Available online at www.sciencedirect.com
SCIENCE DIRECT
DNA Repair 5 (2006) 71–79
DNA REPAIR
www.elsevier.com/locate/dnarepair

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

Alexis B. Hansen [a], Nicholas B. Griner [a], Jon P. Anderson [a,1], Greg C. Kujoth [b], Tomas A. Prolla [b], Lawrence A. Loeb [a,*], Eitan Glick [a]

[a] *The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, Washington 98195-357705, USA*
[b] *Departments of Genetics & Medical Genetics, University of Wisconsin, Madison, WI 53706, USA*

Received 17 June 2005; received in revised form 21 July 2005; accepted 25 July 2005
Available online 13 September 2005

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-γ (Polγ) 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 Polγ and the other to Polβ, 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 Polβ from mitochondria and to show that intact mitochondria, identified by electron microscopy, lacked Polβ 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 exonuclease 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.

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

* Corresponding author. Tel.: +1 206 543 9360; fax: +1 206 543 3967.
E-mail address: laloeb@u.washington.edu (L.A. Loeb).
[1] Present address: LI-COR, Inc., 4308 Progressive Avenue, P.O. Box 4000, Lincoln, NE 68504, USA.

1568-7864/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.dnarep.2005.07.009

mtDNA, like nuclear DNA, is continuously exposed to exogenous and endogenous damaging agents [3]. However, the rate of nucleotide sequence changes in mammalian mtDNA is 10-fold greater than that of nuclear DNA [4]. This elevation in mutation frequency is likely a result of the proximity of mtDNA to the reactive oxygen species (ROS) generated during oxidative phosphorylation, and the lack of protection of mtDNA 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 mtDNA 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)] site [13]. The Ap site is then cleaved by an Ap endonuclease to create 3′-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 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 $3' \rightarrow 5'$ exonuclease activity [19].

In light of these observations and the proximity of mtDNA 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.

2. 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 Polγ-exo$^-$ homozygous (Polγ D257A/D257A) primary mouse embryonic fibroblast cells [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.

2.2. 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

Mouse liver mitochondria 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 5×10^9 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 $MgCl_2$, 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 $1000 \times g$ for 10 min each. A crude mitochondrial pellet was obtained from the supernatant by centrifugation at $10{,}000 \times g$ for 30 min. The pellet was resuspended and spun down again at $10{,}000 \times g$ 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 $92{,}000 \times g$ 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 mitochon-

dria purified as described above was loaded onto a 7 ml phosphocellulose column (P-11, Whatman) (~5.2 cm × 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 50,000 × *g* for 60 min in a Sorvall 70V6 vertical rotor at 4 °C. For 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 at 140,000 × *g* for 120 min in a Sorvall SW41 Ti 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 μCi α^{32}P-dTTP (3000 Ci/mmole) (NEN), and 2 μl of fractionated protein in 50 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, and 500 μM spermidine. For RT activity, the reaction contained 0.2 μg poly(rA)·p(dT)$_{12–18}$ (Amersham), 10 μM dTTP, 1 μCi α^{32}P-dTTP (3000 Ci/mmole) (NEN), and 2 μl of fractionated protein in 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 500 μM spermidine, and 0.5 mM $MnCl_2$. 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 (Biomeda). 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 $MgCl_2$, 50 μM each dATP, dCTP and dGTP, and 2 μCi α^{32}P-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′-CCTATTTGCATACGCCATTCTAC and 5′-GAGTTTTGGTTCACGGAACATG. 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 *Sac*I, *Eco*RI, and *Kpn*I. 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^{-9} 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′-TTTATAGGCTACGTCCTTCCATGAGG and 5′-GTTTATTGCGTAATAGAGTATG. Reaction mixtures (10 μl) contained 50 mM Tris, pH 9.2, 16 mM $(NH_4)_2SO_4$, 1% Tween 20, 200 μM each dNTP, 2 mM $MgSO_4$, 200 nM of each primer, 0.075 U Taq polymerase

(Promega), and 0.15 U Pfu Turbo (Stratagene). 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′-ATAGGCTACGTCCTTCCATGAGGACA and 5′-GAGTTTTGGTTCACGGAACATG. 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

