Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*

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Summary

Mitochondrial nucleoids (mt-nucleoids) of the yeast, *Saccharomyces cerevisiae*, were isolated from spheroplasts of stationary phase cells and their structure and organization were investigated by fluorescence microscopy, electron microscopy, and biochemical techniques. Isolated mt-nucleoids were spherical or ovoid and 0.3-0.6 μm in diameter, and were about the same size and shape as those observed in the cell by the DAPI staining technique.

Measurement of DNA content of mt-nucleoids, using a video-intensified microscope system, after DAPI staining revealed that a mt-nucleoid in spheroplasts from stationary phase cells contains, on average, 3.9 mtDNA molecules and an isolated mt-nucleoid contains, on average, 3.1.

Negatively stained electron micrographs showed that mt-nucleoids consist of particles 20-50 nm in diameter. SDS–polyacrylamide gel electrophoresis of mt-nucleoids detected 20 species of polypeptides in the molecular weight range from $10 \times 10^3$ to $70 \times 10^3$. Acid–urea/SDS two-dimensional electrophoresis of acid extract from mt-nucleoids indicated that a polypeptide of $20 \times 10^3$ is the only major polypeptide with basic property like histones.

Key words: yeast, *Saccharomyces cerevisiae*, mitochondrial nucleoids, DAPI, isolation.

Introduction

During the past several years considerable efforts have produced great progress in the study of mitochondrial genes and their products. The mitochondrial genome of the yeast, *Saccharomyces cerevisiae*, is at present one of the best characterized organelle genomes in eukaryotic cells.

It encodes the genetic information for essential mitochondrial components: namely, several mitochondrial proteins, mitochondrial rRNAs and tRNAs (Dujon, 1981). These mitochondrial genomes have been known to be localized at the electron-transparent area named mitochondrial nucleoids (mt-nucleoids) in the matrix from electron microscopic examinations. But there is still little valuable information on the state of mitochondrial genomes in *vivo*.

Protein–DNA complex has been isolated in mitochondria of the slime mold *Physarum polycephalum* (Kuroiwa et al. 1976; Suzuki et al. 1982; Kuroiwa, 1982), HeLa cells (Albring et al. 1977), *Xenopus laevis* (Pinon et al. 1978; Rickwood & Jurd, 1978), sea-urchin embryos (Sevaljević et al. 1978, 1979), rat liver (Van Tuyle & McPherson, 1979; Van Tuyle & Pavco, 1985) and *Paramecium aurelia* (Olszewska & Tait, 1980). DNA–protein complex has been isolated from the mitochondria of the yeast, *S. cerevisiae* (Rickwood et al. 1981; Rickwood & Chambers, 1981). However, with the exception of studies on isolated mt-nucleoids from *P. polycephalum*, which revealed that isolated mt-nucleoids have the same shape, size and DNA content as in *vivo*, the question remains ambiguous as to whether isolated DNA–protein complexes correspond to the mt-nucleoids observed in the electron microscope and whether they retain the morphological intactness.

We previously reported on fusing and separating figures of mitochondrial nucleoids during the life cycle.
of yeast using the fluorescence microscope and DNA-binding fluorescent dye, 4',6-diamidino-2-phenyindole (DAPI) (Sando et al. 1981; Miyakawa et al. 1984). By DAPI staining, mt-nucleoids in spheroplasts of stationary phase cells were clearly observed as spherical fluorescent particles dispersed in the cytoplasm. The use of DAPI and fluorescence microscopy also provide a useful means for detection and isolation of DNA-containing particles like mt-nucleoids.

The present paper describes the isolation of morphologically intact mt-nucleoids from spheroplasts and some structural and biochemical characteristics of isolated mt-nucleoids.

Materials and methods

Strain and cultivation
The diploid strain G2-2 of S. cerevisiae was cultured aerobically at 30°C to stationary phase in modified Burkholder’s medium supplemented with 3-6% (v/v) tomato extract as described previously (Miyakawa et al. 1984).

Preparation of mitochondrial nucleoids (mt-nucleoids)
Mitochondria and mt-nucleoids were prepared from spheroplasts of stationary phase cells according to the methods of Suzuki et al. (1982) with slight modification.

