真正粘菌 (*Physarum polycephalum*) の
高次ミトコンドリア核における
染色体機能発現制御の解析

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Studies on the Function of Mitochondrial Nuclei
in *Physarum polycephalum*
as a Model System of DNA-protein Complex

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Acknowledgements

Contents

Preface

The concept of mitochondrial nuclei

Mitochondria are semi-autonomous organelles that posses their own DNA, mitochondrial DNA (mtDNA). Early studies using electron microscopy showed that a small amount of the DNA-like fibers presents in electron-transparent or semi-electron transparent spherical area of their mitochondria, 0.1-0.5 μm in diameter. However, such DNA structures is not always observed in all mitochondria, and the amount of DNA-like fibers is much less than expected. It seems likely that the majority of mtDNA is embedded in semi-electron-dense matrix in mitochondria and remains invisible under conventional conditions of fixation and staining for electron microscopy. In 1970s, very sensitive DNA-binding fluorochrome, 4', 6-diamidino-2-phenylindole (DAPI) was synthesized by Dann et al. (1971) and applied for the observation of organelle DNA. DAPI binds A-T base pairs of DNA and emits strong, blue-white fluorescence under UV irradiation. The combination of DAPI with fluorescence microscopy made it possible to observe the behavior of small amount of organelle DNA *in situ*. When observed by DAPI-fluorescence microscopy, DNA-containing region of mitochondria appears as a tiny fluorescent spot, indicating that the mtDNA is organized to form compact structures *in vivo*. Now, it is well established that such compact structures are composed of mtDNA and proteins. From this point of view, Kuroiwa proposed the term "mitochondrial nuclei" for the compactly organized DNA-protein complexes in mitochondria (Kuroiwa 1982).

Mitochondrial nuclei as a model system for DNA-protein complex

In cell nuclei, DNA is also compactly organized with proteins. The DNA replication and transcription occur in the compactly organized nucleus. To

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elucidate the mechanism of DNA replication and transcription in the highly organized nuclear structure such as chromatin, it is necessary to isolate DNA-protein complex in an intact form, both structurally and functionally. However, the size of cell-nuclear genome is so large that neither the analyses of the entire genome at a time, nor the isolation of the chromatin that contains specific genes, is possible.

When compared with cell-nuclei, mt-nuclei would have several advantages for the analyses of the mechanism of DNA replication and transcription in the nuclear organization if the mt-nuclei were isolated in intact forms, both structurally and functionally. (1) Because the genome size of mitochondria is much smaller (16-570 kb) than that of cell-nuclei, it is possible to analyze the entire genome at a time. (2) Information of the complete nucleotide sequence of mitochondria genome are available for several species (Anderson et al. 1981, Bibb et al. 1981). (3) As the genome size and coding capacity are small, the factors involved in replication and transcription of mtDNA is supposed to be much fewer than those operate in cell-nuclei. Therefore, it may be possible to detect and characterize all the major factors involved in the regulation of replication and transcription of mtDNA. Based on these considerations, I decided to use the mt-nuclei as a model system to analyze the mechanism of DNA replication and transcription in the highly organized nuclear structure.

Isolation of mt-nuclei from *Physarum polycephalum*

Protein-DNA complex has been isolated in mitochondria of HeLa cells (Albring et al. 1977), *Xenopus laevis* (Pinon et al. 1978; Rickwood and Jurd, 1978), sea urchin embryos (Sevaljevic et al. 1978, 1979), rat liver (Van Tuyle and McPherson,1979; Van Tuyle and Pavco, 1985) and *Paramecium aurelia* (Olszewska and Tait, 1980). However, the question remains ambiguous as to

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whether isolated DNA-protein complexes correspond to the mt-nucleoids observed in the electron microscope and whether they retain the morphological intactness. Mt-nuclei have been isolated from the plasmodia of *Physarum polycephalum*, which has a large, rod-shaped mt-nucleus (Kuroiwa et al., 1976, Suzuki et al., 1982). The integrity of their morphology seemed to have been preserved; scanning electron micrographs showed the same three-dimensional rod-shaped structure and the same size distribution as mt-nuclei found in the mitochondria. However, function of mtDNA in these isolated structurally intact mt-nuclei has not been investigated.

The feature of mt-nuclei in *Physarum polycephalum*

The degree of mtDNA organization in one type of organism differs from that in the other eukaryotes. To the best of our knowledge, the mt-nuclei of true slime molds such as *Physarum polycephalum* have higher degree of organization than those of other eukaryotes. MtDNA of *P. polycephalum* is a linear molecule of 86.0 kbp, 6 times larger than mammalian mitochondrial DNA (Takano et al. 1990). The life history of *P. polycephalum* is that of a typical haplodiplont, with two distinct stage: the diploid syncytial plasmodium and the haploid uninucleate myxamoebae. The mt-nuclei of *P. polycephalum* contains about ten times more mtDNA than those from other sources, and the mt-nuclei of amoebae and plasmodium contain about 18-24 and 32 mtDNA molecules respectively in the logarithmic phase of growth (Kawano et al. 1983, Sasaki et al. 1994). A number of mtDNA molecules are packed tightly in the mt-nucleus, and the packing ratio of mtDNA appears to be even higher than that of cell-nuclear DNA.

The purpose of this study

The purpose of this study is to analyze the mechanism of DNA replication in the nuclear organization. To accomplish this, I used the mt-nuclei

of *P. polycephalum* plasmodia as a model system. First, I investigated the manner of mtDNA replication in the mt-nucleus of plasmodia by the visualization of the sites of *in situ* mtDNA replication and suggested that the replication of multiple mtDNA molecules occurred in the mitochondrial replicon cluster, which consists of about 10 mtDNA molecules. This implied that the nuclear structure may be related to the control of the mtDNA replication. Next, I isolated the structurally intact mt-nuclei from the plasmodia that were able to support efficient mtDNA synthesis *in vitro*. Finally, I analyzed the proteins that were released from the mtDNA by treating the isolated mt-nuclei with NaCl and identified three DNA-binding proteins, including histone H1-like protein (41-kDa protein), which may participate in the nuclear organization and mtDNA synthesis.

Chapter 1

The manner of replication and organization of mitochondrial DNA in the mitochondrial nucleus of *Physarum polycephalum* plasmodia

SUMMARY

The mitochondrion of *Physarum polycephalum* plasmodia has a large, rod-shaped mitochondrial nucleus (mt-nucleus), which contains about 32 mitochondrial DNA (mtDNA) molecules. These mtDNA molecules bind tightly to the mitochondrial membrane at specific duplicated regions of the molecule. In this study, we studied plasmodia and visualized the site of mtDNA replication by immunofluorescence or immunoelectron microscopy. The replication of multiple mtDNA molecules only occurs at a few discrete sites in the mt-nuclei. The number of replication sites per mt-nucleus was proportional to the copy number of mtDNA per mt-nucleus. The relationship between the number of replication sites and the copy number of mtDNA indicated that mtDNA replication is regulated in groups of 10 adjacent mtDNA molecules.

We also studied the membrane-binding regions (MBRs) of mtDNA in the mt-nucleus by fluorescence *in situ* hybridization with a DNA probe specific for the MBR. Visualization of the MBR sites in the mt-nucleus suggested that the MBRs were grouped in a few discrete locations in the mt-nucleus. The number of MBR clusters in the mt-nucleus was proportional to the mtDNA copy number; one MBR cluster consisted of the MBRs of about ten mtDNA molecules. The MBR clusters appeared to organize a multiple mtDNA in a mt-nucleus into a few unit, each of which contains about 10 mtDNA molecules.

INTRODUCTION

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Mitochondria are semi-autonomously replicating organelles that have their own DNA, which is known as mitochondrial DNA (mtDNA). It is generally accepted that mtDNA is organized into a highly compact structure with proteins, to form the so-called mitochondrial nuclei (mt-nuclei, synonym for mitochondrial nucleoid, Kuroiwa, 1982; Kuroiwa et al., 1994). Early results obtained with electron microscopic autoradiography of [³H]thymidine-labeled cells showed that mtDNA replication occurs within the mt-nuclei. However, the precise site of mtDNA replication in the mt-nuclei is not detected with the limited resolution of autoradiography. Immunofluorescence detection of incorporated bromo-deoxyuridine (BrdU) or biotin-labeled dUTP allows a much more accurate localization of the sites of DNA replication. In fact, the spatial and temporal nuclear organization of DNA replication in the cell nuclei of several cell types has been characterized using these techniques (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992). Some experiments also detected BrdU incorporation into mitochondria (Thiry, 1992; Suzuki et al, 1992; Davis and Clayton, 1996). However, it is difficult to determine the precise sites of DNA replication in the mt-nuclei because the mt-nuclei in these organelles are too small to observe.

Physarum polycephalum contains a large rod-shaped mt-nuclei with a high mtDNA copy number. The mt-nucleus of the *P. polycephalum* amoebae contains approximately 18 to 24 mtDNA molecules (Sasaki et al., 1994). Previously, we visualized the sites of DNA replication in the mt-nuclei of the amoebae after incubating cells with BrdU (Sasaki et al., 1994). The replication sites were not distributed randomly throughout the mt-nuclei, but were concentrated in a few discrete regions. Furthermore, the number of replication sites along a single mt-nuclei was proportional to the copy number of mtDNA per mt-nuclei; one replication signal corresponded to about 10 mtDNA molecules. Therefore, we proposed that replication of individual mtDNA

molecules in the mt-nuclei is not regulated independently, but that replication of groups of approximately 10 adjacent mtDNA molecules is regulated concertedly, within a structure defined as the mitochondrial replicon cluster (MRC). The presence of the MRC implies that the organization of mtDNA in the mt-nuclei plays an important role in the regulation of mtDNA replication.

The organization of the mtDNA in the mt-nuclei has only been studied in the amoebae of *P. polycephalum*. The mtDNA of *P. polycephalum* is a linear, 86.0 kb molecule (Takano et al., 1990), which contains a specific region that interacts with the mitochondrial membrane. The mitochondrial membrane-DNA complex of *P. polycephalum* was isolated and restriction endonuclease analysis identified a specific 3.5 kb region of mtDNA that interacts with the mitochondrial membrane (Kawano and Kuroiwa, 1985; Kuroiwa et al., 1994). This membrane-binding region (MBR) was mapped to a 19.6 kb tandem duplication located at each end of the linear 86 kb mtDNA molecule (Takano, 1990; Kuroiwa et al., 1994). Visualization of the location of the MBRs in the mt-nucleus by fluorescence *in situ* hybridization (FISH) with a DNA probe specific for the MBR showed that the MBRs of approximately 10 mtDNA molecules were clustered at a few discrete sites along the mt-nucleus (Sasaki, 1995). This result suggested that the mtDNA molecules in the mt-nuclei might be organized rather than randomly distributed.

P. polycephalum has two distinct forms in its life cycle: the haploid uninucleate amoebae and the diploid syncytial plasmodium (Kawano et al., 1995). The mt-nucleus in the plasmodia has the same rod-like shape as in the amoebae, and contains about 32 mtDNA molecules (Kawano et al., 1983). In this chapter, I discuss the visualization of the sites of mtDNA replication by immunofluorescence microscopy and the location of the MBRs in the mt-nucleus of the plasmodia by fluorescence *in situ* hybridization with a DNA probe specific for the MBR.

MATERIALS AND METHODS

Cell cultures

Microplasmodia of the *P. polycephalum*, Colonia isogenic strain KM 182 I KM 187, were cultured in liquid medium at 23°C using the method described by Daniel and Baldwin (1964).

Observation of cells

Cells were fixed on a glass slide in 0.6% glutaraldehyde, stained with 1 mg/mL 4',6-diamidino-2-phenylindole (DAPI) in S buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.7, 1 mM EDTA, pH 7.5, 7 mM 2-mercaptoethanol, 0.75 mM spermidine and 0.4 mM PMSF) and observed under an epifluorescence microscope equipped with a phase-contrast objective (BHS-RFC; Olympus Optical Co., Tokyo, Japan).

Labeling with BrdU and indirect immunofluorescence microscopy with BrdU-specific antibodies

Cultured amoebae and microplasmodia from the logarithmic phase of growth were labeled for 1 h with 10 µM BrdU in the presence of 1 µM 5-fluorodeoxyuridine (an inhibitor of thymidine biosynthesis), which enhances the incorporation of BrdU. Cells were fixed in Carnoy's solution for 10 min. A drop of fixed cells was then spread on a coverslide, and the coverslide was passed through a flame rapidly to rupture and fix the samples. Detection of BrdU was performed using the method of Sasaki et al. (1994). The coverslide was soaked in ethanol for 5 min and then treated with 3 N HCl for 30 min. After denaturing the DNA, the samples on the coverslides were incubated with mouse monoclonal antibodies against BrdU (Becton Dickinson Immunocytometry

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Systems Co., CA, USA) and a second antibody, fluorescein isothiocyanate-conjugated (FITC-conjugated) antibodies to mouse immunoglobulins produced in goats (Tago Inc., CA, USA). Finally, the cells were stained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in S buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.7), 1 mM EDTA (pH 7.5), 7 mM 2-mercaptoethanol, 0.75 mM spermidine and 0.4 mM PMSF] and observed under an epifluorescence microscope equipped with a phase-contrast objective (BHS-RFC; Olympus Optical Co., Tokyo, Japan). Photographs were taken at a magnification of 500x with 35 mm Fuji Neopan 400 film (Fuji Photo Film Co., Tokyo, Japan).

Immunostaining with colloidal gold and electron microscopy

Cultured microplasmodia were labeled for 20 h with 10 mM BrdU in the presence of 1 mM 5-fluorodeoxyuridine. Then 8% glutaraldehyde was added to the culture medium to fix the cells in a final concentration of 2% glutaraldehyde for 1 h at 4 °C. After three washes with distilled water, the fixed cells were embedded in 2% low-melting-temperature agarose and then the agarose was cut into blocks, which were dehydrated in an ethanol series. The ethanol was replaced by propylene oxide and a number of blocks were immersed in LR white resin (London Resin; Woking, Surrey, U.K.) and embedded at 60 °C. Serial thin sections (ca. 100 nm) were cut with glass knives on an ultramicrotome (MT-6000 XL, RMC-Eiko Co., Kawasaki, Japan). Thin sections on grids were treated with phosphate-buffered saline (PBS; pH 7.4) that contained 0.05% Triton X-100 for 15 min at room temperature and blocked in blocking buffer (5% bovine serum albumin in PBS) for 20 minutes at room temperature. The sections were then incubated for 1 h at 37 C with mouse antibodies against BrdU that had been diluted ten-fold with the blocking buffer. After five washes with PBS (pH 7.4), each for 5 min at room temperature, the samples were incubated with goat antibodies against mouse IgG conjugated to 10 nm colloidal gold particles (Zymed Laboratories, CA, USA) that had been

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diluted 160-fold with blocking buffer. The sections were washed for 5 min five times with PBS (pH 8.2) at room temperature, rinsed once with distilled water, stained with 3% uranyl acetate for 30 min at room temperature and examined with a JEOL 1200 EX transmission electron microscope (1200 EX; JEOL, Tokyo, Japan).

Estimation of the amount of mtDNA and the length of the mt-nucleus

Cells were fixed on a glass slide in 0.6% glutaraldehyde. The DNA was stained with 1 µg/mL DAPI in S buffer and the samples were observed under an epifluorescence microscope. The fluorescence intensity emitted from the mt-nuclei was measured with a video-intensified microscope photon-counting system (VIMPCS; Hamamatsu Photonics Ltd. Hamamatsu, Japan) connected to an epifluorescence microscope, as previously described (Kuroiwa et al., 1986). To evaluate the fluorescence intensity of the mt-nuclei, T4 phage was used as a standard DNA marker (1T=170 kb; Freifelder, 1970). Since the *P. polycephalum* mtDNA is 86 kb in length (Takano et al., 1990), 1 T is roughly equivalent to 2 mtDNA copies. At the same time, the length of each mt-nucleus was measured on the VIMPCS video frame.

