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Advances in Brief

DNA Interstrand Cross-Links Induced by Psoralen Are Not Repaired in Mammalian Mitochondria

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Abstract

Although it is generally known that mitochondria are defective in DNA damage processing, little is known about the DNA repair pathways and mechanisms that exist in these vital organelles. Certain lesions that are removed by base excision repair are efficiently removed in mitochondria, whereas some bulky lesions that are removed by nucleotide excision repair are not repaired in these organelles. There has been much interest in whether mitochondria possess activities for recombination repair, and some previous studies have reported such activities, whereas others have not. We have taken the approach of studying the formation and removal of interstrand cross-links (ICLs) in DNA. These lesions are thought to be repaired by a repair mechanism that involves nucleotide excision and recombinational repair. The formation and repair of DNA ICLs by 4′-hydroxymethyl-4,5′,8-trimethylpsoralen was investigated in both the nuclear and mitochondrial genomes in hamster cells. Seven-fold-higher levels of ICLs were generated in mtDNA than in the dihydrofolate reductase gene, clearly indicating that the mitochondrial genome is a preferential target of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen damage. ICLs were removed efficiently from the dihydrofolate reductase gene, but no repair was observed in mtDNA. Our observations support previous work showing efficient gene-specific repair of these lesions in the nucleus but suggest that repair of this type of ICL does not exist in the mitochondria. The preferential damage of mtDNA and the absence of cross-link repair further suggests that mtDNA may be a biologically important target for psoralen.

Introduction

Mammalian mitochondria contain a 16.5-kb circular genome that encodes 22 tRNAs, 2 rRNAs, and 13 subunits of the oxidative phosphorylation complex (1). Each mitochondrion contains up to 10 copies of its genome, whereas each cell contains approximately 10^4 mitochondria per cell. As a consequence, in higher eukaryotes, mtDNA[2] represents more than 1% of the total cellular DNA (2). The mitochondria have been shown to be up to 500-fold more susceptible to damage induced by numerous anticancer agents. Such DNA damage induced by both endogenous and exogenous agents may, therefore, contribute significantly to the cytotoxic and mutagenic properties of such compounds. Indeed, numerous reports are now emerging indicating that several human diseases are associated with mt DNA defects, including DNA deletions (Kearns-Sayre's syndrome and Pearson's syndrome) and point mutations (Leber's hereditary optic neuropathy; Ref. 2). The importance of DNA repair in the maintenance of the nuclear genome is relatively well understood. In contrast, the repair capacity of mitochondria is unclear. An increasing body of evidence suggests that mitochondria are capable of repairing some types of DNA lesions. DNA damage induced by *N*-methyl purines, including streptozotocin and *N*-methyl-*N*-nitrosourea, which, in nuclear DNA, are substrates for the base excision repair pathway, are repaired in mitochondria (3). The efficient *in vivo* removal of O^6-ethyl-guanine lesions induced by ethyl nitrosourea in rat mitochondria has also been clearly demonstrated (4). Oxidative DNA lesions induced by alloxan (5) and bleomycin (6) are also efficiently repaired in mitochondria. In contrast, bulky lesions induced by UVC, nitrogen mustard, and cisplatin are apparently not repaired by mitochondria (7–9). Because DNA damage induced by these agents acts as a substrate for the NER pathway, these findings suggest that this pathway is not functional in mitochondria. Whether mitochondria possess a mechanism for the repair of ICLs remains controversial. Using a denaturation-renaturation electrophoretic assay, ICLs induced by cisplatin have been shown to be repaired as efficiently in the mitochondrial genome as they are in the nuclear *DHFR* gene (9). However, in yeast protoplasts, an incision assay was used to demonstrate that incision of 8-methoxy-psoralen-induced cross-linked DNA was dramatically reduced in mtDNA as compared to nuclear DNA (10). HMT is a linear tricyclic molecule that intercalates between TpA sequences in DNA. Following UVA irradiation (365 nm), the molecule may react with the 5,6 double bond of the pyrimidine bases to form a stable monoadduct. Further irradiation can convert a proportion of monoadducts into ICLs (11, 12). The formation and repair of HMT cross-linkable monoadducts and ICLs has been studied extensively in both human and hamster cells. In human cells, higher levels of HMT lesions are induced in expressed sequences than in transcriptionally silent sequences (13). This may reflect the accessibility of HMT to open chromatin structures within the genome (13, 14). Furthermore, HMT ICLs are repaired more efficiently in expressed genes, such as the *DHFR* gene, as compared with the rest of the genome. In CHO B11 cells, >80% of cross-links are repaired in the *DHFR* gene within 8 h, compared to less than 5% repair in a downstream extragenic fragment (15). In contrast, cross-linkable monoadducts are repaired slowly in both transcriptionally active and inactive sequences (15, 16). The rapid repair of ICLs in transcriptionally active sequences is suggested to reflect transcription coupled repair of this lesion (15). In this study, we used a denaturation-renaturation assay to investigate the repair of DNA ICLs induced by HMT in mitochondrial sequences. Significantly higher levels of DNA damage were induced in mtDNA, as compared to the *DHFR* gene. Although efficient removal of cross-links from the *DHFR* gene was observed, no significant repair was observed in mitochondria. These results suggest the mitochondria are deficient in their ability to repair ICLs induced by HMT.

