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Biochemical Characterization of a Mutant recBCD Enzyme, the recB^{2109}CD Enzyme, Which Lacks χ-specific, but Not Non-specific, Nuclease Activity

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RecBCD enzyme of *Escherichia coli* is a DNA helicase which also possesses ATP-dependent nuclease activities. We have purified a mutant recBCD enzyme, designated recB^{2109}CD enzyme, and have examined the nuclease activities of this protein *in vitro* to determine whether any alteration in these activities is responsible for the recombination-deficient phenotype of the *recB2109* strain. The recB^{2109}CD enzyme possesses all of the non-specific nuclease activities (dsDNA exonuclease and ssDNA exo- and endonuclease) associated with wild-type recBCD enzyme although they are reduced $\sim$ 2 to 3-fold relative to the wild-type enzyme. The ATP-dependent dsDNA exonuclease activity of recB^{2109}CD enzyme requires significantly higher ATP concentrations for optimal activity when compared to the wild-type enzyme. The ATP-independent ssDNA endonuclease activity of the two enzymes is similar, but the ATP-stimulated ssDNA endonuclease and ATP-dependent ssDNA exonuclease activities of the mutant enzyme are reduced relative to those of wild-type recBCD enzyme. Despite its ability to degrade linear dsDNA non-specifically, recB^{2109}CD enzyme lacks sequence-specific nicking activity at χ sites, which are hotspots for genetic recombination. Since this interaction with χ significantly attenuates the non-specific dsDNA exonuclease activity of wild-type recBCD enzyme, these results suggest that the non-specific dsDNA exonuclease activity of the mutant enzyme cannot be attenuated, with the consequence that a DNA substrate which is suitable for recombination is not produced.

Keywords: recBCD enzyme; DNA helicase; ATP-dependent nuclease; genetic recombination

1. Introduction

In *Escherichia coli*, the recombination process is well-characterized genetically, and three pathways of homologous recombination have been identified. The RecBCD pathway is the major pathway for conjugational and transductional recombination (for reviews, see Smith, 1988, 1989), for recombination of λ *red*$^-$ *gam*$^-$ phage (Schulman *et al.*, 1970), and for double-strand break repair (Krasin & Hutchinson, 1977; Sargentini & Smith, 1986) and is primarily dependent on the activities of two proteins: recA protein and recBCD enzyme (Clark, 1973; Smith, 1988, 1991). The recA protein promotes homologous pairing and the formation of heteroduplex DNA (for reviews, see Cox & Lehman, 1987; Radding, 1989; Kowalczykowski, 1991) and is essential to all pathways of recombination except λ recombination, which is carried out by phage-encoded proteins, and plasmid recombination, which occurs *via* the RecE pathway. RecBCD enzyme (exonuclease V) is a heterotrimeric protein with subunits, encoded by the *recB*, *recC*, and *recD* genes, of 134, 129, and 67 kDa, respectively (Finch

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et al., 1986*a,b,c*). This enzyme degrades linear ss- and dsDNA† exonucleolytically; degrades circular ssDNA; has sequence-specific endonuclease activity at χ sites; and unwinds linear dsDNA (for recent reviews, see Taylor, 1988; Smith, 1990). All of these activities of recBCD enzyme are absolutely dependent upon the hydrolysis of ATP except the ssDNA endonuclease activity, which is stimulated by the presence of ATP. The role of recBCD enzyme in this pathway of recombination is clearly critical since null mutations in either the *recB* or *recC* gene reduce recombination frequencies 10^2 to 10^3-fold in the absence of activation of the alternate RecE or RecF pathways (Emmerson, 1968; Willetts & Mount, 1969).

Despite the demonstrated requirement for the *recB* and *recC* gene products in recombination, the function of the enzyme's non-specific nuclease activities in recombination remains obscure. Intuitively, such degradative activities would appear to be anti-recombinogenic. Since recBCD enzyme can degrade both linear (Goldmark & Linn, 1972) and circular (Buttin & Wright, 1968; Goldmark & Linn, 1970) ssDNA as well as dsDNA which contains an end (Goldmark & Linn, 1972) or a gap (Karu *et al.*, 1973; Prell & Wackernagel, 1980), putative recombination intermediates having an end or a region of ssDNA would be susceptible to its degradative activities. Though it is plausible that these nucleolytic activities may act to resolve Holliday junctions, direct biochemical evidence for the participation of recBCD enzyme in the processing of recombination intermediates does not exist. In fact, recBCD enzyme is unable to cleave, in a genetically significant fashion, either D-loops in the presence of recA or SSB protein (Williams *et al.*, 1981; Dixon & Kowalczykowski, 1991), recombination intermediates containing Holliday junctions made by recA protein (Müller *et al.*, 1991), or synthetic cruciforms (Taylor & Smith, 1990) *in vitro*. The participation of recBCD enzyme in this resolution process has recently been cast further in doubt by the isolation of an *E. coli* protein, the product of the *ruvC* gene, which specifically cleaves such substrates *in vitro* (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991).

Since recBCD enzyme does not appear to have a role in processing recombination intermediates, current studies have focused on its effect on the initiation of homologous recombination. As proposed by Smith *et al.* (1981, 1984, 1987), recBCD enzyme is envisioned as a helicase which concomitantly degrades duplex DNA. When the enzyme encounters a χ site in the proper orientation (i.e. the site is encountered from the 3′ side of the strand containing 5′-GCTGGTGG-3′), the enzyme makes a specific nick in this strand. Subsequent unwinding of DNA beyond the site results in the release of a ssDNA molecule having a 3′ end, which is utilized by recA protein to promote heteroduplex DNA formation.

This model does not specifically address how the nuclease activities of recBCD enzyme are compatible with *in vivo* recombination. Recent biochemical studies have shed light on this paradox. Dixon & Kowalczykowski (1991) have examined the formation of joint molecules by the combined actions of the recBCD, recA, and SSB proteins. Using differential labeling of the strands of a linear duplex molecule, they demonstrated that the dsDNA exonuclease activity of wild-type recBCD enzyme is asymmetric, with the 3′-terminal strand, relative to the entry site, being degraded to a greater extent than the 5′-terminal strand, which is infrequently nicked. Since these reactions were performed in the presence of $\geqslant 1$ mM-ATP, this observation agrees with the previous suggestion of Eichler & Lehman (1977) that one strand of a duplex molecule is degraded preferentially by recBCD enzyme at high ATP concentration. Furthermore, the 3′ strand-specific nuclease activity is significantly attenuated after recBCD enzyme encounters a properly oriented χ site. This result suggests that χ acts to switch recBCD enzyme from a highly nucleolytic entity to one having reduced nuclease activity. Dixon & Kowalczykowski (1991) have thus proposed that the productive interaction with χ is the crucial determinant of whether recBCD enzyme is in an anti-recombinogenic, highly nucleolytic state or a recombinogenic, reduced nuclease state. As a consequence of this interaction, a long, intact 3′-terminal strand which is competent for recA protein-mediated joint molecule formation is generated, in accord with the Smith model. These data are also consistent with the hypothesis of Stahl and co-workers that recBCD enzyme nuclease activity is altered when it encounters a χ site, producing a form of the protein which is activated for recombination (Thaler *et al.*, 1988, 1989; Stahl *et al.*, 1990).

Many mutations in the *recB*, *recC*, and *recD* genes have been generated (Schultz *et al.*, 1983; Chaudhury & Smith, 1984*a,b*; Lundblad *et al.*, 1984; Amundsen *et al.*, 1990). Based upon genetic tests and *in vitro* assays using crude lysates of these strains, the mutations have been assigned to various classes based upon their differential properties. One such mutation is the *recB2109* allele (Amundsen *et al.*, 1990). Phenotypically, a *recB2109 recC*$^+$ *recD*$^+$ strain is severely deficient in conjugal recombination and is refractory to the presence of χ sequences (i.e. recombination is not enhanced when χ is present; Amundsen *et al.*, 1990). An *in vivo* assay for the degradation of linear duplex DNA indicated that the mutant enzyme lacked dsDNA exonuclease activity, although dsDNA exonuclease activity, as well as ssDNA exonuclease and helicase activities, could be detected in crude cell lysates

† Abbreviations used: ds, double-stranded; ss, single-stranded; SSB protein, *E. coli* single-stranded DNA binding protein; $MgOAc_2$, magnesium acetate; PMSF, phenylmethylsulfonyl fluoride; PEG, polyethylene glycol; PEP, phospho(enol)pyruvate; TCA, trichloroacetic acid; χ, Chi sequence (5′-GCTGGTGG-3′).

under certain conditions (Amundsen *et al.*, 1990). Consequently, it was concluded that the ability of recBCD enzyme to produce ssDNA ends (through dsDNA exonuclease or χ-nicking activities) might be essential for recombination *in vivo* (Amundsen *et al.*, 1990).

We have purified the recB^{2109}CD enzyme and have examined its properties *in vitro* to determine which characteristics of this protein are responsible for its recombination deficiency. In this paper, we compare the various nucleolytic activities of the wild-type recBCD and recB^{2109}CD enzymes; the accompanying paper (Eggleston & Kowalczykowski, 1993) addresses the helicase activity of the two enzymes. Our studies reveal that the mutant enzyme possesses significant dsDNA exonuclease activity *in vitro*, but this activity has a requirement for higher ATP concentration than does that of wild-type recBCD enzyme. Both the ATP-dependent ssDNA exonuclease and the ATP-stimulated ssDNA endonuclease activities are reduced somewhat compared to those of wild-type recBCD enzyme. Our studies also confirm a previous report which suggested that the mutant recBCD enzyme has no detectable χ-nicking activity (Amundsen *et al.*, 1990). From these results, we propose that the dsDNA exonuclease activity of recB^{2109}CD enzyme is inhibitory to recombination, and that the regulatory process (i.e. the recognition of χ) which would convert the enzyme to a recombination-proficient entity is defective.