In situ hybridization and signal detection

Cells were washed with distilled water three times and fixed with Carnoy's solution (25% glacial acetic acid in ethanol) for 10 min. A drop of fixed cells was then spread on a coverslide, and the coverslide was passed rapidly through a flame to burst and fix the samples. *In situ* hybridization was performed using the method of Hizume et al. (1992). A 4.7 kb *Xba*I/*Sph*I fragment that overlaps a 1.7 kb region of the MBR of *P. polycephalum* mtDNA (Takano, 1990) was used as a probe. DNA was labeled with digoxigenin (DIG)-labeled deoxyuridine-triphosphate, using a random primer DNA labeling system (Boehringer Mannheim Biochemicals, Mannheim, Germany). The

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sample was heat-denatured at 70 °C for 5 min and then hybridized with the DIG-labeled DNA probe at 37 °C for 12 h. After hybridization, the sample was washed with 50 % formamide in 2 x SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for 10 min at 37 °C and twice with 2 x SSC for 10 min. The sample was washed three times in PBS (0.13 M NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.2) and blocked with 15 μL of blocking solution (PBS containing 5% BSA and 0.02% Tween 20) at 25°C for 30 min. Then the sample was incubated at 37°C for 1 h with primary anti-digoxigenin mouse monoclonal antibody (Boehringer Mannheim Biochemicals, Mannheim, Germany) that was diluted to 1:40 in blocking solution. After washing and blocking again, the sample was incubated at 37°C for 30 min with secondary anti-mouse-immunoglobulin goat antibody conjugated with fluorescein isothiocyanate (FITC, Tago Inc., CA, USA) that was diluted to 1:40 in blocking solution. The sample was then washed in PBS, stained with DAPI and observed with an epifluorescence microscope as described above.

RESULTS

Spatial distribution of the sites of mtDNA replication in the mt-nuclei of plasmodia

The replication sites in the mt-nuclei were visualized by light and electron microscopy (Fig. 1). Plasmodia from the logarithmic growth phase were labeled with BrdU and immunostained with antibodies against BrdU and FITC-conjugated or colloidal gold-conjugated second antibodies, for light microscopy and electron microscopy, respectively. When the cells were treated with BrdU for a short time, the sites of DNA replication were seen as a few fluorescent globules distributed along each mt-nucleus under the light

microscope, in spite of the high number of mtDNA molecules in the mt-nucleus (Fig. 1A, B). Incorporation of BrdU into DNA was detected with the electron microscope when the labeling with BrdU was allowed to proceed for a relatively long time (Fig. 1C-E). The particles of colloidal gold were selectively distributed over the cell nuclei and the mt-nuclei (Fig. 1C). The replication sites in the mt-nuclei were not readily distinguished as clusters of colloidal gold particles because of the prolonged period required for labeling (Fig. 1D). However, we sometimes observed a few distinct clusters of colloidal gold particles in a single mt-nucleus, as shown in Figure 1E. Our observations revealed that mtDNA replication in the mt-nuclei of plasmodia is regulated concertedly in groups of adjacent mtDNA molecules.

Next, we studied the relationship between the number of globular replication signals and the mtDNA copy number per mt-nucleus in plasmodia (Fig. 2). Since the length of the mt-nucleus is proportional to the copy number of mtDNA per mt-nucleus, it was possible to calculate the copy number of mtDNA in an mt-nucleus from its length (Sasaki, 1995). The number of globular replication signals per mt-nucleus was proportional to the copy number of mtDNA per mt-nucleus when cells were treated with BrdU for 1 hour. One replication signal corresponded to about 10 mtDNA molecules. Thus, mtDNA replication in the plasmodia was regulated in groups of roughly 10 mtDNA molecules.

Spatial distribution of the sites of mtDNA replication in the mt-nuclei of plasmodia

To visualize the location of the MBRs in the mt-nucleus of plasmodia, we used FISH with a DIG-labeled DNA probe specific for the MBR (Fig. 3). The locations where the MBR probes hybridized were observed as a few fluorescence globules distributed along the mt-nuclei. This indicates that MBRs of mtDNA are not distributed randomly throughout the mt-nucleus but are

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clustered in a few discrete positions along the mt-nucleus.

The number of fluorescent sites in an mt-nucleus increased with the length of the mt-nucleus. Then I investigated the relationship between the number of MBR clusters and the copy number of mtDNA per mt-nucleus (Fig. 4). The number of MBR clusters per mt-nucleus was proportional to the amount of mtDNA per mt-nucleus. One MBR cluster contained the MBRs of about 10 mtDNA molecules.

DISCUSSION

Previous studies of the amoeba of *P. polycephalum* suggested that a cluster of about 10 mtDNA molecules, which is defined as a mitochondrial replicon cluster (MRC), might function as the unit of mtDNA replication in the mt-nucleus (Sasaki et al., 1994; Sasaki, 1995). In this study, I investigated the manner of mtDNA replication in the mt-nucleus of plasmodia. The pattern of BrdU incorporation into the mt-nucleus revealed that mtDNA replication is regulated in groups of adjacent mtDNA molecules (Fig. 1). Furthermore, each group consisted of about 10 mtDNA molecules (Fig. 2). These results suggest that mtDNA replication in the mt-nucleus of plasmodia also occurs in MRC composed of about 10 mtDNA molecules.

MBRs were clustered at several sites along an mt-nucleus in plasmodia and one MBR cluster consisted of the MBRs of about 10 mtDNA molecules (Fig. 3, 4). These results agree with a previous study using amoebae (Sasaki, 1995). The MBR clusters appear to organize many mtDNA molecules into a few units, each of which contains approximately 10 mtDNA molecules, in the mt-nucleus. The units organized within an MBR cluster should be the same as the units of mtDNA replication, the MRC, because of the similarity of the mtDNA copy number of each.

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Figure 5 is a model of the replication of multiple mtDNA molecules in the mt-nucleus of *P. polycephalum*. mtDNA replication in the mt-nucleus occurs in the MRC, which consists of approximately 10 mtDNA molecules. Electron microscopy of serial sections of intact cells indicated that some mtDNA molecules were apparently attached to the mitochondrial inner membrane (Nass et al., 1965; Kuroiwa et al., 1977). Therefore, the MBRs of all the mtDNA molecules in an MRC may be clustered at one location on the inner mitochondrial membrane. Thus, each MRC may interact with the membrane at a single site.

The MBR appears to play an important role in the segregation of mtDNA because the association of mtDNA with the membrane is important for the regulation of mt-nuclear division (Kawano and Kuroiwa, 1985). The MBR is very rich in A-T base pairs, like the centromere, which functions in the segregation of chromosomes in the cell nuclei (Alberts et al., 1989). Therefore, the organization of the MBR described here may be important for regulation of the mtDNA segregation in the mt-nucleus.

The presence of MRC implies that DNA replication takes place in specific domains within the mt-nuclei and that the organization of the mtDNA and proteins in the mt-nuclei plays an important role in the regulation of mtDNA replication. Similar functional domains have recently been identified in the cell nuclei. Replicons are found in groups of adjacent replicons, called a replicon cluster, and each cluster contains ~20 replicons (Nakamura et al., 1986; Nakayasu and Berezney, 1989). DNA replication takes place in specific domains, called replication 'factories', attached to the diffuse nucleoskeleton and DNA polymerase and proliferating cell nuclear antigen are concentrated in these replication 'factories' (Hozák et al., 1993; Hozák et al., 1994). My knowledge of the replication domain in mt-nuclei is limited at present, but I believe that the MRC is a good model for studying the functional domains in the mt-nucleus.
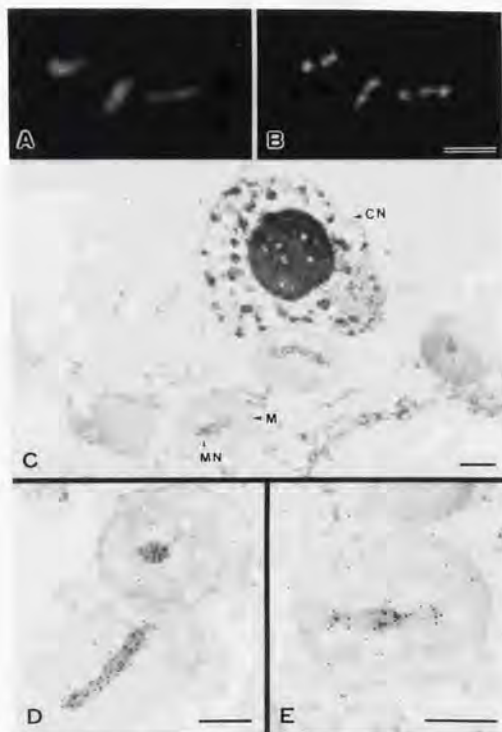
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Fig. 1. The spatial distribution of newly replicated mtDNA in mt-nuclei, as visualized by light (A, B) and electron (C-E) microscopy. For the observations by light microscopy, microplasmodia were labeled with BrdU for 1 hour at 23°C. After fixing cells in Carnoy's solution, incorporated BrdU was immunolabeled using the methods described in the text. A few sites of replicated mtDNA in mt-nuclei (B) are shown together with DAPI-stained DNA fluorescence images (A). For the observations by electron microscopy, microplasmodia were labeled with BrdU for 20 hours at 23 °C. After cutting sections, incorporated BrdU was immunolabeled with 10-nm gold particles and the sections were stained with uranyl acetate. The mt-nuclei are observed as electron-dense structures in the mitochondria. Each mitochondrion contains a single mt-nucleus. Colloidal gold particles are selectively distributed over the cell nucleus and mt-nuclei (C-E). D and E are higher-magnification views of the mitochondria. A typical pattern of the distribution of colloidal gold in an mt-nucleus is shown in (D), but sometimes the particles of colloidal gold formed distinct clusters (E; three clusters in the mt-nucleoid). CN, cell nucleus; M, mitochondrion; MN, mt-nucleoid. Bars, 2 μm (A, B) and 500 nm (C-E).

Fig. 2. The relationship between the copy number of mtDNA and the number of replication signals. Values are given as the mean ± S. D.
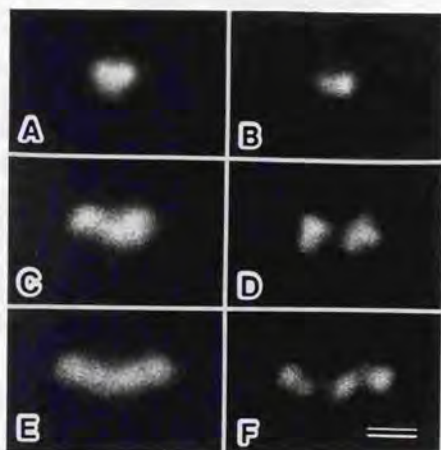
Fig. 3. The spatial distribution of MBR sites along an mt-nucleus. Plasmodium were hybridized *in situ* with DIG-labeled DNA probe corresponding to the MBR of mtDNA from *P. polycephalum*, then stained with DAPI (A, C, E) and anti-DIG antibody (B, D, F). Bar indicates 0.5 μm.

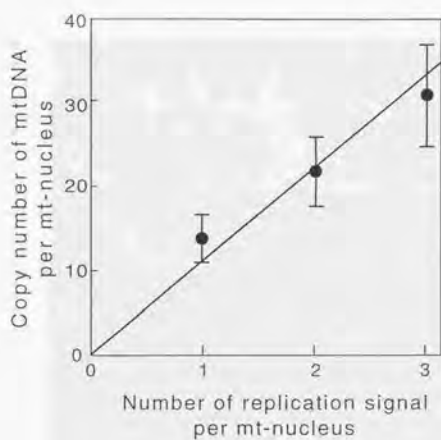**Fig. 4.** The relationship between the copy number of mtDNA and the number of MBR clusters. Values are given as the mean ± S. D.

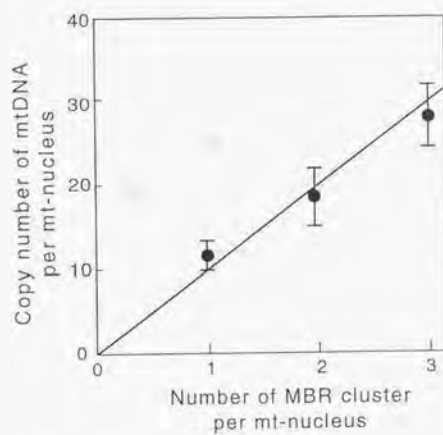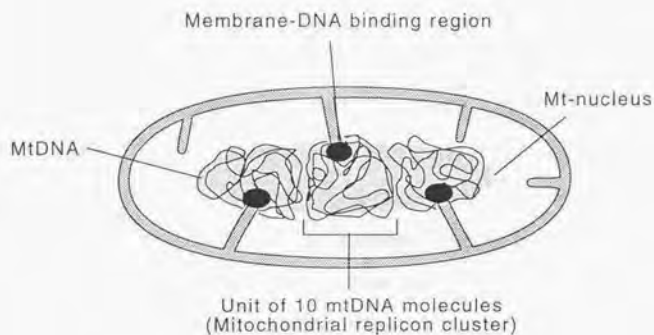**Fig. 5.** A model for the replication and distribution of mtDNA in the mt-nucleus.

Chapter 2

Isolation of structurally intact mitochondrial nuclei from the plasmodia of *Physarum polycephalum* and characterization of their DNA synthesis

SUMMARY

Structurally intact mt-nuclei that were able to support efficient mtDNA synthesis *in vitro* were isolated from the plasmodia of *P. polycephalum* and characterized. I isolated mt-nuclei by dissolving the membranes of highly purified mitochondria with 0.5% Nonidet P-40. Isolated mt-nuclei obtained by treating 4.5 mg of mitochondria protein/mL (MN1) with NP-40 contained 10 times as much inner mitochondrial membrane as mt-nuclei obtained using 0.15 mg mitochondria protein/mL (MN2), as demonstrated by electron microscopy and marker enzymes. This suggests that the protein concentration of the mitochondria being treated with NP-40 affected the solubility of the inner mitochondrial membrane. The isolated mt-nuclei were capable of incorporating dCTP into DNA by endogenous plant-type DNA polymerase in the presence of the four dNTPs, $MgCl_2$. Although the amount of DNA synthesis in MN2 was lower than that in the isolated mitochondria, MN1 retained the same activity as the isolated mitochondria. An *in situ* assay of DNA synthesis following SDS-polyacrylamide gel electrophoresis revealed that MN1 contained the same amount of DNA polymerase as the isolated mitochondria, while MN2 lost about 75% of its DNA polymerase. Furthermore, I demonstrated that MN1 could perform DNA synthesis *in vivo*; DNA synthesis in MN1 occurred in each mitochondrial replicon cluster (MRC) and the activity of DNA synthesis in MN1 isolated from 12 day-old cultures was lower than that from 5day-old cultures. Therefore, the use of MN1 should permit *in vitro* characterization of

the molecular mechanism of mtDNA replication in mt-nuclei.

