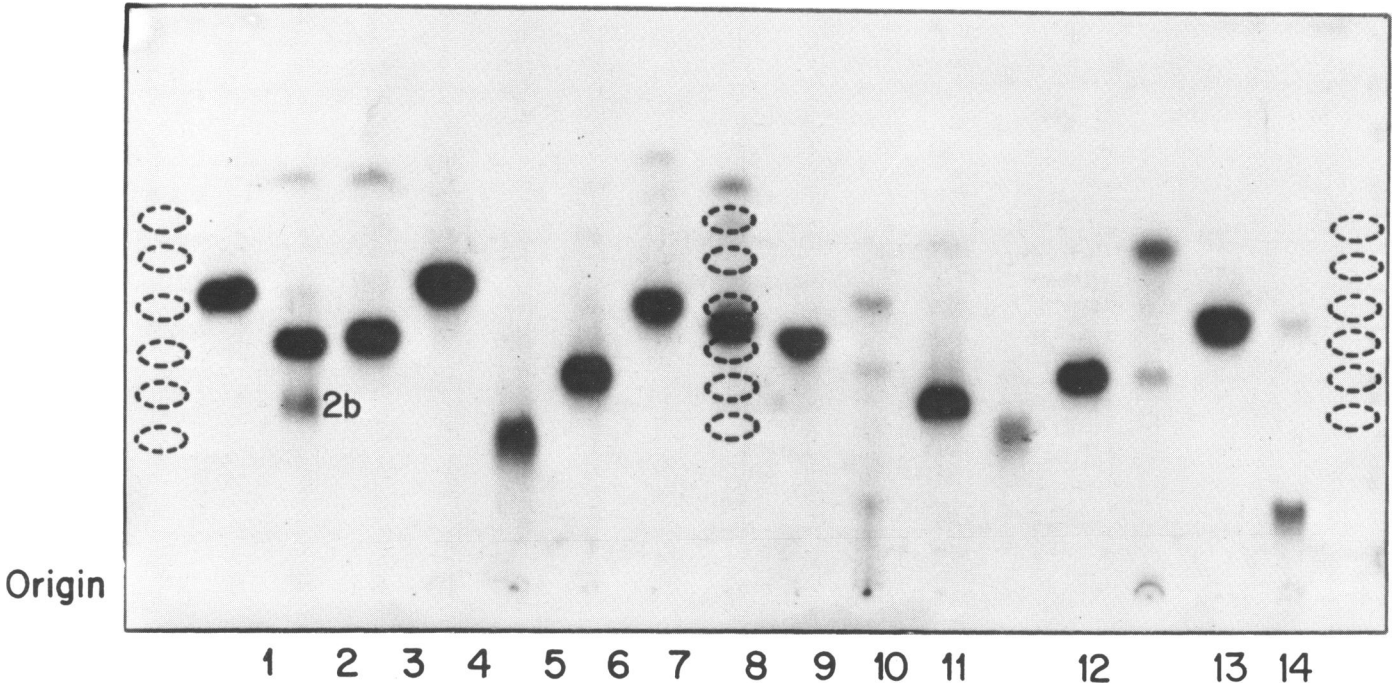

Fig. 8: Thin layer chromatography on spots 1-14 of Fig. 7 in the presence of ultraviolet absorbing markers of oligonucleotides 1-6 units long (see text). Dotted circles indicate the location of the markers. Although these markers were added to each of the spots, only their location in three of the tracks is shown.

