# Chi-activated RecBCD enzyme possesses 5′→3′ nucleolytic activity, but RecBC enzyme does not: evidence suggesting that the alteration induced by Chi is not simply ejection of the RecD subunit

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## Abstract

***Background*: Homologous recombination in *Escherichia coli* is initiated by the RecBCD enzyme, and is stimulated by DNA elements known as Chi (χ) sites. The RecBCD enzyme is both a helicase and a nuclease. Recognition of χ causes both attenuation of the 3′→5′ exonuclease activity of the RecBCD enzyme, and activation of an exonuclease activity with 5′→3′ polarity, while leaving the helicase activity unaffected. A variety of evidence suggests that χ-recognition by RecBCD enzyme is accompanied by ejection of the RecD subunit.**

***Results*: Through examination of RecBCD exonuclease activity under a variety of conditions, we have shown that recognition of χ by the RecBCD enzyme results in a net reduction of nuclease activity. In addition, the exact location of the first cleavage event elicited by χ-activation of the 5′→3′ nuclease is dependent upon the concentration of free magnesium ions. Finally, we have demonstrated that purified RecBC enzyme (i.e. without the RecD subunit) possesses no significant exonuclease activity under conditions where the χ-modified RecBCD enzyme is an active 5′→3′ exonuclease.**

***Conclusions*: We have shown that, despite the activation of a 5′→3′ exonuclease, recognition of χ by the RecBCD enzyme results in a net preservation of DNA. This new χ-activated nucleolytic action shows surprising variability in the exact location of its initial cleavage. We have demonstrated that purified RecBC enzyme is not an exact analogue of the χ-activated RecBCD enzyme, suggesting that the biochemical basis of χ-activation is not simply ejection of the RecD subunit.**

## Introduction

The *recB* and *recC* genes are essential components of the main homologous recombination pathway in *Escherichia coli.* Mutational inactivation of either gene reduces homologous recombination proficiency by up to 1000-fold (Howard-Flanders & Theriot 1966; Emmerson 1968). The products of the *recB* and *recC* genes, along with the *recD* gene product, make up the heterotrimeric RecBCD enzyme (for a review see Kowalczykowski *et al.* 1994). This enzyme is both an ATP-dependent exonuclease, (Telander-Muskavitch & Linn 1981; Smith 1990; Kowalczykowski *et al.* 1994) and an efficient helicase, capable of unwinding DNA at rates of up to 1000 bp/s (Taylor & Smith 1980; Roman & Kowalczykowski 1989a; Roman *et al.* 1992; Eggleston & Kowalczykowski 1993). The RecBCD enzyme is also highly processive. On average, it will unwind and degrade 30 000 base pairs (bp) of DNA per binding event (Roman *et al.* 1992).

The RecBCD enzyme initiates recombination by unwinding and simultaneously degrading the DNA from a double-stranded DNA (dsDNA) end (Taylor & Smith 1980; Telander-Muskavitch & Linn 1981; Taylor & Smith 1985; see Fig. 1). Degradation of the DNA is asymmetric, with the 3′-terminal strand relative to the entry site of RecBCD enzyme being cleaved much

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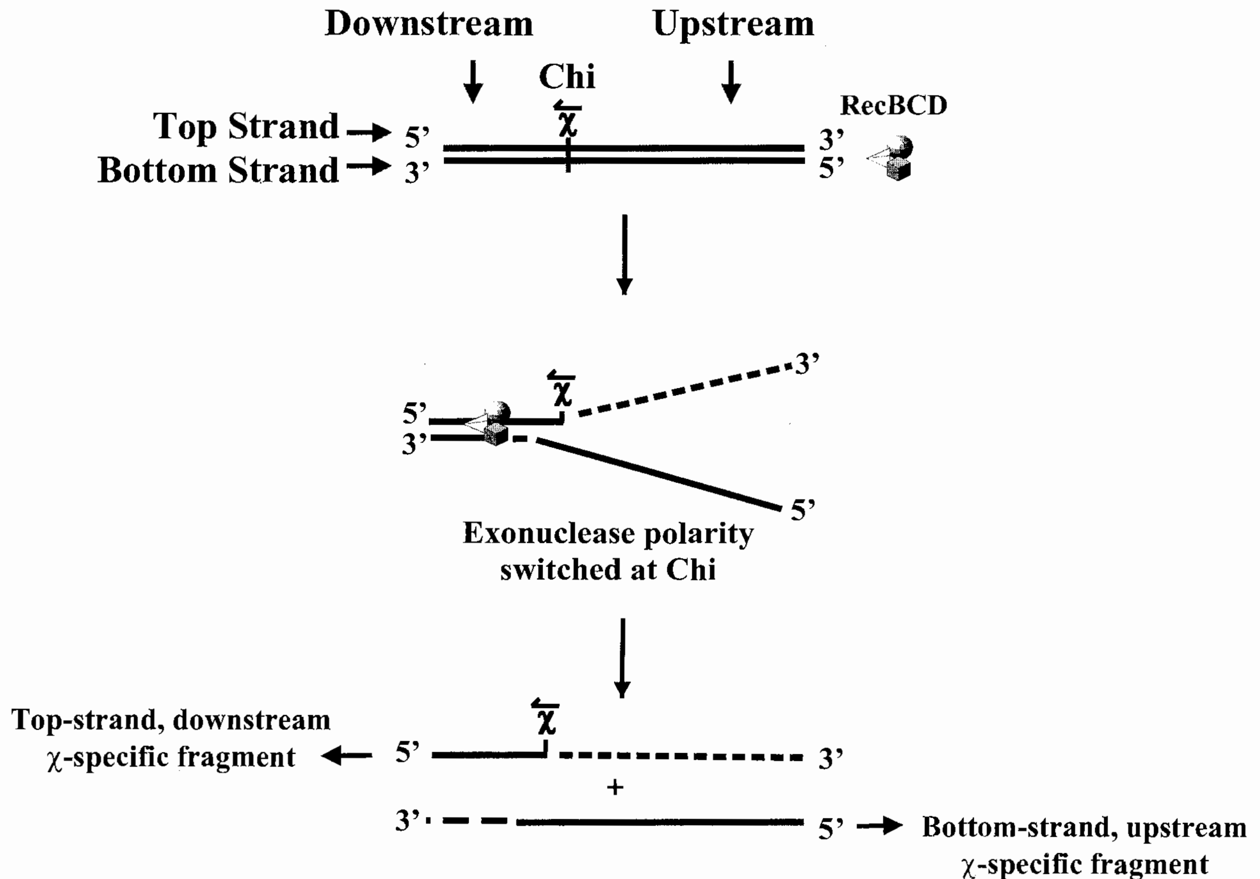


**Figure 1** Processing of $\chi$-containing dsDNA by RecBCD enzyme produces two $\chi$-specific fragments. The strand of DNA that terminates 3′ at the entry site of RecBCD enzyme is the 'top-strand'; the opposite strand is the 'bottom-strand'. The region of dsDNA between $\chi$ and the entry site of RecBCD enzyme is the 'upstream' region, and the region between $\chi$ and the opposite end is the 'downstream' region. The arrow above the $\chi$ site indicates the direction that RecBCD enzyme must travel in order to recognize $\chi$. Unwinding and 3′→5′ exonuclease activity upstream of $\chi$, followed by a switch in the polarity of exonuclease degradation to 5′→3′ leads to the production of both a bottom-strand, upstream $\chi$-specific fragment and a top-strand, downstream $\chi$-specific fragment.

more frequently than the 5′-terminal strand (Dixon & Kowalczykowski 1993). Through processing of dsDNA into single stranded DNA (ssDNA), the RecBCD enzyme creates a substrate suitable for the homologous pairing and DNA strand exchange protein, RecA (Taylor & Smith 1985; Roman & Kowalczykowski 1989b; Dixon & Kowalczykowski 1991). The RecA protein is essential for homologous recombination in *E. coli*. Inactivation of the *recA* gene causes a reduction in homologous recombination by up to $10^5$-fold (Clark & Margulies 1965). *In vitro*, this protein promotes pairing and exchange between ssDNA substrates created by RecBCD enzyme and homologous supercoiled dsDNA counterparts (Dixon & Kowalczykowski 1991; for review see Kowalczykowski & Eggleston 1994). The processing of dsDNA into ssDNA is an essential step, as RecA protein binds dsDNA very poorly under physiological conditions (Kowalczykowski *et al.* 1987; Pugh & Cox 1987).

