THE ROLE OF RECOMBINATION PROTEINS IN MAINTAINING AND PROCESSING ARRESTED REPLICATION FORKS DURING THE RECOVERY OF DNA REPLICATION IN UV-IRRADIATED ESCHERICHIA COLI

By

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CHAPTER I

RESEARCH PURPOSES

INTRODUCTION

Accurate chromosomal replication is critical to ensure that the progeny of each generation inherits an exact copy of the genetic material. The cell's genetic material is vulnerable to assault from endogenous and exogenous DNA-damaging agents, potentially generating DNA strand breaks, interstrand DNA crosslinks, and DNA adducts. These DNA lesions, if left unrepaired, can block DNA replication and transcription leading to aberrant DNA segregation, genomic rearrangements, mutagenesis, or even cell death.

For these reasons, it is crucial to understand the molecular mechanisms that the cell utilizes to overcome lesions and other impediments that may impair or prevent normal progression of the DNA replication machinery on the genomic template. In order to study the effects of DNA lesions on replication process, we employed UV-irradiated *Escherichia coli* as a model system to dissect the molecular reactions of replication machinery in response to the encountering of DNA lesions. UV irradiation at 254nm generates two predominant forms of intrastrand DNA lesions, the cis, syn-cyclobutane pyrimidine dimer (CPD) and the pyrimidine-6-4-pyrimidone (6-4- PP), both which block replication fork progression (5, 35, 36, 43). After a moderate dose of UV, the formation of these lesions transiently inhibits the

replication machinery in wild type *E.coli.* However, replication recovers at a later time (12, 18, 42, 43), indicating the existence of efficient mechanisms that allow the replication machinery to overcome replication-blocking DNA lesions.

The recovery of DNA replication in UV-irradiated *E.coli* has been shown to require the *uvrA*, *uvrB*, and *uvrC* gene products which form an exonuclease that removes a 12-nucleotide patch surrounding the DNA lesion. The damaged segment is then displaced by the *uvrD* gene product and resynthesized by Polymerase I in a process termed nucleotide excision repair (NER) (39). Hence, inactivation of any one of the *uvr* gene products renders cells extremely hypersensitive to UV-irradiation. In addition to NER, the recovery of replication also requires RecA. RecA was originally identified and characterized as a protein required for homologous recombination, and catalyzes DNA strand exchange between homologous DNA sequences`(4). *In vitro* studies have shown that purified RecA protein binds single-strand DNA in a 5'-3' direction to form a filament that can then pair with homologous duplex DNA (15, 25, 44). During DNA replication however, RecA also binds to the single-strand regions generated when replication encounters DNA lesions (38, 40). The binding of RecA to these substrates serves as an activation signal that triggers the upregulation of more than 40 genes in a process that has been termed the SOS response (13, 38, 40). Many of the induced genes have functions that are important for repairing the DNA damage, preventing premature cell division, and restoring the progression of the arrested replication forks (13). Surprisingly, although RecA protein plays such a critical role in orchestrating the response to DNA damage, *recA* mutant are viable in the absence of exogenous sources of DNA damage. However, *recA* mutants are completely

deficient in recombinational processes, and are extremely sensitive to all forms of DNA damage (8). The sensitivity to DNA damage is associated with a failure to recover DNA replication after UV irradiation and extensive degradation of the genomic DNA (10, 11, 22). Thus, while RecA protein is required to change the genetic information during recombinational or sexual cell cycles, it also is the key enzyme required to maintain the genetic information when DNA damage is encountered during replication in asexual cell cycles.

The idea that RecA and other recombination proteins play an important role in rescuing arrested replication forks is supported by recent studies that examine the roles of RecF and RecR proteins in the recovery of replication. Similar to RecA, RecF and RecR were originally characterized for their role in promoting recombination (21). However, more recent studies have demonstrated that the proteins are also required to maintain replication forks arrested by UV-induced DNA lesions and restoring replication (10, 11). In the absence of RecF or RecR, extensive degradation of nascent DNA at arrested replication forks by the RecJ exonuclease and RecQ helicase is observed to occur (9, 12). In addition, the replication machinery fails to resume, eventually leading to cell death (10, 11).

While these observations suggest that the RecF pathway proteins play a prominent role in the recovery process, several candidate genes have yet to be characterized. *recO*, together with *recF*, *recR*, *recJ*, *recQ*, is classified as member of the RecF pathway (24). Among these proteins, one of particular interest is RecO. RecO is epistatic with RecF and RecR with respect to several phenotypes associated with recombination and repair processes. In the absence of RecO, RecF, or RecR,

cells display similar delays in the induction of SOS response, reduced plasmid recombination, reduced conjugational recombination in a *recBCsbcBC* background, and persistent of daughter-strand gaps in the newly synthesized DNA of UV-irradiated *uvrA* mutants (20, 21, 24, 33, 51). In addition, the absence of one or more of the RecF, RecO, or RecR proteins renders cells equally hypersensitive to UV irradiation (31, 33), implying that these proteins act at a common step and are necessary for some aspect during the recovery from DNA damage. Furthermore, *in vitro* studies show that RecF and RecO or RecR and RecO are capable of forming a heterodimer and that RecF can interact with RecR in the presence of RecO to form a heterotrimer that plays role in regulating the loading and stability of RecA filaments on ssDNA (19, 44, 47, 48).

Although a large body of work has focused on the properties of these proteins during recombinational processes, RecF and RecR have also been shown to be necessary for protecting nascent DNA at arrested replication forks from being degraded by RecJ exonuclease and RecQ helicase (9, 10). In the absence of RecF or RecR, replication fails to initiate after DNA damage and nascent DNA at arrested replication forks is extensively degraded by RecJ/RecQ (9-12). These findings indicate that nascent DNA at arrested replication forks is vulnerable to the nucleolytic activity of RecJ/RecQ. The presence of RecF and RecR proteins will ensure the maintenance of the nascent DNA substrate and the recovery of DNA replication after DNA damage. However, despite the likely involvement of RecO in this process, the role that RecO plays in this process remains unclear. Therefore, in the next chapter, we describe experiments designed to ask if RecO is essential, similar to RecF and

RecR, in maintaining nascent DNA at arrested replication forks and ensuring the recovery of DNA replication after DNA damage, and to determine if RecO works together with RecF and RecR at a common point in the recovery process or if it acts at a different point during the recovery process.

A second aspect of this thesis examines the role that DNA degradation plays in the recovery process. The prominent role that RecJ and RecQ play in the processing of arrested replication fork prior to resumption of replication emphasizes the point that DNA degradation, as much as DNA synthesis, can play a critical role in maintaining genome stability. Whereas RecJ and RecQ were found to specifically degrade the nascent lagging strand of DNA at arrested replication forks when the replication proteins RecF or RecR proteins is missing (9, 10, 32), a much more dramatic phenotype can be observed in *recA* mutants. It has been long established that *recA* mutants are hypersensitive to UV irradiation, deficient in recombination activity, and fail to recover DNA replication following exposure to UV irradiation (8, 10, 23, 49). In addition, UV-irradiated *recA* mutants undergo extensive genomic DNA degradation in a process that is termed "recless" degradation (22, 41). Although little information exists as to where or how this massive degradation of the genome initiates, the majority of the degradation in *recA* mutants is known to be catalyzed by the action of Exonuclease V encoded by the RecBCD enzyme (52, 53). The RecBCD enzyme is required to recover viable recombinants during transduction or conjugation in *E.coli* (29). This prominent role has led to the common viewpoint that the RecBCD pathway of recombination is the prominent recombination pathway in *E.coli.* RecB, RecC, and RecD form a trimeric complex (45) that displays ATP-dependent DNA

helicase and nuclease (ssDNA exonuclease, ssDNA endonuclease, dsDNA exonuclease) activities. The RecB and RecD subunits are both DNA-dependent ATPases and control the hydrolysis of 3'-ending strand and 5'-ending strand respectively (6, 7, 34). The C-terminal domain of the RecB subunit has been shown to contain the catalytic site of the nuclease (50, 54, 55). The RecBCD helicase has a preference on double-strand DNA blunt end (46) and initiates exonucleolytic degradation in a 3' to 5' direction, but this activity is inhibited upon encountering a Chi sequence (5'-GCTGGTGG-3') in the appropriate orientation. In this way, the appropriately oriented Chi sequence can attenuate the 3' to 5' exonuclease activity and activate a weaker 5' to 3' exonuclease activity without altering the helicase activity. This results in a 3' single-strand overhang DNA substrate that is coordinately loaded with RecA and utilized in recombinational and strand pairing reactions (1, 16, 17, 29). This Chi-mediated modulation of RecBCD nuclease activity is thought to be a critical step in initiating the recombinational repair of double-strand DNA breaks (29, 30).

The characterization of nascent DNA degradation at arrested replication forks provided significant insights as to the mechanism and processes acting to maintain genomic stability in the presence of DNA damage (9-11, 14). Similarly, a characterization of when and how the dramatic genome degradation occurs may provide valuable insights as to how *recA*-mediated functions are involved in maintaining the genome after DNA damage. Therefore, in the third chapter, we focus on the question of where and how the DNA degradation initiates in DNA-damaged cells when the major recombination protein RecA is absent. Specifically, we examine

several potential substrates for RecBCD loading to determine if these substrates trigger the initiation of genomic DNA degradation in UV-irradiated *recA* mutants. Although there are many different possibilities for how RecBCD may potentially gain entry to the genome in UV-irradiated *recA* cells and initiate its degradative activity, we choose to test four different enzymatic processes that might generate the appropriate DNA substrates or entry sites for RecBCD based upon previous characterizations of these proteins in response to DNA damage will be tested.

Previous studies have shown that the absence of RecF or RecR leads to the processing of the nascent DNA lagging strand at arrested replication forks by the RecJ (5' to 3' exonuclease) and RecQ (3' to 5' helicase) in UV-irradiated *E.coli.* In addition, *recF* and *recR* mutants fail to recover DNA replication after DNA damage (9-12). Hence, RecF and RecR proteins are thought to limit nascent DNA degradation by RecJ/RecQ, a function speculated to facilitate the accurate recovery of DNA replication following arrest by DNA damage. The protective effect of RecF and RecR are thought to result from their ability to promote the loading of RecA filaments onto the single-strand regions generated at arrested replication forks (37, 47, 48). Thus, it is possible that the extensive degradation of the genome may initiate at the arrested replication fork itself, and may require the action the RecJ and RecQ to generate the appropriate DNA substrates for RecBCD to initiate the devastating degradation of the genome.

Alternatively, the degradation of the nascent leading strand could also potentially occur under conditions when RecA is absent which may then generate the appropriate DNA substrates for RecBCD to initiate its degradation of the genome. A

potential candidate for degradation under these conditions would be Exonuclease I (XonA), which exhibits a 3' to 5' single-strand exonuclease activity. Although the function of Exo I in vivo remains poorly defined, several phenotypes suggest that its role may be coordinated with the protective effects of RecA. Early studies showed that Exo I copurifies with RecA, suggesting that Exo I and RecA may associate *in vivo* (2, 3, 26). In addition, specific mutations that inactivate Exo I are known to suppress the impaired recombination and survival of *recBC* mutants, possibly suggesting a functional interaction (27, 28).

Other potential mechanisms for generating a double strand DNA end for RecBCD-mediated genome degradation may exist in the excision of DNA lesions near the branch point of the arrested replication fork or branch migration of the arrested replication structure to either double-strand ends on the regressed replication forks. These possibilities will also be examined experimentally.

