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Defective Production of Mitochondrial Ribosomes in the *Poky* Mutant of *Neurospora crassa*

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ABSTRACT **A strain of *Neurospora crassa* containing a cytoplasmic mutation (*poky*) affecting mitochondrial function is shown to be deficient in small ribosomal subunits in the mitochondrion during the exponential growth phase. In the stationary growth phase, small subunits are more abundant and are present in mitochondrial ribosomal monomers. This change can be correlated with the return of mitochondrial cytochrome content to amounts approaching those of wild type mitochondria. The ribosomal defect shows an extrachromosomal pattern of inheritance in crosses of *poky* with wild type.**

Mitochondrial ribosomes have been isolated from various organisms, including *Neurospora*, yeast, and *Xenopus* (1–6). These ribosomes differ from cytoplasmic ribosomes in their sedimentation properties, their sensitivity to the Mg ion concentration, and their RNA and protein (7) components.

DNA–RNA hybridization studies indicate that mitochondrial rRNAs are probably gene products of mitochondrial DNA (6, 8). In yeast, the existence of erythromycin- and chloramphenicol-resistant mutants showing nonMendelian patterns of inheritance (9–11) suggests that some mitochondrial ribosomal proteins may also be gene products of mitochondrial DNA.

In *Neurospora* the mitochondrial genome is small (8). The determinants for ribosome synthesis would therefore account for a significant fraction of the coding capacity of the DNA. For this reason, we have studied several mitochondrial mutants to see if in any of these strains the primary defect was related to mitochondrial ribosomes. Our findings suggest that the basis of the abnormal mitochondrial phenotype of *poky* (*mi-1*) is an interference in the synthesis or assembly of the small subunits of mitochondrial ribosomes.

MATERIALS AND METHODS

Strains

The following strains of *Neurospora crassa* were used: NSX f+ a (*poky*) (derived from a backcross of 3627-1 with 34486); RL-3-8 A (wild type) for crossing with *poky;* STL-7 (Lys−) for control ribosome and cytochrome determinations.

Growth and preparation of cell fractions

Methods for the growth of *Neurospora* in liquid culture, and the preparation and purification of mitochondria by isopycnic centrifugation in sucrose gradients, have been described (12, 13). To obtain mitochondria completely free of contaminating cytoplasmic ribosomes, we used a sucrose flotation gradient (14).

Mitochondrial ribosomes. Mitochondrial fractions, obtained from sucrose gradients, were diluted with buffer (0.15 M NH_4Cl, 0.01 M $MgCl_2$, 0.02 M Tris·HCl, pH 7.6) and centrifuged for 20 min at 40,000 rpm (Spinco no. 40 rotor). The resulting mitochondrial pellet was resuspended in buffer and the mitochondria were lysed with Triton X-100 according to the method of Küntzel and Noll (2). Ribosome profiles were obtained by layering the lysate on sucrose gradients containing buffer and centrifuging (see Fig. 1).

Cytoplasmic ribosomes. Cytoplasmic ribosomes were prepared as described (1).

Isolation of RNA

Cytoplasmic rRNA was prepared by LiCl precipitation (15) of the postmitochondrial supernatant.

Mitochondrial RNA was prepared either by diethylpyrocarbonate (Eastman)–SDS (sodium dodecyl sulfate, Matheson, Coleman, and Bell) extraction (16) or by phenol–cresol–SDS extraction (1) of purified mitochondria. There was no difference in the RNA prepared by these two methods.

Analytical methods

Protein was determined by the method of Lowry *et al.* (17) with bovine serum albumin as a standard. RNA was estimated by assuming 24 A_{260} = 1 mg RNA/ml.

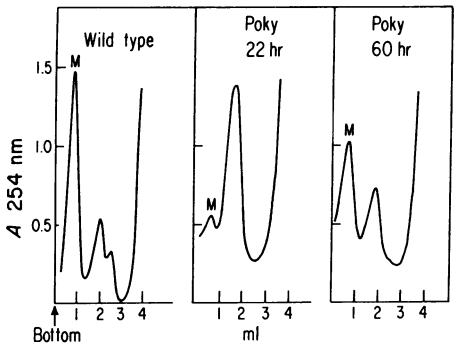

FIG. 1. Sedimentation profiles of mitochondrial lysates. Mitochondria were prepared and lysed with Triton as described in *Materials and Methods.* The lysate was layered on a 5–20% sucrose gradient containing buffer. Centrifugation was carried out at 37,000 rpm for 120 min at 4°C (Spinco SW 39 rotor). After centrifugation, the gradient was analyzed in a flow cell by recording the absorbancy at 254 nm (10-mm light path) using an Isco density gradient fractionator. *M* = monomer.

