

***MMMI* Encodes a Mitochondrial Outer Membrane Protein Essential for Establishing and Maintaining the Structure of Yeast Mitochondria**

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Abstract. In the yeast *Saccharomyces cerevisiae*, mitochondria are elongated organelles which form a reticulum around the cell periphery. To determine the mechanism by which mitochondrial shape is established and maintained, we screened yeast mutants for those defective in mitochondrial morphology. One of these mutants, *mmml*, is temperature-sensitive for the external shape of its mitochondria. At the restrictive temperature, elongated mitochondria appear to quickly collapse into large, spherical organelles. Upon return to the permissive temperature, wild-type mitochondrial structure is restored. The morphology of other cellular organelles is not affected in *mmml* mutants, and *mmml* does not disrupt normal actin or tubulin

organization. Cells disrupted in the *MMMI* gene are inviable when grown on nonfermentable carbon sources and show abnormal mitochondrial morphology at all temperatures. The lethality of *mmml* mutants appears to result from the inability to segregate the aberrant-shaped mitochondria into daughter cells. Mitochondrial structure is therefore important for normal cell function. *Mmm1p* is located in the mitochondrial outer membrane, with a large carboxyl-terminal domain facing the cytosol. We propose that *Mmm1p* maintains mitochondria in an elongated shape by attaching the mitochondrion to an external framework, such as the cytoskeleton.

ALTHOUGH mitochondria are ubiquitous organelles found in virtually all eukaryotic cells, mitochondria show striking variations in their location and structure in different cell types (for review see Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994; Thorsness, 1992). In many cells, mitochondria are dispersed throughout the cytosol as elongated thread-like organelles (Lewis and Lewis, 1915). Other cell types show a more organized distribution of mitochondria. For example, in muscle fibers, mitochondria are organized into arrays and stacked between rows of actin-myosin bundles (Munn, 1974; Tandler and Hoppel, 1972). In spermatozoa, mitochondria often fuse to form a long tube that wraps around the base of the flagellum in a tight spiral (Koehler, 1966; Rohde et al., 1991). Eukaryotic cells apparently position mitochondria near cellular structures that require large amounts of ATP. Very little is known, however, about how mitochondrial structure and position are established or maintained in different cell types.

The yeast *Saccharomyces cerevisiae* dramatically regulates the shape, size, and number of its mitochondria according to the physiological state of the cell. Under anaerobic conditions, mitochondria are barely detectable (Wallace et al., 1968), and form small organelles called "promitochondria" (Criddel and Schatz, 1969; Plattner and Schatz, 1969). When yeast are grown aerobically, particularly under condi-

tions where oxidative phosphorylation is the primary source of ATP (e.g., growth on nonfermentable carbon sources), mitochondria become greatly enlarged and elongated, and are located at the cell periphery (Hoffman and Avers, 1973; Stevens, 1981). This location is proposed to position the mitochondria near the point of entry of oxygen, and the elongated structure is proposed to facilitate the rapid conduction of energy throughout the cell. During meiosis and sporulation, mitochondria fuse to form a branched network that encircles the dividing nuclei (Zickler and Olsen, 1975; Miyakawa et al., 1984). Not only the shape and size, but also the number of mitochondria in a yeast cell can vary. Under most growth conditions, yeast have from one to ten elongated mitochondria per cell (Hoffman and Avers, 1973; Stevens, 1981). Since mitochondria are dynamic organelles, frequently fusing and dividing (Bereiter-Hahn and Vöth, 1994) the absolute number of mitochondria per cells is not constant. When cells enter stationary phase, mitochondria break up to form ~50 separate organelles in each cell (Stevens, 1977).

To determine the mechanism by which mitochondrial shape is established and to understand the role that mitochondrial shape plays in cell function, we screened a collection of yeast mutants for those defective in mitochondrial morphology. One of these mutants, *mmml*, is temperature-sensitive (ts)¹ for the shape of its mitochondria. At the re-

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1. *Abbreviations used in this paper:* Cox IV, cytochrome oxidase; Cyt. b₂, cytochrome b₂; HA, hemagglutinin; ts, temperature-sensitive.

strictive temperature, elongated mitochondria are no longer seen in *mmml* cells. Instead, the mitochondria appear as large sphere-shaped organelles. In time course studies, each elongated mitochondrion appears to quickly collapse into this large spherical structure. Upon return to the permissive temperature, normal mitochondrial structure is restored. The *mmml* defect does not affect the shape of other cellular organelles or major cytoskeletal elements, indicating that *mmml* is specific to mitochondria. The Mmml protein is located in the mitochondrial outer membrane, with a large carboxyl-terminal domain facing the cytosol. *MMMI* is a new gene and our results suggest that the Mmml protein maintains mitochondria in an elongated conformation by mediating the binding of the organelle to the cytoskeleton or some other external framework. Furthermore, *mmml* mutants are defective in transmitting the altered mitochondria to daughter cells, suggesting that mitochondrial shape is critical for normal cell function.

Materials and Methods

Strains and Relevant Genotypes

mmml-1 was identified from a collection of ts yeast mutants generated in *MAT α leu2- Δ 1 trp1- Δ 1 his3- Δ 200* strain YH8 (Xu and Boeke, 1990; a gift from J. Boeke). YSB105 and YSB104 were obtained as follows. First, the original *mmml* mutant was crossed to *MAT α* strain YPH250 (Sikorski and Hieter, 1989). A *MAT α* segregant that carried *mmml-1* (and not the unlinked ts lethal defect) was identified and backcrossed to YPH250. *MAT α mmml-1 ura3-52 leu2- Δ 1 trp1- Δ 1 his3 ade2-101 lys2-801* strain YSB105 and *MAT α mmml-1 ura3-52 leu2- Δ 1 trp1- Δ 1 his3 ade2-101 lys2-801* strain YSB104 were identified among the segregants from the second backcross. YSB106 (*MAT α /MAT α mmml-1/ura3-52/ura3-52 leu2- Δ 1/leu2- Δ 1 trp1- Δ 1/trp1- Δ 1 ade2-101/ade2-101 lys2-801/lys2-801*) was obtained by mating YSB105 to YSB104. *MAT α /MAT α* strain 501 (Sikorski and Hieter, 1989) and strain 410 (Ryan and Jensen, 1994) have been described. Yeast media, including YEP medium and SD medium, have been described (Sherman et al., 1982). Standard yeast molecular genetic techniques were used (Rose et al., 1988).

Identification of *mmml*

Approximately 1,300 ts yeast strains were patched on plates of YEP medium with 2% glucose as the carbon source and grown at 25°C. Patches were replicated to YEP glucose medium and incubated at 37°C for 4–6 h. A small amount of cells from each patch was added to 10 μ l of distilled water containing 1 μ g/ml 3,3'-dihexyloxycarbocyanine (DiOC₆) (Molecular Probes, Eugene, OR). The cell suspensions were immediately transferred to glass slides, and the yeast strains examined using a Zeiss Axiophot fluorescent microscope using a 100 \times objective and an excitation wavelength of 450–490 nm with a barrier filter of 515–565 nm.

Confocal Microscopy of Yeast Cells

Yeast strains were grown to saturation in YEP medium containing 2% glucose or 2% glycerol as the carbon source. Cells were diluted and grown to an OD₆₀₀ of \sim 0.3 in the appropriate medium, and then incubated at either 23 or 37°C. 2.5 μ l of cells were placed on glass slides and 0.5 μ l of a 5 mg/ml solution of DiOC₆ in distilled water was added. The cells were immediately examined using an MRC-600 (BioRad Labs., Hercules, CA) confocal imaging system with a 100 \times objective (Olympus). An argon ion laser source was filtered at 488 nm excitation with a barrier filter of 510–515 nm. Images were collected and processed using CoMOS software (ver. 6.03; BioRad Labs.). Optical sections in the z-axis ranged from 0.7 to 1 μ m. Since diploid yeast cells are larger than haploid cells, we used *MAT α /MAT α mmml-1/mmml-1* strain YSB106 to increase the resolution of our microscopy. No difference in mitochondrial morphology was observed between haploid and diploid cells. To stain cells with MitoTracker (Molecular Probes), cells were incubated with 1 μ M MitoTracker for 30 min at the indicated temperature, washed three times with PBS, and then placed on glass

slides. To immobilize cells for photography or imaging, 4 μ l of a cell suspension were mixed on a glass slide with 4 μ l of 1% low-melting point agarose (GIBCO BRL, Gaithersburg, MD) that had been incubated at 45°C. A glass coverslip was immediately placed on top of the cell-agar mixture, sealed with nail polish and examined.

Isolation and DNA Sequence of the *MMMI* Gene

mmml-1 leu2 strain YSB105 was transformed (Schiestl and Gietz, 1989) with a library of yeast genomic DNA fragments inserted into the BamHI site of the *LEU2*-containing YEp3 plasmid (Nasmyth and Tatchell, 1980; a gift from K. Nasmyth). 30,000 Leu⁺ transformants were selected at 23°C, and then tested for growth at 37°C on YEP medium containing 2% glycerol as the carbon source. Four colonies were isolated that grew at 37°C, and plasmid DNA was obtained from each colony (Hoffman and Winston, 1987). Restriction endonuclease digestions showed that all four plasmids contained identical genomic DNA inserts. To localize the region of *MMMI* activity, restriction fragments from the cloned DNA were inserted into the *LEU2-CEN6* vector, pRS315 (Sikorski and Hieter, 1989). pSB2, which carries a 5-kbp HindIII fragment inserted into pRS315, complemented the *mmml* defect.

The DNA sequence of *MMMI* was determined as follows. First, a series of overlapping deletions of *MMMI* were isolated from the HindIII site through the 3'-end of the gene. pSB2 was digested with ClaI and *MMMI* sequences were removed with exoIII using an Erase-a-Base system (Promega Corp., Madison, WI) according to manufacturers' instructions. To prevent exonuclease digestion of vector sequences, pSB2 was digested with XhoI and the DNA ends filled in with α -thio dNTPs (Stratagene, La Jolla, CA) using DNA polymerase (Putney et al., 1981). Using this collection of deletions, one strand of *MMMI* was sequenced (Sanger et al., 1977). The other strand of *MMMI* and both strands of sequence upstream of the HindIII site were sequenced using a 373A (Appl. Biosystems, Inc., Foster City, CA) automated sequencer and synthetic oligonucleotides spaced approximately 300 bases apart.

MMMI Gene Disruption

mmml::URA3, which replaces a large portion of the *MMMI* open reading frame with the yeast *URA3* gene, was constructed as follows. pSB3 was made by inserting the 5-kbp HindIII fragment, carrying most of the *MMMI* coding sequences, into plasmid pEMBL8 (Dente et al., 1983). pSB3 was digested with NruI and BstXI, and the DNA ends made blunt with DNA polymerase. A 1.1-kbp HindIII fragment containing the *URA3* gene was isolated from YEp24 (Botstein et al., 1979) and the DNA ends were filled in with DNA polymerase. The *URA3* gene was then inserted into NruI–BstXI cut pSB3. *mmml::URA3*, carried on a 5.3-kbp HindIII fragment, was used to replace one of two copies of *MMMI* in *MAT α /MAT α ura3/ura3* diploid strain 410 (Rothstein, 1991). Stable Ura⁺ transformants were isolated, and Southern analysis of one diploid, YSB108, confirmed that one of the two copies of *MMMI* was replaced by *mmml::URA3* (not shown). YSB108 was sporulated and the meiotic products allowed to germinate on medium containing 2% glucose (YEPglucose) at 23°C. These segregants were shown to carry *mmml::URA3* by their ability to grow on medium lacking uracil (SD-URA). We also found that *mmml::URA3* cells were unable to grow on medium containing 2% glycerol (YEPgly) or 2% lactate (YEP lactate) as the sole carbon source, even after incubation for 14 d.

