SPECIFIC AIMS

DNA interstrand crosslinks are a particularly lethal form of DNA damage that represent an absolute block to replication and transcription. Chemicals forming crosslinks have proven to be highly toxic when found in nature, uniquely potent as chemotherapeutics in specific cancers, and effective treatments for a range of diseases states involving hyperplastic or displastic conditions.

Although several genes have been isolated that, when mutated, render cells hypersensitive to crosslinks, many aspects of how these complex lesions are repaired and processed in cells remain unknown. Much of what we know about interstrand crosslink repair has come from eukaryotic studies in extracts and suggest that both replication-dependent and replication-independent mechanisms exist. Both mechanisms are proposed to involve multiple repair pathways, coupling components of nucleotide excision repair with recombination, translesion synthesis, as well as other alternative nuclease complexes. However, after the initial incision event, all these models remain highly speculative, and are hampered by the challenges of reconstituting this multi-step, multi-pathway repair process as well as by the complexity and lack of cellular assays available in mammalian cells.

Here, we propose to directly identify the cellular pathways and structural intermediates that arise during the repair of interstrand crosslinks *in vivo* using the model organism of *E.coli*, where the processes of replication and repair are highly conserved. In *E. coli*, we have established unique cellular assays to monitor the replication fork processing and global repair for these lesions. In addition, in preliminary data, we show that we have identified an alternative endonuclease, similar to mammalian cells, that couples with the nucleotide excision repair complex and is important for crosslink repair. These assays will allow us to directly and definitively identify the repair and progressive intermediates that arise during crosslink repair *in vivo*.
We describe three aims that will be accomplished.

Aim 1. To identify the genes involved in repairing DNA interstrand crosslinks in *E. coli* and determine whether they operate in a replication-dependent or replication-independent (global genomic) repair pathway *in vivo*.

Early prokaryotic models for interstrand crosslink repair proposed coupling between nucleotide excision repair and either translesion synthesis or recombination. Since these models were first proposed, several genes have since been identified in each of these pathways that have not been characterized for their role in crosslink repair. Similarly, work in mammalian cells has identified several genes proposed to participate in either replication-dependent or replication-independent crosslink repair. Many have bacterial homologs, but have not been examined.

Aim 2. To identify the cellular intermediates and biochemical pathway associated with the replication-independent repair of DNA crosslinks *in vivo*.

We describe cellular assays, previously in our lab, to directly monitor lesion repair and visualize DNA structural intermediates that arise *in vivo* during the removal of these lesions from the genome. By comparing wild type cultures to mutants impaired in crosslink repair, we will identify the intermediates that accumulate and determine where in the pathway each gene product acts.

Aim 3. To identify the cellular intermediates and biochemical pathway associated with the replication-dependent repair of DNA crosslinks *in vivo*.

Similar to aim 2, cellular assays developed in our lab will be used to monitor the structural intermediates and genes operating at replication forks during crosslink repair ***in vivo***.

The results of these studies will identify the pathways operating in the repair of this medically relevant lesion *in vivo* and are likely to suggest novel therapeutic approaches that utilize these lesions in the treatment of cancer and other hyperproliferative diseases.

SIGNIFICANCE

DNA interstrand crosslinks are highly cytotoxic lesions that represent an absolute block to replication and transcription due to the covalent linage of the adduct to both DNA strands. Perhaps because of this property, chemicals forming crosslinks are found broadly in nature, often serving a natural defense mechanism in plant species (1-3). Others, including cis-platin, nitrogen mustard, and mitomycin C are widely used as chemotherapeutics (4), and crosslinking agents have proven to be effective treatments for a range of diseases states involving hyperplastic or displastic conditions such as psorasis and white leprosy (5-7).

Interstrand crosslinks also arise endogenously as a by-product of lipid peroxidation and aldehyde processing (8-10) and are thought to be a factor in the cellular cytotoxicity that contributes to aging in humans (11-13).

However, cells are known to develop resistance to crosslinking agents (14-20), suggesting that repair pathways exist in cells for effectively dealing with these lesions and that these pathways can be selected for or upregulated. The observations also imply that a clear understanding of how these medically relevant lesions are repaired could lead to novel targets and strategies for chemotherapeaudics and other treatments for hyerplastic diseases.

BACKGROUND

Prokaryotic models for crosslink repair

Early studies using *Escherichia coli* recognized the challenge of repairing a DNA interstrand crosslink due to the covalent attachment of this adduct to both DNA strands. Researchers inferred that repair will likely require the sequential action of multiple pathways (21-24) and two models were proposed that remain prominent in the literature today. Based on the hypersensitivity of both nucleotide excision repair, *uvrA*, and recombination mutants, *recA*, suggested coupling between these pathways. Initial models suggested that nucleotide excision repair may initiate incisions on one strand, and that recombination from 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 complete the repair process (**Fig1A**). In support of this model, biochemical studies found that the UvrABC nucleotide excision repair complex would recognize and incise one strand of a crosslink *in vitro* (25). Other studies demonstrated that RecA could promote strand

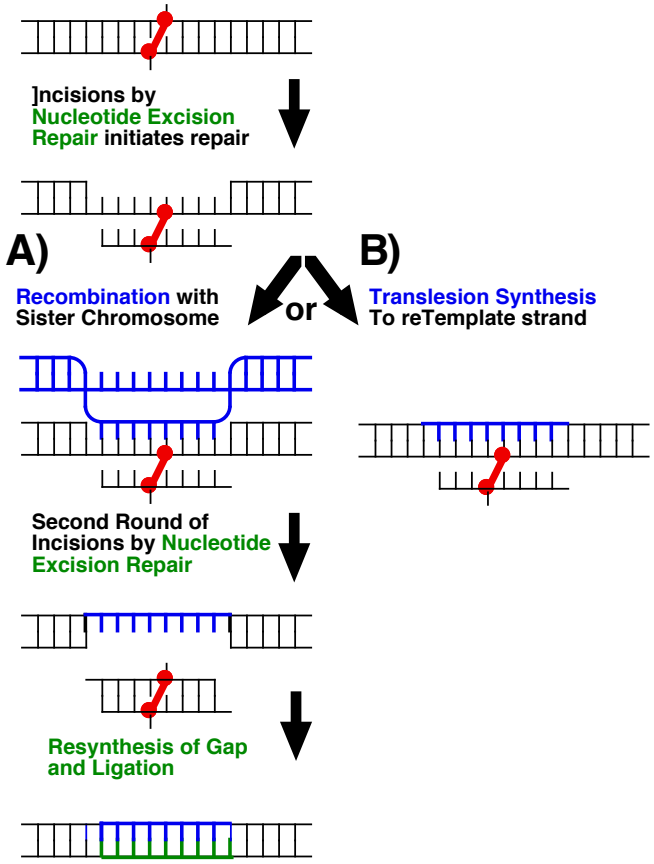

Figure 1. Early Prokaryotic Models for the Repair of DNA Interstrand Crosslinks. Following an initial incision by the Nucleotide Excision Repair Complex, the gap on the incised strand is then filled in by either A) Recombination with a sister chromosome or B) Translesion synthesis past the lesion. Nucleotide Excision Repair could then make a second round of incisions on the opposing strand, which could be filled in using the 'newly formed' complementary strand as a template.

