Mitochondrial Ribosome Assembly in *Neurospora*.  
Structural Analysis of Mature and Partially Assembled Ribosomal Subunits by Equilibrium Centrifugation in CsCl Gradients

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ABSTRACT In *Neurospora*, one protein associated with the mitochondrial small ribosomal subunit (S-5, M, 52,000) is synthesized intramitochondrially and is assumed to be encoded by mtDNA. When mitochondrial protein synthesis is inhibited, either by chloramphenicol or by mutation, cells accumulate incomplete mitochondrial small subunits (CAP-30S and INC-30S particles) that are deficient in S-5 and several other proteins. To gain additional insight into the role of S-5 in mitochondrial ribosome assembly, the structures of *Neurospora* mitochondrial ribosomal subunits, CAP-30S particles, and INC-30S particles were analyzed by equilibrium centrifugation in CsCl gradients containing different concentrations of Mg\(^{2+}\). The results show (a) that S-5 is tightly associated with small ribosomal subunits, as judged by the fact that it is among the last proteins to be dissociated in CsCl gradients as the Mg\(^{2+}\) concentration is decreased, and (b) that CAP-30S and INC-30S particles, which are deficient in S-5, contain at most 12 proteins that are bound as tightly as in mature small subunits. The CAP-30S particles isolated from sucrose gradients contain a number of proteins that appear to be loosely bound, as judged by dissociation of these proteins in CsCl gradients under conditions in which they remain associated with mature small subunits. The results suggest that S-5 is required for the stable binding of a subset of small subunit ribosomal proteins.

Mitochondria contain a distinct species of ribosomes that is used for the translation of proteins encoded by mtDNA (1). Studies in many organisms have shown that mitochondrial rRNAs are encoded by mtDNA, whereas most of the mitochondrial ribosomal proteins are nuclear gene products (1, 5). In *Neurospora*, one mitochondrial ribosomal protein (S-5, M, 52,000) is synthesized intramitochondrially and is assumed to be encoded by mtDNA (7, 8). A similar protein (varl) has been found in yeast (21, 22) and there is evidence that mitochondrial rRNAs are synthesized intramitochondrially and that mitochondrial ribosomal proteins also exist in *Paramecium* (20) and *Tetrahymena* (4, 17). The finding that just a few mitochondrial ribosomal proteins are encoded by mtDNA makes it possible to study the possibility that these proteins have some special role in mitochondrial ribosome assembly or protein synthesis.

In our laboratory we have focused on the *Neurospora* protein, S-5. Previous two-dimensional gel electrophoretic analysis showed that S-5 is present in stoichiometric concentrations in mitochondrial small subunits, that it is among the most basic mitochondrial ribosomal proteins, and that it has a high affinity for RNA under gel electrophoretic conditions in the presence of urea (8). More recently, we showed that S-5 has an extremely high affinity for cation exchange resins and that this property could be exploited to purify S-5 by chromatography on carboxymethyl-Sepharose in a single batch elution step (11). In terms of amino acid composition, S-5 is more similar to *Escherichia coli* and yeast ribosomal proteins than to previously characterized mitochondrial translation products, all of which are hydrophobic membrane proteins (11).

Insight into the role of S-5 in mitochondrial ribosome assembly was obtained by examining the effect of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, on mitochondrial ribosome assembly in wild-type *Neurospora* (8, 11).
9). These studies showed that chloramphenicol rapidly inhibits the assembly of mitochondrial small subunits, but has relatively little effect on the assembly of mitochondrial large subunits, all of whose proteins are synthesized in the cytosol. Wild-type cells grown in chloramphenicol were found to accumulate incomplete small subunits (CAP-30S particles) that sediment more slowly than mature small subunits in sucrose gradients and that are deficient in S-5 and several other proteins. Incomplete small subunits (INC-30S particles), similar to CAP-30S particles, were subsequently found in several nuclear mutants and one extranuclear mutant (ICNJ2) with deficiencies in mitochondrial protein synthesis (2, 3). Considered together, the results led to the tentative conclusion that S-5 is required for the complete assembly of mitochondrial small ribosomal subunits.

In the present work, we analyzed the structures of Neurospora mitochondrial ribosomal subunits, CAP-30S particles, and subribosomal particles from mutant cells by equilibrium centrifugation in CsCl gradients containing different concentrations of Mg$^{2+}$. The results show (a) that S-5 is tightly associated with mature small ribosomal subunits, as judged by the fact that it is among the last proteins to be dissociated in CsCl gradients containing progressively lower concentrations of Mg$^{2+}$, and (b) that CAP-30S and INC-30S particles, which are deficient in S-5, contain at most 12 proteins that are bound as tightly as in mature small subunits. The remaining proteins are either already deficient in the particles isolated from sucrose gradients or are bound less tightly as judged by dissociation in CsCl gradients. The results suggest that S-5 is required for the stable binding of a subset of small subunit ribosomal proteins. A preliminary account of this work has been presented previously (12).

