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T4 DNA polymerase (3'-5') exonuclease, an enzyme for the detection and quantitation of stable DNA lesions: the ultraviolet light example

Paul W.Doetsch, Gerald L.Chan* and William A.Haseltine

Laboratory of Biochemical Pharmacology, Dana-Farber Cancer Institute, Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Ultraviolet light irradiation of DNA results in the formation of two major types of photoproducts, cyclobutane dimers and 6-4' [pyrimidin-2'-one] -pyrimidine photoproducts. The enzyme T4 DNA polymerase possesses a 3' to 5' exonuclease activity and hydrolyzes both single and double stranded DNA in the absence of deoxynucleotide triphosphate substrates. Here we describe the use of T4 DNA polymerase associated exonuclease for the detection and quantitation of UV light-induced damage on both single and double stranded DNA. Hydrolysis of UV-irradiated single or double stranded DNA by the T4 DNA polymerase associated exonuclease is quantitatively blocked by both cyclobutane dimers and (6-4) photoproducts. The enzyme terminates digestion of UV-irradiated DNA at the 3' pyrimidine of both cyclobutane dimers and (6-4) photoproducts. For a given photoproduct site, the induction of cyclobutane dimers was the same for both single and double stranded DNA. A similar relationship was also found for the induction of (6-4)photoproducts. These results suggest that the T4 DNA polymerase proofreading activity alone cannot remove these UV photoproducts present on DNA templates, but instead must function together with enzymes such as the T4 pyrimidine dimer-specific endonuclease in the repair of DNA photoproducts. The T4 DNA polymerase associated exonuclease should be useful for the analysis of a wide variety of bulky, stable DNA adducts.

INTRODUCTION

The location of precise sites of DNA damage in sequences of DNA can provide a wealth of information relevant to an understanding of the chemistry and the biological effects of such DNA modificaions. Several methods for detection of specific sites of DNA modification exist. These include analysis of the location of DNA damage-induced strand breaks, as well as sites of enzymatic and chemical activity that lead either directly or indirectly to strand breaks at modified DNA bases (1-9). The termination of DNA polymerase at DNA damage sites and inhibition of E. coli exonuclease III digestion by modified bases can also be used to localize DNA lesions (10,11). However, none of these techniques provide a general method for detection of bulky adducts on both single and double stranded DNA. The experiments reported here investigate the use of T4 DNA polymerase 3' to 5' exonuclease activity for

such a purpose. Another objective of this study was to determine whether or not there were substantial differences in the induction of UV photoproducts occurring in single and double stranded DNA.

The T4 DNA polymerase associated exonuclease is a good candidate for such studies. The enzyme is active on both single and double stranded DNA (12). In contrast E. coli exonuclease III, an enzyme used previously for similar investigations, is active only on double stranded DNA (10). Digestion of both single and double stranded DNA by the T4 polymerase associated exonuclease proceeds in the absence of deoxynucleotide triphosphates via sequential hydrolysis of phosphodiester bonds (12-14). The commercial availability of a stable enzyme of high purity and activity also recommended the use of T4 DNA polymerase associated exonuclease.

We examined the ability of T4 DNA polymerase 3' to 5' exonuclease to hydrolyze UV-irradiated single and double-stranded 5'-end labeled DNA fragments with the expectation that UV lesions might serve as blocks to the exonuclease at or near the adduct sites. Ultraviolet light was selected as the DNA damaging agent as we have previously established independent methods for quantitative determination of specific lesions within DNA fragments of defined sequence (1,2,4,5).

MATERIALS AND METHODS

Enzymes and Chemicals

[γ-^{32}P]ATP (specific activity 3000Ci/mmol) was obtained from either Amersham or New England Nuclear. Eco RI was purchased from New England Biolabs. Hae III, T4 Polynucleotide Kinase and T4 DNA polymerase were from Bethesda Research Laboratories. Calf alkaline phosphatase and proteinase K were obtained from Boehringer Mannheim. Micrococcus luteus UV-specific endonuclease [endodeoxyribonuclease (pyrimidine dimer) EC 3.1.25.1] was prepared as described by Riazuddin and Grossman (15). All other chemicals and reagents were of the highest purity available.

Growth and Isolation of M13 mp8 RF

The M13 coliphage strain mp8 was used as the source of defined sequence DNA. The double stranded circular replicative form (RF) DNA (7229 base pairs) was isolated from M13 mp8-infected E. coli JM103 as described (16). Replicative form DNA was purified by cesium chloride gradient centrifugation followed by gel filtration on Sepharose 4B (17).

Preparation of 5'[32P]end-labeled Single and Double Stranded DNA Fragments

The M13mp8 strain contains an insert region with 9 unique restriction sites located between a unique Eco RI site (6232) and a Hae III site (6274).

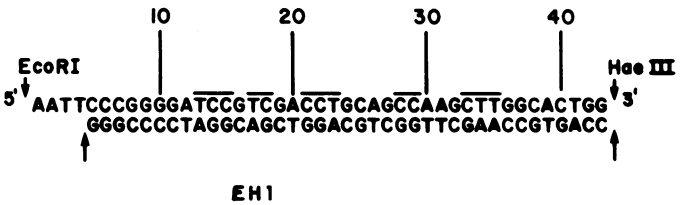


Figure 1. Generation of EH1 DNA damage probe fragment of defined sequence. The replicative form (RF) of the E. coli phage M13 mp8 was isolated and digested with EcoRI. Following 5'-end-labeling with [γ^{32}P] ATP and polynucleotide kinase, the DNA was digested with HaeIII to produce a 43 base pair fragment. This fragment spans the region between bases 6232 and 6274 of the phage RF and contains five regions of potential photoproduct sites suitable for analysis (overlined). Bases are designated by number starting from the 5' end of the labeled strand. EH1 was irradiated as either single or double stranded DNA as described in the text.

This 43 base pair region (EH1) was used as a single or double stranded DNA damage probe of defined sequence (Fig. 1).

Purified M13mp8 RF (10-50µg) was digested with Eco RI (2 hr, 37°C) followed by treatment with calf alkaline phosphatase (2 X 10^{-2} units) and proteinase K (10µg, 50 µl final volume). The double stranded DNA was precipitated with ethanol, lyophilized, and then 5'-end-labeled in a volume of 50µl using T4 polynucleotide kinase and [γ^{32}P]ATP (300µCi) as described (18). The 5'-end-labeled DNA was reprecipitated in ethanol (-20°C, 6 hr.), lyophilized and digested with HaeIII (2 hr., 37°C) that cleaves M13mp8 DNA at positions 6138 and 6274 to produce two 5'-end-labeled double stranded DNA fragments of 43 (EH1, damage probe) and 197 (opposite labeled strand) base pairs, respectively. The fragments were isolated by electrophoresis (800V, 120 min) on a preparative, non-denaturing, 8% polyacrylamide gel (20cm X 40 cm X 2mm). DNA 5'-end-labeled fragments were located as bands on the gel following autoradiography (Kodak XAR-5 film) and were excised and eluted with 0.3M NaOAc as described (18). Following ethanol precipitation the ^{32}P-5'-end-labeled DNA damage probe fragments were suspended in 10-50µl of 10mM Tris-HCl, pH7.5, 1mM EDTA (TE).

