BIFILAR ENZYME-SENSITIVE SITES IN ULTRAVIOLET-IRRADIATED DNA ARE INDICATIVE OF CLOSELY OPPOSED CYCLOBUTYL PYRIMIDINE DIMERS

LUN H. LAM AND RICHARD J. REYNOLDS
Laboratory of Radiobiology, Department of Cancer Biology, Harvard University School of Public Health, Boston, Massachusetts 02115

ABSTRACT Incubation of UV-irradiated DNA with pyrimidine dimer-DNA glycosylase in cell-free lysates prepared from *Micrococcus luteus* results in the appearance of double-strand breaks. It has previously been assumed that such double-strand breaks result from cleavage at closely opposed dimers. We have used hybrid molecules of bacteriophage T7 DNA comprised of two unirradiated strands, two UV-irradiated strands, or one unirradiated and one UV-irradiated strand to test this hypothesis. Bifilar cleavage was observed only with molecules consisting of two irradiated strands and no bifilar cleavage was observed after the monomerization of pyrimidine dimers by enzymatic photoreactivation. Our results indicate that at least 80% of the double-strand breaks result from cleavage at closely opposed dimers and that the induction of dimers in one strand does not influence the induction of dimers at closely opposed positions in the complementary strand of a DNA double helix.

INTRODUCTION

Strauss and co-workers (1966) first reported the appearance of double-strand breaks in UV-irradiated DNA treated with an enzyme extract prepared from *Micrococcus luteus*. Such extracts are now known to contain pyrimidine dimer-DNA glycosylase (PD-DNA glycosylase) (Haseltine et al., 1980). Bifilar cleavage of UV-irradiated DNA upon incubation with an *M. luteus* extract has also been observed by Meneghini and coworkers (1981), and upon incubation with PD-DNA glycosylase purified from bacteriophage T4 infected *Escherichia coli* by Minton and Friedberg (1974) and by Lloyd and coworkers (1980). Both T4 and *M. luteus* PD-DNA glycosylases, together with 3′ apurinic/apyrimidinic endonuclease activities that may be associated as part of the same polypeptides (Tomilin et al., 1976; Gordon and Haseltine, 1980; Haseltine et al., 1980; Seawell et al., 1980; Nakabeppu and Sekiguchi, 1981), act by a two step mechanism to make single-strand incisions in the DNA phosphodiester backbone at the positions of pyrimidine dimers (reviewed by Haseltine, 1983). Double-strand breaks in PD-DNA glycosylase treated, UV-irradiated DNA might therefore be the result of single-strand incisions at closely opposed pyrimidine dimers, as previously suggested (Strauss et al., 1966; Meneghini et al., 1981; Minton and Friedberg, 1974; Lloyd et al., 1980). Double-strand breaks, however, might also arise from occasional nicking of the complementary strand opposite incisions at isolated pyrimidine dimers or opposite UV-induced single-strand breaks (Patrick and Rahn, 1976), nicking of both strands at UV-induced DNA-DNA interstrand crosslinks (Glisin and Doty, 1967), or shear-induced breakage of the complementary strand opposite single-strand nicks (Haward, 1974).

Evidence supporting closely opposed dimers as the source of bifilar enzyme-sensitive sites is limited and relies primarily on the specificity of the enzyme preparations used. The vast majority of far-UV induced sites recognized by the *M. luteus* enzyme are sensitive to enzymatic photoreactivation (Paterson et al., 1973; Reynolds and Lohman, 1978; Lam and Reynolds, unpublished data), a result consistent with the specificity of this activity for pyrimidine dimers (Setlow et al., 1965; Setlow, 1966). Activities against other known far-UV induced DNA lesions, including spore photoproducts, pyrimidine hydrates and bipyrimidine photoadducts of the 4-(pyrimidin-6-yl)pyrimidin-2-one type are apparently absent (Patrick and Harm, 1973; Haseltine et al., 1980; Franklin et al., 1982); however, activity against DNA-DNA interstrand crosslinks has not been excluded. A similar specificity for pyrimidine dimers has been shown for the T4 PD-DNA glycosylase (Friedberg, 1975; Radany and Friedberg, 1980).

Since bifilar cleavage might arise from nicks introduced

Dr. L. H. Lam's present address is Department of Genetics, Stanford University School of Medicine, Stanford, California 94305.

Dr. R. J. Reynolds' present address is Mail Stop M886, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545.

Address all correspondence to Dr. R. J. Reynolds.

into the complementary strand near an incision at a DNA photoproduct, the strand specificities of the activities used are also important. Carrier and Setlow (1970) examined the strand specificity of the *M. luteus* PD-DNA glycosylase in a cell-free extract and found that at least 90% of the incisions made in a hybrid DNA duplex comprised of one UV-irradiated and one unirradiated strand were made in the irradiated strand. A similar strand specificity has been demonstrated by Grossman and coworkers (1968) and Paribok and Tomilin (1971) for PD-DNA glycosylase prepared from *M. luteus* and by Simon and coworkers (1975) for activity isolated from bacteriophage T4 infected *E. coli*. Upper estimates for the amount of nicking in the dimer-free strand that might remain undetected in these studies ranged from once for every 20 dimers to once for every 70 dimers contained in the irradiated strand. At UV doses less than 30–50 J/m^2, it is expected that fewer than one bifilar site will be induced for every 100 dimers (Freifelder and Trumbo, 1969). Thus previous attempts to examine PD-DNA glycosylase strand specificity have lacked the specificity to exclude nonspecific second strand cutting as a source of double-strand breaks in enzyme-treated, UV-irradiated DNA.

To determine if bifilar enzyme-sensitive sites result from the presence of closely opposed DNA lesions, we prepared purified complementary strands of bacteriophage T7 and constructed hybrid duplexes in which neither, only one, or both strands had been UV-irradiated. These hybrid molecules were then used to determine if the formation of bifilar sites required UV-induced photoproducts in both DNA strands and to examine the relationship of bifilar sites to UV-induced interstrand crosslinks. We have also used in vitro enzymatic photoreactivation to determine if pyrimidine dimers are indeed involved in the formation of bifilar enzyme-sensitive sites.

MATERIALS AND METHODS

Preparation of Bacteriophage T7 DNA

Wild-type bacteriophage T7 and a permissive host, *E. coli* strain 011′(thy$^-$), were kindly provided by Dr. F. William Studier (1969). Bacteria were grown in tryptone broth supplemented with calcium-free M9 (Studier, 1969) and either 1 μCi/ml [methy-^{3}H]thymine (20 Ci/mmol; 1 ci = 37 GBq; New England Nuclear, Boston, MA) or 0.5 μCi/ml [methyl-^{14}C]thymine (51 mCi/mmol; New England Nuclear). Late log phase cultures were infected with T7 at a multiplicity of approximately 10 and then incubated at 30°C until phage-induced cell lysis had occurred.

Phage particles were purified from cell lysates by precipitation with polyethylene glycol (Yamamoto and Alberts, 1970) followed by isopycnic sedimentation in CsCl step gradients (Studier, 1969). CsCl was removed from phage preparations by dialysis against TES buffer (50 mM NaCl, 1 mM ethylene-diaminetetraacetate (EDTA) and 10 mM tris(hydroxymethyl)aminomethane-(Tris-) HCl, pH 7.6). T7 DNA was released from purified phage particles by the addition of N-lauroylsarcosine (Sigma Chemical Co., St. Louis, MO) to a final concentration of 0.2% (wt/vol). Contaminating protein was hydrolyzed by the addition of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) to a final concentration of 10 μg/ml followed by incubation at 37°C for 1 h. DNA was further purified by phenol extraction and extensive dialysis against TES buffer.

