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Processivity of the DNA Helicase Activity of *Escherichia coli* recBCD Enzyme*

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A fluorescence assay was used to measure the processivity of *Escherichia coli* recBCD enzyme helicase activity. Under standard conditions, recBCD enzyme unwinds an average of 30 ± 3.2 kilobase pairs (kb)/DNA end before dissociating. The average processivity (P_{obs}) of DNA unwinding under these conditions is 0.99997, indicating that the probability of unwinding another base pair is 30,000-fold greater than the probability of dissociating from the double-stranded DNA. The average number of base pairs unwound per binding event (N) is sensitive to both mono- and divalent salt concentration and ranges from 36 kb at 80 mM NaCl to 15 kb at 280 mM NaCl. The processivity of unwinding increases in a hyperbolic manner with increasing ATP concentration, yielding a K_N value for ATP of 41 ± 9 μM and a limiting value of 32 ± 1.8 kb/end for the number of base pairs unwound. The importance of the processivity of recBCD enzyme helicase activity to the recBCD enzyme-dependent stimulation of recombination at Chi sites observed *in vivo* is discussed.

RecBCD enzyme is a complex protein consisting of three nonidentical subunits and possessing DNA helicase, DNA-dependent ATPase, and ss- and dsDNA[1] nuclease activities (for reviews, see Telander-Muskavitch and Linn (1981), Smith (1988), and Taylor (1988)). Genetic analysis has demonstrated the need for the *recB* and *recC* genes in general genetic recombination in *Escherichia coli*. Mutations in either gene reduce conjugal or transduction recombination frequencies to values as low as 0.1% of wild-type levels (Howard-Flanders and Theriot, 1966; Emmerson and Howard-Flanders, 1967).

The ss- and dsDNA nuclease activities of recBCD enzyme are greatly inhibited by the presence of calcium ions, SSB protein, and high concentrations of ATP, whereas the helicase and ATPase activities of recBCD enzyme are relatively unaffected by these conditions (Mackay and Linn, 1976; Eichler and Lehman, 1977; Rosamond *et al.*, 1979; Roman and Kowalczykowski, 1989a, 1989b). Since the latter two conditions are physiologically significant, the helicase activity, rather than the nuclease activity, of recBCD enzyme is assumed to play an important functional role *in vivo*. A specific proposal by Smith *et al.* (1984) postulated that recBCD enzyme helicase activity creates a ssDNA substrate to which recA protein can bind and subsequently use to catalyze DNA strand invasion. Further unwinding of the dsDNA by recBCD enzyme may then extend the length of the heteroduplex DNA region. To test this hypothesis, we have established *in vitro* reactions that require the helicase activity of recBCD enzyme to initiate the formation of heteroduplex DNA by recA protein (Roman and Kowalczykowski, 1989c; Kowalczykowski and Roman, 1990; Roman *et al.*, 1991; Dixon and Kowalczykowski, 1991). Thus, the study of recBCD enzyme unwinding activity is crucial to the understanding of the mechanistic role of recBCD enzyme both *in vitro* and *in vivo*.

recBCD enzyme is also required for the stimulation of recombination at Chi sites, which are "hot spots" for recombination. Recombination is stimulated as far as 10 (Ennis *et al.*, 1987) to 20 kb (McMilin *et al.*, 1974; Stahl *et al.*, 1983) from the Chi site. The distance over which recombination is stimulated may be related to the extent to which recBCD enzyme continues unwinding the dsDNA after acting at the Chi site; that is, it may be a function of the processivity of enzymatic unwinding. Consistent with this expectation, Chi-dependent formation of joint molecules *in vitro* requires continued DNA unwinding after the recBCD enzyme has cut at the Chi site (Dixon and Kowalczykowski, 1991). Thus, the study of the processivity of recBCD enzyme unwinding may elucidate the mechanism for Chi action at a distance.

Electron microscopic studies have suggested that the processivity of recBCD enzyme unwinding is high because bacteriophage T7 DNA, which is 40 kb in length, can be completely unwound (Telander-Muskavitch and Linn, 1982; Taylor and Smith, 1985). Since recBCD enzyme can only bind to and initiate unwinding at a dsDNA end with a ssDNA tail shorter than about 25 nucleotide residues (Taylor and Smith, 1985), reinitiation cannot occur at an end that has been unwound by more than 25 bp. Assuming that unwinding occurs from both ends, this implies that a recBCD enzyme molecule is capable of unwinding at least 20 kb before dissociating from the duplex DNA.

We have previously determined the enzymatic parameters of recBCD enzyme helicase activity by using a novel helicase assay that is based on the quenching of SSB protein intrinsic fluorescence upon its binding to ssDNA. In this assay, recBCD enzyme unwinds the dsDNA, forming ssDNA that is instantaneously trapped by SSB protein. This binding results in an easily measured decrease in fluorescence that is proportional

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); kb, kilobase pair(s); SSB protein, *E. coli* single-stranded DNA-binding protein.

to the amount of DNA unwinding (Roman and Kowalczykowski, 1989a). We now use this assay to examine the processivity of recBCD enzyme helicase activity under a variety of experimental conditions.

EXPERIMENTAL PROCEDURES

Protein and DNA Isolation—M13 replicative form DNA was prepared either by banding in a CsCl-ethidium bromide density gradient as described by Messing (1983) or by chromatography on Sephacryl S-1000 (Pharmacia LKB Biotechnology Inc.); the DNA was linearized by digestion with *Eco*RI restriction endonuclease. Bacteriophage N4 DNA was prepared by phenol extraction of purified virions (Zivin *et al.*, 1980) and was a gift from G. Lindberg and L. Rothman-Denes of the University of Chicago. N4 DNA is a linear dsDNA molecule that is 72 kb in length (Zivin *et al.*, 1980). Bacteriophage λ DNA was either purchased from U. S. Biochemical Corp. or purified as described in Maniatis *et al.* (1982). Bacteriophage T4 DNA was purchased from Sigma. The molar nucleotide concentration of dsDNA was determined using an extinction coefficient of 6500 M^{-1} cm^{-1} at 260 nm. The molar molecule concentration was determined by dividing the molar nucleotide concentration by 334,000, 144,000, 97,000, and 14,390 nucleotides/molecule for T4, N4, λ, and M13mp7 DNA, respectively. The molar concentration of DNA ends is 2-fold higher than the molar molecule concentration.

recBCD enzyme was purified as described (based on the procedure of Dykstra *et al.* (1984) as modified by Roman and Kowalczykowski (1989a)). Protein concentration was determined using an extinction coefficient of 4.0×10^6 M^{-1} cm^{-1} at 280 nm (Roman and Kowalczykowski, 1989a). The specific activity of the preparation used was 9.4×10^4 nuclease units/mg or 2.1×10^4 helicase units/mg. Nuclease and helicase units were measured as described by Eichler and Lehman (1977) and Roman and Kowalczykowski (1989a), respectively. For the recBCD enzyme preparation used in this paper, the experimentally observed DNA binding stoichiometry as determined by helicase activity was 5.4 recBCD enzyme molecules/dsDNA end (Roman and Kowalczykowski, 1989a).

