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Mmf1p, a Novel Yeast Mitochondrial Protein Conserved throughout Evolution and Involved in Maintenance of the Mitochondrial Genome

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A novel protein family (p14.5, or YERO57c/YJGFc) highly conserved throughout evolution has recently been identified. The biological role of these proteins is not yet well characterized. Two members of the p14.5 family are present in the yeast *Saccharomyces cerevisiae*. In this study, we have characterized some of the biological functions of the two yeast proteins. Mmf1p is a mitochondrial matrix factor, and homologous Mmf1p factor (Hmf1p) copurifies with the soluble cytoplasmic fraction. Δ*mmf1* cells lose mitochondrial DNA (mtDNA) and have a decreased growth rate, while Δ*hmf1* cells do not display any visible phenotype. Furthermore, we demonstrate by genetic analysis that Mmf1p does not play a direct role in replication and segregation of the mtDNA. rho$^+$ Δ*mmf1* haploid cells can be obtained when tetrads are directly dissected on medium containing a nonfermentable carbon source. Our data also indicate that Mmf1p and Hmf1p have similar biological functions in different subcellular compartments. Hmf1p, when fused with the Mmf1p leader peptide, is transported into mitochondria and is able to functionally replace Mmf1p. Moreover, we show that homologous mammalian proteins are functionally related to Mmf1p. Human p14.5 localizes in yeast mitochondria and rescues the Δ*mmf1*-associated phenotypes. In addition, fractionation of rat liver mitochondria showed that rat p14.5, like Mmf1p, is a soluble protein of the matrix. Our study identifies a biological function for Mmf1p and furthermore indicates that this function is conserved between members of the p14.5 family.

Proteins with a key role in central cellular pathways are highly conserved throughout evolution. Recently, a novel family of small proteins (p14.5, or YERO57c/YJGFc) has been identified in bacteria and in lower and higher eukaryotes (14, 19, 23, 25). They have similar primary structures and molecular masses of approximately 15 kDa. Several independent studies have provided conflicting evidence concerning the hypothetical biological function(s) of these proteins. Initial studies suggest that the rat member of this family, rp14.5, may belong to the high-mobility group (HMG) proteins, the major nonhistone components of chromatin. This hypothesis was based on the fact that rp14.5 is soluble in perchloric acid, a feature of HMG proteins, and copurifies with these proteins when isolated from rodent liver cells (14). However, other features of rp14.5 do not meet the general criteria that characterize the HMG protein family, e.g., bipolar distribution of charged amino acids and monomeric behavior in solution (3). Indeed, biochemical studies have demonstrated that native rp14.5 is able to homodimerize (14). Analysis of the rp14.5 amino acid sequence revealed that the N-terminal half of the molecule has approximately 30% similarity to a domain of the 83- to 90-kDa heat shock protein (hsp) (14), which is highly conserved in lower and higher eukaryotes (1, 8, 9, 24). The prediction that p14.5 is related to hsp's was confirmed in isolated rodent hepatocytes and hepatoma cells, in which the levels of rp14.5 are increased upon heat shock (23). However, there is no evidence that this property is conserved among the other members of the p14.5 family. For instance, the human homologue, hp14.5, in contrast to rp14.5, does not show any amino acid sequence similarity with hsp's (25).

A novel biological function of the p14.5 protein family was recently suggested by two independent studies, in which it was shown that p14.5 may be implicated in regulation of protein translation (19, 25). Both the human and the rat p14.5 proteins are able to inhibit cell-free protein synthesis in the rabbit reticulocyte lysate system. Schmiedeknecht et al. have shown that the hp14.5 gene is weakly expressed in freshly isolated monocytes, but high levels of hp14.5 mRNA are detected

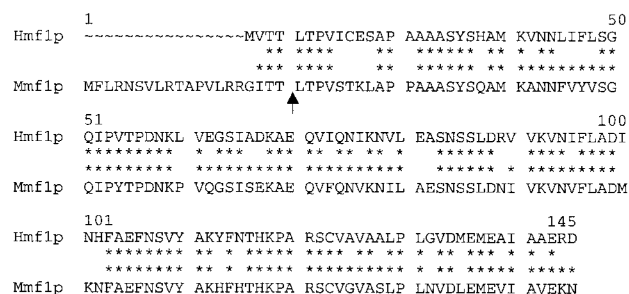

FIG. 1. Alignment of Hmf1p and Mmf1p amino acid sequences. One or two stars indicate conserved or identical amino acids, respectively. The arrow indicates the cleavage site of the leader peptide. The determination of the N-terminal amino acid sequence of the Mmf1p mitochondrial form was performed as described in Materials and Methods.

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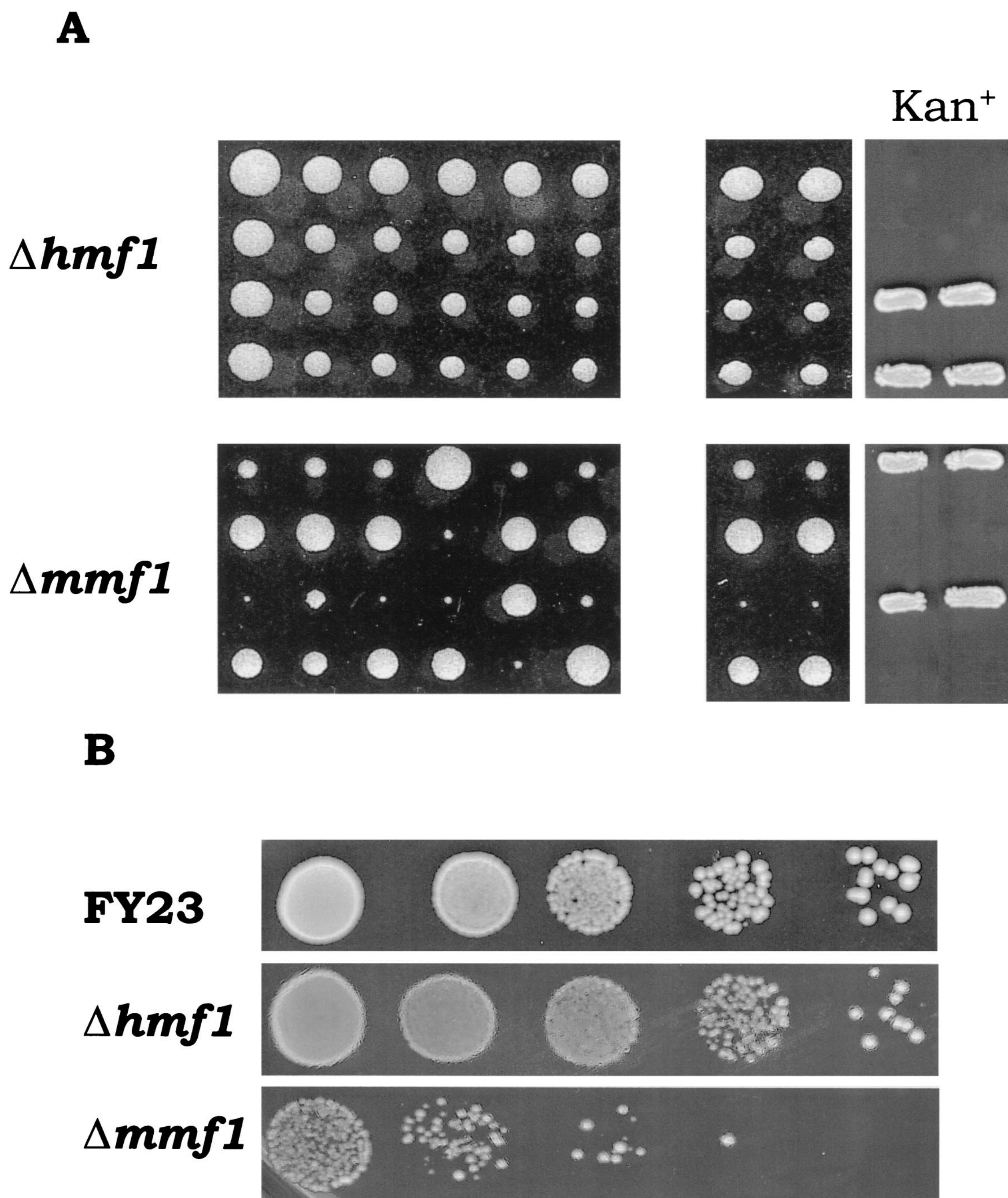

FIG. 2. Δ*hmf1* and Δ*mmf1* cells have different phenotypes. (A) Disruption of *Hmf1* and *Mmf1*. Diploid strain FY1679 was transformed with DNA fragments, comprising a selectable marker (kanamycin) fused to 50-bp flanking regions of *Hmf1* and *Mmf1* (see Materials and Methods). After sporulation, asci were dissected on YPD plates and haploid cells were grown for 5 to 6 days at 30°C. The replacement of the two yeast genes was confirmed by growing the haploid cells on medium containing kanamycin. (B) Serial dilution drop test of wild-type, Δ*hmf1*, and Δ*mmf1* cells. Samples of 10 μl containing decreasing numbers of yeast cells (10^5, 10^4, 10^3, 10^2, 10^1) were plated on SD and grew for 3 to 5 days at 30°C.

in monocytes undergoing differentiation (25). Similar results were obtained by immunocytochemical analysis (25). Based on the inhibitory effect of hp14.5 on protein translation and its differentiation-dependent expression, these authors proposed a role of p14.5 in the regulation of protein synthesis during differentiation (25). However, inhibition of protein translation by rp14.5 and hp14.5 in an in vivo model system has not been demonstrated.