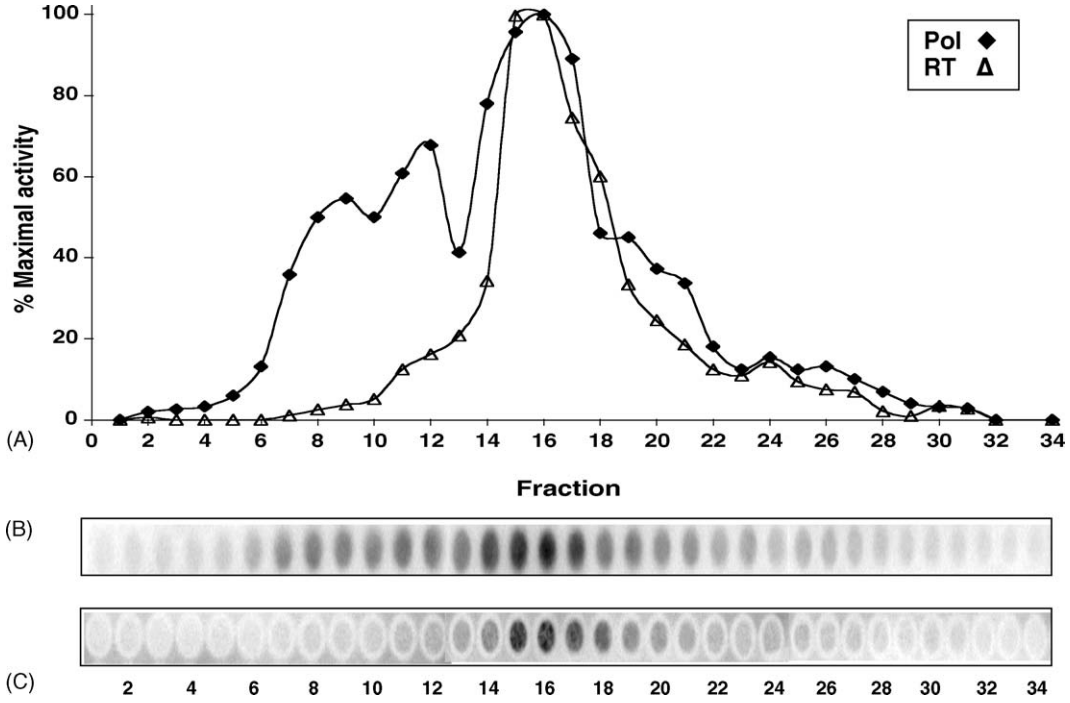

Fig. 1. DNA polymerase and reverse transcriptase activity in a HeLa cell mitochondrial lysate fractionated on phosphocellulose. Mitochondrial proteins were resolved by gradient elution from a phosphocellulose column as described in Section 2. (A) DNA polymerase (♦) and reverse transcriptase (RT) (△) activities were measured in a filter-binding assay employing gapped DNA or poly(dA)·oligo(dT) as template-primer, respectively. (B) Phosphorimager analysis of filter-bound radioactivity in DNA polymerase assay. (C) Phosphorimager analysis of filter-bound radioactivity in RT assay.

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 Polγ, we took advantage of the ability of Polγ 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 Polγ (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

