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The Recombination Hotspot χ Is a Regulatory Sequence That Acts by Attenuating the Nuclease Activity of the E. coli RecBCD Enzyme

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Summary

The RecBCD enzyme is a multifunctional enzyme that is essential for homologous recombination in E. coli. In vitro, the RecBCD enzyme degrades linear double-stranded DNA nonspecifically during the process of unwinding the double-stranded DNA. Here we demonstrate that this DNA degradation is asymmetric, with the strand that is 3′ terminal at the entry site of RecBCD enzyme being degraded much more vigorously than the 5′ terminal strand. Furthermore, interaction with the recombination hotspot χ causes an attenuation of the nuclease activity but not of the helicase activity and is accompanied by a pause of RecBCD enzyme at the χ site. These results demonstrate that χ is a unique regulatory element that acts by controlling the degradative function of RecBCD enzyme and, thereby, enhancing its recombination function.

Introduction

The RecBCD enzyme is an essential component of the main pathway of homologous recombination in Escherichia coli, the RecBCD pathway of generalized recombination (for a review see Smith, 1988). Homologous recombination in wild-type E. coli requires functional *recB* and *recC* genes (Howard-Flanders and Theriot, 1966; Emmerson and Howard-Flanders, 1967).

The multifunctional RecBCD enzyme is composed of three nonidentical subunits, the RecB, RecC, and RecD polypeptides (for reviews see Telander-Muskavitch and Linn, 1981; Taylor, 1988; Smith, 1990). In vitro, RecBCD enzyme degrades both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) by means of its ATP-dependent ssDNA and dsDNA exonuclease activities and its ATP-stimulated ssDNA endonuclease activity. In addition to these degradative activities, the enzyme is a DNA helicase that can processively unwind large tracts of dsDNA (>30 kb) at rates of up to 1000 bp/s (Roman and Kowalczykowski, 1989; Roman et al., 1992). It is presumed that the ATP-dependent degradation of dsDNA is coincident with the unwinding of dsDNA and occurs as a result of the endonucleolytic cleavage of the unwound ssDNA (MacKay and Linn, 1976; Telander-Muskavitch and Linn, 1981).

In addition to these nonspecific nuclease activities, the RecBCD enzyme demonstrates site-specific nicking at χ sites (Ponticelli et al., 1985). χ sites are DNA sequence elements that stimulate RecBCD enzyme–dependent recombination in their vicinity (Lam et al., 1974; Stahl et al., 1974; Dower and Stahl, 1981; for a review see Smith, 1988). Genetic and physical analysis has determined that these recombination hotspots are composed of the sequence 5′-GCTGGTGG-3′ (Smith et al., 1981a). Stimulation of recombination by χ occurs primarily to the 5′ side of the χ site and requires a functional RecBCD enzyme. In vitro, recognition of χ by RecBCD enzyme results in a site-specific nick in the DNA strand containing the χ sequence 4–6 nt to the 3′ side of the χ sequence. This specific nucleolytic event occurs during the unwinding of dsDNA and is orientation dependent; RecBCD enzyme must approach the χ site from the 3′ side for the specific nick to occur (Taylor et al., 1985).

A model incorporating most of the known biochemical and genetic properties of the components participating in the RecBCD pathway of recombination proposes that the interaction between RecBCD enzyme and χ acts to initiate the DNA strand exchange process (Smith et al., 1981b, 1984). The RecBCD enzyme is proposed to initiate RecA protein–dependent DNA strand exchange by virtue of its site-specific nicking of the DNA at χ; subsequent DNA unwinding by the enzyme allows for the production of ssDNA, which is then used by RecA protein to promote homologous pairing and DNA strand exchange. However, the existence of the potent nonspecific nuclease activity of RecBCD enzyme remained paradoxical, and its function in recombination could only be viewed as antirecombinogenic (Thaler et al., 1988, 1989).

Using a reconstituted in vitro recombination reaction, we demonstrated the formation of homologously paired joint molecules that is dependent on the presence of RecA, RecBCD, and E. coli ssDNA-binding (SSB) proteins, is stimulated by the interaction between RecBCD enzyme and χ, and shows the expected polarity of χ site activation (Dixon and Kowalczykowski, 1991). We concluded, though we had not determined directly, not only that the degradation of dsDNA by the RecBCD enzyme is asymmetric, but also, and most significantly, that the interaction with the χ site causes an attenuation of the DNA degradative activity of the enzyme but not of its unwinding activity. These conclusions suggest that the function of χ in the RecBCD pathway of recombination is the regulation of the nucleolytic action of RecBCD enzyme and, consequently, that recombination hotspot activity is not a result of enzymatic activation but rather of enzymatic inactivation.

In this paper we demonstrate directly that, during the process of DNA unwinding, RecBCD enzyme degrades dsDNA asymmetrically, with the DNA strand that is 3′ terminal at the entry site being degraded at a greater frequency than the 5′ terminal strand. Furthermore, we show that, upon encountering χ in the proper orientation, this nucleolytic activity of RecBCD enzyme is attenuated. We propose that the specific protein–DNA interaction between RecBCD enzyme and χ causes the enzyme to

