Proc. Nat. Acad. Sci. USA
Vol. 69, No. 6, pp. 1371–1374 June 1972

Cooperation of Mitochondrial and Nuclear Genes Specifying the Mitochondrial Genetic Apparatus in *Neurospora crassa*

(chloramphenicol/ethidium bromide/repressor control/model)

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Communicated by David E. Green, March 22, 1972

ABSTRACT **Enzymes involved in the expression of the mitochondrial genome in *Neurospora crassa* are induced by chloramphenicol and ethidium bromide, which block transcription and translation of mitochondrial DNA. It is concluded that most, if not all, proteins of the mitochondrial genetic apparatus are coded by nuclear genes, synthesized on cytoplasmic ribosomes, and controlled by a repressor-like mitochondrial gene product. A model explaining the coordination of nuclear and mitochondrial division cycles by repressor control is discussed.**

In some aspects mitochondria behave like endosymbiotic bacterial cells, because they proliferate by division, growth, and distribution to the progeny (1–3) and because they contain a circular genome that is expressed by enzymes of bacterial specificity (4). On the other hand, mitochondria can also be considered as giant multienzyme complexes that are almost completely built up by the nuclear-cytoplasmic protein synthesizing system. Less than 10% of the mitochondrial proteins are synthesized on mitochondrial ribosomes; this fraction consists of probably not more than eight species of hydrophobic proteins that are incorporated into the inner membrane where they assemble cytochromes, cytochrome oxidase, and ATPase (4). A mitochondrial genetic origin of these proteins is highly probable, but has not been proven.

The only mitochondrial gene products that have positively been identified are ribosomal RNA and transfer RNA (4). Although the coding capacity of mitochondrial DNA would be large enough to code for at least an additional 20 proteins, there is no evidence that enzymes involved in the expression of the mitochondrial genome are coded by this genome, with the possible exception of a replication factor (5, 6). On the other hand, most workers agree that not only all proteins of the outer mitochondrial membrane and most proteins of the inner membrane, but also the proteins of the mitochondrial genetic apparatus are synthesized on cytoplasmic ribosomes (4, 7–9). Furthermore, a few enzymes involved in mitochondrial protein synthesis have been suggested to be coded by nuclear DNA, namely a mitochondrial leucyl-tRNA synthetase from *Neurospora* (10) and the two mitochondrial peptide chain elongation factors from yeast (11). We have studied the biosynthesis of mitochondrial RNA and ribosomes and of two enzymes of bacterial specificity involved in mitochondrial protein synthesis in *Neurospora*. The finding that specific inhibitors of mitochondrial transcription and translation stimulate the biosynthesis of mitochondrial enzymes strongly suggests a nuclear origin of these proteins and a control by mitochondrial gene products.

Abbreviations: EF-G, elongation factor G; EF-T, elongation factor T.

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METHODS

Growth Conditions. Neurospora crassa (wild type, Em 5256) was grown at 30° for 14 hr. The inoculum was 2×10^6 conidia per ml. Chloramphenicol (2 mg/ml) was added together with conidia; 75 μM ethidium bromide was added 4 hr after inoculation. The hyphae were homogenized in a carborundum mill (12, 13). The methods for cell fractionation (7) and for isolation of mitochondrial RNA (14), and mitochondrial and cytoplasmic ribosomes (15) have been described.

Assay of Elongation Factors G(EF-G). Hyphae were washed with 20 mM Tris·HCl (pH 7.8)–5 mM $MgCl_2$–14 mM 2-mercaptoethanol–10 mM KCl, ground with sea sand, and extracted with half their wet weight of the above buffer. Debris were removed by centrifugation for 5 min at 3000 rpm in a Christ centrifuge. The supernatant was sonified four times in ice with a Branson sonifier for 15 sec at full power and centrifuged for 20 min at 17,000 rpm in a Sorvall centrifuge. The supernatant was centrifuged for 1 hr at 65,000 rpm in a Spinco centrifuge. Peptide chain elongation factors were isolated from the high-speed supernatant by $(NH_4)_2SO_4$ precipitation and analyzed by filtration on Sephadex G-150 as described (16).

