

Yeast mitochondrial DNA mutators with deficient proofreading exonucleolytic activity

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The *MIP1* gene which encodes yeast mitochondrial DNA polymerase possesses in its N-terminal region the three motifs (*Exo1*, *Exo2* and *Exo3*) which characterize the 3'–5' exonucleolytic domain of many DNA polymerases. By site directed mutagenesis we have substituted alanine or glycine residues for conserved aspartate residues in each consensus sequence. Yeast mutants were therefore generated that are capable of replicating mitochondrial DNA (mtDNA) and exhibit a mutator phenotype, as estimated by the several hundred-fold increase in the frequency of spontaneous mitochondrial erythromycin resistant mutants. By overexpressing the mtDNA polymerase from the *GAL1* promoter as a major 140 kDa polypeptide, we showed that the wild-type enzyme possesses a mismatch-specific 3'–5' exonuclease activity. This activity was decreased by ~500-fold in the mutant D347A; in contrast, the extent of DNA synthesis was only slightly decreased. The wild-type mtDNA polymerase efficiently catalyses elongation of singly-primed M13 DNA to the full-length product. However, the mutant preferentially accumulates low molecular weight products. These data were extended to the two other mutators D171G and D230A. Glycine substitution for the Cys344 residue which is present in the *Exo3* site of several polymerases generates a mutant with a slightly higher mtDNA mutation rate and a slightly lower 3'–5' exonucleolytic activity. We conclude that proofreading is an important determinant of accuracy in the replication of yeast mtDNA.

Key words: mutators/mtDNA polymerase/proofreading/processivity

Introduction

The polypeptides encoded by mitochondrial DNA (mtDNA) are small in number but essential to respiration and oxidative phosphorylation. Therefore the integrity of mitochondrial genetic information must be maintained. There are two main mechanisms by which DNA polymerases ensure accurate DNA replication: high selectivity of nucleotides during the polymerization reaction and proofreading. Animal mtDNA polymerases have a physically associated 3'–5' exonuclease proofreading activity (Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989; Kunkel and Mosbaugh, 1989). It is unknown, however, whether this activity belongs to the polymerase subunit and what role it plays *in vivo*.

We have previously cloned and sequenced the nuclear *MIP1* gene encoding the catalytic subunit of yeast mitochondrial DNA polymerase (Foury, 1989). Although it is highly divergent from other known eukaryotic and prokaryotic DNA polymerases, the yeast mtDNA polymerase seems to share a common ancestor with prokaryotic DNA polymerases (Ito and Braithwaite, 1989; Blanco *et al.*, 1991; Ito and Braithwaite, 1991) and is also related to the DNA polymerases of the *Kluyveromyces lactis* killer factors (Tommasino *et al.*, 1988; Foury, unpublished results). Moreover, we and others (Foury and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco *et al.*, 1991) have found that the three consensus sequences which characterize the 3'–5' exonucleolytic domain of many prokaryotic and eukaryotic DNA polymerases (Bernad *et al.*, 1989) are present in the N-terminal part of the *MIP1* gene. In this work, we have addressed the following questions: does yeast mtDNA polymerase possess a 3'–5' exonucleolytic domain and, if so, does 3'–5' exonuclease activity play an important role in the correction of errors produced during mtDNA replication *in vivo*?

Results

Overproduction of the mtDNA polymerase from the *GAL1* promoter

We have previously reported that the gap filling activity of the mtDNA polymerase was 30- to 40-fold higher in yeast transformants carrying the *MIP1* gene on a multicopy plasmid than in a wild-type strain harbouring a single copy of the *MIP1* gene (Foury, 1989). Therefore, in order to increase further the mtDNA polymerase activity in soluble mitochondrial extracts, we decided to overproduce the *MIP1* product from the strong inducible *GAL1* promoter on a multicopy plasmid. The *MIP1* gene was fused to the *GAL1* promoter, 45 bp upstream of the *MIP1* translation initiator codon, as described in Materials and methods (Figure 1A). Because yeast mitochondria possess a potent endo-exonuclease activity (encoded by the *NUC1* gene) (Zassenhaus *et al.*, 1988) which might interfere with the mtDNA polymerase assays, transformation experiments were achieved in the strain A25 that carries a null *nucl* allele. After a 16 h induction in galactose minimal medium, soluble mitochondrial extracts were prepared in the presence of a cocktail of protease inhibitors. Electrophoresis in SDS–polyacrylamide gels revealed the presence of major polypeptide at ~140 kDa (the expected size), while no polypeptide was detected with the insertless pLGALZ3 vector used as a negative control (Figure 1B, panel a). Thus, the *MIP1* gene is translated as a single 140 kDa polypeptide.

3'–5' exonuclease activity on a mismatched substrate

The 3'–5' exonuclease activity on a mismatched 3'-hydroxyl terminus was measured by using two different primer

