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Genetic and chemical rescue of the *Saccharomyces cerevisiae* phenotype induced by mitochondrial DNA polymerase mutations associated with progressive external ophthalmoplegia in humans

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The human POLG gene encodes the catalytic subunit of mitochondrial DNA polymerase γ (pol γ). Mutations in pol γ are associated with a spectrum of disease phenotypes including autosomal dominant and recessive forms of progressive external ophthalmoplegia, spino-cerebellar ataxia and epilepsy, and Alpers–Huttenlocher hepatocerebral poliodystrophy. Multiple deletions, or depletion of mtDNA in affected tissues, are the molecular hallmarks of pol γ mutations. To shed light on the pathogenic mechanisms leading to these phenotypes, we have introduced in *MIP1*, the yeast homologue of POLG, two mutations equivalent to the human Y955C and G268A mutations, which are associated with dominant and recessive PEO, respectively. Both mutations induced the generation of *petite* colonies, carrying either rearranged (ρ^-) or no (ρ^0) mtDNA. Mutations in genes that control the mitochondrial supply of deoxynucleotides (dNTP) affect the mtDNA integrity in both humans and yeast. To test whether the manipulation of the dNTP pool can modify the effects of pol γ mutations in yeast, we have overexpressed a dNTP checkpoint enzyme, ribonucleotide reductase, *RNR1*, or deleted its inhibitor, *SML1*. In both mutant strains, the *petite* mutability was dramatically reduced. The same result was obtained by exposing the mutant strains to dihydrolipoic acid, an anti-oxidant agent. Therefore, an increase of the mitochondrial dNTP pool and/or a decrease of reactive oxygen species can prevent the mtDNA damage induced by pol γ mutations in yeast and, possibly, in humans.

INTRODUCTION

Different mutations in POLG, the gene encording the catalytic subunit of the mitochondrial DNA polymerase (polymerase gamma, pol γ) (1) are associated with a spectrum of human mitochondrial disorders, including autosomal dominant (ad) and autosomal recessive (ar) progressive external ophthalmoplegia (PEO), juvenile spino-cerebellar ataxia and epilepsy (2) and infantile hepatopatic poliodystrophy, or Alpers–Huttenlocher syndrome (AHS) (3). Adult-onset adPEO and arPEO syndromes are characterized by the accumulation of multiple mtDNA deletions in affected tissues (4), whereas the most severe syndrome, AHS, shows marked reduction in the mtDNA copy number in liver (5) and, possibly, brain. Beside mutations in POLG, adPEO can also be caused by heterozygous mutations in ANT1, encoding the ADP/ATP translocator (6), and TWINKLE, encoding a mitochondrial helicase (7). ANT1 and POLG genes,

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but not TWINKLE, are highly conserved from humans to the facultative budding yeast *Saccharomyces cerevisiae*: the yeast *AAC2* gene corresponds to ANT1 (8), whereas *MIP1* corresponds to POLG (9). This makes it possible to introduce and study in *AAC2* and *MIP1* the mutations that are equivalent to those affecting mtDNA stability in humans (10–12).

Human pol γ is composed of a 140 kDa catalytic subunit A and a 55 kDa accessory subunit B, which increases the processivity of mtDNA synthesis. Pol γA comprises a polymerase domain and an exonuclease proofreading domain, separated by a linker region of 482 amino acids. Over 60 PEO-associated mutations have been found in POLG (13,14). Most of the dominant POLG mutations are in the polymerase domain, whereas most of the recessive mutations are in the exonuclease or in the spacer domains. Only one mutation has been found in pol γB (14).

In the present work, we have introduced in *MIP1* two mutations that are equivalent to two pathogenic mutations in pol γA, the Y955C and the G268A. The first mutation is associated with adPEO (1), and the second is associated with arPEO (15). We have demonstrated that the equivalent yeast mutations behave as dominant and recessive traits, respectively, and, by inducing severe damage to, or the loss of mtDNA, determined an increase of either ρ^- or ρ^0 *petite* colonies. We have also shown that the yeast phenotype can be suppressed by increasing the mitochondrial dNTP pool, through the overexpression of *RNR1*, encoding the ribonucleotide reductase (*RNR1*) (16,17), or the deletion of its inhibitor, *SML1* (18,19). Finally, we have studied the effect of a reactive oxygen species (ROS) scavenger on our mutants. Our results indicate that ROS damage contributes to mtDNA instability in yeast.

RESULTS

Effect of *MIP1* alleles equivalent to PEO-associated hPOLG mutants on oxidative growth phenotype

Phenotypic analysis showed that the haploid $mip1^{Y757C}$ mutant strain (Δ*mip1*//$mip1^{Y757C}$) was unable to grow on 2% ethanol, indicating the absence of respiration. As exemplified by the experiment shown in Figure 1, the spectrum profile of mitochondria isolated from this strain consistently lacked the peaks specific to cytochromes b and aa3, which are part of the mtDNA-dependent complexes III and IV of the respiratory chain, whereas the peak specific to cytochrome c, a nucleus-encoded protein, was normal. Contrariwise, the haploid $mip1^{G224A}$ mutant strain (Δ*mip1*//$mip1^{G224A}$) was able to grow on ethanol, displayed a normal cytochrome profile (Fig. 1) and retained a normal respiratory activity (data not shown). The *MIP1*/Δ*mip1* heterozygous diploid strain, carrying either the $mip1^{Y757C}$ or the $mip1^{G224A}$ mutant alleles (*MIP1*/Δ*mip1*//$mip1^{Y757C}$ and *MIP1*/Δ*mip1*//$mip1^{G224A}$) both failed to show a significant growth defect; the cytochrome profile and respiration were normal in both strains.

