Mitochondrial DNA integrity is not dependent on DNA polymerase-β activity

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Abstract

Mutations in mitochondrial DNA (mtDNA) are involved in a variety of pathologies, including cancer and neurodegenerative diseases, as well as in aging. mtDNA mutations result predominantly from damage by reactive oxygen species (ROS) that is not repaired prior to replication. Repair of ROS-damaged bases occurs mainly via base excision repair (BER) in mitochondria and nuclei. In nuclear BER, the two penultimate steps are carried out by DNA polymerase-β (Polβ), which exhibits both 5′-deoxyribose-5-phosphate (5′-dRP) lyase and DNA polymerase activities. In mitochondria, DNA polymerase-β (Polβ) is believed to be the sole polymerase and is therefore assumed to function in mitochondrial BER. However, a recent report suggested the presence of Polβ or a “Polβ-like” enzyme in bovine mitochondria. Consequently, in the present work, we tested the hypothesis that Polβ is present and functions in mammalian mitochondria. Initially we identified two DNA polymerase activities, one corresponding to Polβ and the other to Polγ, in mitochondrial preparations obtained by differential centrifugation and discontinuous sucrose density gradient centrifugation. However, upon further fractionation in linear Percoll gradients, we were able to separate Polγ from mitochondria and to show that intact mitochondria, identified by electron microscopy, lacked Polγ activity. In a functional test for the presence of Polβ function in mitochondria, we used a new assay for detection of random (i.e., non-clonal) mutations in single mtDNA molecules. We did not detect enhanced mutation frequency in mtDNA from Polβ null cells. In contrast, mtDNA from cells harboring mutations in the Polγ exoribonuclease domain that abolish proofreading displayed a ≥17-fold increase in mutation frequency. We conclude that Polβ is not an essential component of the machinery that maintains mtDNA integrity.

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Keywords: Polβ; Polγ; Mitochondrial DNA; Mitochondria; Base excision repair; Single molecule DNA sequencing

1. Introduction

Each human cell contains, on average, a few thousand copies of the mitochondrial genome. Replication of the 16–17 kb circular mitochondrial DNA (mtDNA) is independent of the cell cycle and occurs in dividing as well as non-dividing cells. In liver and brain, tissues consisting of predominantly non-dividing cells, the half-life of mtDNA is approximately 2–4 weeks. It has been estimated that, during a human life-span, mtDNA in non-dividing tissues undergoes a thousand times more replicative cycles than does nuclear DNA [1]. As a consequence, damage to mtDNA, if not repaired prior to DNA replication, is more likely to result in mutations. In fact, mitochondrial mutations have been demonstrated in a variety of mitochondrial dysfunction diseases [2].

Abbreviations: Ap site, abasic site; BER, base excision repair; 5′-dRP, 5′-deoxyribose-5-phosphate; mtDNA, mitochondrial DNA; Polβ, DNA polymerase-β; Polγ, DNA polymerase-γ; ROS, reactive oxygen species; RT, reverse transcriptase; SMS, single molecule sequencing

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Mitochondrial BER is similar to short patch BER in nuclei. All the BER proteins are encoded in the nucleus, most of them as an alternative DNA polymerase activity [15,16]. Briefly, cells were homogenized in swelling buffer (10 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol) with a Dounce homogenizer, followed by the addition of 2.5 × MSH to a final concentration of 1 × (220 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA) to stabilize mitochondria. Unbroken cells and nuclei were removed by two centrifugations at 1000 × g for 10 min each. A crude mitochondrial pellet was obtained from the supernatant by centrifugation at 10,000 × g for 30 min. The pellet was resuspended and spun down again at 10,000 × g for 30 min.

Mitochondria isolated from both liver and HeLa cells were further enriched by centrifugation in a discontinuous 1/1.5 M sucrose gradient in 5-ml tubes in a Sorvall SW 50.1 rotor at 92,000 × g for 60 min. Mitochondria, located at the interface of the sucrose layers, were lysed by homogenization in 0.05 M potassium phosphate buffer, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5% Nonidet P-40, and 0.1 M NaCl supplemented with protease inhibitors [22]. Alternatively, for gel electrophoresis, mitochondria were lysed in loading buffer as described below.