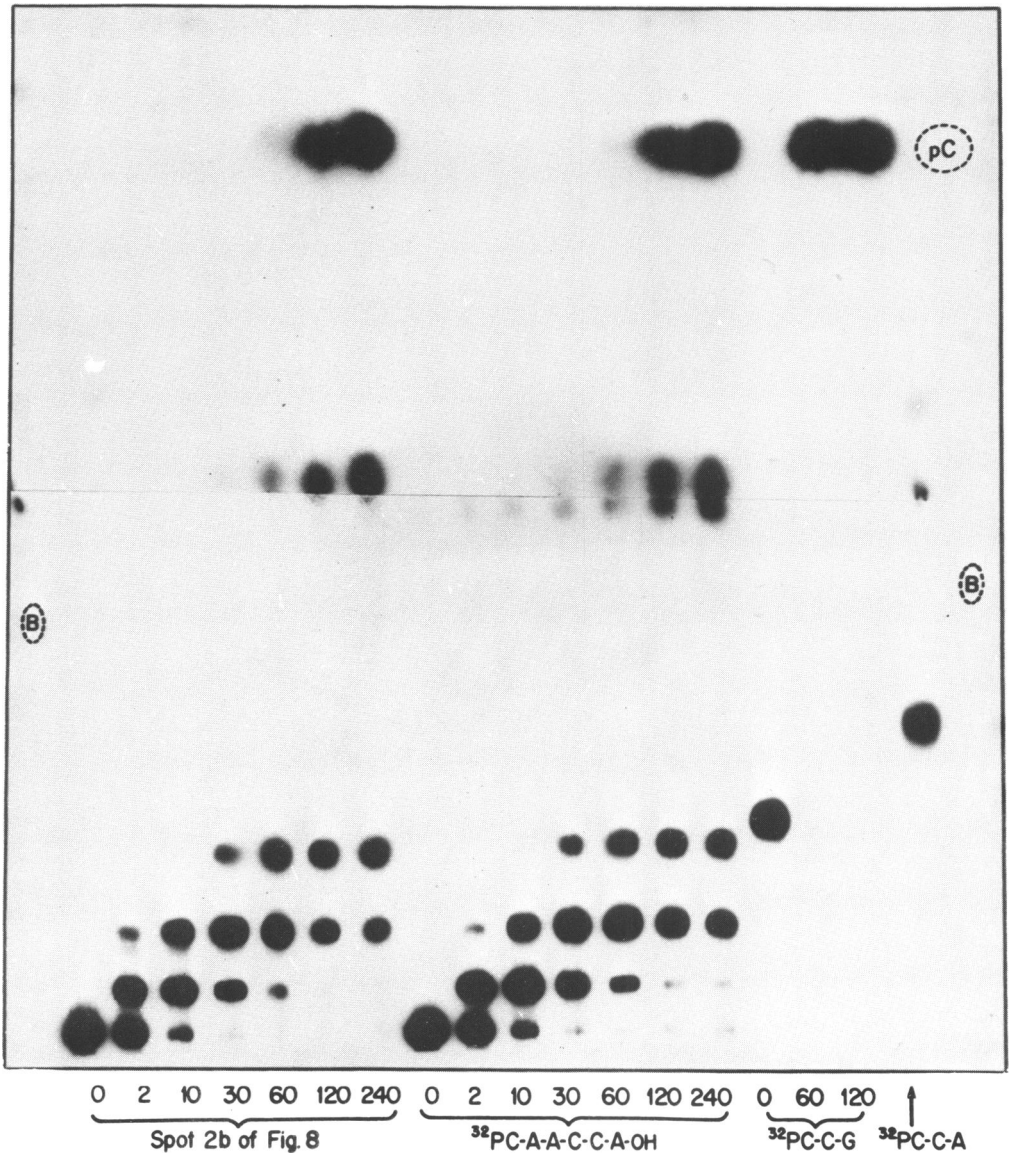

Fig. 9: Autoradiograph of partial digests with snake venom phosphodiesterase on [^{32}P]C-A-A-C-C-A obtained from spot 2b of Fig. 8 and E. coli initiator tRNA. Neither of these digests contains a spot corresponding to a marker of [^{32}P]C-C-A. This also rules out the possibility that the sequence of the 3'-terminal fragment is C-C-A-C-C-A.[10] Electrophoresis was on DEAE-cellulose paper at pH 3.5.[5] B, blue dye marker; pC, a marker added to all the digests and located by UV absorbance. The numbers represent minutes of incubation with snake venom phosphodiesterase at room temperature.

