A STRUCTURE AND FUNCTION ANALYSIS OF RecF IN PROMOTING RECOVERY OF REPLICATION FORKS DISRUPTED BY DNA DAMAGE

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CHAPTER I

INTRODUCTION

RecA IN RECOMBINATION AND REPAIR

DNA damage encountered during replication can generate gaps in newly synthesized DNA, arrest the progression of replication, or lead to an eventual collapse of the replication fork if the block to replication cannot be overcome [1-4]. Recombination proteins play critical roles in maintaining the template and restoring replication in the presence of DNA damage [56-8]. In *Escherichia coli,* the primary gene product required for recombination during sexual events and for maintaining genomic integrity in the presence of DNA damage is encoded by *recA* [5]. In 1965, *recA* was originally isolated in a screen for mutants defective in recombination during the sexual cycle of conjugation. Subsequently, these mutants were also shown to be hypersensitive to UV-irradiation [5].

The RecA protein consists of a large core domain and two smaller domains at the N- and C-termini [9, 10]. Active RecA proteins are bound to DNA and form nucleoprotein filaments. A single monomer of RecA encompasses 3 nucleotides, with ATP bound at each subunit-subunit interface between monomers [11]. The binding serves a two-fold effect. Bound RecA filaments are capable of pairing and exchanging strands with homologous duplex DNA which serves to rearrange or incorporate DNA during sexual events and maintain or restore DNA at replication forks or DNA breaks in the asexual cell cycles [6, 12-14]. A second function mediated by this bound form of RecA is the upregulation of a suite of more than 40 gene products, collectively referred to as the SOS response [15-18]. Upon binding to DNA, RecA undergoes a conformational change that

promotes the autocatalytic cleavage of LexA, de-repressing the induction of the SOS response [19-21]. Included in the suite of genes upregulated during the SOS response are the *uvrA* and *uvrB* genes, the products of which are involved in nucleotide excision and are required for removal of UV-induced DNA lesions as well as the resumption of replication following exposure to UV [17, 22-26]. Other genes upregulated in the SOS response include *polB, dinB,* and *umuCD*, which encode the specialized translesion polymerases Pol II, Pol IV, and Pol V, respectively. These polymerases allow for the bypass of DNA lesions that otherwise block replication [17, 27-34]. The RecA-mediated induction of the SOS response is crucial for the efficient restoration of the DNA template and survival in *E. coli* following UV-irradiation [35-37].

During sexual events, the RecA nucleoprotein filament binds to the invading foreign DNA that has entered the cells by conjugation or transduction, and catalyzes the pairing of the single-stranded DNA to a homologous duplex DNA. Through an ATP-dependent strand-exchange reaction, a plectonemically joined molecule is generated that can be extended by polymerases at the DNA ends, or processed by branch migration prior to resolution to generate a recombinant molecule. [38-40].

In response to the arrest of replication, RecA binds to single-stranded regions of DNA produced by a stalled replication fork and pairs it with homologous duplex DNA. In this case, the homologous duplex DNA is found on the sister chromatid at the stalled fork. The pairing and strand exchange reaction is thought to promote processing of the replication fork such that the impediment can be repaired or bypassed. In addition, these

activities function to protect and maintain the structural integrity of the replication fork until it can resume [14, 41-43].

TARGETING RecA TO ITS SUBSTRATES, RECOMBINATIONAL MEDIATORS

In order to initiate the cellular response to DNA damage as outlined above, RecA requires accessory proteins to recruit and target RecA to the proper substrates during recombination and repair. The targeting of RecA occurs through two primary pathways; the RecBCD pathway or the RecFOR pathway.

RecBCD

The RecBCD pathway of *E. coli* is required for efficient recombination during conjugation or transduction [5, 44, 45]. *recB* and *recC* were originally identified as being impaired for recombination during conjugation in a screen similar to that which identified *recA* [5]. *recB* or *recC* mutants exhibited a lower frequency of recombination during conjugation and transduction and were also hypersensitive to UV-irradiation [46]. Additional studies demonstrated that the RecBCD pathway proteins play critical roles in the repair of double-stranded DNA breaks [47, 48]. These observations led to the proposal that RecBCD functions as an enzyme that mediates the processing, targeting, and loading of RecA at double-strand DNA ends, a proposal that is well-supported by biochemical characterizations [49-54]

RecBCD is a heterotrimeric enzyme composed of the RecB, RecC, and RecD subunits. The holoenzyme possesses ATP-dependent helicases (RecB and RecD) and a

nuclease (RecC) that can unwind and degrade duplex DNA from a double-strand end [44, 55, 56]. The processing of these ends by the RecBCD enzyme is modulated by short sequences of DNA (5'-GCTGGTGG-3') that are hotspots of recombination, known as Chi sites [57-59]. Prior to recognition of a Chi site by the translocating RecBCD enzyme, RecB unwinds the DNA from the 3'-end while RecD unwinds the DNA from the 5'-end [53, 60]. Upon recognition of a Chi site by the RecBCD enzyme, the nucleolytic activity encoded in the RecC subunit shifts from the degradation of both DNA strands to the predominant degradation of the 5'-end, resulting in a 3'-end overhang [58, 61, 62]. This 3'-end overhang is thought to be a target for the loading of RecA and is critical for the initiation of homologous recombination and repair of DNA double-strand breaks in *E. coli* [52, 63]

RecFOR

The RecFOR pathway is required in *E. coli* for the resumption of replication following disruption by DNA damage [14, 43, 64]. *recF* was identified in a screen designed to isolate gene(s) responsible for the remaining recombination that occurred in *recBC* mutants [65]. A number of researchers observed that second site suppressor mutations in *sbcCD* or *sbcA* and *sbcB* could reactivate the ability of *recBC* mutants to undergo conjugational recombination to near wild type levels [65-68]. Thus, *recF* was identified as a gene required for conjugational recombination in *recBC sbcCD* mutants. However, in an otherwise wild type background, this mutant exhibited nearly wild type frequencies of conjugational recombination. Curiously, however, *recF* mutants were hypersensitive to UV-induced damage [65, 69].

RecF, RecO, and RecR form an epistatic group that are thought to act by targeting the RecA protein to the single-stranded gaps or regions of DNA at sites of stalled replication forks and by displacing single-stranded binding (SSB) protein [70-74].

The RecF protein forms an ATP-dependent dimeric clamp that can interact with single-stranded and double-stranded DNA [75, 76]. The crystal structure of the *Deinococcus radiodurans* RecF protein reveals a high level of structural similarity to the Eukaryotic DNA repair protein Rad50. Both Rad50 and RecF exhibit ABC-type ATPase activities conserved among Structural Maintenance of Chromosome (SMC) proteins [76].

The RecO protein exhibits a DNA strand annealing activity that is proposed to be similar to that seen in the Eukaryotic Rad52 protein and can promote annealing between two single-stranded DNA molecules that are coated by SSB protein. It has been suggested that protein-protein interactions occur between RecO and SSB protein that may be relevant to RecO function within the RecFOR pathway [77, 78]. Additionally, the crystal structure of the RecO protein reveals potential sites for protein-protein interactions with RecR and RecA and protein-DNA interactions with double-stranded DNA [71, 79, 80].

The RecR protein can interact with both RecO and RecF, suggesting RecR may act to tether the proteins together and mediate the binding of a RecA filament to DNA [8170, 82]. Additionally, the crystal structure of the *Deinococcus radiodurans* RecR protein reveals that it forms a tetrameric clamp that can encircle double-stranded DNA [83].

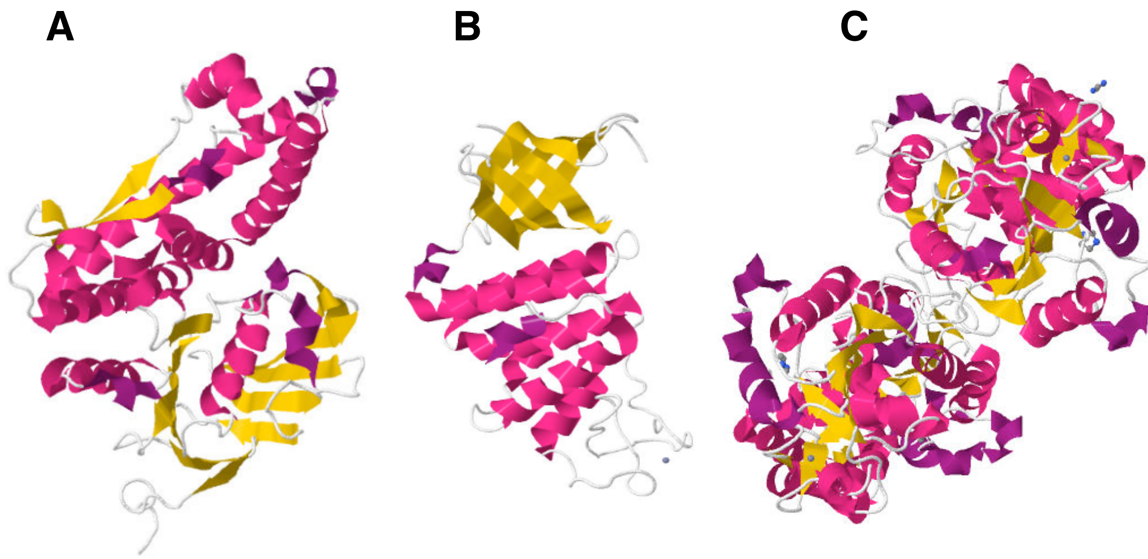

Figure 1.1: Crystal structures of the *Deinococcus radiodurans* RecF, RecO, and RecR proteins. A. The RecF monomer forms a homo-dimeric complex that has a clam-like structure and contains an ABC ATPase domain [76]. The protein contains three conserved motifs, the Walker A and Walker B motifs, located in ATPase domain 1 at the N-terminus or C-terminus, respectively, and the signature motif which resides in domain 2 [76]. B. The RecO monomer forms an elongated globule containing an N-terminal domain with an OB-fold, a C-terminal domain alpha-helical domain with 6 alpha-helices, and a zinc binding domain [79]. C. The RecR monomer forms a two-domain structure, which includes an N-terminal domain with a helix-hairpin-helix motif and a C-terminal domain with a zinc-finger motif, a Walker motif, and a Toprim domain. Four of these monomers are capable of forming a tetrameric ring in solution [83] (Images adapted from the RCSB Protein Databank).

Other proteins associated with the RecFOR pathway of DNA repair include RecQ and RecJ [84, 85]. RecQ, a 3' – 5' helicase, and RecJ, a 5' – 3' nuclease [86, 87], were found to partially degrade the nascent lagging strand at arrested replication forks [43, 88]. It is thought that this limited degradation restores the lesion-containing DNA to a double-stranded form that can be repaired by nucleotide excision repair (NER) and the processing is essential for the rapid recovery of replication following disruption [25, 26].

Historically, since *recF* and the RecFOR pathway genes were identified as being required for recombination to occur during sexual cycles, models and experiments generally assumed that recombinant products would also be generated during the repair that occurred in asexual cell cycles. As a result, most experiments used strains that were repair defective, to enable researchers to characterize the recombinant products in the absence of any repair products [7, 46, 89-91].

RecF PATHWAY FUNCTION FOLLOWING DNA DAMAGE

Early studies, investigating the observed hypersensitivity of *recF* mutants, found that following very low doses of UV-irradiation, nucleotide excision repair mutants accumulated gaps in the nascent DNA that persisted in the absence of RecF [92]. The lack of repair also led to high rates of DNA strand exchanges [93]. The persistent gaps and strand exchanges observed in repair mutants led to a model in which the replication machinery could bypass lesions encountered during replication. In these models, the gaps opposite to these lesions were subsequently filled in using a RecF-mediated exchange of DNA from the sister chromosomes in a process that was termed post-replication recombinational repair [94]. This process was later renamed daughter-strand gap repair to more accurately reflect the lack of lesion repair in the proposed model [92].

While aspects of these original models remain intact, recent studies focusing on the functional role of RecF in wild type cultures have led to alternative models for RecF in which the primary role of these gene products function to process and restore replication without producing recombinant products. These studies focused on the

synergistic enhancement of survival that occurs when the RecF pathway operates in repair proficient cells [95]. These genetic observations imply that both RecF and nucleotide excision repair function in a common pathway to promote cell survival, an aspect that was not considered in early models. In comparing the UV-sensitivity of *recF* mutants to wild type cells, it was noted that the UV hypersensitivity in *recF* mutants correlates with active replication at the time of UV-irradiation [14]. The hypersensitivity of RecF deficient cultures irradiated in stationary phase or pre-treated with antibiotics that inhibit new initiations of DNA replication, was greatly ameliorated [14]. These results were interpreted to indicate that the defect in *recF* mutants was specific to a failure to process DNA damage encountered by replication forks, rather than a failure to process the DNA damage itself.

Subsequent work demonstrated that upon encountering DNA damage, RecFOR pathway mutants were severely impaired in their ability to restore DNA synthesis [1488]. The failure to resume DNA synthesis correlated with extensive degradation of the nascent DNA strands and a failure to maintain the integrity of the replication fork following UV-induced DNA damage [14, 25, 43, 64, 73]. Based on the pulse-labeling time and rate of replication, up to 20 kb of the nascent DNA is estimated to be degraded following UV-irradiation in *recF* mutants [14, 43]. The degradation is preferentially targeted to the nascent lagging strand and is carried out by the RecJ nuclease and RecQ helicase [88]. Similar studies revealed that RecO and RecR functioned together with RecF in the progressive steps to restore replication following disruption by DNA damage [64]**.** Following UV-induced damage, deficiencies in any one of these genes results in extensive nascent DNA

degradation, failure to process stalled replication forks, and an impaired ability to resume DNA synthesis [14, 43].

These findings, in combination with the biochemical characterization of the RecFOR proteins, led to the following model for the RecFOR pathway: Following the arrest of replication, RecF is proposed to direct RecO and RecR to DNA junctions at the arrested fork. RecF, RecO, and RecR are able to displace SSB proteins and enhance the RecA nucleoprotein filament formation at the arrested replication fork where it maintains fork stability [75, 82, 96-98]. RecQ and RecJ act in concert, either coordinately with or subsequent to RecF-O-R binding, to process nascent DNA at the replication fork, exposing the DNA region containing the damage [43, 88]. Following the processing of the stalled replication fork, lesion removal or bypass must occur prior to the reestablishment of an active replisome and resumption of DNA synthesis [24-26]. Removal of these lesions occurs by nucleotide excision repair (NER) [99]. In this pathway, the UvrABC endonucleolytic complex recognizes and excises lesions by making dual incisions 12-14 bp on either side of the damaged nucleotide. The UvrD helicase then removes the DNA fragment containing the lesion. DNA polymerase I fills in the resulting gap and ligase seals the nicks [100-102]. In the absence of repair, the recovery of replication is severely impaired in both extent and efficiency, leading to elevated levels of nascent strand gaps, strand exchanges, and lethality [7, 24, 93].

If the lesion cannot be removed or if NER function is unavailable, an alternative process, DNA translesion synthesis (TLS), can occur that allows for DNA synthesis to resume, although this occurs with much lower efficiency and an elevated frequency of

mutation [33, 103, 104]. The TLS polymerases, PolII, PolIV, and PolV, contain modified active sites that are able to accommodate altered nucleotides, thereby allowing for synthesis to continue past the lesion [105-107]. After UV-induced damage, in the absence of nucleotide excision repair or processing by the RecJ nuclease, translesion synthesis by Polymerase V can restore DNA synthesis at the site of the UV-induced lesion [25, 26].

Most known bacteria encode homologs of the RecFOR pathway proteins and functional homologs are speculated to exist in eukaryotes and mammals. Some functional candidate genes for RecF pathway homologs in mammalian cells include WRN, BLM, RAD52, RAD50, and BRCA2 [78, 108-113]. Since sequence conservation between these genes and the RecF pathway genes is limited, functional homologs will likely have to be identified using cellular assays similar to those developed in the *E. coli* system. In some cases, deficiencies in these candidate proteins are linked to premature aging and a predisposition to cancer, making the prokaryotic pathway an appropriate and relevant model for DNA repair in higher organisms [114-117].

Despite the recent advances outlined above, the nature of how RecF, RecO, and RecR form a complex on DNA remains unknown. In addition, the precise substrates recognized by these proteins or how they recruit RecA to the DNA regions at the damaged forks remains uncharacterized. Finally, the intimate association of the RecF pathway proteins in processing and restoring DNA synthesis suggests that the proteins may play a much more direct role in restoring an active replisome to the DNA at later stages in the recovery process. To begin to address these issues, I employed a structure and function analysis of the RecF protein. In the next chapter, I describe targeted

mutations that impair specific biochemical properties of RecF. The overall goal of this approach was to identify mutations that may arrest the recovery process at unique stages, thereby illuminating the steps that these proteins catalyze or participate in during the processing of lesions encountered during replication.

