IN VITRO TRANSLATION OF MITOCHONDRIAL DNA FROM *NEUROSPORA CRASSA*

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1. Introduction

Our current knowledge of the informational content of mitochondrial DNA is still poor [1]. The circular mitochondrial genome of different organisms has been shown to contain a minimal set of cistrons coding for ribosomal and transfer RNA, and in the case of yeast mitochondria some genetic markers have been obtained but not yet correlated to gene products.

On the other hand, several proteins have been identified as products of intramitochondrial protein synthesis that are incorporated in the inner membrane where some of them seem to integrate cytochromes, cytochrome oxidase and ATPase (for references see [1]). However, the genetic origin of these proteins remains to be determined. A mitochondrial origin of the message is likely but the alternative possibility has also been considered, namely that mitochondrial ribosomes translate imported messenger RNA and that mitochondrial DNA may not contain a single structural gene coding for protein although it has the capacity to code for at least 20 proteins [2].

In this communication we demonstrate that the *in vitro* transcription products of mitochondrial DNA from *Neurospora crassa* contain messenger RNA's that are translated by a submitochondrial system in at least four distinct protein species of molecular weight ranging between 11 000 and 180 000 daltons.

This strongly suggests that mitochondrial DNA codes not only for stable RNA but also for some proteins.

2. Methods

Buffer solutions: Buffer A is 10 mM Tris-HCl pH 7.5, 100 mM NH_4Cl, 10 mM Mg acetate, 0.44 M sucrose. Buffer B is 100 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.44 M sucrose. Buffer C is 50 mM Tris-HCl pH 8, 50 mM KCl, 10 mM Mg acetate, 5 mM $CaCl_2$, 2 mM dithiothreitol.

Growth of *Neurospora* (wild type Em 5256) and isolation of mitochondria has been described [3, 4]. The following modifications have been introduced: hyphae were collected in mid log phase and homogenized in buffer A, the crude mitochondrial pellet was suspended in buffer B and immediately centrifuged in a discontinuous sucrose gradient containing buffer C for 1 hr at 25 000 rpm, 2°. Purified mitochondria were suspended in buffer C (ca. 35 mg/ml); a 5 ml portion was sonified 4 × 15 sec with a Branson sonifier at full power in an ice-salt bath keeping the temperature below 4°. The extract was centrifuged for 10 min at 45 000 *g*, 0°, and the slightly turbid supernatant (S-30) was stored in small portions in liquid nitrogen.

Linear mitochondrial DNA was prepared as described [5] (method B). RNA polymerase was prepared from *E. coli* according to Burgess [6]. The specific activity was 55 mU/mg protein.

N-Formyl-tetrahydrofolic acid (FTHF) was kindly supplied by Dr. Göpel.

The incubation mixture for transcription contained 50 mM Tris-HCl pH 8, 100 mM NH_4Cl, 50 mM KCl, 11 mM Mg acetate, 1 mM dithiothreitol, 2 mM each of ATP, GTP, UTP and CTP, 45 μg/ml mitochondrial DNA and 0.5 mg/ml RNA polymerase.

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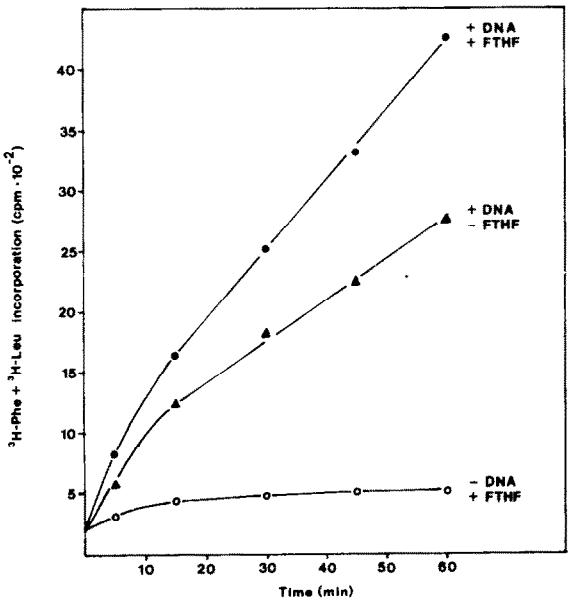

