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Biochemistry

Total synthesis of a RNA molecule with sequence identical to that of *Escherichia coli* formylmethionine tRNA†

(chemical synthesis of tRNA fragments/RNA ligase/methionyl-tRNA synthetase)

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ABSTRACT **A RNA molecule has been synthesized that is identical in sequence to *Escherichia coli* $tRNA_f^{Met}$ except that it lacks the base modifications present in the *E. coli* tRNA. This was achieved by enzymatic joining of chemically synthesized oligonucleotides with chain lengths of 3–10 which were synthesized by the phosphodiester or phosphotriester method. First, quarter molecules of tRNA were constructed by joining of chemically synthesized fragments with RNA ligase. The 5′-quarter molecule (bases 1–20) served as an acceptor in joining reactions with the 3′,5′-bisphosphorylated donor molecule (bases 21–34). The 5′-half molecule thus obtained was treated with phosphatase and joined to the 3′-half molecule which was prepared by ligation of the other quarter molecules (bases 35–60, acceptor; bases 61–77, donor) followed by 5′-phosphorylation with polynucleotide kinase. The synthetic tRNA was characterized by oligonucleotide pattern and was partially active in aminoacylation with *E. coli* methionyl-tRNA synthetase.**

Chemical synthesis of nucleic acids has been a challenging problem in organic chemistry since the structure of the nucleic acids was elucidated. Chemical methods to synthesize short ribo- and deoxyribopolynucleotides with defined sequences were established in early 1960s, and those oligonucleotides were important in the elucidation of the genetic code (1). Discovery of DNA ligase allowed the synthesis of bihelical DNAs from chemically synthesized deoxyribopolynucleotides. With this chemical-enzymatic method the genes for yeast alanine tRNA (2) and *Escherichia coli* tyrosine tRNA precursor (3) have been synthesized; the latter was the first synthetic functional DNA molecule. Genes for peptides have also been synthesized by the same approach, and the methods for joining double-stranded DNA pieces with protruding ends have been used in various recently developed reactions for genetic manipulations.

Although tRNAs are the smallest nucleic acids with unique functions, their synthesis has been difficult until recently, mainly because of the lack of good synthetic methods for larger oligoribonucleotides as well as a lack of joining enzymes. After the primary structure of yeast alanine tRNA had been determined (4), the nona- and hexanucleotide corresponding to the terminal sequence of this tRNA were synthesized by phosphodiester block condensation. These fragments in turn were used to form reconstituted molecules with natural tRNA fragments derived by RNase digestions. However, aminoacylation was not possible because the synthetic fragments were too small to form sufficiently stable complexes for recognition by the alanyl-tRNA synthetase (5). The discovery of RNA ligase (6) and its ability to join single-stranded oligoribonucleotides (7) made it possible to elongate synthetic RNA fragments to yield larger molecules such as tRNAs.

The initiator methionine tRNA of prokaryotes has a special role in protein biosynthesis, which manifests itself in several unique properties of that tRNA (8). It was also the subject of detailed modification studies to explain its structure–function relationship (9). Because a RNase T1-digested one-quarter molecule of *E. coli* $tRNA_f^{Met}$ reconstituted methionine acceptor activity when mixed with the corresponding three-quarter molecules (10), this tRNA seemed an appropriate target for chemical synthesis. The final aim would be to modify systematically the functionally important parts of the molecule.

We began by synthesizing terminal fragments of the tRNA (11–16) and examined the ability of RNA ligase (17–19) to join these fragments. The 5′-terminal icosanucleotide (20), the tetradecanucleotide (bases 21–34) (21), and the 3′-heptadecanucleotide (22) have been obtained by this method. The 5′-quarter molecule here was found to reconstitute methionine acceptor activity when it was combined with the natural RNase T1-generated three-quarter molecule (20). Oligonucleotides corresponding to the rest of the molecule and certain of their analogs have been synthesized either by the phosphodiester method (23, 24) or by the triester method (25, 26).

In this paper we report total synthesis of a RNA molecule with a sequence identical to that of *E. coli* $tRNA_f^{Met}$ obtained by the enzymatic joining of chemically synthesized fragments with RNA ligase.

MATERIALS AND METHODS

Enzymes. T4 RNA ligase was purified as described (27). Polynucleotide kinase and *E. coli* alkaline phosphatase were gifts of M. Sugiura. 3′-Phosphatase-free kinase was isolated from *E. coli* infected with T4 PseT1-amN82SP62 as described (20). Other enzymes for characterization of the products were obtained as described (17–20).