Materials and Methods

Materials

Radionucleotides [α-^{32}P]CTP, [α-^{32}P]dCTP (3000 Ci/mmol), and [^{3}H]thymidine were from ICN Biochemicals. Cell culture medium was from Life

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[2] The abbreviations used are: mtDNA, mitochondrial DNA; NER, nucleotide excision repair; ICL, interstrand cross-link; *DHFR*, dihydrofolate reductase; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen.

Technologies, Inc., and fetal bovine serum from Upstate Biotechnology, Inc. (Lake Placid, NY). HMT was purchased from HRI Associates (Concord, CA).

Methods

The mitochondrial HI probe, which recognizes the rRNA region of the mitochondrial genome, was a generous gift from Dr. C. Filburn (National Institutes on Aging, NIH). The single-stranded probe was labeled with [α-^{32}P]CTP using a T7/SP6 transcription kit from Boehringer Mannheim. The pMB5 probe used to detect a 14-kb fragment of the *DHFR* gene was prepared as described previously (17) and labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using a Random Primed Labeling Kit (Boehringer Mannheim).

Cell Culture. Wild-type CHO-9 cells were maintained in Ham's F-10 and DMEM (1:1) supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc.), 100 units/ml penicillin, 100 units of streptomycin, and 1% glutamine. Cells were incubated in humidified 5% CO_2-95% air at 37°C.

Cells were prelabeled with [^{3}H]thymidine by seeding 5×10^5 cells in complete medium containing 0.3 Ci/mmol [^{3}H]thymidine and 10 μM cold thymidine and culturing for approximately 60 h. The cells were subsequently replated in fresh medium at a density of 2.5×10^6 cells/dish and incubated overnight prior to drug treatment.

HMT Treatment. HMT was dissolved in ethanol at a concentration of 1 mg/ml and stored in the dark at 4°C. A working solution of HMT was prepared in PBS immediately prior to use.

The method of HMT treatment of the cells was as described by Islas *et al.* (13). Briefly, the cells were washed twice with ice-cold PBS. A 2-ml aliquot of the PBS-diluted HMT solution was added directly to the cells, and the dish was incubated on ice in the dark for 10 min. The plates were placed on a glass plate to remove any short-wave emissions above a long-wave transilluminator (UV Products, San Gabriel, CA) and irradiated for 5 min at a fluence of 20 J/m^2/s, as measured with a UVA dosimeter (model IL1400a; International Light) with a UVA detector to measure intensity. The drug solution was then removed, 5 ml of cold PBS were added, and the dish was incubated on ice in the dark for 5 min. The buffer was removed, and the washing step was repeated twice more. Ten ml of cold PBS were then added, and the plates were subsequently reirradiated as described above for 15 min at a fluence of 45 J/m^2/s to facilitate conversion of cross-linkable monoadducts into ICLs.

