Cytosine Photoproduct-DNA Glycosylase in *Escherichia coli* and Cultured Human Cells†

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ABSTRACT: Ultraviolet irradiation of DNA produces a variety of pyrimidine base damages. The activities of *Escherichia coli* endonuclease III and a human lymphoblast endonuclease that incises ultraviolet-irradiated DNA at modified cytosine moieties were compared. Both the bacterial and human enzymes release this cytosine photoproduct as a free base. These glycosylase activities are linear with times of reaction, quantities of enzyme, and irradiation dosages of the substrates. Both enzyme activities are similarly inhibited by the addition of monovalent and divalent cations. Analysis by DNA sequencing identified loci of endonucleolytic incision as cytosines. These are neither cyclobutane pyrimidine dimers, 6-(1,2-dihydro-2-oxo-4-pyrimidinyl)-5-methyl-2,4(1*H*,3*H*)-pyrimidinediones, nor apyrimidinic sites. This cytosine photoproduct is separable from unmodified cytosine by high-performance liquid chromatography. This separation should facilitate identification of this modified cytosine and elucidation of its biological significance.

Escherichia coli endonuclease III has been shown to be responsible for the repair of various monomeric pyrimidine damages in DNA, including both damages produced by UV[1] irradiation (Radman, 1976; Gates & Linn, 1977) and chemical oxidation caused by chemical or physical agents (Gates & Linn, 1977; Demple & Linn, 1980, 1982; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). An endonuclease activity that incises ultraviolet-irradiated DNA was found in cultured human cells (Brent, 1972; Duker & Teebor, 1975). It was demonstrated that this activity, termed the human UV endonuclease, is directed against non-pyrimidine dimer lesions in UV-irradiated DNA. This human endonuclease, like *E. coli* endonuclease III, also recognizes base damages in OsO_4-oxidized or X-irradiated DNAs (Brent, 1973, 1976, 1983; Doetsch et al., 1987). This activity was suggested to be the human counterpart to endonuclease III (Teebor et al., 1978). Thymine glycol had been thought to be the common endonuclease-sensitive lesion produced by all of these treatments and therefore the site of incision of all these DNA substrates, both by endonuclease III and by the human activity (Teebor et al., 1978). However, it has recently been established that a DNA cytosine photoproduct is a substrate for both endonuclease III (Doetsch et al., 1986; Helland et al., 1986; Weiss & Duker, 1986, 1987) and a calf thymus endonuclease following UV irradiation of the DNA (Doetsch et al., 1986; Helland et al., 1986).

The bacterial enzyme endonuclease III was previously demonstrated to act as a DNA glycosylase, releasing 5,6-saturated thymines and their derivatives as free bases from oxidized DNA. These include thymine glycol, 5-hydroxy-5-methylhydantoin, methyltartronylurea, and urea (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984, 1985; Higgins et al., 1987). This is coupled with a DNA apurinic/apyrimidinic site endonuclease activity on the same molecule, which incises DNA at the resultant apyrimidinic sites (Gates & Linn, 1977; Brent, 1983). To investigate the mechanism of action of *E. coli* endonuclease III and the human UV endonuclease against a UV-irradiated substrate and to further characterize these enzyme activities, we developed a radiolabeled photoproduct release assay for the cytosine photoproduct substrate. This cytosine photoproduct was separated from unmodified cytosine by using high-performance liquid chromatography (HPLC). We therefore demonstrate that both endonuclease III and the human UV endonuclease release the modified cytosine as a free base and that this cytosine photoproduct is chromatographically separable from unmodified cytosine.

Experimental Procedures

Preparation of Radiolabeled Substrates. The human alphoid segment, obtained by *Eco*R1 endonuclease digestion of a pUC9 plasmid containing the inserted sequence, was 3′-end-labeled with [^{32}P]dATP and recut as previously described (Weiss et al., 1983; Gallagher & Duker, 1986). The radiolabeled double-stranded polynucleotide poly(dG-dC)·poly(dG-dC) (length > 10 kb) was synthesized by nick translation with [5-^{3}H]dCTP (New England Nuclear, NET-369; specific activity = 25 Ci/mmol) according to the method of Maniatis et al. (1982). The specific activity of the radiolabeled polymer was approximately 1.5×10^6 cpm/μg.

