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Situational Repair of Replication Forks

ROLES OF RecG AND RecA PROTEINS*

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Replication forks often stall or collapse when they encounter a DNA lesion. Fork regression is part of several major paths to the repair of stalled forks, allowing nonmutagenic bypass of the lesion. We have shown previously that *Escherichia coli* RecA protein can promote extensive regression of a forked DNA substrate that mimics a possible structure of a replication fork stalled at a leading strand lesion. Using electron microscopy and gel electrophoresis, we demonstrate that another protein, *E. coli* RecG helicase, promotes extensive fork regression in the same system. The RecG-catalyzed fork regression is very efficient and faster than the RecA-promoted reaction (up to 240 bp s^{-1}), despite very limited processivity of the RecG protein. The reaction is dependent upon ATP hydrolysis and is stimulated by single-stranded binding protein. The RecA- and RecG-promoted reactions are not synergistic. In fact, RecG functions poorly under the conditions optimal for the RecA reaction, and vice versa. When both RecA and RecG proteins are incubated with the DNA substrate, high RecG concentrations inhibit the RecA protein-promoted fork regression. The very different reaction profiles may reflect a situational application of these proteins to the rescue of stalled replication forks *in vivo*.

A substantial fraction of replication forks stall or collapse at sites of DNA damage during bacterial DNA replication (1–3). Potential blocks to replication include non-coding lesions in the DNA template, DNA strand breaks, or proteins bound to DNA ahead of the replication fork. There are multiple pathways for repair of stalled replication forks, and these have recently become a major focus of research on bacterial DNA metabolism (3–9). Both *in vitro* and *in vivo* data indicate that some major pathways of fork repair involve the regression of stalled forks (1, 10–16). The process of fork regression could protect single-stranded DNA (ssDNA)[1] at the forks against degradation by nucleases (1). More importantly, fork regression allows repair or bypass of the lesion in a nonmutagenic process that can proceed without cleavage of pathway intermediates (7, 9, 17, 18).

The major stalled fork structure identified in two recent studies is one in which the leading strand has halted DNA synthesis at the site of a lesion, and lagging strand synthesis has continued for some distance (1 kbp or more) beyond that point (19, 20). The resulting fork structure has a long single strand gap on the leading strand (Fig. 1*A*). Fork regression involves the reannealing of parental strands, coupled to the extrusion and annealing of daughter strands to eventually create a daughter strand duplex (Fig. 1*A*). Once regression has proceeded past the lesion and the daughter strand duplex is formed, the regressed structure constitutes a Holliday junction (Fig. 1*A*). The DNA lesion, now present within a duplex region, can be repaired by nucleotide or base excision repair. The ssDNA gap on the extruded daughter DNA could be filled in by a repair DNA polymerase. The reversed fork can then branch migrate in the opposite direction (fork progression) to reconstitute a structure leading to reassembly of a functional replication fork as originally proposed by Higgins (10). An important feature of this model is its avoidance of the DNA exchange events. In this sense, fork regression is not a true recombination event, even if proteins classically designated as recombination proteins are involved. Limiting the amount of DNA exchange in the chromosome may be beneficial to the cells, decreasing the probability of deleterious genomic rearrangements.

DNA strand polarity is important in these reactions. The fork regression shown in Fig. 1*A* begins with a gap on the leading strand. It creates a Holliday junction with a short arm, and a 5′-single strand extension. The 3′ end can thus be used for the DNA polymerization alluded to above. If the gap in the starting molecule was instead on the lagging strand, it could in principle undergo a similar regression reaction. However, the final product would have a short arm with a 3′ single strand extension, and the enzymatic processing of this intermediate would be quite different as detailed by Michel and colleagues (1).

Replication fork regression can in principle be spontaneous or enzyme-driven. Positive torsional stress ahead of replication fork could cause fork regression (14), although it is difficult to evaluate how likely this process is *in vivo* where the chromosomal environment features numerous topoisomerases (21). The alternative is to catalyze the regression reaction. Proteins that might actively promote fork regression *in vivo* include the RecA and RecG proteins.

RecA, the central recombination protein in bacteria, operates as a DNA-bound filament that pairs and exchanges DNA strands. *In vitro*, RecA promotes strand exchange and strand invasion reactions that mimic its presumed roles in conjugational recombination and double strand break repair (18). RecA has also been implicated in the process of fork regression both genetically and biochemically. As demonstrated by Michel and co-workers (22), inactivation of the replicative helicase DnaB leads to the formation of cleavable Holliday junction intermediates (the products of regression) in a process that is RecA-de-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; kbp, kilobase pair(s); dsDNA, double-stranded DNA; nt, nucleotide(s); SSB, single-stranded binding protein; MM, model molecule; ATPγS, adenosine 5′-*O*-(thiotriphosphate); gDNA, gapped DNA; GdnHCl, guanidine hydrochloride.

pendent. Additionally, RecA protein promotes an extensive regression of a DNA structure mimicking a stalled fork, with a gap on the leading strand, in an *in vitro* system (12). The RecA reaction documented *in vivo* (22) is distinct in terms of strand polarity to that studied *in vitro* (12). However, the processes are complementary and both may occur (18). *Escherichia coli* cells lacking RecA function are extremely sensitive to UV and ionizing radiation, reflecting the many roles of this protein in DNA repair and in the regulation of the SOS response (23, 24).

RecG protein is a very different enzymatic activity, an ATP-dependent helicase that binds and unwinds a variety of branched DNA substrates *in vitro*. The protein appears to act as a monomer (25, 26). The minimal DNA structure bound efficiently by RecG is a flayed-end duplex (Fig. 1*B*, structure *a*). However, RecG requires at least two duplex arms in a three-way DNA junction for efficient unwinding (Fig. 1*B*, structures *b* and *c*) (27). RecG binds and unwinds DNA forks with completely double-stranded (ds) DNA arms (Fig. 1*B*, structure *d*) (11, 28) in a reaction that mimics replication fork regression. RecG protein also promotes branch migration in Holliday junctions, the first activity reported for RecG (Fig. 1*B*, structure *e*) (27, 29). In the case of DNA fork models with ssDNA arms, RecG protein binds and unwinds the fork structures with ssDNA leading arms (modeling a leading strand gap, Fig. 1*B*, structure *b*) more efficiently than forks with ssDNA lagging arms (Fig. 1*B*, structure *c*) (13, 30). These results implicate RecG protein in regression of replication forks with leading strand gaps. All *in vitro* experiments demonstrating fork regression-like reactions in model structures with ssDNA arms used small forks assembled from synthetic oligonucleotides, creating arms that are 25–26 nt long and are heterologous to each other (13, 27, 30). In the case of RecG-mediated unwinding/branch migration of forks with no ssDNA gaps, the DNA substrates have been designed to permit a reaction encompassing from a few dozen (13) to a few hundred (11, 28) base pairs.

The crystal structure of *Thermatoga maritima* RecG protein bound to a fork containing a ssDNA leading arm gap (26) has been recently solved. The structure reveals a novel motif in the RecG protein, called a wedge domain, which seems to be involved in stripping the daughter strands from the parental strands, thus enabling regression of a replication fork. This structural information corroborates with biochemical evidence that suggests a novel and very interesting mechanism for RecG-promoted DNA unwinding (13, 31). Most helicases translocate on ssDNA until they find a duplex region to unwind (32). RecG helicase seems to translocate on dsDNA (31) and bind the two arms of the fork in narrow grooves that run along the wedge domain.

The presence in *E. coli* of two very different enzymes, RecA and RecG, that can both promote fork regression, leads directly to questions of whether the function of these enzymes in fork repair is complementary, redundant, or mutually exclusive. The phenotypes of *recG* mutants are complex, and implicate RecG in a range of reactions.

