THE ROLE OF ENDONUCLEASE III, ENDONUCLEASE IV, AND EXONUCLEASE III IN THE PROTECTION AGAINST NEAR-UV MEDIATED DNA DAMAGE IN *ESCHERICHIA COLI*

By

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A Thesis submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE
(Biology)

McMaster University,
Hamilton, Ontario

TITLE: **The role of endonuclease III, endonuclease IV, and exonuclease III in the protection against near-UV mediated DNA damage in *Escherichia coli***

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NO. OF PAGES: **xi, 137**

ABSTRACT

The largest single risk factor for the development of skin cancer is exposure to ultraviolet light. Speculation that increasing levels of ultraviolet (UV) radiation might be reaching the Earth's surface began following observations of ozone depletion in the Earth's stratosphere. This concern has been substantiated by studies showing that increases in UVB irradiation (290 nm < λ < 320 nm) are correlated with ozone depletion. The cause and effect relationship between UVB induced dipyrimidine photoproducts and tumor formation is well established. However, less is known about the effects of longer wavelengths on DNA repair. The effects of near-UV (UVA: 320 nm < λ < 400 nm) are complicated by the fact that these wavelengths are absorbed by photosensitizers, which can indirectly damage DNA through the production of reactive oxygen species (ROS). When studying complex biological phenomena, it is sometimes advantageous to use a relatively simple organism. It is for this reason that much of our present knowledge on the subject of DNA repair has come from studies using the enteric bacterium *Escherichia coli* as a model.

E. coli enzymes known to be important in the repair of oxidative DNA damage may play a role in protecting the cell from the lethal effects of near-UV. Isogenic strains deficient in one or more of exonuclease III (*xthA*), endonuclease IV (*nfo*), and endonuclease III (*nth*) were exposed to increasing fluences of far-UV and near-UV to evaluate the contribution of these enzymes to the repair of near-UV mediated DNA damage. A far-UV dose of 50 J/m^2 and a near-UV dose of 400 kJ/m^2 were found to be equally lethal, reducing survival of the wildtype strain to similar levels (approximately

10%). All strains, with the exception of the *nth* single mutant, were hypersensitive to the lethal effects of near-UV. Double mutants were no more sensitive to near-UV mediated lethality than single mutants. However, a triple mutant strain (*nth nfo xthA*) exhibited the greatest sensitivity to the lethal effects of near-UV compared to wildtype. DNA repair mutants lacking both exonuclease III and endonuclease IV (*nfo xthA* and *nth nfo xthA*) were hypersensitive to the mutagenic and lethal effects of far-UV. Endonuclease IV and exonuclease III were required for the protection against near-UV and far-UV. Endonuclease III appears to be specific for the protection against near-UV mediated DNA damage, and we therefore propose a previously unrecognized role for this enzyme.

ACKNOWLEDGEMENTS

I would like to thank Dr. H. E. Schellhorn for allowing me the opportunity to study under his supervision. For many helpful discussions, I would like to thank Dr. G. J. Sorger, Dr. R. A. Morton, as well as my co-workers, Dr. S. Mukhopadhyay, Jon Audia, and Lily Chang.

I am especially indebted to Claire Palmer for her help, support, and constructive criticism. Thanks for being a truly great friend.

I am thankful for the unconditional love and support of my parents, and therefore, I would like to dedicate this thesis to my mom and dad.

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List of Abbreviations

AP	apurinic/apyrimidinic
C^^C	cytosine-cytosine cyclobutane pyrimidine dimer
CIE	Commision International de l'Eclairage
CS	Cockayne syndrome
DMSO	dimethyl sulfoxide
8oxoG	8-oxo-7,8 -dihydro-2'deoxyguanine (or 8-hydroxyguanine)
FapyAde	4,6-diamino-5-formamidopyrimidine
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
far-UV	far ultraviolet radiation
5-OHdC	5-hydroxy-2'-deoxycytidine
5-OHdU	5-hydroxy-2'-deoxyuridine
LB	Luria-Bertani
MMS	methylmethaneosulfonate
near-UV	near ultraviolet radiation
ROS	reactive oxygen species
(6-4) photoproduct	pyrimidine-pyrimidone (6-4) photoproduct
SCC	squamous cell carcinoma of the skin
$t_{1/2}$	half-life
ts	temperature sensitive
UVA	320 nm < λ < 400 nm
UVB	290 nm < λ < 320 nm
UVC	λ < 290 nm
WT	wildtype

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CHAPTER 1. LITERATURE REVIEW

1.1 Overview

Solar ultraviolet radiation is comprised of three parts. UVC, or far-UV, is the designation for wavelengths between 100 nm and 290 nm. Longer wavelengths are classified as either UVB (290 nm < λ < 320 nm) or UVA (320 nm < λ < 400 nm), which are collectively referred to as near-UV. Since far-UV is effectively filtered out by stratospheric ozone, wavelengths in the near-UV range are more biologically relevant. Recent declines in the levels of protective ozone, which are attributable to the use of fossil fuels and chlorofluorocarbon containing products, have resulted in an increase in surface near-UV. In Toronto, Canada, seasonal increases in UVB radiation as high as 35% were observed between 1989 and 1993, and correlate well with a decline in total ozone over the same period (Kerr and McElroy, 1993). The UV component of sunlight is the major risk factor in the development of squamous cell carcinoma of the skin (SCC) (Brash, 1991), and therefore, it has been predicted that further increases in exposure to near-UV will result in elevated levels of skin cancer (Doll and Peto, 1981). The carcinogenic potential of sunlight is due to the genotoxic effect of near-UV (Brash *et al.*, 1991). Near-UV is similar to far-UV, since it can effectively induce the formation of both cyclobutane

pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts (Matsunaga *et al.*, 1991). However, near-UV is distinct from far-UV in that a significant portion of DNA damage induced by the former is oxygen dependent. Near-UV has previously been shown to generate lesions more characteristic of oxidative DNA damage (Hattori-Nakakuki *et al.*, 1994). Enzymes important in the repair of far-UV and oxidative damage may also be important in the repair of damage caused by near-UV. UvrABC excinuclease and formamidopyrimidine DNA glycosylase have previously been shown to have a role in the protection of *Eshcherichia coli* cells from the lethal effects of near-UV (Shennan *et al.*, 1996).

1.2 DNA Damage

1.2.1 Far-UV mediated DNA damage

Far-UV photons are absorbed directly by DNA (λ_{max} = 260 nm) The action spectra for mutagenesis and lethality peaks at approximately 254 nm, which is the wavelength of common laboratory germicidal lamps. Far-UV radiation of DNA typically results in the formation of pyrimidine dimers (Setlow, 1966), (6-4) pyrimidine-pyrimidone photoproducts (Brash and Haseltine, 1982), and monomeric base damage, such as hydrated pyrimidines (Demple and Linn, 1982) and fragmented purines (Doetsch *et al.*, 1995) (Figure 1).

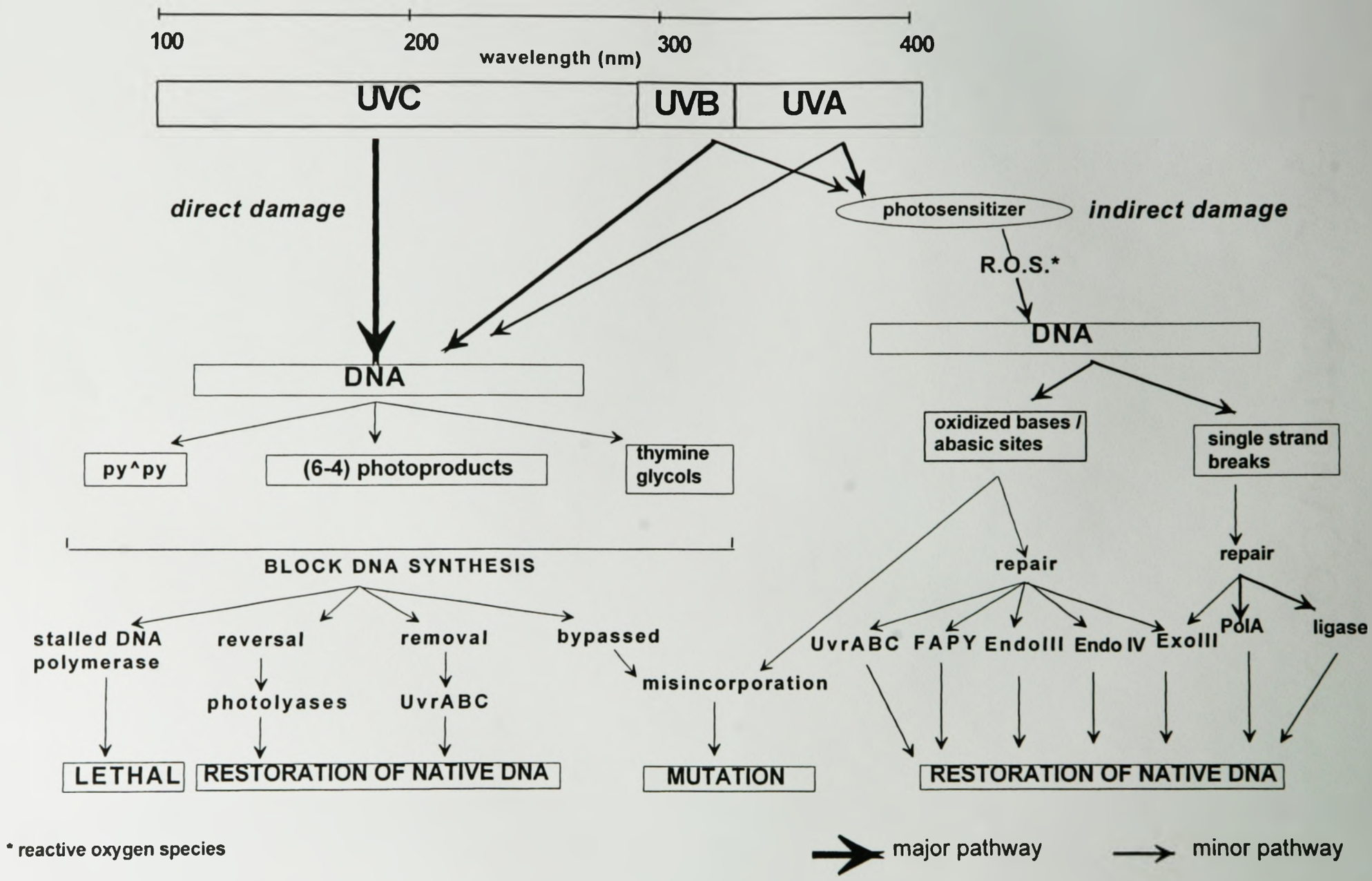


Figure 1. Overview of DNA damage caused by ultraviolet radiation (adapted from Palmer, 1996)

1.2.1.1 Cyclobutane Pyrimidine dimers

One of the major types of UV photoproducts is the pyrimidine dimer. Dimerization of adjacent pyrimidines occurs through the saturation of the C_5 -C_6 double bonds resulting in the formation of a cyclobutane ring (Wacker *et al.*, 1964; Setlow, 1966; Friedberg *et al.*, 1995). Twelve possible isomers of pyrimidine dimers exist, but the most common is the *cys-syn* conformation (Wacker *et al.*, 1964).

Pyrimidine dimers can be removed by photoreactivation or by the nucleotide excision repair pathway. Photoreactivation is the splitting of dimers by photolyase, which is activated in the presence of near-UV and visible light, during or after a far-UV exposure (Jagger, 1983). The formation of pyrimidine dimers can be measured using a bacteriophage T4 pyrimidine dimer (PD) DNA glycosylase / AP endonuclease assay (Gordon and Haseltine, 1982). By radiolabelling the 5' end of a DNA substrate that has been irradiated, DNA dimers can be measured by the addition of the PD DNA glycosylase. This enzyme forms single strand breaks at the 3' pyrimidine of a dimer, which can be visualized by denaturing polyacrylamide gel electrophoresis (Gordon and Haseltine, 1982). This method was used to demonstrate the inability of cells from Xeroderma pigmentosum (XP) patients to repair pyrimidine dimers (Setlow, 1969). Individuals suffering from XP are prone to developing skin cancer, illustrating the mutagenic and carcinogenic potential of this type of photoproduct. Phage lacking the PD DNA glycosylase (*denV*$^-$) are insensitive to non-pyrimidine dimer damage, indicating the high specificity of this enzyme for cyclobutane pyrimidine dimers (Friedberg *et al.*, 1995). This enzyme is particular to

T4 bacteriophage and *Micrococcus luteus* (Friedberg *et al.*, 1995); however, *E. coli* has evolved a more generalized repair mechanism in the nucleotide excision repair system that recognizes pyrimidine dimers as well as a wide variety of other lesions (Friedberg *et al.*, 1995).

Like *E. coli*, mammalian cells also repair pyrimidine dimers through nucleotide excision repair. The importance of pyrimidine dimer repair is illustrated by the human disorders Xeroderma pigmentosum and (XP) Cockayne's syndrome (CS). XP patients lack NER, and have a genetic predisposition to the formation of skin cancer (Tornaletti and Pfeiffer, 1996). There are seven complementation groups to which an affected individual may belong: XPA, XPB, XPC, XPD, XPE, XPF, and XPG (Tornaletti and Pfeiffer, 1996). Lymphoblast cell lines from patients belonging to A,C,D, and E complementation groups exhibit an inability to repair UVB-induced pyrimidine dimers compared to cells from normal donors (Runger *et al.*, 1995). The presence of UV-induced pyrimidine dimers is suggested by the predominance of GC–>AT transitions in fibroblasts from XPA and XPF patients exposed to UV light (Yagi *et al.*, 1991). The C to T transition is the defining mutational event of UV irradiation, since it is the predominant base change that occurs in cells following exposure to UV (Brash *et al.*, 1991). Since C to T transitions occur most frequently at dipyrimidine sites when cells are exposed to UV light, dipyrimidine photoproducts are the most common pre-mutagenic lesions (Armstrong and Kunz, 1990; Brash *et al.*, 1991). Analysis of the p53 tumour supressor gene in squamous cell carcinoma of the skin (SCC) reveals a mutational spectrum that

includes C --> T transitions (62%), CC --> TT transitions (23%) and C --> A transversions (31%) (Brash *et al.*, 1991). The high proportion of C --> T transitions identifies UV light as the carcinogen, and in one tumour (SI 13), the pre-mutagenic lesion was inferred to be a cyclobutane pyrimidine dimer (Brash *et al.*, 1991).

CS is a neurological disorder that results in developmental abnormalities in affected patients. The mechanistic defect in CS patients' cells appears to be the lack preferential repair of actively transcribed genes. In the active adenosine deaminase gene, cells from CS individuals repair pyrimidine dimers with less efficiency than normal control cell lines (Venema *et al.*, 1990). However there is no difference in repair of the inactive dihydrofolate reductase gene between normal and CS individuals (Venema *et al.*, 1990).

The study of pyrimidine dimers as pre-mutagenic lesions in bacteria is complicated by the ability of dimers to induce the error-prone (SOS) repair pathway (Friedberg *et al.*, 1995). The SOS system depends on functional *lexA* and *recA* gene products (Defais *et al.*, 1971) and can cause mutations by the induction of the *umuDC* operon (see below). Nevertheless, pyrimidine dimers have been shown to cause mutations independent of SOS induction by conjugation experiments (Kunz and Glickman, 1984). Irradiated strains carrying the *lacI* gene on an episome were mated into SOS-induced host cells. In this system, mutations at the site of DNA damage (targeted) can be assessed independently of mutations fixed as a result of the UV-induced SOS system (untargeted). Firstly, *recA* dependent recombinational repair can be ruled out in this system, because the transferred F' DNA to a recipient is not a substrate for *recA*, since the F- strain lacks homologous

DNA (*Δlac*). Secondly, the UvrABC complex is not involved in this system, because the F' is transmitted as a single strand, and therefore, UV-induced lesions can not be removed by NER (Kunz and Glickman, 1984). When the episome was transferred to an SOS-induced cell, 95 % of the mutations occurred at potential TC and CT sites, suggesting that the pre-mutagenic lesions were mainly pyrimidine dimers. When the F' strain was exposed to photoreactivating light prior to conjugation, mutations in the transconjugant were reduced by 92 %, which strongly suggests that pyrimidine dimers are the main pre-mutagenic lesion caused by UV in this system (Kunz and Glickman, 1984).

The mutagenic potential of pyrimidine dimers is dependent on both base composition and stereochemistry. The use of single stranded phage possessing different pyrimidine dimers in different conformations has revealed a great deal of information on the mutagenic ability of these lesions. *Cis-syn* T^^T pyrimidine dimers efficiently block DNA polymerase (Banjeree *et al.*, 1988). This was demonstrated using UV-irradiated single stranded M13 phage. When transfected into an *E. coli uvrA* mutant, these phage produce little or no progeny (Banjeree, *et al.*, 1988). However, transfection of single stranded M13 phage into an SOS induced *uvrA* mutant results in an increase in the number of phage progeny by as much as one-hundred fold, indicating that trans-lesion synthesis can bypass otherwise lethal lesions (Friedberg *et al.*, 1995). Mutations occur in 7 percent of phage progeny transfected into SOS-induced cells. Interestingly, all mutations occurred at the 3' thymine of the *cis-syn* T^^T pyrimidine dimer (Banjeree *et al.*, 1988). The mutational spectrum of a *trans-syn* T^^T pyrimidine dimer differs from that of the *cis-syn*

form. Although mutagenesis is similarly low for both conformations, in contrast to the *cis-syn* T^^T dimers, the *trans-cyn* isoform results in mutations exclusively at the 5' thymine (Banjeree *et al.*, 1990).

A comparison of the mutagenic properties of *cys-syn* and *trans-syn* T^^T pyrimidine dimers in *E. coli* and *Saccharomyces cerevisiae* was conducted using a single stranded shuttle vector harbouring these lesions. Replicative bypass of the *cys-syn* T^^T dimer occurred in 16% of the vector molecules when transformed into SOS-induced *E. coli* cells, which is substantially less than the 80% lesion bypass observed in *S. cerevisiae*. Replication past the lesion in *S. cerevisiae* results in fixation of mutations at a rate of 0.4%, while translesion synthesis in *E. coli* was less accurate, resulting a 7.2% mutation rate for bypass events (Gibbs *et al.*, 1993). When the vector harboured a *trans-cyn* T^^T dimer, replicative bypass was equivalent in both organisms, however, the *E. coli* host repaired these lesions with greater accuracy than *S. cerevisiae*. Therefore, the mutagenic nature of this pyrimidine depends on conformation as well as the nature of DNA repair proteins which differs for the two organisms (Gibbs *et al.*, 1993). Although homologous repair processes may exist in *E. coli* and eukaryotes, yeast is likely a better model for mutagenic studies, since many of the genes involved in *S. cerevisiae* NER have homologs in humans (see below).

UV-induced mutagenesis has been demonstrated in mammalian cells using the plasmid shuttle vector pZ189 (Protic-Sabljic *et al.*, 1986). Transfection of UV exposed vector DNA into monkey cells results in a decrease in plasmid replication and an increase

in mutagenesis. When the vector is treated with photoreactivating light prior to transfection, there is a significant increase in viability as well as a marked decrease in mutagenesis, implicating pyrimidine dimers as the pre-mutagenic UV photoproduct (Protic-Sabljic *et al.*, 1986).

Results from experiments using viral genomes as the UV target should be interpreted with caution, since normal replication is much faster in viruses than in higher organisms. It is likely that C^^C pyrimidine dimers are more mutagenic in mammals where longer life cycles are more likely to result in deamination of cytosine (Lawrence *et al.*, 1993; Barak *et al.*, 1995). When cytosine is deaminated, the resulting uracil residue base pairs with adenine, resulting in a C to T transition. Deamination of cytosine occurs frequently at C^^C pyrimidine dimers ($t_{1/2}$ = 2 years), while the rate of deamination of cytosine monomers is negligible ($t_{1/2}$ = 30 000 years)(Barak *et al.*, 1995). However, the half-life of C^^C pyrimidine dimers in *E. coli* is still longer than the normal rate of mutation fixation in this organism (~30 minutes), suggesting that deamination of cytosines may only be relevant in slower replicating organisms (Barak *et al.*, 1995).

1.2.1.2 Pyrimidine-pyrimidone (6-4) photoproducts

Another major UV-induced lesion is the pyrimidine-pyrimidone (6-4) photoproduct, which can be more mutagenic than pyrimidine dimers. In *E. coli*, (6-4) photoproducts are the principal pre-mutagenic lesion (LeClerc *et al.*, 1991; Horsfall and Lawrence, 1994), while mutagenesis in mammalian cells is effected primarily by pyrimidine dimers (Brash,

1988). The linkage of the 5' C_6 to the 3' C_4 of adjacent bases in a (6-4) photoproduct severely distorts the DNA helix, and blocks progression of DNA polymerase . Mass spectroscopy and proton nuclear magnetic resonance have demonstrated that the 3' cytosine in a T (6-4) C photoproduct is bonded 90 degrees to the plane of the 5' thymine (Franklin *et al.*, 1985). The predominant types of (6-4) photoproducts resulting from UV irradiation are CC and TC. The TT adduct occurs less frequently, and the CT adduct does not form at all. It is likely that the presence of the C_5 methyl group in the 3' thymine is less conducive to the formation of the necessary C_6-C_4 bond (Brash and Haseltine, 1982; Pfeiffer *et al.*, 1991)

The ability of (6-4) photoproducts to function as pre-mutagenic lesions was established when single stranded M13-based vectors harbouring this lesion were transformed into *uvrA*$^-$ *E. coli* cells. In the absence of SOS induction, vectors containing the T(6-4)T photoproduct were rarely replicated, yielding few M13 progeny. However, when the SOS system was induced, the number of progeny phage increased to approximately ten fold that of the uninduced host, with mutagenesis occurring in over 90% of surviving phage (LeClerc *et al.*, 1991). All mutations were 3' T --> C transitions, which suggests the formation of a stable T:G mispair at the 3' pyrimidine in this isomer. The Dewar isomer of the T (6-4) T photoproduct was found to be less mutagenic in SOS induced *E. coli* cells than the normal isomer. Approximately 50% of surviving phage carried mutations, of which, only half were T --> C transitions. The lack of specificity of the Dewar isomer is probably due to the reduced efficiency of the pyrimidone to form

hydrogen bonds with guanine (LeClerc *et al.*, 1991).

The mutagenic spectrum of a T(6-4)C photoproduct in its normal and Dewar configuration was studied using a single stranded M13mp7-based vector. Both adducts efficiently block DNA polymerase, since the proportion of surviving phage in SOS-uninduced cells is below 1 % of the level for the lesion free vector. Transfection of the vector containing the normal T (6-4) C adduct into SOS-induced *E. coli uvrA* mutants resulted in mutation rates well below that observed for the T (6-4) T adduct (Horsfall and Lawrence, 1994). Mutagenic bypass occurred in about one third of the surviving phage carrying the T (6-4) C adduct, of which, 80 % were C --> T transitions (Horsfall and Lawrence, 1994). The T (6-4) C Dewar isomer was more mutagenic, but less specific for C-->T transitions than that of the normal isomer.

1.2.1.3 Monoadduct base damage

Damage to individual pyrimidines by 254 nm radiation can result in the formation of cytosine hydrate or thymine glycol (reviewed in Friedberg *et al.*, 1995). Cytosine hydrate is formed by the saturation of the C_5-C_6 double bond, and can exist stably in DNA. This adduct can cause mutations through a dehydration reaction to produce uracil, which has the base pairing properties of thymine (Boorstein *et al.*, 1990). In addition, the methylated form of cytosine hydrate can become deaminated to form thymine hydrate, which can be subsequently dehydrated to form thymine (Vairapandi and Duker, 1994). Thus, cytosine hydrate can result in GC to AT transitions if not removed. Thymine glycols are produced

when exposed to oxidants and ionizing radiation (Basu *et al.*, 1989; see below). This lesion is also produced by far-UV irradiation (Demple and Linn, 1982). In contrast to cytosine hydrate, thymine glycols are generally not mutagenic (Evans *et al.*, 1993). Thymine glycols block the progression of DNA polymerase *in vitro* (Clark and Beardsley, 1986; Evans *et al.*, 1993) and *in vivo* (Evans *et al.*, 1993; Moran and Wallace, 1985) and are therefore lethal if not removed.

Irradiation of DNA in aqueous solution at 254 nm results in the production of the fragmented purines: 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), as well as 5-hydroxy-5,6-dihydrothymine (Doetsch *et al.*, 1995). The addition of the ROS scavenger dimethyl sulfoxide (DMSO) had no effect on the yield of any of these lesions, which eliminates the possibility of a confounding oxidative mechanism for their production. These data indicate that damage to individual bases occurs as a result of far-UV irradiation, which may contribute to the mutagenic potential of far-UV (Doetsch *et al.*, 1995).

1.2.2 Oxidative DNA damage

DNA is an important target for oxidative damage in aerobic organisms. The production of reactive oxygen species (ROS) occurs as a result of successive reductions of molecular oxygen. Products of this electron transfer include singlet oxygen, hydroxyl radicals, hydrogen peroxide, and superoxide ions (Imlay and Linn, 1988; McBride *et al.*, 1991). Intracellularly, the largest source of ROS is the partial reduction of oxygen to

water within the electron transport chain. ROS can be generated extracellularly by near-UV, ionizing radiation, or redox cycling compounds such as paraquat.

Singlet oxygen and hydroxyl radicals are both capable of damaging DNA directly. The former attacks specifically at guanine residues (Sies and Menck, 1992), while the latter can attack all bases as well as the phosphoribosyl backbone of DNA. Superoxide ion and hydrogen peroxide have low oxidative potentials, but can lead to the production of the more reactive hydroxyl radicals through iron-catalyzed Fenton reactions. Firstly, superoxide ion causes the release of iron (II) from [4Fe-4S] clusters. Subsequently, free iron (II) can then be oxidized to iron (III) by hydrogen peroxide in a Fenton reaction resulting in the generation of hydroxyl radicals (Imlay and Linn 1988; Liochev and Fridovich 1994; Henle and Linn, 1997) as shown here:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + \cdot OH + OH^-$$

The major types of oxidative damage include: single strand breaks, which are caused by hydroxyl radicals as well as singlet oxygen (Friedberg *et al.*, 1995, Sies and Menck 1992); apurinic/apyrimidinic sites, which can result from ROS mediated proton abstraction from a DNA deoxyribose moiety; and various forms of monomeric base damage. Damage to particular bases by hydroxyl radical attack can take the form of ring fragmented pyrimidines such as 4,6-diamino- 5-formamidopyrimidine (FapyAde) or 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Hydroxyl radical attack on a thymine C_5-C_6 double bond results in the formation of a thymine glycol, which is predominantly lethal (Hariharen *et al.*, 1977, Moran and Wallace, 1985, Hayes *et al.*, 1988)., but can mispair

with guanine resulting in T to C transitions (Basu *et al.*, 1989). Since thymine glycols are a substrate for endonuclease III, mutants lacking this enzyme may have an elevated level of T to C transitions if these lesions are generated by near-UV. Increases in T to C mutations for phage DNA exposed to ionizing radiation (produces thymine glycols) were found to be independent of a mutation which inactivates the endonuclease III gene *nth* (Basu *et al.*, 1989); however, this may be due to overlapping substrate specificity by other repair enzymes. Therefore, the mutational spectrum of an *nth* mutant strain would have to be examined in combination with other DNA repair enzyme mutants.

