Deamination of 5-Methylcytosines within Cyclobutane Pyrimidine Dimers Is an Important Component of UVB Mutagenesis*

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UVB mutagenesis is characterized by an abundance of C → T and 5-methylcytosine → T transitions at dipyrimidine sequences. It is not known how these mutations might arise. One hypothesis is that UV-induced mutations occur only after deamination of the cytosine or 5-methylcytosine within the pyrimidine dimer. It is not clear how methylation of cytosines at the 5-position influences deamination and how this affects mutagenesis. We have now conducted experiments with a CpG-methylated supF shuttle vector that was irradiated with UVB and then incubated at 37 °C to allow time for deamination before passage through a human cell line to establish mutations. This led to a significantly increased frequency of CC → TT mutations and of transition mutations at 5'-PymCG-3' sequences. A spectrum of deaminated cis-syn cyclobutane pyrimidine dimers in the supF gene was determined using the mismatch glycosylase activities of MBD4 protein in combination with ligation-mediated PCR. Methylation at the C-5 position promoted the deamination of cytosines within cis-syn cyclobutane pyrimidine dimers, and these two events combined led to a significantly increased frequency of UVB-induced transition mutations at 5'-PymCG-3' sequences. Under these conditions, the majority of all supF mutations were transition mutations at 5'-PymCG-3', and they clustered at several mutational hot spots. Exactly these types of mutations are frequently observed in the p53 gene of nonmelanoma skin tumors. This particular mutagenic pathway may become prevalent under conditions of inefficient DNA repair and slow proliferation of cells in the human epidermis.

The UV component of sunlight is responsible for the induction of skin tumors, most notably basal cell and squamous cell carcinomas and most likely also melanomas (1, 2). The most frequent UV-induced mutations are C → T and CC → TT mutations involving pyrimidine dinucleotide sequences. These mutations are considered as a characteristic fingerprint that can be ascribed to solar UV irradiation (3). Such mutations are often found in the p53 gene of sunlight-associated skin cancers (4, 5).

The two most abundant UV-induced DNA photoproducts are the cis-syn cyclobutane pyrimidine dimers (CPDs)\(^1\) and the pyrimidine (6–4) pyrimidine photoproducts ((6–4)-photoproducts). Most of the mutagenic and carcinogenic action of sunlight has been attributed to the UVB portion of the solar spectrum (6), with a possible, but controversial, role for UVA (320–400 nm) in the induction of melanoma (7). The CPD is considered the most important UV-induced lesion based on its relatively high abundance, slow repair, and known mutagenicity (8–11).

In an attempt to dissect the individual contributions of CPDs and (6–4)-photoproducts to UVB mutagenesis, we have previously introduced foreign photolyase genes into a mouse cell line that carries two transgenic mutation reporter genes. We studied the mutations produced after photoproduct-specific photo-reactivation and showed that the CPD is responsible for a great majority of the mutations induced by UVB irradiation (11).

Earlier, we and others found that sequences that contain 5-methylcytosine within a dipyrimidine are up to 15 times more susceptible to CPD formation after exposure to natural sunlight (12) or a UVB light source (13) compared with 254-nm UVC irradiation. Methylation of cytosines enhances CPD formation by sunlight by 5–15-fold (12). This difference may be explained by the higher energy absorption of 5-methylcytosine compared with cytosine in DNA in the UVB range at wavelengths between 295 and 320 nm (12, 14). In previous work, we have examined UV-induced mutational events at methylated CpG sequences using the lacI and cII transgenes as a mutational target. Dipyrimidines that contain 5-methylcytosine were preferential targets for sunlight-induced mutation hot spots (15, 16).

Plasmid constructs containing defined UV photoproducts have been used to study the mutagenic specificities of CPDs and (6–4)-photoproducts. The mutation frequency obtained with site-specific 5'-TT-CPDs is generally very low (17–19). This is consistent with the infrequent recovery of mutations at 5'-TT sequences in UV-irradiated cells and is probably due to the action of DNA polymerase \(\eta\), which correctly bypasses these lesions (20, 21). DNA polymerase \(\eta\) is encoded by the RAD30 gene in yeast and by the XPV gene in humans. The mutagenicity of a site-specific CPD containing the 5'-TC sequence also is very low, with \(>95\%\) accurate lesion bypass, but this has been studied only in Escherichia coli (22). Studies with yeast mutants indicate that RAD30 may correctly bypass 5'-TC and 5'-C-C dimers (23). If cytosine-containing CPDs are indeed bypassed mostly error-free, then what is the origin of C → T mutations at such lesions?

