MITOCHONDRIAL-SPECIFIC AMINOACYL-RNA SYNTHETASES*

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Numerous lines of evidence1-14 indicate that protein synthesis occurs within mitochondria, and tRNA8, 15, 16 and aminoacyl-RNA synthetases8 have been prepared from these organelles. More recently, two very interesting aspects of mitochondrial protein synthesis have been described: (a) Although amino acid incorporation by yeast cytoplasmic ribosomal systems is not inhibited by chloramphenicol,17 protein synthesis by yeast mitochondria in vivo and in vitro is sensitive8, 18 to this antibiotic. (b) Mitochondrial protein synthesis in vitro, and therefore presumably in vivo, is apparently restricted to the assembly of a very limited number of proteins; that is, the amino acids are incorporated preferentially into mitochondrial structural protein(s).11, 12 19-21 Thus, mitochondrial protein synthesis differs from cytoplasmic protein synthesis in response to specific inhibitors and in the spectrum of proteins synthesized.

In this report, evidence will be presented that the translational macromolecules, i.e., tRNA's and aminoacyl-RNA synthetases, found in Neurospora mitochondria are different from those isolated from the cytoplasm. In earlier reports,15, 16 we demonstrated that the mitochondria of Neurospora contain a full complement of tRNA's and that the two which were examined in detail (aspartic acid and phenylalanine tRNA) were distinct from their cytoplasmic counterparts in acylation specificities. The experiments reported here indicate that Neurospora mitochondria contain a full complement of aminoacyl-RNA synthetases and that at least three of these, the aspartyl-, phenylalanyl-, and leucyl-RNA synthetases, together with their respective tRNA's, are exclusively associated with the mitochondria.

Experimental Procedures.—Strains: Neurospora crassa wild-type strain OR23-1a was used.

Preparation of mitochondrial and cytoplasmic fractions: Hyphae in the exponential phase of growth were collected from enriched Vogel's8 synthetic medium. Mitochondria were prepared as described previously18 by the methods of Hall and Greenawalt19 followed by zonal sucrose-gradient centrifugation. After removal of the nuclei and debris from the disrupted hyphae by low-speed centrifugation (1500 X g), the mitochondria were pelleted by centrifugation at 8000 X g. The supernatant from the latter centrifugation was recentrifuged at 30,000 X g for 30 min to remove residual mitochondria; the resulting supernatant is considered as the cytoplasmic fraction. The mitochondria were resuspended and reisolated by zonal centrifugation prior to use.

Preparation of tRNA: tRNA was prepared from the cytoplasmic and mitochondrial fractions as described previously18 (essentially by the method of Holley et al.19).

Preparation of enzymes: (a) Cytoplasmic: The cytoplasmic fraction was centrifuged at 78,000 X g for 2 hr and the supernatant adjusted to 0.2 M KPO4 (pH 7.5) and 0.01 M β-mercaptoethanol; the material was then passed over a DEAE-cellulose column (equilibrated with the same buffer) to remove nucleic acids,28 and the protein precipitated by the addition of (NH4)2SO4 crystals to a final concentration of 3.0 M. After standing overnight at ~3°C, the precipitate was collected by centrifugation at 10,000 X g for 30 min. The ammonium sulfate precipitates may be stored at 0°C for at least several months without loss of enzyme activity. Ammonium sulfate and phosphate were removed prior to use by passage through Sephadex G-25 which had been equilibrated with the appropriate buffer. For use as an unfractionated synthetase preparation, the equilibrating buffer consisted of 0.01 M Tris-HCl buffer (pH 7.5), 0.1 M KCl, 0.01 M β-mercaptoethanol, and 20% glycerol, in which aliquots may be frozen and stored at −20°C without loss of activity.