Conversion of double stranded ^{32}P-end-labeled EH1 DNA fragment to a single stranded fragment was accomplished by suspending the DNA preparation in 40µl of denaturing dye solution (80% deionized formamide v/v, 0.2% aqueous bromphenol blue (BB) xylene cyanol (XC) and heating at 90°C for 10 min., followed by loading onto a denaturing 20% polyacrylamide gel (20cm X 40cm X 1mm) and electrophoresis at 1000V, for 12 hrs. Under these conditions, 5'-end-labeled, single stranded DNA (43 nucleotides) is completely resolved

from the double stranded fragment and the unlabeled opposite strand (39 nucleotides). The single stranded 5'-end-labeled EH1 fragment was excised from the gel and recovered as described above.

UV Irradiation (313nm) of Double Stranded DNA

Photosensitization of double stranded DNA was performed in 20 µl of 0.1 mM silver nitrate that contained approximately 2 X 10^5 cpm of carrier-free EH1 DNA. DNA-containing droplets were irradiated (on ice) at 313nm using a monochromatic light source, 5000W Xenon-mercury lamp (Optical Radiation Corp., Azusa, CA); fluence, 45J/m2/s.

UV-Irradiation (254nm) of Single and Double Stranded DNA

Single and double stranded DNA damage probes (20µl droplets, on ice) were exposed to 254nm light from two GE 15T8 germicidal lamps at a fluence of 30J/m2/s as determined by a Black-Ray ultraviolet intensity meter (Ultraviolet Products, San Gabriel, CA). Following irradiation, DNA samples were suspended in 0.3M NaOAc (200µl) and ethanol precipitated.

Digestion of UV-Irradiated DNA with M. luteus UV-Specific Endonuclease

UV-irradiated DNA samples were resuspended in 18µl of assay buffer (10mM Tris-HCl, pH7.5, 5mM $MgCl_2$, 1mM EDTA, 50mM NaCl) and incubated with 2 µl of M. luteus UV-specific endonuclease (0.11 mg/ml) for 60 min. at 37°C. The reaction was terminated by addition of 1µl salmon sperm carrier DNA (1µg) and 0.3M NaOAc (200µl) followed by three extractions with phenol-chloroform-isoamyl alcohol (20:19:1 v/v/v) and ethanol precipitation.

Hot Alkali Treatment of UV-Irradiated DNA

Irradiated DNA samples were suspended in 1M piperdine (100µl) and heated at 90°C for 20 min. The samples were frozen and lyophilized.

Digestion of UV-Irradiated DNA with T4 DNA Polymerase (3'-5') Exonuclease

DNA samples were suspended in 22.5µl of T4 DNA polymerase associated exonuclease assay buffer (33mM Tris-acetate, pH7.8, 10mM $Mg(OAc)_2$, 66mM KOAc, 0.5mM dithiothreitol, 0.1mg/ml BSA) and incubated with 5 units (2.5µl) 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 M. luteus enzyme digestions.

DNA Base-Specific Sequence Reactions

The Maxam-Gilbert G+A and C+T sequencing reactions (18) were performed on the 5'-end-labeled EH1 43 base pair DNA fragment. The reaction products were loaded on gels in parallel lanes with UV-irradiated, hot alkali, or enzyme treated DNA samples.

Gel Electrophoresis

Ethanol precipitated DNA samples were lyophilized and dissolved in 3µl of

loading buffer (80% deionized formamide v/v, 0.2% BB, XC dyes w/v, 10mM NaOH, 1mM EDTA), heated to 90°C for 5 min, and quick-chilled in an ice bath. The samples were loaded onto prerun, denaturing 20% polyacrylamide, 7M Urea gels (20cm X 40cm X 0.38mm) and electrophoresed at 1000V for 5 hr. Autoradiography was performed at -70°C.

Quantitation of UV-Induced Photoproducts in Single and Double Stranded DNA

The amount of hot alkali breaks, M. luteus UV-specific endonuclease cleavage, and T4 DNA polymerase associated exonuclease termination products were determined by measuring the amount of Cerenkov radiation in each gel band relative to the total Cerenkov radiation in the lane. To account for the possibility that a single DNA molecule may contain more than one photoproduct, a correction factor was applied to obtain the actual percent contribution at a given breakage or termination site (1,4,10). The derivation of this factor has been previously described (1).

RESULTS

Identification of Ultraviolet-Light Induced Photoproducts

UV irradiated, 5' end labeled double stranded DNA of defined sequence was digested with T4 DNA polymerase associated exonuclease. The T4 exonuclease reaction products were compared to the DNA scission products obtained by enzymatic and chemical treatments previously established for the detection of cyclobutane pyrimidine dimers and (6-4)photoproducts (1,2,4,5). In these experiments a DNA fragment 43 base pairs long was irradiated with doses of 254 nm light that range from 500-10,000 J/m2. The DNA was treated either with the M. luteus UV-specific endonuclease that cleaves the glycosidic bond between the 5' pyrimidine of the dimer and its corresponding sugar (DNA glycosylase) followed by phosphodiester bond cleavage by an apyrimidinic endonuclease activity (1). The resultant cleavage product contains a base free sugar at the 3' terminus (Figure 2). Alternatively, the DNA was treated with hot alkali, a procedure that cleaves the glycosidic bond between the 3' pyrimidine moiety of the (6-4) product followed by beta elimination of the deoxyribose at the 3' position (3). The resultant fragment contains a 3' phosphate (Figure 2). The same irradiated DNA fragment was also digested with T4 DNA polymerase associated exonuclease. This enzyme produces DNA fragments containing 3' hydroxyl groups as the digestion products (19). The DNA products were analyzed on high resolution polyacrylamide gels.

