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Translocation by the RecB Motor Is an Absolute Requirement for χ-Recognition and RecA Protein Loading by RecBCD Enzyme*

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RecBCD enzyme is a heterotrimeric helicase/nuclease that initiates homologous recombination at double-stranded DNA breaks. The enzyme is driven by two motor subunits, RecB and RecD, translocating on opposite single-strands of the DNA duplex. Here we provide evidence that, although both motor subunits can support the translocation activity for the enzyme, the activity of the RecB subunit is necessary for proper function of the enzyme both *in vivo* and *in vitro*. We demonstrate that the RecBCD^{K177Q} enzyme, in which RecD helicase is disabled by mutation of the ATPase active site, complements *recBCD* deletion *in vivo* and displays all of the enzymatic activities that are characteristic of the wild-type enzyme *in vitro*. These include helicase and nuclease activities and the abilities to recognize the recombination hotspot χ and to coordinate the loading of RecA protein onto the ssDNA it produces. In contrast, the RecB^{K29Q}CD enzyme, carrying a mutation in the ATPase site of RecB helicase, fails to complement *recBCD* deletion *in vivo*. We further show that even though RecB^{K29Q}CD enzyme displays helicase and nuclease activities, its inability to translocate along the 3′-terminated strand results in the failure to recognize χ and to load RecA protein. Our findings argue that translocation by the RecB motor is required to deliver RecC subunit to χ, whereas the RecD subunit has a dispensable motor activity but an indispensable regulatory function.

RecBCD enzyme is a heterotrimeric protein complex consisting of the three non-identical polypeptides, RecB (134 kDa), RecC (129 kDa), and RecD (67 kDa) (1, 2). The three subunits constitute a multifunctional enzyme that possesses DNA-dependent ATPase, DNA helicase, and both single-stranded DNA (ssDNA)[3] and double-stranded (dsDNA) nuclease activities (for review see Refs. 3 and 4). Working in concert, these activities allow the enzyme to initiate homologous recombination by (*a*) processing dsDNA ends to produce long ssDNA overhangs terminated with the χ sequence at the 3′-end and (*b*) facilitating assembly of the RecA nucleoprotein filament on this ssDNA product (5).

Two of the three subunits of RecBCD enzyme, RecB and RecD, contain motifs characteristic of the Superfamily I DNA helicases. The purified RecB protein is an ssDNA-dependent ATPase and a 3′→5′ DNA helicase (6), whereas, the RecD protein is also an ssDNA-dependent ATPase but a 5′→3′ helicase (7, 8).

The enzyme displays a high affinity for blunt or nearly blunt dsDNA ends. In the initiation complex, RecB subunit is bound to the 3′-terminated ssDNA strand, whereas the RecC and RecD subunits are bound to the 5′-terminated strand (9–11). Thus, both the structural arrangement and the enzymatic movement of the enzyme reflect the antiparallel nature of the DNA duplex. Consequently, the simultaneous translocation of both motor subunits, with their corresponding opposite polarities, results in the unidirectional movement of the holoenzyme. This bipolar motor arrangement explains many of the unique attributes of RecBCD enzyme, such as the high processivity (Refs. 12 and 13, and accompanying report (43)), high affinity for DNA ends (14), and its capacity to displace proteins bound to DNA (15). Intriguingly, only one of either motor subunit is needed for the helicase function of the holoenzyme *in vitro* (8, 16). Thus, the need for two separate motor subunits remains unexplained.

DNA unwinding by RecBCD enzyme is accompanied by degradation of the newly produced ssDNA. The nuclease activity of RecBCD enzyme is asymmetric, occurring preferentially on the 3′-terminated strand, with respect to the DNA end at which the enzyme enters the duplex (17, 18). The nuclease activity is attenuated (17), and its strand bias is switched (19) when RecBCD enzyme interacts with the recombination promoting sequence χ (Chi = crossover hotspot instigator), which is 5′-GCTGGTGG-3′ (20). Recognition of χ occurs when RecBCD enzyme approaches from its 3′-side (21). Chi is recognized in its single-stranded form, and only the single strand containing the GCTGGTGG sequence is required (22). Interaction with χ also affects translocation by RecBCD enzyme: upon χ-recognition, the enzyme briefly pauses then resumes translocation, but at a reduced rate (23). This pause ensures that, before attenuation of nuclease activity, a final cleavage event occurs in the vicinity of χ (24). Finally, the χ-modified RecBCD enzyme displays the capacity to load the DNA strand exchange protein, RecA, onto the χ-terminated ssDNA produced by RecBCD helicase/nuclease activity (25).

The existence of RecC mutants that enable the holoenzyme to recognize an altered χ sequence (26, 27), argues that RecC subunit is involved in χ-recognition. However, the RecD subunit also plays a role in χ-recognition, because the RecBC enzyme, which lacks the RecD subunit, does not recognize χ, implying that it acts by either translocating the RecC subunit along the ssDNA or regulating the recognition of, and response to, χ. The RecBC enzyme is recombinationally proficient both

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[3] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Chi, crossover hotspot instigator; SSB, *E. coli* single-stranded DNA-binding protein; PEP, phosphoenol pyruvate; DTT, dithiothreitol.

in vivo (28) and *in vitro* (29), and it mediates constitutive RecA loading; thus, the RecD motor subunit appears to be at least partially dispensable. Interestingly, the nuclease activity of RecBCD enzyme, whose active site resides within the RecB subunit, depends on the presence of the RecD subunit (30). RecD also stimulates the helicase activity of RecBC enzyme (7, 30) and increases its affinity for dsDNA ends.

To understand the functions of the two motors that comprise the RecBCD enzyme, we analyzed two RecBCD mutant enzymes: the RecB^{K29Q}CD and RecBCD^{K177Q} enzymes, each carrying a lysine to glutamine substitution in the Walker A motif of either the RecB or the RecD subunit, respectively (8, 16, 31, 32). Each mutation inactivates ATP hydrolysis in the respective subunit and eliminates its ability to translocate along ssDNA. We demonstrate that the ability to interact with a χ sequence, to undergo χ-induced modification, and to facilitate RecA loading depends on a functional RecB motor subunit. In contrast, with the exception of a lower processivity, loss of RecD motor function makes little difference in a variety of *in vitro* assays. As might be expected based on these biochemical properties, the RecBCD^{K177Q} also supports recombinational DNA repair *in vivo*, but the RecB^{K29Q}CD enzyme does not.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Chemicals were of a reagent grade. Pyruvate kinase, lactate dehydrogenase, PEP, and ATP were purchased from Sigma. Restriction endonucleases, shrimp alkaline phosphatase, T4 polynucleotide kinase, and Klenow Fragment of DNA polymerase I were from New England Biolabs; [α-^{32}P]ATP and [γ-^{32}P]ATP were from PerkinElmer Life Sciences; and Hoechst 33258 was from Molecular Probes Inc. ATP was dissolved as concentrated stock solutions at pH 7.5, and its concentration was determined spectrophotometrically using $\epsilon_{260} = 1.54 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$.

Covalently closed circular pBR322 and χ^+-3F3H dsDNA (a pBR322 derivative, containing two sets of three tandem χ sequences (33)) were purified using a Qiagen "Maxi kit" followed by cesium chloride gradient centrifugation. Plasmid dsDNA was linearized with NdeI restriction endonuclease, dephosphorylated with shrimp alkaline phosphatase, and 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP using methods given by the vendor. For the 3′-end labeling, pBR322 dsDNA was linearized with EcoRI restriction endonuclease, and labeled with Klenow Fragment of DNA-polymerase I and [α-^{32}P]ATP.

Escherichia coli RecBCD enzyme was expressed and purified as described previously (22). RecBC enzyme and the mutant RecB^{K29Q}CD and RecBCD^{K177Q} proteins were expressed in a *recBCD*Δ background and purified as described in a previous study (8). *E. coli* RecA and SSB proteins were purified as described before (34–36), respectively (the purity of the wild-type and mutant proteins is shown as supplementary information to the accompanying report). Wild-type RecBCD, RecB^{K29Q}CD, and RecBCD^{K177Q} enzymes were 100% active, as determined from titrations using the fluorometric helicase assays, which showed that the enzyme activity saturated at a 1:1 molar ratio of enzyme to dsDNA ends (data not shown).