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(b) Mitochondrial: Mitochondria were collected from the appropriate fractions after zonal centrifugation, diluted to 1/4 the sucrose concentration, and then centrifuged at 20,000 x g for 45 min. After suspension in 0.2 M KPO₄ buffer (pH 7.5) containing 0.01 M β-mercaptoethanol, the mitochondria were disrupted by sonication in a Branson Sonifier (model SI125) at maximal output for five 1-min periods in a total volume of about 60 ml. The disrupted material was then treated exactly as the cytoplasmic fraction, except that the (NH₄)₂SO₄ precipitate was collected by centrifugation at 78,000 x g for 1 hr.

Measurement of tRNA and aminoacyl-tRNA synthetases: The assay reaction mixture contained (in addition to enzyme and tRNA) per ml: 50 μmoles HEPES (N-2-hydroxyethylpiperazone-N-2-ethanesulfonic acid, Calbiochem Corporation) buffer (pH 7.5); 0.5 μmole ATP; 10.0 μmoles magnesium acetate; 5.0 μmoles β-mercaptoethanol; and 1.0 μC₁₄-amino acid. Reactions were performed at 30°C in a final volume of either 0.25 ml or 0.5 ml. C₁₄-aminoacyl-tRNA was assayed by a modification of the filter paper disk method of Bollum.²⁷

Radioactive materials: Uniformly labeled C₁₄-L-amino acids were obtained from the New England Nuclear Corporation.

Results and Discussion.—A comparison of mitochondrial and cytoplasmic synthetase activities for aspartic acid and phenylalanine is shown in Table 1. The striking preference of the crude mitochondrial synthetase preparation for mitochondrial tRNA's suggests that the enzymes found in these organelles are quite different from those located in the cytoplasm. In order to examine this possibility more carefully, cytoplasmic and mitochondrial preparations were subjected to column chromatography. As seen in Figure 1, the phenylalanine-RNA synthetase activities of the two fractions are chromatographically dissimilar, as are their tRNA specificities. Similar chromatographic differences between the mitochondrial and cytoplasmic aspartyl-tRNA synthetases are shown in Figure 2. It should be noted that the cytoplasmic preparation has a minor aspartryl-RNA synthetase activity.

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<tr>
<th>tRNA Specificities of Mitochondrial and Cytoplasmic Aspartyl- and Phenylalanyl-RNA Synthetases</th>
<th>Mitochondrial Synthetases</th>
<th>Cytoplasmic Synthetases</th>
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<tbody>
<tr>
<td>tRNA</td>
<td>tRNA</td>
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<tr>
<td>Phenylalanine</td>
<td>1.0</td>
<td>0.0</td>
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<tr>
<td>Aspartic acid</td>
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<td>&lt;0.1</td>
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FIG. 1.—Elution profiles for mitochondrial and cytoplasmic phenylalanyl-RNA synthetases. Fractions were eluted from hydroxylapatite columns (2 x 30 cm) with linear gradients of potassium phosphate (pH 7.0) from 0.005 M (1200 ml) to 0.5 M (1200 ml), both containing 0.01 M β-mercaptoethanol. One hundred mg of protein were applied to the columns, and 0.05 ml aliquots were assayed as described in Procedures in a final volume of 0.25 ml by using 1.25 A₅₅₀ units of mitochondrial tRNA or 2.9 A₅₅₀ units of cytoplasmic tRNA.
which chromatographs identically with the mitochondrial aspartyl-RNA synthetase. This is believed to reflect contamination of the cytoplasmic fraction with the mitochondrial enzyme, since the hyphal disruption procedure undoubtedly disrupts some fraction of the mitochondria and since acylation occurs only with mitochondrial tRNA.

Crude or unfractionated mitochondrial preparations have been examined for other aminoacyl-RNA synthetases (Table 2), and activities for all amino acids tested are present. The acylation specificity data, however, do not permit the

### Table 2

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<tr>
<th>tRNA Specificities of Mitochondrial and Cytoplasmic Aminoacyl-RNA Synthetases</th>
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<tr>
<td><strong>µMoles Aminoacyl-RNA Formed/A₂₆₀ Unit/5 Min</strong></td>
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<td><strong>Mitochondrial Synthetases</strong></td>
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<td>tRNA</td>
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<td>Asparagine</td>
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<td>Valine</td>
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unambiguous differentiation of mitochondrial and cytoplasmic synthetases for amino acids other than phenylalanine and aspartic acid. We therefore selected the leucyl-RNA synthetase for chromatographic comparisons, and it is apparent from Figure 3 that the two enzymes are distinct. These data also demonstrate the nonidentity of mitochondrial and cytoplasmic leucine tRNA's.

