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The induction and repair of ultraviolet light-induced (6-4) photoproducts in single copy genes

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by

Jin Jen

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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Jin Jen, B.S. (Medicine)

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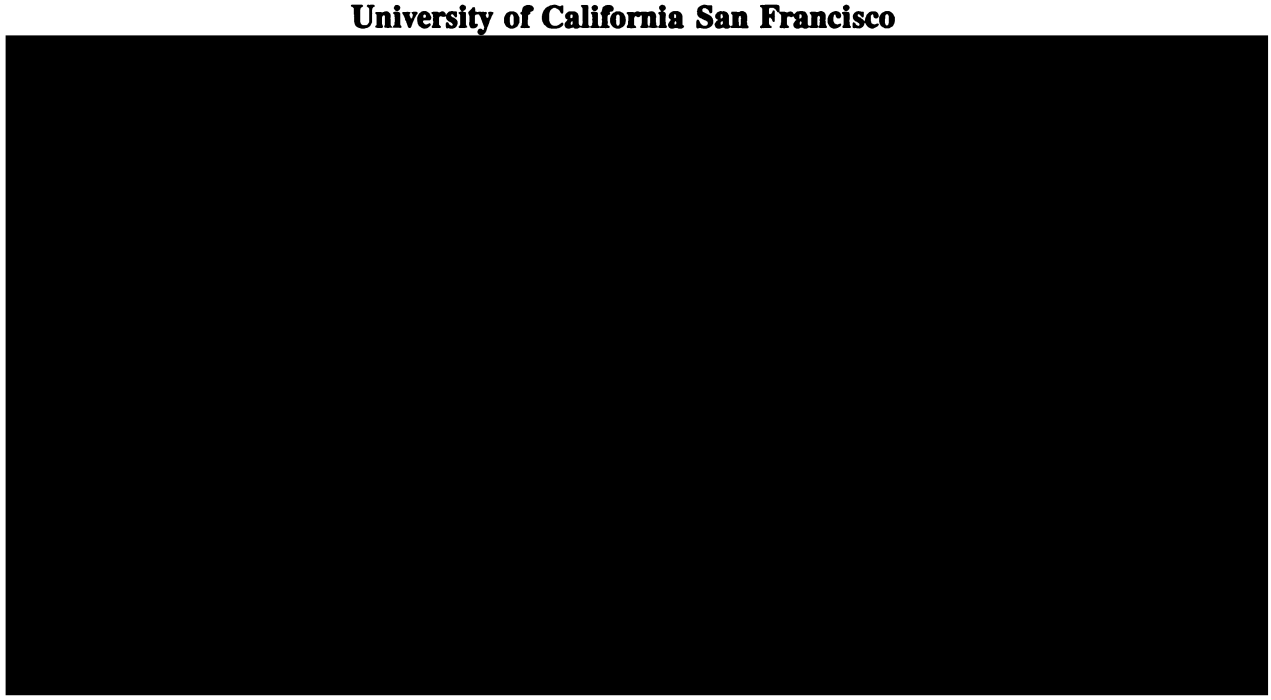

Dedicated to

my parents and

to the memory of

my grandparents

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ABSTRACT

The Induction and Repair of Ultraviolet Light-Induced (6-4) Photoproducts in Single Copy Genes

Jin Jen

University of California, San Francisco, 1991

Thesis Adviser: James E. Cleaver, Ph.D.

Ultraviolet light (UV) irradiation causes damage to human cells by inducing different photoproducts in the DNA. Dipyrimidine cyclobutane dimers and (6-4) photoproducts are the major lesions responsible for the mutagenic and lethal effects of UV irradiation. In mammalian cell systems, evidence suggests that the relatively minor (6-4) photoproducts may be more responsible for the cytotoxic effects attributed to UV irradiation. This research attempts to develop an assay for sensitive detection of the (6-4) photoproduct induction and repair in single copy genes of mammalian cells.

E. coli endonuclease III (endo III) was initially used as a probe to detect (6-4) photoproducts. Sequencing analysis showed that the enzyme cleaved cytosine hydrates among other photoproducts. UV-induced endo III sensitive sites having novel characteristics were also detected. These endo III sensitive sites were primarily induced at cytosine-containing dipyrimidine sites and were stable at 55 °C after 24

hours. Structurally, these photoproducts appeared to be distinct from cyclobutane dimers and (6-4) photoproducts.

A controlled alkaline hydrolysis assay was characterized for the specific detection of (6-4) photoproducts. We showed that this assay can specifically cleave UV-irradiated DNA at the site of (6-4) photoproducts and we subsequently used it in conjunction with Southern blot analysis to measure the induction of (6-4) photoproducts in single copy genes at a biologically relevant low UV dose. We determined that (6-4) photoproducts were primarily induced at the transcribed DHFR gene region but not the nontranscribed delta-globin gene region. In contrast, cyclobutane dimers were formed uniformly throughout the genome. Preliminary results further showed that at the DHFR gene locus, the revertant XP129 cells repaired more than 70% of the (6-4) photoproducts in 6 hours but only about 20-30% of cyclobutane dimers 20 hours after UV irradiation.

Our findings suggested that the induction of (6-4) photoproducts may be favored at the essential and/or more accessible regions of the genome, possibly due to the chromatin structure of the DNA. Consequently, the rapid repair kinetics and the higher cytotoxicity observed for (6-4) photoproducts may be the result of their preferential induction in the mammalian genome. The alkaline hydrolysis assay described here provided a relatively simple and direct method for studying the formation and repair of (6-4) photoproduct in specific genes.

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LIST OF ABBREVIATIONS

(6-4) photoproduct: pyrimidine(6-4)pyrimidone photoproduct

AP: apurinic/apyrimidinic

CHO: Chinese Hamster Ovary

DHFR: dihydrofolate reductase

DNA: deoxyribonucleic acid

Endo III: *E. coli* endonuclease III

ERCC: excision repair complementing CHO cells

ESS: endonuclease sensitive sites

HPLC: high-performance liquid chromatography

LM-PCR: ligation mediated polymerase chain reaction

p1.8: plasmid PBH31R1.8

PALs: photoinduced alkali-labile site

PCR: polymerase chain reaction

RIA: radioimmunoassay

T4 endo V: phage T4 endonuclease V

UV: ultraviolet

UVA: ultraviolet wavelength between 320 nm to visible light

UVB: ultraviolet wavelength between 290 and 320 nm

UVC: ultraviolet wavelength between 240 and 290 nm

XP: xeroderma pigmentosum

CHAPTER ONE: INTRODUCTION

1.1. The Interaction of Ultraviolet Radiation with DNA

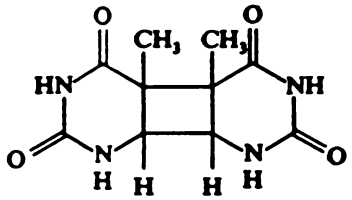
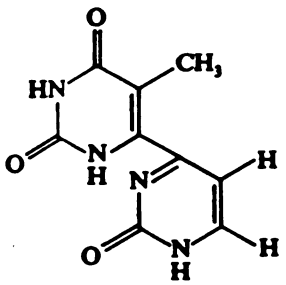
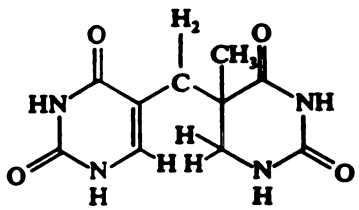
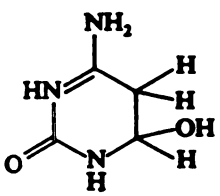
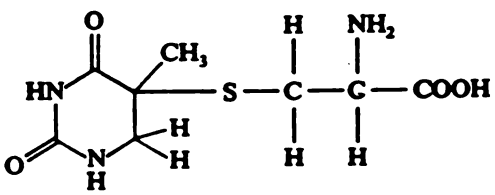
Sunshine, so vital to human life, induces detrimental effects upon over exposure. It may cause erythema and even induce skin cancer (Harm, 1980). Much evidence has suggested that these detrimental effects are primarily due to the formation of DNA photoproducts in cells exposed to the ultraviolet (UV) portion of the sunlight (Jagger, 1967; Setlow, 1968; Murphy, 1975; and Friedberg, 1985). Cells from xeroderma pigmentosum (XP), a rare autosomal recessive disease, are defective in the early steps of cellular repair process for UV-induced DNA damage (Cleaver, 1968, Cook, et al., 1975). Patients with XP exhibit extreme sensitivity to sunlight and usually develop skin cancer at an early age (Cleaver and Kraemer, 1990).

Biologically, DNA is the principal molecule for transmitting genetic information among all organisms. The DNA molecule contains adenine, guanine, cytosine and thymine bases, all of which strongly absorb UV light with a maximal absorbance around 260nm (Wang, 1976). Photons absorbed by DNA generate electronically excited bases that can undergo various photochemical reactions with neighboring bases, water molecules, and proteins, resulting in the formation of UV photoproducts (Kittler and Lober, 1977). Photoproducts commonly found in UV-irradiated DNA are: cyclobutane pyrimidine dimers, pyrimidine (6-4) pyrimidone

photoproducts [(6-4) photoproducts], pyrimidine photohydrates, spore photoproducts, and DNA protein cross-links as illustrated in Figure 1. Unusual photoproducts at purine sites (Gallagher and Duker, 1986) and adenine residues (Gasparro and Fresco, 1986) have been reported. UV-induced photoproducts at individual cytosines recognized and cleaved by *E. coli* endonuclease III have also been reported (Demple and Linn, 1980; Weiss and Duker, 1986, 1987). However, the structure and biological significance of these photoproducts are not yet determined.

Photochemically, the formation of a particular UV photoproduct depends upon the wavelength of the irradiation as well as the chemical composition of the DNA (Setlow and Carrier, 1966; Unrau, 1973), the environment in which the DNA is irradiated (Patrick and Rahn, 1976), and the neighboring sequence of the potential photoproduct sites (Lippke et al., 1981; Mitchell et al., 1991). Spore photoproducts are the major lesions found in DNA irradiated under dehydrated conditions (Jagger, 1976). Cyclobutane dimers can undergo spontaneous reversion upon further UV irradiation (Setlow and Carrier, 1966). The (6-4) photoproducts on the other hand are converted to a photoisomer, the Dewar photoproduct, when subsequently irradiated with 313nm light (Patrick, 1970; Taylor and Chors, 1987; 1990; Mitchell and Rosenstein, 1987). Cytosine hydrates are formed at a significant rate in DNA under physiological conditions but are readily reverted back to original monomers under heated conditions (Fisher and Johns, 1976; Boorstein et al., 1990).

Figure 1. Examples of UV-induced DNA photoproducts (from Harm, 1980).

Class of photoproduct	*Representative photoproduct*
Cyclobutane-type dimers	 cys-syn thymine-thymine dimer
Pyrimidine adducts	 6-4′-[pyrimidin-2′-one] pyrimidine
Spore photoproduct	 5-thyminyl-5,6-dihydrothymine
Pyrimidine hydrates	 6-hydroxy-5,6-dihydrocytosine
DNA-protein crosslinks	 5-*S*-cysteine-5,6-dihydrothymine

1.2. Cyclobutane Dimers and their Biological Importance

Cyclobutane-type dimers are formed between the adjacent pyrimidine bases of the same DNA strand (Brukers and Berends, 1960; Setlow, 1966). Historically, pyrimidine cyclobutane dimers were considered the primary lesions responsible for the mutagenic and lethal effects associated with UV in *E. coli* because:

1) cyclobutane dimers are the major DNA photoproducts found in UV-irradiated cells (Setlow, 1966, 1968);

2) the UV spectrum of dimerization for adjacent thymines coincides with the biological effects of UV light (Jagger, 1985);

3) photoreversal of cyclobutane dimers by photolyase in the presence of visible light abolishes the lethal and most of the mutagenic effects of UV light (Hanawalt et al., 1982); and,

4) cells from repair-deficient *E. coli* mutants that are unable to excise cyclobutane dimers are highly sensitive to UV light (Howard and Tessman, 1966).

In mammalian systems, the cytotoxic effects of cyclobutane dimers is demonstrated in the highly UV-sensitive XP cells that were deficient in repairing these photoproducts (Cleaver, 1968). The mutagenic effect of cyclobutane dimers was demonstrated using a plasmid vector that can propagate in both mammalian and *E. coli* cells (Protic-sabljic et al., 1986). Plasmid DNA was UV irradiated, treated with or without yeast photolyase, and transfected into monkey cells where replication

and mutagenesis occurs. The site and frequency of mutation were analyzed when the plasmids were recovered and used to transform bacterial cells. The removal of ~90% of cyclobutane dimers by photoreactivation increases the biological activity of the plasmid by 75% and reduces the mutation frequency by 80%.

The intensive study on the biological effects of cyclobutane dimers was also helped by the availability of dimer-specific enzymes and our increasing ability to study DNA at higher resolution (Ehmann et al., 1978; Haseltine et al., 1980; and Bohr et al., 1985). Dimer-specific UV endonuclease (T4 endo V) encoded by the *den* V gene of T4 phage has been the most widely used. This enzyme has a DNA glycosylase activity that hydrolyses the bond between the 5' pyrimidine of the dimer and its corresponding sugar residue, and a subsequent AP endonuclease activity which incises DNA at the phosphodiester bond on the 3' side of the abasic sugar (Gordon and Haseltine, 1980; Nakabeppu and Sekiguchi, 1981). The cleavage of cyclobutane dimers by UV endonuclease introduces single stranded breaks in UV-irradiated DNA which is then measurable by a number of different methods (Paterson et al., 1973, Wilkins, 1973; Brash and Haseltine, 1982).

Using the T4 endo V and Southern blot hybridization, Bohr et al. (1985) analyzed the formation and removal of dimers from the dihydrofolate reductase (DHFR) gene in mammalian cells. CHO cells carrying an amplified (50x) DHFR gene were irradiated and allowed to repair for 0 or 24 hours. The unreplicated

parental DNA was purified from CsCl gradient and treated with T4 endo V. DNAs treated with the enzyme were electrophoresed in parallel with the untreated samples; and the presence of dimers in the DHFR gene was calculated from the loss of full length (dimer-free) fragment in the treated samples after hybridization with a genomic DHFR probe. Using this method, it was demonstrated that in mammalian cells the removal UV endonuclease sensitive sites (ESS) was more efficient in the DHFR gene coding region than its neighboring noncoding region and the genome overall (Bohr et al., 1985, 1986a). Furthermore, the efficient removal of ESS from DHFR gene corresponds with the survival of UV-irradiated cells (Bohr et al., 1986b). Similar preferential repair have also been observed for the active *c-abl* but not the inactive *c-mos* oncogene in tumor cells (Madhani et al., 1986); for the adenosine deaminase (ADA) gene but not for a non-transcribed DNA sequence (Van Zeeland, 1986). In addition, cyclobutane dimers are repaired much faster in the transcribed strand of the DHFR gene (Mellon et al., 1986). The transcriptional activation of the *E. coli lac*I gene increases the removal of the dimers from the gene (Mellon and Hanawalt, 1989). These findings suggested the biological relevance of cyclobutane dimers and a possible strategy used by mammalian cells in coping with UV-induced DNA damage: instead of removing all the damage at once, cells attain maximum survival by selectively removing damage from essential regions of the genome.

However, the role of cyclobutane dimers as the major cause of mutation and cytotoxicity was challenged when it was determined that UV-induced mutagenesis

depended upon the expression of the *umu*CD genes which are under SOS control in *E. coli* (Kato and Shinoura, 1977). Wood (1985) further showed that the abolishment of the SOS response signal by photoreactivation rather than the direct reversal of the cyclobutane dimers in damaged genes was responsible for the effect of photoreactivation observed in *E. coli*. In mammalian cell systems, T4 endo V stimulates DNA repair in isolated nuclei from UV-irradiated cells (Smith and Hanawalt, 1978); the introduction and expression of T4 phage *den* V gene into UV-sensitive XP cells enhanced UV-induced unscheduled DNA synthesis (Yamaizumi et al., 1989) and increased cellular removal of pyrimidine dimers (Bohr and Hanawalt, 1987) but only moderately reduced the UV sensitivity of the cell (Valerie, 1987). These findings suggested that photoproducts other than cyclobutane dimers may also be responsible for UV induced mutagenic and cytotoxic effects seen in biological systems. In deed, many evidence have suggested that the relatively minor (6-4) photoproduct may have important biological relevance in this regard.

1.3. Photochemistry and the Biological Importance of (6-4) Photoproducts

The pyrimidine(6-4)pyrimidone photoproduct [(6-4) photoproduct)] is formed between the C6 and the C4 of adjacent pyrimidine bases of the same DNA strand (Wang and Varghese, 1967; Varghese and Wang, 1968). The unique photochemical properties that distinguishes (6-4) photoproducts from cyclobutane dimers are their fluorescent properties and the additional UV absorption peak above 300nm

(Hanswirth and Wang, 1973) which led to their first isolation in mid 1960's (Johns, 1964; Varghese and Wang, 1967). The most commonly formed (6-4) photoproduct is at TC sites followed by CC and TT (Patrick and Rahn, 1976, Gordon and Haseltine, 1982). The CT type of (6-4) photoproduct is rarely formed, possibly due to the presence of a methyl group at C5 of the thymine ring, because methylation of the 3' cytosine at this position inhibits the formation of a CC type (6-4) photoproduct (Franklin, et al., 1982).

Photochemically, the spectrum of (6-4) photoproduct formation is almost indistinguishable from that of pyrimidine dimer at wavelength below 310nm; after which photolysis of the (6-4) photoproduct is known to occur (Johns et al., 1964; Patrick, 1970; Chan et al., 1986; and Matsunaga et al., 1991). Unlike cyclobutane dimers which undergo spontaneous reversion directly to monomers, continuous exposure to UV light converts (6-4) photoproducts into the secondary Dewar photoproducts with maximal efficiency at about 313nm. The structure of this photolysis photoproduct has been determined as a valence isomer of the (6-4) photoproduct called Dewar pyrimidinone photoproduct, or the Dewar product (Taylor and Cohrs, 1987). It is believed that all types of (6-4) photoproducts are quantitatively converted to their Dewar valence isomers upon irradiation with sunlight and that Dewar photoproducts are stable under standard physiological conditions (Taylor et al., 1990). Recent research also suggested that Dewar photoproducts are more alkaline labile than (6-4) photoproducts and that once

photoinduced, they can be specifically cleaved under mild alkali conditions (Mitchell et al., 1990a). Figure 2 illustrates the scheme of the photochemical interactions between two adjacent pyrimidine bases leading to the formation of cyclobutane dimers, (6-4) photoproducts, and Dewar photoproducts.

Biochemically, (6-4) photoproducts can be detected in UV-irradiated DNA by chromatographic methods as a minor photoproduct at about 1/10 the yield of dimers (Patrick, 1977). However, the detection of a photoproduct is governed by its stability and the method of detection. Chromatographic methods requires extreme temperature and pH which could reduce the yield of (6-4) photoproducts. Radioimmunoassay using antiserum raised against UV-irradiated DNA can specifically detect and quantify (6-4) photoproducts by monitoring the competition of antibody binding sites between the labeled UV-DNA and the unlabeled sample DNA (Mitchell and Clarkson, 1981, 1984). It is shown that the rate of induction for (6-4) photoproducts in purified DNA irradiated *in vitro* was as high as one-half the quantity of the immunoprecipitated cyclobutane dimers and about one-forth *in vivo* (Mitchell et al., 1989a; 1990a).