The overall goal of this project is to increase our understanding of the molecular mechanisms that occur in DNA-damaged *E.coli* to maintain the structural stability of the genome and the DNA at arrested replication forks, which are important in allowing cells to survive stress induced by DNA damage. Several experimental assays described in the following chapter will be employed to characterize processing events that occur in response to DNA damage. Chapter II describes our investigation of RecO function in maintaining replication forks arrested by UV-induced DNA damage and allowing replication to recover following DNA damage. In chapter III, we describe our characterization of the DNA degradation that occurs in UV-irradiated *recA* mutants and our investigation of potential candidate

genes that may generate substrates for RecBCD to initiate the degradation of the genome.

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CHAPTER II

RecO ACTS WITH RecF AND RecR TO PROTECT AND MAINTAIN REPLICATION FORKS BLOCKED BY UV-INDUCED DNA DAMAGE IN ESCHERICHIA COLI

ABSTRACT

In *Escherichia coli*, *recF* and *recR* are required to stabilize and maintain replication forks arrested by UV-induced DNA damage. In the absence of RecF, replication fails to recover and the nascent lagging strand of the arrested replication fork is extensively degraded by the RecQ helicase and RecJ nuclease. *recO* mutants are epistatic with *recF* and *recR* with respect to recombination and survival assays following DNA damage. In this study, we show that RecO functions with RecF and RecR to protect the nascent lagging strand of arrested replication forks following UV-irradiation. In the absence of RecO, the nascent DNA at arrested replication forks is extensively degraded and replication fails to recover. The extent of nascent DNA degradation is equivalent in single, double, or triple mutants of *recF, recO,* or *recR* and the degradation is dependent on RecJ and RecQ functions. Since RecF has been shown to protect the nascent lagging strand from degradation, these observations indicate that RecR and RecO function with RecF to protect the same nascent strand of the arrested replication fork and are likely to act at a common point during the

recovery process. We discuss these results in relation to the biochemical and cellular properties of RecF, RecO, and RecR and their potential role in loading RecA filaments to maintain the replication fork structure following the arrest of replication by UV-induced DNA damage.

INTRODUCTION

The failure to accurately replicate the genomic template in the presence of DNA damage is believed to be a primary cause of mutagenesis, genomic rearrangements, and lethality in all cells. Irradiation with near UV (254nm) light induces DNA lesions that block replication (31, 33). In wild type *E. coli*, replication is inhibited following a moderate dose of UV irradiation, but it efficiently recovers at a time correlating with the removal of the lesions by the nucleotide excision repair proteins (4, 30, 31).

The recovery of replication in *E. coli* requires RecA to stabilize and maintain the integrity of replication forks following arrest by DNA lesions. Mutants lacking RecA fail to recover replication following UV-irradiation and exhibit a rapid and eventually complete degradation of the chromosome (3, 14). The degradation initiates at the blocked replication forks and regresses back from these points (14). *In vitro*, RecA proteins will bind to form a filament on single strand DNA and pairs the single strand region with homologous duplex DNA (6, 16, 32), an activity which would be consistent with maintaining the DNA at blocked replication forks (17, 27).

Similar to *recA*, *recF* and *recR* mutants also fail to maintain replication forks that are blocked by DNA damage and do not recover replication (3, 4). In contrast to

recA mutants however, the DNA degradation is less extensive and is limited to approximately 50% of the nascent DNA localized at the blocked replication fork (3, 4). *In vitro*, RecF, RecO, and RecR interact with and stabilize RecA filaments bound to DNA (38), a role that would be consistent with the *in vivo* observation of limiting DNA degradation at the replication fork. Mutants lacking RecF and RecR also exhibit a delayed induction of the SOS response, consistent with the idea that these genes may help stabilize the RecA filaments which are required for SOS induction (12, 32, 34, 41).

The nascent DNA degradation that occurs in *recF* mutants has been shown to result from the combined action of RecQ, a 3'-5' helicase, and RecJ, a 5'-3' exonuclease (2, 5). Based upon the extent of nascent DNA degradation in *recF* mutants (3), the polarity of the helicase and nuclease *in vitro* (19, 37), and the preferential loss of the nascent lagging strand DNA at the fork (2), RecJ and RecQ are thought to degrade the nascent lagging strand of blocked replication forks prior to the recovery of replication as depicted in the model shown in Figure 6. In the absence of either gene product, the nascent DNA degradation does not occur irrespective of whether RecF is present to protect the lagging strand DNA (2) and the frequency of illegitimate recombination is altered (9, 35), suggesting that these enzymes may affect the frequency with which replication resumes accurately when it is disrupted.

recO is classified with *recF*, *recR*, *recJ*, and *recQ* as genes belonging to the RecF pathway. Like *recF* and *recR*, *recO* mutants exhibit a similar delay in SOS induction, hypersensitivity to UV irradiation, reduced plasmid recombination, reduced conjugational recombination in a *recBCsbcBC* background, and the

persistence of daughter-strand gaps in the nascent DNA of UV-irradiated *uvrA* mutants (13, 15, 23). Based upon these phenotypes, *recF*, *recR*, and *recO* have been suggested to form an epistatic group, RecFOR. *In vitro*, RecO physically interacts with both RecF and RecR to form either RecFO, RecRO, or RecFRO complexes, a role which is believed to be important for RecA stabilization (11, 32, 36, 38). These observations, taken together, suggest that RecO may be required with RecF and RecR to maintain arrested replication forks and promote the resumption of DNA synthesis following arrest. However to date, the structures that RecO and RecR act upon at DNA damage-blocked replication forks *in vivo* have not been examined. To identify the relationship between RecF, RecO, and RecR at arrested replication fork structures *in vivo*, we have characterized the role that RecO plays during the recovery of replication following UV-induced DNA damage to determine when and where this protein functions during the recovery process.

MATERIALS AND METHODS

Bacterial strains

SR108 is a *thyA36 deoC2* derivative of W3110 (7). HL946 (SR108 *recF332*::Tn*3*), CL544 (SR108 *recR6212*::cat*883*), and HL973 (SR108 *recF332*::Tn*3*; *recJ284*::Tn*10*) have been reported previously (2, 4, 5). CL584 (SR108 *recO1504*::Tn*5*) was made by P1 transduction of the *recO1504*::Tn*5* alleles from RDK1541 (15) into SR108. CL546 (SR108 *recF332*::Tn*3*; *recR6212*::cat*883*) and CL588 (SR108 *recF332*::Tn*3*; *recO1504*::Tn*5*) were made by P1 transduction of the *recR6212*::cat*883* and *recO1504*::Tn*5*alleles from strains TP647 (Murphy, 2000 #1)

and RDK1541 (15), into HL946. CL592 (SR108 *recR6212*::cat*883*; *recO1504*::Tn*5*) was made by P1 transduction of the *recO1504*::Tn*5* allele from RDK1541 (15) into CL544. CL628 (SR108 *recF332*::Tn*3*; *recQ6215*::cat*883*) was made by P1 transduction of the *recF332*::Tn*3* allele from HL946 (4) into CL581 (5). CL691 (SR108 *recR6212*::cat*883*; *recQ1803*::Tn*3*) was made by P1 transduction of the *recR6212*::cat*883* allele from strain TP647 (Murphy, 2000 #1) into HL944 (2). CL684 (SR108 *recR6212*::cat*883*; *recJ284*::Tn*10*) was made by P1 transduction of the *recR6212*::cat*883* allele from strain TP647 (Murphy, 2000 #1) into HL942 (2). CL666 (SR108 *recO1504*::Tn*5*; *recJ284*::Tn*10*) and CL668 (SR108 *recO1504*::Tn*5*; *recQ1803*::Tn*3*) were made by P1 transduction of the *recJ284*::Tn*10* and *recQ1803*::Tn*3* alleles from HL924 (2) and HL944 (2), respectively, into CL584. CL590 (SR108 *recF332*::Tn*3*; *recO1504*::Tn*5*; *recR6212*::cat*883*) was made by P1 transduction of the *recO1504*::Tn*5* allele from CL544 into CL546.

UV irradiation

Cultures were UV-irradiated in DGCthy medium in Petri dishes on a rotating orbital shaker using a Sylvania 15-W germicidal light bulb (254 nm; 0.9 J/m^2/s).

Density labeling of replicated DNA

A fresh overnight culture was diluted 1:100 and grown in Davis minimal media with 0.4% glucose, 0.2% casamino acids, 10 μg/ml thymine (DGCthy) medium, supplemented with [^{14}C] thymine (0.1 μCi/ml) to an OD_{600} of 0.45 (spectrophotometer) in a 37° C shaking water bath. The culture was then split in two halves and either mock UV-irradiated or UV-irradiated with 27 J/m^2, before the cells

were filtered on 0.4-μm membranes (Fisherbrand General Filtration) and resuspended in DGC medium containing 20 μg/ml 5-bromouracil in place of thymine and 0.5 μCi/ml of [^{3}H] thymine (60.5 Ci/mmol). Cultures were allowed to recover for 1 hour at 37° C in a shaking water bath. Then, 2 volumes of ice-cold NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris; pH 8.0) were added. Cells were pelleted, resuspended in 150 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA), and lysed by the addition of 150 μl K_2HPO_4/KOH (pH 12.5) and 20 μl 20% sarcosyl. The lysate was then subjected to isopycnic alkaline CsCl gradient centrifugation by combining 0.3g lysate, 3.31g 0.1 M K_2HPO_4/KOH (pH 12.5), and 2.23g CsCl (refractive index 1.4055) in a 5 ml polyallomer tube and centrifuged to equilibrium. Gradient fractions were collected on Whatman No. 17 paper, precipitated in 5% TCA, washed in 95% Ethanol, and the amount of ^{14}C and ^{3}H in each fraction was quantitated in a Wallac 1409 liquid scintillation counter.

Degradation assay

A fresh overnight culture was diluted 1:100 and grown in DGCthy media supplemented with [^{14}C] thymine (0.1 μCi/ml) to an OD_{600} of 0.4 in a 37° C shaking incubator. Cultures were then pulse-labeled with 1 μCi/ml [^{3}H] thymidine for 10 seconds (to label the nascent DNA at the replication fork) before being filtered on Fisherbrand General Filtration 0.45 μm membranes, washed with 2 – 5 ml NET buffer, resuspended in prewarmed non-radioactive DGCthy media, UV-irradiated with 27 J/m^2 and then incubated in a 37° C shaking incubator. Duplicate 200-μl aliquots of the culture (triplicate at time 0) were collected at 20-minute intervals and

lysed / precipitated in cold 5% TCA before they were filtered on Millipore glass fiber prefilters. The amount of radioactivity in each filter was determined in a scintillation counter as before.

2D gel and southern analysis

Fresh overnight cultures of cells that contain the plasmid pBR322 were grown in the presence of 100 µg/ml ampicillin. The overnight cultures were diluted 1:100 and grown without ampicillin selection in a shaking incubator at 37° C to an OD_{600} of 0.5 (~ 5 x 10^8 cells/ml) and UV-irradiated with 50 J/m^2. At the indicated time points, 0.75 ml samples were placed into 0.75 ml cold 2X NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA). Each sample was pelleted, resuspended in 150 µl of 1 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10 mM Tris, pH 8.0, 1 mM EDTA), lysed at 37°C for 20 min. At this time, proteinase K (10 µl, 10 mg/mg) and sarcosyl (10 µl, 20%) was added and incubated at 50°C for 1 hr. Samples were then extracted twice with 4 volumes of phenol/chloroform/isoamyl alcohol (25/24/1), once with 4 volumes of chloroform/isoamyl alcohol (24/1), and dialysed for 3 hours on 47 mm Whatman 0.05 µm pore disks (Whatman #VMWP04700) floating on a 250 ml beaker of TE. Samples were then digested with PvuII (New England Biolabs), extracted with chloroform/isoamyl alcohol (24/1), and equal volumes were loaded onto the gel. Restricted genomic DNA samples were run in the first dimension in 0.4%agarose, 1X TBE at 1 V/cm. Gel lanes were cut out, recast, and run in the second dimension in 1.0% agarose, 1X TBE at 6.5 V/cm. Gels were transferred to Hybond N+ nylon

membranes and probed with pBR322 that had been labeled with 32P by nick translation according to the protocol supplied by Promega using alpha [32-P]dCTP (ICN). Radioactivity was visualized and quantitated using a Storm 820 and its associated ImageQuant Software (Molecular Dynamics).

