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# UV Light-Induced Cyclobutane Pyrimidine Dimers Are Mutagenic in Mammalian Cells

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**We used a simian virus 40-based shuttle vector plasmid, pZ189, to determine the role of pyrimidine cyclobutane dimers in UV light-induced mutagenesis in monkey cells. The vector DNA was UV irradiated and then introduced into monkey cells by transfection. After replication, vector DNA was recovered from the cells and tested for mutations in its *supF* suppressor tRNA marker gene by transformation of *Escherichia coli* carrying a nonsense mutation in the β-galactosidase gene. When the irradiated vector was treated with *E. coli* photolyase prior to transfection, pyrimidine cyclobutane dimers were removed selectively. Removal of approximately 90% of the pyrimidine cyclobutane dimers increased the biological activity of the vector by 75% and reduced its mutation frequency by 80%. Sequence analysis of 72 mutants recovered indicated that there were significantly fewer tandem double-base changes and G · C → A · T transitions (particularly at CC sites) after photoreactivation of the DNA. UV-induced photoproducts remained (although at greatly reduced levels) at all pyr-pyr sites after photoreactivation, but there was a relative increase in photoproducts at CC and TC sites and a relative decrease at TT and CT sites, presumably due to a persistence of (6-4) photoproducts at some CC and TC sites. These observations are consistent with the fact that mutations were found after photoreactivation at many sites at which only cyclobutane dimers would be expected to occur. From these results we conclude that UV-induced pyrimidine cyclobutane dimers are mutagenic in DNA replicated in monkey cells.**

Genetic mutations appear to play a fundamental role in cellular transformation and carcinogenesis (1, 28) as well as in many inheritable diseases and developmental anomalies. Although numerous mutagenic agents are known to interact with DNA, the molecular mechanisms by which these agents may cause mutations in mammalian cells are not well understood. One of the most extensively studied mutagens, UV radiation, has been shown to cause mutations in both bacterial and mammalian cell systems (reviewed in reference 7), and its ability to induce skin cancers in animals is well established (reviewed in reference 31). Although many UV-induced photoproducts in DNA have been well characterized (3, 19), those responsible for mutagenesis have not been identified with certainty, particularly in mammalian systems. Furthermore, the molecular events that occur as a consequence of UV damage to the DNA and that lead ultimately to mutation fixation are not fully defined. To approach this problem, we used a simian virus 40 (SV40)-based shuttle vector to analyze the sequence specificity of point mutations induced in mammalian cells by UV-induced DNA damage (8, 30). We reasoned that by comparing the types and locations of UV photoproducts induced in the DNA to the types and sites of UV-induced mutations, we would be able to derive information on the specific photoproducts involved in mutagenesis and perhaps on the mechanism of mutagenesis itself.

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The two most prevalent (and best-characterized) UV-induced photoproducts in DNA are the cyclobutane pyrimidine dimer and the (6-4) photoproduct (3, 19). Both of these products involve dimerization of adjacent pyrimidines on the same DNA strand. Recently it was suggested, contrary to previous thinking, that the (6-4) photoproduct and not the cyclobutane pyrimidine dimer is responsible for the majority of UV-induced mutations (3). Support for this contention has been obtained from studies with bacteriophage lambda (34, 35), although in other bacterial systems cyclobutane pyrimidine dimers do appear to play a major role in UV mutagenesis (10, 11). To study this problem in a mammalian system, we used the *Escherichia coli* photolyase isolated by Sancar et al. (27) which specifically resolves pyrimidine cyclobutane dimers, leaving normal DNA structures without affecting (6-4) photoproducts (2). Thus, we can compare the type, location, and frequency of mutations in UV-irradiated DNA that has been treated with *E. coli* photolyase with the spectrum and frequency of mutations in untreated, UV-irradiated DNA (8).

For these experiments, we used the pZ189 shuttle vector plasmid (30) which contains the pBR327 replication functions and β-lactamase gene, together permitting growth and selection in bacterial cells, the SV40 early region, which permits replication in mammalian cells, and the *E. coli supF* suppressor tRNA gene, which serves as a mutagenesis target. The vector DNA is UV irradiated in vitro, treated with *E. coli* photolyase, and then transfected into mammalian cells. After DNA replication and mutagenesis occurs in these cells, the vector DNA is recovered and used to transform bacterial cells to test for mutations in the *supF*

