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Cho Endonuclease Functions During DNA Interstrand Crosslink Repair in *Escherichia coli*

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ABSTRACT

DNA interstrand crosslinks are complex lesions that covalently link both strands of the duplex DNA. Lesion removal is proposed to initiate via the UvrABC nucleotide excision repair complex, however less is known about the subsequent steps of this complex repair pathway. In this study, we characterized the contribution of nucleotide excision repair mutants to survival in the presence of psoralen-induced damage. 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*. We show that Cho, an alternative endonuclease, acts with UvrAB and is responsible for the reduced hypersensitivity of *uvrC* mutants. We find that Cho's contribution to survival correlates with the presence of DNA interstrand crosslinks, rather than monoadducts, and operates at a step after, or independent from, the initial incision during the global repair of psoralen DNA adducts from the genome.

IMPORTANCE

DNA interstrand crosslinks are complex lesions that covalently bind to both strands of the duplex DNA and whose mechanism of repair remains poorly understood. In this study, we show that Cho, an alternative endonuclease, acts with UvrAB and participates in the repair of DNA interstrand crosslinks formed in the presence of photoactivated psoralens. Cho's contribution to survival correlates with the presence of DNA interstrand crosslinks and operates at a step after, or independent from, the initial incision during the repair process.

INTRODUCTION

Psoralens are tricyclic asymmetrical compounds containing furan and pyrone rings, and bind DNA nonspecifically with a preference for pyrimidines to form noncovalent bonds (39, 40, 84). Upon absorption of UV-A light, a covalent bond forms through photoaddition between the C5=C6 double bond of the pyrimidine and the C4'=C5' furan double bond or C3'=C4' pyrone double bond of psoralen . Absorption of a second photon results in photoaddition on the remaining furan or pyrone with a second pyrimidine, creating a DNA interstrand crosslink. Thus, psoralen-induced damage consists of both monoadducts and DNA interstrand crosslinks. Because DNA interstrand crosslinks covalently bind both strands of the duplex DNA, they inhibit cellular processes that require strand denaturation, including transcription and replication. This inhibition is generally considered to be the reason for the potency of psoralens, and this class of compounds is used in treating different skin diseases such as vitiligo, psoriasis, and as a chemotherapeutic for some forms of cancer (36, 50, 73).

Several models have been proposed for DNA interstrand crosslink repair. A feature common to most 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, which function to provide an undamaged template that replaces the incised sequence. In these models, a second round of nucleotide excision repair then is able to complete the repair of DNA interstrand crosslinks (6, 16, 32). However, no intermediates for the events following the initial incision by nucleotide excision repair enzymes have been characterized or observed in vivo, and the subsequent steps in this repair pathway remain highly speculative.

Nucleotide excision repair is the primary pathway for repairing bulky DNA lesions in cells (reviewed in (78, 85). In *Escherichia coli*, the incision complex for this pathway is made up of UvrA, UvrB and UvrC. UvrA forms a homodimer that has a high affinity for damaged DNA relative to non-damaged DNA. In the presence of a distorting lesion, a complex consisting of UvrA2B specifically binds to the strand containing the lesion (71, 72). Binding of the UvrA2B complex then recruits the UvrC endonuclease which makes an initial incision located on the fourth or fifth phosphodiester bond 3' to the lesion, followed by a second incision at the eighth phosphodiester bond on the 5' side

of the adduct (65, 79, 89). After the incision step, the UvrD helicase displaces the UvrA2BC complex along with the 12-13 bp segment containing the lesion, before DNA polymerase I and ligase re-synthesize and seal this short gap using the undamaged DNA strand as a template (2, 87).

Several studies support the idea that nucleotide excision repair is involved in the repair of interstrand crosslinks (14-19). In vivo, mutants defective in any one of the nucleotide excision repair genes are hypersensitive to crosslinking agents (16, 18). Furthermore, all three nucleotide excision repair mutants are defective in their ability to incise DNA containing DNA interstrand crosslinks in vivo, as measured in alkaline CsCl gradients (16, 18). In vitro, UvrA, UvrB and UvrC are capable of and required for incising oligonucleotides or plasmids containing a psoralen-induced DNA interstrand crosslink, with dual incisions occurring predominantly on the strand containing the furan moiety (81, 82, 86, 87).

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 are required and contribute equally to DNA interstrand 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 (56, 57). While this represents a potentially important observation, it also appears to conflict with a number of earlier studies that suggest *uvrA* and *uvrB* mutants are equally sensitive to psoralen-induced DNA damage and are required for incisions to occur (11, 18, 30).

In mammalian cells, the repair of DNA interstrand crosslinks also depends upon nucleotide excision repair (53, 67, 75), however, a number of alternative nucleases have also been implicated in the repair process. XPF-ERCC1, a nuclease subunit of the nucleotide excision repair complex, is postulated to function in a replication-coupled pathway of DNA interstrand crosslink repair that is separate from its role in the general nucleotide excision repair pathway (59, 61). Other nucleases, such as MUS81-EME1, SLX1-SLX4, and FAN1 are also proposed to participate in DNA interstrand crosslink repair based upon studies using oligonucleotide or plasmid substrates in cell extracts (4, 33, 37, 49, 51, 69, 83). The molecular mechanisms and intermediates for both replication-coupled and global genomic repair pathways in eukaryotes remain

speculative. Similar to mammalian cells, *E. coli* also exhibits replication-coupled repair pathways for removing DNA damage (8, 20-23). Thus, the presence of specialized replication-coupled nucleases for DNA interstrand crosslink repair in eukaryotes raises the possibility that similar mechanisms may also operate in *E. coli*. To date however, the potential involvement of alternative nucleases operating during DNA interstrand crosslink repair in bacteria has not been explored.

To further characterize the role of nucleases in the processing and repair of DNA interstrand crosslinks, we constructed isogenic mutant strains lacking each of the nucleotide excision repair subunits and characterized their ability to survive and incise psoralen-induced DNA crosslinks in vivo. In doing so, we observed that a mutant lacking the nuclease subunit, UvrC, was less sensitive to psoralen-induced damage than the recognition proteins UvrA or UvrB. We found that an alternative endonuclease, Cho, accounts for the reduced hypersensitivity, and that Cho function contributes to the repair of DNA interstrand crosslinks at a step after, or independent from, the initial incision of psoralen crosslinks during global genomic repair of these lesions.

MATERIALS AND METHODS

Bacterial Strains. Strains used in this study are listed in Table 1.

Psoralen-UVA and Angelicin-UVA Survival Assays. Fresh overnight cultures were diluted 1:100 in Davis medium (31) supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10 µg/ml thymine (DGCthy) and grown at 37°C to an optical density at 600 nm (OD600) of 0.3. At this time, 10 µg/ml of 8-methoxypsoralen or 20 ⌈g/ml of angelicin was added to the cultures and incubation continued for 5 min. Cells were then irradiated using two 32-watt UVA bulbs (Sylvania) with a peak emittance of 320 nm at an incident dose of 6.9 J/m2/s. At the times indicated, 100-µL aliquots were removed from each culture and serially diluted in 10-fold increments. Triplicate 10-⌈l aliquots of each dilution were spotted onto Luria-Bertani agar plates supplemented with 10 ⌈g/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). An NMR spectra and an HPLC profile with/without a psoralen-injected control, provided by Sigma, revealed no detectable contamination of other prosalen derivatives in the angelicin preparation.

