386–393 Nucleic Acids Research, 1996, Vol. 24, No. 2

Analysis of mitochondrial DNA nucleoids in wild-type and a mutant strain of *Saccharomyces cerevisiae* that lacks the mitochondrial HMG box protein Abf2p

Scott M. Newman, Olga Zelenaya-Troitskaya, Philip S. Perlman and Ronald A. Butow*

Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235, USA

Received July 6, 1995; Revised and Accepted November 21, 1995

ABSTRACT

DNA–protein complexes (nucleoids) are believed to be the segregating unit of mitochondrial DNA (mtDNA) in *Saccharomyces cerevisiae*. A mitochondrial HMG box protein, Abf2p, is needed for maintenance of mtDNA in cells grown on rich dextrose medium, but is dispensible in glycerol grown cells. As visualized by 4′,6′-diamino-2-phenylindole staining, mtDNA nucleoids in mutant cells lacking Abf2p (Δ*abf2*) are diffuse compared with those in wild-type cells. We have isolated mtDNA nucleoids and characterized two mtDNA–protein complexes, termed NCLDp-2 and NCLDs-2, containing distinct but overlapping sets of polypeptides. This protocol yields similar nucleoid complexes from the Δ*abf2* mutant, although several proteins appear lacking from NCLDs-2. Segments of mtDNA detected with probes to *COXII*, *VAR1* and ori5 sequences are equally sensitive to DNase I digestion in NCLDs-2 and NCLDp-2 from wild-type cells and from the Δ*abf2* mutant. However, *COXII* and *VAR1* sequences are 4- to 5-fold more sensitive to DNase I digestion of mtDNA in toluene-permeabilized mitochondria from the Δ*abf2* mutant than from wild-type cells, but no difference in DNase I sensitivity was detected with the ori5 probe. These results provide a first indication that Abf2p influences differential organization of mtDNA sequences.

INTRODUCTION

In situ observations of mitochondrial DNA (mtDNA) in a variety of organisms reveal that mtDNA is highly organized within the organelle. Electron microscopic analysis of animal mtDNA often shows clumped or thickened fibers (1) in two to six spatially distinct centers, called nucleoids (2), by analogy with the organized DNA structures in bacteria (3,4). Similar observations have been made from serial sections of stationary phase cells of the yeast *Saccharomyces cerevisiae*: bundles of filaments are distributed among one to 20 discrete areas within mitochondria (5). Examination of intact yeast cells by fluorescence microscopy with the DNA binding dye 4′,6′-diamidino-2-phenylindole (DAPI) indicates that mtDNA exists as small, discrete spots (called nucleoids or chondriolites) in mitochondria, usually at the periphery of the cell (5,6). These spots, each containing an estimated two to eight mtDNA molecules (5), are characteristic of the mtDNA staining pattern in vegetative cells. During meiosis and sporulation the number of spots decreases and mtDNA is reorganized into a branched network surrounding the nucleus (5).

Wild-type haploid yeast strains have been estimated to contain 25–50 mtDNA molecules that are stably maintained during mitotic growth. In sexual crosses, mtDNA from each parent is faithfully transmitted to the progeny and segregates rapidly to yield homoplasmic cells (7). It is generally believed that the nucleoid is the unit of mtDNA segregation (8). Recently a mutant of the *MGT1* gene, which is defective in resolving yeast mtDNA recombination junctions (10), was found to have alterations in the number of mtDNA nucleoids and in the pattern of mtDNA transmission (9–11).

MtDNA nucleoids, which have been isolated and characterized from several organisms (12,13), are isolated from sucrose gradients as rapidly sedimenting complexes. Based on electron and fluorescence microscopy, these complexes appear to retain their morphological structure *in vitro* (12,14). Fluorescence intensity analysis of isolated nucleoids from yeast suggests that they contain the same number of mtDNA molecules as *in vivo* estimates (14). Proteins were shown to be required to maintain the structural integrity of isolated yeast nucleoids and only a small subset of total mitochondrial protein is recovered in isolated nucleoids (14).

One yeast mtDNA binding protein that functions in mtDNA stability is a 20 kDa basic protein called Abf2p. This protein, initially called HM, was first purified from isolated mitochondria by DNA–cellulose chromatography (15). Abf2p was later identified in yeast extracts based on its interaction with origins of nuclear DNA replication (16). Abf2p is a relatively abundant mitochondrial protein, which, based on DNA binding assays, could bind to every ~30 bp of mtDNA (17). Abf2p is a member of the family of chromosomal non-histone high mobility group (HMG) proteins (18) and contains two HMG box domains (16). A similar HMG box protein (mtTFA) is also present in animal cell mitochondria (19,20). Null mutations of the *ABF2* gene lead to ρ^+ mtDNA instability in yeast cells grown on dextrose medium, where respiration is dispensable (16,21). However, mutant cells

* To whom correspondence should be addressed

lacking Abf2p grow on glycerol medium (where respiration is obligate), indicating that the protein is not essential for mtDNA replication, expression or transmission. At least one HMG box domain of Abf2p is required for maintenance of ρ^+ mtDNA (22).

As a class these proteins can bend and wrap DNA and, given the other known properties of HMG box proteins (23), are likely to function in DNA packaging. DNA packaging is probably an important function of Abf2p, since the *Escherichia coli* DNA packaging protein HU, which is not an HMG box protein, when expressed and targeted to mitochondria can partially restore ρ^+ mtDNA stability in yeast cells lacking Abf2p (21). In addition, HU and HMG box proteins are functionally interchangeable for a variety of DNA transactions (24).

As an initial approach to characterizing the role of Abf2p in mtDNA organization, we have examined mtDNA in a wild-type (*ABF2*) strain and an *abf2* null mutant (Δ*abf2*) by microscopic and biochemical approaches. We find that Abf2p affects the *in vivo* morphology of mtDNA nucleoids as seen by DAPI staining. The absence of the protein in Δ*abf2* cells was found to have little effect on the sedimentation properties or nuclease sensitivity of isolated nucleoids. In contrast, using toluene-permeabilized mitochondria from Δ*abf2* cells some mtDNA sequences, but not all, are ~4-fold more sensitive to DNase I compared with the wild-type. These experiments indicate a role for Abf2p in the differential organization of mtDNA sequences.

MATERIALS AND METHODS

Strains and growth conditions

The wild-type yeast strain used is 14WW (MAT**a** *ade2 trp1 leu2 ura3-52 cit1*::*LEU2*). The *ABF2* gene of strain 14WW was disrupted by transformation with a 2.0 kb *Eco*RI fragment isolated from plasmid pAM1A::TRP1 (16). These strains contain ρ^+ mtDNA from strain D273-10B (25). ρ° derivatives were generated by passage through YPD medium containing 10 μg/ml ethidium bromide. ρ^+ and ρ° strains were grown on YPG and YPD respectively, at 30°C as previously described (26), except that 2% Bacto-peptone was used.

Visualization of DAPI stained yeast cells

Cells were grown overnight in YPD or YPG medium and stained with DAPI as previously described (26,27).

