# G-to-T Transversions and Small Tandem Base Deletions Are the Hallmark of Mutations Induced by Ultraviolet A Radiation in Mammalian Cells†

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ABSTRACT: Ultraviolet A (UVA) radiation received from the sun and from the widespread use of tanning beds by populations residing in areas of northern latitude represents a potential risk factor for human health. The genotoxic and cancer-causing effects of UVA have remained controversial. A mutagenic role for UVA based on DNA damage formation by reactive oxygen species as well as by generation of photoproducts such as cyclobutane pyrimidine dimers (CPDs) has been suggested. Here, we investigated the mutagenicity of UVA in relation to its DNA damaging effects in transgenic Big Blue mouse embryonic fibroblasts. We determined the formation of a typical oxidative DNA lesion, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), and of CPDs, as well as quantified the induction of mutations in the *cII* transgene in cells irradiated with a 2000 W UVA lamp. UVA irradiation at a dose of 18 J/cm$^2$ produced significant levels of 8-oxo-dG in DNA ($P < 0.03$) but did not yield detectable CPDs. UVA irradiation also increased the *cII* mutant frequency almost 5-fold over background ($P < 0.01$) while showing moderate cytotoxicity (70% cell viability). UVA-induced mutations were characterized by statistically significant increases in G-to-T transversions and small tandem base deletions ($P = 0.0075$, $P = 0.008$, respectively) relative to spontaneously derived mutations. This mutational spectrum differs from those previously reported for UVA in other test systems; however, it corresponds well with the known spectrum of mutations established for oxidative base lesions such as 8-oxo-dG. We conclude that UVA has the potential to trigger carcinogenesis owing to its mutagenic effects mediated through oxidative DNA damage.

Solar ultraviolet (UV)[1] radiation is a well-characterized physical carcinogen, and has long been implicated in basal and squamous cell carcinomas of the skin and more recently in cutaneous malignant melanoma in humans (*1*−*3*). The UV portion of sunlight is comprised of UVC (<280 nm), UVB (280−320 nm), and UVA (320−400 nm). Stratospheric oxygen ($O_2$) generated during photosynthesis in plants absorbs the entire UVC fraction of the sunlight and decomposes in the process. It then recombines to form ozone ($O_3$), which in turn absorbs most of the UVB before it can reach the earth's surface. The remaining portion of the solar UV to which living organisms on earth are exposed is mainly composed of UVA (∼95%) and UVB (∼5%) (*4*, *5*). Unlike UVB that induces promutagenic cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)PPs) (*6*, *7*), the carcinogenicity of UVA has mostly been ascribed to endogenous photosynthesizers causing reactive oxygen species-mediated induction of DNA damage and mutations (*1*, *8*−*14*). However, more recently, it has been reported that UVA induces CPDs in rodent cells as well (*15*−*17*). Altogether, the poor absorbance of UVA by DNA favors the idea that photosensitization reactions occurring within cells are primarily responsible for UVA carcinogenicity. Presumably, the cellular chromophores excited by UVA generate reactive intermediates, which are capable of forming DNA lesions. DNA lesions refractory to repair have the potential to trigger mutagenesis as they promote miscoding during translesion synthesis. UVA has been shown to induce 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a typical oxidative DNA lesion, as well as generate single- and double-strand DNA breaks in a variety of experimental systems (*8*, *12*, *14*, *18*−*20*). Also, UVA-induced mutagenesis has been documented in various reporter genes in bacteria and mammalian cells (*9*−*11*, *20*, *21*). Nevertheless, a direct link between UVA-induced DNA damage and mutagenesis has not been clearly established.

In the present study, we investigated the DNA damaging effects of UVA in relation to mutagenesis in Big Blue mouse embryonic fibroblasts. In this transgenic in vivo system, genomic DNA containing a recoverable lambda shuttle vector, which carries the mutational *cII* target gene, can be screened for DNA lesions, as well as assayed for mutation induction at the nucleotide level (*22*). Here, the UVA-irradiated cells were evaluated for the formation of 8-oxo-dG by high-pressure liquid chromatography/tandem mass spectrometry (HPLC-MS/MS), for CPDs by a T4-endonuclease V digestion assay, and for DNA strand breaks and/

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[1] Abbreviations: (6-4)PPs, pyrimidine (6-4) pyrimidone photoproducts; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; CHO, Chinese hamster ovary; CPDs, cis−syn cyclobutane pyrimidine-dimers; DMEM, Dulbecco's Modified Eagle's medium; HPLC-MS/MS, high-pressure liquid chromatography/tandem mass spectrometry; PBS, phosphate buffered saline; TD-PCR, terminal transferase-dependent polymerase chain reaction; UV, ultraviolet radiation.

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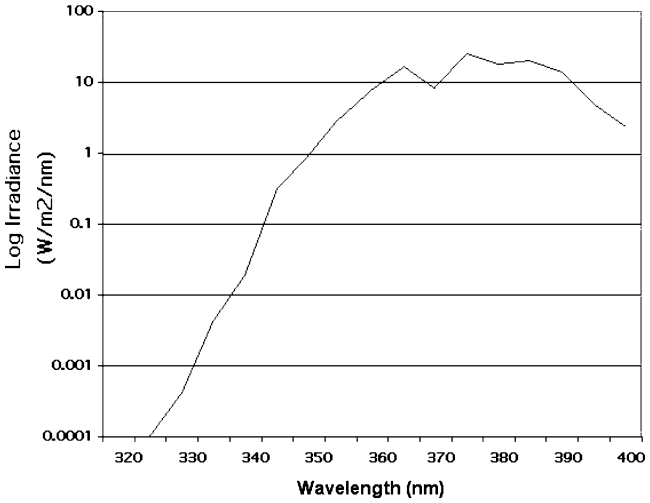


FIGURE 1: Emission spectrum of UVA source.

or bulky DNA adducts by a terminal transferase-dependent polymerase chain reaction (TD-PCR) procedure. Simultaneously, the induction and spectrum of *cII* mutations were determined by the lambda phage-based mutation detection system.