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[1] Abbreviations: UV, ultraviolet; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; AP endonuclease, DNA endonuclease for apurinic/apyrimidinic sites; buffer A, 40 mM KH_2PO_4, 1 mM EDTA, pH 7.4; 6-4 photoproduct, 6-(1,2-dihydro-2-oxo-4-pyrimidinyl)-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione.

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Ultraviolet Irradiation of Substrates. The radiolabeled polynucleotide was irradiated with monochromatic 280-nm light by a 200-W Osram mercury lamp through a Bausch and Lomb high-intensity monochromator as previously described (Duker et al., 1981; Weiss & Duker, 1987). Ultraviolet fluence was determined by using an International Light IL500A radiometer with a calibrated photodetector. All samples were irradiated in 50 μL of 40 mM KH_2PO_4, pH 7.4, and 1 mM EDTA (buffer A) in a 2-mm quartz cell. Marker [2-^{14}C]-cytosine (MC131; specific activity = 58 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Enzyme Preparations. Endonuclease III was purified from *E. coli* strain BW531 as previously described (Weiss & Duker, 1986). Fraction IV was used in all experiments. The human nondimer UV endonuclease was partially purified from cultured lymphoblasts according to the method of Brent (1983). Fraction IVA was used in all experiments. Bacteriophage T4 endonuclease v was purified by the procedure of Friedberg et al. (1980). Fraction IV was used in the experiments.

Assays for Endonuclease and DNA Glycosylase Activities. Enzymic activity was analyzed by separation of radiolabeled reaction fragments on polyacrylamide gels. Enzyme fractions were incubated with irradiated or control 92 base pair segments in buffer A for 60 min at 37 °C. This DNA was ethanol-precipitated and the pellet heated for 1 min at 90 °C after being dissolved in gel-leading buffer (90% formamide, 10 mM NaOH, 1 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). DNA aliquots were also incubated in 25 μL of 1.0 M piperidine at 90 °C for 30 min, twice redissolved in 10 μL of distilled water, and lyophilized before being dissolved in the above buffer. Standard DNA sequencing reactions were performed according to the method of Maxam and Gilbert (1980), except that 25 μL of 1.0 M piperidine was used.

The enzymic release of the radiolabeled cytosine photoproduct into an ethanol-soluble fraction was assayed by using UV-irradiated poly(dG-dC)·poly(dG-dC); 10 ng of radiolabeled, UV-irradiated polymer was incubated at 37 °C in buffer A with 0.5 μg of endonuclease III or 3.0 μg of the human UV endonuclease in a total reaction volume of 100 μL. Following the reaction, 100 μL of a 1 mg/mL solution of calf thymus DNA was added as carrier. The mixture was brought to 0.3 M sodium acetate, pH 7.0, and 450 μL of ethanol was added to precipitate the polymer. Samples were frozen in a dry ice–ethanol bath for a minimum of 30 min and centrifuged in an Eppendorf microfuge for 15 min; 450 μL of the ethanol supernatant was recovered, and the radioactive content was determined by liquid scintillation counting in 20% Biosolve (Beckman).

Ion-exchange chromatography was used to investigate the nature of the enzyme-released cytosine photoproduct (Duncan et al., 1978). Dowex 1-X8 resin (Bio-Rad Laboratories) was swelled overnight in distilled water. Approximately 0.5 mL of resin was packed in a Pasteur pipet that had been plugged with glass wool. The column was washed with 5 mL of 5% formic acid, followed by 10 mL of distilled water. After incubation of the UV-irradiated substrate poly(dG-dC)·poly(dG-dC) with either purified endonuclease III or the human lymphoblast UV endonuclease, the entire mixture was immediately applied to the Dowex column and eluted with 1.35 mL of distilled water. The quantity of eluted material was determined by liquid scintillation counting as above.

