

## Full Paper

# Simple detection of a yeast mitochondrial DNA-binding protein, Abf2p, on SDS-DNA gels

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**Abf2p, a mitochondrial DNA-binding protein of yeast *Saccharomyces cerevisiae*, was selectively detected among mitochondrial nucleoid proteins by SDS-DNA polyacrylamide gel electrophoresis (SDS-DNA PAGE) followed by ethidium bromide staining. This method is simple and specific for the detection of Abf2p, and it may be used to identify an Abf2p-like protein that is present in mitochondrial nucleoids from other yeasts.**

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

## Introduction

Mitochondrial DNA (mtDNA) does not exist free from proteins in vivo. Rather, several mtDNAs are folded together by means of the binding of specific proteins and RNAs to form a mitochondrial nucleoid (mt-nucleoid) (Kuroiwa, 1982). In yeasts, we previously isolated morphologically intact mt-nucleoids from *Saccharomyces cerevisiae*, and used acid/urea-SDS polyacrylamide gel electrophoresis (SDS-PAGE) to detect a 20-kDa protein that was associated with mt-nucleoids and that demonstrated histone-like properties (Miyakawa et al., 1987).

We subsequently reported the presence of six different DNA-binding proteins that are thought to be involved in the assembly of mt-nucleoids (Miyakawa et al., 1995). Among these proteins, the 20-kDa protein, which has been assumed to play key roles in mt-nucleoid formation, was the most abundant and had the highest affinity for mtDNA (Miyakawa et al., 1995;

Newman et al., 1996).

The 20-kDa protein was first purified from mitochondria of *S. cerevisiae* as a unique histone-like protein, HM (Caron et al., 1979), recently designated as Abf2p (Diffley and Stillman, 1991). Abf2p has two HMG boxes in its domain structure, which are closely related to the vertebrate nonhistone chromosomal high-mobility group protein HMG1. Abf2p is essential for the maintenance and expression of mtDNA in the presence of the fermentable substrate, glucose (Diffley and Stillman, 1991). The function of Abf2p can be complemented by human mitochondrial transcription factor (mtTF1), by a bacterial histone-like HU, and by the NHP6A protein from the nuclei of yeast cells (Kao et al., 1993; Megraw and Chae, 1993; Parisi et al., 1993). At present, however, the exact function of Abf2p is unknown. In addition, it is not evident whether the participation of Abf2p in packing of mtDNA is a common feature of the mt-nucleoids in yeasts as well as in other organisms.

SDS-DNA PAGE was first developed to detect the nuclease activity of bacteria and HeLa cells on gels (Rosenthal and Lacks, 1977). Since then, this method has been used to detect nuclease from several organ-

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isms including the fungi *Neurospora crassa* (Chow and Fraser, 1983), higher plants (Nakamura et al., 1987), and algae (Ogawa and Kuroiwa, 1985). On the other hand, SDS-DNA PAGE has also been used to detect nuclear histones of mammalian cells (Rosenthal and Lacks, 1978). Nuclear histones renature and strongly bind to DNA in SDS-DNA gels after the removal of SDS from the gels. In consequence, the intercalation of ethidium bromide into DNA is inhibited, and the DNA-histone complexes appear as dark bands under UV illumination.

Like the histone, the Abf2p introduces negative supercoils into circular plasmid DNA in the presence of topoisomerase I (Caron et al., 1979). Therefore, it is expected that Abf2p-DNA complexes may interfere with the intercalation of ethidium bromide into DNA. To date, however, SDS-DNA PAGE has not been employed for the detection of DNA-binding proteins in mitochondria. In the present study, we demonstrate that SDS-DNA PAGE is an effective method for detecting Abf2p in *S. cerevisiae*.