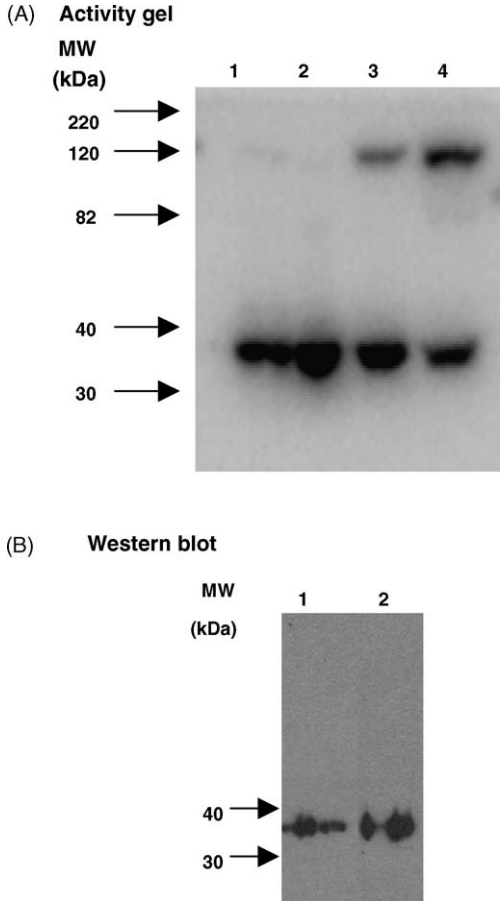

Fig. 2. Identification of Polβ in fractionated mitochondria by activity gel and Western analysis. Mitochondria were isolated from mouse liver by differential centrifugation and further purified by centrifugation in a 1.0 M/1.5 M discontinuous sucrose gradient. (A) DNA polymerase activity was detected in an "in gel" assay that permits correlation of activity with the molecular weight of the enzyme. Each lane of the 10% SDS-polyacrylamide activity gel contained 15 μg of protein, as follows: Lane 1, total cell extract; lane 2, cytoplasmic fraction; lane 3, mitochondria isolated by differential centrifugation; lane 4, sucrose gradient-purified mitochondria. (B) Western blot using monoclonal anti-Polβ antibodies. Lane 1, rat Polβ; lane 2, lysate of mouse liver mitochondria fractionated on a sucrose gradient.

for Polβ using DNA polymerase activity gels (Fig. 4B) and for Polγ reverse transcriptase activity using denaturing gel electrophoresis for product analysis (Fig. 4C). The results showed that Polγ 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 Polγ 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-

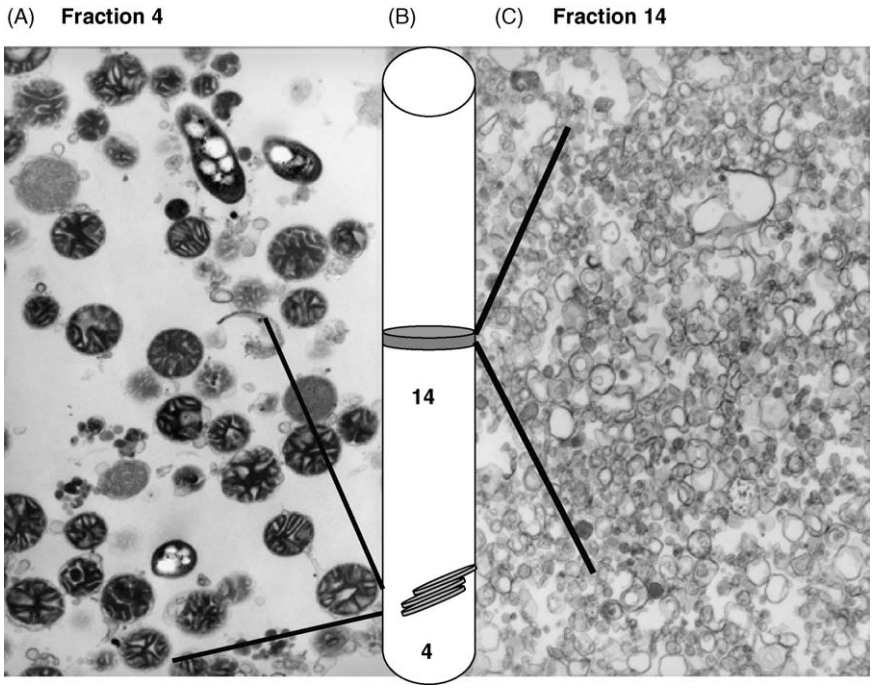

Fig. 3. Electron microscopy of Percoll gradient fractions. The two visible bands in the Percoll gradient of Fig. 4, corresponding to fractions 4 and 14, were collected and examined by electron microscopy. (A) Fraction 4 is composed of mitochondria. (B) Schematic illustration of banding in the Percoll gradient. (C) Fraction 14 is composed mostly of polysomes, microsomes, and liposomes.