An interesting facet of homologous recombination initiated by the RecBCD enzyme is that it is stimulated at DNA sequences known as $\chi$ sites (5′-GCTGGTGG-3′) (Lam *et al.* 1974; Stahl *et al.* 1975; Smith *et al.* 1981). *In vivo*, $\chi$ stimulates homologous recombination 5–10-fold unidirectionally, with maximal stimulation occurring at $\chi$ and decaying downstream relative to the entry site of RecBCD enzyme (Stahl *et al.* 1980; Ennis *et al.* 1987; Cheng & Smith 1989; Myers *et al.* 1995a,b). Recognition of $\chi$ by a translocating RecBCD enzyme molecule results in attenuation of 3′→5′ exonucleolytic degradation approximately 4–5 nucleotides upstream of the $\chi$ site (Ponticelli *et al.* 1985; Dixon & Kowalczykowski 1993, 1995; Taylor & Smith 1995) after which a nuclease activity of the opposite polarity, 5′→3′, is now

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activated (Anderson & Kowalczykowski 1997). However, χ-modification of the RecBCD enzyme does not affect helicase activity. Thus, unwinding and 5′→3′ degradation of the dsDNA continues downstream of χ, producing an ssDNA substrate with a 3′-overhang which can now be utilized by the RecA protein (Dixon & Kowalczykowski 1991; Anderson & Kowalczykowski 1997).

The molecular basis of χ-mediated alteration of the RecBCD enzyme is still unclear, but a variety of experiments *in vivo* have led to the hypothesis that χ-recognition results in the ejection of the RecD subunit from the RecBCD holoenzyme (Thaler *et al.* 1988; Stahl *et al.* 1990; Koppen *et al.* 1995; Myers *et al.* 1995a). Examination *in vitro* revealed that under conditions of limiting the $Mg^{2+}$ ion, the RecBCD enzyme is reversibly inactivated by χ-recognition, leading to a form of the enzyme which is unable to reinitiate unwinding (Dixon *et al.* 1994). This behaviour of the χ-modified RecBCD enzyme is also a characteristic of the RecBC enzyme (i.e. without the RecD subunit), and was taken as further evidence that recognition of χ leads to the functional inactivation or ejection of the RecD subunit. In addition, the RecBC enzyme has been reported to possess some undefined level of exonuclease activity (Masterson *et al.* 1992; Korangy & Julin 1993). Could this nuclease activity be the same as that observed following χ-activation of the RecBCD enzyme?

In this paper we examine χ-activated 5′→3′ exonuclease under a variety of free $Mg^{2+}$ ion concentrations. Since the nucleolytic functions of RecBCD enzyme are strongly influenced by alterations in the free $Mg^{2+}$ ion concentration (Eggleston & Kowalczykowski 1993; Dixon & Kowalcyzkowski 1995), we took advantage of this property to define the nature of this novel activity (Anderson & Kowalczykowski 1997). We demonstrate that the location of the first cleavage event mediated by the up-regulated 5′→3′ exonuclease activity is dependent upon the free $Mg^{2+}$ ion concentrations. Despite activation of this novel nucleolytic activity, there is a net reduction of RecBCD enzyme nuclease activity upon recognition of χ. Finally, examination of purified RecBC enzyme reveals no significant exonuclease activity under conditions where the χ-activated RecBCD enzyme is an active 5′→3′ nuclease. Thus, the RecBC enzyme is not a direct analogue of the χ-activated RecBCD enzyme. We propose that recognition of χ does not lead to the ejection of the RecD subunit, but rather to the activation of a putative 5′→3′ exonuclease domain in the RecD protein.

## Results

### Initial cleavage by the χ-activated 5′→3′ nuclease activity occurs at or downstream of χ

The strand of dsDNA which terminates 3′ at the entry point of RecBCD enzyme is defined as the 'top-strand'; the opposite strand is defined as the 'bottom-strand'. In addition, we define the region between the χ site and the end of the DNA at which RecBCD enzyme initiates unwinding to be the 'upstream' region, and the DNA between the χ site and the opposite end of the DNA to be the 'downstream' region (see Fig. 1). As previously mentioned, recognition of χ by a translocating RecBCD enzyme molecule leads to a switch in the polarity of exonuclease activity from 3′→5′ to 5′→3′ (Fig. 1; Anderson & Kowalczykowski 1997). Thus, RecBCD enzyme-processing of DNA containing a χ site generates two distinct χ-specific ssDNA fragments: a top-strand, downstream fragment and a bottom-strand, upstream fragment.

The location of the initial strand scission on the bottom strand is 300–500 nucleotides downstream of the χ-site, under the conditions previously reported (Anderson & Kowalczykowski 1997). Since the frequency of exonuclease cleavage is sensitive to free $Mg^{2+}$ ion concentration (Eggleston & Kowalczykowski 1993; Dixon & Kowalczykowski 1995), we examined the $Mg^{2+}$ ion dependence of the χ-activated 5′→3′ exonuclease activity, using linear pBR322 $\chi^{+}$F Figs 2A and B) over a range of free $Mg^{2+}$ ion concentration (obtained by varying the relative concentrations of $Mg^{2+}$ ions and ATP; see Eggleston & Kowalczykowski 1993).

The products of DNA unwinding reactions using linear, 5′ end-labelled, pBR322 $\chi^{+}$F are shown in Figs 2A and B. Only 20% of RecBCD enzyme–χ site interactions result in a switch in the polarity of nuclease (Taylor *et al.* 1985; Dixon & Kowalczykowski 1993). Thus, the majority of χ-containing DNA molecules are processed as if they do not contain a χ-site. Processing of dsDNA by RecBCD enzyme in the absence of χ results in the production of full length ssDNA due to the asymmetric (3′→5′) degradation of DNA during unwinding (Dixon & Kowalczykowski 1993). Recognition of χ results in the formation of both the top-strand, downstream χ-specific fragment (due to degradation of the 3′-terminal strand up to χ) and the bottom-strand, upstream χ-specific fragment (due to degradation of the 5′-terminal strand after χ) observed in Fig. 2. The yield of the bottom-strand, upstream χ-specific fragment is low at the highest free $Mg^{2+}$ ion