2. Materials and Methods

(a) *Strains*

E. coli strains V1000, which contains a derivative of pDWS2 (Ponticelli *et al.*, 1985) into which was cloned the 18·5 kb chromosomal *Bam*HI fragment spanning the *thyA-argA* region of a *recB2109* strain, and V320, which carries a plasmid containing this *Bam*HI fragment from a wild-type strain (Roman & Kowalczykowski, 1989*a*), were a kind gift from Drs G. Smith and S. Amundsen of the Fred Hutchinson Cancer Research Center. *E. coli* strain RLM727 was provided by Dr R. McMacken, The Johns Hopkins University.

(b) *DNA isolation*

Unlabeled plasmid DNAs were prepared according to the alkaline lysis and cesium chloride gradient procedures of Sambrook *et al.* (1989). Unlabeled M13mp7 ss- and dsDNAs were isolated using the methods of Messing (1983). Phage λ DNA was purchased from New England Biolabs. The concentration of nucleotides was determined using extinction coefficients of 6500 and 8780 M^{-1} cm^{-1} at 260 nm for dsDNA and ssDNA, respectively.

To prepare ^{3}H-labeled M13 dsDNA, overnight cultures of JM101 were grown at 37°C in M9 media (Sambrook *et al.*, 1989) supplemented with 0·2% (w/v) glucose and 0·0001% (w/v) thiamine and were subcultured at a 1 : 250 dilution into TPG media (per l: 1·1 g NH_4Cl, 1 g KH_2PO_4, 0·5 g NaCl, 8 g KCl, 0·8 g sodium pyruvate, 5·2 g $Na_2SO_4 \cdot 10\ H_2O$, 10 g Casamino acids, 100 mM-Tris·HCl (pH 7·5), 1 mM-$MgCl_2$; pH to 7·4 with NaOH) containing 0·1 μg $FeCl_3 \cdot 6\ H_2O$/ml, 0·2% (w/v) glucose, 1 mM-$CaCl_2$, 1 μg thymine/ml, and 0·0001% (w/v) thiamine. When these cultures reached an A_{595} of 0·4 to 0·6, the cells were infected with M13mp7 phage at a multiplicity of infection of 10, and the following solutions were added: 0·1 vol. 10 mM-adenosine, 0·1 vol. 10 mM-deoxyadenosine, and 1·25 mCi [^{3}H]methyl thymidine (5 Ci/mmol; ICN). After 2 h of growth, the cells were harvested and were processed as described above for unlabeled DNA preparations. Alternatively, tritiated M13 DNA was prepared by incubating 12·5 μg of *Eco*RI-linearized M13 dsDNA, 56 μCi of *S*-adenosyl L-[methyl-^{3}H]methionine (73 Ci/mmol; NEN), and 4 units of *Sss*I methylase (New England Biolabs) in 1 × NEB2 buffer (10 mM-Tris·HCl (pH 7·9), 10 mM-$MgCl_2$, 50 mM-NaCl, 1 mM-DTT) at a final volume of 250 μl for 2 h at 37°C. The DNA was precipitated once with ammonium acetate and ethanol and was resuspended in TE.

^{3}H-labeled *E. coli* chromosomal DNA was isolated as a by-product of a ^{3}H-labeled M13 dsDNA preparation. In this case, the cell pellet was lysed according to the detergent method of Sambrook *et al.* (1989), and the cellular debris was collected by centrifugation. This pellet, which contained the chromosomal DNA, was resuspended in 2 vol. sucrose buffer (25% (w/v) sucrose, 50 mM-Tris·HCl (pH 8·0), 10 mM-EDTA) and was treated with 10 mg RNase at 37°C for 1 h. Next, a 20 mg/ml solution of protease (type XIV; Sigma) was added to a final concentration of 1 mg/ml, and the mixture was incubated at 37°C for 1 h. After centrifugation at 48,000 ***g*** for 30 min, the supernatant was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and was precipitated with ethanol. The DNA pellet was resuspended in TE and was sheared by sonication into fragments predominantly 1 to 4 kb in length as judged by ethidium bromide staining of a 1% (w/v) agarose gel.

(c) *Proteins*

Wild-type recBCD enzyme was purified from strain V320 as previously described (Roman & Kowalczykowski, 1989*a*), except that the protein was eluted from the ssDNA cellulose column with 2 M, rather than 700 mM, NaCl. RecB^{2109}CD enzyme was purified as described below. The concentration of both enzymes was determined using an extinction coefficient at 280 nm of $4{\cdot}0 \times 10^5$ M^{-1} cm^{-1} (Roman & Kowalczykowski, 1989*a*). The specific activity of the wild-type enzyme preparation used in these experiments was $3{\cdot}1 \times 10^5$ nuclease units/mg protein using the assay conditions (i.e. 50 mM-Tris·HCl (pH 8·0), 10 mM-$MgCl_2$, 1 mM-DTT, 25 μM-ATP, 1 mg BSA/ml, 5 pmol (16·7 μM-nucleotide) sonicated, ^{3}H-labeled chromosomal DNA) of Eichler & Lehman (1977); under these conditions, the activity of the mutant enzyme is essentially zero. To obtain a relative specific activity for the mutant enzyme, the assay conditions were altered by raising the ATP concentration to 10 mM to accommodate its requirement for higher ATP concentrations. Under the modified conditions, wild-type recBCD enzyme has a specific activity of $1{\cdot}2 \times 10^5$ nuclease units/mg protein, compared to $5{\cdot}4 \times 10^3$ nuclease units/mg protein for recB^{2109}CD enzyme. (It should be noted that the data presented in Table 1 were obtained under slightly different conditions, as indicated.)

To permit a direct comparison of the kinetic parameters, the fraction of active enzyme in each protein preparation was determined from protein titrations of enzymatic activity. Enzyme titrations were performed using the fluorometric helicase assay to determine the

Table 1
Purification of recB^{2109}CD enzyme

Fraction	Volume (ml)	Total protein (A_{280})	Concentration (A_{280}/ml)	Total activity (units)†	Specific activity (units/A_{280})	Purification (-fold)
I. Ammonium sulfate dialyzate	97	$1\cdot3\times10^4$	$1\cdot3\times10^2$	$1\cdot1\times10^5$	8·8	1
II. Q-sepharose	44	$1\cdot2\times10^2$	2·8	$2\cdot9\times10^4$	$2\cdot4\times10^2$	27
III. Hydroxylapatite	66	56·8	0·86	$4\cdot1\times10^4$	$7\cdot2\times10^2$	82
IV. ssDNA cellulose	12·3	0·39	0·32	$5\cdot2\times10^3$	$1\cdot3\times10^4$	$1\cdot5\times10^3$

† One unit digests 1 pmol DNA in 20 min at 37°C in buffer containing 25 mM-Tris-acetate (pH 7·5), 3 mM-ATP, 5 mM-MgOAc$_2$, 1 mM-DTT, and 6 pmol (20 μM-nucleotide) sonicated chromosomal DNA.

fraction of each preparation that was competent for duplex DNA unwinding (Roman & Kowalczykowski, 1989*a*). In brief, 10 μM-nucleotide (0·69 nM-molecule) linearized M13 DNA was incubated with various concentrations of enzyme. At concentrations of protein less than 4·1 nM for the wild-type enzyme or 6·9 nM for the mutant enzyme, the rate of unwinding increases linearly; in excess of these concentrations, the rate is unchanged, indicating saturation of the DNA substrate (see Eggleston & Kowalczykowski (1993), for details). These titrations indicate that the wild-type recBCD enzyme preparation saturates the DNA at an apparent stoichiometry of binding of 3 molecules/end, while that of the recB^{2109}CD enzyme preparation is 5 molecules/end. All reactions and the derived rate constants in this paper comparing the wild-type and mutant enzymes have been normalized for these values, assuming that one recBCD heterotrimer is the functional unit. It should be noted that normalization of enzymatic results in such a manner yields values for kinetic parameters (e.g. k_{cat}) that are invariant for preparations of wild-type recBCD enzyme that range in activity from ~6% active (i.e. 16 recBCD enzyme molecules/end) to ~50% active (i.e. 2 recBCD enzyme molecules/end) (Roman & Kowalczykowski, 1989*a*; Dixon & Kowalczykowski, 1991; Eggleston & Kowalczykowski, 1993).

SSB protein was purified from strain RLM727 as described (LeBowitz, 1985); its concentration was determined using an extinction coefficient at 280 nm of $3\cdot0\times10^4$ M^{-1} cm^{-1}. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs, Pharmacia, and Boehringer Mannheim Biochemicals.

Standard reaction buffer conditions, unless otherwise noted, consisted of: 25 mM-Tris-acetate (pH 7·5), 1 mM-ATP, 1 mM-MgOAc$_2$, and 1 mM-DTT.