SSB protein was isolated from strain RLM727 as described (LeBowitz, 1985). Protein concentration was determined using an extinction coefficient of 3.0×10^4 M^{-1} cm^{-1} at 280 nm (Ruyechan and Wetmur, 1975).

Reaction Conditions—Standard conditions for the helicase assay consisted of 25 mM Tris acetate (pH 7.5), 1 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, and an ATP-regenerating system consisting of 1.5 mM phosphoenolpyruvate and 16 units/ml of pyruvate kinase. Due to the required addition of a large volume of SSB protein, the final reaction buffer for assays using T4 or N4 DNA also contained either 12.3% glycerol, 64 mM NaCl, and 0.64 mM EDTA or 5.8% glycerol, 30 mM NaCl, and 0.3 mM EDTA, respectively. Control experiments indicate that, under these conditions, EDTA has no effect on the observed processivity; however, the observed rate of unwinding is inhibited by 5.5, 34, and 52% at 0.1, 0.3, and 0.64 mM EDTA, respectively.[2] The fluorescence helicase assays were performed in a total volume of 300 μl at 25 or 37 °C, as indicated.

Unless otherwise noted, the concentrations of dsDNA and proteins were 1.4 nM DNA ends (equal to 240 μM nucleotide of T4 DNA, 103 μM nucleotide of N4 DNA, 68 μM nucleotide of λ DNA, and 10 μM nucleotide of M13 DNA), an SSB protein concentration equal to 20% of the DNA nucleotide concentration (*i.e.* 48, 20.6, 13.6, and 2 μM for T4, N4, λ, and M13 DNAs, respectively), and 7.6 nM recBCD enzyme (53.6 helicase units/ml). This concentration of recBCD enzyme is sufficient to saturate all DNA ends present in the assay, given the experimentally determined stoichiometry for recBCD enzyme binding to dsDNA derived from the helicase assay.

Fluorescence Helicase Assay—This assay was performed and the raw data were treated as described previously (Roman and Kowalczykowski, 1989a). Reactions were initiated by the addition of either recBCD enzyme or DNA after all other components were equilibrated to the correct temperature. The percentage of DNA unwound was calculated from the raw fluorescence data by dividing the observed fluorescence change by the total fluorescence quenching of SSB protein obtained in the presence of an equivalent concentration of heat-denatured dsDNA. This total fluorescence quenching is assumed to represent 100% unwinding of the DNA. The percentage of dsDNA unwound at the final plateau of the reaction is designated as the final extent of unwinding.

To ensure that the measured extent of enzymatic DNA unwinding was unaffected by the nucleolytic activities of recBCD enzyme, which might produce ssDNA fragments too small to be bound by SSB protein, the ability of the unwound DNA products to bind SSB protein and to effect the control level of fluorescence quenching was assessed. The unwound DNA present after a helicase reaction at 25 °C using either standard reaction conditions (1 mM magnesium ion and 1 mM ATP) or conditions that resulted in the highest levels of nuclease activity (*i.e.* 1 mM magnesium ion and 50 μM ATP or 8 mM magnesium ion and 1 mM ATP) was heat-denatured at 95 °C for 5 min. This step inactivates the SSB protein present during the unwinding reaction so that the denatured SSB protein does not contribute to the observed fluorescence quenching. Upon the addition of native SSB protein (20.6 μM for an N4 DNA reaction), the total fluorescence decrease obtained with the heat-denatured helicase reaction mixtures was equivalent to that observed with unreacted heat-denatured DNA under the same conditions. Thus, under these conditions, recBCD enzyme is not creating ssDNA that is too short to be bound by SSB protein. However, there are conditions under which the nuclease activity partially interferes with this assay; these conditions include assays at 37 °C in the absence of calcium or at 25 °C in the absence of SSB protein when the ATP concentration is low (40 μM). Under these conditions, approximately 10–20% of the DNA is degraded to oligonucleotides that fail to completely quench SSB protein fluorescence, thereby leading to an underestimation of the true extent of unwinding by an equivalent amount. Consequently, the assays were typically conducted at 25 °C in the presence of SSB protein; for the few reported experiments conducted at 37 °C, the observed extent of unwinding underestimates the true processivity by 10–20%, which, as will be seen below, is within the experimental variation.

The rate of N4 DNA unwinding was comparable with that reported previously for M13 DNA (Roman and Kowalczykowski, 1989a). The linear M13 DNA used has a 4-nucleotide overhang, and N4 DNA has, at most, a 7-base overhang (Zivin *et al.*, 1980). The initial unwinding rates for λ DNA were approximately 2–5-fold lower, depending on the DNA concentration, than those for N4 and M13 DNAs; these rates increased to those reported with N4 and M13 DNAs when the 12-base overhang was removed with S1 nuclease[2]. This suggests that either the ssDNA overhang itself or the SSB protein bound to this overhang is responsible for the decreased rate of unwinding, perhaps by limiting recBCD enzyme initiation.

Pulsed-field Agarose Gel Electrophoresis—The helicase reaction was performed in 220 μl using standard reaction conditions, except that the ATP concentration was either 40 μM or 10 mM; the concentrations of N4 DNA, SSB protein, and recBCD enzyme were 1.4 nM ends, 20.6 μM, and 0.95 nM, respectively. All components, except recBCD enzyme, were incubated at 37 °C for 3 min, and unwinding was initiated by the addition of recBCD enzyme. Aliquots (30 μl) were removed at the indicated times, added to 0.1 volume of 1% sodium dodecyl sulfate, and stored on ice. S1 nuclease buffer (10×: 50% glycerol, 300 mM sodium acetate (pH 4.6), 10 mM $ZnCl_2$, 500 mM NaCl) was added to a final concentration of 1×, and 0.025 unit of S1 nuclease was added per μg of N4 DNA. The nuclease digestions were incubated for 10 min at 37 °C and were stopped by the addition of 10× loading buffer (50% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol) and 20% sodium dodecyl sulfate to 1.5× and 0.6%, respectively. Under these conditions, there is no detectable degradation of intact N4 DNA, yet heat-denatured N4 DNA is fully digested (see Fig. 4). A portion of each aliquot was run on a 1% agarose gel (20 × 20 cm) made with modified TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA). The pulsed-field gel was run in modified TBE at 12 °C using a double inhomogeneous arrangement (anodes at positions 90 N and W; cathodes at positions 5, 95, and 180 S and E) on a Pharmacia LKB Biotechnology Inc. Pulsaphor Plus apparatus. Electrophoresis was carried out at 330 V (~200 mA) for 3 h and 20 min at a 1-s pulse time followed by 17 h at a 3-s pulse time. The gel was stained in modified TBE containing 2 μg/ml ethidium bromide for 45 min and was destained in water for 15 min before being photographed.