Isolation of mitochondria

Mitochondria were isolated from sphereoplasts from 9–18 l YPG cultures (OD_{600} 0.8–1.1). All procedures were carried out at 4°C except where noted. Cells were harvested, resuspended in ~200 ml 0.1 M Tris–HCl, 0.02 M EDTA, pH 9.3 and shaken with 5 μl/ml β-mercaptoethanol (14.4 M stock) for 15 min at 30°C. The cells were collected, washed once with SCE, pH 5.8 (0.6 M sorbitol, 0.3 M mannitol, 20 mM K_2HPO_4, 20 mM citric acid, 1 mM EDTA) and resuspended in 1 ml/g cells SCE, pH 5.8. Yeast lytic enzyme (+70 000 U/g; ICN Biomedicals) was added to 0.5 mg/ml and the cells treated at 30°C for 30 min, mixing gently every 10 min. Spheroplasts were washed twice with SCE, pH 7.0, containing 1 mM spermidine, 7 mM β-mercaptoethanol and 1 mM phenylmethyl sulfonyl fluoride (PMSF) (~20 ml/wash) and broken by vigorous shaking at 4°C with glass beads in the same buffer. Cellular debris and unlysed cells were removed by three 5 min centrifugations twice at 1600 *g* and once at 2500 *g*. Crude mitochondria were collected by centrifugation at 12 000 *g* for 20 min. These mitochondria were further purified by flotation (28), with the following changes: mitochondria were resuspended in Tricine buffer containing 80% sucrose (w/v) before being floated; after centrifugation mitochondria at the 45/55% sucrose interface were collected, diluted with 2 vol. ice-cold Tricine buffer and pelleted as above. These purified mitochondria were stored frozen at –80°C.

Isolation and characterization of mtDNA nucleoids

We modified a published isolation procedure for mtDNA nucleoids from yeast. Purified mitochondria were thawed on ice, resuspended in NE2 buffer (0.25 M sucrose, 20 mM Tris–HCl, pH 7.6, 2 mM EDTA, 7 mM β-mercaptoethanol) (14) and diluted with an equal volume of 0.5× NE2 buffer to a final concentration of 5–7 mg mitochondrial protein/ml. Spermidine (1.0 M) was added to a final concentration of 3 mM and mitochondria were lysed by adding 20% NP-40 to a final concentration of 0.5%. After 5 min on ice with gentle mixing the lysate was fractionated at 12 000 *g* for 20 min into supernatant (S) and pellet (P) fractions. The P fraction was resuspended as above.

Aliquots from both S and P fractions were layered on top of step gradients, initially comprised of 3.5 ml 20%/2.5 ml 40%/1.8 ml 60%/0.9 ml 70% sucrose, but later comprised of 4 ml 20%/2 ml 60%/1 ml 80% sucrose in gradient buffer (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 1 mM spermidine, 7 mM β-mercaptoethanol, 1 mM PMSF) in SW41 tubes and centrifuged at 111 000 *g* (30 000 r.p.m.) for 75 min. Gradients were fractionated and analyzed for distribution of mtDNA and protein. MtDNA peaks derived from the S and P fractions from an initial NP-40 extraction are hereafter referred to as NCLDs-1 and NCLDp-1 respectively. NCLDs-1 and NCLDp-1 nucleoid fractions banding at the 20/60% interface (see Fig. 1) were collected, diluted with 2 vol. ice-cold gradient buffer, treated with 0.5% NP-40 and centrifuged through a second (20/40/60/70% sucrose) step gradient (49 000 *g*, 3 h) to yield NCLDs-2 and NCLDp-2.

Biochemical analyses

The position of mtDNA in gradients was determined by dot blots using CsCl-purified ρ^+ mtDNA as probe. MtDNA in purified and lysed mitochondria was extracted with PCIA (phenol/chloroform/isoamyl alcohol, 25:24:1), precipitated, resuspended and the concentration determined using dot blots, with known concentrations of mtDNA, purified in a CsCl–bisbenzimide gradient (29) as a standard and quantitated by PhosphorImager analysis. Protein concentration was determined by the Bradford assay (BioRad), using bovine serum albumin as a standard. SDS–PAGE was performed according to Laemmli (30) using 12.5 or 15% gels or 7.5–17.5% gradient gels containing 29.2:0.8% acrylamide:bis-acrylamide. Some gels were stained with Coomassie blue. Others were electrotransferred to nitrocellulose and blocked with 5% non-fat milk in TBS (25 mM Tris, pH 7.6, 0.2 M NaCl, 0.02% KCl) for at least 1 h. Polyclonal antisera against porin (provided by G.Schatz) and monoclonal antibodies against CoxIIIp (Molecular Probes, Eugene, OR) were used at 1:10 000 and 1:1250 dilution respectively. Antisera against Abf2p were prepared in rabbits using protein derived from the cloned gene in a pT7-7 bacterial expression system and used at a 1:10 000 dilution.

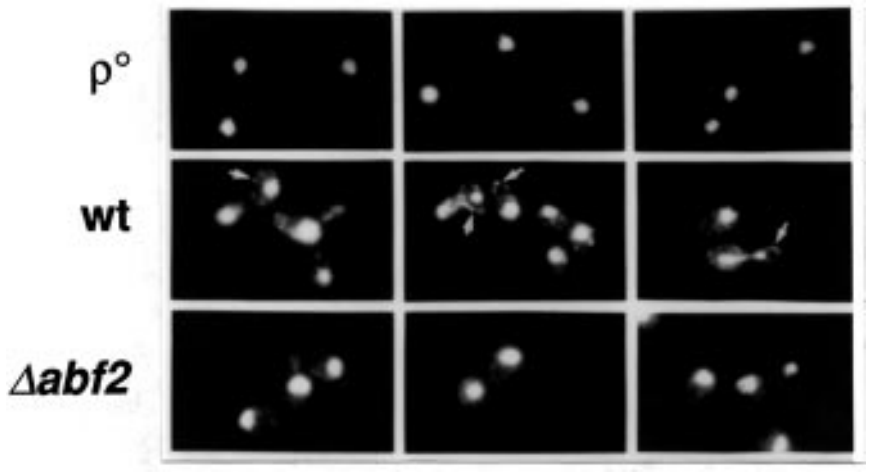

Figure 1. Yeast mtDNA nucleoids have an altered morphology in Δ*abf2* cells. Spheroplasts, prepared from wild-type and Δ*abf2* ρ^+ cells grown on YPG medium and a ρ° derivative of the wild-type grown on YPD medium, were stained with DAPI, examined by fluorescence microscopy and photographed. Representative fields are shown and mtDNA nucleoids in the wild-type (wt) are indicated by arrows.

Antisera against MAS70, cytochrome c, malate dehydrogenase and the ATPase complex were provided by Drs L.Pon, F.Sherman, L.Henn and M.Douglas respectively. All blots were incubated at room temperature in TBS and 5% non-fat milk with primary antisera (overnight) and secondary (goat anti-rabbit or goat anti-mouse) antisera (2–3 h). Blots were washed extensively with TBS after each incubation, prior to treatment with ECL reagents (Amersham) and exposure to film for 1–5 s.

