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Use of the *nuc1* null mutant for analysis of yeast mitochondrial nucleoids

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Mitochondrial DNA (mtDNA) of the yeast *Saccharomyces cerevisiae* is organized to form mitochondrial nucleoids (mt-nucleoids) by association with specific proteins. The sensitivity of DNA-protein complexes to nuclease digestion is a useful means for examining DNA packaging and organization. However, the mt-nucleoids isolated from wild-type cells of *S. cerevisiae* demonstrated a significant amount of endogenous nuclease activity. In order to minimize the nuclease activity associated with the isolated mt-nucleoids, we isolated the mt-nucleoids from a mutant strain that lacked the mitochondrial nuclease, Nuc1p. In this manner, we succeeded in isolating mt-nucleoids that showed a low level of the nuclease activity. Micrococcal nuclease treatment of these mt-nucleoids led to the continuous digestion of mtDNA in the presence of Ca^{2+} ions. MtDNA in the mt-nucleoids also showed the continuous digestion pattern when treated with DNase II. These results suggest that mtDNA in the mt-nucleoids is protected from nuclease digestion by association with proteins, but the organization of the mtDNA-protein complexes is different from that of nuclear chromatin, in which the unit of DNA packaging is regularly repeated.

Key Words—micrococcal nuclease; mitochondrial nucleoids; NUC1 nuclease; *Saccharomyces cerevisiae*; yeast

Introduction

Mitochondrial DNA (mtDNA) is three-dimensionally organized to form mitochondrial nucleoids (mt-nucleoids) by association with specific proteins (Kuroiwa, 1982; Sakai et al., 2004). In yeast *Saccharomyces cerevisiae*, the mt-nucleoids were first isolated intact from stationary-phase cells with the same size and shape as those appearing in cells (Miyakawa et al., 1987). The isolated mt-nucleoids contain a number of proteins, including Abf2p, which is a major DNA-binding protein of mitochondria, subunits of α -ketoglutarate

dehydrogenase, and Mnp1p, which is a putative mitochondrial ribosomal protein, L7/L12 (Miyakawa et al., 1995; Sato et al., 2002; Sato and Miyakawa, 2004). As another approach, DNA-cross linking experiments of mtDNA-protein complexes have also revealed the association of aconitase (Aco1p), Ilv5p, and more than 20 other components with the mt-nucleoids (Chen and Butow, 2005; Chen et al., 2005; Kaufman et al., 2000). These findings introduced a novel model, in which some citric acid enzymes have dual function both as metabolic enzymes and as DNA-stabilizing proteins (Chen et al., 2005). Homologues of the mt-nucleoid proteins have been also identified in the genome sequence of other yeasts (Nosek et al., 2006).

In addition to those nucleoid proteins, we detected DNase activity in a zymographic assay of mt-nucleoid proteins on SDS-DNA gels (Miyakawa et al., 2000,

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2003; Umezaki and Miyakawa, 2002). The DNase associated with mt-nucleoids was assumed to be Nuc1p, a major nonspecific nuclease in mitochondria, based on the molecular mass of 38 kDa. The Nuc1p accounts for more than 90% of total nuclease activity in mitochondria (Dake et al., 1988). The Nuc1p shows both RNase activity on a single-stranded RNA and endonuclease activity on single- and double-stranded DNA (Vincent et al., 1988; Zassenhaus et al., 1988).

The sensitivity of DNA-protein complexes to nuclease digestion has provided useful information for examining the mechanisms of DNA packaging and organization of nuclear chromatin. Micrococcal nuclease digests yeast chromatin into a series of bands, the lengths of which correlate to the nucleosome repeat length (ca. 160 bp) (Lohr et al., 1977). On the other hand, it has been reported that DNase II digestion of the yeast mt-nucleoids formed DNA fragments with multimers of 200 bp (Rickwood and Chambers, 1981). However, so far, the nuclease sensitivity of the mt-nucleoids has not been studied in detail. In this report, we have isolated the mt-nucleoids from wild-type and *nuc1* null mutant ($\Delta nuc1$) cells of *S. cerevisiae*, and have examined the DNase activity associated with the mt-nucleoid fraction. Subsequently, in order to examine the organization of the mt-nucleoids, we analyzed the nuclease sensitivity of the mt-nucleoids isolated from the $\Delta nuc1$ mutant.