Spontaneous deamination of methylated cytosines is commonly proposed as the mutational mechanism responsible for C to T transition mutations at CpG sites in mammalian genes (14, 24, 25). Deamination of cytosine or 5-methylcytosine is expected to occur more rapidly within a CPD as opposed to

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\(^1\) The abbreviations used are: CPD, cyclobutane pyrimidine dimer; (6–4)-photoproduct, pyrimidine-(6–4) pyrimidine photoproduct; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; LMPCR, ligation-mediated PCR.
within normal double-stranded DNA (26, 27). This process may play an important role in UV mutagenesis.

We have shown previously that the deamination of cytosine within CPDs does occur in human cells at a significant rate (28). Here we have studied the mutagenic consequences that are associated with such deamination events, in particular with respect to the involvement of 5-methylcytosine.

**MATERIALS AND METHODS**

**Cell Lines and Plasmids—**SV-40-transformed DNA repair-deficient human XP-A fibroblasts (XP12BE) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The pSP189 plasmid was transfected into the XP12BE cells (including a randomly generated 8-base pair signature sequence at the 3’-end of the supF gene) was kindly provided by Michael Seidman (29). The E. coli tyrosine amber suppressor transfer RNA gene, supF, enables read-through of a UAG stop codon in the lacZ gene, resulting in synthesis of functional β-galactosidase. The bacterial strain MBM7070 is cultured on agar containing ampicillin and isopropyl β-D-thiogalactoside, an inducer of β-galactosidase, and X-gal as a color indicator. Colonies resulting from transformation with a functional supF gene will be blue, whereas those containing mutant supF genes will be white.

**Methylation and UVB Irradiation—**The pSP189 plasmid was methylated in vitro using the CpG-specific DNA methylase SssI (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions. Control DNA was mock-methylated in the absence of S-adenosylmethionine. Completion of methylation was confirmed by digestion of an aliquot of the reaction mixture with the methylation-sensitive restriction endonuclease HpaII. Methylated and unmethylated pSP189 were irradiated in TE buffer (10 mM Tris-HCl, pH 7.5) with a UVB source (a Philips TL 20W/12RS lamp filtered through cellulose acetate; peak emission, 312 nm; lower wavelength cut-off, 295–300 nm) at a dose of 8000 J/m² (about 10 min). The UV dose was determined with a UVX radiometer and a UVB sensor (Ultraviolet Products, Upland, CA). After UVB exposure, DNA was incubated in TE buffer, pH 7.5, at 37 °C for various time intervals to allow deamination to occur.

**Mutagenesis Assay—**The pSP189 shuttle vectors were transfected into cultures of nucleotide excision repair-deficient human fibroblasts (XP12BE). Briefly, 5 × 10⁴ cells were plated into 10-cm tissue culture dishes in Dulbecco’s modified Eagle’s medium. Following a 16-h incubation of the cells, a mixture of plasmid and FuGene 6 transfection reagent (Roche Molecular Biochemicals) was added. After a 72-h incubation of the cells, the plasmid was rescued from the human cells by alkaline lysis. Cells were trypsinized, washed, and resuspended in suspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), mixed with 1 volume of lysis buffer (0.2 M NaOH, 1% (w/v) SDS), and incubated on ice for 3–5 min, followed by the addition of neutralization buffer (3 M potassium acetate, pH 5.5). After a 15-min incubation at room temperature, the mixture was centrifuged at 14,000 g for 15 min at 16,000 × g, and the supernatant was extracted once with phenol/chloroform. Following ethanol precipitation, the DNA was re-suspended in DpnI reaction buffer, and unreplicated plasmid was removed by digestion with DpnI, which recognizes the bacterial adenine methylation pattern. Then the plasmid was electroporated into MBM7070 bacteria, which carry a lacZ gene with an amber mutation (29, 30). The transformed bacteria were diluted in 1 ml of SOC medium and plated on agar plates containing 50 µg/ml ampicillin, 1 µg isopropyl-1-thio-β-D-galactopyranoside, and 100 µg/ml X-Gal. After an overnight incubation at 37 °C, wild-type (blue) and mutant (white) colonies were counted to determine the mutant frequency. Colonies containing a plasmid with a mutated supF gene were identified, and the plasmids were purified using Wizard plasmid purification kits (Promega, Madison, WI) and sequenced. It is unlikely that any significant number of the mutations will arise in E. coli. DpnI treatment removes the unreplicated and still lesion-containing plasmids. It should be noted that the replication-deficient plasmid used in this experiment is low-molecular weight, and there will be an enormous dilution of the added plasmids. Tens of thousands of progeny molecules are recovered from a single cell. The plasmid replication cycle is about 15–20 min. Even if there are a few single cycle progeny, they will be diluted by a huge excess of nonadducted progeny. In addition, no mutations can be recovered upon direct transformation of MBM7070 with UV-treated plasmids (30) because of the high repair capacity of the strain.²