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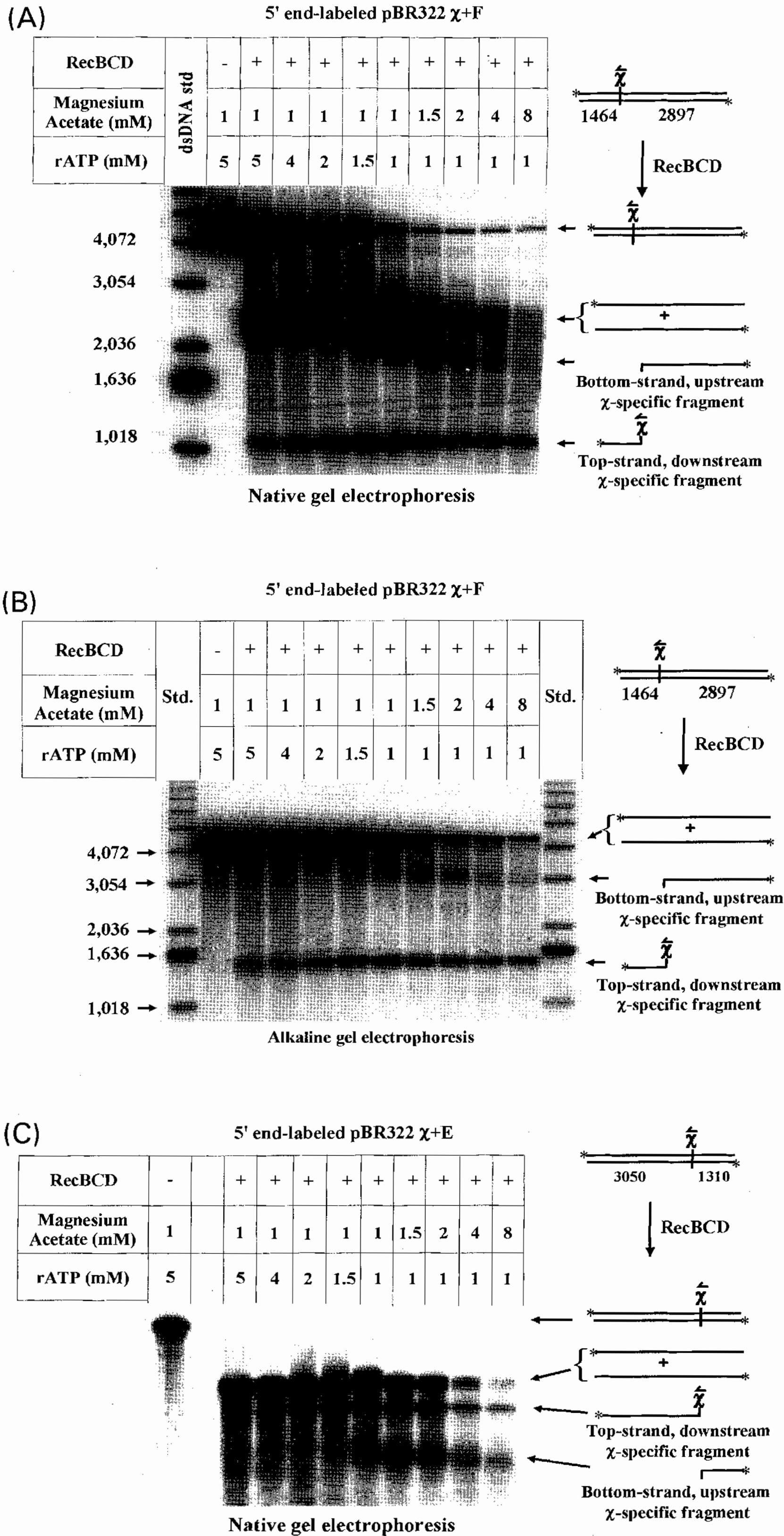


**Figure 2** The length of the bottom-strand χ-specific fragment is dependent on the free $Mg^{2+}$ ion concentration. The linear χ-containing dsDNA substrates were created by restriction of pBR322 $\chi^{+}$F with *Hin*dIII (panels A and B) or pBR322 $\chi^{+}$E with Nde I (C). The location of each χ site is given in nucleotides. The DNA was labelled at the 5′-end with $^{32}$P and treated with one functional RecBCD enzyme (0.115 nM) per 20 dsDNA ends (panels A and B) or one functional RecBCD enzyme (0.46 nM) per five dsDNA ends (C). Full length ssDNA and the 5′-end labelled, χ-specific fragments are indicated. $Mg^{2+}$ ion and ATP were varied as indicated. Reaction products were analysed on a native 1% agarose gel (A and C). Aliquots of reaction (A) were loaded onto an alkaline agarose denaturing gel (B). The data from the 2 mM $Mg^{2+}$, 1 mM ATP lanes appeared in Anderson & Kowalczykowski (1997).

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concentration (right-most lane)—as the free $Mg^{2+}$ ion concentration is decreased (from right to left), the yield of both the full length ssDNA and this upstream $\chi$-specific species increases, reflecting the decrease in nonspecific 5′→3′ exonuclease activity upstream of the $\chi$ site. In addition to an increase in yield, further lowering of the free $Mg^{2+}$ ion concentration leads to a change in the apparent size of the upstream $\chi$-specific fragment; its size increases with decreasing free $Mg^{2+}$ ion concentration until the band is no longer distinguishable from the full length ssDNA.

The exact cleavage location on the bottom-strand was determined by analysing the reaction products using denaturing alkaline agarose gel electrophoresis (Fig. 2B). Again, under a variety of free $Mg^{2+}$ ion concentrations, the formation of a 5′ end-labelled, upstream $\chi$-specific fragment was observed. Comparison of the size of this fragment with the 1 kb ladder shows that, at the highest free $Mg^{2+}$ ion concentration (8 mM $Mg^{2+}$/1 mM ATP), the fragment migrates where it was expected to, based on cleavage occurring at the $\chi$ sequence (within 100 bp accuracy), in agreement with the location established previously (Taylor & Smith 1995). As the free $Mg^{2+}$ ion concentration is lowered, the size of the bottom-strand, upstream $\chi$-specific fragment becomes significantly larger than expected if the initial cleavage were occurring exactly at $\chi$.

To verify that this variation in bottom-strand $\chi$-specific fragment size is not unique to $\chi^+$F, a different $\chi$-containing DNA substrate, $\chi^+$E, was examined (Fig. 2C). Figure 2C shows unwinding reactions that are the same as those in Fig. 2A, but use a different starting dsDNA substrate: *Nde*I-linearized 5′ end-labelled pBR322 $\chi^+$E. In Fig. 2A, a portion of the linear dsDNA substrate is not unwound at low free $Mg^{2+}$ ion concentrations. This incomplete utilization of the starting dsDNA substrate under these conditions is due to $\chi$-dependent reversible inactivation of RecBCD enzyme (Dixon *et al.* 1994). To allow for complete unwinding at all conditions, the linear pBR322 $\chi^+$E was treated with fourfold more RecBCD enzyme than the reactions in Fig. 2A. Unwinding reactions using linear pBR322 $\chi^+$E DNA reveal that it has the same sensitivity to reaction conditions that the pBR322 $\chi^+$F DNA showed. For both of these DNA substrates, the optimum condition for the production of the bottom-strand, upstream $\chi$-specific fragment is 1–2 mM $Mg^{2+}$ ion and 1 mM ATP, conditions which approximate intracellular concentrations (Alatossava *et al.* 1985).

## A bottom-strand, downstream $\chi$-specific fragment is not produced under any reaction conditions

In addition to the top-strand, downstream $\chi$-specific fragment produced at all reaction conditions tested, interaction of the RecBCD enzyme with $\chi$ leads to the production of a top-strand, upstream $\chi$-specific ssDNA fragment (derived from the 3′-terminal strand at the entry site) at conditions of low nucleolytic activity (Ponticelli *et al.* 1985; Dixon & Kowalczykowski 1993; Dixon & Kowalczykowski 1995). To examine whether the downstream 5′→3′ exonuclease activity is similarly sensitive to free $Mg^{2+}$ ion concentration, $\chi$-containing DNAs were 3′ end-labelled instead of 5′ end-labelled to see whether a bottom-strand, downstream $\chi$-specific fragment is produced.