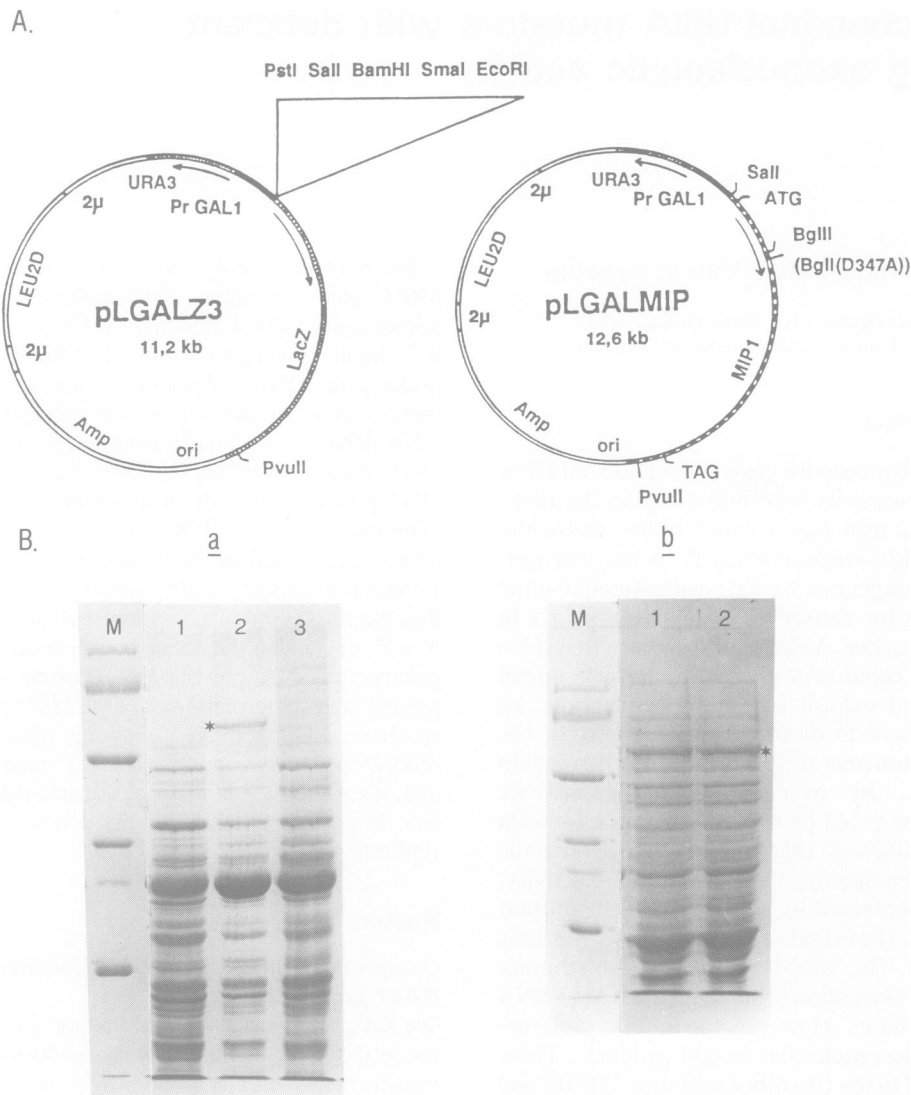


Fig. 1. Overproduction of wild-type and D347A mutant mtDNA polymerases from *GAL1* promoter. **A.** Yeast expression vectors with *GAL1* promoter. The pLGALZ3 (no insert) vector was constructed by Lahaye *et al.* (1991). The construction of pLGALMIP1 and pLGALD347A vectors is described in Materials and methods. Only relevant restriction sites are indicated. The new *Bgl*I site created in the mutant D347A is indicated in brackets. **B.** Overexpression of mtDNA polymerase in soluble mitochondrial extracts. Proteins were separated by electrophoresis in 7% SDS-polyacrylamide gels and stained with Coomassie blue. a: lane 1, strain A25 grown in galactose medium with vector pLGALZ3 (100 μg protein); lane 2, strain A25 grown in galactose medium with pLGALMIP1 vector (40 μg protein); lane 3, non-induced strain A25 grown in raffinose medium with pLGALMIP1 vector (100 μg protein); the intense band at 67 kDa corresponds to BSA that was added during extracts preparation; M, molecular weight markers, 212, 170, 116, 76, 67 and 53 kDa, respectively. b: lane 1, strain A25 with pLGALMIP1 vector (50 μg protein); lane 2, strain A25 with pLGALD347A vector (50 μg protein); M, molecular weight markers. The 140 kDa mtDNA polymerase peptide is indicated with a star.

template substrates in parallel. The first consisted of a perfectly complementary 17mer oligonucleotide annealed with an M13mp19 ssDNA template; the second substrate was identical except that the primer had a single mismatch (dAMP.dAMP) at its 3'-hydroxyl terminus. In a strain harbouring a single copy of the *MIP1* gene, the 3'-5' exonuclease activity was under the limits of detection (data not shown). In extracts of the strain A25 which was overexpressing the mtDNA polymerase from the *GAL1* promoter, excision of both correctly paired and mismatched nucleotides at the 3'-hydroxyl terminus occurred (Figure 2A). After an incubation for 1 min in the presence of 0.01 units of enzyme 5% of the correctly paired primer and 15% of the mismatched one were converted to 16mer or lower molecular weight products (Figure 2B). Thus, the mismatched

nucleotide was excised more rapidly than the correctly paired nucleotide, as expected for an editing function of the 3'-5' exonuclease. These results show that yeast mitochondrial DNA polymerase has an associated 3'-5' exonuclease activity that preferentially removes mismatched nucleotides. Products of 13-14 nucleotides in length accumulated at four consecutive cytosine/guanosine bases. This indicates that although frayed DNA is not necessarily required, the exonucleolytic activity might be impeded at GC-rich regions which have stronger hydrogen bondings.

Isolation of *mip1* mutants with a mutator phenotype

It has been shown that the sequences of prokaryotic and eukaryotic DNA polymerases exhibiting 3'-5' exonuclease activity share three motifs with the 3'-5' exonucleolytic

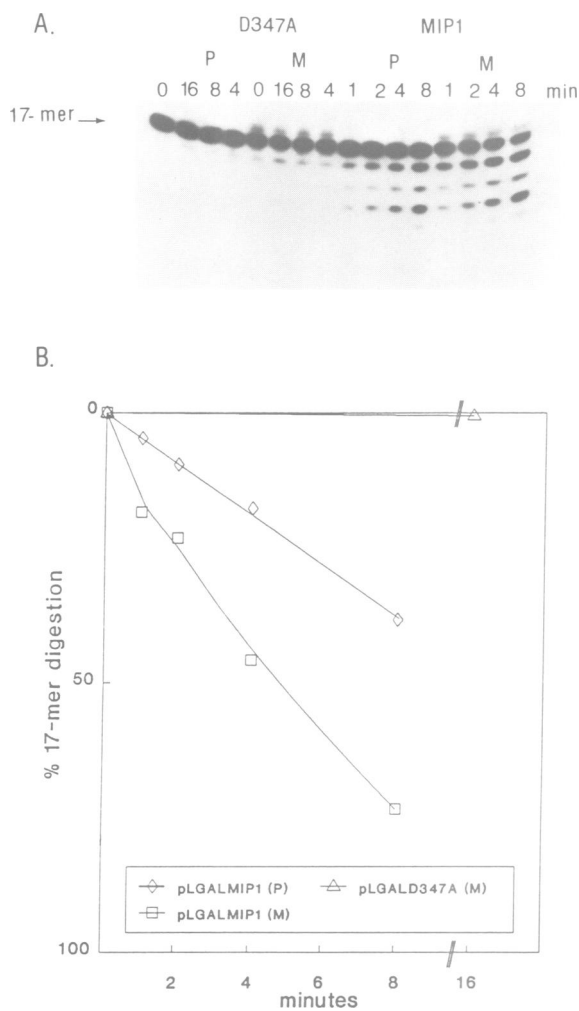


Fig. 2. Mismatch-specific 3'-5' exonuclease activity of wild-type and D347A mutant polymerases overexpressed from *GAL1* promoter. Extracts were prepared from strain A25 with pLGALMIP1 or pLGALD347A plasmids as in Figure 1. The reactions were performed as described in Materials and methods with 4 μ M M13mp19 ssDNA substrate annealed with paired (P, 280 c.p.m./pmol) or mismatched (M, 210 c.p.m./pmol) oligonucleotides. Aliquots of 5 μ l (~3000 c.p.m.) were subjected to electrophoresis. Polymerase activities in the 50 μ l assays were as follows: wild-type (0.01 units), D347A (0.02 units). A. Autoradiography of the gel. It must be noted that the controls at time zero are slightly contaminated with 16mer and 18mer products. B. Quantification of the disappearance of the 17mer substrate. The data are expressed as the percentage of digested 17mer oligonucleotide per 0.01 units of polymerase. After 16 min, only ~0.5% of the 17mer had been digested in the mutant. Quantification of the data was carried out by elution of the silver grains from the film as reported in Materials and methods.

domain of *Escherichia coli* DNA polymerase I (*Poll*) (Bernad *et al.*, 1989). These motifs, named *Exo1*, *Exo2* and *Exo3*, are located in the N-terminal region of the Klenow fragment and mainly characterized by conserved tyrosine/phenylalanine residues or glutamate/aspartate residues. The acidic residues, whose carboxylate groups are involved in dNMP and divalent metal ion binding, play an essential role in the catalytic activity of the 3'-5' exonuclease (Ollis *et al.*, 1985; Joyce and Steitz, 1987; Derbyshire *et al.*, 1988; Freemont *et al.*, 1988; Derbyshire *et al.*, 1991).