UV Irradiation of T7 DNA

T7 DNA in TES buffer at 10 (^{3}H-labeled) or 18 (^{14}C-labeled) μg/ml was irradiated with UV light emitted by an array of five G8T5 germicidal lamps (General Electric Company, Cleveland, OH) at an incident fluence rate of 1.19 W/m^2 as determined by an IL254 germicidal photometer (International Light, Inc., Newburyport, MA). Samples were maintained at a depth of 2 mm and exposures were adjusted to compensate for internal absorption (Morowitz, 1950).

Separation and Isolation of T7 DNA Complementary Strands

The complementary strands of bacteriophage T7 DNA were isolated by a procedure modified from that of Summers and Szybalski (1968) in which CsCl step gradients (Brunk and Leick, 1969) were used to reduce buoyant density banding times. ^{3}H-labeled and ^{14}C-labeled samples contained approximately 22.5 μg and 40.5 μg, respectively, of DNA in 2.25 ml of TES buffer. Prior to isopycnic sedimentation, DNA molecules were denatured by dialysis at room temperature against 100 ml and then 50 ml of formamide (Sigma Chemical Co.) for 16 and 3 h, respectively. Samples were incubated an additional 2 h at 37°C to ensure denaturation. Formamide was removed by dialysis against 2 l of TES buffer for 3 h at 4°C.

To effect strand separation, an excess phosphate molar ratio of poly(G,U) (Sigma Chemical Co.), which binds preferentially to T7 H-strands, was added to the denatured T7 DNA and samples were loaded onto CsCl step gradients (Brunk and Leick, 1969), with an average gradient density of 1.73 g/ml. Step gradients were centrifuged at 25,000 rpm and 25°C in a Sorvall OTD-2 preparative ultracentrifuge (DuPont Instruments, Inc., Newtown, CT) equipped with an SW 50.1 swinging-bucket rotor (Beckman Instruments, Inc., Palo Alto, CA). Under these conditions satisfactory strand separation was attained in as few as 24 h.

Gradients were fractionated by pumping the contents, bottom first, into test tubes with the aid of a Rainin Rabbit peristaltic pump (Rainin Instruments Co., Inc., Boston, MA). A small portion of each fraction was spotted onto Whatman 17 paper strips (Whatman, Inc., Clifton, NJ) marked into fractions (Carrier and Setlow, 1971). Strips were washed once in 5% (wt/vol) TCA, twice in 95% ethanol and air dried. The amount of radioactivity in each fraction was determined with the aid of a dual channel scintillation counter (Delta 300, Searle Analytic, Inc., Des Plains, IL). Peak fractions, determined from the distribution of radioactivity through a gradient, were then pooled. The purity of each strand preparation was verified by isopycnic sedimentation in the presence of poly(G,U) as described above.

Construction of ^{3}H/^{14}C T7 DNA Heteroduplexes

Heteroduplex molecules containing one ^{3}H-labeled and one ^{14}C-labeled DNA strand were constructed by reannealing the appropriate complementary strands in 50% (wt/vol) formamide (Davis and Hyman, 1971). Annealing mixtures contained approximately 4 and 5 μg of L and H strands, respectively, in 600 μl of CsCl solution as taken directly from the CsCl gradients. Excess H strand DNA was used in each annealing mixture to favor the inclusion of L strands in hybrid molecules since residual, unhybridized H strands were more readily eliminated during the purification of hybrid molecules. 20 μg of poly(C) (Sigma Chemical Co.) were added to each mixture to bind poly(G,U) carried-over from the isolation of the individual T7 DNA strands (Szybalski et al., 1971). Mixtures were dialyzed 2 h against 100 ml of formamide at room temperature and then transferred to 50 ml of fresh formamide. Dialysis was continued for 2 h at 37°C to free the H strands from bound poly(G,U). Mixtures were then dialyzed overnight against 50 ml of 50%

(vol/vol) formamide, 10 mM EDTA, 200 mM Tris-HCl, pH 8.5, at 23°C to allow poly(C) to bind poly(G,U) and to allow annealing of the complementary T7 strands. Formamide was removed by successive dialysis at 4°C against 0.5 l (0.5 h) and 2 l (2.5 h) of TES buffer. 50 μg of poly(G,U) were reintroduced into the mixtures to bind unannealed T7 H-strands and the hybrid molecules were then purified by isopycnic sedimentation as described above. Material banding at the buoyant density of native DNA was pooled and dialyzed twice against 250 ml of TES buffer for durations of approximately 2 and 16 h to eliminate CsCl. Dialysis was in nitrocellulose dialysis bags (Parlodion, Mallinckrodt, Inc., Paris, KY) (Reynolds, 1978) to eliminate residual single-stranded nucleic acids through adsorption to the nitrocellulose (Billen and Hewitt, 1966).

Detection and Quantification of Unifilar Enzyme-Sensitive Sites

The detection of unifilar enzyme-sensitive sites was modified from the procedure of Reynolds (1978). PD-DNA glycosylase was prepared from *M. luteus* as described by Carrier and Setlow (1970) except that nucleic acids were removed by precipitation with streptomycin sulfate (ICN Pharmaceuticals, Inc., Cleveland, OH). Unifilar sites were expressed as single-strand breaks by incubation of ~0.2 μg of DNA in 90 μl TES buffer with 20 μl of *M. luteus* PD-DNA glycosylase in 10% (vol/vol) ethylene glycol, 0.1 mM 2-mercaptoethanol and 10 mM Tris-HCl (pH 8.0), for 60 min at 37°C. Parallel samples incubated in the absence of enzyme were run for each determination. Reactions were stopped by the addition of 110 μl of 20 mM EDTA, 1 M NaOH.

DNA single-strand molecular weight distributions were determined by velocity sedimentation through calibrated alkaline sucrose gradients. Two hundred microliter samples were layered onto 4.9 ml 5–20% (wt/vol) alkaline sucrose gradients (10 mM EDTA, 500 mM NaCl, 200 mM NaOH) in 13 × 51 mm polyallomer ultracentrifuge tubes (Beckman Instruments, Inc.). Sedimentation was in Sorvall model OTD-2 preparative ultracentrifuges equipped with SW50.1 rotors (Beckman Instruments, Inc.) for 245 min at 35,000 rpm and 20°C. Gradients were fractionated onto Whatman #17 strips, which were processed and analyzed for the presence of radioactivity in each fraction as described above. The resulting data were analyzed and plotted with the aid of a computer.

Detection and Quantification of Bifilar Enzyme-Sensitive Sites

Bifilar enzyme-sensitive sites were detected by procedures similar to those used for the detection of unifilar enzyme-sensitive sites except that nondenaturing conditions were maintained throughout. PD-DNA glycosylase reactions were terminated by the addition of 110 μl of 2% (wt/vol) N-lauroylsarcosine, 20 mM EDTA, 2 M NaCl and 50 mM Tris-HCl (pH 7.6). Two hundred microliters of the stopped reaction were then loaded onto a 5–20% (wt/vol) neutral sucrose gradient (700 mM NaCl, 10 mM EDTA and 50 mM Tris-HCl, pH 7.6). Sedimentation in OTD-2 ultracentrifuges equipped with SW50.1 rotors was for 245 min at 25,000 rpm and 20°C. Gradients were fractionated and processed as described for the determination of unifilar enzyme-sensitive sites.

Enzymatic Photoreactivation of Bifilar Enzyme-Sensitive Sites

The susceptibility of UV-induced bifilar sites to enzymatic photoreactivation was examined by a procedure similar to that of Reynolds and Lohman (1978). DNA was isolated from Chinese hamster ovary cells grown in the presence of either 0.5 μCi/ml [methyl-^{3}H]thymidine (77.1 Ci/mmol; New England Nuclear) or 0.25 μCi/ml [methyl-^{14}C]thymidine (53.4 mCi/mmol; New England Nuclear). When indicated, cells were exposed to 50 J/m^2 of 254 nm UV light emitted by a bank of five germicidal lamps (described above) at a dose rate of 0.35 W/m^2 prior to lysis and DNA extraction. Purified DNA was dialyzed against TES buffer.

