A Simple Method of Characterizing Mitochondrial Ribonucleic Acid

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A simple method of isolating and characterizing RNA from L-cell mitochondria is described. The mitochondrial fraction is lysed by sodium dodecyl sulphate, and the RNA fractionated by sucrose-density-gradient centrifugation. The efficacy of proteinase K in preventing ribonuclease activity is also demonstrated.

In the isolation of RNA from mitochondria, several problems are encountered. First, repeated washing and density-gradient centrifugation or treatment with digitonin are necessary to prepare a mitochondrial fraction free from contamination by cytosol ribosomes (Bartoov *et al.*, 1970; Malkin, 1971; De Vries & Van de Koogh-Schuuring, 1973). Secondly, there is often much RNAase‡ activity associated with the mitochondrial fraction, resulting in degradation of the mitochondrial RNA. Further, the commonly used methods for the extraction of RNA are often laborious and time-consuming (Penman, 1966; Dubin & Montencourt, 1970; Attardi & Attardi, 1971; Bartoov *et al.*, 1970).

In the present paper, we describe a simple method of isolation and partial characterization of high-molecular-weight mitochondrial RNA from mammalian cells in which mitochondrial but not cytosol rRNA has been labelled. It has been reported by Wieggers & Hilz (1971) that proteinase K can efficiently inactivate RNAase. In the present method, a mixture of proteinase K and sodium dodecyl sulphate has been used to protect the mitochondrial RNA from RNAase action, and the RNA was fractionated on an isokinetic sucrose density gradient.

Methods

The proteolytic activity of proteinase K was measured by following the release of free amino groups (Habeeb, 1966) from bovine serum albumin (1 mg/ml in 0.01 M-Tris-HCl, pH 7.4), at room temperature usually with 200 µg of enzyme per ml. The amount of self-hydrolysis in the presence of sodium dodecyl sulphate is small. Proteinase K,

chromatographically purified, was obtained from E. Merck (Darmstadt, Germany). Methods of growth of L cells, labelling of RNA with [5-³H]uridine in the presence of actinomycin D and the isolation of a crude mitochondrial fraction have been described previously (Bartoov *et al.*, 1970). Mitochondria were further purified on a 1.0–1.5 M-sucrose step gradient; the band at the lower interface was diluted and recovered from 0.3 M-sucrose–2 mM-EDTA, pH 7.4, by centrifugation at 12000g for 15 min.

Standard method of RNA extraction. For extracting mitochondrial RNA, the mitochondrial pellet (4 mg of protein) from 2.5×10^8 cells was resuspended in 0.1 M-NaCl–0.01 M-Tris-HCl, pH 7.4, and digested with 0.5 mg of proteinase K/ml, in the presence of 1 mg of dextran sulphate/ml and 0.5% sodium dodecyl sulphate in a final volume of 1 ml at 25°C for 1 h. One-half of the solution with 10000 c.p.m. of ¹⁴C-labelled cytosol rRNA was layered on top of a 12 ml convex sucrose density gradient, from 15 to 33% sucrose in 0.1 M-NaCl–0.001 M-EDTA–0.01 M-Tris-HCl, pH 7.4, and 0.5% sodium dodecyl sulphate (Noll, 1967). RNA was separated by centrifugation at 25°C in an SW 41 rotor in a Beckman L2-65B ultracentrifuge at 69400g for 16 h. After piercing the bottom of the tube, 15-drop fractions were collected directly into mini-shell vials. They were then mixed with 0.2 ml of water and 3 ml of PCS Solubilizer (Amersham/Searle, Don Mills, Ont., Canada), and counted for radioactivity in a Nuclear-Chicago Mark 1 liquid-scintillation counter.

When RNAase was used to digest extramitochondrial RNA, the RNA was extracted as above except that 1% sodium dodecyl sulphate and 1 mg of proteinase K/ml were used. These higher concentrations were found necessary in this case to prevent RNAase action.

Bovine pancreatic RNAase was type IIA from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and electrophoretically purified bovine pancreatic DNAase was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

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‡ Abbreviations: RNAase, ribonuclease; DNAase, deoxyribonuclease.

Fig. 1. Extraction and characterization of mitochondrial RNA

L cells were labelled for 16h with $[5\text{-}^3\text{H}]\text{uridine}$ (29 Ci/mmol; $1\text{ }\mu\text{Ci/ml}$) in the presence of $0.05\text{ }\mu\text{g}$ of actinomycin D/ml. After purification through a step gradient, the mitochondrial pellet from 2.5×10^8 cells was digested with 0.5 mg of proteinase K/ml, 1 mg of dextran sulphate/ml and 0.5% sodium dodecyl sulphate in 1 ml of $0.1\text{ M-NaCl-}0.01\text{ M-Tris-HCl}$, pH 7.4, for 1 h at 25°C , and then 0.5 ml was characterized by centrifugation as described under 'Methods'.