## Materials and Methods

**Strain and cultivation.** *Saccharomyces cerevisiae* G2-2 strain was cultured in modified Burkholder's medium supplemented with 3.6% (v/v) tomato extract (Miyakawa et al., 1994; Sando and Miyake, 1971). *Pichia jadinii* CBS 1600, *P. anomala* IFO 0122, *P. petersonii* CBS 5555, *Williopsis mrakii* CBS 1707, and *W. saturnus* IFO 0132 were also used for the isolation of mt-nucleoids. With the exception of *S. cerevisiae* G2-2, all strains were cultured aerobically at 30°C in YPD medium (1% yeast extract, 2% glucose, 2% peptone) to the early stationary phase. For the culture of *W. mrakii*, Antifoam 289 (Sigma, St. Louis, MO, USA) was added in a ratio of 50 µl/L YPD medium. In order to isolate mt-nucleoids, cells (ca. 40 g wet wt.) were harvested and converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) as described previously (Miyakawa et al., 1987).

**Isolation of mt-nucleoids.** Mt-nucleoids were isolated according to the method described previously (Miyakawa et al., 1987). Mitochondria were prepared from disrupted spheroplasts by differential centrifugation. The mt-nucleoids were isolated from mitochondria after lysis of mitochondrial membranes with 0.5% (w/v) NP40 and subsequent sucrose density gradient cen-

trifugation.

**Chromatography on DNA-cellulose.** Chromatography on DNA-cellulose was performed as described previously (Miyakawa et al., 1995). Each pellet of mt-nucleoids was treated with DNase I, and the extracted proteins were loaded onto a DNA-cellulose column. After the column was washed with TAN buffer [17% (w/v) sucrose, 20 mM Tris-HCl (pH 7.6), 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 mM PMSF], proteins were eluted first with TAN buffer that contained 0.2 M NaCl and then with TAN buffer that contained 2 M NaCl to elute Abf2p.

**Immunoblotting.** The band of the 20-kDa protein, Abf2p, was excised from the gels after SDS-PAGE of *S. cerevisiae* mt-nucleoids. The gel slices were crushed, mixed with a small amount of PBS (0.13 M NaCl, 2 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2), and supplemented with an equal volume of Freund's complete adjuvant (for the first injection) or of Freund's incomplete adjuvant (for all subsequent injections). Each sample was homogenized, and the rabbits were initially immunized with about 100 µg of the protein. Booster injections, also containing about 100 µg of the protein, were given at intervals of 3–4 weeks. Antisera were tested by immunoblotting of strips of PVDF membranes that carried yeast mt-nucleoid proteins, which had been transferred from SDS-polyacrylamide gels (Towbin et al., 1979). Antibodies that had reacted with antigens on PVDF membranes were detected by means of the immunoperoxidase procedure using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA).

**SDS-PAGE and SDS-DNA PAGE.** An aliquot of each sample (15 µl) was mixed with 5 µl of 4×SDS sample buffer and loaded on a 15% polyacrylamide gel (Laemmli, 1970). SDS-DNA PAGE was performed according to the method of Rosenthal and Lacks (1977, 1978) with slight modifications. Unless specified otherwise, the stacking and separating gels contained 10 µg/ml native calf thymus DNA. After SDS-PAGE, SDS was removed from the gels by three 30-min washings with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The gels were then suspended in TE buffer and supplemented with 10 mM MgCl<sub>2</sub> and 7 mM 2-mercaptoethanol. After overnight incubation at 30°C, the gels were stained with ethidium bromide (1 µg/ml) for 30 min. The band of Abf2p was detected by illuminating the gels under UV irradiation. Proteins were detected on SDS gels with or without DNA by the silver-

staining method described by Oakley et al. (1980). In the experiments described in Fig. 1C and Fig. 3, lanes 2–7, bovine serum albumin (BSA) was added to the gels at a concentration of 10  $\mu$ g/ml, because BSA has been shown to enhance the detection of histones and nucleases at this concentration (Rosenthal and Lacks, 1978). In the experiments described in Fig. 3, the gel represented in lane 6 contained 10  $\mu$ g/ml denatured calf thymus DNA, which was prepared by boiling calf thymus DNA for 5 min and rapidly cooling it on ice. The gel represented in lane 7 contained 10  $\mu$ g/ml yeast RNA (Sigma), in place of native DNA. The molecular-mass markers (Pharmacia, Uppsala, 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  $\alpha$ -lactalbumin (14.4 kDa).