² M. Seidman, personal communication.

**Ligation-mediated PCR (LMPCR)-based Deamination Assay—**For measuring deamination of cytosines or 5-methylcytosines within cyclobutane pyrimidine dimers, a sample of the treated plasmids was used to map deamination rates by LMPCR after a 48- and 96-h incubation period at 37 °C. The CPDs in UVB-irradiated DNA were repaired to completion using Escherichia coli CPD photolyase (1 µg of photolyase for 10 µg of UVB-damaged DNA) in a 100-µl reaction containing 50 mM Tris-HCl, pH 7.4, 5.0 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 50 µg/ml bovine serum albumin, 5% glycerol. The photolyase reaction conditions were as described (31). After phenol/chloroform extraction and ethanol precipitation, DNA was redissolved in water. The DNA was then incubated with 1 µg of recombinant His-tagged human MBD4 protein at 37 °C for 1 h in a 100-µl reaction containing 25 mM Hepes, pH 7.8, 80 mM KCl, 1 mM EDTA, 10 mM dithiothreitol, 50 µg/ml bovine serum albumin, 5% glycerol. The photolyase reaction conditions were as described (31). After phenol/chloroform extraction and ethanol precipitation, the DNA was incubated in 1 M piperidine at 37 °C for 15 min to convert the abasic sites into DNA strand breaks.

LMPCR was performed on the enzyme-treated DNA as described (34). Oligonucleotide primers for LMPCR were as follows: 5′-AAAAAAGGAATAAAGG-3′ (supF-1), 5′-CTTAAAGTCTCGTAAGG-3′ (supF-4), 5′-TAACCGCAACGCGAAT-3′ (supF-2), 5′-TGGTAAAGTCCGGGATAGG-3′ (supF-5), 5′-GAACTGTGATTCATCACTTCC-3′ (supF-3), 5′-AAGGATTCGGTACCAGGA-3′ (supF-6). In brief, oligonucleotide primer supF-1 or supF-4 was annealed to 0.5 µg of DNA after enzyme treatment, followed by primer extension of this primer at 48 °C with Sequenase 2.0 (U.S. Biochemical Corp.). The oligonucleotide linker, consisting of a 25-mer annealed to an 11-mer oligonucleotide, was then ligated to the blunt-ended, primer-extended molecules. After ligation and precipitation of the DNA, gene-specific DNA fragments were amplified with Taq polymerase (Roche Molecular Biochemicals) by using the 25-mer of the linker and a gene-specific PCR primer (primer supF-p2 or supF-5) under conditions given previously (34). After 21 cycles of PCR, the samples were phenol/chloroform-extracted and ethanol-precipitated, and the amplified fragments were separated on 8% (w/v) polyacrylamide gels containing 7 M urea. The samples were run until the xylene cyanol dye reached the bottom of the sequencing gel, and the bottom 40 cm of the gel was electroblotted onto nylon membranes by using an electrotransfer device (Owl Scientific; Cambridge, MA). The sequences were visualized by autoradiography after hybridization with a single-stranded gene-specific PCR probe. The hybridization probes were made by repeated run-off polymerization using primer supF-3 or supF-6 and the respective PCR products as templates (35). Chemical DNA sequencing reactions (36) were used with all LMPCRs to provide sequence markers. As a control, the persistence of CPDs was measured along with the deamination experiments. In this assay, T4 DNA ligase was annealed to the UVB-irradiated DNA which was subjected to LMPCR analysis as described in previous studies (31, 34).