RecG can either facilitate or prevent homologous recombination reactions *in vivo*. During conjugation, RecG promotes the formation of recombinant products in cells that lack the main Holliday junction processing system, RuvABC (33). In contrast, RecG appears to reverse or disrupt recombination events in stationary phase cells (34, 35). *In vitro*, purified RecG protein can catalyze migration of 3- or 4-way junctions like those in Fig. 1*B* toward the ends of the molecules, yielding non-recombinant linear products (36).

In addition to its action on DNA substrates, RecG removes R-loops *in vivo* and *in vitro* (37). RecG-mediated R-loop removal renders the *recG* gene essential for the viability of RNase H mutants (*rnh*), and *recG* inactivation triggers the use of R-loops as alternative replication initiation points, a phenomenon termed constitutive stable DNA replication (38).

RecG protein is thus active on a multitude of branched nucleic acids: D-loops, R-loops, 4- and 3-way DNA junctions, and forked structures. This suggests that RecG plays multiple roles *in vivo*. The many potential roles of RecG are not reflected in the phenotype of *recG* null mutants, which exhibit only a modest sensitivity to DNA damaging agents (17, 39). RecG protein may be active only under certain specific growth conditions, or other enzymes may promote reactions with overlapping functions.

The processing of branched DNA structures is also mediated by the RuvABC system, and many recombination pathways can be delineated along RecG- or RuvAB-dependent lines. In contrast to *recA* mutants, cells lacking *recG* or *ruvABC* function are only mildly affected by UV irradiation (15, 17, 33). The effects of *ruvABC* mutants are greater, suggesting that the *ruv* contribution to normal DNA metabolism is the more important one (15, 17, 33). The *recG* and *ruvABC* mutations act synergistically in double mutants, producing cells with severe defects in repair similar to those seen in *recA* mutants (15, 17, 33). This suggests that the *recG* and *ruv* act in different pathways. The fork repair pathways mediated by RuvABC also require the function of the RecBCD nuclease/helicase. If RecBCD is absent, the forks may be cleaved (1). Repair pathways that include fork regression do not, in principle, require DNA cleavage (1, 9, 17, 22, 40).

The *recG* gene is found at the distal end of an operon composed of four open reading frames, *rpoZ*, *spoT*, *spoU*, and *recG* (41, 42). The *rpoZ* gene encodes the ω subunit of RNA polymerase. The *spoT* gene encodes a bifunctional enzyme catalyzing either (p)ppGpp synthesis or hydrolysis (43). Guanosine tetraphosphate and pentaphosphates (ppGpp and pppGpp) are two unusual nucleotides that accumulate during cellular amino acid starvation, as part of a process called the stringent response. SpoT modulates the steady state levels of (p)ppGpp in response to starvation conditions and modifications of the intracellular energy pool (reviewed in Ref. 44). The *spoU* gene, immediately upstream of *recG*, encodes a protein of unknown function. Two promoters have been identified upstream of the *spo* operon and no obvious promoter sequence can be identified upstream of *recG*, suggesting that all four genes in the operon are co-expressed. Accordingly, an internal *spoT* deletion conferred a mild sensitivity to mitomycin C and UV irradiation, similar to that conferred by *recG* inactivation (41). Increases in the cellular concentration of ppGpp or certain mutations in the β subunit of RNA polymerase greatly improve survival of cells lacking *ruv* function in a RecG-dependent fashion (11). This suggests that RecG has an important role in the repair of forks that encounter stalled RNA polymerase complexes.

When bacterial cells are starved, there is a transient induction of the SOS response that helps bring about the genome-wide hypermutation called adaptive mutation. Adaptive mutation requires both the lesion bypass DNA polymerase IV (the product of the SOS-induced *dinB* gene) and some recombination functions (45–47). During the stationary phase, the accumulation of adaptive mutations is stimulated in *recG* mutants, whereas it is suppressed in *ruvABC* mutants (34, 35). The stimulation seen in *recG* mutants is largely explained by the increase in DNA polymerase IV observed in those strains, because of a partial induction of the SOS response seen when RecG function is not present (47). However, the increase in DNA polymerase IV is also dependent on RpoS (the stationary phase σ factor), and thus associated with the stationary phase (47).

In the present study, we sought to investigate whether RecG

protein can promote fork regression of a larger substrate that mimics a stalled replication fork with a ssDNA gap on the leading strand, and to compare any activity observed with that promoted by the RecA protein. We show here that RecG helicase can promote a very efficient fork regression reaction extending over more than 7 kbp. The reaction is at least 20 times faster than the RecA protein-promoted regression reaction, despite the fact that the enzyme has a very low processivity. When both RecA and RecG proteins are incubated with the substrate DNA, RecG exhibits an inhibitory effect on the RecA-promoted reaction, but only at relatively high RecG concentrations. Importantly, the two proteins promote the regression reaction under quite different sets of conditions, suggesting that these two proteins may play quite different situational roles *in vivo*.

MATERIALS AND METHODS

Enzymes and Reagents—E. coli RecA (48) and SSB (49) proteins were purified as described. The concentration of each protein was determined by absorbance at 280 nm using their extinction coefficients: $\epsilon_{280} = 2.23 \times 10^4$ M^{-1} cm^{-1} for RecA protein (50), and $\epsilon_{280} = 2.83 \times 10^4$ M^{-1} cm^{-1} for SSB (51).

Restriction enzymes and bovine serum albumin were purchased from New England Biolabs. T4 DNA ligase was from Fermentas. ATP, dATP, phosphocreatine, and potassium glutamate were from Sigma. Creatine phosphokinase was purchased from Roche Applied Science. ATPγS and leupeptin were from Roche Molecular Biochemicals. Ammonium sulfate was from Invitrogen. Agarose low EEO was from Fisher Scientific and GenePure LE-agarose was from GeneMate. SP-Sepharose, phenyl-Sepharose 6 Fast Flow (low sub), Sephacryl S-200 HR, and S-300 HR resins and the 5-ml HiTrap heparin column were from Amersham Biosciences. Oligonucleotides were synthesized by Operon Technologies (oligos 1M and 2M) or IDT (oligo 1C).

DNA—Model DNA molecules (MM) were designed, prepared, and purified as previously described (12), with the following modifications in the purification procedures. Supercoiled ds M13mp8.32 DNA (7261 bp) (12) was purified using a QIAFilter Plasmid Mega kit (Qiagen), followed by ethanol precipitation. The supercoiled DNA was then further purified by banding twice in cesium chloride.

Linear dsDNA (5210 bp), gDNA (2051 nt gap, 7261 nt circle), and MM DNA with 6 mismatches at the branch were prepared as described (12) and gel purified on 1% agarose gels. In the case of linear dsDNA (5210 bp) and gDNA, the DNA was visualized on a long wave UV transilluminator after staining with ethidium bromide and the bands corresponding to the DNA of interest were cut out with a scalpel. In the case of MM DNA, the purification gel was cut and only the part with the markers was stained with ethidium bromide and visualized on a long wave UV transilluminator. The band corresponding to the MM DNA marker was cut out, and then the unstained band of MM DNA forming the rest of the gel was also cut. The gel was stained then to ensure accurate and complete removal of MM DNA from the gel. The DNA was extracted from gel slices by electroelution in dialysis bags (SnakeSkin from Pierce, 7,000 molecular weight cutoff), typically for 5 h at 100 V, 4 °C, followed by concentration using Centricon 30 or Microcon 10 (Amicon). In the case of linear dsDNA (5.2 kb) and gDNA, the DNA was also ethanol precipitated to remove ethidium bromide. The concentrations of dsDNA and ssDNA solutions were measured by absorbance at 260 nm, using 50 and 36 μg ml^{-1}, respectively, as conversion factors. The conversion factors used to calculate the concentration of gDNA and MM DNA were based on the fractions of ssDNA and dsDNA in the molecules and were 46 and 48.9 μg ml^{-1}, respectively. DNA concentrations are expressed in terms of total molecules. The MM branched DNA molecules represent over 95% of the total DNA in all but one of the preparations (as noted below).