UVC Survival Assay. Fresh overnight cultures were diluted 1:100 in DGCthy medium and grown at 37°C to an OD600 of 0.4. Ten-⌈l 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 pBR322 was treated with 10µg/ml of 8-methoxypsoralen or 20µg/ml of angelicin and irradiated with increasing doses of UVA light. Treated plasmid DNA was digested with PvuII (Fermentas) overnight at 37°C to linearize the plasmid. Samples were electrophoresed on a 0.5% alkaline agarose gel in 30 mM NaOH, 1 mM 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). Southern blots were 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 OD600 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. 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, 10 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0]) buffer, 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 at 37°C. 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), 1mM EDTA (pH 8.0) for 45 min using 47-mm Millipore 0.025-µm pore disks. The DNA was then digested with PvuII (Fermentas) overnight at 37°C. Samples were then electrophoresed on a 0.5% alkaline agarose gel in 30 mM NaOH, 1 mM 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 as described for the in vitro plasmid crosslinking assay.

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.

$$\text{Fraction Crosslinked DNA} = (XD_{timex}/TD_{timex})-(1/2(XD_{untreated}+XD_{preirrdation})/(TD_{untreated}+TD_{preirradiation}))$$

where XD represents crosslinked DNA and TD represents total DNA.

RESULTS

Irradiation with UVC generates two predominant lesions in DNA− the cis, syn cyclobutane pyrimidine dimer and the pyrimidine 6-4-pyrimidine photoproduct (62, 63). Repair of these lesions in *E. coli* requires UvrA, UvrB and UvrC to initiate incisions of these lesions (79). Mutants lacking any of these gene products fail to remove these lesions and are equally hypersensitive to UVC irradiation (42, 43, 80), an observation that we confirmed (Fig. 1A).

To examine the contribution each of these genes has to the survival of psoralen-induced DNA damage, 10 µg/ml 8-methoxypsoralen was added to growing cultures of the parental and mutant strains before they were UVA irradiated for increasing time

periods. The fraction of cells surviving to form colonies was then determined as shown in Figure 1B. In contrast to UVC irradiation, the *uvr* genes did not contribute equally to survival of psoralen-induced lesions. *uvrC* mutants were significantly less hypersensitive and required approximately twice as much UVA irradiation to reduce the survival of cultures to levels observed in either *uvrA* or *uvrB* mutants. Importantly, no loss of viability was observed in wild-type or *uvrA* cultures treated either with UV-A irradiation alone (Fig. 1C) or when incubated with psoralen alone, indicating that the hypersensitivity and loss of viability in these cells was specific to the photoactivated forms of psoralen and not due to the intercalation of psoralen in DNA or UVA-irradiation. Thus, we observed that mutants lacking UvrC are less sensitive to psoralen-induced damage than are mutants lacking UvrA or UvrB.

While these results are consistent with most studies in the literature, they differ with one report by Lage et. al. (2010) which showed that a *uvrB5* mutant was severely sensitive to psoralen-induced DNA damage, whereas *uvrA6* and *uvrC34* mutants were nearly as resistant as their parental strain (57). The differences between our results and those of Lage et. al. could either be due to the strain backgrounds, the alleles used, or the experimental conditions, all of which differed significantly. Previous studies have reported that various strains of *E. coli* can vary significantly in their sensitivity to DNA crosslinks (12). Further, the *uvr* mutants used in the Lage et. al. study were direct isolates from nitrous acid-mutagenized cultures (41, 44), making it possible that secondary mutations occurred in these strains. Finally, whereas we treated cultures with 10 μg/ml 8-methoxypsoralen and used short UVA exposure times, the Lage et al (2010) study treated cultures with 1000-fold lower psoralen concentrations and then used long UVA exposure times to achieve lethal levels of DNA interstrand crosslinks. This could potentially lead to growth or UVA-specific effects in cultures during the irradiation period that may account for our observed differences.

In order to differentiate between these possibilities, we repeated our survival assays with the strains used in the Lage et al study. As shown in Figure 2A, we were able to reproduce their observation, demonstrating an extreme sensitivity of strain AB1885, containing the *uvrB5* mutation, to psoralen-induced DNA damage. The results argue against the idea that the observed differences are due to the experimental 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 into an isogenic SR108 background using standard P1 transduction and then repeated the experiments as before. As shown in Figure 2B, once the alleles were moved into an isogenic background, the hypersensitivity of the *uvrB5* mutant was similar to that of *uvrA6* allele. These results indicate that extreme hypersensitivity of strain AB1885 is likely due to secondary mutations that occurred in the original mutagenized isolate, rather than to a direct effect of the *uvrB5* allele. However, consistent with our initial observations in Figure 1B, the hypersensitivity of the *uvrC34* mutant was more modest than that of either *uvrA6* or *uvrB5* in all strains examined, arguing that the loss of *uvrC* is not as lethal as the loss of *uvrA* or *uvrB* when psoralen-induced DNA damage is present in the cell.

Although the UvrA, -B and -C excision is often considered to functionally act as a complex (79, 86, 87), the survival assays suggest that UvrC is dispensable for some activity carried out by UvrA and UvrB during the repair of psoralen-induced DNA damage. Considering that the UvrA and UvrB subunits contain the lesion-recognition and binding activities (86, 87), while UvrC contains the dual nuclease (60, 88), we hypothesized that an alternative endonuclease might be participating in the repair of psoralen-induced DNA damage. Cho (UvrC homolog), was initially identified as a putative nuclease that is upregulated following DNA damage (26, 34, 58). Subsequent biochemical studies showed that in the presence of the UvrAB, Cho is able to make a single 3' incision four bases further away than UvrC and that this activity could act on a variety of lesions in vitro including cyclobutane pyrimidine dimers, cholesterol, menthol, cis-platin, and 2-acetylaminofluorene adducts (66). However, its function in vivo remains unclear as *cho* mutants are not hypersensitive to UV or other forms of damage that have been examined (66). To test whether Cho was responsible for the increased resistance of *uvrC* to psoralen-induced DNA damage, we characterized *cho* mutants and *cho uvrC* double mutants for their ability to survive psoralen-induced DNA damage. As shown in Figure 3A, *cho* single mutants were only modestly sensitive to psoralen-induced DNA damage, relative to other *uvr* mutants. However, the absence of Cho increased the hypersensitivity of *uvrC* mutants to a level that was similar to *uvrA* and *uvrB* mutants

(Fig. 3A). The observation indicates that Cho accounts for the reduced sensitivity of *uvrC* mutants in the presence of psoralen-induced lesions.

8-methoxypsoralen creates both DNA monoadducts and DNA interstrand crosslinks upon exposure to the UVA light (1). 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 often reported to form exclusively monoadducts due to its angular structure (1, 3, 9, 10, 29). In contrast to 8-methoxypsoralen, in the presence of angelicin, the hypersensitivity of *uvrC* mutants was similar to that of both *uvrA* and *uvrB* mutants (Fig. 3B). Although a slight resistance remained in *uvrC* mutants relative to *uvrA* and *uvrB* mutants at high UVA doses, we believe this is likely due to a low level of DNA interstrand crosslinks forming in the angelicin-treated samples (Fig. 4B). Although angelicin is often reported to only form monoadducts, some studies have reported that low levels of DNA interstrand crosslinks can form in the presence of angelicin (35, 52). To examine this possibility, linearized plasmid DNA was treated with angelicin and UVA light in vitro, and analyzed following alkali agarose gel electrophoresis. Under denaturing conditions, DNA molecules that contain DNA interstrand crosslinks are prevented from separating and can be detected due to their slower migration pattern relative to linear single strands (47, 90). We observed that crosslinks were detectable in the angelicin-UVA-treated samples, although based on densitometric analysis they formed at a rate that was more than an order of magnitude less efficient than that of 8-methoxypsoralen (Fig. 4). Taken together, the results show that Cho's contribution to survival correlates directly with the presence and proportion of DNA interstand crosslinks, rather than monoadducts in the cell.

