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Homogenous repair of singlet oxygen-induced DNA damage in differentially transcribed regions and strands of human mitochondrial DNA

R. Michael Anson[+], Deborah L. Croteau[§], Rob H. Stierum, Charles Filburn[1], Robin Parsell and Vilhelm A. Bohr*

Laboratory of Molecular Genetics and [1]Laboratory of Biological Chemistry, National Institute on Aging, Baltimore, MD, USA

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ABSTRACT

Photoactivated methylene blue was used to damage purified DNA and the mitochondrial DNA (mtDNA) of human fibroblasts in culture. The primary product of this reaction is the DNA lesion 7-hydro-8-oxo-deoxyguanosine (8-oxo-dG). The DNA damage was quantitated using *Escherichia coli* formamido-pyrimidine DNA glycosylase (Fpg) in a gene-specific damage and repair assay. Assay conditions were refined to give incision at all enzyme-sensitive sites with minimal non-specific cutting. Cultured fibroblasts were exposed to photoactivated methylene blue under conditions that would produce an average of three oxidative lesions per double-stranded mitochondrial genome. Within 9 h, 47% of this damage had been removed by the cells. This removal was due to repair rather than to replication, cell loss or degradation of damaged genomes. The rate of repair was measured in both DNA strands of the frequently transcribed ribosomal region of the mitochondrial genome and in both strands of the non-ribosomal region. Fpg-sensitive alkali-resistant oxidative base damage was efficiently removed from human mtDNA with no differences in the rate of repair between strands or between two different regions of the genome that differ substantially with regard to transcriptional activity.

INTRODUCTION

The DNA of aerobic organisms is continuously damaged by reactive oxygen, resulting in lesions that could lead to mutagenesis or cell death if left unrepaired. One of the most common lesions is 7-hydro-8-oxo-deoxyguanosine (8-oxo-dG) (1). 8-oxo-dG will often adopt the *syn* conformation (2), allowing it to mispair with deoxyadenosine. Most DNA polymerases fail to recognize this mismatch, resulting in G→T transversions (3). Mitochondrial polymerase γ is no exception (4). Despite reports that steady-state levels of 8-oxo-dG are 10-fold higher in mitochondrial DNA (mtDNA) than in nuclear DNA and increase dramatically with age (5), G→T transversions are infrequent in mtDNA (6). The lack of such mutations suggests that the lesion is repaired before DNA replication.

After the discovery that there is no repair of UV-induced pyrimidine dimers in mtDNA (7) and in view of the high copy number, the mitochondrial genome came to be regarded as disposable. It was generally assumed that mitochondria have no DNA repair capacity. Since then, however, several mammalian mtDNA repair enzymes have been detected, including a uracil DNA glycosylase (8,9), AP endonucleases (10), a methyltransferase (11), a pyrimidine hydrate DNA glycosylase (12) and a mitochondrial oxidative damage endonuclease (mtODE) which is specific for 8-oxo-dG (13). A recombinational pathway was also recently reported (14). The types of damage which are now known to be removed from mammalian mtDNA include alkylation base damage induced by various agents (11,15–17), cisplatin interstrand cross-links (17) (but not intrastrand crosslinks; 17,18), uvrABC excinuclease-sensitive sites induced by the carcinogen 4-nitro-quinolone-1-oxide (19) and bleomycin-induced strand breaks (20). In addition, oxidatively induced single-strand breaks or abasic sites are repaired in the mtDNA of rodent (21) and human (22) cells and oxidative lesions induced by a 15 min exposure to hydrogen peroxide are repaired within hours, as measured by their ability to block a DNA polymerase in a PCR reaction (23).

Two *Escherichia coli* repair enzymes, endonuclease III and formamidopyrimidine DNA glycosylase (Fpg), have been used to measure oxidative base damage. Endonuclease III excises oxidatively damaged pyrimidines, while Fpg has a high affinity for oxidatively modified deoxyguanosine (24,25), particularly 8-oxo-dG. When paired with deoxycytidine on the opposite strand, 8-oxo-dG is excised by Fpg and the resulting apurinic sugar is then removed to generate a single-strand break. Both endonuclease III and Fpg have been used to measure the removal of oxidative base damage from rat and hamster mtDNA (21,26). Very little is currently known, however, about the removal of such damage from human mtDNA. In one study human cells did repair oxidatively induced strand breaks and alkali-sensitive sites (22). However, 8-oxo-dG is resistant to mild alkali (27) but readily cleaved by Fpg and, by these criteria, the agent used (alloxan)

*To whom correspondence should be addressed. Tel: +1 410 558 8162; Fax: +1 410 558 8157; Email: bohrv@grc.nia.nih.gov

[+]Also affiliated with the Department of Biology, Johns Hopkins University

[§]Also affiliated with the Department of Pharmacology and Molecular Sciences, Johns Hopkins University

failed to induce 8-oxo-dG (see table 1 in reference 22). Thus repair of this lesion in human mtDNA has yet to be demonstrated.