MATERIALS AND METHODS

Strains of Neurospora and Growth Conditions

Wild-type strain Em 5256A (FGSC #626) was used in these studies. The mutant strains, 289-56 and 299-9, were isolated by Pittenger and West (19). Procedures for maintaining strains, growing cells, and labeling cells with radioactive precursors have been described previously (2, 7-9).

CsCl Gradient Centrifugation

Mitochondrial ribosomal subunits and subribosomal particles were prepared as described previously (6, 10). CsCl gradient centrifugation was carried out by a modification of the method of Maglott and Staehelin (13). Stock solutions containing 65% (w/v) CsCl (optical grade, Bethesda Research Laboratories, Rockville, MD), 30 mM Tris-HCl, pH 7.1, and 0.1-50 mM Mg$^{2+}$ or 1 mM EDTA were prepared and the pH readjusted to 7.1 (25°C) with HCl. For analysis of small subunits, 2.5 ml of the CsCl stock solution were adjusted to density 1.5395 g/cm$^3$ (25°C) by addition of the appropriate buffer and then placed in a 10-ml Oakridge-type polycarbonate centrifuge tube. Purified small ribosomal subunits were dissolved in 0.5 ml of a solution containing 30 mM-Tris-HCl, pH 7.1, 0.1-50 mM Mg$^{2+}$ or 1 mM EDTA, and added to 4.0 ml of a diluted CsCl stock solution containing the same buffer. The density of the solution was then adjusted to 1.4700 g/cm$^3$ (25°C) by addition of buffer and the solution was layered over the denser CsCl solution in the centrifuge tube. Gradients were centrifuged in a Beckman 50 Ti or 65 rotor (226,000 g, 18-20 h, 4°C; Beckman Instruments, Inc., Spinc Div., Palo Alto, CA). The procedures for analyzing other ribonucleoprotein particles were the same except that the final densities of the solutions were as follows: (a) large subunits at 10-50 mM MgCl$_2$, lower solution = 1.508 g/cm$^3$; upper solution = 1.5297 g/cm$^3$; (b) large subunits at 0.1-1.0 mM MgCl$_2$, or 1 mM EDTA, lower solution = 1.6334 g/cm$^3$; upper solution = 1.6571 g/cm$^3$; (c) CAP-30S and INC-30S particles, lower solution = 1.6416 g/cm$^3$; upper solution = 1.5547 g/cm$^3$. The gradients were fractionated by pumping the contents from the bottom of the tubes. Absorbance was monitored at 254 nm using an ISCO Instrumentation Specialties Co., Lincoln, NE density gradient fractionator, and 0.2-ml fractions were collected. Densities were determined by measuring refractive index at 25°C, and correcting for the lower temperature (4°C) during centrifugation. Gradients were found to be linear over most density ranges used. There was occasionally some flattening of the gradients at the bottoms of the tubes containing the higher CsCl concentrations.

Analysis of Mitochondrial Ribosomal Proteins

The protein compositions of particles from CsCl gradients were determined by two-dimensional gel electrophoresis. Pooled fractions from CsCl gradients were dialyzed exhaustively against deionized, distilled water (4°C). Fractions were then lyophilized and proteins were extracted using a modification of the acetic acid procedure (6, 8). Two-dimensional gel electrophoresis was carried out using a modification of the system of Mej and Bogorad (16) as described previously (6, 8). Identification of mitochondrial ribosomal proteins was based on previously published maps of two-dimensional gel patterns (8). Mitochondrial ribosomal proteins are defined as major proteins present reproducibly in many experiments (8).

RESULTS

Core Particles of Mitochondrial Small Ribosomal Subunits

Mitochondrial small ribosomal subunits from wild-type strain Em 5256A were centrifuged through CsCl gradients containing different concentrations of Mg$^{2+}$. Core particles were isolated and their protein compositions were determined by two-dimensional gel electrophoresis. CsCl gradient profiles and gel patterns are shown in Figs. 1 and 2 and the protein compositions of different core particles are summarized in Table I. Three species of core particles ($p = 1.508$ g/cm$^3$, $p = 1.554$ g/cm$^3$, and $p = 1.610$ g/cm$^3$) were identified in CsCl gradients at different Mg$^{2+}$ concentrations. At 50 mM Mg$^{2+}$, the gradient profiles show a single peak at $p = 1.508$ g/cm$^3$ (Fig. 1 a). Two-dimensional gel analysis shows that the $p = 1.508$ g/cm$^3$ particles contain 17 proteins that were consistently present in stoichiometric concentrations and six additional proteins that were present in stoichiometric concentrations in some experiments. Only two proteins (S-6 and S-20) were consistently absent (Fig. 2 b, Table I). The core particles formed at 10 and 25 mM Mg$^{2+}$ are essentially equivalent to the $p = 1.508$ g/cm$^3$ core particles, as judged by density and protein composition (data not shown). A further decrease to 1 mM Mg$^{2+}$ results in the disappearance of the peak at $p = 1.508$ g/cm$^3$. 