The PBS was removed, and the cells were allowed to repair by the addition of complete medium containing 10^{-5} M bromodeoxyuridine and 10^{-6} M fluorodeoxyuridine (to density-label any replicated DNA). At various time intervals, the cells were washed twice with PBS and lysed by the addition of a lysis solution containing 0.5 mg/ml proteinase K, 1% SDS, 0.5 M Tris (pH 8.0), 20 mM EDTA, and 10 mM NaCl. The lysed cells were incubated at 37°C overnight prior to DNA extraction.

DNA Extraction. The cell lysate was extracted once with an equal volume of Tris-saturated phenol (pH 8.0), once with phenol:chloroform (1:1), and once with chloroform. The nucleic acids were subsequently precipitated with ethanol and resuspended in 10 mM Tris (pH 8)-1 mM EDTA. RNase A was added to a final concentration of 0.1 mg/ml, and the samples were incubated at 37°C for 3 h. The DNA was ethanol-precipitated, resuspended in 10 mM Tris (pH 8)-1 mM EDTA, and subsequently digested with *Kpn*I (5 units/μg DNA) to linearize the mitochondrial genome and release a 14-kb fragment of the *DHFR* gene. The parental DNA was separated from any replicated DNA by density gradient ultracentrifugation. The parental DNA was used in subsequent analyses.

DNA ICL Assay. Thirty-μl samples containing 1.5 μg of parental DNA and 50 mM NaOH were incubated at 37°C for 20 min before being placed on ice. Three μl of an alkaline loading dye (26% Ficoll, 10 mM EDTA, and 0.25% bromocresol green) were then added, and the denatured DNA electrophoresed through a 0.5% neutral agarose gel in Tris-acetate buffer at 30 V overnight. The DNA was subsequently transferred to a nylon membrane under alkaline conditions using a positive pressure transfer apparatus (Posiblot; Stratagene). The membrane was hybridized at 45°C overnight in Hybrisol I (Oncor, Gaithersburg, MD) with the ^{32}P-labeled probes, prepared as described above. The blots were washed at high stringency and were exposed to Kodak XAR film overnight. Quantitation was performed using a Molecular Dynamics PhosphorImager. Assuming a random distribution of cross-links in the DNA, the mean number of ICLs per fragment was calculated from the Poisson equation]= $-$ln(fraction of DNA molecules free of ICLs); Ref. 18].

Results

Optimization of Drug Concentration for Mitochondrial Treatment. Initial experiments sought to optimize the frequency of HMT ICLs induced in the mitochondrial genome. CHO cells were incubated with increasing concentrations of HMT, from 0 to 0.5 g/ml, and were exposed to UVA light, as described previously (16), to induce ICLs. Cells were lysed, and the total genomic DNA was extracted and analyzed for the frequency of ICLs induced in the mtDNA. The effect of increasing HMT concentration on the formation of DNA ICLs is shown in Fig. 1. DNA from cells incubated in the absence of HMT was completely denatured, evident by the mtDNA migrating at its single-stranded molecular weight. In contrast, DNA from HMT-treated cells showed a concentration-dependent increase in the amount of mtDNA migrating at double-stranded molecular weight, consistent with the presence of ICLs. At a concentration of 500 ng/ml, all of the