Figure 3 shows that no bottom-strand $\chi$-specific bands are detected when 3′-end labelled dsDNA is used; instead only a very broad smear, which decreases in size with increasing free $Mg^{2+}$ ion concentration, is present. This product corresponds to nonspecific nucleolytic fragments whose size decreases with increasing free $Mg^{2+}$ ion, and is completely $\chi$-independent (Eggleston & Kowalczykowski 1993; Dixon & Kowalczykowski 1995; data not shown). As noted previously (Ponticelli *et al.* 1985; Dixon & Kowalczykowski 1995), low free $Mg^{2+}$ ion concentrations sufficiently attenuate the 3′→5′ exonuclease activity of RecBCD enzyme to permit the detection of a $\chi$-specific fragment corresponding to the 3′ end-labelled upstream ssDNA. Under no conditions, however, is a 3′ end-labelled $\chi$-specific fragment corresponding to the ssDNA downstream from $\chi$ observed. The absence of this 3′ end-labelled downstream $\chi$-specific fragment is not unique to pBR322 $\chi^+$E; analysis of RecBCD enzyme-treated, 3′ end-labelled pBR322 $\chi^+$F, which could yield a shorter 3′-end labelled downstream $\chi$-specific fragment, reveals identical results (see Fig. 3B). Again, at the lowest free $Mg^{2+}$ ion concentrations, the only $\chi$-specific band observed is that corresponding to the 3′ end-labelled region upstream of $\chi$.

## The RecBC enzyme does not possess dsDNA exonuclease activity

A number of experiments have suggested that recognition of $\chi$ by the RecBCD enzyme results in the ejection of the RecD protein subunit. Since we have demonstrated that the $\chi$-activated RecBCD enzyme is a 5′→3′ exonuclease (Anderson & Kowalczykowski 1997), the

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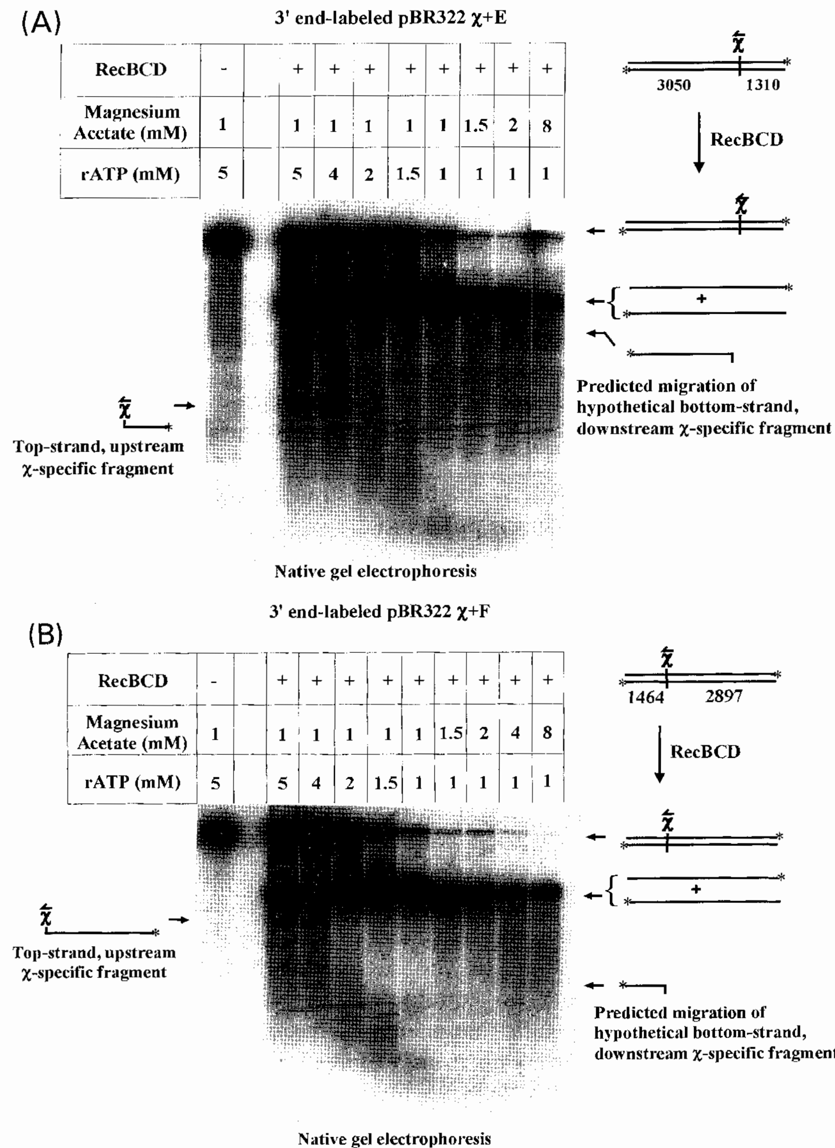


**Figure 3** The bottom-strand, downstream χ-specific fragment is not detected under any conditions. The linear χ-containing dsDNA substrates were created by cutting the plasmids pBR322 $\chi^{+}$E with Nde I (A) and pBR322 $\chi^{+}$F with *Hin*dIII (B). The DNA substrates each contain a χ site in a different location. The DNA was labelled at the 3′-end with $^{32}$P and treated with one functional RecBCD enzyme/20 dsDNA ends (A and B). $Mg^{2+}$ and ATP were varied as indicated. The reactions were analysed on a 1% native agarose gel. The data from the 2 mM $Mg^{2+}$, 1 mM ATP lanes appeared in Anderson & Kowalczykowski (1997).

RecD-ejection hypothesis implies that the RecBC enzyme should be a 5′→3′ exonuclease. It was reported that RecBC enzyme (without RecD protein subunit) possesses an undefined level of exonuclease activity (Masterson *et al.* 1992; Korangy & Julin 1993), but it was not clear whether this activity is comparable to that of the χ-activated RecBCD enzyme. To clarify this question, DNA unwinding reactions with 5′ end-labelled DNA were performed using RecBC enzyme.

Figure 4 shows unwinding reactions with RecBC enzyme using 5′ end-labelled linear pBR322. These were performed with saturating RecBC enzyme (five functional RecBC enzymes per dsDNA end) at conditions that exhibit the highest level of RecBCD enzyme 5′→3′ exonuclease activity (8 mM $Mg^{2+}$, 1 mM ATP; Figs 4A and B), and at conditions that are optimal for the formation of the bottom-strand, upstream χ-specific fragment (2 mM $Mg^{2+}$, 1 mM ATP; Figs 4C and D). These reactions were analysed on native and alkaline agarose gel electrophoresis to control for the presence of nicks in the starting dsDNA substrate (unwinding of nicked DNA causes an apparent loss of signal which could be misinterpreted as nuclease activity). Quantification of the alkaline agarose gel shows no significant degradation over the course of the reaction at either condition (Figs 4B and D). Identical results were obtained with χ-containing DNA, showing that χ does not activate a