Kinase Treatment, Ligation, and Dephosphorylation. 5′-Phosphorylation by using polynucleotide kinase and [γ-^{32}P]ATP was performed as described (20). All 5′-phosphorylations of 3′-phosphorylated oligonucleotides were performed by using the 3′-phosphatase-free kinase unless otherwise specified. Ligation was carried out in the presence of a 2-fold excess of ATP with respect to donor molecules in 50 mM Hepes (made pH 8.3 with NaOH)/10 mM dithiothreitol/10 mM $MgCl_2$/10% (vol/vol) dimethyl sulfoxide containing bovine serum albumin at 10 μg/ml. The 3′-phosphate was removed by treatment with *E. coli* alkaline phosphatase in 50 mM Tris·HCl (5 μl, pH 8.1) at 55°C for 30 min. The enzyme was inactivated by treatment with 1 μl

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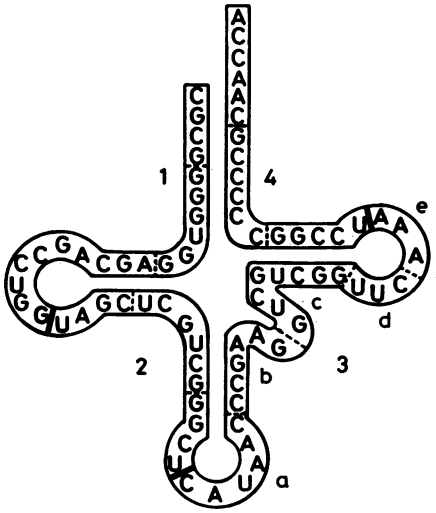

FIG. 1. Structure of *E. coli* $tRNA_f^{Met}$. The quarter molecules 1, 2, and 4 were obtained by joining chemically synthesized oligonucleotides with RNA ligase. Quarter molecule 3 was joined as shown in Fig. 2.

of 40 mM EDTA at room temperature for 30 min and then at 100°C for 2 min (28) and extracted twice with 2 μl of phenol saturated with 50 mM Tris·HCl (pH 8.1). The phenol layer was washed twice with water (20 μl) and the aqueous phase was subjected to gel filtration on a Sephadex G-50 column (1.1 × 21 cm).

Isolation and Characterization of Joined Products. Paper chromatography was performed with 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol), as the solvent system. Paper electrophoresis was performed at 900 V/40 cm with 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5). Homochromatography (29) was performed with Homo-mix I-IV (30). Two-dimensional chromatography on cellulose plates was as described (31). Polyethylenimine-cellulose plates (Macherey-Nagel, Polygram Cell 300 PEI) were treated as described (32). Polyacrylamide gel electrophoresis was performed on slab gels or on a disc apparatus as described (20).

Nearest-neighbor analysis (17), 3′- and 5′-terminal analysis (20), and partial nuclease P1 digestion (22) for mobility shift analysis were as described. For complete RNase T1 digestion of the product (1 pmol), RNase T1 (1 unit) was used in the presence of phosphatase (180 microunits) in 10 mM Tris·HCl (pH 7.5) at 27°C for 4 hr.

Aminoacylation of the Joined Product. The purified *E. coli* methionyl tRNA synthetase (a gift of J. P. Waller) was used at

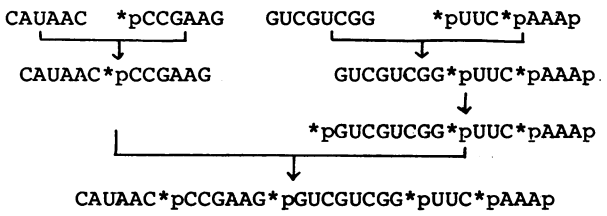

FIG. 2. Synthesis of quarter molecule 3 (bases 35–60).