Singlet oxygen reacts preferentially with guanine residues, resulting in the oxidation products FapyGua and 8-hydroxydeoxyguanosine (8oxoG) (Sies and Menck, 1992). Fapy lesions can block DNA polymerase if not removed, while 8oxoG lesions are mutagenic since they can pair equally well with adenine as with cytosine (Michaels *et al.*, 1992a). Since 8oxoG lesions are removed by Fapy-DNA glycosylase, mutants lacking this enzyme have an elevated rate of G to T transversions when exposed to near-UV (Palmer *et al.*, 1997).

Oxidation products of cytosine include cytosine hydrate, 5-hydroxy-2'-deoxycytidine (5-OHdC) and 5-hydroxy-2'-deoxyuridine (5-OHdU) (Purmal *et al.*, 1994; Hatahet, 1994). Misincorporation of an adenine opposite the oxidized cytosine results in C to T transitions. Since these oxidized pyrimidines are all substrates for endonuclease III, *nth* mutants would be expected to have an elevated level of C to T transitions when exposed to near-UV. Oxidative attack on DNA can also lead to CC to TT tandem transitions,

which is normally considered a signature far-UV mutation (Reid and Loeb, 1993; Brash *et al.*, 1991).

Changes in cytosine methylation patterns have been observed in carcinogenesis. It has been suggested that the oxygen radicals might interfere with methylation patterns of DNA. Oligonucleotides containing 8oxoG instead of guanine result in an altered methylation pattern in adjacent cytosines. Therefore, it is possible that reactive oxygen species may be responsible for changes in methylation patterns observed during carcinogenesis (Weitzman *et al.*, 1994).

1.2.3 Near-UV induced DNA damage

1.2.3.1 The subclasses of near-UV: UVA and UVB

The current definition of UVB set by the Commission International de l'Eclairage (CIE) (1935) is a range of 280 nm - 315 nm (Peak and van der Leun, 1993). The subject of a recent workshop at the 11th International Congress on Photobiology was the wavelength boundary between UVA and UVB (Peak and van der Leun, 1993) . There is a general consensus that the lower boundary is 290 nm, since none of these photons are present in the solar spectrum reaching earth. It has been suggested that the boundary should be based on the action of different photons on DNA (Peak and van der Leun, 1993). DNA has an absorption maximum of approximately 260 nm (Setlow, 1974), therefore, short wavelength photons interact directly with DNA, causing the formation of cyclobutane pyrimidine dimers, (6-4) pyrimidine-pyrimidone photoproducts, and other

monoadduct changes. Longer wavelengths interact with DNA indirectly after absorption by a photosensitizing molecule (as reviewed in Eisenstark, 1989). Peak *et al.* (1984) have found that there is a close correspondence between DNA absorption and the measurement of a biological endpoint (the formation of pyrimidine dimers) between 254 nm and 320 nm. Mutagenicity over this range, as measured by a reverse mutation assay, was found to be constant relative to lethality (Peak *et al.*, 1984). However, at wavelengths above 320 nm, the ratio of mutagenesis to lethality begins to diminish. Oxygen dependence at a wavelength of 330 nm was demonstrated by Peak *et al.* (1983), and implicates the involvement of an endogenous photosensitizer in near-UV mediated lethality. The oxygen enhancement ratio (OER) is the ratio of the fluence required to inactivate cells to 37% survival aerobically to the fluence which produces the same lethality under anaerobic conditions, and has been calculated for a near-UV sensitive strain (*nur*) and a near-UV resistant strain (*nur*$^+$) (Webb and Brown, 1979). The effect of oxygen on lethality in both strains was observed as an increase in the OER at wavelengths above 313 nm, confirming earlier data implicating oxygen in near-UV mediated lethality (Webb and Brown, 1979; Webb and Lorenz, 1970).

1.2.3.2 The role of stray UVB wavelengths in studies of UVA mediated DNA damage

The extent of contamination from wavelengths between 300 nm and 320 nm is an important consideration for studies of UVA mediated DNA damage. There exists numerous brands of filters which transmit wavelengths of light below 320 nm. The extent

to which they transmit these wavelengths depends on the thickness of the glass and the concentration of colourants used in the manufacturing process (Dobrowolski *et al.*, 1977). The contamination of short wavelengths has been demonstrated to be filter-dependent when monochromatic 365 nm radiation is used (Webb and Peak, 1981). The use of Corning 0-53 filter resulted in three and six fold reductions in survival for WT and *uvrA* mutant strains relative to the levels obtained with a Corning 0-52 filter over the same dose range. The Corning 0-52 filter removes wavelengths of light below 330 nm, while the Corning 0-53 filter transmits a larger percentage of these wavelengths (Webb and Peak, 1981). The Corning 0-53 filter has similar spectral properties as that of a Mylar filter used by Harm (1978), accounting for the 10 % higher sensitivity observed with this filter (Harm, 1978) compared with that obtained by others using the same strains (Tyrrell, 1973; Webb and Brown, 1976, Webb and Peak, 1981).

Confounding effects from stray light in the UVB range can be ruled out by employing more stringent filter types or by increasing the thickness of short wave blocking filters. Webb and Brown (1976) exposed DNA repair mutants to a 365 nm monochromatic light source with a Corning 7-51 filter, which transmits less than 10 % of 310 nm wavelengths. *E. coli* strains deficient in excision repair (*uvrA*) and recombination (*recA*) were compared with a repair proficient wildtype strain. All strains were equally sensitive to this light source when passed through more stringent filters such as Baird-Atomic RD/UV (<1 % of λ = 350 nm) or Corning 0-52 (single or double thickness) Therefore, the possibility of short wavelength light contributing to the observed lethality at 365 nm could be eliminated

(Webb and Brown, 1976). At 365 nm, both *uvrA* and *recA* mutations contributed to greater lethality than that of WT, which implicates a role for both nucleotide excision repair and recombination in the repair of UVA mediated DNA damage (Webb and Brown, 1976). Photoreactivation was prevented by irradiating cultures at 4 °C, which suggests that pyrimidine dimers may have caused an increased lethality in the DNA repair deficient mutants during the UVA exposure. This is not surprising in light of the observation by Matsunaga *et al.* (1991), who demonstrated that UVA irradiation of DNA produces pyrimidine dimers, albeit with less efficiency than shorter wavelengths.

1.2.3.3 Direct DNA damage by near-UV

The absorption maximum of DNA is approximately 260 nm, which explains the peak in pyrimidine dimer induction at this wavelength (Setlow, 1974; Peak *et al.*, 1984). (6-4) photoproducts have a similar action spectrum to pyrimidine dimers within the far-UV wavelength range of 240 nm to 280 nm (Chan *et al.*, 1986). The action spectrum for (6-4) photoproducts was determined by treating DNA fragments with hot alkali, which causes cleavage of the 3' base and subsequent β-elimination (Franklin *et al.*, 1985). The result is a strand break at the position of the (6-4) photoproduct (Mitchell and Nairn, 1989). Pyrimidine dimers and (6-4) photoproducts are also formed following near-UV exposure, demonstrating a fluence-dependent induction for all wavelengths between 150 nm and 360 nm (Matsunaga *et al.*, 1991). Pyrimidine dimers and (6-4) photoproducts were measured by the authors using lesion specific antibodies in a competitive enzyme-linked

immunosorbent assay (ELISA). Action spectra for pyrimidine dimers and (6-4) photoproducts were determined by percent inhibition; that is, the ability of the antibodies to bind a competing irradiated DNA molecule (Matsunaga *et al.*, 1991). Both lesions yielded similar action spectra over the range of fluences tested, exhibiting equivalent maxima at 260 nm. The only difference in the observed spectra occurred at wavelengths greater than 313 nm. The action spectrum value for pyrimidine dimers was approximately 1.4 fold higher than that of (6-4) photoproducts, which was attributed to photoisomerization of the latter to the Dewar (6-4) configuration (Matsunaga *et al.*, 1991)

1.2.3.4 Indirect DNA damage by near-UV

Although DNA absorbs maximally at 260 nm, there are numerous non-nucleic acid components of cells which have absorption maxima at higher wavelengths in the near-UV range. (Eisenstark, 1989). Evidence for the involvement of a sensitizing chromophore comes from numerous studies on DNA damage caused by various UV wavelengths. All of these studies indicate a change in slope around 330 nm in the action spectra for lethality, mutagenesis, and dimer induction, which suggests a different mode of DNA damage is occurring for near-UV wavelengths (Webb and Lorenz, 1970; Setlow, 1974; Peak *et al.*, 1983; Peak *et al.*, 1984; Setlow *et al.*, 1993). The action spectrum is determined by the number of lethal or mutagenic events per quantum, and is often displayed graphically as a function of wavelength (Setlow *et al.*, 1993). Action spectra for mutagenesis, dimer induction, and lethality peak at 254 nm, and demonstrate a constant

decline in events per quantum on a log scale for wavelengths up to 330 nm (Peak *et al.*, 1984).

A chromophore is a molecule which causes a biological effect after absorbing light (Jagger, 1983). Many non-nucleic acid molecules have been implicated as chromophores for near-UV mediated DNA damage based on their absorption spectra and critical function in cells (Jagger, 1983). Some of the possible endogenous chromophores include 4-thiouridine (λ = 330 nm), and members of the respiratory chain: for example, NADH (λ_{lmax} = 340 nm), porphyrins (λ_{lmax} = 380 nm), riboflavin, (λ = 375 nm) and menaquinone (λ = 330 nm) (Jagger, 1983; Eisenstark, 1989).

DNA damage following absorption by a near-UV photosensitizing molecule is likely to occur through the production of reactive oxygen species. The involvement of oxygen in near-UV mediated damage was postulated based on observations of increased sensitivity of DNA repair mutants when irradiated at wavelengths above 320 nm in the presence of oxygen (Webb and Lorenz, 1970; Webb and Brown, 1979; Peak *et al.* 1983). Enzymes important in the repair of oxidative damage have been demonstrated to be important in the repair of near-UV mediated DNA damage; for example, exonuclease III (Sammartano and Tuveson, 1983) and superoxide dismutase (Hoerter *et al.*, 1989).

Hydrogen peroxide can be converted to highly reactive hydroxyl (·OH) radicals through iron-catalyzed Fenton reactions (see above). Similar responses have been observed in *E. coli* after H_2O_2 and near-UV exposures, suggesting that near-UV produces ROS. *E. coli* cells scavenge hydrogen peroxide via the action of its two catalases, HPI

(*katG*) and HPII (*katE*) (for review, see Schellhorn, 1995). Pretreatment of cells with 30 μM H_2O_2 for one hour results in a decrease in sensitivity to subsequent H_2O_2 or UVA, exposures, suggesting that similar defense enzymes protect cells from both types of stress (Tyrell, 1985). Similarly, pre-treatment of cells with UVA results in resistance to subsequent H_2O_2 exposures (Tyrell, 1985). One explanation proposed by the author is that catalase levels are elevated as a result of the initial H_2O_2 treatment. The generation of H_2O_2 from near-UV irradiation is likely, since exogenous catalase added to plating media prior to a UVA exposure results in a rescuing effect (Sammartano and Tuveson, 1984; Palmer *et al.*, 1997).

Near-UV results in the production of superoxide (Ahmad, 1981), which reduces cellular iron according to the equation :

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad \text{(Henle and Linn, 1997)}$$

Ferrous iron stimulates the production of hydroxyl radicals from H_2O_2 through Fenton reactions (see above) (Imlay and Linn, 1988). Alternatively, superoxide dismuatase can convert the superoxide radical to H_2O_2, which can be damaging if not removed by the cellular catalases. When both superoxide dismutases (*sodA* and *sodB*) are inactivated in *E. coli* through mutation, cells are hypersensitive to the mutagenic effects of near-UV (Hoerter *et al.*, 1989). The accumulation of superoxide in *sod* mutants allows for the reduction of Fe^{3+} to Fe^{2+}, which can react with H_2O_2 to form DNA damaging hydroxyl radicals (Fenton reaction) (Henle and Linn, 1997). When excess endogenous catalase is

included, through a *katG* overexpressing plasmid, the mutation rate in *sodAsodB* strains is reduced to WT levels. Catalase reduces cellular H_2O_2, which results in a decrease in the intracellular level of ROS (Schellhorn and Hassan, 1988). Therefore, it is likely that the near-UV induced mutagenesis in *sod* mutants is due to Fenton mediated production of hydroxyl radicals (Hoerter *et al.*, 1989).

1.3 DNA Repair in *Escherichia coli*

1.3.1 The SOS regulon

The SOS system involves a number of operons whose member genes are induced as a result of DNA damage (for review, see Friedberg *et al.*. 1995). Prior to such an event, SOS genes are repressed by the binding of the product of the *lexA* gene. Exposure of bacterial cells to a DNA damaging agent, such as UV radiation, results in the cleavage of the LexA protein by the activated product of the *recA* gene (Horii *et al.*, 1981). The RecA protease is thought to become activated after binding to single stranded DNA and ATP (Phizicky and Roberts, 1981). Regions of single stranded DNA are formed where bulky adducts preclude DNA polymerase progression (Friedberg *et al.*, 1995). As a result of the conversion of RecA to its active form, levels of the LexA repressor decrease and are no longer available to bind to the operator regions of SOS-inducible genes. When the availability of LexA becomes low, SOS genes are derepressed. A return to their constitutive state of repression occurs following repair processes which effectively eliminate regions of single stranded DNA (Friedberg *et al.*, 1995). Two of the repair

Table 1. Properties of DNA repair proteins

Repair Enzyme	gene name	gene location[a]	Substrates and activity	structural features	sensitivity of mutants	Human homolog
Exonuclease III	*xthA*	38.5'	- 5' AP endonuclease[b] - 85% of cell's AP activity[c] - 99% of cell's 3' phosphatase function[d] - 3' exonuclease activity[e]	- a 28 kDa protein[l] - requires Mg^{2+} and inhibited by EDTA[m]	- UVA sensitive[n] - H_2O_2 sensitive[v] - alkylating agents (*eg* methyl-methanesulfonate)[w]	- significant similarity with Human AP endonuclease (HAP1)[z]
Endonuclease IV	*nfo*	48'	- 5' AP endonuclease[b] - 5% of cell's AP activity[c] - 3' phosphatase activity[d]	- a 33 kDa protein[n] - inhibited by EDTA[o]	- alkylating agents, oxidants (eg bleomycin), and gamma rays[w]	- not known
Endonuclease III	*nth*	36'	- DNA glycosylase for ring saturation (*eg* thymine glycol), and ring fragmentation (*eg* urea)[f] - AP lyase activity[b,g]	- 25 kDa[p] - no cofactors[q] - contains [4Fe-4S]$^{2+}$ cluster[r]	- not known	- human endonuclease III-like homolog (hNTH1) recognizes thymine glycols [aa]
UvrABC excinuclease	*uvrA* *uvrB* *uvrC*	92' 17' 42'	- multienzyme complex of the inducible SOS response[h] - excision nuclease[i] - UvrA, UvrB, and UvrC bind dsDNA and hydrolyze phosphodiester bonds[i] - repair of numerous substrates including pyrmidine dimers, AP sites, 6-4 photoproducts, and thymine glycols[j]	- 114 kDa (UvrA); 76 kDa (UvrB); 66 kDa (UvrC)[a,h]	- UVC sensitive[a,h]	- human nucleotide excision repair protein ERCC1 shares homology with UvrA and UvrC[bb]
Fpg glycosylase	*fpg*	81.7'	- removes ring opened purines caused by DNA damage as well as oxidized guanine (8oxoG lesions)[k]	- 31 kDa protein[s] has zinc finger domain[t]	- UVA sensitive[x] - *uvrA fpg* double mutants are sensitive to methylene blue plus visible light[y]	- a human DNA glycosylase has been isolated due to its ability to remove 8oxoG lesions[cc]

a) Friedberg *et al.*, 1995 (review); see text; **b)** Warner *et al.*, 1980 ;**c)** Weiss *et al.*, 1988; Levin *et al.*, 1988; **d)** Demple *et al.*, 1983 ;**e)** Richardson *et al.*, 1964; **f)** Breimer and Lindahl, 1984; Hatahet *et al.*, 1994; **g)** Warner *et al.*, 1980; Bailly and Verly, 1987; **h)** Van Houten, 1990; **i)** Orren and Sancar, 1989; Oh and Grossman, 1987, 1989; Sancar and Rupp, 1983; Svoboda *et al.*, 1993; Friedberg, 1995 (review); **j)** Howard-Flanders *et al.*, 1966; Lin and Sancar, 1989; **k)** Boiteux, 1993; **l)** Weiss, 1976; **m)** Gossard and Verly, 1978; Black and Cowan, 1997; **n)** Ljunqvist, 1977; **o)** Levin *et al.*, 1988; **p)** Weiss and Cunningham, 1985; **q)** Radman, 1976; Wallace, 1988; **r)** Cunningham, 1988; Krokan et al., 1997 (review); **s)** Boiteux et al., 1987; **t)** Tchou *et al.*, 1993; **u)** Demple *et al.*, 1983; Demple *et al.*, 1986; **v)** Sammartano and Tuveson, 1983; **w)** Cunningham *et al.*, 1986; **x)** Shennan *et al.*, 1996; Palmer *et al.*, 1997; **y)** Czeczot *et al.*, 1991; **z)** Robson *et al.*, 1991; **aa)** Aspinwall *et al.*, 1997; Hilbert *et al.*, 1997; **bb)** Van Duin *et al.*, 1988; **cc)** Nagashima *et al.*, 1997

pathways that are under SOS control, nucleotide excision repair and the damage inducible *umuD*$^{+}$*C*$^{+}$ operon, are discussed below.

1.3.1.1 Nucleotide Excision Repair

Excision of bulky lesions such as pyrimidine dimers, (6-4) photoproducts, or chemical cross-links is accomplished by nucleotide excision repair (NER) (Howard-Flanders *et al.*, 1966; Selby and Sancar, 1990). The NER pathway also repairs oxidative lesions such as AP sites and thymine glycols (Lin and Sancar, 1989). In *E. coli*, this process is dependent on the products of the *uvr A,B,C,*and *D* genes. Initially, cleavage of LexA results in the derepression of the *uvrA and uvrB* genes , allowing for an increase in cellular concentrations of their respective gene products (Selby and Sancar, 1990; Van Houten, 1990). The reaction mechanism begins when two molecules of UvrA join with a UvrB monomer in an ATP dependent reaction (Orren and Sancar, 1989). The resultant heterotrimer binds DNA and catalyzes unwinding through its 5' to 3' helicase activity. This translocation step is also dependent on ATP hydrolysis (Oh and Grossman, 1987, 1989). After encountering a lesion, the UvrA dimer is released and replaced by the *uvrC* gene product. The UvrBC complex cuts the DNA on both sides of the lesion (Sancar and Rupp, 1983). The incisions occur 8 nucleotides upstream and 4 (or 5) nucleotides downstream to the damage site (Orren and Sancar, 1989; Svoboda *et al.*, 1993), and the resulting 12-13 base pair oligonucleotide is released in part by the helicase activity of UvrD (Lin and Sancar, 1992). The single strand gap is then repaired by DNA polymerase

I and sealed by DNA ligase.

1.3.1.2 umuDC damage inducible operon

Bacterial strains that are unable to induce the SOS sytem, *recA (def)* or *lexA (Ind-)*, are unable to carry out SOS induced mutagenesis. However, results of experiments involving *recA (def)* or *lexA (Ind-)* mutations are complicated by the concominant inactivation of downstream targets of the *recA* and *lexA* gene products. Genes more directly involved in SOS mutagenesis were identified using a papillation assay. *E. coli his-4* mutants treated with EMS were grown on rich media, and then transferred to minimal media, and colonies which were devoid of *his-4* revertants (papillae) were potential antimutator mutants. As expected, two types of these antimutators were of the *recA (def)* or lexA *(Ind-)* type. However, a third class of non-mutable mutants was discovered, which mapped to one of either the *umuD* or *umuC* loci. The *umuD* and *umuC* mutants exhibit diminished rates of mutation induction in the presence of DNA damaging agents such as UV (Kato and Shinoura, 1977) or MMS (Walker and Dobson, 1979). In a *lexA (Ind-)* background, operator constitutive mutants of $umuD^{+}C^{+}$ are hypersensitive to the mutagenic effects of UV irradiation relative to a WT $umuD^{+}C^{+}$ strain, which confirms a role for the *umuDC* operon in active mutagenesis. Furthermore, when the level of the RecA protein was increased by a constitutive *recA* mutant (O^{c}_{98}-*recA*), no further increase in mutation rate was observed relative to the $umuD^{+}C^{+}$ strain (Sommer *et al.*, 1993). The *umuDC* operon is the only locus in the SOS regulon necessary for SOS mutagenesis

(Sommer *et al.*, 1993). Although the role of the *umuD* and *umuC* proteins in the mutagenic process remains to be determined, it is thought to involve translesion bypass. Translesion bypass is the insertion of a base by opposite a non-instructional lesion (Friedberg *et al.*, 1995). Translesion synthesis of DNA damage was originally proposed to be the mechanism responsible for the reactivation and mutagenesis of irradiated phage observed when the host cell was pre-irradiated (Radman, 1974).

1.3.2 Apurinic/Apyrimidinic Enzymes.

The cleavage of the N-glycosyl bond between the nitrogenous base and the deoxyribose of the DNA backbone leaves an apurinic/apyrimidinic (AP site). In mammalian cells, the spontaneous cleavage of a N-glycosyl bond is a frequent event. It has been estimated that up to 10^4 bases are removed per cell per day in humans (Barzilay and Hickson, 1995). In addition to spontaneous base loss, AP sites can occur as a result of exposure to chemicals and radiation. Since AP sites are noninstructional to DNA polymerase, they are lethal if not repaired. An *E. coli* strain carrying a mutation in the *dut* gene, which encodes dUTPase, accumulates uracil in its DNA. Subsequently, the removal of uracil by uracil DNA glycosylase results in the formation of AP sites. The loss of the major cellular AP endonuclease (exonuclease III) in a strain carrying a temperature sensitive *dut* mutation results in inviability when this double mutant (*xthA dut*(ts)) is shifted to the non-permissive temperature. (Cunningham *et al.*, 1986). If replication should proceed past an AP site, an incorrect base may be inserted, resulting in a mutation.

Therefore, AP sites pose a significant threat in terms of mutagenesis and cell viability (Barzilay and Hickson, 1995).

Generally, AP sites exist as a mixture of four different configurations. The opened chain aldehyde form exists in equilibrium with each of the cyclic hemiacetal (α and β) as well as the open chain hydrate. However, the majority of base losses result in the formation of a cyclic hemiacetal, with the α and β anomers occurring in a 1:1 ratio. The open chain aldehyde represents approximately 1 % of all AP sites, but is the most reactive of the four species (Barzilay and Hickson, 1995).

The generation of AP sites can occur through several mechanisms. Firstly, a base can be altered by the addition of an electrophilic alkyl group, weakening the N-glycosidic bond, and increasing the likelihood of breakage. Often, the result is an AP site of one of the configurations listed above. Secondly, reactive oxygen species can abstract a proton from the deoxyribose moiety of DNA, leading to the formation of an oxidized AP site (Barzilay and Hickson, 1995). An oxidized AP site can exist in either the open or cyclic configurations depending on the site of radical attack. Thirdly, base loss can be catalyzed by the action of DNA glycosylases. Examples include MutY-DNA glycosylase, formamidopyrimidine (Fpg)-DNA glycosylase, endonuclease III, and uracil-DNA glycosylase (Friedberg *et al.*, 1995).

The majority of DNA glycosylases are small proteins (~30 kDa), and are specific for a particular base modification. All DNA glycosylases are capable of removing AP sites through a β-elimination reaction. A β-elimination reaction requires a nucleophile, and may

involve the attack of the sugar at the carbon α to the carbonyl group at C1. In the case of endonuclease III, β elimination involves the formation of a Schiff base between an amine and the carbonyl group at C1 (Friedberg *et al.*, 1995). The result is the cleavage of the phosphodiester backbone 3' to the AP site in what is referred to as AP lyase activity or Class I AP endonuclease activity (Wallace, 1988). In addition to the action of DNA-glycosylases, AP sites can also be repaired by: AP endonucleases, which cleave 5' to AP sites (class II AP endonucleases); and the nucleotide excision repair pathway, which cuts the DNA on either side of the AP site (Barzilay and Hickson, 1995).

1.3.2.1 Exonuclease III

E. coli exonuclease III is a 28 kDa protein encoded by *xthA* at 38.5' on the current linkage map (Weiss, 1976, White *et al.* 1976). The enzyme was originally identified by its 3' to 5' exonuclease function (Richardson *et al.*, 1964). It is now known that exonuclease III is the cell's major endonuclease accounting for as much as 85% of all AP activity (Weiss *et al.*, 1988; Levin *et al.*, 1988). Unlike endonuclease III, exonuclease III is a class II AP endonuclease, which cleaves 5' to AP sites, resulting in a terminus with a 3' hydroxyl group (Warner *et al.*, 1980). Such termini do not require further processing to prime DNA synthesis. The enzyme accounts for 99% of the cell's 3' phosphatase function, with the remainder provided by endonuclease IV (Demple *et al.*, 1983). The 3' phosphatase function of exonuclease III represents a major cellular defence against oxidative damage, since it removes the 3' phosphate group at AP sites allowing the priming of DNA

polymerization. *E. coli* mutants lacking exonuclease III are twenty fold more sensitive to the lethal effects of H_2O_2 (Demple *et al.*, 1983). Exposure to H_2O_2 results in hydroxyl radical mediated strand breaks that can not be repaired by DNA polymerase I in a *xthA* mutant. Purified exonuclease III allows for DNA polymerase I directed synthesis of H_2O_2 damaged DNA *in vitro*, which implies that 3' blocking groups are removed by this repair enzyme (Demple *et al.*, 1986).

E. coli xthA mutants are sensitive to alkylating agents which result in an increase in the number of AP sites (Cunningham *et al.*, 1986). Exposure of *xthA* mutants to broad band near-UV results in a decrease in survival compared to wild-type cells. It is likely that the lesions generated by near-UV, and recognized by exonuclease III, are single-strand breaks. The loss of the 3' phosphatase function of exonuclease III would result in the inability of DNA polymerase I to proceed past 3' blocking groups at nicks in the DNA (Sammartano and Tuveson, 1983).

Mutations in the *rpoS (katF)* gene, which encodes a stationary phase sigma factor (RpoS), result in an increased sensitivity to broad band near-UV (Sammartano *et al.*, 1986). Insertional mutagenesis of *rpoS* also results in the elimination of exonuclease III activity (Sak *et al.*, 1989). These results suggested that exonuclease III may be under the control of *rpoS*. The observation that *xthA rpoS* double mutants are no more sensitive than either single mutant implies that it is the loss of exonuclease III that results in *rpoS*$^-$ sensitivity. Furthermore, a plasmid with a functional *rpoS*$^+$ restores exonuclease III activity when transformed into *rpoS*$^-$ cells (Sak *et al.*, 1989).

