PROTECTION FROM NEAR-ULTRAVIOLET RADIATION IN *E. COLI*

FAPY GLYCOSYLASE AND UVRABC EXCINUCLEASE PROTECT *ESCHERICHIA COLI* FROM NEAR-ULTRAVIOLET RADIATION

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ABSTRACT

In contrast to the damage caused by far-UV, the damaging effects of UVA (320-400 nm) in living cells are not well understood. The damage caused by UVA irradiation is largely oxygen-dependent, suggesting UVA-mediated DNA damage involves reactive oxygen species produced through the action of an endogenous photosensitizer. Previous studies examining cellular responses to UVA irradiation in *E. coli* have been hindered by the fact that, at sublethal fluences, wild-type cells undergo a transient inhibition of cell growth termed a "growth delay". This effect is absent in *nuvA*$^-$ strains, thereby facilitating the study of DNA repair factors required for the repair of UVA-mediated damage. Formamidopyrimidine (Fapy) glycosylase (encoded by *fpg*) and the UvrABC excinuclease are both capable of excising oxidatively damaged DNA bases. An *fpg*::kan mutation was placed into isogenic *uvrA*$^+$ and *uvrA*$^-$ strains of *E. coli* to evaluate the relative importance of these repair enzymes in the recovery from UVA-induced stress. In a *nuvA*$^-$ background, the survival of *fpg*$^-$ mutants exposed to UVA was significantly reduced relative to isogenic *fpg*$^+$ control strains. This effect was enhanced in the absence of the UvrABC excinuclease, suggesting a role for both of these enzymes in repairing UVA-generated lesions. Survival of isogenic *nuvA*$^+$ repair-deficient strains was significantly lower than *nuvA*$^-$ strains, suggesting a role for the modified base 4-thiouridine in UVA-mediated lethality. An *in vitro* plasmid DNA irradiation assay in the presence and absence of 4-thiouridine was used to examine this possibility. When irradiated DNA was subsequently used to transform the *fpg*$^-$ and *uvrA*$^-$ mutant strains, no

increase in DNA damage (as measured by a decrease in transformational efficiency) in the presence of 4-thiouridine was observed, suggesting that when present in solution this base does not play a photosensitizing role in UVA-mediated lethality.

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LIST OF ABBREVIATIONS

cm	centimetre
°C	degrees Celsius
far-UV	far-ultraviolet
kDa	kilodalton
kJ	kilojoule
mg	milligram
ml	millilitre
mM	millimolar
mW	milliwatt
near-UV	near-ultraviolet
ng	nanogram
nm	nanometre
OD_{600}	optical density at 600 nm
ROS	reactive oxygen species
rpm	revolutions per minute
µg	microgram
µl	microlitre
µM	micromolar
µW	microwatt
UVA	near-ultraviolet light >320 nm
UVB	near-ultraviolet light 290-320 nm
UVC	far-ultraviolet light (<290 nm)

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

Near-ultraviolet light (near-UV; 290-400 nm) represents the most energetic component of solar radiation reaching the ground. Sunlight reaching the surface of the earth is composed of a major near-UV component (UVA) with wavelengths between 320 and 400 nm, and a minor near-UV component between 290 and 320 nm (UVB). UVC radiation (290 nm), or far-ultraviolet (far-UV) light, is efficiently filtered by the earth's ozone layer and atmosphere (Eisenstark, 1989). As the levels of atmospheric ozone decrease (Kerr and McElroy, 1993; Madronich and de Gruijl, 1994), near-UV fluences (especially those in the UVB region), as measured at the earth's surface, are gradually increasing (Kerr and McElroy, 1993).

There is considerable epidemiological evidence indicating that ultraviolet light is directly involved in the induction of basal and squamous carcinomas of the skin in humans (as reviewed in Doll and Peto, 1981), and is a critical factor in the development of malignant melanomas (Setlow *et al*, 1993) and tumorigenesis (Nishigori *et al.*, 1994). UVA wavelengths have recently also been shown to play an important role in the mutagenicity imparted by solar radiation (Drobetsky *et al.*, 1995). In humans, deficiences in DNA repair activities that are functionally similar to bacterial enzymes can result in the manifestation of diseases such as xeroderma pigmentosum and Cockyane's syndrome (for review see Hanawalt, 1994). Many agents that are mutagens in bacteria have been shown also to be carcinogens in mammals (McCann *et al.*, 1975). As a result, the identification

of bacterial factors that are important in repair of near-UV induced DNA damage should increase our understanding of comparable repair processes in higher organisms.

FAR-UV INDUCED DNA DAMAGE

Exposure of bacterial DNA to wavelengths of ultraviolet radiation approaching its absorption maximum (ie. 260 nm) results primarily in the formation of adducts between neighbouring pyrimidines through the formation of a four-membered ring structure resulting from the saturation of their respective 5,6 double bonds (Freidberg *et al.*, 1995). These lesions, termed cyclobutane pyrimidine dimers can be lethal if not repaired, as they interfere with DNA synthesis (Setlow *et al.*, 1963). Another type of far-UV induced premutagenic lesion, the pyrimidine-pyrimidone (6-4) photoproduct, results from the linkage of the C-6 position of one pyrimidine in a DNA strand and the C-4 position of an adjacent pyrimidine (Freidberg *et al.*, 1995). As with cyclobutane pyrimidine adducts, the non-cyclobutane (6-4) photoproducts can result in mutations if not repaired by host cell processes (Mitchell and Nairn, 1989). The repair of far-UV induced DNA lesions in bacteria has been extensively studied (for reviews see van Houten, 1990; Sancar and Tang, 1993; Sancar, 1994). In *E. coli*, the majority of damage generated by far-UV radiation can either be reversed through the action of the near-UV activated enzyme photolyase (via photoreactivation) or repaired by the UvrABC excision nuclease (Grossman *et al.*, 1988), a component of the DNA damage-inducible SOS response.

Photoreactivation

Photoreactivation refers to the reversal of far-UV-mediated DNA damage through concurrent or subsequent exposure to near-UV/visible light (300-500 nm). The *E. coli* DNA photolyase (*phr*; photoreactivating enzyme) is a 49 kDa protein which removes cyclobutane dimers using the energy supplied by near-UV/visible light (Sancar, 1994). DNA photolyase binds to pyrimidine dimers in DNA in a light-independent step and subsequently is activated through absorption of a single near-UV photon to split the cyclobutane ring, restoring the individual pyrimidines, and then dissociates from the DNA (Sancar, 1994). A photoreactivating enzyme that specifically repairs (6-4) photoproducts has been identified in *Drosophila* (Todo *et al.*, 1993). This is the first evidence of a photoreactivating activity specific for these lesions in any organsim, although the presence of an enzyme with similar activity has been suggested to be present in *E. coli* (Dorrell *et al.*, 1993).

SOS-Inducible nucleotide excision repair

One of the best-characterized and most widely studied DNA repair pathways in *E. coli*, as in most organisms, is nucleotide excision repair. The process involves the action of six proteins, UvrA (104 kDa), UvrB (78 kDa), UvrC (69 kDa), UvrD (70 kDa), DNA polymerase I (103 kDa), and DNA ligase (70 kDa) (Sancar and Tang, 1993). Three of these proteins, the products of the *uvrA*, *uvrB* and *uvrC* loci, are components of the damage inducible SOS regulon, under the control of the RecA (38kDa) and LexA (23kDa) gene products (as reviewed in Walker, 1987). In an uninduced cell, the product

of the *lexA* gene represses expression of a large number of unlinked genes, including the *recA* and *lexA* genes, through binding to similar operator sequences in front of each gene. When an *E. coli* cell is subject to agents or conditions which damage DNA or interfere with DNA replication, an inducing signal is generated, which activates the RecA protein in a reversible manner. The interaction of activated RecA with LexA causes a proteolytic cleavage of LexA, which derepresses the expression of those genes under control of LexA. As the amount of LexA gradually decreases, various SOS genes, including *uvrA*, *uvrB* and *uvrC*, are expressed at an increased level, and the SOS responses controlled by RecA and LexA are initiated. As the cell gradually begins to recover from the inducing treatment, (eg. as a result of the repair of damaged DNA) the inducing signal is eliminated, and the proteolytic activity of RecA molecules is no longer activated. The continued synthesis of LexA once again results in repression of the SOS genes and the cell returns to an uninduced state (Walker, 1987). The nature of the inducing signal is not yet known, although *in vitro* studies have indicated that activation of RecA occurs when it forms a ternary complex with single-stranded DNA in the presence of a nucleoside triphosphate (Roberts *et al.*, 1982).

The process of excision repair of damaged nucleotides consists of five steps: damage recognition, incision, excision, repair synthesis and ligation (van Houten, 1990). The first three steps of this process are carried out by the UvrA, UvrB and UvrC proteins, which complex to form the UvrABC excision nuclease or "excinuclease" (Sancar and Rupp, 1983). The UvrABC complex recognizes the damage site, binds, and hydrolyzes two phosphodiester bonds, one 7 nucleotides 5' and the other 3-4 nucleotides 3' of the

modified nucleotide(s). This results in a 12 or 13 nucleotide oligonucleotide being removed from the damaged strand. The UvrD protein (Helicase II) is thought to help dissociate the both the oligonucleotide and UvrABC complex from the DNA once the incision is complete. The gap left by the excised oligonucleotide is subsequently repaired by DNA polymerase I and DNA ligase (as reviewed in van Houten, 1990).

The UvrABC excinuclease recognizes and repairs a broad range of DNA damage. As mentioned previously, the predominant lesions removed by this system are those generated following exposure to far-UV, specifically pyrimidine dimers (Sancar and Rupp, 1983) and 6-4 photoproducts (Franklin and Haseltine, 1984). In addition, strains of *E. coli* lacking UvrABC activity (*uvrA*$^-$), have been shown to be sensitive to near-UV, suggesting a possible role of nucleotide excision repair in removing lesions generated by longer UV wavelengths (Turner and Webb, 1981). The UvrABC excinuclease also removes DNA lesions generated under conditions of oxidative stress, including thymine glycols and AP (apurinic/apyrimidinic) sites (Lin and Sancar, 1989; Kow et al., 1990). When combined with a mutation in the *fpg* gene, encoding formamidopyrimidine (fapy) glycosylase (see below), an oxidative repair enzyme, *uvr* mutations decrease the transforming efficiency and increase mutagenesis in plasmid DNA treated with methylene blue plus visible light, which oxidatively damages DNA (Czeczot *et al.*, 1991). Although these activities are relatively weak compared to the incision rates for pyrimidine dimers (Sancar and Tang, 1993), it appears that the UvrABC excinuclease may act as a secondary defense against oxidative damage.

NEAR-UV INDUCED DNA DAMAGE

DNA damage caused by low fluence near-UV light is generally thought to be less severe than that caused by shorter wavelengths (Turner and Webb, 1981), although prolonged exposure can generate pyrimidine dimers (Tyrrell, 1973), single strand breaks (Peak and Peak, 1982b) and can lead to the induction of the SOS repair system (Caldeira de Araujo and Favre, 1986). In contrast to far-UV, near-UV irradiation does not conform to the reciprocity law, which is characteristic of simple photochemical reactions. The law states that the effect of radiation is a function of the total radiant energy, and is independent of intensity and time (as cited in Peak and Peak, 1982a). Peak and Peak (1982a) demonstrated that the survival of wild-type *E. coli* cells was significantly reduced following long-term, low-intensity irradiation with 365 nm near-UV, relative to the same dose given at higher intensity over a shorter period of time.

Near-UV-mediated lethality is not thought to be as a result of direct DNA damage (Eisenstark, 1989), as is the case for far-UV, and shows an oxygen dependency (Webb and Lorenz, 1970). This requirement for oxygen has led many investigators to suggest that the lethal effects of near-UV may involve the action of reactive oxygen species (ROS). Near-UV effects may involve the absorption of light by endogenous photosensitizers (either cytoplasmic or membrane-bound) and the subsequent transferral of energy to O_2, leading to the production of ROS (Kramer and Ames, 1987). The nature of the endogenous near-UV photosensitizer is as yet unknown, although cytochromes (Sammartino and Tuveson, 1987) and thiolated nucleotides such as 4-thiouridine (Ito *et al.*, 1988) have been shown to confer a near-UV-sensitive phenotype on cells when these

molecules are overexpressed. Several studies (Boiteux *et al.*, 1993; Paramio *et al.*, 1991) have also shown that psoralens, in concert with near-UV, can cause significant DNA damage as measured by a reduction in plasmid transformational ability into DNA repair-deficient strains.

