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

ROBERT J. LAPOLLA and ALAN M. LAMBOWITZ

The Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104. Dr. LaPolla's present address is the Department of Chemistry, California Institute of Technology, Pasadena, CA 91125.

ABSTRACT In *Neurospora*, one protein associated with the mitochondrial small ribosomal subunit (S-5, M_r 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_r 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 mitochondrially synthesized, 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 raises the possibility that these proteins have some special role in mitochondrial ribosome assembly or protein synthesis.

Studies in our laboratory 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,

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 267-277

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 ([*C93*]) 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 5256*A* (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% (wt/vol) 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., Spinco 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 = 1.6003 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 = 1.6524 g/cm^3, upper solution = 1.5677 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 Mets 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 5256*A* 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 (ρ = 1.508 g/cm^3, ρ = 1.554 g/cm^3, and ρ = 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 ρ = 1.508 g/cm^3 (Fig. 1*a*). Two-dimensional gel analysis shows that the ρ = 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 ρ = 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 ρ = 1.508 g/

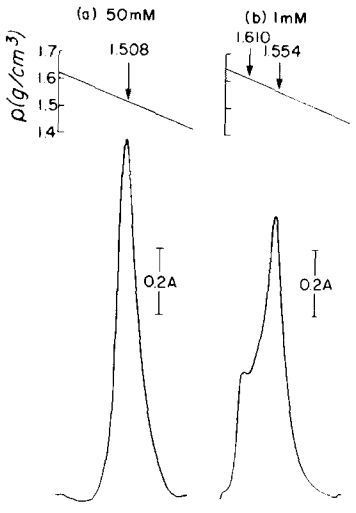


FIGURE 1 CsCl gradient profiles of core particles of mitochondrial small ribosomal subunits. Ribonucleoprotein particles were prepared from 2–4 l of Em5256*A* culture. CsCl gradients in (*a*) and (*b*) contained 50 mM and 1 mM $MgCl_2$, respectively. Profiles show A_{254}. Recoveries of core particles were determined in separate experiments using particles labeled in vivo with [^{32}P]-orthophosphoric acid. Recoveries were ρ = 1.508 g/cm^3, 40%; ρ = 1.554 g/cm^3, 20–30%; ρ = 1.610 g/cm^3, 10–20%. In repeats of the gradient shown in (*b*) the mass ratio of light (ρ = 1.554 g/cm^3) to dense (ρ = 1.610 g/cm^3) core particles varied from 7.59 to 0.62 (nine independent experiments). Densities are averages from at least four independent experiments (Table I).

