A Dynamic RecA Filament Permits DNA Polymerase-catalyzed Extension of the Invading Strand in Recombination Intermediates

Liewei Xu‡ and Kenneth J. Marians‡§

From the ‡Graduate Program in Biochemistry and Structural Biology, Graduate School of Medical Sciences of the Weill College of Medicine of Cornell University and §Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Recombination-dependent replication is an essential housekeeping function in prokaryotes and eukaryotes, serving, for example, to restart DNA replication after the repair of a double-strand break. Little is known about the interplay between the recombination and replication machinery when recombination intermediates are used as substrates for DNA replication. We show here that recombination intermediates formed between linear duplex and supercoiled plasmid DNAs are substrates for a generalized strand displacement DNA synthesis reaction in which the 3'-OH of the invading strand in the recombination intermediate is used as a primer. DNA synthesis is driven by negative superhelicity and is inhibited if disassembly of the RecA filament is prevented. Thus, assembly and disassembly of RecA filaments in the same direction facilitates filament clearance from the 3'-end of the invading strand, allowing DNA synthesis to occur from recombination intermediates.

Maintenance of genomic integrity is dependent on the intersection of DNA replication and recombination (1–3). Many forms of recombinational repair of DNA damage involve limited DNA synthesis and, as has become clear over the past few years, the rescue of stalled replication forks requires the action of the recombination proteins. This latter point is best illustrated in bacteria, in which the replication forks that form at the origin of replication are generally not the ones that complete replication of the chromosome (4). Instead, with high frequency, either they run into frozen proteins or template DNA damage such as thymidine dimers, abasic lesions, or other bulky adducts that cause the replication fork to stall, or they encounter template nicks that cause the replication fork to collapse completely, generating a double-strand break in one of the sister chromosomes. Both types of events require that the template damage be repaired and that DNA replication be restarted.

The role of the recombination proteins in replication restart varies. In some instances, such as daughter strand gap repair, it is thought that DNA strand exchange acts to provide a template strand for repair of the lesion and that subsequent replication restart takes place at a replication fork that is regenerated by the combined action of proteins such as the branch migration enzymes RuvAB and RecG, the Holliday junction resolvase RuvC, and DNA helicases such as RecQ (5–7). On the other hand, during double-strand break repair in bacteria, replication restart proceeds directly from a recombination intermediate (8). This intermediate is most likely a D loop that has been formed between the broken and intact sister chromosome arms by the action of RecA and RecBCD. Replication restart is effected by the assembly of the replication restart primate, which is directed by the action of PriA (9). PriA recognizes the D loop specifically (10, 11), and subsequent primosome assembly leads to the deposition of DnaB, the replication fork DNA helicase, on the lagging-strand template, allowing replisome assembly.

Inactivation of priA leads to the generation of spontaneous suppressor mutations that map in dnaC (12), another restart primosomal protein (13) that forms a complex with DnaB in solution (14) and is involved in its transfer to DNA (15). We have reconstituted PriA-directed replisome assembly on a nicked, double-stranded DNA template that was engineered to contain a D loop (16). Using this system, we showed that DnaC810, which is encoded by the priA suppressor allele dnaC810 (12), had gained the ability to bypass PriA function and load DnaB directly to the D loop. This observation underscores that replication restart is an essential function.

Genetic studies have demonstrated multiple pathways of replication restart (17, 18), the use of which might reflect the different types of substrates for replication restart generated by the recombination proteins. To understand how the enzymatic pathway for processing of recombination intermediates is chosen, we have initiated an effort to reconstitute concerted recombination and replication reactions in vitro. We demonstrate here a generalized DNA polymerase-catalyzed strand displacement synthesis reaction that utilizes as a primer the 3'-OH end of the invading strand in a D loop generated by the action of RecA and RecBCD. The nascent strand synthesis reaction does not require a DNA helicase and is driven by negative supercoiling. We also show that the availability of the 3'-end of the invading strand in a D loop generated by the action of RecA and RecBCD. The nascent strand synthesis reaction does not require a DNA helicase and is driven by negative supercoiling. We also show that the availability of the 3'-end of the invading strand is dictated by the nature of the RecA filament that is formed during strand exchange. If the filament is static, the 3'-end is buried within and cannot be accessed by a DNA polymerase, whereas if the filament is dynamic, any DNA polymerase can bind to the 3'-end and extend it.