### 2.4. Phosphocellulose chromatography of a lysate of fractionated HeLa cell mitochondria

Phosphocellulose chromatography was carried out as previously described [23]. Briefly, a lysate (10 ml) of mitochondria was loaded onto a phosphocellulose column (Whatman P-11, 1 × 15 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, and eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The active fractions were pooled and concentrated by vacuum dialysis.

### 2.5. Phosphocellulose chromatography of a lysate of unfractionated HeLa cell mitochondria

Phosphocellulose chromatography was carried out with unfractionated HeLa cell mitochondria as described above.

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**Materials and methods**

### 2.1. Cell culture

**MB16tsA wild-type (CRL-2307) and MB19tsA Polβ−/− (CRL-2308) mouse embryonic fibroblast cell lines were purchased from the American Type Culture Collection (ATCC).** MEF-50 wild-type and MEF-38 Poly-eso−/− homozygous (Poly D257A/D257A) primary mouse embryonic fibroblast lines [20] were grown in Dulbecco’s Modified Eagle’s Medium with high glucose in the presence of 10% fetal calf serum, penicillin (100 unit/ml), and streptomycin (100 μg/ml). HeLa cells were purchased from the National Cell Culture Center.

**Mice**

Mice were maintained according to protocols approved by the University of Washington Animal Care Committee. Mice were sacrificed in a carbon dioxide atmosphere, decapitated, and livers were harvested.

### 2.3. Preparation of mitochondria by discontinuous sucrose density gradient centrifugation

Mitochondria isolated from both liver and HeLa cells were isolated by differential centrifugation based on the protocol published by Fernandez-Vizarra et al. that employs several low- and high-speed centrifugation steps [21]. Mitochondria from 5 × 10⁶ HeLa cells were also isolated by differential centrifugation [22]. Briefly, cells were homogenized in swelling buffer (10 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol) with a Dounce homogenizer, followed by the addition of 2.5 × MSH to a final concentration of 1 × (220 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA) to stabilize mitochondria. Unbroken cells and nuclei were removed by two centrifugations at 1000 × g for 10 min each. A crude mitochondrial pellet was obtained from the supernatant by centrifugation at 10,000 × g for 30 min. The pellet was resuspended and spun down again at 10,000 × g for 30 min.

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Mitochondria from both liver and HeLa cells were isolated by differential centrifugation as previously described [23]. Briefly, a lysate (10 ml) of mitochondrial DNA, like nuclear DNA, is continuously exposed to exogenous and endogenous damaging agents [3]. However, the rate of nucleotide sequence changes in mammalian mitochondrial DNA is 10-fold greater than that of nuclear DNA [4]. This elevation in mutation frequency is likely a result of the proximity of mitochondrial DNA to the reactive oxygen species (ROS) generated during oxidative phosphorylation, and the lack of protection of mitochondrial DNA by histones [5].

In contrast to the relatively well-known mechanisms of lesion repair in nuclear DNA, repair processes in mitochondria are less understood. Until recently, it was believed that mitochondria do not repair their DNA [6]. However, increasing evidence suggests that, although there is no removal of bulky adducts by nucleotide excision repair, small adducts are efficiently removed by short patch base excision repair (BER) [7]. Other evidence also suggests the presence of mismatch repair and recombination [8,9] in mitochondria, but the contribution of these mechanisms to mitochondrial DNA repair needs to be established.

