J. Mol. Biol. (1993) **231**, 621–633

The Mutant recBCD Enzyme, recB^{2109}CD Enzyme, Has Helicase Activity but Does Not Promote Efficient Joint Molecule Formation *in Vitro*

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(Received 26 August 1992; accepted 20 January 1993)

The *Escherichia coli* recB^{2109}CD enzyme displays a defect in homologous recombination. *In vitro*, it possesses significant levels of non-specific nuclease activity but is deficient in χ-dependent nicking activity. To determine whether an alteration in helicase activity contributes further to its *in vivo* defect, the ability of recB^{2109}CD enzyme to unwind dsDNA was examined. The mutant enzyme is able to unwind DNA but has a k_{cat} which is one-third that of the wild-type enzyme. While the K_m for DNA ends of the wild-type and mutant enzymes at low NaCl concentration are essentially equivalent, the K_m for ATP of recB^{2109}CD enzyme is nearly six times greater. The processivity of unwinding (i.e. the average length of DNA unwound before recB^{2109}CD enzyme dissociates from the DNA substrate) at 1 mM-Mg^{2+} ion and 1 mM-ATP is approximately 13 kb/end, whereas that of wild-type recBCD enzyme is 30 kb/end. In an assay which requires the co-ordinate actions of the recBCD, recA, and SSB proteins, joint molecule formation in the presence of recB^{2109}CD enzyme is up to sixfold slower and proceeds to a lower extent than that mediated by the wild-type enzyme. We conclude that although the reduced helicase activity of the mutant recBCD enzyme may contribute to its recombination deficiency, its defect in the χ-dependent attenuation of non-specific nuclease activity is primarily responsible for the recombination-deficiency of *E. coli* strains bearing the *recB2109* mutation.

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

1. Introduction

RecBCD enzyme is a three subunit, 330 kDa protein involved in genetic recombination in *Escherichia coli*. Although first identified as an ATP-dependent nuclease active on several types of substrates, recBCD enzyme is also a highly active DNA helicase (for reviews, see Taylor, 1988; Smith, 1990). As demonstrated in the accompanying paper, a mutant recBCD enzyme, designated recB^{2109}CD enzyme, has all of the known non-specific nuclease activities (ssDNA‡ endonuclease, ds- and ssDNA exonuclease) of wild-type recBCD enzyme but fails to specifically nick at χ sites *in vitro* (Eggleston & Kowalczykowski, 1993). Because the ability to unwind dsDNA is required for the recognition of χ by recBCD enzyme (Taylor *et al.*, 1985), we have

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‡ Abbreviations used: ds, double-stranded; ss, single-stranded; SSB protein, *E. coli* single-stranded DNA binding protein; $MgOAc_2$, magnesium acetate; PEP, phospho(enol)pyruvate; χ, Chi sequence (5′-GCTGGTGG-3′); form I, supercoiled DNA; form II, nicked circular dsDNA; form III, linear dsDNA; TCA, trichloroacetic acid.

0022-2836/93/110621-13 $08.00/0

extended our characterization of this enzyme to encompass an examination of its helicase activity in a further attempt to account for the *rec*$^-$ phenotype of *recB2109* strains.

Wild-type recBCD enzyme initiates unwinding on linear duplex DNA substrates having blunt ends or ssDNA tails less than 25 nucleotides in length (Taylor & Smith, 1985). As it unwinds the DNA, recBCD enzyme forms one of two structures (Taylor & Smith, 1980; Telander-Muskavitch & Linn, 1982). In the absence of SSB protein, a twin-loop structure results from reannealing of the unwound strands behind the enzyme. When SSB protein is present, a loop-tail structure having two ssDNA tails is produced. The helicase activity of wild-type recBCD enzyme is both rapid ($\sim$1000 bp unwound s^{-1} at 37°C; Roman & Kowalczykowski, 1989*a*) and highly processive, with an average of 30 kb unwound per binding event (Roman *et al.*, 1992). Unwinding is an ATP-dependent process, and two to three ATP molecules are hydrolyzed for each base-pair unwound (Roman & Kowalczykowski, 1989*b*).

RecBCD enzyme nicks DNA specifically at the χ sequence, which enhances recombination in a polar fashion (Ponticelli *et al.*, 1985; Taylor *et al.*, 1985). Since, as noted above, this nicking is dependent upon the ability of the enzyme to translocate through the χ-containing DNA, both the helicase activity and the sequence-specific nicking activity are presumed to be involved in promoting recombination (Smith & Stahl, 1985). The only recBCD enzyme mutants yet identified which are recombination-proficient yet apparently have no nuclease activity and minimal helicase activity are the members of the ‡ class, which are proposed to act by a different mechanism (Smith, 1987; Lovett *et al.*, 1988; Lloyd *et al.*, 1988); Thaler *et al.* (1989) have presented evidence to suggest that the activity of the ‡ enzyme is similar to that of the wild-type enzyme after it has encountered χ. Smith and co-workers first proposed a model which incorporated these two activities of recBCD enzyme (Smith *et al.*, 1981,1984,1987). In this model, recBCD enzyme gains entry to a DNA molecule *via* a double-strand break and initiates unwinding. When the enzyme encounters a χ site in the correct orientation (i.e. from the 3′ side of a χ sequence oriented as 5′-GCTGGTGG-3′), it nicks the DNA 4 to 6 bp to the 3′ side of the site. As recBCD enzyme continues to unwind beyond the site, this strand is extruded as a 3′ single-stranded tail. RecA protein, the DNA strand exchange protein of *E. coli*, binds to this ssDNA, and the recA protein–ssDNA complex then invades a recipient duplex molecule, producing a joint molecule. This heteroduplex DNA is extended by recA protein-mediated branch migration and by the continued unwinding of the donor dsDNA by recBCD enzyme.

In vitro studies support and extend the tenets of this recombination initiation model (Roman & Kowalczykowski, 1989*c*; Wang & Smith, 1989; Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). RecA protein can promote the formation of heteroduplex DNA between DNA substrates that are homologous and that contain a region of ssDNA within the homologous sequences (West *et al.*, 1981; Cox & Lehman, 1987); if both DNA molecules are duplex, however, unwinding of a linear dsDNA molecule is a prerequisite to recA protein-dependent pairing. One assay which models the linkage between recBCD enzyme- and recA protein-dependent activities in the formation of heteroduplex DNA employs linear dsDNA and circular ssDNA molecules (Roman & Kowalczykowski, 1989*c*). In the presence of recBCD enzyme, the linear dsDNA is unwound, and the liberated ssDNA is trapped by recA and SSB proteins. The renaturation activity of recA protein then reanneals the complementary ssDNA, producing joint molecules. Along a similar vein, recBCD enzyme incubated in the presence of two homologous but distinguishable linear dsDNA substrates produces ssDNA fragments that can be bound by SSB protein; the subsequent addition of recA protein promotes rapid renaturation of the ssDNA with concomitant formation of heteroduplex DNA (Wang & Smith, 1989). When linear and supercoiled dsDNA molecules are incubated in the presence of recA protein, recBCD enzyme, and SSB protein, recBCD enzyme helicase activity is required to generate the ssDNA fragments which can be utilized by recA protein to form D-loop molecules with the supercoiled substrate (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). Additionally, joint molecule formation in this reaction is influenced by the presence of a χ sequence in the donor molecule, further supporting the stimulatory role of χ in the recombination process (Dixon & Kowalczykowski, 1991). Since each of these reactions is dependent upon the presence of recBCD enzyme, such studies have provided strong evidence for a role of the helicase and χ-cutting activities of recBCD enzyme in the initiation of homologous recombination.

Our studies using purified recB^{2109}CD enzyme have found that the helicase activity of the mutant enzyme is $\sim$threefold slower than that of wild-type recBCD enzyme and is more sensitive to the concentration of ATP cofactor. The unwinding of duplex DNA by recB^{2109}CD enzyme is also less processive than that catalyzed by wild-type recBCD enzyme. Although recB^{2109}CD enzyme has what would appear to be significant levels of helicase activity, it nevertheless generates ssDNA which is not utilized efficiently in the formation of joint molecules by recA protein. The relationship between these *in vitro* observations and the *in vivo* recombination-deficiency of *recB2109* strains is discussed.

2. Materials and Methods

(a) *Nucleic acids*

M13mp7 ss- and dsDNAs, pBR322 (χ^0 and χ^+F) (Taylor *et al.*, 1985), and ^{3}H-labeled pBR322 were puri-

fied as described in the accompanying paper (Eggleston & Kowalczykowski, 1993). Phage T7 DNA was purchased from Sigma. Phage N4 DNA was a gift from Dr L. Rothman-Denes, University of Chicago. The molar nucleotide concentration was determined at 260 nm using an extinction coefficient of 6500 M^{-1} cm^{-1} for dsDNA and 8780 M^{-1} cm^{-1} for ssDNA. The molar molecule concentration was determined by multiplying the molar nucleotide concentration by 8722 (pBR322), 14,390 (M13mp7), 79,872 (T7), or 144,000 (N4) nucleotides per DNA molecule.

(b) *Proteins*

Wild-type recBCD enzyme, recB^{2109}CD enzyme, and SSB protein were purified as described in the accompanying paper (Eggleston & Kowalczykowski, 1993). RecA protein was purified from strain JC12772 (Uhlin & Clark, 1981) using a protocol (S. C. Kowalczykowski, unpublished observation) based on spermidine precipitation (Griffith & Shores, 1985); its concentration was determined using an extinction coefficient at 280 nm of 2.7×10^4 M^{-1} cm^{-1}. S_1 nuclease and pyruvate kinase were purchased from Pharmacia and Sigma, respectively. DNA restriction and modification enzymes were purchased from New England Biolabs, Pharmacia, and Boehringer Mannheim Biochemicals.