EXPERIMENTAL PROCEDURES

DNAs and Proteins—RecA (19) and RecBCD (20) were prepared as described. single-stranded DNA-binding protein (SSB),1 DNA gyrase, topoisomerase IV, and the DNA polymerase III holoenzyme (Pol III HE, as Pol III*) and β were prepared as described previously (21). RecA

1 The abbreviations used are: SSB, single-stranded DNA-binding protein; HE, holoenzyme; Pol III, polymerase III; BIR, break-induced replication.
K72R was the kind gift of Michael Cox (University of Wisconsin). pBR1 DNA was purified after alkaline lysis by buoyant density CsCl centrifugation and velocity sedimentation in sucrose gradients containing 1 M NaCl. The 800-mer was prepared by PCR using a forward primer with the sequence 5′-TGATCTTCGTCCCGGC-3′ corresponding to nucleotide coordinates 1724–1743 of pBR1 DNA and a reverse primer with the sequence 5′-ATGCCGTGAAGGTAAAGGTG-3′ corresponding to nucleotide coordinates 2549–2530 of pBR1 DNA. The DNA fragment was gel-purified before use.

DNA Synthesis from Joint Molecules—Standard reaction mixtures (20 µl) containing 50 mM HEPES-KOH (pH 8.0), 16 mM MgOAc, 10 mM dithiothreitol, 100 µM bovine serum albumin, 2 mM ATP, 40 µM dNTPs, 7.5 mM creatine phosphate, 62 ng/ml creatine kinase, 12.5 mM 5′-[32P]-labeled 800-mer, 1.1 mM pBR1 DNA, 3.5 µM RecA, 1 mM RecBCD, 0.8 µM SSB, 16 mM dNTPs, 20 mM Pol III*, and 20 mM β-subunit of the HE were incubated at 37 °C for 5 min for joint molecule formation or 20 min for form X formation and DNA elongation. Reactions were terminated by the addition of EDTA, SDS, and proteinase K to 25 mM, 1%, and 100 µg/ml, respectively, followed by incubation at 37 °C for 30 min. The samples were analyzed by electrophoresis through vertical 1% agarose gels at 1.5 V/cm for 15 h using 50 mM Tris-HCl (pH 8.3 at 23 °C), 40 mM NaOAc, 1 mM EDTA as the electrophoresis buffer. Gels were dried and autoradiographed.

Two-dimensional Gel Electrophoresis—Two DNA elongation reaction mixtures were mixed together and then applied equally to two adjacent lanes of a neutral agarose gel as described above. After electrophoresis, the two lanes were excised from the gel. One lane was dried to serve as a reference. The other lane was soaked in 50 mM NaOH, 1 mM EDTA and then inserted into a 0.5% horizontal alkaline agarose gel. Electrophoresis was at 1.5 V/cm for 15 h using 30 mM NaOH, 1 mM EDTA as the electrophoresis buffer. The gel was neutralized by soaking in 5% trichloroacetic acid, dried, and autoradiographed.

Isolation of Joint Molecules—Joint molecules were formed as described above in a standard reaction mixture increased 5-fold in a volume that contained only RecA, RecBCD, and SSB. After treatment with proteinase K, joint molecules were isolated by gel filtration through a Bio-Gel A-5m column (2.5 ml) formed in a 2-ml Falcon pipette using 50 mM Tris-HCl (pH 8.0), 1 mM EDTA as the column buffer. The excluded volume was identified by determining the Cerenkov radiation present in the column fractions and pooled. Typically, 12 or 13 µl of this pool were used in subsequent reactions.