High-Performance Liquid Chromatography. The enzyme-released photoproduct was demonstrated to be separable from unmodified cytosine by HPLC. Following its release from UV-irradiated poly(dG-dC)·poly(dG-dC) by either endonuclease III or the human endonuclease, the cytosine photoproduct was recovered in the ethanol-soluble fraction. The volume was reduced from 450 to 100 μL under vacuum, and 100 μL was injected into a 8 mm × 10 cm Waters C_{18} μBondapak cartridge in a Waters radial compression Z-module. The column was eluted with a 10-mL gradient of distilled water to 10% methanol (Waters gradient controller, curve 10) at a flow rate of 1.0 mL/min. The radioactive content of fractions, collected at 0.5-min intervals, was determined by liquid scintillation counting in 20% Biosolve.

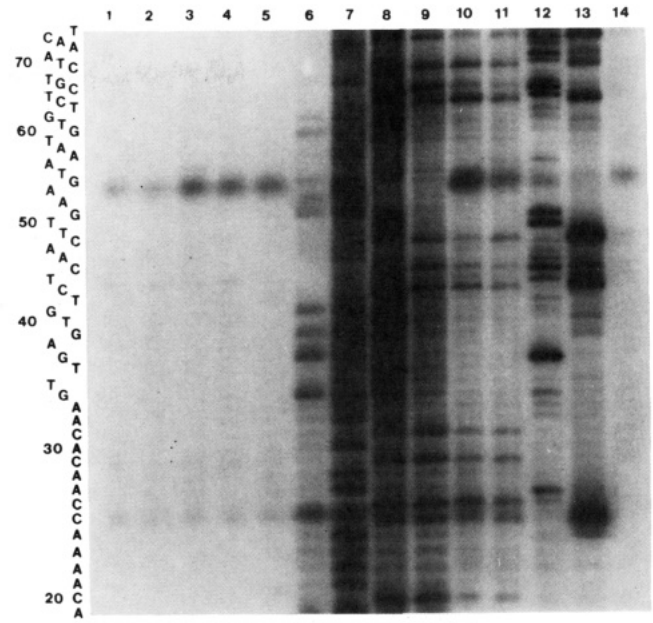

FIGURE 1: Endonuclease-sensitive sites of photochemical modification. Aliquots of 3′-end-labeled DNA were lyophilized (lane 1), heated in piperidine (lane 2), incubated with bacteriophage T4 endonuclease v (lane 3), incubated with *E. coli* endonuclease III (lane 4), incubated with human UV endonuclease (lane 5), subjected to base-specific chemical cleavages via guanine (lane 6), guanine–adenine (lane 7), thymine–cytosine (lane 8), and cytosine (lane 9), UV irradiated and then incubated with human UV endonuclease (lane 10), UV irradiated and then incubated with *E. coli* endonuclease III (lane 11), UV irradiated and then incubated with bacteriophage T4 endonuclease v (lane 12), UV irradiated and then heated in piperidine (lane 13), and UV irradiated and then lyophilized (lane 14).

Results

The sites of human UV endonuclease cleavage were determined by comparison of enzyme-generated reaction fragments with the DNA sequence. As shown in Figure 1, bands corresponding to all possible cytosine sites were generated after reaction of the human enzyme with UV-irradiated DNA (lane 10). Such reaction products were not found with substrate control unirradiated DNA (lane 5). The identical pattern of reaction cytosine loci was produced by reaction with bacterial endonuclease III (lane 11). Comparison of the products of these enzymes with those obtained by reaction with bacteriophage T4 endonuclease v clearly show them not to be cyclobutane pyrimidine dimers (lane 12). For instance, bands at cytosines 20, 29, and 31 were produced by incubating UV-irradiated DNA with the human enzyme or endonuclease III (lanes 10 and 11); no such bands were present in the sample reacted with endonuclease v (lane 12). Similarly, reaction of UV-irradiated DNA with hot alkali produced no cleavage at these three cytosine loci. Therefore, these cytosines cannot be 6-(1,2-dihydro-2-oxo-4-pyrimidinyl)-5-methyl-2,4-(1*H*,3*H*)-pyrimidinediones (6–4 photoproducts). No enzyme-generated bands were observed at any sites of adenines, guanines, or thymines following incubation of the human UV

