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Cho Endonuclease Functions during DNA Interstrand Cross-Link Repair in *Escherichia coli*

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ABSTRACT

DNA interstrand cross-links are complex lesions that covalently link both strands of the duplex DNA. Lesion removal is proposed to be initiated 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 cross-links, rather than monoadducts, and operates at a step after, or independently from, the initial incision during the global repair of psoralen DNA adducts from the genome.

IMPORTANCE

DNA interstrand cross-links 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 cross-links formed in the presence of photoactivated psoralens. Cho's contribution to survival correlates with the presence of DNA interstrand cross-links and operates at a step after, or independently from, the initial incision during the repair process.

Psoralens are tricyclic asymmetrical compounds containing furan and pyrone rings and bind DNA nonspecifically, with a preference for pyrimidines to form noncovalent bonds (1–3). Upon absorption of UV-A light, a covalent bond forms through photoaddition between the C-5═C-6 double bond of the pyrimidine and the C-4′═C-5′ furan double bond or C-3′═C-4′ 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 cross-link. Thus, psoralen-induced damage consists of both monoadducts and DNA interstrand cross-links. Because DNA interstrand cross-links 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 (4–6).

Several models have been proposed for DNA interstrand cross-link 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 cross-links (7–9). 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 references 10 and 11). 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 nondamaged DNA. In the presence of a distorting lesion, a complex consisting of UvrA2B specifically binds to the strand containing the lesion (12, 13). Binding of the UvrA2B complex then recruits the UvrC endonuclease, which makes an initial incision located on the fourth or fifth phosphodiester bond at the 3′ end of the lesion, followed by a second incision at the eighth phosphodiester bond on the 5′ side of the adduct (14–16). After the incision step, the UvrD helicase displaces the UvrA2BC complex along with the 12- to 13-bp segment containing the lesion, before DNA polymerase I and ligase resynthesize and seal this short gap, using the undamaged DNA strand as a template (17, 18).

Several studies support the idea that nucleotide excision repair is involved in the repair of interstrand cross-links (8, 19–23). *In vivo*, mutants defective in any one of the nucleotide excision repair genes are hypersensitive to cross-linking agents (8, 23). Furthermore, all three nucleotide excision repair mutants are defective in their ability to incise DNA containing DNA interstrand

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cross-links *in vivo*, as measured in alkaline CsCl gradients (8, 23). *In vitro*, UvrA, UvrB, and UvrC are capable of and required for incising oligonucleotides or plasmids containing a psoralen-induced DNA interstrand cross-link, with dual incisions occurring predominantly on the strand containing the furan moiety (18, 24–26).

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 cross-link 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 (27, 28). 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 (23, 29, 30).

In mammalian cells, the repair of DNA interstrand cross-links also depends upon nucleotide excision repair (31–33); 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 cross-link repair that is separate from its role in the general nucleotide excision repair pathway (34, 35). Other nucleases, such as MUS81-EME1, SLX1 to SLX4, and FAN1, are also proposed to participate in DNA interstrand cross-link repair based upon studies using oligonucleotide or plasmid substrates in cell extracts (36–42). 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 (43–47). Thus, the presence of specialized replication-coupled nucleases for DNA interstrand cross-link repair in eukaryotes raises the possibility that similar mechanisms also operate in *E. coli*. To date, however, the potential involvement of alternative nucleases operating during DNA interstrand cross-link repair in bacteria has not been explored.

To further characterize the role of nucleases in the processing and repair of DNA interstrand cross-links, we constructed isogenic mutant strains lacking each of the nucleotide excision repair subunits and characterized their ability to survive and incise psoralen-induced DNA cross-links *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 protein 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 cross-links at a step after, or independently from, the initial incision of psoralen cross-links during global genomic repair of these lesions.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Psoralen–UV-A and angelicin–UV-A survival assays. Fresh overnight cultures were diluted 1:100 in Davis medium (48) 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 (OD_{600}) of 0.3. At this time, 10 μg/ml 8-methoxypsoralen or 20 μg/ml angelicin was added to the cultures, and incubation was continued for 5 min. Cells were then irradiated using two 32-W UV-A 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). A nuclear magnetic resonance (NMR) spectrum and a high-performance liquid chromatography (HPLC) profile with and without a psoralen-injected control, provided by Sigma, revealed no detectable contamination of other psoralen derivatives in the angelicin preparation.

