MITOCHONDRIAL RIBOSOME ASSEMBLY IN *NEUROSPORA*

Two-Dimensional Gel Electrophoretic Analysis of Mitochondrial Ribosomal Proteins

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ABSTRACT

Recent results with *Neurospora crassa* show that one protein (S-5, mol wt 52,000) associated with the mitochondrial (mit) small ribosomal subunit is translated within the mitochondria (Lambowitz et al. 1976. *J. Mol. Biol.* **107**:223–253). In the present work, *Neurospora* mit ribosomal proteins were analyzed by two-dimensional gel electrophoresis using a modification of the gel system of Mets and Bogorad. The results show that S-5 is present in near stoichiometric concentrations in high salt (0.5 M KCl)-washed mit small subunits from wild-type strains. S-5 is among the most basic mit ribosomal proteins (pI >10) and has a high affinity for RNA under the conditions of the urea-containing gel buffers.

The role of S-5 in mit ribosome assembly was investigated by an indirect method, making use of chloramphenicol to specifically inhibit mit protein synthesis. Chloramphenicol was found to rapidly inhibit the assembly of mit small subunits leading to the formation of CAP-30S particles which sediment slightly behind mature small subunits (LaPolla and Lambowitz. 1977. *J. Mol. Biol.* **116:** 189–205). Two-dimensional gel analysis shows that the more slowly sedimenting CAP-30S particles are deficient in S-5 and in several other proteins, whereas these proteins are present in normal concentrations in mature small subunits from the same cells. Because S-5 is the only mit ribosomal protein whose synthesis is directly inhibited by chloramphenicol, the results tentatively suggest that S-5 plays a role in the assembly of mit small subunits. In addition, the results are consistent with the idea that S-5 stabilizes the binding of several other mit small subunit proteins.

Two-dimensional gel electrophoresis was used to examine mit ribosomal proteins from [*poky*] and six additional extra-nuclear mutants with defects in the assembly of mit small subunits. The electrophoretic mobility of S-5 is not detectably altered in any of the mutants. However, [*poky*] mit small subunits are deficient in S-5 and also contain several other proteins in abnormally low or high concentrations. These and other results are consistent with a defect in a mit ribosomal constituent in [*poky*].

KEY WORDS mitochondrial ribosome · mitochondrial ribosomal proteins · extra-nuclear mutant · [*poky*] · *Neurospora crassa* · two-dimensional gel electrophoresis

Mitochondrial (mit)[1] ribosome assembly requires the participation of both the nuclear and mitochondrial genetic systems. In all organisms studied so far, mit rRNAs are transcribed from mit DNA whereas most of the mit ribosomal proteins are almost certainly nuclear gene products synthesized in the cytosol and transported into the mitochondria for assembly (7, 25). In the fungus *Neurospora crassa*, one protein (mol wt 52,000; designated S-5 in the present study[2]) associated with the mit small subunit has been found to be translated within the mitochondria (13). Kuriyama and Luck (10) provided evidence that the mature mit rRNA species (19S and 25S) are synthesized via a common 32S RNA precursor. At least 15 newly synthesized small and large subunit proteins appear to bind early, probably to high molecular weight precursor RNA(s), whereas the remaining proteins, including S-5, appear to be added at a later stage of mit ribosome assembly (13, 17).

A possible role of S-5 in mit ribosome assembly was inferred from the finding that chloramphenicol rapidly inhibits the maturation of mit small subunits in wild-type *Neurospora*, thus leading to the formation of CAP-30S particles which sediment slightly behind mature small subunits (17). The CAP-30S particles are enriched in a precursor of 19S RNA and deficient in several mit small subunit proteins. Because inhibition of assembly is relatively specific for the mit small subunit and because it is observed within 15 min after addition of chloramphenicol, it seems likely to be a direct result of inhibiting mit protein synthesis. Considered together, the results suggest that a mitochondrial translation product, presumably S-5, plays a role in the assembly of mit small subunits, in facilitating processing of 19S RNA, and in stabilizing the binding of other mit small subunit proteins (13, 17).

The present report describes continued studies on S-5 in *Neurospora*. At the outset, procedures were developed for two-dimensional gel electrophoresis of *Neurospora* mit ribosomal proteins. These procedures were then applied to examine the relationship of S-5 to the maturation of CAP-30S particles and to look for mutational alterations in S-5 in extra-nuclear, small subunit-deficient mutants. The results give additional insight into the properties of S-5 in wild-type and mutant strains.

[1] *Abbreviations used in this paper:* mit, mitochondrial; TEMED, N,N,N′,N′-tetramethylethylenediamine.

[2] This protein was previously designated S-4a (13). The new nomenclature is based on the position of the protein in the two-dimensional gel system.

MATERIALS AND METHODS

Materials

Wild-type strains were 74-OR23-1*A* (FGSC No. 987), abbreviated 74*A*, and Em 5256*A* (FGSC No. 626). [*Poky*] (13-6), obtained from D. J. L. Luck (The Rockefeller University, New York) has the nuclear background of RL3-8A. The other extra-nuclear mutants have been described by Bertrand and Pittenger (2): [*exn-1*] (N4-9-4), [*exn-2*] (X-21), [*exn-4*] (P85), [*stpB1*] (17-2a-1), [*SG-1*] (RL3202-23), [*SG-3*] (RL3120-10). The mutations were transferred by heterokaryosis into strains with nuclear backgrounds derived from 74*A*.

Procedures for maintaining strains and preparing conidia have been described by Luck (20). Heterotrophic strains were maintained by vegetative growth on slants of Vogel's minimal medium plus 2% (wt/vol) sucrose (27). Vitamin-requiring strains were grown on minimal medium supplemented with 10 μg pantothenic acid/ml and 10 μg nicotinamide/ml.

Sucrose was density gradient grade (ribonuclease-free; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.J.). Urea and Tris were ultrapure grade (Schwarz/Mann). All other chemicals were reagent grade.

Growth of Mycelia and Purification of Mitochondria

Mycelia were grown in 800-ml or 1,600-ml liquid cultures contained in 2- or 4-l Ehrlenmeyer flasks. 5–10 l of culture were required to obtain material for each two-dimensional gel. Cultures were inoculated with 7×10^5 conidia/ml and shaken at 200 rotary reciprocations/min (25°C). Mycelia were harvested in mid- to late-log phase, 14-h cultures for wild-type strains and 24-h cultures for extra-nuclear mutants. The mycelia were disrupted by grinding with sand and mitochondria were isolated by the flotation gradient method (18; with modifications described by Lambowitz [12]).

Labeling with Radioactive Precursors

L[4,5-^{3}H]leucine (40 Ci/mmol) and [5-^{3}H]uridine (8 Ci/mmol) were obtained from Schwarz/Mann. [^{35}S]sulfuric acid (carrier-free) and [^{32}P]phosphoric acid (carrier-free) were obtained from New England Nuclear (Boston, Mass.). Labeling with L[4,5-^{3}H]leucine and [5-^{3}H]uridine

was carried out in standard Vogel's medium. Labeling with [^{35}S]sulfuric acid was carried out in Vogel's medium containing 32 μM $MgSO_4$ (1/25 the normal concentration of $MgSO_4$) but supplemented with $MgCl_2$ to the normal Mg^{+2} concentration. Labeling with [^{32}P]phosphoric acid was carried out in modified Vogel's minimal medium of low phosphate concentration (0.2–1 mg KH_2PO_4/ml) (15).

Extraction and Analysis of Mit RNA

RNA was extracted from whole mitochondria using the SDS-diethylpyrocarbonate procedure (18, 26). Yeast tRNA (Sigma Chemical Co., St. Louis, Mo.) was added as carrier so that the final RNA concentration was ~1.0 OD_{260} U/ml. Electrophoretic analysis of RNAs was carried out as described by Peacock and Dingman (22) on 6-mm thick gels containing 2.4% acrylamide/0.8% agarose in 30 mM NaH_2PO_4, 3 mM EDTA, 0.05% SDS, and 40 mM Tris·HCl, pH 7.9. The gels were run at 140 V (20°C) until the bromophenol blue marker had migrated two-thirds of the way through the gels. Gels were dried by suction on Whatman 3 MM paper (Whatman, Inc., Clifton, N.J.) and autoradiographs were obtained with RP X-Omat Film (Kodak).