RESULTS

RecO, but not RecJ or RecQ, is required to resume replication following UV-induced DNA damage.

Both *recF* and *recR* are required for the recovery of replication following disruption by UV-induced DNA damage (5). The ability of *recO* to recover replication following UV irradiation was compared to that of *recF* and *recR* and visualized by density labeling the post irradiation DNA synthesis with 5-bromouracil. Cultures that were either UV-irradiated with 27 J/m^2 or mock-irradiated were allowed to recover for a period of one hour in media containing 5-bromouracil in place of thymine so that any DNA replicated during this period would be of a greater density than the DNA synthesized before irradiation. The denser, replicated DNA in each culture was then separated from the rest of the DNA by centrifugation in isopycnic alkaline CsCl gradients and quantitated. As shown in figure 1.1, one hour after irradiation, UV-irradiated wild-type cells had replicated nearly as much DNA as their unirradiated counterpart, demonstrating that replication had fully recovered within this time frame. However, the amount of DNA synthesized in either *recF, recR,* or *recO* mutants was inhibited to similar extents following this dose of UV irradiation. By contrast, although both *recJ* and *recQ* process the nascent DNA following UV irradiation (2)

and affect the time that replication recovers (unpublished observations), these genes are not essential for replication to resume and significant amounts of DNA synthesis are observed in the absence of these gene products. It is of interest to note that, in an otherwise wild type background, mutations in *recJ* or *recQ* do not render cells sensitive to UV irradiation (18, 25).

The lack of recovery in *recF* and *recR* mutants is associated with a failure to maintain replication forks blocked by DNA damage. This can be visualized by examining the structural intermediates that are associated with arrested replication forks on plasmids such as pBR322 using 2-dimensional (2D) agarose gel analysis (5, 8). To determine if *recO* mutants also fail to maintain the replication fork, we characterized the structural intermediates that occurred on replicating plasmid molecules of pBR322 after UV irradiation with 50 J/m^2 in growing *E. coli* cultures. Previous studies by our group have shown that this dose produces 0.5 lesions per plasmid strand and that greater than 90% of the parental cells survive the irradiation to form colonies (5). Cells containing the plasmid pBR322 were UV-irradiated and the genomic DNA was purified, digested with Pvu II which cuts the plasmid just downstream of the unidirectional origin of replication, and analyzed by 2D agarose gels at the times indicated. In this technique, nonreplicating plasmids migrate as linear 4.4-kb fragments whereas replicating fragments form Y-shaped structures and migrate more slowly due to their larger size and nonlinear shape. These replicating fragments form an arc that extends out from the linear fragment (Figure 1.2A). In wild type cells, a transient reversal of the replication fork has been shown to occur on plasmids following UV irradiation (5). The regressed fork intermediate is maintained

by the RecF, RecR, and RecA genes until a time that correlates with the removal of the lesions by nucleotide excision repair and the recovery of replication. The extrusion of the nascent DNA converts the three-arm replication fork structure into a four-arm, replication intermediate that further retards its mobility in the gel. These 4-arm structures migrate in a cone region beyond the normal replication arc (Figure 1.2A). Consistent with our previous studies, UV-irradiated *recF* and *recR* mutants did not accumulate the cone region intermediates to any significant extent (5). When we examined the replicating plasmids in UV-irradiated r*ecO* mutants, the replication fork intermediate also failed to accumulate (Figure 1.2B), indicating that RecO, like RecF and RecR, is required to maintain the replication fork following arrest by UV-induced DNA damage.

RecO is required with RecF and RecR to protect the nascent DNA at blocked replication forks from degradation by RecQ and RecJ.

The failure to recover replication in UV-irradiated *recF* or *recR* mutants is associated with the extensive loss of nascent DNA made just prior to irradiation (3). The failure of *recO* mutants to recover replication could indicate that RecO is required at a step similar to RecF and RecR and is needed to limit the nascent DNA degradation. Alternatively, RecO could be required at a subsequent step in the recovery process once the nascent DNA strands have been stabilized or protected. To differentiate between these possibilities, we examined the nascent degradation pattern in *recO* mutants. To this end, exponentially growing, [^{14}C] thymine-prelabeled cultures were pulse-labeled with [^{3}H] thymidine for 10 seconds to label the DNA at replication

forks. Then, the culture was transferred to nonradioactive medium and immediately UV-irradiated with 27 J/m^2. The ^{14}C prelabel allowed us to compare the degradation occurring in the overall genome to that in the ^{3}H-labeled DNA made at replication forks just prior to UV irradiation. As shown previously, UV-irradiated wild type cells degrade very little of their overall genomic DNA following irradiation. However, some limited degradation of the nascent DNA was detected at times prior to the recovery of replication (Figure 1.3A) (3, 4, 10). In contrast to the limited degradation in wild type cells, *recF* or *recR* mutants degraded approximately half of the nascent DNA made just prior to UV irradiation (Figure 1.3B). When the degradation pattern was examined in *recO* mutants, we found that the nascent DNA was extensively degraded, and that the extent of degradation was similar to that occurring in *recF* and *recR* mutants (Figure 1.3B). The result indicates that RecO, like RecF and RecR contributes to protecting the DNA at blocked replication forks in UV-irradiated cells.

Although extensive degradation occurs in all three *recF*, *recR*, and *recO* single mutants, it is possible that these gene products protect different strands of the blocked replication fork *in vivo*. If this is the case, then we would predict that the nascent DNA degradation would increase when more than one of these gene products is absent. To examine this possibility, we examined the nascent DNA degradation in double mutants of *recF recR*, *recF recO*, and *recR recO*, as well as the corresponding *recF recO recR* triple mutant. All double mutants exhibited nascent degradation patterns that were similar in extent to the *recF* or *recR* single mutants (Figure 1.4A). Furthermore, the nascent DNA degradation in the triple mutant was also limited to approximately half of the nascent DNA (Figure 1.4B) suggesting that RecO functions

together with RecF and RecR to protect the same strand (or structural aspect) of the blocked replication fork.

If RecO acts together with RecF and RecR to protect the nascent lagging strand of the arrested replication fork, then the observed nascent DNA degradation should be dependent on RecJ and RecQ (2). To test this idea, we examined the nascent DNA degradation in *recO* mutants that also lacked either the RecQ helicase or RecJ nuclease (Figure 1.3B). Consistent with this idea, the nascent DNA degradation was reduced to a similar extent in *recF, recR* or *recO* mutants when either RecJ or RecQ was inactivated (Figure 1.5). These observations indicate that the nascent DNA degradation in *recO*, *recF*, and *recR* mutants result from the same enzymatic degradation of the lagging strand by RecJ and RecQ.

DISCUSSION

RecF, RecO, and RecR are proposed to form an epistatic group based upon several shared biochemical and genetic characteristics. All three mutations render cells equally hypersensitive to UV irradiation, reduce the frequency of recombinant progeny in conjugation or transduction assays in *recBC* or *recD* backgrounds, and delay the induction of the SOS response following DNA damage (12, 15, 23, 41). A recent study suggested that the UV hypersensitivity of *recO* is associated with a delayed recovery of replication similar to *recF* and *recR* (26). The results presented here indicate that RecF, RecR, and RecO function together to maintain replication forks arrested by UV-induced DNA damage. In addition, all three proteins are

required together in order to limit the degradation of the nascent lagging strand by RecJ and RecQ following UV irradiation.

In vitro, all three proteins bind DNA and RecO has been shown to promote annealing between homologous DNA strands (20). RecO and RecR promote RecA protein-mediated D-loop formation at the 5' end of linear ssDNA and stabilize RecA filaments to prevent their disassembly (32, 36). Complexes of RecF and RecR bind double strand DNA and gapped DNA substrates and limit how far RecA filaments are able to extend into double strand regions (40). Furthermore, the RecFOR proteins in combination have been shown to facilitate RecA loading onto gapped DNA substrates (24, 38). These *in vitro* characterizations are consistent with the *in vivo* observations that RecF, RecO, and RecR may recognize and bind to nascent DNA at blocked replication forks and serve to stabilize the RecA filaments at these regions as proposed previously (1, 3). The data presented here and in previous work indicate that binding by these proteins limits the nascent degradation on the lagging strand by the RecQ-RecJ helicase-nuclease *in vivo* (Figure 1.5). However to date, the binding and activity of these gene products has not been examined on replication fork-like structures *in vitro*.

A role for RecF, RecO, and RecR in stabilizing RecA filaments at blocked replication forks is also consistent with several genetic observations. *recF*, *recO,* and *recR* mutants exhibit a delayed SOS induction (12, 41). Since RecA filaments bound to single strand DNA function as the inducing signal for upregulation of the SOS genes, the delay in upregulation of SOS genes may reflect the reduced ability of RecA to bind to the replication fork substrates in the absence of RecFOR.

Additionally, certain mutant alleles of RecA that increase its affinity to bind DNA are able to partially bypass the requirement for RecFOR and partially suppress the UV sensitivity of *recF*, *recR*, and *recO* mutants (21, 22, 39). To incorporate these observations, we have placed RecFOR at the nascent lagging strand junction of the block replication fork (Figure 1.6). However, the precise arrangement and stoichiometry of this complex will require further investigation and it remains possible that RecF, RecO, and RecR independently bind and recognize different portions of the replication fork structure to achieve its task limiting the nascent lagging strand degradation and stabilizing RecA filaments at arrested replication fork structures. This possibility is supported by genetic studies that show overexpression of RecR or RecO alone can partially suppress the UV sensitivity of *recF* mutants (28, 29). However, the stoichiometry and constitution of the functional complex (or complexes) on the nascent lagging strand remains an important aspect that has not yet been identified.

It is clear from this and previous studies that these genes are required for maintenance of replication forks blocked at DNA lesions until a time corresponding to when the lesions are repaired and replication can resume. In the absence of DNA damage when replication is not frequently disrupted, inactivation of RecF, RecO, or RecR does not appear to affect the growth rate or viability of growing *E.coli* cultures. However, in the event that replication arrests before the duplication of the genome has been completed, RecF, RecO, and RecR play a critical role in recognizing arrested fork structures as a proper substrate and facilitating the stabilization of the

RecA filament to protect and promote the resumption of replication, allowing the processive duplication of the chromosome to be completed.

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FIGURE LEGENDS

Figure 1.1. recO, but not recJ and recQ, fails to recover replication following UV irradiation.

[^{14}C] thymine-prelabeled cultures were split and either UV-irradiated (27 J/m^2) [right panel] or mock-irradiated [left panel], and allowed to recover for 1 hour in media containing 5-bromouracil with a trace amount of [^{3}H] thymine prior to isopycnic CsCl gradient analysis. (Square) ^{14}C -pretreatment DNA; (opened circle) ^{3}H post-treatment DNA synthesized in unirradiated cultures; (filled circle) ^{3}H replicated DNA in irradiated cultures.