The contribution of Cho to crosslink repair could either occur at the initial incision step or at a later stage in the repair process. To address whether the absence of Cho affects the ability to initiate repair of crosslinks, we compared the rate that DNA interstrand crosslinks were incised in each mutant in vivo. To this end, cultures containing the plasmid pBR322 were treated with 8-methoxypsoralen and UVA light and then allowed to recover. At various times during the recovery period, aliquots of the

culture were taken, and total genomic DNA was purified and restricted with PvuII, which linearizes the plasmid. The DNA was then electrophoresed in an alkali denaturing agarose gel and the plasmid DNA forms were quantified by Southern analysis to determine the amount of unincised DNA interstrand crosslinks that remained over time. In wild-type cultures immediately following UVA irradiation, approximately 3% of the plasmid DNA contained a DNA interstrand crosslink (Fig. 5). The fraction of DNA migrating in the crosslink region of the gel decreased by more than half within the first 15 min of the recovery period and was completely removed by the end of the 90-min time course. In *uvrA* and *uvrB* mutants, approximately 6.0% of the plasmid molecules initially contained DNA interstrand crosslinks following UVA irradiation. In these cultures, the crosslinks remained throughout the recovery period and no decrease in the shifted DNA band was observed, indicating that these mutants are defective in their ability to make the initial incision. In cultures of *uvrC*, the crosslinks formed and persisted similar to that seen in *uvrA* and *uvrB* cultures. Since Cho is able to make 3' incisions in the absence of UvrC (66), the persistence of crosslinks in the *uvrC* mutant suggests that Cho is not able to efficiently incise crosslinks during global repair in vivo. In contrast, *cho* mutants remained proficient at incising crosslinks. Although the rate of incision in *cho* mutants initially occurred at a slower rate than in wild-type cells, all crosslinks were incised by the end of the 90-min time course, similar to wild-type cells. In the *uvrC cho* double mutant, DNA interstrand crosslink incision was impaired to a similar extent as observed in *uvrC* mutants.

The initial frequency of crosslinks detected in wild-type cells was lower than other strains (Fig. 5C). This is likely to be due to the incision of crosslinks occurring during the 15-min UVA irradiation period. Consistent with this interpretation, the level of initial crosslinks detected in each mutant correlated with their impaired rate of incision. Taken together, we interpret these results to indicate that although Cho participates and contributes to DNA interstrand crosslink survival, it does not appear to be essential for the initial incision step of the global repair process in vivo.

DISCUSSION

Here, we investigated the role of nucleotide excision repair proteins in repairing psoralen-induced DNA damage and show that not all subunits of the repair complex contribute equally to survival. Mutants lacking the endonucleolytic subunit, UvrC, are less sensitive than mutants lacking the recognition proteins UvrA or UvrB. The increased resistance of *uvrC* was found to depend upon Cho, a second UvrAB-dependent endonuclease that is upregulated after DNA damage (26, 34, 66). Cho's contribution to survival correlates with the presence of interstrand crosslinks in the DNA, and its absence only modestly affects the rate of the initial crosslink incision in vivo.

Many aspects of how DNA interstrand crosslinks are repaired remain speculative. Early studies using *E. coli* recognized the challenge of repairing DNA interstrand crosslinks due to the covalent attachment of this adduct to both DNA strands. Researchers inferred that repair would likely require the sequential action of multiple pathways and two related models were proposed (6, 16). At the time of these studies, both *uvrA* and *recA* had only recently been identified, and based on the hypersensitivity of these nucleotide excision repair and recombination mutants, initial models proposed that nucleotide excision repair may initiate incisions on one strand. Recombination with a sister chromosome would then provide an undamaged template to replace the incised region. A second round of incisions by nucleotide excision repair could then, in theory, complete the repair process (16, 82). Other models noted that DNA interstrand crosslinks occurring in nonreplicating cells or in unreplicated regions of the genome would not have a sister chromosome available for recombination. To account for this, a subsequent but related model was proposed in which translesion synthesis by alternative DNA polymerases would replicate across the incised oligo-lesion product to provide the template for the second round of incisions (5, 6, 55). While both of these models remain possible and are prominent in the literature today, no intermediates for the events following the initial incision have been characterized or observed in vivo.

Within the context of these models, two potential roles for Cho are apparent (Fig 6). The first possibility is that Cho could act as a secondary nuclease that increases the efficiency of the initial dual incisions by UvrA, -B and -C at psoralen-induced crosslinks. In vitro, the incision of the DNA interstrand crosslinks by the nucleotide excision repair

343 complex is influenced by the sequence context (48), and Cho has been shown to incise
344 certain bulky lesions more efficiently than UvrC (66). Such a function could be consistent
345 with the increased sensitivity of *uvrC* mutants lacking Cho (Fig. 3) as well as the
346 modestly reduced incision rate of *cho* mutants *in vivo*. However, we also observed that
347 Cho's contribution to survival correlated with the presence of DNA interstrand crosslinks
348 and not psoralen monoadducts as seen when angelicin was used in place of 8-
349 methoxypsoralen (Fig. 3 and 4). If Cho functions to enhance the initial incision at bulky
350 psoralen adducts of all classes, then one might expect it to contribute similarly to survival
351 in the presence of both 8-methoxypsoralen and angelicin, a congener that forms
352 predominantly monoadducts. Although this argues against Cho acting at the initial
353 incision step, we cannot rule out the possibility that subtle structural differences between
354 these adducts renders Cho unnecessary for incision at angular psoralens or monoadducts.
355 A second possibility is that Cho acts late during DNA interstrand crosslink repair,
356 perhaps during the second round of nucleotide excision repair that is proposed in most
357 crosslink repair models (Fig. 6C). Most models propose that after the initial incision, the
358 resulting gap is filled in by either recombination or translesion synthesis. This would
359 generate a bulky 12-basepair oligo-adduct attached to the DNA that would require a
360 second round of nucleotide excision repair to restore the integrity of the DNA. It is
361 possible that Cho is required with UvrC to make the second round of incisions on this
362 bulky substrate and allow repair to be completed. Such a function would also be
363 consistent with the increased sensitivity of *uvrC* mutants lacking Cho. Additionally, such
364 a function could also result in the observed reduction of incision rate in *cho* mutants if the
365 stalled second incision impairs the turnover rate of UvrC. A similar reduced rate of
366 incision at UV-induced pyrimidine dimers is observed in otherwise nucleotide excision
367 repair proficient cells that lack UvrD (28, 46). In the absence of the UvrD helicase, UvrC
368 is not released from the incised template and fails to turnover, slowing the overall rate of
369 repair significantly. Cho acting in this manner would explain why Cho contributes to
370 survival in the presence of DNA interstrand crosslinks but not in the presence of
371 monoadducts (Fig. 3).
372 Models for crosslink repair in eukaryotic cells suggest that a replication-
373 dependent repair pathway exists in addition to the global repair pathway (13, 54, 74, 76).