Preparation of Mit Ribosomal Subunits

Mit ribonucleoprotein pellets were prepared in high salt, Ca^{+2}-containing media as described previously (12, 15). Monomer pellets were dissolved in $HKMTD_{500/25}$ buffer (500 mM KCl, 25 mM $MgCl_2$, 25 mM Tris·HCl, pH 7.5, 5 mM dithiothreitol) and dissociated into subunits by incubation in 1 mM puromycin (adjusted to pH 7.6) for 15 min at 35°C (3). 0.3- to 0.4-ml aliquots containing a maximum of 5 OD_{260} U were layered over linear gradients of 5–20% sucrose containing $HKMTD_{500/25}$ and centrifuged in a Beckman SW 41 rotor (Beckman Instruments, Inc., Spinco Div., Fullerton, Calif.) (40,000 rpm, 3 h, 3°C). Gradients were fractionated by monitoring absorbance at 254 nm with an ISCO density gradient fractionator (ISCO [Instrumentation Specialties Co.], Lincoln, Nebr.). Pooled subunit fractions were diluted with $HKMTD_{500/25}$ and the subunits were pelleted by overnight centrifugation in a Beckman Ti50 rotor (50,000 rpm, 3°C). To determine the distribution of radioactivity in the gradients, 0.3-ml fractions were collected into scintillation vials, diluted by addition of 0.5 ml of water, and counted in 10 ml of a xylene-based scintillation fluid containing 5% (wt/vol) butyl-PBD and 30% (vol/vol) Triton X-114 (1).

One-Dimensional Gel Electrophoresis

One-dimensional gel electrophoresis of mit ribosomal proteins was carried out using SDS gels containing a linear gradient of 7.5–15% polyacrylamide (6, 13).

Two-Dimensional Gel Electrophoresis

Mit ribosomal proteins were extracted using the acetic acid procedure (9) and analyzed by two-dimensional gel electrophoresis using a modification of the system of Mets and Bogorad (21). Mit ribosomal subunits from pooled gradient fractions were collected by overnight centrifugation (Beckman Ti 50 rotor, 50,000 rpm, 16 h, 3°C). Subunit pellets containing 2–10 OD_{260} U were rinsed and then resuspended in 100 μl of cold, distilled water. In later experiments, 5 μl of 1 M Tris·HCl, pH 7.5, were added and the subunits were treated with ribonuclease (0.8 U Sigma RNase A Type XII-A and 24 U Sigma RNase T_1, Grade III) for 30 min at 25°C. The suspension was cooled to 3°C and proteins were extracted by addition of 0.5 ml of a mixture containing 80% (vol/vol) acetic acid, 40 mM Mg acetate, and 4 mM Tris·HCl, pH 7.5. The suspension was drawn up and down in a Pasteur pipet ~20 times, and then the RNA residue was pelleted in a Sorvall SS 34 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) (10,000 rpm, 15 min, 3°C). The protein-containing supernate was recovered using a Pasteur pipet with the tip drawn to a fine capillary to exclude clumps. The residue was reextracted, the supernates were pooled, and the proteins were precipitated by addition of 8 vol of cold acetone. After overnight incubation at −20°C, the precipitated proteins were pelleted at 3,000 rpm (15 min, 3°C) in an International Model K centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). The protein pellet was washed twice with cold acetone to remove residual acetic acid. It was then dried under a stream of filtered air and finally dissolved in 40 μl of urea sample buffer containing 8 M urea, 10 mM bisTris, 7 mM mercaptoethanol, 10 mM dithiothreitol, adjusted to pH 4.0 with acetic acid. Control experiments showed that the extraction procedure yields >85% of the ^{35}S-labeled protein initially present in the mit ribosomal pellets. Less than 1% of the radioactivity was found in either the RNA pellet or the acetone supernate. Electrophoretic analysis showed that the acetone supernate did not retain specific proteins but that one protein (mol wt ~45,000) was enriched in the RNA pellet.

Gel loads contained at least 100 μg of protein as estimated from the amount of subunits present at the beginning of the extraction. Electrophoresis in the first dimension was carried out on thin slab gels (19 cm × 15 cm × 0.8 mm) with 1.5-cm slots. The gels contained 8 M urea, 4% acrylamide, 0.1% bisacrylamide, and 57 mM bisTris adjusted to pH 5.0 with acetic acid. Polymerization was induced by addition of N,N,N′,N′,-tetramethylethylenediamine (TEMED) (3 μl/ml) and ammonium persulfate (0.3 mg/ml). The upper buffer was 10 mM bisTris adjusted to pH 4.0 with acetic acid and the lower buffer was 0.18 M potassium acetate, adjusted to pH 5.0 with acetic acid. Electrophoresis was toward the cathode at a constant current of 35 mA for ~5.5 h until the pyronine Y tracking dye had migrated 1.3 × through the

gel. Slots from the first dimension were cut out, rinsed with transfer buffer (55 mM Tris·SO_4, pH 6.1), placed over the second-dimension gels, rinsed again with transfer buffer, and then electrophoresed without additional equilibration. Second dimensions were the same SDS-polyacrylamide gradient gels which were used for one-dimensional gel analysis. The gel dimensions were 34 cm × 25 cm × 1 mm so that two first-dimension gels could fit over a single second-dimension gel. Electrophoresis was toward the anode at constant current of 40 mA until the bromophenol blue tracking dye had reached the bottom of the gels. Gels were stained with Coomassie brilliant blue and dried by suction on Whatman 3 MM filter paper. For determination of radioactivity, the spots were cut from the dried gels, placed into scintillation vials and incubated with 0.5 ml of 20% (vol/vol) H_2O_2 overnight at 55°C. Samples were counted in 10 ml of the xylene-based scintillation fluid described above (1).

RESULTS

Two-Dimensional Gel Electrophoresis of Mit Ribosomal Proteins

Figs. 1 and 2 show two-dimensional gels and maps of mit ribosomal proteins from wild-type strains Em 5256*A* and 74*A*. Table I lists the proteins by molecular weight and also attempts to correlate proteins resolved in the two-dimensional gel system with those resolved in the previous one-dimensional system. The patterns show that the minimum numbers of mit ribosomal proteins are 25 for the small subunit and 34 for the large subunit compared to 23 and 30 resolved previously using the one-dimensional gel system (13). The large subunit patterns show a background of small subunit proteins presumably reflecting aggregation of small subunits on sucrose gradients. The background could be reduced by more restrictive fractionation of the gradients, but loss of material made such fractionation impractical for routine two-dimensional gel analysis. It should be noted that the identification of the large subunit proteins is confirmed by patterns for mit small subunit-deficient mutants (see below) and that there are relatively few overlaps between the mit small and large subunit proteins (Fig. 2*B*). Table I shows that the *Neurospora* mit ribosomal proteins range in mol wt from 15,000 to 60,000 and that the largest proteins are found in the mit small subunit.

The patterns for the two wild-type strains were compared to assess the frequency of mit ribosomal protein polymorphisms as a prelude to the analysis of mutants derived from these strains. To maximize sensitivity, the comparisons included mixing experiments in which unlabeled 74*A* and ^{35}S-labeled Em 5256*A* mit ribosomal proteins were co-isolated in proportions such that the Coomassie blue stain showed only proteins from 74*A* and the autoradiograms only proteins from Em 5256*A*. The autoradiograms and staining patterns were superimposable except for one protein, L-11, which appeared to be more basic in Em 5256*A* than in 74*A* (indicated by the arrows in Fig. 1 *B* and *D*).