Materials and Methods

Strain and cultivation. *Saccharomyces cerevisiae* strains BY4743 (*MAT a/α, his3 Δ1/his3 Δ1, leu2 Δ0/leu2 Δ0, met15 Δ0/+ , +/lys2 Δ0, ura3 Δ0/ura3 Δ0*), YJL208c (*MAT a/α, his3 Δ1/his3 Δ1, leu2 Δ0/leu2 Δ0, met15 Δ0/+ , +/lys2 Δ0, ura3 Δ0/ura3 Δ0, nuc1::KanMX4/ nuc1::KanMX4*) (Research Genetics) and G2-2 strains (*MAT a/α*) were used. Cells were cultured to the stationary phase at 30°C in YPD medium that contained 1% yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 2% peptone (Kyokuto Co., Ltd., Tokyo, Japan), and 2% glucose.

Isolation of mt-nucleoids. The mt-nucleoids of *S. cerevisiae* were isolated as described previously (Miyakawa et al., 1987). In brief, whole cells of 13 g–15 g wet weight were converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan). Mitochondria were prepared from disrupted spheroplasts by differential centrifugation. 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%, 40%, and 60% (w/v) sucrose. The mt-nucleoids that formed a band at the boundary between 20% and 40% sucrose were pooled, precipitated by centrifugation at 100,000 × *g* for 1 h, thoroughly suspended in 0.3 ml of NES2 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.

Purification of mtDNA. Mitochondria were lysed by the addition of 0.5% (w/v) SDS in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). MtDNA was extracted with a mixture of phenol and chloroform (1 : 1, v/v) and was then precipitated with cold ethanol. MtDNA was purified by isopycnic centrifugation in CsCl in the presence of Hoechst 33258 (Miyakawa et al., 1995) and used as a probe for Southern hybridization.

SDS-PAGE. For SDS-PAGE of fractions during the mt-nucleoid isolation, an aliquot of each sample (15 μl) was mixed with 5 μl of 4×SDS sample buffer and loaded on a 15% polyacrylamide gel. Proteins on gels were detected by the silver-staining method. A low-molecular-mass calibration kit from Pharmacia was used for the estimation of molecular mass.

Fluorescence microscopy. Spheroplasts of stationary-phase cells were fixed with 5% 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 MgCl₂, 0.1 mM ZnSO₄, 0.1 mM CaCl₂, 0.8 mM PMSF, and 0.05% 2-mercaptoethanol; Miyakawa et al., 1987) and stained with DAPI (2 μg/ml) in NS buffer on a glass slide. The isolated mt-nucleoids were mixed directly with an equal volume of DAPI (2 μg/ml) solution without fixation. All observations were made with an epifluorescence microscope (BHS-RFK; Olympus Optical Co., Ltd., Tokyo, Japan) equipped with appropriate objectives (Dplan Apo 100UVPL and 100UV; Olympus).

Preparation of nuclear fraction. The nuclear fraction was prepared from the G2-2 strain by the method of Ide and Saunders (1981) with slight modifications, as described previously (Miyakawa and Sato, 2001). Spheroplasts of stationary-phase cells were suspended in 18% (w/v) Ficoll solution dissolved in 20 mM KH₂PO₄, 0.5 mM CaCl₂, and 0.4 mM PMSF, pH 6.5, and were then homogenized using a Teflon Potter-Elveh-

jem homogenizer. The nuclear fraction was obtained by differential centrifugations.

Nuclease digestion of the mt-nucleoids. The mt-nucleoid suspensions were incubated in the presence of 10 mM MgCl_2 , 3 mM CaCl_2 , or 10 mM MnCl_2 in NES2 buffer at 25°C for up to 40 min. Then, the mt-nucleoids were treated with SDS-proteinase K (final concentration; 0.7% SDS, 20 mM Tris-HCl, pH 8.0, 14 mM EDTA, 20 mM NaCl, and 0.2 mg/ml proteinase K) for 2 h at 37°C, and were loaded on 0.6% agarose gels. For the extraction of mtDNA from the mt-nucleoids, the mt-nucleoids were treated with 0.5% SDS, and mtDNA was extracted by phenol : chloroform (1 : 1), and precipitated by the addition of ethanol. After washing with 70% ethanol, mtDNA was dissolved in TE buffer and used for *EcoRI* digestion.

