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CHAPTER I

INTRODUCTION

Cells actively undergoing replication confront unique obstacles when faced with DNA damage. When DNA polymerase encounters a lesion on template DNA, it is often unable to pass over the damaged nucleobases, causing the replication fork to stall (Witkin, 1976). In the event that the damaged nucleobases cannot be repaired or bypassed the replication fork may collapse or the integrity of the DNA may be compromised. This event can lead to loss of viability, mutagenesis or gross chromosomal rearrangements (reviewed in Courcelle *et al.*, 2004). Following arrest of the replication fork, the DNA is processed and stabilized by recombination proteins using the homologous template strand to repair or bypass the obstruction (reviewed in Courcelle and Hanawalt, 2003). The cell's basal repair systems are often capable of handling DNA damage at low levels from normal metabolic processes and low doses of environmental mutagens before replication is disrupted. However, when higher levels of damage are incurred on an actively replicating chromosome, the cell initiates a global mechanism, known as the SOS response, to facilitate repair of the damage and restore DNA synthesis (reviewed in Kuzminov, 1995; Cox *et al.*, 2000; Courcelle, 2005). In *Escherichia coli*, RecA, a recombinase protein involved in DNA strand exchange and homologous recombination, catalyzes the SOS response (Kowalczykowski and Eggleston, 1994; d'Ari, 1985). RecA and most of the associated recombinational repair proteins are ubiquitous among prokaryotes (Kowalczykowski, 2000), and homologous proteins have also been found in higher organisms. Rad51 has been identified as a

eukaryotic protein with homologous recombinational functions to RecA in *E. coli* (Shinohara *et al.*, 1992). Regulation of this recombinase is important because recombination that is unregulated can lead to genome instability (reviewed in Courcelle *et al.*, 2004). Therefore, the genes and proteins responsible for this response mechanism are of great interest.

Induction of the SOS response mechanism in *E. coli* is initiated when RecA binds ATP and the single stranded DNA (ssDNA) created at the replication fork following encounters with DNA damage (Kuzminov, 1995). When RecA is bound to ssDNA and ATP, it becomes activated as a co-protease, inducing the autocatalytic cleavage of the LexA repressor (Little *et al.*, 1980), which is known to be responsible for the repression of approximately 30 SOS response genes, including *recA* itself (Yaguchi et al., 2011). Hence cleavage of LexA causes the up-regulation of RecA and other regulatory proteins involved in recombination repair.

The LexA-induced recombination repair is controlled by a host of regulatory proteins. Among those that negatively regulate the homologous recombination repair mechanism is RecX, a protein with a molecular weight of approximately 20 kDa (Yang *et al.*, 2009). *recX* was first identified in *E. coli* as a 366 nucleotide sequence and was originally named *oraA*, which identified its position within the open reading frame between *recA* and *alaSp* (Zaitsev *et al.*, 1994). *recX* is found directly downstream of *recA* in *E. coli*, and is co-transcribed with *recA* by read-through of a palindromic hairpin sequence found between the two genes during vegetative growth as well as under SOS conditions (Renzette *et al.*, 2007). Read through occurs at a rate of approximately 5-10% (Drees *et al.*, 2004b). Overexpression of RecX also inhibits SOS response and decreases

resistance to irradiation with ultra-violet light (Stohl *et al.*, 2002; Mishra *et al.*, 2003). RecX has been observed to alleviate the deleterious effect of RecA overexpression in *Mycobacterium smegmatis* (Papavinasasundaram *et al.*, 1998). It has been observed in *Deinococcus radiodurans* that *recX*⁻ mutants show an increased recombinational efficiency during conjugation and a higher resistance to mutagenesis induced by UV irradiation than wild-type cells, but that wild type cells appear to experience a greater level of genomic stability (Sheng *et al.*, 2005). RecX also shares a partial functional homology with the BRC repeat sequences of breast cancer type 2 susceptibility protein (BRCA2), a breast cancer gene, in its ability to disassemble nucleoprotein filaments on ssDNA (Ragone *et al.*, 2008).

