Replication forks that collapse upon encountering a leading strand lesion are reactivated by a recombinative repair process called replication restart. Using rolling circle DNA substrates to model replication forks, we examine the fate of the helicase and both DNA polymerases when the leading strand polymerase is blocked. We find that the helicase continues over 0.5 kb but less than 3 kb and that the lagging strand DNA polymerase remains active despite its connection to a stalled leading strand enzyme. Furthermore, the blocked leading strand polymerase remains stably bound to the replication fork, implying that it must be dismantled from DNA in order for replication restart to initiate.

Genetic studies have identified at least four gene products required for replication restart, RecF, RecO, RecR, and RecA. We find here that these proteins displace a stalled polymerase at a DNA template lesion. Implications of these results for replication fork collapse and recovery are discussed.

Rapid duplication of genomic DNA is performed by a multiprotein replisome complex (reviewed in Ref. 1). In the bacterium *Escherichia coli*, the replisome is comprised of the DnaB helicase, DnaG primase, and DNA polymerase (Pol) III holoenzyme, which contains two DNA polymerases for simultaneous replication of both the leading and lagging strands of the parental duplex DNA. The holoenzyme component of the replisome is itself a multiprotein machine that is organized into distinct subcomplexes (2). The polymerizing and editing functions reside in the Pol III core, composed of the α, ε, and θ subunits. The β clamp is a ring-shaped dimeric protein that encircles DNA and binds the Pol III core, thereby endowing the polymerase with high processivity via topological linkage to the DNA template. The clamp loader complex uses the energy of ATP binding and hydrolysis to open the β clamp and load it onto a primed site.

The Pol III holoenzyme also contains, as part of the clamp loader complex, at least two copies of the τ subunit. The τ subunit, in addition to its role in the clamp loading reaction, also serves as the “organizing center” of the entire replisome (3). Each τ subunit binds one Pol III core polymerase and also binds to the DnaB helicase, coupling the helicase to the polymerase (4, 5). Thus, when the helicase is coupled to a moving polymerase, the rate of unwinding by helicase alone (~35 bp/s) is markedly increased to ~1000 bp/s, a rate that is consistent with that of normal chromosomal replication (6).

Whereas the replisome machinery appears to be optimized for the task of rapid duplication of the entire chromosome, the life cycle of a cell is filled with events that are potential blocks to the replisome. For example, the chromosome is under a constant barrage of damaging agents that may damage or break the DNA, and if an advancing replisome encounters these damaged sites, it may stall or collapse (reviewed in Refs. 7 and 8). Furthermore, a myriad of proteins are bound to the DNA, including repressors, transcription complexes, and DNA condensing agents, any of which may be expected to interfere with replication fork progression (9). Indeed, Cox *et al.* (7) have estimated that 10–50% of all replication forks may be subject to collapse in one generation of a single cell. Since cell survival requires duplication of the entire genome, a stalled or collapsed replisome must be reactivated. Replication restart reestablishes a replication fork and allows the cell to complete the task of copying the genome (10, 11).

Many studies have addressed the various pathways involved in recombination-mediated repair of blocked replication forks. The absolute requirement for RecA was observed in the earliest studies of cellular responses to DNA damage (12), as well as numerous subsequent investigations (13, 14). Further work showed that proteins of the RecF pathway, including RecF, RecO, RecR (referred to here as RecFOR), RecJ, and RecQ, play roles in reactivation of blocked replication forks (15). Various models have been proposed to explain how these proteins may act, but most mechanistic proposals include strand pairing by RecA, facilitation of RecA binding by RecFOR proteins, and processing of nascent strands by RecQ (reviewed in Ref. 16).

Recent work has demonstrated that specialized systems exist for helicase loading on DNA substrates that mimic stalled replication fork intermediates (11). In *E. coli*, the PriA and PriC pathways, comprising DnaB helicase, DnaG primase, and subsets of primosomal proteins (PriABC), have been reconstituted in vitro and have been demonstrated to assemble a replisome on a blocked replication fork substrate (11, 17–19). The PriA and PriC pathways differ predominantly in their requirements for particular DNA substrates; the PriA system functions with a small size of ssDNA gap on the leading strand template, whereas the PriC system is active in the presence of a large...
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region of ssDNA on the leading strand template. Significantly, these pathways not only load helicase onto a blocked fork substrate but also allow priming on the leading strand (11). This important observation indicates that recombination events need not occur for recovery of an active replication fork; simple downstream priming on the leading strand template and helicase reloading can effectively bypass a leading strand lesion, leaving an ssDNA gap behind that can be repaired later.

Leading strand repriming appears to be the most economical pathway for replication fork reactivation, and the PriAC system appears capable of supporting this pathway in the absence of any recombination proteins. Nonetheless the in vivo requirement for RecA in recovery of replication is absolute, and other recombination proteins play significant roles as well. To further elucidate the actions of recombination proteins at blocked replication intermediates, we use a biochemical approach to further investigate the fates of replisome components when the leading strand polymerase is blocked. In addition, we propose a new function for RecA and RecFOR proteins based on their ability to clear away protein components at a blocked replication fork.

EXPERIMENTAL PROCEDURES

Materials—Labeled nucleotides were from PerkinElmer Life Sciences; unlabeled nucleotides were from Amersham Biosciences. T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were from New England Biolabs. Gel-purified DNA oligonucleotides were from Invitrogen or Integrated DNA Technologies, Inc. The abasic oligonucleotide was synthesized and purified by Integrated DNA Technologies, Inc. Plasmid pUC19 DNA was purchased from New England Biolabs or purified from cells using QiaGen columns according to the manufacturer's instructions. For reactions using primed M13mp18 ssDNA, the M13mp18 ssDNA was purified (20) and primed with a 30-mer DNA oligonucleotide as described (21).