For micrococcal nuclease digestion of mtDNA, the mt-nucleoids (0.26 μg of nucleoid mtDNA) were treated with 6.5 units/ml micrococcal nuclease (Worthington Biochemical Corp., Freehold, NJ) in 20 μl of a buffer containing 1.5 mM CaCl_2 , 0.1 M sucrose, 3 mM Tris-HCl, pH 7.5, and 3 mM KCl at 37°C for up to 5 min. The enzyme reaction was stopped by the addition of a final concentration of 20 mM EDTA, and samples were then treated with SDS-proteinase K for 2 h at 37°C. For DNase II digestion of mtDNA, the mt-nucleoids were treated with 162 units/ml DNase II (Worthington Biochemical Corp.) in 0.1 M CH_3COOH , 1 mM EDTA, 0.14 M NaCl, pH 4.6 at 25°C for up to 40 min. The enzyme reaction was stopped by treatment with SDS-proteinase K for 2 h at 37°C. The samples were then subjected to 3% (w/v) acrylamide gel electrophoresis. Gels were stained with ethidium bromide, and DNA was transferred to a Hybond N⁺ membrane.

Southern hybridization and detection were performed with the ECL direct nucleic acid labeling and detection system (GE Healthcare UK Ltd., Little Chalfont, UK) according to the instruction manual, using the purified mtDNA as a probe. As a control, the nuclear fraction and the purified mtDNA were also used for micrococcal nuclease digestion under the same conditions.

Results

Nuclease activity associated with mt-nucleoids

Both the wild type and the Δnuc1 mutant showed the same growth curve in YPD liquid medium (data not shown). The mt-nucleoids were isolated from each

strain that reached stationary phase by sucrose density centrifugations. DAPI staining photomicrographs of the spheroplasts and the isolated mt-nucleoids are shown in Fig. 1. The mt-nucleoids appeared as many discrete particles along the cell periphery in the cytoplasm (Fig. 1 (A) (B)). The mt-nucleoids isolated from each cell had intact size and shape compared with those appearing in the spheroplasts (Fig. 1 (C) (D)). The mt-nucleoids from wild-type cells and the Δnuc1 mutant had the same morphological properties, at least as far as observed by fluorescence microscopy.

The protein composition of mitochondria was almost the same between the wild type and the Δnuc1 mutant (Fig. 2, lanes 1 and 2). The mt-nucleoids from each strain contained more than 20 polypeptides, including 20 kDa Abf2p, and the protein composition of the mt-nucleoids was also very similar between the wild type and the Δnuc1 mutant, except for the abundance of polypeptides at above 94 kDa in the Δnuc1 mt-nucleoids (Fig. 2, lanes 3 and 4). In our previous study, zymographic assay of the mt-nucleoid proteins from G2-2 strain detected a faint band of a 38-kDa nuclease on SDS-DNA gels, which corresponded to the molecular mass of Nuc1p (Miyakawa et al., 2000). However, in this study, we could not detect the band of nuclease

Fig. 1. Morphology of mt-nucleoids of the wild type and the Δnuc1 mutant.

Spheroplasts of the wild type (A) and the Δnuc1 mutant (B) were fixed with glutaraldehyde and stained with DAPI. Isolated mt-nucleoids of the wild type (C) and the Δnuc1 mutant (D) were stained with DAPI without fixation. The bar represents 5 μm .

Fig. 2. Comparison of mitochondrial and mt-nucleoid proteins by SDS-PAGE.

Lane 1: mitochondria from the wild type, lane 2: mitochondria from the $\Delta nuc1$ mutant, lane 3: mt-nucleoids from the wild type, lane 4: mt-nucleoids from the $\Delta nuc1$ mutant. In the mt-nucleoid fractions, Abf2p is indicated by an arrow.

by SDS-DNA PAGE of the wild-type mt-nucleoids (data not shown). Subsequently, DNase activity associated with the mt-nucleoid fraction was compared on agarose gels between the mt-nucleoids from the wild type and the $\Delta nuc1$ mutant (Fig. 3). MtDNA in the mt-nucleoids from the wild type was extensively digested after incubation of the mt-nucleoids at 25°C for 40 min in the presence of 10 mM $MgCl_2$, 3 mM $CaCl_2$, and 10 mM $MnCl_2$, respectively (Fig. 3 (A)). This result indicated that extensive DNase activity is involved in the isolated mt-nucleoid fraction. On the other hand, mtDNA in the mt-nucleoids from $\Delta nuc1$ mutant did not show apparent degradation under the same conditions, and the sharp band of mtDNA remained on upper parts of the gels even after incubation (Fig. 3 (A)). Although Nuc1p is a minor component among the mt-nucleoid proteins, these results indicate that degradation of mtDNA during incubation of the mt-nucleoids is primarily due to Nuc1p.