The aims of the current study were: first, to determine if oxidative base damage induced by singlet oxygen, which is predominantly 8-oxo-dG, is removed from human mtDNA; second, to determine whether DNA damage removal is truly due to repair. This addresses concerns regarding replication and degradation frequently raised in the literature (5,12,28). A third objective was to investigate the possibility of strand specificity in the repair of mtDNA. Although full-length transcripts are made from both strands of the mitochondrial genome, the two strands are not transcribed with the same frequency (29) and if repair only occurs during transcription a strand bias in repair rate or efficiency would be expected. While mitochondria lack nucleotide excision repair of damage induced by UV radiation, transcription coupled repair pathways that do not involve nucleotide excision repair could theoretically be present. A novel form of transcription coupling was recently described, one that is suggested to involve base excision repair rather than nucleotide excision repair: mutant cells lacking nucleotide excision repair nevertheless repaired the oxidative lesion thymine glycol in a transcription coupled manner (30). A role for transcription coupled repair has never before been investigated in the repair of mtDNA. It has been suggested that transcription is limited to a small fraction of the mtDNA molecules at any given time (31). If repair of 8-oxo-dG only occurred during transcription, this might suggest that repair is also limited to a small fraction of mtDNA molecules at any given time. This might allow damage to accumulate between transcription cycles to levels that would be compatible with the high levels of 8-oxo-dG which have frequently been reported for mtDNA (5). The fourth objective was to determine, for the first time, whether repair of mtDNA occurs with regional heterogeneity. The mitochondrial ribosomal sequences are transcribed more frequently than other sections of the mitochondrial genome (29). We therefore compared the rate of repair in both the ribosomal and in the less frequently transcribed non-ribosomal regions of the mitochondrial genome to determine whether the repair rate correlated with the expected transcription frequency.

MATERIALS AND METHODS

Cell culture

WI38 human fetal lung fibroblasts obtained from the Coriell Institute for Medical Research (Camden, NJ) were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with essential and non-essential amino acids, vitamins, glutamine and 10% fetal bovine serum (FBS) and containing penicillin and streptomycin. Cells used in these experiments were of mid population doubling level (PDL 30–35) and were treated at ~80% confluence.

DNA replication was measured by addition of 10 μM bromodeoxyuridine and 1 μM fluorodeoxyuridine to the medium during the repair period. Cells were lysed after 0, 12, 20 or 44 h. Following DNA isolation and restriction parental and replicated DNA were separated by neutral CsCl centrifugation. Fractions (400 μl) were collected and total DNA localized by staining 2.5 μl of each fraction with 2.5 μl of 40 μg/ml ethidium bromide. Fluorescence was then measured using a Fotodyne system (Fotodyne Inc., Hartland, WI) and analyzed with ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). DNA was quantitated by comparison with a standard curve (0–80 ng λ phage DNA *Hin*dIII digest). Aliquots were also withdrawn from each fraction and used in dot blots to measure replication of mtDNA.

To measure the effect of methylene blue (MB) on cellular functions other than DNA replication, cells were exposed to MB for 1 h and then (without light exposure) placed in fresh medium overnight, at which time they were trypsinized and transferred to a new plate. The ability of cells to form new attachments to the culture dish was then monitored.

To determine if cells remained attached to the culture dish throughout the repair period, 2500 cells/well were plated in 96-well plates and allowed to attach overnight. They were then treated with photoactivated MB as described below and allowed to repair for 0, 1, 3, 6, 9, 12, 20 or 44 h. At the appropriate times the medium was removed and cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) prior to fixation. Upon completion of the experiment wells were rinsed thoroughly with water, allowed to dry and stained with crystal violet prior to spectrophotometric analysis (32).

Damage induction in purified DNA

Total DNA, isolated by salt extraction (33) from WI38 human fibroblasts, was diluted in 10 mM Tris–HCl, 1 mM EDTA, pH 8, (TE) to 125 μg/ml. One ninth volume of 20 μM MB (Ricca Chemical Company, Arlington, TX; λ_{max} = 655 nm, ε = 89125) was added in the dark, mixed rapidly and the mixture placed in a 10 cm plastic tissue culture dish on ice. The solution depth was ~2 mm. The uncovered dish was exposed to light from a 100 W tungsten bulb positioned 18 cm from the surface (34) for 7–10 min. The light exposure was 50–70 kJ/m^2, measured with an IL1400A Radiometer/Photometer (International Light Inc., Newburyport, MA). To remove MB the DNA was ethanol precipitated, washed with 70% ethanol and then resuspended in TE. Following restriction with *Pvu*II (Boehringer Mannheim Corp., Indianapolis, IN) the DNA was used as a substrate in the Fpg assay as described below. Control DNA was from the same original DNA isolation, but was not treated with MB. All work was done under a dim blue light while MB was present in solution with the DNA.

Damage induction *in situ*

Cells at 70–90% confluence were treated for 1 h with 100 μM MB in DPBS containing 1% glucose. MB was replaced with DPBS lacking calcium and magnesium and the cells were exposed to visible light (56 kJ/m^2) from a 300 W tungsten bulb situated beneath the plate. To prevent heating, tissue culture plates were separated from the light source by a ventilated chamber. After light exposure they were washed twice with DPBS (minus calcium and magnesium) and either lysed or allowed to repair in medium. For repair time points >9 h parental and replicated DNA were separated as described above.