Bio-Gel A-15m resin was purchased from Bio-Rad. Nitrocellulose membrane circles were purchased from Schleicher and Schuell. Buffer A is 20 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 5 mM DTT, 10% glycerol. Replication buffer is 20 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 5% glycerol, 40 μg/ml bovine serum albumin, 0.5 mM ATP, and 8 mM MgCl₂. LK buffer is 50 mM Tris-Cl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP, and 25 μg/ml bovine serum albumin. Gel filtration buffer is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10 mM MgCl₂, 100 μg/ml bovine serum albumin, and 4% glycerol (v/v).

Minicircle DNA Preparation—The oligonucleotide substrates and method to construct the tagged form II duplex minicircle DNA (TFII DNA) were described previously (22). Briefly, the 100-mer ssDNA circle was prepared by annealing 5'-phosphorylated linear 100-mer (70 nm) with a 20-mer (140 nm) scaffolding oligonucleotide complementary to 10 bases at both the 5' and 3' termini of the 100-mer. Annealing was performed (22) by bringing the reaction to 37°C in 50 ml of LK buffer, followed by slow cooling to room temperature. 6000 units of T4 ligase were added, the reaction was incubated overnight at 16°C, and then DNA was precipitated using ethanol. The circular product was gel-purified from a 10% denaturing polyacrylamide gel. The 140-mer complementary strand contains a 40-nucleotide noncomplementary 5' terminus. The 140-mer was formed by joining two 5'-phosphorylated 70-mers (15 μM each) that were brought together using a 20-mer scaffold (37.5 μM) that bridged the 5' and 3' 10 bases of the two oligonucleotides in 450 μl of LK buffer. Ligation was performed overnight at 16°C using 4000 units of T4 DNA ligase, ethanol-precipitated, and the 140-mer product was purified from a denaturing 10% polyacrylamide gel. TFII substrate was formed upon mixing 140-mer/100-mer in a 2:1 molar ratio in a solution of 10 mM Tris-Cl (pH 7.5), 0.3 mM NaCl, 30 mM sodium citrate followed by heating to 95°C and slow cooling to 23°C. Duplex TFII DNA was purified from a 10% native polyacrylamide gel. Abasic versions of the TFII were formed similarly. The abasic site on the leading strand is positioned such that the polymerase encounters the abasic site after extension of the 3' terminus by 90 nucleotides. The position of the abasic site on the lagging strand is directly opposite the position of the abasic site on the leading strand.

Abasic TFII Plasmid DNA Template—The abasic pUC19-based rolling circle template was prepared by digesting duplex DNA with Xbal and HindIII, then inserting a synthetic gapped oligonucleotide duplex. The synthetic gapped duplex is formed from three oligonucleotides: oligonucleotide 1 is a 40-mer containing a single abasic site (5'-AGC TTG CAT GCC TGC AGG TCG XCG CTC TTT TAA AAT GTC TCT-3'), where X designates a dSpacer abasic site, a tetrahydrofuran derivative with a methylene group modifying the 1-position of the 2'-deoxyribose); oligonucleotide 2 is a 60-mer (5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3'), which is complementary to oligonucleotide 1 (and the Xbal site of the plasmid). The three oligonucleotides were annealed, and the resulting hybrid was purified from a 10% native polyacrylamide gel. The purified synthetic gapped duplex was mixed with digested pUC19 DNA. Typical ligation reaction conditions were 125 pmol of pUC19 DNA and 375 pmol of purified synthetic gapped duplex in 100 ml of LK buffer, incubated with 6000 units T4 DNA ligase overnight at 16°C. DNA was concentrated with a Centriprep 50 concentrator, and TFII product was resolved from other ligation products on a 1% agarose gel developed in 1× TBE buffer. The band containing TFII was excised, and DNA was eluted and purified by extraction with phenol, followed by ethanol precipitation. Final yields were typically ~10% with respect to input pUC19 DNA.

Proteins—Subunits of Pol III holoenzyme (α, ϵ, θ, γ, δ, δ', χ, ψ, and β) were purified as described (23, 24). DnaB, DnaG, and SSB were purified as described (5). Pol III core was reconstituted by mixing α, ϵ, and θ subunits; Pol III core was resolved from unbound proteins by chromatography on MonoQ as described (25). Pol III' (all holozyme subunits except β) was reconstituted as follows. A mixture of γ and τ (2.5 γ:1 τ molar ratio) was incubated for 90 min at 15°C followed by the addition of δ, δ’, χ, and ψ (3-fold molar excess of each subunit over τ) and a further 60-min incubation. The τ₂γδδ’χψ complex was resolved from other species as described (26), using a 1-ml MonoS column developed with a 0–0.5 m NaCl gradient. To
form Pol III*, the \( \tau_{\gamma} \delta \delta' \chi' \psi \) clamp loader was incubated with an excess of core (2:1, core/\( \tau \) ratio), and Pol III* was purified from excess Pol III core by MonoQ anion exchange chromatography as described (23). \(^{3} \)-Labeled Pol III core and Pol III* were prepared as above using \(^{3} \)-labeled \( \theta \) subunit labeled by reductive methylation (27).

RecF, -O, and -R Proteins—The gene encoding RecR was cloned into the NdeI/BamHI sites of PET11a. The PET11RecR expression plasmid was transformed into BLR(DE3) cells and grown at 37 °C in LB containing 100 \( \mu \)g/mL ampicillin to an OD of 0.6, whereupon isopropyl \( \beta \)-d-galactopyranoside was added to 0.8 mM followed by incubation at 37 °C for 2 h. Cells were collected by centrifugation and resuspended in an equal volume of 50 mM Tris (pH 7.5), 10% sucrose, 2 mM DTT, 1 mM EDTA, 1 mM NaCl, and 30 mM spermidine. Cells were lysed using a French press, and cell debris was removed by centrifugation. The cell lysate was diluted to a conductivity equal to 230 mM NaCl and loaded onto a 200-mL heparin-agarose column. The column was eluted with a 2-liter linear gradient from 0.15 to 1 mM NaCl in buffer A. Fractions were analyzed for RecR by SDS-PAGE, pooled, and loaded onto an 8-mL MonoQ column. The column was eluted using a 200-mL linear gradient from 0.15 to 1 mM NaCl in buffer A. Fractions containing RecR were diluted to 100 mM NaCl and then loaded onto a 1-mL MonoQ column, followed by elution using a linear 20-mL gradient from 0.15 to 1 mM NaCl in buffer A. Fractions containing RecR were aliquoted and stored frozen at −70 °C.

