Studies on Polynucleotides

XCI. YEAST METHIONINE TRANSFER RIBONUCLEIC ACID: PURIFICATION, PROPERTIES, AND TERMINAL NUCLEOTIDE SEQUENCES*

(U-G) and poly (A-U-G) as messengers show that, similar to the aminoacylation of yeast tRNA by Escherichia coli aminoacyl tRNA synthetases. Met-tRNA from Peak II is identical with those present in E. coli aminoacyl-tRNA synthetases. At 15 mM Mg++, however, tRNAMet (Peak II) is aminoacylated by crude E. coli methionyl-tRNA synthetase. It has the following properties. (a) It can be aminoacylated with methionine by crude yeast or Escherichia coli aminoacyl tRNA synthetases. (b) The methionyl-tRNA thus formed can be further converted to N-formyl-methionyl-tRNA by 5'- and 3'-terminal nucleotide sequences of yeast tRNA are P-A-G-, and C-U-A-C-C-A, respectively; the terminal sequences thus derived are not identical with those present in E. coli tRNA.

Under conditions of magnesium ion concentration (5 mM) used for the aminoacylation of tRNA (Met) (Peak I), tRNA (Met) (Peak II) is aminoacylated by crude E. coli aminoacyl tRNA synthetase at a slow rate. At 15 mM Mg++, however, tRNA (Met) (Peak II) is aminoacylated with methionine to the same extent by crude E. coli aminoacyl tRNA synthetases as by yeast aminoacyl tRNA synthetases. Met-tRNA from Peak II cannot be converted to fMet-tRNA by E. coli extracts.

Following the discovery of N-formyl-methionyl-tRNA in yeast by Marcker and Sanger (2) subsequent work from a number of laboratories (3-9) has shown the unique role of this tRNA in the initiation of protein synthesis in E. coli. In a further characterization of this tRNA, it has been shown that E. coli contains at least two major species of methionine tRNA, only one of which could be enzymatically formylated to tRNA (Met) (5). Little is known, however, about the process of initiation of protein synthesis in yeast. The studies reported in this paper were prompted by the consideration that the methionine tRNAs of yeast—if found analogous to those of E. coli—might provide some indication of the nature of tRNA involved in the initiation of protein synthesis in yeast. Furthermore, in view of previous reports (10) that unfractionated yeast tRNA was partially aminoacylated with methionine by crude E. coli aminoacyl tRNA synthetases, studies on individual species of yeast methionine tRNAs would be expected to clarify certain aspects of recognition of tRNAs by homologous and heterologous aminoacyl tRNA synthetases.

This paper reports on the fractionation of yeast tRNA mixture such that tRNA (Met) is separated into two discrete peaks. One of these (tRNA (Met), Peak I) has been further purified to homogeneity and used for sequence analysis. In addition, this paper also describes studies concerning the recognition of the two peaks by (a) E. coli aminoacyl tRNA synthetases and (b) E. coli methionyl-tRNA synthetase.
coli methionyl-tRNA synthetase and the coding properties and terminal nucleotide sequences of tRNA\textsuperscript{Met}.

Similar results have been obtained by Takeishi, Ukita, and Nishimura (11) on the coding properties and the recognition of the two species of yeast tRNA\textsuperscript{Met} by these E. coli enzymes.

MATERIALS AND METHODS

General

Uniformly labeled L-\textsuperscript{14}C-amino acids were obtained commercially and had the following specific activities (mCi per mmole): methionine, 200; valine, 267; cysteine, 150; glutamic acid, 195; and threonine, 107. \textsuperscript{3}H-Fornyl tetrahydrofolic acid was prepared as described previously (9). Aliquot 204 was a gift from K and K Laboratories. Chromosorb W was supplied by Johns-Manville Products Corporation, DEAE-Sephadex A-50 by Pharmacia, and DEAE-cellulose (Whatman DE-23) by Reeve Angel and Company.

Counting and Scanning of Radioactivity—Paper discs or paper chromatograms after cutting into 1 cm strips were counted in a Packard Tri-Carb liquid scintillation counter (model 3224); the scintillation medium consisted of 2,5-diphenyloxazole (PPO), 4 g, and 1,4-bis[(4-methyl-5-phenyloxazolyl)]benzene (diphenyl POPOP), 0.1 g, in toluene (1 liter). The efficiency of counting for tritium was 2 to 3\% and that for \textsuperscript{14}C was 55 to 60\%.

