

- Eoyang, L., & August, J. T. (1971) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) Vol. 2, pp 829-839, Harper and Row, New York.
- Holmes, D. S., & Quigley, M. (1981) *Anal. Biochem.* 114, 193-197.
- Kramer, F. R., & Mills, D. R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 5334-5338.
- Kramer, F. R., Mills, D. R., Cole, P. E., Nishihara, T., & Spiegelman, S. (1974) *J. Mol. Biol.* 89, 719-736.
- McAllister, W. T., & McCarron, R. J. (1977) *Virology* 82, 288-298.
- McAllister, W. T., Morris, C., Rosenberg, A. H., & Studier, F. W. (1981) *J. Mol. Biol.* 153, 527-544.
- McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol.* 110, 119-146.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res.* 12, 7035-7056.
- Mills, D. R., & Kramer, F. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 2232-2235.
- Oakley, J. L., & Coleman, J. E. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4266-4270.
- Oakley, J. L., Strothkamp, R. E., Sarris, A. H., & Coleman, J. E. (1979) *Biochemistry* 18, 528-537.
- Rosa, M. D. (1979) *Cell (Cambridge, Mass.)* 16, 815-826.
- Sanger, F., & Coulson, A. R. (1978) *FEBS Lett.* 87, 107-110.
- Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467.
- Stahl, S. T., & Chamberlin, M. T. (1977) *J. Mol. Biol.* 112, 577-601.
- Studier, F. W. (1969) *Virology* 39, 562-574.
- Studier, F. W. (1975) *J. Bacteriol.* 124, 307-316.
- Studier, F. W. (1979) *Virology* 95, 70-84.
- Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* 43, 77-90.
- Walton, E., Holly, F. W., Boxer, G. E., & Nutt, R. F. (1965) *J. Am. Chem. Soc.* 8, 659-663.
- Walton, E., Holly, F. W., Boxer, G. E., & Nutt, R. F. (1966) *J. Am. Chem. Soc.* 31, 1163-1169.
- Wieslander, L. (1979) *Anal. Biochem.* 98, 305-309.

Cyclobutane Pyrimidine Dimers and (6-4) Photoproducts Block Polymerization by DNA Polymerase I[†]

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ABSTRACT: Bipyrimidine cyclobutane dimers and 6-4'-(pyrimidin-2'-one)-pyrimidine photoproducts are the major adducts formed in DNA following exposure to ultraviolet light. The relationship between the type and frequency of UV-induced DNA damage and the effects of such damage on DNA replication were investigated. UV-irradiated M13 phage DNA was employed in polymerization reactions with the Kenow fragment of *Escherichia coli* DNA polymerase I. The locations and frequencies of polymerase termination events occurring within a defined sequence of M13 DNA were compared with measurements of the locations and frequencies of UV-induced DNA damage of the same DNA sequence by using UV-specific enzymatic and chemical methods. The results indicate that both cyclobutane dimers and (6-4) photoproducts quantitatively block polymerization by DNA polymerase I.

Damage to DNA may have lethal, mutagenic, and carcinogenic consequences (Hanawalt et al., 1978; Friedberg & Bridges, 1983). One event central to such biological effects is DNA replication on a damaged template. Failure to replicate past a site of DNA damage will lead to cell death. Advances in nucleic acid technology make it possible to analyze, at the DNA sequence level, events that occur when DNA polymerases encounter sites of DNA damage (Moore & Strauss, 1979; Moore et al., 1981).

We have used ultraviolet light induced DNA damage as a model for such studies. At low UV doses (10-500 J/m²) the major UV-induced DNA photoproducts are cyclobutane py-

rimidine dimers (Lippke et al., 1981). If unrepaired, a single cyclobutane dimer constitutes a lethal event in bacterial or phage genomes (Howard-Flanders & Boyce, 1966; Radman et al., 1978; Benbow et al., 1974). These observations suggest that cyclobutane dimers, under some circumstances, may constitute quantitative blocks to DNA replication. Although studies have suggested that cyclobutane pyrimidine dimers inhibit polymerization by several different DNA polymerases (Moore & Strauss, 1979; Yoshida et al., 1981; Miyaki et al., 1983), precise quantitative measures of termination events at individual dimer sites have not yet been made.

