Molecular Biology of the Cell
Vol. 8, 1233–1242, July 1997

Mitochondrial Transmission during Mating in *Saccharomyces cerevisiae* Is Determined by Mitochondrial Fusion and Fission and the Intramitochondrial Segregation of Mitochondrial DNA

Jodi Nunnari,*† Wallace F. Marshall,* Aaron Straight,‡ Andrew Murray,‡ John W. Sedat,* and Peter Walter*

Departments of *Biochemistry and Biophysics, and ‡Physiology, University of California Medical School, San Francisco, California 94143-0448

Submitted January 27, 1997; Accepted April 29, 1997
Monitoring Editor: Thomas Fox

To gain insight into the process of mitochondrial transmission in yeast, we directly labeled mitochondrial proteins and mitochondrial DNA (mtDNA) and observed their fate after the fusion of two cells. To this end, mitochondrial proteins in haploid cells of opposite mating type were labeled with different fluorescent dyes and observed by fluorescence microscopy after mating of the cells. Parental mitochondrial protein markers rapidly redistributed and colocalized throughout zygotes, indicating that during mating, parental mitochondria fuse and their protein contents intermix, consistent with results previously obtained with a single parentally derived protein marker. Analysis of the three-dimensional structure and dynamics of mitochondria in living cells with wide-field fluorescence microscopy indicated that mitochondria form a single dynamic network, whose continuity is maintained by a balanced frequency of fission and fusion events. Thus, the complete mixing of mitochondrial proteins can be explained by the formation of one continuous mitochondrial compartment after mating. In marked contrast to the mixing of parental mitochondrial proteins after fusion, mtDNA (labeled with the thymidine analogue 5-bromodeoxyuridine) remained distinctly localized to one half of the zygotic cell. This observation provides a direct explanation for the genetically observed nonrandom patterns of mtDNA transmission. We propose that anchoring of mtDNA within the organelle is linked to an active segregation mechanism that ensures accurate inheritance of mtDNA along with the organelle.

INTRODUCTION

The accurate transmission of subcellular organelles during eukaryotic cell division is an essential and regulated process. Before the completion of cell division, every organelle doubles its mass and is partitioned to a daughter cell (Warren and Wickner, 1996). For progeny to be respiratory-competent, both the mitochondrial organelle and its genome must be accurately transmitted. Although it is widely accepted that the partitioning of mitochondria during cell division is an active process involving the cytoskeleton, it remains unclear whether additional mechanisms are required for the partitioning of mitochondrial DNA (mtDNA) or whether its partitioning occurs randomly (Birky, 1994).

The simple eukaryote *Saccharomyces cerevisiae* is an excellent model system for examining the cellular and molecular mechanisms involved in mitochondrial inheritance because genetic and morphological techniques can be readily combined. A yeast cell contains from 1 to 10 mitochondria that form reticular structures localized at the cortex of the cell (Hoffman and

† Corresponding author and present address: Section of Molecular and Cellular Biology, University of California, Davis, CA 95616.

Avers, 1973; Stevens, 1979, 1981). Segregation of yeast mitochondria occurs at a specific point in the cell cycle, when a portion of the mitochondrial reticulum moves into the emerging daughter bud (Stevens, 1981; McConnell *et al.*, 1990). The cortical localization and reticular structure of mitochondria are important for their inheritance because mutants with abnormal mitochondrial morphology fail to distribute mitochondria into daughter cells (McConnell *et al.*, 1990). To date, the analysis of these mutants suggests that normal morphology requires specific mitochondrial outer membrane proteins and a protein homologous to the mammalian intermediate filament protein vimentin (McConnell and Yaffe, 1992, 1993; Burgess *et al.*, 1994; Sogo and Yaffe, 1994). Components distinct from those involved in maintaining mitochondrial morphology may be required for the movement of mitochondria into a daughter cell. The actual movement of mitochondria into daughter cells is most likely actin mediated, because in vitro yeast mitochondria can drive actin microfilament sliding and in vivo cells harboring a mutation in the nuclear gene *MDM20* that disrupts actin cables also blocks mitochondrial movement into buds (Simon *et al.*, 1995; Hermann *et al.* 1997).

There is an average of 50–100 copies of mtDNA contained within the organelle, depending on growth conditions and nuclear ploidy (Willamson, 1976). In vivo mtDNA, visualized with the DNA-specific fluorescent stain 4,6-diamidino-2-phenylindole (DAPI), is organized into punctate structures, termed nucleoids, that are distributed throughout the mitochondrial reticulum. It has long been known that heteroplasmic mtDNA populations (a mixture of different mtDNA molecules) present in zygotes sort out very rapidly during vegetative mitotic growth of the zygote (Thomas and Wilkie, 1968; Coen *et al.*, 1970; Birky, 1994). Within roughly 20 generations, virtually all progeny are pure (homoplasmic) for one mitochondrial genotype or the other. Random partitioning of mtDNA would require a significantly greater number of cell divisions before a homoplasmic state is reached. This observation indicates that the segregation of mtDNA during division is not occurring randomly and suggests that active mechanisms may be involved. Additional insight has been gained by examining the patterns of segregation during the first round of zygotic division by pedigree analysis (micromanipulation of cells from first division and subsequent analysis of colonies arising from them). These experiments indicate that the genetic composition of mitochondria in zygotic progeny depends on their position of budding from the zygote (Strausberg and Perlman, 1978; Zinn *et al.*, 1987). Biparental inheritance, in the form of recombinant products, occurs in buds arising from the middle. End zygotic buds, in contrast, often inherit mtDNA from only the parent that gives rise to that end of the zygote. These results indicate that, although mitochondrial fusion is occurring, parental mtDNA mixing is limited in zygotes (Strausberg and Perlman, 1978; Zinn *et al.*, 1987).

