ENZYMATIC PROCESSING OF REPLICATION INTERMEDIATES THAT OCCUR FOLLOWING UV-INDUCED DNA DAMAGE IN *ESCHERICHIA COLI*

By

Janet Renee Donaldson

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2005

ENZYMATIC PROCESSING OF REPLICATION INTERMEDIATES THAT OCCUR FOLLOWING UV-INDUCED DNA DAMAGE IN *ESCHERICHIA COLI*

By

Janet Renee Donaldson

Approved:

Justin Courcelle Assistant Professor of Biological Sciences (Director of Dissertation)	John Boyle Professor of Biochemistry (Committee Member)
Nara Gavini Professor of Biological Sciences (Committee Member)	Mark Lawrence Associate Professor of Veterinary Medicine (Committee Member)
Dwayne Wise Professor and Graduate Coordinator of Biological Sciences (Committee Member)	Phil Oldham Dean of the College of Arts & Sciences

Name: Janet Renee Donaldson

Date of Degree: May 7, 2005

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Dr. Justin Courcelle

Title of Study: ENZYMATIC PROCESSING OF REPLICATION INTERMEDIATES THAT OCCUR FOLLOWING UV-INDUCED DNA DAMAGE IN *ESCHERICHIA COLI*

Pages in Study: 125

Candidate for Degree of Doctor of Philosophy

Ultraviolet irradiation induces DNA damage that can block the progression of the replication machinery or generate gaps in the nascent DNA, depending on which template the lesion is found. Characterization of the mechanisms by which DNA synthesis resumes and the integrity of the template restored following UV-induced damage is important to understand how genome stability is maintained in all organisms. When progression of replication is blocked at a UV-induced lesion, several lines of evidence suggest that the resumption of replication requires that the blocking lesion is repaired. A remaining question is how or if the nascent DNA and replication machinery are displaced so repair enzymes can gain access to the damaged DNA and effect repair. Biochemical studies have shown that RecG and RuvABC can catalyze this reaction on synthetic DNA substrates and it has been proposed that one or both may be required to catalyze this reaction *in vivo*. In this study, it is shown that *ruvAB*, *ruvC*, and *recG*

mutants resume DNA synthesis at a time and rate that is similar to wild-type and that neither gene product is required to maintain the structural integrity of blocked replication forks, indicating that RuvABC and RecG are not essential for the resumption of replication. Although DNA synthesis continued, *ruvAB* and *ruvC* mutants accumulated unresolved Holliday junctions during replication after UV irradiation, leading to a general deterioration in the integrity of the genomic DNA, suggesting a potential role for RuvABC in processing Holliday junctions that accumulate following replication past lesions that do not arrest the replication machinery. Structural intermediates induced by UV-irradiation were also characterized by two-dimensional agarose gel electrophoresis in *recG*, *recJ*, *recQ*, *xonA*, *recBC*, and *recD* mutants. It is shown that *recJ*, *recQ*, and *xonA* mutants form UV-induced intermediates that resemble those observed in wild-type cells, but persist for a modestly longer period prior to their resolution. *recBC* mutants form UV-induced intermediates that appear similar to wild-type, but these intermediates persist throughout the times that were examined. In contrast, *recD* mutants form unique intermediates both in the presence and absence of UV irradiation that are not observed in wild-type.

DEDICATION

This dissertation is dedicated to my grandfather, William Earl Owen. Even though you are no longer with us, you remain an inspiration to me every day. Thank you for teaching me to always strive to do my best, but to never forget the road that brought me here.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Justin Courcelle for accepting me into his laboratory and for his encouragement and guidance throughout this project. I am forever grateful for the opportunities he has given to me. I am also eternally grateful to my committee, collaborating faculty, and staff of the department of Biological Sciences for their endless support, encouragement, and guidance throughout my graduate career.

I would also like to thank my entire family for their thoughts and prayers. I would like to especially thank my parents Don and Linda Parker, my parents-in-law John and Bennie Donaldson, my sister Donna Bishop, and my grandmother Blondell Parker for their continued support and encouragement throughout these past few years. It has been a difficult endeavor, and one that I never could have completed without them. Last, but not least, I express my deepest and sincerest gratitude to my husband, Jeremy, for always believing in me and always being there for me. I never would have come this far without him. Thank you for everything!

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The processes of DNA repair and replication are highly conserved between *Escherichia coli* and humans. Both cells must accurately replicate their genome in order to ensure that each daughter cell inherits an exact copy of the genome. However, DNA damage can block the replication machinery and impair its ability to accurately complete its task (Howard-Flanders et al., 1968; Setlow et al., 1963). DNA damage encountered during replication can lead to cell death, mutagenesis, or genomic rearrangements and is thought to play a primary role in carcinogenic transformation in humans (Friedberg, 2003). To deal with these impediments, all cells contain efficient mechanisms to repair damaged DNA and to ensure that accurate replication is maintained when damaged DNA is encountered (for review see (Courcelle et al., 2004; Michel et al., 2004).

Over the years, several mutants of *E. coli* have been shown to exhibit an impaired ability to replicate in the presence of DNA damage (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Rangarajan et al., 2002). Many of these gene products have functional homologs in humans (Ellis et al., 1995; Shinohara et al., 1993; Yu et al., 1996). For example, humans have five genes that are homologous to the *E. coli* RecQ helicase, which has been shown to act at arrested replication forks following UV-induced DNA damage (Courcelle & Hanawalt, 1999; Ellis et al., 1995; Kitao et al., 1998;

Kitao et al., 1999b; Puranam & Blackshear, 1994; Seki et al., 1994; Yu et al., 1996). Three of these genes, *BLM*, *WRN*, and *RecQL4* have been implicated in Bloom's syndrome (Ellis et al., 1995), Werner's syndrome (Imbert et al., 1996; Yu et al., 1996), and Rothmund-Thomson syndrome (Kitao et al., 1999a; Kitao et al., 1999b), respectively. Each of these syndromes renders patients prone to developing cancers due to mutations in the RecQ homolog (for review, see (Mohaghegh & Hickson, 2001). In part for this reason, the bacterium *E. coli* has proven to be an extremely valuable tool for modeling the genetic events that can lead to the development of cancer in humans.

UV irradiation at ~254 nm induces two predominant forms of DNA damage, the pyrimidine 6-4 pyrimidone photoproduct (6-4 PP) and the cyclobutane pyrimidine dimer (CPD), and represents a useful lesion to approach the general question of how cells cope with DNA damage (Friedberg et al., 1995). Figure 1.1 demonstrates the formation of these lesions using adjacent thymines as an example. Following UV irradiation, CPDs are formed between four to ten times more frequently than 6-4 PPs, yet cause less distortion to the DNA and are recognized by the nucleotide excision repair proteins less efficiently than 6-4 PPs, resulting in slower kinetics of repair (Friedberg et al., 1995; Mitchell et al., 1990; Mitchell et al., 1985).

Historically in *E. coli*, the repair of UV induced DNA damage was characterized through two predominant mechanisms: a light-dependent mechanism and a light-independent mechanism (Friedberg et al., 1995). In the presence of visible light (300-500 nm), thymine dimers can be directly removed by photoreactivation (Jagger, 1958). This light-dependent mechanism utilizes the enzyme photolyase, encoded by the *phr* gene

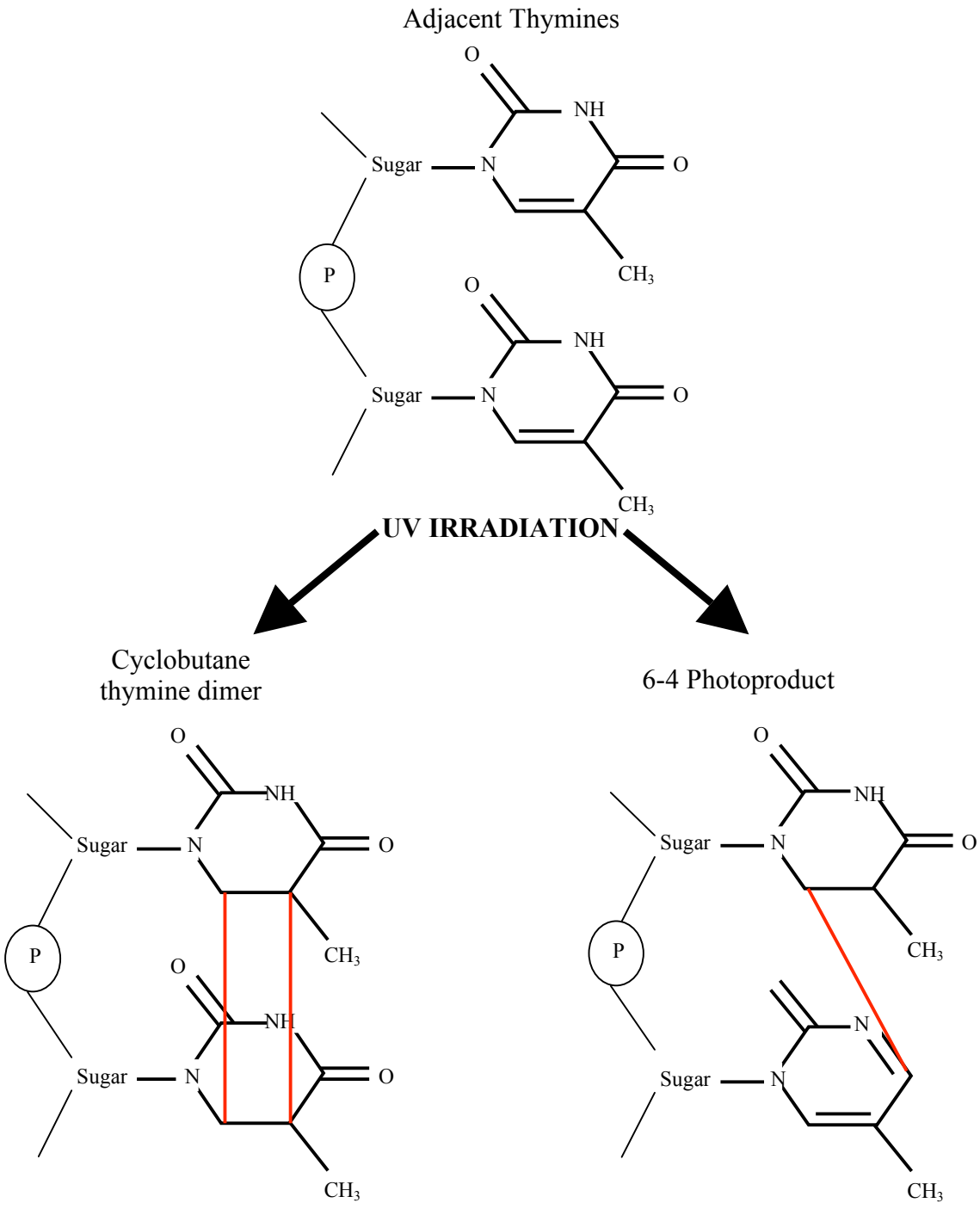


Figure 1.1. UV irradiation induces DNA damage in the form of cyclobutane pyrimidine dimers and 6-4 photoproducts.

(Sancar et al., 1983). Photolyase absorbs light in the range of 300-500 nm and breaks the cyclobutane ring, restoring the base (Sancar, 1994). Cells lacking Phr are modestly hypersensitive to UV compared to wild-type cells (Husain & Sancar, 1987). Photoreactivation is found in many bacteria, archaea, and eukaryotes, but is missing in humans (Li et al., 1993). Due to the minor contribution of Phr to survival of UV irradiated *E. coli* and the absence of homologs in the human genome, the light-independent pathways in *E. coli* may represent a more useful model for understanding mechanisms that operate to repair DNA damage in human cells.

The light-independent repair of UV lesions occurs via the nucleotide excision repair pathway (Friedberg et al., 1995). This pathway involves three principle steps: recognition of the lesion, excision of the region containing the lesion, and resynthesis of the excised strand. The protein UvrA is the lesion recognition protein (Sancar & Hearst, 1993). UvrA binds and recruits UvrB, forming a $UvrA_2UvrB$ complex at the lesion site (Orren & Sancar, 1989). Once the lesion has been found, the $UvrA_2UvrB$ complex dissociates (Moolenaar et al., 2001; Skorvaga et al., 2002). UvrC then binds to UvrB, forming an UvrBC-DNA complex. UvrC introduces one incision at either the fourth or fifth phosphodiester bond from the 3' side of the lesion and another incision at the eighth phosphodiester bond from the 5' side of the lesion (Verhoeven et al., 2000). Another endonuclease, Cho, can introduce an incision on the 3' side of the lesion at a site that is four nucleotides away from the cut site of UvrC, suggesting that this endonuclease is involved in nucleotide excision repair when UvrC is not capable of producing incisions to the 3' side of the lesion (Moolenaar et al., 2002). UvrD helicase releases the region

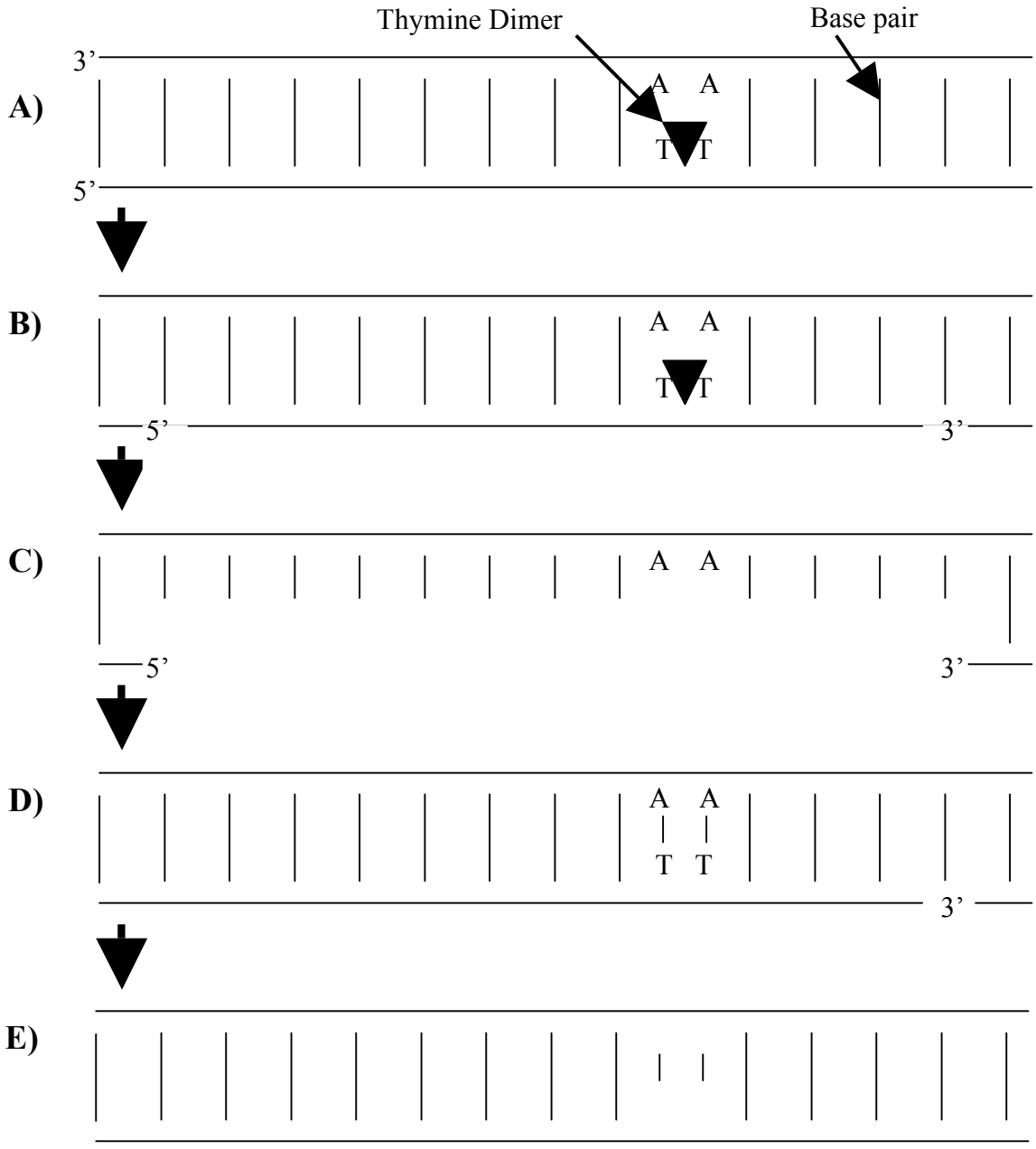


Figure 1.2. Nucleotide excision repair of thymine dimers.

(A) UV irradiation induces the formation of thymine dimers into the DNA. (B) Incisions are made by the UvrABC complex four or five bases to the 3' side of the UV lesion and eight bases to the 5' side of the UV lesion. (C) UvrD helicase releases the section of damaged DNA between the two incisions made by the UvrABC complex. (D) DNA Polymerase I resynthesizes the section of DNA removed. (E) Ligase seals the nick, restoring the DNA.

between the two incisions, DNA polymerase I fills in the twelve to thirteen base gap, and ligase seals the remaining nick (Sancar, 1996) (Fig. 1.2).

UV-induced DNA damage blocks DNA polymerases and RNA polymerases and can prevent the essential processes of replication and transcription (Bonner et al., 1992; Michalke & Bremer, 1969; Setlow et al., 1963). In the absence of photolyase, nucleotide excision repair is essential to remove lesions from the bulk of the genome, which is referred to as global nucleotide excision repair (Hanawalt, 2002). A second subpathway of repair is transcription-coupled nucleotide excision repair. This subpathway was discovered when it was observed that lesions in actively transcribed genes were repaired with more rapid kinetics than those in the overall genome and was later shown that the preferential repair was specific to the transcribed strand of the active gene (Bohr et al., 1985; Mellon & Hanawalt, 1989; Mellon et al., 1987). Consistent with this observation, biochemical studies have shown that lesions in the non-transcribed strand do not arrest the RNA polymerase, whereas UV lesions in the transcribed strand block the RNA polymerase (Donahue et al., 1994; Selby & Sancar, 1990). Transcription-coupled repair in *E. coli* requires the nucleotide excision repair proteins, a functional RNA polymerase, and a transcription-repair coupling factor encoded by the gene *mfd* (Hanawalt, 2002; Selby et al., 1991). Mfd recognizes and binds to the stalled RNA polymerase-DNA template-RNA complex and then recruits nucleotide excision repair proteins to the lesion (Selby & Sancar, 1994; Selby & Sancar, 1995; Selby et al., 1991). Mfd displaces the RNA polymerase and the nascent transcript to allow the nucleotide excision repair proteins to gain access to and remove the damaged DNA, and it has been proposed that

the arrested RNA polymerase acts as a signal that recruits Mfd (Selby & Sancar, 1993; Selby & Sancar, 1994).

UV-induced DNA damage also blocks DNA polymerases (Chan et al., 1985; Cordeiro-Stone et al., 1997; Livneh, 1986; Setlow et al., 1963; Svoboda & Vos, 1995; Taylor & O'Day, 1990; Veaute et al., 2000). Immediately after a moderate dose of UV irradiation, DNA synthesis in *E. coli* is transiently inhibited, but resumes at a time that correlates to when the UV lesions have been removed by the nucleotide excision repair proteins (Courcelle et al., 2003; Setlow et al., 1963). Restarting replication following arrest is known to require several proteins, including the nucleotide excision repair proteins, RecA, and several gene products of the *recF* pathway, including RecF, RecR, RecO, RecJ, and RecQ (Chow & Courcelle, 2004; Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2003; Courcelle & Hanawalt, 1999). The ability of these proteins to protect or degrade the DNA at arrested replication forks was characterized by radioactively labeling the DNA synthesized immediately before UV irradiation in mutant strains of *E. coli* to monitor whether the labeled DNA was maintained or degraded following UV-induced damage (Courcelle et al., 1997; Horii & Suzuki, 1970). Using this assay, it was found that the RecQ helicase and RecJ exonuclease act together to degrade the nascent DNA synthesized at blocked replication forks (Courcelle & Hanawalt, 1999). This degradation was further shown to preferentially occur on the nascent lagging strand of DNA at the blocked replication fork (Courcelle & Hanawalt, 1999). It was also found that the nascent DNA degradation at the blocked replication fork is limited by RecF-O-R and it was suggested that RecF-O-R act to facilitate the binding of the RecA filament to

the three-strand DNA region produced at the blocked replication fork following the processing of the nascent DNA (Chow & Courcelle, 2004; Courcelle et al., 1997). In addition to these gene products, the recovery of DNA synthesis also depends upon nucleotide excision repair proteins (Courcelle et al., 1999; Kato et al., 1977). In the absence of nucleotide excision repair, the nascent DNA at the replication fork is protected, but replication does not resume. These observations have led to the idea that RecJ and RecQ and RecF-O-R act to process and stabilize the replication fork at a DNA lesion until nucleotide excision repair proteins can gain access and remove the blocking UV lesion (Courcelle et al., 2003). This type of model implies that after repair has occurred, the replication holoenzyme could then simply resume at the point where the disruption occurred. Importantly, the resumption of replication following lesion removal would suggest that no strand exchanges or recombinational products would be required or produced when replication resumes in this manner (Courcelle et al., 1997; Courcelle et al., 2003).

When the replication machinery encounters a blocking lesion, the DNA polymerase stalls at the site of the lesion (Chan et al., 1985; Cordeiro-Stone et al., 1997; Livneh, 1986; Setlow et al., 1963; Svoboda & Vos, 1995; Taylor & O'Day, 1990; Veaute et al., 2000). It has been proposed that before the blocking lesion can be removed, the nascent DNA and the replication machinery at the replication fork will be displaced. Displacement of the nascent strand would, in effect, move the branch point of the replication fork backwards and generate a 4-arm branched structure at a point prior to where replication arrested (Courcelle et al., 2003). The existence of such a structure was

examined *in vivo* by characterizing the replication intermediates that form following UV irradiation in mutant strains of *E. coli* containing the plasmid pBR322 (Courcelle et al., 2003). pBR322 utilizes the host's replication machinery, making it a useful tool to identify intermediates observed on replicating molecules (Kingsbury & Helinski, 1973). It was found that a transient, X-shaped structure formed in wild-type cells within 15 min after a UV dose of 50 J/m^2 and began to wane ~30 min following UV at a time that correlated with when the lesions had been removed and robust replication had resumed (Courcelle et al., 2003). *recF*, *recO*, and *recR* mutants, which fail to maintain the arrested replication forks, did not accumulate the X-shaped intermediates following UV irradiation (Chow & Courcelle, 2004; Courcelle et al., 2003). However, the subsequent removal of RecJ or RecQ, which degrade the nascent DNA at arrested replication forks, partially restored the UV-induced intermediate, strongly suggesting that a portion of these X-structures represented the displacement of the nascent DNA at blocked replication forks (Courcelle et al., 2003). 4-arm branched molecules have also been shown to form in partially replicated plasmid molecules stalled at termination sites (Postow et al., 2001). Since these regressed intermediates occurred in reconstituted replication reactions *in vitro*, it is likely that the reversal of the replication fork in plasmids may be analyzed by an increase in positive supercoiling ahead of arrested replication forks.

A remaining question of this model is does the recovery of replication *require* replication fork reversal and if so, are enzymes required to catalyze this reaction *in vivo*? Two candidate enzymes for this activity are the branch migration proteins RecG and RuvAB. RecG was initially discovered in a screen for mutants with increased sensitivity

to UV irradiation and a reduced frequency of recombination during conjugation (Storm et al., 1971). RecG has regions of homology to Mfd and has been proposed to promote the formation of Holliday junctions at arrested replication forks *in vivo* based upon *in vitro* assays with synthetic molecules and UV survival studies (Mahdi et al., 2003; McGlynn & Lloyd, 2000; McGlynn & Lloyd, 2001b; McGlynn et al., 2001). *In vitro*, purified RecG has been shown to bind to and unwind synthetic 3- and 4-arm DNA oligomeric structures as measured by the rate that the synthetic substrates were dissociated (McGlynn & Lloyd, 1999). Using plasmids with reconstituted replication forks stalled by excessive supercoiling, it was further shown that RecG can act at stalled replication forks to form a 4-arm, Holliday junction by displacing the nascent DNA (McGlynn et al., 2001).

RuvABC is also a candidate for acting at arrested replication forks to form Holliday junctions. The *ruv* locus, consisting of *ruvA*, *ruvB*, and *ruvC*, was originally identified through a genetic screen for UV-sensitive mutants (Otsuji et al., 1974). These mutants also were found to have a reduced ability to form recombinant molecules during conjugation and transduction assays (Lloyd et al., 1984; Otsuji et al., 1974). Purified RuvA binds as a tetramer to Holliday junctions and recruits the binding of two hexametric rings of RuvB (Dickman et al., 2002; Rafferty et al., 1996). Together, RuvA and RuvB act as a helicase that catalyzes the branch migration of the Holliday junction in either the 5'-3' or 3'-5' direction, depending on reaction conditions (McGlynn & Lloyd, 2001a; Parsons et al., 1992; Parsons & West, 1993). RuvAB was also shown to be able to catalyze the conversion of synthetic 3-arm oligonucleotide structures to 4-arm branch substrates *in vitro*, although the reverse reaction preferentially occurs (McGlynn &

Lloyd, 2001a). Purified RuvC is an endonuclease that binds to and resolves synthetic Holliday junctions by introducing symmetric nicks at the junction point, producing nicked-duplex products (Connolly et al., 1991). RuvABC proteins were found to have phenotypes that arise in temperature sensitive alleles of *dnaB* at the restrictive temperature (Seigneur et al., 1998). Incubation at the restricted temperature inactivates the DnaB replicative helicase and arrests replication (Wechsler & Gross, 1971). Under these conditions, double-strand breaks were seen to accumulate in their genome in the absence of the double-strand break repair proteins RecBCD (Michel et al., 1997). If RuvABC is additionally inactivated in this background, the formation of double-strand breaks was significantly reduced, suggesting that Holliday junctions form after inactivation of DnaB and are subsequently cleaved by the RuvABC resolvasome (Seigneur et al., 1998).

Even though both RuvAB and RecG proteins have been shown to act at arrested replication forks to form Holliday junctions *in vitro*, this activity has not yet been identified *in vivo* at replication forks arrested at UV-induced DNA damage. Mutant strains of *E. coli* deficient in RuvAB or RecG are hypersensitive to UV irradiation, indicating that these proteins have an essential role following UV-induced DNA damage. It is therefore important to identify where and when these proteins are required in the cell.

Significance

The well established link between inaccurate replication, DNA damage, and cancer makes it important to identify the processes and proteins involved in repair and replication in the presence of DNA damage. However, the mechanisms involved in

processing and repairing DNA lesions that are encountered during replication remain poorly understood. This dissertation characterizes several candidate gene products for their potential role in processing and restoring the DNA template following replication in the presence of UV-induced DNA damage. The major objective of this research was to identify the cellular roles of RuvAB and RecG in promoting cell survival after UV induced damage, as well as other potential candidate gene products. Many of these candidate gene products were initially identified as proteins that would affect the frequency of recombination during conjugation or transduction. Subsequent characterization revealed that many of these gene products also play critical roles in maintaining the integrity of the genome in the presence of DNA damage, yet their functional roles often remain poorly defined. In characterizing the functional roles of RuvABC and RecG, this dissertation sought to further our understanding of the mechanisms that operate to maintain the genomic stability in both microbial and eukaryotic organisms.

REFERENCES

Bohr, V. A., Smith, C. A., Okumoto, D. S. & Hanawalt, P. C. (1985). DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. *Cell* 40, 359-69.

Bonner, C. A., Stukenberg, P. T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H. & Goodman, M. F. (1992). Processive DNA synthesis by DNA polymerase II mediated by DNA polymerase III accessory proteins. *J Biol Chem* 267, 11431-8.

Chan, G. L., Doetsch, P. W. & Haseltine, W. A. (1985). Cyclobutane pyrimidine dimers and (6-4) photoproducts block polymerization by DNA polymerase I. *Biochemistry* 24, 5723-8.

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, 3492-6.

Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991). Resolution of Holliday junctions *in vitro* requires the Escherichia coli *ruvC* gene product. *Proc. Natl. Acad. Sci. U S A* 88, 6063-7.

Cordeiro-Stone, M., Zaritskaya, L. S., Price, L. K. & Kaufmann, W. K. (1997). Replication fork bypass of a pyrimidine dimer blocking leading strand DNA synthesis. *J Biol Chem* 272, 13945-54.

Courcelle, J., Belle, J. J. & Courcelle, C. T. (2004). When replication travels on damaged templates: bumps and blocks in the road. *Res Microbiol* 155, 231-7.

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, 3714-9.

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, 916-22.

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, 1064-7.

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, 543-51.

Dickman, M. J., Ingleston, S. M., Sedelnikova, S. E., Rafferty, J. B., Lloyd, R. G., Grasby, J. A. & Hornby, D. P. (2002). The RuvABC resolvasome. *Eur J Biochem* 269, 5492-501.

Donahue, B. A., Yin, S., Taylor, J. S., Reines, D. & Hanawalt, P. C. (1994). Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. *Proc Natl Acad Sci U S A* 91, 8502-6.

Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., Proytcheva, M. & German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* 83, 655-66.

Friedberg, E. C. (2003). DNA damage and repair. *Nature* 421, 436-40.

Friedberg, E. C., Walker, G. C. & Siede, W. (1995). *DNA Repair and Mutagenesis*, ASM Press, Washington, DC.

Hanawalt, P. C. (2002). Subpathways of nucleotide excision repair and their regulation. *Oncogene* 21, 8949-56.

Horii, Z. I. & Suzuki, K. (1970). Degradation of the DNA of recA mutants of Escherichia coli K-12 after irradiation with ultraviolet light. II. Further studies including a recA uvrA double mutant. *Photochem Photobiol* 11, 99-107.

Howard-Flanders, P., Rupp, W. D., Wilkins, B. M. & Cole, R. S. (1968). DNA replication and recombination after UV irradiation. *Cold Spring Harb. Symp. Quant. Biol.* 33, 195-207.