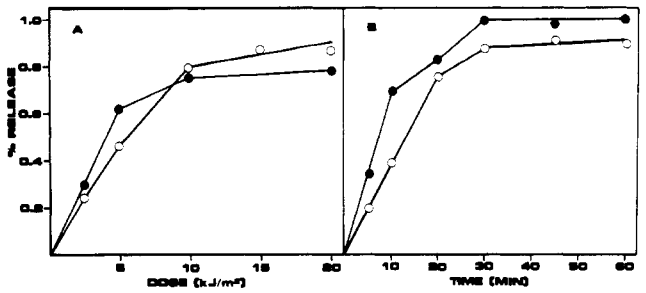

FIGURE 2: Release of cytosine photoproduct from irradiated polydeoxyribonucleotide by endonuclease III and human UV endonuclease. (A) Radiolabeled poly(dG-dC)·poly(dG-dC) was irradiated at 280 nm with the indicated doses and incubated with 0.5 μg of endonuclease III (●) or 3.0 μg of human UV endonuclease (○) at 37 °C for 10 min. (B) Radiolabeled poly(dG-dC)·poly(dG-dC) was irradiated at 280 nm with 10000 J/m² and incubated with 0.5 μg of endonuclease III (●) or 3.0 μg of human UV endonuclease (○) at 37 °C for the time indicated. Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and its measurement by liquid scintillation counting.

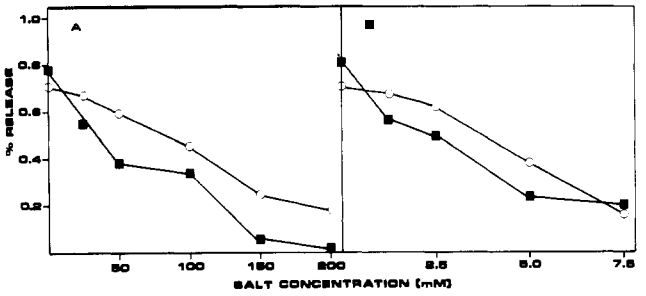

FIGURE 3: Inhibition of the release of cytosine photoproduct from irradiated polydeoxyribonucleotide by NaCl and $MgCl_2$. Radiolabeled poly(dG-dC)·poly(dG-dC) was irradiated at 280 nm with 10000 J/m² and incubated with 0.5 μg of endonuclease III (●) or 3.0 μg of human UV endonuclease (○) at 37 °C for 10 min with (A) increasing concentrations of NaCl or (B) increasing concentrations of $MgCl_2$. Release of radiolabeled material was assayed by ethanol precipitation and recovery of the ethanol-soluble fraction and its measurement by liquid scintillation counting.

endonuclease or *E. coli* endonuclease III with irradiated DNA.

The radiation dose dependences of both endonuclease III activity and the human nondimer UV endonuclease activity directed against this UV-irradiated substrate were examined. The results are shown in Figure 2A. Enzymic release of labeled material into the ethanol supernatant increased with dosage from 0 to 10000 J/m². Approximately 0.8% of the label was released by both the *E. coli* and human enzymes into the ethanol supernatant following a substrate irradiation dose of 10000 J/m². Irradiation of the substrate at higher UV doses did not result in significantly greater enzymic release of ethanol-soluble material.

Measurement of enzymic release of the cytosine photoproduct with increasing time of enzyme reaction (Figure 2B) showed release of radioactive material to be linear for the first 20 min at 37 °C, giving approximately 0.6% release with poly(dG-dC)·poly(dG-dC) irradiated at 280 nm with 10000 J/m² as substrate. There was some additional release between 20 and 30 min before photoproduct release finally plateaued at about 1% for the bacterial enzyme and 0.9% for the human endonuclease. Enzymic release of cytosine photoproduct from the same substrate was linear with increasing concentrations of endonuclease III (0–1.0 μg) and the human UV endonuclease (0–20 μg), both releasing approximately 1% of the total label (data not shown). These studies demonstrate that release of radiolabeled material increased with (1) increasing UV dose to the irradiation of the polymer, yielding substrate photoproduct, (2) increasing time of enzyme reaction, and (3) increasing enzyme concentration, thus confirming the enzymic nature of the reaction.