Standard helicase buffer consisted of 25 mM-Tris-acetate (pH 7·5), 1 mM-ATP, 1 mM-$MgOAc_2$, and 1 mM-DTT.

(c) *Fluorometric helicase assay*

This assay, described by Roman & Kowalczykowski (1989*a*), is based on the quenching of SSB protein intrinsic fluorescence upon binding ssDNA. SSB protein was included at the following concentrations for the different DNA substrates: 2 μM (pBR322 and M13 DNAs); 11·1 μM (T7 DNA); and 19.8 μM (N4 DNA). These concentrations correspond to a 3-fold excess concentration over ssDNA, and are calculated as 0·2 the nucleotide concentration in the reaction. RecBCD enzyme was added as noted. These components, in standard helicase buffer, were equilibrated at the indicated temperature, and the reaction was initiated by the addition of linear dsDNA to the desired concentration. Reactions were conducted in a 350 μl volume at either 15°C, 25°C, or 37°C; lower temperatures were used to decrease the rate of unwinding when high concentrations of wild-type recBCD enzyme were used (see Table 1). The data were collected using a Shimadzu RF5000U spectrofluorophotometer, with excitation and emission wavelengths of 290 and 340 nm, respectively, and excitation and emission slit widths of 1·5 and 10 nm, respectively. Enzymatic parameters were determined by fitting the initial rate data *versus* substrate concentration to a hyperbola using the DAES80 program (R and L Software).

Due to the presence of recBCD enzyme-dependent nuclease activities, oligonucleotides which are unable to bind SSB protein efficiently are produced; the extent of their production is highly dependent on the reaction conditions and is more severe in the presence of recB^{2109}CD enzyme (unpublished observation). This phenomenon results in an underestimation of both the rate and the extent of DNA unwinding if the raw data are used without correction. Consequently, control reactions were performed in the following manner. A 40 μl reaction containing the appropriate buffer components and 87·5 μM-nucleotide (6·04 nM-molecule) *Eco*RI-digested M13 DNA (an amount of DNA equivalent to 10 μM nucleotide or 0·69 nM-molecule in a 350 μl vol.) was pre-incubated at 37°C; DNA unwinding was initiated by the addition of recBCD enzyme. At a time just sufficient for the completion of unwinding (as determined by performing the same reaction in the fluorometric assay), the reaction was stopped by the addition of 2 μl of 0·5 M-EDTA. The unwound DNA was then heat-denatured at 95°C for 8 min and was quenched in an ice water bath. This denatured DNA (42 μl) was added to a cuvette containing 308 μl of the same buffer with 2·27 μM-SSB protein (equivalent to 2 μM in 350 μl). The change in fluorescence after addition of the denatured DNA (accounting for the decrease in SSB protein fluorescence due to dilution) was measured and was compared to that obtained when heat-denatured linear dsDNA not exposed to recBCD enzyme was used. This difference yielded the percentage of maximal possible quenching under a particular set of conditions. These controls were conducted for each extreme of condition (high and low concentration of ATP; high and low concentration of Mg^{2+}; with and without 1 mM-Ca^{2+}; with and without SSB protein present during the unwinding; varying temperature). The percentage of maximum possible quenching obtained for recB^{2109}CD enzyme ranged from 100% in the presence of Ca^{2+} to 40% in the presence of 4 mM-Mg^{2+}. The values for wild-type recBCD enzyme ranged from 100% in the presence of Ca^{2+} to 70% in the presence of 4 mM-Mg^{2+}. For any particular set of conditions, DNA exposed to the mutant enzyme was consistently degraded to a greater extent as judged by the ability to bind SSB protein.

These results appear to contradict our studies in which we found that the rate of TCA solubilization of duplex DNA by the mutant enzyme was less than that of the wild-type enzyme (Eggleston & Kowalczykowski, 1993). As we stated in that paper, however, we believe that the TCA solubility assay measures nuclease activities which occur during unwinding as well as subsequent to it. It is possible that the mutant enzyme generates smaller, although not necessarily TCA soluble, oligonucleotides under the conditions used in the helicase assay, in which SSB protein is present to reduce subsequent degradation of the unwound strands by the ssDNA specific nuclease activities of the enzyme. This situation could account for the apparent greater degradation of the substrate by the mutant enzyme in the helicase assay controls than are indicated by the TCA solubility assay.

(d) *Unwinding processivity assays*

The processivity of unwinding by recB^{2109}CD enzyme was determined in 2 ways as previously described for wild-type recBCD enzyme (Roman *et al.*, 1992). For the first method, the fluorometric assay was performed under standard conditions using DNA molecules of varying lengths: phage T7 DNA (39·9 kb) and phage N4 DNA (72 kb). The concentration of DNA molecules (0·69 nM) in the reaction was constant for each DNA substrate; consequently, the molar nucleotide concentration (55·5 μM-nucleotide for T7 and 99 μM-nucleotide for N4) and the concentration of SSB protein (11·1 μM and 19·8 μM, respectively), vary with the length of the DNA substrate. We consider the values for the processivity of the mutant enzyme obtained by this method to be approximate, as opposed to the more precise values obtained with the wild-type enzyme (Roman *et al.*, 1992), because on these DNA molecules, recB^{2109}CD enzyme sometimes displays a short lag before steady-state

unwinding is reached, unwinds at a slower rate than is observed with shorter DNA molecules such as pBR322 and M13, and does not reach a distinct end point (unpublished observation).

Second, a pulsed-field gel assay was used as a complementary means of examining the processivity of recB^{2109}CD enzyme by allowing direct visualization of the intact duplex DNA remaining at the completion of an unwinding reaction. As noted previously (Roman *et al.*, 1992), this assay circumvents any potential artifacts introduced by recBCD enzyme-dependent nucleolytic activities. N4 DNA (99 μM-nucleotide; 0·69 nM-molecule) was incubated in standard buffer containing 19·8 μM-SSB protein. The reaction was equilibrated to 37 °C, and unwinding was initiated by the addition of a saturating concentration of recB^{2109}CD enzyme (1·25 functional molecules/end). At the indicated times, a sample was removed, was added to 0·1 vol. 1% (w/v) SDS, and was stored on ice. After completion of the time course, 0·1 vol. 10 × S_1 nuclease buffer (300 mM-sodium acetate (pH 4·6), 500 mM-NaCl, 10 mM-$ZnCl_2$, 50% glycerol) and 0·6 unit S_1 nuclease/ml (Pharmacia) were added to each sample. The samples were incubated at 37 °C for 10 min, and the reaction was quenched by adding 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) to 1 ×. The dsDNA remaining after this treatment was separated by pulsed-field gel electrophoresis through a 1% (w/v) agarose gel in modified TBE (100 mM-Tris, 100 mM-boric acid, 2 mM-EDTA) at pulse times of 1 s for 3 h 20 min and 3 s for 17 h (see Roman *et al.*, 1992, for details). To confirm that all of the DNA present was susceptible to the S_1 nuclease treatment, an equivalent amount of heat-denatured N4 DNA was treated in the same manner and was separated by pulsed-field gel electrophoresis. Essentially 100% of the heat-denatured DNA was degraded by this treatment (data not shown).

(e) *Joint molecule formation assays*

Two assays for the recBCD-enzyme dependent formation of joint molecules by recA protein have been described (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). For the nitrocellulose filter binding assay, the buffer contained 25 mM-Tris-acetate (pH 7·5), 1 mM-DTT, 5 mM-ATP, 8 mM-$MgOAc_2$, 1·5 mM-PEP, 4 units pyruvate kinase/ml, 10 μM-nucleotide (1·04 nM-molecule) *Pvu*II-digested pBR322 (χ^0 or χ^+) DNA, 5 μM-nucleotide (0·52 nM-molecule) ^{3}H-labeled supercoiled pBR322 (χ^0) DNA, 1·33 μM-SSB protein, and 5 μM-recA protein. After pre-equilibration of the reaction mixture to 37 °C, a subsaturating concentration of recBCD enzyme (0·1 functional molecule/end) was added to initiate the reaction. At the indicated time, 10 vol. D-loop buffer (2 M-NaCl, 150 mM-sodium citrate) was added to quench the reaction, which was then stored on ice. Within 10 min, the reaction was filtered through a nitrocellulose disc (HAWP 025; Millipore) which had been soaked in D-loop buffer; the filter was then washed with 3 ml D-loop buffer. After drying, the filters were counted in 5 ml non-aqueous scintillation fluid (RPI). Since the reactions were not stopped by the addition of SDS and incubation at 37 °C, the presence of both paranemic and plectonemic molecules was measured (Riddles & Lehman, 1985). All values reported are the average of at least 2 experiments and have been corrected for the percentage of counts which are retained in the absence of both recA and recBCD proteins.

For the agarose gel assay, the reaction contained 25 mM-Tris-acetate (pH 7·5), 5 mM-ATP, 8 mM-$MgOAc_2$, 1 mM-DTT, 2 mM-PEP, 16 units pyruvate kinase/ml, 40 μM-nucleotide (2·76 or 4·16 nM-molecule) linearized M13 or pBR322 (χ^0 or χ^+) DNA, 20 μM-nucleotide (1·38 or 2·08 nM-molecule) supercoiled M13 or pBR322 (χ^0 or χ^+) DNA, and 5·33 μM-SSB protein. After initiating the reaction by simultaneously adding 10 μM-recA protein and a substoichiometric amount of recBCD enzyme (0·025 functional molecule/end), time points were withdrawn at the indicated times and were added to 0·2 vol. 5 × loading buffer. To account for the lower helicase activity of recB^{2109}CD enzyme, the time points for the mutant enzyme reaction are longer than those for wild-type recBCD enzyme. The DNA was separated by electrophoresis through a 0·75% (w/v) agarose gel run in 1 × TAE at 2·1 V/cm for 8 h and was visualized by ethidium bromide staining.