To confirm that we had cloned and disrupted the *bona fide* *MMMI* gene, we crossed a *MAT α mmml::URA3* segregant to the temperature-sensitive *mmml* mutant strain YSB105 and analyzed the meiotic products. In 14 tetrads, we found no recombination between *mmml-1* and *mmml::URA3*, indicating that *mmml::URA3* integrated within 3.6 cM of *mmml-1*. Hybridization of the cloned *MMMI* gene to separated yeast chromosomes (Clontech Laboratories, Inc., Palo Alto, CA) indicated that *MMMI* is located on chromosome XII. Hybridization to an overlapping set of lambda and cosmid clones of genomic yeast DNA (Riles et al., 1993) showed that *MMMI* maps adjacent to the chromosome XII centromere (page 3609).

Construction of An Epitope-tagged Version of Mmmlp

pSB11, which contains a unique NotI site immediately preceding the termination codon of *MMMI*, was constructed as follows. Using pSB2, oligonucleotide No. 106 (5'-CCAGCGGCCGATAACTCTGTAGGCTTTTC-3') and oligonucleotide No. 99 (5'-AATACGACTCACTATAG-3'), we isolated a 1.3-kbp DNA fragment using the polymerase chain reaction (Saiki et al., 1985). We digested this PCR fragment with EcoRV and NotI. pJE7 is a

LEU2-CEN6 vector which carries a 2.2-kbp *SacI*-*BamHI* fragment containing the *MAS6* gene with a unique *NotI* site preceding the *MAS6* termination codon (Emtage and Jensen, 1993). We digested pJE7 with *SacI*, made the DNA ends blunt with DNA polymerase, and digested the plasmid with *NotI*. We ligated the *MMMI*-containing PCR product into pJE7 to form pSB11. pSB12, which encodes *Mmmlp* with the hemagglutinin (HA) epitope at its carboxyl terminus was constructed by inserting a 114-bp *NotI* fragment containing three tandem copies of the HA epitope (Field et al., 1988; Tyers et al., 1992; a gift from B. Futcher) into pSB11. Since pSB12 lacks upstream *MMMI* sequences, we reconstituted *MMMI* by integration into the yeast chromosome. A 2.3-kbp *HindIII* fragment, carrying the *MMMI-HA* fusion, was inserted into the *LEU2* integrating vector pRS305 (Sikorski and Hieter, 1989) to form pSB13. pSB13 was transformed into *MAT α leu2* strain YPH252 (Sikorski and Hieter, 1989). We targeted the integration to the chromosomal *MMMI* gene by digesting pSB13 with *NruI*. Stable *Leu*⁺ transformants were isolated and tested for expression of *Mmmlp-HA*. We showed that immunoblots of proteins isolated from strain YSB107 contained a single protein of 52 kD when decorated with antibodies specific to the HA epitope (Niman et al., 1983). Integration of the pSB1 plasmid inactivates the chromosomal *MMMI*. Strain YSB107 thus expresses only the *Mmmlp-HA* fusion protein and does not express wild-type *Mmmlp*. We found that YSB107 grew on both glucose and glycerol-containing medium at 23, 30, and 37°C indicating that the *Mmmlp-HA* fusion protein is fully functional.

Subcellular and Submitochondrial Localization of *Mmmlp-HA*

Strain YSB107, which expresses the *Mmmlp-HA* fusion protein, was grown to OD₆₀₀ of 1.0 in synthetic complete medium containing 2% galactose and supplemented with the appropriate amino acids. Yeast extract was added to 1% and glycerol to 2%, and the cells were grown to an OD₆₀₀ of ~2.0. Yeast cells were converted to spheroplasts, homogenized, and separated into a 9,600 g mitochondrial pellet and a post-mitochondrial supernatant as described (Daum et al., 1982). Preparation of mitochondrial membrane vesicles, and the separation of outer membrane and inner membrane were as described (Emtage and Jensen, 1993). Proteins were separated by SDS-PAGE (Haid and Suissa, 1983; Laemmli, 1970), and transferred (Haid and Suissa, 1983) to Immobilon filters (Millipore Corp., Bedford, MA). The *Mmmlp-HA* fusion protein was identified by incubation of filters with a 1:25 dilution of culture supernatant from 12CA5 cells (Niman et al., 1983). Marker proteins were identified by incubation with antisera to the following proteins: the β -subunit of the F₁-ATPase (a gift from M. Yaffe), subunit IV of cytochrome oxidase (Jensen and Yaffe, 1988), hexokinase (a gift from M. Yaffe), and OM45p (Yaffe et al., 1989). Immune complexes were visualized using HRP-conjugated secondary antibody (Amersham Corp., Arlington Heights, IL) followed by chemiluminescence (ECL, Amersham Corp.). To further purify mitochondria, 10 mg of mitochondria from the 9,600 g fraction (see above) were added to a 25-ml solution containing 40% Percoll™ (Pharmacia LKB Biotechnology, Piscataway, NJ), 0.6 M Mannitol, and 20 mM Hepes-KOH, pH 7.4, and centrifuged at 60,000 g for 45 min in a fixed angle rotor. 1-ml fractions were taken from the Percoll gradient and proteins from a 40- μ l aliquot were transferred to Immobilon filters using a 96-well vacuum blotter (BioRad Labs.). The *Mmmlp-HA* fusion protein and the β -subunit of the F₁-ATPase were identified by chemiluminescence as described above. Immunoblots were quantitated using a UMAX UC630 scanner and Collage (Fotodyne) analysis software.

To determine the orientation of *Mmmlp* in the outer membrane, mitochondria isolated from strain YSB107 were suspended in Breaking buffer (0.6 M Mannitol; 20 mM Hepes-KOH, pH 7.4) at 10 mg/ml. Aliquots representing 100 μ g mitochondria were digested with 50 μ g/ml or 500 μ g/ml trypsin (Sigma Chem. Co., St. Louis, MO) for 30 min on ice, followed by the addition of soybean trypsin inhibitor (Sigma Chem. Co.) to 2 mg/ml. To disrupt the outer membrane, mitochondria were diluted with 9 vol of OS buffer (20 mM Hepes-KOH, pH 7.4) and incubated at 0°C for 20 min. Mitochondria and mitoplasts were recovered after trypsin digestion by centrifugation at 9,600 g for 10 min, and subjected to SDS-PAGE. Proteins transferred to Immobilon membranes were decorated with antibodies to the HA epitope, cytochrome b₂ (Cyt. b₂) (a gift from G. Schatz), and subunit IV of cytochrome oxidase, and detected by chemiluminescence as described above.

Electron Microscopy

Yeast cells were grown in YEP medium containing 2% glycerol to an

OD₆₀₀ of 0.5, and the cultures were then incubated at 23 or 37°C for an additional three hours. Cells were isolated by centrifugation and prepared for electron microscopy as described (McDonald, 1984) with the following modifications. Cells were fixed by adding an equal volume of 8% paraformaldehyde and 4% glutaraldehyde to suspended cells, followed by incubation at 23°C for 3 h. Cells were postfixed by adding a solution of 4% potassium permanganate in 0.1 M sodium cacodylate, pH 7.4, and rotating suspended cells gently at 4°C for 60 min. Cells were washed in distilled water and cell pellets were stained with 2% uranyl acetate for 1 h. Before infiltration of the Spurr's resin, cell pellets were dehydrated with a graded series of ethanol. Infiltration of the resin was increased by incubation under vacuum (15 psi) for 4 h, followed by incubation at 15 psi overnight at 60°C. 70-nm ultrathin sections were cut with a low angle diamond knife (Diatome U.S., Fort Washington, PA), and placed on 400-mesh copper grids. Grids were stained for 5 min with 0.3% lead citrate and examined using a Zeiss 10 transmission electron microscope at 60 and 80 kV.

Miscellaneous

The rate of respiration-driven protein synthesis was determined as described (Yaffe and Schatz, 1984) with the following modifications. Yeast strains were grown at 23°C in YEP medium containing 2% lactate as the sole carbon source to an OD₆₀₀ of 0.6. Cells were collected by centrifugation, resuspended at a concentration of 10 OD₆₀₀/ml, and incubated at either 23°C or 37°C for 90 min. [³⁵S]Methionine (Amersham Corp.; 1,000 Ci/mmol) was added to 5 μ Ci/ml and 0.1 ml aliquots of cells were taken at various times. Cells were lysed in 2N NaOH/1 mM β -mercaptoethanol (Yaffe and Schatz, 1984). Proteins were precipitated with ice-cold 5% TCA and collected on GF/C filters (Whatman). The [³⁵S]methionine incorporation was determined using a Beckmann LS 7000 scintillation counter. The rate of incorporation was determined from the slope of the plot of incorporation vs time.

Oxygen consumption rates were measured polarographically using a Clarke-type electrode (Yellow Springs Instruments, Yellow Springs, OH) inserted into a water-jacketed sealed glass chamber. The oxygen electrode was connected to a chart recorder which was calibrated between 0 and 100% with atmospheric oxygen. Cells were grown to an OD₆₀₀ of 0.5 in YEP medium containing 2% glycerol as the sole carbon source. An aliquot of the culture was then shifted to 37°C and grown for 120 min. 1.6 ml of cells grown at either 23 or 37°C were added to the chamber and the temperature was maintained using a circulating water bath. Rates of oxygen consumption in change in percentage of saturation/min were read directly from the chart recording and converted to ng atoms of oxygen/min as described (Nakashima et al., 1984).

Results

mmml Mutants Are Temperature-Sensitive for Normal Mitochondrial Morphology

To identify gene products required for normal mitochondrial morphology, we screened a collection of yeast mutants for those with abnormal mitochondrial structure. Since mitochondria are essential for yeast viability even when cells are grown on glucose-containing medium (Jensen and Yaffe, 1988; Kováčová et al., 1968), we anticipated that defects in mitochondrial morphology might also be lethal, and therefore screened conditional lethal mutants. Approximately 1,300 yeast mutants were grown at the permissive temperature (23°C), replicaplated to the restrictive temperature (37°C), and allowed to grow for ~4 h. Individual mutant colonies were then incubated with DiOC₆, a fluorescent dye that when present at low concentrations preferentially stains mitochondria (Koning et al., 1993; Pringle et al., 1989; Weisman et al., 1990). Examination by fluorescence microscopy identified 24 candidates that showed abnormal mitochondrial morphology. One of these mutants defective in maintenance of mitochondrial morphology, *mmml*, was chosen for further study.