Oxidative Stress

Reactive oxygen species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and the superoxide anion (O_2^-), all of which can threaten cellular integrity (Eisenstark, 1989), can arise from a variety of sources, both endogenous and exogenous. Of the above reactive species, only singlet oxygen is capable of directly damaging DNA (Brawn and Fridovich, 1981). During normal aerobic respiration, oxygen is used as the terminal electron acceptor in the electron transport chain, and subsequently oxygen is normally reduced to water. However, H_2O_2, O_2^- and the highly reactive hydroxyl radical (•OH) can be generated as by-products of the incomplete reduction of oxygen to water:

$$O_2 \text{ ---> } O_2^- \text{ ---> } H_2O_2 \text{ ---> } \bullet OH + H_2O \text{ ---> } H_2O$$

In *E. coli*, superoxide radicals can also be generated through exposure to oxidation-reduction (redox) drugs such as paraquat or released as a means of defense from stimulated macrophages (Storz *et al.*, 1990). Levels of superoxide anion have also been shown to increase following exposure to near-UV (Ahmad, 1981). The major sources of superoxide in *E. coli* are those reactions mediated by NADH dehydrogenase,

succinate dehydrogenase and D-lactate dehydrogenase (Imlay and Fridovich, 1991). Glutathione reductase, which uses NADH as an electron source, is also thought to generate significant amounts of superoxide anion (Imlay and Fridovich, 1991). In addition, several cellular constituents, such as ubiquinols, catechols and thiols can easily be reduced to semiquinones, which in turn reduce O_2 to O_2^- (Farr and Korogama, 1991).

Hydrogen peroxide is generated as a by-product of the breakdown of superoxide anion by superoxide dismutases (see below), and also as a consequence of exposure to near-UV irradiation (Eisenstark, 1989).

Singlet oxygen, the lowest electronically excited energy state of molecular oxygen, has a relatively long lifetime and the potential to react with a variety of biological substrates, including DNA (Brawn and Fridovich, 1981). Biological sources of singlet oxygen may include photosensitization reactions involving compounds like methylene blue when irradiated with visible light (Epe *et al.*, 1989), or as a result of the decomposition or interconversion of other reactive oxygen species (Eisenstark, 1989). Among the nucleic acid constituents of DNA, only guanine residues appear to show any reactivity towards singlet oxygen (Piette, 1990).

Hydroxyl radicals or ferryl radicals (hydroxyl radicals complexed with iron),are produced primarily via the iron-catalyzed Fenton reaction (Storz *et al.*, 1990):

$$O_2 + Fe^{2+} + H^+ \text{-----} > \bullet OH + H_2O + Fe^{3+}$$

The hydroxyl radical reactivity is so great that it will react almost immediately with

almost any molecule in its vicinity (Brawn and Fridovich, 1981; Eisenstark, 1989). Radiolysis of cellular water by γ irradiation also results in the production of hydroxyl radicals (Repine *et al.*, 1981).

Oxidative damage

Reactive oxygen species cause a wide range of damage to cellular constituents both *in vitro* and *in vivo*, including damage to DNA and cell membranes (Storz *et al.*, 1990). As a result of studies using strains of *E. coli* with mutations in DNA repair functions which have been shown to be hypersensitive to oxidative stress, DNA is considered to be the primary target for reactive oxygen species attack (Imlay and Linn, 1988). As mentioned above, it is now thought that O_2^- and H_2O_2 do not directly interact with DNA, rather that the reaction of H_2O_2 with iron-complexed DNA leads to the production of •OH, which directly damages DNA. The attack of reactive oxygen species, such as •OH, can generate a variety of products, ranging from abasic (apurinic or apyrimidinic; "AP") sites to modified nucleotides. For example, an •OH attack at the double bond of thymine at C-5 or C-6 can, in the presence of O_2, lead to the production of thymine glycol (Demple and Linn, 1982). Another important oxidation product is 8-hydroxyguanine (8-oxoguanine), which results from the addition of •OH to C-8 of guanine (Jovanovic and Simic, 1989), which can also be generated by the action of methylene blue plus visible light in the presence of oxygen (Floyd *et al.*, 1989; Schneider *et al.*, 1990), a singlet oxygen generating treatment (Epe *et al.*, 1989). Among the nucleic acid constituents of DNA, only guanine residues appear to be sensitive to singlet oxygen

(Piette, 1990). Fragmented purines, with ruptured imidazole rings, are also significant oxidative products resulting from hydroxyl radical attack (Demple and Harrison, 1994). These formamidopyrimidine (Fapy) residues are derived from both adenine and guanine, and can arise as secondary products of N7-alkylated guanines (and adenines) through the base-catalyzed hydrolysis of N7-methylguanine generating N-methyl Fapy (Haines *et al.*, 1962).

OXIDATIVE STRESS ENZYMES (POTENTIAL NEAR-UV STRESS ENZYMES)

To date, no DNA repair enzymes specific for near-UV mediated DNA damage have been identified in *E. coli*, although several repair enzymes (both inducible and constitutive) which may act upon near-UV generated DNA lesions have been identified based on mutations resulting in near-UV sensitivity. In agreement with the concept that near-UV mediated DNA damage involves reactive oxygen species, many of the putative near-UV recovery enzymes have previously been implicated in repair of lesions generated under conditions of oxidative stress (Table 1). In response to oxidative stress, bacteria such as *E. coli* can either; i) remove or "scavenge" the reactive oxygen species before they cause damage to cellular constituents such as DNA, or i) repair the damage to DNA caused by reactive oxygen species before it becomes mutagenic.

SCAVENGING ENZYMES

Enteric bacteria, such as *E. coli*, produce several enzymes such as superoxide dismutase and catalase to protect themselves from the potentially damaging effects of

Table 1. Genes that affect near-ultraviolet sensitivity in *E. coli*. See details in text.

Gene	Enzyme	Near-UV Source	Wavelength[d] (nm)	Fluence Rate (W/m^2)	Reference(s)
rpoS (*katF*)	Sigma factor S (σ^s)	BLB	300-400	24.0	Sammartano and Tuveson (1986)
		BLB	>290	500.0[a]	Eisenstark and Perrot (1987)
xthA	Exonuclease III	BLB	310-405	10.0	Sammartano and Tuveson (1983)
		BLB	>290	500.0[a]	Eisenstark and Perrot (1987)
sodA, *sodB*[c]	Superoxide dismutases	BLB	300-420	26.7[a]	Hoerter *et al.* (1989)
uvrA	Subunit A of UvrABC complex	BLB	313-405	65.0	Turner and Webb (1981)
recA	RecA protein	BLB	313-405	65.0	Turner and Webb (1981)
		BLB	>290	500.0[a]	Eisenstark and Perrot (1987)
fpg (*mutM*)	Fapy DNA glycosylase	XAL	>320	145.0	Shennan *et al.* (in press)
polA	DNA polymerase I	BLB	313-405	65.0	Turner and Webb (1981)
		BLB	>290	500.0[a]	Eisenstark and Perrot (1987)
oxyR[b]	OxyR regulator	BLB	n/a	35.0	Kramer and Ames (1987)

Abbreviations: BLB - black light bulb; XAL - xenon arc lamp: n/a - values not provided in references
[a]as calculated from values presented in paper
[b]in *Salmonella typhimurium*, Δ*oxyR* strains are hypersensitive
[c]the *sodA sodB* double mutant is hypersensitive, the single mutants are not
[d]filtered wavelength of near-UV (see individual reference(s) for description of filter used)

reactive oxygen species. Superoxide dismutase (SOD) and catalase (HP) act to detoxify superoxide anion and hydrogen peroxide, respectively, by the following mechanism:

$$4H^+ + 4O_2^- \xrightarrow{SOD} 2O_2 + 2H_2O_2 \xrightarrow{HP} 2H_2O + 3O_2$$

Superoxide Dismutases

E. coli possesses two forms of superoxide dismutase: a manganese-containing SOD (MnSOD) and an iron-containing SOD (FeSOD), the products of the *sodA* and *sodB* loci, respectively. Both MnSOD and FeSOD are small metallo-proteins capable of catalyzing the detoxification of superoxide by the reaction shown above. The FeSOD is constitutively expressed under both aerobic and anaerobic conditions, while MnSOD is not normally produced under anoxic conditions (Dougherty *et al.*, 1978; Moody and Hassan, 1984). As a component of the SoxRS regulon (Tsaneva and Weiss, 1990; see below), MnSOD is induced by the superoxide anion and by superoxide-generating redox compounds such as paraquat (Hassan and Fridovich, 1980; Hassan and Moody, 1987). Superoxide dismutase has attracted much attention as a possible near-UV recovery enzyme (Ahmad, 1981; Farr *et al.*, 1986). Plasmids carrying an inducible MnSOD provide the cell with limited resistance to near-UV but not far-UV (Carlioz and Touati, 1986), while a plasmid carrying the FeSOD does not endow the cell with near-UV resistance (Scott *et al.*, 1987). A double *sodA sodB* mutant is, however, hypersensitive to near-UV (Carlioz and Touati, 1986; Hoerter *et al.*, 1989), and shows increased mutability

(Hoerter *et al.*, 1989).

Catalases

Two different catalases, HPI (hydroperoxidase I) and HPII (hydroperoxidase II) are also produced in *E. coli* in response to oxidative stress. HPI (encoded by *katG*) is produced both aerobically and anaerobically and is induced by hydrogen peroxide (Loewen *et al.*, 1985). The *katG* gene, encoding HPI, is a member of the oxidative stress inducible OxyR regulon (Morgan *et al.*, 1986; see below). In contrast, HPII (encoded by *katE*) is expressed maximally upon entry of cells into stationary phase (Loewen *et al.*, 1985). The *katE* gene is part of a large number of genes under the control of sigma S (σ^s), the stationary phase-specific sigma factor encoded by the *rpoS* (originally designated *katF*) locus (Sak *et al.*, 1989; Schellhorn and Hassan, 1988).

As H_2O_2 is generated *in situ* following near-UV irradiation (Ananthaswamy and Eisenstark, 1976; McCormick *et al.*, 1976), it was originally thought that catalase would be an important enzyme in conferring resistance to near-UV-induced stress. Despite similarities in the effects of near-UV and H_2O_2 (Ananthaswamy and Eisenstark, 1976; Sammartano and Tuveson, 1983), it is now thought that H_2O_2 plays only a minor role in near-UV lethality (Eisenstark and Perrot, 1987; Kramer and Ames, 1987). The near-UV-sensitive pheonotype of *katF* (*rpoS*) mutants (Eisenstark and Perrot, 1987; Sammartano *et al.*, 1986) suggests that H_2O_2 is a photoproduct of near-UV, as RpoS is necessary for the synthesis of HPII. The sensitivity of *rpoS* mutants is attributable to the fact that *xthA* (encoding Exonuclease III) is also part of the RpoS regulon, and *xthA* mutants are

hypersensitive to near-UV (Sammartano and Tuveson, 1983). It is possible that catalase may actually act as a photosensitizer when present in excess as strains carrying *katG* on a plasmid are actually more sensitive to near-UV (Eisenstark and Perrot, 1987).

DNA REPAIR ENZYMES

E. coli possesses several distinct DNA repair activities (both constitutive and inducible) that remove oxidized bases that are produced as a consequence of exposure to oxidants, and therefore may also be involved in the repair of near-UV induced DNA lesions.

DNA Glycosylases

Escherichia coli possesses two repair systems to eliminate oxidized bases in DNA, the aforementioned nucleotide excision repair pathway mediated by the UvrABC excinuclease (Walker, 1987) and the base excision repair pathway (reviewed in Freidberg *et al.*, 1995). Base excision repair is a multi-step process initiated by the action of a DNA glycosylase which cleaves the (N-1'C) chemical bond to release the damaged base from the DNA, producing an abasic (apurinic/apyrimidinic; AP) site (Boiteux, 1993). This first step is catalysed either by the Fapy glycosylase or endonuclease III depending on the nature of the oxidized base. In *E. coli*, Fapy glycosylase acts to excise oxidized purines, while endonuclease III releases oxidized pyrimidines from DNA.