**RESULTS**

To study the mutational spectrum induced by UVB irradiation in a CpG-methylated gene, we used the shuttle vector pSP189, which contains the supF gene as a mutational target. The vector was methylated in vitro at all CpG sequences, using the CpG-specific DNA methyltransferase SssI. In parallel experiments, pSP189 was mock-methylated in the absence of S-adenosylmethionine to produce an unmethylated counterpart. Completion of the methylation reaction was confirmed by digesting an aliquot of the reaction mixture with the methylation-sensitive restriction endonuclease HpaII.

These methylated and unmethylated shuttle vectors were irradiated with UVB at a dose of 8000 J/m². At this dose, approximately one CPD is produced every 0.5–0.8 kilobases. Nonirradiated DNA was used as a control. After irradiation, an aliquot of the methylated and unmethylated pSP189 vectors was transfected immediately into nucleotide excision repair deficient XP-A fibroblasts. The other aliquots of the UVB-irradiated plasmid were incubated at 37 °C in TE buffer to allow time for deamination of cytosine and 5-methylcytosine to occur within CPDs. After the different incubation times, the plasmids were transfected into XP-A fibroblasts. The cells were...
allowed to grow for 72 h after transfection. Then the plasmids were rescued, and the DNA was cleaved with DpnI to remove unreplicated plasmids still containing the bacterial GATC methylation pattern. Using HpaII and HhaI digestion, we found that the level of CpG methylation was largely (>80%) maintained in DpnI-resistant DNA 72 h after transfection, indicating that there is no active removal of methyl groups from the methylated plasmids and that the methylation pattern is at least partially conserved during DNA replication (data not shown). Conversely, the unmethylated plasmid did not undergo de novo methylation. The rescued plasmids were then electroporated into MBM7070 bacteria, which carry a lacZ gene with an amber mutation. Plasmids were isolated from white colonies, and the supF gene was sequenced. Siblings, indicated by repeated appearance of the signature sequence, were about 2% of all plasmids and were excluded.

The mutant frequencies in unmethylated and methylated UBV-treated supF vectors, incubated for different amounts of time before transfection, were determined (Table I). The mutant frequency in the absence of UV irradiation was generally below 1 × 10⁻³, and it was not different between unmethylated and methylated targets. After UBV irradiation and immediate transfection, the mutant frequency increased to 8–12 × 10⁻³ (a more than 13–18-fold increase). However, the mutant frequencies became even higher with increasing incubation times at 37 °C before transfection (Table I). At the longest incubation times, the mutant frequencies were almost 30 times higher than in the nonirradiated controls and were over 2 times higher than in DNA that was transfected immediately after irradiation. There was no major difference in the mutant frequency trends between unmethylated and methylated DNA. We confirmed by agarose gel electrophoresis that no significant DNA degradation had occurred after a 96-h incubation at 37 °C.

Given the increases in mutant frequencies after the pretransfection incubation, we proceeded to determine the mutational spectra obtained under various conditions. Although there were few colonies obtained in the absence of UBV treatment, we sequenced the available mutants (Fig. 1). Most of the mutations were deletions rather than single nucleotide changes. Further, we selected the samples obtained by immediate transfection and those obtained with the 96-h preincubation period for the sequencing analysis. This was done for both the unmethylated and the methylated plasmids (Figs. 2 and 3).

As expected from the known mutagenic action of UBV irradiation, most of the mutations were C → T transition events (74–91%), and a sizable fraction (5–15%) were CC → TT tandem mutations (Table II). When the DNA was incubated at 37 °C for 96 h before transfection, the frequency of tandem mutations, mostly CC → TT, increased. In the unmethylated samples immediately transfected, the percentage of tandem mutations was 9.3% (7 of 75). This value increased to 15.1% (13 of 86) in the sample that was transfected after 96 h. In the methylated samples immediately transfected, the percentage of tandem mutations was 6.4% (5 of 78). This value increased to 14.9% (13 of 87) in the sample that was transfected after 96 h. Although individually these differences did not reach statistical significance, the difference did become statistically significant (p < 0.05; χ² test) when the values from methylated and unmethylated plasmids were combined (26 of 173 versus 12 of 153 mutants were tandem mutations after delayed transfection). Thus, there is a clear trend toward an increase in tandem mutations, predominantly of the CC → TT type, after time is allowed for deamination to occur within CPD lesions.