Small DNA forks used in RecG processivity experiments were designed to have a similar structure and sequence to the branch region in MM DNA molecules. They were assembled from 3 oligonucleotides: oligo 1M, 5′-CACCGAAGAGCGCACGGTGCCGCGCGCGGCGCCTCGACGGATCCCCGGG-3′ (Operon), oligo 2M, 5′-GGCGCCGCGCGCGGCACCGTGCGCTCTTCG-3′ (IDT), and oligo 1C, 5′-CCCGGGGATCCGTCGA(T)$_{34}$-3′ (IDT). Oligos 1M and 2M are the same oligonucleotides used to construct the branch region in the MM DNA molecule (12). The three short DNAs, 200 pmol of oligo 1M, 300 pmol of oligo 2M, and 300 pmol of oligo 1C, were annealed by incubation at 90 °C for 5 min, then allowed to cool slowly to room temperature for more than 150 min. The small forks obtained were used immediately in experiments.

RecG Protein Purification—This purification protocol was adapted from previously published protocols (29, 36). The detection method used to follow RecG protein during purification was SDS-PAGE, using the known molecular weight of RecG, 76,000. RecG protein was purified from *E. coli* strain STL2669 (gift from Susan Lovett, Brandeis University) transformed with the pEAW111 plasmid that has the *recG* gene under control of an isopropyl-1-thio-β-D-galactopyranoside-inducible promoter. 10 liters of cells were grown in LB in five 2-liter shaker flasks at 37 °C up to $A_{600\ nm} = 0.67$. Then cells were induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) and grown for 20 h more at 30 °C. The cells were pelleted by centrifugation and washed with 50 ml of RecG lysis buffer (40 mM Tris-HCl, 80% H$^+$, 2 mM EDTA, 5% (v/v) glycerol). The yield was 31.4 g of cells. These cells were then resuspended in 94 ml of the same lysis buffer, frozen in liquid nitrogen, and stored at −80 °C.

The cell suspension was subsequently thawed overnight at 4 °C. The cells were then lysed by addition of NaCl to 0.1 M, dithiothreitol to 10 mM, and lysozyme to 0.2 mg/ml and incubated 15 min at 37 °C. Triton X-100 (1% final concentration) and leupeptin protease inhibitor (0.5 μg/ml final concentration) were added and the solution was incubated for another 15 min at room temperature, then placed on ice. Cells were sonicated on ice, then ultracentrifuged for 1 h at 40,000 rpm at 4 °C in a Beckman Ti60 rotor. The supernatant was Fraction I (122 ml).

From this point all steps were carried out at 4 °C. RecG was precipitated by addition of ammonium sulfate to 40% saturation. The mixture was stirred overnight and spun 10 min at 20,000 rpm in a Beckman JA20 rotor. Floating lipids were removed with Kim wipes. Protein pellets were resuspended in 5 ml of buffer A each (50 mM Tris-HCl, 80% H$^+$, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol). Leupeptin was then added to 0.5 μg/ml and the solution was dialyzed against buffer A + 0.5 μg/ml leupeptin. After dialysis, the solution was slightly cloudy and was spun 10 min at 15,000 rpm in a Beckman JA20 rotor. The supernatant (Fraction 2, 30 ml) was then divided in two, frozen in liquid nitrogen, and stored at −80 °C.

Half of Fraction 2 (15 ml) was fractionated successively on SP-Sepharose (1.6 × 24.2 cm, 49-ml column volume), a phenyl-Sepharose 6 Fast Flow column, a HiTrap heparin column, and finally a 1.6 × 89-cm Sephacryl S200HR size exclusion column. Peak fractions from this final column containing RecG were pooled (Fraction 6) and dialyzed against buffer A with 50% (v/v) glycerol before flash-freezing in liquid nitrogen and storage at −80 °C. For the extinction coefficient determination, part of Fraction 6 was concentrated in Centricon Plus-20, 8000 Da molecular weight cutoff concentrators (Amicon) before dialysis into storage buffer. The protein preparation was free of any detectable nucleases. The identity of the purified protein was confirmed by N-terminal sequencing, carried out at the Protein and Nucleic Acid Chemistry Laboratories, Washington University, St. Louis.

Determination of the Extinction Coefficient for RecG Protein—The extinction coefficient for RecG protein was determined using a modification of a published procedure (52). UV absorbance spectra were measured by using a Cary 300 dual-beam spectrophotometer (Varian). The temperature was maintained using a circulating water bath. Cell path length and band width were 1 cm and 0.5 nm, respectively. The extinction coefficient for native RecG was determined in buffer E (50 mM Tris-HCl, 80% H$^+$, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol), by comparing the absorbance spectra of the native protein to the absorbance spectra of the protein denatured in 6 M guanidine hydrochloride (GdnHCl) in buffer E. Using the 1280 M^{-1} cm^{-1} and 5690 M^{-1} cm^{-1} extinction coefficients at 280 nm for glycyl-L-tyrosylglycine and *N*-acetyl-L-tryptophanamide in 6 M GdnHCl, respectively (53), the extinction coefficient at 280 nm for denatured RecG in 6 M GdnHCl was calculated as $\epsilon_{denat,\ 280\ nm} = 4.95 \times 10^4$ M^{-1} cm^{-1}. Absorbance spectra of native and denatured (6 M GdnHCl) RecG were scanned at 25 °C, from 320 to 240 nm, for eight different dilutions and with two different protein preparations. RecG was diluted in buffer E or buffer E + 6 M GdnHCl (final concentration) in a total volume of 80 μl and was preincubated at 25 °C for 10 min before scanning. Each dilution was carried out in triplicate and the absorbance values at 280 nm were averaged. The concentrations of native and denatured protein were equal to each other in each scan at each dilution. Using the expression $\epsilon_{nat,\ 280\ nm} = \epsilon_{denat,\ 280\ nm} \times Abs_{nat,\ 280\ nm}/Abs_{denat,\ 280\ nm}$ (54), the extinction coefficient of native RecG protein at 280 nm was determined as being $\epsilon_{nat,280\ nm} = 4.63 \times 10^4 \pm 0.14$ M^{-1} cm^{-1} in buffer E at 25 °C. The A_{280}/A_{260} ratio for the native RecG is 1.84 ± 0.08. The error in both cases is 1 S.D.

RecG Protein Reactions—Reactions containing RecG protein were

carried out in buffer G (50 mM Tris acetate, 60% H^+, pH 8.0, 5 mM $Mg(OAc)_2$, 20 mM KOAc, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin) in a final volume of 10 μl. Some reactions had extra $Mg(OAc)_2$, as indicated. ATP, dATP, or ATPγS were included as indicated. An ATP or dATP regenerating system (10 units/ml creatine phosphokinase and 12 mM phosphocreatine) was also included. Typically, RecG protein was incubated with MM DNA in reaction buffer, nucleotide cofactor, and a regenerating system for the indicated amounts of time. When SSB protein was used, it was added after 5 min of RecG incubation. All incubations were carried out at 37 °C. Reactions were stopped and deproteinized by incubating 30 min at 37 °C with 1% sodium dodecyl sulfate, 12 mM EDTA, and 1 mg/ml proteinase K (all final concentrations), predigested 30 min at 37 °C. Control experiments in the absence of RecG protein contained all the components except RecG protein.

Reaction mixtures were electrophoresed on 1% agarose gels. The gels were run at 25–30 mV, at room temperature, typically for 16 h. DNA was stained with ethidium bromide and visualized on a UV transilluminator. Gel images were captured with a digital CCD camera using GelExpert software (Nucleotech). The intensity of DNA bands was quantified with TotalLab software (Phoretix) and the efficiency of the reaction was expressed by percentage of products relative to the total amount of DNA in the lane.