RESULTS

recBCD Enzyme Can Unwind 30 kb Before Dissociating from dsDNA—Our assay for the processive behavior of recBCD enzyme helicase activity is based on the following

[2] L. J. Roman, A. K. Eggleston, and S. C. Kowalczykowski, unpublished observations.

observations (see Fig. 1; for clarity, unwinding is shown occurring only from one end but, in fact, occurs from both ends). To initiate unwinding, recBCD enzyme must first bind to an end of a dsDNA molecule (Fig. 1, *a* and *b*). The dsDNA is unwound and, in the presence of SSB protein, is maintained as ssDNA (*c*); renaturation of the ssDNA strands does not occur within the time frame of these experiments (Roman and Kowalczykowski, 1989a). Since recBCD enzyme can only bind to and initiate unwinding at a dsDNA end possessing a ssDNA tail that is shorter than 25 nucleotide residues (Taylor and Smith, 1985), reinitiation cannot occur at an end that has been unwound by more than 25 bp (*d*). Because reinitiation is blocked, the average length of dsDNA unwound from each end (*i.e.* per recBCD enzyme-binding event) is limited either by the length of the dsDNA substrate or by the intrinsic processivity of the recBCD enzyme. Free recBCD enzyme, however, can initiate unwinding on another intact dsDNA molecule, until eventually all of the DNA molecules will be at least partially unwound (Roman and Kowalczykowski, 1989a). The average length of unwound DNA per end is a measure of recBCD enzyme helicase activity processivity, provided that the DNA substrate is longer than twice the average processive distance for recBCD enzyme translocation. In our assay, the fraction of total dsDNA unwound is simply the fraction of the maximum possible quenching of SSB protein fluorescence obtained with heat-denatured DNA.

Fig. 2 shows data derived from a typical helicase assay using N4 DNA. The percent maximum SSB protein fluorescence quenching observed at the reaction plateau is equal to the percentage of total DNA unwound, which, in turn, is equal to the average percent unwinding per dsDNA molecule. In this example (Fig. 2, *solid line*), the observed fluorescence quenching at the plateau region is 84% of the fluorescence quenching observed using an equivalent amount of heat-denatured N4 DNA. Therefore, 84% of the input dsDNA, or 60.5 kb (0.84 × 72 kb), is unwound per N4 DNA molecule. Since recBCD enzyme can bind to and initiate unwinding on both ends of the molecule, the average number of base pairs unwound by a recBCD enzyme molecule before dissociation (defined as *N*) is 30.3 kb.

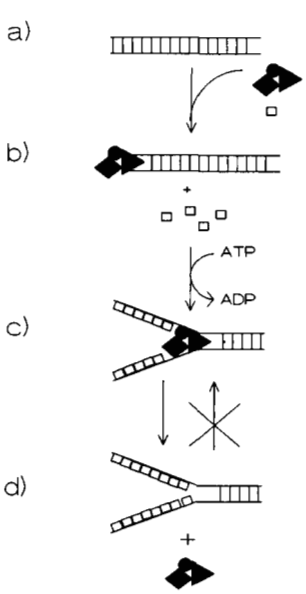

FIG. 1. **Illustration of the fluorescence assay used to determine the average number of base pairs unwound per DNA end by recBCD enzyme before dissociation (*N*).** For simplicity, only a forked DNA molecule is illustrated, although the major DNA species is actually a loop-tail structure. recBCD enzyme is pictured as unwinding only one end of the dsDNA molecule, but unwinding is assumed to occur from both ends. *Triangle/diamond/circle*, recBCD enzyme; *square*, SSB protein.

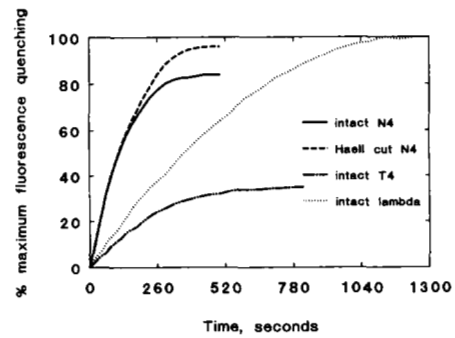

FIG. 2. **Extent of DNA unwinding by recBCD enzyme.** Standard buffer and component concentrations were used at 25 °C. The *traces* represent unwinding of various types of DNA. *Solid trace*, intact N4 DNA; *dashed trace*, N4 DNA fragments generated by restriction with *Hae*II enzyme; *dashed-dot trace*, intact T4 DNA; and *dotted trace*, intact λ DNA.

TABLE I

Processivity of DNA helicase activity as a function of recBCD enzyme concentration

Concentrations of N4 DNA and SSB protein were 1.4 nM ends and 20.6 μM, respectively. Standard buffer conditions were used at 25 °C. Saturation of the DNA helicase activity occurs at 7.6 nM recBCD enzyme for this enzyme preparation. The experimental uncertainty for the *N* values is ±15%.

[recBCD enzyme]	*N*
nM	*kb/end*
1.0	30
2.0	32
3.0	29
4.0	30
5.0	31
6.0	30
7.6	31

To ensure that this limited extent of unwinding is related only to DNA length and is not due to N4 DNA, which, for some unknown reason, cannot be unwound by recBCD enzyme, the extent of unwinding was also tested using either N4 DNA that was restricted with *Hae*II (yielding fragments of 59 and 13 kb (Zivin *et al.*, 1980)), λ DNA (which is 35% shorter than N4 DNA), or T4 DNA (which is 2.3-fold longer than N4 DNA). In the first two cases, nearly all (100 ± 10%) of the DNA is unwound (Fig. 2), demonstrating that both the shorter λ DNA and the shorter lengths of N4 DNA can be almost fully unwound by recBCD enzyme. Using T4 DNA, only 35% of the DNA is unwound, resulting in an average value for *N* (29 ± 2.6 kb/end) that is invariant over a 4-fold range of recBCD enzyme concentration (data not shown). Thus, consistent results are obtained with DNA molecules of different lengths.

