A cluster of pathogenic mutations in the 3′–5′ exonuclease domain of DNA polymerase gamma defines a novel module coupling DNA synthesis and degradation

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Mutations in DNA polymerase gamma (pol g), the unique replicase inside mitochondria, cause a broad and complex spectrum of diseases in human. We have used Mip1, the yeast pol g, as a model enzyme to characterize six pathogenic pol g mutations. Four mutations clustered in a highly conserved 3′–5′ exonuclease module are localized in the DNA-binding channel in close vicinity to the polymerase domain. They result in an increased frequency of point mutations and high instability of the mitochondrial DNA (mtDNA) in yeast cells, and unexpectedly for mutator mutations in the exonuclease domain, they favour exonucleolysis versus polymerization. This trait is associated with highly decreased DNA-binding affinity and poorly processive DNA synthesis. Our data show for the first time that a 3′–5′ exonuclease module of pol g plays a crucial role in the coordination of the polymerase and exonuclease functions and they strongly suggest that in patients the disease is not caused by defective proofreading but results from poor mtDNA replication generated by a severe imbalance between DNA synthesis and degradation.

INTRODUCTION

Mitochondria contain their own DNA (mtDNA) which encodes a small number of components that are absolutely required for respiration and ATP synthesis. Correct replication of the mitochondrial genome is therefore central to mitochondrial function and health. DNA polymerase gamma (pol g), the unique replicase inside the mitochondria, is a key enzyme in mtDNA replication and repair (1). Human pol g is a heterotrimer composed of a 140 kDa catalytic subunit (pol gA) and a dimer of the p55 accessory subunit (pol gB) which increases the activity and processivity of the enzyme (2). The catalytic subunit, which is distantly related to the prokaryotic PolA family, contains two main domains. The 3′–5′ exonuclease proofreading domain in the N-terminal part eliminates mispaired bases and the polymerase domain in the C-terminal part is responsible for DNA synthesis (1). These two domains are connected by a linker region which plays a role in DNA binding and processivity through its contacts with the proximal accessory subunit (3). The recently published crystal structure of human apo-pol g (3) shows that the catalytic subunit displays the canonical right-hand polymerase configuration with the ‘fingers’, ‘thumb’ and ‘palm’ subdomains that form a cleft accommodating the DNA and incoming dNTP. The catalytic reaction takes place in the palm subdomain at the bottom of the cleft (4). It is predicted that the DNA-binding channel occupies part of the exonuclease, linker and thumb domains (3).

Mutations in the human nuclear POLG1 gene which encodes the catalytic subunit of pol g are an important cause of mitochondrial disorders. Since 2001, more than 150 pathogenic POLG1 alleles have been identified in human (5,6). Progressive external ophthalmplegia (PEO) is a frequent pathology associated with multiple deletions of mtDNA coexisting with the intact mtDNA molecules. Nevertheless, many other severe diseases such as myopathy, mental disorders, various neuropathies, premature menopause and Parkinsonism symptoms have also been associated with POLG1 mutations. Several mutations associated with mtDNA depletion result in the Alpers syndrome, a fatal disease in young children characterized by brain deterioration and hepatic failure (7). Some POLG1 mutations are dominant or homozygous; however, the majority of the patients are compound heterozygotes and harbour one or several mutations on each chromosome.
The catalytic subunit of *Saccharomyces cerevisiae* pol g, which is encoded by the *MIPI* gene (8), displays ~40% amino acid sequence identity with human pol gA in their shared regions (9,10). There is no evidence for an accessory subunit in yeast pol g (11). Based on the hypothesis that conserved residues in conserved regions of different pol g should play a similar role, Mip1 has previously been used as a tool to determine the *in vivo* phenotypes conferred in yeast by several pathogenic pol g mutations (12–15). Here we have investigated the impact of five mutations (human L244P, L304R, R309L, R309H and W312R) in the 3′–5′ exonuclease domain and one mutation (R574W) in the linker region (Supplementary Material, Fig. S1). It has generally been assumed that the pathogenicity of 3′–5′ exonuclease mutations is associated with proofreading defects. However, in contrast with studies on the 3′–5′ exonuclease mutations residing at the active site (16,17), we found that several of these exonuclease mutations result in severe impairment of the polymerase function and DNA binding and have no effect on the 3′–5′ exonuclease activity. In most cases, Mip1 is a good model to estimate the contribution of each mutation to the disease and the mechanism which is defective in the mtDNA maintenance. Moreover, this model has helped us identify novel subdomains that play important roles in pol g function and disease onset.

**RESULTS**

**Selection and description of the disease-associated pol g mutations**

The first group of pol g mutations (Table 1) is represented by L244P which was identified in two brothers presenting with fatal hepatocerebralopathy (18,19). Leu244 is a conserved pol g residue between the Exol and ExoII sites in a coil localized at the periphery of the 3′–5′ exonuclease domain (Fig. 1A and B).

The second group comprises four mutations [L304R, R309H(L) and W312R] that are clustered downstream of the ExoII site (Fig. 1A). These mutations are often associated with PEO, myopathy and a broad spectrum of neuropathies (Table 1). Moreover, the L304R and R309H substitutions were fatal in several patients as homozygous (L304R) or in association with other mutations in trans (Table 1) (20–28). Based on the pol g three-dimensional structure, this cluster of mutations is localized in a coil–helix segment (Fig. 1B and C) which is not present in the prokaryotic PolA family but is strongly conserved in all pol g (Supplementary Material, Fig. S1). This area forms part of the DNA-binding channel. Arg309, a strictly conserved residue localized in the α-helix, points towards the lumen of the DNA-binding channel. In contrast, Leu304, a highly conserved residue localized in the coil, and Trp312, which is localized in the distal part of the helix, do not face the channel (Fig. 1C).