Paper Chromatography—The desiccating technique was used at room temperature with Whatman No. 1 paper. Solvent systems used were Solvent A, 2-propanol-concentrated ammonium hydroxide-water (7:1:2, v/v); Solvent B, 1-propanol-concentrated ammonium hydroxide-water (55:10:35, v/v), and Solvent C, isobutyric acid-concentrated ammonium hydroxide-water (60:1:33, v/v) pH 3.7.

Elution of ultraviolet-absorbing material from paper was carried out with water. Paper strips from chromatograms developed in Solvent C were washed with ethanol-ether (50:50, v/v) before elution as described above. In all cases a corresponding area from a blank chromatogram was also eluted and used as blank. Optical density units,\textsuperscript{2} as used in this paper, were measured against appropriate blanks at neutral pH in a Zeiss PMQ II spectrophotometer. Ultraviolet absorption spectra were recorded with a Cary Model 14 spectrophotometer.

Paper Electrophoresis—This was performed on Whatman No. 3MM paper with a voltage gradient of 50 volts per cm in 0.03 M paper with a voltage gradient of 50 volts per cm in 0.03 M Tris-HCl (pH 7.1). The paper was immersed in Varsol (a high boiling petroleum fraction) kept cold with running water.

Identification of Nucleotides and Oligonucleotides—Nucleotides and nucleosides were identified by their ultraviolet absorption spectra at pH 1, 7, and 13 and by their mobilities in the various solvent systems. Oligonucleotides were characterized by degradation with various enzymes as described previously (12, 13).

Countercurrent Distribution—This was carried out as described previously (14) in a 200-tube EC580 fractionator. Tubes were pooled in consecutive groups of two and a total of 100 fractions was thus obtained.

Column Chromatography—Except for enzyme preparations, all chromatographic operations were at room temperature. Oligonucleotides obtained by degradation of tRNA\textsuperscript{Met} were fractionated on DEAE-cellulose in the presence of 7 m urea (15).

\textsuperscript{3} One optical density unit is defined as that amount of material per ml of solution which produces an absorbance of 1 in a 1 cm light path cell at 260 m\textmu.

The nucleotidic material from the pooled fractions was separated from urea and salt by passage through columns (2.0 x 95 cm) of polyacrylamide gel (Bio-Gel P2) (16).

DEAE-Sephadex A-50 beads (particle size 40 to 120 \mu) were processed according to Nishimura et al. (17) before packing onto a column. Appropriate fractions from the column were pooled, concentrated approximately 3-fold by evaporation under reduced pressure, and precipitated by the addition of 2 volumes of ethanol. The tRNA was collected by centrifugation, washed with ethanol, and dialyzed before use.

Reverse phase chromatography was carried out essentially as described by Kelmers, Novelli, and Stulberg (18) except that the Chromosorb W (AW 100 to 200 mesh) was suspended in a large excess of isoamylacetate containing 4\% dimethyl sulfoxide-monium chloride (Aliquot 204). The suspension of Chromosorb W was packed onto a column and was washed thoroughly with a solution containing 0.36 M NaCl, 0.01 M MgCl\textsubscript{2}, and 0.02 M Tris-HCl (pH 7.3) saturated with isoamylacetate. All solutions used during the chromatography were saturated with isoamylacetate. The workup of the pooled fractions was essentially similar to those described above.

Polynucleotides

Poly r(U-G) and poly r(A-U-G) were obtained by the reaction of DNA-dependent RNA polymerase with poly d(T-G):poly d(C-A) and poly d(A-T-G):poly d(C-A-T) (19), respectively, as templates as reported previously (9, 20).

Enzymes

Pancreatic RNase, snake venom phosphodiesterase, and E. coli alkaline phosphatase (electrophoretically purified) were purchased from Worthington; T1-RNase was obtained from Sankyo Chemical Company Ltd., Tokyo, and was further purified by column chromatography before use (21). Micrococcus lysodeikticus polynucleotide phosphorylase was a generous gift of Dr. R M. Bock. RNA polymerase was prepared according to the procedure of Chamberlin and Berg (22). Yeast aminoaoyl tRNA synthetases were prepared as described previously (14) and were stored in 50\% glycerol at -20\% before use.