INTRODUCTION

Mitochondria are semi-autonomously replicating organelles that have their own DNA, known as mitochondrial DNA (mtDNA). It is generally accepted that mtDNA does not exist in a naked form but is organized into a compact structure, the mitochondrial nucleus (mt-nucleus, synonym for mitochondrial nucleoid; Kuroiwa, 1994). Regulation of mtDNA replication occurs in the mt-nuclei. Many researchers have studied the replication of mtDNA. The mechanisms for the initiation of new DNA daughter strands have been clarified (for a review, see Clayton, 1991), and a number of mtDNA replication enzymes have been characterized: DNA polymerase γ (Yamaguchi et al., 1980), DNA primase (Wong and Clayton, 1985a and 1986), topoisomerase (Castora and Simpson, 1979; Lazarus et al., 1987) and DNA helicase (Hehman et al., 1992). However, no attempt has been made to analyze the mechanism of the mtDNA replication in the intricate nuclear structure.

The isolation of structurally and functionally intact mt-nuclei that are capable of mtDNA synthesis *in vitro* facilitates study of the mechanism of DNA replication in the nuclear organization. Some systems for mtDNA synthesis *in vitro* have been established. These systems use a fraction of mitochondrial protein extract (Wong and Clayton, 1985b), mitochondrial lysates (Dunon-Bluteau et al., 1987; Daniell et al., 1995) or permeabilized mitochondria (Jui and Wong, 1991; Enríquez et al., 1994). However, these earlier studies failed to consider the compact structure of the mt-nucleus. In fact, the compact mt-nuclei observed *in vivo* relax spontaneously under the conditions that are generally used for the isolation of mitochondria (Kuroiwa, 1982). Mt-nuclei

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have been isolated from the plasmodia of *Physarum polycephalum* (Suzuki et al., 1982). Electron and fluorescence microscopy show that the isolated mt-nucleoids appear to retain their morphological structure *in vitro*. However, DNA synthesis in these structurally intact, isolated mt-nuclei has not been investigated. In this study, I characterized DNA synthesis in isolated mt-nuclei from the plasmodia of *P. polycephalum*, as the initial step in a study of DNA replication in mt-nuclei.

MATERIALS AND METHODS

Isolation of mitochondria

I used a modified version of the method described by Suzuki et al. (1982) to isolate mitochondria from the microplasmodia of *P. polycephalum*. The microplasmodia were washed twice with distilled water by centrifugation at 100^{l}g for 5 sec each and then suspended in an equal volume of chilled NE1-S buffer [0.5 M sucrose, 20 mM Tris-HCl (pH 7.7), 1 mM EDTA (pH 7.5), 7 mM 2-mercaptoethanol, 0.4 mM spermidine and 0.4 mM PMSF]. All subsequent manipulations were carried out at between 2 and 4°C. The suspension was homogenized by two strokes with a Potter-type homogenizer. The homogenate was diluted by the addition of three to four volumes of NE1-S buffer and centrifuged at 740^{l}g for 5 min. The supernatant was filtered through a sheet of coffee filter paper placed between two layers of 20-μm nylon mesh. The filtrate was centrifuged at 1,000^{l}g for 5 min to remove the cell nuclei. The supernatant was recovered and centrifuged at 4,700^{l}g for 15 min and the resulting pellet was suspended in NE1-S buffer. After the addition of 5 mg of α-amylase (Sigma Chemical Co., MO, USA) to the suspension, the mixture was allowed to stand for 10 min and brought to a concentration of 10% (v/v) Percoll in NE1-S buffer. Then 5.5 mL aliquots were overlaid on discontinuous Percoll

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(Pharmacia, Uppsala, Sweden) density gradients (2 mL of 40% and 4 mL of 20% Percoll in NE1-S buffer) in 11.5 mL centrifugation tubes. After centrifugation at 63,000|g for 60 min in a swinging-bucket rotor (swing rotor RPS40T; Hitachi Koki Co., Ltd., Tokyo, Japan), the band of mitochondria in each tube was recovered. The isolated mitochondria in Percoll were diluted by the addition of two volumes of NE1-S buffer and pelleted in a microcentrifuge at 18,500|g for 2 min. The mitochondrial pellet was washed twice in NE1-S buffer to remove residual Percoll. The isolated mitochondria were resuspended in S buffer.

Isolation of mt-nuclei from mitochondria

I isolated mt-nuclei from mitochondria that had been resuspended in NE1-S buffer at various protein concentrations. To remove the mitochondrial membrane, I treated the suspension with 0.5% Nonidet P-40 (NP-40) for 1 minute, with subsequent centrifugation at 1,300|g for 5 min in a microfuge to remove debris. The supernatant was recovered and centrifuged at 18,500|g for 10 min. The mt-nuclei were resuspended in S buffer and stored on ice until use. Both the isolated mitochondria and the isolated mt-nuclei were observed under an epifluorescence microscope after staining with DAPI.

Assay of DNA synthesis *in vitro*

DNA synthesis was monitored by measuring the incorporation of tritium into polynucleotides after incubating the samples with [5-^{3}H]dCTP. The isolated mitochondria or mt-nuclei were mixed with 1.5 volumes of concentrated reaction mixture to initiate DNA synthesis. The final reaction mixture contained 40 mM Tris-HCl, pH 7.7; 3 mM $MgCl_2$; 0.01% (w/v) NP-40; 180 μM dATP, dGTP and dTTP; 4 μM dCTP and 1 μM [5-^{3}H]dCTP (about 111 TBq/mmol; Amersham, Buckinghamshire, England). After incubation at 26°C,

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aliquots were spotted directly onto discs of paper (DE-81; Whatman International Ltd., Maidstone, U.K.) to stop the reaction. The filter discs were dried, washed four times for 10 min each in 5% Na_2HPO_4, twice in distilled water, twice in 99% ethanol and dried again. Radioactivity was measured with a liquid scintillation counter. Departures from standard reaction conditions are indicated in the legends for the figures.

Assay of DNA polymerase activity *in situ*

Sedimentary mitochondria and mt-nuclei were dissolved in standard SDS sample buffer. The samples were incubated for 3 min at $37°C$ and subjected to electrophoresis following the method described by Spanos and Hübscher (1983). The stacking gel contained 7.5% acrylamide, 0.2% bisacrylamide, 0.375 M Tris-HCl (pH 8.8), 2 mM EDTA (pH 8.0), 0.1% SDS, 0.1 mg/mL heat-denatured salmon testis DNA, 0.05% ammonium persulfate and 0.05% N,N,N',N'-tetramethyl-ethylenediamine (TEMED). The resolving gel contained 3.9% acrylamide, 0.1% bisacrylamide, 65 mM Tris-HCl (pH 6.8), 2 mM EDTA (pH 8.0), 0.1% SDS, 0.1% ammonium persulfate and 0.1% TEMED. After loading the samples, electrophoresis was performed at room temperature at 100 V for 2 hours in reservoir buffer [50 mM Tris-HCl (pH 7.6), 384 mM glycine, 0.1% SDS and 2 mM EDTA (pH 8.0)]. Following electrophoresis, the gel was rinsed in renaturation buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 7.5), 5 mM 2-mercaptoethanol] and then soaked in 1 L of this buffer at room temperature with shaking. The buffer was changed after 30 and 60 min. Then the gel was stored overnight in renaturation buffer at $4°C$. The gel was incubated in the assay mixture [0.5 M sucrose, 1 mM EDTA (pH 7.5), 0.4 mM spermidine, 7 mM 2-mercaptoethanol, 0.4 mM PMSF, 40 mM Tris-HCl (pH 7.7), 3 mM $MgCl_2$, 0.01% (w/v) NP-40, 180 µM dATP, dGTP and dTTP, 4 µM dCTP and 1.85 MBq of [^{32}P]dCTP (about 111 TBq/mmol;

Amersham, Buckinghamshire, England)] at 37°C for 24 h. It was then washed ten times with 100 mL of washing buffer (5% trichloroacetic acid, 1% sodium pyrophosphate) over the course of two days. The gel was dried on filter paper and subjected to autoradiography. DNA polymerase activity was detected as a dark band that represented the [^{32}P]-dCTP incorporated into DNA. For an analysis of polypeptides, the procedure was repeated using another gel without salmon testis DNA. This was then silver-stained using the method described by Oakley et al. (1980).

Labeling with BrdUTP *in vitro* and indirect immunofluorescence microscopy with BrdU-specific antibodies

Isolated mitochondria or isolated mt-nuclei were mixed with 1.5 volumes of concentrated reaction mixture to initiate DNA synthesis. The final reaction mixture contained 40 mM Tris-HCl, pH 7.7, 3 mM MgCl$_2$, 0.01% (w/v) NP-40, 180 μM dATP, dGTP and dCTP and 300 μM bromodeoxyuridine triphosphate (BrdUTP). After incubation for 1 hour, samples were fixed in Carnoy's solution for 10 min. Then the BrdU was immunolabeled in the manner described above.

Quantification of the incorporated BrdU into mtDNA by slot blot analysis

Five and twelve day old cultures were labeled for 2 h with 10 mM BrdU in the presence of 1 mM 5-fluorodeoxyuridine. Then mitochondria were isolated from the cultures using the methods described above. 450 μL of DNA extraction buffer [50 mM Tris-HCl (pH. 8.0), 100mM EDTA, 300 mM NaCl, 2% (w/v) Sarkosyl, 4% (w/v) SDS] were added to 50 μL of the mitochondria fraction, mixed by vortexing and then incubated at 65°C for 15 min. Then 10 μL of 20 mg/mL Proteinase K was added, and incubation was continued for 15 h. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). The mtDNA was slot blotted onto nitrocellulose filters and incubated with anti-BrdU antibody and alkaline phosphatase-conjugated second antibody.

Immuno-blot detection was performed with an immuno-assay kit under the conditions recommended by the manufacturer (Immuno-Blot Assay Kit, Bio-Rad Laboratories, California).

RESULTS

Improved purification of mitochondria from the plasmodia of *P. polycephalum*

I isolated the mt-nuclei from the plasmodia of *P. polycephalum*. Initial isolation of mt-nuclei from plasmodia used a published purification procedure (Suzuki et al. 1982). They isolated mitochondria by dissolving the membranes of the purified mitochondria with the detergent NP-40. However, I found that these mt-nuclei fractions contained cell nuclei, which would have hampered our biochemical analysis. To obtain a very pure mt-nuclei fraction, I improved the method used for isolating mitochondria. Figure 1 summarizes the fractionation procedure I developed. The crude mitochondria obtained by differential centrifugation were purified on a discontinuous gradient of Percoll. After centrifugation, the mitochondria were recovered as a single band located in the centre of the tube (Fig. 7).

Light microscopy confirmed the homogeneity and integrity of the mitochondrial preparation. There was no contamination with cell nuclei in the mitochondrial fraction (Fig. 8B, C). Each mitochondrion contained a rod-shaped mt-nuclei similar to those observed *in vivo* (Fig. 8A, D, E).

Observation of the isolated mt-nuclei by light and electron microscopes

The mt-nuclei were obtained by dissolving the membranes of the highly purified mitochondria with the detergent NP-40 (Fig. 8F, G). It is known that

the concentration of the membrane protein affects the solubility of the membrane when the concentration of detergent is fixed. For example, the solubility of the membrane is lower when the concentration of membrane proteins is high. When observed by light microscopy, all the isolated mt-nuclei that were obtained by treatment of various concentrations of isolated mitochondria with 0.5% NP-40 retained their rod-shaped mt-nuclei and had no mitochondrial membrane (Fig. 9).

Using electron microscopy after negative staining, however, I found that there was a difference between the mt-nuclei isolated from mitochondria with concentrations of 4.5 and 0.15 mg mitochondrial protein/mL. Hereafter these samples will be referred to as MN1 and MN2 respectively. Though the mtDNA was organized into a compactly folded and chromatin-like structure in MN1 and MN2, some mitochondrial membrane-like patches remained attached in MN1 (Fig. 10).

To identify the origin of these membrane-like patches, I measured the specific activity of mitochondrial membrane marker enzymes (Table 1). Isolated mitochondria with intact membranes contain kynurenine hydroxylase (outer membrane), adenylate kinase (intermembrane space) and cytochrome c oxidase (inner membrane). Both MN1 and MN2 had no specific activity for kynurenine hydroxylase or adenylate kinase (Table 1). The specific activity of cytochrome c was determined for MN1 (20%) and MN2 (1%). Therefore, the membrane-like patches observed in MN1 were identified as inner mitochondrial membrane.

DNA and RNA synthesis activity in the isolated mt-nuclei

The isolated mt-nuclei were able to incorporated dCTP into polynucleotides. The time course for the incorporation of dCTP in isolated mitochondria and MN1 is shown in Figure 11A. The incorporation of dCTP in MN1 was similar to that in isolated mitochondria. In both cases, incorporation of dCTP began immediately after the addition of the sample and increased at a

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constant rate for 15 min, thereafter continuing at a slightly decreased rate for up to 2 h. The mitochondria and mt-nuclei incorporated approximately 30 and 34 pmol of dCTP per microgram of DNA during a 60-min incubation, respectively.

The respective effects of various treatments on the incorporation of dCTP by isolated mitochondria and isolated mt-nuclei were similar (Table 2). The level of incorporated radioactivity decreased upon the addition of DNase I to the reaction mixture after the standard assay. In contrast, a similar treatment with RNase A had no effect. These results prove that the material labeled with [³H]-dCTP was DNA. The incorporation of dCTP was completely inhibited by treating the samples with proteinase K before the assay. Intercalating agents, such as ethidium bromide and actinomycin D, also inhibited DNA synthesis. These results prove that the incorporation of dCTP was catalyzed by DNA polymerase. Aphidicolin, which inhibits the activity of DNA polymerase α, had no effect at 25 μg/mL. Under similar conditions, *P. polycephalum* DNA polymerase α loses 40% of its activity (Matsuzawa et al., 1987). On the other hand, mitochondrial polymerase from *P. polycephalum* was slightly sensitive to dideoxycytidine triphosphate (ddCTP) and N-ethylmaleimide (NEM), inhibitors of animal mitochondrial polymerase γ (Wernette and Kaguni, 1986; Yamaguchi et al., 1980; Insdolf and Borgenhagen, 1989). Although inhibition was observed at low concentrations of ddCTP (5 μM) and NEM (2.5 mM), it was not observed at high concentrations of ddCTP (100 μM) and NEM (25 mM). Sensitivity to low concentrations of ddNTPs has been reported for mitochondrial DNA polymerase from many plant sources (Daniell et al., 1995; Christophe et al., 1981; Heinhorst et al., 1990; Sato and Fukuda et al., 1996). Similar sensitivity to low concentrations of NEM has also been reported for mitochondrial DNA polymerase from a few plant sources (Daniell et al. 1995, Christophe et al. 1981). Therefore, the mitochondrial polymerase of *P. polycephalum* should be classified as a plant-type DNA polymerase, rather

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than animal DNA polymerase γ.

The isolated mt-nuclei were able to incorporate UTP into polynucleotides. The time course for the incorporation of UTP in the isolated mitochondria and MN1 is shown in Figure 11B. The incorporation of UTP began immediately after the addition of the sample, and increased for 15 min in both MN1 and the isolated mitochondria. The incorporation of UTP in MN1 was lower than that in isolated mitochondria. The mitochondria and mt-nuclei incorporated about 48 and 74 pmol of UTP per microgram of DNA during a 15-min incubation, respectively.

The respective effects of various treatments on the incorporation of UTP by isolated mitochondria and the isolated mt-nuclei were similar (Table 3). The material labeled with [^{3}H]-UTP was shown to be RNA, because the level of incorporated radioactivity decreased upon the addition of RNase A to the reaction mixture after the standard assay. The incorporation of UTP was completely inhibited by treating the samples with Proteinase K or DNase I before the assay. Moreover, actinomycin D also inhibited RNA synthesis. These results are consistent with DNA-dependent RNA polymerase. Transcription by mt-nuclei was slightly sensitive to rifampicin, which binds to bacterial RNA polymerase and prevents initiation. Furthermore, heparin, which competes with DNA for initial binding of the polymerase, did not affect the transcriptional activity, suggesting that little or no transcription initiation occurred under our experimental conditions. Therefore, it appears that the incorporation of UTP in isolated mt-nuclei was the result of chain elongation of nascent transcripts, which were formed by mitochondrial RNA polymerase before the isolation.

