Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin

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Nuclear gene products replicate and partition mitochondrial DNA (mtDNA) molecules in the yeast *Saccharomyces cerevisiae*. However, few of the relevant genes have been identified. A mutation that results in temperature-sensitive loss of mtDNA identifies one of these genes, *MGM1*. Deletion of *MGM1* shows that aside from its role in the mitochondrion, the gene has no essential cellular function. The *MGM1* protein has a 200-amino-acid region that is highly related to a family of GTP-binding proteins of apparently diverse function that includes the microtubule-binding protein, dynamin D100. The temperature-sensitive strain partitions mtDNA molecules at the restrictive temperature, but a defect in mtDNA synthesis results in a reduction in the number of molecules per cell at each cell division. On the basis of the results of this study, we conclude that cells can partition single mitochondrial genomes, and that when a cell receives a single molecule at division it is able to restore the normal complement of multiple copies.

[Key Words: *Saccharomyces cerevisiae*; mitochondrion; DNA replication; DNA segregation; GTP binding; dynamin]

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The mitochondrial genome of eukaryotic organisms is maintained by the products of nuclear genes that supply the machinery for replication and partition to daughter cells at division. The mitochondrial genome of *Saccharomyces cerevisiae* has been studied extensively because cells can grow by fermentation in the absence of respiratory function. The ability to grow without a functional mitochondrial genome has greatly facilitated mutant analysis. Several laboratories have identified mutations in nuclear genes, called PET genes, that affect respiratory competence (for review, see Tzagoloff and Dieckmann 1990). Thus far, only a small subset of the PET genes have been found to be involved in the maintenance of the mitochondrial genome: *MIP1* coding for DNA polymerase (Foury 1989), *PPA2* encoding a pyrophosphatase (Lundin et al. 1991), and *ABF2* coding for a DNA-binding protein (Diffley and Stillman 1991).

We are using a simple mutant screen to identify additional nuclear genes required for the maintenance of mitochondrial DNA (mtDNA) in *S. cerevisiae*. Here, we report the analysis of one of these mitochondrial genome maintenance genes, *MGM1*. The predicted *MGM1* protein contains a 200-amino-acid region that is highly related to a family of putative GTP-binding proteins and

includes the microtubule-binding protein dynamin D100. A mutation in *MGM1* results in the temperature-sensitive loss of mtDNA molecules. At the restrictive temperature, mutant cells appear to partition their mtDNA molecules normally but mtDNA synthesis is blocked.

Results

Identification and mapping of the MGM1 gene

We used a novel, simple screen to identify mutants defective in mitochondrial genome maintenance. Certain adenine-requiring strains produce a red pigment and form red colonies on YEPD medium, provided that the cells can respire. Cells that have lost the ability to respire form white colonies (Reaume and Tatum 1949). The change in colony color accompanying the loss of respiration competence allows an easy first step to identify the mutants of interest. An *ade1 ade2* strain, BS127, was mutagenized with ethylmethanesulfonate (EMS) and plated at 23°C. Individual colonies were streaked onto plates and incubated at 36°C. Clones that produced a high frequency of white sectors at 36°C were subsequently tested for their inability to grow on glycerol at 36°C to confirm that they give rise to respiration-deficient cells. Cells in the white colonies formed by these

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temperature-sensitive mutants were screened by DAPI staining for the absence of chondriolites, the bright spots of cytoplasmic fluorescence indicative of mtDNA (Williamson 1976; Williamson and Fennel 1979). (Cells lacking mtDNA are called ρ^0 .) This paper characterizes one of these mutations, *mgm1-1*. It is recessive and, in meiosis, segregates 2 : 2 for the temperature-sensitive phenotype, the pattern expected for a nuclear gene.

Hybridization of the cloned *MGM1* gene (see below) to yeast chromosomal DNAs separated by pulse-field gel electrophoresis (Carle and Olson 1984) indicated that the gene maps to one of two unresolved chromosomes, VII or XV (data not shown). Genetic crosses revealed linkage to markers on the right arm of chromosome XV, and a four-factor cross with *his3*, *met7*, *ade2*, and *mgm1-1* showed that *MGM1* is 12 cM centromere-distal of *HIS3* and 27 cM proximal of *MET7* (88 tetrads examined). Southern analysis and sequence comparisons with other cloned genes in this region show that *MGM1* is a previously unidentified gene located in a cluster of known genes. Beginning with the gene closest to the centromere, the order of the genes in the cluster is *PET56-HIS3-DED1-RET1-PTP1-MGM1-STE4-MET7* (Fig. 1; James et al. 1991).