Site of Synthesis of the Mit Ribosomal Proteins

In the previous work, the site of synthesis of the mit ribosomal proteins was investigated in double-label experiments in which cells were mass-labeled with [^{14}C]leucine and then pulse-labeled with [^{3}H]leucine in the presence of an inhibitor of either cytosolic or mit protein synthesis (13). One protein (mol wt 52,000) associated with the mit small ribosomal subunit was found to be synthesized within the mitochondria. In separate experiments, the synthesis of this protein was shown to be resistant to anisomycin, an inhibitor of cytosolic protein synthesis, and specifically inhibited by chloramphenicol, an inhibitor of mitochondrial protein synthesis (13). To locate this protein in the two-dimensional gel patterns, wild-type cells were treated with cycloheximide to inhibit cytosolic protein synthesis and then pulse-labeled with L[4,5-^{3}H]leucine for 30 min. However, because of the paucity of radioactivity that could be recovered after two-dimensional separation, the previous labeling protocol had to be modified in two respects: (*a*) log phase wild-type cells were concentrated 10-fold before pulse-labeling, and (*b*) the [^{14}C]leucine mass label used in the previous study was omitted to facilitate counting of the relatively small amount of ^{3}H-radioactivity that could be recovered. Omission of the ^{14}C-mass label is justified by the previous work which showed that only one protein in the appropriate molecular weight range incorporates substantial L[4,5-^{3}H]leucine radioactivity when cytosolic protein synthesis is inhibited (13).

Fig. 3 shows the results of two experiments. In the first, radioactivity was incorporated predominantly into a single protein, S-5, on the gels (Fig. 3*A*; protein indicated by arrows in Fig. 1 *A* and *C*). In the second, radioactivity was again found in S-5, but also in a parallel spot at the origin of the first-dimension gels (Fig. 3*B*; indicated by

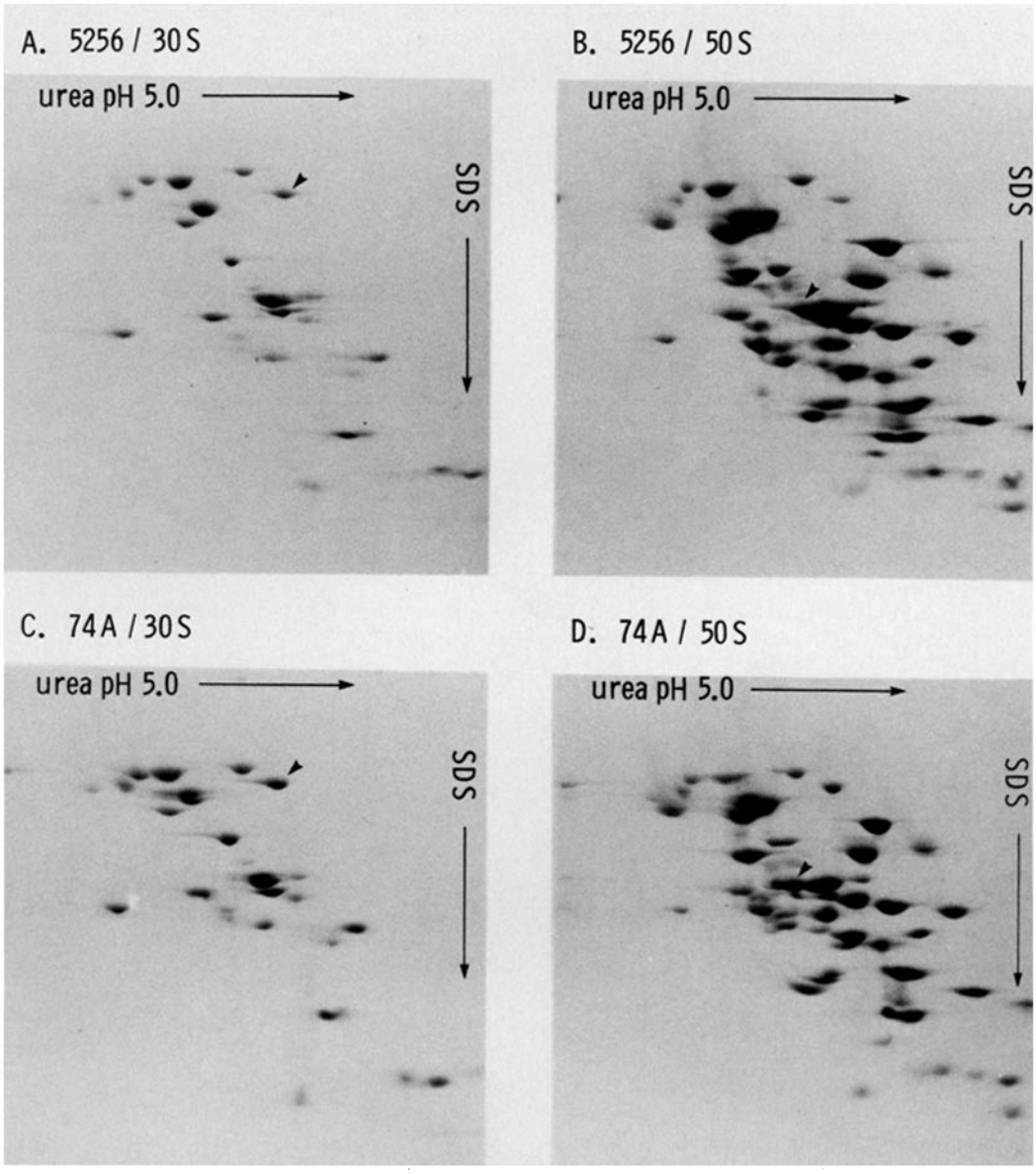

FIGURE 1 Two-dimensional gel electrophoresis of proteins from mit ribosomal subunits. (*A*) Em 5256*A*, 30S; (*B*) Em 5256*A*, 50S; (*C*) 74*A*, 30S; (*D*) 74*A*, 50S. Small arrows in (*A*) and (*C*) point to S-5; small arrows in (*B*) and (*D*) point to L-11. The direction of electrophoresis is indicated by the outside arrows.

arrow in Fig. 4*A*). Because many other experiments showed an inverse correlation in the appearance of these two spots, the results suggest that radioactivity is incorporated into one protein, S-5, some variable proportion of which does not enter the first-dimension gels. As seen in Fig. 1*A*, S-5 is a major protein, roughly stoichiometric with other mit ribosomal proteins as judged by Coomassie blue staining (and in other experiments by incorporation of [^{35}S]sulfate mass-label). The same stoichiometry was observed in >20 experiments with wild-type strains. The high mobility of S-5 in the first dimension places it among the most basic mit ribosomal proteins, probably with a pI >10 by

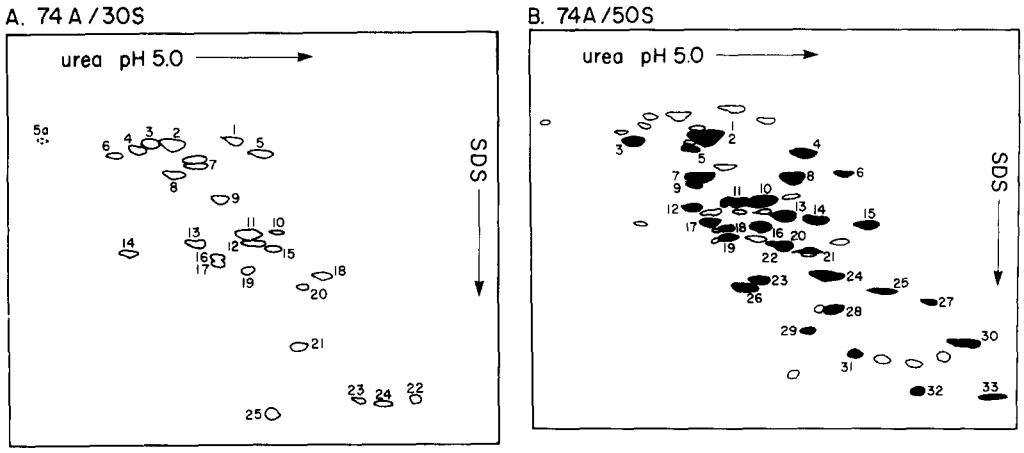

FIGURE 2 Maps of (*A*) small subunit and (*B*) large subunit mit ribosomal proteins. Spots were traced from Fig. 1 *C* and *D*. The numbered spots correspond to major proteins resolved reproducibly in many experiments. The unfilled spots in Fig. 2 *B* correspond to small subunit proteins (see text). To improve resolution of the majority of proteins, the first dimension was over-run in some experiments, resulting in the loss of low molecular weight basic proteins S-22, L-30, L-33, and L-34 (see Fig. 1). Spots for certain proteins (e.g., S-7, and S-11 in Fig. 1 *C*) were occasionally "split" in the second dimension, an effect attributable to interaction with boric acid present in the upper buffer (5). Spot S-5a (Fig. 2 *A*) corresponds to S-5 retained at the origin of the first dimension gels.

comparison with the measured pI's of other mit small subunit proteins (data of Lizardi and Luck [19]).