DNA photolyase reaction mixes contained 0.2 μg/ml DNA, 100 μg/ml bovine serum albumin (nuclease free; Boeringer Mannheim Biochemicals), 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.6. When indicated, *E. coli* DNA photolyase, kindly provided by Dr. A. Sancar (Univ. of North Carolina), was present at a final concentration of 1 μg/ml (4,000 units/ml; 1 unit monomerizes 1 pmol of dimers in 1 h at 20°C; Sancar *et al.*, 1984). Exposure of samples to photoreactivating light was preceded by a 10 min incubation in the dark at 20°C with or without DNA photolyase to allow binding of the photolyase to pyrimidine dimers. Photoreactivating light was provided by two Sylvania F15T8 cool white fluorescent lamps situated 15 cm from the DNA samples and was filtered through 9 mm of window glass to eliminate shorter wavelengths. Photoreactivation was at 20°C and dark held samples were kept in a light tight box at the same temperature. Reactions were terminated by placing samples on ice in the dark. After the addition of proteinase K to a final concentration of 100 μg/ml, photoreactivating enzyme was hydrolyzed by incubation at 37°C in the dark. The protease was inactivated by the addition of phenylmethylsulfonylfluoride (Sigma Chemical Co.) to a final concentration of 2 mM. Each

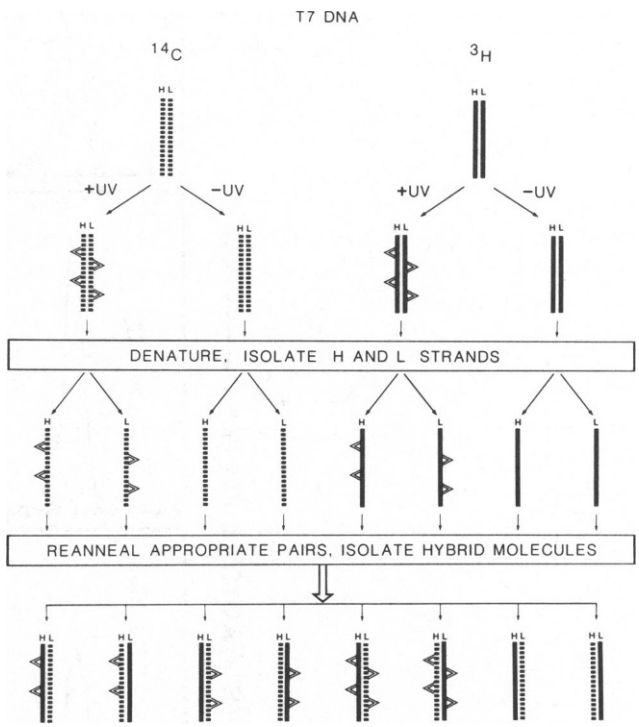

FIGURE 1 Scheme for the construction of hybrid DNA duplexes with UV-induced lesions in both, neither, or only one of the two complementary strands. Separate preparations of T7 DNA labeled with either ^{3}H or ^{14}C were divided and either left unirradiated or irradiated with UV light (500 J/m^2 at 254 nm). Parental duplexes were denatured with formamide and individual strands were separated by isopycnic sedimenation in CsCl density gradients containing poly(G,U). The eight single-strand species recovered were mixed in appropriate combinations of differentially-labeled, complementary strands and reannealed in 50% (vol/vol) formamide to generate the eight hybrid molecules indicated. Four hybrid duplex species contained UV-induced lesions in only one strand, two contained lesions in both strands and two contained lesions in neither strand. Each hybrid species was then purified by isopycnic sedimentation in CsCl density gradients (see Fig. 2). ^{3}H-labeled DNA (▬▬); ^{14}C-labeled DNA (▬ ▬ ▬); heavy strand (H); light strand (L); Uv-irradiated DNA (▬◢◣▬, ▬◢◣▬).

sample was divided in half and assayed for the presence of unifilar and bifilar PD-DNA glycosylase sensitive sites as described above except that sedimentation conditions were adjusted so as to place DNA distributions near the center of each gradient. Controls incubated in the absence of PD-DNA glycosylase were run for all samples.

RESULTS

Construction of Hybrid Molecules

The scheme for the construction of hybrid molecules is presented in Fig. 1. Separate preparations of T7 DNA were uniformly labeled with either ^{3}H or ^{14}C. One-half of each preparation was left unirradiated and one-half was exposed to UV (500 J/m^2). The four resulting parental duplexes were independently denatured in formamide and the complementary strands, designated H and L, were resolved by isopycnic sedimentation. In this way, eight distinct species of purified, single-stranded DNA were isolated. The eight species represent the eight possible combinations of three dichotomous parameters: label (^{3}H or ^{14}C), strand (H or L), and irradiation (^{+}UV or ^{-}UV).

Hybrid molecules were prepared by mixing and reannealing portions of the isolated strands in different paired combinations. The individual strand types for each pair were chosen such that the radionuclides would be different and the strands complementary in each case. This pairing yielded eight heteroduplex species: four with one unirra-

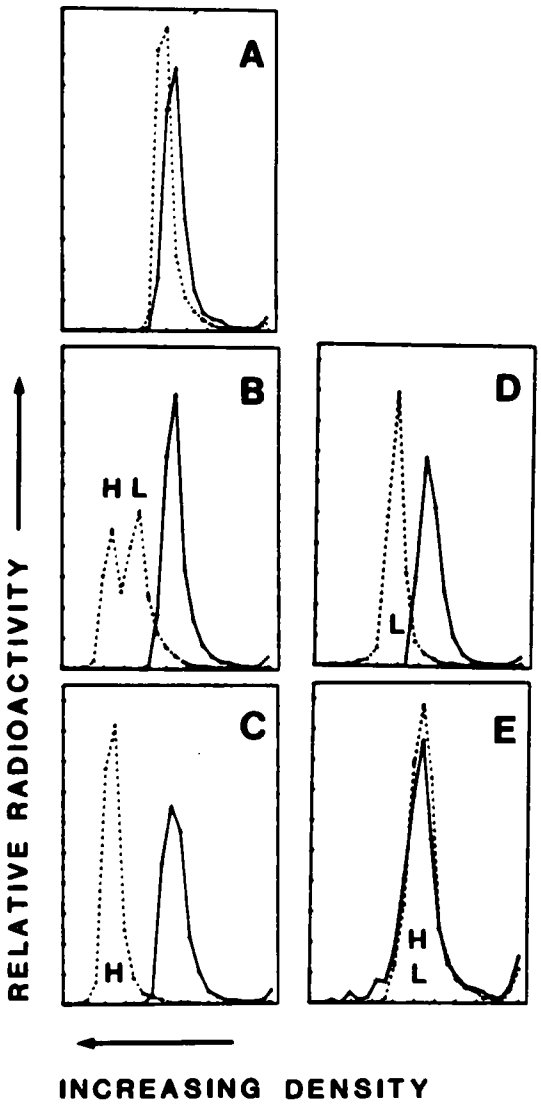

FIGURE 2 Separation of bacteriophage T7 DNA heavy (H) and light (L) complementary strands and purification of reannealed hybrids. *Panel A:* Isopycnic co-sedimentation of native and denatured T7 DNA in the absence of poly(G,U). Native DNA, (——); denatured T7 DNA, (----). *Panel B:* Isopycnic cosedimentation of native and denatured T7 DNA in the presence of poly(G,U). Native DNA, (——); denatured T7 DNA, (----). *Panel C:* Isopycnic sedimentation rebanding with poly(G,U) of T7 H-strand obtained by pooling fractions from peak "H" of panel *B*. Native DNA, (——); T7 H-strand DNA, (----). *Panel D:* Isopycnic sedimentation rebanding with poly(G,U) of T7 L-strand obtained by pooling fractions from peak "L" of panel B. Native DNA, (——), T7 L-strand DNA, (----). *Panel E:* Isopycnic sedimentation of reanealed hybrid DNA in the presence of poly(G,U). L-strand, (——); H-strand, (----). *Panels A–E:* ^{3}H-labeled DNA (——); ^{14}C-labeled DNA (----).

