MOLECULAR PROCESSING OF REPLICATION INTERMEDIATES IN *ESCHERICHIA COLI* AFTER DNA DAMAGE

By

Jerilyn Jalana Belle

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Approved:

_____	_____
Karen Coats Professor of Biological Sciences (Director of Dissertation)	Dwayne Wise Professor and Graduate Coordinator of Biological Sciences (Committee Member)
_____	_____
Nara Gavini Professor of Biological Sciences (Committee Member)	Donald Downer Professor of Biological Sciences (Committee Member)
_____	_____
John Boyle Professor of Biochemistry (Committee Member)	Philip Oldham Dean of the College of Arts and Sciences

Name: Jerilyn Jalana Belle

Date of Degree: May 5, 2007

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Dr. Karen Coats

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Accurate replication of the genome is essential for reproduction in all cells. However, even under normal conditions, the replication machinery may face a variety of impediments that can prevent it from completing its task. The mechanism by which cells overcome these hurdles is likely to vary depending upon the nature of the obstacle. Both UV irradiation and inactivation of replicative proteins in DnaB can inhibit the progression of the DNA replication machinery. However, the mechanism by which replication recovers following UV irradiation is different from the mechanism of recovery following the inactivation of the replicative proteins. Previous results show that following UV-induced damage in *Escherichia coli*, the replication fork is maintained and protected from extensive degradation by RecF, RecO, and RecR until replication can resume. By contrast, replication does not recover following inactivation of the replication protein DnaB, and the nascent DNA is extensively degraded irrespective of whether RecF is present.

In this study, we verified DNA replication arrest by monitoring the total DNA accumulation and rate of DNA synthesis following UV-induced DNA damage and inactivation of thermosensitive replication alleles, such as *dnaB266*. We measured the amount of nascent DNA degradation, allowing us to determine how the newly synthesized strand of DNA is affected following replication fork arrest. Our data indicate that following inactivation of DnaB266, the replication fork is not maintained and is subject to extensive degradation. The degradation that occurs after DnaB266 inactivation is partially reduced in the absence of RecF-O-R, RecJ, and ExoI, suggesting that DNA processing by these enzymes occurs after DnaB arrest. In addition, two-dimensional agarose gel analysis revealed that unique structural intermediates accumulated following inactivation of DnaB266. These observations indicate that the recovery of replication when impeded by DNA lesions, such as those produced by UV-irradiation, is maintained and processed through mechanisms that do not resemble the events occurring when replication proteins are inactivated.

DEDICATION

This dissertation is dedicated to my family. To my parents, Cecil and Angie Belle, who have always motivated and encouraged me to set goals and strive to complete them. To my brother, Jeremy, who has always been my biggest fan. To my nephew, Cameron, and nieces, Cinsley and Cerigan, who are the reasons for me not giving up when times got hard. Hopefully, I will continue to be your role model. To my fiancé, Jamire, who always gives me that extra push when the road to my goals becomes cloudy. I love you!

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Lastly, but definitely not least, I thank my dearest friends and fellow graduate students, Kelvin Harris and Josiah Wilcots, for their endless support and encouragement. They have made me feel like their role model; and no matter where our roads lead us, I will always remember them.

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CHAPTER I

INTRODUCTION

The genetic material is responsible for inheritance; therefore, to ensure normal function, it must be accurately duplicated and separated into two daughter cells during the process of DNA replication. However, even under normal conditions, replication forks encounter a variety of biologically important impediments that might hinder their progression through the genome, including DNA damage, strand breaks in the template (Michel et al., 1997), unusual secondary structures in the DNA sequence (Sinden et al., 1999), DNA bound proteins (Kaplan & Donnel, 2002), or potential instability of the replication machinery itself (Lovett, 2003). Each of these impediments imposes structurally diverse constraints on the DNA and can block, arrest, or stall replication. Therefore, it is likely that the replication fork will be processed and recover through different mechanisms depending on the nature by which replication was disrupted.

In order to understand how the DNA damage is processed and removed, it is important to understand that the chromosome is duplicated by the coordinated replication of both the leading-and lagging-strand templates (reviewed in Marians, 1992). Since DNA polymerization on both strands occurs only in a 5'- 3' direction, the coordinated and simultaneous replication of both templates requires that unique enzymatic dynamics occur on each strand. Following a single priming event, the leading-strand template can be synthesized in a continuous, processive 5'- 3' manner. However, the lagging strand

template is synthesized in a direction opposite to the progress of the ongoing fork, and requires a primase activity that must constantly reprime the lagging strand template, resulting in discontinuous synthesis on the template (Okazaki fragments) (review in Talaro, 2005). These alternative mechanisms of synthesis on each template strand present different problems for the replication machinery when it encounters a DNA lesion. Higuchi and colleagues (2003), using a reconstituted system, examined how the replication holoenzyme behaves when it encounters a blocking lesion, an AP-site, in either the leading-or lagging-strand template of a plasmid (Higuchi et al., 2003). They observed that when the DNA lesion was found on the leading strand, the entire replication machinery was arrested. The substrate that resulted was a forked DNA structure that arrested with the nascent leading strand at the site of the lesion and the nascent lagging strand at, or slightly beyond, the lesion location (Fig. 1.1). Interestingly, when the lesion was placed in the lagging-strand, no disruption of replication was observed, although the polymerase was blocked and failed to complete the Okazaki fragment in which the lesion was found. This resulted in the production of one intact daughter molecule and one gapped molecule containing the arresting DNA lesion. Similar products were observed when plasmids containing a site-specific lesion in either the leading-or the lagging-strand template were transformed into repair-deficient cells (Pages & Fuchs, 2003). These observations correlate well with our understanding of the mechanics of how leading and lagging strands are coordinately synthesized. Blockage of the leading-strand polymerase might be expected to arrest replication

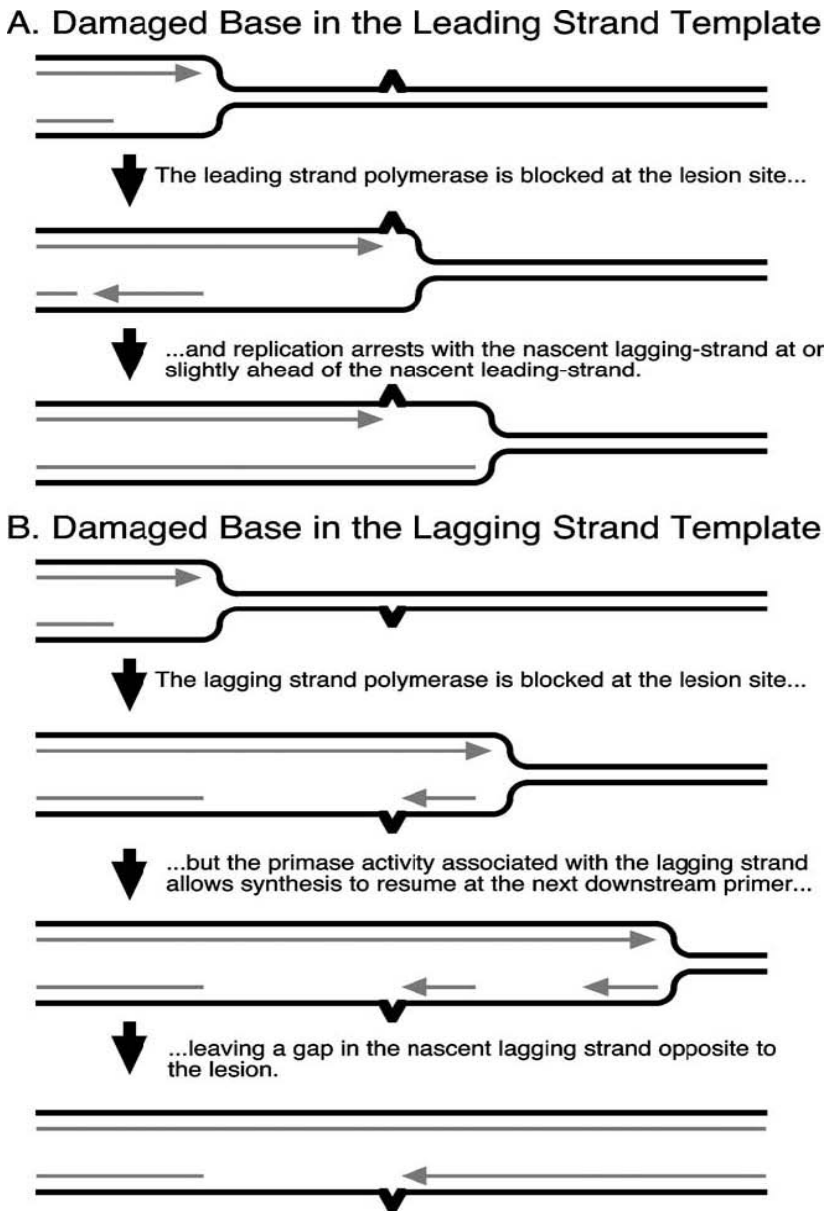

Figure 1.1. Substrates generated when replication encounters a blocking DNA lesion in (A) the leading-strand template and (B) the lagging-strand template.

due to the lack of any mechanism to prime and resume replication downstream of the lesion. By contrast, the primase activity associated with the lagging-strand polymerase allows replication to constantly reinitiate synthesis as new primers arise on the lagging-strand template, suggesting that when the lagging-strand polymerase is blocked at the DNA lesion, it may be able to simply reinitiate downstream when the next primer is synthesized, leaving the observed gap in the nascent lagging strand (Higuchi et al., 2003).

In vivo, it has long been observed that replication is transiently inhibited following DNA damage such as that induced by UV irradiation (Setlow et al., 1963). In addition, it was later shown that although replication is severely reduced, the limited DNA synthesis that does occur during this period of inhibition is in the form of short gapped fragments (Rupp & Howard-Flanders, 1968). Following a moderate dose of UV irradiation, DNA lesions would be randomly distributed between the leading and lagging strands. Thus, half of the replication forks would encounter lesions in the lagging strand template first, generating some gapped DNA substrates before all the replication forks are arrested at lesions in the leading-strand template. An important prediction from these observations, which remains to be tested in vivo, is that the gapped nascent DNA strands produced immediately after UV irradiation should be specific to the nascent lagging strand. The differences observed in vitro for leading versus lagging strand lesions implies that lesions are likely to require unique enzymatic processing events to repair and process the substrates produced in each situation. In addition, it also implies that lesions in either the leading or lagging strand may carry different biological consequences with respect to lethality and mutagenesis for the cell. In order to understand how genomic stability is

maintained throughout the lifespan of the organism, it is important to understand how each of these situations is processed and repaired.

Of these various impediments that block DNA replication, the best understood thus far is that of replication disruption following UV-induced DNA damage. In this case, lesion removal is very important for the recovery and survival of the cell. In *Escherichia coli*, replication is transiently inhibited until the UV lesions are repaired by the nucleotide excision repair proteins (Courcelle et al., 2002; Courcelle et al., 1999). In addition to nucleotide excision repair, several *recF* pathway genes, RecA, RecF, RecO, RecR, RecJ, and RecQ, have been shown to protect and maintain the DNA at the replication fork until the lesion can be removed (Courcelle et al., 1999; Courcelle et al., 1997; Horii & Clark, 1973). In vitro studies of RecA, RecF, RecO, and RecR show that these proteins are able to stabilize replication fork intermediates by promoting the pairing of single stranded (ss) DNA and homologous duplex DNA (reviewed in Shibata, 1980). In vivo, the nascent DNA at blocked replication forks is partially degraded by RecQ and RecJ at times prior to the recovery of replication (Courcelle & Hanawalt, 1999). In the absence of RecA, RecF, RecO, and RecR proteins, which are required to maintain the blocked replication fork, the RecQ and RecJ helicase-nuclease degradation of the intermediates is much more extensive (Courcelle & Hanawalt, 1999). It has been previously observed that replication does not recover in the absence of the *recF* gene (Courcelle et al., 1999).

Less is known about events following replication disruption by other impediments. One alternative method that can be used to experimentally disrupt

replication involves the use of temperature sensitive replication mutants. Over the years, several *E. coli* mutants have been isolated that are thermosensitive for DNA replication (Carl, 1970; Fangman & Novick, 1968). In these mutants, cellular DNA synthesis stops following a shift to the restrictive temperature of 42˚C (Hirota et al., 1968). Temperature sensitive mutants in the replicative helicase (DnaB) recently were used to model replication disruption events and, it was suggested that observations in this system relevant to thermal disruption may be extended to replication disruption by DNA lesions (Hanada et al., 2001; Michel et al., 1997). In *dnaB*^{ts} mutants, double strand breaks accumulate in the genome following extended incubations at the restrictive temperature as measured by sucrose gradient analysis (Hirota et al., 1968) and pulse-field agarose electrophoresis (Michel et al., 1997). The level of double strand breaks in *dnaB* mutants is greater in the absence of the RecBC protein (Michel et al., 1997) and may require the action of RuvABC to form (Seigneur et al., 1998).

These observations have led some researchers to speculate that replication forks blocked by DNA secondary structures, DNA damage, or DNA-bound proteins may commonly produce double strand breaks that are frequently repaired by recombination (Michel et al., 1997). It is speculated that the replication fork may regress through the actions of RuvABC by recognizing a four-way junction called a Holliday junction and catalyze branch migration (Seigneur et al., 1998; Sharples et al., 1994; Tsaneva & West, 1994). However, it remains unclear if these events are occurring at the replication fork directly or if the breaks accumulate at other points in the genome. It is also speculated that the events occurring in this model mimic events occurring at DNA lesions (Veomett

& Kuempel, 1973), but this proposal has not been examined directly. Although it is proposed that there are pathways which reactivate an arrested replication fork, the actual events at the replication fork at the point of replication disruption have yet to be examined directly. Therefore, this research characterizes the events occurring after disruption to thermosensitive mutants as compared to the events that occur following disruption by DNA lesions.

Significance

It is well established that inaccurate replication due to DNA damage and other impediments is a primary cause of mutagenesis, rearrangements, and lethality in all cells. Several cancer prone and premature aging genetic disorders are associated with an impaired ability to accurately replicate the DNA in the presence of damage, clearly demonstrating the important contribution of replication accuracy in both cancer and aging (Modrich & Lahue, 1996). By understanding the mechanism by which replication normally recovers following a stall or disruption event, the circumstances that compromise this otherwise faithful process should be better understood. *E. coli* has proven to be an excellent model for studying the mechanisms involved in restarting replication in part because of the high degree to which processes of DNA repair and replication are conserved throughout evolutionarily divergent organisms. The molecular events that occur during the recovery of replication in *E. coli* are likely to be similar to those occurring in human cells. The studies reported herein characterize the role of several gene products in processing and recovering the DNA template following replication disruption by UV-induced DNA damage and replication protein inactivation

caused by a temperature shift. The major objective of this research was to characterize the molecular mechanisms that occur at the replication fork in *Escherichia coli* following protein inactivation as compared to UV-induced DNA damage. This information will contribute to an understanding of the how the structural stability of the genome at arrested replication forks is maintained.

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CHAPTER II

INACTIVATION OF THE DnaB HELICASE LEADS TO THE COLLAPSE AND DEGRADATION OF THE REPLICATION FORK: A COMPARISON TO UV-INDUCED ARREST

Abstract

Replication forks face a variety of structurally diverse impediments that can prevent them from completing their task. The mechanism by which cells overcome these hurdles is likely to vary depending on the nature of the obstacle and the strand in which the impediment is encountered. Both UV-induced DNA damage and thermosensitive replication proteins have been used in model systems to inhibit DNA replication and characterize the mechanism by which it recovers. In this study, we examined the molecular events that occur at replication forks following inactivation of a thermosensitive DnaB helicase and found that they are distinct from those that occur following arrest at UV-induced damage. Following UV-induced DNA damage, the integrity of replication forks are maintained and protected from extensive degradation by RecA, RecF, RecO, and RecR until replication can resume. By contrast, inactivation of DnaB results in extensive degradation of the nascent and leading strand template DNA and a loss of replication fork integrity as monitored by two-dimensional agarose gel analysis. The degradation that occurs following DnaB inactivation partially depends on

several genes, including *recF, recO, recR, recJ, recG,* and *xonA*. Furthermore, the thermosensitive DnaB allele prevents UV-induced DNA degradation from occurring following arrest even at the permissive temperature, suggesting a role for DnaB prior to loading the RecFOR proteins. We discuss these observations in relation to potential models for both UV-induced and DnaB-mediated replication inhibition.

Introduction

All cells must accurately duplicate their genomes in order to reproduce. However, even under normal conditions, a variety of biologically important impediments such as base alterations, DNA adducts, DNA strand breaks, DNA-bound proteins, secondary structures in the DNA, or even limitations in the processivity of the replication machinery itself may impair the ability of the replication machinery to complete its task (reviewed in Courcelle & Hanawalt, 2003). Each of these impediments poses unique challenges for the cell and may stall, block, or disrupt the replication machinery. Although the specific structure and nature of how the replication holoenzyme arrests in each of these situations is not known, it is reasonable to assume that the mechanisms by which replication recovers may vary, depending on the nature of the obstacle. In order to understand how genomic stability is maintained throughout the lifespan of an organism, it is important to characterize how replication accurately processes and resumes in each of these situations.

UV-induced DNA damage has been used frequently as a model to address the general question as to how replication recovers when it is blocked by DNA damage and

has been fairly well characterized. Irradiation with 254 nm light induces DNA lesions that block the progression of the replication machinery (Courcelle et al., 1997; Chan et al., 1985; Setlow et al., 1963) In *Escherichia coli*, RecA and several of the RecF pathway gene products are required to maintain and process blocked replication forks until the lesion can be repaired by nucleotide excision repair or bypassed by translesion DNA polymerases (Courcelle et al., 2006; Courcelle et al., 2005; Chow and Courcelle, 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). In the absence of RecA, RecF, RecO, or RecR, the arrested replication forks are extensively degraded and replication fails to resume (Chow & Courcelle, 2004; Courcelle et al., 1997; Rothman et al., 1975; Horii & Suzuki, 1968). A number of biochemical approaches suggest that RecF, RecO, and RecR function together to promote the binding and formation of a RecA filament at the arrested fork, which in turn, protects and maintains the replication fork DNA by pairing ssDNA with homologous duplex DNA at the arrested fork (Bork et al., 2001; Webb et al., 1997; Webb et al., 1995; Umezu et al., 1993). Other RecF pathway proteins, 3'-5' RecQ helicase and 5'-3' single strand RecJ nuclease, partially degrade the nascent lagging DNA strand at blocked replication forks at times prior to the recovery of replication (Courcelle & Hanawalt, 1999). The processing is proposed to facilitate RecA loading and promote the regression of the replication fork to restore the lesion in double stranded form and repair enzymes to access the offending lesion. In the absence of RecJ, and to a lesser extent RecQ, the recovery of replication is delayed, consistent with the idea that repair enzymes cannot access the DNA lesion to effect repair (Courcelle et al.,

2006). Under these conditions, cell survival and the recovery of replication becomes dependent on translesion synthesis by PolV (Courcelle et al., 2006).

A second experimental approach for characterizing the molecular events that occur following replication disruption involves the use of thermosensitive replication mutants. Over the years, a large number of *E. coli* mutants have been isolated that appear normal for DNA replication at 30ºC, but fail to continue DNA synthesis following a shift to the restrictive temperature of 42ºC (Carl, 1970; Hirota et al., 1968; Fangman & Novick, 1968; Kohiyama et al., 1966). Several of these thermosensitive mutations occur in the *dnaB* gene which encodes an essential hexameric 3'-5' DNA helicase that functions to unwind duplex DNA during replication (LeBowitz & McMacken, 1986; Sclafani & Wechsler, 1981; Wechsler & Gross, 1971; Carl, 1970; Kohiyama et al., 1966). Previous studies using either sucrose gradient analysis or pulse-field gel electrophoresis have shown that double-strand breaks accumulate in the genome of $dnaB^{ts}$ mutants following a period of incubation at the restrictive temperature (Michel et al., 1997; Hirota et al., 1968). The double strand breaks accumulate to a greater extent in *recBC* mutants which cannot repair double strand breaks, and are significantly reduced in *ruvABC* mutants which lack an enzyme complex that catalyzes branch migration and resolution at DNA Holliday junctions (Seigneur et al., 2000; Seigneur et al., 1998; Michel et al., 1997). To explain these observations, it has been proposed that following replication arrest in $dnaB^{ts}$ mutants, RuvABC catalyses the displacement and regression of the nascent DNA at the replication fork (Flores et al., 2001; Seigneur et al., 2000; Seigneur et al., 1998; Michel et al., 1997). In this model, the nascent DNA of the regressed

fork/Holliday junction could then be degraded by the RecBCD helicase-nuclease or collapse to from a double strand break if RuvABC resolves this intermediate. However, it remains unclear if the observed double strand breaks arise at the replication fork directly or if the breaks accumulate at other replicated portions of the genome. Interestingly, following arrest by UV-induced damage, it has been shown that neither RecBCD nor RuvABC process the nascent DNA or are required for replication to resume (Donaldson et al., 2006; Donaldson et al., 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997; Khidhir et al., 1985), suggesting that the events occurring after arrest in these two situations may be unique.

The accumulation of double strand breaks on the chromosome has been observed following inactivation of other replication proteins as well, including DnaN, DnaE, DnaG, PriA, and HolD (Baharoglu et al., 2006; Flores et al., 2004; Grompone et al., 2003; Flores et al., 2002; Grompone et al., 2002; Flores et al., 2001) and it is possible that these events may mimic the events that occur when replication is arrested by UV-induced damage or other impediments such as DNA-bound proteins, DNA secondary structures, or alternative lesions. Therefore, to better characterize the events that occur following the disruption of replication, we monitored the progression of replication, nascent DNA processing, and structural intermediates that occurred at the replication fork following inactivation of a thermosensitive DnaB. These events have been compared to those which occur at replication forks disrupted by UV-irradiation. We find that replication forks disrupted by inactivation of DnaB contain structural intermediates, and are processed by enzymes that are unique from those occurring at forks arrested by UV-induced lesions.