Segregation of mtDNA in the absence of synthesis

When a culture of *mgm1-1* cells is transferred from 23 to 34°C, cells that yield white (respiration-deficient) colonies at 23°C are not produced for 4.0–4.25 generations (~5 hr); subsequently, they appear at a rate of 50% per cell per division (Fig. 2). Samples of the culture transferred from 23 to 34°C were also stained directly with DAPI. Cells lacking chondriolites appear with kinetics similar to those for white colonies (Fig. 2), indicating that the mitochondrial genotype is already ρ^0 at the time a cell destined to form a white colony is returned to the permissive temperature. These observations are consistent with a defect in which mtDNA replication ceases at 34°C and the preexisting mtDNA molecules are diluted

Figure 2. Rate of white (ρ^0) colony formation when cells are grown at the restrictive temperature. The fraction of the population that still forms red (respiration-competent) colonies is plotted vs. the amount of time at the restrictive temperature. During the course of the experiment, both the *MGM1-1* and *mgm1-1* strains grew at the same rate, 75–80 min/generation. The apparent frequency of cells containing mtDNA in the *mgm1-1* culture, as determined by DAPI staining, is also shown (chondriolite + cells).

by subsequent cell divisions. Direct support for this interpretation is provided by two experiments. First, a *mgm1-1* culture was transferred from 23 to 34°C, samples were taken at different times and stained with DAPI, and the number of chondriolites per cell was counted. Figure 3 shows that the average number of chondriolites per cell begins to decline during the first hour of incubation, well before white colony-forming cells arise. If the mutation resulted in the failure of chon-

Figure 1. Map of the region of chromosome XV that contains *MGM1*. The clone containing *MGM1* overlapped with clones containing *STE4* (Whiteway et al. 1989) and *PTP1/RET1* (James et al. 1991). The solid line represents the original clone containing *MGM1*, and the broken lines represent the flanking DNA defined by the other two clones. Restriction sites that can be used to align the clones are shown above the line. Below the line are several additional restriction sites relevant to the construction of the three plasmid inserts. *pΔI* and *pΔ54* were among a series of plas-

mids generated by cutting at the *BglII* site and making a series of deletions using exonuclease III. *pΔI* was the largest deletion that was still able to complement the *mgm1-1* defect. The insert in this plasmid was sequenced (see Materials and methods). *pΔ54* was the smallest deletion that could no longer complement the *mgm1-1* defect. The right end of the deletion lies between the *PstI* and the *PvuII* sites (22 bp apart, 80–88 amino acids from the carboxyl terminus). The *URA3* substitution-insert in *pBJ16* is not drawn to scale. A *MluI*–*AhaII* digest of *pBJ16* liberates a linear fragment that can be used to delete the chromosomal copy of *MGM1* (see Materials and methods). (V) *PvuII*; (E) *EcoRI*; (P) *PstI*; (N) *HindIII*; (B) *BglII*; (H) *HpaI*; (M) *MluI*; (A) *AhaII*.

Figure 3. Reduction in the number of chondriolites per cell on incubation at the restrictive temperature. All samples in the experiment were "blind" coded and scored before they were identified.

driolites to segregate, with one product of a cell division receiving all or most of the chondriolites and the other cell receiving few or none, a bimodal distribution of chondriolites per cell would be observed after growth at 34°C. That the mtDNA is approximately evenly partitioned between dividing cells was confirmed by fluorescence microscopy of DAPI-stained mitotic cells (defined as cells with a large bud, where the nucleus is located in the neck between the mother and daughter cells or is elongated, extending between the mother and daughter cell). Figure 4A shows an example of a wild-type cell, and Figure 4B shows an example of a *mgm1-1* cell from a culture that has been at 34°C for 2.5 generations. Both show approximately equal mtDNA staining in the mother cell and in the smaller daughter bud. During the course of this work, >2000 mitotic *mgm1-1* cells taken during the first one to six generations after transfer to 34°C were examined; not a single example was observed with an asymmetric distribution of mtDNA. [As a control, we examined wild-type cells treated with acriflavine. Treatment of yeast with acriflavine produces cell divisions in which the daughter cell frequently becomes ρ^0 (Ephrussi and Hottinguer 1951; Mattick and Nagley 1977). In cultures growing in the presence of acriflavine, we observed a large fraction of mitotic cells with an asymmetric distribution of chondriolites (an example is shown in Fig. 4D) consistent with a failure of mtDNA segregation.]