Fpg (Fapy) glycosylase

Fapy glycosylase (encoded by *fpg*) is a 30.2 kDa protein that excises a range of DNA lesions generated during conditions of oxidative stess. Although originally characterized as an activity that removes the lethal imidazole ring-opened form of N7-methylguanine (Chetzanga and Lindahl, 1979), Fapy glycosylase has been shown to remove a variety of ring-opened purines resulting from oxidative DNA damage (Boiteux *et al.*, 1992) as well as 8-hydroxyguanine (8-oxoguanine), a primary oxidation product (Tchou *et al.*, 1991). Imidazole-ring-opened methylguanine inhibits DNA synthesis by blocking the progress of DNA polymerase I (Boiteux and Laval, 1983), resulting in cell lethality (Tudek *et al.*, 1992). 8-hydroxyguanine, although not as lethal as the bulky imidazole-ring-opened guanine residue, is highly mutagenic and occasionally lethal, as a single residue per single-strand viral genome reduces survival by 10-50% in an *E. coli* host (Wood *et al.*, 1990). This oxidized form of guanine (also called a GO lesion) readily pairs with adenine, leading to an increase in G to T transversions if not corrected prior to DNA replication (Michaels *et al.*, 1992). Strains of *E. coli* lacking Fapy glycosylase activity (ie. *fpg*$^-$) exhibit significantly higher levels of spontaneous mutation, due primarily to the mutagenic nature of the 8-oxoguanine lesion (Michaels *et al.*, 1992). Since 8-hydroxyguanine nucleosides have been shown to accumulate in cells following exposure to near-UV (Hattori-Nakakuki *et al.*, 1994), repair of this oxidized lesion may be important in protecting the cell from the lethal effects of near-UV. Consistent with this hypothesis, it has recently been shown that strains of *E. coli* lacking Fapy glycosylase activity are more sensitive to near-UV than are wild-type strains (Shennan *et al.*, in

press).

Although Fapy glycosylase is a small monomeric protein, it possesses at least three enzymatic activities: a DNA glycosylase which liberates the modified base leaving an abasic site, an AP endonuclease activity which nicks DNA at the 3' and 5' sides of the abasic site and a 5'-terminal deoxyribose phosphate excising activity (Boiteux, 1993). The AP endonuclease reaction proceeds via a β-elimination reaction, resulting in a gap limited at the 5' and 3' ends by phosphoryl groups (Bailly *et al.*, 1989). The 3' phosphoryl group is subsequently excised and the gap filled by other enzymes. Functionally similar repair activities to the *E. coli* Fapy glycosylase have also been identified in yeast (de Oliveira *et al.*, 1994) and mammals (Margison and Pegg, 1981), suggesting this may be a conserved repair mechanism.

Endonuclease III

The product of the *nth* gene, endonuclease III, was initially identified as an activity involved in the removal of far-UV-induced DNA lesions (Radman, 1976). Although the predominant type of DNA lesions generated by such treatment are cyclobutane pyrimidine dimers, other lesions such as thymine glycols and abasic sites are also produced (Gates and Linn, 1977; Demple and Linn, 1980). Endonuclease III is a monomeric protein of 23 kDa which excises a large number of thymine and cytosine-derived lesions in DNA, generated by exposure to ionizing radiation and oxidizing agents including 5,6-dihydrothymine, 5-hydroxy-5-methylhydantoin, thymine glycol, urea, cytosine glycol and many others (Dizdaroglu *et al.*, 1993). Although none of above

lesions have been shown to be strongly mutagenic, the presence of thymine glycol in the DNA template act to block DNA synthesis (Clark *et al.*, 1987). All damaged DNA bases that are excised by endonuclease III are derivatives of pyrimidine bases and show ring saturation, ring contraction or ring fragmentation (Dizdaroglu *et al.*, 1993). These modifications result in the formation of non-planar structures which may be recognized by the enzyme (Boiteux, 1993). In agreement with this hypothesis, ionizing radiation products containing intact pyrimidine rings such as 5-hydroxymethyluracil are not excised by endonuclease III (Dizdaroglu *et al.*, 1993).

Endonuclease III possesses both DNA glycosylase activity and abasic nicking activity (Demple and Linn, 1980; Breimer and Lindahl, 1985). Kow and Wallace (1987) have suggested that these two activities work in a concerted fashion, although the enzyme has been shown to catalyse strand cleavage at pre-existing abasic sites (Boiteux, 1993). The phosphodiester cleavage by endonuclease III occurs via a β-elimination reaction (as with Fapy glycosylase) to generate 5' termini with 5'-phosphate nucleotides and 3' termini bearing the 2,3-unsaturated abasic residue 4-hydroxy-2-pentenal (Bailly and Verly, 1987). These blocked 3' termini require further processing by other enzymes to generate 3'-OH primers, which can be used by DNA polymerase I in repair synthesis. It has been suggested that strains of *E. coli* lacking endonuclease III activity are sensitive to near-UV but not far-UV (Sammartano and Tuveson, 1987).

REGULONS

In addition to the constitutively expressed DNA repair systems, *E. coli* also

possesses several inducible pathways capable of reducing the threat of oxidative damage. Kramer *et al.* (1988) demonstrated that at least 57 proteins in wild-type *S. typhimurium* cells were induced following exposure to near-UV. The regulons that are involved in response to near-UV are activated in response to a variety of different stimuli including reactive oxygen species (eg. H_2O_2 and $O_2^{\cdot}$ induce the OxyR and SoxRS regulons, respectively), DNA damage (SOS regulon) and entry into stationary phase of the cell cycle (RpoS regulon).

OxyR (Oxidative/Peroxide Stress) Regulon

The 34kDa OxyR protein regulates the expression of several genes (eight in *E. coli*) in response to H_2O_2 stress, including *katG* (catalase HPI; see above) and *ahp* (alkyl hydroperoxide reductase). In total, four of the eight proteins under the control of *oxyR* have to date been identified. Alkyl hydroperoxide reductase is thought to provide an additional defense to that provided by the catalases by reducing a variety of organic hydroperoxides (Farr and Kogama, 1991). Single mutations within the *oxyR* locus result in the constitutive expression of the proteins under the control of this regulator (eg. a 50-fold increase in HPI and a 20-fold increase in alkyl hydroperoxide reductase), while deleting the *oxyR* region results in the absence of expression of the regulated gene products (Christman *et al.*, 1985; Kramer and Ames, 1987; Kramer *et al.*, 1988).

Kramer and Ames (1987) demonstrated that *Salmonella typhimurium* strains containing a deletion of the *oxyR* locus were hypersensitive to killing by near-UV irradiation. There is, however, some confusion in the literature as to the response of the

single mutation strain (*oxyR1*) of *S. typhimurium* to near-UV stress. Eisenstark and Perrot (1987) found that the constitutively-expressing *oxyR* strain was more resistant to near-UV than a wild-type strain. This result, coupled to the fact that *katG*$^-$ and *katE*$^-$ strains were found to be only slightly more sensitive to near-UV than wild-type strains, led the authors to the conclusion that catalase played only a minor role in protection against near-UV irradiation (Eisenstark and Perrot, 1987). In contrast, Kramer and Ames (1987) showed that the *oxyR1* mutation rendered cells sensitive to near-UV, compared to wild-type strains. They suggested that the increased sensitivity of the *oxyR1* mutant might be as a result of the overproduction of proteins which absorb near-UV wavelengths. Both HPI catalase and alkyl hydroperoxide reductase absorb significantly in the near-UV range (Kramer and Ames, 1987).

SoxRS (Superoxide Stress) Regulon

Superoxide-generated stress results in the induction of a variety of activities under the control of the SoxRS locus, including MnSOD (*sodA*), glucose-6-phosphate dehydrogenase (*zwf*) and the DNA repair enzyme endonuclease IV (*nfo*). The *soxRS* locus (originally designated *soxR*) encodes two peptides, one of 17 kDa and one of 13 kDa, both of which are essential for induction of SoxRS regulated genes (Tsaneva and Weiss, 1990). The activity of endonuclease IV, a minor AP-endonuclease, increases 20-fold in *E. coli* following treatment with a sublethal dose of the redox cycling (ie. superoxide-generating) compound paraquat. This effect is independent of the H_2O_2-inducible *oxyR* regulon (Chan and Weiss, 1987).

Endonuclease IV is a 5' endonuclease which accounts for approximately 10% of the total AP endonuclease activity found in crude extracts in *E. coli* (Ljungquist *et al.*, 1976). Like the AP endonuclease function of exonuclease III (see below), endonuclease IV attacks phosphodiester bonds 5' to an abasic site produced by DNA glycosylases leaving 3'-OH groups (Bailly and Verly, 1989), and can also remove unusual 3' termini such as phosphates or phosphoglycolates produced by oxidative damage that can block DNA repair synthesis (Demple *et al.*, 1986). Strains of *E. coli* lacking endonuclease IV activity are no more sensitive to 254 nm-UV irradiation or H_2O_2 than wild-type strains (Cunningham *et al.*, 1986). In combination with a *xthA* mutation, however, *nfo*$^-$ strains become more sensitive to both H_2O_2 and near-UV irradiation (Cunningham *et al.*, 1986; Eisenstark, 1989). This increased sensitization may be a result of endonuclease IV and exonuclease III accounting for virtually all of the DNA-specific 3'-phosphatase activity in *E. coli* (Demple and Harrision, 1994).

SOS Regulon

RecA-deficient strains of *E. coli* are highly sensitive to near-UV, more than could be accounted for by the loss of the nucleotide excision repair system (ie. UvrABC) alone. Turner and Webb (1981) demonstrated that *recA*$^-$ and *uvrA*$^-$ single mutant strains are slightly more sensitive to the lethal effects of broad-band near-UV, whereas a *uvrA*$^-$ *recA*$^-$ double mutant is hypersensitive, suggesting the possible role of another RecA-mediated DNA repair system in response to near-UV stress. In addition to its role in the SOS regulatory response, RecA protein also plays a central role in the process of homologous

recombination and in a set of DNA repair and damage tolerance pathways which help cells survive the effects of DNA damage (as reviewed in Freidberg *et al.*, 1995).

Although not an inducible component of the SOS repair system, strains lacking DNA polymerase I (which is involved in DNA repair synthesis in both nucleotide and base excision repair systems) are also highly sensitive to near-UV irradiation (Turner and Webb, 1981; Eisenstark and Perrot, 1987).

RpoS Regulon

The increased sensitivity to near-UV irradiation observed in *rpoS* (*katF*) mutants is believed to be due primarily to the resulting loss of the RpoS-directed exonuclease III activity (Sak *et al.*, 1989). *E. coli* exonuclease III is a single 28kDa peptide with four enzymatic activities: an 3' ->5' exonuclease activity which removes 5' mononucleotides from DNA duplexes; a DNA 3' phosphatase activity which releases 3' terminal phospho-monoesters from DNA (as endonuclease IV); an endonuclease activity which cleaves phosphodiester bonds in DNA treated by acid or methylmethane sulfonate (depurinating agents); and an exonucleolytic RNase H activity, which degrades RNA in DNA-RNA hybrids (Freidberg *et al.*, 1995).

Exonuclease III has been shown to be an important repair enzyme in H_2O_2-mediated oxidative stess (Demple *et al.*, 1983; Demple *et al.*, 1986). As a result, *xthA* mutants are highly sensitive to exposure to near-UV (Sammartano and Tuveson, 1983) and H_2O_2 (Demple *et al.*, 1983; Sammartano *et al.*, 1986), but not far-UV (Sammartano and Tuveson, 1983).

THE NEAR-UV-INDUCED "GROWTH DELAY" IN *E. coli*

In wild-type strains of *E. coli*, exposure to sublethal fluences of near-UV results in an transient inhibition of cell growth and division. This "growth delay" effect following near-UV exposure is attributable to 4-thiouridine (Ramabhadran *et al.*, 1976), a modified photosensitive base found in 70% of bulk transfer RNA molecules (Favre *et al.*, 1985). The modification of uridine to 4-thiouridine in *E. coli* tRNA involves a concerted sulfurtransferase system dependent upon the action of two proteins, factors A and C (Abrell *et al.*, 1971), encoded by the *nuvA* (Lipsett, 1978) and *nuvC* (Ryals *et al.*, 1982) loci, respectively. As a result of the substitution of the oxygen atom at position 4 of uracil by sulfur, the modified base absorbs more strongly in the near-UV (334nm) range (Favre *et al.*, 1971). When irradiated, the 4-thiouridine residue at position 8 forms an adduct with a cytosine residue at position 13, reducing the acylation capacity of several species of tRNA, principally $tRNA^{Pro}$ and $tRNA^{Phe}$ (Blondel and Favre, 1988). As uncharged tRNA molecules accumulate within the cell, protein synthesis is halted.

In wild-type cells, this phenomenon is amplified by the RelA-dependent stringent response (Thiam and Favre, 1984). As uncharged tRNAs are bound at the ribosome A site, synthesis of the RelA-dependent stringent factor guanosine tetraphosphate (ppGpp) is stimulated. The ppGpp binds to RNA polymerase and alters transcription of *rrn* (ribosomal RNA) operons (Little *et al.*, 1983) and consequently there is nearly a complete cessation of RNA synthesis (Thiam and Favre, 1984). Two types of near-UV irradiation "resistant" mutants have been characterized: i) cells which lack 4-thiouridine in their tRNA (Ramabhadran *et al.*, 1976), and thus show no growth delay following irradiation

(*nuv*), and ii) cells which are unable to accumulate high concentrations of ppGpp during amino acid starvation and therefore fail to induce the stringent response (Ramabhadran and Jagger, 1976). As a result of the new tRNA being synthesized, these strains (*relA*) have a shorter growth delay.