diated and one irradiated strand (UV^-/UV^+ hybrids), two with two unirradiated strands (UV^-/UV^- hybrids), and two with two irradiated strands (UV^+/UV^+ hybrids). Hybrid molecules were isolated and purified as described in Materials and Methods. Sedimentation profiles demonstrating the separation of the complementary strands and the purity of the pooled material are presented in Fig. 2. (Profiles are representative of those obtained in actual experiments but were run separately with additional marker DNA's to facilitate comparisons.) In the absence of poly(G,U), the buoyant density of the denatured DNA was slightly greater than that of a native T7 DNA marker (Fig. 2, panel *A*). In the presence of poly(G,U), the denatured DNA was resolved into two peaks, corresponding to the complementary strands of T7 DNA (Szybalski et al., 1971) (Fig. 2, panel *B*). The buoyant density of native DNA was unaffected by the presence of poly(G,U) (Fig. 2, panels *A* and *B*).

The purities of the individual strand preparations were tested by rebanding a sample of the isolated strands in a second CsCl buoyant density gradient. Representative profiles are presented in Fig. 2, panels *C* and *D*. In each case, the rebanded material was found to have the same buoyant density as the original material and to be free of contamination by the complementary strand or by DNA of native density. Similar purities were found for all strand preparations in actual experiments.

Hybrid molecules formed by annealing differentially labeled H and L strands were also purified by isopycnic sedimentation in CsCl density gradients. Successful hybridization was indicated by the banding of hybrid molecules at the position of native, parental T7 DNA. The distribution of radioactivity through a representative gradient, depicted in Fig. 2, panel *E*, demonstrates the relative purity of hybrid molecules that were obtained by this procedure.

Unifilar Enzyme-Sensitive Sites in Hybrid Molecules

The compositions of the eight classes of hybrid molecules and the sensitivity of the individual strands in each class to incision upon incubation with PD-DNA glycosylase were analyzed by alkaline sucrose gradient sedimentation. Denaturation of the hybrid molecules in alkali allowed independent sedimentation of the complementary strands. Differential labeling of the complementary strands enabled the examination of each strand for nicking.

Alkaline sucrose gradient sedimentation profiles for enzyme-treated hybrid molecules are depicted in Fig. 3. UV^-/UV^+ hybrid molecules exhibited marked degradation of the irradiated strand without any detectable effect on the unirradiated strand. UV^+/UV^+ hybrid molecules exhibited marked degradation of both strands. UV^-/UV^-

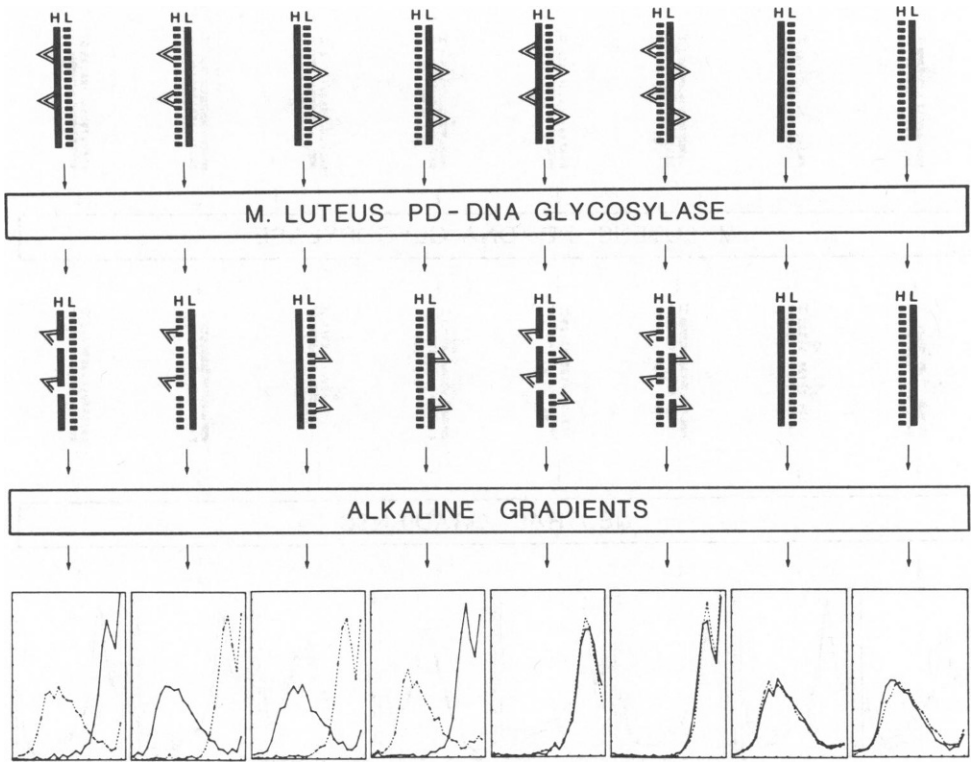

FIGURE 3 Unifilar nicking by *M. luteus* PD-DNA glycosylase acting on hybrid DNA molecules. Eight different species of hybrid molecules, prepared as indicated in Fig. 1, were incubated with PD-DNA glycosylase, denatured with NaOH and sedimented through alkaline sucrose gradients. The direction of sedimentation is from right to left. The complementary strands (H or L) of each hybrid species were differentially labeled with ^3H (——) or ^{14}C (----).

hybrid molecules were not significantly affected by incubation with PD-DNA glycosylase. In each and every case, irradiated strands were degraded upon incubation with PD-DNA glycosylase into fragments that did not sediment significant distances into the gradients while unirradiated strands cosedimented with strands from native T7 molecules with molecular weights of approximately 5×10^6. (Single strand molecular weights were reduced relative to the expected value of 13.2×10^6 (Dunn and Studier, 1983) by radioisotopic decays during the preparation of hybrid molecules.) The degradation of irradiated strands was enzyme dependent as demonstrated by the absence of any significant change in sedimentation rate through alkaline sucrose gradients for either irradiated or unirradiated strands after incubation in the absence of PD-DNA glycosylase (data not shown). These results verify the putative compositions of the individual hybrid molecules and demonstrate that extensive nicking had occurred in all irradiated strands.

Bifilar Nicking of Hybrid Molecules

The eight classes of hybrid molecules and four types of native T7 parental molecules were assayed for the presence of bifilar enzyme-sensitive sites by velocity sedimentation through neutral sucrose gradients. Incubation of UV-irradiated, native T7 DNA molecules with PD-DNA glycosylase resulted in a significant reduction in sedimentation rate through neutral sucrose gradients (data not shown). Hybrid molecules comprised of two irradiated strands and not incubated with PD-DNA glycosylase sedimented at the position of native, parental T7 DNA (data not shown), which has a molecular weight of 26.4×10^6 (Dunn and Studier, 1983). Incubation of UV^+/UV^+ hybrid molecules with PD-DNA glycosylase resulted in a reduction in sedimentation rate (Fig. 4) that was equivalent to that observed upon enzyme treatment of UV-irradiated parental T7 DNA (data not shown). The reduced sedimentation rate of enzyme-treated UV^+/UV^+ hybrid molecules corresponded to number-average molecular weights of $\sim 3.4 \times 10^6$.