We sought to determine directly the cellular basis for the observed nonrandom patterns of mtDNA inheritance in *S. cerevisiae*. It has been observed with indirect immunofluorescence techniques that a haploid parent-derived mitochondrial protein redistributes throughout wild-type yeast zygotes after mating (Azpiroz and Butow, 1993). In light of bud-position–dependent inheritance of mtDNA, this observation suggests that mtDNA transmission is controlled separately from the transmission of the organelle (Azpiroz and Butow, 1993). By using more direct techniques, we have labeled mitochondria in yeast haploids with distinguishable fluorophores so that mitochondria derived from both parents can be followed simultaneously in vivo, allowing us to determine in detail the mechanisms involved in haploid-derived mitochondrial protein redistribution during mating. In addition, we have developed labeling techniques that allow us to examine, by using fluorescence microscopy, the fate of haploid-derived mtDNA to determine directly whether its behavior and transmission are under separate control during mating in yeast.

MATERIALS AND METHODS

Strains and Growth Conditions

Yeast strains used in this study are listed in Table 1. YP and minimal Casamino acids (vitamin assay Casamino acids, Difco, Detroit, MI) media were prepared as described (Nunnari *et al.*, 1993). Cells were grown in YP medium or uracil-deficient Casamino acids medium containing 2% galactose or 2% glucose where indicated.

Strain and Plasmid Construction

The herpes simplex virus type I thymidine kinase gene (*HSVTK*) was cloned by polymerase chain reaction (PCR) from plasmid pJM81 and placed under control of the GPD1 promoter (Sclafani and Fangman, 1986; Schena *et al.*, 1991). This construct was inserted into the yeast genome of strain W303–1A by replacing the coding region of the yeast *CAN1* gene via homologous recombination. Yeast that contained the integrated *GPD-HSVTK* were screened for their ability to grow in the presence of 60 μg/ml L-canavanine and then tested directly for expression of the *HSVTK* gene by monitoring growth on plates containing 5 mg/ml sulfanilamide (Sigma, St. Louis, MO), 100 μg/ml amethopterin (Sigma), and 100 μg/ml thymidine. The resulting strain is AFS98.

The mito-GFP chimeric gene was constructed by introducing a 5′ *Bam*HI site (in-frame and just 5′ to the initiator ATG codon) and a 3′ *Xba*I site after the termination codon into the green fluorescent protein (GFP) gene by PCR with Vent polymerase (New England Biolabs, Beverly, MA). An in-frame *Bam*HI site was introduced at the 3′ end of the DNA encoding the matrix targeting signal from mitochondrial cytochrome c_1 (corresponding to amino acids 1–32) using PCR and Vent polymerase. The DNA encoding the cytochrome c_1 matrix targeting signal was ligated to the modified *GFP* gene and the resulting gene fusion was inserted downstream of the GAL1/10 promoter in the yeast vector pTS210 (YCp50-based vector

from Tim Stearns, Stanford University), containing CEN/ARS sequences and the *URA3* gene (pmito-GFP).

Microscopy

For three-dimensional wide-field fluorescence microscopy, data collection was carried out with a 60 × 1.4 numerical aperture objective (Olympus, Melville, NY) and a scientific grade cooled charge-coupled device camera. All shutters, filter wheels, stage motion, and image acquisition were under computer control. Three-dimensional images were acquired by moving the stage in 0.2-μm intervals. At each focal position, an image was acquired at a maximum of two different wavelengths (605 nm for tetramethylrhosamine methylchloride [TMR-CH_2]-labeled proteins, and 540 nm for mito-GFP, depending on the experiment and the fluorophores used. After data collection, out of focus light was removed with a constrained iterative deconvolution algorithm (Agard *et al.*, 1989).

Standard fluorescence and phase microscopic analyses were performed with a Nikon Microphot-FXA or Nikon, and images were viewed through a 100 × 1.4 numerical aperature objective. The fluorochromes, fluorescein and TMR-CH_2Cl, were either individually or simultaneously viewed using the appropriate filter cube set. Images were recorded on Kodak Ektachrome P1600.

In Vivo Fluorescence Labeling of Mitochondria and Yeast Matings

Labeling Mitochondria with mito-GFP. Cultures of strain W303–1B transformed with pmito-GFP were grown in uracil-deficient Casamino acids medium containing 2% galactose (cas-Gal) to logarithmic phase at 30°C with shaking. Cells were collected by centrifugation and washed into YP medium containing 2% glucose (YPD) at an OD_{600} = 0.2/ml before mating.

Labeling Mitochondria with TMR-CH_2Cl. Cultures of strain W303–1B were grown in complete cas-Gal medium to logarithmic phase at 30°C with shaking. Ten OD_{600} U were collected by centrifugation and resuspended in 1 ml of complete cas-Gal. To label mitochondria, the vital dye TMR-CH_2Cl (Mitotracker, Molecular Probes, Eugene, OR) was added at a final concentration of 500 nM (from a 1 mM stock in dimethyl sulfoxide) and incubated with shaking for 20 min at 30°C. Unincorporated TMR-CH_2Cl was removed from the cells by repeated (10 times) centrifugation and resuspension in cas-Gal. The incubation and wash steps were repeated one additional time. Cells were collected by centrifugation and resuspended in YPD at a concentration of 0.2 OD_{600}/ml. Detergent extraction of fixed and permeabilized TMR-CH_2Cl-labeled cells had no effect on microscopic fluorescence intensity, indicating that TMR-CH_2Cl labeled mitochondrial protein and not lipid components.