## MATERIALS AND METHODS

*Cell Culture and Irradiation.* Big Blue mice were obtained from Stratagene (La Jolla, CA). Early passage Big Blue mouse embryonic fibroblasts (prepared from 13.5-day-old embryos) were grown as monolayers (about 50% confluence) in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were kept in phenol red- and serum-free medium, Opti-MEM I (Invitrogen, Carlsbad, CA), for 12 h before irradiation. The cells were irradiated with doses of 18 and 36 J/cm$^2$ of UVA. The UVA source was a Sellas Sunlight system (Medizinische Geräte GmbH, Gevelsberg, Germany) with an average fluence rate of 60 mW/cm$^2$ mainly emitting wavelengths between 340 and 400 nm (Figure 1). For homogeneous irradiation of the cells, the culture Petri dishes were placed directly under the source at a distance of 1 cm, and were rotated every 30 s during the course of irradiation (5 or 10 min). No excessive heat was generated in the medium throughout the irradiation. Following the irradiation, the cells were harvested by trypsinization and evaluated for survival by the trypan blue dye exclusion technique, and for detection of various DNA lesions using the respective assays. Alternatively, the cells were cultured in complete growth medium for an additional 8 days, and afterward were analyzed for mutant frequency and mutational spectrum of the *cII* transgene. The 8-day growing period is essential for the fixation of all UVA-induced mutations into the genome. All experiments were run in triplicate or quadruplicate.

*Genomic DNA Isolation for HPLC-MS/MS Analysis.* The cell pellets were washed twice with prewarmed phosphate buffered saline (PBS), pH 7.5, and lyzed with 4 mL of a solution containing 0.5 M Tris-HCl, pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, and 0.5 mg/mL proteinase K at 37 °C overnight (*23*). Subsequently, 2 mL of saturated NaCl (~6 M) was added to each sample, and the samples were incubated at 56 °C for 10 min. After centrifugation at 5000*g* for 30 min, the supernatant containing DNA was mixed with 2 volumes of prechilled ethanol (100%), and the DNA was spooled by gently inverting the mix. The DNA was washed thoroughly with 70% ethanol, air-dried, and subsequently dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5). Aliquots of 75 $\mu$g of DNA were dried in a Speedvac, and then dissolved in 100 $\mu$L of a buffer solution containing 10 mM Tris-HCl, pH 7.5 (98 $\mu$L), and 1 M sodium acetate pH 6.0 (2 $\mu$L). Each DNA aliquot was digested with 10 U of nuclease P1 (Sigma-Aldrich) for 1 h, and afterward with 0.004 U of phosphodiesterase 1 (Sigma-Aldrich) together with 34 U of alkaline phosphatase (Roche, Indianapolis, IN) for 2 h at 37 °C. The samples were filter-centrifuged (Millipore Co., Bedford, MA) at 6000*g* for 30 min. The eluents were mixed with an equal volume of methanol and dried in a Speedvac concentrator.

*Genomic DNA Isolation for Enzymatic Digestion Assay, TD-PCR and Mutation Analyses.* Genomic DNA was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol (*24*). The DNA was dissolved in TE buffer, and preserved at −80 °C until further analysis.

*HPLC-MS/MS for Quantification of 8-oxo-dG.* Quantification of 8-oxo-dG was performed using the previously published method of Singh et al. (*25*) with modifications. Briefly, analytical grade 8-oxo-dG was purchased from Cayman Chemical (Ann Arbor, MI) and mass-labeled 8-oxo-dG was kindly provided by Dr. Miral Dizdaroglu of the National Institute of Standards and Technology. Instrumentation consisted of an Agilent 1100 Capillary LC system (Agilent Technologies; Palo Alto, CA) in line with a Micromass Quattro Ultima Triple Quadrupole Mass spectrometer. (Micromass Inc., Beverly, MA). The detector settings were as follows: capillary voltage = 2.20 kV; cone voltage = 16 V; collision cell voltage = 13 V; source temperature = 125 °C; desolvation temperature = 260 °C; cone gas flow = 130 L/h; desolvation gas flow = 500 L/h. The mass transitions monitored for 8-oxo-dG and internal standard were 284 → 168 and 286 → 170, respectively.

Chromatographic conditions consisted of isocratic separation across a Synergi C18 4$\mu$ 150 × 2.0 mm analytical column (Phenomenex, Torrance, CA) using a mobile phase of 8% methanol (Fisher Scientific, USA) in water with 0.1% formic acid (J. T. Baker, USA). The flow rate was 0.2 mL/min, with a total run time of 30 min and a retention time of 8-oxo-dG of 14.9 min. The lower limit of quantitation for 8-oxo-dG was 0.1 ng/mL in the starting solution or 0.2 pg on column. Both precision and accuracy of the assay are within ±10% of target values, respectively,

*T4-Endonuclease V Digestion Assay for Detection of CPDs.* The enzymatic digestion assay is based on the ability of T4 endonuclease V for nicking DNA specifically at CPDs. The cleaved DNA can subsequently be visualized by alkaline gel electrophoresis. Briefly, DNA (1 $\mu$g) was digested with an excess amount of T4 endonuclease V (kindly provided by Dr. T. R. O'Connor, City of Hope) in a buffer solution containing 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 100 $\mu$g/mL bovine serum albumin for 1 h at 37 °C. After phenol/chloroform extraction and ethanol precipitation, the digest was loaded onto a 1.5% alkaline agarose gel, and run for 4 h at 40 V. As a control, known CPD-containing DNA isolated from UVB-irradiated cells was run in all analyses.