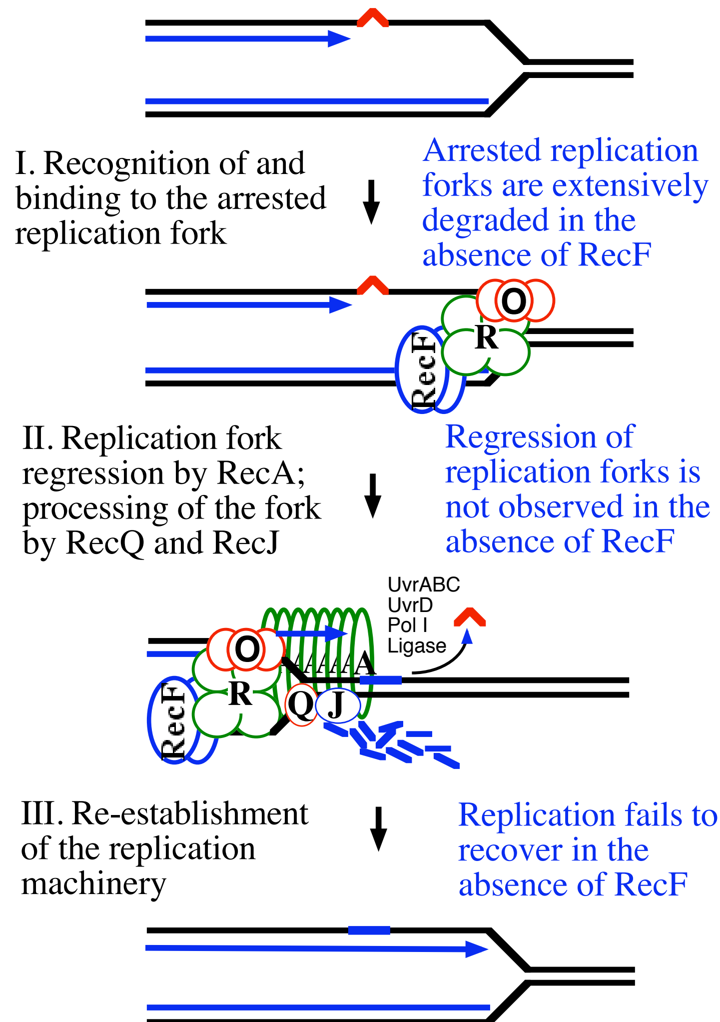

Figure 1.2. Model for the RecFOR pathway of replication recovery following disruption by DNA damage and the consequences to cells deficient in RecF. I. The replication fork is blocked and partially disruped by UV-induced DNA damage. RecF is proposed to direct RecO and RecR to DNA junctions generated at the blocked replication forks and to mediate the loading and formation of RecA nucleoprotein filaments at these sites [75, 96]. The RecQ helicase and RecJ nuclease act in concert to partially degrade the nascent lagging strand DNA at the arrested replication fork which is thought to restore the lesion-containing region to a double-stranded substrate that can be acted on by nucleotide excision repair [26, 88]. Once the lesion is removed and the template is restored, the active replisome can be reassembled or reactivated and replication can resume.

REFERENCES

1. Setlow, R. B., Swenson, P. A. & Carrier, W. L. (1963). Thymine dimers and Inhibition of DNA synthesis by ultraviolet irradiation of cells. *Science* **142**, 1464-1466.
2. Svoboda, D. L. & Vos, J. M. (1995). Differential replication of a single, UV-induced lesion in the leading or lagging strand by a human cell extract: fork uncoupling or gap formation. *Proc Natl Acad Sci U S A* **92**, 11975-11979.
3. Michel, B., Ehrlich, S. D. & Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *Embo J* **16**, 430-438.
4. Seigneur, M., Bidnenko, V., Ehrlich, S. D. & Michel, B. (1998). RuvAB acts at arrested replication forks. *Cell* **95**, 419-430.
5. Clark, A. J. & Margulies, A. D. (1965). Isolation and Characterization of Recombination-Deficient Mutants of Escherichia coli K12. *Proc Natl Acad Sci U S A* **53**, 451-459.
6. Horii, Z. I. & Suzuki, K. (1968). Degradation of the DNA of Escherichia coli K12 rec- (JC1569b) after irradiation with ultraviolet light. *Photochem photobiol* **8**, 93-105.
7. Howard-Flanders, P., Theriot, L. & Stedeford, J. B. (1969). Some properties of excision-defective recombination-deficient mutants of Escherichia coli K-12. *J Bacteriol* **97**, 1134-1141.
8. Satta, G., Gudas, L. J. & Pardee, A. B. (1979). Degradation of Escherichia coli DNA: evidence for limitation in vivo by protein X, the recA gene product. *Mol Gen Genet* **168**, 69-80.
9. Story, R. M. & Steitz, T. A. (1992). Structure of the recA protein-ADP complex. *Nature* **355**, 374-376.
10. Story, R. M., Weber, I. T. & Steitz, T. A. (1992). The structure of the E. coli recA protein monomer and polymer. *Nature* **355**, 318-325.
11. VanLoock, M. S., Yu, X., Yang, S., Galkin, V. E., Huang, H., Rajan, S. S., Anderson, W. F., Stohl, E. A., Seifert, H. S. & Egelman, E. H. (2003). Complexes of RecA with LexA and RecX differentiate between active and inactive RecA nucleoprotein filaments. *J Mol Biol* **333**, 345-354.
12. West, S. C., Cassuto, E. & Howard-Flanders, P. (1981). recA protein promotes homologous-pairing and strand-exchange reactions between duplex DNA molecules. *Proc Natl Acad Sci U S A* **78**, 2100-2104.
13. Howard-Flanders, P. (1968). Genes that control DNA repair and genetic recombination in Escherichia coli. *Adv Biol Med Phys* **12**, 299-317.
14. Courcelle, J., Carswell-Crumpton, C. & Hanawalt, P. C. (1997). recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **94**, 3714-3719.
15. Radman, M. (1974) in *Molecular and Environmental Aspects of Mutagenesis*, eds. Prakash, L., Sherman, F., Miller, M., Lawrence, C. & Tabor, H. W. (Charles C Thomas, Springfield Ill), pp. 128-142.

16. Radman, M. (1975). SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. *Basic Life Sci* **5A**, 355-367.
17. Kenyon, C. J. & Walker, G. C. (1980). DNA-damaging agents stimulate gene expression at specific loci in Escherichia coli. *Proc Natl Acad Sci U S A* **77**, 2819-2823.
18. Courcelle, J., Khodursky, A., Peter, B., Brown, P. O. & Hanawalt, P. C. (2001). Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient Escherichia coli. *Genetics* **158**, 41-64.
19. Little, J. W., Edmiston, S. H., Pacelli, L. Z. & Mount, D. W. (1980). Cleavage of the Escherichia coli lexA protein by the recA protease. *Proc Natl Acad Sci U S A* **77**, 3225-3229.
20. Roberts, J. W., Phizicky, E. M., Burbee, D. G., Roberts, C. W. & Moreau, P. L. (1982). A brief consideration of the SOS inducing signal. *Biochimie* **64**, 805-807.
21. Sassanfar, M. & Roberts, J. W. (1990). Nature of the SOS-inducing signal in Escherichia coli. The involvement of DNA replication. *J Mol Biol* **212**, 79-96.
22. Kenyon, C. J. & Walker, G. C. (1981). Expression of the E. coli uvrA gene is inducible. *Nature* **289**, 808-810.
23. Fogliano, M. & Schendel, P. F. (1981). Evidence for the inducibility of the uvrB operon. *Nature* **289**, 196-198.
24. Courcelle, J., Crowley, D. J. & Hanawalt, P. C. (1999). Recovery of DNA replication in UV-irradiated Escherichia coli requires both excision repair and recF protein function. *J Bacteriol* **181**, 916-922.
25. Courcelle, C. T., Belle, J. J. & Courcelle, J. (2005). Nucleotide excision repair or polymerase V-mediated lesion bypass can act to restore UV-arrested replication forks in Escherichia coli. *J Bacteriol* **187**, 6953-6961.
26. Courcelle, C. T., Chow, K. H., Casey, A. & Courcelle, J. (2006). Nascent DNA processing by RecJ favors lesion repair over translesion synthesis at arrested replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **103**, 9154-9159.
27. Bonner, C. A., Hays, S., McEntee, K. & Goodman, M. F. (1990). DNA polymerase II is encoded by the DNA damage-inducible dinA gene of Escherichia coli. *Proc Natl Acad Sci U S A* **87**, 7663-7667.
28. Iwasaki, H., Nakata, A., Walker, G. C. & Shinagawa, H. (1990). The Escherichia coli polB gene, which encodes DNA polymerase II, is regulated by the SOS system. *J Bacteriol* **172**, 6268-6273.
29. Wickner, R. B., Ginsberg, B. & Hurwitz, J. (1972). Deoxyribonucleic acid polymerase II of Escherichia coli. II. Studies of the requirements and the structure of the deoxyribonucleic acid product. *J Biol Chem* **247**, 498-504.
30. Wickner, R. B., Ginsberg, B., Berkower, I. & Hurwitz, J. (1972). Deoxyribonucleic acid plymerase II. of Escherichia coli. I. The purification and characterization of the enzyme. *J Biol Chem* **247**, 489-497.
31. Wagner, J., Gruz, P., Kim, S. R., Yamada, M., Matsui, K., Fuchs, R. P. & Nohmi, T. (1999). The dinB gene encodes a novel E. coli DNA polymerase, DNA pol IV, involved in mutagenesis. *Mol Cell* **4**, 281-286.

32. Elledge, S. J. & Walker, G. C. (1983). Proteins required for ultraviolet light and chemical mutagenesis. Identification of the products of the umuC locus of Escherichia coli. *J Mol Biol* **164**, 175-192.
33. Bagg, A., Kenyon, C. J. & Walker, G. C. (1981). Inducibility of a gene product required for UV and chemical mutagenesis in Escherichia coli. *Proc Natl Acad Sci U S A* **78**, 5749-5753.
34. Tang, M., Shen, X., Frank, E. G., O'Donnell, M., Woodgate, R. & Goodman, M. F. (1999). UmuD'(2)C is an error-prone DNA polymerase, Escherichia coli pol V. *Proc Natl Acad Sci U S A* **96**, 8919-8924.
35. Dutreix, M., Moreau, P. L., Bailone, A., Galibert, F., Battista, J. R., Walker, G. C. & Devoret, R. (1989). New recA mutations that dissociate the various RecA protein activities in Escherichia coli provide evidence for an additional role for RecA protein in UV mutagenesis. *J Bacteriol* **171**, 2415-2423.
36. Ennis, D. G., Little, J. W. & Mount, D. W. (1993). Novel mechanism for UV sensitivity and apparent UV nonmutability of recA432 mutants: persistent LexA cleavage following SOS induction. *J Bacteriol* **175**, 7373-7382.
37. Crowley, D. J. & Hanawalt, P. C. (1998). Induction of the SOS response increases the efficiency of global nucleotide excision repair of cyclobutane pyrimidine dimers, but not 6-4 photoproducts, in UV-irradiated Escherichia coli. *J Bacteriol* **180**, 3345-3352.
38. Konforti, B. B. & Davis, R. W. (1987). 3' homologous free ends are required for stable joint molecule formation by the RecA and single-stranded binding proteins of Escherichia coli. *Proc Natl Acad Sci U S A* **84**, 690-694.
39. Konforti, B. B. & Davis, R. W. (1990). The preference for a 3' homologous end is intrinsic to RecA-promoted strand exchange. *J Biol Chem* **265**, 6916-6920.
40. Lloyd, R. G. & Sharples, G. J. (1993). Processing of recombination intermediates by the RecG and RuvAB proteins of Escherichia coli. *Nucleic Acids Res* **21**, 1719-1725.
41. Khidhir, M. A., Casaregola, S. & Holland, I. B. (1985). Mechanism of transient inhibition of DNA synthesis in ultraviolet-irradiated E. coli: inhibition is independent of recA whilst recovery requires RecA protein itself and an additional, inducible SOS function. *Mol Gen Genet* **199**, 133-140.
42. Robu, M. E., Inman, R. B. & Cox, M. M. (2001). RecA protein promotes the regression of stalled replication forks in vitro. *Proc Natl Acad Sci U S A* **98**, 8211-8218.
43. Courcelle, J., Donaldson, J. R., Chow, K. H. & Courcelle, C. T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in Escherichia coli. *Science* **299**, 1064-1067.
44. Hickson, I. D. & Emmerson, P. T. (1981). Identification of the Escherichia coli recB and recC gene products. *Nature* **294**, 578-580.
45. Schultz, D. W., Taylor, A. F. & Smith, G. R. (1983). Escherichia coli RecBC pseudorevertants lacking chi recombinational hotspot activity. *J Bacteriol* **155**, 664-680.

46. Howard-Flanders, P. & Theriot, L. (1966). Mutants of Escherichia coli K-12 defective in DNA repair and in genetic recombination. *Genetics* **53**, 1137-1150.
47. Howard-Flanders, P. & Boyce, R. P. (1966). DNA repair and genetic recombination: studies on mutants of Escherichia coli defective in these processes. *Radiat Res* **Suppl 6**, 156-184.
48. Wang, T. C. & Smith, K. C. (1986). Inviability of dam recA and dam recB cells of Escherichia coli is correlated with their inability to repair DNA double-strand breaks produced by mismatch repair. *J Bacteriol* **165**, 1023-1025.
49. Sargentini, N. J. & Smith, K. C. (1986). Quantitation of the involvement of the recA, recB, recC, recF, recJ, recN, lexA, radA, radB, uvrD, and umuC genes in the repair of X-ray-induced DNA double-strand breaks in Escherichia coli. *Radiat Res* **107**, 58-72.
50. Krasin, F. & Hutchinson, F. (1977). Repair of DNA double-strand breaks in Escherichia coli, which requires recA function and the presence of a duplicate genome. *J Mol Biol* **116**, 81-98.
51. Wang, T. C. & Smith, K. C. (1983). Mechanisms for recF-dependent and recB-dependent pathways of postreplication repair in UV-irradiated Escherichia coli uvrB. *J Bacteriol* **156**, 1093-1098.
52. Taylor, A. F. & Smith, G. R. (1985). Substrate specificity of the DNA unwinding activity of the RecBC enzyme of Escherichia coli. *J Mol Biol* **185**, 431-443.
53. Korangy, F. & Julin, D. A. (1993). Kinetics and processivity of ATP hydrolysis and DNA unwinding by the RecBC enzyme from Escherichia coli. *Biochemistry* **32**, 4873-4880.
54. Anderson, D. G. & Kowalczykowski, S. C. (1997). The translocating RecBCD enzyme stimulates recombination by directing RecA protein onto ssDNA in a chi-regulated manner. *Cell* **90**, 77-86.
55. Linn, S. & MacKay, V. (1975). The degradation of duplex DNA by the recBC DNase of Escherichia coli. *Basic Life Sci* **5A**, 293-299.
56. Palas, K. M. & Kushner, S. R. (1990). Biochemical and physical characterization of exonuclease V from Escherichia coli. Comparison of the catalytic activities of the RecBC and RecBCD enzymes. *J Biol Chem* **265**, 3447-3454.
57. Stahl, F. W., Crasemann, J. M. & Stahl, M. M. (1975). Rec-mediated recombinational hot spot activity in bacteriophage lambda. III. Chi mutations are site-mutations stimulating rec-mediated recombination. *J Mol Biol* **94**, 203-212.
58. Taylor, A. F. & Smith, G. R. (1992). RecBCD enzyme is altered upon cutting DNA at a chi recombination hotspot. *Proc Natl Acad Sci U S A* **89**, 5226-5230.
59. Churchill, J. J., Anderson, D. G. & Kowalczykowski, S. C. (1999). The RecBC enzyme loads RecA protein onto ssDNA asymmetrically and independently of chi, resulting in constitutive recombination activation. *Genes Dev* **13**, 901-911.
60. Masterson, C., Boehmer, P. E., McDonald, F., Chaudhuri, S., Hickson, I. D. & Emmerson, P. T. (1992). Reconstitution of the activities of the RecBCD holoenzyme of Escherichia coli from the purified subunits. *J Biol Chem* **267**, 13564-13572.

61. Ponticelli, A. S., Schultz, D. W., Taylor, A. F. & Smith, G. R. (1985). Chi-dependent DNA strand cleavage by RecBC enzyme. *Cell* **41**, 145-151.
62. Dixon, D. A. & Kowalczykowski, S. C. (1993). The recombination hotspot chi is a regulatory sequence that acts by attenuating the nuclease activity of the E. coli RecBCD enzyme. *Cell* **73**, 87-96.
63. Taylor, A. F., Schultz, D. W., Ponticelli, A. S. & Smith, G. R. (1985). RecBC enzyme nicking at Chi sites during DNA unwinding: location and orientation-dependence of the cutting. *Cell* **41**, 153-163.
64. Chow, K. H. & Courcelle, J. (2004). RecO Acts with RecF and RecR to Protect and Maintain Replication Forks Blocked by UV-induced DNA Damage in Escherichia coli. *J Biol Chem* **279**, 3492-3496.
65. Horii, Z. & Clark, A. J. (1973). Genetic analysis of the recF pathway to genetic recombination in Escherichia coli K12: isolation and characterization of mutants. *J Mol Biol* **80**, 327-344.
66. Lloyd, R. G. & Buckman, C. (1985). Identification and genetic analysis of sbcC mutations in commonly used recBC sbcB strains of Escherichia coli K-12. *J Bacteriol* **164**, 836-844.
67. Luisi-DeLuca, C., Lovett, S. T. & Kolodner, R. D. (1989). Genetic and physical analysis of plasmid recombination in recB recC sbcB and recB recC sbcA Escherichia coli K-12 mutants. *Genetics* **122**, 269-278.
68. Bidnenko, V., Seigneur, M., Penel-Colin, M., Bouton, M. F., Dusko, E. S. & Michel, B. (1999). sbcB sbcC null mutations allow RecF-mediated repair of arrested replication forks in rep recBC mutants. *Mol Microbiol* **33**, 846-857.
69. Rothman, R. H., Kato, T. & Clark, A. J. (1975). The beginning of an investigation of the role of recF in the pathways of metabolism of ultraviolet-irradiated DNA in Escherichia coli. *Basic Life Sci* **5A**, 283-291.
70. Shan, Q., Bork, J. M., Webb, B. L., Inman, R. B. & Cox, M. M. (1997). RecA protein filaments: end-dependent dissociation from ssDNA stabilization by RecO and RecR proteins. *J Mol Biol* **265**, 519-540.
71. Umezu, K., Chi, N. W. & Kolodner, R. D. (1993). Biochemical interaction of the Escherichia coli RecF, RecO, and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proc Natl Acad Sci U S A* **90**, 3875-3879.
72. Hegde, S. P., Qin, M. H., Li, X. H., Atkinson, M. A., Clark, A. J., Rajagopalan, M. & Madiraju, M. V. (1996). Interactions of RecF protein with RecO, RecR, and single-stranded DNA binding proteins reveal roles for the RecF-RecO-RecR complex in DNA repair and recombination. *Proc Natl Acad Sci U S A* **93**, 14468-14473.
73. Courcelle, J. & Hanawalt, P. C. (2003). RecA-dependent recovery of arrested DNA replication forks. *Annu Rev Genet* **37**, 611-646.
74. Morimatsu, K. & Kowalczykowski, S. C. (2003). RecFOR Proteins Load RecA Protein onto Gapped DNA to Accelerate DNA Strand Exchange. A Universal Step of Recombinational Repair. *Mol Cell* **11**, 1337-1347.