No difference in the extent of unwinding is observed when either saturating or subsaturating concentrations of recBCD enzyme are used; an average of ~30 kb/end are unwound at all recBCD enzyme concentrations tested using either N4 DNA (Table I) or T4 DNA (data not shown), confirming that recBCD enzyme remains active after passage through a DNA molecule and can act catalytically on those molecules that have not been previously unwound (Roman and Kowalczykowski, 1989a). In agreement with these observations, when an additional aliquot of N4 DNA (1.4 nM ends) is added at the end of an N4 DNA unwinding reaction, this additional dsDNA is unwound at the same rate and to the same final

extent (30 kb/end) as the initial aliquot (data not shown). Thus, the unwinding of only 84% of the N4 DNA is not due to inactivation of recBCD enzyme helicase activity. A saturating amount of recBCD enzyme is used for most of the experiments reported here, since the slopes of both the initial reaction rate and the final extent plateau are more clearly demarcated than those obtained at lower recBCD enzyme concentrations, where the initial rate of unwinding is slower.

To exclude the possibility that the limited extent of N4 DNA unwinding results from the presence of a subpopulation of DNA molecules that cannot be unwound by recBCD enzyme, the products of an N4 DNA unwinding reaction were separated by either conventional (data not shown) or pulsed-field (see Fig. 4, below) agarose gel electrophoresis. Over the range of conditions tested, all of the N4 DNA molecules are at least partially unwound, as evidenced by the decrease in full-length DNA substrate over time.

Since the concentration of DNA molecules in these assays is in the vicinity of, rather than in great excess of, the apparent K_m value for helicase activity (Roman and Kowalczykowski, 1989a), N was determined over a range of DNA concentrations (0.36–2.8 nM ends) to ensure that incomplete unwinding is not due to an inability of recBCD enzyme to bind the DNA substrate as it is depleted during the course of the reaction. We find that 30 ± 3 kb are unwound per DNA end at all concentrations of N4 DNA examined (data not shown). Therefore, although the concentration of DNA ends in these assays is approximately equal to the apparent K_m for DNA unwinding, it must still be well above the K_d for recBCD enzyme binding to DNA ends. Thus, in agreement with the agarose gel electrophoresis results, the lower observed extent of unwinding is not due to a failure of recBCD enzyme to unwind some DNA molecules as a consequence of limited binding affinity.

Nucleotide Cofactor Concentration Affects the Processivity of Unwinding by recBCD Enzyme—The rates of both the dsDNA-dependent ATPase and the helicase activities of recBCD enzyme increase with ATP concentration; the apparent K_m values for ATP are 85 and 130 μM for the ATPase and helicase activities, respectively (Roman and Kowalczykowski, 1989a, 1989b). For comparison, the effect of ATP concentration on the processivity of unwinding N4 DNA was examined (Fig. 3). At low concentrations of ATP (less than 100 μM), the processivity of unwinding is significantly decreased, in an apparently hyperbolic fashion. At 10 μM ATP, N is equal to 3 ± 1 kb/end. Since this value is only 4% of the length of the N4 DNA molecule, M13 DNA (7.2 kb) was used to provide a more accurate result. In agreement, M13 DNA is unwound an average of 2.3 ± 0.2 kb/end at this ATP concentration (Fig. 3, *square*). The data in Fig. 3 were fit to a hyperbola, and the values for the limiting N and K_N (the concentration of ATP at which N is one-half that observed at maximum) were determined to be 32 ± 1.8 kb/end and 41 ± 8.5 μM, respectively. Since the helicase activity of recBCD enzyme is supported by dATP, its effect on processivity was also examined. As shown in Table II (lines 5–7), the dependence of the unwinding processivity on dATP concentration mirrors that of ATP.

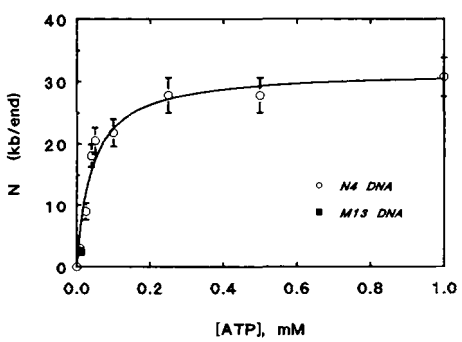

FIG. 3. **Number of kilobase pairs unwound per DNA end as a function of ATP concentration.** Concentrations of DNA and recBCD enzyme were 1.4 nM ends and 7.6 nM, respectively. The concentration of SSB protein was either 20.6 or 2.0 μM for N4 or M13 DNA, respectively. Standard buffer conditions were used at 25 °C, except that ATP was added to the final concentrations indicated; not shown is the value at 15 mM ATP, which is 29 ± 3 kb/end. *Open circles*, data using N4 DNA; *filled square*, data using M13 DNA. The *line* represents the nonlinear least squares fit of the data to a hyperbola using the parameters defined in the text.

In the presence of 1 mM ATP and in the absence of an ATP-regenerating system, the addition of increasing concentrations of ADP results in a corresponding inhibition of the rate of unwinding by recBCD enzyme (Table II, lines 1–4). If the lifetime of the recBCD enzyme-DNA complex is unaffected by ADP, then a recBCD enzyme molecule that is unwinding at a slower rate will not translocate as far along the DNA during the time it is bound and will, therefore, demonstrate a lower processivity. This result is not observed (Table II, lines 1–4); ADP concentrations of 0.2–1 mM, in the presence of 1 mM ATP, do not alter N.

