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Isolation of the mitochondrial nucleoids from yeast *Kluyveromyces lactis* and analyses of the nucleoid proteins

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Mitochondrial (mt) nucleoids were isolated from yeast *Kluyveromyces lactis* with morphological intactness. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed more than 20 proteins that are associated with the mt-nucleoids. However, the protein profile of the mt-nucleoids of *K. lactis* was significantly different from that of the mt-nucleoid proteins from *Saccharomyces cerevisiae*. SDS-DNA PAGE, which detected an Abf2p, a major mitochondrial DNA-binding protein, among the mt-nucleoid proteins of *S. cerevisiae* on a gel, detected only a 17-kDa protein in the *K. lactis* mt-nucleoid proteins. The 17-kDa protein was purified as homogeneous from the mt-nucleoids by a combination of acid extraction, hydroxyapatite chromatography and DNA-cellulose chromatography. The 17-kDa protein introduced a negative supercoil into circular plasmid DNA in the presence of topoisomerase I, as does *S. cerevisiae* Abf2p, and it packed *K. lactis* mtDNA into nucleoid-like particles in vitro. These results, together with the determination of the N-terminal amino acid sequence, suggested that the 17-kDa protein is an Abf2p homologue of *K. lactis* and plays structural roles in compacting mtDNA in cooperation with other nucleoid proteins.

Key Words—Abf2p; DNA-binding protein; *Kluyveromyces lactis*; mitochondria; mitochondrial nucleoids; yeast

Introduction

Mitochondrial nucleoids (mt-nucleoids) of yeast were first successfully isolated from *Saccharomyces cerevisiae* with morphological intactness (Miyakawa et al., 1987). In contrast with the images observed by electron microscopy of ultrathin sections of mitochondria (Stevens, 1981), mtDNA was shown to be packaged into a compact nucleoid structure by association with many protein components (Miyakawa et al., 1987). Subsequently, the six different DNA-binding proteins

were shown to be involved in the assembly of mt-nucleoids (Miyakawa et al., 1995). Among the mt-nucleoid proteins, the most major protein is a 20-kDa DNA-binding protein with basic pI, first designated as HM (Caron et al., 1979) and more recently known as Abf2p (Diffley and Stillman, 1991). Abf2p belongs to the high-mobility group (HMG) protein family and has two HMG box domains. It introduces a negative supercoil into a relaxed circular plasmid in the presence of topoisomerase I. The function of Abf2p has been investigated. Abf2p is essential for the maintenance and expression of mtDNA in the presence of the fermentable substrate, glucose (Diffley and Stillman, 1991). Abf2p also affects the copy number and recombination of mtDNA (Zelenaya-Troitskaya et al., 1998).

The size and number of mt-nucleoids in yeast cells

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vary considerably depending on the respiratory competence of the yeast cells, and mt-nucleoids of different sizes could be isolated. As mt-nucleoids of different sizes have different compositions of protein components (Shiiba et al., 1997), the composition of mt-nucleoid proteins is presumed to determine the degree of mtDNA compaction, and to affect the transcription, replication and transmission of mitochondrial genomes. However, it is still not clear what kinds of proteins are involved in the compaction of yeast mt-nucleoids *in vivo*.

In a previous paper, we reported that SDS-DNA PAGE is a useful method for detecting Abf2p-like proteins in other yeast mt-nucleoids as well as for detecting Abf2p in *S. cerevisiae* mt-nucleoids. The molecular mass of Abf2p-like proteins in mt-nucleoids varied between 20 kDa–26 kDa in several yeasts (Miyakawa et al., 2000).

The yeast *Kluyveromyces lactis* has been extensively investigated for its physiological properties, which are quite distinct from those of *S. cerevisiae*. *K. lactis* is a "petite negative" yeast, and its respiration is not subjected to glucose repression, in contrast with *S. cerevisiae* (Bolotin-Fukuhara et al., 2000; Chen and Clark-Walker, 1999; Ragnini and Fukuhara, 1988). In this study, we isolated the mt-nucleoids from *K. lactis* and subsequently purified an Abf2p homologue from the mt-nucleoids. We discuss the possible roles of Abf2p in mtDNA packaging.

