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RuvABC Is Required to Resolve Holliday Junctions That Accumulate following Replication on Damaged Templates in *Escherichia coli*[*]

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Janet R. Donaldson[1], **Charmain T. Courcelle, and Justin Courcelle**

From the Department of Biology, Portland State University, Portland, Oregon 97207

RuvABC is a complex that promotes branch migration and resolution of Holliday junctions. Although *ruv* mutants are hypersensitive to UV irradiation, the molecular event(s) that necessitate RuvABC processing *in vivo* are not known. Here, we used a combination of two-dimensional gel analysis and electron microscopy to reveal that although *ruvAB* and *ruvC* mutants are able to resume replication following arrest at UV-induced lesions, molecules that replicate in the presence of DNA damage accumulate unresolved Holliday junctions. The failure to resolve the Holliday junctions on the fully replicated molecules correlates with a delayed loss of genomic integrity that is likely to account for the loss of viability in these cells. The strand exchange intermediates that accumulate in *ruv* mutants are distinct from those observed at arrested replication forks and are not subject to resolution by RecG. These results indicate that the Holliday junctions observed in *ruv* mutants are intermediates of a repair pathway that is distinct from that of the recovery of arrested replication forks. A model is proposed in which RuvABC is required to resolve junctions that arise during the repair of a subset of nonarresting lesions after replication has passed through the template.

Irradiation of *Escherichia coli* with 254-nm UV light induces DNA damage that blocks DNA polymerases and can arrest the replication machinery and generate gaps in the newly replicated DNA (1–3). UV-induced DNA lesions that cannot be repaired may lead to mutations if the wrong base is incorporated, rearrangements if replication resumes from the wrong site, or cell lethality if the block to replication cannot be overcome. Despite these challenges, *E. coli* cells are able to survive and replicate following UV doses that produce thousands of lesions per genome, indicating that cells contain efficient mechanisms to deal with these impediments to replication (4). The mechanism(s) that operate to restore the DNA template upon encounters with DNA damage are likely to depend on whether the lesion is found in the leading or lagging strand template of the DNA (5–7). Recent studies using plasmid substrates have shown that lesions in the leading strand template arrest the overall progression of the replication machinery both *in vivo* and *in vitro* (5, 7). Comparatively, lesions in the lagging strand template of plasmids do not arrest the progression of replication and result in fully replicated molecules that contain nascent strand gaps opposite to the lesion sites (6, 7). The arrest and formation of gaps are consistent with the phenotypes observed *in vivo* on the chromosome following UV irradiation. Although the rate of DNA synthesis is severely reduced immediately following a moderate doses of irradiation, a small amount of DNA synthesis can still be detected that contains gaps in the nascent fragments that are made (8, 9).

In the case where replication is arrested by UV-induced damage, several proteins associated with the *recF* pathway are required to protect and maintain the structural integrity of the arrested fork (10–15). RecF, RecO, RecR, and RecA are needed to maintain the DNA at the replication fork until the blocking lesion can be repaired by the nucleotide excision repair proteins or bypassed by translesion DNA polymerases (10–14). Additionally, RecJ, a 5′–3′ single-stranded exonuclease, and RecQ, a 3′–5′ DNA helicase, process or partially degrade the nascent DNA at the fork at times prior to the resumption of DNA synthesis (14, 16). This nascent DNA processing is thought to enhance the ability of RecF, RecO, RecR, and RecA to maintain the arrested fork and restore the lesion-containing region to a form that is accessible to repair enzymes (14, 16, 17). Under conditions where either the nascent DNA processing or repair cannot occur, the recovery of DNA synthesis is delayed and becomes dependent on translesion synthesis by polymerase V (15, 18).

The processing and repair of the nascent strand gaps that are generated by nonarresting lesions are less well understood but depend upon many of the same *recF* pathway gene products that maintain the fork upon arrest (8, 9, 19). Several models propose that the repair of the nascent strand gaps will proceed through a Holliday junction intermediate. Early models proposed that the nascent strand gaps may be restored by exchanging and then resynthesizing these regions with the homologous regions of sister chromosomes (8, 9). Consistent with this type of model, elevated levels of recombination-dependent strand exchanges are observed in repair-deficient mutants during the period when the nascent strand gaps are joined (1, 20, 21). However, experimentally, daughter strand gap repair has been characterized in repair-deficient populations under conditions where high levels of strand exchanges are observed (1, 20, 21). The high levels of lethality under these conditions make it dif-

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[1] To whom correspondence should be addressed: NIDDK, National Institutes of Health, GBB, 5 Memorial Dr. MSC 0538, Bethesda, MD 20892. Tel.: 301-496-4637; Fax: 301-496-9878; E-mail: donaldsonjr@niddk.nih.gov.

ficult to determine the precise efficiency at which this process is able to promote survival (1). Related models have postulated that transient pairing and exchanges between the gaps and sister chromatids may restore the region to a double-stranded form that can then allow the nucleotide excision repair proteins to repair the lesion (22). This type of model would be consistent with the idea that survival may be promoted by a common pathway involving both recombination and repair proteins (4, 10). More recent models have proposed that translesion DNA synthesis by damage-induced polymerases may join these substrates directly without any requirement for strand exchanges (18, 23, 24). The observation that polymerase V contributes to survival, mutagenesis, and the rate of gap joining following modest or high doses of UV irradiation supports the idea that these polymerases may be acting on at least a portion of these substrates (18, 25–28). In addition, reconstitution of translesion synthesis *in vitro* utilizes a gapped substrate that is coated by a RecA filament and contains a β clamp loaded at the lesion site, a substrate very similar to that predicted to occur at nonarresting DNA lesions (29).

Characterization of how nonarresting lesions are processed *in vivo* is complicated by the fact that both arresting and nonarresting lesions are produced by UV irradiation and that the processing of each class of lesion may share common enzymatic steps. In addition, multiple pathways are likely to contribute to the repair of these substrates.

Both RuvABC and RecG have been shown to process synthetic Holliday structures *in vitro* (30–32). The *ruv* locus was originally identified through a genetic screen that isolated mutants that were hypersensitive to UV irradiation (33). RuvABC was also identified biochemically as an enzyme complex that could specifically promote migration and resolve Holliday junctions (30, 34–36). Purified RuvA forms a tetramer that binds Holliday junction structures and recruits two hexameric rings of RuvB (32, 37). Together, RuvA and RuvB act as a helicase that catalyzes an ATP-dependent migration of four-way branched DNA junctions in either the 5′–3′ or 3′–5′ direction, depending on the reaction conditions (30, 38, 39). RuvC is an endonuclease that binds as a dimer to Holliday junctions and resolves these structures by making symmetric incisions in the DNA (35, 40). *In vitro* studies have suggested that RuvC-mediated resolution of Holliday junctions occurs once the RuvAB complex has been removed from the DNA (41). However, other studies have suggested that an equilibrium exists between RuvAB and RuvABC complexes and that efficient resolution may require all three gene products (42–44).