3'-Terminal Sequence of M. capri $tRNA_{f\ II}^{Met}$ is C-A-A-C-C-A_{OH}.
The M. capri $tRNA_{f\ II}^{Met}$ was treated with T_1-RNase, the phosphomonoester groups in the fragments were removed by incubation with E. coli alkaline phosphatase and the 5'-hydroxyl group of each fragment was phosphorylated with [^{32}P] using γ-[^{32}P]ATP and polynucleotide kinase.[5] The 5'-[^{32}P] labeled fragments were then separated by two-dimensional electrophoresis.[19,20] Fig. 7 shows the autoradiogram obtained. Each of the spots was analyzed with respect to their size (Fig. 8), 5'-end group and their sequence by partial digestion with snake venom phosphodiesterase.[5] Table I lists the total data obtained on these fragments.

Table I

Spot number	Size	5'-Terminal nucleotide	Tentative sequence
1	2	C	CG
2	3,5?	C	CCG, CAACCA
3	3	C	CAG
4	2	A	AG
5	6	C	CCCCCG
6	4	C	CUCG
7	3	U*	DAG
8	3	U	UAG
9	-	G	pGp
10†	8?	C	CUCAXCCG
11	5	U	UCCUG
12	4	U	UψCG
13	3	U*	UUG+
14	10 or >10	C	Unknown

*Represents a modified base, X is unknown.

†Most of the material in spot 10 was found to be degraded. The size estimate of 8 is based on the chromatographic mobility of the slowest radioactive material.

+One or both of the U residues are modified. This fragment is likely to be s^4U-s^4U-G which has been partially de-thiolated during electrophoresis to U-U-G.[19]

Spot 2 of Fig. 7 was found to be a mixture of [^{32}P]C-C-G and the 3'-terminal fragment (see Materials and Methods). This 3'-terminal fragment was shown to be identical to an authentic marker of [^{32}P]C-A-A-C-C-A_{OH} (obtained by phosphorylation of C-A-A-C-C-A_{OH} isolated from a T_1-RNase digest of E. coli $tRNA^{fMet}$) as follows: (i) Digestion with T_2-RNase gave [^{32}P]Cp as the only radioactive product, (ii) thin layer chromatography in n-propanol-ammonium hydroxide-water (55:10:35, v/v) showed that the two oligonucleotides possessed identical mobilities and (iii) the radioactive oligonucleotides produced by partial digestion with snake venom phosphodiesterase were identical (Fig. 9).

Modified Nucleotide Composition of Crude M. capri tRNA and $tRNA_{f\ II}^{Met}$. T_2-RNase digest of the crude tRNA followed by two-dimensional thin layer chromatography (see Materials and Methods) showed that the M. capri tRNA contained ψp and probably some m^5Cp but was almost totally lacking in Tp and m^7Gp. The UV spectrum and absorbance at 338 nm indicated that the crude tRNA contained nearly one mole of s^4U per mole of tRNA.

Analysis of T_2-RNase digest of $tRNA_{f\ II}^{Met}$ showed a clear spot corresponding to ψp and also confirmed the absence of Tp, m^7Gp or most other modified nucleotides in this tRNA. The UV spectrum of $tRNA_{f\ II}^{Met}$ showed a peak of absorbance at 337-338 nm in addition to the peak at 260 nm. The observed $A_{338/260}$ of 3.3% correlates well with the maximum value found previously for E. coli $tRNA_{II}^{Tyr}$ of 3.5%[11] which contains two s^4U residues per tRNA and suggests that M. capri $tRNA_{f\ II}^{Met}$ may also contain two s^4U residues. Although the procedure used above cannot detect the presence of D, the data in Table I show that the $tRNA_{f\ II}^{Met}$ contains one mole of D and in the sequence D-A-G- in a T_1-RNase digest.

