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# Repair of oxidative DNA base lesions induced by fluorescent light is defective in xeroderma pigmentosum group A cells

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## ABSTRACT

**Fluorescent light (FL) has been shown to generate free radicals within cells, however, the specific chemical nature of DNA damage induced by FL has not previously been determined. Using gas chromatography/isotope dilution mass spectrometry, we have detected induction of the oxidative DNA lesions 5-hydroxycytosine (5-OH-Cyt), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) in cultured cells irradiated with FL. We followed the repair of these lesions in normal and xeroderma pigmentosum group A (XP-A) cells. 5-OH-Cyt and FapyGua were repaired efficiently in normal cells within 6 h following FL exposure. XP-A cells were unable to repair these oxidative DNA base lesions. Additionally, to compare the repair of oxidative lesions induced by various sources, *in vitro* repair studies were performed using plasmid DNA damaged by FL, γ-irradiation or $OsO_4$ treatment. Whole cell extracts from normal cells repaired damaged substrates efficiently, whereas there was little repair in XP-A extracts. Our data demonstrate defective repair of oxidative DNA base lesions in XP-A cells *in vivo* and *in vitro*.**

## INTRODUCTION

It has been known for a long time that visible light can cause some harmful effects on mammalian cells in culture (1). Fluorescent light (FL) is a very common light source that we are all exposed to quite frequently. It is composed of both visible light (>400 nm) and UVA (320–400 nm). Clastogenic effects of FL in cultured mammalian cells include DNA strand breaks, DNA–protein crosslinks, altered sister chromatid exchange and DNA base damage leading to mutagenesis (2).

Although no specific DNA base changes were shown, the genotoxic effects from exposure to FL have been attributed to oxidative DNA damage mediated by endogenous photosensitizers (3). The most widely used fluorescent bulbs (‘coolwhite’) induce G-C→A-T transitions, which suggests that base damage occurs predominately at guanines or cytosines (4). Additionally, base modifications sensitive to cleavage by bacterial DNA glycosylases were induced in cells by visible light exposure (5). However, the chemical nature of the DNA base damage following FL exposure has not previously been determined.

A primary cellular defense against oxidative DNA base damage is base excision repair (BER) (6). In eukaryotic cells, BER of oxidative DNA base lesions is initiated by DNA glycosylases: the Nth protein (the homolog of bacterial endonuclease III) is responsible for removal of oxidized pyrimidines (7,8) and the Ogg1 (8-oxoguanine DNA glycosylase) protein initiates removal of oxidized purines (9–11). BER then proceeds through two alternative pathways by replacement of either a single nucleotide (12) or a short nucleotide patch (13–15).

Although nucleotide excision repair (NER) is the primary system for removal of bulky nucleotide lesions, oxidative base lesions may also be recognized by this process (16). NER removes an oligonucleotide containing the damaged base, followed by resynthesis of a 25–30 nt patch (17). Transcription-coupled repair (TCR) was first detected as an increased rate of removal of UV-induced DNA damage within actively transcribed genes (18). However, recent evidence suggests that NER and BER may both be involved in TCR of oxidative DNA base lesions such as thymine glycol (19).

Xeroderma pigmentosum (XP) is an autosomal recessive human disease consisting of seven complementation groups. XP proteins are essential for the removal of DNA damage through NER (17) and this leads to elevated rates of skin cancer in these patients (20). XP complementation group A (XP-A) is most defective among the complementation groups in general genome NER, and the patients suffer from severe neurological complications in addition to an increased frequency of cancer (20). Recently, XP-A cells were shown to have altered chromatid break frequencies following exposure to FL, suggesting that XPA protein (XPA) participates in the repair of FL-induced DNA damage (21). *In vitro* repair studies have shown that whole cell extracts (WCE) from XP-A cells are unable to incise substrates containing thymine glycol and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG), indicating an involvement of NER in the processing of these lesions (16).

