Messenger Activity of Ribonucleic Acid from Yeast Mitochondria

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Total yeast mitochondrial RNA was shown to possess messenger RNA activity when injected into oocytes of the frog *Xenopus laevis*. The specific polypeptides formed were precipitated by mitochondrial antisera. A comparison was made of the molecular weights of the proteins obtained from this system with those made by mitochondria *in vivo* in the presence of cycloheximide. No RNA containing poly(A) sequences was detected in yeast mitochondria.

By using different inhibitors, several workers have shown that specific mitochondrial proteins are synthesized on mitochondrial ribosomes (Coote & Work, 1971; Thomas & Williamson, 1971). The relatively small quantity of DNA in mitochondria may therefore code for these proteins, and, if so, the mitochondrial DNA must be transcribed into active mRNA. We have now shown that the RNA extracted from yeast mitochondria behaves as a messenger when injected into *Xenopus* oocytes, and that, moreover, the polypeptides so produced are specifically precipitated by antisera prepared against yeast mitochondrial proteins. The polypeptides synthesized in response to mitochondrial mRNA had much the same range of molecular weights as those polypeptides synthesized within yeast mitochondria, when whole yeast cells were incubated in the presence of cycloheximide (Tzagoloff & Akai, 1972). It has been reported that a poly(A)-rich fraction can be recognized in the RNA from the mitochondria of animal cells (Perlman *et al.*, 1973; Avadhani *et al.*, 1973). The same techniques applied to yeast mitochondrial RNA failed to detect any significant quantity of poly(A) material.

Materials and Methods

Yeast

Saccharomyces cerevisiae strain α DV 147 (original source, R. K. Mortimer) was used. Cultures were grown in Y.E.P. glucose medium [1% yeast extract (Difco, Detroit, Mich., U.S.A.), 2% (w/v) Bacto-peptone (Difco) and 2% (w/v) glucose], at 26°C, to the late-exponential/early-stationary phase.

Preparation of mitochondria

The method described by Thomas & Williamson (1971) was adopted. The cells were broken by using a Braun shaker cooled by a constant flow of CO₂

from a cylinder. The mitochondria were separated by two differential centrifugations, one at 4500g for 10 min, the supernatant then being centrifuged at 7000g for 10 min. The mitochondria were pelleted from this supernatant at 30000g for 20–25 min, and washed with 3 × 30–40 ml of ice-cold TME buffer (0.05M-Tris-HCl, pH 7.4; 0.25M-mannitol; 4mM-EDTA).

RNA preparation

RNA was prepared by the pH 7.0 method of Lee *et al.* (1971), except that tri-isopropyl-naphthalene-sulphonic acid (sodium salt; Eastman Kodak Co., Rochester, N.Y., U.S.A.) was used instead of sodium dodecyl sulphate [a 2% solution in 50mM-Tris-HCl (pH 7.5)–0.1M-NaCl being used as extraction buffer].

Injection of RNA into *Xenopus laevis* oocytes

The method described by Gurdon *et al.* (1971) was used. RNA was dissolved in 'L15 minus methionine' incubation medium (Leibovitz, 1963) and oocytes were injected with a mixture of RNA and [³⁵S]-methionine (The Radiochemical Centre, Amersham, Bucks., U.K.). A solution (usually 10 μ l) of 0.5–0.7 mg of RNA/ml in 'L15 minus methionine' medium and [³⁵S]methionine (sp. radioactivity 160 Ci/mmol) was made up. Between 20 and 30 μ Ci of [³⁵S]methionine were used per 40 oocytes, and each oocyte was injected with approx. 150–200 nl.