Exonuclease III shows significant sequence similarity with the major human AP endonuclease HAP1. This human homolog has been shown to complement certain DNA repair defects of an *E. coli xthA* mutant (Robson and Hickson, 1991). An additional function of exonuclease III not mentioned above is the abilty to degrade DNA-RNA hybrids. This RNaseH type function has been shown to be conserved in the major human AP endonuclease HAP1 (Barzilay and Hickson, 1995).

1.3.2.2 Endonuclease IV

Endonuclease IV is a 33 kDa protein (Ljungquist, 1977), which is encoded by *nfo* located at 46.5' on the current *E. coli* linkage map (Saporito and Cunningham, 1988). This enzyme acts on dsDNA and accounts for approximately 10 % of the cell's apurinic/apyrimidinic activity (Ljungquist, 1976). Unlike endonuclease III, endonuclease IV is a Class II AP enzyme, cleaving 5' to AP sites. The enzyme has 3' phosphatase and 3' diphosphoglyceraldehyde diesterase activities, which likely provide a 3' hydroxyl group to serve as a primer for DNA polymerase I (Levin *et al.*, 1988; Demple and Harrison, 1994)

Weiss and colleagues (Cunningham *et al.*, 1986) constructed a chromosomal endonuclease IV mutant (*nfo::kan*) using a method similar to that used to construct an endonuclease III mutant (Cunningham and Weiss, 1985). A strain deficient for *nfo* is sensitive to killing by alkylating agents such as methylmethanesulfonate (MMS) and mitomycin C, as well as the oxidants H_2O_2 and tert-butyl hydroperoxide. Upon exposure to mitomycin C and MMS, killing is higher in a *xthA* mutant than in an *nfo* mutant, and

was greatest for the *nfo xthA* double mutant (Cunningham *et al.*, 1986). These results suggest that exonuclease III and endonuclease IV recognize similar lesions, and that exonuclease III has a larger role in the repair of alkylated bases. However, *nfo* mutants are more sensitive than *xthA* mutants to the lethal effects of tert-butyl hydroperoxide and bleomycin, which suggesting that endonuclease IV may recognize some lesions not repaired by exonuclease III (Cunningham *et al.*, 1986). Unlike a *xthA* single mutant, *nfo* mutants were not hypersensitive to killing by H_2O_2. However, survival of an *nfo xthA* double mutant was reduced considerably more than the *xthA* single mutant. Regardless of the agent used, the presence of an *nth* mutation has no significant effect on sensitivity alone or in combination with other mutations, which is in agreement with earlier findings (Cunningham *et al.*, 1986; Cunningham and Weiss, 1985).

The type of damage produced by oxidizing agents appears to be that of a 3' blocking moiety rather than an AP site. A mutant allele of endonuclease IV (*nfo-186*) was cloned into pUC18 and introduced into a *xthA nfo* double mutant (Izumi *et al.*, 1992). The product of the *nfo-186* allele was able to complement the sensitivity of the double mutant to MMS but not to H_2O_2. After purification, the mutant protein was determined to have wild-type AP endonuclease activity, but had severely reduced 3' phosphatase activity. Therefore, it was inferred that H_2O_2 must produce 3' blocking damage as opposed to AP site damage (Izumi *et al.*, 1992). Exonuclease III and/or endonuclease IV are required to remove these blocking agents so that DNA synthesis can proceed.

In the presence of the superoxide-generating compound paraquat, endonuclease IV

activity is induced 10 to 20 fold (Chan and Weiss 1987). The inducibility of *nfo* was first demonstrated with an *nfo* overexpressing plasmid, which was shown to have elevated AP endonuclease activity when exposed to paraquat. On the other hand, paraquat-mediated induction was not observed when cells are transfected with an *nth* overexpressing plasmid (Chan and Weiss 1987).

Endonuclease IV is a member of the stress-inducible *soxRS* regulon (Tsaneva and Weiss, 1990). An *nfo-lacZ* transcriptional fusion was constructed to identify mutations affecting the expression of the gene. Two strains were found to harbour mutations in the *soxR* gene (*soxR*$^-$), and were unable to induce endonuclease IV as measured by β-galactosidase levels. Furthermore, in constitutive *soxR* mutants (*sox*^{c}), endonuclease IV transcriptional activity is elevated between five and eight fold above wild-type levels (Tsaneva and Weiss, 1990).

1.3.2.3 Endonuclease III

Endonuclease III is a 25 kDa protein encoded by *nth*, located at 36.5' on the *Escherichia coli* chromosome (Weiss and Cunningham, 1985; Asahara *et al.*, 1989). The enzyme was purified and found to have no requirement for divalent cations (Radman, 1976, Wallace, 1988). Further analysis revealed the presence of an iron-sulfur cluster (Cunningham *et al.*, 1988). The three-dimensional structure of the enzyme has been resolved using crystallography (Kuo *et al.*, 1992). The [4Fe-4S] cluster is bound within the C-terminal domain of the protein, which appears to allow for interaction between basic

amino acids and the DNA backbone. The inhibition binding region, which recognizes the modified base targeted for removal, has also been identified (Kuo *et al.*,1992).

Endonuclease III was discovered as an enzyme that cuts UV irradiated DNA (Radman, 1976). The enzyme was independently discovered in other laboratories as an enzyme that recognizes X-irradiated DNA (Strniste and Wallace, 1975), apurinic DNA (Gates and Linn, 1977), and osmium tetroxide-oxidized DNA (Armel and Wallace, 1977; Gates and Linn, 1977). The enzyme is sometimes referred to as thymine glycol-DNA glycosylase, since it recognizes various oxidized forms of thymine including thymine glycol, urea, methyltartronylurea, and 5-hydroxy-5-methylhydantoin (Breimer and Lindahl, 1984). Recently, the oxidation products 5-hydroxy-2'-deoxycytidine (5-OHdC) and 5-hydroxy-2'-deoxyuridine (5-OHdU) have been added to the list of substrates for endonuclease III (Hatahet, *et al.*, 1994). Both of these substrates are mutagenic *in vitro*, when incorporated into oligodeoxyribonucleotides. In one particular sequence context, dA can be incorporated opposite a 5-OHdC, resulting in a C-->T transition. In another sequence context, dC was the principal base inserted opposite 5-OHdC and 5-OHdU, leading to C-->G transversions in both cases (Purmal *et al.*, 1994).

The activity of endonuclease III is specific for double stranded DNA. The endonuclease activity is class I AP endonuclease-type; that is, cleavage of the DNA backbone occurs 3' to the damaged base (Warner *et al.*, 1980; Bailly and Verly, 1987; Wallace, 1988). The phosphodiester bond is not cleaved hydrolytically, but rather, through a β-elimination reaction; therefore, endonuclease III has since been classified as an AP

lyase (Bailly and Verly, 1987). This type of AP activity may actually be deleterious to the cell, since strand breaks 3' to a base free sugar leave poor primers for DNA synthesis. Another enzyme is required to remove the terminal base free sugar, before DNA polymerase could function in synthesizing the opposing strand (Cunningham *et al.*, 1986; Wallace, 1988). In addition to its endonuclease function, the enzyme has an associated N-glycosylase activity, which releases damaged pyrimidines (Breimer and Lindahl 1984, Boorstein *et al.* 1989).

An endonuclease III mutant was constructed using an overproducing plasmid which was mutagenized with a *Tn*-5 encoding a kanamycin resistance marker (Cunningham and Weiss, 1985). The plasmid was transformed into a DNA polymerase temperature sensitive mutant at 32° C, and then allowed to grow at 42° C. Growth at the higher temperature allows for selection against the plasmid, since its ColE1 origin can not direct replication without a functional DNA polymerase I. The *nth::kan* marker was recombined into the chromosome, and transduced into other strains by selecting for kanamycin resistance. Strains deficient in endonuclease III were not found to be sensitive to H_2O_2 or γ rays (Cunningham and Weiss, 1985). The lack of sensitivity of an *nth* mutant to these treatments, known to produce thymine glycols, is likely due to overlap in the substrate specificity of various repair enzymes and pathways. For example, *E. coli* endonuclease VIII is also known to repair oxidized pyrimidines (Wallace, 1988, Demple and Harrison, 1994).

In contrast to *nth* strains, exonuclease III (*xthA*) mutants are extremely sensitive to

H_2O_2. The presence of the *nth* mutation did not result in a greater sensitivity of a *xthA* strain to the lethal effects of H_2O_2, but rather, offered a small protective effect (Cunningham and Weiss, 1985). The spontaneous mutation rate, as measured by reversion to arginine auxotrophy at *argE3* (ochre), was approximately eleven fold higher in the *nth* strain compared to the wildtype AB1157. Furthermore, the additional presence of *xthA* and endonuclease IV (*nfo*) mutations did not result in a further increase in the spontaneous mutation rate of a *nth* strain compared to wild-type. This suggests that the spontaneous mutations in the *nth* strain were not the result of AP sites (Demple and Harrison 1994).

A homolog of endonuclease III has been identified in the yeast *Saccharomyces cerevisiae* (see below)(Eide *et al.*, 1996). Bovine and human homologs of the bacterial endonuclease III have been isolated through a procedure designed for trapping the enzyme in an intermediate step which is common to all DNA glycosylase / AP lyases (Hilbert *et al.*, 1996; Hilbert *et al.*, 1997; Dodson *et al.*, 1994). Enzymes of this class attack the C_1 of the nucleoside bonded to an altered base, and form an N-acylimine (Schiff base) intermediate. The Schiff base normally undergoes successive elimination and hydrolysis reactions resulting in an α,β unsaturated aldehyde. However, the addition of $NaBH_4$ results in reduction of the Schiff base, and an irreversibly linked enzyme-DNA complex (Dodson *et al.*, 1994, Nash *et al.*, 1996). A 31 kDa protein was identified from calf-thymus using this method (Hilbert *et al.*, 1996), and the amino acid sequence demonstrated homology with *E. coli* endonuclease III , a protein from *C. elegans*, and the

translated amino acid sequence from two incomplete Human cDNA sequences (Hilbert *et al.*, 1996). The partial human cDNA sequences were used to clone the human homolog of *E.coli* endonuclease III. Similar to endonuclease III, the human counterpart contains an iron-sulfur cluster, recognizes thymine-glycols, and becomes irreversibly bound to the DNA substrate in the presence of borohydride (Hilbert *et al.*, 1997). The human homolog of endonuclease III was independently cloned from a gene designated *OCTS3*, which maps to chromosome 16p13.3 (Aspinwall *et al.*, 1997). The purified protein of 34 kDa is an AP lyase, with similar substrate specificity to *E. coli* endonuclease III. The *OCTS3* gene product was designated hNTH1 (human homolog of Nth) since it recognizes thymine glycol and urea, but not 8-oxoG (Aspinwall *et al.*, 1997).

1.3.2.4 Endonuclease VIII

The major enzyme involved in the repair of oxidized pyrimidines is endonuclease III. Endonuclease III (*nth*) mutants are not abnormally sensitive to oxidizing agents or ionizing radiation (Cunningham and Weiss, 1985), which both produce similar types of base damage (Hariharan *et al.*, 1977). Thus it was speculated that another enzyme with similar substrate specificity to endonuclease III existed (Melamede *et al.*, 1994). Endonuclease VIII was purified from an *E. coli nth* mutant, and found to be a novel thymine-glycol DNA glycosylase, that also removes β-ureidoisobutyric acid, urea, dihydrothymine. AP sites are also substrates for this protein, indicating the presence of AP endonuclease activity (Melamede *et al.*, 1994) The cleavage of AP sites by endonuclease VIII occurs 3'

to the site of base loss; therefore, this enzyme is a Class I AP endonuclease or AP lyase (Melamede *et al.*, 1994).

The gene encoding endonuclease VIII (*nei*) was recently cloned, sequenced and characterized (Jiang *et al.*, 1997). The *nei* gene was cloned using degenerate PCR primers obtained from the purified protein amino acid sequence. A 650 bp PCR product was used as a probe in an *in situ* hybridization of the Kohara λ library. One of the phage clones (1H5) hybridized with the probe. Since the location of the 1H5 clone was already known, the *nei* gene was located to 16 min on the *E. coli* chromosome (Jiang *et al.*, 1997). The internal 397 bp region of the *nei* gene was replaced with a chloramphenicol resistance cassette, and the resulting deletion mutation was transferred to the chromosome. The *nei* mutant strain is slightly more sensitive to ionizing radiation. Furthermore, an *nth nei* double mutant is significantly more sensitive to the lethal effects of ionizing radiation than WT (1.4 fold), suggesting that endonuclease III and endonuclease VIII repair lethal pyrimidine lesions that are produced by an X-ray exposure (Jiang *et al.*, 1997). Both *nth* and *nei* single mutants exhibit WT sensitivity to a hydrogen peroxide exposure, while *nth nei* double mutants are extremely sensitive to the lethal effects of this oxidative damaging agent (Cunningham and Weiss, 1985; Saito *et al.*, 1997). The hypersensitivity of the *nth nei* mutant to H_20_2 was equivalent to that of a *xthA* single mutant strain, which further supports the role of endo III and endo VIII in protection against oxidative DNA damage (Saito *et al.*, 1997).

1.3.3 Fpg glycosylase

Fpg (Fapy) DNA glycosylase is a small protein (31 kDa) which maps to 81.7 min on the *E. coli* chromosome (Boiteux *et al.*, 1987). The enzyme was identified from a cloned insert which inactivated MutM. The position of the insert was determined to be within the coding region for Fpg, therefore, MutM and Fpg are the same protein (Michaels *et al.*, 1991). One of the lesions removed by Fpg is 8-oxo-7,8-dihydro-2'-deoxyguanine (8oxoG) (Boiteux, 1993). If 8oxoG lesions are not removed, they can mispair with adenine during replication resulting in G to T transversions (Shibutani *et al.*, 1991) accounting for an increase in this particular mutation in a *mutM* strain (Cabrera *et al.*, 1988). Fpg (Fapy) was originally named for the enzyme's ability to remove formamidopyrimidines from DNA (Chetsanga and Lindahl, 1979; Boiteux *et al.*, 1989). Exposure of *E. coli* cell extracts to dimethyl sulfide at high pH can lead to the production of ring fragmented purines such as 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine lesions (FapyGua) (Chetsanga and Lindahl, 1979).

Another gene that leads to elevated rates of G to T transversions is mutY (Nghiem *et al.*, 1988). MutY is specific for the removal of adenines that have incorrectly mispaired with G residues or 8oxoG lesions after DNA replication *in vitro* (Michaels and Miller, 1992). Both MutM and MutY function as part of the same repair pathway responsible for removal of 8oxoG lesions, which is evident from the synergistic increase in G --> T transversions in a *mutM mutY* double mutant (Michaels and Miller, 1992).

1.4 DNA Repair in *Saccharomyces cerevisiae*

1.4.1 Nucleotide Excision Repair

Many of the genes involved in nucleotide excision repair (NER) in *Saccharomyces cerevisiae* are members of the *RAD3* epistasis group. Strains carrying single mutations for one of the eighteen members of this group are variably sensitive to UV and / or chemical agents that alter base structure (Friedberg *et al.*, 1995). Members of this group are said to be epistatic to one another, since the combination of two mutations does not result in hypersensitivity to DNA damaging agents relative to a single mutant (Friedberg *et al.*, 1995).

Some of the genes involved in NER in *S. cerevisiae* were identified using a temperature sensitive (Ts) *cdc9* mutant, which encodes DNA ligase (Wilcox and Prakash, 1981). When cells harbouring this mutation are shifted to the non-permissive temperature, they are no longer able to repair strand breaks. Previously characterized *RAD* genes were inactivated and individually combined with the *cdc9*(ts) mutation and examined for their ability to cut DNA relative to a *RAD* WT *cdc9*(ts) strain. The inability of some of the *cdc9*(ts) *RAD* mutants to cut DNA was demonstrated by a lack of low molecular weight product at the top of a sucrose gradient for cells that had been shifted to high temperature. In contrast, a *RAD* WT strain or a *RAD* mutant not involved in NER, would be proficient for DNA incision, and would produce low molecular weight products when shifted the non-permissive temperature. Inactivation of *RAD1, RAD2, RAD3, RAD4,* or *RAD10* in a *cdc9*(ts) mutant resulted in a total loss of incision of UV-irradiated DNA (Wilcox and

Prakash, 1981; Reynolds and Friedberg *et al.*, 1981). In contrast, *rad7*, *rad16*, and *rad23* mutants were still able to carry out NER, but at a reduced level (Wilcox and Prakash, 1981; Reynolds and Friedberg *et al.*, 1981).

Two additional members of the *RAD3* epistasis group were discovered serendipitously in a screen for suppressors of an artificially produced stem loop structure in the *HIS4* gene. Two independent mutations were identified from isolates able to grow in the absence of histidine, which were designated *SSL1* and *SSL2* (Yoon *et al.*, 1992; Guylas and Donahue, 1992). The product of the *SSL2* gene which restored HIS4 gene expression was found to be 51% identical to the human NER gene *ERCC3* (*XPB*) (Guylas and Donahue, 1992). *SSL2* was also independently identified as *RAD25* using an *ERCC3* cDNA probe (Park *et al.*, 1992). The *ssl2* mutation results in UV light hypersensitivity in *S. cerevisiae*, providing further evidence that this gene is the functional yeast homolog of *ERCC3* (XPB) (Guylas and Donahue, 1992). *SSL2 (RAD25)* belongs to the subset of genes in the *RAD3* epistasis group that are considered essential for NER: *RAD1, 2, 3, 4, 10, 14, 25, SSL1,* and *TFB1* (for review, see Ramotar and Masson, 1996). In addition, the replication protein A (Rpa) has been shown to be necessary for NER *in vitro* (He *et al.*, 1996). Rpa is also known to be an essential NER protein in human cell extracts (He *et al.*, 1996).

Nucleotide excision repair is initiated in *S. cerevisiae* when a DNA damage site is recognized by Rad14. Rad14 binds (6-4) photoproducts caused by ultraviolet light, but does not appear to bind to pyrimidine dimers (Guzder *et al.*, 1993), suggesting that there

may be other proteins with different substrate specificities that bind the initial damage site (Ramotar and Masson, 1996). Incision at the 5' flanking region of the damage site is effected by the Rad1/Rad10 complex, while Rad2 is thought to cut at the 3' side (Davies *et al.*, 1995; Habraken *et al.*, 1993). Unwinding of the internal DNA segment is carried out by the helicase activities of Rad3 and Rad25, resulting in the removal of an approximately 30 bp oligonucleotide (Ramotar and Masson, 1996).

1.4.2 Apn1: the major apurinic/apyrimidinic endonuclease

Several enzymes in *E. coli* are capable of recognizing AP sites. AP endonuclease activity has been demonstrated for exonuclease III, endonuclease IV (Warner *et al.*, 1980), and the uvrABC complex (Lin and Sancar, 1989). AP lyase (3' AP endonuclease) reactions are catalyzed by endonuclease III (Warner *et al.*, 1980) and endonuclease VIII (Melamede *et al.*, 1994). In contrast, AP sites in *Saccharomyces cerevisiae* are repaired by a single AP endonuclease / 3' diesterase called Apn1 (Popoff *et al.*, 1990). The yeast structural gene *APN1* has been cloned, and has a predicted amino acid sequence that is 41% identical to that of *E. coli* endonuclease IV (Popoff *et al.*, 1990). Apn1 has an additional 80 residues at its C-terminal end which are not present in *E. coli* Endonuclease IV, and are therefore unlikely to be involved in damage recognition. The regions of sequence homology between the two proteins is thought to be critical for the proteins' shared catalytic activities: 3' phosphomonoesterase and 3' phosphodiesterase (Popoff *et al.*, 1990). Endonuclease IV of *E. coli* is able to complement an APN1 mutant for the

repair of AP sites *in vivo* (Ramotar and Demple, 1996). These examples of sequence and functional conservation between a prokaryote and a eukaryote underscore the importance of AP enzymes in DNA repair.

Unlike *E. coli* endonuclease IV, which is a minor AP endonuclease, Apn1 accounts for as much as 97 % of the cell's AP activity, and does not appear to be inducible by any agents (Popoff *et al.*, 1990). Endonuclease IV is responsible for approximately 5 % of AP activity in *E. coli* cells, but can be induced in the presence of superoxide (Chan and Wiess, 1987). The inducibility of endonuclease IV may occur in order to repair lesions not recognized by the major AP endonuclease in *E. coli*, exonuclease III (Popoff *et al.*, 1990).

Apn1 cleaves DNA at AP sites by the same mechanism as that of the *E. coli* enzymes endonuclease IV and exonuclease III. Cleaveage 5' to the AP site in these enzymes result in a 3' hydroxyl group which can serve as a primer for DNA polymerization (Masson and Ramotar, 1997). In contrast, *E. coli* endonuclease III is a 3' AP endonuclease which leaves an abasic sugar residue following cleavage. This cleavage product is not a suitable primer for DNA polymerase (see above). The cloned *APN1* gene has been disrupted with a *URA3* marker (Popoff *et al.*, 1991). *APN1* disruptants are defective in cleavage of methanemethanosulfate-induced AP sites, and are hypermutagenic. The phenotype of the *APN1* disruptant can be rescued by the additon of either endonuclease III or exonuclease III, which suggests that repair of AP sites in *S. cerevisiae* is independent of the catalytic mechanism that effects it (Masson and Ramotar, 1997). Therefore, DNA repair differs between *E. coli* and *S. cerevisiae* in terms of the number of enzymes that recognize AP

sites, as well as the type of processing that occurs subsequent to cleavage.

1.4.3 Base excision repair

A number of DNA repair enzymes have been identified in *Saccharomyces cerevisiae* which do not appear to belong to any of the three major groups: NER, recombinational repair, and mismatch repair. Some of these enzymes are involved in base excision repair (BER), and include an 8-oxoguanine yeast DNA glycosylase (Ogg1) (van der Kemp, 1996; Nash, 1996); endonuclease III-like glycosylase 1 (Ntg1) (Eide *et al.*, 1996); and a DNA glycosylase that removes alkylated bases (AlkA) (Labahn *et al.*, 1996).

Cloning of a 7,8-dihydro-8-oxoguanine DNA glycosylase in *S. cerevisiae* was done by transforming an *E. coli fpg* mutant with a yeast DNA library and subsequent screening for antimutators (van der Kemp *et al.*, 1996). One of the clones resulted in a decrease in the spontaneous mutation rate and was found to harbour enzymatic activity specific for the cleavage of 7,8-dihydro-8-oxoguanine (8oxoG) opposite a cytosine residue in a 34-mer oligonucleotide (van der Kemp *et al.*, 1996). An open reading frame designated *OGG1* was sequenced and mapped to chromosome XIII. The protein, having a molecular mass of 43 kDa, was purified to apparent homogeneity, and demonstrated to possess a DNA glycosylase activity which recognizes 8oxoG and 2,6-diamino-4-hydroxy-5-methylformamidopyrimidine (van der Kemp *et al.*, 1996).

The *OGG1* gene product was isolated concurrently by Nash *et al.* (1996) using a covalent trapping procedure. The mechanism for trapping the protein was based on the

underlying conservative mechamism of DNA glycosylase/lyase function (Dodson *et al.*, 1994). An enzyme containing an amine nucleophile attacks the C-1 bonded to 8-oxoguanine, resulting in a covalent bond between the enzyme and the sugar, and the release of the guanine adduct. A Schiff base (imine) is then generated through the reaction of an aldehyde (sugar fragment) and the amine (amino acid residue of the enzyme). A β-elimination reaction results in the cleavage of the 3' phosphodiester bond, followed by a δ-elimination cut at the 5' C-O bond (Tchou and Grollman, 1995; Nash *et al.*, 1996). Interception of the Schiff base before cleavage of the deoxyribose backbone can be effected by borohydride ($NaBH_4$), which reduces the Schiff base, thus creating a stable enzyme-linked adduct (Nash *et al.*, 1996). Whole cell yeast extracts were reacted with a radiolabeled 8oxoG:C oligonucleotide, yielding two enzyme linked adducts designated complex 1 and complex 2. SDS-PAGE separation of the complexes, peptide sequencing, and BLAST analysis (Altschul *et al.*, 1990) revealed that complex 1 was encoded by the open reading frame for *OGG1*. Competition experiments using excess unlabelled oligonucleotides demonstrated that Ogg1 was specific for 8oxoG:C mispairs (Nash *et al.*, 1996), consistent with the results of others (van der Kemp *et al.*, 1996). Complex 2 was determined to be encoded by a unique gene since it was detected in cell extracts from an *OGG1* disruptant. This complex was found to be specific for 8oxoG:G mispairs, and was designated Ogg2 (Nash *et al.*, 1996). Based on these observations, as well as unpublished observations of distant relatives of *OGG1* in the *S. cerevisiae* database, Verdine and colleagues (Nash *et al.*, 1996) have proposed the existence of a BER superfamily.

Comparson of the primary amino acid sequences of a number of base-excision DNA repair proteins from different organisms suggests that Ogg1 shares structural features with members of this interspecies family (Nash *et al.*, 1996). Oggl contains a helix-hairpin-helix, a glycine / proline rich domain, and a conserved aspartate residue at position 260 (HhHGPD). The same motif is found in glycosylase/AP-endonucleases of other organisms including the *E. coli* protein endonuclease III (Kuo *et al.*, 1992; Thayer *et al.*, 1995), as well as monofunctional glycosylases such as the *E. coli* proteins AlkA (Labahn *et al.*, 1996) and MutY (Michaels *et al.*, 1990). The results of Nash *et al.* (1996) define a family of base-excision DNA repair proteins whose members share three-dimensional structure, but differ in their respective substrate specificities. The Ogg1 protein, although structurally similar to endonuclease III, is believed to be the functional homolog of the bacterial Fpg protein (Thomas *et al.*, 1997). *OGG1* disruptants display a mutator phenotype, with a predominance of GC to TA transversions (Thomas *et al.*, 1997), consistent with the phenotype of an *E. coli fpg (mutM)* mutant (Michaels *et al.*, 1991; Michaels *et al.*, 1992b). Further evidence that Ogg1 is the eukaryotic homolog of Fpg is provided by the demonstration that Ogg1 recognizes 8oxoG and Fapy lesions (van der Kemp *et al.*, 1996; Nash *et al.*, 1996).