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Also, this laboratory has recently demonstrated that WCEs from XP-A lymphoblasts are defective in the repair of 8-oxoguanine (8-oxoGua)-containing substrates that are removed by BER (22). Additionally, extracts from XP-A cells were shown to be deficient in removal of a class of oxygen free radical-induced DNA damage (23). The chemical nature of this DNA damage, which is resistant to removal by bacterial DNA glycosylases, has remained unidentified. Although there are some indications in the literature that XP-A cells might be defective in the repair of some types of oxidative base lesions, the general notion still prevails that XP-A cells repair oxidative DNA damage proficiently.

In this report, we have investigated the specific nature of oxidative DNA base damage induced by FL light exposure using gas chromatography/isotope dilution mass spectrometry (GC/IDMS). We observe that certain lesions are induced. The levels of 5-hydroxycytosine (5-OH-Cyt), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) are all significantly increased after FL irradiation. We next studied the repair of these lesions in normal cells. The nature of this repair was further explored by analyzing the repair process in NER-deficient XP-A cells. Two lesions, 5-OH-Cyt and FapyGua, were repaired efficiently in normal cells, but they were not removed in XP-A cells. These results were further extended to an *in vitro* repair system. Here lesions were introduced into a plasmid by exposure to FL, γ-irradiation and $OsO_4$ treatment. WCEs prepared from XP-A cells were deficient in repair synthesis on substrates damaged by FL exposure. Thus, XP-A cells are defective in both the *in vivo* and the *in vitro* repair of certain oxidative lesions introduced by FL. Our data suggest a possible role for XPA in the repair of specific oxidative DNA base lesions that are traditionally thought to be processed by BER.

## MATERIALS AND METHODS

### Tissue culture methods

Normal (AG10111 and GM9387) and XP-A (GM2250e) lymphoblasts were maintained in RPMI supplemented with 15% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at an average density of $4.0 \times 10^5$ cells/ml at 37°C in a 5% $CO_2$ atmosphere. The cells were grown to 1 l containing ~$8 \times 10^5$ cells/ml, harvested by centrifugation at 300 *g* and resuspended in serum-free RPMI containing L-glutamine and antibiotics. Approximately $1.5 \times 10^6$ cells were transferred to culture dishes and the cells were incubated at 37°C in a 5% $CO_2$ atmosphere for 6 h with exposure to FL ('coolwhite' bulbs) at 10 W/m$^2$. The cells were exposed to FL in covered tissue culture dishes, which effectively shield UVB and UVC. For mock (control) treatments, the cells were incubated in serum-free medium for the duration of the treatment time (6 h). The cells were pelleted by centrifugation, resuspended in 15 ml of complete medium and allowed to incubate in the dark for 6 h for repair studies. The DNA was extracted by modified salt extraction as previously described (24).

The cell viability following 6 h FL exposure was determined by trypan blue exclusion. The viability of the normal (AG1011 and GM9387) and XP-A (AG2250) cells was >75% 6 h after FL exposure as determined by vital staining.

Lymphoblast cells were grown to 1 l containing ~$8 \times 10^5$ cells/ml under the conditions described above. Cells were harvested by centrifugation and WCEs were prepared as previously described (25).

### Gas chromatography/mass spectrometry

Modified DNA bases, their stable isotope-labeled analogs and other materials for gas chromatography/mass spectrometry were obtained as described (26). Labeled 2'-deoxyguanosine ([$^{15}N_5$]2'-deoxyguanosine) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Aliquots of stable isotope-labeled analogs of DNA base products and [$^{15}N_5$]2'-deoxyguanosine were added as internal standards to DNA samples containing ~50 µg of DNA. The samples were dried under vacuum in a SpeedVac. The use of [$^{15}N_5$]2'-deoxyguanosine permits determination of the DNA amount by GC/IDMS. Upon hydrolysis, this compound yields [$^{15}N_5$]guanine, which is used as an internal standard for determination of the amount of guanine in DNA and consequently the amount of DNA (27). DNA samples were hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolysates were frozen in vials, placed in liquid nitrogen and then lyophilized for 18 h. DNA hydrolysates were then derivatized and analyzed by GC/IDMS with selected ion monitoring as described (28).