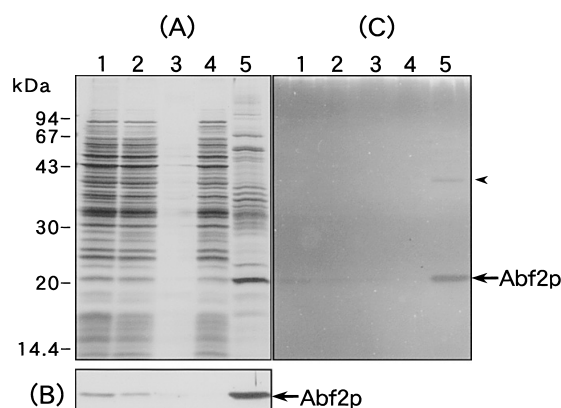


Fig. 1. Detection of Abf2p by immunoblotting and SDS-DNA PAGE.

(A) During the isolation of *S. cerevisiae* mt-nucleoids, each fraction was separated by SDS-DNA PAGE. The gel was silver-stained. Lane 1: mitochondrial fraction; lane 2: the supernatant after lysis of mitochondria by NP40 and subsequent centrifugation (16,000 $\times g$  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. (B) The same samples used in (A) were separated by SDS-PAGE and immunoblotted with anti-Abf2p antibody, which was raised against *S. cerevisiae* Abf2p. (C) The same samples used in (A) were separated by SDS-DNA PAGE. The gel was washed three times with TE buffer, then incubated overnight in TE buffer supplemented with 10 mM MgCl<sub>2</sub> and 7 mM 2-mercaptoethanol. Finally, the gel was stained with ethidium bromide for 30 min. The positions of Abf2p and 38-kDa protein were indicated by an arrow and an arrowhead, respectively.

## Results

### Detection of Abf2p by immunoblotting and SDS-DNA PAGE

During the mt-nucleoid isolation, each fraction was analyzed by SDS-DNA PAGE using 10  $\mu$ g/ml native DNA. Each fraction was then silver-stained (Fig. 1A). The mt-nucleoid fraction contained a subset of proteins whose composition was different from those of the mitochondria. Immunoblotting with anti-Abf2p antibody demonstrated that the 20-kDa protein, Abf2p, was highly concentrated in the mt-nucleoid fraction (Fig. 1B).

The SDS-DNA gel was washed with TE buffer to remove SDS, then was incubated overnight in TE buffer supplemented with 10 mM MgCl<sub>2</sub> and 7 mM 2-mercaptoethanol. The gel was then stained with ethidium bromide. As shown in Fig. 1C, among a number of mitochondrial or mt-nucleoid proteins, a 20-kDa band corresponding to Abf2p was selectively detected on the gel as a dark band. Beside the 20-kDa band, a band of 38 kDa was faintly detectable in the mt-nucleoid fraction. The Abf2p was released from the mt-nucleoids by digestion with DNase I. Subsequently, Abf2p, because of its high affinity for DNA, was separated by DNA-cellulose chromatography and analyzed by SDS-DNA PAGE (Fig. 2). In this trial, Abf2p was detected by both silver-staining (Fig. 2, lane 1) and ethidium bromide staining (Fig. 2, lane 2).

Next, the conditions for detecting Abf2p were examined (Fig. 3). Abf2p was detected after the gels were washed three times, incubated for 1 h in the TE buffer in the presence of 10 mM MgCl<sub>2</sub> and 7 mM 2-mercaptoethanol, and then stained for 30 min with ethidium bromide (Fig. 3, lane 2). The 38-kDa band did not appear soon after the incubation of the gel began, but

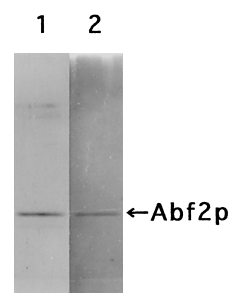


Fig. 2. Detection of purified Abf2p by SDS-DNA PAGE.

The Abf2p was purified from mt-nucleoids by chromatography on DNA-cellulose and loaded on an SDS-DNA gel. Lane 1: silver-staining; lane 2: ethidium bromide staining.

