Repair of Pyrimidine Dimer Ultraviolet Light Photoproducts by Human Cell Extracts

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ABSTRACT: A newly developed method allows human cell extracts to carry out repair synthesis on ultraviolet light irradiated closed circular plasmid DNA [Wood, R. D., Robins, P., & Lindahl, T. (1988) *Cell 53*, 97–106]. The identity of the photodamage that leads to this repair replication was investigated. Removal of stable pyrimidine hydrates from irradiated plasmid pAT153 did not significantly affect the amount of repair replication in the fluence range of 0–450 J/m^2, because of the low yield of these products and their short DNA repair patch size. Photoreactivation of irradiated DNA using purified *Escherichia coli* DNA photolyase to remove more than 95% of the cyclobutane dimers from the DNA reduced the observed repair synthesis by 20–40%. The greater part of the repair synthesis is highly likely to be caused by (6–4) pyrimidine dimer photoproducts. This class of lesions is rapidly repaired by mammalian cells, and their removal is known to be important for cell survival after ultraviolet irradiation.

The photodamage produced when DNA is irradiated with 254-nm ultraviolet light (UV)[1] has been well studied. The two major photoproducts formed are covalent linkages between adjacent pyrimidines: cyclobutane pyrimidine dimers and (6–4) photoproducts. Recent estimates of the relative frequency of these products in DNA give a ratio of cyclobutane dimers to (6–4) photoproducts of about 3:1 (Mitchell, 1988). Both of these photoproducts distort the DNA helix (Husain et al., 1988b; Rao & Kollman, 1985) and are substrates for repair enzymes found in many different organisms. In *Escherichia coli*, the UvrABC incision complex recognizes and cleaves damaged DNA near both types of lesions. The cleavage is more efficient at (6–4) photoproducts than at cyclobutane dimers (Myles et al., 1987).

In addition, UV light induces pyrimidine base hydration products at isolated C and T positions in DNA. Most of these hydration products are unstable and revert to unmodified pyrimidines (Kittler & Löber, 1977); the remaining products are repaired by enzymes with pyrimidine hydrate-DNA glycosylase activity (Wallace, 1988). Such enzymes include the 25-kDa Nth protein of *E. coli*, which removes ring-saturated and ring-fragmented pyrimidines by cleavage of the glycosyl bond, followed by incision at the resulting apyrimidinic site. UV light also forms some photoproducts involving purine bases, but in double-stranded DNA these occur at a very low frequency and account for less than 1% of the total photodamage (Duker & Gallagher, 1988).

Human cells remove pyrimidine dimer photoproducts from DNA by a nucleotide excision repair process. Normal human cells in culture remove 50–70% of the cyclobutane dimers from their DNA within 24 h after irradiation with moderate UV fluences (Ehmann et al., 1978; Park & Cleaver, 1979; Zelle & Lohman, 1979). Intriguingly, (6–4) photoproducts appear to be removed significantly faster, with 70% removal during 2–3 h after irradiation (Mitchell et al., 1985). The rate of removal of (6–4) photoproducts correlates well with the rates of DNA repair synthesis and the disappearance of blocks to semiconservative DNA synthesis (Mitchell, 1988). Recovery from UV damage in human cells is correlated much less well with the overall rate of cyclobutane pyrimidine dimer removal in human cells, although part of this effect may be ascribable to a somewhat faster removal of cyclobutane dimers from regions of transcribed DNA (Mellon et al., 1987).

Cells from patients with the inherited syndrome xeroderma pigmentosum (XP) have defects in the repair of cyclobutane pyrimidine dimers (Park & Cleaver, 1979; Zelle & Lohman, 1979). More recent data indicate that XP cells also are defective in repair of (6–4) photoproducts (Mitchell et al., 1985; Protic-Sabljic et al., 1986). However, both normal and XP cells contain similar overall levels of pyrimidine hydrate-DNA glycosylase activity (Bachetti & Benne, 1975; Breimer, 1983; Doetsch et al., 1987).

Recently, we have described a system in which soluble extracts from normal human cell lines mediate repair replication of UV-irradiated plasmid DNA (Wood et al., 1988a,b). Extracts from cells of xeroderma pigmentosum origin were relatively deficient in repair replication. We are now engaged in experiments to more fully characterize the repair events which take place in this cell-free system.