UV-C 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-microliter aliquots of serial 10-fold dilutions were plated in triplicate onto LBthy agar plates and UV-C irradiated at the indicated doses. UV-C irradiation used a 15-W germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/s. The plates were incubated at 37°C, and colonies were counted the next day to determine the surviving fraction.

***In vitro* plasmid cross-linking assay.** Purified plasmid pBR322 was treated with 10 μg/ml 8-methoxypsoralen or 20 μg/ml angelicin and irradiated with increasing doses of UV-A 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 and 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 >6,000 Ci/mmol [α-^{32}P]dCTP (PerkinElmer). Southern blots were visualized using a Storm 840 phosphorimager (GE Biosciences) and its associated ImageQuant analysis software.

***In vivo* interstrand cross-link incision assay.** Cultures containing the plasmid pBR322 were grown overnight at 37°C in DGCthy medium supplemented with 100 μg/ml ampicillin. A 0.2-ml aliquot from this culture was pelleted, resuspended in 20 ml of 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. The cells were then filtered and collected on Millipore 0.45-μm-pore 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 buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0]), centrifuged for 2 min, resuspended in 140 μl of lysis buffer (1 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris, 1 mM EDTA [pH 8.0]), and incubated at 37°C for 30 min. Ten microliters 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 1 mM Tris (pH 8.0) and 1 mM 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 and 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 cross-linking assay.

The fraction of psoralen cross-links 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 cross-links in untreated and preirradiated samples: fraction of cross-linked DNA = $(XD_{\text{time } x}/TD_{\text{time } x}) - 1/2[(XD_{\text{untreated}} + XD_{\text{preirradiation}})/(TD_{\text{untreated}} + TD_{\text{preirradiation}})]$, where XD represents cross-linked DNA, TD represents total DNA, and $XD_{\text{time } x}$ and $TD_{\text{time } x}$ represent the XD and TD at time *x*, respectively.

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TABLE 1 Characteristics of strains used in this study

Strain	Relevant genotype	Source, reference, and/or construction[a]
Strains used in expts		
SR108 parental	λ⁻ *thyA deo* IN(*rrnD-rrnE*)	Trimethoprim selection of W3110 (91)
HL952	SR108 *uvrA*::Tn*10*	43
CL1735	SR108 Δ*uvrB*::*cat*	P1 transduction of Δ*uvrB*::*cat* from CL1673 into SR108
HL925	SR108 *uvrC297*::Tn*10*	43
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*::Tn*5* from HL759 (92) 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*	93
AB1886	AB1157 *uvrA6*	Nitrous acid mutagenesis of AB1157 (52, 56)
AB1885	AB1157 *uvrB5*	Nitrous acid mutagenesis of AB1157 (52, 56)
AB2498	AB1157 *uvrC34 thy deo*	Nitrous acid mutagenesis and trimethoprim selection of AB1157 (52, 56)
Other strains used in constructions		
DY329 recombineering strain	W3110 Δ*lacU169 nadA*::Tn*10 gal490* λ⁻ Δ*cI857* Δ(*cro-bioA*)	94
CL904	DY329 Δ*cho*::*cat*	PCR primers 5′-GGATAGATAACCAGCATTCGGAGTCAACAGTGGTACGGCGATGAGACGTTGATCGGCAC-3′ and 5′-CTCGCTGGTCATTCGCCGGATCAAGTTCAGTAATTTCATACTTTCGAATTTCTGCCATTC-3′ 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′-ATTACATACCTGCCCGCCCAACTCCTTCAGGTAGCGACTCATGAGACGTTGATCGGCAC-3′ and 5′-GGCTGTTTTCCGTTTGTCATCAGTCTTCTTCGCTATCCTGCTTTCGAATTTCTGCCATTC-3′ 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′-GTTACCGGCTTGCCTGAATAGCAATCAAACCGAAGCCACATGTGACGGAAGATCACTTCG-3′ and 5′-ATGAACAAAGCGCCCTTTGTCAACAATCTGGCCGCGCATAACCAGCAATAGACATAAGCG-3′ were used to amplify *cat*, and the product was transformed into DY329, resulting in the insertion of *cat* 23 bp 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′-CTTAGCAATTAATGATTACATTGTAATAAATCATATTCTTTATGGACAGCAAGCGAACCG-3′ and 5′-CTTGCATAATTAGGCACAACACTGCCTGAAACAATCGATATCAGAAGAACTCGTCAAGAAG-3′ were used to amplify *kan*, and the product was transformed into DY329, resulting in the insertion of *kan* 106 bp upstream of *torY*
CL2341	AB2498 *kan-torY*	P1 transduction of *kan-torY* from CL2280 into AB2498. The *uvrC34* allele was ~60% cotransducible with *torY*::*kan*