4 μg/ml for 10 pmol of the tRNA in 100 mM Hepes, pH 8.0/10 mM $Mg(AcO)_2$/10 mM KCl/4 mM ATP/10 mM 2-mercaptoethanol/6 μM L-[^{14}C]methionine (582 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) in total volume of 20 μl at 37°C for 30 min. Aminoacylation with a crude mixture of *E. coli* synthetases (33) (0.12 mg/ml) was performed in the presence of 0.1 mM CTP at 37°C for 60 min. The reaction was stopped by addition of 1 M pyridinium acetate (pH 5.0; 5 μl) and the mixture was applied to a column (0.8 × 23 cm) of Sephadex G-50 equilibrated with 50 mM pyridinium acetate (pH 5.0). The aminoacylated tRNA was eluted with the equilibration buffer, assayed by Cerenkov's method, desalted by gel filtration on Sephadex G-50 in 0.05 M triethylammonium bicarbonate, and treated with Tris·HCl (pH 9.0) at 37°C for 1 hr to hydrolyze the amino acid. The mixture was applied to a column of Sephadex G-50 to resolve the tRNA and [^{14}C]methionine. Fractions were assayed using a scintillation counter. An aliquot (10 pmol) of the tRNA was assayed for methionine acceptor activity by acid precipitation as described (20) after ^{32}P radioactivity became negligible.

RESULTS

Chemical Synthesis of tRNA Fragments. Most of the synthetic methods used for fragments shown in Fig. 1 have been described previously. The phosphodiester method was used for the synthesis of fragments consisting of bases 1–4 (12), bases 5–10 (13), bases 41–57 (23), bases 58–60 (24), and bases 61–71 (14, 23). The phosphotriester method was applied for synthesis of fragments consisting of bases 11–20 (15), bases 21–34 (unpublished work), bases 35–40 (26), and bases 72–77 (16).

Preparation of Quarter Molecules. Segment 1 (bases 1–20) was prepared by joining three fragments as described (20) and the 3′-terminal phosphate was removed. The next quarter, molecule 2 (bases 21–34), was synthesized by two different approaches (21).

Segment 3 (bases 35–60) was prepared by the joining of five synthetic fragments (Fig. 2). The dodecamer C-A-U-A-A-C-C-C-G-A-A-G (bases 35–46) was synthesized by using 10-fold excess of the acceptor molecule (**3a**, see Fig. 1) as summarized in

Table 1. Reaction conditions for joining of oligoribonucleotides with RNA ligase

Acceptor, nmol (μM)	Donor, nmol (μM)	ATP, μM	Enzyme, μg/ml	Temp., °C	Time, hr	Isolated yield, %
CAUAAC	pCCGAAG					
80 (1000)	8 (100)	200	100	25	1	39
GUCGUCGG	pUUCAAAp					
15 (150)	10 (100)	200	140	25	1	36
CAUAACCCGAAG	pGUCGUCGGUU-					
2.1 (150)	CAAp, 1.5 (100)	200	115	25	1	52
1	2					
0.88 (74)	1.3 (111)	227	535	25	2	31
3	4					
4 (100)	2.5 (63)	200	150	25	2	15
5′-half	p3′-half					
0.14 (4.7)	0.20 (6.7)	100	200	4	17	42

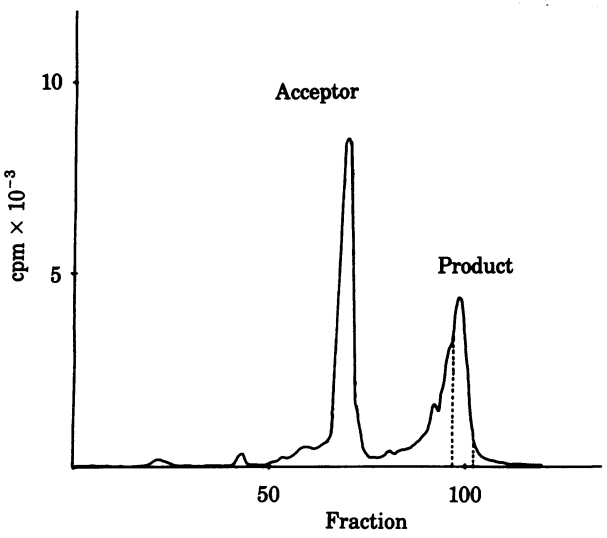

FIG. 3. Disk gel electrophoresis of the 26-nucleotide-long segment 3 (C-A-U-A-A-C*pC-C-G-A*pG-U-C-G-U-C-G-G-U-U-C-A-A-Ap) (Fig. 2) on 20% acrylamide. The first peak contained the acceptor (C-A-U-A-A-C*pC-C-G-A) and the last peak contained the product.

