The recombination hot spot $\chi$ is a regulatory element that switches the polarity of DNA degradation by the RecBCD enzyme

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Homologous recombination in Escherichia coli is stimulated at DNA sequences known as $\chi$ sites. Stimulation requires the multifunctional RecBCD enzyme, which is both a helicase and a 3'→5' exonuclease. Upon recognition of a properly oriented $\chi$ site, the 3'→5' exonuclease activity is attenuated. Here we show that in addition to attenuation of the 3'→5' exonuclease activity, recognition of $\chi$ by the RecBCD enzyme also upregulates a nuclease activity of the opposite polarity, resulting in an enzyme that now preferentially degrades 5'→3'. These results demonstrate that $\chi$ is a unique regulatory element that converts the anticonservative/nicking form of the RecBCD enzyme into a recombination form by causing two distinct enzymatic changes: attenuation of the 3'→5' nuclease activity, and up-regulation of the 5'→3' nuclease activity. The consequence of $\chi$ recognition is the production of a recombination intermediate possessing a 3'-ssDNA end and a 3'-ssDNA overhang parallel to the one used during the initiation of homologous recombination in other pathways in E. coli, and in other organisms such as the yeast Saccharomyces cerevisiae.

[Key Words: Recombination, RecBCD, $\chi$ nuclease, helicase, regulation]

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The RecBCD enzyme is essential for the main pathway of homologous recombination in Escherichia coli. This heterodimeric enzyme is composed of products of the recB, recC, and recD genes (for review, see Smith 1988; Kowalczykowski et al. 1994). Inactivation of either the recB or recC genes leads to a reduction of homologous recombination by up to 1000-fold in vivo (Howard-Flax and Hargit 1966; Emmonson 1968).

The RecBCD enzyme possesses a number of different nuclease activities, it is an ATP-dependent exonuclease and an ATP-stimulated single-stranded DNA (ssDNA) endonuclease (Lengeler et al. 1993). In addition to these degradative properties, the RecBCD enzyme is a highly processive helicase, capable of unwinding up to 30,900 bp per binding event at rates of up to 1000 bp/sec (Roman and Kowalczykowski 1989a, Roman et al. 1992, Eggleston and Kowalcz-}

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ceeds to unwind the DNA duplex while simultaneously degrading the DNA from this end (Telander et al. 1981; Taylor and Smith 1985). Degradation of the DNA is asymmetric, with the 3' terminal strand relative to the entry site of the RecBCD enzyme being degraded much more extensively than the 5' terminal strand (Egan and Kowalczykowski 1993).

Homologous recombination in wild-type Escherichia coli is stimulated at cis-acting DNA sequences known as $\chi$ sites (S'/G-CGGTCCG-S') (Lam et al. 1974; Stahl et al. 1976, Smith et al. 1981). In vivo, these DNA elements stimulate homologous recombination 5 to 10-fold in a recBCD-dependent manner. Stimulation by $\chi$ is polar, with maximum stimulation at $\chi$ and decaying downstream relative to the entry site of the RecBCD enzyme (Stahl et al. 1980; Emmons et al. 1987, Cheng and Smith 1989, Myers et al. 1997a,b). The polar nature of $\chi$ stimulation in vivo is a direct result of the DNA changes in the RecBCD enzyme that occur after recognition. When $\chi$ is recognized by a translocating RecBCD enzyme molecule, DNA degradation backs up 4-8 nucleotides upstream of the $\chi$ site, after which the enzyme continues to unwind the DNA with its 3'→5' exonuclease activity greatly attenuated (Poncet et al. 1989; Egan and Kowalczykowski 1993; Taylor and
Smith 1995). The cotornin unwinding of the dsDNA downstream of χ leads to production of a ssDNA template that can now be utilized by the homologous pairing and strand-exchange protein RecA (Dixon and Kowalczy- kowska 1995).

Perhaps the single most essential enzyme for homologous recombination in E. coli is the RecA protein. Mutation of the recA gene causes a 105- to 106-fold reduction in homologous recombination (Clark and Magalhes 1965). In vitro, this protein is able to promote pairing and exchange between homologous DNA substrates (for review, see Kowalczykowski and Egelston 1994). One re- quirement for RecA protein-promoted pairing in vitro is that at least one of the DNA substrates must possess some ssDNA character. This is in contrast to the obser- vation that the majority of recombination events which occur in vitro, and in particular those that are mediated by the RecBCD pathway, involve donor DNA substrates that are double-stranded (for review, see Kowalczy- kowska et al. 1994). The RecBCD enzyme acts to ini- tiate homologous recombination by processing dsDNA into a ssDNA substrate suitable for RecA protein action (Taylor and Smith 1985; Roman and Kowalczykowski 1986; Dixon and Kowalczykowski 1991).