ATP Hydrolysis Assays—ATP hydrolysis was monitored spectrophotometrically by coupling ATP hydrolysis to NADH oxidation (37, 38) using an Agilent Technologies Model 8452A diode array spectrophotometer. Assay mixtures contained 25 mM Tris acetate (pH 7.5), 1 mM DTT, 2 mM ATP, 1.5 mM PEP, 0.2 mg/ml NADH, 50 μM (nucleotides) poly(dT) ssDNA, pyruvate kinase (30 units/ml), lactate dehydrogenase (30 units/ml), and the indicated concentrations of magnesium acetate. Reactions were initiated by the addition of 0.5 nM RecBCD, RecB^{K29Q}CD, or RecBCD^{K177Q} enzyme after preincubation of all other components at 37 °C for 5 min. The rate of ATP hydrolysis was calculated from the rate of change in absorbance at 340 nm due to oxidation of NADH using the following conversion: rate of A_{340} decrease (s^{-1}) × 9820 = rate of ATP hydrolysis (micromolar/min).

Plasmid DNA Unwinding Assays—Assays were performed as described previously (39). The reaction mixtures contained 25 mM Tris acetate (pH 7.5), 1 or 6 mM magnesium acetate (as indicated), 1 mM DTT, 20 μM (nucleotides) linear pBR322 dsDNA ^{32}P-labeled at either 5′- or 3′-ends (4.5 nM ends), 2 mM ATP, and 2 μM SSB protein. DNA unwinding reactions were started with the addition of, respectively, 0.2 nM RecBCD enzyme, 0.2 nM RecBCD^{K177Q} enzyme, 1 nM RecB^{K29Q}CD enzyme, or 5 nM RecBC enzyme after preincubation of all other components at 37 °C for 5 min. Unequal concentrations of the enzymes were used in all gel-based assays to normalize utilization of the linear dsDNA substrate. Assays were stopped at the indicated times by addition of proteinase K to a final concentration of 0.5 mg/ml, which was dissolved in sample loading buffer (250 mM EDTA, 20% Ficoll 400, 5% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanol). After a 5-min incubation with proteinase K at room temperature, the reaction products were separated on a 1% (w/v) TAE (40 mM Tris acetate (pH 8.2) and 1 mM EDTA) agarose gel at 700 V·h, visualized, and quantified using an Amersham Biosciences Storm 840 PhosphorImager and ImageQuaNT software.

Chi-specific Fragment Production Assays—The assays were performed similarly to the plasmid DNA unwinding assays, except that NdeI-linearized χ^+-3F3H labeled at the 5′-ends was used as a substrate. The reaction mixtures contained 25 mM Tris acetate (pH 7.5), 6 mM magnesium acetate, 1 mM DTT, 20 μM (nucleotides) linear 5′-labeled χ^+-3F3H dsDNA (4.5 nM ends), 2 mM ATP, and 2 μM SSB protein. DNA unwinding reactions were started by the addition of, respectively, 0.2 nM RecBCD enzyme, 0.2 nM RecBCD^{K177Q} enzyme, 1 nM RecB^{K29Q}CD enzyme, or 5 nM RecBC enzyme after preincubation of all other components at 37 °C for 5 min. RecBCD, RecBC, and mutant enzymes at selected concentrations unwind dsDNA substrate to near completion in 10 min.

RecA Loading Assays—The coupled pairing reactions were performed as described (17, 25), using χ^+-3F3H as a χ-containing DNA substrate. The standard "RecABCD" reaction mixture (17) consisted of 25 mM Tris acetate (pH 7.5), 6 mM magnesium acetate, 1 mM DTT, 1.5 mM PEP, 4 units/ml pyruvate kinase, 20 μM (nucleotides) 5′-^{32}P-labeled linear dsDNA (4.5 nM ends), 40 μM supercoiled DNA, 2 mM ATP, 2 μM SSB protein, and 7.5 μM RecA protein. The coupled pairing reactions were started by the addition of either 0.2 nM RecBCD enzyme, 0.2 nM RecBCD^{K177Q} enzyme, 1 nM RecB^{K29Q}CD enzyme, or 5 nM RecBC enzyme after preincubation of all other components at 37 °C for 5 min. Assays were stopped at the indicated times, and the products were resolved and visualized as described for the plasmid DNA unwinding assay.

Fluorometric Helicase Assays—Continuous helicase assays were performed by following the displacement of fluorescent dye (Hoechst 33258) from linear dsDNA upon DNA unwinding (40). The reaction mixtures contained 25 mM Tris acetate (pH 7.5), 1 mM DTT, 2 or 5 mM ATP (as indicated), 1 mM PEP, 1 μM SSB protein, 5 units/ml pyruvate kinase, 300 nM Hoechst 33258, and the indicated concentration of magnesium acetate. The Hoechst 33258 dye fluorescence was monitored at 465 nm upon excitation at 355 nm using an SLM Aminco 8000 spectrofluorometer (SLM Instruments, Inc.). After preincubation of other components at 20 °C for 5 min, the background fluorescence of the Hoechst 33258 dye was measured. NdeI-linearized pBR322 dsDNA (in the assays with wild-type RecBCD enzyme and RecBCD^{K177Q} mutant)

or EcoRI-linearized pBR322 dsDNA (in the case of RecB^{K29Q}CD mutant) was then added to the final concentration of 5 μM nucleotides (1.05 nM ends). The fluorescence of the dsDNA-Hoechst complex was recorded and assigned as 100% dsDNA. Fluorescence corresponding to 0% dsDNA was determined from the heat-denatured dsDNA controls performed at several concentrations of magnesium ion. Under our experimental conditions, the increase of the Hoechst fluorescence in the presence of ssDNA was ~6% of the fluorescence increase in the presence of equimolar concentration of dsDNA. Therefore, the 94% decrease in the dsDNA-Hoechst complex fluorescence relative to the background fluorescence corresponded to 100% unwinding. Helicase reactions were started by the addition of 0.2 nM RecBCD, 0.2 nM RecBCD^{K177Q}, or 1 nM RecB^{K29Q}CD enzyme. The rate and the extent of helicase activity were calculated from the slope of the linear portion of each progress curve and from the difference in the fluorescence before addition of the enzyme and after completion of the unwinding, respectively.

Viability after the UV Irradiation—The *E. coli* strains used in this study were transformants of the *V186* strain (Δ(*argA-thyA*)232), in which a region containing the *recB*, *recC*, and *recD* genes is deleted (41). *V186* cultures were grown in LB media supplemented with 50 μg/ml thymidine, and cells were transformed with the following plasmids: pDWS2 (42), which directs synthesis of the wild-type RecBCD enzyme, pDJ05-D^{K177Q} (32), encoding RecB, RecC, and RecD^{K177Q} polypeptides, and pFS-B^{K29Q} (31), encoding RecB^{K29Q}, RecC, and RecD polypeptides. All three plasmids are present at a similar copy number, and expression of the wild-type and mutant RecBCD enzymes is directed by their native promoters. Approximately equal levels of the wild-type and mutant RecBCD enzymes are synthesized based on our observation that the purification yield for each protein is similar. The bacterial cultures were grown in LB media supplemented with 50 μg/ml thymidine. To maintain the plasmids, 100 μg/ml of ampicillin was added. To measure cell survival after UV irradiation, 50-μl aliquots of appropriate dilutions of exponentially growing cultures (A_{600} = 0.4) were plated on LB plates and irradiated at room temperature for the indicated times. Plates were irradiated by placing a short wave (254 nm) UV lamp (Spectroline) 50 cm above the plates. Immediately after exposure, the plates were covered with aluminum foil and incubated at 37 °C for 16 h. Survival fraction was measured as a fraction of the initial colony-forming units after exposure to the indicated amounts of UV light.

