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Exploration of the functional significance of intra-dimer phosphodiester bond cleavage

by

Hui-Ling Deng

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine

Department of Medicine

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EXPLORATION OF THE FUNCTIONAL SIGNIFICANCE OF INTRA-DIMER PHOSPHODIESTER BOND CLEAVAGE submitted by Hui-Ling Deng in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine.

Dr. Malcolm C. Paterson (supervisor)

Dr. Rufus S. Day III

Dr. Walter T. Dixon

Dr. Andrew R. E. Shaw

Date...April 20, 1995

DEDICATION

This thesis is dedicated to my parents, Mei-Fang Tsui and Zhi-Ting Deng, who are always an inspiration to me, and whose encouragement and support were invaluable.

ABSTRACT

Several findings have been made that clarify the role of intradimer phosphodiester bond cleavage (dimer modification) *in vivo*. Several DNA repair deficient mutants from *Escherichia coli* (*E. coli*) and human fibroblast cell lines underwent dimer modification to the same degree as their wild-type counterparts, indicating that none of the cell lines contained mutations that block dimer modification. Gross dimer modification was not defective in preferential repair deficient *mfd* and Cockayne syndrome complementation groups A and B cell lines. The post-replication repair abnormality in xeroderma pigmentosum variant cells could not be accounted for by dimer modification, nor could the DNA replication bypass of umuCD protein complex. Similarly, it appears that DNA replication arrest in nondividing human fibroblasts could not be directly related to dimer modification in the cells.

As an alternative approach to searching for the function of modified dimers, direct assessment of DNA repair enzymes on intact and modified cyclobutyl dimers has been carried out *in vitro*. Substrates were constructed which contained, at a defined di-thymine site in a 43 bp DNA sequence, a) no lesion, b) an intact cyclobutyl dimer, c) a cyclobutyl dimer with its interpyrimidine phosphodiester linkage severed, or d) a similarly modified cyclobutyl dimer without an intradimer phosphate group. The studies of repair enzymes on these dimer-containing substrates revealed significant differences in enzyme actions. Bacteriophage T4 UV endonuclease cleaves both the intact dimer and modified dimer with a severed intradimer phosphodiester bond at high efficiency, but cleaves inefficiently the modified dimer without an intradimer phosphate, implying that the presence of an intradimer phosphate group is necessary for efficient substrate recognition by T4 UV endonuclease. *E. coli* DNA photolyase, on the contrary, reverses the cyclobutyl bridge in both the intact dimer and the modified dimer without an intradimer phosphate, but does not do so in the modified dimer containing a severed intradimer phosphodiester bond. The *E. coli* UvrABC endonuclease is able to incise, although inefficiently, both modified dimer-containing lesions at a similar rate as the intact one. Together, these results reflect the different mechanisms of action of the three DNA repair enzymes on the different cyclobutyl dimer-containing substrates.

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ABBREVIATIONS

AP	Apurinic/apyrimidinic
AT	ataxia telangiectasia
ATP	adenosine-5'-triphosphate
bp	base pair(s)
CAP	calf alkaline phosphatase
CHO	Chinese hamster ovary
Ci	Curie
cpm	counts per minute
CS	Cockayne syndrome
d-T<p>C	Cytidylyl-(3'>>5')-thymidine dimerized by a cyclobutyl bridge identified from SVP/CAP/NP1 digestion of UV-treated DNA
d-T<p>T	thymidylyl-(3'>>5')-thymidine dimerized by a cyclobutyl bridge identified from SVP/CAP/NP1 digestion of UV-treated DNA
d-T[p]C	thymidylyl-(3'>>5')-cytidine containing a (6-4) photoproduct identified from SVP/CAP/NP1 digestion of UV-treated DNA
d-T[p]Py	includes both d-T[p]C and d-T[p]T
d-T[p]T	thymidylyl-(3'>>5')-thymidine containing a (6-4) photoproduct identified from SVP/CAP/NP1 digestion of UV-treated DNA
ddH_2O	double-distilled water
din	damage-inducible
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
dT	thymidine
dT<>dC	cyclobutyl thymidine-cytidine (modified) dimer identified from SVP/CAP/NP1 digestion of UV-treated DNA
dT<>dT	cyclobutyl thymidine-thymidine (modified) dimer identified from SVP/CAP/NP1 digestion of UV-treated DNA
EDTA	ethylenediaminetetraacetic acid, disodium salt
g	gram(s)
HPLC	high performance liquid chromatography

hr	hour(s)
i.d.	inner diameter
ID	double-stranded DNA containing an intact thymine-thymine dimer
in.	inch
J	joule(s)
kDa	kilodalton(s)
l	litre(s)
lb.	pound(s)
M	molar
mA	milli-amp(s)
MDOH	double-stranded DNA containing a modified thymine-thymine dimer without an intra-dimer phosphate group
MDP	double-stranded DNA containing a modified thymine-thymine dimer with a severed intra-dimer phosphodiester bond
mol	mole(s)
MW	molecular weight
N	normal
ND	double-stranded DNA containing no thymine-thymine dimer
NP1	nuclease P1
nt	nucleotide(s)
OD	optical density
PBS	phosphate-buffered saline
RNA	ribonucleic acid
rpm	revolutions per minute
sq.	square
SVP	snake venom phosphodiesterase
T^C	includes all thymidine-cytidine and cytidine-thymidine cyclobutyl dimers irrespective of molecular configuration
T^T	includes all thymidine-thymidine cyclobutyl dimers irrespective of molecular configuration
TCA	trichloroacetic acid
TEAA	tetraethylammonium acetate

Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
T[]Py	alkali-modified (6-4) photoproduct
UDS	unscheduled DNA synthesis
UV	ultraviolet
v/v	volume per volume
V	volts
w/v	weight per volume
W	watts
XP	xeroderma pigmentosum

CHAPTER ONE: *IN VIVO* STUDIES OF THE FUNCTIONAL SIGNIFICANCE OF PYRIMIDINE DIMER MODIFICATION

INTRODUCTION

Ultraviolet (UV) irradiation is the best studied and most extensively used model environmental agent for investigating DNA damage, its repair and/or tolerance, and biological consequences. Exposure of cells to UV radiation induces two major classes of photoproducts, namely, (5-5,6-6) cyclobutyl pyrimidine dimers, or cyclobutyl dimers, and (6-4) pyrimidine-pyrimidone photoproducts, or (6-4) photoproducts (Setlow *et al.*, 1964; Wang and Varghese, 1967; Mitchell *et al.*, 1985; Basu and Essigmann, 1988). These photoproducts (for structures, see Figure 1) are believed to pose an effective block to both replication and transcription machinery (Benbow *et al.*, 1974; Caillet-Fauquet *et al.*, 1978; Villani *et al.*, 1978; Sauerbier and Hercules, 1978; Moore *et al.*, 1981; Griffiths and Ling, 1987; Selby and Sancar, 1990, 1991).

Living organisms possess a battery of enzymatic DNA repair systems, by which they remove or circumvent the DNA damage induced by UV light. Identification of the biochemical events performed by the various DNA repair systems, and the biological consequences of faulty DNA metabolism, have been the subject of intensive research over the past three decades. Several human cancer-prone disorders have been identified that are characterized by deficiencies in performing DNA repair. These include xeroderma pigmentosum (XP), ataxia telangiectasia (AT), and Fanconi's

anaemia. XP patients, for example, are prone to sunlight-induced skin cancer and cells derived from these patients are hypersensitive to UV light due to a faulty repair mechanism of UV photoproducts (Cleaver, 1986; Hanawalt *et al.*, 1986; Cleaver and Kraemer, 1989; Hoeijmakers, 1993).

In addition to photoreactivation, photoproduct removal depends on DNA repair mechanisms such as excision repair and post-replication repair. Two basic types of excision repair have been described in bacteria: base excision and nucleotide excision (van Houten, 1990). Base excision removes base damage including alkylation products and pyrimidine hydrates. Nucleotide excision is capable of removing bulky DNA adducts such as cyclobutyl dimers, (6-4) photoproducts, cisplatin adducts, and DNA-protein crosslinks. At the molecular level, the excision repair mechanism in prokaryotes has been well characterized. Base excision involves the removal of the damaged base by a damage-specific glycosylase, leaving an apurinic/apyrimidinic (AP) site that is subsequently incised by an AP endonuclease. A well-known example of base excision is that mediated by T4 endonuclease V (T4 endo V), which has both glycosylase and AP endonuclease activities. This enzyme recognizes cyclobutyl dimers, and its combined activities produce a single strand nick in DNA at the site of the dimer. The removal of the lesion-containing fragment is then completed by a conventional excision exonuclease. In contrast, the nucleotide excision process in *Escherichia coli* involves the UvrABC complex (encoded by *uvr*A, *uvr*B, and *uvr*C genes), which recognizes the lesion in the DNA and incises/excises the damaged strand. This process is believed to operate as follows (see Sancar, 1994 for details): First, two UvrA subunits, with associated ATPase activity, and one UvrB subunit, form

$UvrA_2B$ complex, which acts as a 5'→3' helicase to scan for structural abnormalities in the DNA. Once a damaged site is encountered, a UvrC subunit binds to the site. The UvrB and UvrC then catalyze dual incision reaction, releasing a 12-13 nucleotide fragment. The UvrC subunit and the lesion-containing oligonucleotide then dissociate from the DNA by the concerted action of the UvrD protein (helicase II) and DNA polymerase I. The excision repair process is, then, completed by gap filling via DNA polymerase, followed by ligation of the nick via DNA ligase.

The second general repair process, post-replication repair, joins the abnormally short segments of daughter DNA produced by semi-conservative replication on a damaged parental DNA template. This process is poorly understood (Lehmann, 1972; Kaufmann, 1989).

In contrast to the detailed information about the mechanism of excision repair in *E. coli*, the insight of damage-specific recognition and incision of DNA in numans has only begun to emerge. In order to clarify how human cells process cyclobutyl dimers and (6-4) photoproducts induced by UV, this laboratory has attempted to elucidate the primary molecular defects in different forms of xeroderma pigmentosum. XP is a rare autosomal recessive disease, which remarkably predisposes to sunlight-related skin cancers, with high cellular sensitivity to the cytotoxic, mutagenic and carcinogenic actions of UV rays. Eight complementation groups of XP have been classified thus far, of which seven (XP complementation groups A-G) have specific deficits in excision repair (Bootsma *et al.*, 1970; Robbins *et al.*, 1972; Kleijer *et al.*, 1973; Cleaver, 1980; Andrews, 1983). One group, the XP variant (XP-V), is normal in excision repair but has a defect in post-replication repair (Lehmann *et al.*, 1975).

In general, the repair properties of XP strains measured by different methods, such as unscheduled DNA synthesis or DNA repair synthesis (Cleaver, 1968), and the disappearance of UV endonuclease-sensitive sites (Paterson *et al.*, 1981) are consistent, with the exception of XP groups D and F. XP-D cells exhibit a severe defect in recognizing dimer-containing sites, as judged by the removal of UV-endonuclease-sensitive sites (Paterson *et al.*, 1981), yet undergo an appreciable amount of DNA repair synthesis after UV irradiation. XP-F cells show the opposite discrepancy (Zelle and Lohman, 1979; Kraemer, 1983).

To understand the primary defect in XP-D strains, this laboratory initially investigated the fate of dimer-containing sites in genomic DNA of an XP-D strain, stimulated by the aforementioned long-standing inconsistency in the literature regarding the repair deficiency in XP-D cells. Genomic DNAs from post-UV incubated XP-D and normal fibroblasts were treated with excessive amounts of photolyase in the presence of visible light and were then subjected to alkaline sucrose-gradient sedimentation to size the single-stranded DNA. The results showed a decreased molecular weight of DNA in the XP-D strain, compared with that of control. This observation implied that an intradimer phosphodiester bond cleavage had taken place since the single strand breakage should not be seen in genomic DNA that contains intact dimers (Paterson *et al.*, 1984; Paterson *et al.*, 1987). Further studies on dimer-containing excision fragments isolated from post-UV incubated normal human fibroblasts were consistent with the notion that the intradimer sugar-phosphate bond had been severed since the release of both thymidine and its monophosphate dTMP was observed (Weinfeld *et al.*, 1986).

Our working hypothesis supposes that the intradimer backbone cleavage reaction, perhaps by reducing local conformational stress, may affect excision repair or serve to promote DNA replication or transcription on a UV-damaged template. The hypothesis is partially supported by several lines of evidence found in this laboratory. First, the majority of dimers found in excision fragments isolated from human, rodent and *E. coli* cells after post-UV incubation have sustained cleavage of the intradimer phosphodiester bond (Weinfeld *et al.*, 1986; Galloway, 1992; Galloway *et al.*, 1994), indicating widespread occurrence of this intra-dimer backbone cleavage reaction among various phyla. The intra-genomic heterogeneity of modified dimer sites accumulating in UV-treated and incubated XP-D and normal fibroblasts (Middlestadt *et al.*, 1990) suggests the possibility that preferential repair of lesions in active genes, a process that occurs in a wide array of biological systems including human and *E. coli* cells, may actually entail modification of the dimers. This proposition is supported by an *in vitro* study in this laboratory, using pGEM vectors containing either no dimer, an intact or a modified cyclobutyl dimer. Preliminary results demonstrated that T4 endo V, which is usually used to recognize cyclobutyl dimers in gene-specific repair studies (Bohr *et al.*, 1988), failed to recognize modified dimers (Galloway, 1992). This result implies that at least part of gene-specific repair may actually reflect dimer modification without excision of the lesion. Most importantly, the preliminary *in vitro* transcription study, utilizing the aforementioned substrates and Sp6 RNA polymerase, indicates that whereas an intact dimer blocks the progression of the polymerase, a modified dimer allows it to pass (Galloway, 1992).

Therefore, to explore the biological functions of modified dimers, four

experiments have been performed. First, the ability of XP-V and other repair deficient mutants, such as Cockayne syndrome complementation groups A (CS-A) and B (CS-B) and some *E. coli* strains, to sever the intradimer phosphodiester bond of cyclobutyl dimers has been assessed. Secondly, whether dimer modification in diverse biological systems is actually involved in preferential repair after UV exposure has been analyzed. Thirdly, as (i) caffeine dramatically inhibits post-replication repair in XP variant cells but not in normal human cells (Lehmann *et al.*, 1975); and (ii) it also inhibits cell extract to modify dimers *in vitro* (Liuzzi and Paterson, personal communication), the relationship between dimer modification and post-replication repair in XP variant cells in the presence of caffeine has been explored. Finally, the relationship of DNA replication and dimer modification has been studied in non-dividing and rapidly dividing human fibroblasts.

BACTERIAL AND HUMAN FIBROBLAST STRAINS

din mutant strains:

din (damage inducible) genes were first identified as a set of Mud(Ap^R,*lac*) insertions within the *E. coli* chrornosome in which beta-galactosidase (the product of the *lac*Z gene carried by the phage) had been induced in response to treatment with DNA damaging agents, such as mitomycin C and UV (Kenyon and Walker, 1980). The induction of increased *din* gene expression requires both *rec*A and *lex*A gene products and is under their control, as is dimer modification (Galloway, 1992). Therefore,

functionally unidentified *din* gene mutants such as *din*D1, *din*B1 and *din*F1 are viable candidates for altered dimer modification if the incidence of modified dimers is found to be reduced in such mutants, compared to their wild-type counterparts.

_umu_D mutant strain _umu_CD:

*The umu*D gene, together with *umu*C gene, are located in an operon of *E. coli*, which is inducible and regulated by the SOS system (Walker, 1984). When exposed to UV or certain other DNA damaging agents, many of the genes which belong to the SOS network (SOS genes) are induced or up-regulated to participate in the repair or bypass of damaged DNA (Walker, 1984). First of all, recA, upon induction, cleaves *lex*A, which is the repressor of SOS genes such as *uvr*A, *rec*N, *umu*D and *umu*C, etc. As the *lex*A protein pool decreases, the SOS genes are expressed at higher levels. Some of the induced gene products are involved in excision repair, recombinational repair and mutagenic repair (Walker, 1985). Genetic and physiological experiments indicate that the products of the *umu*CD operon and the *rec*A gene are directly required for mutagenic repair (Kato and Shinoura, 1977; Steinborn, 1978; Bagg *et al.*, 1981; Blanco *et al.*, 1982; Ennis *et al.*, 1985; Nohmi *et al.*, 1988).

The individual roles of the SOS gene products are not entirely clear. *rec*A appears to cleave *umu*D to the active form *umu*D' (Bridges and Woodgate, 1985; Shinagawa *et al.*, 1988). *umu*C is a highly basic protein and is tightly associated with *umu*D, in [illegible]t its primary function may be to bring *umu*D to the DNA for cleavage a[illegible] maintain *umu*D' at the lesion site (Woodgate *et al.*, 1989). *umu*D' is the a[illegible]gent for SOS mutagenesis. *umu*CD has been shown to allow the

DNA polymerase, Pol III, to replicate past cyclobutyl dimers with reduced fidelity (Bridges and Woodgate, 1985; Woodgate *et al.*, 1989). These gene products act after insertion of nucleotides across from the lesion (Bridges and Woodgate, 1985). Despite intensive investigation, the mechanism(s) of how *umu*CD allows the DNA replication machinery to bypass damaged template is not yet known. The ongoing study in this laboratory concerning the role of modified dimers in the repair of UV-induced DNA damage has revealed that a *umu*C mutant is capable of dimer modification, while a *rec*A mutant displays a reduced ability to modify cyclobutyl dimers (Galloway, 1992). These findings indicate that the *umu*C gene product may not function in replication bypass through dimer modification mechanism. Therefore, it is of interest to study dimer modification in a *umu*CD mutant for its possible function in replication bypass.

mfd mutant strain WU361045:

MFD (mutation frequency decline) is operationally defined as the rapid and irreversible decrease in the frequency of certain damage-induced suppressor mutations which occurs when protein synthesis is transiently inhibited immediately after irradiation (Doudney and Haas, 1960; Witkin, 1966, 1969). *uvr* strains were found to lack MFD, indicating that MFD may be related to excision repair (Witkin, 1966). *E. coli* cells harbouring the *mfd* mutation, which also abolishes MFD, were found to have a slow rate of dimer excision (George and Witkin, 1974). Thus, the *mfd* gene product may be involved in excision repair. Recent evidence showed that the defective strand-specific repair in an *mfd* mutant can be corrected by a "transcription-repair coupling factor" (Sancar *et al.*, 1991). This *mfd* gene product may be required

to couple repair to transcription. If modified dimers were found at a dramatically lower level in an *mfd* strain than in an *mfd*$^+$ strain, the conclusion would be that dimer modification may play a role in preferential repair, and if this is so, some *mfd* mutants, such as WU361045, might be defective in dimer modification.

Cockayne syndrome cell strains:

Cockayne syndrome is an autosomal recessive human disorder characterized by sun sensitivity, mental and growth retardation, neurological defects and skeletal and retinal abnormalities (Friedberg, 1985). At the molecular level, CS is characterized by an increased sensitivity to the killing effects of UV-irradiation (Brumback *et al.*, 1978; Andrews *et al.*, 1978; Wade and Chu, 1979; Marxhall *et al.*, 1980). Three complementation groups of CS (CS-A, CS-B, CS-C) have been classified (Lehmann, 1982), of which CS-A and CS-B are defective in repair of transcriptionally active sequences (Mullenders *et al.*, 1988; Venema *et al.*, 1990; Barrett *et al.*, 1991), although their ability to carry out global repair of UV-induced DNA damage is normal. Members of our laboratory extended these preferential repair studies by exploring the consequences of photoenzymatic reversal. That is, transient appearance of modified cyclobutyl dimers in the transcriptionally active *c-myc* gene of normal and XP-D human fibroblasts has been observed at very early repair times (Middlestadt *et al.*, 1990). If some or all of gene-specific repair involves dimer modification, CS cells might be unable to modify cyclobutyl dimers. One CS strain belonging to complementation group C (GM2838) is known to be able to modify dimers and to be proficient in gene-specific repair (Galloway, personal communication).