Binding of S-5 to RNA

Surprisingly, the retention of S-5 at the origin of the first-dimension gels was found to reflect binding to residual RNA fragments in protein preparations. As shown in Fig. 4, the entry of S-5 into the gels was promoted by treating ribosomal subunits with ribonuclease before acetic acid extraction, whereas other treatments such as heating the extracted proteins in the urea sample buffer to 65°C in the presence or absence of 20% Nonidet had no effect. Complementary experiments showed that addition of 0.2 OD_{260} U of *Escherichia coli* 16S + 23S RNA to gel samples caused S-5 and seven other proteins to be retained at the first-dimension origin whereas the remaining proteins migrated normally in the gels. Additional proteins were retained as the RNA concentration was increased.

In vitro binding experiments were carried out to determine whether mit ribosomal proteins could bind specifically to RNA in urea-containing buffers. Pooled small and large subunit proteins (~150 μg dissolved in 0.5 ml of urea sample buffer; see Materials and Methods) were incubated (30 min, 25°C) with 3–6 OD_{260} U of purified 19S RNA, 25S RNA, or phage Qβ RNA. The samples were centrifuged at low speed (Sorvall SS-34 rotor, 10,000 rpm, 20 min, 20°C), the supernates recovered, layered over a 6-ml cushion of 15% sucrose in urea sample buffer, and centrifuged at high speed (Beckman 50 Ti rotor, 50,000 rpm, 16 h, 20°C). All three RNAs caused the formation of aggregates which pelleted during the low-speed spin. One-dimensional gel analysis showed that each of the three RNAs bound almost identical mixtures of small and large subunit proteins, suggesting that mit ribosomal proteins bind nonspecifically to RNA under these conditions. (The high-speed supernate contained >50% of the initial protein, and the high-speed pellet <10% of the initial protein as judged by recovery of ^{35}S-radioactivity.) Considered together, the results suggest that S-5 is retained at the origin of the first-dimension gels by nonspecific binding to RNA fragments. The finding that S-5 is often the predominant protein retained at the origin suggests that it has a high affinity for RNA under the urea gel conditions. In subsequent experiments, mit ribosomal subunits were treated with ribonuclease before acetic acid extraction to promote entry of S-5 into the gels.

TABLE I

Neurospora Mit Ribosomal Proteins

Small Subunit			Large Subunit		
Protein	mol wt × 10^{-3}	Correlation with one-dimensional gel	Protein	mol wt × 10^{-3}	Correlation with one-dimensional gel
1	59.3	1, 3	1	49.0	1
2	57.5	2	2	46.5	2
3	56.5	1, 3	3	46.5	2
4	52	4b	4	45.4	3, 4
5	52	4a	5	41.0	4, 5
6	48.0	5	6	37.2	6
7	47.5	6	7	35.8	7
8	44.5	7	8	34.0	8
9	35.4	8	9	34.0	8
10	30.5	9	10	29.8	9, 10
11	29.8	10, 11	11	28.9	10
12	29.7	10, 11, 12	12	28.9	10
13	29.4	11, 12	13	28.8	9, 10
14	28.8	12, 13, 14	14	27.3	9, 10
15	27.5	13, 14, 15	15	26.8	9, 10, 10a, 11
16	27.5	15, 16	16	24.8	12, 13
17	25.8	16, 17, 18	17	24.5	13, 14
18	24.2	18	18	24.4	14, 15
19	22.7	18, 19	19	23.8	16, 17
20	22.0	20	20	22.6	15, 16, 17
21	18.2	21	21	22.1	15, 16, 17
22	17.5	22	22	22.1	16, 17
23	17.4	22	23	20.3	18
24	17.4	22	24	19.8	19
25	17.2	23	25	19.3	20
			26	19.0	21
			27	19.0	22
			28	18.5	23
			29	18.3	24, 25, 26
			30	17.4	26, 27
			31	16.8	28
			32	16.2	29
			33	16.0	30
			34	16.0	30

Neurospora mit ribosomal proteins resolved by two-dimensional gel electrophoresis. The relationship between electrophoretic mobilities and molecular weights was established with the following markers run in parallel slots in one-dimensional gels: bovine serum albumin (68,000), ovalbumin (43,000), DNase (31,000), chymotrypsin (22,600), and myoglobin (17,800). The nomenclature for the one-dimensional gel system is that established in reference 13.

The Effect of Chloramphenicol

After we developed techniques to study S-5 by two-dimensional gel electrophoresis, our next objective was to examine the relationship of S-5 to abnormal mit ribosome assembly in chloramphenicol-treated wild-type cells and in extra-nuclear, mit small subunit-deficient mutants. The previous work had shown that chloramphenicol rapidly inhibits the maturation of mit small subunits in wild-type *Neurospora* leading to the formation of CAP-30S particles which usually sediment behind mature small subunits (17). After prolonged incubation in chloramphenicol, the residual 30S peak was often found to be split, thus suggesting the presence of both CAP-30S particles and mature mit small subunits, the formation of the latter being consistent with the observation that chloramphenicol inhibits only 65–90% of mit protein synthesis in *Neurospora* (19). Again, in the previous work, one-dimensional gel electrophoresis

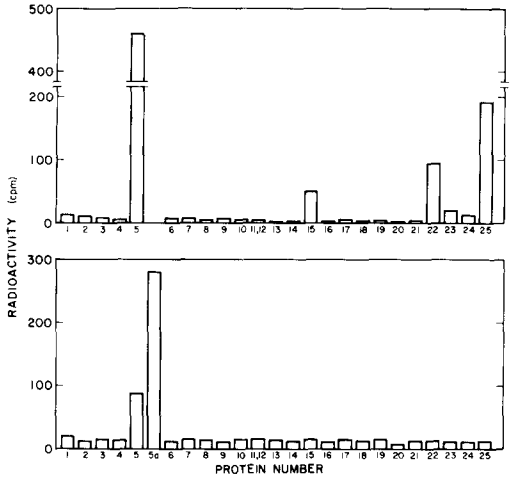

FIGURE 3 Incorporation of L-[4,5-^{3}H]leucine into mit small subunit ribosomal proteins in the presence of cycloheximide. (*A*) and (*B*) show results for two independent experiments. 8.0 l of wild-type (Em 5256*A*) cells were grown for 14 h at 25°C. The cells were then harvested under sterile conditions, resuspended in 800 ml of fresh culture medium, and incubated for a 15-min equilibration period. Cycloheximide was added as a powder to a final concentration of 0.6 mg/ml, and 2 min later the cells were pulse-labeled with 10 mCi L-[4,5-^{3}H]leucine for 30 min. Mit ribosomal proteins were separated by two-dimensional gel electrophoresis and the radioactivity was determined in each spot as described in Methods. The counts associated with S-22 and S-25 in Fig. 3*A* are not reproducible; they may reflect completion of low molecular weight cytosolically synthesized proteins in the presence of cycloheximide in this experiment. S-5a refers to S-5 retained at the origin of the first dimension gels.

was used to determine the protein composition of the combined 30S peak (17), an approach adopted for routine analysis because the two particles occur in low concentrations and are not always well resolved on sucrose gradients. The results suggested that CAP-30S particles are deficient in several proteins including S-5, but the stoichiometry of several proteins was variable in different experiments (17).