One possibility is that Cho is specifically required to make incisions in the subset of DNA interstrand crosslinks encountered by replication forks (Fig. 6D), analogous to what has been proposed for the Fanconi anemia proteins FANCD2 and SLX4/FANCP in humans (54, 77). Fanconi anemia is a rare inherited disease involving more than 15 complementation groups that predisposes patients to cancer, and renders cells hypersensitive to DNA interstrand crosslinks ((45) and references therein). Recent studies have suggested that the defect in Fanconi anemia cells specifically relates to the repair of DNA interstrand crosslinks encountered by the replication fork (70, 74). Fanconi anemia proteins FANCD2 and SLX4/FANCP interact with the 3' endonuclease of the mammalian nucleotide excision repair complex, XPF-ERCC1, to effect repair (38, 53). The participation of XPF-ECRCC1 is independent from its role in nucleotide excision repair, as the remaining subunits of the nucleotide excision repair complex are not required (59, 64). In *E. coli*, it is possible that the alternative nuclease, Cho, functions in a similar manner at the replication fork to effect repair. In vitro, incision of DNA interstrand crosslinks, but not monoadducts occurs more efficiently on underwound superhelical substrates (68). Perhaps superhelical differences in the DNA at replication forks as compared to the overall chromosome necessitate incisions by Cho rather than UvrC. A role in replication-coupled repair would also be consistent with the observation that Cho has only a minor effect on the rate of DNA interstrand crosslink incisions, since the proportion of lesions requiring replication-specific repair is small, relative to the total number of lesions in the genome (21). However, Cho, unlike UvrC, is strongly upregulated following DNA damage (26, 34). If Cho activity was specific to the lesions encountered by replication forks, one might reasonably expect that low levels of Cho expression would be sufficient to deal with these rare events. Thus, Cho's transcriptional regulation would be more consistent with a protein involved in a global repair pathway, than one specifically associated with replication. Although there is strong evidence for the presence of a replication-coupled repair pathway in *E. coli* (7, 8, 20-27), the possibility of a functionally homologous pathway to the Fanconi anemia has not been explored.

Further investigations are required to differentiate between these possibilities. It is also important to consider that few of the molecular intermediates appearing in these

models have been directly observed in vivo. Current models have been generally derived from early studies that assumed DNA interstrand crosslink repair would occur through the general nucleotide excision and recombinational mechanisms that were known at the time. It is possible that genes with functions specific for repairing this unique class of damage exist and have not been characterized. Similarly, it may be that this form of lesion is unique or rare enough such that no specific repair process exists for their repair. In either case, the models could change significantly. The reduced genome size and cellular replication and repair assays available in *E. coli* suggest that it may again provide a valuable model for identifying the basic enzymatic steps and intermediates required to complete DNA interstrand crosslink repair.

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FIGURE LEGENDS

Figure 1. In contrast to UVC-induced damage, *uvrC* mutants are less sensitive to psoralen-induced DNA adducts than either *uvrA* or *uvrB* mutants. The survival of cells following irradiation with UVC (A), UVA in the presence of 10 μg/ml 8-methoxypsoralen (B), and UVA alone (C) is plotted. Parental (squares), *uvrA* (triangles), *uvrB* (inverted triangles), and *uvrC* (circles). Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

Figure 2. The severe hypersensitivity of strain AB1885, containing the *uvrB5* allele, is likely due to secondary mutations that occurred in the original mutagenized isolate. A) The survival of the mutagenized strains AB1886 containing *uvrA6*, AB1885 containing *uvrB5*, and AB2498 containing *uvrC34* is plotted relative to the parental AB1157 following UVA irradiation in the presence of 10 μg/ml 8-methoxypsoralen. B) The survival of SR108 is plotted as in (A) after each *uvr* allele was moved into this strain by standard P1 transduction. Parental (squares), *uvrA6* (triangles), *uvrB5* (inverted triangles), *uvrC34* (circles). Graphs represent the average of three independent experiments. Error bars represent one standard deviation.

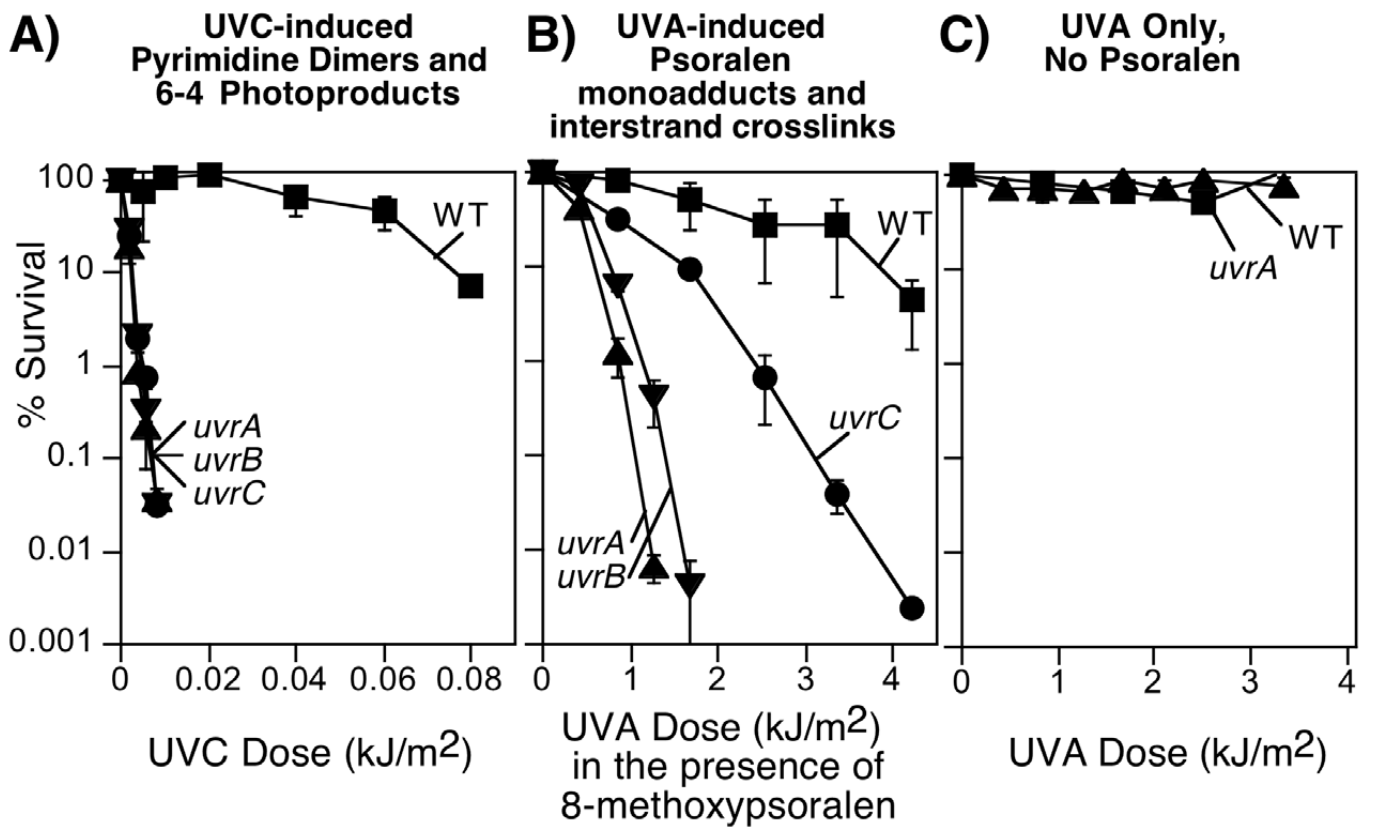
Figure 3. Cho accounts for the reduced sensitivity of *uvrC* mutants and functions predominantly in the presence of DNA interstrand crosslinks. A) The survival of cells following irradiation with UVA in the presence of (A)10 μg/ml 8-methoxypsoralen or (B) 20 μg/ml of angelicin is plotted. Parental (squares), *uvrA* (triangles), *uvrB* (inverted triangles), *uvrC* (circles), *cho* (open squares), and *uvrC cho* (open circles). Graphs represent the average of three or more independent experiments. Error bars represent one standard deviation.