Another class of UV-induced DNA lesions, (6-4) photoproducts,¹ have been described (Lippke et al., 1981; Wang, 1976; Franklin et al., 1982). These photoproducts also form between adjacent pyrimidines, but the structure of these lesions

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¹ Abbreviations: (6-4) photoproducts, 6-4'-(pyrimidin-2'-one)-pyrimidine class of ultraviolet light induced photoproducts; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

differs substantially from that of cyclobutane pyrimidine dimers (Haseltine, 1983). Although these lesions comprise only about 10% of the total UV light induced damage at low fluences, the frequency of formation of the (6-4) photoproducts at some positions exceeds that of the cyclobutane dimers (Brash & Haseltine, 1982). A good correlation between the frequency of formation of the (6-4) photoproducts with the frequency of UV-induced C to T transition mutations has been observed for UV-induced mutations in the *lac I* gene of *Escherichia coli* (Brash & Haseltine, 1982; Wood et al., 1984). Events that occur when DNA polymerases encounter (6-4) photoproducts have not been previously examined.

Here we investigate events that occur when a UV-irradiated DNA template is used in polymerization reactions with the Klenow fragment of *E. coli* DNA polymerase I. The strategy of these experiments was to compare the frequency of polymerase termination events with the frequency of UV-induced damage at specific sites within a defined sequence DNA fragment. The results of these experiments show that both cyclobutane dimers and (6-4) photoproducts may quantitatively block DNA polymerization.

MATERIALS AND METHODS

DNA Templates. The single-stranded coliphage M13 was used as template for in vitro DNA synthesis. A portion of the *lac Z* gene and a DNA fragment that contains a number of different restriction endonuclease recognition sequences was inserted into the original M13 phage (Messing et al., 1977, 1981). Two such constructions of M13 were used in the present study: mp2 and mp8 which contain short insert regions of 39 and 32 bases, respectively. The insert regions possess several unique restriction sites. These phages were propagated in *E. coli* JM103 in YT medium under forced aeration. Phage DNA was obtained by precipitation of the culture supernatant with 4% polyethylene glycol and 0.4 M NaCl and repeated extraction with phenol, chloroform, and isoamyl alcohol (20:19:1).

UV Irradiation. UV irradiation was done on ice with the DNA dissolved in buffer containing 0.01 M Tris-HCl, pH 7.5, and 0.001 M EDTA. A germicidal lamp was used as the light source. The dose rate was 7.2 W/m² as measured by a Lartjet meter.

In Vitro DNA Synthesis. A synthetic 17-mer primer (Collaborative Research, Waltham, MA) that anneals to a unique position on the M13 phage was labeled at the 5' terminus by using the forward kinase reaction with T4 polynucleotide kinase (Bethesda Research Laboratory) and [γ -³²P]ATP (New England Nuclear) according to Maxam & Gilbert (1981). For each polymerization reaction, 10⁻¹³ mol of labeled primer was annealed to 1 μ g of UV-irradiated M13 phage by mixing in 2.5 μ L of buffer H (0.007 M Tris-HCl, pH 7.5, 0.05 M NaCl, 0.007 M MgCl₂, and 0.001 M EDTA), heating for 3 min at 90 °C, and cooling slowly to room temperature. The reaction was initiated by the addition of 3 μ L of buffer H containing 0.1 mM each of dATP, dCTP, dGTP, and TTP (P-L Biochemicals) and 0.4 unit of the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs). The reaction was carried out at room temperature for 15 min and terminated by heating at 90 °C for 1 min. After the addition of 1.5 μ L of a mix containing glycerol, 6 N NaOH, 1% bromophenol blue, and 1% xylene cyanol (35:3:1:1) and heating for 3 min at 90 °C, the total reaction mix was loaded on to a 20% polyacrylamide gel containing 7 M urea. Electrophoresis was carried out at 700 V.

For dideoxy sequencing reactions (Sanger et al., 1977), the reaction conditions were the same as above except that un-

irradiated phage were used as templates. The final concentration of each dideoxynucleoside triphosphate was 75 μ M. The mixture of deoxy- and dideoxynucleotide triphosphates were obtained from New England Biolabs.

Quantitation of Polymerase Stops on UV-Irradiation Templates. For quantitation of each polymerase stop on an UV-irradiated template, the gel slice corresponding to each band was excised, and the radioactivity contained in it was assayed by Cerenkov counting. In order to correct for the bias introduced by polymerase stops proximal to the primer, the true stop frequency at any given band was calculated by using a correction factor. The derivation of this factor has been previously described (Gordon & Haseltine, 1980, 1982).

Quantitation of Cyclobutane Dimers. A restriction fragment of the M13 genome containing the region that was used as a template for the polymerase studies was prepared by cleavage of the M13 RF with BstN1 (New England Biolabs). The 3' termini of the BstN1 restriction fragments were labeled by incubations in reactions that contained the Klenow fragment of *E. coli* DNA polymerase I and [α -³²P]dTTP. The labeled DNA was cut by incubations with *Hpa*II (mp2) or *Alu*I (mp8). The labeled fragments were separated on an 8% nondenaturing polyacrylamide gel and recovered by elution from the gel matrix followed by ethanol precipitation. For mp2, the fragment studied was 148 base pairs long, extending from base 6159 to base 6268 as previously described (Van Weezenbeck et al., 1980). For mp8, the fragment of interest was 94 base pairs (positions 6213-6307). Each of these fragments was labeled only at the 3' terminus of the plus strand.

