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Defective Repair of Oxidative Damage in the Mitochondrial DNA of a Xeroderma Pigmentosum Group A Cell Line[1]

Wesley J. Driggers, Valentina I. Grishko, Susan P. LeDoux, and Glenn L. Wilson[2]

Department of Structural and Cellular Biology, University of South Alabama, Mobile, Alabama 36688

ABSTRACT

Recent evidence has linked mitochondrial DNA (mtDNA) damage to several disease processes, including cancer and aging. An important source of such damage is reactive oxygen species. These molecules can be generated endogenously via the electron transport system or may arise from a host of exogenous sources. It has been reported that extracts from cells of individuals with xeroderma pigmentosum group A (XP-A) do not repair some types of oxidative DNA damage. The current experiments were designed to determine whether there is a correlation between the inadequate repair of oxidatively damaged nuclear DNA in XP-A cells and the capacity of such cells to repair similar damage to their mtDNA. The ability of karyotypically normal human fibroblasts (WI-38) and XP-A fibroblasts to repair alloxan-generated oxidative damage to nuclear and mtDNA was assessed using a quantitative Southern blot method in conjunction with the repair enzymes endonuclease III and formamidopyrimidine DNA glycosylase. The data indicate that both nuclear and mtDNA repair of each damage type investigated is more efficient in the WI-38 cells. These findings suggest a similarity between the process(es) used to repair oxidative damage to nuclear and mtDNA in that both are inhibited by the defect in XP-A.

INTRODUCTION

ROS[3] are a common source of damage to cellular DNA. These species can arise from both endogenous and exogenous sources and produce DNA damage ranging from base modifications to breaks of the helix. mtDNA is especially vulnerable to attack by ROS since: (*a*) these organelles consume ~90% of the body's oxygen, and ~1–2% of the oxygen that they metabolize is converted to superoxide (1); (*b*) the mtDNA is in close proximity to the inner mt membrane where the electron transport system generates ROS; and (*c*) this organelle's DNA is not associated with protective histones. Several studies have linked point mutations and deletions in mtDNA, which could arise from unrepaired DNA damage, to aging and a variety of diseases, including diabetes mellitus, cancer, Parkinson's disease and Alzheimer's disease (2–6).

Prior to the beginning of this decade, it was widely assumed that damaged mtDNA was not repaired; however, over the last 4 years our laboratory has demonstrated that mitochondria have the capacity to repair several types of damage to their genome (7–9). Most recently, we showed that these organelles are particularly efficient at repairing oxidative DNA damage arising from the redox cycler alloxan (10). Although we have documented the ability of mitochondria to repair some lesions, the mechanism by which this repair is accomplished remains to be elucidated. To determine this mechanism(s) we asked whether some of the factors involved in nuclear repair of oxidative DNA damage also were functioning in mtDNA repair. In an effort to answer this question we used the NER-deficient XP-A cells. Patients with XP exhibit increased sensitivity to sunlight and suffer from a high incidence of skin cancers (11). Group A patients (XP-A) have the most severe symptoms, and the defective protein in these individuals (XPAC) is involved in recognizing and binding to damaged DNA in the NER process (12, 13). In the current study, we have investigated repair of oxidative damage in mtDNA from a XP-A cell line whose cellular extracts have been shown to be deficient in removal of a class of oxygen-free radical-induced DNA damage (14). The repair rates from these experiments were compared to those of WI-38 cells. The WI-38 cells were chosen as controls since they are karyotypically and phenotypically normal human fibroblasts and have exhibited normal repair characteristics in previous studies (9). These data support a possible link between nuclear and mtDNA repair processes since the XP-A cells are deficient in the repair of oxidative lesions in their mtDNA.

MATERIALS AND METHODS

Cell Culture. WI-38 and XP-A(20S) human fibroblasts were obtained from American Type Culture Collection and National Institute of General Medical Sciences cell repositories, respectively. This line of SV40-transformed XP-A cells was derived from a patient with multiple severe symptoms of XP-A. Both cell types were maintained in Eagle's MEM with Earle's salts (GIBCO-BRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 50 μg/ml gentamicin (Sigma), 1× nonessential amino acid solution, 1× essential amino acid solution with L-glutamine (GIBCO-BRL), and 1× MEM vitamin solution (GIBCO-BRL). For repair studies, cells were plated in 150 × 25-mm dishes and grown at 37°C in 5% CO_2 until near confluence (5–7 days for the WI-38 cells and 2–3 days for the XP-A cultures).