Two CS cell lines belonging to different complementation groups [A (GM1856B) and B (GM739A)], were selected for study for this purpose. Quantitation of the various photoproducts in CS cells as a function of post-UV incubation time was done to assess the ability of these mutant cells to repair UV-induced damage, with the expectation that few modified dimers would be detected in excision fragments or in genomic DNA of CS cells.

XP variant cell strain XP4BE:

XP variant patients exhibit the typical clinical symptoms of XP but appear to show completely normal excision repair (Burk *et al.*, 1971; Robbins *et al.*, 1972; Cleaver, 1972; Kleijer *et al.*, 1973). Rather, the deficit can be detected by host-cell reactivation or post-replication repair assays (Day, 1974, 1975; Lehmann *et al.*, 1975). After UV-irradiation, the time taken for the newly synthesized DNA (measured as double-strands or single strands) to attain a high molecular weight similar to the size of that in unirradiated controls is much longer than in normal cells. Furthermore, the conversion of low- to high-molecular weight DNA is drastically inhibited by caffeine, which has very little effect on normal human cells (Lehmann *et al.*, 1975). The model to explain these results assumes that XP-V is defective functionally or quantitatively in certain enzymes involved in post-replication repair. Caffeine, therefore, would compete strongly with the enzyme for the dimer or dimer/gap site, thus prolonging the gap-sealing process. Caffeine inhibits cell extracts to perform dimer modification *in vitro* (Liuzzi and Paterson, personal communication); that is, the uncharacterized enzyme in the cell extracts which caffeine inhibits may be related to dimer

modification. If this is the case, a decrease in dimer modification may be observed if UV-irradiated XP-V cells are incubated in the presence of caffeine, even though XP-V cells show normal dimer modification in the absence of this purine analog (Galloway, 1992).

EXPERIMENTAL PROCEDURES

The methods for quantitating photoproducts in excision fragments from UV-treated ^{3}H-thymidine labeled cells have been established in this laboratory (Weinfeld *et al.*, 1986; Liuzzi *et al.*, 1989; Galloway *et al.*, 1994).

To summarize briefly, the procedure for photoproduct analysis in excision fragments involves: 1) labeling cells with ^{3}H-thymidine; 2) irradiating cells with UV_{254nm}; 3) incubating UV-irradiated cells to permit repair; 4) collecting the trichloroacetic acid (TCA)-soluble cellular fraction which contains the excised lesions; 5) removing the vast excess of free ^{3}H-labeled thymidine from the TCA-soluble fraction by dialysis; 6) enzymatically digesting excision fragments with snake venom phosphodiesterase (SVP), calf alkaline phosphatase (CAP), and nuclease P1 (NP1) to release photoproduct-containing dinucleoside monophosphates, dimerized dinucleosides and free nucleosides; 7) separating the reaction products by HPLC; and finally, 8) scintillation-counting of eluted fractions from HPLC (for illustration, see Figure 2).

As the enzymatic digestion/HPLC assay cannot separate (6-4) photoproducts and modified thymidine-cytidine cyclobutyl dimers (dT<>dC) (Galloway, 1992;

Galloway *et al.*, 1994), half of the enzymatic hydrolysate is subjected to hot alkaline treatment. This treatment makes the (6-4) photoproduct (modified) peak move to the front of the HPLC profile, since (6-4) photoproducts are alkali labile. Thus, the amount of (6-4) photoproducts can be calculated from the (dT<>dC + d-T[p]Py) peak of the enzymatic digested sample by subtracting the (dT<>dC) peak in the hot alkaline treated sample (For illustration, see Figure 3).

Materials

All cell culture supplies were obtained from Life Technologies, Inc., glassware from Corning Incorporated (Corning, NY), and all other general laboratory supplies from Fisher Scientific (Pittsburgh, PA). All chemicals were purchased from BDH Chemicals (Toronto, On). Nuclease P1 (NP1) and calf alkaline phosphatase (CAP) were obtained from Boehringer Mannheim (Dorval, PQ), and snake venom phosphodiesterase (SVP) from Sigma Chemical Company (St. Louis, MO). Water used in the experiments was double-distilled and passed through a Nanopure II filtering system (Barnstead/Sybron, Boston, MA).

Cultivation of cells

***E. coli* cell lines:**

GW1000 (F^-, *thr*-1, *leu*-6, *his*-4, *arg*E3, *galK*2, *str*A31, ilv^{is}, *rec*A441, sfiA11, *lac*ΔU169), GW1030 [GW1000, *din*B1::Mud(Ap, *lac*)], GW1040 [GW1000,

*din*D1::Mud(Ap, *lac*)], and GW1070 [GW1000, *din*F1::Mud(Ap, *lac*)] were supplied by Dr. Graham Walker (Massachusetts Institute of Technology, Cambridge, MA). WU3610 (*E. coli* B/r, *tyr*, *leu*) and WU361045 (WU3610, *mfd*-1) were supplied by Dr. Evelyn Witkin (Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ). RW118 [*xyl*-5, *mtl*-1, *gal*K2, λ-*rac*-1, *rps*L31, *kdg*K51, Δ(*gpt-pro*A)62, *lac*Y1, *tsx*-33, *sup*E44, *thi*-1, *leu*B6, *his*G4, *mgl*-51, *arg*-3, *rfb*D1, *ara*-14, *thr*-1, *ara*D139, *sul*A211]) and RW120 [RW118, Δ(*umu*DC)595::*cat*] were supplied by Dr. Roger Woodgate (National Institutes of Health, Bethesda, MD).

The medium used to culture the *E. coli* strains was M9CA containing 1.3% $Na_2HPO_4 \cdot 7H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mM $MgSO_4$, 0.2% glucose, 100 μM $CaCl_2$ and 0.2% casamino acids (Difco Laboratories, Detroit, MI), which was prepared according to Sambrook *et al.* (1989). That is, to make 1 l of medium, 960 ml of ddH_2O, 12.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.05 g of NaCl and 1 g of NH_4Cl were added in a 2 l Erlenmeyer flask. After the salts were dissolved, the flask was autoclaved for 15 min at 121°C (15 lb/square in) on a liquid cycle and then cooled to room temperature. This salt solution was supplemented with 10 ml of a 20% glucose solution (filter sterilized by a 115 ml Nalgene disposable filter unit with a cellulose nitrate membrane of 0.45 μm pore size, Nalge Co. Rochester, NY), 10 ml of 20% casamino acids solution (sterile filtered in the same way as the glucose solution), 1 ml of 1 mg/ml thiamine [sterilized by a syringe filter unit (0.45 μm pore size, Baxter Diagnostics Co.)], 1 ml of 2 M $MgSO_4$ and 1 ml of 0.1 M $CaCl_2$ (autoclaved for sterilization as described previously). The complete M9CA liquid medium was kept sterile and stored at 4°C. To make M9CA agar plates, 15 g/l of

bacto-agar (Difco Laboratories) were added in addition to the salt solution before autoclaving. After being supplemented with the required nutrients as described above, the medium (30-35 ml/plate) was poured into 90 mm bacterial culture plates (Fisher Scientific) under a culture hood. These solidified plates were stored at 4°C for later use.

Culturing *E. coli* cells began by streaking the glycerol stock of each cell strain onto an agar plate. After incubation for 24 hr, a single colony from each plate was then inoculated into a tube containing 5 ml of M9CA medium. These small cultures were kept in an incubator overnight at 37°C, ventilated with 5% CO_2 and 95% air. Each overnight culture was then added to 200-ml culture medium to allow growth of the *E. coli* cells in an incubator at 37°C with vigorous shaking.

Human fibroblast strains:

GM38 (normal), XP4BE (formerly ATCC CRL1162) (XP-V), GM1856B (CS-A) and GM739A (CS-B), were purchased from the NIGMS Human Mutant Cell Repository and the American Type Culture Collection (Rockville, MD). The Ham's F12 medium used in culturing these human fibroblast cells was purchased as dried nutrient mixture which was dissolved in ddH_2O, supplemented with sodium bicarbonate (1.176 g/l), and adjusted to pH 7.0. The medium was routinely subjected to sterilization through a positive-pressure filter and stored in 500-ml sterile bottles at 4°C. All cell culture manipulations were carried out in a sterile environment provided by a laminar flow cabinet (NuAire, Inc., Plymouth, MW).

Cells of each strain (stored in liquid nitrogen) were thawed in 37°C waterbath and quickly transferred into a 150-mm tissue culture dish (Lux Scientific Corp.

Newbury, CA) containing 20 ml prewarmed Ham's F12 medium which was supplemented with 10% (V/V) fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulphate. The cultures were kept in an incubator (Lunaire Incubator, Lunaire Environmental Incorporated, Williamsport, PA) at 37°C, ventilated with 5% CO_2-95% air. The humidity of the incubator was kept at 75-85%. Usually, the medium was changed at intervals of 3 days, and cells were examined under a light microscope every time the medium was changed to ensure that cells grew well in each plate. When cells grew to confluence, they were subcultured. The medium in each dish was aspirated. Cells were washed with PBS (0.2 M NaH_2PO_4/Na_2HPO_4, pH 7.2) at room temperature and then the PBS was aspirated. To detach the cells, 1 ml of 1% trypsin /1 mM EDTA was added to cover the cell surface and incubated for 2-3 min. Upon adding 12 ml of Ham's F12 medium to each plate, cells were subcultured into 4 dishes, each containing 20 ml of medium. The cells were further cultured for experimental use.

Introduction of UV lesions into cellular DNA

Radioactive labeling of mammalian cell DNA: 10^6 cells were incubated with thymidine-free Ham's F12 medium overnight. The medium was then removed thoroughly and replaced with the same medium containing, in addition, 5 µCi/ml of ^{3}H-thymidine (1 mCi/ml, 80 Ci/mmol, NEN Canada, Montreal, PQ). These cultures were allowed to grow for an additional 48 hr so that labeled thymidine could be incorporated

into the genomic DNA of the cells. After removing the radioactive medium, cells were washed with 10 ml of PBS to remove residual medium in each plate, and then cultured for 24 hr in non-radioactive Ham's F12 medium to deplete endogenous precursor pools of tritium label.

UV irradiation and incubation of mammalian cells: Culture medium in each plate was aspirated and drained. Ten ml of PBS was added to rinse out residual medium and the PBS was then removed completely. Each culture was UV-irradiated at a fluence of 40 J/m^2 by two 15 W germicidal lamps (Model GE15T, General Electric, Toronto, ON), which emit mainly 254-nm UV light. The unirradiated control plates were manipulated the same way but without UV exposure (Weinfeld *et al.*, 1986). Post-UV incubations were then carried out for various periods of time to allow cells to repair UV damage to their DNA.

Radioactive labeling of DNA of *E. coli* cells: Five µCi/ml ^{3}H-thymidine and 200 µg/ml 2-deoxyadenosine, which allows the uptake of ^{3}H-thymidine of non-thymidine requiring cells (Galloway, 1992), were added to a 200 ml culture of *E. coli*. The culture was then incubated for about 2 hr with vigorous shaking so that ^{3}H-thymidine could be incorporated into cellular DNA. Cell growth was monitored by measuring OD_{600} before being harvested. Cells were harvested by centrifugation at 6,000g for 1 hr at 4°C in a Sorvall RC-5C refrigerated superspeed centrifuge (Dupont Canada, Inc. Mississauga, ON), and washed with NT buffer containing 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4. The cells were suspended with the same M9CA medium and cell density was adjusted to 0.2 OD_{600}.

UV irradiation and incubation of *E. coli* cells: One-half of the cell

suspension was sham-irradiated and placed on ice until further manipulations were performed. The other half (20 ml at a time in a 150 mm tissue culture plate) was UV-irradiated at a fluence of 40 J/m^2 with continuous shaking. The irradiated bacterial suspensions were pooled and added to 200 ml prewarmed M9CA medium. The irradiated bacteria cultures were incubated for 3 hr at 37°C with vigorous shaking to allow DNA repair to take place.

Isolation of excision fragments

Post-UV incubated mammalian cells were harvested in preparation for the isolation of excision fragments. After removing the medium, 10 ml of PBS was used to rinse the residue medium before the cells were treated with 1% trypsin/1 mM EDTA. After detachment, the cells were collected in 6 ml of Ham's F12 medium and then centrifuged at 1,500g for 4 min at 4°C in a bench-top centrifuge (Accu-Spin FR, Beckman Instruments, Toronto, ON). The cell pellets were washed twice with cold PBS (40 ml) before being resuspended in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5).

Post-UV incubated *E. coli* cells were harvested for isolation of excision fragments in the following manner. Cell cultures were centrifuged at 7,000g for 15 min at 4°C (HB-4 rotor, Sorvall RC-5C Centrifuge). The cell pellets were washed twice with NT buffer (200 ml) and repelleted. The pellets were resuspended in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5).

To isolate excision fragments from both mammalian and *E. coli* cells, 0.5 ml of 10% TCA was added to 0.5 ml of each cell suspension. Two 10-µl aliquots of the samples were counted in a scintillation counter to determine total radioactivity. Each

cell suspension was vortexed thoroughly to ensure complete cell lysis. The TCA-soluble and -insoluble fractions were then separated by centrifugation at 10,000g for 15 min at 4°C in a microfuge (Eppendorf Centrifuge 5415, Brinkmann, Rexdale, ON). The supernatants (i.e., TCA-soluble fractions which contained the excision fragments) were carefully transferred to Eppendorf tubes and the volumes measured. The total radioactivity in each TCA-soluble fraction was obtained through scintillation counting of two 10-µl aliquots of the samples. TCA-soluble samples were subjected to dialysis to remove TCA but retain all of the excision fragments (Galloway *et al.*, 1994). This was achieved using dialysis tubing with a 3.5-kDa molecular weight cut-off (Spectra/Por, Fisher Scientific, Pittsburgh, PA). Excision fragment samples were stored at -20°C until further manipulation.

Enzymatic digestion of excision fragments

The enzymatic digestion procedure adopted was according to Galloway *et al.* (1994). Each excision fragment sample (~10,000 dpm from fibroblasts or ~100,000 from E. coli cells) was treated with 25 units of CAP and 0.025 units of SVP at 37°C overnight in a 1-ml reaction mixture containing 50 mM Tris-HCl pH 6.0. 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 5 mM 2-mercaptoethanol. The next morning, 6 units of NP1 were added and incubation continued for 1 hr. The resultant hydrolysate contained free nucleosides, dinucleotides with a cyclobutyl dimer, a (6-4) photoproduct and two nucleosides joined by a cyclobutyl bridge (i.e., dinucleoside with a modified dimer). One-half of the above SVP/CAP/NP1 hydrolysate was subjected to hot alkali treatment, i.e., supplemented with 55 µl of 2 N NaOH and incubated at 90°C for 90

min, to facilitate separation of dT<>dC from (6-4) photoproducts. Hot alkali hydrolyses both the intralesion phosphodiester bond and the N-glycosyl bond in (6-4) photoproducts (Franklin and Haseltine, 1982) but not in cyclobutyl dimers. As a result, the peak of radioactivity containing the (6-4) photoproduct was more hydrophilic and thus appeared at an earlier elution time in the HPLC C_{18} profile.

Reverse-phase HPLC analysis

The HPLC instrumentation and eluting conditions for separating different ^3H-labeled species have been published (Liuzzi *et al.*, 1989; Galloway *et al.*, 1994). Briefly, the HPLC system included a Waters 840 control station, two Waters 510 dual piston pumps, a Waters U6K universal injector, a Waters 490 Programmable Multiwavelength Detector (Waters Associates, Mississauga, ON), and a Foxy fraction collector (Foxy, Lincoln, NE). A Whatman C_{18} partisil-10 ODS column (250 x 4.6 mm i.d.) was used. The mobile phase consisted of a mixture of buffer A (50 mM NaH_2PO_4, pH 4.5) and Buffer B (50 mM NaH_2PO_4 plus 50% methanol). The eluting gradient consisted of 100% buffer A plus 0% buffer B for 1 min, and a linear gradient from 100% to 0% buffer A and from 0% to 100% buffer B over 30 min. The 100% buffer B was then maintained for 5 min, followed by a 4-min gradient to return to initial conditions. During this 40-min run, the flow rate was kept at 1 ml/min. Seventy-two 0.5 ml fractions were collected automatically and radioactivity counted after adding 5 ml of Beckman EP scintillation cocktail into each fraction vial. The identities of the radioactive species on the HPLC profile had been ascertained previously by people in this laboratory (Weinfeld *et al.*, 1986; Liuzzi *et al.*, 1989; Galloway *et al.*, 1994).

RESULTS AND DISCUSSION

Photoproduct removal in various *E. coli* mutants

Photoproduct profiles of excision fragments isolated from several *E. coli* mutants which were exposed to UV (40 J/m^2) and incubated to allow repair (3 hr) are presented in Tables 1 and 2. The absolute numbers of total UV lesions excised from DNA in the *E. coli* mutants were similar to their wild-type counterparts. For example, the *din*B1 *E. coli* strain excised 2042 dimers in total, compared with 2080 dimers from its wild-type counterpart (Table 1). Therefore, the repair pathways in the *E. coli* mutants were not grossly altered. Comparing the extent of dimer modification, the *E. coli* wild-type strain GW1000 (*din*$^+$ strain) excised 52% of the cyclobutyl dimers in a modified form, while the mutant strains ranged from 50 to 57% (Table 1). In addition, the majority of dimers modified in *din* strains were thymidine-thymidine homodimers, i.e., 69-74% of T^T cyclobutyl dimers were modified, in contrast to 31-38% of T^C cyclobutyl dimers (Table 1). Similarly, there were no obvious deficiencies in dimer modification in the *mfd*$^-$ and *umuCD*$^-$ mutants, compared to their wild-type counterparts (Table 2). Therefore, none of these *E. coli* strains seemed to exhibit an inability to cleave the intradimer phosphodiester bond at cyclobutyl dimer-containing sites, although statistic analysis was not applied because few experiments (n=1 for *din* strains, and n=2 for *mfd* or *umu*CD strains) were done with these *E. coli* strains. These results imply that the *umu*CD complex allows replication past cyclobutyl dimers via mechanisms other than dimer modification, and that the *din* mutants tested are not

noticeably deficient in dimer modification. However, whether dimer modification is involved in preferential repair should be further evaluated by studying dimer modification in a specific active gene in the *mfd* mutant.

Photoproduct removal in Cockayne syndrome cell lines

To assess the ability of Cockayne syndrome cell lines (CS-A and CS-B) to modify dimers, the excision fragments from post-UV (40 J/m^2) incubated (6 or 24 hr) fibroblasts were collected and analyzed by the enzymatic hydrolysis/HPLC assay. Accumulation of total UV lesions excised from these cells was observed with repair time, although there were only two time points tested (Table 3). According to Paterson *et al.* (1973) and Franklin *et al.* (1982, 1986), the induction of 10^5 cyclobutyl dimers and 10^4 (6-4) photoproducts per J/m^2 produced an estimated 4.4 x 10^6 dimers per human cell at a UV fluence of 40 J/m^2. Consequently, at 24 hr, approximately 30 and 38% of the total dimers were removed from each CS-A and CS-B cell, respectively, percentages similar to those obtained with the normal cell line GM38 in this laboratory (Galloway, 1992). These data are also in keeping with an earlier report of others (Venema *et al.*, 1990) demonstrating that CS cells are proficient in global DNA repair. The accumulation of modified thymine-thymine dimers was apparent in both cell lines, giving 70% and 73% of T^T cyclobutyl dimers modified at 24 hr, compared to 36% and 49% at 6 hr. The large proportion of modified thymine-thymine dimers detected in the excision fragments of both cell lines suggests that neither of the cell lines are grossly deficient in dimer modification. However, this preliminary study (n=1) needs to be repeated. Moreover, further investigation into dimer modification in specific

genomic regions such as transcriptionally active versus inactive genes in these two cell lines should be done to evaluate whether their preferential repair defects are due to an inability to modify dimers. Such a study could be performed by using *E. coli* photolyase which is required to cleave modified dimers. If no breaks are revealed in a transcriptionally active gene, which has been reported to be defective in preferential repair in UV-irradiated CS cells, while many are revealed in the same gene in normal cells, this would indicate that defective dimer modification could contribute to defective preferential repair in CS cells.