To quantitate dimer induction, the UV-irradiated restriction fragments were treated with endonuclease V from bacteriophage T4 (gift of Pat Sewall, Stanford University). The enzyme was purified through the phosphocellulose step as previously described (Sewall et al., 1981). Saturating amounts of T4 endonuclease V were used for cleavage of dimers occurring in double-stranded DNA. Increasing the amount of enzyme did not result in an increase in the amount of DNA scission observed. The reaction was carried out in 20 μ L of buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl, 1 mg/mL bovine serum albumin, 0.2 unit of endonuclease V [1 unit being able to nick (2-4) \times 10¹² dimers in 1 min at 37 °C], and 3 ng of irradiated DNA. After a 30-min incubation at 37 °C, the samples were treated with proteinase K and phenol extracted, and the DNA was precipitated with ethanol, resuspended in 80% formamide, and loaded onto 20% polyacrylamide gels containing 7 M urea. The location of each potential site of dimer formation was determined by parallel sequencing reactions using the method of Maxam & Gilbert (1980). After electrophoresis, the radioactivity contained in each band was determined as described above. A correction factor was applied to obtain the true frequency of incision at each site of dimer formation (Gordon & Haseltine, 1980, 1982).

Quantitation of (6-4) Photoproduct. Single-stranded restriction fragments were prepared by cleaving the M13 genome with *Hae*III followed by labeling the 3' termini with terminal deoxynucleotidyltransferase and [α -³²P]dATP. The labeled fragments were separated on 8% polyacrylamide gels. For mp2, the fragment of interest was 203 bases long (positions 6037-6240). For mp8, the fragment of interest was 236 bases (positions 6037-6273).

After UV irradiation, the labeled fragments were treated with hot alkali and subjected to electrophoresis as described (Lippke et al., 1981). Quantitation of breaks at each site of

(6-4) adduct formation was the same as described above for dimer quantitation.

Digestion of UV-Irradiated DNA with T4 DNA Polymerase (3'-5') Exonuclease. DNA samples were suspended in 22.5 μ L of T4 DNA polymerase (3'-5') exonuclease assay buffer (33 mM Tris-acetate, pH 7.8, 10 mM Mg(OAc)₂, 66 mM KOAc, 0.5 mM dithiothreitol, and 0.1 mg/mL BSA) and incubated with 5 units (2-5 μ L) of T4 DNA polymerase (200 units/mL final enzyme concentration) for 120 min at 37 °C. The reaction was terminated and the samples were processed as described above for T4 endonuclease V digestions.

RESULTS

What is the frequency of polymerase termination when DNA polymerase encounters UV-induced cyclobutane dimers and (6-4) photoproducts? Previous studies show that *E. coli* DNA polymerase I, under specific conditions, terminates synthesis either one nucleotide before or at the site of UV photoproducts (Moore & Strauss, 1979). However, no studies to determine the individual contributions of cyclobutane dimers and (6-4) photoproducts to polymerase blockage at individual damage sites has been made.

To address these questions, we have measured the frequency of premature termination events that occur as the Klenow fragment of *E. coli* DNA polymerase I, initiated with the synthetic oligonucleotide primer, replicates along a UV irradiated single-stranded DNA template. The frequency of polymerase termination was then compared with the frequencies of photoproduct formation. The strategy of these experiments requires quantitative measurements of cyclobutane dimers and (6-4) photoproducts occurring on the same fragment of UV-irradiated single-stranded DNA.

Enzymatic and chemical treatments of 3'-end-labeled, UV-irradiated DNA result in the production of short fragments of length extending to the site of photoproduct formation. The nucleotide locations of both cyclobutane dimers and (6-4) photoproducts were determined by comparison of the electrophoretic mobilities of the chemical scission or enzyme digestion products (gel bands) with the product of DNA-sequencing reactions performed on the same, but unmodified, DNA fragment. The extent of photoproduct formation at each position was calculated by measurement of the amount of radioactivity of the individual fragments excised from the gel.