Enzymatic Methylations of M. capri tRNA and Evidence for the Presence of the Sequence G-U-ψ-C-G- in M. capri $tRNA_{f\ II}^{Met}$. Hayashi et al[23] previously showed that Mycoplasma sp. tRNAs lack the modified nucleoside T and that a specific U residue[24] in these tRNAs can be methylated in vitro using E. coli enzymes to form T. Result of a similar experiment shows that crude tRNA from M. capri can also be methylated in the presence of [^{14}C]-methyl-S-adenosyl methionine. Digestion of the methylated tRNA

with T_2-RNase shows that most of the [^{14}C]-methyl group is present in Tp (Fig. 10). Quantitative analysis of the radioactive spots in Fig. 10 indicates that 90% of [^{14}C]-methyl group incorporated is present as Tp, the remaining 10% is present in at least four components and is detected only after prolonged period of autoradiography.

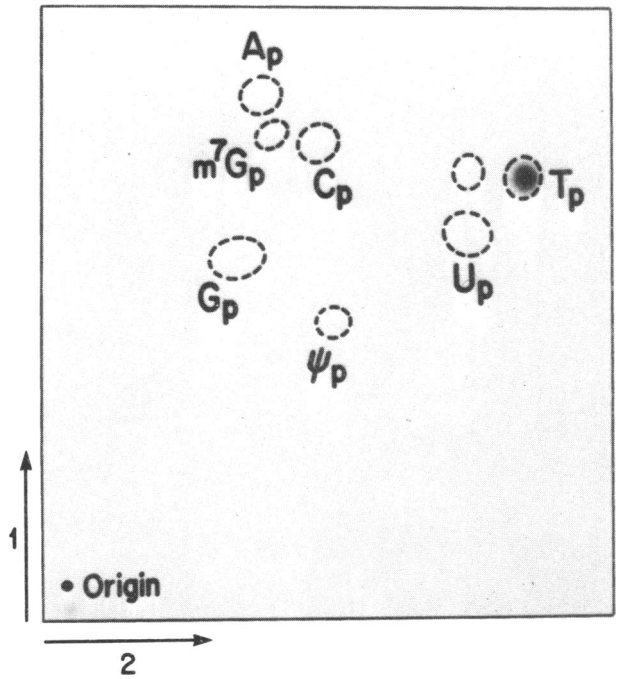

Fig. 10: Two-dimensional thin-layer chromatography of a T_2-RNase digest on [^{14}C]-methyl labeled M. capri $tRNA_{f\ II}^{Met}$ in the presence of carrier E. coli $tRNA^{fMet}$. Exposure time for autoradiography was about 16 hours.

Use of M. capri $tRNA_{f\ II}^{Met}$ in a similar experiment resulted after incubation for 90 min in the incorporation of approximately 0.5 mole of [^{3}H]-methyl group from [^{3}H]-methyl S-adenosyl methionine per mole of tRNA. The kinetics of incorporation showed that the extent of methylation was still increasing at this time. As shown below, treatment of the [^{3}H] methylated $tRNA_{f\ II}^{Met}$ with T_1-RNase yields T-ψ-C-G- as the predominant radioactive product. Thus, in M. capri $tRNA_{f\ II}^{Met}$, a specific U is methylated to T and this U is in the sequence G-U-ψ-C-G-.

The [^{3}H]-methyl labeled $tRNA_{f\ II}^{Met}$ (0.25 O.D. unit, 500,000 cpm) was treated with T_1-RNase and mixed with a marker of T-ψ-

C-G- (2.5 O.D. units) and with a T_1-RNase digest of E. coli $tRNA^{fMet}$ (10 O.D. units). The mixture was chromatographed on an RPC-5 column.[21] Fig. 11 shows that at least 90% of [^{3}H] is coincident with the main A_{260} peak due to T-ψ-C-G- and only negligible amounts of [^{3}H] are present in fractions 115-120 which contain the oligonucleotide T-ψ-C-A-A-A-U-C-C-G- produced by T_1-RNase on E. coli $tRNA^{fMet}$. Fractions 66-69 were pooled, desalted and digested with T_2-RNase and the nucleotides produced were separated by two-dimensional thin-layer chromatography. Of the 40,000 cpm total [^{3}H] applied on the thin-layer plate, at least 98% of the recovered [^{3}H] was found in Tp. Fractions 111-120 were similarly treated and analyzed for their nucleotide composition. The presence of both Tp and ψp in such a digest confirmed that this peak contains the oligonucleotide T-ψ-C-A-A-A-U-C-C-G- from E. coli $tRNA^{fMet}$.