3. Results

(a) *The helicase activity of recB^{2109}CD enzyme is attenuated*

The helicase activity of the recB^{2109}CD enzyme was monitored using a continuous fluorometric assay described previously (Roman & Kowalczykowski, 1989*a*). The amount of functional recBCD enzyme in a protein preparation can be determined by performing a protein titration. The rate of unwinding increases with increasing protein concentration until a point is reached such that all DNA ends are saturated with active enzyme (see Roman & Kowalczykowski, 1989*a*). For recB^{2109}CD enzyme, saturation occurs at a protein concentration of 6·9 nM (data not shown) and at a V_{max} equal to 120 nM-bp unwound/s at 25 °C (Table 1). The concentration of DNA ends in the reaction is 1·38 nM (i.e. 10 μM-nucleotide M13 DNA), resulting in an apparent stoichiometry of five recB^{2109}CD enzyme molecules/DNA end. Using this experi-

Table 1

Characterization of the helicase activity of wild-type recBCD enzyme and recB^{2109}CD enzyme preparations

	Enzyme	
	Wild-type	recB^{2109}CD
Temperature (°C)	15	25
Stoichiometry	3/end	5/end
V_{max}	182 nM-bp s^{-1}	120 nM-bp s^{-1}
$k_{cat,\ corr}$	131 s^{-1} (15 °C)	
	231 s^{-1} (25 °C)†	87 s^{-1} (25 °C)

The reactions contained standard helicase buffer, 2 μM-SSB protein, and varying concentrations of recBCD enzyme. Unwinding was initiated by the addition of 10 μM-nucleotide (0·69 nM-molecule) linearized M13 DNA. The reactions were conducted at the indicated temperature.

† Because the rate of unwinding by the wild-type enzyme is difficult to measure accurately at saturating concentrations of enzyme at 25 °C, this protein titration was performed at 15 °C. Since variation in the reaction temperature affects primarily the rate of unwinding and does not significantly alter the K_m for the DNA substrate (Roman & Kowalczykowski, 1989*b*), the Arrhenius equation was used to calculate the corrected k_{cat} of the wild-type enzyme at 25 °C.

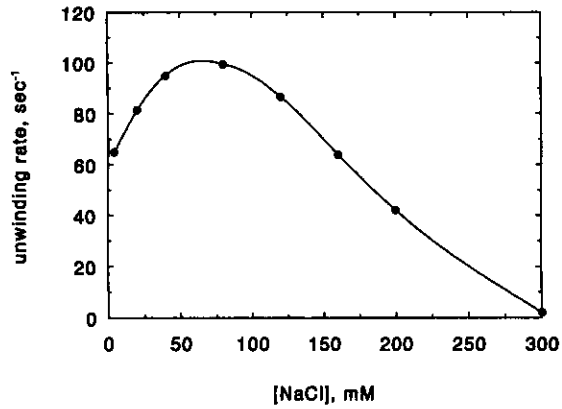

Figure 1. Effect of NaCl concentration on the rate of unwinding by recB^{2109}CD enzyme. The reaction was conducted at 25°C in standard helicase buffer containing 2 μM-SSB protein, 0·4 nM-functional recB^{2109}CD enzyme, and the indicated concentration of NaCl. In the absence of added NaCl, the protein solutions contribute a final concentration of 4 mM-NaCl to the reaction, and the reported NaCl concentration takes this value into account. To start the reaction, 10 μM-nucleotide (0·69 nM-molecule) *Eco*RI-digested M13 DNA was added. The reported rates are corrected for the apparent binding stoichiometry of the enzyme preparation (Table 1).

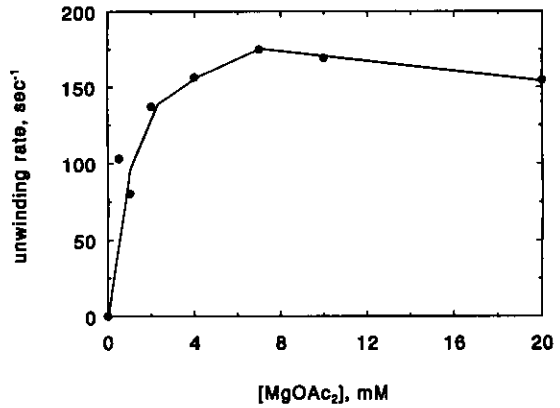

Figure 2. Effect of Mg^{2+} concentration on the helicase activity of recB^{2109}CD enzyme. The reaction was conducted at 25°C in standard helicase buffer except that the $MgOAc_2$ concentration was varied. The reaction contained 2 μM-SSB protein and 0·4 nM-functional recB^{2109}CD enzyme; it was initiated by adding 10 μM-nucleotide (0·69 nM-molecule) *Eco*RI-digested M13 DNA. The reported rates are corrected for the apparent binding stoichiometry of the enzyme preparation and for the production of oligonucleotides which are unable to be bound by SSB protein (see Materials & Methods).

mentally derived stoichiometry, the experimentally determined turnover number for maximum DNA unwinding can be corrected by dividing the observed rate by the observed stoichiometry; consequently, the corrected k_{cat} at 25°C is 87 nM-bp unwound/s per nM-functional recB^{2109}CD enzyme (or 87 s^{-1}). For comparison, the kinetic parameters of the wild-type recBCD enzyme preparation used in these experiments are also given in Table 1. Thus, at 1 mM-ATP, the rate of unwinding by recB^{2109}CD enzyme is approximately 2·7-fold lower than that of the wild-type enzyme. This result confirms the qualitative observation made using a crude cell lysate of a *recB2109* strain that unwinding by the mutant enzyme is slower than that of the wild-type enzyme (Amundsen *et al.*, 1990).

(b) *The concentration of mono- and divalent ions affects the unwinding of DNA by recB^{2109}CD enzyme*

The properties of wild-type recBCD enzyme are affected by changes in the ionic environment. The effect of varying monovalent salt concentration on unwinding was therefore examined. An increase in NaCl concentration up to ~ 60 to 80 mM increases the apparent rate of unwinding catalyzed by recB^{2109}CD enzyme (Fig. 1); above this salt concentration, the apparent unwinding rate decreases until all activity is abolished at or above 300 mM-NaCl. A similar profile, which peaked at 100 mM-NaCl, was observed with wild-type recBCD enzyme (Roman & Kowalczykowski, 1989*a*).

Raising the Mg^{2+} concentration will affect both the stability of the duplex DNA and the dsDNA exonuclease activity of recBCD enzyme (Eggleston & Kowalczykowski, 1993). Due to the enhanced nuclease activity of recBCD enzyme under these conditions, these data must be corrected for changes in oligonucleotide production (see Materials and Methods). The helicase activity of recB^{2109}CD enzyme, like that of wild-type recBCD enzyme (Roman & Kowalczykowski, 1989*a*), is significant across a broad range of Mg^{2+} concentration (Fig. 2); unlike wild-type recBCD enzyme, however, the rate increases twofold when the Mg^{2+} concentration is raised from 1 to 7 mM. This increase corresponds to the conditions at which the dsDNA exonuclease activity is most pronounced (Eggleston & Kowalczykowski, 1993).

(c) *RecB^{2109}CD enzyme has a K_m for DNA ends which is similar to that of wild-type recBCD enzyme at low salt concentration*

Since variation in the monovalent ion concentration affected the apparent rate of unwinding of the mutant enzyme (Fig. 1), the DNA concentration in the helicase assay was varied at several salt concentrations to determine whether the concentration of salt also affects the apparent affinity of the enzyme for duplex DNA (Fig. 3A, B). In the absence of additional NaCl (i.e. at 4 mM-NaCl), the recB^{2109}CD enzyme has an apparent K_m of 0·8 nM-DNA ends and a corrected k_{cat} of 253 s^{-1} at 37°C (Table 2). This apparent K_m value is essentially equal to that obtained for the wild-type enzyme under these conditions (0·9 nM-DNA ends), while the corrected k_{cat} is ~ twofold lower than that of the wild-type enzyme (586 s^{-1}; Table 2). Thus, a

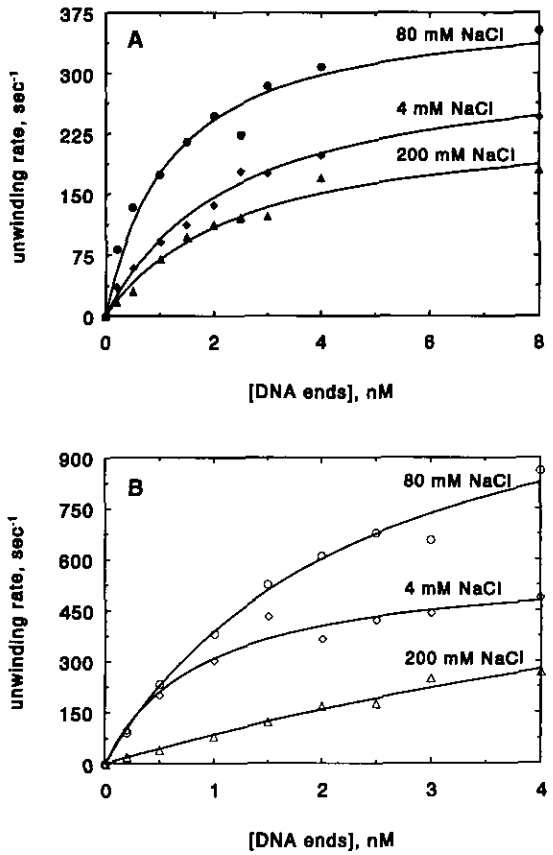

Figure 3. Effect of DNA concentration on the helicase activity of wild-type and mutant recBCD enzyme. In A, the reaction was conducted at 37 °C in standard helicase buffer containing either 0·8 nM-functional recB^{2109}CD enzyme (4 mM-NaCl) or 0·2 nM-functional recB^{2109}CD enzyme (80 and 200 mM-NaCl) and the appropriate concentration of SSB protein (equivalent to 20% of the molar nucleotide concentration). Unwinding was begun by adding *Eco*RI-digested M13 DNA to the desired final concentration. In B, the reaction was conducted as described in A except that 0·07 nM-functional wild-type recBCD enzyme was used. In both panels, the reported rates are corrected for the apparent binding stoichiometry of the enzyme preparations.