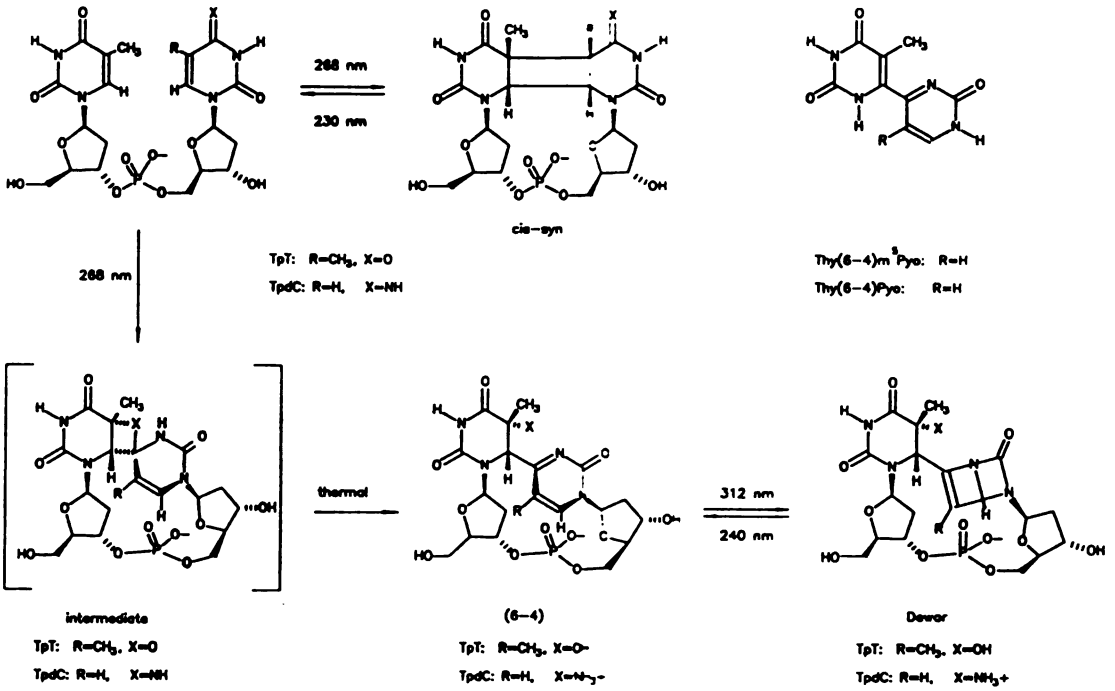


Figure 2. The structure of (6-4) and Dewar photoproducts of TpT and TpdC. Thymidylyl-(3'→ 5')deoxycytidine and its *cis-syn* cyclobutane dimer are drawn in a tautomeric form. The structure of the acid hydrolysis products of the (6-4) products of TpT and TpdC, T(6-4)m^{5}Pyo and T(6-4)Pyo are also given. The wavelength above an arrow at which the forward rate will be at maximum. A wavelength below an arrow refers to a wavelength at which photoreversal is known to occur (from Taylor et al., 1990).

The biological relevance of the relatively minor (6-4) photoproduct has only been appreciated in the past decade after the discovery that (6-4) photoproducts can be detected in a defined sequence (Lippke et al., 1981). While studying the distribution of cyclobutane dimers in DNA, 3'-labeled DNA treated with or without UV light was incubated with hot piperidine to break DNA at the modified sites and separated on a sequencing gel. Unknown lesions sensitive to hot piperidine were identified in UV-irradiated DNA sample and the frequency of these lesions was in the order CC>TC>TT. The biochemical structure of these UV induced alkali-labile lesions were later identified as (6-4) photoproducts (Franklin et al., 1982).

Utilizing the alkaline labile nature of the (6-4) photoproduct, Gordon and Haseltine (1982) determined the site and frequency of photoproduct formation at individual nucleotides in a defined sequence. They first labelled a DNA fragment from the *lacI* gene of *E. coli*, and after heavy UV irradiation treated the sample with UV endonuclease or hot piperidine to introduce single stranded breaks at the site of cyclobutane dimers or (6-4) photoproducts. The site of cleavage on UV-irradiated DNA was analyzed on a sequencing gel. The position and frequency of photoproducts formation were determined by the location and the associated radioactivity of each cleaved band. The sites of (6-4) photoproduct and cyclobutane dimer induction were compared with the nonsense mutation pattern seen in the *lacI* gene. It was shown that, although the overall rate of formation was about 1/10 the rate of dimers in the analyzed fragment, the rate of (6-4) photoproduct formation far

exceeded that of cyclobutane dimers at certain DNA sequences. Furthermore, TT hotspots for dimer formation did not correlate with the mutation hotspots found in the gene while TC sites where (6-4) were potentially formed did correspond to the hotspots for transitional mutations in the gene.

Skopek et al. (1985) further utilized the bacterial phage lambda *cI* gene as a target for UV-induced mutagenesis in *E. coli* cells. They obtained the following results:

1) mutations occurred preferentially at the 3'-base of the pyr-pyr sequence and CT sequences appeared less sensitive to mutation;

2) when phage particles were irradiated with 313nm light under triplet sensitized condition (known to yield mostly TT dimers), few transitional mutations were found; and

3) when photolyase was reacted with UV irradiated phage to remove the dimers before transfecting the host *E. coli* cells, the survival of the phage increased significantly while the number of mutations per surviving phage remained the same.

These findings suggested that although responsible for cytotoxicity in *E. coli*, cyclobutane dimers are not primarily responsible for transitional mutations commonly seen after UV irradiation. In contrast, the (6-4) photoproduct was a more likely candidate because the spectrum of UV induced mutations coincides well with the sites where they are mainly formed and that the structure of (6-4) photoproduct

suggests a destruction of base pairing at 3'-side of the photoproduct.

In mammalian cell systems, radioimmunoassay (RIA) has shown that (6-4) photoproducts are removed from the DNA of UV-irradiated mammalian cells much more efficiently than cyclobutane dimers (Mitchell et al., 1982; 1985) which suggested the possible biological importance of (6-4) photoproducts in UV-irradiated cells. The relevance of (6-4) repair to cell survival was further demonstrated by the characterization of a revertant XP group A cell line, XP129, which displays normal UV sensitivity and removes (6-4) photoproducts at near normal rates but is deficient for cyclobutane dimer repair (Cleaver et al., 1987). Similar properties are also found in another independently generated XPA revertant cell line, XRS70 (see Chapter 4) and in the less UV sensitive CHO UV61 cells (Thompson et al., 1989; Troelstra et al., 1990).

However, the direct relevance of (6-4) photoproduct for cytotoxicity does not seem to coincide with its role of mutagenicity in mammalian cells. This point is presented in the experiment by Brash et al. (1987) using a shuttle vector system to study the mutation spectrum in a UV-irradiated plasmid. After UV irradiation, plasmids were allowed to replicate in normal and UV sensitive (XP) cells. The frequency of photoproduct induction at individual sites was compared with the mutation spectrum of the irradiated plasmid repaired in mammalian cells. In general, the mutation frequency increased with the UV dose and some sites were

indeed more mutagenic than others. However, no correlation was found between the mutation hotspots and the photoproduct hot spots for either cyclobutane dimers or (6-4) photoproducts. It was proposed from these findings that the presence of a photoproduct may contribute to the likelihood of mutation but the site at which mutation occurs is governed by the intrinsic characteristics of the DNA structure. DNA secondary structure and the state of DNA protein binding could be one of the factors determining the formation of mutations. Indeed, it has been shown that the induction of (6-4) photoproduct is more frequent at linker regions than at the core regions of chromatin (Gale and Smerdon, 1989; Mitchell et al., 1990c).

Recently, a ligation-mediated polymerase chain reaction method (LM-PCR; Pfeifer et al., 1989) was used to map the (6-4) photoproduct in unique sequences of the genome. DNA was prepared from heavily UV-irradiated cells and treated with hot piperidine to generate cleavage ends at the site of (6-4) photoproducts. These cleaved fragments were blunt-end ligated with primer sequences which were used for PCR. The amplified region was an X-linked phosphoglycerate kinase (PGK1) gene promotor region and (6-4) photoproducts induction in the gene were determined on a sequencing gel using the amplified product (Pfeifer et al., 1991). Besides confirming the previous notion that (6-4) photoproducts are induced primarily at CC and TC dinucleotide sequences and that their formation depends on the nearest-neighboring bases, the authors showed that (6-4) photoproduct formation was inhibited by protein binding at the promotor region of the gene and was influenced

by the methylation state of the gene as fewer (6-4) photoproducts were formed in the same gene located on the inactivated copy of the X chromosome.

The findings described above suggested that the biological relevance of (6-4) photoproducts may be determined by its site of formation in the genome. In order to better understand this phenomenon, we attempted to develop an enzymatic or biochemical method for detecting UV-induced (6-4) photoproducts in single copy genes of mammalian cells.

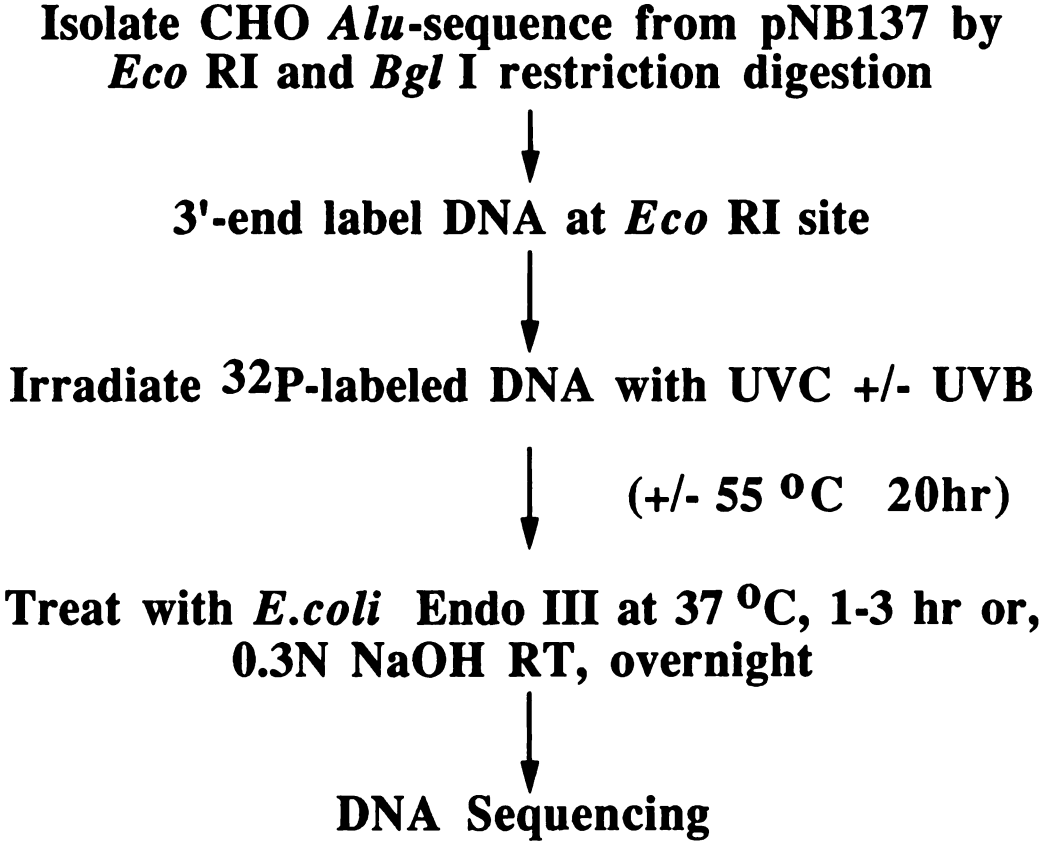
1.4. Outline of the Procedures and Summary of the Research

The main objective of this dissertation was to develop a biochemical assay that allows for sensitive detection of (6-4) photoproducts in single copy genes. Specifically, we searched for methods that can convert (6-4) photoproducts into single stranded DNA breaks which are measurable at their site of formation as has been demonstrated for cyclobutane dimers. We believe that understanding the induction and repair of (6-4) photoproducts in the mammalian genome will help elucidate their role in UV-induced cytotoxicity.

The endonuclease III of *E. coli* (endo III) was initially used to detect (6-4) photoproducts in defined DNA sequences. Endo III is a broad spectrum enzyme capable of recognizing DNA damage induced by UV, X-rays, and oxidative agents.

The main substrates of endo III for UV-irradiated DNA are pyrimidine hydrates and modified cytosines (Weiss and Duker, 1986; Weiss et al., 1987). The specific cleavage by endo III at modified cytosines was particularly relevant to us because of the striking feature from this report indicating that the endo III sensitive C-containing dipyrimidine sites had very similar in sequence specificity with those of the (6-4) photoproducts. We further investigated the biochemical nature of these cytosine-containing photoproducts and found that in the overall genome, endo III sensitive sites were induced at less than 5% of the cyclobutane dimers the (6-4) photoproduct sites and the relative rate of endo III sensitivity did not change with UVB light treatment. The procedures used for studying site specific cleavage by endo III and mild alkali on UV-irradiated DNA are outlined in Figure 3. At the sequence level, endo III cleaved UV-irradiated DNA at the site of cytosines. About 50% of the endo III sensitive sites, primarily those located at individual cytosines, disappeared after heat treatment suggesting that they were cytosine photohydrates. The remaining heat stable endo III sites were exclusively located at cytosine-containing dipyrimidine sites. The insensitivity of these endo III sites to UVB irradiation and the fact that in RIA analysis, they did not share the same antibody binding site with (6-4) photoproducts indicated that they many be a novel type of dipyrimidine photoproduct.

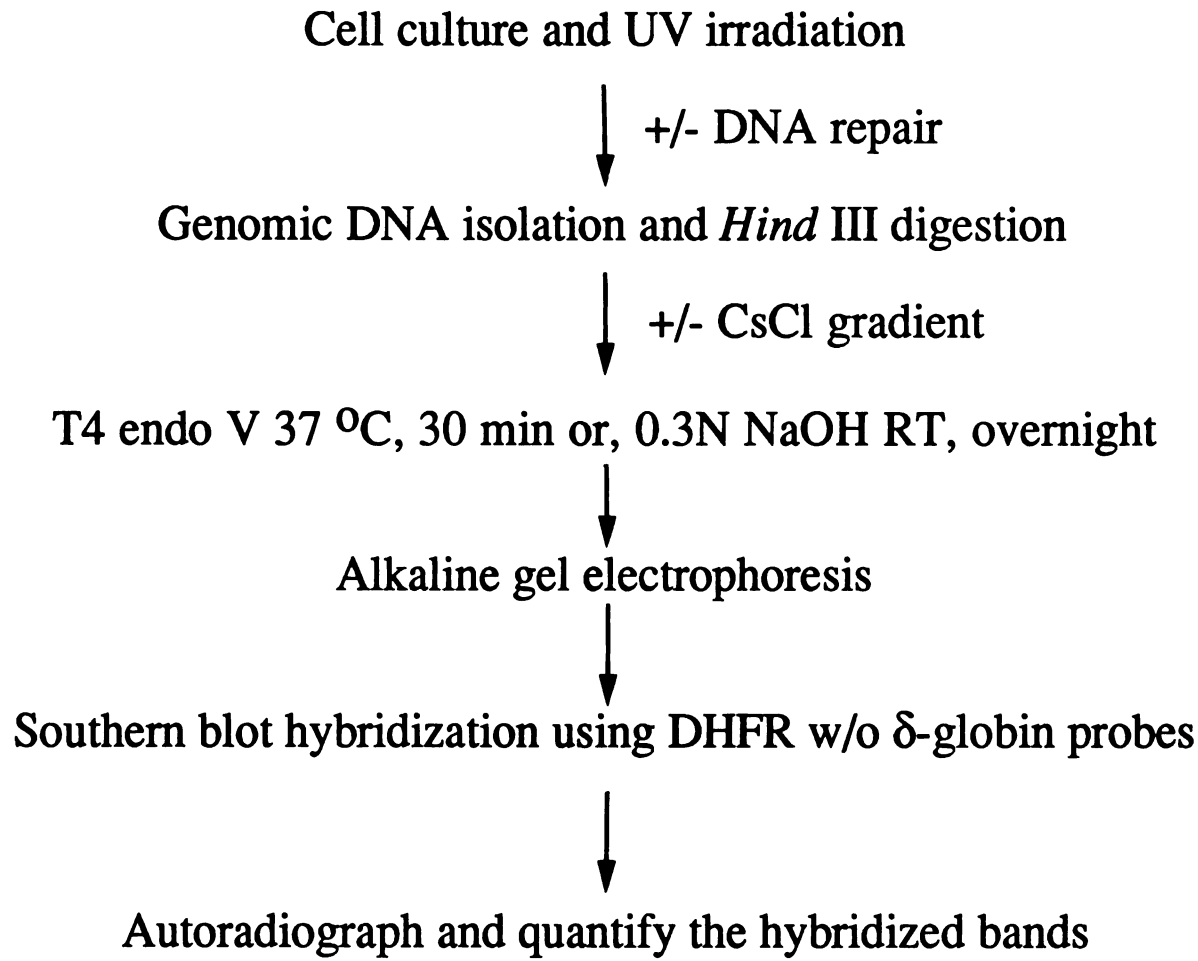
Figure 3. The procedures for studying UV-induced DNA photoproducts in a defined sequence.



Our second approach involved the further characterization of the mild alkaline hydrolysis assay developed by Mitchell and Mitchell et al. (1989a and 1990a). This method utilizes the increased alkaline lability of (6-4) photoproducts when they are photoinduced by longer wavelength UV to become Dewar photoisomers. Using RIA analysis, the authors showed that these photoinduced alkali-labile sites (PALs) coexisted with (6-4) photoproduct binding sites in UV irradiated DNA and that the overall rate of repair of these PAL sites in the genome was similar to the repair of (6-4) photoproducts measured by RIA. We further determined at the sequence level that mild alkaline hydrolysis cleaved UV irradiated DNA at the sites of (6-4) photoproducts and that the cleavage was enhanced when DNA was further irradiated with UVB light. The photoinduced alkaline hydrolysis method also allowed us to detect rare (6-4) photoproducts formed at TT while significantly reducing nonspecific cleavage of the DNA. This assay was therefore used to determine the induction and repair of (6-4) photoproducts in specific genes of mammalian cells genome (Fig. 4).

Human cells were UV irradiated *in vivo* at 0 to 60 J/m^2 and the DNA was immediately extracted and purified. After restriction enzyme digestion, DNA samples were further irradiated with UVB light and treated with increasing concentrations of NaOH. The treated DNA was fractionated by alkaline agarose gel electrophoresis and analyzed by Southern blot hybridization using genomic DNA probes specific for the human DHFR gene and the delta-globin gene. We found that the number of (6-4) photoproducts in the transcribed DHFR gene region increased

Figure 4. The procedures for studying the induction and repair of UV-induced DNA photoproducts in single copy genes of the human genome.



with increasing UV dose, while little (6-4) photoproducts were induced in the non-transcribed delta-globin gene region. In contrast, the induction of cyclobutane dimers as measured by T4 endo V sensitive sites was uniform throughout the genome. Furthermore, XP revertant cells (XP129) removed more than 70% of the (6-4) photoproducts from the DHFR gene 6 hours after UV irradiation but only about 30% of the cyclobutane dimers in the same region in 20 hours.

Our findings suggested that, unlike cyclobutane dimers, the induction of (6-4) photoproducts is affected by the transcriptional state of the gene. This preferential induction of (6-4) photoproduct in the genome suggested that the cytotoxicity and the rapid repair kinetics of (6-4) photoproduct observed in human cell systems may in part be the result of their relatively higher induction at the essential and/or more accessible regions of the genome.

In conclusion, the work presented here demonstrated the presence of a novel *E. coli* endonuclease III sensitive photoproduct that occurs exclusively at cytosine containing dipyrimidine sites; and the preferential induction of (6-4) photoproducts in UV-irradiated human cells. The alkaline assay developed here allowed for the sensitive detection of (6-4) photoproducts in single copy genes of mammalian genome at biologically relevant low UV fluences. It helped us in extending our knowledge on the induction and repair of UV-induced (6-4) photoproducts in human cells.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Plasmid Manipulation

Plasmids

Plasmid pNB137 is a pUC 8 vector containing a Chinese Hamster Ovary (CHO) human *Alu* equivalent sequence (Haynes et al. 1981) at its *Sal* I site and was kindly given by Dr. Janet Arrand (Cambridge, UK). Plasmid pBH31R1.8 (p1.8) was a gift from Dr. Hanawalt's Lab (Stanford University, CA). It is constructed from a pBR322 vector and an 1.8kb *Eco* RI genomic fragment that spans the first and part of the second exon of the human DHFR gene (Mellon et al. 1986). Plasmid pHd 1 was obtained from Dr. Y. W. Kan's laboratory (HHMI, UCSF). It is also of pBR322 origin with an inserted 2.3 kb *Pst* I fragment that covers the entire human delta-globin gene and its surrounding sequences (Collins and Weissman, 1984). Due to the disruption of the *Pst* I site, pHd 1 is only resistant to tetracycline. On Southern blot, p1.8 hybridizes with a 23 kb fragment in *Hind* III digested human genomic DNA, while pHd 1 hybridizes to a 15kb genomic fragment. In addition, both probes apparently hybridize to a common size fragment of about 20kb corresponding to a pseudo-DHFR gene fragment (Yang, et al., 1984).

