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Inactivation of the DnaB Helicase Leads to the Collapse and Degradation of the Replication Fork: a Comparison to UV-Induced Arrest[▽]

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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 DNA damage. Following UV-induced DNA damage, the integrity of replication forks is 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 of the RecFOR proteins. We discuss these observations in relation to potential models for both UV-induced and DnaB(Ts)-mediated replication inhibition.

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 (for reviews, see references 14 and 16). 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 are 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 life span of an organism, it is important to characterize how arrested replication forks are accurately processed and resume in each of these situations.

UV-induced DNA damage has frequently been used as a model to address the general question of how replication recovers when it is blocked by DNA damage, and this damage has been extensively characterized. Irradiation with 254-nm light induces DNA lesions that block the progression of the replication machinery (6, 15, 48). 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 (7, 9, 10, 13, 15). In the absence of RecA, RecF, RecO, or RecR, the arrested replication forks are extensively degraded and replication fails to resume (7, 15, 30, 42). 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 single-stranded DNA with homologous duplex DNA at the arrested fork (3, 28, 50, 52, 53). 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 (13). 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 allow 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 (10). Under these conditions, cell survival and the recovery of replication become dependent on translesion synthesis by polymerase V (Pol V) (10).

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 to be normal for DNA replication at 30°C but fail to continue DNA synthesis following a shift to a restrictive temperature, 42°C (5, 21, 29, 33). Several of the thermosensitive mutations occur in the *dnaB* gene, which encodes an essential hexameric 5′-3′ DNA helicase that functions to unwind duplex DNA during replication (5, 33, 34, 44, 45, 54). Previous studies using either sucrose gradient analysis or pulsed-field gel electrophoresis have shown that double-strand breaks accumulate

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in the genomes of *dnaB*(Ts) mutants following a period of incubation at the restrictive temperature (29, 40). 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 (40, 46, 47). To explain these observations, it has been proposed that following replication arrest in *dnaB*(Ts) mutants, RuvABC catalyzes the displacement and regression of the nascent DNA at the replication fork (23, 40, 46, 47). In this model, the nascent DNA of the regressed fork/Holliday junction could then be degraded by the RecBCD helicase-nuclease or collapse to 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 processes the nascent DNA or is required for replication to resume (13, 15, 19, 20, 32), 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 (1, 22–24, 26, 27), 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 mutant. These events were compared to those which occur at replication forks disrupted by UV irradiation. We found that replication forks disrupted by inactivation of DnaB contain structural intermediates and are processed by enzymes that are different 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 (38). The genotype of CRT266 is *thr leu met thyA deo supE tonA dnaB266* (33, 45). All other strains in this study were derived from either SR108 (*dnaB*$^+$) or CRT266 [*dnaB*(Ts)] by P1 transduction.

HL946 (SR108 *recF332*::Tn*3*), CL579 (SR108 *recF6206*::*tet-857*), CL584 (SR108 *recO1504*::Tn*5*), CL544 (SR108 *recR6212*::*cat-883*), HL924 (SR108 *recJ284*::Tn*10*), HL923 (SR108 *recD1011 argA81*::Tn*10*), and HL1034 (SR108 *xonA*::*cat-300*) have been described previously (7, 13).

CL858 (CRT266 *recF332*::Tn*3*), CL896 (CRT266 *recO1504*::Tn*5*), CL897 (CRT266 *recR6212*::*cat-883*), CL743 (CRT266 *recD1011 argA81*::Tn*10*), CL774 (CRT266 *xonA*::*cat-300*), CL742 (CRT266 *recJ284*::Tn*10*), CL1024 (CRT266 *recG6200*::*tet-857*), CL1026 (CRT266 *ruvAB6203*::*tet-857*), and CL1028 [CRT266 del(*srlR-recA*)*306*::Tn*10*] were constructed by P1 transduction and selection for the indicated alleles from HL946, CL584, CL544, HL923, HL1034, and HL924, TP538, TP540, and HL921, respectively, into CRT266 (7, 13, 15, 41). CL785 (CRT266 *xonA*::*cat-300 recJ284*::Tn*10*) was constructed by P1 transduction of *XonA*::*cat-300* from HL1034 into CL742.

The genotype of PC8 is *leuB6*(Am) λ$^-$ *thyA rpsL*(Str^r) *deoC supH dnaB8*(Ts) (5).

UV irradiation was performed using a 15-W, 254-nm Sylvania germicidal lamp at an incident dose of 0.9 J/m^2/s.