The third type of mutation is represented by R574W which was identified as compound heterozygous in patients showing symptoms of variable severity. PEO, myopathy and dysphagia were observed in an adult patient harbouring the W312R mutation in *trans*, and the Alpers syndrome was diagnosed in three infants harbouring the A467T mutation (Table 1) (29,30). Arg574 is a highly conserved residue residing in a region of the intrinsic processivity (IP) subdomain which is shared by Mip1 and points towards the lumen of the DNA-binding channel (Fig. 1A and B).

This brief overview gives an idea of the extreme complexity and variability of the disease traits, which are the result of the impact of each mutation, alone and in combination with other mutations, as well as of the genetic and environmental parameters specific to each patient. Moreover, studies are complicated by the small number of patients bearing the same mutations and by the absence of familial history for several cases.

**Construction of the *mip1* mutants**

The L210P, L260R, R265H(L), F268W and R467W *mip1* mutations equivalent to the human L244P, L304R, R309H(L), W312R and R574W *POLG1* mutations were obtained by site-directed mutagenesis of the *MIPI* allele (31) harboured by a low copy centromeric plasmid (pFL39*MIP1* or pFL38*MIP1*). The plasmid-borne *mip1* alleles were cloned into the haploid *W303-1B* strain (16) and mtDNA was introduced into *rho<sup>0</sup>* cells by the cytoduction technique (32).

**Cellular respiration and stability of the mitochondrial genome**

Defects in either polymerase or exonuclease proofreading functions can impair mitochondrial biogenesis, leading to production of poorly expressed or functionally defective subunits in the respiratory complexes. Thus, the rate of cellular oxygen consumption is an indicator of the impact of pol g mutations on the mitochondrial function and reflects the state of the mitochondrial genome. All mutants showed, to various extents, impaired respiratory function (Table 2). The *mip1*-L260R,
mip1-R265H and mip1-F268R mutations conferred low respiration levels, whereas the mip1-R265L and mip1-R467W mutations had only a slight effect.

In *Saccharomyces cerevisiae*, mtDNA replication defects result in mtDNA instability characterized by the accumulation of respiratory-deficient cells that either harbour large mtDNA deletions (rho−/−) or have lost mtDNA (rho0/−). In agreement with their low oxygen consumption, the mip1-L260R, mip1-R265H, and mip1-F268R mutants grown at 28°C produced rho−/− (rho− + rho0/−) mutants at a high frequency (more than 50% of the colonies) and most of the respiratory-deficient clones were rho0 (Supplementary Material, Fig. S2I). Only 20–25% of the colonies were rho−/− in the mip1-R265L and mip1-R467W mutants (Table 2). In these five mutants, the percentage of rho−/− was not increased when cells were grown at 37°C (data not shown). In contrast, whereas the percentage of rho−/− mutants produced by the mip1-L210P mutant at 28°C was below 10% (Table 2), the cell population was almost completely converted to rho−/− mutants (>99%) at 37°C. Moreover, 4′-6-diamino-2-phenylidole staining showed that while most mip1-L210P cells grown at 28°C contained mtDNA, cells grown at 37°C were completely (or almost completely) devoid of mtDNA (Supplementary Material, Fig. S2II). These data show that in the mip1-L210P mutant mtDNA, replication defects are temperature-sensitive. All mutations were recessive (data not shown).

**Frequency of mtDNA point mutations**

Accumulation of point mutations reflects the fidelity of mtDNA replication. The mutator properties of mtDNA were estimated by the frequency of erythromycin-resistant (E^R^) mutants. It has previously been shown that E^R^ mutants are only produced by mutations in the mitochondrial 21S rRNA gene. All types of substitutions can affect any of the three adjacent GAA nucleotides at positions 1951–1953 of the gene (17). In all mutants, the frequency of E^R^ mutations was increased (Table 2 and Supplementary Material, Fig. S2III), from 4.5-fold in mip1-R265L to 18-fold in mip1-R467W, and more than 50-fold in mip1-L260R, mip1-R265H, mip1-F268R and mip1-L210P mutants. Furthermore, the actual accumulation of point mutations in mtDNA was probably underestimated due to the high percentage of rho−/− mutants coexisting with rho+ cells in the cultures.
Table 2. Phenotypic analysis in vivo and gap-filling activity of wild-type and mip1 strains

<table>
<thead>
<tr>
<th>MIP1 mutation</th>
<th>mtDNA stability (% rho &lt;0&gt;)</th>
<th>E&lt;sup&gt;R&lt;/sup&gt; mutant frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cell respiration (μL O&lt;sub&gt;2&lt;/sub&gt;/h/mg)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Gap-filling activity&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1</td>
<td>0.9 ± 0.3</td>
<td>1</td>
<td>8.5 ± 2.1</td>
<td>100</td>
</tr>
<tr>
<td>L210P</td>
<td>8.33 ± 0.01</td>
<td>83.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L260R</td>
<td>62.4 ± 5.2</td>
<td>51</td>
<td>1.1</td>
<td>35.5 ± 8.5</td>
</tr>
<tr>
<td>R265L</td>
<td>24.3 ± 3.9</td>
<td>4.5</td>
<td>7.5 ± 1.1</td>
<td>91.1 ± 2.3</td>
</tr>
<tr>
<td>R265H</td>
<td>50.6 ± 2.4</td>
<td>85.8</td>
<td>2.2 ± 0.2</td>
<td>63.5 ± 2.5</td>
</tr>
<tr>
<td>F268R</td>
<td>64.2 ± 10.5</td>
<td>85</td>
<td>2.3 ± 0.2</td>
<td>70.5 ± 8.5</td>
</tr>
<tr>
<td>R467W</td>
<td>21.5 ± 4.1</td>
<td>18.5</td>
<td>6.5 ± 0.5</td>
<td>97.5 ± 1.5</td>
</tr>
<tr>
<td>MIP1/MIP1</td>
<td>2.6 ± 1.7</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F268R/R467W</td>
<td>14.5 ± 3.4</td>
<td>18</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

<sup>a</sup>E<sup>R</sup> mutant frequency was expressed as a fraction of the wild-type frequency arbitrarily set to 1.