Plasmid Propagation

Purified plasmids were used to transform competent *E. coli* strain DH5-alpha (BRL, MD) according to the manufacturer's instructions. Positive transformants were individually selected, analyzed, and maintained on agar plates with appropriate antibiotics.

Plasmid amplification was carried out according to Maniatis et al. (1985) with minor modifications. Briefly, independent bacterial colonies were transferred into sterile tubes containing 10ml Luria-Bertani medium (LB broth) supplemented with appropriate antibiotics and allowed to grow overnight with shaking at 37 °C. These overnight cultures were expanded to 1 L the next day until the OD_{260} reached ~4.0. Amplification of plasmids was achieved by adding 36mg/ml chloramphenicol (in 70% ethanol) to a final concentration of 170*u*g/ml and further incubated at 37 °C overnight. At the end of the incubation, bacterial cells were harvested by centrifugation at 5,000 x g for 10 min at 4 °C and cell pellets were suspended in solution P1 which contains 50mM Tris/HCl, 10mM EDTA, 400*u*g RNaseA/ml (Qiagene, CA) and 1mg/ml lysozyme. The plasmids were released by alkaline lysis with 200mM NaOH plus 1% SDS and separated from the bacterial elements by additional centrifugation at 20,000xg for 30min at 4 °C. The supernatant containing plasmids was loaded onto a pre-equilibrated Q-500 ion exchange column and eluted with QF buffer (1.2 M NaCl, 50mM MOPS, and 10% ethanol, pH 8.0). The eluted plasmid was precipitated by 0.8 volume of isopropanol; the DNA pellet dried under

vacuum in Speedvac, and dissolved in a buffer containing 10mM Tris pH 7.5 and 1mM EDTA (TE). The quality and the concentration of the purified plasmids were routinely examined using 1% mini agarose gel electrophoresis. The concentration of the plasmids was typically about 0.1 to 0.5 mg/ml.

Plasmid Restriction Digestion

The purified plasmid pNB137 was digested with the restriction enzymes *Eco* RI and *Bgl* I to generate asymmetric ends allowing subsequent specific labelling at the 3'-end. The resulting CHO *Alu* equivalent fragment (*Alu* fragment) is 450 base pairs (bp) in length containing the 256bp plasmid insert and part of the pUC 8 sequence. We have determined the complete nucleotide composition of the insert (see below) and used it as a template for determining UV-induced site specific cleavage by *E. coli* endonuclease III (endo III) and mild NaOH.

Typical restriction enzyme digestion conditions were as follows with buffers and enzymes purchased from BRL (Bethesda, MD):

DNA	~30 *u*g
10x reaction Buffer 4	20 *u*l
Eco RI (10 units/*u*l)	10 *u*l
Bgl I (10 units/*u*l)	10 *u*l
H_2O	to 200 *u*l

The restriction digestion was carried out at 37 °C for 2 to 4 hr and the reaction was terminated by heat inactivation at 65 °C for 5min. After enzymes

digestion, the plasmids were either separated on a 5% polyacrylamide gel to separate the plasmid vector sequence from its insert.

Plasmids p1.8 and pHd 1 were digested with either *Eco* RI or *Pst* I to obtain the inserted genomic fragments using the method described above. The use of purified genomic sequences for Southern blot hybridization was necessary to avoid cross hybridization between the plasmid portion of the two probes.

Plasmid DNA Isolation and Purification

The digested plasmid DNA was separated by 5% polyacrylamide gel or 1% low melting agarose gel (Seaplaque, BRL). DNA was visualized by ethidium bromide staining and bands containing the fragment of interest were excised and placed in a 0.5 cm dialysis tubing in 0.2 x TBE buffer (1x = 50mM Tris base, 50mM Boric Acid and 1mM EDTA). The tubing was sealed and placed horizontally in a minigel apparatus with sufficient 0.2 x TBE buffer to cover the tube. The DNA was separated from the gel by electroelution at 200 volts for 20 min (Davis et al. 1986). The elute was collected in an Eppendorff tube and loaded subsequently onto a pre-equilibrated Q-20 column. After 4 washes each with 1 ml QB buffer (750mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0), DNA was eluted by 0.8ml QF buffer and concentrated by isopropanol precipitation. Using this method, the DNA recovery from the gel was about 80% and sample was suitable for labeling as well as sequencing.

The pNB137 Plasmid Structure and its Insert Sequence

The structure of pNB137 plasmid is shown in Figure 5A. The complete sequence of the pNB137 insert was determined using the Maxam and Gilbert sequencing technique (1980) and is shown in Figure 5B. It was evident from our sequence data that the insert contained one CHO *Alu* sequence at the 5'-end and an anonymous sequence of about 110bp at the 3'-end. A GeneBank search did not provide conclusive information about the origin of this sequence but suggested that it may be of *E. coli* origin. The plasmid originally contained two inverted repeats of the CHO *Alu* sequence when it was constructed (personal communication with Dr. Arrand). However, the plasmid was subsequently propagated in a $RecA^+$ bacterial host before we received it. Thus, it is possible that the plasmid rearranged during its propagation in the $RecA^+$ host and that one of its *Alu* sequences was replaced by a random fragment from *E. coli* genomic DNA.

Figure 5. The structure of plasmid pNB137 (A) and the nucleotide composition of its insert (B).

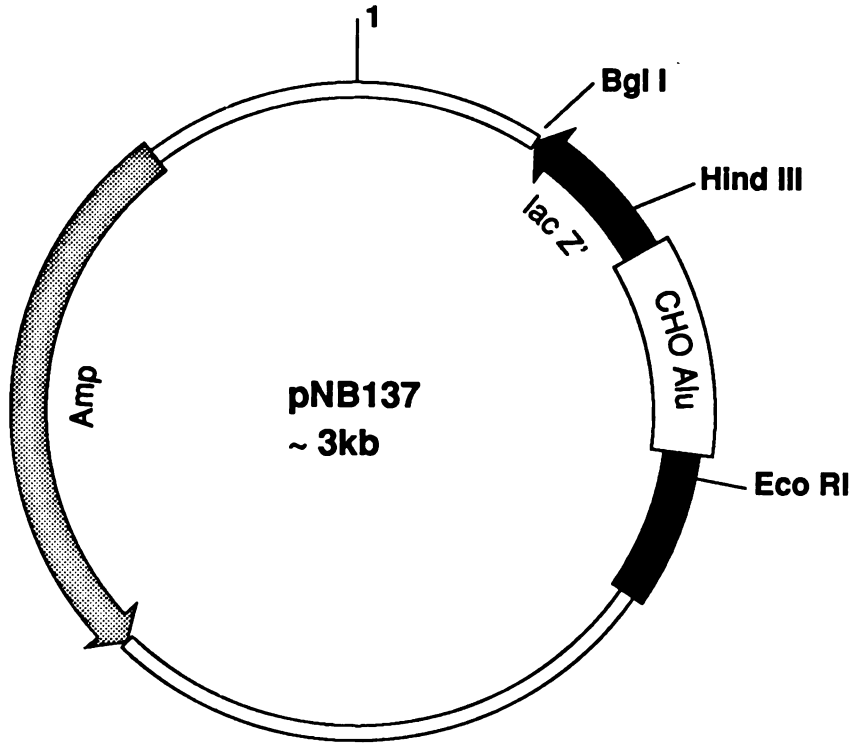


Figure 5B. The nucleotide composition of the pNB137 insert. Underlined portion is a novel sequence.

1 10 20 30 40 50
5'-CCACAGACCC TCCTGTGCAA CAAGATGTAC CCTCTTTTTT CTCTGCGGCT TTGGAGGCTG

60 70 80 90 100 110
CCCTGGAACT AGCTCTTGTA GACCAGGCTG GTCTCGAACT TCACAGAAAT CCTCTGCCTT

120 130 140 150 160 170
TGCCTCCTGA GTGCTGGAAT TAAAGGCATG CGCGACGGGC AATCTTGGTG GCTTCGTCGC

180 190 200 210 220 230
CGATACGCTC AGGTCGATCA CCGACTTGGA GATGCTGATG ATCAAACGCA GGTCGGACGC

240 250
CGCCGGCTGA CGACGGG-3'

2.2. Probe Labeling

End Specific Labeling of the Alu-fragment

The *Eco* RI and *Bgl* I cleavage of plasmid pNB137 resulted in a fragment with one 3' and one 5'-recessed end which allows for ^{32}P end specific labeling at the 3'-end by the large fragment of the *E. coli* polymerase I (Klenow fragment). The labeling reaction was carried out in 50 *u*l volume using the Random Prime Kit from Amersham (USA) with manufacturer's protocol except that the labeling reaction was carried out at 35 °C for 2hr and terminated by dilution with 20*u*l STE buffer (150mM NaCl, 10mM Tris pH 7.5, 10mM EDTA). The unincorporated nucleotides were separated from the labeled fragment by a Push Column (Stratagene, San Diego, CA) equilibrated with 70*u*l STE buffer and eluted with 70*u*l STE buffer. The specific activity of the labeled fragment was typically greater than 10^7 cpm/*u*g DNA.

Random Prime Labeling of Genomic DNA Probes

The purified p1.8 and pHd 1 probes were boiled at 100 °C for 10 min then quickly chilled on ice for 2 min to render them single stranded for efficient labeling with the Random Primer Kit from BRL. The reaction proceeded in a 50 *u*l volume at 35 °C for 4hr and was terminated by the addition of 20*u*l STE buffer. The probes were purified as for *Alu* fragments. The specific activity of the labeled probes was greater than 10^8cpm/*u*g DNA.

2.3. DNA Sequencing

Chemical Cleavage of DNA

DNA was cleaved by the sequencing method developed by Maxam and Gilbert (1980). This method utilizes specific chemical modification of DNA at an average of one nucleotide per fragment followed by a hot piperidine treatment which cleaves DNA at the site of a modified base. Because DNA templates were labelled at one end, gel electrophoresis of these treated DNA samples resulted in a series of ladders corresponding to the site of DNA cleavage. The reaction procedure and chemicals used were from the protocol described in (Frederick et al., 1987).

Polyacrylamide Gel Electrophoresis

The DNA fragments were separated on a 20 x 40 cm sequencing apparatus from BRL. A typical gel was made from 50ml of a solution containing 8% acrylamide (diluted from 20% acrylamide:bisacrylamide stock), 8M urea, 0.1% ammonium persulfate, and 1x TBE. Glass plates used for sequencing were washed with detergent, rinsed with sterile water, wiped with 100% ethanol, and air dried in a chemical hood, after which the inside of the front plate was siliconized with Sigmaco (Sigma, MO) and polished with 100% ethanol. For casting, the two glass plates were clamped together with the siliconized side facing inside and separated by two 0.4mm spacers placed along each side. The bottom of the gel was sealed by water resistant plastic tape (3MM). Fifty microliters of TEMED was added to the

gel mix before it was poured into the space between the two plates. The wells of the gel were made by inserting a 0.4mm comb immediately after pouring the gel and the polymerization of the gel usually took about 30 min. The comb was removed after the gel was set; the plate was assembled onto the sequencing apparatus and allowed to run at 1500V for 30 min in TBE before loading the sample. The temperature of the gel was monitored by a surface thermometer and kept at 55 °C by adjusting the voltage of the electrophoresis.

Before loading, DNA samples having equal amount of radioactivities were put into microfuge tubes and dried in a Speedvac. Two microliters of loading buffer (95% deionized formamide, 5% 1:1 bromophenol blue and xylene cyanol) was added to each sample and mixed thoroughly. These 2ul samples were placed in a 100 °C heating block for 2 min and immediately chilled on ice. After a brief spin to collect the evaporates, samples were loaded onto the gel and run immediately at 1,500 to 2,000 V for ~4 h until the front of the dye was about 1 inch from the bottom of the gel. Stopping the gel at this time retained the radioactivity to the gel only thus greatly reducing the possibility of radioisotope contamination.

Autoradiography

After electrophoresis, the gel plates were removed from the apparatus and carefully separated. One piece of Whatman paper (3MM) was placed smoothly on top of the gel and lifted carefully so that the gel remain attached. The gel was

covered by a plastic wrap, placed in a 35 x 40cm Kodak X-ray film cassette against a sheet of X-ray film and exposed at -70 °C. The time of exposure ranged from 4 to 24 h depending on the amount of radioactivity associated loaded on each lane. Typically, about 100,000 cpm/lane (by Cherenkov counting) was used which gave a good resolution in about 4 to 12 hours. Longer exposure would be needed when the specific activity of the sample was but this could compromise the resolution of the bands. The intensity of the cleavage bands were analyzes using a LKB Ultrascan laser densitometer with Gelscan software.

2.4. Cell Culture and UV Irradiation

Cell Culture

GM637 is an SV40 transformed normal skin fibroblast cell line, purchased from American Type Culture Collection. XP12RO is an SV40-transformed xeroderma pigmentosum (XP) group A line routinely maintained in the laboratory. XP129 is a UV resistant XP revertant cell line derived from UV resistant colonies obtained after treating XP12RO cells to ethyl methanesulfonate (EMS) and repeated exposure to low dose UV irradiation. XRS70 was a gift from Dr. Tanaka (Tokyo, Japan) and is made UV resistant by fusion of mouse DNA with XP12RO cells. All cell lines were maintained at 37 °C in a humidified environment in minimum essential medium (MEM) in the presence of antibiotics streptomycin (50 units/ml) and penicillin (50 μg/ml) plus 10% fetal calf serum. Cells were usually grown in T75

flasks and transferred when confluent. Mycoplasma monitoring was performed every 3 months by Hoestch's staining and fluorescent microscopic examination.

UV Sources

The UVC light source used in these studies was a bank of six 15 w germicidal lamps (General Electric) that emit predominantly 254nm light. The fluency rate of the light source varies depending on the distance. The dose rate was 1.3 $J/m^2/s$ for low dose irradiation and 20 $J/m^2/s$ for high dose. The long wavelength UV was generated from two Westinghouse FS20 fluorescent sunlamps filtered through a Mylar 500D filter (Du Pont). Under these conditions, the light transmitted was above 320nm and the fluency rate was 0.3 $J/m^2/s$. Although this wavelength is at the border of UVB and UVA, for convenience, we refer to this longer wavelength radiation as UVB irradiation. The emission spectra for the UVC and UVB light sources with mylar filters are shown in Figure 6. The fluency rates were measured using spectrophotometers equipped with appropriate photodetectors (Spectonics Corp., Westbury, NY).

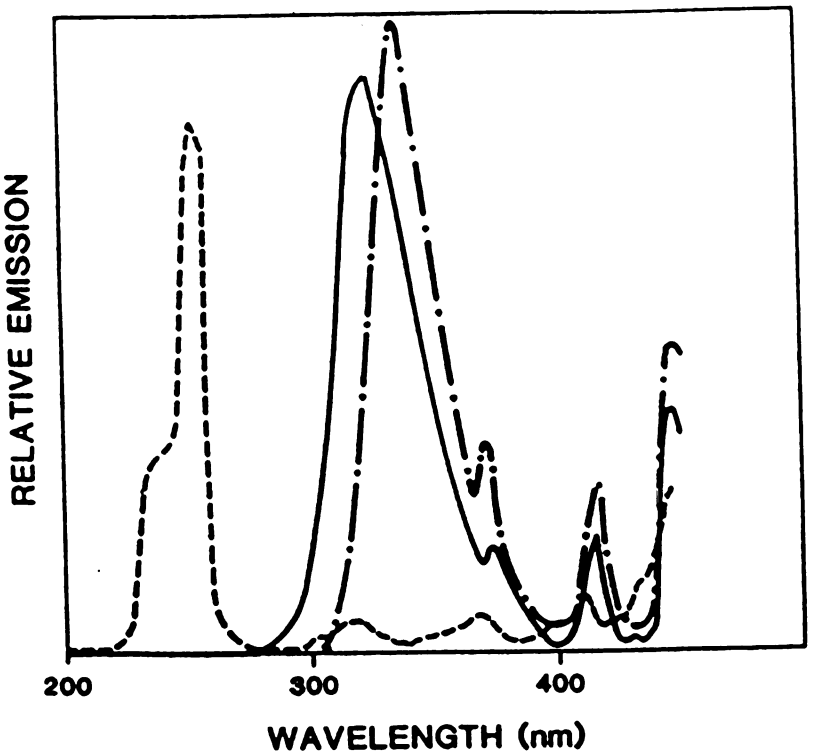


Figure 6. The emission spectra for broad-spectrum light sources. Wavelength spectra for the germicidal lamps (---) and Westinghouse FS20 sunlamps filtered through a Corning Petri dish lid (sunlamp UV>295nm, —) or Mylar 500D (sunlamp UV>320nm, ·····). The relative spectra were detected by a spectrofluorimeter. (From Mitchell and Rosenstein, 1987)

Cell Labeling and UV Irradiation

For experiments that require prelabeling, 10^7 cells were transferred from one T75 flask into a 2L roller bottle and incubated with 0.005uCi/ml ^{14}C-thy and 200ml of fresh medium for 2-4 days. When confluent, the cells were transferred onto 150mm petri dishes at about 10^8/dish and incubated in fresh media for overnight. About 2 hours prior to UV irradiation, cells were incubated in 10^{-5}M bromodeoxyuridine and 10^{-6}M flurodeoxyuridine to allow substitution of BrdU for thymine in newly synthesized DNA (Smith et al., 1981). At the end of the incubation cells were rinsed with 20ml PBS buffer and irradiated with UVC light at predetermined fluences. After UV irradiation, cells were supplemented with fresh medium containing 2mM hydroxyurea, 10^{-5}M bromodeoxyuridine, 10^{-6}M flurodeoxyuridine, 0.01uCi/ml ^{3}H-thymidine and allowed to repair for 0, 6, or 24 hours.

For experiments that measures (6-4) photoproducts induction in DNA, prelabeling was not necessary due to the relatively short duration of the experiment. After UV irradiation, cells were either immediately harvested or allowed to repair in culture medium containing 2mM hydroxyurea for 0, 0.5, 1.5, or 6 hours before being lysed. In experiments that determine the induction of UV photoproducts in a defined DNA sequence, *Alu* DNA fragments were dissolved in TE buffer and irradiated with UVC light or with UVB light in 5% glycerol to prevent nonspecific DNA cleavage by long term light exposure.

2.5. Genomic DNA Manipulation

DNA Purification

Cells were washed with PBS and lysed immediately with 5ml proteinase K buffer/petri dish (150mM NaCl, 10mM Tris, 10mM EDTA, 0.4% SDS) at room temperature (RT) for 10-15 minutes at the end of the repair period. The lysate was collected into a 50ml polypropylene tube; 10mg/ml proteinase K (Sigma, MO) was added to a final concentration of 1mg/ml for overnight digestion at 37°C. The DNA was extracted sequentially with phenol:chloroform:iso-amylalcohol (25:24:1) and chloroform:iso-amylalcohol (24:1). The aqueous phase was transferred after each extraction into a new 50ml polypropylene tube and the DNA was precipitated with 2 volumes of ethanol and 1/10 volume 3M sodium acetate (NaAc) at RT. After precipitation, the DNA was dissolved in 4ml TE for overnight at 37 °C and than incubated with 50*u*g/ml DNase-free RNase for 2h at 37 °C. The RNase treated DNA was extracted and precipitated again as described above. The purified DNA was dissolved in 1ml TE buffer with the final concentration estimated spectrophotometrically at OD_{260} based on the assumption that OD_{260} 1 = 50 *u*g/ml DNA. The recovery of DNA was usually about 300 *u*g for every 10^8 cells.