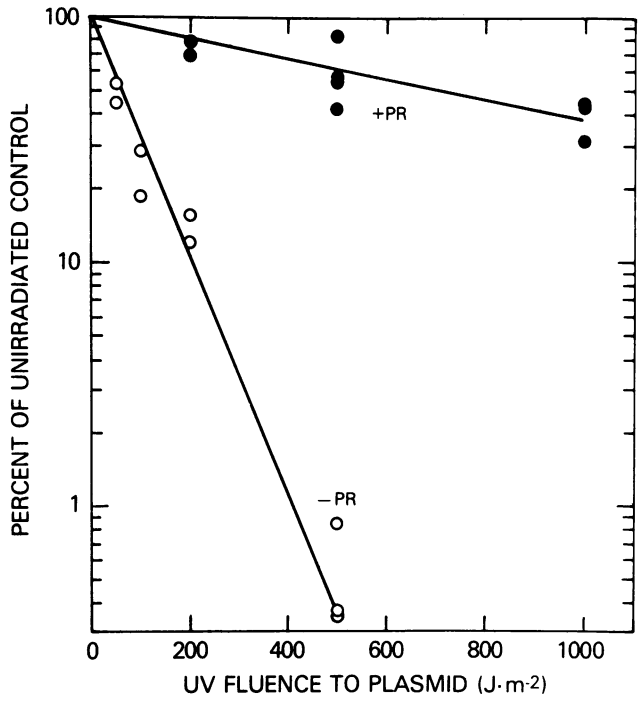


FIG. 1. Effect of photoreactivation on transformation efficiency of UV-irradiated pZ189. Plasmid pZ189 DNA was UV-irradiated and then treated with *E. coli* DNA photolyase as described in Materials and Methods. The DNA was then purified and used to transform competent *E. coli* MBM7070 cells, and the number of ampicillin-resistant transformed colonies was determined. Values are expressed as a percentage of the non-UV-irradiated control. Symbols: ●, with photoreactivation (PR); ○, without photoreactivation.

gene. The *supF* mutants recovered are then analyzed by DNA sequencing.

## MATERIALS AND METHODS

**Cells and plasmids.** The TC7 clone (25) of the African green monkey kidney cell line CV-1 was grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with antibiotics and 10% fetal calf serum. *E. coli* MBM7070 (30) has the genotype $F^-$ *lacZ*(Am)CA7020 *lacY1 hsdR*$^-$ *hsdM*$^+$ *araD139* Δ(*araABC-leu*)*7679 galU galK rpsL thi*. In the presence of isopropyl-β-D-thiogalactoside, an inducer of the *lac* operon, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside, an artificial substrate for β-galactosidase, strain MBM7070 forms blue colonies if it contains an active *supF* suppressor tRNA gene and white colonies if the suppressor is inactive. The plasmid pZ189 (30) and SV40 DNAs were purified by CsCl equilibrium sedimentation by Loftstrand Labs Ltd., Gaithersburg, Md.

**Mutation induction.** Purified plasmid DNA was irradiated with UV light (254 nm) as described previously (21) and then treated or not with *E. coli* photolyase (see below). Subconfluent TC7 cells growing in 100-mm plastic tissue culture dishes were transfected with the plasmid DNA (5 to 25 ng per dish) by the DEAE-dextran method (16). After 48 h, plasmid DNA was harvested from the cells by the Hirt method (9), purified, and treated with *Dpn*I (Bethesda Research Laboratories or New England Biolabs) to remove all nonreplicated DNA (20). After additional purification, the plasmid DNA was used to transform the bacterial tester strain. Usually 1/3 of the plasmid DNA isolated from one 100-mm tissue culture dish was used per transformation in a total volume of 6 μl per 100 μl of competent tester cells. At least 10 separate transfected dishes were used per UV dose, and at least two separate transformations were performed for each transfection.

**Photoreactivation.** Plasmid DNA was photoreactivated as described previously (21a). Briefly, untreated and UV-irradiated DNAs were mixed with *E. coli* photolyase (generously provided by A. Sancar) in a reaction buffer containing 50 mM Tris hydrochloride, pH 7.6, 10 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol (27). After incubation at 0°C for 30 min, the mixture was brought to room temperature and kept in the dark or exposed for 1 h to photoreactivating light (405 nm and 28 J/m$^2$ per s). The extent of photoreactivation was measured by the T4 endonuclease V assay.

**Mutant selection.** Transformation of bacteria, selection of colonies containing the *supF* mutant plasmid, purification of plasmid DNA from bacteria, and DNA sequencing methods have been described previously (8).

**T4 endonuclease V assay for pyrimidine dimers.** T4 endonuclease V was purified by the procedure of Seawell et al. (29) and produced less than 3% nicking of unirradiated DNA. After photoreactivation, plasmid DNA was phenol extracted and ethanol precipitated. The T4 endonuclease V assay was performed as previously described (21, 21a). The number of T4 endonuclease V-sensitive sites per plasmid molecule was calculated by assuming a Poisson distribution of nicks among the molecules. The $D_0$ (the dose that reduces the percentage of form I molecules by 63% along the exponential portion of the dose-response curve) represents the dose that yields an average of 1 T4 endonuclease V-sensitive site per molecule (21, 21a).

**Determination of sites of UV photoproducts.** The positions of UV photoproducts in the tRNA region of UV-irradiated plasmid DNA were determined by a DNA synthesis-arrest assay as described by Moore et al. (18). Two primers were used, the pBR322 *Eco*RI sequencing primer (P-L Biochemicals, Piscataway, N.J.), which hybridizes to positions 5479 through 5494 on the vector DNA (just ahead of position 5504/1) for rightward sequencing through the tRNA gene, and another primer (generously provided by M. Berman) that hybridizes to the opposite strand of the vector at positions 212 through 227 for leftward sequencing.