Nuclease digestion of nucleoids and mitochondria

The concentrations of DNA in nucleoid fractions were determined by fluorimetry and by quantitating Southern blots of mtDNA. Nucleoid fractions and deproteinized mtDNA (PCIA-extracted nucleoid DNA) were treated in 50 mM Tris, pH 7.5, 10 mM $MnCl_2$, 20% sucrose at 30°C with 0.012 U/ml DNase I (Promega). Reactions were terminated by addition on ice of EDTA to 20 mM and treated with 1% SDS and 200 µg/ml proteinase K for at least 2 h at 37°C. Reactions were adjusted to 2.5 M ammonium acetate and 50 µg/ml yeast tRNA prior to PCIA extraction and ethanol precipitation. Samples were collected, dried, restricted with *Hae*III or *Hin*fI and subjected to electrophoresis through 0.8% agarose gels in TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2 mM EDTA). Gels were diffusion blotted to nylon membranes and hybridized in Rapid-Hyb buffer (Amersham) at 65°C to probes of total ρ^+ mtDNA or gel-purified restriction fragments [0.26 kb *Pvu*II fragment from pSB20 (31) for *COXII*, 1.0 kb *Hpa*II fragment for *VAR1* (32), 0.76 kb *Nde*I fragment from the hypersuppressive petite HS40 genome (33) for ori5] random primed (Boehringer Mannheim) with [^{32}P]dATP. Blots were washed according to the manufacturer's recommendations and quantified.

Flotation gradient-purified mitochondria (corresponding to 5–8 mg protein) were resuspended in 1 vol. NE2 buffer and 1 vol. 0.5× NE2 buffer to a final volume of ~1.5 ml and spermidine added to 3 mM. Toluene was added to 1% and mixed gently for 15 min at room temperature. Permeabilized mitochondria were collected in a microfuge tube, resuspended in NE2 buffer, treated with 0.2 U/ml DNase I and processed as described above. MtDNA in mitochondria not treated with toluene was completely resistant to DNase I and the mtDNA in toluene-treated mitochondria not treated with DNase I remained undegraded after 20 min at 30°C (data not shown).

RESULTS

Properties of mtDNA nucleoids in Δ*abf2* cells

As an initial test of whether the mtDNA binding protein Abf2p functions in mtDNA organization, we compared the morphology of mtDNA nucleoids in wild-type and Δ*abf2* cells using fluorescence microscopy. This technique has been employed frequently to determine the presence or absence of mtDNA in mutant yeast strains (6), but has only recently been used to examine the effects of specific mutations on staining of ρ^+ mtDNA (11). Wild-type (wt, *ABF2*) and mutant (Δ*abf2*) ρ^+ cells were grown on glycerol medium and then stained with DAPI. As shown in Figure 1, *ABF2* cells show typical mtDNA nucleoids: brightly staining, punctate cytoplasmic structures (indicated by arrows) often located around the periphery of the cell. As a control cytoplasmic staining is absent in a ρ° petite mutant (which lacks mtDNA) grown on 2% dextrose medium. In contrast to *ABF2* ρ^+ cells, nucleoids in ρ^+ Δ*abf2* cells appear diffuse and clearly different from the bright, punctate staining of wild-type mtDNA nucleoids. When grown on dextrose medium Δ*abf2* cells rapidly lose their mtDNA and lack any fluorescent cytoplasmic staining (16,25). The altered DAPI-staining pattern of ρ^+ mtDNA nucleoids in Δ*abf2* cells suggests that the absence of Abf2p affects the organization of ρ^+ mtDNA nucleoids.

Improved purification of mtDNA nucleoids

We next analyzed mtDNA organization *in vitro* by comparing the properties of mtDNA nucleoids isolated from wild-type and Δ*abf2* cells. Initial studies of yeast mtDNA nucleoids employed a published purification procedure (14), however, we found that only a minority fraction of mtDNA was recovered from the lysed organelles and that the nucleoid fractions were contaminated with mitochondrial membrane proteins (see below). Therefore, we modified that procedure to improve both the properties and yield of mtDNA nucleoids.

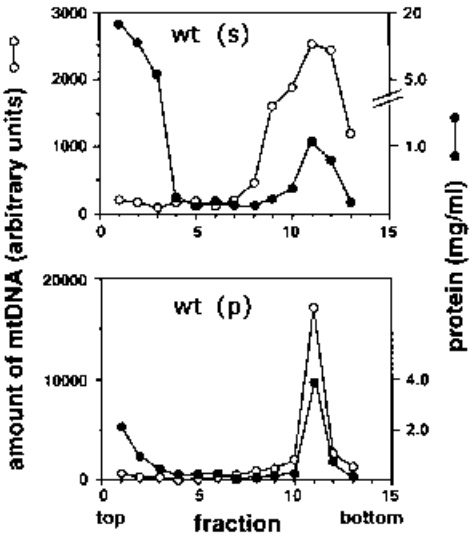

Figure 2. Sucrose gradient centrifugation of mtDNA nucleoids. Aliquots of the NP-40 supernatant (S) and pellet (P) fractions from wild-type cells were applied to a 20/40/60/70% sucrose step gradient and centrifuged at 111 000*g* for 75 min. The gradients were fractionated and aliquots of the fractions analyzed for mtDNA and protein.

As shown in Table 1, the NP-40 soluble fraction (S) contains ~30% of the starting mtDNA, while 70% sediments in the insoluble (P) fraction. The S fraction (as defined here) was used exclusively by Miyakawa *et al.* (14) in their characterization of yeast mtDNA nucleoids, thus it appears that a majority of the starting mtDNA was not examined by them. Figure 2 shows that mtDNA from both the S and P fractions sediments through the sucrose step gradient, banding in both cases at the 40/60% sucrose interface (corresponding to fractions 10 and 11). These mtDNA peaks derived from the S and P fractions from an initial NP-40 extraction are hereafter referred to as NCLDs-1 and NCLDp-1 respectively. We also consistently observe that more mtDNA is recovered in NCLDp-1 and mtDNA and protein sediment together as a much sharper peak than in NCLDs-1 fractions (Fig. 2).

Table 1. Purification of mtDNA nucleoids

Sample	mtDNA (mg)	(%)	Protein (mg)	(%)	Protein:DNA
Purified mitochondria	209	100	64 800	100	310
Supernatant	80	38	44 000	68	550
Pellet	170	81	10 500	16	62
NCLDs-1	61	29	1500	2.3	25
NCLDs-2	46	26	270	0.4	5.9
NCLDp-1	127	61	3500	5.4	28
NCLDp-2	104	55	1500	2.3	14

The results are from a typical isolation from 18 l YPG grown yeast strain 14WW. mtDNA concentration of PCIA extracted samples was determined by dot blots, using CsCl-purified mtDNA as probe and standard.