When wild-type yeast cells grown on nonfermentable

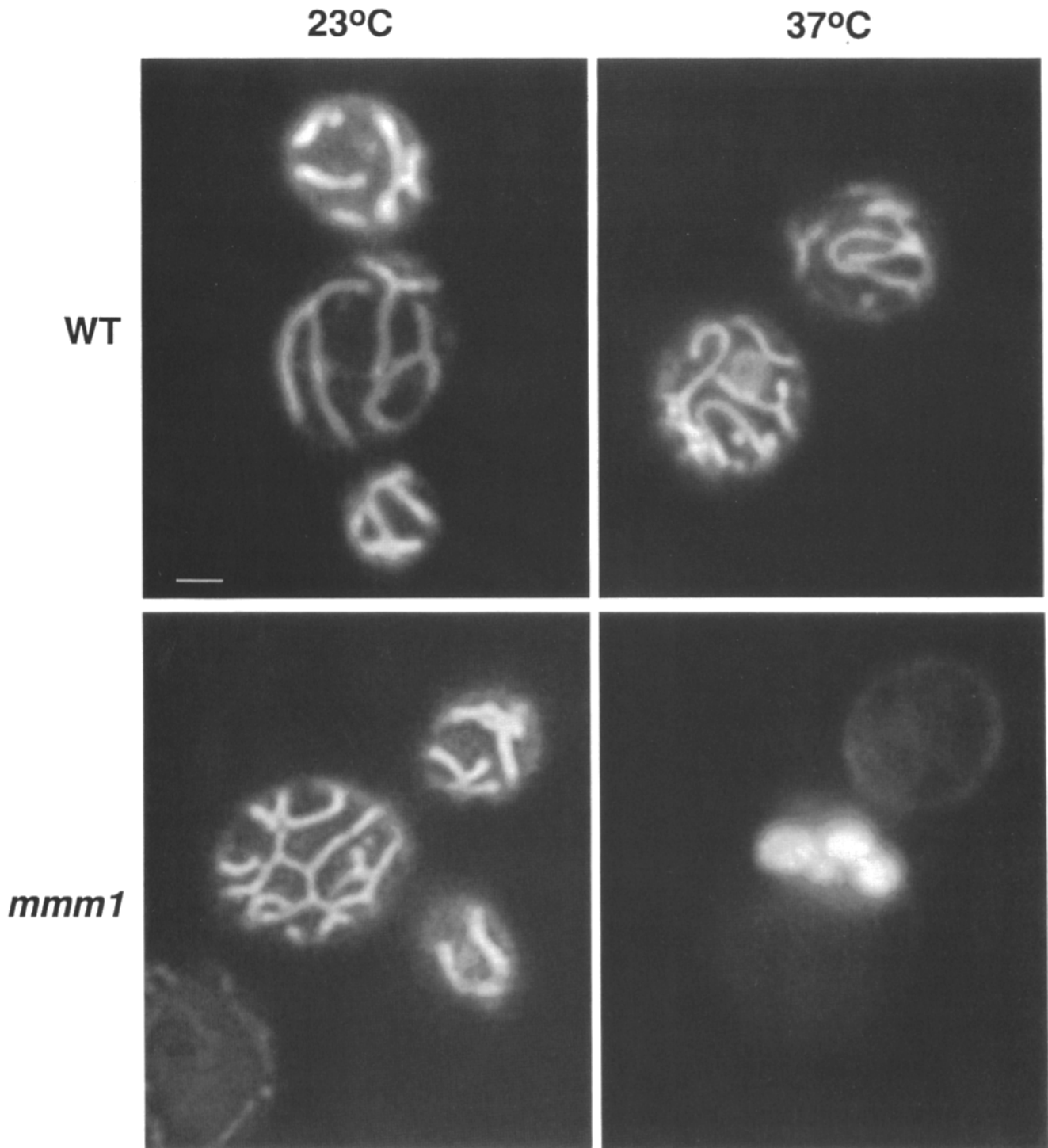


Figure 1. *mmm1* mutants are temperature-sensitive for normal mitochondrial morphology. *mmm1* strain YSB106 and wild-type (*WT*) strain YPH501 were grown at 23°C in YEP medium containing 2% glycerol. Aliquots from both *mmm1* and *WT* cells grown at 23°C were shifted to 37°C for 90 min. Cells were stained with DiOC₆ as described in Materials and Methods, and confocal images of representative cells were taken. Bar in the lower left corner of the top left panel represents 1 μ m.

carbon sources such as glycerol are stained with DiOC₆, mitochondria were seen as long, thread-like organelles with occasional branches (Fig. 1, top row). The number of mitochondria per cell was difficult to determine by light microscopy and appeared to vary among cells. However, we estimate that each cell contained approximately ten elongated mitochondria, and that these organelles were found at the pe-

riphery of the cell. Mitochondria were often seen extending the length of a cell, or even stretching from the mother cell into the daughter bud during cell division. Mitochondria in *mmm1* mutants grown at 23°C were indistinguishable from wild-type mitochondria (Fig. 1, lower left panel). In contrast, we observed that mitochondrial shape was dramatically altered when we grew *mmm1* mutants at 37°C and

stained them with DiOC₆ (Fig. 1, lower right panel). Normal, thread-like mitochondria were completely absent, and instead, 5–15 sphere-shaped organelles were seen in each cell. The average diameter of the spheres was $\sim 1 \mu\text{m}$. Each of these sphere-shaped structures also stained with the DNA-specific fluorescent dye, 4',6-diamidino-2-phenylindole (DAPI; not shown), indicating that they contained DNA and were indeed mitochondria. Although the altered mitochondria are roughly similar in size and shape to normal nuclei, our staining procedures allowed us to distinguish mitochondria from the nucleus. Further evidence that these DiOC₆ and DAPI-staining organelles are indeed mitochondria is described below. We observed that the loss of normal mitochondrial morphology was rapid in *mmml* mutants and the resulting sphere-shaped organelles persisted for hours at the nonpermissive temperature. When a culture of the *mmml* strain YSB106 was shifted to 37°C, we found that within 60 min the mitochondria in greater than 80% of the cells were altered in shape (Fig. 2). By 90 min, all mitochondria in all cells were spherical. The number and the morphology of the mitochondria remained unchanged even after 7 h of incubation at the nonpermissive temperature. The altered mitochondrial morphology in *mmml* mutants was also seen when cells were grown on glucose-containing medium. However, fewer large, sphere-shaped organelles (one to four organelles per cell) were seen as compared to cells grown on glycerol (see Fig. 10 A).

Electron microscopy confirmed that the shape of the mitochondria was dramatically altered in *mmml* mutants. *mmml* and wild-type cells were grown at either 23 or 37°C, and we examined ultrathin sections of these cells under the electron microscope (Fig. 3). In wild-type cells (Fig. 3 B, both panels) and in *mmml* mutants grown at 23°C (Fig. 3 A, top panels), many sections examined showed at least one thread-like mitochondrion which we concluded was cut longitudinally, and several small, round mitochondria that were apparently cut in cross-section. In *mmml* cells grown at 37°C,

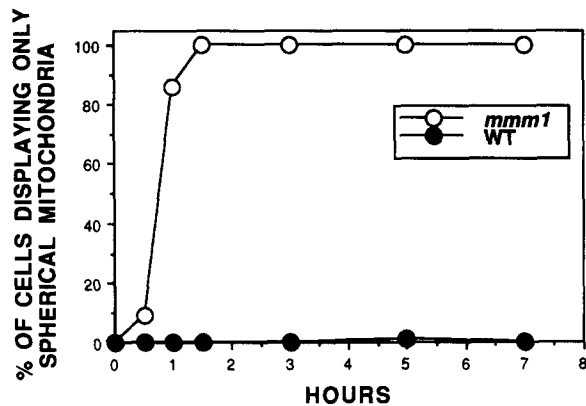


Figure 2. Loss of normal mitochondrial morphology is rapid in *mmml* mutants and the resulting sphere-shaped mitochondria persist at the nonpermissive temperature. *mmml* strain YSP106 and wild-type strain YPH501 were grown at 23°C in YEP medium containing 2% lactate to an OD₆₀₀ of 0.5. Cells were shifted to 37°C, aliquots removed at the indicated times and stained with DiOC₆ as described above. 100 cells were examined at each time point and the percent of cells that contained only spherical mitochondria noted.

however, every mitochondrion observed was large and spherical in shape (Fig. 3 A, lower panels). No elongated structures were seen in any sections. These results compliment our light microscopy studies and show that the *mmml* defect caused mitochondria to take on a round shape. Although the external shape of the mitochondria changes in *mmml* mutants, other aspects of mitochondrial structure were normal. We observed that both the inner and outer membranes were present, and that inner membrane cristae were clearly visible (Fig. 3 A, lower left panel). As described above, we also found that the altered mitochondria contained DNA.

We found that the mitochondrial morphology defect in the *mmml* mutant was caused by a single genetic lesion, and that the *mmml-1* mutation caused its growth on nonfermentable carbon sources. Specifically, we crossed the *mmml* mutant to a wild-type strain, and examined the meiotic segregants from the resulting diploid. We first found that the original *mmml* mutant carried two mutations: one mutation causing a mitochondrial morphology defect at 37°C (called *mmml-1*), and a second mutation conferring a ts growth defect on all carbon sources. When segregants carrying only *mmml-1* were crossed to wild-type strains, we observed 2:2 segregation of the temperature-sensitive mitochondrial morphology defect in all tetrads. Hence the morphology defect was the result of a single mutation. All segregants showed normal mitochondrial shape when grown at 23°C. Cells containing the *mmml-1* mutation would not grow on glycerol or lactate-containing medium at 37°C, but grew at rates comparable to wild-type cells on all carbon sources at 23°C. Therefore, *mmml* mutants are temperature-sensitive for both normal mitochondrial morphology and for growth on nonfermentable carbon sources.

The morphology of cellular structures other than mitochondria was not defective in *mmml* mutants. DAPI staining of *mmml* cells incubated at the restrictive temperature showed that nuclear morphology was not significantly altered (not shown). Furthermore, in contrast to the mitochondria, electron microscopy of *mmml* mutants showed that nuclei and other organelles such as vacuoles were present and normal in their appearance (Fig. 3 A, lower panels). As described below (see Discussion), mitochondria often colocalize with cytoskeletal elements. Consequently, a defect in yeast microtubules or microfilaments might be expected to affect mitochondrial shape. We found, however, that the distribution of actin and tubulin was not significantly altered by the *mmml-1* mutation (Fig. 4). When *mmml* cells were shifted to 37°C for 120 min, the number and length of the mitotic spindles, as well as the appearance of cytoplasmic microtubules, were indistinguishable from those seen in wild-type cells (see Fig. 4, upper panels). Furthermore, *mmml* mutants contained the same average number of actin cortical patches and cables as seen in wild-type cells (see Fig. 4, lower panels). The *mmml* defect therefore appears to be specific to mitochondria.

Normal Mitochondrial Shape Is Rapidly Lost in *mmml* Mutants Shifted to the Restrictive Temperature and Is Regained after Return to the Permissive Temperature

To further probe the function of *MMMI*, we examined mitochondria in cells that carry the *mmml-1* mutation after in-

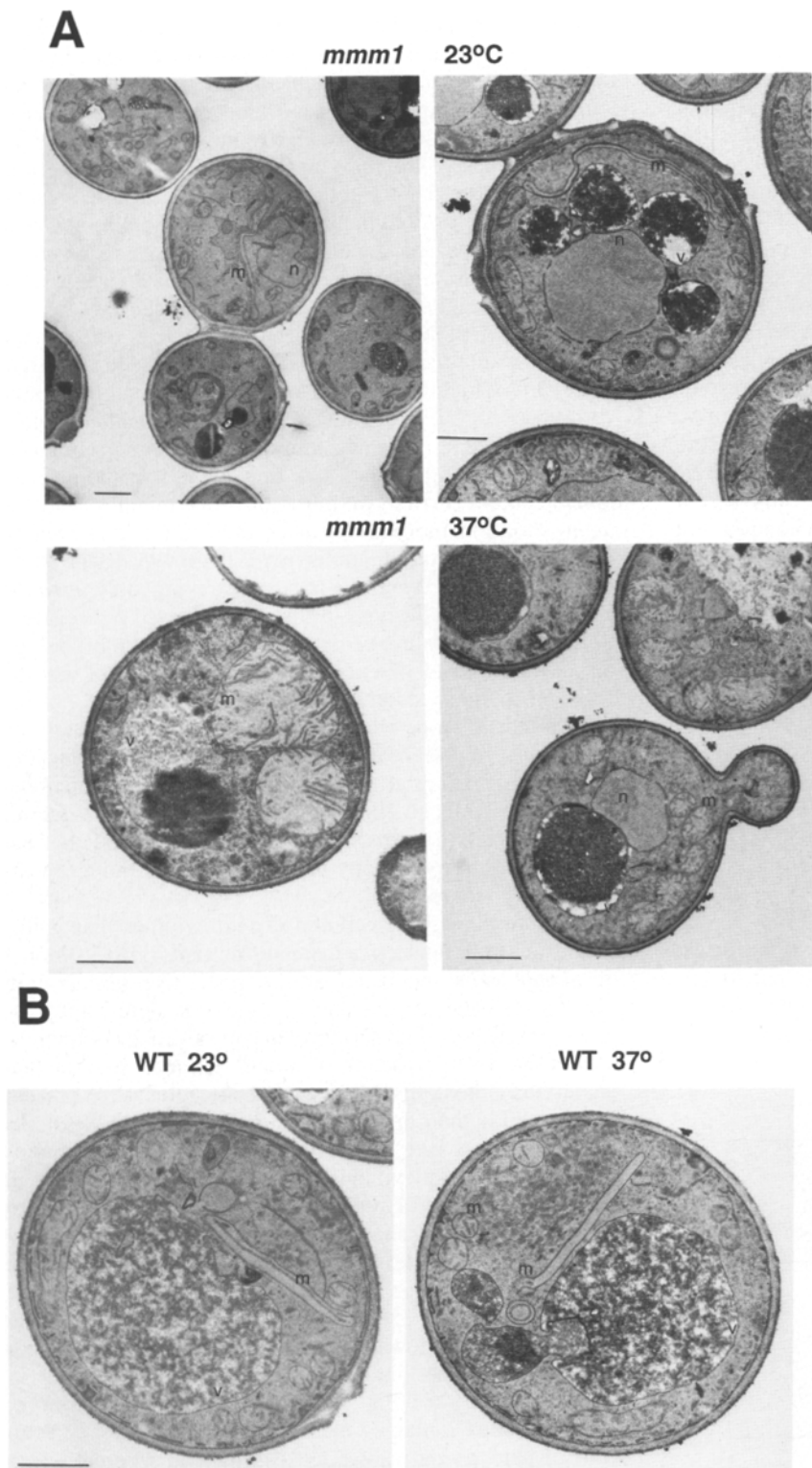


Figure 3. *mmm1* mutants are defective in mitochondrial shape, but other cellular organelles are not affected. *mmm1* strain YSB105 and wild-type strain YPH250 were either maintained at 23°C, or grown at 23°C and shifted to 37°C for 3 h. Cells were fixed and embedded in Spurr's resin, and ultrathin sections were examined by electron microscopy. Mitochondria (*m*), vacuoles (*v*), and nuclei (*n*) are indicated. The bar in the lower left corner of each photograph represents 1 μm . (**A**) *mmm1* mutants grown at either 23 or 37°C. (**B**) Wild-type (*WT*) cells grown at either 23 or 37°C.

