J. Gen. Appl. Microbiol., **56**, 455–464 (2010)

Full Paper

Morphology and protein composition of the mitochondrial nucleoids in yeast cells lacking Abf2p, a high mobility group protein

Isamu Miyakawa,* Miwako Kanayama, Yuka Fujita, and Hiroshi Sato†

Department of Biology, Faculty of Science, Yamaguchi University, Yamaguchi 753–8512, Japan

(Received July 5, 2010; Accepted September 2, 2010)

To elucidate the role of Abf2p, a major mitochondrial DNA-binding protein in the yeast *Saccharomyces cerevisiae*, we examined the morphology of the mitochondrial nucleoids (mt-nucleoids) in an *ABF2*-deficient mutant (*Δabf2*) in vivo and in vitro by 4′,6-diamidino-2-phenylindole (DAPI) staining. The mt-nucleoids appeared as diffuse structures with irregular-size in *Δabf2* cells that were grown to log phase in YPG medium containing glycerol, in contrast to the strings-of-beads appearance of mt-nucleoids in wild-type cells. In addition, DAPI-fluorescence intensity of the mt-nucleoids transmitted to the bud was significantly lower in *Δabf2* cells than in wild-type cells at log phase. However, the lack of Abf2p did not affect the morphology or segregation of mitochondria. The protein composition of the mt-nucleoids isolated from *Δabf2* cells grown to stationary phase in YPG medium was very similar to that of the mt-nucleoids isolated from wild-type cells cultured under the same conditions, except for the lack of Abf2p. These results together suggested that in log-phase cells, the lack of Abf2p influences not only the morphology of mt-nucleoids but also their transmission into the bud. On the other hand, our result suggested that in stationary-phase cells, the lack of Abf2p does not significantly alter the protein composition of the mt-nucleoids.

Key Words——Abf2p; DNA-binding protein; mitochondria; mitochondrial nucleoids; *Saccharomyces cerevisiae*; yeast

Introduction

Mitochondrial DNA (mtDNA) is three-dimensionally organized and packaged into the mitochondrial nucleoids (mt-nucleoids) in association with specific proteins (Kuroiwa, 1982; Sakai et al., 2004). The organization of the mt-nucleoids is closely related to the molecular mechanisms for the replication, transcription, and inheritance of mitochondrial genomes. Packaging of mtDNA into the mt-nucleoids also protects it from attack by reactive oxygen species (ROS) produced by oxidative phosphorylation (O'Rourke et al., 2002). So far, we have successfully isolated mt-nucleoids from spheroplasts of *Saccharomyces cerevisiae* with their size intact, and have identified several proteins associated with the mt-nucleoids (Miyakawa et al., 1987, 1995; Sato and Miyakawa, 2004; Sato et al., 2002). Recently, more than 20 proteins that interact with mtDNA have been identified by mitochondrial DNA-crosslinking experiments (Chen and Butow, 2005; Chen et al., 2007; Kaufman et al., 2000). *S. cerevisiae* is now the best organism for elucidating structure and function of mt-nucleoids.

Abf2p, a nonspecific DNA-binding protein with two

* Address reprint requests to: Dr. Isamu Miyakawa, Department of Biology, Faculty of Science, Yamaguchi University, Yamaguchi 753–8512, Japan.

Tel and Fax: +81–083–933–5716

E-mail: miyakawa@yamaguchi-u.ac.jp

† Present address: Division of Cell Biology, Institute of Life Science, Kurume University, Fukuoka 839–0864, Japan.

HMG box domains, is the most fully characterized yeast mt-nucleoid protein. It was first purified from yeast mitochondria and designated HM (Caron et al., 1979), and later was named Abf2p (Diffley and Stillman, 1991). Abf2p is a unique protein with the most basic p*I* among the mt-nucleoid proteins of *S. cerevisiae* (Miyakawa et al., 1987, 1995) and introduces negative supercoiling to circular plasmids in the presence of topoisimerase I (Diffley and Stillman, 1991). The homologs of Abf2p were detected in other yeast species (Miyakawa et al., 2000; Umezaki and Miyakawa, 2002) and actually purified from the mt-nucleoids of *Kluyveromyces lactis* (Miyakawa et al., 2003), *Pichia jadinii* (Miyakawa and Yawata, 2007; Miyakawa et al., 1996) and *Candida parapsilosis* (Miyakawa et al., 2009). In silico analyses of the yeast genome suggested that the amino acid sequence of Abf2p is relatively divergent in yeast species phylogenetically distant from each other (Miyakawa et al., 2009; Nosek et al., 2006; Visacka et al., 2009).

ABF2-deficient (*Δabf2*) cells can stably maintain mtDNA in YPG medium containing glycerol as the carbon source, but lose their mtDNA during culture in YPD medium containing glucose (Diffley and Stillman, 1991). The instability of mtDNA in *Δabf2* cells grown in YPD medium is suppressed by overexpression of the ILV5 gene, which encodes acetohydroxyacid reductoisomerase, a mitochondrial enzyme that functions in branched amino acid synthesis, but has no obvious DNA-binding motif (Zelenaya-Troitskaya et al., 1995). The mechanism underlying the instability of mtDNA in *Δabf2* cells is associated with regulation by GCN4 (Zelenaya-Troitskaya et al., 1998). Quantitative Southern blot analysis of mtDNA content showed that the mtDNA content in *Δabf2* cells grown in YPG medium was reduced by roughly 50% compared with that of wild-type cells. In contrast, the content of both ρ^+ and ρ^- mtDNAs is increased in cells by 50−150% by moderate (two- to threefold) increases in the *ABF2* copy number, suggesting that Abf2p plays a role in mtDNA copy control (Zelenaya-Troitskaya et al., 1998). Frequency of mtDNA recombination in zygotes is reduced in crosses between *Δabf2* strains (Okamoto et al., 1998; Zelenaya-Troitskaya et al., 1998). In addition, Abf2p promotes or stabilizes Holliday recombination junction intermediates in ρ^+ mtDNA in vivo, suggesting an important role of Abf2p in mtDNA recombination (MacAlpine et al., 1998).