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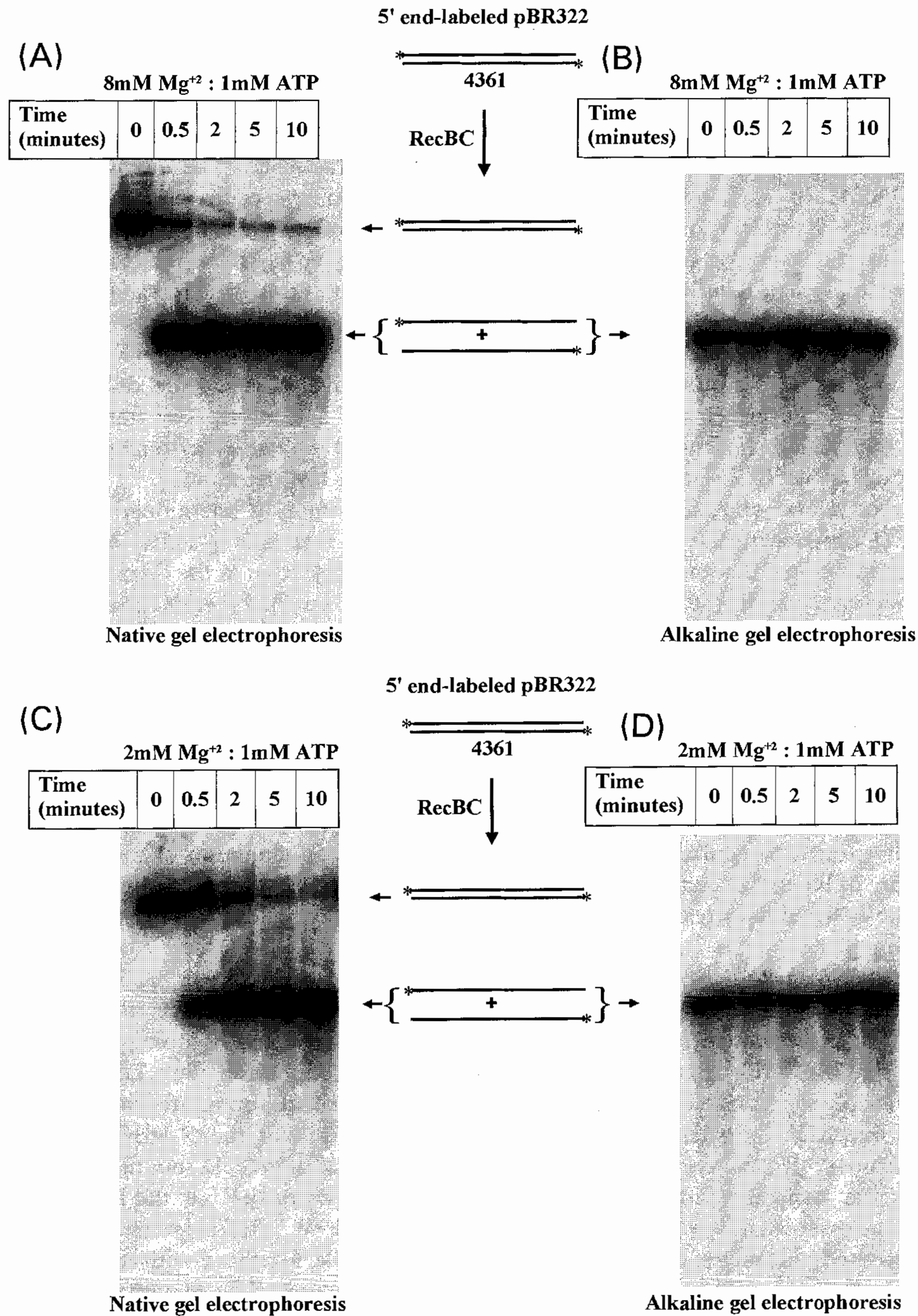


**Figure 4** RecBC enzyme is an active helicase with no significant nuclease activity. Linear pBR322 dsDNA was created by cutting the plasmid with *Nde*I. The DNA was labelled at the 5′-end $^{32}P$ and treated with saturating RecBC enzyme (five functional RecBC enzymes per dsDNA end) in the presence of 8 mM $Mg^{2+}$ (panels A and B) or 2 mM $Mg^{2+}$ (panels C and D) and 1 mM ATP. The reactions were analysed on 1% native (A and C) and alkaline (B and D) agarose gel.

cryptic nuclease activity of RecBC enzyme (J. J. Churchill, unpublished data). Thus, purified RecBC enzyme is not a direct analogue of the χ-activated RecBCD enzyme.

## Discussion

The RecBCD enzyme was originally characterized in crude extracts by its unique property of being an ATP-

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dependent dsDNA exonuclease (Oishi 1969; Barbour & Clark 1970). The vast majority of exonuclease activity in crude extracts is, in fact, due to RecBCD enzyme. This led to the question of how such a voracious nuclease could be essential to homologous recombination, a process that paradoxically requires the preservation of DNA for exchange to occur. The discovery by Dixon & Kowalczykowski (1991, 1993) that the recombination hot spot, $\chi$, acts to attenuate the $3' \rightarrow 5'$ exonuclease activity of RecBCD enzyme without affecting its helicase activity had apparently resolved this conundrum. However, we recently showed that recognition of $\chi$ by RecBCD protein also leads to the activation of a $5' \rightarrow 3'$ exonuclease (Anderson & Kowalczykowski 1997). This finding potentially raises the original paradox. If RecBCD protein is a nuclease before and after recognition of $\chi$, how has $\chi$ acted to stimulate RecBCD enzyme-mediated recombination? One answer to this question is that there is, overall, a net attenuation of nuclease activity after $\chi$ recognition. Treatment of $\chi$-containing DNA with RecBCD enzyme at the higher $Mg^{2+}$ ion concentrations reveals that the top-strand, downstream $\chi$-specific fragment is much less sensitive to the increased nucleolytic activity (i.e. prior to $\chi$) evident at these conditions, since it persists to a greater degree than the other DNA fragments (Fig. 2). Thus, even though interaction with $\chi$ also leads to an up-regulation of the $5' \rightarrow 3'$ exonuclease activity of RecBCD enzyme, there is a net preservation of the DNA downstream of $\chi$ relative to the DNA upstream.

An interesting characteristic of the RecBCD enzyme's $\chi$-activated $5' \rightarrow 3'$ exonuclease is that the exact location of the first cleavage event is dependent upon the level of free $Mg^{2+}$ (Fig. 2). The frequency of $\chi$-independent nucleolytic cleavage is dependent on the concentration of free $Mg^{2+}$ (Eggleston & Kowalczykowski 1993; Dixon & Kowalczykowski 1995). We hypothesize that the variability in the location of $\chi$-activation of nuclease is also a reflection of the sensitivity of $\chi$-activated nucleolytic cleavage frequency to free $Mg^{2+}$ concentration. Thus, while RecBCD enzymes $5' \rightarrow 3'$ exonuclease is activated at $\chi$, the location of the first cleavage on the bottom-strand is closer to $\chi$ at high free $Mg^{2+}$ concentrations. As the free $Mg^{2+}$ concentration is decreased, frequency of cleavage decreases, and the location of the first cleavage after $\chi$ is further downstream, thereby resulting in a larger bottom-strand upstream $\chi$-specific fragment.

In contrast to the size variation of the bottom-strand $\chi$-specific fragment, the size of the top-strand, downstream $\chi$-specific fragment is relatively insensitive to conditions (Fig. 2, Dixon & Kowalczykowski 1995; Taylor & Smith 1995). Previously published evidence suggests that the RecBCD enzyme pauses for a few seconds upon encountering a $\chi$-site (Dixon & Kowalczykowski 1993; D.A. Dixon & S.C. Kowalczykowski, unpublished data). The insensitivity of the location for top-strand $\chi$-specific cleavage to conditions can be interpreted as a cleavage event that occurs with high frequency during the pause at $\chi$ and prior to inactivation. Conversely, the greater variation in position for the bottom-strand $\chi$-specific cleavage event may reflect either the activation of $5' \rightarrow 3'$ exonuclease activity after the putative pause at $\chi$ (i.e. during subsequent translocation), or a relatively low frequency of cleavage while paused at $\chi$. The exact temporal order of these events remains to be determined.

