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Sequence specificity of cyclobutane pyrimidine dimers in DNA treated with solar (ultraviolet B) radiation

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ABSTRACT

Cyclobutane pyrimidine dimers were quantified at the sequence level after irradiation with solar ultraviolet (UVB) and nonsolar ultraviolet (UVC) light sources. The yield of photoproducts at specific sites was dependent on the nucleotide composition in and around the potential lesion as well as on the wavelength of ultraviolet light used to induce the damage. Induction was greater in the presence of 5′ flanking pyrimidines than purines; 5′ guanine inhibited induction more than adenine. UVB irradiation increased the induction of cyclobutane dimers containing cytosine relative to thymine homodimers. At the single UVC and UVB fluences used, the ratio of thymine homodimers (T<>T) to dimers containing cytosine (C<>T, T<>C, C<>C) was greater after UVC compared to UVB irradiation.

INTRODUCTION

Ultraviolet light forms pyrimidine photoproducts in DNA at both UVC (240–280 mn) and UVB (280–320 mn) wavelengths, but there are important practical and experimental differences. The most obvious is that UVC is negligible in solar radiation at the Earth's surface, and it is more important from a practical point of view to know the detailed photochemistry of the longer wavelengths. The mutational spectrum of UVB is different from UVC, containing more deletions and insertions and unique hot spots (1). This may be accounted for by the greater numbers of dimers involving cytosine at UVB wavelengths (2).

Sequence analyses of the spectrum of photochemical changes in DNA by UVC light were performed by Gordon and Haseltine (3), Lippke *et al.* (4), and Brash *et al.* (5), but a similar analysis has not been done for UVB. We therefore determined the distribution of cyclobutane pyrimidine dimers in a defined DNA sequence after irradiation with UVB and UVC, and compared the influence of neighboring bases on the yield of photoproducts.

MATERIALS AND METHODS

Ultraviolet irradiations

UVC irradiations were carried out with six 8 W General Electric germicidal lamps emitting predominantly 254 nm light at a fluence rate of 1.3 J/m^2/s. UVB irradiations were carried out under four Westinghouse FS 20 sunlamps filtered through cellulose acetate (a gift of W. Carrier, Oak Ridge National Laboratory), conditions that closely approximate those of the solar spectrum reaching the earth's surface (6) (lower wavelength cutoff = 295–300 mn). The UVC and UVB fluence rates were determined with a Spectroline radiometer (Spectronics Corp., Westbury, NY) equipped with DM- 254N and DM-300N photodetectors. Spectral sensitivities of these photodetectors were 243–270 mn for DM-254N (peak = 254 mn) and 272–325 nm for DM 300N (peak = 306 nm).

Preparation of DNA substrate

Plasmid pNB137 (a gift of Janet Arrand, CRC Gray Lab., Northwood, UK)) was used for the analysis of site-specific cleavage after UV irradiation. This plasmid was made by subcloning a 257-base pair (bp) fragment containing the Chinese hamster *Alu*-equivalent sequence (7) into the *Hinc*II site of the pUC8 polylinker region; we have sequenced the complete insert region (Figure 1). A 450-bp fragment containing the Alu sequence and part of pUC8 was obtained by double digestion with *Eco*RI and *Bgl*I restriction enzymes, nondenaturing gel electrophoresis, and electroelution (8). The eluted DNA (*Alu* fragment) was further purified by ion-exchange chromatography (Qiagen Corp.) and concentrated by ethanol precipitation.

DNA labeling and sequencing

About 2 μg of the purified *Alu* DNA was labeled at the 3′ end of the *Eco*RI site by Klenow fragment of *Escherichia coli* polymerase I in the presence of α^{32}P-dATP and nonradioactive dTTP. The specific activity of the labeled probe was 5×10^7 cpm/μg; about 10^6 cpm of the labeled probe was used for each enzymatic and sequencing reaction. After enzyme treatment, reactions were terminated by 10×dilution with 0.3 M NaOAc; the DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and concentrated by ethanol precipitation. Sequencing reactions for G+A and T+C were carried out according to standard procedures (9). In a typical sequencing experiment, about 5×10^5 cpm of the sample were loaded onto each lane and electrophoresed at 40 V/cm for about 3 h. After electrophoresis, the gel was transferred immediately onto a sheet of Whatman 3MM paper, covered with plastic wrap, and exposed to X-ray film at −70°C for 4 to 12 h.