### *In vitro* DNA repair synthesis assay

Plasmid DNA, either pBluescript KS(+) II (pKS) (3961 bp) (Stratagene) or pRS (4470 bp), was prepared as previously described (22). For preparation of FL-treated DNA, pKS DNA (50 µg) was resuspended in 10 ml of complete RPMI to provide photosensitizers. The DNA was then exposed to FL in a covered tissue culture dish at 10 W/m$^2$ for 16 h at room temperature. The DNA was precipitated and resuspended in TE buffer. pKS DNA (50 µg) was treated with $OsO_4$ (7 µg/ml) in TE buffer containing 0.1 M NaCl for 90 min at 70°C. The γ-irradiated substrate was prepared by irradiation of 50 µg of pKS in TE with 100 Gy at 0°C. Following treatment of plasmids with oxidative sources the supercoiled plasmid DNA was purified by sucrose density sedimentation as described by Wood *et al*. (25).

DNA repair incorporation reactions were performed basically as described (25). The reactions were performed in 100 µl volume containing 75 µg of WCE and 300 ng each FL-treated pKS and undamaged pRS in 44 mM HEPES–KOH (pH 7.9), 70 mM KCl, 7.5 mM $MgCl_2$, 1.2 mM DDT, 0.05 mM EDTA, 2 mM ATP, 250 µg/ml BSA, 40 mM phosphocreatine, 50 µg/ml creatine phosphokinase, 50 µM each dATP, dGTP and TTP, 5 µM dCTP and 1 µCi of [α-$^{32}$P]dCTP. The reactions were incubated at 32°C for 2 h and stopped by the addition of 2 µl of 0.5 mM EDTA and 2 µl RNase A (2.5 mg/ml). The samples were incubated for 10 min at 37°C. Proteins were removed by proteinase K digestion (0.2 mg/ml) in 0.4% SDS at 50°C for 10 min followed by phenol/chloroform extraction. The DNA was ethanol precipitated and the plasmid DNA was linearized with *Eco*RI. The DNA was separated by electrophoresis using a 1% agarose gel containing 0.5 µg/ml ethidium bromide. The plasmid DNA was visualized and the intensity of each band was calculated using ImageQuant software (Molecular Dynamics). The DNA was dried under vacuum and exposed to a PhosphorImager screen (Molecular Dynamics) for ~18 h.

The number of endonuclease III- and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites were determined by

**Table 1.** Lesion frequency in normal (AG1011) and XP-A (GM2250) cells as measured by GC/IDMS

| Cell type | Lesion | Lesion frequency (lesions/$10^6$ bases) | | |
|---|---|---|---|---|
| | | Control | 6 h FL exposure | 6 h repair |
| Normal (AG10111) | 5-OH-Cyt | 27.84 ± 0.96 | 61.01 ± 2.13 (*P* = 0.0002) | 43.73 ± 2.13 (*P* = 0.003) |
| | FapyGua | 120.96 ± 4.16 | 230.83 ± 24.64 (*P* = 0.006) | 93.33 ± 12.12 (*P* = 0.007) |
| | FapyAde | 16.43 ± 3.36 | 95.04 ± 3.20 (*P* = 0.001) | 65.28 ± 7.46 (*P* = 0.001) |
| XPA (GM2250) | 5-OH-Cyt | 30.40 ± 9.42 | 43.84 ± 15.52 (*P* = 0.224) | 53.33 ± 6.71 (*P* = 0.150) |
| | FapyGua | 69.12 ± 3.52 | 148.27 ± 3.57 (*P* = 0.001) | 143.89 ± 24.38 (*P* = 0.393) |
| | FapyAde | 22.19 ± 7.84 | 59.09 ± 17.22 (*P* = 0.011) | 75.84 ± 10.30 (*P* = 0.065) |