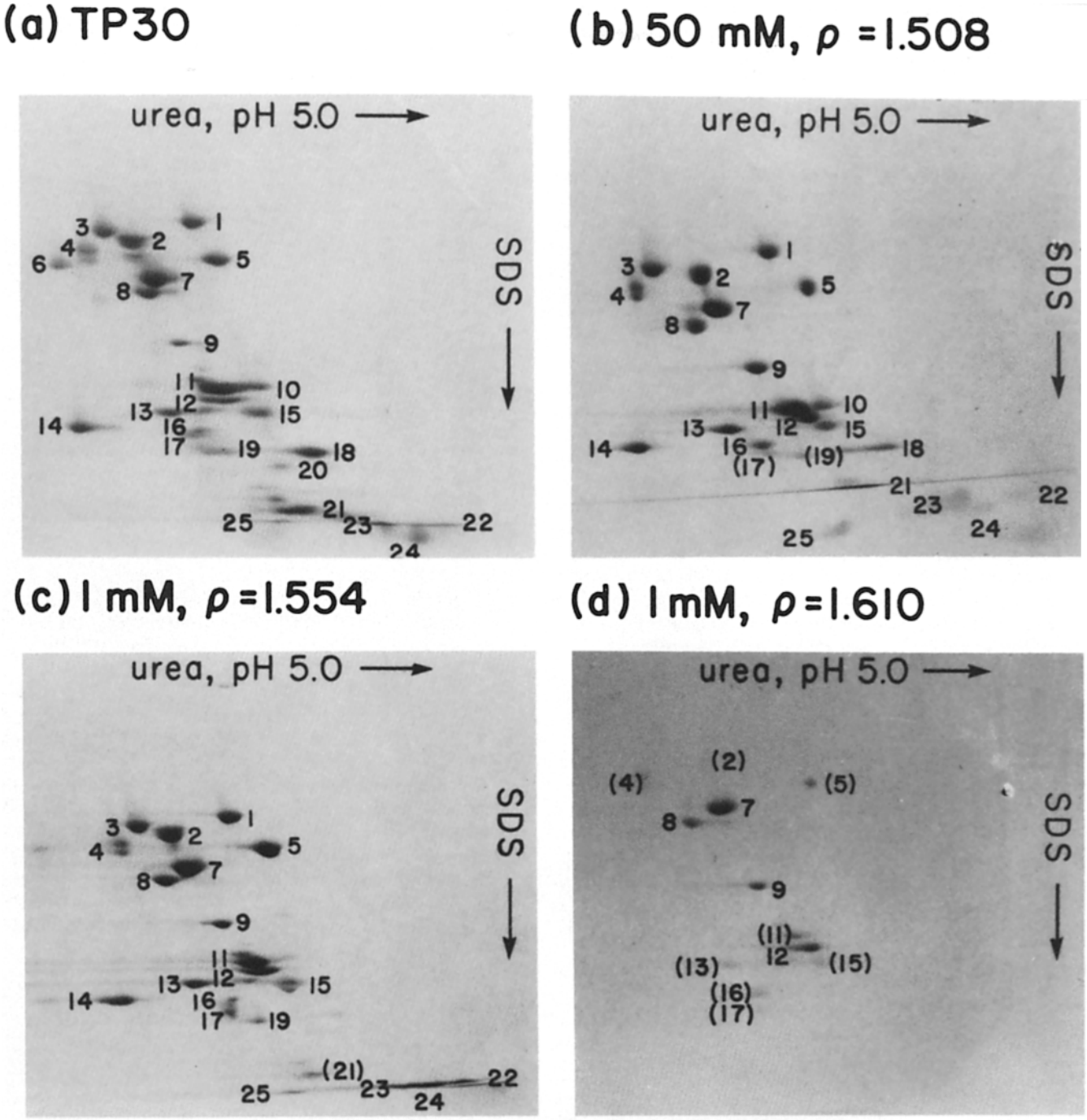


FIGURE 2 Two-dimensional gel electrophoresis of proteins from mitochondrial small ribosomal subunits and core particles. Core particles were prepared from 18–36 l of Em5256*A* culture. (*a*) Total protein, small ribosomal subunit (TP30). (*b*) ρ = 1.508 g/cm^3 particles from CsCl gradients containing 50 mM $MgCl_2$ (Fig. 1 *a*). (*c*) ρ = 1.554 g/cm^3 particles from CsCl gradients containing 1 mM $MgCl_2$ (Fig. 1 *b*). (*d*) ρ = 1.610 g/cm^3 particles from CsCl gradients containing 1 mM $MgCl_2$ (Fig. 1 *b*). 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. 2 *a* shows several additional proteins that appeared occasionally in the gel patterns.

cm^3 and the appearance of new peaks at ρ = 1.554 g/cm^3 and ρ = 1.610 g/cm^3 (Fig. 1 *b*). The ρ = 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. 2 *c*; Table I). The ρ = 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. 2 *d*; 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. 2*d*; Table I).

Core Particles of Mitochondrial Large Ribosomal Subunits

Five different species of large subunit core particles (ρ = 1.562 g/cm^3, ρ = 1.617 g/cm^3, ρ = 1.641 g/cm^3, ρ = 1.655 g/cm^3, and ρ = 1.733 g/cm^3) were identified (Figs. 3 and 4; Table II). At 50 mM Mg^{+2}, the gradient profiles show two peaks: ρ = 1.562 g/cm^3 and ρ = 1.617 g/cm^3 (Fig. 3*a*). The ρ = 1.562 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. 4*b*; Table II). The ρ = 1.617 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. 4*c*; Table II). At 25 mM Mg^{+2}, the ρ = 1.562 and ρ = 1.617 g/cm^3 peaks disappear and the gradients show a major peak at ρ = 1.641 g/cm^3 (Fig. 3*b*). There are only minor changes in protein composition compared to the ρ = 1.617 g/cm^3 particles (Fig. 4*c* and *d*; Table II). In this case, the density shift may in part reflect some change in the conformation of the particles (15). At 10 mM Mg^{+2}, the major peak shifts to ρ = 1.655 g/cm^3 (Fig. 3*c*) and one additional protein (L-16) was consistently absent (Fig. 4*e*; Table II). At 1 mM Mg^{+2}, the major peak is at ρ = 1.733 g/cm^3 (Fig. 3*d*). Again, there are relatively few changes in protein composition and only one additional protein (L-30) was consistently absent. Particles similar to the ρ = 1.733 g/cm^3 were formed in CsCl gradients containing 0.1 mM Mg^{+2} or 1 mM EDTA (data not shown). Calculations using the formula of Perry and Kelley (18) indicate that the differences in protein compositions among the ρ = 1.641, ρ = 1.655, and ρ = 1.733 g/cm^3 particles are not sufficient to account for the differences in density. We infer, therefore, that differences in the conformations of the particles must contribute to the density differences (15).