RecG is a helicase that also catalyzes branch migration *in vitro*. RecG was originally identified through a screen for mutants with increased sensitivity to UV irradiation and subsequently shown to exhibit decreased frequencies of conjugational recombination (45). RecG binds to Holliday junctions as a monomer and, depending on the reaction conditions, can promote ATP-dependent branch migration in either the 3′–5′ or 5′–3′ direction (32, 46, 47). Additionally, RecG was shown to bind and unwind synthetic three-arm structures into four-arm, branched structures that resemble Holliday junctions (48).

Despite their UV hypersensitivity, the absence of either RuvAB or RecG does not impair the resumption of DNA synthesis following UV irradiation, indicating that these enzymes are not essential for the resumption of DNA synthesis following arrest (49). Here, we considered the possibility that these enzymes may process an alternative class of lesions, such as those that fail to arrest the replication machinery. We find that *ruvAB* and *ruvC* mutants, but not *recG* mutants, accumulate unresolved Holliday junctions following replication after UV-induced damage. The accumulation of these unresolved Holliday junctions correlates with a loss of genomic DNA integrity that is likely to result in the lethality observed in these mutants.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Our parental strain, SR108, is a *thyA36 deoC2* derivative of W3110 (50). Strains HL924 (SR108 *recJ284*::Tn*10*), HL946 (SR108 *recF332*::Tn*3*), CL008 (SR108 *recG258*::Tn*5*), CL532 (SR108 *ruvA59*::Tn*10*), CL561 (SR108 *recG258*::Tn*5 ruvA59*::Tn*10*), and CL684 (SR108 *recR6212*:cat883 *recJ284*::Tn*10*) have been described previously (10, 12, 14, 16, 49). CL577 was constructed by P1 transduction of the *ruvC53eda-51*::Tn*10* allele from RDK2615 into SR108 (51). Cells were transformed with plasmid pBR322 by electroporation for experiments involving two-dimensional agarose gel analysis (52). Phenotypes were confirmed by antibiotic resistance and, when appropriate, UV hypersensitivity.

UV Irradiation—All cultures were UV-irradiated in Petri dishes on a rotary platform using a Sylvania 15-watt germicidal lamp (254 nm) at an incident dose of 0.9 J/m^2/s.

Rate of DNA Synthesis—Fresh overnight cultures were diluted 1:100 in 50 ml of Davis medium (53) supplemented with 0.4% glucose, 0.2% casamino acids (DGC medium), and 0.1 μCi/10 μg/ml [^{14}C]thymine and were grown to an A_{600} of 0.3 in a 37 °C shaking incubator. At this time, half of the culture was UV-irradiated with 27 J/m^2, and the other half was mock-irradiated. At the times indicated, 1 μCi/ml [^{3}H]thymidine was added to 0.5-ml aliquots of culture for 2 min at 37 °C. Cells were then lysed, and the DNA was precipitated in 5 ml of 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. Duplicate aliquots were taken at each time point. The amount of ^{3}H- and ^{14}C-labeled DNA on each filter was determined by liquid scintillation counting.

Alkaline Sucrose Gradients—Fresh overnight cultures were diluted 1:100 in DGC medium supplemented with 0.9 μCi/4 μg/ml [^{14}C]thymine to an A_{600} of 0.4. Immediately following UV irradiation with 27 J/m^2, 9 μCi/ml [^{3}H]thymidine (77.8 Ci/mmol) was added to the culture for 5 min at 37 °C. Cells were filtered, washed with 5 ml of 1× NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0), and resuspended in DGC medium supplemented with 10 μg/ml thymine. 0.5-ml aliquots of the culture were removed at each time point, mixed with an equal volume of cold 2× NET, pelleted, and resuspended in 0.1 ml of buffered sucrose (0.01 M Tris, pH. 8.0, 0.01 M EDTA, 0.110 M NaCl, 5.1% sucrose). All samples were kept on ice until the end of the time course. 0.025 ml of each sample (~10^7 cells) was then layered on top of sucrose gradients (5–20% sucrose gradients in 0.1 N NaOH) that had 0.1 ml of 5% Sarkosyl in 0.5 N NaOH layered on top. Gradients were centrifuged for 2 h at 60,000 × *g* at 20 °C. Gradients were collected on Whatman No. 17 chromatography paper, and the amount of ^{3}H- and ^{14}C-

labeled DNA present in each fraction was determined by liquid scintillation counting (18).

Two-dimensional Agarose Gel Electrophoresis—Cultures containing the plasmid pBR322 were grown overnight in the presence of 100 μg/ml ampicillin. 0.2 ml of this culture was pelleted and resuspended in 20 ml of DGC medium supplemented with 10 μg/ml thymine and grown without ampicillin to an A_{600} of 0.5 in a 37 °C shaking incubator. At this time, cultures were irradiated with 50 J/m^2 and transferred to a new, prewarmed flask in the 37 °C shaking incubator. At the indicated times, a 0.75-ml aliquot of culture was transferred to an equal volume of 2× NET, pelleted, resuspended in 0.15 ml of lysozyme solution (1 mg/ml lysozyme, 0.5 mg/ml RNase A in 10 mM Tris, 1 mM EDTA, pH 8.0), and incubated for 20 min at 37 °C. Then 0.025 ml of 20% Sarkosyl and 0.01 ml of 10 mg/ml Proteinase K was added to the samples, and incubation continued for 1 h at 55 °C. Samples were then extracted twice with 4 volumes of phenol/chloroform/isoamyl alcohol (25:24:1), followed by one extraction with 4 volumes of chloroform/isoamyl alcohol (24:1), dialyzed against 200 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0) for 3 h on floating 47-mm Whatman 0.05-μm pore disks (Whatman catalog number VMWP04700), and then digested with PvuII restriction endonuclease (New England Biolabs) overnight at 37 °C. Samples were then extracted with two volumes of chloroform/isoamyl alcohol (24:1) and loaded directly on the gel.

The genomic DNA samples were initially separated in a 0.4% agarose gel in 1× TBE (Tris-borate-EDTA, pH 8.0) at 1 V/cm for 15 h. For the second dimension, the lanes were excised, rotated 90°, and recast in a 1% agarose gel in 1× TBE and electrophoresed at 6.5 V/cm for 7 h. DNA in the gels was transferred to a Hybond N+ nylon membrane by standard Southern blotting, and the plasmid DNA was detected by probing with ^{32}P-labeled pBR322 that was prepared by Nick translation (Roche Applied Science) using dCTP (6000 Ci/mmol; MP Biomedicals) and visualized using a STORM Amersham Biosciences PhosphorImager with its associated ImageQuant analysis software (Amersham Biosciences).

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

Purified DNA was prepared for transmission electron microscopy using either a formamide or aqueous drop technique (54). Formamide samples contained 0.100 μg/ml DNA in 10× TE (pH 8.0) and 50% formamide (v/v). Aqueous samples contained 0.2 μg/ml DNA in 0.25 M ammonium acetate (pH 7.6). For both procedures, cytochrome *c* was added (8 μg/ml final concentration), and a 0.05-ml droplet was transferred to a clean Parafilm sheet in a closed Petri dish. After 90 s, a parlodion-coated 300-mesh copper grid was touched to the surface of the drop, dipped in 75% ethanol for 45 s, 90% ethanol for 5 s, and then rotary shadow-casted with 2.5-cm platinum/palladium (80:20) (EM Sciences) using a Kinney KSE 2A-A evaporator. Samples were observed and photographed under a JEOL JEM-100CXII transmission electron microscope (JEOL USA, Peabody, MA) at 60 kV. Molecules of pBR322 were identified based upon the size of the full-length linear molecule (4.4 kbp) as compared with a known 1-kb standard. Molecules deviating from their predicted size based on electrophoretic migration in the agarose gel were not considered in the analysis.