As previously mentioned, a variety of evidence suggests that the $\chi$-activation of RecBCD enzyme results in ejection of the RecD subunit. We have shown here that purified RecBC enzyme does not completely mimic $\chi$-activated RecBCD enzyme. Under conditions where the $\chi$-activated RecBCD enzyme is an active $5' \rightarrow 3'$ exonuclease, purified RecBC enzyme exhibits no significant DNA exonuclease activity (Fig. 4). Thus, it is unlikely that the biochemical alteration of RecBCD enzyme mediated by $\chi$ is simply the ejection of the RecD subunit. Rather, we propose that $\chi$-recognition results in an alteration of the RecD subunit function, which is reflected in both a $\chi$-dependent inactivation of initiation of helicase activity (i.e. reversible inactivation, Dixon *et al.* 1994) and a $\chi$-dependent activation of nuclease activity ($5' \rightarrow 3'$, Anderson & Kowalczykowski 1997). Intriguingly, the RecD protein contains a sequence that has homology to several $5' \rightarrow 3'$ specific DNA exonucleases (Sue Lovett, Braudeis University, MA, personal communication). In addition, chemical cross-linking of the RecBCD enzyme with dsDNA shows that the RecD subunit binds to the DNA strand $5'$ at the entry site (i.e. the bottom-strand, Ganesan & Smith 1993). This evidence suggests that the domain responsible for the $\chi$-activated nuclease resides in the RecD subunit. This hypothesis is further supported by genetic evidence. Cells with nonfunctional *recD* have normal or elevated levels of recombination that is dependent on *recJ* function (Lovett *et al.* 1988). The RecJ protein is a $5' \rightarrow 3'$ single-strand specific exonuclease (Lovett & Kolodner 1989). Thus, the consequence of RecBC enzyme helicase and RecJ protein nuclease action on a dsDNA end would be to produce a $3'$-ssDNA overhang. In fact, the *recB*C-, *recJ*-dependent degradation of ssDNA occurs *in vivo* (Rinken *et al.* 1992).

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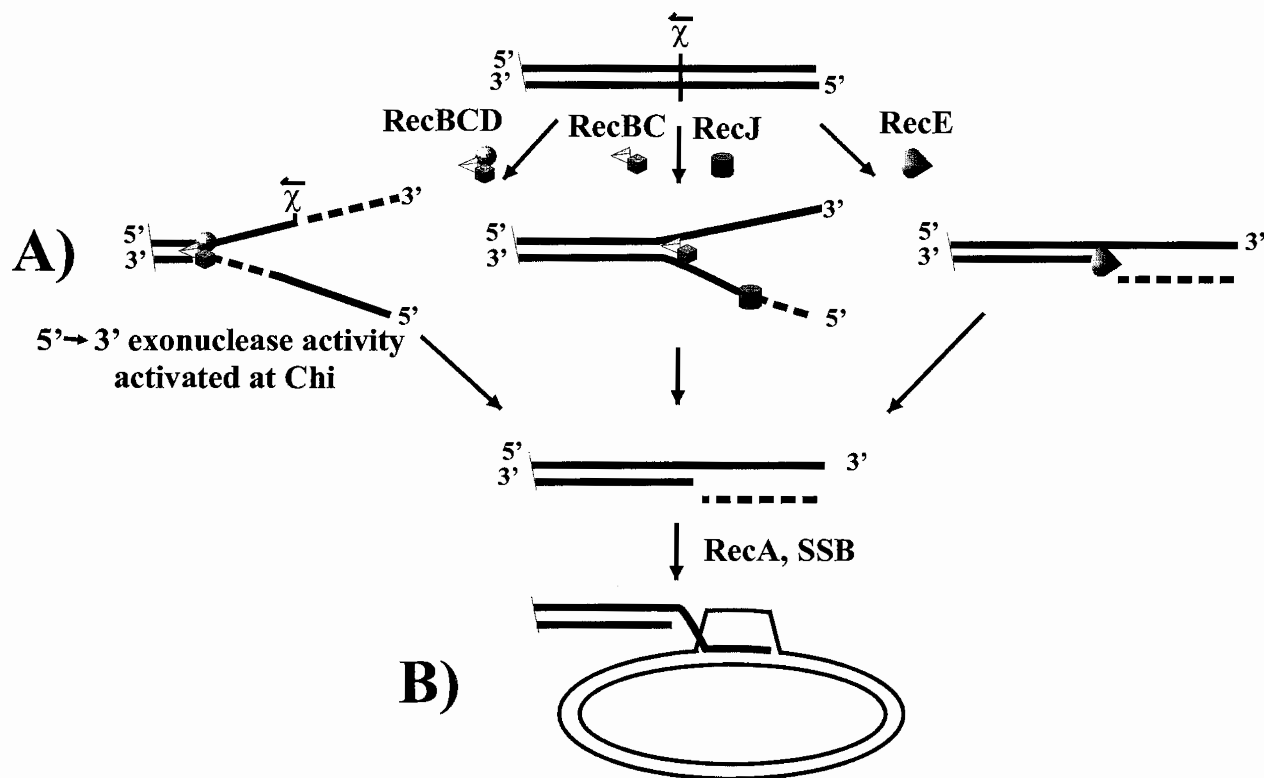


**Figure 5** Biochemical model for genetic recombination initiated from a dsDNA end. Details are discussed in the text. Initiation involves the production of a 3′-ssDNA overhang. (A) Formation of the resected 3′-ssDNA can occur by the combined helicase nuclease activities of the RecBCD enzyme and its interaction with χ; the 5′→3′ exonuclease activity of the RecE protein; or the combined RecBC enzyme helicase and RecJ protein 5′→3′ exonuclease activities. (B) The resultant 3′-ended ssDNA invades a homologous supercoiled recipient to form a D-loop in a RecA protein-dependent reaction. Adapted from Fig. 6, Anderson & Kowalczykowski (1997).

The degradative polarity switch at χ plays an important role in the generation of an appropriate ssDNA substrate for RecA protein action. On long DNA substrates (i.e. those encountered during conjugation or phage recombination), this polarity switch would result in a change from producing 5′-overhanging ssDNA ends to DNA with 3′-overhanging ssDNA ends. RecA protein binds cooperatively to ssDNA, polymerizing in the 5′→3′ direction (Register & Griffith 1985). A consequence of this polar extension is that 3′-ends are more likely to be involved in filament formation than 5′-ends. This results in the bias by RecA protein in joint molecule formation: 3′-ends are at least 10-fold more invasive than 5′-ends (Konforti & Davis 1987, 1990). Thus, by changing its polarity of DNA degradation at χ, RecBCD enzyme processes a random dsDNA break into an intermediate that is optimal for RecA protein action.

Initiation of homologous recombination in *E. coli* has the formation of a 3′-ssDNA overhang as a common intermediate. In *recB* or *recC* null mutants, recombination proficiency can be completely restored by the activation of another nuclease, the RecE protein (Barbour *et al.* 1970; Templin *et al.* 1972). Like χ-activated RecBCD enzyme, the RecE protein is a processive, dsDNA-specific 5′→3′ exonuclease (Gillen *et al.* 1977; Joseph & Kolodner 1983a,b; for review see Kowalczykowski *et al.* 1994). Thus, both RecE protein and χ-activated RecBCD enzyme lead to the production of DNA with 3′-ssDNA overhangs Fig. 5). As elaborated above, this intermediate can also be created by the combined action of the RecBC enzyme and the RecJ protein (Fig. 5).

The formation of a 3′-ssDNA overhang as an initial step of homologous recombination has been elaborated previously (Resnick 1976; Szostak *et al.* 1983). Physical analysis of recombination intermediates in *S. cerevisiae* (White & Haber 1990; Sun *et al.* 1991) and *Xenopus laevis* oocytes (Maryon & Carrol 1989) has revealed that an initial step is the formation of a 3′-ssDNA overhang.