In the experiments described here, repair replication mediated by cell extracts has been measured for UV-irradiated DNA that was subjected to enzymatic photoreactivation. The photoreactivation treatment selectively removes cyclobutane pyrimidine dimers but has no effect on (6–4) photoproducts. The results indicate the relative ability of the two major classes of UV photoproducts to initiate repair events carried out by cell extracts.

Experimental Procedures

Cell Lines. Human lymphoid cell lines were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). All lines used were tested and found to be free of mycoplasma. Cultures were grown in suspension in RPMI 1640 medium supplemented with 15% fetal calf serum.

Cell-Free Extracts. Whole-cell extracts were prepared essentially by the method of Manley et al. (1980), as described in Wood et al. (1988b). One-liter cultures of cells in late-exponential phase at (5–9) × 10^5 cells/mL were used for each preparation.

DNA Irradiation. pAT153 plasmid DNA was prepared from *E. coli* strain DH5 (*recA hsdR*) without chloramphenicol

[1] Abbreviations: UV, ultraviolet light; XP, xeroderma pigmentosum; (6–4) photoproducts, 6-(1,2-dihydro-2-oxo-4-pyrimidyl)pyrimidine class of ultraviolet light induced dimer photoproducts; PR, photoreactivation.

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amplification. DNA was irradiated in a thin stirred layer at 50 μg/mL in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA with 254-nm (peak) germicidal UV light at a flux of 0.5 W/m^2; the UV fluence rate was measured with a Latarjet dosimeter. Introduction of sites sensitive to pyrimidine dimer-DNA glycosylase from *Micrococcus luteus* (obtained from Applied Genetics Inc., Freeport, NY) was monitored by agarose gel electrophoresis. The conversion of closed circular molecules to nicked forms was quantified in the fluence range 0–100 J/m^2 (up to an average of approximately three nicks per molecule). An average of one cyclobutane pyrimidine dimer per molecule was produced by a fluence of 37 J/m^2. The introduction of sites sensitive to *E. coli* Nth protein was measured in a similar way, showing an average of one stable site sensitive to Nth protein produced by a fluence of 450–500 J/m^2. In some experiments, plasmid irradiated with 450 J/m^2 was treated with an excess of *E. coli* Nth protein for 60 min at 37 °C to incise stable UV-induced Nth protein sensitive sites, and closed circular forms were repurified as described (Wood et al., 1988a,b).

Repair Reactions. Standard 50-μL reaction mixtures contained (final concentration) 0.3 μg of unirradiated closed circular form I pBR322, 0.3 μg of pAT153 UV-irradiated substrate, 45 mM Hepes–KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dGTP, dCTP, and TTP, 8 μM dATP, 2 μCi of [α-^{32}P]dATP (3000 Ci/mmol), 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3.4% glycerol, 18 μg of bovine serum albumin, and (typically) 80 μg of extract protein. Reactions were incubated for 6 h at 30 °C.

Visualization of Repair by Autoradiography. Reactions were stopped by the addition of EDTA to 20 mM. After a 10-min incubation at 37 °C with 80 μg/mL RNase A, SDS was added to 0.5% and proteinase K to 190 μg/mL. Tubes were incubated for 30 min at 37 °C, and the mixture was extracted with phenol/chloroform. DNA was precipitated in the presence of 2.5 M ammonium acetate with two volumes of ethanol at −70 °C. Plasmids were digested with *Eco*RI in a 30-μL volume, loaded on a 1% agarose gel cast, and run in buffer containing 0.5 μg/mL ethidium bromide. After electrophoresis overnight, the gel was photographed under near-UV transillumination with Polaroid Type 55 positive/negative film. An autoradiograph of the dried gel was obtained with Kodak XAR-5 film and intensifying screens for 2 h at −80 °C. Band intensities on the autoradiograph and the photographic negative were quantified with an LKB UltroScan XL scanning laser densitometer. In a number of experiments, bands were excised from the gel and analyzed by scintillation counting in order to calibrate the densitometry results with reference to incorporation of radioactive material.

Enzymatic Photoreactivation. Irradiated plasmid at 20 μg/mL in photoreactivating buffer (25 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 300 μg/mL bovine serum albumin) was exposed to 366-nm (peak) black light provided by two fluorescent G.E. T15BLB bulbs. The light was filtered through a Falcon multiwell petri dish lid and 3 mm of glass to remove wavelengths less than 320 nm. Samples were illuminated as 50-μL aliquots in 2-mL microfuge tubes. The flux incident at the sample was 7 J m^{-2} s^{-1} as measured with a Blak-Ray Long Wave ultraviolet meter (J-221, Ultraviolet Products). Reactions contained 0.25 μg (1000 units) of *E. coli* photolyase (a gift from Drs. G. and A. Sancar, University of North Carolina) per microgram of plasmid DNA. Control reactions without photolyase were treated in exactly the same way as those with photolyase.

