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Distribution of UV light-induced damage in a defined sequence of human DNA: Detection of alkaline-sensitive lesions at pyrimidine nucleoside-cytidine sequences

(DNA damage and repair)

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ABSTRACT **The distribution of UV light-induced damage to the highly reiterated α sequence of human DNA was investigated. The results show that the distribution of UV light-induced cyclobutane dimers within a defined sequence is similar whether the DNA is exposed to UV light as part of the chromosome of intact cells or as naked DNA. However, the cellular environment shields the nuclear DNA, resulting in about 50% decrease in apparent dose. A new type of UV photodamage was detected. Treatment of UV light-irradiated DNA with hot alkali results in strand breaks at positions of cytidine located 3′ to pyrimidine nucleosides. The chemical nature and biological significance of the pyrimidine nucleoside-cytidine lesion is discussed.**

The UV portion of the spectrum of the sun induces damage to cellular DNA. In humans such damage can result in cell death or in precancerous lesions in exposed areas of the skin (1). UV irradiation results in similar phenomena in cultured human cells, causing cell death, mutation, and transformation to a malignant phenotype (2, 3). The UV light-induced lesion that has been studied in most detail is the cyclobutane pyrimidine dimer formed between adjacent pyrimidine bases to produce thymine-thymine, cytosine-thymine, and cytosine-cytosine dimers. Attempts to understand the biological effects of UV light have for the most part focused on the enzymatic repair of the pyrimidine photodimers. Here we investigate the distribution of UV light-induced damage in a defined sequence of human DNA. Use of the highly reiterated α DNA sequence (4–8) permits direct comparison of sites of DNA modification within a defined DNA sequence, when either purified DNA or intact human cells are exposed to light. In the course of these experiments, we discovered another major type of photodamage to DNA that may be of biological consequence.

MATERIALS AND METHODS

Cell Lines. HeLa cells, the CEM human lymphoblastic leukemia line, and the TK6 cell line of human B lymphoblasts were used for these experiments. Cells in linear growth phase were used for all experiments.

Enzymes. The enzyme preparation used to cut the DNA at positions of cyclobutane pyrimidine photodimers was the G-75 (9) fraction of the *Micrococcus luteus* pyrimidine dimer-specific endonuclease [endodeoxyribonuclease (pyrimidine dimer), EC 3.1.25.1] supplied by R. Grafstrom and L. Grossman. The human placental apyrimidinic/apurinic (AP) endonuclease [endodeoxyribonuclease (apurinic or apyrimidinic), EC 3.1.25.2] from the second round of G-75 purification (10) was supplied by N. Shaper and L. Grossman.

Preparation of Terminally Labeled DNA Substrates. The α sequence of human DNA was prepared and labeled at the 3′ terminus as described (7, 8). The complete details of the procedure for isolation and labeling of the α DNA sequence of human DNA will be presented elsewhere by Lippke and Haseltine. A 92-base pair (bp) DNA fragment labeled at one terminus was obtained by digestion of the 341-bp α fragment with the restriction endonuclease *Eco*RI*. Other substrates included restriction fragments of the pLJ3 (11) or pMCl (12) plasmids labeled at their 5′ or 3′ termini as described (13, 14).

UV Irradiation. Cells or DNA were irradiated with light from a germicidal lamp (primarily 254-nm light) at 10 J/m^2 per sec. DNA was irradiated in 0.01 M Tris·HCl, pH 7.0/1 mM Na_2EDTA. Cells were irradiated in phosphate-buffered saline (1×10^6 cells/ml). Irradiation was done on ice with constant agitation of the irradiated sample.