<sup>b</sup>Microlitres of O<sub>2</sub> per milligram of yeast cells (wet weight) per hour.

<sup>c</sup>Gap-filling activity was expressed as the average percentage of the wild-type activity (100%) estimated from three experiments.

**In vivo phenotype of the F268R/R467W mip1 mutant**

The heteroallelic F268R/R467W mip1 mutant, equivalent to the W312R/R574W human combination (26), was also analysed. Both the percentage of rho <0> clones and the frequency of E<sup>R</sup> mutants were similar to those of the singly substituted mip1-R467W mutant (Table 2), indicating that the deleterious phenotype conferred by the F268R mutation was attenuated in the presence of the R467W mutation.

**Expression of wild-type and mutant Mip1**

The biochemical properties of wild-type and mutant Mip1 proteins were determined in a strain that was devoid of the potent mitochondrial Nuc1 nuclease (33) and harboured the MIP1 (mip1<sup>+</sup>) gene under the control of the strong inducible GAL1 promoter on a high copy number plasmid (16). Mip1 levels were estimated in soluble mitochondrial extracts from cells grown at 28°C by western blotting using a polyclonal Mip1 antibody. With the exception of Mip1-L210P, the expression levels of wild-type and mutant Mip1 proteins were rather similar (Fig. 2A), suggesting that polymerases are stable and properly folded. In contrast, Mip1-L210P was almost undetectable in the extracts (Fig. 2B). Since in mip1-L210P cells grown at 28°C mtDNA is stable (8% rho <0> only), it must be admitted that the amount of Mip1-L210P protein is sufficient in mitochondria to replicate mtDNA. A likely cause to the loss of Mip1-L210P in the mitochondrial extracts is the high temperature (37°C) required for cell wall digestion during spheroplast preparation. As suggested, by the temperature-sensitive phenotype of the mip1-L210P mutant, the folding defect of the polymerase is certainly enhanced at this temperature and leads to the degradation of the protein by the mitochondrial quality control machinery (34,35).

Nucleic acids and many contaminating proteins were eliminated from the Mip1-containing fraction by diethylaminoethyl cellulose (DEAE) chromatography (Fig. 2C and Supplementary Material, Fig. S3). The purification was not pursued further to avoid potential inactivation of the mutant enzymes. As previously shown in studies with proofreading-deficient mutants (16), after the DEAE step, Mip1 is sufficiently pure with negligible contaminating nuclease activities to get a reliable estimation of its biochemical properties.

**Gap-filling activity and processive DNA synthesis**

Non-processive gap-filling activity measures the capacity of a polymerase to fill in short gaps in a duplex DNA. A decrease in this robust activity often indicates a catalytic defect or low affinity for dNTP or DNA. However, an excess exonuclease activity may also lead to a decreased gap-filling activity. Gap-filling activity was significantly affected in the three mutants that had the highest mtDNA instability in vivo, with only 35% of the wild-type activity for Mip1-L260R and 63 and 70% of the wild-type activity for Mip1-R265H and Mip1-R265R, respectively (Table 2). The activity was similar to that of wild-type for Mip1-R265L and Mip1-R467W (Table 2).

Processive DNA synthesis represents the capacity of a polymerase to elongate a nascent chain to a full-length product without dissociation from the DNA substrate. An excess of single-stranded DNA template paired with an oligonucleotide primer was used as the substrate. Two types of assays were performed. First, the amount of dNTPs incorporated into the...
nascent DNA chain (total DNA synthesis) was measured by using an unlabelled primer and [α-32P]dATP in the assay. Secondly, the fraction of full-length DNA molecules was determined by using a 5′ end [32P]-labelled oligonucleotide primer. Total DNA synthesis was decreased to less than 5% of the wild-type level for Mip1-L260R, Mip1-R265H and Mip1-F268R mutants, while it reached 35 and 59% of the wild-type level for Mip1-R265L and Mip1-R467W, respectively (Fig. 3A). In agreement with the poor total DNA synthesis, full-length products could barely be detected for Mip1-L260R, Mip1-R265H and Mip1-F268R (Fig. 3B). These mutant Mip1 enzymes are not processive, suggesting frequent arrests of the polymerase in vivo followed by the dissociation of the enzyme from the mtDNA. This defect was less pronounced for Mip1-R265L and Mip1-R467W (Fig. 3B).

DNA-binding affinity

Based on the crystal structure of human pol g, all of the mutated residues were predicted to be in the vicinity of the DNA-binding channel (3). Therefore, an electrophoretic mobility shift assay (EMSA) was used to determine the DNA-binding activity of wild-type and mutant Mip1 polymerases. The assay was performed with a 21/45mer primer-template substrate in the presence and absence of 3 mM MgCl2. The DNA-binding activity of wild-type Mip1 was 3–4-fold stimulated by MgCl2 (Fig. 4A and B), suggesting that Mip1 adopts a more biologically relevant conformation in the presence of this cation. In the absence of MgCl2, the DNA-binding activity was similar to that of wild-type for Mip1-R265L and ~2-fold lower for the other mutants. However, binding was not stimulated by magnesium in the mutants clustered downstream of the ExoII site, such that the DNA-binding activity was ~3-fold lower for Mip1-R265L and ~10-fold lower for Mip1-L260R, Mip1-R265H and Mip1-F268R in comparison to wild-type (Fig. 4A and C). In contrast, Mip1-R467W DNA-binding activity was stimulated by MgCl2.

