

# Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts

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## ABSTRACT

Mammalian mitochondria contain several 16.5 kb circular DNAs (mtDNA) encoding electron transport chain proteins. Reactive oxygen species formed as byproducts from oxidative phosphorylation in these organelles can cause oxidative deamination of cytosine and lead to uracil in mtDNA. Upon mtDNA replication, these lesions, if unrepaired, can lead to mutations. Until recently, it was thought that there was no DNA repair in mitochondria, but lately there is evidence that some lesions are efficiently repaired in these organelles. In the study of nuclear DNA repair, the *in vitro* repair measurements in cell extracts have provided major insights into the mechanisms. The use of whole-cell extract based DNA repair methods has revealed that mammalian nuclear base excision repair (BER) diverges into two pathways: the single-nucleotide replacement and long patch repair mechanisms. Similar *in vitro* methods have not been available for the study of mitochondrial BER. We have established an *in vitro* DNA repair system supported by rat liver mitochondrial protein extract and DNA substrates containing a single uracil opposite to a guanine. Using this approach, we examined the repair pathways and the identity of the DNA polymerase involved in mitochondrial BER (mtBER). Employing restriction analysis of *in vitro* repaired DNA to map the repair patch size, we demonstrate that only one nucleotide is incorporated during the repair process. Thus, in contrast to BER in the nucleus, mtBER of uracil in DNA is solely accomplished by single-nucleotide replacement.

## INTRODUCTION

Mammalian mitochondria possess several copies of a circular 16.5 kb supercoiled genomic DNA. This extranuclear, self-replicating mitochondrial DNA (mtDNA) encodes components of the electron transport chain including 13 structural genes, 22 tRNAs and two rRNAs. The majority of cellular oxygen (~85%) is consumed by the mitochondrial electron transport chain (reviewed in 1). Since mtDNA is localized in close proximity to the electron transport chain, DNA bases can be oxidized by

reactive oxygen species (2,3). Oxidized DNA bases can be mutagenic and can interfere with DNA and RNA polymerases. Uracil can arise in mtDNA from oxidative deamination of cytosine or incorporation of dUMP which, when left unrepaired, will give rise to GC-AT transition mutations. Although mitochondrial copy number and turnover are high per cell, permanent structural mtDNA alterations, including deletions and point mutations, have been shown to occur in aging and disease (4,5). Accumulation of mtDNA damage may be responsible for these mitochondrial genomic changes (6).

An early report (7) demonstrated the absence of repair of cyclobutane pyrimidine dimers from mtDNA which led to the notion that mitochondria had no DNA repair capacity. However, more recent reports have described the removal of other types of mtDNA damage including alkylation lesions (8) and oxidative base damage (9–12). Whole mitochondrial protein extracts obtained from *Xenopus laevis* oocytes have been shown to carry out repair synthesis for HCl, H<sub>2</sub>O<sub>2</sub> and UV-induced DNA damage (13). A limited number of mitochondrial repair enzymes involved in base excision repair (BER) have been characterized. Mitochondrial uracil-DNA glycosylase (14,15) has similar properties to its nuclear counterpart and removes the uracil from DNA without incision of the resulting apyrimidinic (AP)-site. Tomkinson *et al.* identified three mitochondrial UV-endonucleases (16). Recently, we have purified repair enzymes specific for 8-oxodeoxyguanosine (8-oxodG) (17) and thymine glycol (18) from rat liver mitochondria. These two enzymes appear to remove the oxidized base and are also able to incise the phosphodiester bond 3' to the resulting AP-site. Thus, mitochondria possess enzymes that remove oxidized and other damaged DNA bases leaving AP-sites that need to be processed in order to complete repair. Several mitochondrial enzymes that may participate in processing of AP-sites have been described. Tomkinson *et al.* found two class II mitochondrial AP-endonuclease activities in mouse mitochondrial extracts that incised the phosphodiester bond 5' to the AP-site (19). Pinz and Bogenhagen purified *X.laevis* class II mitochondrial AP-endonuclease, mtDNA ligase and mtDNA polymerase  $\gamma$  (pol  $\gamma$ ) (20).

Although these studies suggest the existence of mitochondrial BER (mtBER), mechanistic knowledge regarding the repair process is limited. In contrast, nuclear BER, particularly the repair of uracil, is well characterized. Uracil-DNA glycosylase removes the uracil by hydrolytic cleavage of the base-sugar bond (21). The resulting AP-site is thought to be removed by two different pathways. Short-patch BER involves replacement of

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a single nucleotide (22). In this pathway, the AP-site is further processed by the AP-endonuclease APE1. This enzyme incises 5' to the AP-site, generating a DNA nick with 5'-deoxyribose phosphate (dRp) and 3'-OH ends. The resulting dRp residue is removed by DNA pol  $\beta$ , which also fills in the arising one nucleotide gap. DNA ligase seals the phosphodiester bond (23). Recently, an alternative BER pathway has been reported. This pathway involves AP-endonuclease, DNA polymerase, DNA ligase, proliferating cell nuclear antigen (PCNA) and the structure-specific endonuclease FEN1, and is characterized by a repair patch of 2–6 nt (24,25). It is unknown whether a similar branching of BER pathways exists in mitochondria.