[a] Transductants were verified by antibiotic sensitivity and hypersensitivity to UV-C irradiation, when appropriate.

RESULTS

Irradiation with UV-C generates two predominant lesions in DNA, the *cis*,*syn* cyclobutane pyrimidine dimer, and the pyrimidine 6-4-pyrimidine photoproduct (49, 50). Repair of these lesions in *E. coli* requires UvrA, UvrB, and UvrC to initiate incisions of these lesions (16). Mutants lacking any of these gene products fail to remove these lesions and are equally hypersensitive to UV-C irradiation (51–53), 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 UV-A irradiated for increasing time periods. The fraction of cells surviving to form colonies was then determined, as shown in Fig. 1B. In contrast to UV-C irradiation, the *uvr* genes did not contribute equally to the survival of psoralen-induced lesions. *uvrC* mutants were significantly less hypersensitive and required approximately twice as much UV-A irradiation to reduce the survival of cultures to levels observed in either the *uvrA* or *uvrB* mutants. Importantly, no loss of viability was observed in wild-type or *uvrA* cultures either treated 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 UV-A-irradiation. Thus, we observed that mutants lacking UvrC are less sensitive to psoralen-induced damage than are mutants lacking UvrA or UvrB.

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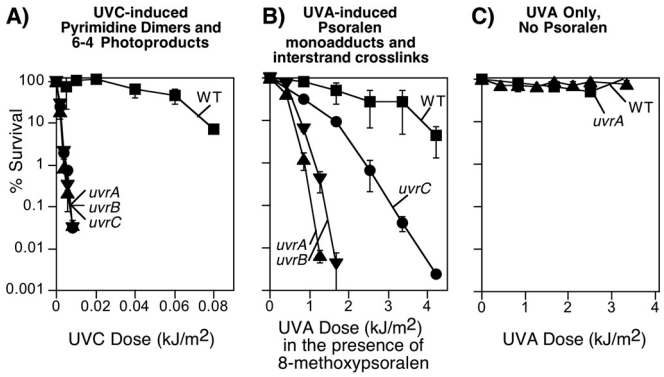

FIG 1 In contrast to UV-C-induced damage, *uvrC* mutants are less sensitive to psoralen-induced DNA adducts than either *uvrA* or *uvrB* mutants. The survival of cells following irradiation with UV-C (A), UV-A in the presence of 10 μg/ml 8-methoxypsoralen (B), and UV-A alone (C) is plotted. WT, wild type. Graphs represent the averages of the results from three independent experiments. Each error bar represents one standard deviation.

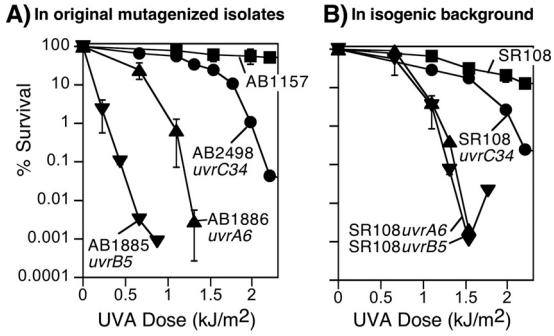

FIG 2 The severe hypersensitivity of strain AB1885, containing the *uvrB5* allele, is likely due to secondary mutations that occurred in the original mutagenized isolate. (A) The survival of the mutagenized strains AB1886 containing *uvrA6*, AB1885 containing *uvrB5*, and AB2498 containing *uvrC34* is plotted relative to the parental AB1157 following UV-A irradiation in the presence of 10 μg/ml 8-methoxypsoralen. (B) The survival of SR108 is plotted as in panel A after each *uvr* allele was moved into this strain by standard P1 transduction. Graphs represent the averages of the results from three independent experiments. Each error bar represents one standard deviation.