The fractions were assayed in two different reaction mixtures. Both mixtures contained 96 mM Tris·HCl (pH 7.8), 13 mM KCl, 5 mM phosphoenolpyruvate, 11 mM 2-mercaptoethanol, 0.8 mM GTP, 16 μg/ml of pyruvate kinase, 100 μg/ml poly(U), and 100,000 cpm/ml [^{3}H]phenylalanyl-tRNA. Assay mixture A (G_{70}-activity) was supplemented with 70S ribosomes (28 *A* units/ml) and 33 μg/ml of elongation factor T (EF-T) (19), both from *Escherichia coli*. Assay mixture B (G_{80}-activity) was supplemented with cytoplasmic 77S ribosomes from *Neurospora* (35 *A* units/ml). After incubation for 30 min at 32°, the radioactivity in the hot Cl_3CCOOH-insoluble fraction was determined.

Assay of N^{10}-Formyltetrahydrofolate: Methionyl-tRNA transformylase. Hyphae were washed with 10 mM Tris·HCl (pH 7.5)–10 mM $MgCl_2$–10 mM 2-mercaptoethanol, ground with sea sand, and extracted with the same buffer. High-speed supernatants were prepared from sonified crude extracts as de-

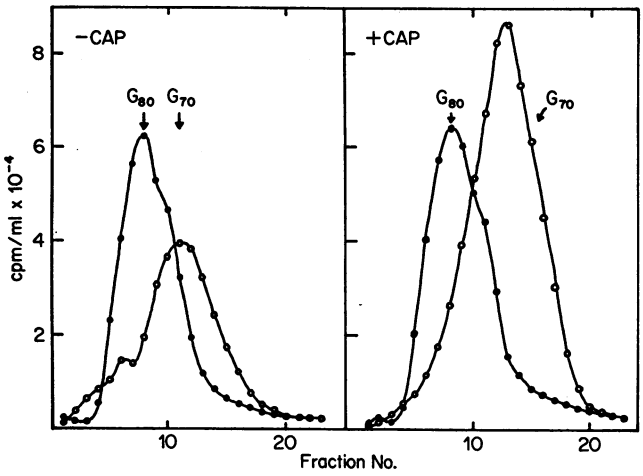

FIG. 1. Filtration on Sephadex G-150 of crude peptide chain elongation factors from whole cell extracts of *Neurospora* grown in the absence and presence of chloramphenicol. For details see *Methods* and ref. 16. 10-μl aliquots of the 1.5-ml fractions were assayed in 50-μl mixtures containing either 70S ribosomes from *E. coli* (O——O) or 77S ribosomes from *Neurospora* (●——●).

scribed above and dialyzed overnight against the extraction buffer. [^{3}H]Methionyl-tRNA (from *E. coli*) was prepared as described (17), except that the formyl donor was omitted.

Transformylase was tested in a mixture containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.01 mM N^{10}-formyltetrahydrofolic acid, and 1.2 mg/ml [^{3}H]methionyl-tRNA (600 cpm/μg). After incubation at 37°, aliquots were treated with 0.1 volume of 25% ammonia for 5 min at room temperature and subjected to paper electrophoresis for 2 hr at 1500 V on Whatman 3 MM sheets. The dried electropherograms were cut into strips and counted in a liquid scintillation counter in toluene–2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl-2)-benzene.

RESULTS

Table 1 shows that the amount of mitochondrial RNA in relation to postmitochondrial supernatant protein has about doubled in cells grown in the presence of 2 mg/ml of chloramphenicol. The mass of mitochondrial protein relative to nonmitochondrial protein has increased 1.3-fold; this explains why the RNA content of mitochondria has increased only 1.5-fold, but also suggests that the increased formation of mitochondrial proteins by cytoplasmic ribosomes may compensate for the loss of cytochrome assembling proteins and the reduction of repiratory efficiency. We have previously shown that the biosynthesis of mitochondrial RNA polymerase in *Neurospora* is stimulated by chloramphenicol and ethidium bromide (18). This suggests that not the rate of transcription, but rather the formation of the mitochondrial transcription complex is stimulated by the antibiotic.