cupating cells for different times at the nonpermissive temperature. YSB106 was grown in glycerol-containing medium at 23°C, and then shifted to 37°C. Aliquots were taken at different times, stained with DiOC₆ and examined with the fluorescence confocal microscope (Fig. 5, top row). Ten minutes after the shift to 37°C, we found that most of the mitochondria had changed from their normal, thread-like ap-

pearance to a shorter and fatter shape. By 40 min, *mmm1* cells contained many large and irregularly shaped mitochondria. By 60 min, essentially all the mitochondria were spherical in shape. This round appearance did not change even after six hours at the nonpermissive temperature (see Figs. 2 and 9). That *mmm1* mutants rapidly lose normal mitochondrial shape after a shift to the nonpermissive temperature

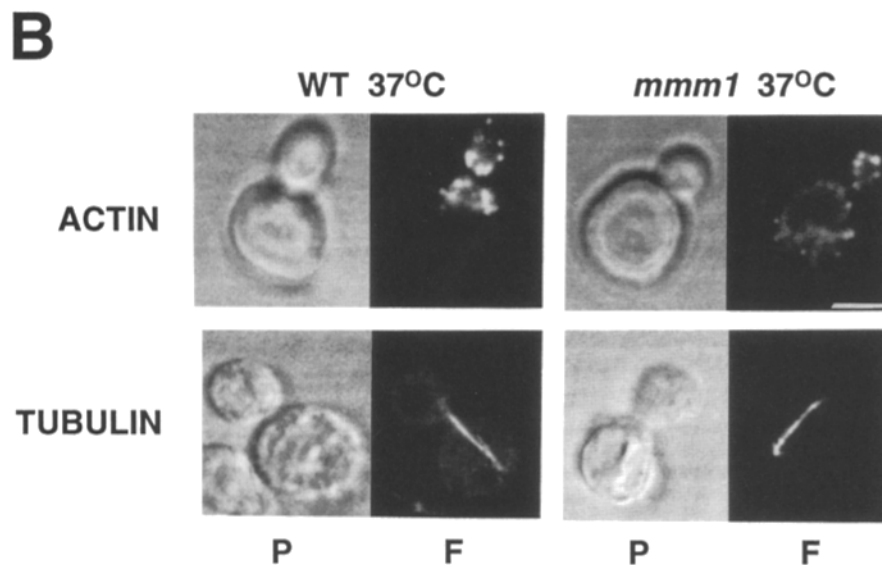
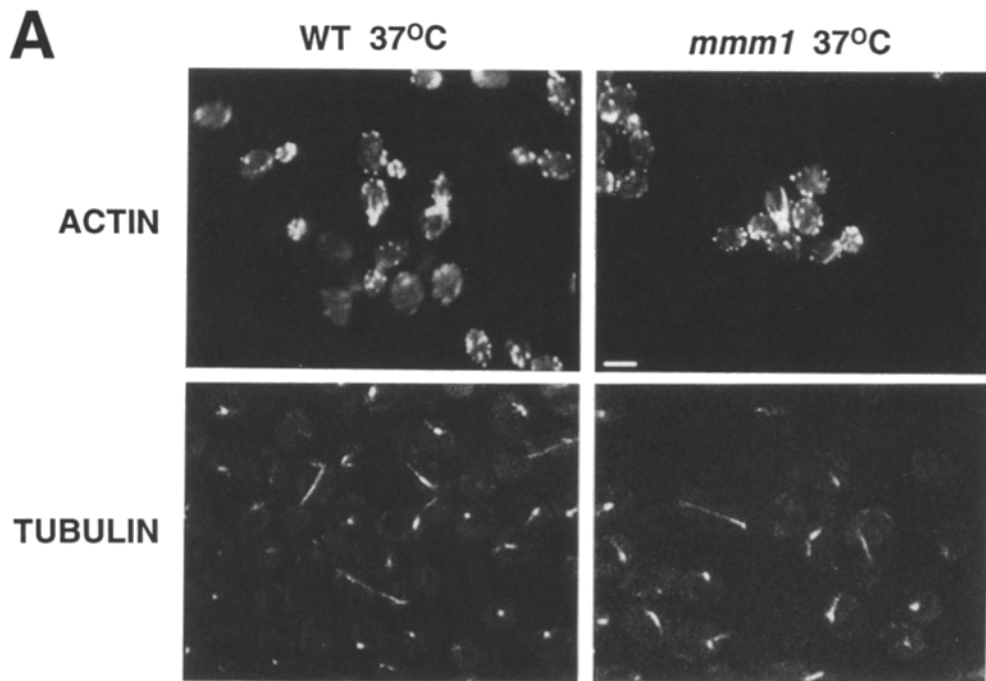


Figure 4. The distribution of actin and tubulin is not disrupted in *mmm1* mutants. *mmm1* strain YSB105 and wild-type strain YPH250 were grown at 23°C in YEP medium containing 2% glycerol to OD₆₀₀ of 0.4, and then shifted to 37°C for 2 h. Cells were fixed and stained for either actin or tubulin. Confocal images were taken using a 510–515-nm filter or by phase contrast. (*Tubulin staining*) Fixed cells were converted to spheroplasts and incubated with anti-tubulin and FITC-conjugated anti-rabbit antibodies as described (Pringle et al., 1989). (*Actin staining*) Actin was visualized as described (Chowdhury et al., 1992) except that 3 U/ml BODIPY FL phalloidin (Molecular Probes) was used instead of rhodamine-phalloidin. (A) Wild-type and *mmm1* cells stained for either actin or tubulin are shown at 100×. The bar in the lower left corner of the top right figure represents 5 μm. (B) 360× images of representative wild-type and *mmm1* cells are shown in phase contrast (P) or fluorescence (F). The bar in the lower right corner of top right figure represents 2 μm.

suggests that *MMM1* plays a direct role in maintaining normal mitochondrial morphology. When mitochondria were in their elongated state, it was difficult to determine the precise number of mitochondria per cell. By examining many different cells, however, our time course studies indicated that the *mmm1* defect was not altering mitochondrial number. We propose that each threadlike mitochondrion seen at 23°C coalesces into a distinct sphere at 37°C.

We also found that normal mitochondrial structure was restored when *mmm1* cells grown at 37°C were returned to the permissive temperature (Fig. 5, bottom row), indicating that the *mmm1* defect is reversible. YSB106 cells were grown at 37°C for 60 min and shifted to 23°C. Aliquots taken at different times were then examined. Approximately 20% of the cells regained normal mitochondrial morphology within 80 min. In these cells the return of normal mitochondrial

shape after these *mmm1* cells were shifted from 37°C to the permissive temperature mirrored the loss of normal morphology after a shift from 23°C to the nonpermissive temperature. Furthermore, all the mitochondria in a given cell would return to normal. The spherical mitochondria seen at 37°C changed to irregularly shaped organelles after 10 min of incubation at 23°C. By 40 min the mitochondria had begun to elongate. After 80 min at the permissive temperature, we observed normal mitochondria. We noted that the mitochondria in approximately three fourths of the cells examined did not immediately regain their normal mitochondrial shape. However, by 24 h after return to 23°C, the shape of all the mitochondria in all cells had returned to normal. Why most cells showed a lag in the reversal of the *mmm1* phenotype is not clear. Perhaps most of the Mmm1 protein cannot recover from inactivation at 37°C and must be newly synthe-

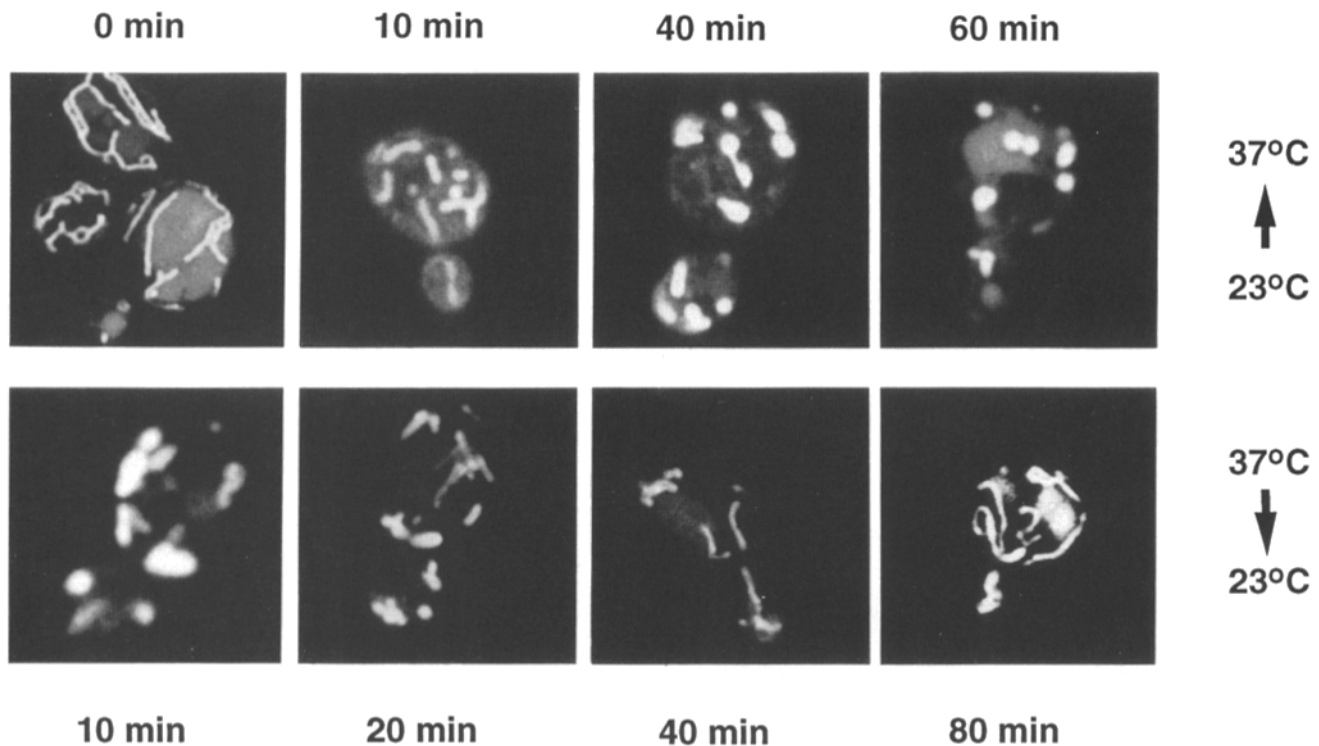


Figure 5. Mitochondrial shape is rapidly and reversibly altered in *mmm1* mutants. (Top row) *mmm1* strain YSP106 was grown at 23°C to an OD₆₀₀ of ~0.3 in YEP medium containing 2% glycerol, and then shifted to 37°C for 60 min. At the indicated times, aliquots were stained with DiOC₆, and imaged by confocal microscopy. Representative cells from each time point are shown. (bottom row) Strain YSB106 was grown at 23°C in YEP glycerol medium, shifted to 37°C for 60 min, and then returned to 23°C. Aliquots were taken at the indicated times after return to 23°C, stained with DiOC₆, and representative cells from each time point are shown.

sized. Alternatively, it is possible that only cells at a particular stage in the cell-division cycle at the time of the temperature shift can immediately remodel their mitochondria.