The conserved helix-hairpin-helix domain of the DNA glycosylase superfamily was compared with the DNA database in a BLAST search, revealing homology to an open reading frame on *S.cerevisiae* chromosome I (Eide *et al.*, 1996). The ORF translated to a protein exhibiting partial identity with that of *E. coli* endonuclease III, and was named

endonuclease III-like glycosylase 1 (NTG1). The NTG1 gene product cleaves DNA containing thymine glycols caused by exposure to OsO_4 (Eide *et al.*, 1996). The mechanism of removal of this lesion is thought to be similar to the sequential glycosylase and 3' AP endonuclease steps performed by *E. coli* Endonuclease III. Unlike Endonuclease III, NTG1 recognizes formamidopyrimidine lesions, and does so with similar efficiency to that of *E. coli* Fpg glycosylase. However, the yeast protein does not recognize 8oxoG in ^{32}P labelled duplex DNA (Eide *et al.*, 1996). Another important difference between these homologs is that the yeast protein is part of a damage inducible regulon, while its bacterial counterpart (endonuclease III) has not been shown to be regulated. Northern blot analysis revealed that the NTG1 gene is induced at the transcriptional level by the DNA damaging agents H_2O_2, 4-nitroquinolone-1-oxide, and methanemethanosulfate (Eide *et al.*, 1996). However, more characterization of the *NTG1* gene product and other proteins involved in yeast BER is still required. It is possible that the recently cloned *NTG1* is identical to the gene which encodes a redoxyendonuclease that was previously isolated (Gossett *et al.*, 1988). This protein of approximately 40 kDa cleaves OsO_4-damaged DNA and UV-irradiated DNA with substrate specificity similar to *E. coli* endonuclease III (Gossett *et al.*, 1988).

1.5 Project Outlines and Objectives

1.5.1 Hypothesis

The goal of the present study is to examine the involvement of three *E. coli* apurinic/

apyrimidinic endonucleases in the repair of near-UV mediated DNA damage. These enzymes have been shown previously to be involved in the repair of oxidative DNA lesions (Breimer and Lindahl, 1984; Cunningham *et al.*., 1986; Hatahet *et al.*, 1994). Strains deficient in one or more of exonuclease III (*xthA*), endonuclease IV (*nfo*), or endonuclease III (*nth*) were exposed to broad band near-UV, and the effect on survival and mutagenesis was determined (Chapters 3 and 4).

The hypothesis that AP endonucleases may be involved in the cell's near-UV defense repertoire stems from our previous observations that Fpg gylcosylase is important in protecting against near-UV mediated lethality and mutagenesis (Shennan *et al.*, 1996; Palmer *et al.*, 1997). Fpg glycosylase recognizes the oxidative lesion 8-hydroxoguanine (8oxoG), and cells lacking this DNA repair enzyme display a mutator phenotype (Boiteux, 1987; Michaels *et al.*, 1992b). Since 8oxoG pairs equally well with adenine as it does with cytosine, the principal mutational event in *fpg*- cells is that of G --> T transversions. Using a specific mutation reporter system, we have found that exposure of *fpg*- cells to UVA results in a predominance of G to T transversions (Chapter 2, Palmer *et al.*, 1997). In contrast, exposure of *fpg*- cells to UVC resulted in predominantly C –> T transitions, implicating dipyrimidine photoproducts as the pre-mutagenic lesion. These data suggest that the type of DNA damage caused by near-UV differs from that of far-UV, and that the pre-mutagenic lesion caused by the former is likely to be 8oxoG.

A similar approach to that used to study the role of Fpg glycosylase in near-UV protection has been employed for endonuclease III, endonuclease IV, and exonuclease III

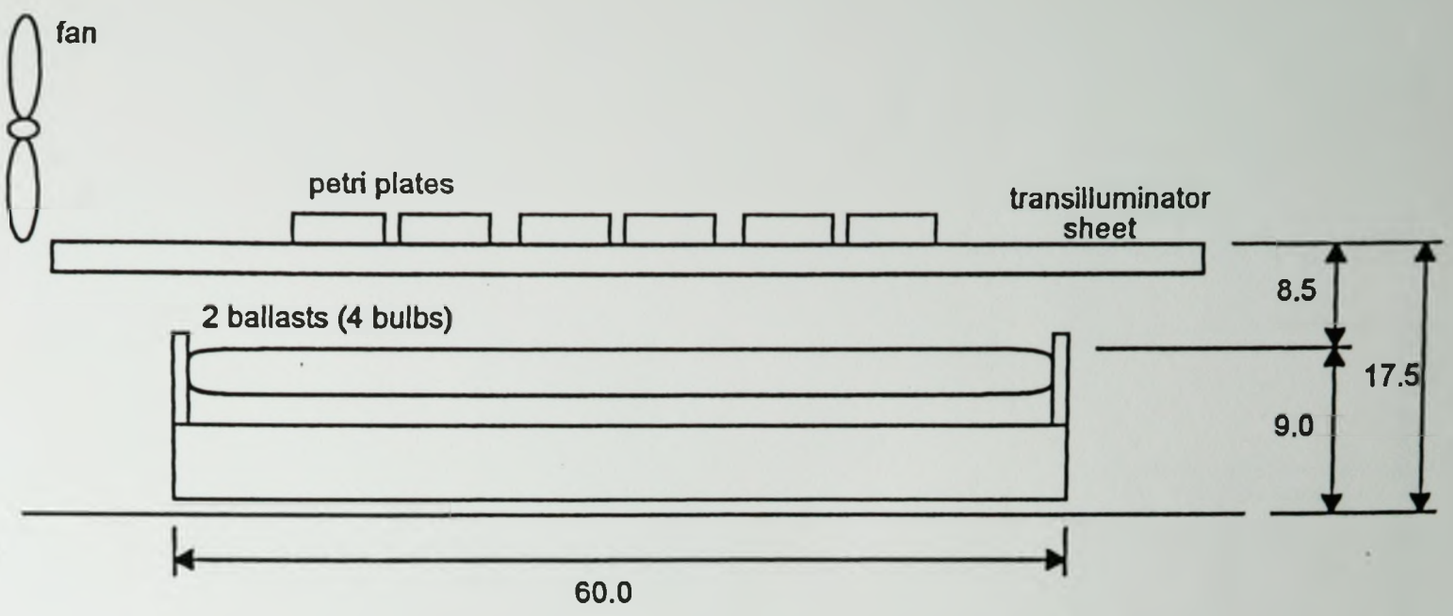
and can be summarized as follows. Firstly, various DNA repair mutants were exposed to UVA in order to determine their role(s) in protecting *E. coli* from the lethal effects of this stress. Secondly, a forward mutation assay was employed to examine the role of endonuclease III, endonuclease IV, and exonuclease III in protection from the mutagenic effects of near-UV. Thirdly, a reporter system was used to determine the specificity of mutations caused by near-UV, to determine by inference, what are the pre-mutagenic lesions responsible for this mutagenesis.

1.5.2 Light source employed in this study

The potential for contaminating UVB wavelengths in studies of UVA-mediated DNA damage has been demonstrated by significant differences in independently reported survival rates for a *uvrA*- strain (see chapter 1). The light source for the present study was chosen to circumvent the problem of contaminating UVB wavelengths. The light apparatus used in this study was as shown in Figure 2. The transmission of various wavelengths by the transilluminating filter is illustrated in Figure 3. The filter used transmits a high proportion of wavelengths in the UVA range (320 nm < λ < 400 nm), and to a lesser extent, those wavelengths below 320 nm. However, the spectral output for the Philips F20T12 bulbs employed is maximal at 360 nm, while the radiant for wavelengths below 310 nm is less than 2 % (Figure 3, inset). The combination of the F20T12 bulbs and the transilluminating filter results in a UVB fluence rate of less than 2 % of the UVA rate, which is confirmed by the measured fluence rates at 360 nm and 310 nm were 1500

$\mu W/cm^2$ and 31 $\mu W/cm^2$. The UVA output of these bulbs represents a reasonable simulation of the UVA component of solar irradiation. The solar fluence rate recorded at Hamilton, Ontario, Canada (43° 15'00" N - 79° 51'00" W) at mid-afternoon was determined to be 1150 $\mu W/cm^2$ using a 360 nm detector.

Figure 2. Near-UV irradiation chamber. Plates were irradiated face down, without lids, on a UV-transmitting transilluminator sheet (Fisher Scientific, Ottawa, ON, Cat. No. FB-TI-105A) above four parallel Philips F20T12/BL (20 W) bulbs. All distances are in centimeters. The temperature on the surface of the sheets was kept at 25°C with fans. The fluence rate was determined to be 1500 $\mu W/cm^2$ using a UV digital radiometer and 360 nm sensor (Ultraviolet Products Inc., San Gabriel, CA). The fluence rate at 310 nm was determined to be 31 $\mu W/cm^2$. Contaminating UVC radiation was determined to be less than 1 $\mu W/cm^2$ (the detection limit of the radiometer).


fan
petri plates
transilluminator sheet
2 ballasts (4 bulbs)
8.5
17.5
9.0
60.0

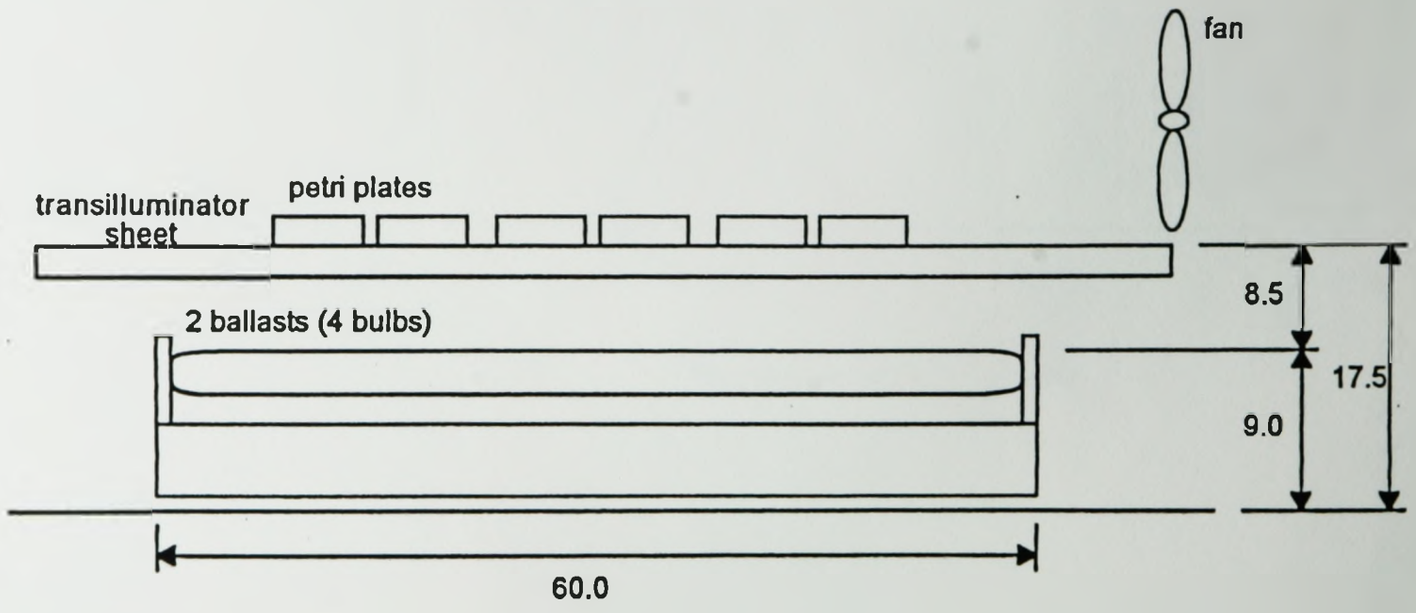
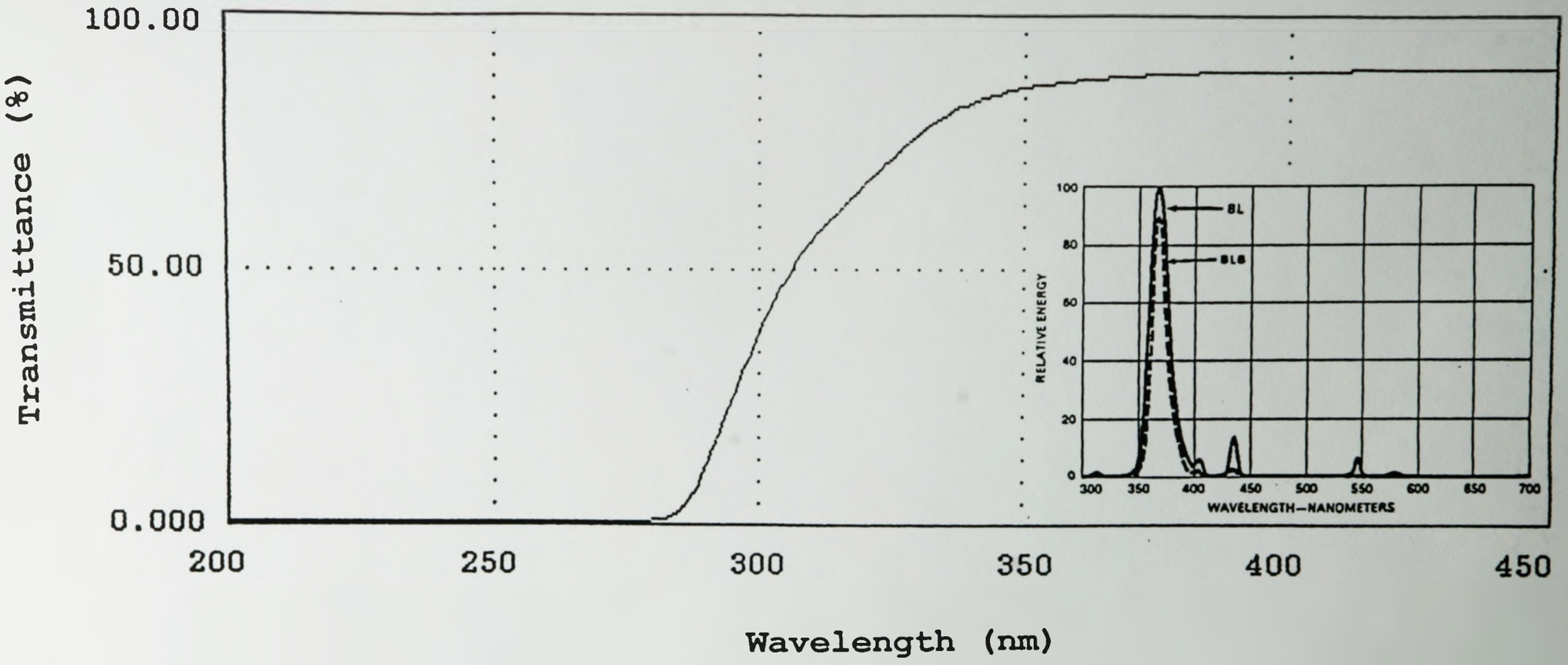

fan
transilluminator sheet
petri plates
2 ballasts (4 bulbs)
8.5
17.5
9.0
60.0

Figure 3. Transmission of UV wavelengths by the UV transilluminating filter used in the present study (Fisher Scientific, Ottawa, ON, Cat. No. FB-TI-105A). **Inset)** Spectral radiation of near-UV bulbs: Philips BL (this study) and Philips BLB.


100.00
50.00
0.000
Transmittance (%)
200
250
300
350
400
450
Wavelength (nm)
RELATIVE ENERGY
100
80
60
40
20
0
BL
BLB
300
350
400
450
500
550
600
650
700
WAVELENGTH—NANOMETERS

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CHAPTER 2

Preface

The following article entitled "Near-Ultraviolet Radiation (UVA) Causes a Fapy Glycosylase Dependent Increase in G to T transversions" was published in *Photochemistry and Photobiology* **65**, 543-549.

D.M. Serafini performed the catalase assay and wrote the section 'Addition of exogenously added catalase rescues WT and *fpg*- strains from the lethal effects of near-UV'. D.M. Serafini was also involved in the writing and editing of the manuscript. Graphical results of the catalase assay were removed from the manuscript prior to publication and appear in the Appendix.

The following pages have been removed.
Please use the following citation to access them.

Palmer C, Serafini D, Schellhorn H. 1997. Near Ultraviolet Radiation (UVA and UVB) Causes a Formamidopyrimidine Glycosylase-Dependent Increase in G to T Transversions. Photochemistry and Photobiology. 65(3): 543-549.

Photochemistry and Photobiology, 1997, 65(3): 543–549

Near Ultraviolet Radiation (UVA and UVB) Causes a Formamidopyrimidine Glycosylase–Dependent Increase in G to T Transversions

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Received 17 July 1996; accepted 21 October 1996

ABSTRACT

In contrast to far-UV (<290 nm) DNA damage, a large fraction of the DNA damage caused by near-UV is oxygen-dependent, suggesting the involvement of reactive oxygen species (ROS). The oxidized base 8-oxo-7,8-dihydroguanine (GO) is characteristic of ROS-induced DNA damage and is removed by Fapy (formamidopyrimidine) glycosylase. We have recently shown that *Escherichia coli* strains deficient in Fapy glycosylase (*fpg*) are hypersensitive to the lethal effects of UVA but not far-UV (UVC), suggesting lesions recognized by this enzyme may be important premutagenic or lethal lesions generated by near-UV radiation. In this study, we have found that while the far-UV-induced mutation rates of Fapy-deficient and wild-type strains were similar, near-UV (UVA and UVB) was hypermutagenic to a Fapy-deficient strain, causing a dose-dependent increase in induced mutation relative to wild type (up to five-fold at 200 kJ/m^2). Using a plasmid back mutation assay, the predominant near-UV-induced mutations in both wild-type and Fapy-deficient strains were found to be C → T transitions and G → T transversions. The former is probably due to replicative bypass of pyrimidine dimers or (6-4) photoproducts that are known to be generated by near-UV, whereas the latter may be due to mispairing of GO lesions with adenine during replication. Consistent with this, the frequency of near-UV-induced G → T transversions was 16-fold higher in a Fapy-deficient strain than a wild-type strain.

INTRODUCTION

Solar UV radiation consists of UVC ($\lambda < 290$ nm), UVB (290 nm $< \lambda <$ 320 nm) and UVA ($\lambda > 320$ nm) components (1). Ultraviolet C or far-UV is effectively filtered out by the earth's atmosphere, and as a result, the UV radiation reaching the earth's surface is primarily in the UVB and UVA ranges, which are collectively called near-UV (1). As a result of the use of fossil fuels and chlorofluorocarbon (CFC)†-containing aerosols, atmospheric ozone levels are decreasing, leading to a rise in the level of UVB radiation at the earth's surface (2). Because near-UV wavelengths effectively induce basal and squamous cell carcinomas of the skin (3), this rise in near-UV radiation has been linked to increases in the incidence of skin cancer (4). If the upward trend in near-UV levels continues, future increases in the incidence of skin cancer are expected (5).

The mechanism by which near-UV damages DNA is more complex than that of far-UV. Absorption of far-UV photons by DNA results in formation of characteristic lesions including pyrimidine dimers, pyrimidine pyrimidone (6-4) photoproducts as well as various other minor photoproducts such as thymine glycols and pyrimidine hydrates (reviewed in Friedberg *et al.* (6)). Although near-UV wavelengths, like far-UV, can also efficiently induce the formation of both pyrimidine dimers and (6-4) photoproducts (7), these lesions appear to be less important in near-UV-mediated lethality (1). Irradiation of DNA with broadband near-UV results in the production of lesions more characteristic of oxygen radical-mediated DNA damage (1). Evidence for the involvement of oxygen radicals in near-UV-mediated damage comes from studies in *Escherichia coli* that show near-UV-mediated lethality is strongly enhanced by oxygen (8). *Escherichia coli* mutant strains that are deficient in enzyme functions that protect the cell against oxidative stress are generally more sensitive to the lethal and mutagenic effects of near-UV but not necessarily far-UV. Examples include superoxide dismutase (9), which catalyzes the conversion of superoxide anion to hydrogen peroxide and oxygen (10); exonuclease III (11), involved in the repair of OH radical-mediated strand break; and σ^S, a regulator of catalase and exonuclease III (1). *In toto*, these results suggest a possible role for superoxide and hydrogen peroxide in near-UV-mediated DNA damage. Oxidized guanine residues, including 8-oxo-7,8-dihydroguanine (GO), are considered indicator lesions for reactive oxygen species (ROS)-induced DNA damage. The level of GO detected in mammalian cells following near-UV irradiation is elevated (12), suggesting this may be

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†*Abbreviations*: CFC, chlorofluorocarbon; DMSO, dimethyl sulfoxide; Fapy, formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; GO, 8-oxo-7,8-dihydroguanine; LB, Luria–Bertani; MutT, 8-hydroxy-2′-deoxyguanine dGTPase; 4-NQO, 4-nitroquinoline 1-oxide; Pgal, phenyl-β-D-galactoside; ROS, reactive oxygen species; WT, wild type; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Table 1. Bacterial strains used in this study

Strain	Relevant genotype*	F plasmid	*lacZ* allele†	Base change required to revert	Source
CSH100	*ara* Δ(*gpt-lac*)*5*	F′ 100	*lacZ*⁺	—	(7)
CSH101	*ara* Δ(*gpt-lac*)*5*	F′ 101	*lacZ* (Am-461)	AT to CG	(7)
CSH102	*ara* Δ(*gpt-lac*)*5*	F′ 102	*lacZ* (Gly-461)	GC to AT	(7)
CSH103	*ara* Δ(*gpt-lac*)*5*	F′ 103	*lacZ* (Gln-461)	GC to CG	(7)
CSH104	*ara* Δ(*gpt-lac*)*5*	F′ 104	*lacZ* (Ala-461)	GC to TA	(7)
CSH105	*ara* Δ(*gpt-lac*)*5*	F′ 105	*lacZ* (Val-461)	AT to TA	(7)
CSH106	*ara* Δ(*gpt-lac*)*5*	F′ 106	*lacZ* (Lys-461)	AT to GC	(7)
HS1190	Same as CSH100 except *fpg*::kan	F′ 100	*lacZ*⁺	—	This study
HS1191	Same as CSH101 except *fpg*::kan	F′ 101	*lacZ* (Am-461)	AT to CG	This study
HS1192	Same as CSH102 except *fpg*::kan	F′ 102	*lacZ* (Gly-461)	GC to AT	This study
HS1193	Same as CSH103 except *fpg*::kan	F′ 103	*lacZ* (Gln-461)	GC to CG	This study
HS1194	Same as CSH104 except *fpg*::kan	F′ 104	*lacZ* (Ala-461)	GC to TA	This study
HS1195	Same as CSH105 except *fpg*::kan	F′ 105	*lacZ* (Val-461)	AT to TA	This study
HS1196	Same as CSH106 except *fpg*::kan	F′ 106	*lacZ* (Lys-461)	AT to GC	This study

*Refers to the genotype of the chromosome.
†Refers to the *lacZ* allele on the F plasmid (episome).

an important near-UV induced lesion. Failure to remove GO from DNA can lead to an increase in G to T transversions (13) because GO pairs equally well with adenine and cytosine (13). Because formamidopyrimidine (Fapy) glycosylase removes GO lesions from DNA as well as imidazole ring-opened forms of guanine and adenine (14), *E. coli* strains deficient in Fapy glycosylase exhibit an elevated frequency of spontaneous G to T transversions (15). A functionally similar glycosylase activity has been detected in both yeast and mammalian cells (14), suggesting a conservation of this repair activity between prokaryotes and higher organisms. Because bacterial mutagens are often mutagenic and carcinogenic in humans (16), the study of factors involved in near-UV-mediated mutagenesis in bacteria can facilitate understanding of the effects of stress on higher organisms.

We have previously shown that mutants deficient in Fapy glycosylase are hypersensitive to the lethal effects of near-UV but are no more sensitive to far-UV than wild type (WT) (17), supporting the hypothesis that near-UV causes a lethal form of damage not significantly represented after far-UV exposure. In the present study, we examined the mutational spectrum caused by near-UV using isogenic WT and *fpg*⁻ strains of *E. coli*, predicting that the mutational spectrum of near-UV would be distinct from that of far-UV. In addition, we expect that if GO is an important near-UV-induced lesion, the frequency of induced G to T transversions should be elevated in a strain deficient in Fapy glycosylase relative to a WT strain.

MATERIALS AND METHODS

Bacterial strains, phage and growth conditions. The genotypes of *E. coli* strains used in this study are listed in Table 1. The *fpg*::kan′ was transduced into the CSH100 to CSH106 using a P1vir lysate prepared on BH20 (*fpg*::kan′) as described elsewhere (18). All cultures were grown aerobically to saturation in Luria–Bertani (LB) (Gibco BRL, Burlington, ON) broth at 37°C. The LB agar plates were prepared by adding 1.5% (wt/vol) agar to LB broth. Minimal agar plates contained M9 minimal salts (Gibco BRL) supplemented with 27 μg/mL thiamine hydrochloride (vitamin B1), 1 m*M* $MgSO_4$, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (50 μg/mL) and either 75 μg/mL phenyl-β-D-galactoside (Pgal) (Diagnostic Chemicals Limited, Charlottetown, PEI), 0.2% glucose or 0.2% lactose as indicated. The LB agar plates were incubated at 37°C overnight, while minimal agar plates were incubated at 37°C for 2 days before being examined.

Far-UV irradiation. Overnight cultures were centrifuged and resuspended in an equal volume of M9 minimal salts. An appropriate dilution was spread onto either glucose minimal agar, lactose minimal agar or Pgal minimal agar. The plates were irradiated using two parallel germicidal lamps (NIS, G15T8GL-15) at a distance of 30 cm from the uncovered plate surface. Using a UV digital radiometer and a 254 nm sensor (Ultraviolet Products Inc., San Gabriel, CA), the fluence rate was determined to be 4.7 W/m². Unirradiated control plates were incubated in parallel. Results are expressed as the mean of three independent irradiations. Percent survival was calculated by comparing the number of colonies on an irradiated plate with that of an unirradiated control. Mutational frequencies were corrected for survival and are reported as mutants per 10^8 surviving cells. To evaluate differences between the WT and *fpg* response to far-UV, the logarithm of surviving fraction or number of *lacI* mutants per 10^8 cells were regressed against UV fluence, and the resultant slope regression estimates were compared using Student's *t* test (19).

Near-UV irradiation. Near-UV irradiations were performed as described elsewhere with slight modification (20). Overnight cultures were centrifuged and resuspended in M9 minimal salts. Appropriately diluted aliquots of resuspended cultures were added directly to 2.5 mL minimal soft agar (0.75% agar [wt/vol]) and poured in a soft agar overlay into 60 × 15 mm petri plates and allowed to dry at room temperature for 2 h. Plates were placed face down, without lids, on a near-UV-transmitting (nominal cutoff = 290 nm) filter (Fisher Scientific, Ottawa, ON; cat no. FB-TI-105A) suspended above four parallel Phillips F20T12/BL (20 W bulbs, peak emission = 360 nm), and the plates were maintained at approximately 30°C. The fluence rate at 360 nm was determined to be 1300 μW/cm² using a UV digital radiometer equipped with a 360 nm sensor while the fluence rate at 310 nm was 33 μW/cm². The levels of contaminating UVC radiation were determined to be less than 0.1 μW/cm² (the detection limit of the radiometer). Unirradiated plates were incubated in parallel and results are the mean of three independent irradiations. Percent survival and mutation frequencies were determined as described above. To test if the mutational spectra caused by near-UV, far-UV and 4-nitroquinoline 1-oxide (4-NQO) were significantly different, chi-square tests (19) were performed using 2 × 6 contingency tables in which columns were treatments and rows were the type of induced mutation (i.e. all classes of transversions and transitions).