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Detection and quantification of photoproducts

Cyclobutane dimers were analyzed by digestion of UV-irradiated DNA with purified T4 endonuclease V (endo V) (a gift of R.S.Lloyd, Vanderbilt University) in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA for 2 h at 37°C. SV40 DNA (BRL, Gaithersburg, MD) was diluted to 50 μg/ml (0.5 μg/10 μL) and treated with 0.8 ng (1.6 units) endonuclease (1 unit = amount of enzyme that nicks 1 μg of plasmid DNA containing 25 cyclobutane dimers per plasmid in 30 min at 37°C).

Determination of cyclobutane dimer frequencies in SV40 DNA

After T4 endo V digestion, samples were mixed with loading buffer, and intact form I and nicked form II molecules were separated by electrophoresis on 0.8% neutral agarose gels (8). After electrophoresis, the gels were stained with ethidium bromide and photographed with Polaroid Type 55 film. The negatives were scanned with an LKB 2222−020 laser densitometer (Pharmacia). The intensity of bands representing forms I and II DNA was analyzed with Ultrascan XL (Pharmacia), and the values for the form I DNA were multiplied by 1.42 to correct topologically restricted reduction in ethidium bromide binding (10). The percentage of unnicked molecules remaining after UV irradiation and digestion with endo V was then determined.

RESULTS

The frequencies of cyclobutane dimers induced by UVC and UVB light sources were determined by damage-specific nicking (relaxation) of irradiated supercoiled SV40 DNA (Figure 2). Form I SV40 DNA was irradiated with UVC light and converted to open circular form II DNA by digestion with T4 endo V. The fluence that reduced the number of form I molecules to 37% of the unirradiated control (the D_{37} value) induced an average of one endonuclease-sensitive site per molecule. The D_{37} calculated by linear regression indicated that on average one T4 endo V-sensitive site was induced per plasmid after 12 J/m^2 UVC light measured at 254 nm. From the size of the plasmid and the D_{37} value, the induction rate was calculated to be 2.4 cyclobutane dimers per 10^8 daltons per J/m^2. This value is in close agreement with similar determinations made with other plasmids irradiated with UVC light *in vitro*, such as pSV2catSVgpt (11). The D_{37} value for induction of T4 endo V-sensitive sites after UVB irradiation was 95 J/m^2 (Figure 2). From this value, the induction rate for UVB light was calculated to be 0.3 cyclobutane dimer per 10^8 daltons per J/m^2.

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                1                  10                  20                  30                  40
5'-CCACAGACCCTCCTGTGCAACAAGATGTACCCTCTTTTTT
   CTCTGCGGCTTTGGAGGCTGCCCTGGAACTAGCTCTTGTA
                50                  60                  70                  80
             90                 100                 110                 120
GACCAGGCTGGTCTCGAACTTCACAGAAATCCTCTGCCTT
             130                 140                 150                 160
TGCCTCCTGAGTGCTGGAATTAAAGGCATGCGCGACGGGC
             170                 180                 190                 200
AATCTTGGTGGCTTCGTCGCCGATACGCTCAGGTCGATCA
             210                 220                 230                 240
CCGACTTGGAGATGCTGATGATCAAACGCAGGTCGGACGC
                250
CGCCGGCTGACGACGGG -3'
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Figure 1. DNA sequence of part of the *Alu*I fragment containing the 257 base pairs used to locate and quantify cyclobutane dimers. This sequence is deposited with GenBank (accession no. M32129).