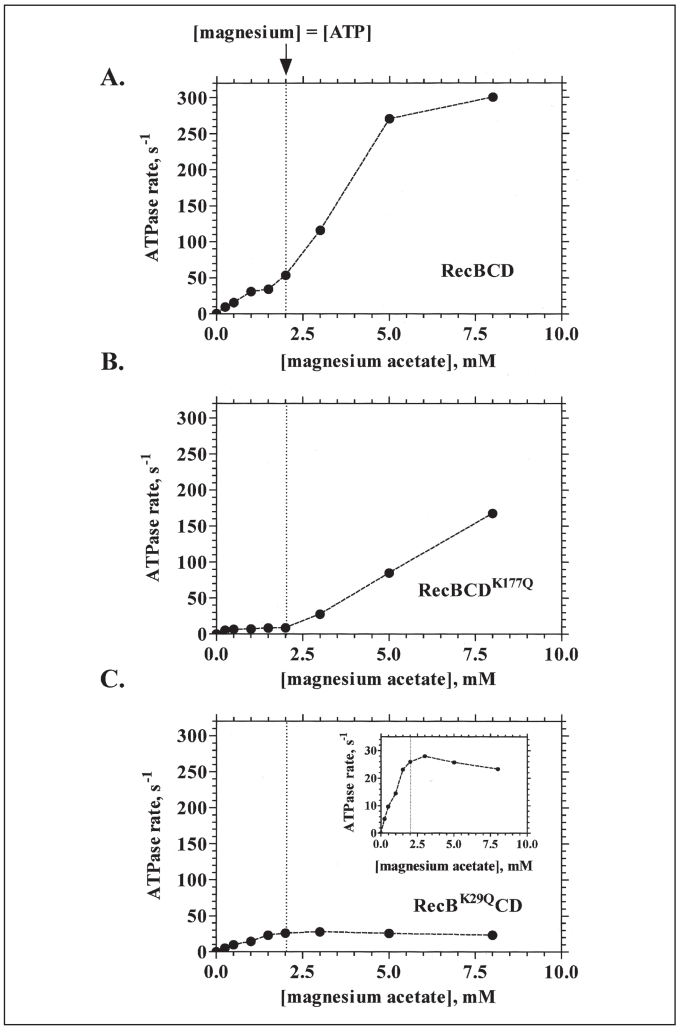

FIGURE 1. **RecB^{K29Q}CD and RecBCD^{K177Q} enzymes are ssDNA-dependent ATPases that display different dependences on free magnesium ion concentration.** ATP hydrolysis by wild-type RecBCD (*A*), RecBCD^{K177Q} (*B*), and RecB^{K29Q}CD (*C*) enzymes was analyzed in the presence of different magnesium ion concentrations as described under "Experimental Procedures." All reactions contained 2 mM ATP, 50 μM nucleotides poly(dT) ssDNA, and 50 nM of the respective enzyme. The *dotted line* indicates the ATP concentration. The *inset* in *C* shows a blow-up of the same data in *C* using an expanded *y*-scale.

RESULTS

Both the RecB^{K29Q}CD and RecBCD^{K177Q} Mutant Enzymes Display ATPase and Helicase Activities—The ssDNA-dependent ATPase activity of the individual RecB and RecD proteins is sensitive to the free magnesium ion concentration (7). Consequently, we analyzed the ssDNA-dependent ATPase activity of the heterotrimeric RecB^{K29Q}CD and RecBCD^{K177Q} mutant enzymes as a function of magnesium ion concentration (Fig. 1). At high magnesium ion concentrations, the ATPase activity of RecBCD^{K177Q} enzyme approaches that of the wild-type enzyme. However, in contrast to the wild-type enzyme, the RecBCD^{K177Q} mutant is essentially inactive when the concentration of ATP in the reaction mixture exceeds the concentration of magnesium ion. The rate of ATP hydrolysis by RecBCD^{K177Q} enzyme increases with increasing concentrations of magnesium acetate in a manner similar to that of the purified RecB subunit (7). In contrast, maximal ATP hydrolysis by the RecB^{K29Q}CD enzyme is ~10-fold lower than that of wild-type enzyme, despite the fact that this mutant enzyme is 100% active based on titration of helicase activity (see "Experimental Procedures"). In addition, ATP hydrolysis displayed an optimum that saturated at an approximately equimolar concentration of ATP and magnesium ion. Qualitatively, the ATPase activity of the wild-type protein seems to represent the sum of activities for each mutant enzyme, suggesting that the magnesium ion dependence of RecBCD enzyme can be deconvoluted into the sum of ATPase activities for the individual motor subunits.

Because of the differences in optimum reaction conditions for RecB^{K29Q}CD and RecBCD^{K177Q} mutant enzymes, we analyzed dsDNA unwinding under both low (limiting relative to ATP) and high (excess relative to ATP) magnesium ion concentrations (Fig. 2). We compared the dsDNA unwinding and nuclease activities of RecB^{K29Q}CD and RecBCD^{K177Q} mutants to those of wild-type RecBCD and RecBC enzymes by analyzing the reaction products using agarose gel electrophoresis (39). Fig. 2 (*A* and *B*) shows the results obtained when the plasmid-length linear dsDNA was labeled at the 5′-end. When the magnesium ion was present in excess over ATP (*A*), both mutant enzymes mediated unwinding of the dsDNA, which can be observed by the disappearance of the dsDNA. Because the nuclease activity of RecBCD enzyme, and apparently of the mutant enzymes as well, is greatest when

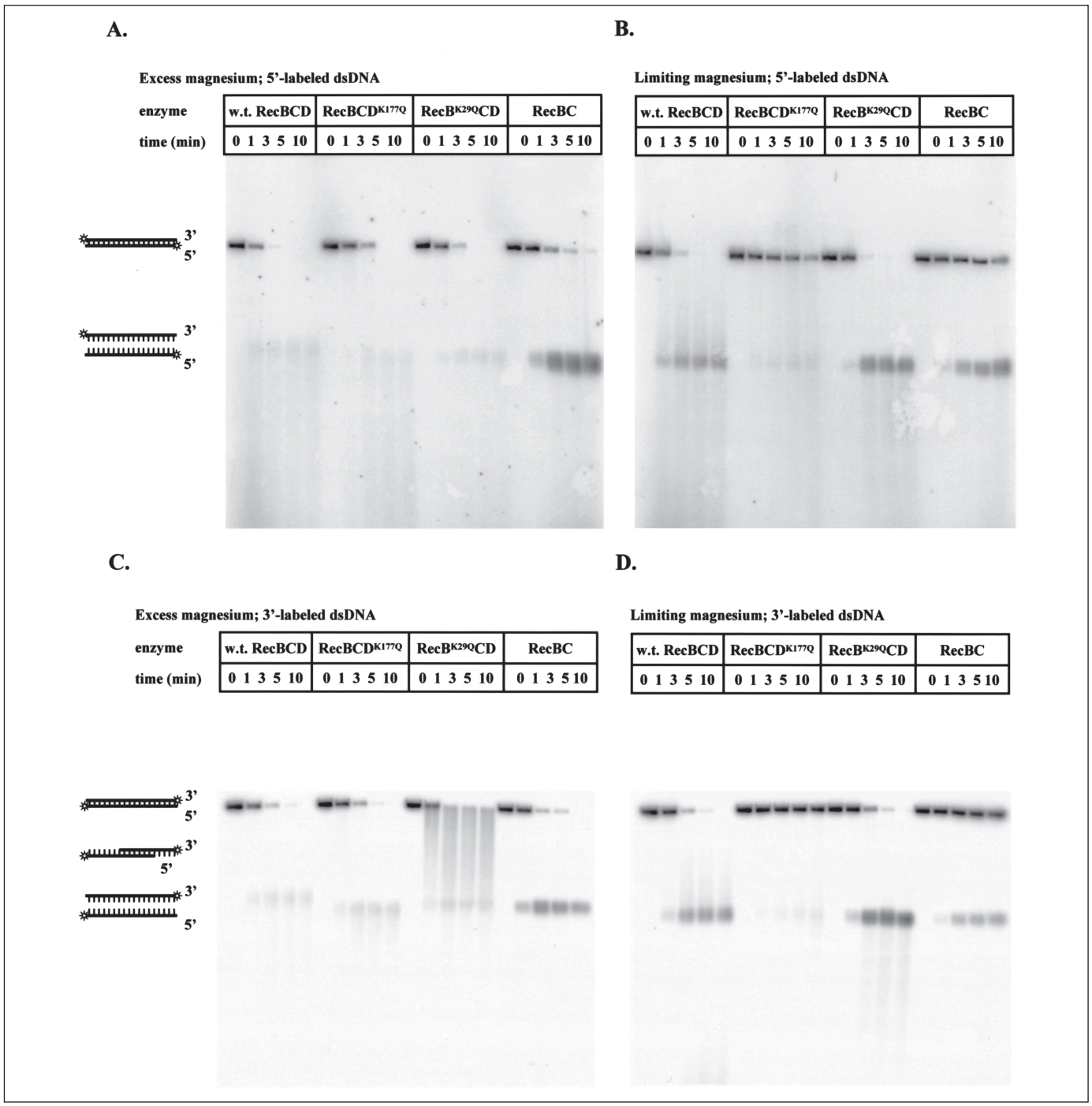

FIGURE 2. **Both RecB^{K29Q}CD and RecBCD^{K177Q} enzymes display helicase and nuclease activity.** Time courses for dsDNA processing by 0.2 nM RecBCD, 0.2 nM RecBCD^{K177Q}, 1 nM RecB^{K29Q}CD, and 5 nM RecBC enzymes. Plasmid length DNA (pBR322) unwinding reactions were carried out in the presence of 2 mM ATP and either 6 mM ("*Excess magnesium*" (*A* and *C*)) or 1 mM ("*Limiting magnesium*" (*B* and *D*)) magnesium acetate as described under "Experimental Procedures." The enzymes, and the times at which the reactions were terminated, are indicated in the tables above the respective gels. The positions of dsDNA and ssDNA species are indicated schematically on the *left*. The reactions in which NdeI-linearized pBR322 dsDNA labeled at 5′-ends was used as a substrate are presented in *A* and *B*. *C* and *D* show reactions containing EcoRI-linearized pBR322 dsDNA labeled at 3′-ends.

the free magnesium ion is high, only a slight amount of the dsDNA is converted into full-length ssDNA. The final amount of full-length ssDNA produced by both mutants is comparable to that generated by the wild-type enzyme but much less than that produced by the nuclease-deficient RecBC enzyme, supporting the conclusion that both RecB^{K29Q}CD and RecBCD^{K177Q} mutant enzymes retain nuclease activity. Note that the aim of the experiments presented here was to qualitatively ascertain the level of activity for the mutant enzymes. Therefore, we used different concentrations of wild-type and mutant enzymes to achieve the same rate of dsDNA utilization (a more quantitative measurement of helicase activity is provided in the accompanying report (43)).