Mating Assays. A 1-ml aliquot (OD_{600} = 0.2) of each labeled haploid strain culture was mixed together by vortex mixing and collected by centrifugation. The cell pellet was resuspended with 0.5 ml of YPD, concentrated by vacuum filtration on a nitrocellulose filter, and placed cell side up on a YPD plate. Cells were recovered from the filters at different times by placing the filter into 1 ml of YPD in an Eppendorf tube and vortexing. For microscopic analysis, cells were visualized by adhering them to polylysine-treated (0.1 mg/ml polylysine for 2 min) slide wells.

Bromodeoxyuridine (BrdUrd) Labeling and Detection of mtDNA

Cultures of strain AFS98 were grown to logarithmic phase (OD_{600} = 0.2) in YP medium containing 2% galactose (YPGal) at 30°C with shaking. α-Factor was added to a 5-ml aliquot at a concentration of 10 μg/ml and cells were incubated with shaking at 30°C for 60 min. G_1 cell cycle arrest was determined by monitoring the percentage of cells that were unbudded and was complete after the 60-min incubation period. Cells were pelleted by centrifugation and transferred into fresh YPGal at OD_{600} = 0.5, containing 10 μg/ml α-factor, 5 mg/ml sulfanilamide (Sigma), and 100 μg/ml amethopterin (Sigma, from a 1000× stock in dimethyl sulfoxide; labeling medium). BrdUrd was then added to a final concentration of 500 μg/ml, and cells were incubated at 30°C for 10 min. After this incubation, cells were pelleted by centrifugation and washed two times with labeling medium and finally resuspended into labeling medium containing 500 μg/ml thymidine. The mixture was incubated with shaking for 2.5 h at 30°C with one exchange into fresh labeling medium at 1.5 h. Cells were then pelleted and washed into YP medium containing 2% glucose and were mixed with 1 OD_{600} U of TMR-CH_2Cl-labeled W303–1B and placed under conditions that promoted mating as described above.

After zygotes appeared in the mating mixture (3 h), the cells were recovered from the filters as described above and fixed in YPD containing 3.7% formaldehyde at room temperature for 2 h. After fixation, the cells were washed three times with 1 ml of phosphate-buffered saline (PBS), pH 7.2, and two times with 1 ml of 0.1 M potassium phosphate, pH 7.4. Cells were resuspended into 1 ml of 0.1 M potassium phosphate, pH 7.4, and 50 μg/ml Zymolyase 100T was added. Cells were incubated at 30°C for 20 min to produce spheroplasts. Fixed spheroplasted cells were washed in 0.1 M potassium phosphate, pH 7.4, followed by PBS and resuspended into 0.5 ml of PBS. Cells were placed on polylysine-coated slide wells and allowed to adhere for 5 min in a humidified chamber. To prepare the cells for incubation with antibody to detect BrdUrd, the cells were incubated as follows: PBS containing 0.5% Tween 20 (Sigma) for 30 min; 0.6 N HCl for 5 min; 0.1 M sodium tetraborate, pH 8.5, for 5 min; 2× PBS for 5 min; PBS containing 5% bovine serum albumin (blocking buffer) for 10 min. Each treatment was performed in a humidified chamber at room temperature and excess liquid was aspirated from the cells between each step.

BrdUrd labeling was detected by incubating the cells with a 1:200 dilution of anti-BrdUrd antibody (Becton-Dickinson, San Jose, CA) in blocking buffer for 3 h at room temperature, followed by two washes with blocking buffer. Fluorescein-conjugated anti-mouse secondary antibody (Kappel) at a dilution of 1:100 in blocking buffer was incubated with the cells at room temperature for 1 h, followed by two washes with blocking buffer. Mounting medium (FITC-Guard, Testgog, Chicago, IL) was placed on fixed cells and coverslips were sealed with nail polish.

RESULTS

Mitochondrial Fusion Occurs during Yeast Mating

To directly follow the behavior of mitochondrial constituents originating from their respective parent cells during yeast mating, we labeled mitochondrial proteins of both haploid parents with fluorescent dyes of distinct emission wavelengths. In brief, haploid cells of one mating type were labeled in vivo with the red dye TMR-CH_2Cl, a so-called "vital dye" that, when driven by the mitochondrial membrane potential, selectively localizes to mitochondria where it becomes covalently attached to resident proteins. The resulting TMR-CH_2-labeled proteins are stably retained in mitochondria and can be directly observed in living cells by fluorescence microscopy. Haploid cells of the opposite mating type were labeled with a mitochondrial matrix-targeted form of the GFP (mito-GFP; Chalfie *et al.*, 1994). mito-GFP was created by fusing the mitochondrial matrix targeting signal from yeast cytochrome c_1 (corresponding to amino acids 1–32) to the amino terminus of GFP. To ensure that mito-GFP was

Table 1. Yeast strains

Strain	Genotype	Reference
W303-1B	*ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100, Matα*	R. Rothstein, Columbia University
W303-1A	Same as W303-1B, except *Mata*	R. Rothstein, Columbia University
AFS98	Same as W303-1A, except *can1::GPD-HSVTK*	This study

produced only in the haploid parent before mating, the gene encoding the fusion protein was placed under control of the GAL1/10 promoter. Mitochondria from parent cells were labeled during growth on galactose and transferred to glucose for mating. Two hours after switching to glucose, mito-GFP mRNA was no longer detectable by Northern blot analysis. This time point preceded the formation of zygotes. We, therefore, conclude that any mito-GFP detected after mating must be derived exclusively from a pool that was contained in parental mitochondria.