Whole-cell extract repair systems developed in recent years have been widely used to study nuclear nucleotide and BER of well-defined substrates containing single lesions under conditions that mimic *in vivo* physiology. The use of these systems has greatly contributed to our understanding of nuclear DNA repair mechanisms. However, progress in the understanding of mammalian mtDNA repair has been hampered by the lack of similar systems for studying mtBER using mitochondrial protein extracts. Here, we report the development of an *in vitro* mammalian mtDNA repair system. Using rat liver mitochondrial protein extracts, in combination with a substrate containing a single lesion, we have examined the complete mtBER process of uracil. We observe that repair of uracil is solely accomplished by a single-nucleotide replacement mechanism. In contrast to the situation for nuclear BER, we find no evidence for long patch BER of uracil in mitochondria.

## MATERIALS AND METHODS

### Materials

All chemicals were obtained from Sigma (St Louis, MO), unless otherwise specified. Livers were obtained from 12-month-old male Wistar rats (Animal Colony of Gerontology Research Center, Baltimore, MD). Percoll was from Pharmacia Biotech. Protease inhibitors and *Hind*III restriction endonuclease (40 U/ $\mu$ l) were from Boehringer Mannheim. *Hpa*II restriction endonuclease (10 U/ $\mu$ l) was from Gibco BRL. [ $\gamma$ - $^{32}$ P]ATP was from NEN Life Science Products and [ $\alpha$ - $^{32}$ P]dCTP was from Amersham. T4-polynucleotide kinase was from US Biochemical Corp. Nensorb™ 20 nucleic acid purification cartridges were from NEN Dupont Research Products. Centricon-10 and Centriprep-10 concentrators were from Amicon, Inc. Gel electrophoresis equipment was from Bio-Rad.

### Isolation of mitochondria and preparation of mitochondrial protein extract

Mitochondria were isolated as described (17). Procedures were carried out at 4°C. Mitochondrial protein extracts were prepared by resuspending isolated mitochondria obtained from each rat (~2.5 g) in 3 vol of 20 mM Hepes pH 7.6, 300 mM KCl, 1 mM EDTA, 5% glycerol, 0.015% Triton X-100, 5 mM dithiothreitol (DTT). The following protease inhibitors were added: 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml chymostatin A, 2  $\mu$ g/ml leupeptin, 2  $\mu$ M benzamide hydrochloride, 1  $\mu$ M phenylmethylsulfonyl fluoride and 1  $\mu$ M E-64. Resuspended mitochondria were microhomogenized and lysed by slowly adding 10% Triton X-100 to a final concentration of 0.5%. The mitochondrial lysate was subsequently clarified by centrifugation for 1 h at

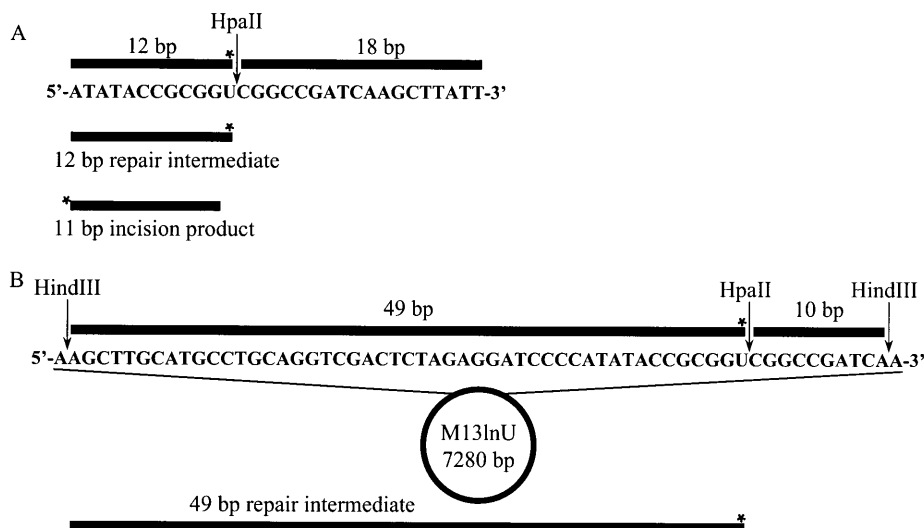
130 000 g in a Beckman SW-50.1 rotor. The supernatant was transferred to a Centriprep-10 and the buffer was exchanged for 40 mM Hepes pH 7.6, 100 mM KCl, 1 mM EDTA, 25% glycerol, 0.015% Triton X-100, 5 mM DTT and protease inhibitors. Upon further concentration to 10 mg/ml protein, the supernatant was aliquoted and stored at –80°C until use. Mitochondrial protein extract produced in this way has been shown to be free of nuclear and lysosomal contamination and extra-mitochondrial BER enzymes (17,18).

### Uracil-containing substrates

30mer oligonucleotides containing either a uracil or cytosine at position 12 relative to the 5'-end, and complementary oligonucleotide were described previously (22). Control and single-uracil-containing M13 DNA (M13InU) were prepared according to methods described previously (26). Briefly, double-stranded closed circular DNA containing a single uracil was constructed by priming 30  $\mu$ g of single-stranded M13In DNA with a 6-fold molar excess of the oligonucleotide 5'-pATATACCGCGGUCGGCCGATCAAGCTTATT-3' for the single-uracil construct (M13InU) or with 5'-pATATACCGCGGCCGCGCCGATCAAGCTTATT-3' for the undamaged control DNA (M13InC). The 250  $\mu$ l reaction mixture containing 50 mM Tris-HCl pH 7.9, 50 mM NaCl, 7 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM EDTA, 100  $\mu$ M ATP, 500  $\mu$ M each of dATP, dCTP, dGTP and TTP, 40 U of T4 DNA polymerase, 35 Weiss units of T4 DNA ligase and 100  $\mu$ g of T4 gene 32 protein (all enzymes from Boehringer Mannheim) was incubated for 4 h at 37°C. Closed circular DNA was isolated by CsCl/ethidium bromide density gradient centrifugation, purified by butanol extraction, desalted and concentrated by centrifugation in a Centricon-10 concentrator. DNA substrates were stored at –20°C in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Substrates were designed to allow mapping of the repair patch size using restriction endonucleases *Hind*III and *Hpa*II. Figure 1 displays sequences and restriction sites of employed substrates. *Hind*III recognizes 5'-A $\downarrow$ AGCTT-3'. Replacement of the uracil residue with cytosine [ $\alpha$ - $^{32}$ P]deoxyribonucleoside monophosphate during the repair process converts the 5'-UCGG-3' sequence into a 5'-C $\downarrow$ CGG-3' *Hpa*II recognition sequence.