4-NQO assay. The 4-NQO assay was performed as described by Ruiz-Laguna *et al.* (21). Briefly, overnight cultures were resuspended in M9 minimal salts to an approximate density of 5 × 10^4 cells/mL. A 1 mL aliquot of resuspended culture was incubated for 20

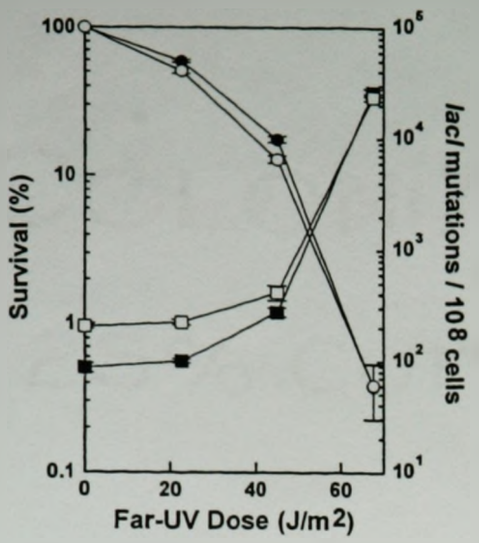

Figure 1. Effect of far-UV irradiation on the survival and mutation frequency of CSH100 and an *fpg* derivative. assays were performed as described in the Materials and Methods. Symbols: circles, survival; squares, mutation frequency; closed, WT; open, *fpg*. Each point represents the average of three independent experiments. Error bars represent the standard error (±) of the mean of three trials.

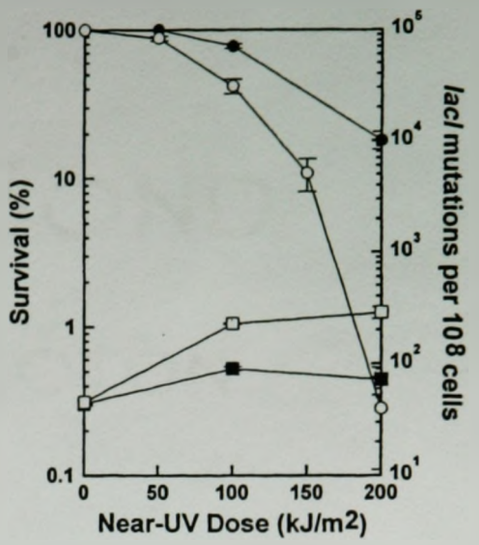

Figure 2. Effect of near-UV irradiation on the survival and mutation frequency of CSH100 and an *fpg* derivative. Irradiation was performed as described in the Materials and Methods. Symbols: circles, survival; squares, mutation frequency; closed, WT; open, *fpg*. Each point represents the average of three independent experiments. Error bars represent the standard error (±) of the mean of three trials.

min at 37°C with the indicated concentrations of 4-NQO (Aldrich Chemical Co., Milwaukee, WI), dissolved in dimethyl sulfoxide (DMSO). Cultures were gently agitated during the incubation period. A 100 μL aliquot was withdrawn and plated onto glucose minimal plates to assess viability. Results reported are the average of three independent trials. Control cultures (containing DMSO alone) were incubated in parallel. Percent survival was calculated by comparing 4-NQO-treated samples with DMSO controls. The mutational assays for 4-NQO were performed essentially as described above. The overnight cultures were resuspended to a density of 5×10^9 cells per mL and aliquots were plated on either lactose minimal media or Pgal minimal media as appropriate.

RESULTS

Mutation assay

To assess the importance of Fapy glycosylase in protecting the cell against the lethal and mutagenic effects of near-UV, far-UV and 4-NQO, we transduced an *fpg*::kan marker into an isogenic series of *E. coli* strains containing an episome with the *lacZ* gene inserted into it. Each strain harbors an episome with a different single base pair substitution in codon 461 of the *lacZ* gene, the structural gene for β-galactosidase (22). *Escherichia coli* strains containing a *lacZ* gene mutated at this glutamic acid codon are unable to utilize lactose as a carbon source (23). Restoration of the active site glutamic acid occurs *via* a specific transition or transversion event dependent upon the episome used and can be easily scored on minimal plates containing lactose as the sole carbon source and supplemented with the indicator dye, X-gal (22). This mutational assay has been previously used to examine near-UV mutagenesis in superoxide dismutase-deficient strains of *E. coli* (24). Consistent with previously published results (15), a deficiency in Fapy glycosylase resulted in an elevated rate of spontaneous G to T transversions. The spontaneous mutational spectra of each treatment were compared using a χ^2 test and found not to be significantly different (WT $P = 0.4069$, *fpg* $P = 0.7613$). Differences in the spontaneous reversion frequency for strains containing F'102 and F'104 may be due to differences in method between experimental treatments. Such variation in spontaneous control rates prior to different mutagenic treatments has been previously observed (22).

Strains lacking Fapy glycosylase are more sensitive to the lethal and mutagenic effects of near-UV

Strains lacking Fapy glycosylase were more sensitive to the lethal ($P = 0.011$) and mutagenic effects of near-UV (Fig. 2) compared to WT, suggesting Fapy glycosylase may be important in the repair of near-UV-induced lesions. The Fapy glycosylase mutants were no more sensitive than WT to the lethal effect of either far-UV ($P = 0.10$) or 4-NQO ($P = 0.23$). Although Fapy glycosylase mutants showed an elevated *lacI* mutation frequency relative to WT after either treatment, this increase was significant for 4-NQO ($P = 0.0028$) but not for far-UV ($P = 0.10$) (Figs. 1 and 3), similar to previously published results (21). The predominant mutations generated by far-UV are C to T transitions (Table 2), considered indicative of either a CC dimer or CC (6-4) photoproduct as the premutagenic lesion. Although near-UV does cause a significant increase in the number of C to T transitions in both WT ($P = 0.027$) and *fpg* ($P = 0.03$) strains following 50 kJ/m² irradiation, the fluence levels required were approximately 2000 times higher. The observed increases in C to T transitions in either the far-UV or the near-UV experiments were independent of Fapy glycosylase ($P = 0.083$) (Tables 2 and 3). Unlike far-UV, the predominant mutation generated by near-UV was a G to T transversion. In a Fapy glycosylase-deficient mutant, the frequency of this mutation was 16-fold higher. These results are consistent with a premutagenic role for GO lesions following near-UV irradiation. The number of G to T transversions

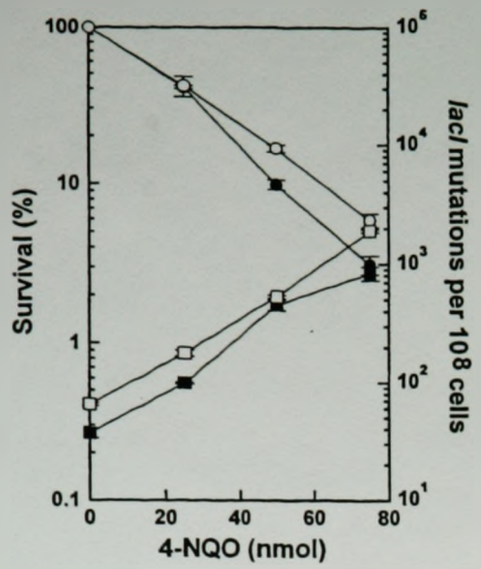

Figure 3. Effect of 4-NQO treatment on the survival and mutation frequency of CSH100 and an *fpg* derivative. Assays were performed as described in the Materials and Methods. Symbols: circles, survival; squares, mutation frequency; closed, WT; open, *fpg*. Each point represents the average of three independent experiments. Error bars represent the standard error (±) of the mean of three trials.

was elevated four-fold in an *fpg* mutant compared to wild type following far-UV irradiation. Far-UV generates a number of photolesions that can be excised by Fapy glycosylase, including 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (25). Such lesions may account for the observed increase in G to T transversions following far-UV irradiation. Unlike far-UV, near-UV did not cause a detectable increase in the number of G to C transversions or A to G transitions. Because the overall mutational spectrum caused by near-UV was significantly different from that caused by far-UV in either WT ($P < 0.0001$) or *fpg* ($P < 0.0001$) backgrounds, the types of premutagenic lesions generated by near-UV are probably different from those generated by far-UV.

Consistent with previously published results (24), the frequency of G to T transversions and C to T transitions observed at codon 461 of the episome-borne β-galactosidase gene was elevated relative to spontaneous levels following irradiation of WT *E. coli* with low doses of broadband near-UV. However, unlike Knowles *et al.* (24), we did not observe a decline in the mutation frequency at higher near-UV doses—this may be due to a difference in light sources used.

Strains lacking Fapy glycosylase have elevated levels of C to T transitions, G to C transversions and G to T transversions following treatment with 4-NQO

Treatment of cells with 4-NQO resulted in elevated levels of C to T transitions, G to C transversions and G to T transversions (Table 4), similar to previously published results (21). The induction of these mutations was elevated 5-, 20- and 25-fold in a strain deficient in Fapy glycosylase. These results suggest that the two major guanine adducts generated by 4-NQO, dGuo-N2-AQO and dGuo-C8-AQO (21) may be substrates for Fapy glycosylase. Because 4-NQO induces the formation of GO lesions *via* a ROS-independent mechanism (21), this may be the premutagenic lesion causing the G to T transversions.

Addition of exogenously added catalase rescues WT and *fpg*⁻ strains from the lethal effects of near-UV

Inclusion of 10 μg/mL bovine liver catalase in plate media resulted in a significant decrease in sensitivity to the lethal effects of near-UV for WT cells ($P < 0.001$) and for *fpg* cells ($P < 0.01$) in accordance with previously published results (26). In the absence of catalase, a dose of 400 kJ/m² resulted in over 99% lethality for both WT and *fpg* cells. When catalase was included in the media, killing was reduced to less than 60% for both strains (results not shown). These results suggest that hydrogen peroxide is involved in near-UV-mediated lethality. Because hydrogen peroxide does not interact directly with DNA (27), it is likely that the observed lethality is due to hydroxyl radicals produced from hydrogen peroxide in iron-catalyzed Fenton reactions (28). The presence of catalase had no effect on far-UV-mediated lethality for either the WT ($P = 0.90$) or the *fpg* ($P = 0.18$) strain (results not shown) consistent with the idea that the damage caused by far-UV is not ROS-dependent.

DISCUSSION

Though the mechanism by which near-UV damages DNA is not completely understood, evidence suggests that it is more

Table 2. Effect of *fpg* on the frequency of *lacZ* reversion in response to far-UV*

Genotype	Mutagen	Dose (J/m²)	Reversion event					
			AT to CG	GC to AT	GC to CG	GC to TA	AT to TA	AT to GC
Wild type	Far-UV	0	0.7 (0.3)	7.14 (0.88)	0.00	1.53	0.00	0.00 (0.00)
		22.8	3.2 (1.6)	85 (6.5)	2.4 (1.2)	17 (3.4)	6.3 (1.0)	19 (1.2)
		45.6	9.6 (2.7)	326 (3.6)	4.1 (0.0)	64 (17)	30 (12)	56 (1.4)
		68.4	79 (0.0)	4600 (550)	52 (26)	314 (45)	576 (138)	863 (120)
fpg	Far-UV	0	0.4 (0.2)	10.9 (1.3)	0.00	57 (4.1)	1.5 (0.6)	0.00 (0.00)
		22.8	7.1 (0.5)	74 (1.5)	0.51	96 (7.7)	12 (2.2)	22 (10)
		45.6	15 (7.7)	548 (53)	15 (4.4)	342 (71)	71 (22)	130 (23)
		68.4	196 (122)	8970 (1000)	28 (28)	1200	251 (84)	2200 (440)

*Mutagenesis with far-UV was performed as described in the Materials and Methods. Mutation rates were corrected for survival and are expressed as *lacZ*⁺ per 10^8 cells (±standard error).

Table 3. Effect of *fpg* on the frequency of *lacZ* reversion in response to near-UV*

Genotype	Mutagen	Dose (kJ/m²)	Reversion event					
			AT to CG	GC to AT	GC to CG	GC to TA	AT to TA	AT to GC
Wild type	Near-UV	0	0.17	0.14	0.0043	1.4 (0.7)	0.5 (0.1)	0.016
		25	—	0.49 (0.1)	—	4.6 (0.9)	0.5 (0.09)	—
		50	—	0.8 (0.26)	—	7.0 (0.8)	0.7 (0.08)	—
		75	—	1.1 (0.5)	—	9.8 (2.8)	1.8 (0.9)	—
		100	0.35	23 (4.6)	—	58 (8.3)	20 (3.8)	—
		125	0.33	30 (1.5)	—	212 (80)	33 (1.5)	—
fpg	Near-UV	0	0.18	0.17	0.00 (0.00)	7.5 (0.8)	0.8 (0.1)	0.00 (0.00)
		25	—	0.4 (0.03)	—	20 (3.0)	0.8 (0.2)	—
		50	—	0.9 (0.18)	—	46 (1.0)	1.5 (0.2)	—
		75	—	1.2 (0.24)	—	193 (25)	6.7 (0.9)	—
		100	0.29	39 (8.2)	—	620 (42)	12.7 (1.3)	—
		125	0.2 (0.07)	81 (15)	—	3400 (111)	93 (16)	—

*Mutagenesis with near-UV was performed as described in the Materials and Methods. Mutation rates were corrected for survival and are expressed as *lacZ*⁺ mutants per 10^8 cells (±standard error).

complex than that of far-UV. The mechanism appears not only to involve the production of pyrimidine dimers and (6-4) photoproducts but also other lesions that are produced by ROS. The most common types of mutations observed in far-UV-irradiated DNA are C to T transitions at dipyrimidinic sites, followed by T to C transitions and T to A transversions (6). The C to T transitions arise following replicative bypass (translesion synthesis) of a cytosine–pyrimidine dimer or (6-4) photoproduct (6). During translesion synthesis in *E. coli*, the UmuDC proteins help catalyze the insertion of a base opposite a miscoding or noninstructional lesion, allowing DNA polymerase to continue replication. If an adenine is inserted opposite the cytosine–pyrimidine dimer or cytosine-containing (6-4) photoproduct, C to T transitions can arise at cytosine–pyrimidine sites. Increases in the frequency of C to T transitions relative to spontaneous levels have previously been observed in near-UV-irradiated cells (29,30). Irradiation of Chinese hamster ovary cells with simulated solar light increases the number of C to T transitions that occur at dipyrimidine sites compared to spontaneous levels (29). Likewise, C to T transitions are the predominant mutation in plasmid DNA irradiated *in vitro* with monochromatic 313 nm light and then transfected into simian cells (30). In addition, the number of tandem C to T transitions is elevated in p53 genes isolated from nonmelanoma tumors of xeroderma pigmentosum patients (31). Consistent with these results, we observed a significant increase in the number of C to T transitions in both WT and *fpg* backgrounds following 50 kJ/m² exposure to broadband near-UV irradiation. This increase in C to T transitions is independent of Fapy glycosylase, suggesting the premutagenic lesion is not a modified guanine residue (such as the GO lesion). Because the C to T transition in this study occurs at a dipyrimidine site (cytosine–cytosine) and near-UV wavelengths induce both pyrimidine dimer and (6-4) photoproduct formation (7), cytosine–cytosine dimers or (6-4) photoproducts are the most likely lesion resulting in the observed increase in C to T transitions in the near-UV-irradiated cells. This, however, does not preclude the involvement of oxidized cytosine residues in the observed increase in C to T transitions. 5-Hydroxycytosine and 5-hydroxyuracil, stable oxidation products of cytosine, have previously been shown to stimulate C to T transitions (32, 33). Both 5-hydroxycytosine and 5-hydroxyuracil are efficiently excised by endonuclease III and Fapy glycosylase (34); 5-hydroxyuracil is also a substrate for uracil DNA *N*-glycosylase (34). An examination of the mutational spectrum induced by near-UV in *E. coli* strains deficient in one or more of these enzymes should clarify the role of near-UV in the formation of these premutagenic lesions.

Table 4. Effect of *fpg* on the frequency of *lacZ* reversion in response to 4-NQO*

Genotype	Mutagen	Dose (nmol)	Reversion event					
			AT to CG	GC to AT	GC to CG	GC to TA	AT to TA	AT to GC
Wild type	4-NQO	0	0.7 (0.1)	1.0 (0.1)	0.0 (0.0)	1.7 (0.5)	1.9 (0.4)	0.1 (0.1)
		25	0.5 (0.2)	1.4 (0.5)	1.1 (0.3)	7.5 (1.2)	2.2 (0.6)	0.4 (0.0)
		50	1.2 (0.3)	2.8 (0.2)	4.2 (2.7)	52 (17)	1.4 (0.4)	0.4 (0.2)
		75	0.8 (0.0)	4.0 (0.4)	7.4 (1.5)	81 (16)	3.2 (0.5)	1.1 (0.3)
fpg	4-NQO	0	0.3 (0.0)	1.3 (0.2)	0.0 (0.0)	5.1 (0.2)	1.5 (0.2)	0.1 (0.1)
		25	0.2 (0.1)	3.1 (0.6)	3.9 (1.9)	57 (8.0)	2.1 (0.6)	0.4 (0.4)
		50	0.4 (0.2)	11 (4.5)	25 (7.0)	645 (49)	2.9 (0.3)	0.3 (0.0)
		75	0.9 (0.5)	21 (4.9)	163 (13)	2246 (190)	3.5 (0.0)	0.4 (0.0)

*Mutagenesis with 4-NQO was performed as described in the Materials and Methods. Mutation rates were corrected for survival and are expressed as *lacZ*⁺ mutants per 10^8 cells (±standard error).

Other types of mutations also arise as a consequence of exposure to near-UV irradiation, including G to C and T to G transversions (29,35). The G to C and T to G transversions are indicative of ROS-mediated DNA damage and can arise *via* the formation of the stable mispairs 8-OHdG:dG and 8-OHdG:dA, respectively (36). *Escherichia coli* strains deficient in MutT (8-hydroxy-2′-deoxyguanine dGTPase) have an elevated spontaneous rate of T to G transversions (as reviewed in Michaels and Miller (37)). Recently, human (38), mouse (39) and rat (40) homologs of MutT have been identified. The observed increase in T to G transversions reported by Drobetsky *et al.* (29) supports a role of GO as a near-UV premutagenic lesion. A previously observed increase in G to C transversions is also consistent with a premutagenic role for GO following near-UV irradiation (35). Although we observed an almost two-fold increase in the number of T to G transversions following irradiation with 100 kJ/m^2 of broadband near-UV, this was not found to be statistically significant.

The predominant near-UV-induced mutation class observed in this study following near-UV irradiation was the G to T transversion. The near-UV-induced mutational frequency was elevated in an *fpg*-deficient strain, suggesting the premutagenic lesion is excised by Fapy glycosylase. Taken with previously observed increases in GO levels following near-UV irradiation (12) is likely that this mispairing base is an important near-UV lesion because G to T transversions result when GO mispairs with adenine during DNA replication.

Other *E. coli* repair mutants that recognize and repair oxidatively damaged DNA, such as exonuclease III, endonuclease IV and endonuclease III, may also protect the cell against near-UV damage. We are currently testing the hypothesis that one or more of these enzymes is important in protecting the cell from near-UV-mediated lethality and/or mutagenesis. Preliminary results indicate that strains lacking these DNA repair enzymes are sensitive to the lethal effects of near-UV but not far-UV. Evaluation of the importance of these enzymes may ultimately help in the identification of human homologs that protect against near-UV-mediated mutation.

Acknowledgements—This work was supported by a Terry Fox New Investigator grant from the National Cancer Institute of Canada to H.E.S. We thank Suman Mukhopadhyay, Jonathon Audia and Michael Shennan for many helpful discussions and Marg Biggs for help in preparation of the manuscript.

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CHAPTER 3

Preface

The following manuscript, entitled "Endonuclease III and Endonuclease IV Protect *Escherichia coli* from the Lethal and Mutagenic Effects of Near-UV Irradiation", is intended for submission to *Photochemistry and Photobiology*. The paper describes the role of endonuclease III, endonuclease IV, and exonuclease III in the repair of DNA damage by the UVA component of sunlight. This work is an extension of our previous studies on the effects of near-UV on DNA repair: Near-Ultraviolet light causes a Fapy Glycosylase-Dependent Increase in G to T transversions (*Photochem. Photobiol.* **65**, 543-549.); and "Role of Fapy Glycosylase and UvrABC Excinuclease in the Repair of UVA (320-400 nm)-mediated DNA damage in *Escherichia coli* (*Photochem. Photobiol.* **63**, 68-73).

The work presented in this study was performed by D.M. Serafini. The paper was written by D.M. Serafini.

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August 18, 1997

ABSTRACT

In contrast to the DNA damage caused by far-UV ($\lambda < 290$ nm), near-UV ($290<\lambda<400$) induced DNA damage is partially oxygen-dependent, suggesting the involvement of reactive oxygen species (ROS). Not surprisingly, enzymes that are known to be important in the repair of oxidative DNA damage also have a role in protecting the cell from the lethal effects of near-UV. To test the hypothesis that enzymes that protect cells from oxidative DNA damage are also involved in preventing near-UV mediated DNA damage, isogenic strains deficient in one or more of exonuclease III (*xthA*), endonuclease IV (*nfo*), and endonuclease III (*nth*) were exposed to increasing fluences of far-UV and near-UV. A far-UV dose of 50 J/m^2 and a near-UV dose of 400 kJ/m^2 were found to be equally lethal, reducing survival of the wildtype strain to similar levels (7.19 % and 15.6 % respectively). All strains, with the exception of the *nth* single mutant were found to be hypersensitive to the lethal effects of near-UV relative to a wild type strain. Double mutants were no more sensitive to near-UV mediated lethality than single mutants. However, a triple mutant strain (*nth nfo xthA*) exhibited the greatest sensitivity to near-UV mediated lethality. The triple mutant was more sensitive than the *nfo xthA* double mutant to the lethal effects of near-UV. The additional absence of endonuclease III in a strain lacking exonuclease III and endonuclease IV, resulted in a synergistic increase in lethality for a near-UV exposure, but not far-UV. A near-UV specific protective role for endonuclease III was confirmed using a forward mutation assay, which also revealed a synergistic sensitivity for the triple mutant compared to the *nfo xthA* disrupted strain.

However, the triple mutant was no more sensitive to the mutagenic effects of far-UV than the *nfo xthA* double mutant. These data suggests that exonuclease III, endonuclease IV, and endonuclease III are important in protection against near-UV induced DNA damage.

INTRODUCTION

Solar ultraviolet radiation is comprised of three parts. UVC, or far UV, is the designation for wavelengths between 100 nm and 290 nm. Longer wavelengths are classified as either UVB (290 nm < λ < 320 nm) or UVA (320 nm < λ < 400 nm), which are collectively referred to as near-UV. Since far-UV is effectively filtered out by stratospheric ozone, wavelengths in the near-UV range are of greater biological relevance. Recent declines in the levels of protective ozone, which can be attributed to the use of fossil fuels and chlorofluorocarbon containing products have resulted in an increase in surface near-UV (Kerr and McElroy 1993). The UVB component of sunlight is genotoxic, and considered the major risk factor in the development of squamous cell carcinoma of the skin (SCC) (Brash *et al.*, 1991). Therefore, it has been predicted that further increases in exposure to UVB will result in elevated levels of skin cancer (Doll and Peto, 1981). In addition, a role for UVA in solar mutagenesis has been demonstrated. Wavelengths greater than 320 nm are mutagenic and carcinogenic in rodents (Drobetsky et al., 1995; Sterenborg and van der Leun, 1990). The contribution of UVA to the mutational spectrum of sunlight has been demonstrated in Chinese hamster ovary cells. When exposed to UVA, these cells exhibit a large induction in T –> G transversions (Drobetsky *et al.*, 1995).

Near-UV can , like far-UV, can induce the formation of both cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts (Matsunaga *et al.*, 1991). However, near-UV is distinct from far-UV in that much of the DNA damage induced by

the former is oxygen-dependent and is similar to that caused by oxidative insult (Hattori-Nakakuki *et al.*, 1994). Enzymes known to be important in the repair of far-UV and oxidative DNA damage may, therefore, also be part of the cell's near-UV repair repertoire. Consistent with this idea, we have found UvrABC excinuclease and formamidopyrimidine (Fapy) DNA glycosylase are important in protecting *Escherichia coli* from the lethal effects of near-UV (Shennan *et al.*, 1996). Near-UV also causes an increased mutation rate in Fapy deficient cells compared to a wildtype (WT) strain, primarily due to increased C to T transitions and G to T transversions (Palmer *et al.*, 1997).

Other enzymes, especially those involved in the repair of oxidative DNA lesions, may also be relevant. Mutants deficient in exonuclease III, which is a multifunctional repair enzyme that possesses apurinic/apyrimidinic (AP) endonuclease activity, are near-UV hypersensitive (Sammartano and Tuveson, 1983). AP sites can result from the oxidation of the DNA backbone or through the removal of an oxidized base by a glycosylase. (Barzilay and Hickson, 1995). To further test the idea that AP endonucleases protect the cell from the lethal and mutagenic effects of near-UV, we assayed the near-UV response of strains deficient in one or more of exonuclease III (*xthA*), endonuclease IV (*nfo*), or endonuclease III (*nth*) relative to an appropriate wild type strain. Since these three enzymes share the common function of AP site repair, it is advantageous to simultaneously assess the effect of multiple mutations in isogenic strains. We have examined the near-UV response of a set of isogenic DNA repair mutants that were

developed previously (Cunningham *et al.*, 1986) to infer the involvement of particular lesions in near-UV mediated lethality and mutagenesis. Since bacterial mutagens are often human carcinogens (McCann *et al.*, 1975), and homologous repair enzymes are present in both organisms (Table 1), the study of near-UV mediated lethality and mutagenesis in *E. coli* should enhance our understanding of near-UV mediated DNA damage in human cells.

METHODS

Bacterial Strains and Growth Conditions

The *E. coli* strains used in this study are listed in Table 2. Cultures were grown from single colonies in Luria-Bertani (LB) (Gibco BRL, Burlington, ON) broth supplemented with antibiotics as appropriate. Cultures were grown aerobically (unless otherwise stated) at 37 °C in flasks with a 1:5 volume to volume ratio of liquid media to air ratio on a shaking platform at 200 rpm. Minimal agar plates containing M9 minimal salts (Gibco BRL) were supplemented with 0.2 % glucose, 27 μg/mL thiamine hydrochloride (vitamin B1), 1 mM $MgSO_4$, 0.004% uracil, and appropriate amino acids (Miller, 1992). All antibiotics and amino acids were obtained from Sigma Chemical Company (St. Louis, MO). Optical densities (OD) of cultures were measured using a Shimadzu UV-1201 spectrophotometer at a wavelength of 600 nm.

