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Genes Affecting the Repair and Survival of Escherichia coli Following Psoralen-Induced Damage: a DNA Interstrand Crosslinking Agent

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Genes Affecting the Repair and Survival of *Escherichia coli* Following Psoralen-Induced Damage: a DNA Interstrand Crosslinking Agent

by

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ABSTRACT

Photoactivated psoralens and other agents that form DNA interstrand crosslinks are highly cytotoxic and are useful in treating a range of diseases, including vitiligo, psoriasis, and some forms of cancer. Unlike many lesions that damage only one strand of the duplex DNA, DNA interstrand crosslinks form covalent bonds with both strands. Thus, repairing these lesions is complicated both by the lack of an undamaged strand to serve as a template for resynthesis following excision ,as well as the potential to form double strand breaks if both strands are incised. A number of models have proposed that repair is likely to couple nucleotide excision repair with other repair pathways such as recombination, and/or translesion synthesis. However, several aspects of these models remain speculative, and how these medically relevant lesions are repaired by cells still remains elusive. In this study, I use *Escherichia coli* as a model organism to characterize which gene products contribute to survival in the presence of psoralen-induced DNA interstrand crosslinks.

In Chapter II, I demonstrate that although nucleotide excision repair initiates repair, not all subunits contribute equally to survival. Notably, *uvrC* is less sensitive to psoralen-induced damage than either *uvrA* or *uvrB*. I found that Cho, an alternative endonuclease, accounts for the increased resistance of *uvrC* mutants and contributes to survival in the presence of UvrABC. Cho was not required following angelicin treatment, a psoralen derivative that only forms monoadducts, suggesting that Cho function is specific for interstrand crosslink repair. However, Cho, by itself, is not required for the initial incision and only modestly enhances the rate that psoralen crosslinks are incised

in vivo.

Following incision, many of the intermediates in the repair process remain speculative. In Chapter III, I examine how recombination and translesion synthesis mutants contribute to survival of psoralen-induced damage. I show that both *recBC* and *recF* contribute to survival, but that neither mutant is as hypersensitive as *recA*, potentially suggesting that pathways involving either single strand gaps or double strand break intermediates can occur during repair. Finally, I show that Polymerase V is responsible for the translesion synthesis that contributes to survival in the case of psoralen-induced damage in *E.coli*.

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CHAPTER I

Psoralens are photoactive chemicals that belong to a family of plant chemicals called furocoumarins that naturally occur in the roots, leaves, and fruits of five plant families: *Umbelliferae, Rutaceae, Moraceae, Leguminosa*e and *Orchidaceo*us (1) These phototoxic chemicals have evolved in plants as a host defense mechanism against plant pathogens and herbivores (2). In plants, in addition to self-defense, these chemicals are also involved in stress response pathways and hormonal regulation (2). It has been found that these chemicals can act as alleochemical compounds, which inhibit seed germination and control growth under unfavorable environmental conditions (2). The phototoxicty of these compounds has not only been beneficial for plants, but has also been useful for humans since the ancient times. The therapeutic effects of these compounds combined with sunlight have been known for thousands of years treating various skin diseases. The most common furocoumarin, psoralen has been used in Egypt and India since 1200-2000 BC to treat skin disfiguring diseases such as vitiligo and leukoderma (1).

The modern medical applications of psoralen began with the isolation of the crystalline structure of 8-methoxypsoralen from the plant *Ammi majaus* in 1940s in Egypt (1). 8-methoxypsoralen combined with sunlight was first used in late 1940s to treat vitiligo (1, 3). Subsequently, it was found that artificial long wavelength ultraviolet light (UV-A, 320-400nm) activated 8-methoxypsoralen and inhibited epidermal DNA synthesis (1, 3, 4). Although, the phototoxic effect of psoralen was known since early 1950s, the photochemotherapy or PUVA (psoralen+UVA), as it came to be known, was

developed and extended to the treatment of psoriasis only in the early 1970s (1, 3-6). In recent years, PUVA therapy has been applied to the treatment of other medical conditions including cutaneous T cell lymphoma, prostate cancer, and stenosis (7-10).

Psoralen and DNA Interstrand Crosslink Formation

Psoralen, the most commonly found furocoumarin, is a tricyclic linear aromatic compound that contains a furan ring and a pyrone ring at each end of the molecule and (Figure 1-1) (11). Psoralen DNA interstrand crosslinks are asymmetrical and contain cycloaddition products of the pyrimidines on the furan and pyrone sides of the molecule. While psoralen does not exhibit sequence specificity for DNA binding, it preferentially forms covalent bonds with thymine. In the absence of ultraviolet light, psoralen intercalates between base pairs of the DNA helix and forms hydrogen bonds with pyrimidines. Upon exposure to the ultraviolet light (>320nm), photoaddition occurs between the C5=C6 double bond of the pyrimidine and the C4'=C5'bond of the furan or C3'=C4' of the pyrone (Figure 1-2) (11-13). After the first photoaddition, furan side monoadducts can absorb a second photon and become converted to a crosslink by cycloaddition between the pyrone double bond and a thymine on the opposite strand (Figure 1-2) (12-14). In contrast, nonlinear psoralen derivatives such as angelicin are only able to form monoadducts due to their angular structure (Figure 1-1) (15, 16). Thus, although angelicin is similar to psoralen in structure, it usually requires higher doses to have similar therapeutic effects (15, 16).

Interstrand crosslinks covalently binds to two DNA strands preventing DNA strand separation, and represent an absolute block to processes such as DNA replication and transcription. The mutagenic and cytotoxic nature of photoadducted 8-methoxypsoralen was demonstrated in the 1950s and these crosslinking agents remain among the most genotoxic compounds in use today (1, 6, 8, 9).

Repair of the DNA interstrand crosslinks

DNA interstrand crosslinks involve both strands of the duplex DNA and present a unique challenge for the cell to repair. Incisions on both strands of the DNA fragment to remove the crosslink would result in a loss of genetic information, but if the damage is unrepaired, would result lethality. Current models of the interstrand crosslink repair have proposed that multiple repair pathways are likely to be involved (17-21). However, whether and how these multiple repair pathways process these lesions is poorly understood and remains to be characterized. Therefore, in the next chapters I describe experiments designed to test aspects of two predominant models.

In both models, the first step of the interstrand crosslink repair involves nucleotide excision repair with subsequent steps performed either by recombination or translesion DNA synthesis pathways (17-22). The individual repair pathways and how they fit in the context of present models are described in the following sections.

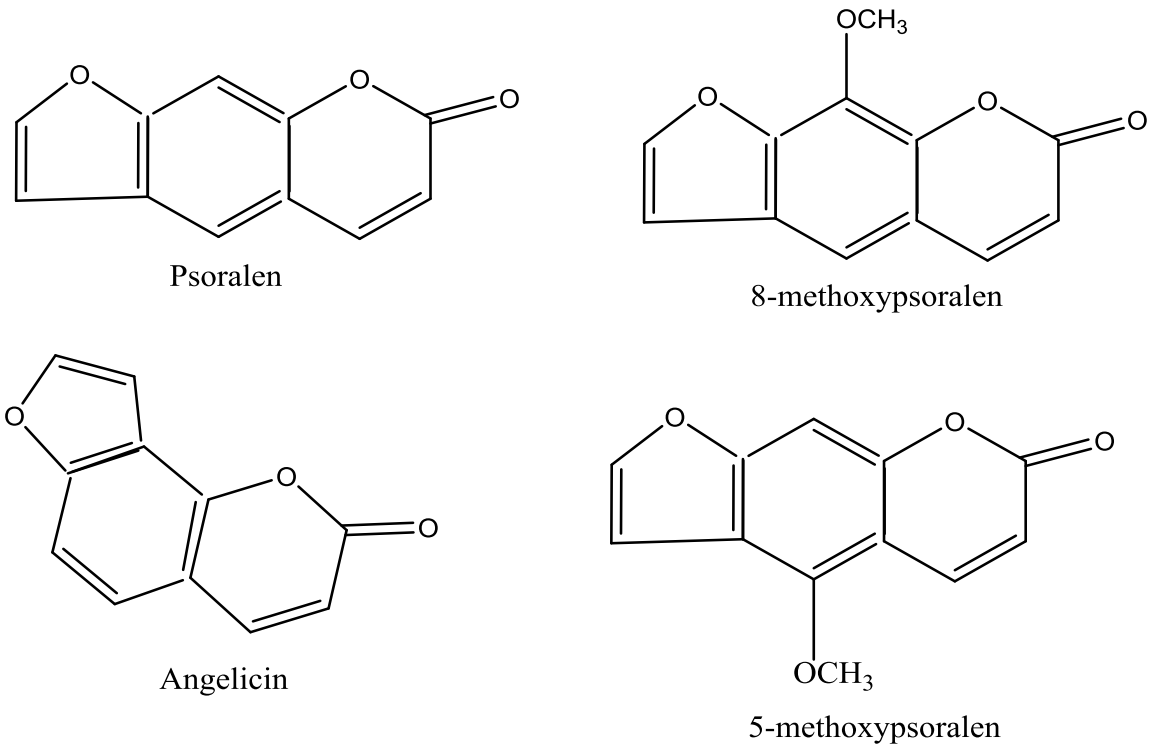

Figure 1-1: Structure of furocoumarins

Furocoumains are tricyclic compounds containing furan and pyron rings. Linear forms of furocoumarins include psoralen and its derivatives. Angelicin is an example of an angular furocoumarins.

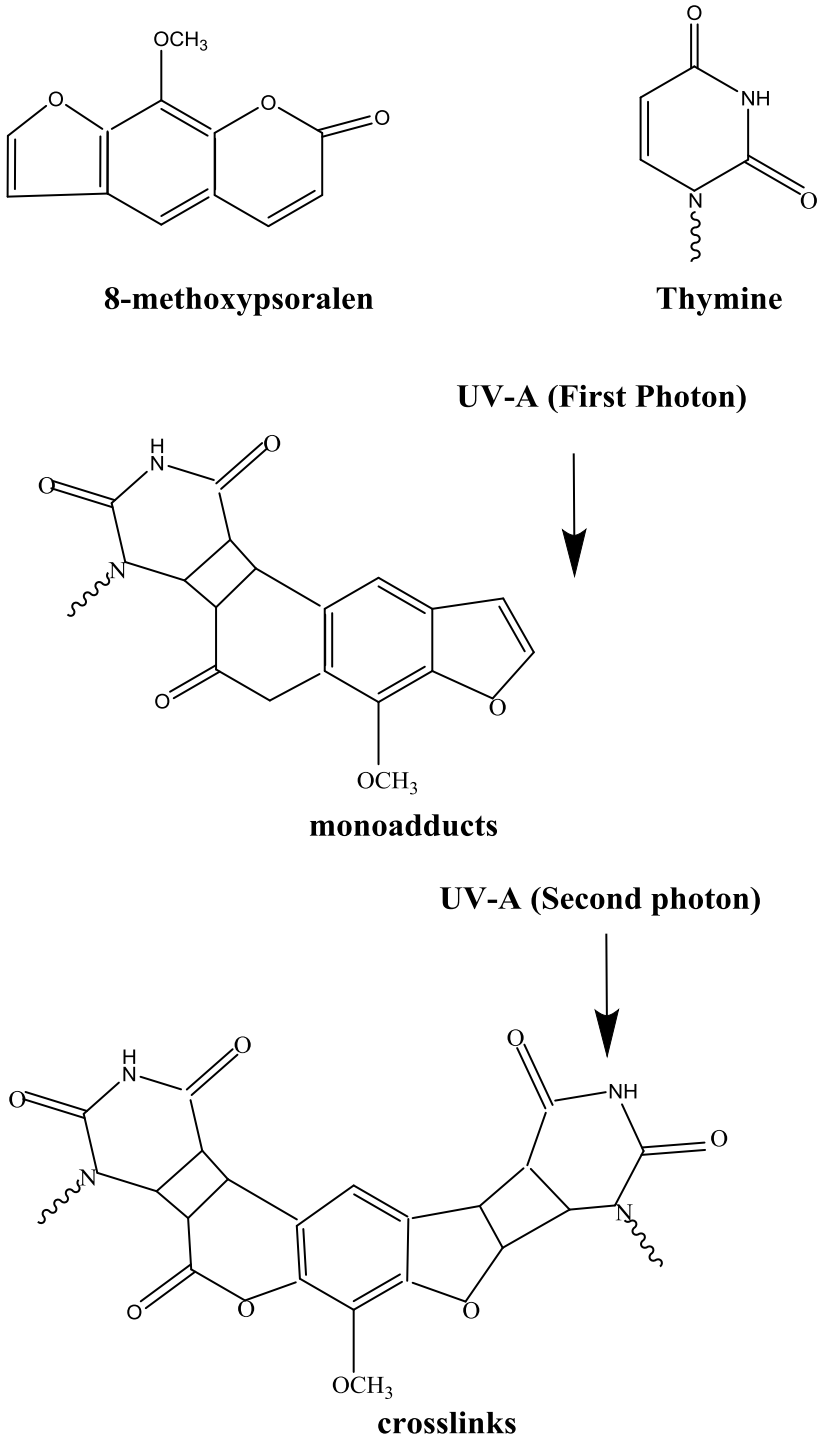

Figure1-2: Formation of DNA crosslinks in thymine (Asymmetrical lesion). Absorption of first photon covalently binds psoralen to the thymine in one strand and creates a monoadduct. Absorption of the second photon can convert monoadducts in to the crosslink by cycloaddition to the adjacent thymine. Adapted from (17)