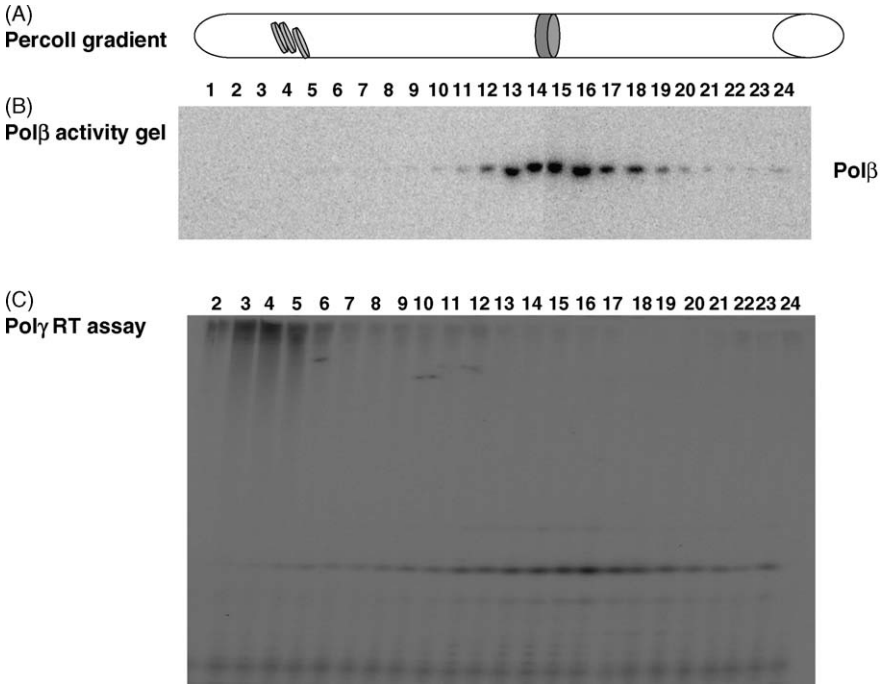

Fig. 4. Separation of Polβ and Polγ activities in a continuous Percoll gradient. Mouse liver mitochondria isolated by differential centrifugation and fractionated on a discontinuous sucrose gradient were further purified by centrifugation in a 35% continuous Percoll gradient. (A) Schematic illustration 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 Polγ. Radiolabeled products formed on a poly(rA)·p$(dT)_{12–18}$ template-primer were analyzed in a denaturing gel. Radioactivity at the top of the gel represents extensive synthesis by Polγ (fractions 2–7). Radioactivity in the center of the gel reflects minimal incorporation, probably by Polβ (fractions 11–21).

Table 1
Mutation frequency and spectrum in mtDNA from mouse embryonic fibroblasts[a]

	PCR and cloning		Single molecule sequencing			
	WT[b]	Polβ$^{-/-}$	WT[b]	Polβ$^{-/-}$	WT[c]	Polγ–exo^{-}
Nt's sequenced	69,500	60,250	48,000	50,000	54,500	57,800
No. mutations	22	12	3	2	1	20
Frequency ($\times 10^{-5}$)	32	20	6.3	4.0	1.8	35
AT → GC	16	9	2	2		5
GC → AT	6	2	1		1	6
AT → TA						4
TC → GA		1				4
Deletions						1

[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 Polβ-deficient MB19tsA Polβ^{-} (Polβ$^{-/-}$). See Section 2.
[c] MEF-50 (WT) is paired with proofreading-deficient MEF-38 (Polγ-exo^{-}) [20]. See Section 2.

ature [19], while Fig. 4 shows that additional purification resolves detectable Polβ from mitochondria and from the mitochondrial polymerase Polγ. These findings suggest that the observed association is adventitious. However, an undetectably small amount of Polβ might be sufficient to catalyze the limited amount of DNA synthesis required for mitochondrial BER. To pursue this possibility, we assayed for the potential function of Polβ in mitochondria in vivo. Specifically, we asked if the frequency of mutation differs in the mtDNA of wild-type and Polβ null cells. DNA was isolated from wild-type and Polβ$^{-/-}$ mouse embryonic fibroblasts and the mutation frequency in mtDNA was measured by two methods. In the first, a 1500-bp fragment that includes the variable D-loop region was PCR-amplified, cloned and sequenced. Sequencing of more than 60,000 bp for each genotype revealed a similar mutation frequency (i.e., 32×10^{-5} and 20×10^{-5} mutations/base for wild-type and Polβ$^{-/-}$cells, respectively (Table 1)). A portion of these mutations could be the result of misincorporation during the PCR reaction, and these errors could obscure an underlying difference in mutation frequency in the unamplified DNA. Therefore, we isolated single molecules of mtDNA, prior to amplification and sequencing, using a new procedure we call single molecule sequencing. SMS is based on serial dilution of the DNA template, such that only 1 in 10 replicate samples of the diluted DNA is amplified; in such circumstances, the probability is high that every PCR product represents amplification of a single DNA molecule. Single molecule sequencing of 50,000 bp of mtDNA from both wild-type and Polβ$^{-/-}$ cells revealed a comparable frequency of mutation (i.e., 6.3×10^{-5} and 4.0×10^{-5} mutations/base, respectively (Table 1)). These frequencies, which are not subject to PCR mutation artifacts, are about five-fold lower than those obtained by direct PCR-amplification and cloning. We conclude that there was no detectable difference in mtDNA mutation frequency between wild-type and Polβ-deficient cells. These results suggest that Polβ is not an essential component of mitochondrial BER.