3′–5′ exonuclease activity

Most mutations mapping to the 3′–5′ exonuclease domain confer mutator properties resulting from defects in the 3′–5′ exonuclease function (36,37). Therefore, the mutator properties in vivo exhibited by the 3′–5′ exonuclease mutants studied here suggested that the 3′–5′ exonuclease activity could be impaired. The 3′–5′ exonuclease activity of wild-type and mutant Mip1 was determined in the presence of 50 mM MgCl2 (optimal concentration) using a template primed with a 17mer oligonucleotide which was either complementary to the DNA template or contained a single nucleotide A:A mispair at its 3′ end. Unexpectedly, none of the mutants in the 3′–5′ exonuclease domain showed substantially decreased 3′–5′ exonuclease activity on correctly paired or mispaired substrates (Fig. 5A–D and G). The exonuclease activity was similar to that of wild-type for Mip1-R265L and Mip1-F268R, reached 76% of the wild-type activity for
Mip1-R265H and was even two times higher for Mip1-L260R. In contrast, Mip1-R467W showed defective exonuclease activity at 50 mM MgCl2 (27% of wild-type) (Fig. 5E and G). Whereas the excision rate of wild-type Mip1 was strongly stimulated upon increasing MgCl2 concentrations, no effect of MgCl2 concentration was observed in Mip1-R467W (Fig. 5F), suggesting that the Mip1-R467W properties might be dependent on the conditions prevailing inside mitochondria.

Since the mip1-L260R, mip1-R265H and mip1-F268R mutants showed mutator properties in vivo and robust 3′–5′ exonuclease activity in vitro, it could not be excluded that the high point mutation rate in vivo was produced by increased dNTP misincorporation during DNA synthesis. Misincorporation assays were performed using an oligonucleotide-primed ssDNA as the substrate in the presence of increasing concentrations of dGTP which is correctly incorporated opposite to C at position +18 and can give G:T mispairing at position +19 (Fig. 6). The assay was performed for Mip1-L260R, which shows a 2-fold increase in 3′–5′ exonuclease activity, and Mip1-R265H in which exonuclease activity is slightly decreased (Fig. 5). Whereas wild-type Mip1 readily formed the G:T mispair, neither Mip1-L260R nor Mip1-R265H displayed misincorporation (Fig. 6A and B), excluding that the mutator trait is caused by increased dNTP misincorporation.

**Coupling of 3′–5′ exonuclease and polymerase functions**

Since pol g has both polymerase and 3′–5′ exonuclease activities, the DNA primer strand can be localized at either of the two active sites and the balance between these two localizations is an important parameter of the replicative properties (38). The data obtained for the total DNA synthesis (in the presence of 20 mM MgCl2, the optimal concentration) and 3′–5′ exonuclease activity (in the absence of dNTP and in the presence of 50 mM MgCl2) provide an estimate of the relative polymerization and degradation (exo/pol) capacities. For an exo/pol ratio set to 1 for wild-type Mip1, all exonuclease mutants exhibited ratios significantly higher than 1, from 2.7 for Mip1-R265L to 16 and 26 for Mip1-R265H and...
Mip1-F268R, respectively, and up to 52 for Mip1-L260R (Fig. 7A). These data were confirmed by polymerase/exonuclease coupling experiments (39). DNA synthesis versus degradation was analysed using a PTZ18U DNA template paired with a 5′ end [32P]-labelled oligonucleotide as the substrate and increasing dNTP concentrations. In the absence of dNTP, only degradation occurs. Upon addition of increasing dNTP concentrations, there is a progressive competition between synthesis and degradation and preferential positioning of the primer strand at the polymerase site. Efficient elongation of the nascent chain was initiated for wild-type Mip1 upon addition of nanomolar range dNTP concentrations with concomitantly decreased 3′–5′ exonuclease activity. In contrast, 10–20 times higher dNTP concentrations were required to elongate the primer strand in the Mip1-L260R, Mip1-R265H and Mip1-F268R mutants (Fig. 7B–D). Elongation efficiency was also notably decreased for Mip1-R265L (Fig. 7E). In contrast, the elongation efficiencies were quite similar for wild-type and Mip1-R467W (Fig. 7F).

These data show that all mutant Mip1 polymerases except Mip1-R467W favoured excision over polymerization as a result of preferential positioning of the primer strand at the exonuclease site.

DISCUSSION

This work had two goals: the first was to shed light on the dysfunction caused by mutations localized in new pol g areas; the second was to determine to which extent the yeast model was valid. To this end, we have generated and characterized the mip1 equivalents of six disease-associated POLG1 mutations. As discussed below, all impaired mtDNA replication in vivo and polymerase function in vitro, implying that their human equivalents play a crucial role in the disease onset. Despite a few exceptions, there is generally a good correlation between yeast phenotypes and disease severity (Supplementary Material, Fig. S4). Whereas the symptoms observed in the patients did not allow the classification of the pol g mutations, the yeast model has allowed us to range them into three categories.

