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The Reduced Levels of χ Recognition Exhibited by the RecBC^{1004}D Enzyme Reflect Its Recombination Defect *in Vivo**

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Homologous recombination in *Escherichia coli* is initiated by the RecBCD enzyme and is stimulated by an 8-nucleotide element known as Chi (χ). We present a detailed biochemical characterization of a mutant RecBCD enzyme, designated RecBC^{1004}D, that displays a reduced level of χ site recognition. Initially characterized genetically as unable to respond to the χ sequence, we provide evidence to indicate that the ability of this mutant enzyme to respond to χ is reduced rather than lost; the mutant displays about 20-fold lower χ recognition than wild-type RecBCD enzyme. Although this enzyme exhibits wild-type levels of double-stranded DNA exonuclease, helicase, and ATPase activity, its ability to degrade single-stranded DNA is enhanced 2–3-fold. The data presented here suggest that the reduced recombination proficiency of the *recBC^{1004}D* strain observed *in vivo* results from a basal level of modification of the RecBC^{1004}D enzyme at both χ-specific, as well as nonspecific, DNA sequences.

The RecBCD enzyme (exonuclease V) is a 330-kDa protein composed of three subunits, the products of the *recB*, *recC*, and *recD* genes (1, 2). The RecBCD holoenzyme is an ATP-dependent DNA helicase that possesses nuclease activities on both dsDNA[1] and ssDNA and plays a central role in the primary recombination pathway of *Escherichia coli*, the RecBCD pathway (3–5). Null mutations in either the *recB* or *recC* genes reduce conjugational and transductional recombination 10^2- to 10^3-fold, reduce cell viability, and decrease resistance to damage by UV irradiation (2, 6, 7). Also essential to this pathway are the activities of the RecA and SSB proteins (8, 9).

Recombination hotspots in *E. coli*, known as Chi sites (χ = 5′-GCTGGTGG-3′) enhance the frequency of recombination in their vicinity 5–10-fold (10–12). This stimulation is dependent on the RecBCD enzyme and is not observed in strains lacking a functional RecBCD enzyme. The mechanism by which χ promotes recombination is through a direct interaction with the RecBCD enzyme that results in both an attenuation of the 3′- to 5′-nuclease activity on the strand possessing the χ sequence, as well as a stimulation of the 5′- to 3′-nuclease activity responsible for degrading the opposite strand of the duplex (13–17).

RecBCD-mediated recombination begins with the RecBCD enzyme binding a blunt, or nearly blunt, dsDNA end; the enzyme proceeds to unwind the substrate while vigorously degrading the 3′-terminal strand (16, 18, 19). Upon recognizing a χ site within the DNA substrate, the nuclease activity on the 3′-terminal strand is attenuated approximately 500-fold, resulting in a 3′-strand terminating 4–6 bp upstream of the χ sequence (16, 18, 20). In addition, a weaker 5′- to 3′-exonuclease activity is stimulated that, upon subsequent unwinding, serves to degrade the 5′-terminal strand downstream of χ (16, 17). This modification of nuclease activities results in the production of a 3′-terminal ssDNA, a suitable substrate for strand invasion mediated by RecA protein. In this way, the χ site serves as a regulatory switch to modify a highly destructive enzyme so that it serves as a critical protein in recombination.

The recognition of χ by RecBCD enzyme is contingent upon the orientation of χ within the dsDNA molecule, such that recognition by the translocating RecBCD enzyme occurs only when the enzyme approaches the sequence from the 3′ direction during unwinding of a duplex substrate (Fig. 1). *In vitro*, χ recognition is observed as the production of two χ-specific fragments, the downstream (top strand) fragment and the upstream (bottom strand) fragment. The amount of each fragment produced is dependent on the efficiency of χ recognition. Thus, χ-specific fragment production provides a measure for the efficiency of χ recognition by the RecBCD enzyme.

Reversible inactivation is another χ-specific modification of RecBCD enzyme observed *in vitro* (21). χ-Dependent inactivation of RecBCD enzyme has been observed *in vivo* as well (22, 23). Upon recognition of χ, RecBCD enzyme undergoes the aforementioned nuclease modifications that persist until the enzyme dissociates from the DNA molecule. Typically, upon dissociation, the enzyme reverts to its "pre-χ" state and is able to reinitiate unwinding and degradation on subsequent duplex DNA substrates. However, when the amount of free magnesium ion in solution is limiting, as is the case when the magnesium ion concentration is less than the ATP concentration, this reversion and reinitiation is inhibited. The catalytic nature of the enzyme is restored by addition of magnesium ion to these arrested reactions, thus the term "reversible inactivation." This assay provides another measure of χ recognition, and more importantly, this assay provides a potential means of separating χ recognition from the modification of nuclease activity at χ that is required to detect χ-specific ssDNA fragments.

Given the pivotal role that χ recognition plays in the conversion of RecBCD into a recombination-proficient enzyme, a mutant enzyme that lacks χ recognition but retains recombination function is potentially very informative. To identify such mutants, a screen was developed to isolate *recBCD* mutants that lacked χ recombinational hotspot activity but maintained

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[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSB, *Escherichia coli* single-stranded DNA-binding protein; DTT, dithiothreitol; PEP, phosphoenolpyruvate; [^{3}H]SAM, *S*-[*methyl*-^{3}H]adenosyl-L-methionine; χ^0, non-χ-containing; χ^+, χ-containing; bp, base pair(s); AU, absorbance units.

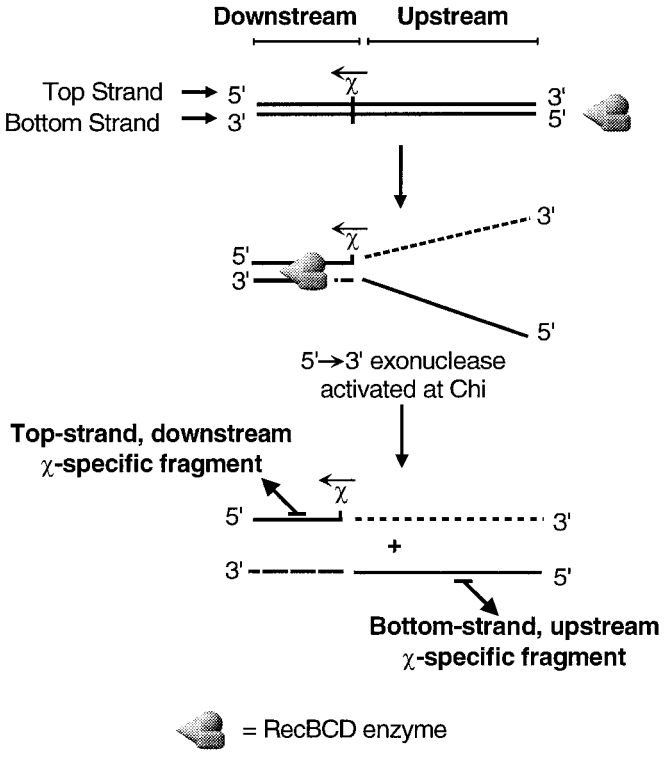

FIG. 1. **Model for RecBCD enzyme processing of a linear duplex containing a χ site.** Upon encountering a properly oriented χ site, the preferred polarity of degradation of the RecBCD enzyme is reversed, thereby preserving a 3′ ssDNA tail containing the χ site and resulting in the production of the top strand, downstream χ-specific fragment and the bottom strand, upstream χ-specific fragment. This process is further described in the text.

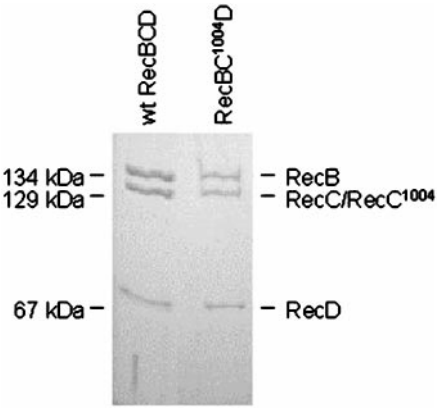

FIG. 2. **SDS-polyacrylamide gel analysis of wild-type RecBCD and mutant RecBC^{1004}D enzyme preparations.** Enzyme samples (2 μg for wild-type RecBCD and 0.5 μg for RecBC^{1004}D enzyme) were separated on an 8% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The individual subunits and their molecular masses are indicated at the *right* and *left*, respectively.

nearly wild-type levels of recombination (24). From this screen, the C* class of mutants was identified. These mutants represent pseudorevertants of the recombinationally deficient *recC*73 null mutant that partially restore recombination by the *recBCD* pathway (24). The mutations responsible for this phenotype map to the *recC* gene for all C* mutants: *recC*^{1001}, *recC*^{1002}, *recC*^{1003}, and *recC*^{1004}. The RecBC*D enzymes lack χ recombinational hotspot activity; however, they retain nearly wild-type levels of nuclease activity (24). Thus, the enigma of this mutant class hinges on their recombination proficiency in the absence of detectable levels of χ stimulation.