We then focused on the role of CpG methylation in UBV mutagenesis. When comparing the unmethylated and CpG-methylated mutational targets, there was a noticeable trend for the mutation hot spots to occur at dipyrimidines involving 5-methylcytosine (i.e. PymCG trimucleotides). For the unmethylated plasmid, 24% of the mutations involved CpG dinucleotides when the DNA was transfected immediately. This value was 21% when the transfection was done after 96 h (Table II). A higher percentage of the mutations (36%, or 28 of 78) were present at methylated CpG sites in the CpG-methylated plasmid. This was particularly striking in the CpG-methylated plasmid that was transfected after 96 h. In this case, 52% (45 of 87 mutations) involved 5-methylcytosine. Thus, when comparing the unmethylated and methylated plasmids transfected after 96 h, there is a shift toward a mutational spectrum that is dominated by mutations involving 5-methylcytosines at CpG dinucleotides, and this difference is highly significant (p < 0.001; χ² test). When we compared the fraction of mutations at methylated CpGs between the DNA that was immediately transfected and the one that was incubated for 96 h, the difference was 36% (28 of 78) versus 52% (45 of 87) (p = 0.05; χ² test). This difference is also evident when the location of mutational hot spots is considered. In the mutational spectrum of the unmethylated plasmid (Fig. 2), which is similar between the 0- and 96-h transfection, there is only one moderate mutation hot spot at a CpG sequence. All of the other hot spots (defined as five or more mutations at the same nucleotide position) involve either CpC or TpC sequences in which the cytosine is converted to thymine. However, in the mutation

| Treatment | No. of total colonies | No. of mutant colonies | Mutant frequencies |
|-----------|-----------------------|------------------------|--------------------|
| No UBV + deamination (0 h) | 8230 | 5 | 0.61 × 10⁻³ |
| No UBV + deamination (24 h) | 12,348 | 9 | 0.73 × 10⁻³ |
| No UBV + deamination (48 h) | 7514 | 7 | 0.93 × 10⁻³ |
| No UBV + deamination (96 h) | 9624 | 8 | 0.93 × 10⁻³ |
| UBV (8000 J/m²) + deamination (0 h) | 10,802 | 84 | 7.92 × 10⁻³ |
| UBV (8000 J/m²) + deamination (24 h) | 3674 | 62 | 17.19 × 10⁻³ |
| UBV (8000 J/m²) + deamination (48 h) | 1404 | 27 | 19.23 × 10⁻³ |
| UBV (8000 J/m²) + deamination (96 h) | 4036 | 90 | 22.30 × 10⁻³ |
| Unmethylated pSP189 | | | |
| No UBV + deamination (0 h) | 10,936 | 7 | 0.64 × 10⁻³ |
| No UBV + deamination (24 h) | 19,624 | 12 | 0.61 × 10⁻³ |
| No UBV + deamination (48 h) | 10,648 | 7 | 0.66 × 10⁻³ |
| No UBV + deamination (96 h) | 7384 | 7 | 0.95 × 10⁻³ |
| UBV (8000 J/m²) + deamination (0 h) | 2427 | 29 | 11.95 × 10⁻³ |
| UBV (8000 J/m²) + deamination (24 h) | 7089 | 121 | 17.07 × 10⁻³ |
| UBV (8000 J/m²) + deamination (48 h) | 3407 | 54 | 15.85 × 10⁻³ |
| UBV (8000 J/m²) + deamination (96 h) | 4236 | 101 | 23.84 × 10⁻³ |
spectrum of the methylated supF plasmid transfected after 96 h (Fig. 3), four of the six mutation hot spots are at CpG sequences.

There may be two independent pathways by which methylation of cytosine can promote mutagenesis after UVB irradiation. The first one is the documented enhancement of CPD formation at methylated cytosines in the UVB range (12, 13). The second pathway may be that 5-methylcytosines efficiently undergo deamination within CPDs to form dimers containing...
thymine. Upon bypass, such lesions would result in C → T transitions. In order to test these possibilities, we have devised an assay that can measure the deamination of cytosines and 5-methylcytosines within CPDs.