RESULTS

The Integrity of Genomic DNA Deteriorates at Late Times after Replication Has Recovered in UV-irradiated ruvAB Mutants—Previous work (that was repeated and shown in Fig. 1*A* for the purpose of controls) found that mutants lacking *ruvAB* or *recG* resume DNA replication after DNA damage similar to wild-type cells (49). The rate of DNA synthesis was followed by measuring the amount of [^{3}H]thymidine incorporated during a 2-min pulse into ^{14}C-prelabeled cultures. Following exposure to 27 J/m^2 of UV irradiation, the rate of DNA synthesis was initially inhibited by ~90% before replication began to recover ~20 min after irradiation, with virtually no reduction in cell survival (Fig. 1*A*) (49). Although this dose severely reduced survival of *ruvAB* and *recG* mutants, DNA synthesis resumed in these mutants with kinetics that were similar to wild-type cells (Fig. 1*A*). By contrast, *recJ* and *recF* mutants, which have been shown to directly participate in the resumption of DNA synthesis following arrest, exhibit a significant delay in the recovery of DNA synthesis (10, 12) (Fig. 1*A*).

To further characterize the nature of why *ruvAB* and *recG* mutants are hypersensitive to UV irradiation, we monitored the integrity of the DNA over this same period of recovery using alkaline sucrose gradient analysis. To this end, [^{14}C]thymine-prelabeled cultures were labeled with [^{3}H]thymidine for the first 5 min after UV irradiation before the cultures were transferred to nonradioactive media and allowed to recover. Then, at various times during the recovery period, the relative size of the ^{14}C-labeled genomic DNA and ^{3}H-labeled postirradiation DNA synthesis was determined by sedimentation in alkaline sucrose gradients. A large body of work by a number of investigators has shown that the limited DNA synthesis that occurs after irradiation is made up of small fragments containing gaps that are subsequently restored (or filled in) during the recovery period (1, 8, 9, 20, 21). Consistent with this, immediately following UV irradiation, the ^{3}H-labeled postirradiation DNA synthesis produced smaller sized fragments that migrated more slowly and separated from the large ^{14}C-labeled genomic DNA at the bottom of the gradient. In UV-irradiated wild-type cultures, the genomic DNA remained primarily intact throughout the recovery period, and the postirradiation DNA fragments were gradually joined,

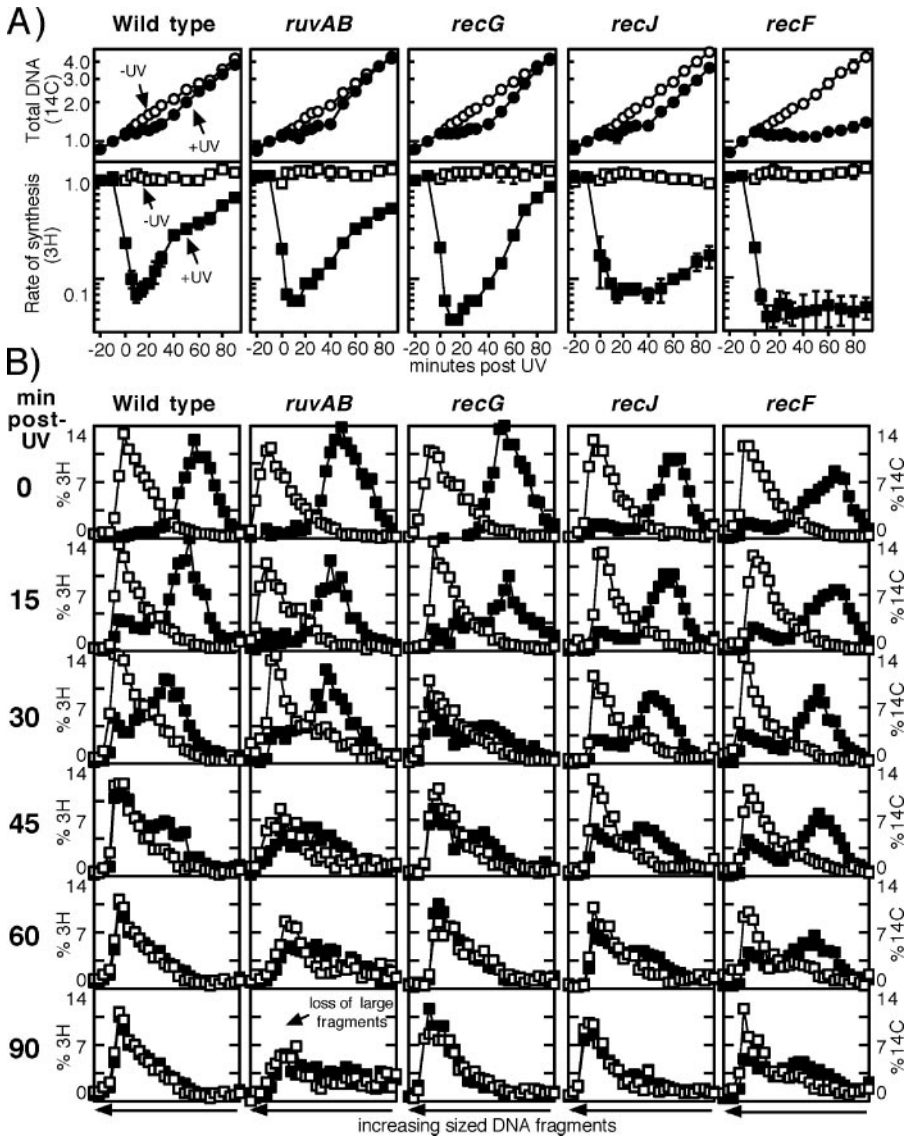


FIGURE 1. ***ruvAB* mutants resume DNA synthesis, but the integrity of the genomic DNA deteriorates at late times following UV irradiation.** *A*, *ruvAB* mutants resume DNA synthesis with kinetics similar to that of wild-type cultures. Cultures grown in the presence of [^{14}C]thymine were pulse-labeled with [^{3}H]thymidine for 2 min at the indicated times following either 27 J/m^2 UV irradiation or mock irradiation. The relative amount of ^{14}C and ^{3}H incorporated into the DNA is plotted over time. Cultures were irradiated at time 0. Graphs represent an average of two independent experiments. *Error bars*, one S.D. ○, total [^{14}C]DNA in mock-irradiated cultures; ●, total [^{14}C]DNA in irradiated cultures; ■, [^{3}H]DNA synthesis in 2 min in mock-irradiated cultures; □, [^{3}H]DNA synthesis in 2 min in irradiated cultures. ^{3}H cpm and ^{14}C cpm at −10 min were as follows: wild type, 16,234.05 and 3165.64; *ruvAB*, 13,789.59 and 1697.10; *recG*, 20,528.5 and 1970.14; *recJ*, 13,838.27 and 1685.74; *recF*, 24,000 and 9000. *B*, the integrity of the genomic DNA in *ruvAB* mutants begins to deteriorate at late times following UV-induced DNA damage. Cells grown in the presence of [^{14}C]thymine were labeled for 5 min with [^{3}H]thymidine following 27 J/m^2 UV irradiation and examined at the indicated times by alkaline sucrose gradient analysis. Larger DNA fragments sediment more rapidly to the bottom of the gradient. The percentage of the total ^{14}C genomic DNA (◇) and ^{3}H DNA made during the first 5 min post-UV (■) for each fraction is plotted. Each time course represents one of at least two independent experiments. Total ^{3}H cpm and ^{14}C cpm in each gradient at time 0 were as follows: wild type, 2600 and 3800; *ruvAB*, 2300 and 3400; *recG*, 2600 and 2800; *recJ*, 4300 and 2100; *recF* 5200 and 2800.