Pulsed-field Agarose Gel Analysis of the Products Generated by recBCD Enzyme Unwinding Confirms the Fluorescence Assay Results—To verify that the measurements of processivity obtained from the fluorescence assay are indicative of the intrinsic processivity of recBCD enzyme helicase activity, the size distribution of the products resulting from unwinding reactions was assayed directly (Fig. 4). The fluorescence assays indicated that processivity is greatest at high ATP concentrations and is reduced at low ATP concentrations; consequently, the product size distribution of N4 DNA after unwinding by recBCD enzyme at either 40 μM or 10 mM ATP (concentrations of cofactor that result in extremes of the observed processivity (see above)) was analyzed. For each time point, the DNA was treated with S1 nuclease to digest the ssDNA tails that were generated by recBCD enzyme unwinding, leaving the dsDNA that was not unwound intact. This dsDNA product population was separated by pulsed-field agarose gel electrophoresis to resolve the large product molecules (Fig. 4). Thus, this assay provides a complementary means of determining recBCD enzyme processivity by measuring not the DNA that is encountered by the enzyme, as

TABLE II

Effect of nucleotide cofactors on the processivity of recBCD enzyme-catalyzed unwinding of dsDNA

Concentrations of N4 DNA, SSB protein, and recBCD enzyme were 1.4 nM ends, 20.6 μM, and 7.6 nM, respectively. Standard buffer conditions were used, except that the reactions in the presence of ADP contained 1 mM ATP in the absence of the ATP-regenerating system, whereas the reactions in the presence of dATP contained no ATP. The experimental uncertainty is ±15% for the values of N and ±20% for the rate values.

Line	Cofactor	[Nucleotide]	N	Rate of unwinding[a]
		μM	*kb/end*	s^{-1}
1	ADP	0	30	420
2		200	28	290
3		500	29	238
4		1000	27	147
5	dATP	100	23	159
6		500	32	423
7		1000	33	371

[a] Rate of unwinding (μM bp/s/μM recBCD enzyme) is corrected for the observed stoichiometry of recBCD enzyme binding to dsDNA.

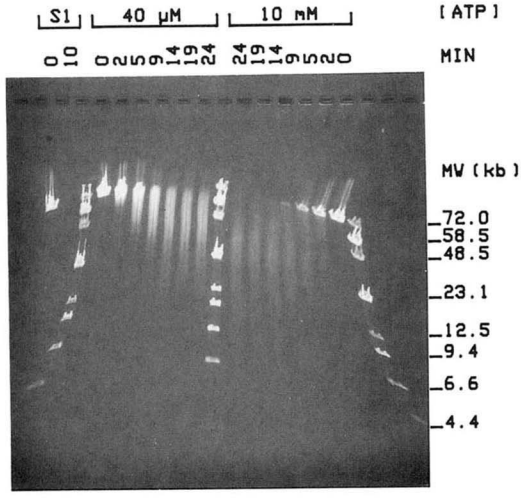

FIG. 4. **Pulsed-field agarose gel electrophoresis of N4 DNA molecules unwound by recBCD enzyme.** The reactions were conducted in standard buffer at 37 °C, except that the ATP concentration was either 40 μM or 10 mM. The concentrations of N4 DNA, SSB protein, and recBCD enzyme were 1.4 nM ends, 20.6 μM, and 0.95 nM, respectively. The molecular weight standards are N4 DNA (72 kb), N4 *Hae*II restriction fragments (58.5 and 12.5 kb), λ DNA (48.5 kb), and λ *Hind*III restriction fragments (23.1, 9.4, 6.6, and 4.4 kb). The lanes have been loaded so that the final time point of each reaction is present at the center of the gel to minimize size distortion due to lane curvature.

with the fluorescence assay, but that which remains untouched by the enzyme.

In the presence of 40 μM ATP, the distribution of product molecules (*i.e.* the dsDNA that is not unwound) is predominantly in the region of ~30–72 kb. With time, the distribution appears to extend to lower sizes (~20 kb); however, this is simply a consequence of the appearance of a greater number of unwound DNA molecules within the same size range. At longer times (*e.g.* at 19 and 24 min), when all of the substrate DNA has been acted upon, the distribution is invariant. It is also evident that all of the substrate N4 DNA is (partially) unwound. In addition, the reaction shown in Fig. 4 contained a substoichiometric concentration of recBCD enzyme (0.13 functional recBCD enzyme molecule/DNA end); consequently, the disappearance of essentially all of the full-length N4 DNA graphically supports the fluorescence assay result in that it demonstrates that recBCD enzyme can reinitiate unwinding on intact (but not unwound) DNA molecules.

At 1 mM ATP or higher, the fluorescence assay indicated that the processivity of unwinding (N) is 30–32 kb/end (data not shown). Under such conditions, the breadth of the product distribution should be extended to include more extensively unwound DNA molecules. Fig. 4 shows that, at 10 mM ATP, a wider distribution of product molecules (ranging from ~4–72 kb) is generated, as expected for more processive helicase action. This broad distribution and the disappearance of the intact N4 DNA cannot be attributed to nucleolytic degradation by recBCD enzyme, since this activity is substantially reduced at this concentration of ATP (Eichler and Lehman, 1977); on the contrary, if nuclease activity were responsible for the decrease in DNA length, the decrease would be more prominent at 40 μM ATP. Thus, we conclude that the unwinding activity of recBCD enzyme is responsible for the disappearance of the substrate DNA and that, consistent with the fluorescence helicase assay, the processivity of recBCD enzyme is strongly influenced by the concentration of ATP.

Increasing Concentrations of Sodium Chloride Decrease the Processivity of recBCD Enzyme Unwinding—Most protein-nucleic acid interactions are sensitive to the concentration of salt present. Therefore, it was expected that an increase in the sodium chloride concentration would increase the probability that recBCD enzyme would dissociate from the dsDNA, thus decreasing the observed processivity. As shown in Fig. 5, this is the case. At low NaCl concentration (30 mM for N4 and 20 mM for λ DNA reactions), an average of 31 ± 3.2 kb/end are unwound before dissociation using N4 DNA, and λ DNA is fully unwound (N = 24 kb/end), whereas only 15 ± 2 and 13 ± 1.5 kb/end, respectively, are unwound before dissociation at the highest NaCl concentration (280 mM for N4 and 270 mM for λ DNA reactions). At the optimal NaCl concentration (80 mM), recBCD enzyme can unwind essentially all the N4 DNA (N = 36 ± 4 kb/end).

Raising the reaction temperature from 25 to 37 °C increases the maximum rate of unwinding by recBCD enzyme 2.5-fold, although it does not change the apparent K_m value for M13 DNA (Roman and Kowalczykowski, 1989a). As shown in Fig. 5, the observed N for N4 DNA is the same, within experimental error, at both 25 and 37 °C in the presence of either 30 mM (N = 28 ± 2.7 kb/end) or 280 mM (N = 15 ± 2 kb/end) NaCl; due to the recBCD enzyme-dependent production of ssDNA oligonucleotides that fail to bind SSB protein, the true processivity is actually 10 and 20% greater, respectively, than these observed values (see "Experimental Procedures"). Thus, both the processivity of recBCD enzyme unwinding and its decrease in the presence of high salt concentration are unaffected by temperature.