Crude E. coli aminoaoyl-tRNA synthetases were prepared from E. coli B according to the procedure of Muench and Berg (23). E. coli B (24 g) was ground in small batches with 3 times its weight of alumina 305 and the paste was then suspended in 50 ml of a solution containing 0.01 M Tris-HCl (pH 8.0), 0.01 M MgCl\textsubscript{2}, and 10\% glycerol. After a brief centrifugation to remove alumina, the supernatant was centrifuged at 100,000 \times g for 2 hours in a Spincio ultracentrifuge. The clear supernatant was then applied to a column (2.6 x 22 cm) of DEAE-cellulose previously equilibrated with a buffer containing 0.02 M potassium phosphate (pH 7.5), 0.02 M 2-mercaptoethanol, 0.001 M MgCl\textsubscript{2}, and 10\% glycerol, and the column was thoroughly washed with the same buffer. The mixture of aminoaoyl-tRNA synthetases was eluted with a solution containing 0.25 M potassium phosphate (pH 6.5), 0.02 M 2-mercaptoethanol, 0.001 M MgCl\textsubscript{2}, and 10\% glycerol. The peak of protein was pooled (total volume, 96 ml) and concentrated by dialysis against a solution containing 0.002 M potassium phosphate (pH 7.0), 0.04 M 2-mercaptoethanol, 10\% glycerol, and 15\% polyethylene glycol, mol wt 6000 (1 liter) for 11 hours. The concentrated enzyme solution (14

\textsuperscript{2} We thank Dr. S. Nishimura for suggesting this modification in the procedure of Kelmers et al. (18).
with 2 ml of phenol saturated with 0.02 M Tris-HCl (pH 7.5), 10 pmoles of MgCl₂, 20 pmoles of i⁴C-methionine, 20 pmoles of fMet-tRNA (Peak I) and Fractions 45 to 58 for methionine tRNA (Peak II). The pooled fractions were concentrated by evaporation and precipitated by the addition of 2 volumes of ethanol. The precipitate was then dissolved in water (25 to 30 ml) and dialyzed against 6 liters of 0.02 M Tris-HCl (pH 7.5) for 16 hours.

FIG. 1. A 200-cycle countercurrent distribution of crude yeast tRNA. The incubation mixture for the assay of methionine acceptor activity contained (in 0.5 ml) ³H-methionine (specific activity 1.2 mCi per pmole), tRNA (0.5 OD unit) from various fractions, and yeast aminoacyl tRNA synthetases (1 mg) in Tris-HCl buffer (see "Materials and Methods"). Fractions 27 to 35 were pooled for methionine tRNA (Peak I) and Fractions 45 to 58 for methionine tRNA (Peak II). The pooled fractions were concentrated by evaporation and precipitated by the addition of 2 volumes of ethanol. The precipitate was then dissolved in water (25 to 30 ml) and dialyzed against 6 liters of 0.02 M Tris-HCl (pH 7.5) for 16 hours.

8% was acid-soluble, and probably represents some methionine which had not been completely dialyzed out.

Ribosomes, Supernatant Factors, and Initiation Factors

These were isolated from E. coli MRE600 by procedures described previously (9).

Assay for In Vitro Amino Acid Incorporation

As in previous work (9, 25) a two-step procedure was used; Stage I involved the synthesis of polyribonucleotide with DNA-like polymers as template for RNA polymerase; in Stage II, the polyribonucleotide was then used without isolation for polypeptide synthesis. The reaction mixture in Stage I contained, per 1 ml: 40 pmoles of Tris-HCl (pH 7.8), 4 pmoles of MgCl₂, 1 pmole of MnCl₂, 12 pmoles of 2-mercaptoethanol, 0.6 mg of RNA polymerase (2000 units), 0.64 pmole of the appropriate ribonucleoside triphosphates, and 50 pmoles of the DNA template. The amount of ribopolynucleotide formed was determined by carrying out a small scale parallel experiment in which one of the ribonucleosides was used as the ¹⁴C-labeled form. After incubation at 37°C for 45 min to 1 hour, the components of Stage I were chilled in ice and to this were added the components of Stage II, such that the final concentrations per ml were: 60 pmoles of Tris-HCl (pH 7.8), 5 pmoles of MgCl₂, 0.3 pmole of MnCl₂, 16 pmoles of 2-mercaptoethanol, 45 pmoles of KCl, 2 pmoles of ATP, 0.25 pmole of GTP, 5 pmoles of 2-phosphoethanolpyruvate, 20 μg of phosphoethanolpyruvate kinase, 30 OD units of ribosomes, 0.25 mg of initiation factor proteins, aminoacyl-tRNAs as described, the ribopolynucleotide template, the DNA, excess nucleoside triphosphates, and RNA polymerase as introduced from Stage I. To this mixture was added 100,000 × g supernatant protein (0.4 mg per ml) and aliquots were taken at various time intervals after incubation at 37°C. The incorporation of radioactive amino acids into acid-insoluble polypeptide material was followed as previously described (9).