These three motifs are clearly present in the *MIP1* gene within a region spanning amino acid residues 170-347

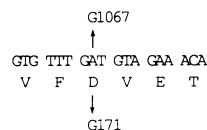
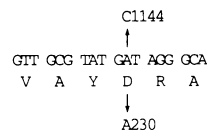
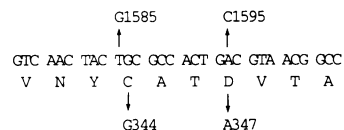
Exo 1 : mutant D171G**Exo 2 : mutant D230A****Exo 3 : mutants C344G and D347A**

Fig. 3. Site directed mutagenesis of conserved residues in the 3'-5' exonucleolytic consensus sequences. The nucleotide sequence is numbered according to Foury (1989). The previously published sequence had to be corrected: nucleotides 1576 and 1577 are G and T, respectively. Nucleotide substitution is indicated above the sequence and amino acid substitution is indicated below the sequence. The names and alleles of the mutants that were generated are detailed in Table III in Materials and methods.

Table I. Frequency of mitochondrial DNA mutations in wild-type strain and mutants

Strain	Number of E ^R colonies per 10 ⁸ cells	%rho ⁻
MIP1	0.3 ± 0.1 (4)	0.5
D171G	63 ± 18 (3)	4.1
D230A	77 ± 12 (3)	5.6
D347A	39 ± 13 (3)	7.9
C344G	3.5 ± 0.7 (2)	0.6

The experiments, carried out with the *rho*⁺ strain W303-1B/LΔ*mip1*-01 harbouring MIP1/PFL39 (wild-type) or *mip1*/PFL39 (mutant) plasmids, were performed as described in Materials and methods. Mean and standard deviation of E^R mutation frequency are given. Numbers in parentheses indicate the number of independent experiments. The percentage of *rho*⁻ mutants was estimated from scoring between 1000 and 2000 colonies.

(Foury and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco *et al.*, 1991). In order to determine whether they are really involved in the 3'-5' exonuclease activity, site directed mutagenesis of conserved residues was carried out (Figure 3). Alanine substitution was generated at Asp230 and Asp347 residues (*Exo2* and *Exo3*, respectively) and a glycine residue was substituted for Asp171 and Cys344 residues (*Exo1* and *Exo3*, respectively). The cysteine residue is conserved among viral DNA polymerases (see Bernad *et al.*, 1989) and is present in the DNA polymerase of *K.lactis* killer factors. It is absent, however, from prokaryotic DNA polymerases. After mutagenesis, each mutant *mip1* allele was introduced into a centromeric vector and the resulting plasmids were used to transform a null *mip1* strain. Since a null *mip1* strain has no mtDNA (*rho*⁰), transformants remained devoid of mtDNA whether their mtDNA polymerase was functional or not. It was therefore necessary to introduce mtDNA into the cells by cytoduction using a

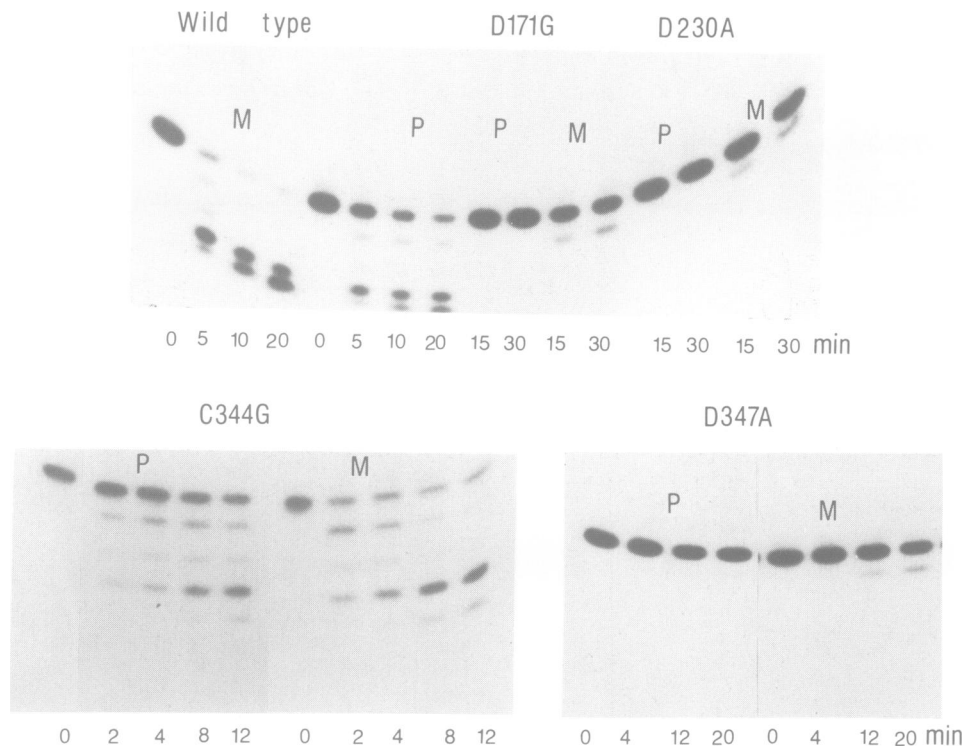


Fig. 4. Mismatch-specific 3'–5' exonuclease activity in wild-type strain and mutants. The mtDNA polymerase was overproduced in the *rho*⁺ strain A25Δ*mip1-02* harbouring the *MIP1* (or *mip1*) gene on the multicopy T7-3 plasmid (Table III). The reaction mixture contained 1.5 μM M13mp18 DNA substrate (200–400 c.p.m./pmol). Polymerase activities in the 60 μl assays were as follows: wild-type (0.06 units); D171G (0.16 units); D230A (0.11 units); D347A (0.17 units); C344G (0.03 units).

kar1 mutant (Conde and Fink, 1976). We found that all *mip1* mutants could grow on glycerol medium and are thus capable of replicating mtDNA. Moreover, the production of cytoplasmic petites (*rho*[−]) was only slightly higher in the mutants than in the wild-type strain (Table I). However, we found that the growth on glycerol medium at 36°C was significantly reduced as compared with the wild-type strain (not shown).

Substitution of alanine or glycine for the aspartate residues D171, D230 and D347 elicited a several hundred-fold increase in the frequency of spontaneous mutations leading to erythromycin resistance (Table I). Erythromycin resistant (*E*^R) mutants are invariably of mitochondrial inheritance and corresponding mutations have been mapped at two distinct alleles of the mitochondrial 21S rRNA gene. (Netter *et al.*, 1974; Sor and Fukuhara, 1983). The *mip1* mutants have therefore a mutator activity. In contrast, replacement of the Cys344 residue by a glycine residue only increased the frequency of *E*^R mutants by 10-fold. This residue must thus contribute to ensuring faithful replication without playing a critical role. These results show that the mutated residues play an important role in mtDNA replication fidelity *in vivo* and strongly suggest that they are located in the 3'–5' exonucleolytic domain of the yeast mtDNA polymerase gene.

In vitro 3'–5' exonuclease activity of the mutants

The 3'–5' exonuclease activity was measured in the mutator strain A25[pLGALD347A] (*mip1-5*) which overproduces the mtDNA polymerase from the *GAL1* promoter. It was shown by SDS–polyacrylamide gel electrophoresis that mutant and wild-type mitochondrial extracts contained the same amount

Table II. Mitochondrial DNA polymerase activity in wild-type and mutant strains

	Gap filling (units/mg protein)	Elongation (units/mg protein)
Experiment 1		
pLGALZ3	1.5	2
pLGALMIP1	620	1300
pLGALD347A	488	700
Experiment 2		
MIP1/PFL39	5	3
D347A/PFL39	4	2

Experiment 1 was carried out with the *rho*⁺ strain A25 grown in galactose medium and harbouring either pLGALZ3, pLGALMIP1 or pLGALD347A plasmids. Experiment 2 was carried out with the strain A25Δ*mip1-01* grown in complete ethanol (YE) medium and harbouring centromeric MIP1/PFL39 or D347A/PFL39 plasmids (*rho*⁺). Gap filling activity on activated DNA and extent of elongation on singly-primed M13 DNA were measured as described in Materials and methods.

of mtDNA polymerase per mg of protein (Figure 1B, panel b). Thus, the results can be readily compared.