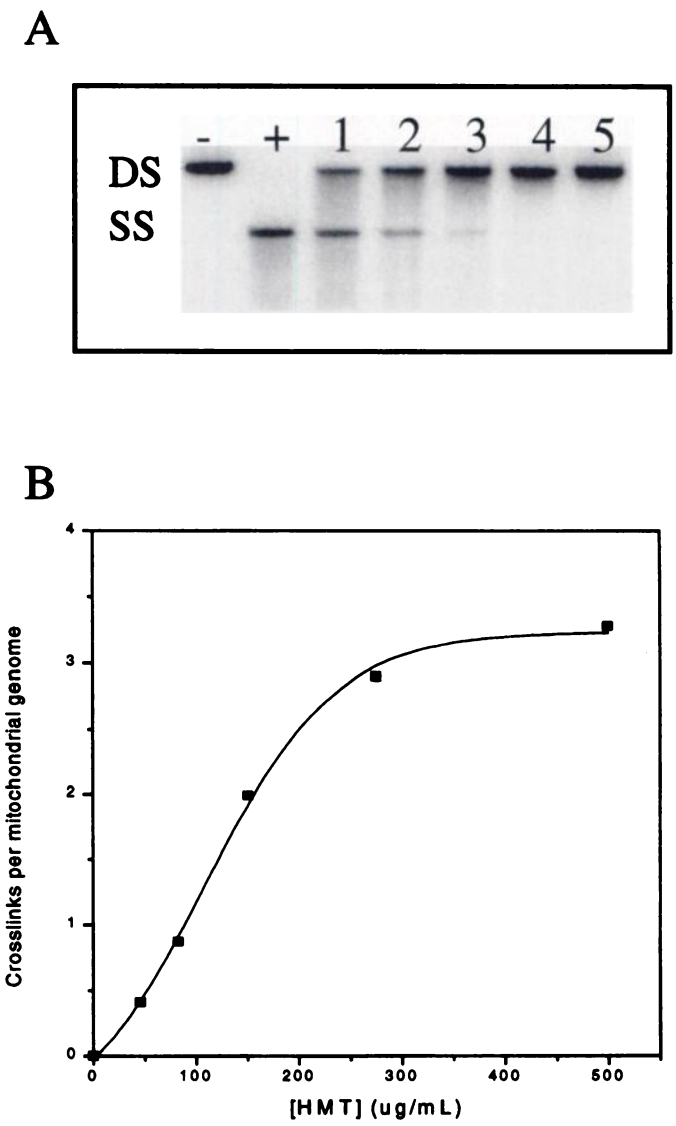

Fig. 1. Dose dependence of HMT ICLs. *A*, CHO-9 cells were treated with 0 ($-$, +), 46 (*Lane 1*), 83 (*Lane 2*), 151 (*Lane 3*), 275 (*Lane 4*), or 500 (*Lane 5*) ng/ml HMT, as described in "Materials and Methods." The cells were lysed, and the parental DNA component was purified. *Kpn*I-restricted DNA (1.5 μg) was denatured in 50 mM NaOH, resolved on a 0.5% agarose gel, blotted onto a nylon membrane, and hybridized to the mtDNA probe. *Lane* $-$, control nondenatured DNA; *Lane* +, control denatured DNA. *DS*, double-stranded DNA; *SS*, single-stranded DNA. *B*, the number of ICLs per mitochondrial genome was quantitated by PhosphorImager analysis of the blot shown in *A* and is expressed as a function of HMT concentration.

mtDNA migrated at a double-stranded molecular weight, indicating that each mitochondrial genome contained at least one ICL.

The gel was quantitated, and the ICL frequency was calculated using the Poisson distribution. Fig. 1*B* shows the calculated number of cross-links present as a function of HMT concentration. At a concentration of 75 ng/ml, a cross-link frequency of approximately 1 per mitochondrial genome was observed. This drug concentration was, therefore, used in subsequent repair assays.

Repair of HMT ICLs. Cells were treated with 75 ng/ml HMT and subjected to UVA irradiation to induce DNA ICLs. The cells were either lysed immediately or were incubated in complete repair medium for periods up to 24 h. At various times, the cells were lysed, and the DNA was assayed for the presence of ICLs. The membranes were hybridized with probes to detect cross-linking in both the nuclear genome (*DHFR* gene) and in the mitochondrial genome.