Table 1 also demonstrates that the amount of mitochondrial ribosomes has increased more than 2-fold in comparison to cytoplasmic ribosomes. The ribosome preparations from cells grown in the presence and absence of chloramphenicol did not differ in their activity in poly(U)-dependent cell-free systems and in their specific response to fusidic acid (16). This result confirms the previous finding that all ribosomal proteins essential for the function of mitochondrial ribosomes are synthesized on cytoplasmic ribosomes (7–9). In addition, it demonstrates that the biosynthesis of mitochondrial, but not of cytoplasmic, ribosomal proteins is stimulated by blockage of mitochondrial protein synthesis, suggesting a control of nuclear genes coding for mitochondrial ribosomal proteins by mitochondrial gene products (see *Discussion*).

Fig. 1 shows the activity pattern of mitochondrial and cytoplasmic peptide chain elongation factors after filtration through a Sephadex G-150 column (16). The enzyme fractions have been isolated from high-speed supernatants containing both mitochondrial soluble matrix proteins and cytosolic proteins. The two complementary elongation factors, EF-G and EF-T, are not separated by this procedure; however, because of the extreme lability of the mitochondrial EF-T (19), the mitochondrial EF-G was complemented with EF-T from *E. coli* for assay. As can be seen from Fig. 1, the enzyme fractions from cells grown in the absence and presence of chloramphenicol contain about the same activity of cytoplasmic elongation factors, whereas the activity of the mitochondrial EF-G is about doubled in the fraction from cells treated with chloramphenicol.

The finding that the biosynthesis of mitochondrial proteins is unaffected, or even stimulated, by chloramphenicol excludes an intramitochondrial site of synthesis but does not exclude a mitochondrial origin of messenger RNA coding for these proteins. The transcriptional origin of mitochondrial enzymes can be determined with ethidium bromide, a drug known to induce mitochondrial "petite" mutants in yeast and to inhibit repair, transcription, and, indirectly, translation of mitochondrial, but not of nuclear, DNA (4). This method cannot be used to study the origin of mitochondrial ribosomal proteins because these proteins are only detectable after assembly of the mitochondrial ribosome, a step that is directly

TABLE 1. *Stimulation of mitochondrial RNA and ribosome synthesis by chloramphenicol*

Growth conditions	Mitochondrial RNA (μg) per mg Mitochondrial protein	Mitochondrial RNA (μg) per mg Postmitochondrial supernatant protein	Mitochondrial ribosomes (μg/mg cytoplasmic ribosomes)	Mitochondrial protein (mg/mg postmitochondrial supernatant protein)
Without chloramphenicol	31	3.5	39	0.11
With chloramphenicol	48	7.3	90	0.14
Stimulation factor	1.5	2.1	2.3	1.3

Protein was determined by the method of Lowry *et al.* (33). The mitochondrial ribosomes had a A_{260}/A_{280} ratio of 1.8, the cytoplasmic ribosomes a ratio of 2.0. 1 mg of ribosomes was taken as 10 A_{260} units.

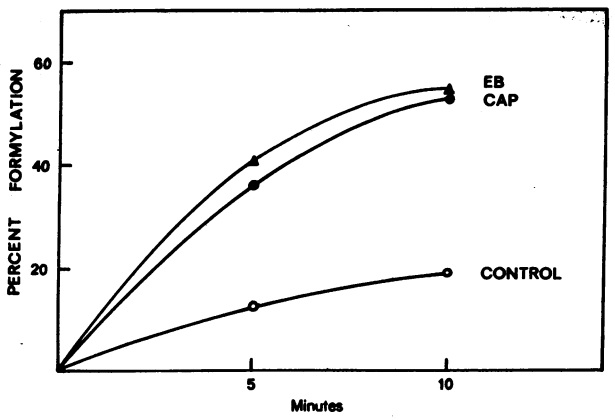

FIG. 2. Methionyl-tRNA transformylase activity of high-speed supernatant fractions from whole cells of *Neurospora* grown in the absence and presence of chloramphenicol (CAP) or ethidium bromide (EB). For details see *Methods*. The amount of protein added per ml assay mixture was 0.63 mg (*Control*), 0.57 mg (*CAP*), and 0.76 mg (*EB*). Previous experiments have shown that the initial formylation rate increased linearly with the protein concentration.

controlled by ethidium bromide-sensitive transcription of mitochondrial ribosomal RNA genes.