Damage in cells and in mitochondria results predominantly from ROS, and repair of ROS-damaged bases in DNA occurs mainly via BER [10,11]. Mitochondrial BER occurs similarly to short patch BER in nuclei. All the BER proteins are encoded in the nucleus, most of them as an alternatively spliced form of the nuclear enzyme, and are selectively imported to the mitochondria [12]. During BER the damaged base is removed by a DNA glycosylase leaving an abasic [apurinic/apyrimidinic (Ap) base] site [13]. The Ap site is then cleaved by an Ap endonuclease to create 5′-OH and 5′-deoxyribose-5-phosphate (dRP) termini. Ap site cleavage is followed by excision of the dRP by dRP lyase activity, gap filling by a DNA polymerase, and ligation of the free ends [14]. In nuclear BER, the two penultimate steps are carried out by DNA polymerase-β (Polβ), which exhibits both 5′-dRP lyase and DNA polymerase activities [15,16].

DNA polymerase-γ (Polγ) is currently believed to be the only DNA polymerase in mammalian mitochondria, and to be solely responsible for both replication and repair of mtDNA. However, while Polγ exhibits dRP lyase activity in vitro, an essential function for mitochondrial BER, the catalytic efficiency is very low [17]. Therefore, another enzyme in mitochondria with stronger dRP lyase activity could be required for BER. Noting its 5′-dRP lyase activity in the nucleus, Polβ might be considered a suitable candidate for such a mitochondrial dRP lyase, and indeed, a second DNA polymerase, a homolog of Polβ, has been identified in trypanosome mitochondria [18]. In addition, a detailed study suggested that there is a “Polβ-like” enzyme expressed in bovine heart mitochondria [19]. This polymerase was purified from isolated mitochondria, and, like Polβ, it was characterized as a 38 ± 2 kDa enzyme with low processivity and no 3′ → 5′ exonuclease activity [19].

In light of these observations and the proximity of mitochondrial DNA to ROS, as well as the involvement of Polβ in BER repair of nuclear DNA, we asked if Polβ is involved in BER in mammalian mitochondria.
DNA purified as described above was loaded onto a 7 ml phosphocellulose column (P-11, Whatman) (~5.2 cm x 1.3 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.05 M NaCl. The column was washed with three column volumes of buffer, and bound proteins were eluted with four column volumes of a linear gradient of 0.05-0.25 M potassium phosphate, pH 7.5 in the same buffer. Fractions (0.75 ml) were collected and were either assayed immediately for DNA polymerase and reverse transcriptase (RT) activities or stored at −80 °C.

2.5. Further purification of mitochondria in a continuous Percoll gradient

Mitochondria prepared by differential centrifugation and subsequent discontinuous sucrose gradient centrifugation were further purified in a continuous linear gradient of Percoll (Amersham) or Nycodenz (Histodenz; Sigma), as described previously [24]. Briefly, mitochondrial pellets were resuspended in 0.5 ml 10% Percoll/0.25 M sucrose and layered onto 5.5 ml 35% Percoll/0.25 M sucrose in a 6-ml tube. Percoll gradients were formed in situ by centrifuging at 20,000 g for 60 min in a Sorvall SW41 Ti rotor at 4 °C. The Nycodenz gradient, the mitochondrial pellet was resuspended in 0.5 ml 0.25 M sucrose and layered onto a 12.5 ml linear 15–35% Nycodenz gradient and centrifuged for 60 min in a Sorvall 70V6 vertical rotor at 4 °C. Fractions of 0.25 ml were collected and either assayed immediately for DNA polymerase and RT activities or stored at −80 °C.