As summarized in Table 1, <5% of the total protein from the S fraction applied to the gradient co-sedimented with mtDNA in the NCLDs-1 peak. In contrast, 30–50% of the protein in the P fraction was recovered with mtDNA in the NCLDp-1 peak.

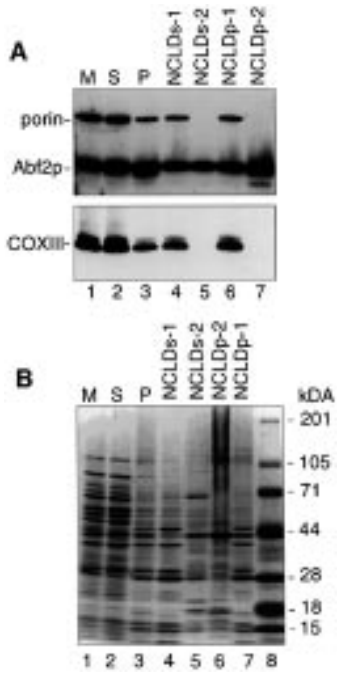

Figure 3. Analysis of proteins associated with mtDNA nucleoids. (**A**) Aliquots of 2 µg protein from purified mitochondria (M), S and P fractions (from NP-40 lysis) and NCLDs-1 and -2 and NCLDp-1 and -2 fractions were analyzed on 15% SDS–polyacrylamide gels for the presence of porin, Abf2p and CoxIIIp by Western blotting. (**B**) Aliquots of 20 µg protein from purified mitochondria (M), NP-40 S and P fractions and nucleoids (NCLDs-1 and -2 and NCLDp-1 and -2) were fractionated on a 7.5–17.5% SDS–polyacrylamide gradient gel and stained with Coomassie blue. Molecular weight markers (Bethesda Research Laboratories) appear in lane 8. Western blotting confirmed the 18 kDa band in NCLDs-2 and p-2 as Abf2p.

Western blot analysis was performed on the NCLDs-1 and NCLDp-1 peak fractions to determine the relative amounts of known mitochondrial proteins in these nucleoid fractions. High concentrations of Abf2p were found in both the NCLDs-1 and NCLDp-1 fractions (Fig. 3A, lanes 4 and 6), demonstrating that Abf2p co-sediments with mtDNA nucleoids. In addition, Abf2p was also abundant in the fractions at the top of the gradient following sedimentation of the NP-40 supernatant (S) fraction (data not shown), suggesting that either some Abf2p is stripped from the nucleoids following NP-40 treatment or that a pool of Abf2p (not associated with DNA) exists in the mitochondria.

Lanes 4 and 6 (Fig. 3A) also reveal the presence in both NCLDs-1 and NCLDp-1 of the inner and outer mitochondrial membrane proteins CoxIIIp and porin respectively. Conversely, the matrix enzyme malate dehydrogenase, the peripheral membrane ATPase holoenzyme, the outer membrane protein MAS70 and cytochrome c were preferentially recovered at the top of the gradients after sedimentation of the S and P fractions (not shown). These data indicate that although effective lysis of mitochondria and substantial solubilization of membranes was achieved, there is some contamination of nucleoids by proteins, such as CoxIIIp and porin, not likely to be associated with mtDNA.

To attempt removal of those contaminating proteins we re-extracted the NCLDs-1 and NCLDp-1 fractions with NP-40 and centrifuged that material through a second step gradient. The sedimentation profile of nucleoids following two NP-40 extractions (termed NCLDs-2 and NCLDp-2) is indistinguishable from the profile generated after a single NP-40 extraction shown in

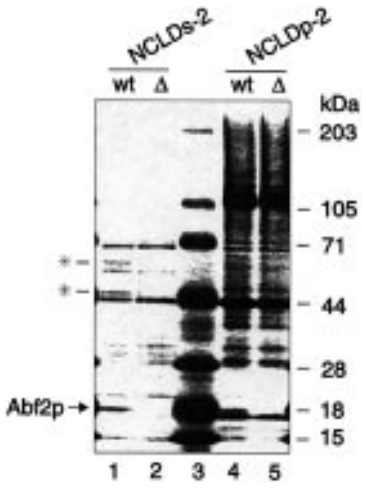

Figure 4. Proteins associated with mtDNA nucleoids from wild-type and Δ*abf2* cells. Aliquots of NCLDs-2 and NCLDp-2 from wild-type (wt) and Δ*abf2* cells (Δ) were applied to an SDS–polyacrylamide gradient gel and stained with Coomassie blue. The position of Abf2p is indicated by the arrow. Polypeptides absent or reduced in abundance in NCLDs-2 from Δ*abf2* cells are indicated by the asterisks (*) to the left of lane 1. Molecular weight markers are in lane 3.

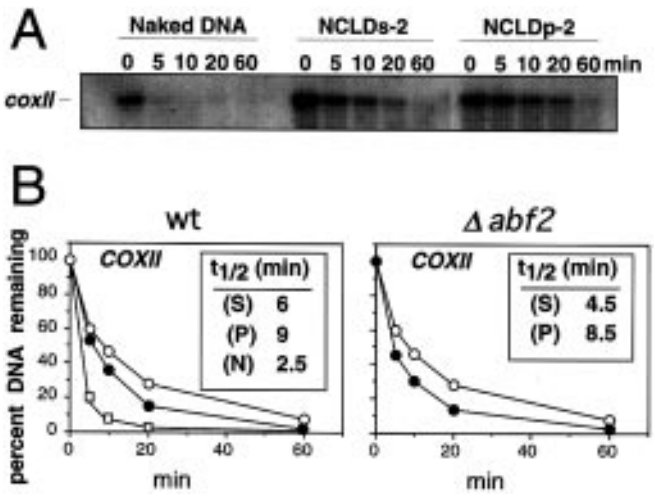

Figure 5. DNase I digestion of mtDNA in NCLDs-2 and NCLDp-2 nucleoids from wild-type and Δ*abf2* cells. (**A**) Approximately 2.0 µg deproteinized mtDNA (DP mtDNA), NCLDs-2 and NCLDp-2 were digested with DNase I for the times indicated and an aliquot extracted with PCIA, ethanol precipitated, cleaved with *Hae*III and fractionated by electrophoresis on a 0.8% gel. The blot was hybridized with a ^{32}P-labeled *COXII*-specific probe. (**B**) Comparison of the kinetics of DNase I digestion of the *COXII* mtDNA sequence in mtDNA nucleoids from wild-type and Δ*abf2* cells. The percent DNA remaining at each time point for DNase I-digested mtDNA in NCLDs-2 (S, ●) and in NCLDp-2 (P, ◑) fractions from wild-type (left panel) and Δ*abf2* cells (right panel) was determined. DNase I digestion of deproteinized mtDNA (N, ❑) is shown in the top left panel. The apparent half-times ($t_{½}$) of digestion, given in the inserts, represent the time in which half of the input DNA is digested.