While these results are consistent with most studies in the literature, they differ with one report by Lage et al. (27), 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 (27). The differences between our results and those of Lage et al. (27) 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 cross-links (54). Further, the *uvr* mutants used in the Lage et al. study were direct isolates from nitrous acid-mutagenized cultures (55, 56), making it possible that secondary mutations occurred in these strains. Finally, whereas we treated cultures with 10 μg/ml 8-methoxypsoralen and used short UV-A exposure times, the Lage et al. study (27) treated cultures with 1,000-fold lower psoralen concentrations and then used long UV-A exposure times to achieve lethal levels of DNA interstrand cross-links. This could potentially lead to growth or UV-A-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 (27). As shown in Fig. 2A, we were able to reproduce their observation, demonstrating the 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 the *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 Fig. 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 the 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 Fig. 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 UvrA, UvrB, and UvrC excision is often considered to functionally act as a complex (16, 18, 26), 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 (18, 26), while UvrC contains the dual nuclease (57, 58), we hypothesized that an alternative endonuclease might be participating in the repair of psoralen-induced DNA damage. Cho (a UvrC homolog) was initially identified as a putative nuclease that is upregulated following DNA damage (59–61). 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, cisplatin, and 2-acetylaminofluorene adducts (62). However, its function *in vivo* remains unclear, as *cho* mutants are not hypersensitive to UV or other forms of damage that have been examined (62). 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 Fig. 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 that of *uvrA* and *uvrB* mutants (Fig. 3A). This 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 cross-links upon exposure to UV-A light (63). 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 has a structure similar to that of 8-methoxypsoralen but is often reported to form exclusively monoadducts due to its angular structure (63–67). In contrast to 8-methoxypsoralen, in the

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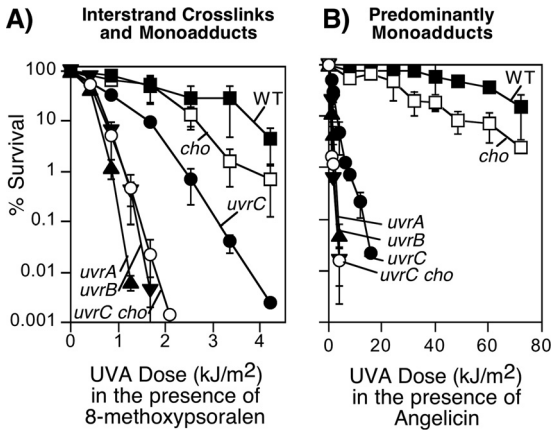

FIG 3 Cho accounts for the reduced sensitivity of *uvrC* mutants and functions predominantly in the presence of DNA interstrand cross-links. The survival of cells following irradiation with UV-A in the presence of 10 μg/ml 8-methoxypsoralen (A) or 20 μg/ml angelicin (B) is plotted. WT, wild type. Graphs represent the averages of the results from three or more independent experiments. Each error bar represents one standard deviation.

presence of angelicin, the hypersensitivity of *uvrC* mutants was similar to that of both the *uvrA* and *uvrB* mutants (Fig. 3B). Although a slight resistance remained in *uvrC* mutants relative to *uvrA* and *uvrB* mutants at high UV-A doses, we believe this is likely due to a low level of DNA interstrand cross-links forming in the angelicin-treated samples (Fig. 4B). Although angelicin is often reported to form only monoadducts, some studies have reported that low levels of DNA interstrand cross-links can form in the presence of angelicin (68, 69). To examine this possibility, linearized plasmid DNA was treated with angelicin and UV-A light *in vitro* and analyzed following alkaline agarose gel electrophoresis. Under denaturing conditions, DNA molecules that contain DNA interstrand cross-links are prevented from separating and can be detected due to their slower migration pattern relative to linear single strands (70, 71). We observed that cross-links were detectable in the angelicin–UV-A-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 interstrand cross-links, rather than monoadducts in the cell.

