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RecBCD and RecJ/RecQ Initiate DNA Degradation on Distinct Substrates in UV-Irradiated *Escherichia coli*

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After UV irradiation, *recA* mutants fail to recover replication, and a dramatic and nearly complete degradation of the genomic DNA occurs. Although the RecBCD helicase/nuclease complex is known to mediate this catastrophic DNA degradation, it is not known how or where this degradation is initiated. Previous studies have speculated that RecBCD targets and initiates degradation from the nascent DNA at replication forks arrested by DNA damage. To test this question, we examined which enzymes were responsible for the degradation of genomic DNA and the nascent DNA in UV-irradiated *recA* cells. We show here that, although RecBCD degrades the genomic DNA after UV irradiation, it does not target the nascent DNA at arrested replication forks. Instead, we observed that the nascent DNA at arrested replication forks in *recA* cultures is degraded by RecJ/RecQ, similar to what occurs in wild-type cultures. These findings indicate that the genomic DNA degradation and nascent DNA degradation in UV-irradiated *recA* mutants are mediated separately through RecBCD and RecJ/RecQ, respectively. In addition, they demonstrate that RecBCD initiates degradation at a site(s) other than the arrested replication fork directly.

INTRODUCTION

In *Escherichia coli*, exposure to 254 nm UV light induces two predominant forms of DNA lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-6-4-pyrimidones (6-4 PPs), both of which block replication fork progression (*1–4*). After UV irradiation of wild-type cells, a transient inhibition of replication is observed (*3, 5–7*). During this period of inhibition, the integrity of the replication fork DNA is protected and remains primarily intact until a time that correlates with when the DNA lesions are removed and robust replication recovers (*5, 6, 8*). In contrast, in UV-irradiated cells lacking RecF, RecO or RecR, DNA replication fails to recover, and the nascent DNA at arrested replication forks is extensively degraded by the RecJ nuclease and RecQ helicase (*5, 6, 9, 10*). Similar to *recF, recO* and *recR* mutants, UV-irradiated *recA* mutants also fail to recover DNA replication and extensively degrade the nascent DNA at arrested replication forks (*9, 11, 12*). Unlike *recF, recO* and *recR* cells however, the degradation of DNA in UV-irradiated *recA* mutants is much more extensive, and in addition to the nascent DNA, the entire genome is also rapidly degraded (*9, 12, 13*). The genomic degradation in UV-irradiated *recA* cells requires Exonuclease V (the RecBCD enzyme) (*14–17*), which is involved in the repair of double-strand DNA breaks (DSBs) and degrades foreign linear DNA transformed into *E. coli* (*18–20*). Early studies also showed that the genomic degradation in UV-irradiated *recA* cells required active replication (*11, 12*). These observations led a number of investigators to speculate that RecBCD may initiate degradation at blocked replication forks and to the conceptual idea that replication forks may collapse to form double-strand breaks when they encounter DNA damage (*21–23*).

It is not known how RecA protects the genomic DNA from the catastrophic degradation of the genome after UV-radiation-induced DNA damage. RecA is a multifunctional protein that was originally identified based on its requirement for strand exchange to occur during recombinational processes (*24*). *In vitro*, RecA monomers bind and form a helical filament around single-strand DNA before pairing it with homologous duplex DNA [reviewed in ref. (*25*)]. In addition to its essential role in mediating homologous recombination, RecA also functions during replication in the presence of DNA damage. RecA binding to single-strand DNA also serves as the inducing signal to up-regulate the SOS response (*26, 27*). After DNA damage, RecA binds to the single-strand regions generated when replication forks encounter DNA lesions. This binding results in the up-regulation of more than 40 SOS genes, including *recA* (*26–28*). The binding and homologous pairing activities of RecA also serve a structural role at the lesion-arrested replication forks by maintaining and processing the replication fork DNA in a manner that allows DNA replication to resume once the lesion has been removed or overcome (*5, 9,*

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TABLE 1
Strains Used in The Study

