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 [Difflrey 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 32°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
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 p°.) 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 p° 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 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-
34°C were examined; not a single example was observed. We observed a large fraction of mitotic cells with an asymmetric distribution of mtDNA. Treatment of yeast with acriflavine produces cell divisions in which the daughter cell frequently becomes p° (Ephrussi and Hottinguer 1951; Mattick and Nagley 1977). In cultures growing in the presence of acriflavine, >2000 mitotic cells taken course of this work, consistent with a failure of mtDNA segregation.

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.

dirolites 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 p° (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 2μ 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 2μ 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 [pΔ1; 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 Hpal–Hpal 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 MhuI–Ahal fragment [see Fig. 1], now containing the URA3 substitution, was transformed into diploid yeast cells. Twenty-eight meiotic tetrads from
The only phenotypes observed in the deletion strains MGM1 are the expected deletion-substitutions (data not shown). In all tetrads, the Ura* phenotype, which signals the deleted gene has no essential cellular function other than maintaining respiratory competence. These results indicated that the deleted 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, pA54 (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.

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, pA54 (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 [Arne­heit 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.

The cloning vector is a high-copy-number plasmid 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 {p+} mitochondrial genome gives rise to deletion variants at high frequency. Some of these {p−} genomes may be replicated by a mechanism that cannot be used efficiently by the {p−} genome [Fangman et al. 1990]. Therefore, their maintenance might not be dependent on MGM1. We examined the replication of two {p−} genomes, {p−} HS3324 and {p−} 4a. {p−} HS3324 contains one of the rep {or ori} sequences thought to be a major replication origin; {p−} 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 {p−} mtDNAs {data not shown}, indicating that at least some {p−} genomes require MGM1 for their maintenance.
Figure 5. [See facing page for legend.]
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 (Difflley and Stillman 1991). MIP1 is required for maintenance of both $\rho^+$ and $\rho^-$ mtDNAs, but whether PPA2 and ABF2 are required by $\rho^-$ 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 $\rho^+$ genome but not for the cellular maintenance of $\rho^-$ 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 (Masters 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 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.

| Protein    | A Block | B Block | C Terminal Tail |
|------------|---------|---------|-----------------|
| MGM1       | G       | X       | D               | 843 aa |
| Mx         | G       | X       | D               | 631 aa |
| VPS1 (SPO15) | G   | X       | D               | 704 aa |
| Dynamin D100 | G   | X       | D               | 851 aa |

GTP consensus: GXXGXK, DXG, TKXD

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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| A BLOCK |
|--------|
| MGM1 (170-216) |
| D100 (29-75) |
| VPS1 (27-73) |
| MX (34-79) |
| GTP CONSENSUS |

| B BLOCK |
|--------|
| MGM1 (253-415) |
| D100 (110-274) |
| VPS1 (152-316) |
| MX (118-284) |
| GTP CONSENSUS |

Figure 7. Alignment of the MGM1 amino acid sequence with dynamin D100, VPS1 (SP015), and mouse Mx. The A block is 46 amino acids long and is separated from the B block by a nonhomologous region that is 37 amino acids long. The single exception is VPS1, which has a total of 79 amino acids between the two blocks of homology. An asterisk (*) is placed below the sequence when all four forms of Mx and the Drosophila dynamin are not shown. Their homologies with the MGM1 protein are not significantly different from those with the Mx and dynamin proteins that are shown.

leads us to propose a different model in which the common function performed by the related amino-terminal halves of these proteins is transmission of a signal to the carboxy-terminal halves by GTP binding and hydrolysis. In this view, the carboxy-terminal half of each protein would be responsible for a function that is specific to that protein and whose activity is controlled by interactions with the amino-terminal half (Fig. 6). The common amino-terminal region corresponds to a protein of ~32 kD, similar in size to two other GTP-binding families, G proteins (~40 kD), and Ras proteins (~20 kD). The non-related carboxy-terminal halves range in size from 39 kD for Mx to 64 kD for dynamin D100, large enough to have enzymatic activities or structural roles of their own. In fact, the carboxy-terminal part of the MGM1 protein is required for function, as deletion of 80-88 amino acids from the carboxyl terminus eliminates complementation of the mgm1-1 mutation (pA54, see Fig. 1). By allowing many large gaps in the sequence comparison, it has been proposed that the carboxyl terminus of VPS1 and D100 are significantly related (Obar et al. 1990). Therefore, of those proteins in the family known currently, only VPS1 and D100 are likely to have a related function.

Implications for mtDNA metabolism

The analysis of mutant mgm1-1 shows that net synthesis of mtDNA ceases at the restrictive temperature, with chondriolites being partitioned more or less equally during subsequent cell divisions. The simplest explanation for these observations is that the mgm1-1 mutation results in a defect in mtDNA replication. Balanced degradation and synthesis of mtDNA remains a possibility, but seems unlikely. Treatments with agents, such as ethidium bromide (Nagley and Linnane 1972), that cause degradation of mtDNA, result first in the production of stable \( p^- \) deletion variants. After plating onto medium without ethidium bromide, \( p^- \) variants are observed as respiration-deficient colonies containing normal mtDNA molecules, depending on whether individual \( p^- \) sequences are contained in mtDNA but lacking most \( p^- \) sequences. With continued incubation with ethidium bromide, cells completely lacking mtDNA (\( p^- \)) are produced. If the \( p^- \) strain at 34°C may produce intermediates; every clone examined was \( p^- \). Examination of many respiration-deficient mgm1-1 colonies provided no evidence for such \( p^- \) intermediates, then the first white colonies to be produced after incubation at 34°C should be \( p^+ \). Examination of many respiration-deficient mgm1-1 colonies provided no evidence for such \( p^- \) intermediates, every clone examined was \( p^- \).

The properties of the mgm1-1 strain at 34°C may provide insights into the nature of mtDNA segregation and amplification in yeast. The first appearance of respiration-deficient colonies should correspond to the time when there is on average only one functional unit remaining in each cell in the population. A functional unit is defined as the material sufficient to restore respiration competence. This unit could consist of one or more mtDNA molecules, depending on whether individual molecules segregate or a cluster of a few clonally derived
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 r- background: BS127 [MATa, ade1, ade2, ura3-52, leu2-3,112, tyr1, lys2, his3] from R. Sclafani; 4a and HS3324 [MATa, hist, trp2, leu2, his3, met5, can5] and BS132 [MATa, ade1, ade2, ura3-52, leu2-3,112, tyr1, lys2, his3] from R. Sclafani; 4a and HS3324 [MATa, his1, his2, leu2, r-] [Fangman et al. 1989]; BJ96-13b [MATa, tyr1, met5, 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-glycerol 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-glycerol 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 x 10⁶ cells/mL and allowed to grow until they reached 2 x 10⁷ 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 dichromic 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/m mole, 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 Lightening 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-
posing 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 alignment was lost during the construction of p110–122, 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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