For determinations of the sequence specificity of cyclobutane dimer induction, the *Alu* fragment was irradiated with either 1 kJ/m^{2}UVC or 8 kJ/m^{2}UVB light, digested with various concentrations of T4 endo V, and size fractionated on a sequencing gel (Figures 3 and 4). UVC and UVB fluences were selected to reflect the relative induction of the various cyclobutane dimers at lower, biologically relevant fluences and yet allow adequate visualization of bands on sequencing gels. Ellison and Childs (2) showed that the relative induction of T<>T, C<>T, and C<>C cyclobutane dimers in *E.coli* DNA irradiated with monochromatic UVC and UVB light was the same at fluences up to and above those used in our protocol. However, their chromatographic data indicated that the rates of C<>T and C<>C formation were reduced at higher fluences, especially after UVC irradiation. Hence, our results may reflect an underestimation of C<>T and C<>C induction relative to T<>T.

Titration of the UV endonuclease optimized band resolution and avoided differences between light treatments resulting from enzyme overdigestion, e.g., increased background values, selective loss of bands at the top of the gel, or cleavage at nonspecific sites. Cleavage at several purine residues (e.g., G_{192}, G_{196}' A_{210}, G_{211}, A_{218}, G_{220}, G_{231}, G_{236}, and G_{249}) was observed in lanes 3, 4, 7, and 8 and may correspond to the purine photoproducts cleaved by T4 endo V that were reported by Gallagher and Duker (12). The relative intensities of the bands determined by densitometry of lanes 4−6 showed no significant differences in digestion at individual sites (data not shown). Hence, within the range of endonuclease concentrations used, it is assumed that all putative cyclobutane dimers were cleaved stoichiometrically. Lanes 5 and 9 of the autoradiograph (Figure 3) were selected for comparative analysis because they displayed optimal digestion with no purine cleavage. The relative intensity of each band that could be resolved from the sequencing gel is shown for UVC and UVB light treatments in Figure 5.

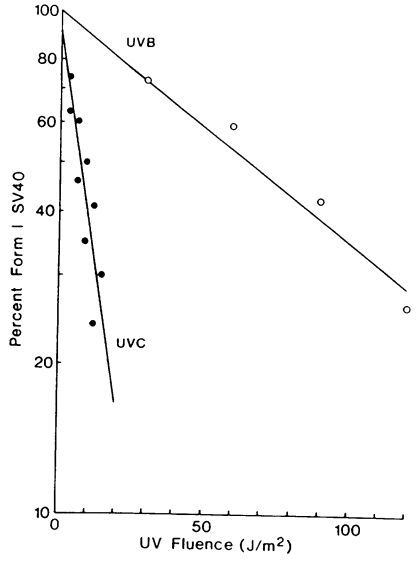

Figure 2. Induction of T4 endonuclease V-sensitive sites (cyclobutane dimers) in UV-irradiated supercoiled SV40 DNA. The loss of form I molecules by nicking with T4 endo V is shown for increasing fluences of UVC and UVB light. D_{37} values were calculated from regression lines; correlation coefficients were 0.9582 (n = 11) for UVC-irradiated DNA and 0.9900 (n = 5) for UVB-irradiated DNA-

UV endonuclease-sensitive sites were induced at all dipyrimidines within the region analyzed; photoproducts that could not be resolved by scanning densitometry (e.g., CT_{165} and CT_{173}) were not used in subsequent calculations. Cyclobutane dimers were induced in the *Alu* sequence most frequently at thymine homodimers (e.g., TT_{166}, TT_{174}, and TT_{207}) by both UVC and UVB irradiations (Figure 5). Thymine-cytosine heterodimers were also induced in significant amounts (e.g., TC_{164}, TC_{175}, and CT_{189}), constituting about 30 and 40% of the total cyclobutane dimer damage after UVC and UVB irradiations, respectively (Table I). Cytosine homodimers were induced in this sequence with the least efficiency, totaling less than 5 and 10% of the damage cleaved by T4 endo V after UVC and UVB light treatments.