Genomic Digestion and Gel Electrophoresis

To determine the repair of UV-irradiated DNA in a specific gene fragment, genomic DNA was digested with *Hind* III restriction enzyme. This digestion allows

the detection of a 23kb DHFR fragment by hybridization with the p1.8 probe and a 15kb delta-globin fragment with the 2.3kb pHd 1 probe. The typical assay for *Hind* III digestion was:

Purified genomic DNA	up to 500 *u*g
10 x React 2 buffer	100 *u*l
Hind III(10 units/*u*l)	100 *u*l
H_2O	to 1000 *u*l

The React 2 buffer was obtained from BRL and the reaction was carried out at 37 °C for overnight. The completeness of the restriction digestion was monitored by mini gel electrophoresis of 10*u*l aliquots. When DNA were used for measuring the induction of T4 endo V sensitive sites, the digested sample was loaded onto CsCl isopycnic gradient centrifugation. For the determination of mild alkali-sensitive sites, DNA samples were precipitated after restriction digestion and then treated with NaOH.

CsCl Isopycnic Gradient Centrifugation and Purification of Parental DNA

The CsCl gradient centrifugation was used to separate the parental unreplicated DNA from the replicated DNA during the postirradiation incubation period (Smith et al., 1981). The digested genomic DNA was diluted with 10mM Tris and EDTA until the weight reached 4.5 gm and then mixed with 5.6gm CsCl. After the CsCl was dissolved, the mixture were transferred into 10ml Beckman quick seal

tubes and topped with heavy mineral oil. The sealed tubes were balanced and placed in a Beckman Ti50 rotor for centrifugation at 37,000rpm for 36 to 48 hours. At the end of the centrifugation, each gradient was collected from the bottom of the tube as 0.5ml fractions and the position of the replicated heavy-light and nonreplicated light-light prelabelled DNA was identified by counting 20*u*l aliquots of each fraction. Fractions corresponding to the parental unreplicated DNA were pooled and dialyzed against 4 liters of TE buffer at 4°C overnight. The DNA was precipitated with ethanol and dissolved in TE to a final concentration of about 1*u*g DNA/*u*l.

Alkaline Sucrose Gradient Sedimentation and Radioimmunoassay

These assays were performed by Dr. David Mitchell using a previously described protocol (1989a and 1990a).

2.6. Biochemical Assays

T4 Endonuclease V Digestion Assay

T4 Endonuclease V (endo V) was a gift from Dr. Steven Lloyd (University of Texas, Houston) which was purified from *E. coli* K12 cells that harbors a plasmid containing the T4 den V structural gene (Asahara et al., 1989). The enzyme's specific activity was 2 x 10^8 dimers/*u*g when incubated in a buffer containing 10mM Tris, 8.0, 10mM EDTA, 150mM NaCl, and 5mg/ml BSA at 37 °C for 30 min (data not shown). One microliter of T4 endo V was used to react with every 10*u*g of DNA

in a 50ul volume. The reactions were terminated by adding 10x alkaline loading buffer (1x = 32mM NaOH, 1mM EDTA, 1% bromophenol blue); the DNA samples were loaded in pairs with the control unreacted DNA and separated on 0.6% alkaline agarose gel that ran at 20-25V for 16 hr. Southern blot analysis was used to determine the number of T4 endo sensitive sites in specific genes.

E.coli Endonuclease III Assay

The *Alu* fragment DNA and plasmid pUC19 (BRL) was used to analyze the induction of endo III sensitive sites *in vitro*. This enzyme was supplied by Dr. Richard Cunningham (SUNY, Albany) in a concentrated form and stored at 4 °C. The Endo III buffer contains 40mM KH_2PO_4 and 1mM EDTA. For sequencing analysis, the reactions were carried out in 20ul volume with various concentrations of the enzyme for 1 to 3 h at 37 °C and terminated by dilution with 0.3M NaAc to 200 ul followed by phenol/chloroform extraction and ethanol precipitation as described earlier. In order to measure the induction of endo III sensitive sites in plasmid, excess endo III was used to react with UV-irradiated supper coiled pUC19 for 1 hr at 37 °C prior to gel electrophoresis.

Alkaline Hydrolysis Assay

The induction of (6-4) photoproducts in a defined sequence was analyzed *in vitro* using the end labelled *Alu* sequence. After UVC and UVB treatment, the *Alu* fragments were precipitated and treated with 0.3M NaOH at RT overnight, then

extracted and analyzed on a sequencing gel. Maxam and Gilbert sequencing reactions specific for A+G and C+T reactions were routinely used as sequence markers to determine the site of cleavage after the complete insert sequence was known.

In order to detect (6-4) photoproduct in specific genes, restriction enzyme digested, UVC and UVB irradiated genomic DNA was precipitated with isopropanol and dissolved in TE buffer at 45*u*l/10*u*g DNA. For alkaline hydrolysis, 5*u*l H_2O or a solution containing 50mM Tris and 0.15mM, 0.3mM, or 0.45mM NaOH was added to each pair of DNA samples irradiated with 0, 20, 40, or 60J/m^2 UVC light. The incubation was allowed to proceed in the dark for 24 hours at 24 °C and the reaction was terminated by the addition of 5*u*l alkaline loading buffer followed by DNA electrophoresis on an alkaline gel. Restriction enzyme *Hind* III-cleaved lambda DNA fragments were used as DNA size markers.

Hot Piperidine Assay

Hot piperidine was used as a control for the induction of (6-4) photoproducts in UV-irradiated *Alu* fragments. Aliquots of ^{32}P end-labeled *Alu* fragments were treated with 50*u*l of 1M piperidine at 90 °C for 20 min and immediately transferred to dry ice/ethanol bath to stop the reaction. The condensate was collected by a brief spin 12,000rpm and the piperidine residues were evaporated under vacuum using a speedvac by 2 times rehydration and lyophilization. This purified DNA was mixed

with 2*u*l sequencing loading buffer and loaded on an 8% denaturing sequencing gel as described in 2.3.

2.7. Southern Blot Analysis

Agarose Gel Electrophoresis

The 0.6% agarose gel was prepared as described in Maniatis et al.(1985). Treated DNA samples were mixed with 1/10 the volume alkaline loading dye and loaded on the gel with lambda/Hind III DNA as molecular size marker. The gel was run at 25 V for 16 h in alkaline electrophoresis buffer (32mM NaOH and 1mM EDTA).

DNA Transfer

At the end of electrophoresis, the gel was placed in a tray; neutralized with 1.5M NaCl and 1M Tris, pH 8.0 for 30 min; and stained by ethidium bromide. After photography, the gel was soaked in 0.25N HCl for 15 min, and then in 1 M NaOH, 1.5 M NaCl for 1 hr before being transferred. For proper transfer, the gel was inverted and placed on top of a sheet of blotting paper (Schleicher and Schuell, NH) with two ends submerged in a reservoir containing 1 M NaOH and 1.5 M NaCl. A sheet of Oncor Sure-blot nylon membrane (Oncor, MD) was placed in complete contact with the gel and which was then covered by two more sheets of blotting paper. A stack of highly absorbent blotting paper were placed above the blotting

paper to ensure proper transfer of DNA to the nylon filter. After at least 4 hours, the filter was retrieved, rinsed with 6x SSC (150mM NaCl, 15mM Na_3 Citrate, pH 7.0), dried briefly, and baked under vacuum for 2 h at 80 °C.

Southern Blot Hybridization

The hybridization of the filters was carried out in a rotating hybridization chamber in siliconized beta-save tubes (Robinson Co., CA). The vacuum dried filter was placed inside the tubes and prehybridized with 5-10ml Hybrisol I (Oncor, MD) depending on the size of the filter at 45 °C for at least 1 hr. The ^{32}P labeled probe was added at a final concentration of 0.5-1 x 10^7 cpm/ml and hybridization was carried out for 20 h at 45 °C. At the end of the incubation, the hybridization solution was drained out and the filter was washed with 0.1 X SSC, 0.1% SDS twice at 45 °C for 1 h while inside the hybridization tube. The filter was then removed from the tube and washed in a plastic container with 500ml 0.1 x SSC and 0.1% SDS at 65 °C for at least 30 min with moderate agitation.

Autoradiography

At the end of the washing, the filter was patted dry with clean paper towels, covered with a plastic wrap, and placed in contact with X-ray film for 1-3 days. The exposed film was developed by an automatic X-ray film processor from Kodak and analyzed by the Facility for Gel Analysis (UCSF) using a two dimensional laser densitometer from Molecular Dynamics (CA).

CHAPTER THREE: THE ENZYMATIC AND BIOCHEMICAL DETECTION OF (6-4) PHOTOPRODUCTS IN DNA

3.1. Background

E. coli endonuclease III (endo III) was first identified as an activity that incises X-irradiated DNA (Strinste and Wallace, 1975) and subsequently purified using heavily UV-irradiated DNA as substrate (Radman, 1976). The gene encoding endo III has been cloned (Asahara et al., 1989) and its mechanism of action well studied (Doetsch et al., 1986; Kow and Wallace, 1987; Bailly and Verly, 1987, Kim and Linn, 1988). The substrates of endo III on DNA exposed to X-rays or oxidizing agents have been shown as thymine glycols and cytosine hydrates (Gates and Linn, 1977; Demple and Linn, 1982; Breimer and Lindahl, 1984; and Dizdaroglu et al., 1986). In UV-irradiated DNA, Weiss and Duker (1986, 1987) showed that endo III incision occurred at modified single cytosines although the enzyme also cleaved at dipyrimidine sites containing cytosine. The induction of (6-4) photoproducts occurs primarily at TCs and CCs (Brash et al., 1982) which is very similar to the dipyrimidine sequences recognized by endo III. Although endo III does not directly recognize the (6-4) photoproduct, we thought it may recognize its photoisomer, the Dewar photoproduct. If this is the case, endo III could serve as a probe for the enzymatic detection of (6-4) photoproducts.

Dewar photoproducts can be induced in UV-irradiated DNA upon secondary UV (>320nm) irradiation by photoisomerization of the (6-4) photoproducts (Mitchell and Rosenstein, 1987; Taylor et al., 1988). Since no specific enzyme has been identified for these photoproducts, we examined the ability of endo III to recognize and cleave (6-4) and/or Dewar photoproduct. Our approach was to develop a chemical or enzymatic method for sensitive detection of (6-4) photoproducts by using DNA irradiated *in vivo* inside the cells and *in vitro* using plasmid pUC19 and purified *Alu* DNA fragments. Our results showed that endo III cleaves UV-irradiated DNA at cytosine hydrates as well as heat stable dipyrimidine sites containing cytosines but these later photoproducts appeared to be structurally distinct from any of the known photoproducts.

Biochemically, (6-4) photoproducts can be detected in a defined DNA sequence by hot alkali treatment (Lippke, 1981). However, the hot piperidine used in these studies breaks DNA into very small fragments resulting in a much reduced sensitivity. On the other hand, Dewar photoproducts are more alkali labile than (6-4) photoproducts. Mitchell et al., (1990a) used photoisomerization and low NaOH concentration to demonstrate that photoinduced alkali-labile sites (PALs) were very similar to those recognized by hot piperidine in a UV-irradiated DNA sequence and that the removal of (6-4) photoproducts in the genome was in close agreement with the radioimmunoassay (RIA) measurements. Based on these findings, we further characterized and confirmed at single base level that the mild alkali treatment

cleaves UV-irradiated DNA at the site of (6-4) photoproducts with much reduced background compared to hot piperidine. This method allowed us to detect (6-4) photoproducts in single copy genes of the mammalian genome.

3.2. Results

Cellular DNA was isolated from UV irradiated cells and incubated with either endo III, T4 endo V, or 0.3M NaOH. The extent of DNA cleavage by these treatments was measured with alkaline sucrose gradient sedimentation. The number of breaks induced by these treatments were calculated as changes in average molecular weight of the total DNA (Table I). UV-induced endo III sensitive sites were induced at the rate of $0.06/10^8 Da/J/m^2$ which was 4.2% of the cyclobutane dimers as measured by T4 endo V sensitive sites. Mild alkaline sensitive sites that correspond to the number of (6-4) photoproducts in the genome were induced at 7.7% of the dimers in this experiment. Additional UVB irradiation prior to endo III treatment did not increase the endo III sensitive sites in the DNA suggesting either that endo III could not recognize either (6-4) or Dewar photoproducts, or it had the same substrate specificity for both lesions under the conditions used (data not shown).

The induction of endo III sensitive sites in purified DNA was measure using supercoiled pUC19 DNA. Plasmids were first irradiated with UVC light and then

Table I. The induction of UV-induced endo III sensitive sites in genomic and plasmid DNA.

	Genomic DNA	pUC19 DNA
Endo III sites	0.06 (4.2%)	0.03 (1.2%)
Mild alkaline sites	0.11 (7.7%)	0.56* (20%)
T4 endo V sites	1.42	2.45*

Unit = 10^8 daltons/J/m^2;

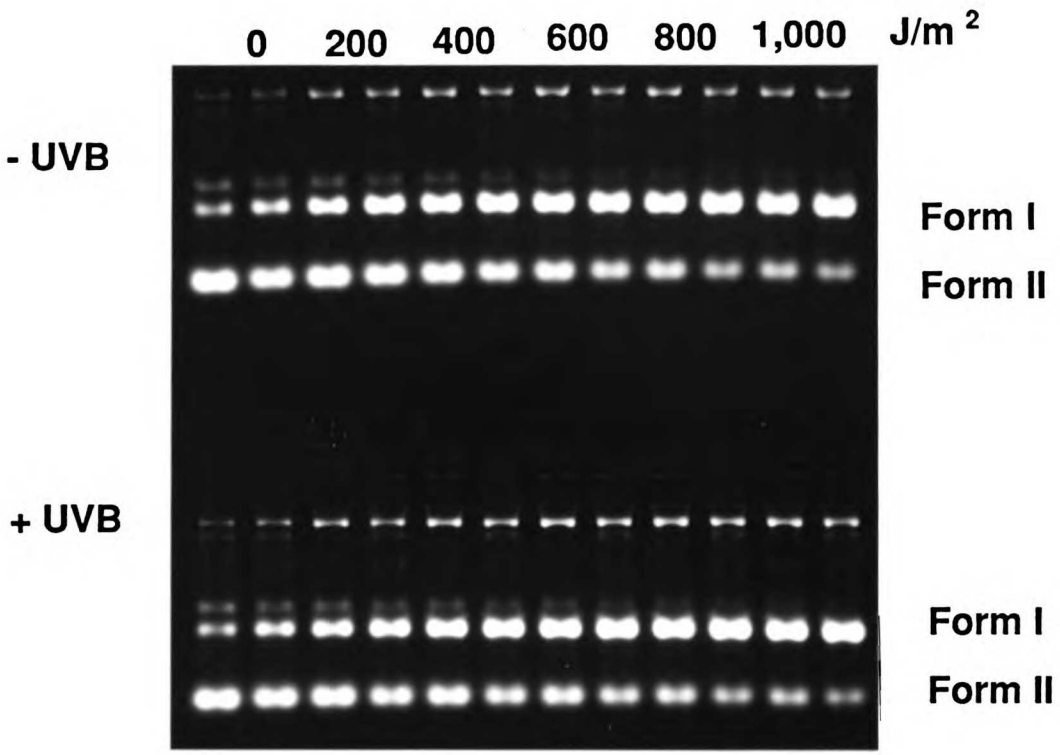
* = data from Mitchell et al.(1990a).

( ) = percent of T4 endo V sensitive sites.

irradiated with or without UVB before being treated with endo III; the enzymatic incision of the plasmid was analyzed by agarose gel electrophoresis (Fig. 7). The number of endo III induction sites was shown as the percentage of conversion from supercoiled form I DNA to the relaxed circular form II DNA with increasing UV dose, determined by a scanning laser densitometer. In this analysis, endo III sites were induced at the rate of 0.03 sites/10^8 Da/J/m^2 for UVC light which was compatible with the genomic measurement (Table I). The D_{37} values with or without secondary UVB treatment were 1,898 J/m^2 and 1,497 J/m^2 respectively and were not significantly different.

Figure 8 illustrates the induction of endo III sensitive sites in ^{32}P-labeled CHO *Alu* fragments. The *Alu* fragments were generated from plasmid pNB137 by double digestion with restriction enzymes *Bgl* I and *Eco* RI, and labeled at the 3' end. After being irradiated with UVC or UVC plus UVB light, the DNA samples were incubated with endo III, mild alkali, or hot piperidine and then analyzed by sequencing. Hot piperidine was used as a control for sites of (6-4) photoproduct induction (Brash et al., 1980). The cleavage at UV-irradiated DNA by these three different treatments are shown. Sequence identification showed that endo III cleaved UV-irradiated DNA at individual cytosines as well as dipyrimidines containing cytosines. These later sites were also potential sites for (6-4) photoproducts as detected by hot piperidine (Fig. 8, lane 8). The intensity of the cleaved bands induced by endo III and mild alkali were scanned by a densitometer and compared

7A.


0
200
400
600
800
1,000
J/m 2
- UVB
+ UVB
Form I
Form II
Form I
Form II

7B.