RESULTS

Distribution of Pyrimidine Dimers in α DNA. We wished to compare the distribution of UV light-induced pyrimidine dimers within a defined sequence of DNA irradiated either as naked DNA or as part of the chromosome of intact cells. The α DNA sequence that comprises 1% of human DNA provides a convenient substrate for such studies (4–8) and is prepared by treatment of the total DNA extract of human cells with the restriction endonuclease *Eco*RI. The α sequence contains a single *Eco*RI site. Because the α sequence exists in tandem arrays in the chromosomal DNA (7), cleavage of human DNA with *Eco*RI releases the α fragment as a DNA molecule 341 bp long. This fragment is readily separated from the bulk of cellular DNA that is cleaved into much larger fragments (3000–4000 bp) by *Eco*RI. For these experiments, the 341-bp-long α fragment was separated from the bulk DNA by sucrose gradient centrifugation and labeled at both termini by incorporation of [α-^{32}P]dATP and [α-^{32}P]dTTP at the *Eco*RI cleavage sites. A DNA fragment labeled at only one terminus is prepared by treatment of the 341-bp-long α DNA with the restriction enzyme *Eco*RI* that cleaves the DNA 92 bp from one end. The 3′ end-labeled 92-bp-long fragment was the substrate used for most of the experiments. The sequence of the 92-bp-long fragment obtained by this procedure is sufficiently homogeneous to permit unambiguous assignment of all residues (6–8).

To determine the distribution of pyrimidine dimers in purified irradiated DNA, the 3′ end-labeled 92-bp-long fragment of α DNA was prepared from unirradiated cells. To determine the location of the pyrimidine dimers, the irradiated DNA was treated with a preparation of the *M. luteus* pyrimidine dimer-

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Abbreviations: Pyd, pyrimidine nucleoside; PydC lesion, pyrimidine nucleoside-cytidine lesion; bp, base pair; AP, apurinic or apyrimidinic.

specific endonuclease that contains both a dimer DNA glycosylase activity and an AP endonuclease activity (15). Reactions were done under conditions of enzyme excess such that increased amounts of enzyme did not lead to increased strand scission. After treatment with the *M. luteus* enzyme, the DNA was denatured in neutral pH and layered directly onto a high resolution urea/polyacrylamide gel. The location of each pyrimidine dimer within the DNA sequence was determined by comparison of the mobilities of the scission products on this gel with the products of DNA sequence determinations (16). An autoradiograph of such a gel is shown for DNA irradiated with 5000 J/m^2 of UV light (Fig. 1). Treatment of irradiated DNA with the *M. luteus* pyrimidine dimer endonuclease resulted in scission products at sites of potential dimers. The frequency of scission at each site was determined by measurement of the amount of radioactivity in each band. Previous experiments indicate that enzymatic cleavage at pyrimidine dimers is quantitative under the conditions used (14, 15). The percentage of incision at potential dimer sites within the 92-bp-long α fragment is given as a function of dose for several potential dimer sites (Fig. 2*A*).

To determine the distribution of pyrimidine dimers in the chromosomal DNA, intact cells were irradiated with UV light immediately prior to DNA extraction. After irradiation, the DNA was extracted and cleaved with the restriction endonuclease *Eco*RI. The 3′ end was labeled and cleaved with *Eco*RI*, and the 92-bp-long fragment was purified. This DNA was analyzed for the distribution of pyrimidine dimers as before. In this sequence of DNA, the distribution of pyrimidine dimers was similar to that observed when purified DNA was irradiated (Fig. 1).

A quantitative comparison of the extent of dimerization at potential dimer sites is given in Fig. 2*A*. In both cases, the exent of dimer formation at a potential dimer site reached a plateau at high doses. The plateau value probably reflects the equilibrium Pyr Pyr $\overset{UV}{\rightleftharpoons}$ Pyr <> Pyr (15). The plateau value for each potential dimer site varied depending upon the sequence of the DNA as reported (15). However, the same plateau levels were reached regardless of whether the DNA was irradiated in solution or within intact cells. The parameter that did vary was the dose at which the photosteady state was reached. Pyrimidine dimers attained an equilibrium value at about 1000 J/m^2 when purified DNA was the substrate as compared to 2000 J/m^2 when DNA in intact cells was irradiated. We conclude that under the experimental conditions used, the effective dose to DNA in intact cells was about half that received by naked DNA. By correcting for this scaling factor, the relative distribution of pyrimidine dimers was the same in the two experiments.