The M. luteus UV-specific endonuclease incised UV-irradiated DNA at sites of adjacent pyrimidines. In the experiments reported here saturating concentrations of enzyme were used, as increasing the amount of enzyme did not

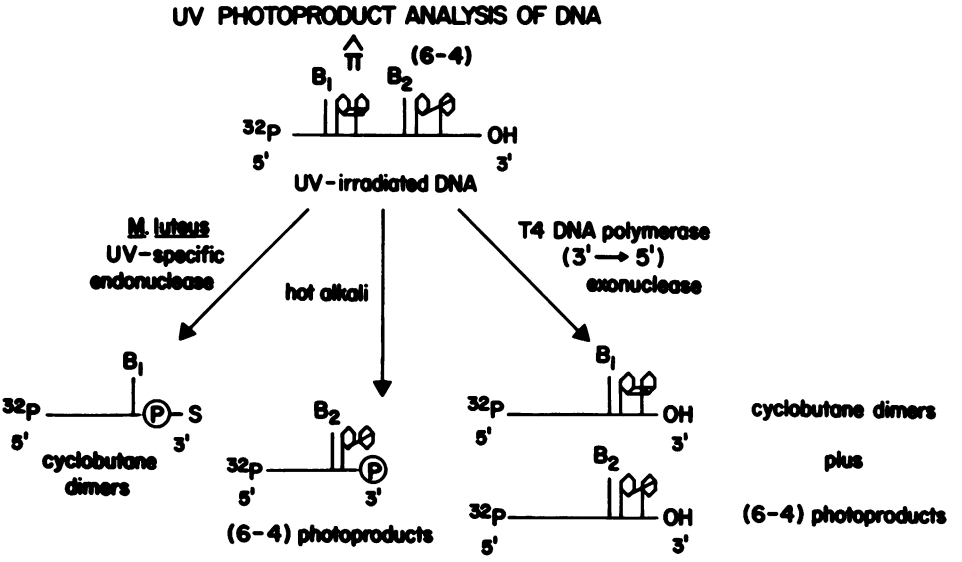


Figure 2. Scheme for analysis of UV-induced photoproducts in DNA. Single and double stranded DNA (5'-end labelled) was irradiated at 313 nm or 254 nm and subjected to the indicated photoproduct analyses for the detection of cyclobutane dimers ($\hat{\pi}$) and (6-4) photoproducts ((6-4)). The resulting fragments were analyzed by polyacrylamide gel electrophoresis as described in the text. M. luteus UV-specific endonuclease was used to treat double stranded DNA only and results in the generation of a fragment containing a base-free sugar at the 3' end of a cleaved strand (P-S).

lead to an increase in the extent of cleavage at adjacent pyrimidine sites (not shown). Hot alkali treatment of UV irradiated DNA revealed the formation of (6-4) photoproducts at sites of adjacent pyrimidines. However, the extent of formation of (6-4) photoproducts at individual sites differed from the extent of formation of the cyclobutane pyrimidine dimers at the same sites. The eletrophoretic mobilities of the fragments produced at the pyrimidine sites by M. luteus enzyme digestion differ from those produced at the same site by treatment with hot alkali. The difference in the structure of the 3' terminus of the scission product accounts for the slightly slower migration of the M. luteus scission products relative to that produced by hot alkali (Fig. 2).

Figure 3 shows that incubation of the T4 DNA polymerase associated exonuclease with UV irradiated DNA produces a series of fragments that are not evident upon digestion of the unirradiated control. Inspection of the autoradiogram reveals that the amount of premature termination products increases with increasing UV doses. Moreover, all the termination products

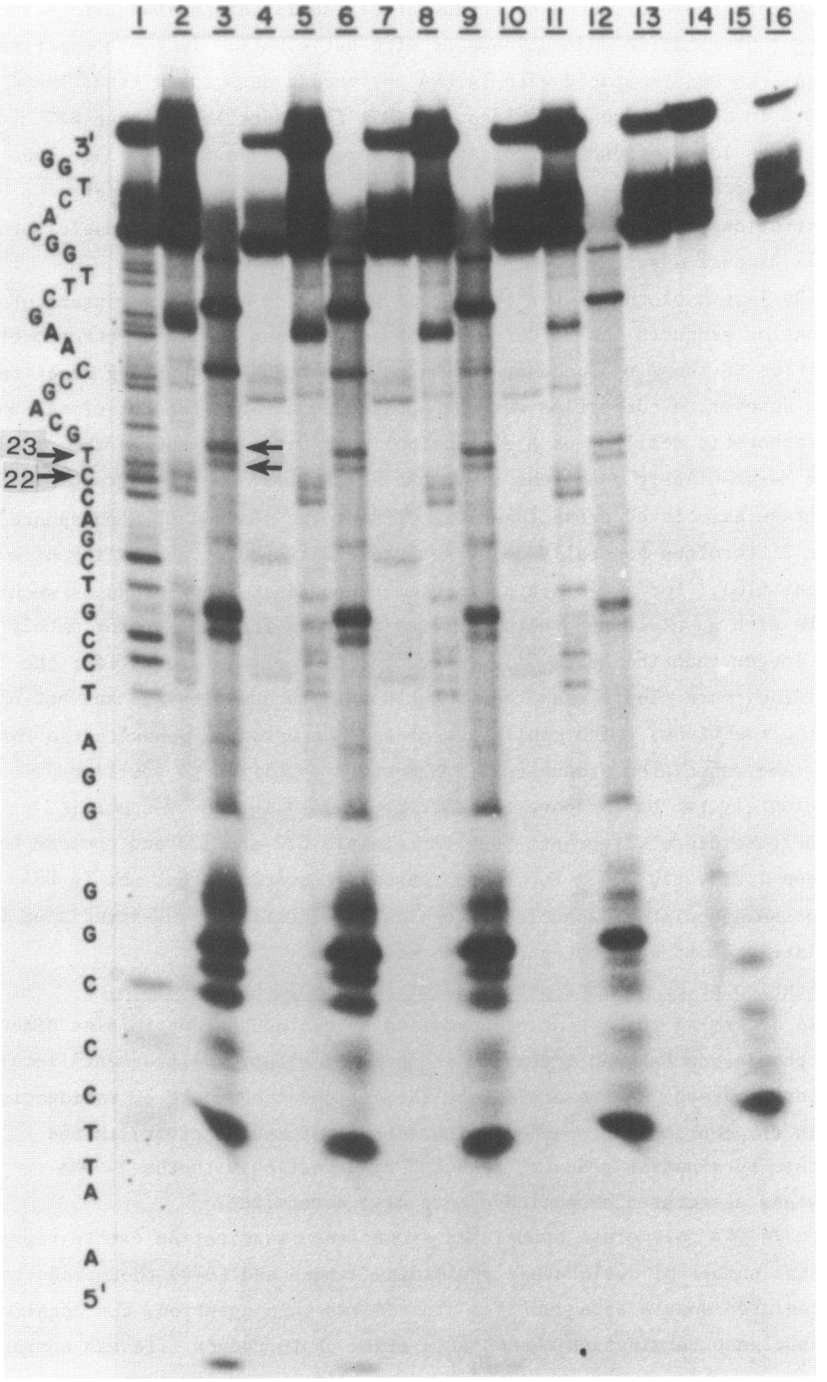
migrate near those produced by treatment of the irradiated DNA with either the M. luteus UV-specific endonuclease or with hot alkali. In these experiments unirradiated DNA incubated with T4 DNA polymerase associated exonuclease was degraded to short oligonucleotides. No DNA fragments larger than 8 nucleotides long, products of digestion, were observed (Figure 3). The absence of premature termination products upon digestion with high concentrations of this enzyme on undamaged templates is a very useful property of this exonuclease.