Materials and Methods

Strain and cultivation. *Kluyveromyces lactis* strain CLIB210, originally called 2359/152 (*MATa*, *metA1-1*, K^+ R^+) (generously donated by Dr. Fukuhara) and *Saccharomyces cerevisiae* G2-2 strain (Miyakawa et al., 1984) were used. Cells were cultured aerobically at 30°C to the early stationary phase in YPD medium that contained 1% yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 2% peptone (Kyokuto Co., Ltd., Tokyo, Japan) and 2% glucose. Cells that reached stationary phase were harvested and converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan).

Isolation of mt-nucleoids. The mt-nucleoids of *S. cerevisiae* were isolated by the methods described previously (Miyakawa et al., 1987). The isolation protocol of *K. lactis* mt-nucleoids was modified to minimize the contamination of nuclear DNA in the mt-nucleoid

fraction. Spheroplasts from ca. 40 g *K. lactis* cells were suspended in 90 ml of NE1 buffer (0.3 M sucrose, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.4 mM spermidine, 7 mM 2-mercaptoethanol, and 0.4 mM PMSF) and homogenized with a Teflon Potter-Elvehjem homogenizer. The homogenates were centrifuged successively at 950×*g* for 5 min, 1,600×*g* for 5 min, 2,500×*g* for 5 min and at 12,000×*g* for 20 min. The pellet was suspended in 400 ml of NE2 buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.8 mM spermidine, 7 mM 2-mercaptoethanol, and 0.4 mM PMSF), and centrifuged twice at 15,000×*g* for 20 min to obtain the mitochondrial pellet. The mitochondrial pellet was resuspended in 80 ml of DL buffer (0.25 M sucrose, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.4 mM spermidine, 7 mM 2-mercaptoethanol, and 0.4 mM PMSF), and 20% Nonidet P-40 (NP-40) was dropped into the solution to a final concentration of 0.5% with gentle stirring. The solution was centrifuged at 16,000×*g* for 10 min. Each 14 ml of the clear supernatant was layered on 8 ml of 15%, 8 ml of 40%, and 6 ml of 60% (w/v) sucrose discontinuous density gradient in the presence of 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.4 mM spermidine, 7 mM 2-mercaptoethanol, and 0.4 mM PMSF, then centrifuged in a Hitachi RPS27-2 swing bucket at 40,000×*g* for 1 h. The fractions equivalent to the boundary layer between 15% and 40% sucrose were collected. The mt-nucleoid fraction was diluted with 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.4 mM spermidine, 7 mM 2-mercaptoethanol, and 0.4 mM PMSF, and was centrifuged at 100,000×*g* for 1 h to precipitate the mt-nucleoids. The mt-nucleoids were thoroughly suspended in 0.5 ml of NE2 buffer, and were stocked at –85°C until use.

The mt-nucleoid fraction for purifying *K. lactis* 17-kDa protein was obtained by directly centrifuging the mitochondrial supernatant at 70,000×*g* for 1 h after lysis with NP-40 and removal of the insoluble materials. In this preparation, the purification step using sucrose density gradient centrifugation was omitted.