Materials and Methods

Bacterial strains and UV irradiation

SR108 is a *thyA36 deoC2* derivative of W3110 (Mellon & Hanawalt, 1989). The genotype of CRT266 is *thr*, *leu*, *met*, *thyA*, *deo*, *supE*, *tonA*, *dnaB266* (Sclafani & Wechsler, 1981; Kohiyama et al., 1966). All other strains in this study were constructed directly from either SR108, $dnaB^{+}$, or CRT266, $dnaB^{ts}$, by P1transduction.

HL946 (SR108 *recF332::*Tn*3*), CL584 (SR108 *recO1504::*Tn*5*), CL528 (SR108 *recR6212::cat883*), HL924 (SR108 *recJ284::*Tn*10*), HL923 (SR108 *recD1011 argA81::*Tn*10*, and HL1034 (SR108 *xonA::cat300*) have been described previously (Chow & Courcelle, 2004; Courcelle & Hanawalt, 1999).

CL858 (CRT266 *recF332::*Tn*3*), CL896 (CRT266 *recO1504::*Tn*5*), CL897 (CRT266 *recR6212::cat883*), CL743 (CRT266 *recD 1011 argA81::*Tn*10*), CL774 (CRT266 *xonA::cat300*), CL742 (CRT266 *recJ284::*Tn*10*), CL1024 (CRT266 *recG6200::tet857*), CL1026 (CRT266 *ruvAB6203::tet857*), CL1028 (CRT266 del(*srlRrecA*)306:Tn*10* and were constructed by P1 transduction and selection for the indicated alleles from HL946, CL584, CL528, HL923, HL1034, and HL924, TP538, TP540, and HL 921, respectively, into CRT266 (Chow & Courcelle, 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997;).

The genotype of PC8 is *leuB6*(*Am*), *lambda-*, *thyA*, *rpsL*(*strR*), *deoC*, *supH*, $dnaB8^{ts}$ (Carl, 1970).

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

Cell viability

Fresh overnight cultures of cells were diluted 1:100 and grown in Davis medium (Davis, 1949) supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy media) to an OD_{600} between 0.4 and 0.5 in a 32°C shaking water bath. At this time, the cultures were collected on Fisherbrand 0.45 μm 47mm general membrane filters and resuspended in pre-warmed 42°C media. At various times following the temperature shift, 100 μl aliquots were serially diluted and plated on Luria-Bertani agarose plates, supplemented with 10μg/ml thymine. The plates were incubated overnight at 30°C and the number of colony-forming cells per milliliter was determined.

Rate of DNA synthesis

Fresh overnight cultures were diluted 1:100 in 50 ml DGCthy medium supplemented with 0.1μCi/ml [^{14}C]thymine and grown to an OD_{600} of precisely 0.3 in a 32°C shaking water bath. At this time, half of the culture was filtered on Fisherbrand 0.45 μm 47mm general membrane filters and either resuspended in DGCthy media supplemented with 0.1 μCi/ml [^{14}C]thymine and irradiated with 27 J/m^2 (for UV treatment), or resuspended in 42ºC DGCthy media supplemented with 0.1 μCi/ml [^{14}C]thymine (for temperature shift). In each case, the other half of the culture was mock treated, filtered, and resuspended in 32ºC pre-warmed DGCthy media supplemented with

0.1 μCi/ml [^{14}C] thymine. At 5 min intervals, duplicate 0.5 ml aliquots of culture were pulse-labeled with 1μCi/ml [^{3}H]thymidine for a period of 2 min. The cells were then lysed and the DNA was precipitated by adding 5 ml ice-cold 5% trichloroacetic acid (TCA). The precipitated DNA was collected on Millipore glass fiber prefilters, washed with ethanol, and the amount of [^{3}H]- and [^{14}C]-labeled DNA on each filter was determined by liquid scintillation counting.

DNA degradation

Fresh overnight cultures were diluted 1:100 in DGCthy media supplemented with 0.1μCi/ml [^{14}C]thymine and grown to an OD_{600} of 0.4 in a 32°C shaking water bath. At this time, cultures were pulse-labeled with 1μCi/ml [^{3}H]thymidine for 5 seconds, collected on Fisherbrand general filtration 0.45 μm membranes, washed with 1X NET buffer (100mM NaCl, 10mM Tris-pH 8.0, 10mM EDTA-pH 8.0), and either resuspended in pre-warmed 42°C non-radioactive DGCthy media (for temperature shift) or resuspended in pre-warmed 32°C non-radioactive DGCthy media and immediately UV-irradiated with 27 J/m^2 of 254nm UV light (for UV treatment). At the indicated times, duplicate 200μl aliquots (triplicate for time 0) of cells were lysed and the DNA precipitated by the addition of 5 ml ice-cold 5% TCA. Samples were then collected on Millipore glass fiber prefilters, washed with ethanol, and the amount of [^{3}H]- and [^{14}C]-labeled DNA on each filter was determined by liquid scintillation counting.

Two-dimensional agarose gel analysis

Fresh overnight cultures containing the plasmid, pBR322, were grown in the presence of ampicillin (100μg/ml). The overnight cultures were pelleted and resuspended in 100 times the volume of fresh DGCthy media without ampicillin and grown in a shaking 32°C water bath to an OD_{600} of 0.5. At this time, the cultures were collected on 0.45 μm 47mm Fisherbrand general filters and either resuspended in 32ºC DGCthy media and UV-irradiated with 50 J/m^2 (for UV treatment) or resuspended in 42°C DGCthy media (for temperature shift). At the indicated times, 0.75 ml aliquots of each culture were placed into 0.75 ml cold 2X NET buffer. Each sample was immediately pelleted in a microcentrifuge and resuspended in 150 μl of 1 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10mM Tris [pH 8.0], 1mM EDTA). Samples were then left on ice for the duration of the time course. All samples were then incubated at 37°C for 30 min before 10 μl proteinase K (10 mg/ml) and 20 μl 20% sarkosyl was added, and incubation continued for 1 hr at 55°C. Samples were extracted twice with 4 volumes (600 μl) of phenol:chloroform:isoamyl alcohol (25:24:1), and once with 4 volumes of chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed for 3 hours on 47 mm Whatman 0.05 μm pore disks against 250 ml of 0.2X TE (10 mM Tris - pH 8, 10 mM EDTA) buffer. Samples were digested with Pvu*II* (New England Biolabs), extracted once with chloroform:isoamyl alcohol, and loaded onto the gel. For the 1st dimension, restricted genomic DNA samples were electrophoresed in 0.4% agarose in 1X Tris-Borate-EDTA (TBE) at 1 V/cm for 15 hr. For the 2nd dimension, the gel lanes were cut out, rotated 90°, recast in 1.0% agarose in 1X TBE, and electrophoresed at 7 V/cm for 7

hr. The DNA in the gels was transferred to Hybond N+ nylon membranes and probed with either pBR322 that had been labeled with ^{32}P by nick translation according to the protocols supplied by Roche using [α-^{32}P]-dCTP (MP Biomedicals), or in the case of strand specific probes, with oligonucleotides that had been labeled with ^{32}P by T4 polynucleotide kinase according to the protocols supplied by New England Biolabs using [γ-32P]-ATP (MP Biomedicals). Radioactivity was visualized and quantitated using a Storm 820, and its associated ImageQuant Software (Molecular Dynamics/Pharmacia).

Strand specific oligonucleotides for pBR322

pBR322 1348-1398 (detects lagging strand template)

5'TGTGAATGCGCAAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCC;

and pBR322 1398-1348 (detects leading strand template)

5'GGCGGACGCGATGGATATGTTCTGCCAAGGGTTGGTTTGCGCATTCACA.

Results

Both UV-irradiation and inactivation of DnaB block the progression of replication

The DnaB protein of *Escherichia coli* encodes an essential helicase responsible for unwinding the duplex DNA at the replication fork during DNA replication. A temperature sensitive allele of this protein, *dnaB266* in strain CRT266, was utilized to inactivate the replication helicase and therefore, disrupt replication progression. In these mutants, cellular DNA synthesis occurs normally at 30ºC, but fails to continue after a shift to the restrictive temperature of 42ºC (Hirota et al., 1968).

We verified that DNA replication arrests in this mutant following a shift to the restrictive temperature and compared it to the arrest observed after UV-irradiation by monitoring the total DNA accumulation and rate of DNA synthesis over time. To this end, cultures grown at 32ºC in media containing [^{14}C]thymine were either UV-irradiated or shifted to a temperature of 42ºC. To monitor how the rate of DNA synthesis was affected by these treatments, duplicate 0.5 ml aliquots of the [^{14}C]-labeled cultures were pulse-labeled with [^{3}H]thymidine for 2 min at periodic intervals before and after each 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 each time. When we examined *dnaB*^{ts} mutants by this assay, we observed that both UV-irradiation and a shift in temperature to 42ºC inhibited the rate of DNA synthesis immediately following treatment (Fig. 2.1A). In the case of UV irradiation, the rate of DNA synthesis was inhibited by

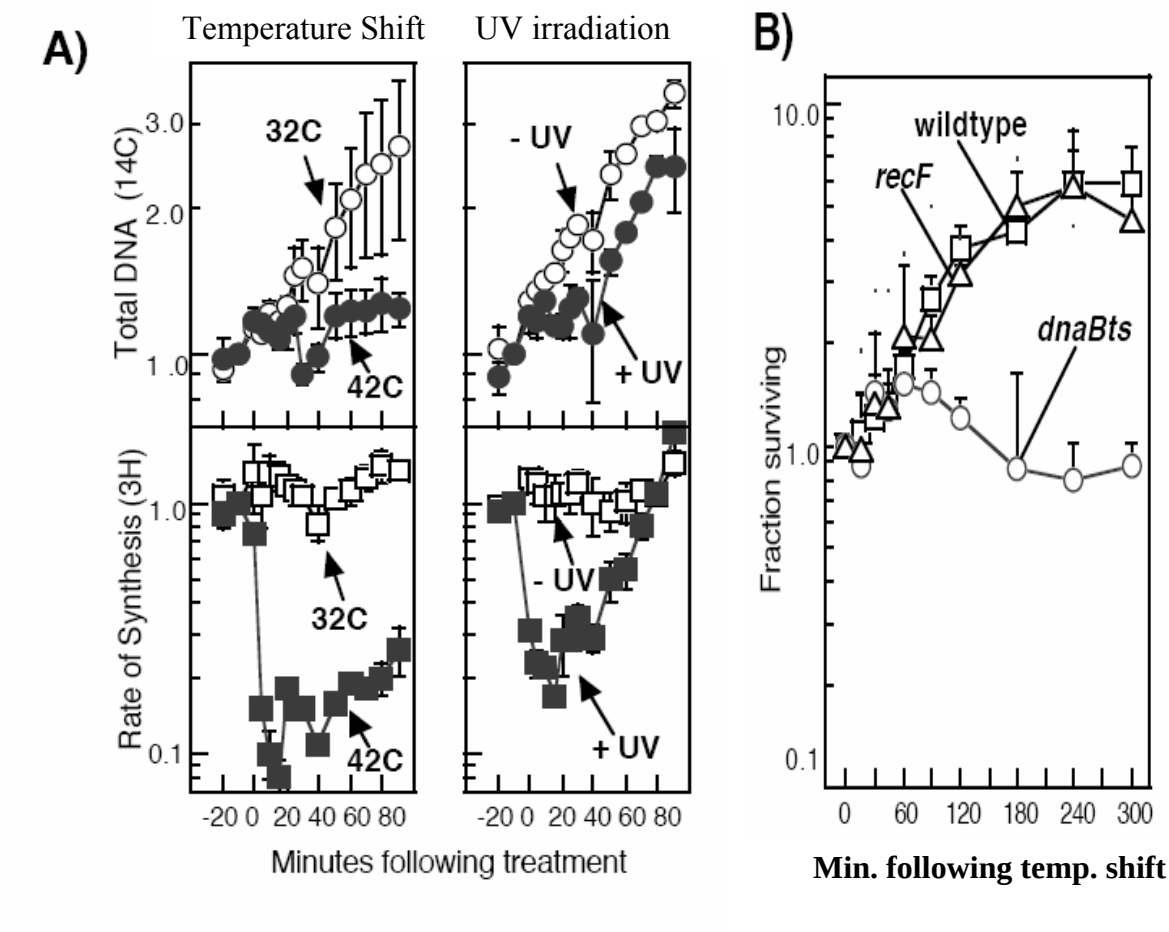

Figure 2.1. Both UV irradiation and inactivation of DnaB266 at 42°C arrest DNA synthesis; however, *dnaB*^{ts} mutants remain viable following the temperature shift.

(A) Cultures of *dnaB*^{ts} (CRT266) grown at 32°C in medium containing [^{14}C]thymine were either UV-irradiated with 27 J/m^2, shifted to 42°C, or mock treated. At the indicated times, duplicate aliquots of each culture were pulse-labeled for 2 min with [^{3}H]thymine and the relative amount of ^{14}C and ^{3}H in the DNA is plotted over time. Total DNA in mock treated cultures (○); total DNA in UV irradiated or temperature shifted cultures (●); rate of DNA synthesis in mock treated cultures (□); rate of DNA synthesis in UV irradiated or temperature shifted cultures (■). **(B)** The fraction of cells surviving per ml of culture following incubation at 42°C for the indicated time is plotted. *dnaB*^{ts} (CRT266) (○); wildtype (SR108) (□); *recF* (CL007) (Δ). Plots represent an average of 3 independent experiments. Error bars represent one standard deviation.

approximately 90%, but began to recover 40 min following UV treatment and had completely recovered to the rate before treatment by 80 min. This result was similar to that observed in other strains at 37ºC (Courcelle et al., 2005; Donaldson et al., 2004). Following inactivation of DnaB at 42ºC, the rate of DNA synthesis was inhibited to a similar extent as that seen with UV-irradiation, and did not resume when held at 42ºC, consistent with the view that DnaB is essential for replication to resume following disruption.

While the *dnaB*^{ts} mutant recovered from UV irradiation, we did not observe a resumption of synthesis following a shift to 42ºC. Therefore, we wished to examine whether the shift in temperature resulted in a loss of viability in *dnaB*^{ts} mutants. To examine this possibility, cultures of CRT266, the *dnaB*^{ts} strain, and the non-temperature sensitive strains, SR108, and CL007, a *recF* derivative SR108, were grown in minimal media at 32ºC. The cultures were then collected and resuspended in media at 42ºC. After the culture had incubated for various times at 42ºC, samples were collected, serially diluted, and plated on LB plates at 30ºC. The number of colony-forming cells per mL was determined and plotted over time (Fig. 2.1B). In the non-temperature sensitive strains, the number of colonies per ml continued to increase during the incubation period at 42ºC. By contrast, in the *dnaB*^{ts} mutants, the culture ceased growing shortly after they were shifted to 42ºC. Importantly, the *dnaB*^{ts} mutants remained viable throughout the 5 hr incubation period we examined (Fig. 2.1B), suggesting that although DNA synthesis is disrupted in these mutants, the cells are capable of recovering from this stress when returned to 32ºC.

DnaB266 leads to extensive degradation at the replication fork when inactivated at 42ºC.

Both UV-induced DNA damage and DnaB inactivation arrest replication forks. Previous work in our laboratory has shown that following UV-induced arrest, the replication fork is maintained and protected from extensive degradation by RecF, RecO, and RecR (Chow & Courcelle, 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). To determine if replication forks disrupted following DnaB inactivation are protected and maintained similarly to that which occurs after UV irradiation, we compared the amount of nascent DNA degradation that occurred at the replication fork following DnaB inactivation to that observed after UV-irradiation. Cultures labeled with [^{14}C]thymine were pulse-labeled with [^{3}H]thymidine for 5 seconds immediately before they were transferred to non-radioactive medium and either shifted to 42ºC or UV irradiated with 27 J/m^2. The amount of [^{3}H] and [^{14}C] remaining in the DNA was then followed over time. This assay allowed us to compare the loss of ^{3}H-labeled DNA at the arrested replication fork to the loss of [^{14}C]-labeled DNA that occurred over time (Fig. 2.2A). In our parental cells (SR108), or in *recF* mutants, following a shift to 42ºC, no degradation of the nascent DNA was detected; consistent with the idea that replication is not disrupted in those cells at this temperature (Fig. 2.2B). The increase in ^{3}H-labeled DNA that occurs in these strains immediately after the temperature shift is due to the incorporation of the remaining intracellular pools of [^{3}H]thymidine as replication continues (Courcelle & Hanawalt, 1999; Courcelle et al., 1997). By contrast, following a shift to 42ºC in cultures of *dnaB*^{ts}, approximately half of the nascent DNA was degraded,

suggesting that the replication forks are not maintained following the DnaB-mediated arrest (Figure 2.2B).

Similar to our previous studies at 37ºC, following UV irradiation at 32ºC, our parental cells (SR108), exhibited a limited amount of nascent DNA degradation for the first 20 min, but unlike the *dnaB*^{ts} -induced arrest, the nascent DNA was maintained and protected (Figure 2.2C) (Courcelle et al., 1999; Courcelle & Hanawalt, 1999). After this time, the levels of ^{3}H-labeled DNA began to increase due to the re-incorporation of remaining [^{3}H]thymidine in intracellular pools when replication resumes (Courcelle & Hanawalt, 1999; Courcelle et al., 1997). As seen previously, in the absence of RecF, replication forks disrupted by UV-induced damage were not maintained and the nascent DNA degradation continued until approximately half of the nascent DNA was degraded (Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). Thus, unlike *dnaB*^{ts}-mediated arrest, forks arrested by UV-induced lesions are maintained and protected from degradation by RecF.

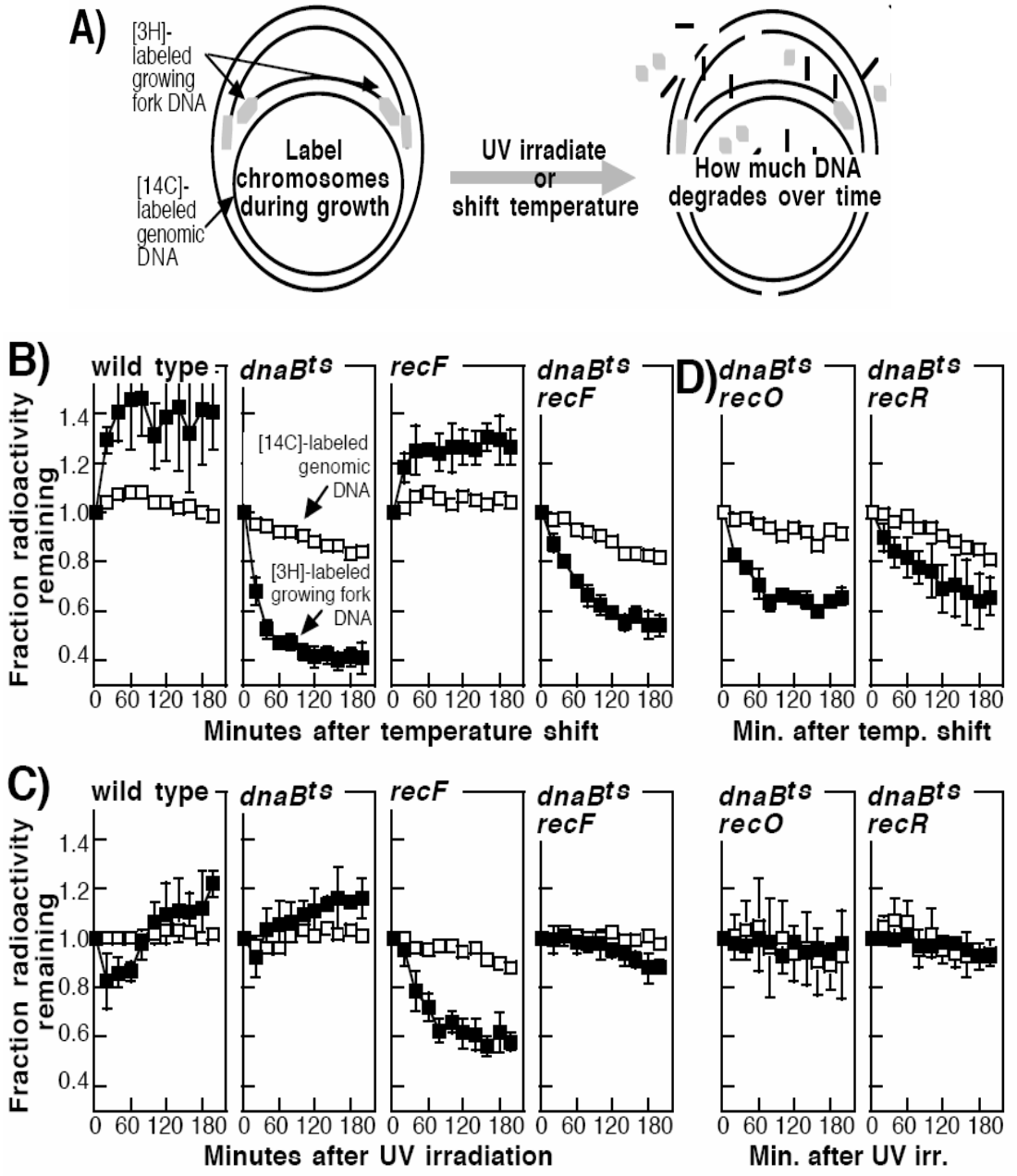

Figure 2.2. Inactivation of DnaB^{ts} at 42°C leads to extensive degradation of the nascent DNA at the arrested replication fork but prevents nascent DNA degradation from occurring after UV irradiation at the permissive temperature.

Figure 2.2. (continued)

(A) Schematic of how the chromosome was labeled before UV-irradiation or temperature shift to 42°C. [^{3}H]thymidine was added to [^{14}C]thymine prelabeled cultures for 5 sec immediately before cells were filtered, resuspended in nonradioactive medium, and then either UV irradiated with 27 J/m^2 or shifted to 42°C. To measure the extent of DNA degradation, the fraction of acid precipitable radioactivity remaining in the DNA was followed over time. **(B)** The relative amount of degradation in the nascent DNA (■) and total genomic DNA (□) is plotted following a temperature shift to 42°C. **(C)** The DnaB^{ts} allele prevents the nascent DNA degradation that occurs at replication forks arrested by UV-induced damage. Degradation is plotted after UV irradiation with 27 J/m^2. **(D)** Similar to *recF*, *recO* and *recR* mutants reduce or prevent the nascent DNA degradation from occurring at replication forks in *dnaB^{ts} recO* and *dnaB^{ts} recR* after a temperature shift to 42°C or UV irradiation with 27 J/m^2. Graphs represent an average of 3 independent experiments. Error bars represent one standard deviation.