The gene encoding RecO was cloned into the NdeI/BamHI sites of PET11A, transformed into BL21(DE3) cells, and grown, induced, collected, and lysed as described above for RecR, except that incubation was at 15 °C for 8 h. The cell lysate was diluted to a conductivity equal to 190 mM NaCl and then loaded onto a 100-mL heparin-agarose column. The column was eluted with a 1-liter linear gradient from 0.15 to 1 mM NaCl in buffer A. Fractions containing RecO were diluted to 300 mM NaCl, and then loaded onto a 10-mL Fast Flow Q column. The column was eluted using a 100-mL linear gradient from 50 mM to 1 mM NaCl. Fractions containing RecO were pooled and dialyzed to 300 mM NaCl in buffer A and then diluted to 220 mM NaCl, followed by loading onto a 10-mL Fast Flow Q column. The column was eluted using a 100-mL linear gradient from 50 mM to 1 mM NaCl. Fractions containing RecO were pooled and dialyzed to 300 mM NaCl in buffer A and then diluted to 220 mM NaCl, followed by loading onto a 1-mL MonoQ column, followed by elution using a linear 20-mL gradient in buffer A from 0.15 to 1 mM NaCl. Fractions containing RecO were aliquoted and stored frozen at −70 °C.

The gene encoding RecF was cloned into the NdeI/BamHI sites of PET11A, transformed into BL21(DE3) cells, and grown, induced, collected, and lysed as described above for RecR. The cell lysate was treated with 70% ammonium sulfate, and the pellet was collected by centrifugation and dialyzed against cell lysate was treated with 70% ammonium sulfate, and the induced, collected, and lysed as described above for RecR. The recombinant RecF, -O, and -R proteins were prepared as above using \(^{3} \)-labeled \( \theta \) subunit labeled by reductive methylation (27).

Replisome Stability Experiments—Replisome stability on DNA was examined by blocking either the leading or lagging strand polymerase and then diluting the reaction. In experiments in which the lagging strand is blocked, ddATP was added to a final concentration of 4 \( \mu \)M 30 s after initiation. After further 1 min of incubation, reactions were diluted 1:50 as above and incubated for 15 min. Quenched reactions were analyzed on a 0.6% alkaline agarose gel. In reactions where the leading strand polymerase is blocked, reactions were initiated and allowed to proceed for 2 min. Leading strand polymerase was blocked by the addition of ddTTP to a final concentration of 1 \( \mu \)M, and where indicated, DnaB helicase was blocked with a mixture of 0.75 units of hexokinase and glucose (final concent-

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DnaB helicase was assembled on the minicircle TFII DNA template by incubating 100 fmol of DNA with 4 pmol of DnaB (as hexamer) in 15 \( \mu \)L of buffer A with a 60 \( \mu \)M concentration each of dGTP and dATP for 30 s at 37 °C, at which time Pol III* (100 fmol) and \( \beta \) (350 fmol) were added and incubated for a further 6.5 min. Reaction was initiated upon adding 1 \( \mu \)g of SSB, 60 pmol of DnaG, 50 \( \mu \)M each rNTP, and a 60 \( \mu \)M concentration each of dTTP and dCTP to a final volume of 25 \( \mu \)L. Leading and lagging strand synthesis were monitored in separate reactions containing either [\( \alpha^{32} \)P]dTTP (leading) or [\( \alpha^{32} \)P]dATP (lagging) (specific activity, 3,000–5,000 cpm/pmol). All reactions were quenched upon the addition of an equal volume of 1% SDS, 40 mM EDTA. DNA synthesis was quantitated as described (25) by spotting reactions onto DE81 filters and washing with ammonium formate/sodium pyrophosphate solution. Products were analyzed in 0.6% agarose gels. In time course reactions using a strand-specific polymerization block, ddTTP (leading strand block) or ddATP (lagging strand block) was added to a final concentration of 24 \( \mu \)M.
tration of 10 mM) as previously described (28, 29). At the indicated times after the addition of the block, 2 µl of reaction was removed and added to 98 µl of mixture as above, except lacking ddTTP, and incubated for a further 15 min. To determine the amount of product for the t = 0 point, 2 µl of reaction was removed just before the addition of blocking agent into 98 µl of mixture as above, except containing 20 nM ddTTP to control for the ddTTP brought into the reaction. Quenched reactions were analyzed on a 0.6% alkaline agarose gel, and leading strand product, which migrates at the position of the gel wells, was quantitated using a GE Healthcare laser densitometer.

Abasic pUC19 TFII Rolling Circle Replication—Reactions were performed as described above for the minicircle TFII assays with the following modifications. 60 fmol of abasic template DNA (or 5–10 fmol of control nonabasic template) was incubated with 750 fmol of PolIII*, 700 fmol of β, and 5 µg of SSB for 5 min at 37 °C in buffer A containing a 60 µM concentration of each of the four dNTPs. Reactions were initiated upon the addition of 10 pmol of DnaB (as hexamer), a 50 µM concentration of each of the four rNTPs, 58 pmol of DnaG, and [α-32P]dTTP (5,000–10,000 cpm/pmol specific activity) and then incubated for 15 min at 37 °C. Quenched reactions were analyzed on a 1% alkaline-agarose gel or a 0.8% neutral gel, dried, and exposed to film. For diluted reactions, rolling circle replication was initiated as above but in the absence of radiolabel. After 30 s, 2 µl of the reaction was removed and added to 98 µl of mixture containing [α-32P]dTTP, and all reaction components except DNA, Pol III*, and DnaB. Reactions were allowed to proceed for a further 15 min and then analyzed as above. Control reactions to assess the efficacy of dilution contained 1.2 fmol of [3H]Pol III core in 180 µl of all other components in a 25-µl reaction volume. Quenched reactions were analyzed on a 0.6% alkaline agarose gel, and leading strand product, which migrates at the position of the gel wells, was quantitated using a GE Healthcare laser densitometer.