Although the ability of χ to attenuate the 3′–5′ exonuclease of the RecBCD enzyme has been demonstrated, the effect of χ recognition on the weaker 5′–3′ exonuclease activity is unclear (Dixon and Kowalczykowski 1995). In this paper we directly demonstrate that recogni- tion of χ results in the up-regulation of the 5′–3′ exonuclease activity, in addition to the attenuation of the 3′–5′ exonuclease activity. Thus, the consequence of χ recognition is the production and net preservation of a combination intermediate possessing a 3′-ssDNA overhang terminating at the χ sequence. This processing of a dsDNA end to a 3′-ssDNA overhang parallels that which occurs in other pathways of homologous recom- bination in E. coli and in other organisms such as the yeast S. cerevisiae (for review, see Kowalczykowski et al. 1994).

Results
Recognition of χ leads to the production of χ-specific DNA fragments derived from both strands of dsDNA

As mentioned previously, recognition of χ by a trans- locating RecBCD enzyme molecule leads to attenuation of 5′–3′ exonuclease activity. The continued unwinding of the DNA after χ produces a downstream ssDNA fragment of a length corresponding to the distance be- tween χ and the distal end of the DNA (Dixon and Kowalczykowski 1995). In addition, Dixon and Kowalczy- kowska (1995) observed that another 5′-end-labeled DNA fragment was created, possibly corresponding to the re- gion of DNA that is upstream of χ but derived from the opposite DNA strand. For clarity, we define the region between the χ site and the end of the DNA at which the RecBCD enzyme initiated 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). In addition, the strand of dsDNA that termi- nates at χ at the entry point of the RecBCD enzyme is defined as the top strand. The DNA strand opposite the top strand is defined as the bottom strand. Thus, the unwinding and specific cleavage of DNA containing a χ site by the RecBCD enzyme has the potential to generate four distinct χ-specific ssDNA fragments: top strand, up- stream fragment; top strand, downstream fragment; bot- tom strand, upstream fragment; and bottom strand, downstream fragment. To establish whether the DNA fragment observed by Dixon and Kowalczykowski (1995) was χ-specific and derived from the bottom strand, un- winding reactions using linear pBR322 χF [Fig. 2A, B] were compared to those using linear pBR322 χE [Fig. 2C, D], both of which contain a single χ site at a different location in pBR322. If the production of this new 5′-end- labeled species is χ-specific, then the size of this species should change as the location of χ is varied. In addition, these reactions were compared to unwinding reactions using linear pBR322, which contains no χ sites [Fig. 2D].

Figure 2A shows the products of an unwinding reac- tion using linearized pBR322 χF, which was 5′-end-la- beled. The efficiency of χ recognition by the RecBCD enzyme is only ~20% (Taylor et al. 1985), Dixon and
Kowalczykowski 1993). Processing of dsDNA by the RecBCD enzyme without recognition of χ results in the production of full-length ssDNA because of the asymmetric degradation of DNA during unwinding [Dixon and Kowalczykowski 1993]. Interaction of the RecBCD enzyme with χ apparently results in the formation of two χ-specific fragments, the top strand, downstream χ-specific fragment reported previously by Dixon and Kowalczykowski (1993), and another band, presumably corresponding to the bottom strand upstream of χ. To determine the size of this putative upstream χ-specific fragment, the reaction products were analyzed by denaturing alkaline agarose gel electrophoresis (Fig. 2B). Comparison of the size of this fragment with the 1-kb ladder showed that cleavage occurred ~300–500 nucleotides downstream of the expected location on the basis of cleavage occurring precisely at the χ sequence. The precise size of the bottom-strand upstream χ-specific fragment is dependent on the frequency of 5′ → 3′ exonuclease activity, which is a function of free magnesium levels (Anderson et al. 1997).

To verify that the formation of the putative bottom-strand, upstream fragment is χ-specific, a different χ-containing DNA substrate was examined (Fig. 2C). The unwinding reaction shown in Figure 2C is the same as that in Figure 2A but uses a different starting dsDNA substrate. Nol-1-linearized χ-ended labeled pBR322 χ/E. Unwinding reactions using linear pBR322 χ/E DNA reveal the same sensitivity to reaction conditions that the pBR322 χ E DNA showed (Anderson et al. 1997); most significantly, a novel χ-ended ssDNA fragment corresponding to the region upstream of χ can be visualized (Fig. 2C). For all of these reactions, the conditions used approximate intracellular free Mg²⁺ ion concentrations (Altamorsa et al., 1985). Examination of unwinding reactions using wild-type pBR322, which does not contain a χ site, reveals that the E strain full-length ssDNA is produced, further verifying that this novel species is χ-specific (Fig. 2D).