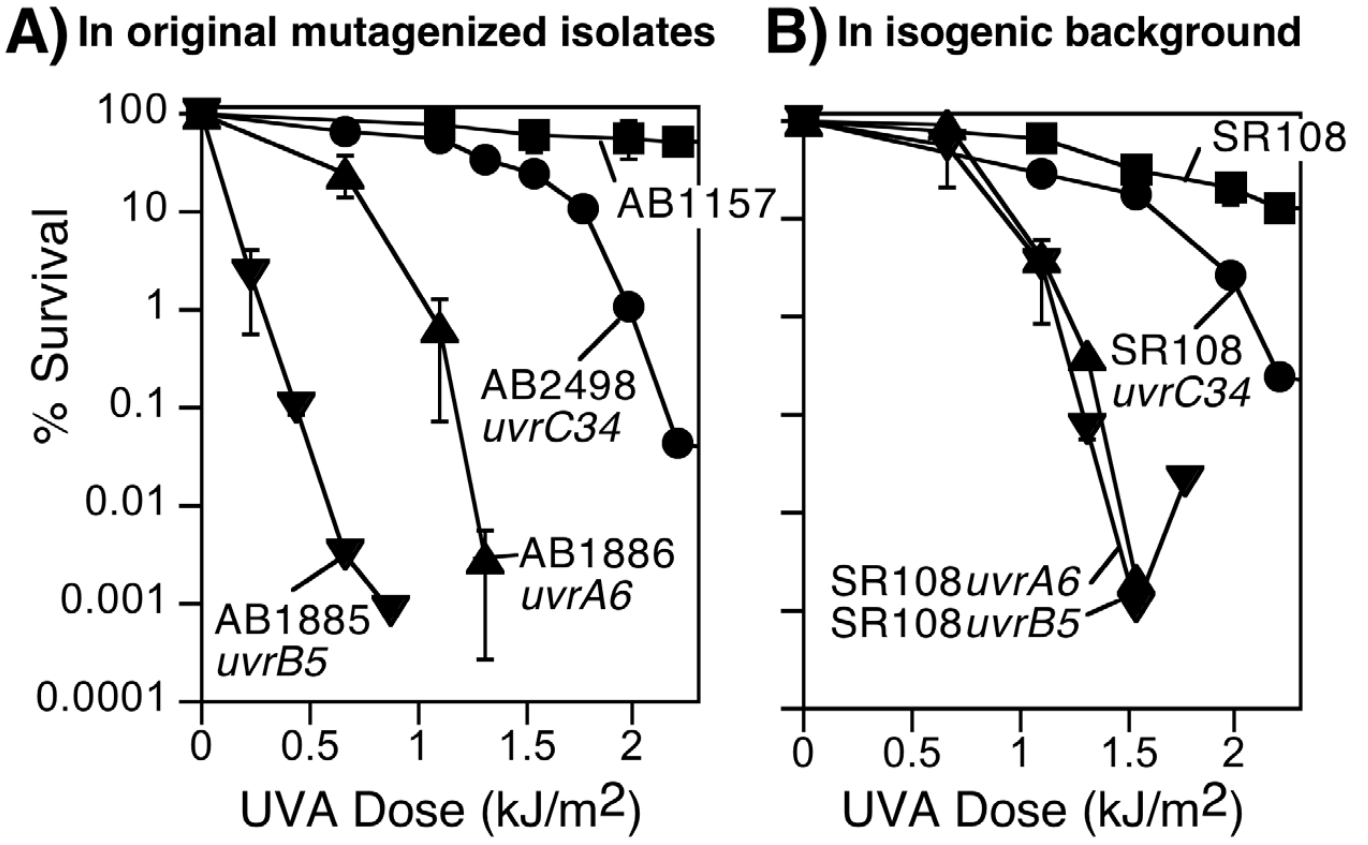
Figure 4. Low levels of DNA interstrand crosslinks are formed in DNA treated with angelicin and UVA light. Purified plasmid pBR322 was treated with (A) 10 μg/ml 8-methoxypsoralen or (B) 20 μg/ml angelicin and irradiated with increasing doses of UVA. The treated DNA was linearized by digestion with PvuII and analyzed by Southern blot following alkali-agarose gel electrophoresis. The positions of linear and crosslinked DNA are indicated. HindIII-digested lambda DNA was used as a size marker.

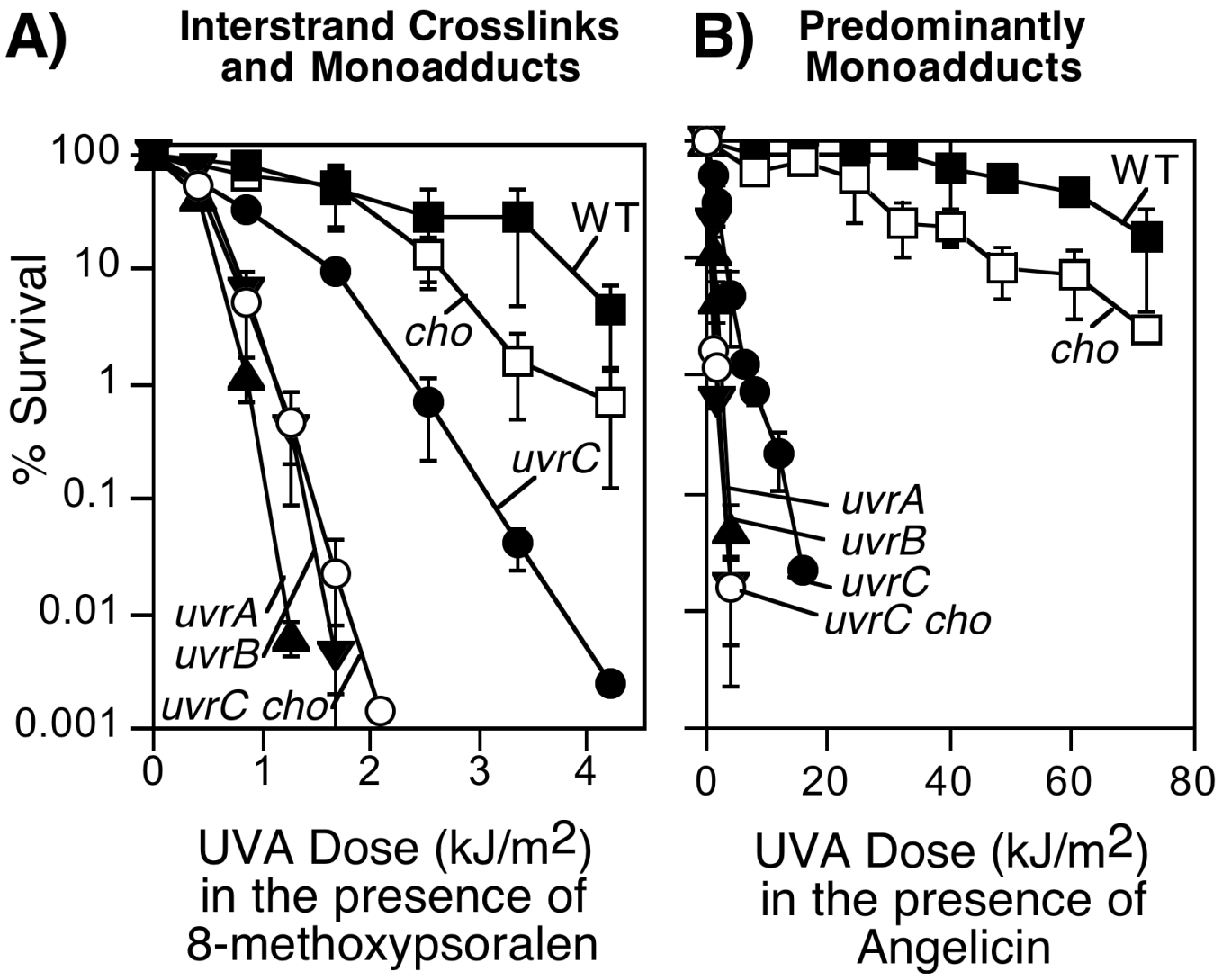
Figure 5. UvrC, but not Cho, is required for the initial incision of the crosslink *in vivo*. A) Cultures containing the plasmid pBR322 were irradiated with 6.2 kJ/m^2 UVA in the presence of 10 μg/ml 8-methoxypsoralen and allowed to recover. At the indicated times, total genomic DNA was purified, restricted with PvuII, and analyzed by Southern blot following alkali-agarose gel electrophoresis using pBR322 as a probe. Representative gels for parental, *uvrA*, *uvrB*, *uvrC*, *cho*, and *uvrC cho* strains are shown. The positions of linear and crosslinked DNA are indicated. HindIII-digested lambda DNA was used as a size marker. B) The relative amount of crosslinks remaining in the plasmid DNA over time is plotted. Parental (squares), *uvrA* (triangles), *uvrB* (inverted triangles), and *uvrC* (circles), *cho* (open squares), and *uvrC cho* (open circles). Plots represent the average of two or more independent experiments. Error bars represent the standard error of the mean. C) The percent of plasmid DNA containing interstrand crosslinks immediately after irradiation with 6.2 kJ/m^2 UVA in each strain ± the standard error of the mean is shown.