### Repair synthesis assay using a single-uracil-containing oligonucleotide substrate

Repair synthesis reactions were performed in a 50  $\mu$ l reaction mixture containing 40 mM Hepes pH 7.8, 75 mM KCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.2 mg/ml BSA, 4  $\mu$ Ci (~25 nM) [ $\alpha$ - $^{32}$ P]dCTP, 2  $\mu$ M dCTP, 20  $\mu$ M dGTP, dTTP and dATP, 2 mM ATP, 25 mM phosphocreatine, 50  $\mu$ g/ml phosphocreatine kinase, indicated amount of mitochondrial protein extract, 2.5 pmol uracil-containing oligonucleotide duplex and 20  $\mu$ g/ml of 53mer single-stranded carrier oligonucleotide. The reactions were incubated at 32°C for the durations indicated in the figure legends. To stop the reaction, samples were extracted with 50  $\mu$ l phenol:chloroform:isoamylalcohol (25:24:1, v/v/v), followed by a chloroform extraction. An equal volume of formamide loading dye (90% formamide, 0.002% bromophenol blue, 0.002% xylene cyanol) was added. After heating for 2–5 min at 80°C, samples were electrophoresed on a denaturing 20% polyacrylamide gel containing 7 M urea, 89 mM Tris-borate pH 8.0 and 2 mM EDTA. After electrophoresis, gels were exposed at 4°C to Phosphor Screens



**Figure 1.** Sequence and restriction sites of single-uracil-containing substrates employed. A single uracil opposite a guanine was located within a 30mer oligonucleotide duplex (A) or a 7280 bp closed circular DNA substrate (M13InU) (B). Only the uracil-containing strands are shown. Substrates were designed to allow mapping of the repair patch size using restriction endonucleases.

and quantified using a Molecular Dynamics PhosphoImager with ImageQuant<sup>TM</sup> software.

#### Determination of repair patch size using restriction endonucleases on a single-uracil-containing oligonucleotide substrate

For determination of the repair patch size oligonucleotides were purified from the reaction mixture by phenol–chloroform extraction and precipitated by the addition of 5 M NaCl to a final concentration of 300 mM and 3 vol of ice-cold 100% ethanol. Samples were washed twice with ice-cold 100% ethanol, dried under vacuum and dissolved in TE (pH 7.8). About 2 pmol of the repaired uracil-containing oligonucleotide were digested for 1 h at 37°C with 30 U *HpaII*. To terminate the digestion, an equal volume of formamide loading dye was added. After heating for 2–5 min at 80°C, samples were electrophoresed on a denaturing 20% polyacrylamide gel and processed for imaging as described above.

#### Repair synthesis assay using a single-uracil-containing closed circular DNA substrate and determination of repair patch size using restriction endonucleases

Repair synthesis reactions were carried out as described above, except that 200 ng of control or single-uracil-containing M13 DNA was used as substrate. Since the circular substrate was more susceptible to non-specific degradation than the linear oligonucleotide substrate [presumably due to the mitochondrial endonuclease (27,28)], non-specific degradation was minimized by incubating the reaction for only 2 h with 25–50 µg protein. For determination of the repair patch size, repair reactions were quenched by addition of EDTA to a final concentration of 20 mM. Samples were treated with 0.1 µg/µl RNaseA for 10 min at 37°C, followed by proteinase K treatment (0.2 µg/µl) in presence of 0.4% SDS. NaCl was added to a final concentration

of 300 mM and samples were extracted with an equal amount of phenol:chloroform:isoamylalcohol (25:24:1, v/v/v), followed by a chloroform extraction. DNA substrates were precipitated by addition of 3 vol of 100% ice-cold ethanol, washed with 80% ethanol, dried under vacuum and dissolved in TE (pH 7.8). About 100 ng of the repaired substrates were then digested with 20 U *HindIII* or with 20 U *HindIII* and 20 U *HpaII*, according to the conditions recommended by the supplier. An equal volume of formamide loading dye was added. After heating for 2–5 min at 80°C, samples were electrophoresed on a denaturing 20% polyacrylamide–7 M urea–TBE gel and processed for imaging as described above.