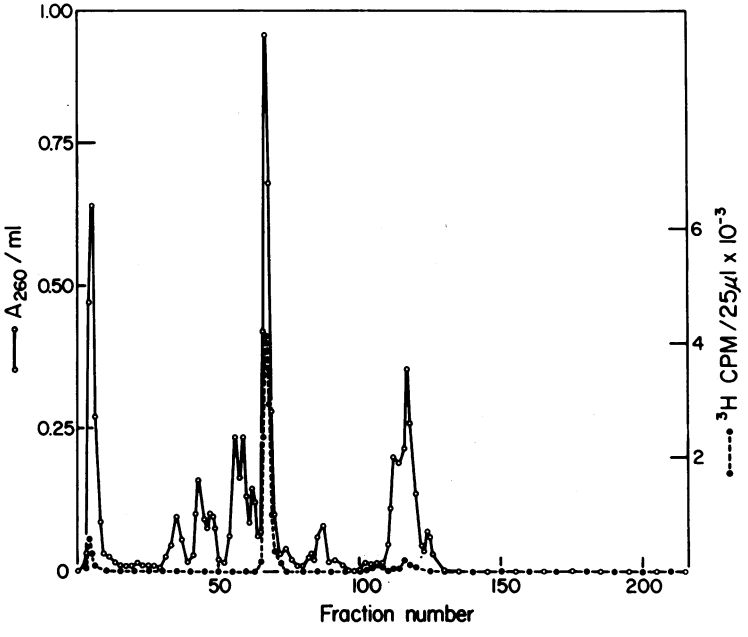

Fig. 11: RPC-5 chromatography of a T_1-RNase digest on 0.25 O.D. of [^{3}H]-methyl labeled M. capri $tRNA_{f\ II}^{Met}$ (see text for details).

The above findings suggest strongly the presence of a sequence G-U-ψ-C-G- in M. capri $tRNA_{f\ II}^{Met}$ in which the U residue can be enzymatically methylated to G-T-ψ-C-G. Further support for this is derived from the identification of U-ψ-C-G- as one of the products in T_1-RNase digests of the $tRNA_{f\ II}^{Met}$ (spot 12 of Fig. 7). Thus, (i) it contains U at the 5'-end (Table I), (ii) it behaves like a tetranucleotide upon thin-layer chromatography (Fig. 8), (iii) its position on the fingerprint is consistent with a nucleotide composition of 1C, 1G and 2U or U derivatives (Fig. 7) and (iv) digestion with snake venom phosphodiesterase (0.5 μg enzyme/pmole oligonucleotide) for one hour, conditions under which most oligonucleotides are totally degraded to mononucleotides, yield only a trace of the mononucleotide [^{32}P]U and a resistant dinucleotide possessing an electrophoretic mobility identical to that expected for [^{32}P]U-ψ Fig. 12). The resistance of the dinucleotide towards further digestion also suggests the presence of ψ at the 3'-end of the dinucleotide. Finally, the total sequence of this oligonucleotide is established as U-ψ-C-G based on the M values of the products of digestion with snake venom phosphodiesterase.