Nucleotide Excision Repair

Nucleotide excision repair (NER) is the primary repair pathways in *Escherichia coli* for repairing bulky DNA lesions (23-26). NER is highly conserved from bacteria to mammals and recognizes a wide range of chemically distinct lesions (Reviewed in (27-29)). Removal occurs through a process of dual incisions in the lesion containing strand followed by a resynthesis step using the complementary strand as a template. UvrA is involved in the initial recognition of lesions in DNA(28). The protein is part of the ATP binding cassettes superfamily of ATPases that act as molecular matchmakers to affect several cellular processes, including DNA repair (30, 31). UvrA dimers form a groove that interacts with duplex DNA. Damage containing DNA distorts the helical backbone and causes a conformational change in the UvrA dimer, which in turn increases its DNA binding affinity (30, 32). $UvrA_2$ binding to distorted DNA is transient and requires association with UvrB(31, 33). The $(UvrA)_2UvrB$ complex associates stably with lesion containing DNA and recruits the third protein UvrC (31). UvrC possess two catalytic domains responsible for the 5'and 3' incisions of the damage containing DNA (34). The N-terminus of UvrC contains the 3' endonuclease that makes its incision at the fourth or fifth phosphodiester bond 3' from the lesion (34-37). The 3' incision is thought to precede the 5' incision, which is catalyzed by the C-terminal domain and occurs at the eighth phosphodiester bond 5' from the lesion (34-37). The C-terminal domain also contains a helix hairpin helix motif that is essential for this reaction and shares 33% sequence homology with human ERCC1. ERCC1 together with XPF catalyze the 5' incision reaction during human nucleotide excision repair (38, 39). Following dual

incisions of the lesion containing fragment, UvrD helicase unwinds the damaged DNA and displaces the $(UvrA)_2$UvrB UvrC complex. The gap that is left is filled in by DNA polymerase I using the complementary strand as the template and is sealed by DNA ligase (40, 41).

In addition, *E coli* also encodes a second endonuclease called Cho (UvrC homolog) that is functions with the nucleotide excision repair pathway and is upregulated along with *uvrA* and *uvrB* in response to DNA damage as part of the SOS response (42, 43) . Notably *uvrC* is not upregulated by the SOS response (43). Cho shows homology with the N- terminus of the UvrC and *in vitro*, catalyzes the 3' incision reaction at the eighth or ninth phosphodiester bond 3' to the lesion, four nucleotides downstream from where UvrC incises DNA(35, 36, 42). Similar to UvrC, Cho also requires UvrAB for its nuclease activity and is recruited to the DNA by UvrB; however, Cho binds to the UvrB at a different domain than UvrC (42). It is unclear why only a subset of bacteria encodes both UvrC and Cho or even what the function of Cho might be in these species and its role *in vivo* remains undefined (43). Several *in vitro* studies have suggested that Cho might make incisions at bulky lesions when UvrC is unable make the appropriate contacts with DNA (42). In support of this, bulky lesions such as cholesterol and menthol have been shown to be more efficiently incised by Cho than UvrC *in vitro*. However, whether Cho plays such a role in cells is unknown. In chapter II, I examine the contribution of Cho and the various nucleotide excision repair proteins to survival and repair of psoralen-induced lesions.

Recombinational Repair

Homologous recombination is the main pathway by which cells repair double-strand DNA breaks and daughter strand gaps in *E coli (44-46).* The primary protein of the homologous recombination is RecA, which binds single stranded DNA and initiates strand exchange with homologous duplex DNA *in vitro*.(47-49) RecA loading onto single stranded DNA is facilitated by one of two pathways, RecBCD or RecF(44, 50-52). RecBCD is a helicase/nuclease that unwinds and preferentially degrades the 5' ends of DNA at double-strand DNA ends to generate 3' single strand overhanging DNA molecules which serve as substrates for RecA loading(49, 50, 52-54). Alternatively, RecF, RecO, and RecR, are thought to act primarily at single strand gaps and single to double strand DNA junctions and operate to recruit RecA loading at these sites (46, 50, 52, 55-57).

Translesion synthesis

Translesion synthesis is an alternative to repair and allows replication to occur on templates containing lesions that have not been repaired (18, 58-60). In vitro, translesion DNA polymerases are able to incorporate nucleotides opposite to DNA lesions at higher efficiency than the replicative polymerase (DNA polymerase III) (61-66). While cell survival after DNA damage increases as a result of translesion synthesis, it is often associated with mutagenesis (58, 61, 63, 65, 67, 68).

E coli encode three translesion DNA polymerases, Pol II (*polB*), Pol IV *(dinB*) and Pol V (*umuD* and *umuC*) (61, 69) . Pol IV and PolV are Y family polymerases, while

Pol II polymerase is a B family polymerase (61, 69). These DNA polymerases are damage inducible and their expression is upregulated as a part of the SOS response (64, 68). *In vitro*, the *E coli* translesion DNA polymerases show specificity for the type of DNA lesion they are able to bypass as well as sequence context variation in each activity (58, 61, 62, 70). For example, Pol V, but not Pol II or Pol IV increases cell survival following UV irradiation, while Pol IV contribute to viability after nitrofurazone treatment (60, 70).

Fanconi anemia pathway and DNA interstrand crosslink repair in mammals

In mammalian cells, Fanconi anemia pathway is the main DNA interstrand crosslink repair pathway during S phase (71-73). Fanconi anemia is a rare recessive disease that is characterized by hypersensitivity to interstrand crosslinks(71). Patients having mutations in Fanconi anemia genes are highly susceptible to cancer, present with bone marrow failure, growth retardation, hyperpigmentation, and kidney malfunction(71). Fanconi anemia includes more than 15 complementation groups and genes that can be divided into three main functional groups. Eight proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM and two associated proteins , FAAP24 and FAAP100 form the core complex, which functions as a ubiquitin E3 ligase which mono-ubiquitinates the second functional complex, FANCD2/FANCI, known as ID complex(71, 74) . Ubiquination of ID initiates the repair process following replication blockage by DNA interstrand crosslinks (71, 74). A third functional group consisting of FANCD1, FANCJ and FANCN(75). FANCM/FAAP24 within the core complex are thought to act as molecular sensors to detect replication fork blockage (76).

Detection of replication fork arrest leads to FA core complex formation and ubiquitination of subsequent complexes, which is required to recruit downstream proteins for repair proteins (71, 73, 74). The Fanconi anemia pathway is thought to recruit several endo- and exo-nucleases from other repair pathways to initiate the incision of crosslinks (72). However, it still remains unclear how these various proteins collaborate with each other to complete the repair process and their exact role in the FA pathway are not known. One of the main endonucleases recruited during replication fork blockage is XPF-ERCC1 endonuclease (77). This endonuclease is responsible for making the 5' within the NER pathway. However, XPG, the 3' endonuclease is not recruited by FA pathway (78-82). In addition, two other endonucleases are also thought to be involved in the later stages of the FA pathway, FAN1- a structural dependent endonuclease having 5' endonuclease activity and weak 5' to 3' exonuclease activity (83-85) , and Mus81-Eme1- a structure-dependent 3' flap endonuclease associated with mismatch repair (86) .

The FA pathway is speculated to be specific for repairing lesions encountered during replication, and most models suggest that distinct mechanism may exist to repair crosslinks independent from replication (71-73). Similar to mammalian cells, prokaryotes may have a unique pathway designated to repair lesions that block DNA replication. I discuss this in relation to Cho function in in *E* chapter II of my thesis.

Models of DNA Interstrand Crosslinks

Two models for prokaryotic interstrand crosslink repair involve nucleotide excision repair coupled with either recombinational (Figure 1-3A) or translesion

synthesis (Figure 1-3B) (17).

Cole first proposed the nucleotide excision repair-homologous recombination model of interstrand crosslink repair (Figure 1.3A) based on survival assays of *uvrA* and *recA* mutants in *E coli.* Using psoralen interstrand crosslinks as his model lesion, Cole found that wild type cells were able to survive up to 65 lesions in their genomes (20, 21). In contrast, mutants in either *uvrA* or *recA* were able to survive 16 and 7 genomic lesions, respectively, while *uvrA recA* mutant was able to tolerate less than one interstrand crosslink per genome. According to the model, UvrABC first makes dual incisions on the strand bearing the furan side adduct, leaving the oligonucleotide containing the crosslink covalently bound to its complementary strand. This intermediate is then proposed to serve as a substrate for RecA- mediated strand exchange. However, in vitro studies have demonstrated, that if true, it is likely to require significant exonucleolytic processing before RecA can recognize and initiate strand exchange (22, 87-89). The resulting three stranded structure is then proposed to a substrate for a second round of incisions by UvrABC to remove the remaining pyrone side adduct, leaving a single strand gapped molecule that is filled and sealed by DNA Pol I and DNA ligase activities (22, 87-90).

An alternative model was proposed to account for the possibility that no homologous DNA sequence is present, such as in cells that have just divided or are in stationary phase (18, 19, 91). In this model, translesion synthesis substitutes for recombination to provide a template prior to the second round of incisions, however the remaining steps remain similar (Figure 1.3B) (87, 89). In vitro, DNA Pol II or Pol IV have been shown to be capable of bypassing different forms of DNA crosslinks (18, 19,

91).

It is important to note that the proteins proposed in these models are based primarily on the *in vitro* hypersensitive of mutants and the *in vitro* reconstitution of partial reaction steps. However, no intermediates have been observed *in vivo* to support these models Therefore, the overall goal of this thesis is to characterize the proteins and cellular pathways involved in the repair and survival of psoralen plus UVA-induced damage in *E coli*. Chapter II describes experiments that determine which nucleotide excision repair proteins contribute to the removal of psoralen DNA interstrand crosslinks *in vivo*. In chapter III, I address the contribution of recombination proteins and translesion DNA polymerases to cellular survival following psoralen-UVA treatment.

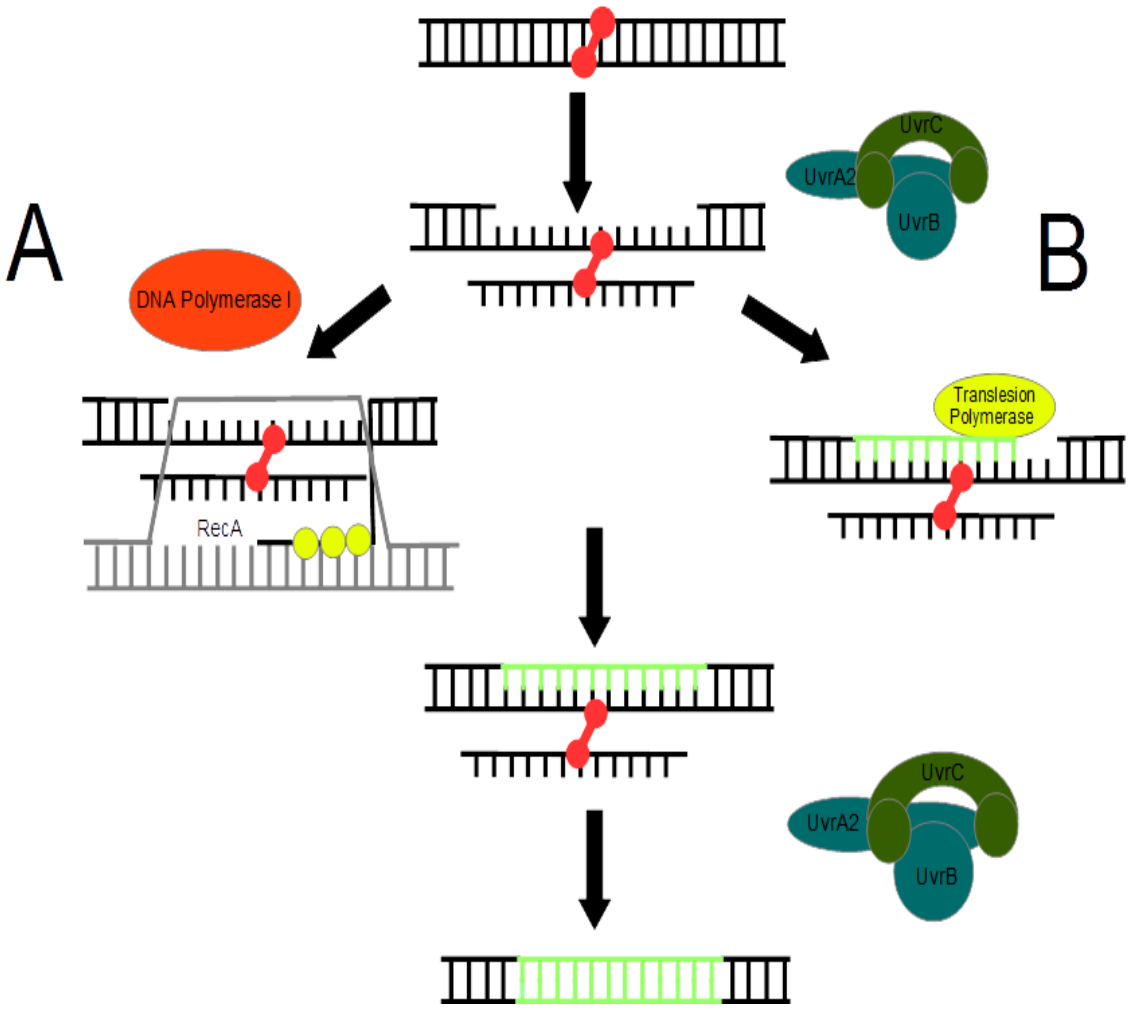

Figure 1-3: Models of Interstrand Crosslink Repair. A) Recombination dependent DNA interstrand crosslink repair. B) Translesion polymerases dependent interstrand crosslink repair. Adapted from. (17)