Table 1. The tetradecamer (bases 47–60) was synthesized by joining 3c to the hexanucleotide *pU-U-C-A-A-Ap which had been obtained by phosphorylation with polynucleotide kinase and [γ-^{32}P]ATP of the joined product from U-U-C (3d) and pA-A-Ap (3e). After 5′-phosphorylation, the tetradecamer was joined to the dodecamer under the condition shown in Table 1. The 26-nucleotide-long segment 3 was isolated by electrophoresis on a 20% acrylamide gel disc as illustrated in Fig. 3. The nearest-neighbor analysis of the product is shown in Fig. 4. The chain length was confirmed by slab gel electrophoresis.

The 3′-phosphorylated heptadecamer 4 was prepared as described (22). The 3′-phosphorylation of C-A-A-C-C-A (16) was done with P1-adenosine-P2-(*o*-nitrobenzyl) pyrophosphate and RNA ligase (19).

Joining of Quarter Segments to Yield the tRNA Molecule. The 5′-half molecule was synthesized by joining of quarter molecules 1 and 2. The reaction conditions are summarized in Table 1. The mixture was separated by polyacrylamide gel electrophoresis, and the product was detected by autoradiography (Fig. 5*A*). The extent of the reaction was 73% as measured by assaying gel slices at the appropriate positions. However, the isolated yield after elution from the gel was 31% based on 1.

For the synthesis of the 3′-half molecule, heptadecamer 4 was phosphorylated and joined to the 3 by using the conditions shown in Table 1. The product was isolated as described for the 5′-half molecule in a yield of 20%. It was characterized by nearest-neighbor and terminal analyses. The 3′-half was then 5′-

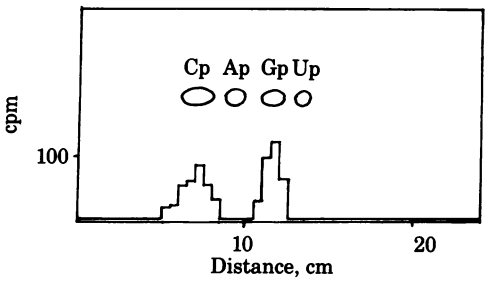

FIG. 4. Nearest-neighbor analysis of 3 after digestion with RNase T2 and paper electrophoresis at pH 3.5.

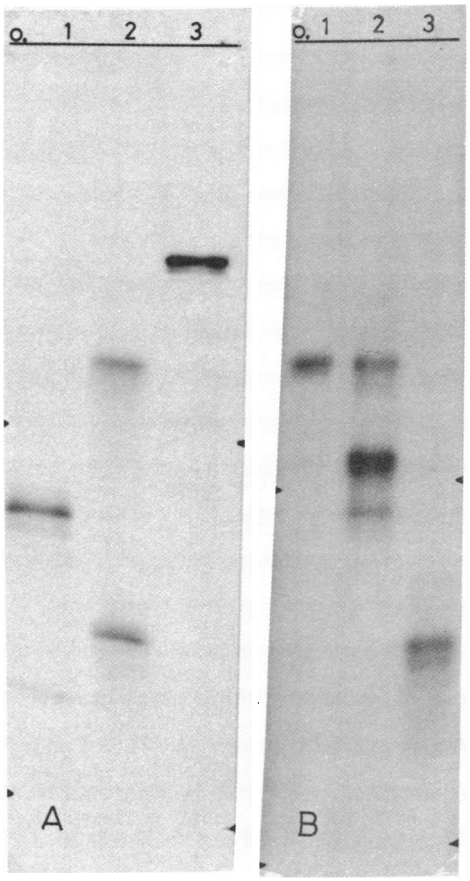

FIG. 5. Polyacrylamide gel electrophoresis of products. (*A*) Synthesis of the 5′-half molecule from 1 and 2 (lane 2). Lane 1: the 5′ quarter molecule, 1. Lane 3: the three-quarter molecule. (*B*) Synthesis of the 3′-half molecule from bases 35–46 and bases 41–77 (lane 2). Lane 1: bases 35–77. Lane 3: bases 47–60.

phosphorylated with polynucleotide kinase and [γ-^{32}P]ATP and joined to the 5′-half molecule at reduced temperature (Table 1).