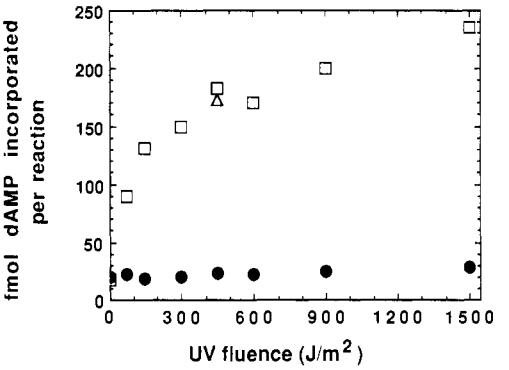

FIGURE 1: Fluence response for repair in cell-free extracts. Irradiated pAT153 plasmid DNA was used in repair reactions with 100 μg of GM1953 whole-cell extracts under standard conditions. After gel electrophoresis and autoradiography, the DNA amounts and radioactive material present in the plasmid were quantified by densitometric scanning and direct counting of bands from the gel, and the ficomoles of dAMP incorporated per reaction were calculated. (□) Purified supercoiled pAT153 plasmid DNA at 50 μg/mL irradiated with UV light as 10-μL droplets in 10 mM Tris-HCl and 1 mM EDTA, at a flux of 0.5 W/m^2; 300 ng of plasmid per reaction. (△) pAT153 plasmid DNA irradiated with 450 J/m^2 and then treated with *E. coli* Nth protein to nick molecules containing stable hydrated pyrimidines. Intact irradiated plasmid was repurified as described (Wood et al., 1988a) and 300 ng was used per reaction. (●) Unirradiated supercoiled pBR322 plasmid DNA, 120 ng incubated in reactions simultaneously with the irradiated pAT153 plasmid DNA.

Samples were incubated in the dark for 5 min at 30 °C to allow enzyme binding and then under photoreactivating light for the indicated times. After photoreactivation, samples were extracted in dim light sequentially with phenol/chloroform, chloroform, and diethyl ether; ether was removed with a stream of N_2 gas. The sample was drop-dialyzed against 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 M NaCl overnight and then against 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA for 2 h.

To analyze for photoreactivation of transformability, 100-ng samples were used to transform competent *E. coli* CSR603 *recA*$^-$ *uvrA*$^-$ *phr*$^-$. Samples from 1-mL transformation mixes were plated on LB plates containing 15 μg/mL tetracycline. After incubation for 24 h at 37 °C, drug-resistant colonies on duplicate plates were counted.

To analyze directly for removal of cyclobutane pyrimidine dimers, 300 ng of DNA was cleaved with 0.4 μg (370 units) of *M. luteus* UV endonuclease for 60 min at 37 °C, and the products were loaded on an agarose gel. The amounts of closed circular and nicked DNA were determined by densitometry of a photographic negative, and the extent of photoreactivation was calculated from the amount of plasmid uncut by the enzyme.

RESULTS

Fluence Response. To observe repair replication of UV-irradiated DNA in vitro, the previously described procedure was followed (Wood et al., 1988b). In this scheme, UV-irradiated purified plasmid DNA is incubated with whole-cell extracts in a reaction mixture which includes the four deoxynucleoside triphosphates and [α-^{32}P]dATP. During incubation at 30 °C, enzymes in the extract incise the damaged plasmid DNA, and repair patches are formed. The plasmid DNA is then extracted from the reaction mixture, isolated by gel electrophoresis, and analyzed for the presence of radioactively labeled DNA repair patches by autoradiography.