Cell viability. Fresh overnight cultures of cells were diluted 1:100 and grown in Davis medium (17) supplemented with 0.4% glucose, 0.2% Casamino Acids, and 10 μg/ml thymine (DGCthy medium) to an optical density at 600 nm (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 47-mm general membrane filters and resuspended in prewarmed 42°C medium. At various times following the temperature shift, 100-μl aliquots were serially diluted and plated on Luria-Bertani agarose plates supplemented with 10 μg/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 0.1 μCi/ml [^{14}C]thymine and grown to an OD_{600} of precisely 0.3 in a 32°C shaking water bath. At this time, one half of each culture was filtered on Fisherbrand 0.45-μm 47-mm general membrane filters and either resuspended in prewarmed 32°C DGCthy medium supplemented with 0.1 μCi/ml [^{14}C]thymine and irradiated with 27 J/m^2 (for UV treatment) or resuspended in 42°C DGCthy medium 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 32°C prewarmed DGCthy medium supplemented with 0.1 μCi/ml [^{14}C] thymine. At 5-min intervals, duplicate 0.5-ml aliquots of a culture were pulse-labeled with 1 μCi/ml [^{3}H]thymidine for 2 min. The cells were then lysed, and the DNA was precipitated by adding 5 ml ice-cold 5% trichloroacetic acid. The precipitated DNA was collected on Millipore glass fiber prefilters and 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 medium supplemented with 0.1 μCi/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 1 μCi/ml [^{3}H]thymidine for 5 s, collected on Fisherbrand 0.45-μm general filtration membranes, washed with 1× NET buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 10 mM EDTA [pH 8.0]), and either resuspended in prewarmed 42°C nonradioactive DGCthy medium (for temperature shift) or resuspended in prewarmed 32°C nonradioactive DGCthy medium and immediately UV irradiated with 27 J/m^2 (for UV treatment). At the times indicated below, duplicate 200-μl aliquots (triplicate aliquots for time zero) of cells were lysed and the DNA precipitated by the addition of 5 ml ice-cold 5% trichloroacetic acid. Samples were then collected on Millipore glass fiber prefilters and 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 plasmid pBR322 were grown in the presence of ampicillin (100 μg/ml). The overnight cultures were pelleted, resuspended in 100 volumes of fresh DGCthy medium 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 47-mm Fisherbrand 0.45-μm general filtration membranes and either resuspended in 32°C DGCthy medium and UV irradiated with 50 J/m^2 (for UV treatment) or resuspended in 42°C DGCthy medium (for temperature shift). At the times indicated below, 0.75-ml aliquots of each culture were placed into 0.75 ml cold 2× NET buffer. Each sample was immediately pelleted in a microcentrifuge and resuspended in 150 μl of a solution containing 1 mg/ml lysozyme and 0.2 mg/ml RNase A in TE buffer (10 mM Tris [pH 8.0], 1 mM 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 were added, and incubation was continued for 1 h 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 h on 47-mm Whatman 0.05-μm-pore-size disks against 250 ml of 0.2× TE buffer. Samples were digested with PvuII (New England Biolabs), extracted once with chloroform-isoamyl alcohol, and loaded onto the gel. For the first dimension, restricted genomic DNA samples were electrophoresed in 0.4% agarose in 1× Tris-borate-EDTA at 1 V/cm for 15 h. For the second dimension, the gel lanes were cut out, rotated 90°, recast in 1.0% agarose in 1× Tris-borate-EDTA, and electrophoresed at 7 V/cm for 7 h. The DNA in the gels was transferred to Hybond N+ nylon membranes and probed either with 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-^{32}P]ATP (MP Biomedicals). Radioactivity was visualized and quantitated using a Storm 820 and the associated ImageQuant software (Molecular Dynamics/Pharmacia).

Strand-specific oligonucleotides for pBR322. The strand-specific oligonucleotides for pBR322 were pBR322 1348-1398 (5′CTGTGAATGCGCAAACCAA CCCTTGGCAGAACATATCCATCGCGTCCGCC), which detected a lagging-strand template, and pBR322 1398-1348 (5′GGCGGACGCGATGGATATGT TCTGCCAAGGGTTGGTTTGCGCATTCACAG), which detected a leading-strand template.