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CHAPTER II

Cho IS SPECIFICALLY REQUIRED FOR THE REPAIR OF DNA INTERSTRAND CROSSLINKS

ABSTRACT

DNA interstrand crosslinks are complex lesions that covalently bind to both strands of the duplex DNA. The repair of interstrand crosslinks is proposed to be initiated by the NER repair pathway. In this study, we characterized the role of NER pathway in the presence of psoralen-induced lesions, which forms both interstrand crosslinks and monoadducts. Unexpectedly, we observed that the nucleotide excision repair mutants exhibit differential sensitivity to psoralen-induced damage, with *uvrC* mutants being less sensitive than either *uvrA* or *uvrB.* Furthermore, we found that Cho, an alternative endonuclease, is responsible for the reduced hypsersensitivity of *uvrC* mutants, and is required specifically in the presence of DNA interstrand crosslinks.Three models are discussed for the mechanism by which Cho may be acting in this pathway.

INTRODUCTION

Psoralens are tricyclic asymmetrical compounds containing furan and pyron rings, and bind DNA nonspecifically with a preference for pyrimidines forming noncovalent bonds(17, 18). Upon absorption of UV-A light, a covalent bond forms through photoaddition between the C5=C6 double bond of the pyrimidine and the psoralen's C4'=C5' bond of the furan or C3'=C4' of the pyrone moiety (17, 18, 44) . Absorption of a second photon results in photo addition on the remaining furan or pyrone with a second pyrimidine, creating a DNA interstrand crosslink (17, 18, 23). Thus, psoralen-induced

damage consists of both monoadducts and DNA interstrand crosslinks. Interstrand crosslinks covalently bind both strands of the duplex DNA, thereby inhibiting any cellular process that requires strand denaturation, such as transcription and replication. This inhibition is generally considered to be the reason for the potency of their toxicity, and use in treating different skin diseases such as vitiligo, psoriasis, and as a chemotherapeutic for some forms of cancer (16, 22, 32, 36, 37).

Several models have been proposed as to how DNA interstrand crosslinks are repaired, although many aspects of these models still remain unknown. A feature common to all these models is that the repair process is initiated by nucleotide excision repair, followed by the sequential action of other DNA repair processes, such as recombination or translesion synthesis (2, 7, 15).

Nucleotide excision repair pathway is the primary pathway for repairing bulky DNA lesions in cells (39, 45, 46). In *Escherichia coli* the incision complex for this pathway is made up of UvrA, UvrB and UvrC(45). UvrA forms a homodimer that has a high affinity for damaged DNA relative to non-damaged DNA (34, 39, 45). In a presence of a distorting lesion, a complex consisting of $UvrA_2B$ specifically binds to the strand containing the lesion (34, 35). Binding of the $UvrA_2B$ complex recruits the UvrC endonuclease, which makes an initial incision located on the fourth or fifth phosphodiester bond 3' to the lesion, immediately followed by a second incision at eighth phosphodiester bond on 5' side (29, 40, 50). After the incision step, the UvrD helicase displaces the UvrA2BC complex and along with the 12-13 bp segment containing the lesion before DNA polymerase I and ligase re-synthesize and seal this segment using the

undamaged strand as a template (47).

Several studies support the idea that NER is involved in the repair of interstrand crosslinks (4-7). *In vivo*, mutants defective in any one of the nucleotide excision repair genes are hypersensitive to crosslinking agents (6, 7). Furthermore, all three NER mutants are defective in their ability to incise DNA containing interstrand crosslinks *in vivo*, as measured in alkaline CsCl gradients (6, 7) . *In vitro*, UvrA UvrB and UvrC are capable of incising oligonucleotides or plasmids containing a psoralen-induced interstrand crosslink, with dual incisions occurring predominantly on the strand containing the furan moiety (40, 42, 43, 49).

Based on these observations, and other lesions known to be repaired by nucleotide excision repair, it was inferred that all three subunits of the repair complex were required and contributed equally to the crosslink repair. However, a recent study reported that *uvrB* mutants were more sensitive to psoralen-induced damage than either *uvrA* or *uvrC*, suggesting a potentially unique mechanism of repair for these lesions (26) . While this represents a potentially important observation, they also appeared to contradict a number of earlier studies that suggested *uvrA* and *uvrB* mutants are equally sensitive to psoralen treatment (3, 13) .

Thus to characterize the comparative roles of the NER proteins in the processing and repairing of DNA interstrand crosslinks, we constructed isogenic mutant strains lacking each of the nucleotide excision genes and characterized their ability to survive and incise psoralen-induced DNA damage. In do so, we observed that a mutant of the

nuclease subunit, UvrC, was less sensitive to psoralen-induced damage.We found that an alternative nuclease, Cho, could account for the reduced hypersensitivity, and that Cho function appears to be specific for the repair of interstrand crosslinks.

MATERIALS AND METHODS

Bacterial Strains

The parental strain used in this study was SR108, a *thyA36 deoC2* derivative of W3110. Isogenic strains lacking *uvrA* (HL952), *uvrC* (HL925), and *uvrD* (CL1302), were constructed by standard P1 transduction and have been reported previously (19, 20, 33) . The *cho* gene was replaced from codons 4 to 280 with *cat*, conferring chloramphenicol resistance, using PCR replacement with the primers,

5'-ggatagataaccagcattcggagtcaacagtggtacggcgatgagacgttgatcggcac-3’

and

5'-ctcgctggtcattcgccggatcaagttcagtaatttcatactttcgaatttctgccattc -3’,

followed by recombineering into DY329 to generate CL904(19) .The *uvrB* gene was replaced from codons 1 to 672 with *cat* using PCR replacement with the primers, 5’-attacatacctgcccgcccaactccttcaggtagcgactcatgagacgttgatcggcac-3’

and

5’-ggctgttttccgtttgtcatcagtcttcttcgctatcctgctttcgaatttctgccattc-3’,

followed by recombineering into DY329 to generate CL1673(53). CL908 (SR108 cho::cat) and CL1735 (SR108 uvrB::cat) were constructed by P1 transduction of cho::cat

and uvrB::cat from CL904 and CL1673, respectively, into SR108. CL2155 (SR108 cho::cat uvrC297::Tn10) was generated by P1 transduction of the uvrC297::Tn10 allele from HL925 into CL908.

The parental strain, AB1157 (*thr-1 leuB6 proA2 his-4 argE3 thi-1 lacY1 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44 galK2*) has been reported previously(14).AB1886 (*uvrA6*), AB1885 (*uvrB5*), and AB2498 (*uvrC34*) are nitrous acid-mutagenized derivatives of AB1157 and have been previously reported HL759 (*uvrA6 zjd*::Tn*5*) has been reported previously (14) .The *uvrB5* allele from AB1885 was linked to kanamycin resistance in two steps. First, the *kanR* gene was inserted 23 bp downstream from *mngB* using PCR insertion with the primers,

5’-gttaccggcttgcctgaatagcaatcaaaccgaagccacatgtgacggaagatcacttcg-3’

and

5’-atgaacaaagcgccctttgtcaacaatctggccgcgcataaccagcaatagacataagcg-3’,

followed by recombineering into DY329 to generate CL2301. *mngB*::*kan*, from CL2301, was then transduced into AB1885 using standard P1 transduction to generate CL2337. The *uvrB5* allele is ~50% cotransducible with *mngB*::*kan*. The *uvrC34* allele from AB2498 was linked to kanamycin resistance in two steps. First, the *kanR* gene was inserted 106 bp upstream from *torY* using PCR insertion with the primers,

5'-ggatagataaccagcattcggagtcaacagtggtacggcgatgagacgttgatcggcac-3’

and

5'ctcgctggtcattcgccggatcaagttcagtaatttcatactttcgaatttctgccattc-3’

followed by recombineering into DY329 to generate CL2280. *torY*::*kan*, from CL2280, was then transduced into AB2498 using standard P1 transduction methods to generate CL2341. The *uvrC34* allele is ~60% co-transducible with *torY*::*kan*. CL2343 (SR108 *uvrB5 mngB*::*cat*) and CL2472 (SR108 *uvrC34 torY*::*cat*) were constructed by P1 transduction of *uvrB5 mngB*::*cat* and *uvrC34 torY*::*cat* from CL2337 and CL2341, respectively, into SR108. CL23 (SR108 *uvrA6 zjd*::Tn*5*) was constructed by P1 transduction of *uvrA6 zjd*::Tn*5* from HL759 into SR108. Transductants were verified by antibiotic sensitivity and sensitivity to UV irradiation at 254 nm where appropriate. Strains were transformed with plasmid pBR322 for interstrand crosslink incision experiments.

Psoralen-UVA and Angelicin-UVA Survival Assays

Overnight cultures grown in Davis medium supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10 μg/ml thymine (DGCthy). Fresh overnight cultures were diluted 1:100 in DGCthy medium and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.3. At this time, 10 μg/ml of 8-methoxypsoralen or 20 μg/ml of angelicin was added to the cultures and incubation continued for 5 min before irradiation with UV-A light. Cells were irradiated using two 32-watt UVA bulbs (Sylvania) with a peak emittance of (320 nm) at an incident dose of 6.9 $J/m^2/s$. At the times indicated, 100-μL aliquots were removed from each culture and serially diluted in 10-fold increments.

Triplicate 10-µl aliquots of each dilution were spotted onto Luria-Bertani agar plates supplemented with 10 µg/ml thymine (LBthy) and incubated at 37°C. Viable colonies were counted the next day to determine the surviving fraction.

8-methoxypsoralen was purchased from Acros Organics, item 298-81-7 lot A0143457. Angelicin was purchased from Sigma-Aldrich, item A0956 lot 042M4054V. Both the NMR spectra and an HPLC profile with/without psoralen spike revealed no detectable contamination of other prosalen deriviaties in the angelicin preparation,

UVC Survival Assay

Fresh overnight cultures were diluted 1:100 in DGCthy medium and grown at 37°C to an OD_{600} of 0.4. Ten-µl aliquots of serial 10-fold dilutions were plated in triplicate onto LBthy agar plates and UVC-irradiated at the indicated doses. UVC irradiation used a 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 $J/m^2/s$. Plates were incubated at 37°C and colonies were counted the next day to determine the surviving fraction.

In vitro Plasmid Crosslinking Assay

Purified plasmid was treated with 10µg/mL of 8-methoxy-psoralen or 20µg/mL of angelicin and irradiated with increasing doses of UV-A light (320 nm). Treated plasmid DNA was digested with PuvII (Fermentas) over night at 37C. Samples were electrophoresed on a 0.5% alkaline agarose gel (30mM NaOH and 1mM EDTA) at 30 V for 16 h. DNA in the gels was then transferred to Hybond N+ nylon membranes (GE Healthcare) using standard Southern blotting techniques. The plasmid DNA was detected

by probing with ^{32}P-labeled pBR322 that was prepared by nick translation (Roche) using alpha^{32}P-dCTP >6000Ci/mmol (Perkin-Elmer) and visualized using a Storm 840 phosphorimager (GE Biosciences) and its associated ImageQuant analysis software.