As mentioned previously, the top-strand, downstream χ-specific fragment results from degradation of the top DNA strand from the enzyme entry site to χ and attenuation of 5′ → 3′ exonuclease at and beyond χ (see Dixon and Kowalczykowski 1993). The simplest explanation for the novel bottom-strand, upstream χ-specific fragment is a single cleavage event on the bottom strand opposite that of χ. This would result in the formation of a bottom-strand, downstream χ-specific
A bottom-strand, downstream \(\chi\)-specific fragment is not produced.

In the conditions obtained with 5'-end-labeled d\(\Phi\)DNA, no bottom-strand \(\chi\)-specific bands are detected when 3'-end-labeled d\(\Phi\)DNA is used (Fig. 3). Under no conditions is a 3'-end-labeled \(\chi\)-specific fragment corresponding to the region downstream from \(\chi\) observed (Anderson et al. 1997). The conspicuous absence of this 3'-end-labeled downstream \(\chi\)-specific fragment under conditions in which a 5'-end-labeled fragment is produced is not unique to pBR322 \(\chi\)'E (Fig. 3A), analysis of the RecBCD enzyme-treated 3'-end-labeled pBR322 \(\chi\)'E, which could yield a shorter 3'-end-labeled downstream \(\chi\)-specific fragment, reveals identical results (Fig. 3B). For both of these reactions, the only product of the RecBCD enzyme unwinding is full-length s\(\Phi\)DNA. These results suggest that the hypotrophic bottom-strand, downstream \(\chi\)-specific fragment was degraded, whereas the bottom-strand, upstream fragment was not.

Interaction of the RecBCD enzyme with \(\chi\) up-regulators of the 5' -> 3' exonuclease activity

The previous experiments showed that interaction of the RecBCD enzyme with \(\chi\) leads to the production of a bottom-strand, upstream \(\chi\)-specific fragment, but not the corresponding bottom-strand, downstream fragment. There are three possible explanations for these observations, one of which is that the 5' -> 3' nuclease activity is up-regulated after interaction with \(\chi\) site. The second possibility is that \(\chi\) might be recognized in a "nonstandard" orientation by the RecBCD enzyme, traveling from the opposite direction. A third alternative is that the production of bottom-strand, upstream \(\chi\)-specific fragments is attributable to DNA that was acted upon by two RecBCD enzymes, one of which degraded the DNA 3' -> 5' up to \(\chi\) and then dissociated, followed by the action of a second RecBCD enzyme that degraded 3' -> 5' up to \(\chi\) from the opposite direction. To distinguish between these alternatives, \(\chi\)-containing substrates were designed that limit the entry of the RecBCD enzyme to only one end. The RecBCD enzyme can be prevented from entering a d\(\Phi\)DNA end by a 3' overhang longer than 25 bases (Taylor and Smith 1985). Such a tailed DNA substrate was produced by treatment of linear pBR322 \(\chi\)'F DNA with exonuclease III [Exo III] to create 5' ssDNA overhangs >50 nucleotides in length at both ends (see Materials and Methods). The tailed DNA end upstream of \(\chi\) was then removed using the restriction enzyme \(\lambda\)StI, creating a substrate that is blocked at one of the two ends and contains a \(\chi\) site oriented such that \(\chi\) recognition will occur in the standard orientation for a RecBCD enzyme entering at the accessible end.

The products of DNA unwinding depend on the total ratio of the RecBCD enzyme to DNA ends (Dixon and Kowalczykowski 1998). At saturating amounts of enzyme, full-length 5'-end-labeled ssDNA is produced by two RecBCD enzymes that meet

**Figure 3.** The bottom-strand, downstream \(\chi\)-specific fragment is not detected when 3'-end-labeled \(\chi\)' DNA is used. The linear \(\chi\)-containing d\(\Phi\)DNA substrates were created by cutting the plasmid pBR322 \(\chi\)'E with N\(\alpha\la\)I and pBR322 \(\chi\)'F with HindIII (B). The DNA substrates each contain a \(\chi\) site in a different location. The DNA was labeled at the 3' end with \(\gamma\)P and treated with one functional RecBCD enzyme per 20-d\(\Phi\)DNA ends (A, B). The reactions were analyzed on a 1% native agarose gel.