Husain, I. & Sancar, A. (1987). Photoreactivation in phr mutants of Escherichia coli K-12. *J Bacteriol* 169, 2367-72.

Imbert, A., Chaffanet, M., Essioux, L., Noguchi, T., Adelaide, J., Kerangueven, F., Le Paslier, D., Bonaiti-Pellie, C., Sobol, H., Birnbaum, D. & Pebusque, M. J. (1996). Integrated map of the chromosome 8p12-p21 region, a region involved in human cancers and Werner syndrome. *Genomics* 32, 29-38.

Jagger, J. (1958). Photoreactivation. *Bacteriol Rev* 22, 99-142.

Kato, T., Rothman, R. H. & Clark, A. J. (1977). Analysis of the role of recombination and repair in mutagenesis of Escherichia coli by UV irradiation. *Genetics* 87, 1-18.

Kingsbury, D. T. & Helinski, D. R. (1973). Temperature-sensitive mutants for the replication of plasmids in Escherichia coli. I. Isolation and specificity of host and plasmid mutations. *Genetics* 74, 17-31.

Kitao, S., Lindor, N. M., Shiratori, M., Furuichi, Y. & Shimamoto, A. (1999a). Rothmund-thomson syndrome responsible gene, RECQL4: genomic structure and products. *Genomics* 61, 268-76.

Kitao, S., Ohsugi, I., Ichikawa, K., Goto, M., Furuichi, Y. & Shimamoto, A. (1998). Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. *Genomics* 54, 443-52.

Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M. & Furuichi, Y. (1999b). Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat Genet* 22, 82-4.

Li, Y. F., Kim, S. T. & Sancar, A. (1993). Evidence for lack of DNA photoreactivating enzyme in humans. *Proc Natl Acad Sci U S A* 90, 4389-93.

Livneh, Z. (1986). Replication of UV-irradiated single-stranded DNA by DNA polymerase III holoenzyme of Escherichia coli: evidence for bypass of pyrimidine photodimers. *Proc Natl Acad Sci U S A* 83, 4599-603.

Lloyd, R. G., Benson, F. E. & Shurvinton, C. E. (1984). Effect of *ruv* mutations on recombination and DNA repair in *Escherichia coli* K12. *Mol. Gen. Genet.* 194, 303-9.

Mahdi, A. A., Briggs, G. S., Sharples, G. J., Wen, Q. & Lloyd, R. G. (2003). A model for dsDNA translocation revealed by a structural motif common to RecG and Mfd proteins. *Embo J* 22, 724-34.

McGlynn, P. & Lloyd, R. G. (1999). RecG helicase activity at three- and four-strand DNA structures. *Nucleic Acids Res.* 27, 3049-56.

McGlynn, P. & Lloyd, R. G. (2000). Modulation of RNA polymerase by (p)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell* 101, 35-45.

McGlynn, P. & Lloyd, R. G. (2001a). Action of RuvAB at replication fork structures. *J. Biol. Chem.* 276, 41938-44.

McGlynn, P. & Lloyd, R. G. (2001b). Rescue of stalled replication forks by RecG: simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and Holliday junction formation. *Proc. Natl. Acad. Sci. U S A* 98, 8227-34.

McGlynn, P., Lloyd, R. G. & Marians, K. J. (2001). Formation of Holliday junctions by regression of nascent DNA in intermediates containing stalled replication forks: RecG stimulates regression even when the DNA is negatively supercoiled. *Proc. Natl. Acad. Sci. U S A* 98, 8235-40.

Mellon, I. & Hanawalt, P. C. (1989). Induction of the Escherichia coli lactose operon selectivity increases repair of its transcribed DNA strand. *Nature* 342, 95-98.

Mellon, I., Spivak, G. & Hanawalt, P. C. (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell* 51, 241-9.

Michalke, H. & Bremer, H. (1969). RNA synthesis in Escherichia coli after irradiation with ultraviolet light. *J Mol Biol* 41, 1-23.

Michel, B., Ehrlich, S. D. & Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *Embo J.* 16, 430-8.

Michel, B., Grompone, G., Flores, M. J. & Bidnenko, V. (2004). Multiple pathways process stalled replication forks. *Proc Natl Acad Sci U S A* 101, 12783-8.

Mitchell, D. L., Brash, D. E. & Nairn, R. S. (1990). Rapid repair kinetics of pyrimidine(6-4)pyrimidone photoproducts in human cells are due to excision rather than conformational change. *Nucleic Acids Res* 18, 963-71.

Mitchell, D. L., Haipek, C. A. & Clarkson, J. M. (1985). (6-4)Photoproducts are removed from the DNA of UV-irradiated mammalian cells more efficiently than cyclobutane pyrimidine dimers. *Mutat Res* 143, 109-12.

Mohaghegh, P. & Hickson, I. D. (2001). DNA helicase deficiencies associated with cancer predisposition and premature ageing disorders. *Hum Mol Genet* 10, 741-6.

Moolenaar, G. F., Hoglund, L. & Goosen, N. (2001). Clue to damage recognition by UvrB: residues in the beta-hairpin structure prevent binding to non-damaged DNA. *Embo J* 20, 6140-9.

Moolenaar, G. F., van Rossum-Fikkert, S., van Kesteren, M. & Goosen, N. (2002). Cho, a second endonuclease involved in Escherichia coli nucleotide excision repair. *Proc Natl Acad Sci U S A* 99, 1467-72.

Orren, D. K. & Sancar, A. (1989). The (A)BC excinuclease of Escherichia coli has only the UvrB and UvrC subunits in the incision complex. *Proc Natl Acad Sci U S A* 86, 5237-41.

Otsuji, N., Iyehara, H. & Hideshima, Y. (1974). Isolation and characterization of an *Escherichia coli ruv* mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. *J. Bacteriol.* 117, 337-44.

Parsons, C. A., Tsaneva, I., Lloyd, R. G. & West, S. C. (1992). Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc. Natl. Acad. Sci. U S A* 89, 5452-6.

Parsons, C. A. & West, S. C. (1993). Formation of a RuvAB-Holliday junction complex *in vitro*. *J. Mol. Biol.* 232, 397-405.

Postow, L., Ullsperger, C., Keller, R. W., Bustamante, C., Vologodskii, A. V. & Cozzarelli, N. R. (2001). Positive torsional strain causes the formation of a four-way junction at replication forks. *J. Biol. Chem.* 276, 2790-6.

Puranam, K. L. & Blackshear, P. J. (1994). Cloning and characterization of RECQL, a potential human homologue of the Escherichia coli DNA helicase RecQ. *J Biol Chem* 269, 29838-45.

Rafferty, J. B., Sedelnikova, S. E., Hargreaves, D., Artymiuk, P. J., Baker, P. J., Sharples, G. J., Mahdi, A. A., Lloyd, R. G. & Rice, D. W. (1996). Crystal structure of DNA recombination protein RuvA and a model for its binding to the Holliday junction. *Science* 274, 415-21.

Rangarajan, S., Woodgate, R. & Goodman, M. F. (2002). Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA and RecFOR proteins. *Mol. Microbiol.* 43, 617-28.

Sancar, A. (1994). Structure and function of DNA photolyase. *Biochemistry* 33, 2-9.

Sancar, A. (1996). DNA excision repair. *Annu Rev Biochem* 65, 43-81.

Sancar, A. & Hearst, J. E. (1993). Molecular matchmakers. *Science* 259, 1415-20.

Sancar, G. B., Smith, F. W. & Sancar, A. (1983). Identification and amplification of the E. coli phr gene product. *Nucleic Acids Res* 11, 6667-78.

Seigneur, M., Bidnenko, V., Ehrlich, S. D. & Michel, B. (1998). RuvAB acts at arrested replication forks. *Cell* 95, 419-30.

Seki, M., Miyazawa, H., Tada, S., Yanagisawa, J., Yamaoka, T., Hoshino, S., Ozawa, K., Eki, T., Nogami, M., Okumura, K. & et al. (1994). Molecular cloning of cDNA encoding human DNA helicase Q1 which has homology to Escherichia coli Rec Q helicase and localization of the gene at chromosome 12p12. *Nucleic Acids Res* 22, 4566-73.

Selby, C. P. & Sancar, A. (1990). Transcription preferentially inhibits nucleotide excision repair of the template DNA strand in vitro. *J Biol Chem* 265, 21330-6.

Selby, C. P. & Sancar, A. (1993). Molecular mechanism of transcription-repair coupling. *Science* 260, 53-8.

Selby, C. P. & Sancar, A. (1994). Mechanisms of transcription-repair coupling and mutation frequency decline. *Microbiol Rev* 58, 317-29.

Selby, C. P. & Sancar, A. (1995). Structure and function of transcription-repair coupling factor. I. Structural domains and binding properties. *J Biol Chem* 270, 4882-9.

Selby, C. P., Witkin, E. M. & Sancar, A. (1991). Escherichia coli mfd mutant deficient in "mutation frequency decline" lacks strand-specific repair: in vitro complementation with purified coupling factor. *Proc Natl Acad Sci U S A* 88, 11574-8.

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.

Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. & Ogawa, T. (1993). Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and recA. *Nat Genet* 4, 239-43.

Skorvaga, M., Theis, K., Mandavilli, B. S., Kisker, C. & Van Houten, B. (2002). The beta -hairpin motif of UvrB is essential for DNA binding, damage processing, and UvrC-mediated incisions. *J Biol Chem* 277, 1553-9.

Storm, P. K., Hoekstra, W. P., deHaan, P. G. & Verhoef, C. (1971). Genetic recombination in *Escherichia coli*. IV. Isolation and characterization of recombination-deficiency mutants of *Escherichia coli* K12. *Mutat. Res.* 13, 9-17.

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, 11975-9.

Taylor, J. S. & O'Day, C. L. (1990). cis-syn thymine dimers are not absolute blocks to replication by DNA polymerase I of Escherichia coli in vitro. *Biochemistry* 29, 1624-32.

Veaute, X., Mari-Giglia, G., Lawrence, C. W. & Sarasin, A. (2000). UV lesions located on the leading strand inhibit DNA replication but do not inhibit SV40 T-antigen helicase activity. *Mutat Res* 459, 19-28.

Verhoeven, E. E., van Kesteren, M., Moolenaar, G. F., Visse, R. & Goosen, N. (2000). Catalytic sites for 3' and 5' incision of Escherichia coli nucleotide excision repair are both located in UvrC. *J Biol Chem* 275, 5120-3.

Wechsler, J. A. & Gross, J. D. (1971). Escherichia coli mutants temperature-sensitive for DNA synthesis. *Mol Gen Genet* 113, 273-84.

Yu, C. E., Oshima, J., Fu, Y. H., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J. & Schellenberg, G. D. (1996). Positional cloning of the Werner's syndrome gene. *Science* 272, 258-62.

CHAPTER II

RuvAB AND RecG ARE NOT ESSENTIAL FOR THE RECOVERY OF DNA SYNTHESIS FOLLOWING UV-INDUCED DNA DAMAGE IN *ESCHERICHIA COLI*

(Published in Genetics 2004; 166**(4)**:1631-40)

Abstract

Ultraviolet light induces DNA lesions that block the progression of the replication machinery. Several models speculate that the resumption of replication following disruption by UV-induced DNA damage requires regression of the nascent DNA or migration of the replication machinery away from the blocking lesion to allow repair or bypass of the lesion to occur. Both RuvAB and RecG catalyze branch migration of three- and four-stranded DNA junctions *in vitro* and are proposed to catalyze fork regression *in vivo*. To examine this possibility, we characterized the recovery of DNA synthesis in *ruvAB* and *recG* mutants. We find that in the absence of either RecG or RuvAB, arrested replication forks are maintained and DNA synthesis resumes with kinetics that are similar to that in wild-type cells. The data presented here indicate that RecG- or RuvAB-catalyzed fork regression is not essential for DNA synthesis to resume following arrest by UV-induced DNA damage *in vivo.*

Introduction

All cells must accurately replicate their entire genome each time they reproduce. Although the replication machinery is extremely processive, DNA damage such as that induced by far-ultraviolet light (254 nm) can block the progression of the DNA replication machinery and prevent it from completing its task (Howard-Flanders et al., 1968; Setlow et al., 1963). The failure to accurately resume replication following disruption by DNA damage can result in mutation if an incorrect nucleotide is incorporated, rearrangement if replication resumes from the wrong site, or lethality if the blocking lesions cannot be overcome. In *Escherichia coli*, the recovery of replication following UV irradiation correlates with the time at which the lesions have been repaired by nucleotide excision repair (Courcelle et al., 1999; Courcelle et al., 2003). Cells deficient in nucleotide excision repair are unable to remove UV-induced DNA lesions, fail to recover replication, and exhibit elevated levels of mutagenesis, rearrangements, and cell lethality (Courcelle & Hanawalt, 2001; Hanawalt, 2002; Howard-Flanders, 1968; Howard-Flanders et al., 1968; Rothman & Clark, 1977).

The recovery of replication also depends on RecA and several gene products of the RecF pathway (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle & Hanawalt, 2001; Rothman & Clark, 1977). In the absence of RecA, RecF, RecO, or RecR, the blocked replication fork is not maintained, replication fails to recover, and extensive degradation of the nascent DNA at the replication fork occurs (Chow & Courcelle, 2003; Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2003). The RecQ helicase and RecJ nuclease also belong to the RecF pathway and partially degrade the nascent

lagging strand of the arrested replication fork prior to the resumption of replication (Courcelle et al., 2003; Courcelle & Hanawalt, 1999). These observations have led to the general model that RecA and these RecF pathway gene products function to maintain and process blocked replication forks until the blocking lesion can be repaired by nucleotide excision repair or bypassed by translesion DNA polymerases (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2003; Rangarajan et al., 2002). It is proposed that RecF, RecO, and RecR help stabilize activated RecA filaments at the arrested replication fork structure, thereby maintaining the replication fork DNA and limiting the degradation of the nascent DNA by RecJ and RecQ (Chow & Courcelle, 2003; Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2003). The genetic observation that mutations in *recF*, *recO*, or *recR* delay the induction of LexA regulated gene expression following DNA damage is consistent with the idea that there is less activated RecA present at early times when RecF-O-R is absent (Hegde et al., 1995; Thoms & Wackernagel, 1987).

Based upon this model, it has been proposed that the repair of the DNA lesions in this situation may require displacement of the arrested replication machinery and nascent DNA to allow repair enzymes to gain access to the damaged region (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2001). The displacement of the nascent DNA would allow the parental template strands in that region to reanneal, in effect reversing the branch point of the replication fork to generate a four-arm regressed intermediate (Fig. 2.1). Other models have speculated that replication fork regression could facilitate a recombination-mediated template switch that allows synthesis to occur

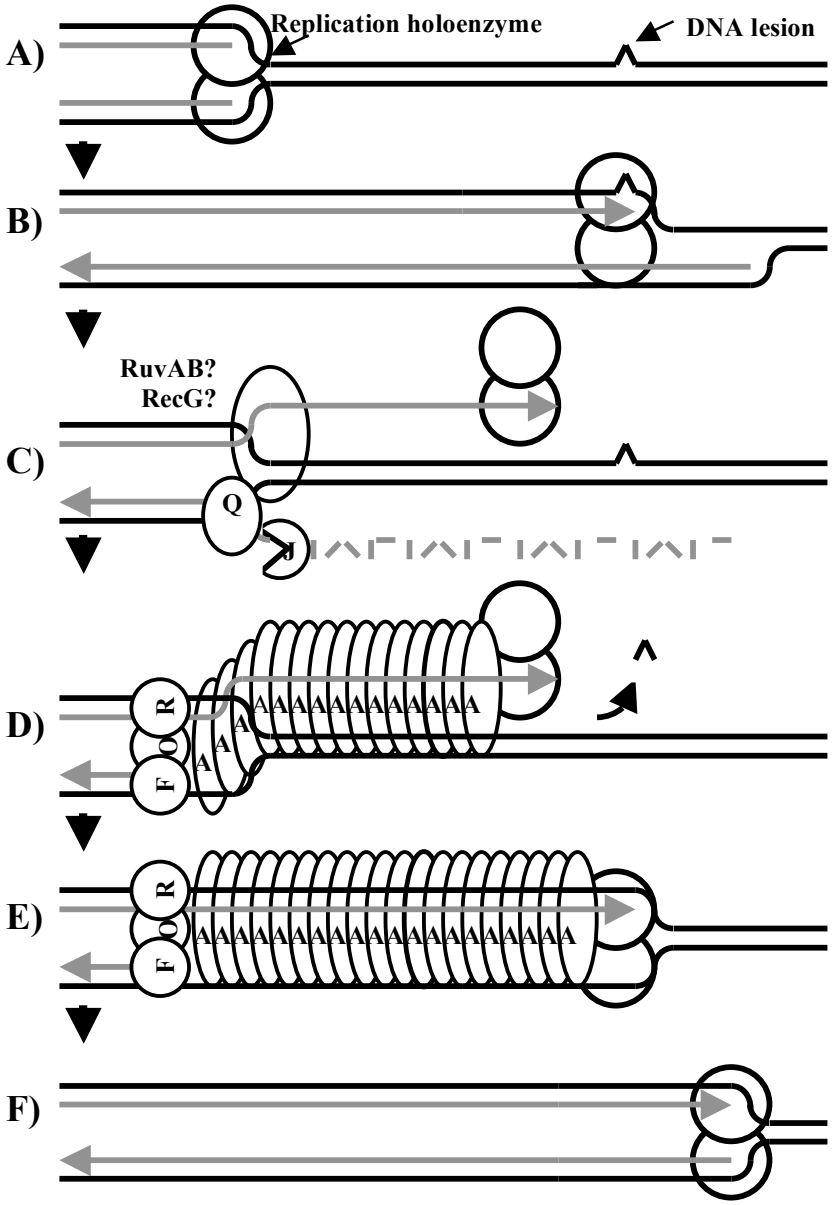


Figure 2.1. Proposed function of RecG or RuvAB during the recovery of replication forks arrested at UV-induced lesions.

(A) Replication is normally extremely processive (B) but is arrested by DNA lesions in the leading strand template (Higuchi et al., 2003; Pages & Fuchs, 2003). (C) RecQ displaces the nascent lagging strand for degradation by RecJ. Based on *in vitro* characterizations, it has been suggested that RecG or RuvAB catalyze fork regression as part of the process required for the recovery of replication. (D) RecFOR and RecA limit nascent DNA degradation and maintain the replication fork until the blocking lesions can be repaired or bypassed. (E) Then, the replication fork can be re-established (F) and processive replication can resume.

past the blocking lesion (Cox, 2002; Higgins et al., 1976; Jaktaji & Lloyd, 2003; Kuzminov, 2001; Lusetti & Cox, 2002; Michel et al., 2001; West, 2003).

Direct evidence for regressed replication fork intermediates has been observed following replication arrest on plasmids. Plasmid replication forks blocked by the DNA binding protein Tus form a reversed intermediate both *in vivo* and *in vitro* (Olavarrieta et al., 2002; Postow et al., 2001). In this case, replication fork regression occurs spontaneously following arrest due to the unwinding of positive supercoils ahead of the replication fork (Postow et al., 2001). A transient regression of the replication fork also occurs following arrest by UV-induced DNA damage on plasmids *in vivo* (Courcelle et al., 2003). The regressed replication fork persists until a time correlating with lesion removal and the resumption of DNA replication. Similar to arrested replication forks on the chromosome, the arrested replication fork intermediates on the plasmid are maintained by RecA, RecF, RecO, and RecR, and are processed by RecQ and RecJ (Courcelle et al., 2003).

Although UV-induced replication fork reversal occurs on plasmids, it is not known whether fork regression also occurs on the bacterial chromosome or whether fork regression is required for replication to resume following disruption. Both RecG and RuvAB have been proposed to catalyze fork reversal *in vivo* based on their *in vitro* activities (Courcelle et al., 2001; McGlynn & Lloyd, 2001a; McGlynn & Lloyd, 2001b). The *ruv* locus, consisting of *ruvA*, *ruvB*, and *ruvC*, was originally identified in a genetic screen for UV-sensitive mutants (Otsuji et al., 1974). In addition to their hypersensitivity to UV, *ruv* mutants also exhibit lower recombination frequencies during conjugation and

transduction and abnormalities in cell division following UV irradiation as seen by the accumulation of long filamentous cells that fail to undergo septation (Lloyd et al., 1984; Otsuji et al., 1974). Purified RuvA and RuvB form a complex that binds to Holliday junctions and promotes ATP-dependent branch migration (Parsons et al., 1992; Parsons & West, 1993). RuvC interacts with RuvAB at Holliday junctions and produces symmetric endonucleolytic incisions at the crossover point to resolve joint molecules (Connolly et al., 1991). *In vitro*, RuvAB can promote branch migration on synthetic replication fork structures to form Holliday junctions (McGlynn & Lloyd, 2001a). However, the enzyme complex preferentially catalyzes the reverse reaction, converting a four-arm Holliday junction into a three-arm, replication fork-like structure (McGlynn & Lloyd, 2001a).

Mutations that inactivate RecG also render cells moderately sensitive to UV and reduce the frequency of conjugational recombination (Storm et al., 1971). Purified RecG is a helicase that is also capable of promoting branch migration of Holliday junctions (Lloyd & Sharples, 1993). In addition, RecG catalyzes the conversion of synthetic three-arm replication fork substrates into four-arm molecules in a manner that preferentially displaces what would represent the nascent lagging strand (McGlynn & Lloyd, 1999; McGlynn & Lloyd, 2001b).

These biochemical characterizations have led to the general view that RecG and potentially RuvAB are required for the recovery of replication following UV-induced DNA damage. We examined this possibility directly and observed that following replication disruption by UV-induced DNA damage, the replication fork is maintained

and DNA synthesis resumes at a time comparable to wild-type when either RecG or RuvAB is absent.

Materials and Methods

Bacterial strains and UV irradiation

Our parental strain, SR108, is a *thyA36 deoC2* derivative of W3110 (De Lucia & Cairns, 1969). The strains HL946 (SR108 *recF332::*Tn*3*) and HL921 (SR108 *recA306::*Tn*10*) have been described previously (Courcelle et al., 1997; Courcelle et al., 1999). The strains CL008 (SR108 *recG258::*Tn*5*), CL532 (SR108 *ruvA59::*Tn*10*), and CL578 (SR108 *ruvAB6204::kan*) were constructed by P1 transduction of the *recG258::*Tn*5*, *ruvA59::*Tn*10*, and *ruvAB6204::kan* alleles from JC19245 (gift from S. Sandler), RDK2641 (Lombardo & Rosenberg, 2000), and TP541 (Murphy et al., 2000) respectively into SR108. The strains CL628 (SR108 *recQ6215::cam recF332::*Tn*3*), CL011 (SR108 *recG258::*Tn*5 recF332::*Tn*3*), and CL744 (SR108 *ruvAB6204::kan recF332::Tn3*) were constructed by P1 transduction of the *recQ6215::cam*, *recG258::*Tn*5*, and *ruvAB6204::kan* alleles from TP648 (Murphy et al., 2000), CL008, and CL578 respectively into HL946. The strain CL561 (SR108 *recG258::*Tn*5 ruvA59::*Tn*10*) was constructed by P1 transduction of the *recG258::*Tn*5* allele into CL532. *ruvA59::*Tn*10* is reported to be a polar mutation affecting both *ruvA* and *ruvB* (Sharples et al., 1990). Phenotypes were confirmed by antibiotic resistance and, when appropriate, UV hypersensitivity or nascent DNA degradation.

UV irradiation for all experiments was performed using a Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/sec.

UV survival studies

Fresh overnight cultures were diluted 1:100 in 10 ml of Davis medium (2.0 g KH_2PO_4, 7.0 g K_2HPO_4, 0.5 g $Na_3C_6H_5O_7$, 0.1 g $MgSO_4$, 1.0 g $(NH_4)_2SO_4$ per liter, pH 7.0) supplemented with 0.4% glucose, 0.2% casamino acids, and 10 µg/ml thymine (DGCthy medium) and grown to an OD_{600} of 0.5 in a 37ºC shaking incubator. Serial dilutions of each culture were plated in triplicate on Luria-Bertani plates supplemented with 10 µg/ml thymine and UV irradiated at the indicated doses. Plates were incubated overnight at 37ºC and colonies were counted the next day.

Growth rates

Fresh overnight cultures were diluted 1:1000 in DGCthy medium and 200 µl aliquots were plated on a 96 well microtiter plate. The optical density at 600 nm for each culture was measured with Molecular Devices SPECTRAmax Plus and analyzed with SOFTmax Pro 4.0.

Total DNA accumulation

Fresh overnight cultures were diluted 1:100 in 40 ml DGCthy medium supplemented with 0.1 µCi/ml [^{3}H] thymine (60.5 Ci/mmol) and grown to an OD_{600} of 0.4 in a 37ºC shaking incubator. At this time, half the culture was UV irradiated with 27 J/m^2 and the other half was mock irradiated. At 5 min intervals, duplicate 200 µl aliquots were precipitated in 5 ml of 5% trichloroacetic acid (TCA) and filtered onto Millipore

glass fiber prefilters. The amount of ^{3}H-labeled DNA on each filter was determined by liquid scintillation counting (Courcelle et al., 1999).

Density labeling and CsCl analysis

Fresh overnight cultures were diluted 1:100 in 20 ml of DGCthy medium supplemented with 0.1 µCi/ml of [^{14}C] thymine (53 mCi/mmol) and were grown to an OD_{600} of 0.5 (approximately 10^8 cells/ml) in a 37ºC shaking incubator. At this time, half the culture was UV irradiated with 27 J/m^2 and the other half was mock irradiated. Cultures were then filtered onto FisherBrand general filtration 0.45 µm membranes, washed with 1X NET buffer (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0), resuspended in 10 ml DGC medium supplemented with 20 µg/ml 5-bromouracil in place of thymine and 0.5 µCi/ml [^{3}H] thymine (60.5 Ci/mmol), and allowed to recover for a period of 1 hr in a 37ºC shaking incubator. Two volumes of ice cold NET buffer were added to the 10ml cultures, and the cells were then pelleted, resuspended in 150µl TE (10mM Tris, 1mM EDTA, pH 8.0), and lysed in 170 µl of 0.5 M H_2KPO_4/KOH, pH 12.5 and 1.25% Sarkosyl. Isopycnic alkali CsCl gradients composed of 0.3 g of a DNA lysate solution, 2.23 g CsCl and 3.31 g of a 0.1M H_2KPO_4/KOH, pH 12.5 solution (refractive index 1.4055) were centrifuged to equilibrium at 80,000 g for 96 hr at 20ºC. Gradients were collected in approximately 30 fractions onto Whatman No. 17 paper, washed in 5% TCA, and then in 95% ethanol. The quantity of ^{3}H and ^{14}C in each fraction was determined by liquid scintillation counting (Courcelle et al., 1997).

Rate of DNA synthesis

The assay to measure the rate of DNA synthesis was modified from previous studies (Khidhir et al., 1985; Rangarajan et al., 2002). Fresh overnight cultures were diluted 1:100 in 50 ml of DGCthy medium supplemented with 0.1 μCi/ml of [^{14}C] thymine (53 mCi/mmol) and grown to an OD_{600} of 0.4 in a 37ºC shaking incubator. At this time, half the culture was UV irradiated with 27 J/m^2 and the other half was mock irradiated. At the indicated times, 1 μCi/ml [^{3}H] thymidine (77.8 Ci/mmol) was added to duplicate 0.5 ml aliquots for 2 min at 37ºC, before the cells were lysed and DNA precipitated in 5 ml of 5% TCA, and filtered onto Millipore glass fiber prefilters. The amount of ^{3}H and ^{14}C on each filter was determined by liquid scintillation counting.

Degradation of nascent and genomic DNA

Fresh overnight cultures were diluted 1:100 in 10 ml DGCthy medium supplemented with 0.1 μCi/ml [^{14}C] thymine (53 mCi/mmol) and grown to an OD_{600} of 0.4 in a 37ºC shaking incubator. Cultures were labeled for 5 sec with 1 μCi/ml [^{3}H] thymidine (77.8 Ci/mmol), filtered onto FisherBrand general filtration 0.45 μm membranes, washed with NET buffer, and resuspended in non-radioactive DGCthy medium. Cultures were immediately irradiated with a UV dose of 27 J/m^2. At the indicated times, duplicate 0.2 ml aliquots (triplicate for the 0 time point) were precipitated in 5 ml of 5% cold TCA and filtered on Millipore glass fiber prefilters. The amount of ^{3}H and ^{14}C on each filter was determined by liquid scintillation counting (Courcelle et al., 1997).

Results

RuvAB and RecG are not required for the recovery of DNA synthesis following UV-induced DNA damage

Isogenic strains lacking either RecG, RuvAB, or both gene products were constructed by standard P1 transduction. As previously reported, the *recG* and *ruvAB* mutants were moderately hypersensitive to UV irradiation (Fig. 2.2A) (Bolt & Lloyd, 2002; Gregg et al., 2002; Ryder et al., 1994). In the *ruvAB recG* double mutant, the hypersensitivity was more severe than in either single mutant and was comparable to that of *recA* (Fig. 2.2A). However, unlike *recA*, the *ruvAB recG* double mutant grew poorly even in the absence of exogenous DNA damage (Fig. 2.2B). Previous studies have documented that RecA is absolutely required for the recovery of replication following inhibition of DNA synthesis (Howard-Flanders, 1968), yet *recA* mutants grow at rates comparable to wild-type cells in the absence of DNA damage. This observation then suggests that replication is not frequently inhibited in the absence of DNA damage. Furthermore, the poor growth of the *ruvAB recG* mutant relative to that of *recA* under these same conditions indicates that RuvAB or RecG is required to process DNA structures other than disrupted replication forks that arise during the normal replication cycle. However, this observation alone does not preclude the possibility that in addition to these alternative roles, they may also be required to process replication forks prior to their recovery.