The treatment of single-stranded DNA with hot alkali results in quantitative cleavage at sites of all (6-4) photoproducts (Lippke et al., 1981). In contrast, determination of cyclobutane dimers in single-stranded DNA is not straightforward as single-stranded DNA is a poor substrate for UV-specific endonucleases that cleave DNA at dimer sites. Quantitative measurements of cyclobutane dimers occurring in single-stranded DNA must be obtained by using large excesses of UV-specific endonucleases or by making comparisons to dimers occurring on double-stranded DNA irradiated with an equivalent dose of UV light. These approaches may not result in precise measurements of cyclobutane dimer formation on the template strand as some cyclobutane dimer sites in single-stranded DNA are resistant to cleavage by UV-specific endonucleases regardless of the amount used. The induction of cyclobutane dimers by UV light is not equivalent for all potential photoproduct sites in single- and double-stranded DNA (Hosszu & Rahn, 1967). Measurements of cyclobutane dimer formation in single-stranded DNA can also be made by utilizing T4 DNA polymerase associated exonuclease (Doetsch et al., 1985). T4 DNA polymerase associated exonuclease hydrolysis of UV-irradiated single- or double-stranded DNA is terminated immediately 3' to positions of cyclobutane

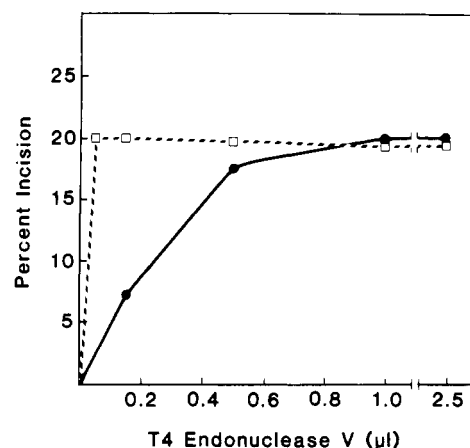


FIGURE 1: Cleavage of single- and double-stranded DNA by T4 endonuclease V. Single- and double-stranded 3'-end-labeled M13 mp2 DNA, irradiated with 5000 J/m² UV light, was treated with increasing concentrations of T4 endonuclease V. The reaction conditions as well as the determination of percent incision were as described in the text. The data presented here are the percent incisions at the site of ATTTCA (base positions 6198-6201) as a function of enzyme concentration for double-stranded (□) and single-stranded (●) DNA.

dimers and (6-4) photoproducts. The termination of T4 exonuclease in the vicinity of UV photoproducts is equal to the actual amount of damage occurring at a given photoproduct site (Doetsch et al., 1985).

Measurement of Cyclobutane Dimers and (6-4) Photoproducts. The T4 UV-specific endonuclease has only limited activity on single-stranded DNA (Gordon & Haseltine, 1980). For a number of sites of potential dimer formation, we were not able to demonstrate saturating enzyme levels. However, for the sites that we were able to show a saturating amount of enzyme, we found that the frequency of dimer formation was the same regardless of whether the DNA was irradiated in the single- or double-stranded form. The result on one such site containing TTTC is shown in Figure 1. Only data for sites at which complete cleavage occurred (saturating amounts of enzyme) are reported in this study. On the basis of the similarities shown in Figure 1, we measured the frequencies of cyclobutane dimer formation on double-stranded DNA fragments containing the region that was used as a template for the polymerase studies (Figure 2). For some of the sites containing more than two adjacent pyrimidines that were located at the upper parts of the gel, it was not possible to resolve the individual bands. Consequently, the results were expressed as the total frequency for all the bands within the cluster. The results with eight different photoproduct sites are shown in Figure 3. Several features are clear from these results. The induction of cyclobutane dimers saturated with UV dose for all sites measured reached a steady-state plateau level at a dose of about 2000 J/m². The level of saturation, however, varied greatly from site to site, depending on the sequence of the adjacent pyrimidines. In general, sequences of TT were 4-5 times more susceptible to dimer formation than sequences of TC, CT, or CC (Figure 3).

Measurement of (6-4) photoproduct formation was achieved by hot alkali treatment of UV-irradiated single-stranded DNA (Figure 4). The quantitation of (6-4) photoproduct formation for eight sites is shown in Figure 3. It is evident that in contrast to cyclobutane dimers (6-4) photoproduct formation did not show saturation and increased steadily with dose. The hot spots for (6-4) photoproduct formation also have sequence composition different from that of hot spots for cyclobutane dimer formation. (6-4) photoproducts occur preferentially at

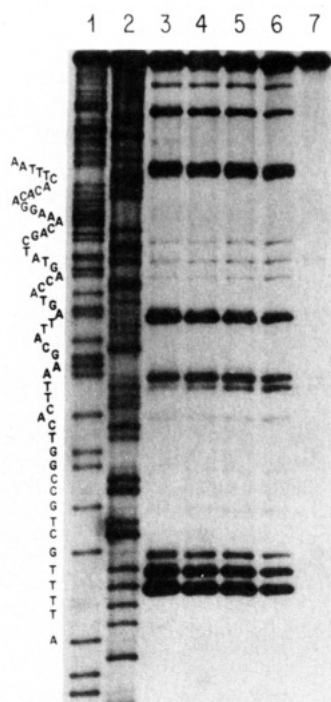


FIGURE 2: Cleavage of 3'-end-labeled double-stranded M13 mp2 DNA by T4 endonuclease V at sites of cyclobutane dimers. Lane 1, G+A DNA sequencing reaction; lane 2, C+T DNA sequencing reaction; lanes 3-6, DNA irradiated with 10 000, 5000, 2000, and 500 J/m² UV light, respectively, and treated with T4 endonuclease V; lane 7, unirradiated DNA, treated with T4 endonuclease V. Electrophoresis was performed on a 20% sequencing gel as described in the text.