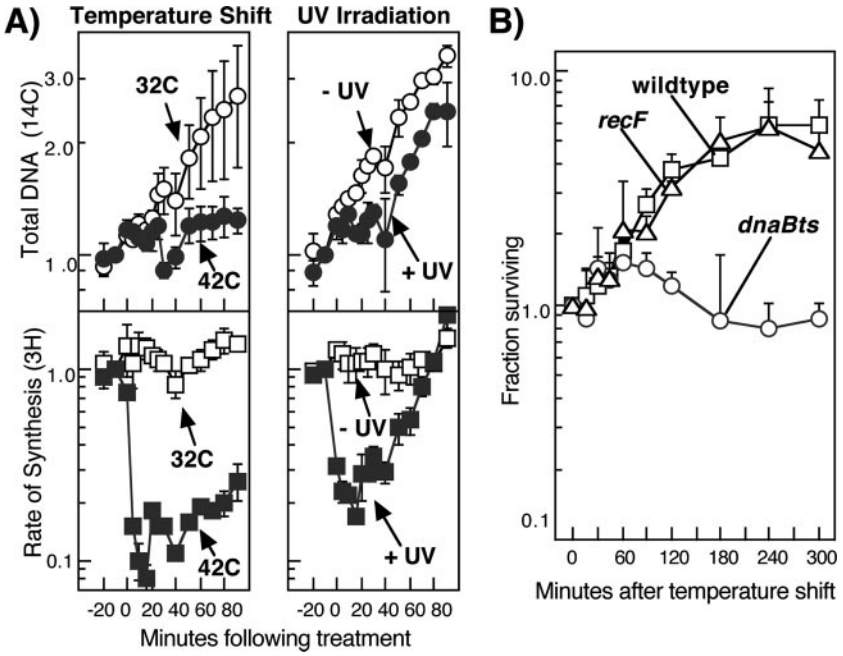

FIG. 1. (A) Both UV irradiation and inactivation of DnaB266 at 42°C arrest DNA synthesis. Cultures of *dnaB*(Ts) strain CRT266 grown at 32°C in medium containing [^{14}C]thymine were either UV irradiated with 27 J/m^2, shifted to 42°C, or mock treated. At the indicated times, duplicate aliquots of each culture were pulse-labeled for 2 min with [^{3}H]thymidine, and the relative amounts of ^{14}C and ^{3}H in the DNA were determined and plotted over time. Open circles, total DNA in mock-treated cultures; filled circles, total DNA in UV-irradiated or temperature-shifted cultures; open squares, rate of DNA synthesis in mock-treated cultures; filled squares, rate of DNA synthesis in UV-irradiated or temperature-shifted cultures. The symbols indicate averages of three independent experiments. The error bars indicate one standard deviation. The ^{14}C and ^{3}H counts at the time of treatment ranged between 1,281 and 2,871 cpm and between 722 and 3,205 cpm, respectively, in all experiments. (B) *dnaB*(Ts) mutants remain viable following a temperature shift to 42°C. The fractions of cells surviving per ml of culture following incubation at 42°C for the indicated times are plotted. Circles, *dnaB*(Ts) strain CRT266; squares, wild-type strain SR108; triangles, *recF* strain HL946.

RESULTS

Both UV irradiation and inactivation of DnaB block the progression of replication. The DnaB protein of *E. 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 this mutant, cellular DNA synthesis occurs normally at 30°C but fails to continue after a shift to the restrictive temperature of 42°C (29).

We verified that DNA replication arrests in DnaB(Ts) strain CRT266 following a shift to the restrictive temperature and compared this arrest 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 32°C in media containing [^{14}C]thymine were either UV irradiated or shifted to a temperature of 42°C. 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 42°C inhibited the rate of DNA synthesis immediately following treatment (Fig. 1A). In the case of UV irradiation, the rate of DNA synthesis was inhibited by 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 37°C (9, 19). Following inactivation of DnaB at 42°C, the rate of DNA synthesis was inhibited to an extent similar to that seen with UV irradiation and DNA synthesis did not resume when the culture was held at 42°C, 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 42°C in this strain. 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 HL946, a *recF* derivative of SR108, were grown in minimal media at 32°C. The cultures were then collected and resuspended in media at 42°C. After a culture had been incubated for various times at 42°C, samples were collected, serially diluted, and plated on Luria-Bertani plates at 30°C. The number of CFU per ml was determined and plotted over time (Fig. 1B). For the non-temperature-sensitive strains, the number of colonies per milliliter continued to increase during the incubation at 42°C. By contrast, cultures of the *dnaB*(Ts) mutants ceased growing shortly after they were shifted to 42°C. Importantly, the *dnaB*(Ts) mutants remained viable throughout the 5-h incubation period that we examined