Analysis of Pol III Stability on M13mp18 ssDNA Containing an Abasic Site—The MutY protein removes the adenine base at an A-G mispair. MutY was used to introduce an abasic site into M13mp18 circular ssDNA by annealing a 30-mer oligonucleotide that produces an A-G mismatch. Wild type MutY contains both glycosylase and AP lyase activities; therefore, we used MutY–K142A (a kind gift from A-Lien Lu, University of Maryland, Baltimore, MD) which retains the glycosylase activity but lacks AP lyase activity (30). This is important, since we did not want to nick the template strand. Reactions contained 9 µg of A-G mismatch primed M13mp18 ssDNA, 90 µg of SSB, 6.7 µg of MutY in 450 µl of buffer A. After 10 min at 37 °C, the reaction was extracted using phenol, precipitated with ethanol, and dissolved in 50 µl TE buffer. Reactions contained 1.8 µg (0.76 pmol) of M13mp18 ssDNA (either undamaged or abasic site), 18 µg of SSB, 1 pmol of γ complex, 1.5 pmol of [32P]β, 0.7 pmol of [3H]Pol III core in 180 µl of dilution buffer. Incubation for 15 min at 37 °C resulted in removal of the primer due to the 3’–5’ exonuclease activity inherent in the Pol III core. Then a 60 µM concentration each of dCTP and dGTP was added, along with a 150 µM concentration of the 30-mer primer. Primer annealing and Pol III holoenzyme assembly were complete after 12 min at 37 °C. Reactions that monitor the stability of [3H]Pol III holoenzyme on DNA under idling conditions were filtered over a 5-ml BioGel A15M column using gel filtration buffer containing 40 mM NaCl and 60 µM each dCTP and dGTP. Reactions that monitor stability of [3H]Pol III holoenzyme on DNA under replication conditions were treated further with 60 µM each dATP and dTTP, incubated for 1 min at 37 °C, and then filtered as above, except the column buffer included a 60 µM concentration of each of the four dNTPs. Fractions of 180 µl were collected and quantitated by liquid scintillation, and the fmol of [3H]Pol III (as core) were calculated from the known specific activity of [3H]θ (21 cpm/fmol) and known stoichiometry of one θ per molecule of Pol III core (ωθ) complex. Replication extension products of reactions containing either undamaged DNA or the abasic site DNA were analyzed side-by-side in a 0.8% native agarose gel developed in TBE buffer.

Effect of RecA and RecFOR Proteins on Stability of Pol III on DNA—Reactions that analyze the effect of RecA, RecF, RecO, and RecR on the stability of [3H]Pol III holoenzyme on DNA were performed using recombinant RecA, -F, -O, and -R proteins that were expressed and purified without tags. We have also examined RecF with a C-terminal hexahistidine tag and RecO containing an N-terminal hexahistidine tag, which after cleavage leaves only a +1 glycine; both proteins were active (tagged proteins prepared by Dr. Dan Kaplan). Reactions in the absence of SSB were assembled in 100 µl of replication buffer containing 1 mM ATP, 2.5 mM phosphocreatine, 3 units of creatine kinase, 100 mM NaCl, 0.5 pmol of [3H]Pol III* (exo mutant), 20 nM β, and 0.94 pmol of primed M13mp18 ssDNA. Reactions were incubated for 9 min at 37 °C to allow [3H]Pol III holoenzyme assembly, and then either protein dilution buffer or 2.4 nmol of RecA was added. After 5 min at 37 °C, reactions were loaded onto 5-ml columns of BioGel A15M equilibrated in buffer A plus 150 mM NaCl. Fractions of 180 µl were collected and quantitated by liquid scintillation. To analyze the effect of RecA and RecFOR proteins in the presence of SSB, reactions were assembled in 100 µl of replication buffer containing 1 mM ATP, 2.5 mM phosphocreatine, 3 units of creatine kinase, 100 mM NaCl, 0.5 pmol of [3H]Pol III* (exo mutant), 20 nM β, 0.94 pmol of primed M13mp18 ssDNA, and 21.6 µg of SSB. Reactions were incubated for 9 min at 37 °C to allow [3H]Pol III holoenzyme assembly and then either protein dilution buffer or 2.4 nmol of RecA was added. After 5 min at 37 °C, reactions were loaded onto 5-ml columns of BioGel A15M equilibrated in buffer A plus 150 mM NaCl. Fractions of 180 µl were collected and quantitated by liquid scintillation. Although protein preparations were routinely checked for nuclease contamination, additional control reactions were performed using the possible combinations of RecA, RecF, RecO, and RecR, followed by analysis in a neutral agarose gel, to ensure that the DNA substrate remained intact under the conditions used in the polymerase stability assays.

RESULTS

Rolling circle DNA substrates are useful tools for mechanistic studies of replication fork action in vitro (31). The initial experiments in this report utilized the minicircle DNA template described in our earlier studies (22). The minicircle tail form II (TF II) substrate (Fig. 1A) consists of a 100-mer circular
ing strand polymerase within the Pol III holoenzyme was blocked. Fig. 1A is a control experiment to show that the E. coli replisome functions on the minicircle substrate to produce concurrent leading and lagging strand synthesis, as demonstrated in our earlier study (22). In Fig. 1, B and C, we examined the fate of the lagging strand upon blocking the leading strand using either a dideoxynucleoside triphosphate (ddNTP) or an abasic site lesion. The result using an abasic site is shown in Fig. 1B. No DNA synthesis was detected on either strand using the minicircle template that contains an abasic site on the leading strand. In contrast, when the abasic site was located on the lagging strand, replication of both leading and lagging strands was essentially unaffected, consistent with earlier studies of replisome advance in the presence of lagging strand blocks (22, 32, 33).