To begin to elucidate the interactions between RecX, RecA and ssDNA, RecX has been structurally characterized (Galvao *et al.*, 2004; Mishra *et al.*, 2003; Ragone *et al.*, 2008; Yang *et al.*, 2009). Purified RecX has a highly positively charged surface and is capable of binding ssDNA (Ragone *et al.*, 2008). RecX interacts with RecA at it's ATP binding site, and can limit the ATPase activity of RecA (Mishra *et al.*, 2003). The 17 C-terminal amino acid residues of RecA are required for RecX to mediate the detrimental effects of RecA overexpression (Renzette *et al.*, 2007). These observations implicate the C-terminal residues in binding RecX to DNA (Drees *et al.*, 2004b).

It has been observed *in vitro* that ATP hydrolysis by RecA, as well as RecA mediated strand exchange, is impeded by the presence of RecX (Venkatesh *et al.*, 2002). It is proposed that RecX impedes RecA filament formation by a capping mechanism on the 3' end of the RecA-ssDNA complex (Drees *et al.*, 2004a). RecA nucleoprotein filaments form on ssDNA in the 5' to 3' direction, with six RecA monomers spanning

one turn of the DNA helix (Cox, 2007). Disassembly of the filament occurs in the same direction with the concomitant hydrolysis of ATP. In the proposed 3' capping mechanism model, RecX binds transient gaps in the RecA filament, blocking further extension of the filament. Dissociation of RecA monomers occurs at a constant but slow rate from the 5' end. Therefore, when RecX is bound to the 3' end, it prevents filament extension. The dissociating end eventually "catches up" to the RecX-RecA-ssDNA complex, resulting in complete dissolution of the filament.

In addition to RecA, RecX also directly interacts with RecF *in vitro* (Lusetti *et al.*, 2006), though the function of this observation has not been elucidated. During the SOS response, RecF, in conjunction with RecO and RecR, promotes RecA filament assembly and disassembly (Shan *et al.*, 1997).

Both RecA and RecF are required for replication to recover following UV-induced arrest (Courcelle and Hanawalt, 2003). In log phase *recA* mutants, genomic and DNA is almost completely degraded following UV irradiation by the RecBCD helicase/nuclease. Degradation of nascent DNA by the RecJ/RecQ helicase/nuclease is also observed in mutants lacking a functional copy of the *recA* gene (Chow and Courcelle, 2007).

Extensive DNA degradation also occurs in *recF* mutants, similar to *recA* mutants, but the degradation is predominantly limited to the nascent DNA at the arrested fork. (Chow and Courcelle, 2004). The RecF-mediated recovery of replication requires the ATPase activity and dimerization by RecF (Michel-Marks *et al.*, 2010).

Although the biochemical interactions of RecX have been characterized, their affects on DNA repair and replication recovery following DNA damage has not yet been

characterized *in vivo.* In this study, we constructed a mutant lacking the *recX* gene and characterized its UV-sensitivity, its ability to protect arrested replication forks from degradation, and its ability to recover DNA synthesis following UV irradiation. In addition, we also characterized how RecX affects recombination during conjugational events.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains

All bacterial strains used for radio-labeling with [^{14}C]-thymine and [^{3}H]-thymidine are derived from SR108 *thyA36 deoC2* derivative of W3110 (Mellon and Hanawalt, 1989). CL579 SR108 *recF6206::tetR* and CL002 *recA::tetR* have been previously described (Courcelle *et al.*, 2003; Courcelle *et al.*, 1997) CL1935 (*S*R108*recX::*kan) was constructed by standard P1 transduction of AB1157Δ*recX::kan* (Stohl *et al.*, 2003) into SR108.

For the conjugational recombination assay, Cl552, an Hfr strain of PK3 (xyl thr leu thi lac) was used as a donor (Kahn, 1968). Recipients were derived from the strain AB1157 (thr-1, ara-14, leuB6, D(gpt-proA)62, lacY1, tsx-33, supE44, galK2, lambda-, rac-,hisG4(Oc), rfbD1, mgl-51, rpsL31, kdgK51,xyl-5, mtl-1, argE3, thi-1). CL 1237 AB1157 Δ(srlR- *recA*)306::Tn10, CL1216 AB1157 *recF*6206::tet and CL1934 *AB1157ΔrecX::kan* have been previously described (Al-Hadid *et al*., 2008; Stohl *et al*.,

2003).