75. Makharashvili, N., Mi, T., Koroleva, O. & Korolev, S. (2009). RecR-mediated modulation of RecF dimer specificity for single- and double-stranded DNA. *J Biol Chem* **284**, 1425-1434.
76. Koroleva, O., Makharashvili, N., Courcelle, C. T., Courcelle, J. & Korolev, S. (2007). Structural conservation of RecF and Rad50: implications for DNA recognition and RecF function. *EMBO J* **26**, 867-877.
77. Luisi-DeLuca, C. & Kolodner, R. (1994). Purification and characterization of the Escherichia coli RecO protein. Renaturation of complementary single-stranded DNA molecules catalyzed by the RecO protein. *J Mol Biol* **236**, 124-138.
78. Kantake, N., Madiraju, M. V., Sugiyama, T. & Kowalczykowski, S. C. (2002). Escherichia coli RecO protein anneals ssDNA complexed with its cognate ssDNA-binding protein: A common step in genetic recombination. *Proc Natl Acad Sci U S A* **99**, 15327-15332.
79. Makharashvili, N., Koroleva, O., Bera, S., Grandgenett, D. P. & Korolev, S. (2004). A novel structure of DNA repair protein RecO from Deinococcus radiodurans. *Structure (Camb)* **12**, 1881-1889.
80. Leiros, I., Timmins, J., Hall, D. R. & McSweeney, S. (2005). Crystal structure and DNA-binding analysis of RecO from Deinococcus radiodurans. *EMBO J* **24**, 906-918.
81. Umezu, K. & Kolodner, R. D. (1994). Protein interactions in genetic recombination in Escherichia coli. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. *J Biol Chem* **269**, 30005-30013.
82. Bork, J. M., Cox, M. M. & Inman, R. B. (2001). The RecOR proteins modulate RecA protein function at 5' ends of single- stranded DNA. *Embo J* **20**, 7313-7322.
83. Lee, B. I., Kim, K. H., Park, S. J., Eom, S. H., Song, H. K. & Suh, S. W. (2004). Ring-shaped architecture of RecR: implications for its role in homologous recombinational DNA repair. *EMBO J* **23**, 2029-2038.
84. Nakayama, H., Nakayama, K., Nakayama, R., Irino, N., Nakayama, Y. & Hanawalt, P. C. (1984). Isolation and genetic characterization of a thymineless death-resistant mutant of Escherichia coli K12: identification of a new mutation (recQ1) that blocks the RecF recombination pathway. *Mol Gen Genet* **195**, 474-480.
85. Lovett, S. T. & Clark, A. J. (1985). Cloning of the Escherichia coli recJ chromosomal region and identification of its encoded proteins. *J Bacteriol* **162**, 280-285.
86. Lovett, S. T. & Kolodner, R. D. (1989). Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the recJ gene of Escherichia coli. *Proc Natl Acad Sci U S A* **86**, 2627-2631.
87. Umezu, K., Nakayama, K. & Nakayama, H. (1990). Escherichia coli RecQ protein is a DNA helicase [published erratum appears in Proc Natl Acad Sci U S A 1990 Nov;87(22):9072]. *Proc Natl Acad Sci U S A* **87**, 5363-5367.
88. Courcelle, J. & Hanawalt, P. C. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. *Mol Gen Genet* **262**, 543-551.

89. Ganesan, A. K. & Hanawalt, P. C. (1985). Effect of a lexA41(Ts) mutation on DNA repair in recA(Def) derivatives of Escherichia coli K-12. *Mol Gen Genet* **201**, 387-392.
90. Ganesan, A. K. & Smith, K. C. (1969). Dark recovery processes in Escherichia coli irradiated with ultraviolet light. II. Effect of uvr genes on liquid holding recovery. *J Bacteriol* **97**, 1129-1133.
91. Wang, T. V. & Smith, K. C. (1984). recF-dependent and recF recB-independent DNA gap-filling repair processes transfer dimer-containing parental strands to daughter strands in Escherichia coli K-12 uvrB. *J Bacteriol* **158**, 727-729.
92. Hanawalt, P. C., Cooper, P. K., Ganesan, A. K. & Smith, C. A. (1979). DNA repair in bacteria and mammalian cells. *Annu Rev Biochem* **48**, 783-836.
93. Rupp, W. D., Wilde, C. E. I. I. I., Reno, D. L. & Howard-Flanders, P. (1971). Exchanges between DNA strand in uvtraviolet-irradiated Escherichia coli. *J Mol Biol* **61**, 25-44.
94. West, S. C., Cassuto, E. & Howard-Flanders, P. (1981). Mechanism of E. coli RecA protein directed strand exchanges in post-replication repair of DNA. *Nature* **294**, 659-662.
95. Courcelle, J., Ganesan, A. K. & Hanawalt, P. C. (2001). Therefore, what are recombination proteins there for? *Bioessays* **23**, 463-470.
96. Sakai, A. & Cox, M. M. (2009). RecFOR and RecOR as distinct RecA loading pathways. *J Biol Chem* **284**, 3264-3272.
97. Webb, B. L., Cox, M. M. & Inman, R. B. (1999). ATP hydrolysis and DNA binding by the Escherichia coli RecF protein. *J Biol Chem* **274**, 15367-15374.
98. Hegde, S. P., Rajagopalan, M. & Madiraju, M. V. (1996). Preferential binding of Escherichia coli RecF protein to gapped DNA in the presence of adenosine (gamma-thio) triphosphate. *J Bacteriol* **178**, 184-190.
99. Reardon, J. T. & Sancar, A. (2005). Nucleotide excision repair. *Prog Nucleic Acid Res Mol Biol* **79**, 183-235.
100. Orren, D. K., Selby, C. P., Hearst, J. E. & Sancar, A. (1992). Post-incision steps of nucleotide excision repair in Escherichia coli. Disassembly of the UvrBC-DNA complex by helicase II and DNA polymerase I. *J Biol Chem* **267**, 780-788.
101. Sancar, A. & Rupp, W. D. (1983). A novel repair enzyme: UVRABC excision nuclease of Escherichia coli cuts a DNA strand on both sides of the damaged region. *Cell* **33**, 249-260.
102. Rupp, W. D., Sancar, A. & Sancar, G. B. (1982). Properties and regulation of the UVRABC endonuclease. *Biochimie* **64**, 595-598.
103. Kato, T. & Shinoura, Y. (1977). Isolation and characterization of mutants of Escherichia coli deficient in induction of mutations by ultraviolet light. *Mol Gen Genet* **156**, 121-131.
104. Witkin, E. M., Roegner-Maniscalco, V., Sweasy, J. B. & McCall, J. O. (1987). Recovery from ultraviolet light-induced inhibition of DNA synthesis requires umuDC gene products in recA718 mutant strains but not in recA+ strains of Escherichia coli. *Proc Natl Acad Sci U S A* **84**, 6805-6809.

105. Jarosz, D. F., Beuning, P. J., Cohen, S. E. & Walker, G. C. (2007). Y-family DNA polymerases in Escherichia coli. *Trends Microbiol* **15**, 70-77.
106. Ling, H., Boudsocq, F., Woodgate, R. & Yang, W. (2001). Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. *Cell* **107**, 91-102.
107. Silvian, L. F., Toth, E. A., Pham, P., Goodman, M. F. & Ellenberger, T. (2001). Crystal structure of a DinB family error-prone DNA polymerase from Sulfolobus solfataricus. *Nat Struct Biol* **8**, 984-989.
108. Cox, M. M. (1999). Recombinational DNA repair in bacteria and the RecA protein. *Prog Nucleic Acid Res Mol Biol* **63**, 311-366.
109. Rocha, E. P., Cornet, E. & Michel, B. (2005). Comparative and evolutionary analysis of the bacterial homologous recombination systems. *PLoS Genet* **1**, e15.
110. Kowalczykowski, S. C. (2005). Cancer: catalyst of a catalyst. *Nature* **433**, 591-592.
111. Mohaghegh, P. & Hickson, I. D. (2001). DNA helicase deficiencies associated with cancer predisposition and premature ageing disorders. *Hum Mol Genet* **10**, 741-746.
112. Karow, J. K., Wu, L. & Hickson, I. D. (2000). RecQ family helicases: roles in cancer and aging. *Curr Opin Genet Dev* **10**, 32-38.
113. Yang, H., Li, Q., Fan, J., Holloman, W. K. & Pavletich, N. P. (2005). The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. *Nature* **433**, 653-657.
114. Rossi, M. L., Ghosh, A. K. & Bohr, V. A. (2010). Roles of Werner syndrome protein in protection of genome integrity. *DNA Repair (Amst)* **9**, 331-344.
115. Ellis, N. A. & German, J. (1996). Molecular genetics of Bloom's syndrome. *Hum Mol Genet* **5 Spec No**, 1457-1463.
116. Symington, L. S. (2002). Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* **66**, 630-70, table of contents.
117. Casey, G. (1997). The BRCA1 and BRCA2 breast cancer genes. *Curr Opin Oncol* **9**, 88-93.

CHAPTER II

ATP BINDING, HYDROLYSIS, AND PROTEIN DIMERIZATION ARE REQUIRED FOR RecF TO CATALYZE AN EARLY STEP IN THE PROCESSING AND RECOVERY OF REPLICATION FORKS DISRUPTED BY DNA DAMAGE

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ABSTRACT

In *Escherichia coli*, the recovery of replication following disruption by UV-induced DNA damage requires the RecF protein and occurs through a process that involves stabilizing the replication fork DNA, resection of the nascent DNA to allow the offending lesion to be repaired, and re-establishment of a productive replisome on the DNA. RecF forms a homodimer and contains an ATP binding cassette (ABC) ATPase domain that is conserved among eukaryotic structural maintenance of chromosome (SMC) proteins, including cohesin, condensin, and Rad50. Here, we investigated the function that RecF dimerization, ATP binding, and ATP hydrolysis have on the progressive steps involved in recovering DNA synthesis following disruption by DNA

damage. RecF point mutations with altered biochemical properties were constructed into the chromosome. We observed that protein dimerization, ATP binding, and ATP hydrolysis were essential for maintaining and processing the arrested replication fork, as well as restoring DNA synthesis. In contrast, stabilization of the RecF protein dimer partially protected the DNA at the arrested fork from degradation, although the overall processing and recovery remained severely impaired.
Keywords: RecF, DNA binding, DNA Replication, Structural Maintenance of Chromosome (SMC) proteins, ATP Binding Cassette (ABC) ATPases

INTRODUCTION

RecF is part of a ubiquitous family of recombination mediator proteins that includes RAD52, BRCA2, BLM, and WRN. These proteins are required to maintain genomic stability, but their precise cellular functions remain poorly understood [1-7]. The structure of the RecF protein of *Escherichia coli* reveals a strong similarity to the globular head domain of human Rad50, a protein involved in detection and repair of double-strand DNA breaks [8]. Both RecF and Rad50 contain a conserved ATP binding cassette (ABC)-type ATPase, which is conserved among many structural maintenance of chromosome (SMC) proteins, DNA repair enzymes, and membrane transporters [9-13].

In *E. coli*, RecF function is required together with RecO and RecR, for replication to resume following disruption by DNA damage [14-17]. In the absence of any one of these genes, the replication forks are not maintained following arrest, the nascent DNA at the arrested fork is extensively degraded, and DNA synthesis fails to resume [15, 16, 18]. Either

coordinately or subsequent to RecF-O-R binding, the nascent lagging strand of the arrested fork is partially degraded by the combined action of RecQ, a 3' – 5' helicase, and RecJ, a 5' – 3' nuclease [16, 18]. The nascent DNA degradation is thought to restore the lesion-containing region to a double-stranded form that can be repaired by nucleotide excision repair and it is essential for the rapid recovery of DNA synthesis [19, 20]. In the absence of either processing or repair, the recovery of replication remains dependant on the RecF pathway but occurs through the action of translesion synthesis polymerases [19, 20]. Once the lesion is removed or bypassed, an active and functional replisome must be restored to allow replication to resume [19, 20]. The precise role RecF has in processing the disrupted replication fork remains unclear and could occur at any of several steps during the recovery process, including the initial binding and recognition of the disrupted fork, the processing or regression of the fork structure away from the offending lesion, or the re-establishment of an active replisome once the lesion has been removed or bypassed (Figure 1).

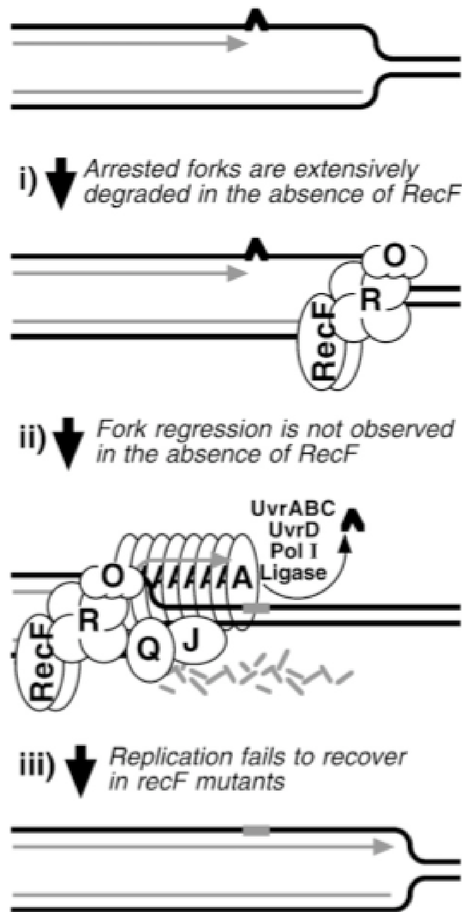

Figure 2.1. Processing events involved in the recovery of replication following disruption by DNA damage and the phenotypes observed in the absence of RecF. i) Replication is arrested following UV-induced DNA damage. In vitro, RecF is proposed to direct RecR and RecO to DNA junctions and initiate the formation of a RecA filament at these sites, an activity that has been proposed to help target RecA to the arrested replication fork in vivo [21, 22] ii) Either subsequent to or concurrent with this occurring, the RecQ helicase and RecJ nuclease partially degrade the nascent lagging strand of the arrested DNA fork. The partial degradation is required for the rapid recovery of replication and is thought to restore the region containing the lesion to a double strand form that allows nucleotide excision repair to remove of the obstructing lesion [18, 20]. In the absence of RecF, this degradation is much more extensive and eventually leads to the loss of the replication fork's integrity [23] iii) Once the lesion has been removed, an active replisome must be re-established. It is not clear which components of the replisome are disrupted upon encountering a UV-induced lesion, although some evidence suggests that the replicative helicase remains bound [24], suggesting that the replisome may remain at least partially intact. It is possible that RecF, along with RecO-R and RecA, may stabilize the nascent leading strand on the template to allow the replisome to resume from this structure. In the diagram, RecA, RecO, RecR, RecQ, and RecJ are denoted as A, O, R, Q, and J, respectively.

The crystal structure and solution studies of the RecF protein revealed that the protein forms a dimeric clam structure and contains an ABC ATPase domain [8]. Similar to the ABC ATPase domains found in other SMC-like proteins, RecF contains three conserved motifs termed the Walker A, Walker B, and signature motif. The signature motifs of ABC ATPases mediate ATP-dependent dimerization, with ATP bound at the interface of two opposing molecules [25-28]. On RecF, the Walker A and Walker B motifs are located in ATPase domain 1 at the N-terminus and C-terminus, respectively, while the signature motif resides in domain 2 on the protein [8]. RecF lacks the striking coiled-

coil region between the N- and C-terminal of the globular head domain that is seen in other SMC-like proteins and Rad50, but is otherwise structurally similar to Rad50 and exhibits both the ATP-dependent DNA binding and DNA-dependent ATP hydrolysis characteristic of SMC proteins [8, 29-31].

Table 1. Targeted mutations in RecF

Amino acid change	Location in protein	Functional prediction based on other SMC proteins	Confirmed for RecF *in vitro*
K36>M	Walker A motif	Prevents ATP binding	Yes
K36>R	Walker A motif	Prevents ATP hydrolysis	Yes
D303>N	Walker B motif	Stabilizes protein dimerization	Yes
S270>R	Signature motif	Prevents protein dimerization	Yes
Q273>A	Signature motif	Prevents protein dimerization	No

The precise catalytic function(s) of the conserved RecF motifs in processing and restoring arrested replication forks remain uncharacterized, although a number of biochemical characterizations are consistent with the idea that they could participate in any of the several progressive steps associated with the recovery process. Purified RecF, RecO, and RecR are able to displace single-stranded DNA-binding protein (SSB) and enhance the nucleation of a RecA filament on DNA [32-35]. Although RecO and RecR proteins are sufficient to perform this reaction *in vitro*, RecF greatly stimulates the process in the presence of dsDNA fragments [5, 21, 22]. Further, RecF appears to play an important role in targeting this nucleation to regions that contain a single to double strand DNA junction and that have a 5' DNA end [5, 21, 29]. These observations are consistent with the idea that RecF may act to recognize the arrested replication forks and catalyze the loading of a RecA filament.

Other studies have suggested that RecF modulates both the ability of RecA filaments to form on single strand regions and the RecA-mediated strand exchange

reaction in a way that would enhance fork regression [34, 36, 37]. Consistent with this idea, *in vitro*, RecF cycles through a complex pathway that involves ATP-dependent dimerization, DNA binding, and repeated interactions with RecR in a DNA substrate-dependent manner [21, 31, 34].

Still other studies support the idea that RecF-O-R along with RecA may functionally interact with the replication machinery and have a direct role in removing the polymerase from its arrest site or re-establishing the replisome after the lesion has been repaired. In reconstituted replication assays, RecF, -O, and –R along with RecA are able to displace a stably bound polymerase from SSB-bound DNA to expose the arresting lesion [38]. Also, suggestively, both *recF* and *recR* are co-regulated and transcribed on the same operon with the replisome's *dnaN* and *dnaX* genes, respectively [39, 40]. These observations are consistent with the possibility that the conserved RecF motifs may function together with RecA to catalyze disassembly or re-establishment of the replisome at the site of replication disruption.