#### Incision assay

The control or uracil-containing oligonucleotide was 5'-end labeled using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Nensorb<sup>TM</sup> 20 nucleic acid purification cartridges were used to separate labeled oligonucleotides from unincorporated nucleoside triphosphates and enzymes. Oligonucleotides were annealed to the complementary oligonucleotide by heating to 80°C in 100 mM KCl and subsequent slow cooling to room temperature. Incision reactions were performed in a final volume of 20 µl in a mixture containing 40 mM Hepes pH 7.8, 75 mM KCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.2 mg/ml BSA, 2 µM dCTP, 20 µM dGTP, dTTP and dATP, 2 mM ATP, 25 µg mitochondrial protein extract, 2.5 pmol 5'-end labeled uracil-containing oligonucleotide duplex and 50 µg/ml of single-stranded carrier oligonucleotide. The reaction was incubated at 32°C for times indicated in the figure legends. The reaction was stopped by addition of 0.8 µl of 10% SDS and 0.8 µl of 5 mg/ml proteinase K and incubated for 15 min at 55°C. Oligonucleotides were precipitated by addition of 4 µl of 11 M ammonium acetate, 1 µl of 20 µg/µl glycogen and 62 µl of 100% ethanol and pelleted by centrifugation. Pellets were

washed with 70% ethanol and dissolved in formamide loading dye and heated for 2–5 min at 80°C. Electrophoresis and autoradiography were performed as described above.

### DNA polymerase inhibitors

To study the involvement of DNA polymerases in the repair process, reactions were carried out in presence of aphidicolin or *N*-ethylmaleimide. Aphidicolin and *N*-ethylmaleimide were dissolved in DMSO and ethanol respectively.

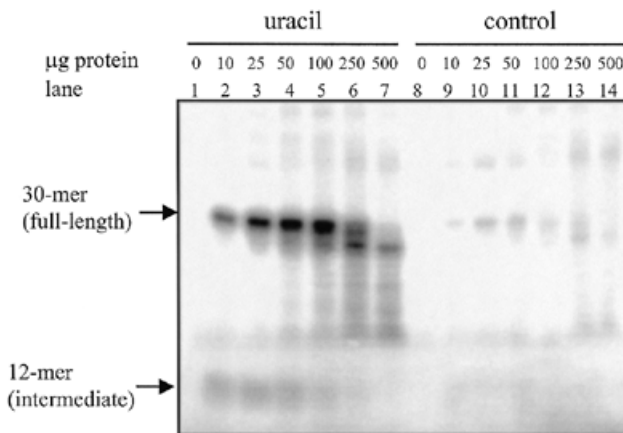
## RESULTS

### BER of uracil supported by mitochondrial protein extracts

To establish an *in vitro* system for BER supported by mitochondrial protein extract, we tested various reaction conditions using a 30-bp oligonucleotide duplex containing a single uracil residue positioned opposite a guanine residue. Repair of the uracil can be assessed by monitoring [ $\alpha$ - $^{32}$ P]dCMP incorporation into the oligonucleotide, upon incubation with mitochondrial protein extract and cofactors. Initial attempts to perform the BER reaction at 37°C with mitochondrial protein extract were unsuccessful due to degradation of the substrate by mitochondrial nucleases. To optimize the reaction conditions, we added a single-stranded carrier oligonucleotide to the reaction mixture and lowered the reaction temperature to 32°C. We also observed that at a concentration of KCl <40 mM, the repaired full-length 30mer oligonucleotide substrate was progressively degraded, whereas at 70–80 mM KCl degradation was minimal (data not shown). The mitochondrial endonuclease has been shown to randomly degrade DNA substrates at lower KCl concentrations (27,28). Thus, an oligonucleotide containing a single uracil was incubated for 3.5 h at 32°C with varying amounts of mitochondrial protein extracts in the presence of radiolabeled [ $\alpha$ - $^{32}$ P]-dCTP, dNTPs, single-stranded carrier oligonucleotide, 75 mM KCl and co-factors. The results are shown in Figure 2. A protein concentration-dependent repair incorporation of [ $\alpha$ - $^{32}$ P]dCMP into the full-length uracil-containing 30mer oligonucleotide was observed, at up to 100  $\mu$ g of protein per reaction. At >100  $\mu$ g protein per reaction, we observed increased oligonucleotide degradation (Fig. 2, lanes 5–7). Figure 3 displays BER of the uracil-containing oligonucleotide by mitochondrial protein extracts as a function of time. An approximate linear increase in the amount of full length repaired product was seen with increasing time of incubation (Fig. 3A, top panel, and B, closed diamonds). Only a very low level of incorporation into control oligonucleotide, presumably because of exonuclease action and repolymerization, was observed (Figs 2A, lanes 8–14, and 3A, lower panel). These experiments demonstrate that rat liver mitochondrial protein extracts contain all factors required to support complete BER of uracil.

### Recognition and incision of uracil proceed rapidly, whereas later steps in mtBER are rate limiting

Removal of uracil from an oligonucleotide by a uracil-DNA glycosylase will create an AP-site. This site is then processed by an AP-endonuclease that hydrolyzes the phosphodiester bond 5' to the AP-site. During nuclear BER, pol  $\beta$  adds one nucleotide to the 3'-end of the nick, and removes the interfering dRp residue. DNA ligase seals the nick. The removal of dRp residue is a rate-limiting step in nuclear BER (29). We