exchange at this site *in vitro*, if the gapped region were first expanded through exonucleolytic degradation (26). Although this model could in principle repair a crosslink, it should also be noted that *uvrA* and *recA* mutants are hypersensitive to almost all forms of DNA damage, not simply crosslinks. And currently, no intermediates for the events following the initial incision have been characterized or observed *in vivo*.

Other models noted that DNA 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, it was proposed that alternative polymerases may replicate across the incised oligo-lesion product to provide a template for the second round of incisions (**Fig1B**) (27, 28). Support for this idea came from some early reports that plasmids containing an interstrand crosslink displayed reduced survival when transformed into a *polB* (Polymerase II) mutant, (28). However, a range of phenotypes were also reported for this *polB* strain (28-30), which several labs have since been unable to verify, implying the effect may have been due to secondary mutations within this particular strain (31-33). A later biochemical study also showed that Pol IV, the *dinB* gene product, could synthesize through templates containing an unhooked oligo bound crosslink *in vitro*, supporting the possibility that translesion synthesis could carry out this hypothetical step in cells (33). However, to date the potential role for the translesion synthesis during crosslink repair *in vivo* has not been systematically examined in bacteria.

Mammalian Interstrand Crosslink Repair

Much of what we have learned about crosslink repair in recent years has come from mammalian and eukaryotic studies using cell extracts. Most evidence suggests that two pathways operate to repair DNA interstrand crosslinks- those that occur when replication encounters the DNA crosslink and those that

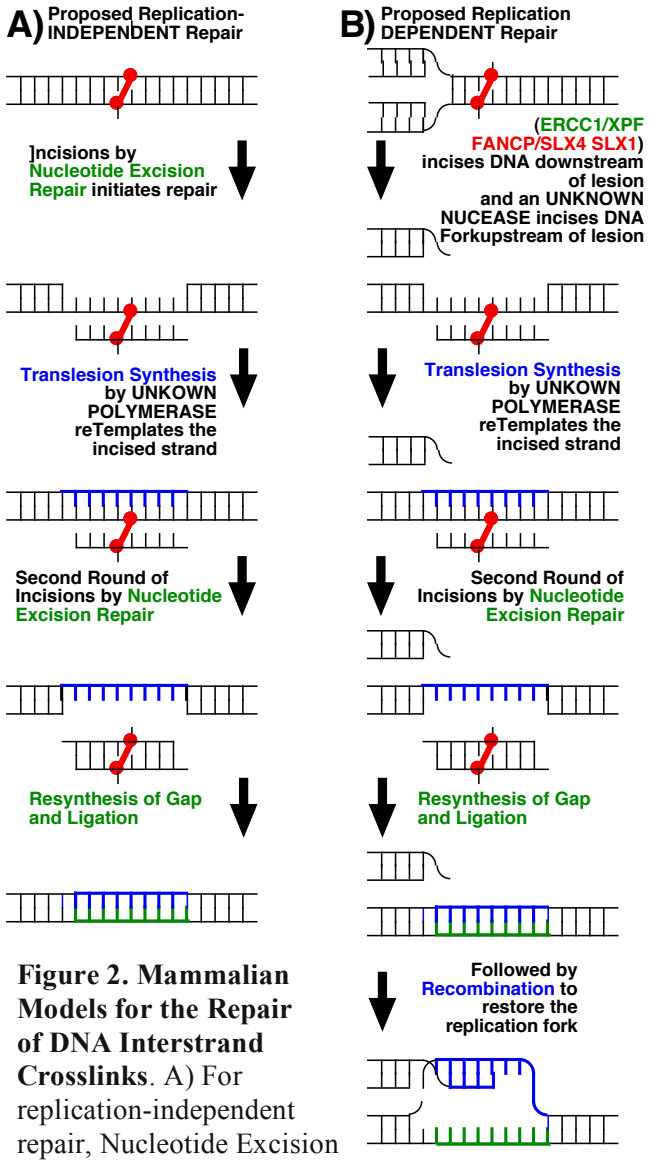

Figure 2. Mammalian Models for the Repair of DNA Interstrand Crosslinks. A) For replication-independent repair, Nucleotide Excision Repair Complex is proposed to make the initial incisions, unhooking the DNA crosslink from one strand. Then, translesion synthesis by an as yet undefined polymerase to re-Templates the incised strand by synthesizing across the lesion. Nucleotide Excision Repair could then, in principle, make a second round of incisions on the opposing strand releasing the crosslink. The gap could then be filled in and ligated to complete repair. B) For replication-dependent repair, a component of the Nucleotide Excision Repair Complex (ERCC1/XPF) is proposed to couple with FANCP/SLX4 and SLX1 or another nuclease to make incisions on one strand of the fork and downstream of the crosslink, creating a double strand break at the fork and unhooking the DNA crosslink. The crosslink could then be repaired similar to the replication-independent mechanism, before recombination restores the replication fork.

occur independent of replication (11, 14, 34-40).

Many models of replication independent crosslink repair in humans mirror those of the prokaryotic translesion synthesis model. Nucleotide excision repair is generally thought to recognize the lesion and make the initial incisions (36, 37, 39). Some evidence suggests that mismatch repair or base excision repair may also contribute to this step (37, 41-44). Following incision, translesion is thought to occur. Several of the mammalian polymerases have been implicated in the translesion synthesis step based on genetic evidence, while others have been shown to be capable of synthesizing past these lesions in vitro (19, 45-48). The remaining steps are more speculative, and are proposed to involve nucleotide excision repair or alternative, uncharacterized nucleases based on the inferred substrates that would result from translesion synthesis (14, 37-40). However, the potential role of translesion synthesis in vivo remains unclear, and no intermediates have been observed directly in cells (**Fig 2A**).