TABLE 1. T4 endonuclease V-sensitive sites in UV-irradiated pZ189 after treatment with *E. coli* photolyase

| UV fluence to plasmid (J/m$^2$) | Photoreactivation | | | |
|---|---|---|---|---|
| | Dark | | 405-nm UV light | |
| | % Form I[a] | Dimers/plasmid[b] | % Form I[a] | Dimers/plasmid[c] |
| 0 | 100 | 0 | 100 | 0 |
| 100 | 0 | 11 | 93 | 0.07 |
| 500 | 0 | 57 | 79 | 0.23 |
| 1,000 | 0 | 115 | 67 | 0.38 |

[a] Percent remaining after T4 endonuclease V treatment.

[b] Calculated from percent form I remaining at UV fluences up to 30 J/m$^2$ and $D_0$ = 8.7 J/m$^2$ for pZ189, assuming a linear relationship between UV fluence and dimers per plasmid at higher fluences.

[c] Calculated from percent form I remaining as: % form I remaining /100 = $e^{-(\text{dimers/plasmid})}$.

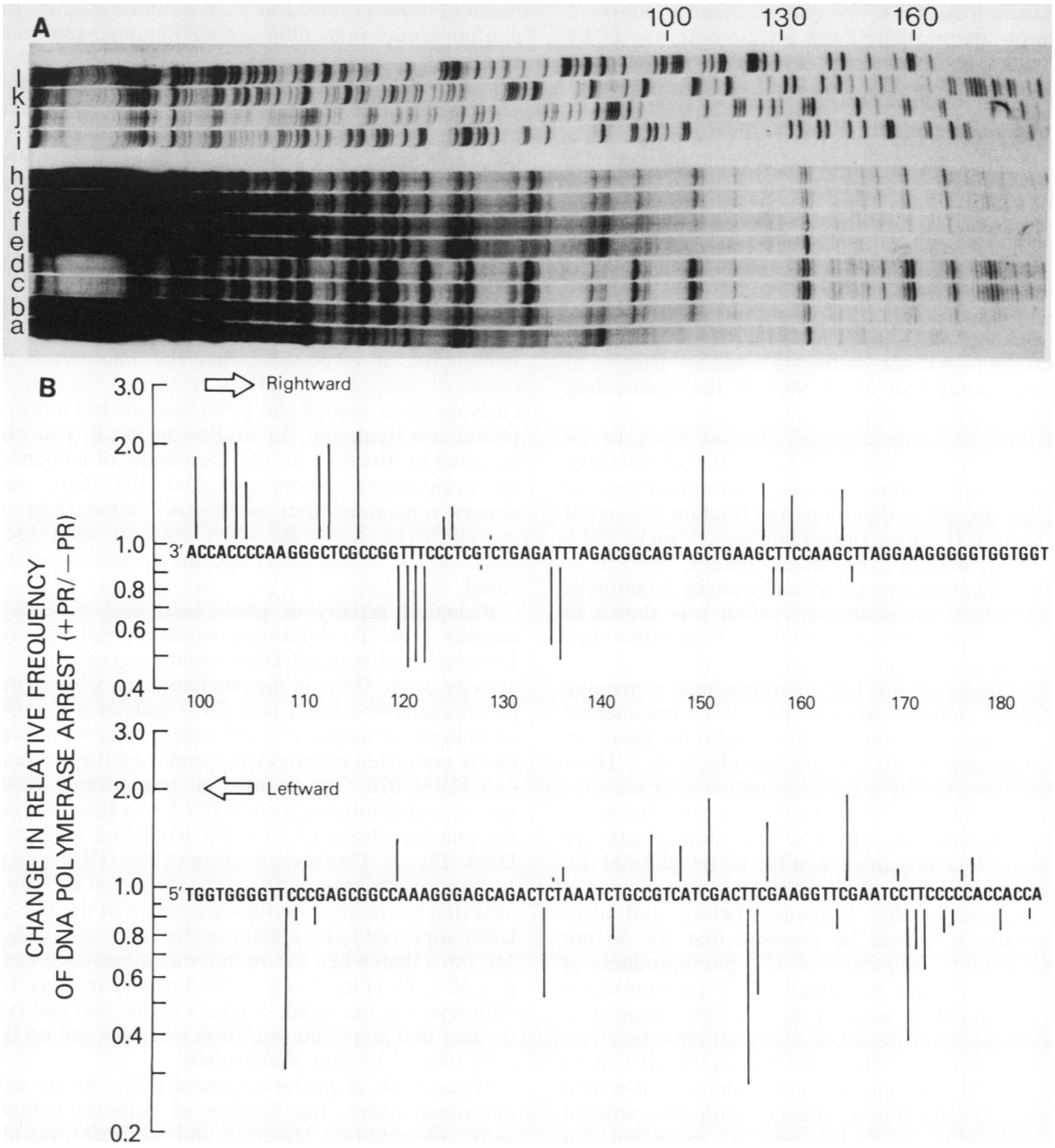


FIG. 2. Relative change in the frequency of UV photoproducts in the *supF* gene of pZ189 after photoreactivation. (Panel A) Polyacrylamide gel of DNA polymerase stop assay (lanes a through h) and DNA sequencing (lanes i through l) with leftward primer. Plasmid pZ189 DNA was UV-irradiated and then treated with *E. coli* DNA photolyase as described in Materials and Methods. The DNA was purified and used as a template for the DNA synthesis arrest assay. The newly synthesized DNA fragments were electrophoresed on a polyacrylamide gel. Lanes: a and e, no UV; b and f, 100 J/m$^2$; c and g, 500 J/m$^2$; d and h, 1,000 J/m$^2$; a–d, no photoreactivation; e–h, photoreactivation. DNA sequencing lanes: i, A; j, T; k, G; l, C. (Panel B) Autoradiograms of gels such as those shown in panel A were scanned by densitometry, and the relative frequency of UV photoproducts (500 J/m$^2$) at each pyr-pyr dinucleotide was then calculated, assuming that each arrest site (indicated by the positions of the vertical bars along the DNA sequence) occurs just 3′ to a pyrimidine dimer on the template DNA, and making corrections for the increase in labeling of longer DNA chains and the probability that more than one dimer occurs in the region of the template examined. The relative change in the frequency of UV photoproducts after photoreactivation (indicated by the lengths of the vertical bars) was calculated as the relative frequency of UV photoproducts at a particular site with photoreactivation divided by the relative frequency without photoreactivation. Arrows indicate the direction of DNA synthesis on the template strands shown.

## RESULTS

**Photoreactivation of plasmid DNA.** Our initial experiments were aimed at determining the extent and specificity of photoreactivation of the UV-irradiated plasmid DNA. By using a biological assay, Sancar et al. (27) have shown that the *E. coli* photolyase is capable of reversing at least 90% of the pyrimidine cyclobutane dimers in DNA irradiated at 200 J/m$^2$. Brash et al. (2) have shown that this enzyme is extremely specific for cyclobutane dimers and does not reverse (6-4) photoproducts. Three assays were used to determine the activity of the photolyase under our conditions. First, we measured the biological activity of the plasmid DNA in a bacterial transformation assay; second,

we measured the loss of T4 UV endonuclease V-sensitive sites; and third, we measured the positions in the DNA sequence at which photoproducts remained in a DNA polymerase stop assay (using avian myeloblastosis virus reverse transcriptase).