(d) *Purification of recB^{2109}CD enzyme*

RecB^{2109}CD enzyme was purified using a modification of a previously described procedure (Roman & Kowalczykowski, 1989*a*). The strain V1000 was grown at 37°C in 10 l of LB broth (Sambrook *et al.*, 1989) supplemented with 0·2% glucose and 50 μg ampicillin/ml until it reached early stationary phase. The cells (~50 g wet weight) were resuspended in 4 vol. buffer A (100 mM-Tris·HCl (pH 8·0), 1 mM-EDTA, 1 mM-DTT) and were lysed by 2 passages through a French pressure cell (Aminco) at 14,000 lb in^2. After the first passage, PMSF (0·1 M in ethanol) was added to 1 mM final concentration. Cellular debris was pelleted by centrifugation at 48,000 ***g*** for 90 min. The soluble protein was precipitated by adding 0·28 g solid ammonium sulfate/ml followed by centrifugation at 7700 ***g*** for 20 min. The pellet was resuspended in buffer B (20 mM-Tris·HCl (pH 7·5), 0·1 mM-EDTA, 0·1 mM-DTT) containing 100 mM-NaCl and was dialyzed against the same buffer. The protein was applied to a 90 ml (4·91 cm^2 × 18·3 cm) Q-sepharose column (Pharmacia) and was eluted with a 900 ml gradient of 100 mM to 600 mM-NaCl in buffer B. The peak fractions, which eluted at ~350 mM-NaCl, were pooled and concentrated against solid PEG (20,000 M_r; Sigma). The concentrated sample was dialyzed against buffer C (50 mM-potassium phosphate (pH 7·0), 0·1 mM-DTT) and was applied to a 25 ml (4·91 cm^2 × 5·1 cm) hydroxylapatite column (BioRad) equilibrated in buffer C. Unlike wild-type recBCD enzyme, the mutant enzyme did not bind to this resin and was recovered in the flow-through fraction; this step was retained, however, since it effectively removes residual ATP-independent nuclease activity (unpublished observation). The pooled flow-through fractions were dialyzed against 100 mM-NaCl in buffer B and applied to a 10 ml (4·91 cm^2 × 2 cm) ssDNA cellulose column (made according to the procedure of Litman, 1968). This column was washed sequentially with buffer B containing 100 mM, 700 mM, or 2 M-NaCl. At various times, both wild-type recBCD and recB^{2109}CD enzymes have eluted in either the 700 mM or the 2 M-NaCl fraction; greater purity is observed when the protein elutes with higher salt (unpublished observation). The appropriate fraction was immediately dialyzed against buffer B containing 100 mM-NaCl to prevent the loss of activity through dissociation of the subunits at high salt concentration (Lieberman & Oishi, 1974). The dialyzate was then concentrated with a Centriprep30 filtration device (Amicon) and was made 50% in glycerol by adding 1·5 vol of buffer B containing 100 mM-NaCl and 80% glycerol; samples were stored at −80°C until needed. Once thawed, the protein was stored at 4°C, with no loss in activity observed for at least one month.

Initial purifications of the mutant enzyme employed a fluorometric helicase assay (Roman & Kowalczykowski, 1989*a*; Eggleston & Kowalczykowski, 1993) to monitor column fractions for activity. In later preparations, once we had determined that the mutant enzyme possessed significant ATP-dependent dsDNA exonuclease activity, column fractions were assayed for this activity using modified nuclease conditions (25 mM-Tris-acetate (pH 7·5), 3 mM-ATP, 5 mM-MgOAc$_2$, 1 mM-DTT, 20 μM-nucleotide ^{3}H-labeled, sonicated chromosomal DNA). Both methods for monitoring activity gave similar profiles throughout the course of the purification. The final yield of recB^{2109}CD enzyme is generally 7 μg/g cells with an approximately 1500-fold purification achieved (Table 1). From Coomassie-stained SDS/polyacrylamide gels, we estimate that the homogeneity of various recB^{2109}CD enzyme preparations ranges from 50 to

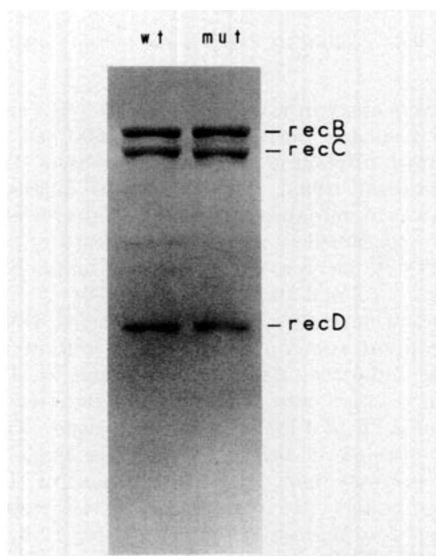


Figure 1. SDS/polyacrylamide gel analysis of wild-type and mutant recBCD enzyme preparations. Samples (2·5 μg) of each protein preparation were separated on a 7·5% SDS/polyacrylamide gel and stained with Coomassie blue. The individual subunits are indicated for the wild-type recBCD enzyme (wt) and recB^{2109}CD enzyme (mut).

>95%. The experiments reported in this paper and in the accompanying paper (Eggleston & Kowalczykowski, 1993) employed mutant enzyme which was > 95% pure (Fig. 1). Two points can be made concerning the mutant enzyme on the basis of this gel. First, although the *recB2109* mutation has been neither mapped nor sequenced, this gel indicates that the mutation does not give rise to a truncated recB subunit. Second, since the 3 subunits of the purified mutant enzyme are present in approximately stoichiometric amounts (1 : 1 : 1), similar to wild-type recBCD enzyme, the protein–protein interactions of the mutant subunit within the heterotrimer have not been apparently altered.

(e) *TCA solubility nuclease assays*

The release of TCA soluble oligonucleotides was used to assay both the ss- and dsDNA exonuclease activities of the enzymes. For the dsDNA exonuclease assay, the reactions were conducted in a 300 μl volume at either 25°C or 37°C, using standard buffer conditions unless otherwise indicated. The ^{3}H-labeled DNA substrate (sonicated chromosomal DNA or linear M13 dsDNA) was present at 20 μM-nucleotide. The reaction was preincubated in the absence of recBCD enzyme for 4 min at the appropriate temperature, and the assay was initiated by the addition of recBCD enzyme to the concentration indicated. To allow direct comparison, equivalent functional concentrations (based on the ability to bind the ends of duplex DNA; see above) of wild-type and mutant recBCD enzyme were used. The reaction was stopped by the addition of 15 μl of a 2·5 mg/ml calf thymus DNA solution and 300 μl of a 15% TCA solution, followed by storage on ice for at least 5 min. This concentration of TCA exceeds that which will precipitate oligonucleotides ⩾ 16 bases with 50% efficiency (Cleaver & Boyer, 1972). The samples were centrifuged at 4°C for 15 min at 13 000 *g*, and the radioactivity present in 500 μl of supernatant was determined in 5 ml of aqueous scintillation fluid (RPI).

The ssDNA exonuclease assays were performed with heat-denatured phage λ DNA that was 5′ end-labeled with [γ-^{32}P]ATP (4000 Ci/mmol; ICN) using T4 polynucleotide kinase according to the manufacturer's directions. Unincorporated nucleotide was removed by 2 sequential ammonium acetate/ethanol precipitations, and the DNA was resuspended in TE. The reaction (25 mM-Tris-acetate (pH 7·5), 1 or 10 mM-ATP, 1 or 10 mM-MgOAc$_2$, 1 mM-DTT, 20 μM-nucleotide (0·21 nM-molecule) heat-denatured, 5′ end-labeled λ DNA, and 4 μM-SSB protein) was equilibrated at 37°C. After adding 0·5 nM-functional recBCD enzyme (i.e. 1·5 nM-wild-type recBCD enzyme or 2·5 nM-recB^{2109}CD enzyme) to start the reaction, 30 μl portions were withdrawn at the indicated times and were added to 300 μl of a 0·125 mg/ml calf thymus DNA solution. These samples were then processed as described above. We do not believe that our detection of TCA soluble counts in this assay is due to phosphatase contamination since incubation of end-labeled substrates with enzyme in the absence of ATP does not result in loss of signal when the substrate is examined on an agarose gel (e.g. Fig. 8B).

In all of these assays, the raw data were corrected for the TCA soluble radioactivity present before the addition of recBCD enzyme; the reported values for % TCA soluble counts were determined by subtracting the soluble counts present in a comparable reaction to which no enzyme was added from the raw value, dividing this corrected value by the total number of counts present in 500 μl of supernatant (obtained by substituting H_2O for the TCA solution or by adding the TCA solution but omitting the centrifugation), and multiplying by 100. These values represent the average of 2 or more experiments and have an error of ±10%.

(f) *Determination of free Mg^{2+} ion concentration*

The concentration of free Mg^{2+} was determined using the CHELATOR program (Schoenmakers *et al.*, 1992). The reaction conditions used in the equilibrium calculation were as follows: temperature, 37°C; buffer, Tris (pH 7·5) at 20 mM; ionic strength, 4 mM; 1 or 10 mM-ATP; varying MgOAc$_2$ concentration. The free Mg^{2+} concentration values were plotted against the rate data from Fig. 4A to give Fig. 4B.

(g) *ssDNA endonuclease agarose gel assay*

The 250 μl reaction contained 25 mM-Tris-acetate (pH 7·5), 10 mM-MgOAc$_2$, 100 μg BSA/ml, 1 mM-DTT, 1·5 mM-PEP, 4 units pyruvate kinase/ml, 60 μM-nucleotide (8·3 nM-molecule) M13 ssDNA, and 1 mM-ATP when indicated. After preincubation at 37°C, the assay was begun with the addition of 0·5 nM-functional recBCD enzyme. At the indicated times, a 40 μl sample was removed and added to 8 μl of 5 × loading buffer (25% (w/v) Ficoll, 0·4% (w/v) bromophenol blue, 0·4% (w/v) xylene cyanol, 5% (w/v) SDS, 50 mM-EDTA). The samples were electrophoresed on a 1% (w/v) agarose gel in 1 × TBE (89 mM-Tris-borate, 1 mM-EDTA) at 1·1 V/cm for 30 h in order to separate the circular and linear ssDNA species present in the starting substrate. The amount of circular ssDNA remaining at the indicated times was determined by scanning and quantification of photographic negatives using a BioImage system (Millipore). The values reported were normalized to the zero time point and represent the average of at least 3 independent experiments; the error of the values is indicated in Fig. 5.