Newman et al. (1996) first isolated the mt-nucleoids from *Δabf2* cells grown in YPG medium and demonstrated that these mt-nucleoids were organized differently than those from wild-type cells. They suggested from fluorescence microscopic observations that the mt-nucleoids in *Δabf2* cells were more diffuse than those in the wild-type cells. So far, many studies have demonstrated important roles of Abf2p for maintenance of the mitochondrial genome. However, morphological studies of the mt-nucleoids in *Δabf2* cells have not been performed in detail, except for a study of behavior of mt-nucleoids during zygote formation (Okamoto et al., 1998; Zelenaya-Troitskaya et al., 1998). In the present study, we examined the morphology and protein composition of the mt-nucleoids in *Δabf2* cells in vivo and in vitro, in comparison with those in wild-type cells.

Materials and Methods

Strain and cultivation. *Saccharomyces cerevisiae* strain YAM101 (*MAT a/α, ade2-1/ade2-1, ura3-1/ura3-1, his3-11,15/his3-11,15, trp1-1/trp1-1, leu2-3,112/leu2-3,112, can1-100/can1-100, ABF2/abf2::TRP1* ; Diffley and Stillman, 1991) was used for tetrad dissection onto 1% yeast extract, 2% peptone and 2% glycerol (YPG) plates, and the resultant *Δabf2* haploid strain (*MATα, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, abf2::TRP1*) and the *ABF2* haploid strain (*MATα, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, ABF2*) were used in the present study. The strains were routinely maintained on YPG agar slants and were cultured in YPG medium, or in 1% yeast extract, 2% peptone, 2% glucose (YPD) medium, or in 1% yeast extract, 2% peptone, 2% galactose (YPGal) medium at 30°C with reciprocal shaking.

Isolation of mt-nucleoids. The mt-nucleoids of *S. cerevisiae* were isolated from spheroplasts by the methods described previously (Miyakawa et al., 1987, 1995). In brief, whole cells of 13−15 g wet weight from the early stationary phase were converted to spheroplasts in SP buffer (0.8 M sorbitol, 50 mM K-phosphate buffer, pH 7.5) by treatment with Zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan). Using differential centrifugation, mitochondria were prepared from disrupted spheroplasts in NE1 buffer (0.3 M sucrose, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.4 mM spermidine, 7 mM mercaptoethanol and 0.4 mM PMSF; Miyakawa et al., 1987). The mt-nucleoids were isolated from mitochondria after the lysis of mitochondrial

membranes with a detergent, 0.5% (w/v) Nonidet P-40 (NP40), and subsequent sucrose density gradient centrifugation with 20% and 60% (w/v) sucrose. The mt-nucleoids that formed a band at the boundary between 20% and 60% sucrose were pooled, precipitated by centrifugation at 100,000×*g* for 1 h, thoroughly suspended in NE2 buffer (0.5 M sucrose, 20 mM Tris-HCl (pH 7.6), 2 mM EDTA, 0.8 mM spermidine, 7 mM β-mercaptoethanol and 0.4 mM PMSF), and stored at −85°C until use.

SDS-PAGE and 2D-gel electrophoresis. For SDS-PAGE of the fractions obtained at each step of mt-nucleoid isolation, mitochondrial proteins (2 μg) and mt-nucleoid proteins (1 μg) were loaded on a 15% polyacrylamide gel. Proteins on the gels were detected by the silver-staining method. A low-molecular-mass calibration kit from Pharmacia (Uppsala, Sweden) was used to estimate molecular mass. For 2D-gel electrophoresis, the mt-nucleoid proteins (2 μg) were dissolved in 8 M urea, 2% (w/v) Nonidet P-40, 2% ampholines (a mixture of 1.6% of p*I* 3.5−10 and 0.4% of p*I* 9−11), and 5% 2-mercaptoethanol. The first dimension gels of non-equilibrium pH gradient electrophoresis contained a mixture of 1.6% p*I* 3.5−10 and 0.4% p*I* 9−11 ampholines in acrylamide. After samples were loaded, the gels were run at 200 volts for 15 min; 300 volts for 30 min; 400 volts for 2 h 15 min; and 800 volts for 30 min. The separation gel of the second dimension contained 12% acrylamide.

Fluorescence microscopy. Whole cells and spheroplasts were fixed with 5% (w/v) glutaraldehyde for 1 h at room temperature and were washed twice with NS buffer (20 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 0.1 mM $CaCl_2$, 0.8 mM PMSF, and 0.05% 2-mercaptoethanol; Miyakawa et al., 1987). Samples were suspended in NS buffer and mixed with an equal volume of 4′,6-diamidino-2-phenylindole (DAPI) (2 μg/ml) in NS buffer on a glass slide. For visualization of mt-nucleoids in living cells, cells were vitally stained with DAPI in YPG medium at a final concentration of 0.6 μg/ml for 20 min (Miyakawa et al., 1994). The isolated mt-nucleoids were mixed directly with an equal volume of DAPI (2 μg/ml) solution without chemical fixation. To visualize mitochondria, cells were quickly washed with NS buffer twice and suspended in NS buffer containing 0.5 μg/ml 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] for 10 min at 30°C. Cells were immediately observed under B excitation.

Immunofluorescence microscopy was performed according to the methods of Pringle et al. (1991) with slight modifications. Cells were converted to spheroplasts and fixed with 3.7% (w/v) formaldehyde. The fixed spheroplasts were applied to polylysine-coated coverslips. The coverslips were treated for 6 min in methanol and for 30 s in acetone at −20°C. After a wash with phosphate-buffered saline (PBS), the samples were incubated with the anti-Abf2p antibody at a 1∶1,000 dilution for 1 h at 30°C (Miyakawa et al., 2000). After a second wash with PBS, a rhodamine-conjugated secondary antibody was added at a 1∶80 dilution. After incubation for 1 h at 30°C, the coverslips were washed with PBS, stained with DAPI, and mounted in mounting medium.

For visualization of mitochondria and mt-nucleoids in the same cells, *Δabf2* and the wild-type cells were transformed with pVT100U-mtGFP plasmid (Westermann and Neupert, 2000) by one-step transformation methods (Akada et al., 2000). Cells grown to log phase in YPG medium were fixed with 3.7% (w/v) formaldehyde for 1 h at room temperature and washed twice with NS buffer. Mitochondria and the mt-nucleoids were observed under B and UV excitation in the same field, respectively. All observations were made with an epifluorescence microscope (BHS-RFK; Olympus Optical, Tokyo, Japan) equipped with appropriate objectives (Dplan Apo 100UVPL and 100UV; Olympus). Photographs were taken with Fuji Neopan 1600 film.