Because the nuclease activity of the RecBCD enzyme is sensitive to the concentration of free magnesium ion (44), we examined the behavior of each enzyme at a limiting magnesium ion concentration (relative to the ATP concentration) (Fig. 2*B*). In agreement with previously pub-

lished data (45), dsDNA unwinding by the wild-type RecBCD enzyme was almost equally efficient both in the presence of limiting or excess concentrations of magnesium ion (Fig. 2, compare *A* with *B*). However, due to the lower nuclease activity, more ssDNA product is evident at the lower magnesium concentration. In contrast, the rate of dsDNA unwinding by RecBCD^{K177Q} mutant was significantly slower at 1 mM magnesium ion than at 6 mM magnesium ion, and almost no ssDNA was detected. This lower helicase activity is in full agreement with the greatly reduced ATP hydrolysis activity observed at lower concentrations of magnesium ion (Fig. 1*B*). RecBC enzyme, which is also driven exclusively by the RecB motor, also displays a reduced rate of DNA unwinding under these conditions (Fig. 1, compare *A* with *B*), but most of the ssDNA is preserved. These results suggest that the RecBCD^{K177Q} mutant enzyme has nuclease activity, despite the defective RecD subunit, whereas the RecBC enzyme, which lacks the RecD subunit, does not.

These helicase assays did not reveal any effect of magnesium ion concentration on the DNA unwinding by RecB^{K29Q}CD mutant. Similar to the wild-type RecBCD enzyme, RecB^{K29Q}CD enzyme produced a significantly greater amount of the full-length ssDNA when the magnesium ion concentration was limited, consistent with a reduction in nuclease activity (Fig. 2*B*).

To confirm and extend these observations, we conducted identical assays, but using 3′-end-labeled dsDNA instead (Fig. 2, *C* and *D*). Wild-type RecBCD, RecBCD^{K177Q}, and RecBC enzymes produced unwinding products identical to those observed in assays using the 5′-end-labeled dsDNA (Fig. 2, *A* and *B*). At the limiting magnesium ion concentration (Fig. 2*D*), RecB^{K29Q}CD enzyme produced mostly full-length ssDNA, but some shorter ssDNA was also observed. At high magnesium ion concentration (Fig. 2*C*), however, DNA species migrating as a continuous smear between dsDNA and ssDNA bands was observed. The presence of these intermediates suggests that firstly, the processivity under these conditions is low, causing RecB^{K29Q}CD enzyme to dissociate with high probability before it completes unwinding of the linearized plasmid dsDNA. This inference is quantitatively confirmed in the accompanying report (43). Secondly, comparison to Fig. 2*A* suggests that RecB^{K29Q}CD enzyme is digesting the 5′-terminated strand with a higher frequency than the 3′-terminated strand. Interestingly, this bias is opposite to that of the wild-type enzyme but is identical to the behavior of the RecB^{2109}CD mutant enzyme (44, 46), which we believe has a defective RecB motor subunit (see "Discussion"). These two characteristics of RecB^{K29Q}CD enzyme result in dsDNA molecules with 3′-overhangs (depicted on the *left* of the gel), which are not substrates for further DNA unwinding.

Wild-type RecBCD, RecBCD^{K177Q}, and RecB^{K29Q}CD Enzymes Display Different Dependences on Solution Conditions— To further define the helicase activity of the mutant RecBCD enzymes, we carried out fluorometric helicase assays that provide quantitative information about both the rate and the extent of dsDNA unwinding (40). At saturating magnesium ion concentrations, the rate of helicase activity of the RecBCD^{K177Q} mutant was approximately one-half that of the wild-type enzyme (Fig. 3, *A* and *B*). Interestingly, the two enzymes displayed different dependences on magnesium ion concentration. In agreement with previous data (45), wild-type RecBCD enzyme displayed a gradual increase in DNA helicase activity that saturated when the magnesium ion concentration exceeded the ATP concentration (Fig. 3*A*). Except at the lowest concentration of magnesium ion, the processivity of RecBCD enzyme was sufficient to fully unwind 4.36 kb of pBR322 plasmid dsDNA (Fig. 3*D*). Note that because of the extremely high unwinding rate of RecBCD enzyme, limiting concentrations of enzyme were used relative to the DNA molecule concentrations. The unwinding rates determined in these experiments are multiple turnover rates and, therefore, can be sensitive to the DNA association kinetics. In contrast to the wild-type RecBCD enzyme, the helicase activity of RecBCD^{K177Q} mutant showed biphasic behavior (Fig. 3*B*). A discontinuity in the unwinding rate appeared when the concentrations of ATP and magnesium acetate were approximately equal (Fig. 3*B*). The processivity of the RecBCD^{K177Q} mutant was also affected in a similar manner, being less processive at low concentrations of magnesium ion (Fig. 3, compare *D* with *E*). Fig. 3*E* shows that at the limiting magnesium ion concentrations, the mutant enzyme unwinds 25–75% of the pBR222 DNA, indicating that an average of only 1.1–3.3 kbp are being unwound per binding event (the large uncertainty associated with extents of unwinding of $<$25% or $>$75% does not allow for a reliable estimate of the processivity). These data indicate that the RecBCD^{K177Q} mutant enzyme, in which RecB subunit is the sole motor, requires high free magnesium ion concentrations for maximum speed and processivity of DNA unwinding.

The RecB^{K29Q}CD mutant behaved differently from the wild-type and RecBCD^{K177Q} enzymes (Fig. 3, *C* and *F*). The initial rate of DNA unwinding catalyzed by RecB^{K29Q}CD enzyme increased continuously as the magnesium ion concentration was increased to about 2 mM, but then its activity declined. Interestingly, the processivity profile of the RecB^{K29Q}CD mutant is the complement of the RecBCD^{K177Q} mutant: above 2 mM magnesium acetate, the extent of unwinding decreases continuously from 100% ($>$4.4 kbp unwound per binding event) to 45% at 10 mM, which corresponds to about 2 kbp unwound per binding event. Consequently, the RecB^{K29Q}CD enzyme and, by inference, the RecD motor display optimal activity when ATP is present in excess of the magnesium ion. Thus, this complementary behavior of the two motor subunits with regard to magnesium ion concentration raises the interesting possibility that the two motors provide a homeostatic function to helicase activity. Regardless, the opposite magnesium ion dependences clearly show that the speed of each motor is sensitive to reaction conditions and that the lead (*i.e.* faster) motor in RecBCD enzyme may switch between RecB and RecD, depending on reaction conditions.