The primer–template substrates described above were used to measure the 3'–5' exonuclease activity on mismatched 3'-hydroxyl terminus. Even after long incubation times, hardly any substrate digestion was detected (Figure 2) and it was estimated that in the mutant, the 3'–5' exonuclease activity was ~500-fold lower than in the wild-type strain.

In the three other mutator strains, the 3'–5' exonuclease

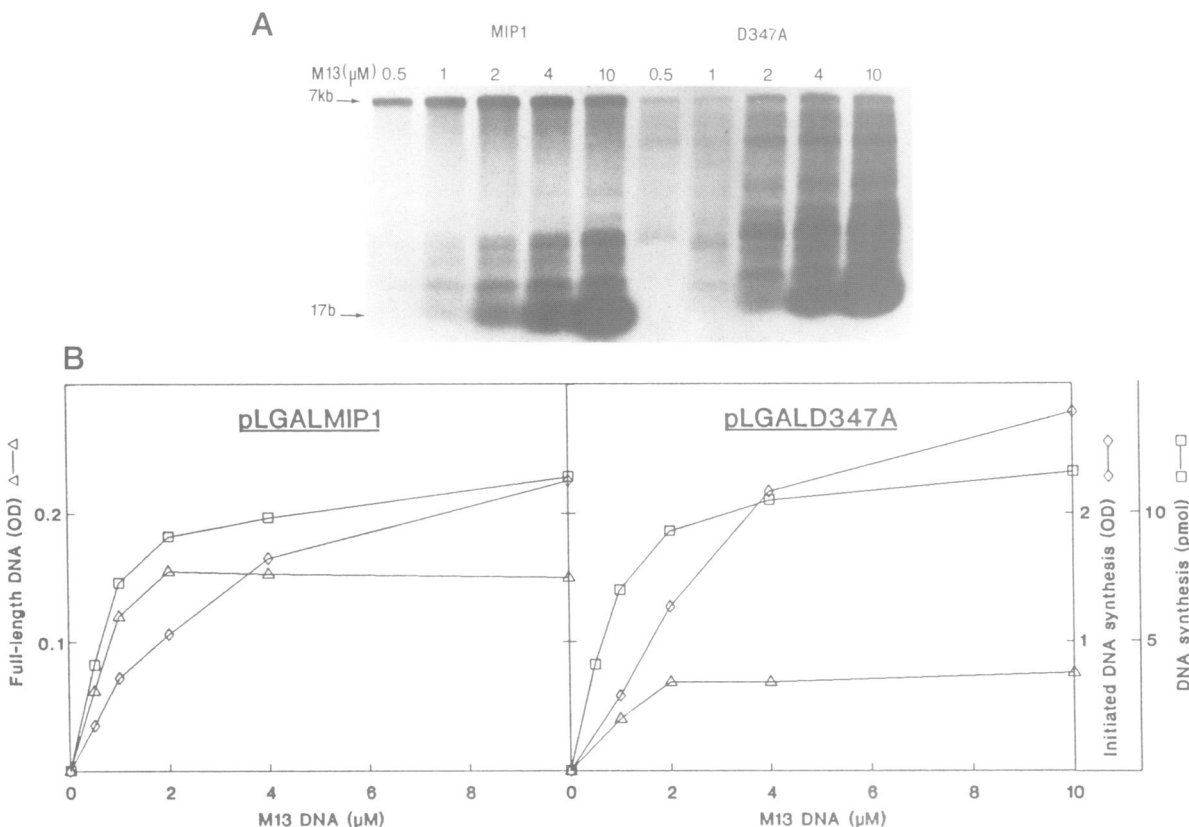


Fig. 5. Singly-primed M13 DNA synthesis by wild-type and D347A mutant mtDNA polymerases expressed from *GAL1* promoter. M13mp19 ssDNA synthesis was initiated from the 5' end ^{32}P -labelled primer AB353. After an incubation of 4 min in a 90 μl reaction mixture containing 0.02 units (34 ng protein) wild-type or 0.03 units (58 ng protein) of D347A mtDNA polymerase, DNA products were analysed by alkaline agarose gel electrophoresis as described in Materials and methods. Under these conditions, all of the DNA molecules are equally radioactive, independently of the product size. **A.** Autoradiography of the gel. **B.** Quantification of the DNA products. The extent of DNA synthesis (pmol of copied template) was measured by the incorporation of ^3H dTTP into acid insoluble material as described in Materials and methods. Estimation (O.D. $_{500\text{ nm}}$) of the fraction of fully elongated molecules (7 kb) or fully elongated plus partially elongated (initiated DNA synthesis) was performed by elution of silver grains from the film as described in Materials and methods. The K_m of the mtDNA polymerase for singly-primed M13 DNA irrespective of the size of the synthesized products, was $\sim 1\ \mu\text{M}$ for both wild-type and mutant enzymes.

activity was estimated using mitochondrial extracts from strains harbouring multicopy plasmid-borne *mip1* genes under the control of their own promoter. It was thus verified that in the mutant A25[D347A/T7-3] (*mip1-5*) as well as in the mutants D171G (*mip1-2*) and D230A (*mip1-3*), even after long incubation times (30 min) and in the presence of an excess of mtDNA polymerase units, the extent of substrate digestion was at least 100-fold lower than in the wild-type strain when standardized with the same amount of polymerase units (Figure 4). These mutator strains are thus practically devoid of 3'–5' exonuclease activity. In the slight mutator C344G (*mip1-4*), the mismatched-specific 3'–5' exonuclease activity was ~ 30 to 40% of that of the wild-type strain (Figure 4).

DNA polymerase gap filling activity

The activity of the mtDNA polymerase on DNase I-activated DNA was measured in soluble mitochondrial extracts from wild-type and D347A mutant strains (Table II). When the mtDNA polymerase was expressed from a PFL39 centromeric vector-borne *MIP1* (or *mip1*) allele and thus was not overproduced, the specific activity of the polymerase in the mutant D347A was still 80% of that of the wild-type enzyme. When the mtDNA polymerase was expressed from the *GAL1* promoter, the specific activity of wild-type enzyme was

increased upto 200 to 500-fold. This activity was only slightly decreased in the mutant (Table II). It was concluded that the catalytic activity of the mutant enzyme on activated DNA is not severely affected.

The polymerase activity of the slight mutator C344G was measured in a strain harbouring the *mip1* gene on the T7-3 multicopy plasmid and under the control of its own promoter. The very slight decrease of its specific activity (85% of wild-type activity) was hardly significant (not shown).