![Figure 1 CsCl gradient profiles of core particles of mitochondrial small ribosomal subunits. Ribonucleoprotein particles were prepared from 2-4 I of Em5256A culture CsCl gradients in (a) and (b) contained 50 mM and 1 mM MgCl$_2$, respectively. Profiles show $A_{254}$. Recoveries of core particles were determined in separate experiments using particles labeled in vivo with $^{32}$P-labeled orthophosphoric acid. Recoveries were $\rho = 1.508$ g/cm$^3$, 40%; $\rho = 1.554$ g/cm$^3$, 20-30%; $\rho = 1.610$ g/cm$^3$, 10-20%. In repeats of the gradient shown in (b) the mass ratio of light ($p = 1.554$ g/cm$^3$) to dense ($p = 1.508$ g/cm$^3$) core particles varied from 0.75 to 0.62 (nine independent experiments). Densities are averages from at least four independent experiments (Table I).

![Figure 2 Two-dimensional gel electrophoresis profiles of core particles of mitochondrial small ribosomal subunits. Ribonucleoprotein particles were prepared from 2-4 I of Em5256A culture CsCl gradients in (a) and (b) contained 50 mM and 1 mM MgCl$_2$, respectively. Profiles show $A_{254}$. Recoveries of core particles were determined in separate experiments using particles labeled in vivo with $^{32}$P-labeled orthophosphoric acid. Recoveries were $\rho = 1.508$ g/cm$^3$, 40%; $\rho = 1.554$ g/cm$^3$, 20-30%; $\rho = 1.610$ g/cm$^3$, 10-20%. In repeats of the gradient shown in (b) the mass ratio of light ($p = 1.554$ g/cm$^3$) to dense ($p = 1.508$ g/cm$^3$) core particles varied from 0.75 to 0.62 (nine independent experiments). Densities are averages from at least four independent experiments (Table I).
FIGURE 2 Two-dimensional gel electrophoresis of proteins from mitochondrial small ribosomal subunits and core particles. Core particles were prepared from 18-36 l of Em5256A culture. (a) Total protein, small ribosomal subunit (TP30). (b) $\rho = 1.508$ g/cm$^3$ particles from CsCl gradients containing 50 mM MgCl$_2$ (Fig. 1a). (c) $\rho = 1.554$ g/cm$^3$ particles from CsCl gradients containing 1 mM MgCl$_2$ (Fig. 1b). (d) $\rho = 1.610$ g/cm$^3$ particles from CsCl gradients containing 1 mM MgCl$_2$ (Fig. 1b). Parentheses indicate proteins present in less than stoichiometric amounts as judged by Coomassie-Blue staining relative to standard two-dimensional gel patterns of small subunit proteins (e.g., a). Mitochondrial ribosomal proteins are defined as major proteins present reproducibly in many experiments (8). Fig. 2a shows several additional proteins that appeared occasionally in the gel patterns.

cm$^3$ and the appearance of new peaks at $\rho = 1.554$ g/cm$^3$ and $\rho = 1.610$ g/cm$^3$ (Fig. 1b). The $\rho = 1.554$ g/cm$^3$ particles contain 13 proteins that were consistently present in stoichiometric concentrations and five additional proteins that were present in stoichiometric concentrations in some experiments (Fig. 2c, Table I). The $\rho = 1.610$ g/cm$^3$ particles contain four proteins (S-7, S-8, S-9, and S-12) in stoichiometric concentrations and at most eight additional proteins including S-5 in lower concentrations (Fig. 2d; Table I). CsCl gradient centrifugation at 0.1 mM Mg$^{2+}$ or 1 mM EDTA results in the formation of essentially the same two particles, judged by density and protein composition (data not shown). The data show that S-5 is tightly bound to mature small subunits since it is present in core particles in CsCl gradients containing low
Mg\(^{2+}\) concentrations or EDTA. However, S-5 is not among the four proteins (S-7, S-8, S-9, and S-12) that are completely resistant to dissociation by CsCl (Fig. 2d; Table I).