Strain	Relevant genotype	P1 donor × recipient
SR108	*thyA36 deoC2* derivative of W3110	(*38*)
CL542	SR108 *recA*::cam	JJC432 (*39*) × SR108
CL718	SR108 Δ(*srlR-recA*)*306*::Tn*10 xonA*::cat*300*	HL921 (*9*) × HL1034 (*35*)
CL700	SR108 *recD1011 argA81*:Tn*10*; *recQ1803*::Tn*3*	HL923 (*35*) × HL944 (*35*)
CL893	SR108 *recD1011*	HL923 (*35*) cured of Tn*10*
CL894	SR108 *recJ284*	HL924 (*35*) cured of Tn*10*
CL752	SR108 *recJ284*::Tn*10 recD1011*	HL924 (*35*) × CL893
CL720	SR108 *recA*::cam *recJ284*::Tn*10*	CL542 × HL924 (*35*)
CL724	SR108 *recA*::cam *recQ1803*::Tn*3*	CL542 × HL944 (*35*)
CL726	SR108 *recA*::cam *recD1011 argA81*::Tn*10*	CL542 × HL923 (*35*)
CL851	SR108 *recA*::cam; *recB21 recC22 argAB1*::Tn*10*	CL542 × HL922 (*35*)
CL853	SR108 *recA*::cam; *ruvAB6204*::kan*858*	CL542 × CL578 (*41*)
CL783	SR108 *recA*::cam *recG*::Tn*5*	CL542 × CL008 (*41*)
CL854	SR108 *recA*::cam *uvrA*::Tn*10*	CL542 × HL952 (*8*)
CL736	SR108 *recA*::cam *uvrC297*::Tn*10*	CL542 × HL925 (*8*)
CL730	SR108 *recA*::cam *recD1011 argA81*::Tn*10 recQ1803*::Tn*3*	CL542 × CL700
CL781	SR108 *recA*::cam *recD1011 argA81*::Tn*10 recJ284*::Tn*10*	CL542 × CL752

12, 29, 30). In the absence of RecA, cells are unable to induce the SOS response and are extremely sensitive to DNA damage (*11, 24, 31*).

RecBCD is required for the DNA degradation to occur in UV-irradiated *recA* cells. It is a trimeric enzyme composed of RecB, RecC and RecD subunits that form a dual ATP-dependent helicase and nuclease that is capable of unwinding and degrading duplex DNA from a double-strand end (*18–20*). The nucleolytic activity of RecBCD switches upon encountering a Chi sequence, from the degradation of duplex DNA to predominantly target the strand containing a 5′ end, thus generating a 3′ single-strand overhang. The 3′ single-strand overhang is a target for loading by RecA and is thought to be an important step in the initiation of repair of double-strand DNA breaks in *E. coli* (*18, 20*).

Although several studies have established that RecBCD mediates the extensive DNA degradation in UV-irradiated *recA* cells (*14–17*), it remains unclear how and upon what sites the RecBCD enzyme initiates upon in these cells. In addition, it remains unclear what relationship, if any, exists between the nascent DNA degradation that occurs in UV-irradiated wild-type cells and the extensive degradation that occurs in RecA mutants. Several reviews and studies have speculated that RecBCD may directly target nascent DNA substrates generated at a stalled replication fork either by the regression of the arrested replication fork or by breakage of the replication fork (*21, 22, 32–34*), although no study has examined this question directly. In contrast, recent studies characterizing the nascent DNA processing that occurs in UV-irradiated wild-type cells have shown that the RecJ nuclease and RecQ helicase, but not RecBCD, partially degrades the nascent DNA at the blocked forks, suggesting that RecBCD does not work at these sites when RecA is present (*5, 9, 10, 35*). The observations that *recBC* mutants exhibit hypersensitivity to DNA damage, low viability in culture, and impaired recombination activity indicate that RecBCD processing plays a critical role in the normal cell cycle and in maintaining genomic stability (*36, 37*). Characterizing when and where RecBCD processing occurs will likely provide critical insights into how these fundamental aspects of genome stability are maintained. Therefore, in this study, we sought to investigate what roles RecBCD plays in the processing of the nascent DNA and genomic DNA in UV-irradiated *recA* mutants and to directly examine its potential function in processing the arrested replication fork. In addition, we also examined several other candidate genes that may be involved in generating a substrate for the initiation of RecBCD degradation in *recA* cells.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this work are presented in Table 1. SR108 is a *thyA36 deoC2* derivative of W3110 (*38*). CL542 (SR108 *recA*::cam) was made by P1 transduction of the *recA*::cam allele from JJC432 (*39*) into SR108. CL718 [SR108 Δ(*srlR-recA*)*306*::Tn*10*; Δ*xonA*::cat*300*] was made by P1 transduction of the *recA*::Tn*10* allele from HL921 (*35*) into HL1034 (*9*). CL700 (SR108 *recD1011 argA81*::Tn*10*; *recQ1803*::Tn*3*) was made by P1 transduction of the *recD1011 argA81*::Tn*10* allele from HL923 into HL944 (*35*). CL752 (SR108 *recJ284*::Tn*10*; *recD1011*) was made by P1 transduction of *recJ284*::Tn*10* allele from HL924 (*35*) into CL893. CL720 (SR108 *recA*::cam; *recJ284*::Tn*10*), CL724 (SR108 *recA*::cam; *recQ1803*::Tn3), CL 726 (SR108 *recA*::cam; *recD1011 argA81*::Tn*10*), CL851 (SR108 *recA*::cam; *recB21 recC22 argAB1*::Tn*10*), CL853 (SR108 *recA*::cam; *ruvAB6204*::kan*858*), CL783 (SR108 *recA*::cam; *recG*::Tn*5*), CL854 (SR108 *recA*::cam; *uvrA*::Tn*10*), CL736 (SR108 *recA*::cam; *uvrC297*::Tn*10*), CL730 (SR108 *recA*::cam; *recD1011 argA81*::Tn10; *recQ1803*::Tn3), CL781 (SR108 SR108 *recA*::cam; *recD1011*; *recJ284*::Tn*10*) were made by P1 transduction of the *recA*::cam allele from CL542 into HL924 (*40*), HL944 (*35*), HL923 (*35*), HL922 (*35*), CL578, HL945 (*41*), HL952 (*41*), HL925 (*41*), CL700, and CL752, respectively.