This assay is based on enzymatic photoreversal of CPDs using CPD photolyase followed by cleavage of the resulting DNA with the mismatch-specific DNA glycosylase MBD4 (Fig. 4A). MBD4 recognizes T/G as well as U/G mismatches in DNA (32, 33) and will thus detect the appearance of deaminated CPDs containing either 5-methylcytosine or cytosine. An excess of MBD4 was used so that mismatches at both CpG and non-CpG sites were efficiently cleaved (33) (and data not shown). After MBD4 cleavage, the DNA was then treated with alkali to produce strand breaks at the resulting abasic sites. Ligation-mediated PCR was used to detect and analyze supF sequences (Fig. 4, B and C). In control lanes, we established that the pattern of T4 endonuclease V-cleaveable sites was largely unchanged between the 0-h samples and the samples incubated for 96 h. Using the photolyase/MBD4 assay, we observed that there was a clear time-dependent increase in signals with incubation times proceeding up to 96 h. As expected from the scheme outlined in Fig. 4A, signals were observed only at cytosines and 5-methylcytosines that are part of a dipyrimidine sequence. Many of the MBD4-induced signals coincided with mutational hot spots, such as the ones at nucleotide positions 124, 129, 150, 155, and 163. No mutations were observed at position 149. The mutational hot spots at positions 168 and 172 on the upper strand could not be measured by LMPCR due to the presence of the random signature sequence 3’ to the tRNA gene. However, not all of the strong signals obtained with the deamination assay, such as the one at position 164, correlated with mutational hot spots. This is not surprising, since it has often been difficult to correlate UV damage hot spots in the supF gene with mutation hot spots (29). Sequence context, perhaps as a consequence of the abundance of hairpin structures in this tRNA gene, appears to play an important role in determining which sequence appears as a mutational hot spot. However, it is apparent that in the MBD4 deamination assay, the strongest signals were observed at methylated CpGs in the UVB-irradiated plasmids incubated for 96 h. Even when taking into account that a somewhat higher frequency of CPDs was produced at methylated PymCG sequences in the methylated plasmid, the extent of deamination seemed to be much higher compared with the unmethylated plasmid, in particular at sequence positions 156, 159, 163, 155, and 149 (Fig. 4, B and C). The stronger signals obtained at deaminated 5-methylcytosine-containing dimers are not a consequence of preferential cleavage by MBD4, since this enzyme equally cleaves or even prefers U/G over T/G mismatches (32, 33). At positions 155, 156, and 163, the increased deamination observed with the methylated DNA was accompanied by an increased C → T mutation frequency at the same nucleotide position (Figs. 3 and 4).

These data show that 5-methylcytosines efficiently undergo deamination at 37°C when they are part of a cyclobutane pyrimidine dimer. The majority of the deaminated sites correspond to UVB-induced mutational hot spots.

### Discussion

CPDs and (6–4)-photoproducts are the most abundant DNA photoproducts produced by UVC and UVB irradiation (8, 9). Previous results using in vivo photoenzymatic removal of specific photoproducts provided evidence that CPDs are responsible for the majority of UVB-induced mutations in mammalian cells (11). The data were consistent with a higher level of formation, slower repair, and increased mutagenicity of CPDs compared with (6–4)-photoproducts (8–11). The low mutagenicity of (6–4)-photoproducts in mammalian cells may be a consequence of their low level of induction, efficient repair (although in this study we used an XP-A cell line), and possibly also a bypass tolerance by DNA polymerase η for the most abundant (6–4)-photoprotect, the one that forms at 5′-TC sequences. DNA polymerase η preferentially incorporates a guanine opposite the 3′-TT of (6–4)-photoproducts, although it is unable to extend from the inserted nucleotide (37). If, due to structural features of the lesion or due to deamination of the 3′ base, this polymerase would have the same incorporation specificity opposite the 3′-C of a 5′-TC (6–4)-photoprotect, then no mutation would be produced.

The mutagenic mechanism involving CPDs is still unclear. T-T dimers, although induced at high levels, are not very mutagenic. This is probably a consequence of their correct replication bypass by the lesion-tolerant enzyme DNA polymerase η (20, 21). CPDs containing cytosines, and most notably 5-methylcytosines, are also abundantly produced in the UVB range (12, 13, 38). Dipyrimidines containing cytosines, particularly 5′-TC, 5′-Tmc, 5′-CC, and 5′-Cmc, are the preferential targets of UV-induced transition mutations in mammalian cells (8, 9, 39). There are at least two possible pathways through which CPDs containing cytosines or 5-methylcytosines can cause mutagenicity: (i) 5-methylcytosines within CPDs.

