Proceedings of the National Academy of Sciences
Vol. 68, No. 2, pp. 294–298, February 1971

Isolation of *Saccharomyces cerevisiae* Mitochondrial Formyltetrahydrofolic Acid:Methionyl-tRNA Transformylase and the Hybridization of Mitochondrial fMet-tRNA with Mitochondrial DNA

A. HALBREICH AND M. RABINOWITZ

Departments of Medicine and Biochemistry, The University of Chicago Pritzker School of Medicine, and the Argonne Cancer Research Hospital (operated by The University of Chicago for the United States Atomic Energy Commission), Chicago, Illinois 60637

Communicated by Leon O. Jacobson, November 18, 1970

ABSTRACT Formyltetrahydrofolic acid:methionyl-tRNA transformylase was isolated from *Saccharomyces cerevisiae* mitochondria and used to prepare yeast mitochondrial [^{3}H]formylmethionyl-tRNA. This fMet-tRNA hybridizes with mitochondrial DNA but not with yeast nuclear or *E. coli* DNA. Unlabeled mitochondrial, but not extramitochondrial, tRNA competes in this reaction. tRNA was eluted from the hybrid and found to contain the label almost exclusively in fMet-tRNA. Yeast cytoplasmic fMet-tRNA formylated with *Escherichia coli* enzyme, and *E. coli* fMet-tRNA, do not hybridize with mitochondrial DNA. It is concluded that yeast mitochondrial tRNA^{fMet} is a gene product of the mitochondrial genome.

N-formylmethionyl-tRNA has been detected in yeast and rat liver mitochondria (1) but not in the soluble cell fractions. Takeishi *et al.* could demonstrate no formyl $(H)_4$folate: methionyl-tRNA transformylase activity in yeast extracts, but were able to separate a methionyl-tRNA species that could be formylated by an *Escherichia coli* enzyme preparation (2, 3). This tRNA species accounted for a large portion of the methionine-acceptor activity of the whole cell tRNA and therefore was unlikely to be of mitochondrial origin. It has recently been identified, in the nonformylated form, as the initiator of extramitochondrial protein synthesis (4).

Recently our laboratory has identified several yeast mitochondrial RNA species as gene products of mitochondrial DNA (5–7) as part of a study of the roles of the mitochondrial and nuclear genomes in mitochondrial biogenesis (8, 9). The known participation of *N*-formylmethionyl-tRNA in initiating protein synthesis in bacteria, combined with the similarity in the characteristics of the mitochondrial and the bacterial protein synthetic systems, prompted our examination of mitochondrial DNA as the source of the genetic information specifying the synthesis of this tRNA species.

We describe in these experiments the isolation of a fraction that contains transformylase activity from yeast mitochondria and the evidence that the mitochondrial tRNA^{fMet} is a gene product of the mitochondrial DNA.

Abbreviations: (H_4) folate tetrahydrofolic acid; βMe, β-mercaptoethanol; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate).

METHODS

A respiratory-sufficient haploid yeast strain, D243-2B-R1 (*a*, *lys*$^-$, *ad*$_1^-$ ρ^+), obtained from Prof. P. P. Slonimski, was used in this study. Yeast was grown in a medium containing 1% yeast extract, 2% bactopeptone, and 2% galactose, and collected in the late exponential phase of growth. Mitochondria were isolated by digestion of the cell wall with glusulase (Endo Laboratories), homogenization, and centrifugal separation (8). Mitochondrial DNA was isolated from DNase-treated mitochondria by lysis in 0.01 M Tris·HCl, pH 7.5, containing 0.01 M EDTA and 1% sodium dodecylsulfate, deproteinization by chloroform–*n*-octanol 9:1, and passage through a hydroxylapatite column (7). The DNA was tested for purity by isopycnic CsCl ultracentrifugation in the analytical ultracentrifuge (10).

