

Mapping frequencies of endogenous oxidative damage and the kinetic response to oxidative stress in a region of rat mtDNA

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ABSTRACT

Genomic DNA is constantly being damaged and repaired and our genomes exist at lesion equilibrium for damage created by endogenous mutagens. Mitochondrial DNA (mtDNA) has the highest lesion equilibrium frequency recorded; presumably due to damage by H₂O₂ and free radicals generated during oxidative phosphorylation processes. We measured the frequencies of single strand breaks and oxidative base damage in mtDNA by ligation-mediated PCR and a quantitative Southern blot technique coupled with digestion by the enzymes endonuclease III and formamidopyrimidine DNA glycosylase. Addition of 5 mM alloxan to cultured rat cells increased the rate of oxidative base damage and, by several fold, the lesion frequency in mtDNA. After removal of this DNA damaging agent from culture, the single strand breaks and oxidative base damage frequency decreased to levels slightly below normal at 4 h and returned to normal levels at 8 h, the overshoot at 4 h being attributed to an adaptive up-regulation of mitochondrial excision repair activity. Guanine positions showed the highest endogenous lesion frequencies and were the most responsive positions to alloxan-induced oxidative stress. Although specific bases were consistently hot spots for damage, there was no evidence that removal of these lesions occurred in a strand-specific manner. The data reveal non-random oxidative damage to several nucleotides in mtDNA and an apparent adaptive, non-strand selective response for removal of such damage. These are the first studies to characterize oxidative damage and its subsequent removal at the nucleotide level in mtDNA.

INTRODUCTION

DNA damage arising from reactive oxygen species (ROS) is the most frequent type of injury encountered by nuclear DNA in aerobic cells (1) and is even more frequent for mitochondrial DNA (mtDNA) (2–4). This damage includes frank strand breaks

and oxidative base damage. The oxidative base damage is sensitive to strand cleavage by the *Escherichia coli* repair enzymes, endonuclease III (Endo III) and formamidopyrimidine (FAPy) DNA glycosylase (FPG). ROS, including hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen (5), are all products of normal oxidative phosphorylation (6,7) and are presumed responsible for the high steady state levels of oxidative lesions. mtDNA is particularly vulnerable to attack by these molecules since it lacks protective histones and is in close proximity to the electron transport chain which leaks 1–2% of the oxygen metabolized as superoxide and hydrogen peroxide (8). The strands of DNA in the circular mitochondrial genome have been designated as ‘heavy’ and ‘light’ based on their buoyant density in CsCl density gradients primarily resulting from differences in the distribution of guanines between the strands (9). The genetic information for the 13 mRNA transcripts is also asymmetrically distributed with 12 of the 13 being found on the heavy strand; however, both strands are transcribed and mitochondrial genes have no introns (for reviews see 10,11). While these organelles are unable to repair UV-induced cyclobutane dimers and several other bulky lesions predominantly repaired via the nucleotide excision repair pathway in the nucleus, they are proficient at removing oxidized and alkylated lesions, damage which is typically repaired by base excision repair (12). While the high rate of production of oxidative DNA lesions in mitochondria is known, knowledge of their repair kinetics remains uncertain.

Ideally, a repair experiment is carried out with a mutagen such as UV light. In seconds, this agent can introduce high frequencies of a novel lesion such as cyclobutane pyrimidine dimers into the DNA of a population of cells. By sampling the population’s lesion frequency as it decreases with time, one obtains a measure of lesion repair wherein the rate constant for such repair remains constant during the repair experiment. Measuring repair of oxidative DNA damage, oxidized bases and single strand breaks in mtDNA of rat cells deviates from the ideal repair experiment primarily because the steady state level of oxidative DNA lesions is already rather high, about three 8-oxo-deoxyguanines per 10 000 bases in rat liver mtDNA, before the experiment begins (4). This is 10-fold higher than that of nuclear DNA (13) and is well within the sensitivity range for quantification by the Southern blot technique (14) or the

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sensitivity limit of one lesion per 20 kb required for lesion frequency mapping by ligation-mediated PCR (LMPCR) (15).

Lesion equilibrium frequency (Leq) describes the steady state condition during which the rate at which lesions are generated is equal to the rate at which they are removed. The repair rate for oxidative base damage in mitochondria is roughly similar to that for nuclei (16–18), so the high Leq values found in mitochondria (2–4) are presumably due to a high rate of damage. Previously, we transiently increased the oxidative stress in cultured rat cells with alloxan. This agent enters the cell, undergoes redox cycling and generates many of the same ROS that are normally generated in mitochondria (19). We showed that alloxan treatment elevated the frequency of oxidized bases sensitive to Endo III and FPG. After alloxan removal, the frequency of oxidized bases decreased rapidly over a few hours and returned to that of mitochondria from untreated cells. This indicates that repair of these lesions is extremely efficient (16). In fact, this repair occurs at a faster rate than does repair of any other type of damage observed previously in mitochondria (18,20,21). We now address whether or not such repair occurs in a strand-specific manner and, through the use of LMPCR, measure damage and repair at the nucleotide level of resolution along a 200 bp sequence of mtDNA. The region analyzed includes a breakpoint involved in the 5 kb ‘common’ deletion associated with populations of mitochondria from aged or diseased individuals.