To characterize the role the conserved motifs have in the process of replication recovery following UV-induced arrest, we constructed five *recF* point mutations into the *E. coli* chromosome. Using the highly conserved RecF from *D. radioadurans*, these mutations have been characterized biochemically demonstrated to generate proteins that either disrupt or stabilize RecF dimerization, prevent ATP binding or prevent ATP hydrolysis (Table 1). We then characterized the molecular events that occur during the progressive steps of restoring replication following disruption in strains containing these altered RecF proteins.

EXPERIMENTAL PROCEDURES

BACTERIAL STRAINS

SR108 is a thyA36 deoC2 derivative of W3110 [41]. CL579 (*recF*6206::tet-857) has been previously described [16]. Gene replacements of *recF* were constructed using the recombineering strain DY329 [42]. A cat-sacB cassette was PCR amplified from plasmid pEL04 [43] using primers 5'CGGCTTATGTTGTCATGCCAATGAGACTGTAATGTCCCTCCCTGTGACGGAA GATCACTTCG3' and 5'CATCAACGTTTCTCGCTCATTTATACTTGGGTTAATCC GTCTGAGG TTCTTATGGCTCTTG3'. The PCR product was then transformed into DY329 to generate CL1204 (*recF*::cat-sacB) selecting for chloramphenicol resistance. The kanamycin resistance cassette was then inserted into the region downstream from *tnaA* in this strain using primers 5'CACTTCACCGCAAAACTTAAAGAAGTTTAATTAATACTACTATGGACAGCA AGCGAACCG3' and 5'TAGAGGAAGGCTATTTTTGTTATTGAGGATGTAGGGTAAGTCAGAA GAACTCGTCAAGAAG3' to amplify the *kan*^{R} cassette from Tn5. The PCR product was transformed into CL1204 to generate CL1206 (*recF*::cat-sacB *tna*::kan).

Point mutations were initially generated using the Quick-change II site-directed mutagenesis kit (Stratagene) on the plasmid pMalF6 that had the *recF* gene cloned into the BamHI site as previously reported [8]. Gene replacements on the chromosome were then made by amplifying the *recF* point mutations from the plasmids using primers 5'GCCAGAGCGCGGCTTATGTTGTCATGCCAATGAGACTGTAATGTCCCTCAC

CCGCTTGTTG and

5'AGAATTCGACATCAACGTTTCTCGCTCATTTATACTTGGGTTAATCCGT

TATTTTACCCTT. The products were then transformed into CL1206 to generate CL1375 (*recF*K36M *tna:kan*), CL1377 (*recF*K36R *tna:kan*), CL1379 (*recF*S270R *tna:kan*), CL1402 (*recF*D303N *tna:kan*) and CL1535 (*recF*Q273A *tna:kan*) by selecting for sucrose resistance. The point mutations were then moved into our parental background SR108 by P1 transduction and selecting for kanamycin resistance to generate CL1412 (*recF*(K36M *tna:kan*), CL1414 (*recF*(K36R) *tna:kan*), CL1416 (*recF*(S270R) *tna:kan*), CL1418 (*recF*(D303N)*tna:kan*), and CL1569 (*recF*(Q273A) *tna:kan*). In each case, the presence of the *recF* mutation conferred hypersensitivity to UV irradiation.

DEGRADATION OF NASCENT DNA

Fresh overnight cultures were diluted 1:100 and grown in Davis medium, supplemented with 0.4% Glucose, 0.2% Casamino acid, 10 μg/mL thymine (DGCthy) and 44-thymine (0.1 μCi/10 μg/mL) to an OD_{600} of 0.4 in a 37°C shaking water bath. Cultures were then pulse-labeled with 3H-thymidine (1 μCi/10 μg/mL) for 5 seconds and then filtered on Whatman 0.4-μm membrane filters and washed twice with 3-mL of cold NET (100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA) buffer. The filter was then resuspended in pre-warmed non-radioactive DGCthy media and immediately UV-irradiated with 30 J/m^2 and incubated in a 37°C shaking water bath. At the times indicated, duplicate 200-μL aliquots of the culture (triplicate at time 0) were taken. Cells were lysed and the DNA was precipitated by the addition of 5-mL ice cold 5%

trichloroacetic acid and then collected on Fisherbrand 2.5-cm glass fiber filters. The amount of radioactivity in each filter was determined using a liquid scintillation counter.

TWO-DIMENSIONAL AGAROSE GEL ELECTROPHORESIS

The 2-dimensional gel electrophoresis technique for *E. coli* containing the pBR322 has been described previously [16]. Briefly, fresh cultures containing the pBR322 plasmid were diluted 1:100 in 10 mL of DGCthy medium and grown to an OD_{600} of 0.4 at 37°C. The cultures were then irradiated with 50 J/m^2 of UV irradiation (254 nm). 750-μL aliquots of the irradiated cultures were taken at 0-, 15-, and 30-min after UV irradiation and placed in 750-μL ice-cold NET buffer. The cells were then pelleted in a microcentrifuge and lysed in 140 μL of TE (10 mM Tris pH 8.0, 1 mM EDTA) containing 2 mg/mL lysozyme and 100 μg/mL RNAse A and incubated at 37°C for 30 min. Then, 10-μL each of 10 mg/mL of proteinase K and 20% Sarkosyl were added to the suspension and incubated at 37°C for another 30 min. Following extraction with 4 volumes of phenol:chloroform, samples were dialyzed for 2 hours in TE buffer on 47-mm Whatman 0.05-μm pore disks (Whatman #VMWP04700) floating on a 250-mL beaker of TE. The samples were then digested with PvuII (Fermentas) and extracted with chloroform before loading onto an agarose gel. Samples were run in a 0.4% agarose gel with 1X TBE buffer for 17 hours at 25 V. For the second dimension, the gel lanes were sliced out, rotated 90°, and recast in a 1.0% agarose gel with 1X TBE buffer and run for 7 hours at 200 V. Gels were transferred to Hybond N+ nylon membranes and probed with pBR322 that had been labeled with ^{32}P by nick translation according to the protocol

supplied by Roche using alpha-32PdCTP (MP Biomedicals). Radioactivity was visualized and quantified using a Storm 820 Phosphorimager and ImageQuant software (Molecular Dynamics).

RECOVERY OF DNA SYNTHESIS

Fresh overnight cultures were diluted 1:100 in 20-mL DGCthy medium supplemented with 44-thymine (0.1 μCi/10 μg/mL) and grown to an OD_{600} of 0.3 in a 37°C shaking water bath, at which time the culture was split. Half of the culture was irradiated with 30 J/m^2 UV, and the other half was mock-irradiated. At the times indicated, duplicate 0.5-mL aliquots of the culture were pulse-labeled with 3H-thymidine (1 μCi/10μg/mL) for 2 minutes. The cells were then lysed and the DNA precipitated by the addition of 5-mL ice-cold 5% trichloroacetic acid. The precipitate was collected on Fisherbrand 2.5-cm glass fiber filters and the amount of radioactivity in each sample was measured using a liquid scintillation counter.

UV SURVIVALS

Fresh overnight cultures were diluted 1:100 and grown in DGCthy medium to an OD_{600} of 0.4 in a 37°C shaking water bath. At this time, cultures were serially diluted and plated in triplicate on Luria Bertani plates supplemented with 10 μg/mL thymine and irradiated on a rotary platform using a Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 $J/m^2/sec$ at the indicated doses. Plates were incubated overnight at 37°C and colonies were counted the next day to determine the surviving fraction.

RESULTS

The crystal structure of RecF revealed that a strong structural similarity to human Rad50, a protein required to recognize and catalyze the repair of double strand breaks in humans [8]. The biochemical analysis demonstrated that RecF contains a conserved ATP binding cassette (ABC) ATPase domain and forms a dimeric clam-like structure that is capable of accommodating single- or double-strand DNA. At the time, we hypothesized that, upon dimerization, the protein could function as a clamp loader that targets a tetrameric RecR ring to regions of single- to double-strand DNA junctions, and subsequently RecO monomers [8, 21]. However, RecF could participate in any or several of the progressive steps associated with the recovery process.

To characterize the functional role of the ABC ATPase domain in the recovery process, we constructed several point mutants that were predicted to have altered RecF functions based on their homology to Rad50 and other SMC-like proteins (Table 1). In previous work, many of these altered proteins have been characterized biochemically using the RecF protein from *D. radiodurans* and confirmed to produce their predicted effect [8].

We initially cloned these *recF* mutants into expression plasmids pQE9 and pMalp2. When these over-expression plasmids were transformed into *E. coli* deleted for *recF*, the altered proteins rendered the cells hypersensitive to UV irradiation to varying extents ([8], data not shown). However, previous studies have shown that altering the intracellular ratio of RecF, RecO, and RecR can affect their normal function and *in vitro* and *in vivo*, and that an excess of RecF can have an inhibitory effect in presynaptic

complex formation [36,45]. Therefore, we chose to reconstruct the altered *recF* genes directly into the chromosome using gene replacements, rather than characterizing their effects when expressed from plasmids. This avoids the possibility that an observed effect is due to abnormal expression levels and ensures that all regulatory elements controlling its endogenous expression are functional.

We have developed a number of molecular assays to monitor the effect that RecF has on replication fork maintenance, replication fork regression, limiting the fork processing by RecJ and RecQ, and restoring DNA synthesis, *in vivo*. An advantage of this type of cellular approach is that it allows us to directly observe RecF function in an environment that contains all of RecF's natural endogenous substrates and multiple protein partners, which would otherwise not be possible to reconstruct biochemically.

The ABC ATPase activity of RecF is required to recognize and protect the nascent DNA ends at arrested replication forks.

Following disruption by UV-induced damage in wild type cells, the nascent DNA at the replication forks is partially degraded by the combined action of the RecQ 3'–5' helicase and the RecJ 5'–3' nuclease [16,18]. RecF is required to maintain the replication fork DNA and limit the nascent DNA processing that occurs following the disruption of replication [15,16]

To examine whether the altered RecF proteins retain the ability to carry out this step in the recovery process, cultures grown in the presence of 44-thymine were pulse-labeled with 3H-thymidine for 5 seconds and then immediately transferred to pre-

warmed, non-radioactive media and irradiated with 30 J/m^2 of UV irradiation. At 20-min intervals, aliquots of the culture were lysed and the radioactivity in the DNA was quantified to determine whether the nascent DNA remained protected and to determine how much degradation occurred in both the overall genomic DNA (^{14}C) and the nascent DNA synthesized just prior to the arrest of replication (^{3}H).

Similar to previous studies, when we examined the degradation that occurred following UV irradiation in wild type cultures, a limited degradation of the nascent DNA occurred at early times after irradiation (Figure 2). The amount of 3H-labeled DNA decreased approximately 10% initially, but began to increase at later times, even exceeding 100% of the initial DNA labeled. In principle, the amount of 3H-labeled DNA should only be able to decrease over time. In previous work, we've shown that the increase at later times is likely due to re-incorporation of the intracellular pools once replication has resumed [15, 18]. In cultures lacking the *recF* gene, we observe that the degradation of the nascent DNA was more extensive and continued for the first 100 minutes until approximately 50% of the pulse label in the nascent DNA had been degraded (Figure 2). This observation has previously been interpreted to indicate that RecF is required to recognize and protect the replication fork DNA following disruption.

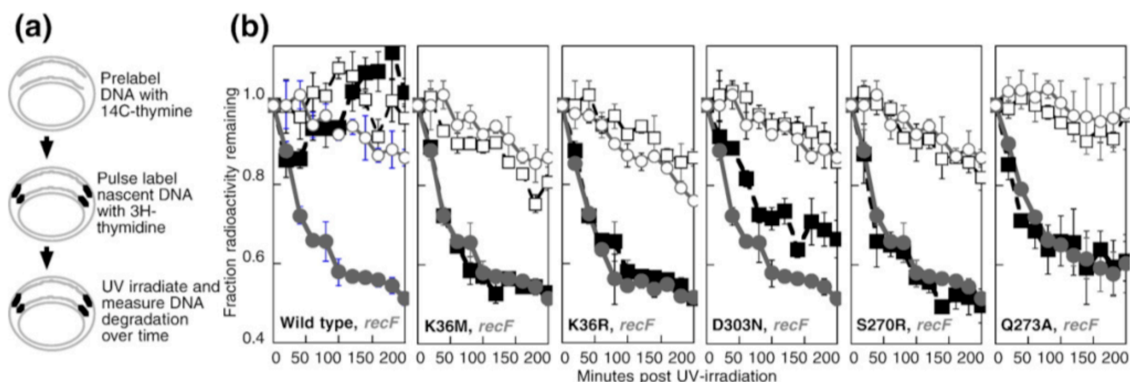

Figure 2.2 A *recF* D303N mutation that stabilizes RecF in its dimeric form partially retains the ability to protect the nascent DNA from degradation following disruption by UV-induced damage. However, *recF* mutations that prevent dimerization (S270R Q273A), ATP-binding (K36M), or ATP hydrolysis (K36R) are as defective as a null mutation. (A) Schematic diagram of the approach used to monitor degradation occurring at the nascent DNA and in the overall genome. Cultures prelabeled with ^{44}-thymine were pulse-labeled for 5 seconds with ^{3H}-thymidine before being resuspended in non-radioactive medium and UV irradiated with 30 J/m2. Aliquots of the culture were taken at various times following UV irradiation and the fraction of radioactivity remaining in DNA was plotted over time. (B) The fraction of radioactive nucleotides that remain in the total DNA (^{14}C, open squares) and newly synthesized DNA (^{3}H, filled squares) in wild type and five *recF* point mutants following disruption by UV-induced damage is plotted. The amount of degradation occurring in the total DNA (^{14}C, open circles) and newly synthesized DNA (^{3}H, filled circles) of the *recF* deletion mutant is plotted in grey in each panel for comparison. Initial counts per minute were between 5,000 – 6,000 for ^{3}H and 1,000 – 2,000 for ^{14}C in each experiment. Each graph represents at least two independent experiments. Error bars represent the standard error of the mean.

When we examined the altered *recF* mutants in this assay, we observed that the pattern of nascent DNA degradation was nearly identical to the *recF* deletion mutant for the K36M, K36R, S270R, and Q273A mutations. The results indicate that ATP binding and hydrolysis, as well as RecF dimerization, are essential to the ability of RecF to protect and limit the degradation of nascent DNA ends at disrupted replication forks from extensive degradation. In contrast, the *recF* D303N point mutation, which has been shown biochemically to trap the ATP-bound RecF protein in its dimeric form, partially retained the ability to protect the nascent DNA from degradation, relative to the deletion mutant. However, the amount of nascent DNA degradation that occurred in the D303N

point mutation still exceeded that occurring in wild-type cells. Taken together, the results could be consistent with the idea that RecF dimer formation but not ATP hydrolysis-dependent disassociation are required to initiate recruitment of the protein partners to protect the disrupted replication fork. The inability of the D303N mutant to restore nascent DNA protection to wild-type levels suggests that the ATP-bound dimeric form of the protein is loaded onto the DNA less efficiently. This type of function would be consistent with the idea that RecF plays an initiating role in the recruitment of factors to protect the nascent DNA.

Alternatively, the partial restoration of nascent DNA protection could also be consistent with the idea that RecF must repeatedly dimerize and disassociate on the DNA during the recovery process. Equivalent mutations to the D303N change in other SMC proteins can retain some residual ATP hydrolysis activity [26]. Although the *recF* D303N mutant did not retain any ATP hydrolysis *in vitro* [8], we cannot rule out the possibility that *in vivo*, in the presence of RecR or other protein partners, some residual ATP-hydrolysis may occur that allows the stabilized dimer to cycle, albeit with greatly reduced efficiency. This type of role would be more consistent with the idea that RecF plays a more active role in the processing and regression of the replication fork DNA during the recovery process.

The ABC ATPase activity of the RecF protein is required for processing replication forks disrupted by DNA damage.

In vivo, the processing of replication forks following disruption by DNA lesions involves a transient regression of the fork structure that persists until a time that correlates with when the lesions are repaired and replication resumes. The processing and intermediate structures that occur at the replication fork during the recovery process can be visualized on plasmids such as pBR322 *in vivo*. In previous work, the formation of these processing intermediates has been shown to depend on the RecF protein [16].

To examine whether the replication fork processing and intermediates occurred in strains expressing the altered RecF proteins, we examined the structural intermediates that occurred on replicating plasmids of pBR322 in each strain using two-dimensional agarose gel analysis. Strains containing the plasmid were irradiated with 50 J/m^2 UV irradiation and then sampled immediately following irradiation and at 15 and 30 min post-UV irradiation. This dose and recovery period produces an average of one lesion per plasmid strand [16]. At the times indicated, genomic DNA was purified from each sample, and digested with PvuII, which linearizes the plasmid just downstream from its origin of replication. The intermediate structures were then visualized by Southern analysis following separation in two-dimensional agarose gels. In this technique, non-replicating plasmids migrate through the gel as a linear 4.4 kb fragment, forming a prominent large spot. Replicating plasmids are observed as Y-shaped structures that migrate more slowly through the gel, due to their larger size and non-linear shape, and form an arc that extends out from the linear fragments (Figure 3). Following UV

irradiation, transient replication intermediates are observed that have a double-Y or X-shaped structure. The non-linearity of these structures causes the intermediates to migrate even more slowly and are observed as a cone region above the arc of replicating Y-structures.

In the absence of UV irradiation, only Y-shape replication intermediates are observed. Following UV irradiation in wild type cultures, however, both Y-shaped replication intermediates and cone region intermediates are observed to accumulate. Previous work has shown that a portion of the cone region intermediates are associated with a RecF-dependent processing of the replication fork, prior to the time that replication resumes [16]. In cells without a functional copy of *recF*, the processing intermediates in the cone region are not observed and replicating intermediates remain and accumulate as simple Y-shaped structures. When we examined each of the strains containing an altered *recF* gene, we observed that, in each case, the cone region intermediates were absent or greatly reduced (Figure 3). This observation indicates that the RecF-protein's ATP hydrolysis and dimerization dynamics are required for the accumulation of replication fork intermediates and processing that occurs following disruption. Unlike the previous assay where the stabilized RecF dimer (*recF* D303N) mutant retained some modest protective functions with respect to degradation at the replication fork, no increase in replication fork intermediates were observed relative to the other *recF* mutants. However, it is possible that a modest retention of activity, such as was seen with the *recF* D303N mutant's ability to protect the nascent DNA in the previous assay, would be below a level that could be detected by this type of assay.

