Homologous recombination in Escherichia coli is initiated by the RecBCD enzyme and is stimulated by an 8-nucleotide element known as Chi (\(\chi\)). We present a detailed biochemical characterization of a mutant RecBCD enzyme, designated RecBCD\(^{1004D}\), that displays a reduced level of \(\chi\) site recognition. Initially, characterized genetically as unable to respond to the \(\chi\) sequence, we provide evidence to indicate that the ability of this mutant enzyme to respond to \(\chi\) is reduced rather than lost; the mutant displays about 20-fold lower \(\chi\) 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 RecBCD\(^{1004D}\) strain observed in vivo results from a basal level of modification of the RecBCD\(^{1004D}\) enzyme at both \(\chi\)-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 nucleolytic activities on both DNA substrates. However, when the amount of free magnesium ion concentration is less than the ATP concentration, the catalytic nature of the enzyme is restored by addition of magnesium ion to these reactions, thus the term “reversible inactivation.” 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 \(\chi\) 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 \(\chi\) by RecBCD enzyme is contingent upon the orientation of \(\chi\) 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, \(\chi\) recognition is observed as the production of two \(\chi\)-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 \(\chi\) recognition. Thus, \(\chi\)-specific fragment production provides a measure for the efficiency of \(\chi\) recognition by the RecBCD enzyme.

Reversible inactivation is another \(\chi\)-specific modification of RecBCD enzyme observed in vitro (21). \(\chi\)-Dependent inactivation of RecBCD enzyme has been observed in vivo as well (22, 23). Upon recognition of \(\chi\), RecBCD enzyme undergoes the aforementioned nucleolytic modifications that persist until the enzyme dissociates from the DNA molecule. Typically, upon dissociation, the enzyme reverts to its “pre-\(\chi\)” 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 \(\chi\) recognition, and more importantly, this assay provides a potential means of separating \(\chi\) recognition from the modification of nucleolytic activity at \(\chi\) that is required to detect \(\chi\)-specific ssDNA fragments.

Given the pivotal role that \(\chi\) recognition plays in the conversion of RecBCD into a recombination-proficient enzyme, a mutant enzyme that lacks \(\chi\) recognition but retains recombination function is potentially very informative. To identify such mutants, a screen was developed to isolate recBCD mutants that lacked \(\chi\) recombinational hotspot activity but maintained...
**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. [3H]SAM, [γ-32P]ATP, and [α-32P]ATP were purchased from NEN Life Science Products. Proteinase K was purchased from Boehringer Mannheim.

**Strains**—E. coli strains expressing wild-type (V320; ΔargA-thyA Δ323, 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 RecBC1004D enzymes (24, 26). The expression plasmids are under runaway replication control and carry chloramphenicol (recBCD) or ampicillin (recBC1004D) antibiotic markers.

**Protein Isolation**—The RecBC1004D enzyme was purified by the method of Eggleton 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 recC1004 mutation did not alter the chromatographic properties of the mutant enzyme (data not shown). The recC1004 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 RecBC1004D enzyme with a greater than 930-fold purification.

Protein concentrations were determined spectrophotometrically using an extinction coefficient of 4.0 × 10³ M⁻¹ cm⁻¹ at 280 nm. The specific activity of the mutant enzyme preparation was determined to be 3.8 × 10⁵ nucleic 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 × 10⁵ 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 RecBC1004D heterotrimers per dsDNA end, indicating that the enzyme preparation is 45% active (Table I). RecBCD and RecBC1004D 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 × 10⁴ M⁻¹ cm⁻¹. SSB protein was purified from E. coli strain RLM727 as described (30) and concentration determined using ε = 3.0 × 10⁴ M⁻¹ cm⁻¹.
TABLE I

| Enzyme       | dsDNA exonuclease | Helicase | ATPase |
|--------------|-------------------|----------|--------|
|              | enzyme activity  | apparent | activity |
|              | (units/mg)        | stoichiometry | (units/mg) |
|              | (per dsDNA end)   | (±50)    | (±100) |
| RecBCD       | 2.4 x 10^6       | 3.4 ± 0.5 | 242    |
| RecBC1004D   | 3.8 x 10^6       | 2.2 ± 0.5 | 226    |