MATERIALS AND METHODS

Cell culture

RINr 38, a rat insulinoma cell line with a doubling time of ~52 h (22) was maintained in Medium 199 (GIBCO) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 2 mg/ml dextrose and 0.05 mg/ml gentamicin. For the repair studies, $\sim 1.5 \times 10^7$ cells were plated per 150×25 mm dish. Cultures were used for experiments ~3 days after plating when RINr 38 cells had grown to confluency.

Cell viability studies

Cell viability was determined via an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay that measures cell viability based on the ability of the mitochondrial enzyme succinate dehydrogenase to cleave MTT and thereby produce visible formazan crystals (23). For these studies cells were seeded in 24 well plates at 5×10^4 cells per well and maintained in culture for 2–3 days. Cells then were treated with 5 mM alloxan (5,6-dioxyuracil; Sigma) for 1 h in Hank’s balanced salt solution (HBSS) + 1% citrate buffer (pH 4.2) (20). Control cultures were exposed to HBSS + 1% citrate only. After 1 h, the cells were rinsed with HBSS and given fresh media for 2, 4 or 24 h or were immediately incubated for 20–30 min with MTT (0.5 mg/ml in HBSS). Additionally, since RINr 38 is an insulin secreting β -cell line, we have previously performed studies measuring insulin secretion via radio-immunoassay (RIA). These studies served as an indicator of cell viability based on a physiological event (i.e. insulin secretion) and are reported elsewhere (16).

Drug preparation and exposure

Alloxan was dissolved in HBSS + 1% citrate buffer at various concentrations from 1 to 10 mM. RINr 38 cells were rinsed with HBSS, then exposed to alloxan for 1 h in a 5% CO_2 , 37°C incubated environment. Control cultures were exposed to HBSS

+ 1% citrate buffer under the same conditions. After incubation for 1 h, cells were either lysed immediately or rinsed in HBSS then allowed to repair in cell culture medium.

Assay for strand-specific damage and repair assessment

RINr 38 monolayer cultures were exposed to alloxan as described above. Cells then were incubated in fresh culture media and lysed in 10 mM Tris, 1 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 0.3 mg/ml Proteinase K overnight at 37°C. High molecular weight DNA was carefully extracted in the absence of phenol to reduce artifactual introduction of oxidative damage. Briefly, the lysates were adjusted to 1 M NaCl with 5 M NaCl and were rocked gently for 10 min. DNA was extracted three times using an equal volume of chloroform:isoamyl alcohol (24:1) with 10 min rocking and centrifugation at 800 g between each extraction. The DNA was precipitated with 2.5 M ammonium acetate and 2 vol ethanol, resuspended in d- H_2O , treated with DNase-free RNase (~1.0 μ g/ml) for 3 h at 37°C and reprecipitated. Following resuspension in d- H_2O , purified DNA was terminally digested with *Bam*HI (5 U/ μ g DNA), precipitated, resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and quantified using Hoechst 33258 dye and a Hoefer TKO 100 Mini-Fluorometer and TKO standards kit (Hoefer Scientific Instruments, San Francisco, CA). Samples containing 7 μ g DNA were heated at 65°C for 20 min and then cooled at room temperature for 20 min. Predetermined amounts of Endo III or FPG were added to appropriate samples which then were incubated at 37°C for 15 min (16). These enzymes produce single strand breaks at specific sites in oxidatively damaged DNA. Specifically Endo III recognizes an array of oxidized, reduced, fractured and ring contracted forms of pyrimidines, while FPG detects purines with ruptured imidazole rings (FAPy lesions) and 8-oxo forms of adenine and guanine (24,25). These enzymes were kindly provided by Drs Yoke W. Kow and Richard Cunningham (Endo III, Emory University, Atlanta, GA and University at Albany, State University of New York, Albany, NY, respectively) and Dr Arthur Grollman (FPG, State University of New York, Stony Brook, NY). A sodium hydroxide solution then was added to a final concentration of 0.1 N and samples were incubated an additional 15 min at 37°C. This produced single strand breaks at all abasic or sugar-modified sites in the DNA. Gel electrophoresis and vacuum transfer were carried out as described previously (16). Following a prehybridization, membranes were hybridized with single-strand, PCR-generated mitochondrial probes complementary to either the heavy or light strand for ~16 h at 60–65°C. Membranes then were washed according to the manufacturer and placed under phosphor-imaging screens and scanned for detection of hybridization bands. Resultant band images were scanned using a BioRad GS-250 molecular imager. Break frequency was determined using the Poisson expression ($s = -\ln P_0$, where s is the number of breaks per fragment and P_0 is the fraction of fragments free of breaks) (14). Percent repair at time t was calculated by subtracting the breaks at time t from the breaks present at 0 h and dividing by 0 h breaks. The resulting value then was converted to % by multiplying by 100. Membranes were stripped in several cases and reprobbed with the opposite strand to ensure exact comparisons and to compensate for any interexperimental variability.

Slot blot analysis

To ensure that after alloxan exposure samples were not selectively enriched in mtDNA, slot blot analysis was performed. Samples

of DNA from individual repair experiments were quantitated fluorometrically and adjusted to 10 ng/ μ l in d-H₂O. Samples were aliquotted into 100 ng fractions and treated with a final concentration of 0.3 M NaOH to denature the DNA. Aliquots were then blotted onto a Nytran plus nylon membrane (Schleicher and Schuell Inc., Keene, NH) using a Schleicher and Schuell Minifold II slot blotting apparatus. The DNA was linked to the membrane in a GS Gene Linker (BioRad) and probed with a mitochondrial or insulin probe at 65°C for ~20 h. Hybridization images were scanned as described above and band intensities were determined. This allowed direct comparison between the amount of nuclear and mtDNA present at each time point of repair and in the 'controls'.