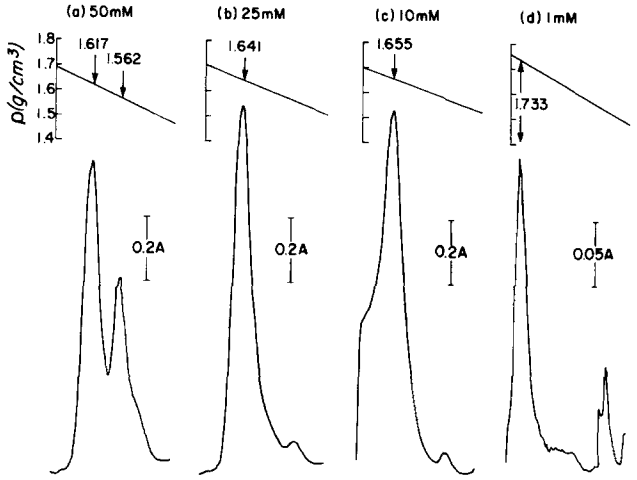


FIGURE 3 CsCl gradient profiles of core particles of mitochondrial large ribosomal subunits. Ribonucleoprotein particles were prepared from 2-8 l of Em5256*A* culture. CsCl gradients contained the following concentrations of $MgCl_2$: (*a*) *50 mM*, (*b*) *25 mM*, (c) *10 mM*, and (*d*) *1 mM*. Profiles show A_{254}. Recoveries of core particles were determined in separate experiments using particles that had been labeled in vivo with [^{32}P]orthophosphate. Recoveries were ρ = 1.562 g/cm^3, 10-20%, ρ = 1.617 g/cm^3, 30-40%, ρ = 1.641 g/cm^3, 50% ρ = 1.655 g/cm^3, 40%, ρ = 1.733 g/cm^3, <5%. In gradients similar to that shown in (*a*), the mass ratio of ρ = 1.562 to ρ = 1.617 g/cm^3 particles varied from 1.31 to 0.09 (12 independent determinations). Densities are averages from at least four independent experiments (Table II).

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	+/(+)	±	–

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, "–" 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. 2 a).

Core Particles of CAP-30S and INC-30S Particles

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 ([*C93*]) 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