Figure 1.2. recO fails to maintain arrested replication forks following arrest by UV-induced DNA damage

A) Diagram of the migration pattern of Pvu II digested pBR322 during 2D agarose gel analysis. Nonreplicating plasmids run as a linear 4.4-kb fragment, (i). Normal replicating fragments form Y-shaped structures and migrate more slowly due to their larger size and nonlinear shape, forming an arc that extends out from the linear fragment, (ii). Double Y- or X-shaped molecules migrate in the cone region, (iii). B) Blocked replication forks and cone region intermediates are not maintained in the absence of *recF*, *recR*, or *recO* after 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 at the times indicated.

Figure 1.3. Extensive nascent DNA degradation occurs in recO following UV irradiation

[^{14}C] thymine-prelabeled cultures were pulse-labeled with [^{3}H] thymidine for 10 seconds immediately before being filtered and irradiated with 27 J/m^2 in nonlabeled medium. The relative amount of radioactivity remaining in DNA is plotted over time. Degradation of the ^{14}C genomic DNA (open symbols) can be compared with the degradation of the ^{3}H labeled nascent DNA (filled symbols) synthesized at the growing fork just before irradiation. (A) (square) Parental cells; (B) (diamond) *recF*, (circle) *recR*, (triangle) *recO*.

Figure 1.4. The absence of two or more of the recF, recR and recO products does not increase the extent of nascent DNA degradation

The assay was performed as in figure 3 for (A) (diamond) *recFrecO*, (circle) *recRrecO*, (triangle) *recFrecR*, and (B) the triple mutant of *recF*, *recR*, and *recO*. (square) *recFrecRrecO* [opened symbols, ^{14}C genomic DNA; filled symbols, ^{3}H nascent DNA]

Figure 1.5. Nascent DNA degradation in recO mutants is mediated by the RecJ/RecQ, nuclease/helicase

The assay was performed as in figure 3 for (A): (diamond) *recF*, (circle) *recFrecJ*, (triangle) *recFrecQ* (B): (diamond) *recR*, (circle) *recRrecJ*, (triangle) *recRrecQ* (C): (diamond) *recO*, (circle) *recOrecJ*, (triangle) *recOrecQ* [opened symbols, ^{14}C genomic DNA; filled symbols, ^{3}H nascent DNA]

Figure 1.6. Model for RecF, RecO, and RecR function during the recovery of replication following UV-induced DNA damage

(A) Replication is blocked by DNA lesions. (B) The nascent DNA is degraded by RecQ and RecJ. (C) RecF, RecO, and RecR limit the degradation by RecJ and RecQ (D) and promote the loading and stabilization of a RecA filament to maintain the integrity of the replication fork DNA until the lesion can be repaired or bypassed. (E) and replication can resume. (F) Thereby, maintaining the processive replication of the genomic template.

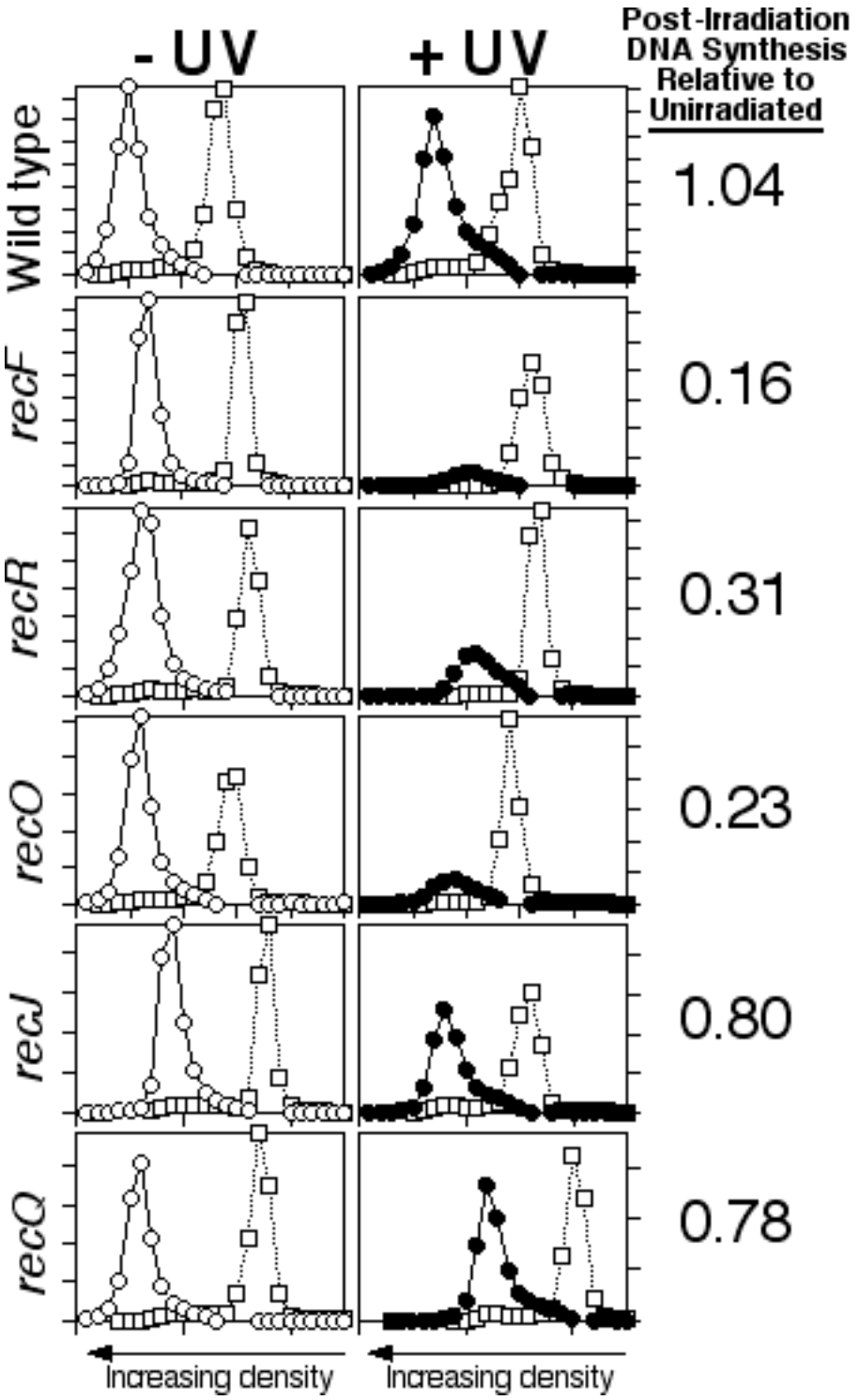

Figure 1.1 *recO*, but not *recJ* and *recQ,* fails to recover replication following UV irradiation

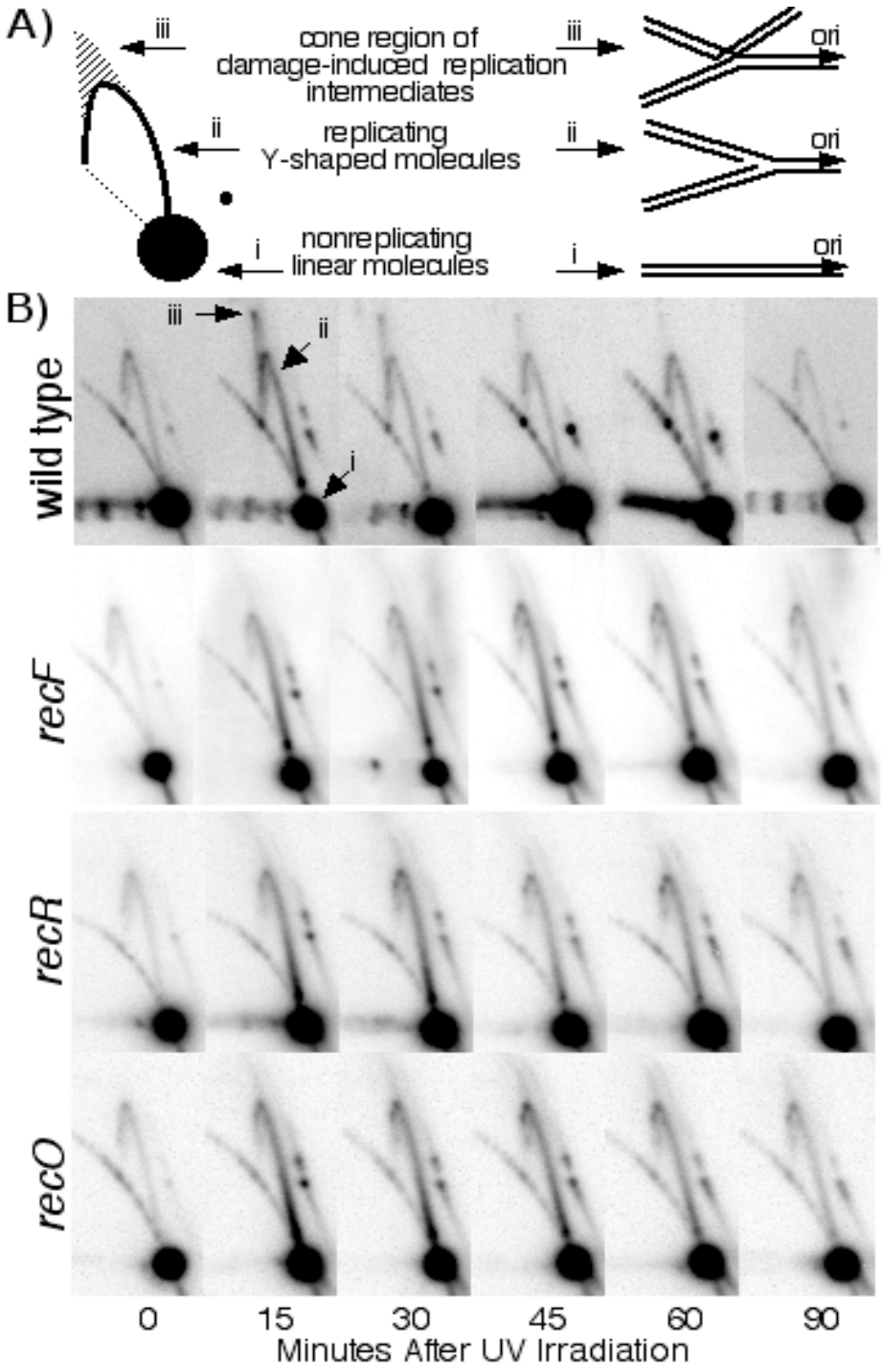

Figure 1.2 *recO* fails to maintain arrested replication forks following arrest by UV-induced DNA damage