UV Survival

Fresh overnight cultures were diluted 1:100 in 10 mL of Davis medium supplemented with 0.4% Glucose, 0.2% Casamino acids and 10 µg/mL thymine (DGCthy medium) then grown to OD_{600} of 0.4 at 37°C with aeration. Serial dilutions were plated in triplicate on Luria-Bertani media plates supplemented with 10µg/mL thymine. Plated cultures were then UV irradiated at doses ranging from 0 to 80 J/m^2 at an incident dose of 1.0 J/m^2•s using a 254 nm GE germicidal lamp. Plates were incubated overnight at 37°C and colonies were counted the following day.

Recovery of DNA Synthesis Following UV Irradiation

The rate of incorporation or removal of the DNA nucleobase thymine in a metabolically active cell can be efficiently measured. In this study, the *E. coli* strains observed were derivatives of SR108, a mutant that lacks the thymidilate synthetase (*thyA*) gene. This gene is responsible for the production of thymidine monophosphate, the precursor of thymidine triphosphate, which is utilized during DNA replication for the incorporation of thymine nucleobases into nascent DNA strands. SR108 derivatives incubated with media enriched with radio-isotopically labeled thymine utilize these molecules for DNA replication, and the relative amount of thymine incorporated into DNA can be measured by liquid scintillation counting. More specifically, incorporation

of [^{14}C]-thymine can be used for labeling over long periods, while [^{3}H]-thymidine can be used for pulse labeling over a shorter period of time. The differences in time courses for which each reagent can be used are attributed to the linearity of incorporation, or lack thereof, of each molecule, respectively (Courcelle and Courcelle, 2006).

For analysis of the recovery of DNA synthesis following UV irradiation, fresh overnight cultures were diluted 1:100 in 50 mL DGCthy media, containing 0.1 μCi/mL [^{14}C]-thymine. Cultures were then grown to OD_{600} 0.3 at 37°C with aeration. At this time, half of the culture was irradiated with 30 J/m^2 and the other half was mock irradiated. At the times indicated, duplicate 0.5 aliquots of irradiated and mock-irradiated culture were pulsed with 0.5 μCi/mL [^{3}H]-thymidine for 2 minutes at 37°C in a shaking water bath, then lysed in ice-cold 5% trichloro-acetic acid. The DNA was then captured on 0.45 μm glass fiber filters. The amount of ^{3}H and ^{14}C was quantified by liquid scintillation counting.

Nascent DNA Degradation

Fresh overnight cultures were diluted 1:100 into 10 mL DGCthy media containing 0.1μCi/mL [^{14}C]-thymine. Cultures were grown to OD_{600} of 0.4 in a 37° shaking water bath. Cells were then labeled with 1μCi/mL [^{3}H]-thymidine, for seven seconds and vacuum filtered through a 0.45μm general nylon membrane. Cells were washed with 3 mL of 1X NET (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 8), and then re-suspended in 10 mL of non-radioactive pre-warmed 37° DGCthy media. Cultures were immediately irradiated with 30 J/m^2 254 nm UV light before being returned to a

37°C shaking water bath. Aliquots of 0.5 mL of culture were collected and precipitated in ice cold 5% TCA every 20 minutes for 200 minutes. The DNA was collected by filtration on 0.45 μm glass fiber filters. The amount of ^{3}H and ^{14}C in the DNA was quantified by liquid scintillation counting.

Conjugational recombination

Fresh overnight cultures of the *arg*$^{+}$ Str^{S} donor (PK3) and the *arg*- Str^{R} recipients were grown to an OD_{600} of ~0.4 in a 37° shaking water bath. To determine cell numbers at the time of conjugation, serial dilutions of the donor and recipient cultures were spotted in triplicate in 10 μL drops on LBthy plates, incubated overnight at 37°C, and counted the following day. For conjugation, one mL each of the donor and recipient cultures were mixed together with an additional mL of LBthy media, and the cells were collected on a 0.45 μm glass membrane filter. The filters were placed on pre-warmed LBthy plates and incubated at 37° for one hour. Filters were then re-suspended in 5 mL of 1X Davis media and serial dilutions were spotted in triplicate in 10 μL drops on DGthy media (1X Davis, 0.4% Glucose, 10 μg/mL thymine) supplemented with 100 μg/mL histidine,100 μg/mL leucine, 100 μg/mL proline, 100 μg/mL threonine, 30 μg/mL thiamine and 50 μg/mL streptomycin, then incubated overnight at 37°C. Transconjugates were counted following a two-day incubation period.

CHAPTER III

RESULTS

RecX does not effect cell survival following UV induced DNA damage.

