Stabilization and Isolation of Mitochondrial Nuclei from *Physarum polycephalum*

Kazuo Ogawa, Shigeyuki Kawano and Tsuneyoshi Kuroiwa

Department of Cell Biology, Institute for Basic Biology, National Center for Biological Sciences, Okazaki 444, Japan

ABSTRACT. Mitochondrial nuclei (mt-nuclei) were isolated from the microplasmodia of *Physarum polycephalum* in buffer containing polyamines and chelator. The individual mt-nucleus retained the native structure observed *in vivo*. The DNA of these mt-nuclei was the non-fragmented, native one. These characteristics indicate that the native structure is preserved during isolation. In contrast to the preparation isolated with buffer exclusive of polyamines and chelator, the present mt-nuclei were stable during storage and did not aggregate. Thus, they provide useful material for the study of the morphology and biochemistry of the individual mt-nucleus.

Since Nass and Nass (23) found DNA-like fibers in the mitochondria, the presence of DNA and RNA has been firmly established, and the biogenesis of mitochondria has been shown to be the result of the joint workings of two genetic systems; the nucleo-cell sap system and a system localized within the mitochondrial matrix (2). We wanted to know whether the mitochondrial system could be separated from mitochondrial matrix as in the case of the cell nucleus from the cell sap. Guttes *et al.* (7, 8) observed mitochondria with a rod-shaped “nucleoid” in the center of the inner matrix in the multinucleated slime mold *Physarum polycephalum*. (We prefer to call this a “nucleus”.) It was composed of a large amount of DNA (15, 16, 25), RNA (13, 25), and protein (18). Our previous papers have described a procedure for isolating the mitochondrial nuclei (abbreviated mt-nuclei) (20, 21). However, those preparations readily swelled and the DNA fibers unfolded. To obtain information on the native, folded DNA in the mt-nucleus, we must develop a procedure that prepares mt-nuclei which retain the morphology observed *in vivo*. We here present a procedure for obtaining stabilized mt-nuclei, using a buffer system which contains polyamines to stabilize the folded DNA structure and a heavy metal chelator to suppress nuclease activity.

MATERIALS AND METHODS

Preparation of Mitochondria and Mt-nuclei. Microplasmodia of *P. polycephalum* were grown by the method of Guttes and Guttes (6). Usually 100 g of packed microplasmodia was used to isolate the mitochondria. The present procedure particularly was developed to stabilize the mt-nuclei during the isolation of mitochondria. The following processes were done at 4°C, unless otherwise stated.

The collected microplasmodia were washed with water, then mixed with 0.1 vol. of buffer

A (10 mM Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose) and homogenized three strokes with a Teflon homogenizer. The homogenate immediately was poured into 10 vol. of buffer B (10 mM Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose, 1 mM EDTA, 0.2 mM spermine, 0.4 mM spermidine, 0.25% NaCl and 0.05% 2-mercaptoethanol). The diluted homogenate was stirred with a glass rod, then centrifuged at $500 \times g$ for 5 min. The supernatant was saved and filtered through a sheet of nylon mesh with a $31 \mu\text{m}$ pore size (NBC Ind., Japan). The filtrate was centrifuged at $650 \times g$ for 5 min. Again the supernatant was saved and filtered through a double sheet of nylon mesh separated by one sheet of tissue paper. The filtrate was centrifuged at $1,100 \times g$ for 5 min. Its supernatant was centrifuged at $5,000 \times g$ for 5 min to sediment the mitochondria. The mitochondria were washed three times with buffer B by repeated centrifugation. The isolated preparations were found to be contaminated with a small amount of cell nuclei when checked by azure B and ethidium bromide staining. One and a half grams of packed mitochondria was obtained from 100 g of packed microplasmodia.

The mt-nuclei were prepared from this mitochondrial preparation by modifying the previous method (19, 20) as follows: Freshly prepared mitochondria (1.5 g wet weight) were suspended in 25 ml of buffer B, then a Pasteur pipette was used to disperse the aggregated mitochondria. Small portions of buffer A (25 ml) were added to the stirred mitochondrial suspension, after which 1.25 ml of 20% (w/v) Nonidet P-40 in water was added in small doses to the stirred suspension. The suspension became clear on the addition of the detergent. After stirring it for 10 min at room temperature, we centrifuged the clear solution at $5,800 \times g$ for 5 min. The supernatant was saved and again centrifuged at $19,000 \times g$ for 5 min. The white pellet was washed with buffer B by centrifugation and used as the mt-nuclei.

Azure B and Ethidium Bromide Staining of the Mt-nuclei. Cell nuclei, mitochondria and the mt-nuclei were stained with 0.5% azure B dissolved in buffer A and observed under a light microscope (21). The mt-nuclei also were stained with one drop of 100 $\mu\text{g/ml}$ ethidium bromide in water, after which the fluorescent mt-nuclei were examined under an Olympus epifluorescence microscope with fluorescence-phase contrast equipment (16). An excitation filter of G (IF545 + BG-35) and an absorption filter of R610 were used for observations which were photographed on Tri-X film (ASA 400).