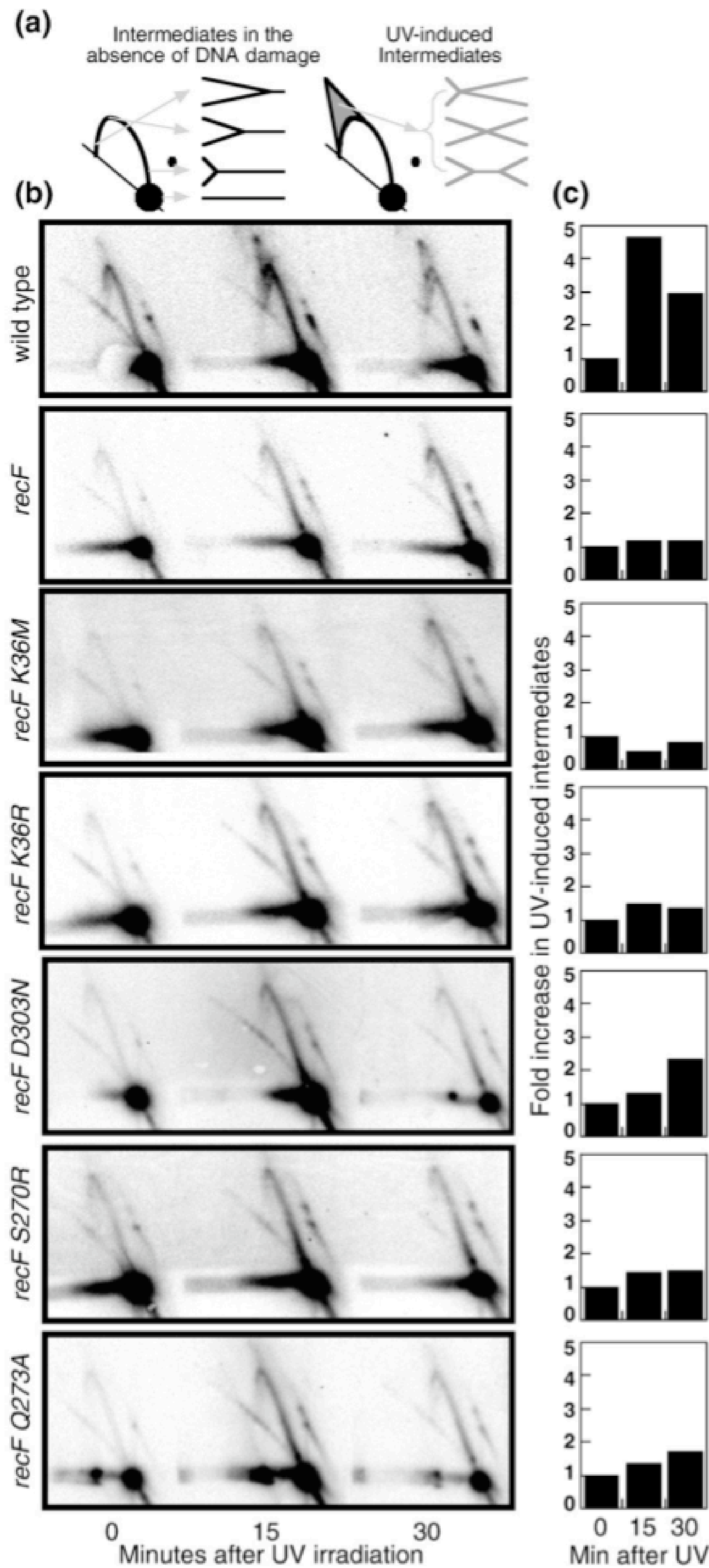

Figure 2.3 Mutations that affect RecF protein dimerization (S270R Q273A), ATP-binding (K36M), and ATP hydrolysis (K36R) are unable to process replication forks following disruption by UV-induced DNA damage. A) Diagram depicting the replication intermediates observed in the absence or presence of UV-induced DNA damage. B) Cells containing the plasmid pBR322 were exposed to 50 J/m^2 of UVC-irradiation. At the indicated times, the genomic DNA was purified, digested with PvuII, and the structural intermediates for each strain were observed using a 2D-agarose gel. C) The increase in UV-induced intermediates at each time point is plotted relative to time 0. UV-induced intermediates were quantified by determining the amount of radioactivity migrating in the cone region of UV-induced intermediates, normalized against the total amount of non-replicating linear DNA.

The ABC ATPase activity of the RecF protein is required to re-establish the replication machinery and resume DNA synthesis following DNA damage that blocks replication.

Following the processing and restoration of the damaged region, survival requires the re-establishment of a functional replisome at, or proximal to, the site of disruption. This latter step fails to occur in the absence of RecF and can be monitored by adding radioactive nucleotide precursors to the media and following their rate of incorporation into the DNA [15, 19].

To examine the DNA synthesis in the *recF* point mutants, we monitored the overall DNA accumulation and the rate that synthesis recovered following UV-induced DNA damage. To this end, duplicate aliquots of cultures grown in the presence of 44-thymine were pulse-labeled with 3H-thymidine for 2 min at various times following either 30 J/m^2 UV irradiation or mock-irradiation. In this way, the rate of DNA synthesis occurring (^3H incorporation/2min) and the overall DNA accumulation (^{14}C incorporation) at various times during the recovery period could be determined relative to that occurring in un-irradiated cultures.

By this assay, the rate of DNA synthesis of irradiated wild-type cultures initially decreased by approximately 90% but had recovered to a rate nearing pre-irradiation levels within 100 min post-irradiation. Similarly, the overall accumulation of DNA in the irradiated cultures increased within this time to approach that of the un-irradiated culture. By comparison, although the rate of synthesis in irradiated *recF* deletion strain cultures was reduced to a similar extent, the rate did not recover and little further DNA was observed to accumulate within the time course that was examined (Figure 4). When we

examined the recovery of DNA synthesis in cultures containing the altered forms of *recF*, all mutants were severely impaired in their ability to resume DNA synthesis. Similar to the initial assay, the possible exception to this was observed for the *recF* D303N mutant, which stabilizes RecF in its dimeric form. In this mutant, we observed a very modest increase in the rate of synthesis over the time course of recovery. However, although the trend was repeatedly observed in individual assays, the overall amount of synthesis was not significant when compared to other point mutations.

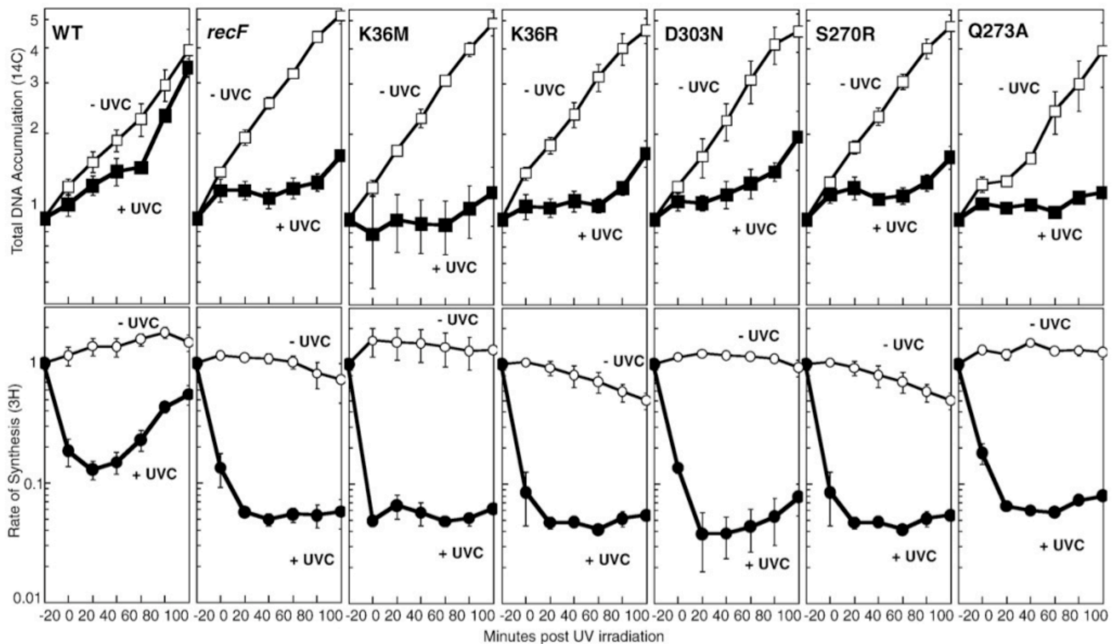

Figure 2.4 RecF protein dimerization, ATP-binding, and ATP hydrolysis is required to resume DNA synthesis following disruption by UV-induced DNA damage. ^{44}-thymine labeled cultures were pulse labeled with ^{3H}-thymidine for 2 minutes at the indicated times following either 30 J/m^2 UVC irradiation (filled symbols) or mock irradiation (open symbols) at time 0. The relative DNA accumulation (^{14}C, squares, top panel) and the rate of DNA synthesis (^{3}H, circles, bottom panel) are plotted. Each graph represents at least 3 independent experiments. Error bars represent the standard error of the mean.

Finally, the survival of each strain expressing an altered RecF protein was compared to wild-type cultures and strains deleted for *recF*. Fresh overnight cultures were spot diluted onto LB agar plates, exposed to UV at the indicated doses, and incubated at 37°C overnight. Cells that survived to form colonies were counted and the fraction of colonies surviving was determined.

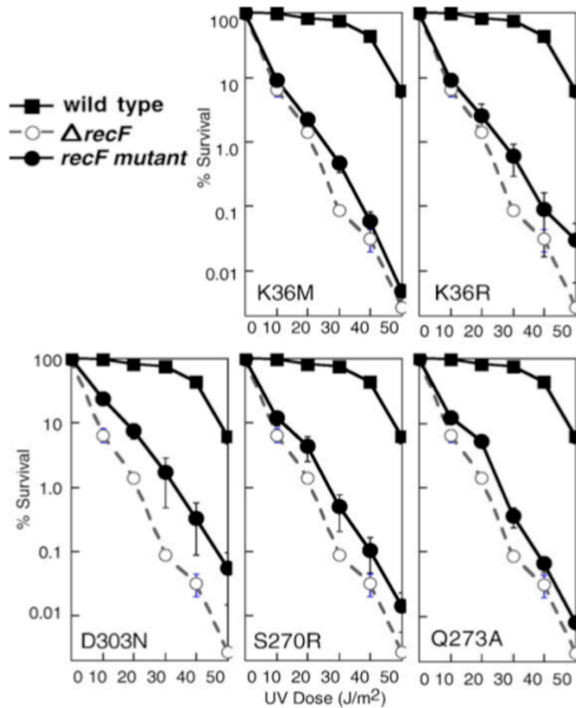

Figure 2.5 RecF protein dimerization, ATP-binding, and ATP hydrolysis is required for resistance to UV-induced DNA damage. The percent of cells surviving the indicated doses of UVC-irradiation is plotted for the following *recF* point mutations K36M, K36R, S270R, D303N, and Q273A (filled squares) as indicated. The survival for wild type (filled squares) and the *recF* deletion strain, (open circles) is plotted on each graph for comparison. Survival curves represent at least 3 independent experiments. Error bars represent the standard error of the mean.

Cells containing the *recF* K36M, K36R, S270R, or Q273A point mutations were as hypersensitive to UV-irradiation as a mutant that was deleted for *recF*. By comparison, the *recF* D303N point mutant was approximately one order of magnitude more resistant to UV at each of the examined doses. Taken together with the previous assays, these results indicate that ATP binding and hydrolysis, as well as the ability of RecF to form protein dimers are essential for RecF function in processing and restoring

replication after disruption by UV-induced damage. By contrast, although the effect was modest, the *recF* D303N mutant, which forms stable RecF-protein dimers, appeared to retain some minimal activity relative to a *recF* deletion mutant with respect to protection of the nascent DNA of the disrupted fork, allowing DNA synthesis to resume, and, ultimately, allowing cells to survive. The result would suggest that in this mutant, the initial round of RecF loading and interactions can occur, and in a small fraction of cases, this may be sufficient to allow survival and recovery to occur. However, it suggests that RecF dimerization and ATP hydrolysis involves repeated cycling *in vivo* as has been observed to occur *in vitro* when interacting with RecR [21, 31]. This cycling appears to be important for full resistance and function *in vivo*.

DISCUSSION

The RecF protein is required for survival following the disruption of replication by DNA damage, a process that includes maintaining the structure of the replication fork DNA, protecting and processing the DNA ends at arrested replication forks to allow repair enzymes to access and repair the damage, and, finally, the re-establishment of an active replisome at the site of disruption [15, 16, 18, 46]

Here, we investigated how altering the ATP binding, hydrolysis, and protein dimerization activities of RecF affects each of the progressive steps involved in restoring replication following arrest of the replication fork. We initially hypothesized that one or more of these altered RecF proteins may retain the ability to bind and protect the replication fork, but fail to proceed further into the recovery process. Instead, we

observed that ATP binding, ATP hydrolysis, protein dimerization, and, to a lesser extent, the ability of the protein dimers to dissociate upon ATP hydrolysis, each rendered cells deficient in every step associated with the recovery of replication. The inability of these mutants to maintain the replication fork DNA or limit the nascent DNA processing argues that RecF is involved in catalyzing an early step in the recovery process. Although it does not preclude the possibility that RecF could also participate in a later steps of the recovery process.

Two possible initiating roles for the RecFOR complex in the recovery process are consistent with biochemical studies. *In vitro,* RecFOR is able to displace SSB from single-strand DNA to allow for RecA to bind and initiate filament formation [5, 33, 47, 48]. *In vivo,* the failure of *recF* deletion strains to maintain the replication fork DNA and to limit the nascent DNA degradation support this type of role, since DNA ends are rapidly degraded in the absence of RecA binding [15, 16, 18]. Similarly, *recF* deletion mutants exhibit a delayed induction of the SOS response, which requires loading RecA filaments on to single strand DNA before activation can occur [49-52].

In addition to displacing SSB, other studies have shown that RecFOR along with RecA is capable of displacing a DNA polymerase from its template when it is arrested at a DNA lesion [38]. This type of activity may be critical to orient the RecA-catalyzed fork regression and allow repair enzymes to gain access to the offending lesion. *In vivo*, after UV-induced damage, replication forks remain stalled in cells that lack the enzymes to repair or resect the nascent DNA away from the lesion [19, 20]. In these cells, the recovery of DNA synthesis depends entirely on translesion synthesis by Polymerase V, which is

induced in an active form only 50 min after SOS induction. It has also been reported that Polymerase V function is also depends directly on RecF function in addition to RecA [53], although other studies have reached different conclusions as to whether this effect is direct or indirect [51, 54, 55].

In vitro, RecO and RecR interact and, at high concentrations, these proteins are able to displace SSB and load RecA in the absence of RecF [22, 32, 47, 56]. The presence of RecF appears to be important for reducing the protein concentrations required for this reaction to occur and for targeting the RecFR complex to appropriate single- to double-strand DNA junctions, such as those found at arrested replication forks, although this targeting appears to occur through complex interactions with RecF protein partners [5, 21, 31, 34]. In solution, *Deinococcus radiodurans* RecR forms a tetrameric clamp capable of encircling DNA, and interacts with RecF in a 4:2 RecR:RecF stoichiometry [21, 57]. RecF ATP-dependent dimers form a clam-like structure whose mouth is capable of accommodating single- or double-strand DNA [8]. Thus, the ATP-dependent interaction of RecF and RecR suggests a role for RecF in initial steps of damage recognition. Interestingly, Koroloev's group recently demonstrated ATPase-dependent selectivity of RecF/RecR complex towards dsDNA substrates [21]. Thus, RecF may provide specificity towards initial placement of RecA loading at boundaries of ss- and dsDNA, and it may be important for limiting RecA filament formation within ssDNA regions [5, 22, 31, 34, 58]. The observation that RecF D303N mutants, stabilized in their dimeric form, partially protect nascent DNA at arrested replication forks may also be consistent with the idea that the dimeric clam-like structure is critical and could play a more dynamic role in later steps of

the recovery process. Although the purified D303N RecF protein dimer did not display any residual ATPase activity, we cannot rule out the possibility that it may retain some ability to hydrolyze ATP when in the presence of other protein partners such as RecR [8, 21, 26].

RecF displays a strong degree of structural similarity to the human Rad50 protein and has homology to several eukaryotic SMC proteins whose biochemical function remains poorly understood but that are critical for maintaining genome stability and resistance to DNA damage [1-4, 6, 7, 59, 59]. Taken together, the results presented here support the idea that RecF plays an initiating role in the process by which disrupted replication forks are processed and restored. Further, they demonstrate that ATP binding and hydrolysis, as well as RecF dimer formation and dissociation are critical activities that allow the RecF protein to initiate the recovery process *in vivo*. The conservation between RecF and Rad50 provides an opportunity to use structure-guided biochemical and cellular approaches with RecF to dissect the mechanism by which this important class of proteins function in both prokaryotes and eukaryotes.