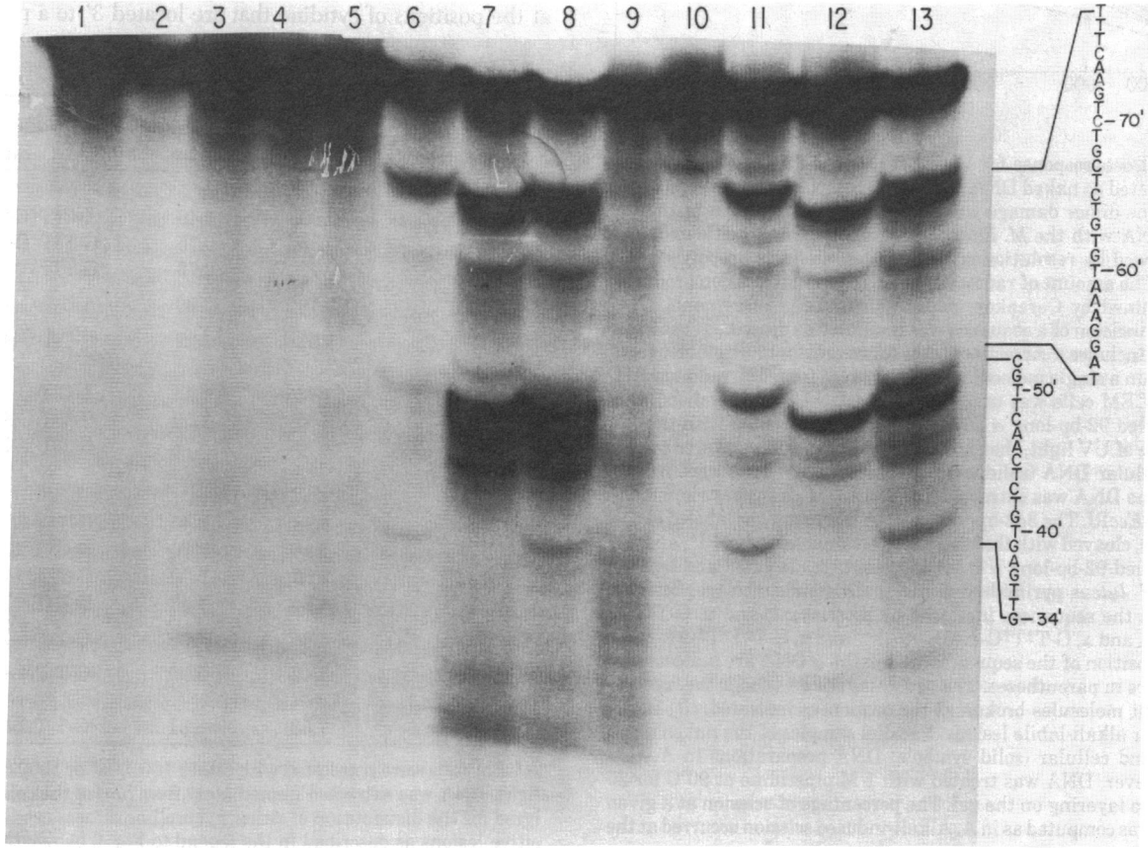

FIG. 1. Comparison of UV light-induced damage to the α sequence irradiated as naked DNA or as cellular DNA. The 342-bp-long α DNA fragment of human DNA was prepared from HeLa cells before (lanes 1–9) or after (lanes 10–13) irradiation with 5000 J/m^2 of UV light. The DNA was labeled at the 3′ terminus in reactions that included [α-^{32}P]dATP and [α-^{32}P]dTTP and the Klenow fragment of *Escherichia coli* DNA polymerase I. A 92-bp fragment was separated from other labeled DNA fragments. The DNA was treated in the following manner prior to layering on a urea-containing 8% (wt/vol) polyacrylamide gel. The sequence of the unirradiated DNA fragment determined by the chemical DNA sequence determination is on the right. Lanes 1–4: DNA prepared from unirradiated cells that were untreated (lane 1), treated with *M. luteus* pyrimidine dimer endonuclease (lane 2), treated with 1 M piperidine at 90°C for 20 min (lane 3), and treated with *M. luteus* pyrimidine dimer endonuclease followed by treatment with 1 M piperidine at 90°C for 20 min (lane 4). Lanes 5–8: DNA extracted from unirradiated cells that was exposed to 5000 J/m^2 of UV light and then subjected to the same four treatments in the same order prior to layering as described for lanes 1–4. Lanes 10–13: DNA purified from cells exposed to 5000 J/m^2 of UV light was treated as described for DNA of lanes 1–4 prior to layering. Lane 9: DNA purified from unirradiated cells treated with neocarzinostatin as described (17).