Singly-primed M13 DNA synthesis

As shown by alkaline agarose gel electrophoresis, singly-primed M13 DNA was efficiently elongated to the full-length product (7 kb) by wild-type mtDNA polymerase over-expressed from the *GAL1* promoter (Figures 5, 6B and 6C). However, when the concentration of M13 DNA was increased, the number of fully elongated molecules reached a maximum at $\sim 2\ \mu\text{M}$ substrate while lower molecular weight intermediates started to be produced (Figure 5). These data reflect the degree of processivity of the polymerase (processivity is defined as the extent of polymerization in a single binding event) and can be explained as follows. Under conditions of substrate limitation, dissociation of the mtDNA polymerase from the substrate is followed in most instances by reassociation of the enzyme to a previously

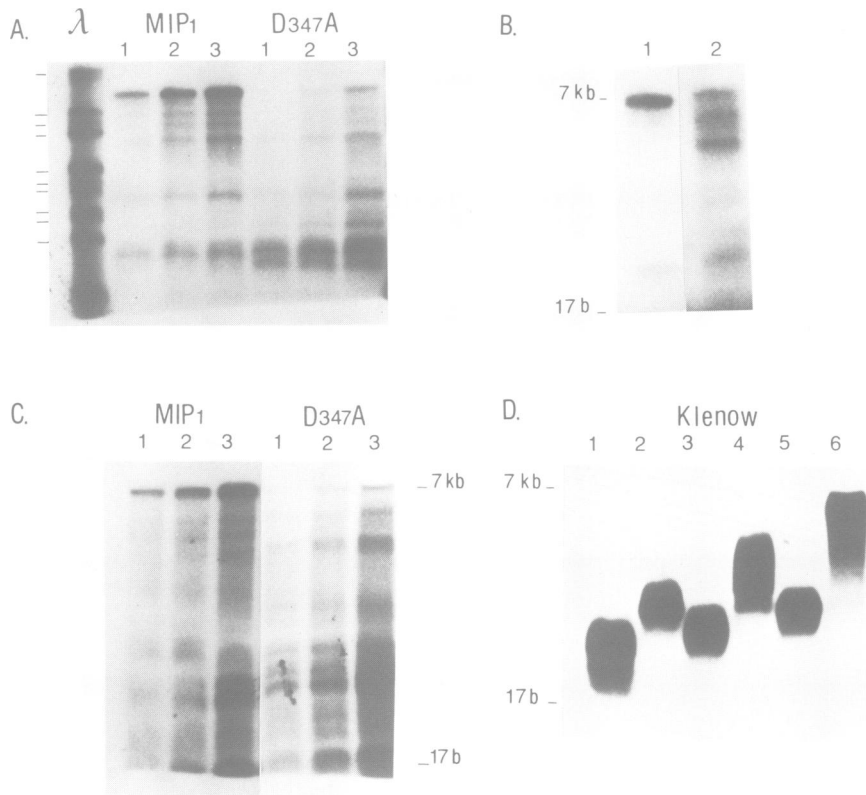


Fig. 6. Singly-primed M13 DNA synthesis by overproduced or non-overproduced wild-type and D347A mutant mtDNA polymerases and by *E. coli* DNA polymerase I. Panels A and B. Singly-primed M13 DNA (5 μ M) synthesis was performed with unlabelled substrate in the presence of [α - 32 P]dCTP as reported in Materials and methods. Under these conditions, the level of radioactivity (and intensity of the bands) is proportional to the length of the synthesized product. A. DNA synthesis by the mtDNA polymerase from non-overproducing *rho*⁺ strains A25 Δ mip1-01[MIP1(or D347A)/PFL39]. The amount of enzyme units and extent of DNA synthesis (% copied template) were as follows. Wild-type, 0.036 units: 5 min, 1.6%; 10 min, 3.1%; 20 min, 6.1%; D347A, 0.025 units: 5 min, 1.4%; 10 min, 2.0%; 20 min, 2.7%. λ , molecular weight markers obtained by digestion of λ phage DNA with *Hind*III and *Eco*RI (21226, 5148 + 4978, 4277, 3530, 2027 + 1904, 1584, 1375, 980, 830, 564, 125 bp). B. DNA synthesis (8 min of incubation) by wild-type (lane 1, 0.011 units) and D347A (lane 2, 0.027 units) mtDNA polymerases overproduced from *GAL*I promoter. Panels C and D. M13 DNA (10 μ M) synthesis was initiated from the 5' end 32 P-labelled primer AB353 as in Figure 5. C. DNA synthesis (4 min of incubation) in function of protein concentration. pLGALMIP1: lanes 1–3, 24, 48 and 96 ng protein; pLGALD347A: lanes 1–3, 32, 64 and 128 ng protein. D. DNA synthesis by *E. coli* DNA polymerase I (Klenow fragment, from Amersham). Lanes 1, 3 and 5: 0.4 units, 5, 10 and 20 min, respectively; lanes 2, 4 and 6: 2 units, 5, 10 and 20 min, respectively; 1 unit catalyses the incorporation of 10 nmol of nucleotides into acidic insoluble material in 30 min at 37°C.

extended 3'-hydroxyl terminus; the dissociation event is not detected because, finally, a full-length product is made. Under conditions of substrate excess (10 μ M), there are more 3'-hydroxyl termini whose extension is initiated; however, after dissociation from the substrate there is a high probability (because of the excess of substrate) that the polymerase binds to 3'-hydroxyl termini which have not been extended previously, giving rise to products of intermediate size. In contrast, a perfectly processive enzyme would not dissociate from the substrate, only synthesizing full-length DNA molecules. On the contrary, a poorly processive enzyme such as *E. coli* Pol I (Klenow fragment) which associates with and dissociates from the template very rapidly (Tabor *et al.*, 1987) elongates the DNA chain at a rate that is strongly dependent on protein concentration (Figure 6D). In some respects, the yeast mtDNA polymerase resembles that of bacteriophage T7 (Tabor *et al.*, 1987) as it fully elongates a significant number of the DNA chains under conditions of substrate excess and at a rate that is independent of protein concentration (Figure 6C). However, the mitochondrial enzyme halts DNA chain elongation at certain sites along the M13 DNA molecule. These are not randomly distributed and constitute hot spots of DNA synthesis arrest. Altogether,

our data suggest that the yeast mtDNA polymerase is moderately processive; it is sensitive to template secondary structure that elicits pauses in DNA synthesis.

Because we were working with a non-purified enzyme, it cannot be excluded that processivity is enhanced by specific proteins present in the mitochondrial extracts. This implies that these processivity factors should be present in such a large excess in the mitochondrial extracts as to stoichiometrically react with mtDNA polymerase when the latter is 500-fold overexpressed from the *GAL*I promoter. A more plausible hypothesis would be that processivity is enhanced by a non-stoichiometric factor which catalytically modifies the polymerase or interacts with the DNA substrate. However, it is also possible that yeast mtDNA polymerase itself is processive.

The extent of singly-primed M13 DNA synthesis in mitochondrial extracts of the mutant D347A was \sim 2-fold slower than in the wild-type strain under overproduction and non-overproduction conditions (Table II). Moreover, under assay conditions where the extent of DNA synthesis was the same in the mutant and wild-type strains, the fraction of fully elongated DNA was considerably decreased in the mutant (Figures 5, 6A, 6B and 6C). This was not due to a modifica-

tion of the K_m of the mutant enzyme for M13 DNA substrate as compared with the wild-type polymerase. These observations were extended to the 3'-5' exonuclease-deficient mutants D171G and D230A, overproducing the mtDNA polymerase from their own promoter (not shown). Thus, it can be concluded that 3'-5' exonuclease deficiency elicits a dramatic decrease in the size of the products that are synthesized from singly-primed M13 DNA. This suggests that the mutant mtDNA polymerase easily dissociates from its DNA substrate because it is less processive and more sensitive to secondary structure. In contrast, the DNA products pattern of the mutant C344G was similar to that of the wild-type strain (not shown).