The effects of caffeine on dimer modification in XP variant cells

The effects of caffeine on dimer excision repair in GM38 (normal human fibroblast) and XP4BE (XP-V) cells are shown in Figure 4 (each data point was from one determination). In general, the amounts of (6-4) photoproducts excised per cell in both cell lines were similar and reached a plateau at about 6 hr of post-UV incubation, indicating proficient (6-4) photoproduct removal in XP4BE cells, in keeping with data obtained by Galloway (1992). The amounts of cyclobutyl dimers excision increased at similarly linear rates within 24 hr of post-UV incubation, indicating that XP variant and normal cells also possess similar abilities in excision repair of UV-induced dimers. After caffeine treatment, the excision repair patterns of both GM38 and XP4BE cells were not greatly changed, indicating that caffeine did not inhibit excision repair in these two cell lines, as has been found in yeast and rodent cells (Kihlman, 1974). Comparing the percentages of modified cyclobutyl dimers excised from normal and XP variant fibroblasts, in the presence or absence of caffeine, very

little differences in dimer modification were found in either normal human fibroblasts or XP variant cells (Figure 5). Therefore, it would appear that the inhibition of post-replication repair observed in XP variant fibroblasts can not be related to dimer modification. Moreover, the unpublished observation (Liuzzi and Paterson) that dimer modification was sensitive to caffeine *in vitro* was not observed *in vivo* in this experiment. One explanation for this discrepancy could be that intracellularly, caffeine was metabolized to form ineffective metabolites, thereby decreasing the concentration of caffeine to a level lower than that required to elicit pharmacological effects. Further studies need to be done with several XP-V cell strains so that statistic analysis can be applied to confirm the findings.

The relationship of *de novo* DNA replication and dimer modification in normal human fibroblasts

The accumulation of UV lesions and distribution of intact and modified cyclobutyl dimers from post-UV incubated confluent and rapidly dividing normal human fibroblasts are shown in Table 4. This preliminary study (n=1) indicates that no difference in dimer modification was found in confluent versus rapidly dividing GM38 cells, implying that dimer modification occurs independently of DNA replication.

CONCLUSIONS

In summary, these *in vivo* studies, by measuring photoproducts [intact and

modified pyrimidine dimers as well as (6-4) photoproducts] of excision fragments of post-UV incubated cells, have yielded the following results. Firstly, all of the DNA repair deficient mutants from *E. coli* and human fibroblast cell lines examined were found to undergo dimer modification to the same extent as their wild-type counterparts, implying that the repair deficiency of the cell lines is not due to dimer modification. Secondly, gross dimer modification was not found defective in *E. coli mfd*, CS-A or CS-B, three cell lines known to be deficient in preferential repair. Thirdly, caffeine did not affect dimer modification in XP-V cells, and the post-replication repair abnormality characteristic of XP-V cells could not be explained by dimer modification, nor could the DNA replicative bypass enabled by the UmuCD protein complex. Finally, dimer modification does not appear to be directly related to DNA replication arrest in non-dividing normal human fibroblasts.

strain	relevant genotype	UV lesions excised per cell						T^C cyclobutyl dimers modified	T^T cyclobutyl dimers modified	total cyclobutyl dimers modified
		d-T[p]Py	d-T<p>C	dT<>dC	d-T<p>T	dT<>dT	total	(%)	(%)	(%)
GW1000	*din*$^+$	472	55?	308	246	546	2080	38	69	52
GW1030	*din*B1	463	476	289	211	603	2042	33	74	57
GW1040	*din*D1	526	661	300	249	620	2356	31	71	50
GW1070	*din*F1	360	387	225	159	440	1571	37	74	55

Table 1. Accumulation of UV lesions and distribution of intact and modified cyclobutyl dimers from post-UV (40 J/m^2) incubated (3 hr) *E. coli* strains defective in the *din* (damage-inducible) genes. d-T[p]Py refers to d-T[p]T and d-T[p]C (6-4) photoproducts; d-T<p>C and dT<>dC refer to intact and modified thymidine-cytidine dimers, respectively, and d-T<p>T and dT<>dT refer to intact and modified thymidine-thymidine dimers, respectively. For each strain, ~50,000 dpm were passed through the column. The numbers of dimers and (6-4) photoproducts were calculated according to Galloway (1992), with the following four assumptions and qualifications: i) all excised UV lesions remain inside the cell and are recovered in the acid-soluble material; ii) the genome size of *E. coli* is 4.2 x 10^6 bp and the thymine content is 25% (Lewin, 1985), and therefore there are 2.1 x 10^6 thymine residues per *E. coli* genome; iii) the percentage of radiolabel is divided by two in order to compute T^T since either thymine can be labelled; and finally, iv) since the vast majority of thymidine-containing (6-4) photoproducts are d-T[p]C (Lippke *et al.*, 1981; Brash and Haseline, 1982; Franklin, *et al.*, 1982), no correction has been made for d-T[p]Py.

strain	relevant genotype	UV lesions excised per cell					T^T cyclobutyl dimers modified
		d-T<p>C	dT<>dC & d-T[p]Py	d-T<p>T	dT<>dT	total	(%)
WU3610	*mfd*$^+$	491	529	176	593	1789	77
WU361045	*mfd*$^-$	412	563	192	399	1566	68
RW118	*umu*CD$^+$	422	682	203	632	2043	76
RW120	*umu*CD$^-$	494	618	209	532	1853	72

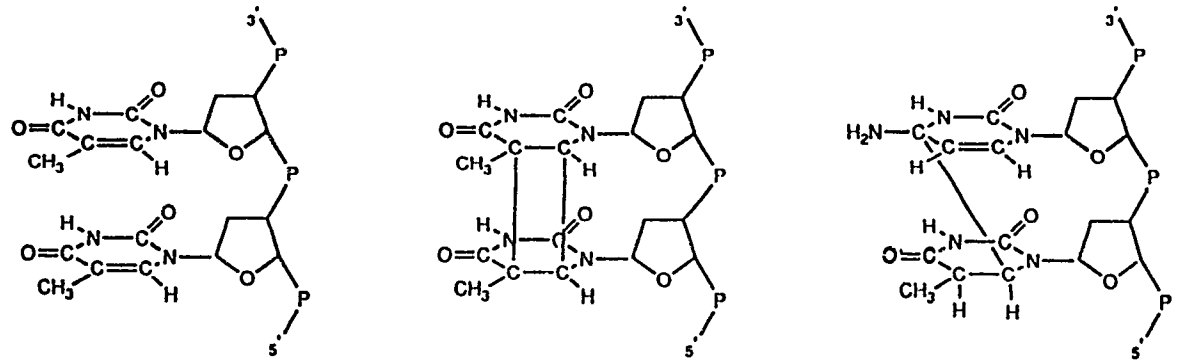
Table 2. Accumulation of UV lesions and distribution of intact and modified cyclobutyl dimers from post-UV (40 J/m^2) incubated (3 hr) *E. coli* strains characterized by different genetic backgrounds. For each strain, ~100,000 dpm were passed through the column. See Table 1 for details concerning the calculation of UV lesions.

strain	relevant genotype	repair time (hr)	UV lesions excised per cell ($\times 10^{-4}$)					T^T cyclobutyl dimers modified
			d-T<p>C	dT<>dC & d-T[p]Py	d-T<p>T	dT<>dT	total	(%)
GM38	normal control	6	5.39	36.94	3.36	3.59	49.29	52
		24	14.35	85.89	7.65	33.96	141.85	82
GM739A	CS-B	6	1.32	25.40	1.19	1.16	29.07	49
		24	10.50	97.60	6.68	18.10	132.88	73
GM1856B	CS-A	6	3.89	43.00	5.04	2.89	54.82	36
		24	12.50	116.50	11.50	26.70	167.20	70

Table 3. Accumulation of UV lesions and distribution of intact and modified cyclobutyl dimers from post-UV (40 J/m^2) incubated (6 and 24 hr) Cockayne syndrome (CS) and normal human cell lines. For each strain, ~10,000 dpm were passed through the column. Calculations were performed as outlined in Table 1, except that genome size of human cells is 6.38 x10^9 bp (Galloway, 1994).

culture state	repair time (hr)	UV-lesions excised per cell (x 10^{-4})	total cyclobutyl dimers modified (%)
rapidly dividing	6	47.75	54
	24	117.20	68
non-dividing	6	58.76	63
	24	126.71	86

Table 4. Accumulation of UV lesions and distribution of intact and modified cyclobutyl dimers from post-UV (20 J/m^2) incubated (6 and 24 hr) confluent and rapidly dividing normal human fibroblasts (GM38). For each strain, ~10,000 dpm were passed through the column. Calculations were made as outlined in Table 3.



A: Two undamaged thymine group

B: (5-5, 6-6) cyclobutane pyrimidine dimer

C: Pyrimidine-pyrimidone (6-4) photoproducts between a thymine and a cytosine

Figure 1. Structures of major DNA photoproducts induced by UV rays. **A**. Two undamaged adjacent thymine groups in a DNA strand; **B**. (5-5, 6-6) cyclobutane pyrimidine dimer; **C**. pyrimidine-pyrimidone (6-4) photoproducts between a thymine and a cytosine.

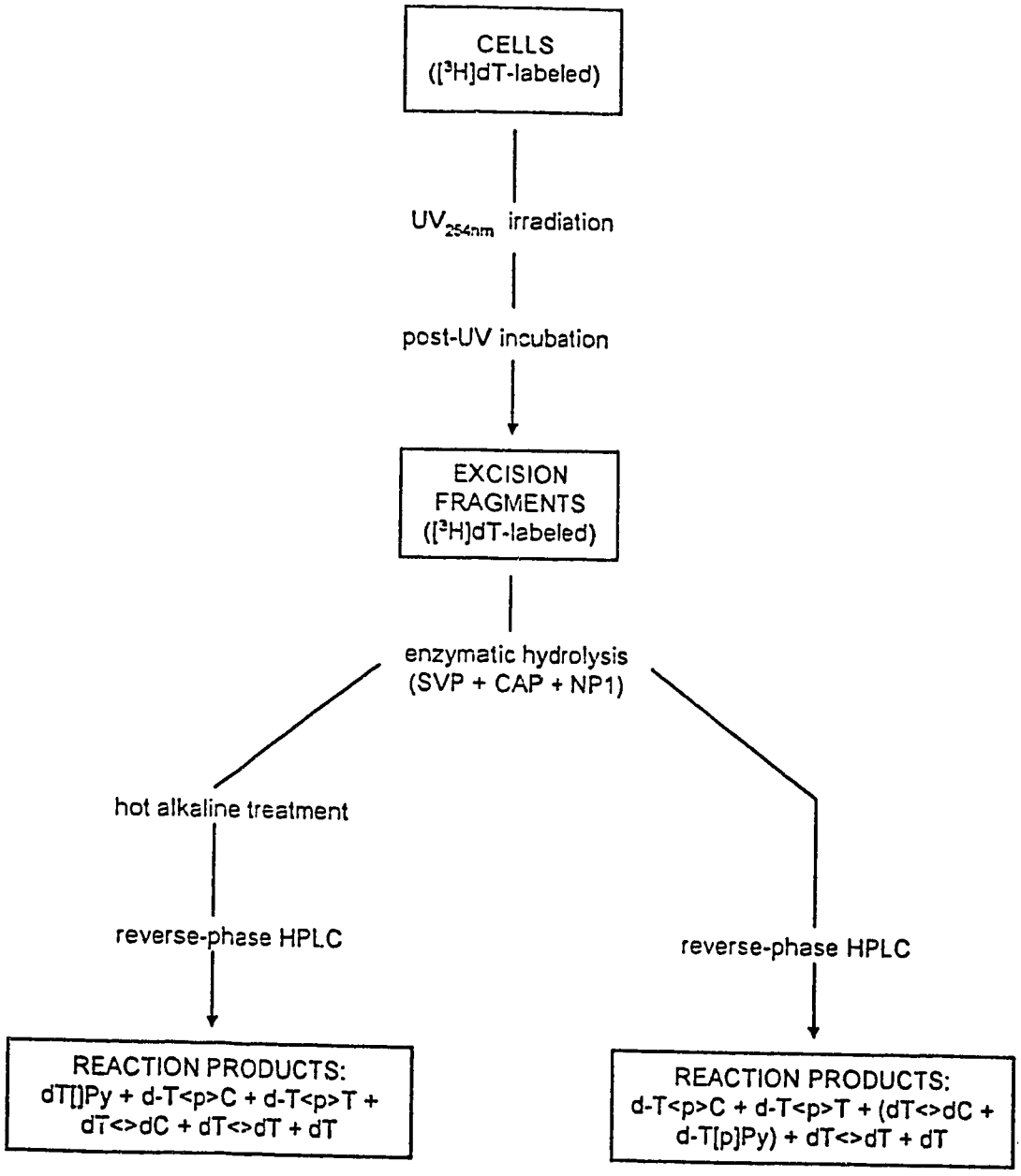


Figure 2. Schematic illustration of measuring cyclobutyl dimers and (6-4) photoproducts in oligomeric excision fragments isolated from post-UV incubated *E. coli* cells and human fibroblasts. The reaction products are listed in order of their retention time from the HPLC chromatogram profiles. SVP, CAP, and NP1 refer to snake venom phosphodiesterase, calf alkaline phosphatase, and nuclease P1, respectively.

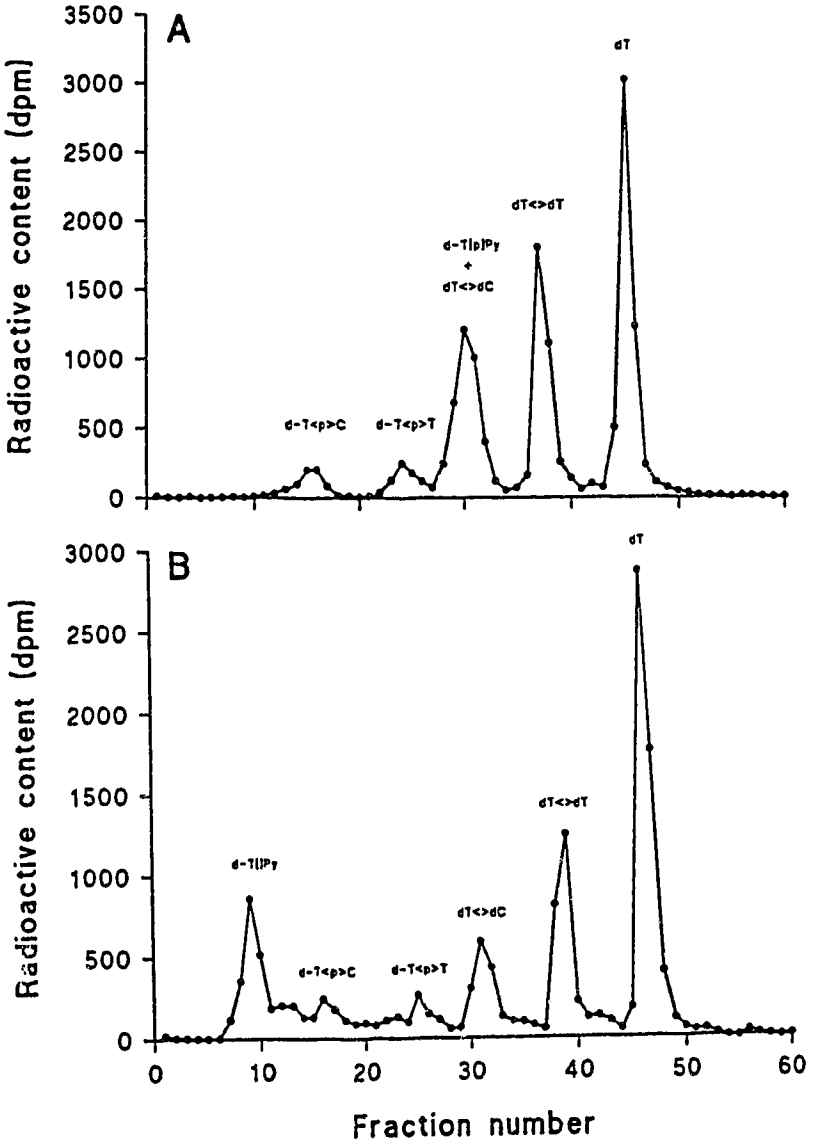


Figure 3. Illustration of photoproduct profiles of excision fragments from post-UV incubated normal human fibroblasts after enzymatic digestion (A) and hot alkaline treatment plus enzymatic digestion (B).

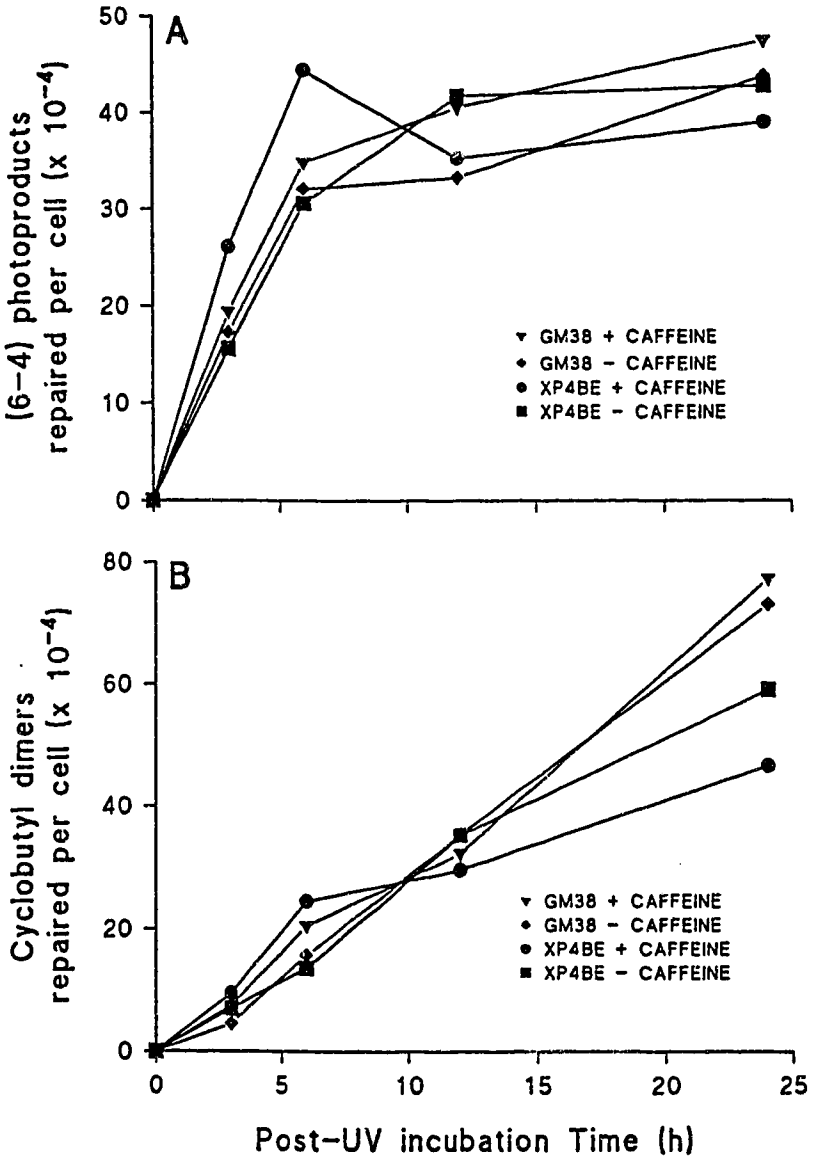


Figure 4. Accumulation in excision fragments of (6-4) photoproducts (A) and cyclobutyl dimers (B) in normal and XP variant cells post-UV (40 J/m^2) incubated in the presence or absence of caffeine (1.5 mM).