Additional experiments using this release assay were performed to further characterize both endonuclease III and the human enzyme. The effects of monovalent and divalent cations on the release of radiolabeled material from UV-irradiated poly(dG-dC)·poly(dG-dC) were examined. Increasing concentrations of NaCl, from 0 to 200 mM, had an inhibitory effect on enzymic activity (Figure 3A). Endonuclease III activity was completely abolished at 150 mM, while the human enzyme was inhibited by greater than 60% at that concentration. Similarly, enzyme activity was inhibited by 75% by the addition of 50 mM KCl (data not shown). Increasing concentrations of $MgCl_2$, from 0 to 7.5 mM, also inhibited the enzymic release of the cytosine photoproduct (Figure 3B). Both enzyme activities were reduced by approximately 75% by the addition of 7.5 mM $MgCl_2$. Similarly, the addition of

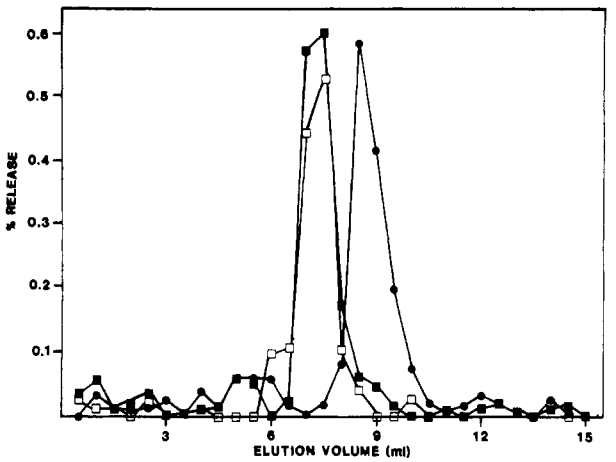

FIGURE 4: Separation of the cytosine photoproduct substrate from cytosine using high-performance liquid chromatography. Radiolabeled poly(dG-dC)·poly(dG-dC) was irradiated at 280 nm with 10000 J/m² and incubated at 37 °C for 60 min with 1.0 μg of endonuclease III or 3.0 μg of human UV endonuclease. Enzyme-released cytosine photoproduct was recovered by ethanol precipitation. The volume was reduced under vacuum to 100 μL and applied to an 8 mm × 10 cm C_{18} μBondapak cartridge. *E. coli* endonuclease III released ^{3}H-labeled material (■), human UV endonuclease released ^{3}H-labeled material (□), or unirradiated [^{14}C]cytosine marker (●) were eluted with a microprocessor-generated 10-mL 0–10% methanol gradient at a flow rate of 1 mL/min, and the radioactivity was determined by liquid scintillation counting.

Table I: Recovery of the Cytosine Photoproduct as Free Base[a]

substrate	enzyme	% recovered radioactivity
unirradiated	endonuclease III	0.04
unirradiated	human UV endonuclease	0.02
irradiated	endonuclease III	0.81
irradiated	human UV endonuclease	0.71

[a] Radiolabeled poly(dG-dC)·poly(dG-dC) was irradiated by 10000 J/m² at 280 nm and incubated with 0.5 μg of *E. coli* endonuclease III or 3.0 μg of human UV endonuclease for 30 min at 37 °C. The reaction mixtures were then applied to Dowex columns that were eluted with 1.35 mL of water. The radioactive content of the eluted material was measured by liquid scintillation counting as described and the percentage of radioactivity recovered from the column calculated.

comparable concentrations of $MnCl_2$ also inhibited both the bacterial and human endonucleases (data not shown). The optimum pH for the *E. coli* and human lymphoblast activities against the UV-irradiated substrate was broad, between 7.0 and 8.0 (data not shown). Enzyme activities was reduced by almost 75% at pH 6.5; a marked decrease in activity with pH

values above 8.0 was also observed.