To determine whether RecX contributes to survival following UV-induced damage, the survival of mutants lacking *recX* was examined and compared to wild type cultures. *recF* and *recA* mutants, which are known to be moderately and highly hypersensitive to UV damage, were also examined as controls (Courcelle *et al.*, 1997).

The UV sensitivity of *recX* mutants was similar to wild type cells at doses below 80 J/m^2 (Figure 1). In both wild type and *recX* cultures, viability was reduced by almost two orders of magnitude at a dose of 60 J/m^2. *recX* mutants appeared to be modestly resistant to UV irradiation compared to wild type cultures at a dose of 80 J/m^2. However, this result was not observed at any other doses. As higher doses were not examined, it remains possible that *recX* mutants may exhibit increased resistance at higher doses of UV irradiation than wild type cells.

RecX does not contribute to the processing of the nascent DNA following arrest by UV-induced damage.

Following the arrest of replication by UV-induced damage, RecF is required to load RecA onto DNA at the site of arrest and to stabilize the replication fork (Courcelle *et al.*, 2003). In the absence of either protein, the nascent DNA undergoes extensive degradation and replication fails to recover. Since RecX is postulated to interact with and

affect RecA filaments, it is possible that it may contribute to the stability or processing of the DNA at the arrested fork. To examine this possibility, the amount of degradation occurring in the nascent ^{3}H labeled DNA and the overall accumulation of ^{14}C labeled DNA was observed following UV irradiation. In brief, cultures pre-labeled with [^{14}C]-thymine were pulse labeled for 10 seconds with [^{3}H]-thymidine. The radioactivity was then removed from the media, the culture was irradiated, and the amount of degradation occurring in the nascent ^{3}H labeled DNA and the overall ^{14}C labeled genomic DNA were observed.

In wild type cells, about 20% of the nascent DNA was degraded following UV irradiation (Figure 2). Fifty to seventy minutes after UV irradiation, the amount of ^{3}H labeled DNA present began to accumulate, presumably following resumption of DNA replication. By comparison, in *recF* cultures, degradation of nascent DNA is observed at the same rate as in wild type cells for the first fifty minutes, but degradation of the nascent strand continues to occur for the duration of the assay. In *recA* cultures, even more extensive degradation is observed, leading to approximately 70% of the nascent DNA being degraded by the end of the observed period at 200 minutes. ^{3}H labeled DNA did not begin to re-accumulate at any point over the observed time period. In the case of *recA*, degradation of genomic DNA is also observed in addition to degradation of nascent DNA.

Given that RecX both inhibits the formation of RecA filaments and has the capacity to physically interact with RecF, potentially causing some degree of sequestration, it was expected that a mutant lacking the *recX* gene would enhance the ability of these proteins to protect the replication fork, and that it was possible that less

degradation of nascent DNA might be observed. However, in the absence of RecX, the amount of degradation that occurs resembles that of wild type cells. When we examined cultures of *recX* mutants, we observed that less than 20% of nascent DNA was degraded by t-50 minutes (Figure 2). At 20 minutes post-irradiation, the amount of nascent DNA detected had returned to the original fraction. Following this time point, nascent DNA continued to accumulate in the *recX*⁻ mutant for the duration of the assay. No degradation of genomic DNA occurred following UV irradiation. These results mimic those observed in wild type cells under the same conditions, suggesting that the absence of RecX does not affect the processing or stability of the fork following arrest.

RecX is not required for DNA replication to resume following UV induced DNA damage.

Both RecF and RecA are required for replication to resume following UV-induced DNA damage (Courcelle *et al.*, 2003). To determine if the biochemical interactions of RecX with either of these proteins are also required for replication to resume, we monitored the rate of synthesis and accumulation of DNA following UV-irradiation in wild type, *recA*, *recF* and *recX* cultures over time. To this end, cultures growing in the presence of ^{14}C labeled thymine were UV-irradiated with 30 J/m^2. At the indicated times during the recovery period, aliquots of the culture were pulse labeled for 2 minutes with [^3H]-thymidine. Then, the amount of ^{14}C labeled DNA that had accumulated in the culture and the rate of [^3H] labeled DNA per 2 minutes of pulse synthesized at that time was determined.