Unexpectedly and in contrast to the increased degradation observed after the temperature shift, *dnaB*^{ts} cultures exhibited reduced degradation of the nascent DNA following UV irradiation as compared to wild type cultures (Figure 2.2C). The observation suggests that DnaB function may be required for the nascent DNA processing to occur at replication forks after UV-induced arrest, and that the DnaB^{ts} disrupts this function even at temperatures that allow the helicase to retain its essential functions in replication. To investigate this possibility further, we examined the effect that the *dnaB*^{ts} allele had on the nascent DNA degradation in a *recF* mutant. Following UV irradiation, RecF, RecO, and RecR are required to protect and limit the degradation of the arrested fork by the RecJ nuclease and RecQ helicase (Chow & Courcelle, 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). When replication was arrested by UV-induced damage, we observed that the DnaB^{ts} allele prevented the nascent DNA degradation from occurring even in the absence of RecF. By contrast, following arrest by inactivation of DnaB^{ts} at 42ºC, the absence of RecF only modestly reduced the extent of nascent DNA degradation that occurred (Figure 2.2C and 2.2B). Similar effects of the *dnaB*^{ts} allele were observed following UV-induced arrest or DnaB^{ts}-mediated arrest in the absence of RecO or RecR (Figure 2.2D). Although the presence of DnaB^{ts} was able to suppress the extensive nascent DNA from occurring, it did not alleviate the hypersensitivity of the *recF* mutants (data not shown). These observations indicate that the processing and recovery of replication forks arrested at UV-induced damage are distinct from that occurring following inactivation of DnaB. In addition, the lack of nascent DNA processing following UV irradiation in *dnaB*^{ts} mutants suggests that

unwinding by the DnaB helicase may be necessary for RecFOR to open up the surrounding region so that subsequent processing enzymes can access the arrested replication fork DNA.

Exonuclease I and the RecF pathway gene products contribute to the degradation of the nascent DNA at replication forks disrupted following DnaB^{ts} inactivation

The results presented above suggest that replication forks disrupted either by UV irradiation or by inactivation of DnaB are likely to be distinct in each case. Following UV irradiation, it has been shown that the nascent DNA degradation that occurs at the arrested fork is mediated by the RecQ helicase and RecJ nuclease (Courcelle et al., 2003; Courcelle & Hanawalt, 1999). To characterize the enzymatic activities that act at the replication fork following inactivation of DnaB^{ts}, we examined the degradation that occurred after a temperature shift to 42ºC in strains of *dnaB^{ts}* that were also deficient in one or more of these candidate nucleases or helicases. In an otherwise wildtype background, inactivation of DnaB^{ts} at 42ºC resulted in roughly half of the nascent DNA being degraded at the disrupted fork within 60 min of the temperature shift. By comparison, following inactivation of DnaB^{ts} in strains that also lacked the RecD nuclease (*recD*), Exonuclease I (*xonA*), or the RecJ exonuclease (*recJ*), we observed that although RecD did not affect the extent of degradation, the absence of either Exonuclease I or RecJ partially prevented the nascent DNA degradation from occurring (Fig. 2.3A). RecJ is a 5'-3' single-strand exonuclease that is associated with the *recF* pathway and known to process the nascent DNA at replication forks blocked by UV-induced

damage (Courcelle et al., 2003; Courcelle & Hanawalt, 1999; Lovett & Kolodner, 1989). The diminished degradation in *recJ* is similar to that observed in the other *recF* pathway mutants (compare Fig. 2.2 and 2.3), suggesting that the diminished degradation in this case may reflect an overall inability of the RecF pathway enzymes to gain access the disrupted site, in contrast to the case following UV-induced arrest. Comparatively, although Exonuclease I-mediated degradation is not detected at replication forks arrested by UV-induced damage (Courcelle & Hanawalt, 1999), it accounts for a substantial amount of the nascent DNA degradation occurring at the fork after $DnaB^{ts}$ inactivation. Exonuclease I degrades DNA in a 3'-5' direction, opposite to that of RecJ and has been reported to associate biochemically with RecA during purification (Lehman & Nussbaum, 1964; Bedale et al., 1993).

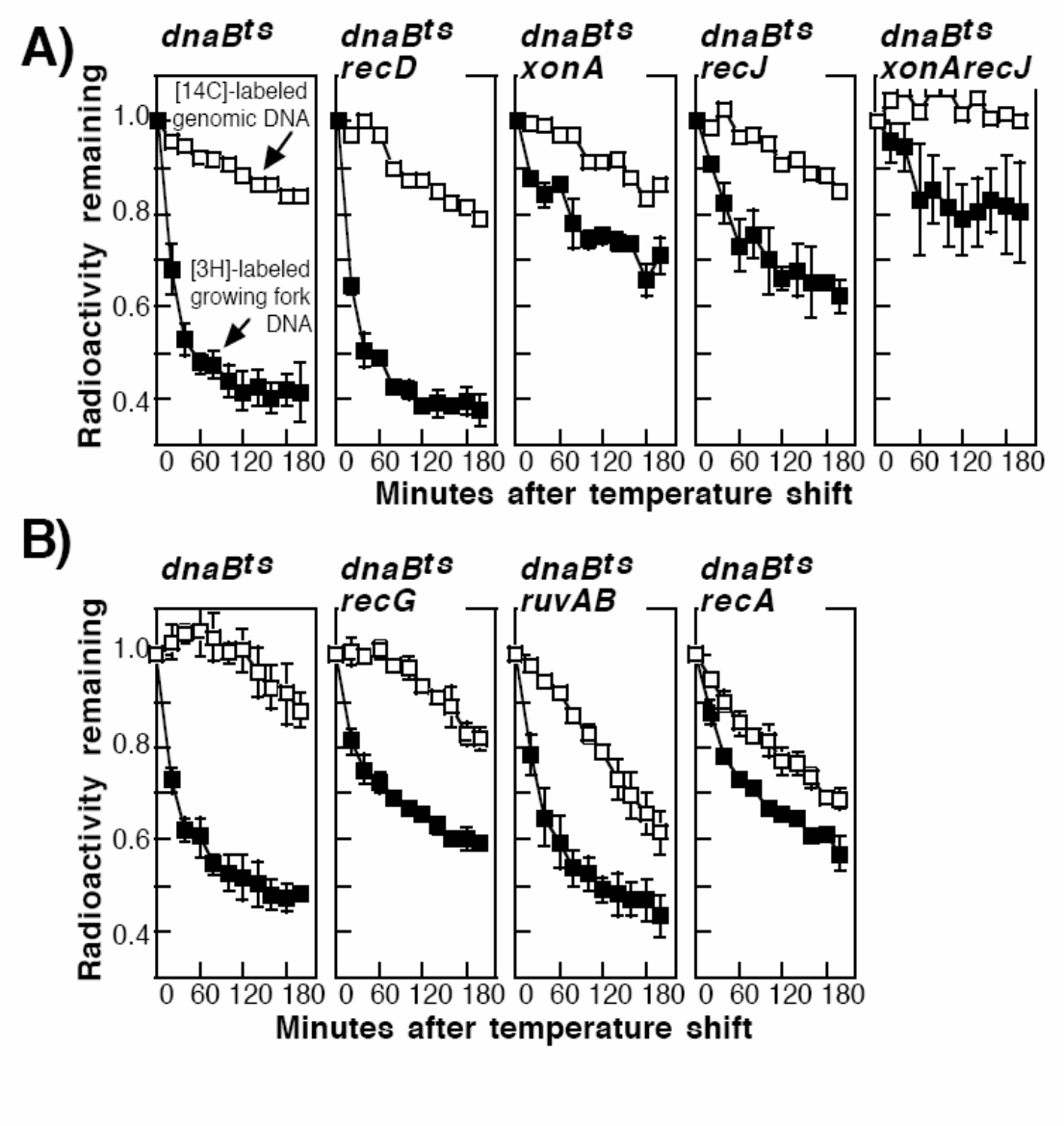

Figure 2.3 Exonuclease I and the RecF pathway gene products contribute to the nascent DNA degradation at replication forks disrupted following DnaB^{ts} inactivation

(A & B) The relative amount of degradation in the nascent DNA (■) and total genomic DNA (□) is plotted following a temperature shift to 42°C. Graphs represent an average of 3 independent experiments. Error bars represent one standard deviation. The *dnaB*^{ts} (CRT266) plots in panel B were generated by Andrew Casey, Portland State University.

We also examined the effect that mutants lacking the RecG helicase, the RuvAB branch migration complex, and the RecA strand-pairing enzyme had on degradation following DnaB^{ts} inactivation (Figure 2B). These enzymes all render cells sensitive to UV-induced damage and have been associated with processing arrested replication forks (reviewed in (Michel et al., 2004; Courcelle & Hanawalt, 2003) and see discussion). We observed that the absence of RecG partially reduced the nascent DNA degradation to an extent that resembled other *recF* pathway mutants. The absence of RecA also partially abrogated the nascent DNA degradation following DnaB^{ts} inactivation. Curiously, although the level of degradation at the arrested fork was reduced, the overall level of DNA degradation in the total genomic DNA was significantly higher. When we examined *ruvAB* mutants following DnaB^{ts} inactivation, we also observed elevated levels of degradation occurring in the genomic DNA, even though the nascent DNA degradation was not affected by the presence or absence of the RuvAB complex.

We were unable to construct a *recBC dnaB^{ts}* double mutant that would grow well enough in culture to characterize by these assays. RecB and RecC form a helicase complex that contains exo- and endo-nucleolytic activity when associated with RecD (Dillingham et al., 2003; Taylor & Smith, 2003). In the absence of RecD, the helicase remains active but does not degrade DNA. *recBC* mutants have pleiotropic phenotypes, exhibiting reduced viability and growth in culture, compromised recombination frequencies during conjugation and transduction, and hypersensitivity to DNA damage (Capaldo & Barbour, 1975; Howard-Flanders, 1975; Clark & Chamberlin, 1966).

With the exception of the excessive degradation associated with *recA* mutants, no significant degradation of the nascent or genomic DNA was observed in any of these nuclease, helicase, or branch migration enzymes following UV-induced arrest (data not shown) (Donaldson et al., 2004; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). Therefore, we interpret these observations to indicate that, in contrast to UV-induced arrest, the nascent DNA at replication forks disrupted by $DnaB^{ts}$ inactivation is subject to degradation by XonA and modestly enhanced by the action of the RecF pathway genes. Additionally, the presence of RuvAB is required to maintaining the integrity of the overall genomic DNA, although its protective function does not affect the nascent DNA at the arrest site directly.

Unique structural intermediates accumulate following the arrest of replication on plasmid molecules after $DnaB^{ts}$ inactivation as compared to UV-irradiation

The distinct enzymatic activities that process replication forks disrupted by UV-induced damage as compared to those disrupted by $DnaB^{ts}$ inactivation suggested that the structure of the arrested fork may be unique in each case. To address this possibility, we used two dimensional agarose gel electrophoresis, a technique that allows the structural properties of replicating DNA fragments to be identified by their unique size and shape, to characterize and compare the intermediates that occur on replicating molecules of the plasmid pBR322 following $DnaB^{ts}$ –induced arrest and UV-induced arrest. The plasmid pBR322 maintains a copy number of ~15 plasmids per cell and utilizes the host's machinery for replication, making it a useful tool to examine rare events such as

replication through a specific DNA sequence (Martin-Parras & Hernandez, 1991). To compare the replication intermediates that occur following $DnaB^{ts}$ -mediated arrest to those after UV-induced arrest, cultures containing the plasmid pBR322 were either UV-irradiated with 50 J/m^2 or shifted to the restrictive temperature of 42ºC. At various times after treatment, the genomic DNA was then purified, digested with the restriction endonuclease, Pvu*II*, and analyzed by two-dimensional agarose gel electrophoresis. Pvu*II* linearizes pBR322 just downstream of its unidirectional origin of replication. In the absence of impediments to replication, this produces a migration pattern in which the non-replicating linear plasmids form a prominent spot and the replicating plasmid molecules migrate as an arc that extends out from the linear spot (sometimes referred to as a simple Y-shaped arc pattern) (Martin-Parras et al., 1991; Friedman & Brewer, 1995; and Figure 2.4A). Following UV-irradiation in wildtype cells at 32ºC, elevated levels of Y-structures and a transient cone region of molecules containing two branch points appears (Courcelle et al., 2003). Previous work from our lab carried out at 37ºC has shown that these Y-structures and a portion of the cone region intermediates represent arrested replication forks (Courcelle et al., 2003; Donaldson et al., 2006). The replication intermediates are stabilized and protected from RecQ RecJ-mediated degradation by RecFOR and RecA until a time that correlates with when the lesions are repaired and DNA synthesis resumes (Chow & Courcelle, 2004; Courcelle et al., 2003; Donaldson et al., 2006). Based on the genetic requirements needed for DNA synthesis to resume, it has

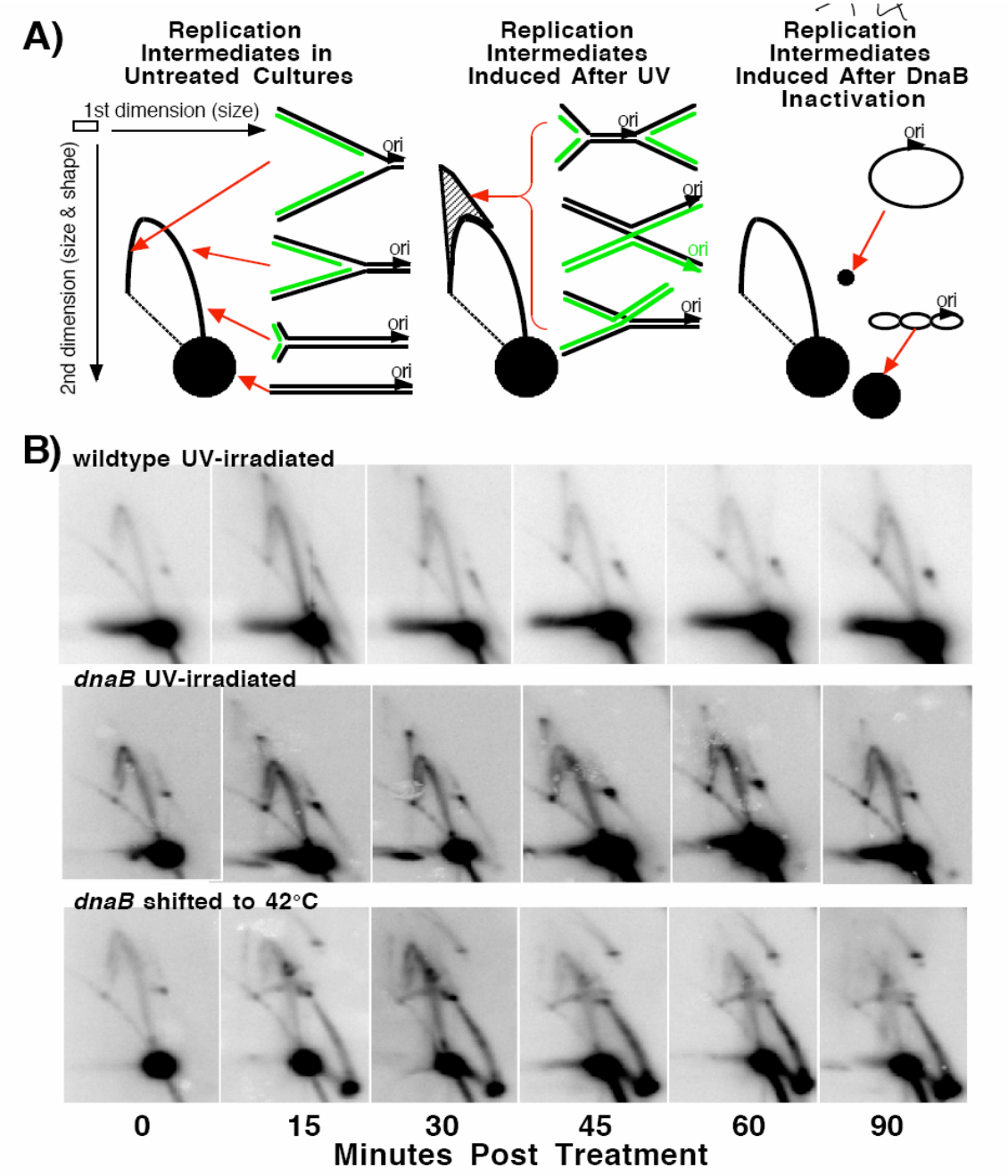

Figure 2.4. The structure of plasmid replication intermediates observed following either DnaB^{ts} inactivation or UV-irradiation are distinct.

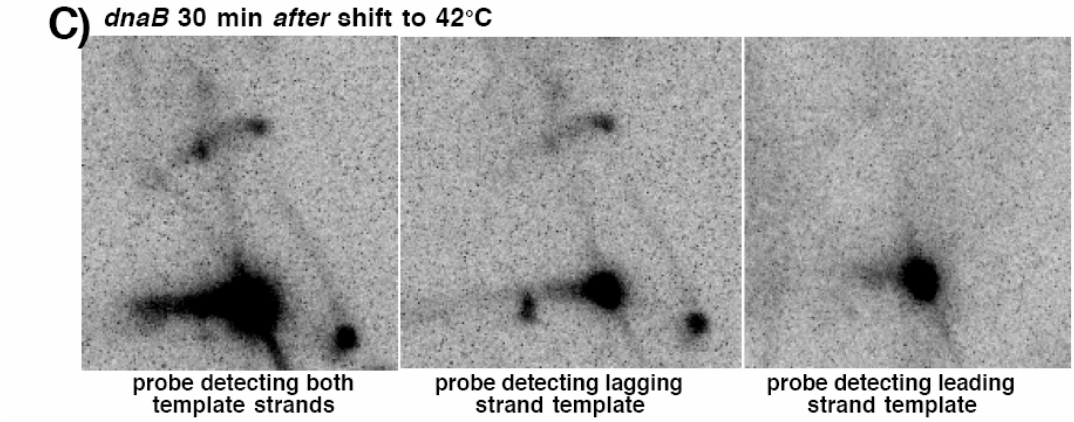

Figure 2.4. (continued)

(A) A diagram of the migration pattern for Pvu*II*-digested pBR322 observed by 2-D agarose gel analysis in (i) untreated cultures, (ii) cultures following UV-irradiation, and (iii) cultures following $DnaB^{ts}$ inactivation. Non-replicating molecules form a prominent spot that migrates as a linear 4.4 kb fragment. In untreated cultures, replicating molecules migrate more slowly due to their larger size and nonlinear shape, forming an arc that extends out from the linear fragment (approximating a simple Y-arc consisting of Y-shaped molecules). Following UV irradiation, transient replication intermediates migrating in a cone- shaped region, beyond the Y-arc are observed at times prior to the recovery of replication that are made up of double Y- and X-shaped molecules. Following inactivation of DnaB, the accumulation of an intermediate is observed that migrates similar to circular, supercoiled plasmid molecules that are resistant to digestion by Pvu*II*. **(B)** Cone region intermediates are observed following UV-induced arrest whereas DnaB inactivation leads to a distinct circular replication intermediate that is resistant to digestion by restriction enzymes. Cultures of wildtype (SR108) or *dnaB*^{ts} (CRT266) containing the plasmid pBR322 were either UV-irradiated or shifted to 42ºC. At the times indicated, DNA was purified, digested with Pvu*II* and analyzed by 2-dimensional agarose gel analysis using pBR322 plasmid as a probe. **(C)** The circular pBR322 replication intermediate that accumulates following $DnaB^{ts}$ inactivation is single stranded DNA matching the lagging strand template of the plasmid. Cultures of *dnaB*^{ts} (CRT266) containing the plasmid pBR322 were shifted to 42ºC for 30 minutes before the DNA was purified and digested with Pvu*II*. Samples were then split and analyzed by 2-dimensional agarose gel analysis using either pBR322 plasmid, an oligo that is complimentary to the lagging strand template, or an oligo that is complimentary to the leading strand template as a probe.

been proposed that the processing by RecF pathway proteins effectively moves the branch point of the replication fork back away from the arresting lesion, restoring the region to a form that allows repair enzymes to gain access and remove the lesion (Courcelle et al., 2006; Courcelle et al., 2003; Courcelle et al., 1999; Courcelle et al., 1997; Courcelle et al., 2005).

When we examined cultures of UV-irradiated *dnaB*^{ts} at 32ºC, we observed similar intermediates and patterns to those observed in wildtype cells (Figure 2.4B). However, following inactivation of DnaB^{ts} at 42ºC, the intermediates observed on the replicating molecules were clearly distinct from those seen after UV-induced damage. In contrast to UV, no intermediates migrating in the cone region were observed, suggesting either that a regressed fork intermediate does not occur or that it is rapidly degraded. Instead, an intermediate that migrated similar to that of unrestricted supercoiled circular plasmid molecules appeared within 15 minutes of the temperature shift and continued to accumulate throughout the time course (Figure 2.4B). The intermediate species ran slightly below the position where double strand supercoiled plasmids appear in the gel and were resistant to restriction digestion, suggesting that these molecules may contains regions of single stranded DNA. To test the possibility and further characterize the substrate formed at forks disrupted by DnaB inactivation, we probed the 2-D gels with strand-specific probes that were complimentary to the leading and lagging strand template of pBR322. As shown in Figure 2.4C, only the probe complimentary to the lagging strand template, but not the leading strand template hybridized to the intermediate formed following DnaB inactivation. The accumulation of a species of

single-strand supercoiled DNA corresponds to the lagging strand template could be produced in either of two ways. The first possibility is that the arrested replication fork results in the degradation of the leading strand template and both daughter strands. These observations would be consistent with the extensive degradation detected in our other assays. Alternatively, the intermediate could represent new leading strand synthesis in the absence of lagging strand replication similar to that with occurs during rolling circle replication. Although new synthesis of this type is generally believed to require DnaB function, we cannot rule out the possibility that our $DnaB^{ts}$ retains some partial function or that this represents some novel DnaB independent replication of the plasmid. Regardless of the mechanism, it is clear from this data that replication forks inactivated by $DnaB^{ts}$ inactivation are structurally distinct from those that occur following UV-induced arrest.