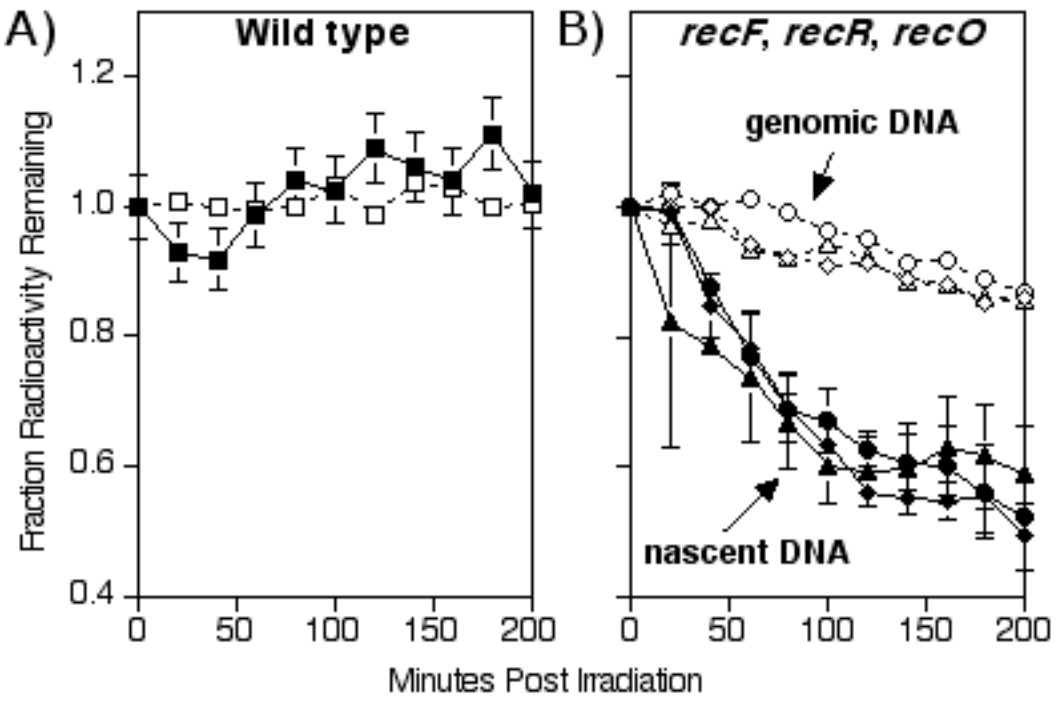

Figure 1.3 Extensive nascent DNA degradation occurs in *recO* following UV irradiation

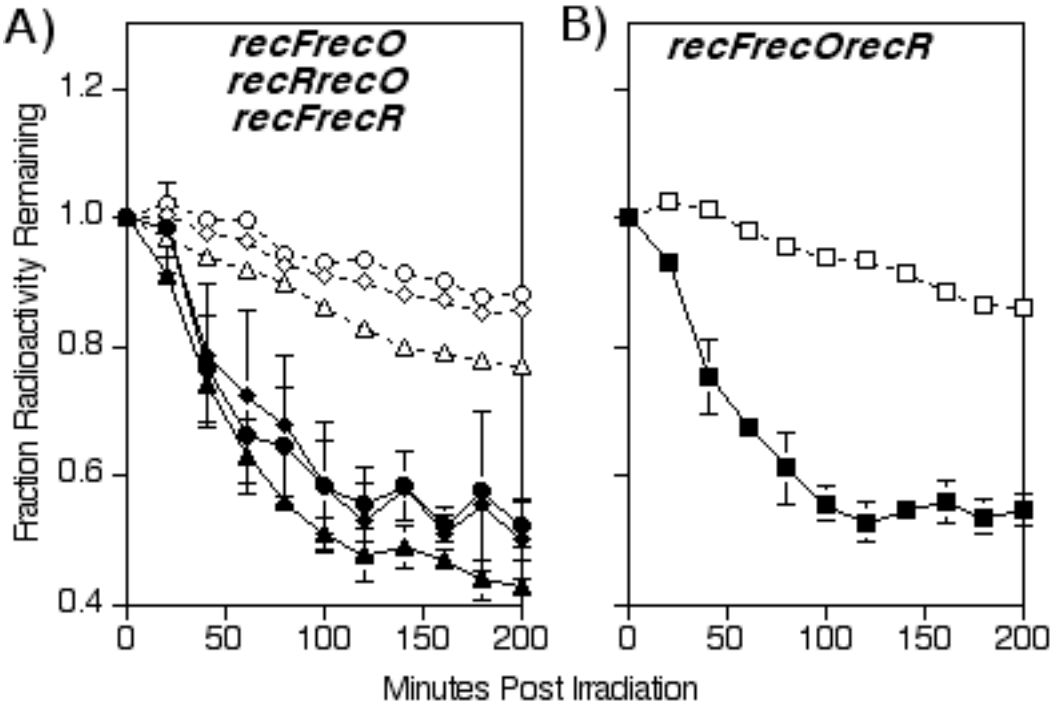

Figure 1.4 The absence of two or more of the *recF*, *recR* and *recO* products does not increase the extent of nascent DNA degradation

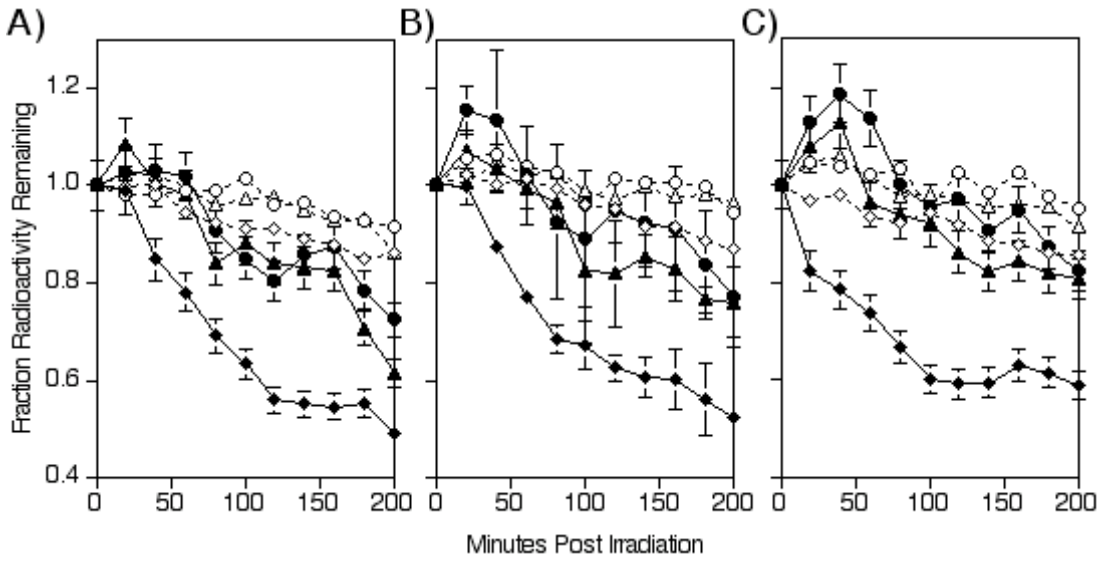

Figure 1.5 Nascent DNA degradation in recO mutants is mediated by the RecJ/RecQ, nuclease/helicase

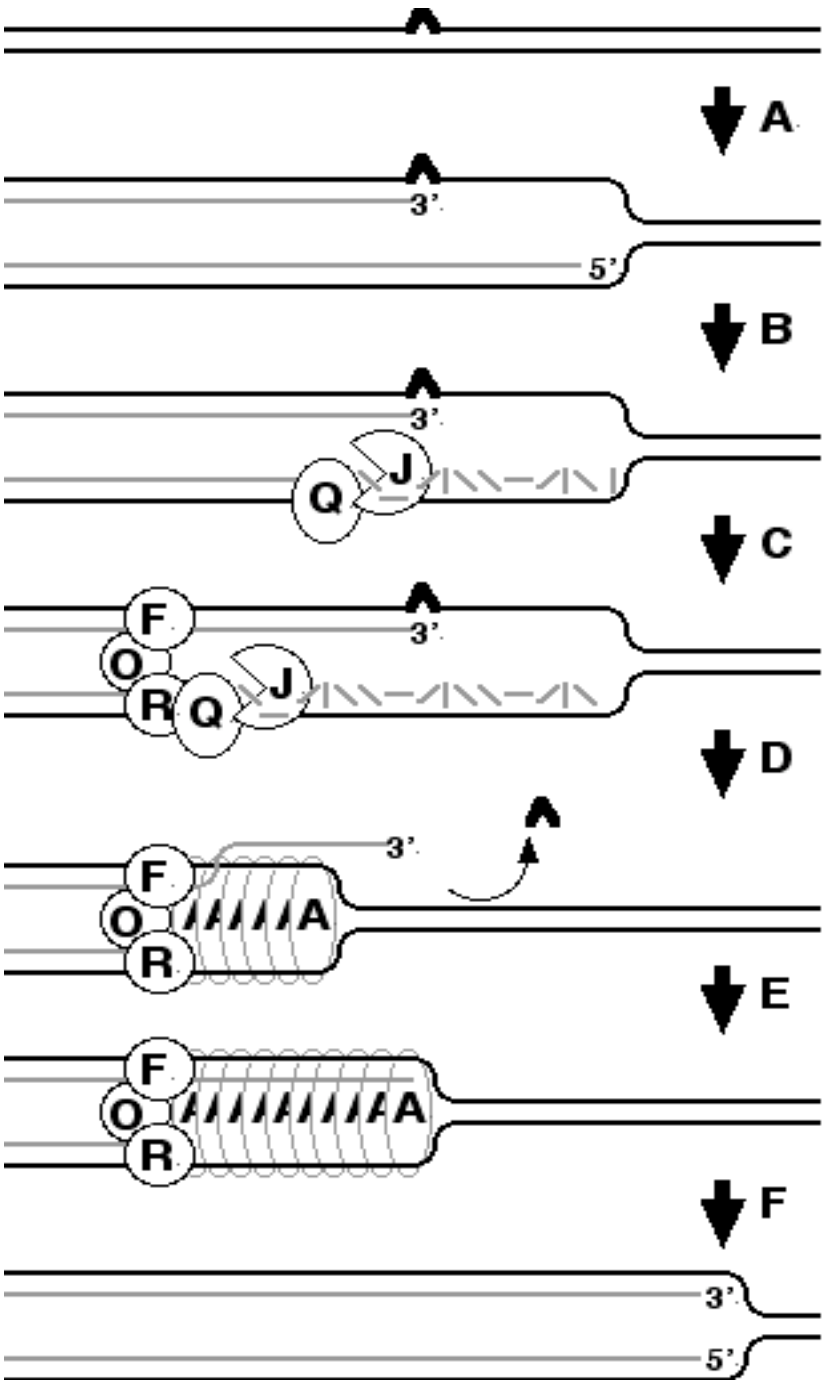

Figure 1.6 Model for RecF, RecO, and RecR function during the recovery of replication following UV-induced DNA damage

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CHAPTER III

RecBCD AND RecJ/RecQ DEGRADE DNA THROUGH SEPARATE PATHWAYS IN UV-IRRADIATED *recA* MUTANTS OF *ESCHERICHIA COLI*

ABSTRACT

Following UV irradiation, *recA* mutants fail to recover replication and a dramatic and complete degradation of the genomic DNA occurs. Although the RecBCD helicase/ nuclease complex is known to mediate this catastrophic DNA degradation, it is still not clear where or upon what substrate it initiates the degradation. Previous studies have speculated that RecBCD targets the nascent DNA at arrested replication forks to initiate degradation. To test this question, we examined which enzymes were responsible for the degradation of genomic DNA and nascent DNA in UV-irradiated *recA* cells. We show here, that although RecBCD degrades the genomic DNA following UV-irradiation, it does not target the nascent DNA at arrested replication forks. By contrast, the nascent DNA at arrested replication forks in *recA* cultures is targeted by RecJ/ RecQ, similar to that which occurs in wild type cells. These findings indicate that the genomic DNA degradation and the nascent DNA degradation at arrested replication forks in UV-irradiated *recA* mutants are mediated separately by RecBCD and RecJ/ RecQ respectively. In addition, they

suggest that RecBCD is initiating its degradation at other sites other than the arrested replication fork directly.