returning to a size that was approximately equal to that of the overall genomic DNA within 45 min after UV (Fig. 1*B*).

When we examined *ruvAB* mutants, we observed that the postirradiation DNA synthesis and genomic DNA initially sedimented with a pattern that was similar to wild-type cultures (Fig. 1*B*). However, at later times when the DNA had been fully restored in wild-type cultures, the integrity of the overall genomic DNA began to deteriorate in *ruvAB* cultures. This was observed as a general loss of the peak corresponding to the large, ^{14}C-labeled DNA in the 60- and 90-min gradients. By contrast, genomic integrity was maintained throughout the recovery period in *recG* cultures, and the postirradiation DNA synthesis was joined and restored with kinetics that was similar to wild-type cultures (Fig. 1*B*). The observation that *recG* is able to maintain genomic integrity despite a UV sensitivity similar to that of *ruvAB* argues that the loss of genomic integrity in *ruvAB* mutants is not simply due to the elevated levels of lethality that occur in these populations but is specifically related to a function carried out by RuvA and RuvB (Fig. 1*B*) (49).

Comparatively, in mutants that are impaired in their ability to resume replication, such as *recJ* or *recF*, we observed that although the joining of the postirradiation nascent DNA fragments was impaired, the integrity of the genomic DNA primarily remained intact throughout the recovery period (Fig. 1*B*). Thus, *ruvAB* mutants exhibit a defect in maintaining the integrity of the genome that is distinct from the class of mutants that are associated with the recovery of replication following UV-induced arrest.

ruvAB Mutants Accumulate Holliday Junctions That Fail to Resolve following Replication on Damaged Templates—The loss of genomic DNA in *ruvAB* suggested the possibility that structural abnormalities may exist in the DNA following replication in these mutants. To test this possibility, we examined the structural intermediates that occurred during replication in the presence of DNA damage using two-dimensional agarose gel electrophoresis. This technique is able to differentiate and identify the structural properties of replicating DNA fragments (14, 56). Cultures of *E. coli* containing the plasmid pBR322 were UV-irradiated with 50 J/m^2, a dose that generates an average of 1 lesion/plasmid. At various times following UV irradiation, total genomic DNA was purified, digested with PvuII to linearize the plasmid slightly downstream from its unidirectional origin of replication, and analyzed by two-dimensional agarose gel electrophoresis. In the absence of DNA damage, nonreplicating plasmid molecules

migrated as a linear 4.4-kb fragment, whereas replicating molecules, which form Y-shaped structures, migrated more slowly due to their larger size and nonlinear shape. This appeared as an arc that extended out from the prominent linear spot (Fig. 2, *A* and *B*). Following UV-induced DNA damage in wild-type cultures, a class of more slowly migrating intermediates accumulated in the cone region of two-dimensional agarose gels that were consistent with molecules having four arms or two branch points (Fig. 2). The resolution of the cone region intermediates was dependent upon nucleotide excision repair and occurred at a time that approximately correlated with when robust replication resumed and the UV-induced lesions were repaired (14) (Fig. 2, *B* and *C*). Previous work from our group has shown that these intermediates are maintained by RecF, RecO, and RecR. In the absence of these proteins, the intermediates are degraded by the RecQ-RecJ helicase-nuclease (14, 57).

When we examined *ruvAB* mutants using this technique, we observed that a class of intermediates also accumulated in the cone region (Fig. 2, *B* and *C*). These intermediates persisted throughout the time course and migrated as fully replicated branched molecules, twice the molecular size of the plasmid. At later times, higher order branched intermediates also accumulated that appeared to be dimers and higher multimeric forms of the plasmid, suggesting that a portion of these intermediates continued to replicate more than once during the time course (Fig. 2, *B* and *C*). Interestingly, a significant portion of these branched intermediates resolved into linear molecules *in situ* during electrophoresis in the second dimension of the gel, as evidenced by the *line* from the fully replicated molecule containing four equal branches that extended down to linear molecules (Fig. 2*B*). Since branch migration of symmetrical four-way junctions is an isoenergetic process (31, 56), the resolution of these intermediates in *ruvAB* mutants may suggest that the molecules contain a symmetrical Holliday junction. The branched intermediates in these mutants were specific to UV-induced damage, since no intermediates were observed in the absence of DNA damage or immediately following UV irradiation (data not shown) (Fig. 2). In *recG* mutants, although the UV-induced replication intermediates that were observed were similar to those that appeared in wild-type, we did observe elevated levels of both Y-shaped and cone region intermediates relative to wild-type cells at early times after UV. Unlike *ruvAB*, however, these intermediates were processed and resolved with kinetics that were similar to the wild-type cultures, and no abnormal intermediates were observed to accumulate in these mutants (Fig. 2, *B* and *C*).

The Intermediates That Accumulate in ruvAB Mutants Are Distinct from Those Associated with Arrested Replication Forks—Our previous work has shown that a portion of the cone region intermediates are generated following the arrest of replication by UV-induced damage (14). Thus, in the absence of the nascent DNA-processing enzymes RecQ or RecJ, mutants unable to restore replication after arrest, such as *recF*, *recO*, or *recR*, accumulate cone region intermediates following UV-induced DNA damage (Fig. 3, *recR recJ*). Comparatively, however, in UV-irradiated *ruvAB* mutants we observed that cone region intermediates accumulated and persisted long after DNA synthesis had fully recovered (Fig. 3 *ruvAB*), suggesting that the