To determine whether the hypersensitivity of *ruvAB* or *recG* mutants results directly from a failure to resume DNA synthesis following disruption by UV irradiation,

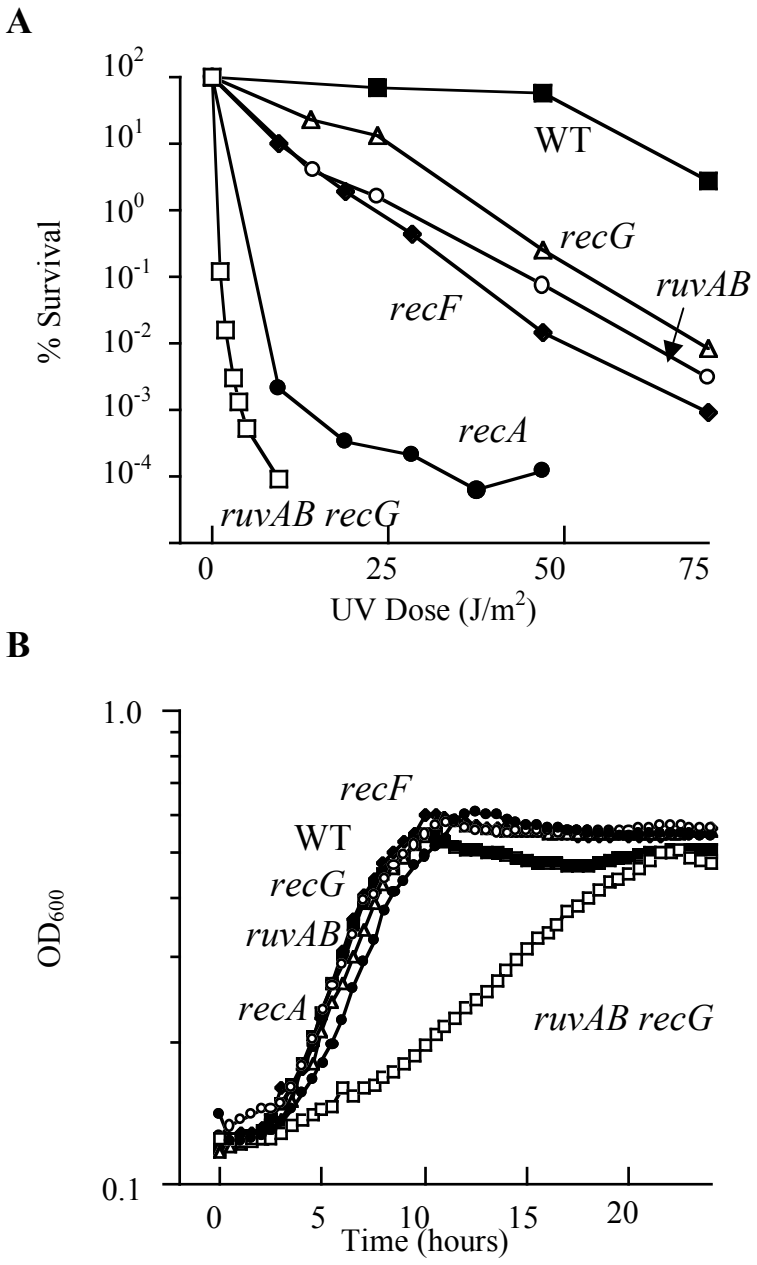


Figure 2.2. Survival following UV irradiation and growth in the absence of DNA damage of wild-type, *recF*, *recG*, *ruvAB,* and *ruvAB recG* strains of *Escherichia coli*.

(A) The percent of cells surviving the indicated dose of UV irradiation is plotted for each strain. Survival curves represent an average of at least two independent experiments. (B) The optical density of each strain at 600 nm is plotted over time. wild-type (■), *recF* (◆), *recG* (Δ), *ruvAB* (❍), *recA* (●), and *ruvAB recG* (□).

we monitored DNA synthesis after UV irradiation in these mutants by [^{3}H] thymine incorporation. Following a UV dose of 27 J/m^2, wild-type cultures exhibited a transient arrest of replication before synthesis resumed at a rate comparable to that in unirradiated cultures (Fig. 2.3). In contrast, *recF* mutants, which are deficient in the resumption of disrupted replication forks (Courcelle et al., 1997; Courcelle et al., 1999), exhibited no further increase in the amount of [^{3}H]-labeled DNA following UV irradiation. When we examined UV irradiated cultures of *ruvAB* or *recG*, we observed that both mutants resumed replication at a time comparable to that in wild-type cultures (Fig. 2.3).

We also examined *ruvAB recG* double mutants to determine if the absence of both gene products prevented the recovery of replication following UV-induced DNA damage. In these mutants, the rate of DNA synthesis recovered to an extent that was comparable to unirradiated *ruvAB recG* cultures. However, the slow growth that occurs in unirradiated *ruvAB recG* cultures makes it inappropriate to compare the recovery observed in this mutant directly to wild-type cells.

The recovery of replication in *ruvAB* and *recG* mutants was also monitored by density labeling the DNA synthesized during the first hour following UV irradiation. Irradiated or mock irradiated cultures were incubated in medium containing 5-bromouracil in place of thymine for one hour such that the DNA made during this period was greater in density than that of the DNA synthesized before treatment. DNA synthesized during the recovery period was then isolated and quantitated in isopycnic alkali CsCl gradients. By this measure, wild-type cultures had almost completely recovered replication one hour after UV irradiation, as seen by the nearly equivalent

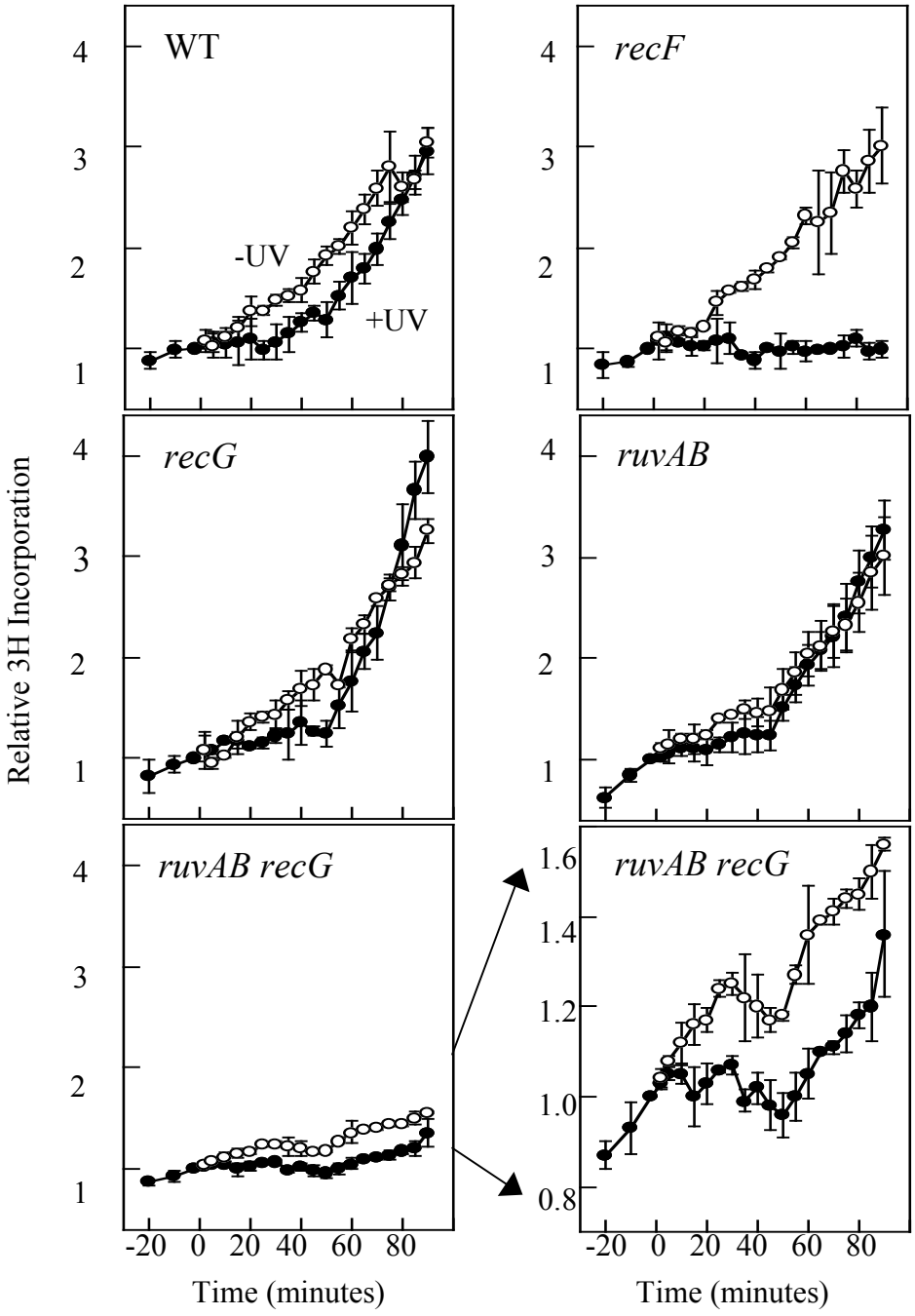


Figure 2.3. *recG* and *ruvAB* recover robust replication at a time similar to that in wild-type cells.

Cultures grown in the presence of [^{3}H] thymine were either UV irradiated with 27 J/m^2 (●) or mock irradiated (❍). The amount of ^{3}H incorporated over time is plotted. Cultures were irradiated at time 0. Each graph represents an average of three independent experiments. Error bars represent one standard deviation.

amounts of DNA synthesis in the irradiated and unirradiated cultures (Fig. 2.4). By contrast, very little DNA synthesis occurred following UV treatment in *recF* mutants. When we examined post-irradiation DNA synthesis in *ruvAB* and *recG* mutants, we observed an amount of DNA synthesis that was comparable to the unirradiated controls, indicating that DNA synthesis was resuming similar to that in wild-type cultures (Fig. 2.4).

In *ruvAB recG* double mutants, we observed an intermediate amount of DNA synthesis in the irradiated culture relative to the unirradiated culture. However, both irradiated and nonirradiated cultures exhibited abnormal patterns of replication, with a significant amount of the DNA synthesis migrating at densities in the intermediate and light regions of the gradient. DNA migrating in these regions may indicate elevated levels of recombination or repair synthesis. The detection of this type of synthesis in unirradiated *ruvAB recG* mutants may be due in part to the toxicity associated with the 5-bromouracil that is used to density label the DNA in this assay. The toxicity of 5-bromouracil is thought to be due in part to the lower incorporation efficiency of this base analog compared to thymine and also because the bromine group on the analog is labile, leading to elevated levels of uracil and uracil glycolyase-induced nicks in the DNA. Incubation in media containing 5-bromouracil results in elevated levels of sister chromatid exchanges and cell death within approximately two rounds of replication (Hackett & Hanawalt, 1966; Krasin & Hutchinson, 1978; Little, 1976). Thus, similar to the previous assay, a direct comparison between wild-type and *ruvAB recG* mutants should be interpreted with caution. However, some DNA synthesis occurs in the UV

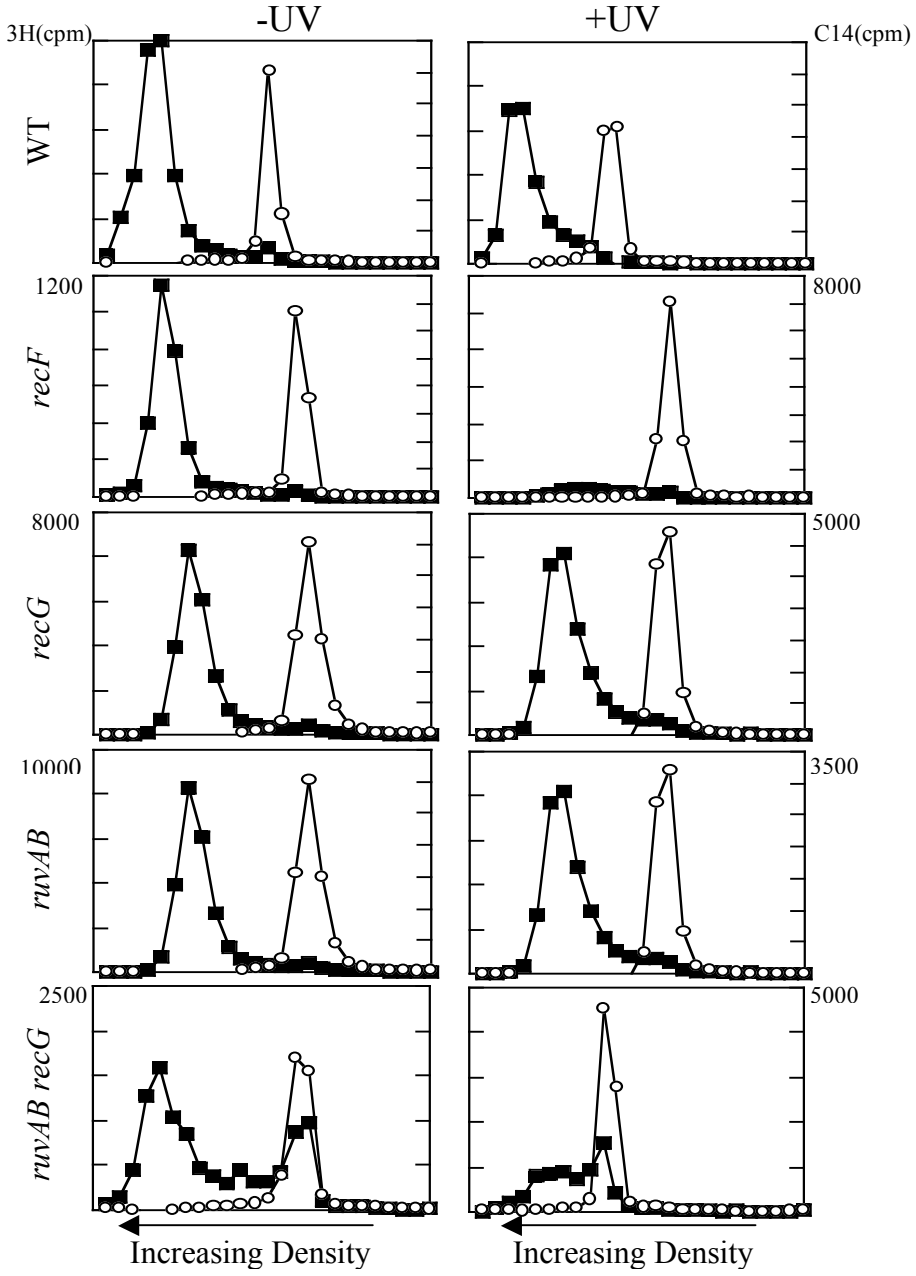


Figure 2.4. *recG* and *ruvAB* cultures synthesize a similar amount of DNA as wild-type cultures during the first hour after UV-irradiation.

The amount of DNA synthesized in UV irradiated (25 J/m^2) or mock irradiated cultures was determined by density labeling the DNA with 5-bromouracil and subsequent isolation in alkali CsCl gradients. DNA synthesized before treatment [^{14}C] (❍); DNA synthesized following treatment [^{3}H] (■). Each graph represents one of at least two, independent experiments.

irradiated *ruvAB recG* mutants, although the viability of these cells is clearly compromised and abnormal relative to wild-type cells even in the absence of UV irradiation.

The previous two assays indicate that replication recovers in the absence of either *recG* or *ruvAB*. However, it remains possible that although robust replication resumes in *ruvAB* or *recG* mutants, the time at which DNA synthesis recovers may be delayed relative to wild-type. To examine this possibility in *recG* and *ruvAB* mutants, we measured the rate of DNA synthesis following UV irradiation by incubating [^{14}C] thymine labeled cultures for 2 min with [^{3}H] thymidine at various times after treatment. The rate of DNA synthesis (^{3}H incorporation/min) could then be determined relative to the total amount of DNA present (^{14}C incorporation) at specific times following treatment. Using this assay, we observed that the rate of DNA synthesis was reduced by approximately 90% in wild-type cells at early times following UV irradiation (Fig. 2.5). Within 20 min, the rate of DNA synthesis began to recover, and by 40 min, the rate of replication was nearly restored to pre-irradiation levels and there was a detectable increase in total DNA accumulation. In UV-irradiated *recF* mutants, the reduction in DNA synthesis was more severe and, consistent with our previous assays, the rate of synthesis did not recover. However, following UV irradiation of *recG* or *ruvAB* mutants, we observed that the time and efficiency with which DNA synthesis recovered were similar to those in wild-type. These observations indicate that RuvAB or RecG function is not essential for replication to resume following disruption by UV-induced DNA damage. In the *ruvAB recG* double mutants, the rate of DNA synthesis recovered to a

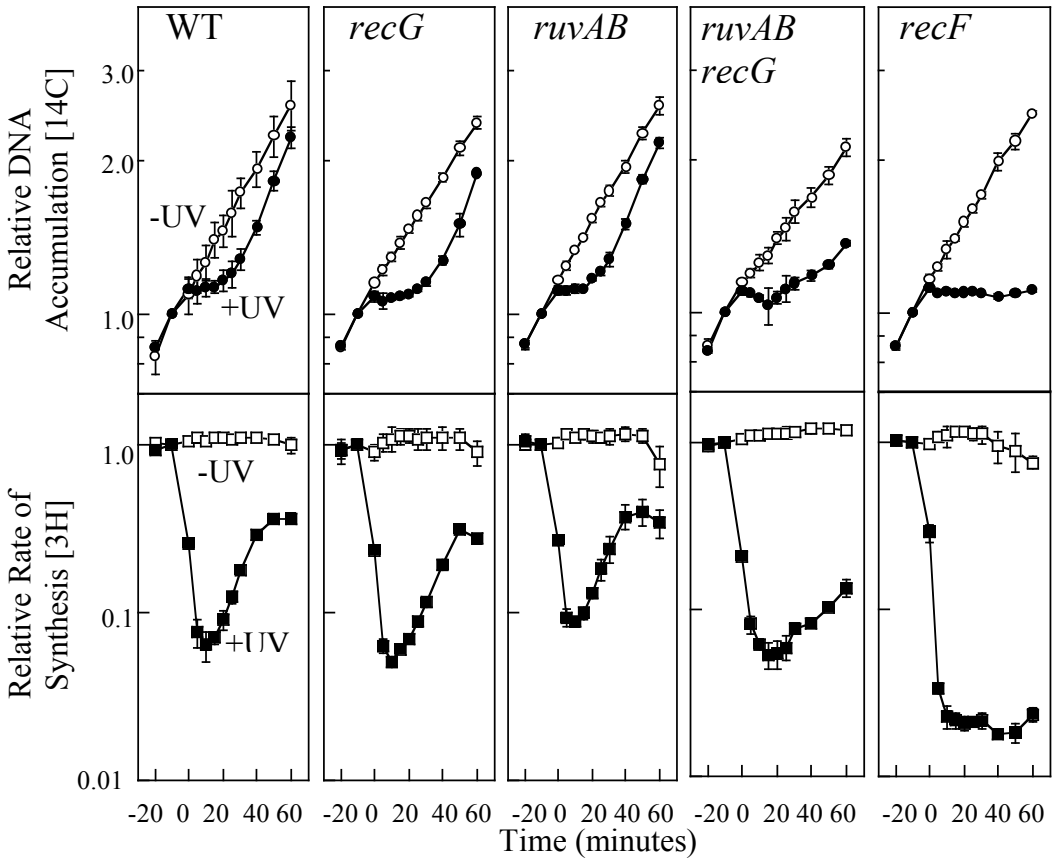


Figure 2.5. *recG* and *ruvAB* resume DNA synthesis with kinetics similar to that of wild-type following UV irradiation.

Cultures grown in the presence of [^{14}C] thymine were pulsed-labeled with [^{3}H] thymidine for 2 min at the indicated times following either 27 J/m^2 of UV irradiation or mock irradiation. The relative amount of ^{14}C and ^{3}H incorporated into the DNA is plotted over time. Cultures were irradiated at time 0. Graphs represent an average of three independent experiments. Error bars represent one standard deviation. Total DNA in mock irradiated cultures (❍); total DNA in irradiated cultures (●); rate of DNA synthesis in mock irradiated cultures (□); rate of DNA synthesis in irradiated cultures (■).

significant extent and approximated the recovery observed in wild-type cultures much more closely than that in *recF* mutants. Although direct comparisons between these strains should be made with caution, the observation that DNA synthesis is inhibited to a greater extent in *recF* mutants than in *ruvAB recG* double mutants suggests that the recovery of DNA synthesis in the single mutants is not due to the simple interpretation that RecG and RuvAB serve redundant functions in this respect. The double mutant recovers to a greater extent than the *recF* mutant despite the fact that it is much more sensitive to DNA damage and grows more poorly than the *recF* mutant (Fig. 2.2).

RuvAB and RecG are not required to maintain the replication fork after UV irradiation

Strains lacking RecF, RecO, or RecR fail to maintain disrupted replication forks, resulting in extensive degradation of the nascent DNA at the replication fork (Courcelle et al., 1997; Courcelle et al., 2003). Both RecG and RuvAB have also been proposed to act on arrested replication fork structures *in vivo* (McGlynn & Lloyd, 2001a; McGlynn & Lloyd, 2001b). To determine whether RuvAB or RecG are required to maintain replication forks arrested at UV-induced DNA damage *in vivo*, we measured the amount of degradation that occurred in the nascent DNA at the replication fork. To this end, [^{14}C]-labeled cultures were pulsed-labeled with [^{3}H] thymidine for 5 sec, transferred to non-radioactive media, and immediately UV irradiated. Then, the amount of radioactivity remaining in the DNA was followed over time. This assay allowed us to compare the amount of degradation that occurred in the nascent strands of the replication fork directly to the total DNA in the cell. In UV irradiated wild-type cells, we observed a limited amount of nascent DNA degradation at times prior to the recovery of replication,

consistent with our previous studies (Fig. 2.6) (Courcelle & Hanawalt, 1999). In *recF* mutants, the replication fork was not maintained and approximately half of the nascent DNA was degraded. By comparison, in *ruvAB* or *recG* mutants, the nascent DNA was not extensively degraded following UV irradiation. In addition, *ruvAB recG* double mutants did not exhibit extensive degradation of DNA following UV irradiation, indicating that these gene products are not required to maintain or protect the nascent DNA at replication forks.

The nascent DNA degradation that occurs prior to the resumption of replication is dependent on RecQ helicase and RecJ nuclease (Courcelle & Hanawalt, 1999). RecQ helicase is required to displace the nascent lagging strand for degradation by RecJ nuclease *in vivo*. Based on *in vitro* characterizations, it has been proposed that RecG and potentially RuvAB also displace the nascent lagging strand of arrested replication forks (McGlynn et al., 2001; Michel et al., 2001). If true, then we would predict that inactivation of RecG or RuvAB should also prevent nascent DNA degradation from occurring similar to when RecQ is inactivated. To test this possibility, we examined the degradation that occurred in UV irradiated *recF* mutants that were also deficient in either RuvAB or RecG. As shown in Figure 2.6, the absence of RuvAB or RecG did not prevent the extensive degradation of the nascent DNA in *recF* mutants. In contrast, in *recF* mutants that also lacked RecQ, the degradation of nascent DNA was significantly reduced. The lack of nascent DNA processing in the *recF recQ* mutant was most evident during the first hour following UV irradiation (Fig. 2.6). This result indicates that RuvAB

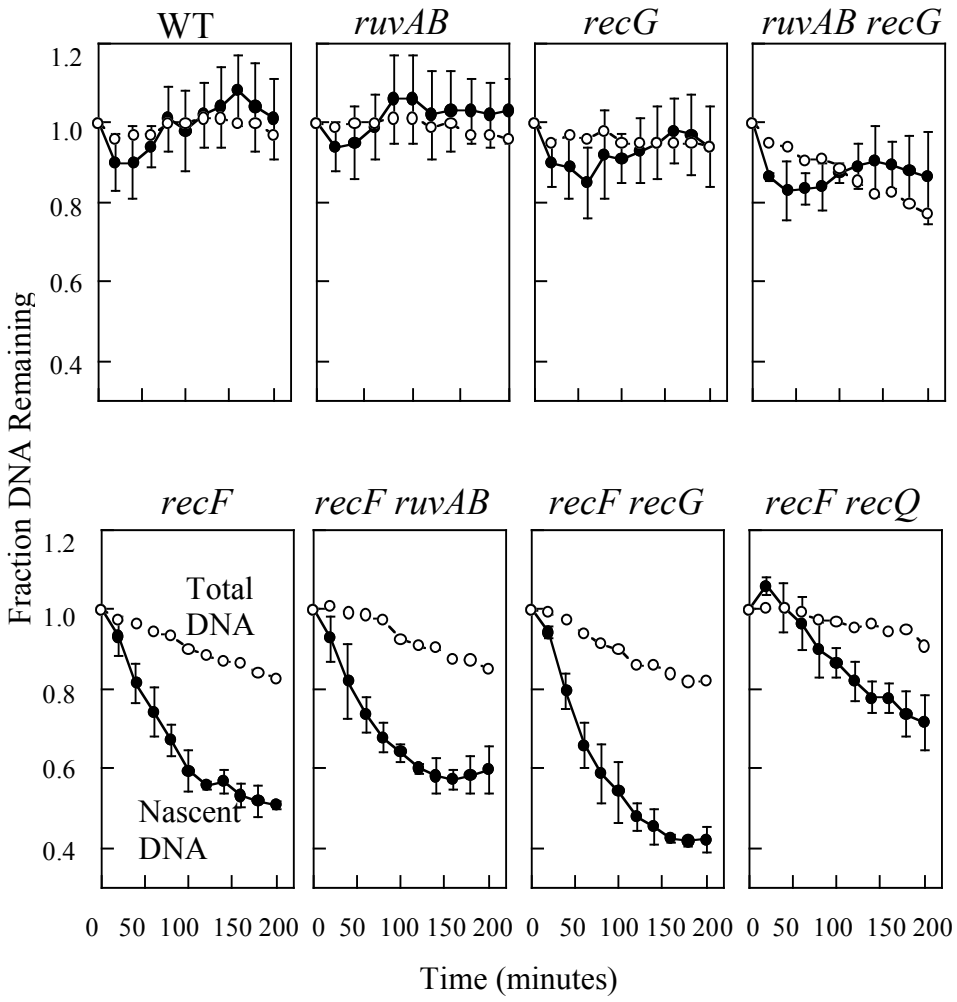


Figure 2.6. RuvAB or RecG is not required to maintain arrested replication forks or displace the nascent DNA prior to degradation following UV irradiation.

[^{3}H] thymidine was added to [^{14}C] thymine pre-labeled cells for 5 sec, washed, and UV irradiated in non-labeled medium. The relative amount of ^{3}H and ^{14}C remaining in the DNA is plotted over time. Graphs represent an average of at least three independent experiments for each strain. Error bars represent one standard deviation. Total DNA [^{14}C] (❍); nascent DNA [^{3}H] (●).

and RecG are not required to displace the nascent lagging strand prior to degradation *in vivo*.

Since this assay specifically measures nascent DNA degradation, and previous studies have shown this degradation occurs preferentially on the nascent lagging strand (Courcelle & Hanawalt, 1999), it remains possible that RuvAB or RecG facilitates the displacement of the nascent leading strand or portions of the lagging strand that are not subject to degradation and therefore are not detected in this assay.

Discussion

Based on biochemical data, several studies have speculated that either RecG or RuvAB catalyze replication fork regression *in vivo* and play a critical role in promoting the recovery of replication when it is blocked by DNA damage (Bolt & Lloyd, 2002; Gregg et al., 2002; McGlynn & Lloyd, 1999; McGlynn & Lloyd, 2001a; McGlynn & Lloyd, 2001b). Using a number of cellular assays, we examined the contribution of RuvAB and RecG to the ability of cells to recover replication following UV irradiation *in vivo*. We found that the absence of either RecG or RuvAB does not affect the cell's ability to resume DNA synthesis. In addition, unlike RecF, RecO, or RecR, we observe that RuvAB or RecG is not required to maintain replication forks following arrest by DNA damage and that neither protein prevents the extensive nascent DNA degradation that occurs in the absence of RecF.

Although these results cannot exclude the possibility that RuvAB or RecG proteins catalyze fork regression *in vivo*, they demonstrate that their function is not required for DNA synthesis to resume following UV-induced DNA damage. It remains

possible that RuvAB- or RecG-catalyzed replication fork regression increases the accuracy or fidelity of replication recovery, but that the regression is not essential for the resumption to occur. By analogy, both RecJ and RecQ process, or partially degrade, the nascent DNA at arrested replication forks in a manner that is believed to increase the frequency that replication resumes from the proper location (Courcelle & Hanawalt, 1999). However, the absence of either RecJ or RecQ does not prevent replication from resuming following UV irradiation, although it does effect the time at which DNA synthesis resumes (Courcelle & Hanawalt, 1999). A second possibility is that fork regression catalyzed by RecG and RuvAB is required for recovery in only a small subset of the total arrested-fork substrates and therefore is below our limit of detection in these cellular assays. Another possibility is that alternative or redundant activities may allow replication to recover in the absence of RecG or RuvAB. Along this line of reasoning, RadA was recently speculated to offer a third potentially redundant activity for replication fork processing based upon survival studies (Beam et al., 2002). However, if either RecG- or RuvAB-catalyzed fork regression is a predominant mechanism by which arrested replication forks normally resume, it seems reasonable to expect that we would have observed a delay in the timing, or a reduction in the efficiency, of the resumption of DNA synthesis. Even allowing for potential redundancies, one might expect that the secondary activity would promote recovery with different (or reduced) kinetics when the primary activity is absent. Our observations show that even though *ruvAB* and *recG* mutants are more sensitive to UV irradiation than wild-type cells, *ruvAB* and *recG* mutants are able to recover DNA synthesis as efficiently as wild-type cells, arguing

against a *requirement* for either of these enzymes in a prominent pathway that allows DNA synthesis to resume.