Quantitative hybridization was used in a second experiment to show directly that net mtDNA synthesis ceases quickly in the *mgm1-1* culture at the restrictive temperature. Total DNA was prepared from samples taken at different times after the transfer to 34°C. The DNA was slot-loaded onto filters and hybridized to a probe for a single-copy nuclear sequence (*MGM1*) and, after stripping, to a probe for mtDNA (*COB*) (data not shown). Densitometric analysis showed that the amount of mtDNA per cell (mtDNA/nuclear DNA signal) begins to decline immediately after transfer of *mgm1-1* cells to 34°C: It decreased to 34% of the initial value by 6 hr and to 14% by 12 hr. There was no effect on the level of the 2 μ plasmid, a native, nuclear, extrachromosomal element. The decline in mtDNA per cell in the *mgm1-1* mutant is explained most simply by a defect in mtDNA replication.

Quantitative hybridization analysis (Materials and methods) of *mgm1-1* cells growing at the permissive temperature showed that they contain 14 ± 5.2 (three determinations) mtDNA molecules per cell. The approximately four-generation lag in appearance of respiration-defective cells at 34°C (see Fig. 2), combined with the observations of an immediate decline in chondriolites per cell (see Fig. 3) and of a cessation in mtDNA synthesis, must mean that there is nearly equal partitioning of these molecules at each cell division. Therefore, cells that remain capable of forming respiration-competent colonies after four generations contain only one or a few mtDNA molecules. To determine whether the normal amount of mtDNA is restored in clones formed from these cells, nine respiration-competent colonies were picked from 23°C platings of cultures that had been incubated for 9.5 generations at 34°C. A single quantitative analysis on cultures grown up from each of these colonies gave copy numbers similar to those found initially (range: 8–22 per cell; 14 ± 4.0).

Effects of MGM1 deletion

The *MGM1* gene was cloned by transforming a *mgm1* strain with a 2 μ plasmid library (Carlson and Botstein 1982) and selecting for complementation of the *mgm1-1* mutation by plating on glycerol plates at 36°C. Subcloning and deletion analysis of the insert indicated that the complementing activity was confined to a 4.5-kb fragment (p Δ I; see Fig. 1). This fragment was sequenced (Materials and methods) and found to contain an open reading frame of 2.5 kb encoding an 843-amino-acid protein (Fig. 5). The only other open reading frames in the fragment coded for no more than 60–70 amino acids. To determine the phenotype of a cell deleted for this gene, a 2.7-kb *HpaI*–*HpaI* fragment containing all codons except those for the carboxy-terminal 55 amino acids was removed from the cloned insert and replaced with a 1.3-kb fragment containing the *URA3* gene (pBJ16; see Fig. 1). A 2.1-kb linear *MluI*–*AhaII* fragment (see Fig. 1), now containing the *URA3* substitution, was transformed into diploid yeast cells. Twenty-eight meiotic tetrads from

Figure 4. DAPI-stained cells undergoing mitosis. (A) An *MGM1* cell displays a brightly staining nucleus in the neck of the bud. The chondriolites are evenly distributed between the mother and the daughter. (B) An *mgm1-1* cell grown at the restrictive temperature for 2.5 generations. Chondriolites are evenly distributed between the mother and the daughter cell. (C) An *mgm1-1* cell that has become irreversibly respiration deficient. The bright-staining nucleus is still present, but the chondriolites are no longer visible. (D) *MGM1* cells grown in the presence of 5 μ g/ml of acriflavine have an asymmetric distribution of chondriolites when the nucleus is in the neck of the bud.

six independent diploid transformants were examined. In all tetrads, the Ura^+ phenotype, which signals the *MGM1* deletion, segregated with respiration deficiency and the loss of chondriolites. Southern analysis of two complete tetrads showed that the Ura^+ spores contained the expected deletion-substitutions (data not shown). The only phenotypes observed in the deletion strains were the loss of mtDNA, respiration deficiency, and a reduced rate of growth on glucose medium that is typical of respiration-deficient cells. These results indicated that the deleted gene has no essential cellular function other than maintaining respiration competence.

Because genetic mapping shows that the *mgm1-1* mutation is located between *HIS3* and *MET7*, and the restriction map and the sequence of the cloned insert that complements *mgm1-1* includes part of the *STE4* gene that maps to the same region (see Fig. 1), it seemed likely that the cloned sequence is the structural gene for *MGM1*. The cloning vector is a high-copy-number plasmid, however, and the possibility remained that the clone does not contain *MGM1* but, instead, a closely linked gene that can suppress the *mgm1-1* defect when its product is overproduced. We proved that the cloned sequence corresponds to *MGM1* by showing that a chromosome with the *URA3* deletion-substitution does not complement the defective *mgm1-1* allele.