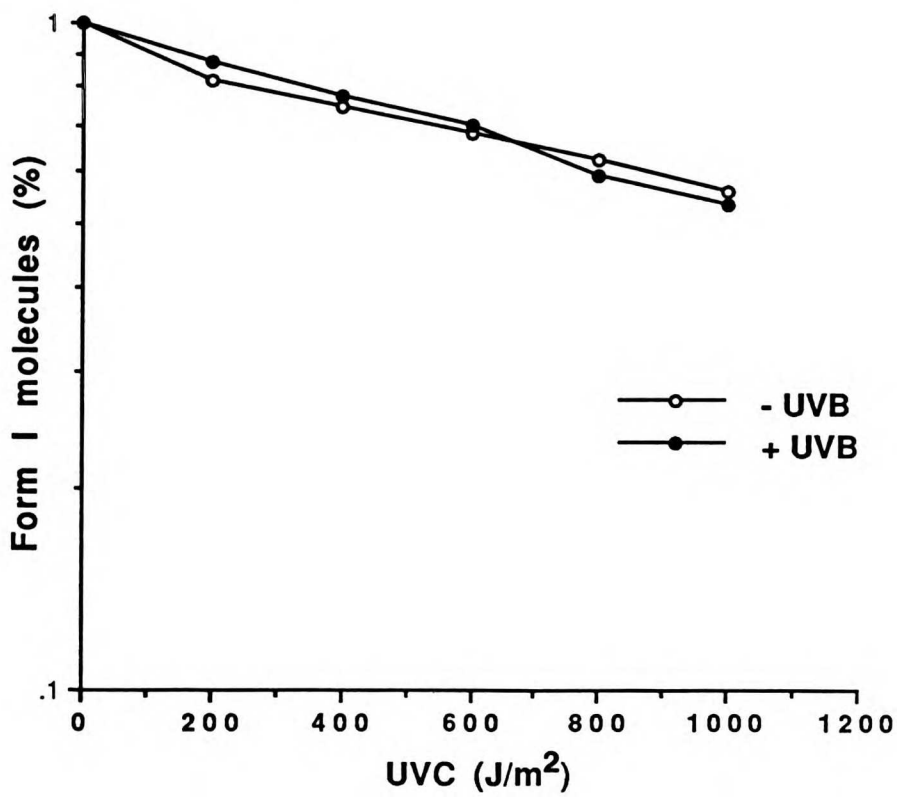
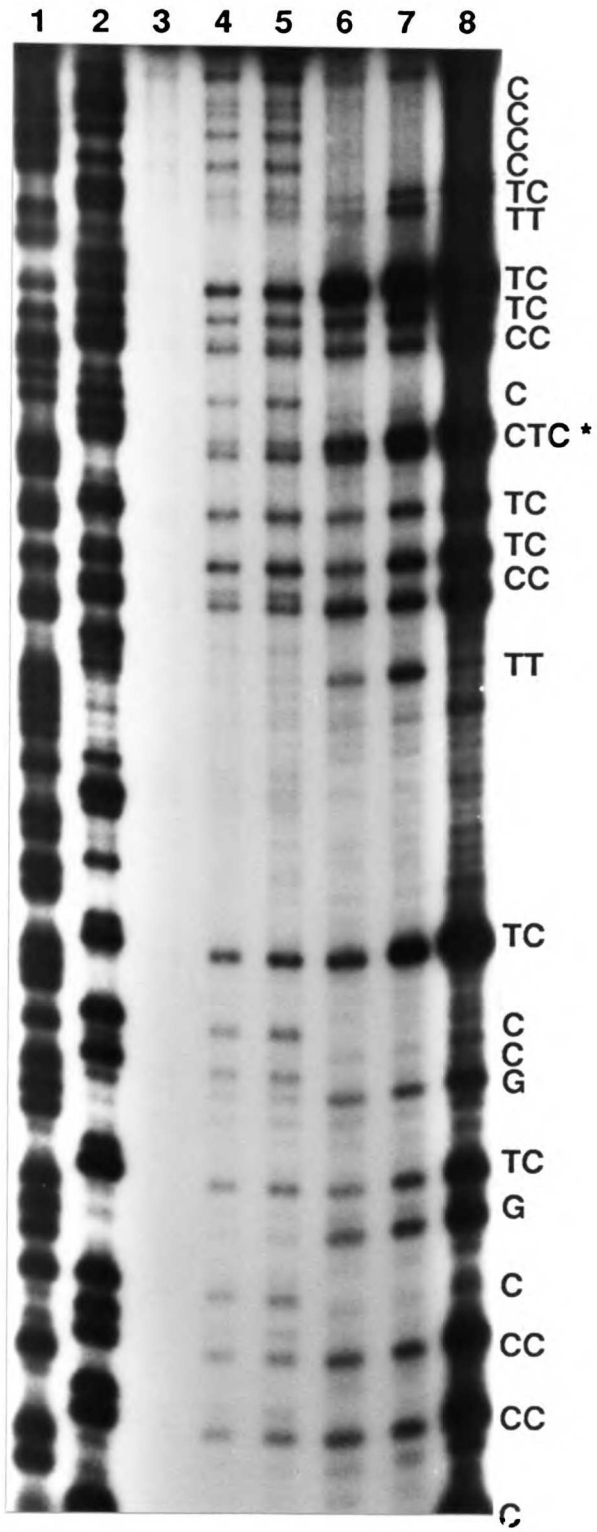

1
.1
Form I molecules (%)
0
200
400
600
800
1000
1200
UVC (J/m2)
- UVB
+ UVB

Figure 7. The induction of endo III sensitive sites in pUC19. Supercoiled pUC19 were first irradiated with UVC light at indicated doses and then irradiated with or without >320nm light for 4 hr followed by incubation with endo III at 37 °C for 1 hr. The loss of form I molecules by nicking with the enzyme treatment is presented on semi-log scale with increasing UVC dose. Each data point in panel B represents the average of two separate experiments. The D_{37} values used for determining the induction rate of endo III sensitive sites in the plasmid were calculated from the regression lines of the data shown.



Lane 1: A + G

Lane 2: T + C

Lane 3: Endo III

Lane 4: UVC + Endo III

Lane 5: UVC + UVB + Endo III

Lane 6: UVC + NaOH

Lane 7: UVC + UVB + NaOH

Lane 8: UVC + Hot piperidine

Figure 8. UV-induced endo III and alkali-labile sites in a defined DNA sequence. ^{32}P 3'-end labeled *Alu* fragment was used as DNA template. Samples were treated as shown and separated on 5% polyacrylamide gel. Letters to the right indicating the sequence of the cleaved sites; * : indicates the unique photoproduct site. Alkali cleavage at TT sites (TT_{166}, TT_{207}) were evident in lanes 6 and 7, suggesting the presents of rare (6-4) photoproducts. The nucleotide sequence of the region shown is as follows. The underlined sequences indicating endo III sensitive sites.

150 160 170
5'-GGCATG CGCGACGGGC AATCTTGGTG GCTTCGTCGC

180 190 200 210
CGATACGCTC*AGGTCGATCA CCGACTTGGA GATGCTGATG

220 230 240 250
ATCAAACGCA GGTCGGACGC CGCCGGCTGA CGACGGG-3'

Figure II. The relative induction of endo III and mild alkali-sensitive sites by UVB and UVC light.

Cleavage Site	Cleavage Sequence	Endo III			NaOH		
		+UVB (Rel.area)	-UVB (Rel.area)	+UVB/ -UVB	+UVB (Rel.area)	-UVB (Rel.area)	+UVB/ -UVB
150/152	CGCG	6.47	6.52	0.99	-	-	-
155	GACG	5.92	5.0	1.18	-	-	-
159	GGCA	5.0	5.9	0.85	-	-	-
163/165	ATCT/CTTG	7.06	5.92	1.19	8.37	7.23	1.16
174	TTCG	10.74	10.90	0.99	18.10	21.50	0.84
177	GTCG	3.97	5.12	0.78	6.10	6.50	0.94
180	GCCG	5.30	6.58	0.81	5.13	5.39	0.95
185	TACG	4.78	4.32	1.11	-	-	-
188/189	CTC*A	7.43	6.98	1.06	15.95	15.63	1.02
194	GTCG	5.85	5.45	1.07	4.55	5.56	0.82
198	ATCA	7.42	7.18	1.03	7.03	5.08	1.38
201	ACCG	6.29	5.68	1.11	6.05	7.63	0.79
206	CTTG	-	-	-	5.22	4.54	1.15
222	ATCA	6.58	7.91	0.83	9.09	6.70	1.36
226	AACG	3.27	3.32	0.98	-	-	-

228	CGCA	4.63	2.73	1.70	-	-	-
231	AGGT	-	-	-	2.13	2.78	0.77
233	GTCG	3.05	3.06	1.00	2.74	2.0	1.37
235	CGGA	-	-	-	3.5	2.64	1.33
237	GACG	2.24	2.66	0.84	-	-	-
240	GCCG	1.73	1.9	0.91	3.17	3.77	0.84
243	GCCG	2.2	1.9	1.16	2.89	3.18	0.91
Total relative change per cleavage site:				0.95			1.15

Underlined bases correspond to the site of cleavage.

Rel. area = relative intensity of the cleaved bands.

"-" = no cleavage.

CTC* = unique photoreactive site.

with the same site treated with or without UVB (Table II). Our data showed that secondary UVB irradiation enhanced the efficiency of cleavages by both endo III and NaOH but the extended of such increase was much greater for alkaline treated samples. Furthermore, in mild alkali treated lanes (Figure 8, lanes 6 and 7), the cleavage pattern was very similar to that of the hot piperidine treated lane 8. Secondary irradiation with UVB light only served to enhance alkaline cleavage at the sites specificity of (6-4) photoproducts because photoinduced Dewar photoproducts were more alkali labile. The degree of nonspecific cleavages were also significantly reduced by mild alkali treatment compared to hot piperidine treated DNA sample. In addition, a highly photoreactive site ($CTC_{187\text{-}189}$) was observed. This site was excluded from endo III and alkaline analysis because we have evidence suggesting that the photochemical properties of this site were unique (see discussion).

The heat sensitivity of UV-induced endo III sites was examined by incubating the UVC-irradiated DNA at 55 °C for 24 hours before digestion with endo III (Figure 9). It has been reported that cytosine hydrates are induced by UV irradiation and that they have a half life of 6 h at 55 °C in DNA (Boorstein et al., 1990, 1991). The heat treatment conditions used here should eliminate greater than 90% of the photohydrates. As shown in Figure 9, almost all of the endo III sensitive sites located at individual cytosines become endo III resistant after heat treatment. While some cytosine-containing dipyrimidine sites also became less sensitive many became more sensitive to endo III cleavage after heating. Table III summarizes the

change in relative intensity of endo III sensitive sites at single cytosines and dipyrimidines with TC and CC sequences. Increasing concentrations of endo III enzyme were used for each treatment in order to optimize cleavage specificity and to avoid artefact. Similar to the induction of (6-4) photoproducts, no endo III sensitive sites were found at CT sequences.

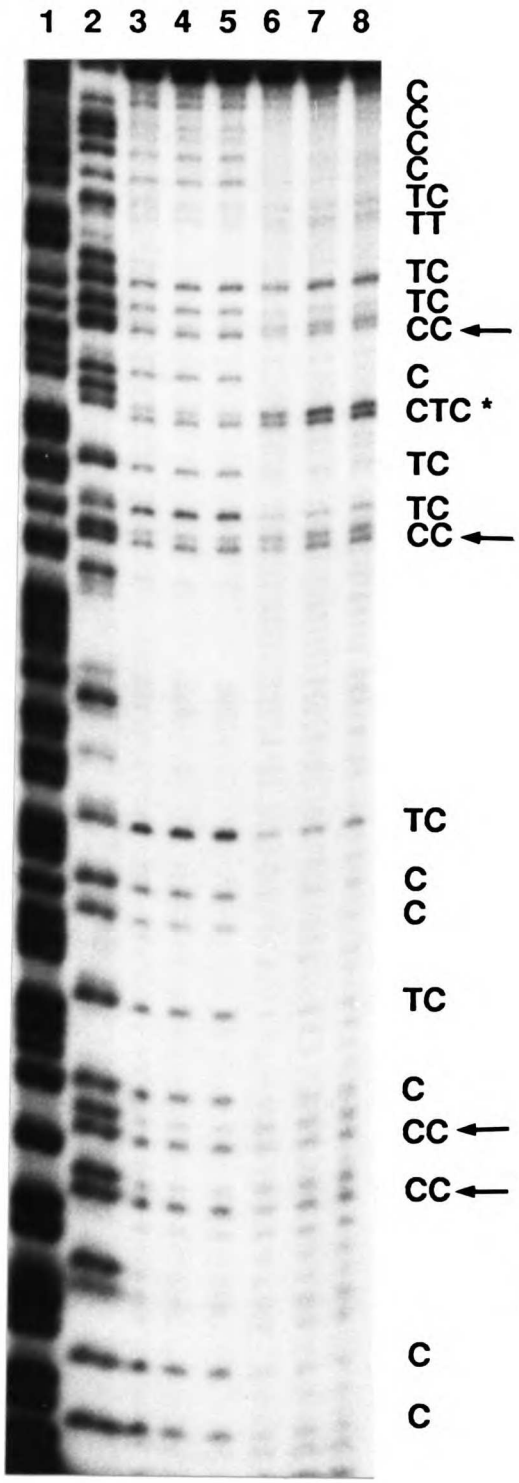

1 2 3 4 5 6 7 8
C
C
C
C
TC
TT
TC
TC
CC
C
CTC *
TC
TC
CC
TC
C
C
TC
C
CC
CC
C
C
Lane 1: A + G
Lane 2: T + C
Lanes 3, 4, 5: UVC + Endo III
Lanes 6, 7, 8: UVC + 55°C 24 h + Endo III

Figure 9. Heat stable endo III sensitive sites in UV-irradiated DNA. After UVC treatment, ^{32}P end-labeled *Alu* fragment was incubated at 55 °C or 0 °C for 24 hr and then treated with endo III at the concentrations of 1/3 *u*l, 1 *u*l, or 3 *u*l before being separated on a sequencing gel. Arrows indicating sequences where endo III cleavage occurred at both pyrimidines. "*" indicating a photochemically unique site. The region analyzed is shown below with the sequences of the heat stable endo III sites underlined.

150 160 170
5'-GGCATG CGCGACGGGC AATCTTGGTG GCTTCGTCGC

180 190 200 210
CGATACGCTC* AGGTCGATCA CCGACTTGGA GATGCTGATG

220 230 240 250
ATCAAACGCA GGTCGGACGC CGCCGGCTGA CGACGGG-3'

Table III. The relative induction of endonuclease III sensitive sites with or without heat treatment.

Pyr	0 °C	55 °C	% reduction
C	4.79	1.31	72.7 %
TC	5.72	2.87	49.8 %
CC	4.03	3.27	18.8 %
Total	14.54	7.45	47.1 %

Values = total optical absorbance of endo III cleaved bands at the indicated sequences in arbitrary unit.

% reduction = [(0 °C - 55 °C) ÷ 0 °C] x 100%

3.3. Discussion

Our results suggest that about 50% of the endo III sensitive sites in UV irradiated DNA were cytosine-hydrates readily reversible by heat treatment. However, stable photoproducts specific for endo III cleavage were also induced by UV irradiation. These photoproducts were primarily located at dipyrimidine sites containing cytosine with sequence specificity very similar to that of (6-4) photoproducts. RIA analysis suggested that these endo III sensitive sites do not represent epitopes recognizable by antibodies specific for (6-4) photoproducts (data not shown). Furthermore, in every instance, the 5'-cytosine of CC sequences in the fragment analyzed became more labile to endo III after heat treatment (Fig. 9, arrows). No similar property have been observed for (6-4) photoproducts.

Weiss and Duker (1986) reported the identification of a UV-induced putative modified cytosine photoproduct that is a substrate of endo III. In their analysis, endo III cleavage was mainly referred to as individual cytosines flanked by purines. Endo III sensitive sites located at C-containing dipyrimidine sites were considered the same as those at individual cytosines. Our work demonstrates that cytosine hydrates are formed in UV irradiated DNA at predominantly single cytosine sites and were substrates for endo III; they are the most likely candidates for those described by Weiss and Duker (1986). However, photohydrates can be eliminated from the DNA by heat treatment (Boorstein et al., 1989); yet about half of the endo III sensitive

sites are heat stable with some became even more sensitive to the enzyme treatment. They are located exclusively at cytosine-containing dipyrimidine sites indicating that they are dipyrimidine photoproducts.

Although not directed at resolving the biochemical nature of the photoproducts, our results showed that these non-hydrate *E. coli* endo III sensitive sites have the following properties:

1) they are induced at C-containing dipyrimidine sites and accounts for about half of the endo III sensitive sites in purified UV-irradiated DNA;

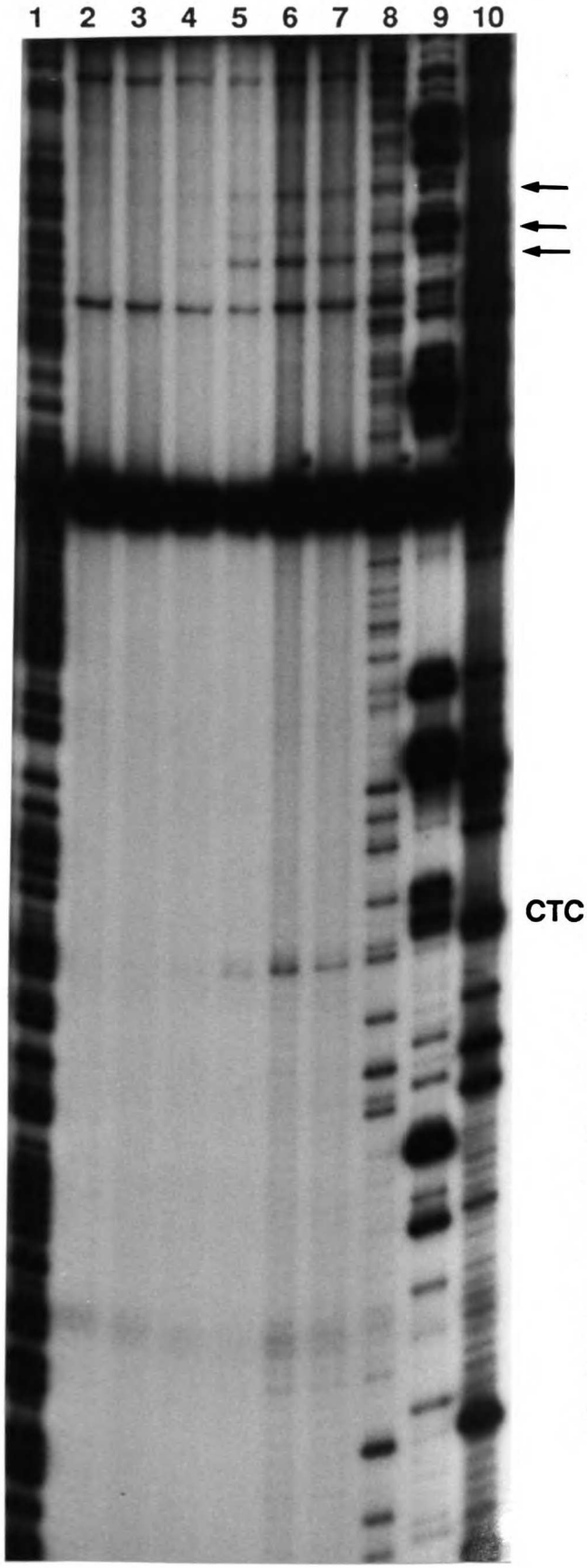
2) they are heat stable and they do not respond to further UVB irradiation;

3) they do not share the same antibody binding site as those for (6-4) photoproducts; and finally,

4) when a CC sequence is present, the 5'-cytosine also becomes sensitive to endo III after heat treatment.

These findings suggested that while having almost identical sequence specificity, the stable endo III sensitive sites we identified are not likely to be (6-4) photoproducts but could represent a novel version of dipyrimidine photoproduct that have not been previously described.

A unique DNA sequence site having remarkable photochemical activities was also observed in our analysis (Figure 10, site $CTC_{187\text{-}189}$, and "*"s in Figures 8 and 9). This site was very photoreactive as seen by the sequence gel. It has a low



Lane 1: A + G

Lane 2: - UVC - AP endo IV

Lane 3: - UVC + AP endo IV

Lane 4: + UVC - AP endo IV

Lanes 5-7: + UVC + AP endo IV

Lane 8: + UVC + endo III

Lane 9: + UVC + T4 endo V

Lane 10: + UVC + Hot piperidine

Figure 10. The detection of a unique UV-induced DNA photoproduct. Samples were treated as shown. Analysis was carried out described in Fig. 8 and 9. Lanes 5 to 7 were treated with *E. coli* endonuclease IV at 1/100 *u*l, 1/10 *u*l, and 1 *u*l dilutions. Arrows indicating sites having similar photochemical properties as $CTC_{187\text{-}189}$.

level of site specific breakage upon UVC irradiation. It was a hot spot for mild alkali, endo III, T4 endo V, and hot piperidine cleavage. This site became more labile to T4 endo V and hot piperidine after UVB irradiation; or to endo III after heat treatment with certain preferences at either $CT_{187\text{-}188}$ or $TC_{188\text{-}189}$ (Fig. 8 and 9; Mitchell et al., 1991b). This unique site was sensitive to *E. coli* AP endonuclease IV suggesting that it is also a potential apyrimidinic (AP) site. DNA secondary structure may have played a role resulting in the complex photochemical reactivity for this site because CTC sequences at other locations did not appear to show the same hyper-photoreactivity.

Photoproducts involving three thymines is known to form in frozen-solution irradiation of thymine (Varghese, 1972). This product exhibits properties characteristic of a thymine dimer containing cyclobutane and an oxetane ring system which is a precursor of (6-4) photoproduct. Furthermore, X-ray crystallographic analysis indicated the structure of this thymine product as the water addition product of a thymine trimer. Based on our analyses using T4 endo V, endo III, and alkali, the $CTC_{187\text{-}189}$ site we described here exhibits all characteristics of a trimer photoproduct. Significantly, unlike the thymine trimer, this CTC trimer was formed under physiological conditions when DNA sample was irradiated in water at room temperature. Thus, the formation of this photoproduct may have important biological implications.

Brash et al (1987) have reported that in mammalian cells, mutation hot spots are not directly a consequence of photoproduct formation but may depend on the secondary structure of the DNA. The unique photoproduct we have describe may have demonstrated one such site that could lead to high frequency of UV induced mutations at this site. Although the particular sequences involved were not identifiable, close inspection of the sequence gel indicates that there are other sites having similar properties in the fragment analyzed (Fig. 10, arrows). It would be interesting to determine the mutation specificity at these sites *in vivo*.