To investigate the way in which these mutants promote recombination, we purified and characterized one member of the C* class, the RecBC^{1004}D enzyme. Of all the C* mutants examined, the strain bearing the *recBC*^{1004}*D* mutation possessed levels of χ-independent recombination most like that of the wild-type strain (24). Thus, in the interest of defining activities that the enzyme might have gained in lieu of χ activation, allowing it this basal level of recombination proficiency, we selected this mutant for further study. Our results show that this enzyme retains wild-type levels of dsDNA exonuclease, ATPase, and helicase activities, and enhanced levels of ssDNA nuclease activities. Furthermore, although *in vivo* data strongly imply a complete absence of χ recognition (24, 25), our experiments reveal that the RecBC^{1004}D enzyme retains the ability to recognize and respond to χ *in vitro*, although at one-twentieth the efficiency of the wild-type enzyme, and also suggest that it may respond to weakly acting non-canonical "χ-like" sequences *in vivo*.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers—All solutions were made using Barnstead NANOpure water and reagent-grade chemicals. ATP was purchased from Amersham Pharmacia Biotech; PEP, β-nicotinamide adenine dinucleotide (NADH), pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs and U. S. Biochemical Corp. Inc. and used as described by the specific vendor. [^{3}H]SAM, [γ-^{32}P]ATP, and [α-^{32}P]ATP were purchased from NEN Life Science Products. Proteinase K was purchased from Boehringer Mannheim.

Strains—E. coli strains expressing wild-type (V320; Δ(*argA-thyA*)323, IN(*rrnD-rrnE*)1, pDWS103), and mutant enzyme (V194; Δ(*argA-thyA*)323, IN(*rrnD-rrnE*)1, pDWS2 derivative) were generously provided by A. F. Taylor, S. K. Amundsen, and G. R. Smith (Hutchinson Cancer Research Center, Seattle, WA). These strains bear a chromosomal deletion from *thyA* to *argA* (which includes the *recB, recC,* and *recD* genes) and contain plasmid derivatives encoding the RecBCD or RecBC^{1004}D enzymes (24, 26). The expression plasmids are under runaway replication control and carry chloramphenicol (*recBCD*) or ampicillin (*recBC*^{1004}*D*) antibiotic markers.

Protein Isolation—The RecBC^{1004}D enzyme was purified by the method of Eggleston and Kowalczykowski (27) with modifications (28). Ten liters of V194 cells were lysed by treatment with 5 mg/ml lysozyme, 50 mM EDTA, and 1% Brij 58. Subsequent chromatographic separations via Q-Sepharose, hydroxylapatite, ssDNA cellulose, and Mono Q columns were performed; the elution profiles for the mutant enzyme were similar to those previously observed for wild-type RecBCD enzyme, indicating that the *recC*^{1004} mutation did not alter the chromatographic properties of the mutant enzyme (data not shown). The *recC*^{1004} mutation does not appear to affect the yield of the RecC subunit, since all three subunits are present in approximately stoichiometric amounts (Fig. 2). The purified protein was stored at −80 °C in 20 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 0.1 mM EDTA, 0.1 mM DTT, and 45% glycerol, final. The final yield was 0.82 mg of RecBC^{1004}D enzyme with a greater than 930-fold purification.

Protein concentrations were determined spectrophotometrically using an extinction coefficient of 4.0×10^5 M^{-1} cm^{-1} at 280 nm. The specific activity of the mutant enzyme preparation was determined to be 3.8×10^5 nuclease units/mg of total protein, measured as described by Eichler and Lehman (3). This is comparable to wild-type RecBCD enzyme for which a specific activity of 2.4×10^5 units/mg of protein was measured. Functional enzyme concentration was determined by performing a protein titration in the fluorometric helicase assay (see below); the observed stoichiometry for the mutant enzyme preparation was determined to be 2.2 RecBC^{1004}D heterotrimers per dsDNA end, indicating that the enzyme preparation is 45% active (Table I). RecBCD and RecBC^{1004}D enzyme were diluted in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 0.1 mM EDTA, and 0.1 mM DTT immediately prior to use.

RecA protein was purified from *E. coli* strain JC12772, generously provided by Dr. A. John Clark, using a protocol based on spermidine acetate precipitation (29). The concentration of RecA protein was determined using a molar extinction coefficient of 2.7×10^4 M^{-1} cm^{-1}. SSB protein was purified from *E. coli* strain RLM727 as described (30) and concentration determined using $\epsilon = 3.0 \times 10^4$ M^{-1} cm^{-1}.

DNA Substrates—The DNA substrate used for the dsDNA exonucle-

TABLE I
Biochemical activities of RecBC^{1004}D enzyme

Assays were performed as described under "Experimental Procedures" for both the wild-type and mutant enzymes in parallel trials.

Enzyme	dsDNA exonuclease	Helicase		ATPase	
	Specific activity[a]	Apparent stoichiometry (per dsDNA end)[b]	$k_{cat,corr}$[c] (±50)	Specific activity[d]	$k_{cat,corr}$[e] (± 100)
	units/mg		s^{-1}	*units/mg*	s^{-1}
RecBCD	2.4×10^5	3.4 ± 0.5	242	1.1×10^5	616
RecBC^{1004}D	3.8×10^5	2.2 ± 0.5	226	1.1×10^5	497

[a] One unit digests 1 nmol of DNA in 20 min at 37 °C in buffer containing 50 mM Tris acetate (pH 7.5), 50 μM ATP, 10 mM magnesium acetate, 0.67 mM DTT, and 20 μM nucleotides of tritiated, linearized plasmid DNA (3).

[b] The apparent stoichiometry is the experimentally observed number of enzyme molecules required to saturate helicase activity at a fixed DNA concentration (2.3 nM dsDNA ends).

[c] The corrected k_{cat} ($k_{cat,corr}$) is the experimentally determined rate of unwinding in base pairs per s per functional enzyme concentration as determined by saturation of helicase activity.

[d] The specific activity for ATPase activity is the number of units per milligrams of enzyme. One ATPase unit hydrolyzes 1 nmol of ATP per min under standard conditions (35).

[e] The corrected k_{cat} values for the hydrolysis of ATP are the observed rate of ATP hydrolysis (ATP per s per total enzyme concentration) multiplied by the apparent stoichiometry as determined by the helicase assays.

ase assays was a linear pYES2 derivative (~14 kilobase pairs total length), uniformly labeled using 55 μCi of [^{3}H]SAM (NEN Life Science Products) and 8 units of *Sss*I methylase (New England Biolabs); the labeling reaction was performed at 37 °C for 1 h in a buffer containing 200 mM Tris acetate (pH 8), 50 mM sodium chloride, and 1 mM DTT. Circular M13 ssDNA, the substrate for the ssDNA nuclease assay, was isolated using the method of Messing (31). The purification of pBR322 (χ-free, χ^0) and pNH92 (χ-containing, χ^+) was based on previously published alkaline lysis and cesium chloride gradient procedures (32, 33). pNH92 is a pBR322 derivative in which the *Bam*HI-*Hin*dIII fragment was replaced with a fragment containing a χ site (a gift from N. Handa, S. Ohashi, K. Kusano, and I. Kobayashi (University of Tokyo, Japan)) (25).

The χ^0 substrate (414 bp) used for reversible inactivation assays was amplified from pBR322 using primers PB08 (5′-TCACTGGTCCCGCCAC-CAAA-3′) and PB13 (5′-AGGTGCCGCCGGCTTCCATT-3′). The χ^+ substrate (438 bp) was amplified from pNH92 using primers SKDH400U (5′-CCACCTGACGTCTAAGAAAC-3′) and SKDH400L (5′-CTGACTG-GGTTGAAGGCTCT-3′). After amplification, the polymerase chain reaction products were purified in a 2% MetaPhor gel (FMC Bioproducts) and recovered from the gel by electroelution. The concentration in nucleotides for all DNA preparations was determined using the molar extinction coefficients of 6500 and 8784 M^{-1} cm^{-1} at 260 nm for dsDNA and ssDNA, respectively. All concentrations of DNA are given in nucleotides, unless otherwise indicated.

Plasmid DNAs were linearized with appropriate restriction enzymes in the presence of shrimp alkaline phosphatase and end-labeled either at the 5′-end by T4 polynucleotide kinase and [γ-^{32}P]ATP or at the 3′-end using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. Subsequent purification of labeled DNA was accomplished by passage through a MicroSpin S-200 HR column (Amersham Pharmacia Biotech) or an ELUTIP mini-column (Schleicher & Schuell).

dsDNA Exonuclease Assay—The exonuclease activity on dsDNA was monitored using a modified trichloroacetic acid solubility assay (3). Reactions (100 μl) consisted of 50 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 0.67 mM DTT, 50 μM ATP, and 20 μM tritiated, linearized plasmid DNA (see above) and were initiated by addition of RecBCD or RecBC^{1004}D enzyme. Aliquots (20 μl) were taken at 20-s intervals and treated as described previously (3). The rate of dsDNA nuclease activity (picomoles nucleotides released per min) was determined by multiplying the number of picomoles of nucleotides per reaction by the percentage of trichloroacetic acid-soluble counts produced per min. The specific activity was then determined by dividing the rate by the number of milligrams of total protein present in the reaction.