The poor growth of *ruvAB recG* double mutants is often interpreted to suggest that replication is frequently disrupted by DNA damage or other impediments during replication which then requires processing by branch migration enzymes in order to resume (Mandal, 1993; Ryder et al., 1994). However, cell viability is an extremely broad criterion by which to measure a specific question such as the ability for DNA synthesis to resume. The survival of a cell could also be compromised by any of a large number of alternative DNA processing events such as chromosome partitioning, replication termination, or resolution of joint dimer chromosomes, among others. The observation that wild-type cells recover from UV doses that reduce the viability of *recG* or *ruvAB* mutants by greater than 99% highlights the observation that these enzymes are essential for some DNA processing event that arises in these cells following moderate levels of DNA damage. If any of the several processing events mentioned above were to function as the preferred targets for these branch migration enzymes, the normal resumption of DNA synthesis would not be expected to be impaired, but could result in elevated levels of lethality in the presence of DNA damage. A second possibility is that the DNA synthesis that occurs in *recG* or *ruvAB* mutants represents an aberrant form of DNA synthesis, potentially resuming from the wrong template, and leads to lethality in the absence of RecG or RuvAB processing. However, it is clear from the observations presented in this study that the lethality is not the result of a failure to resume DNA synthesis, such as occurs in *recF* or *recA* mutants.

Other genetic studies have previously been interpreted to support a role for RuvAB or RecG at arrested replication forks. Following prolonged incubation of a thermosensitive *dnaB* mutant at the restrictive temperature, elevated levels of double-strand breaks accumulate in the genome of *recBC* mutants as observed by pulsed-field gel electrophoresis (Michel et al., 1997). The accumulation of double-strand breaks in *dnaB recB* mutants requires RuvABC function (Seigneur et al., 1998). Based on these observations, it has been speculated by others that inactivation of replication proteins such as DnaB may mimic the disruption that occurs following replication fork encounters with DNA damage (Gregg et al., 2002; Jaktaji & Lloyd, 2003). It was further speculated that if this interpretation were true, then the RuvAB-dependent double-strand breaks could be explained if RuvAB catalyzed the formation of Holliday junctions at stalled replication forks which are then cleaved by RuvC endonuclease or degraded by RecBCD (Bolt & Lloyd, 2002; Cox et al., 2000; Gregg et al., 2002). Based on these observations in thermosensitive replication mutants, it has been inferred from several subsequent studies that RuvABC and RecBCD are required to resume replication following arrest by DNA damage. However, our observations indicate that the resumption of DNA synthesis following UV-induced DNA damage does not require RuvAB, and several previous studies have shown that replication resumes normally in *recBC* and *recD* mutants following UV-induced DNA damage (Courcelle et al., 1997; Courcelle & Hanawalt, 1999; Khidhir et al., 1985). Furthermore, although RecJ and RecQ process the nascent DNA at lesion arrested replication forks, RecBCD does not degrade the nascent DNA at arrested replication forks (Courcelle et al., 1997; Courcelle & Hanawalt, 1999; Khidhir et

al., 1985). Therefore, we believe these observations indicate that the events and enzymes operating at lesion-blocked replication forks are different from those that occur following the removal of specific proteins of the replication machinery.

The basis for the proposal that RecG may promote the rescue of arrested replication forks *in vivo* comes primarily from survival studies following UV irradiation (Gregg et al., 2002; Jaktaji & Lloyd, 2003). By examining the survival of *recG* mutants following UV irradiation in various genetic backgrounds, it has been widely speculated that RecG promotes the rescue of stalled replication forks through a number of different recovery pathways (Dillingham & Kowalczykowski, 2001; Gregg et al., 2002; Jaktaji & Lloyd, 2003; McGlynn & Lloyd, 2000; McGlynn & Lloyd, 2002). However, if this were true, one would predict that the absence of RecG would have an effect on the cell's ability to recover DNA synthesis following UV irradiation. Our observation that *recG* recovers DNA synthesis with kinetics similar to that in wild-type cells argues against the interpretation that RecG has an essential role in promoting the rescue of arrested replication forks following UV-induced DNA damage. However, this does not necessarily exclude the possibility that RecG participates in the recovery process or possibly enhances strand displacement at arrested replication forks.

Although many gene products have been intensely studied for how they affect recombinational processes over the years, the conceptual realization that many of the "*rec*" gene products function to maintain the strands of genetic information rather than rearrange them during chromosome replication has been suggested previously and investigated recently (Campbell, 1984; Courcelle et al., 1997). This perspective has

generated significant amounts of discussion and renewed enthusiasm in the question of how replication recovers following disruption. As a result, in some cases, genes have been speculated to be required for this process based primarily on the observation that their respective mutants grow poorly or render cells hypersensitive to UV. RuvAB and RecG have both been characterized for their recombinational roles and only recently were proposed to promote the recovery of replication based on the observation that the mutants are moderately hypersensitive to UV irradiation (Bolt & Lloyd, 2002; McGlynn & Lloyd, 2001a; McGlynn & Lloyd, 2001b; McGlynn et al., 2001). Although this represents a good starting point for investigation, the recovery of replication is not the only process required for successful reproduction, and UV hypersensitivity does not always correlate with a defect in the recovery of replication. For example, both *recF* and *recBC* mutants are equally hypersensitive to UV irradiation, but while *recF* mutants fail to recover replication following UV irradiation, *recBC* mutants recover replication normally (Courcelle et al., 1997; Courcelle & Hanawalt, 1999). By comparison, *recJ* and *recQ* mutants are not sensitive to UV irradiation, yet these gene products participate in the processing of nascent DNA at replication forks prior to the recovery of replication (Courcelle & Hanawalt, 1999). Although the results presented here cannot exclude the possibility that RuvAB or RecG participate in the regression of the arrested replication forks, we can conclude that neither gene product is essential for DNA synthesis to resume. This implies that the hypersensitivity of these mutants arises from an event(s) that potentially occurs after replication resumes or because replication resumes from the wrong template or the wrong place in their absence. It will be important to develop novel

cellular assays to determine whether replication fork regression is required for the resumption of replication on the bacterial chromosome. In light of these observations, it may also be useful to consider potential alternative roles for RecG and RuvAB function following UV-induced DNA damage.

Acknowledgements

We thank Anthony Poteete, Susan Rosenberg, and Steve Sandler for providing bacterial strains used in this study. This work was supported by award MCB0130486 from the National Science Foundation and a National Research Service Award F32 GM068566 from the National Institutes of Health to CTC.

REFERENCES

Beam, C. E., Saveson, C. J. & Lovett, S. T. (2002). Role for *radA/sms* in recombination intermediate processing in *Escherichia coli*. *J. Bacteriol.* 184, 6836-44.

Bolt, E. L. & Lloyd, R. G. (2002). Substrate specificity of RusA resolvase reveals the DNA structures targeted by RuvAB and RecG *in vivo*. *Mol. Cell.* 10, 187-98.

Campbell, A. (1984). Types of recombination: common problems and common strategies. *Cold Spring Harb. Symp. Quant. Biol.* 49, 839-44.

Chow, K. H. & Courcelle, J. (2003). RecO acts with RecF and RecR to protect and maintain replication forks blocked by UV-induced DNA damage in *Escherichia coli*. *J. Biol. Chem. in press*.

Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991). Resolution of Holliday junctions *in vitro* requires the Escherichia coli *ruvC* gene product. *Proc. Natl. Acad. Sci. U S A* 88, 6063-7.

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, 3714-9.

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, 916-22.

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, 1064-7.

Courcelle, J., Ganesan, A. K. & Hanawalt, P. C. (2001). Therefore, what are recombination proteins there for? *Bioessays* 23, 463-70.

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, 543-51.

Courcelle, J. & Hanawalt, P. C. (2001). Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated *Escherichia coli* need not involve recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8196-202.

Cox, M. M. (2002). The nonmutagenic repair of broken replication forks via recombination. *Mutat. Res.* 510, 107-20.

Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J. & Marians, K. J. (2000). The importance of repairing stalled replication forks. *Nature* 404, 37-41.

De Lucia, P. & Cairns, J. (1969). Isolation of an *E. coli* strain with a mutation affecting DNA polymerase. *Nature* 224, 1164-66.

Dillingham, M. S. & Kowalczykowski, S. C. (2001). A step backward in advancing DNA replication: rescue of stalled replication forks by RecG. *Mol. Cell* 8, 734-36.

Gregg, A. V., McGlynn, P., Jaktaji, R. P. & Lloyd, R. G. (2002). Direct rescue of stalled DNA replication forks via the combined action of PriA and RecG helicase activities. *Mol. Cell* 9, 241-51.

Hackett, P., Jr. & Hanawalt, P. (1966). Selectivity for thymine over 5-bromouracil by a thymine-requiring bacterium. *Biochim. Biophys. Acta.* 123, 356-63.

Hanawalt, P. C. (2002). Subpathways of nucleotide excision repair and their regulation. *Oncogene* 21, 8949-56.

Hegde, S., Sandler, S. J., Clark, A. J. & Madiraju, M. V. (1995). *recO* and *recR* mutations delay induction of the SOS response in *Escherichia coli*. *Mol. Gen. Genet.* 246, 254-8.

Higgins, N. P., Kato, K. & Strauss, B. (1976). A model for replication repair in mammalian cells. *J. Mol. Biol.* 101, 417-25.

Higuchi, K., Katayama, T., Iwai, S., Hidaka, M., Horiuchi, T. & Maki, H. (2003). Fate of DNA replication fork encountering a single DNA lesion during *oriC* plasmid DNA replication *in vitro*. *Genes Cells* 8, 437-49.

Howard-Flanders, P. (1968). Some properties of excision-defective recombination-deficient mutants of *Escherichi coli* K-12. *J. Bacteriol.* 97, 1134-41.

Howard-Flanders, P., Rupp, W. D., Wilkins, B. M. & Cole, R. S. (1968). DNA replication and recombination after UV irradiation. *Cold Spring Harb. Symp. Quant. Biol.* 33, 195-207.

Jaktaji, R. P. & Lloyd, R. G. (2003). PriA supports two distinct pathways for replication restart in UV-irradiated *Escherichia coli* cells. *Mol. Microbiol.* 47, 1091-100.

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.

Krasin, F. & Hutchinson, F. (1978). Strand breaks and alkali-labile bonds induced by ultraviolet light in DNA with 5-bromouracil in vivo. *Biophys. J.* 24, 657-64.

Kuzminov, A. (2001). DNA replication meets genetic exchange: chromosomal damage and its repair by homologous recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8461-8.

Little, J. W. (1976). The effect of 5-bromouracil on recombination of phage lambda. *Virology* 72, 530-5.

Lloyd, R. G., Benson, F. E. & Shurvinton, C. E. (1984). Effect of *ruv* mutations on recombination and DNA repair in *Escherichia coli* K12. *Mol. Gen. Genet.* 194, 303-9.

Lloyd, R. G. & Sharples, G. J. (1993). Processing of recombination intermediates by the RecG and RuvAB proteins of *Escherichia coli*. *Nucleic Acids Res.* 21, 1719-25.

Lombardo, M. J. & Rosenberg, S. M. (2000). *radC102* of *Escherichia coli* is an allele of *recG*. *J. Bacteriol.* 182, 6287-91.

Lusetti, S. L. & Cox, M. M. (2002). The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. *Annu. Rev. Biochem.* 71, 71-100.

Mandal, T. N., A. A. Mahdi, G. J. Sharples, and R. G. Lloyd. (1993). Resolution of Holliday intermediates in recombination and DNA repair: indirect suppression of *ruvA*, *ruvB*, and *ruvC* mutations. *J. Bacteriol.* 175, 4325-34.

McGlynn, P. & Lloyd, R. G. (1999). RecG helicase activity at three- and four-strand DNA structures. *Nucleic Acids Res.* 27, 3049-56.

McGlynn, P. & Lloyd, R. G. (2000). Modulation of RNA polymerase by (p)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell* 101, 35-45.

McGlynn, P. & Lloyd, R. G. (2001a). Action of RuvAB at replication fork structures. *J. Biol. Chem.* 276, 41938-44.

McGlynn, P. & Lloyd, R. G. (2001b). Rescue of stalled replication forks by RecG: simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and Holliday junction formation. *Proc. Natl. Acad. Sci. U S A* 98, 8227-34.

McGlynn, P. & Lloyd, R. G. (2002). Genome stability and the processing of damaged replication forks by RecG. *Trends Genet.* 18, 413-9.

McGlynn, P., Lloyd, R. G. & Marians, K. J. (2001). Formation of Holliday junctions by regression of nascent DNA in intermediates containing stalled replication forks: RecG stimulates regression even when the DNA is negatively supercoiled. *Proc. Natl. Acad. Sci. U S A* 98, 8235-40.

Michel, B., Ehrlich, S. D. & Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *Embo J.* 16, 430-8.

Michel, B., Flores, M. J., Viguera, E., Grompone, G., Seigneur, M. & Bidnenko, V. (2001). Rescue of arrested replication forks by homologous recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8181-8.

Murphy, K. C., Campellone, K. G. & Poteete, A. R. (2000). PCR-mediated gene replacement in *Escherichia coli*. *Gene* 246, 321-30.

Olavarrieta, L., Martinez-Robles, M. L., Sogo, J. M., Stasiak, A., Hernandez, P., Krimer, D. B. & Schvartzman, J. B. (2002). Supercoiling, knotting and replication fork reversal in partially replicated plasmids. *Nucleic Acids Res.* 30, 656-66.

Otsuji, N., Iyehara, H. & Hideshima, Y. (1974). Isolation and characterization of an *Escherichia coli ruv* mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. *J. Bacteriol.* 117, 337-44.

Pages, V. & Fuchs, R. P. (2003). Uncoupling of leading- and lagging-strand DNA replication during lesion bypass *in vivo*. *Science* 300, 1300-3.

Parsons, C. A., Tsaneva, I., Lloyd, R. G. & West, S. C. (1992). Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc. Natl. Acad. Sci. U S A* 89, 5452-6.

Parsons, C. A. & West, S. C. (1993). Formation of a RuvAB-Holliday junction complex *in vitro*. *J. Mol. Biol.* 232, 397-405.

Postow, L., Ullsperger, C., Keller, R. W., Bustamante, C., Vologodskii, A. V. & Cozzarelli, N. R. (2001). Positive torsional strain causes the formation of a four-way junction at replication forks. *J. Biol. Chem.* 276, 2790-6.

Rangarajan, S., Woodgate, R. & Goodman, M. F. (2002). Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA and RecFOR proteins. *Mol. Microbiol.* 43, 617-28.

Rothman, R. H. & Clark, A. J. (1977). The dependence of postreplication repair on *uvrB* in a *recF* mutant of *Escherichia coli* K-12. *Mol. Gen. Genet.* 155, 279-86.

Ryder, L., Whitby, M. C. & Lloyd, R. G. (1994). Mutation of *recF*, *recJ*, *recO*, *recQ*, or *recR* improves Hfr recombination in resolvase-deficient *ruv recG* strains of *Escherichia coli*. *J. Bacteriol.* 176, 1570-77.

Seigneur, M., Bidnenko, V., Ehrlich, S. D. & Michel, B. (1998). RuvAB acts at arrested replication forks. *Cell* 95, 419-30.

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.

Sharples, G. J., Benson, F. E., Illing, G. T. & Lloyd, R. G. (1990). Molecular and functional analysis of the *ruv* region of *Escherichia coli* K-12 reveals three genes involved in DNA repair and recombination. *Mol. Gen. Genet.* 221, 219-26.

Storm, P. K., Hoekstra, W. P., deHaan, P. G. & Verhoef, C. (1971). Genetic recombination in *Escherichia coli*. IV. Isolation and characterization of recombination-deficiency mutants of *Escherichia coli* K12. *Mutat. Res.* 13, 9-17.

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, 1731-6.

West, S. C. (2003). Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol* 4, 435-45.

CHAPTER III

RuvABC IS REQUIRED TO RESOLVE HOLLIDAY JUNCTIONS THAT ACCUMULATE FOLLOWING REPLICATION ON DAMAGED TEMPLATES IN *ESCHERICHIA COLI*

Abstract

RuvABC is a helicase-endonuclease that promotes branch migration and resolution at Holliday junctions on DNA. Mutants deficient in RuvABC are hypersensitive to UV irradiation, yet the molecular event(s) that require RuvABC processing in UV-irradiated cells are not known. In order to characterize the cellular role of the RuvABC complex, we utilized two-dimensional agarose gel electrophoresis in combination with transmission electron microscopy and alkaline sucrose gradients to monitor the integrity and the structural intermediates that occur in UV-irradiated *ruvAB* and *ruvC* mutants. We show here that although *ruvAB* and *ruvC* mutants resume replication following UV-induced arrest, the replicated molecules contain unresolved Holliday junctions. The formation of Holliday junctions correlates with an eventual loss in the integrity of the genomic DNA as monitored by alkaline sucrose gradients. The strand exchange intermediates that accumulate in the absence of RuvAB are distinct from those observed at arrested replication forks and are not resolved by the branch migration enzyme RecG. Potential roles for RuvABC in processing non-replication arresting lesions

or in resolving structures generated following replication on damaged templates are discussed.

Introduction

Irradiation of *Escherichia coli* with far-ultraviolet light (254 nm) induces DNA damage that blocks DNA polymerases and can arrest the replication machinery (Chan et al., 1985; Setlow et al., 1963). UV-induced DNA lesions that cannot be repaired may lead to mutations if the wrong base is incorporated, rearrangements if replication resumes from the wrong site, or cell lethality if the block to replication cannot be overcome. Yet despite these challenges, *E. coli* cells are able to survive and faithfully pass on their genome following UV doses that produce thousands of lesions per genome, indicating that cells contain efficient mechanisms to deal with these impediments to replication (Howard-Flanders et al., 1969). The mechanism(s) that operate to restore replication or the DNA template upon encounters with DNA damage are likely to depend on whether the lesion is found in the leading or lagging strand template of the DNA (Higuchi et al., 2003; McInerney & O'Donnell, 2004; Pages & Fuchs, 2003). Recent studies using plasmid substrates have shown that lesions in the leading strand template arrest the overall progression of the replication machinery both *in vivo* and *in vitro* (Higuchi et al., 2003; Pages & Fuchs, 2003). Comparatively, lesions in the lagging strand template do not arrest the progression of replication, but instead result in nascent strand gaps at the sites opposite to the lesions (Higuchi et al., 2003; McInerney & O'Donnell, 2004).

In the case where replication is arrested by UV-induced damage, several proteins associated with the *recF* pathway are required to protect and maintain the structural

integrity of the arrested fork. RecF, RecO, RecR, and RecA, are needed to maintain the DNA at the replication fork until the blocking lesion can be repaired by the nucleotide excision repair proteins or bypassed by translesion DNA polymerases (Courcelle et al., 1997; Courcelle, 1999; Courcelle et al., 2003; Horii & Suzuki, 1970; Rangarajan et al., 2002). Additionally, RecJ, a 5'-3' single-strand exonuclease, and RecQ, a 3'-5' DNA helicase, process or partially degrade the nascent DNA at the replication fork before replication resumes (Courcelle et al., 2003; Courcelle & Hanawalt, 1999). This nascent DNA processing is thought to enhance the ability of RecF-O-R and RecA to bind to and maintain the fork and to allow repair enzymes to gain access to the lesion by backing up the replication fork, resulting in the formation of a 4-arm branched structure similar to a Holliday junction (Courcelle et al., 2003; Courcelle & Hanawalt, 1999; Hishida et al., 2004). Under conditions where either the nascent DNA processing or repair cannot occur, the recovery of DNA synthesis is delayed and becomes dependent on translesion synthesis by Pol V (Courcelle et al., 2005a).

The events that process and repair lesions and gaps that are generated by damage that does not arrest replication are less well understood. Early models proposed that the nascent strand gaps may be restored by exchanging and then resynthesizing these regions with the homologous regions from sister chromosomes (Ganesan, 1974; Rupp et al., 1971). Consistent with this type of model, a large body of work has shown that in repair deficient mutants, UV irradiation leads to elevated levels of recombination-dependent strand exchanges during the period when the nascent strand gaps are joined (Johnson, 1977; Rothman & Clark, 1977). Although Holliday junctions are formed during these

exchanges, the high level of lethality associated with replication deficient mutants after UV make it difficult to determine if the exchanges are associated with a process that promotes survival (Rupp & Howard-Flanders, 1968). Similar models speculated that transient pairing and exchanges between the gaps and sister chromatids may restore the region to a form that can be repaired by the nucleotide excision repair proteins (Bridges & Sedgwick, 1974). This type of model would be consistent with the idea that survival may be promoted by a common pathway involving both recombination and repair proteins (Courcelle, 1999). More recent models have proposed that translesion DNA synthesis by the damage-induced polymerases may restore these substrates (Courcelle et al., 2005a; Courcelle et al., 2004; Shen et al., 2003). This type of model is supported by the observation that these polymerases are not essential for replication to resume, but contribute to survival and mutagenesis following UV irradiation (Courcelle et al., 2005a; Fuchs et al., 2001; Napolitano et al., 2000; Reuven et al., 1999; Servant et al., 2002). In addition, reconstitution of translesion synthesis *in vitro* utilizes a gapped substrate that is coated by a RecA filament and contains a beta clamp loaded at the lesion site, a substrate very similar to that predicted to occur at non arresting DNA lesions (Tang et al., 1999). However, characterization of how these lesions are processed *in vivo* is complicated by the fact that both arresting and non-arresting lesions are produced by UV irradiation and the processing of each form may in fact share common enzymatic steps.

The formation and resolution of a Holliday junction intermediate has been proposed to occur in models that deal with both arrested replication forks and gaps containing single-strand DNA lesions (Courcelle et al., 2004; Marians, 2004; Michel et

al., 2004). The similarity in structure between the recently characterized fork substrates at DNA damage and Holliday junctions led to the concept that branch migration may be required following arrest in order to move the replication fork away from the lesion before repair can occur. Similarly, Holliday junctions are a central intermediate required to repair nascent gaps in many of the early post-replication repair models (Ganesan et al., 1988; Howard-Flanders, 1975; Rothman & Clark, 1977; West et al., 1982).

Both RuvABC and RecG have been shown to act on synthetic Holliday structures *in vitro* (Lloyd & Sharples, 1993; Parsons et al., 1992; Tsaneva et al., 1992). The *ruv* locus was originally identified through a genetic screen that isolated mutants that were hypersensitive to UV irradiation (Otsuji et al., 1974). RuvABC was also identified biochemically, as enzymes that would specifically resolve and promote migration of Holliday junctions (Connolly et al., 1991; Connolly & West, 1990; Dunderdale et al., 1991; Parsons et al., 1992). Purified RuvA forms a tetramer that specifically binds Holliday junction structures and recruits two hexameric rings of RuvB (Lloyd & Sharples, 1993; Rafferty et al., 1996). Together, RuvA and RuvB act as a helicase that catalyzes an ATP-dependent migration of four-way branched DNA junctions in either the 5'−3' or 3'−5' direction, depending on reaction conditions (McGlynn & Lloyd, 2001a; Parsons et al., 1992; Parsons & West, 1993). RuvC is an endonuclease that binds as a dimer to Holliday junctions and resolves these structures by making symmetric incisions in the DNA (Connolly et al., 1991; Shah et al., 1997). *In vitro* studies have suggested that RuvC-mediated resolution of Holliday junctions occurs once the RuvAB complex has been removed from the DNA (Dickman et al., 2002). However, other studies support the

idea that an equilibrium exists between RuvAB and RuvABC complexes and efficient resolution appears to require all these gene products (Eggleston et al., 1997; Eggleston & West, 2000; Shah et al., 1994).

RecG is also a helicase that exhibits branch migration *in vitro*. RecG was originally identified through a screen for mutants with increased sensitivity to UV irradiation and subsequently shown to exhibit decreased frequencies of conjugational recombination (Storm et al., 1971). RecG binds to Holliday junctions as a monomer and, depending on the reaction conditions, can promote ATP-dependent branch migration in either the 3'−5' or 5'−3' directions (Lloyd & Sharples, 1993; McGlynn & Lloyd, 2001b; Whitby, 1993). Additionally, RecG was shown to preferentially bind to synthetic three-arm structures and unwind these structures into four-arm branched structures that would resemble a Holliday junction (McGlynn & Lloyd, 1999).

Due to the ability of RuvABC and RecG to branch migrate Holliday junction structures *in vitro* and the similarities between the branched structures that form at blocked replication forks and Holliday junctions, recent studies have focused on the possibility that RuvAB or RecG processing may operate at arrested replication forks following DNA damage (Courcelle & Hanawalt, 2001; McGlynn et al., 2001; Seigneur et al., 1998). However, following arrest by UV-induced DNA damage, the absence of either RuvAB or RecG does not impair the resumption of DNA synthesis, indicating that neither protein is essential for replication to resume following arrest (Donaldson et al., 2004). To further characterize the cellular role of RuvABC and RecG that is required for survival after DNA damage, we monitored the structural integrity and intermediates that

occurred in these mutants following UV irradiation. We found that although DNA synthesis resumed following arrest in *ruvAB* or *ruvC* mutants, the replicated DNA accumulated Holliday junctions. The failure to resolve the Holliday junctions correlated with an eventual loss of genomic DNA integrity that is likely to result in the observed lethality in these mutants.

Materials and Methods

Bacterial strains

Our parental strain, SR108, is a *thyA36 deoC2* derivative of W3110 (De Lucia & Cairns, 1969). Strains HL952 (SR108 *uvrA*:Tn*10*), CL008 (SR108 *recG258::*Tn*5*), CL532 (SR108 *ruvA59::*Tn*10*), CL561 (SR108 *recG258::*Tn*5 ruvA59::*Tn*10*), and CL684 (SR108 *recR6212*:cat883 *recJ284*:Tn*10*) have been described previously (Courcelle, 1999; Courcelle et al., 2003; Donaldson et al., 2004). CL577 was constructed by P1 transduction of the *ruvC53eda-51::*Tn*10* allele from RDK2615 into SR108 (Harris et al., 1996). Cells were transformed with plasmid pBR322 by electroporation for experiments involving two-dimensional agarose gel analysis (Sambrook & Russell, 2001). Phenotypes were confirmed by antibiotic resistance and, when appropriate, UV hypersensitivity.

All cultures were UV irradiated on a rotating platform using a Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/sec.

Rate of DNA synthesis

Fresh overnight cultures were diluted 1:100 in 50 ml of Davis medium supplemented with 0.4% glucose, 0.2% casamino acids (DGC media), and 0.1 μCi/10 μg/ml of [^{14}C] thymine and were grown to an OD_{600} of 0.3 in a 37ºC shaking incubator. At this time, half the culture was UV irradiated with 27 J/m^2 and the other half was mock irradiated. At the times indicated, 1 μCi/ml [^{3}H] thymidine was added to 0.5 ml aliquots of culture for 2 min at 37ºC. Cells were then lysed, and the DNA was precipitated in 5 ml 5% trichloroacetic acid (TCA) and filtered onto Millipore glass fiber filters. Duplicate aliquots were taken at each time point. The amount of ^{3}H- and ^{14}C-labeled DNA on each filter was determined by liquid scintillation counting.

Alkaline sucrose gradients

Fresh overnight cultures were diluted 1:100 in DGC media supplemented with 0.9 μCi/4 μg/ml [^{14}C] thymine to an OD_{600} of 0.4. Immediately following UV irradiation with 27 J/m^2, 9 μCi/ml [^{3}H] thymidine (77.8 Ci/mmol) was added to the culture for 5 min at 37ºC. Cells were filtered, washed with 5 ml 1X NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0), and resuspended in DGC media supplemented with 10 μg/ml thymine. 0.5 ml aliquots of the culture were removed at each time point, mixed with an equal volume of cold 2X NET, pelleted, and resuspended in 0.1 ml of buffered sucrose (0.01 M Tris, pH. 8.0, 0.01 M EDTA, 0.110 M NaCl, 5.1% sucrose). All samples were kept on ice until the end of the time course. 0.025 ml of each sample (~10^7 cells) was then layered on top of sucrose gradients (5-20% sucrose gradients in 0.1 N NaOH) that had 0.1 ml of 5% Sarkosyl in 0.5 N NaOH layered on top. Gradients were

centrifuged for 2 hr at 60,000 g at 20ºC. Gradients were collected on Whatman No. 17 chromatography paper and the amount of ^{3}H- and ^{14}C- labeled DNA present in each fraction was determined by liquid scintillation counting (Courcelle et al., 2005a).

Two-dimensional agarose gel electrophoresis

Cultures containing the plasmid pBR322 were grown overnight in the presence of 100 µg/ml ampicillin. 0.2 ml of this culture was pelleted and resuspended in 20 ml of DGC medium supplemented with 10 µg/ml thymine and grown without ampicillin to an OD_{600} of 0.5 in a 37ºC shaking incubator. At this time, cultures were irradiated with 50 J/m^2 and transferred to a new, prewarmed flask in the 37ºC shaking incubator. At the indicated times, a 0.75 ml aliquot of culture was transferred to an equal volume of 2X NET, pelleted, resuspended in 0.15 ml of lysozyme solution (1 mg/ml lysozyme, 0.5 mg/ml RNase A in TE, pH 8.0,) and incubated for 20 min at 37ºC. Then, 0.025 ml of 20% Sarkosyl and 0.01 ml of 10 mg/ml Proteinase K was added to the samples and incubation continued for 1 hr at 55ºC. Samples were then extracted twice with four volumes of phenol:chloroform:isoamyl alcohol (25:24:1), followed by one extraction with four volumes of chloroform:isoamyl alcohol (24:1), dialyzed against 200 ml of TE (100 mM Tris, 1mM EDTA, pH 8.0) for 3 hr on floating 47 mm Whatman 0.05 µm pore disks (Whatman #VMWP04700) and then digested with *Pvu*II restriction endonuclease (New England Biolabs) overnight at 37ºC. Samples were then extracted with two volumes chloroform:isoamyl alcohol (24:1) extraction and loaded directly on the gel.