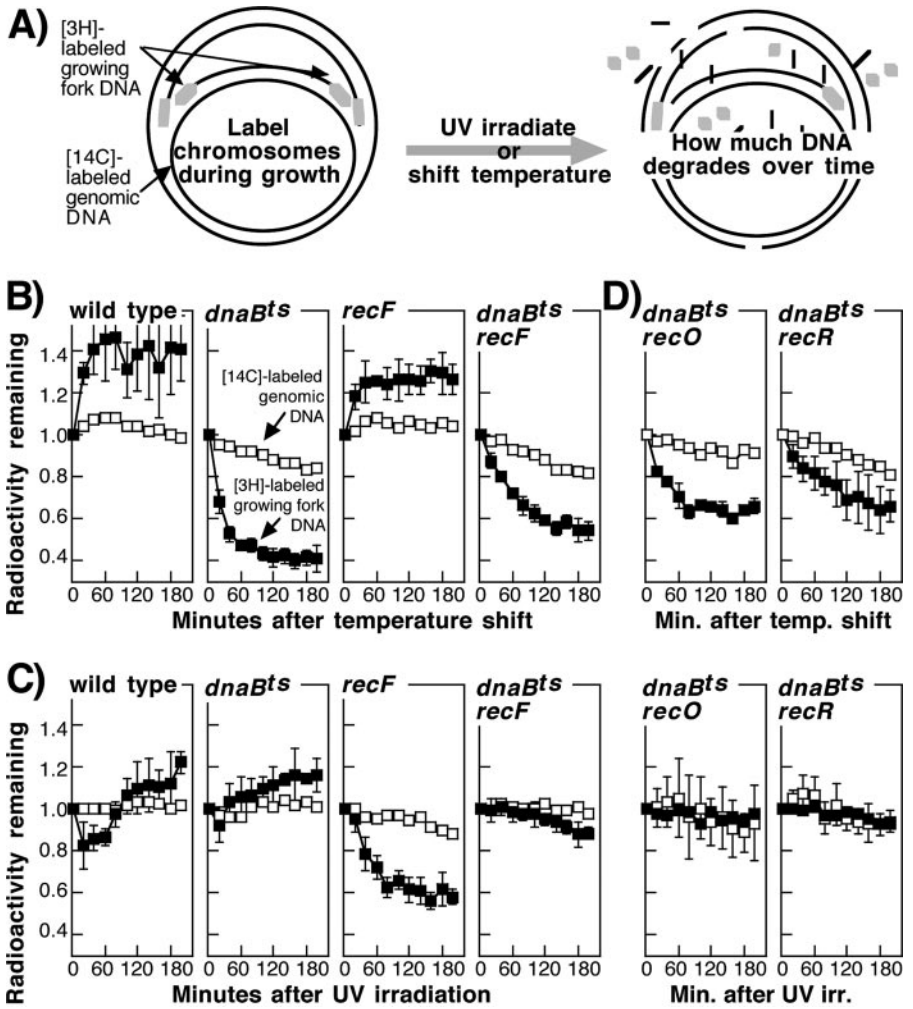

FIG. 2. Inactivation of DnaB(Ts) at 42°C leads to extensive degradation of the nascent DNA at the arrested replication fork but prevents nascent DNA degradation from occurring after UV irradiation at the permissive temperature. (A) Schematic diagram of how the chromosome was labeled before UV irradiation or a temperature shift to 42°C. [^{3}H]thymidine was added to [^{14}C]thymine-prelabeled cultures for 5 s 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) Inactivation of DnaB(Ts) leads to extensive nascent DNA degradation following inactivation at 42°C. The relative amounts of degradation in the nascent DNA (filled symbols) and total genomic DNA (open symbols) are plotted for wild-type strain SR108, *dnaB*(Ts) strain CRT266, *recF* strain CL579, and *dnaB*(Ts) *recF* strain CL858 cultures following a temperature shift to 42°C. (C) *dnaB*(Ts) allele prevents the nascent DNA degradation that occurs at replication forks arrested by UV-induced damage. Degradation is plotted as described above for panel B for wild-type strain SR108, *dnaB*(Ts) strain CRT266, *recF* strain CL579, and *dnaB*(Ts) *recF* strain CL858 cultures after UV irradiation with 27 J/m^2. (D) Similar to *recF* mutants, *recO* and *recR* mutants reduce or prevent the nascent DNA degradation from occurring at replication forks in *dnaB*(Ts) mutants following a temperature shift or UV irradiation. Degradation is plotted for *dnaB*(Ts) *recO* strain CL896 and *dnaB*(Ts) *recR* strain CL897 cultures after a temperature shift to 42°C or UV irradiation with 27 J/m^2. The symbols indicate the averages of at least three independent experiments. The error bars indicate one standard deviation.

(Fig. 1B), suggesting that although DNA synthesis is disrupted in these mutants, the cells are capable of recovering from this stress when they are returned to 32°C.

DnaB(Ts) in activation leads to extensive degradation at the replication fork. 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 (7, 13, 15). To determine if replication forks disrupted following DnaB inactivation are protected and maintained in a similar manner, 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 s immediately before they were transferred to nonradioactive medium and either shifted to 42°C or UV irradiated with 27 J/m^2. The amounts of ^{3}H and ^{14}C remaining in the DNA were 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. 2A). In our parental cells (SR108) or in cells of *recF* mutants, following a shift to 42°C,

no degradation of the nascent DNA was detected, consistent with the idea that replication is not disrupted in these cells at this temperature (Fig. 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 (13, 15). By contrast, following a shift to 42°C in cultures of *dnaB*(Ts) mutants, approximately one-half of the nascent DNA was degraded, suggesting that the replication forks are not maintained following inactivation of DnaB (Fig. 2B). Similar to our previous studies at 37°C, following UV irradiation at 32°C, 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 (11, 13) (Fig. 2C). After this time, the levels of ^{3}H-labeled DNA began to increase due to the reincorporation of remaining [^{3}H]thymidine in intracellular pools when replication resumed (13, 15). 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 one-half of the nascent DNA was degraded (11, 13, 15). Thus, while RecF is required to protect and maintain replication forks following UV-induced damage, it does not appear to have this effect following DnaB-mediated arrest.

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 compared to wild-type cultures (Fig. 2C). This observation suggests that the DnaB function may be required for the nascent DNA processing to occur at replication forks after UV-induced arrest and that the DnaB(Ts) mutation 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 (7, 13, 15). 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 42°C, the absence of RecF only modestly reduced the extent of nascent DNA degradation that occurred (Fig. 2B and 2C). 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 (Fig. 2D). Although the presence of DnaB(Ts) was able to suppress the extensive degradation of nascent DNA from occurring following UV irradiation, 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 the processing and recovery 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 (12, 13). 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 42°C in *dnaB*(Ts) strains that were also deficient in one or more of the candidate nucleases or helicases. In an otherwise wild-type background, inactivation of DnaB(Ts) at 42°C resulted in roughly one-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. 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 (12, 13, 36). The diminished degradation in *recJ* mutants is similar to that observed in the other *recF* pathway mutants (Fig. 2 and 3), which may reflect an overall inability of the RecF pathway enzymes to gain access to 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 (13), 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, which is opposite the direction of RecJ, and has been reported to associate biochemically with RecA during purification (2, 35).