The contribution of Cho to cross-link repair might occur at either the initial incision step or a later stage in the repair process. To address whether the absence of Cho affects the ability to initiate the repair of cross-links, we compared the rate at which DNA interstrand cross-links were incised in each mutant *in vivo*. To this end, cultures containing the plasmid pBR322 were treated with 8-methoxypsoralen and UV-A 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 alkaline denaturing agarose gel, and the plasmid DNA forms were quantified by Southern analysis to determine the amount of unincised DNA interstrand cross-links that remained over time. In wild-type cultures immediately following UV-A irradiation, approximately 3% of the plasmid DNA contained a DNA interstrand cross-link (Fig. 5). The fraction of DNA migrating in the cross-link 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 cross-links following UV-A irradiation. In these cultures, the cross-links 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 cross-links formed and persisted in a manner similar to that seen in *uvrA* and *uvrB* cultures. Since Cho is able to make 3′ incisions in the absence of UvrC (62), the persistence of cross-links in the *uvrC* mutant suggests that Cho is not able to efficiently incise cross-links during global repair *in vivo*. In contrast, *cho* mutants remained proficient at incising cross-links. Although the rate of incision in *cho* mutants initially was lower than that in wild-type cells, all cross-links were incised by the end of the 90-min time course, similar to wild-type cells. In the *uvrC cho* double

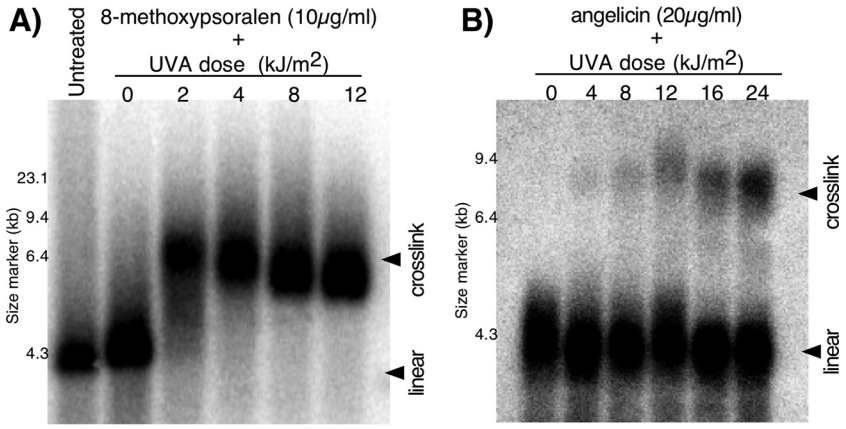

FIG 4 Low levels of DNA interstrand cross-links are formed in DNA treated with angelicin and UV-A light. Purified plasmid pBR322 was treated with 10 μg/ml 8-methoxypsoralen (A) or 20 μg/ml angelicin (B) and irradiated with increasing doses of UV-A. The treated DNA was linearized by digestion with PvuII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

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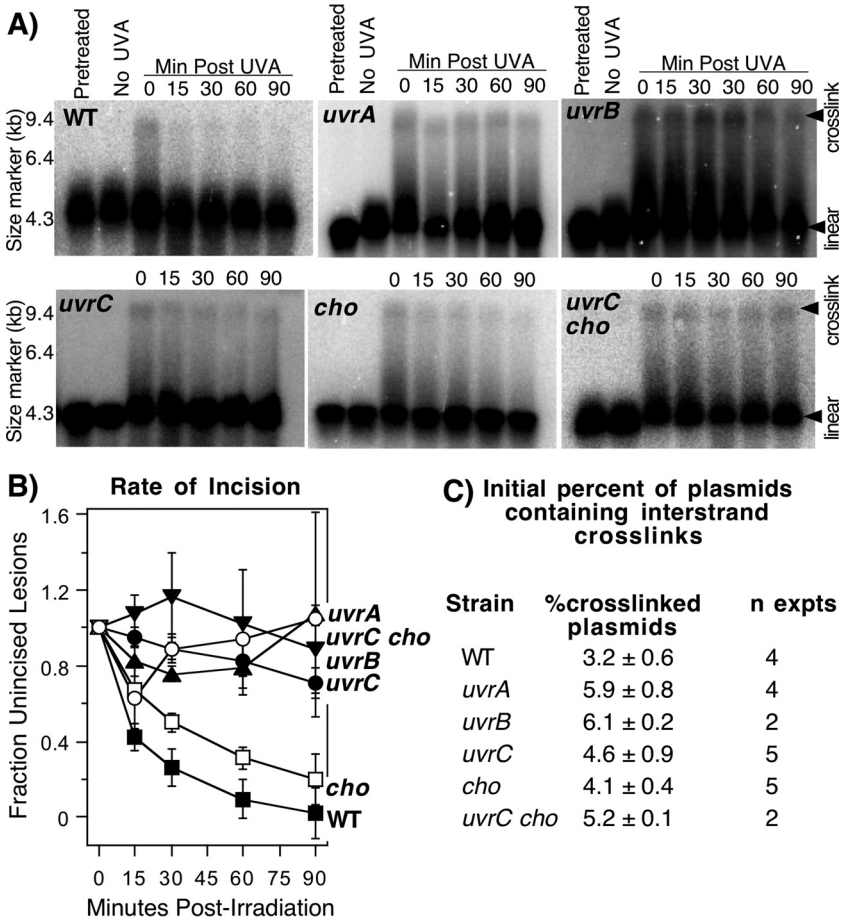