The wild-type (ρ^+) mitochondrial genome gives rise to deletion variants at high frequency. Some of these ρ^- genomes may be replicated by a mechanism that cannot be used efficiently by the ρ^+ genome (Fangman et al. 1990). Therefore, their maintenance might not be dependent on *MGM1*. We examined the replication of two ρ^- genomes, ρ^- HS3324 and ρ^- 4a. ρ^- HS3324 contains one of the *rep* (or *ori*) sequences thought to be a major replication origin; ρ^- 4a lacks *rep* sequences and consists of a simple 64-bp 100% A + T repeated sequence (Fangman et al. 1989). mtDNA was lost when *MGM1* was deleted from strains containing each of these ρ^- mtDNAs (data not shown), indicating that at least some ρ^- genomes require *MGM1* for their maintenance.

Features of the MGM1 protein sequence

The polypeptide predicted from the sequence of the *MGM1* gene is 843 amino acids long (Fig. 5) with a mass of ~ 94 kD. The carboxy-terminal 80–88 amino acids appear to be essential for function, as deletion of this material from the cloned gene results in a plasmid, p Δ 54 (see Fig. 1), that fails to complement the *mgm1-1* mutation even at a high copy number. The amino acid sequence of the predicted amino terminus of the *MGM1* protein is consistent with that of proteins targeted to the mitochondrion. Targeting sequences are 10–70 amino acids long. They are rich in positively charged and hydroxylated amino acids and contain a potential amphiphilic helix (Gavel et al. 1988; Hartl and Neupert 1990). The first 50 amino acids of the *MGM1* protein fits this description, with amino acids 18–36 potentially forming an amphiphilic helix with a hydrophobic moment of 13.8 (von Heijne 1986), among the highest of proteins known to be imported into mitochondria.

Part of the deduced *MGM1* protein is highly related to three other proteins of apparently diverse function: the dynamin D100 protein from vertebrates and *Drosophila* (Obar et al. 1990; Chen et al. 1991; van der Blik and Meyerowitz 1991), *Mx* protein found in vertebrates (Arheiter and Meier 1990), and the *VPS1*, or *SPO15*, protein found in yeast (Rothman et al. 1990; Yeh et al. 1991). All four proteins are highly related within two closely spaced blocks (A and B) that span ~ 200 amino acids (Figs. 6 and 7). The A block, near the amino terminus, is 45 amino acids long and 52% identical among all four proteins, allowing no gaps. Comparison of *MGM1* with each of the other three proteins shows 61–67% identities. Between the A block and the B block there is a region of ~ 35 amino acids (70 amino acids in *VPS1*) where the proteins share little similarity. After this short gap, the B block extends for the next 165 amino acids and is 23% identical among all four proteins, with *MGM1* ranging from 30% to 41% identical in pairwise comparisons.