RecBCD^{K177Q} Enzyme Recognizes χ, but RecB^{K29Q}CD Enzyme Does Not—To determine whether the helicase-deficient mutants of RecBCD enzyme can recognize χ, we assayed for the χ-specific fragment production using 5′-labeled NdeI-linearized χ^+-3F3H dsDNA as a substrate (Fig. 4). As previously reported, at 6 mM magnesium acetate and 2 mM ATP, unwinding and degradation of this substrate by the wild-type RecBCD enzyme result in the production of full-length ssDNA and of χ-specific ssDNA fragments (17, 18, 47). RecBCD enzyme converted ~28% of the dsDNA substrate into χ-specific ssDNA fragments. In comparison, RecBC enzyme unwound this dsDNA producing mostly full-length ssDNA (Fig. 4 and Ref. 29). Despite having a defective RecD subunit, the RecBCD^{K177Q} mutant behaved similarly to the wild-type protein and not like the RecBC enzyme; RecBCD^{K177Q} mutant enzyme recognized χ sequence and produced χ-specific fragments. The amount of χ-specific fragments produced by the mutant enzyme was 16%, which was lower than that produced by the wild-type enzyme (28%). The ability of RecBCD^{K177Q} enzyme to recognize χ was confirmed in several independent experiments. Furthermore, when an EcoRI-linearized plasmid DNA was used as a substrate instead of the NdeI-linearized substrate (data not shown), χ-specific fragments (800 and 1400 bp long) were also produced (with yields of 26 and 15%, respectively) by both the RecBCD and RecBCD^{K177Q} enzymes. These findings demonstrate that RecBCD^{K177Q} enzyme not only recognizes χ, but also both attenuates and switches the polarity of its nuclease activity, to produce ssDNA with χ at its 3′ terminus. This result was unexpected, because it shows that

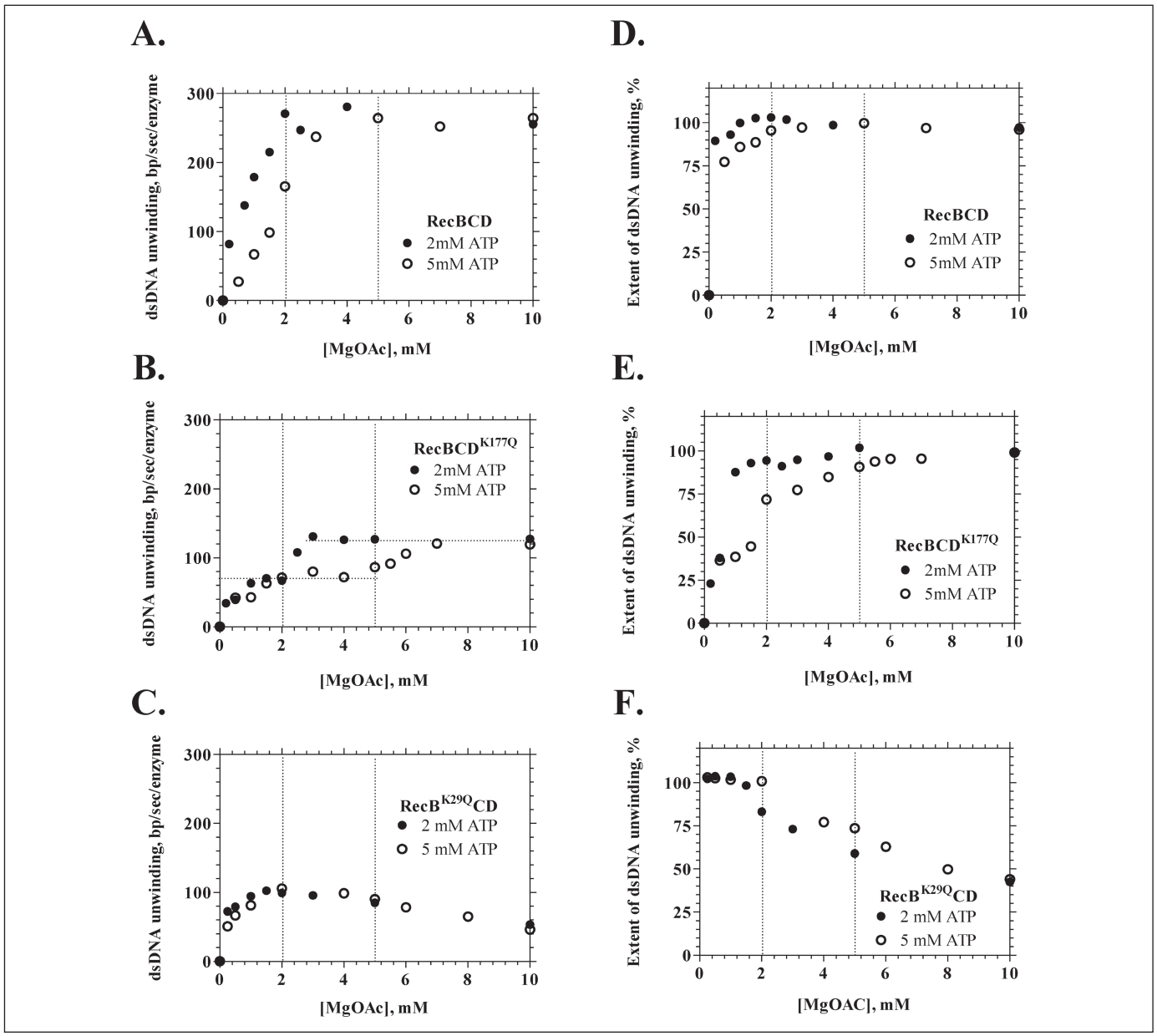

FIGURE 3. **The helicase activities of wild-type and mutant RecBCD enzymes display different dependences on magnesium ion concentration.** Continuous helicase assays were performed using the fluorescent dye-displacement method as described in the "Experimental Procedures." Reaction mixtures contained 5 μM nucleotides (2.1 nM ends) NdeI- or EcoRI-linearized pBR322 dsDNA, the indicated concentrations of magnesium acetate and either 2 mM (*solid circles*) or 5 mM (*open circles*) ATP, 1 μM SSB protein, an ATP regeneration system, and 0.2 nM RecBCD (*A* and *D*), 0.2 nM RecBCD^{K177Q} (*B* and *E*), or 1 nM RecB^{K29Q}CD enzyme (*C* and *F*). The initial rates (*A–C*) and extents (*D–F*) of DNA unwinding were calculated from progress curves recorded at each concentration of magnesium acetate.

inactivation of the RecD motor by mutagenesis is different from the complete removal of this subunit (*i.e.* the RecBC enzyme), indicating that the role of RecD subunit in the nuclease polarity switch does not depend on its ability to translocate or hydrolyze ATP.

In contrast to the other mutant enzyme, RecB^{K29Q}CD enzyme displayed no χ-induced modification of nuclease activity (Fig. 4). Instead, this mutant enzyme behaved as though the DNA were devoid of a χ sequence (see Fig. 2*A*), producing only some ssDNA (~12% of the dsDNA substrate was converted into full-length ssDNA). This finding shows that the two motor subunits are not equivalent with respect to χ-recognition and that a functional RecB motor is required for manifestation of χ-dependent changes in enzyme activity. A functional RecD motor is clearly not a substitute for RecB subunit function, even though it can provide helicase function.

To verify these results, the reactions were also conducted at conditions of limiting magnesium ion (1 mM magnesium acetate and 2 mM ATP; data not shown). As reported previously (48), higher concentrations of both wild-type RecBCD enzyme and RecBCD^{K177Q} mutant were required to unwind χ^+-3F3H dsDNA, indicating χ-induced inactivation as described before (48). Under these conditions, as expected, both wild-type and RecBCD^{K177Q} enzymes produced χ-specific fragments and full-length ssDNA. As described above, however, the RecB^{K29Q}CD mutant did not produce any χ-specific fragments, and its behavior was indistinguishable from that observed using pBR332 dsDNA, which lacks χ sequences.

RecBCD^{K177Q} Enzyme Can Load RecA Protein onto the Processed χ-Containing ssDNA—We also tested the ability of the RecBCD helicase mutants to facilitate the coordinated loading of RecA protein onto

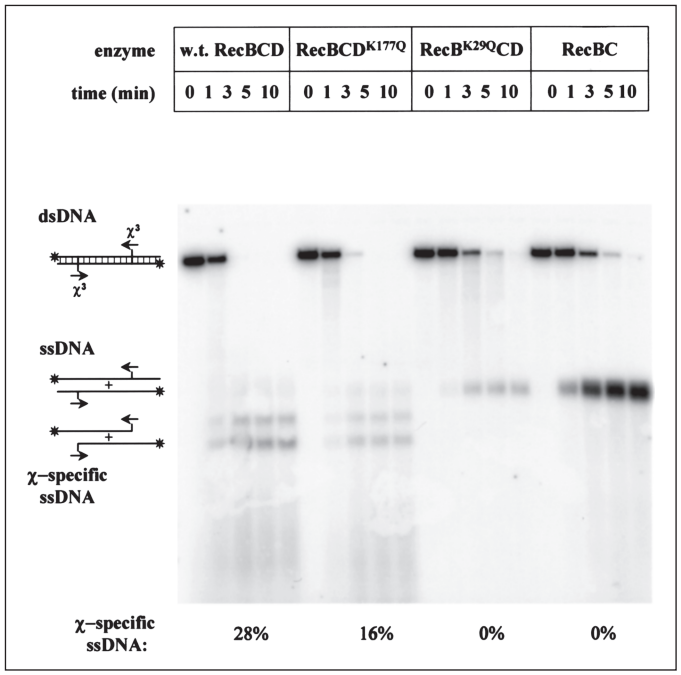

FIGURE 4. **RecBCD^{K177Q}, but not RecB^{K29Q}CD, enzyme recognizes χ and modifies its nuclease activity in response to χ.** Chi-specific fragment production assays were carried out using ^{32}P-labeled NdeI-linearized χ^+-3F3H as a substrate (schematically shown on the *left* of the gel) as described under "Experimental Procedures." All reactions were initiated by addition of the enzyme (0.2 nM RecBCD, 0.2 nM RecBCD^{K177Q}, 1 nM RecB^{K29Q}CD, and 5 nM RecBC enzymes). At the indicated times, aliquots were taken from the reaction mixture and analyzed by electrophoresis on a 1% TAE-agarose gel. For each reaction, the enzyme, and the times at which the reactions were terminated are indicated in the table above the gel. The structures of the full-length ssDNA and of the χ-specific ssDNA fragments are depicted on the *left*. The amount of χ-specific fragments produced by the wild-type and RecBCD^{K177Q} enzymes were quantified using ImageQuaNT software and are indicated under the gel.