Assay for detecting damage and repair at the nucleotide level

The LMPCR procedure (Pfeifer *et al.*) (26,27) was adapted for mtDNA studies. Following treatment with alloxan, extraction, digestion and quantitation as described previously, 5 μ g aliquots of samples were treated with Endo III and FPG for 1 h at 37°C. Untreated samples were also incubated at 37°C for 1 h in buffer only. Extractions, primer extension and ligation were all performed according to Pfeifer *et al.* (26,27) with the exceptions that 100–500 ng of sample and 800 fmol/ μ l primer 1 (see Fig. 4 for sequences of primers 1, 2 and 3) were used. Primer set 'A' is used to obtain information about damage and repair in the heavy strand, while set 'B' is used for the light.

Following ligation, the reactions were precipitated with 0.3 vol 10 M ammonium acetate, 6.6 mM EDTA, 20 μ g glycogen and resuspended in 50 μ l d-H₂O. PCR amplification was performed as described (26,27) with the exception of the use of 2.5 pmol/ μ l of primer 2 and the longer oligonucleotide of the asymmetric linker. The increased amounts of primers used in the ligation and amplification steps are designed to compensate for the increased number of mtDNA templates present in cells as compared to single copy nuclear genes. Following amplification, each reaction received 25 μ l of a mix containing 60 mM EDTA, 1.6 M sodium acetate (pH 5.2). Chloroform extractions then were performed and samples were precipitated via addition of 2.5 vol 100% ethanol. Pellets were air-dried and resuspended in a formamide dye solution (57% deionized formamide, 7 mM EDTA, 0.7 mg/ml xylene cyanol FF and 0.7 mg/ml bromophenol blue). Samples then were electrophoresed, blotted and UV cross-linked to the membrane (Hybond-N+, Amersham) as previously described (26,27). Hybridization was performed overnight at 60–65°C with a single-strand probe generated using primer 3 for the appropriate strand and the PCR program described for single-strand probe preparation below. The probe is 202 bp in length and will allow observation of the complementary sequence in RINr 38 mtDNA. The membrane was washed the following day at 62°C until background levels were sufficiently reduced (typically 10 min in 20 mM NaHPO₄ buffer, pH 7.2, 2.5% SDS, 1 mM EDTA, 0.25% BSA followed by two 7 min washes in 20 mM NaHPO₄ buffer, pH 7.2, 1% SDS, 1 mM EDTA). The membrane was exposed to Kodak XAR film which was developed after 1–4 h at –70°C. Subsequently, it was exposed to a phosphor-imaging screen as described for the Southern blots and specific bands analyzed using the Phosphor-Analyzer™ (BioRad) program.

Table 1. RINr 38 cell viability studies based on MTT assays

Time pts (h)	Viability (%)
Control	96.5 \pm 0.9
0	94.3 \pm 1.0
2	95.1 \pm 1.6
4	94.4 \pm 1.2
24	95.1 \pm 0.7

Cells were seeded, maintained and treated with 5 mM alloxan. Following 1 h exposure, cells were either incubated immediately for 20–30 min with MTT (0.5 mg/ml in HBSS) or placed in media for 2, 4 or 24 h. Percent viability was determined by dividing the # of live cells by the total # of cells and then multiplying by 100.

Probes

The probes used to hybridize to specific strands of mtDNA were generated via single-primer PCR from a mouse mtDNA sequence. Briefly, a 745 bp template was generated and gel purified from a low melt agarose gel (1.8%) using the following primers: 5'-GCAGGAACAGGATGAACAGTCT derived from the light strand and 5'-GTATCGTGAAGCACGATGTCAAGG-GATGAG derived from the heavy strand. One of the above primers then was used in PCR with the purified template to generate a ³²P-labeled single strand probe complementary to the mtDNA strand of interest. The PCR protocol used to generate these probes was modified from Mullenders (28) and consists of 30 cycles of the following steps: 1 min denaturing at 95°C, 2 min annealing at 65°C and 3 min extension at 72°C. The 745 bp product recognizes a 10.8 kb restriction fragment when hybridized to rat mitochondrial DNA digested with *Bam*HI. For the slot blot studies both primers were used to generate double strand probes. The insulin probe generated for the slot blot studies was obtained using the above PCR protocol and an insert in *pBR322* which contains the rat insulin I gene sequence. The primers used were 5'-CCGTCGTGAAGTGGAGGACCCGCAA from the sense strand and 5'-GTTGCAGTAGTCTCCAGTTGGTAG derived from the antisense strand.

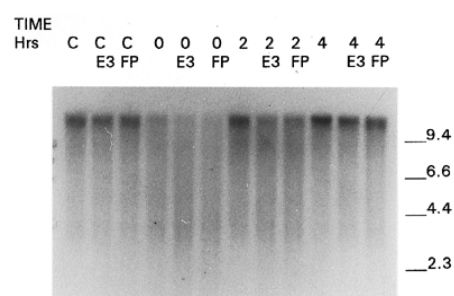


Figure 1. Repair of heavy strand mtDNA in RINr 38 cells following exposure to 5 mM alloxan. RINr 38 cells were exposed to 5 mM alloxan for 1 h and lysed immediately or rinsed and placed in culture media to allow time for repair. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Bam*HI. Samples were treated as described in the text prior to Southern blot analysis and hybridization with a PCR generated probe complementary to the mtDNA heavy strand. 'C' lanes contain control samples, while '0', '2' or '4' above lanes represents repair time allotted following drug exposure. E3 or FP below repair times, samples incubated with Endo III or FPG, respectively. These data are summarized in Table 2.