The *P* values indicate significance for increase in lesion frequency following FL treatment as compared to control and for differences in lesion frequency following a 6 h repair period as compared to the level following 6 h FL exposure.

nicking the supercoiled DNA with repair enzymes. FL-treated pKS DNA (5 µg/ml) was treated with increasing concentrations of Fpg (sp. act. $4 \times 10^7$ U/mg; provided by A. Grollman) in 50 mM Tris–HCl (pH 8.0), 50 mM KCl and 1 mM EDTA in a 20 µl reaction volume at 37°C for 30 min. Endonuclease III-sensitive sites were measured in FL-treated samples under similar conditions using endonuclease III (10 U/µl) and reaction buffer (5 mM HEPES, pH 7.0, and 0.1 M KCl) from Trevigen Inc. (Gaithersburg, MD). Following incubation, the DNA was separated by electrophoresis on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. The intensity of the supercoiled and nicked DNA was determined with a FluorImager (Molecular Dynamics) and analyzed using ImageQuant software (Molecular Dynamics). The average number of nicks per plasmid was determined based on a Poisson distribution of DNA damage as previously described (22).

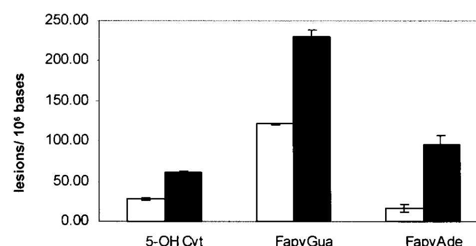


**Figure 1.** Formation of oxidative DNA base lesions in cellular DNA following FL exposure. The number of adducts after no treatment and 6 h FL exposure represents endogenous and induced adduct levels, respectively. Data represent the means ± SD from three biological replicates.

## RESULTS

### Formation of oxidative DNA base lesions following exposure to FL

The cells were exposed to FL ('coolwhite' bulbs) at 10 W/m$^2$ for 6 h in serum-free media. Induction of oxidative DNA base lesions in normal cells was measured by GC/IDMS analysis. Endogenous levels of five oxidative lesions, 5-OH-Cyt, Fapy-Gua, FapyAde, 8-hydroxyadenine and 8-oxoGua, were detected and reliably quantified from the multitude of DNA base lesions that can be measured by GC/IDMS (26). Of these five endogenous lesions, 5-OH-Cyt, Fapy Ade and Fapy Gua were induced by FL exposure, as shown in Figure 1, with more detailed quantitation in Table 1. The level of 5-OH-Cyt and FapyGua each increased ~2-fold while the level of FapyAde exhibited a greater increase of 5.7-fold (Fig. 1).

### Repair of oxidative DNA base lesions

Repair of 5-OH-Cyt, FapyGua and FapyAde in normal and XP-A cells was next determined using GC/IDMS. These data are shown in Figure 2, with more detailed quantitation in Table 1. It can be seen from Figure 2 that all three lesions were repaired to some extent during the 6 h repair incubation. The lesions 5-OH-Cyt and FapyGua (Fig. 2A and B) were fully repaired in that time period whereas FapyAde was only partially removed (Fig. 2C). In contrast, the levels of all three lesions continued to increase in the XP-A cells during the 6 h repair incubation (Fig. 2A–C). The difference in repair efficiency between normal and XP-A cells was most dramatic for the lesions 5-OH-Cyt and FapyGua. The increase in lesions during the repair period in XP-A cells indicates the presence of a long-lived species producing DNA damage. Lipid peroxide molecules or some other unidentified long-lived species may contribute to generation of oxidative DNA damage following FL exposure (29). In the presence of a long-lived species, decreased catalase activity, which has been reported in XP-A cells, may also contribute to the increase in oxidative lesions during the repair incubation (30).