Assay for Amino Acid Acceptor Activity

Unless otherwise mentioned, the incubation mixture contained 0.006 μg MgCl₂, 0.023 μg Tris-HCl (pH 7.5), 0.0025 μg ATP, 1 to 2 pmoles of radioactive amino acid, tRNA, and crude yeast or E. coli B aminoacyl tRNA synthetases. After incubation at 37°C, aliquots were used for assay of acid-precipitable radioactivity with the filter paper technique (14).

RESULTS

Purification of tRNA Met—Starting from crude yeast tRNA, this included three steps and consisted of (a) countercurrent distribution, (b) ion exchange column chromatography of the appropriate pooled fractions on DEAE-Sephadex, and (c) reverse phase chromatography on Chromosorb W. Fig. 1 shows a 200-cycle countercurrent distribution on 8 g of crude yeast tRNA. As can be seen two clearly resolved peaks (I and II) of methionine acceptor activity are present. This step normally yields a 5- to 10-fold enrichment of methionine acceptor activity for both of the peaks and is useful for separation of the two peaks of methionine tRNAs on a large scale. Peak I (Fig. 1) represents the tRNA which can be converted to fMet-tRNA Met by E. coli.
FIG. 2. Chromatography of yeast methionine tRNA (from Peak I of Fig. 1) on a column (4.0 × 115 cm) of DEAE-Sephadex previously equilibrated with 0.375 M NaCl, 0.01 M MgCl₂, and 0.02 M Tris-HCl (pH 7.3). The methionine tRNA (20,000 OD units) in 34 ml of 0.02 M Tris-HCl (pH 7.3) and 0.01 M MgCl₂ was slowly adsorbed on the column at a flow rate of about 0.5 ml per min; the column was then washed with 0.375 M NaCl containing Tris-HCl and MgCl₂ as above and then eluted with a linear gradient (0.375 M → 0.425 M) of NaCl (total volume of gradient was 12 liters) containing 0.02 M Tris-HCl (pH 7.3) and 0.01 M MgCl₂. Fractions (13.5 to 14 ml) were collected every 10 min. No nucleotidic material came off the column before Fraction 450. Aliquots (10 μl) were used for the assay of methionine acceptor activity (with 20 μg of E. coli enzyme) and of glutamic acid acceptor activity (O→O) (with 0.2 mg of yeast enzyme). Incubation was in a total volume of 0.12 ml and at 37°C. Fractions 664 to 707 were pooled and concentrated approximately 3-fold; the tRNA<sup>Met</sup> was then precipitated by addition of 2 volumes of ethanol and used for further chromatography (Fig. 3). Fractions 715 to 785 were pooled and worked up as above. The tRNA<sup>Met</sup> thus obtained (2,900 OD units) was 30 to 35% pure with respect to Glu acceptor activity.

From Fig. 3, it is seen that the distribution of methionine acceptor activity obtained upon assaying with either crude yeast or E. coli aminocetyl tRNA synthetases is superimposable on a column (1.5 × 145 cm) of Chromosorb W previously equilibrated with a solution containing 0.3 M NaCl, 0.01 M MgCl₂, and 0.02 M Tris-HCl (pH 7.3) and saturated with isoamylacetate. The tRNA (290 OD units) from the DEAE-Sephadex chromatography (Fig. 2) was dialyzed against the above solution containing 0.3 M NaCl, Mg++, and Tris-HCl and this solution was then slowly applied to the column. Elution was with a linear gradient (0.3 M → 0.6 M) of NaCl (total volume of gradient was 12 liters) saturated with isoamyl acetate and containing 0.01 M MgCl₂ and 0.02 M Tris-HCl (pH 7.3). Fractions (16 ml) were collected every 15 min. No nucleotidic material was eluted from the column before Fraction 230. Aliquots (10 μl) were taken for the assay of methionine acceptor activity with either crude E. coli enzyme (40 μg) (O→O) or crude yeast enzyme (0.2 mg) (O→O). Incubation was at 37°C for 10 min, and the assay was in a volume of 0.12 ml. Fractions 335 to 355 were pooled as pure tRNA<sup>Met</sup> (611 OD units); Fractions 321 to 334 were pooled (408 OD units and 30 to 40% with respect to methionine acceptor activity) and combined with similar fractions from other columns for rechromatography.
Effect of ammonium chloride on charging of purified yeast tRNA^Met by crude yeast aminoaacyl tRNA synthetases

The incubation mixture (0.23 ml) contained 0.058 OD unit of yeast tRNA^met, ATP, ^4C-methionine, Mg++, and buffer as described under "Materials and Methods." Yeast enzyme (0.5 mg), and ammonium chloride as indicated. After incubation at 37°, aliquots (50 μl) were taken out at the times indicated and used for the assay of acid-insoluble radioactivity.