The length of the T4 DNA polymerase associated exonuclease premature termination products can be determined by comparison of the electrophoretic mobilities to those produced by the Maxam-Gilbert DNA sequencing reactions (18). However, a correction must be applied to account for the effect on the electrophoretic mobility of a 3' terminal phosphate, present in the products of the Maxam-Gilbert reactions (18), but absent from the products of T4 DNA polymerase associated exonuclease digestion. The absence of a phosphate group at the 3' terminus generally decreases the electrophoretic mobility of a fragment (10). For a given bipyrimidine photoadduct, such products should migrate with an electrophoretic mobility of a DNA fragment approximately two bases longer than the Maxam-Gilbert sequencing reaction product for the 3' pyrimidine (more slowly than the products of M.luteus cleavage and hot alkali scission reactions). The mobilities of the photoproduct generated T4 DNA polymerase associated exonuclease fragments are shifted to positions approximately two bases above the correspondng M.luteus uv-specific endonuclease digest fragments (see for example C22 and T23 and compare bands in lanes 1,2,3, Fig. 3). For these reasons we conclude that the T4 DNA polymerase associated exonuclease terminates digestion of UV-irradiated DNA immediately 3' to adjacent pyrimidine sequences.

Quantitation of UV-induced photoproducts on double stranded DNA.

To determine the extent of formation of cyclobutane pyrimidine dimers and (6-4) photoproducts quantitatively at specific sites, DNA fragments localized by autoradiography were excised from the gel and the amount of radioactivity in each fragment was determined. The amounts of radioactivity in the premature termination products produced by digestion with the T4 DNA polymerase associated exonuclease were also determined.

Do T4 DNA polymerase associated exonuclease termination events represent the total number of cyclobutane pyrimidine dimers and (6-4) photoproducts in UV irradiated double stranded DNA? To address this question, the magnitude of T4 exonuclease termination events at a given photoproduct site was compared to


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
3'
23
22
5'

the sums of the M. luteus enzyme and hot alkali-induced scission events occurring at that site. For example, at the sequence G-C-C-A, the amount of T4 exonuclease terminations are equal to the sum of M. luteus enzyme incisions and hot alkali breaks (Fig. 4). A similar relationship was found for photoproducts occurring at other sites of adjacent pyrimidines. We conclude that the T4 DNA polymerase associated exonuclease termination events on UV irradiated double stranded DNA provide a quantitative measure of the total DNA damage at any particular site of adjacent pyrimidines.

Termination Events at Cyclobutane Dimers in the Absence of (6-4) Photoproducts.

The results shown in Figure 4 indicate that the termination of the T4 enzyme is quantitative for both cyclobutane pyrimidine dimers and (6-4) photoproducts. As a further test of this possibility we investigated the activity of the T4 DNA polymerase associated exonuclease on DNA that contained cyclobutane pyrimidine dimers but not (6-4) photoproducts. Such substrates can be prepared by exposure to DNA to long wave UV-light in the presence of a photosensitizing chemical (20). For this purpose a 5' end-labeled double stranded DNA fragment (EH1) was exposed to 313 nm light in the presence of silver nitrate. Under these conditions, cyclobutane dimer formation is favored for sites of adjacent pyrimidines for TT, TC, and CT sequences, but not for CC sequences (20). Moreover, under these conditions, formation of the (6-4) photoproduct does not occur at appreciable rates (not shown). The distribution of cyclobutane dimers on such a fragment was determined by treatment with the M. luteus UV-specific endonuclease. After irradiation the DNA fragments were also digested with the T4 enzyme. The location and extent of formation of the premature termination events due to cyclobutane dimers was measured as described above.

Figure 3. Analysis of T4 DNA polymerase associated exonuclease terminations, hot alkali breaks and M. luteus UV-specific endonuclease incisions at sites of cyclobutane dimers and (6-4) photoproducts occurring in double stranded DNA. EH1, 5'-end labeled double stranded DNA was irradiated at 254nm at doses of 0 (lanes 1,14-16), 500 (lanes 11-13), 2000 (lanes 8-10), 5000 (lanes 5-7), and 10,000 J/m^2 (lanes 2-4) and treated with M. luteus UV-specific endonuclease (lanes 2,5,8,11,14); T4 DNA polymerase associated exonuclease (lanes 3,6,9,12,15); hot alkali (lanes 4,7,10,13,16); or the C+T Maxam-Gilbert base specific sequencing reaction (lane 1). The amount of DNA loaded into the lanes corresponding to T4 DNA pol exo digestions was greater relative to the amounts loaded for M. luteus enzyme digestions and hot alkali treatments. Arrows indicate shifts in mobility of T4 DNA pol exo termination fragments relative to the pyrimidine-specific sequencing fragments (lane 1) and the M. luteus UV-specific endonuclease fragments (lane 2). Electrophoresis and autoradiography was performed as described in the text.

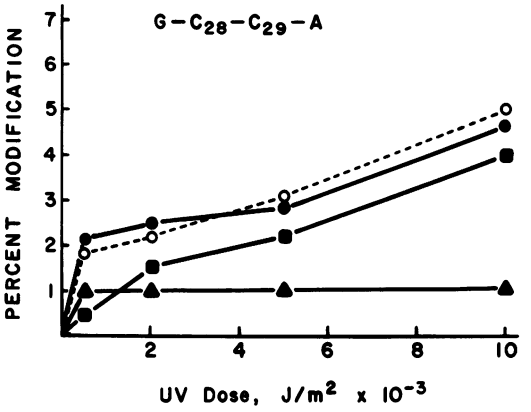


Figure 4. Dose response of UV-induced photoproduct formation occurring at the sequence C_{28}-C_{29} within EH1 double stranded DNA. End-labeled DNA was irradiated with 0-10,000 J/m^2 of ultraviolet light (254nm) and subjected to either T4 DNA polymerase associated exonuclease digestion or M. luteus UV-specific endonuclease digestion or hot alkali treatment. The treated fragments were analyzed on denaturing polyacrylamide gels as shown in Fig. 3. Following autoradiography, the amount of radioactivity in each band was determined by the Cerenkov cpm of the gel slices. The percent contribution of each band occurring at a photoproduct site relative to the entire lane was calculated as described in Materials and Methods. Symbols correspond to percent T4 DNA polymerase associated exonuclease terminations (●); M. luteus UV-specific endonuclease incisions (▲); hot alkali breaks (■); and the sum of M. luteus enzyme incisions and hot alkali breaks (○).