The experiment in Fig. 1C examines the result of adding a leading strand-specific dideoxynucleoside triphosphate (ddTTP) to block the leading strand polymerase. In this experiment, rolling circle reactions were first initiated with all four dNTPs, and then after 2.5 min the leading strand polymerase was blocked by the addition of ddTTP, which is only incorporated on the leading strand. The result shows an immediate cessation of synthesis of both leading and lagging strand synthesis (Fig. 1C, left). As a control, ddATP was used in a similar experiment to block the lagging strand (Fig. 1C, right). The result demonstrates that lagging strand synthesis ceased, but the leading strand continued unabated, consistent with our earlier study (22).

At first glance, the results of Fig. 1 suggest that the activity of the leading and lagging strand polymerases may be functionally coupled. In other words, the lagging strand polymerase stalls because the blocked leading strand enzyme “communicates” its arrested status to the lagging strand polymerase. However, there exist other possible mechanisms that may explain why both DNA polymerases appear to be inactive. For example, the stalled leading strand polymerase may freeze the helicase, thereby stopping DNA synthesis on the lagging strand for lack of ssDNA template. It is also possible that the helicase contin-
ues unwinding ahead of the stalled leading strand polymerase. Since the minicircle is small, it would be completely unwound by a helicase that advances only 100 nucleotides, and this would destroy the TFII DNA substrate and produce only a insignificant amount of lagging strand ssDNA in the process (see diagrams in Fig. 2). The next series of experiments was designed to distinguish among these scenarios.

In the experiments of Fig. 2, the minicircle substrate was used to determine whether the helicase continued unwinding DNA ahead of a stalled leading strand polymerase, thereby destroying the substrate. In Fig. 2B, the leading strand polymerase was blocked by the addition of ddTTP, and then at different times the ddTTP was diluted out 50-fold by transferring an aliquot of the reaction into a mixture containing the four dNTPs but lacking ddTTP. Pol III holoenzyme contains a proofreading 3′–5′ exonuclease, enabling it to remove the 3′ ddT residue and continue synthesis after the ddTTP is diluted out. If stalling of the leading strand polymerase stops DnaB helicase, then the minicircle substrate will not be unwound, and DNA synthesis will continue. However, if the helicase continues to unwind DNA while the leading strand polymerase is arrested, then the minicircle template will be unwound, and further DNA synthesis will not occur. Diluting out the ddTTP at various times after the block should therefore generate a decay curve that reflects the stability of the replisome after arrest of the leading strand polymerase. The time-dependent decay of the minicircle replisome after blocking the leading strand polymerase shows an approximate half-life of 13 s (Fig. 2B). This implies a slow DnaB helicase rate of 8 bp/s, similar to, or even less than, than the earlier estimate of about 35 bp/s for the action of DnaB in the absence of other proteins (6).

The inability to restart synthesis after removing the block to the stalled leading strand polymerase may be caused by continued helicase unwinding, as suggested above, but could also be explained by spontaneous dissociation of Pol III holoenzyme or DnaB helicase from the DNA. To address this, replisome stability on the minicircle template was measured directly by blocking both DnaB helicase and the leading strand polymerase. Helicase activity was blocked by removing ATP using a mixture of glucose and hexokinase, which rapidly consumes the ATP required for helicase unwinding (29). We also added ddTTP to stall the leading strand DNA polymerase. At various time points, the polymerase and helicase blocks were reversed by dilution of the ddTTP and glucose while maintaining the concentration of dNTPs and rNTPs. The decay curve in Fig. 2C shows that preventing helicase movement significantly extends the lifetime of the replisome on the minicircle DNA template. Hence, the rapid decay observed when only the leading strand polymerase is blocked was probably due to continued helicase unwinding. Consistent with DnaB helicase unwinding, we observe complete unwinding of a 5′-32P-end-labeled minicircle template upon stalling the polymerase with a single abasic residue on the leading strand template (data not shown). We also observe ATP-dependent unwinding of the 5′-32P-end-labeled TFII DNA in
reactions containing only DnaB helicase (see Fig. 2D). We conclude that DnaB helicase continues unwinding at least 100 bp ahead of a blocked lagging strand polymerase, although the leading strand polymerase is blocked. This conclusion was confirmed in experiments with a larger DNA template, described below.

**Lagging Strand Synthesis Continues Past a Stalled Leading Strand Polymerase**—Complete unwinding of the DNA template by helicase after the leading strand polymerase is blocked explains the absence of lagging strand synthesis using the minicircle TF II DNA substrate. Specifically, the TF II DNA substrate is unwound and destroyed by DnaB helicase action. Therefore, we devised a different strategy to determine whether the lagging strand polymerase remains active when the leading strand enzyme is stalled. Experiments with Pol III holoenzyme using larger plasmid substrates in vitro and in vivo have indicated that lagging strand replication continues even when the leading strand polymerase is blocked (32, 33). However, these earlier studies did not take steps to prevent excess Pol III holoenzyme from interacting with the plasmid substrates; therefore, it remains possible that the observed lagging strand synthesis was performed by a different molecule of Pol III holoenzyme from the one that is stalled on DNA.

To eliminate the possibility that a separate Pol III holoenzyme molecule provides the source of the observed lagging strand replication products when the leading strand polymerase is stalled, we designed the experiment illustrated in Fig. 3A. Rolling circle replication reactions were initiated using the minicircle DNA template in the absence of primase and radio- 

![Image](http://www.jbc.org/)
Replication Fork Events at a Leading Strand Block

Replisome Advance Is Limited to Less Than 3 kb—To study the extent to which DnaB helicase unwinds a replication fork containing a stalled leading strand polymerase, we developed a larger TF II DNA substrate that contains a damaged nucleotide on the leading strand template. A circular 2.7-kb TFII DNA substrate was constructed as illustrated in the scheme of Fig. 4A. A synthetic oligonucleotide containing an abasic site was hybridized to two oligonucleotides to form a replication fork structure with a single abasic site located 3 nucleotides downstream of the 3’ terminus on the leading strand. B. denaturing gel analysis of rolling circle replication reactions using the abasic site μC19-based TF II plasmid DNA template. Products in lanes 1–3 contain helicase (DnaB) and primase (DnaG) as indicated.