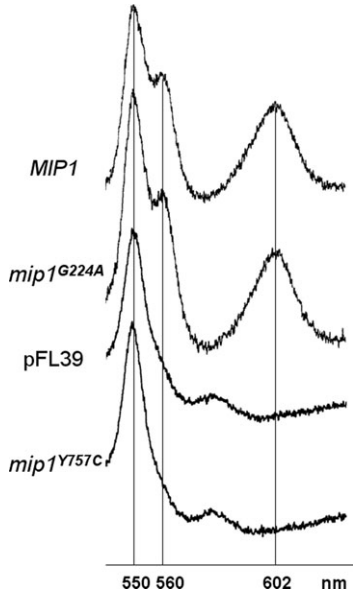

Figure 1. Oxidized versus reduced cytochrome spectra of the Δ*mip1* strain (DWM-5A), transformed with wt *MIP1*, pFL39 plasmid (no insert), $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles cloned in pFL39. The peaks at 550, 560 and 602 nm (vertical bars) correspond to cytochromes c, b and aa3, respectively. The height of each peak relative to the baseline of each spectrum is an index of cytochrome content.

Determination of *petite* frequency and evaluation of the nature of the *petite* mutations

In humans, both G268A and Y955C mutations induce the accumulation of multiple deletions of mtDNA. In *S. cerevisiae*, deletions of mtDNA are known as *petite* mutations. Conditions that increase mtDNA mutability also increase the frequency of *petites* (20). In order to evaluate whether an effect similar to that observed in humans was produced by the equivalent mutations in yeast, we measured the frequency of *petite* mutants in haploid Δ*mip1* strains carrying either the $mip1^{G224A}$ or the $mip1^{Y757C}$ mutant allele. The results reported in Figure 2 indicate that, in the $mip1^{Y757C}$ haploid strain, the *petite* frequency was 100%, as previously described (12). In the $mip1^{G224A}$ haploid strain, the *petite* frequency was ~2.5-fold the frequency determined in Δ*mip1* haploid strains transformed with the *MIP1* wild-type allele.

In the *MIP1*/Δ*mip1* diploid strain carrying the $mip1^{G224A}$ mutant alleles, the *petite* frequency was equivalent to that of the background level observed in the same diploid strain carrying the *MIP1* allele. This result indicates that $mip1^{G224A}$ behaves as a recessive allele in yeast, like the corresponding G268A mutation does in humans. In the same strain carrying the $mip1^{Y757C}$ mutant allele, the *petite* frequency was ~21-fold, indicating that the mutation is dominant in yeast, as it is in humans.

Petite mutants can be either ρ^- (that is, cells in which the mtDNA is partially deleted) or ρ^0 (that is, cells in which the mtDNA is completely lost). When ρ^- mutants are crossed with a mit^- mutant carrying a point mutation, ρ^+ cells are produced by recombination, provided that the mit^- point mutation maps in a region conserved in the ρ^- mtDNA. Contrariwise, ρ^0 mutants, which are completely devoid of

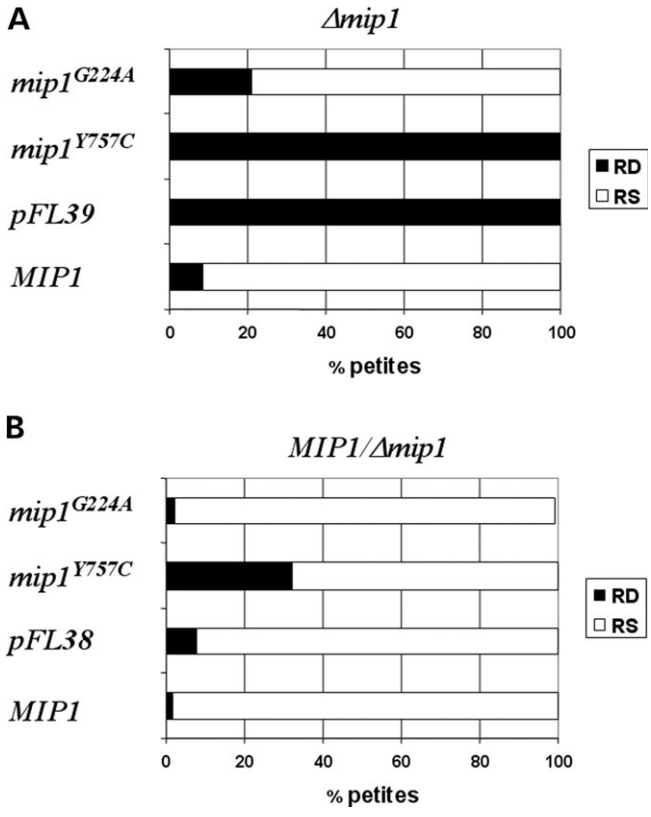

Figure 2. Determination of *petite* frequency in (**A**) haploid Δ*mip1* strain transformed with wt *MIP1*, pFL39 plasmid without insert, $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles cloned in pFL39; (**B**) heterozygous diploid *MIP1*/Δ*mip1* transformed with wt *MIP1*, pFL38 plasmid without insert, $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles cloned in pFL38. More than 4000 colonies/strain were scored. All values are means of three independent experiments. In no case, the variation was higher than 15%. RD, respiratory deficient cells; RS, respiratory sufficient cells.