We also examined the effect that mutations in the RecG helicase, the RuvAB branch migration complex, and the RecA strand-pairing enzyme had on degradation following DnaB(Ts) inactivation (Fig. 3B). Mutations inactivating these enzymes all render cells sensitive to UV-induced damage, and these proteins have been associated with processing arrested replication forks (for reviews, see references 14 and 39; see Discussion). We observed that the absence of RecG partially reduced the nascent DNA degradation to an extent that resembled what was observed with 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 in the *recA* mutant, 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 endonucleolytic activities when it is associ-

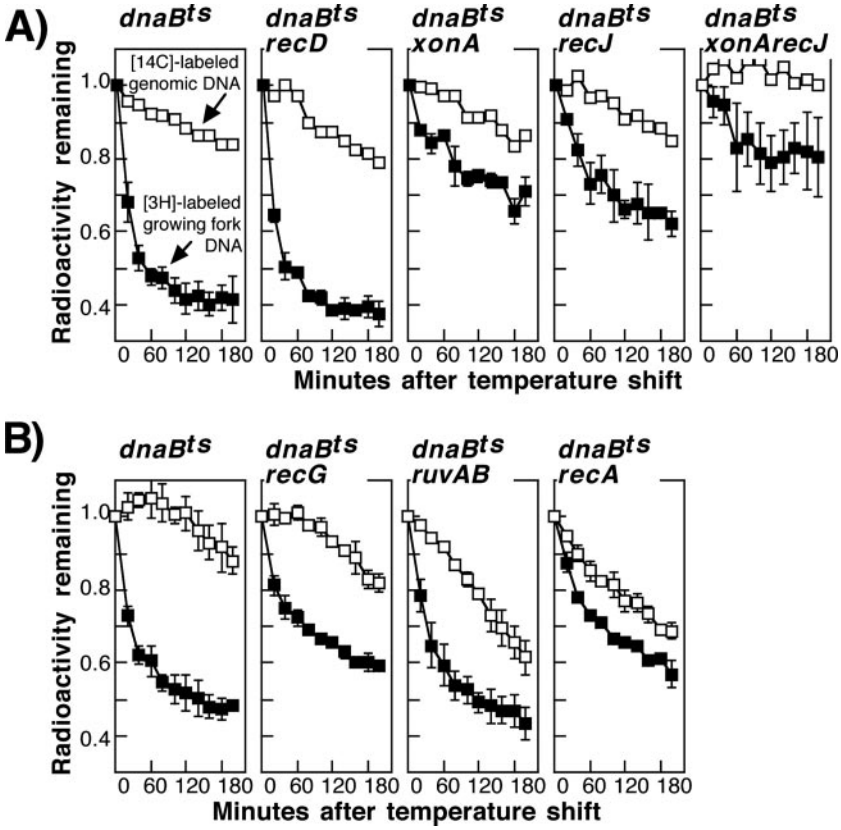

FIG. 3. Exonuclease I and the RecF pathway gene products contribute to the nascent DNA degradation at replication forks disrupted following DnaB(Ts) inactivation. Degradation was measured as described in the legend to Fig. 2B. (A) Relative amounts of degradation in the nascent DNA (filled symbols) and total genomic DNA (open symbols) plotted over time for *dnaB*(Ts) strain CRT266, *dnaB*(Ts) *recD* strain CL743, *dnaB*(Ts) *xonA* strain CL774, *dnaB*(Ts) *recJ* strain CL742, and *dnaB*(Ts) *xonA recJ* strain CL785 following a temperature shift to 42°C. (B) Relative amounts of degradation in the nascent DNA (filled symbols) and total genomic DNA (open symbols) plotted over time for *dnaB*(Ts) *recG* strain CL1024, *dnaB*(Ts) *ruvAB* strain CL1026, and *dnaB*(Ts) *recA* strain CL1028 following a temperature shift to 42°C. The symbols indicate the averages of three independent experiments. The error bars indicate one standard deviation. The *dnaB*(Ts) strain CRT266 plots in panels A and B were generated by separate investigators.

ated with RecD (18, 49). 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 (4, 8, 31).