Other $DnaB^{ts}$ alleles exhibit extensive nascent DNA degradation at the restrictive temperature

The *dnaB266* allele used in this study, like many of the *dnaB* alleles that have been isolated, contains a stop codon mutation that requires the presence of secondary tRNA suppressor mutations. This made it difficult to construct comparable isogenic strains and raised possibility that the phenotypes may be unique to this strain or allele. To examine this possibility, we examined the degradation that occurred in a second strain PC8, which contains a different thermosensitive *dnaB* allelle, *dnaB8* (Saluja & Godson, 1995). When we examined the degradation that occurred in the nascent DNA and genomic DNA of this

strain, we observed a degradation pattern that was identical to that of the *dnaB266* allele (Fig. 2.5). Furthermore, a similar degradation of the nascent DNA has been reported previously for another DnaB^{ts} allele that was isolated independently (Veomett & Kuempel, 1973), consistent with the idea that the observations described here are a general phenomenon occurring after the replication is disrupted by the inactivation of the DnaB helicase.

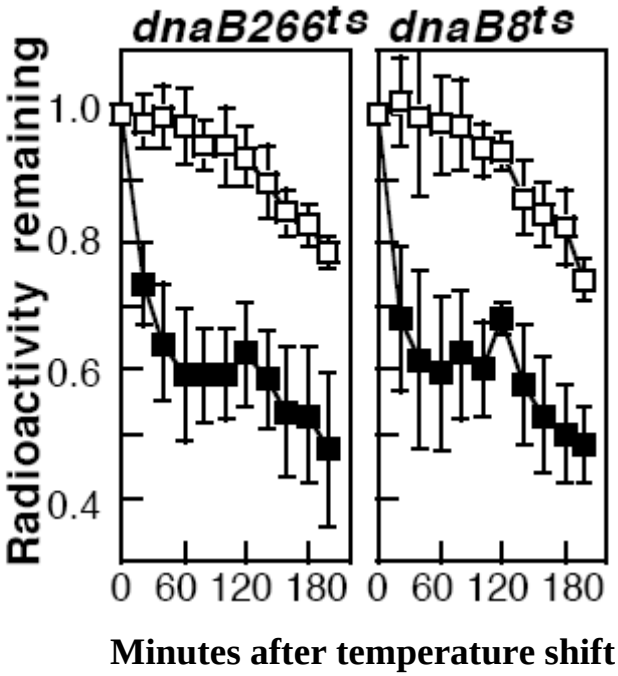

Figure 2.5. Both DnaB266 and DnaB8 alleles result in degradation of the nascent DNA following inactivation at 42°C.

Degradation was measured as described in Figure 2.2B. The relative amount of degradation in the nascent DNA (filled symbols) and total genomic DNA (open symbols) is plotted over time for *dnaB266* (CRT266) and *dnaB8* (PC8) following temperature shift to 42°C. Graphs represent an average of 2 independent experiments. Error bars represent one standard deviation.

Discussion

In this study, we characterized replication forks arrested following UV-irradiation and compared them to those arrested following inactivation of DnaB^{ts}, the replicative helicase. We found that the structure of the arrested fork and the enzymes that process them in each case are unique. In the case where replication is arrested at UV-induced damage, this and previous work from our lab has shown that the arrested replication fork is maintained and protected by RecA which is loaded and stabilized by RecF, RecO, RecR (Courcelle et al., 1997; Courcelle et al., 2003; Chow & Courcelle, 2004). The RecQ helicase and RecJ exonuclease process and partially degrade the nascent lagging strand at the blocked replication fork prior to the time replication resumes (Courcelle & Hanawalt, 1999; Courcelle et al., 2003; Courcelle et al., 2006). The nascent DNA degradation is thought to effectively move the branch point of the arrested fork back, restoring the region to a double stranded form that allows repair of the blocking lesion (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle et al., 2005; Courcelle et al., 2006). In the absence of this processing, the recovery of replication is delayed and becomes dependant on translesion synthesis by Pol V (Courcelle et al., 2006).

Unlike the limited degradation observed after UV-induced arrest, replication arrested by DnaB^{ts} inactivation led to extensive degradation of approximately half the nascent DNA. In this case, rather than limit the nascent DNA degradation, loading of RecA at the arrest site by RecF, RecO, and RecR modestly increased the extent of degradation that occurred. Additionally, we found that Exonuclease I was primarily

responsible for the nascent DNA degradation that occurred following DnaB^{ts} inactivation, whereas RecJ was the nuclease acting after UV-induced arrest.

The inactivation of DnaB^{ts} also resulted in very different structural intermediates on replicating plasmids from those generated after UV-induced damage. In the case of UV, where the nascent DNA degradation is limited following arrest, elevated levels of Y-shaped fragments and double Y-shaped structures are transiently observed to accumulate, consistent with the idea that the arrested forks are maintained until replication can resume (Courcelle et al., 2003; Donaldson et al., 2006). By contrast, no such replication intermediates were seen to occur after DnaB^{ts} inactivation. Instead, the extensive nascent DNA degradation that occurs in *dnaB*^{ts} mutants is accompanied by the accumulation of large amounts of single-strand plasmid which is likely to either represent the degradation of both the nascent DNA and leading strand template or uncoupled leading strand synthesis to generate the circular single strand plasmids.

Using pulse-field gel electrophoresis to measure the integrity of whole chromosomes, previous studies have demonstrated that in the absence of RecBC or RecA, elevated levels of chromosome breaks accumulate when incubated at the restrictive temperature (Michel et al., 1997). The formation of double strand breaks in the chromosome of *dnaB*^{ts} *recB* mutants required RuvABC, RecA, and occurred in cells where active replication was occurring (Seigneur et al., 1998; Seigneur et al., 2000). To explain the presence of breaks on the chromosome, it was proposed that replication forks arrested by DnaB^{ts} inactivation lead to fork regression and the formation of a Holliday junction at the arrested fork with the annealing of the two nascent DNA strands. In this

model, RecBCD was proposed to degrade the nascent double-stranded tail, effectively regenerating the replication fork. In the absence of RecBCD, the Holliday junction could be cleaved by the action of RuvABC, generating the observed chromosome breaks. By pulse-labeling the DNA at the arrested replication fork directly, we did not detect any difference in the processing or degradation of the nascent DNA in *dnaB*^{ts} cultures when either RuvAB or RecD was absent. However, consistent with these previous studies, we did detect elevated levels of degradation occurring in the overall chromosome of *ruvAB* and *recA* cultures, which would be consistent with the presence of chromosome breaks occurring in these mutants. The results presented here would suggest that chromosome breaks in these mutants may occur either in front of the replication fork or in replicated regions farther beyond the pulse-labeled region at the arrest site. Similar to the degradation occurring in *dnaB*^{ts} *recA* cultures, the RecD-mediated degradation that occurs in *recA* cultures following UV irradiation initiates at sites other than the arrested fork (KH Chow, unpublished). While the precise substrates of RuvABC and RecBCD that are generated during replication remain to be elucidated, a number of mutants with impaired replication have been characterized that are prone to chromosome breakage (Michel et al., 1997; Flores et al., 2001; Grompone et al., 2002; Baharoglu et al., 2006), suggesting that the breaks are associated with impaired replication in general, rather than a unique phenotype associated with *dnaB*^{ts}.

Unexpectedly, we also observed that the DnaB^{ts} allele prevented any nascent DNA degradation from occurring following UV-induced damage, irrespective of whether RecFOR was present to protect and maintain the fork following arrest. Given that this

effect occurs at the permissive temperature, we would propose that the mutation in *dnaB266* alters the function of the protein when it encounters DNA damage, despite the fact that the protein remains functional for replication. Speculatively, the unusual phenotype associated with the *dnaB266* mutation could suggest that the DnaB helicase plays a role in opening up the region where replication arrests prior to RecF -O –R loading. If true, the *dnaB266* mutation would have impaired this ability while retaining enough activity for it essential role during replication.

The mechanism by which cells recovery replication following arrest is of critical importance to cell survival in the presence of DNA damage and maintaining genomic integrity. However, replication forks encounter a variety of structurally diverse impediments that can block or impede the progress of the replication holoenzyme, raising the possibility that the mechanism of recovery may vary depending on the nature of the disruption. In this study, we characterized and compared two forms of arrested replication forks that have been used as models to study the mechanism of recovery (Fig 2.6). We demonstrate that replication forks arrested by UV-induced DNA damage and those arrested following inactivation of the DnaB helicase are structurally distinct and are processed by unique enzymes prior to the time that replication recovers. In some cases, these results may help to explain seemingly contradictory or conflicting results that have been obtained by different groups characterizing the enzymes involved in the recovery of replication. Further, they suggest that when trying to understand the mechanism by which cells recover replication in the presence of DNA damage, it will be important to clarify it in the context of the structure of the impediment and the arrested fork.

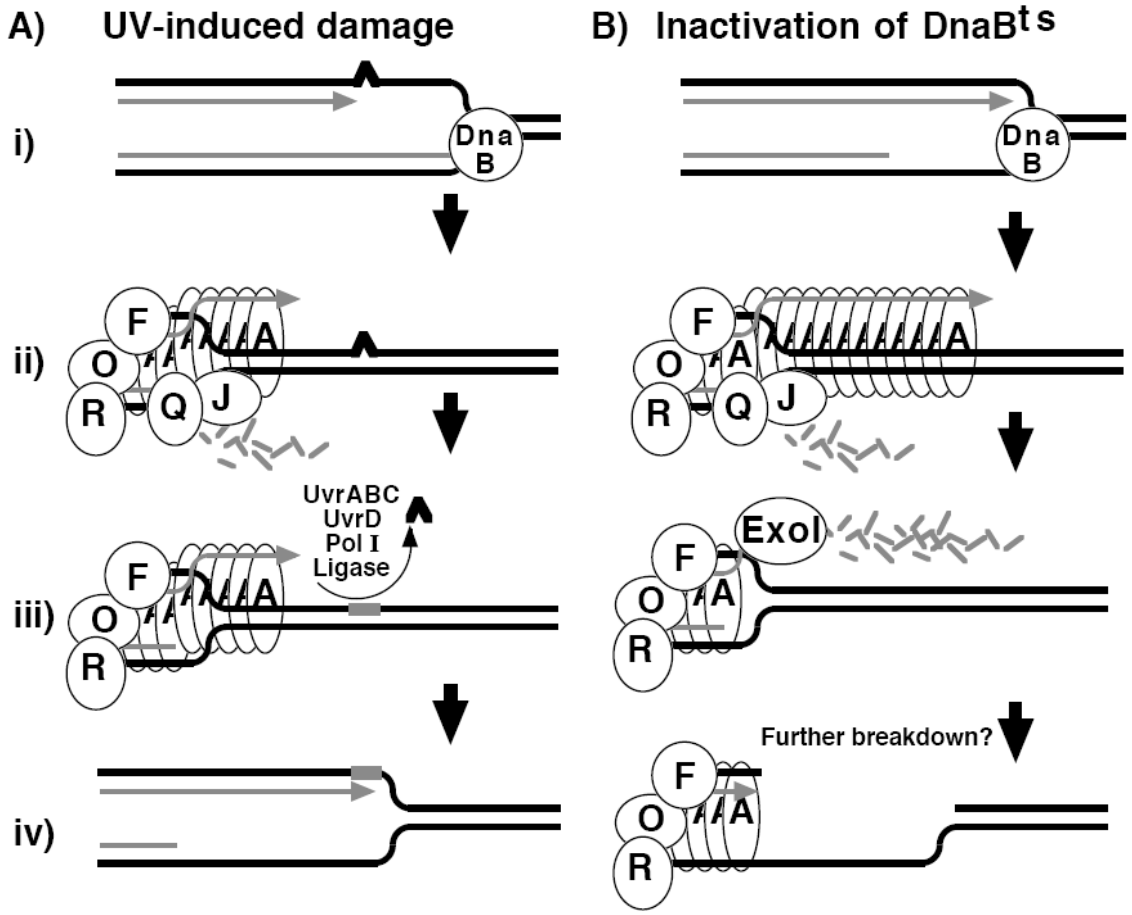

Figure 2.6. Model of enzymatic activities detected at replication forks arrested A) by UV-induced damage or B) following inactivation of DnaB^{ts}.

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CHAPTER III

WHAT HAPPENS WHEN REPLICATION STOPS: THE AFFECT OF PROTEIN INACTIVATION ON DNA REPLICATION

Abstract

DNA replication is among the most important functions occurring in the cell. The process is very specific and susceptible to impediments to its progression. These impediments may lead to mutagenesis, DNA rearrangements and/or cell death. To understand the events occurring at the replication fork following DNA damage, we examined two proteins of the replication machinery, DnaB and DnaE, to get a closer look at what happens at the point of replication arrest. We used thermosensitive mutants, *dnaB*^{ts} and *dnaE*^{ts} to disrupt replication at the replication fork and then characterized the necessity of each protein to replication recovery. We found that replication is disrupted in the absence of either DnaB or DnaE. However, DnaE does not seem to contribute to replication recovery.

Introduction

Thermosensitive mutants are used to study the affect protein inactivation has on replication. Over the years, several *E. coli* mutants have been isolated that are thermosensitive for DNA replication (Carl, 1970; Fangman & Novick, 1968). In these

mutants, normal DNA replication is observed at 32°C; however, cellular DNA synthesis stops following a shift to the restrictive temperature of 42˚C (Hirota et al., 1968). The *dnaB* gene, which encodes a 3'-5' helicase that unwinds duplex DNA during replication, has been shown to contain a thermosensitive mutation (LeBowitz & McMacken, 1986; Sclafani & Wechsler, 1981; Wechsler & Gross, 1971; Carl, 1970; Kohiyama et al., 1966). This $dnaB^{ts}$ mutant has been observed to produce double strand breaks which occur as a result of incubation at the restrictive temperature (Michel et al., 1997; Hirota et al., 1968). The accumulation of double strand breaks is greater in *recBC* mutants, which are unable to repair this defect, and are reduced in *ruvABC* mutants, which are deficient in an enzyme complex to catalyze branch migration and resolution at DNA Holliday junctions (Seigneur et al., 2000; Seigneur et al., 1998; Michel et al., 1997). Several researchers have attempted to explain these observations, and have proposed that following replication arrest in $dnaB^{ts}$ mutants, RuvABC catalyses the displacement and regression of the nascent DNA at the replication fork (Flores et al., 2001; Seigneur et al., 2000; Seigneur et al., 1998; Michel et al., 1997). The nascent DNA of the regressed fork/Holliday junction can then be degraded by the RecBCD helicase-nuclease or collapse to form a double strand break if RuvABC resolves this intermediate. It is still unclear, however, if the observed double strand breaks arise at the replication fork directly or if the breaks accumulate at other replicated portions of the genome. Double strand breaks on the chromosome are also known to accumulate following the inactivation of other replicative proteins, including DnaN, DnaE, DnaG, PriA, and HolD (Baharoglu et al., 2006; Flores et al., 2004; Grompone et al., 2003; Flores et al., 2002;

Grompone et al., 2002; Flores et al., 2001). We assumed that inactivation of these replicative proteins would mimic the events that occur in the *dnaB*^{ts} mutants following a temperature shift to the restrictive 42°C temperature. The inactivation of the *dnaE*^{ts} mutant is compared to the inactivation of *dnaB*^{ts} following a temperature shift.

The *dnaE* gene encodes the gene for the primary polymerase (Pol III) that travels along the 3'-5' leading and lagging strand template joining deoxynucleotides together. Pol III is responsible for carrying out the process of synthesizing a new daughter strand of DNA using the parental strand as a template (reviewed in Talaro, 2005). In *E. coli*, three damage-inducible DNA polymerases, Pol II, Pol IV, and Pol V are able to incorporate bases opposite specific lesions in template DNA with a higher efficiency than the replicative polymerase, Pol III (Napolitano et al., 2000; Reuven et al., 1999; Tang et al, 1999). However, the way in which these polymerases contribute to the resumption of DNA synthesis at arrested forks is yet to be agreed upon in the literature (Courcelle et al., 2005).

It is important to understand what happens to the replication fork when different obstacles impede its progression in order to identify the genes necessary for replication recovery. Some of the defects encountered by the replication fork following DNA damage that impede the progression through the genome include unusual secondary structures in the DNA sequence (Sinden et al., 1999), DNA-bound proteins (Kaplan & O'Donnell, 2002), or strand breaks in the template (Michel et al., 1997). Each of these impediments can block, arrest, or stall replication. How the replication fork reacts to and recovers in these situations is significant in order to maintain genomic stability

throughout the lifespan of the organism. To understand what happens at the replication fork when different obstacles impede its progression, we characterized the events that occur when replication is arrested by DNA damage or disrupted by unstable replication proteins. In the present study, the following processes were determined: the degree to which the blocked impediment prevents further replication, whether the blocked fork is maintained or degraded, whether the replication backs up prior to recovery, and if the blockage results in cell death. (Fig 3.1).

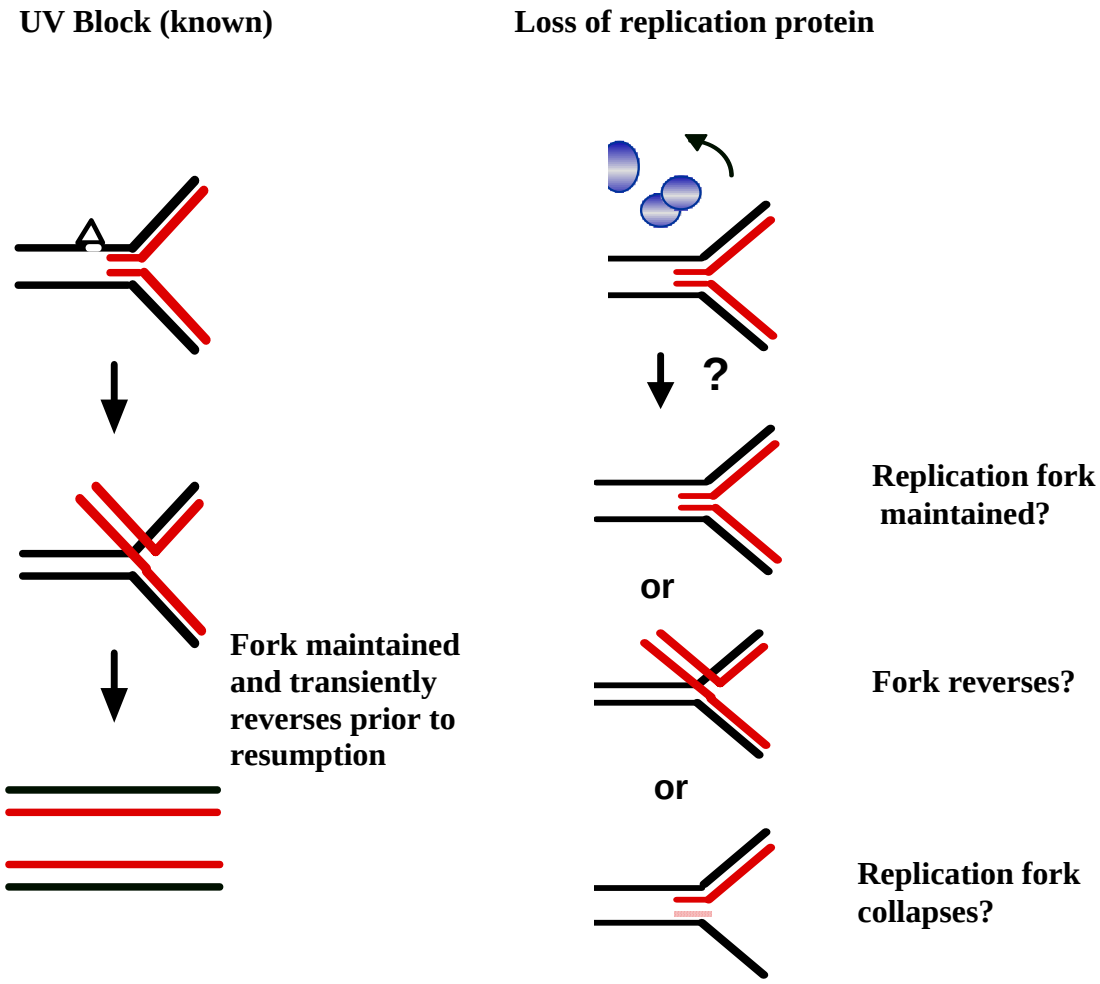

Figure 3.1. Events that may occur following DNA damage of the replication fork.

Replication events that occur following UV irradiation are known. Following DNA damage by UV irradiation, a lesion causes the replication machinery to arrest. RecF pathway genes maintain the replication fork and the nascent strands are transiently displaced prior to resumption of replication. The events occurring following inactivation of a replication protein are unknown. The following events may occur in this case: the fork may be maintained, reverse, or collapse.

Materials and Methods

Bacterial Strains

All bacterial strains are in an SR108 background. SR108 is a *thyA36 deoC2* derivative of W3110 (Mellon and Hanawalt, 1989). The genotype of CRT266 is *thr, leu, met, thyA, deo, supE, tonA, dnaB266* (Sclafani and Wechsler, 1981; Kohiyama et al., 1966). The genotype of E486 is *thr, leu, thyA, deo, lac, rpsL, tonA, Sull+, dnaE486* (Wechsler & Gross, 1971).

Rate of DNA synthesis

Fresh overnight cultures were diluted 1:100 in 50 ml DGCthy medium supplemented with 0.1μCi/ml [^{14}C]thymine and grown to an OD_{600} of precisely 0.3 in a 32°C shaking water bath. At this time, half of the culture was filtered on Fisherbrand 0.45 μm 47mm general membrane filters and either resuspended in DGCthy media supplemented with 0.1 μCi/ml [^{14}C]thymine and irradiated with 27 J/m^2 (for UV treatment), or resuspended in 42ºC DGCthy media supplemented with 0.1 μCi/ml [^{14}C]thymine (for temperature shift). In each case, the other half of the culture was mock treated, filtered, and resuspended in 32ºC pre-warmed DGCthy media supplemented with 0.1 μCi/ml [^{14}C] thymine. At 5 min intervals, duplicate 0.5 ml aliquots of culture were pulse-labeled with 1μCi/ml [^{3}H]thymidine for a period of 2 min. The cells were then lysed and the DNA was precipitated by adding 5 ml ice-cold 5% trichloroacetic acid

(TCA). The precipitated DNA was collected on Millipore glass fiber prefilters, washed with ethanol, and the amount of [^{3}H]- and [^{14}C]-labeled DNA on each filter was determined by liquid scintillation counting.