TABLE 1. *Mitochondrial cytochrome content and ribosomal monomer to large subunit ratio*

Strain	Cytochromes (nmol/mg mitochondrial protein)			Ribosomes monomer/large subunit
	a	*b*	$c + c_1$	
A. wild type	0.40	0.84	1.07	(4.1–7.4)*
poky (22 hr)	0.01	0.30	1.60	0.27
poky (60 hr)	0.03	0.92	1.38	1.25
B. abn-1	0.01	0.63	2.44	2.02
mi-3 (20 hr)	0.03	0.78	1.89	4.98
C117 (47 hr)	0.16	1.11	0.34	3.32
lys-5 (17 hr)	0.05	0.34	1.84	2.24

Oxidized–reduced spectra of purified mitochondria were recorded as described by Luck (13). The concentration of each cytochrome in a given sample was estimated by the method of Williams (19) with the following modifications. The following wavelength pairs (in nm) were used: 610–630, 560–575, 550–535. The differences in millimolar extinction coefficients at each of the wavelength pairs were: cytochrome *a:* 12.0, −0.326, 0.63; cytochrome *b:* 0, 14.3, −3.12; cytochrome $c + c_1$: −0.08, 0, 21.0, respectively.

* Although in wild type this ratio is generally between 4 and 7, it may be as low as 2.7.

RESULTS

Cytochrome content of *poky* mitochondria

In confirmation of the earlier observations of Mitchell and Mitchell (18), mitochondria from log-phase *poky* cultures show diminished cytochrome *a* and *b* content, and excessive cytochrome *c* when compared to wild type cultures (Table 1*A*). The cytochrome *c* content in *poky* mitochondria is 1.6–2-fold higher than in wild type.

This picture changes during the stationary growth phase when the *poky* mitochondrial cytochrome content approaches wild type amounts (Table 1*A*). Cytochrome *b* content increases first; later, cytochrome *a* appears, and with time both continue to increase. The change is characteristic and reproducible although the exact time course is variable. Thus, in mitochondria from a group of 60–62-hr-old *poky* cultures, the cytochrome *a* content varied from 0.03 to 0.14 nmol/mg mitochondrial protein.

Mitochondrial ribosomes

Mitochondrial ribosomes from *poky* cultures differ from those of wild type cultures. At 20 hr in the *poky* growth cycle (Fig. 1), the major mitochondrial ribosomal component appears to be the large subunit. There are few monomers and small subunits are not detected at all. In wild type mitochondria, ribosomes appear mainly as monomers; large subunits are present in variable numbers but are always accompanied by small subunits. Under the conditions used here, mitochondrial polysomes are not preserved, and the ribosomes are recovered as derived monomers. For this reason, the profiles shown in Fig. 1 are a good representation of the total mitochondrial ribosome content.

The identification of the major component in *poky* as large subunits was supported by two additional tests: (*1*) the buoyant density, measured in CsCl after formaldehyde fixation, was 1.517 (the value for wild type large subunits was 1.520); (*2*) RNA extracted from this peak sedimented mainly as 25 S. The presumptive monomer peak, *M*, showed a buoyant density of 1.508 in CsCl compared with the density of the wild type mitochondrial monomer of 1.510.

The abnormal mitochondrial ribosomal profile in *poky* is not due to an altered Mg^{++} dependency. The ribosome profile in sucrose gradients was not changed when lysates were prepared and analyzed in the presence of increasing amounts of $MgCl_2$ up to 50 mM.

The cytoplasmic ribosomes of 20-hr *poky* cultures appeared exclusively as monomers when examined under the same conditions used for the lysis of mitochondria.

60-hr *poky* mitochondrial lysates (Fig. 1) show a relative increase in monomers, a decrease in large subunits, and barely detectable amounts of small subunits. Under dissociating conditions (2 mM $MgCl_2$), however, the small subunit peak expected from the monomer content could be demonstrated.