ATG	TGT	CTT	TTT	TTT	ACG	CAG	TAT	CTC	TTG	CTG	AAA	CAT	TTT	TCA	ACG	CGT	GTA	AAA	ATT	TGC	ATC	TGA	CGA	ANG	TGC	GTA	CAA	AGG	GTT	-913	
GTG	GTC	CCA	AAA	GAG	GTA	GTA	CAC	GCC	GAG	AAT	TTG	CAC	AGG	GTA	CTG	GTT	CAA	AAA	GGT	ANG	TAC	CCC	GCA	GGG	ACG	GTT	GAA	GCT	ATT	-723	
GAA	TCT	TCG	TCT	CAT	CGG	CTG	ATA	CAA	TCC	GAT	CAT	ACA	TAC	GAA	CTG	GTT	CAA	TAA	TTC	GAT	TTC	GAT	TTA	GCC	GAA	GAA	GAA	GAA	GAA	-833	
CAT	CGA	CAA	ATT	GAG	TGT	TCT	TCT	GCT	GCT	CGG	TGG	CAG	TAA	ATA	ACG	TAG	TCG	TTA	AGT	AAT	TAT	AAA	TAG	TAC	ATC	TCT	CTC	AGC	GGA	-733	
TGA	CAT	ATT	GTG	ATA	AAA	TTT	TAA	CGC	AGG	TAT	CAG	TAA	ATA	ACA	GAG	TTG	TAA	TTT	TTT	TTA	ACT	GCA	AAA	GAC	TTC	GCA	AGC	AACT	GTA	-553	
TGG	CGG	GGT	TTA	TCC	TTG	AAA	AAA	CGG	TTT	CCA	TAG	GAA	GCC	TAA	AAT	ATG	AAT	GGA	TCT	AGT	GTA	CTC	TTT	GAG	GAA	ATA	TAT	TTT	AAA	-463	
TAT	TCT	TGT	GTA	TGA	AAG	ATT	TAC	TAT	GAA	ATT	ACC	CCY	CAT	TCA	GCG	CTT	CGT	ATT	CGA	TGA	TCA	ATA	TGC	ANG	TTG	TTA	ATA	TTT	CGT	-373	
TGT	ACC	GTT	AAA	AAA	AAA	ATA	GTA	CGG	TTC	TAT	TGA	ANG	GCA	ACA	TAC	CGC	CAT	GAG	CTA	GAG	TTA	ACA	GAT	AAA	GGG	AGC	AGA	ACA	OCT	-283	
AAA	AGC	CTC	TTT	TGT	GGT	AGT	ACA	GAT	ATC	ATT	TTA	TTC	TGA	GCG	TTT	TCC	ATT	CTT	CTT	GTA	CTT	ATA	ATA	TAC	AGT	CTC	CGC	ATA	TTC	AGC	-193
ATA	ATA	CTA	TTG	TGA	ANG	CAT	GAG	TAA	TTC	TTT	ACT	ANG	GGC	CAT	CCG	ANG	AGT	GCG	GAA	CTA	TAA	CAT	ATT	AGT	ANG	GAT	GAA	TTC	GAC	-103	
CCA	GTA	CGG	CTT	TTA	ATT	CTG	AGA	AGA	CAG	CTT	GCT	ACG	CAT	CCG	GCG	ANG	CTA	TAT	AGC	TCT	CCT	TAC	ATC	AAA	AGT	CCG	TTG	GTG	CAC	-13	
TTA	CAC	AGC	CGC	ATG	AGC	AAT	GTT	CAC	AGA	TCA	GCA	CAT	GCC	AAT	GCT	TTG	AGT	TTT	GTT	ATC	ACC	AGG	AGA	TOG	ATA	TCA	CAT	TTT	CCT	78	
M S N V H R S A H A N A L S F V I T R R S I S H F P																														26	
AAA	ATT	ATA	TCT	AAA	ATT	ATT	AGG	TTA	CCC	ATA	TAT	GTG	GGT	GCG	GGG	ATG	GCT	GCT	GCA	GGG	AGT	TAT	ATA	GCT	TAT	ANG	ATG	GAA	GAA	168	
K	I	I	S	K	I	I	R	L	P	I	Y	V	G	G	G	M	A	A	A	G	S	Y	I	A	Y	K	M	E	E	56	
GCT	AGC	TCT	TTT	ACT	ANG	GAC	AAA	CTA	GAT	CGA	ATT	ANG	GAT	TTA	GGT	GAA	TOG	ATG	ANG	GAA	ANG	TTT	AAT	AAA	ATG	TTT	TCC	GGT	GAT	258	
A	S	S	F	T	K	D	K	L	D	R	I	K	D	L	G	E	S	M	K	E	K	F	N	K	M	F	S	G	D	86	
AAG	TCA	CAA	GAC	TGT	GGT	CAT	GGA	ANC	GAT	GGA	ACG	GTG	CCA	ACT	GCT	ACT	CTA	ATA	GCC	GCT	ACA	TCA	CTC	GAC	GAT	GAC	GAA	AGT	AAG	348	
K	S	Q	D	C	G	H	G	N	D	G	T	V	P	T	A	T	L	I	A	A	T	S	L	D	D	D	E	S	K	116

Figure 6. Schematic drawing showing the important functional regions of the four homologous proteins. In the mature form of *MGM1*, the import signal is probably proteolytically removed so that the A block would be near the amino terminus of the mature form of the protein as it is in the other three members of this family.

An interesting feature of these four proteins is that they are almost 100% identical in three regions within the A and B blocks that contain the consensus motif (**GXXXXGK—DXXG—TKXD**) of GTP-binding proteins (Dever et al. 1987; Rothman et al. 1990). The A block contains the first component **VXGXQ****SXGKSSVLE**, and the B block contains the other two, **LXLXDLPG** and **RTIGVX****TKXDLXD** (Fig. 7). These sequences have the same spacing relative to each other in all four proteins.

Discussion

The MGM1 protein is related to a family of proteins of diverse function

The *MGM1* gene product could function conceivably in the nucleus, controlling the synthesis of one or more mitochondrial proteins that must be supplied constantly to allow continued mtDNA replication. The *MGM1* open reading frame, however, reveals an amino-terminal structure that is consistent with the protein being targeted to the mitochondrion. Based on map location and sequence, *MGM1* is distinct from three other genes that are required for mtDNA maintenance in yeast: *MIP1* codes for a mtDNA polymerase (Foury 1989), *PPA2* encodes a mitochondrial pyrophosphatase (Lundin et al. 1991), and *ABF2* specifies a DNA-binding protein (Difley and Stillman 1991). *MIP1* is required for maintenance of both ρ^+ and ρ^- mtDNAs, but whether *PPA2* and *ABF2* are required by ρ^- mtDNAs is unknown. *MGM1* is also distinct from the gene for mitochondrial RNA polymerase, *RPO41*, which is required for maintenance of the integrity of the ρ^+ genome but not for the cellular maintenance of ρ^- mtDNA (Fangman et al. 1990). It is unlikely that *MGM1* specifies a polymerase because no similarity is observed between the *MGM1* protein and any of the conserved sequences present in other eukaryotic DNA polymerases (Argos 1988; Wong et al. 1988; Boulet et al. 1989) or RNA polymerases (Mas-