Polarity of dsDNA Exonuclease—The polarity of dsDNA exonuclease activity was examined using a method previously described (16). This protocol involved examination of the products of degradation observed under varying concentrations of RecBC^{1004}D enzyme; the substrate was *Eco*RI-linearized pBR322 dsDNA (χ^0) that was either 3′- or 5′-end-labeled. Enzyme was added to reactions lacking ATP over a range of concentrations from subsaturating (one functional enzyme per 5 dsDNA ends) to saturating (5 functional enzymes per 1 dsDNA end). Reactions were preincubated at 37 °C for 5 min to allow binding of wild-type and mutant enzymes to DNA ends and were initiated by addition of 1 mM ATP. Aliquots (40 μl) taken at 1, 2, and 4 min were stopped by addition of 10 μl of 5× loading buffer (2.5% SDS, 125 mM EDTA, 25% glycerol, 0.125% bromphenol blue, and 0.125% xylene cyanol, final) and 14 μg of proteinase K (Boehringer Mannheim). Samples were subjected to electrophoresis (600 V·h) in 1% (w/v) agarose gels which were subsequently dried and analyzed on a Betascope 603 blot analyzer, as well as by autoradiography. The assignment for half-length ssDNA products is based on previously published work (34).

ssDNA Nuclease Assay—The nuclease activities of the mutant enzyme on ssDNA were examined using a circular M13 ssDNA substrate as described previously (27). Aliquots were taken from the 37 °C reactions at the indicated times and separated on native 1% (w/v) agarose gels in 1× TAE buffer (40 mM Tris acetate, 2 mM EDTA) for 1100 V·h. The gels were stained with 0.5 mg/ml ethidium bromide for 30 min, and the bands were visualized by exposure to UV light. Photographs taken of the gels using a Gel Print 2000i gel documentation system (BioPhotonics) were analyzed on a Macintosh computer using the public domain program NIH Image version 1.52 (developed at the National Institutes of Health).[2] The degradation of circular ssDNA was normalized to the total amount of starting circular substrate. The degradation of linear ssDNA was calculated based on both the initial amount of ssDNA present and the amount of linear ssDNA produced by cleavage of circular M13.

Fluorometric Helicase Assay—Conversion of dsDNA to ssDNA was measured using a fluorometric helicase assay that monitors the quenching of the intrinsic fluorescence of SSB protein binding to ssDNA (5). The reaction mix, including 2 μM SSB protein and 10 μM *Eco*RI-linearized pBR322 dsDNA, was preincubated for 2 min at 23 °C before initiation with enzyme; the decrease in SSB fluorescence at 340 nm was continuously monitored, and the data were collected using a model 8100 Spectrofluorometer from Spectronic Instruments (SLM Aminco). The change in fluorescence signal expected for complete unwinding was determined by adding heat-denatured dsDNA to a standard reaction containing SSB protein (no wild-type or mutant RecBCD enzyme) and measuring the total fluorescence change. The rate of helicase activity was determined by multiplying the initial slope (% fluorescence change/s) by the total DNA concentration (nM, bp). The apparent k_{cat} was determined by dividing the rate by the enzyme concentration (nM). By using this assay, the functional stoichiometry of the mutant enzyme preparation was also determined as the number of enzyme molecules required to saturate helicase activity at a fixed dsDNA concentration of 2.3 nM ends.

ATPase Assay—Hydrolysis of ATP was measured using a coupled, spectrophotometric assay (35) with the following modifications. The dsDNA substrate used was pBR322, χ^0, present at a concentration of 30 μM nucleotides. The amounts of PEP and pyruvate kinase were increased to 3 mM and 30 units/ml, respectively, and the amounts of NADH and lactate dehydrogenase were increased to 200 μg/ml and 30 units/ml, respectively, to ensure that substrates for the coupled reactions would not become limiting. SSB protein was present in excess with a concentration 3-fold higher than would be required to saturate 30 μM nucleotides of ssDNA, assuming a site size of 15 nucleotides per monomer. The reactions were initiated by the addition of RecBCD or RecBC^{1004}D enzyme after preincubation at 25 °C and were monitored for 400 s. The rate of hydrolysis (μM ATP/min) was determined by multiplying the initial slope (change in absorbance units (AU) per s, ΔAU/s) of the time course by the conversion factor 9864 μM ATP·s·min^{-1}·AU^{-1}. The corrected k_{cat} values were determined by dividing this rate by the amount of functional enzyme (micromolar) in the reaction. The specific activity was determined by dividing the number of ATPase units (nanomoles of ATP hydrolyzed per min) in the reaction by the number of milligrams of total enzyme present. The efficiency of ATP hydrolysis was also calculated using data from the coupled ATPase assays by averaging the total change in absorbance during unwinding for both the wild-type and mutant enzymes, converting it to "μM ATP hydrolyzed" with the conversion factor 0.16 mM ATP hydrolyzed per AU and dividing this value by the concentration of base pairs in the reaction (15 μM) (35).

[2] Available on-line at the following address: http://rsb.info.nih.gov/nih-image/.

χ-Specific Fragment Production Assay—Standard reaction conditions consisted of 25 mM Tris acetate (pH 7.5), 2 mM magnesium acetate, 1 mM ATP, 1 mM DTT, 1 mM PEP, pyruvate kinase (4 units/ml), 2 μM SSB, and 10 μM nucleotides of *Ava*I-linearized, 5′-end-labeled dsDNA (χ^0 or χ^+). The resulting substrate contains an asymmetrically placed χ site located 1 kilobase pair from the end where RecBCD must enter to encounter χ in the correct orientation, resulting in downstream χ-specific fragments that are 3 kilobase pairs in length. To determine the optimal conditions for χ-specific fragment production, reactions (30 μl) were performed at various concentrations of magnesium acetate, ranging from 1 to 8 mM. The maximum production of downstream χ-specific fragments was observed at 2 mM magnesium acetate, so this concentration was used in all subsequent χ-specific fragment production assays. Reactions were incubated for 2 min at 37 °C before initiating with 0.10 nM functional RecBCD or RecBC^{1004}D enzyme, corresponding to one functional enzyme per 25 dsDNA ends (using the stoichiometry determined in the fluorescence helicase assay). Aliquots (30 μl) were removed from ongoing reactions and stopped by the addition of 10 μl of 5× loading buffer (see above) and 14 μg of proteinase K. The samples were subjected to electrophoresis in a 1% (w/v) agarose gel in 1× TAE buffer for 600 V·h, dried, exposed to PhosphorImager screens, and analyzed using a Molecular Dynamics STORM 840 PhosphorImager and ImageQuant software. The values for χ-specific fragment production efficiency were then normalized to the amount of starting duplex unwound at each time point and corrected for the amount of background radiation present in the lane immediately below the band of interest; since ssDNA has one-half the amount of label as dsDNA, and the enzyme has only a 50% chance of encountering χ in the correct orientation, this value was then multiplied by 4 to yield the reported efficiencies of χ recognition.

Reversible Inactivation Assays—The level of inactivation of the RecBCD and RecBC^{1004}D enzymes upon encountering a χ sequence at conditions of limiting magnesium was examined by gel assay (21). Initial reaction conditions contained 25 mM Tris acetate (pH 7.5), 1 mM magnesium acetate, 5 mM ATP, 1 mM DTT, 6.25 μM plasmid DNA (χ^+ or χ^0, 1.55 nM dsDNA ends), 1.25 μM SSB protein. After equilibration for 2 min at 37 °C, reactions were initiated by addition of 0.04 nM RecBCD or RecBC^{1004}D enzyme, and time points (30 μl) were taken as indicated. After 40 min, magnesium acetate was added to 10 mM final concentration, and the time course was continued for an additional 40 min. These experiments were also performed with 4.7 nM dsDNA ends of either plasmid DNA or ~400-bp polymerase chain reaction product (with and without a χ site), in the presence of 6.25 or 0.41 μM SSB protein, respectively. The 400-bp substrates are amplified regions of either pBR322 (414 bp; χ^0) or pNH92 (438 bp; χ^+). The time points for all reversible inactivation assays were treated and analyzed as described above for the χ-specific fragment production assays.

Joint Molecule Formation Assay—This assay monitors the ability of RecBCD (or RecBC^{1004}D) enzyme to stimulate the production of χ-dependent and χ-independent joint molecules by RecA protein (18, 34, 36). Standard reactions (200 μl) contained 25 mM Tris acetate (pH 7.5), 6 mM magnesium acetate, 3 mM ATP, 1 mM DTT, 1 mM PEP, 4 units/ml pyruvate kinase, 1 μM SSB protein, and 10 μM nucleotides of *Ava*I-linearized, 5′-end-labeled dsDNA (χ^+ or χ^0). In addition, 10 μM supercoiled plasmid (pNH92) and 5 μM RecA protein were included prior to preincubation at 37 °C. The reactions were initiated with 0.17 nM functional RecBCD or RecBC^{1004}D enzyme (one functional enzyme per 15 dsDNA ends). Aliquots were treated and analyzed as described above for the χ-specific fragment production assays.

RESULTS

The RecBC^{1004}D Enzyme Possesses Wild-type Levels of 3′ to 5′ dsDNA Exonuclease Activity—By using conditions that maximize the degradative activity of RecBCD enzyme on a tritiated duplex substrate (10 mM $MgCl_2$ and 50 μM ATP) (3), we detected similar levels of dsDNA exonuclease activity for RecBCD and RecBC^{1004}D enzymes. Initial rates measured for time course reactions were plotted against the functional wild-type and mutant enzyme concentrations (Fig. 3). The mutant enzyme has levels of dsDNA exonuclease comparable to that of the wild-type enzyme, with specific activities of 3.8×10^5 and 2.4×10^5 units/mg of total enzyme, respectively (Table I).