Purification of a 17-kDa protein from *K. lactis* mt-nucleoids. *K. lactis* 17-kDa protein was purified by a combination of acid extraction of mt-nucleoids, hydroxapatite chromatography (Fisher et al., 1991) and DNA-cellulose chromatography. The mt-nucleoids were mixed with an equal volume of 0.4 N HCl and left for 30 min on ice. The sample was centrifuged at 20,000×*g* for 30 min to remove insoluble materials. The supernatant was then mixed with 6 vol. of cold acetone and left for 1 h on ice. The precipitate was col-

lected by centrifugation at $13,000\times g$ for 10 min, washed twice with cold acetone and dried. The precipitate was dissolved with 0.1 M sodium phosphate buffer, pH 6.8, 2% SDS, 20 mM DTT, and 0.5 mM PMSF (Fisher et al., 1991) and boiled for 5 min. The sample was diluted 10-fold with 0.1 M sodium phosphate buffer, pH 6.8 and 1 mM DTT, then loaded on a hydroxyapatite column equilibrated with 0.1 M sodium phosphate buffer pH 6.8, 0.1% SDS, and 1 mM DTT. After washing the column with 0.1 M sodium phosphate buffer pH 6.8 and 1 mM DTT, the 17-kDa protein was eluted with 0.2 M sodium phosphate buffer pH 6.8 and 1 mM DTT. The fractions that contained the 17-kDa protein were pooled and mixed with 2 vol. of buffer A (4% Triton X-100, 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 2 mM DTT, 0.25 mM PMSF, and 15% glycerol; Fisher et al., 1991). The sample was loaded on a native DNA-cellulose column equilibrated with buffer A containing 0.1 M NaCl. After washing the column with buffer A that contained 0.1 M NaCl, the proteins were eluted stepwise with buffer A containing 0.2 M NaCl, then with buffer A containing 0.6 M NaCl. The 17-kDa protein was eluted as a single band with buffer A containing 0.6 M NaCl.

Purification of mtDNA. Spheroplasts were lysed in EDTA-saline (0.15 M NaCl, 0.1 M EDTA, and 0.1 M Tris-HCl, pH 7.6) and 2% Sarkosyl. After insoluble materials were removed by centrifugation, proteinase K was added at 200 $\mu\text{g/ml}$ and incubated at 30°C overnight. 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). DNA was extracted with a mixture of phenol and chloroform and was then precipitated with cold ethanol. DNA that was extracted from spheroplasts and mitochondria was loaded on an isopycnic centrifugation in CsCl in the presence of Hoechst 33258 to separate mtDNA from nuclear DNA (Hudspeth et al., 1980). The pellet of mt-nucleoids was suspended in a solution of 0.07 M EDTA, 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 3.5% (w/v) SDS, and 1 mg/ml proteinase K, then digested for 30 min at 37°C. DNA was extracted with phenol and chloroform and precipitated with ethanol. After being washed with ethanol, the DNA was dissolved in a small amount of TE buffer. The DNA was digested with a restriction enzyme *Hha* I, subjected to electrophoresis in 0.6% agarose gel and stained with ethidium bromide.

Fluorescence microscopy. Whole cells of *K. lactis* in culture medium were fixed with 2.5% glutaraldehyde at room temperature for 1 h. Spheroplasts of *K. lactis*

were fixed with 2.5% glutaraldehyde in 0.8 M sorbitol, and 20 mM potassium phosphate buffer, pH 7.5, at room temperature for 1 h. Whole cells and spheroplasts, which had been 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) were mixed with an equal volume of a solution of DAPI (2 $\mu\text{g/ml}$) in NS buffer on a glass slide. The isolated mitochondria and mt-nucleoids were mixed directly with an equal volume of the solution of DAPI (2 $\mu\text{g/ml}$). 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).

SDS-PAGE and SDS-DNA PAGE. For SDS-PAGE, an aliquot of each sample (15 μl) was mixed with 5 μl of 4 $\times$ SDS sample buffer and loaded on a 15% polyacrylamide gel as described by Laemmli (1970). SDS-DNA PAGE was performed as described previously (Miyakawa et al., 2000). The stacking and separating gels contained 10 $\mu\text{g/ml}$ native calf thymus DNA. After overnight incubation of the gels in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, 10 mM MgCl_2 and 7 mM 2-mercaptoethanol at 30°C, the gels were stained with ethidium bromide (1 $\mu\text{g/ml}$) for 30 min. Proteins on gels were detected by the silver-staining method (Oakley et al., 1980). The molecular-mass markers (Pharmacia, Upsala, Sweden) used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa).