Preparation of Whole Mitochondrial DNA. Mitochondria contaminated with a small amount of cell nuclei were prepared by the method of Bohnert (1), then treated with phenol to extract the DNA. Pure mitochondrial DNA was obtained by repeated CsCl density gradient centrifugations of the crude DNA and served as the control.

CsCl Density Gradient Centrifugation. Mitochondria and mt-nuclei obtained by the present method were treated with phenol to extract their DNA. Finally, DNA was dissolved in SSC (0.015 M sodium citrate buffer, pH 7.0, containing 0.15 M NaCl). Solid CsCl (8.5 g) was added to 7 ml of the DNA solution, and this solution was centrifuged at 38,000 rpm for 50 h in a Hitachi RP 65 rotor at 10°C . Eight-drop fractions were collected, and samples (2.5 μl) of each were used to determine the refractory index. Density was calculated from the equation given by Schildrant *et al.* (24). The remaining fractions were combined with 0.3 ml portions of SSC and the absorbance at 260 nm was determined.

Agarose Gel Electrophoresis. The precipitated mt-nuclei were dissolved in 1 ml of 1% SDS-0.015 M NaCl-1 mM EDTA-0.02% pronase and incubated for 30 min at 60°C . After incubation, the solution was dialyzed against $0.1 \times \text{SSC}$ containing 0.1 mM EDTA. A portion (20 μl) was electrophoresed on a 0.7% agarose gel by the method of Fangman (5) with some modification; a $0.5 \times 14 \text{ cm}$ agarose gel was used. Electrophoresis was carried out at 1 mA per tube for 2.5 h at room temperature. The DNA band on the gel was stained with 0.5 $\mu\text{g/ml}$

of ethidium bromide in water after electrophoresis. The DNA purified from whole mitochondria served as the control.

RESULTS AND DISCUSSION

Mt-nuclei Isolation. To isolate mt-nuclei, we introduced polyamines to stabilize the folded DNA structure in the mt-nucleus and a heavy metal chelator to suppress

Fig. 1. Light micrographs of mitochondria and mt-nuclei. a, isolated mitochondrial fraction stained with azure B. b, pelleted mt-nuclear fraction stained with azure B. The scale is 5 μ m.

nuclease activity. Some nuclear contamination was noticed in the mitochondrial preparation obtained by the present method, though the mitochondria had stabilized mt-nuclei. Azure B staining of the mitochondrial preparation showed dense bodies in the central region of mitochondria (Fig. 1a). These dense bodies have been called both mitochondrial nucleoids (16, 19, 22) and mt-nuclei (17, 20). The staining of these mt-nuclei by azure B was much more dense in the present mitochondria than in previous ones (22). Spherical, oval, rod- or V-shaped mt-nucleus were seen in a mitochondrion. The smaller mitochondrion had a spherical body and the larger one a rod- or V-shaped body. A mitochondrion with a V-shaped mt-nucleus is shown by the arrow in Fig. 1a. The resolution of the mitochondrial substructure by azure B staining was comparable to that of the HCl-thionine method (22). Fig. 1b shows isolated mt-nuclei stained with azure B. They had spherical, oval, rod-, dumbbell- and V-shaped morphologies. The arrow in Fig. 1b shows an mt-nucleus with a V-shaped structure. Mitochondria from Fig. 1a and other corresponding fields were placed in division cycle order as described in a previous paper (22). Fig. 2a-d shows the

Fig. 2. Light micrographs of mitochondria and mt-nuclei corresponding to each stage of the mitochondrial division cycle. a-d, mitochondria stained with azure B. e-h, mt-nuclei stained with azure B. a and e, mG₁; b and f, mS; c and g, mG₂; d and h, mM. The scale is 2 μ m.

mitochondria at G_1 (mG_1), S (mS), G_2 (mG_2) and M (mM), respectively. The division cycle was determined by the morphology of the mt-nucleus in the mitochondrion, since some stages of the mitochondria (e.g., the dividing mitochondrion) lost the original dumbbell-shaped structure after isolation. The result was a large spherical mitochondrion with a V-shaped nucleus, as described previously (13, 21). Fig. 2e-h shows mt-nuclei isolated from mitochondria at G_1 , S, G_2 and M, respectively. The isolated mt-nucleus has a morphology resembling that of the mt-nucleus *in situ*, as shown by the two series of photographs. Accordingly, we concluded that the present buffer system is advantageous for isolating an mt-nucleus that retains its original structure.