Table 2

Effect of NaCl concentration on enzymatic parameters of wild-type recBCD and recB^{2109}CD enzyme helicase activity

Enzyme	[NaCl] (mM)	$K_{m,\ app}$ (nM-DNA ends)	$k_{cat,\ corr}$† (s^{-1})
Wild-type recBCD	4	0·9±0·2	586±45
	80	2·4±0·5	1322±131
	200	12·5±7·0	1142±520
recB^{2109}CD‡	4	0·8±0·3	253±24
	80	0·9±0·2	369±27
	200	1·9±0·3	227±16

The reactions contained standard helicase buffer, SSB protein at the concentration appropriate to the DNA concentration (see Materials and Methods), recBCD enzyme (0·2 nM-wild-type enzyme; either 1·0 or 4·1 nM-mutant enzyme), and the indicated concentrations of NaCl. Unwinding was initiated by the addition of linearized M13 DNA to the indicated concentrations. The reactions were conducted at 37 °C.

† The reported rate values are corrected for the experimentally determined apparent binding stoichiometry of the enzyme preparations (Table 1).

‡ Because these experiments were not conducted at concentrations of substrate which were in vast excess to that of the mutant enzyme (e.g. the concentration of functional mutant enzyme (0·8 nM) was within 4-fold of the K_m for the DNA substrate at 4 mM-NaCl), the reported values for this enzyme at all salt concentrations have been determined by plotting the rate data from Fig. 3 *versus* the concentration of free DNA ends. We have assumed that the K_d of the mutant enzyme for DNA ends is similar to that of the wild-type enzyme. Consequently, since K_d for DNA ends is significantly lower than the K_m for the DNA substrate (Roman *et al.*, 1992), we have calculated the concentration of free DNA ends ([DNA ends]$_{free}$) as: [DNA ends]$_{tot}$—[functional enzyme]$_{tot}$ for those reactions in which the functional enzyme concentration did not exceed the concentration of DNA ends available.

lower intrinsic unwinding rate rather than a lower apparent affinity for DNA ends contributes to the reduced unwinding activity of the mutant enzyme. Because it is likely that, as with the wild-type enzyme, $K_d \ll K_m$ (Roman *et al.*, 1992), these conditions should allow stoichiometric binding of the recB^{2109}CD enzyme to the DNA substrate. The effect of increased concentrations of NaCl on both the apparent binding affinity (K_m) and rate of unwinding (k_{cat}) was also examined (Table 2); like wild-type recBCD enzyme, moderate concentrations of salt (60 to 100 mM) increase the rate of unwinding as well as slightly reduce the affinity of the mutant enzyme for the ends of duplex DNA molecules, although the extent of this stimulation is not as great for the mutant enzyme.

Although it appeared that the mutant enzyme did not have an enhanced affinity for internal base-pairs which might compete for binding to the ends of the linear DNA, the effect of the addition of increasing concentrations of DNA having no ends (i.e. supercoiled DNA) was measured to test this possibility directly. If enhanced binding to internal DNA sites were a factor, then a progressive decrease in the observed rate of unwinding would be obtained upon increasing the concentration of supercoiled DNA. Unwinding reactions performed using a defined concentration of linear M13 DNA (10 μM-nucleotide; 0·69 nM-molecule) with varying concentrations of supercoiled DNA showed no significant decrease in either the rate or the extent of unwinding, however (data not shown).

(d) *RecB^{2109}CD enzyme helicase activity requires significantly higher concentrations of ATP than that of wild-type recBCD enzyme*

The K_m for ATP of the mutant enzyme cannot be determined at saturating concentrations of DNA substrate because the K_m for DNA ends is too high to permit the use of DNA concentrations that are five- to tenfold in excess of the K_m. Consequently, as was employed for wild-type recBCD enzyme (Roman & Kowalczykowski, 1989*a*), the method of Florini & Vestling (1957) was used to derive the

Table 3

Effect of varying ATP concentration on enzymatic parameters of recB^{2109}CD enzyme helicase activity at different DNA concentrations

[DNA] (nM-molecules)	$K_{m,app}$ (mM-ATP)	$k_{cat,corr}$† (s^{-1})
0·35	1·0±0·2	111±12
0·69	0·9±0·1	123±5
1·04	2·2±0·2	245±15

The reactions contained 25 mM-Tris-acetate (pH 7·5), varying ATP concentration, 1 mM-$MgOAc_2$, 1 mM-DTT, SSB protein (1, 2, or 3 μM), and 2 nM-(0·4 nM-functional) recB^{2109}CD enzyme. Unwinding was initiated by the addition of linearized pBR322 DNA to 5, 10, or 15 μM-nucleotide (0·35, 0·69, or 1·04 nM-molecule) final concentration. The reactions were conducted at 25°C.

† The reported rate values are corrected for the apparent binding stoichiometry of the enzyme preparation (Table 1).

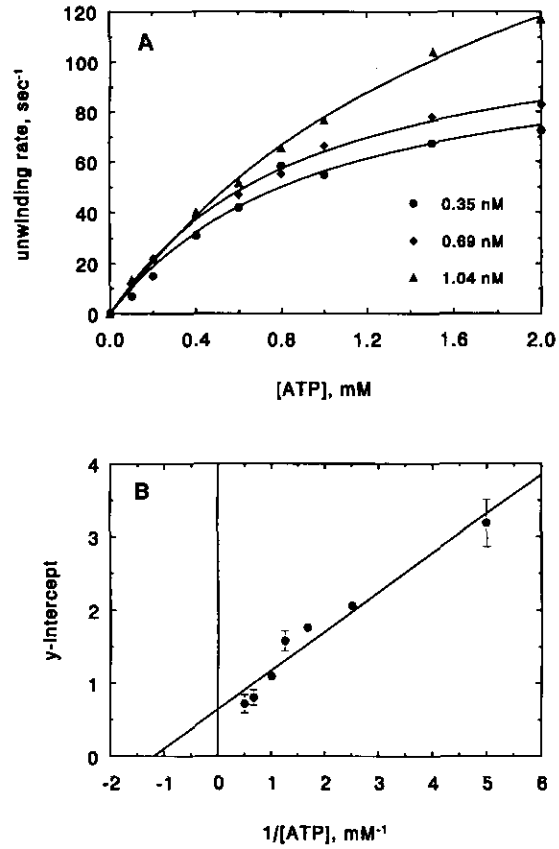

Figure 4. Effect of ATP concentration on the helicase activity of recB^{2109}CD enzyme. The data for A were generated from reactions conducted at 25°C in standard helicase buffer (except that ATP concentration was varied) with an appropriate concentration of SSB protein (equivalent to 20% of the molar nucleotide concentration) and 0·4 nM-functional recB^{2109}CD enzyme. Unwinding was initiated by the addition of *Eco*RI-digested M13 DNA to a final concentration of 0·35 nM-molecule (filled circles), 0·69 nM-molecule (filled diamonds), or 1·04 nM-molecule (filled triangles). These concentrations correspond to 5, 10, and 15 μM-nucleotide, respectively. The reported rates are corrected for the stoichiometry of the enzyme preparation. The results from A were plotted as rate^{-1} *versus* ATP^{-1} (data not shown). The *y*-intercept from each double reciprocal plot was then plotted against ATP^{-1} in B. The *x*-intercept of this graph is equivalent to K_m^{-1}.

apparent K_m for this nucleotide cofactor. ATP titrations were conducted at three concentrations of DNA (all below the K_m for DNA ends of recB^{2109}CD enzyme; Fig. 4A). As expected, the apparent k_{cat} increases with increasing DNA concentration (Table 3). From a plot of rate^{-1} *versus* [DNA molecules]$^{-1}$ for each ATP concentration in Figure 4A, a *y*-intercept was obtained (data not shown). These *y*-intercept values were then replotted against [ATP]$^{-1}$ (Fig. 4B) to yield a graph in which the negative reciprocal of the *x*-intercept is equivalent to the K_m. For the mutant enzyme, this value is 800 μM (with a range in error as low as 500 μM and as high as 2 mM) and is ~sixfold higher than that of wild-type recBCD enzyme (130 μM) (Roman & Kowalczykowski, 1989*a*).

(e) *The processivity of recB^{2109}CD enzyme is significantly less than that of wild-type recBCD enzyme*

DNA unwinding by recBCD enzyme is processive, that is, more than a single base-pair is unwound per binding event. Two factors allow us to easily calculate the processivity of unwinding. First, each end of a DNA molecule can be acted upon only once, because recBCD enzyme cannot initiate unwinding on substrates having ssDNA tails > 25 nucleotides long (Taylor & Smith, 1985). Second, the presence of SSB protein traps the unwound strands and prevents their reannealing, which would otherwise allow the starting substrate to be reformed.