TC, at CC, and to a lesser extent at TT sequences. Little (6-4) photoproduct formation occurs at CT sequences. Previous studies have suggested that the 5-methyl group of thymine located 3' to a cytosine sterically hinders the formation of the (6-4) photoproduct (Franklin et al., 1982).

By comparison of the frequencies of cyclobutane dimer and (6-4) photoproduct formation for the eight different adjacent pyrimidine sites shown in Figure 3, it is clear that the contribution of these two adducts relative to total photoproduct formation differs from site to site, depending on the base sequence as well as the UV dose.

Measurement of DNA Polymerase Termination Sites. A 5'-end-labeled, 17 nucleotide long single-stranded DNA molecule was used as a primer for DNA synthesis. The primer was annealed to a single complementary site on the M13 template. The Klenow fragment of *E. coli* DNA polymerase I was used. This enzyme lacks the 5' to 3' exonuclease activity that might otherwise have degraded the 5'-end-labeled primer. A high ratio of template to primer was used. A molar excess of DNA polymerase I to template was also used to drive the polymerization reactions to completion.

Four additional reactions were done by using the same template primer in the presence of all four unlabeled nucleotide triphosphate precursors and one dideoxynucleotide triphosphate. These reaction products were also analyzed in the same gel. Figure 5 illustrates such an experiment. Under the conditions used, the primer extension reaction was efficient as judged by the presence of high molecular weight species at the top of the gel (not shown). Radioactivity at the position of the primer at the bottom of the gel can be attributed to primer that was not elongated in this reaction (about 10% of the total). No significant termination events occurred within the first 50 nucleotides when unmodified template was used in these reactions.

In contrast, elongation of the labeled primer along UV-ir-

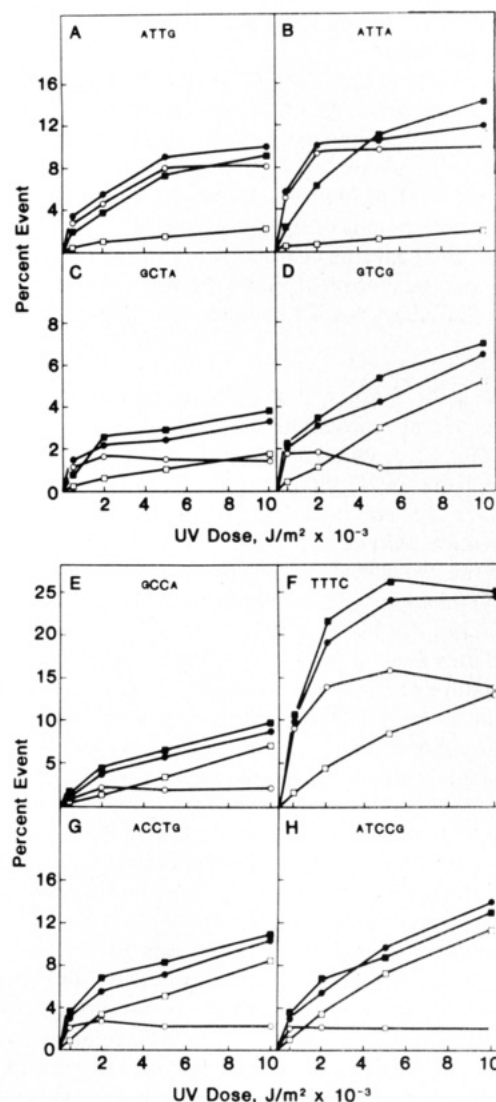


FIGURE 3: UV dose response of photoproduct formation and DNA polymerase I terminations. The induction of cyclobutane dimers (O) and that of (6-4) photoproducts (□) were determined for 3'-end-labeled M13 *Bst*NI/*Alu*I restriction fragments for eight different sites of adjacent pyrimidines as described in the text. The sum of the frequencies of cyclobutane dimers and (6-4) photoproducts (●) were compared to the frequency of DNA polymerase I terminations (■) on UV-irradiated, primed M13 single-stranded DNA.

radiated DNA resulted in production of a number of DNA fragments that range in size from 18 to 80 nucleotides (Figure 5). We attribute such premature termination products to UV-induced damage of the template strand. A dose response for formation of these products is evident. The length, and, therefore, the site of termination, of these products was deduced by comparison of the electrophoretic mobility of each fragment with that of the products produced in the dideoxynucleotide terminated reactions (Figure 5).