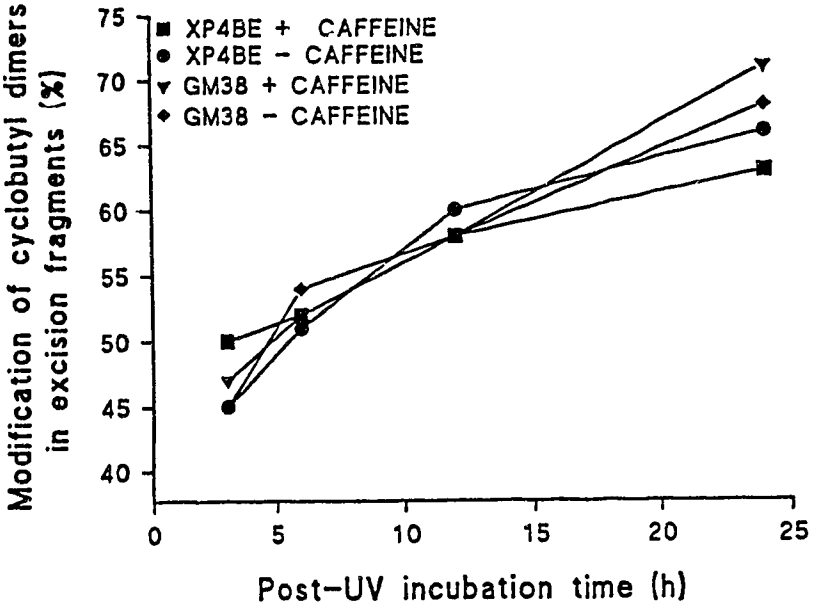


Figure 5. The effects of caffeine on dimer modification in normal and XP variant cells after post-UV (40 J/m^2) incubation. The percentage of modified cyclobutyl dimers is calculated as the total modified T^C and T^T dimers divided by the total number of cyclobutyl dimers (modified and intact) excised from the excision fragments.

CHAPTER TWO: THE ACTIVITIES OF REPAIR ENZYMES TOWARDS OLIGOMERIC SEQUENCES CONTAINING INTACT OR MODIFIED THYMINE-THYMINE DIMERS *IN VITRO*

INTRODUCTION

As noted in Chapter 1, repair of a great variety of damaged and other modified bases is achieved by excision repair (for illustration, see Figure 6). Excision processes remove various kinds of DNA damage such as (i) the damaged base only, creating an AP site, (ii) a fragment containing an AP site, (iii) the nucleotide lesion and a neighbouring region of DNA, or (iv) an interstrand crosslink. The gap generated by excision is filled by DNA polymerase and the nick covalently joined by DNA ligase. This so-called excision repair operates by two closely related but distinct mechanisms for the first step, an incision step. In *E. coli*, a repair endonuclease recognizes the distortion of the DNA (for example, a thymine dimer), and makes cuts in the sugar-phosphate backbone: one is 8 nucleotides to the 5' side of the dimer and the other is 4-5 nucleotides to the 3' side (Sancar and Tang, 1993). It is known, as stated in the first part of this thesis, that this repair endonuclease activity in *E. coli* is determined by the protein complex UvrABC, the products of *uvr*A, *uvr*B, and *uvr*C genes, which work in a concerted manner (Grossman and Yeung, 1990; Sancar and Tang, 1993). In several other systems, such as the bacterium *Micrococcus luteus* and the bacteriophage T4, the incision step occurs in two distinct stages. The first is the

cleavage of the N-glycosidic bond in the 5'-thymine of a dimer by a pyrimidine dimer DNA glycosylase. Incision of the strand is then catalyzed by an endonuclease activity, making a single cut at the 5' side of the remaining thymine in the dimer site. (Friedberg, 1987; Haseltine *et al.*, 1980). T4 endonuclease V (T4 endo V, the product of *den*V gene), as stated in more detail in Chapter 1, is such a protein, mediating both a glycosylase and an endonuclease activity. The removal of the lesion-containing fragment is then completed by a conventional excision exonuclease.

Photoreactivation, an alternative to excision repair corrects only UV-induced cyclobutyl dimers. This reaction is catalyzed by DNA photolyases, which are found in species such as *E. coli* and the yeast *Saccharomyces cerevisiae*, and operate in cells from many sources, ranging from mycoplasmas, to mammalian cells such as those in potoroo and opossum (Walker, 1985; Jorns *et al.*, 1990). Two steps are involved in the reaction. Initially, photolyase binds specifically to a dimer-containing substrate, independent of illumination, and then subsequently uses the energy of visible light (300-600 nm) to reverse the dimer to two normal pyrimidines (for illustration, see Figure 6).

In contrast to the detailed information about the mechanism of excision repair in *E. coli*, the insight of damage-specific recognition and incision of DNA in humans has only begun to emerge. Studies on DNA repair in XP cells have revealed gross similarities between these human cells and microbial and fungal systems. For example, following microinjection of either *M. luteus* or T4 endo V into UV-irradiated XP cells, cell survival increases and unscheduled DNA synthesis, a parameter indicating repair synthesis, reaches near-normal levels (Tanaka *et al.*, 1975, 1977;

Hayakawa *et al.*, 1981; de Jonge *et al.*, 1985). Also, in *den*V-transformed XP cells, 50% of the dimers are removed within 3-6 hr after UV exposure, as compared to no measurable removal in the non-transformed XP cells (Ley *et al.*, 1989). In addition, several of the proteins purified from wild-type cells by complementation of XP cell extracts, have been identified as having UV-repair functions. For example, *ERCC*2 (excision repair cross complementing) and *ERCC*3 gene products are believed to have DNA binding and helicase activities and are essential for nucleotide excision repair (Hoeijmakers and Bootsma, 1990; Friedberg, 1991). The XP-E protein, which binds to damaged DNA, seems functionally analogous to a yeast protein linked to photolyase (Chu and Chang, 1988, 1990; Patterson and Chu, 1989; Friedberg, 1991). The *XPAC* and *XPCC* gene products have damage-specific DNA binding and DNA binding activities, respectively (Hoeijmakers and Bootsma, 1990; Fleitjer *et al.*, 1992). The cell extracts of XP cells defective in complementation groups A, C, D or G are complemented by the *E. coli* UvrABC endonuclease complex in DNA repair synthesis, implying that these cells are deficient in the incision step, similar to the *uvr* mutants of *E. coli* (Hansson *et al.*, 1990).

Although undoubtedly there are similarities between human cells and microbes, the exact biochemical nature of the incision step in the DNA repair process in humans is still unknown. During the process of attempting to elucidate the primary defects in different XP strains, this laboratory discovered photoreactivating enzyme-dependent single strand breaks in the DNA of post-UV incubated XP-D cells, as stated in the first part of this thesis. This phenomenon, which should not seen in genomic DNA containing intact dimers, led us to postulate the existence of intradimer

phosphodiester bond cleavage (Paterson *et al.*, 1984; Paterson *et al.*, 1987). This type of dimer modification has been found in *E. coli*, Chinese hamster ovary and human cells (Weinfeld *et al.*, 1986; Paterson *et al.*, 1992; Galloway, 1992, Galloway *et al.*, 1994; this study). In dimer-containing excision fragments isolated from post-UV incubated normal human fibroblasts, as many as 85% dimers are modified (Weinfeld *et al.*, 1986; Paterson *et al.*, 1992; Galloway *et al.*, 1994; this study).

One hypothesis entertained by our laboratory was that this backbone cleavage event may alleviate the structural distortion caused by dimerization. Several lines of experimentation which have been carried out in this laboratory imply that the intradimer cleavage reaction may be related to preferential DNA repair in transcriptionally active genes and hence facilitation of transcriptional bypass (Middlestadt *et al.*, 1990; Galloway, 1992).

The distribution of modified dimer sites in defined genes of XP-D cells has been studied in view of the fact that preferential repair of transcriptionally active genes has been found in a wide variety of biological systems (Middlestadt, *et al.*, 1990). This study was accomplished by modifying the protocol initially designed by Hanawalt and Bohr (1987). In short, (1) XP-D cells were UV-irradiated (15 J/m^2) and allowed to repair for different periods of time (4-25 hr); (2) genomic DNA was extracted and digested with the restriction enzyme BamH I; (3) restriction fragments were then treated with an excessive amount of photolyase to convert any modified dimer-sites to strand breaks before electrophoresis in alkaline agarose gels; (4) the denatured DNA strands on the gel were transferred to membranes and hybridized with either c-*myc* or c-*mos* ^{32}P-labeled probes; (5) densitometric determination of the full-length

restriction bands was performed; and finally, (6) modified dimer incidence in c-*myc* and c-*mos* sequences were calculated by application of the Poisson analysis. The results of this study showed that backbone-nicked dimer sites appeared to accumulate more rapidly by 12 hr post-UV incubation in the c-*myc* sequence (40%) than in the c-*mos* sequence (16%), the former representing a transcriptionally active gene, and the latter, a silent gene. This intragenomic heterogeneity of modified dimer site accumulation suggests the possibility that preferential repair of lesions in active genes could entail dimer modification.

An *in vitro* transcription experiment was carried out in an attempt to reveal what impact the dimers might have on the activity of RNA polymerase (Galloway, 1992). In principle, this assay system involves (1) preparation of a pGEM vector sequence, (2) construction of a cyclobutyl dimer-containing oligonucleotide in which an intact thymine-thymine dimer or a modified dimer is introduced at a defined site in the sequence, (3) ligation of the specific oligomers into the pGEM vector, and (4) *in vitro* transcription reaction by Sp6 RNA polymerase. Preliminary results demonstrated that an intact dimer blocked the progression of Sp6 RNA polymerase, whereas the modified dimer did not (Galloway, 1992). Using this same pGEM vector system, a preliminary study on DNA replication bypass revealed that both the intact and modified dimer served as a block to the DNA replication machinery (Galloway, 1992). In addition, a preliminary result showed that T4 endo V was unable to incise DNA at the modified dimer site (Galloway, 1992).

To further address the ability of T4 endo V, as well as other DNA repair enzymes including DNA photolyase and UvrABC endonuclease, to act on an intact

versus modified dimers, another experimental design was adopted. A set of substrates were constructed, i.e., a 43-bp oligonucleotide sequence containing a) no lesion, b) an intact cyclobutyl dimer at a defined position, c) the cyclobutyl dimer with its interpyrimidine phosphodiester linkage severed, producing 3' OH and 5' PO_4 termini, or d) the cyclobutyl dimer containing a cleaved intradimer backbone having 3'OH and 5'OH termini. The relative activities of the repair enzymes towards these dimer-containing substrates were measured out *in vitro.*

EXPERIMENTAL PROCEDURES

Materials

All oligonucleotides were purchased from the DNA Synthesis Laboratory, Department of Microbiology, University of Alberta; T4 ligase was obtained from Promega (Madison, WI); T4 polynucleotide kinase was from Pharmacia; [γ-^{32}P] ATP was purchased from Amersham (Burlington Heights, IL); T4 endo V and *E. coli* DNA photolyase were kindly supplied by my colleagues, Dr. Konrad Famulski and Randy Barley; *E. coli* UvrABC endonuclease and concentrated reaction buffer were kindly supplied by Dr. Moon-shong Tang, Department of Carcinogenesis, University of Texas. All other chemicals were procured from BDH Chemicals (Toronto, ON). All buffers were prepared according to Sambrook *et al.* (1989), unless otherwise stated.

Substrate construction

Purification of oligonucleotides:

The oligonucleotide sequences used are shown in Figure 7. DHL11 is a 16-mer with a 5'-phosphorylated end and the sequence GAATTCGTACTGAGTC; DHL12 has a non-phosphorylated 5'-end with the sequence CTATCGATGGCCTGCAT; both of these served as "arm" oligonucleotides in the ligation of dimer-containing substrates. DHL13, a 44-mer strand complementary in different places to DHL11, DHL12 and a dimer-containing 11-mer, has the sequence TGACTCAGTACGAATTCCTCCAAGTTGCCTGCAGGCCATCGATA and a non-phosphorylated 5'-end. DHL16 and DHL17, which served to produce the intact dimer-containing 11-mer (Figure 8), have exactly the same sequence, namely, GCAAGTTGGAG, but the former has a non-phosphorylated 5'-end, and the latter a phosphorylated 5'-end. DHL16 was used as a radioactive marker in the preparation of intact dimer-containing 11-mer after 5'-end phosphorylation with [γ-^{32}P] ATP. Similar to DHL16 and DHL17, DHL14 and DHL15 have the same sequence (GCAAGT), and were used in the preparation of the two kinds of modified dimer-containing 11-mer, where DHL15 served as a radioactive marker which was produced by ^{32}P-end-labeling of DHL14. DHL8 has a non-phosphorylated 5'-end and the sequence TGGAG, which was used in photoligation with DHL15 to form the modified dimer without an intradimer phosphate group. DHL5, which has the same sequence as DHL8 but with a 5'-

phosphorylated end, was used in photoligation with DHL15 to form another modified dimer containing a severed intradimer phosphodiester bond (Figure 8). AGA7 with the sequence CTCCGGACCTCCAACTTGCCATG, as well as DHL12 and DHL13, were ^{32}P-labeled at the 5'-end and served as molecular markers.

Approximately 1 µmol of each oligonucleotide described above, received in lyophilised form from the DNA Synthesis Laboratory, was dissolved in 1 ml of ddH_2O. After 100 x dilution with ddH_2O, the OD_{260} was measured and the concentration of each oligonucleotide solution was calculated (1 OD_{260nm} = 33 µg/ml).

Purification of the oligonucleotides was done before any further manipulation. DHL11, DHL12 and AGA7 were purified on a NAP-5 column (Pharmacia), which is a disposable Sephadex G-25 gel filtration column. This column was equilibrated with 3 volumes of TE buffer. Then 0.5 ml of the oligonucleotide solution (DHL11, DHL12, or AGA7) was loaded onto the column. The first 0.5 ml of eluate was discarded, and then 1 ml of TE buffer was used to elute each oligonucleotide, leaving any smaller molecules on the column (Galloway, 1992). DHL5, DHL8, DHL14, DHL15, DHL16 and DHL17 were purified by HPLC reverse-phase chromatography, according to the method of Galloway (1992). The purification/separation of the oligomers from smaller molecules was achieved solely by their different hydrophobicities on an end-capped Nova-Pak C_{18} reverse-phase column (250 x 4.6 mm i.d., Waters, Mississauga, ON). The mobile phase used was 0.1 M tetraethylammonium acetate (TEAA) and 10% 0.1 M TEAA in 90% acetonitrile. A programmed acetonitrile gradient was used over a 40-min run, i.e., acetonitrile from 7.2% to 9.0% over the first 20 min, 9.0% to 69.2% for the next 10 min, 69.2% for 5 min, and finally from 69.2% back to the initial state in 5

min. Seventy-two 0.5 ml fractions were collected and fractions corresponding to the oligomer UV_{260nm} absorption peak were pooled and dialysed overnight against 2 l of ddH_2O. The dialysed samples were then dried in a Savant SpeedVac Concentrator (model SVC 100H; Savant Instruments, Inc., Farmingdale, NY) and resuspended in 0.5 ml of TE buffer. These solutions were then assayed to determine the concentration of the purified oligomers.

Purification of DHL13 was performed by ultrafiltration with Centricon-10 (Amicon, Beverly, MA), which has a nucleotide cut-off for single stranded RNA/DNA of 30 nt. The ultrafiltration membrane in the sample reservoir was rinsed with 2 ml of ddH_2O to remove trace amounts of glycerin on the membrane by spinning at 5,000g for 1 hr in a fixed angle rotor and then inverting the sample reservoir and spinning at 300g for 2 min to remove the remaining rinse. After the membrane was washed, 0.5 ml of DHL13 solution was loaded in the sample reservoir and centrifuged at 5,000g until approximately 25 µl remained in the sample reservoir. The purified and concentrated solution was then recovered by inverting the device and spinning at 300g for 2 min. The nucleotide solution was brought up to a total volume of 0.5 ml with TE buffer and the concentration measured. All the purified oligomers were stored at 4°C.

Construction and purification of intact dimer-containing oligomer:

End-labeling of DHL16: This 11-mer was labeled to serve as a marker to trace the effects of UV-irradiation on DHL17. Two µg of DHL16 were mixed with 8 µCi of [γ-^{32}P] ATP (3,000 Ci/mmol, 1 mCi/ml) and 10 units of T4 polynucleotide kinase, in

a total volume of 20 µl of reaction buffer containing 10 mM Tris-acetate, 10 mM magnesium acetate and 50 mM potassium acetate. After incubation for 45 min at 37°C, 10 units of polynucleotide kinase were added to the reaction mixture, which was then incubated for an additional 30 min. An aliquot of the labeled DHL16 was then used as a radioactive marker.

UV-irradiation of DHL17: A UV transilluminator (Model TM-36, UVP Inc., San Gabriel, CA) with 2 lamps emitting mainly 302-nm UV light was used. A mixture of 244 µg of DHL17, 0.5 µg of ^{32}P-labeled DHL16, 10 mM of acetophenone and 450 µl of ddH_2O (total volume 600 µl) was prepared in a quartz cuvette and irradiated at 0°C with 302-nm UV light from the UV transilluminator. A 40% acetone solution was used as a liquid filter to remove UV light below 290 nm. This mixture was subjected to HPLC analysis after being irradiated for 7 hr. The total UV fluence was 1,000 kJ/m^2.

Purification of dimer-containing 11-mer: To separate the dimer-containing oligomer from the parent DHL17 and from oligomers bearing other possible photoproducts, the protocol of Galloway (1992) was used as described in more detail in the oligomer purification section. Briefly, the end-capped Nova-Pak C_{18} reverse-phase column was employed. The mobile phase used was 0.1 M TEAA and 10% 0.1 M TEAA in 90% acetonitrile. The same programmed acetonitrile gradient was used over a 40-min run. Seventy-two 0.5 ml fractions were collected for each run and 5 runs were required so as not to overload the analytic column. Collected fractions were assayed for radioactivity by Cerenkov counting. The corresponding dimer-containing fractions and those containing the parent oligomer, which were identified by the radioactivity distributions, were pooled and dialysed against 2 l of ddH_2O overnight

to remove TEAA. The dialysed samples were then dried by the Savant SpeedVac concentrator and resuspended in 50 µl of ddH_2O.

Confirmation of the product: To confirm the identity of the thymine-thymine dimer species, both samples recovered from previous steps were subjected to photochemical and photoenzymatic reversal. For photochemical reversal, ~10.9 pmol of each sample was diluted with 1 ml of ddH_2O and UV-irradiated at 254 nm with a total fluence of 5.5 kJ/m^2. The irradiated samples were then analyzed by HPLC, as described above. For photoenzymatic reversal, the same amount of each sample was mixed with 2.7 nmol of photolyase in a reaction mixture (40 µl) containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT and 0.1 µg/µl BSA (Sourena *et al.*, 1992). This reaction mixture was incubated at 37°C for 30 min in the dark before being exposed to fluorescent light for 1.5 hr. These photoenzymatically treated samples were then subjected to HPLC analysis. For both photochemical and photoenzymatic reversal experiments, the novel radioactive peak shifted to the position of the parent oligomer, indicating that the product contained cyclobutyl dimers.

Construction and purification of modified dimer-containing oligomers:

End-labeling of DHL14: This 6-mer was labeled to serve as a marker to monitor the photoligation reaction. The same procedures were followed as for labeling DHL16. An aliquot of the final reaction mixture was used as a marker to trace the extent of the photoligation reaction.