To assay the transforming efficiency of photoreactivated DNA, plasmid DNA was irradiated at several different UV fluences and then treated with *E. coli* photolyase as described in Materials and Methods. This DNA was used to transform *E. coli* MBM7070, and the efficiency of production of ampicillin-resistant colonies was measured. Photoreactivation efficiently restored the biological activity of the plasmid DNA (Fig. 1). This level of photoreactivation corresponds to a change in $D_0$ (the UV fluence that reduces the biological activity by 63% or yields one lethal hit) from 95 to 1,020 J/m$^2$ and removal of about 90% of the inactivating photoproducts.

The sensitivity of photoreactived plasmid DNA to T4 endonuclease V was assayed by treating the photoreactivated DNA with this enzyme and then electrophoresing the DNA on an agarose gel to determine the fraction converted from form I to form II. Since T4 endonuclease V nicks DNA at the sites of pyrimidine cyclobutane dimers, removal of these dimers by photoreactivation can be easily monitored. By this assay (Table 1), photoreactivation was shown to remove at least 99% of the T4 endonuclease V-sensitive sites on the plasmid DNA.

The site specificity of the UV photoproducts remaining after photoreactivation was assayed by a DNA polymerase arrest assay. Moore et al. (18) have shown that the positions and relative frequencies of UV photoproducts in a DNA template can be assayed by the determination of arrest sites for DNA synthesis in vitro. We have already used this assay to determine the sites at which UV photoproducts are formed in the *supF* gene sequence in the pZ189 plasmid (8). Thus, we can compare the sites and relative frequencies of UV photoproducts in this sequence before and after photoreactivation. It should be stressed that we do not measure the absolute frequency of UV photoproducts at each site, only the relative frequency of UV photoproducts at one site compared to another in the DNA sequence. However, an estimate of the level of overall photoreactivation of lesions that cause arrest of DNA synthesis can be made by comparing the amount of high-molecular-weight DNA migrating at the top of the gel with and without photoreactivation (Fig. 2). In this way we estimated that photoreactivation reversed approximately 90% of the UV photoproducts. This estimate is in good agreement with the results of the biological assay (Fig. 1). In Fig. 2 we have plotted the change in relative frequency with which the DNA polymerase was arrested at the site of each photoproduct, on each of the two DNA strands of the tRNA coding region of the plasmid, as a consequence of photoreactivation. It is clear that photoreactivation of the DNA preferentially removed UV photoproducts at some sites on the DNA sequence. In general, there was a relative increase in photoproducts at TC and CC sites and a decrease at CT and TT sites (Table 2). However, UV photoproducts remained at all pyr-pyr sites in the sequence, and the photoproducts remaining did not appear to be restricted to (6-4) photoproducts. By using an independent assay in which alkali-labile sites were measured in end-labeled DNA (3), Brash and Seidman (personal communication) determined the positions at which (6-4) photoproducts are formed in the *supF* DNA sequence; these sites are predominantly TC and CC sequences, with no CT or TT sites detectable at the UV fluence used (1,000 J/m$^2$). We assume that the other photoproducts remaining are cyclobutane pyrimidine dimers.