INTRODUCTION

In *Escherichia coli*, exposure to near-UV light (254nm) induces two dominant forms of DNA lesions – the cyclobutane pyrimidine dimer (CPD) and the pyrimidine-6-4-pyrimidone (6-4- PP), both of which block replication fork progression (7, 56, 57, 72). In wild type cells, replication is transiently inhibited following a moderate dose of UV. However, the replication fork is maintained until the DNA lesions are removed and replication efficiently recovers (10, 13-15, 18). In contrast, in UV-irradiated cells lacking RecF, RecO, or RecR, DNA replication fails to recover and the nascent DNA at arrested replication forks is extensively degraded by the RecJ/ RecQ nuclease/ helicase (10, 13, 16). Similar to *recF* and *recR* mutants, UV-irradiated *recA* mutants also fail to recover DNA replication and extensively degrade the nascent DNA at arrested replication forks (11, 14, 34). Unlike *recF* and *recR* cells however, the degradation of DNA in UV-irradiated *recA* mutants is much more extensive, and in addition to the nascent DNA, the entire genome is also rapidly degraded (32). The genomic degradation in UV-irradiated *recA* cells requires Exonuclease V (the RecBCD enzyme), which is involved in the repair of double-strand DNA breaks (DSBs) and degrades foreign linear DNA transformed into *E. coli* (40, 45, 63, 88). Early studies also showed that the genomic degradation in UV-irradiated *recA* cells required active replication, leading some to speculate that

RecBCD may initiate at regions near the replication forks and progressively recede back through the rest of the genome (32).

It is not known how RecA protects the DNA from the catastrophic degradation of the genome after UV exposure. RecA is a multifunctional protein that was originally identified as a protein required for strand exchange to occur during recombinational processes (11, 36, 64). *In vitro*, RecA monmers bind and form a helical filament around single-strand DNA (ssDNA) which can then pair it to homologous duplex DNA (40, 45, 63, 74). In addition to its essential role in mediating homologous recombination, RecA also functions during replication in the presence of DNA damage. RecA binding to ssDNA also serves as the inducing signal to upregulate the SOS response (62, 68). Following DNA damage, RecA binds to the single-strand regions generated at replication forks that encounter DNA lesions and which then indirectly derepresses and upregulates more than 40 SOS genes, including itself (17, 62, 68). The binding and homologous pairing activities of RecA also serve a structural role at the lesion-arrested replication forks by maintaining and processing the replication fork DNA in a manner that allows DNA replication to resume once the lesion has been removed (17, 62, 68). In the absence of RecA, cells are unable to induce SOS response and are extremely sensitive to DNA damage (6, 11, 25, 33, 83).

RecBCD is required for the DNA degradation to occur in UV-irradiated *recA* cells. It is a trimeric enzyme composed of RecB, RecC, and RecD subunits that form a dual ATP-dependent helicase and nuclease that is capable of unwinding and degrading duplex DNA from a double-strand end (45). The DNA degradation by RecBCD is altered upon encountering a Chi sequence, which switches the

degradation of the duplex DNA to predominantly target the 5' end, generating a 3' single-strand overhang. The 3' single-strand overhang is a target for loading by RecA and is thought to be an important step in the initiation of double-strand DNA break repair in *E. coli* (45).

Although several studies have established that RecBCD mediates the extensive DNA degradation in UV-irradiated *recA* cells (88, 89), it remains unclear how and upon what sites the RecBCD enzyme initiates from in UV-irradiated *recA* cells. In addition, it remains unclear what relationship, if any, exists between the nascent DNA degradation that occurs in UV-irradiated wild type cells and the extensive degradation that occurs in RecA mutants. Several studies have speculated that RecBCD may directly target nascent DNA substrates generated at stalled replication fork either by the regression of arrested replication fork or by the breakage of replication fork (44), although no study has been able to demonstrate this directly. In contrast, recent studies characterizing the nascent DNA processing that occurs in UV-irradiated wild type cells have shown that RecJ and RecQ but not RecBCD process the nascent DNA, suggesting that RecBCD does not work at these sites when RecA is present (10, 13, 14). Clearly, the hypersensitivity of *recBC* mutants to DNA damage, its low viability in culture, and its impaired ability to carry out recombination indicate that RecBCD processing plays a critical role in the normal cell cycle and in maintaining genomic stability. Characterizing when and where RecBCD processing occurs will likely provide critical insights into how these fundamental aspects of genome stability are maintained. Therefore in this study, we sought to investigate what roles RecBCD plays in the processing of the nascent DNA and

genomic DNA in UV-irradiated *recA* mutants and compare it with the functional activity of RecJ and RecQ at replication fork. In addition, we also examined several other candidate genes that may be involved in generating a substrate for RecBCD to initiate degradation in *recA* cells.

MATERIALS AND METHODS

Bacterial strains

SR108 is a *thyA36 deoC2* derivative of W3110 (20). CL542 (SR108 *recA*::cam) was made by P1 transduction of the *recA*::cam allele from JJC432 into SR108 (5). CL718 (SR108 Δ(*srlR-recA*)*306*::Tn*10*; *DxonA*::cat*300*) was made by P1 transduction of the *recA*::Tn*10* allele from HL921(14) into HL1034 (13). CL578 (SR108 *ruvAB6204*::kan*858*) was made by P1 transduction of the *ruvAB6204*::kan*858* allele from TP541 (59) into SR108. CL700 (SR108 *recD1011 argA81*::Tn*10*; *recQ1803*::Tn*3*) was made by P1 transduction of the *recD1011 argA81*::Tn*10* allele from HL923 (13) into HL944 (13). CL752 (SR108 *recJ284*::Tn*10*; *recD1011*) was made by P1 transduction of *recJ284*::Tn*10* allele from HL924 (13) into CL893. HL952 (SR108 *uvrA*::Tn*10*) was made by P1 transduction of the *uvrA*::Tn*10* allele (35) into SR108. HL925 (SR108 *uvrC297*::Tn*10*) was made by P1 transduction of the *uvrC297*::Tn*10* allele (35) into SR108. CL720 (SR108 *recA*::cam; *recJ284*::Tn*10*), CL724 (SR108 *recA*::cam; *recQ1803*::Tn3), CL 726 (SR108 *recA*::cam; *recD1011 argA81*::Tn*10*), CL851 (SR108 *recA*::cam; *recB21 recC22 argAB1*::Tn*10*), CL853 (SR108 *recA*::cam; *ruvAB6204*::kan*858*), CL783 (SR108 *recA*::cam; *recG*::Tn*5*),

CL854 (SR108 *recA*::cam; *uvrA*::Tn*10*), CL736 (SR108 *recA*::cam; *uvrC297*::Tn*10*), CL730 (SR108 *recA*::cam; *recD1011 argA81*::Tn10; *recQ1803*::Tn3), CL781 (SR108 SR108 *recA*::cam; *recD1011*; *recJ284*::Tn*10*) were made by P1 transduction of the *recA*::cam allele from CL542 into HL924 (48), HL944 (13), HL923 (13), HL922 (13), CL578, HL945, HL952, HL925, CL700, and CL752 respectively.

Selection of Tetracycline-sensitive alleles of recJ284 and recD1011

CL893 (SR108 *recD1011*) and CL894 (SR108 *recJ284*) were cured of their tetracycline resistance marker by the selection of tetracycline-sensitive clones of HL924 (SR108 *recJ284*::Tn*10*) and HL923 (SR108 *recD1011 argA81*::Tn10) based on a previously described method (54). Briefly, cultures were grown overnight in LB medium. Cultures were then diluted to 1000 fold in M9 minimal medium before 100 µl were plated on Tc^S plates containing 15 g/L agar, 5 g/L tryptone broth, 5 g/L yeast extract, 4 ml/L of Chlortetracycline hydrochloride (12.5 mg/ml), 10 g/L NaCl, 10 g/L $NaH_2PO_4.H_2O$, 6 ml/L fusaric acid (2 mg/ml), and 5 ml/L $ZnCl_2$ (20 mM) as described in (54). Plates were incubated for 24 to 48 h at 37° C. Colonies that appeared within this time were then screened to identify cells that acquired sensitivity to 20 µg/ml tetracycline.

UV irradiation.

Bacterial cultures were UV irradiated in DGCthy media (1x Davis, 0.4% glucose, 0.2% casamino acids, 10 µg/ml thymine) in Petri dishes on a rotating orbital shaker using a Sylvania 15-W germicidal light bulb (254 nm; 0.9 $J/m^2/s$).

Degradation assay.

A fresh overnight culture was diluted 1:100 and grown in DGCthy medium supplemented with 0.1 μCi/ml [^{14}C] thymine to an OD_{600} of 0.4 in a 37° C shaking incubator. Cultures were then pulse-labeled with 1 μCi/ml [^{3}H] thymidine for 10 sec (to label the nascent DNA at the replication fork) before cells were filtered on Fisherbrand General Filtration 0.45 μm membranes, washed with 2 – 5 ml NET buffer, resuspended in prewarmed non-radioactive DGCthy media, and UV irradiated with 27 J/m^2 before returning to a 37° C shaking incubator. At 20 min intervals, duplicate 200 μl aliquots of culture (triplicate at time 0) were lysed and the DNA was precipitated in cold 5% TCA, and then filtered on Millipore glass fiber prefilters. The amount of radioactivity in each filter was determined in a scintillation counter.

RESULTS

Degradation of the nascent DNA at stalled replication forks is not dependent on RecBCD.

Recent studies have highlighted the role that nucleolytic DNA degradation plays in the recovery of replication following UV irradiation (14-16). To examine the degradation that occurs at the arrested replication fork and compare it to that occurring in the overall genome, [^{14}C] thymine-prelabeled cultures are pulse-labeled with [^{3}H] thymidine for 10 sec to label the nascent DNA at replication fork. Then, cells are placed into non-radioactive medium, and immediately UV-irradiated at a dose of 27 J/m^2 (Figure 2.1A). The ^{14}C-labeled DNA and the ^{3}H-labeled DNA allow

us to directly compare the degradation that occurs in the overall genome and to that occurs at the nascent DNA synthesized at replication forks just prior to UV irradiation. Consistent with previous studies, we observed that following UV irradiation of wild type cells, a limited amount of nascent DNA degradation occurred at arrested replication forks at times prior to when replication resumed. Overall however, both the nascent DNA and genomic DNA remained protected and little degradation was observed (Figure 2.1B) (14, 15). In contrast, when we examined UV-irradiated *recF* mutants, the nascent DNA degradation was much more extensive. In this case, the degradation was primarily limited to the nascent DNA and the genomic DNA remained relatively protected (Figure 2.1B) (10, 14, 15). As shown previously, we observed that the nascent DNA degradation in both UV-irradiated wild type and *recF* cells was mediated by the RecJ/ RecQ nuclease/helicase (13) (Figure 2.1C). Some models have speculated that RecBCD may target the newly synthesized DNA at the arrested replication forks as part of the recovery process (44). In contrast to these model however, when we examined UV-irradiated *recFrecD* (13) or *recFrecBC* mutants, no inhibition of the nascent degradation was observed (Figure 2.1C). These findings indicate that the newly synthesized DNA at UV-arrested replication forks is not accessible to RecBCD-mediated degradation in $recA^{+}$ cells. In addition, the results do not support models that speculate that arrest leads to double-strand breaks as the result of regression of stalled replication forks or the breakage of replication forks (44).