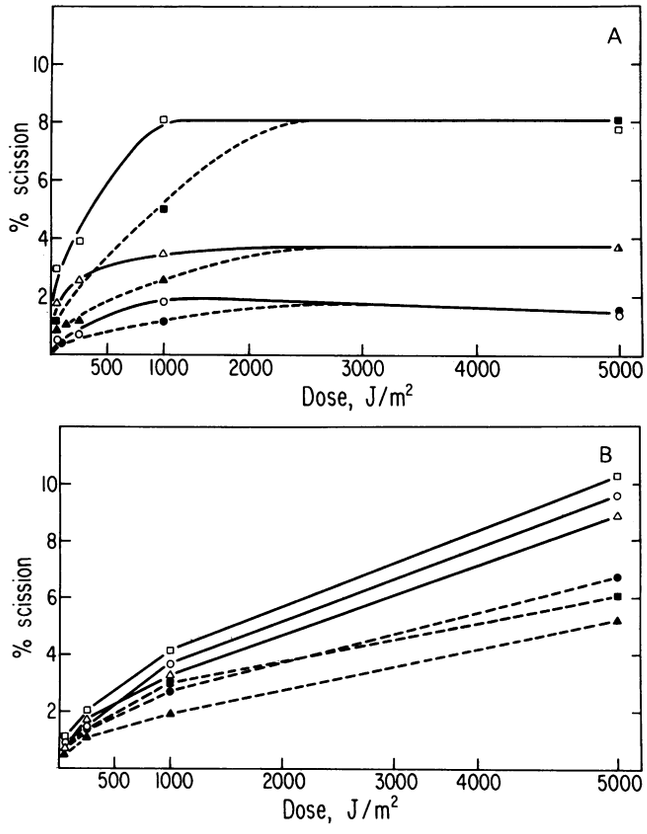

FIG. 2. Dose–response for UV light-induced damage to human α DNA irradiated as naked DNA or as cellular DNA. (*A*) Dose–response of pyrimidine dimer damage was determined by treatment of the irradiated DNA with the *M. luteus* pyrimidine dimer-specific endonuclease followed by resolution of the scission products on polyacrylamide gels. The amount of radioactivity of individual scission products was determined by Cerenkov measurements of gel fragments. The percentage incision of a sequence was computed as described (14). This calculation includes a correction factor to account for multiple cleavage events within a single molecule. DNA prepared from unirradiated and irradiated CEM cells was used. For naked DNA (open symbols), the 3′ end-labeled 92-bp-long α DNA fragment was treated with the indicated dose of UV light, then treated with enzyme, and layered on the gel. For cellular DNA (solid symbols), intact CEM cells were irradiated, and the DNA was extracted and cleaved with the restriction endonuclease *Eco*RI. The 342-bp-long α DNA fragment was labeled at the 3′ terminus, cleaved with the restriction endonuclease *Eco*RI*, and the 3′ end-labeled 92-bp-long α DNA fragment was isolated and treated with the *M. luteus* pyrimidine dimer-specific endonuclease. Scission occurred at the sequences indicated by asterisks: ○ and ●, G-T*T-G (37′–34′); △ and ▲, G-T*T*C-A (51′–47′); □ and ■, G-T*T*T*C-A (79′–74′). The position of the sequences within the α DNA are indicated by the numbers in parentheses. The percentage of scission is the fraction of the input molecules broken at the sequences indicated. (*B*) Dose–response for alkali-labile lesions. Parallel samples of the naked (open symbols) and cellular (solid symbols) DNA preparations in *A* were used. However, DNA was treated with 1 M piperidine at 90°C for 20 min prior to layering on the gel. The percentage of scission at a given sequence was computed as in *A*. Alkali-induced scission occurred at the sequences indicated by asterisks: ○ and ●, A-C-T-C*-T-G (46′–41′); △ and ▲, G-T-T-C*-A (51′–47′); □ and ■, G-T-T-T-C*-A (79′–74′). Similar results were obtained with α DNA extracted from HeLa cells for both types of DNA damage.