The genomic DNA samples were initially separated in a 0.4% agarose gel in 1X TBE (Tris-Borate-EDTA, pH 8.0) at 1 V/cm for 15 hr. For the second dimension, the

lanes were excised, rotated 90º, and recast in a 1% agarose gel in 1X TBE, and electrophoresed at 6.5 V/cm for 7 hr. DNA in the gels was transferred to a Hybond N+ nylon membrane by standard Southern blotting and the plasmid DNA was detected by probing with ^{32}P-labeled pBR322 that was prepared by Nick translation (Roche Applied Science**)** using dCTP (6000 Ci/mmol, MP Biomedicals) and visualized using a STORM Molecular Dynamics PhosphorImager with its associated ImageQuant analysis software (Molecular Dynamics).

Transmission electron microscopy

DNA was prepared as described for the two-dimensional gel analysis except that 60 ml cultures were grown and UV irradiated. The entire culture was then pelleted at the indicated time and resuspended in 4.8 ml lysozyme solution. Rather than dialysis, the DNA was precipitated by adding 0.3 volumes of 10 M ammonium acetate and 3 volumes of ethanol, then pelleted by centrifugation, resuspended in 0.06 ml TE (pH 8.0) and digested with *Pvu*II restriction endonuclease as before. The sample was split and analyzed in parallel by two-dimensional agarose gel electrophoresis. One half of the sample was transferred and analyzed by Southern analysis, and the second half was stained with 0.5 µg/ml ethidium bromide, and the intermediates were excised from the gel, extracted from the agarose using GeneClean Spin Filters Extraction Kit (Qbiogen), and resuspended in 0.01 ml of TE (pH 8.0).

Purified DNA was prepared for transmission electron microscopy using either a formamide or aqueous drop technique (Thresher & Griffith, 1992). Formamide samples contained 0.100 µg/ml DNA in 10X TE (pH 8.0) and 50% formamide (v/v). Aqueous

samples contained 0.2 µg/ml DNA in 0.25 M ammonium acetate (pH 7.6). For both procedures, cytochrome c was added (8 µg/ml final concentration) and a 0.05 ml droplet was transferred to a clean Parafilm sheet in a closed Petri dish. After 90 sec, a parlodian-coated 300 mesh copper grid was touched to the surface of the drop, dipped in 75% ethanol for 45 sec, 90% ethanol for 5 sec, and then rotary shadow casted with 2.5 cm Platinum: Palladium (80:20) (EM Sciences) using a Kinney KSE 2A-A evaporator. Samples were observed and photographed under a JEOL JEM-100CXII transmission electron microscope (JEOL USA, Peabody, MA) at 60 kV.

Results

The integrity of genomic DNA deteriorates at late times following the recovery of replication after UV irradiation in ruvAB and ruvC mutants

Although *ruvAB* mutants are hypersensitive to UV irradiation, our previous work has shown that DNA synthesis resumes normally following UV-induced DNA damage (Donaldson et al., 2004). This observation suggests that the UV sensitivity in these mutants is not due to a failure to restore replication following arrest. To further characterize the nature of why *ruvABC* mutants are hypersensitive to UV irradiation, we monitored the integrity of the DNA made before and after a UV dose of 27 J/m^2 and compared it to the recovery of DNA synthesis in these mutants. The recovery of DNA synthesis was monitored by following the rate that [^{3}H] thymidine was incorporated into [^{14}C]-prelabeled cultures after an identical 27 J/m^2 dose of UV irradiation. In wild-type cells, this dose initially inhibits the rate of DNA synthesis by ~90% before replication

begins to recover ~20 min after irradiation without any significant reduction in survival (Donaldson et al., 2004) (Fig. 3.1A). Consistent with our previous observations, although this dose severely impaired the survival of *ruvAB* and *recG* mutants, DNA synthesis resumed with kinetics that were similar to wild-type cells (Donaldson et al., 2004) (Fig. 3.1A). In contrast, *recJ* and *uvrA* mutants, which have been shown to directly participate in the resumption of DNA synthesis, exhibited a significant delay and failed to recover DNA synthesis, respectively (Courcelle et al., 2005b; Courcelle, 1999) (Fig. 3.1A).

To follow the integrity of the post-irradiation DNA synthesis and genomic DNA during this same time period, we monitored the size of these fragments in alkaline sucrose gradients. To this end, [^{14}C]thymine prelabeled cultures were labeled with [^{3}H]thymidine for 5 min following UV irradiation. Cultures were then transferred to non-radioactive media and allowed to recover. At various times during the recovery period, the relative size of the ^{14}C-labeled genomic DNA and ^{3}H-labeled post-irradiation DNA synthesis was determined by sedimentation in alkaline sucrose gradients. Immediately following UV irradiation, the ^{3}H-labeled post-irradiation DNA synthesis produced smaller sized fragments that migrated more slowly, separating from the large ^{14}C-labeled genomic DNA which migrated to the bottom of the gradient. In UV irradiated wild-type cultures, the genomic DNA remained primarily intact throughout the recovery period and the post-irradiation DNA fragments were gradually joined, returning to a size that was approximately equal to that of the overall genomic DNA within 45 min after UV (Fig. 3.1B).

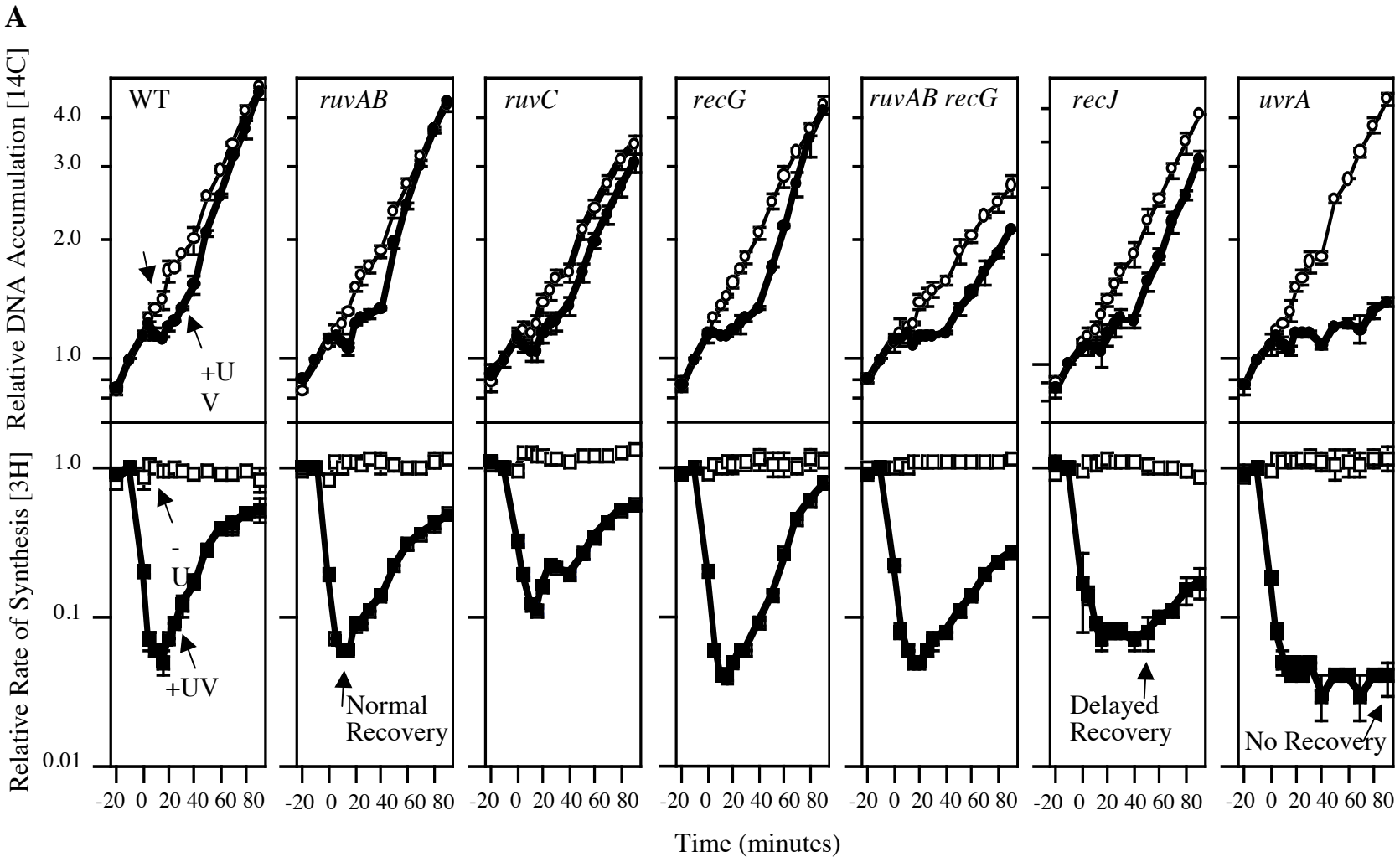


Figure 3.1. *ruvAB* and *ruvC* mutants resume DNA synthesis, but the integrity of the genomic DNA deteriorates at late times following UV irradiation.

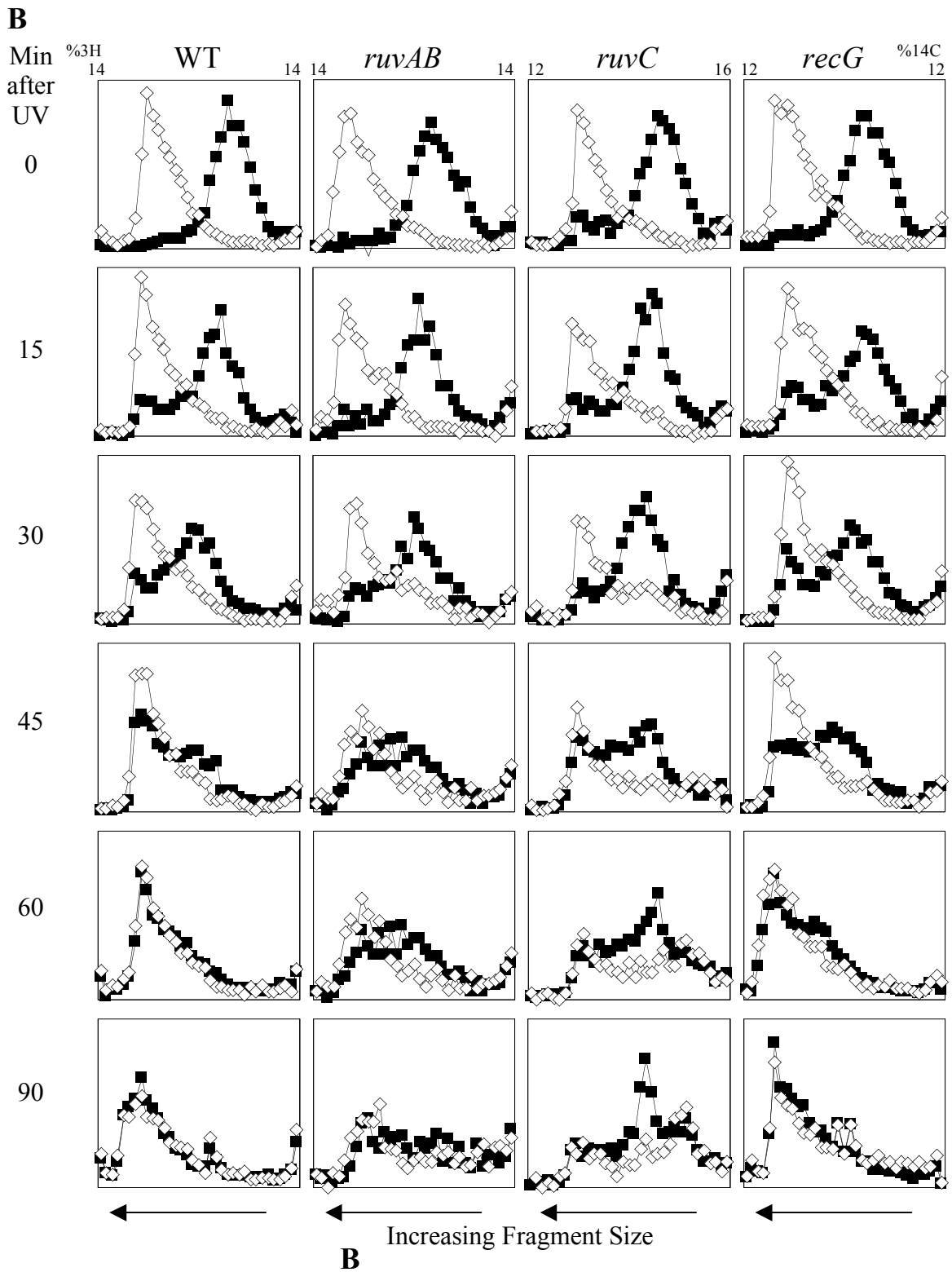


Figure 3.1 (continued)

C

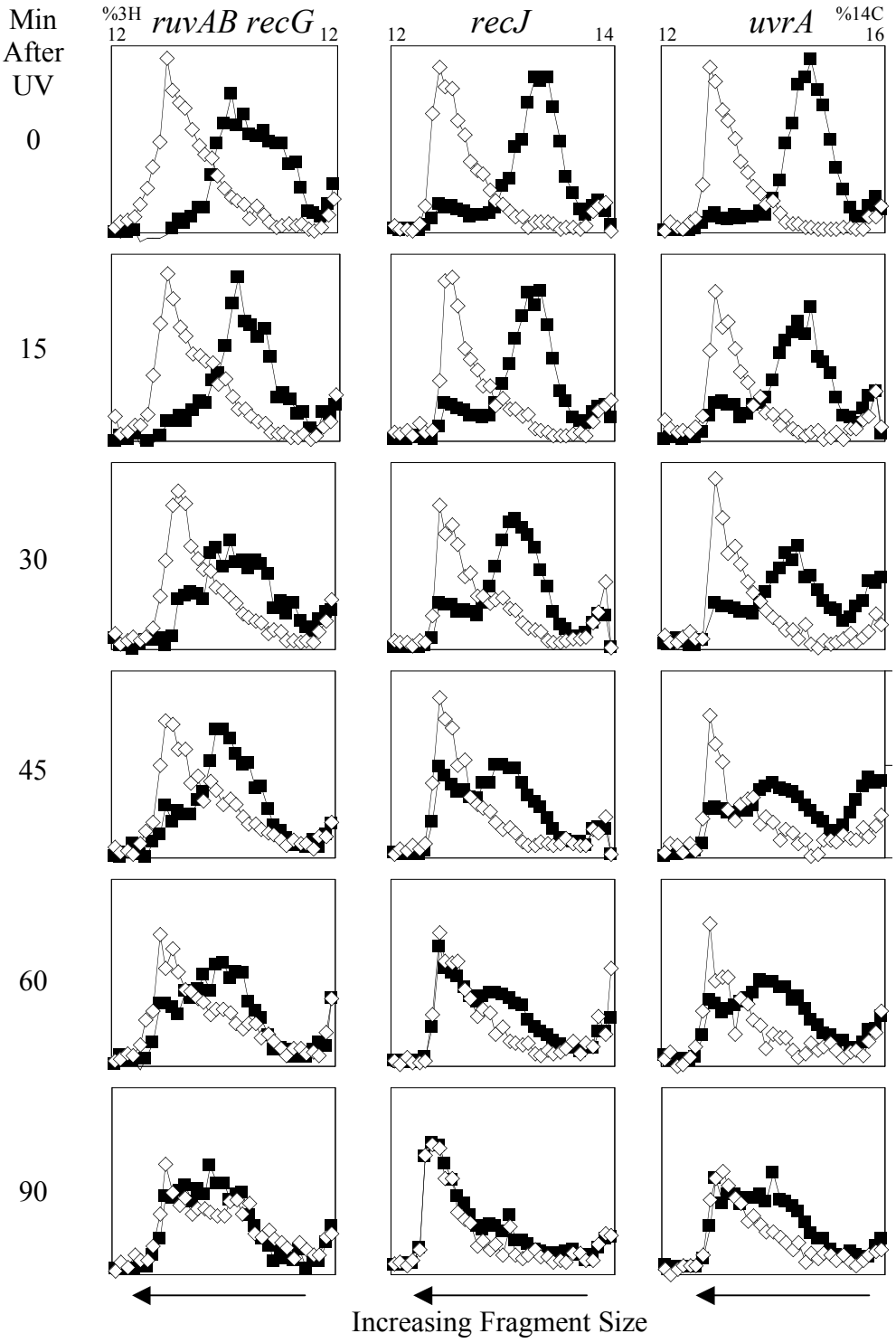


Figure 3.1 (continued)

Figure 3.1 (continued)

A: *ruvAB* and *ruvC* mutants resume DNA synthesis with similar kinetics as wild-type. Cultures grown in the presence of [^{14}C] thymine were pulsed-labeled with [^{3}H] thymidine for 2 min at the indicated times following either 27 J/m^2 of UV irradiation or mock irradiation. The relative amount of ^{14}C and ^{3}H incorporated into the DNA is plotted over time. Cultures were irradiated at time 0. Graphs represent an average of two independent experiments. Error bars represent one standard deviation. Total [^{14}C]-DNA in mock irradiated cultures (❍); total [^{14}C]-DNA in irradiated cultures (●);[^{3}H]-DNA synthesis in 2 min in mock irradiated cultures (□);[^{3}H]-DNA synthesis in 2 min in irradiated cultures (■). ^{3}H and ^{14}C at -10 min: WT 16234.05 and 3165.64, *ruvAB* 13789.59 and 1697.10, *recG* 20528.5 and 1970.14, *ruvAB recG* 14178.18 and 2043.61, *uvrA* 14670.93 and 1767.17, and *ruvC* 3705.82 and 2133.75. The low ^{3}H values in *ruvC* are due to the utilization of ^{3}H thymidine with a lower specific activity. B: The integrity of the genomic DNA in *ruvAB* and *ruvC* mutants begins to deteriorate at late times following UV-induced DNA damage. Cells grown in the presence of [^{14}C] thymine were labeled for 5 min with [^{3}H] thymidine following 27 J/m^2 of UV irradiation and examined at the indicated times by alkaline sucrose gradient analysis. Larger DNA fragments sediment more rapidly at the bottom of the gradient. The percentage of the total ^{14}C genomic DNA (◊) and ^{3}H DNA made during the first 5 min post UV (■) for each fraction is plotted. Each time course represents one of at least two, independent experiments. Total ^{3}H and ^{14}C in each gradient at time 0: WT 2668.5 and 3799.19, *ruvAB* 2321.8 and 3367, *ruvC* 2121.1 and 2265, and *recG* 2605.57 and 2789.21. C: Deterioration of the genomic DNA does not occur in *recJ*, or *uvrA* mutants, but does occur in *ruvAB recG* double mutants. Experiments were performed as in B. Percentage of the total ^{14}C genomic DNA (◊) and percent of ^{3}H DNA made during the first 5 min post UV (■) for each fraction is plotted. Each time course represents one of at least two, independent experiments. Total ^{3}H and ^{14}C in each gradient at time 0: *ruvAB recG* 1113.49 and 2926.93, *recJ* 4344.72 and 2124.62, and *uvrA* 4701.49 and 2096.22.

When we examined *ruvAB* and *ruvC* mutants, we observed that the post-irradiation DNA synthesis and genomic DNA sedimented with a similar pattern to that of wild-type cultures at early times after UV irradiation (Fig. 3.1B). However, at times when the DNA had been fully restored in wild-type cultures, the integrity of the genomic DNA began to deteriorate in *ruvAB* and *ruvC* cultures. This was observed as a general loss of the peak corresponding to the large, ^{14}C-labeled DNA fragments that sedimented throughout 60 and 90 min gradients. By contrast, genomic integrity was maintained throughout the recovery period in *recG* cultures and the post-irradiation DNA synthesis was joined and restored with kinetics that were similar to wild-type cultures (Figure 3.1C). *recG* mutants are approximately as hypersensitive to UV as *ruvAB* or *ruvC* mutants, suggesting that the defect in maintaining the genomic DNA integrity is specific to *ruvABC* mutants and not simply due to the elevated levels of lethality that occur in these populations. In the absence of both RuvAB and RecG, the genomic DNA deteriorated with a pattern that was similar to the *ruvAB* single mutant, indicating that the phenotype in *ruvAB* mutants is dominant and cannot be compensated for by the presence or absence of RecG. In addition, although UV-irradiated *recJ* and *uvrA* mutants exhibited a delay in joining the post-irradiation DNA fragments that roughly correlated with the delayed resumption of DNA synthesis, the size of the genomic DNA primarily remained intact throughout the recovery period (Figure 3.1A, C). These observations suggest that the failure to maintain the integrity of the genomic DNA in *ruvAB* and *ruvC* mutants is distinct from the events that are associated with the recovery of replication following UV-induced arrest.

ruvAB and ruvC mutants accumulate Holliday junctions that fail to resolve following replication on damaged templates

The deterioration of genomic DNA at late times after UV irradiation in *ruvAB* and *ruvC* mutants suggested that structural abnormalities may exist in the DNA following replication in these mutants. To examine this possibility, we examined the structural intermediates that occur on the plasmid pBR322 during replication in the presence of DNA damage by two-dimensional (2D) agarose gel electrophoresis. This technique is able to differentiate and identify the structural properties of replicating DNA fragments (Friedman & Brewer, 1995). Cultures of *E. coli* containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and analyzed by 2D agarose gel electrophoresis. In previous studies, we have shown that this dose generates, on average, 1 lesion per plasmid and that ~90% of wild-type cells are able to survive and form viable colonies (Courcelle et al., 2003). At various times following UV irradiation, total genomic DNA was purified, digested with *Pvu*II, and analyzed by 2D agarose gels. *Pvu*II linearizes pBR322 in a region ~400 bp downstream from its unidirectional origin of replication and generates a migration pattern that approximates a simple Y arc (Fig. 3.2A).

By this assay, in the absence of any DNA damage or at times immediately following UV irradiation, only normal Y-shaped molecules were observed in wild-type, *ruvAB*, *ruvC*, *recG*, and *uvrA* mutants (Fig. 3.2A, B and data not shown). Following UV-induced DNA damage in wild-type cultures, previous work from our group has shown that a class of more slowly migrating intermediates accumulate in the cone region of two-dimensional agarose gels (Courcelle et al., 2003). The intermediates that migrate in this

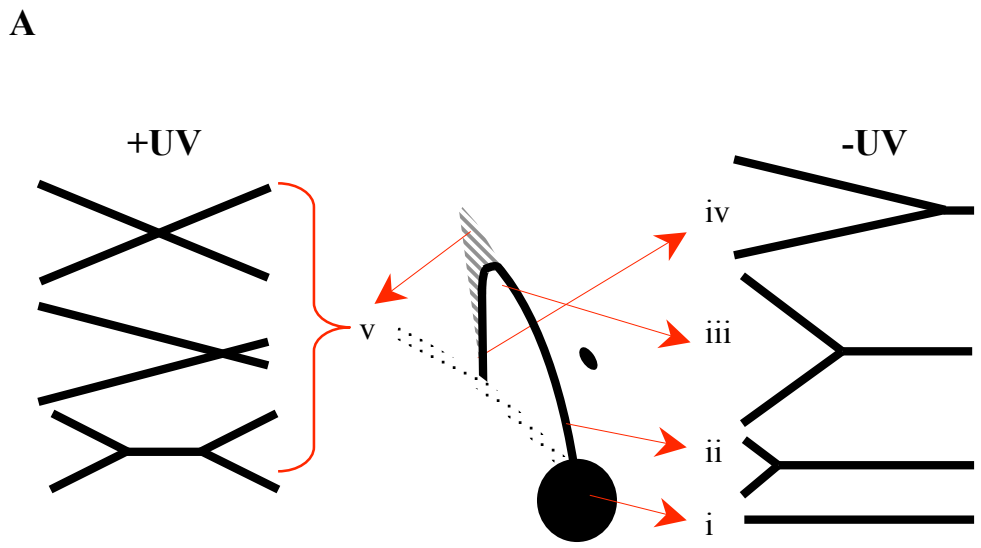


Figure 3.2. *ruvAB* and *ruvC* mutants accumulate branched structures following replication on damaged templates after UV irradiation.

A: Diagram of the migration pattern of *Pvu*II digested pBR322 observed by 2D agarose gel analysis in the absence and presence of UV-induced DNA damage. In the absence of UV irradiation, plasmids migrate as prominent linear, 4.4 kb fragments (*i*) and Y-shaped replicating molecules that migrate more slowly due to their larger size and non-linear shape (*ii, iii, iv*). These Y-shaped molecules form an arc that extends out from the main, linear fragment. In the presence of UV damage, molecules that contain more than one branch point (double Y or X-shaped structures) are observed and migrate more slowly in the cone region of the gel (*v*).

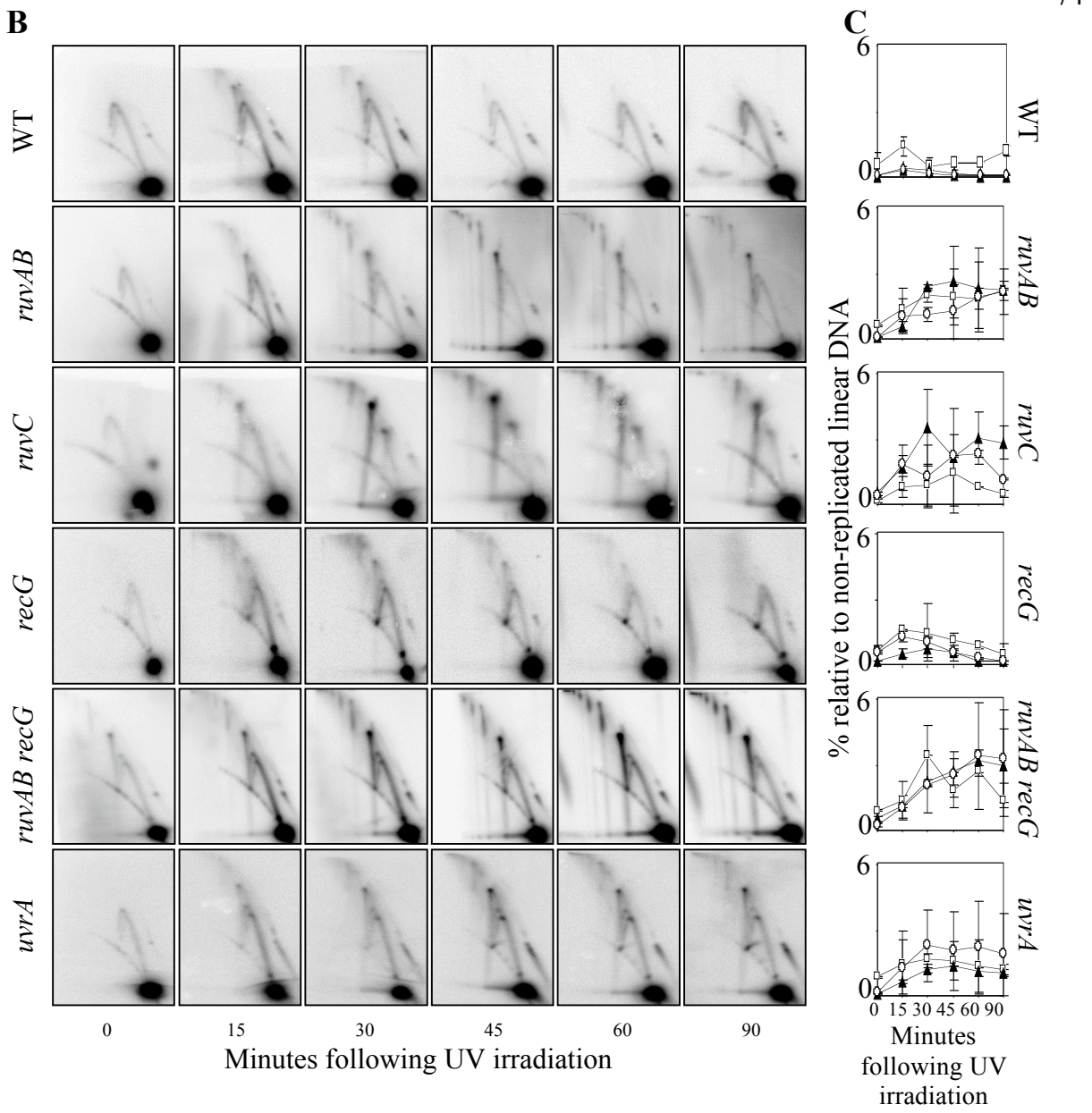


Figure 3.2 (continued)

B: 2D agarose gels of WT, *ruvAB*, *ruvC*, *recG*, *ruvAB recG*, and *uvrA* mutants at the indicated times following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and genomic DNA was purified, digested with *Pvu*II, and analyzed by 2D agarose gels. Gels shown represent one of at least two, independent experiments. C: The average percent of Y-shaped replicating molecules (□), cone region intermediates (▲), and higher order branched intermediates (o) relative to the amount of non-replicated linear molecules is plotted. Plots represent an average of two independent experiments. Error bars represent one standard deviation.

region are consistent with molecules having four arms or two branch points (Fig. 3.2A). In previous work, we demonstrated that a portion of these intermediates are formed at arrested replication forks following displacement of the nascent DNA (Courcelle et al., 2003). The nascent strand displacement and fork regression on the plasmid is probably catalyzed by positive supercoiling induced by replication on the plasmid molecule (Postow et al., 2001). Consistent with this interpretation, the cone region intermediates are not observed in *recF-O-R* mutants, which fail to protect the nascent DNA from degradation by the RecJ nuclease (Chow & Courcelle, 2004; Courcelle et al., 2003). However, inactivation of the RecJ nuclease restores the cone region intermediates in *recF* mutants (Courcelle et al., 2003).

Consistent with our previous studies, in wild-type cells, the intermediates that migrate in the cone region were resolved at a time that correlated with when robust replication resumed and the UV-induced lesions were repaired (Courcelle et al., 2003) (Fig. 3.2B, C). In contrast, *uvrA* mutants were not able to resume DNA synthesis within 90 min following UV irradiation and intermediates continued to accumulate in the cone region throughout this time period (Fig. 3.2B, C). Surprisingly, when we examined *ruvAB* and *ruvC* mutants, we observed that a class of intermediates also accumulated in the cone region (Fig. 3.2B, C). These intermediates persisted throughout the time course and migrated as fully replicated branched molecules, twice the molecular size of the plasmid. At later times, higher order branched intermediates accumulated that were four, eight, or sixteen times the molecular size of the plasmid, suggesting that a portion of these intermediates continued to replicate more than once during the time course (Fig.