Damage detection

DNA was isolated by salt extraction (33), with work performed under a dim blue light until after ethanol precipitation and resuspension. At this point DNA was resuspended in TE and treated with 100 μg/ml RNase A (Sigma Chemical Co., St Louis, MO) together with an appropriate restriction enzyme at 37°C. Following this it was loaded onto a CsCl gradient (for the 0, 12, 20 and 44 h time points) or purified by one extraction with 24:1

chloroform:isopentyl alcohol, precipitated and resuspended in TE (for the 0, 1, 3, 6 and 9 h time points).

The Fpg assay has been described previously (26), but was modified for this study. In brief, Fpg is used to generate a single-strand nick at the site of damage in a restricted DNA fragment. In a denaturing gel the cleaved strand moves forward, away from the main band. After blotting and probing this loss of damaged DNA from the main band allows quantitation of undamaged DNA. The number of incisions is calculated using the Poisson distribution: incisions = –ln(Fpg treated/untreated). Reaction conditions were as follows: concentrated reaction buffer was mixed with DNA dissolved in TE (final 1× concentrations: 0.5 mg/ml BSA, 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM EDTA) and 15 µl aliquots containing DNA were placed in 0.5 ml Eppendorf tubes. The reaction was begun by addition of 5 µl Fpg (obtained from Dr Arthur Grollman, State University of New York, Stony Brook, NY) in 1× reaction buffer, with re-pipetting to mix. All incubations were at 37°C. After Fpg treatment the DNA was fully denatured by a 37°C incubation in 0.06 M NaOH for 15 min, then the entire reaction run on an alkaline gel and analyzed by Southern blot. For assay refinement each tube contained 0.5 µg DNA and 0.005, 0.05 or 0.5 µg Fpg was added to start the reaction. For measurement of damage induced to DNA in cultured fibroblasts the protocol was modified as follows: a master mix of DNA was prepared in 1× reaction buffer for each sample. One aliquot of this mix was then treated with Fpg and a second with 1× reaction buffer. The Fpg:DNA ratio was held between 0.01 and 0.1 µg Fpg/µg DNA, while the amount of DNA in each reaction varied from 0.5 µg/reaction, when only mitochondrial probings were planned, to 5 µg/reaction, when nuclear probings were planned.

Southern blotting and probing

Following Fpg treatment DNA was loaded on a 30 mM NaOH, 1 mM EDTA, 0.75% agarose gel and run overnight at ~1.5 V/cm. After acid depurination and alkaline nicking DNA was transferred to Sureblot nylon membrane (Oncor, Rockville, MD) in 1 M NaOH by positive pressure (Posiblot apparatus; Stratagene, La Jolla, CA), neutralized in 2× SSPE and then baked for 2 h at 80°C. Membranes were prehybridized in Hybrisol (Oncor, Rockville, MD) and then hybridized to ^{32}P-labeled probes generated as riboprobes (SP6/T7 Transcription Kit; Boehringer Mannheim Corp., Indianapolis, IN) or by random primed labeling (Random Primed DNA Labeling Kit; Boehringer Mannheim Corp., Indianapolis, IN). Riboprobes for two mitochondrial and one nuclear sequence were used. The probe for the mitochondrial ribosomal region consisted of the sequence between 652 and 3226 (numbering based on the Anderson sequence; 35) and the probe for the non-ribosomal region of the sequence between 5905 and 7433. They were prepared by cloning restriction fragments into the PCR II vector (Invitrogen, Carlsbad, CA) between the T7 and SP6 promoter sequences. Single-stranded riboprobes can be made from either promoter, allowing strand-specific measurements of repair to be made in both mitochondrial regions. The probe for exon III (*Eco*RI–*Cla*I fragment) of the nuclear c-*myc* sequence was obtained from Lofstrand Labs Ltd (Gaithersburg, MD). The nuclear ribosomal DNA probe is part of plasmid pEE6.7 (36) and consists of the 6.7 kb *Eco*RI fragment containing the 5.8S and 28S sequences of Sprague–Dawley rat ribosomal DNA. Radioactivity in the bands was quantitated using a PhosphorImager (Molecular Dynamics Inc. Sunnyvale, CA) and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

RNA synthesis assay

Cells that had been pre-labeled for ~20 h in medium containing 0.025 µCi/ml [^{14}C]thymidine (NEN, Boston, MA) were treated with 100 µM MB for 1 h followed (or not) by a 56 kJ/m^2 light exposure as described above. Cells were then returned to normal medium and incubated for 1, 8 or 24 h. One hour prior to each time point the medium was replaced with fresh medium containing 8 µCi/ml [^{3}H]uridine (NEN, Boston, MA). At the appropriate time points cells were washed with DPBS and lysed by addition of 2% SDS. Ice-cold TCA was added to a final concentration of 10%. After 1 h at 4°C the lysates were spotted onto GF/C Whatman glass filters. Filters were washed once with 5% TCA, then with 70% ethanol and finally with acetone, dried and counted in a liquid scintillation counter. The [^{14}C]thymidine served as a control for DNA content of each sample.