To observe the fate of mitochondria during mating, haploid cells of different mating types bearing either TMR-CH_2-labeled or GFP-labeled mitochondria were mixed. At different times, live cells were examined directly by fluorescence microscopy. At early times (1.5–2 h after cell mixing), we observed pairs of agglutinated cells of opposite mating type (Figure 1A). Cell pairs contained distinct nonoverlapping populations of mitochondria that fluoresced either red (corresponding to TMR-CH_2-labeled proteins) or green (corresponding to mito-GFP). In contrast, at later times (3–4 h after cell mixing), we found that both the parent-derived mitochondrial protein populations were completely intermixed and distributed throughout individual zygotes (Figure 1, B–D). Indeed, detailed comparisons of the distribution of TMR-CH_2-labeled proteins and mito-GFP within zygotes consistently showed their complete colocalization (Figure 1, B–D, Overlay). Optical sections were examined (see MATERIALS AND METHODS) throughout the entire zygote and colocalization in every 0.2-μm serial section examined was confirmed. These observations strongly support the notion that the redistribution of the parental fluorophores was the result of mixing of mitochondrial contents, indicating that mitochondrial fusion and not movement of parent-derived mitochondria was responsible for the identical distribution of mitochondrial marker proteins.

To determine the timing of mitochondrial fusion, we compared it to nuclear fusion (or karyogamy), an event that occurs rapidly after plasma membrane fusion. For this analysis, we scored nuclear fusion by staining nuclei with the fluorescent dye DAPI, which stains DNA. In the majority of unbudded zygotes, a single nucleus was present, indicating that nuclear fusion had occurred (83%, n = 114), whereas in a minor but significant number (17%, n = 23) of unbudded zygotes, the nuclei were either bilobed or completely separate, indicating that karyogamy in these latter zygotes was not complete. These cells are likely to represent karyogamy intermediates because 100% of zygotes that had already budded contained a single nucleus. In contrast, no intermediates in mitochondrial fusion were observed in the population. These results suggest that, during mating, mitochondrial fusion occurs rapidly after fusion of the plasma membranes, at a time that precedes or accompanies karyogamy.

In Vivo Visualization of Yeast Mitochondria as One Continuous Network

Probably the most surprising feature of the mitochondrial fusion events described is the complete rapid mixing of mitochondrial content from each haploid. In fact, no mitochondrial segments that were derived from only one parent were observed in zygotes (Figure 1, B–D). These results could be explained if mitochondria from each haploid were connected in a network. In this case, fusion at one or a few points between parental mitochondria would create a single contiguous organelle in which marker proteins could freely and rapidly intermix by diffusion.

To address this possibility directly, we used mito-GFP labeled cells to observe and reconstruct the three-dimensional disposition of mitochondria. Mito-GFP is a bright fluorophore that is particularly resistant to photobleaching. This property allowed us to use wide-field fluorescence microscopy to acquire images in three dimensions over long intervals (Agard *et al.*, 1989). Two representative examples of a total of 30 reconstructed mitochondrial structures are shown as stereopairs in Figure 2.

From our data, it is apparent that mitochondria form a continuous reticular structure localized at the cell cortex. This view of mitochondrial structure is similar to that determined by reconstructing serial thin sec-

Figure 1 (facing page). Mitochondrial fusion occurs during conjugation. Prezygotic paired haploid cells (A) and zygotes (B–D). Mitochondria from haploid *Mata* yeast cell populations were labeled with mito-GFP and mitochondria from haploid *Matα* yeast cell populations were labeled with TMR-CH_2Cl and mated as described. Images were obtained from live cells using wide-field fluorescence microscopy and out-of-focus light was removed by using