The T4 DNA polymerase associated exonuclease terminates prematurely at the sites of some adjacent pyrimidines upon digestion of DNA treated under photosensitizing conditions (Figure 5). The DNA termination products correspond to each of the positions of the cyclobutane pyrimidine dimers as detected by M. luteus UV-specific endonuclease treatment of the same fragment of DNA. As before, the electrophoretic mobility of the T4 exonuclease digestion products indicates that termination events have occurred immediately adjacent to the 3' terminal pyrimidine of the cyclobutane dimer. Hence, the T4 exonuclease termination event occurring at C18 of the T17-C18 pyrimidine dimer pair contains a 3' terminal hydroxyl group. Such a fragment migrates to a position in the gel approximately two bases above the C28 sequencing fragment (Fig. 5). Visual inspection of Figure 5 indicates that the relative amounts of the enzymatic termination (T4 DNA polymerase associated exonuclease) or cleavage (M. luteus enzyme) products appear to be identical. This observation is substantiated by comparison of the percentage dimer formation for four different sites of adjacent pyrimidines determined by the

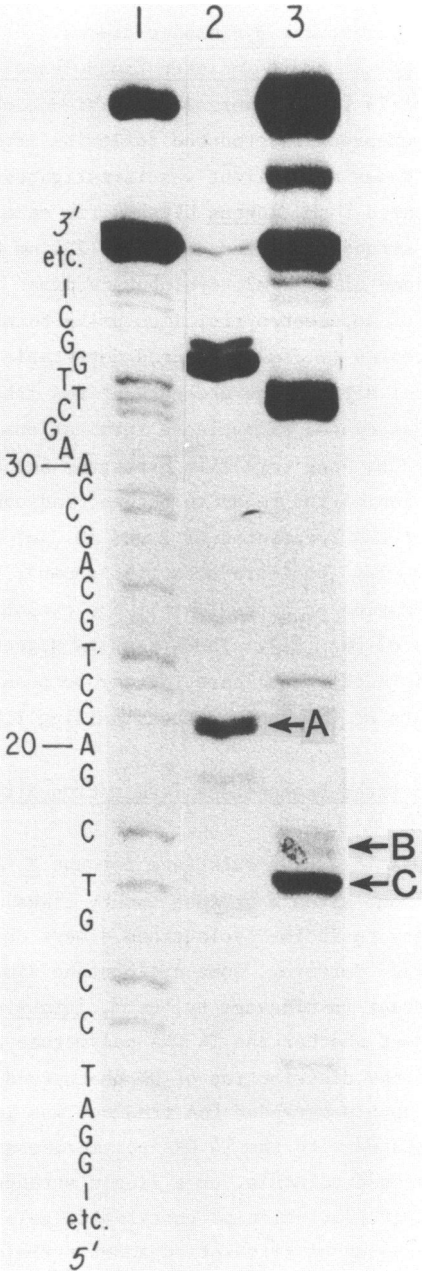
T4 enzyme or M. luteus enzyme methods (Table 1). These experiments demonstrate that the T4 DNA polymerase associated exonuclease quantitatively stops at positions of cyclobutane pyrimidine dimers.

Sensitivity of T4 DNA Polymerase Associated Exonuclease to Photoproducts.

The sensitivity of T4 DNA polymerase associated exonuclease with respect to the detection of photoproducts produced following irradiation of double stranded DNA with low doses of UV light was investigated. T4 enzyme terminations were compared to M. luteus UV-specific endonuclease incisions of 5' end-labeled double stranded DNA irradiated at 254 nm with doses of 2 and 10 J/m2 (Fig. 6). At a dose of 10 J/m2, cyclobutane dimer formation was clearly visible at five sites of adjacent pyrimidines using both enzymes (lane 3, Fig. 6). The lowest dose tested (2 J/m2) produced detectable cyclobutane dimers (faint bands) at several sites. The presence of DNA fragments corresponding to T4 DNA polymerase associated exonuclease terminations and M. luteus enzyme incisions was confirmed by long term film exposures and excision of the bands followed by determination of the relative percent radioactivities (not shown). Hot alkali treatment of DNA irradiated at 2 and 10 J/m2 produced no detectable bands corresponding to (6-4) photoproducts (not shown). A UV dose of 2 J/m2 corresponds to the formation of approximately one cyclobutane dimer occurring within a 50 kb segment of DNA (21). These results suggest that by employing T4 DNA polymerase associated exonuclease, it may be possible to detect relatively small amounts of DNA photoproducts arising from low dose UV irradiation of cells.

T4 DNA Polymerase Associated Exonuclease Termination Sites on UV-irradiated Single Stranded DNA.

Single stranded DNA is a poor substrate for the M. luteus UV-specific endonuclease (4). At some sites more than twenty times the concentration of this enzyme is necessary to incise cyclobutane dimers on single as compared to double stranded DNA (4). Moreover, some cyclobutane dimer sites on single stranded DNA are resistant to cleavage by the M. luteus enzyme regardless of the amount used. To test whether the T4 DNA polymerase associated exonuclease can be used to measure the distribution of UV photoproducts on single stranded DNA, a 5' end-labeled double stranded DNA fragment was irradiated at 2000 J/m2. The DNA was digested with the T4 DNA polymerase associated exonuclease as either a double stranded molecule, or a single stranded molecule following strand separation on high resolution polyacrylamide gels. Figure 7 shows that the frequency of termination at sites of adjacent pyrimidines were the same for UV-irradiated DNA digested in either the single or double stranded form.


1
2
3
3'
etc.
I
C
G
G
T
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G
A
30
A
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C
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C
20
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T
A
G
G
I
etc.
5'
A
B
C

Table 1
Percent T4 DNA polymerase associated exonuclease termination and percent M. luteus UV-specific endonuclease incision on double stranded DNA containing cyclobutane dimers.

Sequence	Position	M. luteus UV-specific Endonuclease	T4 DNA polymerase associated exonuclease
G CTT G	33-35	25.5	28.6
A CCT G	21-23	3.5	2.2
G TC G	17-18	11.2	9.6
A TCC G	13-15	1.8	3.7

The 5'-end-labeled EH1 43bp fragment was irradiated at 313nm (3.6 X 10^5 J/m^2) in the presence of silver nitrate and treated with either T4 DNA polymerase associated exonuclease or M. luteus UV-specific endonuclease. The values represent the summation of photoproducts measured at positions of the underlined adjacent pyrimidines.