This decrease in extent with increasing salt concentration could be due to a decreased affinity of recBCD enzyme for dsDNA ends. This possiblity was eliminated by measuring the extent of M13 dsDNA unwinding at the increased NaCl concentrations. Since M13 dsDNA (7.2 kb) is much smaller than N, complete DNA unwinding should occur but, if recBCD enzyme were unable to bind DNA ends and initiate unwinding at the higher NaCl concentrations, a salt concentration-dependent decrease in the extent of M13 DNA unwinding would be observed. The final extent of M13 DNA unwinding, however, is unaltered over this range of NaCl concentration (data not shown); thus, the affinity of recBCD enzyme for DNA ends is not limiting at high concentrations of salt.

Divalent Cation Concentration Affects the Processivity of recBCD Enzyme Unwinding—The concentration of magnesium acetate affects the rate of dsDNA-dependent ATP hydrolysis by recBCD enzyme but has little effect on the rate of

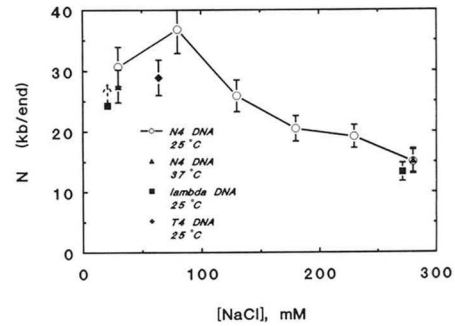

FIG. 5. **Number of kilobase pairs unwound per DNA end as a function of NaCl concentration.** Concentrations of DNA and recBCD enzyme were 1.4 nM ends and 7.6 nM, respectively. The concentration of SSB protein was 20.6 or 13.3 μM for N4 or λ DNA, respectively. Standard buffer conditions were used except that NaCl was added to the final concentration indicated. *Circles*, N4 DNA at 25 °C; *squares*, λ DNA at 25 °C; *diamond*, T4 DNA at 25 °C; *triangles*, N4 DNA at 37 °C.

unwinding of DNA (Roman and Kowalczykowski, 1989a, 1989b). To determine whether the processivity of unwinding was affected, N was determined at various magnesium acetate concentrations. As shown in Fig. 6, an increase in the magnesium ion concentration results in a decrease in N. As the magnesium ion concentration is raised from 1 to 10 mM, the number of base pairs unwound decreases by 42% (from 31 to 18 kb/end).

Calcium acetate also greatly affects some activities of recBCD enzyme. In the presence of 1 mM calcium ion, the nuclease activity of recBCD enzyme is almost completely inhibited (Rosamond *et al.*, 1979), but the helicase activity is reduced only ~25% (Roman and Kowalczykowski, 1989a). As shown in Fig. 6, variations in the concentration of calcium ion affect the processivity of recBCD enzyme unwinding in a manner similar to that seen with magnesium ion, confirming the interpretation that these effects on the processivity parameter N are general divalent ion effects rather than indirect effects mediated through alterations of recBCD enzyme nuclease activity.

DISCUSSION

We have used an assay based on the quenching of SSB protein intrinsic fluorescence (Roman and Kowalczykowski, 1989a) to investigate the processivity of recBCD enzyme unwinding. Since recBCD enzyme cannot initiate unwinding on a dsDNA substrate that has a ssDNA overhang greater than 25 nucleotides (Taylor and Smith, 1985), it cannot reinitiate unwinding on a partially unwound dsDNA molecule. Therefore, for sufficiently long DNA molecules, the final extent of unwinding is equal to twice the average number of base pairs unwound per recBCD enzyme binding event (*i.e.* per dsDNA end) and so reflects the processivity of enzymatic unwinding.

The average number of base pairs unwound before a recBCD enzyme molecule dissociates from N4 DNA is 31 ± 3.2 kb under our standard conditions. This value is invariant regardless of whether T4 or N4 DNA is used, and in agreement, we find that λ DNA is nearly fully unwound. These results indicate that our assay is measuring a property that is intrinsic to recBCD enzyme activity and that is unrelated to the type or length of the DNA substrate. The average number of base pairs unwound per DNA molecule as measured by our fluorescence assay is consistent with electron microscopic studies. Both Telander-Muskavitch and Linn (1982) and Taylor and Smith (1985) have reported that T7 DNA, which is 40 kb in length, can be completely unwound by recBCD enzyme in the presence of 1 mM magnesium and 1 mM calcium ions, suggesting that N is at least 20 kb/end. Our results confirm this observation and both extend and quantify this facet of recBCD enzyme helicase activity.

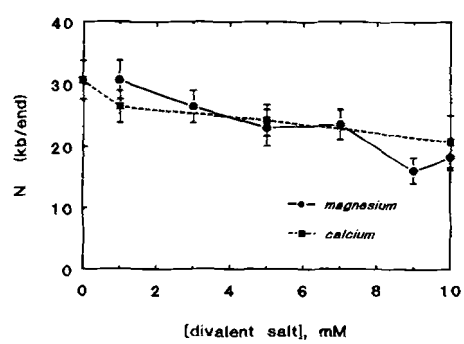

FIG. 6. **Number of kilobase pairs unwound per DNA end as a function of divalent salt concentration.** Concentrations of N4 DNA, SSB protein, and recBCD enzyme were 1.4 nM ends, 20.6 μM, and 7.6 nM, respectively. Standard buffer conditions were used at 25 °C, except that divalent salts were added to the final concentrations indicated. For experiments with calcium acetate, 1 mM magnesium acetate was also present. *Filled circles* and *solid line*, magnesium acetate concentration; *filled squares* and *dashed line*, calcium acetate concentration.

The observed processivity (P_{obs}) is the probability that recBCD enzyme will unwind another base pair of dsDNA rather than dissociate. P_{obs} is calculated from $P_{obs} = (N - 1)/N$, where N is the average number of base pairs unwound per binding event (McClure and Chow, 1980). This relationship assumes that the DNA substrate is homogeneous with regard to recBCD enzyme helicase activity; that is, it assumes that the probability of unwinding is equivalent for each base pair although, in reality, this may not be the case. Under standard conditions (1 mM magnesium acetate, 1 mM ATP, and 30 mM NaCl), $P_{obs} = 0.99997$, indicating that recBCD enzyme is nearly 30,000-fold more likely to unwind another base pair than to dissociate from the DNA. In fact, under all conditions described in this paper, P_{obs} is much greater than 0.99. For comparison, the processivity of *E. coli* DNA polymerase I elongation activity has been shown to range from <0.1 to 0.99 (McClure and Jovin, 1975; Bambara *et al.*, 1978).