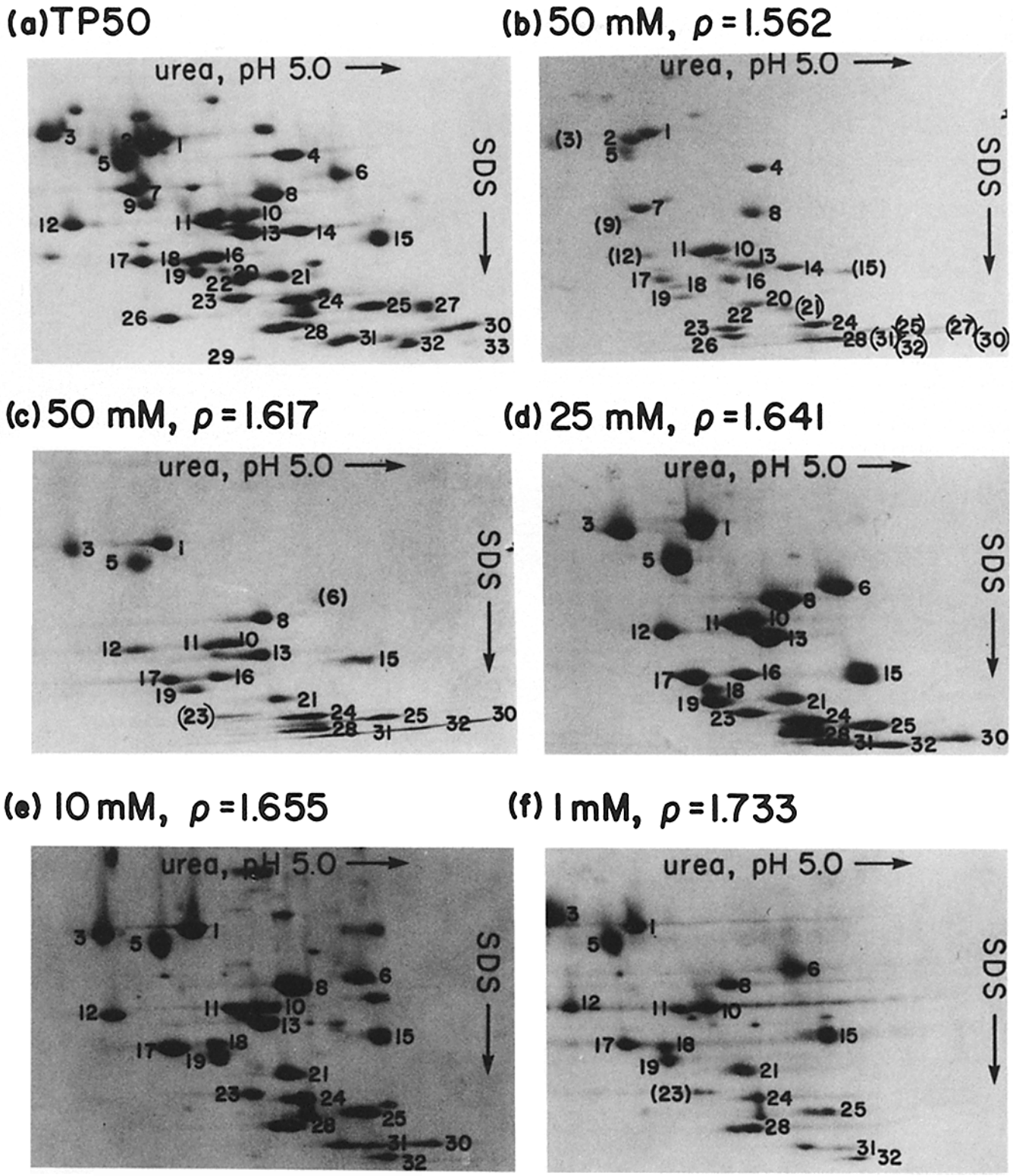


FIGURE 4 Two-dimensional gel electrophoresis of proteins from mitochondrial large ribosomal subunits and core particles. Core particles were prepared from 18 to 72 liters of Em5256*A* culture. (*a*) Total protein, large ribosomal subunit (TP50). The gel pattern shows a background of small subunit proteins 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

[Mg^{+2}]	50 mM	50 mM	25 mM	10 mM	1 mM
Density (g/cm^3)	1.562 ± 0.005	1.617 ± 0.008	1.641 ± 0.012	1.655 ± 0.012	1.733 ± 0.022
Determinations	3	6	7	5	4
Protein					
1	+	+	+	+	+
2	+	−	−	−	−
3	+/(+)	+/(+)	+/(+)	+	+
4	+	−	−	−	−
5	+	+	+	+	+
6	±	±	±	±	±
7	+	±	−	−	±
8	+	+	+	+	+
9	(+)	−	−	−	−
10	+	+	+	+	+
11	+	+/(+)	+/(+)	+	+
12	(+)	+	+/(+)	+/(+)	+/(+)
13	+	+	+	+	±
14	+	−	−	−	−
15	(+)	+/(+)	+/(+)	+	+/(+)
16	+	+	+/(+)	−	−
17	+	+	+	+	+
18	+/(+)	±	+/(+)	+	+
19	+/(+)	+/(+)	+/(+)	+	+/(+)
20, 22	+	−	−	−	−
21	+/(+)	+	+	+	+
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, "−" indicates a protein that was consistently absent. Stoichiometries were judged by Coomassie-Blue staining relative to standard two-dimensional gel patterns of large subunit proteins (e.g., Fig. 4 *a*). 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. 5 *a*). 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. 5 *b* 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. 5 *c*). These dense particles contain only five proteins (S-1, S-7, 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. 6 *a*; Table III). The S-5 protein is completely absent from these core particles (Fig. 6 *a*). 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

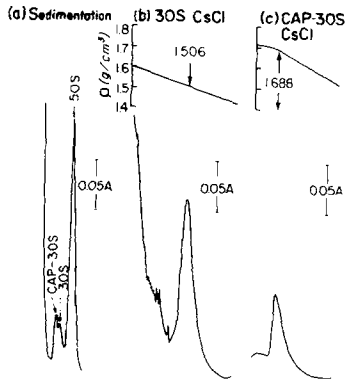


FIGURE 5 Analysis of mitochondrial ribonucleoprotein particles from chloramphenicol-treated wild-type cells. Mitochondrial ribonucleoprotein particles were prepared from 18 l of Em5256*A* grown for 17 h (25°C) in medium containing chloramphenicol (4 mg/ml). Ribonucleoprotein particles were centrifuged through linear gradients of 5–20% sucrose. Particles were collected from sucrose gradients and centrifuged through CsCl gradients containing 50 mM $MgCl_2$. Profiles show A_{254}. (*a*) Sucrose gradient profile. (*b*) Putative mature small subunits centrifuged through *CsCl* gradients. (*c*) *CAP-30S* particles centrifuged through *CsCl* gradients.

25 mM Mg^{+2}, give a particle whose density (ρ = 1.631 g/cm^3) and protein composition are essentially the same as that derived from standard large subunits (data not shown). The results provide additional evidence that chloramphenicol has no effect on the assembly of mitochondrial large subunits.