DISCUSSION

The most significant result from the present work is the finding that in contrast to most tRNAs, in Mycoplasma $tRNA_{f\ II}^{Met}$, the first nucleotide at the 5'-end cannot form a Watson-Crick type of base-pair to the fifth nucleotide from the 3'-end. The same unique feature is present in initiator tRNAs of E. coli,[9] the blue-green alga Anacystis nidulans[10] and possibly also of Streptococcus faecalis.[25] It would thus appear that the absence of the first base-pair in the acceptor stem is a feature common to all prokaryotic initiator tRNAs. Studies involving chemical modification of E. coli initiator tRNA have led Shulman et al[26,27] to propose that this structural feature is probably responsible for two of the unique properties of the E. coli initiator tRNA: (i) the inability of E. coli met-$tRNA^{fMet}$ to bind the E. coli T_u factor[28] and (ii) the known resistance of E. coli fmet-$tRNA^{fMet}$ towards peptidyl-tRNA hydrolase.[29,30] The finding[9,10,25] that this structural feature may be general to all prokaryotic initiator tRNAs also provides independent evidence for the notion that

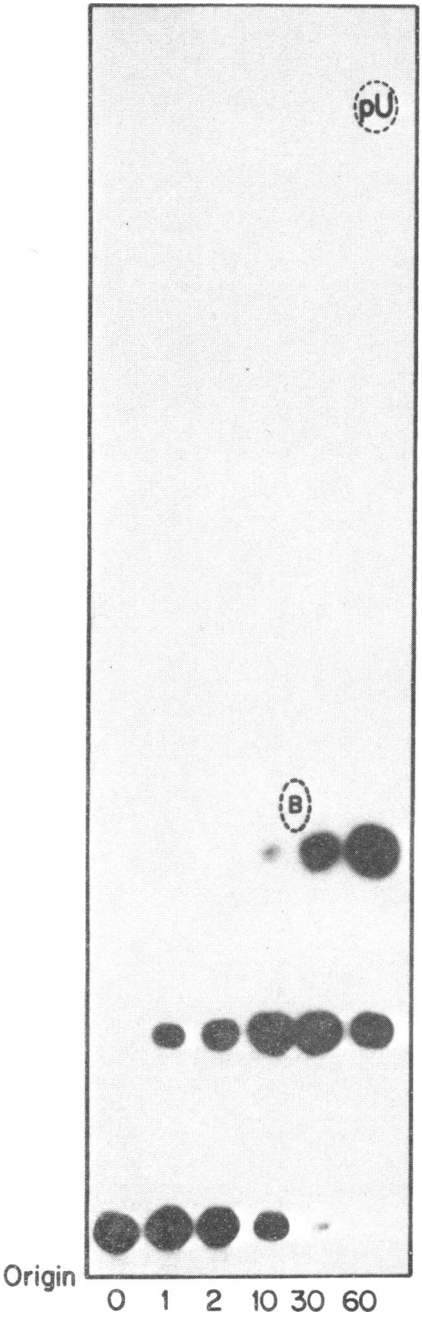

Fig. 12: Autoradiograph of partial digests with snake venom phosphodiesterase on spot 12 of Fig. 7. B, blue dye marker; pU, marker added to all the digests and located by UV absorbance. The numbers represent minutes of incubation with snake venom phosphodiesterase at room temperature. Electrophoresis was on DEAE-cellulose paper at pH 3.5.[5]

this structural feature is related to a very important property or properties of prokaryotic initiator tRNAs.

Although not unique to initiator tRNAs, another common feature of E. coli and Anacystis nidulans initiator tRNAs is the presence of the sequence T-ψ-C-A---- instead of the more common T-ψ-C-G- in loop IV. Initiator tRNA of Streptococcus faecalis[25] grown in the presence of folate also contains the sequence T-ψ-C-A----. The absence of T in Mycoplasma $tRNA_{f\ II}^{Met}$ plus the observation that U which replaced T in this tRNA could be methylated by E. coli extracts enabled us to selectively label and sequence this region of the tRNA. Digestion of [^{3}H] methyl labeled $tRNA_{f\ II}^{Met}$ with T_1-RNase yielded [^{3}H]T-ψ-C-G- and not [^{3}H]T-ψ-C-A-A-A-U-C-C-G (Fig. 11). Thus, M. capri $tRNA_{f\ II}^{Met}$ contains the sequence U-ψ-C-G- in loop IV and this is methylated to T-ψ-C-G-. Unlike the unique structural feature at the acceptor stem, the presence of T-ψ-C-A instead of T-ψ-C-G- in loop IV is, therefore, not a structural requirement general for all prokaryotic initiator tRNAs.