Selection of Tetracycline-Sensitive Alleles of recJ284 and recD1011

CL893 (SR108 *recD1011*) and CL894 (SR108 *recJ284*) were cured of their tetracycline resistance marker by the selection of tetracycline-sensitive clones of HL923 (SR108 *recD1011 argA81*::Tn10) and HL924 (SR108 *recJ284*::Tn*10*) based on a method described previously (*42*). Briefly, cultures were grown overnight in LB medium. Cultures were then diluted to 1000-fold in M9 minimal medium before 100 μl were plated on Tc^S plates containing 15 g/liter agar, 5 g/liter tryptone broth, 5 g/liter yeast extract, 4 ml/liter of chlortetracycline hydrochloride (12.5 mg/ml), 10 g/liter NaCl, 10 g/liter $NaH_2PO_4.H_2O$, 6 ml/liter fusaric acid (2 mg/ml), and 5 ml/liter $ZnCl_2$ (20 m*M*). Plates were incubated for 24 to 48 h at 37°C. Colonies that appeared within this time were then screened to identify cells that had acquired sensitivity to 20 μg/ml tetracycline.

UV Irradiation

Bacterial cultures were UV-irradiated in DGCthy medium [1× Davis (*43*), 0.4% glucose, 0.2% casamino acids, 10 μg/ml thymine] in petri dishes on a rotating orbital shaker using a Sylvania 15-W germicidal light bulb (254 nm; 0.9 J/m²/s).

Degradation Assay

A fresh overnight culture was diluted 1:100 and grown in DGCthy medium supplemented with 3.7 kBq/ml [^{14}C]thymine to an OD_{600} of 0.4 in a 37°C shaking incubator. Cultures were then pulse-labeled with 37 kBq/ml [^{3}H]thymidine for 10 s to label the nascent DNA at the replication fork before cells were filtered on Fisherbrand General Filtration 0.45-μm membranes, washed with 2–5 ml NET buffer (100 m*M* NaCl, 10 m*M* EDTA, 10 m*M* Tris, pH 8), and resuspended in prewarmed non-radioactive DGCthy medium. Cells were then UV-irradiated with 27 J/m² before they were returned to a 37°C shaking incubator. At 20-min intervals, duplicate 200-μl aliquots of culture (triplicate at time 0) were lysed and the DNA was precipitated in cold 5% trichloroacetic acid and then filtered on Millipore glass fiber prefilters. The amount of radioactivity in each filter was determined in a scintillation counter. The ^{14}C and ^{3}H counts at the time of irradiation ranged between 160–2100 cpm and 730–1100 cpm, respectively.