Replication-dependent repair of crosslinks in mammalian cells involves the Fanconi anemia pathway (40, 49, 50). Fanconi anemia is a rare autosomal recessive disorder associated with chromosomal instability and high sensitivity to interstrand crosslinks in cells. Patients present with congenital abnormalities, anemia, and are predisposed to a range of cancers (51-55). More than 15 Fanconi anemia complementation groups have been identified, with most genes encoding subunits of a core ubiquitin complex that serves to functionally activate and recruit the incision complex that initiates crosslink repair (56-62). The initial incisions during replication-dependent repair are unique in that they involve components of the Nucleotide excision repair complex (the ERCC1/XPF endonuclease) as well as alternative structure-specific nucleases

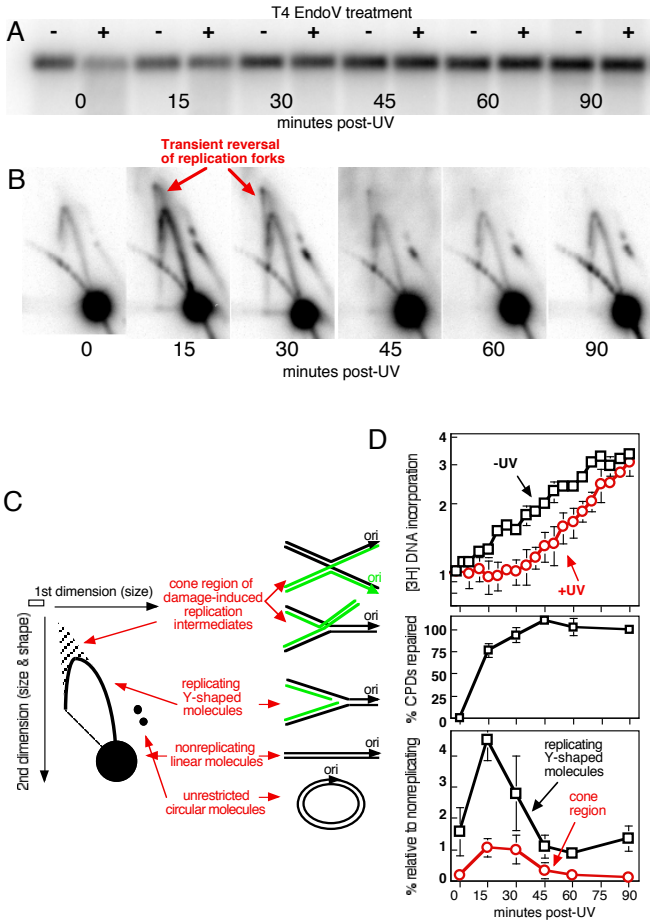

Fig 3. A transient reversal of the replication fork occurs following arrest by UV-induced DNA damage. Replication then resumes at a time when these intermediates are resolved and the blocking lesions have been repaired. A) UV-induced lesions are repaired from the plasmid within 30 minutes following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50J/m2 and genomic DNA was purified, digested with PvuII, and analyzed at the times indicated to measure the rate that the predominant UV-induced lesion, the cyclobutane pyrimidine dimer (CPD), was repaired. Lesion removal was determined by fragment sensitivity to T4 endonuclease V (TEV) which cleaves DNA containing CPDs. B) Blocked replication forks and regressed fork intermediates transiently accumulate following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50J/m2 and genomic DNA was purified, digested with PvuII, and analyzed by 2D agarose gels at the times indicate. C) Diagram of the migration pattern of PvuII digested pBR322 during 2D analysis. Nonreplicating plasmids run as a linear 4.4 kb fragment. Normal replicating fragments form Y-shaped structures and migrate more slowly due to their larger size and nonlinear shape, forming an arc that extends out from the linear fragment. Double Y, or X-shaped molecules migrate in the cone region. D) The replication intermediates persist until a time correlating with replication recovery and lesion removal. Replication recovery, lesion repair, and the relative amount of replicating fragments (squares) and cone region intermediates (circles) are plotted. Replication recovery was assayed by [3H]thymine incorporation for UV irradiated (circles) or mock-irradiated (squares) cultures.

FANCP/SLX4, MUS81–EME1, and/or FAN1 (**Fig 2B**). Although even the initial nucleases are not definitively known *in vivo* (40-43, 63-66). The dual incisions are proposed to occur on one side of the fork and downstream of the lesion, essentially creating a double strand break at the fork while simultaneously unhooking the lesion on that strand. Once this occurs, translesion synthesis is thought to be carried out by REV1 and Pol zeta or potentially other translesion polymerases, to 're-template' the incised strand (47, 48, 62, 67, 68). Similar to the replication independent mechanism, the nucleotide excision repair and homologous recombination pathways are then speculated to complete lesion removal and restore the replication fork integrity.

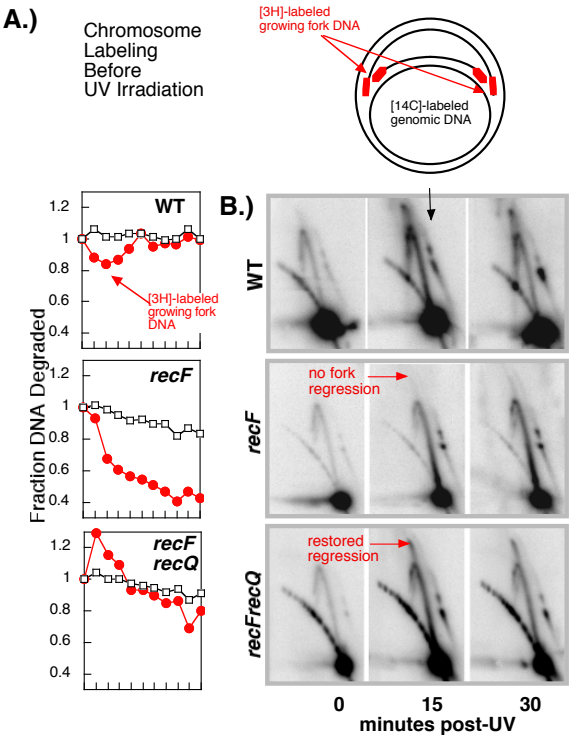

Figure 4. Following arrest at UV-induced damage, replication fork regression is catalyzed by RecF and the nascent DNA is partially degraded by RecJ/RecQ. (A) Diagram depicting the differential labeling of total (open squares) and newly synthesized DNA (filled circles) with [^{14}C]thymine and [^{3}H]thymidine, respectively. (B) The fraction of radioactivity remaining in the DNA after arrest by UV-induced damage from wild-type, *recF* and *recJ* cells is plotted over time. In the absence of *recF*, the nascent DNA at the fork is extensively degraded by the RecJ/RecQ helicase/nuclease and no regressed fork structure is observed. If RecJ or RecQ is inactivated, the nascent DNA degradation does not occur and the regressed fork structure is restored.