To clarify the biological function(s) of members of this family, we have characterized the p14.5-related proteins in the budding yeast *Saccharomyces cerevisiae*. In *S. cerevisiae*, two members of the p14.5 family have been identified. In the present study, we show that mitochondrial matrix factor 1 (Mmf1p) is a mitochondrial protein, while homologous Mmf1 factor 1 (Hmf1p) is localized in the soluble cytoplasmic fraction. We also show that Mmf1p influences the maintenance of

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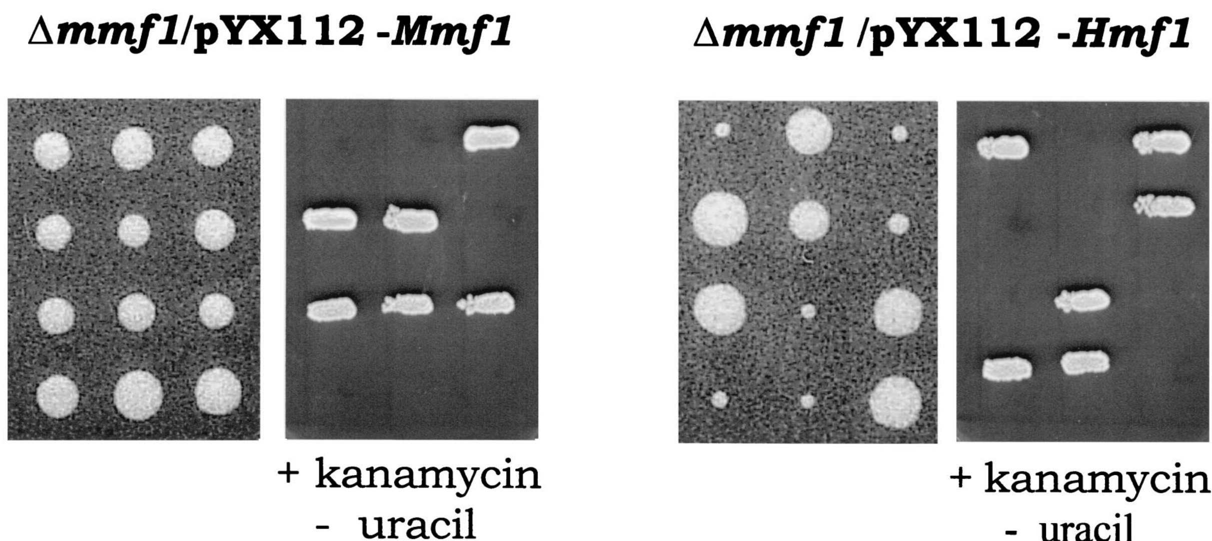

FIG. 3. Overexpression of *Hmf1* in Δ*mmf1* cells does not restore the wild-type phenotype. Heterozygote FY1679 diploid cells (*Mmf1*/*mmf1*::*KAN*) were transformed with pYX112-*Mmf1* or pYX112-*Hmf1* constructs. After sporulation, asci were dissected on YPD plates and haploid cells were grown for 5 to 6 days at 30°C. Segregation of selectable markers, kanamycin resistance for gene replacement and uracil for the pYX112 vector, are also shown.

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mitochondrial DNA (mtDNA) and the cell division time, while no visible phenotype was observed in Δ*hmf1* cells. Our data indicate that Mmf1p and Hmf1p have similar biological functions in pathways which are localized in different cellular compartments. Targeting of Hmf1p into mitochondria by fusion to Mmf1p leader peptide is sufficient to rescue the Δ*mmf1*-associated phenotypes. Furthermore, we demonstrate that the yeast and mammalian members of the p14.5 family are functionally related. Our study provides the first in vivo evidence for a biological function of members of this protein family.

MATERIALS AND METHODS

Media and yeast and bacterial strains. The following media were used for growth of yeast cells: rich medium (YP) (1% yeast extract and 2% bactopeptone) supplemented with different carbon sources, 2% glucose (YPD) or 3% glycerol (YPG). Synthetic minimal medium (SM) (0.67% yeast nitrogen base without amino acids) was supplemented with 2% glucose (SD) or 3% glycerol (SG) and different nutrients as required. Solid medium was obtained by adding 2% agar to the components listed above. Sporulation solid medium was composed of 2% agar and 1% potassium acetate. The yeast strains used were the haploid strains FY23 (*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 GAL2*) and FY73 (*MAT*α *ura3-52 his3 Δ200 GAL2*) and the diploid strain FY1679, generated by crossing FY23 and FY73 (29).

The *Escherichia coli* strains used were DH5α for general molecular cloning and BL 21 for production of bacterial recombinant proteins.

Yeast and bacterial vectors. The vectors pYX112 and pYX212 (Ingenius), which contain a centromeric (pYX112) or a 2μm plasmid (pYX212) replication origin, respectively, the triose phosphate isomerase promoter, and the URA 3-selectable marker were used to express *Mmf1* and *Hmf1* in yeast. The bacterial vectors Bluescript II (Stratagene) and pGEX-4T (Pharmacia Biotech), respectively, were used to amplify the genes for further subcloning and for production of bacterial recombinant proteins, such as *Schistosoma japonicum* glutathione *S*-transferase (GST) fusion products.

Cloning of the p14.5 family members. The Hmf1p and Mmf1p open reading frames were directly amplified by PCR using purified yeast genomic DNA as template, while hp14.5 was PCR amplified from cDNA of human liver cells (kindly provided by K. Schröder, DKFZ, Heidelberg, Germany). The three genes were cloned into the pBluescript vector, and the nucleotide sequence was verified.

Replacement of *Mmf1* and *Hmf1*. The disruption procedure of *Mmf1* and *Hmf1* and subsequent tetrad analysis were performed as described by Jaquet and Jauniaux (12). Briefly, two linear DNA fragments comprising the 50 bp upstream and downstream of *Mmf1* or *Hmf1* separated by the kanamycin resistance gene were generated by two consecutive PCRs using as templates genomic *S. cerevisiae* DNA and the kanamycin resistance gene of the pFA6a-KanMX4 vector (27). After transformation of the FY1679 strain, kanamycin-resistant clones were isolated, genomic DNA was purified, and the replacement driven by homologous recombination was verified by PCR. The kanamycin-resistant clones with the correct replacement were further processed for tetrad dissections and analysis.

Production of bacterial recombinant Mmf1p and Hmf1p. *Mmf1* and *Hmf1* were cloned into the PGEX-4T vector (Pharmacia Biotech) in frame with the carboxy-terminal sequence of GST. The constructs were transformed into *E. coli* BL 21, and fusion protein synthesis was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culturing medium. GST fusion proteins were purified on glutathione-Sepharose (Pharmacia Biotech), and the GST domain was removed by Thrombin (Sigma) cleavage according to the protocols from Pharmacia. Purified Mmf1p and Hmf1p proteins were dialyzed in phosphate-buffered saline (PBS) and used to immunize rabbits for the production of specific antibodies.

Subfractionation of yeast cells. Total yeast extracts were prepared according to the protocol described by Sambrook et al. (22). Mitochondria were isolated according to the procedure described by Newman et al., with some modifications (18). Yeast cells were grown to early exponential phase in YP medium containing 3% glycerol and 0.1% glucose (or rho^0 Δ*mmf1* cells in YP containing 2% glucose), harvested by centrifugation at 2,000 × g, and washed once with deionized water. After washing, the cells were resuspended in 0.1 M Tris-SO_4 (pH 9.4) and 10 mM dithiothreitol and incubated at 30°C for 10 min. The cells were then collected and washed once with 1.2 M sorbitol and resuspended in 1.2 M sorbitol, 20 mM K_3PO_4 (pH 7.4). Lyticase (Sigma) was added to a final concentration of 0.5 mg/ml, and the cells were incubated for 60 min at 30°C with gentle shaking. The protoplasts were harvested at room temperature, washed twice with 1.2 M sorbitol, and resuspended in ice-cold homogenization buffer (0.6 M mannitol, 10 mM Tris-HCl [pH 7.4], 0.1% bovine serum albumin [BSA]), and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The mixture was transferred to a Dounce tight-fitting homogenizer and homogenized on ice by 15 strokes. The lysate was diluted with 1 volume of ice-cold homogenization buffer and centrifuged at 1,000 × *g* at 4°C for 5 min to spin down the cell debris. The mitochondria were collected from the supernatant by centrifugation at 8,000 × *g* for 10 min, resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM morpholinepropanesulfonic acid [MOPS]-KOH [pH 7.2]), and applied on a step gradient consisting of 20, 30, 40, 50, and 60% (wt/wt) sucrose in 10 mM MOPS-KOH (pH 7.4), 100 mM KCl, 1 mM EDTA, and 1 mM PMSF. After centrifugation (27,000 rpm for 30 min in a Beckman SW28 rotor), mitochondria, which banded between the 40 and 50% sucrose layers, were collected, diluted in SEM buffer, and concentrated by centrifugation.

The mitochondrial soluble matrix fraction and the membrane fraction were prepared as described by Rowley et al. (21). Purified mitochondria were resuspended in 20 mM HEPES-KOH (pH 7.4), 100 mM KCl, and 1 mM PMSF, sonicated for 50 s with a Branson Sonifier (5 pulses of 10 s interrupted by 10-s intervals, 80% duty cycle), and centrifuged at 50,000 rpm for 45 min at 4°C in a Beckman TLA 100.3 rotor to isolate the soluble and membrane fractions. Mitochondrial nucleoids were prepared as described by Meeusen et al. (15).

Production and purification of antibodies and immunoblot analysis. Mmf1p and Hmf1p antibodies were generated in rabbits by using purified bacterial recombinant proteins as antigen. After three injections of antigen (100 μg), the blood was taken from the rabbits and left to coagulate and antibodies were purified from the plasma by affinity chromatography. For antibody purification, Mmf1p or Hmf1p was coupled to CNBr-activated Sepharose 4B and the resin was incubated batchwise with the plasma for 1 h at room temperature. After extensive washing with PBS, the bound antibodies were eluted with a glycine