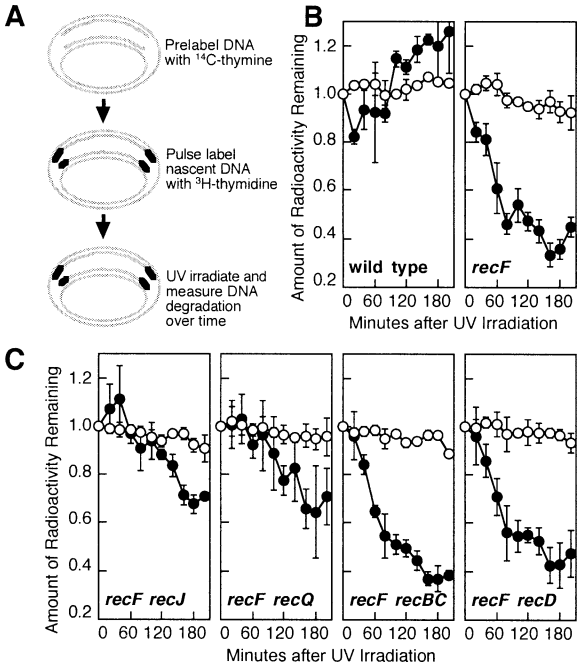

FIG. 1. RecJ and RecQ, but not RecBCD, degrade the nascent DNA at arrested replication forks after UV-radiation-induced damage in $RecA^+$ cells. Panel A: The procedure used for monitoring the degradation occurring at the nascent DNA and in the overall genome. Cultures prelabeled with [^{14}C]thymine were grown to mid-log phase and then pulse-labeled for 10 s with [^{3}H]thymidine before being resuspended in non-radioactive medium and UV-irradiated with 27 J/m². Aliquots were taken at various times after UV irradiation, and the fraction of radioactivity remaining in DNA was determined. Panel B: The ^{14}C-labeled genomic DNA (open symbols) and the ^{3}H-labeled nascent DNA (filled symbols) was monitored as described for panel A for wild-type and *recF* cultures. Panel C: The genomic and nascent DNA was monitored as described for panel A for *recFrecJ, recFrecQ, recFrecD* and *recFrecBC* cultures.

RESULTS

Degradation of the Nascent DNA at Stalled Replication Forks is not Dependent on RecBCD

Recent studies have highlighted the role that nucleolytic DNA degradation plays in the recovery of replication after UV irradiation (*5, 6, 8, 35*). To examine the degradation the arrested replication fork and to compare it to that occurring in the overall genome, cultures prelabeled with [^{14}C]thymine were pulse-labeled with [^{3}H]thymidine for 10 s to label the nascent DNA at the replication fork. Then cells were placed into non-radioactive medium and immediately UV-irradiated at a dose of 27 J/m² (Fig. 1A). The ^{14}C label and the ^{3}H label allowed us to directly compare the degradation that occurs in the overall genome to that which occurs at the nascent DNA synthesized at replication forks just prior to UV irradiation. Consistent with previous studies, we observed that after UV irradiation of wild-type cells, a limited amount of degradation of nascent DNA occurred at arrested replication forks at times prior to when replication resumed (*5, 8, 35*). Overall, however, both the nascent DNA and genomic DNA remained protected, and little degradation was observed (Fig. 1B). In contrast, in UV-irradiated *recF, recO* or *recR* mutants, the nascent DNA degradation was much more extensive. In this case, the degradation was limited to the nascent DNA and the genomic DNA remained relatively protected (Fig. 1B) (*9, 10*). As shown previously, we observed that the nascent DNA degradation in both UV-irradiated wild-type and *recF, recO* or *recR* cells was mediated by the RecJ/RecQ nuclease/helicase (Fig. 1C) (*5, 8, 35*). In the absence of RecJ or RecQ, no degradation of the nascent DNA is observed for the first hour after irradiation. At times longer than 120 min after irradiation, some nascent DNA degradation is still detected in *recJ* or *recQ* mutants lacking RecF. This eventual degradation is likely to be due to the eventual deterioration of the replication forks when they are prevented from recovering normally (*9, 35*). Consistent with this interpretation, in an otherwise wild-type background in which replication does recover, no nascent DNA degradation is detected in *recJ* or *recQ* mutants (*35*). As described in the Introduction, a number of models have speculated that RecBCD may target the newly synthesized DNA at the arrested replication forks as part of the recovery