Discussion

This is the first report that the 3'-5' exonuclease activity associated with mitochondrial DNA polymerase plays an important role in correcting replication errors *in vivo*. It is in agreement with the low mutation rate of yeast mtDNA which accumulates less base substitutions than nuclear DNA (Clark-Walker, 1991). Moreover, these data obtained with yeast also strongly suggest an *in vivo* proofreading function for the 3'-5' exonuclease activity associated with animal mtDNA polymerases which exhibit high accuracy *in vitro* (Kaguni *et al.*, 1988; Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989).

The yeast mitochondrial DNA polymerase overexpressed from the strong inducible *GALI* promoter was detected as a major 140 kDa polypeptide in soluble mitochondrial extracts. Under these conditions, we could show that it exhibits a potent and classical 3'-5' exonuclease activity that is more active on mispaired 3'-hydroxyl termini. In contrast with *Drosophila* mtDNA polymerase (Kaguni and Olson, 1989) and like the chicken and *Xenopus* enzymes (Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989), yeast mtDNA polymerase is able to excise correctly paired bases from a duplex. It is not clear whether in animal mtDNA polymerases the exonucleolytic activity belongs to the polymerase polypeptide (Kaguni and Olson, 1989; Longley and Mosbaugh, 1991). The *MIP1* gene is translated as a single 140 kDa polypeptide. Thus, in yeast the 3'-5' exonucleolytic domain belongs to the N-terminal part of the polymerase.

It has been found that many eukaryotic and prokaryotic DNA polymerases share three motifs (named *Exo1*, *Exo2* and *Exo3*) with the 3'-5' exonucleolytic domain of *E. coli* PolII DNA polymerase (Bernad *et al.*, 1989). We and others have found these motifs in the N-terminal part of the *MIP1* gene (Foury and Vanderstraeten, 1990; Ito and Braithwaite, 1990; Blanco *et al.*, 1991). Interestingly, *MIP1* consensus 3 shares with viral DNA polymerases and *K. lactis* killer factor DNA polymerases a cysteine residue (Tommasino *et al.*, 1988; Bernad *et al.*, 1989). Although these motifs have been detected in many DNA polymerases, in many cases it remains unclear whether they belong to the 3'-5' exonucleolytic domain. Amino acid substitutions in the consensus sequences dramatically decrease 3'-5' exonuclease activity in PolII, T7 and Φ 29 bacteriophages DNA polymerases (Joyce and Steitz, 1988; Bernad *et al.*, 1989; Tabor and Richardson, 1989; Derbyshire *et al.*, 1991; Patel *et al.*, 1991) as well as in yeast DNA polymerases δ and ϵ (Morrison *et al.*, 1991; Simon *et al.*, 1991). This contrasts,

however, with a recent report concerning bacteriophage T4 DNA polymerase (Reha-Krantz *et al.*, 1991). In that particular report, amino acid substitutions in the presumed *Exo1* site only slightly decreased the 3'-5' exonuclease activity and had only a small effect on the spontaneous mutation rate.

We have used site directed mutagenesis, replacing conserved aspartate residues with neutral alanine or glycine residues, in order to demonstrate that the three consensus sequences belong to the 3'-5' exonuclease domain of yeast mitochondrial DNA polymerase. Taking advantage of the fact that the mtDNA polymerase of the wild-type and D347A mutant strains are similarly overproduced from the *GALI* promoter, we determined that the 3'-5' exonuclease activity is 500-fold lower in the mutant than in the wild-type strain when standardized to the same number of polymerase units. A similar deficiency was observed in the other substitution mutants that exhibited a strong mutator phenotype. The ability of 3'-5' exonuclease-deficient mutants to synthesize DNA both *in vivo* and *in vitro* shows nucleotide excision to be the primary defect. Deficiency in 3'-5' exonuclease activity is reflected *in vivo* by a several hundred-fold higher mtDNA mutation frequency. This represents a normal contribution of proofreading to DNA replication accuracy (Fersht *et al.*, 1982).

The effect of substituting a glycine for Cys344 residue is relatively minor; 3'-5' exonuclease activity and the mutation rate are both slightly but significantly affected. This indicates that this conserved residue is not critical to catalysis but contributes to proofreading efficiency.

We also analysed whether 3'-5' exonuclease deficiency was altering polymerization and replication activities of yeast mtDNA polymerase. When *mip1* alleles are harboured on a centromeric vector and transformed into cells, production of cytoplasmic petites by the 3'-5' exonuclease-deficient mutants at 30°C is only slightly increased as compared with wild-type strain. In other words, mtDNA replication and segregation into daughter cell are not severely affected in the mutants and their mutator activity is certainly mostly limited to point mutations. However, growth on glycerol at an elevated temperature is severely impaired.

In mitochondrial extracts and under conditions of substrate excess, yeast mtDNA polymerase expressed from the *GALI* promoter efficiently catalyses the elongation of singly-primed M13 DNA to full-length 7 kb product. The presence of DNA bands of intermediate size indicates, however, that the polymerase is sensitive to template secondary structure. This is also true for *Drosophila* mtDNA polymerase (Wernette *et al.*, 1988). The elongation process in the mutant C344G is not altered. In 3'-5' exonuclease-deficient mutants, whether *mip1* alleles were harboured on centromeric or multicopy plasmids, low molecular weight DNA products accumulate preferentially to fully elongated DNA, suggesting that the mutant mtDNA polymerases fall off the expanding 3' end of the DNA chain very easily, probably because they are highly sensitive to template secondary structure and less processive than the wild-type enzyme.

This points to tight coupling *in vitro* between the 3'-5' exonucleolytic and polymerisation domains of yeast mtDNA polymerase. In contrast, the polymerising activity of 3'-5' exonuclease-deficient PolII, T7 and Φ 29 polymerases is not altered (Joyce and Steitz, 1987; Bernad *et al.*, 1989; Tabor and Richardson, 1989; Derbyshire *et al.*, 1991; Patel *et al.*,

1991). This also holds for the yeast nuclear DNA polymerases δ and ϵ (Morrison *et al.*, 1991; Simon *et al.*, 1991). In PolI, X-ray crystallographic studies have revealed that exonucleolytic and polymerisation domains are separated by 30 Å and behave as distinct entities (Ollis *et al.*, 1985). It has been shown that when the highly processive T7 DNA polymerase (Tabor *et al.*, 1987) meets a mismatch, DNA is channelled to the exonuclease site as a consequence of delayed chain elongation at a mispaired terminus and does not dissociate from DNA polymerase in the absence of excision (Donlin *et al.*, 1991). In contrast, mutations in the conserved regions of the 3'–5' exonuclease domains of the α -like DNA polymerase from Herpes simplex virus severely impair viral DNA replication and polymerase activity (Gibbs *et al.*, 1991), suggesting that in this polymerase, the exonuclease and polymerase domains are not well separate entities. Even though from primary structure comparisons the yeast mtDNA polymerase has been classified among type A polymerases (Blanco *et al.*, 1991; Ito and Braithwaite, 1991), our data indicate that in contrast with these polymerases the 3'–5' exonuclease domain plays some role in processivity. This is also the case for *E.coli* DNA polymerase III whose processivity is strictly dependent on the presence of the exonuclease subunit in the holoenzyme (Studwell and O'Donnell, 1990).