*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).*

*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).*

*The corrected k_cat=corr (k_cat=corr ≥ ±50) is the experimentally determined rate of unwinding in base pairs per s per functional enzyme concentration as determined by saturation of helicase activity.*

*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).*

*The correction 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.*

*The χ substrate (414 bp) used for reversible inactivation assays was amplified from pBR22 using primers PB08 (5'-TCATCGTTCCGGCCACCAA-3') and PB13 (5'-AGGTGCGCGGCGTTCCATT-3'). The χ′ substrate (438 bp) was amplified from pNH92 using primers SKDH92 (5’-CCCTGCTGCTTAAAGACAC-3’ and SKDH40L (5’-CTGAGCTGGTTAAGGTCT-3’). After amplification, the polymerase chain reaction products were purified in a 2% MetaPhor gel (FMC Bioproducts) and recovered from the gel by electrosorption. 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.*

*Fluorometric helicase assay—Conversion of dsDNA to ssDNA was measured using a fluorometric helicase assay that monitors the quenching of the intrinsic fluorescence of ssDNA protein binding to ssDNA (5). The reaction mix, including 2 μM SSB protein and 10 μM EcoRI-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 S100 Spectrofluorometer from Spectronic Instruments (SLM Aminco). The changes 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). Baseline (n=3) subtraction of intrinsic fluorescence 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′, present at a concentration of 30 μM nucleotides. The amounts of PEP and pyruvate kinase were increased to 300 μM 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 size of 15 nucleotides per monomer. The reactions were initiated by the addition of RecBCD or RecBC1004D enzyme 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/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 protein present in the reaction.

**ATPase Assay**—Hydrolysis of ATP was measured using a coupled, spectrophotometric assay (35) with the following modifications. The dsDNA substrate used was pBR322, χ′, present at a concentration of 30 μM nucleotides. The amounts of PEP and pyruvate kinase were increased to 300 μM 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 size of 15 nucleotides per monomer. The reactions were initiated by the addition of RecBCD or RecBC1004D enzyme 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/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 protein present in the reaction. The efficiency of ATP hydrolysis was also calculated using data from the coupled ATPase assay by averaging the total change in absorbance during unwinding of both wild-type and mutant enzymes, with the efficiency of ATP hydrolyzed” with the conversion factor 0.16 μM ATP hydrolyzed per AU and dividing this value by the concentration of base pairs in the reaction (15 μM) (35).
χ Recognition by the RecBC\textsuperscript{1004D} Enzyme

RESULTS

The RecBC\textsuperscript{1004D} 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\textsubscript{2} and 50 μM ATP) (3), we detected similar levels of dsDNA exonuclease activity for RecBCD and RecBC\textsuperscript{1004D} 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\textsuperscript{6} and 2.4 × 10\textsuperscript{6} 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\textsuperscript{1004D} enzyme, we used the gel assay described previously (16). The substrate was χ\textsuperscript{9} 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 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\textsuperscript{1004D} 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\textsuperscript{9} or χ\textsuperscript{5}), 1.55 mM dsDNA ends), 1.25 μM SSB protein. After equilibration for 2 min at 37°C, reactions were initiated by addition of 0.04 μM RecBCD or RecBC\textsuperscript{1004D} enzyme, and time points (30 μl) were taken as indicated. After the 10 μM magnesium acetate was added to 10 μM final concentration, and the time course was continued for an additional 40 min. These experiments were also performed with 4.7 mM 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\textsuperscript{9}) or pNH29 (438 bp, χ\textsuperscript{5}). 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\textsuperscript{1004D}) 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\textsuperscript{I}-linearized, 5’-end-labeled dsDNA (χ\textsuperscript{9} or χ\textsuperscript{5}). In addition, 10 μM supercoiled plasmid (pNH29) 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\textsuperscript{1004D} enzyme (one functional enzyme per 15 dsDNA ends). Aliquots were treated and analyzed as described above for the χ-specific fragment production assays.
pendent ssDNA nuclease activities of both wild-type and mutant enzyme, presumably by coating the substrate and thereby blocking the binding of RecBCD or RecBC1004D 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 RecBC1004D 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. 6A, saturation for the RecBC1004D 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. 6B). 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 enzymes 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 recC1004 Mutation Does Not Alter the ATPase Activity of RecBC1004D 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 1004D enzyme was tested using pBR322 (6) 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 RecBC1004D enzymes were 1.1 $\times$ 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 recC1004 mutation does not significantly alter the in vitro activity of the RecBC1004D enzyme with respect to dsDNA unwinding or ATP hydrolysis.