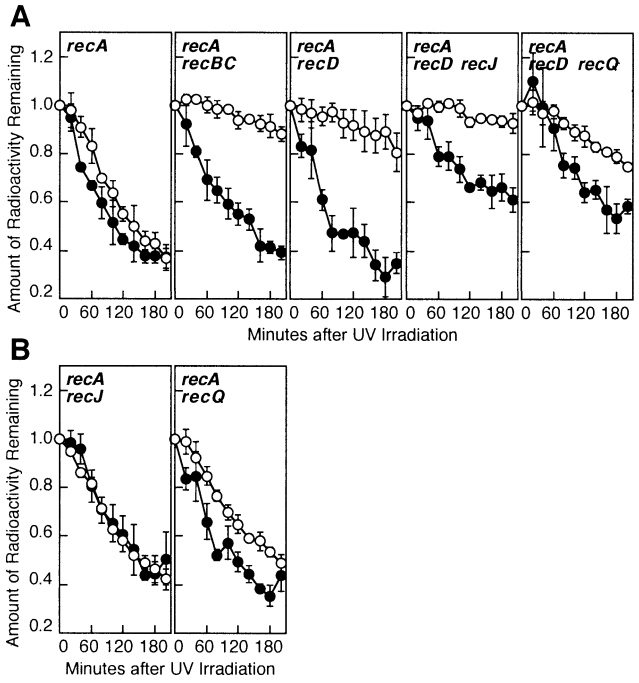

FIG. 2. RecBCD is required for the degradation of the genome to occur in *recA* mutants but does not target the nascent DNA at the arrested replication fork. Panel A: RecBCD is required for genomic DNA degradation but not nascent DNA degradation after UV irradiation. Degradation in the ^{14}C-labeled genomic DNA (open symbols) and the ^{3}H-labeled nascent DNA (filled symbols) was monitored as described in Fig. 1 for *recA, recArecBC, recArecD, recArecDrecJ* and *recArecDrecQ* cultures after UV irradiation with 27 J/m^2. Panel B: Prior processing by RecJ and RecQ is not required for RecBCD to initiate the degradation of the genome in *recA* cultures. Data are for *recArecJ* and *recArecQ* cultures after UV irradiation with 27 J/m^2.

process. To assess this possibility directly, we examined the degradation that occurred in UV-irradiated *recF* mutants that also lacked either RecD or RecBC and observed that the absence of either of these genes did not affect the nascent degradation (Fig. 1C). These findings indicate that the newly synthesized DNA at UV-radiation-arrested replication forks is not accessible to RecBCD-mediated degradation in *recA*$^+$ cells. In addition, the results do not support the idea that replication forks arrested at UV-radiation-induced damage collapse to form double-strand breaks.

Although RecBCD does not initiate the degradation of newly synthesized DNA in wild-type cells, it is possible that an abnormal or unique substrate is generated at arrested replication forks in the absence of RecA, where the RecBCD-mediated degradation is much more extensive. To examine this possibility, we examined the DNA degradation that occurred in UV-irradiated *recA* mutants. It has been established that the failure of UV-irradiated *recA* cells to recover DNA replication correlates with a nearly complete degradation of the genomic DNA (*9, 12, 14*). Consistent with this, when we examined UV-irradiated *recA* mutants, we observed extensive degradation of the genomic DNA as well as the degradation of the nascent DNA (Fig. 2A). The remaining 10–30% of the DNA in *recA* mutants that is not degraded is likely to represent the DNA of cells within the culture that are either not replicating or for statistical reasons did not encounter DNA damage prior to completing their replication cycle, since previous studies have shown that *recA* mutants under these conditions are resistant to DNA degradation (*11, 12*). It has also been established that the genomic degradation in UV-irradiated *recA* cells is mediated by RecBCD (*14–17*). *recBC* mutants lack both the helicase and nuclease activities of the RecBCD enzyme and these mutants exhibit hypersensitivity to UV radiation and reduced recombination frequencies during conjugation or transduction (*45*). *recD* mutants retain helicase function but lack nuclease activity, and these mutants remain resistant to UV radiation and proficient in recombination (*46*). Based on this observation, several subsequent reviews have postulated that the degradation by RecBCD may initiate at the arrested replication forks and then progress back to the bulk of the DNA (*21–23, 33, 44*). If this is true, then one would predict that the degradation of the genome by RecBCD in UV-irradiated *recA* mutants would first initiate and degrade the nascent DNA at stalled replication forks. Surprisingly, however, although UV-irradiated *recA* cells did not degrade the genomic DNA in the absence of RecBC or RecD, we observed that the nascent DNA at arrested replication forks remained susceptible to exonucleolytic digestion (Fig. 2A). Interestingly, under these conditions, the nascent DNA was partially degraded by RecJ nuclease and RecQ helicase, similar to the mechanism occurring in UV-irradiated wild-type cells (Fig. 2A). Although DNA degradation is reduced in *recA* mutants lacking either RecBC, RecD, RecJ or RecQ, the absence of these enzymes does not rescue the UV-radiation hypersensitive phenotype of *recA* cultures (data not shown). These results imply that the genomic degradation and the nascent DNA degradation in UV-irradiated *recA* cells are mediated through separate enzymatic pathways and that RecBCD does not target the nascent DNA for degradation directly.