Strains lacking the thiolation activity responsible for the modification of uracil to 4-thiouridine (ie. *nuv*) are not growth inhibited by near-UV irradiation, thus facilitating identification of DNA repair factors (both constitutive and inducible) required for the repair of near-UV mediated DNA damage. The presence of the growth delay has proven to be a complication in previous studies. Turner and Eisenstark (1984) used a *recA*::*lacZ* fusion in a wild-type *nuvA*$^+$ strain to determine whether the SOS response is induced during conditions of near-UV stress. They concluded that near-UV wavelengths did not induce the SOS response. In contrast, when Caldeira de Araujo and Favre (1986) removed the growth delay by using a *nuvA*$^-$ strain containing a *lacZ* fusion to the SOS inducible *sfiA* gene (encoding a factor responsible for inhibition of cell division), they found that the SOS system was indeed induced. Clearly, the growth delay effect may mask the induction of DNA repair systems, and removal of the effect by using *nuvA*$^-$ strains eliminates the confounding effects of the growth delay phenomenon.

PURPOSE OF CURRENT STUDY

The purpose of my work was three-fold. First, I wanted to isolate a *nuvA*$^-$ mutant harbouring a selectable marker (kanamycin resistance) by transposon insertion, using the method of transposon-directed mutagenesis outlined by Kleckner *et al.* (1991). Strains

deficient in the ability to synthesize 4-thiouridine had been generated previously through chemical mutagenesis (Thomas and Favre, 1980), however a *nuvA* mutant carrying a selectable marker has yet to be identified. Once isolated, I was to examine the effects of broad-band near-UV (UVA; >320 nm) on *nuvA*$^-$ strains of *E. coli* to assess the relative importance of the UvrABC exicnuclease and Fapy glycosylase in the repair of UVA-mediated DNA damage. Finally, the putative role of 4-thiouridine as an endogenous photosensitizer in near-UV induced lethality was to be addressed, through the use of an *in vitro* plasmid DNA irradiation assay, followed by transformation into competent derivatives of the repair deficient strains constructed in the second part of my work.

CHAPTER 2. ISOLATION OF A SELECTABLE *nuvA* MUTANT

INTRODUCTION

In *Escherichia coli*, sublethal fluences of near-UV radiation elicit a transient physiological response which has come to be known as the growth delay (as reviewed in Chapter 1). Strains lacking either of the two thiolation factors required for the modification of uracil residues to 4-thiouridine are not growth inhibited following exposure to near-UV, nor are strains incapable of synthesizing the alarmone molecule (ppGpp) required for the induction of the stringent response. By removing the intrinsic growth delay through mutating any one of the *nuvA*, *nuvC* or *relA* loci, a confounding variable that is involved when considering DNA damage caused by exposure to near-UV is eliminated, and the investigation of the responses involved in protecting the cell from near-UV-induced lethality and mutagenesis is possible.

The initial component of my work was to isolate a *nuvA* mutant harbouring a selectable marker (kanamycin resistance) by transposon insertion, using the method of transposon-directed mutagenesis outlined by Kleckner *et al.* (1991). Strains deficient in the ability to synthesize 4-thiouridine have been generated previously through chemical mutagenesis (Thomas and Favre, 1980). However, a *nuvA* mutant carrying a selectable marker has yet to be identified. The advantage of a having a selectable marker in a gene of interest is that it allows easy movement of the mutation into established backgrounds to contrast established responses with those seen when the gene of interest is knocked out.

MATERIALS AND METHODS

Bacterial Strains, Phage and Growth Conditions

The strains and phage used in this work are shown in Table 1. The Kohara λ clones (9G9, 4F3, 19F6 and 2H5; see Kohara *et al.*, 1987) spanning the *nuvA* region were gifts from N. Daniels and F. Blattner. All cultures were grown in Luria-Bertani (LB) rich medium (Miller, 1992) supplemented with streptomycin (50 μg/ml) and/or kanamycin (50 μg/ml). All antibiotics and amino acids used were from Sigma Chemical Company (St. Louis, MO). LB agar and M9 minimal glucose agar plates were prepared as described previously (Miller, 1992). Bacterial cultures were grown in a shaker at 37°C, and cell growth was monitored by measuring the optical density at 600nm (OD_{600}).

Construction of a *nuvA* Mutant

I) Mutagenesis

The method of transposon-directed mutagenesis used was as described in Kleckner *et al.* (1991). Host cells to be mutagenized (AB1157) were grown overnight at 37°C in TBMM medium (10 g tryptone, 5 g NaCl, after sterilization, add maltose to final concentration of 0.2% (w/v), 10 mM $MgSO_4$,1 μg/ml thiamin). The following morning, the culture was concentrated by centrifugation and resuspended in 1/10 volume LB medium. Phage (λ1316::Tn*10* (kan)) was added to the culture in an moi (multiplicity of infection) of approximately 0.2 (ie. phage:cell = 1:5) and allowed to adsorb for 15 min at room temperature , followed by 15 min at 37°C (without shaking). The cells were

Table 1. Bacterial strains and bacteriophage used in this study.

Strain	Sex	Genotype	Source
AB1157	F-	*thr1 leu6 proA2 his4 argE3 lacY1 galK2 xyl5 mtl2 ara14 tsx33 strA1*	A. Favre
PM2	F-	as AB1157 but *nuvA*	A. Favre
MC4100	F-	*araD139 Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rbsR flb5301*	G. Weinstock
SE5000	F-	as MC4100 but *recA*	G. Weinstock
Phage			
λNK1316		Tn*10*::kan	N. Kleckner
P1*vir*			Lab stock

pelleted by centrifugation and washed with LB containing 50 mM sodium citrate to inactivate any unadsorbed phage. The pellet was then resuspended in 500 µl LB containing 50 mM sodium citrate and incubated at 37°C for 1 h to allow for the expression of kanamycin resistance. The culture was subsequently diluted in LB containing 50 mM sodium citrate and kanamycin (50 µg/ml) and allowed to grow at 37°C for approx. 20 h, to remove the effects of residual 4-thiouridine (see below). The following day, the overnight culture was spun down and resuspended in 1 ml M9 liquid medium (Miller, 1992). 0.1 ml aliquots of the resuspended culture were plated onto 10 LB agar plates containing kanamycin (50 µg/ml) for selection of transposon insertions (ie. kanamycin resistant). The plates were subsequently irradiated at 30°C overnight at fluence of ~80-100 kJ/m^2. The following day, putative *nuv* (ie. growth delay absent) mutant colonies were picked and re-screened for near-UV and kanamycin resistance. (A second method that was used with limited success involved allowing the kanamycin containing plates to grow for 8-10 h at 37°C, then generating replica plates using a replica plating block and velveteen. The original plate served as a control, while the replica was irradiated overnight at 30°C as above. Kanamycin resistant colonies which were uninhibited by near-UV irradiation were selected for subsequent re-screening.)

ii) Growth delay characterization

Characterization of growth delay mutants was achieved using a modification of the method outlined by Ryals *et al.* (1982). Individual colonies were inoculated into 96-well microtiter plates and replica plated onto LB agar plates containing streptomycin and

0.5% glycerol (Thomas and Favre, 1980). One of these plates served as a control, while the other was inverted and placed on a Plexiglass cover suspended 6 cm above four parallel near-UV lamps (NIS, F20T12/BL). The lamps were cycled on for 2.5 h and off for 3.5 h (one light/dark cycle) and the chamber temperature was maintained at 30°C. The dose at the Plexiglass surface was measured using a UV digital radiometer (UVP Inc., San Gabriel, CA) and was determined to be 1500 $\mu W/cm^2$ at 360 nm, providing the cells with approximately 100 kJ/m^2 over a 24 h period (4 cycles).

Screening Methods

i) P1*vir* Co-transduction of Markers (as per Miller (1992))

Lysate Preparation: Mutant strains were grown up in LB containing 5 mM $CaCl_2$ to approximately 1-2 x 10^8 cells/ml. Approx. 10^7 phage/ml from a P1*vir* lab stock was then added to 1ml of donor cells in a 13 x 100 mm Pyrex test tube and allowed to adsorb at 37°C for 20 min. Next, 2.5 ml of R-soft agar (Miller, 1992) containing 10 mM Mg^{2+} was added to each of the tubes. The tubes were then vortexed. The R-soft agar was carefully poured onto R agar plates (Miller, 1992) and incubated face up at 37°C overnight. Two plates were prepared in this manner for each lysate. The following morning, the soft agar was scraped off the plates and added to a 15 ml polypropylene centrifuge tube (Becton Dickinson, Lincoln Park, NJ). The plates were then washed with 2 ml LB and the washings added to the tube. 200 µl of chloroform was added and the tube was vortexed vigorously for 30 sec. The tube was then centrifuged and the supernatant transferred to a 1.5 ml screw-cap polypropylene tube (Diamed, Mississauga,

ON). A few drops of chloroform were added and the lysate was stored at 4°C.

P1 Transduction: Recipient cells (AB1157) were grown up overnight at 37°C with aeration in LB containing 5 mM $CaCl_2$ and resuspended in 1/10 volume MC buffer (0.1 M $MgSO_4$, 5 mM $CaCl_2$). Three 1.5 ml polypropylene tubes were prepared as follows: i) 100 µl of the concentrated cell suspension alone (control for spontaneous mutants); ii) 50 µl of the P1 lysate alone (control for contaminated lysate); and iii) 100 µl of concentrated cell suspension plus P1 lysate to an moi of 0.1-1.0 (max). Tubes were incubated at 37°C for 20 min. to allow for adsorption of phage. After 20 minutes, 200 µl citrate buffer (pH 5.5; Miller, 1992)) was added and the tubes were vortexed and then centrifuged to pellet the cells. The cells were subsequently washed three times with LB containing 50 mM sodium citrate to prevent future P1 adsorption. After the third washing, the pellet was resuspended in 200 µl LB containing 50 mM sodium citrate and 100 µl was plated on R-plates containing kanamycin for selection of transductants. The plates were incubated overnight at 37°C. The following morning, several transductants from each plate were selected for near-UV screening. The colonies to be screened were streaked out onto LB plates containing kanamycin and incubated at 37°C overnight. The next day, colonies from purified transductants were selected and inoculated into microtiter plate wells containing 200 µl LB (plus streptomycin) for replica plating. Following 4-5 hrs. of growth, the cultures were replica plated (using a forked replica plater) onto LB plates containing streptomycin and 0.5% glycerol for final growth delay characterization screening.