After incubation overnight at room temperature (20°C), the oocytes were taken up in 1% (v/v) Nonidet P.40 (BDH, Poole, Dorset, U.K.) in phosphate-buffered saline (0.15M-NaCl–4mM-KCl–2mM-KH₂PO₄–8mM-Na₂HPO₄) and gently homogenized. This homogenate was centrifuged at 4500g for 10 min, and the supernatant carefully pipetted off, avoiding the lipid layer floating on top. Mitochondrial antisera were used to precipitate specific proteins

from this supernatant. The precipitate was formed by adding 20 μ l of mitochondrial antisera to the supernatant. The mixture was incubated at 37°C for 30 min, then 200 μ l of goat anti-rabbit serum was added, and the mixture incubated for a further 45 min. To complete the precipitation, the tubes were left either on ice for 1–2 h, or at +4°C overnight. The precipitate was centrifuged down at 9000 *g* for 10 min and washed with 3 $\times$ 3 ml of phosphate-buffered saline.

Gel electrophoresis of proteins

For this 7% polyacrylamide–sodium dodecyl sulphate gels were prepared by the method of Weber & Osborn (1969). Ethylene diacrylate replaced bis-acrylamide (0.46%, w/v) as the cross-linker. The gels were electrophoresed at 5 mA/tube for 16 h. The gels were sliced into 1 mm sections, digested for 2 h with 0.2 ml of NH₃ (sp.gr. 0.880); after 2 h, 8 ml of dioxan scintillation fluid [250 g of naphthalene and 10 g of PPO (2,5-diphenyloxazole) in 2.5 litres of dioxan] was added to each vial, and samples were counted for radioactivity for 5–10 min in a Packard liquid-scintillation counter (89% efficiency).

Electrophoresis in formamide gels

The method described by Duesberg & Vogt (1973) was used. After electrophoresis, the gels were frozen and sliced into 1 mm sections; the frozen slices were placed in vials, and digested in Protosol (New England Nuclear Corp., Boston, Mass., U.S.A.) scintillation mixture (125 ml of Protosol in 2.5 litres of toluene, plus 10 g of PPO). The vials were then counted for radioactivity as described above.

Preparation of antisera

Antisera against mitochondrial proteins were prepared in Sandylop rabbits [mitochondria were prepared as described above, and sonicated by using a Soniprobe (Dawe Instruments Ltd., Acton, London W.3, U.K.; type 7530A) at mark 5 for 15 s, two to four times until the pitch changed, indicating complete breakage]. Antigen (2–3 mg) was mixed with an equal volume of complete Freund's adjuvant (Difco). This was injected subcutaneously into two sites on the hindquarters of the rabbit, and after 7 days the procedure was repeated. Then 5 weeks later, a boost of 3–5 mg of alum-precipitated antigen was given, injected intravenously over a period of 10 days in increasing doses (e.g. 0.5 mg, 1.0 mg, 1.5 mg and 2.0 mg) and the rabbit was bled on days 7 and 10 after boosting. The serum was decanted by heating at 57°C for 30 min; the titre (Humphrey & White, 1970) was then calibrated.

Poly(A) digest

[³H]Adenine-labelled mitochondrial RNA and cytoplasmic RNA were dissolved in MSB buffer (10 mM-Tris-HCl, pH 7.4; 100 mM-NaCl; 10 mM-MgCl₂), as described by Perlman *et al.* (1973), and treated with deoxyribonuclease (20 μ g/ml; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 1 min at 37°C. The mixture was adjusted to 0.3 M-NaCl–0.3 M-sodium citrate, and RNA was digested with T₁ ribonuclease [50 units/ml, 1 unit being defined as that sufficient to produce acid-soluble oligonucleotides equivalent to an *E*₂₆₀ of 1.0 in 15 min at pH 7.5 and at 37°C (in a reaction mixture of volume 1.0 ml)] and pancreatic ribonuclease (20 μ g/ml) for 30 min at 37°C [both T₁ and pancreatic ribonuclease were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]. The reaction was stopped by adding 5 μ l of diethyl pyrocarbonate and the RNA analysed on 10% (w/v) polyacrylamide–sodium dodecyl sulphate gels.