(h) *χ-nicking assay*

pBR322 DNA without (χ^0) or with (χ^+) the χ^+F sequence (Taylor *et al.*, 1985) was linearized with *Ava*I restriction enzyme and was 5′ end-labeled with [γ-^{32}P]ATP as described above. The resultant linear molecule has the χ sequence located ~ 4290 bp from the duplex end that is properly oriented with regard to χ recognition (Fig. 7A). The reaction was conducted in standard buffer with 10 μM-nucleotide (1·04 nM-molecule) 5′ end-labeled *Ava*I-digested pBR322 DNA (χ^0 or χ^+) and 2 μM-SSB protein. This concentration of SSB protein is in 3-fold excess to the amount required to bind the ssDNA available if all of the DNA is completely unwound; it was included to reduce recBCD enzyme-mediated degradation of the ssDNA fragments generated (MacKay & Linn, 1976). Unwinding was initiated by the addition of 0·5 nM-functional recBCD enzyme; at the indicated times, a 35 μl sample was removed and the reaction was quenched with 9 μl of 5 × loading buffer. The DNA was separated on a 7·5% (w/v) denaturing polyacrylamide gel run in 1 × TBE at 100 V for 7 h. The gel was fixed in 7·5% (v/v) acetic acid for 1 h before being dried onto Whatman 3 mm paper and exposed for autoradiography.

(i) *Single χ substrate dsDNA exonuclease agarose gel assay*

*Bam*HI-digested M13 dsDNA was 5′ end-labeled as described above. 10 μM-nucleotide (0·69 nM-molecule) of this DNA was added to a mixture containing 25 mM-Tris-acetate (pH 7·5), 1 mM-DTT, 2 μM-SSB protein, and the indicated concentrations of ATP and $MgOAc_2$. The reaction was preincubated at 37 °C, and the assay was begun with the addition of 0·5 nM-functional recBCD enzyme. At the indicated times, a 40 μl sample was removed, and the reaction was stopped by the addition of 9 μl of 5 × loading buffer. The samples were separated by electrophoresis through an 0·8% neutral agarose gel in 1 × TAE (40 mM-Tris-acetate, 1 mM-EDTA) at 2·1 V/cm for 8 h and were visualized by autoradiography of the dried gel.

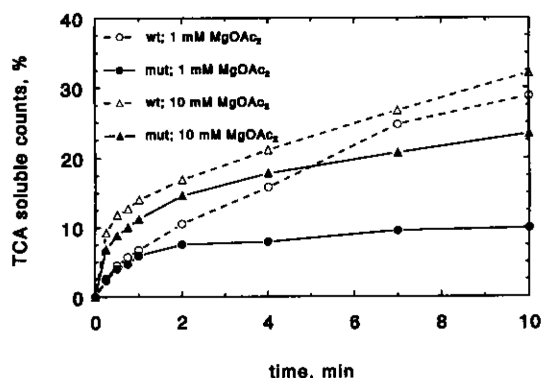


Figure 2. Double-strand DNA exonuclease activity of wild-type and mutant recBCD enzymes. The degradation of uniformly labeled, *Eco*RI-digested M13 dsDNA (20 μM-nucleotide; 1·38 nM-molecule) was assayed at 37 °C in standard buffer containing either 1 mM-ATP, 1 mM-$MgOAc_2$ (triangles) or 1 mM-ATP, 10 mM-$MgOAc_2$ (circles), using the production of TCA soluble oligonucleotides as a measure of nuclease activity. Reactions were initiated by adding 0·5 nM-functional wild-type recBCD enzyme (open symbols) or recB^{2109}CD enzyme (closed symbols).

(j) *Double χ substrate dsDNA exonuclease agarose gel assay*

A χ-cutting assay in which the pBR322 DNA substrate contained χ sites at positions 1493 to 1500 and 3549 to 3556 (termed pBR322 χ^+FH) was also used (Dixon & Kowalczykowski, 1993). This DNA was digested with *Nde*I and was 5′ end-labeled as described above. The 40 μl reaction contained 25 mM-Tris-acetate (pH 7·5), 1 mM-DTT, 1 or 8 mM-$MgOAc_2$, 16 units pyruvate kinase/ml, 2 mM-PEP, 2 μM-SSB protein, 10 μM-nucleotide (1·04 nM-molecule) labeled DNA, and varying concentrations of wild-type or mutant recBCD enzyme as indicated. After preincubation at 37 °C for 4 min, the reaction was synchronously initiated by the addition of ATP to 1 mM final concentration. The reaction was stopped at either 2 (wild-type enzyme) or 5 (mutant enzyme) min by the addition of 10 μl of 5 × loading buffer. The reactions were electrophoresed through a 1·2% agarose gel at 2 V/cm for 12 h; the gel was then dried and exposed for autoradiography.

3. Results

(a) *RecB ^{2109}CD enzyme has dsDNA exonuclease activity*

The dsDNA exonuclease activity of recBCD enzyme generates ssDNA fragments, which are subsequently acted upon by the ssDNA nuclease activities of the enzyme to produce short oligonucleotides, with a limit digest of ~ 4·5 nucleotides under optimal conditions (Goldmark & Linn, 1972). This degradation of linear duplex DNA is maximal at low concentrations of ATP and at high concentrations of Mg^{2+} (Eichler & Lehman, 1977). Several conditions were reported to inhibit this activity: high levels of ATP (Karu *et al.*, 1973; Eichler & Lehman, 1977), the presence of Ca^{2+} (Wright *et al.*, 1971; Rosamond *et al.*, 1979), and the inclusion of SSB protein, which protects the released ssDNA fragments from degradation (MacKay & Linn, 1976). Consequently, the standard conditions that have been adopted for the assay of this activity contained 25 to 50 μM-ATP and 10 mM-$MgCl_2$ (Eichler & Lehman, 1977).

We wanted to compare the nuclease and DNA helicase activities under similar conditions, however. Since the helicase activity of recBCD enzyme has a requirement for higher ATP concentration (K_m ~ 130 μM; Roman & Kowalczykowski, 1989*a*) than the dsDNA exonuclease activity, nuclease assays on both the wild-type and the mutant enzymes were performed under our standard conditions for unwinding (1 mM-ATP, 1 mM-$MgOAc_2$), conditions which are also more physiological (Lowry *et al.*, 1971; Mathews, 1972; Bochner & Ames, 1982). In doing so, we detected significant recB^{2109}CD enzyme-dependent dsDNA exonuclease activity, as shown in the time course of nucleolytic degradation of linear M13 dsDNA (Fig. 2). At equimolar concentrations of ATP and Mg^{2+} (1 mM each), both enzymes degrade the duplex DNA with similar initial rates, although the activity of the mutant enzyme appears to plateau while that of the wild-type enzyme continues to

increase (Fig. 2, circles). When the Mg^{2+} concentration (10 mM) is in excess of the ATP concentration (1 mM), a situation which enhances the nuclease activity (Wright *et al.*, 1971; Friedman & Smith, 1972), the dsDNA is degraded to a greater extent by both enzymes (Fig. 2, triangles). Again, however, the activity of the mutant enzyme is less than that of the wild-type enzyme.

(b) *The ATP concentration dependence of recB^{2109}CD enzyme dsDNA exonuclease activity is altered*

Early studies of wild-type recBCD enzyme reported that the extent of dsDNA exonuclease activity of wild-type recBCD enzyme is inhibited ~ 50% at ATP concentrations greater than 200 μM (Wright *et al.*, 1971; Eichler & Lehman, 1977). Consequently, the effect of varying ATP concentration on the hydrolysis of linear M13 dsDNA was examined. No significant ATP-independent activity (< 1%) was detected in either enzyme preparation. At 1 mM-$MgOAc_2$, the peak of activity with wild-type recBCD enzyme is observed at 50 μM-ATP, the lowest concentration tested; higher ATP levels increasingly inhibit the reaction until the activity is completely abolished as the nucleotide concentration exceeds that of the $MgOAc_2$ concentration (Fig. 3A, open symbols). The profile of recB^{2109}CD enzyme-promoted dsDNA exonuclease activity (Fig. 3A, closed symbols) displays a different ATP dependence, however. This activity does not reach its maximum, which is approximately half that of the wild-type recBCD enzyme, until 500 μM-ATP is added. Concentrations of ATP which exceed that of the $MgOAc_2$ concentration are inhibitory in a manner similar to that observed with wild-type enzyme. At 10 mM-$MgOAc_2$, the same trends are observed for both enzymes, with high levels of activity occurring over a broader concentration of ATP; the maximal rate of degradation, however, is not significantly different from that observed at 1 mM-$MgOAc_2$ (Fig. 3B).

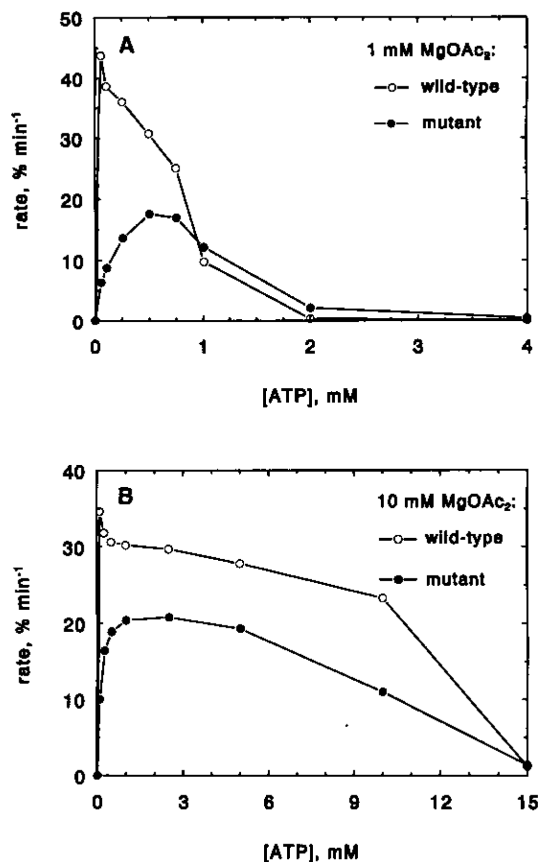


Figure 3. Effect of ATP concentration on the dsDNA exonuclease activity of wild-type and mutant recBCD enzymes. Reactions were performed as described in the legend to Fig. 2, except that ATP concentration was varied at a fixed concentration of $MgOAc_2$, either 1 mM (A) or 10 mM (B). Wild-type recBCD enzyme reactions are indicated by open symbols; recB^{2109}CD enzyme reactions by closed symbols.