I then examined DNA and RNA synthesis in isolated mt-nuclei obtained from various concentrations of mitochondria. DNA synthesis activity decreased as the protein concentration of the mitochondria treated with NP-40

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decreased (Fig. 12A). The level of DNA synthesis in MN2 was about 20% of that in MN1. On the other hand, the level of RNA synthesis was not affected by the protein concentration (Fig. 12B).

Comparison of the amount of mtDNA polymerase in MN1 and MN2

I then compared the amounts of DNA polymerase in isolated mitochondria, MN1 and MN2. This was accomplished by monitoring the incorporation of $[^{32}P]$-dCTP into a gel that contained heat-denatured salmon testis DNA after SDS-PAGE (Fig. 13). Lanes 1-3 show the patterns of proteins in the isolated mitochondria, MN1 and MN2 after silver staining. Each lane contains the same amount of mtDNA. The protein pattern in MN1 was similar to that in MN2 although this pattern was different from that of the mitochondria. mtDNA synthesis was detected as a band in the position of a 120 kDa protein (lanes 4-6). Since the density of the band is proportional to the amount of DNA polymerase, it appears that MN1 retained the same amount of DNA polymerase as present in the isolated mitochondria, while MN2 lost about 75% of its DNA polymerase.

Integrity of the mitochondria replicon cluster during the isolation

In Chapter 1, I visualized the sites of DNA replication in the mt-nuclei of the plasmodia, after incubating cells with 5-bromodeoxyuridine (BrdU). The replication sites are not distributed randomly throughout the mt-nuclei but are concentrated in a few discrete regions. Therefore, I proposed that replication of individual mtDNA molecules in the mt-nucleus is not regulated independently, but that the replication of groups of adjacent mtDNA molecules is regulated concertedly within a structure defined as the mitochondrial replicon cluster (MRC). To examine the structural integrity of the MRC during isolation, mitochondria and mt-nuclei were isolated from cells that had been treated with BrdU for 1 hour and were then immunostained with BrdU-specific antibodies

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(Fig. 14). In both isolated mitochondria and MN1, globular replication signals were observed along the rod-shaped mt-nuclei. Thus, both the rod-shaped structure of the mt-nucleus and the MRC within the mt-nucleus were retained during isolation.

Next, to examine the functional integrity of the MRC during isolation, I visualized the sites of DNA synthesis *in vitro* by immuno-fluorescence microscopy using BrdU-specific antibodies (Fig. 15). *In vivo*, exogenous BrdU is converted to the corresponding triphosphate, which is then incorporated into DNA. Since the conversion reaction does not occur in isolated mitochondria and MN1, I used BrdUTP instead of BrdU. The compact structure of the mt-nuclei in the isolated mitochondria and in MN1 was maintained during the incubation with BrdUTP. In both isolated mitochondria and MN1, several sites of mtDNA synthesis were distributed along the mt-nuclei. Therefore, it appears that DNA synthesis in MN1 might occur in each MRC.

Change in the activity of DNA synthesis in the isolated mitochondria and MN1 during culturing

In a previous study, I showed that mtDNA was synthesized during the log-growth phase, and not during the stationary growth phase in the amoebae of *P. polycephalum* (Sasaki et al. 1994). Similar changes in the level of DNA synthesis during culture were observed in the plasmodia. Figure 16 shows the change in the amount of protein per mL of culture. The microplasmodia grew vigorously until the 7th day, as revealed by the exponential increase in the amount of protein per mL of culture. The amount of protein reached a maximum on the 8th day and then decreased gradually by autolysis. To examine DNA synthesis in 5- and 12-day-old cultures, those cultures were treated with BrdU for 2 hours and were immunostained with BrdU-specific antibodies. In the 5-day-old culture, most mt-nuclei were labeled with BrdU

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(Fig. 17A, C). On the other hand, a few mt-nuclei were slightly labeled in the 12-day-old culture (Fig. 17B, D). The amount of BrdU incorporated into mtDNA was quantified by slot blot analysis. 5- and 12-day-old cultures were treated with BrdU for 2 h and the mtDNA was extracted from the highly purified mitochondria. Then, a serial dilution of mtDNA was slot blotted onto a nitrocellulose filter and probed with the BrdU-specific antibodies (Fig.18). The amount of BrdU incorporated into the mtDNA in the 5-day-old culture was >5 times higher than that in the 12-day-old culture. Such changes in the activity of DNA synthesis observed *in vivo* generally reflect those seen *in vitro*. The isolated mitochondria and MN1 from the 5-day-old culture exhibited more DNA synthesis activity than those from the 12-day-old culture (Fig. 19).

DISCUSSION

I isolated structurally intact mt-nuclei that were able to support efficient mtDNA and RNA synthesis. The isolated mt-nuclei were obtained by dissolving the membranes of highly purified mitochondria with NP-40. Observation by light microscopy after DAPI staining showed the rod-shaped structure of the mt-nuclei was retained during the isolation (Fig. 8, 9). We also showed that the isolated mt-nucleoids retain intact MRCs (Fig. 14). The retention of MRCs strongly suggests that the three-dimensional nuclear structure of the isolated mt-nucleoids reflects the structure *in vivo* and that the isolated mt-nucleoids are structurally intact. Observation by electron microscopy after negative staining showed that some membrane-like patches were attached to the MN1 mt-nuclei, which were obtained from the treatment of highly concentrated mitochondria (4.5 mg protein/mL) with NP-40 (Fig. 9A). Kuroiwa et al (1979) also found membrane-like patches attached to the mt-

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nuclei obtained by mild NP-40 treatment, on electron microscopic observation of thin sections. The membrane enzyme experiment showed that the membrane-like patches were parts of the inner membrane that were not dissolved by the mild treatment with NP-40 (Table 1). These membrane-like patches were not observed in MN2, which was obtained by the treatment of a low concentration of mitochondria (0.15 mg protein/mL) with NP-40. MN2 had a lower cytochrome c oxidase specific activity than MN1 (Table 1). This indicated that the protein concentration of isolated mitochondria being treated with NP-40 affected the dissolution of the inner mitochondrial membrane.

The level of DNA synthesis in the isolated mt-nuclei decreased as the concentration of isolated mitochondria treated with NP-40 decreased. The level of DNA synthesis in MN1 was about 5 times higher than that in MN2 (Fig. 12A). MN1 retained the same capacity for DNA synthesis as the isolated mitochondria, and approximately 34 pmol of dCTP per microgram of DNA was incorporated into the synthesized DNA during a 60-min incubation (Fig. 11). Since 34 pmol of dCTP (M.W. 307.2) corresponds to about 0.010 µg of dCTP, and the CG content of mtDNA is 30%, it was calculated that roughly 0.066 µg DNA, or 6.6% of the template DNA, was synthesized during a 60-minute incubation. This rate of DNA synthesis is comparable to that seen in a good *in vitro* system for mtDNA replication that uses the mitochondrial lysate of *Xenopus laevis* oocytes (Dunon-Bluteau et al. 1987). The assay of DNA synthesis *in situ* after SDS-polyacrylamide gel electrophoresis revealed that the same amount of mitochondrial DNA polymerase found in isolated mitochondria was retained in MN1 (Fig. 13). This suggests that no mitochondrial DNA polymerase was lost during the isolation of MN1. The site of mtDNA synthesis in MN1 was distributed along the mt-nuclei and the pattern of distribution of these replication sites was similar to that observed *in vivo*. This suggested that mtDNA synthesis in MN1 occurred at each MRC. Furthermore, MN1 mt-nuclei isolated from a 5-day-old culture had a greater

level of DNA synthesis than those from a 12-day-old culture (Fig. 19). These results suggest that MN1 mt-nuclei are capable of efficient DNA synthesis and that the DNA synthesis in MN1 reflects that *in vivo*.

In the past, it has been assumed that mtDNA is attached to the inner mitochondrial membrane, based on electron microscopy observations (Nass et al., 1965). In analogy with bacterial chromosomes, it has been suggested that the mitochondrial membrane plays an important role in the replication of mtDNA (Jacob et al., 1964; Nass et al., 1965). There are some reports that the membrane plays an active role in mtDNA replication. *In vivo*, HeLa cell mtDNA is attached to the inner mitochondrial membrane at or near the origin of mtDNA replication (Albring et al., 1977). Inner membrane-DNA complexes, enriched with newly synthesized DNA, were isolated from rat liver mitochondria, and the complex was able to synthesize DNA *in vitro* (Shearman and Kalf, 1977). A high molecular weight complex associated with the mitochondrial membrane was isolated from wheat embryo mitochondria and the complex actively synthesized DNA and RNA *in vitro* (Echeverria et al., 1991). In this study, the mt-nuclei from MN1, which retained a part of the inner membrane, maintained an efficient capacity for DNA synthesis. The level of DNA synthesis in the isolated mt-nuclei decreased as the concentration of isolated mitochondria treated with NP-40 decreased. The level of DNA synthesis in MN2 was about 20% of that in MN1 (Fig. 12A). Since MN2 possessed only a quarter of the DNA polymerase found in MN1, it was clear that this decrease in the level of DNA synthesis was caused by the loss of DNA polymerase (Fig.13). Therefore, it seems reasonable to expect that DNA polymerase is associated with the inner mitochondrial membrane attached to the mt-nuclei in MN1, but further work is necessary to examine this issue.

Unlike DNA synthesis *in vitro*, the level of RNA synthesis in the isolated mt-nuclei was not affected by the protein concentration of the

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mitochondria treated with NP-40. Both MN1 and MN2 had relatively high levels of RNA synthesis (Fig.12 B). These results suggest that RNA polymerase might be associated with the mt-nuclei rather than the mitochondrial membrane.

As described above, both MN1 and MN2 were able to support mtDNA synthesis and mtRNA synthesis. However, MN1 was capable of more efficient DNA synthesis than MN2 and I observed that the level of DNA synthesis in MN1 reflected that seen *in vivo*. Therefore, I believe that MN1 is more useful for the analysis of the mechanism of the replication in the mt-nuclei than MN2. Furthermore, the use of MN1 should permit *in vitro* characterization of the molecular mechanism of mtDNA replication in the MRC, because MN1 retained structurally and functionally intact MRC.

Table 1. The specific activity of mitochondrial membrane marker enzymes in the isolated mitochondria, MN1 and MN2.

Fractions	Kynurenine hydroxyrase (Outer membrane)		Adenylate kinase (Intermembrane space)		Cytochrome oxydase (Inner membrane)	
	Sp. act.	%	Sp. act.	%	Sp. act.	%
Mitochondria	9.1	100	0.14	100	1.57	100
Mt-nuclei (A)	0.0	0.0	0.0	0.0	0.33	21
Mt-nuclei (B)	0.0	0.0	0.0	0.0	0.015	0.95

*Specific activity is expressed as units per µg DNA

Table 2. Characteristics of the incorporation of [³H]dCTP by isolated mitochondria and by isolated mt-nuclei

Condition	Incorporation of [³H]dCTP(%)	
	Isolated mitochondria	Isolated mt-nuclei
Optimal	100	100
With DNase I [b] (400 u/ml)	0	5
RNase [b] (200 µg/ml)	83	97
Proteinase K [a] (4 mg/ml)	4	2
Proteinase K [b] (4 mg/ml)	90	109
Ethidium bromide (25 µg/ml)	10	14
Actinomycin D (25 µg/µl)	38	39
Aphidicolin (25 µg/ml)	115	102
ddCTP (5 µM)	96	108
ddCTP (100 µM)	41	59
NEM (2.5 mM)	104	98
NEM (25 mM)	3	15

The method used to perform the assays is described in the text. Optimal conditions were 40 mM Tris-HCl (pH 7.7), 3 mM MgCl₂, 0.01% (w/v) NP-40, 180 mM each dATP, dGTP and dTTP, 4 mM of dCTP and 1 mM [³H]dCTP, with incubation at 26°C for 60 min.

a: Isolated mitochondria and isolated mt-nucleoids were treated with the indicated enzyme before the assay.

b: Reaction mixtures were treated with the indicated enzyme after the assay.
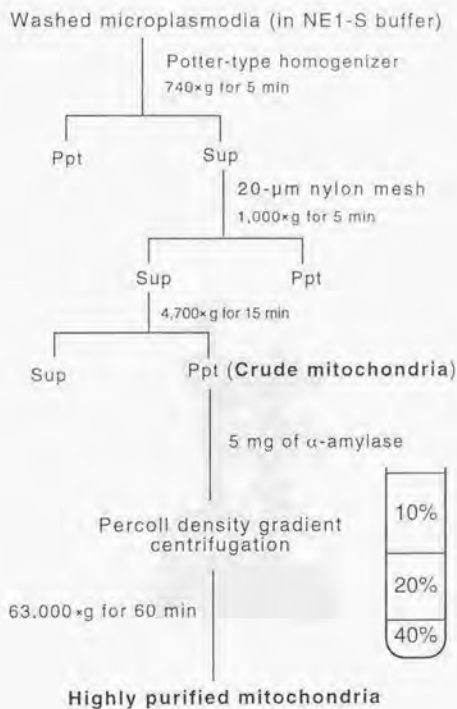
Table 3. Characteristics of the incorporation of [³H]UTP by isolated mitochondria and by isolated mt-nuclei.

Condition	Incorporation of [³H]UTP(%)	
	Isolated mitochondria	Isolated mt-nuclei
Optimal	100	100
With DNase I [a] (400 u/ml)	2.8	1.7
RNase [b] (200 µg/ml)	2.8	2.2
Proteinase K [a] (4 mg/ml)	6.7	0
Proteinase K [b] (4 mg/ml)	92	90
Actinomycin D (25 µg/µl)	42	43
Rifampicin (100 µg/ml)	89	78
Heparin (100 µg/ml)	123	126

The method used to perform the assays is described in the text. Optimal conditions were 40 mM Tris-HCl (pH 7.7), 3 mM $MgCl_2$, 0.01% (w/v) NP-40, 180 mM each ATP, GTP and CTP, 4 mM of UTP and 1 mM [³H]UTP, with incubation at 26˚C for 60 min.

a: Isolated mitochondria and isolated mt-nucleoids were treated with the indicated enzyme before the assay. b: Reaction mixtures were treated with the indicated enzyme after the assay.