mtDNA, are unable to produce ρ^+ cells. In order to evaluate the nature of the *petite* colonies produced by our mutant strains, we crossed 200 *petite* clones from the Δ*mip1*//*MIP1* strain and 200 *petite* clones from Δ*mip1*//$mip1^{G224A}$ with four mit^- mutants. Each mit^- mutant harbours a different deleterious point mutation, namely, in the first and sixth exons of the *cob* gene, in the *cox2* gene and in the *cox3* gene (see Methods). The diploid clones resulting from the crosses were tested for their ability to grow on glycerol. Approximately 56% of the Δ*mip1*//*MIP1* and 54% of the Δ*mip1*//$mip1^{G224A}$ *petite* clones were able to complement at least one of the mit^- mutations, indicating that these clones were ρ^- (Table 1). None of the clones derived from the Δ*mip1*//mip^{Y757C} strain was able to complement any of the mit^- mutations, indicating that they were ρ^0. This was consistently confirmed by Southern-blot analysis, as exemplified in Figure 3 (see also Methods).

The complementation test cannot be used to evaluate the nature of the *petite* mutants in wt/mutant diploid strains. To mimic the diploid condition, we have constructed heteroallelic strains by introducing the wt *MIP1* or the mutated $mip1^{Y757C}$ alleles in the haploid wt strain W303-1B. We first established that the heteroallelic *MIP1*//$mip1^{Y757C}$ and the homoallelic

Table 1. Percentage of *petite* ($\rho^0 + \rho^-$) and percentage of ρ^0 mutants produced in different genetic backgrounds

Relevant genotype	wt		*RNR1*		Δ*sml1*	
	% *petites*	% ρ^0	% *petites*	% ρ^0	% *petites*	% ρ^0
Haploid strains						
Δ*mip1*//*MIP1*	9	4	1.9	0.6	1.8	0.9
Δ*mip1*//$mip1^{G224A}$	21	10	3.2	1.1	3.9	1.3
Heteroallelic strains						
MIP1//*MIP1*	1.5	0.4	0.5	0.1	0.5	0.1
MIP1//$mip1^{Y757C}$	32	16	9.3	3.2	4.5	1.3

Petite frequencies were defined as the percentage of colonies showing the *petite* phenotype after 5 days incubation at 28°C. More than 4000 colonies/strains were scored. All values are means of three independent experiments. In no case the variation was higher than 15%. ρ^0 frequencies were calculated as the percentage of clones unable to complement any of mit^- mutants tested.

MIP1//*MIP1* strains produced *petite* colonies with a frequency similar to that observed in diploid strains carrying the corresponding $mip1^{Y757C}$ and *MIP1* alleles (data not shown). The transformants were then crossed with the mit^- testers. Approximately 74% of 200 *MIP1*//*MIP1* homoallelic *petite* clones was able to complement each of the mit^- mutations but only 49% of 500 heteroallelic *MIP1*//$mip1^{Y757C}$ *petite* clones was able to do so, again indicating that the Y757C mutation induces the loss of mtDNA (Table 1).

Determination of mtDNA point mutations

We next analysed whether our *mip1* mutations increased the mtDNA point mutagenesis, as demonstrated by an increase in the frequency of erythromycin-resistant (Ery^R) mutants. Resistance to the drug erythromycin in yeast is acquired through specific point mutations in the mtDNA-encoded rRNA genes and therefore is a convenient, direct measurement of mtDNA point mutagenesis *in vivo* (21). Using this assay, we have measured mtDNA mutation frequencies in the haploid strain Δ*mip1*//$mip1^{G224A}$ and in the diploid strains *MIP1*/Δ*mip1*//$mip1^{G224A}$ and *MIP1*/Δ*mip1*//$mip1^{Y757C}$. Both $mip1^{G224A}$ in haploid, and $mip1^{Y757C}$ in diploid conditions showed a 10-fold and a 20-fold increase, respectively, in mtDNA point mutagenesis, compared with that observed in the wild-type strain. However, the *MIP1*/$mip1^{G224A}$ heterozygous diploid strain failed to show any increase of Ery^R mutant frequency (Table 2), again indicating that this mutation behaves as a recessive trait also for this phenotype.

Effect of increasing dNTP pool on mitochondrial mutability

We next tested whether an increase in the level of the dNTP pool could rescue the mitochondrial mutability observed in the presence of the two pathological *mip1* mutations. The increase of the dNTP pool was obtained either by overexpressing the *RNR1* gene, which encodes the large subunit of the