No reduction in sedimentation rate was evident after enzyme-treatment of hybrid molecules comprised of either two unirradiated strands or one irradiated strand and one unirradiated strand. Enzyme-treatment was also without effect on the sedimentation rate of unirradiated, native T7 molecules (data not shown), and no significant decrease in sedimentation rate was evident after incubation of any of the hybrid or native molecules in the absence of PD-DNA glycosylase (data not shown).

Sensitivity of Assay for Second Strand Cutting

The lower limit of sensitivity for detection of double-strand breaks in the neutral gradients was estimated by the

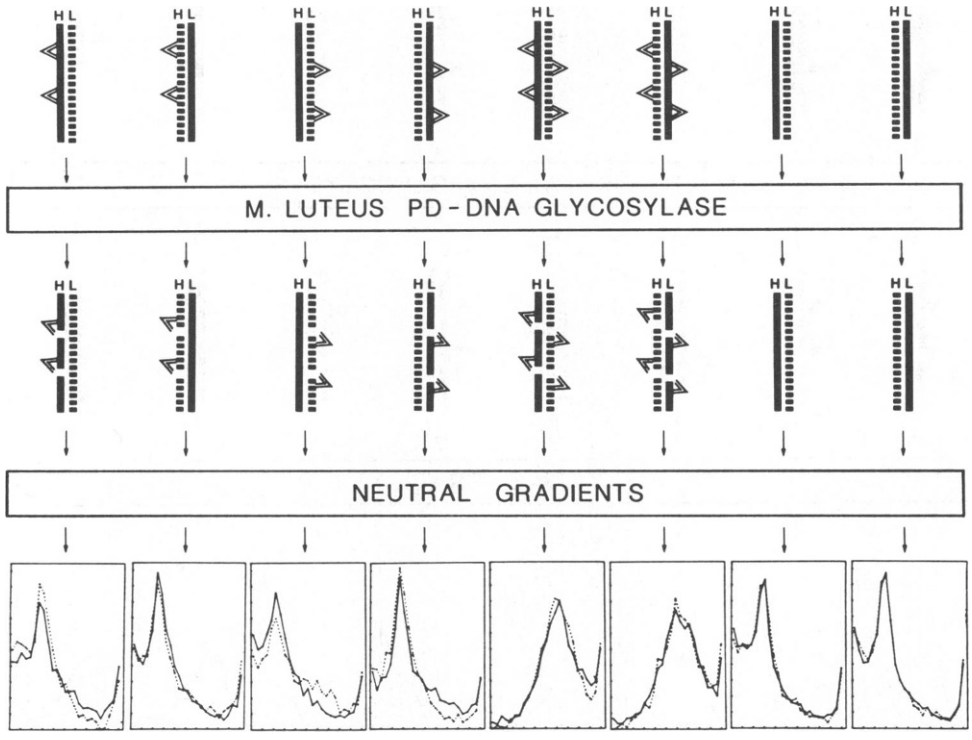

FIGURE 4 Bifilar nicking by *M. luteus* PD-DNA glycosylase acting on hybrid DNA molecules. Hybrid molecules, prepared as indicated in Fig. 1, were incubated with PD-DNA glycosylase and sedimented through neutral sucrose gradients. The direction of sedimentation is from right to left. The complementary strands (H or L) of each hybrid species were differentially labeled with ^3H (——) or ^{14}C (----). Both strands of UV^+/UV^+ hybrid molecules sedimented with number-average molecular weights of approximately 3.4×10^6.

following empirical method. Unirradiated ^{3}H-labeled T7 duplexes and ^{14}C-labeled duplexes irradiated with 50 J/m^2 of UV light were mixed, treated with *M. luteus* PD-DNA glycosylase and sedimented through neutral sucrose gradients as described in Materials and Methods. As can be seen in Fig. 5, panel *A*, an increase in the amount of degraded material and a corresponding decrease in the amount of unit-size material is readily apparent in the irradiated population when compared with the unirradiated population. Similar results were obtained in seven separate gradients.

For statistical analysis, each sedimentation profile was divided into two regions: material sedimenting more than 60% of the distance into the gradient and material sedimenting between 10 and 60% of the distance into the gradient. For each profile, the percentage of material in each region of the gradient was determined gravimetrically. With unirradiated samples, 82.4 ±1.2% of the sample sedimented as high molecular weight material (95% confidence interval). After irradiation with 50 J/m^2 only 70.4 ± 1.4% of the sample sedimented to the high molecular weight region. Homogeneity of variances by the *F* test ($F = 1.3$) permitted application of the *t* test for comparison of two independent means. By this analysis, the difference between size distributions for irradiated and unirradiated T7 molecules after enzyme treatment is shown to be highly significant ($t = 16.4$, $P < 0.001$), and demonstrates our ability to detect this level of breakage.

To ascertain if significant numbers of double-strand breaks resulted from enzyme treatment of UV$^+$/UV$^-$ hybrid molecules, neutral sucrose gradient profiles obtained with enzyme-treated UV$^+$/UV$^-$ hybrids were compared to those of the enzyme-treated UV$^-$/UV$^-$ hybrids and analyzed as described above. Representative profiles are presented in Fig. 5, panel *B*. After enzyme treatment of UV$^+$/UV$^-$ hybrid molecules, 75.3 ±1.9% of the DNA sedimented to the high molecular weight region. After enzyme treatment of UV$^-$/UV$^-$ hybrid molecules, 77.0 ± 1.2% of the DNA sedimented to the high molecular weight region. By this analysis, there is no significant difference between the sedimentation rates of enzyme-treated UV$^-$/UV$^-$ and UV$^+$/UV$^-$ hybrids ($F = 2.6$, $t = 2.1$, $P > 0.05$).

In separate experiments we have observed that the dose response for bifilar sites is nearly linear with dose and that approximatley 0.0004 bifilar sites per 10^6 molecular weight are induced by each J/m^2 of 254 nm UV light. With a molecular weight of 26.43 × 10^6 (Dunn and Studier, 1983), a T7 DNA molecule irradiated with 50 Jm^2 should have, on the average, 0.5 bifilar sites. (Due to the relatively small numbers of sites induced, it was not possible to determine directly the average number of bifilar sites induced per T7 DNA molecule by 50 J/m^2) Since significant numbers of bifilar sites were induced by 50 J/m^2 and since significant numbers of bifilar sites were not present in hybrid molecules comprised of one strand irra-