Washed microplasmodia (in NE1-S buffer)

Potter-type homogenizer
740×g for 5 min

Ppt   Sup

20-μm nylon mesh
1,000×g for 5 min

Sup   Ppt

4,700×g for 15 min

Sup   Ppt (**Crude mitochondria**)

5 mg of α-amylase

Percoll density gradient
centrifugation

63.000×g for 60 min

10%

20%

40%

Highly purified mitochondria

Fig. 6. Flow diagram for the purification of mitochondria from *P. polycephalum* plasmodia. Details are given under Materials and Methods.
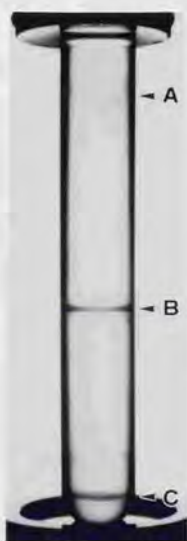
Fig. 7. Photograph of a tube after the Percoll density gradient centrifugation of crude mitochondria. The mitochondria were separated from the cell nuclei, debris and pigment by centrifugation. A band of cell-nuclei and debris was located at the top of the tube (A). The band of mitochondria was situated in the middle of the tube (B). The band of pigments was situated at the bottom of the tube (C).
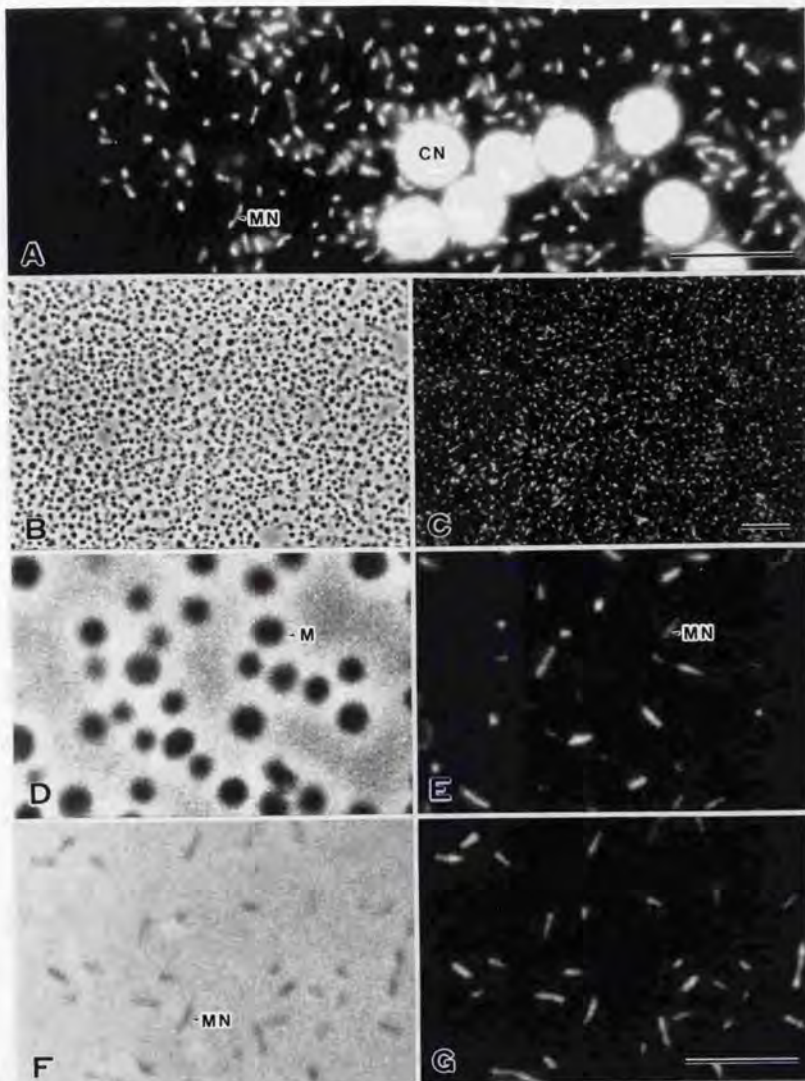
Fig. 8. Light micrographs of whole cell and isolated mitochondria of *P. polycephalum* plasmodia. A, DAPI-stained DNA fluorescence image of plasmodium; B-E, Phase-contrast (B, D) and DAPI-stained DNA fluorescence (C, E) images of isolated mitochondria. D and E are higher-magnification views; F, G, Phase-contrast (F) and DAPI-stained DNA fluorescence (G) images of isolated mt-nuclei. CN, cell nucleus; M, mitochondrion; MN, mt-nucleoid. Bars, 10 μm (A-C) and 5 μm (D-G).
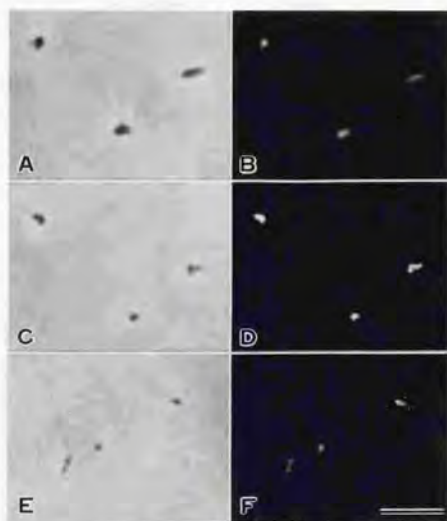
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Fig. 9. Phase-contrast (A, C, E) and DAPI-stained DNA fluorescence (B, D, F) images of isolated mt-nuclei of *P. polycephalum* plasmodia. The mt-nuclei were isolated by the treatment of 4.5 (A, B), 2.25 (C, D) and 0.15 (E, F) μg protein/μL purified mitochondria with 0.5% NP40. Bar, 3 μm.
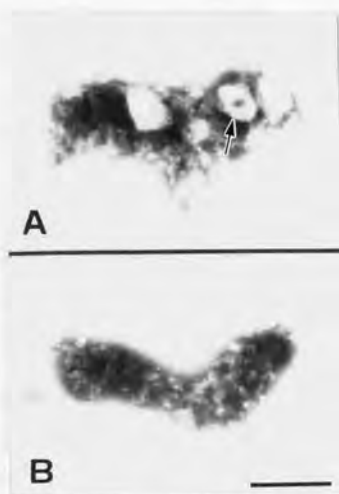
Fig. 10. Electron micrograph of the mt-nuclei after negative staining. The mt-nuclei were isolated by the treatment of 4.5 (A) and 0.15 (B) mg protein/mL purified mitochondria with 0.5% NP40. Membrane-like patches (arrow) were attached to the isolated mt-nuclei (A). Bar, 200 nm.
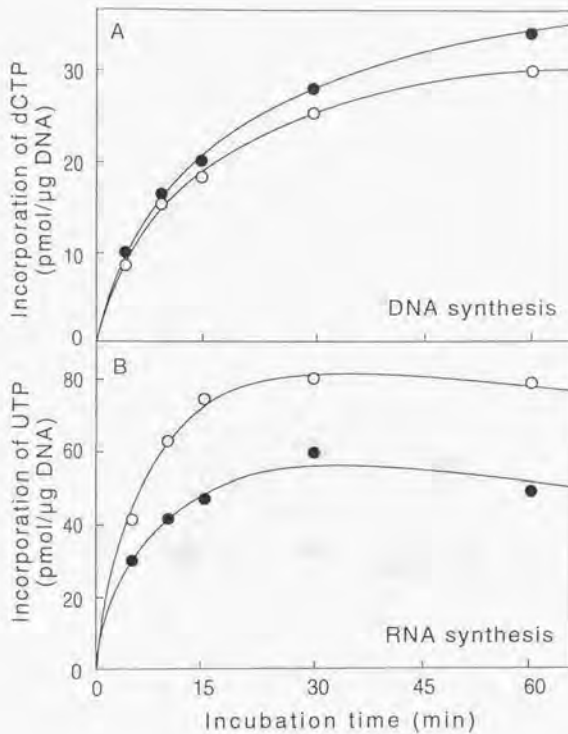
Fig.11. Time courses for mtDNA synthesis (A) and RNA synthesis (B) in isolated mitochondria (O) and in MN1 (●). The assay of DNA synthesis and RNA synthesis *in vitro* was carried out under standard conditions. Radioactivity incorporated into the mtDNA after incubation for different periods was quantified in the manner described in the text.
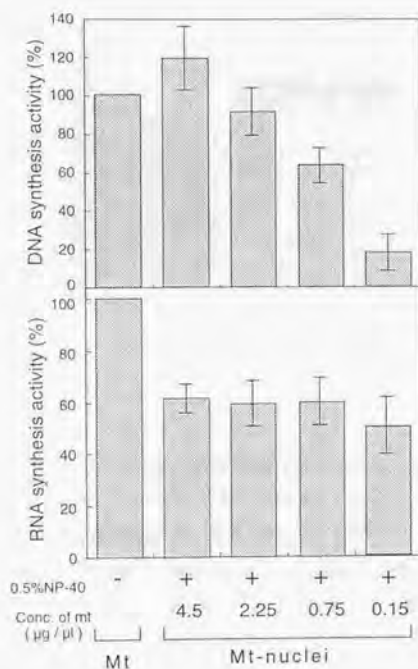
Fig. 12. Change in the activity of DNA synthesis (A) and RNA synthesis (B) in the isolated mt-nuclei. The mt-nuclei were isolated by the treatment of various concentrations of mitochondria with 0.5% NP-40. The assays of DNA synthesis and RNA synthesis *in vitro* were carried out under standard conditions. Radioactivity incorporated into mtDNA after incubation for 15 min was quantified in the manner described in the text. The activity in the isolated mitochondria was arbitrarily set at 100%.
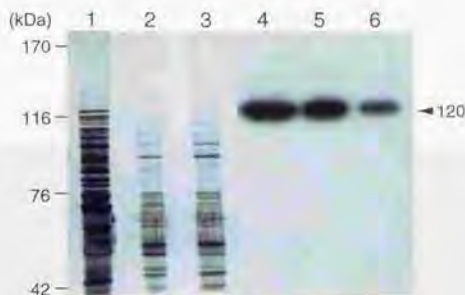
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Fig. 13. Detection of DNA polymerase by an *in situ* assay after SDS polyacrylamide gel electrophoresis. Proteins from isolated mitochondria and isolated mt-nuclei (equivalent to 0.5 µg of DNA) were separated by electrophoresis in an SDS-polyacrylamide gel that contained heat-denatured salmon testis DNA. After renaturation of the enzyme, the DNA polymerase assay was performed *in situ* in the manner described in the text. [^{32}P]dCTP incorporated into the salmon testis DNA was detected as a dark band after autoradiography. To analyze the polypeptides, another gel, without salmon testis DNA, was silver-stained. Lane 1: polypeptides in isolated mitochondria after silver staining. Lane 2: polypeptides in MN1 after silver staining. Lane 3: polypeptides in MN2 after silver staining. Lane 4: an autoradiogram showing the DNA polymerase activity in isolated mitochondria. Lane 5: an autoradiogram showing the DNA polymerase activity in MN1. Lane 6: an autoradiogram showing the DNA polymerase activity in MN2. The numbers on the left and right indicate the mobility of standard proteins and their molecular masses in kDa, respectively.
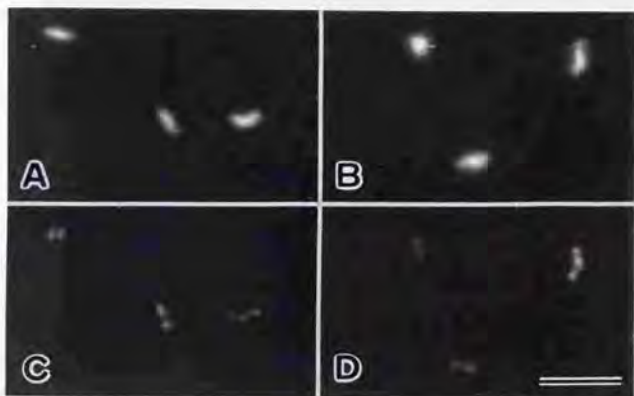
Fig. 14. The site of *in vivo* mtDNA replication in isolated mitochondria and the isolated mt-nuclei (MN1). Mitochondria and mt-nuclei were isolated from microplasmodia labeled with BrdU for 1 h at 23°C. After fixing the isolated mitochondria and isolated mt-nuclei in Carnoy's solution, incorporated BrdU was immunostained using the method described in the text. The site of *in vivo* mtDNA replication in isolated mitochondria (C) and in isolated mt-nuclei (D) is shown, together with DAPI-stained DNA fluorescence images (A and B, respectively). Bar, 3 μm.
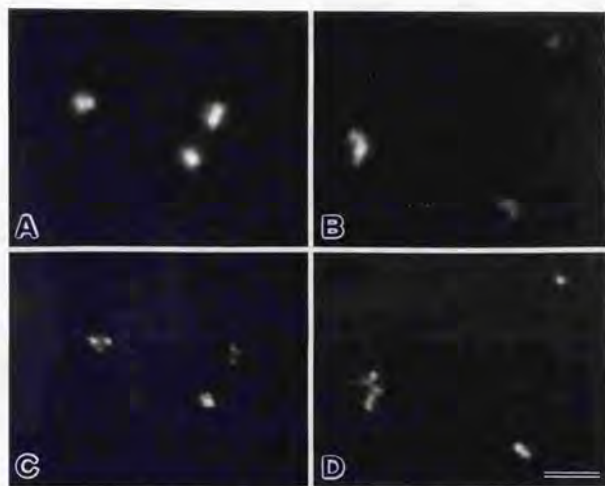
Fig. 15. The sites of *in vitro* DNA replication in isolated mitochondria and the isolated mt-nuclei (MN1). After isolation of mitochondria and mt-nuclei, samples were labeled with BrdUTP for 1 h at 23°C. The incorporated BrdUTP was immunostained using the method described in the text. Sites of *in vitro* mtDNA replication in isolated mitochondria (C) and in isolated mt-nuclei (D) are shown, together with DAPI-stained DNA fluorescence images (A and B, respectively). Bar, 2 μm.
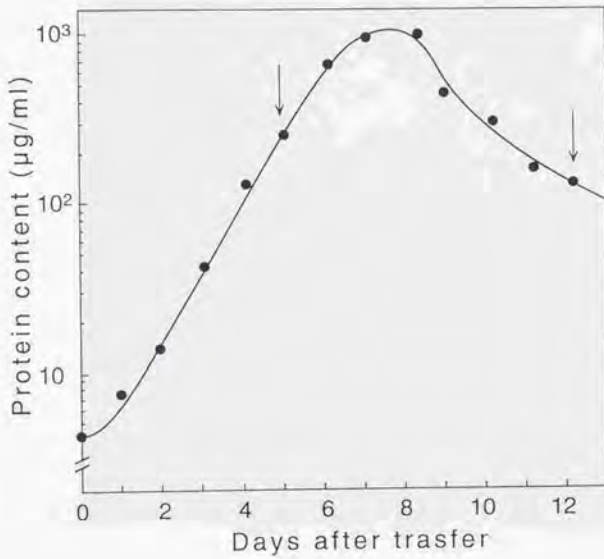
48

Fig. 16. Growth curve for *P. polycephalum* microplasmodia. The protein content per 1 mL of culture was measured by Lowry's method.
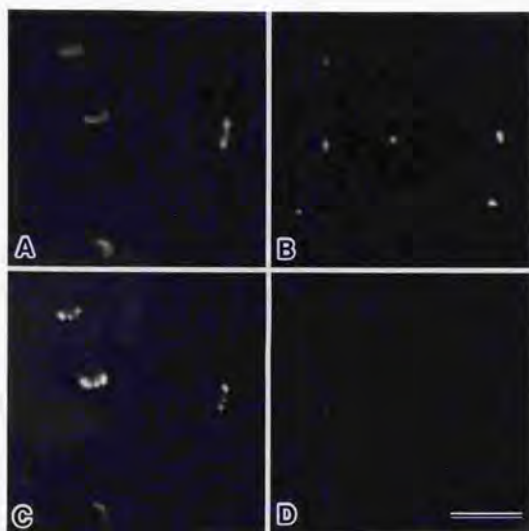
Fig. 17. Light microscopy observation of the BrdU incorporated into the mt-nuclei of the 5- (A, C) and 12-day-old cultures (B, D). Cells were treated with BrdU for 2 h. The incorporated BrdUTP was immunostained using the method described in the text. Sites of DNA replication in the 5-day-old (C) and 12-day-old cultures (D) are shown, together with DAPI-stained DNA fluorescence images (A and B, respectively). Bar, 5 μm.