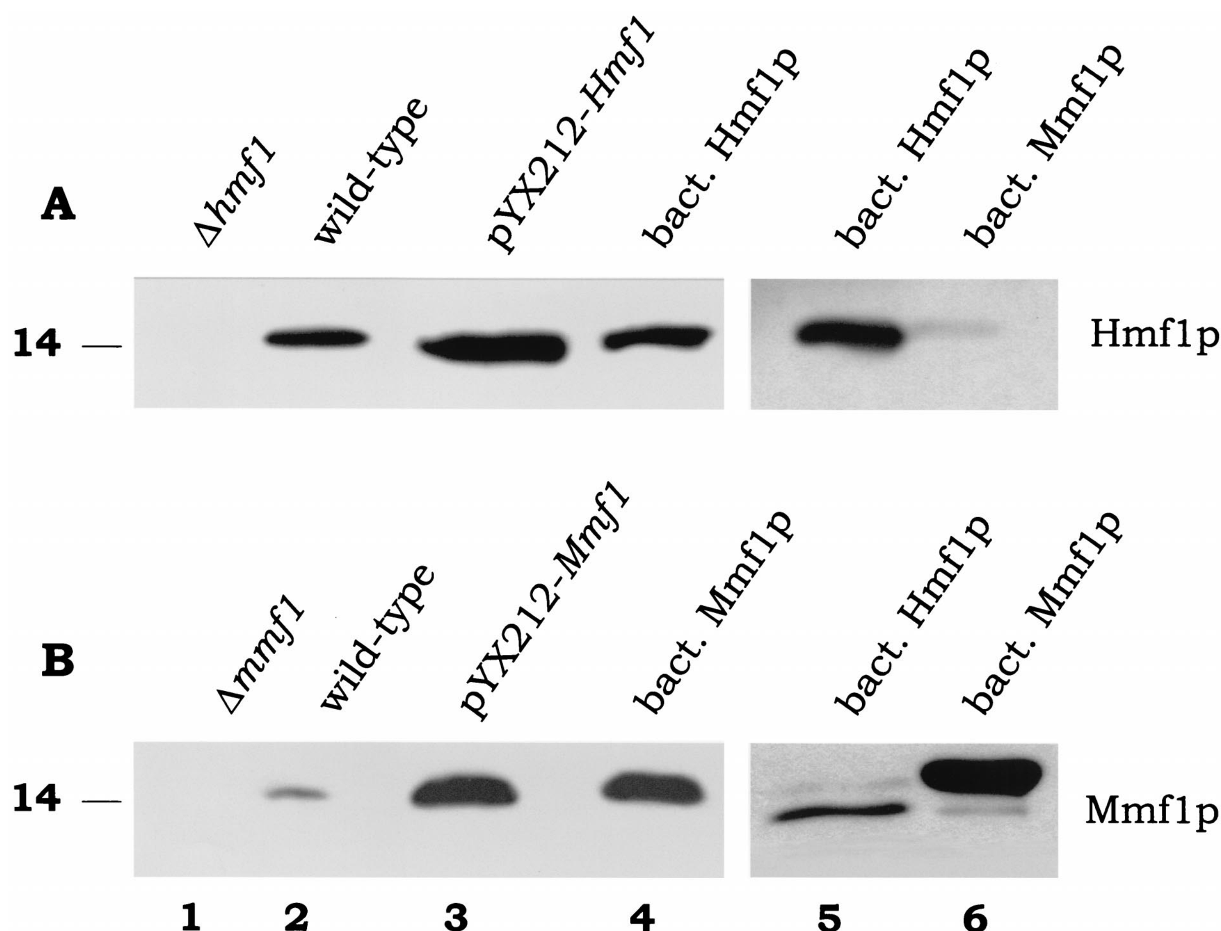

FIG. 4. Detection of Hmf1p and Mmf1p by immunoblot analysis. Total protein extracts were prepared as described in Materials and Methods, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting using anti-Hmf1p (A) or anti-Mmf1p (B) polyclonal antibodies. (A) Lane 1, 50 μg of protein extract of Δ*hmf1* cells; lane 2, 50 μg of protein extract of wild-type cells; lane 3, 50 μg of protein extract of wild-type cells transformed with pYX212-*Hmf1* construct; lanes 4 and 5, 200 ng of Hmf1p bacterial recombinant protein; lane 6, 200 ng of Mmf1p bacterial recombinant protein. (B) Lane 1, 50 μg of protein extract of Δ*mmf1* cells; lane 2, 50 μg of protein extract of wild-type cells; lane 3, 50 μg of protein extract of wild-type cells transformed with the pYX212-*Mmf1* construct; lanes 4 and 6, 200 ng of Mmf1p bacterial recombinant protein; lane 5, 200 ng of Hmf1p bacterial recombinant protein. Molecular mass markers (in kilodaltons) are on the left.

buffer (pH 2.2), neutralized with Tris base, and visualized on a sodium dodecyl sulfate (SDS)-polyacrylamide gel.

For immunoblotting, yeast protein extracts were resolved on an SDS–15% polyacrylamide gel and electroblotted onto a Polyscreen PVDF Transfer Membrane (DuPont). Proteins were detected by enhanced chemiluminescence (ECL; Amersham Life Science) using the following antibodies: anti-Abf2p antibody (dilution, 1:5,000; kindly provided by Jodi Nunnari, Section of Molecular and Cellular Biology, University of California, Davis), anti-Cox IIIp antibody (dilution, 1:1,000; dMoBiTec), anti-Hmf1p antibody (dilution, 1:2,000), anti-Mge1p antibody (dilution, 1:1,000; kindly provided by Benedikt Westerman, Institut für Physiologische Chemie, Munich, Germany), anti-Mgm101p antibody (dilution, 1:2,000; kindly provided by Jodi Nunnari), anti-Mmf1p antibody (dilution, 1:500), anti-porin antibody (dilution, 1:1,000; dMoBiTec), anti-rp14.5 antibody (dilution, 1:1,000; kindly provided by Florence Levy-Favatier, University of Paris, Paris, France), and anti-Tim44p antibody (dilution, 1:1,000; kindly provided by Benedikt Westerman). The polyvinylidene difluoride (PVDF) membranes were incubated with the antibodies overnight at 4°C. The secondary antibody (anti-rabbit or anti-mouse immunoglobulin horseradish peroxidase conjugate; Promega) was diluted 1:5,000.

Purification and sequencing of Mmf1p. Freshly prepared mitochondria were resuspended in 20 mM Tris-HCl (pH 7.4)–200 mM NaCl–1 mM dithiothreitol–2 mM EDTA and broken by freezing and thawing. After centrifugation (40,000 rpm for 45 min at 4°C in a Beckman TLA 100.3 rotor), the supernatant was applied to a Sepharose column with coupled purified anti-Mmf1p antibody. After extensive washing with PBS, Mmf1p protein was eluted from the column with glycine buffer (pH 2.2) and dialyzed in PBS. The purified protein was concentrated by acetone precipitation (1 volume of protein sample/9 volumes of acetone at −20°C for 20 min), applied on an SDS–15% polyacrylamide gel, and electroblotted onto a Polyscreen PVDF Transfer Membrane (DuPont). The membrane was divided in two parts. The first was used for immunoblotting to identify the precise position of Mmf1p on the membrane. The area corresponding to Mmf1p was cut from the second part, and the N-terminal amino acid sequence was determined using a blot cartridge device and a Procise 494A protein sequencer from Applied Biosystems (Weiterstadt, Germany).

Immunoelectron microscopy. Yeast cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Soerensen's phosphate buffer, pH 7.4, for 2 h. Yeast samples were embedded in 10% gelatine which was solidified on ice. Blocks with yeast cells were immersed in 2.3 M sucrose in PBS for 4 h and then frozen in liquid nitrogen. Ultrathin cryosections were cut on an Ultracut S microtome with FC4E cryoattachment and transferred onto Formvar-coated grids and then labeled as follows. After 15 min on a drop of 50 mM glycine and 15 min preincubation in 0.5% BSA in PBS, the grids were transferred onto drops of polyclonal antiserum against Mmf1p (1:250 dilution in 0.1% BSA in PBS) and incubated overnight at 4°C. The sections were then washed three times over a 15-min period with 0.1% BSA in PBS and then immunolabeled with 10 nM protein A-conjugated colloid gold probes (26) by incubation with PAG10 (1:70 dilution in 0.1% BSA in PBS) for 30 min at room temperature. After three rinses in PBS (3 times for 5 min each time), the sections were fixed in 2.5% glutaraldehyde in PBS for 5 min and then washed in PBS (3 times for 5 min each) and distilled water (3 times for 5 min each). After immunolabeling, the sections were stained with uranyl acetate and embedded in metalcellulose as described by Slot and Geuze (26). The sections were examined with a Jeol 1010 FX electron microscope. As a negative control, the primary antibody was omitted from the procedure to visualize any nonspecific binding of gold particles to cell organelles.

DAPI staining. Mid-log-phase wild-type and Δ*mmf1* haploid cells were fixed in 100% methanol for 5 min, washed with PBS, resuspended in PBS containing 1.0 μg of 4′,6′-diamidino-2-phenylindole (DAPI)/ml, and incubated for 1 h in the dark at room temperature under gentle rotation. After three washes in PBS, cells were examined under a fluorescence microscope.

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Determination of Mmf1p-GFP localization in vivo. *Mmf1* was cloned in frame at the C-terminal end with the green fluorescent protein (GFP) gene in the pYX112 vector (Ingenius) and expressed in the yeast strain FY23. Mid-log-phase cells were incubated without fixation in SD medium containing 1 mg of DAPI/ml and 200 nM MitoTracker-Red (Molecular Probes, Eugene, Oreg.) for 45 min at room temperature. After extensive washing of the cells in DAPI and MitoTracker-Red, GFP signals were analyzed using Carl Zeiss confocal laser scanning microscope 510 UV.

RESULTS

Deletions of *Mmf1* and *Hmf1* result in two distinct phenotypes. The p14.5 family comprises several members, which are highly conserved throughout evolution. In the budding yeast *S. cerevisiae*, two members of the p14.5 family have been identified. The two proteins have a high level of homology, being approximately 60% identical (Fig. 1). We termed them Mmf1p and Hmf1p. In the context of the *S. cerevisiae* genome sequencing project, Mmf1p was named YIL051c (accession no. P40185) while Hmf1p was named YER057c (accession no. P40037), and the genes are located on chromosomes IX and V, respectively. To investigate the biological function of the yeast p14.5's, we have generated yeast strains in which *Mmf1* or *Hmf1* is replaced by a gene which confers kanamycin resistance. Two DNA fragments comprising the kanamycin resistance open reading frame fused to 50 bp of the flanking regions of *Mmf1* or *Hmf1* were generated by PCR and used to transform the diploid strain FY1679 (see Materials and Methods). After sporulation of diploid cells transformed with the *Mmf1-Kan* construct, several asci were dissected (Fig. 2A). All tetrads showed a 2:2 segregation into small and large colonies (Fig. 2A). Only the small colonies were able to grow on kanamycin-containing medium, indicating that *Mmf1* replacement cosegregates with the slow-growth phenotype (Fig. 2A). In contrast, tetrad analysis of diploid cells transformed with the *Hmf1-Kan* construct showed that Δ*hmf1* haploid cells do not display any visible phenotype (Fig. 2A). The replacement of the *Mmf1* or *Hmf1* genes was confirmed by PCR (data not shown). The effect on cellular growth of the disruption of *Mmf1* was also observed in a serial dilution drop test. As shown in Fig. 2B, wild-type and Δ*hmf1* cells have a higher growth rate than Δ*mmf1* cells. These initial data suggest that, despite their high amino acid similarity, Hmf1p and Mmf1p are involved in different cellular pathways. To further confirm these findings, we investigated whether the overexpression of *Hmf1* could rescue the Δ*mmf1* phenotype. For this purpose, heterozygote diploid cells (*Mmf1*/*mmf1*::*KAN*) were transformed with episomal plasmids which express low (pYX112) and high (pYX212) levels of *Mmf1* or *Hmf1*. After sporulation and dissection of the asci, we observed that low expression of *Mmf1* is sufficient to reestablish the wild-type phenotype (Fig. 3), while neither low nor high expression of *Hmf1* has any effect on the Δ*mmf1*-associated phenotype (Fig. 3 and data not shown). Thus, the two yeast proteins appear to be implicated in distinct cellular events.