FapyAde was repaired with minimal efficiency during the 6 h following FL treatment in normal cells (Fig. 2C). Inefficient repair of FapyAde is consistent with previous reports of a relatively longer half-life for FapyAde produced by $H_2O_2$ treatment (31). In addition, we observed that FapyAde was induced to greater levels than 5-OH Cyt and FapyGua by FL exposure. This may be a result of decreased rate of repair during the 6 h FL exposure period.

### FL exposure introduces oxidative DNA lesions *in vitro*

The frequency of oxidative DNA base lesions introduced by *in vitro* exposure of DNA to FL was estimated by measuring the number of endonuclease III- and Fpg-sensitive sites. Plasmid DNA (pKS, 3961 bp) was incubated with increasing

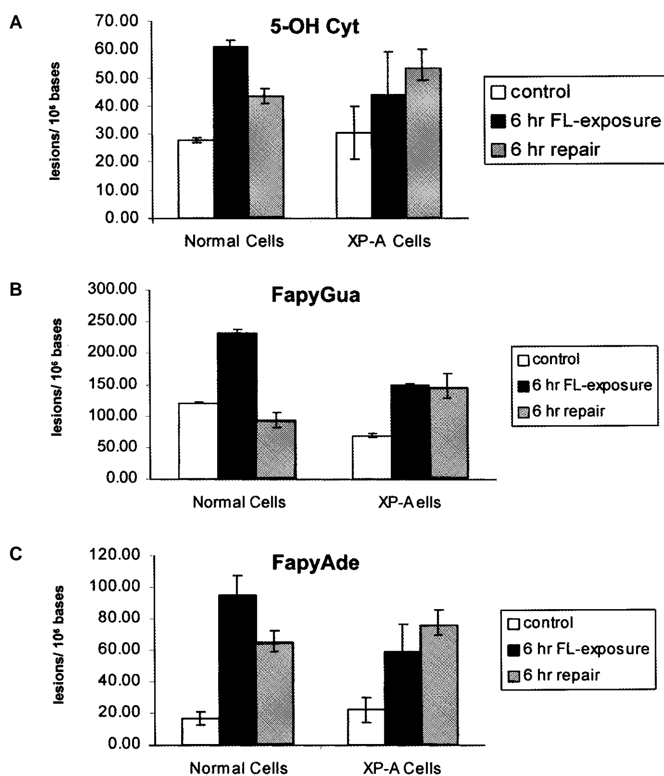


**Figure 2.** Repair of FL-induced base lesions in cellular DNA from normal and XP-A lymphoblasts. The repair of 5-OH-Cyt (**A**), FapyGua (**B**) and FapyAde (**C**) immediately following FL exposure (induced) and those remaining after 6 h of repair are indicated as lesion frequency per $10^6$ bases. Data represent the means ± SD for three biological replicates.