(c) *RecB^{2109}CD enzyme dsDNA exonuclease activity has a free Mg^{2+} concentration requirement similar to that of wild-type recBCD enzyme*

Since the ratio of Mg^{2+} concentration to ATP concentration, rather than the absolute concentrations of these components, appeared to influence the dsDNA exonuclease activity of recBCD enzyme (Fig. 3A, B), the Mg^{2+} dependence of the reaction was examined at two elevated levels of ATP. As shown in Figure 4A, this nuclease activity of both enzymes minimally requires a ratio of at least 0·75 Mg^{2+} per ATP molecule, regardless of whether the ATP concentration is 1 mM (circles) or 10 mM (triangles). The maximal rate of nuclease activity is not affected by the concentration of ATP, but the range of Mg^{2+} over which the activity is exhibited does depend on the concentration of ATP.

These results suggested that Mg^{2+} is not merely required to form a complex with ATP; free Mg^{2+} enhances the nuclease activity of both enzymes, although the extent of this enhancement ultimately saturates. To determine how the concentration of free Mg^{2+} varied under these conditions, the CHELATOR program (Schoenmakers *et al.*, 1992) was used to calculate the equilibrium concentrations of this ion. The results of these calculations were then used to generate Figure 4B, in which the rate of dsDNA exonuclease activity is plotted as a function of free Mg^{2+} in the presence of either 1 or 10 mM-ATP, concentrations of ATP which are sufficient for the mutant enzyme to exhibit activity. This graph clearly demonstrates that, regardless of the ATP concentration, the activity of both the wild-type and mutant enzymes is dependent only on the concentration of free Mg^{2+} present. Concentrations of free Mg^{2+} greater than 10 μM are required to observe a significant increase in the activity of both enzymes, although concentrations greater than ~ 200 μM are required by both enzymes for nearly maximal activity. We have conducted many of our experiments at 1 mM-Mg^{2+},

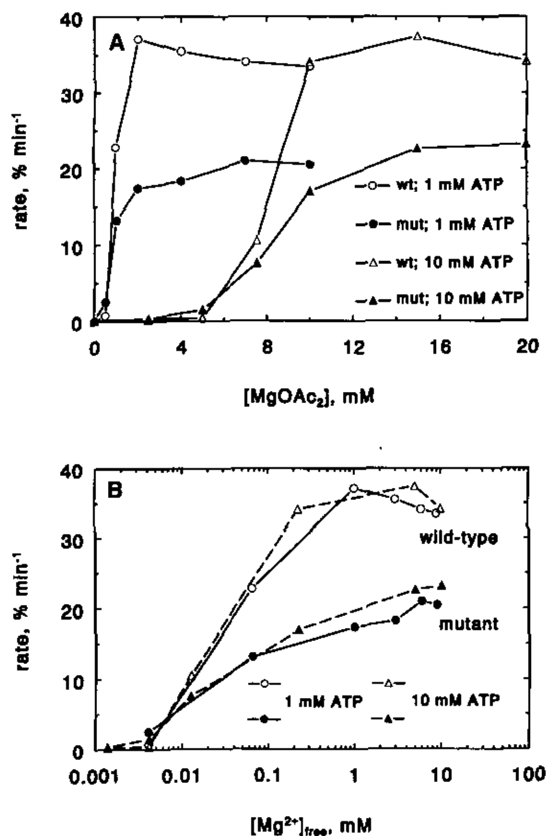


Figure 4. Effect of $MgOAc_2$ concentration on the dsDNA exonuclease activity of wild-type and mutant recBCD enzyme. The reactions in A were performed as described in the legend to Figure 2, except that $MgOAc_2$ concentration was varied at a fixed concentration of ATP, either 1 mM (circles) or 10 mM (triangles). The concentration of free Mg^{2+} was determined as described in Materials and Methods, and these values were used to generate a graph in which dsDNA exonuclease activity is plotted as a function of free Mg^{2+} (B). Wild-type recBCD enzyme reactions are indicated by open symbols, and $recB^{2109}CD$ enzyme reactions by closed symbols.

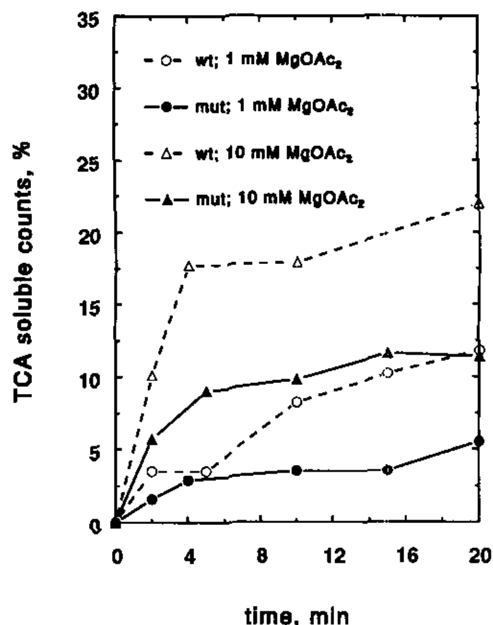


Figure 5. Single-strand DNA exonuclease activity of wild-type and mutant recBCD enzymes. Reactions were conducted as described in the legend to Fig. 2 except that the dsDNA was replaced by 5′ end-labeled, heat-denatured λ DNA (20 μM-nucleotide; 0·21 nM-molecule). The conditions used were: 1 mM-ATP, 1 mM-$MgOAc_2$ (circles) or 1 mM-ATP, 10 mM-$MgOAc_2$ (triangles). Wild-type enzyme reactions are indicated by open symbols, and mutant enzyme reactions are indicated by closed symbols.

but since the intracellular concentration of this ion is not known and may be as high as 5 mM, we have also performed experiments at higher Mg^{2+} concentrations in order that we may bracket the extremes of activity which may be manifest *in vivo*.

(d) *$RecB^{2109}CD$ enzyme has ATP-dependent ssDNA exonuclease activity*

In a manner analogous to the dsDNA exonuclease activity, the ssDNA exonuclease activity of recBCD enzyme produces the same limit digest of ~ 4·3 nucleotides (Goldmark & Linn, 1972) and is believed to occur processively (Goldmark & Linn, 1970). Unlike the dsDNA exonuclease activity, however, the ssDNA exonuclease activity is not sensitive to high concentrations of ATP (Goldmark & Linn, 1972; Eichler & Lehman, 1977). Consequently, we expected that the ssDNA exonuclease activity of the two enzymes might be similar at high ATP concentration if one defect of the mutant enzyme resides in its affinity for ATP, as suggested by Figure 3.

The use of uniformly labeled ssDNA in an assay of recBCD enzyme-promoted ssDNA exonuclease activity may give a falsely high signal due to the presence of its ATP-stimulated ssDNA endonuclease activity. To decrease the probability of this occurring, heat-denatured, 5′ end-labeled λ DNA was employed as the substrate to increase the proportion of internal base-pairs to DNA ends and thus to minimize the possibility that the ^{32}P end-label would be released as a TCA soluble fragment due to an endonucleolytic cut. When this substrate was used, $recB^{2109}CD$ enzyme (Fig. 5, closed symbols) was still found to have approximately half the activity of wild-type recBCD enzyme (Fig. 5, open symbols) under conditions of 1 mM-ATP and either 1 or 10 mM-$MgOAc_2$.

(e) *$RecB^{2109}CD$ enzyme has ATP-stimulated ssDNA endonuclease activity*

Since the ssDNA endonuclease activity of recBCD enzyme is stimulated by, rather than dependent upon, a nucleotide cofactor (Goldmark & Linn, 1972), this activity was examined in both the presence and the absence of ATP by measuring the disappearance of circular M13 ssDNA on an agarose

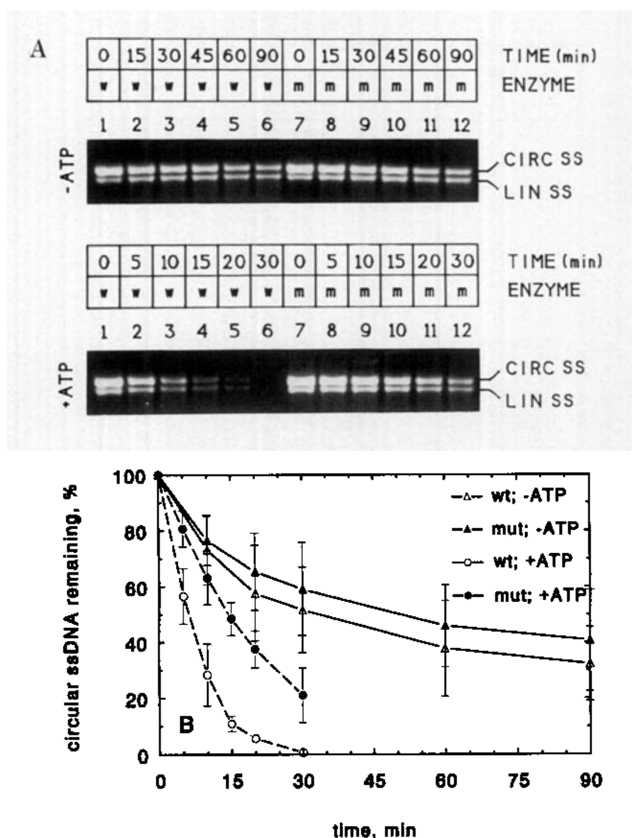


Figure 6. Single-strand DNA endonuclease activity of wild-type and mutant recBCD enzymes. Reactions were conducted at 37 °C in standard buffer which either lacked (A: top panel) or contained (A: bottom panel) 1 mM-ATP; the substrate was M13 ssDNA (60 μM-nucleotide; 8·3 nM-molecule). The positions of the circular and linear ssDNA species are indicated. Photographic negatives of gels such as those in A were quantified, and the graphical results are displayed in B. The reactions performed in the absence of ATP are represented by triangles; those performed in the presence of ATP are represented by circles. In each case, the wild-type enzyme reactions are indicated by open symbols, while those of the mutant enzyme are indicated by closed symbols.