Generation of anti-Mmf1p and anti-Hmf1p antibodies. To characterize the biological role of the two proteins, we have generated rabbit polyclonal antibodies against Mmf1p and Hmf1p bacterial recombinant proteins as described in the Materials and Methods section. Immunoblot analysis showed that the Hmf1p antibody recognizes a protein with a molecular mass of approximately 15 kDa in the total yeast extract which comigrates with the Hmf1p bacterial recombinant protein (Fig. 4A, lanes 2 and 4). Moreover, this protein band is not detected in total extracts of Δ*hmf1* cells (Fig. 4A, lane 1) and is more abundant in cells which overexpress *Hmf1* (pYX212-*HMF1*) than in the wild-type strain (Fig. 4A, compare lane 2 with lane

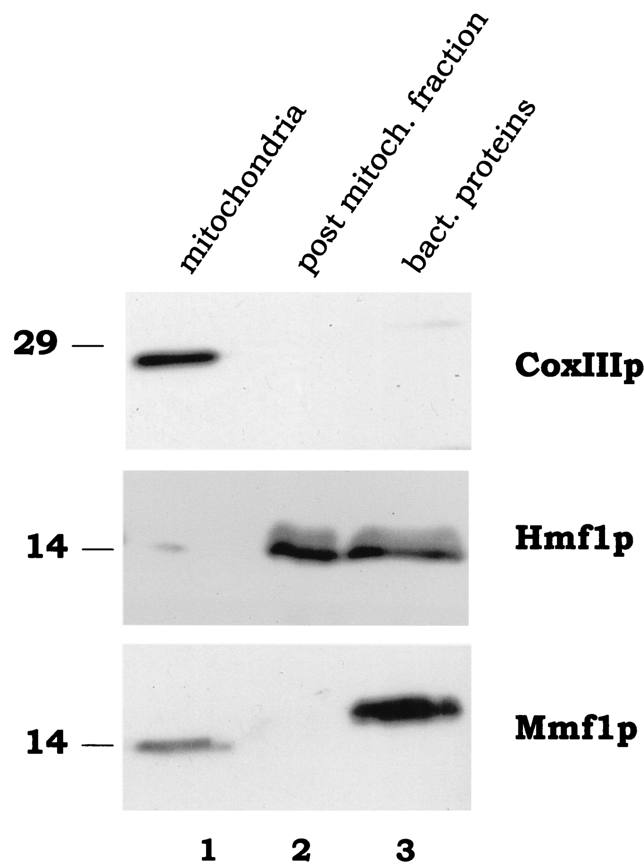

FIG. 5. Subcellular localization of Hmf1p and Mmf1p. Cellular fractionation of FY23 haploid cells. The different cellular extracts were prepared as described in Materials and Methods, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting using anti-Hmf1p, anti-Mmf1p, and anti-CoxIIIp specific antibodies, as indicated. Lane 1, 10 μg of mitochondrial protein extract; lane 2, 10 μg of supernatant of postmitochondrial fraction; lane 3, 200 ng of bacterial recombinant proteins (Hmf1p in the central panel and Mmf1p in the bottom panel). Molecular mass markers (in kilodaltons) are on the left.

3). Analogously to Hmf1p, Mmf1p antibody reacts with a 15-kDa protein band which is absent in Δ*mmf1* cells but overexpressed in cells transformed with the pYX212-*Mmf1* plasmid (Fig. 4B, lanes 1 to 3). The data shown in Fig. 4 (lanes 1 to 4) indicate that the two antibodies are specific. However, we observed that after long exposure, a 15-kDa protein of the Δ*mmf1* cellular extract became visible in the immunoblot analysis with Mmf1p antibody. To determine whether Mmf1p antibody has some cross-reactivity with Hmf1p, we performed an immunoblot analysis using purified Hmf1p and Mmf1p bacterial recombinant proteins. While the Hmf1p antibody appears to be highly specific, Mmf1p antibody weakly cross-reacts with Hmf1p (Fig. 4A and B, lanes 5 and 6).

Mmf1p is a soluble protein of the mitochondrial matrix. To investigate Mmf1p and Hmf1p function(s), we determined their subcellular localization. Cellular fractionation experiments indicated that Mmf1p is associated with mitochondria (Fig. 5, lane 1), while Hmf1p is localized in the cytoplasmic postmitochondrial fraction (Fig. 5, lane 2). The Mmf1p detected in the mitochondrial fraction migrates faster than the bacterial recombinant protein on SDS-polyacrylamide gel (Fig. 5). This difference in migration between the two forms of Mmf1p may be explained by the fact that mitochondrial proteins which are encoded by nuclear genes have a leader sequence responsible for the targeting into the mitochondria.

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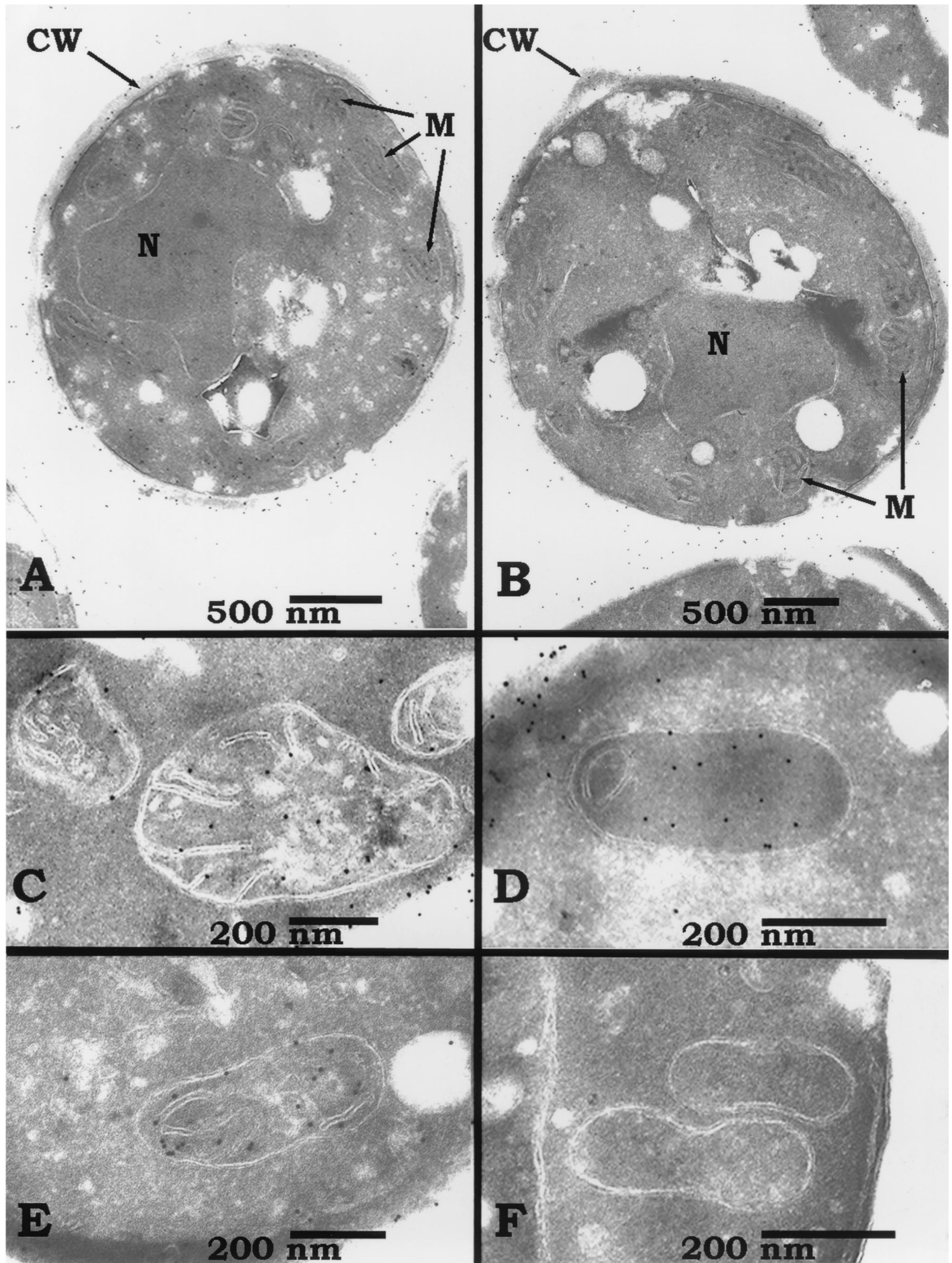

FIG. 6. Mmf1p localizes in mitochondrial matrix. Shown are electron micrographs of wild-type *S. cerevisiae* cells (A to F) fixed with glutaraldehyde-paraformaldehyde and processed for cryosectioning. The ultrathin sections of these cells were incubated with Mmf1p rabbit polyclonal antiserum, followed by immunogold conjugates (protein A, 10 nm size) (A to E) or with the secondary antibody alone (F). Abbreviations: CW, cell wall; N, nucleus; M, mitochondria.

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Once the protein has reached the mitochondrial compartment, the leader peptide is cleaved by specific proteases. A cleavage site motif was predicted at the N-terminal region of Mmf1p by computer analysis (10). To investigate whether Mmf1p has a leader peptide, we purified Mmf1p from yeast mitochondrial protein extract and the N-terminal amino acid sequence was determined as described in the Materials and Methods section. The yeast protein purified from mitochondria is 17 amino acids shorter than the predicted amino acid sequence (Fig. 1), demonstrating the existence of a leader peptide in Mmf1p. To confirm the localization of Mmf1p in mitochondria, we performed electron microscopy immunogold labeling of *S. cerevisiae* cells by using the previously described Mmf1p antibody. Figure 6A to E illustrates that all mitochondria in each section are labeled by an electron-dense immunoreaction product, while the other subcellular compartments remain free of labeling. No labeling was observed when the primary antibody was omitted (Fig. 6F), thus confirming the specificity of the immunogold staining. Soluble mitochondrial proteins can be part of complexes attached to the inner mitochondrial membrane or be localized in the matrix. To discriminate between the two possibilities, we analyzed different mitochondrial fractions. Mitochondria were disrupted by sonication and fractionated into soluble and membrane components. With this procedure, intrinsic proteins (membrane proteins) and nonintrinsic proteins (soluble proteins attached to the membrane) are found in the vesicle fraction (21). As shown in Fig. 7, Tim44p, a peripheral inner membrane protein (20), was detected in the vesicle fraction together with porin, a protein of the outer mitochondrial membrane (lane 3). In contrast, Mmf1p was found in the mitochondrial matrix together with a soluble protein, Mge1p (13, 28), and a nucleoid-associated protein, autonomous replication sequence-binding factor 2 (Abf2p) (4–6). In summary, based on the electron microscopy and mitochondrial fractionation experiments, we can conclude that Mmf1p is a soluble protein of the mitochondrial matrix.