To supplement the analysis of CAP-30S particles, we next analyzed mitochondrial ribosomal particles from two nuclear mutants (289-56 and 299-9) that are deficient in mitochondrial protein synthesis. The use of mutants precludes artifacts that might arise from direct interaction between chloramphenicol and mitochondrial small ribosomal subunits or ribosomal proteins. The degree of inhibition of mitochondrial protein synthesis in the mutants is slightly greater than can be obtained with chloramphenicol (2).

Mutant 289-56 is indistinguishable from wild type when grown at 25°C, but is grossly deficient in mitochondrial small ribosomal subunits and mitochondrial protein synthesis when grown at 37°C (2). As in the case of chloramphenicol-treated wild-type cells, sucrose gradients of ribonucleoprotein particles from 37°C-grown 289-56 show two peaks in the 30S region, corresponding to mature and partially assembled small subunits (Fig. 7*a*). The particles were isolated separately from sucrose gradients and their protein compositions determined by two-dimensional gel electrophoresis. The more rapidly sedimenting particles, putative mature small subunits, have normal two-dimensional gel patterns (data not shown) whereas the more slowly sedimenting INC-30S particles, like CAP-30S particles, lack S-5 and are lacking or deficient in a number of other proteins (S-6, S-8, S-9, S-10, S-12, S-15, S-16, S-17, S-20, S-22, S-23, and S-24; Fig. 6*e*).

The INC-30S particles were centrifuged through CsCl gradients containing 50 mM Mg^{+2}, conditions in which the CAP-30S particles give a peak at ρ = 1.688 g/cm^3 that is skewed toward lower densities (Fig. 5*c*). The behavior of the INC-30S particles is very similar. The gradient profiles show a peak at ρ = 1.685 g/cm^3, with a distinct shoulder at 1.650 g/cm^3 (Fig. 7*c*). The protein compositions of the ρ = 1.685 and 1.650 g/cm^3 fractions were not determined separately because of the small amount of material and the extensive cross contamination that was anticipated on the basis of the absorbance profile. The two-dimensional gel pattern for the combined ρ = 1.685 and 1.650 g/cm^3 fractions shows 11 proteins (S-1, S-2, S-3, S-4, S-7, S-11, S-13, S-14, S-19, S-21, and S-25) that 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. 6*f*; Table III). The 11 major proteins are essentially the same group that remains associated with CAP-30S particles (Fig. 6*a*) 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 ρ = 1.508 g/cm^3 and the other at ρ = 1.574 g/cm^3 (Fig. 7*b*). The former has a density and protein composition equivalent to the core particle derived from wild-type small subunits (Fig. 6*c*; Table III). The latter (ρ = 1.574 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 ρ = 1.574 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-22, S-23, S-24, and S-25 (Fig. 6*d*; Table III). The formation of the ρ = 1.574 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 one 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 ρ = 1.624 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 ρ = 1.624 g/cm^3 particles is the same as that of the particle from wild type (data not shown).

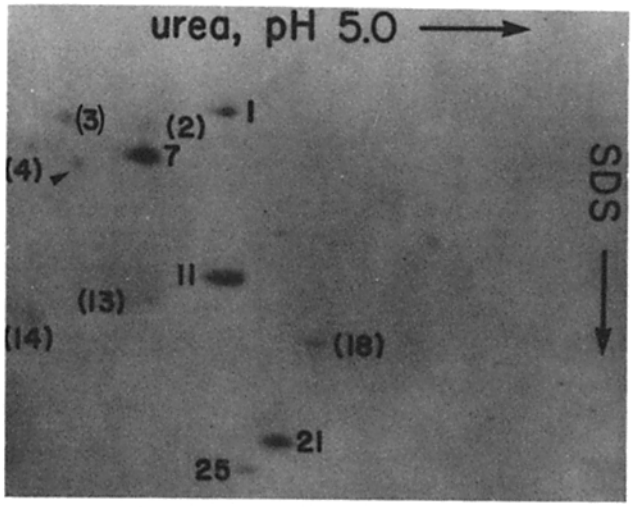
299-9 is a nuclear mutant with a temperature-sensitive defect in splicing the mitochondrial large rRNA (14). When grown at the nonpermissive temperature (37°C), the mutant is strongly deficient in mitochondrial protein synthesis and sucrose gradients of ribonucleoprotein particles show the expected two peaks in the 30S region due to putative mature small subunits and INC-30S particles (Fig. 8*a*). 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 ρ = 1.691 g/cm^3 with a shoulder at ρ = 1.646 g/cm^3 (Fig. 8*b*). The pooled ρ = 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-21, and S-25) in stoichiometric concentrations and four proteins (S-3, S-4, S-18, and S-19) that appear deficient (Fig. 6*b*; 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.