Assay for RecA Filament Stability—An oligonucleotide substrate was prepared by annealing a 65-nt-long top strand of the sequence 5′-ACCGAATACCGAAGCTGCTGAGCAGGCGGAGAATTCGATCGACATCGTGGCCAGACCACGTATCGGAACGT-3′ with a 40-nt-long bottom strand of the sequence 5′-TTCCGATACTGGCTGCTGAGTGAATGTTGCTCGGCGGT-3′ to give a duplex region of 40 bp containing a centrally located RsaI site with a 25-nt-long 5′-ended strand. The top strand was 5′-[32P]-labeled. Reaction mixtures (80 µl) containing 50 mM HEPES-KOH (pH 8.0), 16 mM MgOAc, 10 mM dithiothreitol, 100 µM bovine serum albumin, either 2 mM ATP or dATP, 7.5 mM creatine phosphate, 62 ng/ml creatine kinase, 1.2 mM 5′-[32P]-tailed duplex oligonucleotide, and either 3.5 µM wild-type or K72R RecA were incubated for 15 min at 37 °C. An aliquot (10 µl) was withdrawn to represent the 0 time point (2.5 units) was added, and the incubation was continued. Aliquots (10 µl) were withdrawn at the indicated times. Reactions were terminated by the addition of EDTA to 25 mM followed by extraction with phenol-chloroform and recovery of the DNA by ethanol precipitation. Samples were analyzed by electrophoresis through an 8% polyacrylamide gel (30:1, acrylamide to bisacrylamide) using 100 mM Tris borate (pH 8.3), 2 mM EDTA as the electrophoresis buffer. The gel was fixed by soaking in 10% methanol, 7% HAc, 5% glycerol and then dried.

RESULTS

DNA Synthesis from a Recombination Intermediate—DNA strand exchange during double-strand break-repair is initiated in Escherichia coli by RecBCD locating the double-strand end of the broken chromosome arm and digesting it. Initially, the 3′-ended strand is digested from the break until a properly oriented χ-site is encountered. Nuclease activity then switches to the 5′-ended strand from the break, resulting in the generation of a recombinogenic 3′-single-stranded tail that becomes coated with RecA as a result of the RecA-loading enhancing function of RecBCD (22). Strand invasion into the sister chromosome results initially in the formation of a D loop. This key intermediate is a substrate for both the continued action of recombination proteins and the assembly of a replisome. To study these reactions, we utilized a plasmid template, pBR1, a modified pBR322 DNA into which we had inserted a χ-site at the XcmI site, and an 800-nt-long double-stranded DNA fragment representing the broken chromosome arm that was produced by PCR from the region on pBR1 that included the χ-site (Fig. 1A). Our initial reconstitution efforts included RecA, RecBCD, the SSB, the restart primosomal proteins, the DNA Pol III HE, and DNA gyrase, along with the 800-mer and pBR1. As we developed this system, we found that although we could observe bona fide replisome assembly, which will be described elsewhere, we could also observe a replication reaction that was not dependent on DnaB, the replication fork helicase. This reaction is described here.
Incubation of a linear duplex DNA (the 800-mer) with a plasmid DNA (pBR322) containing complementary sequences in the presence of RecA, RecBCD, SSB, and ATP results in the generation of joint molecules (Fig. 1B, lane 1). In this and all other experiments described herein, $^{32}$P label is present only on the 5' ends of the 800-mer. We observed the formation of both χ-dependent and χ-independent joint molecules. Because the χ-independent joint molecules incorporate all of the 800-mer, whereas in the χ-dependent joint molecules, the 800-mer has been digested by RecBCD to the χ-site (see Fig. 2), which is roughly in the center of the 800-mer, the χ-independent joint molecules have a reduced mobility compared with the χ-dependent joint molecules on the native agarose gels used for analysis of the reaction products.

Inclusion of gyrase and the Pol III HE in the reaction mixture resulted in the appearance of a new band (Fig. 1B, lane 3) that is a consequence of the invading strand in the joint molecule having been extended all the way around the plasmid DNA template by the DNA polymerase (Fig. 2). The formation of this product required RecA, RecBCD, SSB, gyrase, and the Pol III HE (Fig. 1B). Two additional products were apparent when the HE was omitted from the reaction mixture (Fig. 1B, lane 2). The electrophoretic mobility of these products corresponded to that of the plasmid DNA (Fig. 1B, form I-D loop) and to a band that ran faster than the plasmid DNA (Fig. 1B, form X). The presence of SSB inhibited the formation of form X (Fig. 1B, compare lanes 2 and 4). The nature of the $^{32}$P-labeled DNA in these products was investigated by two-dimensional gel electrophoresis (Fig. 2).