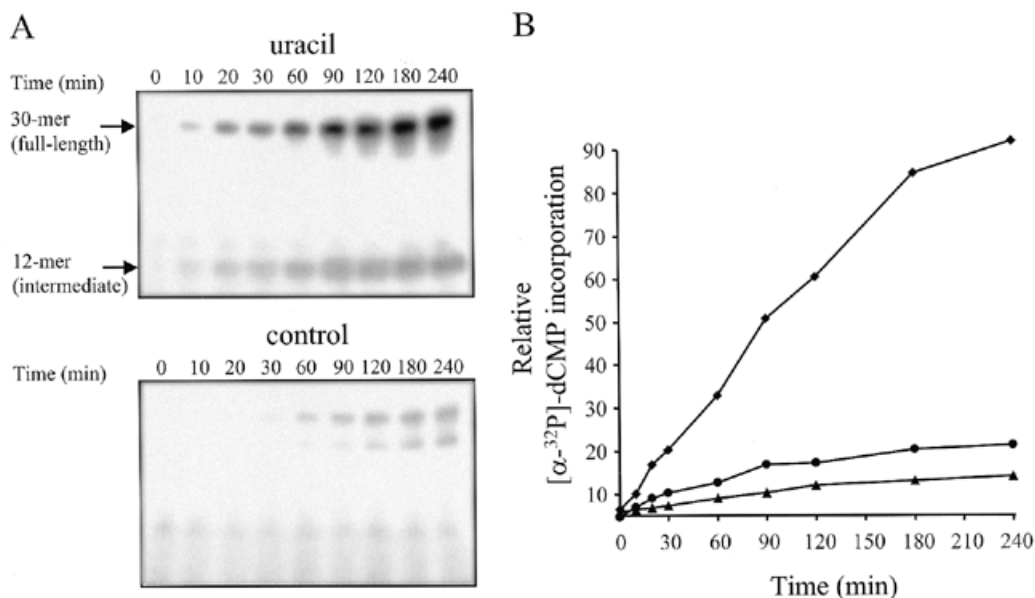


**Figure 2.** Protein concentration-dependent BER of uracil-containing oligonucleotide duplex by mitochondrial protein extracts. Single-uracil-containing (lanes 1–7) or control (lanes 8–14) 30mer oligonucleotide were incubated for 3.5 h at 32°C with the indicated amount of rat liver mitochondrial protein extract, in the presence of [ $\alpha$ - $^{32}$ P]dCTP, dNTPs, single-stranded carrier oligo, 75 mM KCl and co-factors as described in Materials and Methods. Reaction products were purified and separated on a denaturing 20% polyacrylamide gel, exposed to Phosphor Screens and visualized using a Molecular Dynamics PhosphorImager.

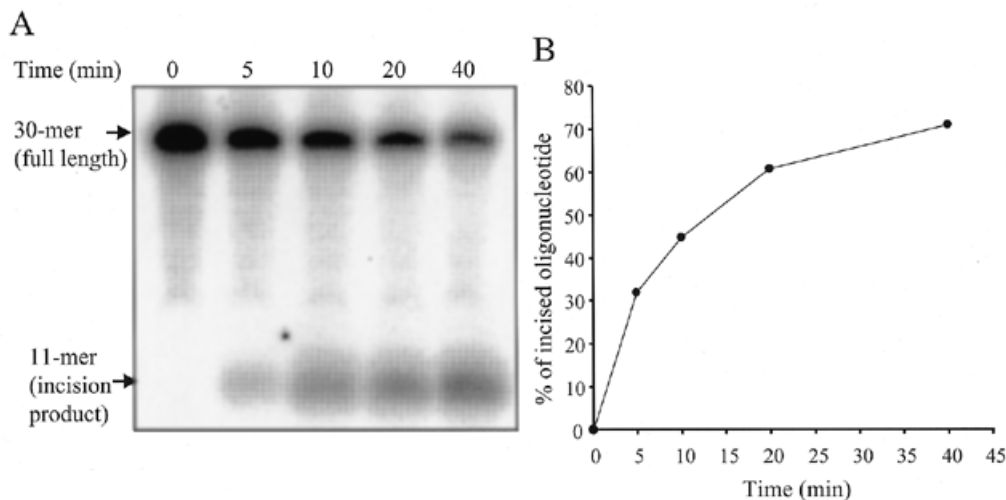
observed that during mitochondrial repair, uracil-specific incision of a 5'-end labeled oligonucleotide was completed by 70% within 40 min (Fig. 4A and B). In contrast, formation of the full-length 30mer repair product increased up to >240 min (Fig. 3A and B). Also, a 12mer labeled intermediate product gradually accumulated during the repair incorporation reaction (Fig. 3A). These data suggest that removal of a dRp residue and/or ligation may be a rate-limiting reaction during mtBER.

### Single-nucleotide patch repair of uracil by mitochondrial protein extract

Nuclear BER is characterized by the existence of short-patch and long-patch mechanisms. To study whether a similar divergence exists in mitochondria, restriction endonucleases were used to map the repair patch size generated during repair of uracil in DNA by mitochondrial extracts. Uracil-containing oligonucleotide was incubated with mitochondrial protein extract in the presence of all cofactors needed, including [ $\alpha$ - $^{32}$ P]dCTP. After incubation, the oligonucleotide was purified from the reaction mixture and subjected to restriction analysis. Figure 5A shows the restriction enzyme analysis of the repaired single-uracil-containing oligonucleotide substrate (Fig. 1A). The signal from each band represents the incorporation of [ $\alpha$ - $^{32}$ P]dCMP into the corresponding restriction fragment during the repair process. Undigested repair synthesis products (Fig. 5A, lane 1) produced two bands, corresponding to the full length (30mer) product and a single unligated repair intermediate (12mer). This repair intermediate is characteristic for one nucleotide extension of the incision product. No repair intermediates of >12 nt were detected in any of the experiments. Since the uracil