**A** 5'-end-labeled pBR322 \(\chi\)'E

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<th>RecBCD</th>
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**B** 3'-end-labeled pBR322 \(\chi\)'F

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<td>1644</td>
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Native gel autoradiogram

Native gel electrophoresis

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in the middle of the dsDNA substrate [Fig. 4B]. If, however, entry at the DNA end downstream of \( \chi \) is blocked by the presence of a ssDNA tail, treatment with a saturating RecBCD enzyme will produce the same DNA fragments that substantiating amounts of enzyme do [Fig. 4C]. If the source of the bottom-strand, upstream \( \chi \)-specific fragment is \( \chi \)-regulation of the \( 5' \rightarrow 3' \) exonuclease activity upon \( \chi \) recognition, then RecBCD enzyme action on this tailed DNA substrate should produce both bottom-strand, upstream and top-strand, downstream \( \chi \)-specific fragments. Finally, dsDNA that has tails at both ends should be completely resistant to the RecBCD enzyme [Fig. 4D].

Figure 5, lanes 7-12, shows the results of reactions with \( \chi \)-containing dsDNA substrates that were not treated with Exo III and, therefore, are accessible to the RecBCD enzyme from both dsDNA ends. Two different \( 5' \)-end-labeled \( \chi \)-containing substrates [pBR322 \( \chi \)-F linearized by HindIII (lanes 0-12) and pBR322 \( \chi \)-F cut with both HindIII and Ascl (lanes 7-9)] were treated with varying amounts of the RecBCD enzyme. At saturating amounts of the RecBCD enzyme, unwinding leads to the production of full-length ssDNA, and both the upstream and downstream \( 5' \)-end-labeled \( \chi \)-specific fragments (lanes 8,11). The size of the bottom-strand, upstream \( \chi \)-specific fragment is smaller for the Ascl-treated DNA substrate, as is the full-length ssDNA, because of cleavage of the region upstream from \( \chi \) at the Ascl site. The region downstream of \( \chi \) was not altered by restriction of the DNA, thus, the migration of the topstrand, downstream \( \chi \)-specific fragment is the same for these two substrates. At saturating enzyme concentrations (lanes 9,12), unwinding was initiated simultaneously from both ends, leading to the production of \( 5' \)-end-labeled ssDNA that is approximately half of full length.

The \( 5' \)-end-labeled pBR322 \( \chi \)-F DNA that had been treated with Exo III was also subjected to RecBCD enzyme treatment (lanes 1-6). This DNA has neither the end downstream of \( \chi \) or both ends blocked to the RecBCD enzyme access by the presence of a ssDNA tail, depending on whether or not the DNA was treated with Ascl. The DNA that has a ssDNA overhang at both ends did not show any evidence of unwinding even at the highest RecBCD enzyme concentrations (lanes 4-6). However, unwinding of the substrate that has a tail only at the end downstream of \( \chi \) [lanes 1-3] produces both \( \chi \)-specific fragments and full-length ssDNA (owing to events where \( \chi \) was not recognized) at both saturating and saturating RecBCD enzyme concentrations. The presence of this full-length ssDNA and the absence of half-length ssDNA at the highest RecBCD concentrations confirm that unwinding occurred from only one of the two ends. Most significantly, unwinding from the upstream end

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Figure 4. Products expected when access of the RecBCD enzyme is restricted to one of the DNA ends. (A) At saturating RecBCD enzyme concentrations (less than one RecBCD enzyme molecule per dsDNA end), RecBCD enzyme initiates unwinding and depauperation from only one of the two ends. Upon recognition of \( \chi \), the \( 5' \rightarrow 3' \) exonuclease activity is attenuated, whereas the \( 3' \rightarrow 5' \) exonuclease is regulated. Therefore, the recognition leads to the production of both top-strand, upstream and bottom-strand, downstream \( \chi \)-specific fragments. (B) At saturating RecBCD enzyme concentrations (more than one RecBCD enzyme molecule per dsDNA end), simultaneous unwinding occurs from both ends of the dsDNA substrate. The \( 5' \rightarrow 3' \) exonuclease activity is synchronous, thus, the meeting of the two RecBCD enzyme molecules in the middle of a DNA substrate leads to production of \( 5' \)-end-labeled DNA that is approximately half of full length. In this case, \( \chi \) is not recognized, as neither RecBCD enzyme encounters \( \chi \) in the correct orientation. (C) At saturating RecBCD enzyme concentrations, when entry at the downstream end is blocked by the presence of a ssDNA tail, RecBCD enzyme unwinding occurs only from the upstream end of the DNA. The products of this reaction are exactly the same as those produced under saturating conditions of the RecBCD enzyme on a ssDNA without a tail. However, the amount of \( \chi \)-specific fragment is doubled, because of the RecBCD enzyme approaching \( \chi \) only from the productive orientation. (D) At saturating RecBCD enzyme concentrations, when both of the available dsDNA ends are blocked by the presence of a ssDNA tail, the RecBCD enzyme is unable to unwind the DNA.
leads to the production of both upstream and down-
stream 3'-end-labeled γ-specific fragments. The forma-
tion of these products excludes the two hypotheses that 
required entry of the RecBCD enzyme from the end of 
the downstream of χ. Therefore, the existence of both bot-
tom-strand upstream and top-strand, downstream γ-spe-
cific fragments when χ is only approached from the up-
stream orientation is attributable to a switch in the po-
larity of nuclease activity at χ.