Subsequently, correlation of mtDNA digestion and the morphology of the mt-nucleoids were investigated by agarose gel electrophoresis and DAPI staining (Fig. 3 (B)). When the mt-nucleoids were incubated in the presence of 10 mM $MgCl_2$ for 20 min at 25°C, mtDNA in the wild-type mt-nucleoids was significantly digested, as revealed on agarose gels. On the other hand, degradation of mtDNA was markedly decreased in the

$\Delta nuc1$ mt-nucleoids under the same conditions (Fig. 3 (B)). Analysis of *Eco*RI digests of mtDNA that was extracted from each mt-nucleoid showed that mtDNA in the wild-type mt-nucleoids is rapidly fragmented below 5 kbp within 20 min, whereas the level of mtDNA digestion in the $\Delta nuc1$ mt-nucleoids is much lower than that of mtDNA in wild-type mt-nucleoids (Fig. 3 (C)). The reason that the smear bands appeared at the upper positions of the *Eco*RI-digested bands in the mtDNA digests of the $\Delta nuc1$ mt-nucleoids is unclear at present (Fig. 3 (C), lane 4). When both mt-nucleoids were observed after 20-min incubation by DAPI staining, both mt-nucleoids were observed as spherical particles of similar size, and dispersal or disappearance of the mt-nucleoids was not observed (Fig. 3 (D)). These results together suggest that extensive digestion of mtDNA in mt-nucleoids does not affect the morphology of the isolated mt-nucleoids, as revealed by DAPI staining. In addition, the presence of DNase activity other than Nuc1p is also suggested in the mt-nucleoids.

Nuclease sensitivity of mt-nucleoids isolated from the $\Delta nuc1$ mutant

Nuclear DNA is packaged to form a nucleosome associating with the histone octamer. Thus, micrococcal nuclease cleaves a linker region of DNA between nucleosomes, resulting in the discrete digestion pattern that is known as a ladder of every ca. 160 bp on acrylamide gels (Lohr et al., 1977). On the other hand, it has not been determined what type of mtDNA digestion pattern the mt-nucleoids exhibit after micrococcal nuclease digestion of the intact mt-nucleoids. As described above, the mt-nucleoid fraction from wild-type cells contains significant endogenous DNase activity in the presence of $CaCl_2$. Accordingly, the effect of micrococcal nuclease, which requires Ca^{2+} for its activity, cannot be accurately examined by use of the wild-type mt-nucleoids. In order to minimize the effects of endogenous nuclease activity, we used the mt-nucleoids from the $\Delta nuc1$ mutant in the following experiments. After treatment with micrococcal nuclease, mtDNA in the mt-nucleoids was loaded for 3% acrylamide gel electrophoresis, stained with ethidium bromide, and detected by Southern hybridization (Fig. 4). First, as a control experiment, micrococcal nuclease treatment of the nuclear fraction caused typical ladders every ca. 160 bp on the acrylamide gels under the experimental conditions used (Fig. 4 (A)). On the

Fig. 3. Detection of nuclease activity in the mt-nucleoids.