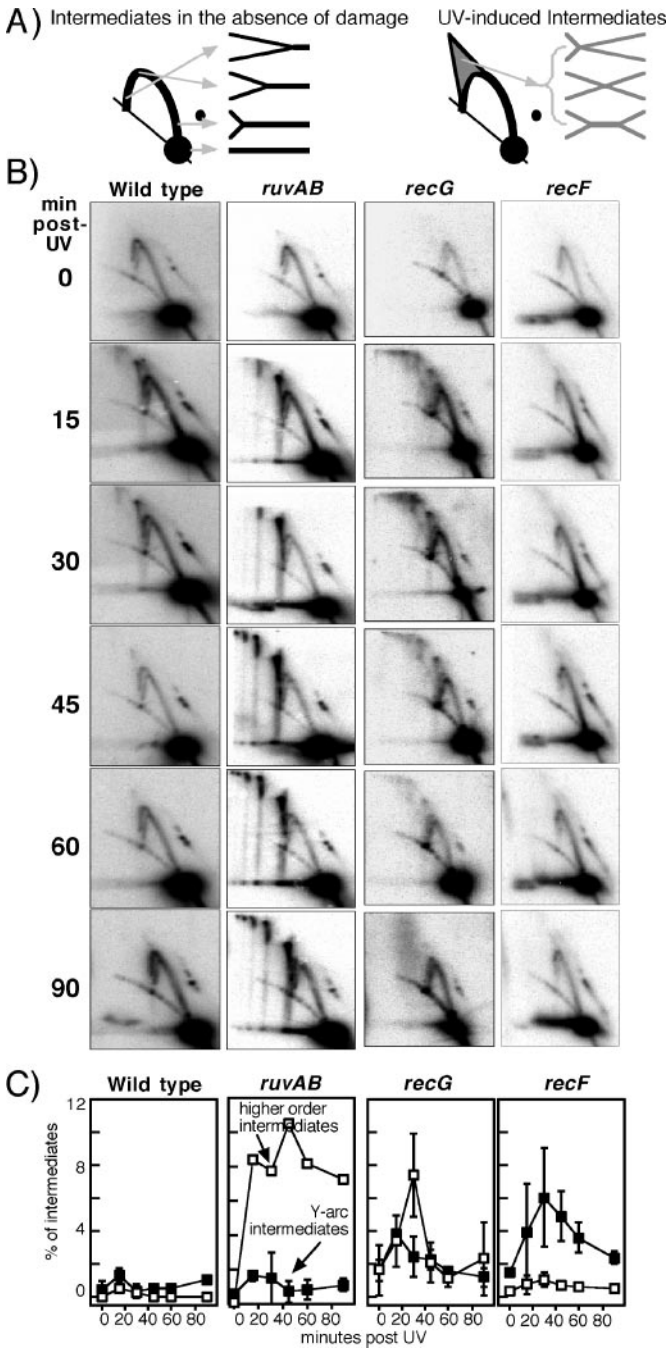
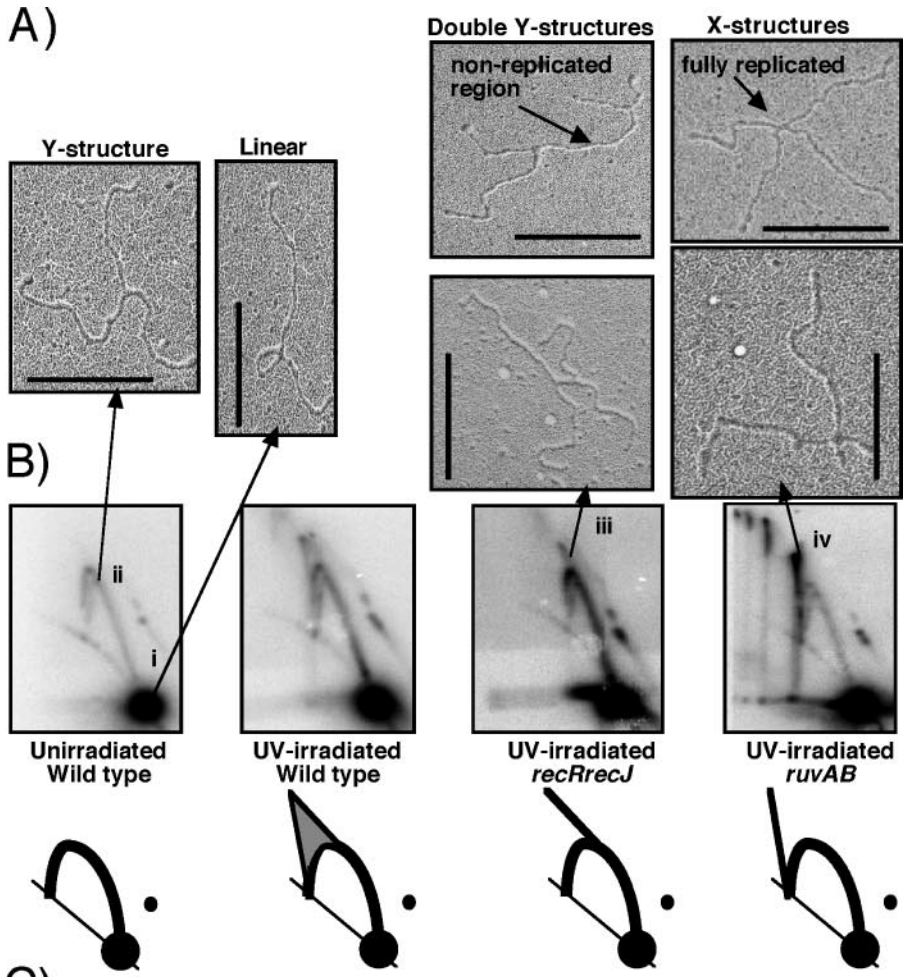
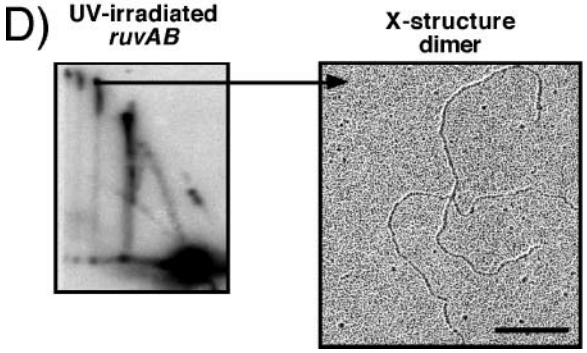


FIGURE 2. ***ruvAB* mutants accumulate branched structures following replication on damaged templates after UV irradiation.** *A*, diagram of the migration pattern of PvuII-digested pBR322 observed by two-dimensional agarose gel analysis in the absence and presence of UV-induced DNA damage. In the absence of UV irradiation, the migration pattern is made up of nonreplicating plasmids that migrate as linear, 4.4-kb fragments and Y-shaped replicating molecules that migrate more slowly due to their larger size and nonlinear shape. These Y-shaped molecules form an arc that extends out from the main, linear fragment. Following UV-induced damage, molecules that contain more than one branch point (double Y- or X-shaped structures) are observed that migrate in the cone region of the gel. *B*, two-dimensional agarose gels of wild type and *ruvAB*, *recG*, and *recF* mutants at the indicated times following UV irradiation. Cells containing the plasmid pBR322 were UV-irradiated with 50 J/m^2, and genomic DNA was purified, digested with PvuII, and analyzed by two-dimensional agarose gels. Gels shown represent one of at least two independent experiments. *C*, the percentage of Y-shaped replicating molecules (□) and the percentage of X-shaped and higher order branched intermediates (▲) relative to the amount of nonreplicating linear molecules is plotted. Plots represent an average of two independent experiments. *Error bars*, one S.D.