Viability Studies. Cell viability was determined using two assays. The first assay uses MTT and is based on the activity of the mt enzyme succinate dehydrogenase (15), whereas the second (trypan blue dye exclusion) is based on cell membrane integrity. For the MTT studies, cells were seeded in 24-well plates at 5×10^4 cells/well and maintained in culture for 2 to 3 days. Cells were then treated with 7.5 mM alloxan (5,6-dioxyuracil; Sigma) for 1 h in HBSS + 1% citrate buffer. Control cultures were exposed to HBSS + 1% citrate only. After 1 h the cells were rinsed with HBSS and received fresh media for 2, 6, or 24 h or were immediately incubated for 20 to 30 min with MTT (0.5 mg/ml in HBSS). Cells were counted at appropriate times to determine the percentage of viability based on their capacity to cleave MTT and thereby produce purple formazan crystals.

In the trypan blue dye studies, cells were plated into 60-mm dishes and maintained in culture media until near confluence (48–72 h). Cultures then were treated with 7.5 mM alloxan in HBSS + 1% citrate buffer for 1 h or with HBSS + citrate buffer only (controls). Following drug exposure, cells were harvested immediately via trypsinization or placed in fresh media for 2-, 6-, or 24-h time periods. After gentle centrifugation and appropriate dilutions in media with trypan blue dye, cells were counted, and the percentage of viability was determined based on the number of live cells/total number of cells × 100. In this assay, live cells maintain membrane integrity and therefore appear to be clear, whereas dead cells stain blue as a result of dye leaking into their cytosol.

Drug Preparation and Exposure. Alloxan was dissolved in HBSS + 1% citrate buffer in subdued light to yield the desired concentrations. Cells were rinsed in HBSS and then exposed to the appropriate amount of alloxan for 1 h in a 37°C/5% CO_2 incubator. Control cultures were exposed to HBSS + 1% citrate buffer under the same conditions. After the 1-h incubation, cells were

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[2] To whom requests for reprints should be addressed, at Department of Structural and Cellular Biology, University of South Alabama, Mobile, Alabama 36688.

[3] The abbreviations used are: ROS, reactive oxygen species; mt, mitochondrial; NER, nucleotide excision repair; XP-A, xeroderma pigmentosum group A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide; FAPy, formamidopyrimidine; DHFR, dihydrofolate reductase.

rinsed and lysed immediately or were allowed repair times of 2 or 6 h in culture media.

Assay for Damage Detection and Repair Analysis in mt or Nuclear DNA. WI-38 or XP-A monolayer cultures were exposed to alloxan as described above. In the repair studies, cells then were incubated in fresh culture media and lysed at appropriate times in 10 mM Tris, 1 mM EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K. Lysates were removed with a rubber policeman, transferred to sterile 50-ml tubes, and incubated overnight at 37°C. High molecular weight DNA was then extracted very carefully in the absence of phenol to prevent introduction of artifactual oxidative damage. In brief, the lysates were adjusted to 1 M NaCl with the appropriate volume of 5 M NaCl and were gently rocked for 10 min. Samples then were extracted three times using an equal volume of SEVAG (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 volumes of ethanol, resuspended in d-H_2O, and treated with DNase-free RNase (~1.0 μg/ml) for 3 h at 37°C. Samples were precipitated as before, resuspended in d-H_2O, and quantified by determining absorbance at 260 nm. The purified DNA was digested with *Kpn*I or *Hin*dIII (5 units/μg DNA) at 37°C for 12 to 16 h. Complete digestion was verified on minigels. Digested samples were precipitated, resuspended in a small volume of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.4), and precisely quantified using a Hoefer TKO 100 Mini-Fluorometer and TKO standards kit (Hoefer Scientific Instruments, San Francisco, CA). Samples containing 7 μg DNA were heated at 65°C for 20 min and then cooled at room temperature for an additional 20 min. Predetermined amounts of endonuclease III or FAPy were added to appropriate samples which were then incubated at 37°C for 15 min. These enzymes produce single-strand breaks at specific sites in oxidatively damaged DNA (16, 17) and were kindly provided by Dr. Yoke W. Kow (endonuclease III; University of Vermont, Burlington, VT) and Dr. Julia Tchou (FAPy; State University of New York, Stony Brook, NY). A sodium hydroxide solution was then 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 any abasic or sugar-modified site in the DNA. Samples were combined with 5 μl loading dye, loaded onto a 0.6% alkaline agarose gel, and electrophoresed at 30 V (1.5 V/cm gel length) for approximately 16 h in an alkaline buffer consisting of 23 mM NaOH and 1 mM EDTA. After standard gel washing, the DNA was transferred via vacu-blotting (Vac-1000; American Bionetics) onto a Zeta-Probe GT nylon membrane (Bio-Rad) and cross-linked to the membrane with a GS Gene Linker (Bio-Rad). Following a 10-min prehybridization in 7 ml 0.25 M sodium phosphate, 7% SDS (pH 7.2) at 65°C, the membrane was hybridized with a PCR-generated, ^{32}P-labeled mt or DHFR probe in 15 ml of the same solution for approximately 20 h at 65°C. The membrane was then washed using the protocol recommended by the manufacturer and placed on a phosphorimaging screen for detection of hybridization bands. The resultant images were scanned on a Bio-Rad GS-250 molecular imaging system, and the amount of DNA damage and repair were determined using the Poisson distribution as previously described (18).