The RecBC1004D Enzyme Processes dsDNA to Produce Reduced, but Detectable, Levels of $\chi$-Specific Fragments in Vitro—In vivo, the RecBC1004D enzyme is unable to promote
\( \chi \) Recognition by the RecBC\(^{1004D} \) Enzyme

**Fig. 6.** The RecBC\(^{1004D} \) enzyme displays wild-type levels of DNA helicase activity. The ability of RecBC\(^{1004D} \) 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.

\( \chi \)-stimulated recombination in response to the canonical \( \chi \) sequence (5'-GCTGCTTG-3') (24). Specifically, in phage lambda vegetative crosses, the mutant enzyme displayed wild-type levels of \( \chi \)-independent recombination, yet showed no stimulation of that basal level in the presence of \( \chi \). These results suggested that the enzyme had lost the ability to recognize or be regulated by the \( \chi \) recombination hotspot. In vitro, the ability of RecBCD enzyme to recognize \( \chi \) can be observed using end-labeled, dsDNA substrates with asymmetrically placed \( \chi \) 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 \( \chi \) site causes the translocating enzyme to pause at \( \chi \) 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 \( \chi \) produces two \( \chi \)-specific fragments, the top strand downstream fragment and the bottom strand upstream fragment (Fig. 1). This is the first step in initiation of \( \chi \)-stimulated recombination since the top strand downstream \( \chi \)-specific fragment is preferentially used by RecA protein to invade a homologous counterpart (34, 36).

For a \( \chi \)-containing duplex, both downstream and upstream \( \chi \)-specific fragments are produced by either enzyme, with the yield of these fragments being substantially lower for RecBC\(^{1004D} \) enzyme. Quantitation of \( \chi \)-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 \( \chi \). Although not clearly visible in the gel shown, upstream (bottom strand) \( \chi \)-specific fragments are observed for both enzymes. Moreover, in a downstream fragment, 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 \( \chi \)-site 2 kilobases further downstream from the point of entry of the enzyme (data not shown).

One possible explanation for the lower yields of \( \chi \)-specific fragments would be that the increased levels of ssDNA nuclease activity described above are degrading these fragments subsequent to unwinding by the enzyme. If this were the case,
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 RecBC1004D 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.

RecBC1004D 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, RecBC1004D 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. 9A show reactions containing either wild-type or mutant enzyme, and the analysis of several such experiments is shown in Fig. 9B. 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 RecBC1004D 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 RecBC1004D 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 χ0 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+ 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. RecBC1004D enzyme does not display reversible inactivation in reactions containing a low concentration of \( \chi \)-containing linear duplex DNA. Reactions containing 6.25 \( \mu \)M (nucleotides) of \( A\)vA1-linearized pNH92 (\( \chi^0 \)) or pBR322 (\( \chi^0 \)) 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 RecBC1004D 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.

To accomplish this, we produced ~400-bp substrates with and without \( \chi \) and used these in reversible inactivation assays. The concentration of dsDNA ends remained at 4.7 nM, keeping the concentration of \( \chi \) sites in the reaction identical to those used in the reactions containing 19 \( \mu \)M (nucleotides) of the plasmid substrates, but the overall concentration of dsDNA (containing the presumed \( \chi \)-like sequences) was increased to 2 \( \mu \)M. 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 \( \chi^0 \) duplex. However, the shorter \( \chi^0 \) substrate reveals that RecBC1004D enzyme can undergo reversible inactivation in a \( \chi \)-dependent manner, unwinding only 46% of the \( \chi \)-containing substrate at 40 min; the level of inactivation is less than that obtained for the wild-type enzyme (16% unwound at 40 min), consistent with lower levels of \( \chi \) 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 \( \chi \)-specific fragment production assays. Therefore, we conclude that the low level of \( \chi \) fragment production by RecBC1004D enzyme is due to both an impaired ability to recognize \( \chi \) during translocation as well as less efficient modification of nuclease activity upon \( \chi \) recognition.