**Core Particles of Mitochondrial Large Ribosomal Subunits**

Five different species of large subunit core particles (\(\rho = 1.562 \text{ g/cm}^3, \rho = 1.617 \text{ g/cm}^3, \rho = 1.641 \text{ g/cm}^3, \rho = 1.655 \text{ g/cm}^3, \rho = 1.733 \text{ g/cm}^3\)) were identified (Figs. 3 and 4; Table II). At 50 mM Mg\(^{2+}\), the gradient profiles show two peaks: \(\rho = 1.562 \text{ g/cm}^3\) and \(\rho = 1.617 \text{ g/cm}^3\) (Fig. 3a). The \(\rho = 1.562 \text{ g/cm}^3\) particles contain 18 proteins that were consistently present in stoichiometric concentrations and nine additional proteins that were present in stoichiometric concentrations in some experiments. Only one protein (L-29) was consistently absent (Fig. 4b; Table II). The \(\rho = 1.617 \text{ g/cm}^3\) particles contain 13 proteins that were consistently present in stoichiometric concentrations. Ten proteins were consistently absent and five additional proteins were present in some experiments but absent in others (Fig. 4c; Table II). At 25 mM Mg\(^{2+}\), the \(\rho = 1.562\) and \(\rho = 1.617 \text{ g/cm}^3\) peaks disappear and the gradients show a major peak at \(\rho = 1.641 \text{ g/cm}^3\) (Fig. 3b). There are only minor changes in protein composition compared to the \(\rho = 1.562 \text{ g/cm}^3\) particles.

**TABLE I**

**Protein Compositions of Core Particles of Mitochondrial Small Ribosomal Subunits**

| [Mg\(^{2+}\)] | 50 mM | 1 mM | 1 mM |
|---------------|-------|------|------|
| Density (g/cm\(^3\)) | 1.508 ± 0.014 | 1.554 ± 0.014 | 1.610 ± 0.019 |
| Determinations | 5 | 8 | 2 |

| Protein | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | ± | + | + | + | + | ± | + | + | + | + | ± | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |

Density is the mean ± SD for at least four independent determinations. Determinations indicate the number of experiments in which protein composition was determined by two-dimensional gel electrophoresis. +/(-) indicates a protein that was consistently present in stoichiometric concentrations, +/+(-) indicates a protein that was present in stoichiometric concentrations in some experiments but deficient in others, and -/- indicates a protein that was consistently absent. Stoichiometries were judged by Coomassie-Blue staining relative to standard two-dimensional gel patterns of small subunit proteins (e.g., Fig. 2a).

In previous work, to gain insight into the role of S-5 in mitochondrial ribosome assembly, we examined the effect of chloramphenicol on mitochondrial ribosome assembly in wild-type *Neurospora* (8, 9). The results showed that chloramphenicol rapidly inhibits the assembly of mitochondrial small ribosomal subunits and leads to the accumulation of incomplete small subunits (CAP-30S particles) that are deficient in S-5 and several other proteins. Similar incomplete small subunits (INC-30S particles) were subsequently found in several nuclear mutants and one extranuclear mutant ([C97]) deficient in mitochondrial protein synthesis (2, 3). It was assumed that the structural alterations in both CAP-30S and INC-30S particles reflected the deficiency of the single mitochondrially synthesized, mitochondrial ribosomal protein, S-5 (8, 9). In the present work, to obtain further insight into these structural
FIGURE 4  Two-dimensional gel electrophoresis of proteins from mitochondrial large ribosomal subunits and core particles. Core particles were prepared from 18 to 72 liters of EmS256A culture. (a) Total protein, large ribosomal subunit (TP50). The gel pattern shows a background of small subunit proteins due to contamination of sucrose gradient fractions. (b) $\rho = 1.562$ g/cm$^3$ particles from CsCl gradients containing 50 mM MgCl$_2$ (Fig. 3 a). The position of L-26 in this gel pattern is anomalous. In both the other gel patterns for these particles, L-26 is present and migrates normally. (c) $\rho = 1.617$ g/cm$^3$ particles from CsCl gradients containing 50 mM MgCl$_2$ (Fig. 3 a). (d) $\rho = 1.641$ g/cm$^3$ particles from CsCl gradients containing 25 mM MgCl$_2$ (Fig. 3 b). (e) $\rho = 1.655$ g/cm$^3$ particles from CsCl gradients containing 10 mM MgCl$_2$ (Fig. 3 c). (f) $\rho = 1.733$ g/cm$^3$ particles from CsCl gradients containing 1 mM MgCl$_2$ (Fig. 3 d). Parentheses indicate proteins present in less than stoichiometric amounts as judged by Coomassie-Blue staining relative to standard two-dimensional gel patterns of large subunit proteins (e.g., a).
TABLE II

Protein Compositions of Core Particles of Mitochondrial Large Ribosomal Subunits

| Protein | Determinations |
|---------|----------------|
|         | 3              |
| 1       | +              |
| 2       | +              |
| 3       | +/-            |
| 4       | +/-            |
| 5       | +/-            |
| 6       | +/-            |
| 7       | +/-            |
| 8       | +/-            |
| 9       | +/-            |
| 10      | +/-            |
| 11      | +/-            |
| 12      | +/-            |
| 13      | +/-            |
| 14      | +              |
| 15      | +/-            |
| 16      | +/-            |
| 17      | +/-            |
| 18      | +/-            |
| 19      | +/-            |
| 20, 22  | +/-            |
| 23      | +/-            |
| 24      | +/-            |
| 25      | +/-            |
| 26      | +/-            |
| 27      | +/-            |
| 28      | +/-            |
| 29      | +/-            |
| 30      | +/-            |
| 31      | +/-            |
| 32      | +/-            |
| 33      | +/-            |