DNA Probes. The probe used to hybridize to mtDNA was generated via PCR from a mouse mtDNA sequence using the following primers: 5′-GCAGGAACAGGATGAACAGTCT-3′ from the sense strand and 5′-GTATCGTGAAGCACGATGTCAAGGGATGAG-3′ from the antisense strand. The 745-bp PCR product recognizes a 13.5-kb restriction fragment when hybridized to human mtDNA digested with *Kpn*I. The 600-bp PCR-generated DHFR probe was derived from human genomic DNA using the primers: 5′-AAGGCATTAAGTACAAATTTGAAGTATAT G-3′ from the sense strand and 5′-ATACTCATTTTCCCATCACTGGACTTCCAG-3′ from the antisense strand. The probe recognizes a 21-kb restriction fragment in *Hin*dIII-digested human DNA.

RESULTS

The initial experiments were designed to determine mtDNA damage in WI-38 and XP-A cells following exposure to varying concentrations of alloxan and to optimize the dose of drug to be used for subsequent repair studies. Cells were exposed to 5, 7.5, and 10 mM concentrations of alloxan. DNA was extracted and digested to completion with *Kpn*I for mtDNA studies or with *Hin*dIII for analysis of the DHFR sequence. After heating, addition of appropriate enzyme, alkali treatment, and alkaline gel electrophoresis, a Southern blot procedure was performed, and data analyses were conducted as described in "Materials and Methods." A concentration of 7.5 mM alloxan was determined to be an appropriate level of drug for study of repair based on initial damage detection. This concentration of alloxan yielded approximately 1.8 breaks/13.5-kb mtDNA restriction fragment in each cell line following alkaline treatment and electrophoresis. Representative autoradiographs from experiments using WI-38 and XP-A cell lines are shown in Figs. 1 and 2, respectively.

To ensure that the level of alloxan chosen for repair studies (7.5 mM) was not overtly toxic to the cells, viability assays involving MTT or trypan blue dye were performed. The MTT assay measures viability based on the capacity of cells to cleave MTT into visible formazan crystals through the action of the mt enzyme succinate dehydrogenase and, therefore, can be used as a measure of mt viability. The trypan blue dye exclusion procedure identifies viable cells based on their ability to exclude the dye from their cytosol and thus measures overall cell viability. WI-38 and XP-A cells were plated and maintained in culture media until near confluence. They were then exposed to 7.5 mM alloxan for 1 h, and checked for viability immediately or returned to normal culture media for 2, 6, or 24 h as described in "Materials and Methods." The results of these studies are shown in Fig. 3 and establish that the 7.5 mM alloxan concentration chosen for the repair studies is not overtly toxic to the WI-38 or XP-A cells.