We can measure the processivity of recBCD enzyme helicase activity using the fluorometric helicase assay. In this assay, the extent of an unwinding reaction indicates the total percentage of DNA unwound on DNA molecules having two ends; thus, it is twice the average length which can be unwound during a single recBCD enzyme binding event (defined as *N*). If the length of the DNA molecule in the reaction is less than or equal to twice the value of *N*, all of the DNA is fully unwound. As the DNA length exceeds twice the value of *N*, however, the extent of the reaction falls below 100% and *N* can be calculated. In the case of wild-type recBCD enzyme, *N* is 30(±3) kb as determined from the unwinding of both phage N4 (72 kb) and phage T4 (160 kb) DNAs (Roman *et al.*, 1992).

For recB^{2109}CD enzyme, *N* was determined at various ATP concentrations using both T7 (39·9 kb) and N4 (72 kb) phage DNAs. As was observed with wild-type recBCD enzyme (Roman *et al.*, 1992), the processivity of recB^{2109}CD enzyme helicase activity is affected in a hyperbolic manner by the concentration of ATP (Fig. 5). *N* does not exceed ~13 kb/end at physiologically relevant ATP concentrations (1 to 3 mM) (Lowry *et al.*, 1971; Mathews, 1972;

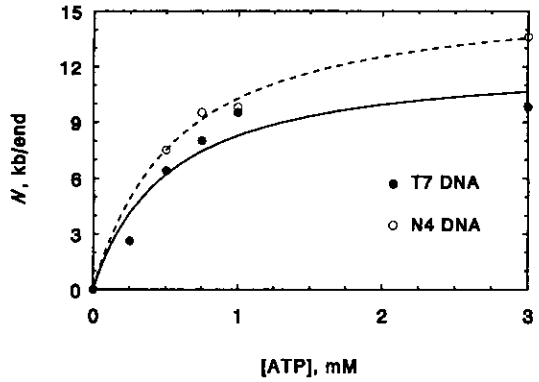

Figure 5. Effect of ATP on recB^{2109}CD enzyme helicase activity processivity. The reaction was conducted at 25°C in standard helicase buffer, except that the ATP concentration was varied. The substrate DNA concentration was 0·69 nM-molecule, and SSB protein was included at a concentration in 3-fold excess to the amount required to bind the total concentration of ssDNA which could be produced. Unwinding was initiated by adding a saturating concentration of recB^{2109}CD enzyme (8·6 nM; 1·25 functional molecules/end).

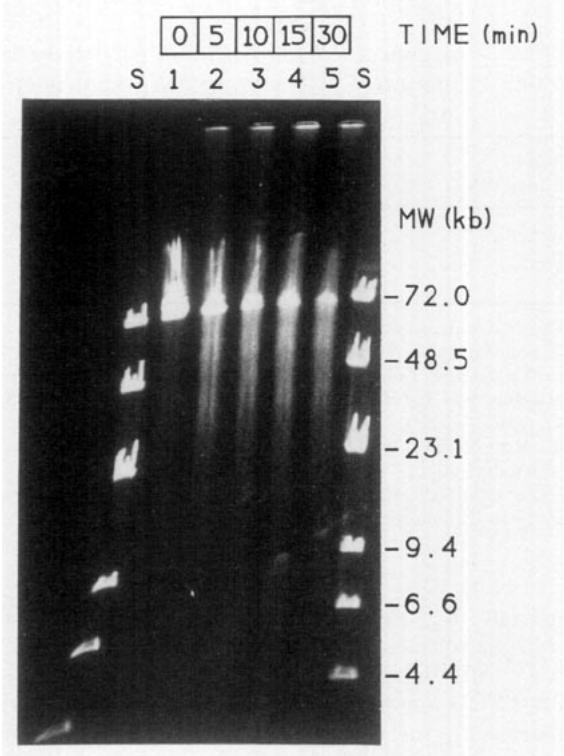

Figure 6. Pulsed field gel analysis of recB^{2109}CD enzyme helicase activity processivity. The reaction was conducted at 37°C in standard helicase buffer containing 99 μM-nucleotide (0·69 nM-molecule) N4 DNA and 19·8 μM-SSB protein. Unwinding was initiated by adding a saturating concentration of recB^{2109}CD enzyme (8·6 nM; 1·25 functional molecules/end). Each time point was treated with S_1 nuclease to remove ssDNA tails; thus, the products separated on the gel are the duplex DNA remaining after the mutant enzyme dissociates. The molecular weight standards are indicated (lane S). Lanes 1 to 5 show the time course of unwinding at 0, 5, 10, 15, and 30 min, respectively.

Bochner & Ames, 1982). Using data from the experiments with T7 DNA, the mutant enzyme has an apparent K_N (i.e. the concentration of ATP at which the processivity is one-half its maximal value) of 500(±210) μM-ATP and a limiting processivity of 12·4(±1·8) kb/end. With N4 DNA, these parameters are 570(±60) μM-ATP and 16·1(±0·6) kb/end, respectively. This K_N value is significantly greater than the K_N value of wild-type recBCD enzyme (41(±9) μM; Roman *et al.*, 1992), while the value for limiting N is reduced ~3-fold (32±(1·8) kb; Roman *et al.*, 1992).

To verify the value of N for recB^{2109}CD enzyme obtained using the fluorometric assay, the size distribution of dsDNA products remaining at the end of an N4 DNA unwinding reaction was measured by pulsed-field gel analysis (Roman *et al.*, 1992). In this assay, samples from a time course of unwinding are treated with S_1 nuclease to digest the unwound ssDNA tails, and the resultant duplex DNA products are separated by pulsed-field gel electrophoresis. This method allows us to examine directly the length of the DNA which has not been unwound; consequently, it is the converse of the fluorometric assay, in which the signal is generated by the presence of the unwound ssDNA. As shown in Figure 6, N4 DNA is partially unwound by recB^{2109}CD enzyme at 1 mM-ATP and 1 mM-$MgOAc_2$, and the peak of the distribution has a mobility less than that of the 48·5 kb standard, at ~50 to 55 kb dsDNA remaining (or 9 to 11 kb unwound/end). This result is in good agreement with that obtained by the fluorometric assay. The amount of intact DNA decreases over time, but the distribution of product molecules does not vary (Fig. 6), suggesting that, as expected, the mutant enzyme cannot reinitiate unwinding on previously unwound DNA molecules (Taylor & Smith, 1985). Although the size distribution extends to ~ 30 to 35 kb (or ~ 20 kb unwound/end), this is not inconsistent with the fluorometric results since N is merely the average, and not the maximum, length which the enzyme can unwind (see Roman *et al.*, 1992).

(f) *Joint molecule formation by recB^{2109}CD enzyme is inefficient and is independent of the presence of χ sequences*

Since the *in vitro* studies of recB^{2109}CD enzyme did not reveal the absence of any nuclease activity except χ-nicking (Eggleston & Kowalczykowski, 1993), and since the mutant enzyme appeared to have considerable helicase activity, the ability of recB^{2109}CD enzyme to participate in the co-ordinated production of joint molecules was determined (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). The assays utilize DNA substrates, linear duplex and supercoiled DNA molecules, which require both the homologous pairing activity of recA protein and, minimally, the helicase activity of

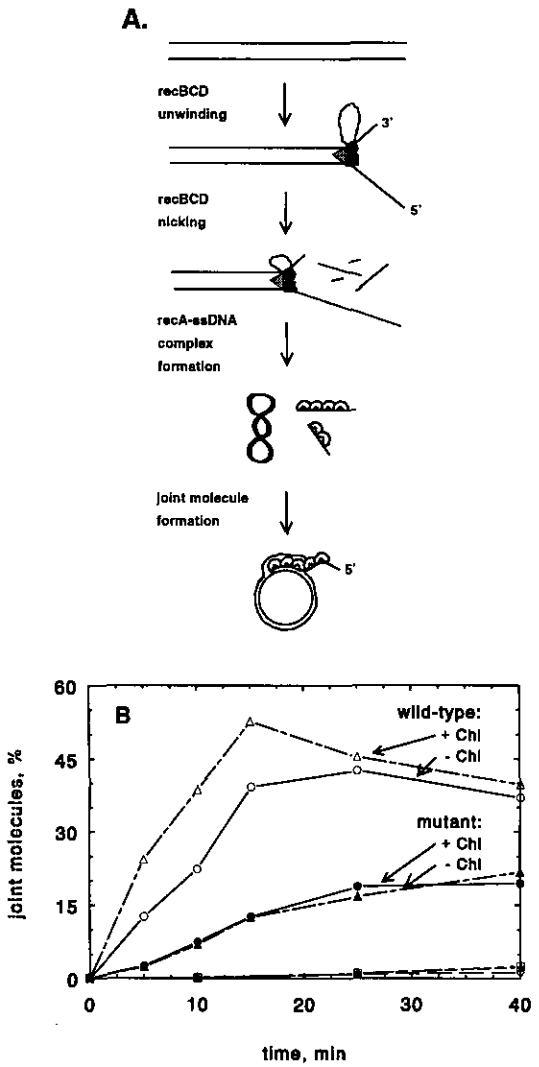

Figure 7. Filter binding analysis of joint molecule formation by the combined actions of wild-type or mutant recBCD enzyme, recA protein, and SSB protein. In A, a model for the formation of joint molecules by the combined actions of recBCD enzyme, recA protein, and SSB protein is presented (see the text for details). In B, filter-binding assays were conducted as described in Materials and Methods, using 10 μM-nucleotide (1·04 nM-molecule) linear pBR322 (χ^0 or χ^+) DNA and 5 μM-nucleotide (0·52 nM-molecule) ^{3}H-labeled supercoiled pBR322 (χ^0) DNA. The reaction was initiated by the simultaneous addition of 10 μM-recA protein and 0·1 functional recBCD enzyme molecule/DNA end. The conditions under which the reaction was performed are as follows: wild-type recBCD enzyme and linear DNA lacking χ, open circles; mutant enzyme and linear DNA lacking χ, filled circles; wild-type enzyme and DNA containing χ, open triangles; mutant enzyme and DNA containing χ, filled triangles; wild-type enzyme in the absence of recA protein, open squares; mutant enzyme in the absence of recA protein, +; and recA protein in the absence of recBCD enzyme, open diamonds.

recBCD enzyme to form joint molecules. The reaction is illustrated in Figure 7A.