In vivo Interstrand Crosslink Incision Assay

Cultures containing the plasmid pBR322 were grown overnight at 37°C in DGCthy medium supplemented with 100 µg/ml of ampicillin. A 0.2-ml aliquot from this culture was pelleted and resuspended in 20-ml DGCthy medium without ampicillin and grown in a 37°C shaking water bath to an OD_{600} of 0.4. At this time, cultures were exposed to 10 µg/ml 8-methoxypsoralen for 5 min at 37°C and subsequently irradiated with 6.2 kJ/m^2 UV-A light (320nm). The cells were then filtered and collected on Millipore 0.45-µm general filtration membranes, resuspended in fresh, prewarmed DGCthy medium and allowed to recover at 37°C. At the times indicated, 0.75-mL aliquots of culture were transferred to an equal volume of ice-cold NET (100 mM NaCl, 10mM Tris [pH 8.0], 20 mM EDTA [pH 8.0]), centrifuged for 2 min, resuspended in 140 µl lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNaseA in 10 mM Tris, 1 mM EDTA [pH 8.0]), and incubated at 37°C for 30 min. Ten µl of 10 mg/ml proteinase K and 10 µl of 20% Sarkosyl were then added to the samples, and incubation continued for a further 30 min. Samples were then extracted with four volumes of phenol-chloroform , followed by four volumes of chloroform and then dialyzed against 200 ml of 1mM Tris (pH 8.0), 2mM EDTA (pH 8.0) for 45 min using 47-mm Millipore 0.025-µm pore disks and digested with PvuII (Fermentas) overnight at 37°C. Samples were then electrophoresed on a 0.5% alkaline agarose gel (30mM NaOH and 1mM EDTA) at 30 V for 16 h. DNA in

the gels was then transferred to Hybond N+ nylon membranes (GE Healthcare) using standard Southern blotting techniques. The plasmid DNA was detected by probing by ^{32}P-labeled pBR322 that was prepared using nick translation (Roche) using alpha^{32}P-dCTP >6000Ci/mmol (Perkin-Elmer) and visualized using a Storm 840 phosphorimager (GE Biosciences) and its associated ImageQuant analysis software.

The fraction of psoralen crosslinks formed at each time point was calculated as the ratio of DNA running above the linear band to the total DNA loaded, and normalized to the average of the fraction of crosslinks in untreated and preirradiated samples. Fraction of Crosslink

$= (XD_{time}x/TD_{time}x)-(1/2(XD_{untreated}+XD_{preirrdation})/TD_{untreated}+TD_{preirradiation}))$ where XD represents crosslinked DNA and TD represents total DNA.

RESULTS

Irradiation with UV-C generates two predominant lesions, the cis, syn cyclobutane pyrimidine dimer and the pyrimidine 6-4-pyrimidine photoproducts in the DNA(27, 28).Repair of these lesions in *E coli* is known to require *uvrA*, *uvrB* and *uvrC* genes to initiate nucleotide excision repair of UV- induced DNA lesions (39). Mutants lacking any of these gene products fail to remove these lesions and are equally sensitive to UV-C irradiation (27). Consistent with earlier studies, we observed that the survival of our *uvrA*, *uvrB* and *uvrC* deletion mutants decreased at the same rate as the UV-C dose was increased, indicating that each protein contributes equally to survival following UV-C-induced damage Figure 2-1A .

We next examined the survival of these strains following psoralen-induced DNA damage. To this end, 10μg/ml 8-methoxypsoralen was added to growing cultures of the parental and mutant strains before they were exposed to 6.9/J/m2/s UV-A irradiation for increasing time periods .The fraction of cells surviving to form colonies was then determined.

In contrast to that observed in UV-C irradiation, the *uvr* strains did not equally contribute to survival of psoralen-induced lesions (Figure 2-1B). Relative to wild type cells, *uvrA* and *uvrB* mutants were equally hypersensitive to psoralen-induced damage. However, the hypersensitivity of *uvrC* mutants was significantly less than either *uvrA* or *uvrB* and required approximately twice as much UV-A radiation to reduce the survival of *uvrC* mutants to the level observed in *uvrA* or *uvrB* mutants. Importantly, no loss of viability was observed in wild type or *uvrA* cultures that were treated either with UV-A irradiation alone (Figure 2-1C) or incubated with psoralen alone, indicating that the hypersensitivity and loss of viability in these cells was specifically due to the photoactivated forms of psoralen and not due to the intercalation of psoralen or UVA-specific damage . Thus, we observed that the *uvrC* gene is less sensitive to psoralen-induced damage than either *uvrA* or *uvrB*.

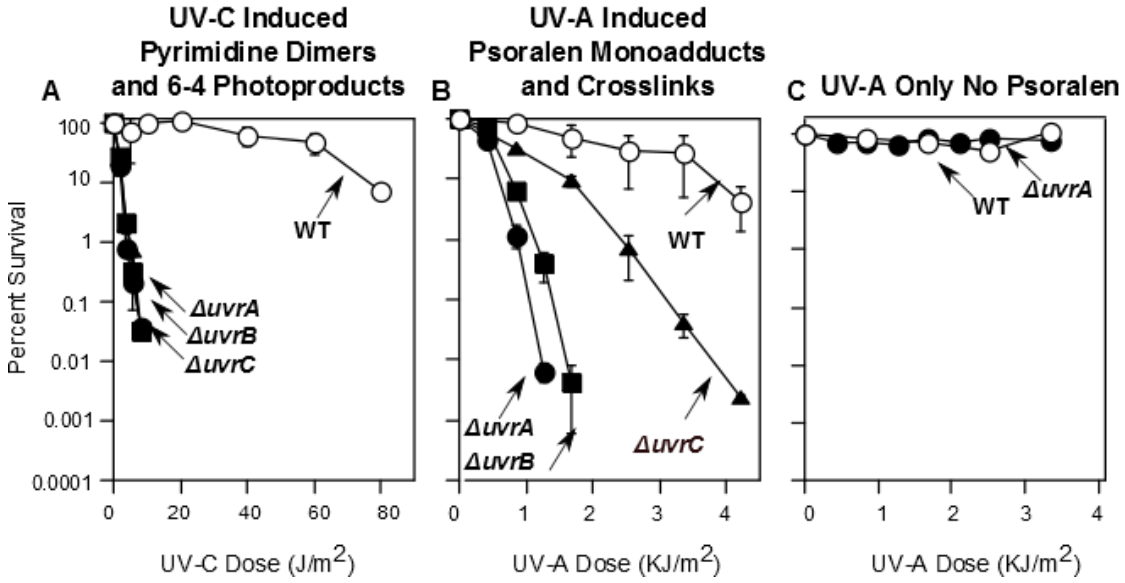

Figure 2-1 In contrast to UV-induced damage, *uvrC* mutants are less sensitive to psoralen-induced adducts than either *uvrA* or *uvrB* mutants.
The survival of cells following irriadiation with UVC light (254nm) (A) or UV-A light (340nm) in the presence of 10μg/ml 8-methoxy-psoralen (B) is plotted. The survival of cells following irradiation with UV-A light (340nm) in the absence of psoralen (C) is plotted. WT (open circles), *uvrA* (closed circles), *uvrB* (closed square), and *uvrC* (closed triangles).Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

These results were unexpected and contrasted with those of Lage et.al. (2010) in which they observed that *uvrB* was markedly more hypersensitive to psoralen-induced damage than either *uvrA* or *uvrC (26)*. One potential reason for the discrepancy between our results and those of Lage et al are the differences between our experimental conditions. Lage et al (2010) used psoralen at concentrations of 0.1µg/ml, and then irradiated cultures for time periods extending up to several hours on ice to achieve lethal levels of psoralen-induced damage. Since the initial goal of our study was to observe the time course of psoralen crosslink repair in living cells, the temperature changes and long exposure times were impractical. Thus we treated our strain cultures with a higher concentration of 8-methoxypsoralen (10µg/ml) similar to other studies. Allowing us to reduce our irradiation times and avoid temperature changes. A second potential reason for the differences seen in our results and those of Lage et al 2010, could be due to the difference in strain backgrounds or the specific *uvr* alleles used in each study. The *uvr* mutants used in the Lage et al study were direct isolates from nitrous acid-mutagenized cultures(20). Previous studies have also reported that various strains of *E.coli* can vary significantly in the sensitivity to DNA crosslinks (4, 20).

In order to test and differentiate between the possibilities, we repeated the survival assays using the strains from the Lage et al study with high 8-methoxypsoralen concentrations (10µg/ml) and short UV-A exposure times. As shown in Figure 2-2A, we were able to reproduce their observations, demonstrating the extreme sensitivity of strain AB1885, containing the *uvrB5* mutation, to psoralen-induced damage. The results refute the idea that underlying differences between our results are due to different treatment

conditions and suggest that *uvrB5* allele or strain background may be the reason for its extreme hypersensitivity. To test this hypothesis, we moved the *uvrA6*, *uvrB5*, and *uvrC34* alleles in the SR108 background using standard P1 transduction to generate isogenic strains. We then repeated the experiments as before. As shown in Figure 2-2B, after moving alleles into isogenic backgrounds, hypersensitivity of the *uvrB5* mutant was similar of that *uvrA6* allele. These results indicate that extreme hypersensitivity of the strain AB1185 is likely due to secondary mutations or differences between the various backgrounds. However, consistent with the initial observation in Figure 1B, the hypersensitivity of *uvrC34* mutant was more modest than that of either *uvrA* or *uvrB* in all strains examined, arguing that the loss of *uvrC* is not as lethal as the loss of *uvrA* or *uvrB* when psoralen-induced damage is present in the cell.

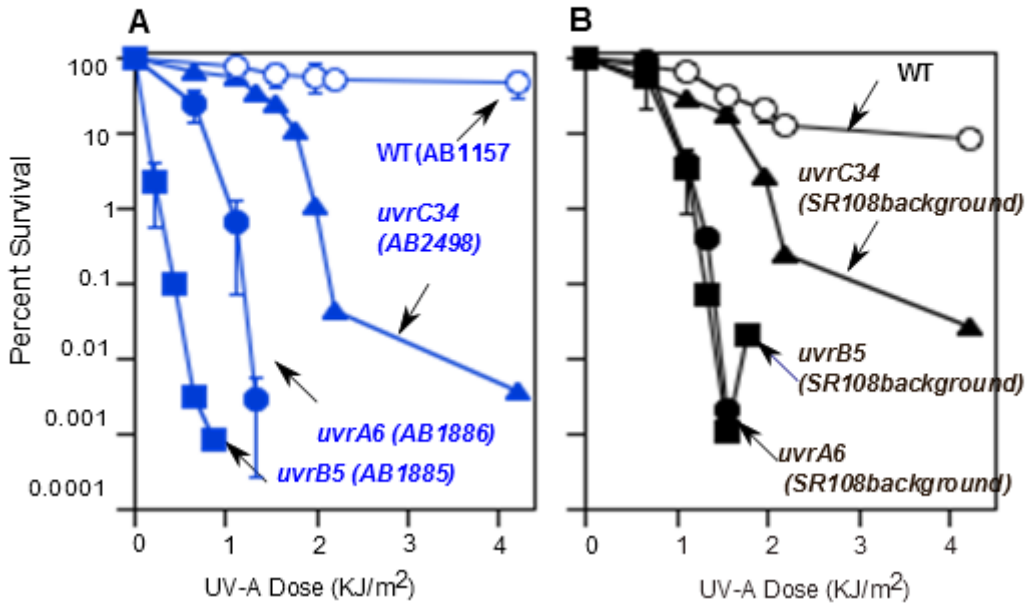

Figure 2-2 Isogenic backgrounds are important when comparing sensitivities of the NER mutants to psoralen

A) The survival of *uvrA6*, *uvrB5* and *uvrC34* mutants following irradiation UV-A (340nm) light in the presence of 10µg/ml 8-methoxy-psoralen is plotted before (A) and after (B) moving the alleles into isogenic backgrounds. (A) AB1157 wild type strain, *uvrA6* in strain AB1886 (closed blue circles), *uvrB5* in strain *AB1885* (blue square), *uvrC34* in strain AB1884 (B) SR108 parental (open blue circles), *uvrA6* in SR108 (closed black circles), *uvrB5* in SR108 (black square closed), *uvrC34* in SR108 (closed triangles) Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

Although the UvrABC excision is thought to function as a complex (40, 41), the survival assays indicated that *uvrC* was less sensitive to psoralen-induced damage than *uvrA* or *uvrB.* Given that UvrA and UvrB act in the initial recognition step of the NER pathway, while *uvrC* functions as the nuclease, I hypothesized that an alternative endonuclease might be participating in the repair of the psoralen-induced damage. Cho (Uvr**C** **ho**molog), was identified as a putative nuclease that is upregulated following DNA damage (30) . Subsequent biochemical studies showed that in the presence of the UvrABC endonuclease, Cho was able to make a 3' incision two bases further away than UvrC and that this activity could act on a variety of lesions in vitro. However, its function *in vivo* remains unclear as *cho* mutants are not sensitive to UV or other forms of damage that have been examined (30).To test whether Cho was responsible for the increased resistance of *uvrC* to psoralen-induced damage, we characterized *cho* mutants and *cho uvrC* double mutants for their ability to survive psoralen-induced damage. As shown in Figure 3A, *cho* mutants were only modestly sensitive to the psoralen-induced damage, relative to other *uvr* mutants (Figure 2-3A). Interestingly however, the absence of Cho increased the hypersensitivity of *uvrC* mutants to a level that was similar to *uvrA* and *uvrB* mutants (Figure 2-3A). The observation indicates that Cho is responsible for the decreased sensitivity of *uvrC* mutants in the presence of psoralen-induced lesions.