The first category results in insufficient pol g levels and is represented by the human L244P mutation localized between the ExoI and ExoII sites in a conserved residue of a peripheric coil. At 28°C, the yeast mip1-L210P mutant does not lose mtDNA but exhibits an 80-fold increase in mtDNA point mutation frequency. Recently, a mutator phenotype was also reported for this mutant (15). However, the authors did not mention the intense loss of mtDNA at 37°C revealing that mtDNA replication is defective at elevated temperatures. This remarkable in vivo temperature-sensitive phenotype is associated with very low levels of Mip1-L210P protein in the mitochondrial extracts from cells grown at 28°C. Based on these data, we propose that the change of Leu210 to proline results in a protein folding defect that is mild enough in cells grown at 28°C to maintain a sufficient amount of Mip1-L210P to replicate mtDNA. However, the Mip1 protein is unstable and degraded during the preparation of the mitochondrial extracts. In addition, the 3′–5′ exonuclease activity is probably not optimal and this results in a mutator phenotype in vivo. In cells grown at 37°C, the folding defect is probably more pronounced, Mip1-L210P is not expressed and mtDNA is lost. These data strongly suggest that in human, L244P mutant form is poorly expressed at 37°C, the physiological body temperature. As a consequence, the disease presentation is most likely governed by the defects produced by the common W748S mutation in trans and amplified by the haploinsufficiency caused by the
structural defect conferred by the L244P mutation. This results in acute Alpers syndrome (19,30) instead of the MIRAS syndrome (mitochondrial recessive ataxia syndrome) that is commonly observed in patients harbouring W748S (40).

The second category is represented by the human R574W mutation. Arg574 is a highly conserved residue residing in a segment of the IP domain which forms a tangent plane to the DNA-binding channel (Fig. 1B). This segment is shared by Mip1 and thus should play a similar function in both organisms. However, the proximity of one accessory pol gB subunit (Arg574 is 7 Å distant from the pol gB Lys477 residue) and the increased length of the human IP domain suggest that the R574W mutation might elicit more complex effects than mip1-R467W. Indeed, the data obtained in yeast only partially correlate with the clinical presentations. Even though the studies with Mip1-R467W validate the expected impact of this mutation on the DNA-binding affinity, the moderately severe defects observed in yeast both in vivo and in vitro do not really explain the fatal Alpers syndrome presented by three infants harbouring the R574W mutation combined with the A467T mutation in trans (29). The discrepancy between the yeast and human phenotypes points to a potential weakness in the yeast model for mutations in the linker region, even for those in highly conserved residues.

The third category is represented by the human L304R, R309H, R309L and W312R mutations downstream of the ExoII motif (Fig. 1). They define a novel subdomain of the 3′–5′ exonuclease that is not present in prokaryotic family A and is strongly conserved in all pol g. This area which is distant from the exonuclease active site and in close proximity to the polymerase domain is localized in the DNA-binding channel and characterized by a coil–helix motif (Fig. 1C).

The mip1-L260R, mip1-R265H and mip1-F268R mutants are characterized by low respiration, high mtDNA instability and a more than 50-fold increased frequency of mtDNA point mutations. It must be noted that the mip1-C261R and mip1-Q264H mutations, which are the equivalents of the human disease-associated S305R and Q308H mutations, also confer high mtDNA instability (Szczepanowska, Cochonneau and Foury, unpublished data). Thus, despite the milder phenotype of the mip1-R265L mutant, these data suggest that the Mip1 fragment encompassing residues 260–268 is of particular importance in mtDNA maintenance. The in vivo genetic properties of the mip1-L260R and mip1-R265H(L) mutants have also been studied by another group (15). They did not find that these mutants were mutator and reported higher mtDNA instability in mip1-R265L than in mip1-R265H mutant. However, our in vivo data support our in vitro data. While all mutants showed decreased polymerase activity associated with low processivity and deeply modified DNA-binding affinity with no stimulation by MgCl2, the defects were more pronounced for Mip1-R265H than for Mip1-R265L. Unexpectedly, for mutator strains, the 3′–5′ exonuclease activity was only slightly decreased in Mip1-R265H, unchanged in Mip1-R265L and Mip1-F268R and even increased in Mip1-L260R. Moreover, exonuclease/polymerase coupling experiments showed that the balance between exonuclease and polymerase functions favours the exonuclease activity. These data suggest that increased exonuclease activity combined with a poor polymerization activity is not an advantage in vivo to efficiently remove the replication mistakes. As neither Mip1-L260R nor Mip1-R265H polymerases displayed tendency to misincorporate nucleotides, it is unlikely that the mutator trait could be explained by an increased
Figure 7. Polymerase/exonuclease-coupled assay and exonuclease/polymerase (exo/pol) ratios for wild-type and mutant Mip1. (A) Estimation of the 3′–5′ exonuclease to polymerase ratio. For each strain, the ratio of the value obtained in Fig. 5G for exonuclease activity to the value obtained in Fig. 3A for total DNA synthesis was reported to the exo/pol ratio of the wild-type Mip1 arbitrarily set to 1. (B–F) Images of the dried gels. Assays were performed at 28°C using ~1.5 ng wild-type and mutant Mip1 from DEAE chromatography-purified fractions as reported in Materials and Methods and increasing dNTP concentrations (lane 0, no dNTP; lane 1, 25 nM; lane 2, 100 nM; lane 3, 500 nM; lane 4, 5 μM and lane 5, 25 μM). The lanes (–) on the left contain the DNA substrate alone. The products were separated by electrophoresis in denaturing 15% polyacrylamide/7 M urea gels. The top panels show the products of higher molecular weight than the 17mer oligonucleotide after an 8 cm Bromophenol blue migration (polymerase activity). The bottom panels show the products of lower molecular weight than the 17mer oligonucleotide after a 30 cm Bromophenol blue migration (exonuclease activity).
incorporation of incorrect nucleotides into mtDNA. However, mtDNA synthesis in yeast is highly dependent on the mitochondrial Msh1-dependent mismatch-repair pathway (17,41). A plausible explanation to the mutator trait of these mutants is that their low DNA polymerase activity is a rate-limiting factor to the mitochondrial mismatch-repair which requires efficient DNA synthesis (17). It must be noted that there is no Msh1 equivalent pathway in human mitochondria.