RecBCD-Mediated Degradation of the Genome does not Require RecJ or RecQ Processing to Initiate

Although the above results indicate that the RecBCD enzyme does not target the nascent DNA at arrested replication forks directly, it remains possible that processing of nascent DNA at arrested replication forks by RecJ and RecQ is required to generate the appropriate substrate for RecBCD degradation to be initiated. If this is true, then one would predict that inactivation of the RecJ/RecQ enzymes should inhibit or delay the degradation of the genome by RecBCD. However, when we examined UV-irradiated *recA* mutants that also lacked either RecJ or RecQ, the kinetics of the genomic degradation was similar to that which occurred in *recA* mutants (Fig. 2B). Interestingly, we also observed that the nascent DNA degradation occurred concurrently with the complete degradation of genome in UV-irradiated *recArecJ* and *recArecQ* cells. These results sup-

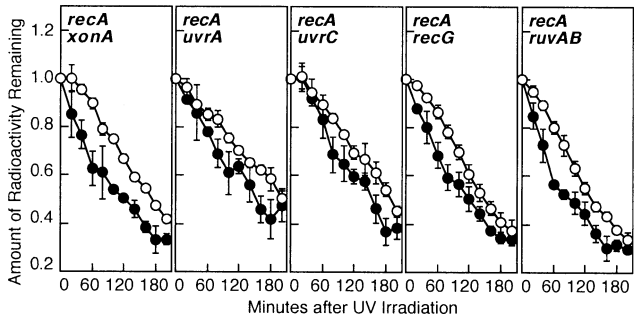

FIG. 3. Inactivation of Exonuclease I, nucleotide excision repair, or the RuvAB or RecG branch migration enzymes does not prevent the degradation of the genome from in UV-irradiated *recA* cultures. Degradation of the ^{14}C-labeled genomic DNA (open symbols) and the ^{3}H-labeled nascent DNA (filled symbols) was monitored as described in Fig. 1 for *recAxonA, recAuvrA, recAuvrC, recArecG* and *recAruvAB* cultures after UV irradiation with 27 J/m^2.

port the idea that RecBCD may initiate at sites other than or independent from the arrested replication fork. If RecBCD initiated on a substrate associated with the nascent DNA degradation, we would expect to observe that the nascent DNA would be degraded prior to the time at which any significant degradation of the genomic DNA occurred. Instead, we observed that both the nascent and the genomic DNA degraded with similar kinetics, suggesting that RecBCD may initiate at a separate substrate, independent of the replication fork itself.

Neither Exo I, Nucleotide Excision Repair, nor Branch Migration by RecG or RuvAB is Required to Generate the DNA Substrate for RecBCD Degradation after UV-Radiation-Induced Damage

In the absence of RecA binding to the single-strand DNA regions at arrested replication forks (i.e. *recA* mutants), the nascent DNA ends might be more vulnerable to nucleolytic activities that would otherwise not have access to these ends. This in turn may generate a DNA substrate that serves as an entry point for RecBCD. One candidate nuclease that is known to target 3 ends that would otherwise be protected by RecA is Exo I, an enzyme that degrades the nascent DNA after inactivation of the DnaB helicase (*47*). However, when we examined cells lacking Exo I, we observed that both the nascent DNA at replication forks and the genome were degraded in UV-irradiated *recA* mutants (Fig. 3).

Other possible candidate enzymatic activities that might generate an appropriate DNA substrate for RecBCD degradation included those that promote branch migration, such as RuvAB or RecG. In support of this idea, it has been observed that in some thermosensitive replication mutants, elevated levels of RuvAB dependent double-strand breaks are observed to arise on the chromosome at the restrictive temperature (*48, 49*). RuvAB is an enzyme complex that together with RuvC promotes migration and resolution of Holliday junctions (*50*). Together, RuvA and RuvB act as a helicase that catalyzes migration of four-way branched DNA junctions. RuvC is an endonuclease that resolves these structures by making symmetric incisions in the DNA. Studies have suggested that efficient resolution of Holliday junctions requires all three gene products (*50, 51*). RecG binds to three- or four-arm branched DNA junctions and promotes branch migration but is not known to have or associate with a protein partner having endonucleotic activity (*52*). We observed that the inactivation of RuvAB or RecG in UV-irradiated *recA* cells did not prevent degradation of the genome (Fig. 3).