gel (Fig. 6A); the resulting linear ssDNA is also degraded by the ssDNA exonuclease activity of recBCD enzyme. In the absence of ATP and at 10 mM-$MgOAc_2$ (Fig. 6B, triangles), the initial rates of degradation of circular ssDNA for both wild-type and mutant recBCD enzymes are approximately equal (0·22 *versus* 0·20 scissions min^{-1}, respectively), and the extents at 90 minutes are similar (41% *versus* 32%, respectively). When 1 mM-ATP is added (Fig. 6B, circles), the initial rate of degradation of the wild-type enzyme increases threefold (to 0·72 scissions min^{-1}) while that of the mutant enzyme increases less than twofold (0·32 scissions min^{-1}). By 30 minutes all of the circular ssDNA has been nicked by wild-type recBCD enzyme, but only ~80% of that in the mutant enzyme reaction has been nicked. Our observation that the ATP-dependent component of this activity of the mutant enzyme is reduced compared to the wild-type enzyme, in a manner similar to the reductions in dsDNA and ssDNA exonuclease activity, suggests that the ssDNA endonuclease activity is intrinsic to recBCD enzyme and is not due to a contaminating protein.

(f) *RecB^{2109}CD enzyme does not nick at the χ sequence*

When wild-type recBCD enzyme encounters the asymmetric χ sequence in the correct orientation (i.e. from the 3′ side of the sequence 5′-GCTGGTGG-3′), it nicks the χ-containing strand 4 to 6 bp to the 3′ side of the site and continues unwinding the DNA (Ponticelli *et al.*, 1985; Taylor *et al.*, 1985). This interaction attenuates the 3′-terminal strand-specific nuclease activity of the enzyme (Dixon & Kowalczykowski, 1991, 1993).

The ability of the mutant enzyme to nick at the χ sequence was examined by Amundsen *et al.* (1990) using a 3′ end-labeled, 930 bp fragment in which the labeled χ-specific bands derived from the 3′ side of χ. On this substrate, a crude cell extract from a *recB2109* strain was found to be deficient in χ-cutting. The observed inactivity of the mutant enzyme in this assay could have resulted from one of two causes: the inability of the mutant enzyme to recognize and/or to cut at the χ sequence, or enhanced non-specific degradation of the DNA 3′ to the χ site by the mutant enzyme (relative to the wild-type) under these conditions, thus causing release of the label as a non-specific ssDNA fragment. Consequently, a more stringent assay was developed. The substrate in our assay is a 5′ end-labeled pBR322 DNA molecule with the χ sequence closer to the "downstream" end, as diagrammed in Figure 7A. If wild-type recBCD enzyme encounters χ in the correct orientation, as indicated, it will liberate an intact ~80 nucleotide, χ-dependent fragment due to attenuation of the 3′ strand-specific nuclease activity and continued unwinding beyond the χ site (Dixon & Kowalczykowski, 1991, 1993). Under the same conditions, a χ-dependent fragment from "upstream" of the χ sequence is never observed (Dixon & Kowalczykowski, 1991, 1993), indicating that the activity which degrades this strand is attenuated after encountering χ. Thus, this assay has the advantage that detection of the χ-dependent fragment is not obscured by the non-specific nuclease activity of the enzyme 'upstream' of χ. In addition, the proximity of χ to the exit site in this substrate enhances the ability to detect the end-label if recB^{2109}CD enzyme were to cut at χ but still retain its dsDNA exonuclease activity.

When both wild-type and mutant recBCD enzymes are reacted with pBR322 (χ^0) DNA, a background of labeled fragments resulting from non-specific degradation is observed, but no distinct bands are generated (Fig. 7B, lanes 2 to 5). When the χ sequence is present, however, two prominent bands having the predicted size (~80 nucleotides) for nicking at χ appear in the reaction containing wild-type recBCD enzyme (Fig. 7B, lanes 7 and 8),

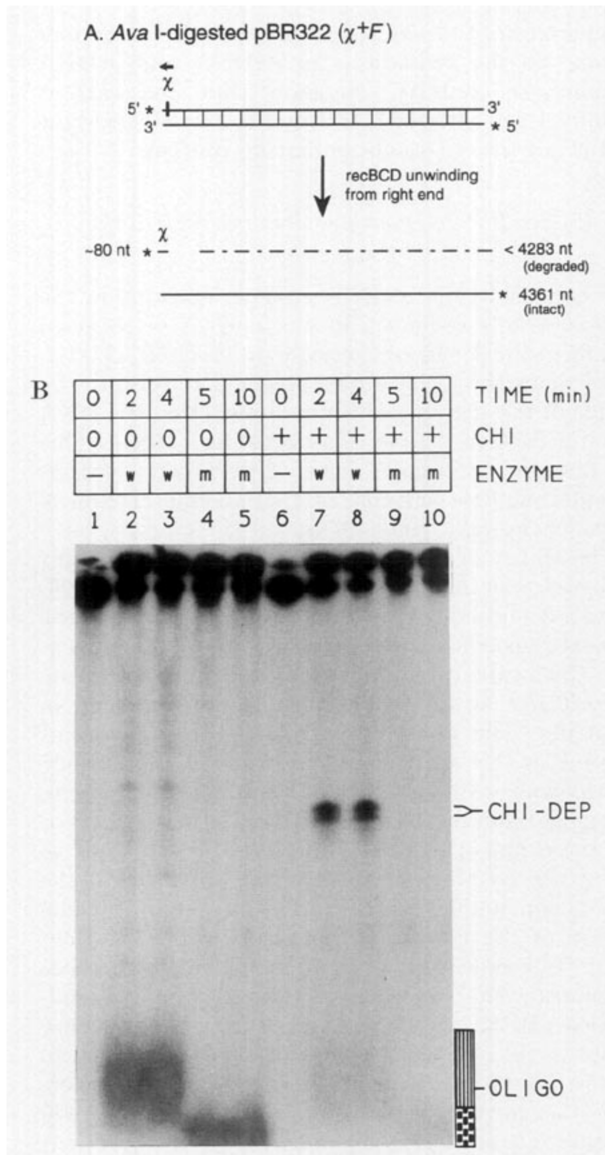


Figure 7. χ-dependent nicking by wild-type and mutant recBCD enzymes. The experimental procedure for detecting cutting at the χ sequence by recBCD enzyme is diagrammed in A. The χ sequence within *Ava*I-digested pBR322 DNA is located at a distance ~ 4290 bp from the end of molecule which will lead to the recognition of χ (i.e. recBCD enzyme must travel through the DNA in the orientation indicated above the χ site). χ-specific cleavage results in the formation of a labeled fragment ~ 80 nucleotides in length. The other products observed are the full-length ssDNA species (generated by either the liberation of the intact 5'-terminal strand which does not contain the χ sequence or by unwinding at the opposite end, which would leave the upper (5'-terminal) strand intact) and ssDNA fragments of shorter lengths, produced by nicking of the 3'-terminal strand (Dixon & Kowalczykowski, 1993). In B, *Ava*I-digested, 5' end-labeled pBR322 DNA (10 μM-nucleotide; 1·04 nM-molecule) which either lacked or contained the χ sequence was reacted under standard conditions with either wild-type or mutant recBCD enzyme as indicated. The intact substrate and full-length unwound ssDNAs are visible at or near the wells of the gel. Fragments produced by non-specific cutting are visible as a light background of discrete bands. The χ-dependent bands are indicated. The oligonucleotide products generated by each enzyme are defined by boxes having either stripes (wild-type) or blocks (mutant).

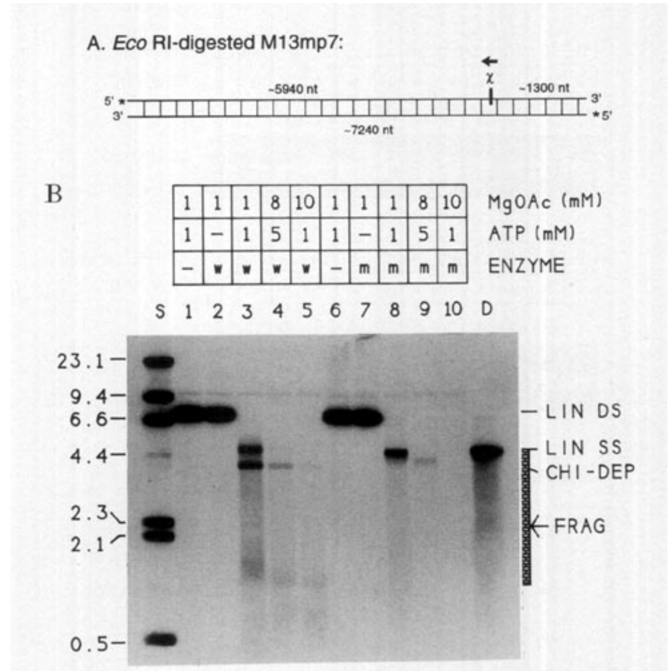


Figure 8. χ-dependent nicking and non-specific nuclease activities of wild-type and mutant recBCD enzymes on M13 DNA containing a single χ site. The DNA substrate was 5' end-labeled *Bam*H1-digested M13 dsDNA (diagrammed in A). Reactions were performed at 37 °C using standard concentrations of components, except as noted below, and were initiated by the addition of recBCD enzyme (wild-type or mutant, as indicated). The unwound and nicked products were separated on a 0·8% agarose gel (B). The molecular weight standards and full-length, heat-denatured substrate are shown in lanes S and D, respectively. The positions of the full-length duplex substrate, χ-dependent fragment, and non-specific ssDNA fragments are also indicated. Conditions were varied as follows: 1 mM-$MgOAc_2$, no ATP (lanes 2 and 7); 1 mM-$MgOAc_2$, 1 mM-ATP (lanes 3 and 8); 8 mM-$MgOAc_2$, 5 mM-ATP (lanes 4 and 9); and 10 mM-$MgOAc_2$, 1 mM-ATP (lanes 5 and 10).

but not in that which contains $recB^{2109}CD$ enzyme (Fig. 7B, lanes 9 and 10).