The phenotypes of the yeast mip1-L260R, mip1-R265H and mip1–R265L mutants fit well with the clinical presentations conferred by their human equivalents. Young adults harbouring the L304R mutation combined with A467T or W748S in trans developed severe neuropathies leading to premature death for two of them (20,25,27). Moreover, early disease onset below 10 years of age was observed for two L304R homozygous patients and was followed by a fatal issue in one subject (27,28). Although the in trans mutations harbouring by the compound heterozygous patients should also be considered, the severe mip1–R265H phenotype is in accordance with the fatal Alpers syndrome developed by a 6-month-old infant harbouring the R309H substitution (26), whereas the mild phenotype conferred by the mip1–R265L mutation agrees with the less severe myopathy/PEO symptoms observed in two patients harbouring the R309L substitution (21). The validity of the yeast model is more questionable for the human W312R/mip1–F268R equivalents. The severe in vivo and in vitro phenotypes conferred by the mip1–F268R mutation do not fit well with the late disease onset in patients harbouring the W312R mutation at the homozygous state or combined with severe mutations such as R574W in trans (26). The discrepancy between yeast and human might be imputable in part to the different properties conferred by Trp312 and Phe268 residues.

Altogether the data obtained in yeast show for the first time that a subdomain of the 3′–5′ exonuclease plays a crucial role in the DNA polymerase function with no major effect on the exonucleolytic activity. In other words, defective proofreading is certainly not the cause of the disease in patients harbouring the L304R, R309H(L) and W312R mutations. Indeed, patients homozygous for L304R did not show any sign of the premature ageing syndrome displayed by proofreading-deficient mutator mice (42). Our studies suggest that in these patients mtDNA replication dysfunction is generated through decreased DNA-binding affinity and a severe imbalance in the coupling between the exonuclease and polymerase functions that favours the exonuclease process. Therefore, the diseases associated with these mutations are caused by poor mtDNA replication.

We would like to propose a function for the 3′–5′ exonuclease subdomain encompassing residues 304–312 in human pol g. All DNA polymerases which can proofread the errors introduced during DNA synthesis possess two distinct catalytic sites and thus must direct the DNA primer strand from one active site to another (38). A subtle balance in positioning of the primer strand at either the polymerase or the exonuclease site is particularly important in replicative DNA polymerases such as pol g which require both fast and faithful DNA replication. The transfer of the 3′ end of the primer strand between polymerase and exonuclease active sites requires precise mechanisms that are not perfectly understood and differ from one polymerase to another. In Escherichia coli DNA polymerase I, the J-helix seems to play a role in the primer strand shuttling between the polymerase and exonuclease sites (43), whereas in several family B polymerases, a β-hairpin loop structure localized in the 3′–5′ exonuclease domain plays an important role in DNA binding during the transfer of the primer end to the exonuclease site (44–48). However, in Φ29 DNA polymerase, the thumb is mainly responsible for the coordination of primer strand switching (49). No switching mechanism has been described so far for pol g. Our data strongly suggest that the 3′–5′ exonuclease segment comprising residues 304–312 plays a crucial role in this mechanism.

This 3′–5′ exonuclease segment belongs to the DNA-binding channel, is at an equivalent distance from the polymerase and exonuclease active sites (26–27A) and is included in a larger module (residues 293–316) that is closer than any other exonuclease region from the polymerase domain. This module which comprises two α-helices (residues 295–301 and 306–318) connected by a short coil is in close proximity to segments localized in a loop of the palm subdomain (residues 845–852) and in the fingers subdomain (residues 1022–1027 and 1047–1091, respectively) (Fig. 8A–C and Supplementary Material, Table S1).

Lee et al. (3) have pointed out that the module encompassing residues 1051–1095 partially blocks the DNA-binding channel, a very unusual feature that has never been reported for a DNA polymerase but is reminiscent of a structural element described for the RNA polymerase from the bacteriophage N4 virion (vRNAP) (50,51). In the vRNAP apo-form, the DNA template-binding channel is blocked by the cooperating action of two modules, the B-loop and the plug localized in the fingers and in the N-terminal subdomains, respectively, whereas in the binary DNA–vRNAP complex, a conformational change associated with the expulsion of the B-loop opens the channel. Based on similarities displayed by the plug /B-loop modules of vRNAP and the 3′–5′ exonuclease/ fingers segments of pol g with respect to positioning in the linear amino acid sequence, secondary structures and localization in the DNA-binding channel, we first thought that the pol g elements could fulfil plug-like and B-loop-like functions in the switching mechanism between the exonuclease and polymerase active sites. However, alignment of the entire protein structures shows that pol g and vRNAP modules cannot be superimposed and display a different spatial organization (Supplementary Material, Fig. S5). In addition, the mechanisms that are involved in the two polymerases are different. The vRNAP requires a mechanism that blocks the entrance of unspecific DNA substrates to the unique polymerase active site, whereas pol g, as an enzyme possessing two distinct but cooperating active sites, requires a switching mechanism that blocks the access of the DNA primer strand to one active site while facilitating its transfer to the other site.