<table>
<thead>
<tr>
<th>Ammonium chloride</th>
<th>Total counts of methionine incorporated</th>
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<tr>
<td></td>
<td>1 min</td>
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<tr>
<td>(m)</td>
<td></td>
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<tr>
<td>0.008</td>
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<td>0.063</td>
<td>15,917</td>
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<td></td>
<td>19,004</td>
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</table>

Effect of Mg++ concentration on charging of yeast tRNA^Met (Peak II of Fig. 1) by E. coli enzyme

The components of the incubation mixture were as described under "Materials and Methods," except for the variation in Mg++ concentration. tRNAMet, 4.4 OD units per ml, was present in all of the mixtures. Yeast enzyme used was 2 mg per ml and E. coli enzyme was 0.8 mg per ml. Incubation was at 37°; aliquots (50 μl) were taken out at the times indicated for the assay of acid-precipitable radioactivity.

<table>
<thead>
<tr>
<th>Mg++</th>
<th>Counts of methionine incorporated</th>
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<tbody>
<tr>
<td></td>
<td>2 min</td>
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<tr>
<td>m</td>
<td></td>
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<tr>
<td>0.005</td>
<td>16,857</td>
</tr>
<tr>
<td>0.005</td>
<td>3,512</td>
</tr>
<tr>
<td>0.015</td>
<td>17,240</td>
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</table>

a Yeast enzyme.
b E. coli enzyme.

tRNA^Met were carried out with the purified tRNA. The results with E. coli aminoaacyl tRNA synthetase show that (a) yeast tRNA^met is charged at about the same rate as E. coli methionine tRNAs, (b) the rate of charging of the three tRNAs is similarly affected by Mg++ concentration, and (c) the kinetic constants K_m and V_max for the three tRNAs are rather similar.

Fig. 4 shows the rate of methionine incorporation into the yeast and E. coli methionine tRNAs with E. coli enzyme and at various Mg++ concentrations. It can be observed that, in all cases except yeast tRNA^met, the rate of charging is maximal at around 5 mM Mg++; increase of Mg++ concentration to 10 and 15 mM resulted in a progressive decrease in the rate of methionine incorporation into the tRNAs. Furthermore, the rate of charging of these three tRNAs by E. coli enzyme are similar (2.5 μmoles per min per mg of enzyme). Yeast tRNA^Met (Fig. 4 a) was used for these studies.

Aminoacyl tRNA Synthetases—When fractions from the counter-current distribution shown in Fig. 1 were assayed for methionine acceptor activity with E. coli aminoaacyl tRNA synthetases, it was found that Peak I (tRNA^Met) could be totally aminoaacylated by E. coli enzyme; under identical conditions (at 5 mM Mg++ concentration) material from Peak II (tRNA^Met) was aminoaacylated only at a slow rate. When the assay was carried out at 15 mM Mg++, the incorporation of methionine into yeast tRNA^Met was rapid and occurred to the same extent as that obtained with yeast enzyme (Table II). The radioactivity incorporated into yeast tRNA^Met under these conditions is quantitatively released under mildly alkaline conditions (pH 10.5 at 37° for 4 min) and hence was most probably in the form of aminoacyl-tRNA. The quantitative release of all of the radioactivity by mild alkali indicates that the incorporation involved only the esterification reaction and that no other reaction such as methylation of hydroxyl groups or heterocyclic rings occurred.

In view of the reports of Barnett and Jacobson (27) that the use of heterologous enzymes can result in the charging of an unexpected species of tRNA, all of the kinetic studies with yeast
E. coli aminoacyl tRNA synthetase. The apparent $K_m$ of the present work, efforts to formylate either of the yeast Met-tRNAs $E. coli$ tRNAMet (1 to 1.1 pmol) Lineweaver-Burk plot of the rate of Met-tRNAfMet formation in the presence of varying amounts of yeast tRNAfMet and with the enzyme were nearly identical and of the order of 2.8 pmol per min per mg of crude enzyme.