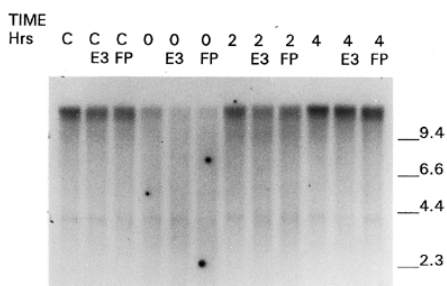


Figure 2. Repair of light strand mtDNA in RINr 38 cells following exposure to 5 mM alloxan. Methods are the same as those described in Figure 1 with the exception that the probe used in these studies was complementary to the mtDNA light strand. These data are summarized in Table 2.

RESULTS

Previously, we have shown that a concentration of 5 mM alloxan produces measurable levels of mtDNA damage without decreasing insulin secretion from the RINr 38 cells (16). Although this finding demonstrates that the cells remained physiologically intact, it does not definitively rule out that there was a significant loss in cell viability. Therefore, to assess cell viability based on mitochondrial function we conducted MTT assays. Table 1 shows that viability at all time points investigated is >90%. Additional viability studies indicated that neither 1 nor 5 mM alloxan was toxic to the cells, while 10 mM produced an LD₇₅ at 24 h (data not shown). Thus, 5 mM alloxan is not overtly toxic to the cells and does not appreciably decrease mitochondrial enzyme function.

To examine the kinetics and strand-specificity of global repair of oxidative damage to mtDNA, cells were treated with 5 mM alloxan for 1 h which, by increasing the rate of DNA damage, forced the lesion frequency toward a greater Leq value. Alloxan was removed and the kinetics of the lesion frequency’s return toward normal equilibrium values was measured at 1, 2, 4 and 8 h (data not shown). Figures 1 and 2 are representative autoradiographs depicting return to Leq at 2 and 4 h in the heavy and light strands, respectively. Return to Leq is quantified in Table 2 from at least four experiments for each strand. Initial strand breaks are

0.67 and 0.74 breaks per 10.8 kb in the alkali-treated samples for the heavy and light strands, respectively. The number of initial breaks in the 10.8 kb mtDNA fragment for the enzyme treated samples range between 0.72 and 0.84 in the heavy strand and between 0.83 and 0.91 in the light strand. No significant differences in initial strand break frequency exist for samples receiving the same treatment on either strand. Repair is extremely rapid for both breaks and oxidized bases, being complete prior to 4 h.

Table 2. Repair of oxidative damage in the heavy and light strands of RINr 38 mtDNA

Repair times (h)		Repair (%) Heavy	Light
1	alk	46.7	43.1
	alk + E3	36.9	25.1
	alk + FP	25.6	14.4
2	alk	62.8	65.4
	alk + E3	63.2	60.2
	alk + FP	75.5	60.3
4	alk	>100	>100
	alk + E3	>100	>100
	alk + FP	>100	>100

Autoradiographic bands representing a 10.8 kb mtDNA fragment were densitometrically scanned. Break frequencies were determined and used to calculate % repair as described in the text. These data represent information from at least four separate experiments. alk, frank strand breaks as either induced breaks or repair intermediates and alkali sensitive sites, including abasic sites and modified sugar residues; alk + E3 or alk + FP, alk lesions + oxidized pyrimidines or purines, respectively.

In an effort to ensure that the measured damage and its subsequent removal did not reflect artifactual changes due to replication or alteration of mtDNA content, we conducted slot blot analysis to quantify changes in the ratio of nuclear gene copy number/mtDNA copy number. For these experiments we used the insulin gene as a nuclear sequence and blotted 100 ng of total genomic DNA onto Nytran plus nylon membranes. Figure 3

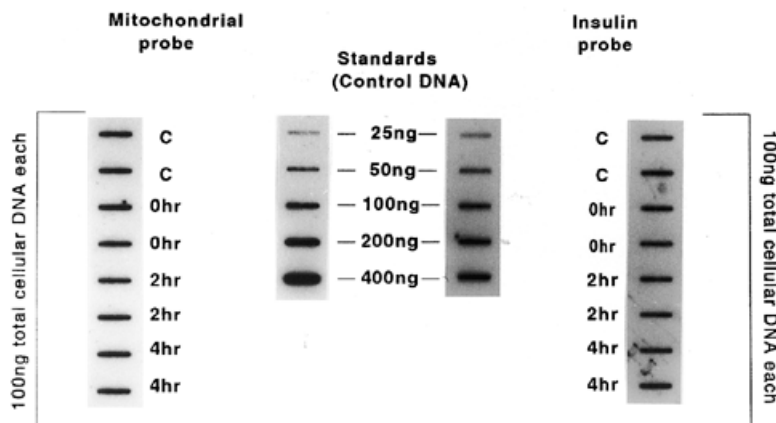


Figure 3. Slot blot analysis of nuclear (insulin) and mtDNA content. Samples were treated with 5 mM alloxan and allowed repair as described in the text. DNA was isolated, restricted and 100 ng of total cellular DNA from each time point was blotted onto a nylon membrane. Varying amounts of DNA were blotted to ensure against nonlinear hybridization patterns. Membranes were hybridized with appropriate probes and scanned with a Phosphor-Imager™ to quantify resultant bands.