To determine if the asymmetric 3′ to 5′ preferential polarity of duplex degradation is retained by the mutant RecBC^{1004}D enzyme, we used the gel assay described previously (16). The

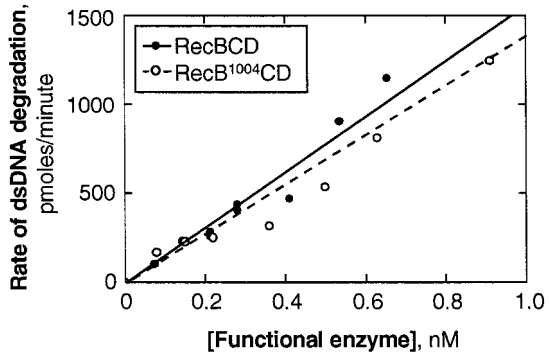

FIG. 3. **Double-stranded DNA exonuclease activity of RecBCD and RecBC^{1004}D enzymes.** The degradation of tritiated linear plasmid DNA was monitored by trichloroacetic acid solubility assay as described under "Experimental Procedures." The *graph* depicts initial rates, as picomoles of nucleotides released per min, from time course reactions plotted against functional enzyme concentration for both wild-type and mutant enzymes.

substrate was χ^0 dsDNA that had been either 3′- or 5′-end-labeled; this DNA was treated by concentrations of enzyme ranging from sub-saturating to saturating (Fig. 4). At sub-saturating concentrations of enzyme, each duplex molecule is acted upon by only one RecBCD enzyme, hence full-length ssDNA is produced regardless of which DNA strand is labeled. In contrast, at saturating concentrations, the duplex is simultaneously unwound and degraded from both ends. Therefore, if the 5′-ends are labeled, half-length ssDNA products are produced; alternatively, if 3′-ends are labeled, no intact ssDNA is observed since the 3′-ends are preferentially degraded. Fig. 4*A* shows that 5′-end-labeled, full-length ssDNA is produced at the sub-saturating ratios of functional RecBC^{1004}D enzyme per dsDNA end (1:5 and 1:3); at the intermediate ratios of 1:2 and 1:1, both full-length and half-length species are detectable; and finally, at greater enzyme concentrations, only half-length ssDNA is produced. In contrast, with the 3′-end-labeled substrate (Fig. 4*B*), only full-length ssDNA is seen at sub-saturating enzyme concentrations (1:5 to 1:2); when the concentration of enzyme is saturating (1:1 to 3:1), no discrete-sized species are present. Thus, we conclude the polarity bias for dsDNA exonuclease activity is not affected by the *recC*^{1004} mutation and remains, like the wild-type enzyme, a 3′ to 5′ polarity.

The RecBC^{1004}D Enzyme Exhibits Enhanced ssDNA Nuclease Activities—RecBCD enzyme possesses both ATP-dependent ssDNA exonuclease and ATP-stimulated ssDNA endonuclease activities that are far weaker than its dsDNA exonuclease activity. To determine if the *recC*^{1004} mutation affected these activities, both the ssDNA endo- and exonuclease activities were examined using an agarose gel assay; the results are shown in Fig. 5. The reactions were initiated with equimolar amounts of functional RecBCD or RecBC^{1004}D enzyme so the rates may be compared directly. It is clear from the gel in Fig. 5*A* that the DNA preparation contained a moderate amount (28%) of linearized substrate at the start of the reaction; the calculations were corrected accordingly, and the mean values for several independent time courses are plotted in Fig. 5*B*. Wild-type enzyme is able to degrade both circular and linear M13 ssDNA in the presence of 1 mM ATP at initial rates of 1.0% min^{-1} and 2.4% min^{-1}, respectively, with 50% of the circular and 20% of the linear substrate remaining after 90 min. In contrast, the activity of RecBC^{1004}D enzyme on these substrates is enhanced 2-fold on linear and 3-fold on circular M13 ssDNA (Fig. 5). These assays were also performed in the absence of ATP, and the ssDNA endonuclease activities observed for both enzymes are similarly reduced (data not shown). A saturating amount (5 μM) of SSB protein inhibits the ATP-de-

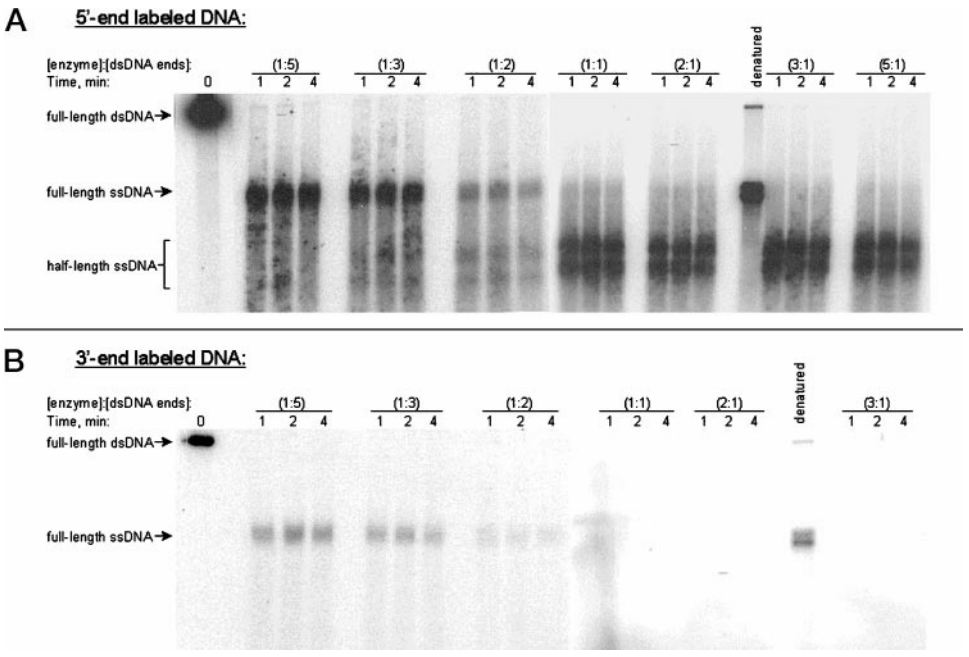

FIG. 4. **RecBC^{1004}D enzyme retains the wild-type polarity of degradation on duplex DNA.** The mutant enzyme was allowed to act on either a 5′- (*A*) or 3′ (*B*)-end-labeled duplex substrate at functional enzyme concentrations ranging from sub-saturating (0.46 nM) to saturating (11.5 nM), with respect to 2.3 nM dsDNA ends present in the reaction. The reactions were initiated by addition of 1 mM ATP, and aliquots taken at 1, 2, and 4 min were separated on a 1% agarose gel and visualized by autoradiography as described under "Experimental Procedures."

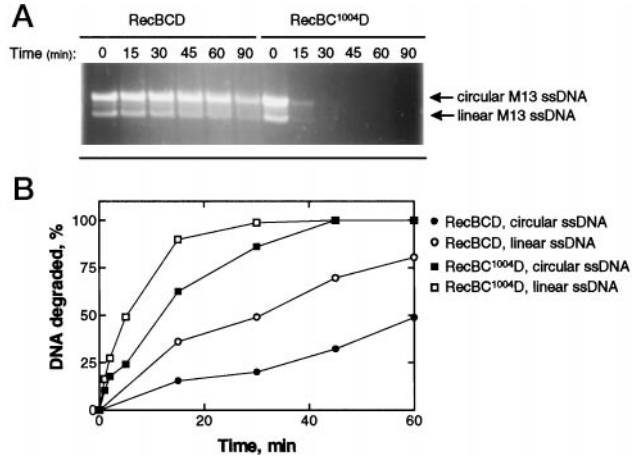

FIG. 5. **RecBC^{1004}D enzyme possesses enhanced ssDNA endo- and exonuclease activities.** Circular M13 ssDNA was subjected to degradation by 0.5 nM functional RecBCD or RecBC^{1004}D enzyme in the presence of 1 mM ATP as described under "Experimental Procedures." *A*, a gel showing the time course of reaction for both wild-type and mutant enzymes. *B*, the combined analyses of four independent assays are shown for the wild-type enzyme and mutant enzyme.

pendent ssDNA nuclease activities of both wild-type and mutant enzyme, presumably by coating the substrate and thereby blocking the binding of RecBCD or RecBC^{1004}D enzyme (data not shown). Thus, the first significant difference found for the mutant enzyme *in vitro* is a modest enhancement of ssDNA nuclease activity compared with wild-type.

The RecBC^{1004}D Enzyme Retains Helicase Activity Comparable with That of RecBCD Enzyme—The ability of the mutant enzyme to unwind duplex DNA was measured using a fluorescent helicase assay which monitors the decrease in intrinsic fluorescence of SSB protein upon binding to ssDNA produced by the helicase activity of RecBCD enzyme (5). A protein titration was performed to determine the fraction of active enzyme in the mutant preparation. Under the conditions used, the rate of unwinding increases with increasing enzyme concentration until saturation, when the amount of active enzyme equals the number of dsDNA ends in the reaction. As shown in Fig. 6*A*, saturation for the RecBC^{1004}D enzyme occurs at 5 nM total protein, corresponding to a stoichiometry of 2.2 enzyme molecules per dsDNA end (45% active) (Table I). To compare the helicase activity of the mutant enzyme to that of wild-type RecBCD enzyme, these assays were also performed over a range of sub-saturating enzyme concentrations, and initial rates were calculated and graphed against the amounts of functional enzyme present in each assay (Fig. 6*B*). These data clearly demonstrate that the helicase activity of the mutant enzyme parallels that of the wild-type enzyme, and the calculated rates of duplex DNA unwinding by wild-type and mutant enzyme were identical within experimental error, producing corrected k_{cat} values of 242 s^{-1} and 226 s^{-1} for the wild-type and mutant enzymes, respectively (Table I and Fig. 6).