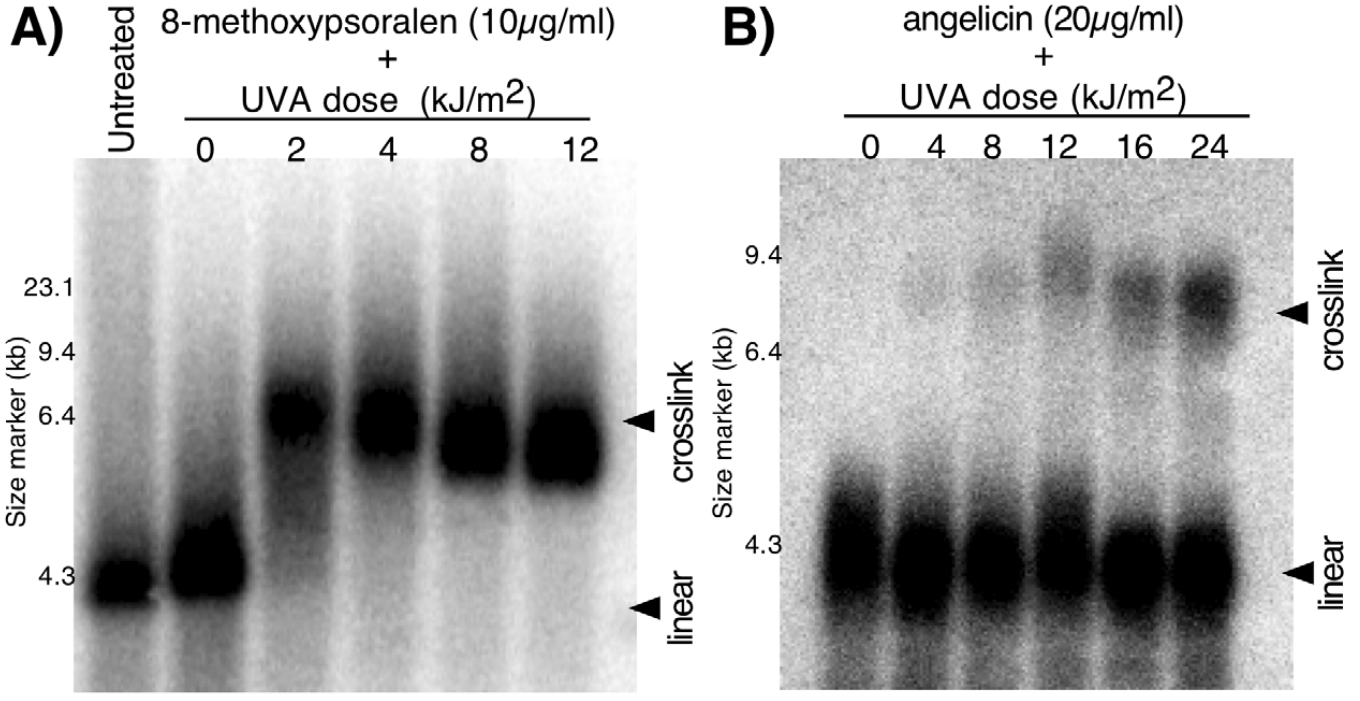
Figure 6: Potential roles for Cho during DNA interstrand crosslink repair. A) Cho is not required for monoadduct repair**.** (i)Dual incisions are made by UvrABC before (ii) the damaged region is resynthesized and ligated to complete the repair process. B) In the presence of DNA interstrand crosslinks, (i) Cho may enhance the ability of UvrABC to make the initial incisions. Current models propose that either (ii) recombination or translesion synthesis may provide a template to replace the incised region. (iii) A second round of nucleotide excision repair then removes the adduct and (iv) the template is then resynthesized and ligated to complete the repair process. C) Similar to (B) except that Cho is required for the second round of nucleotide excision repair, rather than the first