We have, therefore, studied the effect of ethidium bromide on the biosynthesis of methionyl-tRNA transformylase a mitochondrial enzyme of bacterial specificity that does not depend on assembly steps to be tested and that is normally absent from the cytoplasm (20–23). The enzyme was assayed by *N*-formylation of [^{3}H]methionyl-tRNA from *E. coli*, followed by alkaline hydrolysis and electrophoretic separation of methionine and *N*-formylmethionine. Fig. 2 demonstrates that the transformylase activity of high-speed supernatant fractions obtained from whole cells is increased more than 2-fold if cells have been grown in the presence of chloramphenicol or ethidium bromide.

DISCUSSION

Chloramphenicol is known to suppress *in vivo* the synthesis of inner membrane proteins contributed by intramitochondrial protein synthesis and, thus, to prevent the proper assembly of cytochromes *a*, a_3, and *b* and of cytochrome oxidase, resulting in the formation of respiratory-deficient mitochondria characteristic for mitochondrial mutants of yeast (24) and *Neurospora* (25). However, mitochondria isolated from chloramphenicol-treated yeast cells still incorporate amino acids into protein, indicating that the proteins of the mitochondrial translational apparatus in yeast are synthesized on cytoplasmic ribosomes. This agrees with our finding that chloramphenicol does not inhibit biosynthesis of mitochondrial RNA, mitochondrial ribosomes, and mitochondrial enzymes like RNA polymerase (18), ribosomal translocase, and methionyl-tRNA transformylase. Moreover, there is increasing evidence that most, if not all, proteins involved in the expression of mitochondrial DNA are coded by nuclear DNA. A nuclear origin has been suggested for mitochondrial enzymes like DNA polymerase (26), RNA polymerase (18), EF-G (11), and leucyl-tRNA synthetase (10). The results shown in Fig. 2 allow us to add methionyl-tRNA transformylase to this list.

The unexpected finding that the biosynthesis of these enzymes is stimulated by agents blocking transcription and translation of mitochondrial DNA suggests that the nuclear cistrons coding for these proteins are controlled by one or several repressor-like proteins which, in turn, are coded by mitochondrial DNA and synthesized on mitochondrial ribosomes. A similar repressor mechanism has been suggested by Williamson (5) to explain the high efficiency of ethidium bromide in inducing "petite" mutants in yeast. However, in contrast to Williamson who postulates an irreversible repression, we explain the ethidium effect in *Neurospora* by derepression of nuclear genes coding for mitochondrial proteins.

Fig. 3 illustrates the cooperation of nuclear and mitochondrial genes responsible for mitochondrial biogenesis. We have

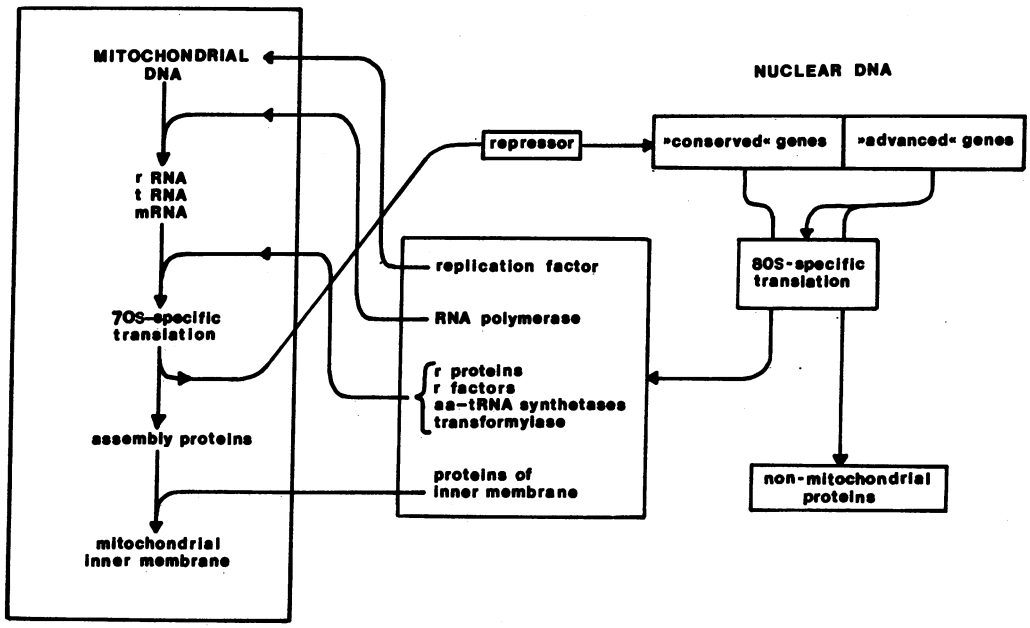

FIG. 3. Schematic representation of the biochemical cooperation between mitochondrial and nuclear genes.