The strains used for irradiation were obtained from B. Weiss (Cunningham *et al.*, 1986) with slight modifications. An Hfr strain, EG333 (*Δgpt-lac car-96::Tn*10), was conjugated with AB1157 and the isogenic DNA repair deficient mutants by standard methods (Miller, 1992). Conjugation was terminated using a mating interrupter as described in Miller (1972). A 100 μL aliquot was plated on LB supplemented with streptomycin (100 μg / mL), tetracycline (15 μg / mL), and X-gal (50 μg / mL) (Diagnostic Chemicals, Charlottetown, PEI). Plates were incubated overnight at 37 °C. A single colony for each *lac*-deletion tetracycline resistant strain was purified and tested to

ensure that all strains were isogenic (Table 2). Each of the *lac* deleted strains were then conjugated with CSH100, which contains an episome carrying the *lacZ* gene (Cupples and Miller, 1989). The F' lac deletion strains (HS1200 - HS1270) were used for all irradiations described here (Table 2).

Far-UV irradiation

Overnight cultures were resuspended in M9 minimal salts. Appropriate dilutions were spread onto glucose minimal agar supplemented with appropriate amino acids. The plates were irradiated by two parallel germicidal lamps (NIS, G15T8GL-15) a distance of 30 cm from the uncovered plate surface. The fluence rate at 254 nm was 4.7 W/m^2, which was determined by the use of a UV digital radiometer and a 254 nm sensor (Ultraviolet Products Inc., San Gabriel, CA). Unirradiated control plates were incubated in parallel. Results are reported as the average of three independent irradiations. Percent survival was determined by comparing the average number of colonies on irradiated plates with that of unirradiated controls. The differences in response of DNA repair mutant strains and WT to far-UV were evaluated by regression of survival curve slopes, and a comparison of the resultant slopes using Student's t test (Sokal and Rohlf, 1995).

Near-UV irradiation

Cultures were treated in the same manner as stated above for far-UV, except that 100 µL aliquots of appropriately diluted resuspended cultures were added to 2.5 mL minimal soft agar (0.75% agar [wt/vol]). The cells were poured in a soft agar overlay onto 60 x 15 mm Petri plates and allowed to dry at room temperature for 2 h prior to

irradiation. Plates were placed face down, without lids on a transilluminator sheet, which was placed 9 cm above 4 parallel Philips F20T12/BL (20 W) bulbs. The surface of the plates was kept at 25°C with a fan. The fluence rate at 360 nm was determined to be approximately 1500 $\mu W/cm^2$, while the fluence rate at 310 nm was 31 $\mu W/cm^2$. Contaminating UVC radiation was determined to be less than 1 $\mu W/cm^2$ (the detection limit of the radiometer). Survival results are reported as stated above for far-UV.

Forward mutation assay

A forward mutation assay was employed to estimate generalized mutation rates (Miller, 1972). *E. coli* cultures will not grow in the presence of trimethoprim unless they have an inactivating mutation in *thyA*, which can be subsequently verified by testing mutants for thymine auxotrophy. Cells were treated as above, except that trimethoprim (15 μg/mL) and thymine (50 μg/mL) were included in the media. Mutation rates were calculated by determining the number of *thyA* mutants per 10^8 surviving cells following exposures to either far-UV or near-UV. Data shown are corrected for spontaneous mutation rates. Induced mutation rates for WT and DNA repair mutants were compared using a Tukey-Kramer test with a fixed significance level of $\alpha = 0.01$ (Sokal and Rohlf, 1995).

RESULTS

Endonuclease III protects E. coli from the lethal effects of near-UV

Exposure of an isogenic series of DNA repair deficient strains to lethal doses of near-UV resulted in survival rates that were markedly different from rates obtained when exposed to a comparable dose of far-UV. At a given UV dose, in which survival for WT was approximately 10%, killing of the DNA repair mutants was greater in the presence of near-UV than for far-UV relative to WT (Figure 1). Survival rates for the *nfo*, *xthA*, *nth nfo*, and *nth nfo* mutant strains exposed to a far-UV dose of 50 J/m^2 were similar to that of the WT strain. Relative to the WT strain, far-UV mediated lethality was only observed for the *nfo xthA* double mutant and the *nth nfo xthA* triple mutant strains. The additional absence of endonuclease III activity in a strain possessing *nfo* and *xthA* mutations did not result in a further increase in lethality, consistent with previously published results (Cunningham *et al.*, 1986).

At a near-UV dose of 400 kJ/m^2, survival rates for all DNA repair deficient strains were lower than WT, with the exception of the *nth* mutant. The *nfo* and *xthA* single mutants were both near-UV hypersensitive relative to the WT strains (P <0.01 and P <0.02 respectively). The additional presence of the *nth* mutation resulted in an additional reduction in survival of an *nfo* mutant (compare *nfo* and *nth nfo* strains in Figure 2B), but not when combined with the *xthA* mutation (compare *nfo* and *nfo xthA* strains in Figure 2A). Nevertheless, all three enzymes are important in protection from near-UV mediated lethality, since the triple mutant strain (*nth nfo xthA*) had the lowest survival rate of any of

the strains in the series. Survival for the triple mutant was significantly lower than all other strains (P <0.005) (Figure2).

Endonuclease IV and exonuclease III protect* E. coli *from the mutagenic effects of far-UV.

The involvement of reactive oxygen species in near-UV mediated DNA damage is inferred from studies showing that cells lacking enzymes that repair oxidative DNA damage, such as exonuclease III and superoxide dismutase, are hypersensitive to the lethal and mutagenic effects of near-UV, but not necessarily far-UV (Sammartano and Tuveson, 1983; Hoerter *et al.*, 1989). We have previously shown that Fapy deficient cells are hypersensitive to the mutagenic effects of near-UV, but not far-UV (Palmer *et al.*, 1997). Since Fapy recognizes the oxidized purine 7, 8-dihydro-8-oxoguanine (8oxoG) (Boiteux, 1993), we expected to find differences in the induced mutation rates of other enzymes that are important in the repair of oxidative DNA damage, such as endonuclease III, endonuclease IV, and exonuclease III.

Exposure of an isogenic series of DNA repair deficient strains to comparably lethal doses of near-UV and far-UV (400 kJ/m^2 and 50 J/m^2 respectively) resulted in differences in the induced mutation rates. Following a far-UV exposure of 50 J/m^2, induced mutation rates for the DNA repair deficient strains, except *nfo xthA* and *nth nfo xthA* strains, were similar to WT (Figure 3). The additional absence of endonuclease III in the triple DNA repair mutant did not result in an increase in far-UV mediated mutagenesis relative to the strain already devoid of endonuclease IV and exonuclease III. This suggests that

endonuclease III is not important in the repair of non-lethal lesions generated by far-UV.

Endonuclease III, endonuclease IV, and exonuclease III protect* E. coli *from the mutagenic effects of near-UV.

All DNA repair deficient strains tested were hypermutable by near-UV (Figure 4). Relative to WT, the *nth* and *nfo* single DNA repair mutants exhibited two and five fold increases in the induced mutation rate respectively. The induced rate of *thyA* mutants in an exonuclease III-deficient strain (*xthA*) was 36 fold higher than that of WT. The additional absence of endonuclease III in an *nfo xthA* DNA repair mutant resulted in a synergistic increase in the induction of *thyA* mutations (compare *nfo xthA* and *nth nfo xthA* strains in Figure 4). Endonuclease III was important in protecting *E. coli* from the mutagenic effects of near-UV, but not far-UV. The triple mutant strain (*nth nfo xthA*) had an induced mutation rate that was 800 fold higher than WT, and was significantly greater than all other strains in the series ($P < 0.01$), supporting a protective role for all three enzymes against the mutagenic action of near-UV.

DISCUSSION

Near-UV is similar to far-UV, since it can cause DNA damage directly through the production of cyclobutane-pyrimidine dimers and (6-4) pyrimidine-pyrimidone photoproducts (Matsunaga *et al.*, 1991). Absorption of near-UV photons by a cellular photosensitizer, such as NADH, results in the production of reactive-oxygen species (ROS), which can damage DNA (Burchuladze and Fraikin, 1991). It is likely that NADH donates electrons for the *in vivo* reduction of Fe (III) to Fe (II), which can potentiate hydroxyl radical formation through Fenton chemistry (Imlay and Linn, 1988). This possibility is supported by *in vitro* evidence that NADH powers Fenton-mediated damage to deoxycytidine residues in DNA (Luo *et al.*, 1996). Hydroxyl radicals cause single strand breaks in DNA exposing a 3' phosphate or 3' phosphoglycolate, which block the replicative polymerase unless they are removed by an enzyme possessing phosphodiesterase activity (eg., exonuclease III or endonuclease IV) (Demple *et al.*, 1986, Cunningham *et al.*, 1986, Imlay and Linn, 1988).

Some reactive oxygen species, namely hydroxyl radicals and singlet oxygen, attack individual bases. Oxidized bases are removed by glycosylases resulting in apurinic / apyrimidinic (AP) sites. If AP sites are bypassed, misincorporation may occur, resulting in the fixation of mutations in subsequent rounds of DNA replication. (Kunkel 1984, Wallace 1988, Barzilay and Hickson, 1995). AP sites can also occur spontaneously or through oxidation of the deoxyribose backbone itself (Haring *et al.*, 1994). The accumulation of large numbers of AP sites in bacteria leads to a loss of viability (Cunningham *et al.*, 1986).

In *E. coli* cell-free extracts, exonuclease III accounts for 85% of total AP endonuclease activity, while endonuclease IV is responsible for approximately 5 % of all AP endonuclease activity (Weiss et al., 1988; Levin *et al.*, 1988) Exonuclease III is known to protect *E. coli* from the lethal effects of near-UV (Sammartano and Tuveson, 1983), while the role of endonuclease IV and endonuclease III in protection against near-UV mediated DNA damage has not been examined. Endonuclease III is a 25 kDa DNA glycosylase, which possesses AP lyase (Breimer and Lindahl, 1984; Baily and Verly, 1987). The present study examines the role of these three enzymes in the protection against the mutagenic and lethal effects of near-UV.

At a near-UV dose of 400 kJ/m^2, survival rates were lower for all repair deficient strains with the exception of the *nth* mutant. This is consistent with the idea that near-UV produces AP sites. AP producing agents such as methylmethanesulfonate and mitomycin C do not result in hypersensitivity of an *nth* mutant alone or in combination with other mutations (Cunningham *et al.*, 1986). The apparent insensitivity of *nth* mutants to the above agents and near-UV (this study) may be explained by the substrate specificity of endonuclease III. Cunningham *et al.*, (1986) have previously hypothesized that endonuclease III may exist solely for its glycosylase function, and that its AP activity may actually be detrimental to the cell, since it leaves a poor primer for DNA polymerization reactions. Unlike endonuclease IV and exonuclease III, which cleave DNA 5' to AP sites, endonuclease III is an AP lyase that cleaves 3' to AP sites (as reviewed in Wallace, 1988). Following 3' cleavage of an AP site, the terminal 3' sugar must be removed to generate a 3'

OH group that can serve as a primer for DNA polymerase I (Warner *et al.*, 1980). Cunningham *et al.* (1986) found that exposure of cells to oxidizing agents resulted in increased lethality for *nfo* and *xthA* mutants. Consistent with this, *nfo* and *xthA* mutants were sensitive to the lethal effects of near-UV, while the *nth* single mutant was not (this study). During an oxidative stress, the additional presence of the *nth* mutation does not result in an increase in lethality in cells deficient in endonuclease IV and exonuclease III (Cunningham *et al.*, 1986). However, a deficency in endonuclease III can sensitize the cell to near-UV when the majority of the cell's apurinic/apyrimidinic (AP) activity has been abolished through *nfo* and *xthA* mutations (this study). All three enzymes are important in protection from near-UV mediated lethality, since the triple mutant strain (*nth nfo xthA*) had the lowest survival rate of any of the strains in the series. Survival for the triple mutant was significantly lower than all other strains ($p < 0.005$) (Figure 4). Therefore, a near-UV protective role for endonuclease III exists when the majority of the cell's AP endonuclease activity has been removed through *nfo* and *xthA* mutations. The near-UV hypersensitivity of the triple mutant may be due to a greater accumulation of AP sites than for an *nfo xthA* double mutant. Alternatively, the accumulation of thymine-glycols, which are the predominant substrate for endonuclease III, may contribute to the observed increase in lethality. Thymine glycols are lethal if not removed, since they block the progression of DNA polymerase (Hariharen *et al.*, 1977, Moran and Wallace, 1985, Hayes *et al.*, 1988).

A far-UV dose of 50 J/m^2 resulted in a significant increase in lethality for the *nfo*

xthA double mutant and the *nth nfo xthA* triple mutant, consistent with the original data of Cunningham *et al.* (1986). Far-UV can produce AP sites *in vitro*, suggesting that the sensitivity of strains lacking both exonuclease III and endonuclease IV may be due to the *in vivo* production of AP sites following exposure to 254 nm radiation (Zuo *et al.* ,1995). The triple mutant (*nth nfo xthA*) was not significantly more sensitive than the *nfo xthA* double mutant to the lethal effects of far-UV ($P > 0.05$), which is consistent with previously published results (Cunningham *et al.*, 1986). Furthermore, the triple mutant was less sensitive to the mutagenic effects of far-UV relative to the *nfo xthA* double mutant. In contrast, the additional loss of endonuclease III activity resulted in a 35 fold increase in the near-UV induced mutation rate compared to the *nfo xthA* double mutant ($P < 0.01$). A spontaneous mutator effect of the *nth* mutant has been previously reported (Cunningham *et al.*, 1986, Jiang *et al.*, 1997), as well as an induced mutator effect at sub-lethal doses of MMS (Cunningham *et al.*, 1986). However, no mutator effect for *nth* has been reported for exposure to oxidizing agents (Weiss *et al.*, 1988, Friedberg *et al.*, 1995). A principal finding of the present study is that the induced mutator effect of the *nth* mutant is specific for longer wavelengths, since equally lethal doses of far-UV and near-UV resulted in an *nth* mutator effect only for the latter Therefore, endonuclease III must repair some non-lethal lesions that are generated by near-UV but not far-UV. For example, oxidized pyrimidines such as 5-hydroxy-2'-deoxycytosine (5-OHdC) and 5-hydroxy-2'-deoxyuracil (5-OHdU) are substrates for endonuclease III (Hatahet *et al.*, 1994), and are mutagenic *in vitro*. Depending on the sequence context, 5-OHdC can

mispair with adenine resulting in C –> T transitions, or with cytosine resulting in C–> G transversions (Purmal *et al.*, 1994).

With the exception of the *nth* single mutant, all DNA repair mutants exposed to near-UV had reduced survival rates relative to WT, demonstrating that endonuclease IV and exonuclease III protect *E. coli* cells from the lethal effects of near-UV. Survival of the *nth nfo xthA* triple mutant was lower than all other strains, which suggests a previously unrecognized role for endonuclease III in the repair of near-UV mediated DNA damage. The relative increase in lethality of an *nth nfo xthA* triple DNA repair mutant strain relative to an *nfo xthA* double mutant exposed to near-UV was not observed for far-UV, which indicates that the mechanism of near-UV damage is distinct from that of far-UV. Endonuclease IV and exonuclease III protect *E. coli* cells from the mutagenic effects of near-UV and far-UV. However, endonuclease III was determined to protect cells from the mutagenic effects of near-UV, but not far-UV.

Another explanation for the response of the triple mutant to the two treatments (near and far-UV) that can not yet be ruled out is the involvement of other repair enzymes that have not been completely characterized. Endonuclease VIII is similar in activity to endonuclease III, since it is an AP lyase that repairs modified pyrimidines (Melamede *et al.*, 1994). Endonuclease III mutants are not hypersensitive to the lethal effects of X-rays (Cunningham and Weiss, 1985), while endonuclease VIII mutants are slightly sensitive to this agent (Jiang *et al.*, 1997). However, a double mutant lacking endonuclease III (*nth*) and endonuclease VIII (*nei*) was more sensitive to killing by X-rays than the single *nei*

mutant. Endonuclease VIII, which has overlapping substrate specificity with endonuclease III, may recognize some X-ray induced lesions that can not be repaired by the latter (Jiang *et al.*, 1997).

Both *nth* and *nei* single mutants exhibit WT sensitivity to H_2O_2, however, the double mutant (*nth nei*) is extremely sensitive to this oxidizing agent (Cunningham and Weiss, 1985; Saito *et al.*, 1997). The ability of endonuclease VIII to compensate for a lack of endonuclease III may depend on the type or amount of oxidative lesions produced. It is possible that endonuclease VIII may compensate for the absence of endonuclease III in an *nth nfo xthA* triple mutant when exposed to far-UV, since this strain is not more sensitive than an *nfo xthA* double mutant. During a near-UV stress, which is known to have a large oxidative component, repair by endonuclease VIII alone may not be sufficient to compensate for a deficiency in endonuclease III, thus explaining the extreme hypersensitivity of the triple mutant (this study). The determination of the types of lesions recognized by these enzymes during a near-UV stress will allow us to better determine the role of each AP endonuclease in DNA repair. We are currently examining the specific mutation rates for the isogenic series of DNA repair mutants using a reverse mutation assay in order to determine , by inference, which pre-mutagenic lesions are caused by near-UV.

Table 1. *E. coli* enzymes important in the repair of near-UV/oxidative DNA damage

Repair Enzyme	Activity	Gene(s)	Induced by...	Effect of loss	Human homolog?
UvrABC	- excinuclease (a)	*uvrABC*	DNA damage (a)	UVC sensitive (a)	*UvrA and UvrC* share homology with human ERCC1 (j)
Fpg glycosylase	- removes 8oxoG and Fapy lesions (b)	*fpg*	ROS (e)	UVA sensitive (h)	protein that cleaves 8oxoG from 8oxoG:C mispairs (k)
Exonuclease III	- 5' AP endonuclease (85% of total activity) (c) - removes 3' blocking groups (d)	*xthA*	starvation (f)	UVA sensitive (i)	AP endonuclease (HAP1) (l)
Endonuclease IV	- 5' AP endonuclease (5% of total activity) (c) - removes 3' blocking groups (d)	*nfo*	ROS (g)	addressed in this study	not known
Endonuclease III	- 3' AP endonuclease (n) - glycosylase (removes oxidized pyrimidines and fragmentation products) (o)	*nth*	not known	addressed in this study	human endonuclease III homolog 1 (hNTH1) (m)

Table 1. References

a) reviewed in Van Houten, 1990 and Friedberg *et al.*, 1995 pp. 191-232

b) Boiteux, 1993

c) Weiss *et al.*, 1988; Levin *et al.*, 1988

d) Demple *et al.*, 1986

e) Kim *et al.*, 1996

f) Sammartano *et al.*, 1986, Sak *et al.*, 1989

g) Chan and Weiss, 1987, Tsaneva and Weiss, 1990

h) Shennan *et al.*, 1996; Palmer *et al.*, 1997

i) Sammartano and Tuveson, 1983

j) van Duin *et al.*, 1988

k) Chung *et al.*, 1991; Lee *et al.*, 1993; Nagashima *et al.*, 1997

l) Demple *et al.*, 1991, Robson and Hickson , 1991

m) Aspinwall *et al.*, 1997; Hilbert *et al.*, 1997

n) Warner *et al.*, 1980

o) Breimer and Lindahl, 1984, Hatahet *et al.*, 1994

Table 2. Bacterial Strains used in this study

Strain	Sex	Genotype	Source
AB1157	F-	*thr1 leu6 proA2 his4 argE3 lac Y1 galK2 xyl5 mtl2 ara14 tsx33 strA1*	B.Weiss
BW372	F-	as AB1157 but *nth::kan*	B.Weiss
BW434	F-	as AB1157 but *nth::kan Δ(xthA-pncA)*	B.Weiss
BW534	F-	as AB1157 but *nth::kan nfo::kan*	B.Weiss
BW535	F-	as AB1157 but *nth::kan nfo::kan Δ(xthA-pncA)*	B.Weiss
BW9109	F-	as AB1157 but *Δ(xthA-pncA)*	B.Weiss
RPC500	F-	as AB1157 but *nfo::kan*	B.Weiss
RPC501	F-	as AB1157 but *nfo::kan Δ(xthA-pncA)*	B.Weiss
EG333	Hfr	*(gpt-lac)X111 metB1 cysG303 car-96::Tn*10 λ-	K. Brooks Low
CSH100	F'	*ara Δ(gpt-lac)5* F'(*lacZ*+)	J. Miller
HS1200	F'	as AB1157 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1210	F'	as BW372 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1220	F'	as RPC500 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1230	F'	as BW9109 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1240	F'	as BW534 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1250	F'	as BW535 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1260	F'	as RPC501 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study
HS1270	F'	as BW535 but *Δ(gpt-lac) car96::Tn*10 F'(*lacZ*+)	this study

Figure 1. Effect of far-UV (UVC) on the survival of a series of *E. coli* AB1157 DNA repair mutants. Cells were irradiated as described in Materials and Methods. **(A)** Symbols: open circles, HS1200 (wildtype); closed circles, HS1210 (*nth*); open squares, HS1230 (*xthA*); closed squares, HS1250 (*nth xthA*); open triangles, HS1270 (*nth nfo xthA*). **(B)** open circles, HS1200 (wildtype); closed circles, HS1220 (*nfo*); open squares, HS1240 (*nth nfo*); closed squares, HS1260 (*nfo xthA*).

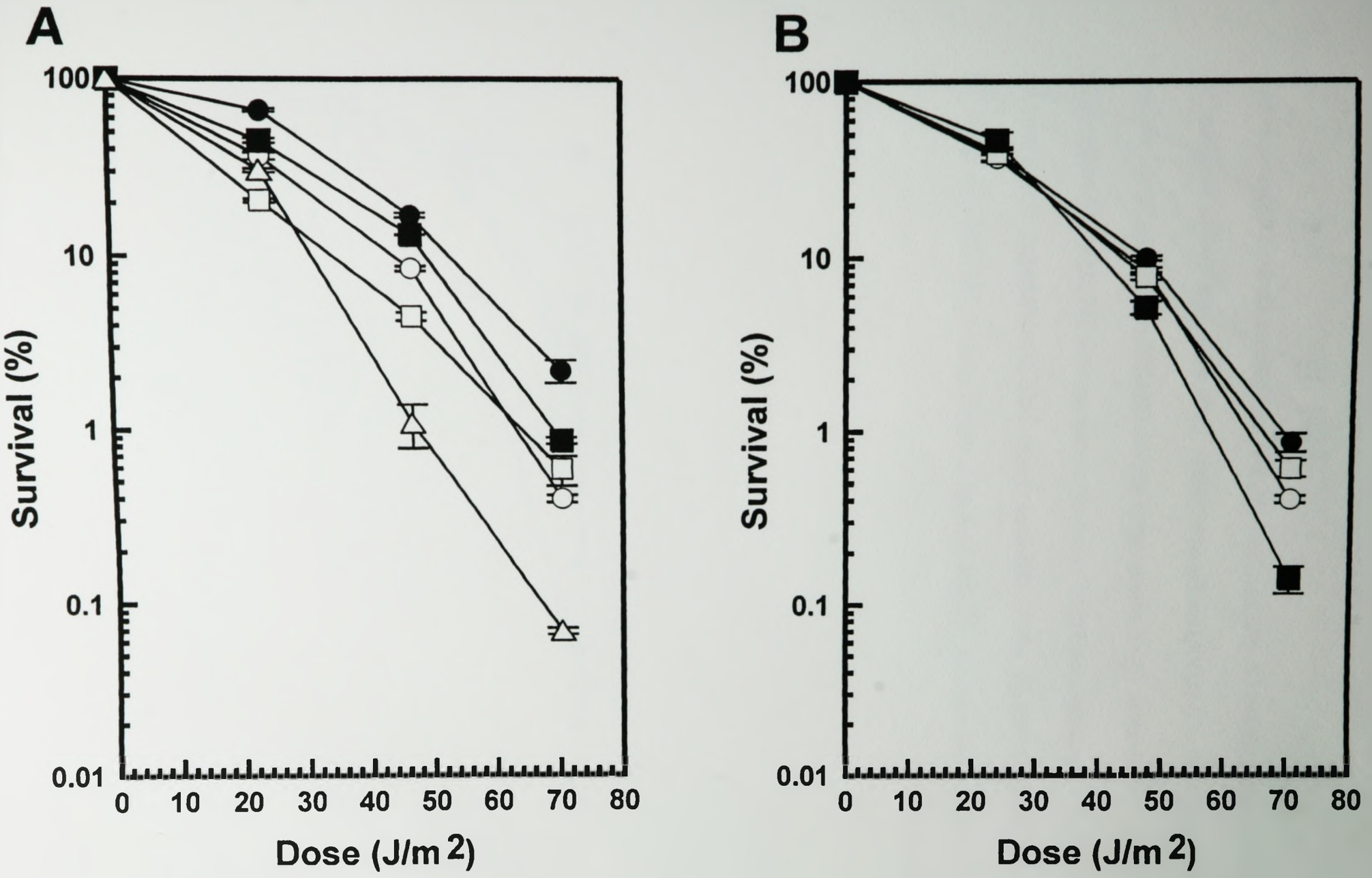
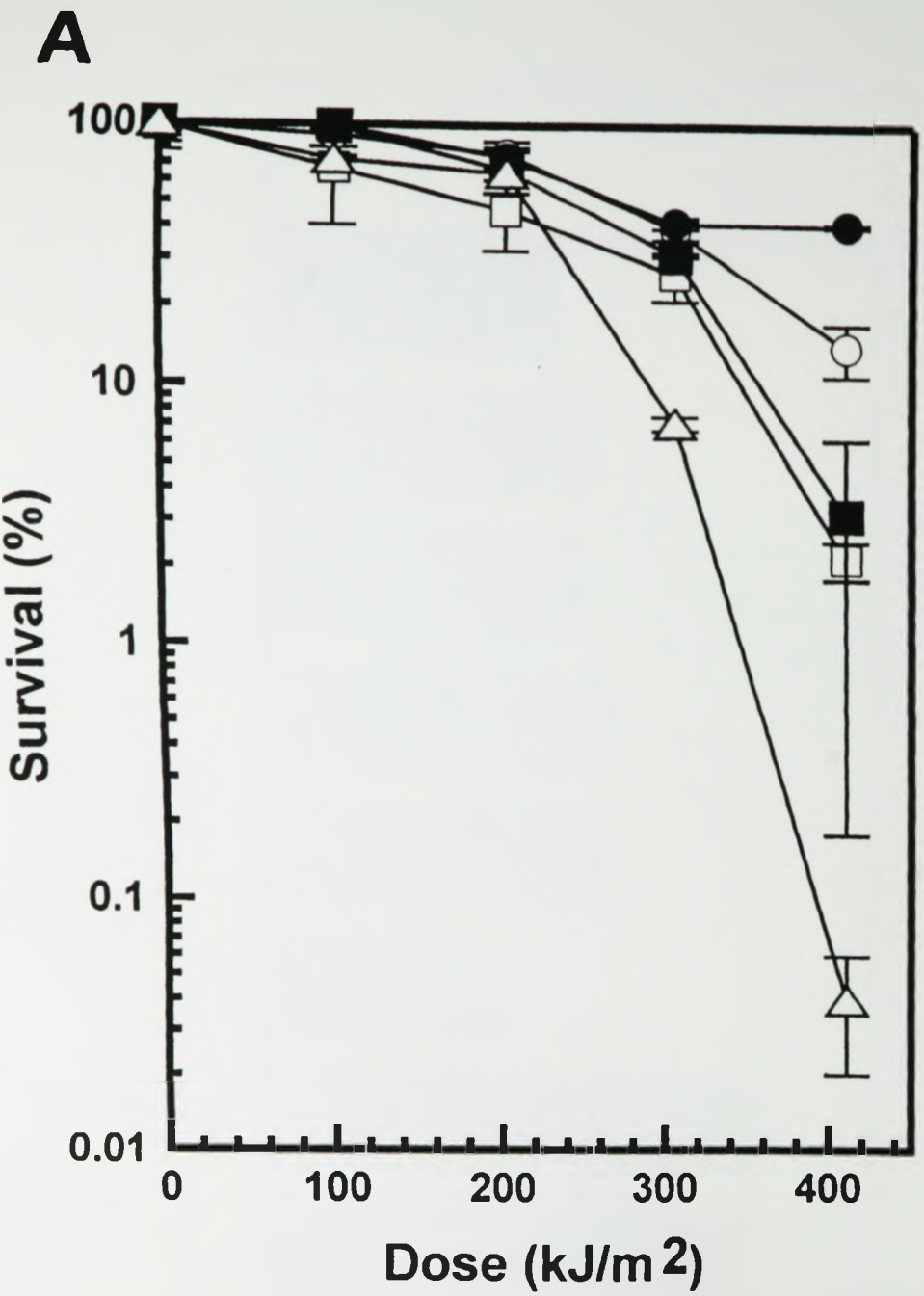

A
B
100
10
1
0.1
0.01
Survival (%)
0
10
20
30
40
50
60
70
80
Dose (J/m 2)

Figure 2. Endonuclease III, endonuclease IV, and exonuclease III are important in the prevention of near-UV (UVA) mediated lethality. Cells were irradiated as described in Materials and Methods. **(A)** Symbols: open circles, HS1200 (wildtype); closed circles, HS1210 (*nth*); open squares, HS1230 (*xthA*); closed squares, HS1250 (*nth xthA*); open triangles, HS1270 (*nth nfo xthA*). **(B)** open circles, HS1200 (wildtype); closed circles, HS1220 (*nfo*); open squares, HS1240 (*nth nfo*); closed squares, HS1260 (*nfo xthA*).