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REFERENCES

1. Gray, M. D., Shen, J. C., Kamath-Loeb, A. S., Blank, A., Sopher, B. L., Martin, G. M., Oshima, J. & Loeb, L. A. (1997). The Werner syndrome protein is a DNA helicase. *Nat Genet* **17**, 100-103.
2. Karow, J. K., Chakraverty, R. K. & Hickson, I. D. (1997). The Bloom's syndrome gene product is a 3'-5' DNA helicase. *J Biol Chem* **272**, 30611-30614.
3. Prince, P. R., Emond, M. J. & Monnat, R. J. J. (2001). Loss of Werner syndrome protein function promotes aberrant mitotic recombination. *Genes Dev* **15**, 933-938.
4. Yamagata, K., Kato, J., Shimamoto, A., Goto, M., Furuichi, Y. & Ikeda, H. (1998). Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast sgs1 mutant: implication for genomic instability in human diseases. *Proc Natl Acad Sci U S A* **95**, 8733-8738.
5. Morimatsu, K. & Kowalczykowski, S. C. (2003). RecFOR Proteins Load RecA Protein onto Gapped DNA to Accelerate DNA Strand Exchange. A Universal Step of Recombinational Repair. *Mol Cell* **11**, 1337-1347.
6. Thorslund, T. & West, S. C. (2007). BRCA2: a universal recombinase regulator. *Oncogene* **26**, 7720-7730.
7. Gasior, S. L., Olivares, H., Ear, U., Hari, D. M., Weichselbaum, R. & Bishop, D. K. (2001). Assembly of RecA-like recombinases: distinct roles for mediator proteins in mitosis and meiosis. *Proc Natl Acad Sci U S A* **98**, 8411-8418.
8. Koroleva, O., Makharashvili, N., Courcelle, C. T., Courcelle, J. & Korolev, S. (2007). Structural conservation of RecF and Rad50: implications for DNA recognition and RecF function. *EMBO J* **26**, 867-877.
9. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P. & Tainer, J. A. (2000). Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **101**, 789-800.
10. Obmolova, G., Ban, C., Hsieh, P. & Yang, W. (2000). Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. *Nature* **407**, 703-710.
11. Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**, 67-113.
12. Aravind, L., Walker, D. R. & Koonin, E. V. (1999). Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Res* **27**, 1223-1242.
13. Hopfner, K. P. & Tainer, J. A. (2003). Rad50/SMC proteins and ABC transporters: unifying concepts from high-resolution structures. *Curr Opin Struct Biol* **13**, 249-255.
14. Chow, K. H. & Courcelle, J. (2004). RecO Acts with RecF and RecR to Protect and Maintain Replication Forks Blocked by UV-induced DNA Damage in Escherichia coli. *J Biol Chem* **279**, 3492-3496.

15. Courcelle, J., Carswell-Crumpton, C. & Hanawalt, P. C. (1997). recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **94**, 3714-3719.
16. Courcelle, J., Donaldson, J. R., Chow, K. H. & Courcelle, C. T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in Escherichia coli. *Science* **299**, 1064-1067.
17. Horii, Z. & Clark, A. J. (1973). Genetic analysis of the recF pathway to genetic recombination in Escherichia coli K12: isolation and characterization of mutants. *J Mol Biol* **80**, 327-344.
18. Courcelle, J. & Hanawalt, P. C. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. *Mol Gen Genet* **262**, 543-551.
19. Courcelle, C. T., Belle, J. J. & Courcelle, J. (2005). Nucleotide excision repair or polymerase V-mediated lesion bypass can act to restore UV-arrested replication forks in Escherichia coli. *J Bacteriol* **187**, 6953-6961.
20. Courcelle, C. T., Chow, K. H., Casey, A. & Courcelle, J. (2006). Nascent DNA processing by RecJ favors lesion repair over translesion synthesis at arrested replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **103**, 9154-9159.
21. Makharashvili, N., Mi, T., Koroleva, O. & Korolev, S. (2009). RecR-mediated modulation of RecF dimer specificity for single- and double-stranded DNA. *J Biol Chem* **284**, 1425-1434.
22. Sakai, A. & Cox, M. M. (2009). RecFOR and RecOR as distinct RecA loading pathways. *J Biol Chem* **284**, 3264-3272.
23. Chow, K. H. & Courcelle, J. (2007). RecBCD and RecJ/RecQ initiate DNA degradation on distinct substrates in UV-irradiated Escherichia coli. *Radiat Res* **168**, 499-506.
24. Belle, J. J., Casey, A., Courcelle, C. T. & Courcelle, J. (2007). Inactivation of the DnaB helicase leads to the collapse and degradation of the replication fork: a comparison to UV-induced arrest. *J Bacteriol* **189**, 5452-5462.
25. Junop, M. S., Obmolova, G., Rausch, K., Hsieh, P. & Yang, W. (2001). Composite active site of an ABC ATPase: MutS uses ATP to verify mismatch recognition and authorize DNA repair. *Mol Cell* **7**, 1-12.
26. Lammens, A., Schele, A. & Hopfner, K. P. (2004). Structural biochemistry of ATP-driven dimerization and DNA-stimulated activation of SMC ATPases. *Curr Biol* **14**, 1778-1782.
27. Moncalian, G., Lengsfeld, B., Bhaskara, V., Hopfner, K. P., Karcher, A., Alden, E., Tainer, J. A. & Paull, T. T. (2004). The rad50 signature motif: essential to ATP binding and biological function. *J Mol Biol* **335**, 937-951.
28. Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J. & Hunt, J. F. (2002). ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol Cell* **10**, 139-149.
29. Hegde, S. P., Rajagopalan, M. & Madiraju, M. V. (1996). Preferential binding of Escherichia coli RecF protein to gapped DNA in the presence of adenosine (gamma-thio) triphosphate. *J Bacteriol* **178**, 184-190.

30. Madiraju, M. V. & Clark, A. J. (1992). Evidence for ATP binding and double-stranded DNA binding by Escherichia coli RecF protein. *J Bacteriol* **174**, 7705-7710.
31. Webb, B. L., Cox, M. M. & Inman, R. B. (1995). An interaction between the Escherichia coli RecF and RecR proteins dependent on ATP and double-stranded DNA. *J Biol Chem* **270**, 31397-31404.
32. Bork, J. M., Cox, M. M. & Inman, R. B. (2001). The RecOR proteins modulate RecA protein function at 5' ends of single- stranded DNA. *Embo J* **20**, 7313-7322.
33. Hegde, S. P., Qin, M. H., Li, X. H., Atkinson, M. A., Clark, A. J., Rajagopalan, M. & Madiraju, M. V. (1996). Interactions of RecF protein with RecO, RecR, and single-stranded DNA binding proteins reveal roles for the RecF-RecO-RecR complex in DNA repair and recombination. *Proc Natl Acad Sci U S A* **93**, 14468-14473.
34. Webb, B. L., Cox, M. M. & Inman, R. B. (1997). Recombinational DNA repair: the RecF and RecR proteins limit the extension of RecA filaments beyond single-strand DNA gaps. *Cell* **91**, 347-356.
35. Webb, B. L., Cox, M. M. & Inman, R. B. (1999). ATP hydrolysis and DNA binding by the Escherichia coli RecF protein. *J Biol Chem* **274**, 15367-15374.
36. Madiraju, M. V. & Clark, A. J. (1991). Effect of RecF protein on reactions catalyzed by RecA protein. *Nucleic Acids Res* **19**, 6295-6300.
37. Handa, N., Morimatsu, K., Lovett, S. T. & Kowalczykowski, S. C. (2009). Reconstitution of initial steps of dsDNA break repair by the RecF pathway of E. coli. *Genes Dev* **23**, 1234-1245.
38. McInerney, P. & O'donnell, M. (2007). Replisome Fate upon Encountering a Leading Strand Block and Clearance from DNA by Recombination Proteins. *J Biol Chem* **282**, 25903-25916.
39. Flower, A. M. & McHenry, C. S. (1991). Transcriptional organization of the Escherichia coli dnaX gene. *J Mol Biol* **220**, 649-658.
40. Ream, L. W., Margossian, L., Clark, A. J., Hansen, F. G. & von Meyenburg, K. (1980). Genetic and physical mapping of recF in Escherichia coli K-12. *Mol Gen Genet* **180**, 115-121.
41. Mellon, I. & Hanawalt, P. C. (1989). Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature* **342**, 95-98.
42. Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. & Court, D. L. (2000). An efficient recombination system for chromosome engineering in Escherichia coli. *Proc Natl Acad Sci U S A* **97**, 5978-5983.
43. Lee, E. C., Yu, D., Martinez, d. V. J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A. & Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**, 56-65.
44. Wentzell, B. & McCalla, D. R. (1980). Formation and excision of nitrofuran-DNA adducts in Escherichia coli. *Chem Biol Interact* **31**, 133-150.

45. Sandler, S. J. & Clark, A. J. (1993). Use of high and low level overexpression plasmids to test mutant alleles of the recF gene of Escherichia coli K-12 for partial activity. *Genetics* **135**, 643-654.
46. Courcelle, J., Crowley, D. J. & Hanawalt, P. C. (1999). Recovery of DNA replication in UV-irradiated Escherichia coli requires both excision repair and recF protein function. *J Bacteriol* **181**, 916-922.
47. Umezu, K., Chi, N. W. & Kolodner, R. D. (1993). Biochemical interaction of the Escherichia coli RecF, RecO, and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proc Natl Acad Sci U S A* **90**, 3875-3879.
48. Umezu, K. & Kolodner, R. D. (1994). Protein interactions in genetic recombination in Escherichia coli. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. *J Biol Chem* **269**, 30005-30013.
49. Knezevic-Vukcevic, J., Vukovic, B. & Simic, D. (1987). Role of rec genes in SOS-induced inhibition of cell division in Escherichia coli. *Mutat Res* **192**, 247-252.
50. Sassanfar, M. & Roberts, J. (1991). Constitutive and UV-mediated activation of RecA protein: combined effects of recA441 and recF143 mutations and of addition of nucleosides and adenine. *J Bacteriol* **173**, 5869-5875.
51. Wood, R. D. & Stein, J. (1986). Role of the RecF gene product in UV mutagenesis of lambda phage. *Mol Gen Genet* **204**, 82-84.
52. Thoms, B. & Wackernagel, W. (1987). Regulatory role of recF in the SOS response of Escherichia coli: impaired induction of SOS genes by UV irradiation and nalidixic acid in a recF mutant. *J Bacteriol* **169**, 1731-1736.
53. Fujii, S., Isogawa, A. & Fuchs, R. P. (2006). RecFOR proteins are essential for Pol V-mediated translesion synthesis and mutagenesis. *EMBO J* **25**, 5754-5763.
54. Ciesla, Z., O'Brien, P. & Clark, A. J. (1987). Genetic analysis of UV mutagenesis of the Escherichia coli glyU gene. *Mol Gen Genet* **207**, 1-8.
55. Liu, Y. H., Cheng, A. J. & Wang, T. C. (1998). Involvement of recF, recO, and recR genes in UV-radiation mutagenesis of Escherichia coli. *J Bacteriol* **180**, 1766-1770.
56. Shan, Q., Bork, J. M., Webb, B. L., Inman, R. B. & Cox, M. M. (1997). RecA protein filaments: end-dependent dissociation from ssDNA stabilization by RecO and RecR proteins. *J Mol Biol* **265**, 519-540.
57. Lee, B. I., Kim, K. H., Park, S. J., Eom, S. H., Song, H. K. & Suh, S. W. (2004). Ring-shaped architecture of RecR: implications for its role in homologous recombinational DNA repair. *EMBO J* **23**, 2029-2038.
58. Lusetti, S. L., Hobbs, M. D., Stohl, E. A., Chitteni-Pattu, S., Inman, R. B., Seifert, H. S. & Cox, M. M. (2006). The RecF Protein Antagonizes RecX Function via Direct Interaction. *Mol Cell* **21**, 41-50.
59. Courcelle, J. (2005). Recs preventing wrecks. *Mutat Res* **577**, 217-227.

CHAPTER III

CONCLUDING REMARKS

Extensive studies have focused on characterizing the role of the RecF pathway proteins in the processing of UVC-induced DNA damage encountered during replication. In *E. coli,* these roles include the stabilization of the replication fork, resection of nascent DNA to expose the lesions to repair enzymes, and re-establishment of a functional replisome [1-4]. Here we examined the response of the RecF protein to two distinct DNA damaging agents.

Using UVC-induced DNA damage as a model, we undertook a structure and function analysis of RecF to identify which enzymatic properties of this protein are required to carry out the progressive steps associated with the recovery of replication. The RecF protein contains three conserved motifs with enzymatic functions including ATP binding, ATP hydrolysis, and protein dimerization [5]. We speculated that mutants altered in each of these activities might be blocked at an intermediate step in the recovery process. Instead, we found that each of these functions is required for the initial steps in the processing and repair of replication forks disrupted by UVC-induced DNA damage.

Considering that *recF, recO,* and *recR* are epistatic, it may prove useful and would be of interest in the future to utilize a similar structure and function approach with RecR and RecO to identify the intermediates associated with the recovery of replication. Of the RecF, RecO, and RecR proteins, RecR is the most conserved among bacterial

species, making it an appropriate candidate protein for further characterization of the RecFOR pathway of DNA repair. The crystal structure of the *D. radiodurans* RecR showed that the monomer consists of two domains: the N-terminal domain, which contains a helix-hairpin-helix (HhH) motif, and the C-terminal domain, which contains a Cys_4 zinc-finger motif, a Toprim domain, and a Walker B motif [6]. Four of these monomers form a tetrameric clamp with a central cavity that can encircle both single- and double-stranded DNA [6]. Additionally, RecR can interact with both RecF and RecO and it has been proposed that RecR acts to tether these proteins together to form a presynaptic complex at sites of stalled replication forks [7-9]. The observation that RecR does not bind DNA by itself is consistent with the idea that a clamp-loading activity may be required to form the presynaptic complex [10]. Additionally, RecR only interacts with RecF when RecF is bound to DNA [11], making it tempting to speculate that RecF may be necessary for this clamp-loading activity.

To characterize RecR's interactions with RecO and RecF and potentially elucidate how these proteins catalyze the recovery of replication, point mutations could be constructed that would disrupt the physical binding of RecR to RecF or RecR to RecO interactions. Using the molecular approaches we have previously described, we could then characterize the cellular events that occur during the progressive steps of repair and replication recovery following disruption by DNA damage in strains with altered RecR function.

Previous *in vitro* studies by Lee et al. have shown that the HhH motif in the N-terminal domain is responsible for coordinating *D. radiodurans* RecR tetramer formation

and is critical for DNA binding. The two conserved basic residues, Lys23 and Arg27, within this motif are found on the surface of the main cavity of the RecR tetramer with their side chains oriented towards the cavity. These residues appear to be essential for RecR's DNA binding properties in *D. radiodurans*, as a RecR double mutant (K23A R27A) has already been found to exhibit greatly reduced DNA binding affinity, *in vitro* [6].

Based on the crystal structure of *D. radiodurans* RecR, Korolev's lab group has constructed two double point mutations, K23A R27A and K23E R27E, corresponding to these residues on plasmid vectors expressing *E. coli* RecR. These double mutants are predicted to similarly disrupt function of the RecR tetramer and inhibit RecR binding to either single- or double-stranded DNA, *in vivo*. It would be of interest to introduce these mutations directly onto the *E. coli* chromosome using the *sacB::cat* cassette method we utilized in the construction of our *recF* point mutants and to carry out cellular assays that would allow us to track whether the binding of RecR to DNA is required for the formation of the presynaptic complex and subsequent restoration of synthesis following disruption by DNA damage, *in vivo*.

A second semi-conserved domain of RecR, the Toprim domain, has been suggested to contain binding sites for both RecF and RecO. Mutations introduced into Toprim domain residues inhibited both RecRRecF and RecRRecO binding of *Thermus thermophilus* proteins, *in vitro* [12]. Similarly, it would be of interest to construct mutations in the conserved acidic residues of the RecR Toprim domain and introduce them onto the *E. coli* chromosome for *in vivo* characterization of the protein-protein interactions between RecR, RecF, and RecO. Specifically, using molecular approaches, we might be

able to determine whether the RecFR and RecOR complexes are both required for the progressive steps of replication restoration following disruption by DNA damage, *in vivo*.

Of particular interest to me, are the mechanisms of DNA damage and repair in the extremely radiation-resistant family of bacteria, *Deinococcaceae*. The first of these to be described was *D. radiodurans*, which was found to be highly resistant to several DNA damaging agents including ionizing radiation, UV light, and hydrogen peroxide [13-16].

Incredibly, *D. radiodurans* can be exposed to over 1,500 kilorads of acute gamma radiation without any loss of viability or induced mutagenesis [17]. In addition, *D. radiodurans* can be exposed to up to 6 kilorads/h of chronic irradiation without having any effect on its growth rate [13, 18, 19]. In contrast, as little as 100 kilorads of gamma radiation is sufficient to sterilize a culture of *E. coli* and at fluencies of less than 6 kilorads/h *E. coli* cultures do not grow and are rendered inviable over time [13].

Studies of *D. radiodurans* have since shown that its extreme radioresistance is largely attributable to a highly efficient DNA repair system (for review see [20]). Ionizing radiation is as efficient in producing double-strand breaks in the DNA of *D. radiodurans* as it is in the DNA of other organisms. However, *D. radiodurans* can efficiently repair more than 100 double-strand DNA breaks per chromosome without mutagenesis or cell lethality, whereas most other organisms can only repair up to three double-strand DNA breaks [21-23].

The complete genome of *D. radiodurans* was sequenced and annotated in 1991 [24]. Intriguingly, while RecFOR pathway protein homologs were present, no RecBC homologs exist [25]. Considering the crucial role that RecBC plays in repair of double-

strand DNA breaks in other organisms, it is quite curious that no such proteins exist in these extremely radioresistant bacteria.

In light of these observations, it would be of interest to characterize the mechanisms by which the *D. radiodurans* RecFOR proteins are able to repair DNA damage with high efficiency and fidelity, *in vivo*. Introducing RecF pathway proteins from *D. radiodurans* into *E. coli* strains lacking these proteins might allow us to determine whether the activity of these repair enzymes is sufficient to confer or contribute to the radioresistance of *D. radiodurans*.

As described in the appendix, we also made an effort to characterize RecF's involvement in the cellular response to DNA damage caused by UVA, an agent that produces lesions distinct from those of UVC. To this end, we employed the cellular assays we described above to compare the contributions of RecF, RecA, and RecBC to the restoration of replication and cell survival after UVA irradiation. We found that, in contrast to what is observed following UVC-induced DNA damage, RecF is not required for survival in the presence of UVA-induced DNA damage, although the recovery of replication is delayed. In contrast, while RecA and RecBC contributed to survival following exposure to UVA, only RecA but not RecBC was needed to resume replication. The finding that recovery of replication is independent of RecBC mimics what is observed after UVC-induced damage and supports a DNA repair role for RecBC that is distinct from that occurring at sites of stalled replication forks [26].