RESULTS

Optimization of Fpg cleavage using damaged and undamaged purified DNA

To optimize use of the Fpg protein in the gene-specific damage and repair assay, DNA that had been damaged after purification using MB and light was incubated with varying amounts of Fpg for varying times. The reaction was complete at low enzyme:DNA ratios within minutes (Fig. 1A). Cutting in undamaged DNA was a linear function of enzyme concentration and a similar dependence on enzyme concentration was observed with damaged DNA (Fig. 1B): no plateau was reached. While this demonstrated the presence of non-specific cutting by the enzyme preparation, it was also observed that at low enzyme:DNA ratios the contribution of this non-specific cleavage to the total amount of cutting was minimal.

To verify that the plateau observed with the two lower enzyme:DNA ratios was due to completion of the reaction rather than enzyme inactivation the amount of specific cutting [(total cutting, damaged DNA) – (total cutting, undamaged DNA)] was calculated from the data in Figure 1B. Specific cutting was not a function of enzyme concentration (Fig. 1C), demonstrating that all accessible sites were cleaved. In addition, human mtDNA has 2.4 times more guanine in one strand, the heavy (H) strand, than in the light (L) strand. The amount of cutting in each DNA strand was proportional to the amount of guanine in each DNA strand, providing direct evidence that specific cutting was, as expected, occurring at sites of oxidized guanine.

These results demonstrated complete cutting with minimal non-specific cutting at enzyme:DNA ratios between 0.01 and 0.1 µg enzyme/µg DNA. This range was therefore used in subsequent gene-specific damage and repair experiments. (The same enzyme preparation was used for all experiments.) In addition, an aliquot of DNA treated with photoactivated MB was included in all subsequent gene-specific damage and repair assays as a positive control (to demonstrate that damage was indeed being recognized by the enzyme). A negative control consisting of DNA purified from untreated cells was also included in all assays.

Treatment of cells in culture

In order to identify an agent that would give high levels of 8-oxo-dG in mtDNA as measured by comparison of identically loaded –Fpg and +Fpg lanes on an alkaline gel followed by Southern blotting, several agents were rapidly screened for their ability to induce oxidative damage in the mtDNA of human fibroblasts. These

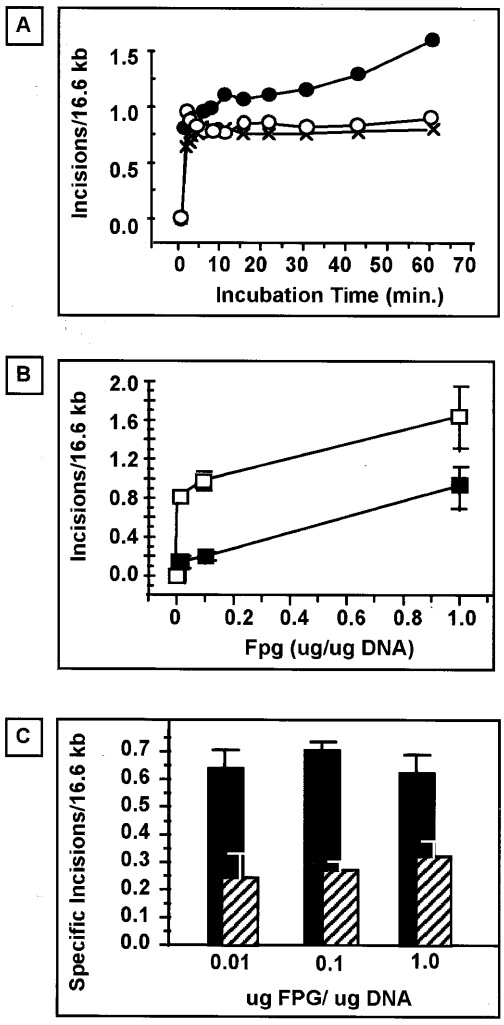

Figure 1. Optimal conditions for the Fpg assay give complete incision at sites of damage without non-specific cutting. Purified DNA was treated with photoactivated MB, then incubated with varying amounts of Fpg for varying times. Control DNA was from the same isolation, but was not treated with MB or light. (**A**) A plateau is reached at low enzyme:DNA ratios within minutes. Shown are mtDNA H strand, DNA damaged *in vitro*, means from 2–3 experiments. Error bars omitted for clarity. Key: X, 0.01 μg/μg; open circle, 0.1 μg/μg; closed circle, 1 μg/μg. (**B**) Total cutting is a function of enzyme concentration in undamaged (closed square) as well as damaged (open square) DNA, although the effect is negligible at the two lower enzyme:DNA ratios. Shown is 60 min mtDNA H strand, mean ± SEM, 3–5 experiments. (**C**) Specific cutting is not a function of enzyme concentration, showing that the reaction is not enzyme limited. This is shown by subtraction of the number of enzyme incisions seen for the undamaged mtDNA from the number seen for damaged mtDNA, as shown for the mtDNA H strand in (B) above. Note that oxidative damage is random and so more 8-oxo-dG occurs on the guanine-rich H strand (solid bars) than on the L strand (striped bars).

included hydrogen peroxide, alloxan, hypoxanthine/xanthine oxidase, photoactivated acridine orange and photoactivated MB (data not shown). Of the agents tested, photoactivated MB generated the most base damage in mtDNA without generation of excessive strand breaks or alkali-labile sites and without damaging cells so severely that they detached from the dish.