Fig. 1. Translation of the *in vitro* transcription product of mitochondrial DNA by a mitochondrial S-30 fraction. 200 μl of the transcription mixture (see Methods) were incubated for 1 hr at 37°. From parallel experiments in the presence of ^{3}H-UTP the amount of cRNA synthesized during this period has been estimated to be 15 μg. The mixture was then added to 300 μl of the translation mixture which was supplemented with 2.7 μCi/ml of [^{3}H]phenylalanine (11 Ci/mmole) and 10 μCi/ml of [^{3}H]leucine (1 Ci/mmole). 50 μl aliquots were treated with hot TCA, the precipitate was collected on GFA filters and counted in toluene scintillation mixture. (●–●–●): Complete system, (▲–▲–▲): FTHF was omitted, (○–○–○): DNA and RNA polymerase was omitted.

The incubation mixture for translation contained 50 mM Tris-HCl pH 8, 100 mM NH_4Cl, 50 mM KCl, 11 mM Mg acetate, 1 mM DTT, 4 mM ATP, 2 mM each of GTP, CTP, UTP, 5 mM PEP, 20 μg/ml PK, 0.6 mg/ml tRNA (*E. coli*, Boehringer), 2.5 mM FTHF, 0.2 mM each of all twenty ^{12}C-amino acids and 4 mg/ml protein of the mitochondrial S-30. ^{3}H-amino acids were added as indicated.

In vivo labelling experiments: Hyphae were grown for 14 hr in a New Brunswick fermenter as described [4]. 200 ml aliquots were transferred into 1 liter erlenmeyer flasks and shaken for 15 min at 30° in the presence of 0.1 mg/ml cycloheximide. After addition of 0.5 μCi/ml [^{3}H]leucine (40 Ci/mmole), 0.5 μCi/ml [^{3}H]alanine (50 Ci/mmole) and 1 μCi/ml [^{3}H]lysine (0.15 Ci/mmole) the cells were allowed to grow for 1 hr at 30°, harvested on a Büchner funnel, washed with buffer A, ground with sea sand and extracted with buffer A. The crude mitochondrial pellet was suspended in a small volume of 0.1 M Na phosphate pH 7, 1% SDS, 1% 2-mercaptoethanol and heated for 2 min in a boiling water bath.

SDS-gel electrophoresis was carried out as described [7]. Gels were run for 4 hr at 8.2 mA per gel. For calibration of the marker proteins the gels were stained with Coomassie brilliant blue. For determination of radioactivity the gels were sliced immediately after electrophoresis in ca. 1.3 mm discs. The discs were placed in Tricarb vials containing 1 ml of 1 mg/ml pronase (Serva, predigested for 2 hr at 37°) in 10 mM Tris-HCl pH 8 and incubated overnight at 37°. After addition of 10 ml Aquasol (NEN) the vials were counted.

3. Results

Fig. 1 shows the amino acid polymerizing activity of a mitochondrial soluble fraction (S-30). The limited endogenous incorporation during the first 10 min probably reflects the run-off of mitochondrial polysomes.

This protein synthesizing activity is dramatically increased if a mixture contaning RNA polymerase from *E. coli*, mitochondrial DNA and about the equal amount of complementary RNA (transcription product) is added. For still unknown reasons the stimulation is less pronounced if mitochondrial DNA and *E. coli* RNA polymerase are added without preincubation in a transcription assay mixture. The transcription dependent protein synthesis proceeds linearly for at least 2 hr and is markedly stimulated by the formyl donor *N*-formyltetrahydrofolic acid, indicating that mitochondrial ribosomes use a bacteria-like chain initiation mechanism involving *N*-formylmethionyl-tRNA [4, 8, 9] to translate mitochondrial messenger RNA synthesized *in vitro*.