In the first assay, a nitrocellulose filter binding assay, the formation of joint molecules containing even short lengths of heteroduplex DNA results in the retention of the labeled supercoiled DNA on the filter (Roman *et al.*, 1991). In the absence of recBCD enzyme, recA protein cannot promote the formation of joint molecules with these substrates (Fig. 7B, open diamond); in the absence of recA protein, no joint molecules are formed with either wild-type or mutant recBCD enzyme (Fig. 7B, open squares and +). When a subsaturating amount of wild-type recBCD enzyme is incubated with recA protein and linear dsDNA lacking a χ sequence, joint molecules are formed at an initial rate of 2·6% min^{-1}, and a maximum of 42% of the supercoiled DNA is taken up in joint molecules (Fig. 7B, open circle). In the presence of χ, the initial rate increases almost twofold to 4·9% min^{-1}, and the extent of the reaction increases to 52% (Fig. 7B, open triangle). RecB^{2109}CD enzyme, on the other hand, is less proficient at promoting joint molecule formation by recA protein. As expected from the absence of any apparent χ-cutting activity by the mutant enzyme (Amundsen *et al.*, 1990; Eggleston & Kowalczykowski, 1993), the presence or absence of a χ site in the donor DNA does not affect the initial rate of joint molecule formation (Fig. 7B, closed circle and closed triangle), which, at 0·8% min^{-1}, is three to sixfold lower than that of wild-type recBCD enzyme; in addition, the extent of the reaction (18%) is up to threefold lower. The magnitude of this rate difference in the presence of χ is greater than can be accounted for by the difference in helicase rate ($\sim$ 3-fold) alone.

Recent studies have suggested that the nonspecific dsDNA exonuclease activity of recBCD enzyme is depressed after the enzyme encounters a properly oriented χ site (Dixon & Kowalczykowski, 1991,1993). Consequently, DNA which is unwound after a χ is recognized and nicked is liberated as an intact ssDNA fragment (Dixon & Kowalczykowski, 1991,1993). In the recBCD enzyme-dependent joint molecule formation assay, therefore, the presence of a χ site in the linear duplex DNA has the effect of generating a discrete subpopulation of joint molecules which have incorporated the χ-dependent fragment, whereas in the absence of χ, recBCD enzyme remains in its nucleolytic mode, generating a heterogeneous population of fragments and, hence, a disperse joint molecule population. This observation has proven useful in the analysis of recABCD protein-dependent joint molecule formation by agarose gel electrophoresis (Dixon & Kowalczykowski, 1991).

M13mp7 DNA, which, as noted in Roman *et al.* (1991), contains a χ site at position 4943 to 4950, was used initially (diagrammed in Fig. 8A). In Figure 8B (lanes 2 to 5), a distinct joint molecule band which corresponds to invasion of the supercoiled DNA by the χ-dependent fragment is seen with the wild-type enzyme; this species is superimposed on a disperse population of joint molecules generated by χ-independent nicking (Roman *et al.*, 1991). In addition, a joint molecule species of lower mobility in which the full-length 5′ strand is incor-

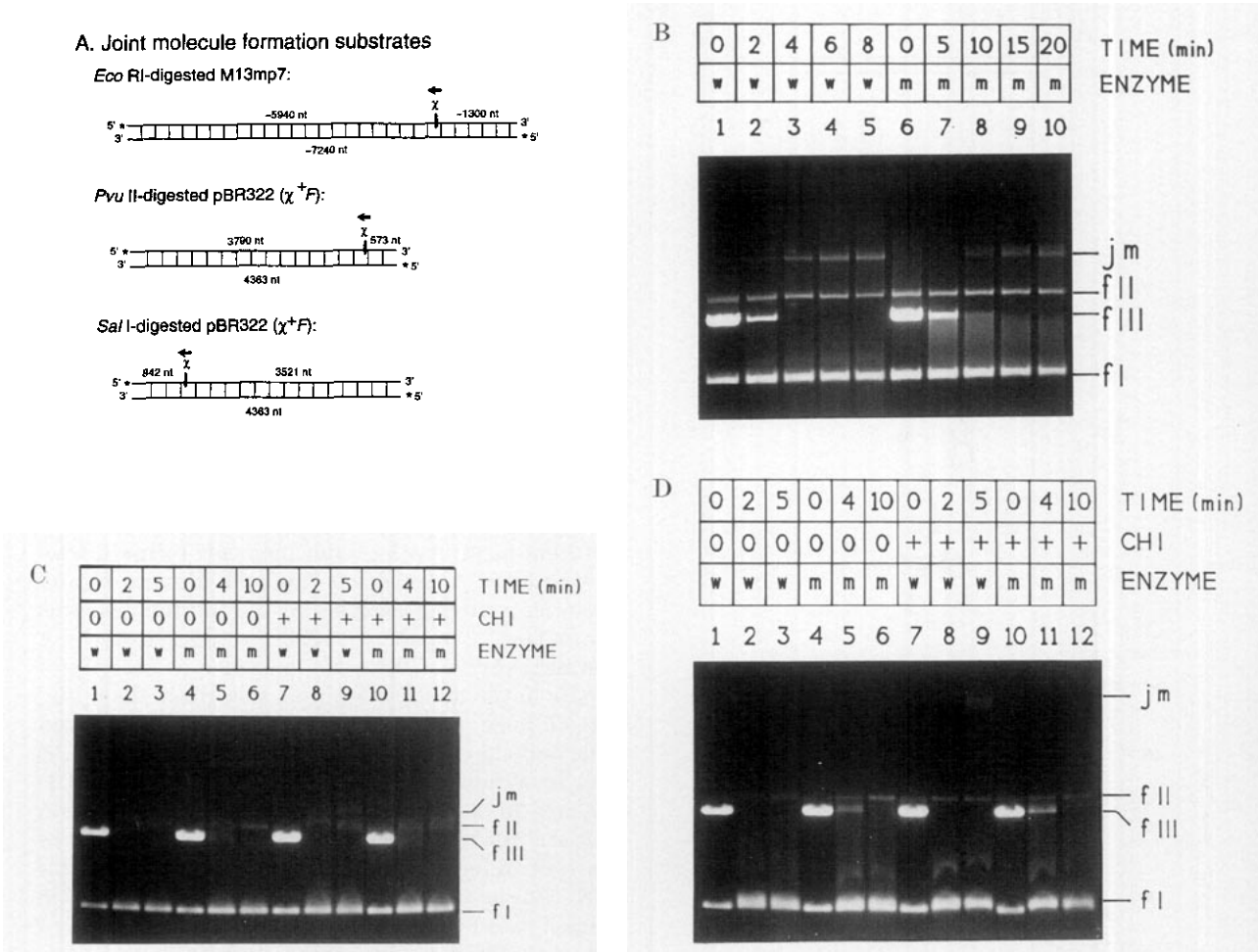

Figure 8. Agarose gel analysis of joint molecule formation by the combined actions of wild-type or mutant recBCD enzyme, recA protein, and SSB protein. The linear DNA substrates used are diagrammed (A). The pairs of DNA substrates were as follows: *Eco*RI-digested M13 DNA and supercoiled M13 DNA (B); *Sal*I-digested pBR322 (χ^0 or χ^+) DNA and supercoiled pBR322 (χ^0) DNA (C); and *Pvu*II-digested pBR322 (χ^0 or χ^+) DNA and supercoiled pBR322 (χ^0) DNA (D). The reactions were performed essentially as described for Fig. 6B except that 40 μM-nucleotide (2·76 nM or 4·16 nM-molecule) of the linear substrate and 20 μM-nucleotide (1·38 nM or 2·08 nM-molecule) of the supercoiled substrate were used. Time points were withdrawn at 0, 2, and 5 min for the wild-type enzyme reactions, and at 0, 4, and 10 min for the recB^{2109}CD enzyme reactions. The presence of a χ sequence is noted above the appropriate lanes. The mobility of the χ-dependent joint molecule is indicated.

porated is also formed irrespective of the presence of χ (data not shown; see Dixon & Kowalczykowski, 1991). In the mutant enzyme reaction (Fig. 8B, lanes 8 to 10), a fairly discrete band of slightly lower mobility is present; as will be demonstrated below, however, this band presumably derives not from cutting at χ but from specific cutting at a cryptic site. This interpretation is supported by results obtained when the nuclease activities of the mutant enzyme is examined. When 5′ end-labeled M13 DNA is treated with recB^{2109}CD enzyme, a discrete band which corresponds neither to full-length M13 nor to the χ-dependent fragment is observed specifically under these conditions (Eggleston & Kowalczykowski, 1993).

To confirm that the discrete band observed in the presence of the mutant enzyme derived from cutting at a cryptic site rather than at χ, the experiment was repeated with pBR322 DNA which either lacked or contained the χ sequence and which was linearized with either *Pvu*II or *Sal*I (illustrated in Fig. 8A). With *Sal*I-digested DNA, no discrete band is observed in either the wild-type (Fig. 8C, lanes 2, 3) or the mutant (Fig. 8C, lanes 5, 6) enzyme reactions in the absence of χ, while in the presence of χ, a discrete joint molecule is visible in the wild-type enzyme reaction (Fig. 8C, lanes 8, 9) but not in the mutant enzyme reaction (Fig. 8C, lanes 11, 12). pBR322 DNA linearized with *Pvu*II was used to eliminate the possibility that recB^{2109}CD enzyme was unable to recognize χ sites that were distant from the entry site; in this molecule, the χ site is positioned closer to the entry site, and hence the length of the intact χ fragment is longer (Fig. 8A). As expected, the χ-dependent joint molecule produced by wild-type recBCD enzyme has a lower mobility due to the greater size of the invading strand (Fig. 8D, lanes 8, 9), whereas no discrete

band is observed in the mutant enzyme reaction (Fig. 8D, lanes 11, 12). Thus, recB^{2109}CD enzyme is unable to produce χ-specific joint molecules.