Density is the mean ± SD for at least four independent determinations. Determinations indicate the number of experiments in which protein composition was determined by two-dimensional gel electrophoresis. "+" indicates a protein that was consistently present in stoichiometric concentrations. "+/(+)" indicates a protein that was present in stoichiometric concentrations in some experiments but deficient in others; "(+)" indicates a protein that was consistently deficient. "-" indicates a protein that was present in some experiments but absent in others. Stoichiometries were judged by Coomassie-Blue staining relative to standard two-dimensional gel patterns of large subunit proteins (e.g., Fig. 4a). Proteins L-20 and L-22 were considered together because of poor separation in this gel system.

alterations, we compared the behavior of CAP-30S and INC-30S particles in CsCl gradients with that of mature mitochondrial small ribosomal subunits.

Fig. 5 shows an experiment in which wild-type cells were grown in chloramphenicol for 17 h and then ribonucleoprotein particles were isolated and centrifuged through sucrose gradients. As expected from the previous work, the sucrose-gradient profile shows two peaks in the 30S region corresponding to putative mature small ribosomal subunits and more slowly sedimenting CAP-30S particles (Fig. 5a). Previous one- and two-dimensional gel analysis showed that the CAP-30S particles are deficient in S-5 and several other proteins. Although there was some variability in the protein composition of the CAP-30S particles, S-5 was consistently deficient and six proteins (S-8, S-9, S-12, S-13, S-14, and S-19) were deficient in >50% of the experiments. By contrast, putative mature small subunits from chloramphenicol-treated cells were found to contain all small subunit proteins in normal concentrations (8, 9).

In the present work, CAP-30S particles and putative mature small subunits from chloramphenicol-treated cells were isolated separately from sucrose gradients and centrifuged through CsCl gradients containing 50 mM Mg\(^{2+}\) (Fig. 5b and c). The putative mature small subunits give a core particle whose density (ρ = 1.506 g/cm\(^3\)) and protein composition, including stoichiometric amounts of S-5 (data not shown), are equivalent to the core particle obtained from standard small subunits (see above). The results support the previous assumption that mature small subunits are synthesized at a slow rate in the presence of chloramphenicol (8, 9).

By contrast, the CAP-30S particles give rise to much denser particles that are reflected in the CsCl gradient profiles by a peak at ρ = 1.688 g/cm\(^3\) that is skewed toward lower densities (Fig. 5c). These dense particles contain only five proteins (S-1, S-7, S-9, S-11, S-21, and S-25) in stoichiometric concentrations and six others (S-2, S-3, S-4, S-13, S-14, and S-18) that are judged to be deficient (Fig. 6a; Table III). The S-5 protein is completely absent from these core particles (Fig. 6a). The results show that CAP-30S particles contain relatively few proteins that are bound as tightly as in mature small subunits.

Mitochondrial large subunits from chloramphenicol-treated wild-type cells, centrifuged through CsCl gradients containing
are present in stoichiometric concentrations and four additional proteins (S-18, S-22, S-23, and S-24) that are present in lower concentrations (Fig. 6f; Table III). The 11 major proteins are essentially the same group that remains associated with CAP-30S particles (Fig. 6a) plus one additional protein (S-19). However, the stoichiometry of some proteins is different between the two species of particles (see Discussion).

CsCl gradient centrifugation of putative mature small subunits from 289-56 unexpectedly gave two peaks, one at $\rho = 1.508 \text{ g/cm}^3$ and the other at $\rho = 1.574 \text{ g/cm}^3$ (Fig. 7b). The former has a density and protein composition equivalent to the core particle derived from wild-type small subunits (Fig. 6c; Table III). The latter ($\rho = 1.574 \text{ g/cm}^3$) is a novel particle that has no equivalent in standard wild-type or chloramphenicol-treated wild-type cells. Gel analysis shows that the $\rho = 1.574 \text{ g/cm}^3$ particles contain S-5, but are missing S-6, S-10, S-15, S-16, and S-20 and are deficient in S-1, S-17, S-18, S-19, S-21, S-23, S-24, and S-25 (Fig. 6d; Table III). The formation of the $\rho = 1.574 \text{ g/cm}^3$ particles may reflect a structural alteration in a component of the small ribosomal subunit, possibly a ribosomal protein, which occurs as a result of the 289-56 mutation. Alternatively, the synthesis of some or more of the proteins S-6, S-10, S-15, S-16, or S-20 could be impaired in the mutant.

Mitochondrial large ribosomal subunits from 37°C-grown 289-56 give a core particle of density $\rho = 1.624 \text{ g/cm}^3$ in CsCl gradients containing 25 mM Mg$^{2+}$ (data not shown). This density is slightly lower than the average density of wild-type mitochondrial large subunits at 25 mM Mg$^{2+}$, but the difference is probably not significant. The protein composition of the $\rho = 1.624 \text{ g/cm}^3$ particles is the same as that of the particle from wild type (data not shown).