A lane excised from a native agarose gel displaying the products of a reaction mixture containing the 5'-32P-labeled 800-mer, pBR322-1, RecA, RecBCD, SSB, gyrase, and the HE was soaked in 50 mM NaOH to denature the DNA products and inserted into a second, alkaline agarose gel (Fig. 2). For reference, another lane from the initial native agarose gel that displayed the products of the reaction is shown at the top of Fig. 2. The HE and gyrase (Fig. 4, lanes 1–3) or form X was first generated in the presence of RecA, RecBCD, and ATP results in the generation of a reaction pathway observed by Shibata and colleagues (25) during their studies of the D loop cycle catalyzed by RecA. Using small oligonucleotides as the invading strand in the presence of RecA and various topoisomerases, these investigators observed the appearance of highly supercoiled form X DNA. In their case, the invading strand was no longer present in the form X product. It is present in the product reported here because the greater length of the invading strand sizes the D loop. Shibata and colleagues (25) hypothesized that extensive RecA filament formation extended the D loop into the duplex portion of the plasmid DNA causing unwinding of the plasmid DNA. Extensive unwinding generates compensatory positive supercoils that DNA gyrase will convert directly to negative supercoils. Following removal of the proteins from the DNA, it will assume the hyper-supercoiled form reported here (form X).

Thus, either joint molecules or form X could be the substrate for the observed DNA synthesis reaction. We therefore investigated this issue. Reactions were executed in two stages (Fig. 4). During the first stage, either joint molecules were first generated in the presence of RecA, RecBCD, and SSB (Fig. 4, lanes 1–3) or form X was first generated in the presence of RecA, RecBCD, and gyrase (Fig. 4, lanes 4–6). In the second stage, the reaction mixtures were supplemented with either the HE and gyrase (Fig. 4, lanes 1–3) or the HE and SSB (Fig. 4, lanes 4–6). Only in the case where joint molecules were formed in the first stage could elongated product be observed in the second stage, although there was a small amount of elon-
gated product present in the reaction in which form X was formed first and then HE was added (Fig. 4, lane 6), it most likely arose from the equivalent amount of \( \gamma \)-independent joint molecules that accumulated during form X formation (Fig. 4, lane 4). Thus, we conclude that the substrate for HE-catalyzed elongation was a joint molecule formed by the action of RecA and RecBCD and not a highly supercoiled DNA that contained a D loop.

**The Elongation Reaction Represents Strand Displacement Synthesis**—Because form X could be generated in the reaction, we considered the possibility that RecA might, in some manner, be acting as a DNA helicase to allow the elongation of the invading strand, perhaps as a result of treadmilling of the filament. To address this question, joint molecules were formed in the presence of RecA, RecBCD, and SSB, and the proteins were then removed by digestion with proteinase K in the presence of SDS followed by isolation of the joint molecules by gel filtration through a Bio-Gel A-5m column. Elongation of the invading strand in these isolated joint molecules required only the HE, gyrase, and SSB (Fig. 5). Some partially elongated material could be observed in the absence of SSB (Fig. 5, lane 3). In addition, in the presence of HE, gyrase, and SSB, elongated product with a lower mobility than the full-length product could be observed. Two-dimensional gel analysis has shown that this elongated product corresponds to DNA that is greater than unit template length (data not shown), presumably produced by rolling circle-type replication.

Thus, the requirement for RecA observed with the concerted reaction in Fig. 1B related only to the requirement that joint molecules must be made before any polymerase-catalyzed elongation of the invading strand could take place and not to the involvement of RecA in the elongation process itself. How, then, was DNA unwinding being driven given that no DNA helicase

**Fig. 3. Requirements for form X (FX) formation.** Standard reaction mixtures containing the indicated assortment of proteins were incubated and analyzed as described under “Experimental Procedures.” Both an autoradiogram (top) and a photograph of the ethidium bromide-stained gel (bottom) are shown. Lane 0 is the form I (FI) plasmid DNA. Gyr, gyrase; \( \gamma \)-indep JM, \( \gamma \)-independent joint molecules; \( \gamma \)-dep JM, \( \gamma \)-dependent joint molecules; FX, form X DNA; FII, form II DNA; JM, joint molecules.