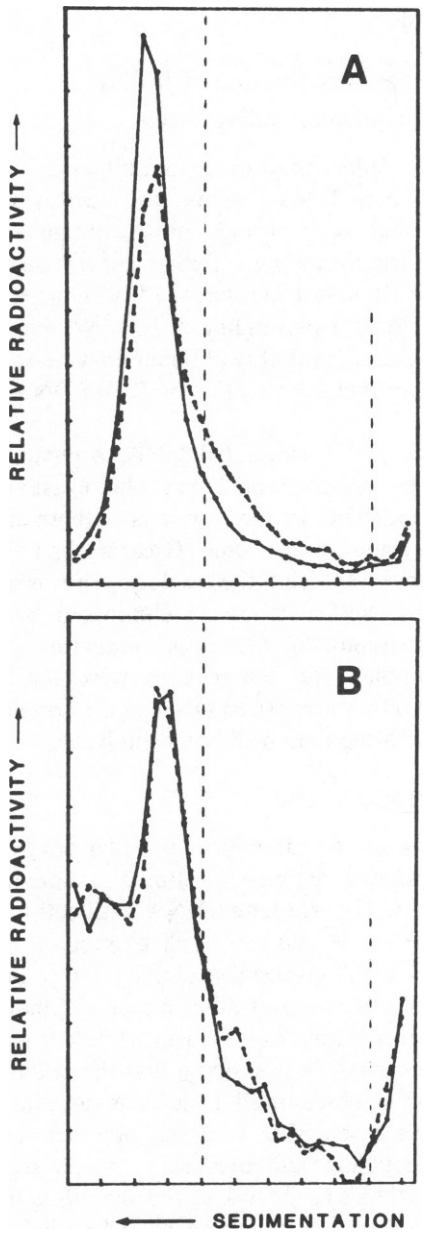

FIGURE 5 Detection of bifilar enzyme-sensitive sites at a frequency of 0.5 sites per T7 DNA molecule. *Panel A:* A mixture of unirradiated ^{3}H-labeled (——) and UV-irradiated (50 J/m^2 at 254 nm) ^{14}C-labeled (----) native T7 DNA was incubated with *M. luteus* pyrimidine dimer-DNA glycosylase to convert bifilar sites to double-strand breaks. Breaks were detected by sedimentation analysis in neutral sucrose density gradients (sedimentation was from right to left). 50 J/m^2 was estimated to induce an average of one bifilar enzyme-sensitive site for every two molecules of T7 DNA. *Panel B:* Profiles of two ^{14}C-labeled L strands of T7 DNA are co-plotted. One (——) had been hybridized to an unirradiated and the other (----) to an irradiated (500 J/m^2) ^{3}H-labeled H strand during incubation with *M. luteus* pyrimidine dimer-DNA glycosylase. The two hybrid preparations were sedimented in neutral sucrose gradients under identical conditions.

diated with 500 J/m^2 and one with unirradiated strand, we have concluded that at least 80% of the bifilar sites detected result from the presence of UV-induced lesions in both strands.

Photoreactivation of Bifilar Enzyme-Sensitive Sites

The above results demonstrate that bifilar sites are formed only when both DNA strands have been irradiated and therefore that both strands must contain UV-induced lesions for the formation of bifilar enzyme-sensitive sites. They do not demonstrate that the UV-induced lesions are in fact cyclobutyl pyrimidine dimers. We have therefore examined the susceptibility of bifilar enzyme-sensitive sites to enzymatic photoreactivation by *E. coli* DNA photolyase in vitro.

Exposure of UV-irradiated DNA to photoreactivating light in the presence of DNA photolyase resulted in marked reductions in the numbers of both unifilar and bifilar enzyme-sensitive sites. Greater than 98% of the unifilar sites and, within the resolution of this experiment, 100% of the bifilar sites were eliminated by enzymatic photoreactivation (Fig. 6). Similar reductions in the numbers of unifilar and bifilar sites were not seen upon incubation of UV-irradiated DNA in the absence of either photoreactivating light or DNA photolyase.

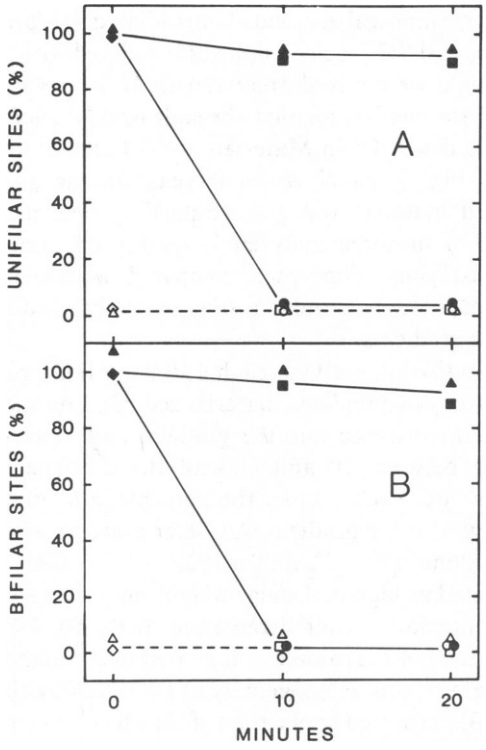

FIGURE 6 *In vitro* enzymatic photoreactivation of unifilar and bifilar enzyme-sensitive sites. ^{3}H- or ^{14}C-labeled DNA's were purified from unirradiated and UV-irradiated (50 J/m^2 at 254 nm) Chinese hamster ovary cells. Unirradiated and UV-irradiated DNA's were mixed and then incubated with or without *E. coli* DNA photolyase (PRE) either in the dark or in the presence of photoreactivating light (PRL). Individual samples were then treated with *M. luteus* PD-DNA glycosylase, divided in half and analyzed for the presence of unifilar (*panel A*) and bifilar (*panel B*) enzyme-sensitive sites as described under Materials and Methods. Unirradiated, ^{3}H-labeled DNA (open symbols); UV-irradiated, ^{14}C-labeled DNA (closed symbols). −PRE, −PRL (◇, ◆); +PRE, −PRL (△, ▲); −PRE, +PRL (□, ■); +PRE, +PRL (○, ●).

DISCUSSION

We have examined the nature of bifilar enzyme-sensitive sites expressed as double-strand breaks subsequent to the incubation of UV-irradiated DNA with cell-free extracts prepared from *M. luteus*. Such extracts are known to contain PD-DNA glycosylase activity and to be devoid of incising activity acting at other major UV-induced DNA photoproducts (Patrick and Harm, 1973). It would therefore seem reasonable to suppose that the bifilar sites arise from closely opposed unifilar incisions introduced by PD-DNA glycosylase acting at closely opposed dimers. Nevertheless, double-strand breaks in enzyme-treated, UV-irradiated DNA's might also arise from infrequent nicking of the second strand opposite a nicked pyrimidine dimer, nicking of the second strand opposite a UV-induced single-strand break (Patrick and Rahn, 1976), nicking of both strands at a UV-induced interstrand crosslink (Glisin and Doty, 1967), or shear-induced degradation (Haward, 1974). If the bifilar sites are truly related to the presence of closely opposed pyrimidine dimers they should (*a*) require the presence of UV-induced lesions in both strands of the DNA helix, and (*b*) be susceptible to enzymatic photoreactivation.

To determine if the formation of bifilar sites required the presence of UV-induced lesions in both strands, in general, and if they were related to nicks created opposite incisions at pyrimidine dimers or single-strand breaks, in particular, UV$^-$/UV$^+$ hybrid molecules were examined for the presence of bifilar enzyme-sensitive sites. None were detected. Double-strand breaks were evident only after enzyme treatment of UV$^+$/UV$^+$ hybrid molecules. Analysis of single-strand nicking demonstrated that all UV-irradiated strands were degraded by the enzyme treatment regardless of whether or not the complementary strand was also irradiated (Fig. 3). Therefore the absence of bifilar sites in UV$^+$/UV$^+$ hybrid molecules is not due to a simple inhibition of nicking activity. Furthermore the numbers of bifilar sites present in UV$^+$/UV$^+$ hybrid molecules and in the original, undenatured UV-irradiated parental molecules were not significantly different. Thus denaturation and rehybridization had no significant effect on the numbers of bifilar sites detected.

We have also found that few, if any, of the bifilar sites arise from incisions at DNA-DNA interstrand crosslinks (Rahn and Patrick, 1976). Due to the experimental protocol used, the complementary strands of UV$^+$/UV$^+$ hybrid molecules had to be from independently irradiated molecules. Crosslinking would have prevented strand separation

during formamide denaturation and crosslinked molecules should have renatured rapidly after being returned to nondenaturing conditions. Reannealed molecules would have banded at the density of native DNA instead of with either H or L strands during isopycnic sedimentation and would have been eliminated prior to the construction of hybrid molecules. But despite the absence of crosslinked molecules, no fewer bifilar sites were observed with UV^+/UV^+hybrid molecules than with undenatured parental molecules (data not shown).