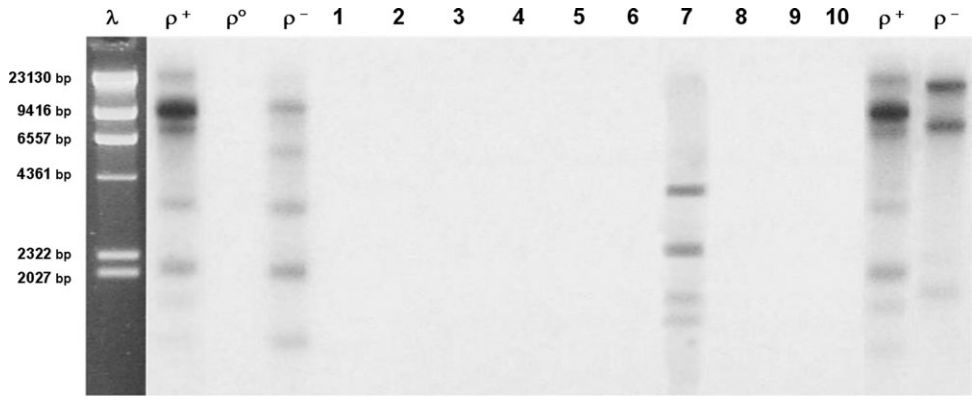

Figure 3. Southern-blot analysis of mtDNA isolated from *petite* clones unable to complement any of the *mit*$^-$ mutants tested (lanes 1–10). mtDNA was prepared and analysed as described in Materials and Methods from cells grown on YNB medium supplemented with 2% glucose. mtDNA (1 μg) was digested with *Eco*RV and hybridized with an mtDNA-specific probe. Lane 7 corresponds to an mtDNA-rearranged ρ$^-$ clone; lanes 1–6 and 8–10 correspond to mtDNA-less ρ^0 clones. The mtDNA hybridization pattern of a respiratory sufficient ρ$^+$ strain is shown as a normal control; examples of abnormal hybridization patterns were obtained from a respiratory-deficient mtDNA-less ρ^0 strain, and from two respiratory-deficient, mtDNA-rearranged ρ$^-$ strains.

Table 2. Frequency of Ery^R mutants

Relevant genotype	wt	Δ*sml1*
Haploid strains		
Δ*mip1*//*MIP1*	1.1×10^{-8}	1.2×10^{-8}
Δ*mip1*//*mip1*^{G224A}	9.8×10^{-8}	11.5×10^{-8}
Diploid strains		
MIP1/Δ*mip1*//*MIP1*	1.0×10^{-8}	0.9×10^{-8}
MIP1/Δ*mip1*//pFL38	3.2×10^{-8}	3.5×10^{-8}
MIP1/Δ*mip1*//*mip1*^{Y757C}	11.4×10^{-8}	5.6×10^{-8}
MIP1/Δ*mip1*//*mip1*^{G224A}	1.1×10^{-8}	1.1×10^{-8}

The frequency of erythromycin resistant mutants was calculated as number of Ery^R colonies/total number of colonies, as described in Materials and Methods.

ribonucleotide reductase (22) or by deleting the *SML1* gene, which encodes an inhibitor of the latter activity (18).

We transformed haploid and diploid strains harbouring the *mip1*^{G224A} or the *mip1*^{Y757C} alleles with a multicopy plasmid carrying the *RNR1* gene (plasmid pWJ841). We also constructed a haploid Δ*sml1*, Δ*mip1* strain and a diploid Δ*sml1*/Δ*sml1 MIP1*/Δ*mip1* strain, which were transformed with either one of three different plasmids: the first plasmid carried the wt *MIP1* allele, the second the *mip1*^{G224A} allele and the third the *mip1*^{Y757C} allele. The results reported in Figure 4 indicate that both conditions caused dramatic decreases of the *petite* frequencies in the mutant strains. The *petite* mutability was reduced approximately to the same extent, suggesting that the protective effect on mtDNA is likely due to the increase of the dNTP pool and not to an unrelated effect of the *RNR1* overexpression or of the *SML1* deletion.

Decreased *petite* mutability was associated with decreased ρ^0 frequency (Table 1). In the presence of *RNR1* overexpression, the proportion of haploid Δ*mip1*//*MIP1* ρ^0 clones was reduced from 46 to 33% and in the presence of the *SML1* deletion was reduced to 28%. Similar results were obtained in the haploid Δ*mip1*//*mip1*^{G224A} strain. The heteroallelic strain, *MIP1*//*mip1*^{Y757C} showed a reduction of ρ^0 clones from 51 to 35% in the presence of overexpressed *RNR1* and to 29% in the presence of Δ*sml1*, whereas no reduction was observed in the *MIP1*//*MIP1* homoallelic strain. The 4–5-fold decrease of *petite* mutability and the 7–10-fold decrease of ρ^0 frequency suggest that the increase of the dNTP pool contributed to maintain mtDNA integrity and stability.

Since the effects obtained by *RNR1* overexpression were similar to those obtained with *SML1* deletion, we evaluated the effect on mtDNA point mutability only in the Δ*sml1* background. The number of Ery^R mutants was unchanged in the presence of the *mip1*^{G224A} mutation, but decreased by ~2-fold in the presence of the *mip1*^{Y757C} mutation (Table 2).

Role of ROS in mtDNA mutability

Increased production of ROS is associated with an increase in the accumulation of mtDNA rearrangements (23,24). To address this issue in our system, we analysed the effect of the ROS scavenger dihydrolipoic acid on the *petite* mutability of the heterozygous *MIP1*/Δ*mip1* diploid strain carrying the *mip1*^{Y757C} allele, as well as of the Δ*mip1* haploid strain carrying the *mip1*^{G224A} allele. Exposure to dihydrolipoic acid decreased significantly the *petite* mutability in both cases by a factor of six to eight. A clear, albeit less prominent, effect was also observed in the *mip1* and *MIP1*/*mip1* strains carrying the wild-type *MIP1* allele (Fig. 5).