2.6. DNA polymerase and reverse transcriptase assays

DNA polymerase and RT activities were measured as previously described [25]. Briefly, reactions were carried out at 37 °C for 15–60 min in 10 μl mixtures. For DNA polymerase activity the reaction contained: 1 μg of activated calf thymus DNA, 50 μM each dNTP, 1 μM α32P-dTTP (3000 Ci/mmole) (NEN), and 2 μl of fractionated protein in 50 mM Tris-HCl, pH 8.3, 10 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol, and 500 μM spermidine. For RT activity, the reaction contained 0.2 μg poly(A) poly(dT)12-18 (Amersham), 10 μM dTTP, 1 μM α32P-dTTP (3000 Ci/mmole) (NEN), and 2 μl of fractionated protein in 50 mM Tris-HCl (pH 8.3), 10 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol, 500 μM spermidine, and 0.5 mM MnCl2. For filter binding assays [25], reactions were terminated by addition of 0.1 M sodium pyrophosphate and 0.05 M EDTA, and radioactive products were collected by filtration in a 96 microwell plate (Biodyne B, NUNC) mounted on a vacuum manifold (Beckman). Radioactivity associated with the filter was quantified by phosphorimager analysis using ImageQuant® software (Molecular Dynamics). For electrophoretic analysis of labeled products in the RT assay, reactions were terminated by addition of 20 μl 98% formamide and 5 μl was analyzed by electrophoresis through 14% denaturing polyacrylamide gels. Extension was quantified by phosphorimager analysis.

2.7. Western blots and activity gel assays

For Western analysis, proteins were resolved by electrophoresis through 10% SDS-polyacrylamide gels, and visualized by immunoblotting using the anti-Polβ monoclonal antibody V10165 (Biomedia). For activity gel analysis, proteins were resolved in 10% SDS-polyacrylamide gels that contained 0.1 mg/ml activated salmon sperm DNA and 0.1 mg/ml fibrinogen in the matrix [26]. Samples were heated for 5 min at 60 °C in loading buffer containing 10% SDS and no reducing agent. After electrophoresis, SDS was extracted from the gel by two washes (15 min each) with 20% isopropanol followed by two washes (15 min each) with 10 mM Tris–HCl, pH 7.5. DNA polymerase activity was detected by incubation for 3–16 h in reaction buffer (50 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 25 mM KCl, 15% glycerol, 400 μg/ml BSA, 5 mM dithiothreitol, 7.5 mM MgCl2, 50 μM each dATP, dCTP and dGTP, and 2 μl α32P-dTTP (3000 Ci/mmole)), followed by 10 washes (10 min each) with 0.1 M sodium pyrophosphate containing 10% TCA. Radioactive bands were visualized by phosphorimager analysis using ImageQuant® software (Molecular Dynamics).

2.8. DNA sequencing

For conventional sequencing, total cellular DNA was prepared from mouse embryonic fibroblasts using a DNeasy isolation kit (Qiagen). A 1300-bp mtDNA fragment including nucleotides 14,967–16,267 was produced by PCR amplification using an Expand High Fidelity PCR System (Roche) or Pfu DNA polymerase (Stratagene) and the primers 5′- CCGTTTTTGATACGCCATGC-3′ and 5′-GAGTTTTGGTTCACGGAAC-3′. The DNA product was cloned into the vector pCR 2.1-TOPO (Invitrogen) and plasmids isolated from independent clones were sequenced. For single molecule sequencing (SMS), DNA was isolated from mouse embryonic fibroblasts using a Wako DNA Isolation Kit (Wako), and 50 μg was digested with SacI, EcoRI, and KpnI. The digested DNA was fractionated by centrifugation in a 5-ml 10–40% sucrose gradient in a SW 50.1 rotor for 16 h at 150,000 × g. Fractions were collected and DNA fragment size was determined by agarose gel electrophoresis. Fractions containing fragments of 4 kb or longer were pooled. Amplification of single molecules was achieved by using serial 10-fold dilutions, ranging from 10 × 10−4 to 10 × 10−8, and two sequential PCR reactions. Diluted DNA was used as a template for the initial amplification of a mtDNA fragment including nucleotides 14,527–16,292; primers were 5′-TCTATTTTGCATACGCCATTCTAC and 5′- GATTTGTTTGCACCGGAAACATG. The DNA product was cloned into the vector pCR 2.1-TOPO (Invitrogen) and plasmids isolated from independent clones were sequenced.