In wild type cultures, immediately following UV-irradiation, the rate of synthesis decreases to less than 1% of the pre-irradiated rate (Figure 3). However, after approximately 20 minutes, replication resumes and the rate of DNA synthesis approaches pre-irradiated levels. Additionally, after 40 minutes, total genomic DNA begins accumulating. By comparison, in *recF*$^-$ and in *recA*$^-$ cultures the rate of replication following UV irradiation never increases beyond background levels, and genomic DNA does not accumulate at any point over the course of the assay.

In *recX*, we observed that the rate of DNA synthesis post-irradiation reached about 1% relative to the rate just prior to UV-irradiation. Similar to wild type, at approximately 20 minutes, the rate of synthesis began to increase and continued to do so for the duration of the assay. Also comparable to wild type, genomic DNA begins accumulating approximately 40 minutes post-irradiation.

RecX is not necessary for proficient conjugational recombination.

RecX has been shown to be an inhibitor of DNA strand exchange during homologous recombination and during the SOS response (Stohl *et al.*, 2003). In *D. radiodurans* an increase in recombinational proficiency has been observed during conjugation in mutants lacking *recX* (Sheng *et al.*, 2005). To determine if a similar effect occurs in *E. coli*, the frequency of recombination during conjugation in a mutant lacking *recX* was compared to wild type, *recF, recA* and *recBC* mutant strains. To this end, *arg*$^+$ Str^S Hfr donors were incubated with *arg*$^-$ Str^R recipients of each mutant background. In wild type cells, the frequency of recombination was approximately 6 *arg*$^+$ recombinants

per 100,000 donor cells, or about 0.006% (Figure 4). Similarly, in *recF*, which does not significantly affect conjugational recombination, the frequency of recombination was 3 *arg*$^+$ recombinants per 100,000 donor cells, or about 0.003%. By comparison, in *recA* or *recBC* mutants, no conjugational recombination was observed. When we examined the frequency of recombination in *recX*, conjugational recombination occurred at a frequency of of 5 *arg*$^+$ recombinants per 100,000 donor cells, or about 0.005%. We concluded that, in contrast to the case of *D. radiodurans*, absence of RecX in *E. coli* does not result in increased conjugational recombination. Rather, the conjugational phenotype expressed by mutants lacking the *recX* gene resembled that of wild type cells.

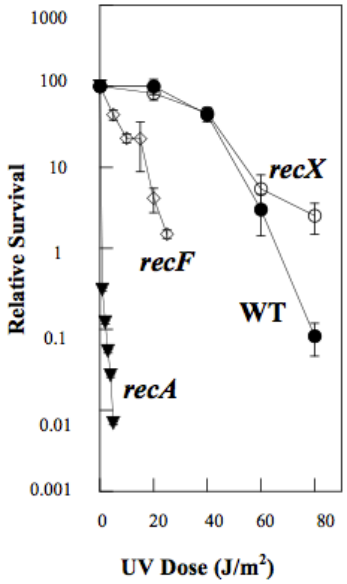

Figure 1. Survival following UV irradiation and growth of wild-type, *recA*, *recF* and *recX*. The percentage of cells surviving the indicated dose of UV irradiation is plotted for each strain. Survival averages represent at least three independent experiments. Error bars represent standard error of the mean.