We propose a model of a switching mechanism which could fulfill such a function (Fig. 8D). As pol g structure has been obtained without bound DNA, we have superimposed the structures of pol g and bacteriophage T7 DNA polymerase complexed with DNA to provide a rough model of the pol g-DNA complex (3). This modelling with docked DNA shows that the fingers module (residues 1049–1095) forms a loop that tightly grabs the template strand through several residues localized at less than 1 Å from the DNA (Fig. 8B and C and Supplementary Material, Table S2). This module which could have a DNA
anchoring function will be referred to as the ‘stabilizer’. The second element is the 3′–5′ exonuclease module (residues 293–316) which could play a role in the orientation of the primer strand towards the active sites in response to pol g conformational changes. This module will be referred to as the ‘orienter’. The ‘orienter’ module could play a double role. First, through a pressure exerted by residues situated near the template strand, the ‘orienter’ module could create spatial constraints leading to a destabilization of the template strand and a modification in the arrangement of the primer strand. Secondly, this module could constitute a mobile barrier preventing the access of the primer strand to the exonuclease site during transition from the open to the closed conformation and, reciprocally, opening the access to the exonuclease site after base mispairing. The disease-associated mutated residues residing in the ‘orienter’ module yield a more tightly packed structure with additional hydrogen bonds (Supplementary Material, Fig. S6). This disturbance of the structural properties of the mutated ‘orienter’ module would leave the exonuclease site widely accessible to the primer strand. Thus, the ‘stabilizer’ and ‘orienter’ modules seem to form a bottleneck in the DNA-binding channel ensuring an optimal environment for positioning of the primer and template strands. However, our model relies on a modelled pol g–DNA complex based on the structure of bacteriophage T7 DNA polymerase which does not contain these modules. It is clear that a crystal structure of pol g bound to DNA is required to test our model.

In conclusion, this work performed with yeast as a model system has revealed that dysfunction in the coordination of the coupling between the polymerase and 3′–5′ exonuclease functions is an important cause of human pol g-associated diseases. A 3′–5′ exonuclease module localized in the DNA-binding channel and in close vicinity to a small fingers subdomain that partially blocks the DNA-binding channel plays a crucial regulatory role in this functional coupling.

**MATERIALS AND METHODS**

**Strains and media**

For *in vivo* studies, the parental strain was W303-1B/Δmip1 (MATα ade2-1 ura3-1 his3-11, 15 trp1-1 can1-100...
mip1Δ::KANr, rho0) (32). The mip1 mutations were generated in the MIP1[S] allele (31) by site-directed mutagenesis using the overlap extension technique (Supplementary Material, Table S1) (52). The mip1 mutation-containing fragments were substituted for their wild-type copy in the MIP1 gene harboured by pFL39 (TRP1) or pFL38 (URA3) centromeric plasmids (Supplementary Material, Materials and Methods) and the plasmids were transformed into W303-1B/Δmip1. MtDNA was introduced into the rho0 transformants in crosses with the JC7 strain (MATa leu1 kar1-1 rho+) using the cytoduction technique (32). For in vitro studies, strain A25 (MATα ade2 ura3 trpl, nucl1::LEU2) was transformed with the multi-copy pGALMIP1 plasmid harbouring either the wild-type MIP1 or the mutant mip1 alleles placed under the control of the strong inducible GAL1 promoter (16).

The following media were used: minimal synthetic medium (0.7% yeast nitrogen base supplemented with a mixture of amino acids and either 2% glucose, raffinose or galactose) (33), rich medium (2% yeast extract Kat supplemented with either 2% glucose or 3% glycerol), ERY medium (1% yeast extract Difco, 2% bactopeptone, 2% glycerol, 3% ethanol, 25 mM sodium phosphate, pH 6.5 and 3 g/l erythromycin).

In vivo techniques

In all experiments, at least three independent transformants from each strain were analysed and they were successively refreshed on rich glycerol and minimal synthetic glucose media before use. The frequency of rho−/− mutants was estimated as follows. Cells were incubated on rich glucose medium for 1 day at 28°C (or 37°C), replica-plated on the same medium the following day and incubated 1 day more at 28°C (or 37°C). Cells were spread for single colonies on synthetic glucose medium weakly measured in adenine (53), and after 3–4 days at 28°C, the rho−/− colonies, which were identified by their small size and white colour, and the pink rho+ colonies were counted. The experiment was repeated four times and at least 30 000 colonies were counted for each mutant. The fraction of rho−/− clones was determined as reported in Supplementary Material, Figure S2I. Oxygen consumption was determined with a Clark electrode on cell suspensions of three independent transformants grown for 16 h in a liquid glucose-rich medium.

MtDNA mutation frequency was determined as follows. Thirty colonies from three independent transformants were grown in liquid glycerol-rich medium for 48 h and spread on an ERY medium. After 8 days at 28°C, the number of EK colonies was determined. Aliquots of each culture were spread on a synthetic glucose medium to determine the fraction of rho−/− mutants. The frequency of EK mutants was normalized to wild-type frequency set to 1.

Preparation and expression level of Mip1 protein

Soluble mitochondrial extracts from strain A25 were obtained after a 15 h MIP1 induction at 28°C in a synthetic galactose medium and Mip1 protein was partially purified through DEAE chromatography as previously reported (17) (Supplementary Material, Materials and Methods). To compare the relative wild-type and mutant Mip1 levels, increasing amounts of protein were loaded on 7% polyacrylamide–SDS gels and the signals were detected by western blotting using a polyclonal antibody raised against Mip1 (Supplementary Material, Materials and Methods) or by colloidal Coomassie blue staining. Mip1 protein levels were determined using Kodak Image Station 4000 R software (Supplementary Material, Fig. S3). The concentration (ng/µl) of reference wild-type Mip1 was estimated after SDS–PAGE and colloidal blue staining of the gel using known amounts of bovine serum albumin as a reference.

DNA polymerase assays

To determine gap-filling activity the reaction medium (100 µl) contained 20 mM Tris–HCl pH 8.0, 2 mM DTT, ~200 µg/ml DNase I-activated calf-thymus DNA as the substrate, 25 µM of each dATP, dGTP, dCTP and 5 µM [3H]dTTTP (3000 c.p.m./pmol). The reactions performed at 28°C were initiated by adding equal amounts of wild-type and mutant Mip1 and aliquots were removed after 4 and 8 min. The radioactivity present in the acid-insoluble material was counted in a liquid scintillation counter. Values were expressed as a percentage of the wild-type Mip1 activity (100%).