(A) The mt-nucleoids from the wild type and the $\Delta nuc1$ mutant were incubated at 25°C for 40 min without addition (lane 1), with 10 mM $MgCl_2$ (lane 2), with 3 mM $CaCl_2$ (lane 3), and with 10 mM $MnCl_2$ (lane 4). After treatment of the mt-nucleoids with SDS-proteinase K, mtDNA was separated by agarose gel electrophoresis. (B) The mt-nucleoids from the wild type (lanes 1 and 2) and the $\Delta nuc1$ mutant (lanes 3 and 4) were incubated for 20 min at 25°C with 10 mM $MgCl_2$. MtDNA was separated by agarose gel electrophoresis after treatment with SDS-proteinase K. (C) After incubation of the mt-nucleoids with 10 mM $MgCl_2$ under the same conditions as (B), mtDNA was extracted from the mt-nucleoids and treated with *EcoRI*. Lane 1: mtDNA from the wild type without incubation, lane 2: mtDNA from the wild type after 20-min incubation, lane 3: mtDNA from the $\Delta nuc1$ mutant without incubation, lane 4: mtDNA from the $\Delta nuc1$ mutant after 20-min incubation. (D) Photomicrographs of the mt-nucleoids after DAPI staining. (a) Mt-nucleoids from the wild type without incubation, (b) mt-nucleoids from the $\Delta nuc1$ mutant without incubation, (c) mt-nucleoids from the wild type after 20-min incubation, (d) mt-nucleoids from the $\Delta nuc1$ mutant after 20-min incubation. The incubation conditions are the same as (B). The bar represents 10 μm .

other hand, mtDNA in the mt-nucleoids, which was extensively digested within a 5-min incubation period, did not show any discrete ladder of digested mtDNA in contrast to nuclear chromatin, and the pattern of mtDNA digestion was rather continuous (Fig. 4 (B) (C)). When the mt-nucleoids were incubated in the presence of 1.5 mM $CaCl_2$ without micrococcal nuclease

under the same conditions, no degradation of mtDNA was discerned within 5 min (Fig. 4 (D) (E)). When the purified mtDNA was digested under the same conditions as the control experiment, mtDNA was completely digested within 30 s (Fig. 4 (F) (G)). The nuclease sensitivity of the mt-nucleoids was also examined with DNase II (Fig. 5). Similar to the case of micrococcal

Fig. 4. Micrococcal nuclease digestion of the mt-nucleoids.

DNA was separated on 3% acrylamide gels. (A) The nuclear fraction was digested with micrococcal nuclease for the indicated time (0, 1, 2.5, and 5 min). Discontinuous ladders every ca. 160 bp are visible after staining with ethidium bromide. (B and C) The mt-nucleoids were digested with micrococcal nuclease for the indicated time (0, 0.5, 1, 3, and 5 min). (D and E) The mt-nucleoids were incubated for the indicated time without micrococcal nuclease. (F and G) The purified mtDNA [0.26 $\mu\text{g}/\text{lane}$, equivalent to the quantity of mtDNA per lane used in experiments (B) and (C)] was digested for the indicated time with micrococcal nuclease as a control experiment. In (A), (B), (D), and (F), gels were stained with ethidium bromide, and in (C), (E), and (G), mtDNA was detected by Southern hybridization using CsCl-purified mtDNA as a probe.

nuclease treatment, mtDNA in the mt-nucleoids was digested continuously without apparent ladders of digested DNA.

Discussion

Detection of NUC1 nuclease activity in the mt-nucleoid fraction

In the present study, the comparison of DNase activity that is associated with the mt-nucleoids demon-

Fig. 5. DNase II digestion of the mt-nucleoids.

The mt-nucleoids were digested with DNase II for the indicated time (0, 10, 20, and 40 min) as described in MATERIALS AND METHODS, and DNA was separated on 3% acrylamide gels. The mtDNA was detected by Southern hybridization using CsCl-purified mtDNA as a probe.

strated that a major DNase in the mt-nucleoids is Nuc1p. As significant amounts of nuclease activity remained in the supernatant after fractionation of mt-nucleoids, it is assumed that only a fraction of Nuc1p is associated with the mt-nucleoid fraction (data not shown). Nuc1p is localized to the inner mitochondrial membrane in yeasts because mitoplasts still retain NUC1 nuclease activity (Dake et al., 1988). Nuc1p is assumed to be involved in the recombination process, as the frequency of mtDNA recombination is reduced in a yeast mutant lacking Nuc1p (Zassenhaus and Denninger, 1994). On the other hand, EndoG nuclease, a mammalian homologue of NUC1, is localized in the intermembrane space and is released from mitochondria during apoptosis. The EndoG plays crucial roles in the digestion of nuclear chromatin independent of the caspase pathway (Li et al., 2001; Low, 2003; Parrish et al., 2001). Recently, the role of Nuc1p in yeast apoptosis has also been reported (Buttner et al., 2007). Nevertheless, it remains ambiguous why significant amounts of nonspecific nuclease localize in mitochondria.