Results

Morphology of the mt-nucleoids in ΔAbf2 and the wild-type cells

Indirect immunofluorescence microscopy using anti-Abf2p antibody demonstrated that Abf2p is localized to the punctate structures in wild-type cells, which well corresponded to the mt-nucleoids as revealed by DAPI staining. On the other hand, no fluorescent structures were visible in the cytoplasm of *ΔAbf2* cells (Fig. 1). When wild-type and *ΔAbf2* cells that reached the stationary phase in YPG medium were inoculated into YPG, YPD and YPGal media, the growth of *Δabf2* cells was delayed in both YPG and YPGal medium compared to the growth of wild-type cells (Fig. 2A and C), while the growth of *Δabf2* cells was almost the same as that of wild-type cells in YPD medium (Fig. 2B). The doubling times of wild-type and *ΔAbf2* cells were 1.5 and 3.4 h in YPG medium, 1.2 and 1.3 h in YPD medi-

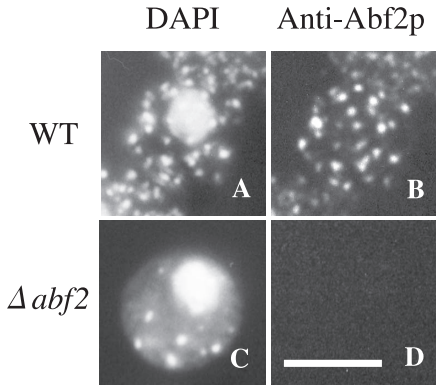

Fig. 1. Immunofluorescence localization of Abf2p.

Spheroplasts of wild-type (A, B) and *Δabf2* cells (C, D) at early stationary phase grown in YPG medium were labeled with anti-Abf2p antibody and rhodamine-conjugated secondary antibody for localization of Abf2p (B, D), and were stained with DAPI for visualization of the mt-nucleoids (A, C). Bar represents 5 μm.

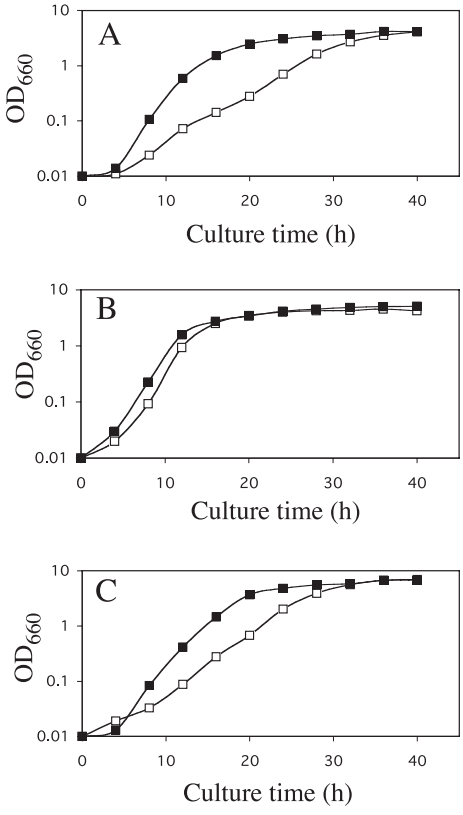

Fig. 2. Growth of wild-type and *Δabf2* cells in three different media.

Wild-type (■)and *Δabf2* (□) cells were grown to the stationary phase in YPG medium, after which, an aliquot of cells was inoculated to YPG (A), YPD (B), and YPGal medium (C) at OD_{660} = 0.01, followed by culturing at 30°C with reciprocal shaking.

um, and 1.5 and 2.7 h in YPGal medium, respectively (Fig. 2).

The morphology of the mt-nucleoids in wild-type and *ΔAbf2* cells during growth was examined with DAPI staining (Fig. 3). Mt-nucleoids in the wild-type cells appeared as strings-of-beads through the log phase in YPG, YPD, and YPGal media (Fig. 3A–C, shown by arrows). In contrast, the mt-nucleoids in *Δabf2* cells at the log phase appeared as punctate structures in all three media (Fig. 3D–F). The number of mt-nucleoids in *Δabf2* cells grown in YPD and YPGal media seemed to be lower than that in wild-type cells (Fig. 3BC and EF). On the other hand, the number of mt-nucleoids in *Δabf2* cells grown in YPG me-

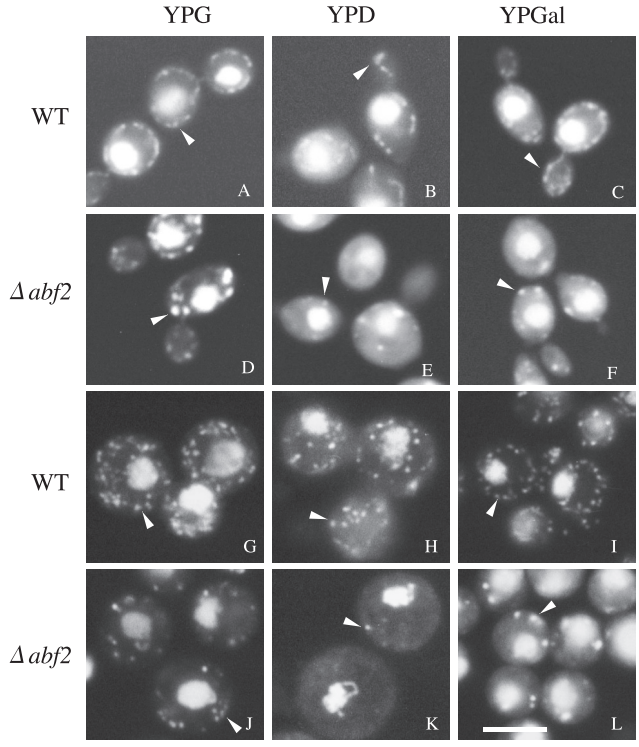

Fig. 3. Morphology of the mt-nucleoids in wild-type and *Δabf2* cells stained with DAPI.

Photomicrographs (A)–(F) show cells at log phase, and (G)–(L) show cells at stationary phase. For observation of cells at log phase, cells were inoculated at OD_{660} = 0.01 to each medium and were fixed at appropriate times. (A–C) Wild-type cells grown for 8 h in YPG (A), YPD (B), and YPGal medium (C). (D–F) The *Δabf2* cells grown for 12 h in YPG (D), for 8 h in YPD (E), and for 12 h for YPGal medium (F). For observation of cells at stationary phase, cells were fixed at 40 h for both wild-type and *Δabf2* cells, and then were treated with zymolyase for spheroplasting. (G–I) Wild-type cells grown in YPG (G), YPD (H), and YPGal medium (I). (J–L) The *Δabf2* cells grown in YPG (J), YPD (K), YPGal medium (L). Arrowheads indicate the mt-nucleoids. Bar represents 5 μm.

dium seemed to be similar to that in wild-type cells; in particular, some mt-nucleoids in *Δabf2* cells appeared more diffuse than those in wild-type cells (Fig. 3A and D).