Yeast nuclear and *E. coli* K 12 DNA were isolated by a similar method, except that the yeast nuclear DNA was further purified by hydroxylapatite chromatography according to Bernardi *et al.* (11). tRNA was isolated from washed mitochondria essentially according to Avital and Elson (12) by lysis with 0.01 M Tris·HCl, pH 7.5–2% sodium dodecylsulfate–0.1% macaloid (National Lead Co.), deproteinization of the lysate with a phenol–cresol mixture (13), mild alkali deacylation, and extraction with 2 M LiCl. The postmitochondrial supernatant was centrifuged for 2 hr at 150,000 × *g* (Spinco ultracentrifuge, Model L2-65, rotor 50 Ti) and the ensuing supernatant was employed in the preparation of supernatant tRNA, essentially by the procedure outlined above.

A mitochondrial fraction containing aminoacyl-tRNA synthetases and transformylase activity was prepared as follows: mitochondria from 100 g of yeast cells were suspended in 20 ml of 1 M KCl containing 0.02 M Tris·HCl (pH 7.5)–0.01 M β-mercaptoethanol (βME)–1 mM EDTA. The suspension was sonicated with a Branson sonicator in the cold for 10 sec at 3 mA, centrifuged first for 10 min at 48,000 × *g* (Sorvall RC2B centrifuge, rotor SS34) and then for 2 hr at 150,000 × *g*. The final supernatant was dialyzed against 50 volumes of 0.01 M Tris·HCl, pH 7.5, containing 0.02 M βME, 0.01 M $MgCl_2$, and 10% glycerol, applied to a DEAE-cellulose column (3 × 11 cm) (14) and eluted with a 0.25 M KH_2PO_4 buffer, pH 6.5, containing 0.02 M βME 0.01 M $MgCl_2$ and 10% glycerol. A fraction exhibiting both amino acid activation and trans-

formylase activity was eluted immediately after the void volume. An *E. coli* enzyme preparation was kindly furnished by Dr. P. Sigler.

Formation of [^{3}H]methionyl (Met)- and *N*-formylmethionyl (fMet)-tRNA was tested according to Marcker and Sanger (15) in a reaction mixture consisting of imidazole (pH 7.5) 0.06 M; $MgCl_2$, 0.01 M; KCl, 0.01 M; βME, 0.05 M; ATP, 2 mM; [methyl^{3}H]L-methionine, 3.3 Ci/mmol, 2.4 μM (Schwarz BioResearch); 0.12 mM Ca-formyltetrahydrofolate (Leucovorin: Lederle); 15–50 μg of tRNA; and 20 μl of enzyme preparation in a final volume of 0.1 ml. The reaction proceeded for 10 min at 33°C, and was stopped by the addition of 0.1 volume of 20% K-acetate, pH 5.2, followed by 100 μg of *E. coli* tRNA, and 20 volumes of 2% K-acetate, pH 5.2. The acylated tRNA was deproteinized with H_2O-saturated phenol and separated from free methionine by repeated alcohol precipitations. Finally Met- and fMet-adenosine were obtained by pancreatic RNase treatment of acylated tRNA and separated by high-voltage electrophoresis in 0.5 M acetic acid, pH 3.5, containing 0.3% pyridine (75 min at 3000 V) (15). 2-cm wide strips of the paper were cut into 1-cm pieces and immersed in 0.2 ml of water in counting vials, 15 ml of Bray's solution was added, and the samples were counted in a Packard liquid scintillation spectrometer.

Tritiated mitochondrial fMet-tRNA was prepared in the above-mentioned reaction mixture in a final volume of 2.5 ml. Met-tRNA was enzymatically deacylated by a second 10-min incubation at 33°C in the presence of 30 mM $MgCl_2$, 15 mM AMP, and 5 mM Na-pyrophosphate, pH 7.0 (16). The reaction was stopped by the addition of 0.1 volume of 20% K-acetate, pH 5.2 and the mixture was diluted with an equal volume of 2% K-acetate, pH 5.2, and extracted with H_2O-saturated phenol. The aqueous phase was repeatedly extracted with ether to remove the phenol; N_2 was bubbled through to remove the ether. RNA was precipitated by adding 2.5 volumes of ethanol, redissolved in 50 mM NH_4-acetate, pH 5.2, and passed through a 3 × 50-cm Sephadex G-100 column with 50 mM NH_4-acetate, pH 5.2. The radioactivity and absorbance at 260 nm of the eluate were monitored, and the acylated tRNA was collected by alcohol precipitation from the fractions exhibiting the highest specific radioactivity. [^{3}H]fMet-tRNA represented more than 85% of the labeled aminoacyl tRNA in these preparations as judged by electrophoresis of RNase digests. Supernatant [^{3}H]fMet-tRNA was prepared in the same manner except that an *E. coli* enzyme was employed for acylation and formylation.