\[ \chi \] -Dependent Joint Molecule Formation Is Stimulated by the RecBC1004D Enzyme—We have shown that the RecBC1004D enzyme can produce a reduced level of \( \chi \)-specific fragments in vitro. The wild-type \( \chi \)-RecBCD enzyme interaction results not only in the production of an invasive 3’-terminal \( \chi \)-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 \( \chi \)-stimulated recombination in vitro. Therefore, we determined whether the mutant could stimulate the incorporation of these \( \chi \)-specific fragments into joint molecules.

\( \chi \)-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 \( \chi \)-specific fragment produced by RecBCD enzyme on a linear, \( \chi \)-containing duplex (the upstream \( \chi \)-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, \( \chi \)-independent and \( \chi \)-dependent, respectively.

The gels in Fig. 12 show that bands corresponding to the downstream \( \chi \)-specific fragment are detectable, as are bands corresponding to both \( \chi \)-dependent and \( \chi \)-independent joint molecules for both wild-type (Fig. 12A) and mutant enzyme (Fig. 12B). Although \( \chi \)-specific fragment production is inefficient as shown in the previous experiments, RecBC1004D 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. 12C. The RecBCD enzyme is able to convert about 5% of total starting substrate into \( \chi \)-dependent joint molecules, an amount comparable to that previously reported (18). Reactions with RecBC1004D enzyme also produce
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 were reanalyzed to normalize the amount of χ-dependent or χ-independent joint molecules formed to the amounts of χ-specific fragments and full-length ssDNA present, respectively: D, RecBCD; E, RecBC\(^{1004}\)D.

**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 activity.
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<sup>1004</sup> 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<sup>1004</sup>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<sup>1004</sup>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 nuclelease 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<sup>1004</sup>D enzyme responds phenotypically to a novel χ-like sequence, which they termed χ<sup>α</sup>, in a manner similar to that of wild-type RecBCD enzyme at χ (25). This interaction in vitro is currently under investigation.

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 by RecBCD enzyme onto the χ-specific fragment is dependent upon its χ-independent manner, we used shorter χ<sup>-</sup> and χ<sup>+</sup> 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<sup>-</sup> 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<sup>1004</sup>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<sup>2109</sup>CD enzyme (27, 40). The purified enzyme, like the RecBC<sup>1004</sup>D enzyme, possesses all the non-χ-related activities of the wild-type RecBCD enzyme. However, unlike the RecBC<sup>1004</sup>D enzyme, the RecB<sup>2109</sup>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 γ 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 γ site or not, further supporting the complete lack of γ recognition by RecB2109CD enzyme.4 In addition, even at high concentrations of DNA there was no inactivation observed for this enzyme, suggesting that not only is γ recognition completely defective but that this enzyme is also unable to recognize or respond to γ-like sequences. Accordingly, the recB2109 strain is phenotypically deficient for recombination (41), whereas the recC1004 strain possesses a reduced, but significant, recombination proficiency. These findings do not exclude the possibility that there are other factors acting to produce the phenotypes observed for the recB2109 strain, and transductional recombination observed at these sites in a RecBCD-like manner (24, 25).3 However, they do reveal that this mutant can respond to γ 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 γ-like sequences, suggesting that RecBC1004D enzyme remains recombination-proficient in vitro as a result of these two properties.

Acknowledgments—We are grateful to our colleagues in the Kowalczykowski laboratory, Daniel Anderson, Frederic Chedin, Jason Churchill, Frank Harmon, Noriko Kantake, Alex Mazin, Jim New, Erica Seitz, Tomohiko Sugiyama, and Robert Tracy, for helpful criticism and discussion of this manuscript.