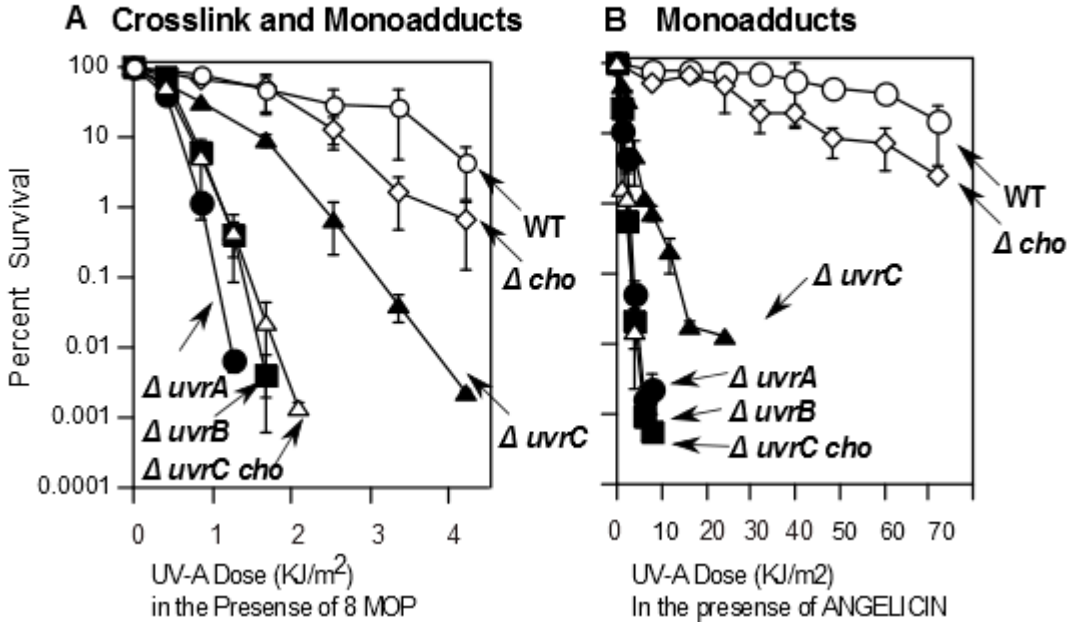

Figure 2-3 Cho accounts for the reduced sensitivity of *uvrC* mutants and is required specifically in the presence of interstrand DNA crosslinks. A) The survival of cells following irradiation with increasing doses of UV-A (340nm) light in the presence of 10μg/ml 8-methoxy-psoralen (A) or B) 20μg/ml of angelicin is plotted. WT (open circles), *uvrA* (closed circles), *uvrB* (closed square), *uvrC* (closed triangles), *cho* (open diamonds), and *uvrC cho* (Open triangles)
Graphs represent the average of the three or more independent experiments. Error bars represent one standard deviation.

8-methoxypsoralen is capable of creating both monoadducts and interstrand crosslinks with exposure to the UV-A light (1, 23) . One possible explanation for the increased sensitivity of Cho in the absence of *uvrC* is that Cho is required to act with UvrC on only one of these two classes of lesions. To test this idea, the experiments were repeated using angelicin in place of the 8-methoxypsoralen. Angelicin shares a similar structure to that of 8-methoxypsoralen, but is thought to only be capable of forming mono adducts due to its angular structure. In contrast to 8-methoxypsoralen, in the presence of angelicin, the relative hypersensitivity of the *uvrC* mutant increased to a level that approached that of the *uvrA* and *uvrB* mutants (Figure 2-3B). The slight resistance that remained between *uvrC* and the *uvrA* and *uvrB* mutant survival is likely to be due a low level of interstrand DNA crosslinks that still form in the angelicin-treated samples (Figure 2-4B). Although angelicin is reported to only be capable of forming monoadducts, we observed that our angelicin-treated DNA could form interstrand DNA crosslinks, although at a rate that was orders of magnitude less efficient than that of 8-methoxypsoralen (Compare Figure 2-4 A and B).

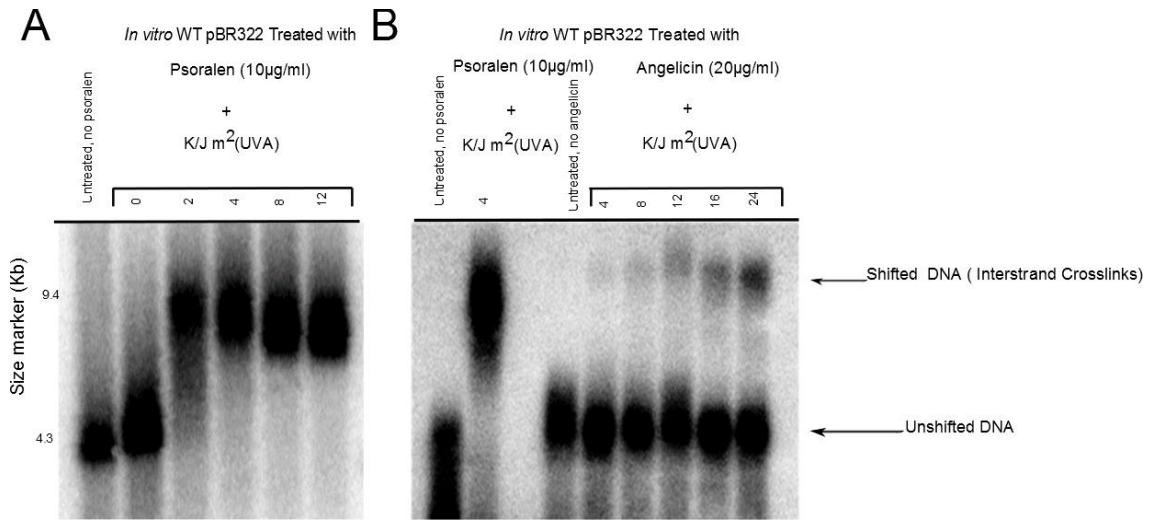

Figure 2-4 Purified plasmid treated with angelicin UV-A light forms minor product of DNA interstrand crosslinks.

A) Purified plasmid pBR322, was treated with 10µg/mL 8-methoxy-psoralen or B) 20µg/mL angelicin and irradiated with UV-A (340nm) light for increasing doses. DNA was restricted with PvuII and analyzed by southern analysis following alkali-agarose gel electrophoresis using pBR322 as a probe to determine the amount of DNA interstrand crosslinks present.

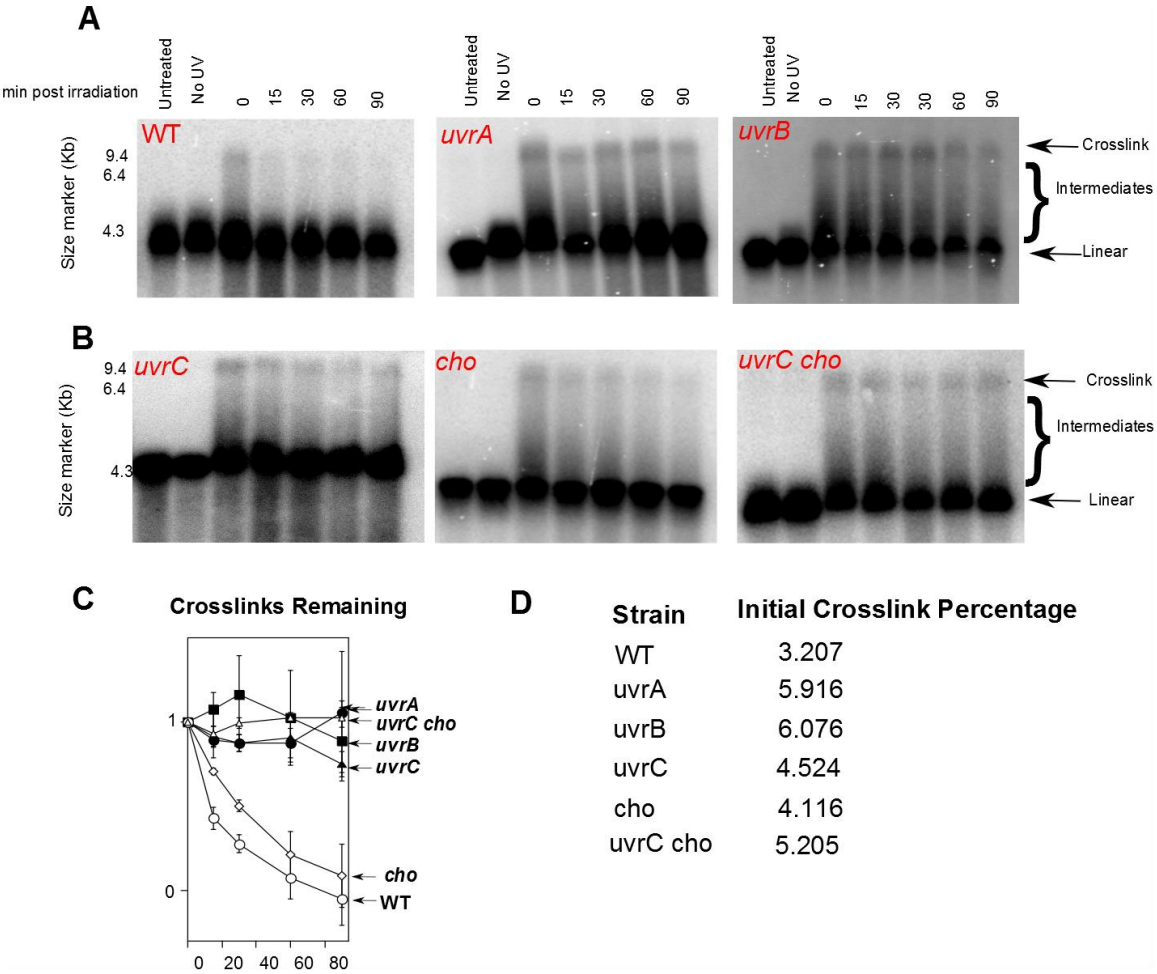

Figure 2-5 UvrC but not Cho is required for the initial incision of the crosslink *in vivo*.

A) Cultures containing the plasmid, pBR322, were irradiated with UV-A (340nm) light for 15 min in the presence of 10μg/ml 8-methoxy-psoralen and allowed to recover. At the indicated time points, total genomic DNA was purified, restricted with PvuII, and analyzed by southern analysis following alkali-agarose gel electrophoresis using pBR322 as a probe to determine the amount of DNA interstrand crosslinks present in the plasmid DNA over the recovery period. A representative gel for WT *uvrA*, *uvrB*, *uvrC*, *cho*, and *uvrC cho* is shown.

B) The relative amount of crosslinks remaining in the plasmid DNA over time is plotted, as normalized to the amount of noncrosslinked plasmid DNA. Band intensities for the DNA crosslinked and uncrosslinked fragments at each time point were Quantified using imageQuant software. Fraction of crosslinks at each time point were determined by calculating the ratio of the DNA crosslink band to linear plasmid. WT (open circles), *uvrA* (closed circles), *uvrB* (closed square), *uvrC* (closed triangles), *cho* (open diamonds), and *uvrC cho* (Open triangles). Plots represent the average of two independent experiments. Error bars represent the standard error of the mean.

Based on the survival assays, Cho appears to contribute to the repair of DNA interstrand crosslinks. To examine whether the absence of Cho affects the ability to initiate repair of the crosslinks *in vivo*, we compared each mutant's ability to incise interstrand crosslinks. To this end, I treated cultures containing the plasmid pBR322 with 8-methyoxypsoralen and UV-A light and then allowed them to recover over a period of time. At various time intervals, aliquots of the culture were taken, and the genomic and plasmid DNA was purified and restricted with PvuII which linearizes the plasmid. The DNA was then electrophoresed in a denaturing agarose gel and plasmid DNA was detected by southern analysis. Under denaturing conditions, DNA molecules that contain interstrand crosslinks are prevented from separating and can be detected due to their slower migration pattern relative to linear strands in alkali gels. In wild type cultures immediately following irradiation, approximately 3.2% of the plasmid DNA contained an interstrand DNA crosslink (Figure 2-4A and C). Over the 90 minute recovery period, the fraction of DNA migrating in the crosslink region of the gel decreased to approximately 0.57%, which we interpret to reflect incision of the crosslinks over time. In cultures of *uvrA*, *uvrB* mutants, 5.9% and 6.0% of the plasmid molecules contained DNA interstrand crosslinks immediately after irradiation. However, in these cultures, the crosslinks remained throughout the recovery period and no decrease in the shifted DNA band was observed, indicating that these cultures are impaired in their ability to make the initial incision (Figure 2-4A and 2-4C). When I examined cultures of *uvrC*, I observed that the crosslinks formed and persisted similar to that seen in *uvrA* and *uvrB* cultures (Figure 2-4B and 2-4C). Thus although the *uvrC* mutant is markedly less sensitive, by this assay, it

appears to be as defective in making the initial incision as either *uvrA* or *uvrB* mutants. In contrast, crosslinks were incised in *cho* mutants at a rate that was similar to wild type cultures, as indicated by the decrease in the intensity of the crossed link DNA band. In the *uvrC cho* double mutant, the initial incision was impaired to a similar extent as in *uvrC* mutants. We interpret these results to suggest that although Cho contributes to survival in the presence of interstrand DNA crosslinks, it is not required for the initial incision the DNA interstrand crosslinks *in vivo*.