Figure 2. After the second NP-40 extraction and centrifugation the recovery of mtDNA is ~90% and a substantial amount of protein (82 and 57% respectively) has been removed from the NCLDs-1 and NCLDp-1 nucleoids (Table 1). Importantly, among the proteins removed are porin and CoxIIIp, which are no longer detectable on the Western blots, while Abf2p is retained in the NCLDs-2 and NCLDp-2 fractions (Fig. 3A, lanes 5 and 7). Thus it is likely that the nucleoids examined by Miyakawa *et al.* (14), which were derived from a single NP-40 extraction, are contaminated with extraneous mitochondrial protein.

The profiles of polypeptides associated with mtDNA nucleoid fractions were examined by SDS–PAGE analysis and compared with starting mitochondria and the S and P fractions (Fig. 3B). In addition to porin and CoxIIIp (Fig. 3A), a number of polypeptides are depleted in NCLDs-2 and NCLDp-2 compared with NCLDs-1 and NCLDp-1. However, a small subset of mitochondrial proteins (see Table 1) are significantly enriched in NCLDs-2 and NCLDp-2. Among these is 18 kDa Abf2p, which is recovered in approximately equal amounts in both NCLDs-2 and NCLDp-2 (Fig. 3A). Prominent polypeptides found preferentially in NCLDs-2 include 70, 28, 21 and 15 kDa species, while polypeptides of 110 and 17 kDa and a doublet at 44 kDa are enriched in NCLDp-2. Therefore, NCLDs-2 and NCLDp-2 fractions contain a high concentration of Abf2p and distinct but overlapping sets of polypeptides, most of which are enriched relative to NCLDs-1 and NCLDp-1. In addition, the NCLDs-2 and NCLDp-2 nucleoid preparations are free of mitochondrial membrane proteins and represent the starting material for biochemical characterization of mtDNA nucleoids in Δ*abf2* cells.

Biochemical properties of mtDNA nucleoids from Δ*abf2* cells

To determine if the *in vivo* morphological alterations of mtDNA nucleoid organization in Δ*abf2* cells (Fig. 1) are reflected in the properties of the isolated nucleoids, NCLDs-2 and NCLDp-2 fractions from wild-type and mutant cells were compared. Sedimentation properties of nucleoids from Δ*abf2* cells (data not shown) were indistinguishable from the wild-type (Fig. 2), indicating that Abf2p is not critical in maintaining the nucleoid as a rapidly sedimenting mtDNA–protein complex. However, a comparison of the protein profiles of NCLDs-2 and NCLDp-2 from wild-type and Δ*abf2* cells reveals some differences in the polypeptides associated with mtDNA nucleoids (Fig. 4). As expected, Abf2p is absent from both nucleoid fractions from Δ*abf2* cells (Fig. 4, lanes 2 and 5). In addition, 60 and 46 kDa polypeptides of comparable abundance to Abf2p are absent or greatly reduced in NCLDs-2 from Δ*abf2* cells relative to the wild-type (Fig. 5, lane 2). Some minor polypeptides are also reduced in abundance in NCLDs-2 fractions from Δ*abf2* cells. While these differences were consistently observed in independent NCLDs-2 nucleoid preparations, no reproducible differences between wild-type and mutant were observed in protein profiles of NCLDp-2 fractions.

DNase I sensitivity of mtDNA nucleoids isolated from wild-type and Δ*abf2* cells

The sensitivity of protein–DNA complexes to nuclease digestion has proven useful for examining DNA packaging and organization (34). Therefore, to further characterize the organization of mtDNA in nucleoids isolated from wild-type and Δ*abf2* cells we analyzed their sensitivity to DNase I digestion. First we determined if mtDNA in isolated NCLDs-2 and NCLDp-2 fractions is more nuclease resistant than mtDNA not complexed with proteins. This analysis should measure the maximum difference that could be observed between nucleoids isolated from wild-type and Δ*abf2* cells. Deproteinized mtDNA, NCLDs-2 and NCLDp-2 were digested with DNase I, treated with SDS and proteinase K, PCIA extracted and precipitated. Samples, either undigested or digested with restriction enzymes, were analyzed by Southern hybridization

using radiolabeled mtDNA restriction fragments as probes. A representative Southern blot of sequences hybridizing to the *COXII* gene (encoding subunit 2 of cytochrome c oxidase) following DNase I and *Hae*III digestion is presented in Figure 5A and quantified results are summarized in Figure 5B.

In the representative experiment shown in Figure 5, the $t_{½}$ of DNase I digestion of *COXII* sequences in deproteinized mtDNA is 2.5 min, compared with $t_{½}$ values of 6 and 9 min for those same sequences in NCLDs-2 and NCLDp-2 respectively. Analysis of three independent preparations of deproteinized mtDNA ($t_{½}$ 2.7 ± 0.3 min), NCLDs-2 ($t_{½}$ 5.5 ± 1.3 min) and NCLDp-2 ($t_{½}$ 7.0 ± 2.3 min) from the wild-type reveal that *COXII* sequences in both nucleoid fractions are 2- to 3-fold more resistant to DNase I digestion than in deproteinized mtDNA. Thus this approach measures the protection of mtDNA by one or more of the proteins present in the isolated nucleoid fractions and represents a level of organization not found in deproteinized mtDNA.

These data show no statistically significant difference in the DNase I sensitivity of *COXII* sequences in the nucleoid NCLDs-2 and NCLDp-2 fractions (Fig. 5A). The DNase I sensitivity of two other mtDNA sequences was also examined: *VAR1* is an A+T-rich gene (unlike *COXII*) that encodes a ribosomal protein (35); ori5, which is also very A+T-rich and is a putative origin of mtDNA replication (36). These loci were chosen to examine any bias that DNase I might have for A+T-rich sequences and to determine if DNase I sensitivity differs between gene and non-coding sequences. As with *COXII*, no statistically significant difference was detected in the DNase I sensitivity ($t_{½}$) of the *VAR1* (7.0 ± 0.5 min and 11 ± 1.0 min) and ori5 (6.5 ± 1.3 min and 8.5 ± 4.0 min) sequences in NCLDs-2 and NCLDp-2 fractions respectively (data not shown). Therefore, no differential nuclease protection was observed among these sequences between NCLDs-2 and NCLDp-2 fractions.

Next we compared the DNase I sensitivity of nucleoids isolated from Δ*abf2* cells with those isolated from wild-type cells, but no significant differences were found. As with wild-type nucleoids, similar DNase I sensitivities ($t_{½}$) were observed for NCLDs-2 (4.0 ± 0.5 min, 3.8 ± 0.3 min and 4.3 ± 0.8 min) and NCLDp-2 (5.2 ± 2.9 min, 5.0 ± 1.0 min and 5.3 ± 0.6 min) from Δ*abf2* cells for all three sequences (*COXII*, *VAR1* and ori5 respectively) analyzed. Also, no statistically significant differences were detected in the DNase I sensitivity among these three sequences. These data indicate that despite the presence of high concentrations of Abf2p in wild-type mtDNA nucleoids, this protein is not a major determinant of nuclease resistance in isolated nucleoids. Therefore, major differences in mtDNA organization that might exist between wild-type and Δ*abf2* cells may not be retained in isolated nucleoids. Alternatively, since deproteinized mtDNA is at most only 2- to 3-fold more DNase I sensitive than mtDNA nucleoids from wild-type cells (Fig. 5), the maximum difference in DNase I sensitivity of isolated nucleoids from wild-type and Δ*abf2* cells must certainly be less. Therefore, small differences in DNase I sensitivity of nucleoids isolated from wild-type and Δ*abf2* cells may not be detectable by this *in vitro* assay.