DNA was immobilized on nitrocellulose filters, according to Gillespie and Spiegelman (17), after alkali denaturation, neutralization, and addition of 10 × SSC to a final concentration of 6 × SSC. DNA and blank (no DNA) filters were hybridized with [^{3}H]fMet-tRNA according to Weiss *et al.* (18) in 0.3 ml of 33% formamide containing 2 × SSC–25 mM NH_4-acetate (final pH 5.2) and various amounts of [^{3}H]fMet-tRNA. After a 4-hr incubation at 33°C, the filters were removed, swirled for 15 min in 1 liter of 2 × SSC, pH 5.2, and washed on each side with 60 ml of this solution. Finally, the filters were dried and counted in a scintillation fluid consisting of 0.1% PPO (2,5-diphenyloxazole) and 0.01% Me_2-POPOP (1,4-bis-2[4-methyl-5-phenyloxazolyl]benzene) in toluene.

fMet-tRNA was eluted from filters containing the hybrid by three 20-min incubations at 33°C in 0.3-ml portions of 95% formamide in 50 mM ammonium acetate, pH 5.2. The filters were washed with 0.3 ml of H_2O, the supernatants were combined, 100 μg *E. coli* tRNA was added, and the mixture was passed through a 1 × 20-cm Sephadex G-25 column and eluted with 50 mM NH_4-acetate, pH 5.2. The tRNA-containing fractions appeared in the void volume; they were pooled and evaporated to dryness. The residue was dissolved in water, treated with pancreatic RNase, and electrophoresed as described above.

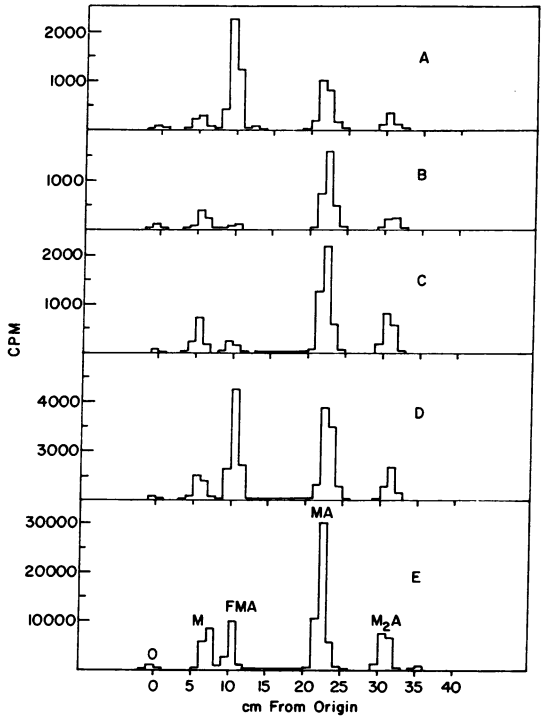

FIG. 1. Identification of products of the reactions between yeast mitochondrial and *E. coli* transformylases and mitochondrial, cytoplasmic, and *E. coli* tRNA preparations. Samples were electrophoresed for 2 hr at 1500 V. The following reactions were tested: (A) mitochondrial enzyme with mitochondrial tRNA; (*B*) *E. coli* enzyme with mitochondrial tRNA; (*C*) mitochondrial enzyme with supernatant tRNA; (*D*) *E. coli* enzyme with supernatant tRNA; (*E*) mitochondrial enzyme with *E. coli* tRNA. The peaks were identified, using as markers [^{3}H]methionine and ^{3}H-labeled *E. coli* fMet- and Met-tRNA, as: M, methionine; FMA, fMet-adenosine; MA, Met-adenosine; and M_2A, an isomeric form of Met-adenosine (15).