The extent of repair replication is dependent on the UV fluence given to the plasmid DNA. Figure 1 shows results for repair mediated by an extract from the normal lymphoid

Table I: Evidence for Photoreactivation by Cyclobutane Dimer Removal and Increased Transformability[a]

PR light (min)	cyclobutane dimers[b]	CSR603 transformants/mL[c]
0	(12)	14
5	1.7	358
10	1.0	1132
30	0.5	1515

[a] pAT153 plasmid DNA was irradiated with 450 J/m^2 and treated with Nth protein, and supercoiled circles were repurified. After addition of photolyase, samples were exposed to photoreactivating light for the indicated times. The DNA was then assayed for extent of photoreactivation. [b] Samples were treated with *M. luteus* UV endonuclease as described and analyzed on agarose gels for the presence of endonuclease-sensitive sites. With no photoreactivation, all molecules were incised by the enzyme. A value of 12 cyclobutane dimers per circle for 450 J/m^2 was calculated from the rate of induction of one dimer per 37 J/m^2 as measured for the range 0–100 J/m^2 (see Experimental Procedures). [c] Samples were used to transform competent *E. coli* CSR603 *recA*$^-$ *uvrA*$^-$ *phr*$^-$ cells. The number of tet^r colony forming units per milliliter is indicated. Samples of unirradiated plasmid in the same reaction showed 2208 CSR603 transformants/mL with 30-min photoreactivating light.

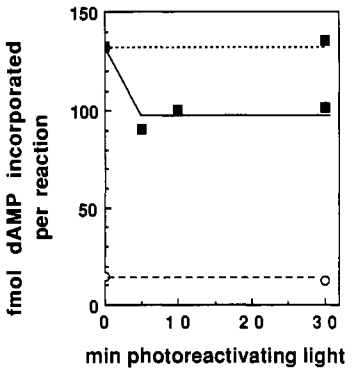

FIGURE 2: Effect of photoreactivation on repair replication catalyzed by soluble human cell extracts. pAT153 plasmid DNA was irradiated with 450 J/m^2 and treated with Nth protein, and supercoiled circles were repurified. After additiuon of photolyase, samples were exposed to photoreactivating light for the indicated times. Samples of the DNA were then used in repair reactions (final 20 mM KCl) with 100 μg of GM1953 cell extract for 2 h at 30 °C. The amounts of DNA and autoradiographic signals were quantified by densitometry. (■) UV-irradiated DNA, illumination with black light in the presence (solid line) or absence (dotted line) of photolyase; (○) unirradiated DNA.

cell line GM1953. Repair incorporation is approximately linear up to 200 J/m^2, with a gradually decreasing slope above this fluence.

Effect of Removing Sites Sensitive to Nth Protein. Cyclobutane pyrimidine dimers are formed in pAT153 plasmid DNA at a rate of one dimer per 37 J/m^2 (see Experimental Procedures), so that a plasmid irradiated with 450 J/m^2 contains an average of 12 cyclobutane dimers as well as three to four (6–4) photoproducts per molecule. In addition, there are 0.5–1.0 stable pyrmidine hydrates per molecule, detectable as sites sensitive to Nth protein (Wood et al., 1988a,b).

For the experiments reported previously (Wood et al., 1988b), UV-irradiated plasmid DNA was prepared free of Nth-sensitive pyrimidine products. This was done by irradiating plasmid pAT153 with 450 J/m^2, treating it with *E. coli* Nth protein to nick molecules containing pyrimidine hydrates, and then repurifying intact molecules for use as the UV-irradiated substrate in repair reactions.

The purification of plasmid free of Nth protein sensitive sites was done in order to ensure that there would be no possibility of detecting irrelevant base excision repair events initiated at pyrimidine hydrates in the in vitro system. In Figure 1, data are included for DNA which had been treated to remove sites sensitive to Nth protein. This result and results from several similar experiments indicate that the average of one pyrimidine hydrate per molecule present at 450 J/m^2 does not substantially increase the repair replication detected in this assay. Other experiments (not shown) indicate that pyrimidine hydrates only make a significant contribution to the total repair replication when several stable products are present per molecule, resulting from irradiation of DNA with fluences greater than 900 J/m^2.

Photoreactivation of UV-Irradiated Plasmids. In order to assess the contribution of cyclobutane dimers to the total repair observed in cell-free extracts, UV-irradiated plasmid was treated with purified photolyase and photoreactivating light. This treatment monomerizes cyclobutane dimers but has no effect on (6–4) photoproducts (Brash et al., 1985).

The first series of experiments was done with plasmid pAT153 DNA that had been UV-irradiated with 450 J/m^2, treated with Nth protein, and repurified free from pyrimidine hydrates as described above. The DNA was then enzymatically photoreactivated, by use of highly purified *E. coli* DNA photolyase and 320–400-nm near-UV light.