Negative supercoiling. Plasmid pUC19 (2.6 μg) was relaxed with 50 units of topoisomerase I (MBI Fermentas) in supercoiling buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 0.5 mM EDTA, 0.4 mM PMSF, 7 mM 2-mercaptoethanol, and 0.03 mg/ml BSA; Diffley and Stillman, 1992) in 105 μl at 37°C for 60 min. The relaxed plasmid was divided among the tubes (0.43 $\mu\text{g/tube}$) and incubated with the indicated amounts of *K. lactis* Abf2p in the presence of 10 units of topoisomerase I in supercoiling buffer at 37°C for 30 min. Reactions were then terminated by adding one-third volume of 1 mg/ml proteinase K, 70 mM EDTA, 3.5% SDS, 0.1 M Tris-HCl, pH 8.0, and 0.1 M NaCl. After 30 min at 37°C, the sample was subjected to electrophoresis in 1% gels in Tris-acetate EDTA buffer that contained the indicated amounts of chloroquine.

Disassembly and reassembly of mt-nucleoids.

Three samples each of (1) mt-nucleoids obtained from the 15–40% sucrose boundary of sucrose gradient centrifugation, (2) mtDNA (0.13 µg) purified from mitochondria by a CsCl density gradient, and (3) the mixture of mtDNA (0.13 µg) and *K. lactis* 17-kDa protein (0.53 µg), were respectively mixed with equal volumes of 4M NaCl in NE2 buffer and then dialyzed against NE2 buffer overnight at 4°C. Each sample was mixed with an equal amount of DAPI (2 µg/ml) and observed immediately under fluorescence microscopy.

Analysis of the amino acid sequence. The 17-kDa protein was transferred to a polyvinylidene difluoride membrane after SDS-PAGE. The membrane was excised, and the N-terminal amino acid sequence was determined using a Shimadzu PPSQ-21 protein sequencer.

Results

The mt-nucleoids of *K. lactis* were visible as discrete dots in the cytoplasm in stationary-phase cells (Fig. 1A) and appeared more distinctly in the squashed spheroplasts (Fig. 1B). The cytoplasm of *K. lactis* cells was dimly stained with DAPI, indicating the presence of killer plasmids pGKL1 and pGKL2 (Fig. 1, A and B). The mt-nucleoid fraction, which was recovered from the boundary between 15% and 40% sucrose, contained a number of fluorescent particles of relatively uniform size that corresponded to those in the spheroplasts (Fig. 1C). The mt-nucleoid fraction was free from contamination by nuclear DNA as shown by a CsCl-density gradient centrifugation (Fig. 2).

Analyses by agarose gel electrophoresis showed that DNA extracted from mt-nucleoids (Fig. 3, lane 4) was free from cytoplasmic killer plasmids as was mtDNA purified from mitochondria by CsCl-density gradient centrifugation (Fig. 3, lane 3). In contrast, mtDNA preparation, which was directly purified from spheroplasts by CsCl-density gradient centrifugation, was significantly contaminated by two killer plasmids, pGKL1 (8.9 kbp) and pGKL2 (13.5 kbp) (Fig. 3, lanes 2 and 5). The pattern of fragments obtained after digestion with restriction enzyme, *Hha* I, was identical for mtDNA that was purified from mitochondria and mtDNA that was purified from mt-nucleoids (Fig. 3, lanes 6 and 7). The total length of mtDNA fragments coincided well with the established genome size of 39 kbp (Ragnini and Fukuhara, 1988), indicating that

Fig. 1. DAPI-fluorescence photomicrographs of whole cells, spheroplasts, and isolated mt-nucleoids of *K. lactis*.