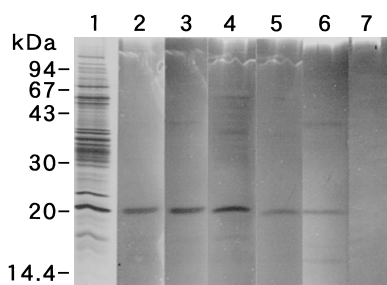


Fig. 3. SDS-DNA PAGE and SDS-RNA PAGE of *S. cerevisiae* mt-nucleoid proteins.

The same amount of *S. cerevisiae* mt-nucleoid proteins was loaded on each of five SDS-native DNA gels (lanes 1–5), an SDS-denatured DNA gel (lane 6), and an SDS-RNA gel (lane 7). Lane 1: silver-staining; lanes 2–7: ethidium bromide staining. The gel was incubated in TE buffer supplemented with 10 mM  $MgCl_2$  and 7 mM 2-mercaptoethanol for 1 h (lane 2) or overnight (lanes 3, 6 and 7). The gel was incubated in TE buffer supplemented with 7 mM 2-mercaptoethanol for 1 h (lane 4) or overnight (lane 5).

rather appeared faintly after overnight incubation of the gels (Fig. 3, lane 3). Abf2p was also detected in the absence of  $MgCl_2$ , although many minor bands other than Abf2p appeared faintly on the gels. The addition of 10 mM  $MgCl_2$  did not increase the sensitivity for detection of Abf2p (Fig. 3, lanes 2 and 4). In the absence of  $MgCl_2$ , the 38-kDa band did not appear in the incubation buffer after overnight incubation (Fig. 3, lane 5). Abf2p was similarly detected on SDS-DNA gels that included 10  $\mu$ g/ml denatured DNA (Fig. 3, lane 6), but was not detected on SDS-RNA gels that included 10  $\mu$ g/ml RNA (Fig. 3, lane 7). The 38-kDa band was more apparent on SDS-denatured DNA gels than on SDS-native DNA gels. The addition of BSA to the gels somewhat improved the detection of the 38-kDa band, but had no effect on the detection of Abf2p (data not shown). These results indicated that the Abf2p of *S. cerevisiae* mt-nucleoids was selectively detectable on SDS-DNA gels.

#### SDS-DNA PAGE of mt-nucleoid proteins from other yeasts

In order to investigate whether the Abf2p-like protein was commonly associated with the mt-nucleoids of other yeasts, mt-nucleoids were isolated from *P. jadinii* and *W. mrakii*, and the mt-nucleoid proteins were analyzed by immunoblotting with anti-Abf2p antibody. However, no proteins of either mt-nucleoid were detected by the antibody (data not shown). Using DNA-

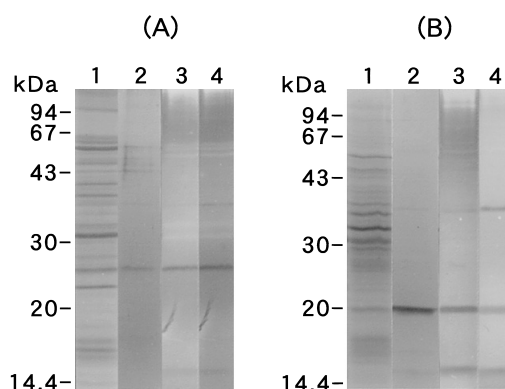


Fig. 4. SDS-PAGE and SDS-DNA PAGE of mt-nucleoid proteins from *Picha jadinii* and *Williopsis mrakii*.

Mt-nucleoid proteins of *P. jadinii* (A) and *W. mrakii* (B) were loaded on an SDS gel (lane 1) and on an SDS-DNA gel (lanes 3 and 4). DNA-binding proteins that had been separated from mt-nucleoids by DNA-cellulose chromatography were loaded on an SDS gel (lane 2). Lanes 1 and 2: silver-staining; lane 3: ethidium bromide staining after overnight incubation; lane 4: ethidium bromide staining after 3-day incubation.