Consistent with previous reports, the frequency of cyclobutane dimers varied considerably at individual dipyrimidine sites (3, 5, 13, 14). The potential for adjacent pyrimidines to dimerize depended on the composition of the reacting bases and the flanking sequences. When cyclobutane dimer sites were ranked in descending order of induction after UVB irradiation (Figure 5), the following pattern emerged: cyclobutane dimer hot spots (i.e., 70% of the damage contained in 33% of the potential sites) were, with one exception (CT_{189}), flanked on the 5′ side by a pyrimidine base (primarily cytosine); cold spots (i.e., 14% of the photoproducts contained in nearly 40% of the potential sites) were flanked in every case by guanine residues; and sites displaying intermediate induction values were flanked on the 5′ side by adenine.

Enhanced dimer induction by solar UV (UVB/UVC > 1.00) was observed at nearly all of the dipyrimidine sequences containing cytosine (TC_{164} and TC_{178} were the exceptions) (Figure 5). The most significant increases after UVB irradiation occurred at cytosine homodimers (CC_{181}, CC_{202}, CC_{241}, and

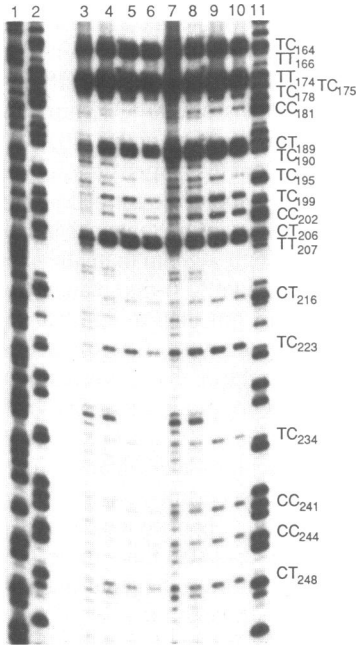

Figure 3. Digestion of UVC- and UVB-irradiated DNA by T4 endonuclease V. The DNA fragment was ^{32}P-end-labeled at the *Eco*RI site, irradiated with UVC (lanes 3–6) or UVB (lanes 7–10) light, digested with decreasing amounts of enzyme, and analyzed on DNA sequencing gels. Lane 1, A + G; lanes 2 and 11, C + T; lanes 3–6, DNA irradiated with 1 kJ/m^2 UVC (254 mn) light and incubated in 20 μl buffer containing 250 ng (lane 3), 83 ng (lane 4), 25 ng (lane 5), or 8.3 ng (lane 6) T4 endo V; lanes 7–10, DNA irradiated with 8 kJ/m^2 UVB light and digested as described for lanes 3–6.

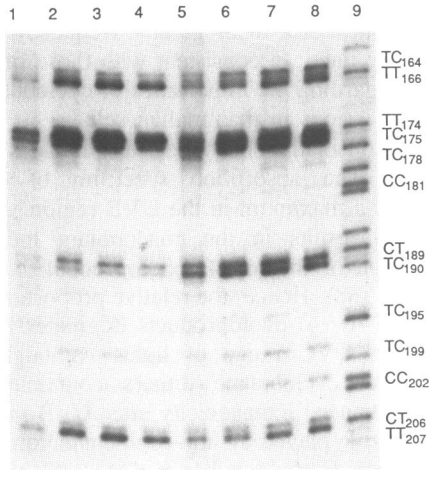

Figure 4. Detail of Figure 3. Lanes 1–9 correspond to lanes 3–11 in Figure 3.

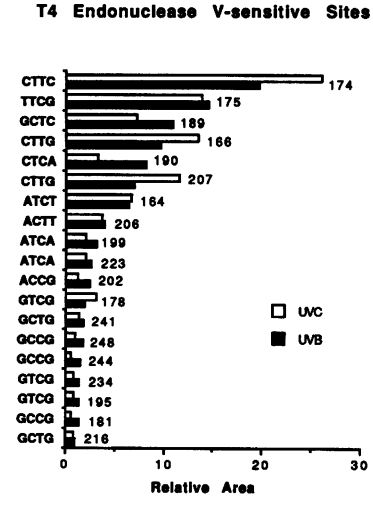

Figure 5. Relative induction of T4 endonuclease V-sensitive sites at selected sequences in DNA irradiated with UVC (open bars) or UVB (solid bars). Relative areas for each band were determined by densitometry of lanes 5 (UVC) and 9 (UVB) in Figure 3.