To assess the repair of ROS-induced damage in the mtDNA of normal (WI-38) and XP-A cells, cultures were exposed to 7.5 mM alloxan for 1 h and harvested immediately or allowed to repair for 2 or 6 h. These time points were chosen based on previous experiments which demonstrated efficient repair of oxidative mtDNA damage during similar time intervals (10). After isolation of DNA, Southern blotting, and hybridization, membranes were washed, scanned on a phosphorimager, and resultant bands used for quantitation of repair based on the Poisson distribution (18). Representative autoradiographs from these experiments are shown in Figs. 4 (WI-38) and 5 (XP-A). Study of initial damage (*Lanes 4–6*) reveals substantially fewer 13.5-kb fragments. During the allotted repair times, the WI-38 cells were able to efficiently repair nearly 40% of the alloxan-induced mtDNA damage by 2 h and approximately 70% by 6 h (Fig. 4 and Table 1), while the XP-A cells exhibited only 18 and 30% repair during the same respective time intervals (Fig. 5 and Table 2). Tables 1 and 2 summarize initial break frequencies and repair rates of both cell types. The repair rates should be unaltered by the difference in replication rates between the two cell lines, since the time points of repair assessed were so short (2 and 6 h), and preliminary studies indicated that only negligible amounts of mtDNA had replicated

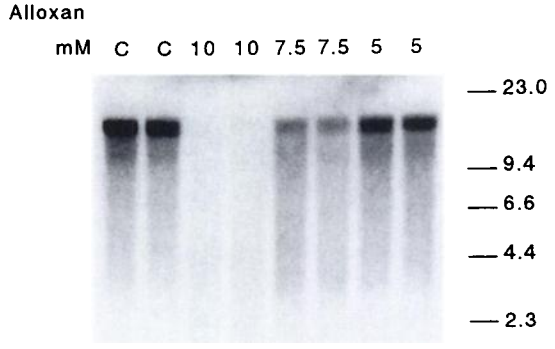

Fig. 1. WI-38-dose response study. WI-38 cells were exposed to 10, 7.5, or 5 mM alloxan for 1 h and lysed immediately. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Kpn*I. Samples were exposed to 0.1 N NaOH prior to Southern blot analysis and hybridization with a mt probe.

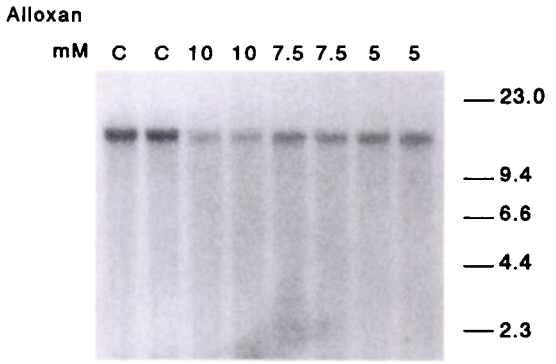

Fig. 2. XP-A dose-response study. XP-A cells were exposed to 10, 7.5, or 5 mM alloxan for 1 h and lysed immediately. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Kpn*I. Samples were exposed to 0.1 N NaOH prior to Southern blot analysis and hybridization with a mt probe.

during the repair intervals. Additionally, if the observed rates were altered by increased replication, producing more full-length intact fragments in a given sample, this would skew the XP-A repair rates toward estimations that were artificially high. Since we saw only minimal repair in these cells, this effect was obviously insignificant.

A final set of experiments was conducted to determine whether the XP-A cells exhibited deficient nuclear repair of alloxan-induced oxidative DNA damage. For these studies cells were drugged with 7.5 mM alloxan and DNA processed as before with the exception of the use of the *Hin*dIII restriction enzyme. The Southern blots were hybridized with a ^{32}P-labeled DHFR probe and analyzed as described above. The data from these studies are shown in graphic form in Fig. 6 and indicate that the XP-A cells also are deficient in repair of the alloxan-induced damage in a similarly sized nuclear gene sequence.