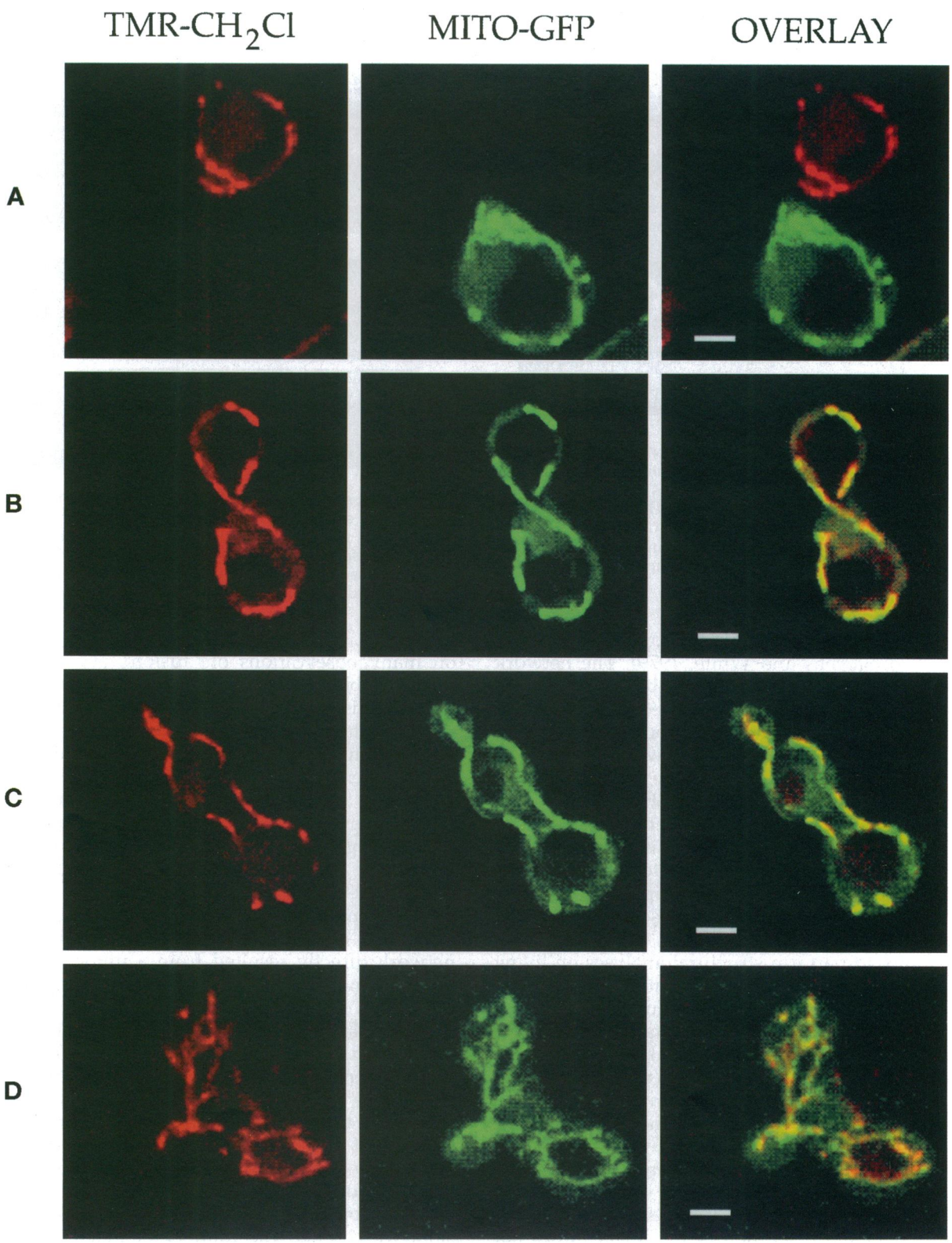

Figure 1 (cont). a constrained iterative deconvolution algorithm. Data obtained from a 0.2-μm optical section of mito-GFP (in green, center) and TMR-CH_2-labeled mitochondria (in red, left) are shown. Overlay (right) indicates the image obtained by merging the signals from mito-GFP and TMR-CH_2-labeled mitochondria. Bar, 1 μm.

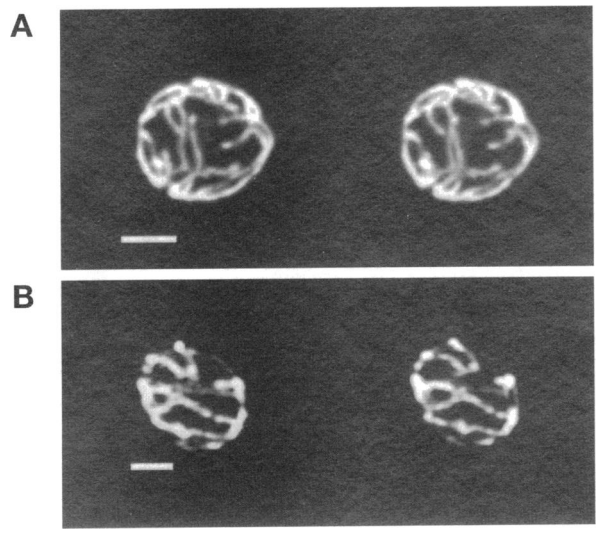

Figure 2. The three-dimensional structure of yeast mitochondria. Wild-type cells were grown to logarithmic phase in medium containing galactose at 30°C. An aliquot (3 μl) was removed and placed on a microscope slide under a coverslip and sealed with nail polish. Three-dimensional images were acquired by using wide-field fluorescence microscopy and moving the stage in 0.2-μm increments. Two representative examples are shown (A and B). Bars, 1 μm.

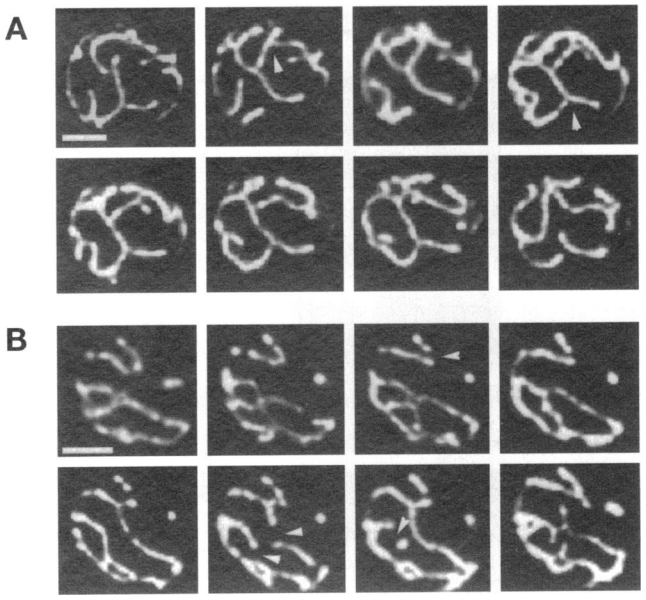

Figure 3. Fission and fusion of mitochondria. Wild-type cells from Figure 2 are shown. Time-resolved images from a single optical section of 0.2 μm. Time increases from left to right in 3-min increments. Representative fusion events are indicated by arrowheads in A and representative fission events are indicated by arrowheads in B. Bars, 1 μm.

tions viewed by electron microscopy (Hoffman and Avers, 1973; Stevens and White, 1979). The network consists primarily of tubules and is branched at multiple points. On average we observed approximately eight bifurcations per cell. Most tubules terminate in branch points at both of their ends; whereas a smaller fraction of tubules is connected to the network only by one end and the other end is free. Shifting cells from aerobic growth conditions to anaerobic glucose-repressed conditions for up to 7 h (limited by the stability of mito-GFP) did not affect the mitochondrial structure, indicating that there was no rapid regulation of mitochondrial dynamics in response to changes in metabolic state of the cell.