E. coli is uniquely suited to dissect the enzymes and intermediates that arise during repair events *in vivo*

Both the prokaryotic and mammalian models for crosslink repair are primarily inferred from the hypersensitivity of mutants, and a partial reconstitution of the initial incision event in extracts. The remaining steps and the proposed intermediates of this multistep pathway remain highly speculative, with little evidence for how these complex lesions are resolved *in vivo*. In part, this is because eukaryotic Nucleotide Excision Repair, Fanconi anemia, Recombination, Mismatch Repair, Base Excision Repair and an Active Replisome each involve dozens of proteins which hampers efforts to resonstitute the process in vitro, and in part because the complexity and lack of cellular assays in eukaryotic cells make dissection of this multi-pathway process challenging to visual *in vivo*.

This proposal addresses this question in the model organism of *E.coli*, where each of these repair pathways is highly conserved. Additionally, in previous work, our lab has developed a number of assays that we have used to effectively visualize and monitor the mechanism by which UV-induced DNA damage is processed and repaired during replication in *E. coli in vivo* (69). Many of these assays can be directly applied to examine the cellular intermediates that occur during the repair of an interstrand crosslink.

Similar to crosslinks, UV irradiation also induces lesions that block the progression of the replication machinery (70-72). The predominant lesion, the cyclobutane pyrimidine dimer (CPD) is repaired by nucleotide excision repair. To visualize the repair of the UV lesions, DNA is purified from cells at various times after UV irradiation and treated with T4 Endo V glycosylase, an enzyme

that specifically cleaves the DNA at sites containing cyclobutane pyrimidine dimers. The rate of lesion removal can then be determined by southern analysis following electrophoresis in an alkali agarose gel. As the lesions are repaired during the recovery period, the chromosome fragment becomes resistant to enzyme cleavage (**Fig 3A**). *In preliminary data, we demonstrate a similar technique for visualizing the removal of DNA interstrand crosslinks*.

Also similar to DNA crosslinks, UV damage blocks DNA replication. In the case of UV, replication arrest induces a transient regression of the replication fork prior to the resumption of replication. The DNA intermediates associated with the regression and processing of replication forks can be observed directly on plasmid molecules purified from cells following UV-induced DNA damage. The resolution of the regressed fork structure correlates with the removal of the lesions by the excision repair proteins and the recovery of replication (**Fig 3B**). In the absence of nucleotide excision repair, the lesions are not removed, the reversed fork structure persists and the recovery of DNA synthesis is severely impaired (32, 73-79). *Similar branched and recombination intermediates are proposed to occur during DNA crosslink processing and can be directly visualized and identified using a similar approach*.

In *Escherichia coli*, we demonstrated that RecA and several of the RecF pathway gene products are required catalyze the regression of the replication fork so that the lesion can be repaired and replication can resume (72, 75, 76, 78, 80). In the absence of RecF, RecO, or RecR, the arrested replication fork structure is not maintained and the DNA at the fork is extensively degraded (**Fig 4A & B**) (72, 76, 78, 80, 81). We additionally identified two proteins, RecQ a 3' -5' helicase and RecJ a 5' -3' single-strand nuclease, that are responsible for degrading the nascent lagging DNA strand of blocked replication forks at times prior to the recovery of replication (75, 76). The degradation effectively pushes the branch point of the replication fork back, restoring the region to a double-stranded form that allows repair enzymes to access the offending lesion. In the absence of this processing by RecQ-RecJ, the recovery of replication is delayed, consistent with the idea that repair enzymes cannot access the DNA lesion to effect repair (32, 79). Under these conditions, we showed that cell survival and the recovery of replication become entirely dependent on translesion synthesis

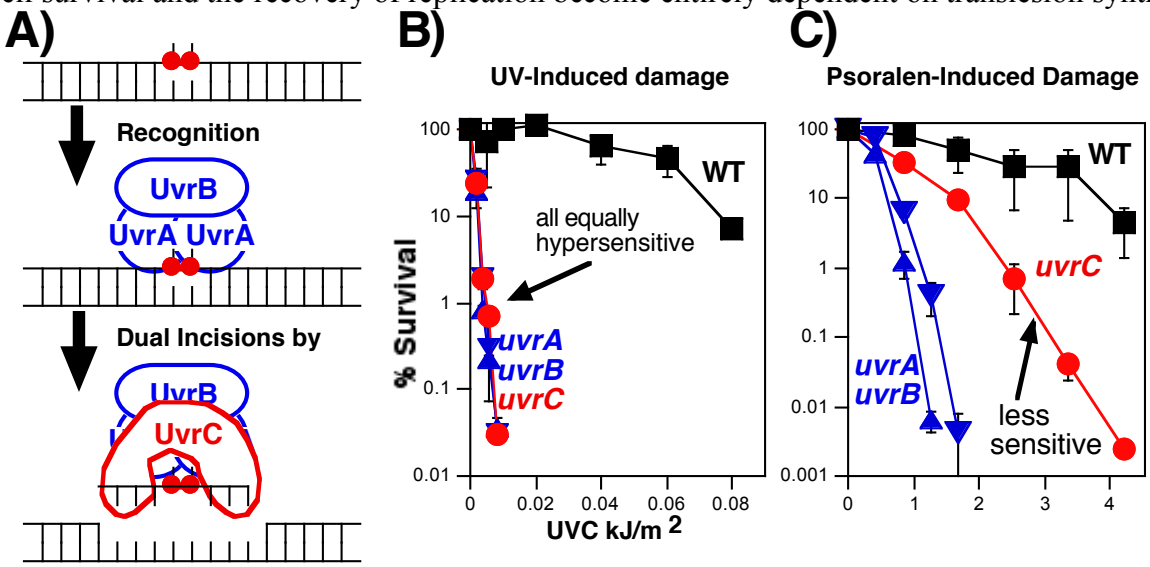

Figure 5. The UvrC endonuclease is less sensitive to psoralen, a crosslinking agent, than the recognition subunits, UvrA and UvrB, suggesting that alternative nucleases may be involved. A) Nucleotide Excision Repair model. $UvrA_2B$ act during the recognition phase of DNA damage, then recruit the UvrC endonuclease, which makes dual incisions 12 bp surrounding the lesion. B) The survival of wildtype, *uvrA*, *uvrB*, and *uvrC* mutants following UVC irradiation at the indicated dose is plotted. are all equally sensitive to UV-induced damage. C) The survival of the same strains following UVA irradiation in the presence of 10 µg/ml 8methoxypsoralen is plotted.