The mt-nucleoids in wild-type cells at the stationary phase appeared as many punctate structures with similar sizes in YPG, YPD, and YPGal media, which were uniformly scattered in the cytoplasm (Fig. 3G–I). In contrast, the number of mt-nucleoids in *Δabf2* cells significantly varied depending on the culture medium (Fig. 3J–L). The mt-nucleoids were stably maintained in *Δabf2* cells grown in YPG medium, but the number of mt-nucleoids was significantly lower in *Δabf2* cells than in wild-type cells (Fig. 3G and J). The mt-nucleoids in a stationary-phase cell were counted in highly squashed spheroplasts (Fig. 4). In YPG medium, the average numbers of mt-nucleoids per cell were 11 in *Δabf2* cells and 37 in the wild-type cells (Fig. 3G and J, Fig. 4A). The *Δabf2* cells grown in YPD medium retained only a few mt-nucleoids or completely lacked them (Fig. 3K). Counting the mt-nucleoids in highly squashed spheroplasts revealed that approximately 50% of *Δabf2* cells lost the mt-nucleoids and thus produced ρ^0 cells in YPD medium, and the average number of mt-nucleoids per cell was only 1.3 in *Δabf2* cells under these conditions (Fig. 4B). These observations well coincided with previous report that demonstrated the significant instability of mtDNA in cells grown in YPD medium (Diffley and Stillman, 1991). On the other hand, the mt-nucleoids in *Δabf2* cells grown to the stationary phase in YPGal medium were more stable than those in *Δabf2* cells grown in YPD medium, and they were visible as even brighter particles than those appearing in cells grown in YPG and YPD media (Fig. 3L, Fig. 4B). Only approximately 20% of *Δabf2* cells lost mt-nucleoids under these conditions, and the average number of mt-nucleoids was 3.4 per cell (Fig. 4B). Average numbers of mt-nucleoids of wild-type cells cultured in YPD and YPGal medium to stationary phase were 30 and 33, respectively.

We found an interesting feature of the mt-nucleoids in *Δabf2* cells grown in YPG medium. Representative images of the mt-nucleoids and mitochondria in wild-type and in *Δabf2* cells during log phase are shown in Fig. 5. In wild-type cells, as the bud size gradually increased, the mt-nucleoids were transmitted from the mother cell to the bud with a strings-of-beads appearance (Fig. 5A, a–d). In these cells, the DAPI-fluorescence intensity of the individual mt-nucleoid was similar between mother cells and bud cells (Fig. 5A, arrowheads). In contrast, the DAPI-fluorescence intensity of an individual mt-nucleoid was distinctly reduced in the buds of *Δabf2* cells, although the mother cells had many spherical mt-nucleoids with irregular sizes (Fig. 5A, e–h, arrowheads). This suggested that the buds receive only small mt-nucleoids, into which lower copy numbers of mtDNA are packaged. The difference in distribution of the mt-nucleoids between mother and bud cells is evident in the comparison of wild-type and *Δabf2* cells, as shown in Fig. 5A. The percentage of budded cells, in which the bud cells have small mt-nucleoids with low fluorescence intensity and the mother cells have relatively large mt-nucleoids, reached 79% in the *Δabf2* mutant, whereas only 2% of budded cells showed this feature in wild-type cells (Table 1). To examine whether or not the morphology

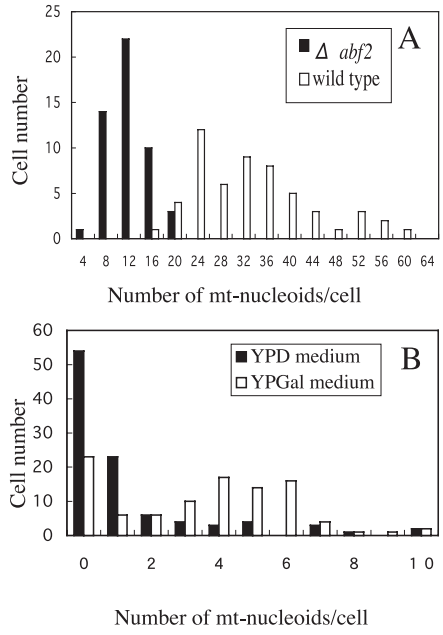

Fig. 4. The number of mt-nucleoids per cell examined by DAPI staining.

(A) The number of mt-nucleoids in wild-type and *Δabf2* cells grown to stationary phase in YPG medium. The cells grown for 40 h were fixed with glutaraldehyde and then subjected to Zymolyase treatment for spheroplasting. Spheroplasts were highly squashed on glass slides and stained with DAPI. The mt-nucleoids contained in a cell were counted under fluorescence microscopy. The total number of cells examined was 52 in each strain. (B) The number of mt-nucleoids per *Δabf2* cell grown in YPD and YPGal media. The stationary-phase cells grown for 40 h in each medium were fixed and subjected to spheroplasting. The total number of cells examined was 100 in each strain.

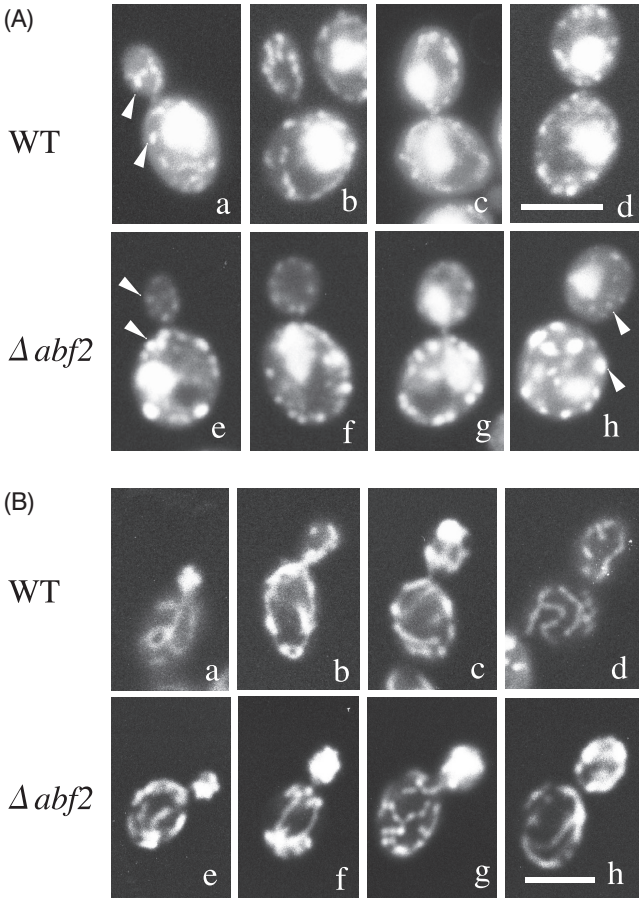

Fig. 5. Morphology of the mt-nucleoids and mitochondria in wild-type and *Δabf2* cells.