ters et al. 1987). There are also no similarities to sequences found in many DNA-binding domains, such as zinc fingers, homeo domains, leucine zippers, or helix-turn-helix regions (Mitchell and Tjian 1989).

The high degree of relatedness of the *MGM1*, *Mx*, *VPS1* (*SPO15*), and dynamin D100 proteins in an extensive region surrounding the GTP-binding motif is striking (Fig. 7). Except for the tripartite consensus itself, most other GTP-binding proteins share only limited homology (Dever et al. 1987). Apparently, the three components of the consensus sequence merely need to be in some proper orientation relative to the tertiary structure of the protein, and many different primary sequences are capable of providing that structure. The proteins of this dynamin family have diverse functions: mtDNA maintenance (*MGM1*; this report), resistance to influenza virus (*Mx*; for review, see Arnheiter and Meier 1990), meiosis I and vacuole protein sorting (*VPS1/SPO15*; Rothman et al. 1990; Yeh et al. 1991), and endocytosis and microtubule sliding (dynamin D100; for review, see Collins 1991). A possible explanation for the more extensive relatedness of this family of proteins is that they share a function beyond GTP binding and hydrolysis. One proposal, based on a presumed mechanochemical motor activity of dynamin D100, is that the GTP-binding region of the proteins associates with microtubules and uses GTP hydrolysis to generate motive force. In this view, the common function of these proteins is to move materials to different cellular compartments as dictated by their unrelated carboxy-terminal halves (Obar et al. 1990). The evidence, however, that dynamin is a microtubule motor is not complete (for review, see Collins 1991). Furthermore, microtubules have not been detected in the mitochondrion where the *MGM1* protein most likely functions in yeast, although fibrous structures have been demonstrated in this organelle (Yotsuyanagi 1988).

Finding a protein that is likely localized to the mitochondrion and that is a member of the dynamin family

Figure 5. The open reading frame of the *MGM1* gene. The underlined nucleotide sequence is ~0.5 kb from the start of the *STE4* gene. *MGM1* and *STE4* have opposite sense strands (Whiteway et al. 1989). The underlined amino acids represent the location of the proposed mitochondrial targeting amphiphilic helix. The double underlined amino acids indicate the location of the three GTP consensus-binding sites.

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molecules segregate together, or on how many molecules are required to establish respiration competence when cells are returned to the permissive temperature. The observed lag of 4.0–4.25 generations would be sufficient to dilute 16–19 units to 1 unit per cell. These values are close to the number of mtDNA molecules in a *mgm1-1* cell grown under our culture conditions. Thus, it would appear that under these conditions, yeast cells partition their complement of mitochondrial genomes until a single mtDNA molecule is being distributed at cell division. This finding is surprising in light of the segregation behavior observed when zygotes are formed by mating two cells with differently marked mitochondrial genomes. The heteroplasmic state is unstable, and within a few generations only homoplasmic cells, those with a single type of mitochondrial genome, remain. This postzygotic segregation is much too rapid to be accounted for by random partitioning of the original mtDNA molecules (for review, see Birky 1978). The segregation could be explained, however, if there was a smaller number of segregating units, each composed of more than one mtDNA molecule. But our results indicate that, at least for vegetative cells, segregating units need not consist of multiple molecules. They also indicate that a single molecule can be detected and amplified by the copy number control system.