From these three assays we conclude that the *E. coli* photolyase reverses cyclobutane pyrimidine dimers in the plasmid DNA with an efficiency of at least 90%. The reversal does not appear to be entirely random, since more dimers remained at some sites than at others. Since the *E. coli* photolyase resolves only cyclobutane dimers (2), other photoproducts that remain in the DNA may account for the difference between the results of the biological assay and the T4 endonuclease V assay. However, it may also be that the T4 endonuclease V assay is giving an overestimate of the reversal of cyclobutane pyrimidine dimers by the *E. coli* photolyase. It is possible that the photolyase treatment rendered the plasmid DNA refractory to endonuclease cleavage even though the DNA was phenol extracted after photolyase treatment. In studies on the *E. coli* photolyase reported by Brash et al. (2), the results of a chemical assay for cyclobutane dimers indicated that there were more dimers remaining after photoreactivation than were observed in an assay in which specific cleavage by the *Micrococcus luteus* dimer-specific endonuclease was measured.

**Biological activity of photoreactivated plasmid DNA in monkey cells.** To determine whether photoreactivation of UV-irradiated plasmid DNA would increase the biological activity of the DNA in mammalian cells, we transfected the photoreactivated DNA into CV-1 cells at a low DNA concentration (25 ng per $2 \times 10^6$ cells). After 48 h, we isolated newly replicated plasmid DNA and used this to transform *E. coli* MBM7070. The number of transformed colonies observed with photoreactivated DNA was then compared with the number observed with UV-irradiated and unirradiated DNA (Fig. 3). Photoreactivation of the UV-irradiated DNA restored much (about 75%) of the biological activity. We also note that the relative biological activity of the UV-irradiated DNA appeared to be greater in the CV-1 cells (Fig. 3; $D_0$ = 340 J/m$^2$) than when the vector was introduced directly into *E. coli* cells (Fig. 1; $D_0$ = 95 J/m$^2$). This may be due to differences in the repair capacity of the two cell types or to the fact that ampicillin selection was imposed on the *E. coli* cells soon (1 h) after transfection.

**Mutagenesis of photoreactivated DNA.** In the experiment described above, the fraction of colonies containing the *supF*$^-$ plasmid (see Materials and Methods) was also determined for photoreactivated and unreactivated DNA (Table 3). The observed mutation frequency decreased (by about 80%) concomitantly with the increase in biological activity of the photoreactivated plasmid. Thus, the pyrimidine cyclobutane dimers that were removed by photoreactivation appeared to contribute to both the biological activity and mutagenesis of the plasmid in CV-1 cells.

To confirm this conclusion, it was necessary to rule out the possibility that the apparent reduction in mutagenesis was due to failure to induce a hypothetical SOS-type response in the CV-1 cells. If, as suggested by the work of Cornelis et al. (5), transfection of mammalian cells with UV-damaged DNA induces a mutagenic recovery pathway like the SOS pathway in bacterial cells (33), and the induction of this pathway is responsible for UV mutagenesis, then reducing the amount of UV damage in the transfected DNA by photoreactivation might reduce the induction of this pathway and lead to an apparent reduction in mutation frequency. To test this possibility, we cotransfected CV-1 cells with UV-irradiated SV40 DNA and photoreactivated

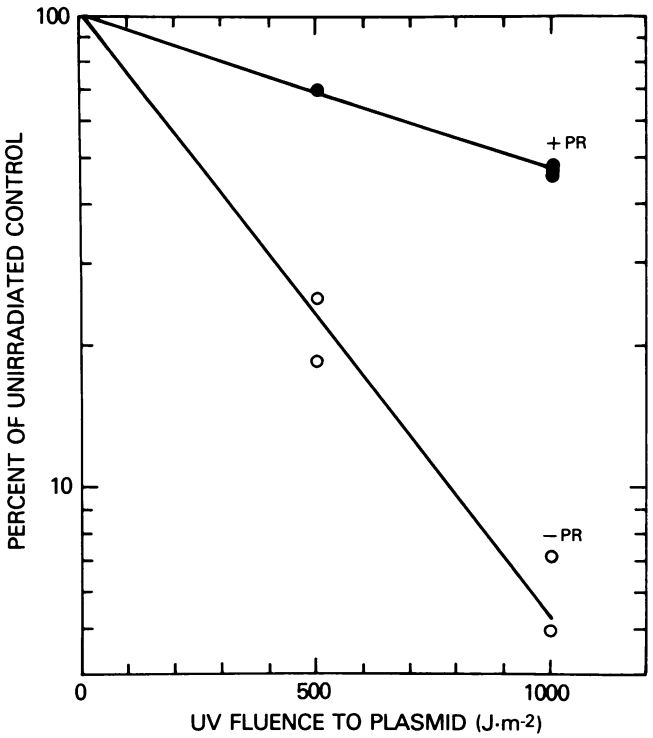


FIG. 3. Effect of photoreactivation on transfection efficiency of UV-irradiated pZ189 in CV-1 cells. Plasmid pZ189 DNA was UV-irradiated and then treated with *E. coli* DNA photolyase as described in Materials and Methods. The DNA was then used to transfect CV-1 cells. After 48 h, plasmid DNA was extracted from the CV-1 cells, purified, and used to transform *E. coli* MBM7070. The yield of transformed bacterial colonies was used as a measure of the transfection efficiency of the plasmid in CV-1 cells. Symbols: ●, with photoreactivation (PR); ○, without photoreactivation.