299-9 is a nuclear mutant with a temperature-sensitive defect in splicing the mitochondrial large rRNA (14). When grown at the nonpermissive temperature (37°C), the mutant is strongly deficient in mitochondrial protein synthesis and sucrose gradients of ribonucleoprotein particles showed the expected two peaks in the 30S region due to putative mature small subunits and INC-30S particles (Fig. 8a). The behavior of INC-30S particles from 299-9 in CsCl gradients containing 50 mM Mg$^{2+}$ was essentially the same as that of CAP-30S particles and INC-30S particles from 289-56. The gradient profiles show a major peak at $\rho = 1.691 \text{ g/cm}^3$ with a shoulder at $\rho = 1.646 \text{ g/cm}^3$ (Fig. 8b). The pooled $\rho = 1.691$ and 1.646 g/cm$^3$ particles contain eight proteins (S-1, S-2, S-7, S-11, S-13, S-14, S-19, S-21, and S-25) in stoichiometric concentrations and four proteins (S-3, S-4, S-18, and S-19) that appear deficient (Fig. 6b; Table III). Again, the proteins are the same group that remains associated with CAP-30S particles and INC-30S particles from 289-56, but with some differences in the stoichiometry (see Discussion).

CsCl gradient analysis of 50–70S particles from 37°C-grown 299-9 was reported previously (10). The results led to the identification of a unique ribonucleoprotein particle containing an unspliced precursor of the large rRNA.

**DISCUSSION**

In the present work, the structures of mature and partially assembled *Neurospora* mitochondrial ribosomal subunits were analyzed by equilibrium centrifugation in CsCl gradients. The results show (a) that the mitochondrially synthesized, mitochondrial ribosomal protein, S-5, is tightly associated with mature small subunits and (b) that incompletely assembled
(a) 5256 + CAP, $\rho=1.688$

(b) 299-9, $\rho=1.691+1.646$

(c) 289-56, $\rho=1.508$

(d) 289-56, $\rho=1.574$

(e) 289-56, INC - 30S

(f) 289-56, $\rho=1.685+1.650$
small subunits (CAP-30S and INC-30S particles) that lack S-5 have structures in which less than half of the small subunit proteins are bound as tightly as in mature small subunits. The results suggest that S-5 is required for stable binding of a subset of mitochondrial ribosomal proteins.

Centrifugation of mitochondrial small and large ribosomal subunits in CsCl gradients containing progressively lower Mg\(^{2+}\) concentrations results in dissociation of increasing numbers of proteins, as expected. Three species of small subunit core particles and five species of large subunit core particles were obtained. In some cases, two core particles of different densities were present in the same CsCl gradient. These may reflect different populations of particles present initially in the preparations (e.g., ribosomal subunits having different conformations or somewhat different protein compositions) or they may be formed by dissociation of different groups of proteins during CsCl gradient centrifugation. We note that the ratio of the different particles varied considerably from one experiment to another. In a number of cases, the core particles contain one group of proteins in stoichiometric concentrations and other proteins in lower concentrations. The two groups of proteins are assumed to reflect the presence of different populations of particles.

Two species of small subunit core particles survive centrifugation in CsCl gradients containing low Mg\(^{2+}\) concentrations or EDTA. The finding that the S-5 protein remains associated with small subunit core particles under these extreme gradient conditions demonstrates that it is tightly bound to mature small

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**Table III**

Protein Compositions of Complete and Partially Assembled Small Ribosomal Subunits from Chloramphenicol-treated Wild Type and Mutants

| Strain      | Density (g/cm\(^3\)) | 5256A + CAP | 299-9 | 289-56 + 289-56 | 289-56 | 289-56 |
|-------------|----------------------|-------------|-------|----------------|--------|--------|
|             |                      | 1.688       | 1.691 + 1.646 | 1.685 + 1.650 | 1.508  | 1.574  |
| Protein     |                      |             |       |                |        |        |
| 1           | +                    | +           | -     | +              | +      | (+)    |
| 2           | (+)                  | +           | +     | +              | +      | (+)    |
| 3           | (+)                  | (+)         | +     | +              | +      | (+)    |
| 4           | (+)                  | (+)         | +     | +              | +      | (+)    |
| 5           | -                    | -           | -     | -              | -      | -      |
| 6           | -                    | -           | +     | -              | +      | +      |
| 7           | +                    | -           | +     | +              | +      | +      |
| 8           | -                    | -           | -     | -              | -      | -      |
| 9           | +                    | +           | +     | +              | +      | +      |
| 10          | -                    | -           | -     | +              | -      | +      |
| 11          | +                    | +           | +     | +              | +      | +      |
| 12          | -                    | -           | -     | -              | -      | -      |
| 13          | (+)                  | (+)         | +     | +              | +      | +      |
| 14          | (+)                  | (+)         | +     | +              | +      | +      |
| 15          | -                    | -           | -     | -              | -      | -      |
| 16          | -                    | -           | -     | -              | -      | -      |
| 17          | -                    | -           | -     | -              | -      | -      |
| 18          | (+)                  | (+)         | (+)   | +              | +      | (+)    |
| 19          | -                    | (+)         | +     | +              | +      | (+)    |
| 20          | -                    | -           | -     | -              | -      | -      |
| 21          | +                    | +           | +     | +              | +      | +      |
| 22          | -                    | -           | (+)   | (+)            | (+)    |
| 23          | -                    | -           | (+)   | (+)            | (+)    |
| 24          | -                    | -           | (+)   | (+)            | (+)    |
| 25          | +                    | +           | +     | +              | +      | (+)    |