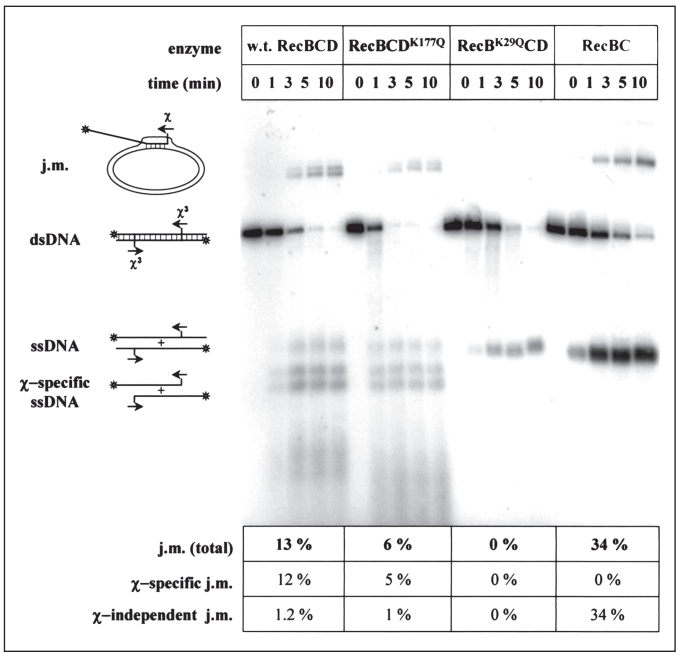

FIGURE 5. **RecBCD^{K177Q} enzyme can load RecA protein onto χ-containing ssDNA to promote homologous pairing.** Coupled RecABCD reactions were carried out as described under "Experimental Procedures." The ^{32}P-labeled substrate (an NdeI-linearized χ^+-3F3H dsDNA), as well as products and intermediates of the reactions (full-length ssDNA, χ-specific ssDNA fragments, and joint molecules (*j.m.*)) are indicated at the *left* of the gel. The three *j.m.* bands produced in the presence of the wild-type RecBCD and RecBCD^{K177Q} enzymes are the following: the two intense faster migrating bands are the χ-specific *j.m.* derived from the two different χ-specific fragments, while the faint band migrating at the same rate as *j.m.* produced by RecBC enzyme corresponds to the χ-independent *j.m.* All reactions were initiated by addition of the enzyme (0.2 nM RecBCD, 0.2 nM RecBCD^{K177Q}, 1 nM RecB^{K29Q}CD, and 5 nM RecBC enzymes). Aliquots were taken from each reaction at the indicated times, and analyzed by electrophoresis on a 1% TAE-agarose gel. The amounts of joint molecules produced by each enzyme were quantified and are presented in the table below the gel.

ssDNA using a coupled DNA unwinding and pairing assay. In this assay, RecBCD enzyme processes linear χ-containing dsDNA to produce χ-specific ssDNA fragments, and also facilitates loading of RecA protein onto these χ-specific fragments. RecA nucleoprotein filaments then invade homologous supercoiled DNA resulting in the production of joint molecules (17, 47). The facilitated loading of RecA protein onto the χ-containing ssDNA by RecBCD enzyme is manifest as an increase in χ-specific joint molecule formation relative to other types of joint molecules (*e.g.* the full-length ssDNA) (25). Under these conditions (Fig. 5), 12% of the label in the starting linear dsDNA is processed by wild-type RecBCD enzyme into χ-specific ssDNA that is assimilated to produce χ-specific joint molecules; of the χ-specific ssDNA produced, ~42% participates in joint molecule formation.

We found that RecBCD^{K177Q} enzyme also loads RecA protein onto χ-specific ssDNA to produce χ-specific joint molecules (Fig. 5). The yield of joint molecules produced is 5% (relative to the dsDNA), which is approximately one-half of that obtained with the wild-type enzyme. Of the χ-specific ssDNA produced by the RecBCD^{K177Q} enzyme, 31% is paired to produce χ-specific joint molecules. Significantly, this mutant enzyme with the defective RecD subunit does not mimic the RecBC enzyme, which loads RecA protein independently of χ onto full-length ssDNA to produce the slowest migrating joint molecules (Fig. 5) (29). Rather, although somewhat impaired, the RecBCD^{K177Q} mutant more closely emulates the wild-type enzyme in that it can clearly respond to χ and load RecA protein onto the processed χ-containing ssDNA.

In contrast, the RecB^{K29Q}CD mutant enzyme did not promote any detectable joint molecule formation, not even using the ssDNA produced (Fig. 5), suggesting that it is completely defective in RecA-loading. As shown above, the mutant enzyme does not produce any χ-specific fragments, even when RecA protein is present.

RecBCD^{K177Q} Enzyme Confers Resistance to UV Irradiation, Whereas RecB^{K29Q}CD Does Not—The ability to both recognize χ and load RecA protein are essential for RecBCD function in vivo (46, 49, 50). Therefore, based on our biochemical observations, we would expect the RecBCD^{K177Q} mutant enzyme to support recombinational repair in *recBCD*Δ host cells, whereas the RecB^{K29Q}CD mutant enzyme would not.

We tested the ability of these helicase-deficient mutants to complement the UV sensitivity of the *V186* strain (Δ(*argA-thyA*)232), in which the *recB*, *recC*, and *recD* genes are deleted (Fig. 6). Plasmid pDJ05-D^{K177Q}, which encodes the *recB*, *recC*, and *recD^{K177Q}* genes, restores UV resistance of *V186* cells to the level obtained with a plasmid (pWS2) carrying wild-type *recB*, *recC*, and *recD* genes. In contrast, cells carrying the plasmid (pFS-B^{K29Q}), which encodes the *recB^{K29Q}*, *recC*, and *recD* genes, are as sensitive to UV irradiation as the *V186* cells that lack RecBCD enzyme. Thus, consistent with our expectations based on the biochemical characteristics, we find that the RecBCD^{K177Q} mutant enzyme is functional for recombination *in vivo*, whereas the RecB^{K29Q}CD mutant enzyme is not.

DISCUSSION

Recently, we demonstrated that RecBCD enzyme is a bipolar DNA helicase that employs two ssDNA motors: the RecB helicase subunit that translocates on the 3′-terminated strand with a 3′→5′ polarity, and the RecD helicase subunit that translocates on the 5′-terminated strand

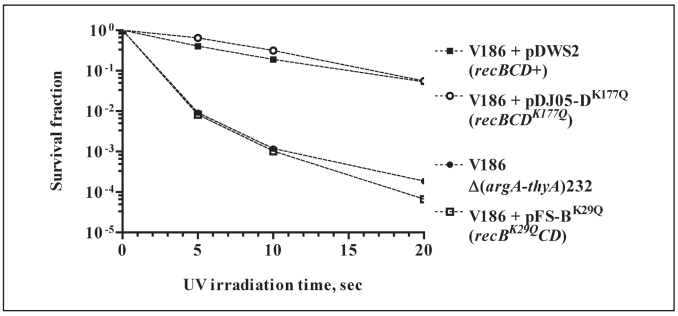

FIGURE 6. **RecBCD^{K177Q}, but not RecB^{K29Q}CD, enzyme can complement the UV radiation sensitivity of cells lacking RecBCD enzyme.** The efficiency of DNA repair was measured as survival of the *E. coli* cells after exposure to different doses of UV light (see the "Experimental Procedures" for details). All strains were transformants of *V186* (Δ(*argA-thyA*)232) with the indicated *recBCD* alleles present on plasmids. Survival was measured in a single experiment as a fraction of the initial colony-forming units after exposure to UV light for the indicated time.