DISCUSSION

These studies were designed to provide insight into the mechanism by which mitochondria repair oxidative damage to their DNA. For these experiments we chose the XP-A(20S) cell line which has been widely used in previous studies involving repair mechanisms (19–22). Most recently, investigators have shown that these cells lack efficient repair of some forms of oxidative damage in their nuclear DNA (14). The results of the study described herein demonstrate that the XP-A(20S) cells also have attenuated repair of oxidative lesions in their mtDNA. This finding suggests that certain repair mechanisms involved in correcting nuclear oxidative DNA damage also participate in repair of mtDNA after oxidative injury. Upon initial consideration such a result may be expected since proteins performing mtDNA repair presumably are encoded by the nucleus, and if such proteins were functioning in both organelles, a nuclear repair deficiency would be expected to lead to a corresponding mtDNA repair problem. Several laboratories have shown that mitochondria contain enzymes, such as uracil-DNA glycosylase (23), apurinic/apyrimidinic endonucleases (24), and a DNA ligase (25), which have been associated with repairing DNA. Moreover, some of these are specific for the mitochondrion (26). This would suggest that a separate set of enzymes is targeted to mitochondria for DNA repair purposes. In support of this notion is the finding from our laboratory that XP-D cells efficiently repair simple alkylation damage (*i.e.*, *N*-methylpurines) in their mtDNA, while exhibiting a repair deficiency for the same type of damage in nuclear DNA (9). The observations of mitochondria-specific enzymes and efficient mtDNA repair in XP-D cells despite the deficiency in nuclear repair appear to be contrary to the results of the current study which support overlap of mt and nuclear DNA repair processes. One explanation may be related to the fact that the current studies deal with oxidative DNA damage, which we have shown to be repaired very rapidly in several nuclear sequences (27) and mtDNA of normal cells (10). Cells are constantly exposed to this type of damage through endogenous metabolic processes and the repair system(s) for such damage, therefore, may function differently than the one correcting simple alkylation damage.

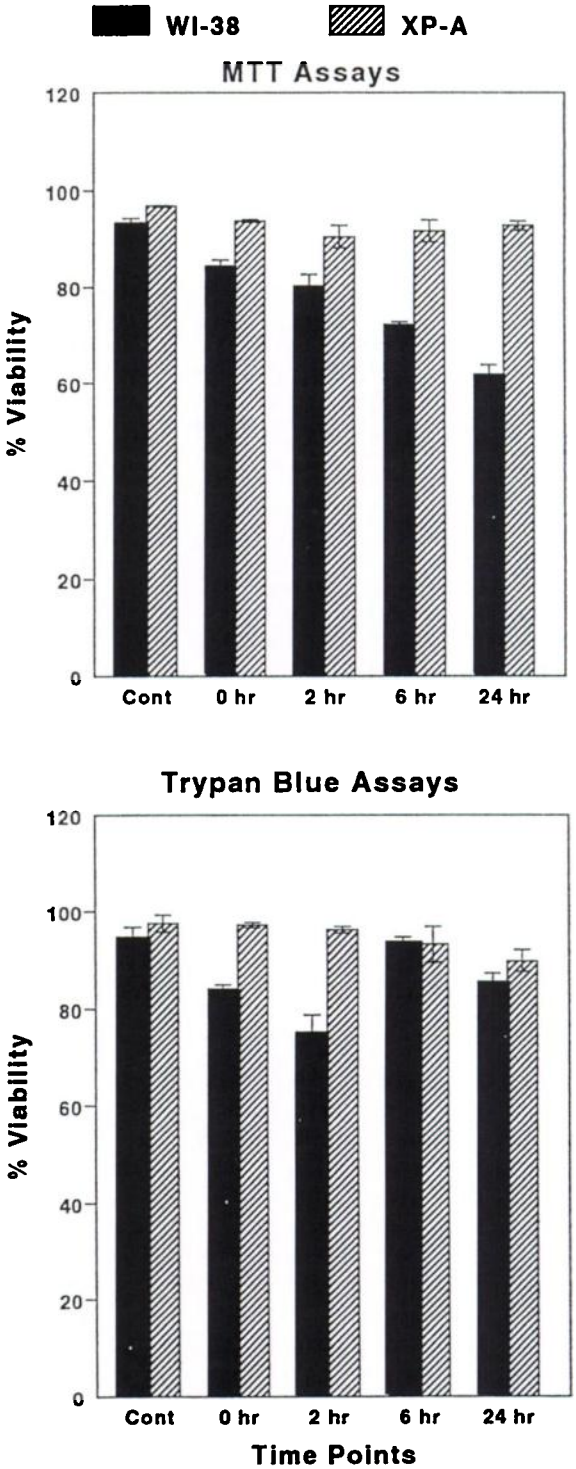

Fig. 3. WI-38 and XP-A cell viability studies based on MTT and trypan blue dye assays. Cells were plated into 24-well plates (MTT assays) or 60-mm dishes (trypan blue dye studies) and maintained in culture medium until near confluence. Cultures were then treated with 7.5 mM alloxan for 1 h or with HBSS diluent only [controls (*Cont*)]. Following drug exposure, the percentage of viability was determined at 2-, 6-, or 24-h time points.