*TD-PCR for Mapping of Bulky DNA Adducts and/or DNA Strand Breaks.* The entire length of the *cII* transgene was subjected to TD-PCR as described earlier with some modifications (*26*, *27*). Genomic DNA (100 ng) was used as a template, and single-stranded products were made by repeated primer extensions. The extension protocol consisted of a custom-made biotinylated primer, U1: 5′-AATC-GAGAGTGCGTTGCTT-3′, $T_m$ = 49.9 °C in a mixture of Vent $^{(exo-)}$ DNA polymerase (New England Biolabs, Beverly, MA) with a thermocycler setting of one cycle of 2 min at 95 °C, 2 min at 61 °C, 3 min at 72 °C, and nine cycles (in which one cycle consisted of 45 s at 95 °C, 2 min at 61 °C, and 3 min at 72 °C). The resultant was mixed with streptavidin-coupled magnetic beads (Dynal ASA, Oslo, Norway), and binding was achieved by gently rotating the mixture for 45 min at room temperature. The streptavidin-bound DNA was denatured by incubation with 0.15 M NaOH at 37 °C for 10 min. After thorough washing of the sample with 1× TE, pH 7.5, in a magnetic particle concentrator (Dynal ASA), the single-stranded DNA was resuspended in 0.1× TE pH 7.5, and afterward was subjected to homopolymeric ribotailing and adapter ligation (*24*, *26*). The ligation product was washed with 1× TE, pH 8.0, in the magnetic particle concentrator, resuspended in 0.1× TE pH 8.0, and amplified in a PCR using primer U2: 5′-GCGTTGCTTAA-CAAAATCGCAATGCT-3′, $T_m$ = 63.1 °C, and the linker primer, in the presence of Expand Long Polymerase (Roche). The thermocycler settings were as follows: 2 min at 95 °C, 2 min at 62 °C, 3 min at 72 °C, 18 cycles (in which one cycle consisted of 45 s at 95 °C, 2 min at 62 °C, and 3 min at 72 °C), 45 s at 95 °C, 2 min at 62 °C, and 10 min at 72 °C. The PCR product was labeled by a fluorescence infrared dyed primer [IRD-700] (LI-COR Inc., Lincoln, NE) U3: 5′-GCAATGCTTGGA-ACTGAGAAGACAGC-3′, $T_m$ = 61.4 °C in a thermocycler setting of 2 min at 95 °C, 2 min at 66 °C, 3 min at 72 °C, one cycle of 45 s at 95 °C, 2 min at 66 °C, and 3 min at 72 °C, 45 s at 95 °C, 2 min at 66 °C, and 10 min at 72 °C. The labeled products were loaded onto a 5% polyacrylamide/urea gel for electrophoresis and simultaneous quantification by an IR$^2$ Long Ranger 4200 system (LI-COR Inc.). The locations of DNA adduct formation and/or DNA strand breaks were identified as the sites in which the presence of the lesions stopped the DNA polymerase from progressing, resulting in an intense dark band (dependent on the lesion frequency) in the sequencing gel.

*cII Mutant Frequency Analysis.* The *cII* mutant frequency was determined by the lambda select-*cII* mutation detection system for Big Blue rodents (Stratagene) (*22*). The lambda LIZ shuttle vectors were recovered from the genomic DNA (∼5 μg) and packaged into viable phage particles by Transpack packaging extract according to the manufacturer's instructions (Stratagene). The phages were then preadsorbed to G1250 *Escherichia coli*, and the bacterial culture was plated on TB1 agar plates. The plates were incubated for 48 h at 24 °C or overnight at 37 °C (selective and nonselective conditions, respectively). The *cII* mutant frequency was expressed as the ratio of the number of plaques formed on the selective plates to that formed on the nonselective plates. A minimum of 3 × 10$^5$ rescued phages was screened for each experimental condition. For quality assurance, control phage solutions containing a mixture of lambda *cII* $^{(+)}$ and lambda *cII* $^{(-)}$ with known mutant frequencies (Stratagene) were assayed in all runs.

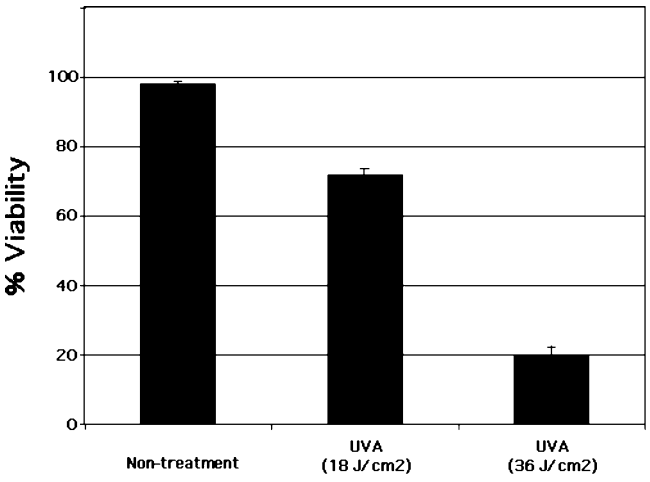


FIGURE 2: Cytotoxicity of UVA in Big Blue mouse embryonic fibroblasts. Confluent cell cultures were irradiated with 18 or 36 J/cm$^2$ UVA, and cell viability was determined by trypan blue dye exclusion assay at 24 h after irradiation. Viability is expressed as a percentage of total cell number. Results are expressed as medians of two independent experiments, with each experiment run in quadruplicate. Error bars = SE.