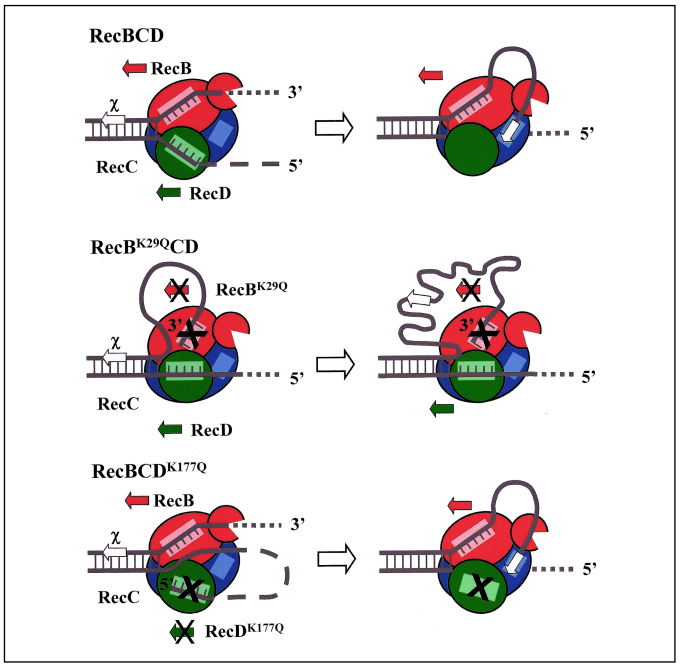

FIGURE 7. **Roles of the RecB and RecD motor subunits in RecBCD enzyme function.** The two helicase subunits of wild-type RecBCD enzyme, RecB and RecD, translocate on the 3′- and 5′-terminated strands, respectively, making both ssDNA strands available to the nuclease active site which is located where the ssDNA exits the enzyme. Binding of χ by the RecC subunit reduces nucleolytic degradation of the 3′-terminated strand but not the 5′-terminated strand. In the case of the RecB^{K29Q}CD enzyme, the 3′-terminated end is bound by the inactive RecB motor and is therefore protected from degradation by nuclease. On the other hand, the inability of the RecB^{K29Q} motor to translocate on the χ-containing strand results in its inability to deliver χ into the χ-recognition site on the RecC subunit. Similar to the wild-type enzyme, RecBCD^{K177Q} mutant translocates along 3′-terminated strand, recognizes χ, and undergoes χ-induced modification.

with a 5′→3′ polarity (8). We speculated that the dual motor organization contributes to the high translocation rate and processivity of the holoenzyme (8). However, the role of the two motor subunits in other activities of the RecBCD enzyme remained unclear. To address the involvement of RecB and RecD subunits in DNA unwinding, nuclease activity, χ-recognition, and RecA loading, we analyzed two RecBCD mutants, RecB^{K29Q}CD and RecBCD^{K177Q}, in which the respective ATP hydrolytic sites were disabled by site-directed mutagenesis. Although both mutant enzymes retain the ability to bind tightly to dsDNA ends, the rate and, in particular, the processivity of translocation are substantially reduced in both helicase mutants (43).

Here, we demonstrated that the activity of either the RecB or RecD motor is sufficient to maintain RecBCD-mediated translocation along and unwinding of dsDNA over a broad range of reaction conditions. However, both the DNA-dependent ATPase and helicase activities of the wild-type and the mutant enzymes were affected differently by changes in magnesium ion concentration, a variable that greatly affects all biochemical activities of RecBCD enzyme. The helicase activity of RecBCD^{K177Q} enzyme increased progressively as the free magnesium ion concentration increased. This behavior is consistent with the preference for high concentration of magnesium ion displayed by the purified RecB helicase (7). On the other hand, both the rate and processivity of dsDNA unwinding by RecB^{K29Q}CD mutant were optimal at lower concentrations of magnesium ion. These opposing dependences are intriguing. Assuming that these phenomena largely reflect the biochemical properties of the respective individual motor subunit, then it may suggest that these motors are "tuned" to provide translocation capability to the wild-type holoenzyme over a broad range of potential physiological conditions. The results also strongly imply that the designation of which motor subunit is the "fast" subunit and which is the "slow" subunit will depend on reaction conditions. Thus, a simple prediction of the results in Fig. 3 is that the RecD motor would be the fast lead motor at low magnesium ion concentrations, whereas the RecB motor would be the fast lead motor at high magnesium ion concentrations; at intermediate concentrations (*e.g.* a low millimolar free magnesium ion concentration, which is the *in vivo* concentration (51)), their speeds would be comparable. Indeed, in agreement with this simple idea, the RecD subunit was shown to be the faster subunit when the free magnesium ion concentration was low (16).

With regard to interaction with χ, we found that the RecBCD^{K177Q} mutant recognized χ and loaded RecA protein onto the χ-terminated ssDNA produced by its helicase/nuclease activity. RecBCD^{K177Q} enzyme was also able to complement the DNA damage repair deficiency of *recBCD*Δ cells *in vivo* indicating that the mutation, which disables the RecD motor, does not interfere with multiple functions of RecBCD enzyme. The helicase activity of the RecB motor is clearly sufficient to deliver the χ-recognition subunit, RecC, to the χ sequence. In contrast, the RecB^{K29Q}CD enzyme failed to both respond to χ and to load RecA protein; its failure to complement the UV sensitivity of the *recBCD*Δ cells corroborated the *in vitro* findings. Therefore, although either motor subunit can sustain RecBCD enzyme translocation, the RecB motor is essential for RecBCD enzyme function in homologous recombination. Translocation by the RecD subunit, on the other hand, is not essential for recombinational repair function; however, as explained below, the RecD subunit is an important component of the regulatory response to χ (Fig. 7).

Our finding that the RecBCD^{K177Q} mutant maintains all of the activities, albeit somewhat reduced, of the wild-type enzyme shows that the role of the RecD subunit in the RecBCD complex does not require its ATPase or translocation activities. The RecD subunit does, however, contribute important capabilities to the holoenzyme. As a structural element of the complex, the RecD subunit increases the affinity of RecBC enzyme for the dsDNA ends (52). In addition, RecD translocation is required for increased translocation rate and processivity of the holoenzyme (43). Furthermore, our work also makes it clear that inactivation of the RecD motor is different from its complete deletion. Similar to the wild-type enzyme, the RecBCD^{K177Q} mutant recognizes χ, responds to χ, and loads RecA protein in a χ-dependent manner, whereas RecBC enzyme is a constitutive RecA loader that does not recognize χ. This comparison indicates that RecD plays an indispensable structural role in χ-recognition and in the χ-dependent regulatory phenomena, but that this role does not require translocation by the

RecD motor (Fig. 7, *A* and *C*). Thus, from a regulatory perspective, the RecD subunit is essential, and its translocation activity is likely maintained evolutionarily to enable the enhanced processivity of the heterotrimeric wild-type enzyme.

In contrast to the wild-type and RecBCD^{K177Q} enzymes that degrade both strands of the unwound DNA, RecB^{K29Q}CD nuclease acted predominantly on the 5′-terminated strand. A recent electron microscopy study (16) demonstrated that during the course of DNA unwinding, the RecB subunit of RecB^{K29Q}CD enzyme remained bound to the 3′-terminal ssDNA at the entry site (Fig. 7*B*). This finding can explain our observation that the RecB^{K29Q}CD enzyme exerts its nuclease activity predominantly on the 5′-terminated strand. An important implication of this observation is that a nuclease switch can be achieved simply by preventing translocation of the enzyme on 3′-terminated strand. This observation also agrees well with the model for switching of the nuclease activity upon χ-recognition proposed based on the crystal structure of the RecBCD enzyme (11). According to this model, reduction of the nucleolytic degradation of the 3′-terminated strand after χ-recognition results from the tight binding of χ (which is at the terminus of this 3′-strand) to the RecC subunit, both preventing its entry into the active site in RecB, and simultaneously permitting access of the 5′-terminated strand to that same site.