The recC^{1004} Mutation Does Not Alter the ATPase Activity of RecBC^{1004}D Enzyme—The dsDNA-dependent ATPase activity was monitored using a coupled, spectrophotometric assay that measures the decrease in absorbance of the reaction as NADH is converted to NAD^+. The ATPase activity of RecBC^{1004}D enzyme was tested using pBR322 (χ^0) as the dsDNA substrate, and reactions with RecBCD enzyme were performed in parallel. The results indicate that the ATPase activity of the mutant enzyme is similar to that of the wild-type enzyme. The specific activities for both RecBCD and RecBC^{1004}D enzymes were 1.1×10^5 units/mg of total enzyme (Table I). The corrected k_{cat} values, normalized to the amount of functional enzyme concentration, are 616 s^{-1} for the wild-type enzyme and 497 s^{-1} for the mutant enzyme, essentially equivalent values (Table I). The efficiency of ATP hydrolysis during unwinding was determined to be 2–3 ATP molecules hydrolyzed per base pair of DNA unwound for both the wild-type and mutant enzymes in these assays; these values agree with previously published data (35). Thus, the *recC^{1004}* mutation does not significantly alter the *in vitro* activity of the RecBC^{1004}D enzyme with respect to dsDNA unwinding or ATP hydrolysis.

The RecBC^{1004}D Enzyme Processes dsDNA to Produce Reduced, but Detectable, Levels of χ-Specific Fragments in Vitro—In vivo, the RecBC^{1004}D enzyme is unable to promote

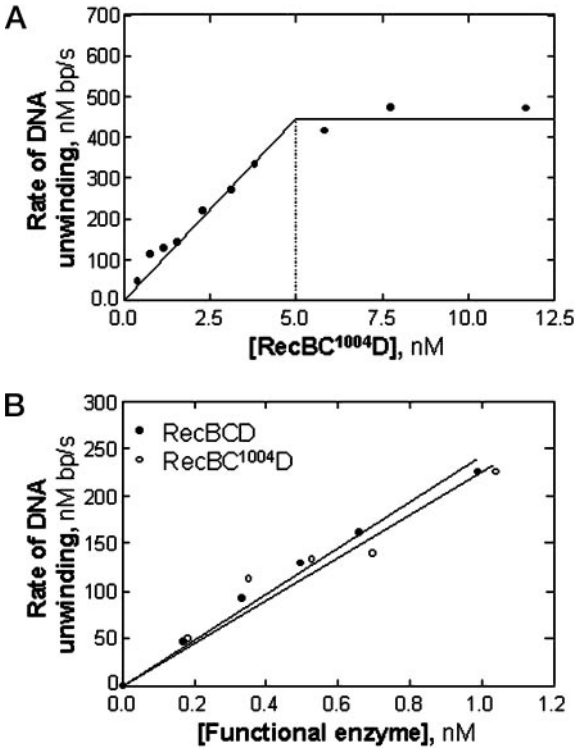

FIG. 6. **The RecBC^{1004}D enzyme displays wild-type levels of DNA helicase activity.** The ability of RecBC^{1004}D enzyme to unwind a linearized plasmid substrate was monitored using a fluorometric helicase assay as described under "Experimental Procedures." *A*, the amount of active enzyme in the preparation is determined by plotting the rate of unwinding against total enzyme concentration. *B*, the rates of unwinding by wild-type and mutant enzyme are plotted against functional enzyme concentration.

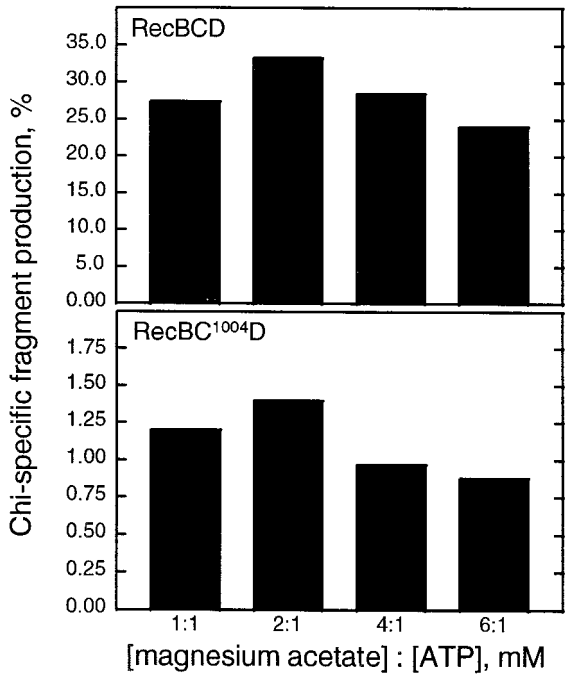

FIG. 7. **The optimal conditions for χ-specific fragment production by RecBC^{1004}D enzyme parallel those for the wild-type enzyme.** The ability of RecBC^{1004}D enzyme to produce χ-specific fragments was tested over a range of conditions varying the concentration of magnesium acetate as described under "Experimental Procedures." The *bar graphs* depict the mean values for χ-specific fragment production at the various reaction conditions. The concentrations of magnesium acetate and ATP are given in millimolar.

χ-stimulated recombination in response to the canonical χ sequence (5′-GCTGGTGG-3′) (24). Specifically, in phage lambda vegetative crosses, the mutant enzyme displayed wild-type levels of χ-independent recombination, yet showed no stimulation of that basal level in the presence of χ. These results suggested that the enzyme had lost the ability to recognize or be regulated by the χ recombination hotspot. *In vitro*, the ability of RecBCD enzyme to recognize χ can be observed using end-labeled, dsDNA substrates with asymmetrically placed χ sites (Fig. 1). The wild-type RecBCD enzyme enters a dsDNA molecule at a blunt, or nearly blunt, end and proceeds to unwind and degrade the strand corresponding to the 3′-end at the point of entry. The specific interaction between wild-type RecBCD enzyme and a properly oriented χ site causes the translocating enzyme to pause at χ where the nuclease activity of the enzyme is modified: the 3′ to 5′ dsDNA exonuclease activity is attenuated, and the 5′- to 3′-nuclease activity is stimulated (16, 17). Continued unwinding following the interaction with χ produces two χ-specific fragments, the top strand downstream fragment and the bottom strand upstream fragment (Fig. 1). This is the first step in initiation of χ-stimulated recombination since the top strand downstream χ-specific fragment is preferentially used by RecA protein to invade a homologous counterpart (34, 36). If the DNA substrate does not contain χ, or if the translocating RecBCD enzyme fails to recognize χ, full-length ssDNA is the major species produced, and no χ-specific fragments are observed.

To determine if the mutant enzyme retained the ability to recognize χ, χ-specific fragment production assays were optimized by varying the ratio of magnesium ions to ATP since the ratio of these components is known to have dramatic effects on the nuclease activity, and hence the χ-specific fragment production ability, of the wild-type enzyme (21, 27, 37). Reactions containing either the wild-type or mutant enzyme were performed in parallel as described under "Experimental Procedures." χ-Specific fragments are observed in reactions containing RecBC^{1004}D enzyme, although the amounts of these species are significantly less than that seen in wild-type reactions. The chart in Fig. 7 presents the quantitation of downstream χ-specific fragment production at each reaction condition, revealing that the mutant enzyme displays optimal χ-specific fragment production at 2 mM magnesium acetate and 1 mM ATP, conditions also favored by the wild-type enzyme. Subsequent experiments examining χ-specific fragment production were performed at these conditions. Shown in Fig. 8 are time course reactions containing either the wild-type or mutant enzyme and either a χ^+- or χ^0-substrate. For pBR322 (χ^0), both enzymes produce equivalent amounts of full-length ssDNA, with the disappearance of dsDNA substrate nearly complete at 5 min. For a χ-containing duplex, both downstream and upstream χ-specific fragments are produced by either enzyme, with the yield of these fragments being substantially lower for RecBC^{1004}D enzyme. Quantitation of χ-specific fragment production for several independently performed experiments demonstrated that the mutant enzyme possesses, on average, 5% of the wild-type ability to recognize and respond to χ. Although not clearly visible in the gel shown, upstream (bottom strand) χ-specific fragments are observed for both enzymes. Moreover, the amount produced by the mutant is reduced compared with wild-type, and the reduction is similar to that observed for the downstream fragment. Similar behavior was observed using a second substrate containing a χ-site 2 kilobases further downstream from the point of entry of the enzyme (data not shown).