The results are shown in Fig. 2. Fig. 2*A* shows a representative blot of HMT-induced ICLs in the *DHFR* gene over a 24-h repair period. Under the assay conditions used, only a low level of HMT-induced cross-linking was detected. The repair of the cross-links was quantitated for the 24 h time period studied and is shown in Fig. 3. The cross-links were efficiently removed from the *DHFR* gene, with 80% of the lesions being removed within 24 h.

A blot representing HMT-induced formation of ICLs in mtDNA is shown in Fig. 2*B*. A higher initial level of ICLs was evident in the mtDNA as compared to the nuclear *DHFR* gene. Quantitation of the initial cross-link frequency in each sequence probed (Table 1) revealed a 7.5-fold-higher level of cross-links in the mitochondrial genome as compared to the nuclear gene, clearly indicating the susceptibility of the mtDNA to HMT damage.

In contrast to the *DHFR* gene, cross-linking in mtDNA remained high throughout the 24-h repair period studied. The repair of the ICLs in mtDNA was quantitated and is summarized in Fig. 3. In sharp contrast to that of the *DHFR* gene, no repair of the ICLs in the mtDNA was evident over the time period studied; rather, an apparent increase in the level of cross-links was observed. This result is consistent with the absence of repair of HMT ICLs.

Under the assay conditions used in this study, only the parental DNA is analyzed for the presence of HMT cross-links. Thus, in the absence of HMT cross-link repair, the absolute amount of cross-linked DNA is not expected to change. In contrast, parental DNA that is free of damage can be expected to replicate. At any particular time point, that population of parental DNA which has undergone replication is removed (by cesium chloride gradient following density labeling of replicated DNA) from the DNA analyzed for repair. As a consequence, in a situation in which no repair occurs, the proportion of parental DNA containing cross-links is expected to increase as a function of time, as observed in Fig. 3.

Discussion

In 1974, Clayton *et al.* (7) clearly demonstrated the inability of UV lesions to be removed from mtDNA. It was, therefore, generally thought that mitochondria were defective in the removal of DNA damage in general. Until recently, therefore, the understanding of the DNA repair capacity of mitochondria was limited. It has since been confirmed that NER is absent from mitochondria (9) but that numerous lesions, including those induced by *N*-methyl purines, bleomycin, and oxidative damage are efficiently removed from mtDNA (4, 6, 9). In addition, the carcinogen 4-nitroquinoline appears to be efficiently removed from the mtDNA (19). This lesion is considered to be a UV mimetic due the pattern of repair in xeroderma pigmentosum cells, and it is generally thought to be repaired via NER mechanisms. Few studies to date have investigated the repair of DNA ICLs in mtDNA. Hence, the repair of such lesions in mitochondria remains unclear. This study, therefore, sought to investigate the fate of HMT-induced DNA ICLs in mtDNA.

Repair of both cisplatin and HMT-induced DNA ICLs in the nuclear genome has been clearly demonstrated (13, 15, 20, 21). The mechanistic details of ICL repair, however, remain poorly understood. In bacteria, a model has been proposed in which the NER and homologous recombination pathways are coordinated to excise a psoralen-induced ICL (22). The results of this study clearly demonstrated that HMT-induced ICLs were rapidly repaired in the endogenous *DHFR* gene in the nucleus. The rate and extent of repair were similar to those observed previously by Islas *et al.* (15) in CHO B11 cells.

A significantly higher level of HMT ICLs was induced in mtDNA, as compared to the *DHFR* gene. In a previous study investigating formation of ICLs in yeast by the closely related compound, 8-methoxypsoralen, a similar disparity in cross-link levels was observed (10). Several explanations may account for this observed effect.