3.5. Mutation frequency in mtDNA from Polγ-exo^{-} and wild-type cells

To verify that impaired polymerase activity in mitochondria would affect mtDNA mutation frequency, we sequenced single molecules of mtDNA obtained from wild type mouse embryo fibroblasts and from mouse embryo fibroblasts harboring two mutations in Polγ that inactivate the proof-reading exonuclease activity [20]. One single base substitution was detected in the 54,500 nucleotides sequenced in mtDNA from the wild-type cells. In contrast 20 single base substitution were detected amongst the 57,800 nucleotides sequenced in mtDNA from the Polγ-exo^{-} cells. The mutation frequencies of the wild-type and Polγ-exo^{-} mouse embryonic fibroblasts were $\leq 1.8 \times 10^{-5}$ mutations/base and 32×10^{-5} mutations/base, respectively (Table 1). Thus, in contrast to Polβ deficiency, impaired Polγ proofreading resulted in a $\geq$17-fold increase in mtDNA mutation frequency.

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, Polβ or "Polβ-like" polymerase.

Using two fractionation steps, we indeed identified Polβ in preparations of isolated mitochondria. Lysis of the mitochondria yielded two DNA polymerase activities, one corresponding to Polγ and the other to Polβ. 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 Polβ 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 mutations in wild type cells and in 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 artifactual 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 mutation frequency on the presence of Polβ, a genetic defect in Polγ that abolishes $3' \rightarrow 5'$ 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 Polβ 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 Polβ. Our data are in accord with the observation that cells deficient in Polβ 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.

Acknowledgements

We thank Dr. Ann Blank for critical reading of the manuscript. This work was supported by a New Scholar in Aging Award from the Ellison Medical Foundation (EG), and grants AG01751 (EG) and CA102029 (LAL) and ES11045 from the National Institutes of Health.

References

[1] N.J. Gross, G.S. Getz, M. Rabinowitz, Apparent turnover of mitochondrial deoxyribonucleic acid and mitochondrial phospholipids in the tissues of the rat, J. Biol. Chem. 244 (1969) 1552–1562.
[2] D.C. Wallace, J.M. Shoffner, I. Trounce, M.D. Brown, S.W. Ballinger, M. Corral-Debrinski, T. Horton, A.S. Jun, M.T. Lott, Mitochondrial DNA mutations in human degenerative diseases and aging, Biochim. Biophys. Acta 1271 (1995) 141–151.
[3] D.E. Barnes, T. Lindahl, Repair and genetic consequences of endogenous DNA base damage in mammalian cells, Annu. Rev. Genet. 38 (2004) 445–476.
[4] W.M. Brown, E.M. Prager, A. Wang, A.C. Wilson, Mitochondrial DNA sequences of primates: tempo and mode of evolution, J. Mol. Evol. 18 (1982) 225–239.
[5] S.P. LeDoux, G.L. Wilson, Base excision repair of mitochondrial DNA damage in mammalian cells, Prog. Nucleic Acid Res. Mol. Biol. 68 (2001) 273–284.
[6] D.A. Clayton, J.N. Doda, E.C. Friedberg, The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 2777–2781.
[7] D.F. Bogenhagen, K.G. Pinz, R.M. Perez-Jannotti, Enzymology of mitochondrial base excision repair, Prog. Nucleic Acid Res. Mol. Biol. 68 (2001) 257–271.
[8] P.A. Mason, E.C. Matheson, A.G. Hall, R.N. Lightowlers, Mismatch repair activity in mammalian mitochondria, Nucleic Acids Res. 31 (2003) 1052–1058.
[9] V.A. Bohr, T. Stevnsner, N.C. de Souza-Pinto, Mitochondrial DNA repair of oxidative damage in mammalian cells, Gene 286 (2002) 127–134.
[10] D.L. Croteau, V.A. Bohr, Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells, J. Biol. Chem. 272 (1997) 25409–25412.
[11] A. Barzilai, K. Yamamoto, DNA damage responses to oxidative stress, DNA Repair (Amst) 3 (2004) 1109–1115.
[12] D. Kang, N. Hamasaki, Maintenance of mitochondrial DNA integrity: repair and degradation, Curr. Genet. 41 (2002) 311–322.
[13] T.K. Hazra, T. Izumi, L. Maidt, R.A. Floyd, S. Mitra, The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation, Nucleic Acids Res. 26 (1998) 5116–5122.
[14] A. Memisoglu, L. Samson, Base excision repair in yeast and mammals, Mutat. Res. 451 (2000) 39–51.
[15] R.W. Sobol, R. Prasad, A. Evenski, A. Baker, X.P. Yang, J.K. Horton, S.H. Wilson, The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity, Nature 405 (2000) 807–810.
[16] Y. Matsumoto, K. Kim, Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair, Science 269 (1995) 699–702.