MtDNA in the isolated mitochondria is not easily subject to digestion by endogenous nuclease even in the presence of Ca^{2+} (data not shown). This means that mtDNA is not accessible to Nuc1p in organelle. Therefore, it is unclear whether Nuc1p is associated with the mt-nucleoids in vivo. It is possible that a frac-

tion of Nuc1p binds to the mt-nucleoids after lysis of the mitochondrial inner membrane by detergent NP40. As shown in Fig. 3 (C) (D), the compact fluorescent particles were visible with DAPI staining after extensive nuclease digestion of mtDNA. MtDNA that underwent extensive double-strand breaks may still be compactly packaged into the mt-nucleoid structure, in association with mt-nucleoid proteins.

Activity from DNases other than Nuc1p was also suggested to be present in the Δnuc1 mt-nucleoids (Fig. 3 (A)–(C)). There are three proteins—Cce1p, Din7p, and Mhr1p—involved in the recombination process of mtDNA, and the level of Din7p nuclease modulates the stability of the mitochondrial genome (Koprowski et al., 2003). Identification of the nucleases other than Nuc1p remains to be carried out in the isolated mt-nucleoids.

Use of the mt-nucleoids from the Δnuc1 mutant in analysis of mt-nucleoids

In order to investigate the effects of exogenous nuclease on the mt-nucleoids, it is important to minimize the effects of endogenous nuclease activity as much as possible. For this purpose, the mt-nucleoids from Δnuc1 mutant are more useful than those from the wild type. The lack of Nuc1p hardly affects the protein composition of the mt-nucleoids.

From the comparison of the nuclease sensitivity of purified mtDNA and mt-nucleoids, it is evident that mtDNA packaged into the nucleoid structure is more resistant to nuclease digestion than naked mtDNA (Fig. 4 (B) (C) (F) (G)). This coincides with a model in which several mtDNA molecules are highly packaged and protected in the mt-nucleoids, in association with specific proteins. Our results suggest two possibilities concerning the protection of mtDNA in the mt-nucleoids. One possibility is that the mt-nucleoid proteins, including Abf2p, bind along the contours of the mtDNA uniformly rather than discontinuously. Alternatively, the positioning of the mt-nucleoid proteins, which protect mtDNA, may be irregular and the protected regions of mtDNA may be of variable size. As shown in Fig. 4 (C), the digested mtDNA fragments seem to be rather concentrated between 600 and 1,000 bp during micrococcal nuclease digestion. These results suggest the possibility that unit-like structures, in which 600–1,000 bp of mtDNA is protected against nuclease digestion, are irregularly positioned in mtDNA that is tightly packaged into the mt-nucleoids.

Previously, Rickwood and Chambers (1981) isolated the mt-nucleoids by metrizamide gradient centrifugation, which is different from our method, and found multimers of a 200 bp fragment on acrylamide gels after digestion of the mt-nucleoids by DNase II. However, there was no direct evidence showing that ladders of DNA fragments are derived from mtDNA. In the present study, we could not detect the multimers of the 200 bp fragment of mtDNA digests in several independent assays, using CsCl-purified mtDNA as a probe for Southern hybridization (Fig. 5).

Among the mt-nucleoid proteins, the role of Abf2p has been most thoroughly investigated. One molecule of Abf2p binds to mtDNA in every 20–30 base pairs and Abf2p introduces a negative supercoil into the mtDNA. As the deletion of Abf2p causes a loss of mtDNA during culture of cells in YPD medium, there is no doubt that Abf2p plays an important role in stabilizing the mitochondrial genome (Diffley and Stillman, 1991, 1992). On the other hand, the results of atomic force microscopy suggest that packaging of DNA by Abf2p is much weaker than that by histones (Brewer et al., 2003). Newman et al. (1996) showed that *COXII* and *VAR1* sequences are 4- to 5-fold more sensitive to DNase I digestion of mtDNA in toluene-permeabilized mitochondria from the $\Delta abf2$ mutant than from wild-type cells. In regard to the mt-nucleoid organization, a model for packaging of mtDNA into the nucleoid structure has been postulated, and the importance of matrix enzymes such as Iiv5p and Aco1p for the protection of mtDNA has been discussed (Chen and Butow, 2005; Chen et al., 2005, 2007). The use of mt-nucleoids from the $\Delta nuc1$ mutant will provide further useful information in examining how mtDNA is packaged.

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