The ribosome profiles of several other slow-growing strains with abnormal cytochrome contents were examined. These strains included: (*1*) *abn-1* (20) and *mi-3* (21), two mitochondrial mutant strains; (*2*) C117 (21), a nuclear mutant; and (*3*) a culture of STL-7 (*lys-5*) which spontaneously developed a slow growth rate. The results (Table 1*B*) show that in all cases the monomer/large subunit ratios are greater than those obtained from early *poky* cultures. Small subunits were always present in the expected ratio to large subunits. It can be concluded that a slow growth rate and an abnormal cytochrome spectrum do not cause an abnormal ribosome pattern.

TABLE 2. *Inheritance of mitochondrial cytochrome content and ribosomal monomer to large subunit ratio*

		Mating type	*f* locus	Cytochromes (nmol/mg protein) *a*	*b*	$c + c_1$	Ribosomal ratio monomer/large subunit
RL-3-8A (♀) × *poky* a(♂)							
	6-2	a		0.49	0.99	1.42	5.74
	6-4	a		0.57	0.89	1.03	5.90
	6-6	A		0.49	0.76	1.44	4.34
	6-8	A		0.59	0.79	1.27	4.11
poky a(♀) × RL-3-8A (♂)							
early	2-1	a	−	<0.01	0.44	1.97	0.78
	2-3	a	+	<0.01	0.25	2.14	0.69
	2-5	A	+	<0.01	0.30	1.61	0.27
	2-7	A	−	<0.01	0.44	1.70	0.25
late	2-1	a	−	0.17	1.09	2.25	2.25
	2-3	a	+	0.14	1.05	1.22	1.75
	2-5	A	+	0.09	1.28	2.20	0.94
	2-7	A	−	0.01	1.74	2.54	0.47

Reciprocal crosses were made (23) and ascospores isolated in order (24). Cultures derived from one member of each spore pair were studied.

Since *poky* partially recovers during its growth cycle, early and late cultures of f^+ and f^- ascospore isolates with ar equivalent mass to the parent *poky* strain at 20–22 hr and 60 hr of growth were examined.

Inheritance of ribosome deficiency

Tetrad analyses of reciprocal crosses between *poky* and wild type demonstrated that the ribosome defect, like the cytochrome content, shows the segregation pattern expected for nonchromosomal inheritance, while mating type and the *f* locus, both nuclear genes, exhibit a normal chromosomal pattern of segregation (Table 2).

In *poky*, the presence of the nuclear suppressor gene, *f*, increases the growth rate both on solid and in liquid medium; it reduces to some extent the accumulation of cytochrome *c* but does not affect the lack of cytochromes *a* and *b*. The *f* gene has no effect on wild type (22). When cultures are examined at equivalent mass there is no special effect of the nuclear suppressor *f* on the mitochondrial ribosome pattern (Table 2).

Correlation of cytochrome content with ribosome deficiency

The tendency for mitochondrial cytochrome content to shift toward wild type amounts in late *poky* cultures provides an opportunity for correlation with mitochondrial ribosomal monomer content. Since the amount of cytochrome *a* increases from barely detectable concentrations, it is the best parameter to use for this correlation. When the results from late *poky* cultures in Table 2 are plotted, there appears a linear relationship between cytochrome *a* content and the ratio of mitochondrial monomer to large subunit. This correlation exists despite the fact that the time course of cytochrome *a* reappearance is quite variable.

Ribosomal RNA

The total RNA content of *poky* mitochondria (45.0 ± 3.1 μg RNA/mg mitochondrial protein) is close to that of wild type mitochondria (49.5 ± 7.8 μg RNA/mg mitochondrial protein). However, the ratio of 25S/19S mitochondrial rRNA was found to be much higher in *poky* cultures than in wild type. RNA obtained from a postmitochondrial supernatant of 20-hr *poky* shows the usual 2:1 ratio of 28S/18S cytoplasmic rRNA.

Accurate 25S/19S RNA ratios were obtained by agarose–acrylamide gel electrophoresis (14). This technique gives good separation of the mitochondrial rRNAs from contaminating cytoplasmic rRNA. The results (Table 3) indicate that during the growth cycle the 25S/19S ratio in *poky* mitochondria decreases from an initially high value to a value closer to the normal wild type value of 2. This change in the mitochondrial rRNA ratios with time corresponds to the shift in mitochondrial ribosome particle content in early and late *poky*.