The nature of the enzyme-released material was determined by ion-exchange Dowex chromatography. The results of a typical experiment are shown in Table I. Both the bacterial and the human enzymes released radiolabeled material that did not bind to the Dowex resin. Endonuclease III released 0.81% of the total UV-irradiated, radiolabeled material, while the human endonuclease released 0.71%. Under these conditions, the Dowex resin retains DNA and nucleotides, whereas free bases are eluted from the column. This demonstrates that both the bacterial and human lymphoblast endonucleases release the cytosine photoproduct as a free base.

HPLC was used to isolate the enzyme-released photoproduct and to demonstrate that it is distinct and separable from unmodified cytosine. The UV-irradiated, labeled polynucleotide was incubated with either endonuclease III or the human UV endonuclease, and the enzyme-released material was analyzed by HPLC. As shown in Figure 3, the tritiated cytosine photoproduct, released by both enzyme activities, was eluted from the column between 6.5 and 8.0 mL with a peak elution at 7.0–7.5 mL. The cytosine photoproduct was not detected by monitoring the absorbance at 254 nm, indicating that aromaticity of the ring has been lost. A ^{14}C-labeled cytosine marker eluted between 8.0 and 10.0 min with peak elution at 9.0 mL. Clearly, the enzyme-released photoproduct is separable from unmodified cytosine. These results support the conclusion that the cytosine photoproduct is released by both enzymes as a free base. Furthermore, the bacterial and human activities appear to excize the same cytosine photoproduct from irradiated DNA. This separation renders possible the eventual chemical characterization of the cytosine photoproduct, necessary for elucidation of its biological significance.

Discussion

The varying effects of UV irradiation on living cells have been generally attributed to DNA pyrimidine alterations. The presence of the major product, the pyrimidine dimer, is thought to result in lethality, mutagenicity, or transformation (Hart et al., 1977), while DNA 6–4 photoproducts are mutagenic (Brash & Haseltine, 1982; Haseltine, 1983). These experiments show another type of pyrimidine photoproduct to be incised by both enzymes at all possible cytosine sites. These moieties were present in regions of DNA lacking adjacent pyrimidines and are therefore neither pyrimidine dimers nor 6–4 photoproducts; this was confirmed by appropriate controls. Both the sequence and chromatographic studies indicate the human UV endonuclease to possess the identical substrate specificity as *E. coli* endonuclease III, with specific incisions at modified cytosines.

This study of endonucleolytic incision was performed with DNA substrates irradiated at 280 nm, since that was demonstrated to be the optimal wavelength for cytosine photoproduct site formation for both endonuclease III and the human lymphoblast enzyme (Weiss et al., 1987; Gallagher et al., 1989). The maximum wavelengths for production of substrate sites for both enzymes are between 270 and 295 nm, and identical sites of cytosine cleavages were found at various UVB and UVC wavelengths (Weiss et al., 1987; Gallagher et al., 1989). This is similar to the finding of maximal incision of UV-irradiated DNA at 280 nm by a partially purified endonuclease preparation from human HeLa cells (Doetsch et al., 1988). Unlike the human lymphoblast enzyme examined here, which incised the human substrate DNA only at cytosines, the HeLa cell activity nicked a UV-irradiated restriction fragment from pUC18 plasmid at many other sites, especially thymines. One thymine locus in particular accounted for 60% of the total incisions in DNA at 280 nm; cytosine photoproducts were less prominent sites of endonucleolytic activity (Doetsch et al., 1988). This could indicate either a greater variety of modified substrates recognized by the HeLa enzyme or the possible presence of other activities within that preparation. Some specific DNA loci may be especially susceptible to particular photochemical modifications. Further investigations are necessary to resolve this problem.

A number of other enzymes with activities similar to that of *E. coli* endonuclease III and the human lymphoblast enzyme examined here have been found in a variety of species. These include *Micrococcus luteus* (Riazuddin, 1980; Jorgensen et al., 1987), calf thymus (Bacchetti & Benne, 1975; Doetsch et al., 1986; Helland et al., 1986), rat liver (van Lancker & Tomora, 1974; Teebor et al., 1977), mouse cells (Nes & Nissen-Mayer, 1978; Helland et al., 1985), and yeast (Gossett et al., 1988). It has been suggested that this apparently ubiquitous enzyme activity initiates the repair of modified pyrimidines through recognition of the loss of the 5,6-double bond, resulting in the nonplanar structure of such moieties (Breimer & Lindahl, 1984).