As before, the extent of photoproduct formation as measured by the T4 enzyme termination reaction is the same as that calculated for the sum of incision at such sites determined using the M. luteus and hot alkali methods. A similar relationship was found at higher doses (5000 J/m2) of UV-irradiation (not shown). We conclude that both cyclobutane dimers and (6-4) photoproducts quantitatively block digestion of single stranded DNA by T4 DNA polymerase associated exonuclease.

The ability of T4 DNA polymerase associated exonuclease to reliably

Figure 5. T4 DNA polymerase associated exonuclease terminations and M. luteus UV-specific endonuclease incisions at sites of cyclobutane dimers. EH1, 5'-end-labeled, double stranded DNA was photosensitized with silver nitrate and irradiated at 313nm with a dose of 3.6 X 10^5 J/m^2 (conditions producing only cyclobutane dimers). Following irradiation, DNA samples were digested with either T4 DNA pol exo (lane 2) or M. luteus UV-specific endonuclease (lane 3), and electrophoresed on a denaturing 20% sequencing gel. Following autoradiography, the T4 DNA polymerase associated exonuclease termination band patterns were compared to M. luteus UV-specific endonuclease incision bands and the C+T base specific Maxam-Gilbert cleavage reaction bands (lane 1). DNA samples were loaded into the sequencing gel under mild alkaline conditions (10mM NaOH). Such conditions result in β-elimination of the 3' terminal base free sugar contained in the DNA scission products produced by M. luteus UV-specific endonuclease cleavage at sites of cyclobutane dimers (see Fig. 2 and reference 4). The resultant fragment possesses a 3' terminal phosphate and hence migrates to a lower position in the gel. Arrow (A) corresponds to the T4 DNA pol exo termination event occurring at C18 of the T17-C18 pyrimidine dimer. Arrow (B) indicates the M. luteus UV-specific endonuclease scission product of the T17-C18 dimer containing a 3' terminal base free sugar. Arrow (C) indicates the corresponding hydrolysis product containing a 3' terminal phosphate. The magnitude of the shift in electrophoretic mobility following β-eliminaton of the base free sugar is dependent upon the relative positon of the fragment in the gel (4).

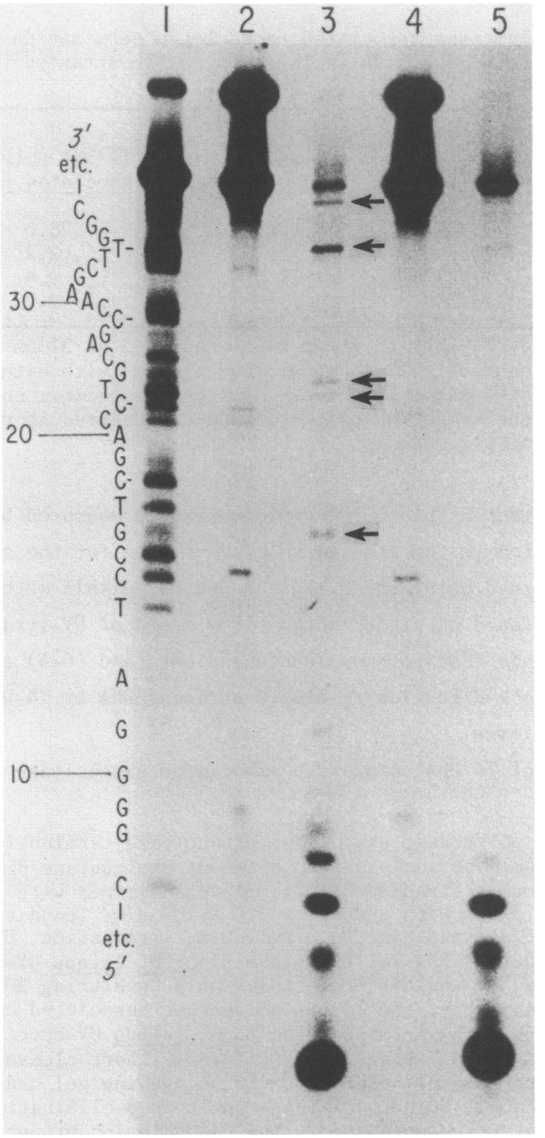


Figure 6. Sensitivity of T4 DNA polymerase associated exonuclease for detecting UV photoproducts. EH1, 5'-end labelled double stranded DNA was irradiated at 254 nm at doses of 2 (lanes 4, 5) and 10 J/m^2 (lanes 2,3) and treated with T4 DNA polymerase associated exonuclease (lanes 3,5); the M.luteus UV-specific endonuclease (lanes 2,4); or the C+T (lane 1) base specific sequencing reaction. Arrows (lane 3, bottom to top) indicate T4 pol exo termination fragments produced from cyclobutane dimers occurring at positions C14-C15, C21-C22, C22-T23, T34-T35, and C40-T41. Electrophoresis and autoradiography were performed as described in Materials and Methods.