Labelling of mitochondrial protein in the presence of cycloheximide in vivo

The method used was based on that described by Thomas & Williamson (1971). Cells were grown to the late-exponential phase, harvested, washed in 0.05 M-potassium phosphate buffer (pH 7.4) and resuspended to give between 10⁸ and 10¹⁰ cells/ml, in 20 ml of media containing 0.05 M-phosphate buffer (pH 7.4)–ethanol (4%)–glucose (0.5%). Cycloheximide was added at a concentration of 100 μ g/ml, and the cells were incubated at 26°C for 15 min. [³⁵S]Methionine (8–10 μ Ci/ml) was then added to the cell culture, and incubated for a further 2 h at 26°C. The cells were harvested, washed, and a mitochondrial pellet was obtained as described above. The mitochondria were sonicated as described for the preparation of antisera.

Results

Formamide gels were used to show the distribution of yeast mitochondrial RNA and cytoplasmic RNA types, and the results of this gel system are shown in Fig. 1. This system has the advantage that the formamide unfolds the secondary structure, so that the different types of RNA separate according to their size.

*Injection of RNA into *Xenopus laevis* oocytes*

Various quantities of mitochondrial RNA and cytoplasmic RNA were injected into oocytes in order to determine the optimum concentrations required to

Fig. 1. Analysis of RNA by formamide-polyacrylamide-gel electrophoresis

Cells were labelled for 16 h with [^{32}P]P_i in a medium containing 1% yeast extract, 2% (w/v) peptone and 2% (w/v) glucose. RNA was extracted and the gel electrophoresis and analysis were performed as described in the Materials and Methods section. (a) Mitochondrial RNA; (b) cytoplasmic RNA; (c) mitochondrial (M) and cytoplasmic (C) RNA. Gels (a) and (b) were electrophoresed for 6–8 h at 80 V until the Bromophenol Blue marker dye was 1–2 cm from the bottom of the gel (total gel length approx. 11–12 cm). Gel (c) was electrophoresed until the dye had reached the bottom of the gel.

stimulate the system. RNA was then injected into the oocytes, and the specific polypeptides formed were precipitated by the antisera to mitochondrial proteins. Samples of these precipitates were counted for radioactivity (Table 1), and the remaining precipitates were electrophoresed on polyacrylamide-sodium dodecyl sulphate gels (Fig. 2).

The apparent molecular weights of proteins obtained when mitochondrial RNA was injected into oocytes were compared with those of mitochondrial proteins synthesized *in vivo* in the presence of cyclo-

heximide. The distribution of the latter is also shown in Fig. 2.

Poly(A) attached to mitochondrial RNA

During the present work, different methods for the isolation of mitochondrial mRNA from total mitochondrial RNA were tried. Most eukaryotic mRNA can be isolated by making use of the characteristic poly(A) sequence at the 3' end of the messenger.

A variety of techniques have been used in this search, including the binding of poly(A)-containing

RNA to various filters (Sheldon *et al.*, 1972; Lee *et al.*, 1971), binding to cellulose columns (Schutz *et al.*, 1972; Kitos *et al.*, 1972), and digestion with nucleases (Perlman *et al.*, 1973).

The filter-binding assay was unsatisfactory, as the amount of binding was variable between different preparations. The cellulose column provided a crude separation of RNA, although it did not cause enhancement of messenger activity.

By using a combination of nucleases, RNA can be digested to leave only adenosine residues. The products of this digestion were run on a 10% (w/v) polyacrylamide-sodium dodecyl sulphate gel. Digestion products from yeast cytoplasmic RNA and mitochondrial RNA were run, together with marker [^3H]ATP, and Bromophenol Blue (Fig. 3).

Table 1. *Precipitation of radioactive proteins with mitochondrial antisera from *X. laevis* oocytes injected with RNA*

RNA and [^{35}S]methionine were injected into oocytes, and incubated at 20°C for 16 h. Each sample consisted of 40 oocytes into which 20–30 μCi of [^{35}S]methionine was injected. After incubation, the oocytes were homogenized, and mitochondrial antisera were added (as described in the Materials and Methods section). The radioactivity recovered in the precipitates was measured as described in the Materials and Methods section.