One possible explanation for the lower yields of χ-specific fragments would be that the increased levels of ssDNA nuclease activities described above are degrading these fragments subsequent to unwinding by the enzyme. If this were the case,

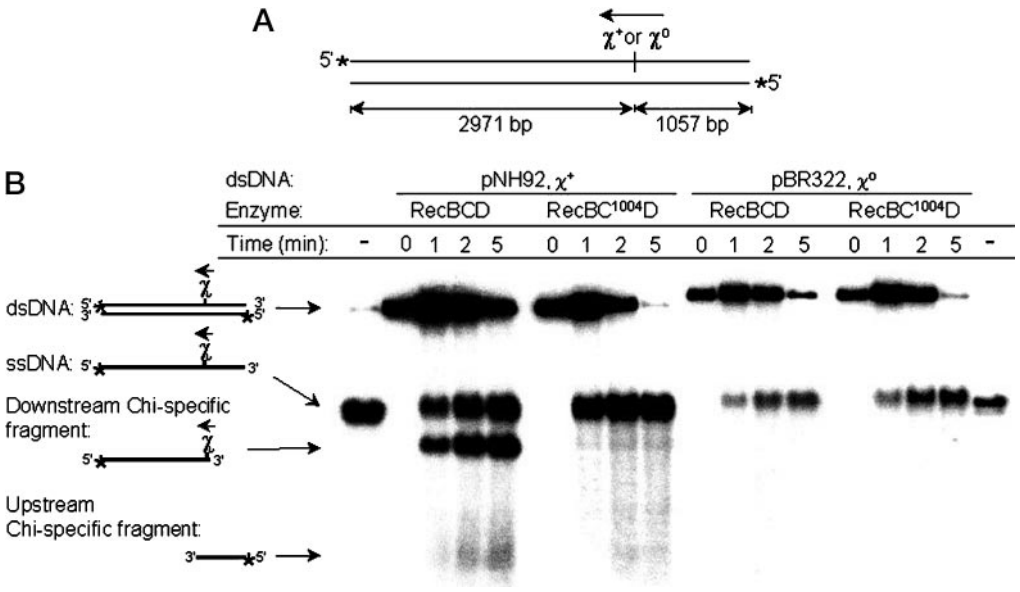

FIG. 8. **RecBC^{1004}D enzyme produces both downstream (*top strand*) and upstream (*bottom strand*) χ-specific fragments.** *A*, a linear dsDNA substrate containing a χ site (pNH92) was 5′-end-labeled and processed by a sub-saturating amount of RecBCD or RecBC^{1004}D enzyme as described under "Experimental Procedures." Control reactions were performed with linear substrate lacking χ (pBR322). *B*, products were separated on an agarose gel and quantified using a Molecular Dynamics STORM PhosphorImager and ImageQuant software. Typical gels are shown with the various bands identified to the *left*; these images have been enhanced to emphasize the χ-specific fragments produced by the mutant enzyme. The zero time points contain one-sixth the amount of DNA as do the accompanying samples; heat-denatured controls (95 °C, 5 min) are shown at the *far left* (χ^+) and the *far right* (χ^0) of the gel as markers for full-length ssDNA.

then the amount of full-length ssDNA produced by the mutant enzyme should be likewise reduced. However, the rate of full-length ssDNA production was found to be very similar for the two enzymes at all times assayed (Fig. 8). The absence of any detectable levels of post-unwinding degradation of ssDNA in these assays is consistent with the protection against degradation by the ssDNA nuclease afforded by the presence of saturating amounts of SSB protein observed in the ssDNA nuclease assays.

The alternative explanation for the lower production of χ-specific fragments is simply that the interaction between RecBC^{1004}D enzyme and the χ site does not efficiently mediate modification of the enzyme. This hypothesis is consistent with the *in vivo* data showing no detectable stimulation of recombination in response to χ (24); however, our *in vitro* data suggest that the ability of the mutant enzyme to recognize and modify nuclease activity at a χ site is not absent but simply less efficient than that of the wild-type enzyme. Thus, the question remained as to whether this reduction in χ-specific fragment production was due to less efficient χ recognition or due to less efficient attenuation of the nuclease activity at χ.

RecBC^{1004}D Enzyme Displays Reduced χ-Dependent Reversible Inactivation—To determine the basis for the decreased χ-specific fragment production, we utilized a second assay for χ recognition. Reversible inactivation measures χ recognition independently of χ-specific fragment production, providing the potential to observe χ recognition without nuclease modification. The RecBCD enzyme undergoes reversible inactivation subsequent to χ recognition at conditions of limiting magnesium ion concentration (21). Under conditions in which magnesium ion is not limiting, the enzyme acts in a catalytic fashion so that upon exit from a molecule in which χ modification occurred, it is able to reinitiate on additional dsDNA molecules. However, at conditions of limiting magnesium, the enzyme cannot reinitiate after a χ recognition event. This cessation of activity can be reversed by the addition of magnesium; catalytic activity of the enzyme is restored, and the remaining duplex substrate is unwound and degraded.

To determine if the mutant enzyme could be inactivated in a χ-specific manner at low magnesium conditions, RecBC^{1004}D enzyme was allowed to act on a 5′-end-labeled χ-containing substrate at conditions of limiting magnesium ion concentration (1 mM), and the disappearance of the duplex DNA substrate was monitored. The gels in Fig. 9*A* show reactions containing either wild-type or mutant enzyme, and the analysis of several such experiments is shown in Fig. 9*B*. These gels show that, at 40 min, reactions containing the wild-type enzyme still have a substantial amount (approximately 40%) of χ-containing substrate remaining; the mutant enzyme reactions, however, have nearly complete unwinding of the dsDNA, with only about 10% remaining at 40 min. These results suggest that χ recognition by RecBC^{1004}D enzyme is diminished. In agreement, under these conditions, the production of χ-specific fragments is observed for only the wild-type but not the mutant enzyme (data not shown). This is not unexpected since these conditions are severely sub-optimal for χ-specific fragment production; the defective χ recognition of the mutant enzyme is further reduced, resulting in no detectable χ-specific fragments at these conditions. Thus, based on the reduced levels of χ-specific fragment production, we reasoned that the reversible inactivation assays might not be sensitive enough to detect the lower levels of inactivation of the RecBC^{1004}D enzyme at χ. In that case, an enzyme with a reduced ability to recognize χ would require more encounters with χ to undergo a detectable level of inactivation.

To test this hypothesis, we raised the concentration of substrate from 6.25 to 19 μM (from 1.55 to 4.7 nM ends). Under these conditions, identical levels of reversible inactivation of the mutant enzyme are observed on both χ^0 and χ^+ substrates with only about 50% of the substrate unwound at 40 min. Unexpectedly, wild-type RecBCD enzyme exhibits the same behavior on χ^0 duplex DNA (Fig. 10). We surmised that this inactivation was due to interaction with "χ-like" sequences embedded within pBR322. If this is true, then this χ-independent effect should be reduced by lowering the amount of χ-like sequences in the substrate relative to the χ site present.

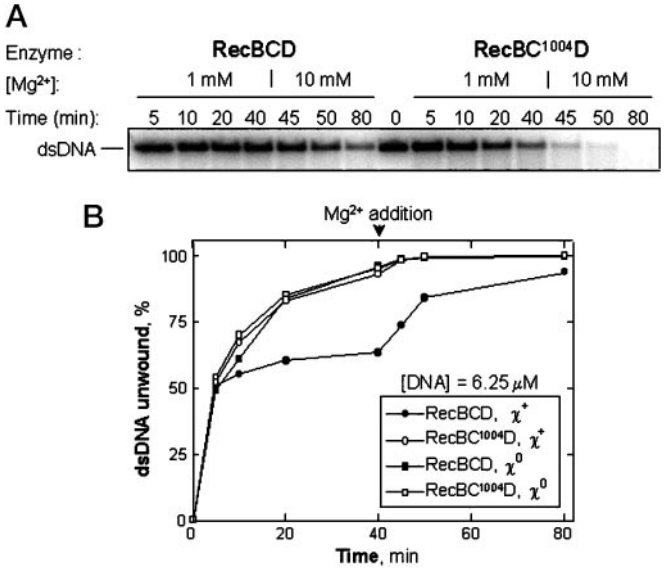

FIG. 9. **RecBC^{1004}D enzyme does not display reversible inactivation in reactions containing a low concentration of χ-containing linear duplex DNA.** Reactions containing 6.25 μM (nucleotides) of *Ava*I-linearized pNH92 (χ^+) or pBR322 (χ^0) DNA (1.55 nM dsDNA ends), 1 mM Mg^{2+}, and 5 mM ATP were initiated by addition of 0.04 nM functional RecBCD or RecBC^{1004}D enzyme as described under "Experimental Procedures." At 40 min, the concentration of magnesium acetate was increased to 10 mM. *A*, the degradation of the duplex substrate was monitored by gel assay. *B*, quantitation of duplex DNA remaining, normalized to the amount present at the start of the reaction. The data shown are representative of several independent assays.

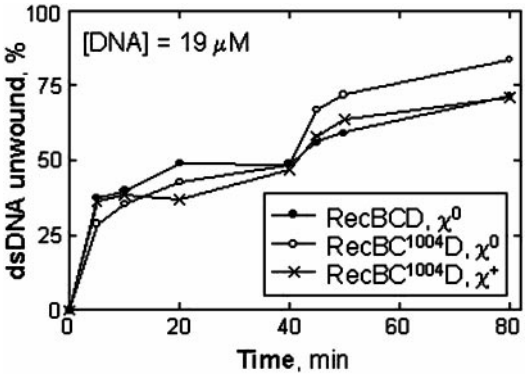

FIG. 10. **RecBCD and RecBC^{1004}D enzyme reversibly inactivate in reactions containing a high concentration of χ^0 dsDNA.** Reactions were performed as described under "Experimental Procedures" with 19 μM nucleotides of pBR322 or pNH92 (4.7 nM dsDNA ends). The degradation of the duplex substrate is plotted as a function of total starting substrate. The data shown are the averages of dsDNA degradation at each time point for several assays performed independently.