Cells of about 50 g wet weight were treated with 0-4 M-2-mercaptoethanol (2-ME) at 30°C in SP buffer (0-8 M-sorbitol, 25 mM-potassium phosphate buffer (pH 7-5). After washing with SP buffer, cells were treated with zymolylase 60 000 or 100T (Kirin Brewery Co., Ltd) at 30°C for 1 h in SP buffer to make spheroplasts. Spheroplasts, washed twice with SP buffer, were suspended in NEI buffer (0-3 M-sucrose, 20 mM-Tris•HCl, pH 7-6, 1 mM-EDTA and 0-4 mM-spermidine, 7 mM-2-ME, 0-4 mM-phenylmethylsulphonyl fluoride (PMSF)). The suspension was homogenized in a Waring blender for 45 s at high speed and centrifuged at 950 g for 5 min to remove cell debris. The supernatant was filtered through coffee filter paper placed between two layers of nylon sheet and centrifuged at 1600 g for 5 min. The supernatant was then centrifuged at 2500 g for 5 min and at 12 000 g for 20 min. The sedimented pellet was suspended in NE2 buffer (0-5 M-sucrose, 20 mM-Tris•HCl, pH 7-6, 2 mM-EDTA, 0-8 mM-spermidine, 7 mM-2-ME, 0-4 mM-PMSF) and 0-6 mM-spermine (2 mg) was added. The mixture was kept for 10 min at 0°C, filtered as described above, and centrifuged twice at 3900 g for 5 min. The supernatant was centrifuged at 15 000 g for 20 min to obtain the mitochondrial pellet.

The mitochondrial pellet was thoroughly resuspended in NE2 buffer, and diluted by adding an equal volume of DL buffer (0-25 M-sucrose, 20 mM-Tris•HCl, pH 7-6, 7 mM-2-ME, 0-4 mM-PMSF). To lyse the mitochondrial membrane, 20% Nonidet P-40 (NP-40) was dropped into the solution at 4°C to a final concentration of 0-5% with gentle stirring. The solution was centrifuged at 16 000 g for 10 min to remove NP-40 insoluble materials. The clear supernatant was layered on 20, 40, 60% (v/v) sucrose discontinuous density gradient in the presence of 20 mM-Tris•HCl, pH 7-6, 1 mM-EDTA, 0-6 mM-spermidine, 7 mM-2-ME, 0-4 mM-PMSF, centrifuged in a Hitachi RPS 27-2 swing bucket at 46 000 g for 1 h. The fractions containing mt-nucleoids equivalent to the boundary layer between 20% and 40% sucrose and the boundary layer between 40% and 60% sucrose were collected and pooled.

Fluorescence measurement
Lyzed mitochondrial solution was mixed with 1 ug ml⁻¹ DAPI and the fluorescence intensity of each fraction after density gradient centrifugation was measured with a Shimadzu spectrophotometer RF-500 with the wavelength of excitation at 350 nm and emission at 450 nm.

Biochemical analysis
The fractions containing mt-nucleoids were pooled and centrifuged at 131 000 g for 1 h. Pellets of isolated mitochondria and mt-nucleoids were suspended in 4% trichloroacetic acid (TCA) dissolved in 50% acetic-water (v/v). After 2 h at 0°C, the suspension was centrifuged and the pellet was washed with 4% TCA. Pellets were washed twice with 0-25 m-perchloric acid and then extracted with 0-5 m-perchloric acid at 70°C for 15 min. After centrifugation, the protein content of the pellets was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. DNA concentration in the extract was determined by Burton’s (1956) method using calf thymus DNA as a standard. RNA concentration in the extract was determined by the orcinol reaction (Ceriotti, 1955), using yeast RNA as a standard.

Hoechst 33258–CsCl density gradient centrifugation
Analysis of DNA by Hoechst 33258–CsCl density gradient centrifugation was carried out by the method of Hudspeth et al. (1980). The mitochondrial and nuclear DNA bands were photographed by illuminating the tubes with long wavelength u.v. irradiation.

Photomicroscopy
All observations were made with an Olympus BHS-RFK epifluorescence microscope equipped with phase-contrast objectives. mt-nucleoids were stained with DAPI as described previously (Miyakawa et al. 1984). Photographs were taken at a magnification of ×850 on 35 mm Fuji Neopan (ASA 400) or Fuji Minicopy film. The number of mt-nucleoids in the fraction was estimated from the ratio of the number of mt-nucleoids to the number of polystyrene latex spheres (1·09 μm in diameter, Dow Chemical Co., Midland, Michigan) in the mixed suspension as described by Suzuki et al. (1982).