***mmm1* Mutants Are Not Defective in Mitochondrial Metabolism or the Permeability of the Mitochondrial Inner Membrane**

mmm1 mutants are unable to grow on nonfermentable carbon sources such as glycerol or lactate at the nonpermissive temperature. It is therefore possible that *mmm1* mutants are defective in some aspect of mitochondrial function or metabolism, and that this metabolic defect leads to a change in mitochondrial morphology. Alternatively, mitochondria in *mmm1* mutants may allow the influx of ions or other molecules from the cytoplasm, causing the organelles to swell from osmotic pressure. To determine whether *mmm1* mutants are defective in mitochondrial metabolism or in the inner membrane potential, we compared mitochondrial function in strains that carry the *mmm1-1* mutation to that of wild-type strains in several different experiments. First, we measured the rate of protein synthesis under conditions where mitochondria provide virtually all cellular energy (Table I). When *mmm1* mutants were grown for 90 min at 37°C on the nonfermentable carbon source L-lactate, all the mitochondria had become spherical. At this time the rate of incorporation of [³⁵S]methionine in *mmm1* mutants into protein at 37°C was virtually identical to that of wild-type cells. A similar pattern of labeling was observed for both wild-type and *mmm1* cells at 23°C. Almost no radioactivity was incor-

porated into protein when the mitochondrial inner membrane potential was dissipated with carbonyl cyanide *m*-chlorophenylhydrazone (Table I) or when the electron transport chain was poisoned with sodium azide (not shown). *mmm1* mutants are thus not defective in respiration-driven protein synthesis. Second, we measured the rate of mitochondrial oxygen consumption in *mmm1* mutants (Table II). Polarographic measurements of oxygen consumption showed that mitochondrial respiration rates in *mmm1* mutants were the same as wild-type cells, even after incubation at 37°C for 120 min when mitochondria shape is completely altered. In-

Table I. Respiration-driven Protein Synthesis Is Not Temperature-Sensitive in *mmm1* Strains

Strain	Incorporation rate*		Ratio of rates 37°C/23°C
	23°C	37°C	
Wild-type	70.8	201.3	2.8
<i>mmm1-1</i>	79.8	277.0	3.4
Wild-type/CCCP‡	ND	<1.0	ND

Wild-type strain YPH501 and strain YSB106 carrying the *mmm1-1* mutation were grown at 23°C in YEP medium containing 2% lactate as the sole carbon source to an OD₆₀₀ of 0.6, resuspended in fresh medium to OD₆₀₀ of 10, and incubated at 23 or 37°C for 90 min. Labeling of cellular proteins was initiated by the addition of [³⁵S]methionine (5 μCi/ml). Aliquots were taken and the radioactivity incorporated was determined as described in Materials and Methods. ND, not determined.

* cpm incorporated into TCA-precipitable material per min.

‡ Labeling in the presence of 50 μM carbonyl cyanide *m*-chlorophenylhydrazone.

Table II. Rate of Mitochondrial Oxygen Consumption Is Not Defective in *mmml* Strains

Strain	Respiration rate*	
	23°C	37°C
Wild-type	41.7	114.3
<i>mmml-1</i>	40.8	116.1
Wild-type/KCN‡	3.3	5.4

Wild-type strain YPH501 and the *mmml* mutant YSB106 were grown at 23°C in YEP medium containing 2% glycerol to an OD₆₀₀ of 0.5, and the culture was incubated at 23 or 37°C for 120 min. The rate of oxygen consumption of the cultures was then measured using a Clarke-type oxygen electrode as described in Materials and Methods.

* ng-atom oxygen/min.

‡ Wild-type cells incubated with 0.38 mM potassium cyanide.

hibition of mitochondrial cytochrome oxidase activity with potassium cyanide, however, eliminated essentially all cellular oxygen consumption. Thus *mmml* mutants are not defective in mitochondrial respiration. Third, we tested the mitochondrial inner membrane potential directly by examining mitochondrial staining with fluorescent dyes dependent upon the membrane potential for their uptake. Wild-type cells and *mmml* mutants were grown for 60, 90, or 120 min at 37°C, and then incubated with varying amounts of three different mitochondrial dyes: 0.01–1.0 µg/ml DiOC₆, 0.25–25 µg/ml dimethylaminostyrylmethylpyridiniumiodide (DASPMI; Bereiter-Hahn, 1976), or 0.1 mM–5 mM MitoTracker (Molecular Probes). No difference in mitochondrial staining was observed between *mmml* mutants and wild-type cells. We also found that mitochondria would stain efficiently in *mmml* mutants even after 6–7 h at 37°C (see Figs. 2 and 9). Fourth, we examined growth rates on glycerol-containing medium at 37°C. We found that for at least 10 h *mmml* mutants grew at the same rate as wild-type strains (with a doubling time of ~4 h). Fifth, we assayed the import of proteins into mitochondria. Immunoprecipitation of proteins from pulse-labeled yeast cells showed that the rate of import of the β -subunit of the F₁-ATPase protein (F1 β) was the same in *mmml* mutants and wild-type cells (not shown). All our observations strongly argue that mitochondrial function is normal in *mmml* mutants grown at 37°C even though the morphology of their mitochondria has been dramatically altered. We therefore argue that the primary defect in *mmml* mutants is in maintaining mitochondrial shape.

Isolation of the *MMMI* Gene

MMMI was cloned by complementation of the ts growth defect on nonfermentable carbon sources of the *mmml* mutant. Specifically, *mmml-1 leu2Δ* strain YSB105 was transformed with a yeast genomic DNA library carried on the *LEU2*-containing plasmid, YEp13. Leu⁺ transformants were selected at 23°C and replicaplated to glycerol-containing medium at 37°C. Four plasmids, each carrying an identical 10-kbp DNA insert, were isolated that allowed *mmml* cells to grow at 37°C. Examination of transformants by fluorescence microscopy after DiOC₆ staining showed that the plasmids also corrected the mitochondrial morphology defect (not shown).

To localize the region of *MMMI* activity, we subcloned different restriction endonuclease fragments from the isolated genomic fragment into the *LEU2-CEN6* vector pRS315

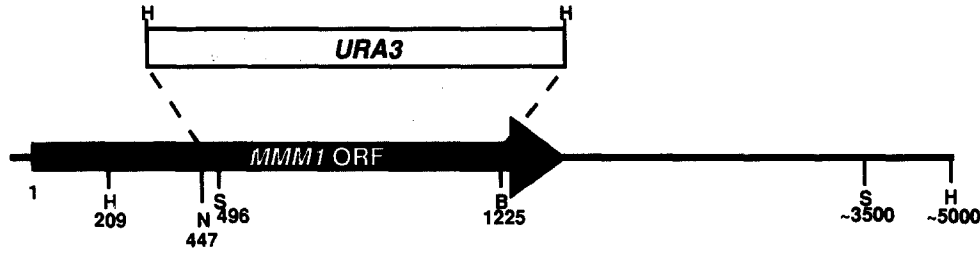
(Sikorski and Hieter, 1989) and tested these plasmids for the ability to correct both the growth and morphology defects of the *mmml* defect (Fig. 6 A). We found that *MMMI* activity was carried on a 5-kbp HindIII fragment, but that a 3.5-kbp SacI fragment internal to the HindIII fragment failed to complement *mmml*. *MMMI* thus extends across one of the two SacI sites. Insertion of the yeast *URA3* gene into a region spanning the leftmost SacI site (see Fig. 6 A, position 496) inactivated complementing activity and thus pinpointed the location of *MMMI*. Approximately 1.8 kbp of DNA surrounding this SacI site were completely sequenced (Fig. 6 B), revealing an open reading frame of 1281 bp, which would encode a protein of 48.7 kD. Interestingly, although *MMMI* activity was carried on a 5-kbp HindIII fragment, we found that the amino-terminal region of the Mmmlp protein extends beyond the original HindIII site (Fig. 6 A, position 209). Apparently the first 73 amino acids of Mmmlp are not required to complement the *mmml-1* mutation, and may not be essential for normal Mmmlp function.

A comparison of *MMMI* to DNA and protein sequences in available databases revealed no significant homologies. Hydropathy analysis (Kyte and Doolittle, 1982) suggests that Mmmlp is a membrane protein, with a single transmembrane-spanning domain near the amino terminus (aa 92 to 116, underlined in Fig. 6 B).

Mmmlp Is Essential for Normal Mitochondrial Morphology and Growth on Nonfermentable Carbon Sources

Yeast strains that carry the *mmml-1* mutation are ts for mitochondrial morphology. To test whether *MMMI* is required at all temperatures, we created a null allele by removing 778 bp of the *MMMI* open reading frame and replacing it with the yeast *URA3* gene (see Fig. 6 A). We then used this *mmml::URA3* construct to disrupt one of the two copies of *MMMI* in a *ura3/ura3* diploid cell. When these diploid cells were sporulated, we found that all four spores in each tetrad were viable on glucose-containing medium (Fig. 7 A), but that two spores in each tetrad formed very small colonies. Cells from the small colonies were found to carry the *mmml::URA3* gene, whereas cells from normal-sized colonies carried the wild-type *MMMI* gene. Although the mitochondria stained poorly with DiOC₆ or MitoTracker, we found that *mmml::URA3* cells were defective in mitochondrial morphology at both 23 and 37°C (not shown). Each cell contained one to four large, sphere-shaped mitochondria similar to those seen in *mmml* cells grown on glucose at 37°C. Therefore, *MMMI* is required for normal mitochondrial morphology at all temperatures, and we assume that our *mmml* strain carries a thermolabile Mmmlp protein.

We found that cells carrying the *mmml::URA3* disruption grew slowly on glucose-containing medium, and failed to grow on glycerol-containing medium. At 23 or 37°C on YEP glucose medium, *mmml::URA3* cells grew at a rate ~2.5-fold less than wild-type cells. As shown in Fig. 7 B, *mmml::URA3* cells failed to grow on glycerol-containing medium (YEPgly), even at 23°C. Examination of single cells showed that *mmml::URA3* cells arrested quickly in their growth and underwent at most one cell division after transfer to glycerol-containing medium. In contrast, cells that carried the *mmml-1* mutation grew for hours at the nonpermissive tempera-

A**B**

TCGTATTCATCACTTGCTGTGTCAAAATTTTATTACTGATCAGCGTATGAT

-452 AGCAAAGCTTTTGTATGTGACATCGTAAGTGGCTTCCATGAGTTTCTTAAATTTAGGATTCAGTAGCACTTTATGTTTTCGAAGCTTTCATCTGGCATCTG

-400 CCTGTTATCGCTTCAATCGCTTATGCGTTATTTGAGGTTACTTTAATCTATTTTCCACTGATGACACAAATGAGTCAATCCAAAGTGGAAACGGGTTGCAC

-300 TTGATACATTCAGTTTACTCTTTTTCATGTAATTTCTTAATAGTTTATTTTTCACTTCTGCTTATCCGTTTCAATTCGGAAGAACCGTCAACATCCA

-200 ATAAAGATCATCTACAAACAATAAGTCCCTCAATAATTTTCCCAATGAGATGAAAGAACTTTGAGAGAGTCAATAATAATACCTGTAGCGCTTTTCTGAAA

-100

1 ATG ACT GAT AGT GAG AAT GAA TCC ACC GAA ACG GAT TCG TTA ATG ACG TTT GAC GAT TAT ATA AGC AAA GAG CTA
 1 M T D S E N E S T E T D S L M T F D D Y I S K E L