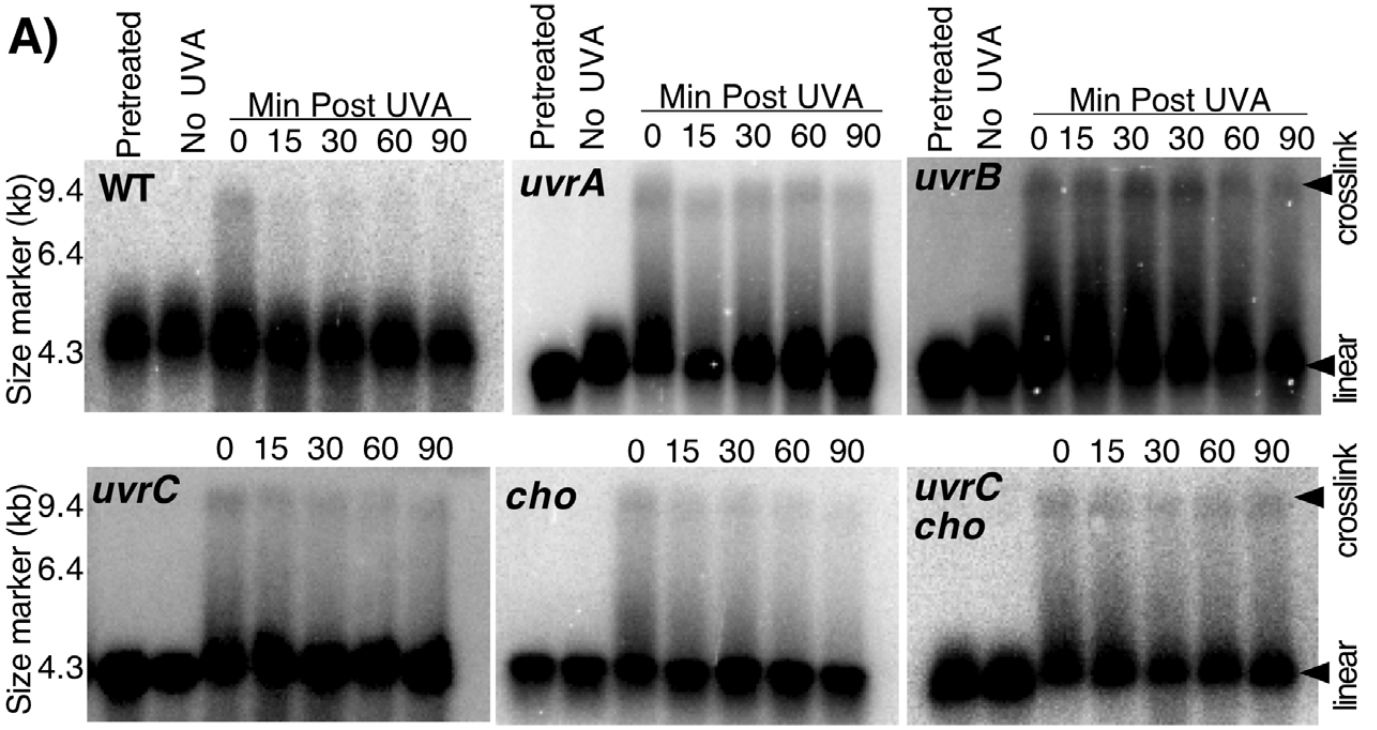
round. D) (i) Cho could function as a specialized nuclease that incises DNA interstrand crosslinks that block DNA replication. Then similar to the previous models, (ii) translesion synthesis or recombination may restore the template of the incised region so that (iii) replication can resume. (iv and v) A second round of nucleotide excision repair may then complete the repair process.

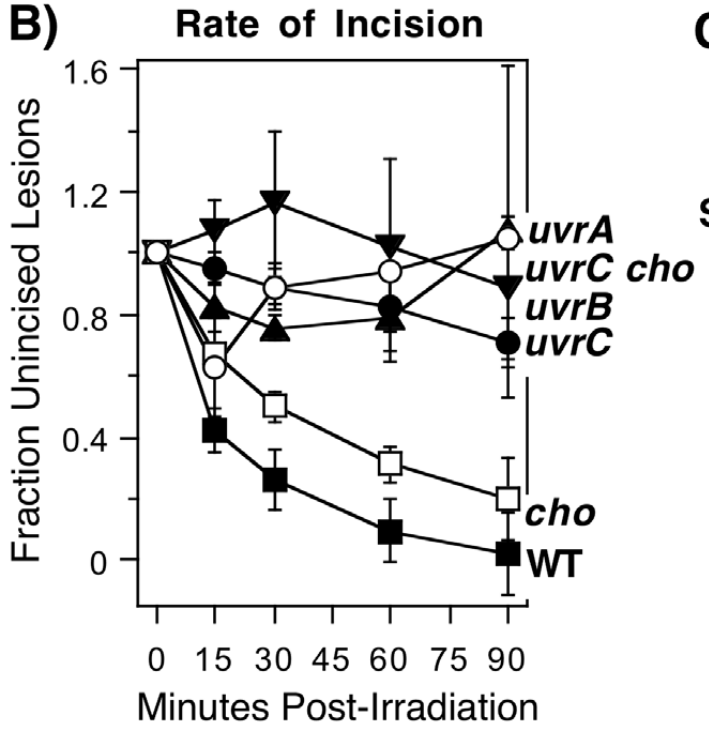

A)
UVC-induced
Pyrimidine Dimers and
6-4 Photoproducts
B)
UVA-induced
Psoralen
monoadducts and
interstrand crosslinks
C)
UVA Only,
No Psoralen
% Survival
100
10
1
0.1
0.01
0.001
WT
uvrA
uvrB
uvrC
0
0.02
0.04
0.06
0.08
UVC Dose (kJ/m2)
0
1
2
3
4
UVA Dose (kJ/m2)
in the presence of
8-methoxypsoralen
UVA Dose (kJ/m2)


A) In original mutagenized isolates
B) In isogenic background
% Survival
100
10
1
0.1
0.01
0.001
0.0001
0
0.5
1
1.5
2
UVA Dose (kJ/m2)
AB1157
AB2498
uvrC34
AB1886
uvrA6
AB1885
uvrB5
SR108
SR108
uvrC34
SR108uvrA6
SR108uvrB5


A)
Interstrand Crosslinks and Monoadducts
% Survival
100
10
1
0.1
0.01
0.001
0
1
2
3
4
WT
cho
uvrC
uvrA
uvrB
uvrC cho
UVA Dose (kJ/m2) in the presence of 8-methoxypsoralen
B)
Predominantly Monoadducts
0
20
40
60
80
WT
cho
uvrA
uvrB
uvrC
uvrC cho
UVA Dose (kJ/m2) in the presence of Angelicin


A)
Untreated
8-methoxypsoralen (10μg/ml)
+
UVA dose (kJ/m2)
0
2
4
8
12
Size marker (kb)
23.1
9.4
6.4
4.3
crosslink
linear
B)
angelicin (20μg/ml)
+
UVA dose (kJ/m2)
0
4
8
12
16
24
Size marker (kb)
9.4
6.4
4.3
crosslink
linear





C) Initial percent of plasmids containing interstrand crosslinks

Strain	%crosslinked plasmids	n expts
WT	3.2 ± 0.6	4
uvrA	5.9 ± 0.8	4
uvrB	6.1 ± 0.2	2
uvrC	4.6 ± 0.9	5
cho	4.1 ± 0.4	5
uvrC cho	5.2 ± 0.1	2