Materials and methods

Yeast strains and genetic manipulations

All of the strains used for this study were congenic to A364A (Hartwell 1967), with the exception of those used for mapping and for deletion of *MGM1* in a ρ^- background: BS127 [*MATa*, *ade1*, *ade2*, *ura3-52*, *leu2-3,112*, *trp1-289*, *CYH^r*, *CAN^r*, *gal (-)*] and BS132 [*MATa*, *ade1*, *ade2*, *ura3-52*, *leu2-3,112*, *tyr1*, *lys2*, *his7*] from R. Sclafani, 4a and HS3324 [*MATa*, *his1*, *trp2*, *leu2*, ρ^-] (Fangman et al. 1989); BJ96-13b [*MATa*, *tyr1*, *met7*, *mgm1-1*], BJ97-3a [*MATa*, *ade1*, *ade2*, *ura3-52*, *his3*, *arg8*, *trp1*, *asp5*], BJ72-10 [*MATa*, *ade1*, *ade2*, *ura3-52*, *leu2-3,112*, *bar1*, *his6*], and BJ89-4d [*MATa*, *ade1*, *ade2*, *ura3-52*, *leu2-3,112*, *bar1*, *his6*, *mgm1-1*] from this work. Except where noted, standard yeast genetics methods were used (Mortimer and Hawthorne 1969).

For the mutant screen, the haploid strain BS127 was mutagenized at 23°C with EMS (Sherman et al. 1979). Cells treated for 30 min, to 50% viability, were plated on YEP–glycerol plates and incubated at 23°C for 5 days. Primary colonies from these glycerol plates were streaked on YEP–glucose plates to obtain individual colonies, and plates were incubated at 36°C for 3–7 days. A secondary white colony from each of the streaks that displayed a high proportion of white colonies and/or sectors at 36°C was restreaked on glucose plates that were then incubated at 23°C for 4–5 days. White colonies from the 23°C plates were stained with 4',6-diamidino-2-phenylindole (DAPI). If cells from the white colony had cytoplasmic staining typical of mtDNA, the primary mutant clone was discarded. If there was no cytoplasmic staining, the original clone was considered a possible mutant with a defect in nuclear gene required for mtDNA maintenance. From the original glycerol plates, each of the potential mutants was used to inoculate a liquid YEP–glycerol culture, which was grown overnight at 23°C. A small sample of each culture was plated at 23°C, and liquid YEP–glucose cultures were grown at 23°C and at 36°C. When the cultures

reached stationary phase (five to eight generations), samples were plated on glucose plates and grown at 23°C. Mutant strains that produced a high frequency (~60–100%) of white colonies when plated from 36°C cultures were selected for further study.

Media and culture conditions

Liquid and solid yeast media are as described previously (Hartwell 1967; Wood 1982). The only exception to the standard media was in the temperature-shift experiments used to determine the rate of petite production: Cells were grown overnight (at least eight generations) in YEP with 10% glucose to minimize the difference in growth rates normally observed between wild-type and respiration-deficient cells. Exponential cultures were diluted in fresh prewarmed YEP/10% glucose and placed at 34–35°C. During the course of the experiment, fresh prewarmed media were added so that the cultures remained in log-phase growth; typically, cells were diluted to 5×10^6 cells/ml and allowed to grow until they reached 2×10^7 cells/ml. At the appropriate time intervals, samples were taken, diluted, and plated on YEPD plates that were incubated at 23°C.

DAPI staining and fluorescence microscopy

Cells were centrifuged out of liquid cultures or picked from colonies, fixed with 100% methanol for 5 min, washed with PBS [0.5 M KPO₄ and 1.0% NaCl (pH 7.4)], resuspended in 1 µg/ml of DAPI, and incubated at 4°C until it was convenient to examine them (5 min to 1 week). DAPI-stained cells were examined with a fluorescence microscope (Nikon Microphot-FX) using a 60× oil immersion objective. The barrier filter was BA420, and the dichromatic mirror was DM400. Pictures were taken with ~10-sec exposures on Kodak Tech-pan film, ASA 160.

DNA isolation and characterization

Total yeast DNA was prepared by the guanidine–HCl protocol (Holm et al. 1986). The DNA was serially diluted twofold in a microtiter dish and incubated for 1 hr at 68°C with 0.4 N NaOH (final concentration) to hydrolyze any RNA present and denature the DNA. An equal volume of 20× SSC (3 M NaCl and 0.3 M sodium citrate) was added to the denatured DNA and filtered by vacuum onto a Nytran filter using slot-blot apparatus (Schleicher and Schuell). DNA was cross-linked to the Nytran with 1200 J of UV light using a UV Stratalinker (Stratagene). DNA for Southern blots of restriction endonuclease-digested genomic DNAs and for recovery of plasmids from yeast clones was prepared by the quick technique of Hoffman and Winston (1987). Isolated DNA fragments were labeled with [α -³²P]dATP (6000 Ci/mmol, New England Nuclear) by random hexanucleotide primer extension (Feinberg and Vogelstein 1983). Unincorporated nucleotides were removed by the Sephadex G-50 procedure (Maniatis et al. 1982). Probes were denatured immediately before hybridization by boiling for 5 min.