To further verify that the presence of the bottom-
strand, upstream γ-specific fragment is attributable to an 
activation of nuclease activity at χ, DNA was also 
blocked only at the end upstream of χ and subjected to 
RecBCD enzyme processing. Untwisting of DNA that 
possesses a ssDNA overhang upstream of χ behaves as if 
there were no sites present at all (data not shown). This 
is further proof that χ must be recognized in the standard 
orientation and that γ-specific fragments are not being 
created following unwinding. Finally, time-course analy-
sis of the production of γ-specific fragments reveals that 
both top-strand, downstream and bottom-strand, up-
stream γ-specific fragments are produced at the same 
rate (data not shown), illustrating further that γ-specific 
fragments are a result of neither two sequential process-
ing events nor post-unwinding recognition. Thus, the 
bottom-strand, upstream γ-specific fragments are produced 
by a single RecBCD enzyme recognizing χ in the stan-
dard orientation. The production of this fragment is a 
direct consequence of the previously unknown facet of 
the interaction of RecBCD enzyme and χ, namely that χ acts 
as a regulatory element to switch the polarity of DNA 
degradation by the RecBCD enzyme.

Discussion

The means by which the recombination hot spot χ acts 
to stimulate homologous recombination lies in the abil-
ity of χ to modify the nucleolytic properties of the 
RecBCD enzyme. One of the first biochemical observa-
tions of this process was the discovery that the RecBCD 
protein can produce a γ-specific ssDNA fragment be-
cause of nicking in the vicinity of χ (Poncetelli et al. 
1985; Taylor et al. 1985). Dixon and Kowalczykowski

![Figure 5](image-url)

Figure 5. Blocking access of the RecBCD enzyme to DNA with ssDNA tails defines the orientation dependence of the novel γ-specific 
fragment. Unwinding reactions were carried out under standard conditions using pBR322 χ'γ DNA that was bacteriolyzed by HindIII. 
Half of this DNA was treated with exonuclease T1 (lanes 1-4) to create a ssDNA tail of ~90 nucleotides in length. The tail at the 
upstream end was removed using Adel, and then the DNA substrates were 5'-end labeled. These DNA substrates were then treated 
with either subterminating (one functional RecBCD enzyme per 17 αDNA ends) or saturating amounts (4x functional RecBCD enzyme 
molecules per 1 αDNA end) of enzyme and incubated for 4 min. The products were analyzed by native agarose gel electrophoresis. 
Lanes 1-3 show the products of an unwinding reaction using DNA that has the downstream end blocked by the presence of a ssDNA 
tail. Recognition of χ in the proper orientation leads to the production of both bottom-strand, upstream and top-strand, downstream 
γ-specific fragments at all concentrations of RecBCD enzyme tested (see Fig. 4C for description of this case). Lanes 4-6 show an 
unwinding reaction in which both ends possess ssDNA tails, this DNA shows no unwinding at any RecBCD enzyme concentration, 
demonstrating the ssDNA tail as an effective block to RecBCD enzyme access (see Fig. 4D). Lanes 7-9 show an unwinding reaction 
using DNA that does not possess a ssDNA tail at either end; however, the upstream end was removed using Adel to give DNA that 
is the same length as the singly tailed DNA used in Lanes 1-3. RecBCD enzyme treatment of this DNA leads to both upstream and 
downstream γ-specific fragments at subsaturating RecBCD enzyme concentrations, and half-length ssDNA at saturating RecBCD 
protein (see Fig. 4, A and B, respectively). Lanes 10-12 show an unwinding reaction using DNA that does not possess a ssDNA tail at 
either end. Again, RecBCD enzyme treatment of this DNA leads to the production of both upstream and downstream γ-specific 
fragments at subsaturating RecBCD protein concentration, as saturating the RecBCD enzyme, the major product is half-length 
ssDNA.