(A) Whole cells at early stationary-phase, (B) spheroplasts of early stationary-phase cells, (C) mt-nucleoids that were obtained from the boundary between 15% and 40% sucrose after sucrose-density gradient centrifugation. Bar represents 10 µm.

Fig. 2. Results of density gradient centrifugation on Hoechst 33258-CsCl of DNA extracted from the isolated mt-nucleoids.

Fig. 3. Analysis of mtDNA by agarose gel electrophoresis.

Lane 1: *Hind* III digest of λDNA; lane 2: mtDNA purified from cell lysate by CsCl density gradient centrifugation; lane 3: mtDNA purified from mitochondria by CsCl density gradient centrifugation; lane 4: DNA extracted from isolated mt-nucleoids; lanes 5, 6 and 7: *Hha* I digests of DNA from lanes 2, 3 and 4.

the degradation of mtDNA did not occur during the course of mt-nucleoid isolation.

Proteins from each step of mt-nucleoid isolation

Fig. 4. SDS-DNA PAGE of protein components of various fractions during the isolation of mt-nucleoids from *K. lactis*.

During the isolation of *K. lactis* mt-nucleoids, each fraction was separated on two SDS gels that contained 10 µg/ml calf thymus DNA. After electrophoresis, one gel was silver-stained (A) and another gel (B) was stained with ethidium bromide after incubation with 10 mM MgCl₂ for 3 h at 30°C as described in MATERIALS AND METHODS. Lane 1: mitochondrial fraction; lane 2: the supernatant after lysis of mitochondria by NP-40 and subsequent centrifugation (16,000×*g* for 10 min); lane 3: the pellet after lysis of mitochondria and centrifugation; lane 4: the soluble fraction after sucrose density gradient centrifugation; lane 5: mt-nucleoid fraction obtained from the 15–40% sucrose boundary; lane 6: mt-nucleoid fraction isolated from *S. cerevisiae*.

were analyzed by SDS-DNA PAGE (Fig. 4). Silver-staining revealed more than 20 bands in the mt-nucleoid fraction from the 15–40% sucrose boundary (Fig. 4A, lane 5). All bands disappeared after treatment of the mt-nucleoids with proteinase K before SDS-PAGE, indicating that those bands corresponded to protein components of the mt-nucleoid fractions. The molecular masses and relative levels of the respective mt-nucleoid proteins differed between *K. lactis* and *S. cerevisiae* (Fig. 4A, lanes 5 and 6). In particular, the presence of a protein that corresponds to Abf2p, a 20-kDa DNA-binding protein in *S. cerevisiae* mt-nucleoids, was not apparent in *K. lactis* mt-nucleoids (Fig. 4A, lanes 5 and 6).

In a previous paper (Miyakawa et al., 2000), we reported that the Abf2p of *S. cerevisiae* is a unique protein among mt-nucleoid proteins. It was detected on a SDS-DNA gel as a dark band after ethidium bromide staining because of its high affinity to DNA and easy renaturation after removal of SDS from the gel (Fig. 4B, lane 6). The SDS-DNA PAGE detected only a relatively minor 17-kDa protein in *K. lactis* mt-nucleoid proteins (Fig. 4B, lane 5). SDS-DNA PAGE also revealed the presence of a 37-kDa protein in the mitochondrial

Fig. 5. Acid extraction of mt-nucleoids.

Lane 1: mt-nucleoids obtained from the 15–40% sucrose boundary; lane 2: precipitate after extraction of mt-nucleoids by 0.4 N HCl; lane 3: proteins extracted from mt-nucleoids by 0.4 N HCl. The gel was silver-stained.

fraction and two other fractions (Fig. 4B, lanes 1, 2 and 4). Dake et al. (1988) purified a 38-kDa NUC1 nuclease from the mitochondria of *S. cerevisiae*, and showed that 90–95% of mitochondrial nuclease activity was due to the NUC1 nuclease. SDS-DNA PAGE of *S. cerevisiae* mt-nucleoids detected a band of 38 kDa, which corresponds to the molecular mass of NUC1 (Miyakawa et al., 2000). The 37 kDa band of *K. lactis* increasingly thickened during the incubation of gels in the presence of Mg²⁺ ion (data not shown) as did the 38 kDa band of *S. cerevisiae*. These results suggested that the 37-kDa protein of *K. lactis* is a mitochondrial nuclease, a homologue of *S. cerevisiae* NUC1.