**Fig. 4. Joint molecules, not form X (FX), are the substrate for the elongation reaction.** Reactions were executed in two stages. In stage 1, either joint molecules (JM) were formed using RecA, RecBCD, and SSB (lanes 1–3) or form X was formed using RecA, RecBCD, and gyrase (lanes 4–6). In stage 2, HE and gyrase were added to the reactions shown in lanes 1–3, and HE and SSB were added to the reactions shown in lanes 4–6. Reactions were processed and analyzed as described under “Experimental Procedures.” An autoradiogram of the dried gel is shown. EP, elongated product; \( \gamma \)-indep JM, \( \gamma \)-independent joint molecules; \( \gamma \)-dep JM, \( \gamma \)-dependent joint molecules.

**Fig. 5. RecA is not required for elongation of the invading strand in joint molecules (JM).** Joint molecules were isolated by gel filtration as described under “Experimental Procedures.” Standard reaction mixtures contained 13 \( \mu l \) of the isolated pool of joint molecules. The complete reaction (Comp) also contained Pol III HE, SSB, and gyrase (gyr). Reactions were processed and analyzed as described under “Experimental Procedures.” An autoradiogram of the dried gel is shown. EP, elongated product; FI-DL, form I D loop DNA.
was involved in the replication reaction? The most likely possibility was that we were observing a slow strand displacement reaction in which the DNA was being unwound by a combination of the DNA being kept negatively supercoiled by the action of DNA gyrase, which will favor unwinding, and SSB binding to the displaced single-stranded DNA that is generated as the polymerase slowly pushes forward. We would expect that in such a reaction, there would not be any specificity for the DNA polymerase, that elongation would be slow, and that other topoisomerases that could relax the positive supercoils generated by the unwinding would not be able to support the elongation reaction because they could not supercoil the DNA template. These predictions were tested directly.

Both Sequenase (Fig. 6A), a version of the bacteriophage T7 DNA polymerase, as well as E. coli DNA polymerase I (data not shown) could substitute for the HE in the reaction. Thus, it was unlikely that elongation of the invading strand in the joint molecule required any specific interaction between the DNA polymerase and either RecA, RecBCD, or SSB.

To measure the speed of the elongation reaction, aliquots were removed from the reaction mixture starting from the beginning of the reaction, and the products were analyzed on denaturing alkaline agarose gels (Fig. 6B). The trailing edge of the smear present in the lanes in Fig. 6B represents the longest elongated invading strands at that time point; thus the speed of elongation can be assessed by observing the change in the size of the largest products as a function of time. It required about 90 s to produce full-length material, giving an elongation speed of about 70 nts/s. This is far slower than the 700–1000 nts/s that we have measured for bona fide replication forks in vitro (26) and is therefore consistent with the progress of a strand displacement reaction.

Elongation of the invading strand in the joint molecule all the way around the circular template creates positive linkages as a result of the unwinding of the parental duplex DNA. If a DNA helicase were driving the elongation reaction, the production of full-length product DNA would require only that the excess positive linkages be removed by a topoisomerase such as topoisomerase IV, which can relax both negatively and positively supercoiled DNA but cannot supercoil DNA (21). We have shown, for example, that this enzyme is capable of supporting oriC replication in vitro (27). However, topoisomerase IV was unable to replace gyrase in elongation reactions using isolated joint molecules (Fig. 7). This test had to be performed...
ATP is required to observe elongation of the invading strand in joint molecules. Standard reactions for either joint molecule formation (JM), form X formation (FX), or invading strand elongation (EI) with either wild-type (WT) or K72R RecA and containing either the standard concentration of ATP and all other nucleotides (ATP) or lacking ATP and containing 2 mM dATP and the standard concentration of all other nucleotides (dATP), as indicated, were incubated, processed, and analyzed as described under “Experimental Procedures.” Both an autoradiogram (top) and a photograph of the ethidium bromide-stained gel (bottom) are shown. EP, elongated product; FI, form II DNA; FI/JM, form I DNA.

The wild-type protein could only support the formation of elongated product in the presence of ATP (Fig. 8, compare lanes 3 and 9). The mutant protein was unable to support the formation of elongated product in the presence of dATP (Fig. 8, lane 12). Although there are other ATP-utilizing enzymes present in the reaction, the failure to observe elongated product in the presence of dATP could be attributed directly to an altered property of RecA. Initiation complex formation by the HE proceeds equally well in the presence of either ATP or dATP (35), and supercoiling by DNA gyrase is supported about a third as well by dATP as by ATP (36). To compensate for this latter deficiency, the concentration of gyrase in the reaction was increased 5-fold.