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Nested PCR was then carried out in reaction mixtures (50 μl) containing 1 μl of the first PCR mixture as a template for amplification of nucleotides 14,530–16,266; primers were 5′-ATAGGCTACGTCCTTCCA TGAGGACA and 5′-GA- GTTTTGTTTCAGGAACATG. PCR products were visualized on a 1% agarose gel. Amplification of single molecules is indicated for any particular DNA dilution when less than 12% of reactions contain visible product. PCR products were purified using a Montage PCR96 Cleanup Plate kit (Millipore) and sequenced directly. Sequences were analyzed using the program Sequencher 4.2. Only those nucleotide substitutions that occurred in both strands of the template DNA were scored as mutations; nucleotide changes observed in only one strand were considered to be potential PCR artifacts and were not scored. By restricting the designation of mutations to nucleotide substitutions documented in both template strands, single-molecule analysis results in virtual elimination of the contribution of PCR errors to mutation frequency. In contrast, the more conventional amplification of bulk DNA, followed by cloning and sequencing does not eliminate the contribution of errors in DNA synthesis due to PCR amplification.

2.9. Electron microscopy

Electron microscopy was performed by the Electron Microscopy Laboratory, Department of Anatomic Pathology, University of Washington Medical Center. After fixation in one-half strength Karnovsky’s fixative, tissues were rinsed twice in 0.1 M phosphate buffer, followed by post-fixation incubation in 1% osmium tetroxide in 0.1 M phosphate buffer for 1.5 h. Tissue was then rinsed in buffer and dehydrated for 10 min each in a series of ethanol solutions of increasing concentration: 50, 70, 90, and 100%. Tissues were placed in propylene oxide for two washes of 10 min each and infiltrated with a 1:1 mixture of propylene oxide and resin for 1.5 h. The resin mixture was replaced with 100% resin, which was in turn replaced with fresh 100% resin after 2 h, and specimens were polymerized overnight in a 60°C oven. Sections (0.1 μm) were then cut with an Ultracut E ultramicrotome, placed on a grid, and stained with uranyl acetate and lead citrate. Specimens were viewed with a Philips 410 electron microscope.

3. Results

3.1. Two DNA polymerase activities associated with mitochondria prepared by discontinuous sucrose density gradient centrifugation

To identify DNA polymerases that participate in maintaining mtDNA integrity, we isolated mitochondria from HeLa cells using differential centrifugation followed by purification on a discontinuous sucrose density gradient. Proteins (Promega), and 0.15 U Pfu Turbo (Stratagene).
associated with the lysed mitochondria were resolved by phosphocellulose chromatography and assayed for DNA polymerase activity using a filter-binding assay. As shown in Fig. 1, two major regions of activity were identified, the first a bifurcated peak in fractions 6–12 and the second a peak centered in fractions 14–18. To determine if these fractions contained Poly, we took advantage of the ability of Poly to synthesize DNA using an RNA template (RT activity). Only low levels of RT activity were observed in the first peak (fractions 6–12). RT activity was highest in fractions 14–18 and eluted with the second polymerase activity peak.

3.2. DNA polymerase-β is associated with mitochondria prepared by discontinuous sucrose density gradient centrifugation

In order to identify the polymerases associated with the mitochondrial fraction, we utilized an “in gel” activity assay with which one can correlate polymerase activity with the molecular weight of the catalytically active polypeptide. Lysates of both mouse liver (Fig. 2) and HeLa cell (data not shown) mitochondria were assayed. One band corresponded to activity at ∼38 kDa observed in the cytoplasm, and the other to a more slowly migrating activity with a molecular weight greater than or equal to 120 kDa, presumptively corresponding to Poly (140 kDa) (Fig. 2A) [27].

The only known eukaryotic DNA polymerase of size similar to that of the low molecular weight activity is Polβ (39 kDa) [28]. Both western blots using anti-Polβ antibodies (Fig. 2B) and activity gels (data not shown) indicated that this activity is in fact Polβ. Comparable results were obtained for a lysate of HeLa cell mitochondria, such as that used for the phosphocellulose chromatography in Fig. 1 (data not shown). Moreover, the band corresponding to Polβ was not detected in lysates of mitochondria prepared from Polβ null mouse embryonic fibroblasts by sucrose gradient centrifugation (data not shown). Taken together, these results establish that Polβ is associated with our preparation of sucrose density gradient-purified mitochondria.