A base release assay using radiolabeled poly(dG-dC)·poly(dG-dC) was developed to characterize *E. coli* endonuclease III and the human nondimer UV endonuclease against an irradiated substrate and to examine their mechanism of action. The alternating purine–pyrimidine sequence prohibits formation of both pyrimidine dimers and 6–4 photoproducts, ensuring maximal cytosine photoproduct yield. The polynucleotide was irradiated with monochromatic 280-nm light since this wavelength produces the maximum number of enzyme-sensitive sites for both endonuclease (Weiss & Duker, 1987; Gallagher et al., 1989). This assay, coupled with ion-exchange Dowex chromatography, was used to establish the nature of the enzyme-released material. The lack of Mg^{2+} or other divalent cations in the reaction buffer rules out release of radiolabeled material by possible contaminating nucleases or nucleotidases. Since all material other than free bases is retained by the Dowex resin, the results demonstrated that both the bacterial and human enzymes release the cytosine photoproduct as a free base (Duncan et al., 1978). This establishes the mechanism of incision of UV-irradiated DNA by both endonuclease III and the human UV enzyme as glycosylic release of the cytosine photoproduct, followed by endonucleolytic incision at the resultant apyrimidinic site. The separation of the cytosine photoproduct by HPLC should facilitate definitive identification of its structure.

Although the DNA cytosine lesions are formed in low yields, requiring higher fluences than needed to produce pyrimidine dimers of 6–4 photoproducts, their biological importance is undetermined. However, since the initial repair step proceeds by a glycosylic incision, this photoproduct might be mutagenic via an apyrimidinic site, which causes mispairing during DNA replication (Loeb, 1985). Mutations at cytosines have been observed following ionizing radiation of the *lac* region of bacteriophage M13 (Ayaki et al., 1986). The structures of these modified cytosines have not been identified, but it is possible that they might be similar to the photoproduct studied here. It is therefore important to determine if cytosine photoproducts are efficiently excised in living cells and to assess the consequences should this photoproduct persist in DNA.

While the sequence poly(dG-dC)·poly(dG-dC) is rare in human DNA, several laboratories have established the presence of stretches of the alternating purine–pyrimidine sequence poly(dG-dT)·poly(dA-dC) in actively transcribed regions of various eukaryotic cells (Kim et al., 1981; Hamada & Ka-

kunga, 1982; Hamada et al., 1982). Since neither pyrimidine dimers nor 6–4 photoproducts could be formed in such regions of DNA, UV-induced photoproducts would necessarily be limited to non-pyrimidine dimer type damages such as the cytosine photoproduct described here. Repair of these DNA lesions might then be essential for accurate transcription of mRNA molecules and eventual protein synthesis. Studies of the removal of pyrimidine dimers from the DNAs of UV-irradiated Chinese hamster ovary cells or mouse cells have demonstrated that repair of these damages is more efficient in actively transcribed genes than in inactive regions of the genome (Bohr et al., 1985; Madhani et al., 1986). Similar analyses of possible differential excision repair of the cytosine photoproduct reported here could help to elucidate the biological importance of this DNA moiety and its repair.

Epidemiological evidence has established a causal relationship between exposure to solar radiation and the formation of cutaneous carcinomas. The skin is the most common site of primary carcinomas of man, representing roughly 40% of all diagnosed human cancers (Jensen & Bolander, 1980). The wide variety of DNA photoproducts formed contribute to the complexity of elucidating the links between ultraviolet radiation and its ultimate cytotoxic, mutagenic, and carcinogenic effects. Definitive characterization of the various DNA photoproducts and elucidation of the enzyme mechanisms responsible for their repair are essential for understanding the biological consequences of actinic radiation.

Registry No. Endonuclease III, 60184-90-9; endonuclease, 9055-11-2; cytosine photoproduct–DNA glycosylase, 118016-98-1.

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