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Fig. 18. Slot blot analysis of the BrdU incorporated into the mtDNA of the 5- and 12-day-old cultures. Cells were treated with BrdU for 2 h. Then, mitochondria were isolated and their DNA was extracted. Serial dilution of the mtDNA (0.9 - 9 µg) from the 5-day-old culture and 9 µg mtDNA from the 12-day-old culture were slot blotted onto a nitrocellulose filter. Anti-BrdU antibody was used to detect the BrdU incorporated into the mtDNA.
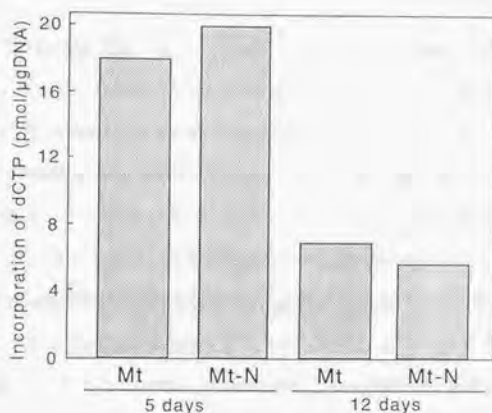
Fig. 19. Change in the activity of DNA synthesis in the isolated mitochondria and the isolated mt-nuclei (MN1) during the culture. The mt-nuclei were isolated from the 5- and 12-day-old cultures. The assay of DNA synthesis *in vitro* was carried out under standard conditions. Radioactivity incorporated into the mtDNA after incubation for 15 min was quantified using the method described in the text. The activity in the isolated mitochondria was arbitrarily set at 100%.

Chapter 3

Analysis of mitochondrial nuclear proteins that may be associated with structure and function of mitochondrial DNA

SUMMARY

I investigated the disruption of the structure and function of mitochondrial DNA (mtDNA) in the isolated mitochondrial nuclei (mt-nuclei) of *Physarum polycephalum* plasmodia by treatment with NaCl and identified the mitochondrial proteins that were released from the mtDNA. Treatment with low concentrations of NaCl (≤0.2 M) separated the mtDNA molecules in the mt-nuclei. When the salt concentration was increased to 0.2 M NaCl, the activity of DNA synthesis decreased and DNA polymerase was released from the mt-nuclei. At a concentration of 0.5 M NaCl, the mtDNA was dispersed and the activity of RNA synthesis decreased. Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that 41- and 56-kDa proteins were released into the supernatant in 0.2 M NaCl, while 39-kDa protein was released in 0.5 M NaCl. A protein gel blot probed with mtDNA indicated that these proteins possess DNA binding activity. This suggests that these proteins are associated with mtDNA in the mt-nuclei.

To characterize the 41-kDa protein, the major protein in the mt-nuclei, I sequenced 617 nucleotides, which encode a part of the 41-kDa protein, by PCR amplification using two degenerate primers corresponding to the N-terminal sequence and the internal amino acid sequence, the 41-kDa protein. The amino acid sequence predicted from the DNA sequence revealed some remarkable properties of the 41-kDa protein, including a high lysine content and significant homology with the N-terminal region of the H1 class of eukaryotic histones.

INTRODUCTION

Mitochondrial DNA (mtDNA) forms a highly organized structure with proteins, the so-called mitochondrial nucleus (Kuroiwa et al., 1994). Visualization of the sites of DNA replication by autoradiography of [3H]thymidine-labeled cells (Kuroiwa et al., 1978) and immunofluorescence detection of incorporated bromodeoxyuridine (Chapter 1) clearly shows that mtDNA replicates within the mitochondrial nucleus (mt-nucleus) *in vivo*. In Chapter 2, I discussed the isolation of mt-nuclei from the *Physarum polycephalum* that retained their nuclear organization and could efficiently synthesize mtDNA. This suggests that the proteins that are essential for nuclear organization and mtDNA synthesis are organized in the mt-nuclei.

To understand the mechanism of mtDNA replication in the organized nuclear structure, it is important to identify the mitochondrial nuclear proteins that play roles in the nuclear organization and mtDNA synthesis. To identify these proteins, I analyzed the proteins that were released from the mtDNA when the nuclear organization was disrupted and the activity of mtDNA synthesis decreased *in vitro*. It is known that heat shock, NaCl, EDTA, EGTA+MgCl$_2$, Pronase E, SDS and HCl will all disrupt isolated mt-nuclei (Suzuki et al., 1982). In this study, I treated the isolated mt-nuclei with NaCl to disrupt the nuclear organization, because neutral salts separate DNA and the DNA binding proteins without affecting the primary structure of either molecule. Treatment with NaCl also decreases the levels of DNA and RNA synthesis. In the analysis of the proteins that are released from mtDNA by treatment with NaCl, I identified three DNA binding proteins in the mt-nuclei and one of these proteins (41-kDa) showed significant homology to histone H1.

MATERIALS AND METHODS

Culture of cells and isolation of mitochondria and mt-nuclei

Mt-nuclei were isolated from the microplasmodia of *P. polycephalum*, Colonia isogenic strain KM 182 / KM 187. The conditions used for cell culture and the procedures used for the isolation of the mitochondria and mt-nuclei were the same as described in Chapters 1 and 2.

Disorganization of isolated mt-nuclei

Isolated mt-nuclei with a protein concentration of 1 mg protein/mL were treated with various concentrations of NaCl to disrupt the interaction between the mtDNA and DNA-binding proteins. An equal volume of NE1-S buffer that contained NaCl at twice the final concentration was added to the suspension of isolated mt-nuclei. In all these treatments, the samples were incubated at 26°C for 1 h.

Microscopic observations

The disorganization of the isolated mt-nuclei was observed by DAPI-fluorescence microscopy using the method described in Chapter 1.

Estimation of the copy number of mtDNA molecule

The disorganized mt-nuclei were stained with DAPI using the previously described method. The intensity of fluorescence emitted by the mt-nuclei was measured with a video-intensified microscope photon-counting system (VIMPCS; Hamamatsu Photonics Ltd. Hamamatsu, Japan) connected to an epifluorescence microscope, as described in Chapter 1.

Assay of DNA and RNA synthesis *in vitro*

Isolated mt-nuclei (4 µg) were treated with various concentrations of NaCl. The disorganized mt-nuclei were pelleted by centrifugation at 110,000 x g at 4°C for 1 h. The pellets were gently washed with NEl-S buffer to remove most of the NaCl and then were gently resuspended in NEl-S buffer. Four microliters of the suspension were then mixed with 6 µL of assay mixture as described in Chapter 2. After a 15 min incubation at 26°C, the amount of [^{3}H]dCTP or [5,6-^{3}H]UTP in the DE-81 bound fraction was determined by scintillation counting.

Assay of DNA polymerase activity *in situ.*

Isolated mt-nuclei (6 µg) were treated with various concentrations of NaCl. Then they were centrifuged at 110,000 l g and 4°C. The supernatant was mixed with 1/10 volume of TCA, cooled on ice for 30 min, and then centrifuged at 18,500 l g for 15 min. The pellet was washed with cold acetone. The pellets from the centrifugations and the supernatant were dissolved separately in standard SDS sample buffer. The samples were incubated for 3 min at 37°C and subjected to electrophoresis. DNA synthesis activity was detected *in situ* using the methods described in Chapter 2.

Polypepide analysis

The methods used to prepare the isolated mitochondria and mt-nuclei, and the pellet and supernatant from the centrifugation have already been described. All the fractions were dissolved in standard SDS sample buffer and incubated in a boiling water bath for 3 min. Electrophoresis was performed using the methods described by Laemmli (1970) with 12% gels. Polypeptides were detected on the gel by Coomassie brilliant blue (CBB) staining.

Southwestern blotting

Proteins in the isolated mt-nuclei were separated by SDS-PAGE. Separated proteins were then transferred to PVDF membrane (Bio-Rad. California) by one of two methods. In the first method, proteins were transferred in SDS containing Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.03% SDS) onto a membrane that was placed beside the anode at 20 V for 30 min. In the second method, the proteins were transferred in 0.7% acetic acid onto a membrane placed beside the cathode at 20 V for 30 min. HindIII-digested mtDNA was end-labeled by phosphorylation with T4 polynucleotide kinase and [^{32}P]ATP. The protein-blotted membrane was blocked in 5% skim milk (Difco), 20 mM Tris (pH 7.7) at 4°C for 1 h and then incubated with end-labeled mtDNA probes dissolved in binding buffer [0.25% skim milk, 20 mM Tris (pH 7.7), 1 mM EDTA and 7 mM 2-mercaptoethanol] at room temperature for 2 h. The membrane was then washed four times in binding buffer containing 50 mM NaCl for 15 min each. The membrane was sealed in a thin polypropylene bag and then autoradiographed.

Protein sequence analysis

Proteins from the isolated mt-nuclei were separated by SDS-PAGE as described above. Separated proteins were transferred in 0.7% acetic acid onto a membrane that was placed beside the cathode at 20 V for 30 min. The membrane was stained with CBB. The membrane was then washed extensively with DW and air-dried. The band containing the 41-kDa protein was excised and the amino-terminal amino acid sequence determined using the HP G1005A protein sequencing system (Hewlett Packard). To determine the internal amino acid sequence, the excised band was treated with lysyl endopeptidase, and the digested peptide was separated by reverse-phase HPLC with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The

peptide of the 37.7' (the number indicates the retention time of the peptide that was eluted by reverse-phase HPLC) fragment was sequenced in a similar manner.

DNA cloning and sequencing

The following underlined portion of the amino-terminal and internal amino acid sequences of the 41-kDa protein were used to construct primers [amino-terminal amino acid sequence, SVGKGKPTPKAVTPAKKAPPPP; internal amino acid sequence, KENPQLPVTAVLG(E)EIAK]. The oligonucleotide primers constructed were 5'-AARGGHAARCCCACIC-CIAARGCIGT-3' and 5'-AAGAGAACCCICARYTBCCCGTIACIGC-3'. Fifty picomoles of each oligonucleotide were used to amplify from total *P. polycephalum* DNA. The amplification protocol consisted of 40 cycles of 1 min at 94°C, 1 min at 58°C and 3 min at 72°C. The PCR products were cloned into the EcoRV site of pT7Blue (Novagen) and subsequently sequenced.

Preparation of polyclonal antibodies

Isolated mt-nuclei were treated with 0.5 M NaCl. Then the mixture was centrifuged at 110,000 I g and 4°C for 1 h. Proteins in the supernatant fraction were separated by SDS-PAGE and then stained with CBB in water. The gel containing the 41-kDa protein was homogenized and injected into the abdominal cavities of six-week-old BALB/c mice. Two boosters were given by subcutaneous injection at intervals of 1 month, and the serum was collected 1 week after the last booster.

Western blot analysis

Proteins in the whole cells, the isolated cell nuclei, the isolated mitochondria and the isolated mt-nuclei were separated by SDS-PAGE. These proteins were then transferred in SDS containing Towbin buffer (25 mM Tris,

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192 mM glycine, 20% methanol and 0.1% SDS) onto a PVDF membrane that was placed beside the anode at 20 V for 30 min. The membrane was blocked in 3% gelatin in TBS [20 m Tris (pH 7.5), 0.5 M NaCl] for 1 h; incubated with primary antibody (anti-41-kDa antiserum diluted 1:200) in TTBS (TBS with 0.05% Tween-20) containing 1% gelatin; washed twice in TTBS; incubated for 1 h in anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 1:3000 in 1% gelatin TTBS; washed twice in TTBS, then twice in TBS; and then finally developed.

RESULTS

The effects of various concentrations of NaCl on the structure of the isolated mt-nuclei

P. polycephalum has large rod-shaped mt-nuclei containing a high mtDNA copy number. I isolated mt-nuclei from P. polycephalum plasmodia by dissolving the membranes of highly purified mitochondria (4.5 mg protein/mL) with 0.5% Nonidet P-40. These isolated mt-nuclei definitely retained their morphological structure and the capacity for DNA and RNA synthesis (Chapter 2). The isolated mt-nuclei were treated with NaCl to separate the mtDNA and proteins. Figure 20 shows the nuclear structure observed by DAPI-fluorescence microscopy when the isolated mt-nuclei were treated with various concentrations of NaCl at 26°C for 1h (Fig. 20). The isolated mt-nuclei retained their rod-shaped structure in 0 and 0.05 M NaCl (Fig. 20A, B). With 0.1 M NaCl, the mt-nuclei became shorter and no longer appeared rod-shaped (Fig. 20C). In 0.2 M NaCl, many fluorescent dots were observed (Fig. 20D). At high concentrations, between 0.5 and 1.0 M NaCl, the compact structure of the mt-nuclei almost completely disappeared and large amounts of DAPI-

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staining materials were released surrounded by long filaments. These filaments were assumed virtually naked DNA molecules.

I used VIMPCS to measure the copy number of mtDNA molecules from the individual compact structures that were produced by treatment with a low concentration of NaCl (≤ 0.2 M). At 0 and 0.05 M NaCl, the copy number of mtDNA molecules within individual fluorescent structures was large (Fig. 21A, B). Approximately half of the fluorescent structures in the fraction contained more than 15 mtDNA molecules. In 0.1 M NaCl, the fluorescent structures with a high copy number of mtDNA molecules gradually disappeared and fluorescent structures with 10-15 molecules were most abundant (Fig. 21C). With 0.2 M NaCl, the copy number of mtDNA molecules rapidly decreased and fluorescent structures containing 0-5 molecules were most abundant (Fig. 21D). On average, individual fluorescent structures in 0.2 M NaCl contained about 4 mtDNA molecules, which is approximately 1/4 the number seen in 0 M NaCl (Fig. 22A). On the other hand, the number of fluorescent structures per microliter of suspension gradually increased as the concentration of NaCl rose. The number of fluorescent structures in the fraction treated with 0.2 M NaCl was about 4 times greater than in the fraction with no NaCl (Fig. 22B). The total copy number of mtDNA molecules per microliter remained constant (Fig. 22C). These results suggest that the treatment with low concentrations of NaCl separated the mtDNA molecules in the mt-nuclei.

The effects of various concentrations of NaCl on DNA and RNA synthesis in the disorganized mt-nuclei

I then investigated the activity of DNA and RNA synthesis after the disorganization of the structure of mt-nuclei by the treatment with various concentrations of NaCl (Fig. 23). The disorganized mt-nuclei were collected by centrifugation to remove the NaCl and mitochondrial nuclear proteins that were

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released from the mtDNA, and then used for the assay. The *in vitro* assays of DNA and RNA synthesis were carried out for 15 min. The level of DNA synthesis decreased slightly in 0.1 M NaCl and decreased markedly in 0.2 M NaCl. On the other hand, RNA synthesis was not affected by the treatment with 0.2 M NaCl, but treatment with 0.5 M NaCl decreased the level of RNA synthesis.

To determine the concentration of NaCl at which the DNA polymerase was released from the mtDNA, the disorganized mt-nuclei were centrifuged and then the levels of DNA synthesis in the pellet and supernatant were monitored by the incorporation of [32P]-dCTP into a gel that contained heat-denatured salmon testis DNA after SDS-PAGE (Fig. 24). DNA synthesis in the gel was detected as a 120 kDa band, as described in Chapter 2. DNA synthesis occurred in the gels containing a suspension of the pellet from the 0, 0.05 and 0.1 M NaCl treatments and the supernatant from the 0.2 and 0.5 M NaCl treatments. Therefore, it appears that DNA polymerase is released from the mtDNA by the treatment with 0.2 M NaCl.