3.3. Separation of Polβ from mitochondria in a continuous Percoll gradient

We next carried out a more extensive purification of mitochondria on a continuous Percoll gradient. Mouse liver mitochondria were prepared by differential centrifugation followed by discontinuous sucrose gradient centrifugation, as before. Mitochondria were then further purified by centrifugation in a continuous 35% Percoll gradient, and 24 fractions were collected. Two bands were visible in the gradient, corresponding to fractions 4–5 and 14–15, respectively, counting from the bottom of the tube (Fig. 3B). Visualization of components in fractions 4 and 14 by electron microscopy revealed that fraction 4 is composed of mitochondria (Fig. 3A). In contrast, fraction 14 is composed mainly of polysomes, microsomes, and liposomes (Fig. 3C). The gradient was analyzed for Polβ using DNA polymerase activity gels (Fig. 4B) and for Poly reverse transcriptase activity using denaturing gel electrophoresis for product analysis (Fig. 4C). The results showed that Poly sediments with the more dense band in fractions 2–7, while Polβ sediments with the less dense band in fractions 12–18. A different centrifugation procedure using a continuous, 15–35% Nycodenz gradient to further purify mouse liver mitochondria obtained in a sucrose gradient likewise showed that Polβ activity does not co-sediment with Poly activity (data not shown).

3.4. Mutation frequency in mtDNA from Polβ null and wild-type cells

Figs. 1–3 illustrate an association of Polβ with partially purified mitochondria, consistent with reports in the liter-
Fig. 3. Electron microscopy of Percoll gradient fractions. The two visible bands in the Percoll gradient of Fig. 4, corresponding to fractions 4 and 14, were collected and examined by electron microscopy. (A) Fraction 4 is composed of mitochondria. (B) Schematic illustration of banding in the Percoll gradient. (C) Fraction 14 is composed mostly of polysomes, microsomes, and liposomes.

Fig. 4. Separation of Polβ and Poly activities in a continuous Percoll gradient. Mouse liver mitochondria isolated by differential centrifugation and fractionated on a discontinuous sucrose gradient were further purified by centrifugation in a 35% continuous Percoll gradient. (A) Schematic illustration of banding in the Percoll gradient. Twenty-four fractions were collected from the bottom of the tube. (B) DNA polymerase activity gel assay for Polβ. (C) Reverse transcriptase assay for Poly. Radiolabeled products formed on a poly(A) poly(dA)12-18 template-primer were analyzed in a denaturing gel. Radioactivity at the top of the gel represents extensive synthesis by Poly (fractions 2–7). Radioactivity in the center of the gel reflects minimal incorporation, probably by Polβ (fractions 11–21).
was isolated from wild-type and Pol H9252 [19], while Fig. 4 shows that additional purification

<table>
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<th>No. mutations</th>
<th>WT</th>
<th>PolH9252</th>
<th>WT</th>
<th>PolH9252</th>
<th>WT</th>
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<tr>
<td>Deletions</td>
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Table 1

<table>
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<tr>
<th>Mutation frequency and spectrum in mtDNA from mouse embryonic fibroblasts a</th>
<th>PCR and cloning</th>
<th>Single molecule sequencing</th>
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<tbody>
<tr>
<td>WT</td>
<td>PolH9252</td>
<td>WT</td>
</tr>
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<td>69,500 bp for each genotype revealed a similar mutation frequency of 32 × 10⁻⁵</td>
<td>60,250 bp</td>
<td>48,000 bp</td>
</tr>
<tr>
<td>4,000 mutations</td>
<td>60,250 bp</td>
<td>50,000 bp</td>
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<tr>
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<td>57,800 bp</td>
<td>57,800 bp</td>
</tr>
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</table>

a Mutation data were obtained by two methods: PCR, followed by cloning and sequencing (left), and by the new SMS method (right).

b MB16tsA (WT) is paired with PolH9252-deficient MB16tsA PolH9252- (PolH9253-). See Section 2.