Inspection of Figure 5 reveals that most of the premature termination photoproducts occurred in the vicinity of adjacent pyrimidines on the template strand. Multiple premature termination products are observed near each photoproduct site. To compare the frequency of polymerase termination events near sites of adjacent pyrimidines with the frequency of cyclobutane dimer and (6-4) photoproduct formation, the amount of radioactivity corresponding to each termination product was measured. The frequency of termination at each site was calculated by applying a corrective factor to account for the possibility of multiple lesions per template (Materials and Methods).

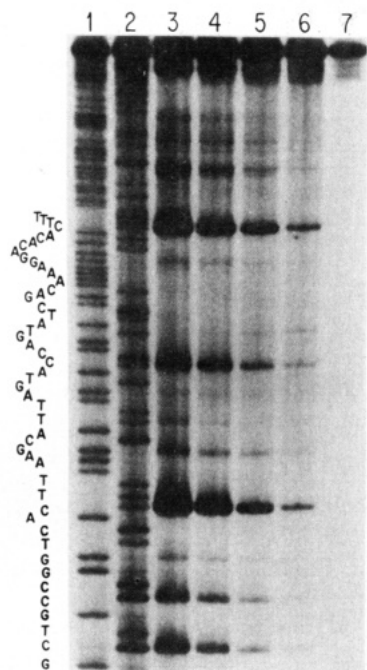


FIGURE 4: (6-4) photoproduct analysis on single-stranded M13 mp2 DNA. Lane 1, G+A DNA sequencing reaction; lane 2, C+T DNA sequencing reaction; lanes 3-6, DNA irradiated with 10 000, 5000, 2000, and 500 J/m² of UV light, respectively, and treated with hot alkali; lane 7, unirradiated DNA treated with hot alkali. Electrophoresis was performed on a 20% sequencing gel as described in the text.

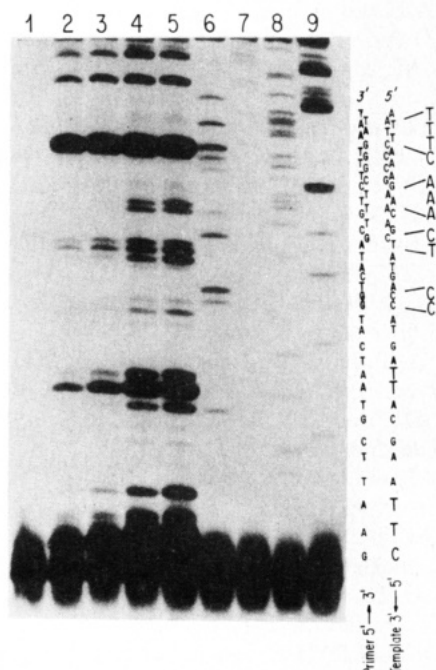


FIGURE 5: Action of the Klenow fragment of DNA polymerase I on UV-irradiated M13 mp2 templates. Lane 1, unirradiated M13 template; lanes 2–5, M13 templates irradiated with 500, 2000, 5000, and 10000 J/m² UV light, respectively. Reactions were initiated by simultaneous addition of a mixture that contained the DNA polymerase and the four deoxynucleotide triphosphate precursors. The reactions were carried out at 20 °C for 15 min. Lanes 6–9, dideoxy sequencing reactions for G, A, T, and C, respectively. Electrophoresis was performed on a 20% sequencing gel as described in the text.

The results of these measurements are shown in Figure 3. The frequency of premature termination given in these figures is the summation of all the premature termination events that occur in the vicinity of the sequence indicated. The frequency

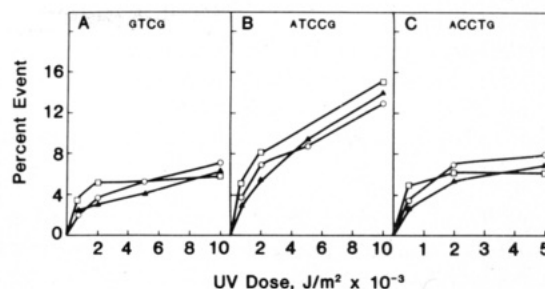


FIGURE 6: T4 DNA polymerase (3'-5') exonuclease determination of UV-induced photoproduct formation. M13 mp8 single-stranded DNA was UV irradiated at the indicated doses and analyzed for photoproducts. Percent event values are expressed as T4 DNA polymerase associated exonuclease terminations (□), DNA polymerase I blocks (▲), or the sum of the frequencies of cyclobutane dimer (T4 endonuclease V incision) and (6-4) photoproduct (hot alkali breaks) formation (○) determined as described in the text. UV-irradiated enzyme or hot alkali-treated samples were subjected to electrophoresis on 20% sequencing gels as described under Materials and Methods.