To more precisely define the protein composition of the CAP-30S particles, CAP-30S particles and mature mit small subunits were isolated separately from the same gradients and their proteins were analyzed by two-dimensional gel electrophoresis. This type of experiment could not be carried out routinely for the reasons cited above; nevertheless, it seemed essential to carry out at least one such experiment. The results in Fig. 5 strikingly show that the more slowly sedimenting CAP-30S particles are deficient in S-5 and several other proteins (e.g., S-8 and S-9; Fig. 5 *B*) whereas mature mit small subunits from the same gradients contain all proteins in nearly normal concentrations (Fig. 5 *C*). (The gel pattern for mature small subunits shows a background of large subunit proteins because of the position of the "cuts" during sucrose gradient fractionation.) Results consistent with these were obtained in one additional experiment in which the protein composition of the separated particles was determined by one-dimensional gel electrophoresis. In both experiments, no alteration could be detected in the gel patterns for large subunit proteins (data not shown), essentially confirming previous findings (17).

To systematically identify the proteins deficient in CAP-30S particles in the experiment of Fig. 5, we labeled the proteins in vivo with [^{35}S]sulfate (10 mCi) and measured the radioactivity in each spot. Stoichiometry was then determined as radioactivity relative to S-1. The yield of proteins from CAP-30S particles is low, and only ~100 cpm were recovered in each spot. For this reason, deficient proteins were defined as those which contained <50% normal amounts of radioactivity and which also appeared deficient on the basis of the Coomassie blue-staining pattern (Fig. 5). By these criteria, S-5 and at least six other proteins (S-8, S-9, S-12, S-13, S-14, and S-19) are deficient in CAP-30S particles (Fig. 5 *B*). Quantitation of ^{35}S-label, even taking into account the small amount of S-5 which may be present at the origin of the first dimension gels (Fig. 5 *B*), shows that the concentration of S-5 is <25% that found in mature mit small subunits. Moreover, the magnitude of the protein deficiencies may be underestimated because of contamination of the CAP-30S fraction by mature mit small subunits (Fig. 5 *A*).

To assess the reproducibility of the protein composition of CAP-30S particles, we carried out >10 additional experiments in which wild-type cells were grown in chloramphenicol for 17 h and the proteins of the combined 30S peak were analyzed by one- or two-dimensional gel electrophoresis. These experiments showed that aberrant mit small subunits deficient in S-5 are consistently formed in the presence of chloramphenicol, but that their exact protein composition and sedimentation rate can vary in different experiments. Physiological parameters such as growth temperature (between 20° and 30°C), degree of aeration, and age of

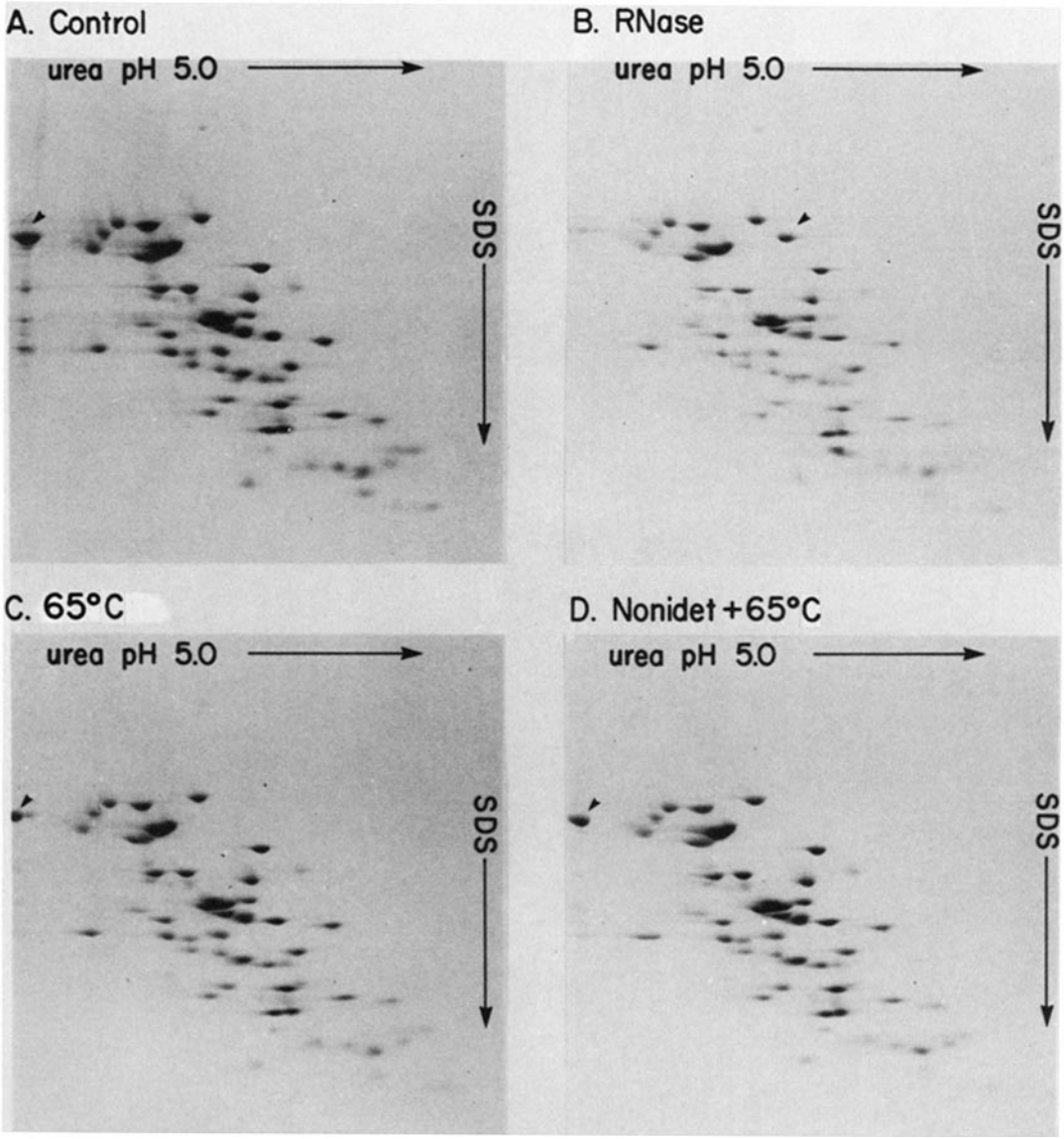

FIGURE 4 Treatment with RNase facilitates entry of S-5 into the first-dimension gels. 19.2 l of wild-type (Em 5256*A*) cells were grown for 14 h at 25°C. Small and large subunit fractions were collected from sucrose gradients, pooled, and centrifuged to give four pellets of washed mit ribosomal subunits. (*A*) Control, the pellet was extracted with acetic acid and analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. (*B*) The pellet was resuspended in 100 μl of 50 mM Tris·HCl, pH 7.5, and treated with ribonuclease (0.8 U Sigma RNase A Type XII-A and 24 U Sigma RNase T_1, Grade III) for 30 min before acetic acid extraction. (*C*) The pellet was extracted with acetic acid as described in Materials and Methods; proteins were heated to 65°C for 5 min in the urea sample buffer before electrophoresis. (*D*) Procedure same as (*C*) but with 20% Nonidet present in the urea sample buffer. The small arrows point to S-5. The direction of electrophoresis is indicated by the outside arrows.