76 CCT GAA CAT TTA CAG AGA CTA ATC ATG GAG AAT TTG AAG GGT TCT ACT ACT AAT GAC TTA AAG CAA ACT TCA AAC
 26 P R H L Q R L I M E N L K G S T T N D L K Q T S N

151 AAC TCA GAG TTT AAT GTC AGT AAA AAC GGG AGC TTC AAA GGT CTC GAT GAT GCA ATT CAA GCT TTG CAA ATG CAA
 51 N S E F N V S K N G S F K G L D D A I Q A L Q M Q

226 AGC GTG TTG CAT CCT TCT TCG TTA GGA TCG TTA GCA ACG TCC TCC AAA TTT TCT GGA TGG TCG TTT GCT CAA GGG
 76 S V L H P S S L G S L A T S S K F E G W S F A Q G

301 TTT TTT GTA GGA CAG CTA AGC ATA GTG TTG TTG TTC ATC TTT CTC TTA AAG TTC TTT ATA TTC AGT GAT GAG CCA
 101 F F V G Q L S I V L L F I F F L K F F I F S D E P

376 TCT AAA AGT AAG AAT CCG AAA CCT GCA GCC TCC CGT CAC AGA TCA AAA TTT AAA GAA TAT CCC TTT ATA TCT CGC
 126 S K S K N P K P A A S R H R S K F E Y I S R

451 GAA TTC CTG ACT TCT CTT GTT AGG AAG GGT GCT AAA CAA CAC TAC GAG CTC AAT GAA GAG GCA GAA AAT GAA CAT
 151 E F L T S L V R K G A K Q H Y E L N E E A E N E H

526 CTT CAA GAA CTA GCT CTT ATT TTA GAG AAA ACC TAT TAT AAT GTC GAC GTG CAC CCT GCA GAG TCA TTG GAC TGG
 176 L Q E L A L I L E K T Y Y N V D V H P A E S L D W

601 TTC AAC GTT TTA GTT GCC CAA ATA ATA CAG CAA TTC CGC AGT GAG GCT TGG CAC AGG GAC AAT ATC CTT CAT TCC
 201 F N V L V A I I Q Y S E A W H R D N I L H S

676 TTG AAT GAT TTT ATT GGA AGA AAA TCA CCC GAT CTG CCT GAA TAT TTG GAT ACC ATA AAA ATA ACT GAA CTG GAT
 226 L N D F I G R K S P D L P E Y L D T I K I T E R L D

751 ACA GGT GAT GAT TTC CCC ATT TTC TCG AAT TGC AGA ATA CAA TAT TCT CCA AAT TCA GGA AAT AAA AAG CTA GAG
 251 T G D I F I Q Y S N C R I Q Y S P N S G N K K L E

826 GCT AAA ATT GAT ATA GAT TTA AAT GAC CAC TTA ACT TTA GGA GTA GAA ACA AAA CTA TTA CTT AAC TAT CCA AAG
 276 A K I D I D L N D H L T L G V E T K L L L N Y P K

901 CGT GGT ATT GCC GCA CTC CCC ATA AAT CTA GTA GTG TCA ATT GTG AGG TTT CAG CGG TTG TTG ACC GTA TCT TTA
 301 P G I A A L P I N L V V S I V R F Q A C L T V S L

976 ACT AAT GCA GAG GAG TTT GCT TCT ACT TCG AAC GGT AGC AGT AOT GAA AAC GGT ATG GAG GGC AAT TCA GGA TAC
 326 T N A E R A S T S S N G S E A C M R G N R C V E

1051 TTT TTG ATG TTT TCT TTT GCT CCT GAA TAT AGA ATG GAA TTT CAA ATC AAG CCG CTA ATT GGC TCA CGG TCC AAA
 351 F L M F S F A P E Y R M E F Q I K P L I G S R S K

1126 CTT GAA AAT ATT CCC AAG ATC GGC AGT GTC ATT GAA TAC CAA ATA AAA AAA TGG TTC GTT GAA CGA TGC GTT GAA
 376 L E N I P K I G S V I H Y V W F P R E E K P T E

1201 CCA AGA TTT CAA TTT GTC AGG TTA CCA AGT ATG TGG CCA CGT AGT AAA AAT ACG AGA GAA GAA AAG CCT ACA GAG
 401 P R F Q F V R L P S M W P R S K N T R E E K P T E

1276 TTA TAA ATATTTATGACAAAATTTTGTTCATCTTTTCTCTATCTCTGCTCAGGGGGGTTCACGTTTGGTTAGTGCACAGTGGCTTGGT
 426 L *

Figure 6. *MMM1* encodes a 48.7-kD protein. (A) Restriction endonuclease map of the *MMM1* gene. The bottom line indicates relevant restriction sites in the cloned *MMM1* gene, and their position in basepairs with respect to the amino terminus of the *Mmml* protein (position 1). The arrow represents the *MMM1* open reading frame, with the arrowhead indicating the 3'-end of *MMM1*. The location of the *URA3* disruption, which inactivates *MMM1*, is also shown. Restriction endonuclease sites: *H*, HindIII; *S*, SacI; *B*, BstXI; *N*, NruI. (B) DNA sequence of *MMM1* and its predicted protein product. The underlined amino acids represent a potential membrane-spanning domain predicted from hydropathy analysis.

ture and would undergo at least five cell divisions before stopping.

Why the growth defect of *mmml::URA3* cells on nonfermentable carbon sources is more severe than of cells carrying the *mmml-1* mutation is not clear. The inability of *mmml::URA3* cells to grow on glycerol or lactate-containing medium is not due to a defect in or a lack of mitochondrial DNA. When *mmml::URA3* strains were crossed to rho^o tester strains, which lack mitochondrial DNA, we found that the resulting diploids grew on glycerol. Hence mitochondrial DNA is present and functional in *mmml::URA3* cells. We propose that mitochondria in cells grown for long periods of time in the absence of the *Mmml* protein eventu-

ally lose normal mitochondrial function. Consistent with this idea we found that the mitochondria in *mmml::URA3* cells stained poorly with potential-sensitive fluorescent dyes, indicating that the inner membrane potential was less than in wild-type cells or in cells that carry the *mmml-1* mutation. Moreover, we found that incubation of *mmml-1* cells at the nonpermissive temperature for prolonged periods led to growth defects similar to those found in *mmml::URA3* cells. For example, at 37°C *mmml-1* cells grew at rates indistinguishable from wild-type on glucose-containing medium for up to 20 h. After 30 h at 37°C, the doubling time of *mmml-1* cells was 2–3-fold less, similar to that seen in *mmml::URA3* cells.

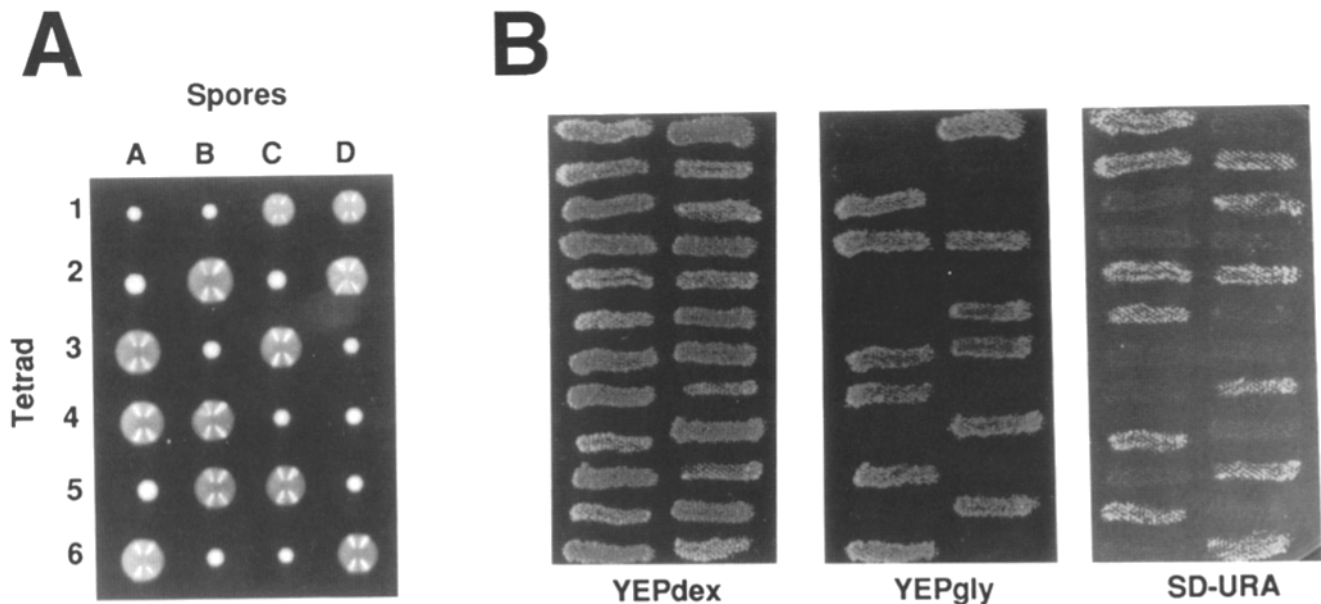


Figure 7. Strains carrying a disruption of the *Mmm1* gene grow slowly on glucose-containing medium and are inviable on nonfermentable carbon sources. (A) Meiotic products from diploid strain YSB108, in which one of the two *Mmm1* genes was replaced by the *mml1::URA3* disruption, were separated by micromanipulation and allowed to grow at 23°C for five days on YEP medium containing 2% glucose. (B) Six tetrads from the sporulated YSB108 diploid were patched out onto medium containing 2% glucose (YEPdex), and then replicaplated to either medium containing 2% glycerol (YEPgly), or to synthetic complete medium containing 2% glucose but lacking uracil (SD-URA). Cells were incubated at 23°C for two days before photography.

The Mmml Protein Is Located in the Mitochondrial Outer Membrane, with a Large Carboxyl-Terminal Domain Facing the Cytoplasm

To explore the mechanism by which the Mmml protein maintains normal mitochondrial morphology, we asked where the Mmml protein is located within the yeast cell. We constructed an epitope-tagged version of Mmmlp by inserting a domain from the influenza HA protein at the carboxyl terminus of Mmmlp, and then integrated this construct into the yeast genome. The HA epitope is recognized by the monoclonal antibody 12CA5 (Niman et al., 1983). Cells that express Mmmlp-HA contain a single protein of ~52 kD that reacts with the 12CA5 antibodies. The size of this protein is consistent with the addition of the 4-kD HA epitope to the 48.7-kD Mmml protein. We also found that the Mmmlp-HA fusion protein is functional. YSB107, which expresses only Mmmlp-HA and not wild-type Mmmlp, grew on glucose or glycerol-containing medium at 23, 30, or 37°C.

Immune-blotting experiments indicate that Mmmlp is a mitochondrial protein (Fig. 8 A). We homogenized yeast cells expressing the Mmmlp-HA fusion protein, and separated them into a mitochondrial fraction and a post-mitochondrial supernatant by centrifugation. We found that Mmmlp cofractionated with F1 β , a mitochondrial protein. Little or no Mmmlp was found in the supernatant with the cytosolic hexokinase protein. When mitochondria were further purified on a Percoll density gradient, we found that Mmmlp still cofractionated with the F1 β protein (Fig. 8 B). Hydropathy analysis predicts that Mmmlp is an integral membrane protein with a single membrane-spanning domain

near its amino terminus. Supporting this prediction, we found that Mmmlp was not extracted from the mitochondrial membrane fraction by treatment with 0.1 M sodium carbonate, 7 M urea, or 1.5 M sodium chloride (not shown). To determine in which of the two mitochondrial membranes Mmmlp resides, we isolated mitochondria from cells expressing the Mmmlp-HA protein and prepared membrane vesicles by sonication. When the mitochondrial outer membrane vesicles were separated from inner membrane vesicles on sucrose density gradients, Mmmlp cofractionated with OM45p, a protein located in the outer mitochondrial membrane, and not with F1 β , an inner membrane protein (Fig. 8 C). Mmmlp is thus located in the mitochondrial outer membrane.