Phase contrast, fluorescence-phase contrast and fluorescence micrographs of mitochondria stained with ethidium bromide, in the same field, are shown in Fig. 3a-c, respectively. The mitochondrial matrix was loosely stained, and the sharply stained nucleus is seen in the central region. Mitochondria at G_1 , S, G_2 and M, respectively, are shown in Fig. 4a-d and the mt-nuclei isolated from corresponding mitochondria are shown in Fig. 4e-h. From a comparison of the isolated mt-nuclei with the corresponding mitochondria, we concluded that the present buffer system is useful for isolating the folded DNA structure of the mt-nucleus.

Fig. 3. Ethidium bromide staining of mitochondria and mt-nuclei. a-c, isolated mitochondrial fraction stained with ethidium bromide: a, phase contrast micrograph; b, fluorescence-phase contrast micrograph; c, fluorescence micrograph of the same field. d, fluorescence micrograph of pelleted mt-nuclei stained with ethidium bromide. The scale is 10 μ m.

Stability. The mt-nuclei appeared to be stable for 1 week at 4°C in a solution of buffer B diluted with buffer A in the presence of 0.5% Nonidet P-40. The addition of ethidium bromide to this solution and a rise in the mt-nuclei concentration did not affect the stability of the mt-nuclei. The mt-nuclei in buffer B also were stable for 1 week at 4°C and did not aggregate during storage.

CsCl Density Gradient Centrifugation. The present procedures gave mitochondria with stabilized nuclei, but with some nuclear contamination. Contamination also was shown by the CsCl density gradient centrifugation pattern of the DNA extracted from them (Fig. 5a). In this preparation two DNA peaks with densities of 1.705 and 1.685 g/cc corresponded to the nuclear and mitochondrial DNAs. The densities of these DNAs were similar to values reported by other groups (1, 3, 4, 9, 10). About 30% of the total DNA preparation was due to nuclear DNA contamination. However, the mt-nuclear fraction was not contaminated with nuclear DNA. CsCl density gradient centrifugation of the DNA extracted from the mt-nuclear fraction gave a single peak with a density of 1.680 g/cc (Fig. 5b). This suggests that the

Fig. 4. Fluorescence micrographs of mitochondria and mt-nuclei corresponding to each stage of the mitochondrial division cycle. a and e, mG₁; b and f, mS; c and g, mG₂; d and h, mM. The scale is 2 μ m.

contaminated cell nuclei were not solubilized by detergent treatment, rather they were precipitated and removed during subsequent isolation steps. We estimated the recovery of mitochondrial DNA as approximately 20% of the total DNA in the mt-nuclei preparation from the results shown in Fig. 5.

Agarose Gel Electrophoresis. The molecular size of mitochondrial DNA has been reported to be 2.0×10^7 – 3.0×10^7 daltons (4, 11). This variation in size is due to the fragmentation of DNA during isolation. Agarose gel electrophoretic patterns of SDS, pronase-treated mt-nuclei and DNA from whole mitochondria (a and b, respectively) are shown in Fig. 6. The mt-nuclear DNA had a sharp stained band,

Fig. 5. CsCl density gradient centrifugation of DNA. a, DNA extracted from the mitochondrial fraction. b, DNA extracted from the mt-nuclear fraction. Each DNA preparation was obtained from mitochondria or mt-nuclei prepared from 80 g of packed microplasmodia as a starting material. —●—, A_{260 nm}.

whereas, the whole mitochondrial DNA had a slightly more diffused pattern. The former appears to have slightly slower mobility than the latter. This was ascertained by co-electrophoresis of both samples (Fig. 6c) in which the DNA band of the gel was diffuse as in gel b, whereas the upper part of band coincided with the band of gel a, not gel b. This is evidence that the DNA from whole mitochondria was fragmented during preparation, whereas the mt-nuclear DNA remained non-fragmented and native. Therefore, we recommend the use of mt-nuclei rather than whole mitochondria as starting material used to obtain unfragmented, native mitochondrial DNA. Faintly stained RNA bands were observed in the region of gels a and c as indicated by the arrows.

The present method that uses polyamines and a chelator is satisfactory for isolating native mt-nuclei with undegraded DNA from *P. polycephalum* mitochondria, as in the case of isolation of bacterial chromosome (12, 26). The isolated mt-nuclei appeared to be stable for 1 week at 4°C and did not aggregate during storage. They also retained the native mt-nuclear structure observed during the mitochondrial division cycle. This method is particularly suitable for obtaining mt-nuclei for studies of DNA

replication and the developmental regulation of gene expression in mitochondria. Biochemical studies of the isolated mt-nuclei are in progress.

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Fig. 6. Agarose gel electrophoretic pattern of mitochondrial DNA. a, DNA from SDS, pronase-treated mt-nuclei. b, DNA from whole mitochondria (0.8 μ g DNA). c, mixture of samples a and b.

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