Our *in vitro* data have to be correlated to observations made *in vivo*. *In vivo* mtDNA replication is not severely altered. This suggests that our *in vitro* system imperfectly reflects *in vivo* physiological conditions. Accessory proteins of the replication complex or concentration of cofactors (such as magnesium) may considerably improve *in vivo* mtDNA replication. It must be noted, however, that when the mutants are grown at an elevated temperature (36°C), their growth on glycerol medium is severely decreased, suggesting that at this temperature *in vivo* replication is better reflected by our *in vitro* system.

Animal mitochondrial DNA polymerases are highly accurate *in vitro*: they select bases with high accuracy and possess a potent proofreading activity. The same accuracy applies to the yeast enzyme, as shown by the mutator phenotype of 3'–5' exonuclease-deficient mutants. The several hundred-fold increase in DNA replication accuracy attributable to the mitochondrial 3'–5' exonuclease editing activity is within the range of its expected contribution. Why such a high accuracy for a multicopy genomic system? Although we have no definitive answer, a justification could be the great biological importance of the mitochondrial genetic information which is essential to life in most eukaryotes and the fact that segregation mechanisms allow but a few copies of mtDNA to enter cells during meiosis. The severity and the wide variety of mitochondrial diseases (Wallace, 1989; Lander and Lodish, 1990) emphasize the reliance of mitochondria on error-free replication systems.

Materials and methods

Strains and media

Yeast strains and plasmids are listed in Table III. Strains were grown on YD (2% glucose, 2% yeast extract Kat), YG (2% glycerol, 2% yeast extract), YE (2.5% ethanol, 2% yeast extract) or minimal medium (either 2% glucose, galactose or raffinose plus 0.1% glucose, 0.7% yeast nitrogen base) supplemented with required amino acids and bases. Solid media were supplemented with 2% agar. When indicated, YG medium was supplemented with 50 mM sodium phosphate, pH 6.5 and 4 mg/ml erythromycin.

Table III. List of yeast strains and plasmids

Strains or plasmids	Relevant characteristics
<i>Saccharomyces cerevisiae</i>	
W303-1B	<i>Matα ade2 ura3 his3 trp1 leu2 rho⁺</i>
W303-1B/ Δ mip1-01	as above, but <i>LEU2 mip1::URA3 rho^o</i>
A25	<i>Matα ade2 ura3 trp1 nuc1::LEU2 rho⁺</i>
A25 Δ mip1-01	as above, but <i>mip1::URA3 rho^o</i>
A25 Δ mip1-02	as above, but <i>mip1::TRP1 rho^o</i>
JC7	<i>Mata leu1 kar1 rho⁺</i>
Centromeric plasmids	
PFL39	PUC19 vector with a <i>Bgl</i> III cassette containing the <i>TRP1</i> gene and a <i>Clal</i> cassette containing <i>CENV1</i> plus <i>ARS</i> (from F.Lacroute)
MIP1/PFL39	<i>MIP1</i> insert in <i>Sma</i> I site of PFL39 polylinker
D171G/PFL39	as above, but <i>mip1-2</i>
D230A/PFL39	as above, but <i>mip1-3</i>
C344G/PFL39	as above, but <i>mip1-4</i>
D347A/PFL39	as above, but <i>mip1-5</i>
2 μ episomal plasmids	
YE24	Yeast multicopy vector
T7-3	Selected from a library constructed in YE24 plasmid (Foury, 1989); contains a 6 kb insert including the <i>MIP1</i> and <i>VMA4</i> genes
D171G/T7-3	as above, but <i>mip1-2</i>
D230A/T7-3	as above, but <i>mip1-3</i>
C344G/T7-3	as above, but <i>mip1-4</i>
D347A/T7-3	as above, but <i>mip1-5</i>
pLGALZ3	Yeast multicopy vector containing the <i>GAL1</i> promoter (Lahaye <i>et al.</i> , 1991)
pLGALMIP1	pLGALZ3 after deletion of <i>lacZ</i> gene and insertion of <i>MIP1</i> gene
pLGALD347A	as above, but <i>mip1-5</i>

The construction of the disrupted *mip1::URA3* gene has been reported by Foury (1989). The disruption *mip1::TRP1* was performed by inserting the *TRP1* gene contained in a *Bgl*III cassette into the unique *Bgl*III site of the *MIP1* gene. The wild-type *LEU2* marker was introduced into the strain W303-1B Δ mip1-01 by integrative transformation, giving rise to the strain W303-1B/ Δ mip1-01. The strain A25 has been isolated as previously reported (Lahaye *et al.*, 1991) and contains a disrupted copy of the *NUC1* gene (Zassenhaus *et al.*, 1988) constructed by P.Zassenhaus (University of Saint Louis). In this study, all strains harbouring plasmids are *rho*⁺. The vector PFL39 is a gift from F.Lacroute (Centre de Génétique Moléculaire, Gif-sur-Yvette, France). The vector YE24 has been described by Botstein *et al.* (1979). The *VMA4* gene has been described by Foury (1990). The vector pLGALZ3 has been described by Lahaye *et al.* (1991).

Nomenclature

The *MIP1* and *mip1* were borne on either multicopy or centromeric plasmids. In all experiments, except those utilizing the *GAL1* promoter, the plasmids were present in a yeast strain that had a deleted copy of the chromosomal *MIP1* gene (Δ mip1-01 or Δ mip1-02, see Table III). However, the transformation experiments using multicopy plasmids with the *MIP1* (or *mip1-5*) gene fused to the *GAL1* promoter (pLGALMIP1 or pLGALD347A plasmids) were carried out in the *rho*⁺ strain A25 which still possesses the chromosomal copy of *MIP1*. Thus after transformation with the pLGALD347A plasmid, a single *MIP1* copy and a large number of *mip1-5* copies are simultaneously present in the cells. Although heterozygous for the *MIP1* gene, these transformants were also designated as 'mutants' in our text for the following reason. The mutant polypeptide expressed from the *GAL1* promoter in the plasmid pLGALD347A is in such large amounts compared with the protein encoded by the chromosomal wild-type *MIP1* that the latter is not detected (or hardly detected) in the enzymatic assays.

Strains whose *MIP1* (or *mip1*) gene is harboured on a centromeric vector

(PFL39) are designated as non-overproducing strains; strains whose *MIP1* (or *mip1*) gene is harboured on a multicopy plasmid are designated as overproducing strains, with the mtDNA polymerase expressed from either the *MIP1* (T7-3 plasmids) or *GAL1* (pLGAL plasmids) promoter.

A *rho*⁺ strain has a wild-type mitochondrial genome or exhibits only point mutations or small deletions in the mtDNA. A *rho*⁻ mutant (or cytoplasmic petite) results from large irreversible deletions of mtDNA leading to total deficiency of respiratory functions. A *rho*^o mutant is irreversibly devoid of mtDNA.

Site directed mutagenesis and construction of mutants

Standard oligonucleotide mutagenesis procedures were used to introduce point mutations in the *MIP1* gene (Sambrook *et al.*, 1989; Stanssens *et al.*, 1989). The *MIP1* nucleotide sequence is numbered according to Foury (1989). The restriction fragments subjected to site directed mutagenesis were entirely sequenced by the dideoxynucleotide chain termination method (Biggins *et al.*, 1983). Thus, it was verified that they did not contain additional nucleotide alterations. The mutated restriction fragments were substituted for their wild-type homologues in the centromeric *MIP1*/PFL39 plasmid or in the multicopy T7-3 plasmid. The disrupted *mip1* strain W303-IB/LΔ*mip1*-01 and A25Δ*mip1*-01 were transformed with the centromeric plasmids and the disrupted *mip1* strain A25Δ*mip1*-02 was transformed with the multicopy plasmids. In order to introduce mtDNA into the cells transformants were crossed on YD medium with the *rho*⁺ strain Jc7 which bears the *kar1* mutation and the mixture of haploids, diploids and cytoductants was streaked onto minimal glucose medium devoid of leucine to eliminate Jc7 haploids and select mutant cytoductants.