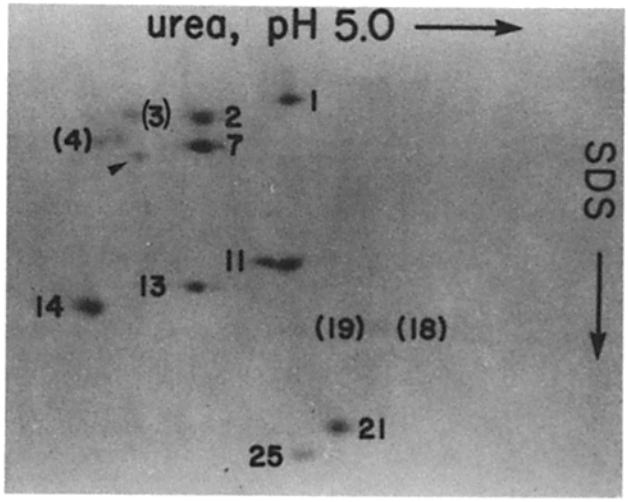
DISCUSSION

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

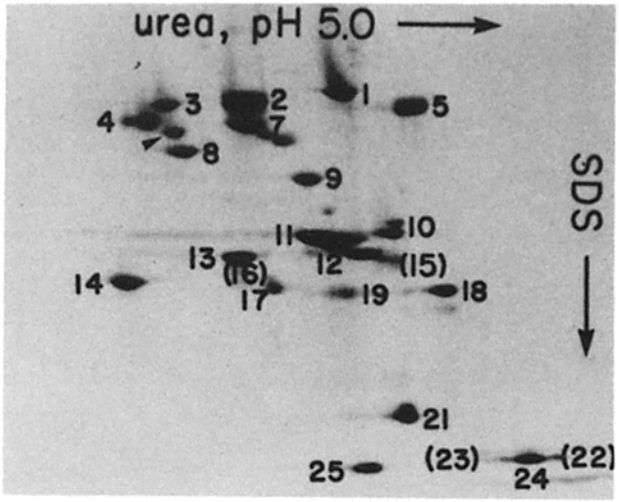
(a) 5256 + CAP, ρ = 1.688



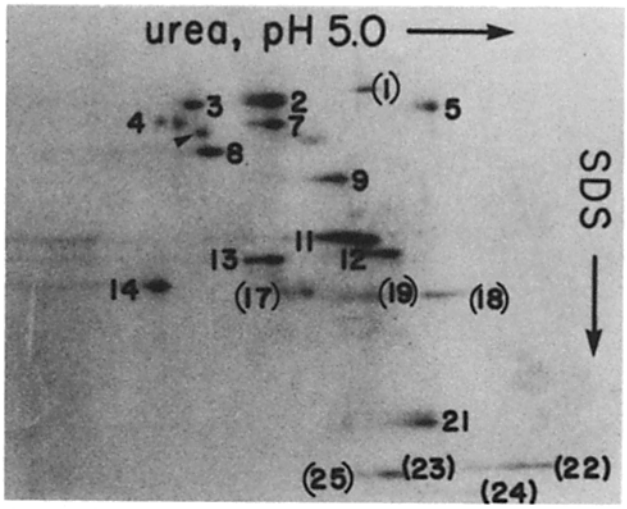
(b) 299-9, ρ = 1.691+1.646



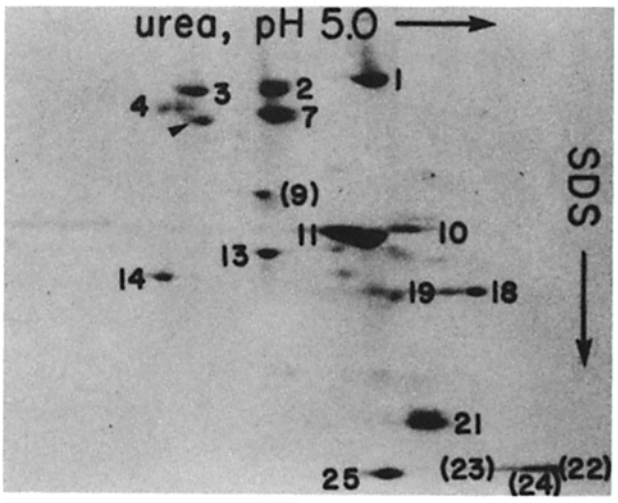
(c) 289-56, ρ = 1.508



(d) 289-56, ρ = 1.574



(e) 289-56, INC - 30S



(f) 289-56, ρ = 1.685+1.650

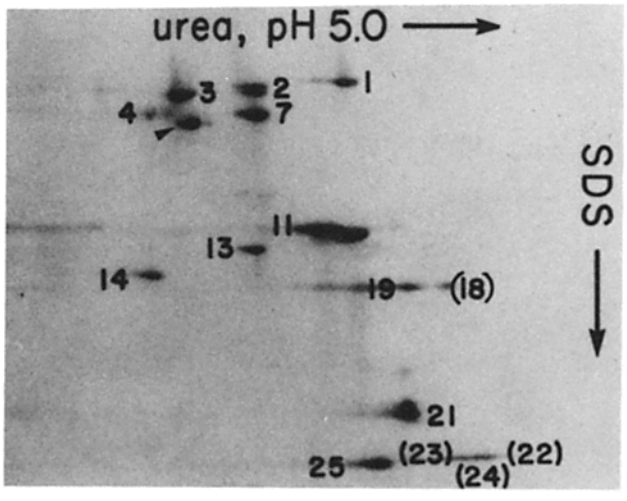


TABLE III

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

Strain	5256A + CAP	299-9	289-56	289-56	289-56
Density (g/cm^3)	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.

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

FIGURE 6 Protein composition of ribonucleoprotein particles from chloramphenicol-treated wild-type cells and mutants. CAP-30S particles were prepared from 21 l of Em5256*A* culture grown for 17 h (25°C) in low sulfate Vogel's minimal medium containing chloramphenicol (4 mg/ml) and 7.5 mCi of [^{35}S]sulfuric acid. *INC-30S* particles from mutant *299-9* were prepared from 21 l of culture grown for 24 h (37°C) in low sulfate Vogel's minimal medium containing 7.5 mCi [^{35}S]sulfuric acid. Putative mature small subunits and *INC-30S* particles from *289-56* were prepared from 34 l of culture grown for 24 h (37°C). Core particles were prepared by centrifugation in CsCl gradients containing 50 mM $MgCl_2$. Particles were dialyzed exhaustively against deionized, distilled water (4°C), and lyophilized. Proteins were extracted by the acetic acid procedure. ^{35}S-labeled proteins were combined with excess, unlabeled total