Total DNA synthesis and processivity were determined on ssDNA primed with the 17mer AB353 oligonucleotide (16). An excess DNA was used to prevent as much as possible the re-association of the polymerase with the same DNA molecule after a dissociation event. For total DNA synthesis, the reaction mixture contained 20 mM Tris–HCl pH 7.5, 5 mM DTT, 20 mM MgCl2, 25 µM of each dGTP, dCTP and dTTP, 5 µM [α-32P]dATP (3000 c.p.m./pmol) and ~10 µM M13 DNA primed with the AB353 oligonucleotide. Reactions were stopped and the products were analysed by electrophoresis in 15% polyacrylamide/7 M urea gel as previously reported (16). At this high acrylamide concentration and after an 8 cm Bromophenol blue migration, DNA was visualized as a single band whose intensity can easily be quantified with a PhosphorImager. For processivity analysis, the mixture contained 20 mM Tris–HCl pH 7.5, 5 mM DTT, 20 mM MgCl2, 25 µM of each dGTP, dCTP and dTTP, 5 µM [α-32P]dATP (3000 c.p.m./pmol) and ~10 µM M13 DNA primed with a 5′ end [32P]-labelled AB353 oligonucleotide as the substrate. The reactions performed at 28°C were initiated by adding equal amounts of wild-type and mutant Mip1. Reactions were quenched and samples were electrophoresed in 1% alkaline agarose gel as previously reported (16).

3′–5′ exonuclease and misincorporation assays

3′–5′ exonuclease activity was determined using PTZU18 DNA primed with a 5′ end [32P]-labelled oligonucleotide which was either complementary to PTZ18U (AB353) or contained a single A:A mispairing at its 3′ end (AB354) (16). The assay mixture contained 20 mM Tris–HCl pH 7.5, 5 mM DTT, ~10 µM DNA substrate and unless otherwise indicated 50 mM MgCl2. The reactions initiated by adding equal amounts of wild-type and mutant Mip1 were quenched after 2, 4, 8 and 12 min at 25°C and the samples were electrophoresed in 15% polyacrylamide/7 M urea gels and processed as previously reported (16). The degradation products were visualized and quantified using a PhosphorImager. The intensity (I) of each band (I16 for the 16mer, I15 for the 15mer, I14 for the 14mer and I13 for the 13mer) was determined and the total
degradation was calculated using the following algorithm: \( I_{16} \times 1 + I_{15} \times 2 + I_{14} \times 3 + I_{13} \times 4 + \ldots + I_{1} \times n \), where \( n = 1, 2, 3 \) and 4 are the number of excision events giving rise to the 16, 15, 14 and 13 mers, respectively. Activities were expressed as a percentage of the wild-type activity (100%). The misincorporation assay was performed as above using the AB353 oligonucleotide, 10 mM MgCl₂ and increasing dGTP concentrations with a 3 min incubation time.

**Gel EMSA**

DNA-binding affinity of wild-type and mutant Mip1 was determined by gel EMSA. The substrate was a \([^{32}P]\)-labelled 21/45mer primer template (Supplementary data) obtained as reported by Luo and Kaguni (54). The binding mixture (9 \( \mu \)l) contained 10 mM Tris–HCl pH 8.0, 5 mM DTT, 90 mM NaCl, 0.05 pmol primer-template DNA and when indicated 3 mM MgCl₂. The assay performed at 28°C was initiated by adding 3 \( \mu \)l (~6 ng) of wild-type or mutant Mip1 (diluted 25 times in a buffer containing 10 mM Tris–HCl pH 8.0 and 5 mM DTT). After 2 or 3 min, 3 \( \mu \)l of loading buffer (0.1% Bromophenol blue, 50% glycerol) was added to the samples which were placed on ice and immediately electrophoresed at 4°C in a 6% native polyacrylamide (1:37.5) gel (20 × 30 × 0.1 cm) in 45 mM Tris-borate pH 8.3 and 1 mM EDTA. The products of the dried gel were visualized and quantified with a PhosphorImager.

**Polymerase/3′–5′ exonuclease-coupled assay**

The reaction mixture contained 20 mM Tris–HCl pH 8.0, 5 mM DTT, 20 mM MgCl₂, ~10 \( \mu \)M PTZ18U ssDNA primed with a 5′ end \([^{32}P]\)-labelled AB353 oligonucleotide and increasing dNTP concentrations. All assay and electrophoresis conditions were those of the 3′–5′ exonuclease assay. Samples were electrophoresed in two independent gels to observe the polymerization mode after an 8 cm migration of Bromophenol blue and the exonuclease mode after a 28 cm migration of the dye. The products of the reactions were visualized with a PhosphorImager.

**Presentation of the structural data**

The bacteriophage T7 DNA polymerase (pdb ID: 18TE) and pol g (pdb ID: 3IKM) structures were superimposed by using the UCSF Chimera software. Closest neighbours from each residue of the 3′–5′ exonuclease module (residues 295–316) were identified using Swiss-Pdb Viewer and UCSF Chimera softwares. The Swiss-Pdb Viewer software was used to estimate the average distance of Leu304, Arg309 and Trp312 from the polymerase and exonuclease active sites. The calculated distance corresponds to the average distance of Leu304, Arg309 and Trp312 from each of Asp890, Asp1135 and Glu1136 (polymerase) and Asp198, Glu200, Asp274 and Asp399 (3′–5′ exonuclease) residues.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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