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Thus, biochemical, genetic and physical analyses strongly support the idea that the production of 3′-overhanging DNA is a common method for the initiation of homologous recombination.

## Experimental procedures

### Enzymes

RecBCD enzyme was purified as described (Roman & Kowalczykowski 1989a). Protein concentration was determined using an extinction coefficient of $4.0 \times 10^5$ /M/cm at 280 nm (Roman & Kowalczykowski 1989a). The specific activity of the enzyme preparation was $5.4 \times 10^4$ U of nuclease per mg of protein or $1.1 \times 10^4$ U of helicase per mg of protein. Nuclease units and helicase units were measured as described by Eichler & Lehman (1977) and Roman & Kowalczykowski (1989a), respectively. RecBC enzyme was purified from strain V186 (*recBCD* deletion; Chaudhury & Smith 1984) transformed with pMS421 (*Lac I$^q$*), pPB700 (*recB*) and pB520 (*recC*) (Boehmer & Emmerson 1991), as described by (Masterson *et al.* 1992) and modified by (J. J. Churchill, in preparation); in brief, RecBC enzyme was purified using Q-sepharose, hydroxylapatite, Sephacryl-200, and ssDNA cellulose chromatography. Purified RecBC enzyme concentration was determined using an extinction coefficient of $3.6 \times 10^5$ /M/cm at 280 nm (Korangy & Julin 1993), and was determined to be 20% active as described previously (Roman & Kowalczykowski 1989a). SSB protein was isolated from strain RLM727 and purified according to LeBowitz, (1985). Protein concentration was determined using an extinction coefficient of $3.0 \times 10^4$ /M/cm at 280 nm (Ruyechan & Wetmur 1975). All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, Pharmacia LKB, Bethesda Research Laboratories, or Promega. The enzymes were used as specified by the vendor.

### DNA Substrates

Plasmids pBR322 $\chi^0$ (wild-type), pBR322 $\chi^+$F, and pBR322 $\chi^+$E (Smith *et al.* 1981) were purified using alkaline lysis as described in (Sambrook *et al.* 1989). The molar concentration of dsDNA in nucleotides was determined using an extinction coefficient of 6290/M/cm at 260 nm. Plasmid DNA was linearized with the appropriate restriction enzyme, and then end-labelled at either the 3′-end using the Klenow fragment of DNA Polymerase I and appropriate [$\gamma$-$^{32}$P]dNTP (NEN), or at the 5′-end by sequential reactions with shrimp alkaline phosphatase and T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]ATP (NEN) (Sambrook *et al.* 1989).

### Reaction conditions

Standard RecBCD reaction conditions consisted of 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 4 U/mL pyruvate kinase, 10 $\mu$M nucleotides linear dsDNA (1.15 nM dsDNA molecules), 2 $\mu$M SSB protein, 0.31 nM total RecBCD enzyme (0.115 nM functional), with the magnesium acetate and ATP concentrations as indicated. In some cases, RecBCD enzyme was varied as indicated. Standard RecBC reaction conditions were the same as standard RecBCD reaction conditions, using 8 mM or 2 mM magnesium acetate, 1 mM ATP, and 57.5 nM total RecBC enzyme (11.5 nM functional). Assays were performed at 37 °C and were initiated with the addition of enzyme after the pre-incubation of all other components for 3 min.

### Analysis of reaction products

After a 4-min incubation with RecBCD enzyme or varying incubation times with RecBC enzyme, each reaction (40 $\mu$L) was stopped by the addition of 10 $\mu$L of stop buffer (0.1 M EDTA, 2.5% SDS, 40% glycerol, 0.125% bromophenol blue, and 0.125% xylene cyanol). For all reactions except those in Fig. 4, the volume of reactions without the RecBCD enzyme were one-half those with RecBCD enzyme. Samples were subjected to electrophoresis through 1% native agarose gels for 15 h at 1.4 V/cm in TAE (40 mM Tris-acetate (pH 8.0), 2 mM EDTA). The gels were dried and exposed to Kodak XAR-5 film at room temperature. Samples (40 $\mu$L) which were analysed by denaturing alkaline agarose electrophoresis were stopped by the addition of stop buffer, followed by 12 $\mu$L of alkaline loading buffer (300 mM NaOH, 6 mM EDTA, 18% ficol, 0.15% bromocresol green, 0.25% xylene cyanol). The samples were mixed and then subjected to electrophoresis through 1% alkaline agarose gels for 15 h at 1.4 V/cm in alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA)

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## References

Alatossava, T., Jutte, H., Kuhn, A. & Kellenberger, E. (1985) Manipulation of intracellular magnesium content in polymyxin B nonapeptide-sensitized *Escherichia coli* by ionophore A23187. *J. Bacteriol.* **162**, 413–419.

Anderson, D.G. & Kowalczykowski, S.C. (1997) The recombination hot spot, Chi, is a regulatory element that switches the polarity of DNA degradation by the RecBCD enzyme. *Genes Dev.*, **11**, 571–581.

Barbour, S.D. & Clark, A.J. (1970) Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. I. Enzymatic activity associated w/*recB*+ and *recC*+ genes. *Proc. Natl. Acad. Sci. USA* **65**, 955–961.

Barbour, S.D., Nagaishi, H., Templin, A. & Clark, A.J. (1970) Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. II. rec+ revertants caused by indirect suppression of rec- mutations. *Proc. Natl. Acad. Sci. USA* **67**, 128–135.

Boehmer, P.E. & Emmerson, P.T. (1991) *Escherichia coli* RecBCD enzyme: Inducible overproduction and reconstitution of the

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ATP-dependent deoxyribonuclease from purified subunits. *Gene* **102**, 1–6.

Chaudhury, A.M. & Smith, G.R. (1984) *Escherichia coli recBC* deletion mutants. *J. Bacteriol.* **160**, 788–791.

Cheng, K.C. & Smith, G.R. (1989) Distribution of Chi-stimulated recombinational exchanges and heteroduplex end-points in phage lambda. *Genetics* **123**, 5–17.

Clark, A.J. & Margulies, A.D. (1965) Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* **53**, 451–459.

Dixon, D.A., Churchill, J.J. & Kowalczykowski, S.C. (1994) Reversible inactivation of the *Escherichia coli* RecBCD enzyme by the recombination hotspot, Chi, *in vitro*: Evidence for functional inactivation or loss of the RecD subunit. *Proc. Natl. Acad. Sci. USA* **91**, 2980–2984.

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) The recombination hotspot, Chi, is a regulatory sequence that acts by attenuating the nuclease activity of the *Escherichia coli recB*CD enzyme. *Cell* **73**, 87–96.

Dixon, D.A. & Kowalczykowski, S.C. (1995) Role of the *Escherichia coli* recombination hotspot, chi, in RecABCD-dependent homologous pairing. *J. Biol. Chem.* **270**, 16360–16370.

Eggleston, A.K. & Kowalczykowski, S.C. (1993) Biochemical characterization of a mutant RecBCD enzyme, the $RecB^{2109}CD$ enzyme, which lacks $\chi$-specific, but not non-specific, nuclease activity. *J. Mol. Biol.* **231**, 605–620.

Eichler, D.C. & Lehman, I.R. (1977) On the role of ATP in phosphodiester bond hydrolysis catalyzed by the *recBC* deoxyribonuclease of *Escherichia coli*. *J. Biol. Chem.* **252**, 499–503.

Emmerson, P.T. (1968) Recombination deficient mutants of *Escherichia coli* K12 that map between thyA and argA. *Genetics* **60**, 19–30.