To accomplish this, we produced ~400-bp substrates with and without χ and used these in reversible inactivation assays. The concentration of dsDNA ends remained at 4.7 nM, keeping the concentration of χ sites in the reaction identical to those used in the reactions containing 19 μM (nucleotides) of the plasmid substrates, but the overall concentration of dsDNA (containing the presumed χ-like sequences) was lowered to 2 μM. The results from these experiments are shown in Fig. 11 and show, as we expected, that there is negligible inactivation for either the wild-type or mutant enzyme on the short χ^0 duplex. However, the shorter χ^+ substrate reveals that RecBC^{1004}D enzyme can undergo reversible inactivation in a χ-dependent manner, unwinding only 46% of the χ-containing substrate at 40 min; the level of inactivation is less than that

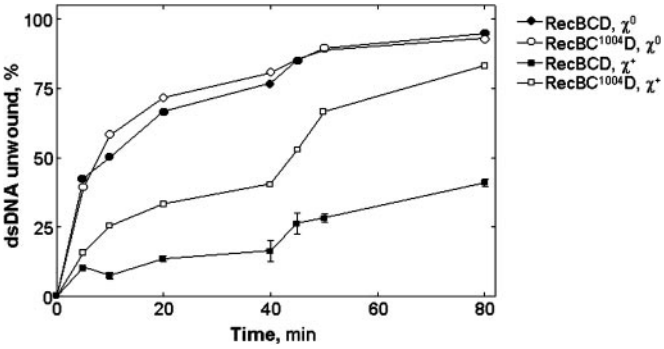

FIG. 11. **RecBC^{1004}D enzyme displays reversible inactivation on a short χ-containing duplex substrate.** Regions of approximately 400 bp were amplified by polymerase chain reaction from pBR322 (χ^0) and pNH92 (χ^+). These substrates (2 μM nucleotides, 4.7 nM dsDNA ends) were used in reversible inactivation assays containing either wild-type or mutant enzyme as described under "Experimental Procedures." The degradation of these short dsDNAs was monitored by gel assay and quantitated as a function of total starting substrate.

obtained for the wild-type enzyme (16% unwound at 40 min), consistent with lower levels of χ recognition by the mutant enzyme. The difference in the levels of inactivation by these enzymes is significantly less than would be predicted based on the χ-specific fragment production assays. Therefore, we conclude that the low level of χ fragment production by RecBC^{1004}D enzyme is due to both an impaired ability to recognize χ during translocation as well as less efficient modification of nuclease activity upon χ recognition.

χ-Dependent Joint Molecule Formation Is Stimulated by the RecBC^{1004}D Enzyme—We have shown that the RecBC^{1004}D enzyme can produce a reduced level of χ-specific fragments *in vitro*. The wild-type χ-RecBCD enzyme interaction results not only in the production of an invasive 3′-terminal χ-specific fragment but also in the preferential loading of RecA protein onto this product (38); potentially, the mutant enzyme could be deficient in this loading function of RecBCD enzyme, resulting in a lack of χ-stimulated recombination *in vivo*. Therefore, we determined whether the mutant could stimulate the incorporation of these χ-specific fragments into joint molecules.

χ-stimulated joint molecule formation was examined using an agarose gel assay (34, 36). Joint molecules are formed when RecA protein coats the 3′-end of a ssDNA molecule produced by the translocating RecBCD enzyme and mediates the invasion of this strand into the homologous region of supercoiled DNA. The two substrates utilized by RecA protein in joint molecule formation assays are full-length ssDNA and the downstream χ-specific fragment produced by RecBCD enzyme on a linear, χ-containing duplex (the upstream χ-specific fragment is not preferentially incorporated into joint molecules). The invasion of these ssDNA products into homologous, supercoiled DNA results in the production of two types of joint molecules, χ-independent and χ-dependent, respectively.

The gels in Fig. 12 show that bands corresponding to the downstream χ-specific fragment are detectable, as are bands corresponding to both χ-dependent and χ-independent joint molecules for both wild-type (Fig. 12*A*) and mutant enzyme (Fig. 12*B*). Although χ-specific fragment production is inefficient as shown in the previous experiments, RecBC^{1004}D enzyme does stimulate the incorporation of these fragments into RecA protein-mediated joint molecules; the extent of joint molecule formation as a percentage of total input linear DNA substrate is plotted in Fig. 12*C*. The RecBCD enzyme is able to convert about 5% of total starting substrate into χ-dependent joint molecules, an amount comparable to that previously reported (18). Reactions with RecBC^{1004}D enzyme also produce

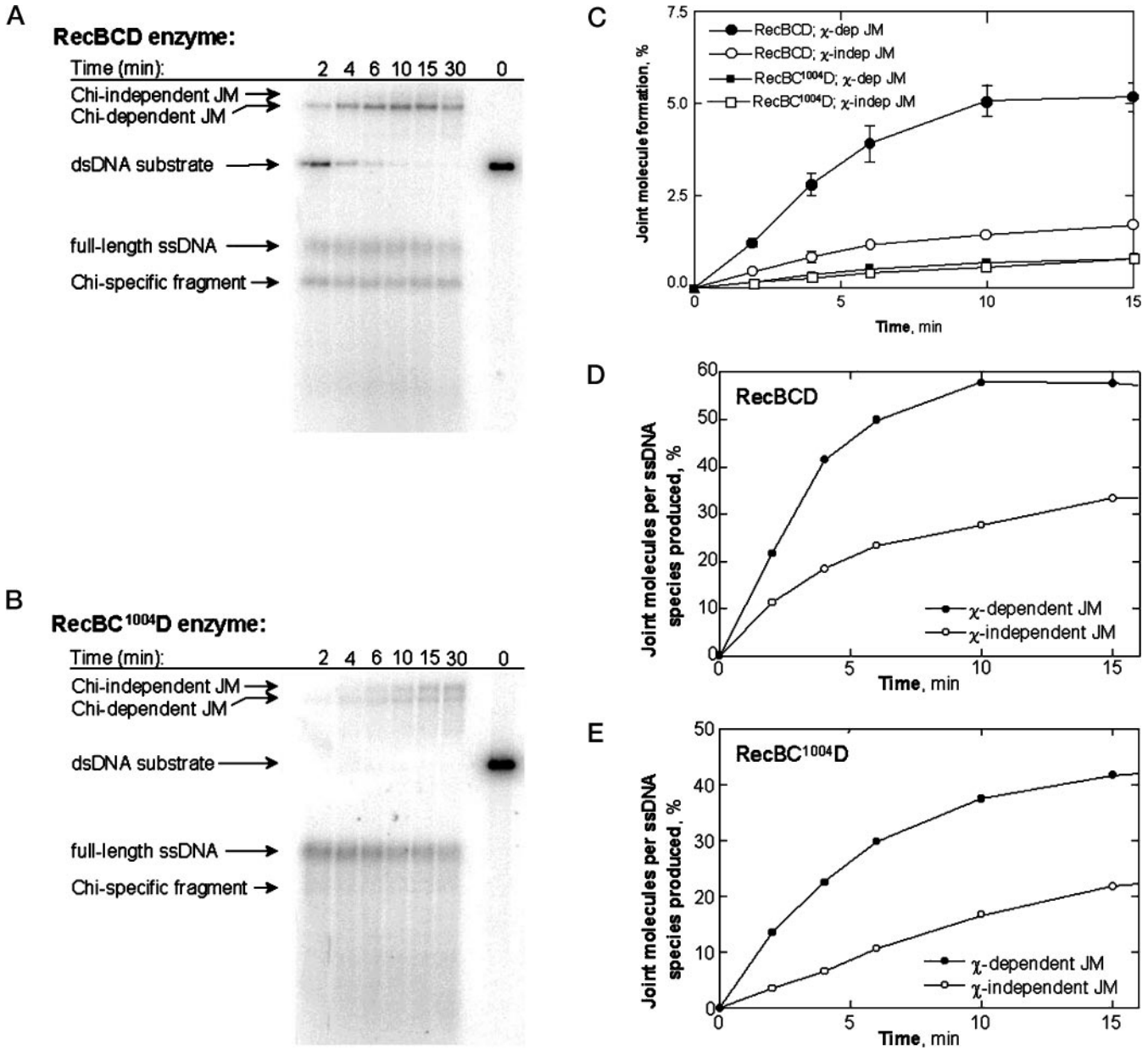

FIG. 12. **RecBC^{1004}D enzyme is capable of promoting χ-dependent joint molecule formation in the presence of RecA protein.** Joint molecule (*JM*) production assays were carried out as described under "Experimental Procedures." *A*, RecBCD; *B*, RecBC^{1004}D; *C*, joint molecule formation from several independent assays was normalized to the amount of starting substrate and is plotted as percent of initial linear dsDNA substrate. To investigate the preference for χ-dependent over χ-independent joint molecule production, the data depicted in *C* were re-evaluated by normalizing the χ-dependent and χ-independent joint molecules to the amounts of χ-specific fragments and full-length DNA present, respectively: *D*, RecBCD; *E*, RecBC^{1004}D.

χ-dependent joint molecules but at only 1% of total input duplex DNA. A reduction in the formation of χ-dependent joint molecules is expected due to the lower yields of χ-specific fragment formation. Therefore, the data from Fig. 12*C* were reanalyzed to normalize the amount of χ-dependent or χ-independent joint molecules formed to the amount of χ-specific fragment or full-length ssDNA produced, respectively. Both wild-type and mutant enzymes produce approximately 2–3-fold more χ-dependent than χ-independent joint molecules, although the production of joint molecules by RecBC^{1004}D enzyme is slightly lower overall (Fig. 12, *D* and *E*). These data clearly demonstrate that the preferential use of χ-specific fragments over full-length ssDNA exhibited by the wild-type enzyme is retained by the mutant. Thus, we conclude that the primary reason for the reduction of χ-stimulated recombination *in vivo* by the RecBC^{1004}D enzyme is a defect in the ability to recognize and modify nuclease activity in response to the recombination hotspot, χ.