plasmid DNA. Inclusion of the damaged SV40 DNA in the transfection did not alter the reduction in mutation frequency observed when the plasmid DNA was photoreactivated (Table 4).

**Spectrum of mutations observed with photoreactivated DNA.** Seventy-two of the *supF* mutants recovered from transfections of CV-1 cells with photoreactivated UV-irradiated DNA were analyzed by DNA sequencing. The types of changes observed in the 160-base-pair segment sequenced included single-base substitutions (38 mutants), tandem double-base substitutions (2 mutants), multiple mutations with two changes (8 mutants), multiple mutations with more than two changes (11 mutants), and deletions (13 mutants). Specific base substitutions are listed in Table 5, and the positions of the base changes are shown in Fig. 4. In Fig. 5 the single and tandem double-base changes that occurred within the tRNA coding sequence are compared

TABLE 2. Change in relative frequency of dimers at specific sequences after photoreactivation

| DNA synthesis direction | Mean frequency of dimer sequence[a] | | | |
|---|---|---|---|---|
| | CC | TC | CT | TT |
| Rightward | 1.42 ± 0.26 | 1.46 ± 0.13 | 0.98 ± 0.24 | 0.60 ± 0.06 |
| Leftward | 0.96 ± 0.11 | 1.24 ± 0.14 | 0.65 ± 0.19 | 0.54 ± 0.13 |

[a] Geometric mean of relative dimer frequency with photoreactivation divided by relative frequency without photoreactivation, ± standard error of the geometric mean. The UV fluence was 500 J/m².

TABLE 3. Effect of photoreactivation on mutation frequency of pZ189 plasmid in monkey cells

| UV fluence to plasmid (J/m²) | Photoreactivation | | | |
|---|---|---|---|---|
| | No | | Yes | |
| | No. of *supF*⁻ colonies/total | % Mutants | No. of *supF*⁻ colonies/total | % Mutants |
| 0 | 17/49,724 | 0.034 | 7/56,879 | 0.012 |
| 500 | 233/22,295 | 1.1 | 93/47,122 | 0.20 |
| 1,000 | 84/4,695 | 1.8 | 49/13;103 | 0.37 |

with those obtained previously with nonphotoreactivated DNA (8); an additional seven single-base changes were observed in the upstream noncoding region near the promotor (two each at positions 43 and 46 and one each at positions 45, 50, and 65). As observed with UV-irradiated plasmid DNA that had not been photoreactivated (8), some of the mutants contained multiple mutations. Since, in the photoreactivated DNA, the induced mutation frequency is 5- to 10-fold higher than the spontaneous background, some of these may actually be spontaneous mutations. Most of the spontaneous mutants that we sequenced contained multiple mutations (8). A striking difference between the photoreactivated and nonphotoreactivated DNA mutation spectra was that very few tandem double mutations (5% of single-site mutations) were observed after photoreactivation, whereas 23% of the UV-induced single-site mutants contained tandem double mutations (mostly at CC sites). This difference was statistically significant at the 0.05 level (chi-squared with 1 degree of freedom, 5.4). There were few major differences in the site specificity of the single-base substitutions induced in photoreactivated and nonphotoreactivated DNA (Fig. 5), although there appeared to be a reduction in the frequency of mutations around position 120 as a result of photoreactivation. However, there was a marked change in the types of base substitutions observed. There was a significant reduction in the frequency of the G · C → A · T transition after photoreactivation (chi-squared with 1 degree of freedom, 7.6; $P < 0.01$) and a concomitant increase in transversion mutations (Table 5). This change in base specificity was particularly striking at position 169 (Fig. 6).

## DISCUSSION

We used an SV40-based shuttle vector system to demonstrate that UV-induced cyclobutane pyrimidine dimers are mutagenic in DNA replicated in mammalian cells. Treatment of UV-irradiated vector DNA with purified *E. coli* photolyase before transfection into mammalian cells increased the biological activity and decreased the mutation frequency of the vector. Cotransfection of the cells with UV-irradiated SV40 DNA that had not been photoreacti-

TABLE 4. Effect of cotransfection with UV-irradiated SV40 DNA on pZ189 mutagenesis[a]

| Treatment of pZ189 DNA | No. of mutants/ total colonies | Mutant frequency (%) |
|---|---|---|
| No UV | 2/12,971 | 0.015 |
| UV (500 J/m²) | 53/5,810 | 0.91 |
| UV (500 J/m²) + PR[b] | 26/16,006 | 0.16 |

[a] CV-1 cells (2 × 10⁶) were transfected with 25 ng of pZ189 DNA and 100 ng of SV40 DNA (UV-irradiated to 500 J/m²).
[b] PR, Photoreactivation.