3.2B, C). Interestingly, a significant portion of these branched intermediates resolved into linear molecules during electrophoresis in the 2nd dimension of the gel, as evidenced by the line from the fully replicated molecule containing four equal branches that migrated down to linear molecules (Fig. 3.2B). This is in contrast to *uvrA* mutants, which accumulated similar, higher order intermediates, but no *in situ* migration of these intermediates were observed during the gel electrophoresis. Since branch migration of symmetrical 4-way junctions is an isoenergetic process (Friedman & Brewer, 1995; Tsaneva et al., 1992), the resolution of these intermediates in *ruvAB* or *ruvC* mutants may suggest that the molecules contain a symmetrical Holliday junction. The branched intermediates in these mutants were specific to UV-induced damage since no intermediates were observed in the absence of DNA damage or immediately following UV irradiation in *ruvAB* and *ruvC* mutants (Data not shown and Fig. 3.2). In *recG* mutants, the UV-induced replication intermediates observed were similar to those that appeared in wild-type. Although the overall quantity of Y-shaped and cone region intermediates was slightly higher than those that occurred in wild-type cells at early times after UV, they were processed and resolved with kinetics that were similar to wild-type and no accumulation of these intermediates was observed (Fig. 3.2B, C).

We also examined *ruvAB recG* mutants to determine if the absence of both gene products would result in the formation of abnormal structural intermediates following UV-induced DNA damage. The intermediates observed in *ruvAB recG* mutants following UV irradiation were similar to those observed in *ruvAB* mutants (Fig. 3.2B, C). This suggests that the accumulation of UV-induced intermediates observed in the *ruvAB*

mutant is specific to RuvAB function and that the intermediates are not acted upon by other branch migration enzymes. It must be noted, though, that immediately following UV damage (time 0) intermediates appear in the cone region of the *ruvAB recG* double mutant that are not observed in *ruvAB* or *recG* single mutants (Fig. 3.2B). Furthermore, *ruvAB recG* mutants exhibit poor growth even in the absence of UV damage, suggesting that at least one of these gene products must be present to process an event or structure that occurs during normal cellular replication, even in the absence of UV damage (Donaldson et al., 2004). However, the intermediates in the cone region only appear to accumulate and convert to higher order branched molecules after UV irradiation, suggesting that the UV-induced intermediates that form in this mutant are, at least in part, attributed to a deficiency in RuvABC processing.

The intermediates that accumulate in ruvAB mutants are distinct from those associated with arrested replication forks

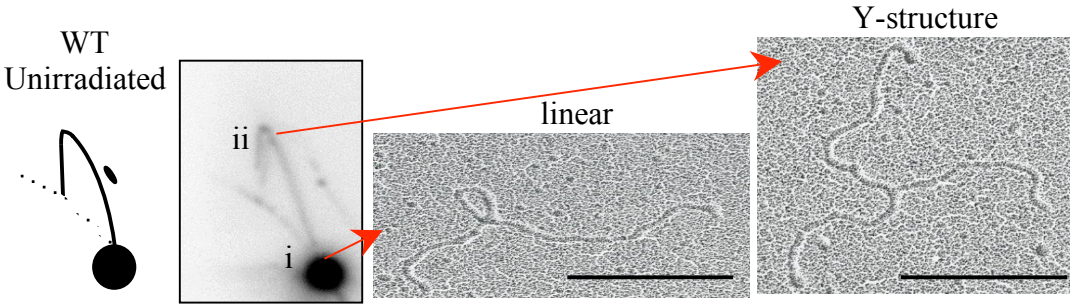
Cone region intermediates in *ruvAB* or *ruvC* mutants accumulate despite the fact that DNA synthesis resumes normally. As described above, intermediates that accumulate in the cone region of mutants lacking *recF* and *recJ* are thought to represent branched molecules that have arrested prior to completion since replication fails to recover following arrest in these mutants (Courcelle et al., 2003). To characterize the cone region intermediates that accumulate in *ruvAB* mutants, we compared the shape and migration pattern of intermediates that formed in wild-type 15 min after UV, *recR recJ* mutants 45 min after UV, and *ruvAB* mutants 45 min after UV by 2D agarose gels and transmission electron microscopy (Fig. 3.3). For electron microscopy, DNA was purified from larger

60 ml cultures then concentrated by ethanol precipitation prior to digestion by *Pvu*II restriction enzyme for the 2D agarose gel analysis. Parallel samples of the concentrated DNA were subjected to 2D agarose gels. One sample was used for Southern analysis and served to identify the locations of the intermediates in the gel, while the other half was stained with ethidium bromide, and the DNA was extracted and purified from the gel for electron microscopic analysis.

As mentioned previously, in the absence of DNA damage, only Y-arc replication intermediates are observed in wild-type, *ruvAB*, and *recR recJ* mutants. When molecules from this region in wild-type were examined by electron microscopy, it was found that the region consisted predominantly of the expected Y-shaped structures (Fig. 3.3A). Molecules examined from this region in *ruvAB* and *recR recJ* mutants also predominantly contained Y-shaped molecules and were indistinguishable from wild-type (data not shown).

Fifteen minutes after UV irradiation in wild-type cells, the damage-induced structures were comprised of intermediates that were evenly distributed throughout the cone region (Fig. 3.3B). By comparison, the damage-induced intermediates that were observed in either *ruvAB* or *recR recJ* mutants 45 min after UV irradiation formed predominantly in specific and distinct areas of the cone region (Fig. 3.3B). In the *recR recJ* mutant, the cone region intermediates accumulated primarily along the side of the cone farthest from the origin, corresponding to smaller molecules. Comparatively, the intermediates in the cone region of *ruvAB* mutants accumulated along the side of the cone that is proximal to the origin, corresponding to larger molecules (Fig. 3.3B).

A

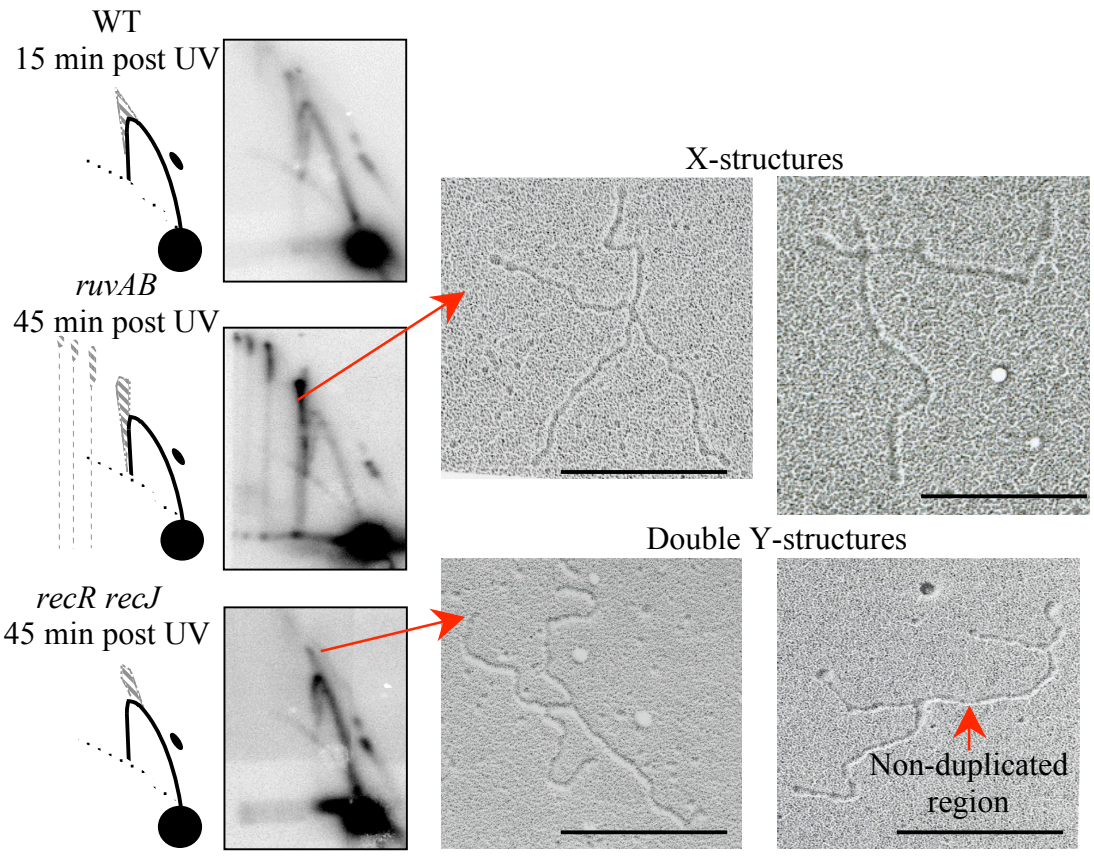


Structures in WT unirradiated	*n*	linear	Y
WT Section i	40	40	0
WT Section ii	40	6	34

Figure 3.3. Electron microscopic analysis of plasmid DNA intermediates in wild-type cultures in the absence of DNA damage and WT, *ruvAB*, and *recR recJ* mutants following UV irradiation.

A: In the absence of UV irradiation, *Pvu*II digested pBR322 prepared from WT cells forms linear, nonreplicated molecules (*i*) and Y-shaped replication intermediates (*ii*). A diagram and an actual 2D agarose gel representing the migration pattern of pBR322 in WT cells is shown. DNA extracted from specific areas of the 2D gel was observed by transmission electron microscopy. Micrographs represent the structure observed for the particular region of the gel. Scales for each micrograph represent 0.5 µm. The number of molecules examined by electron microscopy from the nonreplicated linear fragment and the Y-shaped replication arc are listed in the table and represent the totals from two independent experiments.

B



Non-linear structures in cone region	*n*	Y	X	DoubleY	Length of non-duplicated region in double Y-structures 0.5 kb	1.0 kb	1.5 kb
recR recJ	40	20	4	16	10	4	2
ruvAB	40	10	24	6	5	1	0

Figure 3.3 (continued)

B: The branch intermediates in *ruvAB* mutants are fully replicated molecules, whereas the branched intermediates in *recR recJ* mutants contain unreplicated regions. Diagrams and actual 2D agarose gels representing the migration pattern of pBR322 in WT, *ruvAB*, and *recR recJ* mutants are shown. DNA extracted from specific areas of the 2D gels was observed by transmission electron microscopy. Micrographs represent the structure observed for the particular region of the gel. Scales for each micrograph represent 0.5 µm. The number of molecules examined by electron microscopy from the cone region of *ruvAB* and *recR recJ* mutants and their shape is listed in the table. The numbers represent the totals from two independent experiments in each strain.

When we examined the cone region intermediates from *recR recJ* mutants by transmission electron microscopy, a large portion of the molecules were found to have a double Y-shape, containing four branches that extended from two distinct junction points on the molecule (Fig. 3.3B). While the double Y-shaped molecule is not the shape that we would have predicted would form at the arrested replication fork of a unidirectional plasmid, it is clearly consistent with the idea that replication in these mutants arrested prior to completion. It is not precisely clear how this structure is formed. It is possible that the structure represents the synthesis of the lagging strand back through the origin or replication after the leading strand has arrested. Alternatively, some branch migration may occur during the preparation of the DNA that alters the structure following arrest. We also cannot rule out the possibility that the structure is altered during the concentration or precipitation procedures used in the samples prepared for electron microscopy. Consistent with this latter possibility, the quantity of molecules migrating in the cone region was slightly higher and contained some higher migrating species in the 2D gels that were prepared for electron microscopy analysis as compared to those prepared by our standard method (data not shown).

In contrast to the partially replicated molecules that were seen in *recR recJ* mutants, cone region intermediates from *ruvAB* mutants were predominantly found to be X-shaped molecules that contained four branches extending from a single junction point (Fig. 3.3B). In general, these molecules were equivalent in size to two linear molecules that contained a single Holliday junction. This observed shape is consistent with what would be predicted for this region of the gel (Bell & Byers, 1983; Friedman & Brewer,

1995; Lucas & Hyrien, 2000). When the larger, higher order intermediates that migrated beyond the cone region were examined in the *ruvAB* mutants, they were found to be predominantly made up of molecules that contained a single Holliday junction or were linear dimers that contained four linear molecules that all radiated from a single junction (data not shown). The observation that replication recovers and continues at rate comparable to wild-type cells following UV-induced damage taken together with the full length molecules observed by 2D agarose gels and electron microscopy are consistent with the idea that the unresolved Holliday junctions may arise as an intermediate that occurs during processing and repair of a subset of lesions that are bypassed or skipped over by the replication machinery.

Discussion

ruvAB and *recG* mutants are hypersensitive to UV irradiation, but are not required for replication to resume following arrest at UV-induced DNA damage. Therefore, in an attempt to determine the cellular role of these proteins that is required for resistance to UV irradiation, here we considered the possibility that the proteins may process lesions at sites other than the arrested replication fork. We found that the integrity of the genomic DNA in *ruvAB* and *ruvC* mutants began to deteriorate at late times after UV irradiation and that this correlated with the accumulation of unresolved Holliday junctions *in vivo*. Although the molecular size of these molecules suggests that they were fully replicated, the observation that they were resistant to *Pvu*II digestion suggests that they may contain significant single-strand regions. The higher order intermediates that are seen to accumulate in *ruvAB* and *ruvC* mutants are consistent with the idea that replication was

not able to continue on these molecules and was able to reinitiate even in the absence of resolution of the initial rounds of replication. We did not observe any obvious defects in *recG* mutants as compared to wild-type cells in the assays we utilized in this study. Although we were unable to identify a potential role for the RecG helicase, the lack of a phenotype in these mutants indicates that the abnormalities observed in *ruvAB* and *ruvC* mutants specifically require RuvABC processing and are not able to be acted upon by other branch migration enzymes, such as RecG or RecJ.

The accumulation of unresolved Holliday junctions despite the completion of replication of the chromosome would lead to lethality and a loss of genomic integrity if the chromosomes fail to partition properly. Consistent with this idea are early studies that found that cells lacking RuvABC filamented after UV irradiation and contained high numbers of anucleated or multinucleated cells (Otsuji et al., 1974). Overtime, the *ruvAB* cultures accumulated large numbers of anucleate cells and a few filamentous cells contained non-partitioned, centrally aggregated chromosomes (Ishioka et al., 1998). At late time points, the DNA from UV irradiated *ruvAB* and *ruvC* mutants was also found to aggregate and failed to enter pulsed field agarose gels, an observation that could suggest strand exchanges may be accumulating on the chromosome (Ishioka et al., 1998).

The evidence presented here suggests that RuvABC is involved in processing intermediates that are associated with the repair or tolerance of lesions at sites other than the arrested replication fork. Some recent studies have suggested that lesions on the lagging strand template may not arrest the replication apparatus (Higuchi et al., 2003; McInerney & O'Donnell, 2004; Pages & Fuchs, 2003). If true, it is possible that RuvABC

may be required in this case to process the lesions in the gapped lagging strand template, which are thought to be produced in these events. The Holliday junctions that accumulate in *ruvAB* and *ruvC* mutants may suggest that RuvABC is required to resolve strand exchange intermediates that arise due to the repair of gaps that form on the lagging strand daughter DNA. The formation of these Holliday junctions that do not block replication would potentially result in the partitioning defects and loss of viability, as has been observed by others (Ishioka et al., 1998; Otsuji et al., 1974).

It is also possible that RuvABC may be acting at Holliday junctions that have formed at arrested replication forks. Previous work by others has shown that substrates for RuvABC are also generated in temperature sensitive alleles of *dnaB* at the restrictive temperature (Seigneur et al., 1998). Incubation at the restricted temperature inactivates the DnaB replicative helicase and arrests replication (Wechsler & Gross, 1971). Under these conditions, double-strand breaks arose in the genome in the absence of the double-strand break repair proteins RecBCD (Michel et al., 1997). If RuvABC is additionally inactivated, the formation of double-strand breaks was significantly reduced, suggesting that Holliday junctions form after inactivation of DnaB and are subsequently cleaved by the RuvABC resolvasome (Seigneur et al., 1998). It seems reasonable to speculate that a similar Holliday junction substrate may be generated both following UV irradiation and inactivation of DnaB. The DnaB helicase is thought to track along the lagging strand template and interacts and affects primase, properties that may suggest lagging strand abnormalities occur in *dnaB* mutants. The possibility that unique substrates arise in each case should also not be excluded from consideration. The data presented here suggests

that RuvABC resolves Holliday junctions that form due to repair of damaged DNA that did not significantly impair the ability of replication to resume. Based on current models of replication fork dynamics, we speculate that RuvABC may be required to resolve Holliday junctions that arise as an intermediate in the lagging strand template, which do not arrest replication. It will be important to further explore the possibility of RuvABC acting on lesions that form specifically on the leading strand template or the lagging strand template *in vivo*.

Acknowledgements

We thank Jack Griffith and Bill Monroe for advice and expertise with the transmission electron microscope. This work was supported by Career Award MCB-0448315 from the National Science Foundation and a Sigma Xi Grants-in-Aid of Research to JRD.

REFERENCES

Bell, L. & Byers, B. (1983). Separation of branched from linear DNA by two-dimensional gel electrophoresis. *Anal Biochem* 130, 527-35.

Bridges, B. A. & Sedgwick, S. G. (1974). Effect of photoreactivation on the filling of gaps in deoxyribonucleic acid synthesized after exposure of Escherichia coli to ultraviolet light. *J Bacteriol* 117, 1077-81.

Chan, G. L., Doetsch, P. W. & Haseltine, W. A. (1985). Cyclobutane pyrimidine dimers and (6-4) photoproducts block polymerization by DNA polymerase I. *Biochemistry* 24, 5723-8.

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, 3492-6.

Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991). Resolution of Holliday junctions *in vitro* requires the Escherichia coli *ruvC* gene product. *Proc. Natl. Acad. Sci. U S A* 88, 6063-7.

Connolly, B. & West, S. C. (1990). Genetic recombination in Escherichia coli: Holliday junctions made by RecA protein are resolved by fractionated cell-free extracts. *Proc Natl Acad Sci U S A* 87, 8476-80.

Courcelle, C. T., Belle, J. J. & Courcelle, J. (2005a). Escherichia coli utilize repair to restore UV-arrested replication forks over Pol V-mediated lesion bypass. *submitted*.

Courcelle, C. T., Chow, K. H. & Courcelle, J. (2005b). Replication fork reversal by RecJ determines if DNA damage is reversed or bypassed. *submitted*.

Courcelle, J., Belle, J. J. & Courcelle, C. T. (2004). When replication travels on damaged templates: bumps and blocks in the road. *Res Microbiol* 155, 231-7.

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, 3714-9.

Courcelle, J., D. J. Crowley, and P. C. Hanawalt. (1999). Recovery of DNA replication in UV-irradiated Escherichia coli requires both excision repair and *recF* protein function. *J. Bacteriol.* 181, 916-22.

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, 1064-7.

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, 543-51.

Courcelle, J. & Hanawalt, P. C. (2001). Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated *Escherichia coli* need not involve recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8196-202.

De Lucia, P. & Cairns, J. (1969). Isolation of an *E. coli* strain with a mutation affecting DNA polymerase. *Nature* 224, 1164-66.

Dickman, M. J., Ingleston, S. M., Sedelnikova, S. E., Rafferty, J. B., Lloyd, R. G., Grasby, J. A. & Hornby, D. P. (2002). The RuvABC resolvasome. *Eur J Biochem* 269, 5492-501.

Donaldson, J. R., Courcelle, C. T. & Courcelle, J. (2004). RuvAB and RecG are not essential for the recovery of DNA synthesis following UV-induced DNA damage in Escherichia coli. *Genetics* 166, 1631-40.

Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G. & West, S. C. (1991). Formation and resolution of recombination intermediates by E. coli RecA and RuvC proteins. *Nature* 354, 506-10.

Eggleston, A. K., Mitchell, A. H. & West, S. C. (1997). In vitro reconstitution of the late steps of genetic recombination in E. coli. *Cell* 89, 607-17.

Eggleston, A. K. & West, S. C. (2000). Cleavage of holliday junctions by the Escherichia coli RuvABC complex. *J Biol Chem* 275, 26467-76.

Friedman, K. L. & Brewer, B. J. (1995). Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol* 262, 613-27.

Fuchs, R. P., Koffel-Schwartz, N., Pelet, S., Janel-Bintz, R., Napolitano, R., Becherel, O. J., Broschard, T. H., Burnouf, D. Y. & Wagner, J. (2001). DNA polymerases II and V mediate respectively mutagenic (-2 frameshift) and error-free bypass of a single N-2-acetylaminofluorene adduct. *Biochem Soc Trans* 29, 191-5.

Ganesan, A. K. (1974). Persistence of pyrimidine dimers during post-replication repair in ultraviolet light-irradiated Escherichia coli K12. *J Mol Biol* 87, 103-19.

Ganesan, A. K., Hunt, J. & Hanawalt, P. C. (1988). Temperature dependent survival of UV-irradiated Escherichia coli K12. *Mol Gen Genet* 214, 198-203.

Harris, R. S., Ross, K. J. & Rosenberg, S. M. (1996). Opposing roles of the holliday junction processing systems of Escherichia coli in recombination-dependent adaptive mutation. *Genetics* 142, 681-91.

Higuchi, K., Katayama, T., Iwai, S., Hidaka, M., Horiuchi, T. & Maki, H. (2003). Fate of DNA replication fork encountering a single DNA lesion during *oriC* plasmid DNA replication *in vitro*. *Genes Cells* 8, 437-49.

Hishida, T., Han, Y. W., Shibata, T., Kubota, Y., Ishino, Y., Iwasaki, H. & Shinagawa, H. (2004). Role of the Escherichia coli RecQ DNA helicase in SOS signaling and genome stabilization at stalled replication forks. *Genes Dev* 18, 1886-97.

Horii, Z. I. & Suzuki, K. (1970). Degradation of the DNA of recA mutants of Escherichia coli K-12 after irradiation with ultraviolet light. II. Further studies including a recA uvrA double mutant. *Photochem Photobiol* 11, 99-107.

Howard-Flanders, P. (1975). Repair by genetic recombination in bacteria: overview. *Basic Life Sci* 5A, 265-74.

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, 1134-41.

Ishioka, K., Fukuoh, A., Iwasaki, H., Nakata, A. & Shinagawa, H. (1998). Abortive recombination in Escherichia coli ruv mutants blocks chromosome partitioning. *Genes Cells* 3, 209-20.

Johnson, R. C. (1977). Gap filling during postreplication repair of DNA in recombination deficient Escherichia coli. *Nature* 267, 80-1.

Lloyd, R. G. & Sharples, G. J. (1993). Processing of recombination intermediates by the RecG and RuvAB proteins of *Escherichia coli*. *Nucleic Acids Res.* 21, 1719-25.

Lucas, I. & Hyrien, O. (2000). Hemicatenanes form upon inhibition of DNA replication. *Nucleic Acids Res* 28, 2187-93.

Marians, K. J. (2004). Mechanisms of replication fork restart in Escherichia coli. *Philos Trans R Soc Lond B Biol Sci* 359, 71-7.

McGlynn, P. & Lloyd, R. G. (1999). RecG helicase activity at three- and four-strand DNA structures. *Nucleic Acids Res.* 27, 3049-56.

McGlynn, P. & Lloyd, R. G. (2001a). Action of RuvAB at replication fork structures. *J. Biol. Chem.* 276, 41938-44.

McGlynn, P. & Lloyd, R. G. (2001b). Rescue of stalled replication forks by RecG: simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and Holliday junction formation. *Proc. Natl. Acad. Sci. U S A* 98, 8227-34.

McGlynn, P., Lloyd, R. G. & Marians, K. J. (2001). Formation of Holliday junctions by regression of nascent DNA in intermediates containing stalled replication forks: RecG stimulates regression even when the DNA is negatively supercoiled. *Proc. Natl. Acad. Sci. U S A* 98, 8235-40.

McInerney, P. & O'Donnell, M. (2004). Functional uncoupling of twin polymerases: mechanism of polymerase dissociation from a lagging-strand block. *J Biol Chem* 279, 21543-51.

Michel, B., Ehrlich, S. D. & Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *Embo J.* 16, 430-8.

Michel, B., Grompone, G., Flores, M. J. & Bidnenko, V. (2004). Multiple pathways process stalled replication forks. *Proc Natl Acad Sci U S A* 101, 12783-8.

Napolitano, R., Janel-Bintz, R., Wagner, J. & Fuchs, R. P. (2000). All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis. *Embo J* 19, 6259-65.

Otsuji, N., Iyehara, H. & Hideshima, Y. (1974). Isolation and characterization of an *Escherichia coli ruv* mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. *J. Bacteriol.* 117, 337-44.

Pages, V. & Fuchs, R. P. (2003). Uncoupling of leading- and lagging-strand DNA replication during lesion bypass *in vivo*. *Science* 300, 1300-3.

Parsons, C. A., Tsaneva, I., Lloyd, R. G. & West, S. C. (1992). Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc. Natl. Acad. Sci. U S A* 89, 5452-6.

Parsons, C. A. & West, S. C. (1993). Formation of a RuvAB-Holliday junction complex *in vitro*. *J. Mol. Biol.* 232, 397-405.

Postow, L., Ullsperger, C., Keller, R. W., Bustamante, C., Vologodskii, A. V. & Cozzarelli, N. R. (2001). Positive torsional strain causes the formation of a four-way junction at replication forks. *J. Biol. Chem.* 276, 2790-6.

Rafferty, J. B., Sedelnikova, S. E., Hargreaves, D., Artymiuk, P. J., Baker, P. J., Sharples, G. J., Mahdi, A. A., Lloyd, R. G. & Rice, D. W. (1996). Crystal structure of DNA recombination protein RuvA and a model for its binding to the Holliday junction. *Science* 274, 415-21.

Rangarajan, S., Woodgate, R. & Goodman, M. F. (2002). Replication restart in UV-irradiated *Escherichia coli* involving pols II, III, V, PriA, RecA and RecFOR proteins. *Mol. Microbiol.* 43, 617-28.

Reuven, N. B., Arad, G., Maor-Shoshani, A. & Livneh, Z. (1999). The mutagenesis protein UmuC is a DNA polymerase activated by UmuD', RecA, and SSB and is specialized for translesion replication. *J Biol Chem* 274, 31763-6.

Rothman, R. H. & Clark, A. J. (1977). The dependence of postreplication repair on *uvrB* in a *recF* mutant of *Escherichia coli* K-12. *Mol. Gen. Genet.* 155, 279-86.

Rupp, W. D. & Howard-Flanders, P. (1968). Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. *J Mol Biol* 31, 291-304.

Rupp, W. D., Wilde, C. E., 3rd, Reno, D. L. & Howard-Flanders, P. (1971). Exchanges between DNA strands in ultraviolet-irradiated Escherichia coli. *J Mol Biol* 61, 25-44.

Sambrook, J. & Russell, D. W. (2001). Transformation of E. coli by Electroporation. In *Molecular Cloning: A Laboratory Manual, 3rd Ed.*, pp. 1.119-1.122. Cold Spring Harbor Laboratory Press.

Seigneur, M., Bidnenko, V., Ehrlich, S. D. & Michel, B. (1998). RuvAB acts at arrested replication forks. *Cell* 95, 419-30.

Servant, L., Cazaux, C., Bieth, A., Iwai, S., Hanaoka, F. & Hoffmann, J. S. (2002). A role for DNA polymerase beta in mutagenic UV lesion bypass. *J Biol Chem* 277, 50046-53.

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.

Shah, R., Bennett, R. J. & West, S. C. (1994). Activation of RuvC Holliday junction resolvase in vitro. *Nucleic Acids Res* 22, 2490-7.

Shah, R., Cosstick, R. & West, S. C. (1997). The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts. *Embo J* 16, 1464-72.

Shen, X., Woodgate, R. & Goodman, M. F. (2003). Escherichia coli DNA polymerase V subunit exchange: a post-SOS mechanism to curtail error-prone DNA synthesis. *J Biol Chem* 278, 52546-50.

Storm, P. K., Hoekstra, W. P., deHaan, P. G. & Verhoef, C. (1971). Genetic recombination in *Escherichia coli*. IV. Isolation and characterization of recombination-deficiency mutants of *Escherichia coli* K12. *Mutat. Res.* 13, 9-17.

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, 8919-24.

Thresher, R. & Griffith, J. (1992). Electron microscopic visualization of DNA and DNA-protein complexes as adjunct to biochemical studies. *Methods Enzymol* 211, 481-90.

Tsaneva, I. R., Muller, B. & West, S. C. (1992). ATP-dependent branch migration of Holliday junctions promoted by the RuvA and RuvB proteins of E. coli. *Cell* 69, 1171-80.

Wechsler, J. A. & Gross, J. D. (1971). Escherichia coli mutants temperature-sensitive for DNA synthesis. *Mol Gen Genet* 113, 273-84.

West, S. C., Cassuto, E. & Howard-Flanders, P. (1982). Postreplication repair in E. coli: strand exchange reactions of gapped DNA by RecA protein. *Mol Gen Genet* 187, 209-17.

Whitby, M. C., L. Ryder, and R. G. Lloyd. (1993). Reverse branch migration of Holliday junctions by RecG protein: a new mechanism for resolution of intermediates in recombination and DNA repair. *Cell* 75, 341-50.