Interestingly, behavior similar to that of RecB^{K29Q}CD enzyme was observed for the RecB^{2109}CD mutant enzyme (44, 46, 53), which is completely defective for genetic recombination *in vivo* (54). Biochemical characterization of the RecB^{2109}CD enzyme revealed that it retained most of the biochemical functions associated with the wild-type RecBCD enzyme. The mutant enzyme is a processive DNA helicase; however, its processivity and unwinding rate are reduced compared with that of the wild-type enzyme (53). RecB^{2109}CD enzyme possesses nucleolytic activity that, similar to the RecB^{K29Q}CD mutant, is exerted primarily on the 5′-terminated strand and is not attenuated at χ (44, 46). Also like the RecB^{K29Q}CD mutant, RecB^{2109}CD enzyme does not facilitate loading of the RecA protein onto the 3′-terminated ssDNA it produces. The mutation in RecB^{2109} protein is a change of threonine 807 to isoleucine (46). This residue is strictly conserved in helicases, and it is within helicase motif VI (55). This motif is one of the seven motifs conserved among Superfamily I DNA helicases, which include RecB and RecD. The structures of Superfamily I helicases suggest that residues in motif VI are responsible for transducing conformational changes between the nucleotide- and DNA-binding regions of these proteins (56, 57). The mutated threonine residue in RecB protein interacts with residues in helicase motif III, which are involved in ssDNA binding (11, 58). It is most likely, therefore, that RecB motor activity is disabled in the RecB^{2109}CD enzyme, resulting in a phenocopy of the RecB^{K29Q}CD enzyme. We believe that in both mutants the inactive RecB motor remains bound to the end of the 3′-terminated strand. Sequestration of the 3′-terminated strand, on one hand, protects it from degradation but, on the other hand, prevents channeling of this strand into the χ-recognition site of RecC subunit (Fig. 7*B*).

Binding of the χ-terminated 3′-end by RecC subunit may take place upon χ-recognition resulting in the switch in the nuclease activity (11, 23). The inability of the RecB motor subunit to translocate along the 3′-terminated strand would also explain the failure of RecB^{K29Q}CD enzyme (as well as of the RecB^{2109}CD mutant) both to recognize the χ sequence that is imbedded in this strand and to load RecA protein. Therefore, translocation along the 3′-terminated strand by RecB subunit is needed to deliver the RecC subunit to χ, a function that is essential for the χ-regulated performance of RecBCD enzyme in homologous recombination.

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REFERENCES

1. Myers, R. S., and Stahl, F. W. (1994) *Annu. Rev. Genet.* **28,** 49–70
2. Amundsen, S. K., Taylor, A. F., Chaudhury, A. M., and Smith, G. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5558–5562
3. Arnold, D. A., and Kowalczykowski, S. C. (1999) *Encyclopedia of Life Sciences*, pp. 55–60, Nature Publishing Group, London
4. Spies, M., and Kowalczykowski, S. C. (2005) *The Bacterial Chromosome*, pp. 389–403, ASM Press, Washington, D. C.
5. Kowalczykowski, S. C. (2000) *Trends Biochem. Sci.* **25,** 156–165
6. Boehmer, P. E., and Emmerson, P. T. (1992) *J. Biol. Chem.* **267,** 4981–4987
7. Chen, H. W., Ruan, B., Yu, M., Wang, J., and Julin, D. A. (1997) *J. Biol. Chem.* **272,** 10072–10079
8. Dillingham, M. S., Spies, M., and Kowalczykowski, S. C. (2003) *Nature* **423,** 893–897
9. Farah, J. A., and Smith, G. R. (1997) *J. Mol. Biol.* **272,** 699–715
10. Ganesan, S., and Smith, G. R. (1993) *J. Mol. Biol.* **229,** 67–78
11. Singleton, M. R., Dillingham, M. S., Gaudier, M., Kowalczykowski, S. C., and Wigley, D. B. (2004) *Nature* **432,** 187–193
12. Roman, L. J., Eggleston, A. K., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267,** 4207–4214
13. Bianco, P. R., Brewer, L. R., Corzett, M., Balhorn, R., Yeh, Y., Kowalczykowski, S. C., and Baskin, R. J. (2001) *Nature* (*London*) **409,** 374–378
14. Taylor, A. F., and Smith, G. R. (1995) *J. Biol. Chem.* **270,** 24451–24458
15. Eggleston, A. K., O'Neill, T. E., Bradbury, E. M., and Kowalczykowski, S. C. (1995) *J. Biol. Chem.* **270,** 2024–2031
16. Taylor, A. F., and Smith, G. R. (2003) *Nature* **423,** 889–893
17. Dixon, D. A., and Kowalczykowski, S. C. (1991) *Cell* **66,** 361–371
18. Dixon, D. A., and Kowalczykowski, S. C. (1993) *Cell* **73,** 87–96
19. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Genes Dev.* **11,** 571–581
20. Lam, S. T., Stahl, M. M., McMilin, K. D., and Stahl, F. W. (1974) *Genetics* **77,** 425–433
21. Taylor, A. F., Schultz, D. W., Ponticelli, A. S., and Smith, G. R. (1985) *Cell* **41,** 153–163
22. Bianco, P. R., and Kowalczykowski, S. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6706–6711
23. Spies, M., Bianco, P. R., Dillingham, M. S., Handa, N., Baskin, R. J., and Kowalczykowski, S. C. (2003) *Cell* **114,** 647–654
24. Taylor, A. F., and Smith, G. R. (1995) *J. Biol. Chem.* **270,** 24459–24467
25. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Cell* **90,** 77–86
26. Handa, N., Ohashi, S., Kusano, K., and Kobayashi, I. (1997) *Genes Cells* **2,** 525–536
27. Arnold, D. A., Handa, N., Kobayashi, I., and Kowalczykowski, S. C. (2000) *J. Mol. Biol.* **300,** 469–479
28. Chaudhury, A. M., and Smith, G. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7850–7854
29. Churchill, J. J., Anderson, D. G., and Kowalczykowski, S. C. (1999) *Genes Dev.* **13,** 901–911
30. Korangy, F., and Julin, D. A. (1993) *Biochemistry* **32,** 4873–4880
31. Hsieh, S., and Julin, D. A. (1992) *Nucleic Acids Res.* **20,** 5647–5653
32. Korangy, F., and Julin, D. A. (1992) *J. Biol. Chem.* **267,** 1727–1732
33. Anderson, D. G., Churchill, J. J., and Kowalczykowski, S. C. (1999) *J. Biol. Chem.* **274,** 27139–27144
34. Mirshad, J. K., and Kowalczykowski, S. C. (2003) *Biochemistry* **42,** 5945–5954
35. Griffith, J., and Shores, C. G. (1985) *Biochemistry* **24,** 158–162
36. LeBowitz, J. (1985) *Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication*, Johns Hopkins University, Baltimore, MD
37. Kowalczykowski, S. C., Clow, J. C., Somani, R., and Varghese, A. (1987) *J. Mol. Biol.* **193,** 81–95
38. Kreuzer, K. N., and Jongeneel, C. V. (1983) *Methods Enzymol.* **100,** 144–160
39. Arnold, D. A., Bianco, P. R., and Kowalczykowski, S. C. (1998) *J. Biol. Chem.* **273,** 16476–16486
40. Eggleston, A. K., Rahim, N. A., and Kowalczykowski, S. C. (1996) *Nucleic Acids Res.* **24,** 1179–1186
41. Chaudhury, A. M., and Smith, G. R. (1984) *J. Bacteriol.* **160,** 788–791
42. Ponticelli, A. S., Schultz, D. W., Taylor, A. F., and Smith, G. R. (1985) *Cell* **41,** 145–151
43. Dillingham, M. S., Web, M. R., and Kowalczykowski, S. C. (2005) *J. Biol. Chem.* **280,** 37069–37077
44. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 605–620
45. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2863–2873
46. Arnold, D. A., and Kowalczykowski, S. C. (2000) *J. Biol. Chem.* **275,** 12261–12265

47. Dixon, D. A., and Kowalczykowski, S. C. (1995) *J. Biol. Chem.* **270,** 16360–16370
48. Dixon, D. A., Churchill, J. J., and Kowalczykowski, S. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2980–2984
49. de Vries, J., and Wackernagel, W. (1992) *J. Gen. Microbiol.* **138,** 31–38
50. Amundsen, S. K., Taylor, A. F., and Smith, G. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7399–7404
51. Alatossava, T., Jutte, H., Kuhn, A., and Kellenberger, E. (1985) *J. Bacteriol.* **162,** 413–419
52. Korangy, F., and Julin, D. A. (1994) *Biochemistry* **33,** 9552–9560
53. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 621–633
54. Amundsen, S. K., Neiman, A. M., Thibodeaux, S. M., and Smith, G. R. (1990) *Genetics* **126,** 25–40
55. Gorbalenya, A. E., and Koonin, E. V. (1993) *Curr. Opin. Struct. Biol.* **3,** 419–429
56. Hall, M. C., Ozsoy, A. Z., and Matson, S. W. (1998) *J. Mol. Biol.* **277,** 257–271
57. Soultanas, P., Dillingham, M. S., Velankar, S. S., and Wigley, D. B. (1999) *J. Mol. Biol.* **290,** 137–148
58. Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) *Cell* **90,** 635–647