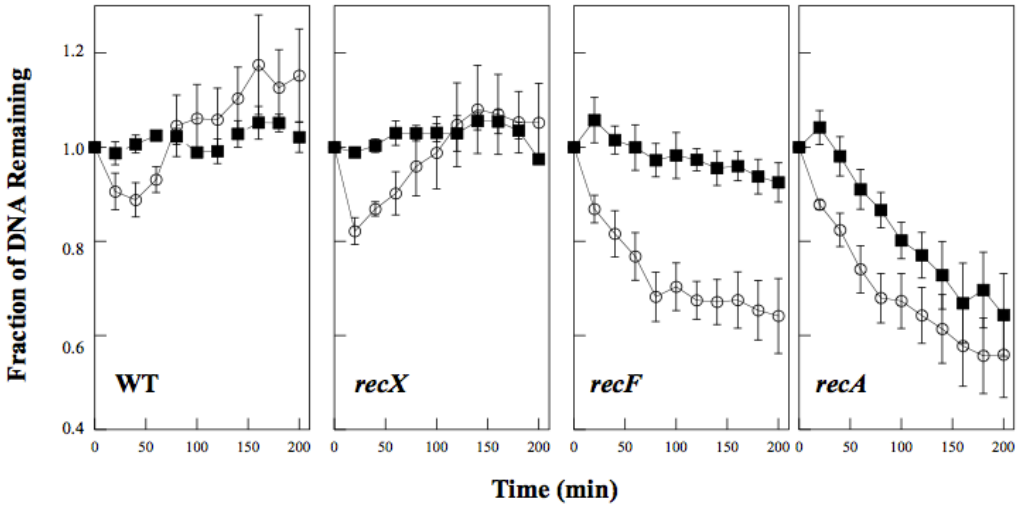

Figure 2. Degradation of nascent and genomic DNA following UV irradiation. Neither 14C labeled genomic DNA (closed squares) nor ^{3}H labeled nascent DNA (open circles) are degraded excessively relative to wild type in the absence of RecX. Degradation of nascent and genomic DNA was monitored as described for wild type, *recX*, *recF* and *recA*. Data shown represents averages for at least three independent experiments. Error bars represent standard error of the mean.

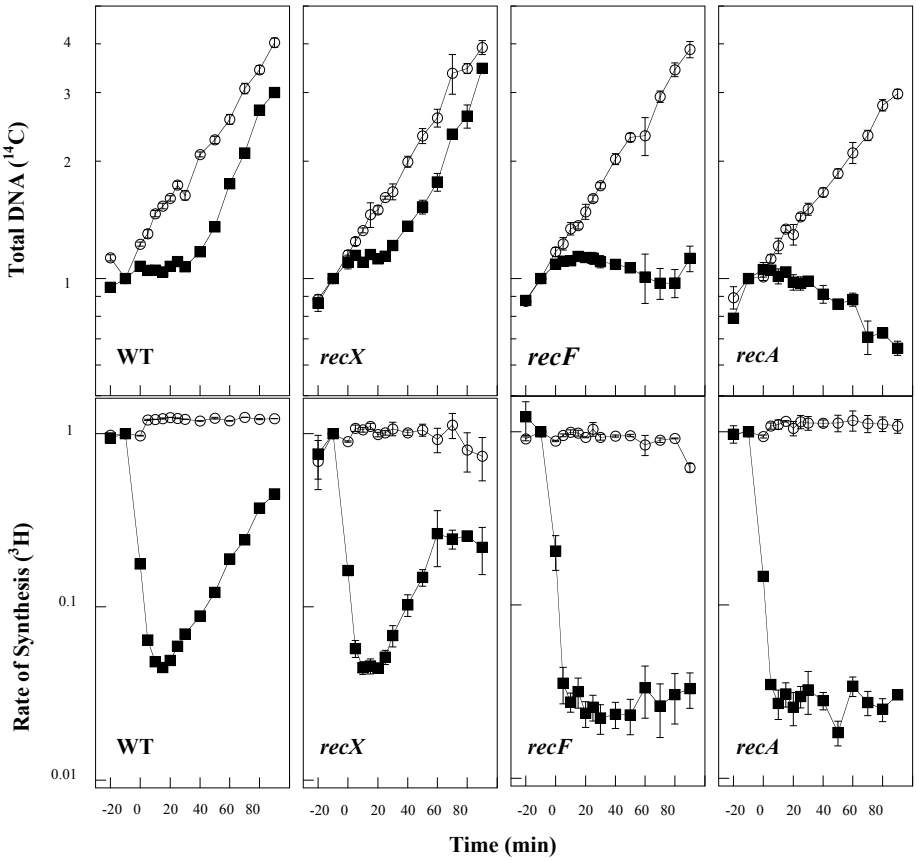

Figure 3. Mutants lacking the *recX* gene recover replication at a rate similar to wild type. Cultures grown in ^{14}C-thymine were either irradiated with 30 J/m^2 (closed squares) or mock irradiated (open circles) and then pulsed with ^{3}H-thymidine at the times indicated. Data shown represents the averages of at least two independent experiments. Error bars represent standard error of the mean.

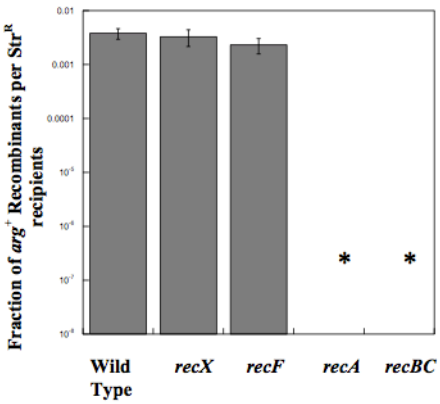

Figure 4. The ability to undergo conjugational recombination is not inhibited in the absence of RecX. Percent *arg*$^+$ Recombinant averages represent at least three independent experiments for wild type, *recX*, *recF*, *recA*, and *recBC*. Error bars represent standard error of the mean.
*The average number of recombinants was less than one for *recA* and *recBC*.