Consistent with the presence of small numbers of UV-induced interstrand crosslinks, small amounts of rapidly renaturing, native density DNA were observed in UV-irradiated samples (data not shown). This rapidly renaturing material was also isolated and analyzed for the presence of bifilar sites. If bifilar sites were to occur preferentially at interstrand crosslinks, the rapidly renaturing DNA should be enriched for these sites. No such enrichment was observed. Similar numbers of bifilar sites were found in rapidly renatured DNA, parental UV-irradiated DNA, and UV^+/UV^+ hybrid molecules. Furthermore, if nicking had been found to occur at interstrand crosslinks its contribution to the numbers of bifilar sites would have been small as indicated by the relatively small amount of rapidly renaturing DNA. In these experiments, approximately five bifilar sites were induced per molecule. Only ~5% of the irradiated DNA was found to be rapidly renaturing. If a Poisson distribution is assumed, these results indicate that one crosslink is induced for every twenty molecules or approximately one crosslink for every one hundred bifilar sites.

Double-strand breaks might also arise from shear-induced breakage occurring preferentially at the positions of single-strand nicks (Harvard, 1974). If this were the source of the bifilar sites then the enzyme-treated UV^+/UV^- hybrid molecules, which contain nicks in one of the two strands, should be sensitive to shear-induced breakage, and should suffer at least half the double-strand breaks seen in their bifilarly nicked counterparts. In fact, no double-strand breaks were detected in the UV^+/UV^- hybrids.

In summary, we have demonstrated that the formation of bifilar sites requires that both strands of a DNA double helix contain UV photoproducts. Nonspecific nicks in the second strand, bifilar nicks at interstrand crosslinks, and shear degradation at single-strand nicks do not contribute significantly to the presence of bifilar enzyme-sensitive sites. We have also demonstrated that the bifilar sites are sensitive to enzymatic photoreactivation and therefore must, at least in part, depend upon the presence of pyrimidine dimers (see Fig. 6).

It should be noted that, in the strictest sense, we have implicated the presence of a pyrimidine dimer in only one of the two complementary strands at a bifilar site. If one of two closely opposed lesions susceptible to glycosylic and/or endonucleolytic attack by activities in the *M. luteus* extract were a pyrimidine dimer, photoreactivation of that dimer would be sufficient to prevent detection of a bifilar site. This model, however, requires the presence of a second UV-induced DNA lesion subject to incision. *M. luteus* extracts similar to that used in this study are remarkably specific for pyrimidine dimers in UV-irradiated DNA's. Activity against other major far-UV DNA lesions is apparently absent (Patrick and Harm, 1973). Activity against minor photoproducts cannot be excluded, but coincidental induction of some such lesion at a position closely opposed to a pyrimidine dimer would be much less likely than the induction of two dimers at closely opposed positions. We have therefore concluded that within the resolution of our experiments the majority of, if not all, bifilar sites result from the presence of closely opposed pyrimidine dimers. This conclusion is also supported by studies employing DNA sequence analysis to examine the nature of unifilar cleavages at bifilar sites (Lam, 1985).

The lower limit for the sensitivity of our analysis was determined in separate control experiments. Irradiation with 500 J/m^2, the dose used to test PD-DNA glycosylase strand specificity, induces approximately five bifilar sites per T7 molecule when both strands are UV-irradiated. However, UV^+/UV^- hybrid molecules contained only half the dimer load since only one of the two strands had been UV-irradiated. In other words, UV^+/UV^- hybrids might be expected to have 2.5 bifilar sites if such sites were due to limited nicking opposite an incision at a dimer. Our assay was found to be able to detect consistently an average of 0.5 double-strand breaks per T7 DNA molecules. If 1 out of every 5 bifilar sites were due to nicking of the complementary strand, we should have been able to detect the resulting molecular weight reduction for UV^+/UV^- hybrids. No such reduction was observed. Based upon these results, we have concluded that greater than 80 percent of bifilar enzyme-sensitive sites represent closely opposed pyrimidine dimers. It should be noted that this is a lower limit for the contribution of closely opposed dimers to bifilar sites and that we were unable to demonstrate any "bifilar sites" arising by mechanisms unrelated to the presence of closely opposed dimers.

Another interesting conclusion concerning the possibility of synergistic interactions between complementary strands during irradiation can also be made from our studies with hybrid molecules. Minton and Friedberg (1974) have observed that many more double-strand breaks result from the incubation of UV-irradiated DNA with PD-DNA glycosylase activity than would be expected from random single-strand events. They proposed that the induction of a dimer in one strand facilitates the induction of a second dimer at a nearby position in the complementary strand. In our experiments, the individual strands of UV^+/UV^+ hybrid molecules were derived from separate populations of molecules and could not have been hydrogen bonded to each other at the time of irradiation. Denaturation and rehybridization, however, was found to be without

effect on the yield of bifilar sites (data not shown). Therefore events in each strand must have occurred independently of events in its complementary strand. We have also found that the maximal distance between closely opposed dimers expressed as bifilar sites is approximately 15 base pairs (Lam and Reynolds, unpublished results), a value in rough agreement with that used by Minton and Friedberg (1974) to estimate the numbers of closely opposed dimers expected from specific UV doses. Despite these observations, yields of bifilar sites at low UV doses were too high to be explained on the basis of their formation from totally random, independent events (Minton and Friedberg, 1974; Lam and Reynolds, unpublished results). We have therefore concluded that certain stretches of DNA must favor the induction of bifilar sites and that the nucleotide sequence itself must be the primary determinant of induction probabilities. Recent results in our laboratory have confirmed a marked sequence dependence for the induction of bifilar enzyme-sensitive sites (Lam and Reynolds, 1985; Lam, 1985).

Closely opposed dimers may play prominent roles in the induction of mutations (Bresler, 1975), genetic recombination (Bradley and Taylor, 1983), and cell killing (Harm, 1968), by UV radiation. We expect that the identity of bifilar sites to closely opposed dimers demonstrated in this study will enable the development of quantitative assays that will facilitate further investigations into the induction and biologic significance of closely opposed dimers.

This work was supported by research grant CA-33949, training grant CA-09078, and center grant ES-00002 from the U.S. National Institutes of Health.

Received for publication 3 October 1985 and in final form 3 March 1986.

REFERENCES

Billen, D., and R. Hewitt. 1966. Selective adsorption of denatured DNA to cellulose nitrate tubes during preparative density-gradient equilibrium sedimentation. *Anal. Biochem.* 15:177–180.

Bradley, M. O., and V. I. Taylor. 1983. Repair-induced DNA double strand breaks after ultraviolet-light and either aphidocolin or 1-β-D-arabinofuranosyl-cytosine/hydroxyurea. *Carcinogenesis.* 14:1513–1517.

Bresler, S. E. 1975. Theory of misrepair mutagenesis. *Mutation Res.* 29:467–474.

Brunk, C. R., and V. Leick. 1969. Rapid equilibrium isopycnic CsCl gradients. *Biochim. Biophys. Acta.* 179:136–144.

Carrier, W. L., and R. B. Setlow. 1970. Endonuclease from *Micrococcus luteus* which has activity toward ultraviolet-irradiated deoxyribonucleic acid: purification and properties. *J. Bact.* 102:178–186.

Carrier, W. L., and R. B. Setlow. 1971. Paper strip method for assaying gradient fractions containing radioactive macromolecules. *Anal. Biochem.* 43:427–432.

Davis, R. W., and R. W. Hyman. 1971. A study in evolution: the DNA base sequence homology between coliphages T7 and T3. *J. Mol. Biol.* 62:287–301.

Dunn, J. J., and F. W. Studier. 1983. The complete nucleotide sequence of bacteriophage T7 DNA, and the locations of T7 genetic elements. *J. Mol. Biol.* 166:477–536.