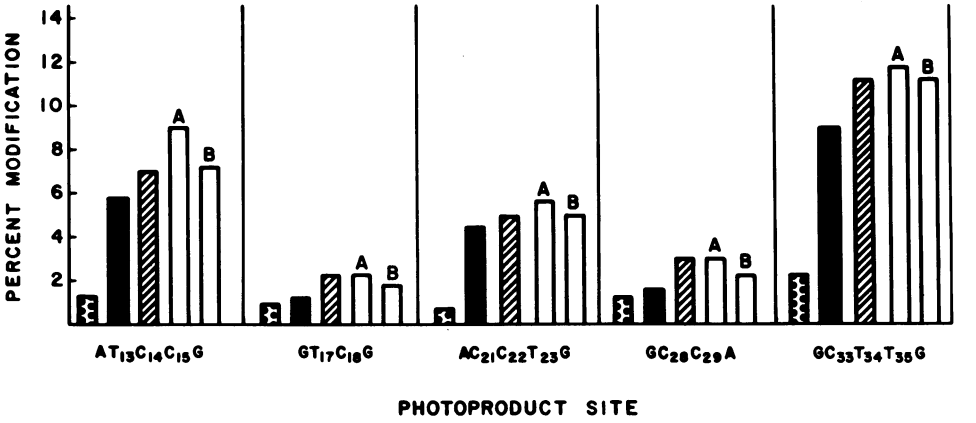
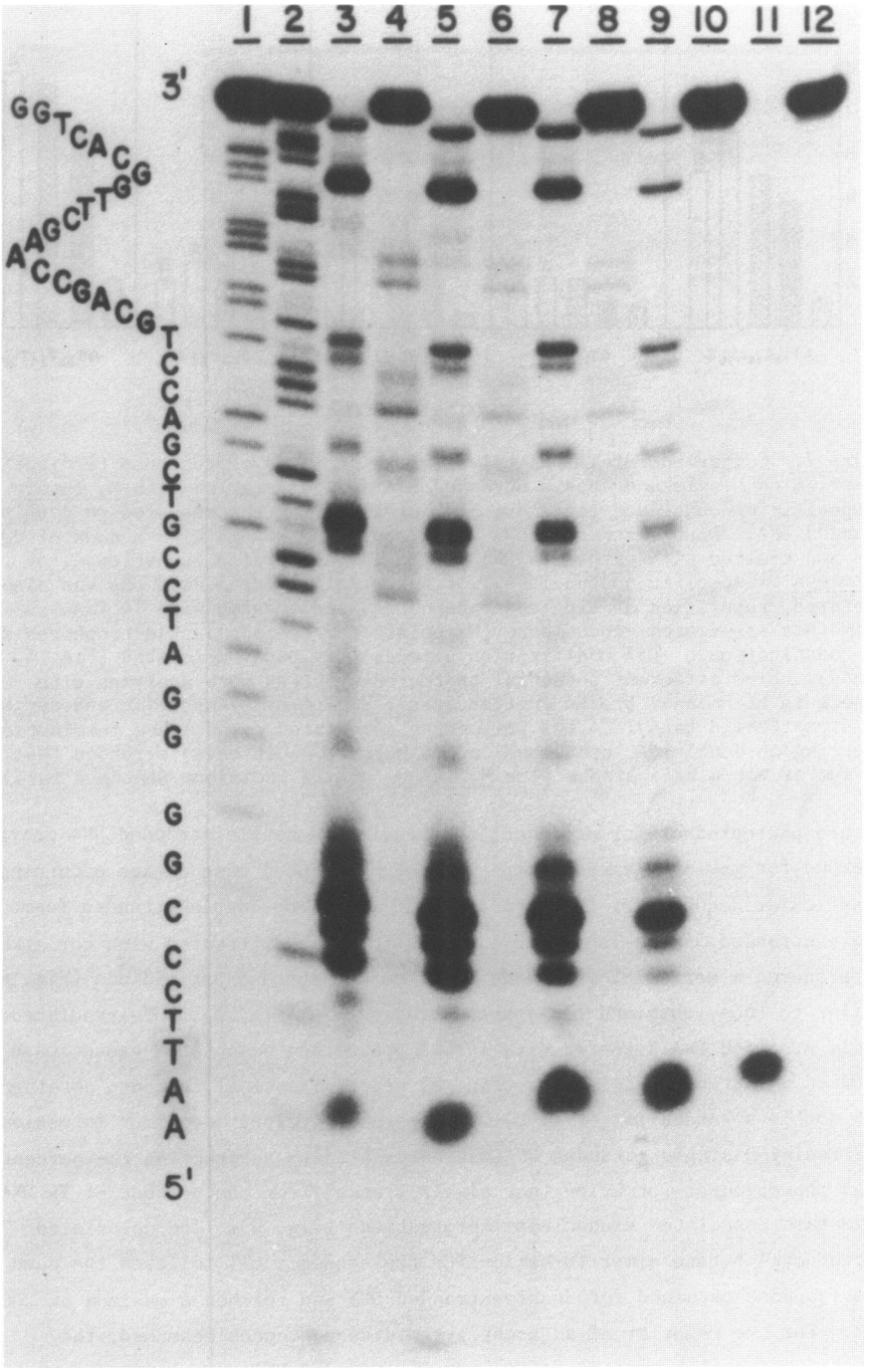


Figure 7. Comparison of T4 DNA polymerase associated exonuclease terminations occurring on single and double stranded DNA and correlation with M. luteus UV-specific endonuclease incisions and hot alkali breaks measured on double stranded DNA. Double stranded DNA was irradiated (254nm) with a dose of 2000 J/m^2 and treated with either T4 DNA polymerase associated exonuclease, or M. luteus UV-specific endonuclease, or hot alkali. Irradiated DNA was also denatured, repurified as single stranded DNA, and treated with T4 DNA polymerase associated exonuclease (Materials and Methods). Electrophoresis and quantitation of DNA modification were as previously described (Fig. 4, legend). Five different potential photoproduct sites were analyzed with respect to hot alkali breaks (dotted bars); M. luteus UV-specific endonuclease incisions (solid bars); T4 DNA polymerase associated exonuclease terminations occurring on double (A, open bars) and single (B, open bars) stranded DNA; and the sum of hot alkali breaks plus M. luteus enzyme incisions (hatched bars).

measure photoproduct formation on both single and double stranded DNA provides a method for making a direct comparison of UV-induced base damage occurring on a particular sequence of DNA irradiated in single or double stranded form. Single stranded 5' end-labeled DNA was UV irradiated, treated with hot alkali and produced a series of gel bands at sites of adjacent pyrimidines (Fig. 8) similar to those obtained for double stranded DNA (Fig. 3). UV-irradiated single stranded DNA digested with T4 DNA polymerase associated exonuclease produced a pattern of gel bands (Fig. 8) nearly identical to those obtained with double stranded DNA (Fig. 3). The percent cyclobutane dimer formation on UV-irradiated single stranded DNA was calculated by subtracting the percent (6-4) photoproduct formation (hot alkali breaks) from the percent of T4 DNA polymerase associated exonuclease terminations (Fig. 9). The calculated percent cyclobutane dimer formation for the sequence CTT followed the same dose response obtained for double-stranded DNA and reached a maximum at 2000 J/m2. For the majority of adjacent pyrimidine sequences measured, the induction of cyclobutane dimers in single stranded DNA was similar to that


1 2 3 4 5 6 7 8 9 10 11 12
3'
GGTCACG
AAGCTTGG
ACCGACG
TCCAGCTGCCTAGGGGCCCTTAA
5'

obtained for double stranded DNA.

DISCUSSION

Ultraviolet light produces lesions in both single and double stranded DNA. Previously no effective method was available to determine the UV dose response at specific sites for single stranded DNA photoproduct formation. The experiments presented here demonstrate that the T4 DNA polymerase associated exonuclease can be used to identify the location and to measure the extent of UV-induced DNA modifications. Both cyclobutane dimers and (6-4) photoproducts inhibit exonuclease hydrolysis of DNA. The site of termination is generally at the 3' pyrimidine of the photoproduct. The frequency of exonuclease termination events observed for a potential UV damage site is equivalent to the sum of frequencies for cyclobutane dimers and (6-4) photoproducts. Thus, the occurrence of cyclobutane dimers on single stranded DNA can be determined by subtracting the percent hot alkali breaks ((6-4) photoproducts) from the percent of exonuclease terminations (total photoproducts). The T4 DNA polymerase associated exonuclease assay is sensitive enough to detect photoproducts formed by low doses of ultraviolet light (2 J/m2). Thus, biologically relevant measurements of UV-induced DNA damage can be made using T4 DNA polymerase associated exonuclease.