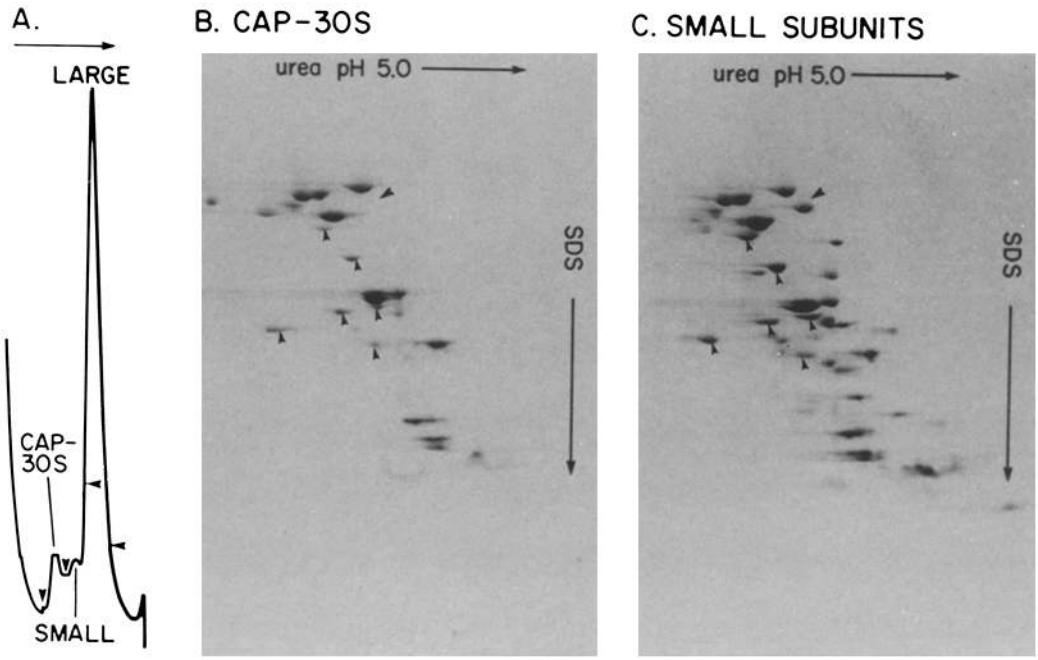

FIGURE 5 Two-dimensional gel electrophoresis of proteins present in CAP-30S particles and putative mature small subunits. 19.2 l of wild-type (Em 5256*A*) cells were grown for 17 h at 25°C in the presence of chloramphenicol (4.0 mg/ml). Ribonucleoprotein particles were separated on high salt-sucrose gradients as described in Materials and Methods. CAP-30S particles and putative mature small subunits were collected from each gradient and material from 12 gradients was pooled for two-dimensional protein analysis. (*A*) Representative gradient; the small arrows show where "cuts" were made for each fraction; the height of the CAP-30S and 50S peaks are 0.15 and 2.5 OD_{260} units, respectively. (*B*) Proteins from CAP-30S particles and (*C*) proteins from small subunit fraction; this fraction also shows a background of large subunit proteins. The small arrows point to proteins deficient in CAP-30S particles. The direction of electrophoresis is indicated by the outside arrows.

conidia used for the inoculation of liquid cultures did not affect the variability. The following points can be made in summary: (*a*) S-5 was consistently present in <50% normal concentration as judged by two-dimensional gel electrophoresis in four experiments; (*b*) nine other mit small subunit proteins were >50% deficient in at least two experiments and six proteins (S-8, S-9, S-12, S-13, S-14, and S-19) were deficient to that extent in >50% of the experiments; and (*c*) the deficiency of S-5 was not absolutely correlated with the deficiency of the other proteins. In one experiment, two-dimensional gel patterns showed S-5 deficient but all the other proteins present in near normal concentrations. The last result shows that the binding of S-5 is not absolutely required for the binding of the other proteins. The deficiency of S-5 in the CAP-30S particles is consistent with the following interpretation: that binding of S-5 is the rate-limiting step in the assembly of mit small subunits in the presence of chloramphenicol and that the appearance of the more defective particles is related to the absence of S-5. However, because the evidence is indirect, this interpretation should be considered tentative.

Extra-Nuclear, Small Subunit-Deficient Mutants

The existence of S-5 came to light as a result of studies on [*poky*], a mit small subunit-deficient mutant (23, 24), after fingerprinting analysis failed to detect any defect in the primary structure of the mit rRNAs (13, 15). More recently, mit ribosome assembly defects similar to that in [*poky*] have been found in six additional extra-nuclear mutants which had previously been considered related to [*poky*] on the basis of mitochondrial phenotype and response to the *f*-gene suppressor (8, 16). Kuriyama and Luck (11) provided evidence that

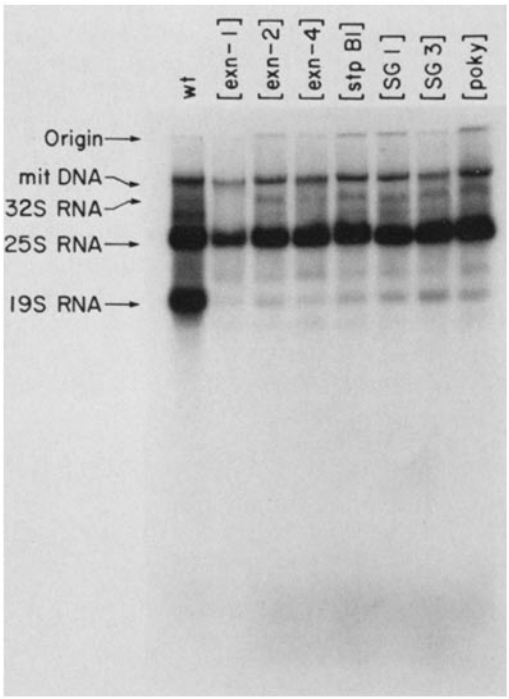

FIGURE 6 Gel electrophoretic analysis of mit rRNAs from wild-type Em 5256*A* and seven extranuclear, small subunit-deficient mutants. Cells were mass-labeled with [^{32}P]orthophosphate (sp act 0.25 mCi/mmol) and then pulse-labeled with 5 mCi [5-^{3}H]uridine for 30 min. Mit rRNAs were isolated by the SDS-diethylpyrocarbonate method and analyzed by electrophoresis on composite agarose-acrylamide gels as described in Materials and Methods. The figure shows an autoradiograph of the dried gel. The direction of electrophoresis is from top to bottom. Although the slot for [*exn-1*] does not show [^{32}P]mass-label corresponding to 32S RNA, it did show a peak of [5-^{3}H]uridine pulse-label at the expected position.

32S RNA is synthesized in [*poky*] but that 19S RNA sequences are rapidly degraded leading to a deficiency of mit small subunits. Fig. 6 shows gel electrophoretic analysis of whole mit rRNAs which extends this result to the other mutants. In all six cases, the gels show predominantly 25S RNA with 19S RNA grossly deficient. In addition, the mutants show bands corresponding to high molecular weight precursor RNAs which comigrate with wild-type precursor RNAs. Pulse-labeling data from this experiment confirm that high molecular weight precursor(s) are synthesized in the mutants, but that 19S RNA is rapidly degraded.[3] The results are consistent with the idea that all of the mutants have a primary defect in the same component.

To determine whether the altered component might be S-5, we examined mit ribosomal proteins from the mutant strains by two-dimensional gel electrophoresis as shown in Fig. 7. Because of the low concentration of mit small subunits in the mutants, protein analysis was carried out on pooled small and large subunit fractions making use of the large subunit proteins as "carriers" for the small subunit proteins. The dark spots in Fig. 7 are large subunit proteins against which the small subunit proteins appear as a background of lighter spots; the position of S-5 is indicated by the arrows. Two points can be made from the gels: first, the electrophoretic mobility of S-5 does not appear altered in any of the mutants. This point was confirmed by mixing experiments in which the electrophoretic mobilities of S-5 from wild-type and each of the mutant strains were shown to be identical by co-migration of unlabeled wild-type and ^{35}S-labeled mutant mit ribosomal proteins (protocol of Fig. 9; data not shown). Second, the stoichiometry of S-5 appears to be near normal in most mutants. However, the results of Fig. 9 and additional gel analyses showed that S-5 is present in <50% normal concentrations in [*poky*] mit small subunits (see below), and there was some indication (data not shown) that the stoichiometry of S-5 might be variable in [*exn-1*] and [*exn-4*]. It should be noted that the inferences about stoichiometry are based on at least three experiments for each strain and that we are taking into account S-5 present at the origin of the first-dimension gels (S-5a; see pattern for [*SG-1*]; S-5a is present in the gel for [*SG-3*] but not visible in the photograph).