CHAPTER IV

UV-INDUCED REPLICATION INTERMEDIATES OF pBR322 VISUALIZED BY TWO-DIMENSIONAL AGAROSE GEL ELECTROPHORESIS

Abstract

UV irradiation induces DNA damage that blocks DNA polymerases and can prevent the continuation of replication. Although several gene products have been identified that, when mutated, impair the ability of replication in the presence of DNA damage, the cellular function of several of these gene products has not been well characterized. To explore potential roles for these candidate gene products following UV irradiation, we utilized two-dimensional agarose gel electrophoresis to examine the structural properties of plasmid pBR322 following UV irradiation in several of these candidate mutants. We observed that replicating plasmids in *recJ*, *xonA*, and *recQ* mutants formed a transient cone region, or X-shaped intermediates, following UV irradiation that resembled those observed in wild-type cultures, although the intermediates persisted approximately 15 min longer in each of these mutants as compared to wild-type cultures. By contrast, the UV-induced cone region intermediates that accumulated in *recBC* and *recD* mutants persisted throughout the 90 min time course. Also, as previously reported, replication of the plasmid pBR322 in *recD* mutants

generated linear multimers and contained unique intermediates that occurred even in the absence of DNA damage. These observations are discussed and compared to other mutants that have been previously characterized by this technique.

Introduction

UV-induced DNA damage blocks DNA polymerases and RNA polymerases and can prevent the essential processes of replication and transcription (Bonner et al., 1992; Michalke & Bremer, 1969; Setlow et al., 1963). UV-induced DNA damage that blocks the progression of the replication machinery can lead to mutations or cell lethality (Howard-Flanders et al., 1968; Setlow et al., 1963). To deal with these impediments, cells are equipped with various mechanisms to ensure that replication can complete its task even in the presence of moderate levels of DNA damage (reviewed in (Courcelle et al., 2004; Marians, 2004; Michel et al., 2004). Several mechanisms have been proposed to operate to process DNA damage encountered during replication, depending on the type of damage encountered and potentially which template strand encounters the damage. Lesions on the lagging strand template do not block replication, while lesions on the leading strand template block the DNA polymerase and stall replication (Cordeiro-Stone et al., 1999; Higuchi et al., 2003; McInerney & O'Donnell, 2004; Pages & Fuchs, 2003).

In the case where replication arrests at the site of the lesion, several lines of evidence suggest that the replication fork regresses, backing away from the blocking lesion to allow for the nucleotide excision repair proteins to gain access to and remove the damaged DNA (Courcelle et al., 1999; Courcelle et al., 2003; Courcelle & Hanawalt,

2001; Higgins et al., 1976; Michel et al., 1997; Robu, 2001). Maintaining the replication fork and processing these intermediate substrates has been shown to depend on RecA and several gene products of the *recF* pathway (Chow & Courcelle, 2004; Courcelle et al., 1997; Courcelle & Hanawalt, 1999; Robu, 2001). Following arrest, the 3'-5' helicase RecQ and the 5'-3' single-strand specific exonuclease RecJ have been shown to preferentially displace and degrade the nascent lagging strand at the replication fork (Courcelle & Hanawalt, 1999). The nascent strand processing by RecJ and RecQ is limited by RecF, RecO, RecR, and RecA. The processing is proposed to facilitate fork regression and enhance the ability of RecA to bind and form a filament at this site, which maintains the arrested replication fork until the UV lesion is repaired by the nucleotide excision repair proteins (Chow & Courcelle, 2004; Courcelle et al., 2003; Hishida et al., 2004). Following repair of the obstructing lesion, the replication fork could reset and normal replication could continue (Courcelle et al., 2003).

In contrast to the arrest at replication, some studies currently suggest that lesions that form on the lagging strand template do not block the replication machinery, but will instead result in the formation of nascent strand gaps opposite to the site of the lesion (Higuchi et al., 2003; McInerney & O'Donnell, 2004). This observation is consistent with early findings on the chromosome that showed that following UV irradiation, some limited replication can still be detected that contains gaps in regions where the lesion formed (Ganesan et al., 1988; Rothman & Clark, 1977; Rupp & Howard-Flanders, 1968). However, the events that process and repair nascent strand gaps that form following UV irradiation are not well understood. We have previously shown that RuvABC is not

essential for DNA synthesis to resume (Donaldson et al., 2004), but is required to resolve Holliday junctions that form due to replication occurring on damaged templates (Donaldson et al., 2005). This role of RuvABC was identified by analyzing the structural intermediates that formed following UV irradiation. The abnormalities observed in *ruvAB* mutants despite the apparent normal resumption of DNA synthesis highlights the need to characterize the structural intermediates that may occur in other UV-sensitive mutants that appear to recover replication normally.

Two-dimensional (2D) agarose gel electrophoresis is a technique that allows for the identification of DNA structural intermediates that form following UV irradiation. The technique of 2D agarose gels was first utilized to separate closed circular plasmid species from nicked and linear molecules (Oppenheim, 1981). The technique was further developed to separate branched structures from linear molecules by Bell and Byers, who found that by increasing the voltage and agarose concentration in the second dimension, X-shaped structures could be resolved from other linear species (Bell & Byers, 1983). This technique has since been refined and applied to both eukaryotic and prokaryotic systems to identify different structures that occur on DNA molecules (Bell & Byers, 1983; Brewer & Fangman, 1987; Courcelle et al., 2003; Friedman & Brewer, 1995; Kuzminov et al., 1997; Martin-Parras et al., 1991; Martin-Parras et al., 1998; Olavarrieta et al., 2002).

Characterizing replicating fragments on the *E. coli* chromosome by 2D agarose gels is complicated by the fact that replication through any given sequence on the chromosome is a rare event. To address this issue, the 4.4 kb plasmid pBR322 can be

used to identify intermediates associated with replicating DNA fragments. The plasmid's moderate copy number and smaller size facilitate the detection of a rare event such as replication through a specific DNA sequence. Following the initiation of replication, pBR322 utilizes the *E. coli* host's replication proteins to replicate its own template (Bremer & Lin-Chao, 1986; Tomizawa, 1984; Tomizawa & Som, 1984), suggesting that intermediates observed on the plasmid may also occur during replication on the chromosome. In addition, previous studies have characterized the replication properties and UV-induced intermediates of pBR322 (Courcelle et al., 2003; Martin-Parras et al., 1991; Martin-Parras et al., 1998).

Here, we extended these previous studies to characterize the replication intermediates that arise in several mutants of *E. coli* that lack proteins proposed to have cellular roles in the presence of DNA damage. We analyzed the structural intermediates that formed following UV irradiation in *xonA* (Exo I), *recJ*, *recQ*, *recBC*, and *recD* mutants to identify potential intermediates that these enzymes may be responsible for processing *in vivo*.

Materials and Methods

Bacterial strains and UV irradiation

Our parental strain, SR108, is a *thyA36 deoC2* derivative of W3110 (De Lucia & Cairns, 1969). The strains HL922 (SR108 *recB21recC22 argA81*::Tn*10*), HL923 (SR108 *recD1011 argA81*::Tn*10*), HL924 (SR108 *recJ284*::Tn*10*), HL1034 (SR108 *xonA*::cat*300*), and CL581 (SR108 r*ecQ6215*::cat883) have been reported previously

(Courcelle et al., 2003; Courcelle & Hanawalt, 1999). Cells were transformed with plasmid pBR322 by electroporation (Sambrook & Russell, 2001). Phenotypes were confirmed by antibiotic resistance and, when appropriate, UV hypersensitivity.

UV irradiation was performed using a Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/sec.

Two-dimensional agarose gel electrophoresis

0.2 ml of overnight cultures grown in the presence of 100 µg/ml ampicillin were pelleted, resuspended in 20 ml of Davis medium supplemented with 0.4% glucose, 0.2% casamino acids, 10 µg/ml thymine (DGCthy media), and grown without ampicillin to an OD_{600} of 0.5 in a 37ºC shaking incubator. Following a UV dose of 50 J/m^2, cultures were transferred to a new, prewarmed flask and returned to the 37ºC shaking incubator. At the indicated times, 0.75 ml aliquots of culture were transferred to an equal volume of 2X NET (200 mM NaCl, 20 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0), centrifuged for 90 sec at 14,000 rpm, resuspended in 0.15 ml of lysozyme solution (1 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris, 1 mM EDTA, (pH 8.0)), and incubated for 20 min at 37ºC. 0.025 ml of 20% Sarkosyl and 0.010 ml of 10 mg/ml Proteinase K were added and incubation continued for 1 hr at 55ºC. Samples were then extracted twice with four volumes of phenol:chloroform:isoamyl alcohol (25:24:1), followed by one extraction with four volumes of chloroform:isoamyl alcohol (24:1), prior to dialysis on 47 mm Whatman 0.05 µm pore disks (Whatman #VMWP04700) floating on a 200 ml beaker of TE (100 mM Tris, 1mM EDTA, pH 8.0) for 3 hr. Samples were then restricted with

either *Pvu*II or *Sac*II restriction enzyme (New England Biolabs) at 37ºC overnight. Samples were then extracted with two volumes of chloroform:isoamyl alcohol (24:1) and loaded directly on the gel.

The genomic DNA samples were initially separated in a 0.4% agarose gel in 1X TBE (Tris, Boric Acid, EDTA, pH 8.0) at 1 V/cm for 15 hr. The lanes were then excised, rotated 90 degrees, and recast in a 1% agarose gel in 1X TBE and electrophoresed at 6.5 V/cm for 7 hr. DNA in the gels was transferred to a Hybond N+ nylon membrane (Amersham) and the membrane was probed with ^{32}P-labeled pBR322 that was prepared by Nick translation (Roche Applied Science**)** using α-dCTP (6000 Ci/mmol, MP Biomedicals). Radioactivity was visualized using a STORM Molecular Dynamics PhosphorImager with its associated ImageQuant analysis software (Molecular Dynamics).

Results and Discussion

A model for studying replication intermediates using two-dimensional agarose gel electrophoresis

To examine UV-induced replication intermediates on the plasmid pBR322 by 2D agarose gel electrophoresis, strains containing the plasmid pBR322 were UV irradiated with 50 J/m^2. We have shown previously that this dose generates an average of 1 lesion per plasmid and reduces survival of wild-type cultures by ~10% (Courcelle et al., 2003). At various times following UV irradiation, total genomic DNA (chromosomal and plasmid) was purified and digested with restriction endonucleases prior to agarose gel

electrophoresis. Two different restriction endonucleases were utilized in this study. The restriction endonuclease *Pvu*II linearizes the plasmid pBR322 at a location that is ~400 bp downstream of the origin of replication and allows us to examine the structural properties of the replication fork as it progresses through the molecule. The restriction endonuclease *Sac*II restricts the genomic DNA, but leaves the plasmid pBR322 intact, allowing us to examine multimeric or catenated molecules that may result from replication in the presence of UV-induced DNA damage.

The first dimension of the 2D gel separated DNA in a low percentage agarose gel (0.4%) at a low voltage (1 V/cm^2) for 15 hr to resolve DNA fragments primarily by size. For the second dimension, the lanes were excised, rotated 90º, recast in a high percentage agarose gel (1.0%), and electrophoresed at a high voltage (6.5 V/cm^2) for 7 hr to separate DNA fragments according to both shape and size. Figure 4.1 is a representative diagram of the migration pattern of pBR322 when it is either undigested (Fig. 4.1A) or restricted near the origin of replication (Fig. 4.1B).

Figure 4.2 shows the migration pattern of structural intermediates that arise in pBR322 purified from wild-type cells at various times following UV irradiation. pBR322 restricted with *Pvu*II from wild-type cells produced only non-replicating linear molecules and replicating Y-shaped molecules immediately after UV irradiation (time 0) (Figs. 4.1B and 4.2A). The non-replicating 4.4 kb linear molecules produced the most prominent spot seen in the 2D agarose gel of the restricted plasmid. The replicating, Y-shaped molecules formed an arc that extended from the linear fragment to a point that correlated to 8.8 kb in length, or a fully replicated linear molecule. After UV irradiation, transient cone region

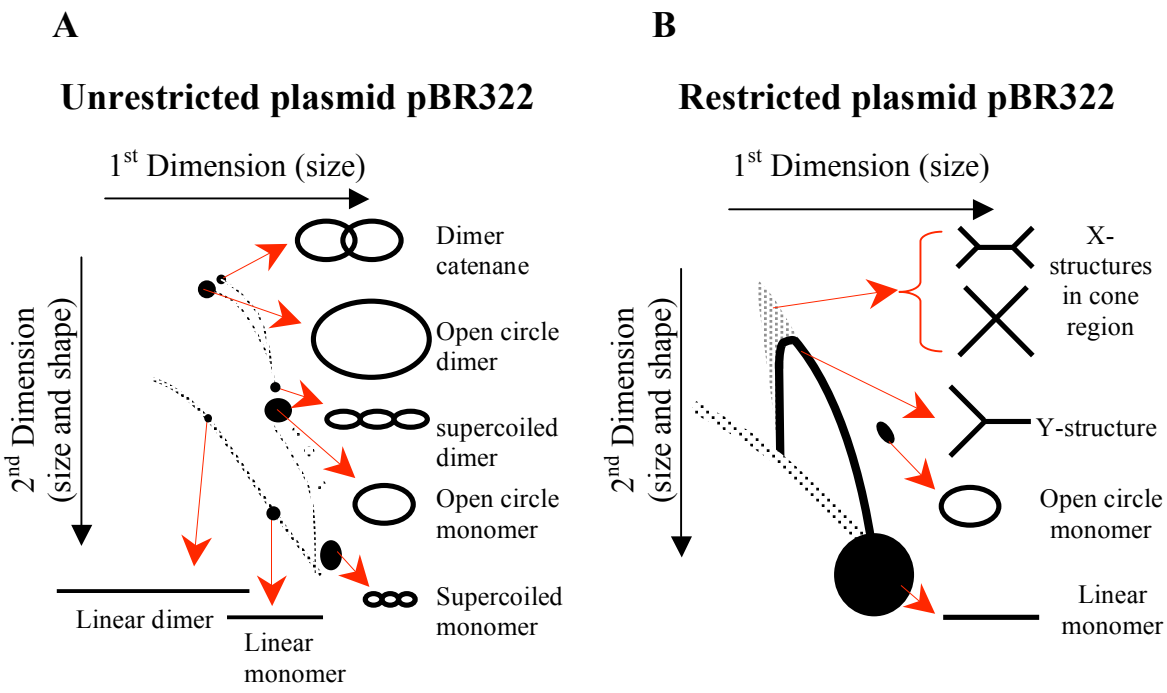


Figure 4.1. Migration pattern of two-dimensional agarose gels.

The first dimension separates DNA by size. This lane is excised, rotated 90º, and electrophoresed in the second dimension to separate DNA by shape. A: The migration pattern of undigested plasmid pBR322 following UV irradiation. The prominent species in the absence of DNA damage migrate as supercoiled monomers and open circle monomers. Smaller quantities of linear and dimer molecules can also be detected. Following UV irradiation, the level of dimer (and higher order) circular molecules increases, as does the level of linear, linear dimer, and catenated molecules. For description of structures and pattern of migration, please see text. B: Migration pattern of *Pvu*II digested plasmid pBR322 following UV irradiation. In the absence of DNA damage, the non-replicating linear molecules migrate as 4.4 kb fragments, while Y-shaped replicating molecules migrate more slowly, forming an arc that extends out from the linear fragments. Molecules that form double-Y structures and X-structures appear in the cone region following UV irradiation.

intermediates were visible that migrated above the Y-arc, peaking between 15 and 30 min after UV irradiation (Courcelle et al., 2003) (Fig. 4.2A). We have shown previously that these cone region intermediates consist of double-Y and X-structures that have formed at arrested replication forks and replication through damaged templates, respectively (Courcelle et al., 2003; Donaldson et al., 2005) (Fig. 4.1B). Previous work has shown that the cone region intermediates persist until a time that correlates with when the UV-induced lesions had been removed from the DNA by the nucleotide excision repair proteins in wild-type cultures (Courcelle et al., 2003).

Figure 4.2B shows the migration pattern of structural intermediates that arise in undigested pBR322 purified from wild-type cells following UV irradiation. These samples were digested with *Sac*II, which does not restrict the plasmid, but digests the chromosomal DNA, making the samples easier to manipulate. The 2D agarose gels of undigested pBR322 were used to identify the end products of replication on damaged molecules and is useful to distinguish between supercoiled and open circle molecules, as well as multimers and catenante structures. The locations of unreplicated supercoiled and open circle monomers and dimers have been identified by others by relaxing supercoiled molecules using DNaseI to produce predominantly open circle molecules (Martin-Parras et al., 1998). These structures migrated in the 2D gels at the positions indicated in Figure 4.1A. The discontinuous arc that appeared between the open circle monomer and supercoiled monomer species is reported to consist of knotted supercoiled molecules (Martin-Parras et al., 1998). Dual faint, but visible arcs radiated from the open circle monomer molecules and extended to the open circle dimers and the catenate structures.

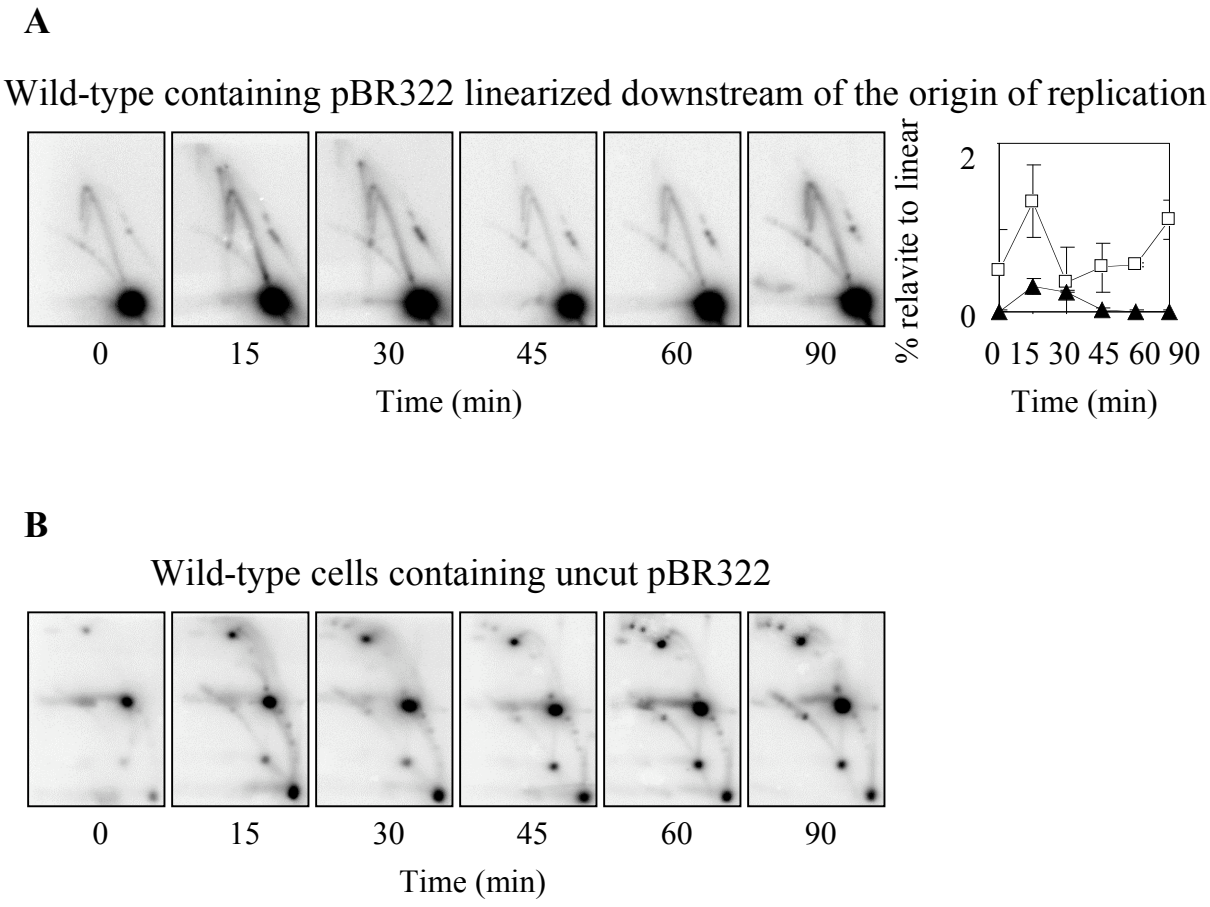


Figure 4.2. UV-induced replication intermediates of pBR322 from wild-type cultures.

A: 2D agarose gels of *Pvu*II digested pBR322 following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and the total genomic DNA was purified, restricted with *Pvu*II, and resolved by 2D agarose gels at the times indicated. The percent of the molecules in the Y-arc (□) and in the cone region (▲) are plotted relative to the amount of linear DNA in the sample and are based upon two independent experiments. Errors bars represent one standard deviation. B: 2D agarose gels of undigested pBR322 following UV irradiation. DNA prepared as in (A) except digested with *Sac*II rather than *Pvu*II. *Sac*II does not restrict the DNA of plasmid pBR322. Gels represent one of at least two independent experiments.

The arc between open circle monomer and open circle dimer has previously been reported to correspond to replication intermediates that had an open circle with a double-strand tail that increased in size, a Cairns or theta-shaped structure (Brewer & Fangman, 1987; Martin-Parras et al., 1998; Sundin & Varshavsky, 1980). The second arc between the open circle monomer and the catenate species is suggested to correspond to catenated dimers of differing linking numbers (Brewer & Fangman, 1987; DiNardo et al., 1984; Martin-Parras et al., 1998; Sundin & Varshavsky, 1980). Previous studies that examined replication on SV40 plasmids in human cells suggested that catenates formed as a normal intermediate during the completion of replication and typically involved interlocked daughter molecules that contained nicks or gaps at the terminus (Sundin & Varshavsky, 1980; Sundin & Varshavsky, 1981). Figure 4.2B shows that in wild-type cells, catenated dimers appeared to accumulate within ~15 min and after UV irradiation. The formation of these species correlated with the time that cone region intermediates were observed in the *Pvu*II digested samples, suggesting that these species may reflect the structures maintained during the repair of gaps and/or lesions that have formed on the molecules.

recJ, recQ, and xonA mutants form intermediates that resemble those of wild-type following UV irradiation

Over the years, several genes have been identified, that when, mutated impair the cell's ability to replicate and survive in the presence of UV-induced DNA damage. Although these gene products promote survival following UV irradiation, in many cases little is known about the specific role(s) these enzymes have in the cell. As mentioned

above, the 3'-5' helicase RecQ and the 5'-3' exonuclease RecJ have been shown to process the nascent lagging strand DNA at arrested replication forks (Courcelle & Hanawalt, 1999). In addition to its role in processing the arrested replication fork, RecQ has also been suggested as to having an important role in suppressing genomic rearrangements (Hanada et al., 1997; Hishida et al., 2004). Although, the removal of RecJ or RecQ does not prevent the cell's ability to resume DNA synthesis, it does result in a delay (for *recJ*) or a reduced rate (for *recQ*) in the recovery of DNA synthesis after UV irradiation (Courcelle et al., 2005). In addition, both *recQ* mutants and *recJ* mutants have altered patterns and frequency of recombination induced by DNA damage, suggesting that abnormal intermediates may accumulate in their absence (Hanada et al., 1997; Ukita & Ikeda, 1996).

Consistent with our previous results, *recJ* and *recQ* mutants formed cone region intermediates that migrated and appeared similar to wild-type cells following UV irradiation (Courcelle et al., 2003) (Fig. 4.3A). While subtle, the cone region intermediates appeared to remain evident in both *recJ* and *recQ* mutants ~15 min longer than in the wild-type cultures, perhaps reflecting the delayed recovery in these mutants (Fig. 4.3A). However, the difference was not quantitatively significant and the intermediates were resolved by the end of the time course, similar to wild-type cultures. Comparatively, when we examined the structural intermediates associated with undigested pBR322 molecules from *recJ* and *recQ* mutants, we did not observe any detectable differences in the multimeric structures or catenates that formed during the

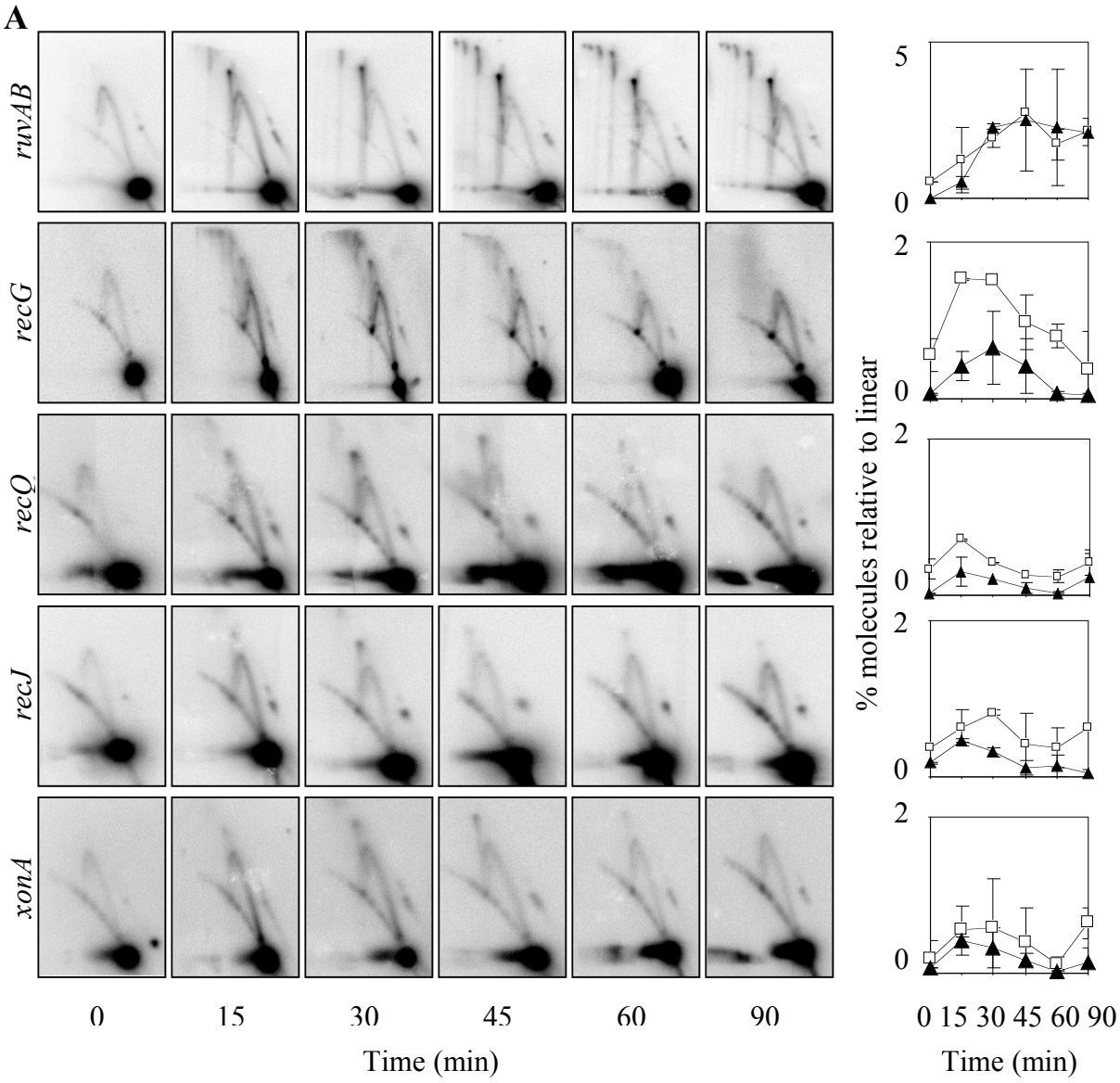


Figure 4.3. UV-induced replication intermediates of pBR322 from *ruvAB*, *recG*, *recQ*, *recJ*, and *xonA* mutants of *Escherichia coli*.

A: 2D agarose gels of *Pvu*II digested pBR322 following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and the total genomic DNA was purified and restricted with *Pvu*II and resolved by 2D agarose gels at the times indicated. Gels shown represent one of at least two independent experiments. The percent of molecules in the Y-arc (□) and in the cone region (▲) are plotted relative to the amount of linear DNA in the sample. Note the different scale on the *ruvAB* graph. Graphs are based on two independent experiments. Error bars represent one standard deviation.

B

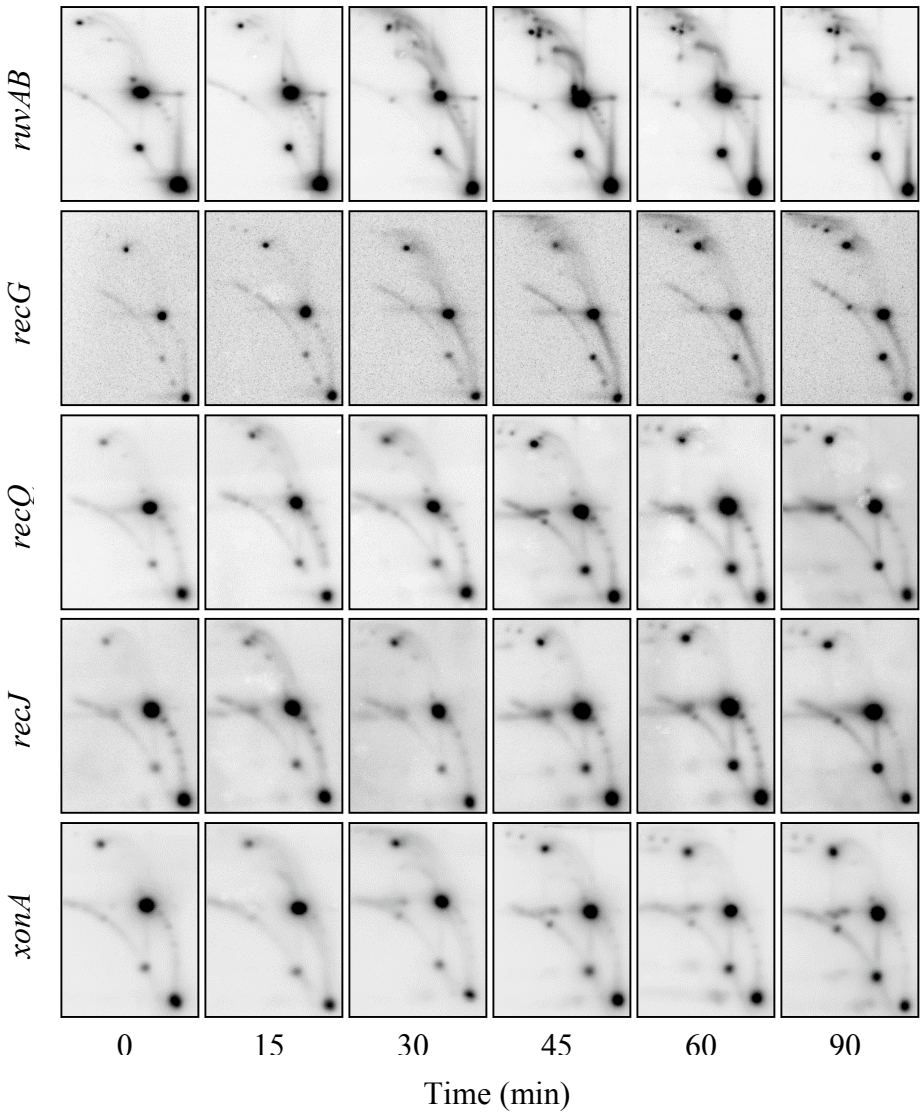


Figure 4.3 (continued)

B: 2D agarose gels of undigested pBR322 following UV irradiation. DNA was prepared as in (A) and restricted with *Sac*II, and resolved by 2D agarose gels at the times indicated. Gels shown represent one of at least two independent experiments.

recovery period of UV irradiated *recQ* or *recJ* mutants when compared to wild-type cells (Fig. 4.3B).