(A) The wild-type (a–d) and *Δabf2* cells (e–h) at log phase in YPG medium were fixed with glutaraldehyde and stained with DAPI. (B) Mitochondria in wild-type (a–d) and *Δabf2* cells (e–h) at log phase in YPG medium were vitally stained with $DiOC_6(3)$. Note that the DAPI-fluorescence intensity of the mt-nucleoids transmitted to the bud is rather lower in *Δabf2* cells than in wild-type cells. Bar represents 5 μm.

Table 1. Distribution of the mt-nucleoids in wild-type and *Δabf2* cells.

	A	B	C	D
Wild type (%)	98	2	0	0
Δabf2 (%)	0	0	21	79

The budded cells (n =211 each culture) grown to log-phase in YPG medium were examined by DAPI-fluorescence microscopy and grouped into 4 types according to the morphology of the mt-nucleoids. (A) Strings-of-beads appearance, (B) strings-of-beads appearance with small mt-nucleoids in the bud, (C) punctate structure, (D) punctate structure with small mt-nucleoids in the bud. The percentage of each group is shown.

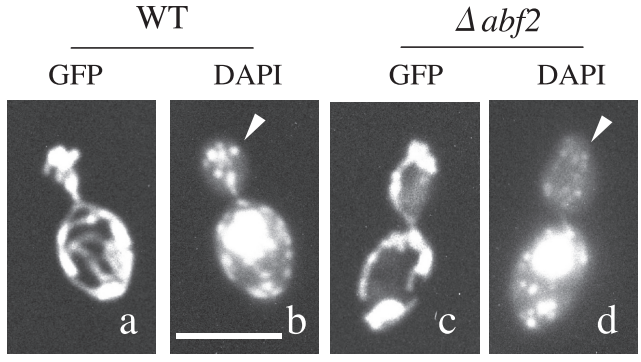

Fig. 6. Visualization of mitochondria and mt-nucleoids in the same cells.

The wild-type and *Δabf2* cells transformed by pVT100U-mtGFP plasmid were grown to log phase in YPG medium and fixed with 3.7% formaldehyde for 1 h at room temperature. The same cells were observed under B excitation for visualization of mitochondria or under UV excitation for mt-nucleoids. Bar represents 5 μm.

and distribution of mitochondria were affected in *Δabf2* cells, mitochondria was vitally stained with $DiOC_6(3)$ (Fig. 5B). However, almost all the wild-type and *Δabf2* cells grown in YPG medium had similar tubular mitochondria that were elongating from mother to bud cells. These results suggested that the lack of Abf2p did not affect the morphology or segregation of mitochondria.

Morphology of mitochondria that were labeled with GFP and the mt-nucleoids stained with DAPI was observed in the same cells in *Δabf2* and wild-type cells (Fig. 6). The mitochondria in *Δabf2* cells showed normal morphology and transmission to the bud. However, it was evident that mitochondria transmitted to the bud of *Δabf2* cells contained smaller mt-nucleoids than those in wild-type cells.

Comparison of mt-nucleoids in vitro between wild-type and Δabf2 cells

To avoid artifacts derived from chemical fixation, the mt-nucleoids in wild-type and *Δabf2* cells at the stationary phase were vitally stained by DAPI (Fig. 7A, a and b). The mt-nucleoids in both cells appeared as discrete fluorescent particles with similar size. The mt-nucleoids were isolated from both wild-type and *Δabf2* cells grown to the stationary phase in YPG medium by methods using sucrose gradient centrifugation. The mt-nucleoids, which were pooled from 20–60% bound-