One possible explanation for the lack of χ-dependent bands in the mutant enzyme reaction is that limited processivity of the mutant enzyme prevents the enzyme from translocating to the site before dissociating. This should not be the case, since the average processive length for $recB^{2109}CD$ enzyme helicase activity is significantly greater than the length of a pBR322 molecule (Eggleston & Kowalczykowski, 1993). Nevertheless, the assay was repeated using *Bam*HI-digested M13 DNA which contains χ, when approached with the proper polarity, only 1·3 kb from the entry site (Fig. 8A). In the absence of ATP (Fig. 8B, lanes 2 and 7), no unwinding or degradation occurs with either enzyme. In the presence of 1 mM-ATP and at a

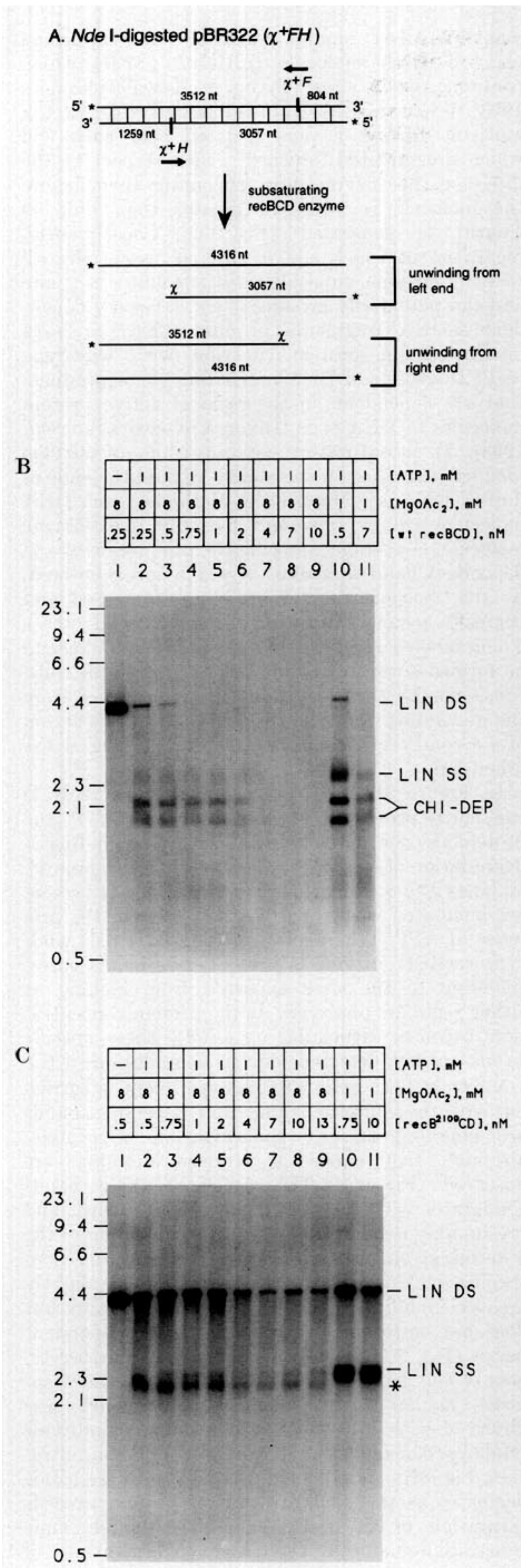
saturating concentration of SSB protein (Fig. 8B, lanes 3 and 8), the DNA is completely unwound by both enzymes, as evidenced by the disappearance of the substrate band. The wild-type enzyme reaction produces two distinct bands, whereas the mutant enzyme reaction produces one band (Fig. 8B, lane 3 *versus* lane 8). The slower migrating band, common to both reactions, comigrates with heat-denatured substrate DNA (Fig. 8B, lane D) and derives from the 5′-terminal strand relative to the entry site (Dixon & Kowalczykowski, 1991, 1993). This demonstrates that the mutant recBCD enzyme also can completely unwind the linear molecule without concomitant nicking of the 5′-terminal strand. In the wild-type enzyme reaction, the faster migrating band, which is approximately 5·9 kb in length, corresponds to the fragment size expected from cutting at the χ site in M13mp7 DNA (Roman *et al.*, 1991). The absence of this band in the mutant enzyme reaction confirms that recB^{2109}CD enzyme does not have χ-cutting activity, even when χ is positioned only 1·3 kb from the entry site. Thus, whether the χ sequence is 1·3 or 4·3 kb from the end which leads to an encounter with χ in the correct orientation for recognition (see Figs 7A and 8A), recB^{2109}CD enzyme does not liberate a χ-dependent fragment.

When the concentration of Mg^{2+} is raised so that it is in excess to ATP (i.e. at conditions which enhance the nuclease activity of the enzyme), the yield of the full-length ssDNA band in both reactions and the χ-dependent band in the wild-type enzyme reaction is severely reduced (Fig. 8B, lanes 4 to 5 and 9 to 10, respectively). In the mutant enzyme reaction (Fig. 8B, lane 9), however, a novel band with slightly greater mobility than the full-length ssDNA band is formed only under conditions optimal for recA protein-promoted joint molecule formation (5 mM-ATP, 8 mM-$MgOAc_2$); we have not yet been able to account for the origin of this apparently specific fragment.

In addition to the full-length and χ-specific bands, non-specific nicking by wild-type recBCD enzyme generates labeled ssDNA fragments 1 to 6 kb in length (Fig. 8B, lanes 3 to 5). The fragments produced by recB^{2109}CD enzyme are apparently fewer in number and less defined in distribution (Fig. 8B, lanes 8 to 10). A time course of both reactions reveals that even at the earliest measurable time point (15 s), the mutant enzyme does not form levels of intermediates comparable to that produced by the wild-type enzyme (data not shown). Therefore, the observed paucity of fragments shorter than full-length in the mutant enzyme reaction is not due to more rapid degradation of these intermediates by subsequent nuclease activity which is active on the unwound ssDNA (see above). Instead, even though the reaction contains SSB protein, the dsDNA substrate is perhaps directly converted to fragments smaller than ~0·5 kb during unwinding, which are not readily detected using this gel system.

In order to increase the sensitivity of the χ-cutting assay, similar reactions were performed using a DNA molecule (pBR322 χ^+FH) which contains two χ sites (Dixon & Kowalczykowski, 1993). When this substrate is digested with *Nde*I, a molecule having χ sites situated near each end which are oriented "inward" (with respect to the direction the enzyme must encounter them before the molecule is unwound greater than half its length) is generated (Fig. 9A). Consequently, regardless of which end is entered, essentially all recBCD enzyme molecules will encounter a χ site and can potentially produce 5′ end-labeled χ-dependent ssDNA fragments of either 3057 or 3512 nucleotides. A protein titration with wild-type recBCD enzyme of DNA unwinding yields products that are dependent on the ratio of active protein molecules to DNA ends (Dixon & Kowalczykowski, 1993). At subsaturating concentrations of enzyme (defined according to the ability to bind the ends of duplex DNA; see Materials & Methods), each DNA molecule will be unwound by only one enzyme molecule on average, allowing the production of a χ-dependent band regardless of which end is entered. As the concentration of protein is increased and begins to achieve saturation of the DNA ends, these 5′ end-labeled χ-dependent species are less likely to be formed since unwinding occurs from both ends; consequently, the χ-dependent band produced from the unwinding of one end is degraded by the action of a second recBCD enzyme molecule acting at the other end.

In Figure 9B, a titration of wild-type recBCD enzyme is presented. In the absence of ATP, the protein alone is unable to catalyze any unwinding or degradation of the DNA substrate (Fig. 9B, lane 1). In lanes 2 to 8, increasing concentrations of protein are incubated with the DNA substrate in the presence of ATP. When subsaturating (< 4 nM) wild-type recBCD enzyme is present, two bands corresponding to the sizes expected from nicking at either χ site are observed. As the protein concentration becomes saturating ($\geqslant 4$ nM), these specific bands are not detected, due to degradation of the DNA from both ends. If this experiment is carried out with the mutant enzyme, no χ-dependent bands are liberated at any concentration of protein, although two bands of reduced mobility are observed (Fig. 9C). The upper of these bands comigrates with the heat-denatured standard, and presumably results from complete unwinding of the 5′-terminal strands without any concurrent non-specific nicking. The lower band has a slightly greater mobility than the full-length strand but does not correspond to either of the χ-dependent bands (Fig. 9C and data not shown). The discreteness of this band suggests that it is a specific fragment, similar to the defined band which was observed with M13 DNA under similar conditions ($MgOAc_2$ concentration in excess of ATP concentration; Fig. 8B). As expected, the yield of these bands decreases as the concentration of protein exceeds saturation of the dsDNA ends. Although some substrate remains at even high concentrations of



mutant enzyme, this is a function of the time frame used; if the reaction is allowed to proceed for a longer period of time, all of the DNA is unwound (data not shown).