Annealing procedures: To achieve efficient photoligation of DHL15 and

DHL5, the 3'-thymine of DHL15 must be juxtaposed with the 5'-thymine of DHL5. This was achieved by annealing these two oligomers to their complementary scaffolding strand DHL13 at a molar ratio of 1:1:2 (Galloway, 1992). To this end, 124 µg of DHL15, 104 µg of DHL5, 0.9 µg of ^{32}P-labeled DHL14, and 1,825 µg of DHL13 were mixed in a total volume of 1 ml containing 500 mM NaCl and 50 mM sodium phosphate buffer, pH 6.8. The mixture was heated at 65°C for 10 min, and the temperature was then allowed to fall gradually to 4°C. The sample remained at the latter temperature overnight for efficient hybridization before being irradiated.

Photoligation procedures: The hybridized oligonucleotides were subjected to irradiation by 302 nm UV light after supplementing with acetophenone. The procedure was as described above for the intact dimer preparation.

Purification of photoligated oligonucleotides: Using the same protocol as for intact dimer separation, the photoligated oligonucleotides were separated from the parent molecules by HPLC chromatography and purified. Seventy-two 0.5-ml fractions of each injection were collected from the HPLC column and radioactivity was ascertained by Cerenkov counting. Fractions corresponding to the dimer-containing 11-mer and the parent 6-mer were pooled, dialysed, dried and resuspended in 60 µl ddH_2O, as described previously.

Confirmation of the product: Approximately 5 pmol of each sample collected was diluted with 0.5 ml of ddH_2O and subjected to 5.5 kJ/m^2 of UV irradiation at 254 nm wavelength and analyzed by HPLC. The regeneration of DHL15 served to identify the novel radioactive peak as being the photoligated dimer-containing 11-mer.

To construct an 11-mer containing a modified dimer without an intradimer phosphate, DHL8, instead of DHL5, was photoligated with DHL15. The protocols employed, such as end-labeling, annealing, photoligation, purification of photoligated oligonucleotides and confirmation of the product, were the same as those described above.

Generation of double-stranded DNA substrates containing a specific dimer:

To generate double-stranded DNA substrates containing different thymine-thymine dimers for monitoring the action of repair enzymes, 43 bp DNA sequences were constructed which contained in one strand a 17-mer at the 5'-end, a dimer-containing 11-mer in the middle and a 16-mer at the 3'-end, with a 44-mer as the complementary strand (Husain *et al.*, 1987).

End-labeling of DHL12: 1.45 μg of DHL12 were incubated with 25 μCi of [γ-^{32}P] ATP and 20 units of polynucleotide kinase in a 40-μl reaction mixture containing 10 mM Tris-acetate, 10 mM magnesium acetate, and 50 mM potassium acetate. After 45 min incubation at 37°C, 10 units of polynucleotide kinase were added and the reaction continued for another 30 min. Removal of unincorporated [γ-^{32}P] ATP was achieved using a Sep-Pak (Waters) C_{18} cartridge as follows: 1) the cartridge was washed with 10 ml of ddH_2O; 2) the samples were carefully loaded onto the cartridge; 3) free ^{32}P-ATP was washed off the cartridge with 10 ml of ddH_2O repeatedly until no radioactivity was present in the eluate; and 4) the radioactivity remaining was eluted and collected from the cartridge with 2 ml of methanol. The eluted sample was dried in a Savant SpeedVac Concentrator.

Ligation procedures: Approximately 65 ng of the three dimer-containing 11-mers and the non-dimer containing 11-mer were added to four separate Eppendorf tubes. About 350 ng of DHL12 (2.7 x 10^6 dpm), 1051 ng of DHL13 and 350 ng of DHL11 were then added to each tube and mixed with a ligation buffer (Promega) and 10 units of T4 ligase in a total volume of 100 µl. The solution was incubated first at 37°C for 30 min, then at 23°C for 90 min, and finally at 4°C for 12-14 hr (Husain *et al.*, 1987).

Purification and gel separation procedures: The ligated DNAs were precipitated by adding 0.1 volumes of 2.5 M sodium acetate and 3 volumes of ethanol and then holding at -20°C overnight. DNA pellets were obtained by spinning the samples at 10,000g for 60 min at 4°C. The supernatants were carefully discarded and the pellets were rinsed with 70% cold ethanol and dried in the Savant SpeedVac Concentrator. Each dried DNA sample was resuspended in 20 µl of loading dye (0.1% xylene cyanol FF, 0.1% bromophenol blue, 10 mM EDTA and 98% formamide), and then loaded on a 12% polyacrylamide/7 M urea gel. The gel was run for 1 hr at 500 V and 45 mA. A photographic film was exposed at room temperature for 1 min and then developed.

Recovery of the ligation products: The corresponding 44-mer bands were cut from the gel and the DNAs were eluted by the following procedure. The gel pieces were smashed into fine particles in Eppendorf tubes. One-half ml of 0.2 M ammonium acetate was added to each tube, which was kept at 4°C for 24 hr to allow the DNA to diffuse from the gel into the solution. The DNA solution was collected by spinning at 10,000g for 5 min. A second extraction was performed in the same way and the

contents were pooled. This solution was cleaned of trace gel particles by passing it through a 0.45 μm pore-size disposable filter unit (Baxter Diagnostics Co.), and the DNA was then phenol:chloroform extracted, ethanol precipitated, dried and resuspended in 50 μl of ddH_2O.

Formation of double-stranded DNA substrates: Approximately 60 ng of each dimer-containing 44-mer as well as the non-dimer control oligomer were mixed with 120 ng of DHL13 in annealing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA). Each sample was heated at 65°C for 10 min and subsequently allowed to cool to room temperature. These annealed dsDNA samples were then desalted by Centricon-10, with four successive washes with 2 ml of ddH_2O. The samples were then collected and the volumes measured. The concentration of each annealed sample was measured by spectrophotometer.

Confirmation of the ligation products: Approximately 0.05 pmol of each annealed 43 bp DNA sequence was treated with 10 units of EcoR I, which incises the substrates at position 29 and generates a 28-bp radioactive fragment. The reaction buffer contained 20 mM Tris-acetate, 20 mM magnesium acetate, and 100 mM potassium acetate. This reaction mixture was incubated at 37°C for 1 hr before phenol:chloroform extraction. The DNA in each sample was then ethanol precipitated, dried, and resuspended in sequencing gel loading dye. A 12% polyacrylamide sequencing gel, which also contained 2 molecular markers, DHL13 (44-mer) and AGA7 (23-mer), was run and then autoradiographed.

T4 endonuclease V incision reactions

Because of its pronounced substrate specificity, T4 endo V has been extensively used for quantification of pyrimidine dimers in DNA in general, and specific transcriptionally active genes subject to preferential repair in particular. However, structurally modified pyrimidine dimers such as dimers with severed intradimer phosphodiester bonds which are thought to exist in cells, may not be acted upon by this enzyme (Lewis and Hanawalt, 1982; Galloway, 1992). Thus, a systematic study with the three kinds of structurally similar but distinct pyrimidine dimers as substrates for T4 endo V would help to clarify the substrate requirement of T4 endo V.

Approximately 0.05 pmol of each of the dimer-containing dsDNA substrates and their control counterpart was treated for 20 min with 10 pmol of T4 endo V in a reaction buffer (50 µl) containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT and 0.1 µg/µl bovine serum albumin (BSA). The samples were then phenol:chloroform extracted, and the DNAs precipitated, dried, and resuspended in 10 µl of loading dye before performing sequencing gel analysis. A T4 endo V concentration-response curve was obtained by incubating the same amount of each substrate with 0.5-50 pmol of T4 endo V.

To assess whether photolyase had any effect on the T4 endo V incision reaction, the dimer-containing substrates were first incubated with 13.5 pmol of photolyase in the dark for 30 min at 37°C, whereupon 10 pmol of T4 endo V was added to each reaction mixture. After continuing incubation for another 20 min, the samples were phenol:chloroform extracted, ethanol precipitated and subjected to electrophoresis in a 12% polyacrylamide/7 M urea gel.

***E. coli* DNA photolyase dimer reversal reactions**

Photolyase also has a specificity for pyrimidine dimers. However, the extent to which this enzyme can act on modified pyrimidine dimers is an intriguing question. The report by Lewis and Hanawalt (1982) showed that this enzyme did not cleave photoligated thymine-thymine dimers. However, later reports (Paterson *et al.*, 1982; Pirsel *et al.*, 1989) provided unequivocal evidence to the contrary. Photolyase produced DNA strand breakage (5-15%) in the genomic DNA from post-UV incubated XP-D cells, which indicated that a portion of pyrimidine dimers underwent modification to become "backbone-nicked" dimers. *In vitro* studies on photoligated thymine-thymine dimers demonstrated that such modified dimers were subject to cyclobutyl ring-splitting by photolyase, although at a 10-fold lower efficiency than found in intact dimers (Weinfeld and Paterson, 1988; Liuzzi and Paterson, 1992). Interestingly, a chemically constructed modified dimer containing no intradimer phosphate was reported to be efficiently cleaved by photolyase (Sourena *et al.*, 1992). It was our intention to reach a conclusion regarding the specificity of photolyase towards modified dimers by comparing, under the same experimental conditions, the action of *E. coli phr*B-encoded photolyase on the intact and modified dimers constructed in this study.

In the photolyase dimer reversal reaction, approximately 0.05 pmol of each of the dimer-containing dsDNAs was incubated with either 1.35 or 13.5 pmol of *E. coli* photolyase in 50 µl of reaction mixture containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT and 0.1 µg/µl BSA at 37°C for 30 min in the dark prior to illumination under fluorescent light for 1.5 hr. Then two ID-containing tubes

were supplemented with 10 pmol of T4 endo V. Afterwards, all tubes were incubated for an additional 20 min. All such treated samples were then phenol:chloroform extracted, ethanol precipitated and subjected to electrophoresis analysis. A photolyase concentration-response curve was obtained in a similar manner, by incubating the same amount of each substrate with 1.35-13.5 pmol of photolyase.

E. coli UvrABC endonuclease incision reactions

The UvrABC endonuclease recognizes and acts on many kinds of structurally diverse DNA lesions. For example, pyrimidine dimers constitute a UvrABC substrate. These lesions induce a significant distortion in the DNA helix, causing strand unwinding, helix destabilization, and a site-specific bend (Pearlman *et al.*, 1985; Sherman and Lippard, 1987; Rice *et al.*, 1988; Husain *et al.*, 1988). Another example is thymine glycols, which do not appear to induce significant distortions but are nevertheless substrates for the UvrABC endonuclease complex (Lin and Sancar, 1989; Kow *et al.*, 1990). Therefore, it seems that gross structural alterations may not be necessary for damage recognition. So far, the precise mode of recognition of the UvrABC endonuclease is still not clear (van Houten and Snowden, 1993). Based on our hypothesis that modified dimers reduce the conformational distortion, it was of interest to know whether modified dimers would serve as substrates for the UvrABC endonuclease.

To assess whether the UvrABC endonuclease incises different dimer-containing substrates with different efficiencies and whether photolyase binding to the substrate affects the UvrABC incision reaction, one set of reactions contained 1 µl (0.05 pmol)

of each substrate, 6 µl of 10x ABC buffer (500 mM Tris-HCl, pH 7.5, 50 mM DTT, 100 mM $MgCl_2$, and 20 mM ATP) and 6 µl of 1 M KCl. Another set contained, in addition, 13.5 pmol of photolyase in the same final volume (60 µl). The tubes were incubated at 37°C for 5 min in the dark, before adding 4.4 pmol of UvrA, 8.4 pmol of UvrB, and 4.4 pmol of UvrC. The samples were then kept at 37°C for another 90 min, before phenol:chloroform extraction. The recovery of the oligomer fragments, including the 14-mer incision products, was achieved by conventional ethanol precipitation with glycogen as a co-precipitator. The complete precipitation of the DNA fragments was verified by radioactive counts. Then the samples were dried and electrophoresed through 12% polyacrylamide/7 M urea gel. Finally, the gel was enclosed in Saran wrap, and placed in a cassette with a Kodak X-ray film. The film was exposed for 19 hr at room temperature.

RESULTS

Production and isolation of intact dimer-containing oligomers

Modification of several established methods (Rahn and Landry, 1971; Lewis and Hanawalt, 1982; Benerjee, 1988; Weinfeld and Paterson, 1988), e.g., irradiation with a large dose (1,000 kJ/m^2), using 10 mM acetophenone as a photosensitizer, and a 40% acetone liquid filter to remove UV light shorter than 302 nm, improved the production of intact dimers 3.2-fold, compared with the method used by Galloway (1992).

The separation of intact dimer-containing DHL17 from its parent molecule by end-capped C_{18} reverse-phase column is shown in Figure 9. The novel peak at fractions 31-37 was well separated from the parent molecule at fractions 51-60. This novel peak contained DHL17 with the intact thymine-thymine dimer and was confirmed by both photochemical and photoenzymatic reversal analyses (Figures 10 and 11). The regeneration of the parent molecule by both photoreversal methods confirmed beyond reasonable doubt that the purified novel peak comprised the intact dimer-containing oligomer.

Production and isolation of modified dimer-containing oligomers

^{32}P-labeled DHL14 (i.e., DHL15) (6-mer) was photoligated with either DHL5 with a 5'-phosphate group or DHL8 with a 5'-hydroxy group to form two different kinds of modified dimers, i.e., dimers with a broken intradimer phosphodiester bond, one with and the other without an intradimer phosphate group (see Figure 8).

The HPLC chromatograms of the two resulting species are shown in Figures 12 and 13, respectively. The new peak at fractions 40-49 and 41-48 presumably represented the two modified dimer-containing 11-mers. Photochemical reversal of the two species, which regenerated a peak at fractions 20-30 where DHL15 elutes, confirmed this deduction (Figures 14 and 15). The amount of product containing the modified dimers was calculated to be 20% and 18% of the starting material, respectively, about 10-fold greater than that (2%) in both cases generated by UV irradiation at 254 nm wavelength (Galloway, 1992).

Ligation of the dimer-containing 11-mer to form a 43-bp dsDNA

The dimer-containing oligomers and their control counterparts were successfully ligated with 2 other oligomers at a molar ratio of 1:5:5:5, in order to make maximum use of the dimer-containing oligomers (Husain *et al.*, 1987). Each ligated 44-mer was separated from incomplete smaller ligation products on a 12% denaturing polyacrylamide gel (Figure 16). The dimer-containing 44-mer bands, which co-migrated with the 44-mer marker, were cut out and the DNA in each gel slice was eluted and purified, as previously described in "Experimental Procedures". Each purified ligation product was then annealed to its complementary strand to form 43 bp dsDNA molecules (one unpaired nucleotide at either end) (Figure 17). Confirmation of the final dsDNA substrates was achieved by EcoR I restriction mapping (Figure 18). These molecules were designated as "ND" for the non-dimer-containing dsDNA, "ID" for the intact dimer-containing dsDNA, "MDP" for the dsDNA containing a modified dimer with a severed intradimer phosphodiester bond, and "MDOH" for the dsDNA containing a modified dimer without an intradimer phosphate group.

T4 endonuclease V incision specificity towards the dimers

To determine optimal enzyme reaction conditions, the initial reaction conditions of Sibgaat-Ullah and Sancar (1992) were used. Approximately 0.05 pmol of ND, ID, MDP or MDOH was incubated with 5 pmol of T4 endo V in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM NaCl and 10 mM $MgCl_2$ in a volume of 100 µl at 37°C for 1 hr. The reaction was stopped by placing the tubes at -70°C. After precipitating the DNA, samples were subjected to electrophoretic analysis. Figure 19 shows that although T4

endo V cleaved ID and MDP, it created a smear. Nevertheless, T4 endo V did not cleave the ND control, and it was inactive towards the MDOH substrate, which showed no non-specific degradation by T4 endo V. To ensure that the smearing was not due to prolonged incubation, the reaction time was reduced to 20 min. This produced essentially the same results (data not shown). Based on the fact that in the presence of Mg^{++}, some other nuclease activities might be activated (Famulski, personal communication), the reaction mixture was modified to 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 20 mM DTT and 0.1 μg/μl BSA. Under these new conditions, T4 endo V cleaved the substrates with much greater specificity. Up to 40 pmol of the enzyme in a 50-μl reaction mixture was used but no smear was seen (data not shown). Therefore, the new reaction conditions were adopted in the study of the T4 endo V incision specificity.

As illustrated in Figure 20, T4 endo V is capable of severing both ID and MDP with similar efficiencies. With 10 pmol of T4 endo V, 90% and 94% of the substrates, respectively, were converted to an incision product (23-mer). On the other hand, MDOH was refractory to T4 endo V, in the presence of the same amount of enzyme.

The T4 endo V concentration-response curves in Figure 21 show that with as little as 0.5 pmol of enzyme, about 65% of the dimer-containing sites are cleaved in both ID and MDP substrates. Because the enzyme preparation loses its activity completely upon further dilution, responses at lower concentrations of the enzyme were not obtained. The shape of the response curves for both ID and MDP were very similar and the incision reaction went almost to completion at 10 pmol of the enzyme. T4 endo V again showed no incision of MDOH at a 5-fold excess of the enzyme which

cleaved the other two substrates completely. Increasing the amount of T4 endo V to 200 pmol also failed to produce any incision in MDOH (data not shown). These results imply that the action of T4 endo V on dimers is dependent on the presence of the intradimer phosphate group.

The effects of photolyase binding on the T4 endo V incision reaction are presented in Figure 22. In the presence of an excessive amount of photolyase (13.5 pmol), the efficiency of the T4 endo V reaction was reduced substantially, i.e., 69% breakage produced in lane 4 compared to 99% in the control (lane 2) with ID as substrate; and 75% (lane 8) compared to 92% (lane 6) with MDP as substrate. The inhibition of T4 endo V incision of pyrimidine dimer-containing sites by photolyase is consistent with the results of Sancar (1984).

E. coli DNA photolyase specificity towards the dimers

Results in Figure 23 demonstrate the effects of photolyase at different concentrations on dimer reversal. Lane 1 corresponds to ID without photolyase or T4 endo V treatment. Lane 2 serves as a positive control, showing that 10 pmol of T4 endo V cleaved most of the intact dimers. Lanes 3 and 4 show that pretreatment of the ID substrate with 1.35 pmol of photolyase plus fluorescent light reversed all the dimers such that the oligomer was no longer subject to T4 endo V incision. At this low enzyme level (1.35 pmol), photolyase monomerized all the dimers in ID (lane 3), but only 9.3% in MDOH (lane 8). A 10-fold increase in photolyase resulted in cleavage of as little as 4.5% of the dimers in MDP (lane 6). Figure 24 displays the photolyase concentration-response relationship for the various substrates. It again reveals the

relative inactivity of photolyase towards MDP substrate, and the concentration-dependent cleavage of MDOH. The dimer reversal of ID by photolyase has the highest efficiency of all enzymes tested. Therefore, it is clear that there are significant differences in the action of photolyase towards these three dimer-containing substrates.

E. coli UvrABC endonuclease incision specificity towards the dimers

As the UvrABC dual incision process involves three protein subunits, which act in a concerted manner, inactivity of any one of the subunits would result in failure to incise the dimers. Therefore, to ensure the success of our endeavour, all enzyme proteins and related reagents including double-distilled water were kindly supplied by Dr. Moon-Shong Tang. We are grateful to Dr. Tang for providing us with these proteins and reagents, as well as the protocol and related references.