by polymerase V (Pol V), a process that under normal conditions does not significantly contribute to the recovery of replication (79). *Using similar assays, we expect that by examining mutants hypersensitive to DNA crosslinks, we will be able to visualize which reactions they catalyze during crosslink repair based on the intermediates that accumulate*.

These experiments show that we are able to monitor lesion removal, identify the structural intermediates on the DNA, and determine which enzymes operating at the replication fork throughout recovery from UV-induced damage. In PRELIMINARY DATA, we describe how we have identified an alternative nuclease, which similar to mammalian cells, participates specifically in the repair to interstrand crosslinks. Then, in the RESEARCH DESIGN section we elaborate in our aims how we will apply similar assays as those described above to visualize and determine the intermediates and enzymes that are necessary for repairing these complex lesions *in vivo*.

PRELIMINARY DATA

An alternative nuclease, Cho is recruited by the Nucleotide Excision Repair complex during interstrand crosslink repair in *E. coli*. In mammalian cells, alternative nucleases are thought to function with components of the nucleotide excision repair complex to initiate the repair of interstrand DNA crosslinks.

In *E.coli*, nucleotide excision repair is carried out by UvrA UvrB and UvrC, which are thought to function as a single complex that acts sequentially to repair DNA adducts (**Fig 5A**). To begin to examine how DNA interstrand crosslinks are repaired in *E. coli*, James Mendenhall, an undergraduate, established conditions and characterized the sensitivity of each of the nucleotide excision repair mutants to 8-methoxy-psoralen (8MOP), a photoactivated crosslinking agent (82-85). Surprisingly, he observed that unlike most forms of damage, mutants lacking the endonuclease subunit of the nucleotide excision repair complex, UvrC, were less sensitive to psoralen-induced damage than were other excision repair mutants (Compare **Fig 5B & C**). To explain this, Vidya Perera, a Masters student, hypothesized that since UvrC encodes the endonuclease subunit of the complex, that an alternative endonuclease might be involved in the repair of psoralen-induced damage. She turned her attention to Cho as a potential candidate. Cho, stands for UvrC homolog, and was first identified as a putative nuclease upregulated following DNA damage (86, 87). Subsequent biochemical studies showed that Cho, in the presence of UvrA B and C, can make an incision on the 5' side of the lesion, 2 bases further away than the 5' incision made by UvrC (88). Similar to Nucleotide Excision Repair complex, Cho was shown to incise a variety of DNA lesions with varying efficiency *in vitro* (88). However, no role for Cho *in vivo* has yet been described, and *cho* mutants are not sensitive to UV-induced damage.

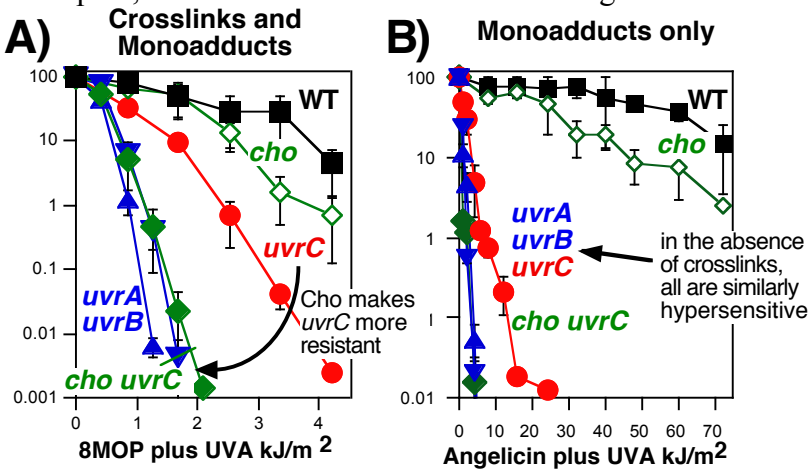

Figure 6. Cho, an altnernative endonuclease that acts with UvrABC, is required for psoralen-resistance and appears to be specific for DNA interstrand crosslinks. A) The survival of each strain following UVA irradiation in the presence of 10 µg/ml 8-methoxypsoralen is plotted. 8-methoxypsoralen forms both monoadducts and crosslinks. B) The survival of each strain following UVA irradiation in the presence of 20 µg/ml angelicin is plotted. Angelicin is a psoralen derivative that can only form monoadducts.

Vidya observed that Cho accounts for the increased resistance of *uvrC* mutants to psoralen damage, and contributes significantly to survival of crosslinks in the presence of UvrABC (**Fig 6A**). To our knowledge, this is the first significant cellular role for Cho observed *in vivo*. 8-methoxypsoralen creates both monoadducts and DNA interstrand crosslinks in the DNA, depending upon whether one or two photons are absorbed by the molecule, respectively. Since *uvrC* mutants remained partially sensitive to psoralen, Vidya hypothesized that Cho may only be required during the repair of one of these two forms of damage. To address this, Vidya used a psoralen derivative, angelicin, that is only able to form monoadducts and re-examined the sensitivity of each strain. She observed that when the psoralen treatment only produced monoadducts, the contributions from Cho were largely diminished and that *uvrA*, *uvrB* and *uvrC* mutants all exhibited similar hypersensitivities (compare **Fig 6A & B**). The observation suggests that Cho may be specifically required in the repair of DNA interstrand crosslinks, but is not required to repair monoadducts.