Franklin, W. A., K. M. Lo, and W. A. Haseltine. 1982. Alkaline lability of fluorescent photoproducts produced in ultraviolet light-irradiated DNA. *J. Biol. Chem.* 257:13535–13543.

Freifelder, D., and B. Trumbo. 1969. Matching of single-strand breaks to form double-strand breaks in DNA. *Biopolymers.* 7:681–693.

Friedberg, E. C. 1975. DNA repair of ultraviolet-irradiated bacteriophage T4. *Photochem. Photobiol.* 21:277–289.

Gordon, L. K., and W. A. Haseltine. 1980. Comparison of the cleavage of pyrimidine dimers by the bacteriophage T4 and *Micrococcus luteus* UV-specific endonucleases. *J. Biol. Chem.* 255:12047–12050.

Glisin, V. R., and P. Doty. 1967. The cross-linking of DNA by ultraviolet radiation. *Biochim. Biophys. Acta.* 142:314–322.

Grossman, L., J. C. Kaplan, S. R. Kushner, and I. Mahler. 1968. Enzymes involved in the early stages of repair of ultraviolet-irradiated DNA. *In* Replication of DNA in micro-organisms. Vol. XXXIII. *Cold Spring Harbor Symp. Quant. Biol.* 229–234.

Harm, W. 1968. Effects of dose fractionation on ultraviolet survival of *Escherichia coli*. *Photochem. Photobiol.* 7:73–86.

Haseltine, W. A. 1983. Ultraviolet light repair and mutagenesis revisited. *Cell.* 33:13–17.

Haseltine, W. A., L. K. Gordon, C. P. Lindan, R. H. Grafstrom, N. L. Shaper and L. Grossman. 1980. Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of *M. luteus*. *Nature (Lond.).* 285:634–641.

Haward, G. S. 1974. Unique double-stranded fragments of bacteriophage T5 DNA resulting from preferential shear-induced breakage at nicks. *Proc. Natl. Acad. Sci. USA.* 71:2108–2112.

Lam, L. H. 1985. *Induction and repair of closely opposed pyrimidine dimers.* Sc.D. dissertation. Harvard University School of Public Health, Boston, Massachusetts. 182 pp.

Lam, L. H., and R. J. Reynolds. 1985. Site specificity of closely opposed dimers. *In* Abstract of Papers for the Thirty-Third Annual Meeting of the Radiation Research Society. Ef-2. 37. (Abstr.)

Lloyd, R. S., P. C. Hanawalt, and M. L. Dodson. 1980. Processive action of T4 endonuclease V on ultraviolet-irradiated DNA. *Nucleic Acids Res.* 8:5113–5127.

Meneghini, R., C. F. M. Menck, and R. J. Schumacher. 1981. Mechanisms of tolerance to DNA lesions in mammalian cells. *Q. Rev. Biophys.* 14:381–432.

Minton, K., and E. C. Friedberg. 1974. Evidence for clustering of pyrimidine dimers on opposite strands of U.V.-irradiated bacteriophage DNA. *Int. J. Radiat. Biol.* 26:81–85.

Morowitz, H. J. 1950. Absorption effects in volume irradiation of microorganisms. *Science (Wash. DC).* 111:229–230.

Nakabeppu, Y., and M. Sekiguchi. 1981. Physical association of pyrimidine dimer DNA glycosylase and apurinic-apyrimidinic DNA endonuclease essential for repair of ultraviolet-damaged DNA. *Proc. Natl. Acad. Sci. USA.* 78:2742–2746.

Paribok, V. P., and N. V. Tomilin. 1971. Recognition of pyrimidine dimers in DNA by the incision enzyme from *Micrococcus lysodeikticus*. *Nature New Biol.* 230:210–211.

Paterson, M. C., P. H. M. Lohman, and M. L. Slyter. 1973. Use of UV endonuclease from *Micrococcus leteus* to monitor the progress of DNA repair in UV-irradiated human cells. *Mutation Res.* 19:245–256.

Patrick, M. H., and H. Harm. 1973. Substrate specificity of a bacterial UV endonuclease and the overlap with *in vitro* photoenzymatic repair. *Photochem. Photobiol.* 18:371–386.

Patrick, M. H., and R. O. Rahn. 1976. Photochemistry of DNA and polynucleotides: Photoproducts. *In* Photochemistry and Photobiology of Nucleic Acids. S. Y. Wang, editor. Vol. II. Academic Press, Inc., New York. 35–95.

Radany, E. H., and E. C. Friedberg. 1980. A pyrimidine dimer-DNA glycosylase activity associated with the v gene product of bacteriophage T4. *Nature (Lond.).* 286:182–185.

Rahn, R. O., and M. H. Patrick. 1976. Photochemistry of DNA; secondary structure, photosensitization, base substitution, and exogeneous molecules. *In* Photochemistry and Photobiology of Nucleic

Acids. S. Y. Wang, editor. Vol. II. Academic Press, Inc., New York. 97–145.

Reynolds, R. J. 1978. Removal of pyrimidine dimers from *Saccharomyces cerevisiae* nuclear DNA under nongrowth conditions as detected by a sensitive, enzymatic assay. *Mutation Res.* 50:43–56.

Reynolds, R. J., and P. H. M. Lohman. 1978. *Micrococcus luteus* UV-endonuclease-sensitive sites in far- and near-UV irradiated Chinese hamster ovary cells. *In* DNA Repair Mechanisms. P. C. Hanawalt, E. C. Friedberg, and E. C. Fox, editors. Academic Press, Inc., New York. 27–30.

Sancar, A., F. W. Smith, and G. B. Sancar. 1984. Purification of *Escherichia coli* DNA photolyase. *J. Biol. Chem.* 259:6028–6032.

Seawell, P. C., C. A. Smith, and A. K. Ganesan. 1980. *denV* gene of bacteriophage T4 determines a DNA glycosylase specific for pyrimidine dimers in DNA. *J. Virol.* 35:790–797.

Setlow, J. K. 1966. The molecular basis of biological effects of ultraviolet radiation and photoreactivation. *In* Current Topics in Radiation Research. M. Ebert and A. Howard, editors. Vol. 2. Elsevier/North Holland Publishing Co., Amsterdam. 195–248.

Setlow, J. K., M. E. Boling, and F. J. Bollum. 1965. The chemical nature of photoreactivable lesions in DNA. *Proc. Natl. Acad. Sci. USA.* 53:1430–1436.

Simon, T. J., C. A. Smith, and E. C. Friedberg. 1975. Action of bacteriophage T4 endonuclease on duplex DNA containing one ultraviolet-irradiated strand. *J. Biol. Chem.* 250:8748–8752.

Strauss, B., T. Searashi, and M. Robbins. 1966. Repair of DNA studied with a nuclease specific for UV induced lesions. *Proc. Natl. Acad. Sci. USA.* 56:932–939.

Studier, W. F. 1969. The genetics and physiology of bacteriophage T7. *Virology.* 39:562–574.

Summers, W. C., and W. Szybalski. 1968. Totally asymmetric transcription of coliphage T7 *in vivo:* correlation with poly G binding sites. *Virology.* 34:9–16.

Szybalski, W., H. Kubinski, Z. Hradecna, and W. C. Summers. 1971. Analytical and preparative separation of the complementary DNA strands. *In* Methods in Enzymology. L. Grossman and K. Moldave, editors. Vol. 21D. Academic Press, Inc., New York. 383–413.

Tomilin, N. V., E. B. Paveltchuk, and T. V. Mosevitskaya. 1976. Substrate specificity of the ultraviolet-endonuclease from *Micrococcus luteus.* Endonucleolytic cleavage of depurinated DNA. *Eur. J. Biochem.* 69:265–272.

Yamamoto, K. R., and B. M. Alberts. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology.* 40:734–744.