DNA degradation

Fresh overnight cultures were diluted 1:100 in DGCthy media supplemented with 0.1μCi/ml [^{3}H]thymine and grown to an OD_{600} of 0.4 in a 32°C shaking water bath. At this time, cultures were pulse-labeled with 1 μCi/ml [^{3}H]thymidine for 5 seconds, collected on Fisherbrand general filtration 0.45 μm membranes, washed with 1X NET buffer (100mM NaCl, 10mM Tris-pH 8.0, 10mM EDTA-pH 8.0), and resuspended in pre-warmed 42°C non-radioactive DGCthy media. At the indicated times, duplicate 200μl aliquots (triplicate for time 0) of cells were lysed and the DNA precipitated in 5 ml of ice-cold 5% trichloroactetic acid (TCA). Samples were then collected on Millipore glass fiber prefilters, washed with ethanol, and the amount of [^{3}H]- and [^{14}C]-labeled DNA on each filter was determined by liquid scintillation counting

One-dimensional agarose gel analysis

Fresh overnight cultures containing the plasmid pBR322 were grown in the presence of ampicillin (100μg/ml). The overnight cultures were pelleted and resuspended in 100 times the volume of fresh DGCthy media without ampicillin and grown in a shaking 32°C water bath to an OD_{600} of 0.5. At this time, the cultures were collected on 0.45 μm 47mm Fisherbrand general filters and either resuspended in 32ºC DGCthy media

and UV-irradiated with 50 J/m^2 (for UV treatment) or resuspended in 42°C DGCthy media (for temperature shift). At the indicated times, 0.75 ml aliquots of each culture were placed into 0.75 ml cold 2X NET buffer. Each sample was immediately pelleted in a microcentrifuge and resuspended in 150 µl of 1 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10mM Tris [pH 8.0], 1mM EDTA). Samples were then left on ice for the duration of the time course. All samples were then incubated at 37°C for 30 min before 10 µl proteinase K (10 mg/ml) and 20 µl 20% sarkosyl was added, and incubation continued for 1 hr at 55°C. Samples were extracted twice with 4 volumes (600 µl) of phenol:chloroform:isoamyl alcohol (25:24:1), and once with 4 volumes of chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed for 3 hours on 47 mm Whatman 0.05 µm pore disks against 250 ml of 0.2X TE (10mM Tris - pH 8, 1 mM EDTA) buffer. Samples were digested with Pvu*II* (New England Biolabs), extracted once with chloroform:isoamyl alcohol, and loaded onto the gel. For the 1st dimension, restricted genomic DNA samples were electrophoresed in 0.4% agarose in 1X Tris-Borate-EDTA (TBE) at 1 V/cm for 15 hr. The DNA in the gels was transferred to Hybond N+ nylon membranes and probed with pBR322 that had been labeled with ^{32}P by nick translation according to the protocols supplied by Roche Applied Science using [α-^{32}P]-dCTP (MP Biomedicals). Radioactivity was visualized and quantitated using a STORM Molecular Dynamics PhosphoImager and its associated ImageQuant Software (Molecular Dynamics/Pharmacia).

Results

DNA replication is disrupted in both dnaB^{ts} and dnaE^{ts} mutants.

The DnaB protein of *E. coli* encodes a replication fork helicase that is responsible for unwinding the duplex DNA at the replication fork during DNA replication. The DnaE protein of *E. coli* encodes the primary DNA polymerase which travels along the leading and lagging template strands and joins deoxynucleotides together. A temperature sensitive allele of the DnaB protein, *dnaB266* in strain CRT266, and DnaE protein, *dnaE486* in strain E486, was utilized to inactive the replication helicase and therefore, disrupt replication progress. Cellular DNA synthesis occurs normally at 30°C, but fails to continue after a shift to the restrictive temperature of 42°C in these mutants (Hirota et al., 1968).

DNA replication arrest is verified in the *dnaB266* and *dnaE486* mutants by monitoring the rate of DNA synthesis over time. Cultures were grown at 32°C in media containing [^{14}C]thymine and then temperature shifted to 42°C. To monitor how the rate of DNA synthesis was affected by the temperature shift, duplicate 0.5 ml aliquots of the [^{14}C]-labeled cultures were pulse-labeled with [^{3}H] thymidine for 2 min at periodic intervals before and after treatment. The rate of synthesis (^{3}H incorporation/min) could then be determined relative to the total amount of DNA present (^{14}C incorporation) at each time. We observed that a shift in temperature to 42°C inhibited the rate of DNA synthesis immediately following treatments in both *dnaB266* and *dnaE486* mutants (Fig 3.2). In DnaB, the rate of DNA synthesis was inhibited approximately 90% and did not

resume when held at 42°C. The rate of DNA synthesis in DnaE was inhibited to a lesser extent (~50%) and was unable to resume replication when held at 42°C. These results are consistent with the view that DnaB and DnaE are essential for replication to resume following disruption.

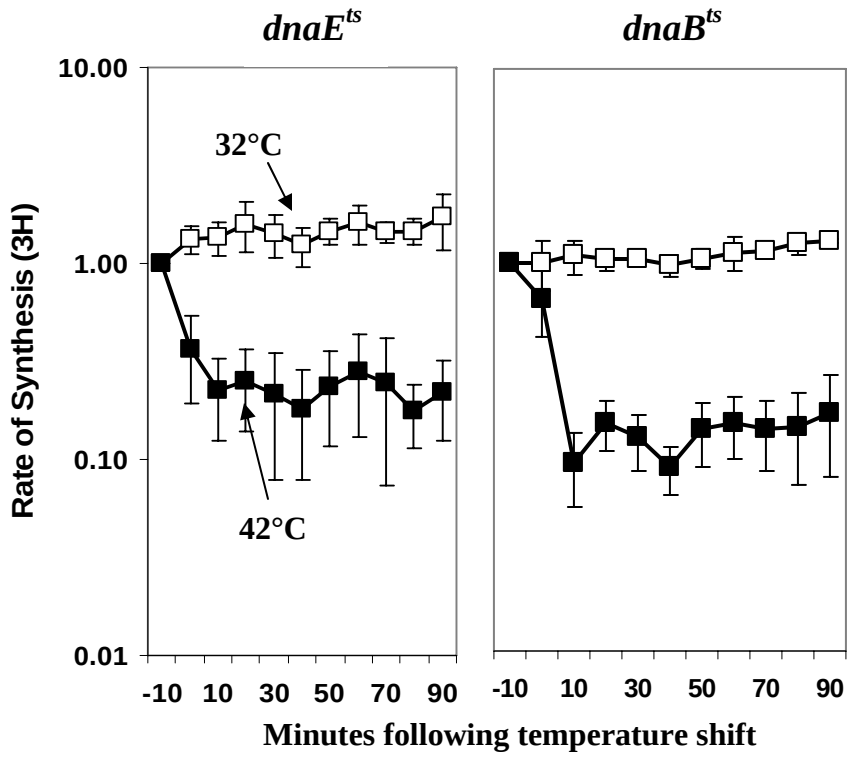

Figure 3.2. Synthesis is disrupted in both *dnaB* and *dnaE* mutants.

Cultures grown in the presence of [^{14}C] thymine were pulse-labeled with [^{3}H] thymidine for 2 min. at the indicated times following a shift to 42°C. The relative amount of ^{3}H incorporated into the DNA is plotted over time. Rate of DNA synthesis in mock temperature shifted cultures (□); rate of DNA synthesis in temperature shifted cultures (■).

Inactivation of DnaE does not lead to degradation of the nascent DNA.

Synthesis is disrupted in both *dnaE*^{ts} and *dnaB*^{ts} mutants after inactivation by a temperature shift to 42°C. To determine if the replication forks disrupted following protein inactivation are protected and maintained from extensive degradation, we observed the degradation patterns of dnaE^{ts} and dnaB^{ts} mutants. Cultures that were pre-labeled with [^{14}C]thymine were pulse-labeled with [^{3}H]thymidine for 5 seconds immediately before they were transferred to non-radioactive medium and shifted to 42°C. The amount of [^{3}H] and [^{14}C] remaining in the DNA was then followed over time. With this assay, we were able to compare the amount of 3H-labeled DNA lost at the arrested replication fork to the loss of [^{14}C]-labeled DNA that occurred over time (Fig. 3.3). Similar to the parental strain (SR108), *dnaE*^{ts} mutants exhibited no degradation of the nascent DNA following a temperature shift to 42°C. The increase in ^{3}H-labeled DNA that occurs in these strains immediately after the temperature shift is due to the incorporation of the remaining intracellular pools of [^{3}H]thymidine as replication continues (Courcelle & Hanawalt, 1999; Courcelle et al., 1997). In contrast, cultures of *dnaB*^{ts} exhibited nascent DNA degradation of approximately 50% following a shift to 42°C. This degradation is consistent with the need for the replicative helicase in nascent DNA processing following other types of damage-induced arrests, like UV-irradiation (Belle et al., submitted).

DnaE^{ts} *does not play a significant role in replication recovery.*

Both DnaB and DnaE protein inactivation disrupt replication. To determine if these proteins play a role in recovering disrupted DNA replication, we compared the total

accumulation of DNA before and after the temperature shift to 42°C. We measured the total DNA accumulation by [^{3}H]thymine incorporation into the DNA over time. DnaB mutants do not synthesize DNA following a temperature shift to 42°C indicating the importance of this protein in replication recovery (Fig 3.3). However, DnaE mutants are still able to synthesize DNA following a temperature shift to 42°C.

Another method of analyzing the ability to synthesize DNA following replication disruption in the thermosensitive mutants is observing the size of the DNA fragments of each mutant following a temperature shift to 42°C using one dimensional agarose gel electrophoresis. With this method, the structural properties of replicating DNA fragments can be identified by their size. The plasmid pBR322 is useful because it has a high copy number of plasmids per cell. To compare the two mutants, *dnaB*^{ts} and *dnaE*^{ts}, cultures containing the plasmid pBR322 were shifted to the restrictive temperature of 42°C. At various times following the temperature shift, the genomic DNA was purified, digested with the restriction endonuclease, Pvu*II* (which linearizes pBR322 just downstream of the unidirectional origin of replication), and separated on an agarose gel. Using a STORM Molecular Dynamics PhosphoImager and its associated ImageQuant analysis software, the relative amount of DNA at each sample time was measured. Figure 3.4A shows the 1-D gel analysis of the *dnaB*^{ts} and *dnaE*^{ts} mutants following a temperature shift to 42°C. DNA replication stops in the *dnaB*^{ts} mutant at nonpermissive temperatures, but is unaffected in the *dnaE*^{ts} mutant following a shift to the restrictive temperature of 42°C (Fig 3.4B).

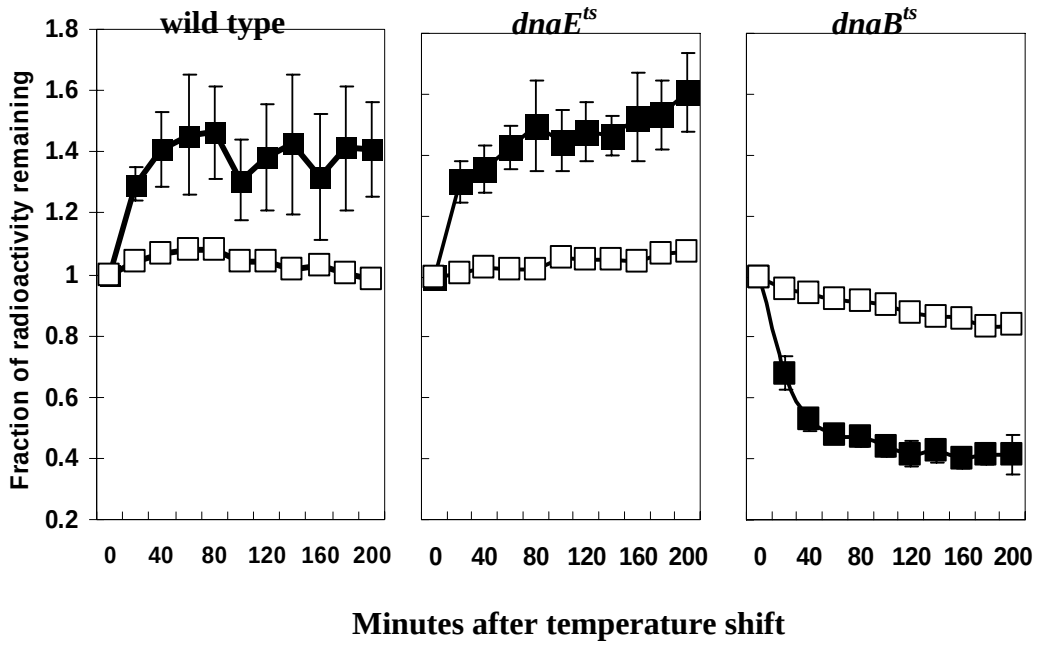

Figure 3.3. The inactivation of DnaB^{ts} leads to extensive nascent strand degradation after replication arrest, but no degradation is observed in dnaE^{ts} mutants.

[^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cells for 5 seconds prior to a temperature shift to 42°C. The relative amount of degradation in the nascent DNA (■) and total genomic DNA (□) is plotted following a temperature shift to 42°C. Graphs represent an average of at least three independent experiments. Error bars represent 1 standard deviation.

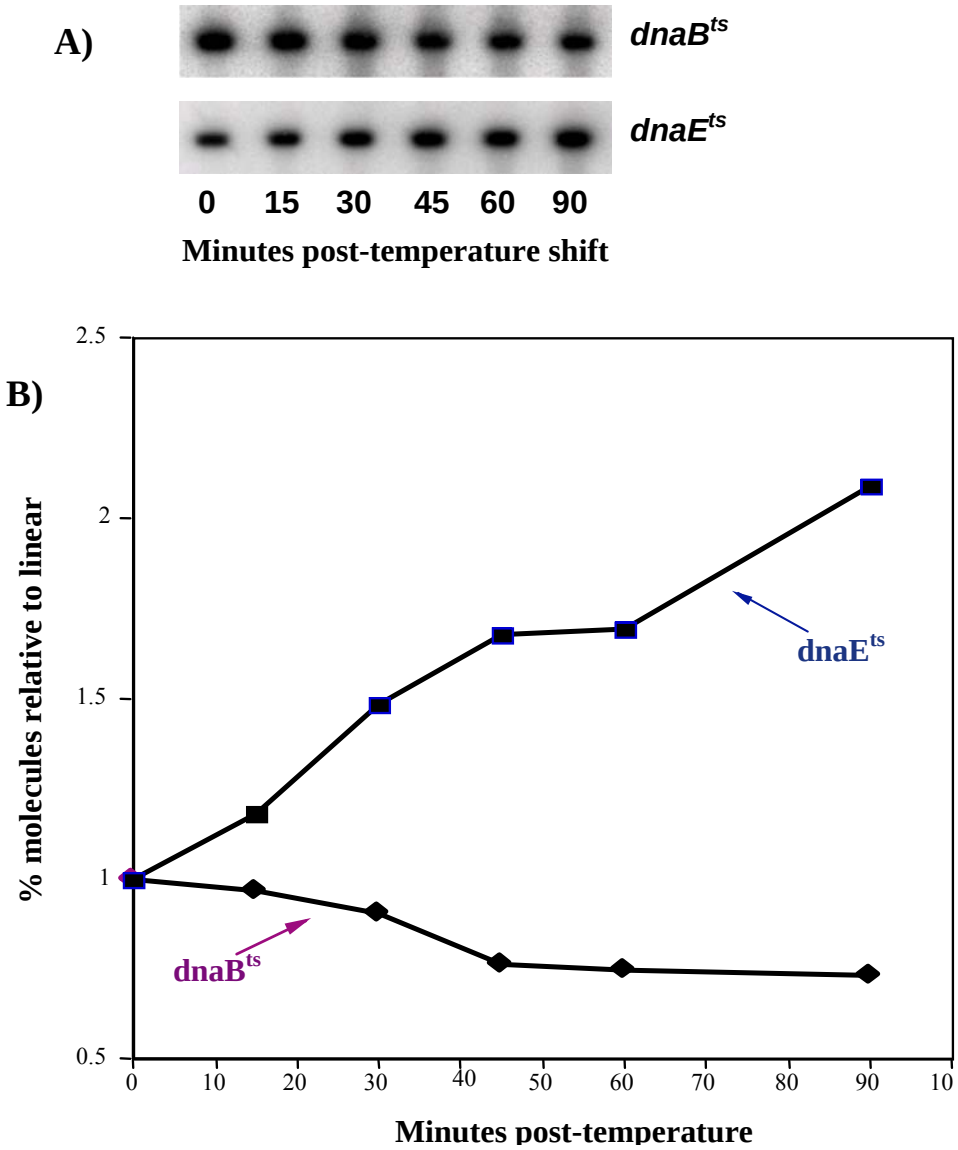

Figure 3.4. Following a shift to nonpermissive temperatures, DNA replication in the *dnaB*^{ts} mutant is blocked, while the *dnaE*^{ts} mutant retains its ability to replicate.

dnaB^{ts} and *dnaE*^{ts} strains containing the plasmid pBR322 were grown in minimal media at 32°C to an OD_{600} of 0.4, and then shifted to the nonpermissive temperature (42°C). Genomic DNA was isolated at various times after temperature shift, restricted with Pvu*II*, which cuts near the origin of replication of the plasmid pBR322, and separated on an agarose gel. The DNA was transferred to a nylon membrane and hybridized with a ^{32}P-labeled probe to pBR322. Results were quantitated using a PhosphorImager.

Discussion

A variety of biologically important impediments such as DNA damage, strand breaks in the template, DNA-bound proteins, unusual secondary structures, or unstable replication machinery may cause inaccurate replication and prevent the successful replication of parental cells into two identical daughter cells. However, the mechanisms by which these impediments are repaired have proven to be varied depending on the way in which replication is arrested. To understand how replication forks recover from different obstacles that impede it progression, it is first important to know which proteins are most essential to replication. In this study, we compared the events occurring at replication forks disrupted due to unstable replication proteins. Mutants of $DnaB^{ts}$ and $DnaE^{ts}$ were used to determine whether these replication machinery proteins are essential in replication recovery

Originally, we assumed that inactivating either replication protein (whether it was the helicase or the polymerase) would result in the same extent of damage. Previously, we reported that inactivation of the DnaB helicase causes an immediate arrest of synthesis that does not recover and is not repaired (Belle et al., submitted). These results are consistent with the idea that the mutants are "quick-stop" mutants in which an immediate arrest of synthesis occurs when the cells are exposed to temperatures above 40°C (Carl, 1970; Hirota et. al, 1968).

To determine if the helicase and polymerase are capable of acting at sites of replication arrest, we examined the ability of DnaB and DnaE to protect the nascent DNA at the arrested replication fork from damage. In previous studies, the nascent degradation

assay has been used to show that the nascent lagging strand of arrested replication forks is subject to degradation by the RecJ nuclease and RecQ helicase at times prior to the resumption of replication (Courcelle & Hanawalt, 1999). Cells that are able to recover replication show a decrease or halt in the degradation of the nascent DNA when replication resumes. Mutants that are unable to recover DNA synthesis, show a more extensive degradation in nascent DNA (Courcelle et al., 2003, Courcelle et al., 1999; Courcelle et al., 1997). Unexpectedly, *dnaB*^{ts} and *dnaE*^{ts} did not have the same pattern of nascent degradation. In the *dnaB*^{ts} mutant, degradation was extensive following incubation at the restrictive temperature; consistent with the accumulation of double strand breaks in the genome (Belle et al., submitted, Michel et al., 1997). The *dnaE*^{ts} mutant did not have a pattern of degradation, but instead, was similar to the parental strain in which there was an increase in the ^{3}H-labeled DNA. This increase is due to the incorporation of the remaining intracellular pools of [^{3}H]thymidine as replication continues (Courcelle & Hanawalt, 1999; Courcelle et al., 1997). DnaE encodes the primary polymerase (Pol III), but is not the only polymerase that is activated in the presence of DNA damage. Polymerase II (Pol II), Pol IV, and Pol V are three other damage-inducible DNA polymerases encoded by *Escherichia coli* in the presence of DNA damage (Courcelle et al, 2005; Napolitano et al., 2000). The activation of one or more of these polymerases would explain why the nascent degradation is limited in the absence of the primary polymerase protein, DnaE.

We find that the DnaB may have a more essential role than DnaE in replication recovery because it serves as the primary helicase to the cell. For the parent leading and

lagging strands to be duplicated, the helicase must first travel along the duplex strands and separate them. In the absence of DnaB, the strands are not unwound and DNA replication cannot proceed. This leads to the decrease in cell survival and eventually to the collapse of the replication fork. However, in the absence of the DnaE polymerase, other cellular polymerases are able to help maintain the integrity of the replication fork until the appropriate repair mechanism can repair or bypass the damage.

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CHAPTER IV

SUMMARY

Although there is a wide array of organisms, both simple and complex, that are different both genetically and phenotypically, processes of DNA replication and repair seemed to be conserved throughout evolutionary divergent organisms. One of the most useful model organisms for studying DNA replication and repair is *Escherichia coli* because it is already well characterized and has been shown to share genes that are homologous to humans (Courcelle & Hanawalt, 1999). Using this organism, a better understanding of the processes of replication recovery can be gained and these findings may be extrapolated to comparable processes in more complex organisms.

To determine the molecular events that can lead to replication arrest and the different repair processes that contribute to the recovery of DNA replication, it was first important to identify the substrates and intermediates produced when DNA damage is encountered. Chapter I introduced the replication fork structure and the repair processes the cell utilizes to repair itself in the event of DNA damage. Several *recF* pathway genes are required to maintain and stabilize the replication fork when DNA lesions are encountered. These genes include RecA, RecF, RecO, and RecR, which are required to restore replication following arrest by UV-induced DNA lesions (Courcelle et al., 1997; Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Horii &Clark, 1973). Other RecF pathway proteins, RecJ and RecQ, selectively degrade the nascent lagging strand at

blocked replication forks prior to the resumption of DNA synthesis (Courcelle & Hanawalt, 1999).