To further examine how Cho function may operate at DNA interstrand crosslinks, we developed a method to monitor the initial incision of the crosslink *in vivo*. To this end, cultures containing an endogenously replicating plasmid were treated with 8methoxypsoralen and irradiated at a dose that produces 1 crosslink per 72kb of DNA or ~60 lesions per genome. At this dose, approximately 40% of the cells survive to form colonies. Following irradiation, the cultures were allowed to recover and the DNA was prepared at various time points. To quantify crosslinks, the DNA was digested with a restriction enzyme that linearizes the plasmid before the samples were run in an alkali agarose gel and the plasmid fragment was detected using southern analysis (**Fig 7**). By this analysis, Vidya observes that the rate of crosslink incision is only modestly reduced in the absence of Cho. For the most part, *cho* mutants are nearly normal with respect to making the first incision during the global genome repair, *in vivo*. We hypothesize and propose to test the idea that Cho is specifically involved in the repair of crosslinks encountered during replication, similar to how FANCP/SLX4 acting with the nucleotide excision repair nuclease, XPF/ERCC1 in mammalian cells.

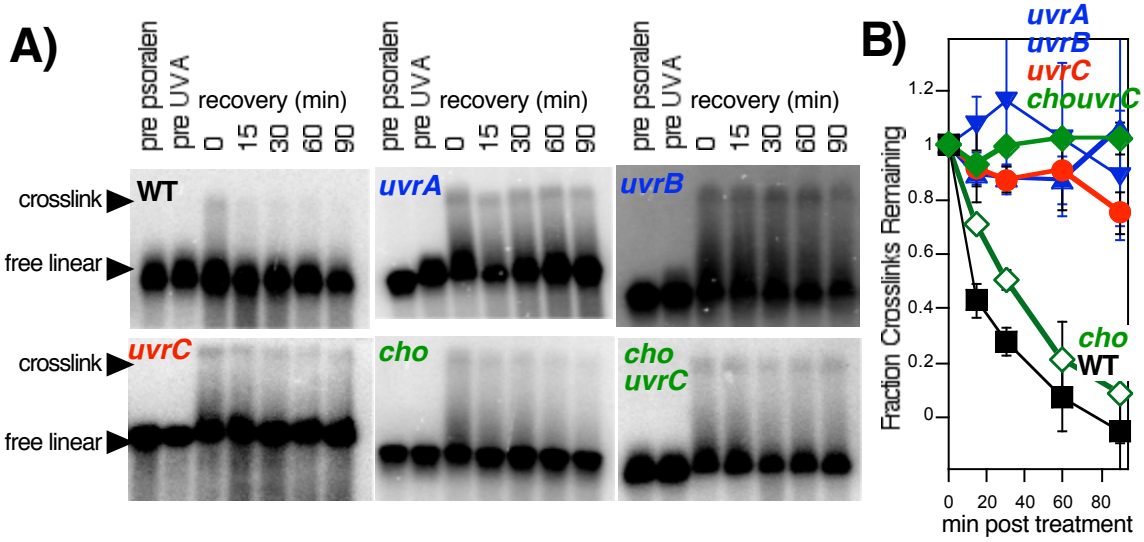

Figure 7. Despite its contribution to survival, *cho* mutants incise crosslinks in the overall genome at a rate that is only modestly reduced relative to wild type cells, potentially suggesting a replication-specific defect. A) Strains were treated with 10µg/ml 8MOP and irradiated with 3 kJ/m^2 UVA, and then allowed to recover for the indicated times. DNA was purified, digested with Pvu II. Samples were electrophoresed through an alkali-agarose gel and probed with a 4.3 kb fragment of DNA by standard Southern analysis. Fragments containing crosslinks are unable to denature and migrate more slowly as shown. B) The fraction of crosslinked DNA remaining over time is plotted. Graphs represent a minimum of three independent experiments. Error bars represent the standard error.

RESEARCH DESIGN AND METHODS

Aim 1. To identify the genes involved in DNA interstrand crosslink repair in *E. coli* and determine whether they participate in replication-dependent or -independent repair *in vivo*.

Early models for prokaryotic interstrand crosslink repair proposed coupling between nucleotide excision repair and either translesion synthesis or recombination. Since these models were first proposed, several additional genes have been identified that operate in each of these pathways, but many have not been characterized for their potential role in crosslink repair. Similarly, work in mammalian cells has identified several genes that are proposed to operate in either a replication-dependent and replication independent manner for repairing crosslinks. Many of these mammalian genes have bacterial homologs, but have not been examined to determine if they also contribute to crosslink repair in bacteria.

To examine this, we will first obtain the basic, but essential, information as to which genes are involved in crosslink repair in bacteria. As shown in preliminary data, we have already identified one novel gene product, Cho, associated with nucleotide excision repair pathway. We propose to examine candidate genes shown in **Table 1**, among others.

TABLE 1 *indicates genes that have not yet been examined for psoralen interstand crosslink sensitivity. Other genes listed below have been examined, but have not been directly compared.

REPAIR PATHWAY	CANDIDATE GENES	RATIONALE
Nucleotide excision repair	***uvrA*** (recognition), ***uvrB*** (recognition), ***uvrC*** (dual nuclease), ***cho*** (nuclease), ***uvrD**** (helicase, enzymatic turnover)	We have initiated this analysis in our preliminary data. The helicase, which is required for repairing CPDs but not 6-4 phtoproducts and is involved in enzyme turnover, has not been examined.
Translesion synthesis	***polB**** (Pol II), ***dinB**** (Pol IV), ***umuCD**** (Pol V)	Translesion synthesis is speculated to participate in both bacterial and eukaryotic crosslink repair. Yet the hypersensitivity of the each polymerase mutants has not been systematically examined in bacteria.
Recombination	***recA*** (strand exchange)***, recB-C-D*** (recombination at double strand breaks)***, recF-O-R*** (recombination at single strand gaps, branched forks)	Some models predict that repair occurs through a double strand break intermediate whereas other propose sequential rounds of gap repair. A replication-associated sensitivity of *recBC* would support a double strand break model, whereas *recF* hypersensitivity would support gap repair.
Branch Migration, DNA End Processing, Alternative Nucleases	***ruvAB**** (4-stranded branch migration)***, ruvC**** (holliday junction resolvase)***, recG**** (3-stranded branch migration)***, rusA**** (resolvase)***, xonA*, sbcCD*, recJ*, xse****	Which branch migration enzymes are involved in the repair of crosslinks may similarly differentiate between double strand break and fork processing models. *rusA* and *sbcCD* represent poorly characterized alternative nucleases, that could operate in crosslink repair similar to the FANC pathway.
Mismatch Repair	***mutS**** (recognition), ***mutL**** (recognition), ***mutH**** (strand specificity),	Mammalian mismatch repair mutants are sensitive to interstrand crosslinks. However, the repair pathway has not been examined in *E. coli*. The association between mismatch repair and replication may suggest coupling to replication if hypersensitivity is observed.
Base Excision Repair	***fpg**** (glycosylase)***, nth**** (glycosylase)***, nei**** (glycosylase), ***nfo**** (AP endo)***, xth*** (AP endo)****	Base excision repair has been reported to be involved in crosslink repair in mammalian cells. It has not been examined in *E. coli*.