"+" indicates a protein that was present in stoichiometric concentrations, "(+)") indicates a protein that was deficient, "-" indicates a protein that was absent. Stoichiometries were judged by Coomassie-Blue staining or autoradiography relative to the appropriate standard two-dimensional gel pattern for small subunit proteins (i.e., Coomassie-Blue stained gel, Fig. 2 a; autoradiogram, Fig. 9 a of reference 8). The data are from Fig. 6.
subunits. Based on previous results, it seems likely that this tight binding reflects strong interaction between S-5 and the subunits by solutions containing 1 M sodium acetate whereas all other mitochondrial ribosomal proteins (8, 11). For example, S-5 solubilized in urea has been shown to have a deficiency of S-5. S-5 is the only known mitochondrial ribosomal proteins could bind to some extent even in solutions containing 50 mM sodium acetate (11). Nevertheless, direct evidence that S-5 binds to rRNA under physiological conditions is still lacking. Previous studies of mitochondrial ribosomal precursor particles suggest that S-5 binds relatively late in mitochondrial ribosome assembly (7). This finding is not contradictory because RNA binding proteins could bind either early or late in assembly so long as RNA binding sites remain open at late stages.

The most important aspect of our results is the additional insight they provide into the structural alterations in partially assembled small subunits. The CAP-30S particles and the two species of INC-30S particles that were examined show very similar behavior in CsCl gradients, confirming that they are in fact structurally related. In all three cases, the gradient profiles show a predominant particle at $\rho = 1.685$ to 1.691 g/cm$^3$ and lighter particles present in lower concentrations. The latter are reflected by skewing toward lower densities in the gradient for CAP-30S particles and by distinct shoulders ($\rho = 1.646$ to 1.650 g/cm$^3$) in the gradients for the INC-30S particles. Two-dimensional gel analysis shows that the CAP-30S particles and both species of INC-30S particles retain essentially the same protein compositions as mature small subunits. The finding of CAP-30S and INC-30S particles isolated from sucrose gradients, or they are loosely bound, as judged by dissociation of the proteins in CsCl gradients under conditions in which they remain associated with mature small subunits. The finding that CAP-30S and INC-30S particles may contain loosely bound proteins readily accounts for the previously observed variability in the protein compositions of these particles (8, 9). Considered together, the results lead to the conclusion that inhibition of mitochondrial protein synthesis affects the binding of at least 12 mitochondrial small subunit ribosomal proteins, in addition to S-5.

By now, it is a reasonable assumption that the structural alterations in CAP-30S and INC-30S particles reflect the deficiency of S-5. S-5 is the only known mitochondrial ribosomal protein and the possibility of an additional, undetected protein is remote. Furthermore, experiments in which CAP-30S or INC-30S particles and putative mature small subunits are isolated from the same cells show that the former are deficient in S-5 whereas the latter contain S-5 in normal concentrations (2, 8). These experiments provide direct evidence that the binding of S-5 is rate-limiting for the maturation of CAP-30S particles. Given this assumption, the results suggest that the binding of S-5 is required for stable binding of at least 12 mitochondrial small subunit ribosomal proteins. In previous work, analysis of the protein composition of CAP-30S particles showed that all of the small subunit ribosomal proteins could bind to some extent even in the absence of S-5 (reference 8). This finding suggests that the stabilized binding of small subunit proteins is due to the binding of S-5 and not to the binding of some other protein whose binding is in turn dependent on binding of S-5. Stabilized binding of small subunit ribosomal proteins could reflect either direct protein-protein interactions or a conformational change in the particles. Given the relatively large number of proteins involved, the second possibility seems more likely.

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REFERENCES

1. Boynton, J. E., N. W. Gillham, and A. M. Lambowitz. 1980. Biogenesis of chloroplast and mitochondrial ribosomes. In Ribosomes: Structure, Function and Genetics. G. Chambon, G. R. Crabbe, J. Davison, K. Davis, L. Kahan, and M. Nomura, editors. University Park Press, Baltimore, MD. 903-950.