The extent of photoreactivation was monitored in two ways. First, the removal of cyclobutane dimers was assessed directly by monitoring the susceptibility of plasmids to cutting by *M. luteus* UV endonuclease before and after photoreactivation (Table I). Initially, an average of 12 cyclobutane dimers was present per plasmid circle. After 30-min illumination an average of 0.5 endonuclease-sensitive site remained per plasmid. Thus about 96% of the cyclobutane dimers were monomerized. Most of the photoreversal took place within 10 min.

Photoreactivation was also monitored by a biological assay, on the basis of the knowledge that unrepaired cyclobutane dimers are lethal for bacterial plasmids. Samples of plasmid DNA were added to competent cells prepared from the repair-deficient *E. coli* strain CSR603 (*uvrA*$^-$ *recA*$^-$ *phr*$^-$). The efficiency of transformation of this strain to drug resistance by a plasmid-encoded gene reflects the amount of toxic damage on the plasmid (Sancar & Sancar, 1984). By this assay, most of the lethal lesions in the plasmid were removed after 10–30-min photoreactivation (Table I). The sector of damage that is not enzymatically photoreactivatable is principally ascribable to (6–4) photoproducts (Husain et al., 1988a; Tang et al., 1986).

Samples of the photoreactivated DNA were used in repair reactions with GM1953 normal cell extract (Figure 2). Extensive photoreactivation of the DNA reduced the UV-dependent repair replication in the reaction by about 25%. This implies that cyclobutane dimers are responsible for some of the repair replication observed. However, most of the repair replication was due to photoproducts other than cyclobutane dimers, probably (6–4) photoproducts.

The relationship between total damage to the plasmid and the amount of repair replication is nonlinear above 200 J/m^2. This could complicate interpretation of the results, by deemphasizing the effects of photoreactivation of more heavily irradiated DNA. With this possible saturation effect in mind, another experiment was performed to examine the photoreactivatability of DNA repair replication at lower UV fluences. Figure 3a indicates the extent of photoreactivation of samples given 100, 200, 300, or 400 J/m^2 as measured by the ability of the DNA to transform *E. coli* strain CSR603. The photoreactivated and nonphotoreactivated samples were used in repair replication reactions with GM1953 extract and analyzed by autoradiography (Figure 3b). The data were quantified by densitometry, yielding the results in Table II.