We expect that several of the proposed genes will exhibit some hypersensitivity, suggesting they participate in psoralen repair in *E. coli*. These genes, along with those previously identified will then be examined to determine whether they operate in a replication-dependent or replication-independent (global repair) pathway. To differentiate between these two possibilities, we will employ two strategies, previously utilized in our lab.

The first method compares the survival of actively replicating cultures to those that lack active replication forks. To examine mutants in the absence of ongoing replication, cultures are pretreated with chloramphenicol for three hours before the antibiotic is removed and cells are exposed to the DNA damaging agent. Chloramphenicol inhibits protein synthesis, which prevents new rounds of replication from initiating in *E.coli*. We have used this method previously to show that the UV hypersensitivity of mutants in the RecF pathway arises from trying to replicate DNA in the presence of damage, indicating the repair mediated by RecF is coupled to replication **(Fig 8A)** (72). By contrast, the hypersensitivity of mutants in the nucleotide excision repair pathway remains unchanged, irrespective of the replication state of the cells **(Fig 8B)**, indicating that the repair catalyzed by these genes can occur independent of replication.

We will use this approach with the mutants previously identified to be hypersensitive to psoralen DNA damage as well as those we identify above, to determine in which of the two pathways each mutant operates to repair psoralen-induced DNA crosslinks.

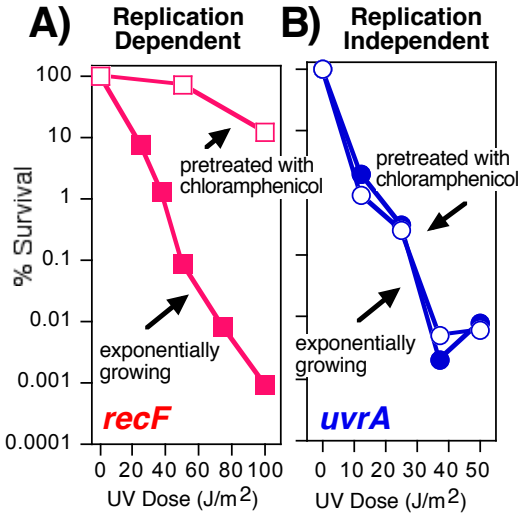

Figure 8. Differentiating between replication-dependent and replication-independent repair pathways. Chlroamphenicol inhibits protein synthesis and prevents new rounds of replication from initiating. The survival of exponentially growing cultures and cultures pretreated with chloramphenicol for 3 hours can be compared to identify genes associated with repair pathways that depend on replication. A) *recF* mutants, defective in a replication-dependent repair pathway are less sensitive to damage in the absence of active replication. B) The sensitivity of *uvrA* mutants, defective in a replication-independent repair pathway, does not change in the presence or absence of active replication. Data from (72).

In a second, alternative approach, we will examine the ability of each mutant to repair DNA crosslinks on an endogenously replicating plasmid, pSF119. This plasmid contains a ColE1 origin and is thermosensitive for replication. By comparing the repair and removal of DNA crosslinks from the plasmid in both wild type and mutants cultures at 30C (replication permissive) and 42C (replication restricted), we will be able to identify which genes operate in repair pathways that depend on active replication. This approach has advantages in that the chromosomal DNA remains able to replicate normally throughout the repair assay, and that further DNA intermediates may subsequently be identified using the analysis outlined below (See Aims 2 and 3).

We expect these results to identify and catagorize which genes in *E. coli* operate in replication-dependent and -independent pathways during the repair of interstrand crosslinks. Based on the known properties of many of these genes, their involvement is likely to suggest intermediates in the repair process. For example, the *recBC* pathway is involved in processing double strand breaks whereas the *recF* pathway is associated with processing DNA gaps and forked structures. If

the *recBC* mutants are shown to be specifically hypersensitive during replication-dependent or independent repair, it would suggest that the repair in this pathway involves a double strand break intermediate. Similarly, hypersensitivity of *recF* mutants would suggest that the repair intermediates are likely to progress though gapped or forked structures, rather than breaks. Similar inferences can be made for many of the mutants listed in **Table 1**.

Additional, more detailed information about the DNA structures, intermediates, and overall progression of the repair pathway will be revealed by examining the plasmid repair directly *in vivo* as outlined in Aims 2 and 3 below.

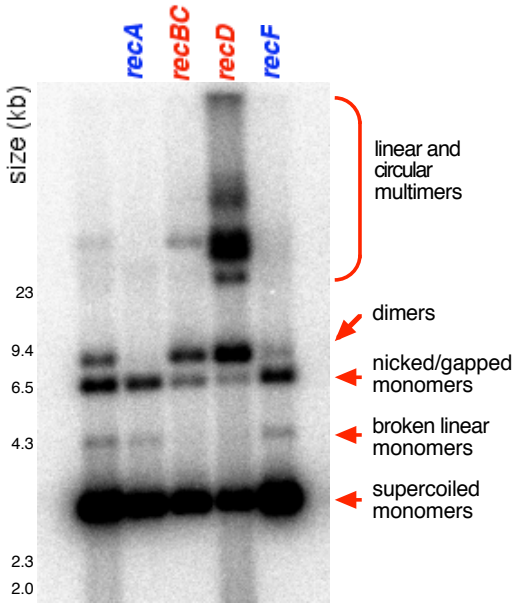

FIGURE 9. Use of neutral agarose gels to detect broken linear and gapped intermediates. Plasmids growing in E. coli mutants can be used to detect the presence of double strand breaks (broken linear molecules), gapped, and strand exchange intermediates can be detected using neutral agarose gels and southern analysis. In this example, *recBC* and *recD* mutants were shown to lack a linear intermediate associated with the completion of DNA replication (89). However, this same technique can be applied to identify the intermediates that arise during DNA crosslink repair by monitoring the intermediates observed over time following the induction of psoralen-induced damage.