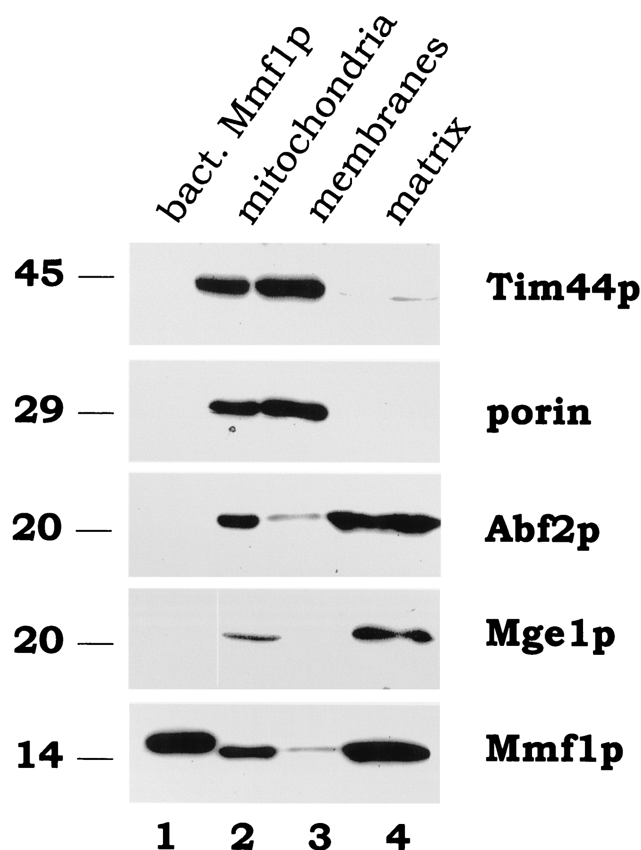

FIG. 7. Mmf1p is not attached to the mitochondrial inner membrane. Purified mitochondria were disrupted by sonication, soluble and membrane fractions were separated by centrifugation as described in Materials and Methods, and the distribution of Mmf1p in the different fractions was determined by immunoblot analysis. Lane 1, 200 ng of Mmf1p bacterial recombinant protein; lane 2, 20 μg of mitochondrial protein extract; lane 3, 20 μg of mitochondrial membrane extract; lane 4, 20 μg of mitochondrial matrix. The following antibodies were used as markers for the submitochondrial compartments: anti-Tim44p, nonintrinsic inner membrane protein; porin, outer membrane protein; Abf2p, mitochondrial nucleoid-associated protein; Mge1p, matrix soluble protein. Molecular mass markers (in kilodaltons) are on the left.

Mmf1p is involved in the maintenance of mtDNA. Since Mmf1p is localized in mitochondria, we determined whether the deletion of its gene has any effect on mitochondrial functions. Yeast cells in which mitochondrial activities are compromised are unable to grow on media containing nonfermentable carbon sources, such as glycerol. Therefore, we compared the abilities of Δ*hmf1*, Δ*mmf1*, and the wild-type strains to grow on glucose and glycerol (Fig. 8A). The Δ*hmf1* and the wild-type strains can efficiently grow on plates containing fermentable and nonfermentable carbon sources. In contrast, the Δ*mmf1* strain grows only on glucose, but not on glycerol-containing medium (Fig. 8A). These findings indicate that mitochondrial respiratory functions are lost in the Δ*mmf1* cells. Surprisingly, reintroduction of *Mmf1* in Δ*mmf1* haploid cells reestablishes the normal growth rate on glucose, but does not restore the growth on glycerol (Fig. 8A). The failure of Mmf1p to reestablish wild-type growth of Δ*mmf1* haploid cells on glycerol may be due to loss of mtDNA upon *Mmf1* disruption. To evaluate this hypothesis, we have crossed Δ*mmf1* haploid cells with rho$^-$ or wild-type haploid cells. Δ*mmf1* was able to grow on glycerol only when mated with wild-type haploid cells (Fig. 8B). Mating Δ*mmf1* with rho$^-$ haploid cells does not restore the ability to grow in glycerol-containing medium (Fig. 8B). To exclude the possibility that the inability of diploid cells (rho$^-$/Δ*mmf1*) to grow on glycerol medium is due to an alteration of *Mmf1* expression, we determined the protein levels of Mmf1p in rho$^-$/Δ*mmf1* and Δ*mmf1*/wild-type diploid cells. Similar levels of protein were detected in both diploid strains (data not shown). Finally, to confirm the loss of mtDNA in the Δ*mmf1* strain, we stained the yeast cells with a DNA-specific dye, DAPI. When cells were examined under a fluorescence microscope, we observed that the small fluorescent cytoplasmic bodies corresponding to chondrolites were visible only in the wild-type cells but not in Δ*mmf1* cells (Fig. 8C). These findings clearly demonstrate that the consequence of *Mmf1* disruption is a loss of mtDNA.

Mmf1p plays a secondary role in mtDNA maintenance. Several studies have shown that different mitochondrial proteins can influence the maintenance of mtDNA. Abf2p is an *S. cerevisiae* protein which associates with mitochondrial nucleoids and is closely related to the nuclear high-mobility group proteins HMG1 and HMG2 from vertebrate cells (4–6). Several data indicate that Abf2p plays an important role in maintenance, but not in replication, of the mitochondrial genome. Disruption of *Abf2* results in a rapid loss of mtDNA only when yeast cells are cultured in glucose-containing medium (6, 16, 17). In contrast, when the *abf2* null haploid cells are isolated directly on a nonfermentable carbon source, the mitochondrial genome is indefinitely maintained. In order to understand whether Mmf1p has an essential role in the maintenance of mtDNA, we determined whether Δ*mmf1* haploid cells obtained by tetrad dissection on a glycerol plate were able to maintain the mtDNA and grow on medium containing a non-

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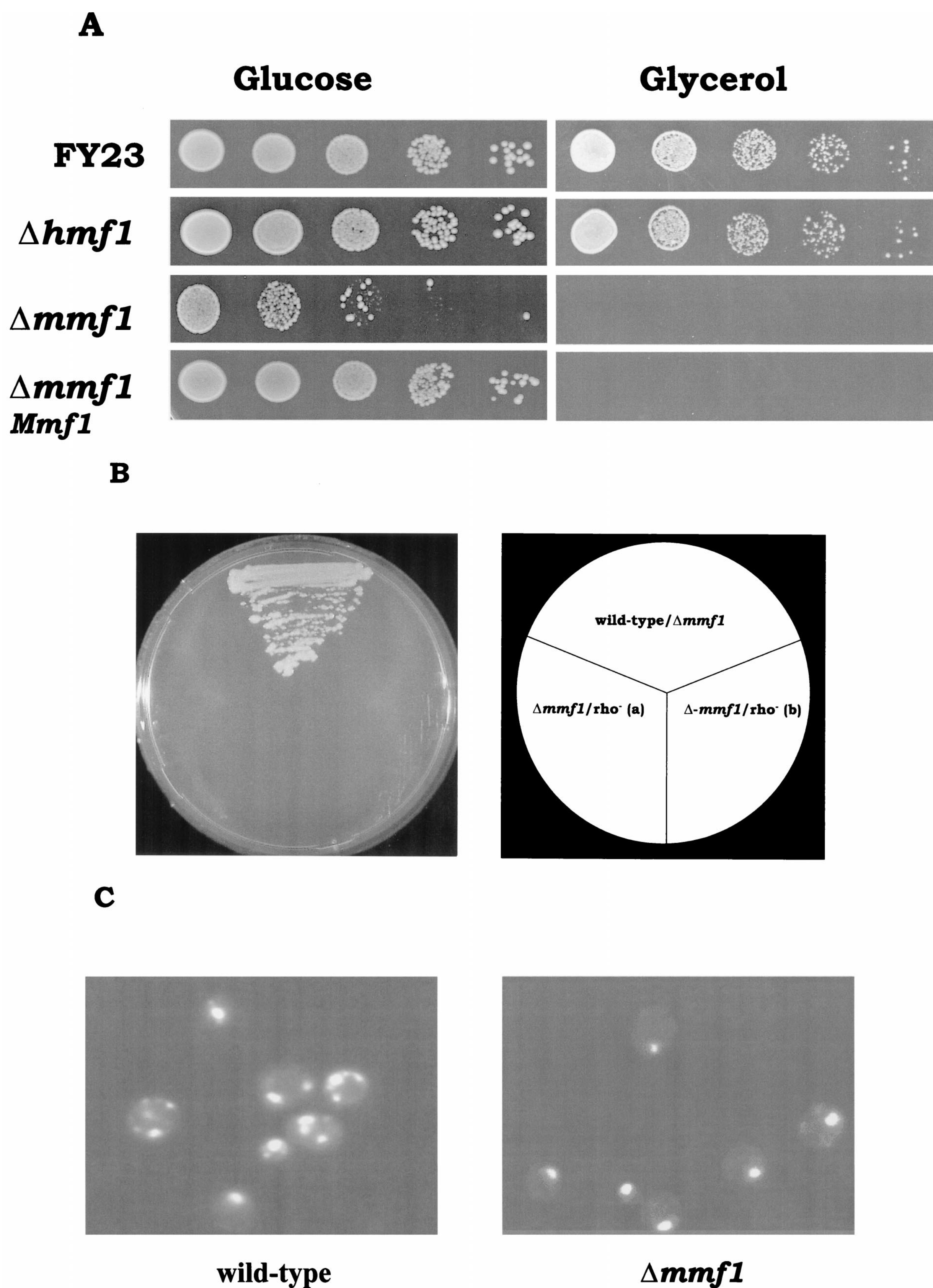

FIG. 8. Mmf1p is involved in the maintenance of mtDNA. (A) Δ*mmf1* cells do not grow in glycerol-containing medium. Shown is a serial dilution drop test of wild-type, Δ*hmf1*, Δ*mmf1*, and Δ*mmf1* pYX112-*Mmf1* transformed cells. Volumes of 10 μl containing decreasing numbers of yeast cells (10^5, 10^4, 10^3, 10^2, 10^1) were plated on SD or SG and grown for 3 to 5 days at 30°C. (B) Δ*mmf1* cells have lost mtDNA. Haploid Δ*mmf1* cells were mated with two different rho^- haploid or wild-type haploid cells. All diploid cells were grown in YPD, and during mid-log phase they were plated on YPG plates and grown for 4 to 5 days at 30°C. (C) DAPI staining of wild-type and Δ*mmf1* haploid cells.

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fermentable carbon source. After sporulation of heterozygote diploid cells (*Mmf1*/*mmf1*::*KAN*) and dissection of the asci on glycerol, we observed that all four haploid cells were able to grow (data not shown). The replacement of *Mmf1* was confirmed by growing the haploid cells on kanamycin medium and verifying the absence of *Mmf1* by PCR (data not shown). In addition, the Mmf1p levels in Δ*mmf1* cells were determined by immunoblot analysis. As expected, Mmf1p was not detected in total-protein extracts of Δ*mmf1* cells (Fig. 9A, lane 2). The weak band visible in lane 2 is due to a cross-reaction of anti-Mmf1p antibody with Hmf1p (Fig. 4B) and is not present in mitochondrial protein extract (data not shown). Next, we determined whether, as is the case for Δ*abf2* cells, preculturing of the rho$^+$ Δ*mmf1* strain in glucose-containing medium results in a loss of mtDNA. As shown in Fig. 9B and C, the number of Δ*mmf1* cells which are able to grow on glycerol medium progressively decreases with the increase of glucose preculture time. Furthermore, we observed that the rho$^+$ Δ*mmf1* cells have a reduced growth rate on glycerol medium in comparison to that of the wild-type strain (FY23) and are unable to survive when cultured at 37°C in the same medium (Fig. 9D). In contrast, when rho$^+$ Δ*mmf1* cells were grown on glucose medium, they did not show a temperature-sensitive phenotype (Fig. 9D). Together, these data demonstrate that Mmf1p does not play an essential role in the maintenance of mtDNA. However, loss of *Mmf1* expression strongly decreases mtDNA stability in cells cultured at 30°C in glucose medium or at 37°C in glycerol medium.