ii) Kohara λ Clone Plaque Complementation Assay

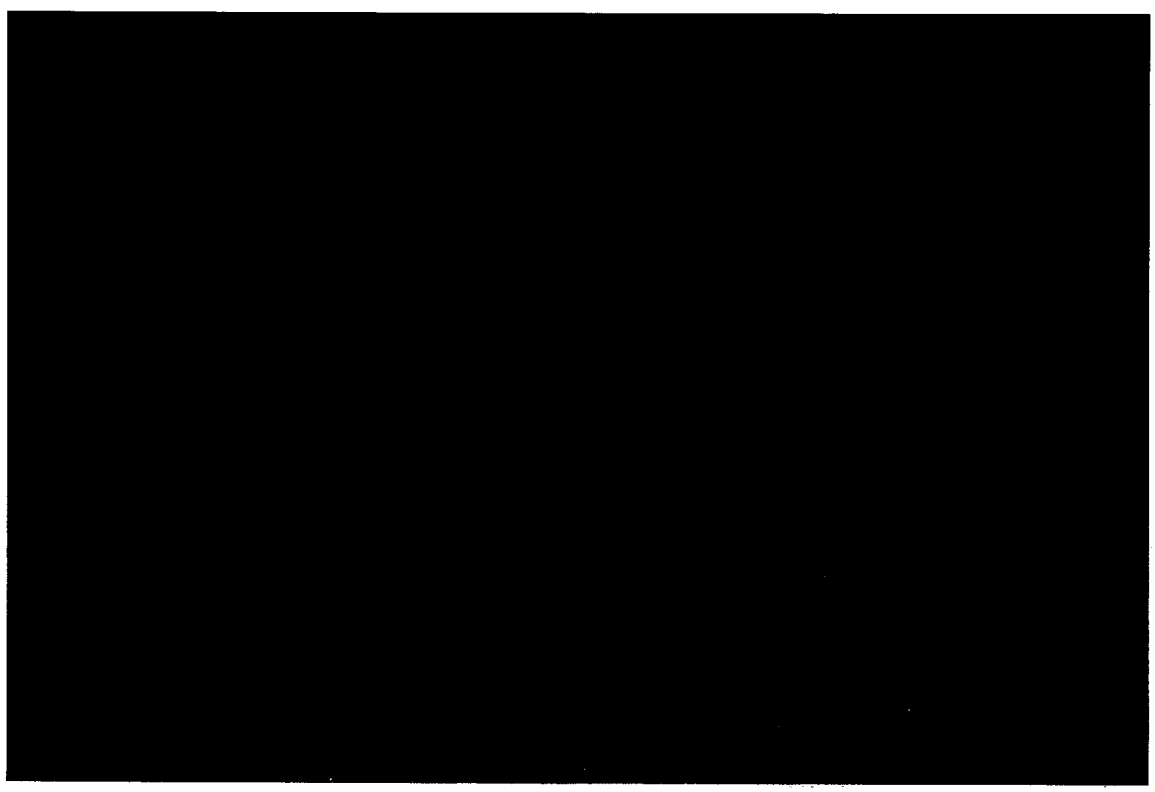
A second method employed to screen potential transposon-insertion *nuvA* mutants involved the use of non-lysogenic λ clones of *E. coli* DNA (see Kohara *et al.* (1987) in the *nuvA* region (9-10 min). The rationale behind this assay was that if a λ clone containing a complete *nuvA* locus was plaqued onto a *nuvA*$^-$ strain, 4-thiouridine levels within the host would increase (as a result of the λ harboured *nuvA*) and following irradiation with non-lethal fluences of near-UV, the intrinsic growth delay would be restored. Under these circumstances, host protein synthesis would cease, and no plaques (indicative of phage proliferation) would appear on the plates. By contrasting the results of plaquing a particular phage clone onto putative *nuvA*$^-$ strains in the presence and absence of irradiation, it theoretically would be possible to isolate strains containing a *nuvA* mutation. Putative *nuvA*$^-$ strains to be tested and PM2 (as a control) were grown overnight in LB, subcultured 1:100 and grown to an OD_{600} of 0.375. The phage stock to be used was diluted 10^{-6}, 10^{-7} and 10^{-8}, and 0.1 ml of each dilution was added to 0.1ml of cell culture in small test tubes. 50 µl of 0.1 M $MgSO_4$ was then added to each tube. The phage was pre-adsorbed by incubating the tubes at 37°C for 20 min. Following that time, 3 ml of R-soft agar was added and the contents of each tube was plated onto R agar plates. Once the agar had polymerized, the plates were irradiated at 30°C as described above. Unirradiated (control) plates were prepared in parallel. Plaques were examined the following morning.

RESULTS

By using multiple cycles of irradiation from a near-UV light source using non-lethal fluences, followed by several hours of incubation under conditions supporting growth, it is possible to select mutants which are deficient in the near-UV-induced growth delay (ie. *nuv*), since such mutants will grow preferentially (Jagger, 1983; see Figure 1). This method was first used to select for mutants lacking the stringent response (*relA*, Ramabhadran, 1976), and variations on this method were subsequently used in the identification of both thiolation factors required for the biosynthesis of 4-thiouridine (*nuvA*, Ramabhadran *et al.*, 1976; *nuvC*, Ryals *et al.*, 1982). Through the use of this enhancement method coupled to a method of random transposon mutagenesis, it was hoped that the isolation of a *nuvA* (or *nuvC*) mutant harbouring a selectable kanamycin marker would be possible which could be used in the future phases of my work.

Following transposon mutagenesis and subsequent growth delay characterization, a total of 408 mutant colonies which both lacked a growth delay and were resistant to kanamycin were identified. The exact nature of these mutants, however, still needed to be determined. There were three possibilities regarding the nature of the isolated mutants. First, and most unlikely, was the possibility that spontaneous mutations had arisen simultaneously at both the *nuvA* (*nuvC* or *relA*) locus and within one of the genes conferring kanamycin resistance (see Vinopal, 1987). A second possibility was that a spontaneous mutation had arisen in the locus imparting the growth delay phenotype on the cell, while the transposon had inserted at another site within the chromosome. The

Figure 1. The near-ultraviolet-induced growth delay in *E. coli*. Photograph showing *nuvA*$^-$ (PM2) and *relA*$^-$ (MC4100) strain responses to the near-UV cycling protocol used to characterize these mutants in this study. Individual colonies were replica plated onto LB agar plates containing streptomycin and 0.5% glycerol. One of these plates was subsequently irradiated as described in Materials and Methods. Also shown for comparison is the wild-type AB1157 (*nuvA*$^+$) and a *recA*$^-$ derivative of MC4100 (SE5000) which is hypersensitive to near-UV irradiation.



final possible scenario was that the transposon carrying the kanamycin resistance marker had been incorporated into the *nuvA*, *nuvC* or *relA* locus. To distinguish between the latter two of these possibilities, a method of characterizing the putative *nuvA*::Tn*10* mutations was necessary.

The method most frequently used in this regard is through the use of the bacteriophage P1*vir*. By generating a P1 lysate on a mutant strain and using this lysate to transduce a wild-type strain, it is possible to evaluate the success of a selection procedure through the co-transduction of the two markers of interest. If kanamycin resistant transductants are subsequently found to be lacking the growth delay, then the transposon has inserted within the *nuvA*, *nuvC* or *relA* gene, or within a closely linked gene (Kleckner *et al.*, 1991). Using this method of screening, 10 of the 408 mutants were examined and the two markers (*nuv*$^-$ and kan^r) were not co-transducible in the strains tested. A second method to screen putative *nuvA*::Tn*10* mutants was to use non-lysogenic λ phage containing *E. coli* DNA in the region of the *nuvA* locus (Kohara *et al.*, 1987) in a plaque complementation assay. Using each of the four clones which encompass the *nuvA* region in this manner, 5 putative *nuvA* mutants were tested. In all 5 cases, plaques were observed on both the irradiated and unirradiated plates, suggesting either that the loss of the growth delay phenotype was due to another locus (ie. *nuvC* or *relA*), or that the levels of 4-thiouridine being expressed were not sufficient to restore the wild-type growth delay response.

DISCUSSION

The identification of a *nuvA* mutant containing a marker for easy selection would be of great use in studies which are otherwise hindered by the growth delay response to sublethal fluences of near-UV exhibited by *E. coli* (see Turner and Eisenstark (1984) for example). In this study, I attempted to isolate and characterize a *nuvA* mutant using a method of transposon mutagenesis coupled to the method of *nuv* mutant enhancement first used by Ramabhadran (1976). Although 408 growth delay-minus, kanamycin-resistant mutants were isolated, none of them have to date been conclusively identified as *nuvA*::Tn*10* mutations. One of the problems that was encountered during the course of this study was the presence of residual 4-thiouridine in both mutagenized and transduced host strains. As this modified nucleotide is found in 70% of bulk transfer RNA molecules (Favre *et al.*, 1985) and has been calculated to be present in a ratio of one per 140 S-RNA nucleotides (Lipsett, 1965), several generations of growth following mutagenesis of the *nuvA* locus were necessary to remove any residual 4-thiouridine from the nucleotide pool. Following 20 h of growth, any spontaneously generated mutants could theoretically be amplified to the point that some, if not all, of the 408 mutants isolated in this work may be the progeny of a single spontaneous mutant. Further characterization of the remaining mutants in the future should act to address this possibility.

CHAPTER 3. MANUSCRIPT

PREFACE

The following manuscript, entitled "Role of Fapy glycosylase and UvrABC excinuclease in the repair of UVA (320-400 nm)-mediated DNA damage in *Escherichia coli*" was submitted to the journal "Photochemistry and Photobiology" for review on June 14, 1995. It was accepted for publication with minor revisions on August 15, 1995. Following the completion of the required revisions, the modified manuscript, which constitutes pages 38 to 65 (inclusive) of this thesis, was returned to the editor on September 12, 1995. At the time of submission of this thesis, the authors were awaiting verification of publication status.

All of the experiments contained within this work, including the construction and characterization of the DNA repair-deficient mutants used in this study, were performed by the primary author, with the exception of the T4 endonuclease V nicking assay, which was performed by Claire Palmer, a fellow graduate student. Claire and Dr. Schellhorn were also involved in the final drafting of the manuscript, and provided valuable insights regarding the interpretation of the results found in this study.

Michael Shennan, B.Sc. (Hons.)
September, 1995.

Pages in this article were replaced with the citation below due to copyright issues.

Michael G. Shennan, Claire M. Palmer & Herb E. Schellhorn (1996) Role of Fapy Glycosylase and UvrABC Excinuclease in the Repair of UVA (320-400 nm)-mediated DNA Damage in Escherichia coli, Photochemistry and Photobiology, 63 (1): 68-73, DOI: https://doi.org/10.1111/j.1751-1097.1996.tb02993.x

APPENDIX

The following appendix contains figures relevant to the work presented in the manuscript making up the body of this chapter which were not included in the final draft of the manuscript.

Figures 1A and 2A represent the growth curves of the *nuvA*$^{+}$ and *nuvA*$^{-}$ repair deficient mutants strains constructed for this work, respectively, and show that mutating the *fpg* and/or *uvr*A locus did not alter the growth rates of the resultant mutants relative to control ("wild-type") strains.

Figure 3A demonstrates that while their responses to near-UV (UVA) irradiation differ, AB1157 and PM2 show similar survival responses when irradiated with far-UV wavelengths (compare with Figure 3 in manuscript). The irradiation procedure used to generate this figure was exactly as described for PM2 in the manuscript.

Figure 1A. Growth curve for AB1157-derived DNA repair-deficient mutants used in this study. Strains were grown overnight in LB medium at 37°C with aeration and were subcultured 1:100 the following morning. At the time points shown, aliquots of the subcultured cells were drawn and growth was monitored by measuring the optical density at 600 nm (OD_{600}). Symbols: ●, AB1157; ○, HS641 (*fpg*$^-$); ■, HS642 (*uvrA*$^-$); □, HS643 (*uvrA*$^-$ *fpg*$^-$).

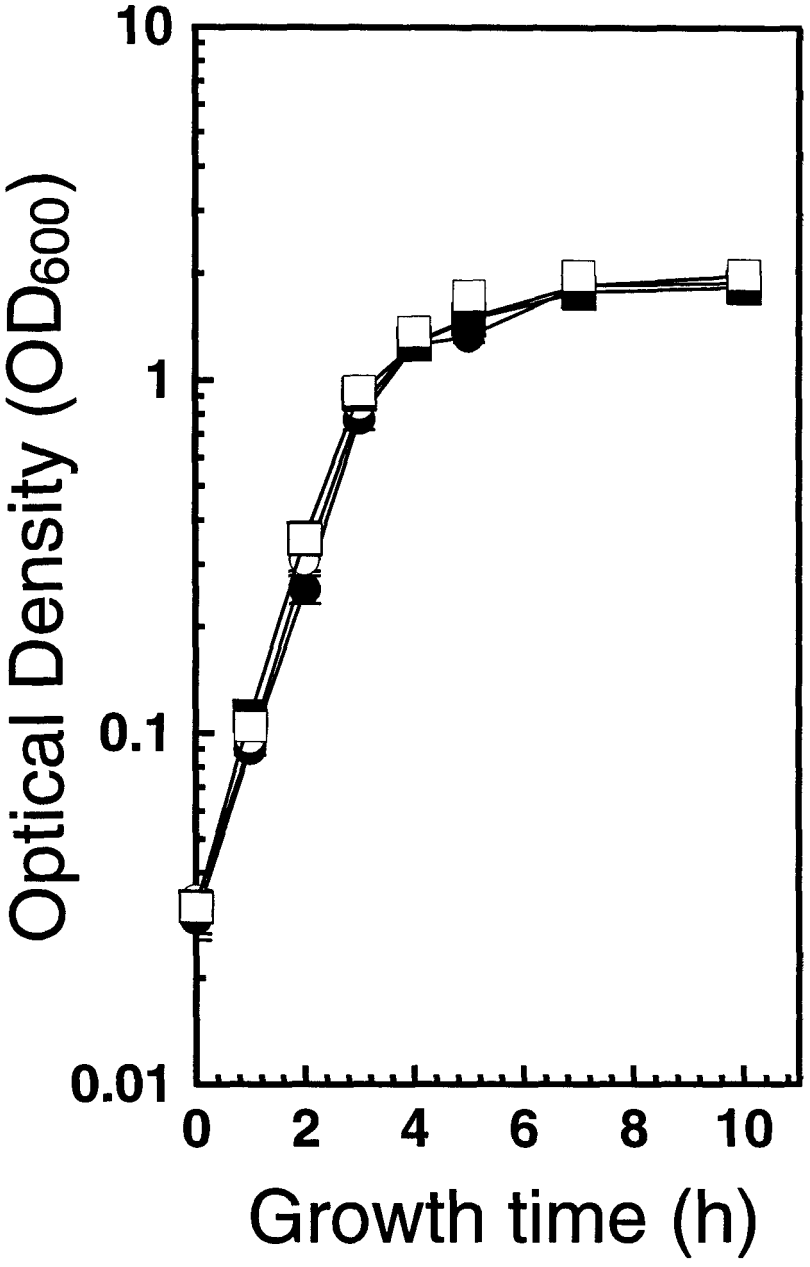

10
1
0.1
0.01
Optical Density (OD600)
0
2
4
6
8
10
Growth time (h)

Figure 2A. Growth curve for PM2-derived DNA repair-deficient mutants used in this study. Strains were grown overnight in LB medium at 37°C with aeration and were subcultured 1:100 the following morning. At the time points shown, aliquots of the subcultured cells were drawn and growth was monitored by measuring the optical density at 600 nm (OD_{600}). Symbols: ●, PM2; ○, HS644 (*fpg*$^-$); ■, HS645 (*uvrA*$^-$); □, HS646 (*uvrA*$^-$ *fpg*$^-$).