When cells were exposed to MB followed by a light exposure of 56 kJ/m^2 high levels of enzyme-sensitive alkali-resistant lesions were generated. This damage was rapidly repaired (Fig. 2). The presence of another type of lesion, perhaps strand breaks or alkali-labile sites, is suggested by the loss of mtDNA immediately after damage. This is seen in the 0 h band (Fig. 2) and in the decreased mtDNA:nuclear DNA ratio seen in all treated samples (Fig. 3). However, the assay is optimized for detection of enzyme-sensitive damage and thus low levels of repair of strand breaks or alkali-sensitive sites would not be detectable in these experiments. No lesions were observed in untreated cells or in cells treated with MB in the absence of light.

To ensure that the increase in the fraction of damage-free mtDNA with time was not caused by mtDNA replication occurring during the repair period, BrdU was included in the culture medium. This allowed separation of parental and replicated DNA by neutral CsCl centrifugation. It was found that treatment with 100 μM MB for 1 h even in the absence of light exposure prevented DNA replication throughout the remainder of the experiment; only the single, parental peak was observed in the CsCl gradient, both for total DNA and for mtDNA measured by dot blot (data not shown). Although it prevented replication, the cells are capable of synthesizing protein, as demonstrated by their ability to re-attach following trypsinization (data not shown). Furthermore, except for a minor initial inhibition, total RNA synthesis continued after treatment with MB in the absence of light (data not shown). From these observations we conclude that MB was not overtly toxic to the cells.

To ensure that a sub-class of cells with highly damaged mtDNA was not being lost during the repair period cell attachment following treatment with photoactivated MB was monitored using crystal violet staining and spectrophotometry, as well as microscopic observation. Cells remained attached to the dish and maintained normal morphology throughout the repair period (data not shown).

To determine if the change in the percentage of damaged mtDNA was due to repair or to selective degradation, the relative amounts of intact mtDNA and nuclear DNA were compared. If degradation was pronounced the amount of full-length mtDNA per cell should decrease. To determine if this was occurring the relative band intensities using nuclear (rDNA and c-*myc*) and mitochondrial probes were compared. By 10 h (during which 50% of the initial damage has been removed) the mitochondrial to nuclear DNA ratio had not changed (Fig. 3).

Human mtDNA restricted with *Eco*RI yields an 8.1 kb fragment containing the 2.6 kb ribosomal sequences and a 7.4 kb fragment containing several genes, including the sequence for cytochrome c oxidase I (shown graphically in Fig. 4A). The L strand is moderately transcribed in both regions, ~20–30 times between mtDNA replication cycles. The H strand, in contrast, is transcribed two times more frequently than the L strand in the ribosomal region and 10 times less frequently in the non-ribosomal region (29). The levels of initial damage are shown in Table 1. (Lesions were quantitated using the Poisson distribution as described in Materials and Methods.) As expected, the G-rich H strand shows more damage than the G-poor L strand, while differences in initial damage between regions were insignificant. Repair was measured in both strands of the fragment containing the ribosomal region using single-stranded probes specific for the ribosomal sequences. In the 7.4 kb non-ribosomal fragment, repair was measured in both strands using single-stranded probes specific for the cytochrome c oxidase I sequence. Repair rates were similar in the two DNA strands in both the ribosomal (Fig. 4B) and non-ribosomal (Fig. 4C) regions and both regions were repaired at the same rate (Fig. 4D). Treatment of the cells with MB in the presence of light resulted in a brief decline