With the exception of the excessive degradation associated with *recA* mutants, no significant degradation of the nascent or genomic DNA was observed for any of these nuclease, helicase, or branch migration enzymes following UV-induced arrest (13, 15, 19; data not shown). 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 requires the action of the RecF pathway genes. Additionally, the presence of RuvAB is required to maintain 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 compared to what occurs after UV irradiation. The distinct enzymatic activities that process replication forks disrupted by UV-induced damage compared to those that process replication forks 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 plasmid pBR322 following DnaB(Ts)-induced arrest and UV-induced arrest. 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 (39). To compare the replication intermediates that occur following DnaB(Ts)-mediated arrest to those that occur after UV-induced arrest, cultures containing plasmid pBR322 were either UV irradiated with 50 J/m^2 or shifted to the restrictive temperature of 42°C. At various times after treatment, the genomic DNA was purified, digested with the restriction endonuclease PvuII, and analyzed by two-dimensional agarose gel electrophoresis. PvuII linearizes pBR322 just downstream of its unidirectional origin of replication. In the absence of

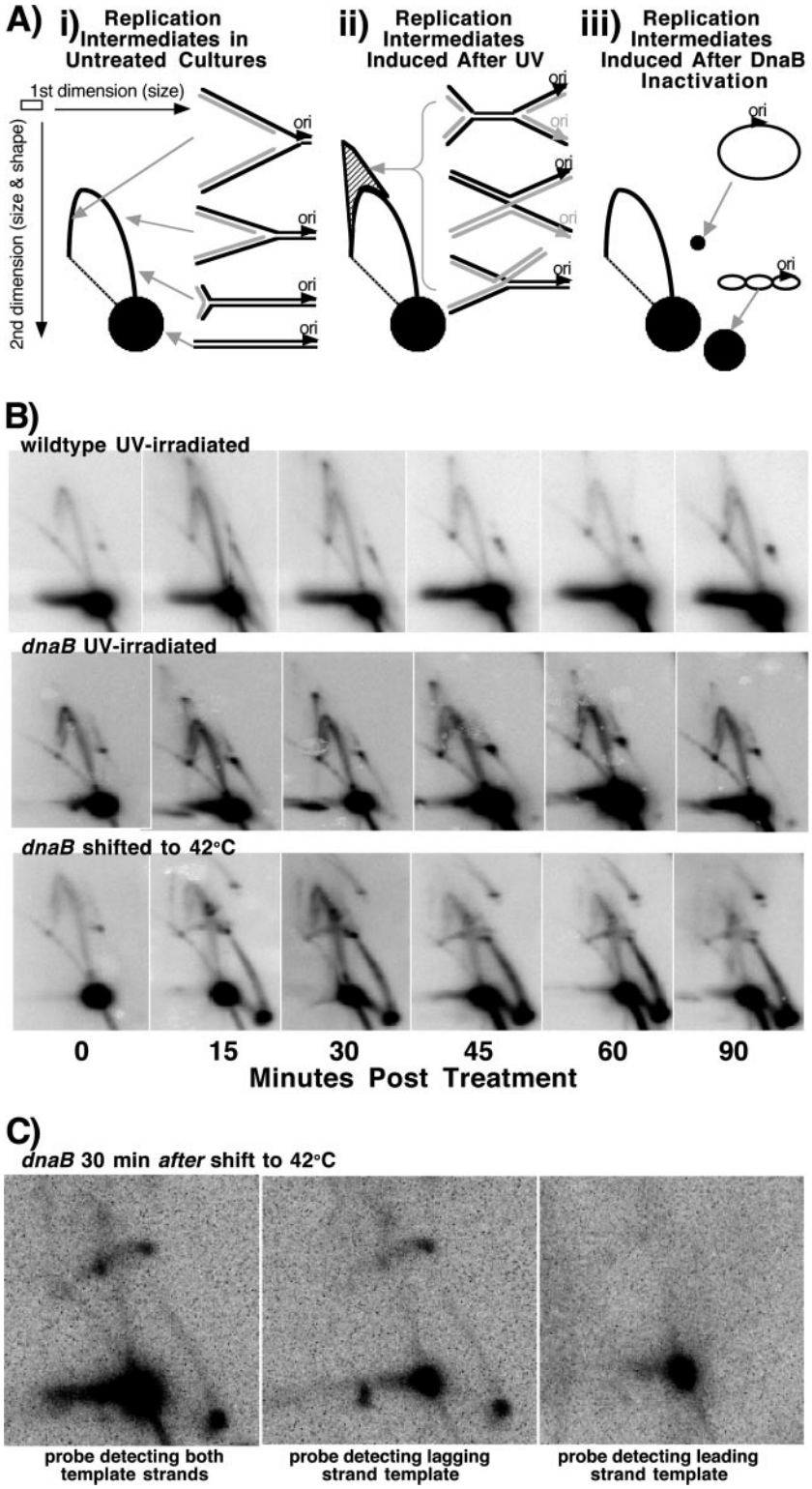

FIG. 4. Structures of plasmid replication intermediates observed following DnaB(Ts) inactivation and following UV irradiation are distinct. (A) Diagram of the migration pattern for PvuII-digested pBR322 observed by two-dimensional agarose gel electrophoresis in (i) untreated cultures, (ii) cultures following UV irradiation, and (iii) cultures following DnaB(Ts) inactivation. Nonreplicating 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 and are made up of double Y- and X-shaped molecules. Following inactivation of DnaB, accumulation of an intermediate that migrates similar to circular, supercoiled plasmid molecules and that is resistant to digestion by PvuII is observed. (B) Cone region intermediates are

impediments to replication, this produces a migration pattern in which the nonreplicating linear plasmid forms 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) (25, 37) (Fig. 4A). Following UV irradiation in wild-type cells at 32°C, elevated levels of Y structures and a transient cone region of molecules containing two branch points appear (12). Previous work from our lab carried out at 37°C has shown that these Y structures and a portion of the cone region intermediates represent arrested replication forks (12, 20). The replication intermediates are stabilized and protected from RecQ- and RecJ-mediated degradation by RecFOR and RecA until a time that correlates with the time when the lesions are repaired and DNA synthesis resumes (7, 12, 20). Based on the genetic requirements needed for DNA synthesis to resume, it has been proposed that the processing by RecF pathway proteins effectively moves the branch point of the replication fork away from the arresting lesion, restoring the region to a form that allows repair enzymes to gain access and remove the damage (9–12, 15).