Measurement of DNA content of mt-nucleoids
The DNA contents of mt-nucleoids were determined by measuring the fluorescence intensity of each mt-nucleoid stained with DAPI, using a video-intensified microscope photon counting system (VIMPCS) (Hamamatsu Photonics Ltd, Hamamatsu, Japan) which was connected to an Olympus epifluorescence microscope BHS-RFK as described previously (Kuroiwa et al. 1986). The fluorescence intensity of T4 phage after DAPI staining was used as the standard to express the relative fluorescence intensity.
Electron microscopy
For electron microscopy, mt-nucleoids were deposited on a mesh-grid coated with carbon film by centrifugation (90,000g for 10 min). Negative staining of the mt-nucleoids was performed with 1% sodium phosphotungstate solution neutralized to pH 7.0 by adding 5 M-NaOH. Protease digestion of isolated mt-nucleoids was carried out as follows. One drop of DL buffer containing pronase E (50 ng ml⁻¹) was added to mt-nucleoids on a mesh grid and samples were incubated at 20°C for 15 min. After fixation by adding 1 drop of DL buffer containing 1% glutaraldehyde, the grid was washed once with distilled water and mt-nucleoids were negatively stained. The specimens were observed with a Hitachi HU 500 transmission electron microscope at an accelerating voltage of 100 kV.

Acid extraction of mt-nucleoids
Acid extraction of proteins from mt-nucleoids was carried out according to the method of Sommer (1978).

Gel electrophoresis
SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli (1970). Acid–urea electrophoresis was carried out according to the method of Panyim & Chalkley (1969) and further resolution was achieved by running samples in the second dimension using the SDS system of Laemmli (1970). Gels were stained with 0.25% Coomassie Brilliant Blue R-250 or by the silver staining technique (Oakley et al. 1980).

Figs 1, 2. Fluorescence and phase-contrast photomicrographs showing the cell nucleus and mt-nucleoids in the spheroplast at stationary phase after DAPI staining. ×5500.

Figs 3–5. The mitochondrial fraction was observed in the same field by fluorescence (Fig. 3), phase-contrast and fluorescence (Fig. 4) and phase-contrast microscopy (Fig. 5). ×5500.
Figs 6, 7. Photomicrographs showing isolated mt-nucleoids observed by fluorescence (Fig. 6) and phase-contrast (Fig. 7) microscopy in the same field. ×5500.

**Fig. 8.** Sucrose density gradient fractionation of yeast mt-nucleoids.

**Results**

*Isolation of mt-nucleoids*

The use of fixed and squashed spheroplasts and the DAPI-staining technique made it possible to measure approximate size of each mt-nucleoid and to count the number of mt-nucleoids in an intact cell (Miyakawa et al. 1984). On a fluorescence microphotograph of a DAPI-stained spheroplast, mt-nucleoids have a diameter ranging from 0.2 to 0.5 μm (Figs 1, 2). As the same size and shape of mt-nucleoids was obtained by vital staining of whole cells and spheroplasts with DAPI, these fluorescent particles were considered to be intact mt-nucleoids *in vivo* in this culture condition.

Mitochondria were released from spheroplasts by homogenization in a Waring blender to effect spheroplast rupture and the release of intact mitochondria. Both phase-contrast and fluorescence photomicrographs of mitochondria showed that each isolated mitochondrion contained one mt-nucleoid (Figs 3–5). Addition of 0.5% NP-40 immediately resulted in lysis of mitochondria and the brown, clear supernatant after precipitation of NP-40-insoluble materials was loaded on a discontinuous sucrose density gradient. Fluorescence measurement and counting of mt-nucleoids showed two peaks corresponding to the boundary between 20% and 40% sucrose and one between 40% and 60% sucrose (Fig. 8). As shown in Figs 6 and 7, fluorescence and phase-contrast microscopy
Fluorescence photographs of Hoechst 33258-CsCl density gradients of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) extracted from: A, spheroplasts; B, isolated mt-nucleoids from the 20 %-40 % sucrose boundary.

Table 1. Chemical analysis of fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>1.0</td>
<td>1.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Mt-nucleoids</td>
<td>1.0</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Mt-nucleoids</td>
<td>1.0</td>
<td>0.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

mt-nucleoids fractions 1 and 2 were obtained from the 20 %-40 % and 40 %-60 % sucrose boundaries, respectively.

showed that isolated mt-nucleoids obtained from the 20 %-40 % sucrose boundary were uniform particles with diameter of 0-3-0-6 μm and were faintly visible in the phase-contrast microscope. Particles, except mt-nucleoids, were hardly detected in the mt-nucleoids fraction. Any contamination by nuclear DNA in this fraction was not detected by Hoechst 33258-CsCl density gradient centrifugation of DNA (Fig. 9). On the other hand, mt-nucleoids from the boundary between 40 % and 60 % sucrose contained larger mt-nucleoids than those of the upper peak. Some aggregated mt-nucleoids were also observed. DASPMI staining used to stain mitochondria (Miyakawa et al. 1984) detected some membranous materials in this fraction (data not shown).