Material injected into oocytes	Radioactivity in sample precipitate (c.p.m.)
Mitochondrial RNA	16 800
Cytoplasmic RNA	9500
Rabbit rRNA	6400
Incubation medium	5000

Fig. 2. Analysis on polyacrylamide-sodium dodecyl sulphate gels of the antisera-precipitated products of the injected oocytes, and the mitochondrial proteins labelled *in vivo* in the presence of cycloheximide

Electrophoresis and analysis was performed as described in the Materials and Methods section. (a) shows the relationship between the log of the molecular weight and the distance migrated. (b) Pattern *in vivo*. (c) Pattern from oocyte system directed by: ■, mitochondrial RNA; ○, cytoplasmic RNA; △, rabbit rRNA; ▲, control, no RNA injected.

Fig. 3. Polyacrylamide-sodium dodecyl sulphate-gel electrophoresis of nuclease-digested RNA

Cells were labelled for 2h with [^3H]adenine ($2\mu\text{Ci/ml}$) and mitochondrial RNA and cytoplasmic RNA were extracted. These RNA types were digested and analysed as described in the Materials and Methods section. Similar amounts of radioactivity were used in each case. ●, Cytoplasmic RNA; ○, mitochondrial RNA; ▲, [^3H]ATP.

Discussion

The present study was undertaken to investigate mRNA activity in yeast mitochondria, and to isolate and identify any products for which mitochondrial mRNA might code.

When subjected to gel electrophoresis, the mitochondrial RNA preparation appears to be almost completely free of cytoplasmic contamination. The nucleic acid types in the mitochondrial fraction, which migrate 11 mm and 14 mm into the mitochondrial gel, and 19 mm and 23 mm into the mitochondrial and cytoplasmic gel, represent DNA and double-stranded RNA respectively (Bevan *et al.*, 1973; M. J. Eggitt, unpublished work), and are characteristic of many yeast strains. These types are always associated with the mitochondrial pellet under the conditions of isolation used. It is necessary to illustrate the clean separation of mitochondrial RNA, as it could be argued that cytoplasmic rRNA contaminating the preparation could cause the observed stimulation of protein synthesis in the oocytes. Kellems *et al.* (1974) have reported the binding of 80S cytoplasmic ribosomes to the outer mitochondrial membrane. This is a possible source of contamination of the mitochondrial RNA prepared. However, when contamination of the mitochondrial preparation occurs, only the cytoplasmic large-subunit ribosomal RNA is observed. This suggests that the 80S ribosomes are attached to the outer

mitochondrial membrane by their large subunit [see Dobberstein *et al.* (1974) for observations about the attachment of ribosomes to membranes by the large subunit] and that the mRNA and small subunit are removed during isolation. However, the results indicate very little cytoplasmic contamination and the injection of cytoplasmic RNA into oocytes did not stimulate the synthesis of discrete polypeptides precipitable with mitochondria antisera.

The injection of total mitochondrial RNA into *X. laevis* oocytes demonstrates the presence of an mRNA activity that is capable of directing the synthesis of polypeptides precipitable by antisera prepared against mitochondrial proteins (Table 1). The antisera may also precipitate polypeptides which are antigenically similar to the mitochondrial proteins. In addition, smaller polypeptides, and possibly free [^{35}S]methionine, may be trapped by the antigen-antiserum complex. The latter is probably the explanation for most of the precipitated radioactivity observed in the controls, and these small polypeptides etc. migrate more rapidly on electrophoresis than does the Bromophenol Blue marker dye, and thus are not detected on subsequent slicing and counting for radioactivity (A. H. Scragg, unpublished work).

The molecular weights of the polypeptides produced when mitochondrial RNA was injected into oocytes compare well with those of the proteins

synthesized *in vivo* in the presence of cycloheximide (Fig. 2). The antigenicity of proteins varies greatly, some being very antigenic, others not at all, so that antisera against a wide range of proteins (e.g. this mitochondrial antiserum), would not necessarily give a true picture of the actual quantity of a particular protein present. In addition, the differences in relative quantities produced can be explained by a variation in the translational efficiency of the oocyte system, and possibly even a variation in the actual quantity of some of the mRNA extracted.