Our initial trial was carried out according to Tang *et al.* (1992). The 25-µl reaction mixture contained either 0.05 pmol of each dimer-containing substrate, or UV-irradiated pBR322 plasmid (15 J/m^2 to produce, on average, one dimer per plasmid), 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP and 100 mM KCl), and 0.4 pmol UvrA, 0.35 pmol UvrB and 0.4 pmol UvrC. Controls contained no Uvr proteins. All tubes were then incubated at 37°C for 90 min. The pBR322-containing samples were subjected to electrophoresis in a 0.8% agarose gel, running in 1x TBE buffer at 35 mA for 1 hr. The gel was then stained with ethidium bromide (0.5 µg/ml in TBE buffer for 30 min) and photographed. The oligomer substrate-containing samples were subjected to phenol:chloroform extraction, ethanol

precipitation, drying and resuspension in sequencing gel loading buffer. Electrophoretic analysis revealed no activity towards dimer-containing oligomer substrates, while 56% of the UV-irradiated pBR322 substrate was acted upon, indicating that the Uvr proteins are active towards the plasmid DNA (data not shown).

The failure of the initial experiment on dimer-containing oligomer substrate prompted us to increase the enzyme concentrations and/or decrease the substrate concentrations in the reaction mixture. Up to a 16-fold increase of the enzyme:substrate ratio had no effect on the ID DNA, while the increased enzyme concentration did result in a greater amount of incisions of the UV-irradiated pBR322 (data not shown).

Considering that our dimer-containing oligomer substrates are only 43 bp long, which is much shorter than the 179-bp and 129-bp DNA fragments used in the studies of Tang and associates, a search for alternative reaction conditions which would be suitable for short oligomer substrates led us to employ the protocol published by Snowden (1990). This protocol was used to compare the effects of the UvrABC endonuclease on a 49 bp DNA duplex containing different lesions. The main differences between this protocol and that of Tang and coworkers were as follows: a) the UvrA to UvrB to UvrC ratio is 3:6:14, compared to that of approximately 1:1:1 in the protocol described above; b) the reaction buffer does not contain EDTA, a common cation chelator; c) the concentration of ATP, which is required for tight UvrB binding to the DNA lesion site and release of UvrA from $UvrA_2B$ complex, is increased two-fold (Sancar and Tang, 1993).

Preliminary experiments with the second protocol were then carried out to

determine whether the incision might now occur in the ID substrate. The 50-µl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 10 mM $MgCl_2$, 2 mM ATP, 140 mM KCl, 100 ng/µl BSA,and 0.05 pmol of ID or UV-irradiated pBR322. After complete mixing, all reaction mixtures were preincubated at 37°C for 5 min before UvrA, UvrB and UvrC were added simultaneously. Two sets of concentrations of Uvr proteins were tested. One set of tubes contained 1.1 pmol of UvrA, 2.1 pmol UvrB and 5.5 pmol UvrC; another set contained 4.4 pmol UvrA, 8.4 pmol UvrB and 22 pmol UvrC. The control tubes contained no Uvr proteins. After thorough mixing, the reaction solutions were incubated for 90 min before phenol:chloroform extraction, ethanol precipitation and electrophoretic analysis. The results are shown in Figure 25. An incised product, which represented 8% of the input ID substrate was generated in the sample treated with the higher amount of enzyme. Therefore, the detection of effects of UvrABC endonuclease on the different dimer-containing substrates, and the effects of photolyase binding on the UvrABC dual incision reaction, were based on the above experimental conditions, as described in the "Experimental Procedures" section of this chapter.

The effects of the UvrABC endonuclease on the different dimer-containing substrates are shown in Figure 26. Each substrate was treated with or without UvrABC, with or without photolyase (13.5 pmol), and with UvrABC alone or with photolyase in the dark plus UvrABC. Lanes 2-5, 6-9, and 10-13 depict reactions with ID, MDP and MDOH as substrate, respectively. First, UvrABC recognized and incised all 3 different dimer-containing oligomers, producing a 14-mer product band (lanes 4, 8, and 12), which migrated slightly faster than the 17-mer marker (lane 1). This

fragment size was about what would be expected as the excision fragment removed from the middle of the substrate by the UvrABC complex should be 12-13 nt long (Sancar, 1994). The rates of substrate incision were calculated to be 5.9%, 3.3%, and 3.6% for ID, MDP, and MDOH, respectively. Photolyase-plus-fluorescent light treatment of the substrates revealed that the enzyme was capable of reversing two of the dimers in the substrates, revealing an unaltered 44-mer band from ID (lane 3), but a 23-mer band from MDP (faint, lane 7) or from MDOH (lane 11). The demonstration of photolyase activity is a prerequisite for the photolyase-plus-UvrABC incision reaction. The intensity of the 14-mer bands produced by UvrABC endonuclease in non-photolyase pretreated (lanes 4, 8, and 12) or photolyase pretreated (lanes 5, 9, and 13) were similar (3-6% of substrate inputs). This indicates that photolyase binding to the substrates in the dark, prior to the addition of UvrABC into the reaction mixture, did not seem to alter the activity of UvrABC towards the dimer-containing oligomers.

DISCUSSION

The interpyrimidine phosphodiester bond cleavage in a cyclobutyl dimer, has been found in both prokaryotic and eukaryotic cells. These modified dimer sites accumulate during post-UV incubation in the genomic DNA of human cells (Paterson *et al.*, 1984, Paterson, 1987; Galloway, 1992), CHO (Piirsel *et al.*, 1989) or *E. coli*

strains that are deficient in DNA repair (Galloway, 1992). Furthermore, modified dimers have been recovered from excision fragments of post-UV incubated human fibroblasts (Weinfeld *et al.*, 1986; Galloway, 1992; this study), CHO cells (Galloway, 1992), and *E. coli* (Galloway, 1992; this study). The nuclease digestion/HPLC assay used to identify modified dimers involves treatment of the samples with CAP, which removes the 5'-phosphate of the dinucleotide containing the dimer. Therefore, DNAs with modified dimers were constructed accordingly, with or without a 5'-phosphate within the thymine-thymine dimer.

To produce large amounts of intact dimer-containing substrate, the parent oligomer, an 11-mer, was first irradiated with 254 nm UV light (Galloway, 1992). The problem encountered was that the dimer-containing 11-mer peak was difficult to identify in the HPLC profile, presumably because of either low dimer yield and/or a vast amount of radioactivity of the parent compound interfering with the separation of the dimer-containing peak from that of the parent molecules. After failure over several months, a modified method was established, which incorporated aspects from several protocols (Lewis and Hanawalt, 1982; Weinfeld and Paterson, 1988; Benerjee *et al.*, 1988). UV light of 302 nm wavelength was used instead of 254 nm light. The latter induced only low levels (7-10%) of dimer-containing products (Rahn and Landry, 1971; Galloway, 1992) due to the equilibrium established between cyclobutyl dimer formation and their subsequent photochemical reversal at high UV doses. The use of 40% (v/v in water) acetone as a filter prevented the sample solution from being irradiated by shorter (< 302 nm) UV light, thereby avoiding photochemical reversal. The use of acetophenone, which serves as a photosensitizer, specifically increased pyrimidine

dimer formation. The operation of the UV transilluminator (Model TM-36, UVP Inc. San Gabriel, CA) as a 302 nm UV light source proved much simpler than a UV monochromotor (Weinfeld and Paterson, 1988), and was comparable in simplicity to that of a germicidal UV (254 nm) light box (Galloway, 1992). The generation of a dimer-containing 11-mer under these conditions was easily detected. This 11-mer was produced in a yield similar that reported by Lewis and Hanawalt (1982) and Benerjee *et al.* (1988), and 3- d greater than that of Galloway (1992).

Photoligation of terminal thymines to generate modified dimers was achieved by Lewis and Hanawalt (1982). In their protocol, double-stranded polymers of oligo $(d(pT)_{10})$.poly(dA) were irradiated with >290 nm UV light under anoxic conditions, generating 20-mer, 30-mer, and 40-mer products, with production rates of 20%, 10% and 5%, respectively. By modification of this protocol and that of Weinfeld *et al.* (1986), two kinds of modified dimers in defined oligomer sequences were produced, with efficiencies similar to that reported (20%) by Lewis and Hanawalt (1982). Our protocol was not only easier to operate but also more efficient in generating modified dimers (20% and 18%, respectively) than that achieved (2%) by UV irradiation at 254-nm wavelength (Galloway, 1992).

The separation of a dimer-containing 11-mer from its progenitor molecules was accomplished by using an end-capped C_{18} reverse-phase column. C_{18} functional groups are attached to a silica matrix, allowing separation of molecules via hydrophobic interaction. Because unreacted silica groups are end-capped, any silanophilic associations with the molecules are avoided. Therefore, the separation is purely based on the hydrophobic differences of the cyclobutyl dimer-containing 11-mer

from its progenitor molecule.

The ligation of the dimer-containing 11-mer with 2 "arm" oligomers to their 44-mer complementary strand formed a 43 bp duplex containing a thymine dimer at the central location. The adopted design of forming a 43-bp duplex with a single noncomplementary base at the termini served to prevent multimer formation by end-to-end ligation (van Houten *et al.*, 1986).

It is clear from our results that T4 endo V cleaves MDP as efficiently as it does ID, but is sluggish in cleaving MDOH even at a 100-fold excess of the enzyme. Preliminary results from this laboratory, by using another dimer-containing substrate system, revealed similar action of T4 endo V towards dimers structurally similar to that of MDOH. That is, modified dimers with severed intradimer phosphodiester bonds were refractory to incision by T4 endo V, after pretreatment with CAP (Galloway, 1992). Our data regarding the inactivity of T4 endo V towards MDOH is consistent with data reported by Sourena *et al.* (1992). Therefore, our discovery of T4 endo V incision specificity towards modified dimers with a broken intradimer phosphodiester bond (as opposed to modified dimers without an intradimer phosphate group), extends our understanding of the action of this enzyme. That is, the action of T4 endo V is highly structure-specific and the intradimer phosphate group is necessary for the incision reaction by T4 endo V. This finding supports the idea that the appearance of preferential repair of lesions in active genes may be caused, in part, by dimer modification, particularly if the modified dimers in the active genes lack an intradimer phosphate group. This hypothesis needs further study.

The finding that photolyase inhibits the T4 endo V incision reaction is in

accordance with that of Sancar (1984). It also correlates with the report of Patrick and Harm (1973), who showed that yeast photolyase inhibits *Micrococcus luteus* UV endonuclease, an enzyme that has a size similar to that of T4 endo V and a similar mechanism of action. As the amount of T4 endo V produced upon infection by T4 phage is in vast excess of photolyase (Nakabeppu and Sekiguchi, 1981), the inhibition of T4 endo V by photolyase may be of little physiological significance in *E. coli*.

E. coli photolyase cleaves a modified dimer with a severed intradimer phosphodiester bond poorly, but can completely cleave a modified dimer without an intradimer phosphate group if excessive amounts of enzyme are used. These reproducible results are consistent with those reported by Lewis and Hanawalt (1982) and Sourena *et al.* (1992). The reduced activity of photolyase towards modified dimers without an intradimer phosphate group, and the relative insensitivity of photolyase towards modified dimers with a severed intradimer phosphodiester bond, could also explain why extremely large amounts of photolyase are required to reverse "nicked" dimers, and to detect single-strand breaks in c-*myc* and c-*mos* genes (Weinfeld and Paterson, 1986; Liuzzi and Paterson, 1992; Middlestadt *et al.*, 1990). It is not currently known whether the cleavage of intradimer phosphates by an exonuclease occurs after dimers are modified by severing intradimer phosphodiester bonds. Such a study would help to clarify the role(s) of dimer modification in preferential repair.

The fact that photolyase can cleave a pyrimidine dimer without an intradimer phosphate but not modified dimers with a severed intradimer phosphodiester bonds leads us to hypothesize that the mechanism of action of *E. coli* photolyase does not require the intradimer phosphodiester bond itself. However, the breakage of the

intradimer phosphodiester bond could induce a local conformational change, causing the enzyme to be inactive. Employing the ethylation interference technique to identify phosphate contacts of this same dsDNA sequence with the enzyme reveals that ethylation of the intradimer phosphodiester bond does not inhibit binding to the enzyme; however, modification of the first or second phosphate 3' to the dimer almost completely eliminates the binding. Modification of the first phosphate 5' to the dimer or third phosphate 3' to the dimer also greatly diminishes the binding (Baer and Sancar, 1989). Lewis and Hanawalt (1982) reported that *E. coli* photolyase could recognize but not cleave a nicked dimer produced by photoligation of $d(pT)_{10}$. These data lend support to the hypothesis described above.

Our initial intention was to determine to what extent modified pyrimidine dimers could serve as substrates for the UvrABC endonuclease, because even intact pyrimidine dimers are known to be poor substrates for the UvrABC endonuclease (Svoboda *et al.*, 1993). As seen in Figure 26, the modified dimers are substrates for the UvrABC endonuclease, although the efficiency is exceedingly low. The intensities of the 14-mer product bands in UvrABC treated dimer-containing oligomers were very similar, being 3-6% of the total radioactivity, as determined by densitometry.

The results shown in Figure 26 also indicate that photolyase does not enhance incision of the dimers by UvrABC. This finding is consistent with the report of Sutherland (1981). Photolyase from yeast, on the contrary, inhibits the UvrABC incision of dimers (Sancar and Smith, 1989). There are also some data to suggest that *E. coli* photolyase stimulates UvrABC excision repair (Harm and Hillebrandt, 1962; Yamomoto *et al.*, 1984; Sancar *et al.*, 1984). One explanation for these results could

be that there are major differences in substrate binding, or substrate-dependent protein-protein interactions involved in the reactions.

CONCLUSIONS

In summary, this study has revealed the following results (Table 5). First, T4 endo V cleaves both an intact dimer and a modified dimer with a severed intradimer phosphodiester bond with similar efficiencies; however, it is essentially refractory to a modified dimer lacking an intradimer phosphate group, implying that the internal phosphate group is necessary for substrate recognition by T4 endo V. Secondly, *E. coli* photolyase is capable of reversing the cyclobutyl bridge in intact dimers and modified dimers lacking an intradimer phosphate group, but it fails to reverse a modified dimer with a severed intradimer phosphodiester bond, even in the presence of a 10-fold higher concentration of the enzyme. This implies that the intradimer phosphate group may inhibit the action of photolyase at a modified dimer site. Lastly, the UvrABC endonuclease cleaves all three substrates with similar, albeit low efficiencies. These results evidently reflect the different modes of action of the three repair enzymes on cyclobutyl dimer-containing substrates.

Reaction product (%) Substrate / Enzyme	ID	MDP	MDOH
T4 endonuclease V	91.4	94.0	2.9
Photolyase (1x)	100.0	2.0	9.3
Photolyase (10x)	100.0	7.0	96.0
UvrABC endonuclease	5.9	3.3	3.6

Table 5. Summary of results of the enzymatic reaction by T4 endonuclease V, *E. coli* DNA photolyase or UvrABC endonuclease, towards various dimer-containing double-stranded DNA substrates.

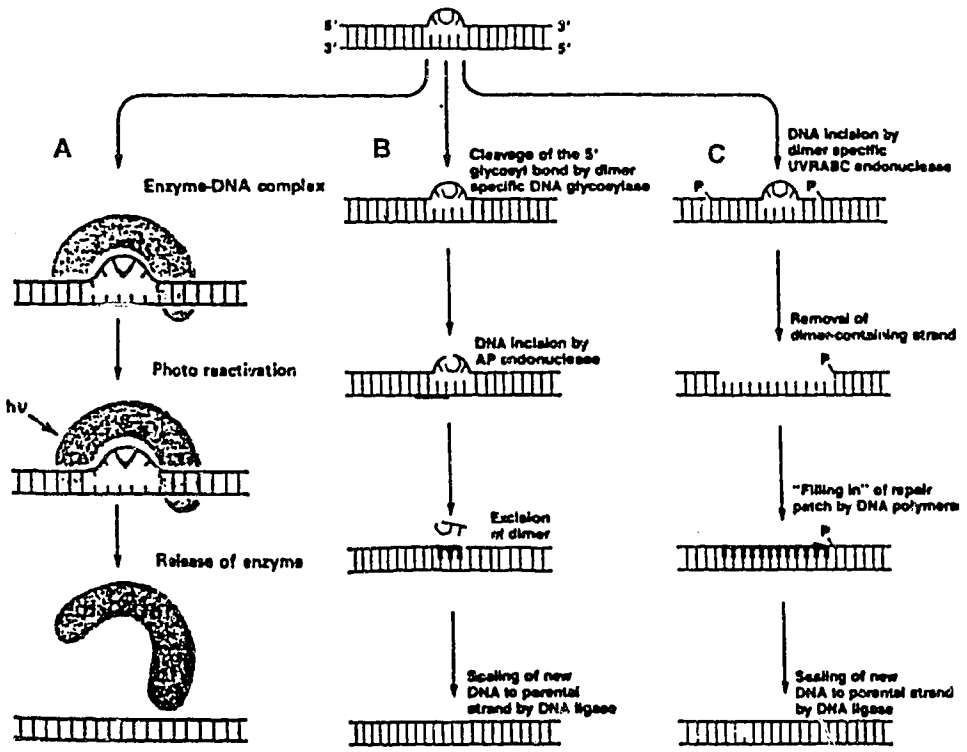


Figure 6. Schematic illustration of modes of action of DNA photolyase (A), T4 endonuclease V (B), and UvrABC endonuclease (C) towards a cyclobutyl pyrimidine dimer.

DHL5/8: $_{P/HO}$TGGAG

DHL14/15: $_{HO/P}$GCAAGT

DHL16/17: $_{HO/P}$GCAAGTTGGAG

DHL11: $_{P}$GAATTCGTACTGAGTC

DHL12: $_{HO}$CTATCGATGGCCTGCAG

AGA7: $_{P}$CTCCGGACCTCCAACTTGCCATG

DHL13: $_{HO}$TGACTCAGTACGAATTCCTCCAACTTGCCTGCAGGCCATCGATA

Figure 7. Oligonucleotides used in this study.

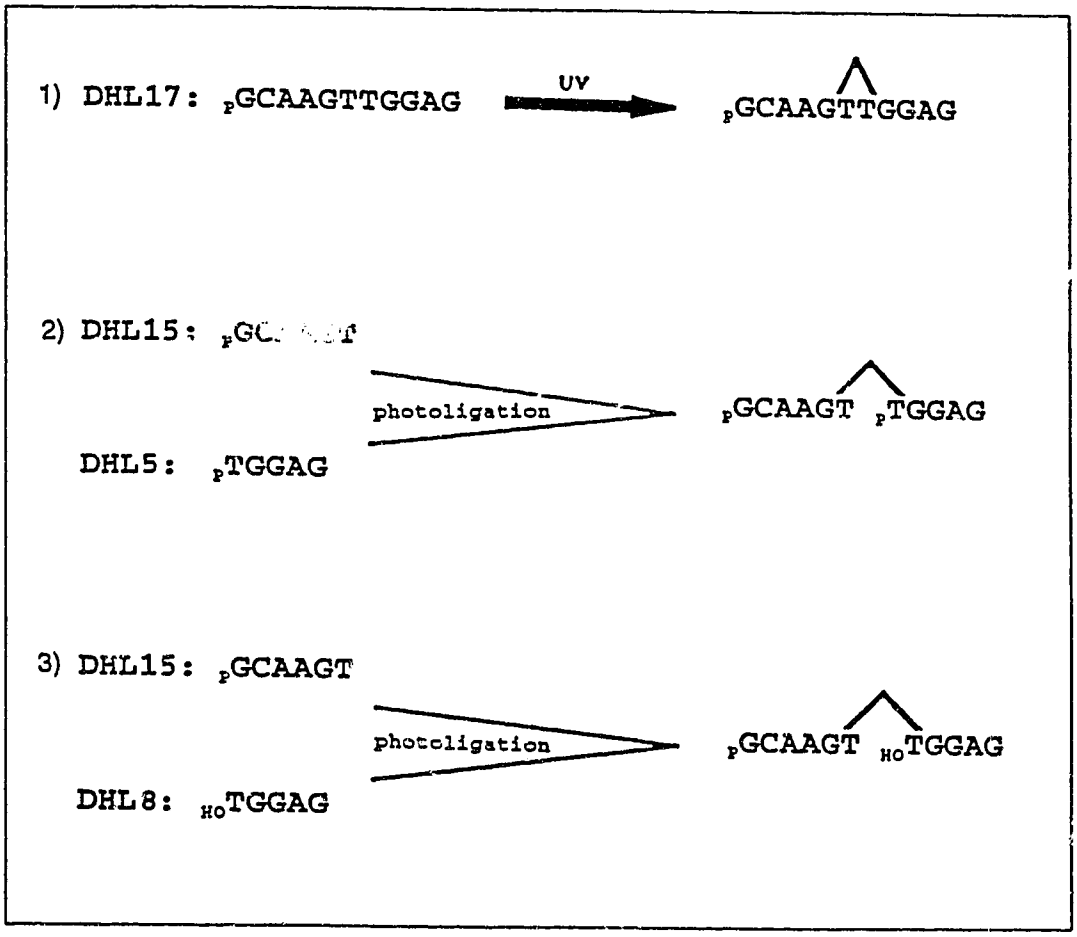


Figure 8. Construction of dimer-containing oligonucleotides containing: 1) an intact dimer; 2) a modified dimer with a "severed" intradimer phosphodiester bond; or 3) a modified dimer without an intradimer phosphate group.