Thus, it appeared that if the RecA filament was stabilized, the HE could not access the 3’-end of the invading strand in the joint molecules. To confirm that it was the stability of the RecA filament that was the variable under these reaction conditions, we developed an assay to test access to DNA coated with a RecA filament. An oligonucleotide substrate was designed that was a 40-nt duplex with an RsaI site in the middle of the duplex and that had a 5’-single-stranded tail of 25 nt. Thus, the RecA filament assembly will nucleate on the single strand and spread onto the duplex portion of the oligonucleotide. We assessed digestion of digestion by RsaI of the duplex portion of the oligonucleotide under the various conditions used in the experiment described in the legend for Fig. 8. After allowing filament formation to reach an equilibrium, RsaI was added, and digestion of the oligonucleotide was followed over time (Fig. 9).

In the absence of RecA, cleavage of the oligonucleotide was rapid, and the reaction was complete 2–3 min after addition of RsaI. Filament formation by wild-type RecA in the presence of ATP slowed the rate of cleavage by RsaI but, notably, it did not prevent it. On the other hand, filament formation by either the wild-type or the K72R protein in the presence of dATP prevented cleavage by the restriction enzyme. These findings are consistent with the data presented in Fig. 8. Thus, a dynamic RecA filament permits access to DNA that it is nominally bound to, presumably, in the case of the experiment shown in Fig. 9, because the cycles of assembly and disassembly that describe the equilibrium position leave the RsaI site uncoated for a certain fraction of time. In the elongation reaction from joint molecules, the equivalent action would result in clearance of the RecA filament from the terminus of the invading strand in the D loop and allow the 3’-OH to be detected and elongated by a DNA polymerase.

**DISCUSSION**

The intersection between the recombination and replication machinery is now well established in prokaryotes and is being revealed in eukaryotes. During recombinational repair of stalled replication forks in bacteria, recombination proteins often act to generate substrates for replication restart. The situation therefore arises in which a recombination intermediate is poised to proceed along a pathway of maturation and resolution that is dictated by the enzymatic specificities of either recombination or replication proteins. Thus, a D loop formed, for example, between the broken and intact sister arms of a replicating circular bacterial chromosome could yield extensive strand exchange by the continued action of RecA and by the additional action of the branch migration DNA helicase RuvAB. On the other hand, the same D loop could become a substrate for replisome assembly directed by the restart helicase. We report here a third possibility, that the invading strand in the joint molecule can be used as a primer by a DNA polymerase in a strand displacement reaction that is driven by negative supercoiling.
Elongation of the invading strand in the joint molecules creates positive windings in the template DNA that compensate for the unwinding of the parental duplex strands. Accrual of excess positive windings will prevent additional template unwinding and stall DNA replication. Topoisomerases are responsible for sustaining DNA replication on topologically constrained templates by relaxing the positive windings. Both DNA gyrase and topoisomerase IV are capable of relaxing positive supercoils, but only DNA gyrase was capable of supporting the elongation reaction. We have to attribute this difference to the fact that, but not topoisomerase IV, can also supercoil DNA. In fact, during the elongation reaction, gyrase will convert the positive supercoils directly into negative ones. Therefore, with gyrase present, the template DNA will retain a net negative supercoiling. Supercoiled DNA prefers to exist in the relaxed state, in which there are no supercoils. Thus, negative supercoils favor the unwinding of the duplex turns in the DNA molecule. This tendency likely acts as a driving force for the unwinding of the duplex template DNA during the elongation reaction. However, it clearly does not provide sufficient energy for the process because SSB is also required. In the absence of SSB, only partial elongation was observed. We presume that the energy of SSB binding cooperatively to single-stranded DNA at the fork contributes to the unwinding reaction.

Although the requirement for RecA in the concerted elongation reaction derives from the need to first make a joint molecule, it is clear, because form X can be produced in the same reaction, that an extensive RecA filament can form on the duplex template DNA. At first glance, it would seem that the RecA filament would actually represent an impediment to any DNA replication reaction seeking to utilize the joint molecule as a substrate, suggesting that the tendency would be for the joint molecule to continue forward to the replication fork, to a small region about the site of cleavage. However, rare events can be observed in which BIR is quite extensive. During the elongation reaction, to a small region about the site of cleavage. However, rare events can be observed in which BIR is quite extensive. This clearance, in turn, allows subsequent reactions to occur on the joint molecules, permitting a choice of enzymatic pathway for resolution. It is particularly interesting that because RecA filaments assemble and disassemble in the same direction, 5′ → 3′, clearance occurs in a manner that specifically encourages subsequent DNA replication that uses the 3′-end of the invading strand as a primer for leading-strand synthesis.