*cII Mutational Spectrum Analysis.* The putative *cII* mutant plaques were all verified after being replated under the selective conditions on a second TB1 agar plate. The verified plaques were amplified in a PCR using the lambda select-*cII* sequencing primers according to the recommended protocol (Stratagene). The PCR products were purified with QIA quick PCR purification kits (Qiagen, Hilden, Germany), and subsequently sequenced by a Big Dye terminator cycle sequencing kit on an ABI-377 DNA sequencer (ABI Prism, PE Applied BioSystems; Foster City, CA).

*Statistical Analysis.* Results are expressed as medians ± SE. All variables in the treated groups were compared to their respective counterparts in the control group using the Wilcoxon signed rank test. The entire mutational spectra and the specific types of mutations in the treated versus control groups were compared by the hypergeometric test of Adams and Skopek (*28*) and chi-square test, respectively. Relationship between different variables was calculated using the Spearman rank correlation coefficient analysis. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

*Cytotoxicity Examination.* As shown in Figure 2, UVA irradiation was cytotoxic in mouse embryonic fibroblasts. The severity of cytotoxicity was dependent on the radiation dose (Figure 2). At physiologically relevant doses of UVA, 18 and 36 J/cm$^2$, respectively, approximately 70 and 20% of the cells remained viable. A UVA dose of 18 J/cm$^2$ is comparable to doses received during a single session in a UVA tanning parlor (up to 60 J/cm$^2$) or a 10-min sun exposure in summer at noon at 45° latitude (*9*).

*Quantification of 8-oxo-dGs and CPDs.* UVA irradiation induced the formation of 8-oxo-dGs in mouse embryonic fibroblasts as measured by HPLC-MS/MS (Figure 3A). The extent of 8-oxo-dG generation was directly related to the radiation dose ($r = 0.97$, $P < 0.03$). At 18 J/cm$^2$, the levels of 8-oxo-dG were induced 5.4-fold over background, and

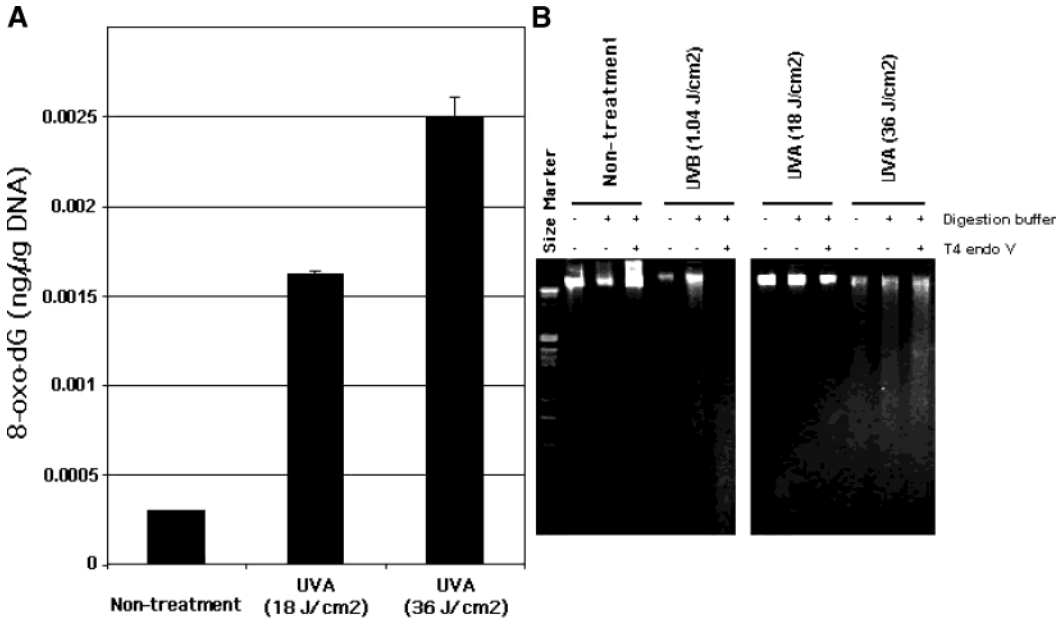


FIGURE 3: UVA-induced DNA damage. (A) Quantification of 8-oxo-dG by HPLC-MS/MS in Big Blue mouse embryonic fibroblasts irradiated with 18 or 36 J/cm$^2$ UVA. Results are expressed as medians of two independent experiments, with each experiment run in triplicate. Error bars = SE. (B) Enzymatic digestion of CPDs by T4 endonuclease V in DNA from mouse embryonic fibroblasts irradiated with 18 or 36 J/cm$^2$ UVA. DNA digests were extracted with phenol/chloroform, precipitated with ethanol, and subsequently run on 1.5% alkaline/agarose gels. Known CPD-containing DNA isolated from UVB-irradiated cells was used as a positive control. Size marker, lambda mixed ladder (HindIII cut + HindIII/EcoRI cut).

they increased to 8.4-fold over background at 36 J/m$^2$ UVA reaching levels of 2.5 pg per microgram of DNA.

In contrast, UVA irradiation of the cells at doses of 18 or 36 J/cm$^2$ did not produce any detectable CPDs as measured by T4 endonuclease V digestion assay (Figure 3B). Elevation of the radiation dose, however, increased the amount of DNA fragmentation independently of T4 endonuclease V digestion (Figure 3B). The increased DNA fragmentation corresponds to the greater cytotoxicity of UVA at the higher dose and may indicate apoptotic cell death.