Although RecBCD does not initiate the degradation of newly synthesized DNA in wild type cells, it is possible that an abnormal or unique substrate is

generated at arrested replication forks in the absence of RecA. To examine this possibility, we examined the DNA degradation that occurred in UV-irradiated *recA* mutants. It has been established that the failure of UV-irradiated *recA* cells to recover DNA replication correlates with a complete degradation of the genomic DNA (14, 32). Consistent with this, when we examined UV-irradiated *recA* mutants, we observed extensive degradation of the genomic DNA as well as the degradation of the nascent DNA (Figure 2.2). Previous studies have also established that the genomic degradation in UV-irradiated *recA* cells is mediated by RecBCD. These studies postulated that the degradation by RecBCD may initiate at the replication forks and then progress back to the bulk of the DNA (32, 44, 88). If true, then one would predict that the degradation of the genome by RecBCD in UV-irradiated *recA* mutants would first initiate and degrade the nascent DNA at stalled replication forks (44). Surprisingly however, although UV-irradiated *recA* cells did not degrade the genomic DNA in the absence of RecBC or RecD, we observed that the nascent DNA at arrested replication forks remained susceptible to nucleolytic digestion (Figure 2.2). Interestingly, under these conditions, the nascent DNA was partially degraded by RecJ/ RecQ, similar to that occurring in UV-irradiated wild type cells (Figure 2.2). These results imply that the genomic degradation and the nascent DNA degradation in UV-irradiated *recA* cells are mediated through separate enzymatic pathways and that RecBCD does not target the nascent DNA for degradation directly.

RecBCD degradation of the genome does not require RecJ or RecQ processing to initiate.

Although the above results indicate that the RecBCD enzyme does not target the nascent DNA at arrested replication forks directly, it remains possible that processing of nascent DNA at arrested replication forks by the RecJ/ RecQ enzymes is required to generate the appropriate substrate for the RecBCD degradation to initiate. If true, then one would predict that inactivation of the RecJ/ RecQ enzymes should inhibit or delay the degradation of the genome by RecBCD. However when we examined UV-irradiated *recA* mutants that also lacked either RecJ or RecQ, the kinetics of the genomic degradation was similar to that which occurred in *recA* mutants (Figure 2.3). Interestingly we also observed that the nascent DNA degraded in UV-irradiated *recArecJ* and *recArecQ* cells concurrently with the complete degradation of genome (Figure 2.3). These results support the idea that RecBCD may initiate at sites other than or independent from the arrested replication fork. If RecBCD initiated on a substrate associated with the nascent DNA degradation, we would expect to observe that the nascent DNA would almost completely degraded before any significant degradation of the genomic DNA occurred. Instead, both the nascent and the genomic DNA degraded with similar kinetics, suggesting that RecBCD may initiate at a separate substrate, independent of the replication fork itself.

Exo I, nucleotide excision repair, and branch migration by RecG or RuvAB are not required to generate the DNA substrate for RecBCD degradation.

In the absence of RecA binding to single-strand DNA regions at arrested replication forks (i.e. *recA* mutants), the nascent DNA ends might be more vulnerable to nucleolytic activities that would otherwise not have access to these ends. This in turn, may generate a DNA substrate that serves as an entry point for RecBCD. One candidate nuclease that is known to target 3' ends that would otherwise be protected by RecA is Exo I. However, when we examined cells lacking Exo I, we observed that both the nascent DNA at replication forks and the genome degraded in UV-irradiated *recA* mutants (Figure 2.4).

Other possible candidate enzymatic activities that we felt might generate an appropriate DNA substrate for RecBCD degradation included those that promote branch migration, such as RuvAB or RecG. In support of this idea, it has been observed that in some thermosensitive replication mutants, elevated levels of RuvAB dependent double-strand breaks are observed to arise on the chromosome at the restrictive temperature (70). However, we observed that the inactivation of RuvAB or RecG in UV-irradiated *recA* cells did not prevent the degradation of the genome from occurring (Figure 2.4).

We also examined the possibility that the incision of UV-induced lesions near the branch point of arrested replication forks by nucleotide excision repair proteins may generate a DSB substrate that allows RecBCD to initiate degradation. Again however, in *uvrA* or *uvrC* mutants, which are unable to incise UV-induced lesions in the genome (23), no inhibition of the degradation in *recA* mutants was observed

(Figure 2.4). These results indicate that the absence of the enzymatic excision of DNA lesions near the branch point at arrested replication forks or branch migration of joint molecules does not prevent the generation of the appropriate DNA substrate for RecBCD degradation.

DISCUSSION

DNA ends in *E. coli* are unstable and vulnerable to nucleolytic digestion if they are not repaired or protected. In *E. coli*, double-strand breaks may be generated directly by exposure to ionizing radiation or oxidizing agents, or indirectly by replicating through nicks in the DNA template, or by the subsequent processing of persistent single-strand DNA gaps that may not be subject to repair (39). Biochemically, RecBCD is thought to require a double-strand end to initiate its helicase and exonucleolytic acticities. It remains unclear how or where this substrate is generated *in vivo* following UV irradiation.

In this study, we sought to determine how the double-strand DNA ends are generated in RecA deficient cells. Although *recA* mutants do not survive the UV irradiation, identifying when and where RecBCD initiates degradation in UV-irradiated cells will provide valuable information as to its role and function in wild type cells. While this enzyme has been speculated to target nascent DNA at arrested replication forks (44), we show here that RecBCD does not have access to the nascent DNA at arrested replication forks *in vivo*. Instead, we observed that RecJ and RecQ were able to target the nascent DNA. The RecBCD degradation of the genomic DNA occurred independently from the degradation of the nascent DNA, suggesting that

RecBCD is initiating upon an alternative substrate generated following UV-induced DNA damage. We cannot entirely rule out the possibility that unrepaired DNA ends or DNA ends that are not normally produced in wild type cells are subject to non-specific nuclease activities in the absence of RecA. Hence the failure to inhibit nascent DNA degradation in our UV-irradiated *recArecD* and *recArecBC* double mutants could be the result of non-specific nucleolytic degradation of nascent DNA. Consistent with this latter interpretation, although the inactivation of both RecBCD and RecJ or RecQ partially reduced the degradation of the nascent DNA in *recA* cells, approximately 40% of nascent DNA remained susceptible to degradation by other, as yet, unidentified nucleases. Although we were not able to conclusively determine how the RecBCD-mediated degradation is initiated, we examined several possible enzymatic activities that might generate an entry site for RecBCD, including the processing of nascent DNA at arrested replication forks by RecJ/ RecQ, XonA (Exo I), DNA strand breaks catalyzed by NER, and branch migration catalyzed by RecG or RuvAB. In all cases, we found that these activities did not affect then degradation by RecBCD. Although we do not understand where RecBCD initiates, our data strongly suggests that it does not target the nascent DNA at the replication fork directly and that it is likely to initiate independently at other sites generated as a result of replication in the presence DNA damage (Figure 2.5). Therefore, it will be important in future studies to consider alternative sites or possibly even alternative substrates for RecBCD as we try to identify the mechanism by which this multifunctional enzyme promotes survival in the presence of DNA damage.

FIGURE LEGENDS

Figure 2.1 Genomic and nascent DNA degradation in RecA$^+$ cells

A) The procedure used for monitoring the degradation occurring at the nascent DNA and in the overall genome. Cultures prelabeled with [^{14}C] thymine were grown to mid-log phase and pulse-labeled with [^{3}H] thymidine before being resuspended in non-radioactive medium and UV irradiated with 27J/m^2. Aliquots of culture were taken at various time points post-UV and the fraction of radioactivity remaining in DNA is plotted over time. B) The ^{14}C-labeled genomic DNA (opened symbols) and the ^{3}H-labeled nascent DNA (filled symbols) was monitored as described in (A) for parental cells (squares), and *recF* cells (circles). C) The genomic and nascent DNA was monitored as described in (A) for *recFrecJ* (triangles), *recFrecQ* (inverted triangles), *recFrecD* (squares), and *recFrecBC* (diamonds).

Figure 2.2. The nascent DNA at arrested replication fork is not targeted by RecBCD

The assay was performed as in Figure 1 for *recA* (squares), *recArecBC* (triangles), *recArecD* (inverted triangles), *recArecDrecJ* (diamonds), and *recArecDrecQ* (squares). ^{14}C-labeled genomic DNA (opened symbols); ^{3}H-labeled nascent DNA (filled symbols).

Figure 2.3. Processing by RecJ and RecQ is not required for RecBCD to degrade the genome

The assay was performed as in Figure 1 for *recArecJ* (triangles) and *recArecQ* (inverted triangles). ^{14}C-labeled genomic DNA (opened symbols); ^{3}H-labeled nascent DNA (filled symbols).

Figure 2.4. Inactivation of Exo I, NER, or branch migration does not prevent the degradation of the genome in UV-irradiated recA cells

The assay was performed as in Figure 1 for *recAxonA* (circles), *recAuvrA* (triangles), *recAuvrC* (inverted triangles), *recArecG* (squares), and *recAruvAB* (diamonds). ^{14}C-labeled genomic DNA (opened symbols); ^{3}H-labeled nascent DNA (filled symbols).

Figure 2.5. Hypothetical substrates that were found not to be targeted by RecBCD

A) i) DNA lesions encountered by replication fork may form a DSB if NER were to incise a lesion at the branch point of the replication fork. ii) Alternatively, a double strand end could be generated by the regression of replication fork if the nascent strands were allowed to re-anneal. iii) It is also possible that the prior degradation of lagging nascent DNA strand by RecJ and RecQ generates a substrate for RecBCD entry. These possibilities were found not to generate substrates for RecBCD. B) The presence of nicks or gaps generated by replication on damaged templates could be processed by the exonuclease XonA to generate DSB. This possibility was also found not to occur or generate a substrate for RecBCD. iii) DSB could also be produced as the result of strand exchange catalyzed by RecG or RuvAB on DNA templates that

contain nicks or gaps. Substrates generated by these enzymes were also found not to be targeted by RecBCD.