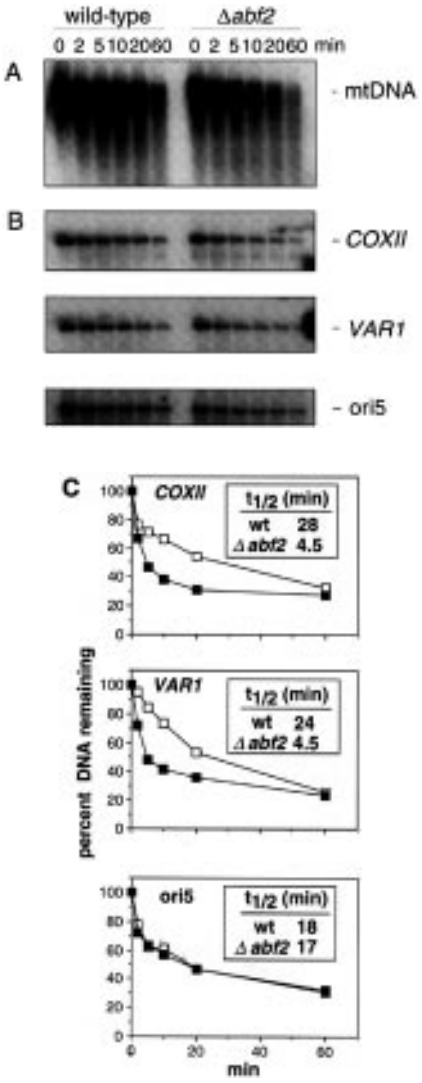

Figure 6. *In organello* DNase I digestion of mtDNA from wild-type and Δ*abf2* cells. Purified mitochondria from wild-type and Δ*abf2* cells were permeabilized by treatment with 1% toluene and incubated with DNase I for 0–60 min prior to Southern blot analysis as follows. Aliqouts from each time point were (**A**) not restricted and probed with CsCl-purified total mtDNA or (**B**) cleaved with *Hae*III or *Hin*fI and hybridized with probes for *COXII*, *VAR1* and ori5 mtDNA sequences as shown. (**C**) PhosphorImager quantitation was used to analyze data shown in (B) and the percent hybridizing DNA remaining at each time point was determined for wild-type (wt, ❑) and Δ*abf2* (Δ*abf2*, ■) strains.

DNase I digestion of mtDNA in isolated mitochondria

In an effort to determine potential differences in mtDNA organization between wild-type and Δ*abf2* cells we next analyzed DNase I sensitivity of mtDNA *in organello*. The use of isolated mitochondria for the analysis of transcription initiation and replication (37,38) has proven successful. Gradient-purified mitochondria were permeabilized with toluene, treated with DNase I and processed as described above. Preliminary experiments used total ρ^+ mtDNA as a probe to determine the effect of DNase I treatment on mtDNA in permeabilized mitochondria from wild-type and Δ*abf2* cells (Fig. 6A). Undigested DNA from isolated mitochondria (Fig. 6A) migrates as a >45 kb species on a 0.8% agarose gel, similar to DNA from isolated nucleoids (not shown). mtDNA in toluene-permeabilized mitochondria from wild-type cells is clearly sensitive to DNase I, although a 10-fold higher concentration of enzyme is required to achieve a comparable extent of digestion to that of isolated nucleoids. MtDNA in mitochondria from Δ*abf2* cells appears more sensitive (~4-fold, data not shown) to DNase I than mtDNA from wild-type cells (Fig. 6A).

Next the kinetics of digestion of specific mtDNA sequences were measured. In these experiments mtDNA was extracted from DNase I-treated mitochondria, digested with a restriction enzyme and analyzed as above for nucleoids. Figure 6B shows Southern blots of restricted mtDNA hybridized with the same *COXII, VAR1* and ori5 probes used above; quantification of those blots is shown graphically in Figure 6C. For all three mtDNA sequences 25–35% of the DNA is resistant to DNase I after a 60 min digestion. This could reflect a population of mitochondria whose mtDNA is not accessible to DNase I, perhaps because of inefficient permeabilization by toluene. Nonetheless, in this experiment the $t_{1/2}$ values for DNase I digestion of *COXII* and *VAR1* sequences are 28 and 24 min respectively in the wild-type and 4.5 min for both *COXII* and *VAR1* sequences in Δ*abf2* cells. Analysis of three independent preparations of toulene-permeabilized mitochondria shows that the $t_{1/2}$ of *COXII* is 17.5 ± 9.6 and 4 ± 2.3 min in wild-type and Δ*abf2* cells respectively. Two independent assays for sensitivity of *VAR1* sequences to DNase I digestion in permeabilized mitochondria resulted in mean $t_{1/2}$ values of 21 and 4 min for wild-type and Δ*abf2* cells respectively. Therefore, *COXII* and *VAR1* sequences in mtDNA of Δ*abf2* cells are ~4- to 5-fold more sensitive to DNase I digestion than in the wild-type. Furthermore, the 4- to 5-fold greater DNase I sensitivity of *COXII* and *VAR1* sequences in mtDNA of Δ*abf2* cells (Fig. 6B and C) is similar to the difference observed between the wild-type and the Δ*abf2* mutant using total mtDNA as a probe (Fig. 6A). These data indicate that Abf2p has a substantial role in conferring DNase I resistance on *COXII* and *VAR1* sequences under these *in organello* assay conditions and that those sequences are likely to be representative of most of the genome with resepect to Abf2p-dependent DNase I resistance.

To determine the DNase I sensitivity of a non-coding region of mtDNA we next hybridized these samples with a probe specific for ori5 (Fig. 6B and C, bottom). The $t_{1/2}$ value for ori5 sequences in mitochondria from the wild-type strain was 18 min, about the same value obtained for *COXII* and *VAR1* sequences. It was striking, however, that ori5 sequences were not more sensitive to DNase I digestion in mitochondria from the Δ*abf2* mutant; there the $t_{1/2}$ for ori5 was 17 min (while the $t_{1/2}$ for both *COXII* and *VAR1* was ~4 min). These data indicate that Abf2p affects *COXII* and *VAR1* sequence organization but not that of ori5. Taken together these data show that portions of mtDNA are organized differently in Δ*abf2* cells than in wild-type cells, providing a biochemical correlation with the *in situ* morphological data (Fig. 1).