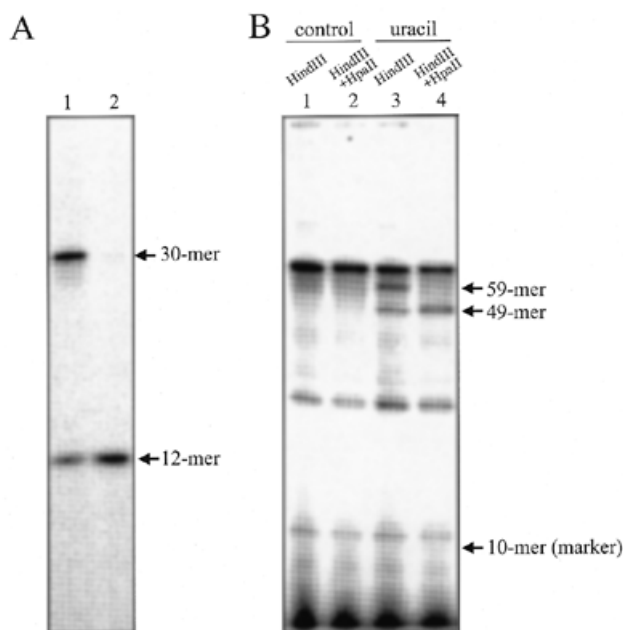
**Figure 3.** BER of a uracil-containing oligonucleotide by mitochondrial protein extract as a function of time. (A) The single-uracil-containing (upper panel) or control (lower panel) oligonucleotides were incubated for different time points with 25  $\mu$ g of whole mitochondrial protein extract under the conditions described in Materials and Methods. (B) Quantitative analysis of the repair synthesis signal. Diamonds: uracil, 30mer full-length repair product; circles: uracil, 12mer repair intermediate; triangles: background incorporation into control oligonucleotide.



**Figure 4.** Incision of a uracil-containing oligonucleotide by whole mitochondrial protein extract. The single-uracil-containing oligonucleotide (Fig. 1A) was 5'-end-labeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP and incubated for different time points with 25  $\mu$ g mitochondrial protein extract under conditions similar to those employed for repair synthesis reactions. (A) Representative gel. (B) Percentage of oligonucleotide incised plotted against time of incubation.

is placed within the *Hpa*II recognition sequence (Fig. 1A), the uracil-containing oligonucleotide used is resistant to *Hpa*II cleavage (30). *Hpa*II is only able to cleave the 30mer oligonucleotide to a

12mer upon replacement of the uracil (and the resulting AP-site) with a single cytosine deoxyribonucleoside monophosphate, as would be expected to occur during repair. Digestion



**Figure 5.** Restriction analysis of uracil-containing DNA substrates repaired *in vitro* by mitochondrial protein extracts. (A) The repaired single-uracil-containing substrate oligonucleotide was purified and digested with restriction endonucleases to map the size of the repaired patch (see Fig. 1A for restriction sites). Samples were processed for imaging as described. Lane 1, repaired undigested oligonucleotide; lane 2, *HpaII* digestion. (B) Single-uracil-containing M13lnU DNA or control DNA containing a normal C:G base pair were allowed to undergo repair synthesis as described and restricted with restriction endonucleases to map the repair patch size. Lane 1, control DNA, *HindIII* digested; lane 2, control DNA *HindIII+HpaII* digested; lane 3, M13lnU DNA, *HindIII* digested; lane 4, M13lnU DNA *HindIII+HpaII* digested.

with *HpaII* converted all the 30mer towards a 12mer (Fig. 5A, lane 2). Thus, sensitivity of the *in vitro* repaired oligonucleotide to *HpaII* hydrolysis indicates that uracil was removed from the oligonucleotide and the [ $\alpha$ - $^{32}$ P]dCMP incorporation occurred upon uracil removal rather than end-labeling of the oligonucleotide during exonuclease cleavage and resynthesis. The 12mer *HpaII* hydrolysis product migrated at a position similar to that of the repair intermediate detected in the unrestricted sample (Fig. 5A, lane 1). No 18-bp fragment was detected, indicating the absence of [ $\alpha$ - $^{32}$ P]dCMP incorporation 3' to the original uracil. These data demonstrate that only one nucleotide is replaced during mtBER of uracil-containing oligonucleotides.

It was shown recently that nuclear long-patch BER is inefficient on linear DNA (31). In order to exclude the possibility that potential long-patch mtBER would remain undetected on the short 30mer oligonucleotide, we conducted similar repair synthesis experiments using a closed circular DNA substrate containing a single uracil residue (Fig. 1B). The substrate DNA was designed such that digestion of the repaired DNA with *HindIII* releases a 59mer containing a [ $\alpha$ - $^{32}$ P]dCMP residue at the position that contained a uracil prior to repair (Fig. 1B). Further cleavage of the 59mer with *HpaII* generates a 49mer and a 10mer. Incorporation of [ $\alpha$ - $^{32}$ P]dCMP into the 49mer