Table I: Summary of cyclobutane dimer induction and distribution at dipyrimidine sequences after irradiation with UVC or UVB light[a]

Cyclobutane dimers	Relative area/site		Relative induction		
	UVC	UVB	UVC	UVB	UVB/UVC
TT	17.0 (4.5)	12.1 (3.9)	0.68 (0.15)	0.52 (0.18)	0.7 (0.5)
CT	3.3 (1.4)	4.4 (2.3)	0.13 (0.09)	0.19 (0.13)	1.3 (1.5)
TC	4.1 (1.6)	4.9 (1.6)	0.16 (0.10)	0.21 (0.12)	1.2 (1.0)
CC	0.8 (.16)	1.7 (.25)	0.03 (0.02)	0.07 (0.04)	2.1 (0.8)

[a]Relative area per site was determined as the mean induction value for each dipyrimidine photoproduct. The standard error of the mean is shown in parentheses. The relative induction and UVB/UVC ratios were calculated from these mean values.

CC_{244}) and at dipyrimidine sites in which cytosine was located 3′ to thymine (TC_{190}, TC_{195}, TC_{199}, TC_{234}). At sites in which thymine was located 3′ to cytosine, some sites were more photoreactive than others (CT_{189} and CT_{248} > CT_{206} and CT_{216}). Reduced dimer induction by solar UV (UVB/UVC < 1.00) was observed at all of the thymine dipyrimidine sites (Figure 5).

DISCUSSION

In studies with ultraviolet light, most biological data have been acquired by using germicidal (254 nm) (UVC) light, and yet the character of DNA damage is significantly different after exposure to solar (UVB) light (2). We quantified induction frequencies of cyclobutane dimers at the sequence level after irradiation with solar (UVB) and nonsolar (UVC) light sources. The dimer yield at specific sites was dependent not only on DNA structure (i.e., composition of dimerizing pyrimidines and effect of flanking bases) but also on the wavelength of the ultraviolet radiation used.

Induction of cyclobutane dimers is significantly influenced by the sequence of neighboring bases (13, 14), in particular, the 5′ flanking base. As in other studies (3, 14), we found that the presence of a pyrimidine 5′ to a dipyrimidine enhanced the potential for dimerization (Figure 5). We further extended this rule to show that, of the 5′ flanking purines, guanine inhibited dimerization to a greater extent than adenine.

Early studies by Setlow and Carrier (15) indicated that cyclobutane dimers are induced in DNA in the order T<>T > T<>C + C<>T > C<>C. Subsequent chromatographic analyses estimated that the ratio of T<>T: T<>C + C<>T: C<>C is 60:24:16 after UVC irradiation (16). Sequence analysis in our study was consistent with this pattern (Table I): T<>T: C<>T: T<>C: C<>C = 68:13:16:3. The spectrum of cyclobutane dimer damage was different in UVB-irradiated DNA compared with UVC-irradiated DNA. The T<>T: C<>T ratio resulting from UVC irradiation has been independently determined in a number of studies and ranges from as low as 1.1 (14, 17, 18) to as high as 1.5 (16, 19). Concurrent studies showed a significantly lower ratio with a mid-UV (UVB) light source, ranging from 0.66 (16) to 1.0 (2, 18, 19). A ratio of T<>T: C<>T + T<>C: C<>C = 40:40:20 (16) was determined in human DNA with a UVB light source identical to that used in our studies (sunlamp filtered through cellulose acetate). The ratio of T<>T: C<>T: T<>C: C<>C determined by analysis of the UVB-irradiated *Alu* sequence was 52:19:21:7 (Table I). Although the induction rates determined at the sequence level were not identical to those determined in human genomic DNA, the same trends were evident; that is, T<>T induction was reduced and dimers containing cytosine increased. Moreover, the UVB/UVC enhancement ratio of 2.1 for C<>C homodimers (Table I) was greater than that determined for C<>T and T<>C (1.3 and 1.2, respectively). These data indicate that cytosine plays a more photoreactive role in cyclobutane dimer formation by UVB wavelengths than by UVC.