Ennis, D.G., Amundsen, S.K. & Smith, G.R. (1987) Genetic functions promoting homologous recombination in *Escherichia coli*: A study of inversions in phage lambda. *Genetics* **115**, 11–24.

Ganesan, S. & Smith, G.R. (1993) Strand-specific binding to duplex DNA ends by the subunits of *Escherichia coli recB*CD enzyme. *J. Mol. Biol.* **229**, 67–78.

Gillen, J.R., Karu, A.E., Nagaishi, H. & Clark, A.J. (1977) Characterization of the deoxyribonuclease determined by lambda reverse as exonuclease VIII of *Escherichia coli*. *J. Mol. Biol.* **113**, 27–41.

Howard-Flanders, P. & Theriot, L. (1966) Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination. *Genetics* **53**, 1137–1150.

Joseph, J.W. & Kolodner, R. (1983a) Exonuclease VIII of *Escherichia coli*. I. Purification and physical properties. *J. Biol. Chem.* **258**, 10411–10417.

Joseph, J.W. & Kolodner, R. (1983b) Exonuclease VIII of *Escherichia coli*. II. Mechanism of action. *J. Biol. Chem.* **258**, 10418–10424.

Konforti, B.B. & Davis, R.W. (1987) 3′ homologous free ends are required for stable joint molecule formation by the RecA and single-stranded binding proteins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **84**, 690–694.

Konforti, B.B. & Davis, R.W. (1990) The preference for a 3′ homologous end is intrinsic to RecA-promoted strand exchange. *J. Biol. Chem.* **265**, 6916–6920.

Koppen, A., Krobitsch, S., Thoms, B. & Wackernagel, W. (1995) Interaction with the recombination hot spot Chi *in vivo* converts the RecBCD enzyme of *Escherichia coli* into a Chi-independent recombinase by inactivation of the RecD subunit. *Proc. Natl. Acad. Sci. USA* **92**, 6249–6253.

Korangy, F. & Julin, D.A. (1993) Kinetics and processivity of ATP hydrolysis and DNA unwinding by the *recBC* enzyme from *Escherichia coli*. *Biochemistry* **32**, 4873–4880.

Kowalczykowski, S.C., Clow, J. & Krupp, R.A. (1987) Properties of the duplex DNA-dependent ATPase activity of *Escherichia coli* recA protein and its role in branch migration. *Proc. Natl. Acad. Sci. USA* **84**, 3127–3131.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D. & Rehrauer, W.M. (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58**, 401–465.

Kowalczykowski, S.C. & Eggleston, A.K. (1994) Homologous pairing and DNA strand-exchange proteins. *Annu. Rev. Biochem.* **63**, 991–1043.

Lam, S.T., Stahl, M.M., McMilin, K.D. & Stahl, F.W. (1974) Rec-mediated recombinational hot spot activity in bacteriophage lambda. II. A mutation which causes hot spot activity. *Genetics* **77**, 425–433.

LeBowitz, J. (1985) *Biochemical mechanism of strand initiation in bacteriophage lambda DNA replication*. PhD thesis, John Hopkins University, Baltimore, MD.

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.

Lovett, S.T. & Kolodner, R.D. (1989) Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the recJ gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**, 2627–2631.

Masterson, C., Boehmer, P.E., McDonald, F., Chaudhuri, S., Hickson, I.D. & Emmerson, P.T. (1992) Reconstitution of the activities of the RecBCD holoenzyme of *Escherichia coli* from the purified subunits. *J. Biol. Chem.* **267**, 13564–13572.

Maryon, E. & Carroll, D. (1989) Degradation of linear DNA by a strand-specific exonuclease activity in Xenopus laevis oocytes. *Mol. Cell. Biol.* **9**, 4862–4871.

Myers, R.S., Kuzminov, A. & Stahl, F.W. (1995a) The recombination hot spot Chi activates RecBCD recombination by converting *Escherichia coli* to a *recD* mutant phenocopy. *Proc. Natl. Acad. Sci. USA* **92**, 6244–6248.

Myers, R.S., Stahl, M.M. & Stahl, F.W. (1995b) Chi recombination activity in phage lambda decays as a function of genetic distance. *Genetics* **141**, 805–812.

Oishi, M. (1969) An ATP-dependent deoxyribonuclease from *E. coli* with a possible role in genetic recombination. *Proc. Natl. Acad. Sci. USA* **64**, 1292–1299.

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.

Pugh, B.F. & Cox, M.M. (1987) Stable binding of recA protein to duplex DNA. Unraveling a paradox. *J. Biol. Chem.* **262**, 1326–1336.

Register, J.C. III & Griffith, J. (1985) The direction of RecA protein assembly onto single strand DNA is the same as the direction of strand assimilation during strand exchange. *J. Biol. Chem.* **260**, 12308–12312.

Resnick, M.A. (1976) The repair of double-strand breaks in DNA: A model involving recombination. *J. Theor. Biol.* **59**, 97–106.

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

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

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

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

Ruyechan, W.T. & Wetmur, J.G. (1975) Studies on the cooperative binding of the *Escherichia coli* DNA unwinding protein to single-stranded DNA. *Biochemistry* **14**, 5529–5534.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

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

Smith, G.R., Kunes, S.M., Schultz, D.W., Taylor, A. & Triman, K.L. (1981) Structure of Chi hotspots of generalized recombination. *Cell* **24**, 429–436.

Stahl, F.W., Crasemann, J.M. & Stahl, M.M. (1975) Rec-mediated recombinational hot spot activity in bacteriophage lambda. III. Chi mutations are site-mutations stimulating rec-mediated recombination. *J. Mol. Biol.* **94**, 203–212.

Stahl, F.W., Stahl, M.M., Malone, R.E. & Crasemann, J.M. (1980) Directionality and nonreciprocality of Chi-stimulated recombination in phage lambda. *Genetics* **94**, 235–248.

Stahl, F.W., Thomason, L.C., Siddiqi, I. & Stahl, M.M. (1990) Further tests of a recombination model in which Chi removes the RecD subunit from the RecBCD enzyme of *Escherichia coli*. *Genetics* **126**, 519–533.

Sun, H., Treco, D. & Szostak, J.W. (1991) Extensive 3′-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the ARG4 recombination initiation site. *Cell* **64**, 1155–1161.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. & Stahl, F.W. (1983) The double-strand break repair model for recombination. *Cell* **33**, 25–35.

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

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

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

Taylor, A.F. & Smith, G.R. (1995) Strand specificity of nicking of DNA at Chi sites by RecBCD enzyme. Modulation by ATP and magnesium levels. *J. Biol. Chem.* **270**, 24459–24467.

Telander-Muskavitch, K.M. & Linn, S. (1981) RecBC-like enzymes: Exonuclease V deoxyribonucleases. In: *The Enzymes* (ed. P.D. Boyer), pp. 233–250. New York: Academic Press.

Templin, A., Kushner, S.R. & Clark, A.J. (1972) Genetic analysis of mutations indirectly suppressing *recB* and *recC* mutations. *Genetics* **72**, 205–215.

Thaler, D.S., Sampson, E., Siddiqi, I., Rosenberg, S.M., Stahl, F.W. & Stahl, M. (1988) A hypothesis: Chi-activation of *recB*CD enzyme involves removal of the *recD* subunit. In: *Mechanisms and Consequences of DNA Damage Processing* (eds E. Friedberg & P. Hanawalt), pp. 413–422. New York: Alan R. Liss, Inc.

White, C.I. & Haber, J.E. (1990) Intermediates of recombination during mating type switching in *Saccharomyces cerevisiae*. *EMBO J.* **9**, 663–673.

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