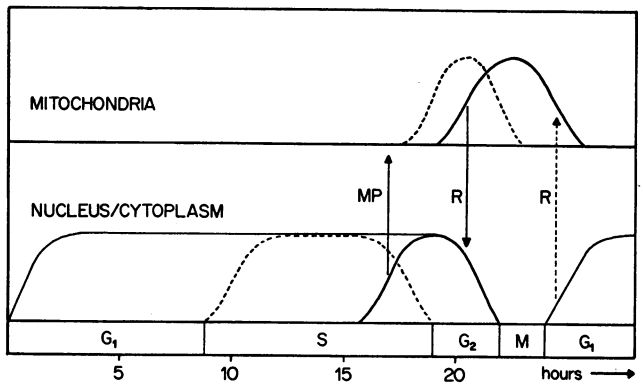

FIG. 4. Possible coordination of nuclear and mitochondrial division cycles by repressor control. The time scale reflects the mitotic cycle of synchronized HeLa cells (31). - - -, DNA synthesis; ——, synthesis of nonmitochrondrial proteins; ——, synthesis of mitochondrial proteins. *R* = repressor; MP = mitochondrial proteins.

to assume that the nuclear genome contains a set of genes that code for mitochondrial proteins and that have been conserved since the origin of the eukaryotic cell, probably because they had to replace functionally the lost genes of the mitochondrial genome. A part of these gene products indeed exhibit a strict specificity for prokaryotic protein synthesis (4). Another set of genes, which probably evolved later by duplication of the early genome, is responsible for the synthesis of the nuclear/cytoplasmic genetic system, of the mitochondrial outer membrane, nuclear membrane, and outer cell membranes, and of other nonmitochondrial proteins. It is suggested that the conserved part of the nuclear genome responsible for mitochondrial biogenesis is controlled by its "partner," the mitochondrial genome, which produces repressor-like proteins binding to the nuclear partner genes.

There is good evidence that in *Neurospora* (2), yeast (3, 27, 28), and HeLa cells (29, 30) all mitochondria divide and grow synchronously and that the replication periods of nuclear and mitochondrial DNA alternate periodically. The possible role of mitochondrial repressor(s) in maintaining the rhythm of nuclear and mitochondrial division is depicted in Fig. 4, showing the mitotic cycle of HeLa cells (31). In HeLa cells, mitochondrial DNA synthesis starts in the S phase and reaches its maximum in the G_2 phase (29), whereas mitochondrial transcription starts in the G_2 phase (30) and proceeds into the metaphase (32). Consequently mitochondrial protein synthesis would start in the G_2 phase and produce a repressor that terminates translation of nuclear genes coding for mitochondrial proteins. After metaphase only the unrepressed nuclear genes would start to be expressed (G_1 phase), whereas expression of the repressed "conserved" genes would be possible only after derepression by a new round of nuclear replication (late S phase).

This model predicts three periods of protein synthesis during the cell cycle: A first period, starting after metaphase, in which nonmitochondrial proteins are produced; a second period, starting in the late S phase, in which the enzymes of the mitochondrial genetic apparatus and most of the inner membrane proteins are synthesized; and a third period of intramitochondrial protein synthesis during the G_2 phase, in which the previously formed "soluble" proteins of the respiratory chain and oxidative phosphorylation are assembled by the insoluble mitochondrial gene products to enlarge the inner membrane during mitochondrial growth. This relatively short period of mitochondrial replication, division, and growth could conceivably be initiated by the nuclear production of a mitochondrial replication factor (5, 6) and be terminated either by a repressor produced at the onset of nuclear gene expression after metaphase or, more likely, by the exhaustion of the pool of soluble mitochondrial proteins leading to a halt of assembly-controlled intramitochondrial protein synthesis.

This investigation was supported by the Deutsche Forschungsgemeinschaft. Z. B. holds a fellowship of the Deutsche Akademische Austauschdienst. We thank Dr. F. Cramer for generous support and Miss A. Helms for expert technical cooperation.

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