DISCUSSION

We have examined the organization of ρ^+ mtDNA in a wild-type and an *abf2* null mutant (Δ*abf2*) strain of yeast. Our experiments show that in the Δ*abf2* strain mtDNA nucleoids, visualized *in situ* by DAPI staining, are morphologically less distinct than nucleoids in wild-type cells: they appear more diffuse and lack the characteristic bright, punctate staining of wild-type nucleoids (Fig. 1). These data indicate that Abf2p has a role in mtDNA organization, in addition to its previously ascribed function in mtDNA stability (16).

To compare mtDNA nucleoids from wild-type and Δ*abf2* cells *in vitro* we modified the published nucleoid isolation procedure (14) in several important ways. First, mitochondrial fractions were prepared by flotation gradient centrifugation: mitochondria generated solely by differential centrifugation contain ~10 times more nuclear DNA contamination than do flotation-purified mitochondria. Second, we found that the soluble NP-40 mitochondrial extract, used by others to prepare mtDNA nucleoids (14), contains only a fraction of the total mtDNA: we routinely recover ~70% of the starting mtDNA in the NP-40 *insoluble* fraction and that material is readily purified as a DNA–protein complex, comparable with the mtDNA nucleoids present in the NP-40 soluble extract. Third, a second NP-40 extraction quantitatively eliminates contamination by inner and outer mitochondrial membrane proteins, without any significant loss of Abf2p or mtDNA from the final nucleoid preparations.

At present the biochemical significance of the partitioning of mtDNA nucleoids between the S and P fractions is not known. NCLDp-2 and NCLDs-2 fractions may reflect mtDNA nucleoids present in the organelle as membrane-bound and -free protein–DNA complexes respectively. Membrane association of bacterial nucleoids has been recognized for some time (3,4). Additionally, studies of HeLa cell mtDNA have suggested a specific membrane attachment at the D-loop origin of replication and the presence of a unique protein structure bound to that region of the mitochondrial genome (39). While proteins present in the NCLDp-2 and NCLDs-2 fractions are similar, one notable difference is the presence of an abundant ~110 kDa species in NCLDp-2 that is essentially absent from NCLDs nucleoids (Figs 3 and 4). It is tempting to speculate that this protein could be a component originating from membranes and be involved in nucleoid membrane attachment. Studies are currently under way to characterize this 110 kDa species, as well as other proteins in these fractions. To date few proteins associated with mtDNA nucleoids have been identified. In the present work Abf2p has been shown to be present in both NCLDs-2 and NCLDp-2 nucleoid preparations. Miyakawa *et al.* (40) identified a 48 kDa polypeptide that is loosely associated with yeast mitochondrial nucleoids and a series of small (15–21 kDa) proteins that were chemically cross-linked to kinetoplast DNA has been described (41), however, in neither case has the function of these proteins been determined.

Given the difference in morphology between DAPI stained mtDNA nucleoids in wild-type and Δ*abf2* cells and the known function of HMG box proteins in DNA organization, we attempted to detect biochemical differences between mtDNA nucleoids of wild-type and Δ*abf2* cells. The partitioning of mtDNA between the S and P fractions and the sedimentation profile difference of NCLDs-2 and NCLDp-2 fractions that we observed for mtDNA nucleoids isolated from wild-type were similar to those observed for mtDNA nucleoids isolated from Δ*abf2* cells. Therefore, Abf2p cannot be a major protein needed for maintaining the presence of fast sedimenting mtDNA–protein complexes in yeast. However, we found several polypeptides in NCLDs-2 fractions of the wild-type strain that were significantly depleted or absent from this fraction from Δ*abf2* cells (Fig. 5). This observation is consistent with studies in both prokaryotes (42–44) and eukaryotes (45,46), indicating that some DNA packaging proteins influence the DNA binding of other proteins.

We have measured DNase I sensitivity of mtDNA nucleoids both *in vitro* (Fig. 5) and *in organello* (Fig. 6). Clearly, the presence of proteins associated with mtDNA in both the NCLDs-2 and NCLDp-2 nucleoid fractions contributes to their 2- to 3-fold increased DNase I resistance compared with deproteinized mtDNA (Fig. 5). However, Abf2p alone cannot be responsible for this increased resistance, since purified nucleoids from Δ*abf2* cells are still 2- to 3-fold more resistant than deproteinized mtDNA. No statistically significant difference was observed in

the nuclease sensitivity between NCLDs-2 and NCLDp-2 or between nucleoids from wild-type and Δ*abf2* cells.

While these nuclease digestion experiments of mtDNA in nucleoid fractions did not provide a correlation with the morphological differences between nucleoids observed in wild-type and Δ*abf2* cells, the *in organello* DNase I digestion experiments revealed possible organizational differences. In this case both *COXII* and *VAR1* sequences were 4- to 5-fold more sensitive to DNase I digestion in mitochondria from Δ*abf2* cells than from the wild-type. It is quite conceivable, therefore, that Abf2p-dependent features of mtDNA nucleoid organization are lost upon extraction and isolation of these structures. However, we believe that differences in nuclease sensitivity *in organello* between the wild-type and mutant is more representative of the actual Δ*abf2* phenotype than the *in vitro* digestion pattern. While purified nucleoids will be valuable in characterizing specific proteins associated with mtDNA, the *in organello* analysis opens up additional possibilities for the experimental evaluation of the organization of mtDNA. Recent studies (37) indicate differences in the *in vitro* and *in organello* binding properties of the human Abf2p homolog, mtTFA, to mtDNA.

The finding that the *in organello* DNase I sensitivity of ori5 sequences is the same in wild-type and in Δ*abf2* cells suggests fundamental differences in the organization of those sequences relative to the *COXII* and *VAR1* genes. *In vitro* Abf2p binding to ori sequences is phased, binding every 25–30 bp and separated by short stretches of poly(dA) or poly(dT) (17). Although both ori5 (36,47) and *VAR1* (35) are ~90% A+T sequences, the increased density of poly(dA) or poly(dT) sequences surrounding ori5, relative to the *VAR1* gene, could result in less efficient binding of Abf2p to ori5 than to *VAR1*. However, our data indicate that Abf2p is not a factor in conferring DNase I resistance to ori5. Therefore, the binding of other proteins to ori5 must confer the observed DNase I resistance. For example, origins of DNA replication in bacteria (48), nuclei (49) and mitochondria (39,50) are probably compartmentalized to membrane- or scaffold-associated regions and hence subject to constraints that may differ from bulk DNA. We anticipate that future experiments comparing the properties of mtDNA nucleoids *in vitro* and *in organello* will be fruitful in defining the functional organization of the mitochondrial genome.

ACKNOWLEDGEMENTS

This work was supported by grant GM33510 from the NIH and grant I-0642 from The Robert A.Welch Foundation. We thank Betty Key for expert technical assistance and Beverly Rothermel for preparation of the Abf2p antibody. We also thank our colleagues for generous gifts of antisera and D.Clayton for the Abf2p expression vector.