We also determined that the carboxyl terminus of Mmmlp faces the cytosol. In particular, we found that the carboxyl-terminal HA tag of Mmmlp-HA fusion protein was extremely sensitive to trypsin digestion of intact mitochondria (Fig. 8 D). In contrast, Cyt. b₂, a protein located in the intermembrane space, or subunit IV of cytochrome oxidase (Cox IV), a protein located in the matrix, were not accessible to trypsin digestion when the outer membrane was intact. Disruption of the mitochondrial outer membrane by osmotic shock released Cyt. b₂ and allowed access of the trypsin to the intermembrane space, but did not allow access to the matrix-localized Cox IV protein. Our results thus indicate that Mmmlp is inserted in the mitochondrial outer membrane with its amino terminus facing the intermembrane space and its carboxyl terminus exposed to the cytosol. This topology is similar to several other mitochondrial outer membrane proteins, including Mas70p (Hase et al., 1983) and OM45p (Yaffe et al., 1989). Mmmlp, like other outer

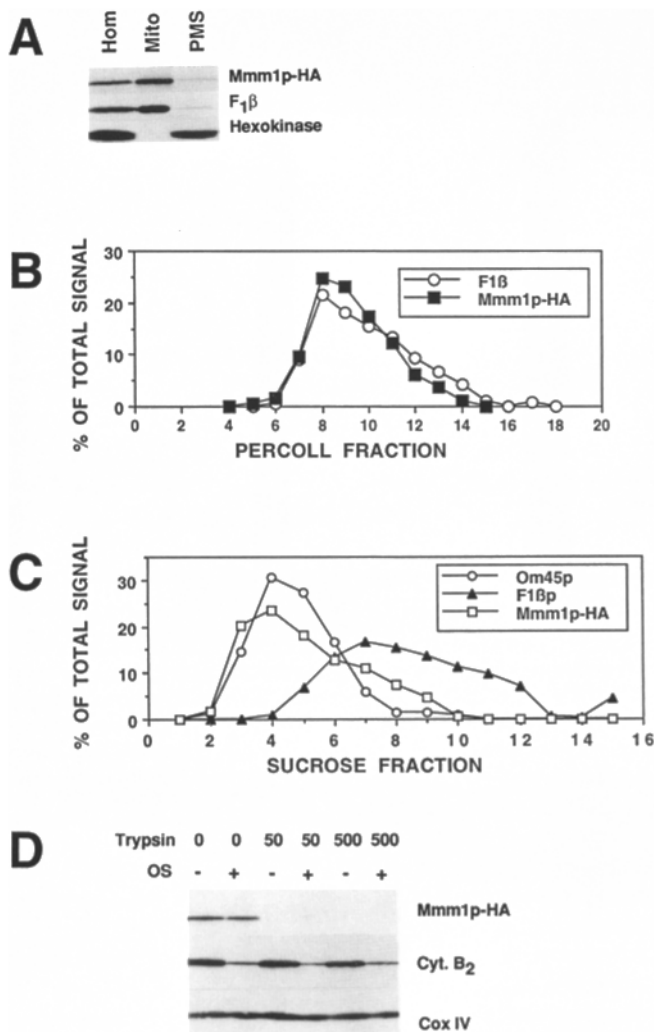


Figure 8. Mmm1p is a mitochondrial outer membrane protein with its carboxyl terminus facing the cytosol. (A) Mmm1p is a mitochondrial protein. YSB107 cells, which express the Mmm1 protein tagged with the HA epitope, were converted to spheroplasts and homogenized as described in Materials and Methods. The homogenate (*Hom*) was centrifuged at 2,500g for 5 min, and the supernatant was separated into a mitochondrial pellet (*Mito*) and a post-mitochondrial supernatant (*PMS*) by centrifugation at 9,600g. Aliquots of homogenate, mitochondria, and PMS representing equivalent numbers of cells were subjected to SDS-PAGE, blotted, and proteins decorated with 12CA5 antibodies to the HA epitope (*Mmm1p-HA*), antiserum to the β -subunit of the F₁-ATPase (*F₁β*), or antiserum to hexokinase. (B) Mmm1p cofractionates with mitochondria on Percoll gradients. 25 mg of mitochondria from above were centrifuged in 40% Percoll. 1-ml fractions were taken from the gradient and analyzed by immune blotting with antibodies to the HA epitope (*Mmm1p-HA*), and the mitochondrial F₁β protein. Immunoblots were quantitated and the percent of total Mmm1p and the F₁β protein in each fraction are indicated. Fraction 1 represents the top of the gradient. (C) Mmm1p is located in the mitochondrial outer membrane. Mitochondria from strain YSB107 were sonicated and the resulting vesicles were loaded onto 5-ml sucrose step gradients. After centrifugation at 100,000g for 16 h, 0.3-ml fractions were collected and analyzed by immune blotting with antibodies to the HA epitope (*Mmm1p-HA*), the inner membrane protein, F₁β, or the outer membrane protein, OM45p. The percent of total Mmm1p-HA, OM45p, and the F₁β protein are indicated. Fraction 1 represents the top of the gradient. (D) The carboxyl terminus of

membrane proteins, does not appear to contain an amino-terminal, cleavable presequence typical of proteins that are imported into the mitochondrial matrix, the inner membrane, or the intermembrane space. The mitochondrial targeting information of Mmm1p therefore resides within the mature protein.

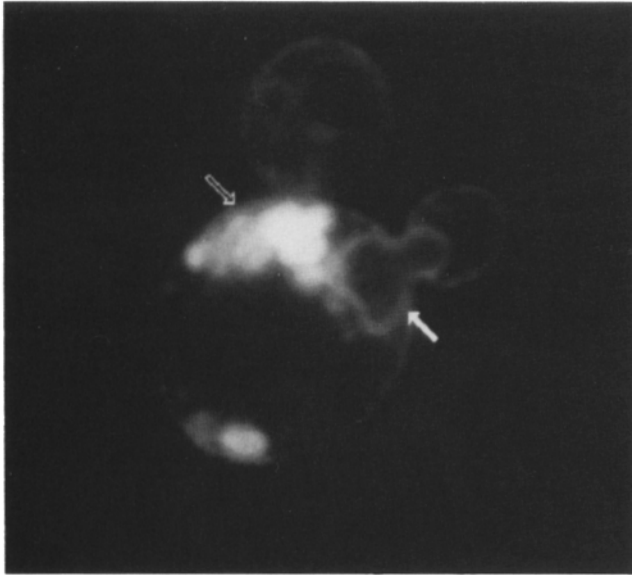
mmm1 Mutants Fail to Transmit Mitochondria to Daughter Cells When Grown on a Nonfermentable Carbon Source

mmm1 mutants fail to grow on nonfermentable carbon sources. To determine the cause of the growth defect, we incubated cells that carried the *mmm1-1* mutation for prolonged times at the nonpermissive temperature on glycerol-containing medium. After six hours of incubation at 37°C, we observed that most cells in the culture contained multiple buds, with more than 90% of the cells carrying two daughter buds. Incubation of *mmm1* cells for 24 h at 37°C would yield cells with three, four, or even five buds. *mmm1* mutants apparently are defective in the completion of cytokinesis. After staining the cells with DiOC₆, we observed that the spherical mitochondria in *mmm1* mutants were contained almost exclusively in the mother cell, and that the mitochondria were located immediately adjacent to one of the bud sites (Fig. 9). We found that fewer than 10% of the daughter buds examined contained mitochondria. Other organelles such as nuclei and vacuoles segregated normally. DAPI staining showed that each daughter bud contained a nucleus and staining with FUN-1 (Molecular Probes) showed that all daughter cells had vacuoles (not shown). Our results suggested that *mmm1* mutants were unable to grow on glycerol-containing medium because they failed to efficiently transmit their altered mitochondria to daughter cells.

To test this possibility, we compared the ability of *mmm1-1* cells to segregate mitochondria to daughter cells at 37°C when grown in medium containing glucose (in which *mmm1-1* mutants are viable) to *mmm1-1* cells grown in glycerol (in which *mmm1-1* mutants are inviable). *MATa mmm1-1* strain YSB105 was grown in glucose or glycerol-containing medium at 23°C, and synchronized at the G₁ (unbudded) stage of the cell division cycle by the addition of α -factor (Pringle and Hartwell, 1981). To inactivate Mmm1p function, *mmm1* cells were then shifted to 37°C, released from α -factor arrest, and allowed to grow for 2 h at 37°C. Daughter buds were then examined for the presence of mitochondria after staining with DiOC₆ or MitoTracker (Fig. 10). We observed that *mmm1* cells grown on glucose-containing medium at 37°C each contained one to four large, spherical organelles (average diameter of 1 μ m) and a variable number

Mmm1p is exposed to the cytoplasm. Mitochondria isolated from strain YSB107 were digested with either 50 μ g/ml or 500 μ g/ml trypsin for 30 min on ice, followed by the addition of 2 mg/ml soybean trypsin inhibitor. Mitochondria were reisolated by centrifugation at 9,600g for 10 min and analyzed by immune blotting with antibodies to the HA epitope (*Mmm1p-HA*), antiserum to subunit IV of cytochrome oxidase (*Cox IV*), or antiserum to cytochrome b₂ (*Cyt. B₂*). To expose proteins located in the intermembrane space, the mitochondrial outer membrane was ruptured by osmotic shock (OS), and proteins were digested with either 50 μ g/ml or 500 μ g/ml trypsin as above.

DiOC₆



Phase

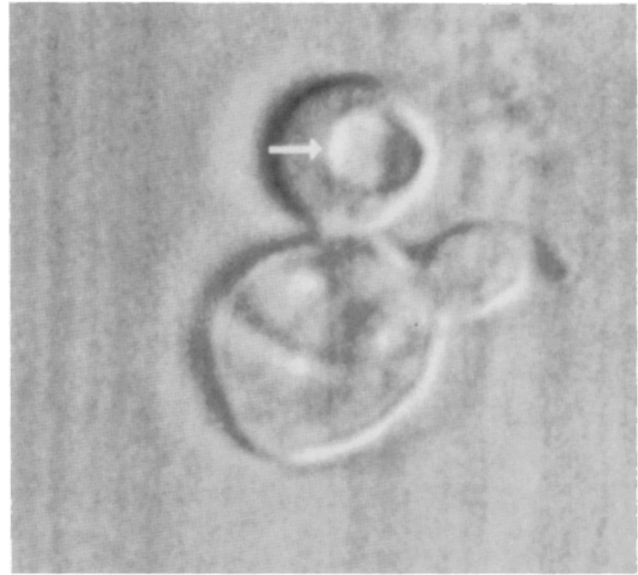


Figure 9. Mitochondria accumulate at the bud site in *mmml* mutants grown on glycerol-containing medium, but other organelles are transmitted normally. *mmml* strain YSB105 was grown in YEP medium containing 2% glycerol at 23°C to an OD₆₀₀ of 0.3, and then shifted to 37°C for 6 h. Cells were stained with 1 μg/ml DiOC₆ and examined using the confocal microscope. (*left*) Fluorescence image showing the brightly staining mitochondria (*open arrow*), and the weakly staining nucleus (*solid arrow*). (*right*) Phase contrast image of the same cell. The arrow indicates a vacuole.

of small organelles that stained very intensely with DiOC₆ or MitoTracker (average diameter of 0.25 μm). The large and small organelles contained DNA (not shown), indicating that both are mitochondria. When *mmml* mutants were grown on glucose-containing medium, all buds contained at least one mitochondrion (Fig. 10, *A* and *B*). Strikingly, the small organelles were always seen in the buds from *mmml* cells grown on glucose, whereas the large, sphere-shaped mitochondria remained in the mother cell in greater than 90% of cells. The large spheres were usually located at the bud site. Our results suggest that there may be a size limit for mitochondrial entry into the bud. Consistent with this idea, we found that when *mmml* cells were grown on glycerol-containing medium, transfer of mitochondria to the bud was defective. Mitochondria were found in only 13 of 100 buds (Fig. 10 *B*). *mmml* mutants grown on glycerol medium contained only the large, sphere-shaped organelles, and no small mitochondria were seen in either mother or daughter cells. Therefore, the inability to transmit mitochondria efficiently into the bud correlated with the growth defect of *mmml* cells on glycerol.