A
100
10
1
0.1
0.01
Survival (%)
0
100
200
300
400
Dose (kJ/m 2)

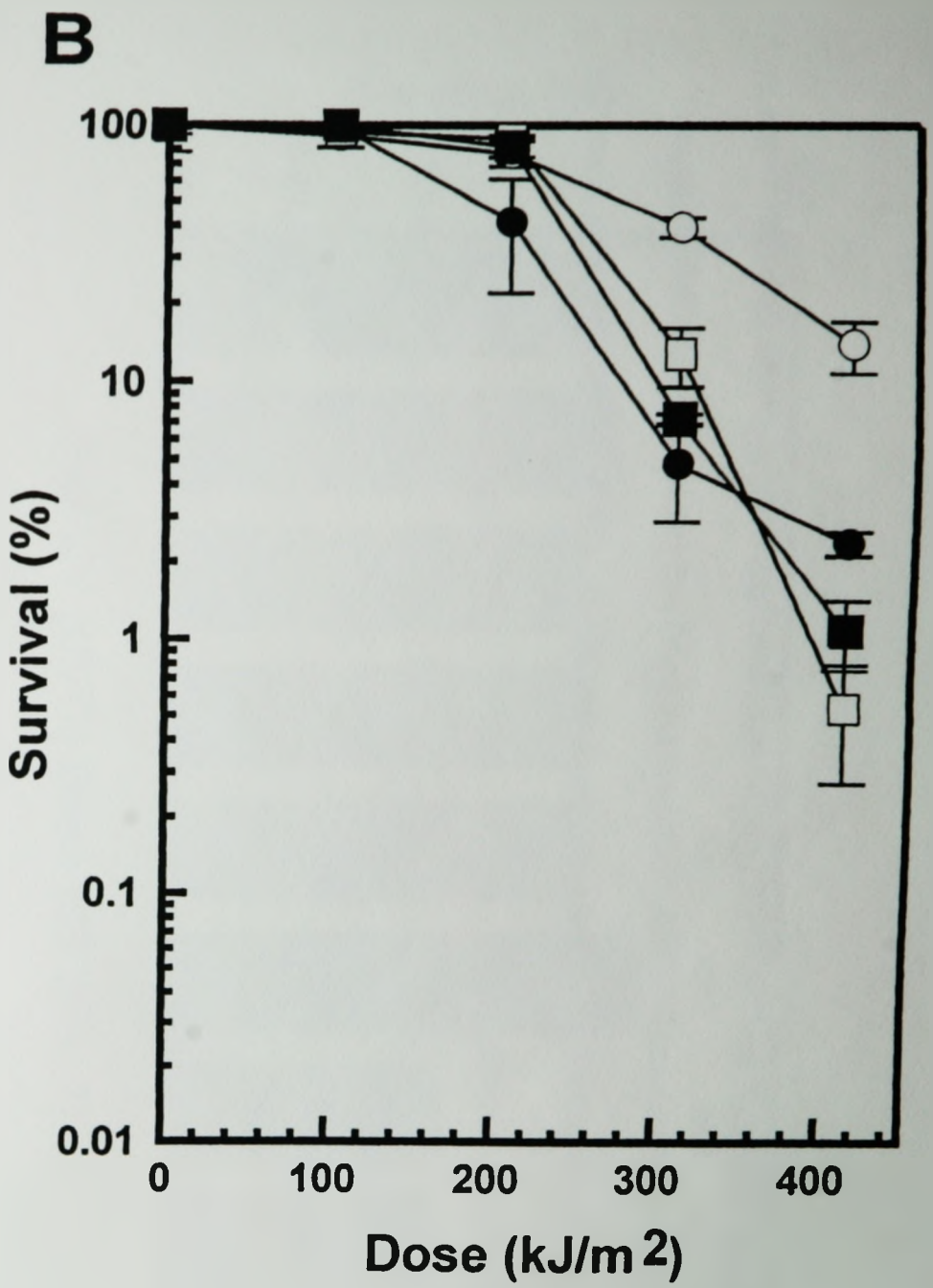
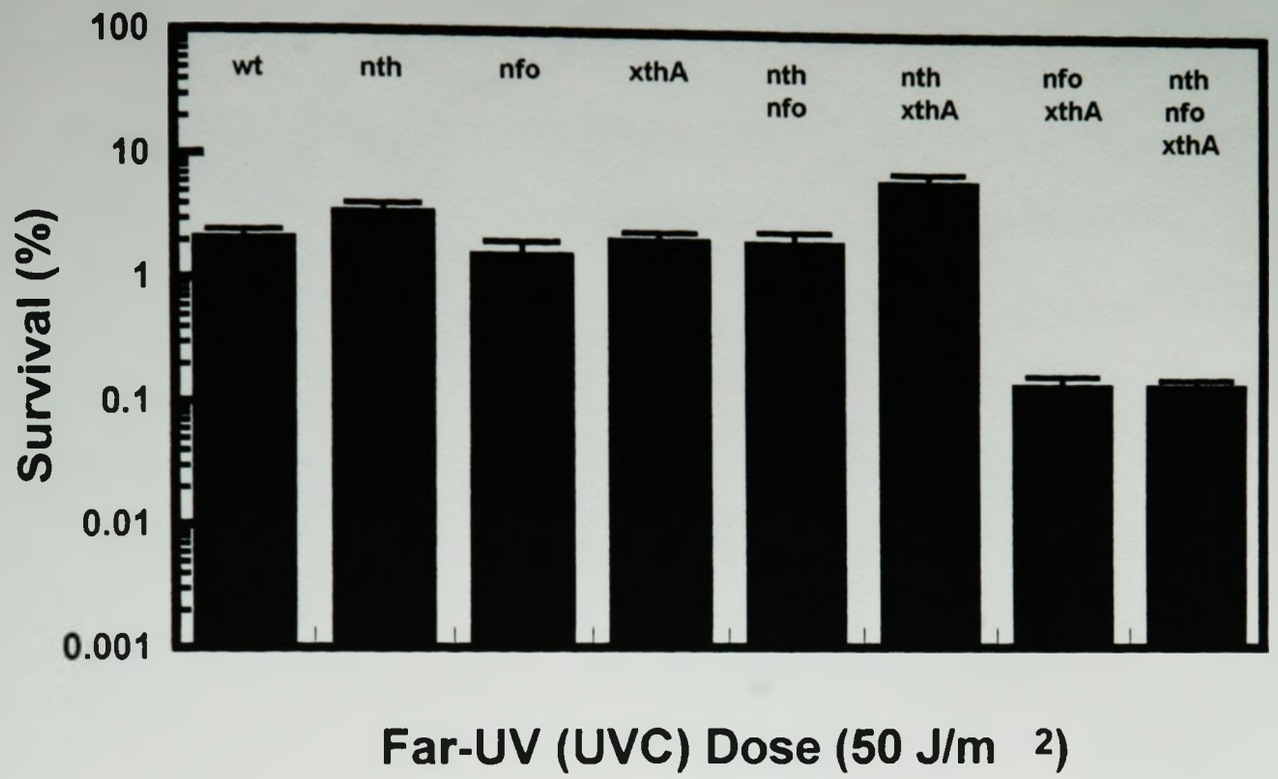

B
100
10
1
0.1
0.01
Survival (%)
0
100
200
300
400
Dose (kJ/m 2)

Figure 3. Effect of far-UV (UVC) on the mutation frequency of strains deficient in one or more of: endonuclease III (*nth*), endonuclease (*nfo*) and exonuclease III (*xthA*) (shown below). Corresponding survival rates are shown above. Cells were irradiated as described in Materials and Methods.


100
10
1
0.1
0.01
0.001
Survival (%)
wt
nth
nfo
xthA
nth nfo
nth xthA
nfo xthA
nth nfo xthA
Far-UV (UVC) Dose (50 J/m 2)

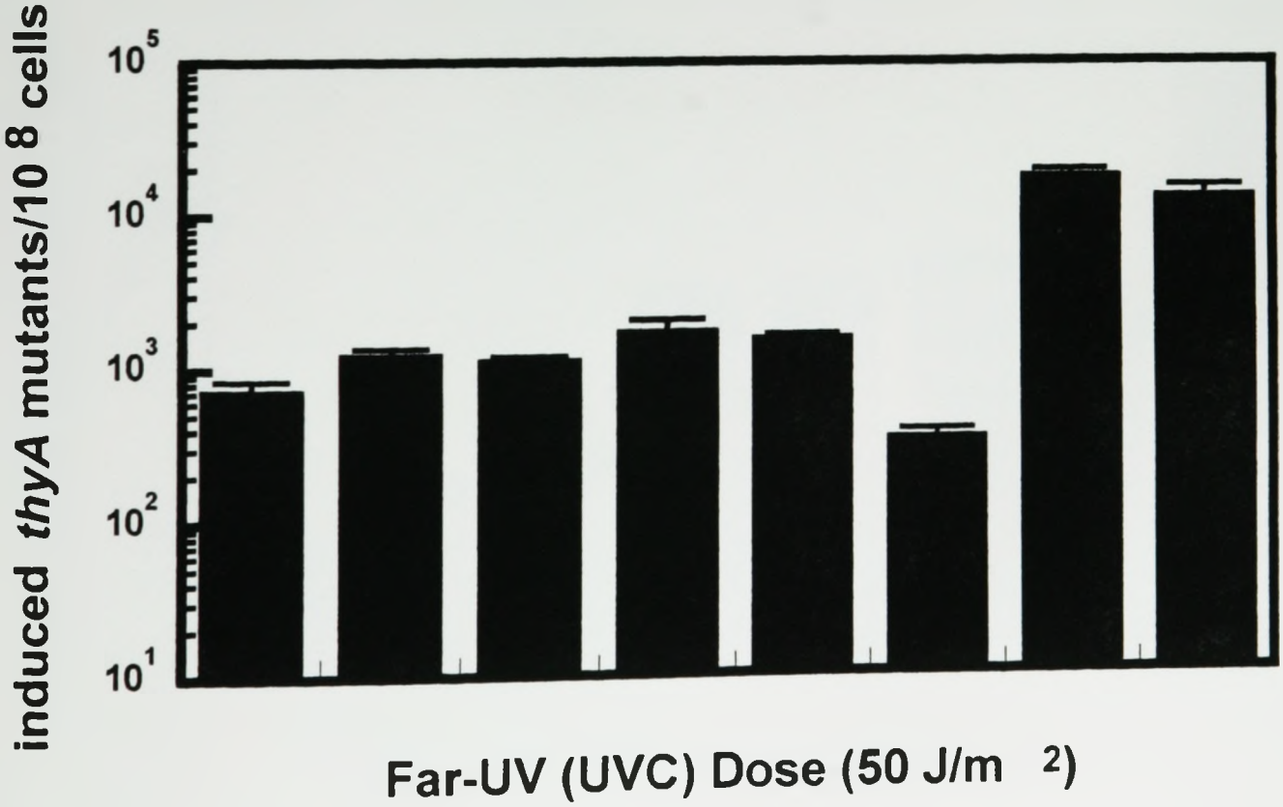
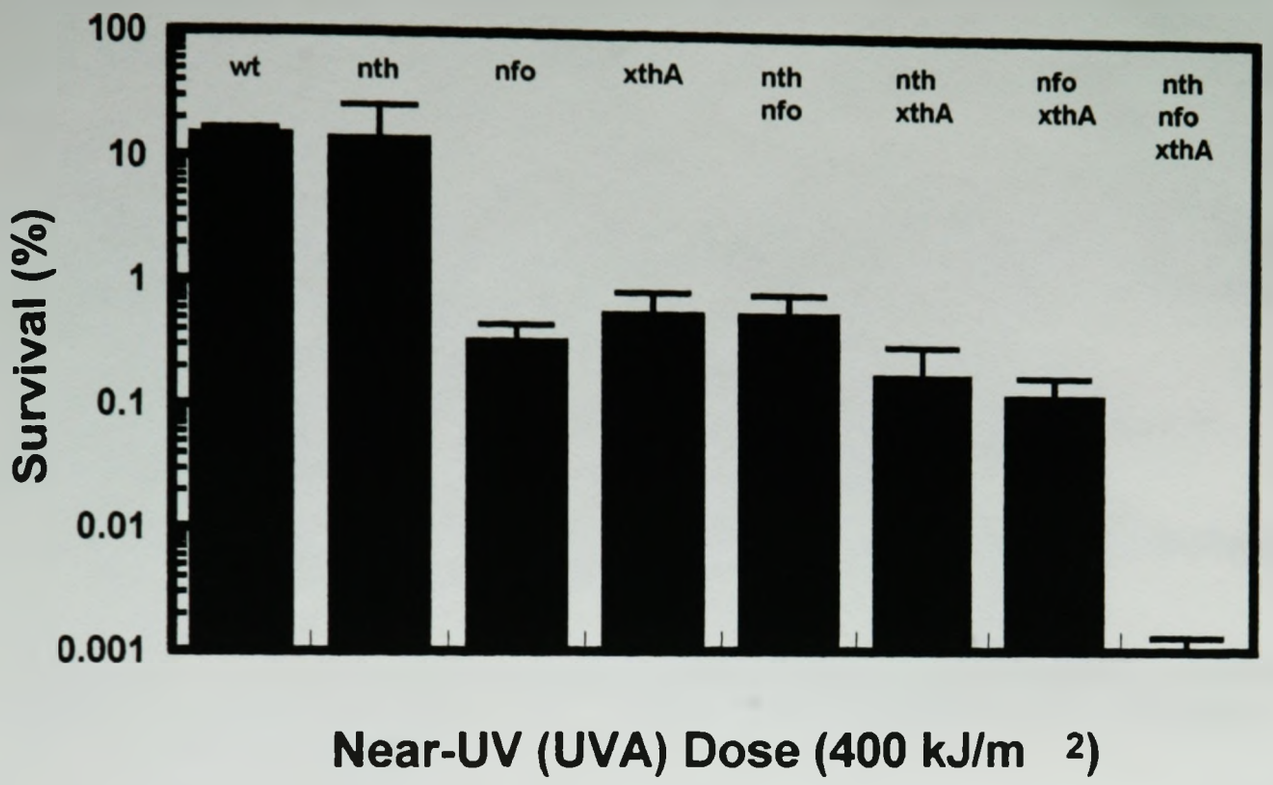
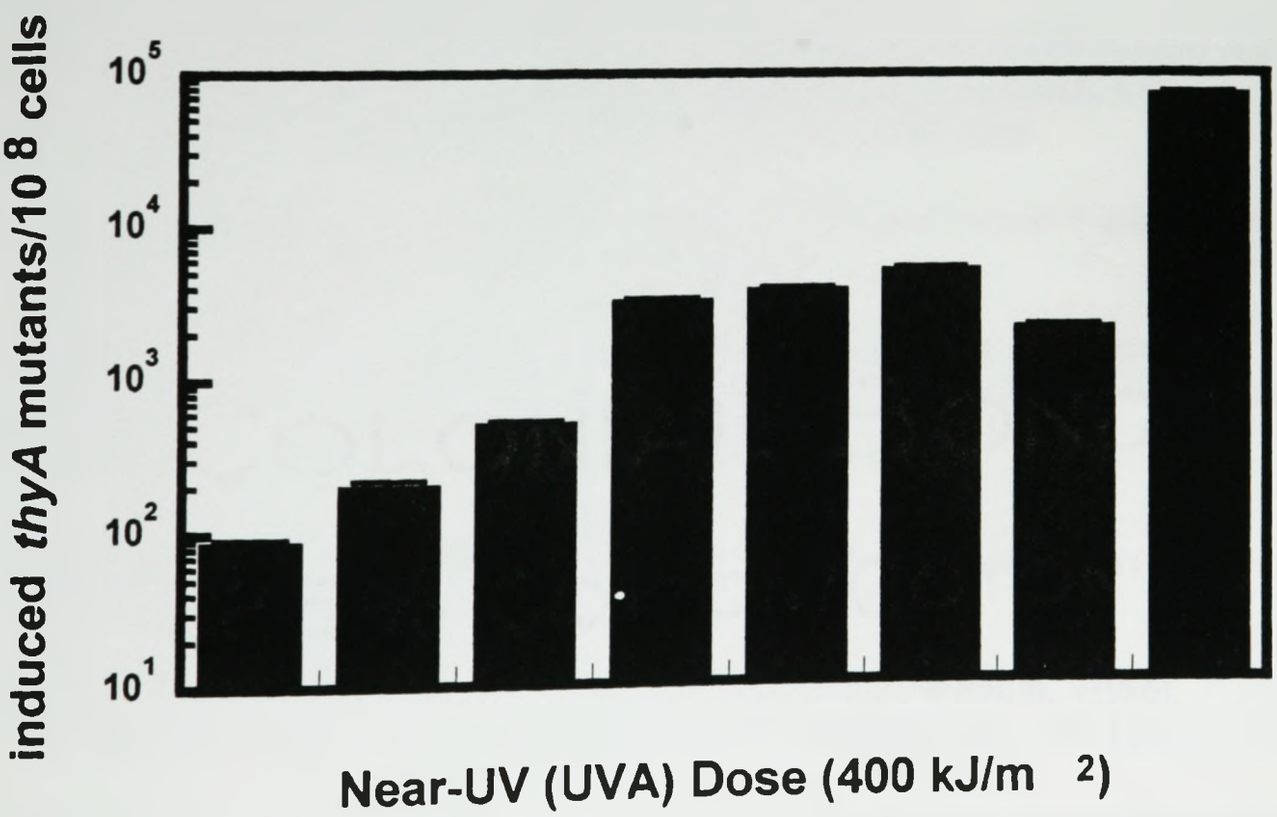

10^5
10^4
10^3
10^2
10^1
induced *thyA* mutants/10 8 cells
Far-UV (UVC) Dose (50 J/m 2)

Figure 4. The role of endonuclease III (*nth*), endonuclease IV (*nfo*), and exonuclease III (*xthA*) in protecting *E. coli* cells from the mutagenic effects of near-UV (UVA) (shown below). Corresponding survival rates are shown above. Cells were irradiated as described in Materials and Methods.


100
10
1
0.1
0.01
0.001
Survival (%)
wt
nth
nfo
xthA
nth nfo
nth xthA
nfo xthA
nth nfo xthA
Near-UV (UVA) Dose (400 kJ/m 2)


induced thyA mutants/10 8 cells
10^5
10^4
10^3
10^2
10^1
Near-UV (UVA) Dose (400 kJ/m 2)

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CHAPTER 4

Preface

The following chapter is a description of the *lacZ* reversion assay used to identify the main types of mutations produced by near-UV in an isogenic series of DNA repair deficient strains. The use of the Cupples/Miller reporter plasmids to detect specific mutations in strains lacking one or more of endonuclease III, endonuclease IV, and exonuclease III is described. The assay detects single base pair changes in the *lacZ* gene, and has been used previously to detect near-UV induced mutations (chapter 2, Palmer *et al.*, 1997)

CHAPTER 4

Base substitution analysis using Cupples and Miller reporter system

4.1 Description of Assay

To calculate the frequency of specific transition and transversion events, plasmids of the Cupples and Miller reporter system were mated into DNA repair mutant strains. The Hfr strain EG333 (*Δgpt-lac car-96::Tn*10) was conjugated with AB1157 and the repair deficient mutants (Table 1) by standard methods (Miller, 1992). Conjugation was terminated using a mating interrupter as described in Miller (1972). A 100 μL aliquot was plated on LB supplemented with streptomycin (100 μg / mL), tetracycline (15 μg / mL), and X-gal (50 μg / mL). Plates were incubated overnight at 37 °C. A single colony for each *lac*-deletion tetracycline resistant strain was purified and tested to ensure that all strains were isogenic.

Each of the *lac* deleted strains were then conjugated with an isogenic series of F' strains containing an episome carrying the *lacZ* gene (Table 2) (Cupples and Miller, 1989). Each strain in the F' series contained an episome with a different single base pair mutation in the glutamic acid residue at codon 461 the β-galactosidase gene. The episome also harbours the *pro* gene allowing for selection of the F^- recipient strains, which are proline auxotrophs. The F' donor strains (str^s) were counterselected using streptomycin. *E. coli* strains harbouring a mutation in the *lacZ* gene are unable to grow on M9 minimal media

when lactose is the sole carbon source (Cupples and Miller, 1989). The reversion event can be easily scored as growth on M9 minimal plates supplemented with lactose.

4.2 Results and Discussion

Far-UV

AP sites are formed by exposure to the alkylating agent methanemethanosulfate (MMS). Exposure of DNA repair mutants of MMS results in high levels of induced mutations for strains lacking the two major AP endonucleases, endonuclease IV and exonuclease III (Cunningham *et al.*, 1986). On mimimal media with MMS and valine, *nfo xthA* and *nth nfo xthA* mutants both exhibited six fold higher rates of induction (val^r) compared to WT. However, induction was less than two-fold for the *nfo* and *xthA* single mutants (Cunningham *et al.*, 1986).

A far-UV dose of 50 J/m^2 resulted in a significant induction of mutations in *nfo xthA* and *nth nfo xthA* DNA repair mutant strains ($P < 0.01$)(this study). However, there was not a significant induction for the same dose among any of the single mutant strains relative to WT ($P > 0.1$)(see chapter 3). The predominant mutational event for far-UV was a C to T transition, which was increased several hundred fold for all DNA repair mutants and the WT strain (Table 3). Since the specific mutation occurred at a CC site, the pre-mutagenic lesion is likely a C^^C pyrimidine dimer or a C(6-4) photoproduct. The induction of C --> T transitions was highest for strains lacking endonuclease IV and exonuclease III. Far-UV induced C to T transitions for *nfo xthA* and *nth nfo xthA* DNA repair mutants were 6000 fold and 1100 fold respectively, while the WT was induced 800 fold (Table 3). The higher rates of induction for strains lacking the two major AP

endonucleases may be due to monomeric base damage, resulting in increased lability of the N-glycosidic bond, and subsequent production of AP sites. AP sites are not efficiently repaired in endonuclease III-exonuclease III-deficient cells (Cunningham *et al.*, 1986). Although it is not clear how the accumulation of AP sites can lead to an increase in C to T transitions, there may be far-UV induced substrates for these enzymes that are not yet identified. For example, Fapy (Gua) and Fapy (Ade) are two modified bases that are caused by far-UV and are potentially mutagenic (Doetsch *et al.*, 1995).

Although C --> T transitions were the predominant mutational event induced by far-UV for all strains, the induction of G --> T transversions was higher among DNA repair mutants relative to WT. The *nfo xthA* and *nth nfo xthA* strain were induced 91and 62 fold respectively, while the WT strain was induced 16 fold. The large increase in G to T transversions for endo IV-exoIII-deficient cells is consistent with the accumulation of AP sites as a result of monomeric base damage. AP sites are misinstructional to DNA polymerase, resulting primarily in the insertion of an adenine (Kunkel, 1984). The incorrect insertion of an adenine in place of the internal cytosine of the *lacZ* codon 461 (GCG) would lead to a G --> T transversion event. Preferential insertion of adenine may also explain the increase in A-->T transversions for strains lacking the two major AP endonucleases (compare *nfo xthA*, *nth nfo xthA* with WT in Table 3). Interestingly, the spontaneous rates of A --> T and G --> T transversions were highest in the triple mutant strain compared to WT and the other DNA repair mutants (Table 3). These data demonstrate the importance of AP endonucleases in the prevention of spontaneous AP site

formation. In human cells, spontaneous AP site formation is estimated at 10^4 / cell / day (Barzilay and Hickson, 1995). The observed increased in spontaneous rates for specific mutations in bacterial cells lacking AP endonuclease activity (this study), as well as the identification of human homologs of exonuclease III (Robson and Hickson, 1991) and endonuclease III (Aspinwall *et al.*, 1997; Hilbert *et al.*, 1997), indicates the universal importance of AP endonucleases in DNA repair.

Near-UV

Using a *lacZ* reversion assay, a sub-lethal dose of near-UV resulted in a two fold increase in C to T transitions in the triple mutant (*nth nfo xthA*) relative to the WT strain (Table 4). However, no increase in C to T transitions was observed in the *nth* mutant relative to WT, suggesting that the pre-mutagenic lesion was an AP site, which may have formed from either a CC pyrimidine dimer or a CC (6-4) photoproduct.

In addition to a near-UV induction of C to T transitions in the triple mutant strain, the *lacZ* reversion assay demonstrated two fold increases in G to C transversions and A to T transversions in the triple mutant relative to WT (Table 4). These specific mutations are consistent with the accumulation of oxidative DNA damage. G to C transversions have been observed in gamma irradiated double stranded M13 DNA under aerobic conditions (Hoebee *et al.*, 1988); sunlight irradiated single stranded M13 DNA (Negishi and Hao, 1992); and in aerobically treated single straned M13 DNA with Fe^{2+} (McBride *et al.*, 1991). Oxidized bases increase the lability of the N-glycosidic bond, which can result in

the formation of an AP site. The oxidation products of cytidine: 5-hydroxy-2'deoxycytidine (5OHdC) and 5-hydroxy-2'-deoxyuridine (5OHdU) are substrates for endonuclease III (Hatahet *et al.*, 1994). In one particular sequence context, deoxycytosine was the principal base inserted opposite 5OHdC and 5OHdU, resulting in G to C transversions (Purmal *et al.*, 1994). The induction of G to C transversions in the triple mutant relative to WT, may be the result of a near-UV induced accumulation of AP sites or oxidized cytosine residues.