Judging from the fluorescence microscope observation, analysis of DNA, and electron microscopic view as described below, it was concluded that the mt-nucleoid fraction obtained from the boundary between 20 % and 40 % sucrose contains large amounts of morphologically intact mt-nucleoids without mitochondrial membranes or cell nuclear contamination.

Chemical analysis showed that mt-nucleoids were composed of DNA, RNA and proteins (Table 1). The mass ratio of RNA/DNA, protein/DNA was similar to the value for Physarum polycephalum mt-nucleoids (Suzuki et al. 1982), yeast nuclear chromatin (Sommer, 1978) and yeast mtDNA-protein complex isolated by Triton X-100 lysis of mitochondria at 30°C in the presence of RNase (Rickwood et al. 1981).

Structure of isolated mt-nucleoids

Fluorescence intensity of each DAPI-stained mt-nucleoid was directly measured using VIMPCS to investigate the preservation of DNA content during the isolation process (Fig. 10). The distribution pattern

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Fig. 11. Fluorescence photomicrograph of isolated mt-nucleoids after DAPI staining. ×8000.

Fig. 12. Electron photomicrograph of mt-nucleoids after negative staining in the same field as Fig. 11. ×27 000.

Fig. 13. An enlargement of an isolated mt-nucleoid observed by negative staining electron microscopy. A membrane vesicle attached to the mt-nucleoid is shown by an arrowhead. ×72 000.

Fig. 14. A mt-nucleoid treated with pronase E, which shows the fibrous structure of DNA spread from the undigested region of a mt-nucleoid. ×62 000.
of fluorescence intensity per nucleoid in isolated mitochondria coincided well with the pattern of fluorescence intensity of nucleoids in spheroplasts. However, the distribution of isolated nucleoids shifted to the smaller side as a whole, indicating that selective isolation of smaller nucleoids might occur in the fraction. DNA content of a mt-nucleoid was calculated from photon numbers on the basis of G+C content of mtDNA and T4 phage used as standard, because DAPI has specific affinity to A-T base-pair (Williamson & Fennell, 1975; Kapucinski & Skoezylas, 1977; Lin et al. 1977; Kapucinski & Szer, 1979). The average number of mtDNA molecules in an mt-nucleoid was estimated as 3-9 in spheroplasts, 4-0 in isolated mitochondria and 3-1 in isolated mt-nucleoids, respectively.

In order to observe the fine structure of the isolated mt-nucleoids, DAPI-stained mt-nucleoids deposited on a mesh-grid were observed with the fluorescence microscope and subsequently observed in the electron microscope in the same field after negative staining (Figs 11 and 12). Electron microscopic observation clearly showed that the mt-nucleoids have a spherical or ovoid configuration 0.4-0.6 μm in diameter into which the chromatin-like structure is three-dimensionally folded. An enlarged figure of one of the mt-nucleoids is shown in Fig. 13. mt-nucleoids consisted of particles measuring 20-50 nm in diameter and in some mt-nucleoids one or two membrane vesicles were observed to attach to the core portion of folded chromatin. Pronase E digestion disrupted the chromatin-like structure of the mt-nucleoid and a DNA-like thin filament (less than 7 nm in diameter) appeared radiating from the undigested region of the mt-nucleoid (Fig. 14). Apart from pronase E, the nucleoids were completely dispersed by treatment with NaOH, HCl, DNase and SDS, tended to expand when heat treated or exposed to high KCl or NaCl concentrations and were resistant to RNase.

**Nucleoid-organizing proteins**

SDS-PAGE of mt-nucleoids was performed to investigate proteins which organize mtDNA into nucleoids (Fig. 15). The mt-nucleoid fraction from the 20-40% sucrose boundary contained at least 20 species of polypeptides mainly in the molecular weight range from 10 to 70(×10^3). As Rickwood et al. (1981) reported, we also found several polypeptides above 70K (K = 10^3 M_r), but these polypeptides were quantitatively minor components of nucleoids. To search for basic, histone-like proteins, acid extract from nucleoids was analysed by acid-urea/SDS two-dimensional electrophoresis (Fig. 16). The results revealed only one major polypeptide of 20K, which suggested that the 20K polypeptide plays an important role in packing of mtDNA similar to nuclear histones.