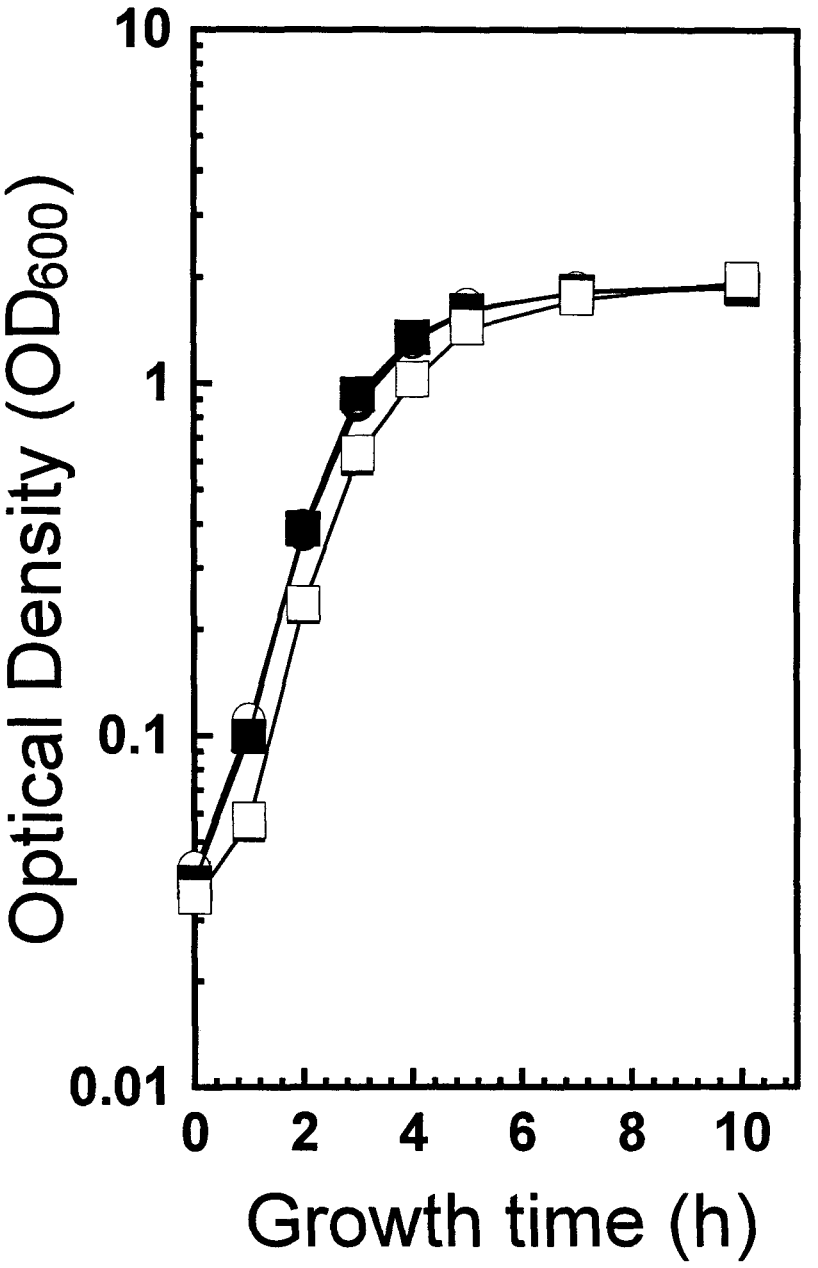
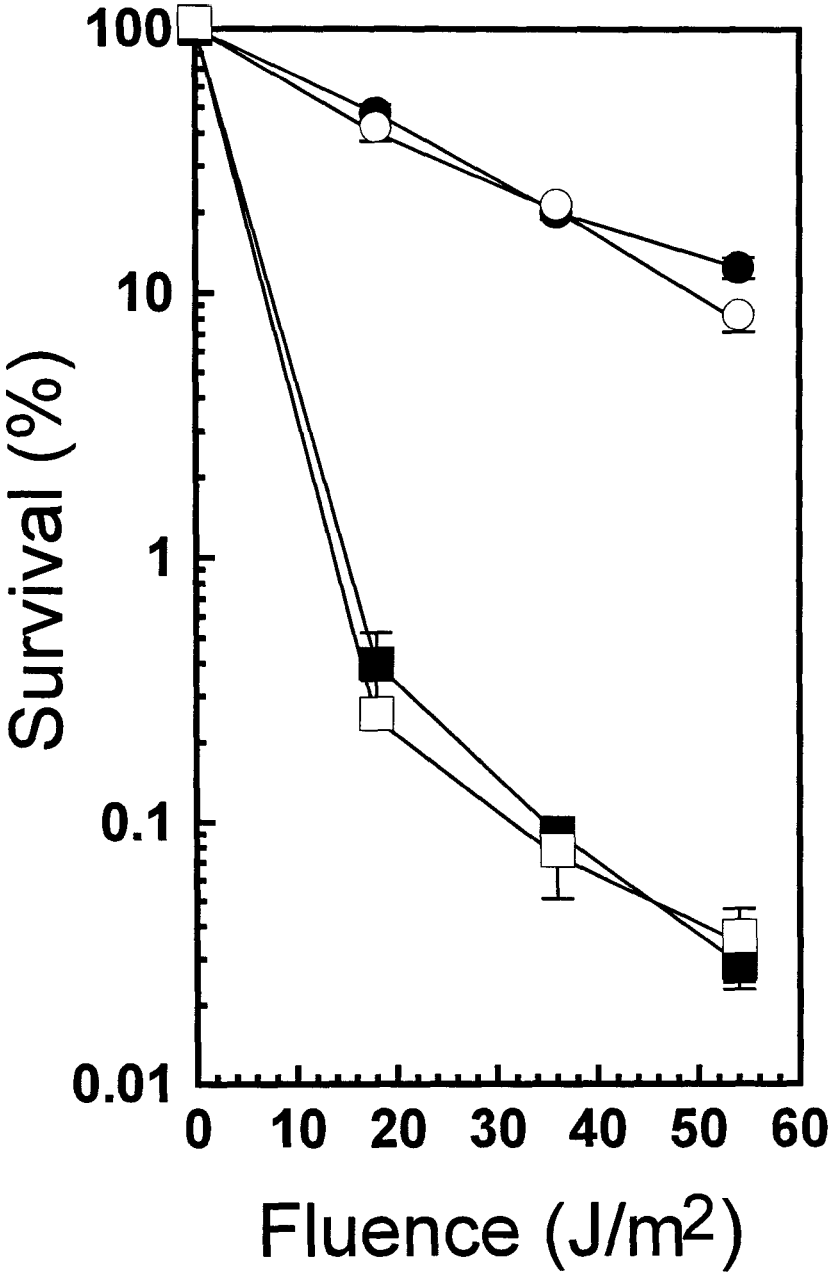

10
1
0.1
0.01
Optical Density (OD600)
0
2
4
6
8
10
Growth time (h)

Figure 3A. Effect of far-UV (254 nm) irradiation on survival of *E. coli* AB1157 repair mutants. Cell suspensions were irradiated as described in Materials and Methods. Each value represents the mean of three individual experiments. Error bars represent the standard error (±) of the mean of three trials. Symbols are as described in Figure 1A.


100
10
1
0.1
0.01
Survival (%)
0
10
20
30
40
50
60
Fluence (J/m2)

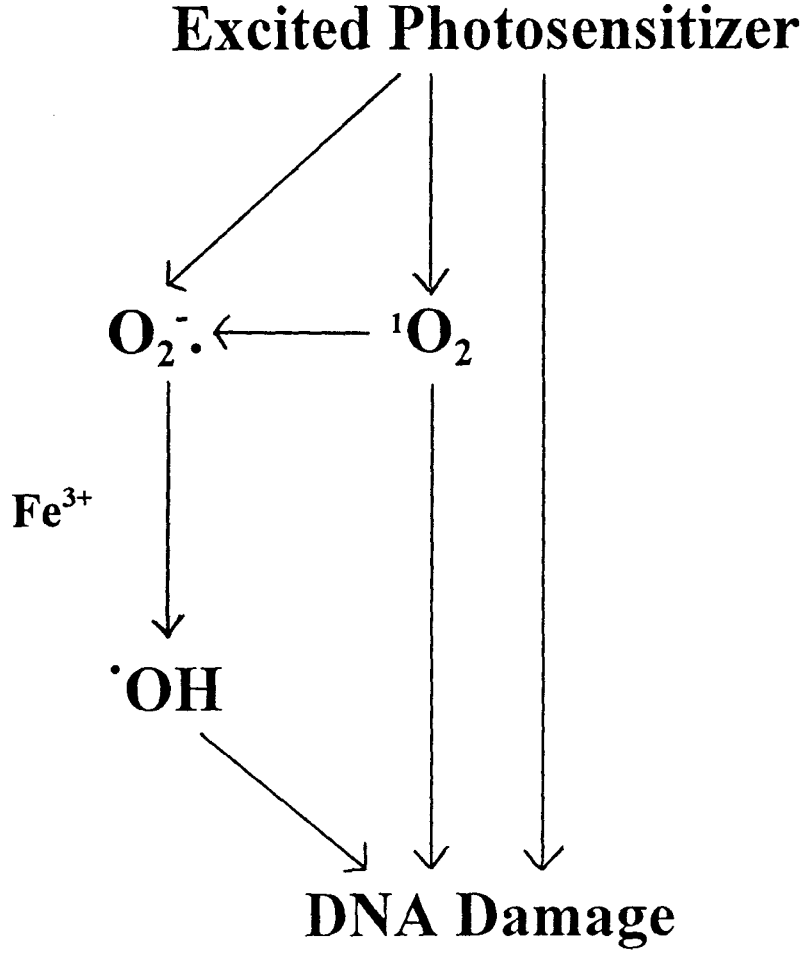
CHAPTER 4. IS 4-THIOURIDINE A PHOTOSENSITIZING CHROMOPHORE IN UVA-MEDIATED LETHALITY?

INTRODUCTION

Perhaps the most pronounced difference between the effects of far-UV and near-UV (UVA) irradiation is in the nature of the damage caused by the two treatments. As the absorption maximum of DNA lies within the far-UV range (ie. approx. 260 nm), DNA itself is the major chromophore (ie. the molecule which absorbs the incident photons), as well as the target (ie. the molecule that is damaged by the energy of the absorbed light) in far-UV mediated DNA damage. In contrast, while DNA is not thought to be the primary chromophore for near-UV inactivation, it is the major target, resulting in mutations, inhibition of DNA synthesis and biochemical alterations (Eisenstark, 1989). The oxygen dependence of UVA-mediated DNA damage has led to the generally accepted conclusion that most of the inactivation by UVA is via a photodynamic process involving the action of (endogenous) photosensitizing chromophores having absorption maximums within the near-UV region. However, the species or mechanisms that modify the DNA and therefore are ultimately responsible for the DNA damage induced by UVA are not yet known.

A photosensitizer which has become activated following excitation by UVA can either damage DNA directly (via a "type I" reaction) or through the generation of reactive oxygen species such as singlet oxygen ($^1O_2$; via a "type II" reaction), which can subsequently damage DNA (Pflaum *et al.*, 1994; Figure 1). In the presence of transition

Figure 1. Mechanisms by which an excited (endogenous) photosensitizer can hypothetically elucidate cellular DNA damage following near-UV exposure.


Excited Photosensitizer
$O_2^{-}\cdot$
$^1O_2$
Fe^{3+}
$^{\cdot}OH$
DNA Damage

metals such as iron (Fe^{3+}), it is also possible that highly reactive hydroxyl radicals (•OH) can be generated from superoxide (O_2^{-}.) via a Fenton reaction (Figure 1) and are responsible for the modifications (recall Chapter 1).

