FUSIDIC ACID RESISTANCE OF MITOCHONDRIAL G FACTOR FROM NEUROSPORA CRASSA

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Summary: Fusidic acid, an antibiotic which inhibits protein synthesis in bacteria and in the cytoplasm of eucaryotic cells by interacting with the G factor (translocase), does not affect the function of the mitochondrial G factor from Neurospora.

Introduction: Mitochondria from Neurospora crassa possess a protein synthesizing machinery (1) which differs from the cytoplasmic counterpart and which is functionally related to the bacterial protein synthesizing system: both mitochondria and bacteria contain ribosomes which are sensitive to chloramphenicol and resistant to cycloheximide (2), they contain transformylase (3), initiation factors recognizing fMet-tRNA (4) and a peptide chain elongation factor G (translocase) specific for 70s ribosomes (5,6).

We present evidence that mitochondrial protein synthesis can be distinguished from both bacterial and cytoplasmic protein synthesis by fusidic acid, which blocks translocation by interacting with G factor (7,8). The mitochondrial G factor, although interchangeable with bacterial G factor (6), is not affected by fusidic acid. We will show also that the cytoplasm from Neurospora contains a 70s specific G factor as a minor component which in contrast to the major 80s specific G factor is not inhibited by fusidic acid.

Methods: Buffer solutions: Buffer A: 20 mM Tris-HCl pH 7.8; 5 mM magnesium acetate; 14 mM ß-mercaptoethanol; 10 mM KCl. Buffer B: 50 mM Tris-HCl pH 7.8; 1 mM dithiothreitol.

Ribosomes were isolated from E. coli A 19 according to Lucas-Lenard and Lipman (10). Cytoplasmic ribosomes from Neurospora were isolated as described (11).

E. coli peptide chain elongation factors were isolated according to Parmeggiani (12). The fraction eluting from hydroxylapatite at 30 mM potassium phosphate was brought to 70% ammonium sulfate saturation, the precipitate was suspended in buffer B containing 50% glycerol and stored at -70° (G factor, Fraction III).

T factor was eluted from hydroxylapatite at 100 mM potassium phosphate and further purified by chromatography on DEAE-Sephadex A-50 using a linear gradient from 150 mM to 350 mM KCl (10). The active fractions were concentrated by ammonium sulfate precipitation (80% saturation), suspended in buffer B containing 50% glycerol and stored at -70°.

Neurospora crassa (wild type, Em 5256) was grown as described (11). Hyphae were homogenized in 10 volumes of buffer A containing 0.44 M sucrose by means of a carborundum mill (13). The homogenate was centrifuged at 1600 rpm for 10 minutes, the supernatant was centrifuged at 17000 rpm for 20 minutes. The supernatant was used for isolation of cytoplasmic elongation factors; the crude mitochondrial pellet was suspended in buffer A containing 0.44 M sucrose and purified by sucrose gradient centrifugation (11). Mitochondrial elongation factors were isolated from purified mitochondria by grinding with 2 volumes alumina and extracting with 2 volumes buffer B. The extract was centrifuged at 17000 rpm for 30 minutes, the

supernatant at 60000 rpm for 1 hour. The supernatant was brought to 70% ammonium sulfate saturation, the precipitate was suspended in buffer B (Fraction I) and applied to a Sephadex G-150 column. The active fractions eluting with buffer B were pooled, concentrated with aquacide I (fraction II) and separated into G and T factor by chromatography on hydroxylapatite (6). The mitochondrial G factor eluted at 40 mM; the active fractions were concentrated by treatment with aquacide I precipitated with ammonium sulfate (70% saturation) and stored in buffer B containing 50% glycerol at -70°,(fraction III).

Cytoplasmic elongation factors were isolated from a postmitochondrial supernatant which was centrifuged at 60000 rpm for 1 hour to remove ribosomes. The supernatant was brought to 70% ammonium sulfate, the precipitate was dissolved in buffer B (fraction I) and applied to a Sephadex G-150 column. The fractions which were active with E. coli ribosomes were purified on hydroxylapatite exactly as described for the mitochondrial G factor (cytoplasmic 70s specific G factor, fraction III). ^{3}H-phenylalanyl-tRNA was prepared according to Kaji, Kaji and Novelli (14).

Polymerizing activity of elongation factors was tested in 75 µl assay mixtures containing (µM/ml): Tris pH 7.8 (96), KCl (13), PEP (4.8), pyruvat kinase (16 µg/ml), ß-mercaptoethanol (11), GTP (0.8), poly U (100 µg/ml), ^{3}H-phenylalanyl-tRNA (2 mg/ml, 45 pM/ml ^{3}H-phenylalanine), and ribosomes and factors as indicated in the legends. After 30 minutes at 32° the radioactivity insoluble in hot TCA was determined.

Results and Discussion: Fig. 1 shows the activity peaks of the unfractionated elongation factors (G and T not separated) derived from mitochondria (A) and from cytoplasm (B) after fil-