RecA and RecG Protein Reactions—Reactions with both RecA and RecG proteins (RecA incubated first) were carried out in buffer AG (25 mM Tris acetate, 80% cation, pH 7.5, 10 mM $Mg(OAc)_2$, 20 mM KOAc, 2 mM dithiothreitol, 5% glycerol) with a 5 mM dATP and dATP regeneration system (8 units/ml creatine phosphokinase and 8 mM phosphocreatine) in a final volume of 10 μl. RecA protein was preincubated with MM DNA (0.28 nM) in reaction buffer with the regeneration system for 10 min. Then dATP and SSB protein (228 nM, 4 SSB monomers per 10 ssDNA nucleotides) were added and the reaction mixtures were incubated again for 20 min. At this point the same volume of RecG protein (at the final concentrations indicated, 1× = 0.28 nM) or RecG storage buffer (for the control reaction) was added to the RecA reactions and the mixtures were incubated for 30 more min (50 min total incubation with RecA protein), before deproteinization and analysis as described above.

Reactions with only RecG under RecA optimal conditions were carried out in buffer AG with the 5 mM dATP and dATP regeneration system (8 units/ml creatine phosphokinase and 8 mM phosphocreatine) in a final volume of 10 μl. Reactions with both RecA and RecG that were initiated with RecG were carried out under RecG optimal conditions (buffer G, 5 mM dATP, 8 units/ml creatine phosphokinase, and 8 mM phosphocreatine, 228 nM SSB) in a final volume of 10 μl.

Electron Microscopy—Samples for electron microscopy (EM) were obtained by spreading the reaction mixtures with the cytochrome technique. The reactions were first deproteinized as described above and dialyzed against 20 mM NaCl and 5 mM EDTA for at least 16 h at 25 °C on Millipore type VM (0.05 mm) filters. Then the samples were spread as described previously (55). Photography and measurements of the DNA molecules were carried out as described (56).

RESULTS

Experimental Design—The purpose of this study was to investigate the action of RecG helicase using branched DNA substrates designed to permit extensive regression/branch migration, and to directly compare this activity with that of the RecA protein. For this we designed a DNA molecule to mimic the stalled replication fork structure observed most prominently in the limited studies carried out to date *in vitro* on the structure of forks stalled at leading strand lesions (19, 20). Our construct features a long (2035 nucleotide) ssDNA gap on the leading strand (Fig. 1*C*). The branched model molecule was designated MM. The complete structure consists of a gapped DNA circle with a homologous dsDNA tail attached at the 5′-proximal end of the gap. Based on previous work, RecG protein is expected to bind to the branched region in the MM DNA (27, 57). The molecule was assembled from a gapped DNA circle and a homologous linear dsDNA joined by a synthetic linker as previously described (12). The potential regression reactions and expected products of a RecG-promoted reaction of MM DNA are shown in Fig. 1*C*. A completed regression reaction entails the movement of the DNA branch over a distance of 7255 bp (the size of the duplex branch only, the size of the circle is 7261 bp). A 6-nucleotide heterology (inserted to prevent spontaneous branch migration in the DNA substrate (12)) must be overcome at the outset of the reaction. After 2035 nt of migration through the gapped region, the reaction transitions to the migration of a Holliday structure.

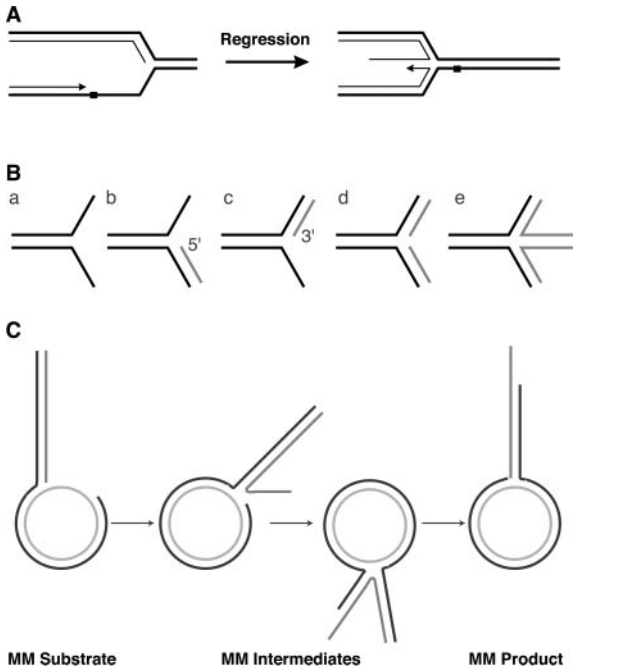

FIG. 1. **Developing a model system to study the mechanics of fork regression.** *A*, schematic of fork regression. Replication forks stalled at lesions (depicted by the *shaded square*) on the DNA can regress by reannealing the parental strands (shown in *bold lines*). The daughter strands (shown in *thin lines*) are also annealed as regression continues, leading to formation of a Holliday junction ("chicken-foot") intermediate. *B*, schematic illustrating the substrate specificity of RecG helicase. The minimal DNA structure required for efficient RecG binding is a flayed-end duplex (*a*). This structure is included in all the other DNA constructs, and is shown with *dark lines* in all cases. For DNA unwinding, RecG requires a least two dsDNA arms in a 3-way junction (*b* and *c*). RecG binds and unwinds better the fork structures with a double stranded lagging strand arm (*b*) (ssDNA gap on leading strand) than the forks with a double stranded leading strand arm (*c*) (ssDNA gap on lagging strand). RecG can also bind and unwind forks with completely dsDNA arms (*d*) or 4-way junctions (Holliday junctions) (*e*). *C*, schematic of the fork regression reaction with the MM DNA substrate. The schematic shows the MM DNA substrate, two intermediates and the final product of the reaction. The substrate has a ssDNA gap in the *circle* that mimics a gap on the leading strand, whereas the final product has the ssDNA region at the end of the dsDNA tail. The substrate, intermediates, and the product can be distinguished by electron microscopy and gel electrophoresis. Construction of this DNA molecule has been described (12). The total lengths of the different DNA segments within the MM substrate are: single strand gap, 2035 nucleotides; DNA circle, 7261 bp total (including gapped region); linear duplex DNA branch, 7255 bp.

RecG Promotes a Rapid Conversion of the MM DNA Substrate to Final Product—RecG protein promotes unwinding of very short DNA molecules (<50 bp) that have the structure of a stalled replication fork with a single-stranded leading arm (13). To determine whether RecG protein can promote much more extensive fork regression, we examined the activity of RecG on the MM DNA molecule. The reaction conditions (buffer G) included 5 mM Mg ion, and 5 mM ATP, in concert with the optimal conditions for RecG activity identified in earlier studies. The MM DNA substrate (0.28 nM in molecules) was incubated with RecG protein (1.4 nM, or a 5-fold excess over the DNA molecules) for 60 min and the reaction was analyzed by electron microscopy. Counts of DNA molecules visualized by EM revealed that 54% of MM DNA substrate molecules (21 of 39 MM molecules examined) were transformed into the expected product under these conditions. Fig. 2 shows the MM

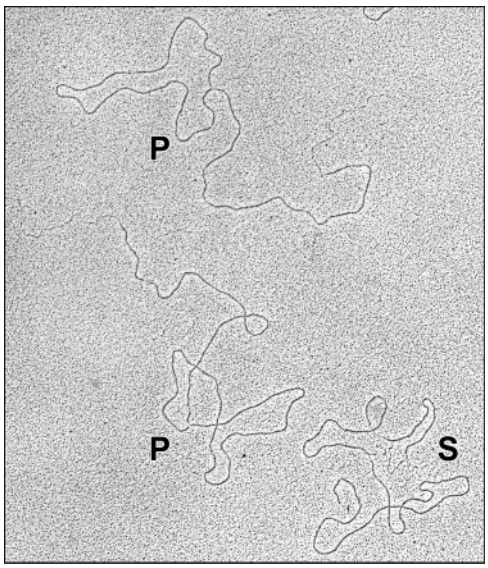

FIG. 2. **Electron micrograph of substrates and products of RecG-promoted MM reaction.** The MM DNA substrate was incubated for 60 min with 28.5 nM RecG (100× excess over the DNA molecules) protein under optimal reaction conditions for RecG (buffer G, 5 mM ATP, 5 mM Mg^{2+}, and ATP regeneration system). The samples were then spread and visualized by electron microscopy. The substrate (*S*) has a ssDNA gap in the circle. The two product molecules shown (*P*) have ssDNA regions at the end of the dsDNA tails.

substrate, an intermediate and two final product molecules in a reaction promoted by RecG protein, demonstrating that RecG protein can readily promote regression of a DNA fork for more than 7 kbp.