2. Collins, R. A., H. Bertrand, R. J. LaPolla, and A. M. Lambowitz. 1979. Mitochondrial ribosome assembly in Neurospora crassa: mutants with defects in mitochondrial ribosome assembly. Mol. Gen. Genet. 177:73-84.

3. Collins, R. A., H. Bertrand, R. J. LaPolla, and A. M. Lambowitz. 1981. A novel extranuclear mutant of Neurospora with a temperature-sensitive defect in mitochondrial protein synthesis and mitochondrial ATPase. Mol. Gen. Genet. 181:13-19.

4. Cury, J.-J., R. Persson, E. Boissonneau, F. Iftode, N. Stelly, and J. André. 1981. The mitochondrial genes of a chloramphenicol-resistant cytoplasmic mutant of Tetrahymena pyriformis differ from those of the wild strain. Can. J. Genet. 24:121-130.

5. Grant, D. M., and A. M. Lambowitz. 1982. Mitochondrial ribosomal RNA genes. In The Cell Nucleus. H. Busch and L. Rothblum, editors. Academic Press, New York. In press.

6. Lambowitz, A. M. 1979. Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. LXI (Pt. G):621-633.

7. Lambowitz, A. M., N.-H. Chua, and D. J. L. Luck. 1976. Mitochondrial ribosome assembly in Neurospora. Preparation of mitochondrial ribosomal precursor particles, site of synthesis of mitochondrial ribosomal proteins, and studies on the "poky" mutant. J. Mol. Biol. 107:223-253.

8. Lambowitz, A. M., R. J. LaPolla, and R. A. Collins. 1979. Mitochondrial ribosome assembly in Neurospora. Two-dimensional gel electrophoretic analysis of mitochondrial ribosomal proteins. J. Cell Biol. 82:17-31.

9. LaPolla, R. J., and A. M. Lambowitz. 1977. Mitochondrial ribosome assembly in Neurospora crassa. Chloramphenicol inhibits the maturation of small ribosomal subunits. J. Mol. Biol. 116:189-205.

10. LaPolla, R. J., and A. M. Lambowitz. 1979. Binding of mitochondrial ribosomal proteins to a mitochondrial ribosomal precursor RNA containing a 2.3-kilobase intron. J. Biol. Chem. 254:11746-11750.

11. LaPolla, R. J., and A. M. Lambowitz. 1981. Mitochondrial ribosome assembly in Neurospora crassa. Purification of the mitochondrially synthesized ribosomal protein, S-5. J. Biol. Chem. 256:7064-7067.

12. LaPolla, R. J., and A. M. Lambowitz. 1981. Mitochondrial ribosome assembly in Neurospora. J. Cell Biol. 91(2):279a (Abstr.).

13. Maglott, D., and T. Stahlhelm. 1971. Fractionation of Escherichia coli 50S ribosomes into various protein-deficient cores and split proteins. J. Bacteriol. 106:406-417.

14. Mannella, C. A., R. A. Collins, M. R. Green, and A. M. Lambowitz. 1979. Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of Neurospora crassa. Proc. Natl. Acad. Sci. U. S. A. 76:2635-2639.

15. McGonkey, E. H. 1974. Composition of mammalian ribosomal subunits: a reevaluation. Proc. Natl. Acad. Sci. U. S. A. 71:1379-1383.

16. Metz, L. J., and L. Bogorad. 1974. Two-dimensional polyacrylamide gel electrophoresis: an improved method for ribosomal proteins. Anal. Biochem. 57:200-210.

17. Persano, R.-J.-J. Cury, F. Hodge, and J. André. 1980. Interactions between mitochondria and their cellular environment in a cytoplasmic mutant of Tetrahymena pyriformis resistant to chloramphenicol. In The Organization and Expression of the Mitochondrial Genome. A. M. Kroon, and C. Saccone, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 355-363.

18. Perry, R. P., and D. E. Kelley. 1966. Buoyant densities of cytoplasmic ribonucleoprotein particles of mammalian cells: distinctive character of ribosomal subunits and the rapidly labeled components. J. Mol. Biol. 16:255-268.

19. Pittenger, T. H., and D. J. West. 1976. Isolation and characterization of temperature-sensitive respiratory mutants of Paramecium tetraurelia. J. Mol. Biol. 107:709-720.

20. Pittenger, T. H., and D. J. West. 1979. Isolation and characterization of temperature-sensitive respiratory mutants of Paramecium tetraurelia. J. Mol. Biol. 126:129-142.

21. Terpstra, P., and R. A. Butow, 1979. The role of var1 in the assembly of yeast mitochondrial ribosomes. J. Biol. Chem. 254:12652-12669.

22. Terpstra, P., E. Zanders, and R. A. Butow. 1979. The association of var1 with the 38S mitochondrial ribosomal precursor RNA containing a 2.3-kilobase intron. J. Biol. Chem. 254:11746-11750.

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