Analysis of the protein released from the mtDNA

The above-discribed results led me to expect that the proteins which were essential for nuclear organization and mtDNA function would be selectively released from the insoluble fraction at concentrations higher than 0.2 M. Figure 25 shows a SDS-PAGE analysis of proteins in the pellets and supernatants after the treatment with various concentrations of NaCl. When the mt-nuclei were treated with 0.2 M NaCl, 41- and 56-kDa proteins were found in the supernatant for the first time. However, the 41-kDa protein was also observed in the pellet fraction at 0.2 M NaCl. When the mt-nuclei were treated with 0.5 M NaCl, all the 41-kDa proteins were released into the supernatant and a 39-kDa protein first appeared in the supernatant.

Identification of DNA-binding proteins in mt-nuclei

The DNA binding activity of the 39-, 41- and 56-kDa proteins was analyzed by Southwestern blotting. I used two effective methods for transferring the proteins onto the membrane. In the first, 0.3% SDS containing Towbin buffer was used and the membrane was placed beside the anode. This method transfers almost all but the basic proteins onto the membrane. To efficiently blot basic proteins, I selected another method that used a 0.7% acetic acid buffer, and the membrane was placed beside the cathode. The 39- and 56-kDa proteins were transferred onto the membrane efficiently with the first method (Fig. 26, lane 1, 2), while the second method was effective for the 39- and 41-kDa proteins (lane 1, 3). Therefore, the 39- and 41-kDa proteins are probably basic. All three proteins were able to bind to mtDNA (lanes 4, 5). However, the DNA binding capability of the 39- and 41-kDa proteins that were blotted using the second method (lane 5) was much lower than that of the 39- and 56-kDa proteins that were blotted with the first method (lane 4). Since the DNA binding activity of the 39 kDa protein in lane 5 was much lower than that of the 39-kDa protein in lane 4, yet the amount of protein in each lane was similar, it appears that the second method decreased the DNA binding capacity.

Sequence analysis of the 41 kDa protein.

The 41 kDa protein is the predominant protein in the mt-nuclei, although it is a minor constituent of the mitochondria (Fig. 25). I analyzed the sequence of the 41 kDa protein to characterize it. The 41 kDa protein was electroblotted onto membranes using the second method described above, and its N-terminal amino acid sequence was determined (Fig. 27). The blotted protein was also digested *in situ* with lysyl endopeptidase and the peptides were separated by reverse-phase HPLC (Fig. 28). The amino acid sequence of the 37.7' fragment was determined (Fig 27). Since the 41-kDa protein was digested into many

smaller peptides by lysyl endopeptidase, it is thought to be a lysine rich protein (Fig. 28). To clone the gene that encodes the 41-kDa protein, PCR was performed with degenerate primers that were designed from the N-terminal amino acid sequence and an internal amino acid sequence described in Materials and Methods. The 617 bp PCR product was identified as a part of the gene encoding the 41-kDa protein. Figure 29 shows the amino acid sequence deduced from the nucleotide sequence of the PCR product. In the figure, the N-terminal deduced amino acid sequence of the protein is underlined; however, one amino acid at the C-terminal end of this region is different from the amino acid sequence determined from the protein sequence (TPAKKAPPLP, Fig. 29).

The deduced amino acid sequence of the 41-kDa protein was compared with sequences in the GenBank and GenomeNET databases using BLAST (Altschul et al., 1990) and Beauty (Kim et al., 1995) programs. The N-terminal region and the C-terminal region shared significant homology with the H1 class of eukaryotic histones and high-mobility groupe (HMG)-box repectively (Fig. 30). As far as histone H1, the best alignment (p=1.2e-9) was found in the C-terminal region of H1 protein of the angulate sea urchin *Parechinus angulosus* with 22% identity in a 126-amino acid overlap (Fig. 30). The region of homology between 41-kDa protein and H1 overlaps of a high persentage of lysine and alanine residues. As far as HMG box, the best alignment (p=8.7e-5) was found in the HMG box of the structure-specific recognization protein (SSRP) of *Saccharomyces cerevisiae* with 55% identity in a 26-amino acid overlap (Fig. 30). Since the C-terminal region of H1 protein and HMG-box are DNA-binding motiefs, those results indicated the presence of at least two DNA-binding regions in the 41-kDa protein.

Immunoblot analysis of the 41-kDa protein

Although highly purified mitochondria were used in these experiments, it was necessary to prove that the 41-kDa protein was not a nuclear

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contaminant. I determined its subcellular localization by immunoblotting experiments using polyclonal antibodies raised against the 41-kDa protein. The subcellular localization indicated that the 41-kDa protein was found in purified mitochondria and mt-nuclei, but not in the cell nuclei (Fig. 31). Furthermore, the 41-kDa protein was concentrated in the mt-nuclei fraction. These results indicate that this protein is not a cell-nucleus protein but a mitochondrial nucleus protein.

DISCUSSION

mtDNA is highly organized with proteins to form mt-nuclei within the mitochondria. The nuclear organization in mt-nuclei isolated from *P. polycephalum* was disrupted by treatment with NaCl (Fig. 20). Treatment with 0.2 M NaCl caused the mtDNA molecules in the mt-nuclei to separate (Fig. 21 and 22). When the isolated mt-nuclei were treated with 0.5 M NaCl, the compact packaged form of mtDNA was destroyed and filamentous mtDNA was observed. This suggests that the proteins that hold groups of mtDNA molecules together and keep the molecules in compact units were released from the mtDNA at NaCl concentrations of 0.2 M and 0.5 M, respectively.

Treatment with NaCl also caused a decrease in the levels of DNA and RNA synthesis in the isolated mt-nuclei (Fig. 23). When the mt-nuclei were treated with 0.2 M NaCl, the DNA synthesis activity in the pelletable fraction decreased and DNA polymerase was released from the mtDNA (Fig. 23, 24). At this concentration of NaCl, the mt-nuclei became a more disorganized, compact structure containing approximately 4 mtDNA molecules (Fig. 22). Since this copy number of mtDNA is smaller than that in the mitochondrial replicon cluster that might function as a unit of mtDNA replication in mt-nuclei

(Chapter. 1), it is possible that disorganization of the structure of MRC might cause the decrease of DNA synthesis activity and the release of DNA polymerase. On the other hand, the disorganization of the MRC did not affect RNA synthesis, because RNA synthesis activity only decreased in 0.5 M NaCl (Fig. 23).

Three proteins (39-, 41-, 56-kDa) were released from the mtDNA when the isolated mt-nuclei were treated with either 0.2 M NaCl or 0.5 M NaCl (Fig. 25). Since all three proteins possess DNA binding activity, these proteins are probably associated with mtDNA in the mt-nuclei (Fig. 26). DNA binding proteins in the mitochondria have been identified in several organisms. Single-stranded DNA-binding proteins have been identified in humans (Genuario and Wong, 1993), rats (Pavco and Van-tuyle, 1985), *Xenopus laevis* (Ghrir et al., 1991), yeast (Van-Dyck et al., 1992), *Drosophila melanogaster* (Stroumbakis et al., 1994), and Paracentrotus lividus egg mitochondria (Roberti et al., 1997). In addition, mitochondrial pause-region binding protein 1 from sea urchin embryo (mtPBP1, Qureshi and Jacobs, 1993), avian mitochondria DNA-binding protein from the chick embryo (aMDP, D'agostino and Nass, 1992), mitochondrial transcription factor 1 from humans (mtTF1, Fisher and Clayton, 1988) and autonomously replicating sequence binding factor 2 from yeast (ABF2, Diffley and Stillman, 1988) have also been identified. Where the function of these proteins has been identified, it indicates that they are essential for DNA replication or transcription. Only two of these proteins play roles in nuclear organization: mtTF1 has the ability to wrap or condense and unwind DNA *in vitro* and bend DNA at specific sites (Fisher et al., 1992); and ABF2, which is identical to HM, appears to have a similar function to the *E. coli* histone-like protein HU (Megraw and Chae, 1993). Those two proteins contains two HMG box DNA binding motifs that have been found in a number of regulatory DNA-binding proteins (Diffley and Stillman, 1991, Fisher et al.,

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1992). In this study, the analysis of the amino acid sequence showed that the 41-kDa protein has not only significant homology with the HMG box, but shares significant homology with the H1 histone family (Fig. 30). In eukaryotes, the H1 histones appear to interact with the DNA between nucleosomes, unlike the core histones, and are thought to be responsible for forming higher-order chromatin structures. H1-like proteins were identified in a prokaryote, *Chlamydia trachomatis* (Hackstadt et al., 1991), and the kinetoplast of the trypanosomatid *Crithidia fasciculata* (Xu et al., 1996). In *C. trachomatis*, the gene of this protein (Hc1) is only expressed during the late stage of the chlamydial life cycle, concomitant with the reorganization of chlamydial reticulate bodies containing a dispersed chromatin into elementary bodies containing a dense core of apparently condensed chromatin. When Hc1 was expressed in *E. coli*, a condensed nucleoid structure was observed with light and electron microscopy (Clifon et al., 1992). In *C. fasciculata*, expression of H1-like proteins in an *E. coli* mutant lacking the HU protein eliminated a defect in chromosome condensation. These results suggest a role for H1-like proteins in the condensation of DNA. Since the mt-nuclei in *P. polycephalum* were also condenced form, the 41-kDa protein may play a similar role in the condensation of mtDNA. Moreover, the 41-kDa protein may also be involved in the interaction between mtDNA molecules, because it was found in the supernatant, not with the pelletable mt-nuclei, when the isolated mt-nuclei were treated with 0.2 M NaCl (Fig. 21, 22).

Although the major function of the 41-kDa protein may be related to the structure of the mt-nuclei, it will interesting to determine its effect on the replication and transcription of mtDNA.
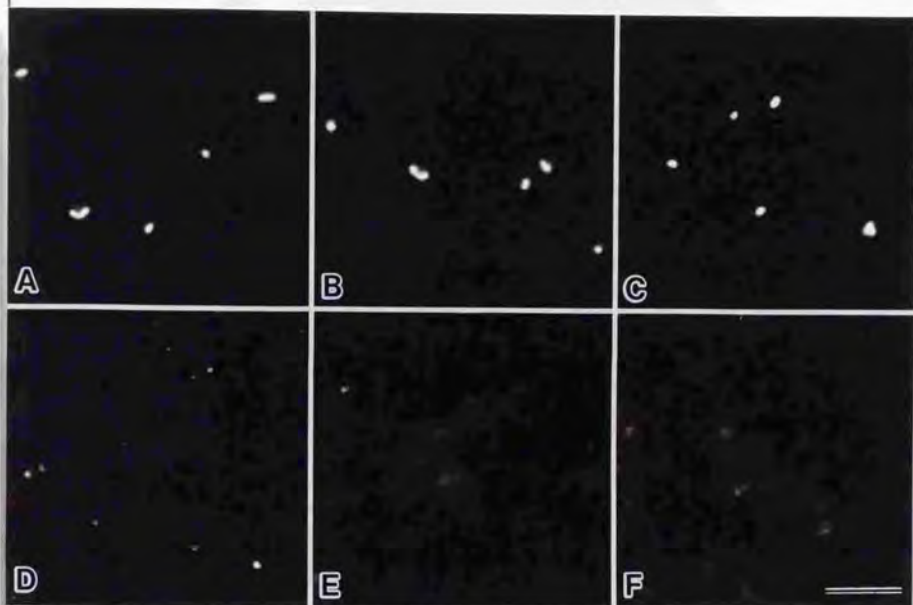
Fig. 20. Disorganization of mt-nuclei by increasing the concentration of NaCl. The isolated mt-nuclei were treated with 0 (A), 0.05 (B), 0.1 (C), 0.2 (D), 0.5 (E), 1.0 (F) M NaCl at 26°C for 1h, stained with DAPI and observed by epifluorescence microscopy. Bar, 5 μm
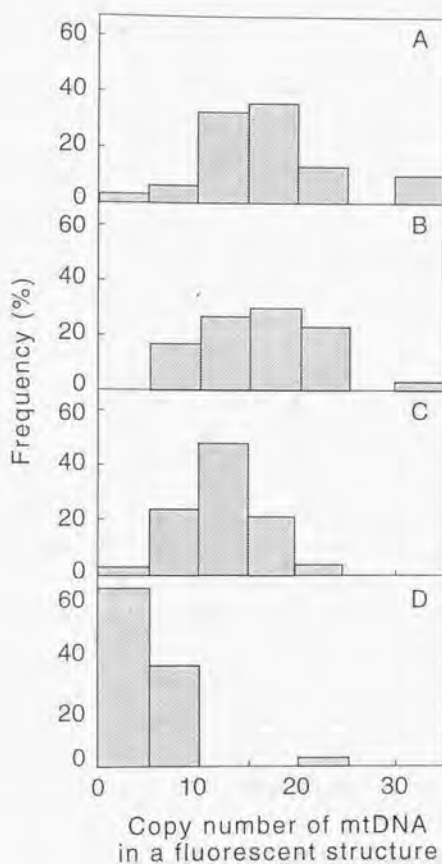
Fig. 21. The copy number of mtDNA molecules within individual fluorescent structures that resulted from the treatment with NaCl. The isolated mt-nuclei were treated with 0 (A), 0.05 (B), 0.1 (C), 0.2 (D) M NaCl at 26°C for 1h. The copy number of mtDNA molecules was calculated from the fluorescence intensity that was measured by VIMPCS, as described in the text.
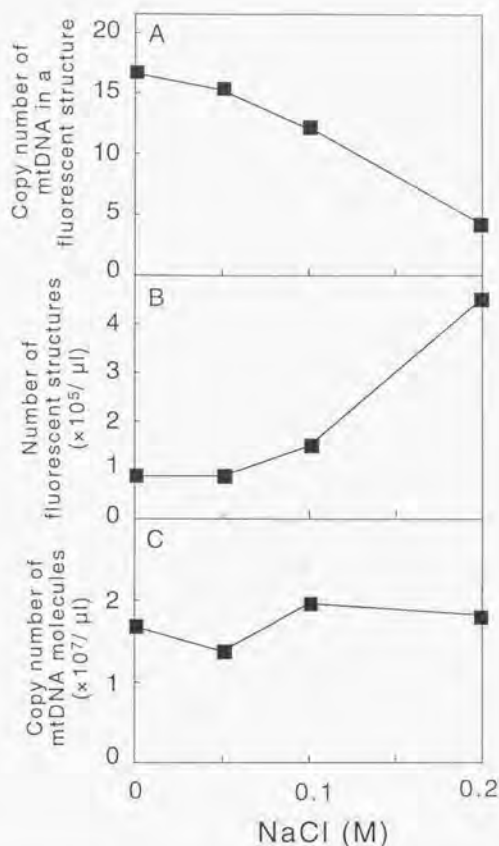
Fig. 22. Change in the copy number of mtDNA molecules within individual fluorescent structures (A), the number of fluorescent structures per 1μl of fraction (B) and the copy number of mtDNA molecules per 1 μl of fraction (C) with treatment with various concentrations of NaCl. The isolated mt-nuclei were treated with various concentrations of NaCl at 26˚C for 1h. The copy number of mtDNA molecules was calculated from the fluorescence intensity that was measured by VIMPCS as described in the text.
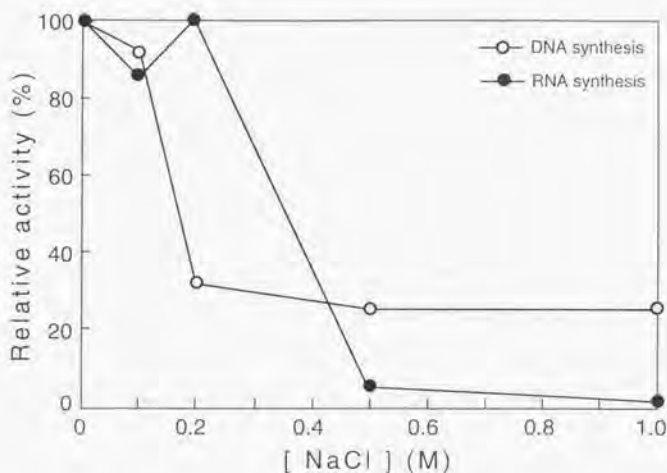
Fig. 23. The change in the activity of DNA synthesis (○) and RNA synthesis (●) resulting from treatment with various concentrations of NaCl. The isolated mt-nuclei were treated with various concentrations of NaCl at 26°C for 1h and then centrifuged to separate the pelletable fraction and the supernatant. The pelletable fractions were used for the assay. The assay of DNA synthesis and RNA synthesis *in vitro* was carried out under standard conditions. Radioactivity incorporated into mtDNA after a 15 min incubation was quantified in the manner described in the text. The activity in the pelletable fractions from 0 M NaCl-treated isolated mitochondria was arbitrarily set at 100%.
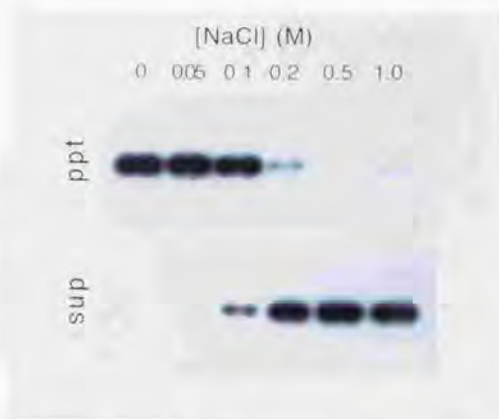
Fig. 24. Detection of DNA polymerase in the pelletable fraction (ppt) and the supernatant fraction (sup) by an *in situ* assay after SDS polyacrylamide gel electrophoresis. Identical amounts of the isolated mt-nuclei were treated with various concentrations of NaCl at 26°C for 1h and then centrifuged to separate the pelletable fraction and the supernatant. Proteins in the two fractions were separated by electrophoresis in an SDS-polyacrylamide gel that contained heat-denatured salmon testis DNA. After renaturation of the enzyme, the DNA polymerase assay was performed *in situ* in the manner described in the text. [^{32}P]dCTP incorporated into salmon testis DNA was detected as a dark band after autoradiography.
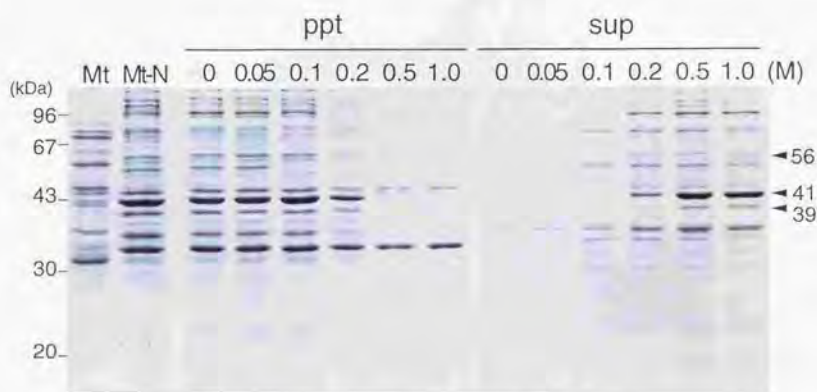
Fig. 25. SDS-PAGE patterns of proteins in the isolated mitochondria (mt) and isolated mt-nuclei (mt-N), pelletable fraction (ppt) and the supernatant (sup). The isolated mt-nuclei (6 μg) were treated with various concentrations of NaCl at 26°C for 1h and then centrifuged to separate the pelletable fraction and the supernatant. Proteins from the isolated mitochondria (6 μg), the isolated mt-nuclei (6 μg), the pelletable fraction and the supernatant were separated by electrophoresis in an SDS-polyacrylamide gel and stained with Coomassie brilliant blue. The numbers on the left and right indicate the mobility of standard proteins and their molecular masses in kDa.
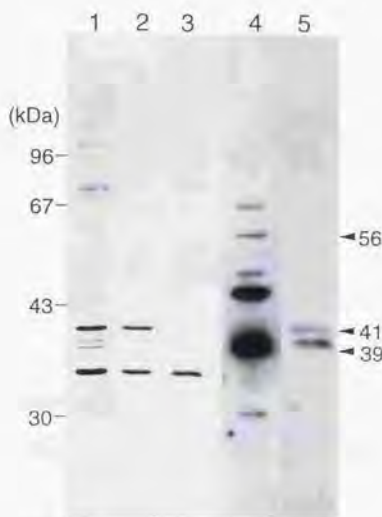
Fig. 26. DNA binding analysis of mitochondrial nuclear proteins. Proteins from the isolated mt-nuclei were separated by electrophoresis in an SDS-polyacrylamide gel, blotted onto a membrane using two methods, (Method A used Towbin transfer buffer containing 0.3% SDS and the membrane was placed beside the anode. Method B used 0.7 % acetic acid buffer and the membrane was placed beside the cathode) and hybridized with 32P-labeled mtDNA. Lane 1, CBB-staining gel after SDS-PAGE of isolated mt-nuclei; lane 2, CBB-staining gel after blotting by Method A; lane 3, CBB-staining gel after blotting by Method B; lane 4, an autoradiogram showing the DNA binding activity of polypeptides blotted by Method A; lane 5, an autoradiogram showing the DNA binding activity of polypeptides blotted by Method B. The numbers on the left and right indicate the mobility of standard proteins and their molecular masses.