RESULTS

[^{3}H]fMet-tRNA may be detected by high voltage electrophoresis of the RNase-treated reaction product (15). Formyl (H)$_4$folate:Met-tRNA transformylase activity was thus demonstrated in a yeast mitochondrial fraction obtained by extraction of the mitochondria with 1 M KCl (Fig. 1A). This enzyme preparation also exhibited Met-tRNA synthetase activity. The presence of other synthetases was not tested. Transformylase was not released into the medium by extraction at low-ionic strength. No transformylase activity was detected in preparation of acylating enzymes obtained from the postmitochondrial supernatant.

Yeast cytoplasmic Met-tRNA may be separated into two species (2, 3) one of which may be formylated by *E. coli*

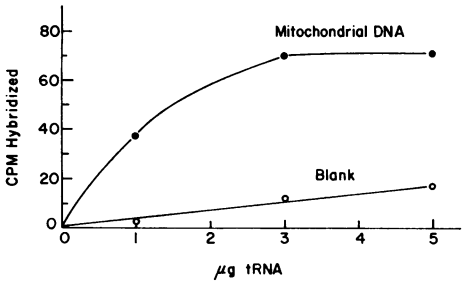

FIG. 2. Saturation of mitochondrial DNA with mitochondrial [^{3}H]fMet-tRNA. Different amounts of [^{3}H]fMet-tRNA (390 cpm/μg RNA) were hybridized with 20 μg of DNA. The blanks consisted of filters without DNA that were incubated concurrently in the reaction mixture.

enzyme. This is confirmed in Fig. 1*D*. It may be seen in Fig. 1*C* that although this tRNA is readily acylated by the mitochondrial enzyme, formylation is poor with the same enzyme preparation. The mitochondrial enzyme also formylates *E. coli* tRNA rather inefficiently (Fig. 1*E*). Conversely, the *E. coli* enzyme exhibited an extremely low formylase activity towards mitochondrial tRNA (Fig. 1*B*). The slight activity seen possibly represents contamination of the mitochondrial tRNA preparation by cytoplasmic tRNA.

Mitochondrial [^{3}H]fMet-tRNA was prepared essentially free of labeled Met-tRNA by an enzymatic deacylation of the latter in the presence of AMP and PP_i (16) and Sephadex G-100 chromatography for hybridization experiments. Approximately 90% of the radioactivity in the RNase digest of these preparations appeared as fMet-adenosine upon electrophoretic analysis (see Fig. 3*B*). Mitochondrial fMet-tRNA hybridized with mitochondrial DNA. Saturation was obtained by hybridizing 5 μg of tRNA with 20 μg of DNA (Fig. 2). Unlabeled mitochondrial, but not supernatant, tRNA competed in this reaction (Table 1). The specificity of the reaction was further attested to by the fact that no detectable mitochondrial fMet-tRNA was bound by either yeast nuclear or *E. coli* DNA. The results of a representative experiment are shown in Table 2. It should be pointed out, however, that the size of yeast nuclear and *E. coli* DNA, relative to that of the yeast mitochondrial DNA, may make it difficult to detect structural homology with tRNA at the specific radioactivity employed. Yeast supernatant [^{3}H]fMet-tRNA prepared with the *E. coli* enzyme and [^{3}H]fMet-tRNA (*E. coli*) did not hybridize with mitochondrial DNA (Table 3).

TABLE 1. *Competition by mitochondrial tRNA in the hybridization of mitochondrial N-formylmethionyl-tRNA with mitochondrial DNA*

Additions	Radioactivity bound to filters, cpm	
	Blank filter	DNA filter
None	17	71
6.75 μg Mitochondrial tRNA	14	37
115 μg Supernatant tRNA	20	84

Filters containing 20 μg of mitochondrial DNA were hybridized with 5 μg of [^{3}H]*N*-formylmethionyl-tRNA (390 cpm/μg) as described in *Methods*.

tRNA was eluted from the fMet-tRNA–mitochondrial DNA hybrid with 95% formamide, treated with pancreatic RNase, and examined by high-voltage electrophoresis. It may be seen in Fig. 3*A* that most of the radioactivity was recovered as fMet-adenosine, proving that mitochondrial fMet-tRNA had actually hybridized with the mitochondrial DNA.