We also examined the possibility that the incision of UV-radiation-induced lesions near the branch point of arrested replication forks by nucleotide excision repair proteins may generate a DSB substrate that allows RecBCD to initiate degradation. Again, however, in *uvrA* or *uvrC* mutants, which are unable to incise UV-radiation-induced lesions in the genome (*53–55*), no inhibition of the degradation in *recA* mutants was observed (Fig. 3). These results indicate that the absence of the enzymatic excision of DNA lesions near the branch point at arrested replication forks or branch migration of joint molecules does not prevent the generation of an appropriate DNA substrate for RecBCD degradation after UV-radiation-induced damage.

DISCUSSION

The extensive degradation that occurs in replicating *recA* mutants of *E. coli* after DNA damage demonstrates that DNA ends are unstable and vulnerable to nucleolytic digestion if they are not repaired or protected. Double-strand breaks may be generated directly by exposure to ionizing radiation or chemical agents such as the gyrase inhibitor nalidixic acid (*56–59*). They have also been shown to arise indirectly after exposure to high levels of UV radiation or oxidizing agents when proximal lesions are incised on both strands of the DNA (*60*). Biochemically, RecBCD is thought to require a double-strand end to initiate its helicase and exonucleolytic activities (*61*). Furthermore, *recBC* mutants are hypersensitive to UV-radiation-induced DNA damage and in replicating *recA* mutants, RecBCD-mediated DNA degradation is extensive (*11, 12, 14–17*). These observations have led to models speculating that double-strand breaks may frequently arise at replication forks if the holoenzyme were to replicate through persistent nicks in the DNA template, or if the replication fork were to collapse and break down when its progress is impeded (*21–23, 33, 44*). However, it remains unclear whether either of these events occur *in vivo* or can account for the hypersensitivity of *recBC* mutants after UV-radiation-induced DNA damage.

In this study, we directly tested the idea that substrates for RecBCD are generated at the replication forks when they encounter DNA damage by labeling the nascent DNA at arrested replication forks and characterizing the nucleases that participate in the degradation that occurs in UV-

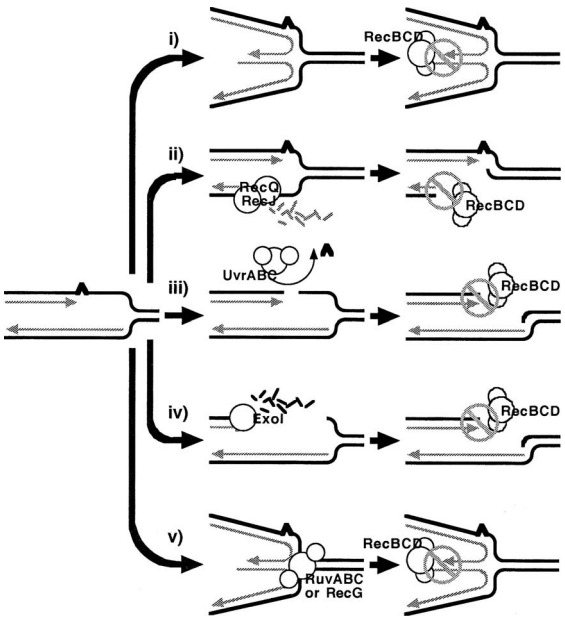

FIG. 4. Hypothetical substrates and enzymatic processing of arrested replication forks that did not affect the ability of RecBCD to degrade the genome after UV-radiation-induced damage. After UV irradiation of *recA* mutants, RecBCD promotes a nearly complete degradation of the genome. The RecBCD-mediated degradation has been widely speculated to initiate at the arrested replication fork. (i) The absence of RecBCD did not affect degradation of the nascent DNA at the arrested fork. (ii) Processing of the nascent DNA by RecJ or RecQ was not required to generate a substrate for RecBCD-mediated degradation. (iii) Incisions generated by the nucleotide excision repair proteins during lesion removal were not required to generated a substrate for RecBCD-mediated degradation. (iv) Processing of the arrested replication fork by Exonuclease I was not required to generate a substrate for RecBCD-mediated degradation. (v) Branch migration of the arrested replication fork as proposed to occur by either RecG or RuvAB was not required to generate a substrate for the RecBCD-mediated degradation.