REFERENCES

1. Courcelle, J., Carswell-Crumpton, C. & Hanawalt, P. C. (1997). recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **94**, 3714-3719.
2. Courcelle, J., Donaldson, J. R., Chow, K. H. & Courcelle, C. T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in Escherichia coli. *Science* **299**, 1064-1067.
3. Courcelle, J. & Hanawalt, P. C. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. *Mol Gen Genet* **262**, 543-551.
4. Courcelle, J., Crowley, D. J. & Hanawalt, P. C. (1999). Recovery of DNA replication in UV-irradiated Escherichia coli requires both excision repair and recF protein function. *J Bacteriol* **181**, 916-922.
5. Koroleva, O., Makharashvili, N., Courcelle, C. T., Courcelle, J. & Korolev, S. (2007). Structural conservation of RecF and Rad50: implications for DNA recognition and RecF function. *EMBO J* **26**, 867-877.
6. Lee, B. I., Kim, K. H., Park, S. J., Eom, S. H., Song, H. K. & Suh, S. W. (2004). Ring-shaped architecture of RecR: implications for its role in homologous recombinational DNA repair. *EMBO J* **23**, 2029-2038.
7. Umezu, K. & Kolodner, R. D. (1994). Protein interactions in genetic recombination in Escherichia coli. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. *J Biol Chem* **269**, 30005-30013.
8. Webb, B. L., Cox, M. M. & Inman, R. B. (1997). Recombinational DNA repair: the RecF and RecR proteins limit the extension of RecA filaments beyond single-strand DNA gaps. *Cell* **91**, 347-356.
9. Bork, J. M., Cox, M. M. & Inman, R. B. (2001). The RecOR proteins modulate RecA protein function at 5' ends of single- stranded DNA. *Embo J* **20**, 7313-7322.
10. Webb, B. L., Cox, M. M. & Inman, R. B. (1995). An interaction between the Escherichia coli RecF and RecR proteins dependent on ATP and double-stranded DNA. *J Biol Chem* **270**, 31397-31404.
11. Webb, B. L., Cox, M. M. & Inman, R. B. (1999). ATP hydrolysis and DNA binding by the Escherichia coli RecF protein. *J Biol Chem* **274**, 15367-15374.
12. Honda, M., Inoue, J., Yoshimasu, M., Ito, Y., Shibata, T. & Mikawa, T. (2006). Identification of the RecR toprim domain as the binding site for both RecF and RecO: A role of RecR in RecFOR assembly at dsDNA-ssDNA junctions. *J Biol Chem*
13. Lange, C. C., Wackett, L. P., Minton, K. W. & Daly, M. J. (1998). Engineering a recombinant Deinococcus radiodurans for organopollutant degradation in radioactive mixed waste environments. *Nat Biotechnol* **16**, 929-933.
14. Minton, K. W. (1994). DNA repair in the extremely radioresistant bacterium Deinococcus radiodurans. *Mol Microbiol* **13**, 9-15.

15. Moseley, B. E. & Evans, D. M. (1983). Isolation and properties of strains of Micrococcus (Deinococcus) radiodurans unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways. *J Gen Microbiol* **129**, 2437-2445.
16. Wang, P. & Schellhorn, H. E. (1995). Induction of resistance to hydrogen peroxide and radiation in Deinococcus radiodurans. *Can J Microbiol* **41**, 170-176.
17. Daly, M. J., Ouyang, L., Fuchs, P. & Minton, K. W. (1994). In vivo damage and recA-dependent repair of plasmid and chromosomal DNA in the radiation-resistant bacterium Deinococcus radiodurans. *J Bacteriol* **176**, 3508-3517.
18. Venkateswaran, A., McFarlan, S. C., Ghosal, D., Minton, K. W., Vasilenko, A., Makarova, K., Wackett, L. P. & Daly, M. J. (2000). Physiologic determinants of radiation resistance in Deinococcus radiodurans. *Appl Environ Microbiol* **66**, 2620-2626.
19. Brim, H., McFarlan, S. C., Fredrickson, J. K., Minton, K. W., Zhai, M., Wackett, L. P. & Daly, M. J. (2000). Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments. *Nat Biotechnol* **18**, 85-90.
20. Battista, J. R. (1997). Against all odds: the survival strategies of Deinococcus radiodurans. *Annu Rev Microbiol* **51**, 203-224.
21. Dean, C. J., Feldschreiber, P. & Lett, J. T. (1966). Repair of x-ray damage to the deoxyribonucleic acid in Micrococcus radiodurans. *Nature* **209**, 49-52.
22. Burrell, A. D., Feldschreiber, P. & Dean, C. J. (1971). DNA-membrane association and the repair of double breaks in x-irradiated Micrococcus radiodurans. *Biochim Biophys Acta* **247**, 38-53.
23. Grimsley, J. K., Masters, C. I., Clark, E. P. & Minton, K. W. (1991). Analysis by pulsed-field gel electrophoresis of DNA double-strand breakage and repair in Deinococcus radiodurans and a radiosensitive mutant. *Int J Radiat Biol* **60**, 613-626.
24. White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., Haft, D. H., Gwinn, M. L., Nelson, W. C., Richardson, D. L., Moffat, K. S., Qin, H., Jiang, L., Pamphile, W., Crosby, M., Shen, M., Vamathevan, J. J., Lam, P., McDonald, L., Utterback, T., Zalewski, C., Makarova, K. S., Aravind, L., Daly, M. J., Minton, K. W., Fleischmann, R. D., Ketchum, K. A., Nelson, K. E., Salzberg, S., Smith, H. O., Venter, J. C. & Fraser, C. M. (1999). Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1. *Science* **286**, 1571-1577.
25. Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V. & Daly, M. J. (2001). Genome of the extremely radiation-resistant bacterium Deinococcus radiodurans viewed from the perspective of comparative genomics. *Microbiol Mol Biol Rev* **65**, 44-79.
26. Chow, K. H. & Courcelle, J. (2007). RecBCD and RecJ/RecQ initiate DNA degradation on distinct substrates in UV-irradiated Escherichia coli. *Radiat Res* **168**, 499-506.

APPENDIX

A. INVOLVEMENT OF THE RecF AND RecBC PATHWAYS OF REPLICATION FOLLOWING UVA-INDUCED DNA DAMAGE

ABSTRACT

In *E. coli*, RecA-mediated events are initiated either through the RecF or RecBC pathway. The absence of either pathway renders cells hyper-sensitive to UVC irradiation, which produces two primary DNA lesions, cyclobutane pyrimidine dimers and 6, 4 pyrimidine -pyrimidone photoproducts. RecA and RecF, but not RecBC, are required for processing of the arrested replication fork and restoring replication. By comparison, less is known about the cellular response to UVA exposure. UVA, which consists of longer wavelengths than UVC, indirectly produces a broad range of oxidative lesions. Here, we begin to investigate the role of RecA, RecF, and RecBC in survival and the recovery of replication following exposure to UVA. We observed that RecA and RecBC are required for resistance to UVA-irradiation; and that RecA, but not RecBC, is required for the recovery of replication. In contrast, RecF does not contribute to cell survival following UVA-induced DNA damage, but its absence does delay the recovery of replication. The delayed recovery in *recF* mutants suggests that RecF may participate in restoring replication following arrest by UVA-induced damage. However, the lack of hypersensitivity in *recF* cells suggests that, in contrast to UVC-induced damage,

alternative or secondary processes may exist in *E. coli* to process UVA-induced DNA damage encountered by replication forks.

INTRODUCTION

Damage to DNA by solar radiation can occur directly through the absorption of radiation by nucleotides or indirectly via interactions between DNA and photosensitizers, non-DNA cellular components that absorb UV energy (reviewed in [1 2]. Exposure to the shorter, higher energy wavelengths, UVB (315 – 280 nm) and UVC (280 – 100 nm), results in direct DNA damage, predominantly cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts [3,4]. In contrast, the longer, lower energy wavelengths within the UVA range (400 – 315 nm) are absorbed by endogenous photosensitizers, resulting in predominantly oxidative damage to DNA either through direct interaction between DNA and the modified photosensitizer or reactive oxygen species (ROS) generated by the modification to photosensitizers [5-8].

The oxidative DNA lesions induced by UVA and other oxidizing agents can result in single-strand DNA breaks, abasic sites, or a range of modifications to the nucleoside bases themselves [9-11]. Some of these base adducts, such as dihydrothymine, have relatively minor consequences to the cell, as they retain base-pairing specificity [12]. Other adducts, such as 7,8-dihydro-8-oxoguanine (8-oxoG) and thymine glycol, can result in mutagenesis or replication arrest and lethality, respectively [13-16].

Not surprisingly, cells have mechanisms to prevent and repair the cellular damage caused by UVA and other oxidizing agents. *E. coli* has at least two global regulators that respond to increased levels of intracellular ROS, SoxRS and OxyRS. The SoxRS regulon is activated by superoxide [17] and upregulates approximately 15 genes, including *sodA* and *sodB* [18, 19, 19-21]. These genes encode dismutases that convert superoxide to the less reactive species, molecular oxygen and hydrogen peroxide (H_2O_2) [22]. The OxyR regulon upregulates approximately 30 genes [23] including *katE* and *katG,* which encode catalases that can reduce H_2O_2 to molecular oxygen and water [23-25].

In addition to scavenging mechanisms, cells possess repair enzymes that act in response to the presence of oxidative DNA lesions. Analogous to nucleotide excision repair (NER), which repairs bulky lesions including those induced by UVC, base excision repair (BER) is the major repair pathway that removes oxidized bases (reviewed in [26]). BER is initiated by one of several DNA glycosylases, which cleaves the glycosidic bond of the modified base to produce an apyrimidinic or apurinic (AP) site. An AP endonuclease then cleaves the sugar-phosphate backbone at the AP site. The resulting gap is then filled in and ligated by DNA Polymerase I and Ligase (reviewed in [26]).

Several evolutionarily conserved glycosylases exist that repair DNA damage induced by ROS. In *E. coli,* these include Formamidopyrimidine glycosylase (Fpg), Exonuclease III, and Endonucleases III, IV, V, and VIII [27-32]. Interestingly, no single glycosylase mutant is hypersensitive to oxidative damage, suggesting either that these glycosylases are redundant or that other repair enzymes may prevent lethality in the absence of repair [33, 34].

Whereas these scavenging and repair pathways have been well characterized, less is known about how UVA-induced DNA damage is processed during replication and some evidence suggests that the genetic requirements to process this form of damage will be distinct from those needed for UVC-induced DNA damage [5-8, 35-37]. Following exposure to UVC-induced DNA lesions, *recA, recF,* and *recBC* mutants all have decreased survival [38-40]. Restoring replication following UVC-induced arrest requires RecA and RecF [41-43]. In contrast, *recBC* mutants process the arrested fork and recover replication normally, but arrest and die at a later stage [44, 45]. *recA* and *recBC* mutants are hypersensitive to H_2O_2, which induces lesions similar in spectrum to those induced by UVA irradiation [46, 47]. However, *recF* mutants are nearly as resistant as wild-type cells [48, 49]. In this chapter, I directly characterized RecA, RecBC, and RecF for their ability to survive and replicate following exposure to UVA to begin to address how this form of damage is processed during replication.

EXPERIMENTAL PROCEDURES

BACTERIAL STRAINS

SR108 is a *thy*A36 *deo*C2 derivative of W3110 [50]. HL921 (SR108Δ(*srlR-recA*) *306::*Tn10), HL922 (SR108 *recB21 recC22 argA81*::Tn10), and CL579 (*recF6206*::tet857) have been previously described [41, 42, 51].

UVA SURVIVALS

Fresh overnight cultures were diluted 1:100 and grown in Davis medium supplemented with 0.4% glucose, 0.2% cassamino acids, and 10 μg/mL thymine (DGCthy) to an OD_{600} of 0.4 in a 37 °C shaking water bath. At this time, cultures were transferred to 6-cm Petri dishes, placed on a rotary platform 5 cm from a Sylvania 32 Watt UVA bulb (model 350 Black Light) and irradiated at an incident dose of 285 $J/m^2/sec$. Trifold serial dilutions were plated in triplicate on Luria-Bertani (LB) plates supplemented with 10 μg/mL thymine at each indicated dose. Plates were incubated overnight at 37 °C, and colonies were counted on the next day to determine the surviving fraction.

RECOVERY OF DNA SYNTHESIS

Fresh overnight cultures were diluted 1:100 in 20 mL of DGCthy medium supplemented with 0.1 μCi/mL [^{14}C] thymine and grown in a 37 °C shaking water bath to an OD_{600} of 0.3, at which time the culture was split. Half of the culture was irradiated for 30 min with UVA, a dose equivalent to 513 kJ/m^2, and the other half was mock-irradiated. At the times indicated, duplicate 0.5 mL aliquots of the culture were pulse labeled with 1 μCi/mL [^3H]thymidine for 2 min. The cells were then lysed, and the DNA was precipitated by addition of 5 mL of ice-cold 5% trichloroacetic acid. The precipitate was collected on Fisherbrand 2.5-cm glass fiber filters, and the amount of radioactivity in each sample was measured using a liquid scintillation counter.

RESULTS

To determine whether *recA, recF* and *recBC* mutants are hypersensitive to UVA, the survival of cultures exposed to UVA were compared to that of an isogenic wild-type culture. Cultures were irradiated with various doses of UVA, serially diluted, spotted on LBthy plates, and incubated at 37°C overnight. Cells that survived to form colonies were counted and the fraction of surviving colonies was determined (Figure A.1).

By this assay, wild-type cells irradiated with UVA showed no decrease in survival at doses up to 350 kJ/m^2, which was the maximum dose tested. In contrast, survival in cells lacking *recA* was reduced by 1000-fold over the same range, with most of the lethality occurring at low doses. Unexpectedly, *recF* cells showed minimal, if any, hypersensitivity to UVA irradiation, with less than an order of magnitude reduction in survival at the highest tested dose. This result contrasts with the hypersensitive phenotype observed in *recF* cells exposed to UVC irradiation [40] and suggests that an alternative mechanism may operate to promote survival following UVA-induced damage.

In *recBC* mutants, survival was reduced by approximately 10-fold at the highest dose of UVA. The hypersensitivity of these mutants is similar to the intermediate phenotype observed in *recBC* cells exposed to UVC irradiation [39] and suggests that RecBC may have a similar function in response to UVA irradiation as it does following UVC exposure.

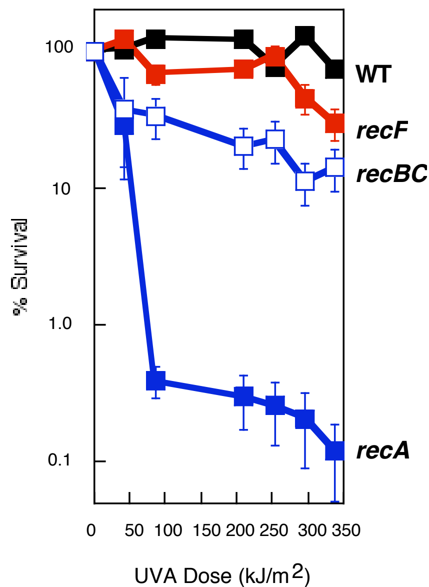

Figure A.1 RecA and, to a lesser extent, RecBC are required for resistance to UVA-induced DNA damage. The percentage of cells surviving the indicated dose of UVA is plotted for wild- type (black-filled squares), *recF* (red, filled squares), *recBC* (blue, unfilled squares), and *recA* (blue, filled squares) strains, as indicated. Survival curves represent at least three independent experiments. Error bars represent the standard error of the mean.

To further characterize the role of *recA, recF,* and *recBC* in the cellular response to UVA, we examined how DNA synthesis was affected in these mutants following exposure to UVA irradiation. To monitor the overall DNA accumulation and rate of DNA synthesis following irradiation, duplicate aliquots of cultures grown in the presence of [^{14}C] thymine were pulse labeled with [^{3}H] thymidine for 2 min at various times following 30 min of UVA irradiation (513 kJ/m^2) or mock-irradiation. In this way, the rate of DNA synthesis (^{3}H incorporation / 2min) and the overall DNA accumulation (^{14}C incorporation) at various times during the recovery period could be determined relative to that occurring in unirradiated cultures.

By this assay, the rate of DNA synthesis in irradiated wild-type cultures initially decreased by approximately 80%, but recovered to a rate near pre-irradiation levels within 100 min post-irradiation (Figure A.2). The overall accumulation of DNA in the irradiated cultures continued to accumulate over the time course at a rate of

approximately 50% of that in unirradiated cultures. By comparison, the rate of synthesis in irradiated cultures of *recA* mutants decreased by more than 90% and did not recover within the time course examined. In addition, no further DNA accumulation was seen. This result suggests that the hypersensitivity of *recA* mutants to UVA is likely to be due, in part, to an inability to restore replication after arrest following UVA-induced damage.

In *recF* mutants, the rate of DNA synthesis following irradiation decreased by more than 90%. Surprisingly, no recovery of replication was observed for a period of 60 min, after which time a rapid increase in the rate of replication was observed and synthesis resumed to near pre-irradiated levels by 100 min. A similar delay in recovery of DNA synthesis occurs in *recF* mutants following UVC-induced damage. Following UVC irradiation, however, the impaired recovery correlates with a loss of viability. The observed delay may suggest that RecF participates in the normal recovery process. However, the eventual resumption of replication and survival of *recF* mutants indicates that a secondary or back-up mechanism may operate following UVA exposure that is distinct from the mechanisms functioning after UVC irradiation.

In *recBC* mutants, the rate of synthesis in irradiated cultures initially decreased by approximately 80 to 90% but recovered at a time and a rate that was nearly equivalent to that seen in wild-type cultures. Additionally, the overall accumulation of DNA in irradiated *recBC* cultures continued to accumulate over the time course at a rate of approximately 50% of that in unirradiated *recBC* cultures. The recovery of replication, despite the hypersensitive phenotype, is similar to the phenotype observed in *recBC* mutants exposed to UVC irradiation [39, 41, 43] and suggests that the hypersensitivity of these

mutants is not directly related to an impaired ability to restore DNA synthesis after damage.