It is possible that the unusually high 25S/19S ratio could result from a block in the assembly of small subunits which prevents the incorporation of newly synthesized 19S RNA into particles. This unincorporated 19S RNA would then be rapidly degraded by intramitochondrial nucleases. On the other hand, feedback control mechanisms for mitochondrial rRNA synthesis might also play a role in limiting the amount of 19S RNA synthesized.

DISCUSSION

Our results indicate that the *poky* phenotype is related to a defect in the synthesis or assembly of mitochondrial ribosomal small subunits. This defect, like the abnormal cytochrome pattern, shows the segregation pattern expected for nonchromosomal inheritance. The lack of small subunits appears to be a relative rather than an absolute deficiency, since in the stationary growth phase of *poky* cultures the content of mitochondrial ribosome monomers increases. The appearance of additional ribosome monomers after prolonged growth can be correlated with a phenotypic change toward wild type, namely the increase of mitochondrial cytochromes *a* and *b* and decrease in cytochrome *c*.

It would appear that during the exponential growth phase small ribosomal subunits are produced at a low rate, inadequate for maintenance of the usual supply of mitochondrial ribosomes necessary for normal autonomous protein synthesis. During stationary phase, when the rates of increase in both cell mass and mitochondrial structures are low, the rate of synthesis of small subunits is more adequate, producing a higher content of ribosome monomers.

Although we have no information concerning the detailed mechanism for the production of defective mitochondrial ribosomes in *poky*, several possible hypotheses can be mentioned. First, since mitochondrial rRNAs are gene products of mitochondrial DNA (8), the relative number of 25S and 19S genes could be altered in *poky*. This seems unlikely since preliminary experiments suggest that there is no difference in the hybridization values obtained between *poky* and wild type. Second, the synthesis of 19S RNA could be the rate-limiting step in the production of mitochondrial ribosomal small subunits. A [^{3}H]uracil dilution experiment during the 19–25 hr growth period (one mass doubling) indicated that the rates of synthesis of mitochondrial 25S, 19S, and 4S RNAs were equivalent. Since this method would not detect small differences in the rates of synthesis of 25S and 19S RNA the experiment does not rule out the synthesis of 19S RNA as the rate-limiting step. Third, another step in the assembly of small subunits, such as the maturation of 19S RNA or the synthesis of a small subunit protein, could be rate limiting. In any case, more complete understanding of this ribosome defect in *poky* will have to await much additional information on mitochondrial RNA synthesis (the possible existence of precursors), maturation of mitochondrial RNA, and the mode of mitochondrial ribosome assembly.

We consider the cytochrome defect in *poky* to be a direct consequence of the ribosome deficiency since other slow grow-

TABLE 3. *Ratio of 25S to 19S* poky *mitochondrial RNA at different stages of the growth cycle*

Hr	Ratio 25S/19S
17 (log phase)	6.85, 6.16*
24 (late log phase)	5.79, 5.62
90 (stationary phase)	3.91, 2.68

At the indicated times, mitochondrial RNA was extracted by the diethylpyrocarbonate–sodium dodecyl sulfate procedure (17) from *poky* cultures growing in medium labeled with 100 μCi of [6-^{3}H]uracil (Amersham-Searle) per 150 ml of medium. Mitochondria were prepared by the sucrose flotation method (see *Methods*). The RNA was run on agarose–acrylamide gels (14). After staining and destaining, the gels were sliced and the slices were solubilized and counted. Quantitative estimates of the amounts of 25S and 19S RNA were obtained from the measured radioactivity.

* The second value of each pair was obtained from gels in which the sample was layered on the gel in a higher ionic strength buffer to minimize aggregation artifacts.

ing strains, similar to or different from *poky* in cytochrome content, show no defect in mitochondrial ribosome profiles. There is uncertainty concerning the exact mechanism. Failure of mitochondrial protein synthesis resulting from a ribosome deficiency could interfere with the biosynthesis of one or more cytochrome proteins or with the biosynthesis of proteins having some function in the organization of the electron transport components in mitochondrial cristae. In any case, our conclusion conflicts with the hypothesis of Woodward and Munkres (25) that the *poky* phenotype results from a single amino acid replacement in mitochondrial "structural protein." On the basis of our findings it is to be expected that such a protein or proteins, if they are products of mitochondrial protein synthesis, would be deficient during exponential growth and would become more abundant during stationary phase when mitochondrial protein synthesis is supported by a higher content of mitochondrial ribosome monomers.

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