C)

Section of Gel	Description	Number of molecules examined	Linears	Y-structures	Double Y-structures	X-structures
i	Unirradiated wild type: linear region	40	40	0	0	0
ii	Unirradiated wild type: Y-arc region	40	6	34	0	0
iii	UV-irradiated *recRrecJ:* cone region	40	0	20	16	4
iv	UV-irradiated *ruvAB:* cone region	40	0	10	6	24



mechanism and defect that leads to the accumulation of cone region intermediates in each case are unique. To further characterize the intermediates that accumulate in each of these mutants, we examined the shape of the cone region intermediates in the two-dimensional agarose gels by transmission electron microscopy (Fig. 3). In order to obtain enough DNA for visualization by electron microscopy, DNA was purified from 60-ml cultures and then concentrated by ethanol precipitation prior to PvuII digestion. Parallel samples were then subjected to two-dimensional agarose gel electrophoresis. One sample was used for Southern analysis and served to identify the locations of the intermediates in the gel, whereas the other half was stained with ethidium bromide, and the DNA was extracted and purified from the gel for electron microscopic analysis.

As mentioned previously, in the absence of DNA damage, intermediates from wild-type, *ruvAB*, and *recR recJ* mutants all form a simple Y arc. When molecules from this region in wild-type were examined by electron microscopy, it was found that the region consisted predominantly of the expected Y-shaped structures (Fig. 3*A*). Molecules examined from this region in *ruvAB* and *recR recJ* mutants also predominantly contained Y-shaped molecules and were indistinguishable from those observed in wild-type cultures (data not shown).

In UV-irradiated wild-type cultures, the damage-induced structures were composed of intermediates that were evenly distributed throughout the cone region (Fig. 3*B*). By comparison, the damage-induced intermediates in UV-irradiated cultures of *ruvAB* and *recR recJ* mutants formed unique structures that migrated in distinct areas of the cone region (Fig. 3*B*). In the *recR recJ* mutant, the cone region intermediates accumulated primarily along the side of the cone farthest from the gel's loading well, corresponding to smaller molecules. Comparatively, the intermediates in the cone region of *ruvAB* mutants accumulated along the side of the cone that is proximal to the gel's loading well, corresponding to larger molecules.

When we examined the cone region intermediates from *recR recJ* mutants by transmission electron microscopy, a large portion of the molecules were found to have a double Y-shape that contained four branches that extended from two distinct junction points on the molecule (Fig. 3, *A* and *C*). The double Y-shaped molecules are consistent with the idea that replication arrested prior to completion in these mutants. One mechanism by which the double Y-structure could be generated in the *recR recJ* mutants is one in which the synthesis of the lagging strand continues back through the origin of replication after the leading strand has arrested. This type of mechanism would also be consistent with the structures observed in *recF*, *recO*, and *recR* mutants, where degradation of the nascent lagging strand by RecJ and RecQ would reduce the double Y-structure back to a Y-structure that is observed in these mutants (14, 57). We cannot rule out the possibility that the regression structure is altered during the concentration/precipitation procedures used for electron microscopy, since we did observe that samples prepared for electron microscopy contained slightly elevated quantities of cone region and higher order species as compared with those prepared by our standard method (data not shown). Nevertheless, it is clear that the intermediates in this case predominantly contain nonreplicated regions, consistent with the inability of these mutants to resume replication following arrest.

In contrast to the partially replicated molecules that were seen in *recR recJ* mutants, cone region intermediates from *ruvAB* mutants were predominantly found to be X-shaped molecules that contained four branches extending from a single junction point (Fig. 3). In general, these molecules were equivalent in size to two linear molecules that contained a single Holliday junction. This observed shape is consistent with what would be predicted for this region of the gel (56, 58, 59). When the larger, higher order intermediates that migrated beyond the cone region were examined in the *ruvAB* mutants, they were found to be predominantly made up of molecules that contained a single Holliday junction made up of linear dimers (Fig. 3*D*). Thus, *ruvAB* mutants recovered replication following UV-induced arrest but failed to maintain genomic integrity at late times when DNA synthesis had been restored. Consistent with these observations, the intermediates that accumulated in the *ruvAB* mutants resembled fully replicated molecules containing unresolved Holliday junctions. This lack of resolution following replication would be predicted to result in a loss of genomic integrity and lethality if similar structures were present and not resolved on the chromosome.

ruvC and ruvAB recG Mutants Exhibit Phenotypes Similar to That of ruvAB Mutants—ruvC encodes an endonuclease that functions with RuvAB and is able to resolve Holliday junction structures. When we examined *ruvC* mutants, we observed that they exhibited a phenotype similar to that of the *ruvAB* mutants (Fig. 4). Following UV irradiation, *ruvC* mutants exhibited a loss of genomic integrity at times after DNA synthesis had fully recovered. In addition, Holliday structures and higher order intermediates persisted on the plasmids following replication, similar to those observed in the *ruvAB* mutants.

ruvAB recG mutants exhibit impaired growth even in the absence of DNA damage and are more sensitive to UV irradiation than either single mutant (49, 60). It is possible that if the cellular function of RecG operates prior to RuvAB, inactivation of RecG would then prevent the Holliday junctions from accumulating. Alternatively, if RecG and RuvAB operate at different sites or unique times in the cell, distinct intermediates may

FIGURE 3. **Holliday junctions accumulate on fully replicated molecules in UV-irradiated *ruvAB* mutants, which are distinct from the arrested fork structures observed in *recF* pathway mutants.** *A*, in the absence of UV irradiation, electron microscopic analysis of PvuII-digested pBR322 prepared from wild type cells reveals predominantly linear, nonreplicating molecules (*i*) and Y-shaped replication intermediates (*ii*). Following UV irradiation, branched DNA intermediates accumulate on fully replicated molecules in *ruvAB* mutants (*iv*), whereas the branched DNA intermediates observed in *recR recJ* mutants contain unreplicated regions (*iii*). Micrographs represent the predominant DNA structures observed by transmission electron microscopy following extraction from the indicated areas of the two-dimensional gels. Scales for all micrographs represent 0.5 μm. Diagrams and two-dimensional agarose gels of PvuII-digested pBR322 in wild type, *ruvAB*, and *recR recJ* mutants are shown in *B*. *C*, the numbers of molecules examined by electron microscopy from the unirradiated wild type linear unreplicated region, unirradiated wild type Y-shaped replication arc, cone region of *recR recJ* mutants, and cone region of *ruvAB* mutants are listed in the *table* and represent the totals from two independent experiments. *D*, a representative electron micrograph of a typical four-arm branched structure isolated from the indicated area of the two-dimensional gel of UV-irradiated *ruvAB* cultures. *Scale bar*, 0.5 μm.