Several UVA-absorbing molecules have been examined in an attempt to identify the photosensitizer(s) responsible for UVA-mediated lethality and mutagenesis in *E. coli*. Heme-containing polypeptides, such as catalase (Kramer and Ames, 1987) and cytochromes (Sammartano and Tuveson, 1987), absorb in the near-UV region of the spectrum, as do porphyrins, flavins and quinones (Jagger, 1983). Overexpression of catalase HPI (Kramer and Ames, 1987) and the membrane-bound cytochromes b_{558}, b_{595} and *d* (Sammartano and Tuveson, 1987) result in an increased sensitivity to near-UV, but not far-UV irradiation , suggesting a near-UV photosensitizing role for these proteins.

In addition to heme-containing proteins, thiolated nucleotides also absorb in the near-UV range, making them good candidates for near-UV photosensitizers. When a uracil nucleotide in *E. coli* transfer RNA is thiolated via a sulfurtransferase system (see Chapter 1) to generate the modified base 4-thiouridine, its absorption maximum shifts from within the far-UV range (approx. 260 nm) to the UVA range (328 nm in solution, 336 nm when present in transfer RNA; Favre *et al.*, 1971). As a result of this shift, 4-thiouridine readily absorbs near-UV photons and is involved in a variety of processes induced by UVA wavelengths. In addition to the growth delay phenotype observed at low fluences (see Chapter 2), *nuvA*$^{-}$ strains are more resistant to near-UV irradiation than wild-type *nuvA*$^{+}$ strains (Tsai and Jagger, 1981; Peak *et al.*, 1983; Shennan *et al.*, in press), suggesting a possible role for this base in near-UV mediated lethality.

The possiblility that 4-thiouridine is a photosensitizing chromophore was first examined by Ito *et al.* (1988), who examined the nature of the damage generated in single-stranded and double-stranded (RF) M13 phage DNA when irradiated using a monochromatic 334 nm near-UV light source in the presence of 4-thiouridine *in vitro.* The results of their study showed that at low fluences (7.5 kJ/m^2) of 334 nm near-UV, thymine bases were preferentially damaged, resulting in a termination of DNA strand synthesis. In contrast, at higher fluences (375 kJ/m^2), damage to guanine bases were found to be the predominant lesions. Based on these results, they hypothesized that at low fluences, 4-thiouridine forms photoadducts with thymine bases which block DNA synthesis (ie. a type I reaction), while at high fluences, the guanine sites are damaged via the action of reactive oxygen species (a type II reaction). Further, when they transformed M13 ssDNA into competent cells following 334 nm irradiation (40 kJ/m^2) in the presence of 4-thiouridine, they found a significant decrease in DNA transforming ability relative to the same experiment done in the absence of 4-thiouridine, suggesting that 4-thiouridine is acting as a photosensitizer for 334 nm-mediated DNA damage (Ito *et al.*, 1988), .

In contrast to the work of Ito *et al.* (1988), Kramer *et al.* (1988) suggested that 4-thiouridine played a protective, rather than a photosensitizing role following near-UV irradiation based on their work with *Salmonella typhimurium.* When they irradiated *Salmonella* strains lacking 4-thiouridine (*nuv*) with a lower fluence, more physiologically relevant broad-band near-UV light source, they found that survival of the growth delay mutant strain was lower than an isogenic strain that produced 4-thiouridine. They hypothesized that 4-thiouridine may act as a sensor for near-UV and mediate cellular

responses to this stress (Kramer *et al.*, 1988).

The purpose of the study described in this chapter was to attempt to clarify the putative role of 4-thiouridine as a photosensitizer in UVA-mediated lethality, through the use of a similar *in vitro* assay to that employed by Ito *et al.* (1988). Plasmid DNA (pGEM-3Z) was irradiated in the presence and absence of 4-thiouridine in the same ratio by mass of DNA:4-thiouridine that would be found in a typical *E. coli* cell, and subsequently was used to transform competent derivatives of the *fpg*$^-$ and *uvrA*$^-$ mutants constructed for the survival assays presented in Chapter 3 of this work.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The strains and growth conditions used in this study were the same as those presented in Chapter 3. Plasmid pGEM-3Z DNA (amp^r tet^r) was obtained from Promega (catalogue # P215A, Madison, WI). Purified 4-thiouridine was obtained from the Sigma Chemical Company (St. Louis, MO).

Preparation of Competent Cells

Competent derivatives of the repair-deficient strains were generated using the method outlined in Seidman (1990), with some modifications. A single *E. coli* colony was inoculated into 10 ml LB medium and grown overnight at 37°C with shaking (200 rpm). The following day, the culture was subcultured 1:100 into LB medium (usually

100 ml) and grown with shaking at 37°C to an OD_{600} of approx. 0.375. The culture was then equally divided into two pre-chilled 50 ml polypropylene tubes (Becton Dickinson, Lincoln Park, NJ) and put on ice for 5-10 min. The cells were pelleted by centrifugation at 3000 rpm, 4°C for 7 min. (Sorvall RT6000B, DuPont, Newtown, CT). Once pelleted, the supernatant from each tube was discarded and the cells were gently resuspended in 10 ml ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM MOPS (pH 7)). The resuspended cultures were transferred to pre-chilled 15 ml polypropylene tubes and centrifuged 5 min. at 2500 rpm, 4°C. The supernatant was discarded from each tube and the pellet was again gently resuspended in 10 ml $CaCl_2$ solution. The cells were left on ice for 30 min. After this time, the cells were once again spun down at 2500 rpm for 5 min. at 4°C and the supernatant discarded. The cellular pellet was resuspended in 2 ml ice-cold $CaCl_2$. The concentrated cells were aliquoted into pre-chilled 1.5 ml polypropylene tubes (Diamed, Mississauga, ON), usually in 600 µl volumes, and the cells were frozen at -80°C until required for transformation experiments.

Irradiation of Plasmid DNA

For the experiment using far-UV irradiation, 50 µl of a 1 ng/µl stock of DNA was streaked onto the centre of a chilled glass petri plate over an area of approximately 1 cm^2. The plate was then irradiated using a germicidal lamp as was used previously in the survival assays (see Chapter 3). For near-UV irradiations, 50 µl of a 2 ng/µl stock of DNA was irradiated in the presence or absence of 4-thiouridine (in 1:20 total mass (4-thiouridine:DNA) ratio outlined in Ito *et al.* (1988)) in a glass microcuvette using the

same Xenon arc lamp used in the survival assays (see Chapter 3).

Transformation Assay

Following irradiation with either near-UV or far-UV, competent derivatives of the *fpg*$^-$ and *uvrA*$^-$ strains were transformed using the irradiated DNA according to the method outlined in Provence *et al.* (1994). In parallel, unirradiated (control) DNA was used to transform the same strains. Any reduction in the ability of pGEM-3Z DNA to transform a particular mutant strain was assumed to be due to the loss of the repair activity coded for by the mutated locus. 10 µl of the plasmid DNA (ie. 10 ng or 20 ng) was added to 200 µl of competent cells and left on ice for 30-40 minutes. After that time, the cells were heat-shocked by placing the tubes in a heat block containing water equilibrated at 42°C for 60-90 seconds, and placed on ice for 2 min. 800 µl of LB medium was then added to each tube and the cells were grown for 1 hr at 37°C with moderate shaking. Aliquots of the transformation culture were then plated on LB agar plates containing ampicillin (100 µg/ml) to select for transformants. The number of transformants obtained using this method ranged from 1.08×10^4 to 1.42×10^5 per microgram of plasmid DNA. The transformational efficiency was then calculated as follows:

$$\%\ \text{transformational efficiency (ie. following irradiation)} = \frac{\#\ \text{transformants / µg DNA after irradiation}}{\#\ \text{transformants / µg DNA (unirradiated)}} \times 100$$

To evaluate differences in response to UV fluence, the logarithm of the transformational efficiency (%) was regressed against UV fluence and the resultant regression estimates were compared using a Student' s t-test.

RESULTS

Far-UV irradiation reduces the ability of pGEM-3Z to transform uvrA⁻ strains.

To test the validity of the transformational assay, plasmid DNA was first irradiated using far-UV and transformed into competent derivatives of the mutant strains used in the survival studies (Chapter 3). As expected, the transforming ability of pGEM -3Z DNA following far-UV irradation was greatly reduced when *uvrA*⁻ strains lacking the nucleotide excision repair pathway were used as transformation hosts (Figure 2). In contrast, *fpg*⁻ strains showed no significant reduction ($p>0.001$) in transformability, relative to *fpg*⁺ isogenic strains (Figure 2). As the primary lesions generated by this treatment are pyrimidine dimers and (6-4) photoproducts, loss of Fapy glycosylase activity was not be expected to reduce the transforming ability of pGEM-3Z into these strains. The percent transformational efficiency values obtained in this experiment are in close agreement with those found by Czeczot *et al.* (1991), who performed a similar assay to validate the protocol used in their study.

4-thiouridine does not reduce the ability of pGEM-3Z to transform fpg⁻ or uvrA⁻ strains following UVA irradiation

As UVA wavelength photons are not directly absorbed by DNA, it was expected that irradiating pGEM-3Z plasmid DNA with near-UV in this range in the absence of any photosensitizing agent should not greatly decrease the ability of pGEM to transform any of the mutant strains. As shown in Figure 3, there was no significant difference ($p>0.001$) between any of the curves generated under these conditions. There was,

Figure 2. Transformational efficiency of pGEM-3Z DNA into repair-deficient mutants of PM2 following irradiation with far-UV. Plasmid DNA was irradiated as described in Materials and Methods. Symbols: ●, PM2; ○, HS644 (*fpg*$^-$); ■, HS645 (*uvrA*$^-$); □, HS646 (*uvrA*$^-$ *fpg*$^-$). Each value represents the mean of three individual experiments. Error bars represent the standard error (±) of the three trials.