The reaction mixture was subjected to gel filtration (Fig. 6*A*). The joined product was found in peak 1 whereas the acceptor and the donor eluted together in peak 2. This was verified by polyacrylamide gel electrophoresis of the fractions. The molecular weight of the product was estimated to be 2.6×10^4 from a plot of logarithm of molecular weight against mobility in polyacrylamide gel electrophoresis. As expected, 5′-end group analysis of the product after ^{32}P-labeling (with polynucleotide kinase and [γ-^{32}P]ATP) and RNase T2 digestion yielded [^{32}P]pCp. The 3′-end analysis was performed by transferring the labeled 5′-phosphate by circularization with RNA ligase followed by hydrolysis with RNase T2. C*p (instead of A*p) was identified as the 3′-end by two-dimensional chromatography. This may be due to removal of pAp by a reverse reaction of RNA ligase which has been observed with large excesses of the enzyme (R. I. Gumport and O. C. Uhlenbeck, personal communication). To avoid this side reaction, the 3′-half molecule was prepared (Fig. 5*B*) and the 3′-phosphate of the 43-unit segment was removed during the 5′-phosphorylation by using polynucleotide kinase with 3′-phosphatase activity. Although the presence of a 3′-OH group on the donor molecule could lead to the donor molecule joining onto itself, it was hoped that the secondary structure would prevent circularization of the 3′-half molecule (donor) during the joining reaction of the halves at the anticodon loop.

Joining of this 43-unit segment to the 5′ half was performed under the same conditions as described above, and the product

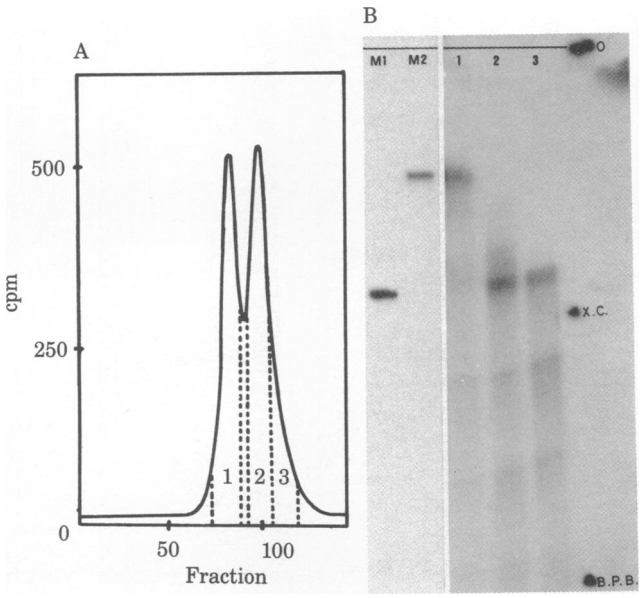

FIG. 6. (*A*) Gel filtration of products in the synthesis of the total molecule from the 3′- and 5′-half molecules on a column (0.7 × 90 cm) of Sephadex G-200 equilibrated with 50 mM potassium phosphate; pH 7.5/0.1 mM EDTA. Elution was at 50°C with flow rate 1.2 ml/hr; 0.22-ml fractions were collected. Column volume, 34.6 ml: void volume, 11.5 ml. Peak 1 contained the total molecule; the half molecules were eluted in peaks 2 and 3. (*B*) Polyacrylamide gel electrophoresis of the compound in each peak. M1 and M2 indicate markers of segment *1* and $tRNA_f^{Met}$, respectively. Lanes 1, 2, and 3 correspond to peak 1, 2, and 3.

was isolated in a yield of 17% by gel filtration. The joined product was analyzed as previously and again partial removal of pA from the 3′ end was observed. Possibly, dephosphorylation did not go to completion during kination. The whole molecule was then treated with phosphatase to remove the 3′-phosphate residue and then phosphorylated at the 5′ end with unlabeled ATP and polynucleotide kinase.

This tRNA molecule was tested for methionine acceptor activity by using L-[^{14}C]methionine and purified or crude methionyl-tRNA synthetase. Aminoacylation was measured by isolating the aminoacyl-tRNA formed by gel filtration in acidic medium followed by hydrolysis and quantitation of the [^{14}C]methionine produced. With the purified enzyme aminoacylation was 6%; with the crude enzyme it was 4%.‡ The deacylated recovered tRNA was then subjected to RNase T1 digestion for further structural analysis. The T1 fragments were labeled by phosphorylation (28) and mapped by two-dimensional thin-layer chromatography on PEI-cellulose. As shown in Fig. 7, the synthetic tRNA gave essentially the same pattern as the natural $tRNA_f^{Met}$. The 3′-fragment *pC-A-A-C-C-A (spot 1) was accompanied by *pC-A-A-C-C (spot 1′) in chromatography of the product.