4. Discussion

These studies on the mutant enzyme, recB[2109]CD enzyme, have demonstrated that the protein is proficient in all known non-specific nuclease activities associated with wild-type recBCD enzyme *in vitro*. The maximal dsDNA exonuclease activity of the mutant enzyme is approximately half that of the wild-type enzyme (Figs 3 and 4), although its optimal requirement for ATP is significantly higher (500 μM *versus* 25 to 50 μM at 1 mM-$MgOAc_2$; Fig. 3). Likewise, the ATP-dependent ssDNA exonuclease and ATP-stimulated ssDNA endonuclease activities of the mutant enzyme are somewhat reduced compared to the wild-type enzyme (Figs 5 and 6). Interestingly, on circular ssDNA, the ATP-independent component of the ssDNA endonuclease activity is virtually indistinguishable between the two enzymes, whereas the ATP-stimulated component of the mutant enzyme is ~ threefold less (Fig. 6). This difference in rate may be due either to slower nicking on the part of the mutant enzyme or to a lower affinity of the mutant enzyme for binding to an internal base. Despite the cause, the observation that the mutant enzyme displays a greater difference in the ATP-stimulated, rather than ATP-independent, component of an activity suggests that an impaired interaction of the protein with ATP cofactor is perhaps one physical defect caused by the *recB2109* mutation.

These slightly lower levels of nuclease activity exhibited by the mutant enzyme do not readily lead to an explanation of the recombination-deficient phenotype of *recB2109* cells, as it is difficult to imagine a model in which a two- to threefold reduction in any or all of the non-specific nuclease activities could affect the process of recombination

Figure 9. χ-dependent nicking and non-specific nuclease activities of wild-type and mutant recBCD enzymes on DNA containing two χ sites. Reactions were conducted at 37 °C in standard buffer (with $MgOAc_2$ varied as noted) with 10 μM-nucleotide (1·04 nM-molecule) 5′ end-labeled, *Nde*I-digested pBR322χ^+*FH* DNA (depicted in A) and the indicated concentrations of wild-type (B) or mutant (C) recBCD enzyme. Unwinding was initiated synchronously by the addition of ATP to 1 mM final concentration. For these enzyme preparations, the stoichiometry of binding to duplex DNA ends saturates at 4 nM (wild-type) or 6·9 nM (mutant) enzyme. The positions of the duplex molecular weight standards, full-length intact and heat-denatured substrate, and χ-dependent fragments are indicated. The position of the specific band generated by the mutant enzyme is indicated by an asterisk in C. Conditions in both panels were as follows: no ATP, 8 mM-$MgOA_2$ (lane 1), 1 mM-ATP, 8 mM-$MgOAc_2$ (lanes 2 to 9); and 1 mM-ATP, 1 mM-$MgOAc_2$ (lanes 10 and 11).

in vivo, which necessarily requires intact DNA strands. Specifically, degradation of the 3′-terminal strand, which is required for recA protein-dependent pairing and strand exchange, would not promote recombination. The studies of Dixon & Kowalczykowski (1991,1993) demonstrate conclusively that the dsDNA exonuclease activity of recBCD enzyme nicks the 3′ strand with much greater frequency than the 5′ strand; thus, the finding that χ acts to downregulate this activity is strong evidence that it is not required for recombination. Instead, it is the attenuation of this activity which allows recombination to occur at increased frequency beyond the recombination hotspot. Also, no recombination-deficient mutant of recBCD enzyme which has been isolated to date, to our knowledge, has exhibited specific loss of the dsDNA exonuclease activity, while retaining the sequence-specific nicking activity at χ.

The finding that the mutant enzyme is apparently wholly defective in recognition of the χ recombination hotspot sequence (Figs 7 to 9), however, does suggest a reasonable explanation for the inability of the mutant enzyme to promote recombination. The presence of χ has long been known to enhance the formation of recombinants both in λ crosses (Lam *et al.*, 1974; McMilin *et al.*, 1974) and in conjugation and transduction (Dower & Stahl, 1981). Recent genetic studies have demonstrated a role for recBCD enzyme in at least the initiation of recombination (Holbeck & Smith, 1992). *In vitro* studies have also strongly indicated a role for recBCD enzyme in initiating the recombination process, with χ-stimulation being manifest in an increased number of joint molecules formed (Roman & Kowalczykowski, 1989*b*; Wang & Smith, 1989; Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). Consequently, the observation that the recB^{2109}CD enzyme does not interact productively with χ appears to be critical in understanding its phenotype.

The inability of the mutant enzyme to be stimulated by χ may result from one of two causes: a defect in the recognition and/or nicking of the sequence or a defect in the attenuation of the 3′-terminal strand-specific nuclease activity once the enzyme has recognized and cleaved at the site. Although we have been unable to distinguish between these two alternatives experimentally, this result, that the non-specific nuclease activity of the mutant enzyme is not attenuated in the presence of χ, has clear implications for the activity of the recB^{2109}CD enzyme in the cell. Given that the mutant enzyme unwinds duplex DNA *in vivo* (as suggested by our ability to measure this activity *in vitro* (Eggleston & Kowalczykowski, 1993) and by the observation that T4 *2*$^-$ phage do not plate on *recB2109* cells (Amundsen *et al.*, 1990), our studies suggest that it concomitantly degrades the DNA. This 3′ strand-specific nuclease activity is constitutive (i.e. is not attenuated upon an encounter with χ) and thus produces ssDNA fragments which in some manner are unable to be utilized by recA protein in the formation of significant lengths of heteroduplex DNA. Consequently, despite the uncertainty as to the precise mechanistic basis of the χ recognition defect of the mutant enzyme, the fact that it does not undergo a change of state to the recombination-promoting mode is likely to have a profound effect on its function in recombination. In fact, the results of the χ-nicking assay (Fig. 7) suggest that if the mutant enzyme is able to recognize and nick at χ, it cleaves the same strand within 80 bp with 100% efficiency. This strongly argues that, even should the mutant enzyme possess the ability to recognize χ, the fragments which it produces are of insufficient size to be recombinationally significant.

This view must be reconciled with the *in vivo* data of Amundsen *et al.* (1990), which appear to conflict with both our and their *in vitro* data. When *recB2109* cells were infected with labeled phage DNA, a minimal amount of *in vivo* DNA degradation, as measured by a TCA solubility assay, was detected; yet, *in vitro*, dsDNA exonuclease activity was detected in crude extracts of *recB2109* cells at elevated ATP concentration (Amundsen *et al.*, 1990). The observation that the mutant enzyme does not allow plating of T4 *2*$^-$ phage (Amundsen *et al.*, 1990) suggests that the enzyme has at least dsDNA exonuclease or helicase activity *in vivo*. We propose that recB^{2109}CD enzyme must retain some dsDNA exonuclease activity in the cell to account for its phenotype, since the enzyme is capable of unwinding significant lengths of DNA, which, in the absence of degradation, would presumably be sufficient for recA protein-mediated heteroduplex DNA formation (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). Though the observations of neither laboratory completely explain the failure to detect TCA soluble products during phage T4 *2*$^-$ infection, it is conceivable that the ssDNA fragments produced in the cell by the dsDNA exonuclease activity of both the wild-type and mutant enzymes are not small enough to be TCA soluble, and that the liberation of TCA soluble counts is the result of subsequent ssDNA nuclease activities of recBCD enzyme which are somewhat more active in the wild-type enzyme than in the mutant enzyme (Figs 5 and 6).

In fact, two results suggest that post-unwinding degradation by recBCD enzyme may be occurring. First, the initial rate of release of TCA soluble fragments from dsDNA is approximately equal for both enzymes, although with increasing time (which exceeds that required to completely unwind the DNA under these conditions as monitored by a fluorescent helicase assay (data not shown)), the wild-type enzyme continues to liberate fragments at a greater rate than does the mutant enzyme (Fig. 2). This difference is more pronounced at the lower Mg^{2+} concentration tested (1 mM-$MgOAc_2$), which presumably is closer to the physiological concentration of this ion. Secondly, while the mutant enzyme generates an approximately equivalent amount of intact ssDNA (derived from the strand

which is 5′ at the entry site) during dsDNA unwinding, fewer ssDNA fragments are observed (Fig. 8). Based on these results, one might expect that mutant enzyme could degrade the DNA to smaller fragments which might be TCA soluble. Instead, TCA solubility assays demonstrate that, for a given functional concentration of enzyme, the activity of the mutant enzyme is less than that of the wild-type enzyme. Thus, the ssDNA-specific nuclease activities of the wild-type enzyme, which act subsequent to unwinding, appear to contribute significantly to the TCA solubilization of dsDNA *in vitro* and, presumably, *in vivo*.

In summary, the recB^{2109}CD enzyme degrades both linear DNA (double- and single-stranded) and circular ssDNA molecules. *In vitro*, none of these non-specific nuclease activities is reduced greater than threefold under optimal conditions when compared to those of the wild-type enzyme. We have, however, been unable to detect any specific recognition of the recombination hotspot sequence, χ, by the mutant enzyme. Thus, the nuclease activity specific for the 3′ strand at the entry site is constitutively expressed by the mutant enzyme. This observation leads to a facile explanation for the recombination-deficiency of this strain, which is that the recB^{2109}CD enzyme, by virtue of its unregulated degradation of the DNA substrate, cannot support the formation of long tracts of heteroduplex DNA. This proposal is contingent upon the ability of the mutant enzyme to unwind dsDNA, and, consequently, we have examined the DNA helicase activity of the mutant enzyme to determine if this activity is affected by the *recB2109* mutation (Eggleston & Kowalczykowski, 1993). Although the helicase activity of the mutant enzyme is also reduced ~threefold compared to the wild-type enzyme, it is still unable to support the efficient formation of joint molecules in a reaction utilizing the *E. coli* recA and SSB proteins (Eggleston & Kowalczykowski, 1993). The results of these studies further uphold the notion that the inability of the mutant enzyme to recognize or be altered by χ is of major importance in causing the recombination defect of *recB2109* cells.

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