#### Table II

5-Methylcytosine Deamination in UV Mutagenesis

| Sequence | methylated CpG control (no UVB) | methylated CpG UVB | unmethylated CpG control (no UVB) | unmethylated CpG UVB |
|----------|----------------------------------|-------------------|-----------------------------------|---------------------|
| C to T   | 0 (0)                            | 2 (25)            | 1 (33)                            | 2 (40)              |
| C to A   | 2 (40)                           | 0 (0)             | 0 (0)                             | 0 (0)               |
| C to G   | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| T to C   | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| T to A   | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| T to G   | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| Deletion | 2 (40)                           | 6 (75)            | 3 (60)                            | 2 (40)              |
| Insertion| 1 (20)                           | 0 (0)             | 1 (33)                            | 1 (20)              |
| Tandem   | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| Multiple | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| Total    | 5 (100)                          | 8 (100)           | 5 (100)                           | 5 (100)             |
| CC → TT  | 0 (0)                            | 0 (0)             | 0 (0)                             | 0 (0)               |
| Percentage of all mutations | 0/5 (0) | 0/8 (0) | 0/3 (0) | 0/5 (0) |

*Percentage of all mutations in parentheses.*
Fig. 4. A, outline of the method used to detect deaminated cytosines and 5-methylcytosines within pyrimidine dimers at specific sequences. This method is based on enzymatic photoreversal followed by removal of uracils or thymines from U/G or T/G mismatches by the MBD4 protein, strand cleavage, and ligation-mediated PCR to detect the resulting strand breaks. B and C, characterization of deaminated CPDs along sequences of the supF gene. The lower strand was analyzed in B, and the upper strand was analyzed in C. DNA was analyzed without UV irradiation (−UV), immediately after UVB irradiation (0 h), and after incubation at 37 °C for 48 and 96 h following irradiation. The UVB dose was 8000 J/m². The lanes labeled T4 endoV show total CPD frequency immediately after irradiation and after a 96-h incubation. CPDs were mapped with T4 endonuclease V and photolyase followed by LMPCR. G, GA, TC, and C lanes are Maxam-Gilbert sequencing reactions used as position markers. Several 5-methylcytosines and cytosines at dipyrimidines showing an increased deamination signal after 96 h are shown by arrows. SupF mutational hot spots are indicated by a box. The asterisks indicate deaminated 5-methylcytosines at CpG sequences. The 5-methylcytosines indicated near the top of the gels are outside of the supF gene. UM, unmethylated supF plasmid; M, methylated supF plasmid.
Deamination of 5-methylcytosine within the CPD followed by error-free mutations may involve direct error-prone replication of the CPD or tetrad CpG sequences after UVB irradiation. A DNA polymerase that incorporates adenines opposite the C or mC within the dimer. The nature of this polymerase is unknown. The second pathway includes a model in which the C or mC first deaminates within the CPD lesion, a reaction that is then followed by correct bypass during DNA replication. The mutagenicity of a site-specific CPD containing the 5'-TC sequence in a plasmid construct is very low, with 95% accurate lesion bypass when studied in E. coli (22). If cytosine-containing CPDs are bypassed mostly error-free, then the origin of C → T mutations at such lesions could be the deamination pathway.

We and others have proposed that most UV-induced transition mutations at dipyrimidines containing cytosine may result from correct DNA polymerase bypass of CPDs containing deaminated cytosine or 5-methylcytosine (16,28–44). Deamination of cytosine or 5-methylcytosine occurs more rapidly within a CPD as opposed to within normal double-stranded DNA (26, 27). Deamination of C in T-C or C-C dimers leads to formation of T-U or U-U dimers, respectively. Adenines are incorporated with high specificity during bypass of site-specific T-T, T-U, or U-U dimers in vivo (41, 45). After deamination of cytosine or 5-methylcytosine within CPDs, DNA polymerase η is probably bypassing these CPDs in an error-free manner (20,21,46). The final outcome would be a C → T or mC → T transition mutation, the most common type of mutation seen after UV irradiation.

It is not known how DNA damage-tolerant DNA polymerases bypass CPDs containing nondeaminated cytosines or 5-methylcytosines. One possibility is that they incorporate adenines (47). However, our expectation is that they incorporate guanines in a mostly error-free pathway and that a mutation occurs only after deamination. There is, in fact, genetic evidence from studies with the yeast homologue showing that DNA polymerase η may bypass CPDs containing cytosine correctly (23).