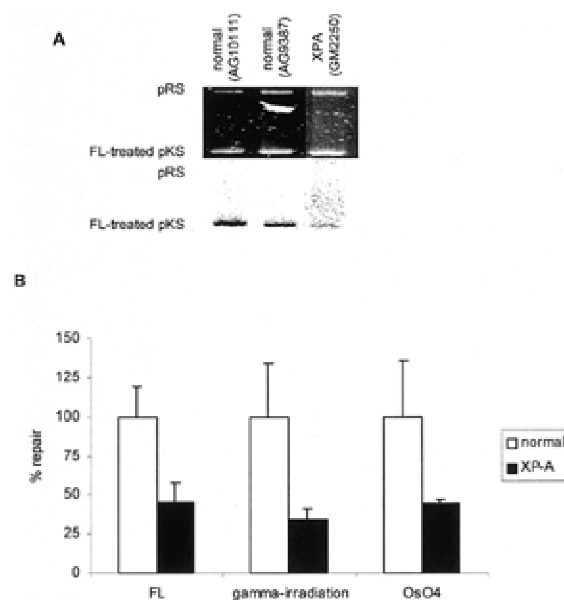


**Figure 3.** Damage-specific repair synthesis by WCEs prepared from normal and XP-A cells. (**A**) Gel images of ethidium bromide staining (top) and radioactivity incorporation (bottom) using untreated pRS and FL-damaged pKS with normal and XP-A extracts. (**B**) Damage-specific DNA repair incorporation using either FL-treated, γ-irradiated or $OsO_4$-treated pKS. The data were normalized to the average incorporation by WCEs prepared from two normal cell lines (AG1011 and GM 9387). Histograms represent the means ± the SE for three independent experiments.

concentrations of either endonuclease III or Fpg to detect the formation of enzyme-sensitive sites. Both endonuclease III- and Fpg-sensitive sites were detected in FL-treated DNA, as shown in Table 2. At a saturating level of endonuclease III (0.02 µg protein), 95 endonuclease III-sensitive sites/$10^6$ bases were detected above background nicking. Similarly, 0.8 U of Fpg glycosylase generated 110 Fpg-sensitive sites/$10^6$ bases over background nicking. There was very little detectable incision of undamaged DNA using either enzyme (Table 2).

### *In vitro* repair synthesis of oxidative lesions

The incorporation of radioactive material into the characterized substrates was measured using the *in vitro* repair synthesis assay with extracts from normal and XP-A lymphoblast cell lines. Damage-specific incorporation was detected with FL-treated DNA using normal extracts (Fig. 3A). Repair synthesis was greatly decreased in XP-A WCE. The three lesions that we found to be induced by FL, 5-OH-Cyt, FapyGua and FapyAde, have also also been shown to be induced by γ-irradiation and $H_2O_2$ treatment of cells (32). We thus exposed plasmids to these other forms of oxidative stress. We observed a decrease in repair capacity in XP-A WCE. The repair was reduced by 60–75% of that in normal WCE and this deficiency was similar for plasmid DNA damaged by either FL, γ-irradiation or $OsO_4$ (Fig. 3B).

## DISCUSSION

The biological effects of visible light have been widely studied. Mutations (4) and endonuclease-sensitive sites have been detected after exposure to visible light (5). The harmful effects of visible light on cells in culture have been known for many years (1) and it has been suggested that there might be DNA

**Table 2.** Enzyme-sensitive sites in undamaged and FL-treated DNA

| Protein | Enzyme-sensitive sites in undamaged DNA | Enzyme-sensitive sites in FL-treated DNA |
|---|---|---|
| No enzyme | 37/$10^6$ bases | 98/$10^6$ bases |
| Endonuclease III (0.20 µg/mg DNA) | 39/$10^6$ bases | 134/$10^6$ bases |
| Fpg protein (0.20 U/mg DNA) | 40/$10^6$ bases | 150/$10^6$ bases |

The endonuclease III- and Fpg-sensitive sites/$10^6$ bases were determined based on the conversion of supercoiled DNA to nicked form.

modifications (3). However, this is the first report of the induction of specific oxidative DNA base lesions after FL. We find that FL induces the lesions 5-OH-Cyt, FapyGua and FapyAde. These lesions are also known to be induced by other forms of oxidative stress, as they have been shown to be increased after $H_2O_2$ treatment and γ-irradiation (31). Our conditions for FL exposure are rather intensive and humans are rarely exposed to FL light for hours at a short distance. However, since FL is so ubiquitous, it may be advisable to begin to think about ways of limiting this exposure.