The induction of A to T transversions in a triple mutant is consistent with the accumulation of AP sites. AP sites, which are non-instructional to DNA polymerase, are often mutagenic (Schaaper *et al.*, 1983). Misincorporation opposite an AP site often occurs, with the most frequent insertions being adenines or thymines, accounting for 59 and 28 percent of all misincorporated bases respectively (Kunkel, 1984). Interestingly, the spontaneous rate of A to T transversions in a triple mutant was fifteen fold higher than that of WT, which is consistent with the misincorporation of adenine or thymine residues opposite AP sites (Kunkel, 1984).

There was no increase in G to T transversions for any of the DNA repair deficient strains, including the triple mutant. Oxidized guanines are known to be the main substrate for Fpg glycosylase (MutM). We have previously shown (Palmer *et al.*, 1997) that the number of G to T transversions is elevated in a Fapy mutant relative to WT. Since oxidized guanines are not substrates for any of endonuclease III, endonuclease IV, and exonuclease IV, the lack of a near-UV induced increase in G to T transversions is

consistent with the idea that oxidized guanine residues are the main pre-mutagenic lesion leading to this base change.

The results of the near-UV treatment should not be over-interpreted. In several instances, the induced mutation rate actually decreased with a near-UV exposure (Table 4). It is likely that the observed decrease was an artefact of the high concentrations of cells plated (~ 4 X 10^9 / aliquot). It was necessary to concentrate saturated cultures 100 fold in order to obtain reproducible cell counts of at least 10 or more colonies / plate. It is possible that the high concentration of cells plated resulted in *lacZ* revertants that were too small to detect. The *lacZ* reversion assay is limited in this case by the low spontaneous mutation rates for the isogenic series. Concentration of cells does not alleviate the problem, but rather, results in an inability to detect blue colonies on a very saturated white background.

It is useful to note that in our previous study on the mutational spectrum of *fpg* mutant cells (Palmer *et al.*, 1997), as well as the original study (Cupples and Miller, 1989), the background was CSH100. In contrast, the DNA repair mutants to which the F' strains were mated were AB1157 derived. Although AB1157 is often used as WT, it is a chemically mutagenized strain that may not be completely characterized. It has been recently shown that AB1157 harbours an amber mutation in *rpoS*, the structural gene for the stationary phase sigma factor (Visick and Clarke, 1997). RpoS is responsible for the regulation of over 30 genes including *katE* (catalase HPII), *katG* (catalase HPI) and *xthA* (exonuclease III) (Hengge-Aronis, 1996). The low spontaneous rates of *lacZ* reversion

events in an AB1157 background, combined with the recent report of an *rpoS*(Am) mutation in this strain, suggests that the Cupples/Miller assay was not an appropriate choice for the measurement of near-UV specific mutational events.

Table 4-1. Bacterial Strains used in this study

Strain	Genotype	sex	Source
AB1157	*thr1 leu6 proA2 his4 argE3 lac Y1 galK2 xyl5 mtl2 ara14 tsx33 strA1*	F-	B.Weiss
BW372	as AB1157 but *nth::kan*	F-	B.Weiss
BW434	as AB1157 but *nth::kan Δ(xthA-pncA)*	F-	B.Weiss
BW534	as AB1157 but *nth::kan nfo::kan*	F-	B.Weiss
BW535	as AB1157 but *nth::kan nfo::kan Δ(xthA-pncA)*	F-	B.Weiss
BW9109	as AB1157 but *Δ(xthA-pncA)*	F-	B.Weiss
RPC500	as AB1157 but *nfo::kan*	F-	B.Weiss
RPC501	as AB1157 but *nfo::kan Δ(xthA-pncA)*	F-	B.Weiss
EG333	*(gpt-lac)X111 metB1 cysG303 car-96::Tn*10 λ-	Hfr	K. Brooks Low

Table 4-2. Bacterial strains constructed in this study

Strain	Genotype
CSH100	*ara Δ(gpt-lac)5*
CSH101	*ara Δ(gpt-lac)5*
CSH102	*ara Δ(gpt-lac)5*
CSH103	*ara Δ(gpt-lac)5*
CSH104	*ara Δ(gpt-lac)5*
CSH105	*ara Δ(gpt-lac)5*
CSH106	*ara Δ(gpt-lac)5*
HS1200	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1201	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1202	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1203	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1204	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1205	as AB1157 but *Δ(gpt-lac) car96::Tn*10
HS1206	as AB1157 but *Δ(gpt-lac) car96::Tn*10

F' plasmid	*lacZ* allele	Base change required to revert	Source
F' 100	*lacZ*+	-	J. Miller
F' 101	*lacZ* (Am-461)	AT to CG	J. Miller
F' 102	*lacZ* (Gly-461)	GC to AT	J. Miller
F' 103	*lacZ* (Gln-461)	GC to CG	J. Miller
F' 104	*lacZ* (Ala-461)	GC to TA	J. Miller
F' 105	*lacZ* (Val-461)	AT to TA	J. Miller
F' 106	*lacZ* (Lys-461)	AT to GC	J. Miller
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study

Table 4-2. Bacterial strains constructed in this study

Strain	Genotype
HS1210	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1211	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1212	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1213	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1214	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1215	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1216	as BW372 but *Δ(gpt-lac) car96::Tn*10
HS1220	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1221	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1222	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1223	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1224	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1225	as RPC500 but *Δ(gpt-lac) car96::Tn*10
HS1226	as RPC500 but *Δ(gpt-lac) car96::Tn*10

F' plasmid	*lacZ* allele	Base change required to revert	Source
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study

Table 4-2. Bacterial strains constructed in this study

Strain	Genotype	F' plasmid	*lacZ* allele	Base change required to revert	Source
HS1230	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 100	*lacZ*+	-	this study
HS1231	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 101	*lacZ* (Am-461)	AT to CG	this study
HS1232	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 102	*lacZ* (Gly-461)	GC to AT	this study
HS1233	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 103	*lacZ* (Gln-461)	GC to CG	this study
HS1234	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 104	*lacZ* (Ala-461)	GC to TA	this study
HS1235	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 105	*lacZ* (Val-461)	AT to TA	this study
HS1236	as BW9109 but *Δ(gpt-lac) car96::Tn*10	F' 106	*lacZ* (Lys-461)	AT to GC	this study
HS1240	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 100	*lacZ*+	-	this study
HS1241	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 101	*lacZ* (Am-461)	AT to CG	this study
HS1242	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 102	*lacZ* (Gly-461)	GC to AT	this study
HS1243	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 103	*lacZ* (Gln-461)	GC to CG	this study
HS1244	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 104	*lacZ* (Ala-461)	GC to TA	this study
HS1245	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 105	*lacZ* (Val-461)	AT to TA	this study
HS1246	as BW534 but *Δ(gpt-lac) car96::Tn*10	F' 106	*lacZ* (Lys-461)	AT to GC	this study

Table 4-2. Bacterial strains constructed in this study

Strain	Genotype
HS1250	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1251	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1252	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1253	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1254	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1255	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1256	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1260	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1261	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1262	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1263	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1264	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1265	as RPC501 but *Δ(gpt-lac) car96::Tn*10
HS1266	as RPC501 but *Δ(gpt-lac) car96::Tn*10

F' plasmid	*lacZ* allele	Base change required to revert	Source
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study

Table 4-2. Bacterial strains constructed in this study

Strain	Genotype
HS1270	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1271	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1272	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1273	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1274	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1275	as BW535 but *Δ(gpt-lac) car96::Tn*10
HS1276	as BW535 but *Δ(gpt-lac) car96::Tn*10

F' plasmid	*lacZ* allele	Base change required to revert	Source
F' 100	*lacZ*+	-	this study
F' 101	*lacZ* (Am-461)	AT to CG	this study
F' 102	*lacZ* (Gly-461)	GC to AT	this study
F' 103	*lacZ* (Gln-461)	GC to CG	this study
F' 104	*lacZ* (Ala-461)	GC to TA	this study
F' 105	*lacZ* (Val-461)	AT to TA	this study
F' 106	*lacZ* (Lys-461)	AT to GC	this study

Table 4-3. Spontaneous and far-UV induced rates of specific mutations using a *lacZ* reversion assay.

Genotype	UVC Dose (J/m^2)	Reversion event					
		AT to CG	GC to AT	GC to CG	GC to TA	AT to TA	AT to GC
wt	0	1.04 (0.12)	1.26 (0.30)	0.02 (0.01)	0.45 (0.08)	0.26 (0.03)	b.d.l.
	50	15.21 (0.30)	1055.21 (35.5)	0.53 (0.23)	7.01 (1.18)	1.68 (0.24)	0.17 (0.04)
nth	0	0.24 (0.04)	3.32 (0.23)	0.04 (0.01)	0.41 (0.15)	0.10 (0.01)	0.00 (0.00)
	50	3.37 (0.36)	1069.98 (43.4)	0.12 (0.06)	5.42 (0.62)	0.50 (0.03)	0.08 (0.02)
nfo	0	0.16 (0.06)	1.04 (0.15)	0.01 (0.01)	0.59 (0.03)	0.03 (0.00)	0.00 (0.00
	50	3.36 (0.35)	507.10 (24.9)	0.82 (0.29)	12.47 (1.36)	0.56 (0.03)	0.76 (0.31)
xthA	0	0.30 (0.03)	0.29 (0.08)	0.02 (0.01)	5.92 (0.16)	0.38 (0.07)	0.01 (0.01)
	50	4.15 (1.44)	1261.90 (105)	1.50 (0.39)	22.73 (2.33	3.02 (1.27)	0.17 (0.09)
nth nfo	0	0.05 (0.03)	3.62 (0.35)	0.01 (0.01)	2.90 (0.18)	0.04 (0.00)	b.d.l.
	50	2.37 (0.85)	2503.02 (107)	0.33 (0.15)	38.71 (0.64)	0.97 (0.18)	0.40 (0.35)
nth xthA	0	0.39 (0.01)	2.83 (0.71)	b.d.l.	0.18 (0.12)	0.27 (0.05)	0.02 (0.00)
	50	5.80 (1.02)	1436.12 (61.7)	b.d.l.	1.54 (0.38)	1.34 (0.45)	0.60 (0.05)
nfo xthA	0	b.d.l.	1.28 (0.19)	b.d.l.	2.50 (0.37)	0.10 (0.01)	0.54 (0.12)
	50	b.d.l.	8507.94 (837)	0.62 (0.62)	222.09 (7.62)	26.83 (2.13)	26.34 (2.93)
nth nfo	0	0.14 (0.06)	2.94 (0.81)	0.13 (0.01)	6.81 (0.07)	2.25 (0.14)	0.08 (0.01)
xthA	50	71.43 (20.6)	3333.33 (275)	5.47 (0.63)	420.51 (13.6)	98.68 (41.1)	10.91(0.00)

Mutagenesis with near-UV was performed as described in the text. Mutation rates were corrected for survival and are expressed as *lacZ* mutants per 10^8 cells (+/- standard error); b.d.l = below detection limit of the assay, ie, less than 1 per 10^{11} cells.

Table 4-4. Spontaneous and near-UV induced rates of specific mutations using a *lacZ* reversion assay.

Genotype	UVA Dose (kJ/m2)	Reversion event					
		AT to CG	GC to AT	GC to CG	GC to TA	AT to TA	AT to GC
wt	0	0.86 (0.02)	0.31(0.06)	0.01 (0.01)	0.69 (0.04)	0.11 (0.01)	b.d.l.
	100	1.18 (0.11)	0.10 (0.05)	0.01 (0.01)	0.18 (0.01)	0.12 (0.02)	0.07 (0.05)
nth	0	0.19 (0.02)	1.20 (0.10)	0.03 (0.02)	0.11 (0.11)	0.06 (0.00)	b.d.l.
	100	0.23 (0.04)	1.20 (0.15)	0.01 (0.01)	0.38 (0.00)	0.06 (0.01)	0.01 (0.01)
nfo	0	0.13 (0.01)	0.35 (0.04)	b.d.l.	2.08 (0.08)	0.02 (0.01)	b.d.l.
	100	0.13 (0.01)	0.32 (0.09)	0.01(0.00)	0.26 (0.03)	0.03 (0.00)	b.d.l.
xthA	0	0.25 (0.02)	0.46 (0.23)	0.03 (0.00)	0.46 (0.05)	0.84 (0.02)	0.03 (0.01)
	100	0.25 (0.01)	0.17 (0.17)	0.02 (0.01)	0.92 (0.02)	0.50 (0.15)	0.06 (0.03)
nth nfo	0	0.07 (0.01)	0.56 (0.14)	b.d.l.	0.06 (0.04)	0.04 (0.00)	b.d.l.
	100	0.03 (0.01)	0.55 (0.12)	b.d.l.	0.12 (0.01)	0.04 (0.00)	0.01 (0.00)
nth xthA	0	0.33 (0.02)	2.22 (0.47)	0.02 (0.02)	0.20 (0.03)	0.23 (0.03)	0.01 (0.00)
	100	0.44 (0.03)	1.68 (0.52)	0.01 (0.00)	0.43 (0.01)	0.28 (0.00)	0.01 (0.00)
nfo xthA	0	b.d.l.	0.24 (0.05)	b.d.l.	0.72 (0.06)	0.12 (0.04)	0.05 (0.01)
	100	b.d.l.	0.62 (0.16)	b.d.l.	0.06 (0.01)	0.18 (0.01)	0.07 (0.00)
nth nfo xthA	0	0.08 (0.02)	0.75 (0.04)	0.05 (0.01)	0.33 (0.05)	1.58 (0.01)	0.05 (0.03)
	100	0.06 (0.01)	1.50 (0.57)	0.12 (0.01)	0.35 (0.04)	3.09 (0.08)	0.08 (0.02)

Mutagenesis with near-UV was performed as described in the text. Mutation rates were corrected for survival and are expressed as *lacZ* mutants per 10^8 cells (+/- standard error); b.d.l = below detection limit of the assay, ie, less than 1 per 10^{11} cells.

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CONCLUSIONS

- With the exception of the *nth* single mutant, all DNA repair mutants exposed to near-UV had reduced survival rates relative to WT, demonstrating that endonuclease IV and exonuclease III protect *E. coli* cells from the lethal effects of near-UV.

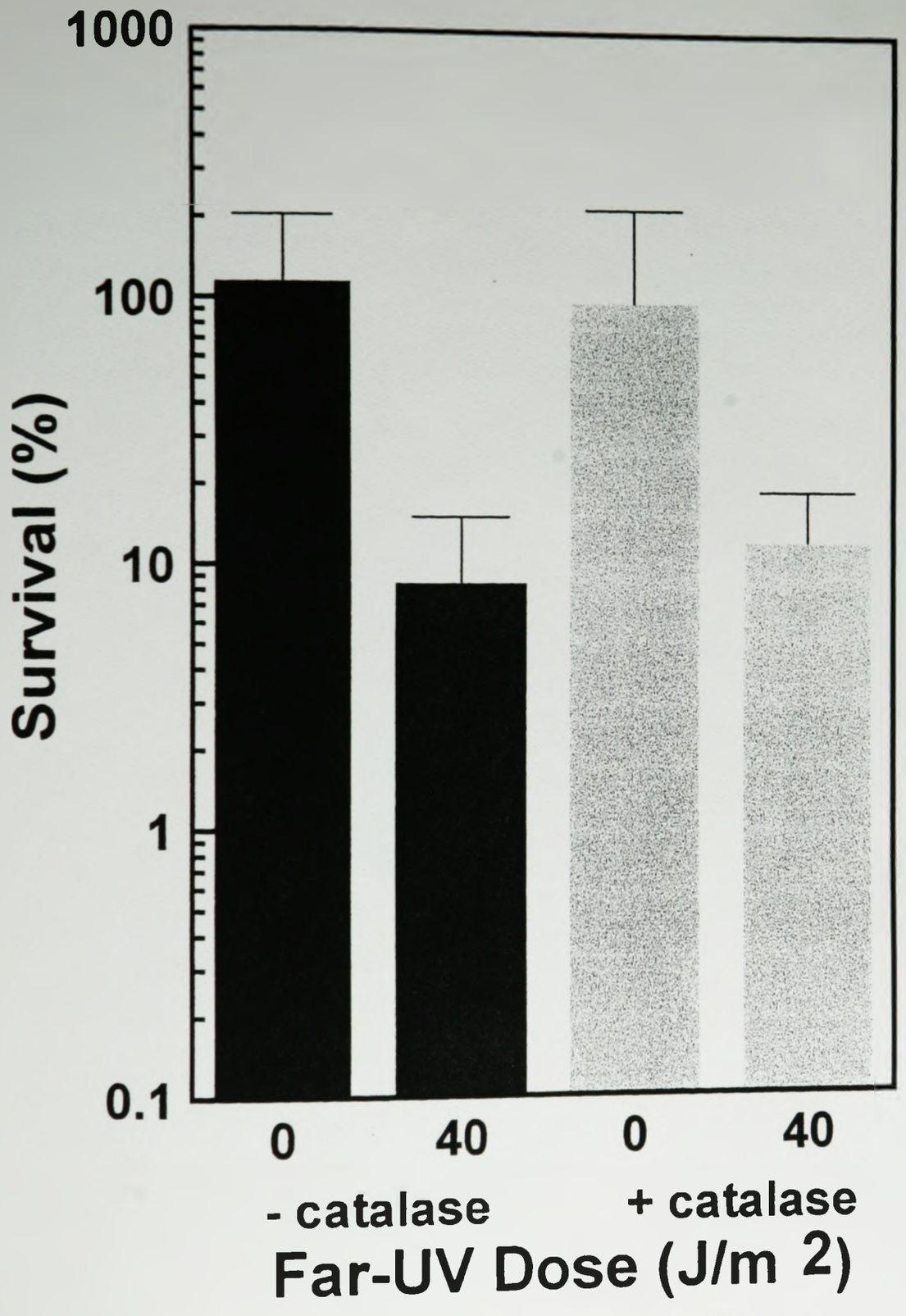
- Survival of the *nth nfo xthA* triple mutant was lower than all other strains (including *nfo xthA*), demonstrating a previously unrecognized role for endonuclease III in the repair of near-UV mediated DNA damage.

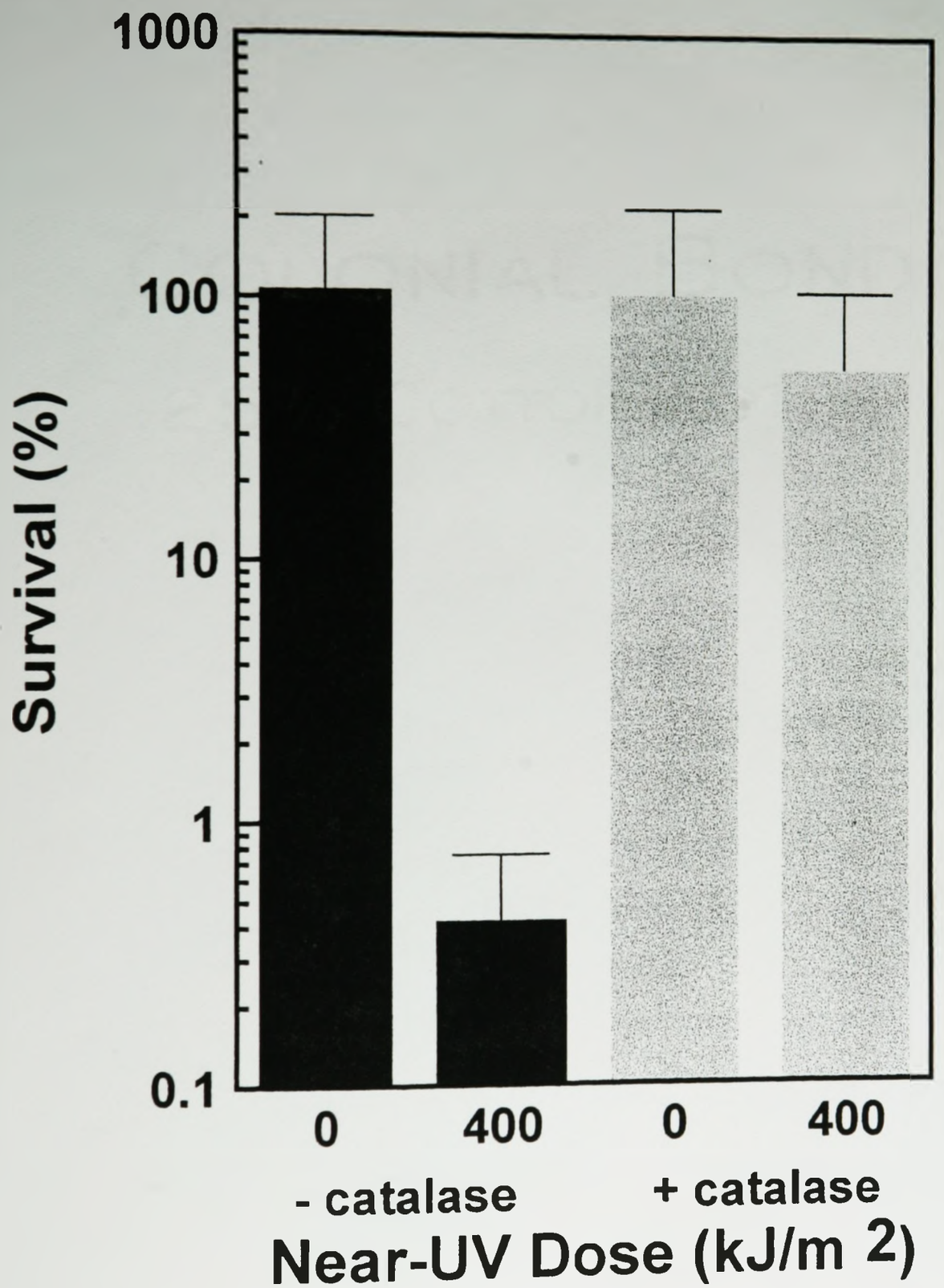
- The hypersensitivity of the DNA repair mutants exposed to near-UV was not observed for far-UV (except for mutants lacking both endonuclease IV and exonuclease III). This suggests that AP sites are an important near-UV lesion.

- Endonuclease III, endonuclease IV, and exonuclease III were all important in the prevention of near-UV induced mutagenesis. The triple DNA repair mutant was more sensitive to the mutagenic effects of near-UV than an *nfo xthA* double mutant, confirming a near-UV protective role for endonuclease III.

APPENDIX I

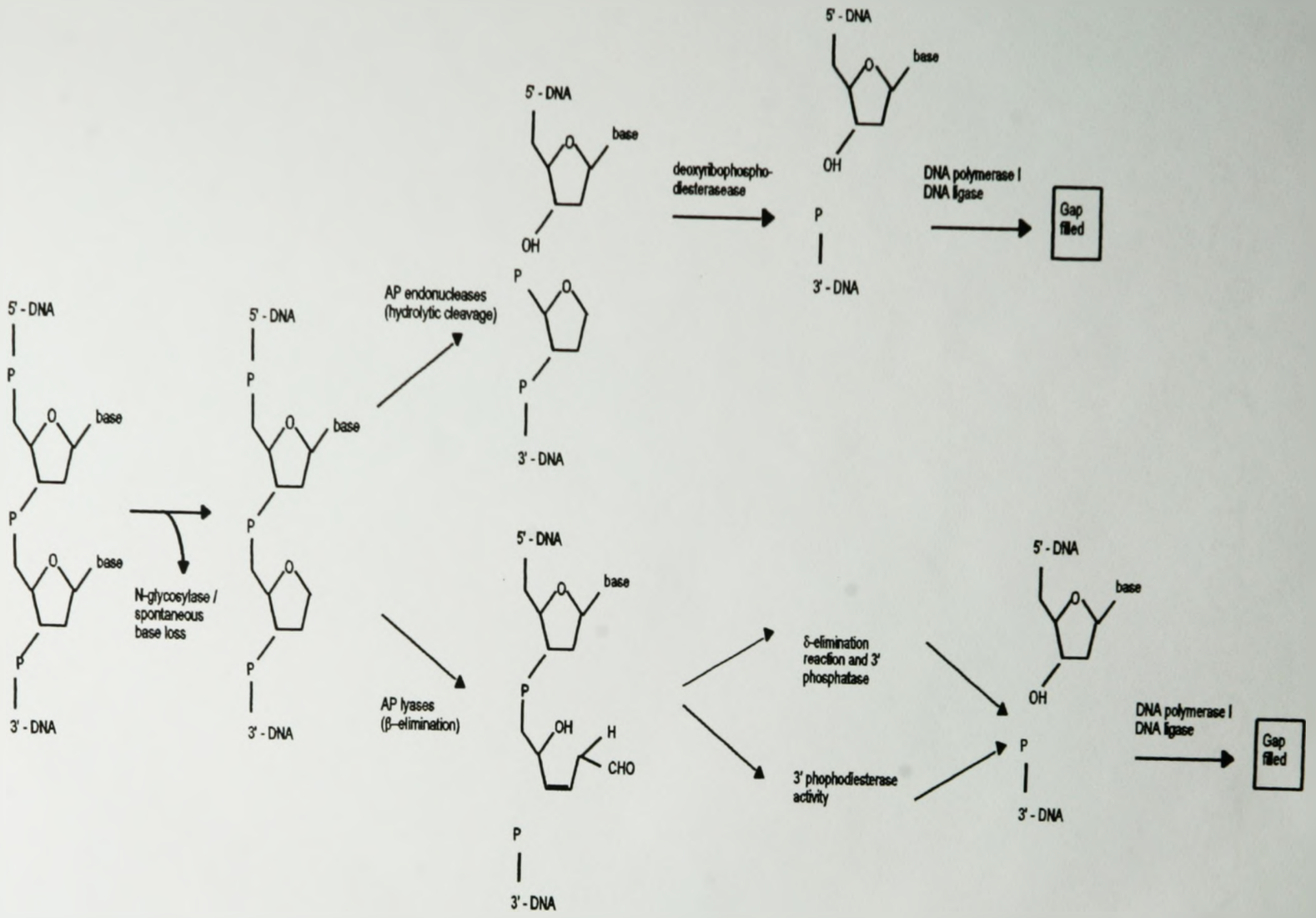
The following figures display graphically the results of the catalase assay described in our publication "Near-Ultraviolet Radiation (UVA) Causes a Fapy Glycosylase Dependent Increase in G to T transversions" (chapter 2).


1000
100
10
1
0.1
Survival (%)
0
40
0
40
- catalase
+ catalase
Far-UV Dose (J/m 2)


1000
100
10
1
0.1
Survival (%)
0
400
0
400
- catalase
+ catalase
Near-UV Dose (kJ/m 2)

APPENDIX II

The following figure is taken from the oral defence of this thesis and does not appear anywhere else above.



Generalized mechanism of base excision repair (adapted from Demple and Harrison, 1994).