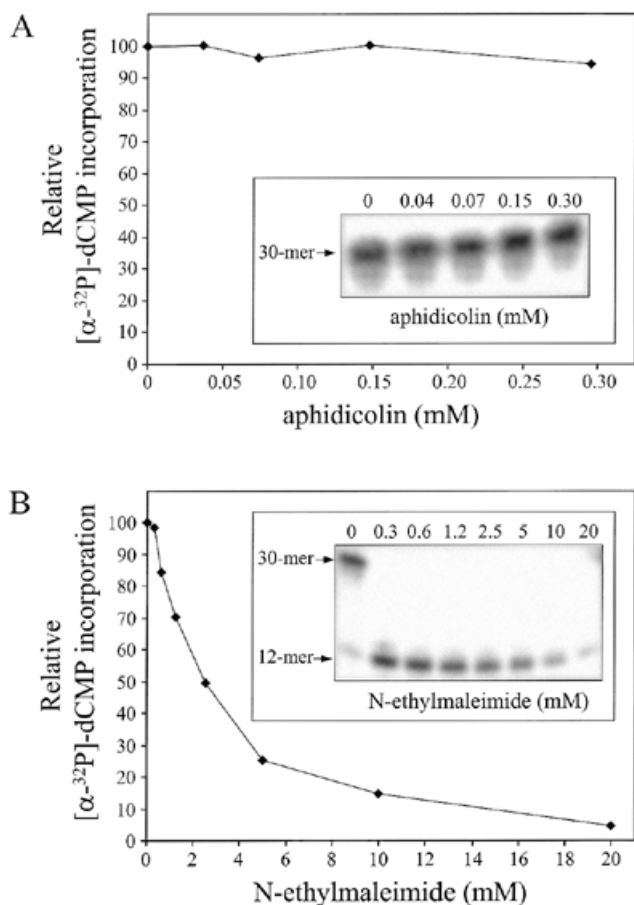
then reflects incorporation following uracil replacement. Any 3' extension of the repair patch beyond one nucleotide will result in incorporation into the *HindIII-HpaII* double-digested 10mer fragment. The results of the restriction analysis are shown in Figure 5B. Digestion of the repaired uracil-containing circular DNA with *HindIII* (lane 3) yielded the expected 59mer full-length repair product and a single 49mer unligated repair intermediate. No longer repair intermediates were detected. Upon further cleavage of the *HindIII*-digested repair product with *HpaII* (lane 4), all of the 59mer full-length product was converted to a 49mer product, migrating similarly to the 49mer repair intermediate. No 10mer fragment was detected, indicating that, as was observed with oligonucleotide substrates (Fig. 5A), the single-nucleotide patch repair is the major repair mechanism in mitochondrial extracts. Three bands at ~66, ~26 and ~11 bp were present in the reactions with both uracil-containing and control DNA. These products are likely due to damage non-specific cleavage and labeling of DNA substrates by mitochondrial enzymes.

#### Single-nucleotide incorporation and ligation is sensitive to *N*-ethylmaleimide but not to aphidicolin, suggesting the involvement of pol $\gamma$ in mtBER

To determine which of the known DNA polymerases were involved in the single-nucleotide replacement repair reaction, different inhibitors were used (Fig. 6). Mammalian DNA polymerases have a differential sensitivity towards DNA synthesis inhibitors. Aphidicolin at the concentration of 60  $\mu$ M inhibits the nuclear pol  $\alpha$ ,  $\delta$  and  $\epsilon$  (32,33). No decrease in the relative amount of [ $\alpha$ - $^{32}$ P]dCMP incorporation in single-uracil-containing oligonucleotides was observed when the repair reaction was carried out in presence of aphidicolin at concentrations up to 0.3 mM (Fig. 6A). Thus, the DNA repair synthesis supported by mitochondrial extracts is not sensitive to aphidicolin and none of the nuclear pol  $\alpha$ ,  $\delta$  and  $\epsilon$  is involved in mtBER. *N*-ethylmaleimide inhibits DNA pol  $\gamma$  (in addition to  $\alpha$  and  $\delta$ ) at low concentrations (2 mM), whereas, even at 10 mM, pol  $\beta$  is not inhibited (32,34). Addition of *N*-ethylmaleimide to the reaction mixture inhibited [ $\alpha$ - $^{32}$ P]dCMP incorporation by 50% at a concentration of 2.5 mM (Fig. 6B). Therefore, the DNA repair synthesis supported by mitochondrial extracts was sensitive to *N*-ethylmaleimide, suggesting that pol  $\gamma$  but not pol  $\beta$  is involved in mtBER. Interestingly, the conversion of the repair intermediate (12mer) to the full-length repair product (30mer) was completely inhibited at a concentration that did not inhibit incorporation (0.3 mM; Fig. 6B). This suggests that, during the mtBER process, the ligation step is more sensitive to *N*-ethylmaleimide than the one nucleotide repair incorporation step. Thus, it is likely that pol  $\gamma$  is the primary DNA polymerase involved in mtBER. These data also demonstrate that the repair observed was not due to potential contamination of the mitochondrial protein extract with nuclear DNA polymerases.

## DISCUSSION

We have investigated the mechanism of mammalian mtBER of uracil. Substrates containing a single uracil lesion were used in combination with rat liver whole mitochondrial protein extracts. We demonstrate that complete BER of uracil in DNA can be achieved by mammalian mitochondrial extracts. We also show that mammalian mitochondrial repair of uracil in



**Figure 6.** Repair synthesis and ligation are sensitive to *N*-ethylmaleimide but not to aphidicolin. Repair synthesis reaction were carried out as described in the presence of (A) aphidicolin (inhibitor of pol  $\alpha$ ,  $\delta$  and  $\epsilon$ ) or (B) *N*-ethylmaleimide (inhibitor of pol  $\gamma$ ).

DNA is accomplished solely through a one nucleotide replacement mechanism. Additionally, we show that pol  $\gamma$  is the polymerase most likely involved in BER supported by mitochondrial protein extracts.