We characterized the reaction of RecG with the MM DNA. First, we carried out a RecG protein titration in the MM reaction to find how much RecG protein is required for this reaction. The MM DNA substrate was incubated with increasing concentrations of RecG protein for 60 min and the reactions were analyzed by gel electrophoresis (Fig. 3*A*). A concentration of 1× RecG represents a stoichiometry of 1 RecG molecule per 1 DNA molecule (with both at 0.28 nM). The reaction is completely dependent upon RecG protein, as no reaction occurs in the absence of added protein. Just one monomer of RecG protein per DNA substrate molecule was required for nearly maximal efficiency of the MM DNA fork regression reaction within 60 min. Notably, there was no evidence of a reversal of the regression reaction. The final products accumulated, and the reaction appeared to go nearly to completion. Reversal was not seen at any RecG concentration, even when RecG was in substantial excess relative to the DNA or when a large excess of RecG protein was incubated with purified MM products obtained by either RecG or RecA proteins (data not shown).

To examine the kinetics of the RecG-promoted *in vitro* fork regression, we incubated the MM DNA substrate with RecG protein (5×) for up to 2 h. At different time points, aliquots were removed from the reaction mixture as indicated and were analyzed by gel electrophoresis (Fig. 3*B*). After only 10 min, more than 40% of substrate was converted to a final product and the reaction was essentially complete after 30 min. Some products were consistently detectable after only 1 min (data not shown). In this reaction, regression is not synchronized on all DNA substrates, and the best rate estimate can be obtained by determining when the first products appear (these are the reactions that initiated first and proceeded with the least interruption to completion). The migration of the branch over 7.3 kbp in 1 min requires a rate of migration in excess of 120 bp s^{-1}. As will be seen below, in some cases we saw products forming even after 0.5 min, indicating a maximum rate of

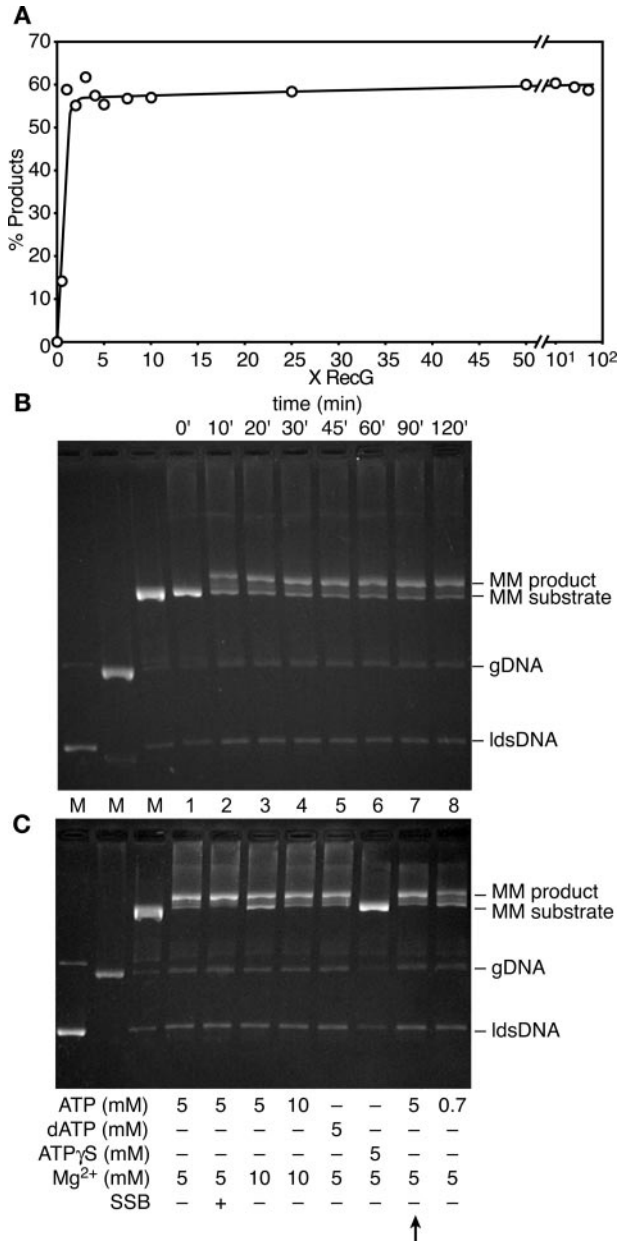

FIG. 3. **Properties of RecG protein-mediated fork regression *in vitro*.** *A*, dependence of the MM reaction on RecG protein concentration. The MM DNA substrate (0.28 nM) was incubated with increasing amounts of RecG protein for 60 min in buffer G with 5 mM ATP, 5 mM Mg^{2+}, and an ATP regeneration system. The reactions were analyzed by gel electrophoresis. 1× RecG represents 1 molecule of RecG per 1 MM DNA molecule (0.28 nM RecG protein). The percentage of reaction products is quantitated and shown as a function of increasing concentrations of RecG. The plot includes data from two experiments, with 8 of the points (spanning the range from 0 to 500 × RecG) being duplicated in both (with the average plotted here). The *right side* of the *x* axis is on a logarithmic scale. *B*, time course of the RecG-promoted regression reaction. The MM DNA substrate (0.28 nM) was incubated with 1.42 nM RecG (5×) for up to 120 min in buffer G with 5 mM ATP, 5 mM Mg^{2+}, and an ATP regeneration system. Aliquots (10 μl) were removed at the indicated time points and were analyzed by gel electrophoresis. *M* represents DNA markers, as indicated. *C*, nucleotide and Mg^{2+} requirements for RecG-promoted MM reaction. The MM DNA substrate (0.28 nM) was incubated with 28 nM RecG (100×) protein for 1 h, in buffer G in the presence of various nucleotide cofactors and with various Mg^{2+} concentrations as indicated. All reactions contained 10 units/ml creatine phosphokinase and 12 mM phosphocreatine, except the reaction indicated with an *arrow*, which contained 8 units/ml creatine phosphokinase and 8 mM phosphocreatine. The second reaction contained 57 nM SSB protein (1 SSB monomer per 10 single-strand nucleotides) and SSB was added after 5 min of RecG incubation with MM DNA. *M* represents DNA markers, as indicated. *ldsDNA*, linear dsDNA.

RecG-promoted fork regression of at least 240 bp s^{-1}. This rate could be even higher, as we did not attempt to investigate time points earlier than 0.5 min. Therefore, RecG protein promotes a very fast fork regression reaction, especially when compared with the reaction promoted by RecA protein (6 bp s^{-1} (12, 58)).

We also investigated whether ATP hydrolysis is required for RecG-promoted regression of the MM model fork, and the nucleotide and Mg^{2+} requirements for the reaction. We incubated the MM DNA substrate with RecG protein (100×) for 60 min in the presence of various nucleotide cofactors and the reactions were analyzed by gel electrophoresis (Fig. 3*C*). We found that ATP and dATP promoted an efficient RecG-catalyzed MM reaction, but ATPγS (a slowly hydrolyzable ATP analog) abolished the reaction completely, even in the presence of this large excess of RecG (Fig. 3*C*). Thus ATP hydrolysis is required for the RecG-promoted reaction, similar to results obtained with various shorter substrates (59).