We found that the 17-kDa protein was effectively extracted by treatment of the mt-nucleoids that were isolated by sucrose density gradient centrifugation with 0.4 N HCl (Fig. 5). Subsequently, in order to obtain higher yields of the protein, we tried to purify the 17-kDa protein from the pellet of mt-nucleoids that were obtained by the omission of sucrose density gradient centrifugation as described in MATERIALS AND METHODS. The proteins in each purification step of the 17-kDa protein are shown in Fig. 6. The acid-extracts (Fig. 6, lane 1) of mt-nucleoids (Fig. 6, lane 2) were loaded to a hydroxyapatite column after denaturing by boiling, and the fractions that contained the 17-kDa protein were obtained by elution of a hydroxyapatite column with the sodium phosphate buffer that contained 0.2 M NaCl (Fig. 6, lane 3). After treatment of the sample for renaturation of proteins (Fisher et al., 1991), the 17-kDa protein was loaded on a native DNA cellulose column and was eluted as a homogeneous band with

Fig. 6. Purification of *K. lactis* 17-kDa protein.

Fractions from different stages of purification were analyzed by SDS-PAGE. Lane 1: mt-nucleoid fractions obtained from the mitochondrial lysate without sucrose density gradient centrifugation; lane 2: proteins extracted from mt-nucleoids by 0.4 M HCl; lane 3: a fraction obtained by chromatography on hydroxyapatite. Elution of proteins with 0.2 M sodium phosphate buffer produced a fraction enriched in the 17-kDa protein; lane 4: a fraction obtained by chromatography on DNA-cellulose. The 17-kDa protein was eluted as a homogeneous band with buffer A that contained 0.6 M NaCl. The gel was silver-stained.

buffer A that contained 0.6 M NaCl (Fig. 6, lane 4). These fractions were pooled, concentrated and used in the supercoiling experiments.

Abf2p is characterized by the ability to induce supercoiling in relaxed, closed circular plasmid in the presence of topoisomerase I. Therefore, various amounts of the purified 17-kDa protein were mixed with relaxed pUC19 plasmid in the presence of topoisomerase I, and the supercoiling activity was investigated by agarose gel electrophoresis. As shown in Fig. 7A, the addition of increasing amounts of the 17-kDa protein introduced an increase of supercoiling in the plasmid. The direction of supercoiling was determined by electrophoresis in the presence of 1 μ M chloroquine. With the addition of chloroquine, supercoiled DNA shifted again to the relaxed form, indicating that the 17-kDa protein induced the formation of negatively supercoiled DNA, as shown using *S. cerevisiae* Abf2p (Diffley and Stillman, 1992) (Fig. 7B).

To determine that the 17-kDa protein has the ability to pack mtDNA molecules, the 17-kDa protein was mixed with purified *K. lactis* mtDNA at a ratio of protein/DNA=4, which is sufficient to introduce supercoiling in plasmid DNA. The degree of mtDNA compaction was observed by DAPI-staining. As a control, *K. lactis* mt-nucleoids and the purified mtDNA were also ob-

Fig. 7. Negative supercoiling of plasmid DNA by 17-kDa protein in the presence of topoisomerase I.