FIG 5 UvrC, but not Cho, is required for the initial incision of the cross-link *in vivo*. (A) Cultures containing the plasmid pBR322 were irradiated with 6.2 kJ/m^2 UV-A in the presence of 10 μg/ml 8-methoxypsoralen and allowed to recover. At the indicated times, total genomic DNA was purified, restricted with PvuII, and analyzed by Southern blotting following alkaline 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 cross-linked DNA are indicated. HindIII-digested lambda DNA was used as a size marker. (B) The relative amount of cross-links remaining in the plasmid DNA over time is plotted. Wild type, filled squares; *uvrA*, triangles; *uvrB*, inverted triangles; *uvrC*, filled circles; *cho*, open squares; *uvrC cho*, open circles. Plots represent the averages of the results from two or more independent experiments. Error bars represent the standard errors of the mean. (C) The percentage of plasmid DNA containing interstrand cross-links immediately after irradiation with 6.2 kJ/m^2 UV-A in each strain ± the standard error of the mean is shown. n expts, number of experiments.

mutant, DNA interstrand cross-link incision was impaired to a similar extent as that observed in *uvrC* mutants.

The initial frequency of cross-links detected in wild-type cells was lower than other strains (Fig. 5C). This is likely to be due to the incision of cross-links occurring during the 15-min UV-A irradiation period. Consistent with this interpretation, the level of initial cross-links 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 cross-link 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 protein 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 (59, 61, 62). Cho's contribution to survival correlates with the presence of interstrand cross-links in the DNA, and its absence only modestly affects the rate of the initial cross-link incision *in vivo*.

Many aspects of how DNA interstrand cross-links are repaired are speculative. Early studies using *E. coli* recognized the challenge of repairing DNA interstrand cross-links 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 (7, 8). 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 (8, 25). Other models

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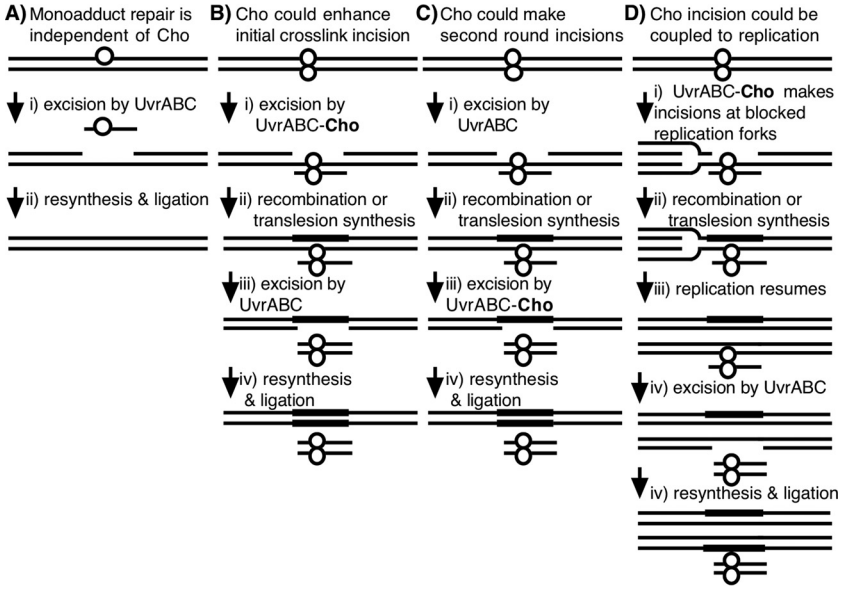