Mmf1p does not copurify with mitochondrial nucleoids and is localized at specific sites within the matrix. The fact that Δ*abf2* and Δ*mmf1* cells have similar phenotypes suggests that the two mitochondrial proteins may share some common properties. Therefore, we determined whether Mmf1p, like Abf2, is associated with the nucleoid structures. To evaluate this possibility, we fractionated the mitochondrial lysate on a step sucrose gradient, which is commonly used for the purification of mitochondrial nucleoids (15). The distribution of Mmf1p and Mgm101p, a nucleoid-associated protein (15), in the sucrose gradient was determined by immunoblotting. Mgm101p was detected at the top of the gradient and in the fractions corresponding to the 37.5%–60% interphase, where nucleoids are normally found. In contrast, Mmf1p was exclusively localized in the upper part of the gradient (Fig. 10).

In order to determine the localization of Mmf1p in vivo, we fused the yeast protein with the GFP at the C terminus. The fusion protein was expressed in the yeast strain FY23, and its localization was analyzed by a confocal fluorescence microscope. The matrix space was visualized using a vital dye, Mito Tracker-Red, while mtDNA structures were made evident by DAPI staining. The Mmf1p-GFP fusion protein is not evenly distributed throughout the matrix, as can be observed by the Mmf1p-GFP/MitoTracker-Red double staining. In addition, Mmf1p-GFP appears to partially colocalize with mtDNA structures (Fig. 11 and data not shown). Together, the results of nucleoid purification experiments and the in vivo colocalization of Mmf1p-GFP indicate that Mmf1p may be part of a protein complex which is not directly in contact with the nucleoids.

Hmf1p can complement the Δ*mmf1* phenotype when targeted into mitochondria. The high homology between Hmf1p and Mmf1p suggests that the two proteins have some functional similarity. However, Hmf1p is not able to rescue the Δ*mmf1*-associated phenotype. This is most likely due to the fact that Hmf1p does not have a leader peptide (Fig. 1) and is not localized in mitochondria (Fig. 5). To establish whether Hmf1p, when targeted into mitochondria, is able to functionally replace Mmf1p, we fused the Mmf1p leader peptide to Hmf1p (LHmf1p). *Mmf1*/*mmf1*::*KAN* heterozygote diploid cells were transformed with a vector (pYX112) expressing LHmf1p. After sporulation and dissection of asci, we observed that the four haploid cells have a similar growth rate on glucose medium (Fig. 12A). A serial dilution drop test confirmed the data illustrated in Fig. 12A and also showed that the Δ*mmf1*/*LHmf1* cells maintain the mtDNA, being able to grow on glycerol medium at the same rate as the wild-type cells (Fig. 12B). To confirm that the ability of LHmf1p to complement the Δ*mmf1*-associated phenotype is indeed dependent on its subcellular localization, we determined the levels of Hmf1p in mitochondria of Δ*mmf1*/*LHmf1* cells by immunoblotting. As shown in Fig. 12C, Hmf1p is detected in Δ*mmf1* mitochondria only in cells transformed with the pYX112-*LHmf1* construct. These data indicate that Hmf1p and Mmf1p have a similar biological activity and are involved in similar cellular pathways, but in different subcellular compartments.

Mmf1p and mammalian p14.5 are functionally related proteins. Mmf1p has approximately 40% similarity to hp14.5 (Fig. 13A). To establish whether the function of members of the p14.5 family is conserved in lower and higher eukaryotic cells, we expressed the hp14.5 gene in heterozygote diploid cells (*Mmf1*/*mmf1*::*KAN*). Dissection of the asci showed that the human protein is able to rescue the phenotype associated with the disruption of *Mmf1* (Fig. 13B and C and data not shown). To investigate whether hp14.5 rescues the Δ*mmf1*-associated phenotype by a mechanism similar to that of the yeast protein, we determined the levels of hp14.5 in yeast mitochondria. Figure 13D shows that hp14.5 copurifies with mitochondria. Also, in the case of hp14.5, computer analysis of its amino acid sequence reveals the presence of a putative mitochondrial leader peptide (Fig. 13A) (10). To extend our analysis to other vertebrate members of p14.5, we determined whether rp14.5 is localized in mitochondria. The soluble and membrane fractions were prepared from rat liver mitochondria and analyzed by immunoblotting. As shown in Fig. 14, rp14.5 is exclusively localized in the mitochondrial matrix. Thus, it is highly likely that Mmf1p and mammalian p14.5's have a similar role in lower and higher eukaryotic cells, respectively.

DISCUSSION

Protein families which are involved in basic cellular pathways are highly conserved throughout evolution. The p14.5 family (YERO57c/YJGFc) is a group of small proteins which

FIG. 9. Mmf1p does not play a direct role in mtDNA replication. (A) Levels of Mmf1p in rho$^+$ Δ*mmf1* haploid cells isolated on glycerol medium. A sample of 50 μg of total protein extracts of wild-type and rho$^+$ Δ*mmf1* cells were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using anti-Mmf1p or anti-Hmf1p (loading control) polyclonal antibody. (B and C) mtDNA is lost after several generations in rho$^+$ Δ*mmf1* cells grown in glucose-containing medium. Wild-type and rho$^+$ Δ*mmf1* cells were precultured in SD for 12, 24, 36, and 48 h and plated on glycerol medium in serial dilution as described in the legend of Fig. 2 (B) or plated (10^4 cells) on 10-cm-diameter dishes containing SG (C). After 5 days at 30°C, the cell number was determined by counting. The results are the mean of three independent platings. (D) Δ*mmf1* cells show a temperature-sensitive defect on nonfermentable carbon sources. Serial dilution of wild-type and rho$^+$ Δ*mmf1* cells were plated on SD or SG medium and grown for 3 to 5 days at 30 or 37°C.

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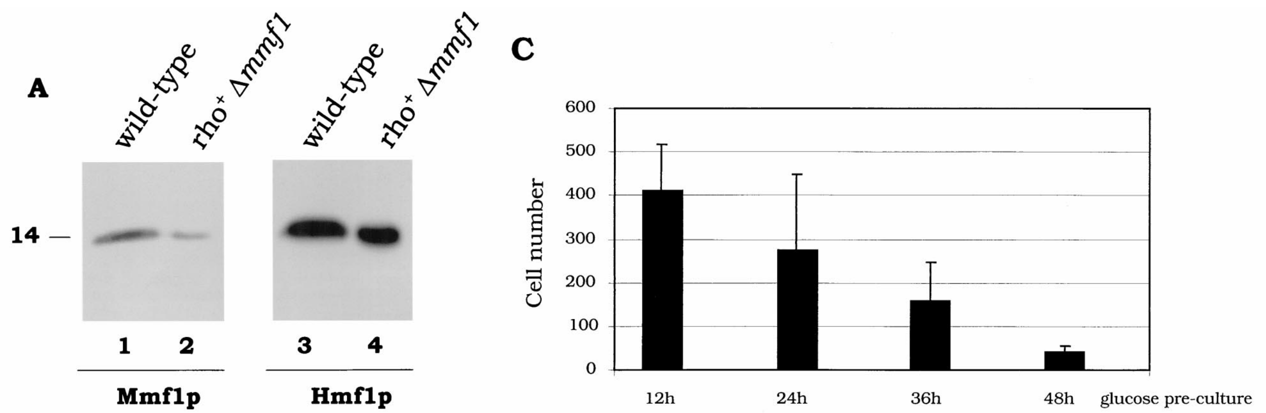

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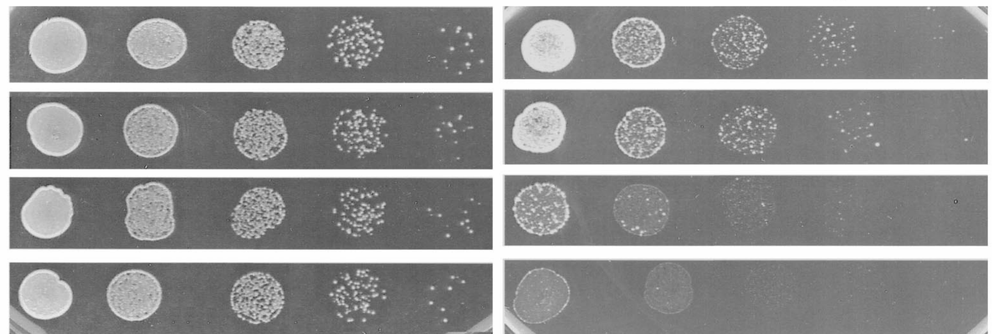

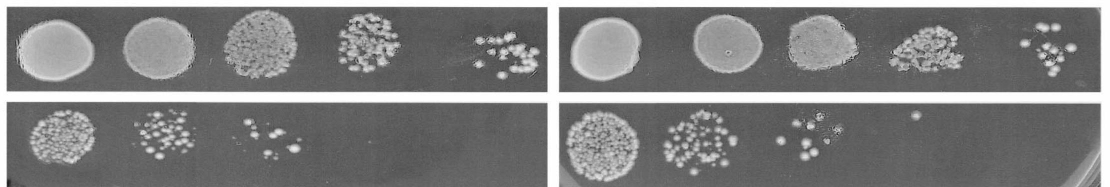

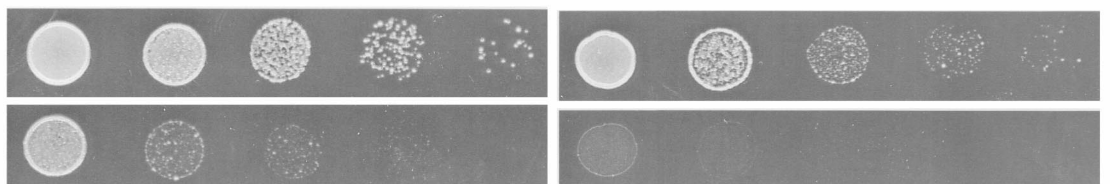

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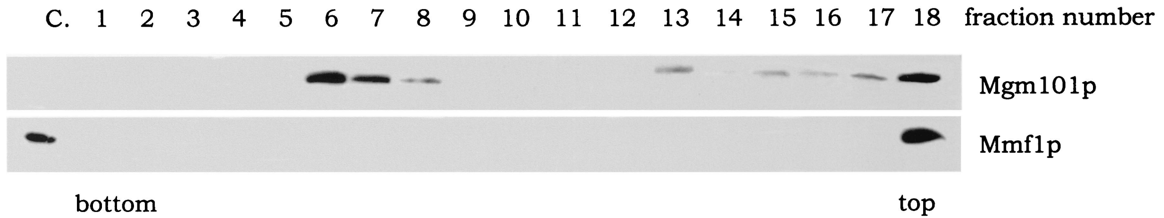