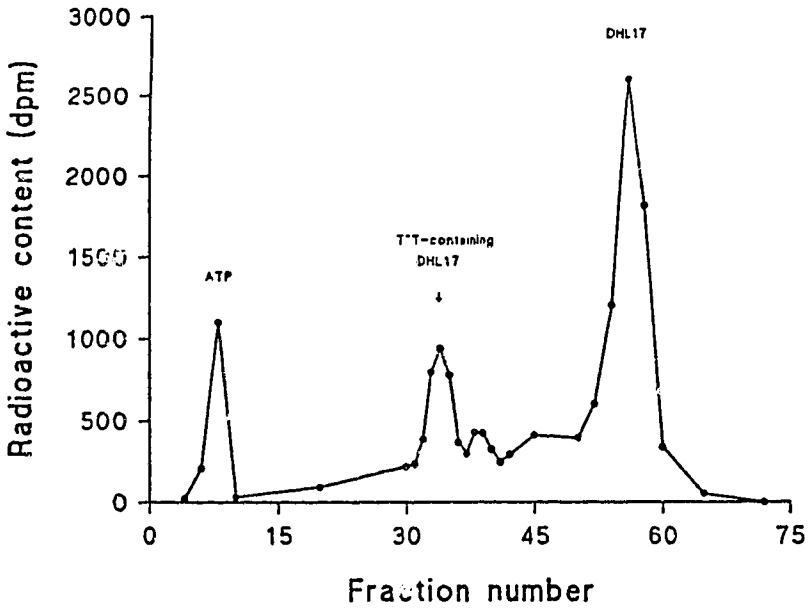


Figure 9. Reverse-phase HPLC chromatogram illustrating the separation of an intact dimer-containing DHL17 from its parent oligomer DHL17.

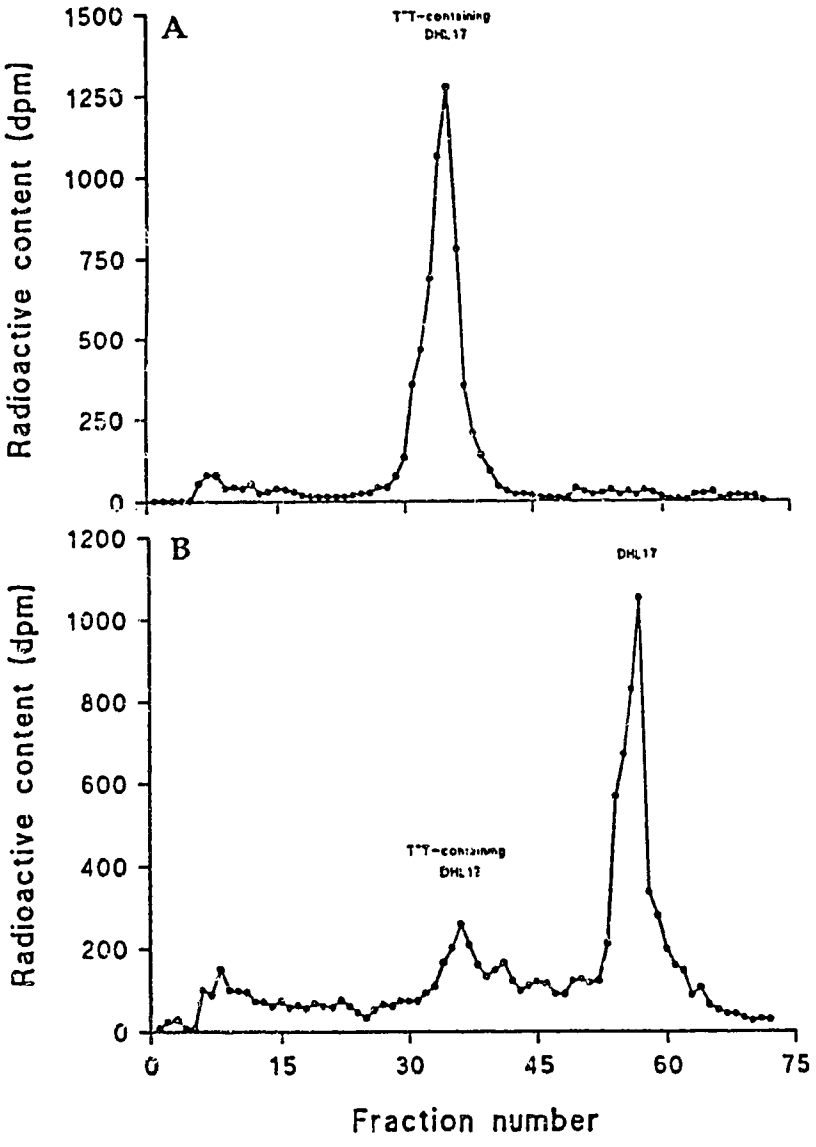


Figure 10. Reverse-phase HPLC chromatogram demonstrating photochemical reversal of intact dimer-containing DHL17 (A) to its parent oligomer DHL17 (B).

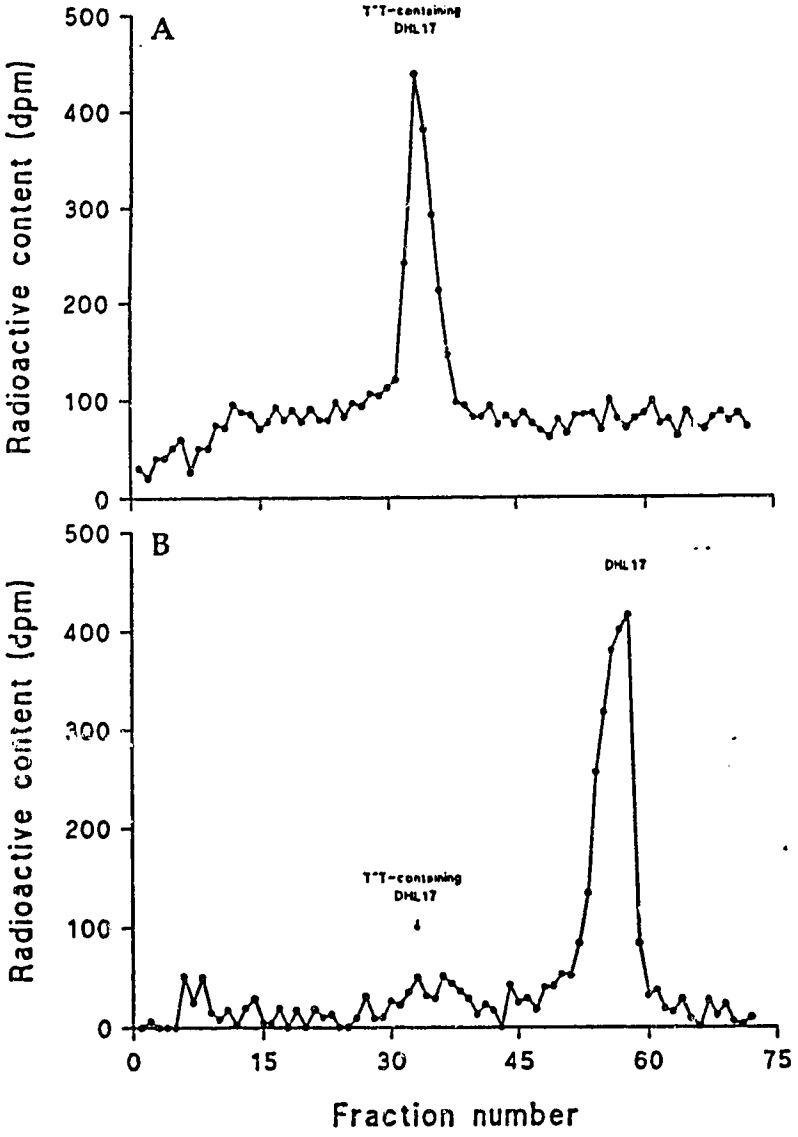


Figure 11. Reverse-phase HPLC chromatogram demonstrating photoenzymatic reversal of intact dimer-containing DHL17 (A) to its parent oligomer DHL17 (B).

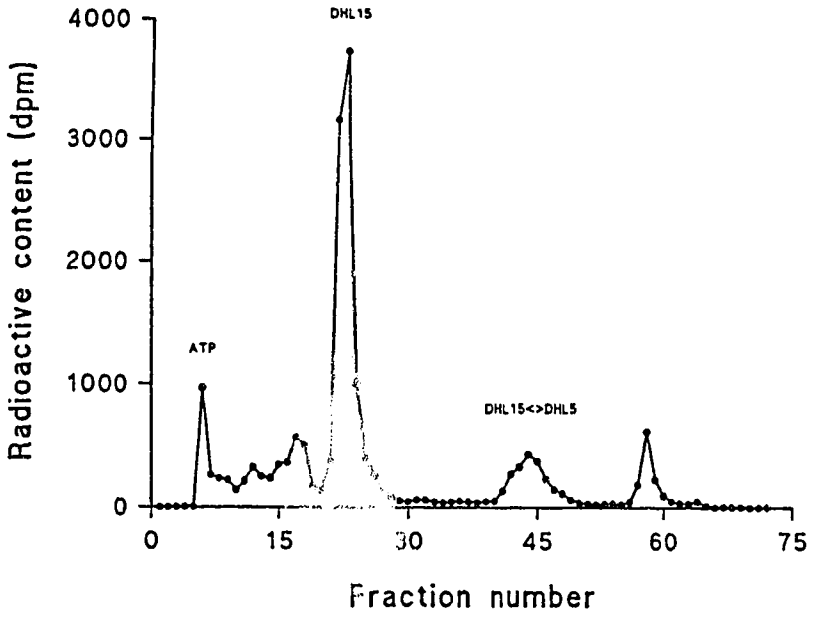


Figure 12. Reverse-phase HPLC chromatogram showing the production and separation of DHL15<>DHL5 containing a modified dimer with a severed intradimer phosphodiester bond from one of the parent oligomer (i.e., DHL15). Note that the second parent oligomer, DHL5, is not labeled and therefore is not seen on the chromatogram.

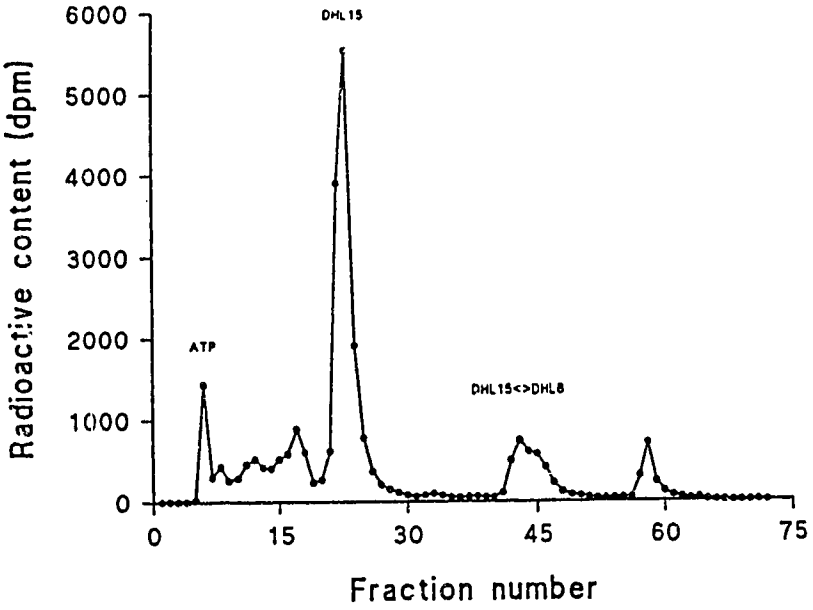


Figure 13. Reverse-phase HPLC chromatogram showing the isolation of DHL15<>DHL8 containing a modified dimer without an intradimer phosphate group from one of the two parent oligomers, DHL15. Note that the second parent oligomer, DHL8, is not labeled and therefore is not seen on the chromatogram.

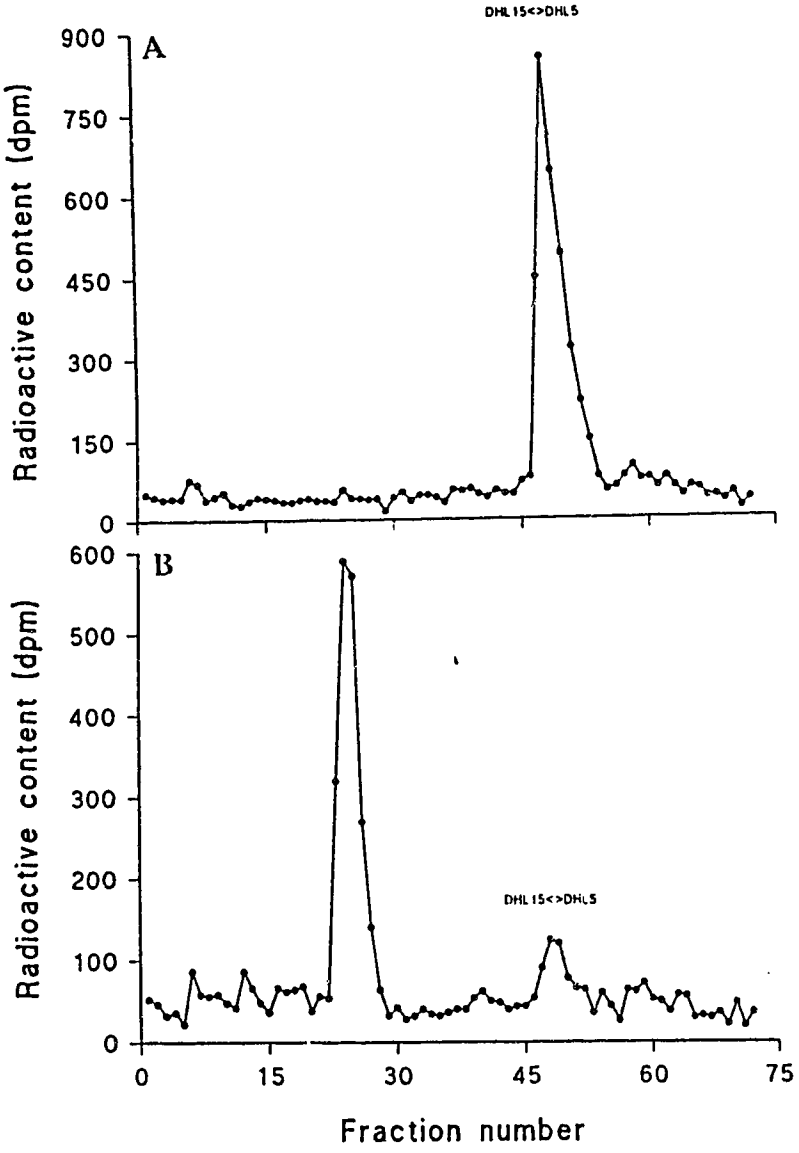


Figure 14. Reverse-phase HPLC chromatogram demonstrating photochemical reversal of DHL15<>DHL5 containing a modified dimer with a severed intradimer phosphodiester bond (A) to one of its parent oligomers, DHL15 (B). Note that the second parent oligomer, DHL5, is not labeled and therefore is not seen on the chromatogram.

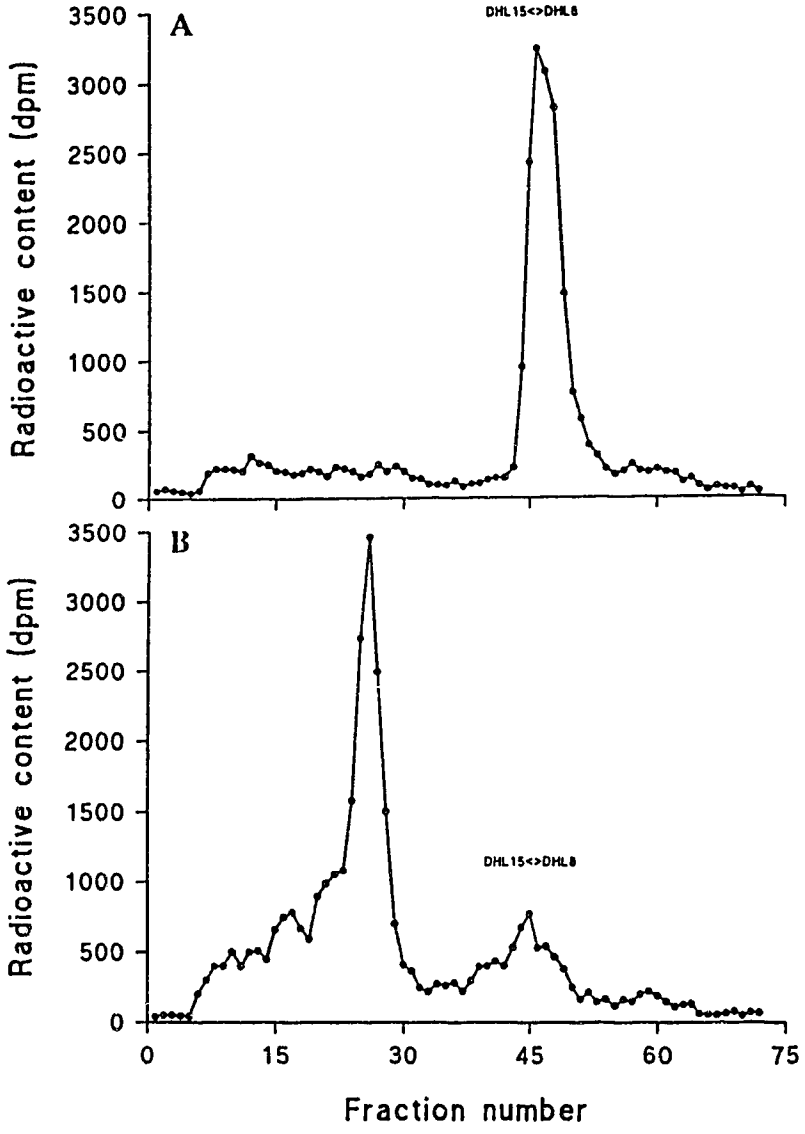


Figure 15. Reverse-phase HPLC chromatogram demonstrating photochemical reversal of modified dimer without an intradimer phosphate group (A) to its parent oligomer, DHL15 (B). Note that the second parent oligomer, DHL8, is not labeled and therefore is not seen on the chromatogram.

Figure 16. Polyacrylamide denaturing gel displaying the products of large ligation reactions containing ^{32}P-end-labeled DHL12, DHL11, DHL13, and different dimer-containing 11-mers. Lane 1: 44-mer marker; lane 2: a ligation reaction involving non-dimer-containing DHL17; lane 3: as in lane T^T-containing DHL17; lane 4: as in lane 2, but using DHL15<>DHL5; lane 2, but using DHL15<>DHL8.