Alkali-labile sites induced by 0.3N NaOH treatment occurred at the 3'-cytosines of (6-4) photoproducts. The cleavage of these sites increased with the subsequent irradiation of UVB which converts the (6-4) photoproducts into the more labile Dewar photoproducts (Fig. 8). The sequence specificities of photoproduct sensitivity to hot piperidine and mild alkali were not identical. Alkaline treatment of UV irradiated DNA also revealed sensitive sites at TTs corresponding to the rare (6-4) photoproduct sites. A few individual guanine sites sensitive to UVB light were also detected by both hot piperidine and alkaline treatment (Fig. 8) but they may be a different class of purine photoproducts from those sensitive to T4 endo V (Gallager and Duker, 1987) and their structure remains to be determined.

In addition, although the low induction rate for endo III sensitive sites in genomic DNA was compatible with its induction rate determined in purified plasmid

DNA, the relative induction of endo III sensitive sites to that of PALs was significantly different (Table I). This difference may in part be due to variations in the sensitivity of different methods used. Mitchell et al. (1990a) showed that using RIA, the induction of (6-4) photoproducts *in vivo* was similar to those observed in the purified plasmid DNA. Furthermore, our sequencing analysis (Fig. 8, lane 6) showed that (6-4) photoproducts were also sensitive to mild alkali treatment; the additional UVB radiation only acted to enhance its cleavage while reducing nonspecific background cleavage (Fig. 8, lane 7). Thus the measurement of (6-4) photoproducts induction *in vivo* should be the accumulation of site specific cleavages by both UVC and UVB irradiation. The genomic (6-4) induction data shown in Table I only represented the induction of UVB sensitive sites in UV-irradiated DNA thus resulting in a much lower value. The reliability of the PALs method for measuring the induction of (6-4) photoproducts in the genome is further substantiated by its measurement for the removal of these photoproducts in human cells. The rate of (6-4) photoproduct repair as measured by PALs method was the same as those obtained by other methods showing the lack of (6-4) photoproduct repair in UV-sensitive XP group A cells but a rapid repair kinetics in normal cells (Cleaver, 1988; and Mitchell, 1989a).

CHAPTER FOUR: THE INDUCTION AND REPAIR OF (6-4) PHOTOPRODUCTS IN SINGLE COPY GENES

4.1. Background

DNA damage and repair events have been studied at the single gene level in mammalian cells. Using Southern blot hybridization technique, Bohr et al. (1985) measured DNA lesions within a restriction fragment by employing T4 endo V which specifically recognizes cyclobutane dimers. Enzymes specific for (6-4) photoproducts are yet to be found. The (6-4) photoproducts are repaired by the *uvr*ABC gene products of *E. coli* (Franklin and Haseltine, 1984). This enzyme system is responsible for the repairing a wide range of DNA damages induced by different physical and chemical agents (Sancar and Sancar, 1985). All genes involved in the *uvr*ABC excision repair pathway have been cloned and the reaction reconstituted *in vitro* (Sancar and Rupp, 1983; Grossman et al., 1988). In order to remove UV induced damage, the UvrA protein forms a dimer by hydrolyzing ATP which initiates the binding of this protein to DNA. Subsequently, UvrB, which is a helicase, binds to the DNA-$UvrA_2$ complex and unwinds DNA at the damaged sites. The unwinding of the DNA allows for a dual incision of the DNA by UvrC at 7 nucleotides 5' and 3-4 nucleotides 3' to the damaged site. After incision, the Uvr proteins remain bound to DNA until UvrD and DNA polymerase I displaces the excised fragment and resynthesizes the DNA. Finally, the newly synthesized DNA is rejoined by

ligase, restoring its original condition.

Utilizing the broad specificity of the *uvr*ABC system that includes the recognition of (6-4) photoproducts and the DNA photolyase which reverts cyclobutane dimers, Thomas et al. (1989) developed a method to quantify (6-4) photoproducts in the amplified DHFR region of the CHO cell genome. After UV-irradiation, repaired DNA were subjected to photolyase treatment to eliminate cyclobutane dimers and then treated with UurABC enzymes. As the (6-4) photoproduct is the second most abundant photoproduct formed in UV-irradiated DNA, the extent of enzyme cleavage was therefore considered primarily a measure of (6-4) photoproducts. They showed that (6-4) photoproducts were repaired more efficiently than pyrimidine dimers and like dimers, there was preferential repair of (6-4) photoproducts in the DHFR gene compared to the downstream noncoding sequences. However, the rate of induction for (6-4) photoproducts was much higher than the induction of dimers in the same region (Bohr et al., 1985). One possible explanation of this discrepancy is the fact that photoproducts other than (6-4) photoproduct are still present after photoreactivation. Furthermore, although a major portion of the pyrimidine dimers are reverted by photolyase, the remaining dimers may still constitute a significant part of the *uvr*ABC substrate (Myles et al., 1987).

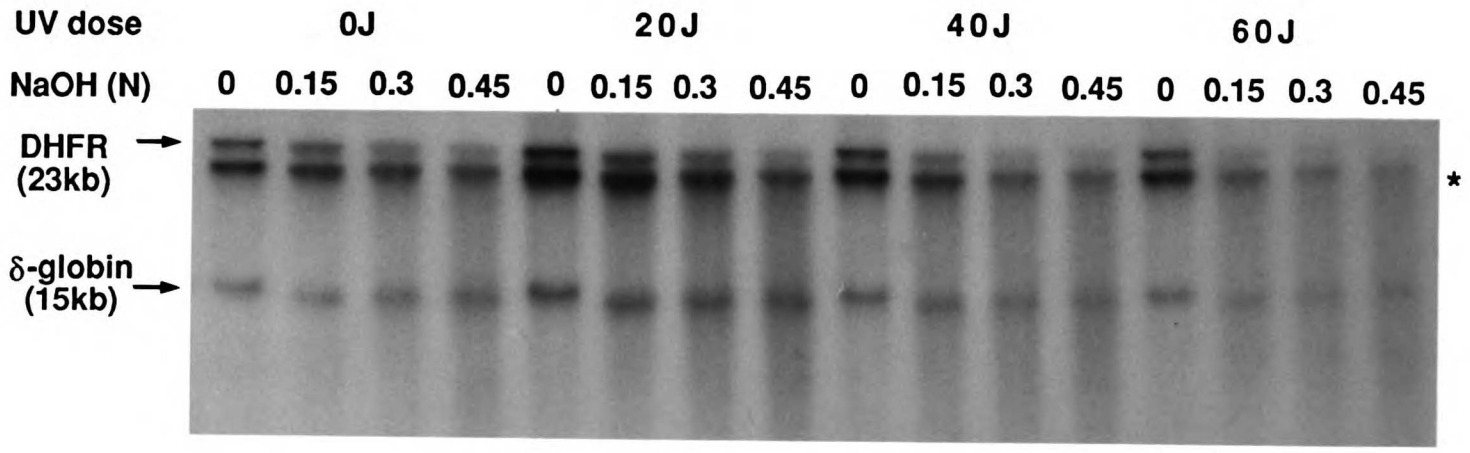
Due to the lack of a sensitive enzymatic assay that can detect (6-4)

photoproducts in cellular DNA at biologically relevant UV fluences, the kinetics for repairing (6-4) photoproducts in specific genes of the genome are not well understood. Mitchell et al. (1990a) showed that the alkali lability of (6-4) photoproducts depended upon both alkali concentration and temperature. The (6-4) photoproducts can be converted quantitatively to Dewar photoproducts which are alkali labile at room temperature and are cleavable by mild alkaline hydrolysis. As described in the previous Chapter, we have characterized the specific cleavage at the site of (6-4) photoproducts by mild alkali in a defined DNA sequence. Here, we used this method in conjunction with Southern blot hybridization to determine the induction and repair of (6-4) photoproducts in single copy genes of human skin fibroblasts.

4.2. Results

Mild alkaline hydrolysis was used to determine the induction of (6-4) photoproducts in genomic regions having different transcriptional activities (Figure 11). Repair deficient XP12RO cells were irradiated with 254nm UV light at 0, 20, 40, 60 J/m^2 and lysed immediately. Genomic DNA was isolated, digested with *Hind* III, precipitated by ethanol, and then dissolved in H_2O at a concentration of 10*u*g/40*u*l. This DNA was irradiated under UVB light in 5% glycerol for 4 hr at RT followed by incubation with increasing concentrations of NaOH for each UV fluencyat 24 ^{0}C for 20 hr. At the end of incubation, samples were separated on a

The induction of (6-4) photoproducts in UV-irradiated cells



DHFR: dihydrofolate reductase gene

δ-Globin: a nontranscribed gene in skin fibroblasts

Figure 11. The induction of (6-4) photoproducts in UV-irradiated cells. Repair deficient XP12RO cells were irradiated with UVC light at indicated doses. DNA were immediately extracted and analyzed for (6-4) photoproducts by PALs method using indicated alkali conditions at RT for 24 hr. An equal mix of p1.8 and pHd 1 probes were used for Southern blot analysis. "*" indicates the 20 kb DHFR pseudogene fragment that was detected by both probes.

alkaline agarose gel in parallel with the untreated controls and analyzed by Southern blot hybridization using a mixture of human DHFR (p1.8) and delta-globin gene (pHd 1) probes.

As shown in Figure 11, the intensity of the hybridized 23kb DHFR band decreased significantly with increasing UV doses indicating an increase in the number of (6-4) photoproducts in this region. In contrast, minimum changes were observed for the intensity of the hybridized 15kb delta-globin fragment. A third band of about 20kb in length was also observed in this experiment. This band was apparently resulted from cross hybridizations between the DHFR probe with DHFR pseudogene (Yang et al., 1984) and the delta-globin gene with a fragment of similar size. When analyzed, the intensity of this cross hybridized band decreased with the increasing UV dose at an intermediate rate. The results were analyzed by a two dimensional laser densitometer that can differentiate the hybridization signals associated with two closely spaced bands. The intensity of the hybridization bands decreased somewhat in none UV-irradiated lanes with the increase in alkali concentration. This background cleavage was compensated by using the bands not treated with alkali as baseline when calculating the percentage of change in hybridization intensity for each UV dose.

The mild alkali sensitive sites in the gene were plotted for each detected band with increasing UV does at various alkali conditions as a measure of (6-4()

photoproduct induction (Figure 12). The induction of (6-4) photoproducts in the gene was determined by the relative hybridization intensity of the bands in alkali treated lanes as compared to their untreated controls. This ratio gave us the value for the fraction of interested fragments that are free of (6-4) photoproducts. The average number of (6-4) photoproducts per fragment was determined based on the relative proportion of fragments free of photoproducts assuming a Poisson distribution. This method is analogous with the one used by Bohr et al. (1985) for determining cyclobutane dimer induction in the gene using T4 endo V.

The relative induction of alkaline sensitive sites in DHFR and Delta-globin genes with increased UV fluence are shown in Figure 12. Of all three NaOH concentrations, the average induction of alkali-sensitive sites per DHFR fragment increased linearly with UV fluence at 0.55/20J/m^2, 1.0/40J/m^2, and 1.8/60J/m^2. This result gave an average rate of (6-4) photoproduct induction at 0.28/10J/m^2/23kb DHFR fragment which equals to 0.2 (6-4)/10^8dalton/J/m^2. In contrast, the induction of alkali-sensitive sites was much lower for the nontranscribed 15kb delta-globin gene fragment at 0.07/60J/m^2 with the corresponding rate of induction at 0.013 (6-4)/10^8dalton/J/m^2. A higher background cleavage was observed with increased alkali concentrations. We have therefore chosen a concentration of 0.3N NaOH and 40J/m^2 for determining the rate of (6-4) photoproduct repair in the DHFR gene.

Figure. 12A. The relative induction of (6-4) photoproducts in DHFR gene.

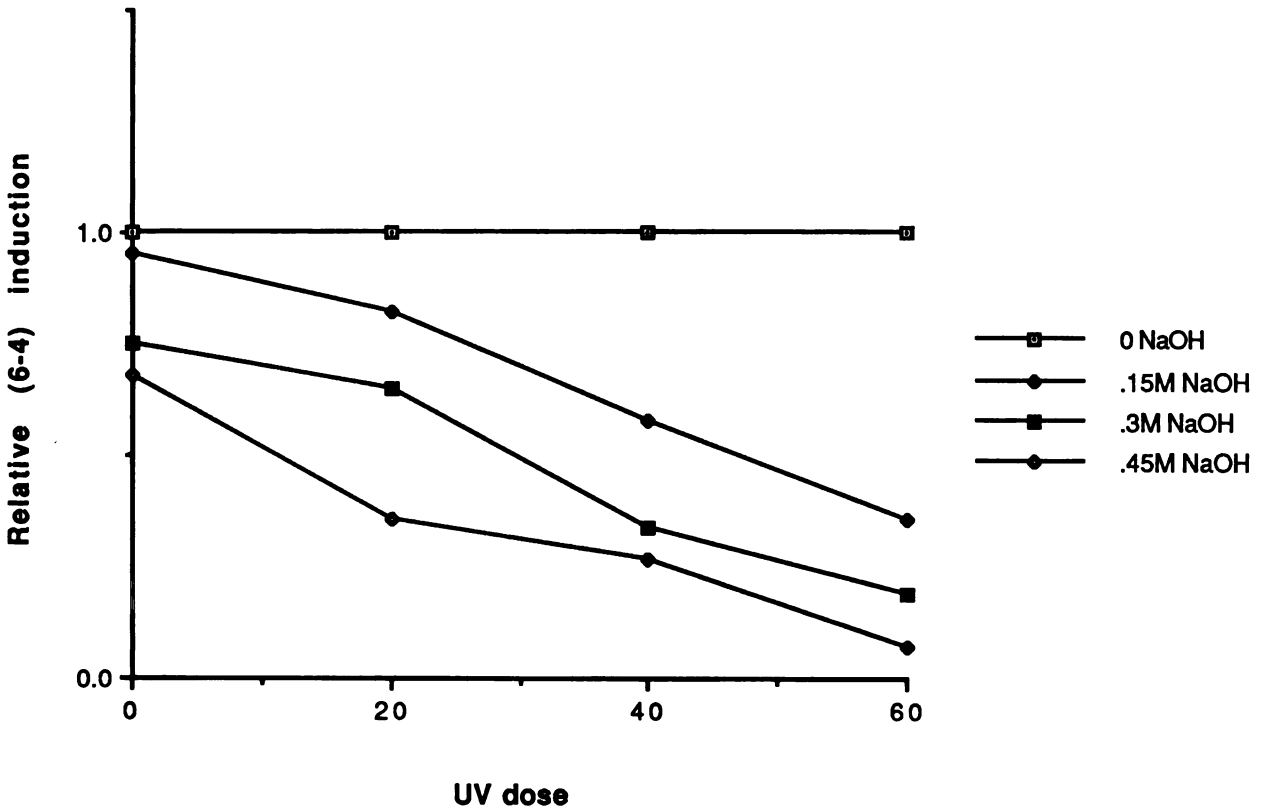


Figure 12B. The relative induction of (6-4) photoproducts in delta-globin gene.

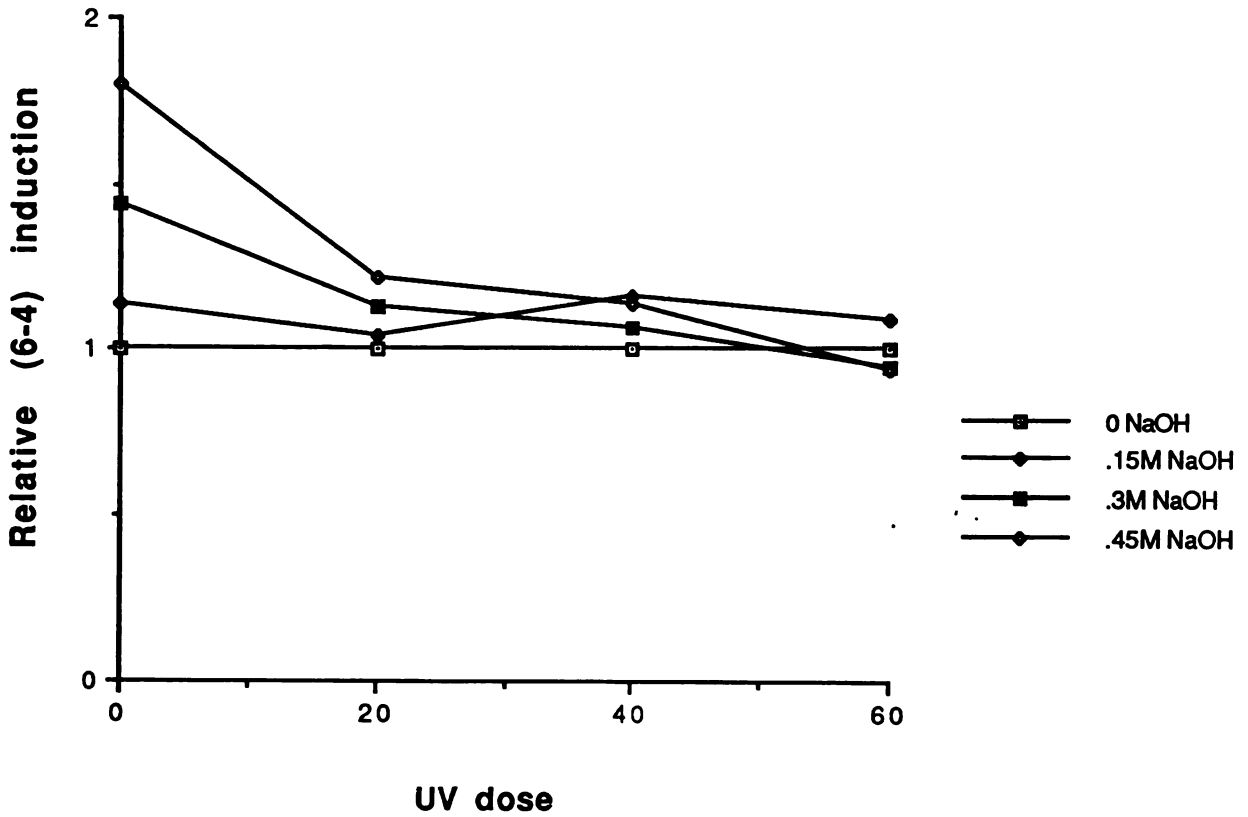
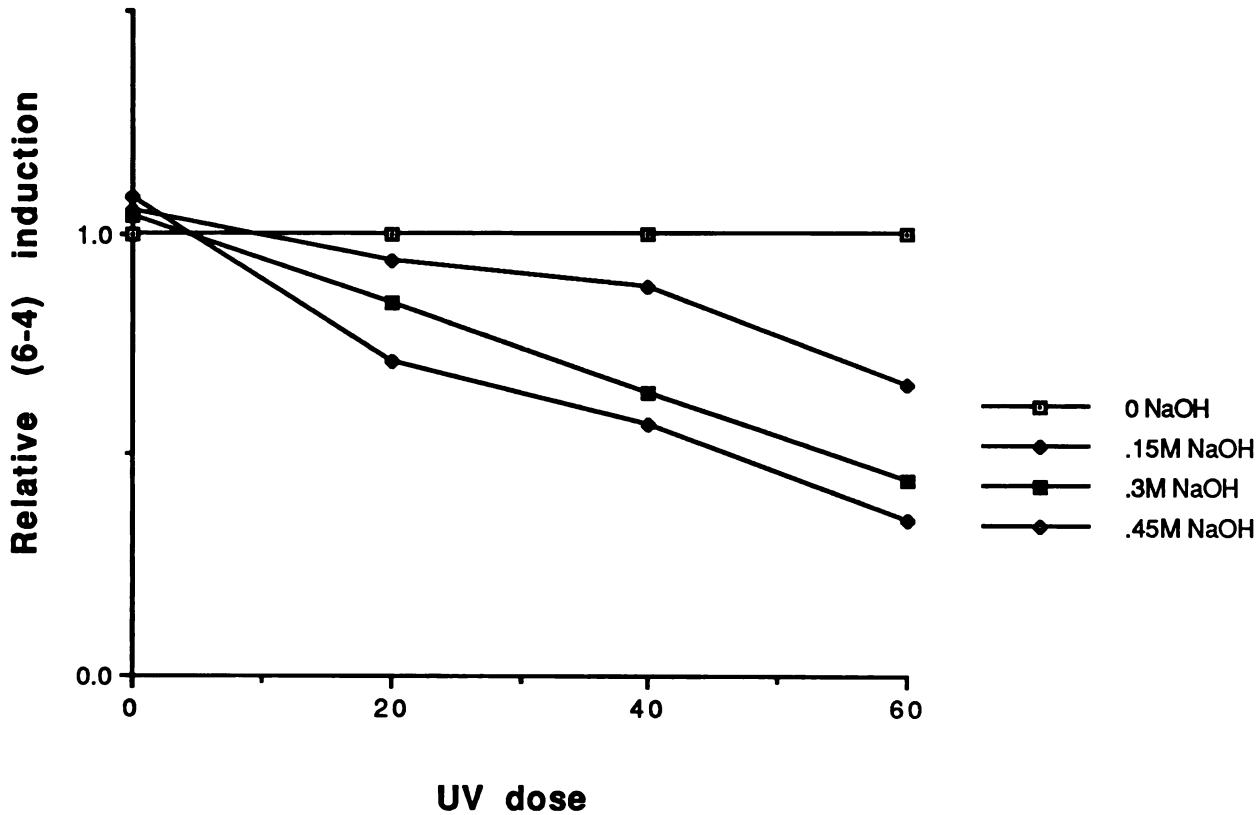