The biological effects of specific photoproducts have been a matter of some interest, especially those resulting from cyclobutane dimers and (6−4) photoproducts (20). However, more subtle structural differences between similar species of photodamage (e.g., base sequence) may also elicit different molecular and biological responses (21). Prokaryotic and eukaryotic repair systems that discriminate between the various types of cyclobutane dimers have evolved (13). For example, *E.coli* photolyase catalyzes the *in situ* light-dependent monomerization of T<>T cyclobutane dimers yet does not efficiently bind or monomerize C<>C and other cytosine dimers under the same conditions (22). In human fibroblasts, the initial rate and extent of removal of cytosine cyclobutane dimers is about 2-fold greater than that of the T<>T homodimer (23). Furthermore, the rate of cytosine dimer excision is about half that of the more distortive (6−4) photoproduct (24). Hence, both sequence composition and surrounding nucleotides, as well as, perhaps, the degree of helical distortion, appear to determine repair efficiency.

Several studies have shown that the action spectrum for lethality correlates with that for pyrimidine dimer induction in *E.coli* (25), in rodent cells (26, 27), and in human cells (28, 29). Other studies suggest that UV wavelengths > 300−313 nm may be more lethal (per quantum) than lower wavelengths (30, 31). Early studies suggested that the wavelengths responsible for mutagenesis may not coincide with those responsible for cell killing (19, 32). The frequency of ouabain-resistant mutants per lethal event is much higher after irradiation with 308 nm than after 254 run (33), and the ratio of ouabain-resistant to 6-thioguanine-resistant mutants is 10-fold greater at 313 nm than at 254 nm (34). These data suggest that either a unique type of premutagenic base damage is induced by 313 nm light or that damages induced at greater frequency by UVB light are more mutagenic than those induced by UVC light.

Identifying the molecular determinants important in UVB (solar) mutagenesis may be pertinent, as the 'A rule' (i.e., DNA polymerase inserts adenine opposite abasic sites associated with DNA damage) (35) suggests that dimerized pyrimidines containing cytosine are more mutagenic than those containing thymine exclusively. In *E.coli*, sites of (6−4) photoproducts occur preferentially at T-C and C-C dipyrimidines and correlate with C → T transition mutations (4, 36). In contrast, shuttle vector experiments in human cells utilizing photoreactivation to monomerize cyclobutane dimers suggest that these photoproducts, and not (6−4) photoproducts, are the primary premutagenic lesions (5). Keyse and coworkers (1) using a shuttle vector to quantify the mutation spectrum in mammalian cells showed that, in addition to increased deletions and insertions, C → T transitions were significantly increased at dipyrimidine sites after UVB compared to UVC radiation. This increase in transition mutations correlates with our observation that cyclobutane dimers containing cytosine occur with greater frequency after UVB irradiation. These data are consistent with those of Brash and coworkers (5) regarding the importance of cyclobutane dimers as premutagenic lesions in mammalian cells.

The UVB region of the solar spectrum displays a complex photochemistry. The absorption spectrum of the (6−4) photoproduct has a maximum in the UVB region. As a result, UVB irradiation results in the concomitant induction and photoisomerization of the (6−4) photoproduct to the Dewar pyrimidinone (37, 38). Hence, the relative proportion of Dewar pyrimidinones to (6−4) photoproducts is greater in the UVB compared to the UVC range of light. Although we have demonstrated that cyclobutane dimers containing cytosine correlate with enhanced mutagenicity after UVB radiation, we do not know the role played by non-cyclobutane dimer damage in this phenomenon. Further studies regarding the effects of UV wavelength on the sequence distribution of the (6−4) photoproduct and Dewar pyrimidinone in DNA are warranted.

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