UV irradiation induces lesions that can either block the progression of the replication machinery or produce nascent-strand gaps depending on which template strand contains the lesion (Higuchi et al., 2003; Carty et al., 1996; Mitchell & Nairn, 1989; Chan et al., 1985; Setlow et al., 1963). Two mechanisms, nucleotide excision repair and translesion DNA synthesis, operate to reduce the frequency of recombination and promote cell survival following DNA damage (Courcelle et al., 1999; Bagg et al., 1981; Kato & Shinoura, 1977; Rupp & Howard-Flanders, 1968) (Fig 4.1).

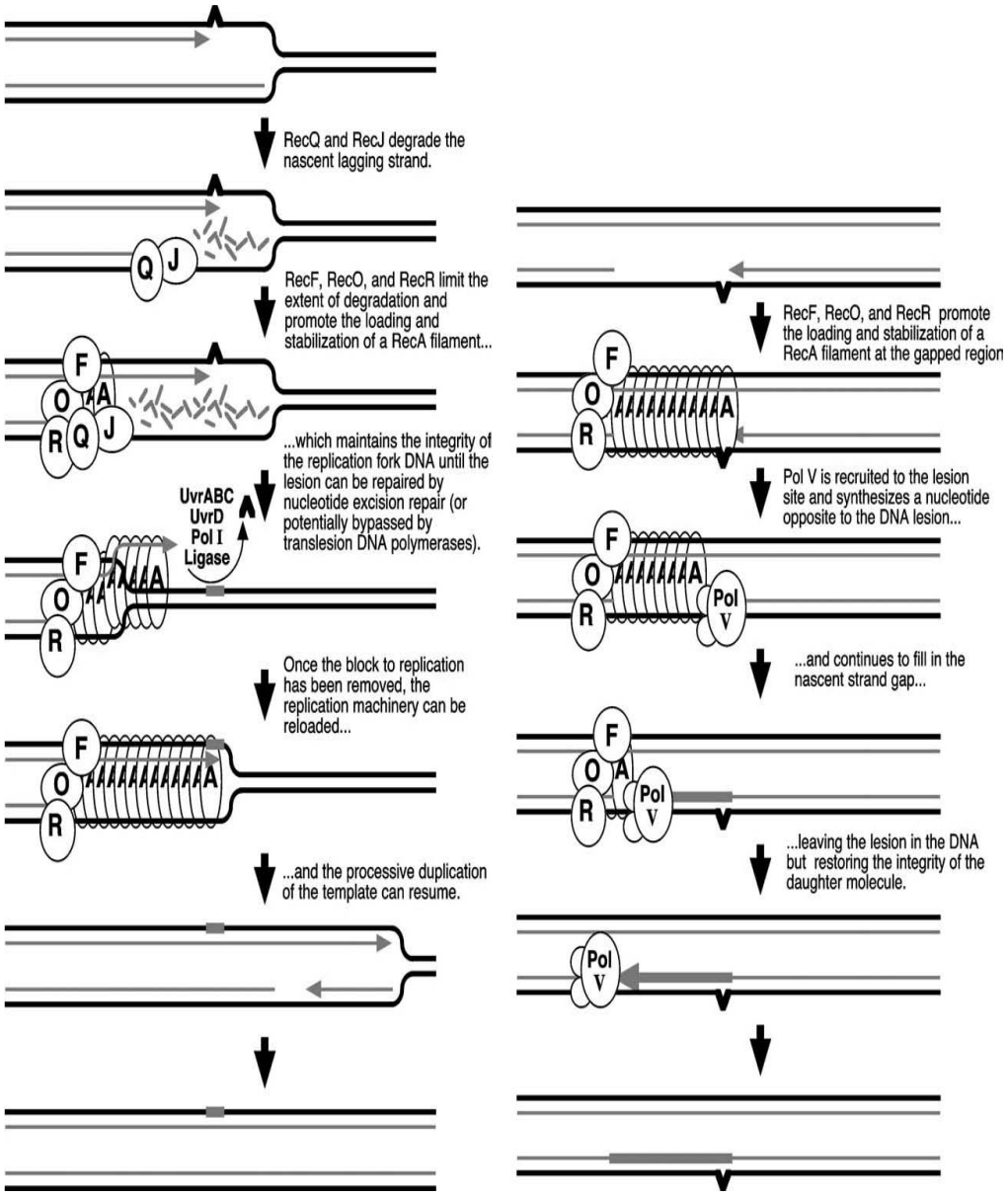

Figure 4.1. Models for (A) the repair of a DNA lesion that arrests DNA replication and (B) the tolerance of a DNA lesion that does not arrest DNA replication.

In Chapter II, we characterized the events that occur when replication is arrested by DNA damage and compared the event to those which occur when replication disruption is caused by unstable replication proteins. This characterization involved determining the degree to which the impediment prevents further replication, whether the blocked fork is maintained or degraded, if the replication fork backs up prior to recovery and if the blockage results in cell death. We found that inactivation of the DnaB helicase causes the replication machinery to collapse and leads to the extensive degradation of the replication fork. Both UV irradiation and thermosensitive replication mutants (*dnaB*^{ts}) were used as models to disrupt replication and characterize the general mechanism by which replication forks recover. Although both UV-irradiation and inactivation of DnaB inhibit the progression of the DNA replication machinery, unique processing events and replication intermediates are observed to accumulate following disruption. Following UV irradiation, replication is only transiently inhibited and the nascent DNA at replication forks is protected from degradation by the RecJ nuclease by the action of the RecF protein. By contrast, replication does not recover following inactivation of the replication protein, DnaB, and the nascent DNA was extensively degraded irrespective of whether RecF was present. In addition, unique structural intermediates were observed to accumulate in each case. These studies indicate that the mechanism and enzymes that operate at arrested replication forks depend on the nature of the impairment to replication progression.

Inactivation of the DnaB helicase leads to the disruption of the replication machinery and extensive degradation of the replication fork. However, chapter III

focuses on the effects of inactivating the primary polymerase, DnaE. This inactivation is compared to inactivation of DnaB. Through our studies, it is shown that inactivation of DnaE causes less detriment to replication recovery, perhaps indicating the possibility of inactivation of other damage-inducible polymerases which can prevent extreme DNA damage to the cell.

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APPENDIX A

PUBLISHED PAPER IN WHICH AUTHOR OF DISSERTATION PERFORMED UV MUTAGENESIS EXPERIMENTS

NUCLEOTIDE EXCISION REPAIR OR POLYMERASE V-MEDIATED LESION BYPASS CAN ACT TO RESTORE UV-ARRESTED REPLICATION FORKS IN *ESCHERICHIA COLI*

Charmain T. Courcelle, Jerilyn J. Belle, and Justin Courcelle

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Abstract

Nucleotide excision repair and translesion DNA synthesis are two processes that operate at arrested replication forks to reduce the frequency of recombination and promote cell survival following UV-induced DNA damage. While nucleotide excision repair is generally considered to be error free, translesion synthesis can result in mutations, making it important to identify the order and conditions that determine when each process is recruited to the arrested fork. We show here that at early times following UV irradiation, the recovery of DNA synthesis occurs through nucleotide excision repair of the lesion. In the absence of repair, or when the repair capacity of the cell has been exceeded, translesion synthesis by polymerase V (Pol V) allows DNA synthesis to resume and is required to protect the arrested replication fork from degradation. Pol II and Pol IV do not contribute detectably to survival, mutagenesis, or restoration of DNA synthesis, suggesting that, in vivo, these polymerases are not functionally redundant with Pol V at UV-induced lesions. We discuss a model in which cells first use DNA repair to process replication-arresting UV lesions before resorting to mutagenic pathways such as translesion DNA synthesis to bypass these impediments to replication progression.

Introduction

Irradiation of cells with 254-nm UV light induces lesions that block DNA polymerases. Lesions that block polymerases are thought to either arrest the progress of the replication machinery or produce nascent-strand gaps depending on which template strand contains the lesion (Higuchi et al., 2003; Veaute et al., 1997; Veaute & Sarasin, 1997; Carty et al., 1996; Svoboda & Vos, 1995; Mitchell & Nairn, 1989; Chan et al., 1985; Setlow et al., 1963). Several studies using plasmid substrates indicate that lesions in the leading-strand template arrest the overall progression of the replication fork, with the nascent lagging strand continuing a short distance beyond the arrested leading strand (Higuchi et al., 2003; Veaute et al., 1997; Svoboda & Vos, 1995). In contrast, lesions in the lagging-strand template are thought to generate gaps in the nascent DNA strand at sites opposite to the lesion, presumably because discontinuous synthesis of the lagging strand allows the blocked polymerase to reinitiate downstream of the lesion site (McInerney & O'Donnell, 2004; Higuchi et al., 2003; Svoboda & Vos, 1995). Events that are consistent with this can also be seen on the chromosome of UV-irradiated *Escherichia coli*. Following a moderate dose of UV irradiation, the rate of DNA synthesis is transiently inhibited before it efficiently recovers at a time that correlates with lesion removal (Courcelle et al., 2003; Setlow et al., 1963). During this period of inhibition, some limited DNA synthesis is still observed that contains gaps, consistent with replication continuing past a subset of the lesions in the template (Ganesan, 1974; Rupp et al., 1971; Rupp & Howard-Flanders, 1968). The repair and restoration of the DNA template in each of these two situations may involve unique

enzymatic pathways and are likely to have different consequences for the cell with respect to survival and mutagenesis.

Lesions that arrest the overall progression of the replication machinery would be expected to prevent the replication of the genome and are likely to result in cell lethality if the block to replication cannot be overcome. The ability of *E. coli* to survive doses of UV irradiation that produce thousands of lesions per genome clearly indicates that efficient mechanisms to deal with replication-arresting lesions exist in the cell. Several proteins associated with the *recF* pathway, including RecA, RecF, RecO, and RecR, are required to restore replication following arrest by UV-induced DNA lesions (Rangarajan et al., 2002; Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Courcelle et al., 1997; Horii & Clark, 1973). In the absence of any of these genes, UV-irradiated cells fail to recover DNA synthesis following arrest, gaps persist in the DNA synthesized post-irradiation, and the nascent DNA at the replication fork is extensively degraded (Courcelle et al., 2003; Rangarajan et al., 2002; Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Courcelle et al., 1997; Rothman & Clark, 1977; Smith & Meun, 1970; Horii & Suzuki, 1968). In vitro, RecA, RecF, RecO, and RecR promote pairing between single-strand DNA and homologous duplex DNA (Kantake et al., 2002; Bork et al., 2001; Webb et al., 1997; Shan et al., 1997; Webb et al., 1995), an activity that was originally characterized for its role in bringing together homologous strands of DNA during recombinationalprocesses (Clark & Margulies, 1965). Cellular assays indicate that the sameenzymatic activity is also required during replication to maintain and process the homologous strands of the replication fork when the normal progression of thereplication machinery is prevented (reviewed in Courcelle et al., 2001). Other *recF* pathway

proteins, RecQ, a 3'-5' DNA helicase, and RecJ, a 5'-3' single-strand exonuclease, selectively degrade the nascent lagging strand at blocked replication forks prior to the resumption of DNA synthesis (Courcelle &Hanawalt, 1999). Degradation of nascent DNA by RecJ and RecQ facilitatesthe timely recovery of DNA synthesis in normal cells and is thought to play a rolein suppressing the frequency of illegitimate recombination, perhaps by generating a more extensive substrate for RecA to bind and stabilize at the blocked replication fork (Courcelle et al., 2003; Courcelle & Hanawalt, 1999; Hanada etal., 1997). Consistent with this interpretation, RecQ homologs in yeast, *Drosophila melanogaster*, and humans have been shown to play critical roles maintaining processive replication and suppressing the frequency of DNA strand exchanges (reviewed in Karow et al., 2000). These observations have led to a general model in which RecA and several *recF* pathway gene products act to maintain and process the arrested replication fork so that repair enzymes or alternative DNA polymerases can gain access to the blocking lesion (Courcelle etal., 2003; Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Courcelle et al., 1997). In this way, processive replication is maintained while avoiding strand exchanges that may lead to recombination or rearrangements.

Two mechanisms that operate to reduce the frequency of recombination and promote cell survival following DNA damage are nucleotide excision repair and translesion DNA synthesis (Courcelle et al., 1999; Bagg et al., 1981; Kato Shinoura, 1977; Rupp & Howard-Flanders, 1968). Both processes have been proposed to operate at lesion-arrested replication forks to allow DNA synthesis toresume following arrest (Courcelle et al., 1999; Napolitano et al., 2000; Rangarajan et al., 1999). In *E. coli*, the *uvrA*, *uvrB*, and *uvrC* gene products form an excinuclease that is required to initiate

nucleotide excision repair of UV-induced lesions (reviewed in Sancar, 1996). Cells deficient in lesion removal areseverely impaired in their ability to resume robust DNA replication and exhibit elevated levels of recombination, genomic rearrangements, and cell lethality(Courcelle et al., 2003; Courcelle et al., 1999; Howard-Flanders e al., 1969; Setlow et al., 1963). In wild-type cells, the time at which robust replication resumes correlates with the removal of the lesions by nucleotide excision repair (Courcelle et al., 2003). These observations have been interpreted to support theidea that nucleotide excision repair is a prominent mechanism that operates at replication-arresting DNA lesions (Courcelle et al., 2003). However, sincenucleotide excision repair is required to remove all lesions throughout the genome, it remains possible that an alternative process, such as translesion DNAsynthesis, predominantly operates at lesion-arrested replication forks and that the failure to observe robust replication resumption in *uvr* mutants occurs due to there-arrest of replication at subsequent downstream lesions.

E. coli encodes three damage-inducible DNA polymerases that have multiple homologs in both prokaryotes and eukaryotes (Sutton & Walker, 2001). In vitro, polymerase II (Pol II) (*polB*), Pol IV (*dinB*), and Pol V (*umuDC*) are able to incorporate bases opposite to specific lesions in template DNA with higher efficiency than the replicative polymerase, Pol III (34, 39, 52).There are differing reports in the literature as to the contribution of these polymerases to the resumption of DNA synthesis at arrested forks (Rangarajan et al, 1999; Witkin et al., 1987; Khidhir et al., 1985). An initial study reported that the recovery of DNA synthesis occurred more slowly in the absence of nucleotide excision repair following UV-induced damage, but was not affected by the absence of Pol V (Khidhir et al., 1985). However, a subsequent study using repair-

deficient mutants found that Pol V was essential and sufficient for DNA synthesis to resume in the absence of repair following exposure to low UV doses in a *recA718* background (Witkin et al., 1987). Still a third study reported that the absence of Pol II delayed the recovery of DNA synthesis after UV-induced damage, even when Pol V and nucleotide excision repair were functional (Rangarajan et al., 1999). However, in an earlier study, this group did not find any contribution of Pol II to lesion bypass following UV irradiation (Kow et al., 1993). In addition, numerous studies have shown that Pol V, but not Pol II or Pol IV, increases the survival and frequency of mutagenesis in UV-irradiated *E. coli* (Elledge & Walker, 1983; Bagg et al., 1981; Steinborn, 1978; Kato & Shinoura, 1977). These observations suggest that translesion DNA polymerases may operate at lesion arrested replication forks and raise the possibility that translesion synthesis could be a predominant mechanism that restores DNA synthesis at lesion-arrested replication forks in wild-type cells. While it is apparent that the translesion DNA polymerases play an important role in cellular mutagenesis and genome stability after DNA damage, exactly where and when they operate in the cell are not clear. To date, no study has directly compared the activities of the translesion DNA polymerases and nucleotide excision repair to determine the time and frequency at which each process occurs following UV-induced arrest in wild-type cells.

Utilization of nucleotide excision repair or translesion DNA synthesis at anarrested fork may have very different biological consequences for the cell. Whereas lesion removal by nucleotide excision repair is generally considered to be error free, translesion DNA synthesis by enzymes such as Pol V is responsiblefor most of the mutagenesis that results from UV-induced DNA damage (Kato & Shinoura, 1977; Kim et

al., 1997). In this study, we characterized the recovery of replication following arrest in mutants lacking either nucleotide excision repair, the three damage-inducible DNA polymerases, or both to identify when these processes are recruited to the arrested fork in wild-type cells in order to gain a better understanding of how DNA replication is restored following disruption by UV-induced DNA damage.

Material and Methods

Bacterial strains

All bacterial strains are in an SR108 background. SR108 is a *thyA36 deoC2* derivative of W3110 (Mellon & Hanawalt, 1989). HL952 (SR108 *uvrA::*Tn*10*)and CL579 (SR108 *recF6206::*Tet^r) have been described previously (Courcelle et al., 2003; Courcelle et al., 1999). CL575 (SR108 *umuC122::*Tn*5*), CL632 (SR108*umuDC595::cat*), CL634 (SR108 *dinB::*Kan^r), and CL636 (SR108 polB::Ω Sm-Sp) were constructed by P1 transduction of *umuC122::*Tn*5*, *umuDC595::cat*, *dinB::*Kan^r, and *polB*::Ω Sm-Sp from GW2100 (Elledge &Walker, 1983), RW82 (Woodgate, 1992), MGZdinB (Napolitano et al., 2000), and MGZpolB(Napolitano et al., 2000), respectively, into SR108. CL637 (SR108 *polB::*Ω Sm-Sp *dinB::*Kan^r) was constructed by P1transduction of *dinB::*Kan^r from MGZdinB (Napolitano et al., 2000) into CL636. CL646 (SR108 *polB::*Ω Sm-Sp *dinB::*Kan^r*umuDC595::cat*) was constructed by P1 transduction of *umuDC595::cat* fromRW82 into CL637. CL681 (SR108 *polB::*Ω Sm-Sp *dinB::*Kan^r *umuDC595::cat uvrA::*Tn*10*) was constructed by P1 transduction of *uvrA::*Tn*10* from HL952 into CL646. Phenotypes were confirmed by antibiotic resistance and, whenappropriate, sensitivity to

UV. Genotypes for *polB*, *umuDC*, and *dinB* strains wereconfirmed by PCR and Southern blot analysis.

UV survival

UV irradiations used a 15-W germicidal lamp (254 nm) at an incident dose of0.9 $J/m^2/s$ (0.2 $J/m^2/s$ for doses below 20 J/m^2). Cells were grown in Davismedium supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10 µg/ml thymine (DGCthy medium). Fresh overnight cultures were diluted 1:100 andgrown to an optical density at 600 nm (OD_{600}) of between 0.4 and 0.5 (approximately 6 x 10^8 cells/ml). Ten-microliter aliquots of serial 10-folddilutions were applied as spots in triplicate on Luria-Bertani plates containing 10 µg/ml thymine and UV irradiated at the indicated doses. Viable colonies werecounted following overnight incubation at 37°C.

UV-induced mutagenesis

Mutagenesis induced by UV was measured by the appearance of rifampin-resistant colonies as a result of UV exposure. At least 69 base substitutions within the rpoB gene have been identified that confer resistance to rifampin, allowing one to monitor numerous UV-induced mutation sites in different sequence contexts (Garibyan et al., 2003). Overnight cultures were diluted 1:100 andgrown in DGCthy medium to an OD_{600} of 0.4, at which point the culture was splitinto three equal fractions and irradiated with an incident dose of 0, 2, or 10 J/m^2UV. Following overnight incubation at 37°C, the cultures were plated on Luria-Bertani plates containing 10 µg/ml thymine and 100 µg/ml

rifampin. Rifampin-resistant colonies were counted following overnight incubation at 37°C.

DNA synthesis and accumulation

Overnight cultures were diluted 1:100 and grown in DGCthy medium supplemented with 0.1 μCi/ml of [^{14}C]thymine to an OD_{600} of precisely 0.3, at which point half of the culture received an incident dose of 27 J/m^2 while the other half was mock irradiated. At the times indicated, duplicate 0.5-ml aliquots of culture were pulse-labeled with 1 μCi/ml [^{3}H]thymidine for 2 min at 37°C. Cells were then lysed, and the DNA was precipitated in cold 5% trichloroacetic acid (TCA) and filtered onto Millipore glass fiber filters. The amounts of ^{3}H and ^{14}C on each filter were determined by scintillation counting.

DNA degradation

Overnight cultures were diluted 1:100 and grown in DGCthy medium supplemented with 0.1 μCi/ml [^{14}C]thymine to an OD_{600} of 0.4. [^{3}H]thymidine (1μCi/ml) was then added to the culture. After 5 s, cells were filtered onto a 0.45-μm membrane, rinsed twice with 5 ml of NET buffer (100 mM NaCl, 10 mMTris, pH 8.0, 10 mM EDTA, pH 8.0), resuspended in prewarmed, nonradioactive DGCthy medium, and irradiated with a UV dose of 27 J/m^2. At the times indicated, duplicate 0.2-ml aliquots (triplicate for 0 min) of the culture were precipitated in cold 5% TCA and filtered onto Millipore glass fiber filters. Theamounts of ^{3}H and ^{14}C were determined as before.

Alkali sucrose gradients

Overnight cultures were diluted 1:100 and grown in DGC medium supplemented with 0.9 μCi/4 μg/ml [^{14}C]thymine to an OD_{600} of 0.4. The culture was then UV irradiated with 27 J/m^2 before the addition of 9 μCi/ml [^{3}H]thymidine for 5 min at 37°C. Cells were filtered onto a 0.45-μm membrane,rinsed with NET buffer, and resuspended in prewarmed, nonradioactive DGCthymedium. At the times indicated, 0.5-ml aliquots of cells were collected into 0.5 mlice-cold 2% NET, pelleted, and resuspended in 0.1 ml buffered sucrose (10mMTris, pH 8.0, 10 mM EDTA, pH 8.0, 110 mM NaCl, 5.1% sucrose), and stored on ice for the duration of the time course. The equivalent of 2.5 x 10^6 cells was layered on 4.9-ml linear gradients of 5 to 20% (wt/vol) sucrose in 0.1 N NaOH with a 0.1-ml top layer of 5% (wt/vol) Sarkosyl in 0.5 N NaOH. Gradients were centrifuged at 30,000 rpm at 20°C for 120 min in an SW55.1 Ti rotor. Eachgradient was dripped onto strips of Whatman no. 17 paper. The strips were washed in 5% TCA and then 95% ethanol, and amounts of ^{3}H and ^{14}C were then determined as before.

Results

The absence of nucleotide excision repair, but not translesion synthesis, impairs recovery of DNA synthesis following UV-induced damage.