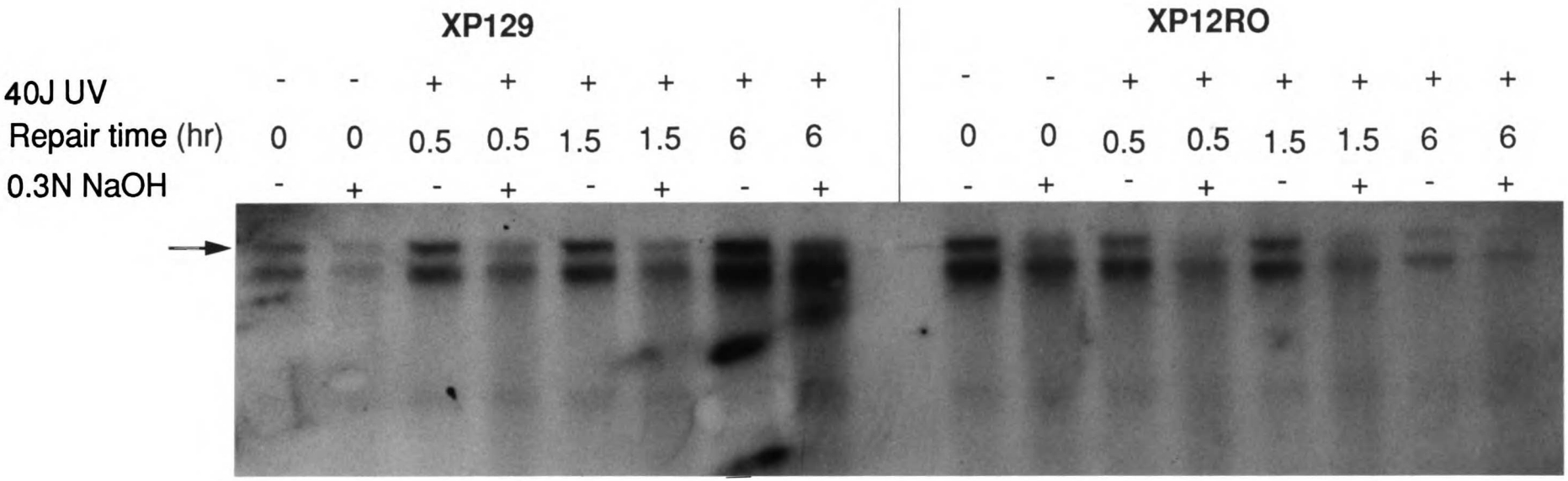
Figure 12C. The relative induction of (6-4) photoproducts in pseudo-DHFR gene.



UV-sensitive XP12RO and the revertant XP129 cells were irradiated with UVC light and allowed to repair for 0 to 6 hours. DNA were further irradiated with UVB after restriction enzyme digestion, and were treated with 0.3N NaOH for 24 hr (Figure 13). The rate of (6-4) photoproduct removal in DHFR gene of the two cells lines were determined by Southern blot hybridization. For revertant XP129 cells, over 70% of the (6-4) photoproducts were removed from DHFR gene 6 hours after UV irradiation while the parental XP12RO cell showed no detectable repair of (6-4) photoproducts in this region during the same time period.

The induction and repair of cyclobutane dimers were also studied using the T4 endonuclease V. Figure 14 shows the induction of cyclobutane dimers in DHFR and delta-globin genes. Repair deficient XP12RO cells were irradiated at 0, 20, 40, or 60 J/m^2 and genomic DNA was immediately extracted from the cells after UV irradiation. *Hind* III digested DNAs were incubated with T4 endo V which specific incisions DNA at cyclobutane dimer sites. The results obtained by Southern blot hybridization were consistent with those previously reported (Bohr et al., 1985) indicating a uniform induction of cyclobutane dimers throughout genome.

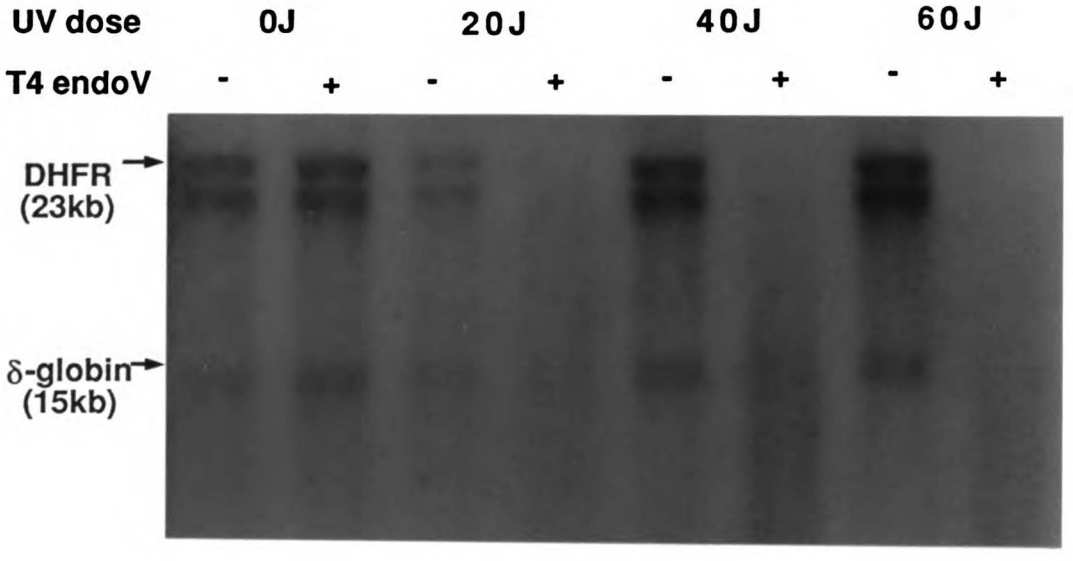
The repair of (6-4) photoproducts in DHFR gene



Arrow = DHFR fragment

Figure 13. The repair of (6-4) photoproducts in DHFR gene. UV sensitive XP12RO cell and the revertant XP129 cells were irradiated with or without 40 J/m^2 UVC light and then allowed to undergo repair for 0 to 6 hr. Five microliter of H_2O or 0.3N NaOH was used to treat DNA samples after they had been treated for 4 hr with UVB light. Probe p1.8 was used for Southern blot hybridization which detects the functional DHFR and one of its pseudogenes but only the hybridization at the functional DHFR gene fragment (arrow) was analyzed.

The induction of cyclobutane dimers in UV-irradiated cells



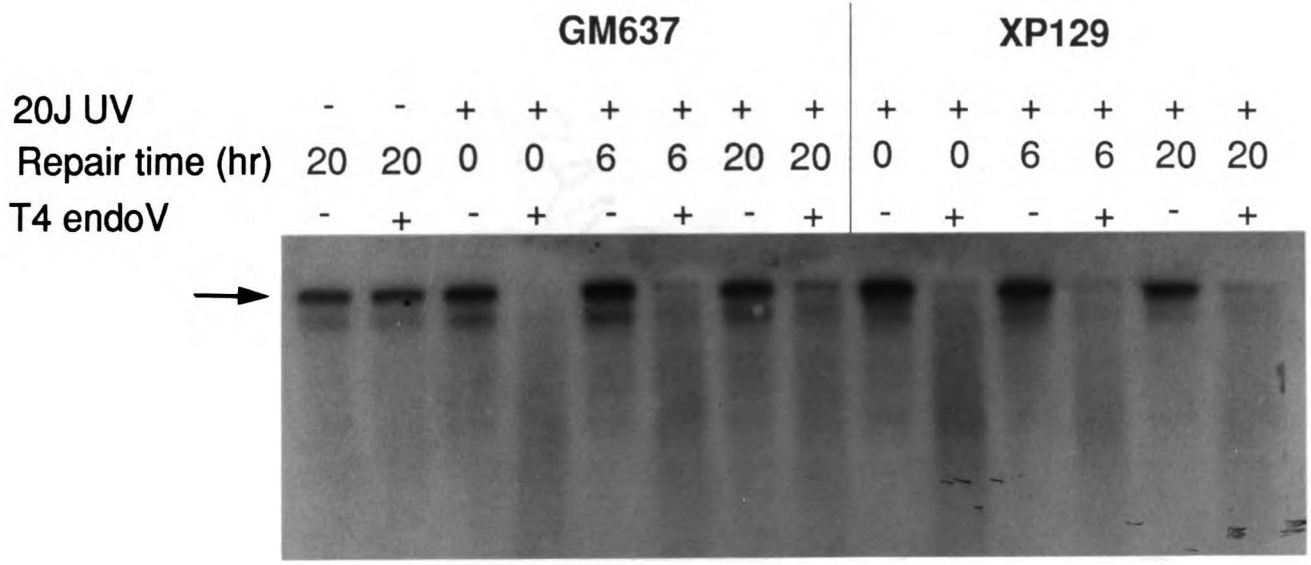
DHFR: dihydrofolate reductase gene

δ-globin: a nontranscribed gene in skin fibroblasts

Figure 14. The induction of cyclobutane dimers in UV-irradiated DNA. Repair deficient XP12RO cells were irradiated with indicated UVC light. Genomic DNA were extracted, digested with *Hind* III and parental fractions were purified from CsCl gradient. One microliter of T4 endo V was used to react with 10*u*g DNA at 37 °C for 30min. A mixture of p1.8 and pHd 1 probes were used for Southern blot analysis. The number of dimers induced were not analyzed due to the near complete loss of the full length fragment at high UV dose.

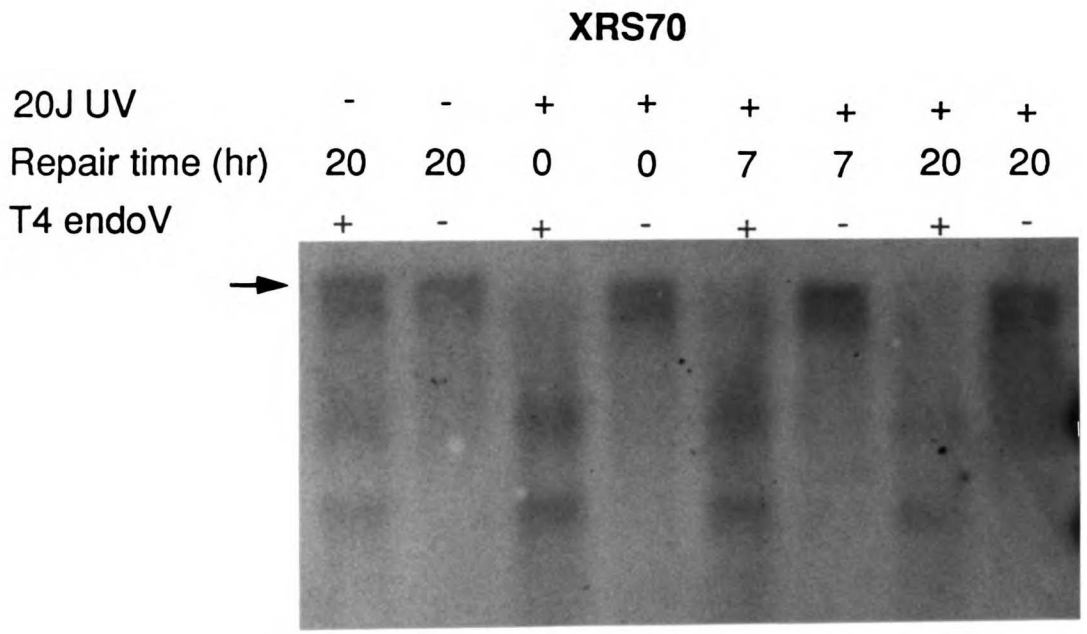
The repair of cyclobutane dimers in SV40 transformed normal skin fibroblasts (GM637) and XP revertant cell lines (XP129 and XSR70) were also analyzed using a similar method (Figure 15). Cells were irradiated at 20 J/m^2 and allowed to repair for 0, 6 and 24 hours. Unreplicated parental DNA were obtained by CsCl gradient centrifugation. After incubation with T4 endo V, the samples were loaded in pairs with or without enzyme treatment and the rate of cyclobutane dimer repair at the DHFR gene were analyzed. In our hand, GM cells showed about 50% of repair at the DHFR gene locus 20 hr after UV irradiation, while XP129 cells consistently repaired less over the same period (Fig. 15A). The rate of cyclobutane dimer removal for another independently generated XPA revertant cell line, XRS70, was even less at only 9% after 20 hr (Fig. 15B).

Cyclobutane dimer repair in DHFR gene (Fig. 15A)



Arrow = DHFR fragment

Cyclobutane dimer repair in DHFR gene (Fig. 15B)



Arrow = DHFR fragment

Figure 15. The repair of cyclobutane dimers in human cells. The indicated human cell lines were irradiated at 20 J/m^2 and allowed to repair at indicated time. The method of analysis was the same as described in Fig. 14 except that only p1.8 probe were used. The top band (arrow) indicates the functional DHFR gene fragment which was analyzed for dimer repair.

4.3. Discussion

Although induced at a relatively lower rate than cyclobutane dimers, (6-4) photoproducts play an important role in the mutagenic and cytotoxic effects inducted by UV irradiation in both prokaryotic and eukaryotic systems (Haseltine, 1983; Franklin and Haseltine, 1986; Brash, 1988; and Mitchell, 1989b). In mammalian cells, the cytotoxic effect (6-4) photoproducts is more prominent because their removal is necessary for the UV survival of the cells (Cleaver et al., 1987; 1988; Mitchell., 1988). The kinetics of (6-4) photoproduct removal is also much more rapid than cyclobutane dimers suggesting their importance for the cellular recovery after UV irradiation. In this chapter, we utilized a mild alkaline hydrolysis assay to determine the induction and repair of (6-4) photoproducts in a single copy gene in order to reveal the underlining mechanisms that led to the cytotoxic effects observed for this photoproducts.

The induction of (6-4) photoproducts in mammalian genome was analyzed using the repair deficient XP12RO cells. The transcriptionally active DHFR gene and the nontranscribed delta-globin gene were used as markers for this analysis. Our result demonstrated that the induction of (6-4) photoproducts was different in different regions of the genome and this is in contrast to cyclobutane dimers which are induced uniformly in the genome. Thus, the induction of the (6-4) photoproducts may be influenced by the transcriptional state, secondary structure, and the

chromosomal organization of the DNA. Although our data are still preliminary, they provided an explanation for the rapid repair kinetics and higher cytotoxicity observed for (6-4) photoproducts which is that they are primarily located at the more essential and more accessible regions of the genome. Similar findings have also been reported by Gale et al., (1989) who showed that although (6-4) photoproducts are induced more randomly than cyclobutane dimers at the chromatin core regions, the overall induction of (6-4) photoproducts is much higher at the linker region. Mitchell et al., (1990) also showed that (6-4) photoproducts is induced at about 6 times as much in the linker region of the nucleosome than core regions. Our data on the repair of (6-4) photoproducts in the gene was consistent with the rapid repair kinetics reported using RIA (Mitchell and Clarkson, 1984, Mitchell et a., 1987) which reaffirms the alkaline hydrolysis method employed

The removal of (6-4) photoproducts in the DHFR gene was determined for the XP129 cells and its parental UV-sensitive XP12RO cells. The XP revertant XP129 cells generated by chemical mutagenesis was studied for such analysis because although normal for UV survival, this cell line has very low level of repair for cyclobutane dimers after 6 hours but near normal level repair of (6-4) photoproducts in the overall genome (Cleaver et al., 1987). These results were further substantiated by our study on the induction and repair of both cyclobutane dimers and (6-4) photoproducts in human cells at the DHFR locus (Table IV). (6-4) photoproducts are induced at about one forth of the rate of cyclobutane dimers in the DHFR gene.

Table IV. The induction and repair of cyclobutane dimers and (6-4) photoproducts in UV-irradiated human cells.

Cyclobutane Dimer Repair*

Repair time		0 hr	6 hr	24 hr
Dimer/DHFR per 20J/m^2	GM637	1.36	1.23 (10%)	0.75 (45%)
	XP129	1.65	1.36 (17%)	1.05 (35%)
	XRS70#	1.17	1.06 (9%)	1.06 (9%)

* DATA generated from the average of 2 and 3 experiments for GM637 and XP129 correspondingly.

Results obtained using *M. luteus* UV endonuclease (Applied Biomedicals, MD).

( ) = percent repair.

(6-4) Photoproduct Repair*

Repair time		0 hr	0.5 hr	1.5 hr	6 hr
(6-4)/DHFR per 40J/m^2	XP129	1.3	0.61 (53%)	0.64 (51%)	0.35 (73%)
	XP12RO	0.52	0.75	1.28	0.55

* Data generated from one experiment only. The removal of (6-4) photoproducts in XP12RO cells could not be determined due to variations.

( ) = percent repair.

As shown in Table IV, the repair of cyclobutane dimers at DHFR gene in normal GM cells was about 45% over a 20 hr period which was significantly lower than the similar analysis obtained by others using primary skin fibroblasts (Bohr et al., 1986b; Keyese and Tyrrell, 1987). This difference may resides on the fact that GM637 is SV40 transformed which may have disrupted the excision repair pathway. Our data are compatible with those obtained by Ley et al. (1989) using the same cell line. In our hands, the repair of cyclobutane dimers in the revertant XP129 cells was consistently lower than in GM637 cells after 20 J/m^2 UVC irradiation. This relatively low level of cyclobutane dimer repair was further supported by the results from another independent revertant cell line, XRS70. However, Lommel and Hanawalt (personal communication) have reported a normal cyclobutane dimer repair for XP129 cells at the transcribed strand of DHFR gene after a much lower does (7-10 J/m^2) UV irradiation. This variation remains to be further investigated but the difference in the UV doses used and the varied sensitivity with or without strand specific detection may have played a important roles. Further analysis is necessary to determine the dosage effect on cyclobutane dimer repair, whether differential induction of (6-4) photoproducts is diminished when purified DNA is irradiated as well as the general state of (6-4) photoproduct induction throughout the genome.

CHAPTER FIVE: CONCLUSIONS

The interaction of UV light with DNA is a complicated photochemical process involving the breakage and rejoining of chemical bonds between DNA bases as well as their surrounding water molecules, or proteins (Wang, 1976). Many different types of photoproducts are generated upon UV irradiation but our understanding of these photoproducts has largely depended upon the availability of specific enzymes and techniques (Friedberg, 1985). Consequently, a large amount of the research has been devoted to the study of the dipyrimidine cyclobutane dimers. The work presented here intended to develop and characterize a sensitive method for the specific detection of (6-4) photoproducts in single copy genes of the mammalian genome. Within this goal, we examined the photochemical and biochemical properties of UV-induced *E. coli* endonuclease III sensitive sites which resulted in the identification of a novel dipyrimidine photoproduct. We characterized a photochemical method for site-specific cleavage at (6-4) photoproduct sites and we further extended this method making it possible to measure the induction and repair of (6-4) photoproducts in human skin fibroblasts exhibiting different repair capabilities.