Finally, it should be mentioned that the annealing mixture may be reused with fresh DNA filters and demonstrates an equal efficiency for hybrid formation. In fact, this use results in lower blank values, indicating the nonspecific nature of the blank radioactivity associated with the blank filters. Similarly, inclusion of several DNA filters in the reaction mixture results in equal binding of tRNA to each of the filters with a consequent increase in the fraction of the input tRNA utilized. These features should be expected in view of the very large excess of RNA required in this reaction.

DISCUSSION

A formyltetrahydrofolic acid:Met-tRNA transformylase activity has been found in extracts of yeast mitochondria. No such activity was present in yeast postmitochondrial supernatant. The enzyme was most active with mitochondrial Met-tRNA, but showed a low-level activity with yeast supernatant, and *E. coli* tRNAs. These findings explain observations of Smith and Marcker (1) that fMet-tRNA is localized exclusively in the mitochondria in yeast and rat liver. The yeast-supernatant fraction is known to contain two species of Met-tRNA, one of which may be formylated by the *E. coli* transformylase. This tRNA, in its nonformylated form, has recently been shown to be an initiator of extramitochondrial protein synthesis in mouse liver (4) and rabbit reticulocytes (19) as well as in yeast (3, 4). It is likely that yeast mitochondrial fMet-tRNA serves in the same capacity for mitochondrial protein synthesis.

We have also shown that the mitochondrial fMet-tRNA hybridizes with, and is probably specified by, mitochondrial DNA. tRNA acylated *in vitro* with labeled amino acid was used in the hybridization studies, which were carried out in formamide, at low temperature and at acid pH to minimize deacylation. This annealing system, which is based on the procedure devised by Weiss *et al.* (18), allows the specific detection of tRNA hybrids without interference from other RNA species. Mild alkaline hydrolysis of the aminoacyl-tRNA completely removes the radioactivity bound to the DNA filters, showing that this binding is dependent on the ester bond

TABLE 2. *Hybridization of mitochondrial N-formylmethionyl-tRNA with yeast mitochondrial and nuclear DNA, and with E. coli DNA*

Type of DNA	μg DNA	Radioactivity bound to filters, cpm
None	—	23
Yeast mitochondrial	10	58
Yeast mitochondrial	20	87
Yeast nuclear	100	20
E. coli	20	26

The annealing mixture contained 10 μg of [^{3}H]*N*-formylmethionyl-tRNA (665 cpm/μg). Results are representative of six similar experiments.

between the amino acid and the tRNA molecule. The specificity is confirmed by electrophoretic identification of [^{3}H]fMet-adenosine in RNase digests of the tRNA hybridized to mitochondrial DNA. The fact that the tRNA, eluted from the hybrid, appears in the void volume of the Sephadex G-25 filtrate indicates that a high molecular weight tRNA was hybridized to mitochondrial DNA. Competition of the hybridization by unlabeled mitochondrial tRNA but not by unlabeled extramitochondrial tRNA is further evidence for the specificity of the results. The extramitochondrial $tRNA^{Met}$ that is formylated by *E. coli* transformylase, and initiates cytoplasmic protein synthesis, does not hybridize to mitochondrial DNA. *E. coli* $tRNA^{fMet}$ also does not hybridize to mitochondrial DNA.

It should be theoretically possible to estimate the number of cistrons for fMet-tRNA present in the mitochondrial genome from the saturation-hybridization data, assuming the specific activity of the tRNA to be that of the [^{3}H]methionine used. Such calculations, however, are rendered invalid by uncertainty regarding degree of acylation of the tRNA, and by the short periods of incubation used for hybridization, which may prevent the reaction from going to completion. Weiss *et al.* (18) had previously noted that there was a considerably lower saturation level when the tRNA was labeled in the amino acid than when the nucleotides were labeled with ^{35}S.