REFERENCES

1 Nass,M.M.K., Nass,S. and Afzelius,B.A. (1965) *Exp. Cell Res.*, **37**, 516–639.
2 Nass,M.M.K. (1969) *J. Mol. Biol.*, **42**, 521–528.
3 Rothfield,L.I. (1994) *Cell*, **77**, 963–966.
4 Pettijohn,D. (1988) *J. Biol. Chem.*, **263**, 12793–12796.
5 Stevens,B. (1981) In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 471–504.
6 Williamson,D.H. and Fennell,D.J. (1979) *Methods Enzymol.*, **56**, 728–733.
7 Birky,C.W.,Jr, Strausberg,R.L., Perlman,P.S. and Forster,J.L. (1978) *Mol. Gen. Genet.*, **158**, 251–261.
8 Dujon,B. and Slonimski,P.P. (1976) In Bucher,Th., Neupert,W., Sebald,W. and Werner,S. (eds.), *Genetics and Biogenesis of Chloroplasts and Mitochondria*. North-Holland Biomedical Press, Amsterdam, The Netherlands, pp. 393–403.
9 Zweifel,S.G. and Fangman, W.L. (1991) *Genetics*, **128**, 241–249.
10 Kleff,S., Kemper,B. and Sterngalanz,R. (1992) *EMBO J.*, **11**, 699–704.
11 Lockshon,D., Zweifel,S.G., Freeman-Cook,L.L., Lorimer,H.E., Brewer,B.J. and Fangman,W.L. (1995) *Cell*, **81**, 947–955.
12 Suzuki,T., Kawano,S. and Kuroiwa,T. (1982) *J. Cell Sci.*, **58**, 241–261.
13 Van Tuyle,G.C. and McPherson,M.L. (1979) *J. Biol. Chem.*, **254**, 6044–6053.
14 Miyakawa,I., Sando,N., Kawano,K., Nakamura,S. and Kuroiwa,T. (1987) *J. Cell Sci.*, **88**, 431–439.
15 Caron,F., Jacq,C. and Rouviere-Yaniv,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4265–4269.
16 Diffley,J.F.X. and Stillman,B. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7864–7868.
17 Diffley,J.F.X. and Stillman,B. (1992) *J. Biol. Chem.*, **267**, 3368–3374.
18 Landsman,D. and Bustin,M. (1993) *BioEssays*, **15**, 1–8.
19 Parisi,M.A. and Clayton,D.A. (1991) *Science*, **252**, 965–969.
20 Fisher,R.P., Lisowsky,T., Parisi,M.A. and Clayton,D.A. (1992) *J. Biol. Chem.*, **267**, 3358–3367.
21 Megraw,T.L. and Chae,C.B. (1993) *J. Biol. Chem.*, **268**, 12758–12763.
22 Kao,L.R., Megraw,T.L. and Chae,C.B. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5598–5602.
23 Grosschedl,R., Giese,K. and Pagel,J. (1994) *Trends Genet.*, **10**, 94–99.
24 Bianchi,M.E. (1994) *Mol. Microbiol.*, **14**, 1–5.
25 Nobrega,F. and Tzagoloff,A. (1980) *J. Biol. Chem.*, **255**, 9828–9837.
26 Zelenaya-Troitskaya,O., Perlman,P.S. and Butow,R.A. (1995) *EMBO J.*, **14**, 3268–3276.
27 Azpiroz,R. and Butow,R.A. (1993) *Mol. Biol. Cell*, **4**, 21–36.
28 Lambowitz,A.M. (1979) *Meth. Enzymol.*, **59**,421–433.
29 Hudspeth,M., Shumard,K., Tatti,K. and Grossman,L. (1980) *Biochim. Biophys. Acta*, **610**, 221–228.Hudspeth,M., Shumard,K., Tatti,K. and Grossman,L. (1980) *Biochim. Biophys. Acta*, **610**, 221–228.
30 Laemmli,U.K. (1970) *Nature*, **227**, 680–68530.
31 Peebles,C.L., Belcher,S.M., Zhang,M., Dietrich,R.C. and Perlman,P.S. (1993) *J. Biol. Chem.*, **268**, 11929–11938.
32 Zassenhaus,H.P., Martin,N.C. and Butow,R.A. (1984) *J. Biol. Chem.*, **259**, 6019–6027.
33 Parikh,V.S., Conrad-Webb,H., Docherty,R. and Butow,R. A. (1989) *Mol. Cell. Biol.*, **9**, 1897–1907.
34 Gross,D.S. and Garrard,W.T. (1988) *Annu. Rev. Biochem.*, **57**, 159–197.
35 Hudspeth,M.E.S., Ainley,W.M., Shumard,D.S., Butow,R.A. and Grossman,L. (1982) *Cell*, **30**, 617–626.
36 de Zamaroczy, M., Marotta,R., Faugeron-Fonty,G., Goursot,R., Mangin,M., Baldacci,G. and Bernardi,G. (1981) *Nature*, **292**, 75–78.
37 Ghivizzani,S.C., Madsen,C.S. and Hauswirth,W.W. (1993) *J. Biol .Chem.*, **268**, 8675–8682.
38 Ghivizzani,S.C., Madsen,C.S., Nelen,M.R., Ammini,C.V. and Hauswirth,W.W. (1994) *Mol. Cell. Biol.*, **14**, 7717–7730.
39 Albring,M., Griffith,J. and Attardi,G. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1348–1352.
40 Miyakawa,I., Tokitaka,M., Shiiba,D., Sawada,T., Fujikura,Y., Fukumoto,T. and Sando,N. (1993) *Plant Cell Physiol.*, **34**, 151–156.
41 Xu,C. and Ray,D.S. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1786–1789.
42 Preobrajenskays,O., Boullard,A., Boubrik,F., Schnarr,M. and Rouviere-Yaniv, J. (1994) *Mol. Microbiol.*, **13**, 459–467.
43 Flashner,Y. and Gralla,J.D. (1988) *Cell*, **54**, 713–721.
44 Bonnefoy,E. and Rouviere-Yaniv,J. (1992) *EMBO J.*, **11**, 4489–4496.
45 Onate,S.A., Prendergast,P., Wagner,J.P., Nissen,M., Reeves,R., Pettijohn,D.E. and Edwards,D.P. (1994) *Mol. Cell. Biol.*, **14**, 3376–3391.
46 Ge,H. and Roeder,R.G. (1994) *J. Biol. Chem.*, **25**, 17136–17140.
47 de Zamaroczy,M., Faugeron-Fonty,G., Baldacci,G., Goursot,R. and Bernardi,G. (1984) *Gene*, **32**, 439–457.
48 Herrick,J., Kern,R., Guha,S., Landoulsi,A., Fayet,O., Malki,A. and Kohiyama,M. (1994) *EMBO J.*, **13**, 4695–4703.
49 Amati,B.B. and Gasser,S.M. (1988) *Cell*, **54**, 967–978.
50 Potter,D., Fostel,J.M., Berninger,M., Pardue,M.L. and Cech,T.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4118–4122.