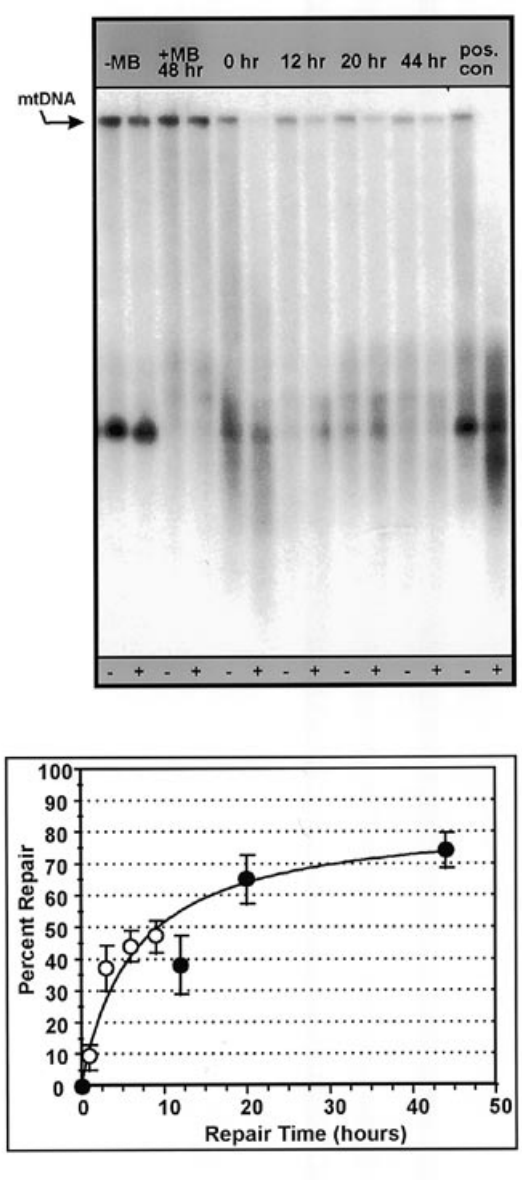

Figure 2. High levels of alkali-resistant Fpg-sensitive damage are rapidly removed from mtDNA by cultured human fibroblasts. (Top) Representative data (mtDNA H strand). Duplicate aliquots of total DNA isolated at various times after treatment of cells with photoactivated MB were either treated with Fpg (+) or not (–). To ensure even loading a master mix containing all components of the reaction was made for each DNA sample, from which two identical aliquots were drawn. Each aliquot, which contained 1 µg DNA/tube, was then loaded in the well in its entirety following a true (+Fpg) or mock (–Fpg) incubation with Fpg. [–MB], untreated cells; [+MB], cells treated with MB without photoactivation. The identity of the lower band is not known. (Bottom) Initial damage averaged 3 lesions/16 kb double-stranded mitochondrial genome and was in all experiments >1.5 lesions/genome. The initial rate of damage removal was 0.2 lesions/genome/h. Data is the mean ± SEM of 3–4 experiments. Averaged data from both strands of two mtDNA regions is shown. For repair points longer than 9 h (closed circles) parental and replicated DNA were separated by incorporation of BrdU and subsequent CsCl centrifugation.

in total RNA synthesis to 50% of control levels, but it returned to 67% after 8 h and to 78% within 24 h. Thus no evidence was seen for transcription coupled repair or of mitochondrial repair heterogeneity, suggesting that repair was not dependent on transcription.

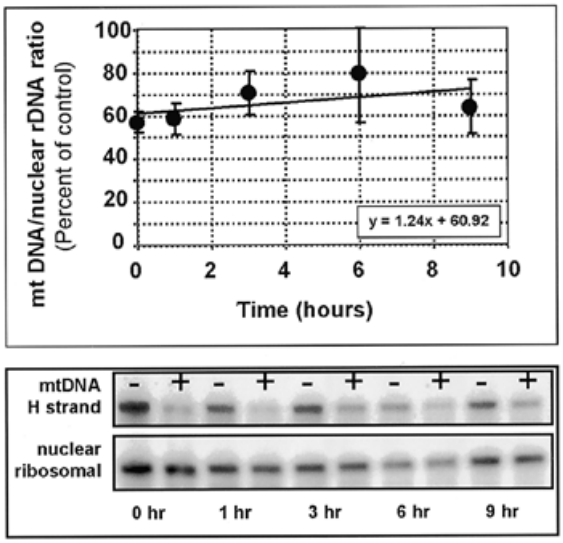

Figure 3. Removal of damage from mtDNA is not due to degradation of damaged mitochondrial genomes. (Top) For DNA that was not subjected to CsCl centrifugation band intensities in nuclear and mitochondrial sequences were compared in each Fpg-untreated lane of Southern blots as repair progressed. Data using the nuclear ribosomal probe is shown; the result does not differ from that obtained using a probe to the nuclear c-*myc* gene (not shown). Points represent mean ± SEM for 3 experiments, expressed as a percentage of control values obtained for untreated cells. Initial damage levels were sufficient to ensure that nearly all mitochondrial genomes contained damage; no decrease in the mtDNA:nuclear DNA ratio was seen within 10 h, during which time 50% of the initial damage had been removed. Thus damage is removed by repair. (Bottom) Representative data. Duplicate aliquots were either treated with Fpg (+) or not (–). Note that the relative intensities of the mitochondrial and nuclear bands in the –Fpg lanes do not decrease with time. Note also that damage was induced in the mitochondrial but not in the nuclear genome, as determined by comparison of the –Fpg and +Fpg band intensities. The top and bottom panels show the same blot; the lack of nuclear damage also demonstrates that the damage detected in mtDNA is not due to differences in loading between –Fpg and +Fpg lanes.

DISCUSSION

The results presented here are the first to show rapid and efficient removal of singlet oxygen-induced base damage from human mtDNA. This was not due to replication or cell loss nor to degradation of damaged genomes. There were no differences in the rate of repair between strands or between two different regions of the genome that differ substantially with regard to transcriptional activity. Although this study focused on oxidative base damage, it was noted that the mtDNA:nuclear DNA ratio was lower in cells treated with photoactivated MB than in untreated cells. There are several possible explanations, including the presence of single-strand breaks or of alkali-labile sites in the mtDNA.

Table 1. Levels of initial damage

Region	H strand (sites/10 kb ± SEM)	L strand (sites/10 kb ± SEM)
Ribosomal	0.94 ± 0.11	0.63 ± 0.05
Non-ribosomal	0.83 ± 0.09	0.54 ± 0.09

For initial damage determinations cells were lysed immediately following light exposure.