Our qualitative results on the modified nucleoside content of M. capri tRNA show that except for ψ, s^4U and probably D, most other modified nucleosides are present in much smaller amounts. T is almost completely absent in M. capri tRNA. Furthermore, since M. capri tRNA is methylated by E. coli extracts to yield T as the predominant product of methylation (Fig. 10) we conclude that T is replaced by its precursor U in the bulk of M. capri tRNA. Hence, the absence of T is most probably due to the absence of a U→T methylase in M. capri and not due to further methylation of T.[31]

Recent structural studies on eukaryotic cytoplasmic initiator tRNAs[1-4] have shown that they all possess a unique sequence in loop IV of the tRNA. The sequence G-T-ψ-C-G(A)-, which has so far been found in all tRNAs that are active in protein synthesis including prokaryotic initiator tRNAs, is absent and is replaced by G-A-U(or U*)-C-G-. Our findings that M. capri $tRNA_{f\ II}^{Met}$ contains the sequence G-U-ψ-C-G- which can be enzymatically methylated to G-T-ψ-C-G combined with the observation that M. capri tRNAs contain the modified nucleoside s^4U, till now found exclusively in prokaryotic tRNAs, clearly distinguishes M. capri from eukaryotes and reaffirms the commonly assumed relationship be-

tween Mycoplasma and prokaryotes. Further evidence for this is also derived from the reports of Hayashi et al,[23] Feldman et al[32] that extracts of Mycoplasma contain methionyl-tRNA formylase, an enzyme so far found only in prokaryotes or prokaryote-like eukaryotic organelles.

During the fractionation of M. capri tRNA on BD-cellulose (Fig. 1), two peaks of methionine acceptor activity were observed. Both of these peaks could be essentially quantitatively formylated by E. coli extracts and presumably represent two species of M. capri $tRNA_f^{Met}$. We have so far failed to locate the $tRNA_m^{Met}$ species during fractionation of M. capri tRNA on BD-cellulose. Among the possible reasons for this are: (i) occurence of $tRNA_m^{Met}$ species in relatively smaller amounts compared to the formylatable species and (ii) separation of $tRNA_m^{Met}$ into several small peaks making their detection at the relatively low level of crude tRNA used in these studies more difficult. An alternative possibility that this is due to the use of heterologous synthetases from E. coli rather than the homologous one from Mycoplasma is unlikely in view of previous observations[23,32] that E. coli synthetases can aminoacylate both the $tRNA_m^{Met}$ and $tRNA_f^{Met}$ species from several different strains of Mycoplasma.

Finally, the 5'- and 3'-terminal sequences deduced for M. capri $tRNA_{f\ II}^{Met}$ are pC-G- and C-A-A-C-C-A_{OH}, respectively. These sequences are identical to those present in E. coli $tRNA^{fMet}$. In addition, a comparison of the sequences of fragments present in a T_1-RNase digest of M. capri $tRNA_{f\ II}^{Met}$ (Table I) with those present in a similar digest of E. coli $tRNA^{fMet}$ reveals several fragments which are common to both tRNAs. Particularly noteworthy is the fragment C-C-C-C-C-G-, which in E. coli $tRNA^{fMet}$ is present in the acceptor stem as part of the sequence C-C-C-C-G-C-A-A-C-C-A_{OH}. If the location of C-C-C-C-C-G- in M. capri $tRNA_{f\ II}^{Met}$ is the same as in E. coli $tRNA^{fMet}$, this would indicate that the entire sequence of the acceptor stem may be identical between these two tRNAs. Further studies on M. capri $tRNA_{f\ II}^{Met}$ are necessary to establish this and to compare the sequence homology between various prokaryotic initiator tRNAs.

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