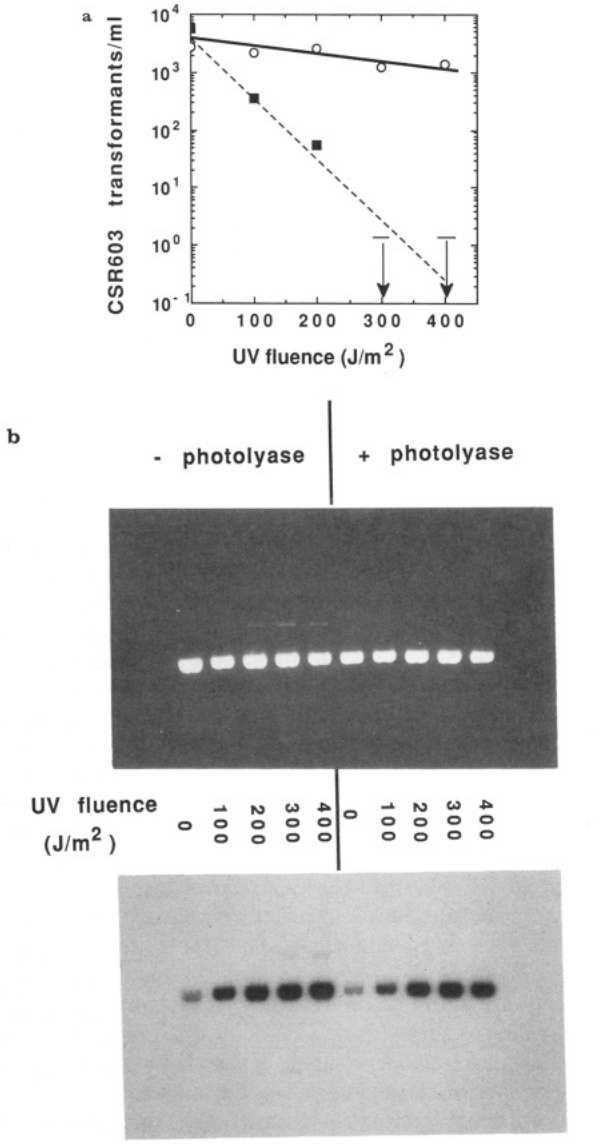

FIGURE 3: (a) Inactivation of transformability by increasing UV fluences. Purified supercoiled pAT153 plasmid DNA was irradiated with the indicated fluences of UV light. Purified photolyase was added to a portion of DNA at each fluence, and samples were then exposed to 30 min of photoreactivating black light. After illumination, the DNA was purified, and samples were used to transform competent *E. coli* CSR603 *recA– uvrA– phr–* cells. (■) No photolyase; (○) plus photolyase. For the transformations without photolyase, no colonies were detected at 300 and 400 J/m²; the arrows represent the upper statistical limits for colony-forming units per milliliters. (b) Effect of photoreactivation on DNA irradiated with increasing UV fluences. The samples described in (a) were used in repair reactions under standard conditions with 190 μg of GM1953 normal cell extract. Top: agarose gel of DNA extracted from the reactions and linearized with *Eco*RI. Bottom: autoradiograph of the agarose gel.

Subtraction of the background incorporation found in the unirradiated plasmid DNA shows that about 20% of the UV-dependent repair was photoreactivatable at 200 J/m² and above, in close agreement with the experiment in Figure 2. A greater proportion of the repair replication (43%) appeared to be photoreactivatable at the lowest fluence, 100 J/m². Thus the data indicate a saturation effect between 100 and 200 J/m².

Photoreactivation of Repair Replication Initiated by UV Endonuclease from M. luteus. The limited photoreactivatability of repair synthesis in normal cell extracts raised the possibility that some unknown limitation in the approach might prohibit detection of more extensive photoreactivation.

Table II: Photoreactivation of in Vitro Repair Replication for Plasmid DNA Given Different UV Fluences

UV fluence (J/m²)	dAMP incorporated per reaction (fmol)		UV-dependent dAMP incorporated per reaction (fmol)		
	–PR	+PR	–PR	+PR	(+PR/–PR)
0	81	66	0	0	
100	215	143	134	77	0.57
200	311	245	230	179	0.78
300	357	287	276	221	0.80
400	437	327	356	261	0.73

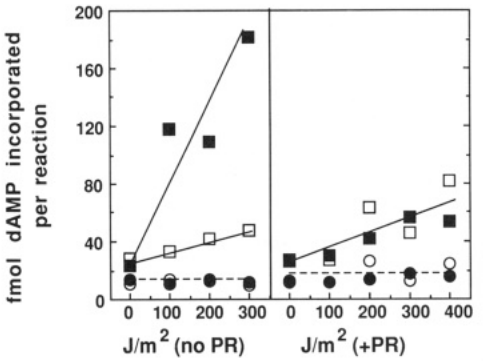

FIGURE 4: Photoreactivation of repair initiated by UV endonuclease from *M. luteus*. Supercoiled pAT153 plasmid was irradiated with the indicated fluences of UV light and then exposed to photoreactivating light for 30 min as described, either without photolyase (left panel) or with photolyase (right panel). The irradiated and photoreactivated pAT153 plasmid was mixed with unirradiated pBR322 DNA and used in standard repair reactions with 200 μg of GM2345 XP-A extract. Square symbols (□, ■): irradiated pAT153. Circles (○, ●): unirradiated pBR322. Closed symbols (■, ●): reactions supplemented with 1 μL (62 units) of *M. luteus* UV endonuclease. Open symbols (□, ○): no *M. luteus* UV endonuclease.

Therefore, photoreactivation of repair was examined for a specialized case where most repair synthesis is known to be dependent on cyclobutane pyrimidine dimers: repair in XP-A cell extracts supplemented with UV endonuclease from *M. luteus*. Addition of this UV endonuclease to repair-deficient XP cell extracts greatly stimulates the introduction of repair patches into UV-irradiated plasmid DNA (Wood et al., 1988). The enzyme incises DNA at sites of cyclobutane dimers to produce an unusual 5′ terminus containing a cyclobutane dimer and a 3′ abasic site (Grafstrom, 1982). Human cells do not introduce nicks at cyclobutane dimers by this mechanism (LaBelle & Linn, 1982). Consequently, repair replication initiated by the *M. luteus* enzyme represents introduction of a new pathway of repair, separate from the normal mechanism.