Detection of Alkali Labile UV Light-Induced Lesions. In studies of the distribution of pyrimidine dimers in bacterial DNA, we have noticed that ultraviolet light also induces lesions that lead to strand scission upon treatment of the DNA at high pH (14, 15). To determine whether or not such lesions also occur upon irradiation of human DNA, the DNA samples used in the previous experiments were also treated at high pH prior to layering on the gel. Exposure to alkaline conditions resulted in substantial breakage of both samples of irradiated DNA. No significant breakage occurred upon similar treatment for unirradiated DNA. The electrophoretic mobilities of the cleavage products produced by alkaline hydrolysis were different from those produced by enzymatic cleavage of the pyrimidine dimers. Combined treatment of the irradiated DNA with the *M. luteus* enzyme followed by treatment with piperidine resulted in scission products characteristic of both reactions. Quantitative measurement of the amount of radioactivity in scission products demonstrated that the effects of the *M. luteus* and piperidine treatments were additive (Table 1). The alkali-labile lesions occurred at the same sites in naked DNA as they did in DNA irradiated in intact cells. If the shielding effect of intact cells in the DNA is taken into account, the relative distribution of the lesions was the same in both cases (Fig. 2*B*). In contrast to the pyrimidine dimers, no plateau value for the extent of formation of the alkali lesions was noted over the dose range 50–10,000 J/m².

The Site of the Alkali-Labile Breaks. What is the sequence at the site of the alkali-labile lesions? Comparison of the electrophoretic mobilities of the alkali-induced scission products of 3′ end-labeled DNA with those produced in the DNA sequence determinations showed that the alkali-induced breaks occurred at the positions of cytidine that are located 3′ to a pyrimidine

Table 1. Stability of the UV light-induced damage

Treatment	UV irradiation, 0 hr	UV irradiation, 24 hr	Site
	100 J/m²		
PyrDEase	0.66	0.49	A-C*T*C-T-G
	0.59	0.75	G-T*T*C-A
	0.77	0.73	G-T*T*T*C-A
Piperidine	3.79	2.60	A-C-T-C*-T-G
	1.51	1.22	G-T-T-C*-A
PyrDEase and piperidine	4.48	4.48	A-C-T-C
	3.03	2.50	G-T-T-C-A
	4.35	5.78	G-T-T-T-C-A
	1000 J/m²		
PyrDEase	0.98	2.40	G-T*T-G
	2.35	2.70	A-C*T*C*T-G
	2.53	2.70	G-T*T*C-A
	4.60	4.43	G-T*T*T*C-A
Piperidine	3.06	2.68	G-T-T-C*-A
	3.42	3.29	G-T-T-T-C*-A
PyrDEase and piperidine	7.63	6.81	G-T-T-T-C-A