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(1993) furthered this characterization by showing that the production of this \( x \)-specific fragment was a consequence of a more general phenomenon: the attenuation of the RecBCD enzyme's 3'→5' exonuclease activity upon interaction with \( x \). However, the effect of \( x \) recognition on the weaker 5'→3' exonuclease activity of the RecBCD protein remained undefined. Here we show that recognition of \( x \) in the standard orientation by a translocating RecBCD molecule not only leads to the production of a bottom-strand, upstream \( x \)-specific fragment, but also the degradation of the bottom-strand, downstream \( x \)-specific region. We demonstrated that this asymmetric production of \( x \)-specific fragments is a direct result of the unexpected up-regulation of the 5'→3' exonuclease activity upon recognition of \( x \) (Fig. 6). This observation demonstrates that \( x \) is a unique regulatory element, which acts to stimulate recombination by controlling the polarity of exonuclease degradation by the RecBCD enzyme.

The switch in the polarity of degradation at \( x \) from 3'→5' to 5'→3' has important biological consequences for the initiation of homologous recombination. Although the processive helicase action of the RecBCD enzyme leads to the complete unwinding of the small, pHBB2-based plasmid used in vitro, the result of this polarity switch on longer DNA substrates (i.e., chromosomal or phase A DNA) would be a change from the production of DNA with 5'→overhanging ssDNA to DNA with 3'→overhanging ssDNA ends. The generation of 3'-ssDNA ends is critical with regard to RecA protein function. As mentioned previously, the RecA protein binds the ssDNA produced by RecBCD enzyme unwinding and then catalyzes the pairing of this DNA to a homologous region in its supercoiled counterpart, forming a recombination intermediate known as a joint molecule (Shibata et al. 1979, McEntee et al. 1980; Romani and Kowalczykowski 1989b; Dixon and Kowalczykowski 1991) (Fig. 6B). The binding of the RecA protein to ssDNA is cooperative and polar in nature, with extension of the RecA protein filament occurring 5'→3' (Register and Griffith 1985). This polarity is reflected in the bias shown in DNA strand invasion: 3' ends are approxi- mately 10-fold more invasive than 5' ends in RecA protein-promoted recombination. This bias is even more exaggerated in the presence of the single-stranded DNA binding (SSB) protein (Koufopulos and Davis 1987, 1990). Thus, the switch in polarity of DNA degra- dation at \( x \) from primarily 5'→3' to 5'→3' changes the RecBCD enzyme from a form that produces a DNA in- termediate that has a poorly recombinogenic 3'→overhanging ssDNA end to a modified form that produces a highly recombinogenic 3'→overhanging ssDNA end. The helicase activity of RecBCD enzyme has long been postulated to produce a 3'-ssDNA end that is used by the RecA protein for DNA strand invasion (see Smith 1988). The discovery that the 5'→3' exonuclease activ- ity is up-regulated upon interaction with \( x \) raises the question of what role this increase in degradation plays in homologous recombination. Perhaps the most reason- able explanation for the existence of this activity is that it removes a possible competitor for binding to the re- combinogenic 3' end of the \( x \)-specific ssDNA. Degradation of the DNA complementary to this 3'-ssDNA end would prevent strand annealing and consequent inactivation of this invasive strand, making the ssDNA more accessible for RecA protein-promoted DNA strand invasion. The hypothesis that initiation of homologous recom- bination from a DNA end requires the formation of a 3'-ssDNA overhang is supported by suppressor analysis of recBCD null mutations. The reduced recombination in these strains can be suppressed completely by the ac- tivation of another nuclease, the RecF protein (Barbour et al. 1970, Tympl et al. 1972). In vitro, the RecF protein is a highly processive dsDNA-specific 5'→3' exonuclease that removes 10,000–30,000 nucleotides from the 5'-terminal strand per binding event (Gillen et al.

![Figure 6](image)

**Figure 6.** Biochemical model for genetic recombination initiated from a dsDNA end. Details are described in the text. Initiation involves the resection of a dsDNA end to produce a 3'-ssDNA overhang. (a) This resection can occur through the ac- tion of the RecBCD enzyme and \( x \), or the RecF protein, or the combined actions of the RecBC and RecD proteins. (b) The 3' end of this ssDNA overhang invades a ho- mologous in a reaction mediated by RecA and SSB proteins to form a D-loop.