Comparison of [poky] Mit Small Subunits and CAP-30S Particles

To determine whether the mit ribosome assembly defect in [*poky*] could be attributed to a diminished capacity for mit protein synthesis, the phenotypes of [*poky*] and chloramphenicol-treated wild-type cells were compared in detail. Fig. 8 shows sucrose gradient analyses in which ^{3}H-labeled mit ribosomal subunits from wild-type cells were cosedimented with ^{32}P-labeled RNP from either chloramphenicol-treated wild type or

[3] Collins, R. A., H. Bertrand, R. J. LaPolla, and A. M. Lambowitz. Submitted for publication.

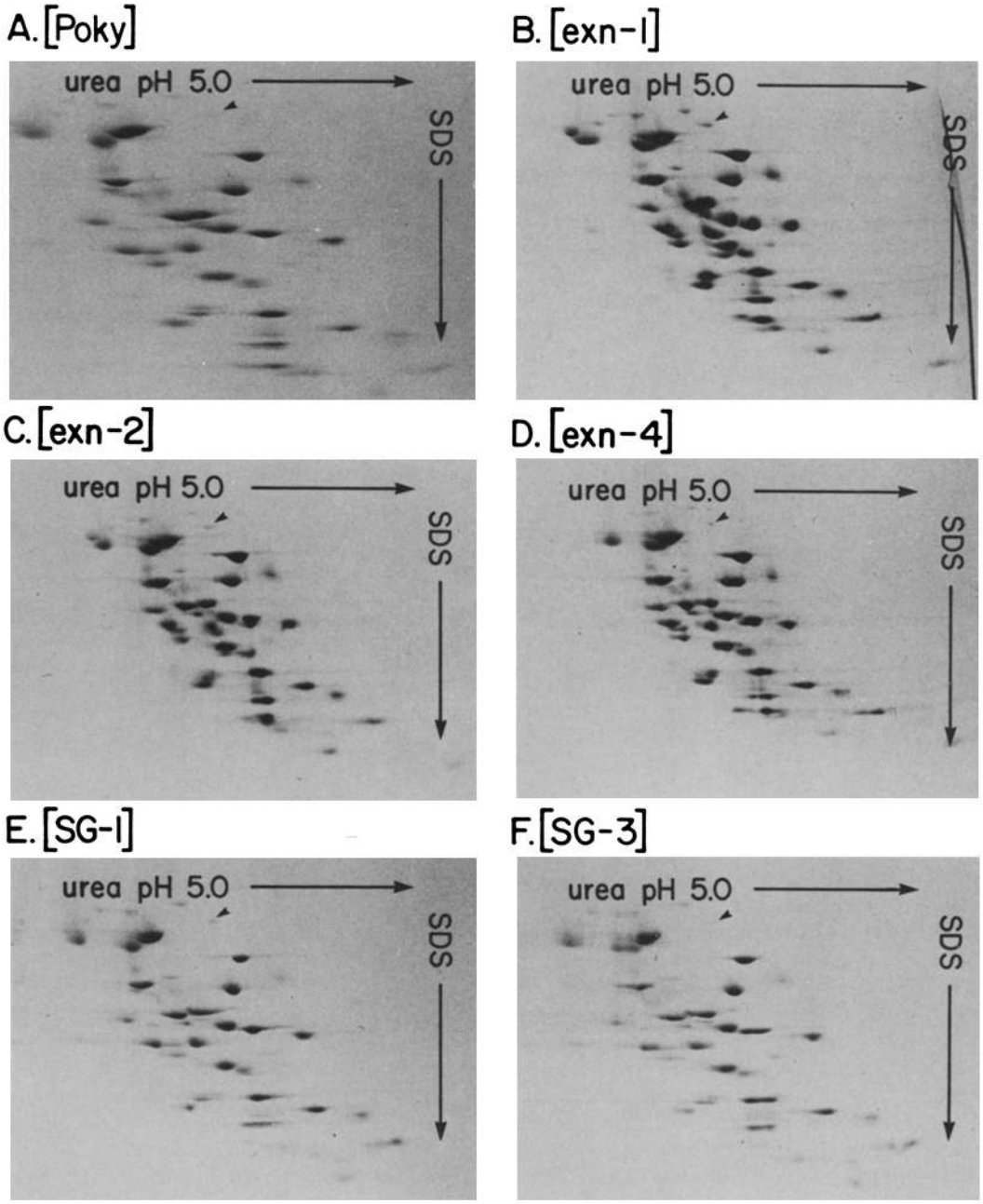

FIGURE 7 Two-dimensional gel electrophoresis of mit ribosomal proteins from six extra-nuclear, small subunit-deficient mutants. Similar results were obtained for [*stpB1*], but the gels are not shown. 9.6 l of cells of each strain were grown for 24 h at 25°C. Gels show proteins present in pooled small and large subunit fractions from high salt-sucrose gradients. The small arrows point to S-5. The direction of electrophoresis is indicated by the outside arrows.

[*poky*]. Fig. 8*A* shows that CAP-30S particles sediment as a symmetrical peak behind wild-type mit small subunits (peaks at fractions 17 and 22, respectively) whereas Fig. 8*B* shows that [*poky*] contains a single 30S particle present in low concentrations but cosedimenting with wild-type mit

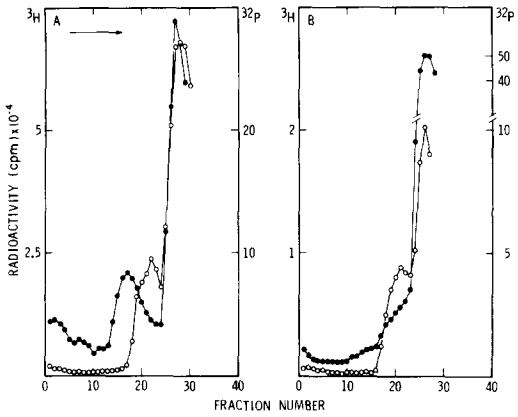

FIGURE 8 Sucrose gradient analysis of (*A*) ^{3}H-labeled RNP from wild-type cells mixed with ^{32}P-labeled RNP from chloramphenicol-treated wild-type cells, and (*B*) ^{3}H-labeled RNP from wild-type cells mixed with ^{32}P-labeled RNP from [*poky*]. Mit RNPs were isolated from labeled cells as described in Materials and Methods and mixed before sucrose gradient centrifugation. The gradients are drawn to emphasize the small subunit fractions. (○—○) ^{3}H-radioactivity; (●—●) ^{32}P-radioactivity. The direction of sedimentation is indicated by the arrow.

small subunits (peak at fraction 21; Fig. 8 *B*). Significantly, the gradient for [*poky*] shows no peak at the position expected for CAP-30S particles (fractions 16 and 17).

The protein composition of [*poky*] mit small subunits was determined in experiments in which unlabeled wild-type and ^{35}S-labeled [*poky*] mit ribosomal proteins were co-isolated. In this type of experiment, the wild-type strain contributes sufficient mass to permit separate analysis of [*poky*] small and large subunit proteins with the unlabeled wild-type proteins serving as carriers for the labeled [*poky*] proteins. Fig. 9 shows autoradiographic patterns of small subunit proteins from wild type and [*poky*]. These patterns along with quantitation of ^{35}S-label in three independent experiments suggest that S-5 is present in <50% normal concentrations in [*poky*] mit small subunits. In the experiment of Fig. 9, S-3 and S-14 are also deficient and S-6 is present in more than twofold excess. However, S-9, which appeared deficient in the previous one-dimensional gel analysis (13), was found in near normal concentrations in the present experiments. The arrows in Fig. 9 *B*