DISCUSSION

The human Y955C pol γ mutation is located in the polymerase domain. Its fidelity is 42-fold decreased in an exonuclease-deficient background and 2-fold decreased in an exonuclease-proficient background (25); its polymerase activity is 3000-fold decreased (26). The yeast mutation *mip1*^{Y757C}, corresponding to Y955C, behaved like the *mip1* null mutation when introduced into a Δ*mip1* haploid strain. It induced a dramatic loss of mitochondrial DNA, as shown by the production of 100% petite mutants that were all ρ^0, that is, mtDNA-less cells. In a *MIP1*/*mip1* heterozygous diploid strain, the

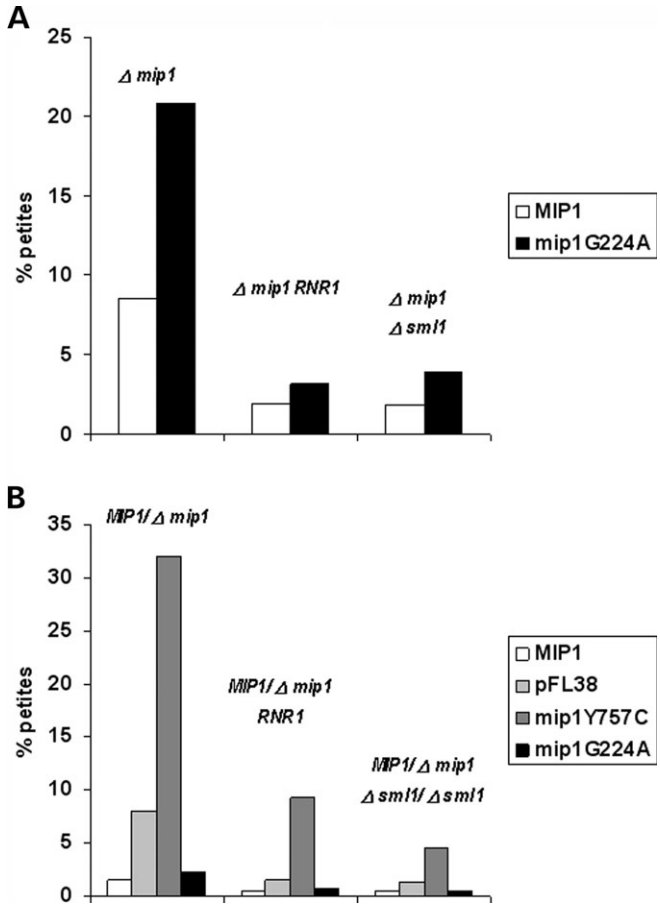

Figure 4. Determination of *petite* frequency in the presence of *RNR1* overexpression and *SML1* deletion. Relevant genotypes of different strains are indicated on top of the bars. Bars indicate transformants with *MIP1* or different *mip1* mutant alleles, as indicated. (**A**) Haploid strains transformed with wt *MIP1* and $mip1^{G224A}$ mutant allele cloned in pFL39; (**B**) diploid transformed with wt *MIP1*, pFL38 plasmid without insert, $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles cloned in pFL38. More than 4000 colonies/strains were scored. All values are means of three independent experiments. In no case the variation was higher than 15%.

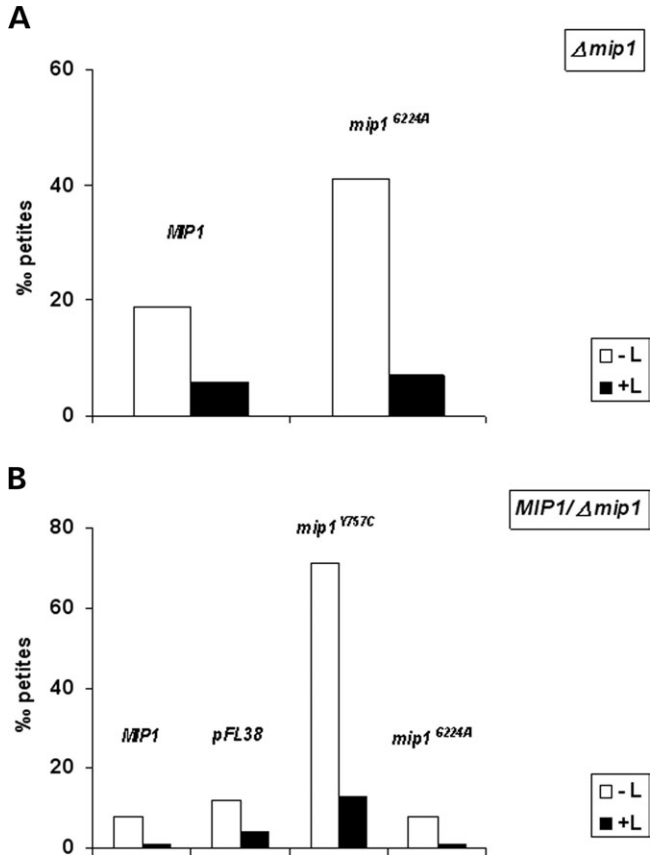

Figure 5. Determination of *petite* frequency in the presence/absence of 30 μM dihydrolipoic acid (**A**) haploid Δ*mip1* strain transformed with wt *MIP1* and $mip1^{G224A}$ mutant allele cloned in pFL39; (**B**) heterozygous diploid *MIP1*/Δ*mip1* transformed with wt *MIP1*, pFL38 plasmid without insert, $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles cloned in pFL38. More than 4000 colonies/strains were scored. All values are means of three independent experiments. In no case the variation was higher than 15%. −L, cells untreated with dihydrolipoic acid; +L, cells treated with dihydrolipoic acid.

$mip1^{Y757C}$ mutation caused a 20-fold increase in *petite* mutability compared with the *MIP1* wt allele and a 4-fold increase compared to the *mip1* null allele. The $mip1^{Y757C}$ mutation caused also an increase of point mutability of mtDNA, leading to a 10-fold increase of Ery^R mutations compared with the *MIP1* wt allele and a 3-fold increase compared with the Δ*mip1* null allele. These results indicate that the $mip1^{Y757C}$ behaves as a dominant allele, since its presence is more deleterious to mtDNA than the absence of the *MIP1* gene itself. This behaviour mimics exactly what is observed in humans.