Because the mitochondrial genome is highly negatively supercoiled and free of the protective histone proteins, it would be expected to readily facilitate the requirement of the drug to intercalate between 5′-TpA sequences prior to alkylation. Consistent with this, HMT has been shown to preferentially bind DNA in linker regions of chromatin in preference to core sequences, reflecting its preference for a more open DNA structure (12). HMT may also accumulate to a higher concentration in mtDNA than in nuclear DNA due to its preferential uptake into the mitochondria. Indeed, the high level of mtDNA damage observed following treatment of cells with other polycyclic

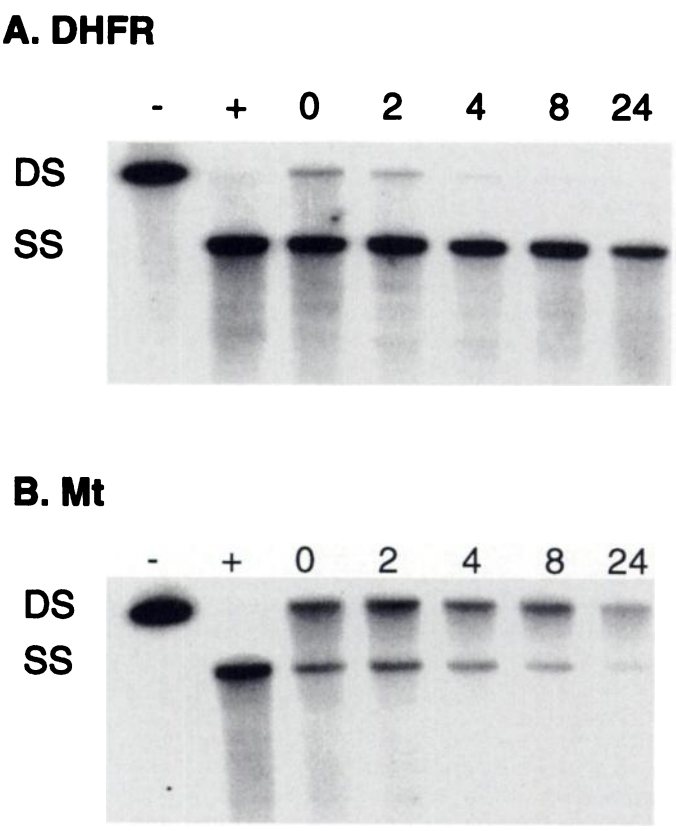

Fig. 2. Repair of HMT ICLs in the *DHFR* gene and mtDNA. CHO-9 cells were treated with 75 ng/ml HMT and were allowed repair for 0–24 h, as shown, before the cells were lysed, and the parental DNA was isolated and digested with *Kpn*I. DNA (1.5 μg) was denatured with NaOH, resolved on an agarose gel, blotted onto nylon, and subsequently probed for the DNA fragment of interest. Representative autoradiograms of blots hybridized to detect the presence of a 14-kb *DHFR* fragment (*A*) and mtDNA (*B*) are shown.

compounds, including benzo(*a*)pyrene and aflatoxin (23, 24) is suggested to result from preferential accumulation of such lipophilic compounds in the mitochondria.

Because NER is thought to be absent in mitochondria (7, 9), it was expected that mitochondria were deficient in ICL repair. A study from our laboratory, however, demonstrated that ICLs induced by cisplatin in hamster cells were removed from mtDNA at a rate comparable to that of such lesions in the nuclear-encoded *DHFR* gene (9). This observation is supported by recent data suggesting that recombinational pathways exist in mammalian mitochondria in human cells (25).

This investigation showed no repair of HMT-induced ICLs in mtDNA in CHO cells. This is in agreement with a study by Magana-Schwenche *et al.* (10), in which no repair of the related compound 8-methoxypsoralen was observed in yeast mtDNA. Therefore, both yeast and mammalian cells appear to be deficient in the repair of psoralen-induced DNA ICLs in mitochondria.