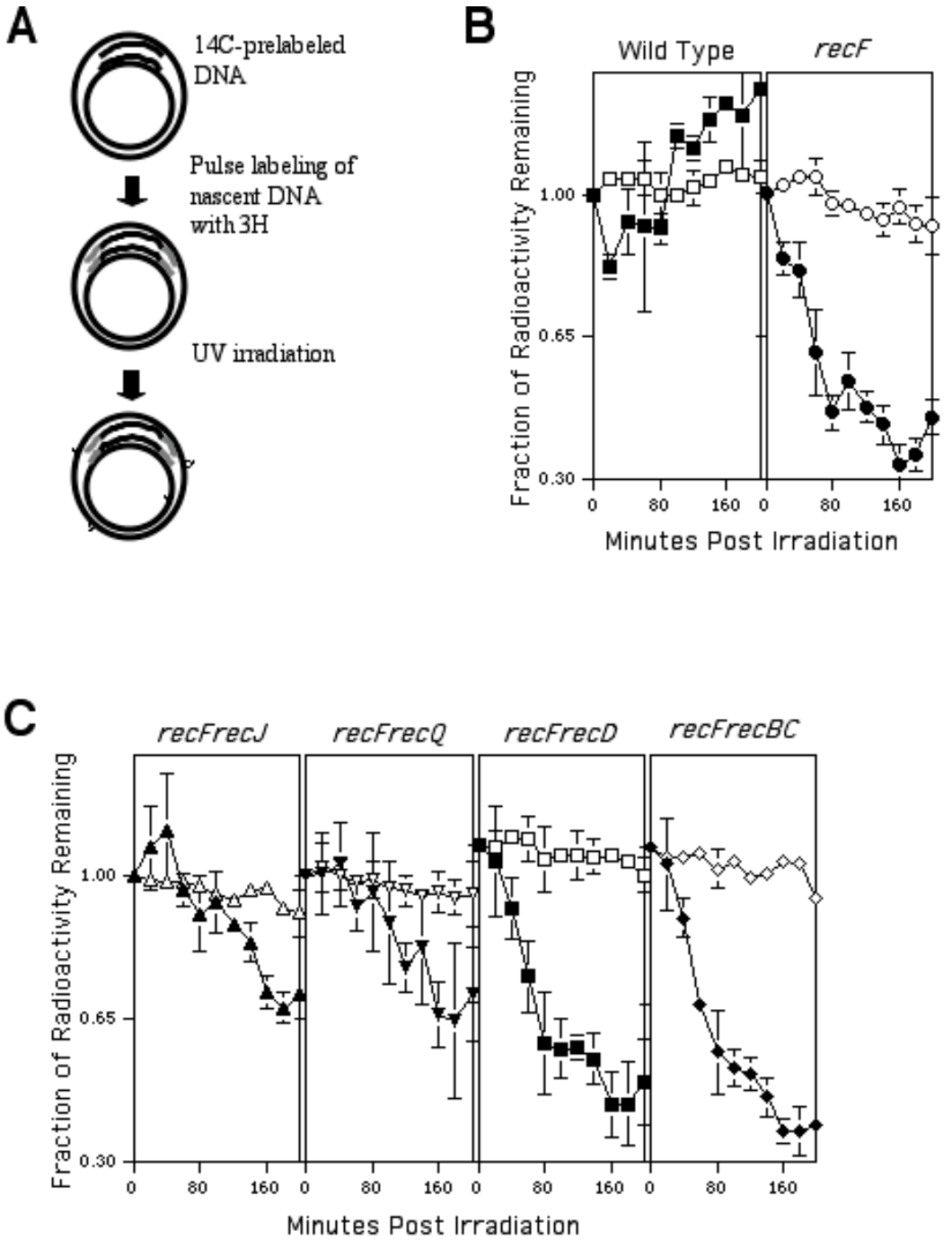

Figure 2.1 Genomic and nascent DNA degradation in RecA^{+} cells

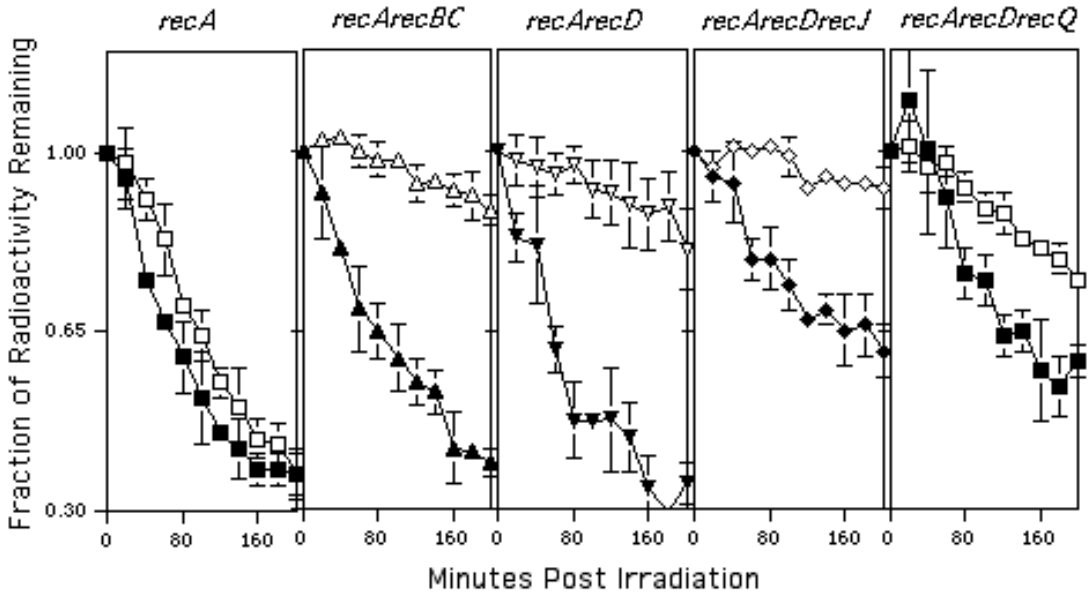

Figure 2.2 The nascent DNA at arrested replication fork is not targeted by RecBCD

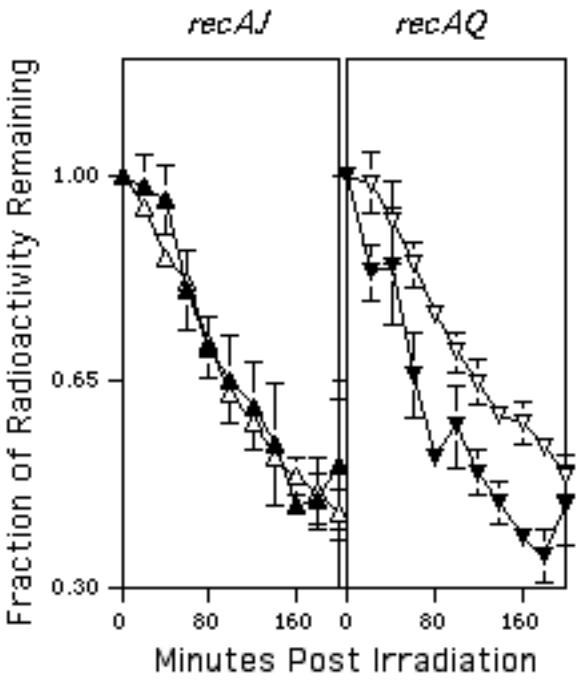

Figure 2.3 Processing by RecJ and RecQ is not required for RecBCD to degrade the genome

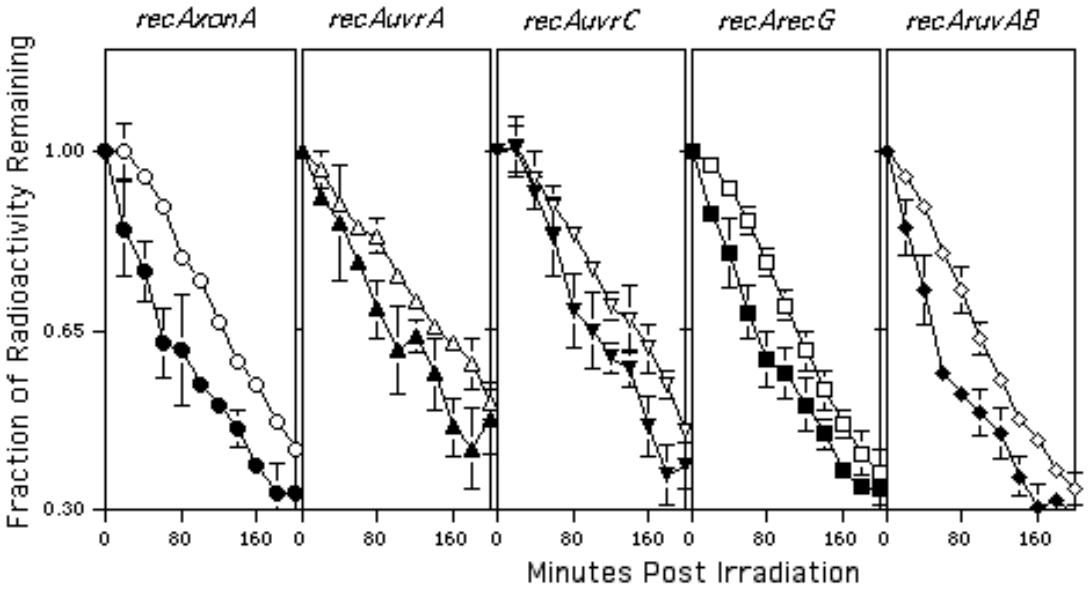

Figure 2.4 Inactivation of Exo I, NER, or branch migration does not prevent the degradation of the genome in UV-irradiated *recA* cells

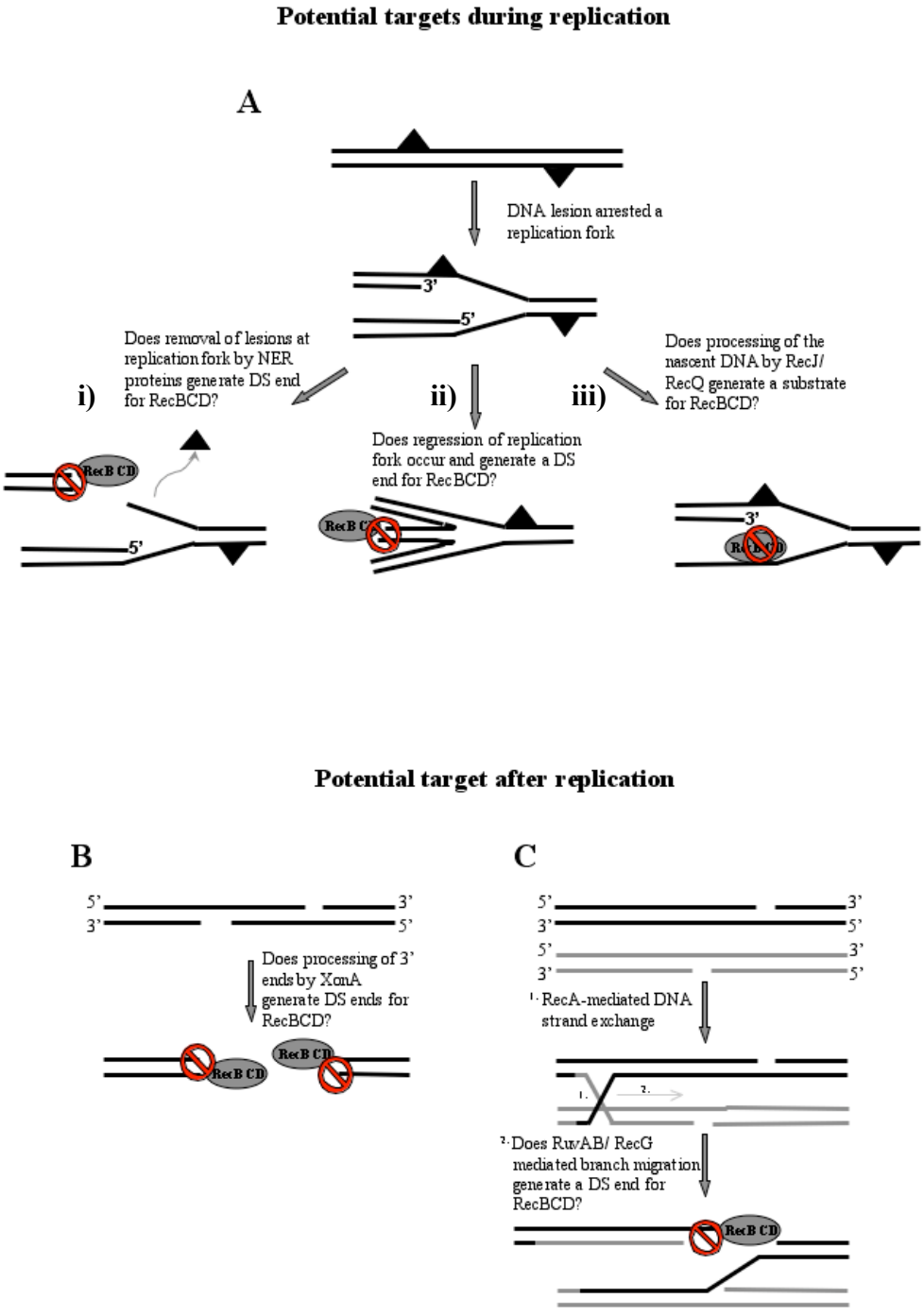

Figure 2.5 Hypothetical substrates that were found not to be targeted by RecBCD

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