**Enzymatic Formation of Yeast fMet-tRNAfMet**—Clark and Marcker (5) have shown that, out of at least two species of methionine tRNAs in $E. coli$, only one (designated tRNAfMet) could be enzymatically converted to fMet-tRNAfMet. In the present work, efforts to formylate either of the yeast Met-tRNAs by crude extracts from either Saccharomyces cerevisiae or Saccharomyces lactis at various stages of growth were unsuccessful. However, as seen in Fig. 6, Met-tRNA from Peak I of Fig. 1 can be formylated by $E. coli$ extracts. With any of the other amino acids and crude yeast tRNA no incorporation of radioactivity from $^3$H-formyl-$^4$H-folate occurs either in the presence of crude yeast or $E. coli$ enzymes; thus only Met-tRNAfMet of yeast can be enzymatically formylated.

The kinetics of incorporation of $^3$H- from $^3$H-formyl-$^4$H-folate into yeast tRNAfMet and yeast tRNAMet in the presence of $E. coli$ enzymes and methionine is shown in Fig. 6. It can be clearly seen that only Met-tRNAfMet and not Met-tRNAMet is formylated. In the above experiments, to ensure that yeast tRNAfMet was completely charged to Met-tRNAfMet, all incubation mixtures were made 10 mM with respect to Mg++. The lack of any incorporation of $^3$H- from $^3$H-formyl-$^4$H-folate in the absence of added tRNA also shows that the crude $E. coli$ enzyme used was essentially free of any $E. coli$ tRNAfMet.

In a parallel experiment (see "Materials and Methods"), purified yeast tRNAfMet was incubated with unlabeled formyl-$^4$H-folate, and $^{14}$C-methionine and the formyl-$^{14}$C-Met-tRNAfMet were isolated. Hydrolysis of this under alkaline condition followed by paper electrophoresis of the hydrolysate showed that all of the radioactive methionine incorporated into tRNA could be recovered as material that behaved like formylmethionine (Fig. 5). The small amount of ($\sim$8%) of the total radioactivity near the origin represents some free radioactive methionine still present in the isolated f-14C-Met-tRNAfMet.

**Coding Properties of Yeast fMet-tRNAfMet**—The coding properties of yeast fMet-tRNAfMet were studied with the $E. coli$ protein-synthesizing system in vitro under the direction of ribopolynucleotides containing repeating sequences. Initiation of protein synthesis was followed essentially as described previously (9).
Characterization of Cytosine-terminal Nucleotides of Yeast tRNA <sup>Met</sup>—Yeast tRNA<sup>Met</sup> (185 OD units) was incubated at 37°C with Tr RNAse (150 units) in 3.3 ml of 0.05 M Tris-HCl (pH 7.3). After 7 hours (increase in total optical density units was 25%), the incubation mixture was made 7 M with respect to urea and chromatographed on a column of DEAE-cellulose (chloride form). The pattern of elution is shown in Fig. 10. Peak 7 contained the 3′-terminal oligonucleotide fragment C-U-A-C-C-A and Peak 8 contained as one of its components the 5′-terminal oligonucleotide fragment PA-G. The oligonucleotide material in these peaks was freed of urea and salt (see "Materials and Methods") and used for sequence analysis.

Characterization of C U A C C A (a) The absence of a 3′-phosphate end group was indicated by the fact that the oligonucleotide had identical mobility in Solvent II before or after treatment with E. coli alkaline phosphatase. (b) The 3′-terminal nucleoside was shown to be adenosine by degradation with pancreatic RNase. Thus, upon chromatography of such a digest in Solvent I, the products were A, C, U, and A-C (2:3:1:1). C and U were separated by chromatography in Solvent III, A-C was characterized by dephosphorylation to A-C and further degradation with snake venom phosphodiesterase to A and pC. (c) C was concluded to be the 5′-terminal nucleoside, since degradation of the oligonucleotide with snake venom phosphodiesterase yielded C as the nucleoside in addition to PC, pA, and pU (Chromatography in Solvent I). The three 5′-nucleotides were separated from each other by first excising the band corresponding to C and then developing the chromatogram in Solvent III. (d) Finally, the sequence of the oligonucleotide could be derived from the finding that degradation of the oligonucleotide with polynucleotide phosphorylase (30) produced C-U-A as the 5′-terminal nucleotide fragment. pA-G was characterized by dephosphorylation to pA-G and further degradation with snake venom phosphodiesterase to pC, pA, and pP (Chromatography in Solvent I). The three 5′-nucleotides were separated from each other by first excising the band corresponding to C and then developing the chromatogram in Solvent III. (e) Finally, the sequence of the oligonucleotide could be derived from the finding that degradation of the oligonucleotide with polynucleotide phosphorylase (30) produced C-U-A as the 5′-terminal nucleotide fragment. pA-G was characterized by dephosphorylation to pA-G and further degradation with snake venom phosphodiesterase to pC, pA, and pP (Chromatography in Solvent I). The three 5′-nucleotides were separated from each other by first excising the band corresponding to C and then developing the chromatogram in Solvent III. (f) Finally, the sequence of the oligonucleotide could be derived from the finding that degradation of the oligonucleotide with polynucleotide phosphorylase (30) produced C-U-A as the 5′-terminal nucleotide fragment. pA-G was characterized by dephosphorylation to pA-G and further degradation with snake venom phosphodiesterase to pC, pA, and pP (Chromatography in Solvent I). The three 5′-nucleotides were separated from each other by first excising the band corresponding to C and then developing the chromatogram in Solvent III. (g) Finally, the sequence of the oligonucleotide could be derived from the finding that degradation of the oligonucleotide with polynucleotide phosphorylase (30) produced C-U-A as the 5′-terminal nucleotide fragment. pA-G was characterized by dephosphorylation to pA-G and further degradation with snake venom phosphodiesterase to pC, pA, and pP (Chromatography in Solvent I). The three 5′-nucleotides were separated from each other by first excising the band corresponding to C and then developing the chromatogram in Solvent III.
FIG. 10. Chromatography of a Ti-RNase digest of yeast tRNA Met (158 OD units) on a column (0.5 × 75 cm) of DEAE-cellulose (chloride form) in the presence of 7 M urea. Elution was with a linear gradient (0 to 0.3 M) of NaCl (total volume of gradient was 900 ml) containing 7 M urea and 0.02 M Tris-HCl (pH 7.3). Fractions, 1.7 to 1.8 ml, were collected every 12 min.