Endogenous Fpg-sensitive damage in the mtDNA of untreated cells was not observed in this study. Although it is a prevalent notion that there is more endogenous damage in mtDNA than in nuclear DNA, this is based on studies of mtDNA isolated directly from tissue. There are very few studies of the levels of endogenous

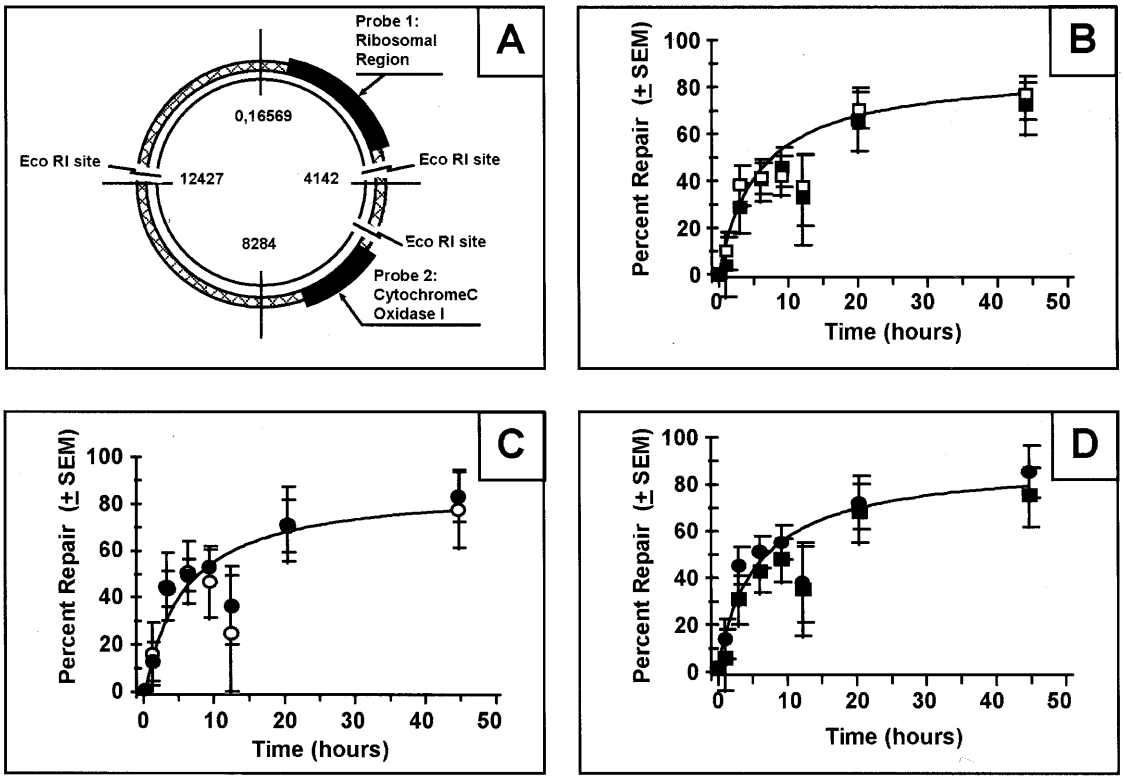

Figure 4. Repair rate does not differ between strands or regions of the mitochondrial genome that differ in transcriptional activity. (**A**) Human mtDNA restricted with *Eco*RI yields two ~8 kb fragments. The L strand is moderately transcribed in both regions, ~25 times between mtDNA replication cycles. The H strand, in contrast, is transcribed ~50 times in the ribosomal region and only twice in the non-ribosomal region in the same time period. Single-stranded probes to the ribosomal and cytochrome c oxidase I genes were used (shown in black). (**B**) Both strands are repaired at the same rate in the ribosomal region. Open symbol, L strand (initial damage 0.63 ± 0.05 lesions/10 kb); closed symbol, H strand (initial damage 0.94 ± 0.11 lesions/10 kb). (**C**) Both strands are repaired at the same rate in the non-ribosomal region. Open symbol, L strand (initial damage 0.54 ± 0.09 lesions/10 kb); closed symbol, H strand (initial damage 0.83 ± 0.09 lesions/10 kb). (**D**) Both regions are repaired at the same rate. Shown is the H strand. Square, ribosomal region; circle, non-ribosomal region.

oxidative damage in the mtDNA of cultured cells, however, relatively low levels have been reported in mtDNA from cultured HeLa cells (37). This is in agreement with the findings presented here. Nevertheless, to demonstrate that oxidative DNA damage would indeed be recognized if present purified DNA was treated with photoactivated MB and assay conditions were established which gave complete and specific cutting. These conditions were used in all subsequent assays.