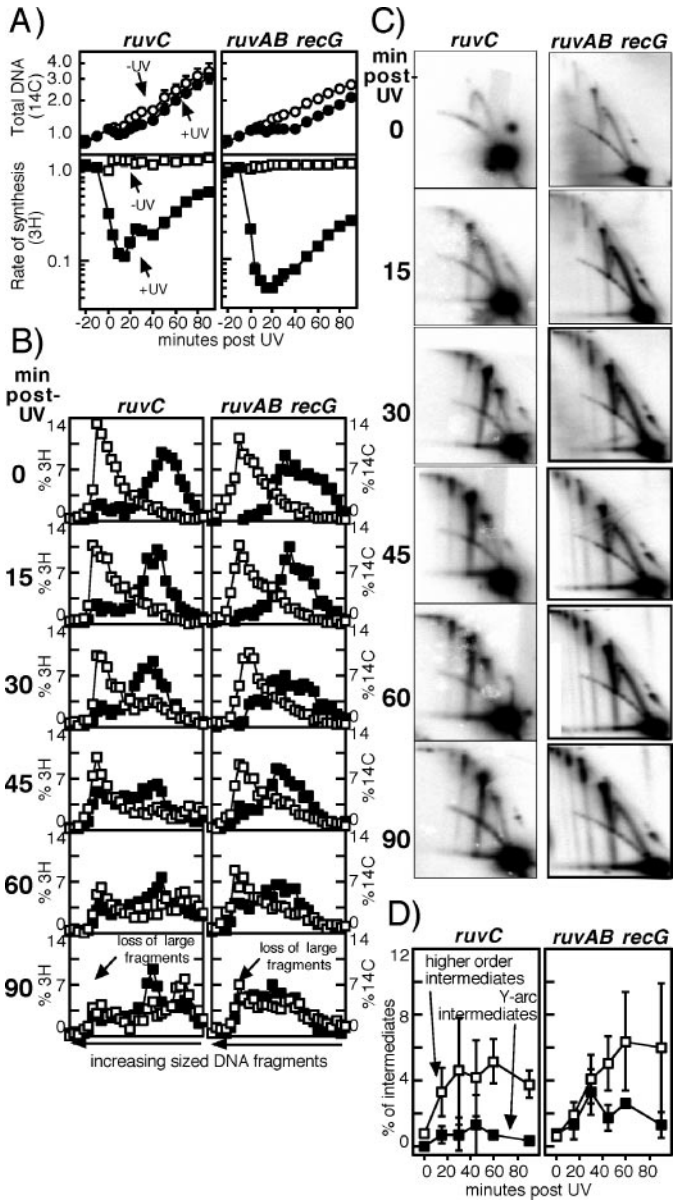


FIGURE 4. ***ruvC* and *ruvAB recG* mutants have similar phenotypes as *ruvAB* mutants following UV-induced DNA damage.** *A*, *ruvC* and *ruvAB recG* mutants resume DNA synthesis with kinetics that are similar to *ruvAB* cultures. The rate of synthesis was the same as indicated in Fig. 1*A*. ○, total [^{14}C]DNA in mock-irradiated cultures; ●, total [^{14}C]DNA in irradiated cultures; □, [^{3}H]DNA synthesis in 2 min in mock-irradiated cultures; ■, [^{3}H]DNA synthesis in 2 min in irradiated cultures. ^{3}H cpm and ^{14}C cpm at −10 min were as follows: *ruvC*, 3700 and 2100; *ruvAB recG*, 14,200 and 2000. *B*, the deterioration of the genomic DNA at late times following UV-induced DNA damage in *ruvC* and *ruvAB recG* mutants is similar to that observed in *ruvAB* mutants. Sucrose gradients were performed the same as in Fig. 1*B*. The percentage of the total ^{14}C genomic DNA (◇) and ^{3}H DNA made during the first 5 min post-UV (■) for each fraction is plotted. Total ^{3}H cpm and ^{14}C cpm in each gradient at time 0 were as follows: *ruvC*, 2100 and 2300; *ruvAB recG*, 1100 and 2900. *C*, *ruvC* and *ruvAB recG* mutants accumulate branched structures similar to those observed in *ruvAB* mutants following UV-induced DNA damage. Two-dimensional gels were performed as in Fig. 2*B*. *D*, the percentage of Y-shaped replicating molecules (□) and the percentage of X-shaped and higher order branched intermediates (▲) relative to the amount of nonreplicating linear molecules is plotted.

accumulate in the double mutant that have not been observed in either single mutant. When we examined the behavior of the *ruvAB recG* double mutant, we observed that it resembled the *ruvAB* single mutant in the assays we utilized (Fig. 4). Although *ruvAB recG* mutants recovered replication after UV irradiation, they accumulated Holliday structures on the replicated DNA, which resulted in a deterioration of genomic integrity at later times in the recovery period.

DISCUSSION

Both *ruvAB* and *recG* mutants are sensitive to UV irradiation, but the absence of these gene products does not impair the ability of the cell to resume replication following arrest at DNA damage. Therefore, in an attempt to determine the cellular role of these proteins that is required for resistance to UV irradiation, we considered the possibility that these proteins may operate at sites other than the arrested replication fork. We found that the integrity of the genomic DNA in *ruv* mutants began to deteriorate at late times after UV irradiation and that this correlated with the accumulation of unresolved Holliday junctions on the replicated DNA. In contrast to *ruvAB* and *ruvC*, no deterioration or unresolved intermediates were observed in the DNA of *recG* mutants. Although the *in vivo* function of RecG remains an interesting question, the inability of RecG to process the Holliday junctions that accumulate in *ruvAB* mutants indicates that although RecG and RuvAB are capable of catalyzing similar biochemical reactions *in vitro*, they may have spatially or temporally nonredundant roles *in vivo*.

The accumulation of unresolved Holliday junctions in *ruv* mutants despite the completion of replication on the chromosome would be expected to lead to lethality and a loss of genomic integrity if the chromosomes fail to partition properly. Previous studies have observed that *ruv* mutants form filaments after UV irradiation and contain a significant number of multinucleated cells with nonpartitioned, centrally aggregated chromosomes (33, 61). These microscopic observations are consistent with our cellular assays demonstrating that replication can continue for several rounds before a crisis, or a physical deterioration of the genomic integrity, occurs. The presence of multiple nonpartitioned chromosomes would also be consistent with our plasmid observations, in which replication could continue on the plasmids in the absence of resolution, resulting in the appearance of multimeric forms of the branched molecules in the two-dimensional gels.

In this report, we show directly that RuvABC is required to process Holliday junctions that accumulate after replication has occurred on damaged templates *in vivo*. Importantly, the phenotypes exhibited by *ruvAB* appear to represent a class of mutants that are distinct from those mutants with an impaired ability to recover replication following arrest. As measured by the ability to replicate DNA, the loss of viability in mutants, such as *recF*, appears to occur after cells initially encounter DNA damage, when they are unable to resume and complete the duplication of the chromosome. In contrast, *ruvAB* mutants retain viability for a significant period of time and continue to replicate until multiple chromosomes have accumulated in the cell. Cell death in these mutants does not appear to occur until much later and is likely to occur when the multiple interlocked chromosomes fail to partition into discreet units.