chromatography in Solvent III for 45 hours. The faster traveling band was eluted and characterized as follows. (a) Dephosphorylation with E. coli alkaline phosphatase yielded A-G (characterized by mobility identical with A-G in Solvent II and by further degradation to A and pG (1:1)). (b) Chromatography of the original oligonucleotide against A-G and A-G in Solvent II showed that the oligonucleotide possessed lower mobility than either A-G or A-G-. (c) Hydrolysis of the oligonucleotide with alkali and subsequent chromatography in Solvent II gave pA and G-, pA- being characterized by its ultraviolet absorption spectrum, paper chromatographic mobility, and enzymatic dephosphorylation to adenosine.

Discussion

In an attempt to elucidate the nature of tRNA involved in protein chain initiation in yeast the present paper has reported on the fractionation and properties of methionine tRNAs of yeast.

Aminoacylation of Yeast Methionine tRNAs—Several lines of evidence including identical Mg++ optimum, K_m, and V_max values indicate that yeast tRNA Met (Peak II of Fig. 1) is as well recognized by E. coli methionyl tRNA synthetase as the homologous E. coli methionine tRNAs.

The complete aminoacylation of the second species of tRNA Met (Peak II of Fig. 1) by E. coli enzyme, however, requires higher Mg++ concentration and a larger amount of enzyme than that required for yeast tRNA Met. It is, therefore, not clear whether another aminoacyl tRNA synthetase present in E. coli or the same E. coli methionyl tRNA synthetase involved in the charging of homologous methionine tRNAs is used in this reaction. Alternatively, since E. coli methionyl tRNA synthetase has been shown to undergo dissociation into active subunits (31), it is conceivable that this enzyme in a different form is involved in the charging of yeast tRNA Met.

Formylation of Yeast tRNA Met and Initiation of in Vitro Protein Synthesis—An interesting aspect of yeast tRNA Met is the ability of the tRNA to form fMet-tRNA Met in the presence of crude E. coli enzyme. While extracts from yeast to date have not shown any methionyl-tRNA formyltransferase activity as judged by the lack of incorporation of H-formyl residue into trichloroacetic acid-insoluble [H]fMet-tRNA, the possibility that such an enzyme does exist in yeast cannot be entirely ruled out, since the only formyl donor examined in the present work was formyl-H4-folate. Other alternatives are (a) the enzyme in question from yeast is relatively labile or (b) fMet-tRNA Met is actually formed during the incubation but that it is very rapidly hydrolyzed to N-formyl methionine and tRNA Met by the N-acylaminoacyl-tRNA hydrolase known to be present in yeast extracts (32).