Primer Sequences

Set A
A1 5'-GACCATTATTATGTTCCCATCAA-3'
A2 5'-ATTCCCATCATCAGAAGCGCTAATCAGC-3'
A3 5'-CAGCAACCGACTACACTCATTTCAACACTG-3'

Set B
B1 5'-TGTGTCGGAAACCTAGAATTACG-3'
B2 5'-GCTCCTGCTCATAGGGGGATGGCT-3'
B3 5'-GGCTATGCTTAGGTCTATAGATAGCTGAGTGG-3'

Linker Primers (together=assymetric linker)
LP25 5'-GCGGTGACCCGGGAGATCTGAATTC-3'
LP11 5'-GAATTCAGATC-3'

Figure 4. Nucleotide sequence of primers used in LMPCR.

displays an autoradiograph following probing for the mtDNA or insulin sequence. Upon scanning of the blots using a Bio-Rad Phosphor-ImagerTM, no difference in insulin/mtDNA ratio was apparent at any of the time points studied. These results indicate no artificial selection of mtDNA and suggest that the alloxan-induced damage has up-regulated the mitochondrial repair proficiency.

LMPCR (Figs 5 and 6) was used to visualize the patterns of breaks and oxidative damage in rat mtDNA along both strands of an ~200 bp sequence. The lesion equilibrium pattern of breaks plus base damage (C+ lane) showed several hot spots and relative signal intensity was G > T > C > A. This pattern is unlike that of nuclear DNA which does not show such prominent hot spots and wherein the signal frequency is G > C >> T > A (unpublished data). The mtDNA pattern at Leq (C+ lane) is quite similar to the lesion pattern after alloxan treatment (0+ lane). A few exceptional positions deviated from this generalization, such as thymidine 8121 on the light strand which is almost undamaged at Leq, but became heavily damaged by alloxan treatment. This position, however, was repaired rather quickly, returning to near Leq levels of damage after only 2 h (2+ lane). A map of the damage frequency is shown in Figure 7. The bars represent arbitrary values on a scale of 1–6 based on intensity of bands scanned with the Phosphor-ImagerTM.

The data show several areas of enhanced damage (i.e. between positions 8234–8241 on the heavy strand and positions 8082–8089 on the light strand). The 'hot spots' registering a 3 or above on the 1–6 scale were used to determine which base(s) were receiving a majority of damage and to surmise if there was a correlation between damaged sites and the nucleotide immediately 5' or 3' to the damaged spot. The data in Table 3 indicate that while T has the highest overall number of severely damaged bases in the 160 bp fragment shown in Figure 5, G > T > C > A is the order of damage frequency based on proportion of nucleotide present. This frequency is obtained by dividing the number of severe damage hits at a particular nucleotide by the total number of that nucleotide present in the sequence analyzed (i.e. # of severely damaged Gs/total # of Gs present in fragment). Additionally, it was determined that although cytosines comprise only 18% of the bases present in both strands, they were present as the base immediately 3' to the damage in 33% of the cases. Conversely, As which represent nearly 1/3 of the total bases, were present as the immediate 3' base only 20% of the time. It was also revealed that Ts were present 30% of the time immediately 5' of the damaged base and in 30% of the cases as the adjacent 3' base. This correlates well with the percent of Ts present (~33%) in the sequence.

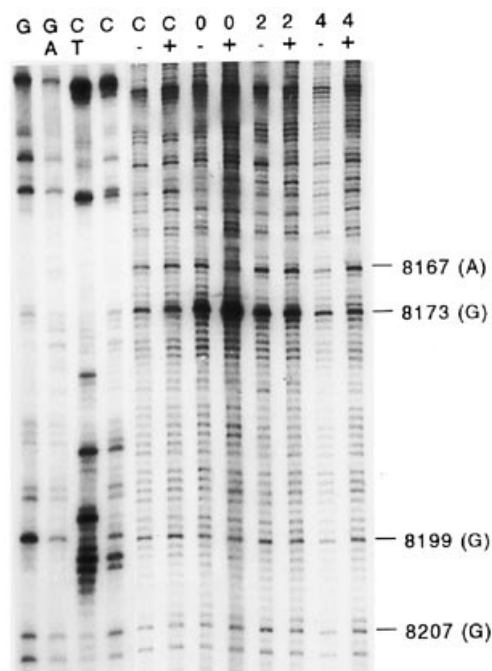


Figure 5. Repair of oxidative damage at nucleotide resolution in a sequence of the 'heavy' strand of mtDNA. RINr 38 cells were treated with 5 mM alloxan for 1 h and lysed immediately or allowed 2 or 4 h repair times. DNA was isolated, digested and 300 ng subjected to LMPCR. C, control samples; 0, 2 and 4, repair time in hours; +, treatment with Endo III and FPG enzymes. Maxam-Gilbert sequence ladders are on the left while the numbers and letters to the right represent position and base within the heavy strand of mtDNA, respectively.

Table 3. Frequency of severe oxidative damage along a 160 bp fragment of mtDNA

Nucleotide damage frequencies	
Base	% of base with severe damage ^a /# of base in fragment
G	18/57
A	11/103
T	15/103
C	16/57

Data was obtained by scanning hybridization patterns of gels following LMPCR of the mtDNA fragment as discussed in the text.