FIG 6 Potential roles for Cho during DNA interstrand cross-link repair. (A) Cho is not required for monoadduct repair. (i) Dual incisions are made by UvrABC before (ii) the damaged region is resynthesized and ligated to complete the repair process. (B) In the presence of DNA interstrand cross-links, (i) Cho may enhance the ability of UvrABC to make the initial incisions. Current models propose that (ii) either recombination or translesion synthesis provides 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 panel 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 cross-links 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.

noted that DNA interstrand cross-links 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 (7, 72, 73). 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, UvrB, and UvrC at psoralen-induced cross-links. *In vitro*, the incision of the DNA interstrand cross-links by the nucleotide excision repair complex is influenced by the sequence context (74), and Cho has been shown to incise certain bulky lesions more efficiently than UvrC (62). Such a function could be consistent with the increased sensitivity of *uvrC* mutants lacking Cho (Fig. 3) as well as the modestly reduced incision rate of *cho* mutants *in vivo*. However, we also observed that Cho's contribution to survival correlated with the presence of DNA interstrand cross-links and not psoralen monoadducts as seen when angelicin was used in place of 8-methoxypsoralen (Fig. 3 and 4). If Cho functions to enhance the initial incision at bulky psoralen adducts of all classes, one might expect it to contribute similarly to survival in the presence of both 8-methoxypsoralen and angelicin, a congener that forms predominantly monoadducts. Although this argues against Cho acting at the initial incision step, we cannot rule out the possibility that subtle structural differences between these adducts render Cho unnecessary for incision at angular psoralens or monoadducts.

A second possibility is that Cho acts late during DNA interstrand cross-link repair, perhaps during the second round of nucleotide excision repair that is proposed in most cross-link repair models (Fig. 6C). Most models propose that after the initial incision, the resulting gap is filled in by either recombination or translesion synthesis. This would generate a bulky 12-bp oligonucleotide adduct attached to the DNA that would require a second round of nucleotide excision repair to restore the integrity of the DNA. It is possible that Cho is required with UvrC to make the second round of incisions on this bulky substrate and allow repair to be completed. Such a function would also be consistent with the increased sensitivity of *uvrC* mutants lacking Cho. Additionally, such a function could also result in the observed reduction of the incision rate in *cho* mutants if the stalled second incision impairs the turnover rate of UvrC. A similar reduced rate of incision at UV-induced pyrimidine dimers is observed in otherwise nucleotide excision repair-proficient cells that lack UvrD (75, 76). In the absence of the UvrD helicase, UvrC is not released from the incised template and fails to turn over, lowering the overall rate of repair significantly. Cho acting in this manner would explain why Cho contributes to survival in the presence of DNA interstrand cross-links but not in the presence of monoadducts (Fig. 3).

Models for cross-link repair in eukaryotic cells suggest that a replication-dependent repair pathway exists in addition to the global repair pathway (77–80). One possibility is that Cho is specifically required to make incisions in the subset of DNA interstrand cross-links encountered by replication forks

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(Fig. 6D), analogous to what has been proposed for the Fanconi anemia proteins FANCD2 and SLX4/FANCP in humans (80, 81). 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 cross-links (reference 82 and references therein). Recent studies have suggested that the defect in Fanconi anemia cells specifically relates to the repair of DNA interstrand cross-links encountered by the replication fork (79, 83). Fanconi anemia proteins FANCD2 and SLX4/FANCP interact with the 3′ endonuclease of the mammalian nucleotide excision repair complex XPF-ERCC1 to effect repair (33, 84). 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 (34, 85). 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*, the incision of DNA interstrand cross-links, but not monoadducts, occurs more efficiently on underwound superhelical substrates (86). Perhaps, superhelical differences in the DNA at replication forks 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 cross-link incisions, since the proportion of lesions requiring replication-specific repair is small relative to the total number of lesions in the genome (43). However, Cho, unlike UvrC, is strongly upregulated following DNA damage (59, 61). 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* (43–47, 61, 87–90), 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 that DNA interstrand cross-link 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 cross-link repair.

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