irradiated *recA* cultures. We found that while RecBCD was responsible for the degradation of the overall genomic DNA in *recA* mutants, the RecBCD-mediated degradation did not initiate at the arrested replication forks *in vivo.* Instead, we observed that RecJ and RecQ target the nascent DNA and that degradation of the genomic DNA occurred independently from the degradation that occurred at the arrested fork. The observations support the idea that RecBCD is initiating degradation upon an alternative substrate generated after UV-radiation-induced DNA damage, though this substrate did not require nucleotide excision repair, Exonuclease I, or branch migration by either RuvAB or RecG to form (Fig. 4). In all cases, we found that these enzymatic activities did not affect the RecBCD-mediated degradation of the genome. Although we did not identify where RecBCD initiates degradation, our data strongly suggest that it does not target the nascent DNA at the replication fork directly and that it is likely to initiate independently at other sites generated as a result of replication in the presence DNA damage.

The results presented here also indicate that the recF pathway gene products do not play a role in the RecBCD-mediated degradation of the genome, an observation that is surprising considering that several lines of evidence have demonstrated RecF-O-R promotes and stabilizes RecA filaments on specific DNA substrates both *in vitro* and *in vivo* (*62–64*). Similarly, recF pathway genes are not essential for other recBCD-mediated processes, such as recombination during conjugation or transduction (*65*). Implied in this observation is that the genomic degradation may be initiated on a substrate mimicking a structural intermediate that occurs during these recombinational processes but does not arise at the replication fork.

While *recBC* mutants are hypersensitive to agents that generate double-strand breaks, several observations suggest that the cellular targets of RecBCD are more specialized and integrated with the process of DNA replication. Notably, mutations inactivating genes such as *recN* or *sbcC* render cells hypersensitive to agents that generate double-strand breaks (X rays and ionizing radiation) but not to agents that generate base adducts or single-strand lesions (such as UV radiation) (*66–69*). In contrast, *recBC* mutants are hypersensitive to all DNA-damaging agents, not just those that generate double-strand breaks, suggesting that its substrate in the case of UV-radiation-induced damage is distinct from those of a simple double-strand break. Further, *recBC* mutants exhibit reduced growth and lower viability even in the absence of DNA damage (*37*). By comparison, *recF* mutants, which are hypersensitive to single-strand lesions encountered during replication, grow as well as wild-type cultures in the absence of DNA damage (*37*). Assuming that single-strand lesions arise far more frequently than double-strand breaks during normal metabolism, these observations imply that the impaired growth of *recBC* mutants may result from a failure to process a substrate associated with the normal replication of the chromosome rather than the double-strand breaks themselves. Last, there is the observation that Chi sites on the *E. coli* chromosome are distributed nonrandomly with respect to the direction of replication (*70*). The octameric Chi sequences, which attenuate the nucleolytic activity of RecBCD and target the site where RecBCD-promoted recombination will occur, are preferentially found on the leading strand template (or nascent lagging strand) of the chromosome (*20, 70, 71*). While this observation has been the subject of much speculation (*22, 72*), the correlation between the Chi sites and the direction of replication clearly suggests a function and substrate that goes beyond repairing random breaks in the genome.

Consistent with the results presented here, previous studies have demonstrated that the recovery of DNA synthesis after UV-radiation-induced arrest does not require RecBCD (*9, 29*). If one assumes that the function of RecBCD is simply to repair double-strand breaks, the recovery of DNA synthesis in recBC mutants would imply that the hypersensitive phenotype of *recBC* mutants is not the result of replication forks collapsing or breaking after arrest at UV-ra-

diation-induced damage. One of several alternative hypotheses for why RecBCD function is required after UV-radiation-induced damage is that strand breaks form predominantly at sites where topoisomerase or gyrase function has been compromised. Inhibition of gyrase by the antibiotic nalidixic acid has been shown to generate double-strand breaks that are processed by RecBCD (*59*). Further, this type of substrate would be consistent with the observed correlation between replication and *recBC* hypersensitivity but would not necessarily be associated directly with the arrested replication fork DNA. Infrequent or spontaneous formation of DNA breaks at sites that maintain the proper superhelical tension within the genome may also explain why *recBC* mutants exhibit poor growth and viability and is a phenotype that becomes exacerbated the normal progression of replication.

The results and discussion presented here highlight the need to consider alternative sites or even alternative substrates for RecBCD in future studies as we try to identify the mechanism by which this multifunctional enzyme is required for full viability during normal growth and for survival in the presence of DNA damage.

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