The fork regression reaction was most efficient when the Mg^{2+} concentration was equal to the concentration of ATP or dATP (5 or 10 mM). When the Mg^{2+} concentration was higher than the ATP concentration (10 mM Mg^{2+} with 5 mM ATP or 5 mM Mg^{2+} with 0.7 mM ATP), the RecG-promoted reaction was inhibited substantially (Fig. 3*C*). Magnesium ions are thought to coordinate (d)ATP in a 1:1 molar ratio (60). We conclude that free Mg^{2+} inhibits the RecG helicase in an extensive fork regression reaction, as previously described with shorter DNA substrates (27).

We investigated the role of SSB protein on the RecG-promoted MM reaction. When SSB (228 nM, 4 SSB monomers per 10 ssDNA nucleotides) was added to an ongoing RecG reaction, the reaction yield was slightly enhanced (Fig. 3*C*). This might occur by virtue of the removal of structural barriers to the RecG reaction, such as secondary structures, that may be present in a subset of the DNA substrates. We also varied the concentration of the components of the regeneration system (12 or 8 mM phosphocreatine, 10 or 8 units/ml creatine phosphokinase, respectively), but this had no effect on the efficiency of the reaction, under our conditions (Fig. 3*C*).

RecG Protein Processivity—As RecG promotes a fast regression of more than 7 kbp DNA, we speculated that perhaps the helicase is very processive on appropriate DNA substrates. RecG helicase has a low processivity when measured with a classical helicase assay in which the protein is incubated with a circular ssDNA substrate with a short oligonucleotide annealed to it. RecG helicase can unwind 26, but not 52 base pairs in this assay (57). However, RecG binds unforked ss or dsDNA with very low affinity (29), and the efficiency of even the shorter unwinding reaction is low (57). RecG processivity was also studied with a Holliday junction substrate that is bound with a higher affinity by RecG protein (27). RecG helicase shows a significant decrease in ability to unwind a Holliday junction with heterologous arms 30 nt long as compared with similarly heterologous junctions with 25-nt long arms (27). The decline occurred even when the RecG protein was present in large excess (up to 1600-fold) relative to the available DNA substrate. However, the decline in unwinding efficiency is much less when the junction arms are homologous (27). These results suggest that RecG can unwind a maximum of 25–26 nt (less than 30 nt) when there are no pairing partners ready to anneal to the unwound DNA strands, but unwinding is improved when a true branch migration can take place. Therefore, we wished to determine whether processivity is improved when RecG is utilizing a substrate shown to be optimal for RecG binding and reaction.

To explore RecG processivity on the MM DNA substrate, we used a DNA challenge protocol featuring a large excess of small

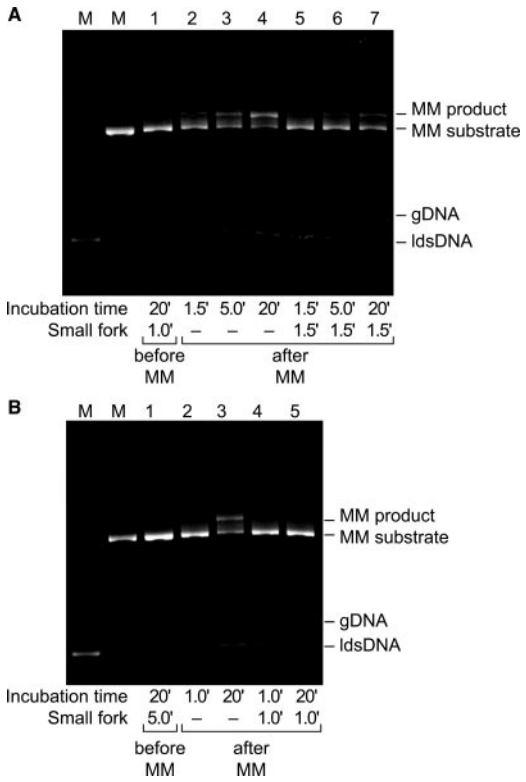

FIG. 4. **RecG helicase processivity.** *A*, RecG protein (1.4 nM, 5×) was incubated with 0.28 nM MM DNA in the presence of 5 mM ATP and 5 mM Mg^{2+} and the reactions were challenged at 1.5 min with a 600-fold excess of small DNA forks or TE. Aliquots were removed at the indicated time points and were analyzed by gel electrophoresis. *B*, RecG protein (1.4 nM, 5×) was preincubated with 0.28 nM MM DNA for 15 min in the absence of ATP and Mg^{2+}, the reactions were then started with 5 mM ATP and 5 mM Mg^{2+} and were incubated for 1 min before the challenge with a 1200-fold excess of small forks or TE. Aliquots were removed at the indicated time points and were analyzed by gel electrophoresis. *M* represents DNA markers, as indicated. *ldsDNA*, linear dsDNA.

DNA forks (constructed with short oligonucleotides) to trap any RecG molecules that dissociate from the MM DNA substrate. The small fork has a very similar sequence to the initial branch junction in the MM DNA molecule, and has a gap on the leading strand. RecG binds this DNA molecule with high affinity, as suggested by the control reactions where RecG was preincubated with a 600-fold excess of the small forks (170 nM) for 1 min, before the addition of MM DNA (Fig. 4*A*). In this case, the MM reaction was almost completely suppressed. We assume this occurred because the RecG protein was effectively sequestered by the small forks. We incubated the MM DNA substrate (0.28 nM) with 1.4 nM (5×) RecG protein for 1.5 min, and then added the small forks in 600-fold excess relative to MM DNA molecules. TE was added in place of the small forks in the control experiment. The reactions were further incubated and aliquots were removed at the indicated time points, deproteinized, and analyzed by gel electrophoresis (Fig. 4*A*). We found that addition of the small fork significantly inhibited the reaction, presumably by trapping RecG molecules that dissociated from the long MM substrate. This result suggests that the RecG protein dissociates frequently from its substrate, thus having a low intrinsic processivity.

However, as the 600× excess of small forks did not completely inhibit the MM reaction when the small forks were preincubated with RecG, we wished to use more stringent challenging conditions. Also, an alternative explanation for the inhibitory effect of the small forks on RecG-promoted MM reaction would be that the binding of RecG to the MM substrate

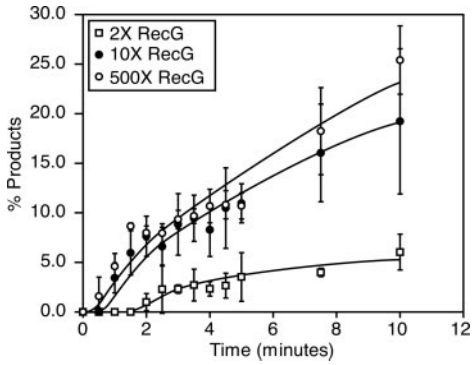

FIG. 5. **Time course experiments of RecG-promoted MM reactions.** The MM DNA substrate (0.28 nM) was incubated with 2×, 10×, and 500× RecG for up to 10 min in buffer G with 5 mM ATP, 5 mM Mg^{2+}, and the ATP regeneration system. To ensure complete binding of RecG to DNA and to synchronize the regression reactions, we preincubated the MM DNA substrate with RecG in the absence of ATP, for 5 min on ice. The reactions were initiated with the addition of ATP. Aliquots (10 μl) were removed at the indicated time points and were analyzed by gel electrophoresis. The DNA bands were quantified and the percentage of reaction products was plotted over time. The plotted values are averages of three independent experiments and the *error bars* indicate the standard deviation.

was slow, and not all MM DNA molecules were bound by RecG during the 1.5-min incubation. Thus, the challenge might be affecting only the RecG not yet bound to MM, and the RecG that was bound might be quite processive. To control for the possibility that RecG binding is rate-limiting, we preincubated RecG protein with MM DNA in the absence of ATP and Mg^{2+} (other conditions as above) for 15 min, to allow full binding of RecG to DNA. RecG protein does not require ATP or Mg^{2+} to bind to branched DNA, but cannot promote unwinding without MgATP (27). The reactions were then initiated with 5 mM ATP and 5 mM Mg^{2+} and challenged after 1 min of incubation with either small DNA fork solution (1200× excess as compared with MM DNA molecules, 336 nM) or TE and further incubated for 20 min. In a control reaction, RecG protein (1.4 nM) was preincubated for 5 min with the small DNA forks (336 nM) in the absence of ATP and Mg^{2+}, then ATP, Mg^{2+}, and MM DNA were added and the mixture was incubated for 20 min before deproteinization. The higher excess of small forks completely inhibited MM reaction when preincubated with RecG in the absence of ATP and Mg^{2+} (Fig. 4*B*). The challenge with small forks also completely inhibited the RecG protein-promoted MM reaction when added to the ongoing reaction. These results strongly suggest that RecG helicase has a very limited processivity even on the long homologous DNA substrate we have used here.