In each tube, the relaxed pUC19 (0.43 μ g) was respectively incubated with 0, 0.3, 0.5, 0.7, 0.9, and 1.2 μ g 17-kDa protein in the presence of excess calf thymus topoisomerase I and processed for electrophoresis as described in MATERIALS AND METHODS. The ratio of 17-kDa protein/DNA is indicated on each lane. 1% agarose gels and gel running buffer contained 0 (A), 1 (B) μ M chloroquine. M is *Hind* III digest of λ DNA. Sc is supercoiled pUC19 plasmid.

served. As in the procedure previously used in *S. cerevisiae* mt-nucleoids (Miyakawa et al., 1995), the *K. lactis* mt-nucleoids (Fig. 8A) were disassembled in 2 M NaCl solution (Fig. 8B) and reassembled again as fluorescent particles after the removal of salt by dialysis (Fig. 8C). The samples of purified mtDNA, mtDNA in 2 M NaCl and mtDNA after dialysis showed extensive Brownian movement, but no fluorescent particles were observed (data not shown). On the other hand, after the addition of the 17-kDa protein to the mtDNA solution, the compaction of mtDNA immediately became visible (Fig. 8D), and the fluorescent particles were disassembled by the addition of 2 M NaCl (Fig. 8E). After dialysis, more distinct small fluorescent particles were also visible (Fig. 8F), indicating that the compact packing of mtDNA occurred with the addition of the 17-kDa protein.

The amino-terminal amino acid sequence of the 17-kDa protein is shown in Fig. 9. In comparison with the sequence of *S. cerevisiae* Abf2p, residues 6–9 (Pro-Lys-Arg-Pro) and residue 12 (Ala) of *K. lactis* 17-kDa protein coincided with residues 17–20 and a residue 23 in HMG box1 of *S. cerevisiae* Abf2p.

Fig. 8. Photomicrographs showing the assembly in vitro of mtDNA upon the association of protein components.

(A) Isolated mt-nucleoids, (B) mt-nucleoids disassembled in 2 M NaCl solution, (C) mt-nucleoids reassembled after the removal of NaCl by dialysis, (D) compaction of mtDNA by the addition of *K. lactis* 17-kDa protein, (E) disassembly of the 17-kDa protein-mtDNA complex in 2 M NaCl solution, (F) reassembly of 17-kDa protein-mtDNA complex after the removal of NaCl by dialysis. Samples were stained with DAPI without fixation and were immediately observed. Bar represents 10 μ m.

		1	5	10	15																					
<i>K. lactis</i>			GA	AVK	PKR	PTA	F	S	Y																	
<i>S. cerevisiae</i>	K	A	S	K	R	T	Q	L	R	N	E	L	I	K	Q	G	P	K	R	P	T	S	A	Y	F	L

Fig. 9. Amino-terminal amino acid sequence of the *K. lactis* 17-kDa protein.

The amino-terminal amino acid sequence of *S. cerevisiae* Abf2p (Diffley and Stillman, 1991) is shown below for comparison. The conserved sequence of HMG box1 is indicated by boxes.

Discussion

It is generally accepted that mtDNA is three-dimensionally folded to form mt-nuclei or nucleoids by association with a number of proteins (Kuroiwa, 1982). In yeast, several proteins have been identified as mt-nucleoid proteins (Miyakawa and Sato, 2001; Miyakawa et al., 1995, 1996; Newman et al., 1996; Sato et al., 2002) or as mtDNA-binding proteins (Kaufman et al., 2000). However, the function of each protein in the organization of the mt-nucleoid structure remains unclear. Some proteins may directly bind to mtDNA, and other proteins may indirectly associate with mt-nucleoids via protein-protein interactions. The possibility that membrane proteins anchor mt-nucleoids in the matrix has also been reported (Aiken Hobbs et al., 2001). In those studies, Abf2p is the most characterized in yeast mt-nucleoids, and the homologous protein, h-mtTFA has also been purified from human mito-

chondria (Parisi et al., 1993).