^aBases designated as having 'severe' damage were those which scored a 3 or above on the scale of 1–6 used to construct the nucleotide damage map shown in Figure 7.

Figures 5 and 6 show that most of the sites are repaired (return to Leq frequencies) quite rapidly. Repair rates are heterogeneous and were calculated for selected, commonly damaged sites (Figs 8 and 9). Percent return to Leq frequencies at 2 h ranges from ~30 to 75% with the exception of G(8173) in the heavy strand, which is almost completely repaired by 2 h. At 4 h several base positions have lesion frequencies lower than they have at Leq (% return >100%). This observation is consistent with our previous

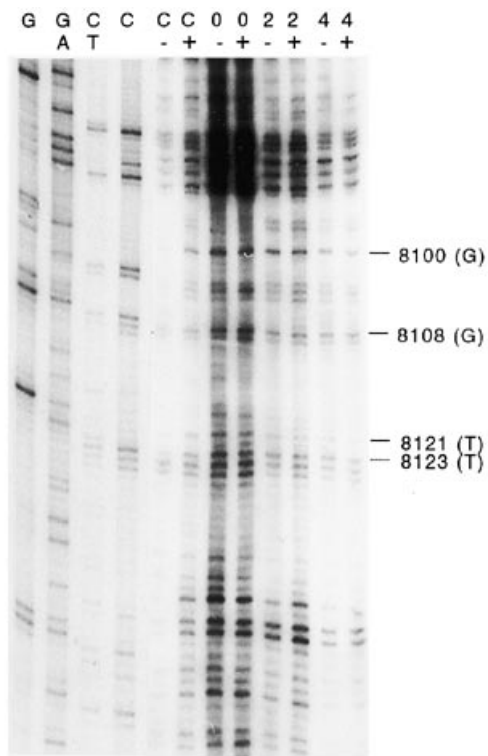


Figure 6. Repair of oxidative damage at nucleotide resolution in a sequence of the 'light' strand of mtDNA. RINr 38 cells were treated with 5 mM alloxan for 1 h and lysed immediately or allowed 2 or 4 h repair times. DNA was isolated, digested and 300 ng subjected to LMPCR. Symbols above the lanes are as those described in Figure 5. Numbers and letters to the right represent position and base within the light strand of mtDNA, respectively.

Southern blot experiments (16) and with data in Figures 1 and 2 and calculations in Table 2.

DISCUSSION

A prerequisite for successful repair analyses is a system in which an exogenous mutagen can introduce a measurable lesion density without being overtly toxic to the cells being studied. The MTT viability studies shown in Table 1 as well as the previously published RIA studies (16) indicate that 5 mM alloxan is not a toxic dose to RINr 38 cells while it induces sufficient levels of mtDNA damage above Leq values so that repair rates can be measured. The issue of viability following oxidative damage has been an obstacle in accessing repair in nuclear sequences. That mtDNA is highly susceptible to oxidative damage at a non-lethal concentration provided a unique opportunity to study not only DNA damage and 'hot spot' formation using LMPCR, but also subsequent removal of this damage at specific nucleotide positions.

Mutagen-induced replication of mtDNA, degradation or selective isolation of mtDNA would make relating changes in breaks per μ g of mtDNA to repair rates difficult. To rule these factors out, we conducted a slot blot analysis to determine alloxan-induced changes in the copy ratio of nuclear/mtDNA (Fig. 3). This ratio was found

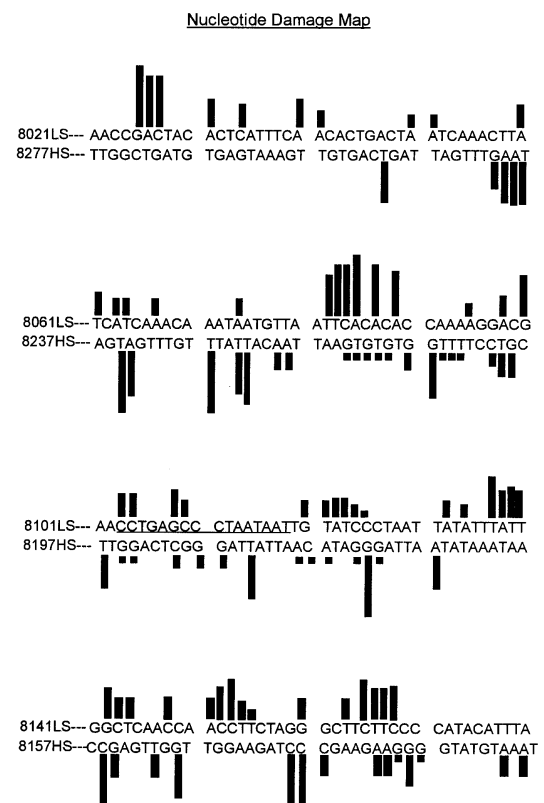


Figure 7. Nucleotide damage map. The above map represents a 160 bp stretch within the 202 bp fragment of mtDNA analyzed. The darkened bars above and below specific nucleotides indicate arbitrary values on a scale from 1 to 6 for both frequency and intensity of damage observed for the particular bases. The damage represented was observed immediately after 1 h exposure to alloxan and depicts the relative frequency of breaks + base modifications. The underlined region denotes the 16 bp repeat previously reported as the site where the 5 kb 'common' deletion begins. Numbers to the left represent base position in the rat mitochondrial genome; LS and HS, light strand and heavy strand, respectively.

to remain constant. To rule out an artifact in the Southern blot technique, we performed assays to test for degradation of samples, artifactual positional effects of blotting and hybridization. The initial tests revealed no degraded 'control' DNA and no artificial effect associated with blotting and hybridizing in different lanes or on different types of membranes (data not shown). We conclude that the alloxan-induced changes of lesion frequency measured herein are entirely due to damage and repair of mtDNA.