DISCUSSION

In this chapter, I investigated the role of nucleotide excision repair proteins in repairing psoralen-induced DNA damage. I found that not all the NER subunits contribute equally to the survival of psoralen-induced damage. Notably, mutants lacking the endonucleotic subunit UvrC are less sensitive to psoralen-induced damage than mutants lacking the recognition proteins UvrA or UvrB. The increased resistance of *uvrC* was found to depend upon Cho, a secondary UvrABC-dependent endonuclease that is strongly induced after DNA damage (30).

The observation that Cho inactivation reduced the partially sensitive *uvrC* mutant to a level similar to that of *uvrA* and *uvrB* mutants, suggested that Cho is only required to participate in a subset of the psoralen-induced repair events. Since psoralen plus UV-A irradiation generates two classes or subsets of DNA lesions, one possibility is that Cho functions with UvrABC to repair psoralen interstrand crosslinks, but not monoadducts. Using a psoralen-derivative that predominantly forms monoadducts, I found that Cho was specifically required for survival in the presence of DNA interstrand crosslinks, but did

not contribute significantly to the repair of monoadducts. Interestingly, although Cho contributes to survival in the presence of crosslinks, its absence did not affect the cell's ability to make the initial incision on plasmids in vitro, suggesting that Cho operates later in the repair process or in only a subset of crosslink repair events.

I consider three possible scenarios that Cho function is required in the cell for repairing DNA interstrand crosslinks. One possibility is that Cho is required for repair of the interstrand crosslinks that block replication is blocked or those occurring in the global genome overall (Figure 2-5). Several studies based upon experiments using mammalian cell extracts have proposed that the repair of interstrand DNA crosslinks can occur through replication dependent and replication independent pathways (25, 51, 52). Fanconi anemia is a rare inherited disease that predisposes humans to cancer, involving more than 15 complementation groups, which renders cells highly sensitive to DNA interstrand crosslinks. Recent studies have suggested that the Fanconi anemia defect is specifically related to the repair of crosslinks encountered by the replication fork (51). In addition, the Fanconi anemia proteins FANCD2 and SLX4/FANCP interact with the 3' endonuclease of the mammalian nucleotide excision repair complex, XPF-ERCC1, to affect repair. The participation of XPF-ECRCC1 is independent from its role in nucleotide excision repair, as the remaining subunits of the NER complex are not required (51) (24). By analogy in *E. coli*, it is possible that the alternative nuclease, Cho, functions specifically at the replication fork to effect repair. *In vitro*, incision of DNA interstrand crosslinks occurs more efficiently on underwound superhelical substrates, perhaps suggesting that superhelicity differences between the DNA at replication forks

and the overall chromosome necessitate a role for Cho to act with UvrC in some cases (31). A role in replication-coupled repair would also be consistent with the observation that Cho has only a minor effect on the rate of crosslink incisions (Fig 2-5). Similar to the case with UV-induced damage, since the proportion of lesions requiring replication-specific repair events is quite small, mutants defective in replication-coupled repair would be expected to appear repair proficient (10). However, other observations do not support such a role for Cho. Defects in replication-coupled repair typically have severe effects on cell survival in both prokaryotes and eukaryotes, even when only low levels of damage are present (8-11).Yet the absence of Cho, by itself, does not have a large effect on cell survival.

A second possible role for Cho is that it acts as a secondary nuclease for the nucleotide excision repair complex that increases the efficiency of incision for some forms of bulky adducts (Figure2- 6). *In vitro*, the incision of the interstrand crosslinks by the nucleotide excision repair complex is influenced by the sequence context with preferential incisions occurring when the strands are GC enriched 5' and 3' to the adducted bases (21, 38). The longer excision patch resulting from Cho incision may enhance incision at poorly recognized or bulky lesions that cause problems for the core UvrABC exinuclease complex. Such a function could be consistent with both the survival we observe. Cho, along with UvrA and UvrB, is upregulated following DNA damage (12). However, UvrC is the only subunit that is not upregulated by DNA damage. If Cho did function to increase the efficiency of excision of psoralen damage, it would be expected to have a more significant effect on survival when high levels of DNA damage

are present, and less of an effect at lower levels of damage, as seen in our survival data. However, one might still expect to see that the rate of incision is affected in the absence of Cho, and this did not appear to be the case (Figure 2-5).

A third possibility is that Cho may function in a later step in the crosslink repair pathway, after the initial incision (Figure 2-7). In vitro, UvrABC exinuclease incises psoralen interstrand crosslinks on the strand containing the furan moiety of the psoralen molecule while leaving the strand containing the pyrone moiety intact (42, 43, 47-49). Replication independent repair models propose that following incision, either recombination or translesion synthesis generates a bulky three stranded structure, with the oligo containing the adduct. It is possible that Cho is required to make this second incision on the pyrone side strand, leading to the second round of repair. Cho protein is known to incise bulky substrates more efficiently than UvrC and Cho acting in this manner would be consistent with results showing that it remains proficient in making the initial incision (Fig 2-4) (30). An inability to carry out the second round of repair could also be less lethal than mutants unable to initiate repair, if alternative pathways, such as translesion synthesis or recombination, were able to compensate and act on the incised substrates when Cho is absent. Considering the angelicin survival data, and the psoralen *in vivo* incision results, I favor the third model as most likely the role of Cho in repairing DNA interstrand crosslinks. All our results are consistent with the model that Cho is required for the second incision step of the repair process, but not the first step. However, more experiments are required to confirm that Cho is responsible for the second incision of the repair process.

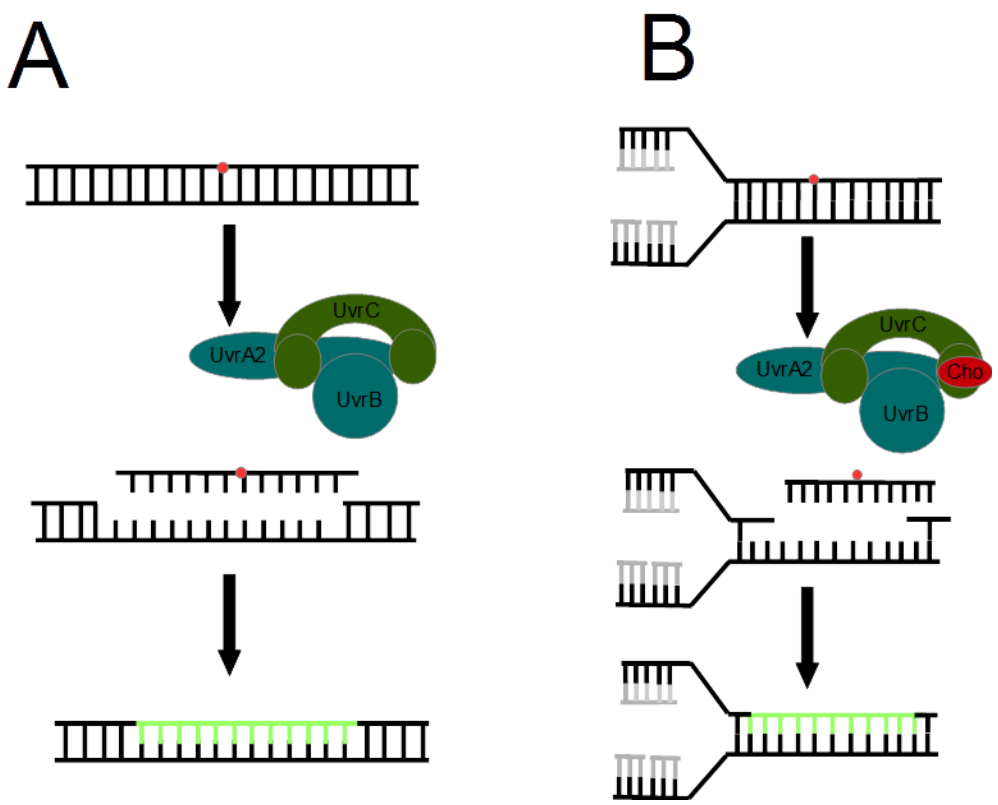

Figure 2-6: Model 1, Cho is required for repair of psoralen-induced lesions in a replication dependent manner. A) Global repair pathway B) Cho protein is involved in repairing psoralen-induced lesions only at replication fork arrest and assist in making incision in addition to UvrC.

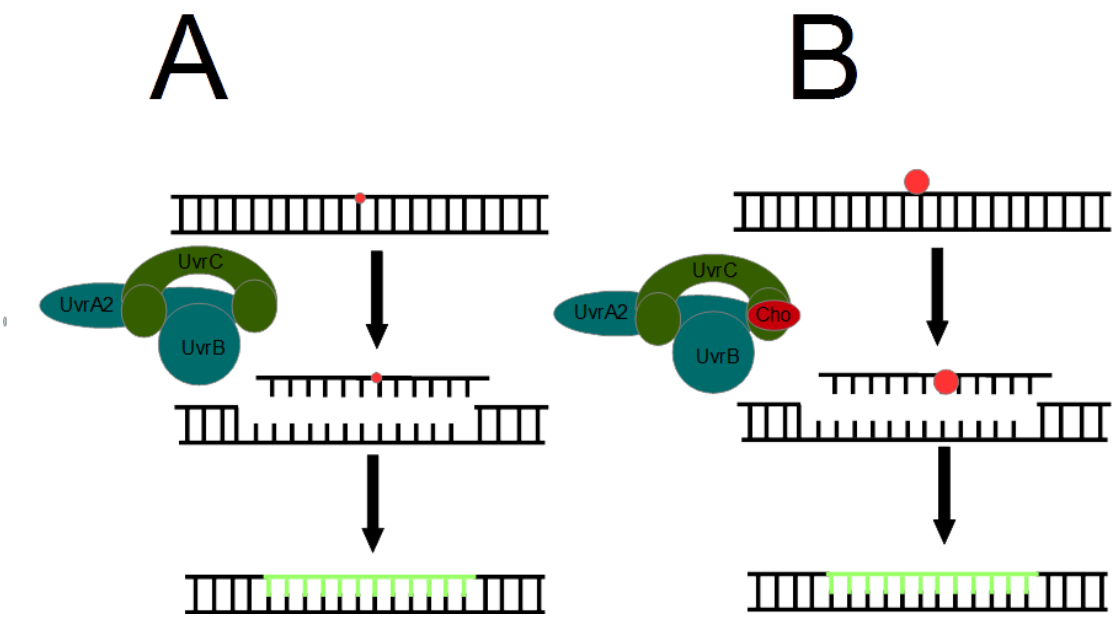

Figure 2-7: Model 2, Cho is required to increase the efficiency of psoralen-induced lesions in addition to UvrC. A) Main NER proteins repairing lesions. B) Cho is required to increase efficiency of incision rate in the presence of psoralen-induced lesions (bulkier lesions).