In response to poly r(A-U-G) or poly r(U-G), yeast fMet-tRNA Met synthesized by the use of E. coli enzyme can bring about the initiation of protein synthesis in vitro in an E. coli system (Figs. 8 and 9). This tRNA is thus similar to E. coli fMet-tRNA Met in that it is coded for by either of the codons A-U-G or G-U-G. It should be recalled that all available experimental data involving multiple codon recognition by a species of tRNA involve degeneracy in the third letter of the codon (33, 34), except E. coli fMet-tRNA Met, in which the two synonym codons A-U-G and G-U-G differ in the first letter of the codon.
the codon. Further evidence that yeast fMet tRNA<sup>fMet</sup> was involved in the initiation of protein biosynthesis in vitro is derived from the work of Takeishi et al. (11), who have shown the transfer of radioactive fMet into bacteriophage f2RNA-directed protein synthesis in an E. coli protein-synthesizing system.

While the similarities between E. coli tRNA<sup>fMet</sup> and yeast tRNA<sup>fMet</sup> appear evident, the question as to whether yeast tRNA<sup>fMet</sup> represents an “initiator tRNA” in yeast analogous to the E. coli tRNA<sup>fMet</sup> must still be left open. A satisfactory answer to this must await the development and further study of a stable protein-synthesizing system of yeast (35). Alternatively, by analogy with the E. coli system, a clear demonstration of the presence of any specific aminoaeryl-tRNA formylase or acylase in the yeast extracts can be expected to provide some indication as to the nature of tRNA involved in the initiation of protein biosynthesis.

Comparison of Terminal Nucleotide Sequences between Yeast tRNA<sup>fMet</sup> and E. coli tRNA<sup>fMet</sup>. As a first step toward the nucleotide sequence analysis of yeast tRNA<sup>fMet</sup>, the 5' and 3' terminal sequences have now been identified as pA-G-7 and C-U-A-C-C-A, respectively. The terminal sequences of yeast tRNA<sup>fMet</sup>, the total nucleotide sequence of which has been recently deduced by Dube et al. (40). Since yeast tRNA<sup>fMet</sup> can be aminoaacylated and subsequently formylated, by E. coli extracts, these results indicate that the terminal sequences of yeast tRNA<sup>fMet</sup> do not constitute unique recognition sites for either the E. coli methionyl tRNA synthetase or E. coli methionyl tRNA formylase (see below).

A distinguishing feature of E. coli tRNA<sup>fMet</sup> noted by Dube et al. (40) was the unusual base pairing between the 5' and 3' end of this tRNA. Thus, in contrast to other tRNAs in which base pairing starts from the fifth base of the acceptor end to the first base of the 5' end (Fig. 11B), in E. coli tRNA<sup>fMet</sup>, base pairing starts only from the sixth base of the acceptor end to the second base of the 5' end (Fig. 11A). The attractive possibility that this unique variation in the structure represents a recognition site for E. coli Met-tRNA formylase is, however, ruled out from the present work, since yeast tRNA<sup>fMet</sup>, which is presumably recognized by the same enzyme, is found not to possess this unusual base-pairing pattern at the end (Fig. 11).

A comparison of the terminal sequences of E. coli tRNA<sup>fMet</sup> and yeast tRNA<sup>fMet</sup> (Fig. 11) also allows certain conclusions as to whether the terminal nucleotide sequences comprise specific recognition sites for the E. coli methionyl-tRNA synthetase. Recently, studies on photoreaction with purified yeast tRNA<sup>Ala</sup> have been carried out by Schuman and Chambers (41). Results obtained from this work, in conjunction with other data, led these authors to propose that the first three base pairs of tRNA from the end might serve as specific recognition sites for alanayl tRNA synthetase. Assuming that the same E. coli methionyl tRNA synthetase is involved in the recognition of yeast tRNA<sup>fMet</sup> and E. coli tRNA<sup>fMet</sup>, it would seem unlikely, at least for this enzyme, that all of the three terminal base pairs are specifically recognized.

One of the features of E. coli tRNA<sup>fMet</sup> noted by Dube et al. (40) was the nature of the nucleotide next to the anticodon. The observed anticodon sequence C-A-U is that expected for recognition by the codons A-U-G or G-U-G in which the last letter of the anticodon “U” can “wobble” by base pairing with either A or G (42). However, in contrast to all other tRNAs of known sequence except yeast tRNA<sup>Val</sup> (43, 44), the nucleoside next to the anticodon was found to be unmodified. Since yeast tRNA<sup>fMet</sup>, like the E. coli tRNA<sup>fMet</sup> also responds to both A-U-G and G-U-G, whether this unique ambiguity in the first letter of the codon for E. coli tRNA<sup>fMet</sup> requires that the nucleoside next to the anticodon be unmodified can now be directly examined from a comparison of the sequences around the anticodon in the two tRNAs.

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