INHIBITION BY ETHIDIUM BROMIDE OF MITOCHONDRIAL PROTEIN SYNTHESIS PROGRAMMED BY IMPORTED POLY(U)

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SUMMARY

Poly(U) stimulates incorporation of phenylalanine by mitochondria isolated from Xenopus laevis, but is without effect on mitochondria from yeast, Tetrahymena, rat or chick liver. Poly(U)-stimulated activity is resistant to cycloheximide and ribonuclease, but partially sensitive to chloramphenicol, consistent with the idea that import, followed by translation of the poly(U) on mitochondrial ribosomes is occurring. Ethidium bromide blocks the incorporation of phenylalanine, without affecting uptake of [^{3}H]poly-(U) into ribonuclease-resistant form, indicating a direct effect of this agent on mitochondrial translation. This effect should be taken into account in the interpretation of experiments involving the use of ethidium as a probe for the existence of nuclear mRNAs within mitochondria in vivo.

INTRODUCTION

The ability of certain synthetic and natural mRNAs to stimulate mitochondrial protein synthesis in vitro has suggested that mitochondria may be able to import mRNAs of nuclear origin in vivo [1,2]. On the other hand, the high sensitivity both in vivo and in vitro of mitochondrial protein synthesis to specific inhibitors of mitochondrial transcription, like the acridines and ethidium bromide [3], implies that protein synthesis is highly dependent on the transcription of mtDNA and that mRNA import by mitochondria is absent or of minor importance [4-6]. Implicit in this interpretation, however, is the assumption that these intercalating dyes are without direct effects on mitochondrial translation. We now present

evidence that this assumption may not be justified. Protein synthesis by isolated Xenopus laevis mitochondria, programmed by an added synthetic mRNA (poly(U)) and thus independent of transcription was found to be highly sensitive to inhibition by ethidium bromide.

METHODS AND MATERIALS

Preparation of mitochondria from X. laevis:

Approximately 2000 fertilised eggs were de-jellied [7], rinsed several times in 0.7% w/v NaCl solution and resuspended in about 40 ml medium I (0.25 M sucrose, 0.007 M $MgCl_2$, 0.03 M Tris-HCl (pH 8.0), diluted with 1/9 volume 0.06 M 2-mercaptoethanol immediately before use). Cell-disruption was achieved by 4-5 passes of a loose-fitting, motor-driven teflon-glass homogeniser. Nuclei and debris were removed by centrifugation for 10 min at 480 x g (Sorvall SS-34 rotor, 2-4°C). A yolk layer which accumulated on the surface of the supernatant was also removed at this stage. The supernatant was decanted and centrifuged at 10 000 x g for 10 min. The resulting pellet consisted clearly of two layers: the upper part (mitochondria) was separated from the lower (pigment granules) by addition of medium I and gentle rubbing with a teflon rod. The mitochondria were recovered by centrifugation at 10 000 x g for 10 min, suspended in medium I at a concentration of approximately 50 mg protein/ml and used immediately.

Protein synthesising activity:

Protein synthesising activity of isolated mitochondria in the presence or absence of poly(U) was measured exactly as described by Swanson [1] using [^{14}C]-U-phenylalanine, spec. act. 492 mCi/mmole (Amersham).

TABLE I: Stimulation of protein synthesis in mitochondria from X. laevis by poly(U) and the effect of ethidium bromide on this activity

Mitochondria were incubated as described in METHODS. Poly(U), when present, was added at a concentration of 100 μg/ml. Mitochondrial protein concentration was approximately 2 mg/ml in Expt. 1 and approximately 5 mg/ml in Expt. 2.

Expt.	Additions	pmoles [^{14}C]phe incorp./mg protein	
		- poly(U)	+ poly(U)
1	None	0.2	5.4
	+ Chloramphenicol (80 μg/ml)	0.1	3.9
	+ Cycloheximide (20 μg/ml)	0.3	5.3
	+ Ribonuclease (40 μg/ml)	0.2	4.8
2	None	0.16	2.25
	+ Ethidium bromide (1 μg/ml)	0.15	2.0
	+ Ethidium bromide (2 μg/ml)	0.14	1.2
	+ Ethidium bromide (5 μg/ml)	0.1	0.54

TABLE II: Lack of effect of ethidium bromide on uptake of ^{3}H-labelled poly(U) into ribonuclease-resistant form by X. laevis mitochondria

Uptake was followed as described in METHODS. Values are the means of duplicate determinations and have been corrected for uptake at zero time (54 cpm).

Additions	Ribonuclease-resistant [^{3}H] cpm per assay
None	3613
+ Ethidium bromide (1 μg/ml)	3456
+ Ethidium bromide (2 μg/ml)	3737
+ Ethidium bromide (5 μg/ml)	3960
+ Ethidium bromide (10 μg/ml)	2840

Uptake of ^{3}H-labelled poly(U):

This was followed by incubation of a mitochondrial suspension (about 15 mg protein/ml) in medium I with 21 μg ^{3}H-labelled poly(U), spec. act. 1.3 x 10^{4} dpm/μg (Miles) in a total volume of 0.2 ml for 3 min at 22°C. 0.4 ml 100 mM K-phosphate buffer (pH 7.0), contain-

ing 400 μg pancreatic ribonuclease was then added and the incubation continued for a further 10 min to degrade extraneous poly(U). The reaction was terminated by addition of 5 ml ice-cold medium I and immediate filtration of the suspension through a Millipore filter (HAWP). Each filter was washed by suction with 3 x 3-ml portions of medium I, followed by 3 x 3-ml portions of 5% trichloroacetic acid. Filters were then dried and counted.