RecG Protein Rebinding to DNA Substrate Is Rate-limiting for Extensive Fork Regression—RecG protein promotes a very efficient and fast reaction, however, it has a low processivity. We investigated whether RecG rebinding after the frequent dissociation events is rate-limiting for fork regression. For this, we carried out time course experiments of MM reactions incubated with 2×, 10×, and 500× RecG. If the amount of time required for detection of the first reaction product was the same, independent of RecG protein concentration, this would suggest that RecG rebinding to the DNA substrate is not rate-limiting. If, however, the first product formed faster when more RecG protein is available, this would suggest that RecG rebinding to DNA after dissociation is rate-limiting. We saw that when MM DNA was incubated with an excess of RecG protein (10 or 500×), detectable reaction products appeared earlier (after 0.5–1 min) than in a reaction with only 2× RecG (1.5–2 min) (Fig. 5). This result suggests that RecG rebinding to DNA is rate-limiting for fork regression. There is not a significant difference between 10× and 500× RecG, probably because the rate of RecG rebinding achieves saturation at 10× RecG.

The time course experiments presented in Fig. 5 were carried out with one MM DNA preparation in which the linear dsDNA[1M–2M] and the gDNA substrates did not complete the ligation step prior to purification. In this case, when the RecG-promoted regression reached the unligated nick in some of the MM DNA substrates, the MM product molecule collapsed into two unique and readily identifiable new products: a nicked circular DNA molecule and a linear gDNA molecule. Because the nick is 16 nt upstream of the starting point of the reaction, appearance of these two products represented an essentially complete reaction of the MM substrate. Therefore, when quantifying the amount of products formed in RecG reactions, the amount of the nicked circular and linear gDNA products was added to the amount of MM product.

RecG Protein Inhibits RecA-promoted MM Reaction Only at High Concentrations—We wished to determine the effects that the RecG and RecA proteins have on each other during the extensive fork regression reaction. RecG helicase has been reported to drive branch migration in a direction opposite to that of RecA protein when proteins were incubated together with the DNA substrates used for RecA protein-promoted DNA strand exchange reaction (61, 62). These experiments contained RecG in a very high concentration as compared with what is usually required to promote an efficient RecG-mediated branch migration reaction (59). We sought to investigate how the two proteins would affect each other when incubated together with the MM DNA molecule.

Any reaction containing both RecA and RecG is complicated by the lack of meaningful overlap in the useful reaction conditions required by the two proteins. The high RecG concentrations used to inhibit RecA in earlier studies represented an attempt to compensate for suboptimal reaction conditions for RecG (62). In the present effort, we focused on conditions that work for RecA-mediated fork regression (buffer AG). Within the parameters of an optimal RecA reaction, some compromises were made in an attempt to provide better conditions for RecG. The free Mg^{2+} level was reduced to 5 mM, and the RecA reaction proficiency was maintained by substituting dATP for ATP. RecG promotes an efficient regression reaction when using a buffer identical to buffer AG, except with no free Mg^{2+} (5 mM Mg^{2+} total) (data not shown). The MM DNA substrate was first incubated with RecA protein for 20 min, a length of time that is insufficient for RecA to generate a detectable level of complete regression products in the fork regression system. Then RecG storage buffer or increasing concentrations of RecG protein were added to the RecA-initiated reactions and incubation was continued for 30 min more, followed by deproteinization and analysis by gel electrophoresis (Fig. 6*A*). Control reactions with only RecG protein incubated for 30 min under these conditions exhibit minimal activity because of the 5 mM free Mg^{2+} (Fig. 6*B*). When RecG is added to an ongoing RecA reaction, a low RecG concentration (5–10×) did not have a significant effect on the reaction. A high RecG concentration (150×, corresponding to 42 nM or 1 RecG for every 52 RecA monomers, or more) significantly inhibits the RecA reaction (Fig. 6*A*). This result is consistent with the previous finding that RecG inhibits RecA protein-promoted DNA strand exchange reactions (61, 62), but only at high RecG concentrations (1 RecG:11.5 RecA in those studies).

When RecA protein was added to a RecG-initiated reaction under conditions optimal for RecG protein, the RecA (at concentrations high enough to bind all of the DNA) stimulated the RecG reaction (Fig. 6*C*), but only slightly. In this case a relatively low concentration of RecG was used (10×), albeit a

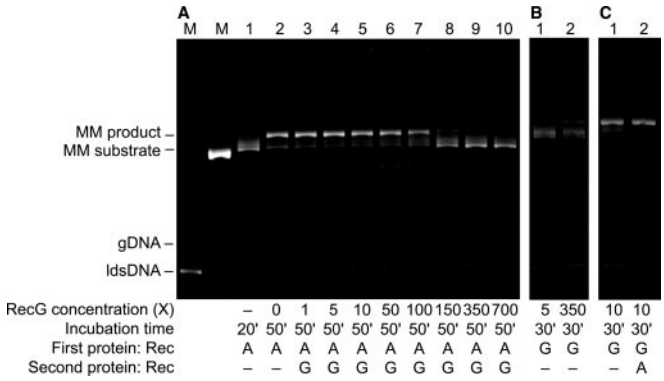

FIG. 6. **Effects of RecG on RecA reactions and of RecA on RecG reactions.** *A*, RecG protein inhibits RecA-promoted MM reaction only at high concentrations. The MM DNA substrate (0.28 nM) was preincubated with 2.2 μM RecA for 10 min in buffer AG with 5 10 mM Mg^{2+}, and a dATP regeneration system, before initiation of the reaction with dATP (5 mM) and SSB protein (0.228 μM). The reaction was allowed to proceed 20 min, when it was challenged with increasing amounts of RecG protein, as indicated. After 30 min of incubation with both RecA and RecG, the reactions were stopped and analyzed by gel electrophoresis. In these reactions, 1× RecG corresponds to 0.28 nM or 1 RecG:7860 RecA, 10× RecG is 1RecG:786RecA, 150× RecG is 1RecG:52.7 RecA, etc. *B*, RecG promotes a poor reaction under conditions optimal for RecA. The MM DNA substrate (0.28 nM) was incubated with 5× or 350× RecG for 30 min under RecA optimal reaction conditions (as in *A*) and reactions were analyzed by gel electrophoresis. *C*, RecA slightly stimulates the RecG-catalyzed MM reaction. The MM DNA substrate (0.28 nM) was incubated with 2.8 nM RecG (10×) for 10 min in buffer G with 5 mM ATP, 5 mM Mg^{2+}, and an ATP regeneration system. At this point the reaction was challenged with 2.2 μM RecA, incubated for 5 min, then SSB protein (0.228 μM) was added and the incubation was continued for 15 more min (30 min total incubation with RecG). In the control reaction the MM DNA substrate was incubated with RecG for 15 min when SSB protein (0.228 μM) was added and the incubation was continued for 15 more min. Both reactions were analyzed by gel electrophoresis. *M* represents DNA markers, as indicated. *ldsDNA*, linear dsDNA.

concentration more than sufficient for a robust reaction under these conditions. RecA alone promotes a very slow fork regression reaction under the RecG conditions (data not shown). After 30 min incubation with RecA under RecG conditions, the reaction exhibited no product generation. After 60 min (total) incubation, there was some product formation, but the yield was less than 20% that observed under RecA optimal conditions.