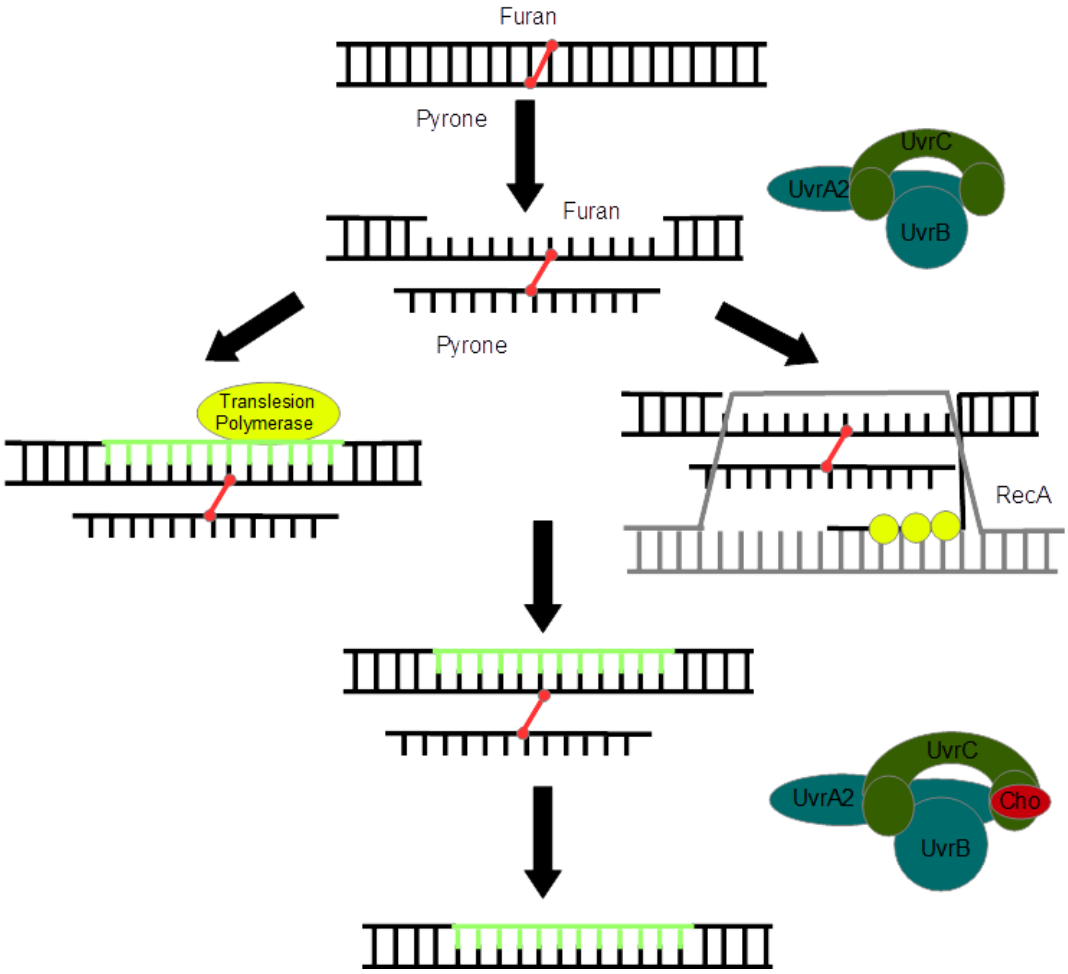

Figure 2-8: Model 3, Cho is responsible for the second incision step of the repair process at pyrone side of psoralen-induced lesions after recombination or translesion synthesis. Cho is responsible for the second incision followed recombination or translesion synthesis. In order for cells to completely remove the lesion, a second incision need to occur after first incision step. Cho makes the incision on pyrone side, while other main NER proteins are required to make the first incision on furan side.

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CHAPTER III

ELEMENTS OF RECOMBINATION PATHWAY AND TRANSLESION POLYMERASES ARE REQUIRED FOR THE REPAIR OF DNA INTERSTRAND CROSSLINKS

ABSTRACT

Agents forming DNA interstrand crosslinks are highly toxic and widely used in the treatment of hyperplastic disease. For these reasons the mechanism by which cells repair these lesions is of intense interest. Most models propose that repair is initiated by the nucleotide excision repair pathway, and coupled with either recombination pathways, translesion synthesis pathways or both. However, following incision, these models remain highly speculative, and the role of recombination and translesion synthesis in the repair of interstrand crosslinks still remains unclear. In order to further characterize how recombination proteins and translesion synthesis polymerases contribute to the repair of interstrand DNA crosslinks, I examined how these gene products contribute to the survival of psoralen damage. I observed that both *recBC* and *recF* mutants were hypersensitive to psoralen-induced lesions; however neither mutant was as sensitive as a *recA* mutant. With respect to translesion synthesis, I found that Polymerase V was the only translesion polymerase that contributes to survival of psoralen damage. I discuss these results in relation to both prokaryotic and mammalian models for crosslink repair.

INTRODUCTION

DNA interstrand crosslinks are a particularly lethal form of DNA damage that represents an absolute block to replication and transcription. A number of chemicals forming DNA crosslinks have proven to be highly toxic in nature, uniquely potent as a

chemotherapeutics, and highly effective in treating a range of diseases states involving hyperplastic or dysplastic conditions (1, 2). Psoralens are asymmetrical, tricyclic aromatic compounds, containing a pyrone and a furan ring (3-5). Psoralen intercalates into DNA and form monoadducts and interstrand DNA crosslinks upon absorption of one or two photons of UVA light, respectively (5).

The mechanism of how cells repair DNA interstrand crosslinks has remained a challenging problem. It is widely accepted that repair involves proteins associated with the nucleotide excision repair pathway, since NER mutants in both bacteria and mammalian systems are hypersensitive to crosslinking agents (6-10) . Further, cellular studies, suggest that in the absence of these gene products, DNA interstrand crosslinks are not incised and persist in the DNA (6, 7). However, nucleotide excision repair alone, as it is currently understood, is unable to account for the repair of these complex lesions. The linkage of both DNA strands to the lesion implies that the normal NER process of displacing the damaged region and resynthesis of cannot occur, since no template strand is available for synthesis. To accommodate this repair, two primary models for interstrand crosslink repair have been proposed (7, 11, 12) . Both propose that the nucleotide excision repair initiates the process, but then speculate that other repair pathways, such as recombination or translesion synthesis are coupled to excision repair to complete repair of the second strand through a multistep, multipathway process (7, 8, 11, 12).

The first model for ICL repair couples the NER pathway to recombination and is based primarily on early *in vitro* characterizations (13-17). In this model, the UvrABC

enzyme makes the initial 3' and 5' incisions on the furan side of interstrand crosslink. Then, the 3' →5' exonuclease activity of DNA polymerase I forms a gap creating a single strand region in which RecA, the central enzyme involved in homologous recombination, binds and promotes strand exchange with a homologous daughter chromosome (18, 19). The strand exchange enables UvrABC to make a second incision on the other strand (pyrone side of the interstrand crosslink) (13-16). In theory, after this occurs, the crosslink could then be removed and the gap re-synthesized using the sister chromosome as a template. RecA-mediated homologous recombination is classically defined as initiating through one of two pathways in *Escherichia coli*, the RecFOR and RecBCD pathway (19-23) . In order for RecA protein to initiate strand exchange reaction, RecFOR or RecBCD proteins are thought to be necessary to assist RecA to bind substrates containing single strand regions or DNA ends, respectively (18, 24). Although it is well established that RecA plays a role in surviving psoralen-induced damage, the comparative contributions of the RecFOR and RecBCD pathways is not known. However, an understanding of which pathway is involved would be useful and could suggest intermediates that arise in the repair process.

In the absence of a daughter chromosome, homologous recombination cannot occur. To account for this, an alternative model proposes that translesion systhesis may be coupled to NER. In this model, after the UvrA-B-C complex makes the initial incisions, the gap opposite to the lesion is resynthesized by translesion polymerases (11, 25, 26). In principle, once this is accomplished, the UvrA-B-C complex could initiate a second round of incisions on the opposite strand and the damaged region could then be

removed and the gap resynthesized.

Escherichia coli contain three damage-inducible polymerases that participate in translesion synthesis. In vitro, polymerase II (Pol II) (*polB*), Pol IV (*dinB*), and Pol V (*umuDC*) are able to incorporate bases opposite to specific lesions in template DNA with higher efficiency than the replicative polymerase (27-34). Pol III and mutants lacking these genes have higher mutation rates in response to different forms of damage, suggesting that they operate similarly in vivo (27-34) . Supporting the translesion synthesis-dependent model of crosslink repair, the survival of plasmids containing interstrand crosslinks suggested that repair could occur independently from recombination and replication (26) .There are also conflicting reports about the hypersensitivity of some translesion polymerase mutants to crosslinking agents. One group has reported multiple phenotypes for polymerase II (PolB), including hypersensitivity to nitrogen mustard, an agent that induces interstrand crosslinks among other lesions (25, 33, 34). However, subsequent studies have failed to confirm these PolB phenotypes (28, 35). Furthermore, a subsequent study reported that Pol IV, but not PolB, was able to synthesize past an oligo containing an acrolein interstrand crosslink in vitro (36). However, the role of the translesion polymerases has not been examined for psoralen-induced damage and no systematic study has examined all three polymerases for their contributions to survival in the presence of DNA interstrand crosslinks.

In the second chapter, we reported the relative sensitivities of the NER pathway mutants to psoralen-induced damage and found that Cho contributes to the repair process with UvrC. Here, we characterize and compare the relative contributions of the

recombination and translesion synthesis pathways in the survival of psoralen-induced damage to better understand the mechanism involved in the repair of these lesions.

MATERIALS AND METHODS

Bacterial Strains

The parental strain used in this study was SR108, a thyA36 deoC2 derivative of W3110(37). Mutant strains HL952 lacking (SR108 uvrA::Tn10)(38), *polB* CL636 (del(polB)::omega spc) (35), *umuDC* CL632 (UmuDC CL632) (35) ,*dinB* CL634 (del(dinB)::Kan) (35).CL646 (SR108 polB::Ω Sm-Sp dinB::Kanr umuDC595::cat) (35), CL681 (SR108 polB:: Ω Sm-Sp dinB::Kanr umuDC595::cat uvrA::Tn10)(35), *recA* HL921 (recA::tetR) (38), *recBC* HL922 (tetR(recBC)) (38), and *recF* CL579 (SR108 recF6206::Tetr) (38) have been previously reported.

Psoralen UV-A Survival Assay

Overnight cultures were grown in Davis medium supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10µg/ml thymine (DGCthy). Subcultures were diluted 1:100 and grown at 37°C shaker in to an optical density at 600 nm (OD600) of 0.3. At this time, 10µg/mL of psoralen was added to the cultures and incubation continued for five more minutes before irradiation with UV-A light. Cells were irradiated using two 32 watt UVA bulbs (Sylvanyia) with a peak emittance of (320 nm) at an incident dose of 6.9 J/m2/s. After irradiation, cells were serially diluted and 10 µl spots of each dilution were plated in triplicate onto LB thy agar plates. Plates were incubated at 37°C and colonies were counted the next day to determine the surviving fraction.

RESULTS

To examine how the two primary recombination pathways affect survival in the presence of psoralen-induced damage, we compared the survival of mutants defective in each pathway to the wild type strain and a *recA* strain, which is defective in all homologous recombination. To this end, 10μg/ml 8-methoxypsoralen was added to growing cultures of the parental and mutant strains before they were exposed to 6.9/J/m2/s UVA irradiation for increasing time periods. The fraction of cells surviving to form colonies at each dose was then determined. Under these conditions, greater than 37% of wild type cells survive UV-A doses up to ~3kJ/m2, which in the presence of 10μg/ml 8-methoxypsoralen produces about 45-50 DNA crosslinks per genome (see Ch 2, Figure 2-4). Although the level of monoadducts could not be determined from this data, these adducts are also likely to contribute to the lethality observed in the population. Consistent with earlier studies, *recA* mutants were highly sensitivity to psoralen-induced DNA damage, and survival was reduced to 37% with at dose ~0.5kJ/m2, which was similar to that of the nucleotide excision repair defective *uvrA* strain (Figure 3-1) under our conditions. This dose would correspond to approximately 7 crosslinks per genome, which is also similar to the lethal dose observed in previous studies (7) .We next examined the sensitivity of *recF* and *recBC* mutants. Both *recBC* and *recF* mutants were hypersensitive to psoralen-induced damage, relative to wild type cultures. However, the hypersensitivity of each mutant was more modest than that observed in *recA* cultures. At higher doses of UV-A light, *recF* cultures demonstrated slightly more sensitivity to psoralen-induced damage than *recBC* cultures. These results suggest that the repair of

psoralen-induced damage involves intermediates, or produces substrates that are processed by both the RecFOR and RecBCD pathways.

To examine the role of translesion synthesis in the repair of psoralen-induced DNA damage, we compared the survival of mutants that lack Pol II, Pol IV, Pol V, or all three of the damage inducible DNA polymerases in *E. coli* following treatment with psoralen plus UVA irradiation as before. Relative to wild type cultures, mutants lacking any single translesion polymerase were generally as resistant as wild type cultures at doses of irradiation below ~1 kJ/m2, but exhibited a modest hypersensitivity at higher doses (Figure 3-2). *umuDC* mutants, lacking Pol V, were significantly more sensitive than either *polB* (Pol II) or *dinB* (Pol IV) mutants.. The sensitivity of the triple polymerase mutant lacking all three damage-inducible DNA polymerases was similar to that of the *umuDC* (Pol V) single mutant, suggesting that Pol V is the predominant polymerase that contributes to survival at psoralen-induced DNA damage.