4. Discussion

In this paper, we have shown that in several respects, the helicase activity of the recB^{2109}CD enzyme is reduced compared to that of the wild-type recBCD enzyme. The rate of unwinding by the mutant enzyme is ~threefold slower than that of wild-type recBCD enzyme ($k_{cat,corr} = 87$ *versus* 231 s^{-1} at 25°C; Table 1). The K_m for DNA ends of both enzymes (0·9 *versus* 0·8 nM; Table 2) at low salt concentrations is in the range of the *in vivo* concentration of DNA ends (~0·9 nM) (Roman & Kowalczykowski, 1989*a*). The K_m for ATP of the mutant enzyme, however, is significantly greater than that of the wild-type enzyme (800 μM *versus* 130 μM; Fig. 4). Since the recB subunit has an ATP-binding consensus sequence (Finch *et al.*, 1986) and is labeled by the ATP photoaffinity analog, azido-ATP (Julin & Lehman, 1987), it is conceivable that the *recB2109* mutation alters ATP binding; this mutation has not been sequenced, however, so this proposal cannot be confirmed. The processivity of the mutant enzyme is also reduced ~threefold, from an average of 30 kb unwound/DNA end by the wild-type enzyme (Roman *et al.*, 1992) to 13 kb/end for the mutant enzyme at physiological ATP concentrations (Fig. 5). The observation that both the rate of unwinding and the processivity of the recB^{2109}CD enzyme are reduced threefold implies that a lower intrinsic rate of unwinding, rather than an increased rate of dissociation, accounts for the decreased processivity of the mutant enzyme.

As shown in the accompanying paper, the non-specific nuclease activities of the mutant enzyme are somewhat reduced compared to those of the wild-type enzyme (Eggleston & Kowalczykowski, 1993). During the initial unwinding and degradation of duplex DNA, however, the rate of solubilization of DNA is nearly equivalent for the two enzymes (Eggleston & Kowalczykowski, 1993). Because the dsDNA exonuclease activity of recBCD enzyme is in actuality a ssDNA endonuclease activity which is manifest only during the unwinding of DNA (Taylor *et al.*, 1985), the rate of non-specific degradation must be considered in the context of the rate at which the DNA is unwound. Therefore, when the apparent initial rate of dsDNA degradation is corrected for the two to threefold reduction in rate of helicase activity of the mutant enzyme (Table 1), the rate of exonuclease activity would be higher for the recB^{2109}CD enzyme, suggesting that this enzyme nicks duplex DNA two to three times more frequently per unit length than does wild-type recBCD enzyme.

One of the more physiologically significant findings in terms of recB^{2109}CD enzyme behavior *in vitro* is its reduced ability to initiate the formation of joint molecules in the presence of both recA and SSB proteins. Filter-binding and agarose gel assays have been used to examine this biologically important function of recBCD enzyme in initiating joint molecule formation in the presence of both recA and SSB proteins (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991). As demonstrated by Dixon & Kowalczykowski (1991), these assays are also responsive to the presence of a χ sequence in the donor (linear duplex) DNA molecule. Wild-type enzyme, which can productively interact with χ, demonstrates heightened activity in the presence of χ (Figs 7 and 8), in qualitative agreement with *in vivo* observations of the stimulatory effect of χ on recombination. Although the mutant enzyme is processive enough to completely unwind the dsDNA substrate used in the assay (Figs 5 and 6), recB^{2109}CD enzyme-initiated joint molecule formation is less efficient, irrespective of the presence or absence of χ (Fig. 7). As noted in the accompanying paper (Eggleston & Kowalczykowski, 1993), this inability of the mutant enzyme to productively interact with χ could result from either failure to recognize the site or failure to attenuate the 3′ strand non-specific nuclease activity.

In the absence of χ, the initial rate of joint molecule formation by the mutant enzyme is, again, approximately threefold less than that of the wild-type enzyme, indicating that, when normalized for helicase activity, the enzymes appear to be similar. If it were true that the apparent difference between the enzymes in the absence of χ was accounted for solely by the slower unwinding rate of the mutant enzyme, however, then the yield of product molecules generated by the mutant enzyme should approach that generated by the wild-type enzyme if the reaction were allowed to proceed for a longer period of time. Since this result is not observed (Fig. 7, and data not shown), it is likely that a subpopulation of the ssDNA products which result from unwinding and concomitant degradation of the linear duplex substrate by recB^{2109}CD enzyme are incompetent for joint molecule formation. This subpopulation of molecules may be ssDNA fragments which are of insufficient length to be efficiently incorporated into stable joint molecules by recA protein, an interpretation which would agree with our proposal that the mutant enzyme nicks duplex DNA more frequently than the wild-type enzyme (see above).

Before proposing a model based strictly on these biochemical findings, the available genetic characterization of the *recB2109* mutation, summarized by Amundsen *et al.* (1990), must also be considered. In several respects, the effects of this allele *in vivo* are as pronounced as those displayed by a *recB* or *recC* null allele. The frequency of conjugal recombination in a *recB2109* strain is reduced by as much as 10^3-fold. Such a strain is also extremely sensitive to the DNA-damaging agent, mitomycin C. The frequency of λ *red*$^-$ *gam*$^-$ recombination and the degree of χ activation of λ recombination in a *recB2109* strain are not significantly different from those of strains bearing *recB* or *recC* null alleles. The observation that recombination of λ *red*$^-$ *gam*$^-$ (χ^0)

phage is lower in a mutant strain than in a wild-type strain suggests that unwinding of duplex DNA by the mutant enzyme results in DNA molecules unsuitable for recombination and/or that the dsDNA exonuclease activity of the wild-type enzyme is partially attenuated by non-canonical χ-like sequences. Although the *in vivo* degradation of T4 2^+ phage by the mutant enzyme is essentially equivalent to that of the null alleles, in the absence of the gene *2* product, twice as much acid-soluble material is released in the *recB2109* strain when compared to the null alleles. Like wild-type recBCD enzyme, however, recB^{2109}CD enzyme does not allow plating of T4 2^- phage. The biological basis of this phenotype is unclear and may result from the helicase and/or the dsDNA exonuclease activities of the enzyme. Recently, Rinken *et al.* (1992) have shown that unwinding activity alone is sufficient to inhibit plating of T4 2^- phage. Thus, we assume that the recB^{2109}CD enzyme must have, minimally, helicase activity *in vivo*. If this mutant enzyme were able to unwind DNA but not degrade the 3′ strand, we would expect, based on our current understanding of how the enzyme acts and is regulated, that the enzyme would not be recombination-deficient. This assumption is reasonable, since either recBCD enzyme mutants of the ‡ class (Chaudhury & Smith, 1984) or the isolated recBC enzyme (Palas & Kushner, 1990), which have no dsDNA exonuclease or χ-nicking activity but do possess reduced helicase activity, are fully capable of forming recombinant products; these results suggest that DNA unwinding alone, in the absence of non-specific degradation or nicking at χ, is sufficient for recombination. Thus, the genetic data and our results suggest that, given an adequate cellular concentration of ATP and Mg^{2+} (Eggleston & Kowalczykowski, 1993), the mutant enzyme must also have dsDNA exonuclease activity.

Taken as a whole, our studies of recB^{2109}CD enzyme provide a groundwork for considering its activity *in vivo*. RecB^{2109}CD enzyme is able to bind the ends of dsDNA and initiate its unwinding. As unwinding proceeds, the duplex molecule is degraded into short ssDNA fragments, even in the presence of χ sequences. At physiological concentrations of ATP (1 to 3 mM) (Bochner & Ames, 1982; Lowry *et al.*, 1971; Mathews, 1972), both the helicase and the nuclease activities of the mutant enzyme are estimated to be only ~ 70% of their maximum since the K_m for ATP by every criteria measured (dsDNA exonuclease, DNA helicase, and processivity of helicase activity) is higher than that of the wild-type enzyme and is comparable to the physiological concentration of this cofactor; therefore, the differences between the wild-type and mutant enzymes *in vivo* may be greater than the *in vitro* k_{cat} comparison suggests. After unwinding an average of 13 kb, the mutant enzyme dissociates from the DNA. Recombination in *recB2109* cells does not occur with high frequency because long, intact ssDNA molecules which can be utilized efficiently by recA protein are not produced. This situation contrasts dramatically with that of the wild-type recBCD enzyme, which is converted by its productive interaction with χ into a recombination-proficient entity that produces significant lengths of 3′ tailed ssDNA (Roman *et al.*, 1991; Dixon & Kowalczykowski, 1991,1993). While the reductions in the nuclease and helicase activities of recB^{2109}CD enzyme observed *in vitro* are reproducible, they are probably less significant *in vivo*. Therefore, the inability of the mutant enzyme to recognize the χ site and/or to attenuate the 3′-terminal strand-specific nuclease activity once a χ sequence is encountered is probably highly significant. This important defect obstructs the regulatory mechanism by which the activities of recBCD enzyme are normally governed.