We have characterized the proteins synthesized by mitochondrial ribosomes *in vitro* in the absence or presence of mitochondrial transcription products by means of SDS-acrylamide gel electrophoresis [7]. All samples were mixed with cytochrome *c* as a visible internal marker. External markers run in separate gels were found to be useless since the rate of migration of mitochondrial proteins strongly depends on the protein input under otherwise identical conditions. The gels were cut in slices, and the labelled

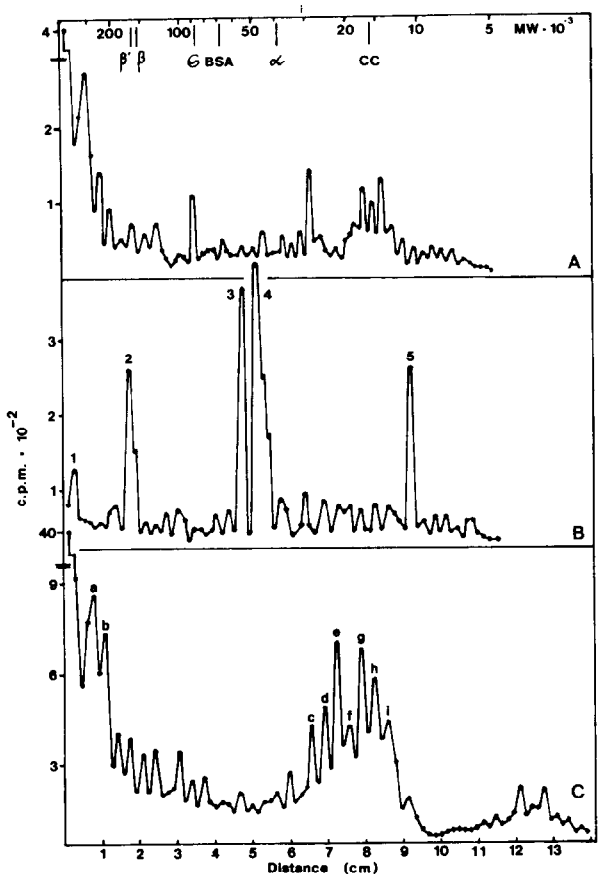

Fig. 2. SDS-acrylamide gel electrophoresis of mitochondrial proteins synthesized *in vitro* and *in vivo*. A mitochondrial S-30 fraction was incubated in the absence (A) or presence (B) of mitochondrial DNA and *E. coli* RNA polymerase for 2 hr under the same conditions as described under fig. 1, except that the following ^{3}H-amino acids were added: alanine, arginine, leucine, lysine, methionine and phenylalanine (5 μCi/ml). The reaction was stopped by adding an equal volume of ice-cold 10% TCA. The precipitate was washed twice with 5% TCA, dissolved in a small volume of 0.1 M Na phosphate pH 7, 1% SDS, 1% 2-mercaptoethanol and heated for 2 min at 100°. The preparation of the proteins labelled *in vivo* in the presence of cycloheximide (C), the conditions of electrophoresis and analysis of the gels are described under Methods. All samples contained cytochrome *c* as visible internal marker.

proteins were solubilized by digestion with pronase. In fig. 2 the patterns of proteins synthesized *in vitro* and *in vivo* are compared. The upper part (A) shows the material synthesized endogenously by the mitochondrial soluble fraction. Addition of mitochondrial transcription product leads to the synthesis of

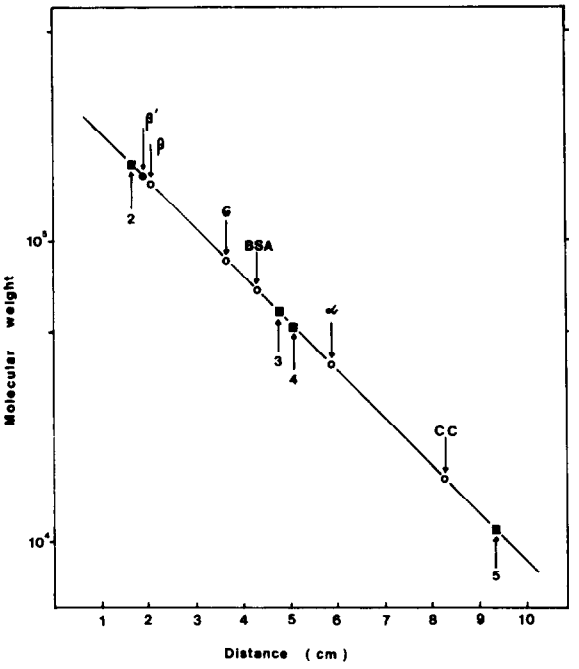

Fig. 3. Determination of the molecular weight of mitochondrial proteins synthesized *in vitro* and *in vivo*. The α, β, β′ and δ subunits of *E. coli* RNA polymerase [6] and bovine serum albumin (BSA) [10] have been used to construct the calibration plot. The filled squares represent the positions of the peak fractions of fig. 2B relative to the internal marker cytochrome *c*(CC).