4. Discussion

The integrity of mitochondrial and nuclear genomes is dependent on avoidance of mutations. DNA lesions, if not repaired prior to replication, will result in misincorporation during copying by DNA polymerases. Considering the exposure of mtDNA to ROS and the more frequent DNA damage in mitochondria, it is surprising that DNA repair is not more extensive in these organelles. Yet, early findings that UV-induced pyrimidine dimers are not repaired in mitochondria led to the general notion that there is no mtDNA repair at all [6]. Indeed, recent studies have confirmed that pyrimidine dimers and other bulky lesions that are removed from nuclear DNA by nucleotide excision repair are not repaired in mtDNA. However, other lesions, including the most abundant base adducts, i.e., those resulting from ROS, are repaired by BER in mitochondria. Although some of the proteins that participate in mitochondrial BER have not been identified yet, it seems likely that the pathway is very similar to short
patch BER in nuclei. The mitochondrial DNA polymerase, Polγ, has been shown to exhibit dRP lyase in vitro, an activity necessary for BER, but this activity is much weaker than that of Polβ [17,29]. It seems unlikely that the inefficient Polγ dRP lyase would be capable of dealing with the high rates of damage in mtDNA. Thus, it could be argued that Polβ, an enzyme responsible for DNA synthesis during BER in nuclei, could also be involved in DNA synthesis in mtDNA repair. It has been suggested that, as in trypanosomid mitochondria, mammalian mitochondria contain another, Polγ or “Polβ-like” polymerase.

Using two fractionation steps, we indeed identified Polβ preparations of isolated mitochondria. Lysis of the mitochondria yielded two DNA polymerase activities, one corresponding to Polβ and the other to Polγ. The identification of Polβ in association with mitochondria was further supported by the absence of this activity in Polβ null cells. However, upon further fractionation in continuous Percoll or Nycodenz gradients, we were able to separate Polγ from mitochondria. Intact mitochondria, as determined by electron microscopy, lacked Polβ activity.

In a functional test for the presence of Polβ in mitochondria, we analyzed the frequencies of mitochondrial mutational events in wild type and cells genetically deficient in Polβ. PCR amplification of a mtDNA fragment that included the highly variable region containing the D-loop yielded a mutation frequency of one in 3000 in mtDNA from both wild type and Polβ null cells. Since the most frequent substitutions were AT → GC transitions, the errors could be artifacts and the result of misincorporation by DNA polymerase during PCR. Analysis of mtDNA by single molecule DNA sequencing yielded a much lower mutation frequency, one in 20,000 in mtDNA from both wild-type and Polβ null cells. Thus, even though only a small amount of Polβ might be required for mitochondrial BER, its absence did not result in detectably altered mitochondrial mutagenesis.

In contrast to the lack of dependence of mitochondrial mutational frequency on the presence of Polβ, a genetic defect in Polγ that abolishes 3′ → 5′ proofreading activity resulted in a greater than 17-fold increase in mutation frequency, as assessed by SMS. The enhancement of mutations in mtDNA from cells containing Polγ-exo− is in accord with previous publications [30] and supports the validity of our new SMS method for measuring mutations in mtDNA. The mutations scored in single DNA molecules from Polγ-exo− cells were predominantly single base substitutions, in accord with the spectrum of mutations catalyzed by Polγ-exo− in vitro [31]. The mutations were different in each molecule that was sequenced, indicating that they were random and not clonal.

Our results indicate that Polγ is not an essential component of the machinery that maintains mtDNA integrity. It is absent from our most highly purified fractions of mitochondria and the mtDNA mutation frequency is not altered in cells lacking Polγ. Our data are in accord with the observation that cells deficient in Polγ exhibit only minor sensitivity to agents that enhance the production of ROS [32]. Nevertheless, our findings do not eliminate a possible role for other DNA polymerases in mitochondrial DNA repair.

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