Amino-terminal sequence

SVGKGKPTPKAVTPASKKAPPPP

37.7' flagment sequence

ENPQLPVTAVLG(E)EIAK

Fig. 27. Partial amino acid sequence of peptides from the 41-kDa protein.
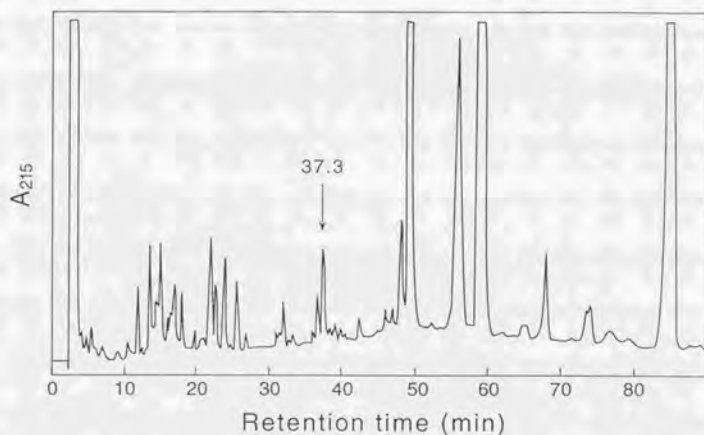
Fig. 28. Reverse-phase HPLC of proteolytic fragments obtained by lysyl endopeptidase digestion. The sample was loaded into a column and eluted with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The arrow indicates the fraction selected for amino acid sequence analysis. The solid curve shows absorbance at 215 nm.

5' <u>AARGGHAARCCCACTCCTAARGCTGT</u> ··· T ACT CCC GCC AAG AAA GCC CCT CCA CTA 28
                                      T   P   A   K   K   A   P   P   L 21

29 CCC CCC GCC AGG AAA GGC ACT CCA CCA CCC CCC GCT AAG AAA TCA ACT CCA CCT 82
22 <u>P   P</u>   A   R   K   G   T   P   P   P   P   A   K   K   S   T   P   P 39

83 CCA CCC ACT GGA AAG AAA CCA GCC CAA AAT ACG GTG GTA GCA AAG AAG CCC GCA 136
40 P   P   T   G   K   K   P   A   Q   N   T   V   V   A   K   K   P   A 57

137 GCG AAG ACC CCA CCC CCA CCC GTC AAG AAA CCC TCC CCT CCA CAA GGA TCT AAG 190
58 A   K   T   P   P   P   P   V   K   K   P   S   P   P   Q   G   S   K 75

191 GGG ACC GTA NTG GTT AAG GCG CAA CAA CAA GCT GCG AAG GAG AAG GCT AAG GAA 244
76 G   T   V   X   V   K   A   Q   Q   Q   A   A   K   E   K   A   K   E 93

245 AAG GCA CAA AAG GAG AAG GAA AGG GAC AAG GAA GCA AGG GAG AGA CAA AGG GAA 298
94 K   A   Q   K   E   K   E   R   D   K   E   A   R   E   R   Q   R   E 111

299 AAG GAG AGG TTA GCC AAG GAG AAA GCA AAG GAG AAA GAG GAC ATG GCT AAA GAG 352
112 K   E   R   L   A   K   E   K   A   K   E   K   E   D   M   A   K   E 129

353 AAG GAG AGG GAT AGG AAG CTA AAG GAA AAA GAG AAG GAG CAA CAA CAT AAA GAA 406
130 K   E   R   D   R   K   L   K   E   K   E   K   E   Q   Q   H   K   E 147

407 AAG GAA AAG GAG AAG GAG AAA AAG CTC ATA AAA AAA GAG AGG GCT GAT AGA GAA 460
148 K   E   K   E   K   E   K   K   L   I   K   K   E   R   A   D   R   E 165

461 AAG CAG GAG ATT GCA GAG TTT GTT CAT TTG TTC ATT TGT TCA TTT GTT CAT TTG 514
166 K   Q   E   I   A   E   F   V   H   L   F   I   C   S   F   V   H   L 183

515 TTC ATT TGT TCA TTT GTT CAT TTG TTA CAT AAC ACA GCC ATG CCC TCG CGC CCC 568
184 F   I   C   S   F   V   H   L   L   H   N   T   A   M   P   S   R   P 201

569 AAA TCC GCG TAC ATC TGT TTC GCC GTA GAA GCG AGG CCC ACC ATC GTG A ··· <u>AAG</u>
202 K   S   A   Y   I   C   F   A   V   E   A   R   P   T   I   V

<u>AGAACCCTCARYTBCCCGTTACTGC</u> 3'

Fig. 29. Nucleotide and predicted amino acid sequences of the 41-kDa protein. Double underlining indicates the degenerate primers corresponding to the N-terminal sequence and the internal amino acid sequence and underlining indicates the corresponding peptide sequence, which was determined with a protein sequencer.

```
41 kDa protein     45 KKPAQNTVVAKKPAAKTPPPPVKKPSPPQGSKGTVXVKAQQQAAKE  90
Histone H1         20 KKAKKTSAAAKAKKAKAAAAKKARKAKAAAAKRKAALAKKKAAAAKR 165
(Parechinus angulosus)

                   91 KAKEKAQKEKERDKEARERQREKERLAKKKAKEKEDMAKEKERDRK 136
                  166 KAAAKAKKAKKPKKKAAKKAKKPAKKSPKKAKKPAKKSPKKKKAKK 211

                  137 LKEKEKEQQHNEKEKEKEKKLIKKERADREKQEIA 170
                  212 SPKKAKKAAGKRKPAAKKARRSPRKAGKRRSPKKA 246

    [P = 1.2e-09, Identities = 29/127 (22%), Positives = 59/127 (46%)]


41 kDa protein    195 TAMPSRPKSAYICFAVEARPTIVKENP 221
HMG-box           431 TLPPKRPSGPFIQFTQEIRPTVVKENP 457
(SSRP, S.cerevisiae)

    [P = 8.7e-05, Identities = 15/27 (55%), Positives = 17/27 (62%)]
```

Fig. 30. Alignment of the amino acid sequence of the 41-kDa protein with the amino acid sequences of angulate sea urchin *Parechinus angulosus* Histone H1 and *Saccharomyces cerevisiae* SSRP (HMG box). Red amino acid residues indicated identities and blue amino acid residues indicated conserved amino acids replacements.

Fig. 31.   Subcellular localization of the 41-kDa protein determined by immunoblotting. Proteins of the whole cell, the isolated cell nuclei, the isolated mitochondria and the isolated mt-nuclei were separated by electrophoresis in an SDS-polyacrylamide gel and blotted onto a membrane. The 41-kDa protein was detected by immunoblotting. Lane 1, polypeptides in the whole cell (60 µg) after CBB-staining; lane 2, polypeptides in the isolated cell nuclei (8 µg) after CBB-staining; lane 3, polypeptides in the isolated mitochondria (20 µg) after CBB-staining; lane 4, polypeptides in the isolated mt-nuclei (8 µg) after CBB-staining; lane 5, immunoblotted whole cell (60 µg); lane 6, immunoblotted cell nuclei (8 µg); lane 7, immunoblotted isolated mitochondria (20 µg); lane 8, immunoblotted mt-nuclei (8 µg).

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Conclusion and Perspective

I studied the replication of mtDNA in *Physarum polycephalum* plasmodia with emphasis on the concept of nuclear organization and revealed following facts.

(1) Multiple mtDNA molecules in a mt-nucleus of plasmodium appeared to replicate in the mitochondrial replicon cluster (MRC), which consisted of approximately 10 mtDNA molecules. I previously proposed that the MRC might be a unit of mtDNA replication in the amoeba of *P. polycephalum* (Sasaki, 1994). The confirmation of the MRC in the plasmodia indicates that this nuclear organization should be important for mtDNA replication in the mt-nuclei at least during the two distinct stages of the life cycle of *P. polycephalum*.

(2) Structurally and functionally intact mt-nuclei were isolated from plasmodia by dissolving the membranes of highly purified mitochondria with NP-40. The mt-nuclei (MN1) that were obtained by treating the high concentration of mitochondria (4.5 mg of mitochondria protein /mL) with 0.5% NP-40 retained their compact nuclear organization and were capable of efficient DNA synthesis. Furthermore, MN1 could perform DNA synthesis as *in vivo*; DNA synthesis in MN1 occurred at each MRC and the level of DNA synthesis in MN1 reflected that seen *in vivo*. These results suggested that the components essential for nuclear organization and mtDNA synthesis should have not been lost from MN1. Therefore, MN1 seems very useful for the *in vitro* analysis to reveal the mechanism of mtDNA replication in the nuclear organization.

(3) Three DNA binding proteins (39-, 41-, 56-kDa protein) in the mt-nuclei were identified, which were released when the structure and function of the MN1 were gradually lost by the treatment with NaCl. One of these

proteins, the 41-kDa protein, had a high lysin content in the amino-terminal region and this region were significantly homologous to the C-terminal region of the H1 class of eucaryotic histones.

The 41-kDa protein is one of the most major protein in the mt-nuclei. The physiological function of 41-kDa protein presently remains undefined. From the analogies with histone H1 and HMG box, however, it is predicted that the function of the 41-kDa protein may be primarily related to the nuclear organization. Moreover, the 41-kDa protein might play the important role for the construction of the MRC because the 41-kDa protein was released from the mtDNA when the structure of the MRC were disorganized by the treatment with 0.2 M NaCl. At the same concentration of NaCl, I found the decrease of the activity of DNA synthesis and the release of mtDNA polymerase. It is interesting to identify whether the 41-kDa protein participates in organization of the MRC and the regulation of the mtDNA replication. The disorganized structure of isolated mt-nuclei can be reassembled by dialyzing NaCl. Combination of the system for an assay of DNA synthesis *in vitro* with the reassembly of the nuclear organization after the removal of the 41-kDa protein by the immunoprecipitation using antibodies will make it possible to reveal the function of the 41-kDa protein in the mt-nuclei. Accurate knowledge of the function of the 41-kDa protein and other components in MN1 should provide a chance for complete understanding of the mechanism of the mtDNA replication in the nuclear organization.

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