[17] M.J. Longley, R. Prasad, D.K. Srivastava, S.H. Wilson, W.C. Copeland, Identification of 5′-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair in vitro, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 12244–12248.
[18] M.M. Klingbeil, S.A. Motyka, P.T. Englund, Multiple mitochondrial DNA polymerases in Trypanosoma brucei, Mol. Cells 10 (2002) 175–186.
[19] S.M. Nielsen-Preiss, R.L. Low, Identification of a beta-like DNA polymerase activity in bovine heart mitochondria, Arch. Biochem. Biophys. 374 (2000) 229–240.
[20] G.C. Kujoth, A. Hiona, T.D. Pugh, S. Someya, K. Panzer, S.E. Wohlgemuth, T. Hofer, A.Y. Seo, R. Sullivan, W.A. Jobling, J.D. Morrow, H. Van Remmen, J.M. Sedivy, T. Yamasoba, M. Tanokura, R. Weindruch, C. Leeuwenburgh, T.A. Prolla, Mitochondrial DNA mutations, oxidative stress and apoptosis in mammalian aging, Science 309 (2005) 481–484.
[21] E. Fernandez-Vizarra, M.J. Lopez-Perez, J.A. Enriquez, Isolation of biogenetically competent mitochondria from mammalian tissues and cultured cells, Methods 26 (2002) 292–297.
[22] D.F. Bogenhagen, Purification of mitochondria for enzymes involved in nucleic acid transactions, Methods Mol. Biol. 197 (2002) 199–210.
[23] M.J. Longley, W.C. Copeland, Purification, separation, and identification of the human mtDNA polymerase with and without its accessory subunit, Methods Mol. Biol. 197 (2002) 245–257.
[24] S. Krajewski, S. Tanaka, S. Takayama, M.J. Schibler, W. Fenton, J.C. Reed, Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes, Cancer Res. 53 (1993) 4701–4714.
[25] E. Glick, J.P. Anderson, L.A. Loeb, In vitro production and screening of DNA polymerase eta mutants for catalytic diversity, Biotechniques 33 (2002), 1136–1142, 1144.
[26] A. Blank, J.R. Silber, M.P. Thelen, C.A. Dekker, Detection of enzymatic activities in sodium dodecyl sulfate-polyacrylamide gels: DNA polymerases as model enzymes, Anal. Biochem. 135 (1983) 423–430.
[27] M.J. Longley, P.A. Ropp, S.E. Lim, W.C. Copeland, Characterization of the native and recombinant catalytic subunit of human DNA polymerase gamma: identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity, Biochemistry 37 (1998) 10529–10539.
[28] J. Abbotts, D.N. SenGupta, B. Zmudzka, S.G. Widen, V. Notario, S.H. Wilson, Expression of human DNA polymerase β in *E. coli* and characterization of the recombinant enzyme, Biochemistry 27 (1988) 901–909.
[29] K.G. Pinz, D.F. Bogenhagen, Characterization of a catalytically slow AP lyase activity in DNA polymerase gamma and other family A DNA polymerases, J. Biol. Chem. 275 (2000) 12509–12514.
[30] A. Trifunovic, A. Wredenberg, M. Falkenberg, J.N. Spelbrink, A.T. Rovio, C.E. Bruder, Y.M. Bohlooly, S. Gidlof, A. Oldfors, R. Wibom, J. Tornell, H.T. Jacobs, N.G. Larsson, Premature ageing in mice expressing defective mitochondrial DNA polymerase, Nature 429 (2004) 417–423.
[31] M.J. Longley, D. Nguyen, T.A. Kunkel, W.C. Copeland, The fidelity of human DNA polymerase gamma with and without exonucleolytic proofreading and the p55 accessory subunit, J. Biol. Chem. 276 (2001) 38555–38562.
[32] J.K. Horton, A. Baker, B.J. Berg, R.W. Sobol, S.H. Wilson, Involvement of DNA polymerase beta in protection against the cytotoxicity of oxidative DNA damage, DNA Repair (Amst) 1 (2002) 317–333.