small subunit protein prior to electrophoresis. (*a* and *b*) Autoradiograms. (*c–f*) Gels stained with Coomassie Blue. (*a*) ρ = 1.688 g/cm^3 particles from chloramphenicol-treated Em5256*A* (Fig. 5 *c*). (*b*) Pooled ρ = 1.691 and ρ = 1.646 g/cm^3 particles from *299-9* (Fig. 8 *b*). (*c*) ρ = 1.508 g/cm^3 particles from *289-56* (Fig. 7 *b*). (*d*) ρ = 1.574 g/cm^3 particles from *289-56* (Fig. 7 *b*). (*e*) *INC-30S* particles from *289-56* (Fig. 7 *a*). (*f*) pooled ρ = 1.685 and ρ = 1.650 g/cm^3 particles from *289-56* (Fig. 7 *c*). Parentheses indicate proteins present in less than stoichiometric amounts as judged by autoradiography or Coomassie-Blue staining relative to the appropriate standard two-dimensional gel pattern of small subunit proteins (i.e., Coomassie-Blue stained gel, Fig. 2 *a*; or autoradiogram, Fig. 9 *a* of reference 8). The arrows in Fig. 6 *a–f* indicate an unidentified protein that appeared occasionally in the gel patterns and that migrates near S-4.

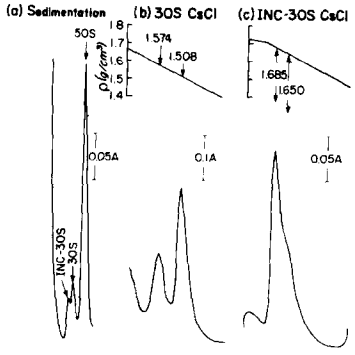


FIGURE 7 Analysis of mitochondrial ribonucleoprotein particles from 289-56. Mitochondrial ribosomal subunits were prepared from 24 l of culture grown for 24 h (37°C). Ribonucleoprotein particles were centrifuged through linear gradients of 5–20% sucrose. Particles were collected from sucrose gradients and centrifuged through CsCl gradients containing 50 mM $MgCl_2$. Profiles show A_{254}. (*a*) Sucrose gradient profile. (*b*) Putative mature small subunits centrifuged through *CsCl* gradients. (*c*) *INC-30S* particles centrifuged through *CsCl* gradients.