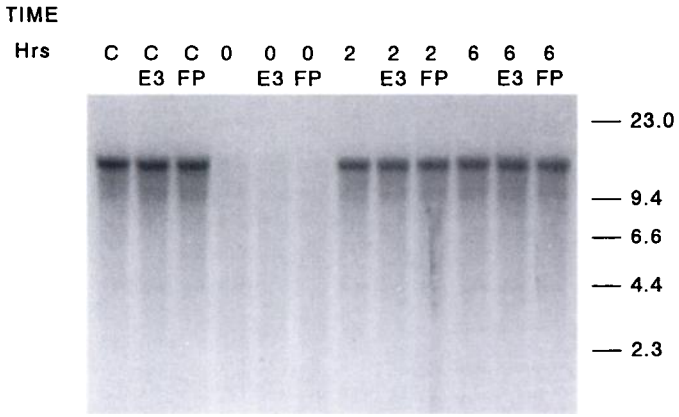

Fig. 4. Repair of mtDNA in WI-38 cells following exposure to 7.5 mM alloxan. WI-38 cells were exposed to 7.5 mM alloxan for 1 h and lysed immediately or rinsed and placed in culture media to allow time for repair. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Kpn*I. Samples were exposed to 0.1 N NaOH alone, 0.1 N NaOH + endonuclease III, or 0.1 N NaOH + FAPy DNA glycosylase prior to Southern blot analysis and hybridization with a PCR-generated mtDNA probe. *0*, *2*, or *6*, repair times allotted following drug exposure. *E3* or *FP*, samples incubated with 0.1 N NaOH + endonuclease III or 0.1 N NaOH + FAPy glycosylase, respectively.

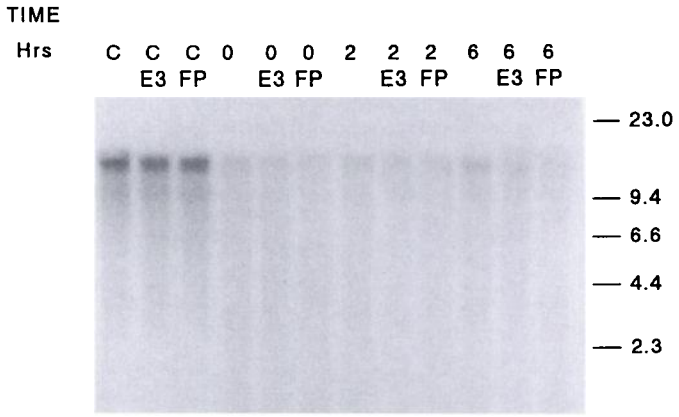

Fig. 5. Repair of mtDNA in XP-A cells following exposure to 7.5 mM alloxan. XP-A cells were exposed to 7.5 mM alloxan for 1 h and lysed immediately or rinsed and placed in culture media to allow time for repair. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Kpn*I. Samples were exposed to 0.1 N NaOH alone, 0.1 N NaOH + endonuclease III, or 0.1 N NaOH + FAPy DNA glycosylase prior to Southern blot analysis and hybridization with a PCR-generated mtDNA probe. *0*, *2*, or *6*, repair times allotted following drug exposure. *E3* or *FP*, samples incubated with 0.1 N NaOH + endonuclease III or 0.1 N NaOH + FAPy glycosylase, respectively.

Another conceivable explanation for these results is that certain steps of nuclear and mtDNA repair processes overlap while others do not. For example, the gene product defective in XP-A cells has been shown to be involved in the recognition and binding of damaged DNA (28), two of the initial steps in repair. The XP-D defective protein, on the other hand, is a DNA helicase (29) which participates in DNA unwinding at a later step after the damage has been "identified." This protein could conceivably be different in mitochondria since the DNA structure to be unwound is more like a bacterial genome conformation and has no protective histones. This explanation along with the previously mentioned data would support the idea of a common recognition factor for both nuclear and mtDNA damage with the repair pathways separating at a later step. These data also raise the possibility of an overlap in recognition of at least certain DNA damage between NER and base excision repair processes, since XP cells are deficient in NER, yet many types of oxidative damage studied to date require base excision repair.