FIG. 10. Mmf1p does not copurify with mitochondrial nucleoids. Mitochondria were lysed by detergent treatment. Mitochondrial extract was applied on a step sucrose gradient and processed as described in Materials and Methods. The distribution of Mgm101p (a nucleoid-associated protein) and Mmf1p in each fraction of the sucrose gradient was determined by immunoblot analysis. Lane C, a positive control (100 ng of bacterial recombinant Mmf1p).

are present in bacteria, yeast, and higher eukaryotes whose function is poorly characterized. The completion of the genome sequence of the budding yeast *S. cerevisiae* has revealed that two members of the p14.5 family are present in this microorganism. Here, we have analyzed the biological properties of the two yeast proteins that we have named Mmf1p and Hmf1p. Replacement of *Mmf1* with a gene that confers resistance to kanamycin results in a loss of mtDNA and in a decreased growth rate. In contrast, similar replacement of *Hmf1* does not give rise to any visible phenotype. We show that Mmf1p and Hmf1p are localized in different subcellular compartments, being present in mitochondria and the cytoplasm, respectively. We demonstrate that, like the majority of mitochondrial proteins encoded by nuclear genes, Mmf1p has a leader peptide, which is 17 amino acids long and cleaved after the protein has reached the mitochondrion. Electron microscopy immunogold labeling shows that Mmf1p is present in the mitochondrial matrix, and analysis of the different submitochondrial fractions demonstrated that Mmf1p is a soluble protein of the matrix. Our findings indicate that Mmf1p does not have a direct role in replication of mtDNA. The mitochondrial genome can be maintained in Δ*mmf1* haploid cells, if the dissection of asci obtained by sporulation of *Mmf1*/*mmf1*::*KAN* heterozygote diploids is directly performed on plates containing a nonfermentable carbon source. However, the maintenance of mtDNA is strongly decreased in the absence of Mmf1p. For instance, the rho$^+$ Δ*mmf1* cells lose the mtDNA when grown under conditions that do not require mitochondrial respiration, e.g., in the presence of glucose. Moreover, on glycerol medium, the rho$^+$ Δ*mmf1* cells display a temperature-sensitive defect, being unable to grow at 37°C. Abf2p is a 20-kDa yeast protein which associates with mitochondrial nucleoids and is closely related to the nuclear high-mobility group proteins HMG1 and HMG2 from vertebrate cells (4–6). Δ*abf2* and Δ*mmf1* cells share some similarity in their phenotype. Analogously to *mmf1* null cells, disruption of *Abf2* causes a rapid loss of mtDNA when cells are grown in glucose, but not in glycerol-containing medium (6, 16, 17). In addition, a rho$^+$ Δ*abf2* strain, as does a rho$^+$ Δ*mmf1* strain, shows a temperature-sensitive defect on glycerol medium. However, our data provide evidence that Mmf1p and Abf2p have different roles in determining mtDNA stability. Indeed, we have shown that Mmf1p, in contrast to Abf2p, does not associate with mitochondrial nucleoids, although it partially colocalizes in vivo with mtDNA structures.

Overexpression of *Hmf1* does not restore the wild-type phenotype in *mmf1* null cells. This is due to the fact that Hmf1p lacks a leader peptide required for mitochondrial localization. Indeed, Hmf1p, when fused to the Mmf1p leader peptide, acquires the ability to localize in mitochondria and to rescue the Δ*mmf1*-associated phenotype. The fact that Mmf1p and

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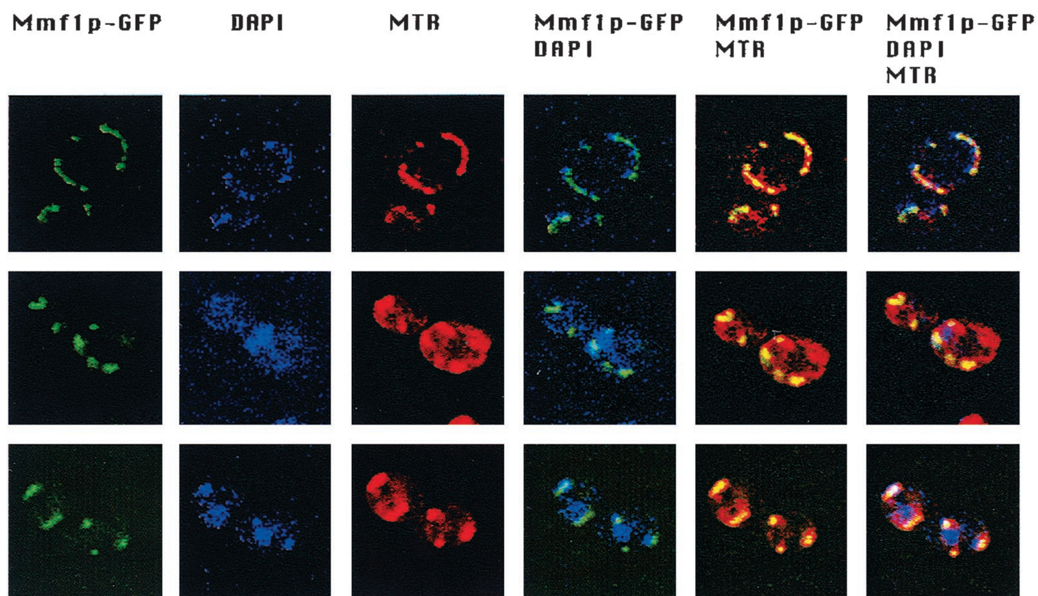

FIG. 11. Mmf1p-GFP fusion protein is located at specific sites of mitochondrial matrix. *Mmf1* has been cloned in frame at the C-terminal end with the GFP gene in the pYX112 vector and expressed in the yeast strain FY23. Live cells were incubated with DAPI or MitoTracker-Red (MTR) as described in Materials and Methods, and the localization of Mmf1-GFP was determined using a confocal microscope.

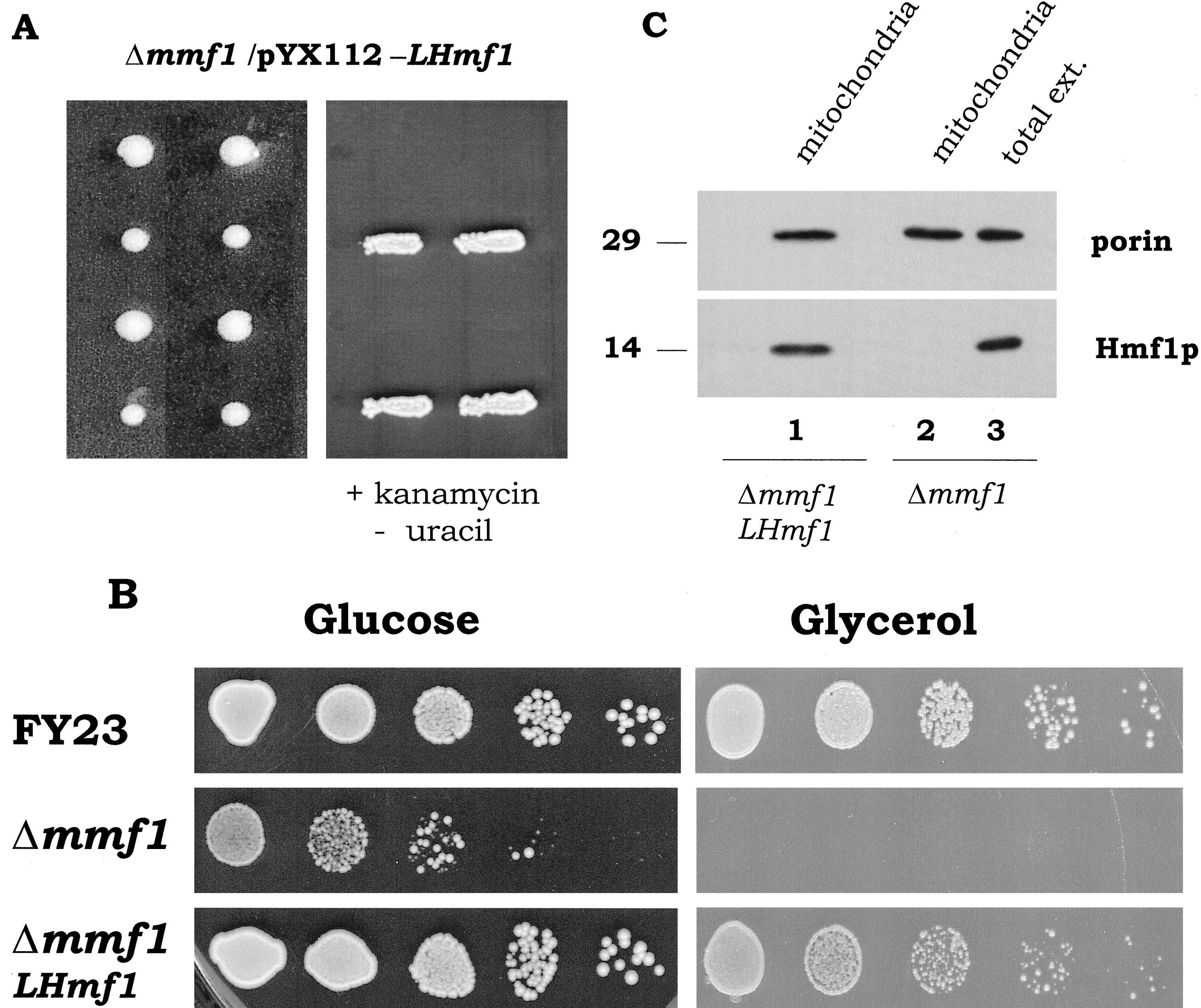

FIG. 12. Targeting of Hmf1p into mitochondria complements the Δ*mmf1* mutation. (A) LHmf1p is able to rescue the Δ*mmf1*-associated phenotype. Heterozygote FY1679 diploid cells (*Mmf1*/*mmf1*::*KAN*) were transformed with the pYX112-*LHmf1* construct. After sporulation, asci were dissected on YPD plates and haploid cells were grown for 5 to 6 days at 30°C. (B) Δ*mmf1*/*LHmf1* cells are able to grow on glycerol medium. Samples of 10 μl containing decreasing numbers of yeast cells (10^5, 10^4, 10^3, 10^2, 10^1) were plated on SD or SG and grew for 3 to 5 days at 30°C. (C) LHmf1p is targeted into mitochondria. Mitochondria were prepared from Δ*mmf1* and Δ*mmf1*/*LHmf1* cells, and protein content was analyzed by immunoblotting using anti-Hmf1p and anti-porin antibodies. Lane 1, 30 μg of mitochondrial protein extract of Δ*mmf1*/*LHmf1* cells; lane 2, 30 μg of mitochondrial protein extract of Δ*mmf1* cells; lane 3, 50 μg of total protein extract of Δ*mmf1* cells.