The plasmids that contain *MIP1* under the control of the inducible *GAL1* promoter were constructed as follows. A *Sall* site was created by site direct mutagenesis (G to C change at position 515), 45 bp upstream of the initiator ATG codon (Figure 1A). A 4.2 kb *Sall*-*PvuII* fragment containing the *MIP1* gene and 3' flanking sequences was substituted for the *Sall*-*PvuII* fragment of the pLGALZ3 vector which contains most of the *lacZ* gene. The D347A mutant plasmid was constructed by substituting the mutated *BglII*-*PvuII* fragment (characterized by a new *BglII* site) for its wild-type homologue.

Mitochondrial DNA mutations frequency

The strain W303-IB/L-Δ*mip1*-01/*rho*⁺ containing *MIP1*/PFL39 or *mip1*/PFL39 plasmids was used to estimate the frequency of spontaneous mtDNA mutations. 10–15 independent cultures for each mutant and 25 independent cultures for the wild-type strain were inoculated from independent colonies and grown in liquid complete glycerol (YG) medium for 2 days at 30°C. Aliquots were spread in lawns on glycerol plates (YG) supplemented with 4 mg/ml erythromycin. The number of erythromycin resistant (E^R) colonies was determined after 5 days at 30°C. This experiment was repeated several times. The mutator activity was estimated as the average number of E^R colonies per 10⁸ cells in the culture. The frequency of *rho*⁻ mutants was estimated as follows. Cells were grown for 24 h in liquid ethanol (YE) medium and spread on YD plates for single colonies. The percentage of *rho*⁻ colonies on YD plates was determined from the size of the colonies. The *rho*⁻ colonies which had lost their plasmid were subtracted.

Mitochondrial DNA polymerase extracts

The different conditions of culture were as follows. The wild-type A25Δ*mip1*-01[*MIP1*/PFL39] and the corresponding mutant D347A were grown in ethanol medium (YE) until late exponential phase of growth. The wild-type strain A25Δ*mip1*-02[*MIP1*/T7-3] *rho*⁺ and the mutant strains A25Δ*mip1*-02[*mip1*/T7-3] *rho*⁺ were grown in minimal raffinose medium until late exponential phase. The A25 strains with the multicopy plasmid-borne *MIP1* (or *mip1*) gene under the control of the *GAL1* promoter were first grown in raffinose minimal medium (without glucose) for 24 h. This culture was used to inoculate the galactose minimal medium at a final density of ~5 × 10⁶ cells/ml and galactose induction was proceeded for 16 h. Soluble mitochondrial extracts enriched in mtDNA polymerase were prepared as previously reported (Foury, 1989), except that protease inhibitors (1 μg/ml leupeptine and aprotinin, 2 mM benzamide and 1 mM PMSF) were added during protoplast lysis and mtDNA polymerase solubilisation steps. PMSF (1 mM) was also added during protoplast formation in galactose-grown cells.

Gap filling activity of mitochondrial polymerases

The DNA polymerase activity was measured at 37°C in a 200 μl reaction mixture containing 20 mM Tris-HCl, pH 8.0, 50 mM MgCl₂, 25 μM each of dATP, dCTP, dGTP and 5.5 μM [³H]dTTP (3000 c.p.m./pmol), 2 mM dithiothreitol and 200 μM DNaseI-activated calf thymus DNA. Aliquots of 40 μl were removed at time intervals and the radioactivity was

measured as previously reported (Genga *et al.*, 1986). The unit of mtDNA polymerase activity is defined as the amount of enzyme which catalyses the incorporation of 1 nmol dNTP into acid-insoluble material in 60 min at 37°C with activated calf thymus DNA as the substrate.

3'-5' exonuclease activity on mismatched 3'-hydroxyl terminus

A 17mer oligonucleotide that was either perfectly complementary to the M13 sequence (AB353, GTAAAACGACGGCCAGT) or contained a single mismatch dAMP:dAMP at the 3' hydroxyl terminus (AB354, GTAAA-ACGACGGCCAGA) was annealed to M13mp19 (or mp18) ssDNA. Oligonucleotides were previously labelled at their 5' end with [³²P]ATP and T4 polynucleotide kinase. An excess of primer was annealed to 5 μg M13 DNA in a 50 μl hybridization buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl) at 70°C for 5 min followed by slow cooling to room temperature. Singly-primed DNA was purified on a Sepharose CL-4B column in a pasteur pipette equilibrated with 80 mM NaCl and 10 mM Tris-HCl, pH 7.5. The exonuclease reaction mixture (50–60 μl) contained 20 mM Tris-HCl, pH 8.0, 50 mM MgCl₂, 0.5 mg/ml bovine serum albumin (BSA), 3 mM dithiothreitol (DTT) and 2–4 μM DNA substrate (~200 c.p.m./pmol). The reaction was started with the mitochondrial DNA polymerase extract and carried out at 30°C. Aliquots of 8 μl were removed at identical times and quenched with an equal volume of 80% formamide, 0.75% sodium dodecylsulfate (SDS) and 3 mM EDTA, pH 8.0. Products were analysed by electrophoresis in 15% polyacrylamide–8 M urea gels and autoradiography of the gels. The extent of 17mer hydrolysis was quantified by cutting the bands from the autoradiography into small pieces followed by elution of the silver grains with 1 N NaOH and measurement of the optical density at 500 nm in the colloidal medium (Suissa, 1983). Blanks were carried out by cutting pieces of the film that have not been exposed to the radioactivity.

Singly-primed M13 DNA synthesis

Annealing with AB353 primer and singly-primed DNA purification were performed as described above. The experiments were carried out either with DNA that was ³²P-labelled at the 5' end of the oligonucleotide as reported above or with unlabelled DNA which was elongated in the presence of [³²P]dCTP. In the latter case, the DNA elongation reaction mixture (90 μl) contained 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.5 mg/ml BSA, 25 mM KCl, 35 mM MgCl₂, 25 μM dATP, dTTP, dGTP, 5 μM [³²P]dCTP (5000 to 20,000 c.p.m./pmol) and singly-primed M13 DNA at the indicated concentration. In the former case, dCTP was unlabelled and its concentration was 25 μM. The reaction was started with the mitochondrial DNA polymerase extracts and carried out at 37°C. Aliquots of 20 μl were removed at indicated times and incubated further at 37°C for 20 min with 5 μl of stopper buffer containing 1 mg/ml proteinase K, 5% SDS and 100 mM EDTA, pH 8.0. Before electrophoresis in a 1% alkaline agarose gel (Sambrook *et al.*, 1989), DNA was phenol-extracted and denatured for 15 min at room temperature in a buffer containing 30 mM NaOH, 1 mM EDTA and 50% glycerol. Gels were fixed, dried and autoradiographed. Quantification of the extent of DNA synthesis was carried out in parallel experiments under the same reaction conditions, except that 5 μM [³H]dTTP (3000 c.p.m./pmol) and 25 μM dCTP were used. Aliquots were removed at indicated times, spotted onto Whatman GF/C filters, precipitated into 5% trichloroacetic acid and washed as previously reported (Genga *et al.*, 1986).

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