The human G268A is a missense mutation in the proofreading exonuclease domain of pol γA. In homozygous conditions, the human G268A mutation causes the accumulation of both mtDNA deletions and point mutations (27). We have observed reduced fidelity of the homologous mutation also in yeast, where the equivalent $mip1^{G224A}$ allele was associated with a 2.5-fold increase in the frequency of *petite* clones and a 10-fold increase in the point mutability of mtDNA. Therefore, the phenotype is recessive in yeast as it is in humans.

The recessive phenotype is formally indicative of a complementation exerted by the wild-type allele and is possibly due to the ability of the wild-type MIP1 polymerase to proofread errors introduced by the mutant polymerase.

Mutations in genes that control the mitochondrial supply of deoxynucleotides (dNTP) affect the mtDNA integrity in both humans (28) and yeast (29).

The level of dNTP pools is under an evolutionary conserved surveillance system that maintains genomic stability. One important checkpoint of this control system is the *RNR1*. In yeast, mutations in *RNR1* increase *petite* mutations (30). In both yeast and humans, the transcription of *RNR1* is induced after DNA damage. In addition, the yeast mtDNA-damage-checkpoint proteins also regulate the *RNR1* inhibitor Sml1p. After DNA damage or at S phase, the *MEC1* and *RAD53* gene products control the phosphorylation and the subsequent degradation of the Sml1 protein (31). This control contributes to increase the synthesis of dNTPs, necessary for DNA replication and repair. Overexpression of *RNR1* (32) or deletion of *SML1* (18), both are able to rescue the *petite*-inducing phenotype of a specific point mutation in yeast pol γ (*mip1-1*).

Here we show that either *RNR1* overexpression or *SML1* deletion, two conditions that increase the dNTP pools of the cell, including the mitochondrial pool (19), induced a 4–8-fold reduction in the production of *petite* colonies of our recombinant mutant strains. However, neither *RNR1* overexpression nor *SML1* deletion decreased the frequency of Ery^R clones, which is an index of the propensity of mtDNA to accumulate point mutations. This result is similar to previous data (33) and indicates that induction of large-scale rearrangements, rather than increased point mutagenesis, is responsible for the *petite* mutability induced by the two *mip* mutations considered in our work. The human Y955C mutation displays a 45-fold reduction in affinity for the incoming nucleotide (25). Reduced affinity could determine a stalling on homopolymeric runs due to frequent reiteration of a single nucleotide. This could result in local depletion of that nucleotide and make it difficult for a defective polymerase to incorporate the subsequent nucleotide (34). By preventing pol γ stalling, an increase of the dNTP pool could decrease the generation of mtDNA rearrangements, and thereby reduce the number of *petite* clones. However, the increase of the dNTP pool could also induce the rescue of *petites* by improving the efficiency of mtDNA repair, especially in the presence of the D268A mutation, which is an error-prone mutation, being contained in the proofreading domain of pol γA. The existence of mtDNA repair is supported by the recent demonstration that mitochondria share several repair systems with the nucleus, in both yeast (35) and humans (36).

MtDNA is attached to the mitochondrial inner membrane in close proximity to the respiratory chain. Mitochondrial respiration is a major source of ROS, which makes mtDNA vulnerable to oxidative damage (23). The latter effect can lead to the production of more ROS by impairing the electron transport chain (37), which can in turn determine further mtDNA damage (38,39).

To evaluate whether the mitochondrial mutability consequent to POLG mutations could result from ROS damage on mtDNA, we analysed the effect of a ROS scavenger, dihydrolipoic acid. Exposure to dihydrolipoic acid caused a 6-fold decrease in the *petite* mutability of the heterozygous *MIP1/mip1* diploid carrying the $mip1^{Y757C}$ dominant allele and of the Δ*mip1* haploid strain carrying the $mip1^{G224A}$ recessive allele. However, we could not establish whether the polymerase mutations increase the amount of ROS or whether they make the mtDNA more sensitive to ROS.

We have previously reported that the presence of ROS scavengers reduced significantly the consequences of a mutation equivalent to an arPEO mutation in the human ANT1 gene (11). Likewise, the data presented in this paper ushers in the development of a rational anti-ROS strategy for patients with pol γA mutations.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains are listed in Table 3. YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (Difco). Minimal medium (YNB) contained 7 g/l yeast nitrogen base without amino acids (Difco) supplemented with appropriate amino acids and bases for auxotrophy. Various carbon sources were added at the indicated concentrations. N1 medium contained 1% peptone, 1% yeast extract, 2% ethanol in 50 mM phosphate buffer at pH 6.3, supplemented with 4 g/l erythromycin (SIGMA).

Construction of yeast strains carrying *mip1* mutations

Saccharomyces cerevisiae strain W303-1B (40) was used for the construction of a null Δ*mip1* mutant by one step gene disruption using KanMX expression cassette (41). *MIP1* ORF was completely deleted and substituted by the Kan^r marker, whose correct target of at *MIP1* locus was verified by PCR. Oligonucleotides used for *MIP1* disruption and verification are listed in Table 3. Δ*mip1* mutant strain (strain OF1), ρ^0, was then crossed with the isogenic strain W303-1A (40), giving rise to the diploid DWM *MIP1*/Δ*mip1* ρ^+. This strain was transformed with wt *MIP1* or $mip1^{Y757C}$ or $mip1^{G224A}$ mutant alleles, cloned in the centromeric vector pFL38 or with the empty plasmid. Diploid clones obtained will be named: *MIP1*/Δ*mip1*//*MIP1*, *MIP1*/Δ*mip1*//$mip1^{Y757C}$, *MIP1*/Δ*mip1*//$mip1^{G224A}$, *MIP1*/Δ*mip1*//pFL38, respectively.