Hmf1p are functionally interchangeable when present in mitochondria indicates that the two proteins play a similar role in different subcellular compartments. Two independent studies have shown that human and rat p14.5 inhibit protein translation in vitro (19, 25). Based on these findings, it can be hypothesized that Mmf1p and Hmf1p are involved in the regulation of mitochondrial and cytoplasmic protein translation, respectively. However, our data exclude the possibility of an inhibitory role of Mmf1p and Hmf1p in protein translation. Mmf1p clearly has a positive function in mitochondria-associated events. Moreover, we observed that overexpression of Mmf1p or Hmf1p in wild-type cells does not result in a detectable inhibition of protein synthesis (data not shown).

Our study also demonstrates that Mmf1p is functionally related to the homologous rat and human proteins. hp14.5, when expressed in *S. cerevisiae*, is able to localize in mitochondria and to restore the growth deficiency of *mmf1* null cells on glycerol or glucose medium. Furthermore, isolation and fractionation of rat liver mitochondria showed that rp14.5, like Mmf1p, is a soluble protein of the mitochondrial matrix. It has been reported that factors involved in basic mitochondrial processes, like protein folding, stabilization, complex assembly, and membrane translocation, are highly conserved throughout evolution (11). Thus, it is possible that Mmf1p and Hmf1p have a chaperone-like role in mitochondria and cytoplasm, respectively. Indeed, it was recently shown that Mdj1p, a chaperone of the DnaJ family, is indirectly involved in mtDNA maintenance (7).

Besides the loss of mtDNA, disruption of *Mmf1* results in an increase of cell division time. Reintroduction of *Mmf1* in Δ*mmf1* cells, which lack mtDNA, restores the wild-type growth on glucose-containing medium. These findings indicate that Mmf1p has an additional mitochondrial function, which is important for the normal progression of the cell cycle and independent of the presence of mtDNA. In agreement with our

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A

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         1                                                   50
hp14.5 p   ~~~~~~~~~ ~~~~~~~ MSSLIRRV ISTAK APGAIGPYSQ AVLVDRTIYI
                                *      **   ** *   *** *       *
                              ***     ***   ** *** *** *  **  ***
Mmf1p     MFLRNSVLRT APVLRRG ITTL...TPVSTKL APPAAASYSQ AMKANNFVYV

          51                                                 100
hp14.5    SGQIGMDPSS GQLVSGGVAE EAKQALKNMG EILKAAGCDF TNVVKTTVLL
          ****   *      * *   *  * *   *    **         * **    *
          ****   *      * * ***  * *** *   ***  *   *  ****  ***
Mmf1p     SGQIPYTPDN .KPVQGSISE KAEQVFQNVK NILAESNSSL DNIVKVNVFL

          101                                                150
hp14.5    ADINDFNTVN EIYKQYFKSN FPARAAYQVA ALPKGSRIEI EAVAIQGPLT
          **   *   *   *   *     ***    **  **     *  *  *
          **  **  **  **   *     ***  * ** ***    **  ******
Mmf1p     ADMKNFAEFN SVYAKHFHTH KPARSCVGVA SLPLNVDLEM EVIAVEKN~~

          151
hp14.5    TASL

Mmf1p     ~~~~
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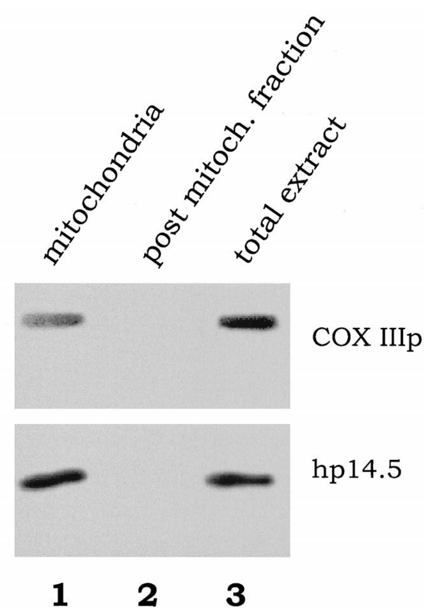

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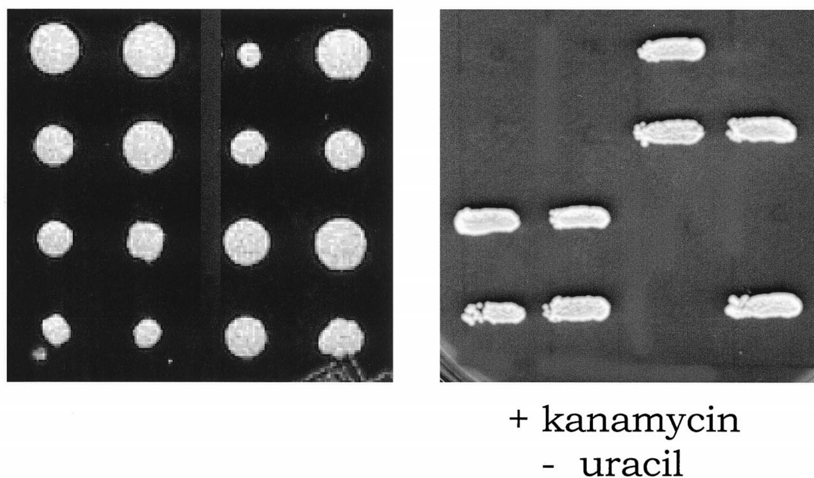

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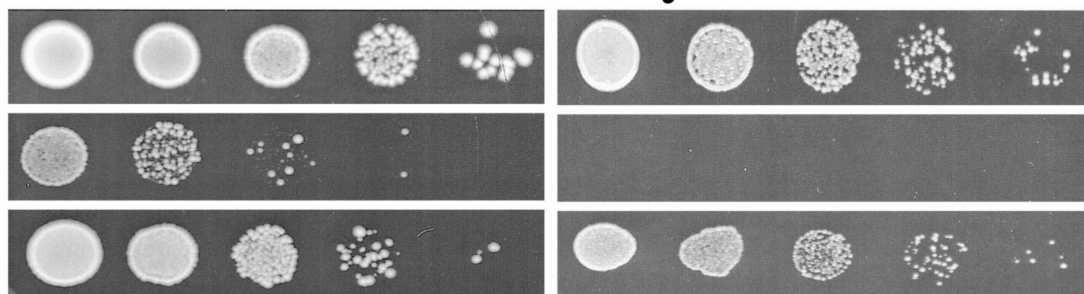

FIG. 13. Human p14.5 is functionally related to Mmf1p. (A) Alignment of hp14.5 and Mmf1p amino acid sequences. One or two stars indicate conserved or identical amino acids, respectively. The arrows indicate the determined and the predicted cleavage sites of the mitochondrial leader peptide in Mmf1p and hp14.5, respectively. (B) hp14.5 is able to rescue the Δ*mmf1*-associated phenotype. Heterozygote FY1679 diploid cells (*Mmf1*/*mmf1*::*KAN*) were transformed with a pYX112-hp14.5 construct. After sporulation, asci were dissected on YPD plates and haploid cells were grown for 5 to 6 days at 30°C. (C) Δ*mmf1*/hp14.5 cells are able to grow on glycerol medium. Samples of 10 μl containing decreasing numbers of yeast cells (10^5, 10^4, 10^3, 10^2, 10^1) were plated on SD or SG and grew for 3 to 5 days at 30°C. (D) Human p14.5 localizes in mitochondria of *S. cerevisiae*. The different cellular extracts were prepared as described in Materials and Methods, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting using anti-CoxIIIp or anti-hp14.5 specific polyclonal antibodies. Lane 1, 20 μg of mitochondrial protein extract; lane 2, 20 μg of supernatant of post-mitochondrial fraction; lane 3, 50 μg of total cellular protein extract.

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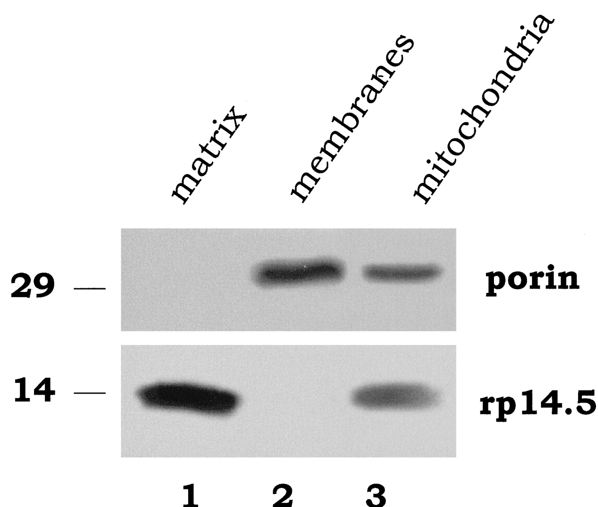

FIG. 14. Rat p14.5 is a soluble protein of mitochondrial matrix. Purified mitochondria from rat liver cells (kindly provided by Cesare Indiveri, University of Bari, Bari, Italy) were frozen and thawed in a buffer containing 250 mM NaCl. After centrifugation, the presence of rp14.5 in the matrix fraction was determined by immunoblot analysis using an anti-rp14.5 antibody. Lane 1, 20 μg of mitochondrial matrix protein extract; lane 2, 20 μg of mitochondrial membrane protein extract; lane 3, 20 μg of total mitochondrial protein extract. Molecular mass markers (in kilodaltons) are on the left.

findings, it has recently been shown in rho^0 human cells that additional mitochondrial functions can influence the growth rate. HeLa S3 and osteosarcoma 143B cells have lost the mtDNA and consequently do not express the mtDNA-coded F_0 subunits 6 and 8 of the mitochondrial ATP synthetase complex. Despite this fact, mitochondria of both cell lines possess normal levels of functional F_1-ATPase (2). Specific inhibition by aurovertin of F_1-ATPase strongly decreases rho^0 cell growth. These results demonstrate that F_1-ATPase contributes to an efficient cellular proliferation. Based on the findings in human cells, we can speculate that Mmf1p may be directly or indirectly involved in F_1-ATPase-associated cellular events.

In summary, in this study we have identified a function of a novel *S. cerevisiae* protein highly conserved throughout evolution. We have determined that Mmf1p has a role in mtDNA maintenance. Further work will be required to elucidate the precise mechanism of this process. In addition, we have demonstrated that members from different species of the p14.5 family are functionally related and involved in mitochondria-associated events.

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