TABLE 5. Characteristics of *supF* mutants[a]

| Base substitution | No. of mutants with substitution | | | |
|---|---|---|---|---|
| | All mutations | | Single and tandem double mutations only | |
| | +PR[b] | −PR[c] | +PR | −PR[c] |
| Transitions | | | | |
| G·C→A·T | 54 | 90 | 15 | 55 |
| A·T→G·C | 5 | 11 | 5 | 5 |
| Transversions | | | | |
| G·C→T·A | 20 | 19 | 10 | 8 |
| G·C→C·G | 11 | 8 | 3 | 4 |
| A·T→C·G | 3 | 6 | 3 | 5 |
| A·T→T·A | 9 | 14 | 6 | 10 |

[a] UV fluences used were 500 and 1,000 J/m$^2$.
[b] PR, Photoreactivation.
[c] Data are from reference 8.

vated did not alter the effects of photoreactivation of the vector DNA; therefore, the effect of photoreactivation cannot be explained by the indirect effects of reducing the induction of a hypothetical SOS-like reactivation mechanism in the mammalian cells. Although pZ189 and other vector systems (4, 8, 12, 13, 17, 23, 24, 30) have been used to analyze mutations induced in mammalian cells, this is the first demonstration of the role of a specific UV photoproduct, the cyclobutane pyrimidine dimer, as a premutagenic lesion in mammalian cells.

Experiments with lambda phage in *E. coli* have suggested that, at least in this system, cyclobutane pyrimidine dimers are not the principal premutagenic photoproduct (34; 35). When phage were irradiated with 313-nm light in the presence of acetaphenone to increase the relative frequency of cyclobutane dimers, the mutation frequency was decreased (35). In addition, photoreactivation of UV-irradiated lambda DNA in vitro before transformation did not substantially reduce the observed mutation frequency (34). However, when photoreactivation of an *E. coli* F factor was allowed to occur in vivo before transfer to a recipient *E. coli* strain, a substantial reduction in mutation frequency was observed (10, 11), even when the recipient strain was maximally induced for SOS functions (10). Thus, in this *E. coli* system, pyrimidine cyclobutane dimers do appear to be mutagenic. Glickman et al. (B. W. Glickman, R. M. Schaaper, W. A. Haseltine, R. L. Dunn, and D. E. Brash, Proc. Natl. Acad. Sci. USA, in press) have demonstrated that (6-4) photoproducts are also mutagenic in this system by methylating cytosine residues in DNA before UV irradiation. This procedure renders the DNA refractory to formation of cyclobutane dimers at the methylated sites but not to (6-4) photoproducts; however, mutagenesis is still observed. As we have discussed previously (8), the observation that many mutational hotspots occur at G · C base pairs rather than A · T base pairs, where the majority of cyclobutane dimers are formed (3), can be explained on the basis of the propensity of procaryotic DNA polymerases to insert an A opposite UV photoproducts or other sites of damage in the template DNA. Thus, base misincorporation is less likely to occur opposite a dimerized T.

We also determined the site specificity of mutations induced with and without photoreactivation of UV-irradiated vector DNA and compared these results with the site specificity of UV photoproducts remaining in the DNA after photoreactivation. We found that photoreactivation of the UV-irradiated DNA did not cause a major change in the site specificitiy of mutagenesis we observed in our system. However, although photoreactivation of the DNA removed at least 90% of cyclobutane dimers, it did not cause major changes in the site specificity of UV photoproducts remaining in the DNA, at least when these sites were determined by the DNA synthesis stop assay. There did appear to be a relative increase in the frequency of photoproducts at some CC and TC sites [presumably due at least in part to the persistence of the (6-4) photoproducts at these sites] and a relative decrease at CT and TT sites; nevertheless, photoproducts remained at most pyr-pyr sites. It is noteworthy that the sites at which mutations were reduced by photoreactivation corresponded, in general, to sites at which