There is a great deal of controversy concerning the actual level of 8-oxo-dG in mtDNA *in vivo* (38). In general, however, the reported levels are quite high, of the order of two per mitochondrial genome, and it is generally thought that there is ~10-fold more 8-oxo-dG in mtDNA than in nuclear DNA (5,39). Another difference between nuclear and mtDNA is that in comparison with nuclear DNA mtDNA is infrequently transcribed. For example, while a mitochondrial ribosomal sequence is transcribed ~50 times between cell divisions, a nuclear ribosomal sequence is transcribed ~5100 times (31). If high levels of 8-oxo-dG are indeed present *in vivo*, repair might occur only when the genome is being transcribed or replicated, with damage accumulating in the bulk of the mtDNA at other times. In this way errors in transcription and replication would be avoided despite a high steady-state level of damage. Transcription coupled DNA repair is known to occur in the nucleus, where there are at least two pathways for this important process. One involves nucleotide excision repair (40). Another pathway, more recently discovered, involves base excision repair: the repair of thymine glycol, another common oxidative DNA lesion, is transcription coupled in mutant cells that are incapable of nucleotide excision repair (30). The possibility that transcription coupling might exist in mitochondria has never before been examined. While mitochondrial transcription is via a proprietary polymerase and repair factors requiring interaction with the nuclear RNA polymerase TFIIH would be unlikely to function in these organelles, novel mechanisms to allow mitochondrial transcription coupled repair might be involved.

Studies in nuclear DNA show that repair rate is related to transcription rate (41). In the mitochondria the ribosomal heavy strand is transcribed ~50 times between replicative cycles, compared with 20–30 times for the opposite strand in that region; the non-ribosomal region of the H strand is transcribed only two times per replicative cycle, compared again with 20–30 times for the L strand (29). Differences in the rate of repair would be expected if repair were transcription coupled in mitochondria. No differences were observed, suggesting that most or all of the lesions are repaired independently of transcription.

While it was impractical to assay the effects of photoactivated MB on mitochondrial RNA synthesis in this system, total RNA synthesis briefly declined to 50% of control levels, returning to 78% within 24 h. Even if the same or greater inhibition is occurring within the mitochondria, the argument that most or all of the lesions are repaired independently of transcription would, in fact, be strengthened. Thus these results do not support the hypothesis that

repair is only occurring in conjunction with transcription or replication.

A useful feature of the photoactivated MB model in the study of mitochondrial repair is that it allows us to mimic the high mitochondrial to nuclear damage ratio reported to exist *in vivo*. MB is a cell permeant cation and is in equilibrium in the cells with uncharged leukomethylene blue (42), suggesting that the photoactive form would tend to localize in the mitochondria. Although we did not compare the relative concentrations of the colored photoactive form of MB in the mitochondria versus the nucleus, we did verify that in intact cells photoactivated MB caused damage in mtDNA without causing enough nuclear DNA damage for it to be detected in this assay. (Fig. 3 shows a representative blot in which mtDNA and, subsequently, nuclear ribosomal DNA were probed. Similar results were obtained when a probe for the single copy c-*myc* gene was used; data not shown.) Although other explanations would be possible for this phenomenon, the simplest is that there is, indeed, preferential mitochondrial localization of the photoactive MB.

Due to the long held belief that mitochondria lack the capacity for DNA repair, concerns have been raised in the literature that damage removal from mtDNA is due to mtDNA replication or to cell detachment and death. In addition, it has been noted that while the removal of damage by a glycosylase could be the first step in repair of a damaged mitochondrial genome, it could also be the first incision in its degradation (5,12,28). Here, for the first time, all three of these concerns are explicitly addressed in a single study. Care was taken that neither replication nor cell loss was occurring. In addition, since the level of damage induced in the current study ensured that nearly every genome was damaged, a clear decrease in mtDNA content of the cells would be observed if removal of these sites was due to degradation. Following damage the mtDNA:nuclear DNA ratio remained constant. Taken together, these results demonstrate that damage removal is via a DNA repair pathway.

It is interesting to compare the rate of repair for base damage seen here for human cells with the rate of repair seen in earlier studies in rodent cells. In 3 h ~0.5 Fpg sites/ss genome were removed in the current study, compared with ~0.8 sites/ss genome in 4 h for rat RINr 38 cells (21) or ~0.6 Fpg sites/ss genome in 4 h for hamster B11 cells (26). Thus although higher levels of base damage were achieved in this study, early repair rates were similar between the studies, averaging between 0.1 and 0.2 sites/ss genome/h.

The rate of repair in different regions of the genome did not correlate with the expected transcription frequency of these regions, suggesting that damage is removed uniformly as well as rapidly. These findings are difficult to reconcile with the high levels of oxidative DNA damage that are generally observed in mtDNA in tissue. Is the oxidative stress in tissue so high that even an efficient repair system is unable to maintain low levels of damage? Alternatively, is the damage confined to a small subset of mitochondria, perhaps those destined for degradation, and thus not representative of the bulk of the genomes? An even more intriguing possibility is that oxidative damage may not, in fact, be higher in mitochondrial than in nuclear DNA. Reported levels of 8-oxo-dG in mtDNA isolated from animal tissues vary by three orders of magnitude (38). Perhaps the measured levels do not reflect the *in vivo* levels; for example, mitochondrial isolation from intact tissue could be postulated to impose an oxidative stress that would, in itself, lead to damage to mtDNA. Studies are underway in our laboratory to address these questions.

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