High-molecular-weight proteins, not detected *in vivo*, are produced when mitochondrial RNA is injected into oocytes. This is interesting in view of the fact that double-stranded RNA and DNA were injected into oocytes, along with the mitochondrial RNA. Reports suggest that this double-stranded RNA is part of a virus particle coding for virus coat protein (Herring & Bevan, 1974). This raises the possibility that the double-stranded RNA and DNA may give rise to a functional messenger in the oocyte system. Alternatively, this high-molecular-weight material could represent products present *in vivo* in quantities too small to be detected other than by the use of antisera.

The lack of poly(A) in mitochondria contrasts with the results obtained in animal-cell mitochondria. Yeast cytoplasmic poly(A) sequences are detectable, but by using a variety of techniques, yeast mitochondrial poly(A) has not been found. In addition, it appears that the mitochondrial mRNA molecules are actively translated, despite their lack of poly(A). However, Cooper & Avers (1974) state that they have found poly(A) sequences in mitochondria, whereas Groot *et al.* (1974) have reported the lack of poly(A) attached to yeast mitochondrial RNA.

Since the original submission of the present paper, total mitochondrial RNA has been used successfully in an *Escherichia coli* cell-free protein-synthesizing system, stimulating the incorporation of [³⁵S]-methionine into discrete polypeptides, giving a good comparison with the results obtained in the oocytes (M. J. Eggitt, unpublished work).

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References

- Avadhani, W., Kuan, M., Van Der Lign, P. & Rutman, R. (1973) *Biochem. Biophys. Res. Commun.* **51**, 1090–1096
- Bevan, E. A., Herring, A. J. & Mitchell, D. J. (1973) *Nature (London)* **245**, 81–86
- Cooper, C. & Avers, C. (1974) *The Biogenesis of Mitochondria* (Kroon A. M. & Saccone, C., eds.), pp. 289–303, Academic Press, New York
- Coote, J. & Work, T. S. (1971) *Eur. J. Biochem.* **23**, 564–574
- Dobberstein, B., Volkmann, D. & Klambt, D. (1974) *Biochim. Biophys. Acta* **374**, 187–196
- Duesberg, P. & Vogt, P. (1973) *J. Virol.* **12**, 594–599
- Groot, G. S. P., Flavell, R. A., Van Ommen, G. J. B. & Grivell, L. A. (1974) *Nature (London)* **252**, 167
- Gurdon, J., Lane, C., Woodland, H. & Marbaix, G. (1971) *Nature (London)* **233**, 177–182
- Herring, A. J. & Bevan, E. A. (1974) *J. Gen. Virol.* **22**, 387–394
- Humphrey, J. H. & White, R. G. (1970) *Immunology for Students of Medicine*, 3rd edn., pp. 350–352, Blackwell Scientific Publications, Oxford and Edinburgh
- Kelless, R. E., Allison, V. F. & Butow, R. A. (1974) *J. Biol. Chem.* **249**, 3297–3303
- Kitos, P., Saxon, G. & Amos, H. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1426–1437
- Lee, S. Y., Mendecki, J. & Brawerman, G. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1331–1335
- Leibovitz, A. (1963) *Am. J. Hyg.* **78**, 173–180
- Perlman, S., Abelson, H. & Penman, S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 350–353
- Schutz, G., Beato, M. & Feigelson, P. (1972) *Biochem. Biophys. Res. Commun.* **49**, 680–689
- Sheldon, R., Jurale, C. & Kates, J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 417–421
- Thomas, D. Y. & Williamson, D. (1971) *Nature (London)* **233**, 196–199
- Tzagoloff, A. & Akai, A. (1972) *J. Biol. Chem.* **247**, 6517–6523
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412