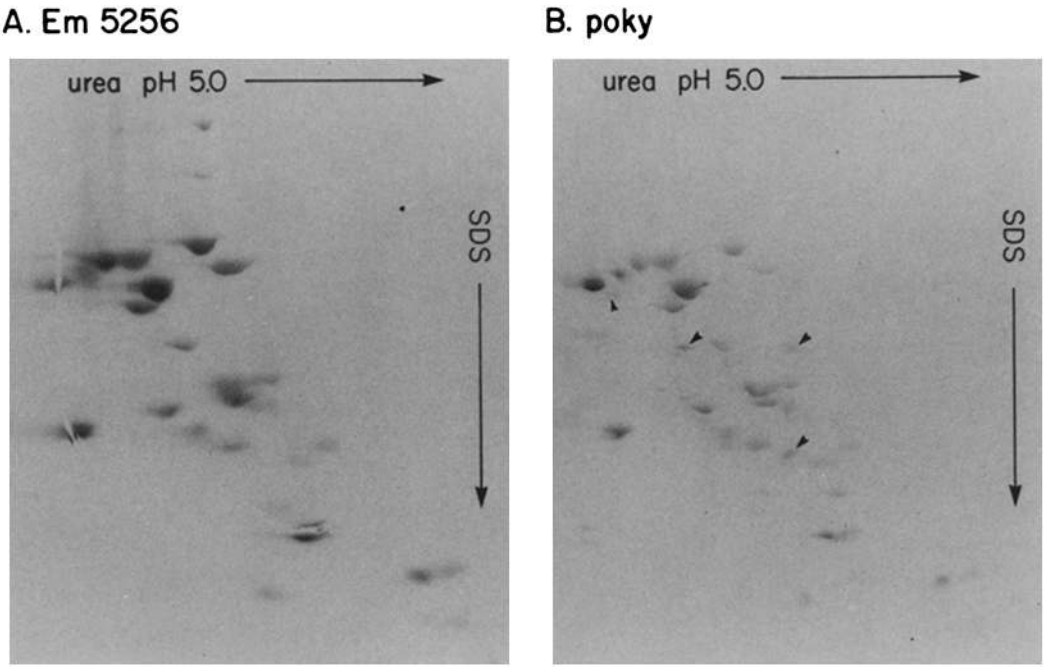

FIGURE 9 Two-dimensional gel patterns of small subunit proteins from wild-type Em 5256*A* and [*poky*] 13-6. 800 ml of wild-type and [*poky*] cells were labeled in low sulfate medium with 5 mCi and 10 mCi [^{35}S]sulfate, respectively (see Materials and Methods). The cells of each strain were harvested and mixed with 6.4 l of wild-type 74*A*. The figure shows autoradiograms of proteins present in the small subunit fractions from high salt-sucrose gradients. The small arrows in the [*poky*] pattern point to "extra" proteins present in [*poky*] (see text). The direction of electrophoresis is indicated by the arrows.

point to several "extra" proteins which consistently appear in [*poky*] mit small subunit patterns. These proteins are tentatively assigned to a precursor of large subunits which was previously found in the 30S fraction (17). The four indicated proteins have electrophoretic mobilities corresponding to L-3, L-7, L-8, and L-20. They are sometimes faintly visible in wild-type mit small subunit patterns (Fig. 9*A*) but are more prominent in [*poky*], presumably because the large subunit precursor comprises a greater proportion of the 30S fraction. Because the alterations cited above in [*poky*] mit small subunits are observed in mixing experiments in which wild-type mit small subunits are present in excess, they are likely to reflect physiological differences rather than artifacts of isolation of proteins from mutant strains. A key finding is that [*poky*] mit small subunits contain normal concentrations of some proteins which are ordinarily deficient in CAP-30S particles (e.g., S-8, S-12, and S-13). No alteration could be detected in gel patterns for [*poky*] large subunit proteins (data not shown), in agreement with previous findings (13).

DISCUSSION

Two-dimensional gel analysis shows that a mitochondrially synthesized protein, S-5, is present in stoichiometric concentrations in mit small subunits from wild-type *Neurospora* strains. The binding of S-5 to mit small subunits is specific by the following criteria: (*a*) the protein remains bound to mit small subunits which have been sedimented through sucrose gradients containing 0.5 M KCl (Fig. 1), (*b*) the concentration of S-5 decreases roughly in parallel with that of other mit small subunit proteins in mit small subunit-deficient mutants (Figs. 7 and 9), and (*c*) in experiments in which CAP-30S particles and mit small subunits are obtained from the same cells, S-5 is found in normal concentrations in mature mit small subunits but deficient in the CAP-30S particles (Fig. 5). The last point is particularly significant because CAP-30S particles and mature small subunits are closely related and would be expected to contain similar sites for nonspecific binding.

The gel analysis shows that S-5 is among the most basic mit ribosomal proteins, probably with a pI >10, and that S-5 has a remarkably high affinity for RNA in urea-containing buffers. We are now making use of the latter property to purify the protein by RNA affinity chromatography. It is tempting to speculate that S-5 binds to 19S RNA in mit small subunits and that this binding is relevant to the role of S-5 in mit ribosome assembly or protein synthesis. So far, however, in vitro binding experiments show only that S-5 and other mit ribosomal proteins bind nonspecifically to RNA in urea-containing buffers. Experiments under physiological conditions have been hindered by the insolubility of *Neurospora* mit ribosomal proteins near neutral pH (unpublished data).

Two-dimensional gel electrophoresis was used to examine mit ribosomal proteins from [*poky*] and six additional extra-nuclear mutants with defects in the assembly of mit small subunits. The electrophoretic mobility of S-5 is not detectably altered in any of the mutants as judged by comigration of S-5 from mutant and wild-type cells in mixing experiments. In addition, S-5 appears to be present in near normal stoichiometry in most of the mutants although S-5 was consistently deficient in [*poky*] mit small subunits. To determine whether the mit ribosome assembly defects in [*poky*] might be secondary effects of a diminished capacity for mit protein synthesis, we compared in detail the phenotypes of [*poky*] and chloramphenicol-treated wild-type cells. Although both are deficient in mit small subunits, several differences were found, including the lack of CAP-30S particles in [*poky*] and the rapid degradation of 19S RNA in [*poky*] compared to its stability in chloramphenicol-treated wild-type cells (see also reference 17). Recent results with nuclear mutants confirm that inhibition of mit protein synthesis can lead to secondary defects in the assembly of mit small subunits which closely resemble those found in chloramphenicol-treated wild-type cells (16).[3] It is still possible, however, that specific types of nonribosomal mutations (e.g., tRNA mutations; cf. reference 4) could mimic the [*poky*] phenotype. The unaltered electrophoretic mobility of S-5 in [*poky*] and the other extra-nuclear mutants does not preclude a mutational alteration because (*a*) a mutation could have occurred without changing the charge of the protein, and (*b*) the electrophoretic system is probably not sensitive enough to detect small charge differences in proteins of mol wt 52,000 (12). If such differences exist, they might be detectable by a more sensitive technique such as polypeptide fingerprint analysis. Further discussion of the relationship of S-5 to the [*poky*] phenotype will be deferred until such experiments have been carried out.

The role of S-5 in the assembly of mit small subunits has been investigated by an indirect method, making use of chloramphenicol to specif-

ically inhibit mit protein synthesis. The results (present work and reference 17) show that chloramphenicol rapidly inhibits the assembly of mit small subunits in wild-type cells leading to the formation of CAP-30S particles which: (*a*) sediment more slowly than normal mit small subunits, (*b*) are enriched in a precursor of 19S RNA, and (*c*) are deficient in S-5 and several other proteins. Because the formation of CAP-30S particles is observed quickly, within 15 min after addition of chloramphenicol, it seems likely to be a direct effect of inhibiting mit protein synthesis (17). The data do not indicate whether CAP-30S particles are a normal intermediate in mit small subunit assembly. However, the pulse-labeling experiments in the previous work do exclude the possibility that CAP-30S particles are derived from preexisting mit small subunits (17). Because S-5 is the only mit ribosomal protein whose synthesis is directly inhibited by chloramphenicol (13), the finding that chloramphenicol inhibits the maturation of mit small subunits tentatively implicates S-5 in this process. In addition, the results are consistent with the idea that S-5 stabilizes the binding of several other mit small subunit proteins. With the completion of the two-dimensional gel analysis described in this paper, the proposed role of S-5 can be tested by direct experiments along several lines: continued studies on S-5 in the mutants, studies on the binding of S-5 to rRNA, the preparation of antibodies to S-5, and partial in vitro reconstitution.

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A preliminary account of this work has been presented elsewhere (14).

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