Discussion

Our results show that Mmmlp is a novel protein that is required for normal mitochondrial shape. In the temperature-sensitive *mmml* mutant, mitochondrial shape is rapidly lost, and elongated mitochondria collapse into large spherical-shaped organelles within 90 min after shift to the restrictive temperature. Upon return of *mmml* cells to the permissive temperature, normal mitochondrial morphology is restored. Mmmlp may therefore play an active role in both establishing and maintaining mitochondrial shape. Our results indi-

cate that the *mmml* mutation is specific to mitochondria. *mmml* cells are not defective in the morphology of other cellular organelles, such as the nucleus or the vacuole, and the distributions of actin and tubulin are not disrupted in the *mmml* mutant. Furthermore, we find that the Mmml protein is located in the mitochondrial outer membrane with the bulk of its carboxyl terminus facing the cytosol.

We found that the altered mitochondria in *mmml* mutants were not efficiently transmitted to daughter cells, and we suggest that this defect in inheritance may explain the growth defect of *mmml* strains on nonfermentable carbon sources. When the temperature-sensitive *mmml* mutant was synchronized with α -factor at the G₁ stage of the cell division cycle, and then allowed to bud at 37°C on glycerol-containing medium, examination of budded cells suggested that mitochondria were only rarely transmitted to the daughter buds. Since mitochondria are essential for cell viability (Kovacová et al., 1968; Jensen and Yaffe, 1988), the lack of mitochondria in daughter buds would explain the growth defect of *mmml* mutants. Our results suggest that the spherical mitochondria that accumulate in *mmml* mutants may simply be too large to pass into the bud. Supporting this hypothesis, we found that small mitochondria were transmitted efficiently to daughter cells when *mmml* mutants were grown on glucose-containing medium at 37°C. On glucose each cell contained several large, spherical mitochondria (average diameter of 1 μm) and a variable number of very small mitochondria (average diameter of 0.25 μm). The small organelles were always seen in the buds from *mmml* cells grown on glucose. However, the large, sphere-shaped mitochondria remained in the mother cell in more than 90% of the cells examined. It is possible that the elongated shape (and narrow diameter) of wild-type mitochondria is impor-

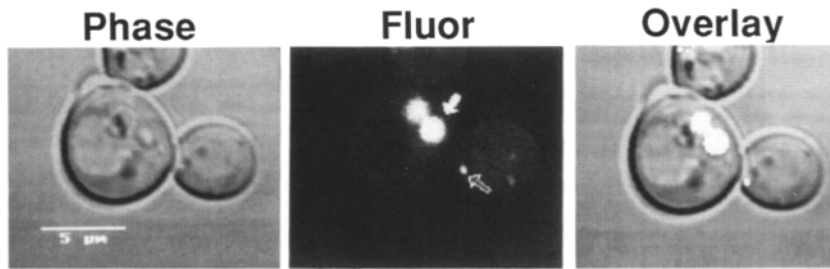
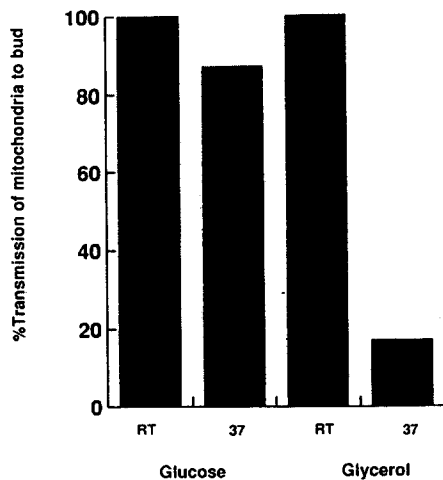
A**B**

Figure 10. *mmm1* mutants are defective in the transmission of the large, sphere-shaped mitochondria to daughter cells. *MATa mmm1* strain YSB105 was grown at 23°C to an OD₆₀₀ of 0.5 in YEP medium containing either 2% glucose or 2% glycerol as the carbon source. To arrest cells in the G₁ phase of the cell division cycle, α -factor (Sigma Chem. Co.) was added to each culture to a final concentration of 100 μ M, and cells were incubated for 2 h at 23°C. Cells were then shifted to 37°C for 60 min to inactivate *mmm1* function. Examination of cells at this time showed that at least 95% were unbudded, confirming that most were arrested in G₁. Cells were centrifuged at 3,500g, and the pellet washed three times to remove the α -factor. Cell pellets were resuspended in YEPglucose or YEPglycerol medium and allowed to grow for 2 h at 37°C. Aliquots from each culture were stained with either DiOC₆ or MitoTracker (Molecular Probes), and the cells examined by fluorescence microscopy. (A) *MATa mmm1* strain YSB105 grown on glucose-containing medium as described above and stained with 1 μ M MitoTracker. (Fluor) Fluorescence confocal image showing two large sphere-shaped mitochondria in the mother cell (filled arrow) and a smaller brightly staining mitochondrion in the daughter bud (open arrow). (Phase) Phase contrast image of the same cell. (Overlay) The fluorescence and phase contrast images were superimposed to show the position of the mitochondria within the mother and daughter cells. Bar at lower left corner of the phase contrast image indicates 5 μ m. (B) 100 cells grown on glucose or glycerol-containing medium were scored for the presence of DiOC₆-staining mitochondria in the daughter bud. The percentage of cells that transmitted at least one mitochondrion to a daughter bud is indicated.

tant to allow efficient entry of the organelles into the bud. Since the large spherical mitochondria were occasionally found in daughter cells, there may not be an absolute block to their segregation. However, because synchronization with α -factor is never complete, the cells in which mitochondria were seen in daughter buds may have been cells that were not arrested at G₁ (e.g., budded cells) at the beginning of our experiment. Nonetheless, the inability to efficiently transmit mitochondria to the bud correlated with the growth defect of *mmm1* cells on glycerol. *mmm1* mutants grown for prolonged periods at the nonpermissive temperature on glycerol arrested in their growth with several buds that failed to separate from the mother cell. Why the separation of daughter cells from mother cells is defective in *mmm1* mutants is unclear. It is possible that the presence of mitochondria could be a “checkpoint” (Weinert and Hartwell, 1990),

and daughter cells without mitochondria may not be permitted to complete cytokinesis.

What is the nature and origin of the small mitochondria seen when cells are grown on glucose-containing medium? Like the larger mitochondria, we found that the small organelles contained DNA, indicating that they are indeed mitochondria. These small, round mitochondria in glucose-grown cells have also been seen in electron microscopy studies (Stevens, 1977, 1981). It is possible that the small organelles are dedifferentiated mitochondria similar to the promitochondria seen in yeast cells grown anaerobically (Criddel and Schatz, 1969; Plattner and Schatz, 1969). On glucose-containing medium, where the demand for mitochondrial function is low, some of the mitochondria in a cell may remain small in size. On nonfermentable carbon sources where the demand for mitochondrial function is

high, cells may enlarge and elongate all their mitochondria. *mmml* apparently affects only the shape of the elongated organelles. The small organelles appear to arise by budding from larger organelles. Supporting this idea, we find that many of the small structures are in close contact with the larger mitochondria and appear to be attached since both large and small organelles were often seen moving or rotating together within unfixed cells.

Our studies suggest that Mmmlp plays a direct role in maintaining mitochondrial morphology. Because mitochondrial structure can be influenced by the metabolic state of the cell (Bereiter-Hahn and Vöth, 1983; Tandler et al., 1968) and since organelle movement within cells is an energy-dependent process (Rebhun, 1972), it was important to show that the mitochondrial morphology and inheritance defects in *mmml* mutants were not due to loss of mitochondrial function. We found that although the shape of mitochondria in *ts mmml* mutants was dramatically altered within 90 min after shift to the nonpermissive temperature, mitochondrial activities such as oxygen consumption, energy production, uptake of potential-sensitive dyes, and mitochondrial protein import were all virtually identical to that seen in wild-type cells. In addition, on glycerol-containing medium cells that carry the *mmml-1* mutation continued to divide at the same rate as wild-type cells for at least five generations after the shift to the nonpermissive temperature. Our observations thus indicate that mitochondrial metabolism is not defective in *mmml* mutants. Moreover, since Mmmlp is a mitochondrial outer membrane protein and since we find no significant change in the mitochondrial inner membrane potential in cells that carry the *mmml-1* mutation, it is unlikely that the alteration of mitochondrial morphology in *mmml* mutants is a consequence of the influx of large amounts of charged molecules into the matrix. We argue that the primary defect in *mmml* mutants is in maintaining mitochondrial shape and that Mmmlp plays a direct role in this morphological process. We do note, however, that *ts mmml* mutants grown for very long periods at the nonpermissive temperature and cells carrying a disruption of *MMML* (*mmml::URA3*) show some defects in mitochondrial function. These problems arise long after the alteration of mitochondrial shape in *mmml* mutants and we argue that they are secondary consequences of the loss of Mmmlp function.

There are several potential mechanisms by which Mmmlp maintains normal mitochondrial structure. For example, *mmml* mutants may be defective in the division or the fusion of mitochondria (Bereiter-Hahn and Vöth, 1994), and this defect may alter the shape of the organelles. Although the exact number of mitochondria per cell is difficult to measure, our studies suggest that mitochondrial number does not change in *mmml* mutants. Rather it appears from our time course experiments with the *ts mmml* mutant that each elongated mitochondrion coalesces into a sphere at the nonpermissive temperature. Alternatively, Mmmlp may be part of an internal framework or "mitoskeleton." Filaments and fibrous structures have been detected within the mitochondria of many different organisms (May, 1974; Price and Gomer, 1989; Yotsuyanagi, 1988), although the composition and precise arrangement of these filaments is not known. We favor a different model, in which mitochondria form elongated structures by binding to an external framework. Supporting this idea we find that mitochondria isolated from

wild-type yeast cells are spherical in shape, similar to those seen in *mmml* mutants (Burgess, S. M., unpublished observations). Mitochondria that contained an internal framework would be expected to maintain their structure after isolation. In addition, we find that Mmmlp, which is essential for mitochondrial shape, is located in the mitochondrial outer membrane, with the majority of the Mmml protein facing the cytosol. This topology suggests that Mmmlp functions outside the mitochondria.

Mitochondria have been shown to colocalize with all three major cytoskeletal elements: actin filaments (Drubin et al., 1993; Pardo et al., 1983), microtubules (Ball and Singer, 1982; Heggeness et al., 1978; Leterrier et al., 1994; Schnapp and Reese, 1982), and intermediate filaments (Collier et al., 1993; Leterrier et al., 1994; Morse-Larsen et al., 1982; Zorn et al., 1990). In addition, yeast mutants defective in actin (Drubin et al., 1993) and a protein homologous to mammalian vimentin (McConnell et al., 1990) show an altered distribution of mitochondria. We suggest that Mmmlp keeps mitochondria in an elongated shape by mediating binding to one specific cytoskeletal element: either actin filaments, microtubules, or intermediate filaments. In our model, *mmml* mutants are defective in this attachment, causing mitochondria to release from the filament and collapse into spherical shapes. This attachment may also be important in the transfer of mitochondria to daughter cells (see above). We do not expect that the interaction of mitochondria with all cytoskeletal elements requires Mmmlp. Consistent with this idea, we find that the migration of mitochondria to the bud site is not affected in *mmml* mutants. Depolymerization of microtubules with nocodazole does not significantly alter normal mitochondrial morphology (Burgess, S. M., unpublished observations). In addition, although minor changes in mitochondrial shape are seen in some actin mutants (Drubin et al., 1993), no actin mutant contains mitochondria nearly as misshapen as those seen in *mmml* strains. Therefore, future studies to pinpoint the mechanism by which the Mmml protein maintains mitochondrial structure will focus on the identification of proteins that interact with Mmmlp. Mmmlp gives us a unique tool with which to reach out from the surface of the mitochondria and ask what additional cellular components are necessary to establish and maintain the shape of a specific organelle. Furthermore, Mmmlp will allow us to determine why the shape of mitochondria is important for cell growth.

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