CEM cells were irradiated with either 100 J/m² or 1000 J/m² of UV light. DNA was extracted immediately from half of the cells and analyzed for the distribution of either pyrimidine dimers or alkaline-sensitive lesions as described in the legend to Fig. 2 by treatment of the DNA with either the *M. luteus* pyrimidine dimer-specific endonuclease (PyrDEase) or with 1 M piperidine at 90°C for 20 min or with PyrDEase followed by piperidine treatment. The remaining cells were resuspended in complete medium and incubated for 24 hr at 37°C prior to extraction of the DNA. In both cases the 3′ end-labeled 92-bp-long α DNA fragment was prepared, and the amount of strand scission is shown as percentage of input molecules broken at the sequences indicated. The asterisks indicate the sites of breakage within the sequence. Sequences studied included G-T-T-G (37′–34′), A-C-T-C-T-G (46′–41′), G-T-T-C-A (51′–47′), and G-T-T-T-C-A (79′–74′), in which the numbers indicate the position on the 3′ end-labeled strand relative to the labeled *Eco*RI end.

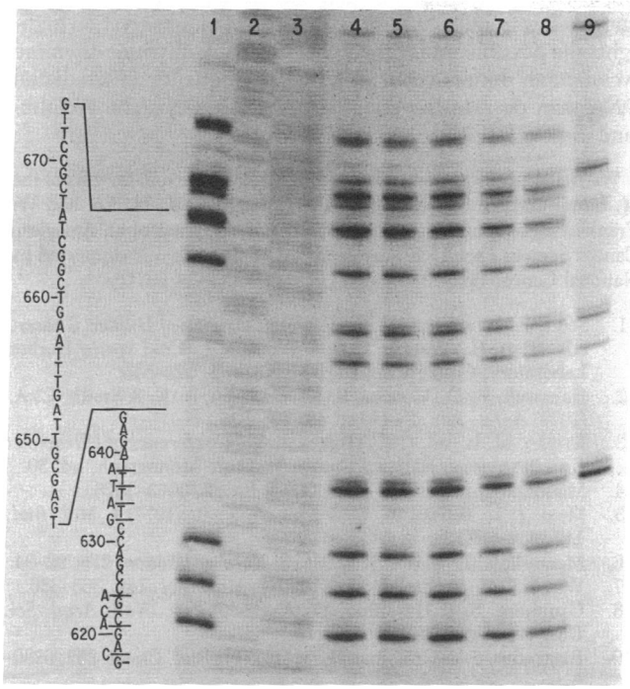

FIG. 3. Disposition of UV light-induced damage in a bacterial DNA fragment. A segment of the *lac i* gene extending from bp 561 to the sequence flanking the *lac i* gene (12, 18) was purified from the pMCl plasmid by digestion of the plasmid with restriction endonuclease *Bst*EII, labeled at the 3′ terminus as described (14), and redigested with restriction endonuclease *Hin*cII. The fragment extending from bp 561 to bp 885 was irradiated with 5000 J/m^2 of UV light and layered on a urea-containing 8% polyacrylamide gel after treatment. Lanes: 1, treatment with 1 M piperidine at 90°C for 30 min; 2 and 3, unirradiated DNA treated for the G+A and C+T DNA sequence analysis (16), respectively; 4–8, treatment with 1, 2, 5, 10, and 25 μl of *M. luteus* pyrimidine dimer-specific endonuclease, respectively, followed by treatment with 1 M piperidine at 90°C for 30 min; 9, treatment with 25 μl of *M. luteus* pyrimidine dimer-specific endonuclease only.

nucleoside (Pyd) (Fig. 3). Thus, breaks occur at the sequence T-C or C-C but not at the sequence C-T or a sequence in which cytidine is flanked by a purine nucleoside on both sides. Therefore, we call this DNA modification the pyrimidine nucleoside-cytidine (PydC) lesion. Alkali-labile lesions also were observed at positions of adjacent thymines but the frequency of these lesions was at least 1/10th that measured for T-C or C-C sequences.