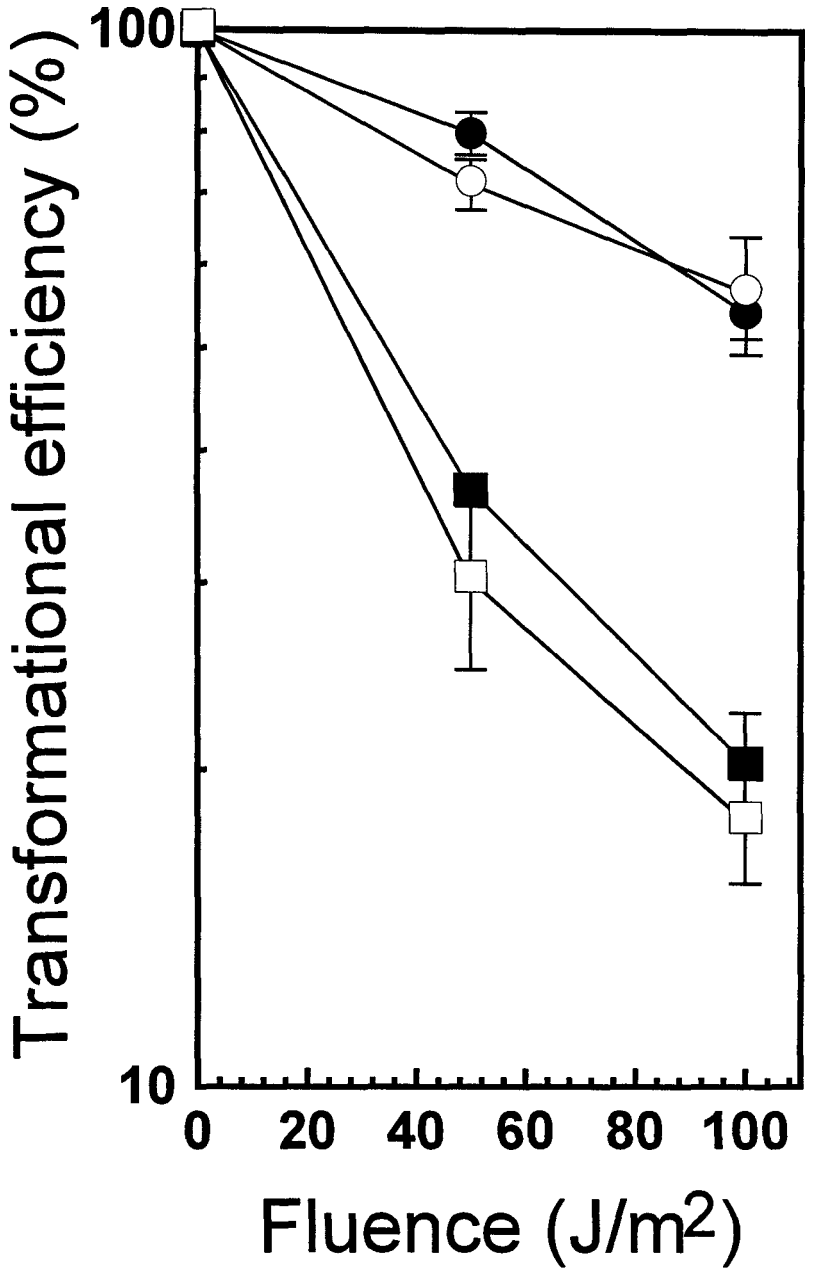
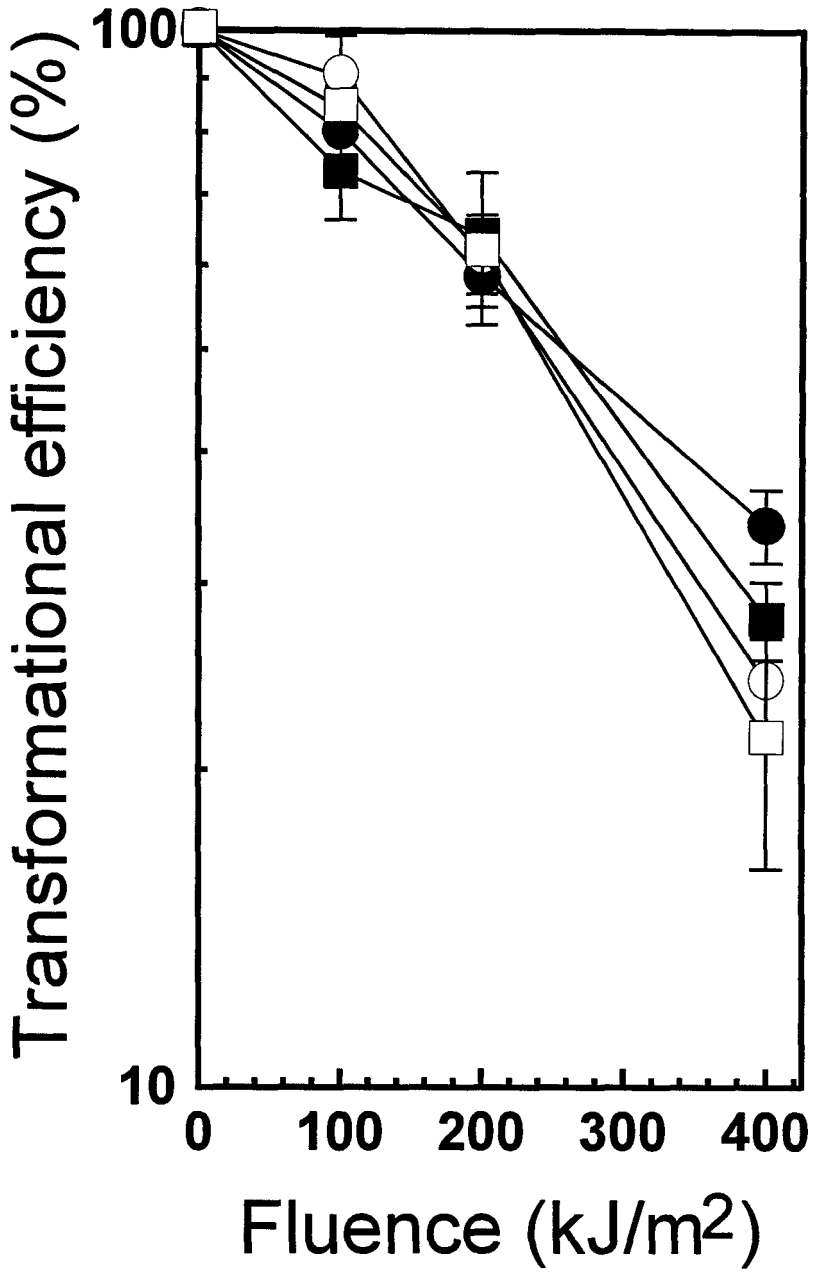

100
10
Transformational efficiency (%)
0
20
40
60
80
100
Fluence (J/m2)

Figure 3. Transformational efficiency of pGEM-3Z DNA into repair-deficient mutants of PM2 following irradiation with UVA. Plasmid DNA was irradiated as described in Materials and Methods. Symbols: ●, PM2; ○, HS644 (*fpg*$^-$); ■, HS645 (*uvrA*$^-$); □, HS646 (*uvrA*$^-$ *fpg*$^-$). Each value represents the mean of three individual experiments. Error bars represent the standard error (±) of the three trials.


Transformational efficiency (%)
100
10
0
100
200
300
400
Fluence (kJ/m2)

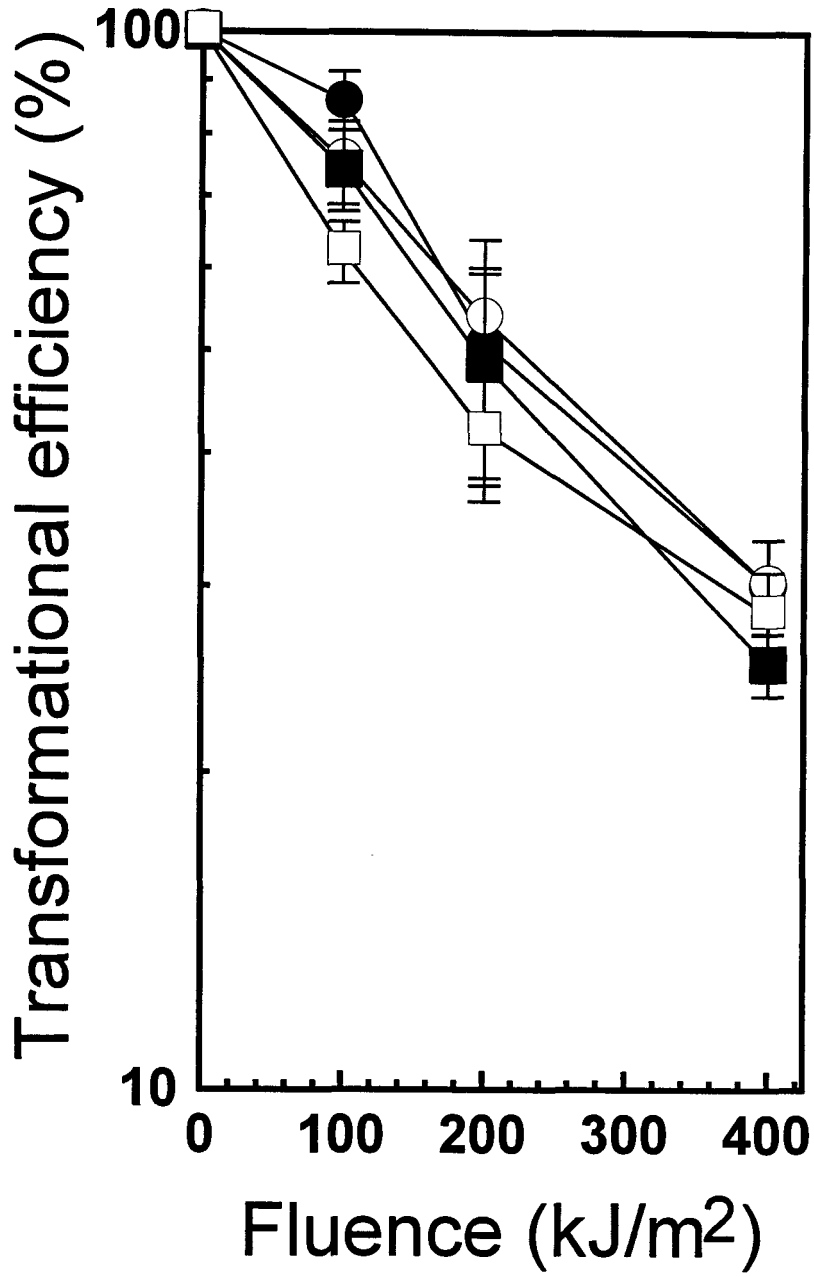
however, a overall decrease in transformational efficiency as the fluence of UVA increased, indicative of some form of DNA damage. A comparable reduction in DNA transforming ability was found previously when M13 phage DNA was irradiated with UVA wavelengths in the absence of 4-thiouridine (Ito *et al.*, 1988).

When purified 4-thiouridine was mixed in with the plasmid DNA in a similar ratio by mass that these two constituents would be found in an intact cell, a similar set of curves to that found in the absence of 4-thiouridine was generated (Figure 4). The transforming abilty of pGEM-3Z was not affected by the presence of 4-thiouridine, and none of the mutants strains showed any significant difference in the ability to repair any lesions generated from the control (PM2) at all fluences tested. Once again, there was a fluence-dependent reduction in the transformational efficiency of pGEM-3Z, suggesting some type of DNA damage was occuring under the experimental conditions (Figure 4). This damage is not recognized by either the UvrABC excinuclease or Fapy glycosylase.

DISCUSSION

The results presented here suggest that 4-thiouridine is not a photosensitizing chromophore for UVA under the *in vitro* conditions used in this study. Through the addition of purified 4-thiouridine to a dilute concentration of plasmid DNA in solution, any photochemical effects of this base on intact DNA could be assayed by measuring transformational ability of the plasmid DNA in the presence and absence of irradiation. The lack of any variation in transforming ability of pGEM-3Z when used to transform

Figure 4. Transformational efficiency of pGEM-3Z DNA in the presence of 4-thiouridine into repair-deficient mutants of PM2 following irradiation with UVA. The plasmid DNA-4-thiouridine mix was irradiated as described in Materials and Methods. Symbols: ●, PM2; ○, HS644 (*fpg*$^-$); ■, HS645 (*uvrA*$^-$); □, HS646 (*uvrA*$^-$ *fpg*$^-$). Each value represents the mean of three individual experiments. Error bars represent the standard error (±) of the three trials.


100
10
Transformational efficiency (%)
0
100
200
300
400
Fluence (kJ/m2)

fpg⁻ and *uvrA*⁻ strains suggests that any DNA damage caused by the UVA-4-thiouridine treatment is not recognized by either Fapy glycosylase or the nucleotide excision repair system.

There are several possible explanations for the differences observed between the results of this study and those of Ito *et al.* (1988). First, for practical purposes, a ten-fold lower concentration of DNA (and consequently 4-thiouridine) was used in this study. The kinetics of any putative interactions between 4-thiouridine and the plasmid DNA may be affected by such a dilution. A second difference is in the nature of the DNA used in the assays. The transformational data of Ito *et al.* (1988) was obtained using single-stranded M13 phage DNA, in contrast to the double-stranded, negatively-supercoiled plasmid DNA used in this study. The use of single-stranded DNA might promote DNA base (eg. thymine)-4-thiouridine interactions (as hypothesized by the authors), which might not normally occur under *in vivo* conditions (ie. in a double-stranded bacterial chromosome).

The most important difference between the two studies, however, involves the nature of the near-UV light sources used. The use of monochromatic near-UV near the absorption maximum of 4-thiouridine (334 nm) leads to an overestimation of this base as a photosensitizer under physiological conditions (Kramer *et al.* 1988). In contrast, the spectral distribution of xenon arc lamps closely mimics that of solar radiation when used in conjunction with the appropriate filters. Therefore, our results may be a more relevant estimation of the (non-)photosensitizing nature of 4-thiouridine on bacterial DNA when this base is present in the nucleotide pool. It is possible that 4-thiouridine must be bound

in transfer RNA to be involved in UVA photosensitization and that in solution or in the cytoplasm it plays no such role. There is also a possibility that a secondary factor which is involved in the generation of reactive oxygen species following activation by 4-thiouridine may be missing from these *in vitro* assays, resulting in an underestimation of the complexity of the system. To date, published studies have produced several hypotheses, but there is no conclusive evidence implicating 4-thiouridine as a photosensitizer involved in harming (or protecting) the cell under conditions of near-UV stress.

CONCLUSIONS

- Strains of *Escherichia coli* lacking 4-thiouridine (*nuvA*$^-$) are less sensitive to the lethal effects of UVA irradiation than wild-type strains

- *E. coli nuvA*$^-$ strains lacking Fapy glycosylase activity (*fpg*$^-$) are more sensitive to UVA irradiation than isogenic control strains

- In the absence of the nucleotide excision repair system (*uvrA*$^-$), this sensitivity is further enhanced suggesting these two enzymes are capable of recognizing and repairing a common DNA lesion(s)

- This is a UVA-specific effect; Fapy glycosylase does not protect *E. coli* from far-UV (254 nm) irradiation

- When irradiated in solution, 4-thiouridine does not damage double-stranded DNA, suggesting it is not a photosensitizing chromophore for UVA-mediated DNA damage

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