*Quantification of DNA Adducts and Strand Breaks.* TD-PCR analyses showed no appreciable formation of polymerase-blocking bulky DNA lesions (CPDs or (6-4)PPs) and/or DNA strand breaks in mouse embryonic fibroblasts irradiated with 18 or 36 J/cm$^2$ UVA (Figure 4). In contrast, UVB-induced CPDs and (6-4)PPs were clearly detectable by TD-PCR (Figure 4).

*cII Mutant Frequency and Mutational Spectrum Analyses.* UVA irradiation at a dose of 18 J/cm$^2$ was mutagenic in mouse embryonic fibroblasts as it significantly increased the *cII* mutant frequency relative to the background [$(28.6 \pm 0.43)$ versus $(6.2 \pm 0.4) \times 10^{-5}$; $P < 0.01$)] (Figure 5). After doubling the radiation dose, however, the increment in induced *cII* mutant frequency was not statistically significant [$(30.4 \pm 3.3) \times 10^{-5}$; $P < 0.1$)] possibly because cell survival was diminished at the higher dose (Figure 2).

For mutational spectra analyses, DNA sequencing was performed on the *cII* plaques formed in samples irradiated with 18 J/cm$^2$ UVA, or in the nontreated control (number of sequenced plaques = 161 and 173, respectively). Overall, DNA sequencing confirmed a mutated *cII* gene in 93−95% of all analyzed plaques. Of these, the vast majority were single base substitutions and less frequently single base insertions/deletions and small tandem base deletions/base substitutions, respectively (Figure 6A,B). In both induced and spontaneous mutational spectra, there were four "jackpot" mutations at nucleotide positions 179−184 [G insertion/deletion], 196 [G to A transition], 211 [G to C transversion], and 221 [T to G transversion] (Figure 6A,B). These jackpot mutations, already reported by us (*27*, *29*) as well as by others (*30*, *31*), are common phenomena in transgenic model systems. Presumably, the jackpot mutations occur in the early development of the transgenic animal and undergo clonal expansion such that many cells from various tissues harbor the same type of mutations. The jackpot mutations might also represent genuine hotspots of spontaneous mutagenesis (*32*, *33*). Regardless of their origin, it is methodologically appropriate to exclude all jackpot mutations from the comparative spectra analyses. Excluding the jackpot mutations, the overall spectrum of mutations induced by UVA radiation was significantly different from that derived spontaneously ($P < 0.001$; Adams-Skopek test).

Because the *cII* transgene is almost certainly not transcribed after being integrated into the genome (*34*), the "strand-biased mutagenesis", a phenomenon caused by transcription-coupled DNA repair in mammalian endogenous genes (*35*, *36*), is an unlikely event in the Big Blue system. Therefore, we combined the strand mirror counterparts of all transitions (e.g., G to A + C to T) and transversions (e.g., G to T + C to A and G to C + C to G) induced by UVA or arisen spontaneously, and made a comparative analysis for each specific type of mutation. As shown in Figure 7, G-to-T + C-to-A transversions and small tandem base deletions were the hallmark of mutations induced by UVA ($P = 0.0075$, $P = 0.008$, respectively). C-to-T transitions targeted to dipyrimidine sites, which are typical of UVB-induced mutagenesis, comprised only 24% of all UVA-induced mutations and over 21% of all mutations in nontreated cells.

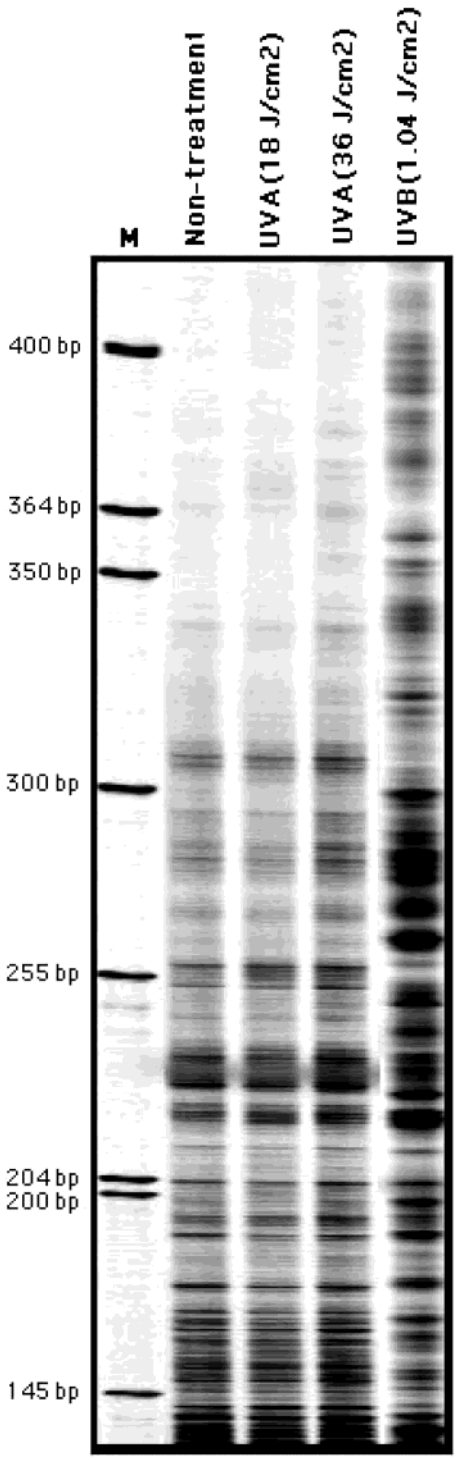


FIGURE 4: Mapping of DNA adducts and/or DNA strand breaks along the lower strand of the cII gene in Big Blue mouse embryonic fibroblasts irradiated with 18 or 36 J/cm$^2$ UVA. Genomic DNA was extracted and subjected to TD-PCR. Isolated DNA from UVB (1.04 J/cm$^2$) irradiated cells was used as a positive control. M = sizing standard; bp = base pair.