Does the reaction described here play a role in the cell? This issue is difficult to assess. Any form of extensive replication from recombination intermediates, be it the result of bona fide recombination assembly or strand displacement synthesis, within the context of double-strand break repair between sister chromosomes is genetically silent. Detection requires the creation of specifically marked chromosome arms and the use of physical analysis. It is, however, interesting to point out that in one of the few cases in which recombination-directed replication has been studied carefully, a reaction similar to the one described here may be operative.

Haber and his colleagues (39) have carefully analyzed break-induced replication (BIR) in yeast originating at the mating type locus as a result of HO endonuclease-catalyzed cleavage of the DNA. Normally, BIR is limited, probably because of the formation of a second Holliday junction that acts to capture the replication fork, to a small region about the site of cleavage. However, rare events can be observed in which BIR is quite

The DNA replication reaction that we observed uses joint molecules formed by the action of RecA and RecBCD as a template. The reaction drew our attention initially because, in the absence of a DNA helicase, full-length DNA was being synthesized by the Pol III HE, which is nominally incapable of strand displacement synthesis (37). Using various oligonucleotide substrates where a primer was annealed upstream to a duplex region, we confirmed that the HE was, indeed, incapable of strand displacement synthesis (data not shown). These observations implied that during the extension of the invading strand in the joint molecules, the unwinding of the template duplex was achieved by novel means.

The fact that a highly unwound form of the plasmid DNA, form X, could also be generated in the reaction raised the possibility that the same driving force for form X production, the unwinding of the DNA by the RecA filament (38), might also be capable of sustaining the DNA replication reaction. However, although it was clear that RecA was required initially in the reaction to catalyze the formation of joint molecules, we found that it could be eliminated from the elongation reaction without effect. This observation left us to consider that superhelicity was driving the unwinding needed for elongation.

The DNA products were analyzed by electrophoresis through a 25-nt 5′-single-stranded tail (schematically diagrammed at the top of the figure). RsaI was then added, and digestion of the oligonucleotide was assessed as a function of time as described under “Experimental Procedures.” DNA products were analyzed by electrophoresis through 8% polyacrylamide gels. Autoradiograms of the dried gels and quantification of the digestion are shown. Only about 60% of the input oligonucleotide could be digested with RsaI. We suspect that this was because of incomplete deprotection during synthesis.

Elongation of the invading strand in the joint molecules creates positive windings in the template DNA that compensate for the unwinding of the parental duplex strands. Accrual of excess positive windings will prevent additional template unwinding and stall DNA replication. Topoisomerases are responsible for sustaining DNA replication on topologically constrained templates by relaxing the positive windings. Both DNA gyrase and topoisomerase IV are capable of relaxing positive supercoils, but only DNA gyrase was capable of supporting the elongation reaction. We have to attribute this difference to the fact that, but not topoisomerase IV, can also supercoil DNA. In fact, during the elongation reaction, gyrase will convert the positive supercoils directly into negative ones. Therefore, with gyrase present, the template DNA will retain a net negative supercoiling. Supercoiled DNA prefers to exist in the relaxed state, in which there are no supercoils. Thus, negative supercoils favor the unwinding of the duplex turns in the DNA molecule. This tendency likely acts as a driving force for the unwinding of the duplex template DNA during the elongation reaction. However, it clearly does not provide sufficient energy for the process because SSB is also required. In the absence of SSB, only partial elongation was observed. We presume that the energy of SSB binding cooperatively to single-stranded DNA at the fork contributes to the unwinding reaction.

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extensive (hundreds of kilobases long). Two genetic pathways have been delineated that differ by their dependence on Rad51. However, in each case, although it can be demonstrated that many of the proteins present at the replication fork, such as polα/primase, polδ, proliferating cell nuclear antigen, and replication protein A, are required for BIR, there is no indication that a helicase is required for the reaction. These observations are consistent with the type of strand displacement synthesis described here.

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