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

REFERENCES

1. Amundsen, S. K., Taylor, A. F., Chaudhury, A. M., and Smith, G. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5558–5562
2. Emmerson, P. T. (1986) Genetics 100, 39–60
3. Eichler, D. C., and Lehman, I. R. (1977) J. Biol. Chem. 252, 499–503
4. Telander-Muskavitch, K. M., and Linn, S. (1989) J. Biol. Chem. 257, 2671–2678
5. Koppen, L. J., and Kowalczykowski, S. C. (1989) Biochemistry 28, 2683–2687
6. Howard-Flinders, P., and Boyce, R. P. (1966) Radiat. Res. 13, 156–181
7. Capaldo-Kimbial, F., and Barbour, S. D. (1971) J. Bacteriol. 106, 204–212
8. Clark, A. J., and Margulies, A. D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 451–459
9. Ennis, D. G., Amundsen, S. K., and Smith, G. R. (1987) Genetics 115, 11–24
10. Lam, S. T., Stahl, M. M., McMillen, K. D., and Stahl, F. W. (1974) Genetics 77, 421–433
11. Stahl, F. W., Crasemann, J. M., and Stahl, M. M. (1975) J. Mol. Biol. 94, 203–212
12. Stahl, F. W., and Stahl, M. M. (1975) Genetics 86, 715–725
13. Smith, G. R., Kunes, S. M., Schultz, D. W., Taylor, A., and Triman, K. L. (1981) Cell 24, 429–436
14. Ponticelli, A. S., Schultz, D. W., Taylor, A. F., and Smith, G. R. (1985) Cell 41, 145–151
15. Taylor, A. F., Schultz, D. W., Ponticelli, A. S., and Smith, G. R. (1985) Cell 41, 153–163
16. Dixon, D. A., and Kowalczykowski, S. C. (1993) Cell 73, 87–96
17. Anderson, D. G., and Kowalczykowski, S. C. (1997) Genes Dev. 11, 571–581
18. Dixon, D. A., and Kowalczykowski, S. C. (1991) Cell 66, 361–371
19. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., and Rehrer, W. M. (1994) Microbiol. Rev. 58, 401–465
20. Cheng, K. C., and Smith, G. R. (1987) J. Mol. Biol. 194, 747–750
21. Dixon, D. A., Churchill, J. J., and Kowalczykowski, S. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2980–2987
22. Myers, R. S., Kuimov, A., and Stahl, F. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6244–6248
23. Koppen, A., Krobitsch, S., Thoms, B., and Wackernagel, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6249–6253
24. Schultz, D. W., Taylor, A. F., and Smith, G. R. (1983) J. Bacteriol. 155, 664–680
25. Handa, N., Ohashi, S., Kusano, K., and Kobayashi, I. (1997) Genes Cells 2, 525–536
26. Chaudhury, A. M., and Smith, G. R. (1984) J. Bacteriol. 160, 788–791
27. Eggleston, A. K., and Kowalczykowski, S. C. (1993) J. Mol. Biol. 231, 605–620
28. Murphy, K. C. (1994) J. Biol. Chem. 269, 22507–22516
29. Griffith, J., and Shores, C. G. (1985) Biochemistry 24, 158–162
30. LeBowitz, J. (1985) Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication, Ph.D. thesis, The Johns Hopkins University, Baltimore
31. Messing, J. (1983) Methods Enzymol. 101, 20–78
32. Birnboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523
33. Vinograd, J., and Leibowitz, J. (1966) J. Gen. Physiol. 49, 101–125
34. Dixon, D. A., and Kowalczykowski, S. C. (1995) J. Biol. Chem. 270, 16360–16370
35. Roman, L. J., and Kowalczykowski, S. C. (1989) Biochemistry 28, 2873–2881
36. Roman, L. J., Dixon, D. A., and Kowalczykowski, S. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3367–3371
37. Anderson, D. G., Churchill, J. J., and Kowalczykowski, S. C. (1997) Genes Cells 2, 117–128
38. Anderson, D. G., and Kowalczykowski, S. C. (1997) Cell 89, 77–86
39. Cheng, K. C., and Smith, G. R. (1984) J. Mol. Biol. 180, 371–377
40. Eggleston, A. K., and Kowalczykowski, S. C. (1993) J. Mol. Biol. 231, 621–633
41. Amundsen, S. K., Neiman, A. M., Thibodeaux, S. M., and Smith, G. R. (1990) Genetics 126, 25–40