Fig. 4B confirms that the lagging strand polymerase and the helicase continue to function when the leading strand enzyme is blocked (lanes 1–3). Okazaki fragments with an average size of ~0.5 kb are observed, and their production requires both primase (lane 1) and helicase (lane 3). The Okazaki fragment size distribution extends beyond 1 kb in size, indicating that the replication fork sometimes continues at least 1 kb ahead of the block. The minor product at ~3 kb, arising independently of both primase and helicase, probably represents limited DNA synthesis on contaminating DNA that is not separated from the desired product during template purification (e.g. nicked circular μC19 lacking the abasic insert).

There exist two possible sources of the radioactive DNA products, as outlined in the scheme of Fig. 5A. In one case, primase targets DnaB on the lagging strand to prime the lagging strand for extension into Okazaki fragments. In the other case, primase targets DnaB on the lagging strand but primes the leading strand, as demonstrated in an earlier study (11). Extension of leading strand primers should continue to the abasic site, displacing the complementary strand and producing a partially duplex strand (see Fig. 5A). These two outcomes can be distinguished upon analysis in a native agarose gel, since one product (leading strand priming) consists of DNA that migrates faster than the original substrate, whereas the other product (lagging strand priming) consists of DNA that migrates slower than the original substrate. The result, in lane 2 of Fig. 5B, demonstrates that most of the replication products migrate slower than the original TF II DNA substrate, consistent with a predominant path involving priming of the lagging strand and extension by the lagging strand polymerase. It is possible, however, that the small but detectable amount of product that migrates ahead of the TF II DNA substrate derives from primase action on the leading strand.

To confirm that the observed lagging strand products derive from the action of a lagging strand polymerase that is attached to the stalled leading strand enzyme, we used a dilution strategy to eliminate possible action from an independent Pol III holoenzyme molecule. In this strategy, proteins are first assembled onto DNA, and then the reaction is diluted 50-fold prior to adding radioactive nucleotide, such that proteins that are not already associated with DNA cannot function. Previous studies have shown that this dilution is sufficient to prevent product formation from exogenous Pol III and is consistent with the results obtained in Fig. 2 (22).

The result of the dilution strategy, in Fig. 5C, shows that the reaction products do not change. Hence, the observed Okazaki fragments, both before and after dilution, are produced by the lagging strand polymerase within the replisome (i.e. attached to the stalled leading strand enzyme) and are not produced by unassociated polymerase molecules. Overall, these results demonstrate that when the leading strand polymerase stalls at a lesion, the replication fork advances for a limited distance, but less than 3 kb, and that the lagging strand polymerase synthesizes Okazaki fragments ahead of the blocked leading strand polymerase.
Pol III Holoenzyme Stalled at an Abasic Site Remains Stably Attached to DNA—Although most DNA lesions are repaired before the replication fork approaches, the replisome will encounter some lesions, and these will need to be repaired. The experiment of Fig. 5 implies that the stalled Pol III apparatus remains attached to DNA, and one may therefore presume that lesion repair will require the removal of the stalled DNA polymerase. In Fig. 6, we devised an experiment to directly test whether Pol III holoenzyme remains attached to DNA upon encountering a DNA lesion. In this experiment, we engineered a site-specific abasic site into a 7.2-kb M13mp18 circular ssDNA (Fig. 6A). The circular ssDNA was then primed with a DNA oligonucleotide, and Pol III holoenzyme was assembled at the primed site using [3H]Pol III core, γ complex, and β with only two dNTPs, where it idles on the primer 3' terminus. Gel filtration analysis indicates that the idling [3H]Pol III holoenzyme remains stably attached to the DNA (squares). Upon adding the remaining dNTPs, [3H]Pol III holoenzyme extends the primer 5.9 kb to the abasic site. Gel filtration analysis indicates that the [3H]Pol III holoenzyme remains attached to the DNA both while idling on the primer (squares) and when stalled at the lesion (circles). B, control reactions using primed undamaged M13mp18 ssDNA indicate that [3H]Pol III holoenzyme dissociates from DNA upon completing replication (circles) but remains stably attached to the DNA while idling with only dCTP and dGTP (squares). C, native agarose gel analysis of replication products using either undamaged primed M13mp18 ssDNA as template (first lane) or the abasic site M13mp18 primed ssDNA template (second lane).
Overall, these results indicate that Pol III holoenzyme remains stably attached to DNA after encountering a DNA lesion.

A Blocked Leading Strand Polymerase Is Cleared From DNA by the RecA and RecFOR Proteins—Provided that a leading strand polymerase remains stably attached to DNA at the site of a lesion, other proteins may be required to clear the DNA of the stalled replicase in order to prepare the DNA for repair. Since the DnaB helicase continues unwinding DNA ahead of the stalled polymerase, there will be a partial single-stranded region on the leading strand template. This template has been proposed to serve as a substrate for a recombination-mediated replication fork repair pathway involving the RecA and RecFOR proteins (16, 37, 38). RecA is unable to form a filament on SSB-coated ssDNA, and the RecFOR proteins are thought to enable the loading of RecA onto SSB-coated ssDNA (39).

In the experiments of Fig. 7, we asked whether RecA protein, which forms filaments on ssDNA but requires the action of the RecFOR proteins to load onto SSB-coated ssDNA, can displace \[^{3}H\]Pol III holoenzyme from DNA.