RESULTS

Results presented in Table I confirm the finding of Swanson [1] that incorporation of phenylalanine by isolated X. laevis mitochondria is greatly stimulated by poly(U). Activity is insensitive to ribonuclease and cycloheximide and, in common with poly(U)-programmed protein synthesis carried out by isolated bacterial [8] or mitochondrial ribosomes [9], is partially inhibited by chloramphenicol. Although Xenopus mitochondrial preparations are difficult to obtain completely sterile, a contribution of contaminating bacteria is improbable, since bacterial cultures derived from mitochondrial preparations do not show stimulation of phenylalanine incorporation by poly(U) (experiments not shown). It therefore seems likely that mRNA import occurs, followed by translation of the poly(U) on mitochondrial ribosomes within the mitochondrial matrix. Poly(U,C) was also found to stimulate protein synthesis, but to a lesser extent than poly(U); RNA from the bacteriophage MS2 was completely inactive (results not shown).

The ability to respond to added mRNA seems so far peculiar to Xenopus; no uptake has been detected with mitochondria from Tetrahymena (2 preparations), yeast (8 preparations), chick liver (1 preparation) or rat liver (1 preparation), using either conditions devised by Swanson [1] for mRNA uptake by X. laevis mitochondria or

those determined to be optimal for protein synthesising activity of the isolated mitochondria from the various organisms. Mitochondria from all sources displayed nuclease activity, but this was insufficient to account for the total absence of uptake activity. Further, previous treatment of mitochondria with puromycin to bring about run-off of existing polysomes had no effect on the lack of response to poly(U).

If mRNA import indeed occurs, the observation (Table I) that ethidium bromide inhibits poly(U)-stimulated incorporation is unexpected and inconsistent with the idea that the sole effect of this compound on protein synthesis is secondary to the inhibition of mitochondrial transcription. Since assay of protein synthesis is conducted in the presence of an ATP-regenerating system, it is unlikely that ethidium blocks activity via an effect on mitochondrial respiration or phosphorylation [see also 6]. Furthermore, as Table II shows, ethidium is without effect on uptake by isolated mitochondria of ^{3}H-labelled poly(U) into a ribonuclease-resistant form. It seems probable, therefore, that ethidium exerts a direct effect on mitochondrial translation.

DISCUSSION

Uptake of synthetic polynucleotides by isolated mitochondria is suggestive evidence for the existence of mRNA import by mitochondria *in vivo*. We have confirmed Swanson's finding [1] that incorporation of phenylalanine by mitochondria isolated from *Xenopus laevis* is stimulated by poly(U). Further, the properties of the stimulated activity are consistent with the idea that the poly(U) is being translated on mitochondrial ribosomes.

The strong inhibition of mitochondrial protein synthesis produced within minutes of addition of ethidium to yeast [4,5] or rat

liver [6] has been considered a strong argument against the existence of mRNA import into mitochondria on any major scale, even though direct effects of ethidium on translation could not be excluded [cf. 10]. Our finding that translation of imported mRNA is sensitive to ethidium reveals a direct effect of this agent on mitochondrial protein synthesis and demonstrates that caution should be used in the interpretation of experiments involving the use of ethidium bromide as a probe for the presence of nuclear mRNAs within mitochondria.

Although Dawid [11] has proposed that RNA import might constitute a major form of information transfer from nucleus to mitochondria, there are several arguments which make this unlikely [12]. Nevertheless, tRNA import would seem to be the only really satisfactory means of compensating for the deficiency of tRNAs within mitochondria and mRNA import might fulfill certain specific roles in mitochondrial biogenesis.

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REFERENCES

[1] Swanson, R.F., Nature 231, 31-34 (1971)
[2] Gaitskhoki, V.S., Kisselev, O.I. and Neifakh, S.A., FEBS Letters 31, 93-96 (1973)
[3] Zylber, E., Vesco, C. and Penman, S., J.Mol.Biol. 44, 195-204 (1969)
[4] Schatz, G., Groot, G.S.P., Mason, T., Rouslin, W., Wharton, D.C. and Saltzgaber, J., Fed.Proc. 31, 21-29 (1972)
[5] Mahler, H.R. and Dawidowicz, K., Proc.Natl.Acad.Sci.U.S. 70, 111-114 (1973)

[6] Kroon, A.M. and De Vries, H., in Autonomy and Biogenesis of Mitochondria and Chloroplasts (Boardman, N.K., Linnane, A.W. and Smillie, R.M., eds.), North-Holland, Amsterdam, pp. 318-327, 1971
[7] Dawid, I.B., J.Mol.Biol. 12, 581-599 (1965)
[8] Kućan, Z. and Lipmann, F., J.Biol.Chem. 239, 516-520 (1964)
[9] Grivell, L.A., Reijnders, L. and Borst, P., Biochim.Biophys. Acta 247, 91-103 (1971)
[10] Lederman, M. and Attardi, G., Biochem.Biophys.Res.Commun. 40, 1492-1500 (1970)
[11] Dawid, I.B., in Control of Organelle Development (Miller, P.L., ed.), Cambridge University Press, Cambridge, pp. 227-246, 1970
[12] Borst, P. and Grivell, L.A., Biochemie 55, in the press (1973)