These two distinct classes of mutants would be consistent with recent studies that suggest two potential substrates can be

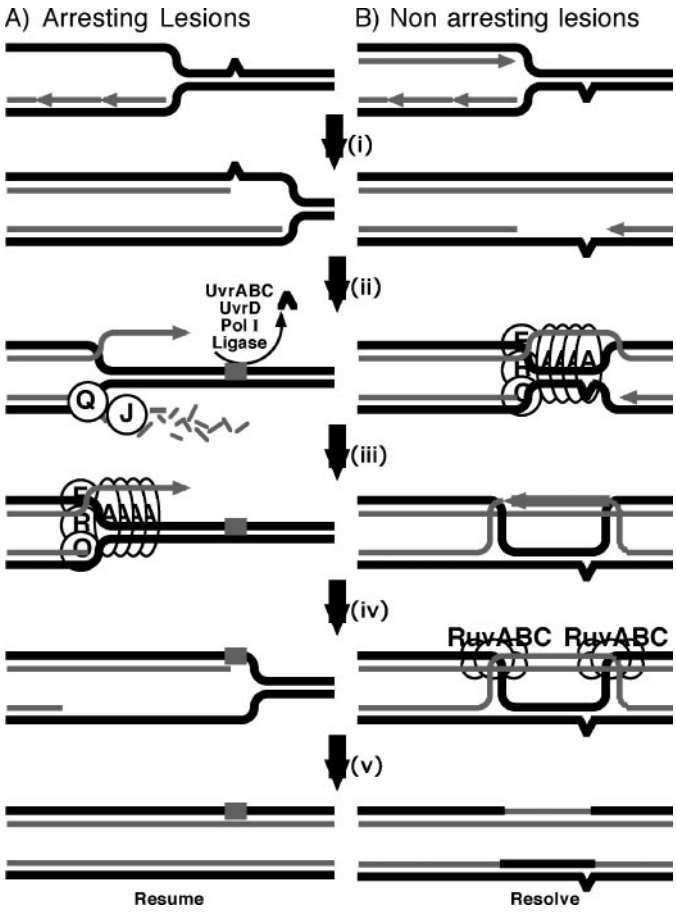


FIGURE 5. **Model for the differential processing of arresting and nonarresting UV-induced lesions encountered during replication.** *A*, lesions that arrest replication. Following the arrest of replication (*i*), partial degradation of the nascent DNA by RecQ and RecJ restores the lesion-containing region to a form that can be repaired by the nucleotide excision repair proteins (*ii*). RecF, RecO, and RecR promote the formation of a RecA filament at the double to single strand DNA junction (*iii*), helping to maintain the integrity of the replication fork until replication can resume (*iv*). *B*, lesions that do not arrest replication may be restored through daughter strand gap repair. Priming events on the lagging strand template allow replication to continue through a subset of lesions, leaving gaps in the nascent DNA (*i*). RecF, RecO, and RecR promote the formation of a RecA filament at the double to single strand DNA junction. *ii*, completion of the unreplicated regions in this case leads to the formation of cross-overs following strand exchange (*iii*). The cross-overs (Holliday junctions) in the restored region are then resolved prior to cell division to maintain the cell viability (*iv*).

generated upon encounters with UV-induced lesions (5–7). Several studies using plasmid substrates have shown that lesions on the leading strand template, but not the lagging strand template, arrest the replication apparatus (Fig. 5, *A versus B*). In the case of a leading strand lesion, the progression of replication is transiently blocked (Fig. 5*A*). *In vivo*, the recovery of DNA synthesis depends on several RecF pathway genes that process and recruit RecA to the blocked replication fork (10–14). The resumption of DNA synthesis also requires either repair or translesion synthesis, consistent with the idea that replication resumes from the site of disruption. Related models based on *in vitro* studies have proposed that DNA synthesis may resume downstream from the site of disruption rather than from the original site of disruption as presented Fig. 5*A* (62). Irrespective of the site of resumption, the role of PriA would be functionally similar in each scenario, and a number of *in vitro* studies are consistent with the idea that the final reestablishment of the replication machinery is likely to involve PriA and several other factors (62–65).

In the case where replication encounters a lesion on the lagging strand template, plasmid studies suggest that the effect on replication is distinct and does not arrest the progression of replication (5–7). If replication on plasmids behaves similarly to replication on the chromosome, then these observations would be consistent with a model in which RuvABC-mediated resolution occurs at a late step in a repair pathway associated with nonarresting lesions during replication (Fig. 5*B*). Based on plasmid studies, this pathway may be primarily associated with lesions in the lagging strand template, as shown in Fig. 5*B*. Such a role would be consistent with the partitioning defects, aggregated chromosomes, and an eventual loss of viability in *ruv* mutants if these chromosomes fail to resolve before cell division occurs (33, 61).

Substrates for RuvABC resolution are also generated in strains that contain thermosensitive alleles of the replicative helicase DnaB (66). Following inactivation of DnaB, elevated levels of RuvAB-dependent double strand breaks accumulate in the genome of *recBC* mutants, suggesting either that DnaB inactivation or RecBC processing after DnaB inactivation leads to the formation of RuvAB substrates (66). It seems reasonable to consider the possibility that both UV-induced DNA damage and DnaB inactivation may generate a similar substrate for RuvABC. With this perspective in mind, it is noteworthy that during replication, the helicase DnaB is believed to unwind the replication fork by moving along the lagging strand template and interacting directly with the primosomal proteins (67, 68). Since inactivation of DnaB leads to RuvAB-mediated double strand breaks, it is tempting to speculate that the processing of nascent lagging strand gaps generates Holliday junctions that require RuvABC for resolution, similar to the proposed model for UV-induced DNA damage. Although speculative, it is of interest to note that a number of replication mutants associated with lagging strand synthesis depend on the activities of the RuvABC resolvasome for viability and replication (69–74).

These observations provide evidence that lesions encountered during replication are processed differentially, depending upon their location and how they affect the progression of the replication fork. This does not suggest that common characteristics may not exist for both arresting and nonarresting UV lesions. However, our studies presented here support the thought that at least one protein complex, RuvABC, may be required for processing nonarresting lesions but not necessarily for processing substrates that arise due to UV lesions that arrest replication. In Fig. 5, we have presented a model to help clarify that differential repair of arresting and nonarresting lesions may occur to hopefully help resolve what appears to be conflicting observations from early studies (1, 2, 20, 21). Although it is well documented that UV-induced lesions can arrest DNA replication, a large number of studies have found that a limited amount of gapped DNA synthesis can still be detected after UV irradiation, suggesting that not all lesions result in the arrest of DNA replication (1, 2, 20, 21). The model we have presented suggests that replication fork reactivation downstream of the damaged DNA results in a gap opposite to the site of damage

that is a substrate for RuvABC processing. Several of these studies proposed related models that suggested that strand exchanges may be involved in the repair or tolerance of lesions that are skipped over by the replication machinery and would require resolution of Holliday junctions at a late step in this repair process (1, 20, 21). Although the early steps in these processing pathways remain to be identified, the observations presented here are consistent with a role for RuvABC in resolving strand exchanges after replication has occurred. More recent studies have centered upon the problem of how replication is restored when it is disrupted or arrested by DNA damage. These results establish that RuvABC is directly required for the repair of a subset of UV-induced lesions encountered during replication but that its function is independent from the process of restoring the replication machinery following disruption. Gaps in the nascent strands of replicated DNA have been observed in many organisms following DNA damage, including yeast and human cells (55, 75). Considering the high evolutionary conservation of the DNA replication process, it seems likely that the differential processing observed in this study may also occur in these organisms.

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