DISCUSSION

In an effort to investigate RecBCD-mediated recombination in the absence of stimulation by χ, a new class of RecBCD mutants was isolated and described (24). The C* mutant class is characterized by the distinctive ability to promote near wild-type levels of homologous recombination but to display no enhancement of these levels in the presence of the recombination hotspot, χ. Four C* mutants were isolated, each exhibiting wild-type function to varying degrees; the mutant that behaved most like the wild-type enzyme in the absence of χ was the RecBC^{1004}D enzyme (24). Cells containing the *recC*^{1004} mutation were found to possess wild-type levels of nuclease activity and moderately reduced (20–40% of wild-type) recombination

function as measured by conjugation and transduction assays (24). However, lambda phage vegetative crosses carried out in the mutant strain demonstrated a complete lack of stimulation of recombination in response to χ, even though the basal level of χ-independent recombination was unaffected (24). These findings suggested that the *recC*^{1004} mutant is able to promote recombination in a χ-independent manner at levels approximating that of wild-type but is unable to enhance recombination events at χ (24).

We purified and further characterized the RecBC^{1004}D enzyme to determine the biochemical basis for the phenotypes observed by Schultz *et al.* (24). This mutant enzyme possesses wild-type levels of helicase, ATPase, and dsDNA exonuclease activities, and it has a moderately enhanced level of both endo- and exonuclease activities on ssDNA. We found that the RecBC^{1004}D enzyme retains the ability to interact with, and be modified by, the χ sequence in a manner analogous to that of the wild-type enzyme, although at a much lower efficiency than wild type. This reduction in χ-specific fragment production may be interpreted as either a defect in χ recognition or a deficiency in the ability of the mutant enzyme to respond to χ upon its recognition, *i.e.* a failure to modify nuclease activity at χ. To distinguish between these possibilities, we used reversible inactivation assays, which measure χ recognition independent of χ-specific fragment production. These assays revealed that recognition of χ by the mutant enzyme was reduced to a lesser degree than was production of χ-specific fragments, suggesting that the defect lies in both the recognition of and the response to a χ site. Furthermore, since this mutation maps to the *recC* gene (24), these findings suggest that the RecC subunit is crucial, if not directly responsible, for χ recognition. This supposition is supported by recent findings from the Kobayashi laboratory at the University of Tokyo, Japan (25). They discovered that the RecBC^{1004}D enzyme responds phenotypically to a novel χ-like sequence, which they termed χ*, in a manner similar to that of wild-type RecBCD enzyme at χ (25). This interaction *in vitro* is currently under investigation.[3]

Joint molecule formation *in vitro* represents the first steps of homologous recombination *in vivo* (34, 36). As described previously, ssDNA produced by RecBCD enzyme is utilized by RecA protein for strand invasion of a homologous, supercoiled DNA molecule. Two types of joint molecules are generated as follows: those containing the downstream χ-specific fragment (χ-dependent) and those containing full-length ssDNA (χ-independent). However, there is a RecBCD-dependent preference for producing χ-dependent over χ-independent joint molecules in these assays. This bias is due to the preferential loading of RecA protein by RecBCD enzyme onto the χ-specific fragment following χ recognition (38). If this ability to facilitate the loading of RecA protein were impaired in the mutant enzyme, then the production of χ-dependent joint molecules would be expected to approximate that of χ-independent joint molecules. However, a lower yield of χ-specific fragments would predict an overall reduction in χ-dependent joint molecule formation. When the raw data are corrected for the lower yields of downstream χ-specific fragments, we find that the mutant enzyme displays a stimulation of χ-dependent over χ-independent joint molecules comparable to that observed with the wild-type enzyme. These data argue that the apparently equivalent amounts of χ-dependent and χ-independent joint molecules formed by the mutant enzyme are due to the reduced yields of χ-specific fragments and not to a defect in the ability of the RecBC^{1004}D enzyme to promote RecA protein-ssDNA complex formation in a χ-dependent manner.

Our reversible inactivation studies with the higher than typically used concentrations of DNA reveal an unexpected feature of RecBCD enzyme, χ-independent reversible inactivation. We suspect that this characteristic χ-mediated modification is not absolutely dependent on the interaction with a canonical χ sequence but may also be stimulated by the presence of χ-like sequences within the duplex DNA substrate. This idea is far from inconceivable: RecBCD enzyme can promote modest levels of recombination in response to χ sites containing single base mutations, one of which (5′-GCTGGTAG-3′) is found in both the χ^+ and the χ^0 plasmids used in our assays (39). There are four additional single base pair mutations of χ (5′-GCTGGCGG-3′, 5′-GCTGGTGA-3′, and two of 5′-GCTGCTGG-3′) present in our plasmids as well, none of which have been characterized; however, there are neither χ* nor single base mutations of χ* within this plasmid DNA. In addition, the observed levels of χ-independent recombination in lambda phage crosses correspond to the calculated amount of hotspot activity that could be obtained from χ-like sequences naturally occurring in the phage, leading to the conclusion that stimulation of recombination by these sites could account for the levels of recombination seen in the absence of χ (39). This implies that what is commonly referred to as "χ-independent recombination" is actually recombination occurring at non-canonical χ sites but at a lower efficiency than that which occurs at χ. If this χ-independent recombination occurs by the same mechanism as χ-dependent recombination, then it would also require the χ-mediated nuclease modifications and RecA protein-loading functions of the RecBCD enzyme, both of which are exhibited by the RecBC^{1004}D enzyme as well. Since the *recBC1004D* strain displays the same level of χ-independent recombination in lambda phage vegetative crosses as does a wild-type strain, we would argue that the mutant enzyme also stimulates recombination at χ-like sites. These data do not exclude the possibility that a sequence-independent mechanism is responsible for the χ-independent inactivation observed with these enzymes, although the complete lack of inactivation of the recombination-deficient RecB^{2109}CD enzyme (see below) implies that the inactivation observed corresponds to an activity of the enzyme required for recombination. This hypothesis is currently under investigation.

To minimize the χ-independent inactivation that we observe *in vitro* and to test further the ability of RecBC^{1004}D enzyme to inactivate in a χ-dependent manner, we used shorter χ^+ and χ^0 substrates at the same high concentration of DNA molecules. These conditions thereby reduce the amount of potentially χ-like DNA by approximately 10-fold, without lowering the concentration of χ sites in the reaction (all five single base pair mutations of χ were excluded). Under these conditions, the χ^0 substrate no longer induced inactivation of either the wild-type or mutant enzyme; however, χ-specific inactivation was detected for both the wild-type and mutant enzymes. These findings, together with the wild-type levels of χ-independent recombination exhibited by the RecBC^{1004}D enzyme in the lambda phage vegetative crosses (24), suggest that the mutant enzyme retains the wild-type ability to respond to χ-like sequences even though its response to the canonical χ is markedly reduced.

Previously, our laboratory characterized the RecB^{2109}CD enzyme (27, 40). The purified enzyme, like the RecBC^{1004}D enzyme, possesses all the non-χ-related activities of the wild-type RecBCD enzyme. However, unlike the RecBC^{1004}D enzyme, the RecB^{2109}CD enzyme does not appear to recognize or respond to χ in χ-specific fragment production assays or in joint molecule formation assays. We recently performed reversible inactiva-

[3] D. A. Arnold, I. Kobayashi, N. Handa, and S. C. Kowalczykowski, manuscript in preparation.

tion assays with this enzyme to examine the potential for χ recognition in the absence of attenuation of nuclease activity. We were unable to detect inactivation on plasmid DNA substrates regardless of whether they contained a χ site or not, further supporting the complete lack of χ recognition by RecB^{2109}CD enzyme.[4] In addition, even at high concentrations of DNA there was no inactivation observed for this enzyme, suggesting that not only is χ recognition completely defective but that this enzyme is also unable to recognize or respond to χ-like sequences. Accordingly, the *recB*^{2109} strain is phenotypically deficient for recombination (41), whereas the *recC*^{1004} strain possesses a reduced, but significant, recombination proficiency. These findings suggest that both the low level of canonical χ recognition and the less effective but more frequent interactions with χ-like sequences by the RecBC^{1004}D enzyme are sufficient to promote the moderate levels of conjugational and transductional recombination observed *in vivo* (24).

In summary, our data show the RecBC^{1004}D enzyme to be very similar to the wild-type enzyme with two exceptions. First, the mutant displays an enhancement of ssDNA nuclease activities that is both ATP-dependent and SSB protein-inhibited. Second, the ability of the mutant to recognize and undergo χ-stimulated modification is reduced 20-fold, too low to account for the recombination proficiency observed for the *recC*^{1004} strain. These findings do not exclude the possibility that there are other factors acting *in vivo* to produce the phenotypes reported by Schultz *et al*. (24), nor do they address the supposition that the RecBC^{1004}D enzyme may recognize altered χ sequences that may promote heightened levels of recombination at these sites in a RecBCD-like manner (24, 25).[3] However, they do reveal that this mutant can respond to χ sites *in vitro* in a manner analogous to, yet less efficiently than, wild type; also, like the wild-type enzyme, it can respond to as yet undefined χ-like sequences, suggesting that RecBC^{1004}D enzyme remains recombination-proficient *in vivo* as a result of these two properties.

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[4] D. A. Arnold and S. C. Kowalczykowski, unpublished observations.

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