**FIGURE 7.** RecFOR proteins assist RecA in dismantling a stalled Pol III holoenzyme from DNA. **A,** the diagram to the right illustrates the finding that RecA displaces \[^{3}H\]Pol III holoenzyme that is idling at a primed site in the absence of SSB. **B,** the plot shows the gel filtration results of \[^{3}H\]Pol III holoenzyme idling at a primed site in the absence of SSB (squares) and the effect of RecA on displacing \[^{3}H\]Pol III holoenzyme from the DNA (circles). **C–H,** results in the presence of SSB. **C,** RecA and RecFOR proteins were not added. **D,** RecA was added. **E,** RecFR proteins were added, and then RecA was added. **F,** RecOR proteins were added, and then RecA was added. **G,** RecFOR proteins were added, and no RecA was added. **H,** RecFOR proteins were added, and then RecA was added.
heloenzyme on primed M13mp18 ssDNA coated with SSB and then added either RecA or buffer, followed by gel filtration analysis (Figs. 7, C and D). Under these conditions, the addition of RecA alone has no significant effect on the $[^{3}H]$Pol III holoenzyme-DNA complex, probably due to the presence of SSB, which prevents RecA from binding to DNA. In Fig. 7, E and F, we tested the ability of the RecFR and RecOR proteins (which form heterooligomers) to displace $[^{3}H]$Pol III holoenzyme from DNA in the presence of RecA, but no significant displacement was observed. Next we tested the effect of the RecFOR proteins on $[^{3}H]$Pol III holoenzyme displacement in the presence or absence of RecA. RecFOR in the absence of RecA did not displace $[^{3}H]$Pol III from DNA (Fig. 7G). However, the addition of RecFOR along with RecA (Fig. 7H) results in nearly complete displacement of $[^{3}H]$Pol III holoenzyme from DNA. Overall these results indicate that the RecFOR proteins help establish the RecA filament on SSB-coated DNA, which dissociates the stalled replicase from the DNA and thus clears the way for recombinative repair.

**DISCUSSION**

This report shows that blocking the leading strand polymerase does not prevent continued unwinding by DnaB helicase, nor does it compromise the activity of the lagging strand polymerase. These results complement and extend observations by others using in vitro (33) and in vivo (32, 33) experimental systems. Further, we demonstrate that the physical integrity of a stalled replisome complex remains intact, the blocked leading strand polymerase remains stably attached at the blocked site, and fork movement continues, albeit at a rate slower than normal. We also find that a blocked leading strand DNA polymerase can be removed by the combined action of the RecA and RecFOR proteins. These observations are discussed in greater detail below and are summarized in Fig. 8.

**Replication Fork Events at a Leading Strand Block**

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via the τ component of the holoenzyme clamp loader, and thus the processivity of the polymerase indirectly conveys additional contacts to DNA onto the helicase. DnaB helicase alone is not highly processive (44), indicating a relatively unstable interaction with DNA, but DnaB helicase is highly processive and very stable on DNA when complexed with Pol III holoenzyme (5). As diagrammed in Fig. 8, C and D, interaction with Pol III may eventually be lost at some point after the two polymerases functionally uncouple in leading and lagging strand synthesis, and this may result in spontaneous dissociation of DnaB from DNA within a short time. Even if DnaB maintains its connection to a blocked Pol III holoenzyme, the build-up of ssDNA (leading) and dsDNA (lagging) between the helicase and polymerases may exert a viscous drag that eventually breaks the τ-DnaB connection. A further stress on indirect attachment of DnaB to DNA through Pol III will occur upon displacement of the leading strand Pol III from DNA by RecA-RecFOR (Fig. 8D).

The observation here that the lagging strand polymerase retains function despite the inactive leading strand enzyme is consistent with reports from other studies (32, 33). However, an important caveat in the earlier work is the possibility that exogenous Pol III holoenzyme molecules could have been responsible for filling in the lagging strand. The current report addresses this concern using a dilution protocol to rule out the possibility that lagging strand synthesis results from another Pol III holoenzyme molecule that is not associated with the replication fork. The results support a model in which the two polymerases within one Pol III holoenzyme have significant flexibility such that they functionally uncouple even while remaining physically coupled. Indeed, the consensus is developing that all dimeric replisomes undergo some form of uncoupling in response to polymerase encounter with DNA damage sites. Both electron microscopy and two-dimensional electrophoresis have been used to detect structures consistent with uncoupled replication forks after UV irradiation of yeast cells (45). Moreover, analysis of UV-irradiated DNA treated with extracts from human cells by biochemical as well as electron microscopic methods has provided evidence for uncoupling of leading and lagging strand synthesis (46–48). For these eukaryotic systems, the architecture of the replisome is far less characterized than that of E. coli, making detailed mechanistic studies more challenging. Nonetheless, the apparent conservation of polymerase uncoupling as a feature of cellular replisomes points to the critical nature of this mechanism as a response to DNA damage.

Dissociation Mechanism of a Stalled Leading Strand Polymerase—The immediate problem posed by a DNA lesion that is encountered by the replisome before it is repaired is that they halt progression by the cellular replicase. Stalling of a replicative polymerase is nontrivial, because chromosomal replicases are typically tightly tethered to the DNA template by means of a processivity clamp (3) and are typically present in the cell in very limited numbers (1). Although cells have numerous pathways available for lesion repair and/or replication fork reactivation, one may presume that an early event common to all of these pathways is a mechanism by which the stalled polymerase is cleared from the lesion site.

The Pol III holoenzyme of E. coli displays an asymmetry in modulating polymerase association with the template during synthesis. The leading strand polymerase does not normally dissociate from the β-DNA complex, because a single binding event is sufficient, in principle, to allow for duplication of the entire template strand. The lagging strand polymerase, however, acts on a template strand with polarity opposite the direction of the advancing replication fork. As a result, the lagging strand polymerase must proceed through repeated cycles of (a) processive synthesis, (b) dissociation from the β clamp, and (c) rebinding to the next RNA primer to which β has been attached by the clamp loader (28). To date, two different mechanisms have been shown to affect the processivity switching that defines the lagging strand replication cycle. In one case, the polymerase-β connection is severed when the 5′ terminus of the preceding Okazaki fragment is encountered (29). In the second case, the polymerase releases prematurely from the β clamp when Okazaki fragments are much longer than normal (49) or when the lagging strand polymerase is blocked (22). Premature release appears to be triggered by accumulation of naked ssDNA upon SSB depletion.