What is the chemical modification of DNA that leads to alkaline sensitivity of the phosphodiester bond at the PydC lesion? UV light is known to induce the formation of cytosine photohydrates (19). Cytosine photohydrates have two diagnostic properties: they are unstable at neutral pH and they are formed at 10-fold greater rate in single- versus double-stranded DNA (19). To measure the stability of the PydC lesions, we incubated the UV-irradiated 92-bp-long α fragment for 7 days at 4°C and 20°C or at 90°C for 30 min. Both the PydC lesions and the cyclobutane pyrimidine dimers were unaffected by these treatments (not shown). To determine if the PydC lesions were also chemically stable to conditions that exist in the nuclei of intact cells, cells were irradiated at doses of 100 J/m^2 and 1000 J/m^2, and the DNA was extracted immediately or after the cells had been incubated at 37°C in fresh medium for an additional 24 hr. The same amount of both the alkali-labile and pyrimidine dimer endonuclease-sensitive lesions were detected in the α DNA fragment in both DNA samples (Table 1). To measure the extent of formation of the PydC lesions in single- and double-stranded DNA, a DNA fragment was irradiated in either the double-stranded or single-stranded forms at a dose of 7500 J/m^2. The state of the DNA did not significantly affect the extent of formation of the PydC lesions (Table 2). From these experiments we conclude that it is unlikely that the PydC photoproduct is a simple photohydrate of cytosine.

Table 2. Distribution of alkali-labile damage in double- and single-stranded DNA

Site	Double-stranded DNA	Single-stranded DNA
C-T-T-C*-C*-G	3.7 ± 1.0	3.6 ± 0.6
G-C-T-C*-G	1.2 ± 0.6	2.1 ± 0.4
A-C-C*-C*-C*-A	3.0 ± 1.2	4.3 ± 2.1

The 5′ end-labeled 117-nucleotide-long DNA fragment prepared from the *lac* promoter–operator insert of the pLJ3 plasmid, prepared as described (15), was used as a substrate. For one experiment, the double-stranded DNA was irradiated with 7500 J/m^2 of UV light and treated with 0.1 M NaOH at 90°C for 30 min prior to layering on urea-containing 8% polyacrylamide gels. For the other experiment, single-stranded DNA was prepared by heat denaturation of the 5′ end-labeled 117-bp DNA fragment followed by electrophoresis in a urea-containing 8% polyacrylamide gel. The labeled DNA fragment was separated by this procedure from the unlabeled complement because it is four nucleotides longer. The single-stranded DNA was treated with 0.1 M NaOH after exposure to 7500 J/m^2 of UV light. The amount of strand scission is expressed as percentage of total cpm per track broken at the indicated sites and was computed as described in the legend to Fig. 2. The sequences G-C-T-C*-G (47–51), C-T-T-C*-C*-G (40–46) and A-C-C*-C*-C*-A (19–24) are numbered from the *Eco*RI terminus of the 117-bp-long fragment (15). The asterisks indicate the actual site of cleavage. The numbers reflect the mean of 3 independent measurements at each sequence ± SEM.

To obtain more information regarding the nature of the PydC lesion, we investigated the sensitivity of irradiated DNA to breakage at cytidine upon other high pH treatments. Our standard high pH treatment involved incubation of the DNA with 1 M piperidine at 90°C for 20 min. The same amount of strand breakage occurred if the DNA was treated with 0.1 M NaOH for 30 min at 90°C, regardless of whether the DNA had been heated to 90°C at neutral pH prior to exposure to NaOH. Heating of the DNA to 90°C for 30 min at neutral pH did not result in significant strand scission of irradiated DNA. Elevated temperature at alkaline pH was required for breakage, as no significant cleavage was observed upon exposure of the DNA to 0.1 M NaOH at 20°C for 30 min.