The mechanisms by which mitochondria repair damage to their genome are still unknown. This is due in large part to early reports that damage to mtDNA was not repaired (30, 31). However, more recent studies have demonstrated that this organelle possesses a remarkable capacity to repair a variety of DNA damages including O^6-ethylguanine lesions (32), alkali-labile sites (7), and oxidative base and sugar-phosphate damage (10). It is evident that more work is needed to determine the exact mechanisms and proteins involved in mtDNA repair and how such factors may be altered in various diseases. The present findings pave the way for a new approach to study mtDNA

Table 1 *Repair of alkali-labile and enzyme-sensitive sites within the mtDNA of WI-38 cells*

Hybridization bands representing a 13.5-kb mtDNA fragment were scanned with a phosphorimager. The break frequency per 13.5-kb fragment 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).

Repair times (h)	Break frequencies		% Repair
0	alk	1.77 ± 0.32	
	alk + E3	1.85 ± 0.34	
	alk + FP	1.87 ± 0.41	
2	alk	1.06 ± 0.60	40.4
	alk + E3	1.10 ± 0.56	37.3
	alk + FP	1.13 ± 0.53	35.1
6	alk	0.505 ± 0.16	69.1
	alk + E3	0.540 ± 0.20	66.5
	alk + FP	0.598 ± 0.18	64.2

[a] alk, alkaline labile; E3, sample incubated with 0.1 N NaOH + endonuclease III, FP, sample incubated with 0.1 N NaOH + FAPy glycosylase.

Table 2 *Repair of alkali-labile and enzyme-sensitive sites within the mtDNA of XP-A cells*

Hybridization bands representing a 13.5-kb mtDNA fragment were scanned with a phosphorimager. The break frequency per 13.5-kb fragment 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).

Repair times (h)	Break frequences		% Repair
0	alk[a]	1.81 ± 0.32	
	alk +E3	1.76 ± 0.32	
	alk + FP	1.65 ± 0.19	
2	alk	1.42 ± 0.29	18.3
	alk + E3	1.40 ± 0.23	19.4
	alk + FP	1.51 ± 0.37	15.2
6	alk	1.23 ± 0.25	29.2
	alk + E3	1.31 ± 0.37	27.3
	alk + FP	1.37 ± 0.41	22.6

[a] alk, alkaline labile; E3, sample incubated with 0.1 N NaOH + endonuclease III; FP, sample incubated with 0.1 N NaOH + FAPy glycosylase.

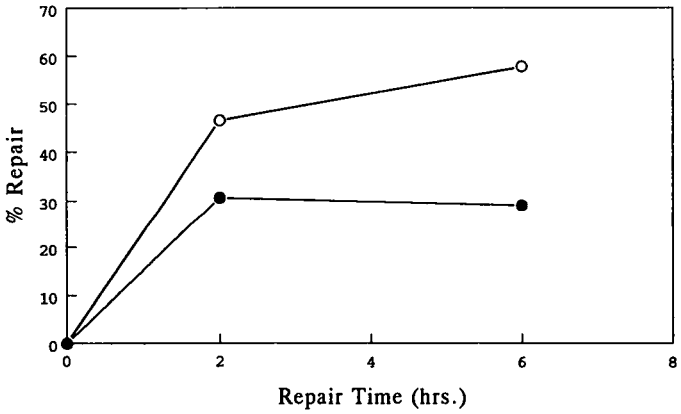

Fig. 6. Repair of oxidative damage in the DHFR sequence of WI-38 (○) and XP-A cells (●). XP-A cells were exposed to 7.5 mM alloxan for 1 h and lysed immediately or rinsed and placed in culture media to allow time for repair. Control cultures were incubated in drug diluent only. High molecular weight DNA was isolated and digested to completion with *Hin*dIII. Repair efficiency was then determined using a PCR-generated DHFR probe and the described quantitative Southern blot procedure.

repair. Complementation analysis can be performed to determine whether the XPAC protein participates in the removal of oxidative damage from mtDNA. Such experiments will be the focus of future investigations.

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