Do either of these mechanisms come into play to dissociate a stalled leading strand polymerase? Although ssDNA may accumulate on the leading strand as the helicase continues unwinding, the slow rate and extent of helicase progression make SSB protein depletion unlikely. Collision with a 5′ terminus of a new DNA strand also seems unlikely. Even in the event of repriming on the leading strand (11), there is probably a distance between the new primer and the blocked polymerase that prevents collision between them, although mechanistic details of the leading strand priming pathway are not yet clear.

This report describes a new mechanism for dissociation of a stalled leading strand polymerase from DNA, specifically the formation of a RecA filament on the template ssDNA strand facilitated by the RecF, -O, and -R proteins. The requirement of the RecFOR proteins, which aid RecA filament formation on SSB-coated ssDNA (39, 50, 51), indicates that polymerase dissociation is mediated by the filament form of RecA. How does RecA clear the stalled polymerase from DNA? Further experiments are clearly required to address this mechanism, but a plausible model involves a mimic of one of the processivity-switching mechanisms of Pol III holoenzyme that normally takes place on the lagging strand. The switch that triggers polymerase dissociation upon finishing an Okazaki fragment could be enabled by the RecA filament simply by pushing the polymerase off the 3′ primed template junction so that the polymerase no longer “sees” ssDNA. Once on double-stranded DNA, Pol III ejects from β via a mechanism involving competitive interactions among polymerase, the β clamp, and the τ subunit (53–55). In this way, RecA may displace a stalled polymerase on the leading strand from the β clamp simply by triggering a switch mechanism that normally occurs repeatedly on the lagging strand.

It is interesting to note that protein-mediated displacement of a stalled polymerase is a well documented mechanism by which to reactivate a stalled RNA polymerase at damaged DNA sites, an enzyme that possesses extremely high processivity (56, 57). The molecular mechanism for release of a stalled RNA
polymerase involves specific protein factors that remove the stalled RNA polymerase from DNA (and also initiate lesion removal by recruitment of excision repair factors). Therefore, it has some similarities to the mechanism of stalled DNA polymerase release proposed here. In the case of a stalled leading strand DNA polymerase at a replication fork, the displacement mechanism relies on recombination proteins. The direct interplay of recombination proteins with a stalled replisome further highlights the intimate connection between recombination, replication, and repair that has led some to propose that recovery of stalled replication forks is the *raison d’être* for evolution of recombination proteins (58).

**Replication Fork Recovery Pathways—**In vivo experiments demonstrate that DNA synthesis stops immediately after UV irradiation of *E. coli*, but then after a lag of 15–20 min, synthesis resumes at the normal rate (13, 14). The term “replication restart” has been used to describe this robust capability of the cell to recover DNA synthesis following UV irradiation. Replication restart absolutely requires elevated levels of RecA, and genetic experiments have further defined the existence of multiple pathways that underlie this phenomenon. For example, a RecBCD-dependent pathway uses a homologous duplex DNA to invade a stalled replication fork DNA substrate (37, 59). The recombination intermediate in this pathway is then acted upon by DnaB helicase-loading factors, PriA and/or DnaC, to assemble a new DnaB helicase onto DNA and initiate a new replication fork using the invading strand as the new leading strand (60). Since the present study addresses immediate consequences of replication fork arrest, as opposed to later events, such as establishment of new replication forks via recombination pathways, we will focus below on RecBCD-independent replication restart mechanisms.

Based on results of the current study, we propose an intermediate replication fork structure similar to that diagrammed in Fig. 8B, with ssDNA on both the leading and lagging strands that is generated by continued DnaB activity beyond a stalled leading strand polymerase. The ssDNA ahead of the blocked leading strand polymerase serves as template for the action of DnaG primase, which can act on the lagging strand but under some instances can also act on the leading strand (11). The lagging strand DNA polymerase remains active, even with a stalled leading polymerase, and it extends the lagging strand RNA primers (Fig. 8C). RecA filament formation on leading strand SSB-coated ssDNA is facilitated by the RecFOR proteins, which disrupt the SSB-ssDNA complex that normally prevents RecA-ssDNA binding (Fig. 8C) (39, 50, 51). The RecA filament then displaces the stalled leading strand DNA polymerase from DNA (Fig. 8D).

Displacement of the stalled leading strand Pol III may be a prerequisite for reestablishment of a new replication fork and/or removal of the DNA lesion. This model accounts for previously observed roles of RecA as a direct factor in replication recovery, as opposed to its role in inducing SOS repair functions (14), as well as the observed UV sensitivity and abnormal replication restart phenotypes of RecFOR mutant cells (38, 61). The displacement of stalled polymerase, along with the proper combination of recombination proteins at the stalled fork, may serve as the initiating step for various fork regression pathways that are proposed to restore the parental duplex in the region ahead of the lesion and convert the fork to a “chicken-foot” structure (43). A RecA filament on the leading strand may catalyze this reaction by pairing it with the duplex lagging strand arm ahead of the block (62). Alternatively, the RecG protein, an enzyme that preferentially binds Y-junctions and catalyzes reverse branch migration, may catalyze the fork reversal reaction. Supporting this hypothesis is the finding that Y structures with ssDNA on the leading strand arm are the optimal substrate for RecG action (63). Keeping in mind the central importance of completing chromosome replication, it seems quite likely that at least two (and probably many) different paths exist to accomplish the same goal.

Finally, the final fate of the polymerase displaced by a RecA filament remains to be further investigated. For example, the displaced polymerase may still retain a grip on the β clamp. Biochemical studies have demonstrated that different polymerases have multiple attachment modes on the β clamp (64, 65). Furthermore, simultaneous residency on the β clamp by Pol III, a replicative polymerase, and Pol IV, a repair polymerase, has also been demonstrated (66). Given the facts that RecA acts at a template lesion to recruit Pol V, the main repair polymerase of *E. coli* (67), and that Pol V requires the β clamp (52), we expect that further work will reveal exquisite fine tuning among the different DNA polymerases, accessory factors, and recombination proteins that act in concert to perform their functions together at a stalled replication fork.

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DNA: Replication, Repair, Recombination, and Chromosome Dynamics:
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