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enzyme before \( \chi \) and the \( 5' \rightarrow 3' \) exonuclease after \( \chi \) re-require functional RecBCD protein, suggesting that the RecD protein is directly responsible for some or both of these activities. Another formal possibility is that inactivation of the RecD subunit after recognition of \( \xi \) activates a nucleosome domain in either the RecC or RecD subunits, which is inactive in the RecBC enzyme. Although the biochemical basis of \( \xi \) recognition remains a mystery, it is interesting to note that the RecD protein possesses a region of sequence similarity with RecE and a number of other \( 5' \rightarrow 3' \) specific DNA endonucleases (S.T. Lovett, pers. comm.).

The finding here that processing of a dsDNA break by the RecBCD enzyme and \( \xi \) leads to the production of a \( 3' \rightarrow 5' \) dsDNA overhang is significant in that it brings all the pathways of recombination in E. coli into conformity. These findings support a universal model for homologous recombination that involves initiation from a \( 5' \rightarrow 3' \) dsDNA overhang and that also pertains to replicative repair processes (Kowalczykowski et al. 1994, Kogoma 1998) (Fig. 6). In this model initiation occurs through resection of a dsDNA end to a \( 3' \)-overhang, which can then be used by the RecA protein to catalyze D-loop formation as described previously.

The idea that initiation of homologous recombination involves a \( 3' \rightarrow 5' \) dsDNA overhang is neither new (Resnick 1976, Szostak et al. 1983) nor unique to E. coli. Characterization of the processing of dsDNA breaks in the yeast S. cerevisiae has revealed that both IRO endonuclease-induced breaks (White and Haber 1990) and the ARG4 initiation site for meiotic gene conversion (Kim et al. 1991), the first processing event that occurs in the asymmetric resection of the DNA producing extensive, dsDNA tails with \( 3' \) overhangs. We have shown that the RecBCD enzyme-dependent processing of dsDNA breaks, mediated by \( \xi \), leads to the same end result, but focused at \( \chi \). Thus, the processing of dsDNA breaks to form DNA ends with \( 3' \rightarrow 5' \) overhangs may represent a universal aspect of the initiation of homologous recombination and recombinational DNA repair.

Materials and methods

Enzyme

The RecBCD enzyme was purified as described (Raman and Kowalczykowski 1989a). Protein concentration was determined using an extinction coefficient of 4.0 \( \times 10^9 \) M \(^{-1}\) cm \(^{-1}\) at 280 nm (Raman and Kowalczykowski 1989b). The specific activity of the enzyme preparation was 5.4 \( \times 10^6 \) units of nuclelease or 1.1 \( \times 10^6 \) units of helicase per milligram of protein. Nuclelease units and helicase units were defined as described (Kisitok and Lehmann 1977). Raman and Kowalczykowski 1989a, respectively). 538 protein was isolated from RIDM727 and purified according to Lehmann (1985). Protein concentration was determined using an extinction coefficient of 3.0 \( \times 10^9 \) M \(^{-1}\) cm \(^{-1}\) at 280 nm (Roysch and Wernet 1977). All restriction enzymes and DNA-modifying enzymes were purchased from New En-

1977, Joseph and Kolodner 1983b, for review, see Kowalczykowski et al. 1994). Thus, the behavior of the RecA protein is very similar to the behavior of the RecBCD enzyme after it has interacted with a \( \chi \) site. They both lead to the production of DNA with \( 3' \rightarrow 5' \) ssDNA tails (Fig. 6). The need to form a \( 3' \rightarrow 5' \) ssDNA overhang is also supported by examination of homologous recombination in recD null mutants. Cells that have functional recF and recG but nonfunctional recD have approximately normal levels of recombination, but they show a genetic dependence on another nuclease, the RecF protein (Lovett et al. 1988). In vivo, the RecBC protein (i.e., without the RecD subunit) is an active helicase with little or no measurable nuclease activity (Palas and Kispert 1992; Koronpe and Jolin 1993, Andersen et al. 1997). The RecF protein is a single-strand specific exonuclease with a polarity that is \( 5' \rightarrow 3' \). It is likely then that in the absence of RecD subunit, the RecBC protein initiates unwinding at a dsDNA end and the RecF protein exerts the assistent \( 5' \)-terminal ssDNA (Fig. 6) degradation by the RecF protein of ssDNA that was formed by the RecBC protein was demonstrated in vivo (Kishimoto et al. 1992). Again, the consequence of this separated helicase/nuclease action is formation of \( 3' \)-overhanging ssDNA that could then be used by the RecA protein to promote DNA strand inva-