Nytran filters were hybridized by the high phosphate method of Church and Gilbert (1984). Exposures were made on Kodak XR-5 film with a Dupont Cronex Lighting Plus intensifying screen at –70°C. Blots were stripped by washing twice at 68°C for 20 min, then with a boiling solution of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1% SDS. Care was taken that filters never dried out so that the probes were not irreversibly bound. Appropriate exposures of the autoradiographs were scanned using a Quick Scan Jr. densitometer (Helena Laboratories). The number of mtDNA molecules per cell was determined by quantitative slot hybridization, using as a standard a total DNA prepa-

ration in which the absolute ratio of mtDNA to nuclear DNA had been determined (Fangman et al. 1989).

Cloning and sequencing manipulations

The yeast strain BJ41-8c (*mgm1-1*) was grown overnight in YEPD at 23°C; plasmids containing random inserts from wild-type yeast chromosomal were introduced by a spheroplast transformation (Hinnen et al. 1978). The library that gave rise to a successful clone was RB112 (*URA3*, 2 μ origin, Amp^r) (Carlson and Botstein 1982). The cells were plated on glycerol plates minus uracil (to select for transformants) at 36°C (to select for *MGM1* function). Subsequent constructs were introduced by the lithium acetate transformation procedure (Ito et al. 1983).

The overlap of the *MGM1* clone with the *RET1/PTP1* and *STE4* clones was recognized initially by restriction site patterns and proven by a combination of sequence analysis and Southern hybridizations using fragments of each clone as a probe. Southern hybridizations showed that the cloned insert containing *MGM1* was present in the yeast chromosome in single copy and with the same restriction map as in the clone. The orientation relative to *HIS3* (centromere side) and *MET7* (telomere side) was determined because the other end of the *RET1/PTP1* clone overlapped with the *HIS3* clone (James et al. 1991).

MGM1 was deleted by the one-step disruption technique of Rothstein (1983). Two of the plasmids used in sequencing, pI12 and pI10, were cut with *HpaI* and *HindIII*. The appropriate bands were isolated from a gel and ligated to form pBJ Δ H. The resulting plasmid was opened up with *HpaI*, and a *HpaI*–*SmaI* *URA3* fragment from YEp352 was ligated in to form pBJ16. The deleted region begins 300 bp 5' of the first methionine and leaves only the carboxy-terminal 55 amino acids of the original protein. When pBJ16 is cut with *MluI* and *AhaII*, it yields a linear fragment containing *URA3* flanked by DNA that normally flanks *MGM1*. This linear fragment was transformed into a *MGM1* strain, and transformants were selected on plates minus uracil.

p Δ I, the complementing plasmid that contained the least amount of non-*MGM1* DNA (see Results), was used to construct a series of plasmids for sequencing the *MGM1* gene. A *PstI* digest of p Δ I cut the *mgm1-1* complementing region into three separate fragments. Each of these fragments was cloned in both possible orientations into pGEM-4 (Promega) to form plasmids I10, I11, I12, I20, I21, and I22. Each of these six plasmids was opened up with an enzyme (*KpnI* or *SstI*) that leaves a 3' overhang that cannot be used as a substrate for exonuclease III degradation. They were then cut with an enzyme (*BamHI* or *XbaI*) that leaves a 5' overhang adjacent to the *MGM1*-coding region. The 5' overhang is a substrate for exonuclease III. Exonuclease III was added, and samples were taken at 30-sec intervals during incubation at 37°C. Any single-stranded regions left from exonuclease III treatment were removed with S1 nuclease, small gaps were filled with Klenow polymerase, and the plasmids were recircularized with T4 DNA ligase and transformed into bacteria (Henikoff 1984). A series of plasmids containing progressively bigger deletions, in ~200-bp increments, was obtained. An oligonucleotide complementary to the SP6 promoter was used as the primer for sequencing. Sequencing reactions with Sequenase and ³⁵S-labeled dATP were performed according to the manufacturer (U.S. Biochemical). Reactions were loaded on a 6% acrylamide gel (Biggin et al. 1983) and run at 1500 V for 2–6 hr. Gels were transferred to Whatman 3MM paper and exposed using Kodak XR-5 film at room temperature. Under these conditions, a typical run would yield 150–250 bases of sequence information. There were a few places where the appropriate deletion was not present in the plasmid collection. To fill in

these gaps, the restriction sites determined by previous runs were used to make plasmids with the desired sequence adjacent to the SP6 promoter. To prove that there were not two closely spaced *PstI* sites that could have resulted in a small fragment being lost during the construction of pI10–I22, oligonucleotides complementary to the appropriate regions were used to prime sequencing reactions across the *PstI* sites in the original plasmid, pKa-1.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession no. X62834 SCMGM DNA.

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