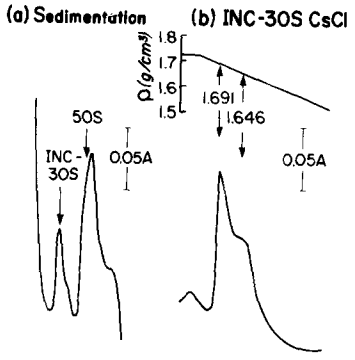


FIGURE 8 Analysis of mitochondrial ribonucleoprotein particles from 299-9. Mitochondrial ribonucleoprotein particles were prepared from 24 l of culture grown for 24 h (37°C). Ribonucleoprotein particles were centrifuged through linear gradients of 5–20% sucrose. Particles were collected from sucrose gradients and centrifuged through *CsCl* gradients containing 50 mM $MgCl_2$. Profiles show A_{254}. (*a*) Sucrose gradient profile. (*b*) *INC-30S* particles centrifuged through CsCl gradients.

subunits. Based on previous results, it seems likely that this tight binding reflects strong interaction between S–5 and the 19S rRNA. S–5 solubilized in urea has been shown to have a much higher affinity for RNA and cation exchange resins than do other mitochondrial ribosomal proteins (8, 11). For example, S–5 could not be eluted from carboxymethyl-Sepharose columns by solutions containing 1 M sodium acetate whereas all other mitochondrial ribosomal proteins were eluted by 0.38 M 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 ρ = 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 (ρ = 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 group of proteins following CsCl gradient centrifugation. Five of these proteins (S–1, S–7, S–11, S–21, and S–25) were consistently present in stoichiometric concentrations, whereas six other proteins (S–2, S–3, S–4, S–13, S–14, and S–19) were present in stoichiometric concentrations in some cases but deficient in others (Table III). We assume that the first group of proteins is associated with the predominant ρ = 1.685 to 1.691 g/cm^3 particles and that the second group is associated with the less dense ρ = 1.646 to 1.650 g/cm^3 particles that are present in variable concentrations. The behavior of CAP–30S and INC–30S particles in CsCl gradients is markedly different than that of mature small subunits. Under the same gradient conditions, the latter give rise to a ρ = 1.508 g/cm^3 particle that contains 17–23 proteins in stoichiometric concentrations. Considering the results for mutant 289–56 and using the criterion of stability to CsCl gradient centrifugation, we estimate that INC–30S particles contain a maximum of 12 proteins that are bound as tightly as in mature small subunits. These include the 11 proteins that remain associated with 289–56 INC–30S particles in stoichiometric concentrations and one additional protein (S–18) that is deficient but that is also deficient from core particles of mature small subunits. We emphasize that 12 is a maximum estimate and that a somewhat lower estimate can be obtained by using the result for CAP–30S particles. The remaining proteins are either already deficient from CAP–30S or 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 mitochondrially synthesized, 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.

We thank Mr. Kenneth Figlioli for technical assistance.

This work was supported by National Institutes of Health grant GM 23961.

Correspondence should be addressed to Dr. Lambowitz.

Received for publication 9 April 1982, and in revised form 2 July 1982.

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. Chambliss, G. R. Craven, J. Davies, 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. Curgy, J.-J., R. Perasso, E. Boissonneau, F. Iftode, N. Stelly, and J. André. 1981. The mitoribosomes of a chloramphenicol-resistant cytoplasmic mutant of *Tetrahymena pyriformis* differ from those of the wild strain. *Curr. Genet.* 4:121–130.
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.* LIX (Pt. G):421–433.
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, Pt.2):279a (*Abstr.*).
13. Maglott, D., and T. Staehelin. 1971. Fractionation of *Escherichia coli* 50S ribosomes into various protein-deficient cores and split protein fractions by CsCl density gradient centrifugation and reconstitution of active particles. *Methods Enzymol.* XX (Pt. C):408–417.
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. McConkey, E. H. 1974. Composition of mammalian ribosomal subunits: a reevaluation. *Proc. Natl. Acad. Sci. U. S. A.* 71:1379–1383.
16. Mets, L. J., and L. Bogorad. 1974. Two-dimensional polyacrylamide gel electrophoresis: an improved method for ribosomal proteins. *Anal. Biochem.* 57:200–210.
17. Perasso, R., J.-J. Curgy, F. Iftode, 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 ribosome subunits and the rapidly labeled components. *J. Mol. Biol.* 16:255–268.
19. Pittenger, T. H., and D. J. West. 1979. Isolation and characterization of temperature-sensitive respiratory mutants of *Neurospora crassa*. *Genetics.* 93:539–555.
20. Tait, A., J. K. C. Knowles, J. C. Hardy, and H. Lipps. 1976. The study of the genetic function of *Paramecium* mitochondrial DNA using species hybrids. *In* Genetics and Biogenesis of Chloroplasts and Mitochondria. Th. Bucher, W. Neupert, W. Sebald, and S. Werner, editors. North-Holland Publishing Co., Amsterdam. 569–572.
21. Terpstra, P., and R. A. Butow. 1979. The role of varl in the assembly of yeast mitochondrial ribosomes. *J. Biol. Chem.* 254:12662–12669.
22. Terpstra, P., E. Zanders, and R. A. Butow. 1979. The association of varl with the 38S mitochondrial ribosomal subunit in yeast. *J. Biol. Chem.* 254:12653–12661.