If translesion synthesis were the predominant mechanism by which cells recover replication following arrest, one would predict that the absence of the translesion DNA polymerases would significantly impair cell survival in the presence of DNA damage.

Although several studies have examined the survival of individual polymerase mutants following DNA damage, it is possible that these proteins are functionally redundant at UV-induced lesions and that a phenotype would only be revealed in the absence of all three damage-inducible polymerases. To examine this possibility, we constructed isogenic mutants lacking Pol II (*polB*), Pol IV (*dinB*), Pol V (*umuC* and *umuDC*), or all three gene products. Consistent with previous studies (Rangarajan et al., 1999), mutations affecting Pol V, but not Pol II or Pol IV, rendered cells modestly hypersensitive to irradiation at higher doses of UV (Fig. A.1A). Interestingly, mutants lacking all three DNA polymerases were no more sensitive to UV irradiation than the Pol V single mutant. By comparison, an *uvrA* mutant was much more sensitive to UV irradiation than the triple-polymerase mutant, and the sensitivity of the deviation.

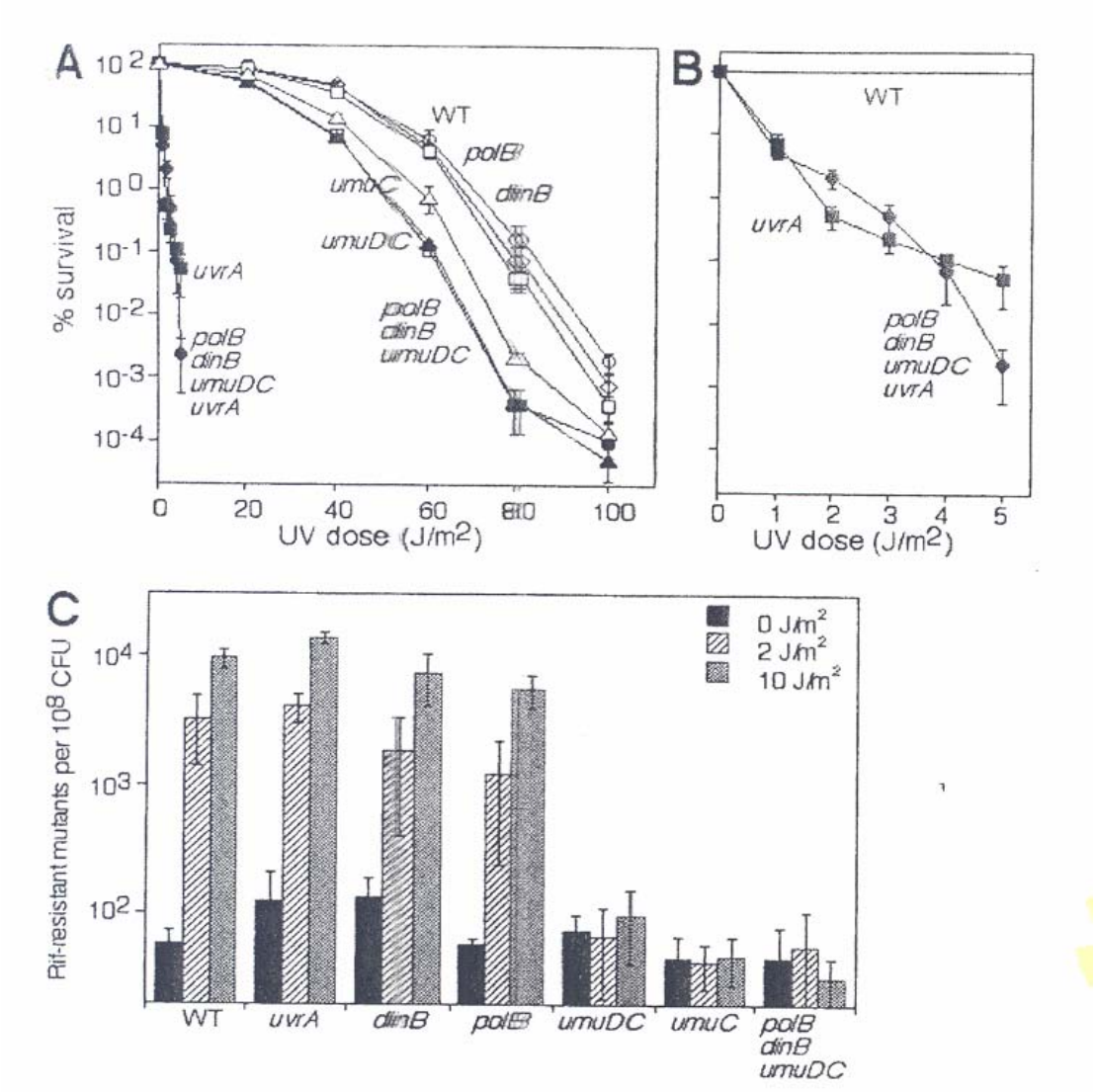

Figure A.1. Pol V is required for resistance and mutagenesis following UV irradiation.

(A) The survival of parental (○), polB (◊), dinB (□), umuDC (▲), umuC (Δ), uvrA (■), polB dinB umuDC (●), and polB dinB umuDC uvrA (♦) cultures is shown after UV irradiation at the indicated doses. (B) The survival of parental (○), uvrA (■), and polB dinB umuDC uvrA (♦) cultures replotted on a different scale. Graphs represent an average of at least three independent experiments. Error bars represent 1 standard deviation. (C) Cultures were irradiated at the indicated doses and examined for the number of rifampin (Rif)-resistant colonies that appeared following an overnight incubation. The number of rifampin-resistant colonies that appeared per 10^8 cells is plotted. Graphs represent an average of four independent experiments. Error bars represent 1 standard .

quadruple-uvrA-polymerase mutant was similar to that of the uvrA mutant alone (Fig. A.1A, B). Similar to previous studies (Tang et al., 2000; Elledge & Walker, 1983; Steinborn, 1978; Kato & Shinoura, 1977), when we measured the frequency of mutagenesis in cultures exposed to UV irradiation as monitored by cells acquiring resistance to the antibiotic rifampin, we found that Pol V, but not Pol II or Pol IV, was responsible for essentially all of the mutagenesis generated by UV-induced DNA damage (Fig. A.1C). Thus, the absence of all three translesion DNA polymerases does not severely impair the survival of UV irradiated E. coli. However, the observation that Pol V contributes to cell survival primarily at high doses of UV could indicate that it plays a critical role in the recovery of replication under conditions when the repair capacity of the cell has been exceeded. Alternatively, the observation that Pol V-dependent mutagenesis increases approximately 100-fold at low doses of UV (2 J/m2) that do not affect cell survival or exceed the repair capacity of the cell suggests that the polymerase is active even at these low doses in wild-type cells. This may imply that its preferred substrate may not relate to lesions that arrest replication forks or impair cell survival. The observation that Pol II and Pol IV do not contribute to survival or mutagenesis and were unable to compensate for the absence of Pol V argues that these polymerases are not functionally redundant with respect to survival or mutagenesis following UV irradiation in vivo.

Impaired survival following DNA damage could result from a deficiency in any of several cellular processes and does not directly address the potential role that translesion synthesis may have at sites of replication arrest. Therefore, to examine the recovery of DNA synthesis in these mutants directly, we monitored the overall DNA

accumulation and rate that synthesis recovered following UV-induced DNA damage. To this end, duplicate aliquots of [^{14}C]thymine-labeled cultures were pulse-labeled for 2 min with [^{3}H]thymidine at various timesafter 27 J/m^2 UV irradiation. The rate of DNA synthesis (^{3}H incorporation/2 min) could then be determined relative to the total amount of DNA present (^{14}C incorporation) at specific times following treatment. Since the rate of DNA synthesis was found to vary significantly with cell density (data not shown),all experiments included a mock-irradiated control that allowed us to directly compare irradiated and unirradiated cultures and ensure that any observed differences were due to UV treatment rather than culture density. These irradiation conditions generated approximately one cyclobutane-pyrimidinedimer per 9-kb single-strand DNA as measured by T4 endonucleaseV-sensitive sites in the DNA (Mellon & Hanawalt, 1989; data not shown), butdid not significantly reduce the survival of wild-type cells (Fig. A.1). By this assay, the rate of DNA synthesis in UV-irradiated wild-type cultures initially decreased by more than 90% but began to recover 15 min post-UV irradiation and continued to increase until it approached unirradiated levels, approximately 80 min post-UV irradiation (Fig. A.2). At this time, the overall DNA accumulation also approached that of the unirradiated cultures. By comparison, while DNA synthesis was inhibited to a similar extent in *uvrA* mutants as in wild-type cells, no recovery in the rate of DNA synthesis occurred during the course of the experiment and very little, if any, further DNA accumulation was observed. Similar results were obtained after low doses of UV (5 J/m^2). Following a low dose of UV, the rate of DNA synthesis in *uvrA* mutants was initially inhibited to a lesser extent, but no recovery in DNA synthesis rates was seen during the 90-min time course (data not shown).

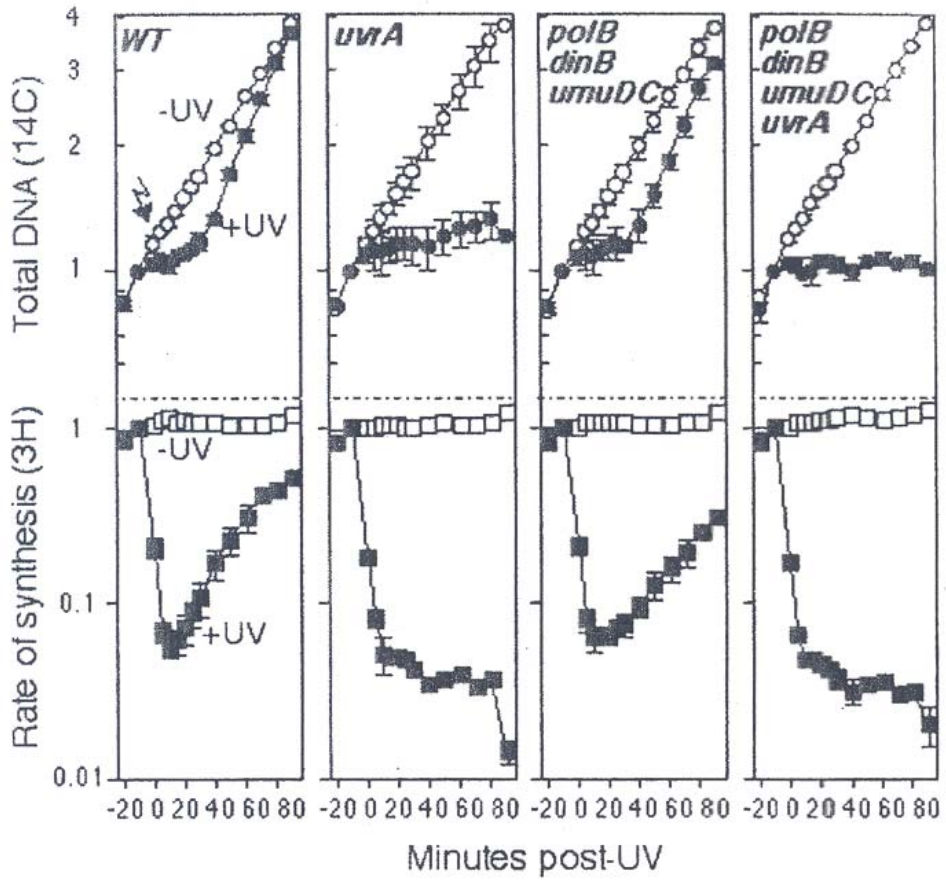

Figure A.2. Nucleotide excision repair, but not translesion DNA synthesis, is required for the recovery of DNA replication after UV irradiation.

[^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 2 min at the indicated times following either 27 J/m^{2} UV irradiation (filled symbols) or mock irradiation (open symbols) at time zero. The relative amounts of total DNA (^{14}C; ○) and DNA synthesis/2 min (^{3}H; □) are plotted. Graphs represent an average of at least three independent experiments. Error bars represent 1 standard deviation.

In contrast to *uvrA*, the absence of the damage-inducible polymerases did not affect the time at which DNA synthesis resumed. The overall rate that DNA synthesis increased occurred with a small but reproducible reduction in kinetics inthe triple-polymerase mutant compared to wild-type cells; however, DNA synthesis began to resume in UV-irradiated *polB dinB umuDC* mutants at a similar time to that observed in wild-type cells (Fig. A.2). In the absence of both uvrA and the polymerases, no recovery in the rate of DNA synthesis or further accumulation of DNA was observed to occur.

The lack of recovery in *uvrA* mutants could be interpreted to support a prominent role for nucleotide excision repair operating at lesion-arrested replication forks. However, this result does not exclude the possibility that translesion synthesis can occur at these sites, since the recovery in *uvrA* mutantsmay remain below the level of detection due to the persistence of lesions in the *uvrA* mutant genome. Despite this shortcoming, the recovery of DNA synthesis in the absence of all three damage-inducible polymerases does indicate that these polymerases are not essential for replication to resume following arrest by UV-induced DNA damage. Furthermore, the lack of any significant delay in the recovery argues against the idea that they are used as a predominant mechanism for replication to resume following arrest at UV-induced lesions.

Damage-inducible polymerases act at arrested replication forks in the absence of repair

While the previous assay indicated that translesion synthesis is not essential or replication to resume following UV-induced damage, it lacked the sensitivity todetermine if the damage-inducible DNA polymerases are capable of acting at sites of replication-arresting DNA lesions. To address this question, we examined the ability of the

polymerases to protect the nascent DNA at the arrested replicationfork from degradation. In previous studies, this assay has been used to show that the nascent lagging strand of arrested replication forks is subject to degradation by the RecJ nuclease and RecQ helicase at times prior to the resumption ofreplication (Courcelle & Hanawalt, 1999). In cells that are able to recover replication, the observed degradation of the nascent DNA ceases at thetime when replication resumes. However, in mutants that fail to resume DNA synthesis, the nascent DNA degradation continues and is much more extensive(Courcelle et al., 2003; Courcelle et al., 1999; Courcelle et al., 1997). To examine the degradation that occurs in the polymerase mutants, cultures grown in [^{14}C]thymine were pulse-labeled with [^{3}H]thymidine for 5 s, transferred to nonradioactive medium, and irradiated with 27 J/m^2 UV. The amounts of ^{3}H and ^{14}C remaining in the DNA after irradiation were then monitored over time. The ^{14}C label allowed us to compare the degradation that occurred in the overall genome to that which occurred specifically at the ^{3}H-labeled DNA at the arrested fork. As seen in previous studies, the degradation ceased in wild-type cells between 20 to 40 min after irradiation and was limited to less than 20% of thenascent DNA (Courcelle et al., 1999; Courcelle & Hanawalt, 1999; Courcelle et al., 1997) (Fig. A.3). The increase in ^{3}H-labeled DNA after 40 min occurs due to the reincorporation of the remaining intracellular pools of [^{3}H]thymidine at the time of replication resumption (Courcelle & Hanawalt, 1999). Comparatively, in *recF* mutants, which fail to resume replication following arrest (Courcelle et al., 2003; Courcelle et al., 1999; Courcelle et al. 1997), the nascent DNA degradation continued for more than 100 min until approximately half of the nascent DNA had been degraded (Fig. A.3).

When we examined cultures of *polB dinB umuDC* mutants, we found that nascent DNA degradation was limited to the first 40 min and was comparable in extent to that observed in wild-type cells (Fig. A.3), consistent with the idea that the time at which replication recovers is not affected by the absence of thepolymerases. In contrast, the nascent DNA degradation continued beyond 60 min in the absence of *uvrA* but then ceased at a point that only moderately exceededthat which occurred in wild-type cells. Interestingly, the extensive nascent DNAdegradation seen in *recF* mutants did not occur in the *uvrA* mutants despite the fact that robust replication does not detectably resume in either mutant. Surprisingly, when the damage-inducible polymerases were also inactivated, thenascent DNA continued to degrade in *uvrA* mutants and now exhibited a degradation pattern that was similar in duration and extent to that seen in *recF*mutants (Fig. A.3). The observation that the damage-inducible polymerases are required to protect the nascent DNA from degradation in the absence of nucleotide excision repair suggests that translesion synthesis can act as analternative to excision repair at sites of blocking DNA lesions. Furthermore, theextensive degradation in *polB dinB umuDC uvrA* mutants suggests that in the absence of either nucleotide excision repair or translesion DNA synthesis, the ability of the cell to elongate past the arresting lesion is severely compromised, leading to the eventual degradation of the nascent DNA.

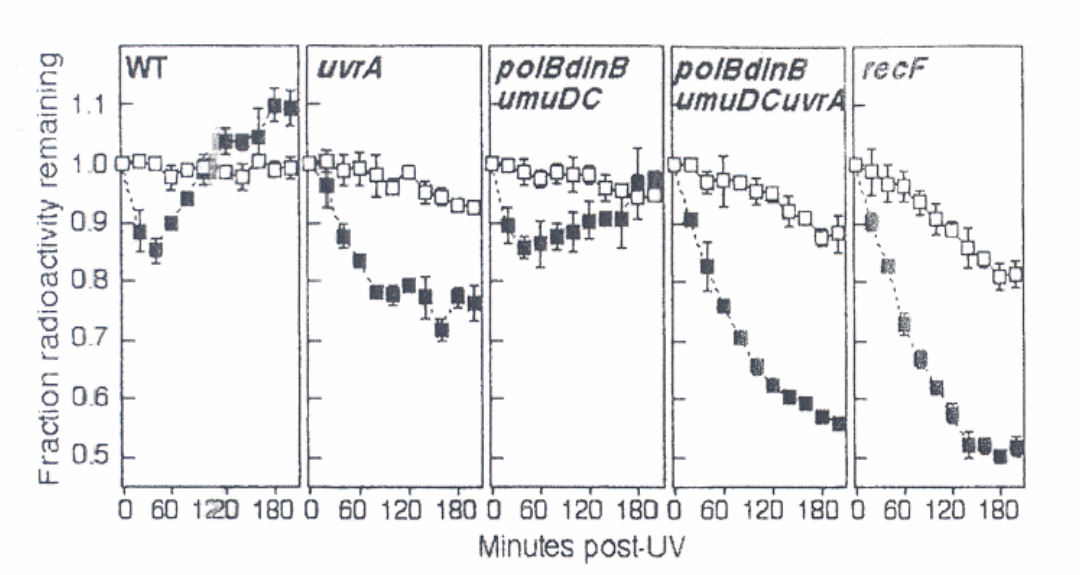

Figure. A.3. Increased degradation occurs at the growing fork after irradiation in *polB dinB umuDC uvrA* cells.

[^{3}H]thymidine was added to[^{14}C]thymine-prelabeled cells for 5 s prior to irradiation with 27 J/m^{2} in nonlabeled medium. The fraction of radioactive nucleotides remaining in the DNA is plotted over time. The initial values for ^{3}H and ^{14}C were between 2,500 to 4,000 and 1,200 to 1,700 cpm, respectively, for all experiments. Graphs represent an average of at least three independent experiments. Error bars represent 1 standard deviation. Results for total DNA (^{14}C; □) and nascent DNA (^{3}H; ■) are shown.

Daughter-strand gap repair is delayed in the absence of the damage-inducible polymerase

The assays described above would be expected to identify enzymes that specifically participate during the recovery of replication following arrest by a DNA lesion. However, several studies using plasmid substrates have suggested that a subset of DNA lesions, such as those in the lagging-strand template, fail to arrest replication and generate gaps in the daughter-strand DNA. It is reasonable to consider that the processing and repair of these gapped substrates may require a different subset of enzymatic pathways than those that act at arrested replication forks. Consistent with the gapped products observed on plasmids, a number of previous studies have used alkali sucrose gradient analysis to show that immediately after UV irradiation, a limited amount of DNA synthesis can be detected on the chromosome that also contains gaps (Ganesan, 1974; Rupp et al., 1971; Rupp & Howard-Flanders, 1968;). The repair (or joining) of the chromosomal gapped DNA fragments appears to depend on at least some of the same gene products that are required for the resumption of DNA synthesis, including RecA, RecF, RecO, and RecR (Rothman & Clark, 1977; Ganesan & Seawell, 1975; Smith and Meun, 1970). Several early studies characterizing the processing and repair of nascent-strand gaps were performed prior to the discovery of the concept that translesion synthesis or nucleotide excision repair may be involved in this pathway. For these reasons, most studies characterizing the repair of nascent-strand gaps were done with *uvr* mutants and the potential contribution that nucleotide excision repair or the damage-inducible polymerases may have in this process has not been considered or examined.

To examine the repair of the post-irradiation DNA fragments in these mutants,a 5-min pulse of [^{3}H]thymidine was added to ^{14}C-prelabeled culturesimmediately after UV irradiation with 27 J/m^{2}. Cultures were then placed into nonradioactive media, and at the indicated times, cells were lysed and the size of the labeled DNA fragments was analyzed on alkaline sucrose gradients.Whereas the pulse-labeled ^{3}H-DNA co-sedimented with the large ^{14}C-labeled genomic DNA in mock-irradiated cultures (data not shown), the DNA synthesized immediately after irradiation consisted of smaller, slower-migrating fragmentsin UV-irradiated cultures (Fig. A.4). The average size of the smaller ^{3}H-labeled fragments was 5 kb, while the size of the ^{14}C-labeled genomic DNA averagedgreater than 150 kb (Ganesan, 1974; data not shown). In wild-type cultures, the size of the post-irradiation DNA fragments returned to that of the overall genomic DNA within 45 min of irradiation. However, in *polB dinB umuDC* mutants, we observed a modest but reproducible delay (of approximately 15 min) before the nascent-strand gaps were repaired, despite the timely recovery of DNA synthesis at the arrested replication forks of these mutants as demonstrated by our previous assays (Fig. A.4). We interpret this observation to indicate that translesion synthesis participates in and at least partially contributes to the joining of the observed nascent-strand gaps in wild-type cells.