The initial repair studies demonstrate that after alloxan induction of additional oxidative DNA damage, there is a rapid return to steady state levels of strand breaks and oxidative base damage in both the heavy and light strands of mtDNA (Figs 1 and 2; Table 2). As shown in Table 2, there is little difference in repair levels in the two strands. The % repair at 1 h seems to indicate an initial lag in repair of enzyme-sensitive sites when compared to alkali-labile sites. However, this apparent disparity is not significantly different based on a Student's *t*-test and the % repair is virtually the same for all detected damage at 2 and 4 h. While this finding cannot be used to rule out the possibility that transcription influences repair in mtDNA, because both strands are transcribed, it does show that there are no conformational

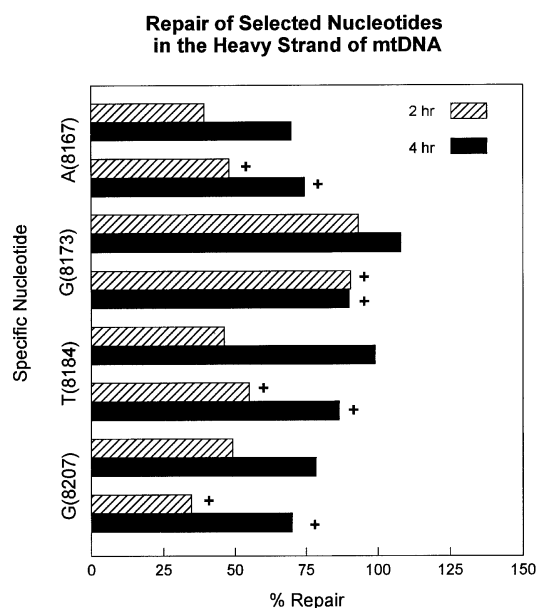


Figure 8. Repair rates for certain damaged nucleotides in the 'heavy' strand of mtDNA. Membranes hybridized with ^{32}P -labeled probes for the 'heavy' strand of the mtDNA sequence investigated were analyzed using a molecular imager. Band intensities were calculated and % repair was determined at 2 and 4 h time points based on the ratio of damaged sample remaining as compared to the intensity of the same nucleotide in the 0 h lanes after subtracting control background levels. +, samples treated with Endo III and FPG. The letters and numbers represent base and location within the heavy strand.

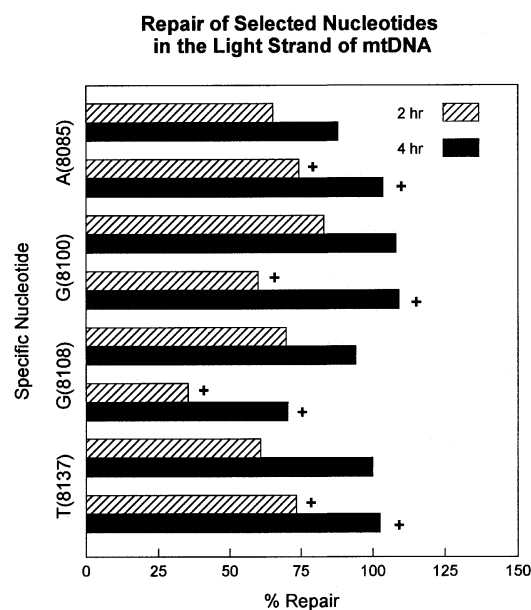


Figure 9. Repair rates for certain damaged nucleotides in the 'light' strand of mtDNA. Membranes hybridized with ^{32}P -labeled probes for the 'light' strand of the mtDNA sequence investigated were analyzed using a molecular imager. Band intensities were calculated and % repair was determined at 2 and 4 h time points based on the ratio of damaged sample remaining as compared to the intensity of the same nucleotide in the 0 h lanes after subtracting control background levels. +, samples treated with Endo III and FPG. The letters and numbers represent base and location within the light strand.

constraints or differences in transcriptional rates that would cause one strand to be repaired preferentially.

The adaptive response reflects the ability of cells to 'sense' the presence of some lesions and induce a repair pathway to remove them; adaptation is the induction of faster DNA repair in response to the damaged DNA itself. It has been documented for alkylation in bacteria and mammals and for oxidative stress in bacteria (29,30). The alloxan-induced oxidative DNA damage appeared to cause a rapid induction of the oxidative DNA damage repair system in the mitochondria of the rat cells used in these studies. The data in Table 2 show that repair of frank breaks and oxidative base damage at 4 h exceed 100% in both strands. These levels were determined by standard algorithm designs for experimental systems where induced damage far exceeds steady state damage. Figures 1 and 2 show that the 4 h post-alloxan samples possessed a lower frequency of frank breaks and oxidative base damage (E3 and FP lanes) than did pre-alloxan samples. We previously reported evidence for this adaptation to oxidative phenomenon using mtDNA (16) and nuclear DNA (17). This adaptation of the repair process(es) in mitochondria allowed faster removal of not only the alloxan-induced damage, but also of the endogenous damage. It has been previously reported that endogenous oxidative damage in mtDNA is extensive (2-4,31). Additional support for the induction of such repair comes from repair studies we have conducted at 8 h following exposure to 5 mM alloxan. The band intensity returns to that seen in 'control' lanes. This indicates that the system returns to its 'normal' status of function following complete repair of the damage.