The ability to perform structural analyses on living cells allowed us to observe and quantitate the dynamics of the mitochondrial network. Figure 3 shows time-lapsed images of mitochondria contained in a single 0.2-μm section from the individual cells shown in Figure 2 (increasing time from left to right, sampled at 3-min intervals). When images from each successive time are compared, changes in the mitochondrial structure that appear to be due to fusion and fission events can be observed (Figure 3, A and B, arrowheads in A indicate representative fusion events and arrowheads in B indicate representative fission events). Although the spatial resolution of the images does not allow us unambiguously to distinguish fusion from close spatial approximation, we consider it very likely that fusion does indeed occur. This conviction is based on many observations of "fusion" events that produced stable connections, which in later times, behaved indistinguishably from other contiguous segments of the network.

Interestingly, such fusion events were only observed when either two mitochondrial ends came together or when one end approached a tubule from the side. Thus, in every case, a mitochondrial end was involved, suggesting that such ends may contain components of a putative fusion machinery. Indeed, end-dependent mitochondrial fusion also has been observed in other organisms (Bereiter-Hahn and Voth, 1994). All new branch points in the mitochondrial network directly resulted from a mitochondrial end approaching a tubule side, but never originated by outgrowth from the side of a tubule, as observed for the endoplasmic reticulum in other systems (Dabora and Sheetz, 1988; Vale and Hotani, 1988). Similarly, fission events originated either within a tubule (thus creating two ends) or at branch points (creating one end and a tubule). These events were not simply the reversal of preceding fusion events but occurred at apparently random positions within the mitochondrial network.

To approximate the relative rates of fission and fusion, we counted the number of fusion and fission events occurring in optical sections, representing 75% of total mitochondrial volume, from each of several cells over time. As summarized in Table 2, fusion and

fission rates were approximately equivalent in each individual cell examined. Extrapolating from these data, the average rate of mitochondrial fusion or fission measured under our conditions is approximately 0.5 event/min. The fact that the rates of fusion and fission are matching suggests that at any given time, mitochondria are likely to be connected in a cell. The presence of a dynamic continuous network, therefore, provides a likely explanation for the rapid mixing of the haploid-derived mitochondrial marker proteins after mating.

mtDNA Remains Segregated after Mitochondrial Fusion

The complete mixing of mitochondrial proteins after mating that we and others have observed is surprising in light of genetic data obtained from pedigree analyses (Azpiroz and Butow, 1993). Such data indicate that mixing of mtDNA from both parental cells is limited (Strausberg and Perlman, 1978; Zinn *et al.*, 1987). To address this paradox, we followed the fate of mtDNA directly during mating.

To this end, we developed a method to label mtDNA with the thymidine analogue BrdUrd that can be subsequently detected with a fluorescently labeled antibody. Yeast lacks a thymidine kinase required to phosphorylate BrdUrd, a step that is a prerequisite for its incorporation into DNA (Leff and Eccleshall, 1978; Sclafani and Fangman, 1986; Dien and Srienc, 1991). We, therefore, constructed a strain (AFS98) that contains an exogenous thymidine kinase gene from HSV-I integrated into the chromosome.

To incorporate BrdUrd into mtDNA selectively, we arrested cells in the G_1 by treatment with the mating phermone α-factor. Punctate labeling was detected at the cortex of cells exposed to BrdUrd after G_1 arrest. This punctate label overlapped with TMR-CH_2-labeled mitochondrial proteins, indicating that it corresponds to mtDNA (Figure 4). The overlap was not complete, however, because mtDNA is organized into compact structures (nucleoids) that are not homogeneously dispersed. Mitochondrial nucleoids detected with anti-BrdUrd-labeled antibodies are spaced throughout the mitochondrial network, resembling the pattern observed with DAPI staining (Miyakawa *et al.*, 1984).

To follow the fate of mtDNA after mitochondrial fusion during mating, haploid cells labeled with BrdUrd were mated with cells of the opposite mating type labeled with TMR-CH_2Cl. In contrast to the redistribution of parental mitochondrial marker proteins (Figure 1, B–D), BrdUrd-labeled mtDNA was not observed throughout the zygote but remained distinctly localized to one lobe (Figure 4, B and C, punctate structures in yellow and green, respectively). The zygotic lobe containing BrdUrd-labeled mtDNA we detected presumably originated from the BrdUrd-labeled parental cells. We observed the redistribution of TMR-CH_2Cl-labeled mitochondrial proteins throughout the same zygotes in which BrdUrd-labeled mtDNA remained localized, confirming that mitochondrial fusion and mixing had occurred (Figure 4, B and C, red). This pattern was observed in the majority of zygotes examined (84%, n = 43). In the remaining zygotes, BrdUrd-labeled mtDNA was distributed throughout the zygote. However, in these latter cases, a significant pool of unincorporated BrdUrd remained in the cells after mating (indicated by a brightly stained nucleus). We consider it probable that the BrdUrd-labeled mtDNA in these zygotes was synthesized after mating.