When we examined cultures of UV-irradiated *dnaB*(Ts) mutants at 32°C, we observed intermediates and patterns similar to those observed in wild-type cells (Fig. 4B). However, following inactivation of DnaB(Ts) at 42°C, the intermediates observed on the replicating molecules were clearly distinct from those seen after UV-induced damage. In contrast to the UV results, 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 similarly to unrestricted supercoiled, circular plasmid molecules appeared within 15 min of the temperature shift and continued to accumulate throughout the time course (Fig. 4C). The intermediate species ran slightly below the position where double-stranded supercoiled plasmids appeared in the gel and was resistant to restriction digestion, suggesting that the molecule may contain regions of single-stranded DNA. To test this possibility and to further characterize the substrate formed at forks disrupted by DnaB inactivation, we probed the two-dimensional gels with strand-specific probes that were complementary to either the leading- or lagging-strand template of pBR322. As shown in Fig. 4D, the probe complementary to the lagging-strand template, but not the probe complementary to the leading-strand template, hybridized to the intermediate formed following DnaB inactivation. The accumulation of a species of single-stranded supercoiled DNA that 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. This 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 what 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 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 these data that the replication forks inactivated by DnaB(Ts) are structurally distinct from those that occur following UV-induced arrest.

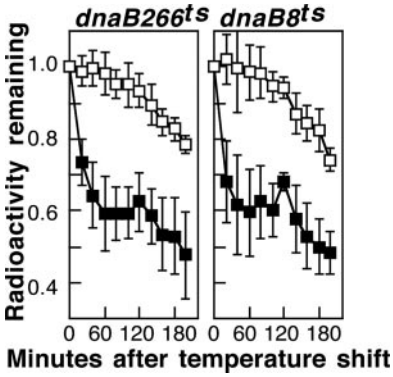

FIG. 5. Both *dnaB266* and *dnaB8* alleles result in degradation of the nascent DNA following inactivation at 42°C. Degradation was measured as described in the legend to Fig. 2B. The relative amounts of degradation in the nascent DNA (filled symbols) and total genomic DNA (open symbols) are plotted over time for *dnaB266* strain CRT266 and *dnaB8* strain PC8 following a temperature shift to 42°C. The symbols indicate the averages of two independent experiments. The error bars indicate one standard deviation.

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 (29, 33, 45). This made it difficult to construct comparable isogenic strains in the W3110 background and raised the possibility that the phenotypes may be unique to the CRT266 strain or *dnaB266* allele. To examine this possibility, we examined the degradation that occurred in a second strain, PC8, which contains a different thermosensitive *dnaB* allele, *dnaB8* (43). 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 strain with the *dnaB266* allele (Fig. 5). Although it remains possible that allelic differences could be revealed by other assays, similar degradation of the nascent DNA has been reported previously

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 wild-type strain SR108 or *dnaB*(Ts) strain CRT266 containing plasmid pBR322 were either UV irradiated or shifted to 42°C. At the times indicated, DNA was purified, digested with PvuII, and analyzed by two-dimensional agarose gel electrophoresis using the pBR322 plasmid as a probe. (C) 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) strain CRT266 containing plasmid pBR322 were shifted to 42°C for 30 min before the DNA was purified and digested with PvuII. Samples were then split and analyzed by two-dimensional agarose gel electrophoresis using either the pBR322 plasmid, an oligonucleotide that is complementary to the lagging-strand template, or an oligonucleotide that is complementary to the leading-strand template as a probe.