This is the first report regarding the purification of ABF2-like protein from yeast species other than *S. cerevisiae*. Several methods for purifying *S. cerevisiae* Abf2p from mitochondria have been reported. These include a combination of SDS-hydroxyapatite chromatography and phosphocellulose chromatography (Fisher et al., 1991), or a combination of heparin-agarose, Blue-Sepharose CL-4B, gel filtration and ion exchange chromatography (Diffley and Stillman, 1992). The Abf2p has also been purified from the mt-nucleoids by a combination of DNase I digestion of mt-nucleoids and subsequent DNA-cellulose chromatography (Miyakawa et al., 1995). For the purpose of identifying mt-nucleoid proteins, it is better to use the isolated mt-nucleoids rather than mitochondria as starting materials. First, we tried to purify *K. lactis* Abf2p from the mt-nucleoids by conventional DNase I digestion and DNA-cellulose chromatography, but the solubilization of the 17-kDa protein was difficult for unknown reasons. Therefore, we combined several previously published procedures for purifying the 17-kDa protein, an Abf2p candidate (Figs. 5 and 6).

DNase I footprinting showed that in a sufficiently high concentration of *S. cerevisiae* Abf2p, every 25–30 bp is protected by binding with Abf2p. Estimation of the intracellular amounts of Abf2p suggests that a monomer of *S. cerevisiae* Abf2p binds to each 25–30 bp along the mtDNA molecule (Diffley and Stillman, 1992). As shown in Fig. 7, supercoiling distinctly

occurred at a ratio above 17-kDa protein/DNA=1.6, and a majority of mtDNA shifted to supercoiled form at a ratio of 2.8. Assuming that one molecule of the 17-kDa protein binds to every 30 bp mtDNA, the 17-kDa protein/DNA ratio of the DNA-protein complex is 1.8. Therefore, our results suggest that the distinct supercoiling was introduced by saturated binding of the 17-kDa protein to plasmid DNA.

The addition of 17-kDa protein sufficient to cover the contour of mtDNA introduced the compaction of mtDNA (Fig. 8). This result suggests that the supercoiling activity of mtDNA by wrapping or bending DNA correlates with an ability for compaction of mtDNA. However, this does not necessarily mean that the 17-kDa protein plays a central role in the organization of mt-nucleoids. The amount of 17-kDa protein among *K. lactis* mt-nucleoid proteins seems to be much lower than that of Abf2p in *S. cerevisiae* mt-nucleoids. Therefore, careful quantitative analysis of the proteins in isolated mt-nucleoids is essential to evaluate the structural importance of the 17-kDa protein and other nucleoid proteins.

As the fluorescent particles (Fig. 8F) that were formed by the association of mtDNA and the 17-kDa protein seem to be smaller than the isolated mt-nucleoids (Fig. 1C), it is possible that the 17-kDa protein packs an mtDNA molecule, whereas the isolated mt-nucleoids may be composed of more than one mtDNA molecule. In fact, it has been shown that in *S. cerevisiae*, a single mt-nucleoid contains 3–4 molecules of mtDNA.

The HMG box contains a highly conserved sequence near the N-terminal. In the mature form of *S. cerevisiae* Abf2p, this conserved sequence corresponds to the 17th–20th amino acids (Pro-Lys-Arg-Pro) (Baxeavanis and Landsman, 1995; Diffley and Stillman, 1991). This sequence was well conserved in the 7th–11th amino acids of *K. lactis* 17-kDa protein, strongly suggesting that the 17-kDa protein is an ABF2 homologue in *K. lactis*. A comparison of both proteins indicates that the N-terminal sequence near HMG box1 is shortened by 11 residues in *K. lactis*.

In this paper, SDS-DNA PAGE has been verified to be useful for detecting ABF2-like proteins in yeasts other than *S. cerevisiae*. Considering the size diversity of ABF2-like proteins among yeast species as revealed by SDS-DNA PAGE (Miyakawa et al., 2000), amino acid sequences other than the two HMG box domains may be significantly varied in yeasts.

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