It was predicted that the *M. luteus* enzyme would no longer be able to stimulate repair replication in xeroderma pigmentosum extracts if the DNA was first photoreactivated to remove cyclobutane dimers. Figure 4 shows the results of an experiment where UV-irradiated plasmid DNA was incubated with extracts from the XP-A cell line GM2345 and supplemented with *M. luteus* UV endonuclease. The enzyme initiated repair replication events which increased with the UV fluence given to the plasmid (Figure 4, left panel). Incubation of plasmid with photolyase and illumination for 30 min with photoreactivating light removed at least 95% of the cyclobutane dimers. When this photoreactivated DNA was incubated with XP-A cell extract, incubation with *M. luteus* enzyme gave no stimulation of repair replication (Figure 4, right panel). The experiment shows that under conditions where extensive repair replication at cyclobutane dimers occurs, a large effect of

photoreactivation can be observed in the in vitro system. This suggests that there is no inherent limitation in the assay that prevents observation of extensive photoreactivation of repair replication.

Discussion

The experiments reported here indicate that UV photoproducts other than cyclobutane dimers [probably (6–4) photoproducts] initiate most of the in vitro repair replication reaction in human cell extracts.

Repair replication by the extracts is not significantly stimulated by the low number (about one per plasmid molecule at 400 J/m^2) of ring-saturated pyrimidine photoproducts present in the irradiated DNA. Several experiments indicate that this is the case. Most directly, removal of these products by treatment of the UV-irradiated DNA with Nth protein and repurification of intact molecules does not significantly alter the amount of repair replication in the extracts (Figure 1). Moreover, the same fraction of repair replication was photoreactivatable in experiments where the hydrated pyrimidines were removed by Nth treatment (Figure 2) and in experiments where they were not removed (Figure 3). Finally, both normal cells and cells of xeroderma pigmentosum origin are known to contain pyrimidine hydrate-DNA glycosylases, but XP cells are generally deficient in the repair replication mediated by cell-free extracts (Wood et al., 1988b).

The low contribution of pyrimidine hydrates to the total repair replication is probably ascribable to a relatively small patch size. Theoretically, excision of a damaged base by a glycosylase/endonuclease base excision repair mechanism requires the replacement of only one nucleotide. There is some evidence to suggest that patches resulting from base excision repair are indeed very short (Regan & Setlow, 1974). Nicking at pyrimidine hydrates does occur in the extracts (Wood et al., 1988a) but extensive nick translation does not occur. Although the contribution of pyrimidine hydrates to the total observed repair replication signal is very small, it should be emphasized that this is not the case if a direct assay for UV-dependent incision is used (Wood et al., 1988a). Experiments to directly detect the incision step at pyrimidine dimers using crude extracts require UV-irradiated DNA lacking pyrimidine hydrates, as previously noted (Wood et al., 1988a,b).

It is possible that conclusions drawn from the photoreactivatability of repair replication could be complicated by different DNA excision repair patch sizes for cyclobutane dimers and (6–4) photoproducts. Estimates of the repair patch length in the cell-free system give average sizes of 30–100 nucleotides per patch (Wood & Robins, 1989). At this point, experimental evidence does not suggest that different types of damage have large differences in nucleotide excision repair patch size.

(6–4) photoproducts are formed in UV-irradiated DNA with approximately one-third the yield of cyclobutane dimers (Mitchell, 1988). From the data in Table II, one can make an estimate of the relative efficiency of repair of (6–4) photoproducts and cyclobutane dimers in the extracts. At 100 J/m^2, where the fluence response for repair is in the linear region (Figure 1), complete photoreactivation of the cyclobutane dimers reduced the repair replication to 57% of the nonphotoreactivated value (Table II). Thus cyclobutane dimers (0.75 of the total photoproducts) account for 0.43 of the repair replication, while (6–4) photoproducts (0.25 of the total) account for 0.57 of the repair. Therefore, (6–4) photoproducts stimulate repair replication in the soluble extract about (0.57/0.25)/(0.43/0.75) = 4-fold more readily than cyclobutane dimers. If the fluence response curve is linear to 200 J/m^2, the relative preference for (6–4) photoproducts over cyclobutane dimers becomes (0.78/0.25/(0.22/0.75) = 12-fold.