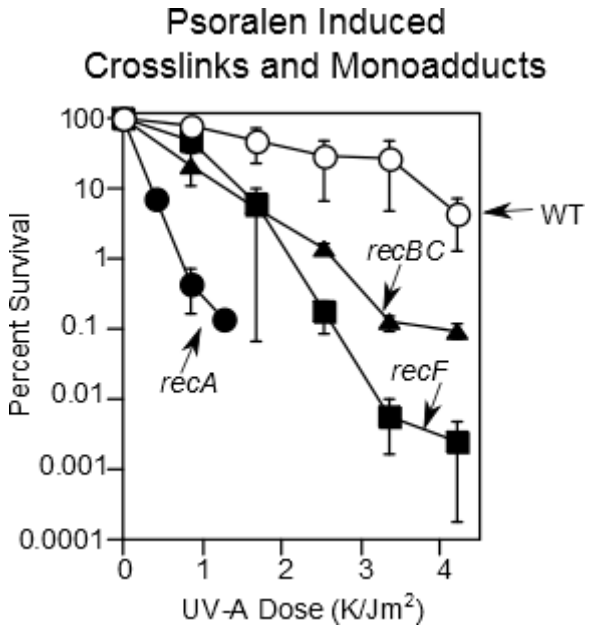

Figure 3-1. Both RecFOR and RecBCD pathways contribute to the survival of psoralen-induced DNA damage. The survival of cells following UV-A (340nm) irradiation in the presence of 10μg/ml 8-methoxy-psoralen is plotted. WT (open circles), *recA* (closed circles), *recF* (closed squares), *recBC* (closed triangles) Graphs represent the average of three or more independent experiments. Error bars represent one standard deviation.

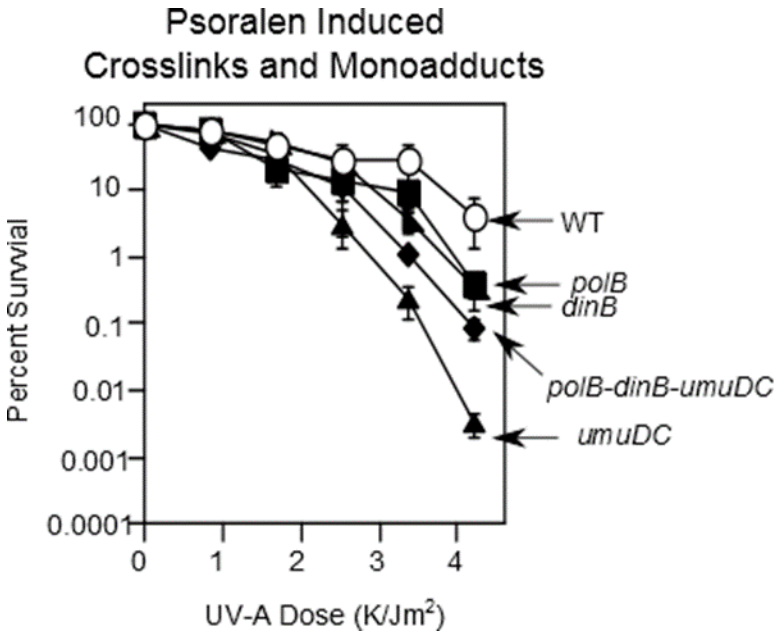

Figure 3-2: Tranlesion Polymerase V, encoded by *umuDC* contributes to survival of psoralen-induced damage. The survival of cells following UV-A (340nm) irradiation in the presence of 10μg/ml 8-methoxy-psoralen is plotted. WT (open circles), *umuDC* (closed isosceles), *polB-dinB-umuDC* triple mutant (closed diamonds), *dinB* (closed squares) and *polB* (closed right triangle). Graphs represent the average of three or more independent experiments. Error bars represent one standard deviation.

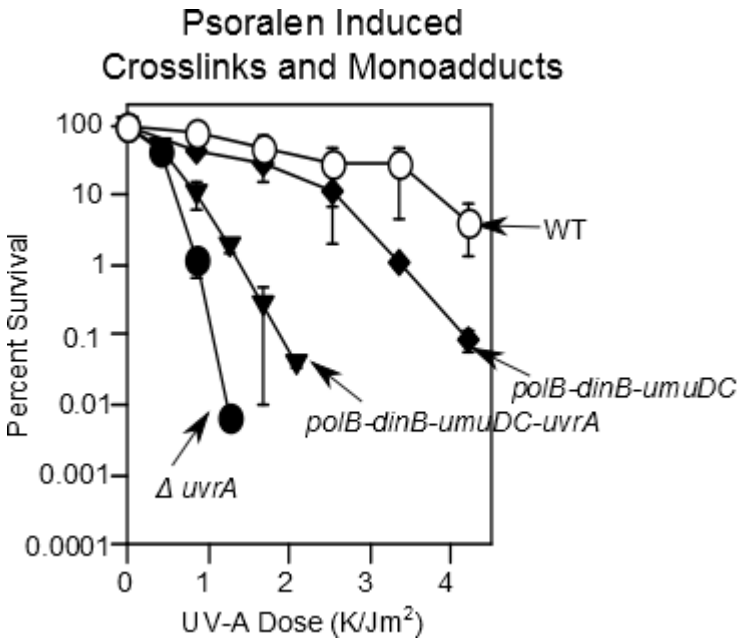

Figure 3-3: UvrA coupled with translesion polymerases increases the sensitivity for the psoralen induced lesions. The survival of cells following UV-A (340nm) irradiation in the presence of 10μg/ml 8-methoxy-psoralen is plotted. WT (open circles), *polB-dinB-umuDC* triple mutants (closed diamonds), *polB-dinB-umuDC-uvrA* quadraple mutant (closed isosceles), and *uvrA* (closed circles).Graphs represent the average of three or more independent experiments. Error bars represent one standard deviation.

I also compared the relative survival of the triple translesion polymerase mutants to that of the nucleotide excision repair, *uvrA* mutant. The hypersensitivity of the polymerase mutants was distinct from that of *uvrA* in that the polymerase mutants were not hypersensitive to low doses, and far less hypersensitive overall. This could be interpreted to suggest that that primary contribution of translesion synthesis occurs when the level of DNA damage in the cell exceeds the capacity of the other repair pathways to deal with the lesions. Curiously, the absence of the translesion polymerases modestly increased the resistance of the *uvrA* mutant, although this mutant remained highly sensitive to psoralen-induced damage (Figure 3-3). This result could indicate that the absence of incisions that initiate repair benefits survival of cells in cases where the polymerases are not present.

DISCUSSION

Current models of DNA interstrand crosslinks propose that coupling of multiple repair pathways are responsible for the repair of DNA interstrand crosslinks (Reviewed in (12)). One model suggested in the presence of replication, interstrand crosslinks are repaired by collaboration between NER and recombination proteins (7, 8, 39) . We demonstrated in our studies that both the RecF and RecBC recombination pathways are required for full resistance to psoralen-induced lesions. Although both pathways exhibited hypersensitivity, the higher sensitivity of the RecFOR pathway may suggest a role for this replication-associated recombination during the repair process. A significant limitation of this study is that 8-methoxy psoralen induces both DNA interstrand crosslinks and monoadducts. Both the *recFOR* and *recBCD* pathways are known to by

hypersensitive to a variety of agents that produce monoadducts, thus, although we can conclude that these pathways contribute to the repair of psoralen-induced damage, we cannot say whether they contribute to DNA interstrand crosslinks.

A second model of crosslink repair suggests that the repair process couples NER with translesion synthesis pathways. According to the model, translesion polymerases assist in bypassing the displaced lesion-containing oligo that remains after the NER proteins make the first incision (11, 12, 25). A prediction of this model is that the translesion polymerases should be required for the survival of cells containing DNA interstrand crosslinks similar to that of the nucleotide excision repair mutants. However, while we observed that translesion polymerases contributed to cell survival of UVA irradiated cultures treated with psoralen treated , particularly with respect to Pol V (*umuDC)*, the survival curve was distinct from that the excision repair mutants in several ways. In contrast to *uvrA*, the absence of the polymerases did not affect survival at lower levels of psoralen-induced damage, and the overall hypersensitivity of the polymerase mutants was much less than that of the *uvrA* mutants. These results would suggest that the polymerase mutants are not acting in the same pathway as nucleotide excision repair in removing these lesions as proposed. Although as noted above, we cannot distinguish between the contributions of the polymerases have in tolerating monoadducts from that of interstrand crosslinks, the differences between the *uvrA* and polymerase mutant survivals allow us to infer that they are not acting in a single linear repair pathway.

In future work, it may prove informative to compare the survival curves of these strains using both 8-methoxypsoralen and angelicin, similar to the approach used for the

Cho mutant. This may allow us to differentiate which of these pathways and genes contribute to the repair of monoadducts and DNA interstrand crosslinks.

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APPENDIX

ROLE OF BASE EXCISION REPAIR PATHWAY AND NER ACCESSORY PROTEINS IN SURVIVAL OF DNA INTERSTRAND CROSSLINKS

UvrD is the helicase associated with nucleotide excision repair. Following dual incisions by UvrABC, UvrD displaces the oligo containing the lesion (1), well as the Nucleotide Excision Repair Complex that is bound to the lesion (2). I compared the survival of *uvrD* mutants to that of wildtype and other nucleotide excision repair mutants following psoralen- and UV-induced DNA damage.

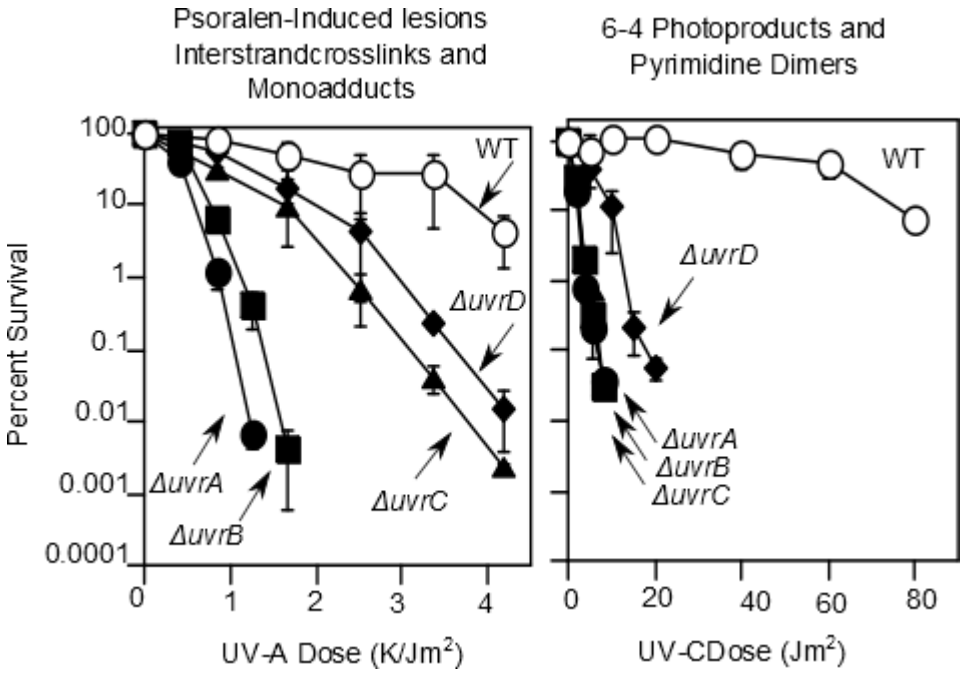

Figure 1: *uvrD* is less sensitive to psoralen- and UV-induced damage than other components of nucleotide excision repair. The survival of cells following irriadiation with (A) UV-A light (340nm) in the presence of 10μg/ml 8-methoxy-psoralen or (B) UVC light (254nm) is plotted. WT (open circles), *uvrA* (closed circles), *uvrB* (closed square), *uvrC* (closed triangles) and *uvrD* (closed diamonds).Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

Irradiation with UVA by itself is known to generate significant levels of oxygen free radicals that lead to oxidative DNA damage (3) and mutants lacking Exonuclease III (*xth*) or Endonuclease IV are hypersensitive to oxidative DNA damage (4).To assess whether the level of oxidative DNA damage generated by the irradiation conditions used in this study could be contributing to the lethality observed on our various strains, we compared the survival of *xth* and *nfo* mutants to wild type cultures.

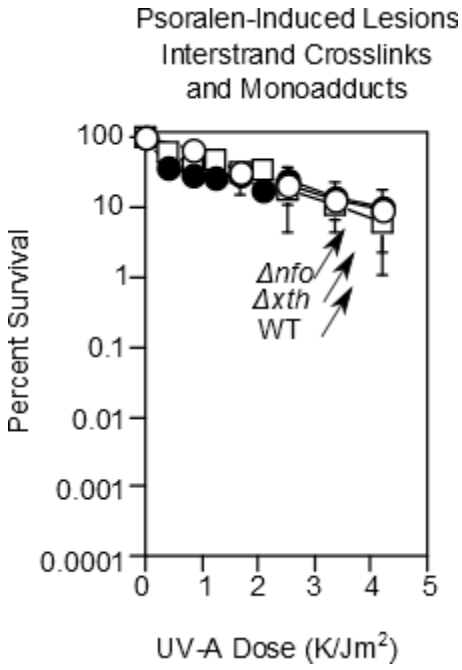

Figure 2: Under the conditions used in this study, nfo and xth mutants are as resistant to psoralen-induced damage as wildtype cultures. The survival of cells following irriadiation with UV-A light (340nm) in the presence of 10μg/ml 8-methoxy-psoralen is plotted..WT (open circles), *nfo* (closed circles) and *xth* (open square).Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

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