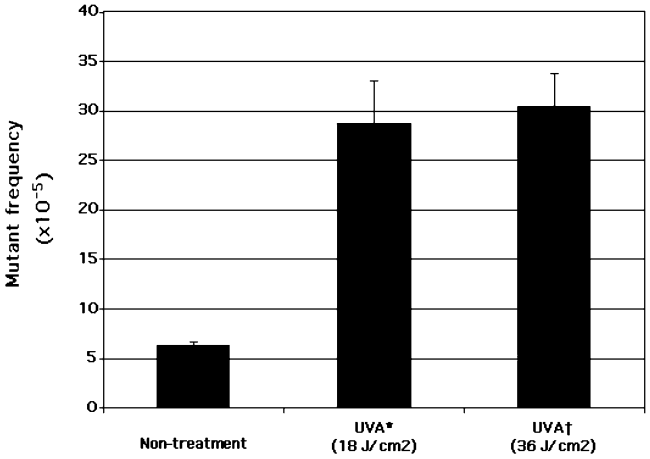


FIGURE 5: Mutant frequency of the cII transgene in Big Blue mouse embryonic fibroblasts irradiated with 18 or 36 J/cm$^2$ UVA. Quantification was done 8 days after treatments using the lambda select-*cII* mutation detection system for Big Blue rodents, a phage-based assay that permits detection of mutations within the transgene on the basis of plaque formation. Mutant frequency was determined from a minimum of $3 \times 10^5$ plaques. Results are expressed as medians of three independent experiments, with each experiment run in triplicate. Error bars = SE. *As compared with nontreatment; $P < 0.01$; †As compared with UVA (18 J/cm$^2$); $P < 0.1$.

## DISCUSSION

Various epidemiological studies have addressed the relative contributions of UVB and UVA wavelengths to skin carcinogenesis (*2*, *5*, *37*−*39*). The conclusions from these studies have been that UVB is clearly linked to nonmelanoma skin cancers and that UVA may play a role in melanoma. UVA is a complete rodent carcinogen (*4*). Although UVB dominates the carcinogenic effect of sunlight, UVA is estimated to contribute 10−20% to the carcinogenic dose (*40*). UVA is capable of inducing melanoma-like lesions in opossums (*41*) and in certain fish species (*42*). Modern high-powered tanning lamps emit mainly long wave UVA (340−400 nm). Exposure to sunlamps or sunbeds has been linked to melanoma with a 2-fold risk increase (*43*). UVA is far more abundant in sunlight than is UVB (>20 times radiant energy).

The mechanisms of UVA carcinogenicity have remained unclear. In addition to a possible tumor promoting effect mediated through chronic stimulation of cell proliferation or immunosuppression, UVA may directly induce DNA damage and mutations. In contrast to UVB, UVA is not significantly absorbed by DNA itself. Hence, the absorption by other molecules (endogenous photosensitizers) becomes more important. UVA may generate reactive oxygen species that can damage DNA (*8*). However, recently it has been reported that the formation of pyrimidine dimers, specifically CPDs forming at TT sequences, is a significant component of UVA mutagenesis (*16*, *17*). Here, we have directly compared the DNA damaging and mutagenic properties of UVA in mouse fibroblasts carrying a mutational reporter gene. We have shown that (I) UVA causes oxidative DNA base lesions, (II) UVA is mutagenic, and (III) the UVA-induced mutational spectrum is consistent with the known mutational spectrum of oxidative DNA base damage.

We have used a highly sensitive and specific HPLC-MS/MS technique with mass-labeled internal standards to demonstrate unequivocally the formation of 8-oxo-dG in UVA-irradiated cells. On the other hand, we were not able to detect the existence of CPDs or (6-4)PPs in UVA-irradiated cells, either by T4 endonuclease V cleavage or by the highly sensitive TD-PCR assay. Very high levels of nondimer DNA photolesions, mostly in the form of 8-oxo-dG, have previously been reported after irradiation of DNA or cells with UVA or solar light-mimicking radiation sources