A 4–12-fold preference for (6–4) photoproducts over cyclobutane dimers is not unreasonable because (6–4) photoproducts appear to be more avidly removed by DNA excision repair in human cells. The figures given by Mitchell (1988) indicate that human cells remove 50% of (6–4) photoproducts in 1 h after irradiation and 50% of cyclobutane dimers in 8 h; removal of both products is approximately linear during this time period, yielding a difference in rate of about 8-fold.

It is conceivable that the incision systems for cyclobutane dimers and (6–4) photoproducts are biochemically separable and that the repair system for cyclobutane dimers is compromised in cell extracts for some reason. For example, human cells might contain a factor that binds to cyclobutane dimers and enhances their recognition by the excision repair system in a way analogous to the stimulation of *E. coli* UvrABC by photolyase (Sancar et al., 1984). Such a factor could be present in limiting amounts in the cell extracts used in these experiments.

Repair of the two types of pyrimidine dimer photoproducts is separable to some extent in experiments with cultured cells. Cleaver et al. (1987) have studied DNA repair in a UV-resistant revertant cell line derived from XP complementation group A. This cell line has apparently normal survival characteristics after UV irradiation. The cells have regained the ability to repair (6–4) photoproducts but are still deficient in repair of cyclobutane dimers. In this situation, (6–4) pyrimidine dimers appear to be the most important photoproducts for determining cell survival after UV irradiation. A complementary example has been found for the Chinese hamster ovary cell mutant UV-61, which is defective in (6–4) photoproduct repair but proficient in cyclobutane dimer repair (Thompson et al., 1989). This mutant cell line is significantly less sensitive to UV light than mutant cell lines from other complementation groups that are defective in repair of both photoproducts.

Neither the above studies nor experiments with cell-free extracts are able to distinguish whether the same enzyme acts on both (6–4) photoproducts and cyclobutane dimers or whether there are two separate repair systems. Most UV-sensitive XP cells and Chinese hamster ovary cells are simultaneously deficient in repair of both photoproducts (Mitchell et al., 1985; Thompson et al., 1989). This fact and the Mendelian inheritance pattern of XP suggest that the same enzyme complex is responsible.

Acknowledgments

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Registry No. DNA photolyase, 37290-70-3.

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Sequence-Dependent Variations in the ^{31}P NMR Spectra and Backbone Torsional Angles of Wild-Type and Mutant *Lac* Operator Fragments†

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ABSTRACT: Assignment of the ^{31}P resonances of a series of six sequenced-related tetradecamer DNA duplexes, $d(TGTGAGCGCTCACA)_2$, $d(TATGAGCGCTCATA)_2$, $d(TCTGAGCGCTCAGA)_2$, $d(TGTGTGCGCACACA)_2$, $d(TGTGACGCGTCACA)_2$ and $d(CACAGTATACTGTG)_2$, related to the *lac* operator DNA sequence was determined either by site-specific ^{17}O labeling of the phosphoryl groups or by two-dimensional ^1H-^{31}P pure absorption phase constant time (PAC) heteronuclear correlation spectroscopy. $J(H3'-P)$ coupling constants for each of the phosphates of the tetradecamers were obtained from ^1H-^{31}P *J*-resolved selective proton flip 2D spectra. By use of a modified Karplus relationship the C4′-C3′-O3′-P torsional angles (ϵ) were obtained. Comparison of the ^{31}P chemical shifts and $J(H3'-P)$ coupling constants of these sequences has allowed greater insight into those various factors responsible for ^{31}P chemical shift variations in oligonucleotides and provided an important probe of the sequence-dependent structural variation of the deoxyribose phosphate backbone of DNA in solution. These sequence-specific variations in the conformation of the DNA sugar phosphate backbone of various *lac* operator DNA sequences can possibly explain the sequence-specific recognition of DNA by DNA binding proteins, as mediated through direct contacts between the phosphates and the protein.

Lac Operator. A number of studies have been carried out both in solution and in the solid state to gain a better understanding of binding interactions between regulatory proteins such as the *lac* repressor and DNA operator sequences. Most efforts directed toward defining the binding specificity between amino acid sequences and DNA sequences have centered on hydrogen bonding to the acceptor/donor groups on the Watson-Crick base pairs in the major groove [cf. Landschulz et al. (1988); see also the critical discussion in Matthews (1988)]. At present we do not understand this "second genetic code" of protein-DNA recognition. Perhaps one reason for the inability to dissect the basis for this specificity is the em-

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