**Discussion**

In the electron microscope mtDNA appears as tiny electron-dense spots, filaments, or bundles of filaments in regions of lower electron density in the mitochondrial matrix. This depends on the fixation method used and it has been assumed that the almost complete

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**Fig. 15.** Analysis of mt-nucleoid proteins by SDS-PAGE. Molecular weight standard is indicated to the left of a gel (×10^-3).

**Fig. 16.** Acid-urea/SDS two-dimensional PAGE of acid extract from mt-nucleoids. The first dimensional acid-urea gel is shown at the top of a two-dimensional SDS gel. Direction of electrophoresis is indicated by an arrow. A 20×10^9 M_r polypeptide is indicated by an arrowhead. Gels were stained with Coomassie Brilliant Blue.
absence of associated protein is responsible for the clumping of DNA (Stevens, 1981). On the other hand, Rickwood et al. (1981) isolated the DNA–protein complex from mitochondria of the yeast, Saccharomyces cerevisiae and detected four species of proteins associated with mt-nucleoids. In the electron microscope the isolated DNA–protein complexes appeared as various sized aggregates composed of spheroidal bodies. However, it was not certain whether DNA–protein complexes remained intact.

The purpose of this study was to isolate morphologically intact mt-nucleoids without disrupting their native structure and to investigate the organization. For this purpose, DAPI seems a useful tool for monitoring the preservation of size, shape and (especially combined with VIMPCS) DNA content of nucleoids during the isolation process.

To obtain highly pure mitochondria from yeast, combined methods of repeated differential centrifugations and density gradient centrifugation have been widely used, and DNase I treatment of mitochondria has also been generally carried out to remove contaminated cell nuclear DNA. However, in this study the prolonged isolation process had to be avoided to protect mt-nucleoids from proteolytic and nucleolytic digestion, which cause the disruption of the intact morphology of mt-nucleoids, and to obtain a good yield. In the present work, we found it possible to isolate mt-nucleoids with high purity without the time-consuming density gradient centrifugation and DNase I digestion of mitochondria. In fact, the mitochondrial fraction contained some membranous structures besides mitochondria. Analysis of DNA by Hoechst–CsCl density gradient also revealed contamination of nuclear chromatin of about 20% of the total DNA in the mitochondrial fraction. However, it was possible to remove almost all cell nuclear chromatin and NP-40-insoluble membranous material from the NP-40 lysate of mitochondria as an NP-40-insoluble fraction. The remaining contamination cosedimented with aggregates of mt-nucleoids in the fraction at the 40%–60% sucrose boundary. As a result, the fraction from the 20%–40% sucrose boundary contained highly pure mt-nucleoid particles as seen in Figs 11 and 12, when investigated by fluorescence and electron microscopy. Analysis by a CsCl density gradient always showed above 98% purity of mtDNA in total DNA as seen in Fig. 9. The rapid method described here enabled us to isolate morphologically intact mt-nucleoids from spheroplasts within 5 h, although we might select somewhat smaller mt-nucleoids during the isolation process. Analysis of nucleoid-organizing proteins produced quite different results from those of Rickwood et al. (1981) who detected only four major polypeptides (68K, 80K, 2 species of 95K) using a [35S]sulphate-labelling technique. Although our results also detected several polypeptides of high molecular weight above 70K, these polypeptides were minor components of mt-nucleoids. The discrepancy might be partly caused by the difference in the method for detection of polypeptides. In the case of yeast, it has been claimed that a basic histone-like protein named HM protein with molecular weight of 20K is present in mitochondrial (Caron et al. 1979; Certa et al. 1984). We confirmed that the 20K protein, which may be identical to HM protein, is really one of the major components of nucleoids with the most basic property. At present, the functions of almost all other polypeptides detected in mt-nucleoids still remain to be determined. Rickwood & Chambers (1981) have reported that the significant fraction of the mtDNA of S. cerevisiae is organized into a nucleosome-like structure. Our preliminary work with DNA-cellulose affinity chromatography indicates that at least five major species of polypeptides including the 20K polypeptide have the ability to bind to double-stranded DNA. Further investigation of isolated mt-nucleoids would bring us new information about how 75 kb circular mtDNAs are compactly folded.

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References


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