As described above, processing by the RecBCD en-
zyme places \( \xi \) at the \( 3' \) end of the ssDNA tail. Analysis of DNA strand invasion reactions catalyzed by RecA and RecBCD proteins reveals that the formation of joint mol-
ecules using the \( \xi \)-containing \( 5' \rightarrow 5' \) ends is favored over the \( 3' \) containing \( 3' \rightarrow 5' \) ssDNA (Dixon and Kowal-
czykowski 1991, 1995). Recent examination of this pro-
cess has revealed that the \( \xi \) sequence not only can act to directly stimulate RecA protein-promoted pairing (Tracy and Kowalczykowski 1996) but also to stimulate the leading of the RecA protein onto the \( \xi \)-containing ssDNA strand in a RecBCD enzyme-dependent reaction (D.G. Anderson and S.C. Kowalczykowski, in prep.). Thus, the ability of the RecBCD protein to translocate a \( 3' \rightarrow 5' \) ssDNA overhang further enhances the efficiency of joint molecule formation.

Although the specific modification of the RecBCD en-
yzme affected by \( \chi \) recognition remains unclear, evi-
dence suggests that interaction of the RecBCD protein with \( \chi \) leads to the reversible inactivation/ejection of the RecD subunit (Thibert et al. 1988; Stahl et al. 1990, Dixon et al. 1994; Koppen et al. 1995; Myers et al. 1995). As mentioned previously, the RecBC protein is a helicase but has little or no nucleolytic activity. The fact that the RecBCD enzyme after interaction with \( \chi \) is not only a helicase, but also a \( 5' \rightarrow 3' \) exonuclease now raises the question of whether the RecD subunit is actually ejected at \( \chi \). Both the \( 5' \rightarrow 3' \) exonuclease activity of RecBCD
DNA substrates
Phospho-pBR322 χ (wild type), pBR22 xBa, and pBR322 xBe (Sinaitik et al. 1989) were purified using alkaline lysis as described in Sambrook et al. (1989). The nucleotides were labeled using the appropriate restriction enzyme and 32P-end-labeled at either the 5' end using the Klenow fragment of DNA polymerase I and appropriate [γ-32P]ATP (New England Nuclear) or by alkaline phosphatase and γ32P-polyribonucleotide (in the presence of γ32P[ATP] (NEI Labs)).

Reaction conditions
Standard reaction conditions consisted of 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5% (v/v) of a 10 mM solution of either 100 mM NaCl or 100 mM KCl, 0.1 M NaCl, 0.01 M Tris-Cl buffer (pH 7.5), and 1 mM ATP. In some tests, RecBCD enzyme was varied as indicated. Assays were performed at 37°C and were initiated with the addition of enzyme after preincubulation of all other components for 3 min.

Analysis of reaction products
After a 4-min incubation with the RecBCD enzyme, each reaction (48 µl) was stopped by the addition of 10 µl of stop buffer (0.2 µl EDTA, 2.5% SDS, 4% glycerol, 0.125% bromophenol blue, 0.125% xylene cyanol). Samples were subjected to electrophoresis through a 1% agarose gel for 15 h at 1.4 V/cm in TAE (40 mM Tris-acetate at pH 8.0, 1 mM EDTA). The gels were dried and exposed to Kodak XAR-5 film at room temperature. Samples (40 µl) were analyzed by densitometry using a laser densitometer (Bio-Rad Laboratories, Inc.). The media were used to determine the percentage of DNA cleaved.

Degradation of ssDNA substrates using Exo III
Plasmid pBR322 χ DNA (10 µg), which was labeled with [32P]γ-ATP, was incubated with 175 units of Exo III using reaction conditions according to Piggot (65 mM Tris·HCl at pH 8.0, 0.1 M NaCl, 10 mM MgCl2) for 15 min at 37°C. The reactions were stopped by the addition of 20 mM EDTA (pH 8.0) and 500 µM NaCl.heat treated at 75°C for 15 min and phenol/chloroform extracted as described in New England Nuclear (1989). Reaction conditions were performed by adding Exo III to the DNA after the addition of stop buffer. The DNA was then precipitated by the addition of 2 volumes of 100% ethanol, washed twice with 70% ethanol, and resuspended in 100 µl of TE buffer. The labeled DNA and the unlabeled DNA were electrophoresed with 32P end labeled to remove the ssDNA tail upstream from the 3' site. or incubated in suspension buffer without Asel. This DNA was then 5' end labeled as described previously, treated with various RecBCD enzymes concentrations at various 0.0, 0.57, 0.6, or 37 mM [γ-32P]ATP (NEI Labs). 1 functional RecBCD enzyme per 17 DNA ends or 6 functional RecBCD enzyme per DNA and for the DNA substrate that were not cut with Asel under optimal conditions and analyzed as described previously.

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References

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