Knowledge of the processivity parameter, P_{obs}, permits a quantitative comparison of the average number of base pairs unwound per enzyme binding event to the reaction product distribution displayed in Fig. 4. The probability of finding a dsDNA molecule that has been unwound a total of exactly n bp by the action of a recBCD enzyme acting at each DNA end is given by $(n + 1)P^n(1 - P)^2$ (the term $(n + 1)$ represents the number of ways of obtaining a DNA molecule that is unwound some distance, x, at one end of the molecule and a distance $n - x$ at the other end, to yield a product that is unwound a total of exactly n bp; since the probability of unwinding exactly x bp is $P^x(1 - P)$ (McClure and Chow, 1980), the probability of unwinding x bp at one end and $n - x$ at the other is the product of $P^x(1 - P)$ and $P^{n-x}(1 - P)$, yielding the above equation). A plot of this function *versus* n yields the number average distribution of DNA unwinding products. However, the dsDNA products shown in Fig. 4 are visualized by ethidium bromide staining, which yields a size-weighted signal. Therefore, the number average distribution was converted to a size average distribution by multiplication with the appropriate factor (for N4 DNA, 72,000 − n). The resultant plot of the size-weighted distribution of DNA unwinding products as a function of distance unwound (in kb) is shown in Fig. 7 for two different values of P_{obs}; one value of P_{obs} is characteristic of the processivity at or above 1 mM ATP (*solid line*), whereas the other is characteristic of the lower processivity observed at 40 μM ATP (*dashed line*). These graphs show that, due to the relatively high processivity of unwinding under either condition, the amount of dsDNA that is unwound just a short distance (*e.g.* less than 1 kb) is negligible. The distribution rises to a maximum that is dependent on the value of P_{obs}, then decreases; at 40 μM ATP, the distribution peaks at approximately 15 kb unwound, whereas at 1 mM ATP or greater, the distribution peaks at approximately 20 kb unwound. A comparison of this calculated distribution with the experimental data in Fig. 4 shows qualitative agreement. At 40 μM ATP, the relatively narrow distribution has a peak at approximately 45–50 kb (*i.e.* 22–27 kb unwound); at 10 mM ATP, the considerably broader distribution has a peak at approximately 30–35 kb (*i.e.* 37–42 kb unwound). The explanation for the systematic difference between the experimentally observed peaks compared with the calculated peaks is unknown, but it may suggest that the

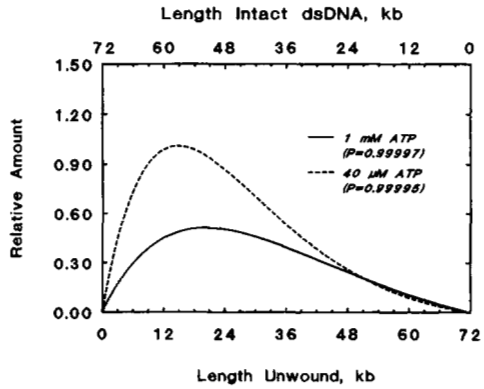

FIG. 7. **Distribution of DNA unwinding products as a function of the length unwound.** The *curves* were calculated for N4 DNA using the equation $(n + 1)P^n(1 - P)^2(72000 - n)$ and were truncated at n = 72,000. *Dashed line*, the distribution for P_{obs} = 0.99995 (*i.e.* at 40 μM ATP); *solid line*, the distribution for P_{obs} = 0.99997 (*i.e.* at or above 1 mM ATP).

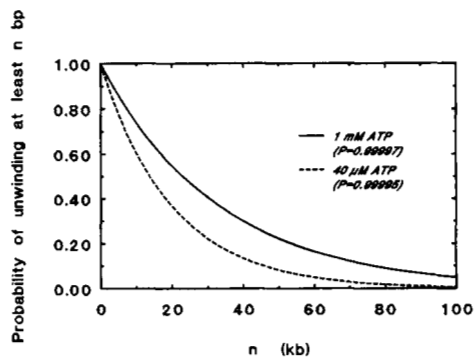

FIG. 8. **Theoretical distribution of the probability (P^n) that recBCD enzyme will unwind at least n bp before dissociation.** *Dashed line*, the distribution for P_{obs} = 0.99995 (*i.e.* at 40 μM ATP); *solid line*, the distribution for P_{obs} = 0.99997 (*i.e.* at or above 1 mM ATP).

processivity parameters determined here are underestimates of the true value; consistent with this possibility, approximately 15% of the λ DNA should remain unwound if P = 0.99997, yet we typically observe 90–100% unwinding. Alternatively, this discrepancy may reflect microheterogeneity of the microscopic processivity parameter (*i.e.* rather than being constant, the probability of translocation may actually depend on nucleotide sequence). Variation of P_{obs} with DNA composition would not be detected by the experiments presented here and would certainly alter the distributions calculated in Fig. 7. Despite this deviation, the agreement between the fluorescence and agarose gel methods is reasonable, thereby validating the suitability of these methods in addressing this question.

The processivity of unwinding shows a sensitivity to reaction conditions. Above 80 mM NaCl, an increase in sodium chloride concentration leads to a corresponding decrease in N. At 280 mM NaCl, whether N4 or λ DNA is used, N is similar and is approximately 49% of the value observed for N4 DNA at 30 mM NaCl. This is not unexpected, since the binding of most proteins to nucleic acids is sensitive to the ionic environment, a sensitivity which is generally also manifest in the dissociation rate of the protein. Thus, if the translocation rate is unaffected by NaCl, then the slope derived from a plot of log N *versus* log [NaCl] may reflect the salt sensitivity of the dissociation rate constant for the recBCD enzyme-DNA complex. Such a plot (data not shown), using N values for NaCl concentrations between 80 and 280 mM, yields a slope of -0.7 ± 0.1. This suggests that the rate-limiting step of the processive unwinding reaction involves the net formation of only one ionic contact with the dsDNA. Assuming that unwinding and dissociation are independent events, an estimate of the actual kinetic lifetime for the recBCD enzyme-DNA complex can be calculated by dividing N (bp unwound/recBCD enzyme) by the k_{cat} value (bp unwound/s/recBCD enzyme) obtained at a particular salt concentration; N/k_{cat} will yield the apparent time (in seconds) for dissociation from the unwound DNA. Using the unwinding rate constants determined previously (Roman and Kowalczykowski, 1989a), we calculate that the dissociation time decreases with increasing NaCl concentration (over the range 30–180 mM). The average recBCD enzyme molecule remains associated with the dsDNA during the unwinding reaction for approximately 100 s at 30 mM NaCl, but for only 65 s at 180 mM NaCl.