of formation of cyclobutane dimer damage and (6-4) photoproduct damage at each site is also shown for comparison. There is good agreement between the frequency of premature polymerase termination and the frequency of total UV-induced damage at each site measured. The frequency of premature terminations cannot be attributed to either the cyclobutane dimers or (6-4) photoproducts alone. Termination events correspond to total damage regardless of whether the (6-4) photoproducts or cyclobutane pyrimidine dimers predominate at an individual site. These data suggest that both cyclobutane dimers and (6-4) photoproducts quantitatively block DNA polymerization.

Location of Sites of Premature Termination. As noted above, multiple DNA polymerase premature termination events occur for each potential damage site. Inspection of Figure 5 reveals that three such products occur in the vicinity of each of the sequences of adjacent pyrimidine. The shortest of the products corresponds to extension of the primer to within two bases of the potential damage site. The next longest product corresponds to termination one base before the dimer site. The third product represents extension of the primer to a position opposite the 3' nucleotide of the pyrimidine pair (Figure 5). Quantitative measure of each product shows that the rate of formation of each of the three terminations varies considerably from site to site. The ratio of termination events as determined by measurement of the amount of radioactivity in each termination product in order of increasing length was 2.4:10:2.3 at the sequence ATTA and 7:11:6 at the sequence GCTA. Such multiple termination events at these sites are attributable to cyclobutane pyrimidine dimers, as (6-4) photoproducts did not occur at measurable frequency at these particular sites. Generally, the major product corresponds to termination opposite the nucleotide that immediately precedes the potential damage site.

Quantitation of Total UV-Induced Damage by T4 DNA Polymerase (3'-5') Exonuclease. T4 DNA polymerase (3'-5') exonuclease hydrolysis of DNA is blocked by both cyclobutane dimers and (6-4) photoproducts (Doetsch et al., 1985). This enzyme was utilized as an additional method to assess the total UV-induced DNA damage on single-stranded templates. For a given photoproduct site, the frequency of T4 DNA polymerase associated exonuclease terminations should equal the sum of the frequencies of cyclobutane dimers and (6-4) photoproducts. The relationship between these independently measured values is shown in Figure 6 for three different sites of adjacent pyrimidines within the M13 mp8 phage genome. The frequencies of either T4 DNA polymerase associated

exonuclease terminations or DNA polymerase blockages are equal to the sum of cyclobutane dimer and (6-4) photoproduct frequencies. These data confirm the notion that cyclobutane dimers and (6-4) photoproducts constitute absolute blocks to DNA polymerization under the experimental conditions used.

DISCUSSION

Our results indicate that both cyclobutane dimers and (6-4) photoproducts are absolute blocks for *E. coli* DNA polymerase I. The similarity between the induction curves for polymerase terminations and the induction curve for the sum of cyclobutane dimers and (6-4) photoproducts suggests it is unlikely that a third major photoproduct also blocks the polymerase (Figure 3). This is further supported by the close correlation between DNA polymerase I stops and T4 DNA polymerase associated exonuclease terminations at sites of photoproduct formation (Figure 6).

The finding that UV-induced photoproducts constitute blocks for DNA polymerase I is in agreement with previous studies by Strauss and co-workers (Moore & Strauss, 1979; Moore et al., 1981; Rabkin et al., 1983). However, no previous studies have correlated polymerase terminations with the actual amount of UV light damage occurring at a potential photoproduct site. Our results differ from those of these investigators in that we detect polymerase terminations occurring two nucleotides prior to the 3' component of a photoproduct (Figure 5). Such results may reflect differences in the equilibrium of the elongation and exonuclease components of the DNA polymerization reaction and may be attributable to differences in our assay conditions.

Cyclobutane dimers alone have been inadequate for explaining all the effects of mutagenesis. Several lines of evidence indicate that (6-4) photoproducts may be the primary premutagenic lesion for transition mutations in *E. coli* (Brash & Haseltine, 1982; Wood et al., 1984). Additionally, mutations in the *E. coli lac Z* locus as well as suppressor mutations of the *trp A* alleles have been found not to be reversed by photoreactivation (Witkin, 1966). Preliminary experiments from our laboratory indicate that the (6-4) photoproduct is not a substrate for the *E. coli* photoreactivation enzyme (D. E. Brash et al., unpublished results).