Absence of *MIP1* determines the complete and irreversible loss of mtDNA. For this reason, we maintained wt *MIP1* expressed by a plasmid during the construction of the haploid mutant strains and eliminated it by plasmid shuffling once the mutant allele was already into the cell. For the construction of haploid strains, DWM strain transformed with the pFL38*MIP1* plasmid was sporulated. By tetrad analysis the haploid spore DWM-5A, carrying the Δ*mip1* disruption and the pFL38*MIP1*, and therefore ρ^+, was selected. This strain was the host for wt *MIP1* or $mip1^{Y757C}$ or $mip1^{G224A}$ mutant allele, cloned in the pFL39 centromeric vector. By plasmid shuffling in the presence of 5-fluoro orotic acid (5FOA, SIGMA), it was then possible to isolate DWM-5A strain devoid of pFL38*MIP1*. Haploid clones obtained will be named: Δ*mip1*//*MIP1*, Δ*mip1*//$mip1^{Y757C}$, Δ*mip1*//$mip1^{G224A}$ respectively.

Strains carrying Δ*sml1* mutation were derived from strain YG855, kindly obtained from Rodney Rothstein. This strain was crossed with DWM-5A (Δ*mip1*) thus obtaining the diploid YO81 heterozygous at Δ*sml1* and Δ*mip1* loci. By sporulation and tetrad analysis of this strain, we selected the spores YO81-3A (Δ*sml1*) and YO81-4B (Δ*mip1*Δ*sml1*). These clones were then crossed to obtain the diploid DYY *MIP1*/Δ*mip* Δ*sml1*/Δ*sml1*, used as the host for wt *MIP1* or $mip1^{Y757C}$ or $mip1^{G224A}$ mutant alleles cloned in pFL38. For the construction of haploid strains, DYY diploid strain, transformed with the pFL38*MIP1* plasmid, was sporulated. By tetrad analysis, the haploid spore DYY-4C Δ*mip1* Δ*sml1* carrying pFL38*MIP1*, therefore being ρ^+, was selected. This strain was transformed by $mip1^{G224A}$ mutant allele, cloned in the centromeric vector pFL39. By plasmid shuffling in the presence of 5-fluoro orotic acid, it was then possible to isolate the DYY-4C strain (Δ*mip1* Δ*sml1*) carrying mip^{G224A} allele and devoid of pFL38*MIP1*. The same has been done also with *MIP1* wt allele in order to obtain strains transformed with the same plasmid pFL39 and consequently carrying the same selectable marker, *TRP1*.

Construction of mutant alleles

The $mip1^{G224A}$ and $mip1^{Y757C}$ mutant alleles were produced by site-directed mutagenesis, using the QuikChange Kit (STRATAGENE). The template DNA was the wt *MIP1* cloned in

Table 3. List of yeast strains

Haploid strains	Genotype	Origin
W303-1B	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*	Thomas and Rothstein (41)
W303-1A	*Mata ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*	Thomas and Rothstein (41)
OF1	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR*	*mip1:KanR* derived from W303-1B
DWM-5A	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR*	Sporulation of DWM diploid strain
YG855	Mata *ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 sml1::HIS3 adh4::URA3*	Rodney Rothstein
YO81-4B	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR sml1::HIS3*	Sporulation of YO81 diploid strain
YO81-3A	Mata *ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 sml1::HIS3*	Sporulation of YO81 diploid strain
YO81-5D	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 sml1::HIS3*	Sporulation of YO81 diploid strain
DYY-4C	Mata *ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR sml1::HIS3*	Sporulation of DYY diploid strain
M9-94-4B	*Mata ade1cox2*	Françoise Foury
M7-40-5B	*Mata ade1cob*	Françoise Foury
M17-162-4D	*Mata ade1cob*	Françoise Foury
M9-3-5B	*Mata ade1cox3*	Françoise Foury
M9-94/A1	*Matα met cox2*	Françoise Foury
M9-3/A3	*Matα met cox3*	Françoise Foury
M7-40/A1	*Matα met cob*	Françoise Foury
M17-162	*Matα met cob*	Françoise Foury
Diploid strains	Genotype	Cross
DWW	*Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100/ Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100*	W303-1B × W303-1A
DWM	*Mata ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100/ Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR*	W303-1A × OF1
Y081	Mata *ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 sml1::HIS3 adh4::URA3/ Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR*	YG855 × OF1
DYY	Mata *ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 sml1::HIS3/ Matα ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100 mip1::KanR sml1::HIS3*	YO81-3A × YO81-4B

pUC19 vector. To obtain this construction, we PCR-amplified a DNA fragment of 5110 bp, containing the ORF and the 5' and 3' flanking regions (851 and 441 bp, respectively), using genomic DNA of strain W303-1B as template and the forward primer MIP1C (containing a *Sac*I site at the 5' end) and the reverse primer MIP1E (containing a *Sal*I site at the 5' end).

The list of base changes, and corresponding modified primers used to generate mutated vectors, is reported in Table 4. In order to maximize the expression of these variants, the preferred yeast codons (42) were used in the oligonucleotide sequences used for mutagenesis: GCT for Alanine and TGT for Cysteine. Mutagenized inserts for each different construct were sequence-verified on both strands. The sequences of the oligonucleotide primers used for cloning and sequencing *MIP1* are also listed in Table 3.