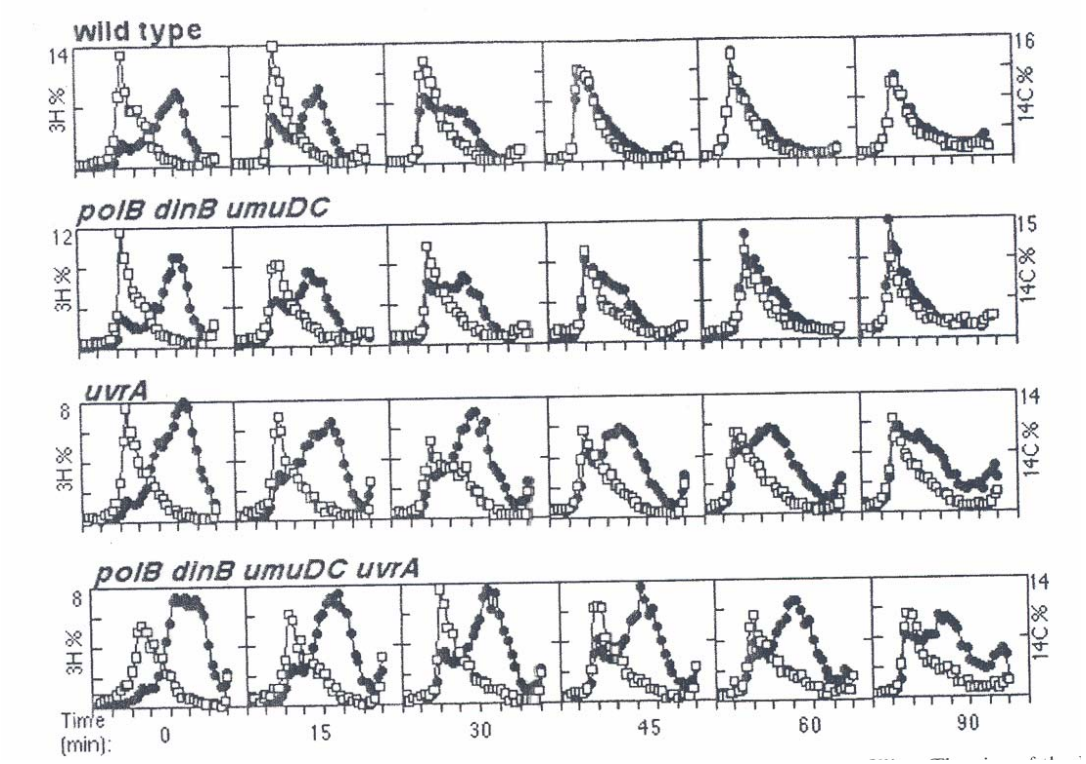

Figure. A.4. Nucleotide excision repair and translesion DNA synthesis are required for nascent DNA gap filling.

The size of the DNA synthesized immediately after irradiation was analyzed by alkali sucrose gradients over time. [^{14}C]thymine-prelabeled cells were irradiated at a dose of 27 J/m^2, pulse-labeled with [^{3}H]thymidine for 5 min, and then filtered into nonlabeled medium. Cultures collected immediately after ^{3}H labeling are referred to here as time zero. Amounts of ^{3}H and ^{14}C in each fraction are plotted as a percentage of the total counts in each gradient. ^{3}H and ^{14}C values were between 4,200 to 9,500 and 1,500 to 4,300 cpm per gradient, respectively. Results for DNA synthesized before irradiation (^{14}C; □) and DNA synthesized after treatment (^{3}H; ●) are shown. Graphs represent one of at least three independent experiments.

Comparatively, a large portion of the DNA synthesized postirradiation in irradiated *uvrA* cultures persisted as small fragments throughout the duration of the experiment. Consistent with earlier studies, the size of the postirradiation DNA fragments began to increase at later times, although the average size remained smaller than that of the genomic DNA throughout the 90-min time course in *uvrA* mutants (Ganesan, 1974; Rupp et al., 1971; and Fig. A.4).This observation may indicate a potential role for nucleotide excision repair in processing lesions prior to nascent-strand gap repair. However, the delayed gap joining in *uvrA* mutants could also suggest that the repair of the nascent-strand gaps is coupled to or dependent on the efficient resumption of replicationfollowing arrest. In *uvrA* mutants that also lacked the damage-inducible polymerases, the impaired gap joining was similar in extent to that observed in the *uvrA* mutants (Fig. A.4).

UmuDC participates in the recovery of DNA synthesis and daughter-strand gap repair following UV irradiation

To identify which of the damage-inducible DNA polymerases acts at arrested replication forks and participates in daughter-strand gap repair following UV-induced DNA damage, we examined mutants that lacked either Pol II (*polB*), Pol IV (*dinB*), or Pol V (*umuDC*) using the assays described above. As shown in Fig. A.5A, both *polB* and *dinB* mutants recovered DNA synthesis with kineticsthat were identical to those of wild-type cultures. By comparison, in *umuDC* mutants, although the recovery began at a similar time, it occurred with a modest reduction in kinetics that was identical to that in the mutant lacking all three damage-inducible polymerases. Identical results were

obtained when weexamined a *umuC* mutant that maintains and expresses a functional copy of the UmuD subunit, but is unable to perform translesion DNA synthesis (Elledge &Walker, 1983; Opperman et al., 1999). In addition, we found that following UV irradiation, the presence of Pol V was able to protect the arrested replication fork from degradation when excision repair was absent, whereas the degradation was not affected by the presence or absence of Pol II or Pol IV (Fig. A.5B). Similarly, both *umuDC* and *umuC* mutants also exhibited a 15-min delay before the postirradiation DNA fragments were fully repaired, whereas no delay was detected in either *polB* or *dinB* mutants (Fig. A.5C). We interpret these observations to indicate that following UV-induced DNA damage, Pol V is able to act at lesion-arrested replication forks and promote daughter-strand gap repair.In addition, the observations argue against a functionally redundant role for Pol II or Pol IV with Pol V at sites of UV-induced DNA damage.

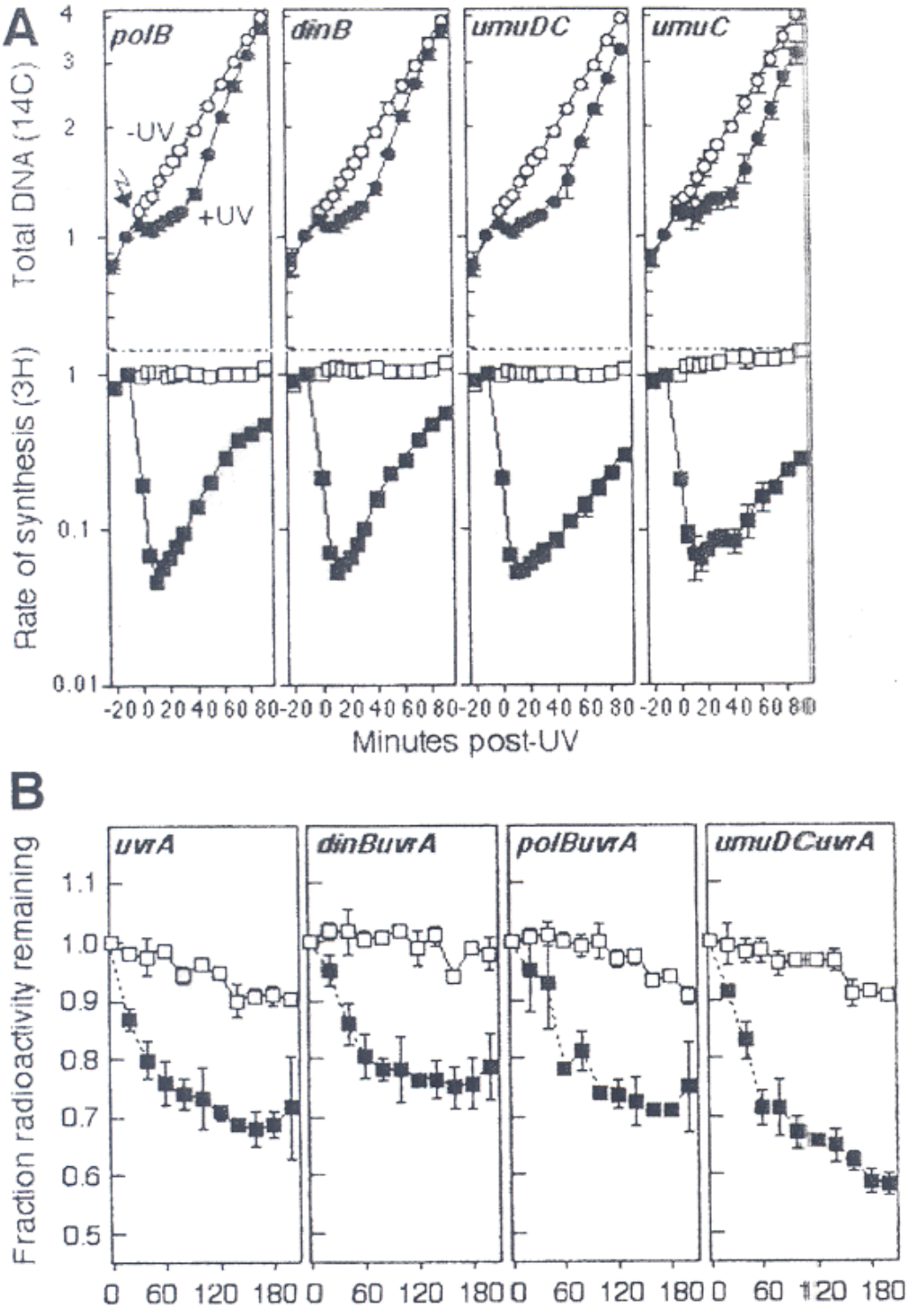

Figure. A.5. Pol V contributes to the rate that DNA synthesis resumes, protection of the replication fork in the absence of repair, and daughter strand gap repair after UV irradiation.

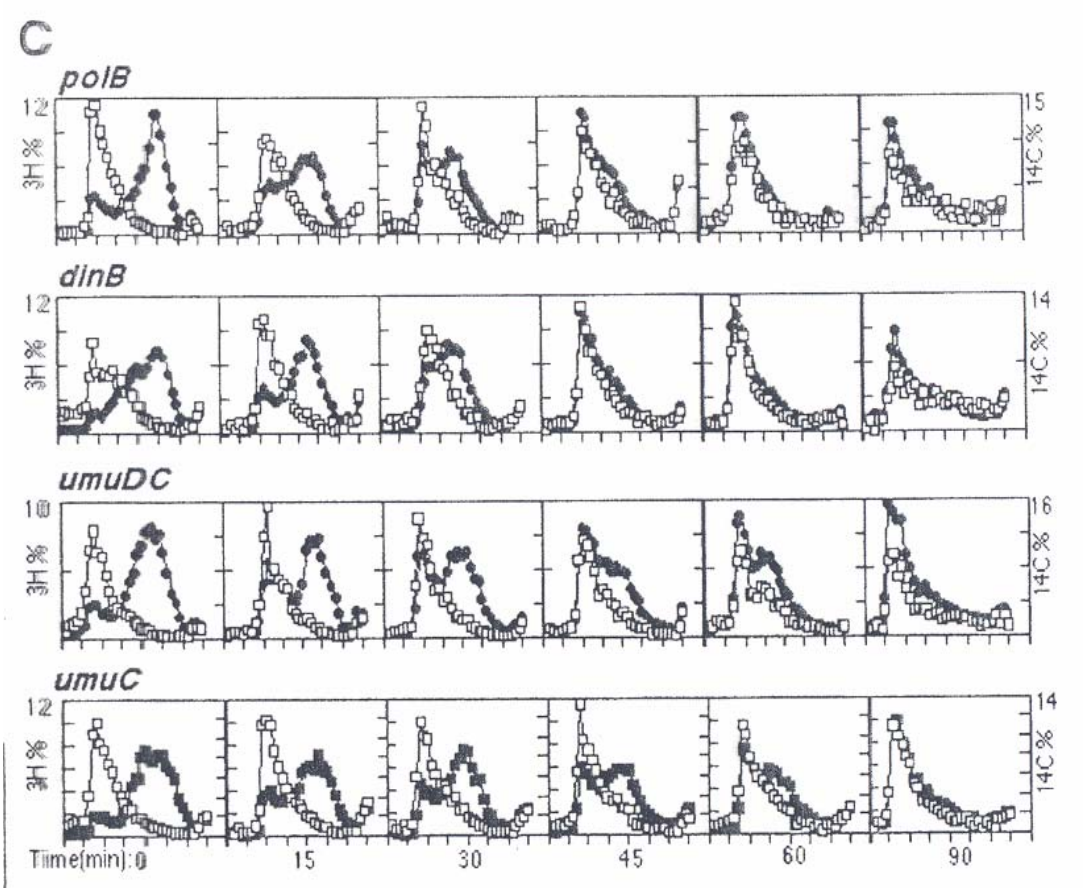

Figure A.5. (continued)

(A) Data were obtained and plotted as in Fig. A.2. Each graph represents an average of at least three independent experiments. Error bars represent 1 standard deviation. Total DNA (^{14}C) in mock-irradiated cultures (○), total DNA in irradiated cultures (●), the rate of DNA synthesis (^{3}H) in mock-irradiated cultures (□), and the rate of DNA synthesis in irradiated cultures (■) are shown.
(B) Data were obtained and plotted as in Fig. 2.3. The initial values for ^{3}H and ^{14}C were between 750 to 1,200 and 800 to 1,200 cpm, respectively, for all experiments. Results for total DNA (^{14}C; □) and nascent DNA (^{3}H; ■) are shown. Each graph represents an average of at least three independent experiments. Error bars represent 1 standard deviation. **(C)** Data were obtained and plotted as in Fig. A.4. ^{3}H and ^{14}C values were between 6,700 to 11,000 and 2,500 to 4,000 cpm per gradient, respectively. Results for DNA synthesized before irradiation (^{14}C; □) and DNA synthesized after treatment (^{3}H; ■) are shown. Graphs represent one of at least three independent experiments.

Discussion

Both translesion DNA synthesis and nucleotide excision repair have been postulated to act on replication-arresting DNA lesions to promote the recovery of DNA synthesis and reduce the frequency of recombination in wild-type cells. In this study, we show that both processes contribute to replication recovery, but with different efficiencies and kinetics. A role for nucleotide excision repair acting at early times during recovery is supported by the observations that the resumption of DNA synthesis following arrest is severely impaired in the absence of repair enzymes but occurs with nearly wild-type kinetics in the absence of all three damage-inducible DNA polymerases (Fig. A.2). Furthermore, the degradation of the DNA at the arrested fork occurs for a longer duration when the recovery process depends solely on translesion synthesis, as in nucleotide excision repair mutants (Fig. A.3).

In contrast, several observations are consistent with the idea that translesionsynthesis by Pol V can function at the arrested replication fork substrates at later times or as an alternative pathway when the repair capacity of the cell has been exceeded. The hypersensitivity of *umuDC* mutants is distinct from that of manyother UV-sensitive mutants in that it only becomes prominent at higher doses of UV irradiation (Fig. A.1A). At lower UV doses, *umuDC* mutants survive as well as the parental strain and exhibit a similar "shoulder" in their survival curves, consistent with the idea that Pol V becomes essential for survival when the repair capacity of the cell has been exceeded. Furthermore, although robust replication did not resume in the absence of lesion removal, the presence of Pol V reduced the degradation that occurred at the replication fork (Fig.

A.3 and A.5B), suggesting that it is capable of synthesizing past arresting UV lesions, albeit with an efficiency that remains far below that seen during normal replication on undamaged templates.

The data we present indicate that nucleotide excision repair contributes to therecovery of replication at early times after UV irradiation. Although Pol V is not essential for replication to resume, a large induction of Pol V-dependent mutagenesis occurs even after low doses of UV irradiation (Fig. A.1C),strongly arguing that Pol V actively participates in some form of DNA synthesisthat occurs after UV.

One attractive model to consider is that the prominent replication substrates targeted by nucleotide excision repair and Pol V may be distinct. The observation that the repair of daughter-strand gaps is delayed in Pol V mutants, despite thetimely resumption of DNA synthesis, is consistent with the idea that Pol V may predominantly target DNA gaps produced by nonarresting UV-induced lesions. The preferential targeting of Pol V to nonarresting lesions is also more consistent with the modest UV hypersensitivity exhibited in the Pol V mutants. In vitro, efficient translesion synthesis by Pol V requires that the gapped substrate contain a RecA-bound filament and a beta clamp loaded at the site of the lesion (Pham et al., 2001; Reuven et al., 1999; Tang et al., 1999; Reuven et al., 1998). Based upon our current understanding of replication fork dynamics, this is similar to the substrate that is expected to be generated following replication through lesions on lagging-strand templates in vivo.

In addition to its role in translesion synthesis, overexpression of UmuD has aninhibitory effect on growth at low temperatures and on exiting stationary phase (Marsh & Walker, 1985; Murli et al., 2000). These observations have led to the proposal that

UmuD may function as a cell cycle checkpoint, preventing replication from resuming prematurely in the presence of DNA damage (Opperman et al., 1999). If the protein did act as a damage checkpoint, one would predict that replication would resume more rapidly in the absence of thecheckpoint protein than in its presence. In the results presented here, the rate of recovery was modestly hindered in the absence of UmuD (Fig. A.5A, B, and C), suggesting that the checkpoint function of UmuD may not be delaying replicationrecovery from the arrested fork substrate. However, this does not rule out that UmuD may delay synthesis from occurring at other substrates or act during other phases of the culture growth, such as appears to occur during the exit from stationary phase (Murli et al., 2000).

We observed that Pol V, but not Pol II or Pol IV, detectably contributes to the survival, mutagenesis, resumption of DNA synthesis, and nascent-strand gap repair following UV irradiation in vivo (Fig. A.5). These observations are consistent with work by Khidir et al. (Khidir et al., 1985) in which they reported as data not shown that *uvrA* mutants but not *umuC* mutants recovered DNA synthesis more slowly than wild-type cells. Using a background that contained a *recA718* allele, Witkin et al. (Witkin et al., 1987) found that in the absence of nucleotide excision repair, *umuC* was essential and sufficient for DNA synthesis to resume. Although it remains difficult to clearly interpret why the phenotype was dependent on the *recA718* allele, which has a complex phenotype (McCall et al., 1987; Witkin et al., 1982), the observation is consistent with the resultspresented here, in which Pol V contributes to the recovery of replication when excision repair cannot occur. A previous study observed that mutations in *polB* delayed the recovery of DNA synthesis by 50 min (Rangarajan et al., 1999). However in an

earlier study, this group did not observe any Pol II-mediated translesion synthesis on UV-damaged templates (Kow et al.,1993), and in this study, we did not detect a contribution by Pol II at the arrested fork asmeasured by either the recovery of DNA synthesis, nascent strand gap repair, orprotection of the nascent DNA following arrest. This difference could either be due to experimental conditions or secondary mutations in the strains utilized. Thepreviously described delay in the recovery of *polB* mutant strain STL1336 was based on a single experiment in which the culture was irradiated early in the growth phase (OD_{450} of 0.08 equivalent to OD_{600} of ~0.06). In our hands, we haddifficulty monitoring growth of the culture at this OD and therefore chose to irradiate cultures at an OD_{600} of 0.3. We also routinely divided cultures at the time of treatment to include an unirradiated control so that the rate of synthesis after irradiation could be directly compared to the rate occurring in an equivalent unirradiated culture. When we irradiated STL1336 at an OD_{600} of 0.3, DNAsynthesis recovered with kinetics similar to those of wild-type cells (data notshown). Previous studies from this group have also reported that the *polB* strain,STL1336, is prone to accumulate suppressor mutations that alter its response to DNA damage (Escarceller et al., 1994). To address the possibility of suppressorsin our study, we constructed a new *polB::tet* deletion and obtained results identical to those shown in Fig. A.4 (data not shown). Furthermore, to reduce the potential of suppressor mutations accumulating, strains were frozen immediately after construction and grown the day prior to each experiment. In constructing our *polB* mutants, we did not observe any reduction in P1 transduction efficiency or growth impairment that may be expected to occur if suppressor mutations arose in culture populations.

The lack of a phenotype for Pol II and Pol IV highlights the need to consider that these polymerases may not be functionally redundant in vivo. The possibilities that they may act during different temporal phases of the cell cycle, on substrates that are unrelated to the recovery of DNA synthesis or specificallyon different forms of DNA damage, should not be excluded from consideration. The last possibility is consistent with the observation that the mutations produced by different forms of DNA damage vary, depending on which translesionpolymerases are present in the cell (Napolitano et al. 2000). Other studies havealso suggested that both Pol II and Pol IV are active on undamaged templates in the absence of the replicative polymerase, Pol III (Rangarajan et al., 1999;Viguera et al., 2003). The mutagenesis, survival, recovery of replication, and daughter-strand gap repair presented here, as well as previous studies on plasmid substrates (Napolitano et al., 2000), are consistent with the idea that Pol V is able to productively bypass lesions generated by 254-nm UV irradiation in vivo.

Although UV-induced lesions appear to specifically require Pol V, there areclear examples, in both *E. coli* and humans, where there is a functional redundancy in the ability of more than one polymerase to bypass a specific formof DNA damage (Napolitano et al., 2000). It is also clear that the competitive or preferential order by which this redundancy occurs can have serious consequenceson genomic stability. In humans, Pol η appears to bypass UV-induced DNA damage with relatively high fidelity. However, in its absence, translesion synthesis is achieved by alternative DNA polymerases with much less accuracy and results in the severe cancer-prone phenotype exhibited by patients with the variant form of xeroderma pigmentosum (Kannouche & Stary, 2003).

By analogy, the results presented here suggest that in *E. coli*, lesionsencountered during replication are initially processed with high fidelity through the nucleotide excision repair pathway. At later times, when the cell either cannot or fails to remove the lesions in a timely fashion, these lesions can be bypassed alternatively by Pol V, in a process that is associated with an increased likelihood of mutagenesis. The delayed expression of the active UmuD' subunit of Pol Vfollowing UV irradiation has been previously hypothesized to allow nucleotideexcision repair more time to remove DNA lesions (Opperman et al., 1999) and would also be consistent with the results presented here. Thus, in the case of UV-induced damage, in both *E. coli* and humans, the pathways operating to process lesions encountered during replication appear to be ordered in such a way as to give nonmutagenic pathways priority over those associated with higher mutation frequencies. It will be interesting to see if the same relationship holds true for other forms of DNA damage that are encountered environmentally ortherapeutically, which may depend on repair enzymes or alternative polymerasesto restore damaged genomic templates.

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