The results in Figure 5 indicate the location of chain termination events when DNA polymerase is blocked at bipyrimidine UV photoproducts. The insertion of a nucleotide opposite the 3'-pyrimidine of a cyclobutane dimer pyrimidine pair is particularly intriguing. For a cyclobutane dimer structure, the atoms of the 3'-pyrimidine that are involved in base pairing are not very much distorted from their native configurations, suggesting that the coding properties of this pyrimidine is not totally lost (Haseltine, 1983). In contrast, the 5'-pyrimidine of a cyclobutane dimer preserves very little of its base-pairing properties (Haseltine, 1983). In the case of a (6-4) photoproduct, the two adjacent pyrimidines are nearly perpendicular to each other, as opposed to being on parallel planes in their native configuration (Haseltine, 1983). We have used chemical photosensitization to induce dimers, but not (6-4) photoproducts on M13 single-stranded templates. The nature of the base inserted opposite the 3'-pyrimidine of a dimer is currently under investigation.

The in vitro DNA synthesis assay system described here should be a general method for detecting DNA adducts induced by many mutagens. Any adduct that renders the bases noncoding should be detectable. Furthermore, this system provides information on the sequence specificity of DNA-damaging agents and allows for the quantitation of the modified bases.

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REFERENCES

- Benbow, R. M., Zuccarelli, A. J., & Sinsheimer, R. L. (1974) *J. Mol. Biol.* 88, 629-651.
- Blakesley, R., Dodgson, J., Nes, I., & Wells, R. (1977) *J. Biol. Chem.* 252, 7300-7307.
- Brash, D. E., & Haseltine, W. A. (1982) *Nature (London)* 298, 189-192.
- Doetsch, P. W., Chan, G. L., & Haseltine, W. A. (1985) *Nucleic Acids Res.* 13, 3285-3303.
- Franklin, W. A., Lo, K. M., & Haseltine, W. A. (1982) *J. Biol. Chem.* 257, 13535-13543.
- Friedberg, E. C., & Bridges, B. A. (Eds.) (1983) *Cellular Response to DNA Damages*, Liss, New York.
- Gordon, L. K., & Haseltine, W. A. (1980) *J. Biol. Chem.* 255, 12047-12050.
- Gordon, L. K., & Haseltine, W. A. (1982) *Radiat. Res.* 89, 99-112.
- Hanawalt, P. C., Friedberg, E. C., & Fox, C. F. (1978) *DNA Repair Mechanisms*, Academic Press, New York.
- Haseltine, W. A. (1983) *Cell (Cambridge, Mass.)* 33, 13-17.
- Hosszu, J. L., & Rahn, R. O. (1967) *Biochim. Biophys. Res. Commun.* 29, 327-336.
- Howard-Flanders, P., & Boyce, R. P. (1966) *Radiat. Res., Suppl.* 6, 156-184.
- Lippke, J. A., Gordon, L. K., Brash, D. E., & Haseltine, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 3388-3392.
- Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol.* 65, 499-560.
- Messing, J., Croenborn, B., Muller-Hill, B., & Hofschneider, P. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 3642-3646.
- Messing, J., Crea, R., & Seeburg, P. (1981) *Nucleic Acids Res.* 9, 309-317.
- Miyaki, M., Suzuki, K., Aihara, M., & Ono, T. (1983) *Mutat. Res.* 107, 203-218.
- Moore, P. D., & Strauss, B. S. (1979) *Nature (London)* 278, 664-666.
- Moore, P. D., Bose, K. K., Rabkin, S. D., & Strauss, B. S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 110-114.
- Rabkin, S. D., Moore, P. D., & Strauss, B. S. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 1541-1545.
- Radman, M., Villain, G., Boiteux, S., Kinsella, A. R., Glickman, B. W., & Spadari, S. (1978) *Cold Spring Harbor Symp. Quant. Biol.* 43, 937-946.
- Sanger, R., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5468.
- Sewall, P. A., Freidberg, E. C., Ganesan, A. K., & Hanawalt, P. C. (1981) in *DNA Repair* (Friedberg, E. C., & Hanawalt, P. C., Eds.) Vol. 1, pp 229-236, Marcel Dekker, New York.
- Van Wexenbeek, P., Hulsebos, T., & Shoenmakers, J. (1980) *Gene* 11, 129-148.
- Wang, S. Y. (1976) in *Photochemistry and Photobiology of Nucleic Acids*, Vol. 1, pp 326-356, Academic Press, New York.
- Witkin, E. M. (1966) *Science (Washington, D.C.)* 152, 1345-1353.
- Wood, R. D., Skopek, T. R., & Hutchinson, R. (1984) *J. Mol. Biol.* 173, 273-292.
- Yoshida, S., Masaki, S., Nakamura, H., & Morita, T. (1981) *Biochim. Biophys. Acta* 652, 324-333.