5.1. Biochemical Nature of UV-induced *E. coli* Endonuclease III Sensitive Sites

E. coli endonuclease III (endo III) is a broad spectrum DNA repair enzyme that reacts with damages induced by UV light, X-ray, acid, and Osmium tetroxide

(Radman, 1976; Gates and Linn, 1977). Although damaged thymine residues induced by X-ray or oxidative agents are shown to be substrates of endo III (Katcher and Wallace, 1983; Breimer and Lindahl, 1984), the biochemical nature of UV-induced endo III sensitive sites is not well understood. Our results indicated that about half of the endo III sensitive sites were presented primarily at individual cytosines located between two purines in the fragment analyzed. These sites became endo III resistant after 55 °C 20hr treatment suggesting that they were cytosine photohydrates. The remaining heat stable photoproducts occurred almost exclusively at TC and CC dipyrimidine sequences indicating they are nonhydrate photoproducts, possibly a new type of dipyrimidine photoproducts.

Weiss and Duker (1986) reported that endo III incises UV-irradiated DNA at single cytosines. Cleavages at dipyrimidine sites were also evident but regarded as the same because of the enzyme recognition at single cytosines. This endo III recognition at single cytosines was further supported by HPLC analysis which showed the release of modified free cytosine bases in UV-irradiated DNA after endo III treatment (Doetsch et al., 1986; Weiss et al., 1988). Similar analysis using UV-irradiated poly(dG-[^{3}H]-dC) indicated that the cytosine bases released by endo III treatment were cytosine hydrates (Boorstein et al., 1989) and that they had a relatively short half life under heated conditions (Boorstein et al., 1991) this later observation was also confirmed by our data (Chapter 3).

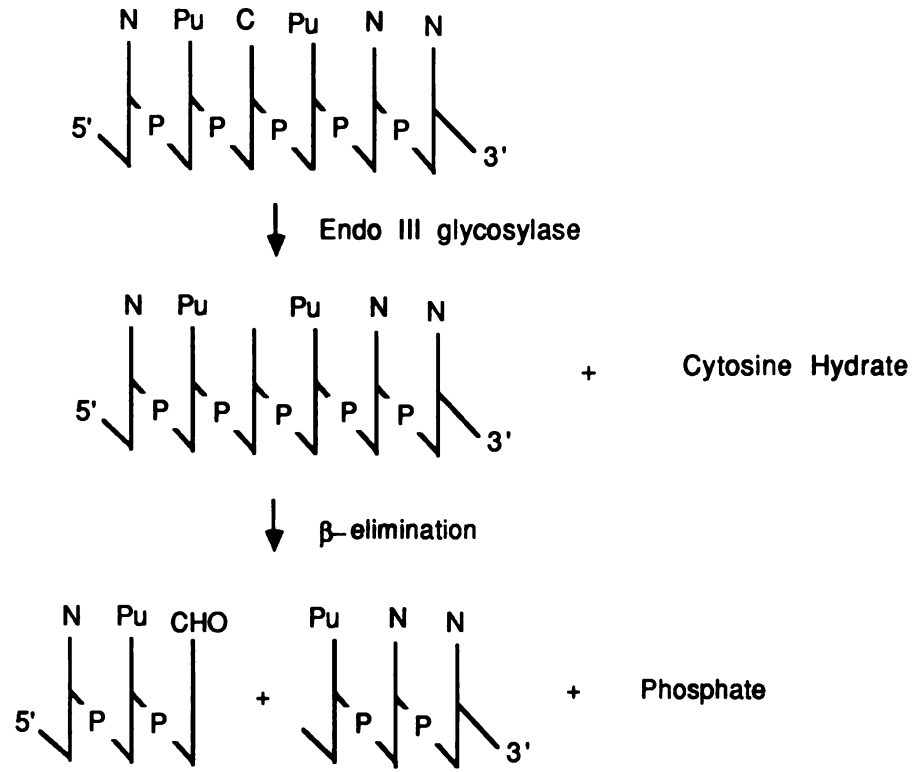
The structure of these endo III sensitive dipyrimidine photoproducts are yet to be determined. Our data suggested that they were not cyclobutane dimers because they were resistant to T4 endonuclease V treatment which specifically cleaves DNA at cyclobutane dimers. *E. coli* endonuclease IV treatment showed that they were not apyrimidinic sites either. Their rate of induction in DNA and RIA analysis further excluded them from being (6-4) photoproducts although they have almost identical sequence specificity. These results were consistent with those reported by Weiss and Duker (1986) who also showed that endo III sensitive sites were not produced by deamination of cytosines to uracils.

The photoproducts formed at dipyrimidine sites could, alternatively, be interpreted as cytosine hydrates whose stability depends on the presence of a neighboring pyrimidine base. The observed dual incisions of endo III at both cytosines when CC sequence is involved supports this possibility. HPLC analysis detected only one single type of modified cytosine photoproducts which was later determined to be cytosine hydrates also support this interpretation. However, the characteristic property of photohydrates is their ability to readily reverse back to original monomers upon heat treatment (Patrick and Rahn, 76). It is also difficult to explain why the presence of a neighboring pyrimidine could stabilize a photohydrate although neighboring bases do affect the formation of a potential UV-induced DNA photoproduct (Mitchell et al. 1991). Therefore, the heat stable endo III sensitive dipyrimidine photoproduct reported here could either be modified single

cytosines which are formed only at dipyrimidine sites, or simply a novel type of dipyrimidine photoproduct.

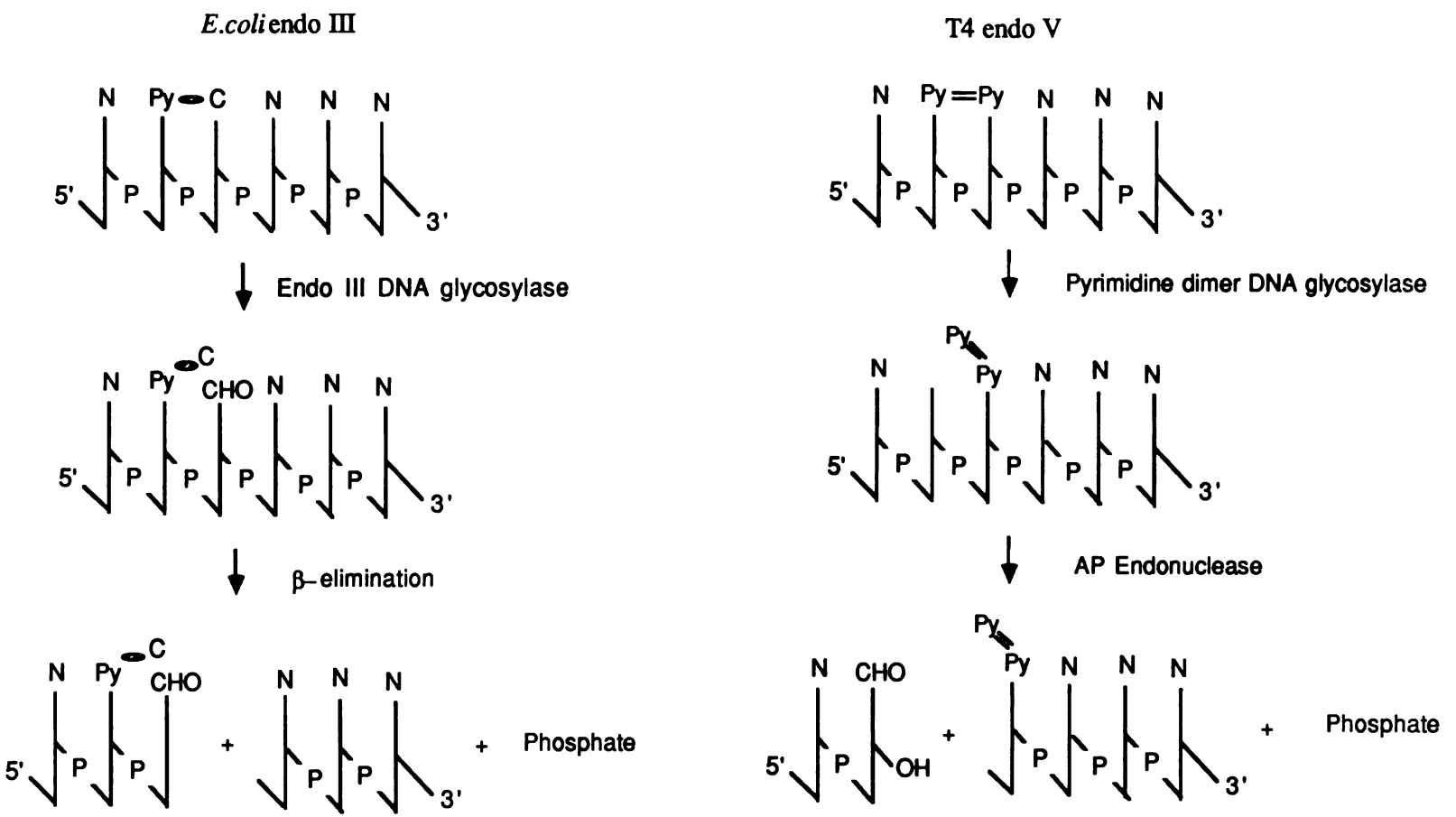
The mechanism of action for endo III on its substrates has been proposed based on its recognition at single base photoproducts (Demple and Linn, 1980; Doetsch et al., 1986; Kim and Linn, 1988). This enzyme cleaves the glycosyl bond between the modified base and its sugar residue followed by a beta-elimination which cleaves DNA at the 3'-side of the photoproduct resulting in the release of a modified cytosine (Fig. 16A). Doetsch et al., (1988) further analyzed the cleavage ends generated by endo III and showed that when the UV-irradiated DNA was labeled at the 5'-end, the fragment migrated some what slower possibly due to the presence of a 3'-abasic sugar. In the case of a dipyrimidine photoproduct, we propose that endo III primarily cleaves the glycosylase bond of the 3' pyrimidine (a cytosine in most cases) and subsequently incises DNA at the 3'-side of the dipyrimidine photoproduct (Figure 16B). This is in contrast to the cleavage at the 5' pyrimidine of the cyclobutane dimer by T4 endonuclease V which results in a 5'-dangling pyrimidine base after endolytic incision between the two involved bases (Haseltine et al., 1980). The cleavage by endo III results in a 3'-overhanging pyrimidine base which is attached to the other pyrimidine via a stable but not yet determined chemical bond. The resulting 3'-overhanging ends will cause a slow down in DNA fragment migration when it is labeled at 5' end as shown by Doetsch et al. (1988).

Figure 16A. The mechanism of action of E. coli endonuclease III on cytosine hydrate(modified from Doetsch et al.1986).



C = cytosine hydrate; N = any base; Pu = purine base; Py = pyrimidine base. P = phosphate.

Figure 16B. Mechanisms of action of endo III and T4 endo V on dipyrimidine photoproducts (the mechanism of T4 endo V action modified from Gordon and Haseltine, 1981).



Py⬬C = heat stable endo III sensitive photoproduct.

Py=Py = cyclobutane dimer.

See Fig.16A for other legends.

The biological importance of endo III specific photoproducts is implicated by the fact that endo III activities have also been found in many different biological systems (Doetsch et al., 1986; 1987; Gossett et al., 1988; Boorstein et al., 1989; Gallagher et al., 1989; and Weiss et al., 1989). They suggest that endo III is required for maintaining the normal cellular function and integrity of the DNA. Mutant *E.coli* strains deficient in endonuclease III have reduced thymine glycol-DNA glycosylase activities as well as reduced incision of a broad-spectrum-irradiated DNA (Cunningham and Weiss, 1985). At least in prokaryotes, mutation hot spots have been mapped to TC and CC sequences and were correlated with (6-4) photoproducts because of their most frequent occurrence at these sites. The endo III sensitive dipyrimidine lesions reported here displays an almost identical sequence specificity as for (6-4) photoproducts. Therefore these photoproducts may have also contributed to the observed UV-induced mutagenic and cytotoxic effects. Further study is warranted to better understand the structure and the biological relevance of these photoproducts.

5.2 The Induction and Repair of (6-4) Photoproducts in Human Cells

The relevance of repairing UV-induced damages in human cells is best illustrate for the DNA repair deficient autosomal recessive disease, xeroderma pigmentosum (XP). At least 7 different excision repair deficient complimentation groups have been identified from XP patients (Cleaver and Kraemer, 1990). It is

hoped that by disassembling and identifying the genes involved, we can better understand the DNA repair process in mammalian systems.

Much progress has been made in recent years in understanding the genetic changes leading to XP. The Genes for XPA, XPB, and XPD have been cloned. XPA gene (XPAC) was first isolated from mouse by co-transfecting pSV2GPT plasmid and mouse genomic DNA into a XP-A cell line (Tanaka et al., 1989). The cDNA of the human counterpart (XPAC) was later identified and assigned onto chromosome 9 (Tanaka et al., 1990). Many XPA patients tested have an abnormal XPAC or absent message. In XP12RO cells, a nonsense mutation at codon 207 of the gene resulted the early termination of the mRNA and the UV-sensitive phenotype (Tanaka et al., 1990). Recent finding from our own laboratory indicates that this codon was further mutated by a substitution mutation in the revertant XP129 cell which resulted in a net amino acid change from arginine to glycine and the reversal to normal UV sensitivity as well as selective repair of (6-4) photoproducts (Cleaver et al., personal communication). XPB gene was cloned from the only known XP group B patient who also exhibits symptoms of another rare excision repair disorder, Cockayne's syndrome. The gene involved was identified as the ERCC-3 gene, a human gene complementing the CHO UV sensitive mutant group 3. In this patient, the only expressed ERCC-3 allele contained a C to A transversion in the splice acceptor sequence resulted in a 4bp insertion in the mRNA and an inactivating frame shift in the C-terminus of the protein (Weeda et al., 1990).

XPD gene was recently identified as the ERCC-2 gene by genetic complimentation (Weber and Thompson, personal communication). A DNA binding protein that is absent in some XPE patient has also been identified and shown to resemble the DNA photolyase in yeast (Chu et al., 1989).

Structurally, all genes identified encode different functions in the DNA repair pathway. XPA gene product has a zinc-finger motif suggesting a DNA binding protein (Tanaka et al., 1990). XPB/ERCC-3 gene belongs to a helicase superfamily (Weeda et al., 1990). XPD/ERCC-2 gene is highly homologous to the yeast RAD3 gene which is an ATP dependent helicase (Weber et al., 1990). The gene whose product incises UV-induced DNA photoproducts is yet to be identified. The biological functions of these genes and their involvement in the cellular repair process for removing DNA photoproducts remain to be elucidated.

Our research focused on understanding the initial induction of photoproducts in the genome and the kinetics of photoproduct removal in human skin fibroblasts having different repair capabilities. Particularly, the induction and repair of (6-4) photoproducts in the cells is the major purpose of this study. We characterized a method first described by Mitchell and Mitchell et al., (1989a, 1990a) which utilizes photoinduction and mild alkali to specifically cleave DNA at the site of (6-4) photoproduct. This method was chosen because it was shown that 1) photoinduced alkali-labile sites (PALS) were induced with the same site-specificity as (6-4)

photoproducts in UV-irradiated plasmid DNA; and 2) the repair of PALS was rapid in normal cells but deficient in XP cells as seen for (6-4) photoproducts repair measured by RIA. We showed that at nucleotide level, the photoinduced mild alkali-sensitive sites were almost identical to those induced by hot piperidine. While rare (6-4) photoproducts at TTs site were also detected by PALS method, the background was significantly reduced compared to hot piperidine treated sample.

The induction of (6-4) photoproduct in specific genes was studied using the mild alkali method. We determined that the induction of (6-4) photoproducts was about 2 per DHFR fragment while only 0.5 for each delta-globin fragment. This was in contrast to cyclobutane dimer induction which was uniform throughout the genome. Although our result remains to be confirmed by further analysis, the preferential induction of (6-4) photoproducts is consistent with many of the properties previously reported. Gale et al. (1989) showed that although (6-4) photoproducts were induced more randomly than cyclobutane dimers in the chromatin core DNA, the overall induction of (6-4) photoproducts was much higher at the linker DNA. Mitchell et al. (1990) reported that (6-4) photoproducts was induced at about 6 times as much in the linker region of the nucleosome than core regions. Pfeifer et al. (1991) observed that (6-4) photoproduct formation was more predominant in the active X chromosome than the inactive X chromosome and that the formation of (6-4) photoproducts was inhibited by protein binding of the DNA.

Structurally, the planes of the pyrimidine and pyrimidone rings of (6-4) photoproduct are separated by an angle of about 90° in DNA (Karle et al., 1969; Franklin et al., 1985; Taylor et al., 1988a). Thus, the formation of (6-4) photoproducts in regions where DNA molecules are tightly packed with low degrees of freedom could be limited because significant distortion of the DNA structure was not possible. Therefore, it is conceivable that (6-4) photoproducts be primarily induced in actively transcribed regions which are generally more accessible and their formation are inhibited in regions tightly bond by proteins.

The large distortions induced in DNA as a result of (6-4) photoproduct formation also provides an explanation for their higher mutagenic effects than the more abundant cyclobutane dimers in *E. coli*. For a (6-4) photoproduct, the 5' pyrimidine ring of the (6-4) photoproduct may still form a Watson-Crick base pair with its complementary base, but the 3' pyrimidone ring can no longer pair with its complementary base (Rycyna and Alderfer, 1985). Thus, unlike cyclobutane dimers whose mutagenic effects can sometimes be suppressed by the innate properties of DNA polymerase which preferentially inserts an adenine when the complementary base is missing; (6-4) photoproducts are primarily formed at TC and CC sites, an A inserted opposite to an C would results in mutation.

Biologically, the differential induction of (6-4) photoproducts can also explain the findings that they are removed much earlier than the cyclobutane dimers from

the over all genome, because they are induced at regions more readily accessible to the cellular repair enzymes. The higher cytotoxicity observed for (6-4) photoproducts can also be better comprehended by their preferential induction at the essential regions of the genome.

We further determined the removal of (6-4) photoproducts in repair deficient XP12RO cells and its revertant XP129 cells. XP129 cells were derived from XP12RO cells by chemical mutagenesis and low fluency UV irradiation (Cleaver et al., 1988). Phenotypically, XP129 cells have near normal UV sensitivity but show little repair in the overall genome. In our hands, the repair of cyclobutane dimers in the DHFR region was consistently lower than its repair at the same region in normal GM637 cells. However, our preliminary result showed that the removal of (6-4) photoproducts was over 70% 6 hr after UV irradiation as compared to undetectable repair in its parental XP12RO cells. This effective repair of (6-4) photoproducts but not cyclobutane dimers is in close agreement with the measurement obtained by RIA analysis (Cleaver, 1988; Mitchell et al., 1988). Similar selective repair have also been demonstrated for another XP revertant XRS70 cells and a revertant of Chinese hamster ovary UV-sensitive cell line, CHO61 cells (Thompson et al., 1989). Our results indicated that PALS method can be used for sensitive measurement of the induction and repair of (6-4) photoproducts in single copy genes.

The biological importance of (6-4) photoproducts in mammalian systems are observed by their cell killing effects after UV irradiation. In mammalian systems, the mechanism of action for (6-4) photoproducts is still unclear. Our report on the preferential induction of (6-4) photoproducts in the genome may have provided a clue as to the cytotoxic effect of UV irradiation in mammalian cells. The clustering of these photoproducts at essential regions of the genome makes them more potent lesions with significant biological consequences and also explains their rapid removal from the genome. It will be necessary to establish that changing chromatin structure alters the preferential induction of (6-4) photoproducts in UV-irradiated DNA. Further research is also required in order to present a more complete view of the general state of (6-4) photoproduct induction throughout the genome. Nevertheless, the mild alkali treatment method used in this dissertation for determining (6-4) photoproducts in specific genes should lead to a better understanding of their induction and repair in mammalian cell genome.

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