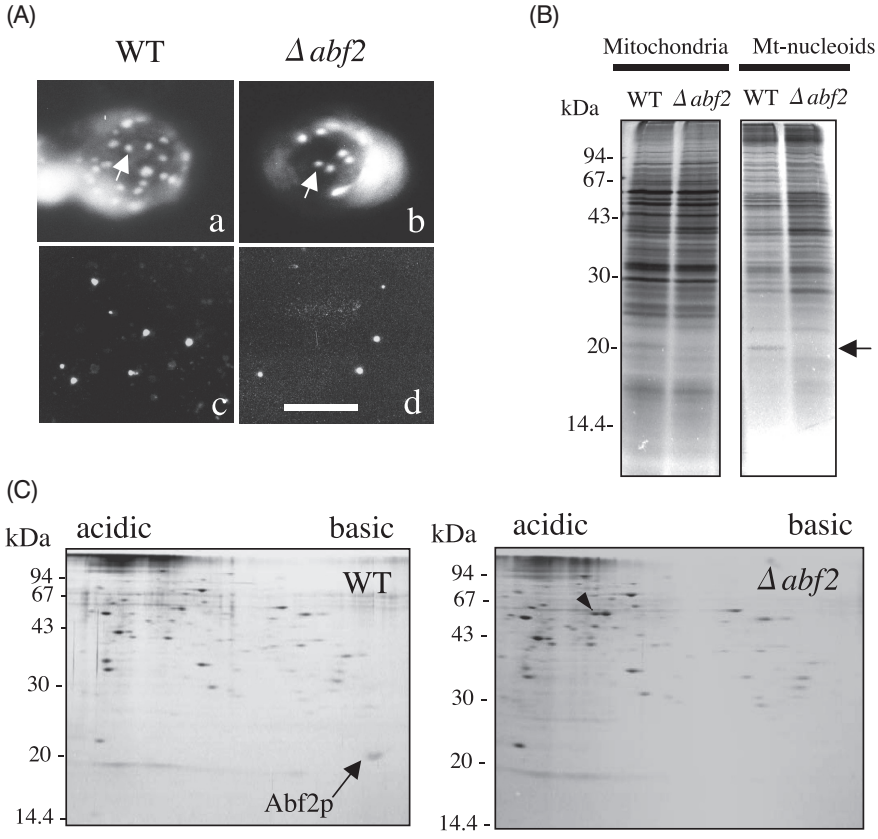

Fig. 7. Analysis of the mt-nucleoids isolated from wild-type and *Δabf2* cells.
(A) Morphology of the mt-nucleoids in vivo and in vitro. The mt-nucleoids were vitally stained with DAPI in wild-type (a) and *Δabf2* cells (b) grown to the stationary phase in YPG medium. The mt-nucleoids isolated from wild-type (c) and *Δabf2* (d) cells were also stained with DAPI. Bar represents 5 μm. (B) Comparison of the protein components of mitochondrial and the mt-nucleoids isolated from wild-type and *Δabf2* cells by SDS-PAGE. Arrow indicates the position of 20 kDa Abf2p, (C) 2D-gel electrophoresis of the mt-nucleoid proteins from wild-type and *Δabf2* cells. Arrow indicates the position of Abf2p and arrowhead indicates a 50-kDa protein.

ary of the sucrose gradient, were visible in vitro as compact fluorescent particles with a size similar to those appearing in vivo after DAPI staining (Fig. 7A, c and d). The protein composition of mitochondria and that of the mt-nucleoids were nearly the same between wild-type and *Δabf2* cells, except for the absence of Abf2p at the position of 20 kDa in mitochondria and the mt-nucleoids from *Δabf2* cells (Fig. 7B). Moreover, more than 40 proteins of mt-nucleoids from two strains were separated and compared by 2D-gel electrophoresis (Fig. 6C, Miyakawa and Sato, 2001). In these analyses, a slight increase of a 50-kDa protein was recognized in the mt-nucleoids from *Δabf2* cells. No distinct decrease or increase of specific mt-nucleoid proteins was recognized in the mt-nucleoids from *Δabf2* cells, except for the lack of Abf2p at 20 kDa.

Discussion

Morphology and stability of the mt-nucleoids in a Δabf2 mutant

During the observation of the mt-nucleoids in *Δabf2* cells, we found interesting features concerning the mt-nucleoid morphology and its distribution between mother and daughter cells during budding. As mentioned in Figs. 3 and 5, mother cells grown to log-phase in YPG medium contain diffuse mt-nucleoids with irregular size. Appearance of the diffuse mt-nucleoids in *Δabf2* cells coincided with the results of Newman et al. (1996). Appearance of diffuse mt-nucleoids was evident in mother cells, but rare in the small bud. The fluorescence intensity of large mt-nucleoids in *Δabf2* cells appeared to be higher than that of mt-nu-

cleoids of wild-type cells. In *Δabf2* cells, strings-of-beads-like mt-nucleoids are disorganized and several mt-nucleoids may aggregate to form large mt-nucleoids with different sizes. At the stationary phase, the mt-nucleoids in *Δabf2* cells appeared as normal punctate structures with sizes similar to those in wild-type cells. This indicates that the lack of abf2p differently affects the morphology of the mt-nucleoids at log phase and stationary phase.

Another feature is that DAPI-fluorescence intensity of the individual mt-nucleoids transmitted to the bud was significantly lower in *Δabf2* cells than that in wild-type cells. This means that the daughter cells of a *Δabf2* mutant receive only a low copy number of mtDNA compared to those of wild-type cells. This unequal distribution of the mt-nucleoids in *Δabf2* cells was obvious from comparison of *Δabf2* and wild-type cells (Figs. 5 and 6, Table 1).

As revealed by observation with $DiOC_6(3)$ staining and mitochondria-targeted GFP, the tubular mitochondria with several branches were elongating from the mother cell to the bud cell even in *Δabf2*, as did the wild-type cells (Figs. 5 and 6). Therefore, it is unlikely that the lack of Abf2p significantly impairs the morphology of mitochondria and their migration to the bud. Considering together the morphology of mitochondria and the mt-nucleoids, our results suggest that the lack of Abf2p induces some defects in the replication of mtDNA and/or in the segregation of mt-nucleoids that occur in tubular mitochondria, which are located at the site of bud emergence. A recent study revealed that the recombination-dependent segregation of mtDNA to the bud occurs near the neck of mother cells via rolling circle mechanisms (Shibata and Ling, 2007). Thus, it is possible that the lack of Abf2p directly affects the recombination-dependent replication of mtDNA coupled with the transmission of mtDNA to the bud. A model for morphology of mt-nucleoids and the role of Abf2p is depicted in Fig. 8. The behavior of mitochondria and mt-nucleoids near the bud neck should be focused on so as to elucidate the correlation between transmission of mitochondria and inheritance of mtDNA.

The *Δabf2* cells undergo respiration-dependent growth in YPG medium. Therefore, if the bud cells receive few copies of mtDNA in *Δabf2* cells, it will take longer to replicate mtDNA at a level that is adequate to complete the next cell cycle with a non-fermentative carbon source. This may explain why *Δabf2* cells require more than twice the doubling time of wild-type cells in YPG medium. The yeast cells can proliferate with glucose independently of respiration; actually, *Δabf2* cells grow at nearly the same rate as wild-type cells in YPD medium (Fig. 2). Under these conditions, the high cell growth rate may finally overcome the rate of replication and segregation of mtDNA, and may result in the gradual decrease in the copy number of mtDNA.

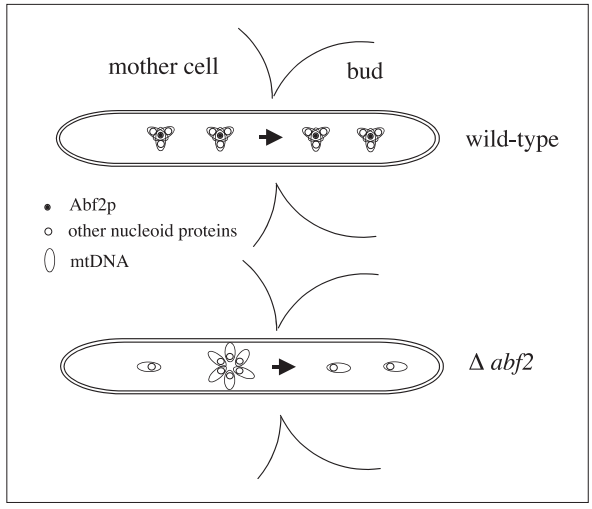

Fig. 8. A model for morphology of the mt-nucleoids and the role of Abf2p.