For the DNA sequences examined, the induction of cyclobutane dimers was the same for both single and double stranded DNA. The induction of (6-4)photoproducts was also similar for single and double stranded DNA. These results suggest that the magnitude of UV light-induced damage in regions of single stranded DNA undergoing replication should be the same as double stranded, non-replicating regions.

The T4 DNA polymerase associated exonuclease method of DNA damage analysis possesses several advantages over other DNA processing enzymes used in previous studies (2,4,5,10,11). Equivalent amounts of T4 enzyme used in previous studies can be used to analyze photoproducts occurring on either single or double stranded DNA. Large excesses of M. luteus UV-specific endonuclease are necessary to achieve equivalent incision of cyclobutane

Figure 8. Analysis of T4 DNA polymerase associated exonuclease terminations and hot alkali breaks at sites of cyclobutane dimers and (6-4) photoproducts occurring in single stranded DNA. EH1, 5'-end labeled single stranded DNA was irradiated at 254nm at doses of 0 (lanes 1, 2, 11, 12), 500 (lanes 9, 10), 2000 (lanes 7, 8), 5000 (lanes 5, 6) and 10,000 J/m^2 (lanes 3, 4) and treated with T4 DNA polymerase associated exonuclease (lanes 3, 5, 7, 9, 11); hot alkali (lanes 4, 6, 8, 10, 12); the G+A (lane 1) or C+T (lane 2) Maxam-Gilbert base specific sequencing reactions. Electrophoresis and autoradiography were as described in the text.

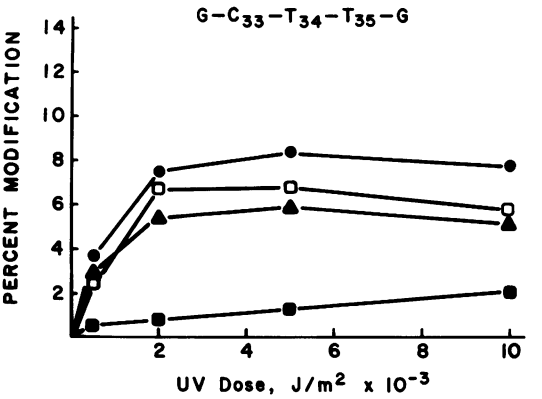


Figure 9. Estimation of cyclobutane dimer formation on UV-irradiated single stranded DNA by use of T4 DNA polymerase associated exonuclease. Dose response of UV-induced photoproduct formation occurring at C_{33}-T34-T35 in EH1 was achieved by irradiation of single stranded DNA with 0-10,000 J/m^2 of ultraviolet light (254nm), treatment with either T4 DNA polymerase associated exonuclease or hot alkali, and electrophoresis. Bands corresponding to either T4 DNA polymerase associated exonuclease terminations or hot alkali breaks were quantitated as described (Fig.5, legend). The percent cyclobutane dimer formation (□); was estimated by subtracting the percent hot alkali breaks (■) from the percent T4 DNA polymerase associated exonuclease terminations (●). The percent M. luteus UV-specific endonuclease incisions obtained for double stranded DNA (Fig. 3) is included for comparison (▲).

dimers occurring on single stranded DNA compared to double stranded DNA (4). Enzymes such as E. coli exonuclease III have been used to reveal sites of DNA modificaion caused by cis-platinum and ultraviolet light (10,11). E. coli exonuclease III analysis of base-modified DNA is confined to double stranded substrates and is further limited by multiple enzyme terminations caused by unmodified sequences (10,22). Under our assay conditions (high enzyme concentrations), T4 DNA polymerase associated exonulcease completely degraded unirradiated substrates to small oligonucleotides indicating a lack of sequence specific hydrolysis of unmodified DNA (Figs. 4 and 8). However, at lower enzyme concentrations, the exonuclease shows pause sites near G-C rich regions that can be overcome by the T4 polymerase accessory proteins (23). DNA photoproducts occurring near (within 8 bases) the 5'-end of a labeled substrate are not easily analyzed due to interference from the end-digest oligonucleotides. T4 DNA polymerase associated exonuclease analysis of photoproduct formation in UV-irradiated single stranded DNA has been successfully applied to studies investigating the relationship between UV damaged single stranded templates and the ability of DNA polymerase to

replicate beyond such damage (Chan, Doetsch, and Haseltine, submitted).

The 3' to 5' exonuclease activity of T4 DNA polymerase serves a proofreading function that is of importance in mediating the fidelity of DNA replication (24-30). The experiments presented here also suggest that the T4 DNA polymerase associated exonuclease activity may play some role in excision of cyclobutane pyrimidine dimers. In phage T4 infected E. coli , a UV-specific endonuclease incises DNA at the 5' pyrimidine of cyclobutane dimers via N-glycosylase and apyrimidinic endonuclease activities (1). This leaves the cyclobutane dimer attached to the 5' end of a nicked molecule. Hydrolysis of the fragment of DNA that contains this 5' cyclobutane dimer by the T4 DNA polymerase associated exonuclease should result in complete excision of the cyclobutane dimer from the DNA. Excision of the DNA might be followed by repair synthesis. The 3' to 5' exonuclease activity assocated with this enzyme may also be involved in repair of other stable bulky DNA adducts in phage T4 DNA.

It is likely that the assay described here will provide a qualitative and quantitative measure for the determination of the distribution of DNA adducts other than cyclobutane pyrimidine dimers and (6-4) photoproducts. Most chemicals that produce bulky stable adducts on DNA can probably be investigated using this enzyme. Preliminary experiments have demonstrated that modification of DNA by either benzo(a)pyrene diolepoxide or cis-dichlorodiamine platinum II, impede the digestion of single and double stranded DNA by the T4 enzyme at specific sequences (unpublished results). Additionally, Fuchs and his colleagues have reported that treatment of double stranded DNA with N-acetoxy-N-2-acetylaminofluorene also blocks the digestion of modified DNA by the T4 enzyme at guanine residues (31). It appears that the T4 DNA polymerase associated exonuclease will provide a general method to both identify and quantitate sites of stable DNA modification on single or double stranded DNA.

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ABBREVIATIONS

The abbreviations used are: bp, base pair; (6-4) photoproducts, 6-4' [pyrimidin-2'-one]-pyrimidine class of ultraviolet light-induced photoproducts.

*Present address: Laboratory of Radiobiology, Harvard University School of Public Health, Boston, MA 02115, USA

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