We have shown previously that deamination of cytosine in CPDs does occur at significant rates in vivo (28). Here, we have studied the mutagenic consequences of these deamination reactions using a mammalian shuttle vector system. The shuttle vector was either transfected immediately or was incubated for 96 h prior to transfection. With the longer incubation times, the mutant frequencies increased by 2-2.5-fold. The increase was not linear but rather a first order increase, a particularly strong early increase followed by a tendency toward leveling. The reason for this nonlinear increase is not clear, but it could indicate that some of the mutations may have been produced by (6-4)-photoproducts. However, it is likely that deamination reactions would also occur in cells that were transfected imme-

diately and that some of this deamination could occur prior to DNA replication. Thus, with the plasmid transfected immediately after irradiation, we cannot clearly distinguish if the mutations were produced by the deamination pathway or by the pathway of directly replicating cytosine-containing CPDs (Fig. 5). The 2-fold and nonlinear increase in mutant frequency after delayed transfection is consistent with the proposal that most mutations after immediate transfection may also have arisen through the deamination pathway.

We find that incubating the shuttle vector plasmids for an extended period of time prior to transfection produces two clear effects. The first is an increase of tandem, mostly CC → TT mutations, and the second is an increased fraction of mutations occurring at 5-methylcytosine bases. How can these two observations be explained? Since both phenomena are dependent on incubating the UVB-irradiated DNA in vitro at 37 °C, the likely explanation is that deamination of cytosines within CPDs is causing these effects. We have confirmed that the deamination reactions do indeed occur, both at cytosines and at 5-methylcytosines (Fig. 4). The data show that 5-methylcytosines efficiently undergo deamination at 37 °C when part of a cyclobutane pyrimidine dimer. This is most likely the mechanism through which UVB irradiation produces mutational hot spots at PymCG sequences. Several mutational hot spots are seen in the p53 gene in skin tumors (5,12). Six of the eight most commonly mutated sites (codons 12,15,196,213, 245, 248, and 282) contain the mutated dipyrimidine in the sequence context 5'-CCG or 5'-TCG. All of these CpG sequences are methylated to contain 5-methylcytosines in normal human keratinocytes (48).

In order to explain the increased frequency of CC → TT mutations after incubation of the plasmid, one can invoke double deamination events in which both cytosines are converted to uracils (42,44). The LMP-PCR assay does not allow the detection of the two uracils simultaneously, but such events have been detected using a sensitive PCR assay (28). Since no other explanation seems likely, the observation of an increased frequency of CC → TT mutations after incubation of the plasmid also supports the UV mutagenesis model involving deamination (Fig. 5). One remarkable finding is the high proportion of CC → TT, and in particular CmC → TT mutations present at CpG sites, in the p53 gene of skin cancers from xeroderma pigmentosum patients (49,50). We have not observed a large number of such mutations in the supF assay. This could be due to the fact that the major UVB hot spots in the methylated supF gene were at the sequence TCGA, which does not allow for the formation of such mutations. The high frequency of tandem mutations in UV-exposed skin of xeroderma pigmentosum patients could be a consequence of complete or almost complete lack of nucleotide excision repair and slow cell division rates providing enough time for double deamination events to occur within CPDs. The deamination pathway of UVB mutagenesis is probably of importance in sun-exposed cells in the human epidermis.

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**Additions and Corrections**

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Deamination of 5-methylcytosines within cyclobutane pyrimidine dimers is an important component of UVB mutagenesis.

_Dong-Hyun Lee and Gerd P. Pfeifer_

Page 10317, Figs. 2 and 3: These figures were not processed properly. The correct figures are shown below. The online version has already been corrected in departure from print.

**FIG. 2.** Mutation spectra induced by UVB irradiation into the unmethylated supF shuttle vectors. The mutations found in the plasmids in the absence of any incubation prior to transfection are shown above the sequence, and the mutations introduced into the plasmids after 96-h incubation prior to transfection are below the sequence. The UVB dose was 8000 J/m². The nucleotide positions are numbered. Tandem mutations are underlined. CpG sequences in the supF gene are also underlined.

**FIG. 3.** Mutation spectra induced by UVB irradiation into the methylated supF shuttle vector. The mutations found in the plasmids in the absence of any incubation prior to transfection are shown above the sequence, and the mutations introduced into the plasmids after 96-h incubation prior to transfection are below the sequence. The UVB dose was 8000 J/m². *, #, and $, two mutations introduced into the same plasmid. Tandem mutations are underlined. CpG sequences in the supF gene are also underlined.

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