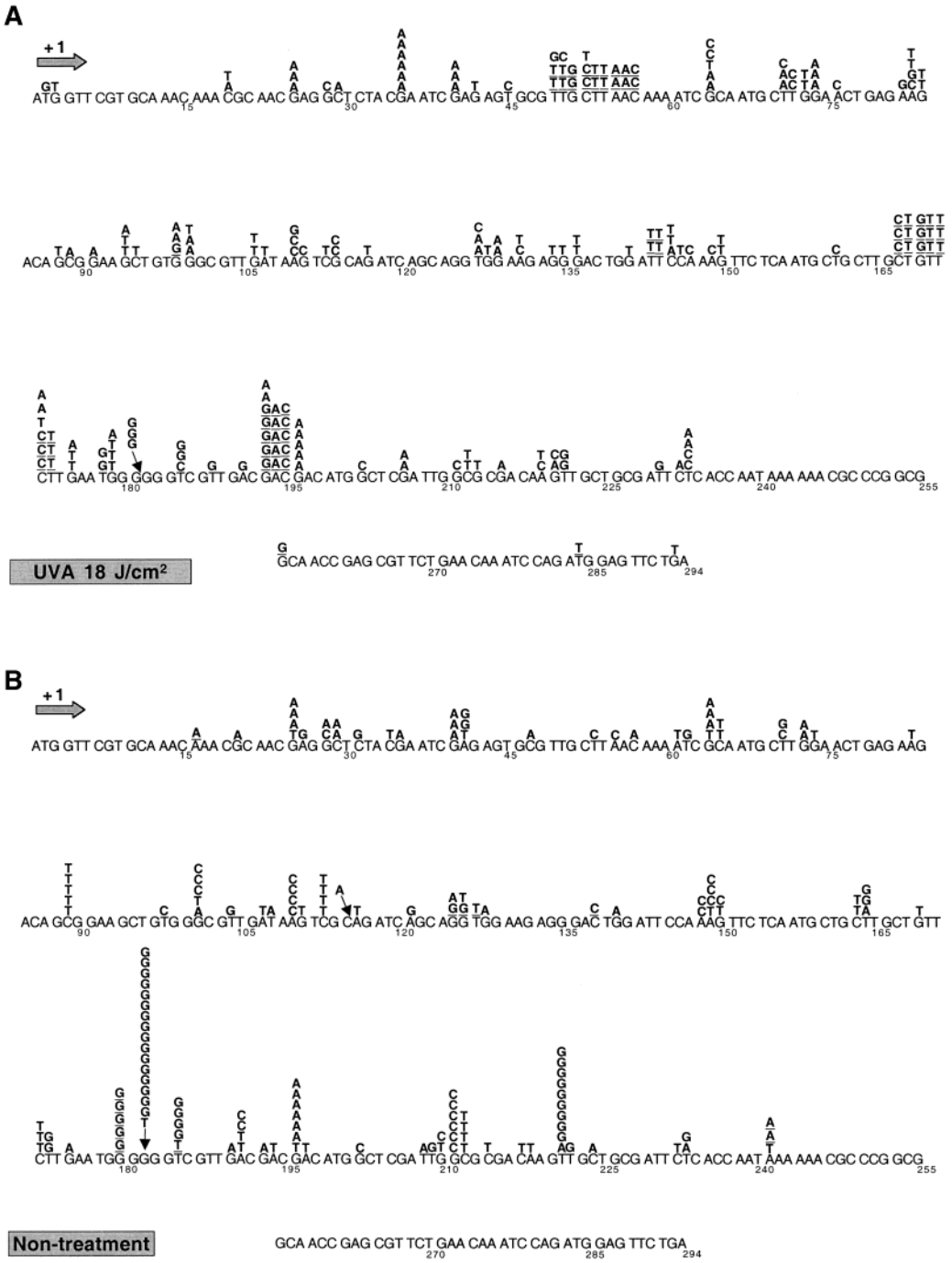


FIGURE 6: Detailed mutational spectra of the cII transgene in Big Blue mouse embryonic fibroblasts irradiated with 18 J/cm$^2$ UVA. (A) UVA irradiated cells and (B) control nonirradiated cells (total number of sequenced mutant plaques = 161 and 173, respectively). Substituted bases are in bold. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

(*12*, *13*, *44*). In determining the levels of 8-oxo-dG, the possibility of an artifact must be considered given the large discrepancies that have been reported when oxidative DNA damage has been measured using different techniques (*14*, *45*−*47*). Our measurements of 8-oxo-dG are more in line with the lower levels that have been reported; however, 8-oxo-dG levels were still significantly increased after UVA irradiation ($P < 0.03$).

The mutational spectrum produced by UVA in our report was characterized by G-to-T transversions and small base deletions. G-to-T transversions are typical mutations induced by 8-oxo-dG (*48*, *49*). Small deletions are also commonly observed in oxidative stress-induced mutagenesis (*50*−*52*). The lack of detectable CPDs is in line with the absence of signature mutations of CPDs, i.e., C-to-T and CC-to-TT transitions. In fact, C-to-T mutations at dipyrimidine sites were only 24% of the mutations in UVA-irradiated cells and almost the same frequency of such mutations (>21%) was found in unirradiated cells. In addition, no tandem CC-to-TT mutations were observed. In a previous study of UVB mutagenesis in transgenic mouse fibroblasts, we observed that C-to-T transitions were 64% of the induced mutations and CC-to-TT were 9% (*53*). Although one might say that our inability to detect CPDs in UVA-irradiated cells could