Although BrdUrd-labeled mtDNA did not distribute throughout the zygote after mitochondrial fusion, BrdUrd-labeled mtDNA entered buds emerging from the midpoints of the bilobed zygotic cells (zygotic bud position indicated by b and arrowheads indicated BrdUrd-labeled mtDNA in bud; Figure 4, B and C). The static distribution of BrdUrd-labeled mtDNA in the zygote suggests that end buds would contain primarily, if not exclusively, mtDNA derived from the particular parent cell giving rise to the portion of the zygote from which an end bud would form.

Table 2. Measure of relative rates[a] of fusion versus fission during vegetative growth

	Total no. of fission events	Total no. of fusion events
Cell 1 (39 min)[b]	11	12
Cell 2 (39 min)	13	15
Cell 3 (33 min)	19	16
Cell 4 (27 min)	9	9
Cell 5 (45 min)	12	18
Cell 6 (45 min)	16	16
Cell 7 (54 min)	32	28
Average rate (events/min)	0.39 ± 0.14	0.40 ± 0.08

[a] Values obtained from 75% of total cell volume.

[b] Values in parentheses are total times.

DISCUSSION

By using three-dimensional wide-field fluorescence microscopy, we have shown that in the yeast *S. cerevisiae*, mitochondria form one continuous dynamic reticular organelle and that this structure affects the pattern of mitochondrial inheritance. In zygotes, fusion between parental mitochondria creates a single continuous organelle that conducts the complete and rapid mixing of mitochondrial protein. Fusion also places parental mitochondrial genomes into one compartment, an event that ultimately results in genetic

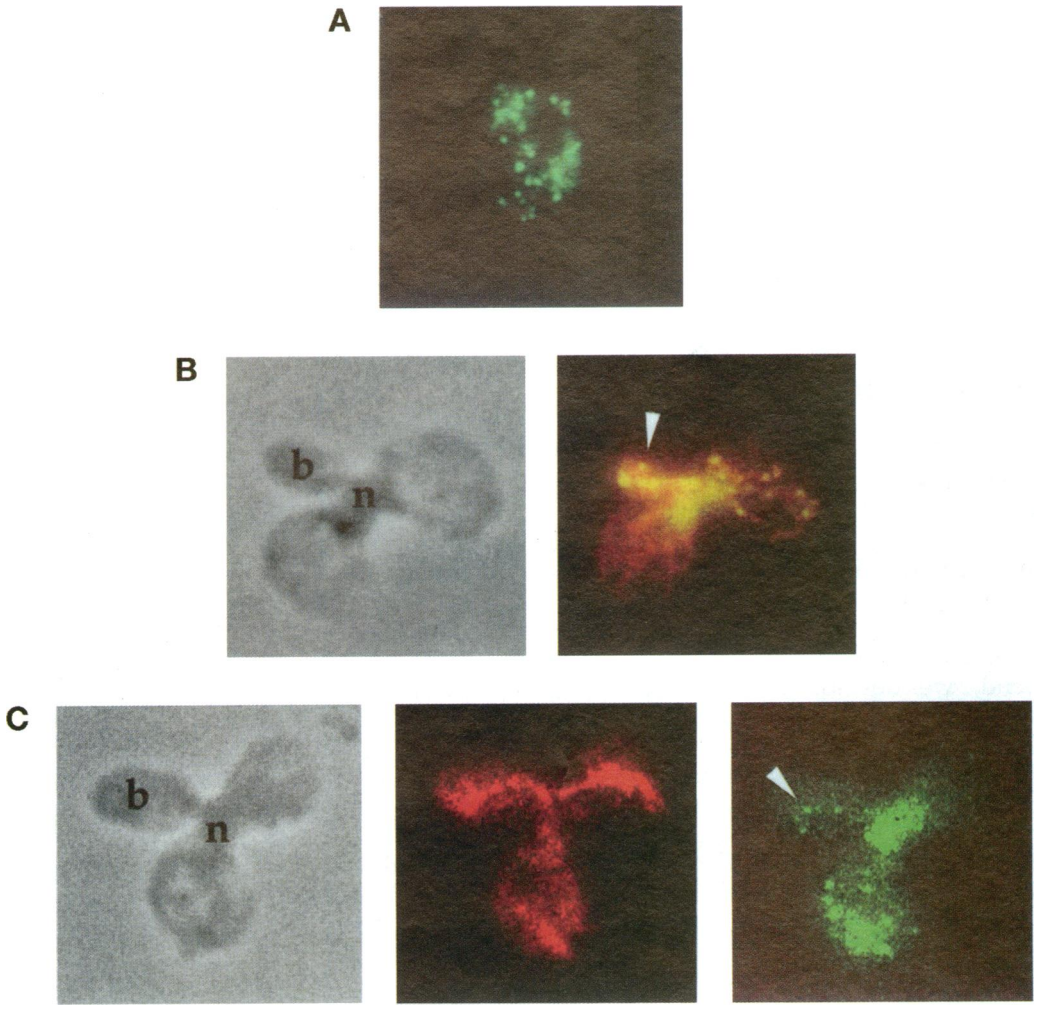

Figure 4. Intramitochondrial segregation of mtDNA in zygotes. mtDNA from *Mat***a** cells were labeled with BrdUrd, mitochondria from *Matα* cells were labeled with TMR-CH_2Cl, and the cells were mated. Cells were fixed with formaldehyde. BrdUrd incorporation was detected by indirect immunofluorescence with anti-BrdUrd antibody and fluorescein-conjugated secondary antibody. Haploid cells labeled with BrdUrd (A) and zygotes from matings with BrdUrd-labeled haploid cells (B and C) are shown. The green (A and C) and yellow (B) punctate fluorescence denotes the BrdUrd labeling and the red fluorescence denotes the TMR-CH_2-labeled mitochondrial proteins (B and C). Phase images of the zygotes are also shown (B and C). The position of the zygotic bud (b) and nucleus (n) are indicated in phase images. Nuclear BrdUrd labeling was due to a pool of unincorporated BrdUrd that was resistant to chase.