CHAPTER IV

DISCUSSION

The focus of this study was to determine if an *E. coli* mutant with a deletion of the *recX* gene expressed a deleterious phenotype under SOS response conditions. A *recX*$^-$ mutant was constructed and characterized for its UV sensitivity, conjugational proficiency and ability to process and restore arrested replication forks following DNA damage. The results of these assays were compared to results from the wild type SR108 strain, as well as mutants with non-functional RecA and RecF proteins, which are known to have defects in these processes. The capacity for *recX* to undergo conjugational recombination was additionally compared to a mutant lacking functional RecB and RecC proteins, as *recBC* mutants exhibit a defect in this process.

While assessing the relative survival of *recX* mutants following DNA damage at doses ranging from 0 to 80 J//m^2, it was observed that the *recX* mutant showed slight UV

resistance at a dose of 80 J/m^2. This observation was not reproducible at doses of 80 ±10 J/m^2. It has been previously reported that *recX* mutants exhibit a less than 2-fold greater UV sensitivity (Renzette, et al, 2007, Stohl, et al., 2003). However, we did not detect this in strain CL1135 *recX::kan*. This discrepancy may be due to differences in the strain backgrounds used in these studies, or simply because differences within this range border on the sensitivity of typical survival assays.

In order for DNA lesions to be repaired, nucleases must first process the damaged strand and remove the chemically altered bases. In UV irradiated cells, the RecJ/RecQ helicase/nuclease mediates the process of nascent DNA degradation, while the RecBCD helicase/nuclease degrades unprotected genomic DNA ends, although its precise substrate *in vivo* remains unknown (Chow and Courcelle, 2007). We found that RecX does not detectably contribute to or prevent the amount of nascent DNA processing that occurs at the arrested fork. Similarly, we observed here that RecX did not affect the time or efficiency of when replication resumed following UV irradiation. Finally, we also did not observe any effect of RecX on recombination efficiency during recombination.

The lack of any dramatic phenotype observed in a *recX* mutant relative to wild type cells may suggest that the RecX protein does not play a significant role in processing UV damage or conjugation in *E. coli*. It is possible that it may have more significant roles in processing other forms of damage, such as double strand breaks or DNA crosslinks, or that it has more prominent roles in other RecA-mediated process that were not detected in our assays. Given that biochemically, RecX interacts with RecA and inhibits RecA filament formation, and interacts with RecF, one would presume that it has a specific cellular role for which its evolutionary conservation has been selected for and

retained. It may be that replication forks arrested by DNA adducts is simply not its primary function.

Alternatively, it is possible that in, in contrast to other organisms, the function of RecX in *E. coli* is either less critical or masked. While the absence of RecX in *E. coli* is associated with only modes phenotypical differences (this study; Stohl *et al.*, 2003; Lusetti *et al.*, 2004; Renzette *et al.*, 2007), in other organisms, RecX has a significant effect on recombination and survival following UV-induced DNA damage (refs!!!!!). For example, *D. radiodurans*, which lacks the RecBCD complex (Makarova *et al.*, 2001), an enzyme critical to genomic stability and resistance to DNA damage in *E. coli* (reviewed in Dillingham and Kowalczykowski, 2008), expresses increased recombinational proficiency during conjugation in the absence of RecX. However, an *E. coli recX* mutant does not exhibit the same capacity for recombination (this study; Sheng *et al.*, 2005; Stohl *et al.*, 2003). It is possible that the presence of RecBC may be either masking or complementing the effects of RecX in *E. coli*. Analogously, it has been suggested that the absence of RecBC in *D. radiodurans* is partially compensated for by the RecFOR pathway (Bentchikou *et al.*, 2010). It is therefore possible that RecBCD or RecFOR are masking any effects of RecX in *E. coli.* This could be directly tested by investigating whether *recX* becomes more important for survival or recovery forlowing DNA damage in cells lacking RecBCD or RecF.

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