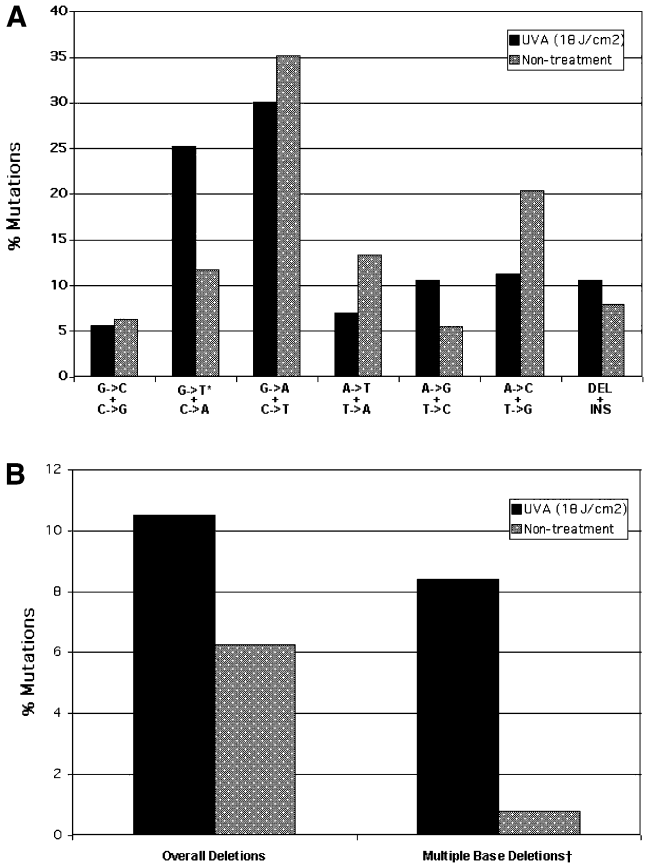


FIGURE 7: Mutational spectra of the cII transgene in Big Blue mouse embryonic fibroblasts irradiated with 18 J/cm$^2$ UVA or nonirradiated. (A) Overall mutational spectrum and (B) overall single base deletions and/or multiple base deletions. For comparison, the strand mirror counterparts of all transitions (e.g., G to A + C to T) and transversions (e.g., G to T + C to A) were combined. Ins = insertion; Del = deletion. *As compared with nontreatment; $P = 0.0075$; †As compared with nontreatment; $P = 0.008$.

reflect an insufficient sensitivity of the applied assays, the observed UVA-induced mutational spectrum argues against this possibility. In our system, no contribution of CPD-derived mutagenesis could be inferred from the mutational spectrum produced by UVA irradiation.

The literature contains only limited information on the mutational specificity of UVA. The most cited report is one by Drobetsky et al. (*10*). These authors have studied UVA-induced mutations in the hamster adenine phosphoribosyltransferase gene. T-to-G transversions, a generally rare class of mutation, were induced at a high frequency (up to 37% of all mutations) in the UVA-exposed cells. The mutant frequency was increased 3−5-fold but with no clear dose response, and the cells were irradiated for 2 h, as opposed to 5 min in our study. In contrast to the situation for repair-competent Chinese hamster ovary (CHO) cells, the mutagenic specificity of UVA in nucleotide excision repair-deficient CHO cells (in which a mutagenic contribution of dipyrimidine photoproducts is expected to be greater) was characterized by a complete absence of T-to-G mutations and by an abundance of C-to-T transitions (*11*). Recently, the UVA-induced T-to-G transversions in the CHO system have been ascribed to CPDs forming at TT dipyrimidine sequences after UVA irradiation (*16*, *17*). However, it is hard to rationalize how these lesions can be so highly mutagenic since DNA polymerase eta is known to accurately and efficiently bypass TT-CPDs by inserting two dAMPs opposite the lesions (*54*, *55*). Another report used an episomal shuttle vector as a UVA mutagenesis target (*9*). Although a large percentage of the mutations occurred at A/T base pairs and G-to-A transitions were common, T-to-G transversions were not observed in that study, either. We observed that the frequency of T-to-G transversions actually decreased upon UVA irradiation (Figure 7A). In another study on the mutagenicity of UVA, the mutational spectrum was almost completely dominated by C-to-T and CC-to-TT transitions at dipyrimidine sites including a large fraction at 5-methylcytosine bases (*56*). No T-to-G or G-to-T transversions were observed, but the UVA source emitted a considerable amount of energy in the 310−320 nm range, which can easily explain the dipyrimidine transitions.

Our report is the first one to demonstrate G-to-T transversions and small deletions as specific UVA mutational signatures. The contribution of CPDs to UVA-induced mutagenesis might depend on radiation source (ours emits only negligibly wavelengths below 340 nm), as well as on the cell type as a consequence of different contents of endogenous photosensitizers. A few pyrimidine dimers may still form consequent to UVA irradiation, at least in the range of 320−340 nm, or else during photosensitization reactions (*16*−*18*, *57*). In our system, however, no contribution from CPDs, either in the form of DNA damage or delineated from the mutational spectrum, was observed. Therefore, the specific features of our UVA-induced mutational spectrum, namely, G-to-T transversions and small tandem base deletions, can entirely be ascribed to oxidative DNA damage, such as 8-oxo-dG, generated via endogenous photosensitization processes. Other types of oxidized base lesions, not yet specifically identified, may also contribute to UVA-induced mutagenicity. In our hands, the lethality but not the extent of mutagenicity of UVA was dose-dependent because doubling the radiation dose, UVA caused severe cytotoxicity without showing any enhanced mutagenicity. Obviously, the radiation dose is of concern in adduct-targeted mutagenesis because excessive and lethal irradiation abolishes the DNA replication process, thereby preventing the translesion synthesis-based induction of mutations. This might partially explain the difference between UVA-induced mutational spectrum established here and those found in studies that had used an excessive radiation dose (up to 25-fold higher than what administered here) (*16*, *17*). Altogether, it appears that the biological effects of UVA in vivo are determined by a finely tuned interplay between UVA dose and the content of intracellular photosensitizers.

Last, the established signatures of UVA-induced mutation are discernible in the mutational spectrum of *p53* in skin cancer patients with known theraputic and/or recreational exposure to UVA e.g., psoriasis patients and tanning bed users (www-p53.iarc.fr/p53DataBase.htm). However, the complexity of exposure in these individuals who are inherently exposed to other environmental and/or theraputic mutagens leads to the occurrence of other types of mutations as well. This is well-documented in our recent work in which we demonstrated that the mutational signatures of mixed or combined mutagens are not always straightforward due to the synergistic and/or multiplicative effects of the mutagenic agents (*58*).

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