LMPCR was used to map damage to specific nucleotides along a ~200 bp fragment of rat mtDNA. This fragment spans from 8017 to 8218 on the light strand and includes a 16 bp repeat

associated with the start on the 5 kb 'common' deletion (32,33). The data reveal several 'hot spots' for damage in both strands of mtDNA (Figs 5 and 6). The damage sites along 160 bp of the fragment are summarized in Figure 7. As indicated, some nucleotides are damaged more severely and frequently than others. For example, the stretch of nucleotides between positions 8082-8089 and 8163-8168 on the light strand suffer consistent damage at a number of bases. Although overall numbers of damaged bases along the individual strands of the 160 bp sequence are surprisingly similar, the light strand appears to have more severe damage spots in clusters of adjacent bases, whereas the heavy strand acquires less severe damage at many nucleotides (i.e. 8208-8214 on the heavy strand). Interestingly, there is no evident increase or persistence of damage within the 16 bp repeat (underlined region in Fig. 7) associated with the 5 kb 'common' deletion. This finding, however, does not rule out the possibility that damage to this or surrounding areas contributes to the formation of the 'common' deletion. Recent findings suggest that this region of mtDNA is associated with underlying cytoskeletal structures (i.e. inner mitochondrial membrane) (34). This association coupled with damage to surrounding nucleotides and the nearby repeat sequence may contribute to a recombination, duplication or deletion event (35). Additional work investigating UV damage indicates that single base changes can modulate 'hot spot' mutational events as much as 80 bases away (36). Therefore, another explanation is that damaged bases that create mutations some distance away from this repeat alter the DNA structure and contribute to the formation of a deletion in this region. Specifically, the most consistently heavily damaged base in this study, G(8173) on the heavy strand, is a mere seven bases away from the 16 bp repeat

and could conceivably contribute to a deletion event in this area through a similar mechanism.

The damage to nucleotides within the sequence occur at different frequencies with G showing the highest frequency of damage based on number of specific bases present (Table 3). The finding that G seems to suffer proportionally more damage correlates well with the finding of Rodriguez *et al.* in the PGK-I promoter and first exon following Cu(II)-H₂O₂-ascorbate treatment (37). However, the remaining order of damage frequency we observe is somewhat different and in our work we find no overwhelming skewing of damage towards a particular base. These differences may reflect enhanced levels of specific damage due to Cu(II) binding in studies by others, localized sequence structure variations, different ROS species or input of surrounding chromatin structure(s).

The repair analyses reveal variable rates of return to steady state damage levels for the selected sites (Figs 8 and 9). In general agreement with the Southern blot experiments, the repair is very rapid with all sites being 70 to >100% repaired by 4 h. The enhanced susceptibility of G(8173) on the heavy strand to damage and its subsequent efficient removal are worthy of note. G(8173) was consistently 'hot' in all experiments and exhibits nearly 90% repair by 2 h. This percent is higher than that seen in some other damaged sites at 4 h. Such a finding may indicate that this particular nucleotide is in a position readily accessible to both damage and repair processes. G(8173) repeatedly sustained damage in the 'control' samples. It is unlikely that this finding is the result of an artifact of the LMPCR procedure since such an artifact would most likely maintain a constant band intensity when treated with alloxan and would not elicit repair. This is not the case for the damage seen at G(8173) and we believe, therefore, this nucleotide to suffer elevated levels of endogenous damage.

The fact that repair is rather efficient at all sites begs the question of how, or if, such damage plays a role in mutations seen in mtDNA. However, it must be remembered that these experiments are conducted in a tissue culture environment under controlled conditions. *In vivo* situations involve complex interactions with other cells and cell types in addition to input from a variety of variables held constant in the culture dish. Additionally, it can be argued that since many of the mutations reported in mtDNA are found in cells from aged individuals or in patients with various mitochondrial myopathies (38,39), the repair capacity or ability of specific polymerases to function efficiently in such cells may be compromised. It also is evident that more factors than DNA repair rates contribute to mutation events. Therefore, attempts to make correlations between rate of repair and mutation frequency must be made with caution and should take into account a host of variables both in the cell and its extracellular environment.

In conclusion, our findings indicate that a very efficient repair system exists for the removal of oxidative damage from mtDNA. The repair does not occur in a strand-specific manner and may be the result of an induced response which repairs both drug induced and endogenous damage present in the mitochondrial genome. Damage to individual nucleotides in a region of mtDNA associated with the 5 kb 'common' deletion varies in intensity and frequency; however, efficient repair of such damage is evident at 'hot spots' throughout the sequence. These are the first studies investigating repair of oxidative mtDNA damage at the strand- and nucleotide-specific levels. The results should contribute to further the understanding of the repair system(s) used to restore oxidatively damaged mtDNA to its native state. Future studies will include investigation of specific damage and repair profiles elicited by

other ROS generating agents and in other areas of the mitochondrial genome.

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