Results

The effect of various conditions on the activity of proteinase K was examined by following the hydrolysis of bovine serum albumin. Sodium dodecyl sulphate up to a concentration of 1% did not affect the activity. The activity was the same between pH 5 and 8, and was 60% greater at pH 9.0. Dextran sulphate at concentrations up to 0.8 mg/ml did not inhibit, but at 1 mg/ml it inhibited 10% . The insensitivity of proteinase K to these changes in the medium allow considerable latitude in its utilization.

When L cells were labelled with $[5\text{-}^3\text{H}]\text{uridine}$ in the presence of actinomycin D, the RNA from the lysed mitochondrial fraction showed the pattern given in Fig. 1. The results clearly demonstrated the presence of mitochondrial rRNA sedimenting at approx. 16S and 14S. In Fig. 2(a) these components were at 15S with a shoulder at 13S, and will be referred to as the 15S and 13S rRNA. The values obtained are similar to that reported earlier from this laboratory (Bartoov *et al.*, 1970). Omission of dextran sulphate during the digestion of the mitochondrial fraction with sodium dodecyl sulphate did not change the sedimentation pattern of the mitochondrial rRNA. When proteinase K was omitted there was either no change in the sedimentation pattern or in some experiments the 15S peak

Fig. 2. Effect on mitochondrial RNA of RNAase treatment of mitochondria with or without subsequent inactivation with proteinase K

L cells (2.5×10^8) were labelled for 1 h with $[5\text{-}^3\text{H}]\text{uridine}$ at $0.5\text{ }\mu\text{Ci/ml}$ in the presence of $0.05\text{ }\mu\text{g}$ of actionmycin D/ml. The crude pellet was then treated with either (a) DNAase at $50\text{ }\mu\text{g/ml}$ for 10 min in the cold or (b) and (c) RNAase at $10\text{ }\mu\text{g/ml}$ at room temperature followed by DNAase. DNAase treatment was in $0.3\text{ M-sucrose-}2\text{ mM-MgCl}_2\text{-}2\text{ mM-Tris-HCl}$, pH 7.4. The mitochondria were further fractionated on a sucrose step gradient as described under 'Methods'. The mitochondria of (c) only were further treated with proteinase K at 1 mg/ml for 20 min at room temperature in $0.1\text{ M-NaCl-}0.01\text{ M-Tris-HCl}$, pH 7.4, before lysis. All mitochondria were lysed in 1.0% sodium dodecyl sulphate, and the RNA was then characterized as described under 'Methods'. ○, Cytosol RNA (^{14}C c.p.m.); ●, mitochondrial RNA (^3H c.p.m.). The arrows give the S values.

was not as sharp and the 13S species was present as a shoulder rather than as a peak.

The method was further tested to test whether proteinase K could inactivate RNAase added to intact mitochondria to remove extramitochondrial RNA. In this experiment, the mitochondrial RNA was labelled for a short time, and, as can be seen in Fig. 2(a), it shows several features different from those in Fig. 1. There is more RNA sedimenting at greater than 18S, including a component at 21S characteristic of mitochondrial RNA labelled for short periods of time (Dubin & Czaplicki, 1970, 1971; Fukamachi *et al.*, 1972), and the 13S component appears as a shoulder on the 15S component. The latter might be expected in the light of the results of Dubin (1972) in which there was a lower ratio of the larger to smaller mitochondrial rRNA components in short-term labelling. As shown in Fig. 2(b), treatment of the mitochondrial fraction with RNAase followed by purification of the mitochondria on a step gradient before lysis with sodium dodecyl sulphate caused a substantial amount of degradation of the mitochondrial RNA, as judged by the higher amount of 13S RNA compared with the 15S peak. Further, RNAase eliminated all RNA sedimenting faster than 25S. This is probably extramitochondrial RNA as, in control experiments in which the synthesis of mitochondrial RNA but not RNA in the nucleus was inhibited with ethidium bromide (Zylber *et al.*, 1969), RNAase treatment was found to remove all RNA which sediments faster than 7S. When mitochondria treated with RNAase and purified on a step gradient were incubated with proteinase K before lysis, the pattern of the mitochondrial rRNA was similar to Fig. 2(a) with the 15S component being greater than the 13S shoulder (Fig. 2c). The component at 21S seen in Fig. 2(a) was also present in Figs. 2(b) and 2(c) although the exact position was variable, perhaps depending on the amount of other species of RNA present.

Discussion

The method described is a simple procedure for the characterization of RNA components in a mitochondrial fraction. Both sodium dodecyl sulphate and

proteinase K were necessary to obtain the least-degraded RNA. Proteinase K was necessary to prevent the degradative effect of RNAase on mitochondrial RNA when it was used to degrade extramitochondrial RNA. Pronase has been used to inactivate DNAase added to remove extramitochondrial DNA (Ojala & Attardi, 1974). RNAase in the present experiments degraded all the extramitochondrial non-ribosomal RNA.

The method presented here for characterizing RNA from mitochondria does not involve laborious and time-consuming extraction of RNA as previously reported (Penman, 1966; Bartoov *et al.*, 1970; Dubin & Montencourt, 1970; Attardi & Attardi, 1971). It should prove useful in experiments requiring a rapid characterization of mitochondrial RNA from a variety of sources especially where RNAase is a problem.

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