DISCUSSION

Examination of tRNA structure–function relationship may lead to an understanding of an interesting example of the specific

‡ Acid precipitation of the amino acid carried by the aliquot of the synthetic tRNA (10 pmol) was measured after ^{32}P radioactivity became negligible with a control (-$tRNA_f^{Met}$, 66 cpm); it was found to be 25/cpm (0.32 pmol after subtraction of background) when the natural $tRNA_f^{Met}$ (10 pmol) accepted methionine (2866 cpm, 4.8 pmol). Thus, the aminoacylation of the synthetic tRNA was 6.7% with respect to the intact tRNA.

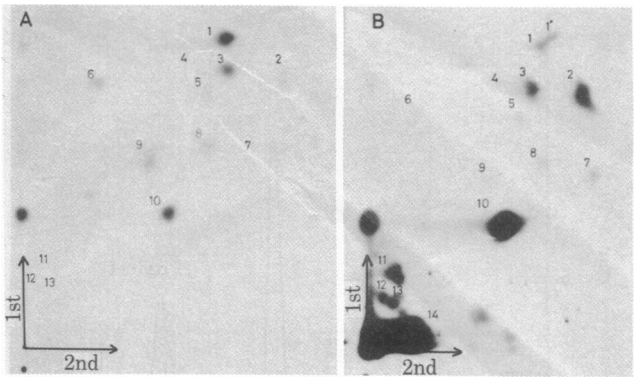

FIG. 7. Two-dimensional thin-layer chromatography of RNase T1 fragments of the natural $tRNA_f^{Met}$ (*A*) and of the synthetic nascent molecule (*B*) on PEI-cellulose plates (20 × 20). The plates were irrigated with 1.4 M lithium formate, pH 3.5/7 M urea for 10 cm, and then with 2.3 M lithium formate, pH 3.5/7 M urea (1st dimension) and with 0.6 M lithium chloride/20 mM Tris·HCl, pH 8.0/7 M urea (2nd dimension) (33). Spots: 1, pC-A-A-C-C-A; 1′, pC-A-A-C-C; 2, ps^4U-P or pU-G; 3, pC-G; 4, pC-A-G; 5, pA-G; 6, pC-C-C-C-G; 7, pD-A-G or pU-A-G; 8, pU-C-G; 9, pC-U-C-G, pC-C-U-G, and pA-U-C-G; 10, P_i; 11, pCmU-C-A-U-A-A-C-C-C-G or pC-U-C-A-U-A-A-C-C-C-G; 12, pm^7G-U-C-G or pG-U-C-G; 13, pT-Ψ-C-A-A-A-U-C-C-G or pU-U-C-A-A-A-U-C-C-G; 14, ATP.

recognition of a nucleic acid by a protein. Chemical modifications of tRNAs or genetic approaches to find mutants with base substitutions have been used previously for recognition studies. However, these approaches have certain limitations. Chemical synthesis should provide defined alterations which would be useful in structure–function relationship studies of tRNAs.

The chemical synthesis of oligoribonucleotides that have sequences of *E. coli* $tRNA_f^{Met}$ and their analogs (e.g. U-G-C-G-G) (25) has provided suitable substrates for the construction of tRNA molecules by joining with RNA ligase. This paper reports the total synthesis of $tRNA_f^{Met}$ from synthetic oligonucleotides with chain lengths 3–10. Oligonucleotides containing modified bases can be joined to other synthetic fragments by methods similar to those described herein. Even though RNA ligase can join short oligonucleotides and is a convenient tool for substituting fragments, it would be desirable to reduce the number of joining steps so as to obtain tRNA molecules in sufficient quantity to provide enough material for biological studies. Chemical synthesis of oligonucleotides as long as quarter molecules of tRNA would yield whole molecules after three ligations. Recently, we synthesized an icosaribonucleotide corresponding to bases 35–54 of the $tRNA_f^{Met}$ by the phosphotriester method (unpublished data) and this fragment will be joined to fragments including modified bases to obtain tRNAs with partial modifications.

The tRNA synthesized in the present work is recognized to a limited extent by *E. coli* methionyl-tRNA synthetase. We do not know the tertiary structure of this tRNA. If modified nucleotides are necessary for forming the correct conformation required for synthetase recognition, then the low acceptor activity would be explained. Otherwise, a particular nucleotide modification may be required for direct interaction with the enzyme. Further synthetic investigation to identify the levels of modification that increase amino acid acceptor activities should aid in elucidating the mechanisms of these interactions.

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