Aim 2. To identify the cellular intermediates and biochemical pathway associated with the replication-independent repair of DNA crosslinks.

and

Aim 3. To identify the cellular intermediates and biochemical pathway associated with the replication-dependent repair of DNA crosslinks.

In both of aims 2 and 3, we will monitor the repair of an endogenous plasmid in *E. coli* cultures, both in the presence and absence of replication, to identify the processive intermediates and mechanism by which DNA crosslinks are repaired *in vivo*. To this end, we have developed techniques to simultaneously and quantitatively follow, over time, a range of the proposed intermediates that occur during crosslink repair. These include assays to monitor the progressive appearance of the initial incision (unhooking) of the crosslink (**Fig 7**), the presence of gapped or broken (double strand breaks) and strand exchange intermediates (**Fig 9**), as well as the formation of regressed forks, recombinational, or branched DNA structures (**Fig 10**).

To this end, time courses of the repair occurring *in vivo* will be monitored for wild type cultures, as well for mutants thought to be involved in crosslink repair. By examining wild type cultures, the progressive steps in the repair process may be partially revealed by their temporal appearance in the repair process (similar to **Fig 3**). By comparing these intermediates to those observed in mutants impaired for crosslink repair, we will be able to identify at which step in the repair process the respective gene products act based upon which intermediated are observed to accumulate (similar to **Fig 4**.

The order in which the gene products operate in the repair process can be further delineated by examining double mutants that exhibit distinct repair intermediates. As in any biochemical pathway, the intermediates associated with the gene product that operate earlier in the repair process would be predicted to accumulate in the double mutant, allowing us to order the progressive steps in the repair process.

We expect these results to distinguish between the various models for DNA interstrand crosslink repair and to identify the mechanisms by which these lesions are repaired *in vivo*. For instance, the eukaryotic replication-dependent model of crosslink repair predicts that repair proceeds through a double strand intermediate, whereas the replication-independent pathway and the prokaryotics models predict that repair occurs through processive gapped intermediates. These intermediates can be directly detected and differentiated through the use of neutral agarose gels as shown in **Fig 10**. These techniques and the use of mutants will also be expected to reveal whether hypothetical double strand breaks arise concurrently or sequentially during the repair process.

Finally, these assays can be easily adapted to examine the repair and intermediates of crosslink repair occurring on the *E. coli* chromosome by using fragments of the chromosome as probes for the southern analysis, rather than the plasmid DNA. However, we prefer to begin with the analysis of the plasmid DNA, which offers several advantages over the chromosome, including its higher copy number and ability to control replication initiation which may allow us to detect rare intermediates that may otherwise be missed on the *E. coli* chromsome.

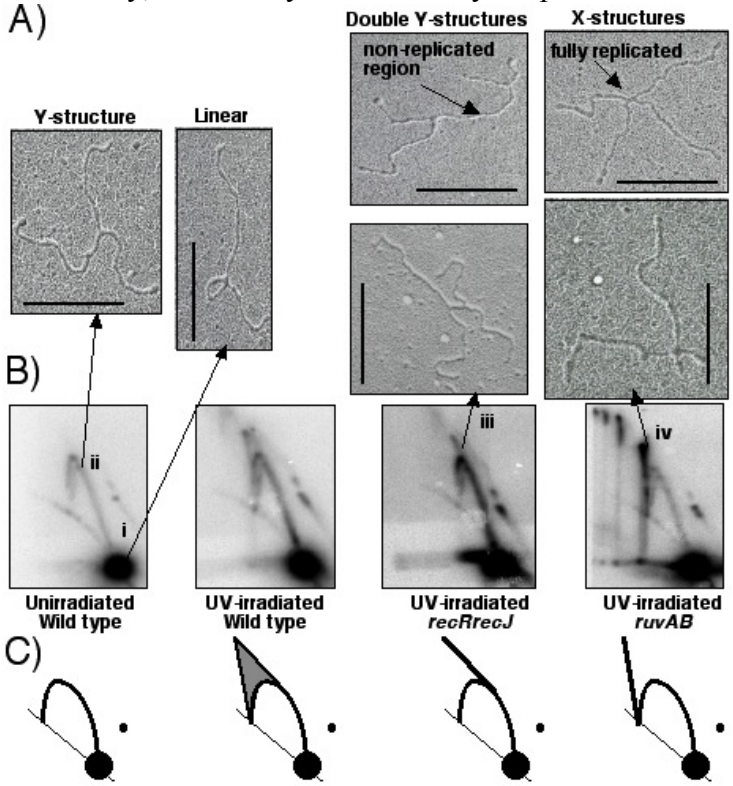

FIGURE 10. Use of 2D agarose gels can detect a variety of intermediates. A) Electron microscopic analysis of PvuII-digested pBR322 prepared from wild type cells reveals predominantly linear, nonreplicating molecules (i) and Y-shaped replication intermediates in the absence of damage (ii). Following UV irradiation, branched DNA intermediates accumulate on fully replicated molecules in ruvAB mutants (iv), whereas the branched DNA intermediates observed in recR recJ mutants contain unreplicated regions (iii). Micrographs represent the predominant DNA structures observed by transmission electron microscopy following extraction from the indicated areas of the two-dimensional gels. Scales for all micrographs represent 0.5 μm. Diagrams and two-dimensional agarose gels of PvuII-digested pBR322 in wild type, ruvAB, and recR recJ mutants are shown in (B) and (C). A similar approach can be applied to identify the intermediates that arise during the DNA crosslink repair by monitoring the intermediates observed over time following the induction of psoralen-induced damage and is likely to reveal key intermediates associated with the repair process *in vivo*.

In addition, once the initial repair intermediates are identified, it may be of use to construct plasmids containing a site-specific psoralen crosslinks. Although tranforming plasmids into cells has specific disadvantages over using endogenously replicating plasmids (90), this approach should allow us to further refine the regions where incisions occur with respect to the lesion and replication forks. We have experience in constructing site-specific UV-lesions on plasmids (90) and the construction of site-specific DNA crosslinks in plasmids was well-established in the lab where I did my PhD thesis and in the labs of Amanda McCullough and Stephen Lloyd at OHSU, with whom we hold joint lab meetings (33, 91).

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