Nass and Buck have shown (20) the hybridization of several mitochondrial tRNA species including tyrosyl-, seryl-, phenylalanyl-, and leucyl-tRNA in rat liver. Leucyl-tRNA was observed to hybridize only with the heavy strand of the mitochondrial DNA. We now have shown that yeast mitochondrial DNA specifies the synthesis of several tRNA molecules including $tRNA^{fMet}$ (this communication), $tRNA^{Met}$ (Halbreich and Rabinowitz, unpublished), $tRNA^{Leu}$ (5) and $tRNA^{Val}$ (6). The two mitochondrial ribosomal RNA species also show sequence homology with mitochondrial DNA (7, 21, 22). Other mitochondrial tRNAs are being tested to determine whether mitochondrial DNA codes for all, or for only a few, of the mitochondrial tRNAs. It is of interest that several cytoplasmic-petite yeast strains retain cistrons for valyl- and leucyl-tRNA (5, 6). It is hoped that the hybridization of several mitochondrial tRNA species with mitochondrial DNA of a series of yeast petite strains, combined with information obtained from the genetic analysis of cytoplasmically controlled antibiotic-resistance markers (23), will offer an opportunity for preliminary mapping of the mitochondrial genome

TABLE 3. *Hybridization of yeast cytoplasmic and E. coli fMet-tRNA with yeast mitochondrial DNA*

Source of [^{3}H]fMet-tRNA	Radioactivity bound to filters, cpm	
	Blank filter	DNA filter
E. coli	21	17
Yeast cytoplasm	22	26
Yeast mitochondrial	17	128

13.5 μg of *E. coli* fMet-tRNA (2870 cpm/μg), 5.5 μg of yeast cytoplasmic fMet-tRNA (160 cpm/μg), and 5 μg of mitochondrial fMet-tRNA (390 cpm/μg) were each incubated with filters containing 50 μg of mitochondrial DNA or blank filters, as described in *Methods*. An *E. coli* enzyme was employed in the preparation of *E. coli* and yeast of cytoplasmic fMet-tRNA. When 13.5–101 μg of *E. coli* fMet-tRNA (2870 cpm/μg) was annealed with 100 μg of *E. coli* DNA, 16–85 cpm were bound to the filters.

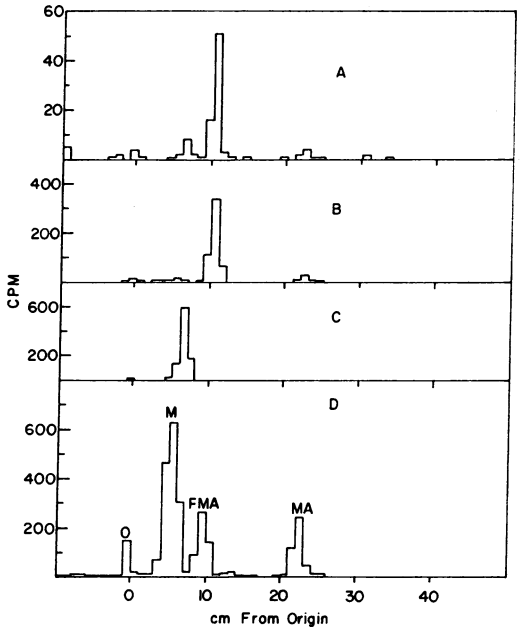

FIG. 3. Analysis of tRNA eluted from the hybrid. tRNA was eluted from three filters containing 50 μg of mitochondrial DNA, to which was bound a total of 396 cpm of [^{3}H]fMet-tRNA. A blank (no DNA) filter bound 23 cpm. Approximately 280 cpm were recovered from the Sephadex column; 225 cpm were applied to the paper. The peaks obtained by paper electrophoresis were identified as in Fig. 1. Counting efficiency of the paper strips was 40% of that of a liquid sample. Approximately 75% of the radioactivity originally on the filters could be accounted for in this experiment when values are corrected for reduced counting efficiency. The following samples were examined: (*A*) [^{3}H]fMet-tRNA eluted from the hybrid with mitochondrial DNA; (*B*) mitochondrial [^{3}H]fMet-tRNA used for hybridization; (*C*) [^{3}H]methionine; (*D*) a mixture of *E. coli* fMet-and Met-tRNA, and free [^{3}H]methionine as a marker.

We wish to thank Dr. G. S. Getz for his stimulating discussions and for his help in aspects of this work. We also thank Drs. S. B. Weiss and R. Haselkorn for their critical review of the paper.

This work was supported in part by grants HE-04442 and HE-90172 from the National Heart Institute, United States Public Health Service.

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