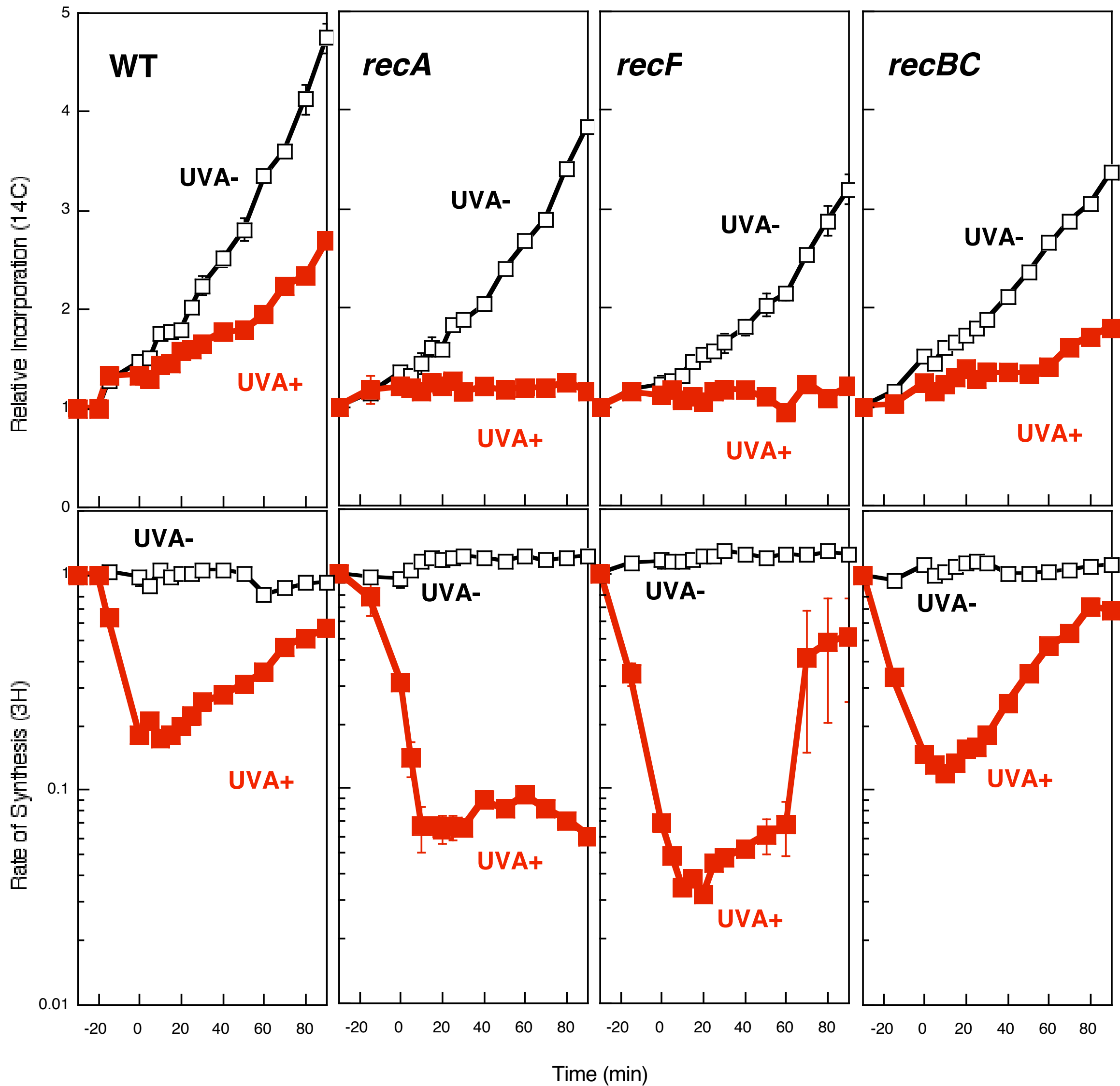

Figure A.2 RecA is required to resume DNA synthesis following disruption by UVA-induced DNA damage. [^{14}C]Thymine labeled cultures were pulse labeled with [^{3}H]Thymidine for 2 min at the indicated times following 30 min of UVA-irradiation (513 kJ/m^2) (filled symbols) or mock irradiation (open symbols). The relative DNA accumulation (^{14}C; top panels) and the rate of DNA synthesis (^{3}H; bottom panels) are plotted. The *recBC* graph represents a single experiment. All other graphs represent at least two independent experiments. Error bars represent the standard error of the mean.

DISCUSSION

Here, we investigated the roles of the RecA, RecF, and RecBC proteins in survival and the reestablishment of a functional replisome following UVA-induced DNA damage. RecA, RecF, and RecBC are each essential for survival after exposure to UVC irradiation [38-40], however, only RecA and RecBC appear to be required for survival following UVA irradiation. Additionally, while RecF is required to resume replication following exposure to UVC irradiation [41], we detected only a moderate delay in recovery of replication in *recF* mutants following exposure to UVA. Both *recA* and *recBC* mutants exhibit similar phenotypes for the recovery of replication following exposure to either UVA or UVC irradiation; *recA* mutants exposed to UVA or UVC irradiation are unable to resume replication while resumption of replication in *recBC* mutants exposed to UVA or UVC irradiation occurs without delay.

The inability of *recA* mutants to resume replication following exposure to UVA irradiation may explain their hypersensitivity under these conditions. RecA is required to stabilize the replication fork and protect genomic DNA from degradation when replication is stalled by UVC-induced DNA lesions [41]. The similar phenotypes of *recA* mutants in the face of either UVC or UVA irradiation may suggest that some lesions produced by both damaging agents require similar processing events that are mediated by RecA functions.

The RecF protein is required for survival in the face of replication-blocking lesions [40, 41], however, *recF* mutants show no sensitivity to UVA-induced DNA damage

despite the fact that oxidative damage is known to produce replication-blocking lesions [14]. Interestingly, although survival is not decreased in *recF* mutants exposed to UVA irradiation, the recovery of replication is delayed, perhaps indicating a secondary mechanism by which lesions are eventually removed. In this scenario, RecF would serve as the primary means by which UVA-induced lesions are efficiently repaired, while in the absence of RecF, an alternative pathway triggered by the SOS response or some other cellular response might serve to repair these lesions prior to the resumption of DNA synthesis at, or proximal to, the site of stalled replication forks.

Although *recBC* mutants are hypersensitive to UVA irradiation, the resumption of replication following disruption by UVA-induced lesions is not impaired. This finding is consistent with previous results showing that while *recBC* mutants are sensitive to UVC-induced DNA damage, the protein complex does not appear to be directly involved in the repair of damage at stalled replication forks as no delay in the recovery of replication in *recBC* mutants is detected following UVC-irradiation [44].

The hypersensitivity and replication recovery phenotypes in *recA* and *recBC* mutants following either UVC or UVA irradiation suggest that UVA-induced replication-blocking lesions may induce a similar response as that following UVC irradiation. However, the lack of sensitivity and eventual recovery of replication in *recF* mutants following exposure to UVA indicates that additional or novel mechanisms exist to promote survival and recovery of replication that are distinct from those observed after UVC.

Considering these results, it would be of interest to confirm the normal recovery of *recBC* mutants, since this is based on a single observation. It would also be of interest to compare these results to H_2O_2-treated cultures to assess whether these observations are specific to UVA irradiation or are a more general response to oxidative stress. Finally, the effect of UVA irradiation on replication could be further characterized by looking at how much degradation occurs at the replication fork following inhibition by UVA and which genes are responsible for the degradation, if it is observed. It may also be useful to look at the structural intermediates that are formed following UVA irradiation. Following UVC irradiation, distinct RecF pathway processing intermediates and degradation patterns are observed prior to replication recovery [42]. If these intermediates were seen following UVA irradiation, it would help to identify the mechanisms allowing for cell survival under these conditions.

REFERENCES

1. Tyrrell, R. M. & Keyse, S. M. (1990). New trends in photobiology. The interaction of UVA radiation with cultured cells. *J Photochem Photobiol B* **4**, 349-361.
2. Moan, J. & Peak, M. J. (1989). Effects of UV radiation of cells. *J Photochem Photobiol B* **4**, 21-34.
3. Setlow, R. B. (1966). Cyclobutane-type pyrimidine dimers in polynucleotides. *Science* **153**, 379-386.
4. Mitchell, D. L. & Nairn, R. S. (1989). The biology of the (6-4) photoproduct. *Photochem Photobiol* **49**, 805-819.
5. Morliere, P., Moysan, A., Santus, R., Huppe, G., Maziere, J. C. & Dubertret, L. (1991). UVA-induced lipid peroxidation in cultured human fibroblasts. *Biochim Biophys Acta* **1084**, 261-268.
6. Kielbassa, C., Roza, L. & Epe, B. (1997). Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis* **18**, 811-816.
7. Takeuchi, S., Zhang, W., Wakamatsu, K., Ito, S., Hearing, V. J., Kraemer, K. H. & Brash, D. E. (2004). Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin. *Proc Natl Acad Sci U S A* **101**, 15076-15081.
8. O'Donovan, P., Perrett, C. M., Zhang, X., Montaner, B., Xu, Y. Z., Harwood, C. A., McGregor, J. M., Walker, S. L., Hanaoka, F. & Karran, P. (2005). Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* **309**, 1871-1874.
9. von Sonntag, C. & Dizdaroglu, M. (1977). The reactions of OH radicals with D-ribose in deoxygenated and oxygenated qqueous solution. *Carbohydr Res* **58**, 21-30.
10. Beesk, F., Dizdaroglu, M., Schulte-Frohlinde, D. & von Sonntag, C. (1979). Radiation-induced DNA strand breaks in deoxygenated aqueous solutions. The formation of altered sugars as end groups. *Int J Radiat Biol Relat Stud Phys Chem Med* **36**, 565-576.
11. von Sonntag, C. (1987). New aspects in the free-radical chemistry of pyrimidine nucleobases. *Free Radic Res Commun* **2**, 217-224.
12. Ide, H. & Wallace, S. S. (1988). Dihydrothymidine and thymidine glycol triphosphates as substrates for DNA polymerases: differential recognition of thymine C5-C6 bond saturation and sequence specificity of incorporation. *Nucleic Acids Res* **16**, 11339-11354.
13. Hariharan, P. V., Achey, P. M. & Cerutti, P. A. (1977). Biological effect of thymine ring saturation in coliphage phiX174-DNA. *Radiat Res* **69**, 375-378.
14. Rouet, P. & Essigmann, J. M. (1985). Possible role for thymine glycol in the selective inhibition of DNA synthesis on oxidized DNA templates. *Cancer Res* **45**, 6113-6118.
15. Ide, H., Kow, Y. W. & Wallace, S. S. (1985). Thymine glycols and urea residues in M13 DNA constitute replicative blocks in vitro. *Nucleic Acids Res* **13**, 8035-8052.

16. Wood, M. L., Dizdaroglu, M., Gajewski, E. & Essigmann, J. M. (1990). Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry* **29**, 7024-7032.
17. Gu, M. & Imlay, J. A. (2011). The SoxRS response of Escherichia coli is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol*
18. Walkup, L. K. & Kogoma, T. (1989). Escherichia coli proteins inducible by oxidative stress mediated by the superoxide radical. *J Bacteriol* **171**, 1476-1484.
19. Tsaneva, I. R. & Weiss, B. (1990). soxR, a locus governing a superoxide response regulon in Escherichia coli K-12. *J Bacteriol* **172**, 4197-4205.
20. Greenberg, J. T., Monach, P., Chou, J. H., Josephy, P. D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli. *Proc Natl Acad Sci U S A* **87**, 6181-6185.
21. Pomposiello, P. J. & Demple, B. (2000). Identification of SoxS-regulated genes in Salmonella enterica serovar typhimurium. *J Bacteriol* **182**, 23-29.
22. Keele, B. B. J., McCord, J. M. & Fridovich, I. (1970). Superoxide dismutase from escherichia coli B. A new manganese-containing enzyme. *J Biol Chem* **245**, 6176-6181.
23. Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell* **41**, 753-762.
24. Loewen, P. C. (1984). Isolation of catalase-deficient Escherichia coli mutants and genetic mapping of katE, a locus that affects catalase activity. *J Bacteriol* **157**, 622-626.
25. Loewen, P. C., Triggs, B. L., George, C. S. & Hrabarchuk, B. E. (1985). Genetic mapping of katG, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in Escherichia coli. *J Bacteriol* **162**, 661-667.
26. Seeberg, E., Eide, L. & Bjoras, M. (1995). The base excision repair pathway. *Trends Biochem Sci* **20**, 391-397.
27. Saporito, S. M., Smith-White, B. J. & Cunningham, R. P. (1988). Nucleotide sequence of the xth gene of Escherichia coli K-12. *J Bacteriol* **170**, 4542-4547.
28. Saporito, S. M. & Cunningham, R. P. (1988). Nucleotide sequence of the nfo gene of Escherichia coli K-12. *J Bacteriol* **170**, 5141-5145.
29. Dizdaroglu, M., Laval, J. & Boiteux, S. (1993). Substrate specificity of the Escherichia coli endonuclease III: excision of thymine- and cytosine-derived lesions in DNA produced by radiation-generated free radicals. *Biochemistry* **32**, 12105-12111.
30. Boiteux, S., Gajewski, E., Laval, J. & Dizdaroglu, M. (1992). Substrate specificity of the Escherichia coli Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry* **31**, 106-110.
31. Jiang, D., Hatahet, Z., Melamede, R. J., Kow, Y. W. & Wallace, S. S. (1997). Characterization of Escherichia coli endonuclease VIII. *J Biol Chem* **272**, 32230-32239.

32. Guo, G., Ding, Y. & Weiss, B. (1997). nfi, the gene for endonuclease V in Escherichia coli K-12. *J Bacteriol* **179**, 310-316.
33. Saito, Y., Uraki, F., Nakajima, S., Asaeda, A., Ono, K., Kubo, K. & Yamamoto, K. (1997). Characterization of endonuclease III (nth) and endonuclease VIII (nei) mutants of Escherichia coli K-12. *J Bacteriol* **179**, 3783-3785.
34. Jiang, D., Hatahet, Z., Blaisdell, J. O., Melamede, R. J. & Wallace, S. S. (1997). Escherichia coli endonuclease VIII: cloning, sequencing, and overexpression of the nei structural gene and characterization of nei and nei nth mutants. *J Bacteriol* **179**, 3773-3782.
35. Setlow, R. B., Regan, J. D., German, J. & Carrier, W. L. (1969). Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA. *Proc Natl Acad Sci U S A* **64**, 1035-1041.
36. Zelle, B. & Lohman, P. H. (1979). Repair of UV-endonuclease-susceptible sites in the 7 complementation groups of xeroderma pigmentosum A through G. *Mutat Res* **62**, 363-368.
37. Mitchell, D. L. & Nairn, R. S. (1988). The (6-4) photoproduct and human skin cancer. *Photodermatol* **5**, 61-64.
38. Clark, A. J. & Margulies, A. D. (1965). Isolation and Characterization of Recombination-Deficient Mutants of Escherichia coli K12. *Proc Natl Acad Sci U S A* **53**, 451-459.
39. Howard-Flanders, P. & Theriot, L. (1966). Mutants of Escherichia coli K-12 defective in DNA repair and in genetic recombination. *Genetics* **53**, 1137-1150.
40. Horii, Z. & Clark, A. J. (1973). Genetic analysis of the recF pathway to genetic recombination in Escherichia coli K12: isolation and characterization of mutants. *J Mol Biol* **80**, 327-344.
41. Courcelle, J., Carswell-Crumpton, C. & Hanawalt, P. C. (1997). recF and recR are required for the resumption of replication at DNA replication forks in Escherichia coli. *Proc Natl Acad Sci U S A* **94**, 3714-3719.
42. Courcelle, J., Donaldson, J. R., Chow, K. H. & Courcelle, C. T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in Escherichia coli. *Science* **299**, 1064-1067.
43. Khidhir, M. A., Casaregola, S. & Holland, I. B. (1985). Mechanism of transient inhibition of DNA synthesis in ultraviolet-irradiated E. coli: inhibition is independent of recA whilst recovery requires RecA protein itself and an additional, inducible SOS function. *Mol Gen Genet* **199**, 133-140.
44. Chow, K. H. & Courcelle, J. (2007). RecBCD and RecJ/RecQ initiate DNA degradation on distinct substrates in UV-irradiated Escherichia coli. *Radiat Res* **168**, 499-506.
45. Al-Hadid, Q., Ona, K., Courcelle, C. T. & Courcelle, J. (2008). RecA433 cells are defective in recF-mediated processing of disrupted replication forks but retain recBCD-mediated functions. *Mutat Res* **645**, 19-26.
46. Hartman, P. S. (1986). In situ hydrogen peroxide production may account for a portion of NUV (300-400 nm) inactivation of stationary phase Escherichia coli. *Photochem Photobiol* **43**, 87-89.

47. Coombs, A. M. & Moss, S. H. (1987). Effects of peroxide and catalase on near ultraviolet radiation sensitivity in Escherichia coli strains. *Int J Radiat Biol Relat Stud Phys Chem Med* **51**, 493-503.
48. Hartman, P. S. & Eisenstark, A. (1978). Synergistic killing of Escherichia coli by near-UV radiation and hydrogen peroxide: distinction between recA-repairable and recA-nonrepairable damage. *J Bacteriol* **133**, 769-774.
49. Imlay, J. A. & Linn, S. (1987). Mutagenesis and stress responses induced in Escherichia coli by hydrogen peroxide. *J Bacteriol* **169**, 2967-2976.
50. Mellon, I. & Hanawalt, P. C. (1989). Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature* **342**, 95-98.
51. Courcelle, J. & Hanawalt, P. C. (1999). RecQ and RecJ process blocked replication forks prior to the resumption of replication in UV-irradiated Escherichia coli. *Mol Gen Genet* **262**, 543-551.