The major advantage of the mitochondrial protein extract-based DNA repair method presented here is that the complete mammalian mtDNA repair process of a well-defined lesion can be studied within a context that comes closer to the *in vivo* physiology. Previous efforts to detect BER of defined lesions in mitochondrial extracts were limited (13), presumably due to nuclease activities present in crude mitochondrial protein extracts. Therefore, *in vitro* reconstitution of the mitochondrial repair with purified enzymes has been used as an alternative approach. Pinz and Bogenhagen reconstituted a non-mammalian *in vitro* mtBER reaction using an AP-site-containing substrate in combination with purified *X.laevis* pol  $\gamma$ , mitochondrial AP-endonuclease and mtDNA ligase (20). Longley *et al.* reconstituted *in vitro* repair on a uracil-containing substrate using human pol  $\gamma$ , human AP-endonuclease and human DNA ligase I (35). The AP-endonuclease they used was, however, shown to be absent

in human mitochondria (36) and the participation of DNA ligase I in mtBER has been questioned (20). Both of these studies show that purified mitochondrial enzymes, under *in vitro* conditions that simulate mtBER, can repair uracil and AP-sites. We have extended these studies and demonstrate that repair of uracil actually takes place in a reaction supported by rat liver mitochondrial protein extract. Thus, mammalian mitochondria possess all enzymes required to complete BER of uracil in DNA. One of the frequently raised concerns regarding mitochondrial protein extracts is their potential contamination with nuclear proteins. In our previous studies, we demonstrated the absence of BER activities for 8-oxodG and thymine glycol in the outer membrane fractions obtained from digitonin-fractionated mitochondria (17,18). Therefore, mitochondria prepared by our protocol are free of extra-mitochondrial BER enzymes.

We have investigated the mechanism of mitochondrial BER. The potential existence of long-patch mitochondrial BER, similar to mechanisms that exist in the nucleus (24) has never been examined. Two studies have demonstrated that pol  $\gamma$ -mediated one nucleotide extension is possible during mtBER-like reactions reconstituted *in vitro* with purified enzymes, indicating that pol  $\gamma$  may participate in a single-nucleotide replacement BER mechanism (20,35). However, since accessory proteins which could modulate pol  $\gamma$  activity were not present, these studies could not exclude the existence of pol  $\gamma$ -mediated long-patch BER mechanisms in mitochondria. In addition, although pol  $\gamma$  is generally believed to be the only mtDNA polymerase, mtDNA polymerases that were distinct from pol  $\gamma$  have been isolated from bovine heart (37) and *Saccharomyces cerevisiae* mitochondria (38). Therefore, the possibility is not excluded that other mammalian mtDNA polymerases exist that participate in other than single-nucleotide repair mechanisms. Employing restriction endonuclease analysis of the *in vitro* repaired DNA, we demonstrate that only one nucleotide is replaced during mitochondrial BER. Thus, in contrast to the nucleus, mitochondria do not possess mechanisms for long-patch BER of uracil. The repair is solely accomplished by single-nucleotide replacement. Our observation that BER of uracil-containing oligonucleotides supported by mitochondrial protein extract is not sensitive to aphidicolin (pol  $\alpha$ ,  $\delta$ ,  $\epsilon$  inhibitor) but only to *N*-ethylmaleimide (pol  $\gamma$  inhibitor) (Fig. 6) suggests that pol  $\gamma$  is indeed involved in the single-nucleotide repair reaction, as predicted by previous studies (20,35).

Detection of long-patch BER of AP-sites in the nucleus has been shown to depend on the length of the substrate used (31). Whereas long-patch PCNA-dependent nuclear BER was functional on circular DNA, this mechanism functioned poorly on short linear DNA. To exclude that potential mitochondrial long-patch BER remained undetected due to such a substrate effect, both a 30mer linear oligonucleotide and a closed circular DNA substrate were tested. Using both substrates, only single-nucleotide replacement was observed during the repair process (Fig. 5).

Analysis of the repair kinetics data indicates that removal of the uracil and incision of the resulting AP-site was much faster than the complete repair reaction [ $\sim 40$  min (Fig. 4) versus  $\sim 240$  min (Fig. 3)]. In addition, a 12mer repair intermediate accumulated. Thus, after formation of the repair intermediate, there is a delay in its processing to the full-length repair product. Longley *et al.* reported that dRp removal mediated by pol  $\gamma$  is a low-rate reaction and proceeds at a lower rate than nucleotide

incorporation ( $K_{\text{cat}}$  nucleotide incorporation:  $4.5 \text{ s}^{-1}$ ;  $K_{\text{cat}}$  dRp lyase:  $0.26 \text{ min}^{-1}$ ) (35). If we assume that pol  $\gamma$  is the only or the major enzyme that catalyzes one nucleotide incorporation and dRp removal, the accumulation of the intermediate (Fig. 3) agrees with these kinetic data. Thus, it is suggested that during mtBER removal of the dRp residue is rate-limiting, as was previously demonstrated for pol  $\beta$ -mediated nuclear BER (29).

Previously, we have shown that mitochondria possess enzymatic activities specific for 8-oxodG (17) and thymine glycol (18). These activities appear to have a glycosylase function which removes the oxidized base. After removal of the oxidized base, the resulting AP-site could be repaired through a single-nucleotide replacement mechanism similar to that we describe here for uracil. At present, we cannot exclude that back-up mechanisms exist for pol  $\gamma$ -mediated single-nucleotide repair. However, our findings may imply that in mitochondria one mechanism of BER for uracil and oxidative DNA damage is sufficient, as opposed to the nuclei where different BER mechanisms act on these lesions (24–26).

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