DISCUSSION

We demonstrate that the RecG protein can promote an extensive fork regression reaction, utilizing a DNA mimic for a stalled replication fork with a long leading strand gap *in vitro*. The RecG-catalyzed fork regression is efficient and fast, proceeding at 240 bp s^{-1} or more. This is ~6× the highest rate reported to date for RecG helicase-promoted branch movement. Even with these optimal DNA substrates, RecG protein exhibits a very limited processivity. Re-binding of dissociated RecG helicase appears to limit the rate of the overall RecG-mediated regression process. The reaction appears to be unidirectional, as the products are not re-converted to substrates even when RecG protein is present in substantial excess or the reaction times are extended. The reaction is dependent upon ATP hydrolysis and is slightly enhanced by SSB protein.

A comparison of the RecA- and RecG-promoted reactions, and an examination of the effects of the proteins on each other, demonstrates that RecA and RecG proteins do not act synergistically to promote the fork regression reaction. RecG can interfere with RecA function if sufficient RecG protein is present. Most importantly, the reaction conditions needed for a robust fork regression reaction are different for the two proteins. RecA requires a significant concentration of free Mg^{2+}, whereas free Mg^{2+} must be minimized in the RecG reaction. The free magnesium requirement in the RecA reaction can be partially supplanted by addition of 4 mM spermidine, but this inhibits the RecG-promoted reaction much as the free Mg^{2+} ion does (data not shown).

We propose that RecA and RecG are not simply redundant activities that represent different paths to the same end. Instead, these proteins appear to promote fork regression most efficiently under different sets of conditions. They are thus likely to function in DNA repair in different structural and metabolic contexts. RecG may play different roles in DNA metabolism under different conditions. These ideas are developed further below.

The RecG Helicase-promoted Fork Regression Reaction—The extensive fork regression reaction promoted by RecG protein is very efficient, with very few RecG monomers per DNA fork substrate needed to generate a high yield of DNA product (Fig. 3*A*). The reaction is very fast, with the products of a 7+ kbp DNA regression reaction detectable in as little as 0.5 min (Fig. 5). The reaction is also non-processive (Fig. 4). Dissociation of RecG that occurs must be compensated for by rebinding to support the rapid regression process (Fig. 5). RecG rebinding to DNA limits the overall reaction rate at low RecG concentrations, but becomes saturated when RecG is present in 10-fold excess to the branched DNA substrates. The 240 bp s^{-1} rate is based on the easily detectable presence of complete regression products after 30 s of reaction. This is merely a lower limit, because times shorter than 30 s were not examined. Even so, the use of longer DNA substrates permits the detection of faster rates and the rate reported here is ~6-fold higher than the highest rate estimated to date for RecG helicase-promoted branch movement (61).

The RecG-promoted fork regression requires ATP hydrolysis (Fig. 3*C*). This result is in concert with expectations for DNA helicases and previous results with RecG (59). The ATP hydrolysis in this case is most likely required for translocating on dsDNA (31). ATP can also be replaced with dATP without a significant change in reaction efficiency (Fig. 3*C*). Interestingly, the RecG helicase activity is maximal when the Mg^{2+} concentration is just enough to coordinate the ATP in the reaction (Fig. 3*C*). Experiments with shorter substrates showed that free Mg^{2+} inhibited RecG binding to 3- and 4-way DNA junctions (27, 36). The authors proposed that Mg^{2+} elicits stacking of the 4-way DNA junctions, thus inhibiting RecG binding (27). It is possible that free Mg^{2+} can also alter the structure of the 3-way DNA junction we are using in a way that prevents RecG from binding. Alternatively, Mg^{2+} could affect the RecG protein directly by inducing an inhibitory conformational change, or indirectly by stabilizing a downstream secondary structure in the DNA substrates.

Comparing RecA- and RecG-mediated Fork Regression Reactions in Vitro—The RecA protein can promote extensive fork regression in MM DNA substrates, yielding the same products as RecG protein (12). SSB protein significantly stimulates the RecA reactions and has a modest positive effect on the RecG reactions (Fig. 6, (12)). The conditions for optimal reactions promoted by the RecA and RecG protein are different, the major difference being in the Mg^{2+} concentration requirement. RecA protein needs 4–6 mM free Mg^{2+} for an efficient reaction (data not shown (63)), whereas the RecG activity is inhibited by free Mg^{2+}. Another notable difference is that RecA protein works as a filament on DNA, therefore the concentration of RecA required for efficient reactions is much higher than the concentration of RecG needed. RecG is thought to act as a monomer (25). The RecG-promoted reaction of MM DNA is up

to 80–100-fold faster than the RecA reaction, when each enzyme is monitored under its optimal conditions.

We sought to investigate the effect that the two proteins have on each other in the MM DNA reaction. When added to an ongoing RecA reaction, low levels of RecG had no effect and high RecG concentrations inhibited the RecA-promoted fork regression (Fig. 6*A*). RecG helicase has been reported to drive branch migration in the opposite direction to RecA protein (61, 62). However, these experiments contained a very high concentration of RecG as compared with what is required to promote an efficient branch migration reaction (59). The proteins did not appear to work against each other in the DNA regression reactions, and certainly did not act cooperatively or synergistically.

Replication Fork Regression in Vivo—RecA does not function under the conditions optimal for RecG helicase-mediated fork regression, and vice versa. Both RecA and RecG proteins can promote an efficient regression of a stalled replication fork with a gap on the leading strand *in vitro*, if the conditions are adjusted appropriately. However, the RecG protein inhibits RecA function when RecG is present at sufficiently high concentrations. How might these proteins cooperate to bring about repair *in vivo*? Based on these and previous results, we propose that RecA and RecG do not offer alternative avenues to repair. Instead they may function most prominently in different metabolic and repair contexts.

Given the limited information available about changes in cellular conditions that may occur in different growth situations, as well as our still incomplete understanding of replication fork repair, it is not possible as yet to define the contexts most appropriate for each protein. However, genetics offers some clues. The genes encoding RecG and RecA are regulated quite differently. RecG appears to be present in low concentrations (42), and is not induced as part of the SOS response. The presence of *recG* within the *spo* operon suggests that RecG may be especially important during starvation conditions, whereas still providing a more general and viable repair alternative in cells deficient in *ruv* function. The *ruvABC* and *recA* genes are part of the SOS system, and may play a more central role in replication fork repair under conditions prevalent during exponential growth. It is possible that RecA might be essential for processing stalled replication forks with a gap on the lagging strand, as suggested by *in vivo* studies with *E. coli* strains containing a mutant DnaB helicase (22). RecG protein binds or unwinds this type of DNA fork much less efficiently than forks with gaps on the leading strand. Both *in vivo* and *in vitro* results (11) suggest that RecG protein might be very important in processing forks stalled where they have collided with other proteins bound to DNA, a situation unlikely to present a ssDNA gap near the stall site. In this situation, RecA protein cannot bind and promote fork regression, as it requires a ssDNA region for nucleation. Also, RecG protein might be essential for inverse branch migration of regressed forks, to promote "progression" of the replication fork and resumption of replication. All of these scenarios are supported by at least some experimental observations, and are not mutually exclusive (3).

Whereas new ways for replication fork repair are being unraveled, it is becoming increasingly clear that cells resort to multiple interrelated pathways to rescue forks stalled at various blocks and complete replication. A full understanding of these pathways will require a better understanding of the condition changes that occur within the cells in response to alteration of growth conditions and challenges with different DNA damaging agents.

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