The sensitivity of the UV light-induced alkali-labile lesions to high pH is similar to that observed at AP sites. To determine if the lesions were AP sites, DNA fragments irradiated at a dose of 7500 J/m^2 were incubated with a human placental AP endonuclease (10). No detectable breakage at the PydC lesions was observed in these experiments. Control experiments done simultaneously demonstrated that the enzyme was active at *bona fide* AP sites. Moreover, inspection of Figs. 1 and 3 reveals that the AP endonuclease activity (15) of the *M. luteus* pyrimidine dimer endonuclease preparation did not recognize the alkali-labile lesions. Therefore, we conclude that the PydC lesion is not a simple AP site.

DISCUSSION

The results presented here show that the α sequence of human DNA can be used to investigate the effects of ultraviolet light on DNA in intact human cells. Use of this sequence permits direct comparison of damage in naked DNA to damage in the same sequence of DNA as part of the chromosome in living cells. The results also show that there is no qualitative difference in the types of UV light-induced damage within the α DNA se-

quence under the two experimental conditions. However, there is a quantitative difference; the cellular environment shields the nuclear DNA from the damaging effects of UV light, resulting in about 50% reduction in the effective dose for the formation of cyclobutane dimers and the PydC lesion. Correcting for the shielding factor, the distribution of UV-induced lesions is the same for each potential damage site regardless of the environment of the DNA.

The data also show that UV light induces alkali-labile lesions at positions of cytidines that are located 3′ to pyrimidine nucleosides. It is unlikely that these lesions are pyrimidine dimers themselves. Chemical analysis of nucleoside dimers demonstrates that although dimer formation does weaken the *N*-glycosyl bond slightly, this bond is stable to treatment with 0.1 M NaOH (20). Moreover, the alkaline-labile lesions are not recognized by the *M. luteus* pyrimidine dimer endonuclease. In contrast to photoinduced dimers, the extent of the alkali-labile lesions is proportional to the total dose over a range of 50–10,000 J/m^2.

It is also unlikely that the alkali-labile lesions are simple cytosine photohydrates of the type described previously (19) because the lesions do not revert to cytosine at neutral pH and are formed at approximately the same rate in single- and double-stranded DNA. These observations also rule out the possibility that the lesion is a photohydrate of uridine, a deamination product of the cytosine photohydrate (19). Although AP sites have been reported to be induced in UV light-irradiated DNA (21), the insensitivity of the alkali-labile lesions to AP endonuclease rules out this possibility.

A variety of photoproducts have been identified in UV-irradiated DNA (22). Of these, the Thy(6–4)Pyo (23, 24) and Cyd(5–4)Pyo (25) products observed in the acid hydrolysates of UV-irradiated DNA are of possible relevance to the alkaline-sensitive lesions reported here. These products could be formed at T-C and C-C sequences. However, the relationship between the alkaline-sensitive PydC sites and the lesions that give rise to the Thy(6–4)Pyo and Cyd(5–4)Pyo products awaits further study.

The PydC lesions detected here may have significant biological consequences. At relatively low doses of UV light, 20 J/m^2, the alkali-labile cytidine lesion occurs at the same frequency at some sites as do some thymine-thymine dimers. The PydC lesion is stable in the cellular environment and does not revert spontaneously *in vitro* at neutral pH. The cytidine lesion may be mutagenic. The great majority of UV light-induced *lac i* amber, ochre, and UGA mutations occur at the position of cytidines located 3′ to adjacent pyrimidine nucleosides. This is the case for the five mutational hot spots that account for 40% of all UV light-induced chain-terminating mutations in the *lac i* gene (26, 27). The most frequent change involves a PydC to PydT mutation (26, 27). None of the hot spots for UV light mutagenesis occur at T-T sequences, although mutations do occur in the *lac i* gene at low frequency at some T-T sites. This observation highlights the possible role of the PydC lesions, as thymine-thymine cyclobutane dimers occur at a higher frequency in irradiated DNA than do cytosine-thymine or cytosine-cytosine cyclobutane dimers (14, 15).

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