Another protein with a similar function to RecJ is Exonuclease I. Exo I, encoded by *xonA*, is an exonuclease that degrades single-strand DNA in the 3'-5' direction, which is opposite in polarity to that of RecJ (Lehman & Nussbaum, 1964). Exo I copurifies with RecA and has been implicated in DNA repair and recombination (Bedale et al., 1993; Burdett et al., 2001; Phillips et al., 1988; Viswanathan & Lovett, 1998). The polarity of Exo I raises the possibility that it could process the nascent leading strand DNA at blocked replication forks. However, *xonA* mutants have a similar pattern of nascent DNA processing as wild-type, suggesting that this gene product is not involved in processing nascent DNA at the arrested replication fork or that the level of degradation is below what can be detected in our assays (Courcelle & Hanawalt, 1999). To expand upon these previous results, we examined the UV-induced intermediates that formed on pBR322 molecules from *xonA* mutants (Fig. 4.3). When we examined the migration pattern of *xonA* mutants digested with *Pvu*II, we found that the cone region intermediates formed were similar to those observed in *recQ* and *recJ* mutants. The intermediates also appeared to persist slightly longer than in wild-type cultures, although again this difference was not statistically significant (Fig. 4.3A). 2D agarose gels of undigested pBR322 molecules from *xonA* mutants after UV irradiation showed that molecular species in *xonA* mutants were similar in kind and quantity to those observed in wild-type cells (Fig. 4.3B).

We have shown previously that strains deficient in the branch migration proteins RuvAB or RecG did not exhibit a delay in the resumption of DNA synthesis following

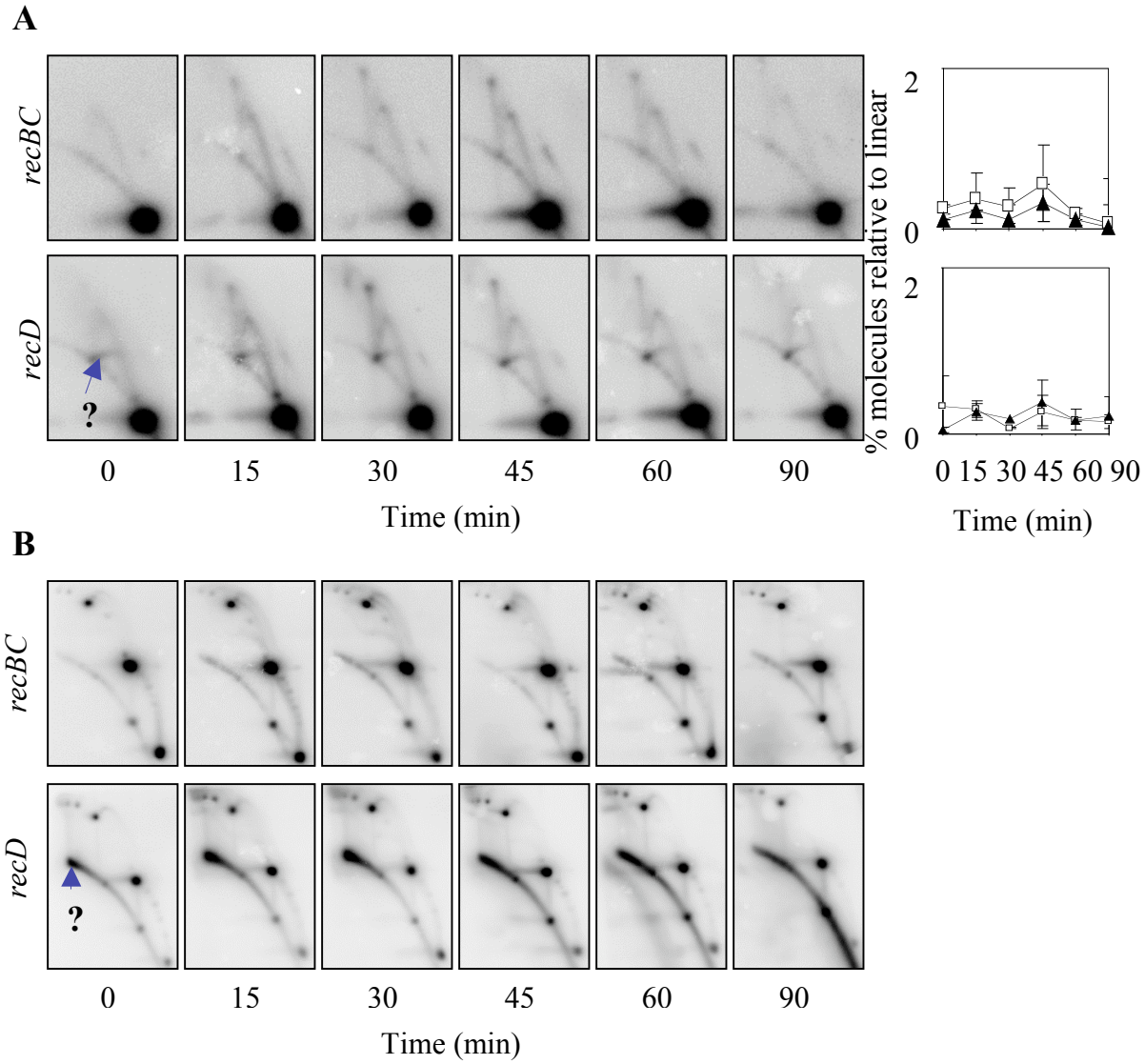
UV irradiation (Donaldson et al., 2004). However, the removal of RuvAB, not RecG, resulted in the accumulation of structures in the cone region of the 2D agarose gel following UV irradiation, indicating an important role for RuvAB in processing Holliday junctions that accumulate due to replication occurring on damaged templates (Donaldson et al., 2005) (Fig. 4.3A). To further characterize these intermediates, we examined 2D agarose gels of undigested plasmid pBR322 from *ruvAB* mutants. We found that these mutants accumulated species that would be consistent with the formation of catenated dimers (Fig. 4.3B). It is reasonable to speculate that the unresolved Holliday junctions and catenated molecules in *ruvAB* mutants may lead to a failure of the chromosome to partition properly and result in the UV hypersensitive phenotype associated with these mutants. Consistent with this view, previous studies have shown that following UV irradiation, *ruvAB* mutants filament extensively. These filaments contain regions of multinucleate and anucleate regions between the locations where septation should occur (Ishioka et al., 1998; Otsuji et al., 1974).

recBC and recD mutants are not able to remove X-structures that arise following UV irradiation

In addition to the proteins mentioned above, RecBC and RecD also have roles in processing DNA in the presence of damage. Purified RecBCD unwinds and degrades duplex DNA from a double-strand end and is important for the repair of double-strand breaks (Kowalczykowski et al., 1994; Kuzminov, 1999; Smith, 1998). The DNA degradation continues in a progressive manner until reaching a specific sequence, the Chi

Site (reviewed in (Kuzminov, 1999). Upon encountering the Chi site, the nuclease activity is attenuated, although the helicase activity continues to unwind DNA. It is thought that RecA is then recruited to this substrate to initiate recombination (Amundsen et al., 2000). *recBC* mutants are deficient in all known activities of RecBCD, have a reduced recombination frequency as measured by conjugation and transduction, have a low plating efficiency, and are sensitive to DNA damage (Chaudhury & Smith, 1984; Clark, 1973). *recD* mutants are deficient in exonuclease activity, but are still able to unwind DNA and are proficient in recombination (Amundsen et al., 1986). Additionally, *recD* mutants have normal cell viability as compared to wild-type following UV irradiation (Chaudhury & Smith, 1984). Strains deficient in RecBC or RecD appear to process the nascent DNA normally following the arrest of replication (Courcelle & Hanawalt, 1999). The absence of RecBC or RecD does not impair the cell's ability to resume replication (Courcelle et al., 1997; KH Chow, unpublished results). However, although *recBC* mutants begin to recover DNA synthesis at a time that is similar to wild-type cultures, the rate of synthesis does not return to pre-UV rates within 90 min (KH Chow, unpublished results).

In an attempt to further understand the cellular roles that RecBC and RecD might have following UV-induced DNA damage, we examined the replication intermediates that occurred on pBR322 molecules from *recBC* and *recD* mutants using 2D agarose gels (Fig. 4.4). In *recBC*, mutants the migration pattern of *Pvu*II digested pBR322 was found to be similar to that observed in wild-type cells (Fig. 4.4A). However, unlike wild-type cells and as previously reported, the intermediates that migrated in the cone region of



A: 2D agarose gels of *Pvu*II digested pBR322 following UV irradiation. Cells containing the plasmid pBR322 were UV irradiated with 50 J/m^2 and the total genomic DNA was purified and restricted with *Pvu*II and resolved by 2D agarose gels at the times indicated. The percent of molecules in the Y-arc (□) and in the cone region (▲) are plotted relative to the amount of linear DNA in the sample. Graphs are based on two independent experiments. Error bars represent one standard deviation. B: 2D agarose gels of undigested pBR322 following UV irradiation. DNA was purified as in (A) and restricted with *Sac*II, and resolved by 2D agarose gels at the times indicated. Gels shown represent one of at least two independent experiments.

recBC mutants persisted throughout the 90 min time course (Fig. 4.4A). To further characterize the structural intermediates that persisted in this strain, undigested pBR322 molecules from *recBC* mutants were also examined. We did not observe any detectable differences in the species or quantities of products generated during the recovery period following UV irradiation, making the events that lead to the persistence of the X-shaped molecules in the *Pvu*II digested samples unclear (Fig. 4.4).

Similar to *recBC* mutants, *Pvu*II digested pBR322 molecules from *recD* mutants also formed cone region intermediates that persisted throughout the 90 min time course in response to UV irradiation (Fig. 4.4A). In contrast to *recBC* mutants, however, *recD* mutants additionally contained a class of unique structural intermediates that were present both in the absence and presence of UV irradiation. These unique intermediates migrated as a tail of shorter products that extended from the 8.8 kb linear spot (Fig. 4.4A). Examination of undigested pBR322 from *recD* mutants indicated that long, linear multimers of pBR322 formed even in the absence of DNA damage (Fig. 4.4B). Previous studies have shown that RecD is required to prevent plasmid replication from occurring via a rolling circle mechanism and that this results in reduced plasmid stability in *recD* mutants (Biek & Cohen, 1986; Niki et al., 1990).

These results indicate that RecBC or RecD are required to process DNA intermediates generated by UV-induced DNA damage. However, the nature of the substrate that requires processing is not clear. The presence of linear multimers in *recD* mutants in the absence of UV irradiation, taken together with the persistence of the cone region intermediates following UV irradiation, suggests that RecD may be processing a

common substrate that occurs during both normal replication and DNA repair. The possibility that the replication defect in *recD* mutants is simply exaggerated by the presence of UV damage also cannot be excluded.

Here we have investigated several mutants for potential intermediates that may indicate the cellular substrate for these gene products *in vivo*. Together with previous data, our results suggest that RecJ, RecQ, and potentially Exo I may have roles in processing UV-induced intermediates that would increase the efficiency by which replication resumes. RecBC and RecD both have roles in processing UV-induced intermediates as well, but may also have a functional roles in the absence of DNA damage. The question of what specific role(s) that RecBC or RecD has during normal cellular replication and how it relates to its role following UV-induced DNA damage is an interesting problem that merits further investigation.

Acknowledgements

This work was supported by Career Award MCB-0448315 from the National Science Foundation.

REFERENCES

Amundsen, S. K., Taylor, A. F., Chaudhury, A. M. & Smith, G. R. (1986). recD: the gene for an essential third subunit of exonuclease V. *Proc Natl Acad Sci U S A* 83, 5558-62.

Amundsen, S. K., Taylor, A. F. & Smith, G. R. (2000). The RecD subunit of the Escherichia coli RecBCD enzyme inhibits RecA loading, homologous recombination, and DNA repair. *Proc Natl Acad Sci U S A* 97, 7399-404.

Bedale, W. A., Inman, R. B. & Cox, M. M. (1993). A reverse DNA strand exchange mediated by recA protein and exonuclease I. The generation of apparent DNA strand breaks by recA protein is explained. *J Biol Chem* 268, 15004-16.

Bell, L. & Byers, B. (1983). Separation of branched from linear DNA by two-dimensional gel electrophoresis. *Anal Biochem* 130, 527-35.

Biek, D. P. & Cohen, S. N. (1986). Identification and characterization of recD, a gene affecting plasmid maintenance and recombination in Escherichia coli. *J Bacteriol* 167, 594-603.

Bonner, C. A., Stukenberg, P. T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H. & Goodman, M. F. (1992). Processive DNA synthesis by DNA polymerase II mediated by DNA polymerase III accessory proteins. *J Biol Chem* 267, 11431-8.

Bremer, H. & Lin-Chao, S. (1986). Analysis of the physiological control of replication of ColE1-type plasmids. *J Theor Biol* 123, 453-70.

Brewer, B. J. & Fangman, W. L. (1987). The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell* 51, 463-71.

Burdett, V., Baitinger, C., Viswanathan, M., Lovett, S. T. & Modrich, P. (2001). In vivo requirement for RecJ, ExoVII, ExoI, and ExoX in methyl-directed mismatch repair. *Proc Natl Acad Sci U S A* 98, 6765-70.

Chaudhury, A. M. & Smith, G. R. (1984). A new class of Escherichia coli recBC mutants: implications for the role of RecBC enzyme in homologous recombination. *Proc Natl Acad Sci U S A* 81, 7850-4.

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, 3492-6.

Clark, A. J. (1973). Recombination deficient mutants of E. coli and other bacteria. *Annu Rev Genet* 7, 67-86.

Cordeiro-Stone, M., Makhov, A. M., Zaritskaya, L. S. & Griffith, J. D. (1999). Analysis of DNA replication forks encountering a pyrimidine dimer in the template to the leading strand. *J Mol Biol* 289, 1207-18.

Courcelle, C. T., Chow, K. H. & Courcelle, J. (2005). Replication fork reversal by RecJ determines if DNA damage is reversed or bypassed. *submitted*.

Courcelle, J., Belle, J. J. & Courcelle, C. T. (2004). When replication travels on damaged templates: bumps and blocks in the road. *Res Microbiol* 155, 231-7.

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, 3714-9.

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, 916-22.

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, 1064-7.

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, 543-51.

Courcelle, J. & Hanawalt, P. C. (2001). Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated *Escherichia coli* need not involve recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8196-202.

De Lucia, P. & Cairns, J. (1969). Isolation of an *E. coli* strain with a mutation affecting DNA polymerase. *Nature* 224, 1164-66.

DiNardo, S., Voelkel, K. & Sternglanz, R. (1984). DNA topoisomerase II mutant of Saccharomyces cerevisiae: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc Natl Acad Sci U S A* 81, 2616-20.

Donaldson, J. R., Courcelle, C. T. & Courcelle, J. (2004). RuvAB and RecG are not essential for the recovery of DNA synthesis following UV-induced DNA damage in Escherichia coli. *Genetics* 166, 1631-40.

Donaldson, J. R., Courcelle, C. T. & Courcelle, J. (2005). RuvABC is required to resolve Holliday junctions that accumulate when replication occurs on damaged templates in *Escherichia coli*. *in process*.

Friedman, K. L. & Brewer, B. J. (1995). Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol* 262, 613-27.

Ganesan, A. K., Hunt, J. & Hanawalt, P. C. (1988). Temperature dependent survival of UV-irradiated Escherichia coli K12. *Mol Gen Genet* 214, 198-203.

Hanada, K., Ukita, T., Kohno, Y., Saito, K., Kato, J. & Ikeda, H. (1997). RecQ DNA helicase is a suppressor of illegitimate recombination in Escherichia coli. *Proc Natl Acad Sci U S A* 94, 3860-5.

Higgins, N. P., Kato, K. & Strauss, B. (1976). A model for replication repair in mammalian cells. *J. Mol. Biol.* 101, 417-25.

Higuchi, K., Katayama, T., Iwai, S., Hidaka, M., Horiuchi, T. & Maki, H. (2003). Fate of DNA replication fork encountering a single DNA lesion during *oriC* plasmid DNA replication *in vitro*. *Genes Cells* 8, 437-49.

Hishida, T., Han, Y. W., Shibata, T., Kubota, Y., Ishino, Y., Iwasaki, H. & Shinagawa, H. (2004). Role of the Escherichia coli RecQ DNA helicase in SOS signaling and genome stabilization at stalled replication forks. *Genes Dev* 18, 1886-97.

Howard-Flanders, P., Rupp, W. D., Wilkins, B. M. & Cole, R. S. (1968). DNA replication and recombination after UV irradiation. *Cold Spring Harb. Symp. Quant. Biol.* 33, 195-207.

Ishioka, K., Fukuoh, A., Iwasaki, H., Nakata, A. & Shinagawa, H. (1998). Abortive recombination in Escherichia coli ruv mutants blocks chromosome partitioning. *Genes Cells* 3, 209-20.

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. & Rehrauer, W. M. (1994). Biochemistry of homologous recombination in Escherichia coli. *Microbiol Rev* 58, 401-65.

Kuzminov, A. (1999). Recombinational repair of DNA damage in Escherichia coli and bacteriophage lambda. *Microbiol Mol Biol Rev* 63, 751-813, table of contents.

Kuzminov, A., Schabtach, E. & Stahl, F. W. (1997). Study of plasmid replication in Escherichia coli with a combination of 2D gel electrophoresis and electron microscopy. *J Mol Biol* 268, 1-7.

Lehman, I. R. & Nussbaum, A. L. (1964). The Deoxyribonucleases of Escherichia Coli. V. On the Specificity of Exonuclease I (Phosphodiesterase). *J Biol Chem* 239, 2628-36.

Marians, K. J. (2004). Mechanisms of replication fork restart in Escherichia coli. *Philos Trans R Soc Lond B Biol Sci* 359, 71-7.

Martin-Parras, L., Hernandez, P., Martinez-Robles, M. L. & Schvartzman, J. B. (1991). Unidirectional replication as visualized by two-dimensional agarose gel electrophoresis. *J Mol Biol* 220, 843-53.

Martin-Parras, L., Lucas, I., Martinez-Robles, M. L., Hernandez, P., Krimer, D. B., Hyrien, O. & Schvartzman, J. B. (1998). Topological complexity of different populations of pBR322 as visualized by two-dimensional agarose gel electrophoresis. *Nucleic Acids Res* 26, 3424-32.

McInerney, P. & O'Donnell, M. (2004). Functional uncoupling of twin polymerases: mechanism of polymerase dissociation from a lagging-strand block. *J Biol Chem* 279, 21543-51.

Michalke, H. & Bremer, H. (1969). RNA synthesis in Escherichia coli after irradiation with ultraviolet light. *J Mol Biol* 41, 1-23.

Michel, B., Ehrlich, S. D. & Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *Embo J.* 16, 430-8.

Michel, B., Grompone, G., Flores, M. J. & Bidnenko, V. (2004). Multiple pathways process stalled replication forks. *Proc Natl Acad Sci U S A* 101, 12783-8.

Niki, H., Ogura, T. & Hiraga, S. (1990). Linear multimer formation of plasmid DNA in Escherichia coli hopE (recD) mutants. *Mol Gen Genet* 224, 1-9.

Olavarrieta, L., Martinez-Robles, M. L., Sogo, J. M., Stasiak, A., Hernandez, P., Krimer, D. B. & Schvartzman, J. B. (2002). Supercoiling, knotting and replication fork reversal in partially replicated plasmids. *Nucleic Acids Res.* 30, 656-66.

Oppenheim, A. (1981). Separation of closed circular DNA from linear DNA by electrophoresis in two dimensions in agarose gels. *Nucleic Acids Res* 9, 6805-12.

Otsuji, N., Iyehara, H. & Hideshima, Y. (1974). Isolation and characterization of an *Escherichia coli ruv* mutant which forms nonseptate filaments after low doses of ultraviolet light irradiation. *J. Bacteriol.* 117, 337-44.

Pages, V. & Fuchs, R. P. (2003). Uncoupling of leading- and lagging-strand DNA replication during lesion bypass *in vivo*. *Science* 300, 1300-3.

Phillips, G. J., Prasher, D. C. & Kushner, S. R. (1988). Physical and biochemical characterization of cloned sbcB and xonA mutations from Escherichia coli K-12. *J Bacteriol* 170, 2089-94.

Robu, M. E., R. B. Inman, and M. M. Cox. (2001). RecA protein promotes the regression of stalled replication forks in vitro. *Proc Natl Acad Sci U S A* 98, 8211-8.

Rothman, R. H. & Clark, A. J. (1977). The dependence of postreplication repair on *uvrB* in a *recF* mutant of *Escherichia coli* K-12. *Mol. Gen. Genet.* 155, 279-86.

Rupp, W. D. & Howard-Flanders, P. (1968). Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. *J Mol Biol* 31, 291-304.

Sambrook, J. & Russell, D. W. (2001). Transformation of E. coli by Electroporation. In *Molecular Cloning: A Laboratory Manual, 3rd Ed.*, pp. 1.119-1.122. Cold Spring Harbor Laboratory Press.

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.

Smith, G. R. (1998). DNA double-strand break repair and recombination in Escherichia coli. In *DNA Damage and Repair, Vol. I: Repair in Prokaryotes and Lower Eukaryotes*, pp. 135-57.

Sundin, O. & Varshavsky, A. (1980). Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. *Cell* 21, 103-14.

Sundin, O. & Varshavsky, A. (1981). Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. *Cell* 25, 659-69.

Tomizawa, J. (1984). Control of ColE1 plasmid replication: the process of binding of RNA I to the primer transcript. *Cell* 38, 861-70.

Tomizawa, J. & Som, T. (1984). Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. *Cell* 38, 871-8.

Ukita, T. & Ikeda, H. (1996). Role of the recJ gene product in UV-induced illegitimate recombination at the hotspot. *J Bacteriol* 178, 2362-7.

Viswanathan, M. & Lovett, S. T. (1998). Single-strand DNA-specific exonucleases in Escherichia coli. Roles in repair and mutation avoidance. *Genetics* 149, 7-16.

CHAPTER V

SUMMARY

The bacterium *Escherichia coli* has served as a useful model organism for characterizing how cells respond to DNA damage. The genetics of this organism are well characterized, allowing for rapid construction of specific mutants. Additionally, the processes of replication, transcription, and repair are highly conserved between *E. coli* and humans, allowing us to dissect how these complicated cellular processes occur in a simpler, more manipulable system such as *E. coli*.

This project utilized DNA damage induced by UV irradiation as a model lesion to dissect the general question of how replication occurs in the presence of DNA damage. Lesions that are induced by UV irradiation have been shown to block the progression of replication machinery (Howard-Flanders et al., 1968; Setlow et al., 1963). Following UV irradiation, DNA synthesis undergoes a brief arrest before resuming again (Courcelle et al., 2003; Setlow et al., 1963). The time at which replication resumes correlates with when the lesions have been removed from the DNA and UV-induced intermediates have been processed (Courcelle et al., 2003). Although several proteins are known to be involved, the specific processes by which replication resumes following arrest at a UV lesion or continues in the presence of a non-arresting lesion are not completely understood. This dissertation focused upon characterizing several candidate gene

products for their potential roles in either the recovery of DNA synthesis following UV irradiation or potentially processing UV-induced lesions that fail to arrest the progression of the replication machinery.

Following UV irradiation, it has been proposed that the nascent DNA at the arrested replication fork will be displaced and processed to generate a 4-arm branched structure that resembles a Holliday junction. This would then expose the blocking lesion and allow the nucleotide excision repair proteins to gain access to and remove the damaged DNA (Courcelle et al., 2003). RuvAB and RecG have both been proposed to potentially form Holliday junctions at arrested replications forks based primarily upon *in vitro* studies and UV survival assays (Courcelle & Hanawalt, 2001; McGlynn & Lloyd, 1999; McGlynn & Lloyd, 2001; Seigneur et al., 1998). Chapter II of this dissertation tested the hypothesis that RuvAB or RecG may be required for DNA synthesis to resume following UV irradiation. We showed that the absence of either RecG or RuvAB did not affect the time or kinetics that DNA synthesis resumed. We also showed that RuvAB and RecG were not required for maintaining the structural integrity of the arrested replication fork following UV irradiation. This suggests that if RuvAB or RecG is involved in catalyzing fork regression, it is not essential for the resumption of DNA synthesis following UV-induced DNA damage.

Chapter III's surprising result that RuvAB or RecG are not essential for the resumption of DNA synthesis following arrest led us to pursue the question of "Why are strains deficient in RuvAB or RecG hypersensitive to UV irradiation?" The impaired survival following UV-induced DNA damage clearly indicates that these gene products

are required for some function that promotes viability when the cells are exposed to UV. Chapter III of this dissertation explored the possibility that RuvABC or RecG are involved in alternative processes following UV irradiation other than the direct processing of arrested replication forks. We explored this possibility using two-dimensional agarose gel electrophoresis, along with transmission electron microscopy and alkaline sucrose gradients to monitor the structural integrity and intermediates of *ruvAB* and *recG* mutants following UV irradiation. We showed that even though DNA synthesis resumed following arrest, mutants lacking RuvAB or RuvC accumulated Holliday junctions following replication on damaged templates. The failure to resolve the Holliday junctions correlated with a loss in the integrity of the genomic DNA at later times following UV. These Holliday junctions were further examined by transmission electron microscopy and were found to be structurally distinct from the intermediates observed to accumulate in mutants that fail to resume replication following arrest. We proposed that RuvABC may be necessary to resolve Holliday junctions that accumulate at a subset of lesions that are skipped over by the replication apparatus.

The technique of two-dimensional agarose gel electrophoresis to reveal structural intermediates associated with the recovery of replication following UV-induced DNA damage has proven to be a useful tool for identifying the potential cellular functions of several candidate gene products (Courcelle et al., 2003; Donaldson et al., 2005). To make use of this, Chapter IV used two-dimensional agarose gels to survey several mutants for potential intermediates that may indicate the cellular substrates for these gene products *in vivo*. The UV-induced intermediates of strains deficient in RecJ, Exo I (*xonA*), RecQ,

RecBC, and RecD were analyzed by two-dimensional agarose gel electrophoresis. We showed that *recJ*, *recQ*, *xonA*, and *recBC* mutants did not accumulate UV-induced intermediates that were distinct from those observed in wild-type cells by this assay (Courcelle et al., 2003). However, in the *recJ*, *recQ*, and *xonA* mutants, the UV-induced cone region intermediates persisted for 15 min beyond the time that they had been resolved in wild-type cultures. By contrast, the cone region intermediates in *recBC* and *recD* mutants persisted throughout the 90 min time period we examined. Additionally, *recD* mutants formed long, linear multimers both the absence and presence of UV-induced DNA damage as has been reported previously.

These results suggest several experimental predictions and questions that could be pursued in the future. First, what is the role of RecG? The assays we utilized in these studies were unable to identify any significant abnormalities in *recG* mutants that could account for its hypersensitivity to DNA damage. RecG catalyzes the formation of Holliday junctions from replication fork-like structures *in vitro*, yet these activities have not been identified *in vivo*. Second, what are the molecular events that generate the DNA cross-overs observed in *ruvAB* and *ruvC* mutants? Third, what are the structures of the intermediates that form in *recBC* and *recD* mutants? These gene products are involved in recombination-mediated repair of double-strand breaks and the roles of these proteins in the cell in the presence of UV-induced DNA damage need to be identified. Are they part of a second pathway that the cell will choose if not able to repair the damaged lesions by nucleotide excision repair? It will be important to further characterize these and other proteins in cellular processes that occur in the presence of DNA damage.

REFERENCES

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, 1064-7.

Courcelle, J. & Hanawalt, P. C. (2001). Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated *Escherichia coli* need not involve recombination. *Proc. Natl. Acad. Sci. U S A* 98, 8196-202.

Donaldson, J. R., Courcelle, C. T. & Courcelle, J. (2005). RuvABC is required to resolve Holliday junctions that accumulate when replication occurs on damaged templates in *Escherichia coli*. *in process*.

Howard-Flanders, P., Rupp, W. D., Wilkins, B. M. & Cole, R. S. (1968). DNA replication and recombination after UV irradiation. *Cold Spring Harb. Symp. Quant. Biol.* 33, 195-207.

McGlynn, P. & Lloyd, R. G. (1999). RecG helicase activity at three- and four-strand DNA structures. *Nucleic Acids Res.* 27, 3049-56.

McGlynn, P. & Lloyd, R. G. (2001). Action of RuvAB at replication fork structures. *J. Biol. Chem.* 276, 41938-44.

Seigneur, M., Bidnenko, V., Ehrlich, S. D. & Michel, B. (1998). RuvAB acts at arrested replication forks. *Cell* 95, 419-30.

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.