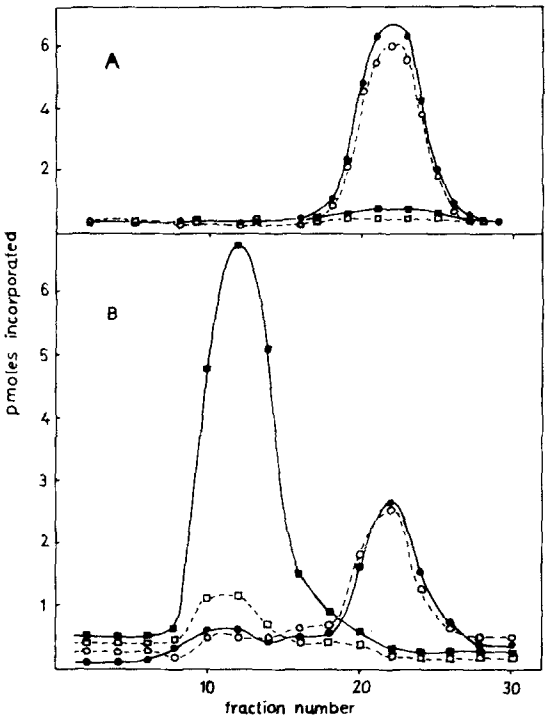

Fig. 1 Sephadex G-150 filtration of unfractionated elongation factors (fraction I) from Neurospora mitochondria (A) and cytoplasm (B). 38 mg of mitochondrial factors and 72 mg of cytoplasmic factors were applied to a 1.5 x 50 cm column, eluted with buffer B and collected in 1.5 ml fractions. 30 µl aliquots were tested with 77s ribosomes from Neurospora cytoplasm (170 µg) and with E. coli ribosomes (150 µg) in the absence and presence of 10^{-4}M fusidic acid.

●——● E. coli ribosomes
○-------○ E. coli ribosomes + fusidic acid
■——■ cyto ribosomes
□- - - - -□ cyto ribosomes + fusidic acid

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tration on Sephadex G-150. The mitochondrial factors which elute as a single peak are active only on E. coli 70s ribosomes and are not affected by fusidic acid. The cytoplasmic factors are separated into two fractions: the first peak elutes earlier than the mitochondrial factors and contains factors which are active only on cytoplasmic 77s ribosomes and which are sensitive to fusidic acid. A second minor peak elutes in the position of the mitochondrial peak and contains fusidic acid resis-

tant factors which are active only on 70s ribosomes like the mitochondrial factors.

From these results we conclude that the 70s specific mitochondrial elongation factors are insensitive to fusidic acid, in contrast to the 80s specific cytoplasmic factors which are sensitive to the drug like the E.coli elongation factors. Furthermore, the cytoplasm contains an additional set of 70s specific factors which behave like mitochondrial factors. Two sets of 80s and 70s specific elongation factors have been detected also in yeast (15). The 70s specific cytoplasmic factors could either be derived from broken mitochondria, or they could represent the intermediate fraction of mitochondrial factors which after synthesis has not yet been taken up by mitochondria. The latter interpretation is supported by the observation that in Neurospora the mitochondrial G factor is synthesized on cytoplasmic ribosomes (manuscript in preparation) and that 70s specific elongation factors have been isolated from the cytoplasm of yeast "petite" mutants which do not contain mitochondrial ribosomes (16).

Fig. 2 shows the effect of increasing concentrations of fusidic acid on the polymerizing activity of purified G factors supplemented by E. coli T factor. Fusidic acid at a concentration of 10^{-5} M completely inhibits bacterial G factor, whereas the mitochondrial factor and also the minor cytoplasmic 70s specific factor are not affected. Only after 100-fold increase of fusidic acid concentration become the Neurospora factors partially sensitive.

Table 1 shows the result of a control experiment which excludes the possibility that the fusidic acid resistance of the mitochondrial G factor is due to an inactivation of the drug by

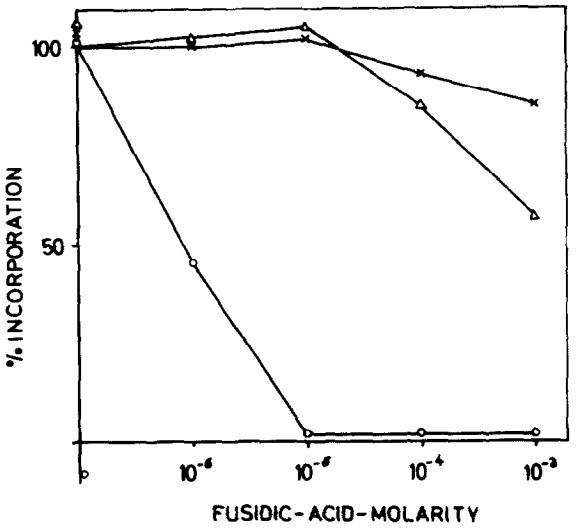

Fig. 2 Inhibition of the purified G factors from Neurospora mitochondria, cytoplasm (70s specific) and E. coli by increasing amounts of fusidic acid. 7 µg of fraction III was tested with 150 µg ribosomes and 5 µg T factor from E. coli.

o——o E. coli G factor
Δ——Δ mitochondrial G factor
×——× cytoplasmic 70s specific G factor

Table I

G factor	pMoles incorporated	
	- fusidic acid	+ fusidic acid
G_{Mito}	5.6	6.2
G_{Coli}	5.6	1.0
$G_{Mito} + G_{Coli}$	9.0	5.3

The test mixture contained 2.3 µg G factor from E. coli and/or from mitochondria (fraction III), 5 µg T factor and 150 µg ribosomes from E. coli, and 10^{-4}M fusidic acid.

the mitochondrial fraction; the bacterial G factor remains sensitive in the presence of the mitochondrial factor. Other controls have shown that replacement of F. coli ribosomes by mitochondrial 73s ribosomes did not lead to different results. Furthermore, protein synthesis in isolated mitochondria from Neurospora is also fusidic acid insensitive (manuscript in preparation).

Thus it seems that fusidic acid is the only drug so far tested which discriminates between bacterial and mitochondrial protein synthesis and which should help to rule out bacterial contamination as a source of incorporating activity of isolated mitochondria or submitochondrial systems.

We also conclude that the mitochondria G factor is a unique species differing from both procaryotic and eucaryotic cytoplasmic G factors.

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