a few distinct protein subunits shown in fig. 2B. This pattern differs not only from the *in vitro* background material but also from the pattern of proteins synthesized on mitochondrial ribosomes *in vivo* in the presence of cycloheximide (fig. 2C).

It should be emphasized that these gel patterns are highly reproducible with respect to the relative mobility of the peak fractions; the method is limited by the fact that the slices are not thin enough to sub divide band regions.

Fig. 3 shows the calibration plot to estimate the molecular weight of the labelled proteins from their mobility relative to internal markers. Using published values for the molecular weight (M.W.) of the subunits of *E. coli* RNA polymerase (β' = 165 000, β = 155 000, α = 39 000) [6] and of bovine serum albumin (69 000) [10] we find a M.W. of 87 000 for sigma factor and 16 000 for cytochrome *c* from horse heart. The molecular weights of the major peak fractions of the DNA-directed incorporation

(fig. 2B) have the following values: 1 = more than 300 000; 2 = 180 000; 3 = 58 000; 4 = 51 000; 5 = 11 000.

The M.W. of mitochondrial proteins synthesized *in vivo* in the presence of cycloheximide (fig. 2C) have been determined to be a = 250 000; b = 215 000; c = 29 000; d = 25 500; e = 23 000; f = 20 000; g = 17 500; h = 16 000; i = 15 000.

4. Discussion

The genetic map of the mitochondrial genome in *Neurospora* has previously been shown to contain a single set of cistrons coding for ribosomal RNA [11] and about 40 cistrons for transfer RNA (unpublished) by hybridization. However, the observation that mitochondrial RNA contains minor species which hybridize with at least 20% of the genome in addition to the stable RNA cistrons [11] suggests that mitochondrial DNA might code also for messenger RNA. Whether the few inner membrane proteins synthesized *in vivo* on mitochondrial ribosomes [14–16] are coded for by mitochondrial DNA or are translated from imported messenger RNA is still an open question. There is no genetical or biochemical evidence for mitochondrial genes coding for proteins; an early claim that the extrachromosomal *Neurospora* mutant mi-1 contains an altered "structural protein" [17] cannot be maintained.

Our finding that the *in vitro* transcription product of mitochondrial DNA can be translated by a submitochondrial system seems to be the first direct evidence for the existence of messenger RNA coded by mitochondrial DNA.

Although the mitochondrial messenger RNA is produced by a heterologous RNA polymerase it is translated by mitochondrial ribosomes in a nonrandom fashion leading to the formation of a few distinct large proteins (fig. 2B). This agrees with our earlier observation that *E. coli* RNA polymerase recognizes specifically mitochondrial promoter sites [5].

By comparing the gel electrophorograms of fig. 2 it becomes obvious that the pattern of the endogenously synthesized proteins (A) resembles that of the *in vivo* products (C), whereas the pattern of the DNA-dependent translation products is completely different. Although these results are well reproducible it cannot be excluded that some of the bands, especially the larger ones (protein 1 of fig. 2B, a and b of fig. 2C) represent aggregates resistant to the drastic denaturating conditions used.

Several explanations for the striking differences between the patterns B and C may be proposed:

1) The DNA-dependent translation products are not related at all to the *in vivo* products and represent translational artefacts.

2) They represent larger precursors of the *in vivo* products.

3) They are identical or related to the *in vivo* products but have different aggregation properties.

4) They represent true mitochondrial gene products not found *in vivo* in mitochondria.

Whatever the proteins synthesized *in vitro* turn out to be, the translation of the small and genetically unexploited mitochondrial genome in a cell free system could be useful to answer some open questions of mitochondrial biogenesis.

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