The data presented in Figures 1–3 were obtained under conditions designed to determine the relative amounts of synthetase activity in various fractions for a particular tRNA, and therefore do not represent the total acylation capacities of these tRNA's. Thus, the possibility existed that the apparent specificities were artifacts of acylation rate differences. It may be seen in Figure 4 that this is not the case, since permitting the reaction to proceed for 30 min (essentially to completion) does not alter the patterns of specificity observed.

Recently, concern has been expressed regarding the role of bacterial contamination in the protein synthesis exhibited by rat-liver mitochondrial preparations. Although it is clear that relatively small numbers of bacteria in such preparations could lead to erroneous interpretations (especially when the sensitivity of radioisotopic incorporation experiments is considered), it has been established that the mitochondria per se do synthesize proteins in vitro.11, 12, 19–21 These studies
have demonstrated that mitochondria incorporate amino acids preferentially into mitochondrial structural protein, in contrast to bacterial incorporation, which directs no radioactive amino acids into this class of proteins. For several reasons, we do not feel that the observations reported here reflect bacterial contamination. The mitochondrial aspartyl- and phenylalanyl-RNA synthetases are identical in
chromatographic properties and acylation specificities with minor synthetase activities found\textsuperscript{29, 30} in whole-cell preparations. These whole-cell preparations have been prepared from pure cultures of several strains of \textit{Neurospora} in this laboratory and independently in the laboratory of Dr. Noboru Sueoka with essentially identical results.\textsuperscript{31} Similarly, phenylalanine and aspartic acid tRNA's which are indistinguishable from these mitochondrial tRNA's have been isolated by phenol extraction of freshly harvested mycelia from pure cultures.\textsuperscript{29, 30} In all procedures sterile solutions were used, and aliquots from all stages of the preparations were plated for bacterial counts. No preparations were used if the bacterial count was over \(~200\) per ml. These numbers are negligible when it is considered that in a typical zonal centrifuge run (from an initial 1000 gm of hyphae) the mitochondria are isolated in a volume of \(~150\) ml and yield \(~15\) mg of tRNA and \(300\) mg of soluble protein.

Woodward and Munkres\textsuperscript{32} have demonstrated that nonchromosomal, maternally inherited mutations, which are believed to occur within mitochondrial DNA, lead to amino acid substitutions within \textit{Neurospora} mitochondrial structural protein, and Luck\textsuperscript{33} has reported detection of a DNA-dependent RNA polymerase in these same organelles. We have also demonstrated the presence of ribosomal RNA's within mitochondria.\textsuperscript{24} Thus, the available evidence strongly suggests that transcription and translation of genetic information occurs within mitochondria, and the data presented in this report indicate that at least the tRNA's and aminoacyl-RNA synthetases involved in translation are distinct from those which participate in cytoplasmic protein syntheses.

**Summary.—** \textit{Neurospora} mitochondria were found to contain aminoacyl-RNA synthetases for all 15 amino acids tested. Comparison of mitochondrial and cytoplasmic aspartyl-, phenylalanyl-, and leucyl-RNA synthetases by column chromatography and utilization of tRNA's isolated from both mitochondria and cytoplasm demonstrated that mitochondria contain unique enzymes and tRNA's for these amino acids.

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\dagger U.S. Public Health Service fellow (fellowship no. 1-F2-GM-28,619-01).


31 Imamoto, F., T. Yamane, and N. Sueoka, these *PROCEEDINGS*, 53, 1456 (1965).