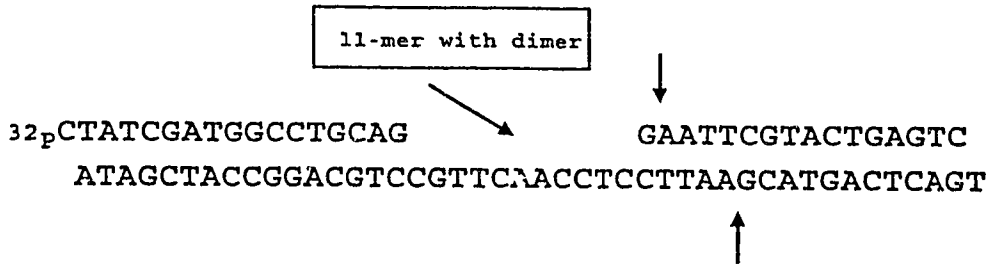


Figure 17. Ligated double-stranded DNA fragment containing a centrally located cyclobutyl dimer. The EcoR I restriction site is indicated by vertical arrows.

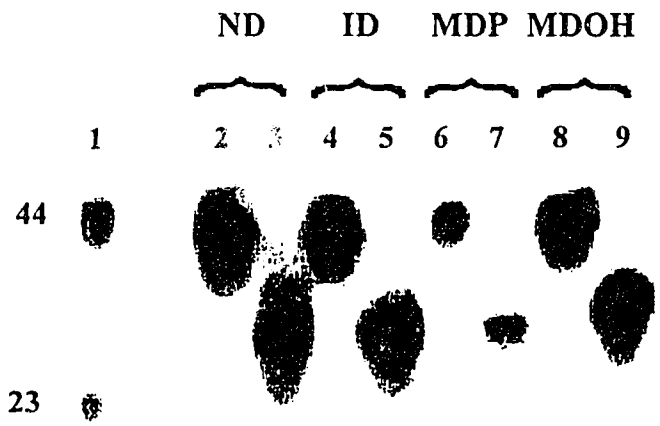


Figure 18. Polyacrylamide denaturing gel displaying the ligation products ND, ID, MDP and MDOH following treatment with EcoR I restriction enzyme. Lane 1: molecular markers 23-mer and 44-mer; lanes 2 and 3: ND treated without or with EcoR I; lanes 4 and 5: ID treated as in lanes 2 and 3; lanes 6 and 7: MDP treated as in lanes 2 and 3; lanes 8 and 9: MDOH treated as in lanes 2 and 3.

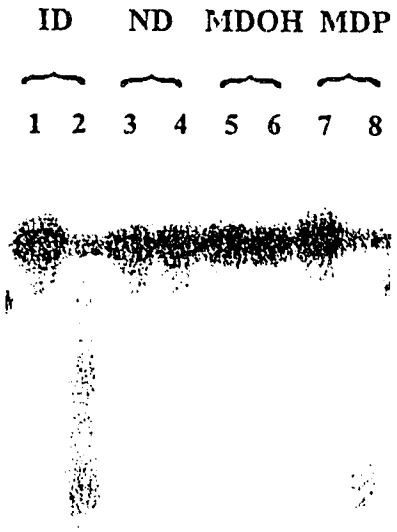


Figure 19. Polyacrylamide denaturing gel displaying the non-specific activity of T4 endonuclease V towards various dimer-containing dsDNA substrates, under the following experimental conditions: 50 fmol of ID, ND, MDOH, or MDP were incubated with 5 pmol of T4 endo V, in a reaction mixture containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM NaCl and 10 mM $MgCl_2$ for 1 hr at 37°C. Lanes 1 and 2: ID treated without or with T4 endo V; lanes 3 and 4: ND treated as in lanes 1 and 2; lanes 5 and 6: MDOH treated as in lanes 1 and 2; lanes 7 and 8: MDP treated as in lanes 1 and 2.

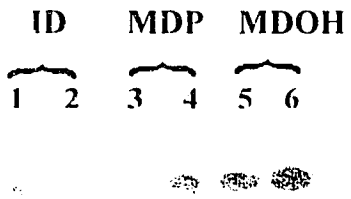


Figure 20. Polyacrylamide denaturing gel demonstrating the improved specific activity of T4 endonuclease V towards various dimer-containing dsDNA substrates, under the following experimental condition: 50 fmol of ID, MDP, or MDOH was incubated with 10 pmol of T4 endo V, in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 20 mM DTT and 0.1 mg/ml BSA for 20 min at 37°C. Lanes 1 and 2: ID treated without or with T4 endo V; Lanes 3 and 4: MDP treated with or without T4 endo V; Lanes 5 and 6: MDOH treated as in lanes 1 and 2.

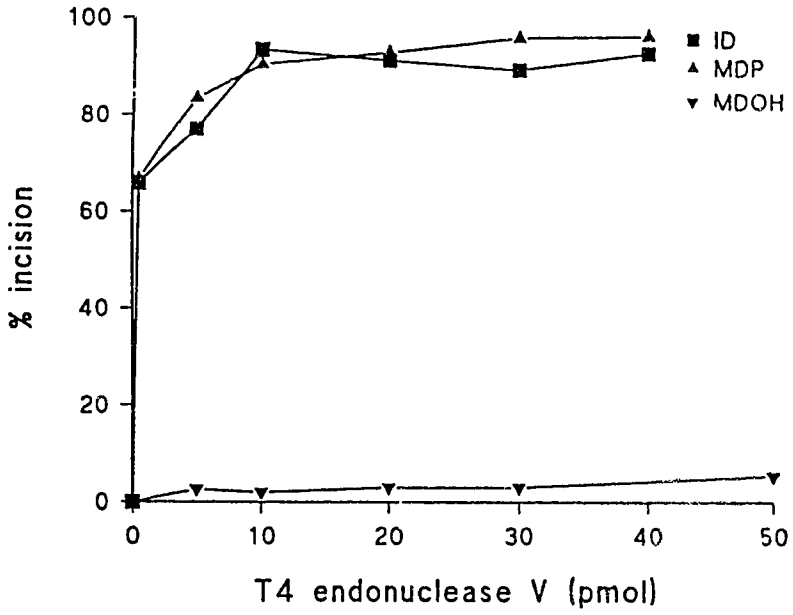


Figure 21. T4 endonuclease V concentration-response relationship. A fixed amount of dsDNA substrate ID, MDP, or MDOH was treated with various amounts of T4 endonuclease V. The incision of the substrates is expressed as percentage of the band density of the incision product divided by that of the substrate untreated with the enzyme.

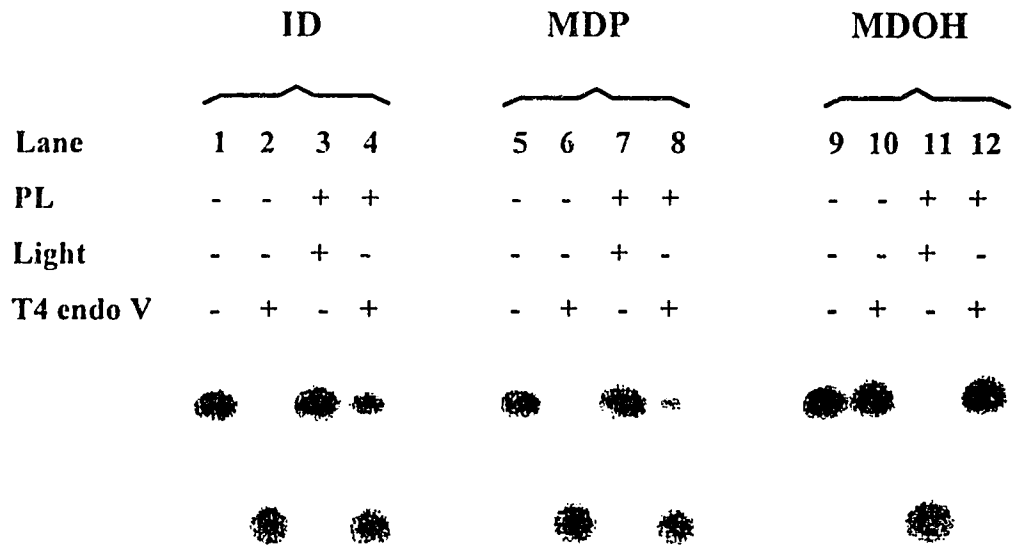


Figure 22. Polyacrylamide denaturing gel displaying the effects of DNA photolyase binding on the T4 endonuclease V incision reaction. Each of the various dimer-containing dsDNA substrates was incubated with an excess amount of photolyase in the dark at 37°C for 30 min to permit binding of photolyase to each substrate; then T4 endonuclease V was added to each reaction mixture for a further 20 min. The incision product (23-mer) of T4 endonuclease V migrates faster and is represented by the lower band. Lanes 1-4: ID as substrate; lanes 5-8: MDP as substrate; lanes 9-12: MDOH as substrate. The + and - signs represent with or without the enzyme or fluorescent light treatment.

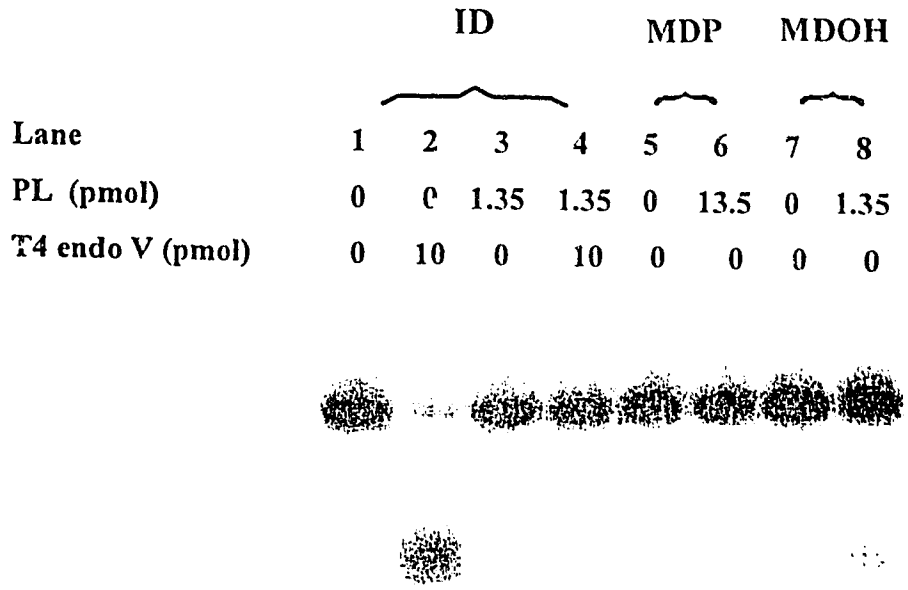


Figure 23. Polyacrylamide denaturing gel displaying the activity of DNA photolyase towards different dimer-containing substrates. Fifty fmol of ID, MDP, or MDOH were incubated with 1.35 or 13.5 pmol of photolyase in a reaction mixture (50 µl) containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT and 0.1 µg/µl BSA, at 37°C for 30 min in the dark before being irradiated with fluorescent light for 1.5 hr. Two ID-containing tubes, as indicated on top of this figure, were then supplemented with 10 pmol of T4 endonuclease V. Afterwards, all tubes were incubated for an additional 20 min. Lane 1: ID without T4 endonuclease V or photolyase treatment; lane 2: ID treated with T4 endonuclease V; lanes 3 and 4: ID treated with 1.35 pmol of photolyase plus fluorescent light, and then without or with T4 endonuclease V; lanes 5 and 6: MDP treated without or with 13.5 pmol of photolyase plus fluorescent light; lanes 7 and 8: MDOH treated as in lanes 1 and 3.

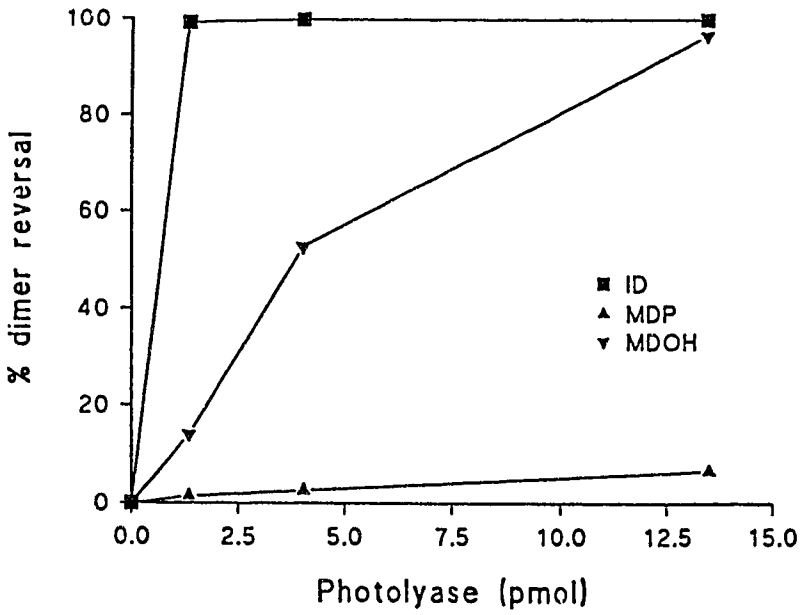


Figure 24. DNA photolyase concentration-response relationship. Fixed amounts of dsDNA substrate ID, MDP, or MDOH were treated with various amounts of photolyase. Dimer reversal is expressed as percentage of the band density of the 44-mer product (in the case of ID as substrate), or the 23-mer product (in the case of MDP or MDOH as substrate), divided by that of the substrate untreated with the enzyme.

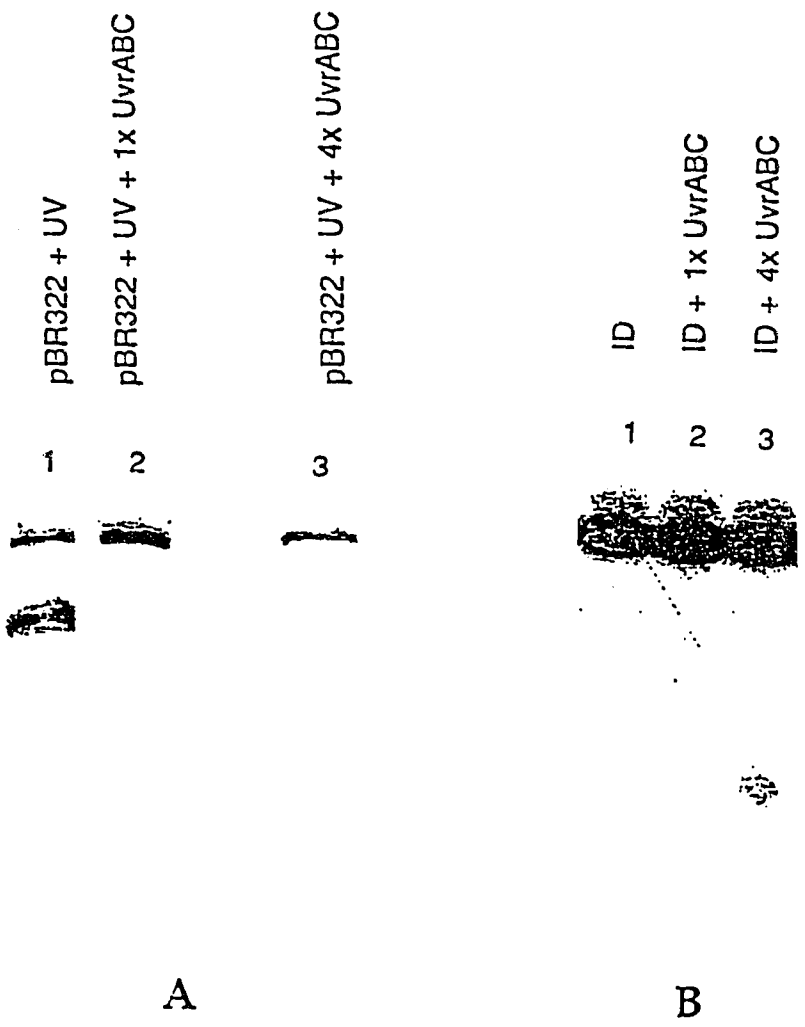


Figure 25. Polyacrylamide denaturing gel displaying the activity of UvrABC towards UV-irradiated pBR322 (A) and intact dimer-containing dsDNA substrate,denoted ID (B). Fifty fmol of UV-irradiated pBR322 or ID were incubated with two different concentrations of UvrABC endonuclease (1x or 4x), as described in "Results". Lane 1: UV-irradiated pBR322 (A) or ID (B); lane 2: as in lane 1, but treated with 1x enzyme complex; lane 3: as in lane 1, but treated with 4x enzyme complex.

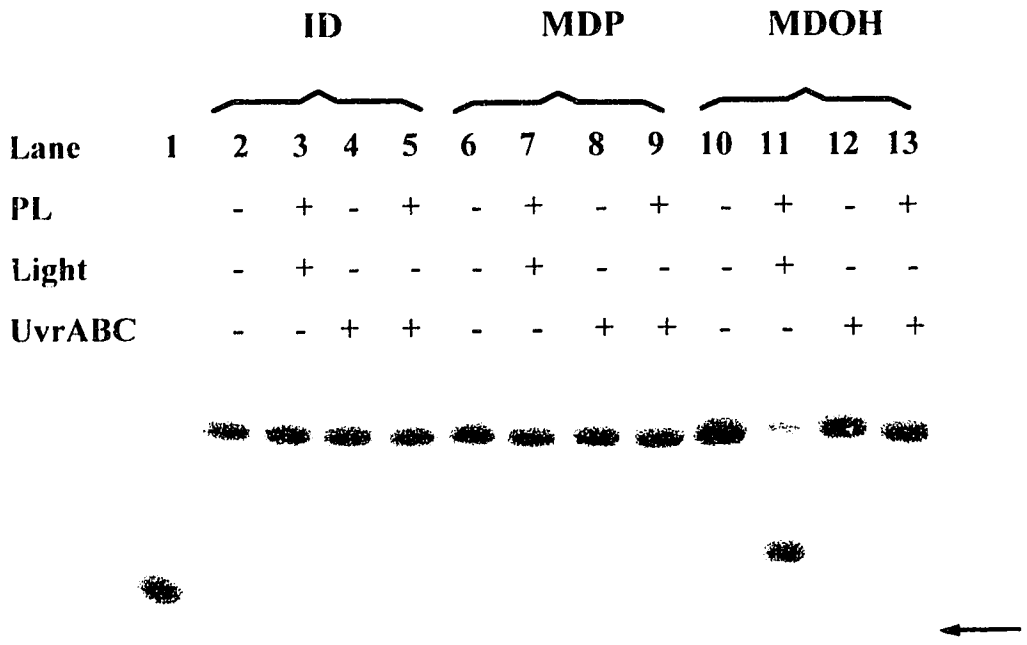


Figure 26. Polyacrylamide denaturing gel displaying the activity of UvrABC endonuclease towards various dimer-containing dsDNA substrates and the effects of DNA photolyase binding on the UvrABC-mediated dual incision reaction. Each of the dimer-containing dsDNA substrates was incubated with an excess amount of photolyase in the dark at 37°C for 30 min to permit photolyase binding to the substrate, whereupon UvrA, UvrB, and UvrC subunits were added concurrently to each reaction mixture and incubation was continued for a further 90-min period. The UvrABC incision product (indicated by an arrow) migrates faster than the 17-mer marker. Lane 1: a 17-mer marker; lanes 2-5: ID treated as indicated; lanes 6-9: MDP treated as indicated; lanes 10-13: MDOH treated as indicated. The + and - signs represent with or without the enzyme or fluorescent light treatment, respectively.

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