The ATP concentration dependence of N is hyperbolic. The K_N value for ATP (or the concentration of ATP at which N is one-half that at maximum) is 41 μM, and the apparent limiting extent of unwinding is 32 kb/end, raising the question of why the unwinding processivity increases with ATP concentration. One possibility is suggested by the observation that the rate of recBCD enzyme-catalyzed dsDNA unwinding increases with higher ATP concentrations (with an apparent K_m value of 130 μM (Roman and Kowalczykowski, 1989a). If the dissociation time of a recBCD enzyme-dsDNA complex remains independent of ATP concentration, then the distance traversed by recBCD enzyme before dissociation will depend on the rate of unwinding. At lower ATP concentrations, the rate of unwinding is slower, resulting in a decrease in the observed processivity. This explanation cannot be totally correct, however, since the rate of unwinding is affected by the concentration of ADP present, whereas the processivity of unwinding is not (Table II), demonstrating that the processivity is independent of the translocation rate and may, instead, be determined by the lifetime of an ATP- (or ADP-) bound species. Interestingly, the apparent K_m and K_N values (130 and 41 μM, respectively) are very similar to the equilibrium dissociation constants for the binding of azido-ATP to the recB and recD subunits (130 and 30 μM, respectively (Julin and Lehman, 1987)). This coincidence may suggest roles for the recB and recD subunits during unwinding. Specifically, since the ATP concentration dependence of translocation (as measured by the processivity of the unwinding reaction) is comparable with that of azido-ATP binding to the recD subunit, it may be the recD subunit that governs the probability of translocation relative to dissociation, whereas the recB subunit may govern the initiation and steady-state rate of unwinding. Thus, it is conceivable that mutations in the *recB* or *recD* genes could result in altered enzymes that are selectively defective in either steady-state unwinding or processivity, respectively.

What relevance does the processivity of recBCD enzyme unwinding have *in vivo*? Chi sites are "hot spots" of recombination that are active only in the recBCD pathway of recombination. These sites stimulate recombination nearby and for distances as great as 10 (Ennis *et al.*, 1987) to 20 kb (McMilin *et al.*, 1974; Stahl *et al.*, 1983) away from Chi, and *in vitro*, recBCD enzyme cleaves at Chi sites (Taylor *et al.*, 1985). Smith *et al.* (1984) have incorporated these *in vivo* and *in vitro* observations into a model for recBCD enzyme action during recombination. They propose that recBCD enzyme travels along the dsDNA, unwinding it, until it encounters a Chi site. recBCD enzyme nicks the DNA and continues unwinding, producing ssDNA that can be used by recA protein

to catalyze DNA strand invasion. Recent *in vitro* results demonstrate that recBCD enzyme helicase activity is indeed capable of producing ssDNA that is a suitable substrate for recA protein-dependent activities (Roman and Kowalczykowski, 1989c; Wang and Smith, 1989; Kowalczykowski and Roman, 1990; Roman *et al.*, 1991; Dixon and Kowalczykowski, 1991). Thus, biochemical evidence supports some of the fundamental tenets of this model.

If the processivity of recBCD enzyme unwinding is an important determinant of Chi stimulation, then the maximum distance over which Chi stimulation occurs *in vivo* cannot exceed the distance traveled by recBCD enzyme before dissociation as measured *in vitro*. Here, again, knowledge of the processivity parameter is beneficial, since the probability of a single recBCD enzyme molecule unwinding at least n bp before dissociating is given by P^n. The plots of this function for both 40 μM ATP (P_{obs} = 0.99995) and 1 mM ATP or higher (P_{obs} = 0.99997) are shown in Fig. 8. They show that some recBCD enzyme molecules are theoretically capable of unwinding at least 100 kb at the higher concentration of ATP (*solid line*); in fact, approximately 15% of the enzyme molecules can unwind more than 50 kb from the entry site (*i.e.* greater than the length of λ DNA) before dissociation occurs. The average distance at which one-half of the recBCD enzyme molecules dissociate ($n_{1/2}$) is related to the value of P ($n_{1/2} = -0.7/\ln P$). Depending on conditions (*i.e.* mono- and divalent ion concentrations), 50% of the recBCD enzyme molecules will dissociate after unwinding 10–23 kb.

For comparison, Chi stimulation of recombination decreases exponentially with distance from the Chi site, decreasing 50% every 2.2–3.2 kb *in vivo* (Ennis *et al.*, 1987; Cheng and Smith, 1989). This comparison demonstrates that the measured *in vitro* processivity is sufficiently great to accommodate the observed *in vivo* action of recBCD enzyme over large distances, but it also suggests that the processivity of recBCD enzyme alone does not limit the distance over which Chi stimulation occurs. Thus, either Chi-stimulated recombination may not be limited by recBCD enzyme helicase activity (*e.g.* exchange may be limited by the action of recA or SSB proteins), or *in vivo*, the processivity of recBCD enzyme unwinding may be reduced by some of the following considerations. First, the ionic environment *in vivo* is not clearly defined, with the possibility that small molecules not yet examined *in vitro* may affect recBCD enzyme activity. Second, translocation of recBCD enzyme *in vivo* may be limited by the presence of other proteins bound to the dsDNA (*e.g.* RNA polymerase, repressor proteins, etc.). Third, recBCD enzyme itself might be altered after encountering a Chi site, perhaps resulting in reduced processivity. A change in recBCD enzyme activity upon interaction with a Chi site has been previously suggested (Thaler *et al.*, 1988), and *in vitro* results demonstrate a reduction in nuclease activity after encountering a properly oriented Chi site (Dixon and Kowalczykowski, 1991); the effects of these sites on processivity are yet to be examined.

This investigation of the processivity of recBCD enzyme unwinding demonstrates another application of the assay based on the quenching of SSB protein fluorescence that we have developed. Defining and characterizing the processivity of the wild-type recBCD enzyme also allows for the investigation both of the effect of Chi sites on unwinding processivity and of the behavior of mutant recBCD enzymes, some of which may demonstrate defects in processivity.[3]

[3] A. K. Eggleston and S. C. Kowalczykowski, manuscript in preparation.

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