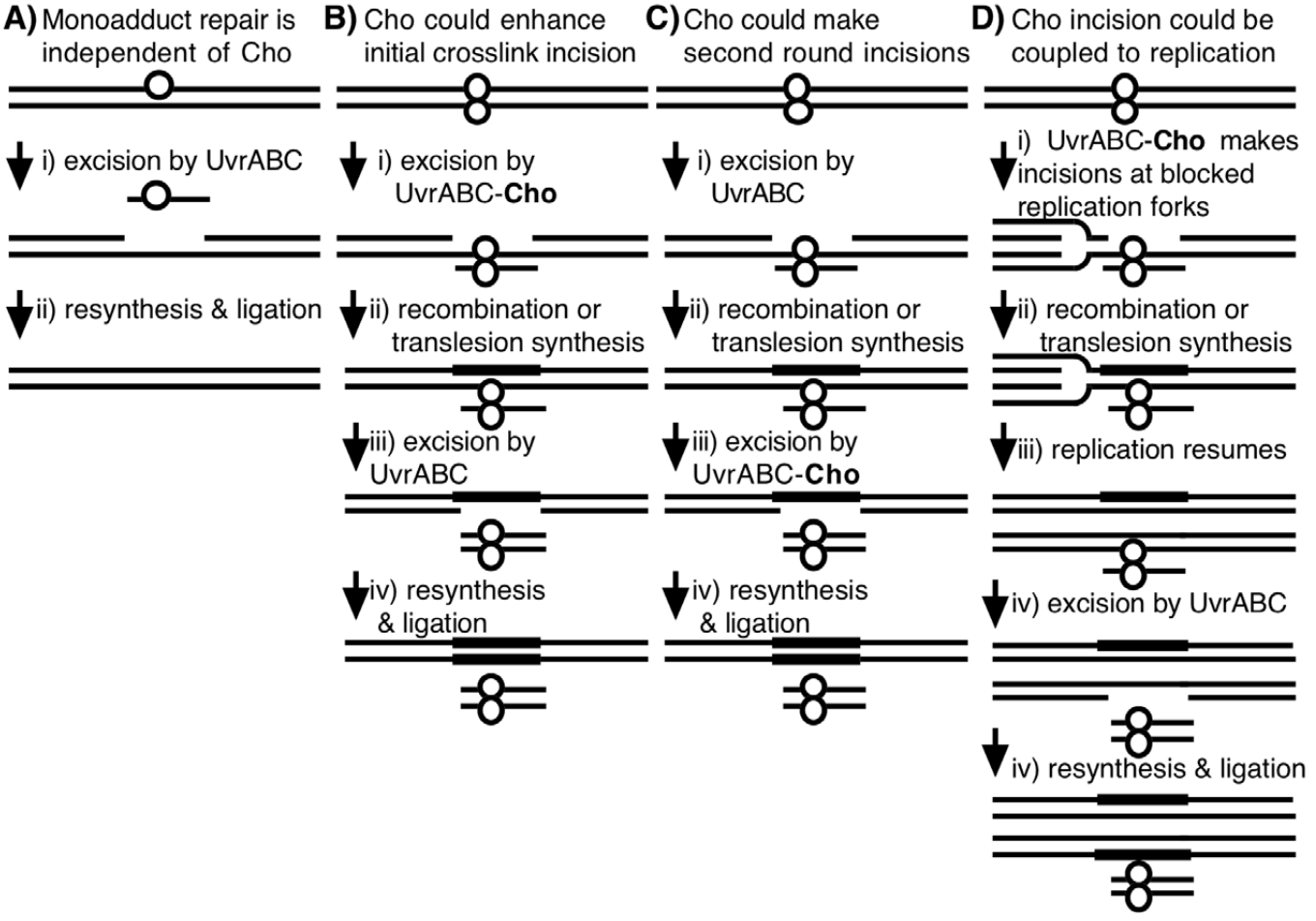

A) Monoadduct repair is independent of Cho
i) excision by UvrABC
ii) resynthesis & ligation
B) Cho could enhance initial crosslink incision
i) excision by UvrABC-Cho
ii) recombination or translesion synthesis
iii) excision by UvrABC
iv) resynthesis & ligation
C) Cho could make second round incisions
i) excision by UvrABC
ii) recombination or translesion synthesis
iii) excision by UvrABC-Cho
iv) resynthesis & ligation
D) Cho incision could be coupled to replication
i) UvrABC-Cho makes incisions at blocked replication forks
ii) recombination or translesion synthesis
iii) replication resumes
iv) excision by UvrABC
iv) resynthesis & ligation

TABLE 1

Strains used in experiments	Relevant Genotype	Source or Construction
SR108 parental	λ-, *thyA*, *deo*, IN(*rrnD-rrnE*)	trimethoprim selection of W3110 (59)
HL952	SR108 *uvrA::*Tn*10*	(22)
CL1735	SR108 Δ*uvrB*::*cat*	P1 transduction of Δ*uvrB*::*cat* from CL1673 into SR108
HL925	SR108 *uvrC297*::Tn*10*	(22)
CL908	SR108 Δ*cho*::*cat*	P1 transduction of Δ*cho*::*cat* from CL904 into SR108
HL972	SR108 *uvrA6 zjd*::Tn*5*	P1 transduction of *uvrA6 zjd*::Tn5 from HL759 (45) into SR108
CL2343	SR108 *kan-mngB uvrB5*	P1 transduction of *cat-mngB uvrB5* from CL2337 into SR108
CL2472	SR108 *uvrC34 kan-torY*	P1 transduction of *uvrC34 kan-torY* from CL2341 into SR108
CL2155	SR108 Δ*cho*::*cat uvrC297*::Tn*10*	P1 transduction of the *uvrC297*::Tn*10* allele from HL925 into CL908
AB1157	*thr-1 leuB6 proA2 his-4 argE3 thi-1 lacY1 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44 galK2*	(4)
AB1886	AB1157 *uvrA6*	Nitrous acid mutagenesis of AB1157 (39, 41)
AB1885	AB1157 *uvrB5*	Nitrous acid mutagenesis of AB1157 (39, 41)
AB2498	AB1157 *uvrC34 thy deo*	Nitrous acid mutagenesis and trimethoprim selection of AB1157 (39, 41)
Other strains used in constructions	**Genotype**	**Source or Construction**
DY329 recombineering strain	W3110 Δ*lacU169 nadA*::Tn*10 gal490* λ-Δ*cI857* Δ(*cro-bioA*)	(87)
CL904	DY329 Δ*cho*::*cat*	PCR primers 5'ggatagataaccagcattcggagtcaacagtggtacggcgATGAGACGTTGATCGGCAC3' and 5'ctcgctggtcattcgccggatcaagttcagtaatttcataCTTTCGAATTTCTGCCATTC3' were used to amplify *cat*, and the product was transformed into DY329, resulting in replacement of *cho* codons 4-280 with *cat*

CL1673	DY329 Δ*uvrB*::*cat*	PCR primers 5'attacatacctgcccgcccaactccttcaggtagcgactcATGAGACGTTGATCGGCAC3' and 5'ggctgttttccgtttgtcatcagtcttcttcgctatcctgCTTTCGAATTTCTGCCATTC3' were used to amplify *cat*, and the product was transformed into DY329, resulting in replacement of *uvrB* codons 1-672 with *cat*
CL2301	DY329 *cat-mngB*	PCR primers 5'gttaccggcttgcctgaatagcaatcaaaccgaagccacaTGTGACGGAAGATCACTTCG3' and 5'atgaacaaagcgccctttgtcaacaatctggccgcgcataACCAGCAATAGACATAAGCG3' were used to amplify *cat*, and the product was transformed into DY329, resulting in the insertion of *cat* 23bp downstream of *mngB*
CL2337	AB1885 *cat-mngB*	P1 transduction of *cat-mngB uvrB5* from CL2301 into AB1885. The *uvrB5* allele was ~50% cotransducible with *cat-mngB*
CL2280	DY329 *kan-torY*	PCR primers 5'cttagcaattaatgattacattgtaataaatcatattcttTATGGACAGCAAGCGAACCG3' and 5'cttgcataattaggcacaacactgcctgaaacaatcgataTCAGAAGAACTCGTCAAGAAG3' were used to amplify *kan*, and the product was transformed into DY329, resulting in the insertion of *kan* 106bp upstream of *torY*
CL2341	AB2498 *kan-torY*	P1 transduction of *kan-torY* from CL2280 into AB2498. The *uvrC34* allele was ~60% co-transducible with *torY*::*kan*

*Transductants were verified by antibiotic sensitivity and hypersensitivity to UVC irradiation when appropriate.