In wild-type cells, mtDNAs are compactly organized to form mt-nucleoids by associating with Abf2p and other nucleoid proteins. Each mt-nucleoid containing newly replicated mtDNAs is normally transmitted to the bud cells. In *Δabf2* cells, the mt-nucleoids are diffuse and partially aggregated. As Abf2p plays a role in mtDNA replication and transmission, bud cells receive small mt-nucleoids containing low copy of mtDNA.

The role of Abf2p in mtDNA packaging into nucleoid structure

Newman et al. (1996) first compared the protein components of mt-nucleoids obtained from wild-type cells and *Δabf2* cells. They showed that the mt-nucleoid fraction was associated with several proteins including Abf2p and other mt-nucleoid-specific proteins (70, 28, 21 and 15 kDa). Moreover, they found that the two mt-nucleoid proteins of 60 and 46 kDa, which are of comparable abundance to Abf2p, were absent in the mt-nucleoids isolated from *Δabf2* cells, concomitant with the lack of Abf2p. However, our results from SDS-PAGE and 2D-gel electrophoresis showed that the protein composition of the mt-nucleoids is almost the same between wild-type and *Δabf2* cells, except

for the lack of Abf2p. Although more than 40 protein components were associated with the mt-nucleoid fractions in our preparation, the absence of the 60 and 46 kDa proteins was not detected in the mt-nucleoids from *Δabf2* cells (Fig. 7C).

Recently, it has been reported that yeast cells alter the protein composition of mt-nucleoids depending on culture medium (fermentative or non-fermentative) (Kucej et al., 2008), and culture conditions (aerobic or anaerobic) (Shiiba et al., 1997, 2005). The size and DNA content of individual mt-nucleoids also vary depending on the culture condition of cells (Miyakawa et al., 2004). In the present study, we have isolated the mt-nucleoids from cells at early stationary phase. On the other hand, Newman et al. (1996) have isolated the mt-nucleoids from cells at log phase. Although we cannot explain why the protein components of the mt-nucleoids differ between the two preparation protocols, the difference in protein components of mt-nucleoids may be derived from the difference in growth phase of cells from which the mt-nucleoids were isolated.

The instability of mtDNA from lack of Abf2p is compensated by overexpression of Ilv5p (Macierzanka et al., 2008; Zelenaya-Troitskaya et al., 1995) and of aconitase (Chen et al., 2005, 2007). In addition, the mt-nucleoids contain many other proteins (Chen and Butow, 2005; Nosek et al., 2006), which include three components of α-ketoglutarate dehydrogenase (Kaufman et al., 2000; Sato et al., 2002) and mitochondrial ribosomal protein Mnp1p (Sato and Miyakawa, 2004). A high-resolution atomic force microscopy indicated that the interaction between Abf2p and mtDNA is rather weak, compared to that between nuclear DNA and histones, and that Abf2p compacts DNA by simply introducing a number of sharp bends into the DNA backbone (Brewer et al., 2003; Friddle et al., 2004). Even if Abf2p plays important roles in mtDNA packaging, other mt-nucleoid proteins may serve as mtDNA-packaging proteins to compensate for the loss of function due to the lack of Abf2p. More detailed investigations are needed to elucidate the correlation between protein components of mt-nucleoids and the morphology of mt-nucleoids.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 22570213) from the Japan Society for the Promotion of Science to I. Miyakawa.

References

Akada, R., Kawahata, M., and Nishizawa, Y. (2000) Elevated temperature greatly improves transformation of fresh and competent cells in yeast. *BioTechniques*, **28**, 854–856.

Brewer, L. R., Friddle, R., Noy, A., Baldwin, E., Martin, S. S., Corzett, M., Balhorn, R., and Baskin, R. J. (2003) Packaging of single DNA molecules by the yeast mitochondrial protein Abf2p. *Biophys. J.,* **85**, 2519–2524.

Caron, F., Jacq, C., and Rouviere-Yaniv, J. (1979) Characterization of a histone-like protein extracted from yeast mitochondria. *Proc. Natl. Acad. Sci. USA,* **76**, 4265–4269.

Chen, X. J. and Butow, R. A. (2005) The organization and inheritance of the mitochondrial genome. *Nat. Rev. Genet.,* **6**, 815–825.

Chen, X., Wang, X., and Butow, R. (2007) Yeast aconitase binds and provides metabolically coupled protection to mitochondrial DNA. *Proc. Natl. Acad. Sci. USA,* **34**, 13738–13743.

Chen, X. J., Wang, X., Kaufman, B. A., and Butow, R. A. (2005) Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science,* **307**, 714–717.

Diffley, J. F. X. and Stillman, B. (1991) A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. *Proc. Natl. Acad. Sci. USA*, **88**, 7864–7868.

Friddle, R. W., Klare, J. E., Martin, S. S., Corzett, M., Balhom, R., Baldwin, E. P., Baskin, R. J., and Noy, A. (2004) Mechanism of DNA compaction by yeast mitochondrial protein Abf2p. *Biophys. J.,* **86**, 1632–1639.

Kaufman, B. A., Newman, S. M., Hallberg, R. L., Slaughter, C. A., Perlman, P. S., and Butow, R. A. (2000) In *organello* formaldehyde crosslinking of proteins to mtDNA: Identification of biofunctional proteins. *Proc. Natl. Acad. Sci. USA,* **97**, 7772–7777.

Kucej, M., Kucejova, B., Subramanian, R., Chen, X. J., and Butow, R. A. (2008) Mitochondrial nucleoids undergo remodeling in response to metabolic cues. *J. Cell Sci.*, **121**, 1861–1868.

Kuroiwa, T. (1982) Mitochondrial nuclei. *Int. Rev. Cytol.*, **75**, 1–59.

MacAlpine, D. M., Perlman, P. S., and Butow, R. A. (1998) The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. *Proc. Natl. Acad. Sci. USA,* **95**, 6739–6743.

Macierzanka, M., Plotka, M., Pryputniewicz-Drobinska, D., Lewandowska, A., Lightowlers, R., and Marszalek, J. (2008) Maintenance and stabilization of mtDNA can be facilitated by the DNA-binding activity of Ilv5p. *Biochim. Biophys. Acta*, **1783**, 107–117.