We are grateful to Drs Gerry Smith, A. John Clark, Roger McMacken, and Lucia Rothman-Denes for providing strains and plasmids essential to these studies. Dr Malcolm Winkler, currently at the University of Texas Health Center in Houston, kindly provided the use of the pulsed-field gel apparatus. Discussions with and comments on the manuscript by D. Dixon, P. Lavery, S. Lauder, J. New, and B. Rehrauer were greatly appreciated. This work was supported by N.I.H. research grants GM-41347 and AI-18987 to S. C. K. and in part by a pre-doctoral fellowship from N.I.H. N.R.S.A. training grant GM-08061 to A. K. E. A preliminary account of this work was presented at the conferences RecA and Related Proteins, held in Saclay, France on September 17 to 21, 1990 and Genetic Recombination and Genome Rearrangements, held at Saxtons River, Vermont on July 28 to August 2, 1991.

References

Amundsen, S. K., Nieman, A. M., Thibodeaux, S. M. & Smith, G. R. (1990). Genetic dissection of the biochemical activities of recBCD enzyme. *Genetics*, **126**, 25–40.

Bochner, B. R. & Ames, B. N. (1982). Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. *J. Biol. Chem.* **257**, 9759–9769.

Chaudhury, A. M. & Smith, G. R. (1984). A new class of *Escherichia coli recBC* mutants: Implications for the role of recBC enzyme in homologous recombination. *Proc. Nat. Acad. Sci., U.S.A.* **81**, 7850–7854.

Cox, M. M. & Lehman, I. R. (1987). Enzymes of general recombination. *Annu. Rev. Biochem.* **56**, 229–262.

Dixon, D. A. & Kowalczykowski, S. C. (1991). Homologous pairing *in vitro* stimulated by the recombination hotspot, Chi. *Cell*, **66**, 361–371.

Dixon, D. A. & Kowalczykowski, S. C. (1993). Degradation of DNA by the recBCD enzyme of *E. coli* is controlled by the recombination hotspot, Chi. *Cell*. **73**, in the press.

Eggleston, A. K. & Kowalczykowski, S. C. (1993). Characterization of a mutant recBCD enzyme, the recB^{2109}CD enzyme, which lacks χ-specific, but not non-specific, nuclease activity. *J. Mol. Biol.* **231**, 605–620.

Finch, P. W., Storey, A., Chapman, K. E., Brown, K., Hickson, I. D. & Emmerson, P. T. (1986). Complete nucleotide sequence of the *Escherichia coli recB* gene. *Nucl. Acids Res.* **14**, 8573–8582.

Florini, J. R. & Vestling, C. S. (1957). Graphical deter-

mination of the dissociation constants for two substrate enzyme systems. *Biochim. Biophys. Acta*, **25**, 575–578.

Griffith, J. & Shores, C. G. (1985). RecA protein rapidly crystalizes in the presence of spermidine: a valuable step in its purification and physical characterization. *Biochemistry*, **24**, 158–162.

Julin, D. A. & Lehman, I. R. (1987). Photoaffinity labelling of the recBCD enzyme of *Escherichia coli* with 8-azidoadenosine 5′-triphosphate. *J. Biol. Chem.* **262**, 9044–9051.

Lloyd, R. G., Porton, M. C. & Buckman, C. (1988). Effect of *recF*, *recJ*, *recN*, *recO*, and *ruv* mutations on ultraviolet survival and genetic recombination in a *recD* strain of *Escherichia coli* K-12. *Mol. Gen. Genet.* **212**, 317–324.

Lovett, S. T., Luisi-DeLuca, C. & Kolodner, R. D. (1988). The genetic dependence of recombination in *recD* mutants of *Escherichia coli*. *Genetics*, **120**, 37–45.

Lowry, O. H., Carter, J., Ward, J. B. & Glaser, L. (1971). The effect of carbon and nitrogen sources on the level of metabolic intermediates in *Escherichia coli*. *J. Biol. Chem.* **246**, 6511–6521.

Mathews, C. K. (1972). Biochemistry of deoxyribonucleic acid-defective amber mutants of bacteriophage T4. 3. Nucleotide pools. *J. Biol. Chem.* **247**, 7430–7438.

Palas, K. M. & Kushner, S. R. (1990). Biochemical and physical characterization of exonuclease V from *Escherichia coli*: comparison of the catalytic activities of the recBC and recBCD enzymes. *J. Biol. Chem.* **265**, 3447–3454.

Ponticelli, A. S., Schultz, D. W., Taylor, A. F. & Smith, G. R. (1985). Chi-dependent DNA strand cleavage by recBC enzyme. *Cell*, **41**, 145–151.

Riddles, P. W. & Lehman, I. R. (1985). The formation of paranemic and plectonemic joints between DNA molecules by the recA and single-stranded DNA-binding proteins of *Escherichia coli*. *J. Biol. Chem.* **260**, 170–173.

Rinken, R., Thoms, B. & Wackernagel, W. (1992). Evidence that *recBC*-dependent degradation of duplex DNA in *Escherichia coli recD* mutants involves DNA unwinding. *J. Bacteriol.* **174**, 5424–5429.

Roman, L. J. & Kowalczykowski, S. C. (1989*a*). Characterization of the helicase activity of the *Escherichia coli* recBCD enzyme using a novel helicase assay. *Biochemistry*, **28**, 2863–2873.

Roman, L. J. & Kowalczykowski, S. C. (1989*b*). Characterization of the adenosine triphosphatase activity of the *Escherichia coli* recBCD enzyme: relationship of ATP hydrolysis to the unwinding of duplex DNA. *Biochemistry*, **28**, 2873–2881.

Roman, L. J. & Kowalczykowski, S. C. (1989*c*). Formation of heteroduplex DNA promoted by the combined activities of *Escherichia coli* recA and recBCD proteins. *J. Biol. Chem.* **264**, 18340–18348.

Roman, L. J., Dixon, D. A. & Kowalczykowski, S. C. (1991). RecBCD-dependent joint molecule formation promoted by the *Escherichia coli* recA and SSB proteins. *Proc. Nat. Acad. Sci., U.S.A.* **88**, 3367–3371.

Roman, L. J., Eggleston, A. K. & Kowalczykowski, S. C. (1992). Processivity of the DNA helicase activity of *Escherichia coli* recBCD enzyme. *J. Biol. Chem.* **267**, 4207–4214.

Smith, G. R. (1987). Mechanism and control of homologous recombination in *Escherichia coli*. *Annu. Rev. Genet.* **21**, 179–201.

Smith, G. R. (1990). RecBCD enzyme. In *Nucleic Acids and Molecular Biology* (Eckstein, F. & Lilley, D. J. M., eds), vol. 4, pp. 78–98, Springer Verlag. Berlin, Heidelberg.

Smith, G. R. & Stahl, F. W. (1985). Homologous recombination promoted by Chi sites and recBC enzyme of *E. coli*. *BioEssays*, **2**, 244–249.

Smith, G. R., Schultz, D. W., Taylor, A. F. & Triman, K. (1981). Chi sites, recBC enzyme, and generalized recombination. *Stadler Genet. Symp.* **13**, 25–37.

Smith, G. R., Amundsen, S. K., Braedt, G., Holbeck, S. L., McKittrick, N. H., Neiman, A. M., Taylor, A. F. & Thibodeaux, S. M. (1984). Roles of recBC enzyme and Chi sites in homologous recombination. *Cold Spring Harbor Symp. Quant. Biol.* **49**, 485–495.

Smith, G. R., Amundsen, S. K., Cheng, K. C., McKittrick, N. H., Ponticelli, A. S., Schultz, D. W., Taylor, A. F. & Thibodeaux, S. M. (1987). Mechanism and control of homologous recombination. In *DNA Replication and Recombination* (McMacken, R. & Kelly, T. J., eds), pp. 567–583, Alan R. Liss, Inc. New York.

Taylor, A. F. (1988). RecBCD enzyme of *Escherichia coli*. In *Genetic Recombination* (Kucherlapati, R. & Smith, G. R., eds), pp. 231–263, American Society for Microbiology. Washington, DC.

Taylor, A. F. & Smith, G. R. (1980). Unwinding and rewinding of DNA by the recBC enzyme. *Cell*, **22**, 447–457.

Taylor, A. F. & Smith, G. R. (1985). Substrate specificity of the DNA unwinding activity of the recBC enzyme of *E. coli*. *J. Mol. Biol.* **185**, 431–443.

Taylor, A. F., Schultz, D. W., Ponticelli, A. S. & Smith, G. R. (1985). RecBC enzyme nicking at Chi sites during DNA unwinding: location and orientration-dependence of the cutting. *Cell*, **41**, 153–163.

Telander-Muskavitch, K. M. & Linn, S. (1982). A unified mechanism for the nuclease and unwinding activities of the recBC enzyme of *Escherichia coli*. *J. Biol. Chem.* **257**, 2641–2648.

Thaler, D. S., Sampson, E., Siddiqi, I., Rosenberg, S. M., Thomason, L. C., Stahl, F. W. & Stahl, M. M. (1989). Recombination of bacteriophage λ in *recD* mutants of *Escherichia coli*. *Genome*, **31**, 53–67.

Uhlin, B. E. & Clark, A. J. (1981). Overproduction of the *Escherichia coli* recA protein without stimulation of its proteolytic activity. *J. Bacteriol.* **148**, 386–390.

Wang, T. V. & Smith, K. C. (1989). The roles of recBCD, SSB, and recA proteins in the formation of heteroduplexes from linear-duplex DNA *in vitro*. *Mol. Gen. Genet.* **216**, 315–320.

West, S. C., Cassuto, E. & Howard-Flanders, P. (1981). RecA protein promotes homologous pairing and strand exchange reactions between duplex DNA molecules. *Proc. Nat. Acad. Sci., U.S.A.* **78**, 2100–2104.

Edited by P. von Hippel