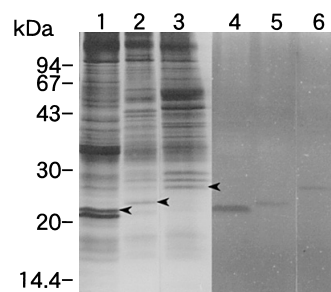


Fig. 5. SDS-DNA PAGE of mt-nucleoids from *Williopsis saturnus*, *Pichia anomala* and *Pichia petersonii*.

Lanes 1–3: silver-staining; lanes 4–6: ethidium bromide staining. Lanes 1 and 4: *W. saturnus*; lanes 2 and 5: *P. anomala*; lanes 3 and 6: *P. petersonii*. The bands that correspond with those detected by ethidium bromide staining are indicated by arrowheads in the left gel.

cellulose chromatography, a single protein of 26 kDa or 20 kDa was separated from each mt-nucleoid of *P. jadinii* or *W. mrakii*, respectively (Fig. 4, A and B, lanes 1 and 2). These two proteins were most intensely detected on SDS-DNA gels (Fig. 4, A and B, lanes 3 and 4). In both yeasts, a 38-kDa band grew increasingly thicker as the incubation time lengthened.

Mt-nucleoid was prepared from three other yeasts, *W. saturnus*, *P. anomala*, and *P. petersonii*, and the mt-nucleoid proteins were analyzed by SDS-DNA PAGE (Fig. 5). Only single bands of 21 kDa, 22 kDa and 24 kDa were detected among the mt-nucleoid pro-

teins of *W. saturnus*, *P. anomala*, and *P. petersonii*, respectively.

## Discussion

In this study, we demonstrated that SDS-DNA PAGE is a simple and effective method for detecting *S. cerevisiae* Abf2p. A single gel can be used for the initial ethidium bromide staining. Then, after being washed with distilled water, the same gel can be used for silver staining. The sensitivity of SDS-DNA PAGE and its specificity for detecting Abf2p were comparable to those of immunoblotting. In addition to Abf2p, SDS-DNA PAGE detected a 38-kDa protein as a faint band. This band may correspond to the mitochondrial nuclease, NUC1, according to an estimate made from the molecular mass and the requirements of the  $Mg^{2+}$  ion (Dake et al., 1988). 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. Although these researchers were not able to detect NUC1 activity by SDS-DNA PAGE, our results suggest that a very small fraction of NUC1 activity may be restored on SDS-DNA gels. The 38-kDa bands, which became intense during the incubation period, were also detected in *P. jadinii* and *W. mrakii* mt-nucleoids. Recently, a 32-kDa endonuclease was partially purified from the fission yeast *Schizosaccharomyces pombe* (Ikeda et al., 1996). The NUC1 nuclease may be commonly present in the mitochondria of yeasts. Other than Abf2p and the 38-kDa protein, several 15–16-kDa bands were faintly visible (Fig. 3, lanes 2–6). Similarly, a single 15-kDa band was discerned in mt-nucleoids of both *P. jadinii* and *W. mrakii* (Fig. 4, A and B, lanes 3 and 4). These bands may represent other DNA-binding proteins that participate in mt-nucleoid formation.

The results of SDS-DNA PAGE when applied to mt-nucleoids from other yeasts suggest that this method may be useful for detecting Abf2p-like proteins in the mitochondria of other yeasts, although the proteins that were detected by SDS-DNA PAGE have not been proven to be Abf2p. Abf2p has two HMG domains in the central region of its amino acid sequence. These domains are conserved in HMG proteins from various organisms (Diffley and Stillman, 1991). It is possible, however, that the franking N-terminal or C-terminal sequences may vary significantly among yeasts. Previously, we suggested that a 16-kDa protein may be a

candidate for Abf2p homolog in *P. jadinii* (Miyakawa et al., 1996). However, in the present study, the 26-kDa protein showed the highest affinity for native DNA in DNA-cellulose chromatography and SDS-DNA PAGE. To conclude definitively that the proteins that are detected in the mt-nucleoid proteins of various yeasts by SDS-DNA PAGE are Abf2p, the amino acid sequences of those proteins must be determined.

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