Miyakawa, I., Fumoto, S., Kuroiwa, T., and Sando, N. (1995) Characterization of DNA-binding proteins involved in the

assembly of mitochondrial nucleoids in the yeast *Saccharomyces cerevisiae*. *Plant Cell Physiol.,* **36**, 1179–1188.

Miyakawa, I., Higo, K., Osaki, F., and Sando, N. (1994) Double staining of mitochondria and mitochondrial nucleoids in the living yeast during the life cycle. *J. Gen. Appl. Microbiol.,* **40**, 1–14.

Miyakawa, I., Kitamura, Y., Jyozaki, T., Sato, H., and Umezaki, T. (2000) Simple detection of a yeast mitochondrial DNA-binding protein, Abf2p, on SDS-DNA gels. *J. Gen. Appl. Microbiol.,* **46**, 311–316.

Miyakawa, I., Miyamoto, M., Kuroiwa, T., and Sando, N. (2004) DNA content of individual mitochondrial nucleoids varies depending on the culture conditions of the yeast *Saccharomyces cerevisiae*. *Cytologia,* **69**, 101–107.

Miyakawa, I., Okamuro, A., Kinsky, S., Visacka, K., Tomaska, L., and Nosek, J. (2009) Mitochondrial nucleoids from the yeast *Candida parapsilosis*: Expansion of the repertoire of proteins associated with mitochondrial DNA. *Microbiology,* **155**, 1558–1568.

Miyakawa, I., Okazaki-Higashi, C., Higashi, T., Furutani, Y., and Sando, N. (1996) Isolation and characterization of mitochondrial nucleoids from the yeast *Pichia jadinii*. *Plant Cell Physiol.,* **37**, 816–824.

Miyakawa, I., Sando, N., Kawano, S., Nakamura, S., and Kuroiwa, T. (1987) Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. *J. Cell Sci*., **8**, 431–439.

Miyakawa, I. and Sato, H. (2001) Analysis of mitochondrial nucleoid proteins of yeast *Saccharomyces cerevisiae* by means of two-dimensional gel electrophoresis. *Cytologia,* **66**, 99–104.

Miyakawa, I., Sato, H., Maruyama, Y., and Nakaoka, T. (2003) Isolation of the mitochondrial nucleoids from yeast *Kluyveromyces lactis* and analyses of the nucleoid proteins. *J. Gen. Appl. Microbiol.,* **49**, 85–93.

Miyakawa, I. and Yawata, K. (2007) Purification of an Abf2p-like protein from mitochondrial nucleoids of yeast *Pichia jadinii* and its role in the packaging of mitochondrial DNA. *Antonie van Leeuwenhoek,* **91**, 197–207.

Newman, S. M., Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1996) Analysis of mitochondrial DNA nucleoids in wild-type and a mutant strain of *Saccharomyces cerevisiae* that lacks the mitochondrial HMG box protein Abf2p. *Nucleic Acids Res.,* **24**, 386–393.

Nosek, J., Tomaska, L., Bolotin-Fukuhara, M., and Miyakawa, I. (2006) Structure and dynamics of the mitochondrial chromosomes: An insight from the complete yeast genomes analysis. *FEMS Yeast Res.,* **6**, 356–370.

Okamoto, K., Perlman, P. S., and Butow, R. A. (1998) The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: Preferential transmission of mitochondrial DNA to the medial bud. *J. Cell Biol.,* **142**, 613–623.

O'Rourke, T. W., Doudican, N. A., Mackereth, M. D., Doetsch, P. W., and Shadel, G. S. (2002) Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. *Mol. Cell Biol.,* **22**, 4086–4093.

Pringle, J. R., Adams, A. E. M., Drubin, D. G., and Haarer, B. K. (1991) Immunofluorescence methods for yeast. In Guide to Yeast Genetics and Molecular Biology, ed. by Guthrie, C. and Fink, G.R., Academic Press, San Diego, pp. 565–602.

Sakai, A., Takano, H., and Kuroiwa, T. (2004) Organelle nuclei in higher plants: Structure, composition, function and evolution. *Int. Rev. Cytol.,* **238**, 59–118.

Sato, H. and Miyakawa, I. (2004) A 22-kDa protein specific for yeast mitochondrial nucleoids is an unidentified putative ribosomal protein encoded in open reading frame YGL068W. *Protoplasma,* **223**, 175–182.

Sato, H., Tachifuji, A., Tamura., and Miyakawa, I. (2002) Identification of the YMN-1 antigen protein and biochemical analyses of protein components in the mitochondrial nucleoid fraction of the yeast *Saccharomyces cerevisiae*. *Protoplasma,* **219**, 51–58.

Shibata, T. and Ling, F. (2007) DNA recombination protein-dependent mechanism of homoplasmy and its proposed function. *Mitochondrion,* **7**, 17–23.

Shiiba, D., Fumoto, S.-I., Miyakawa, I., and Sando, N. (1997) Isolation of giant mitochondrial nucleoids from the yeast *Saccharomyces cerevisiae*. *Protoplasma,* **198**,177–185.

Shiiba, D., Miyakawa, I., and Sando, N. (2005) Dynamic changes in mitochondrial nucleoids during the transition from anaerobic to aerobic culture in the yeast *Saccharomyces cerevisiae*. *Cytologia,* **70**, 287–293.

Umezaki, T. and Miyakawa, I. (2002) Use of SDS-DNA PAGE for detection of mitochondrial Abf2p-like proteins and mitochondrial nuclease in *Saccharomyces* yeasts and *Arxiozyma telluris*. *Cytologia,* **67**, 423–428.

Visacka, K., Gerhold, J. M., Petrovicova, J., Kinsky, S., Joers, P., Nosek, J., Sedman, J., and Tomaska, L. (2009) Novel subfamily of mitochondrial HMG box-containing protein: Functional analysis of Gcf1p from *Candida albicans*. *Microbiology,* **155**, 1226–1240.

Westermann, B. and Neupert, W. (2000) Mitochondria-targeted green fluorescent proteins: Convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast,* **16**, 1421–1427.

Zelenaya-Troitskaya, O., Newman, S. M., Okamoto, K., Perlman, P. S., and Butow, R. A. (1998) Functions of the high mobility group protein, Abf2p, in mitochondrial DNA segregation, recombination and copy number in *Saccharomyces cerevisiae*. *Genetics*, **148**, 1763–1776.

Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1995) An enzyme in yeast mitochondria that catalyzes a step in branched-amino acid biosynthesis also functions in mitochondrial DNA stability. *EMBO J*.,**14**, 3268–3276.