We find that XP-A cells are deficient in the repair of certain oxidative DNA base lesions introduced by FL. Our observations are consistent with previous reports indicating compromised repair of oxidative stress-induced DNA damage in XP-A cells. Sensitivity of some XP-A cells to γ-irradiation indicates a possible repair defect (33). Additionally, alterations in chromatid break frequency in XP-A cells were detected following exposure of cultured cells to X-rays and FL (34). Defects in NER of an unidentified, minor bulky oxidative lesion (23) and of other oxidative DNA base modifications (8-oxoG and thymine glycol; 16) have been demonstrated in XP-A cell extracts. Repair of a shuttle vector containing a single 8-oxoG was deficient after passage through XP-A cells (35). Also, this laboratory has recently demonstrated that repair of 8-oxoG-containing substrates is deficient in XP-A extracts (22,36). Thus, there have been indications in the literature suggesting that XPA protein is involved in the processing of oxidative DNA damage. This study is, however, the first to identify deficient repair of the specific oxidative DNA base lesions 5-OH-Cyt, FapyGua and FapyAde in XP-A cells.

In this report, we have demonstrated that 5-OH-Cyt, FapyGua and FapyAde are induced in cellular DNA by exposure to FL. Additionally, we have shown that 5-OH-Cyt and FapyGua are not efficiently repaired in XP-A cells. If, indeed, XPA protein has a direct involvement in the repair of oxidative DNA base lesions, the removal of these lesions may be more complex than through the classic BER pathway. XPA may participate in the early steps of BER by increasing the affinity of DNA glycosylases for oxidative DNA lesions. This model is consistent with the known role of XPA as a damage recognition protein, including the ability of XPA to bind to substrates damaged by oxidative sources (37). Alternatively, XPA may influence later steps of BER through interactions with proteins such as RPA (38).

The yeast and human homologs of endonuclease III have been shown to release 5-OH-Cyt from substrates damaged by oxidative sources (39,40). Similarly, the eukaryotic Ogg1 protein releases FapyGua from DNA damaged by oxidative sources (41). Therefore, the repair of these lesions is thought to be initiated by DNA glycosylases and repair completed through BER. Since we have demonstrated deficient repair of these lesions in XP-A cells, there might be a role for XPA in processing these lesions. This notion is also supported by our *in vitro* experiments. The damaged plasmid DNA used for the repair synthesis assays primarily contained oxidative DNA base lesions. FL treatment of plasmid DNA produces endonuclease III- and Fpg-sensitive sites; γ-irradiation has been shown previously to induce both pyrimidine and purine base damage (42); $OsO_4$ treatment of DNA produces predominately thymine glycol and to a lesser extent cytosine glycol and 5,6-dihydroxycytosine (43). The decreased *in vitro* repair synthesis capacity of XP-A cell extracts further supports the involvement of XPA in removal of oxidative DNA base damage.

Many disease states, including breast cancer, have been been suggested to have defects in repair of oxidative DNA damage based on assays which evaluate chromatid stability (2). This further suggests that inefficient repair of endogenous oxidative DNA damage may lead to cancer (44). Decreased repair of endogenous DNA damage may also be a factor responsible for the increased level of internal cancers seen in XP-A patients. Exposure to exogenous carcinogens, such as cigarette smoke, has been implicated in a portion of the increased incidence of internal organ cancers in XP patients (20). However, decreased DNA repair of oxidative lesions in XP-A may also lead to increased mutation frequency and ultimately carcinogenesis without exposure to UV light or chemical carcinogens. Recently, evaluation of p53 mutations in tumors from internal organs from XP-C patients suggested that the mutations may be due to DNA damage from endogenous sources (45). Additionally, increased oxidative DNA damage has been implicated in disease states associated with neurodegeneration, including XP. The increased oxidative DNA damage in these conditions is possibly due to deficient DNA repair (46,47). Our data suggest that a deficiency in repair of oxidative DNA base lesions in XP-A patients may lead to accumulation of damage. Persistence of such lesions may result in altered cell function and neurodegeneration.

## ACKNOWLEDGEMENTS

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