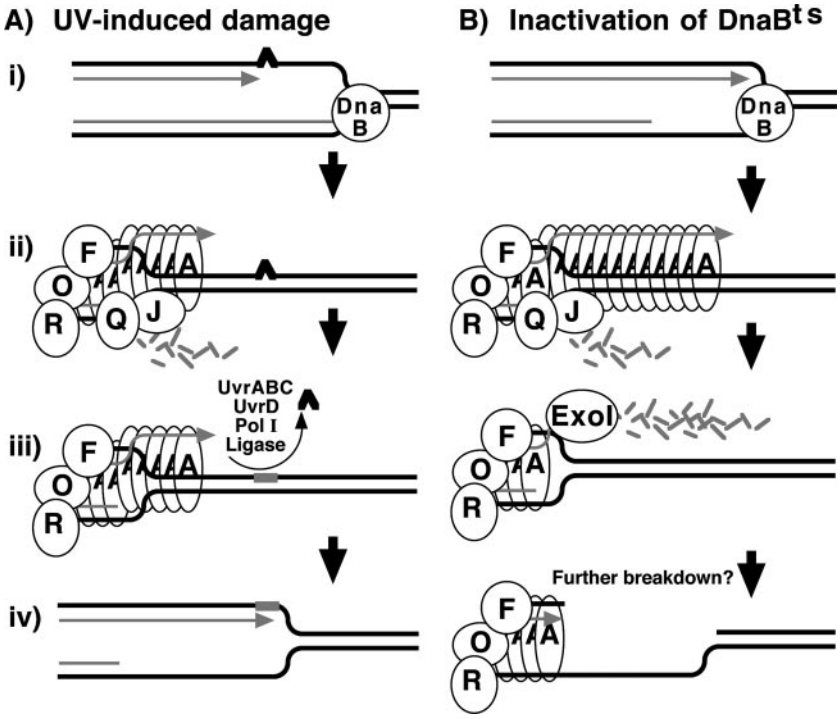

FIG. 6. Model of enzymatic activities detected at replication forks arrested by (A) UV-induced damage or (B) inactivation of DnaB(Ts). (A) (i) Following UV irradiation, the leading-strand polymerase is blocked at the site of a lesion. (ii) RecF (F), RecO (O), and RecR (R) load RecA (A) at the arrested site, limiting the nascent DNA degradation by the RecQ helicase (Q) and RecJ nuclease (J). (iii) Processing by the RecF pathway genes restores the region to a form that allows repair enzymes to remove the blocking lesion. (iv) Replication can then resume once the replication holoenzyme is reloaded. (B) (i) Inactivation of DnaB(Ts) arrests DNA synthesis. (ii) RecF, RecO, and RecR load RecA at the arrested site, limiting the nascent DNA degradation by the RecQ helicase and RecJ nuclease. (iii) Extensive degradation by exonuclease I (ExoI), a 3′-5′ exonuclease, occurs on the nascent leading-strand DNA. (iv) The amount of degradation leads to further breakdown and possible collapse of the replication fork, requiring recombination in order to restore the replication fork.

for another *dnaB*(Ts) allele that was isolated independently (51), consistent with the idea that the observations described here are a general phenomenon occurring after replication is disrupted by the inactivation of the DnaB helicase.

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 it in each case are unique. When 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, and RecR (7, 12, 15) (Fig. 6). The RecQ helicase and RecJ exonuclease process and partially degrade the nascent lagging strand at the blocked replication fork prior to the time that replication resumes (10, 12, 13). 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 (9–11, 15). In the absence of this processing, the recovery of replication is delayed and becomes dependent on translesion synthesis by Pol V (9, 10).

Unlike the limited degradation observed after UV-induced arrest, replication arrested by DnaB(Ts) inactivation led to extensive degradation of approximately one-half the nascent DNA. In this case, rather than limiting 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 required for the nascent DNA degradation that occurred following DnaB(Ts) inactivation, whereas it was not required for the degradation occurring after UV-induced arrest.

The inactivation of DnaB(Ts) also resulted in structural intermediates on replicating plasmids very different 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 (12, 20). 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-stranded plasmid which is likely to represent either the degradation of both the nascent DNA and leading-strand template or uncoupled leading-strand synthesis to generate the circular single-stranded plasmids.

Using pulsed-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 in *dnaB*(Ts) mutants incubated at the restrictive temperature (40). The formation of double-strand breaks in the chromosome of *dnaB*(Ts) *recB* mutants required RuvABC and RecA and occurred in cells that were actively replicating (46, 47). 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 in *ruvAB* and *recA* cultures, which would be consistent with the presence of chromosome breaks occurring in these mutants. The results presented here 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 (K. H. Chow, unpublished data). 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 (1, 23, 27, 40), 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 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 the RecF pathway and may be required for RecF-RecO-RecR to load and the RecJ-mediated degradation to occur. If this is true, the *dnaB266* mutation would be impaired in this ability while retaining enough activity for its essential role during replication.

The observation that distinct forms of processing occur on the replication fork DNA after DnaB inactivation compared to the forms that occur after UV irradiation also suggests that the replication machinery, or at least the DnaB helicase, is not disrupted or inactivated when replication is arrested by UV-induced damage. If the helicase were disrupted or rendered nonfunctional upon encountering UV-induced damage, one might expect that the replication fork DNA would be processed similarly in each case. The distinct processing that is induced by DnaB inactivation is more consistent with the view that the replicative helicase remains partially intact and is required for the normal recovery process to occur. In contrast, following inactivation of a thermosensitive *dnaE* gene, no nascent DNA degradation is observed (data not shown). We are currently investigating whether other DNA polymerases or enzymes are involved in processing these events.

The mechanism by which cells recover replication following arrest is of critical importance to cell survival in the presence of DNA damage and to 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. We demonstrated that the 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. 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 workers try to understand the mechanism by which cells recover replication in the presence of DNA damage, it will be important to clarify this mechanism in the context of the structure of the impediment and the arrested fork.

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