The assay used in this study to detect DNA ICL removal measures the conversion of double-stranded cross-linked DNA to single-stranded DNA. This assay, therefore, reflects the "unhooking" of one arm of the cross-link but not necessarily complete repair of the cross-link. Mitochondria are, thus, deficient in the initial incision steps required for the removal of HMT ICLs. In contrast, mitochondria are capable of performing the initial unhooking event required for cisplatin cross-link repair (9). These findings suggest the involvement of different repair pathways for the initial step of the repair of these two types of lesions.

It has previously been suggested that repair of cisplatin damage is complex and may involve repair pathways in addition to nucleotide excision (26). Repair of cisplatin ICLs *in vitro* has been observed using a repair synthesis assay that detects a common step of all repair pathways (27). In contrast, no NER-specific incision of a template containing a single ICL has observed *in vitro* (28), suggesting the possible involvement of an alternative repair pathway in the removal of such cross-links (28). Indeed, the mismatch repair and NER pathways have recently been shown to overlap. Intrastrand cross-links induced by cisplatin are recognized by a mismatch recognition protein

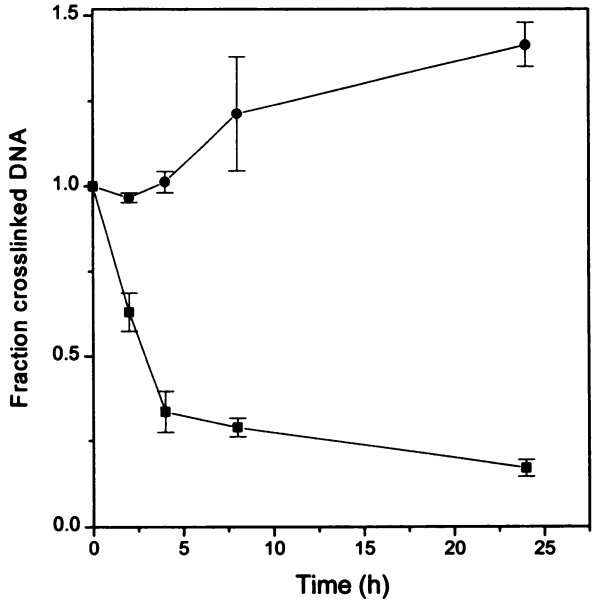

Fig. 3. Quantitation of HMT ICL repair. The removal of HMT ICLs from the 14-kb fragment of the *DHFR* gene (■) and the 16.5-kb mtDNA genome (●) was determined using PhosphorImager analysis and is expressed as a fraction of initial cross-link levels remaining as function of time. *Data points*, means calculated from two blots each of two independent experiments; *bars*, SE.

Table 1 *I-CL Frequency per 10 kb (±SE)*

Time (h)	*DHFR*	mtDNA
0	0.12 ± 0.01	0.71 ± 0.03
2	0.07 ± 0.01	0.69 ± 0.03
4	0.04 ± 0.00	0.71 ± 0.01
8	0.03 ± 0.00	0.85 ± 0.08
24	0.02 ± 0.00	1.00 ± 0.01

in vitro (29). *In vivo*, resistance of cells to cisplatin has been shown to correlate with defects in the mismatch repair pathway (30), implicating mismatch repair in the cytotoxicity of this drug. It is, therefore, possible that repair enzymes present in mitochondria are also capable of repair of cisplatin ICLs.

A recent study has shown that mammalian mitochondria are proficient in homologous recombination (25). Because the general model for ICL repair involves two incision events in the unhooking process and a homologous recombination step (22), the findings of the current study are consistent with mitochondria being deficient in the initial incision of the HMT ICL.

mtDNA has been implicated as a critical target of several carcinogens, including aflatoxin B_1 and benzo(*a*)pyrene, and, more recently, of oxidative damage (31) due to the preferential damage of mtDNA by these agents (23, 24). The high level of mtDNA damage induced by HMT together with the inability of this organelle to process such damage strongly suggests that a biologically relevant target of psoralen is mtDNA.

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