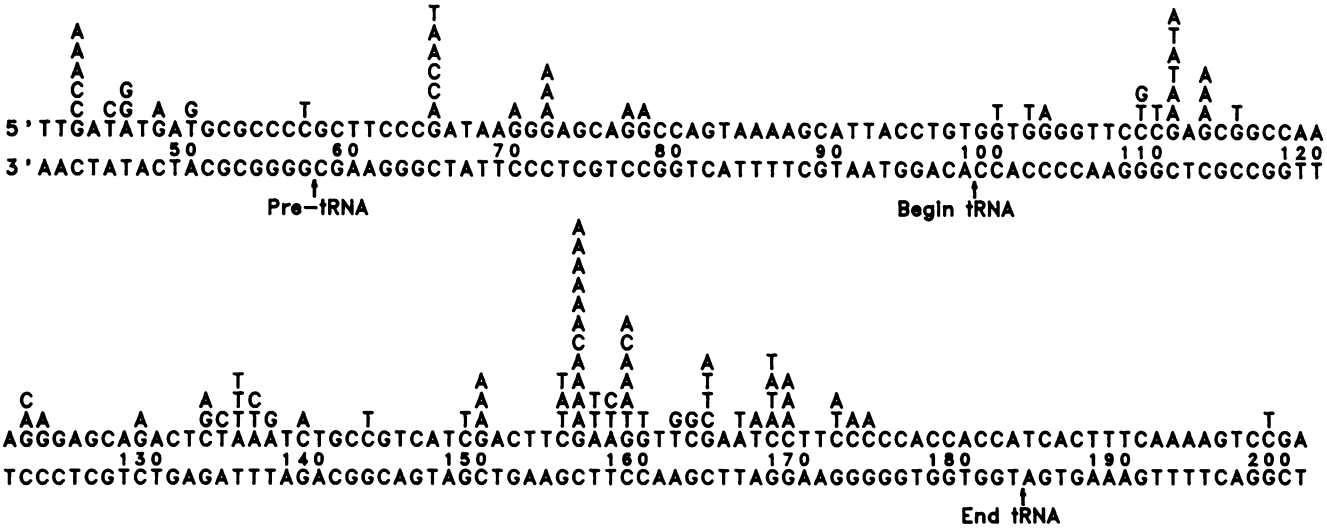


FIG. 4. Distribution of mutations in the tRNA region of pZ189. *supF* mutants induced in UV-irradiated (500 and 1,000 J/m$^2$), photoreactivated pZ189 DNA were sequenced in the region of the *supF* suppressor tRNA gene (from base 40 to base 200 on the pZ189 genome [31]). Base changes are indicated by letters above the strand sequenced. The figure was prepared with the use of the DNADRAW program (32).

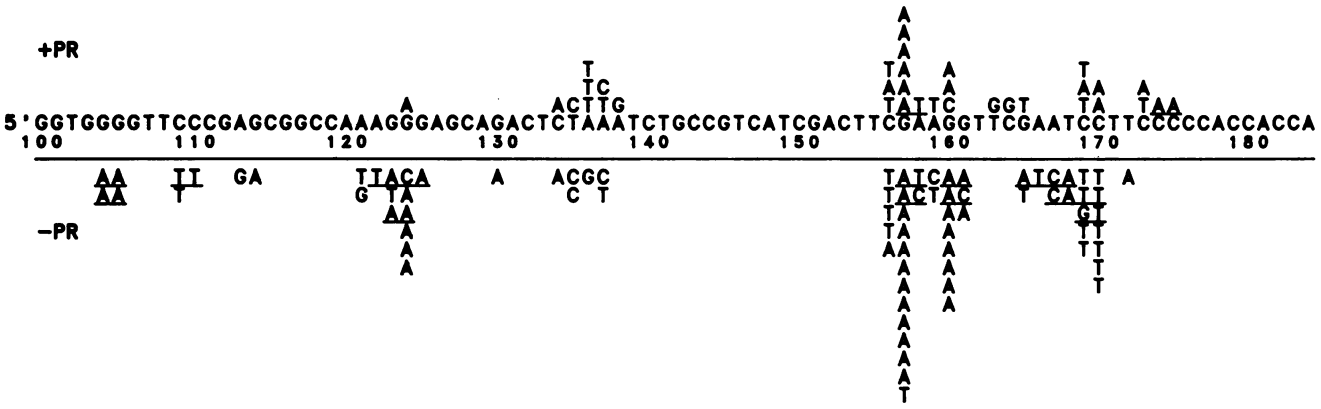


FIG. 5. Positions of single and tandem double-base changes within the *supF* tRNA coding sequence of pZ189 induced by UV radiation (500 and 1,000 J/m$^2$), with and without photoreactivation (PR). Mutational changes observed in mutants with single and tandem double-base changes induced by UV radiation followed by photoreactivation (+PR) or by UV radiation alone (−PR) (data are from reference 8). Tandem double mutations are underlined. The figure was prepared with the use of the DNADRAW program. (32).

the relative frequency of UV photoproducts was reduced (e.g., positions 120 through 124). We assume that many of the photoproducts that remain are cyclobutane pyrimidine dimers despite the failure of the T4 endonuclease V to detect them. Certainly, it is not unreasonable to expect that photoreactivation of pyrimidine cyclobutane dimers would be incomplete. Furthermore, Brash et al. (2) have shown directly by a chemical assay that some cyclobutane pyrimidine dimers remain after photoreactivation.

Photoreactivation of the vector DNA did cause significant changes in the types of base changes observed. There was a marked reduction in the frequency of tandem double-base changes and in the relative frequency of the G · C → A · T transition. These two alterations may indicate that cyclobutane dimers are principally responsible for these types of mutational changes and that (6-4) photoproducts may be more likely to cause single-base transversions. Indeed, it has been postulated that the 5′ base of the (6-4) photoproduct may be able to base-pair almost normally (6).

In summary, we used a shuttle vector system to demonstrate that cyclobutane pyrimidine dimers are mutagenic in DNA replicated in monkey cells. This work is a further demonstration of the utility of shuttle vector systems for investigating the mechanisms of mutagenesis in mammalian cells.

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