Plasmids

MIP1 gene and mutant alleles were cloned in both the centromeric vectors pFL38 and pFL39 (43) at *Sac*I and *Sal*I sites. These vectors carry *URA3* and *TRP1* markers, respectively. *RNR1* gene, cloned in the multicopy vector pRS425 (plasmid pWJ841), was kindly obtained from Rodney Rothstein.

Mitochondrial DNA mutation frequency

Analysis of petite frequencies. Diploid and haploid strains transformed with wt or mutated *mip1* alleles were pregrown overnight in YNB medium supplemented with 2% ethanol and then inoculated in the same medium supplemented with 2% glucose. After 15 generations of growth at 36°C, cells were plated on YNB agar plates supplemented with 2% ethanol and 0.25% glucose at a dilution that gave approximately 200 cells/plate. *Petite* frequencies were defined as the percentage of colonies showing the *petite* phenotype after a 5 day incubation at 28°C.

To test the effect of dihydrolipoic acid, cells were grown in YNB medium supplemented with 2% glucose in the presence or in the absence of 30 μM dihydrolipoic acid (SIGMA) for 15 generations at 28°C. These experiments were performed at 28°C because lipoic acid at 37°C determined high level of cells lethality.

Evaluation of the nature of *petite* clones

In order to evaluate the nature of the *petite* mutants produced by pathologic mutations, *petite* clones were crossed with mit^- strains harbouring point mutations in genes encoding respiratory proteins. The four mit^- mutations used for this analysis map in the first and in the sixth exons of *cob* gene, in the *cox2* gene and in the *cox3* gene, respectively. This choice was based on the observation that in the *petite* ρ^- mutants, the fragments that are most frequently retained encompass cytochrome *b*, *cox2* and *cox3* genes (44,45). The *petite* clones unable to complement any of the mit^- mutants could be either ρ^0 (no mtDNA) or ρ^- carrying rearranged mtDNA

Table 4. List of oligonucleotides used

Amino acid change	Oligonucleotides used for site directed mutagenesis[a]
Y783C	Fw:GGTAGAATTTGTGGCGCTGGTGCTAAATTTGCGAGTCAGTTAC
	Rv:GTAACTGACTCGCAAATTTAGCACCAGCGCCACAAATTCTACC
G250A	Fw:GAATAAAGAGCAAGTAATAATTGCTCACAATGTTGCGTATGATAG
	Rv: TATCATACGCAACATTGTGAGCAATTATTACTTGCTCTTTATTC
	Oligonucleotides used for cloning and sequencing *MIP1*[a]
MIP1C	GGGGGAGCTCCAATCTGATGGCGGGCAATGC
MIP1E	GGCGCGTCGACCTGGAATCATGTGGAGCAGCAG
MIPF	CGT GGC CTG TTC TGT GCC TC
MIPG	GGG CGT TTC AAT TCT GAA CC
MIPH	CGG CCA CAA GCC AAG TGT TTG
MIPI	CGA AGG TAC AGA TTT GCA C
MIPL	CAG TTA GAG ATG GGT TAA ACC
MIPM	GGG AAC ACT GCA GAG TAC AGC C
MIPN	CGA CCC TTG GTT AAA TGT GTC
	Oligonucleotides used for the disruption of *MIP1*[a]
S_1MIP	GCC GCC ACT ACA AGG CG GTC GCT AGA TGA GCA TTT CCA GAA GCG TAC GCT GCA GGT CGA C
S_2MIP	CTC CTG TTC TCC AGC AAT TGC TTT TCT GGG ATT GCT GAA TCG ATC GAT GAA TTC GAG CTC G
K_2	GAA AGA AGA ACC TCA GTG GC
K_3	CTG CCT CGG TGA GTT TTC TCC

molecules which lack the three mutant genes. To distinguish between these two possibilities, we evaluated the presence/absence of mtDNA in a set of these clones by Southern analysis. Mitochondrial DNA was extracted by rapid mitochondrial preparation (46) from clones unable to complement any of the *mit*$^-$, grown in YNB supplemented with 2% glucose. Aliquots of 1 μg of DNA were digested with *Eco*RV (Amersham). Southern-blot analysis was carried out as previously described (47). mtDNAs extracted from three ρ^+, three ρ^- and from three ρ^0 independent strains were also hybridized as a control. Hybridization was performed by standard methods, with a 5′-[γ-^{32}P]ATP end-labelled yeast mtDNA-specific sequence repeat (5′-CTCCTTTCGGGGTTCCGGCTCCCG TGGCCGGGCCCCGG-3′) as a probe. Among the 25 putative ρ^o clones that were analysed with this method, no mtDNA was observed in all but two clones, in which the mtDNA was likely not to encompass any of the *mit*$^-$ mutations (Fig. 3). These results indicate that the absence of *mit*$^-$ complementation is a good indicator of a ρ^0 condition.

Erythromycin resistance assays

Fifteen independent colonies from each strain were inoculated in separate 10 ml cultures of YNB + glucose and allowed to reach the stationary phase. After 24 h, a small sample was removed to determine the total number of respiration-competent cells by plating onto YP supplemented with 2% ethanol. The remainder of each culture was plated onto solid N1 medium containing 4 mg/ml erythromycin (SIGMA) and grown at 28°C for 8 days until drug-resistant colonies formed. The experiment was carried out in duplicate. The mutation frequency was calculated as number of Ery^R colonies/total number of colonies.

Miscellaneous

Transformation of yeast strain was obtained by the lithium chloride method (48). Restriction enzyme digestions, *E. coli* transformation and plasmid extractions were performed using standard methods (47). Cytochrome spectra and respiration were determined as described previously (10).

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