recombination. mtDNA from either parent, however, does not freely diffuse through the zygotic mitochondrial reticulum. Thus, after mating, daughter cells with three different mitochondrial genotypes become possible: those that bud from the midpoint of the zygote and, therefore, are likely to contain mtDNA from both parents and those that bud from either end of the zygote and are, therefore, likely to contain mtDNA from only one of the two parents. The structure of mitochondria, therefore, has direct consequences for cytoplasmic inheritance.

Mitochondrial structure varies significantly from cell type to cell type (Johnson *et al.*, 1980). Some cell types contain an abundance of distinct mitochondria distributed throughout the cytoplasm, whereas others contain a smaller number of long thread-like tubes. We have shown herein that mitochondrial fission and fusion shape the structure of the organelle in yeast. Specifically, the continuous nature of the mitochondrial network appears to result from offsetting rates of fission and fusion. Given that mitochondrial fission and fusion have been observed or suggested from studies in a number of other organisms, it is likely that the regulation of membrane dynamics is a mechanism utilized by all eukaryotic cells to establish the steady-state structure of this organelle (Bereiter-Hahn and Voth, 1994; Hayashi *et al.*, 1994). It is possible that mitochondrial structure is regulated in some cells by

metabolic demand for mitochondrial function (Bereiter-Hahn and Voth, 1994). Elongated mitochondria, for example, might be more efficient for energy production because of their ability to conduct the ATP-generating membrane potential over long distances.

The components that mediate fission and fusion of mitochondria are unknown. From the mixing of the mitochondrial-matrix–targeted GFP, we can infer that fusion of both the mitochondrial outer and inner membranes occurs. Fusion of a double membrane-bounded organelle must involve two fusion steps both of which are likely to require a unique fusion apparatus operating on the respective membrane system. In the outer membrane, such fusion components may be concentrated specifically at the ends of tubules, as suggested by our observation that branched structures in the mitochondrial network always arise from the fusion of a mitochondrial end. We have tested three obvious candidates for proteins that might participate in mitochondrial fusion: the yeast *N*-ethylmaleimide-sensitive factor Sec18p and its homologues Cdc48p and Afg2p (involved in secretory vesicle fusion, homotypic endoplasmic reticulum membrane fusion) and a protein of unknown function (Eakle *et al.*, 1988; Wilson *et al.*, 1989; Thorsness *et al.*, 1993; Latterich *et al.*, 1995). Preliminary studies indicate that temperature-sensitive mutations in the genes corresponding to these proteins have no effect on mitochondrial content mixing during mating. Thus, it is possible that the mechanism of mitochondrial fusion is fundamentally different from fusion events occurring in the secretory pathway.

We have shown that, although the movement of mtDNA within the organelle is restricted, mtDNA is still able to enter emerging buds. Segregation of mitochondria into daughter buds is an ordered nonrandom event that occurs at a specific point in the cell cycle, suggesting that it is coordinated by protein components (Stevens, 1981). Such a putative segregation machine may work hand-in-hand and possibly share components with structures that keep mtDNA anchored within the organelle. mtDNA, for example, may be attached to some cytoplasmic cytoskeleton via structures that traverse both the inner and outer membrane. One example of such an attachment is found in *Trypanosome brucei*, where mtDNA molecules are organized into a single structure, termed the kinetoplast. In this organism, mtDNA is replicated at a discreet phase of the cell cycle, and each copy is attached to a flagellar basal body (Robinson and Gull, 1991). Partitioning of mtDNA is mediated by the microtubule-dependent separation of these basal bodies during cell division in a manner that resembles the partitioning of nuclear chromosomes (Robinson and Gull, 1991; Robinson *et al.*, 1995). In the majority of other eukaryotic cells, including *S. cerevisiae*, however, the requirements for mtDNA segregation may be more relaxed because mtDNA is present in multiple copies. Nevertheless, other types of cytoskeletal attachment sites may exist and be responsible for anchoring and limiting the diffusion of mtDNA within the organelle. Other possibilities that could account for the observed anchoring of mtDNA include cytoskeleton-like structures in the mitochondrial matrix that could create specific membrane attachment sites for mtDNA or generally restrict its diffusion within the organelle. Nuclear mutations that affect the maintenance of mtDNA in vivo have been isolated. Included among these is *MGM1*, a gene encoding a dynamin-like protein, that may play a role in anchoring and/or segregating mtDNA (Jones and Fangman, 1992; Guan *et al.*, 1993; Backer, 1995). Independent of the precise molecular mechanism, however, limiting the diffusion of mtDNA within the mitochondrial network is likely to be an important mechanism by which the cell ensures the inheritance of the mitochondrial genome.

ACKNOWLEDGMENTS

We are grateful to Maho Niwa, Steven Ogg, Ted Powers, and Caroline Shamu for scientific discussions and comments on the manuscript and to members of Bruce Alberts' laboratory and Tim Mitchison for advice. This work was supported by grants from the National Institutes of Health to J.W.S., A.M., and P.W.

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