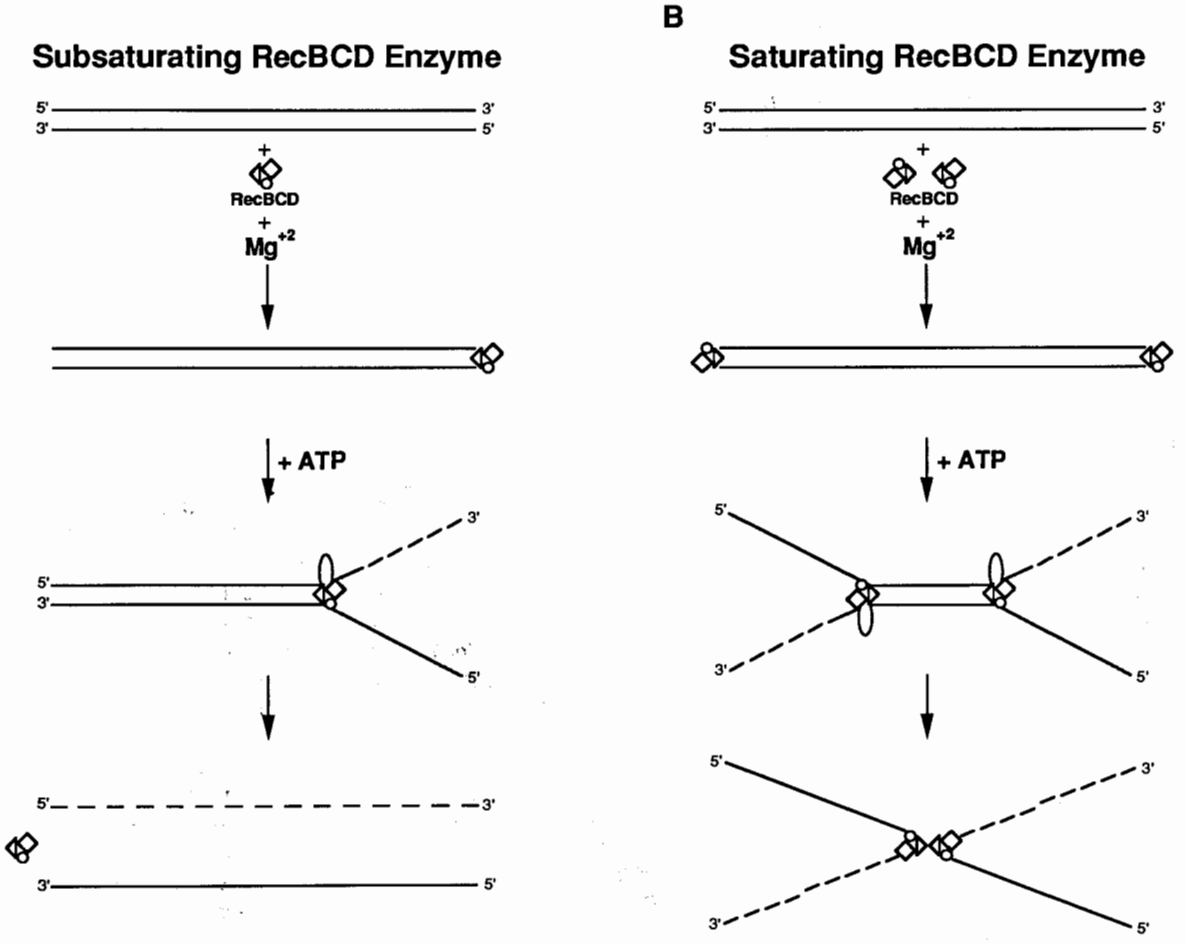

Figure 1. Experimental Model Used to Examine Asymmetric DNA Degradation by RecBCD Enzyme

Details are discussed in the text. RecBCD enzyme is represented by the diamond–triangle–circle. To achieve synchronized unwinding of DNA, RecBCD enzyme is prebound to the ends of the dsDNA in the presence of Mg^{2+}, and the reactions are initiated by the addition of ATP. The unwound ssDNA produced is bound by the SSB protein (not shown).

(A) At subsaturating RecBCD enzyme concentrations (<1 RecBCD enzyme molecule per dsDNA end) two types of ssDNA products are envisioned, owing to unwinding and degradation of linear dsDNA. Owing to frequent cutting by RecBCD enzyme as the dsDNA unwinds, the production of oligomeric ssDNA fragments is depicted as arising from the DNA strand that is 3′ terminal at the entry site, whereas the full-length ssDNA originates from the DNA strand that is 5′ terminal at the entry site, which is left largely intact.

(B) At saturating RecBCD enzyme concentrations (≥1 RecBCD enzyme molecule per dsDNA end), the expected sizes of the ssDNA fragments change. The production of oligomeric sized fragments derived from the DNA strand 3′ terminal at the entry site remains. However, owing to unwinding and degradation from both ends, the DNA strand that is 5′ terminal at the entry site is now only half length; note that no half-length ssDNA fragments are derived from the DNA strand that is 3′ at the entry site.

pause at the χ site. This pause not only reflects the specific recognition event but also may represent the time required for the alteration of the enzymatic functional state and, simultaneously, ensures that a nick occurs at the χ site. Thus, the χ sequence is an unusual regulatory element or allosteric effector that alters the recombinational properties of the RecBCD enzyme.

Results

Experimental Design

To test the hypothesis that χ acts by regulating the activities of RecBCD enzyme and to determine which aspect of dsDNA unwinding and degradation is controlled by χ, it was necessary first to test the proposal that RecBCD enzyme degrades dsDNA asymmetrically (Dixon and Kowalczykowski, 1991). To ascertain whether, in fact, the DNA strand that is 3′ terminal at the entry site is degraded more vigorously than the strand that is 5′ terminal at the entry site, the following experimental model was used to examine the products of enzyme-mediated dsDNA unwinding and exonucleolytic action (Figure 1). RecBCD enzyme, when incubated with dsDNA in the presence of Mg^{2+}, binds to dsDNA ends with high affinity ($K_D \approx 0.5$ nM; Taylor and Smith, 1985; Ganesan and Smith, 1993). Upon addition of the cofactor, ATP, the enzyme can initiate the relatively synchronous unwinding and degradation of the dsDNA (see below). Owing to the high processivity of DNA unwind-

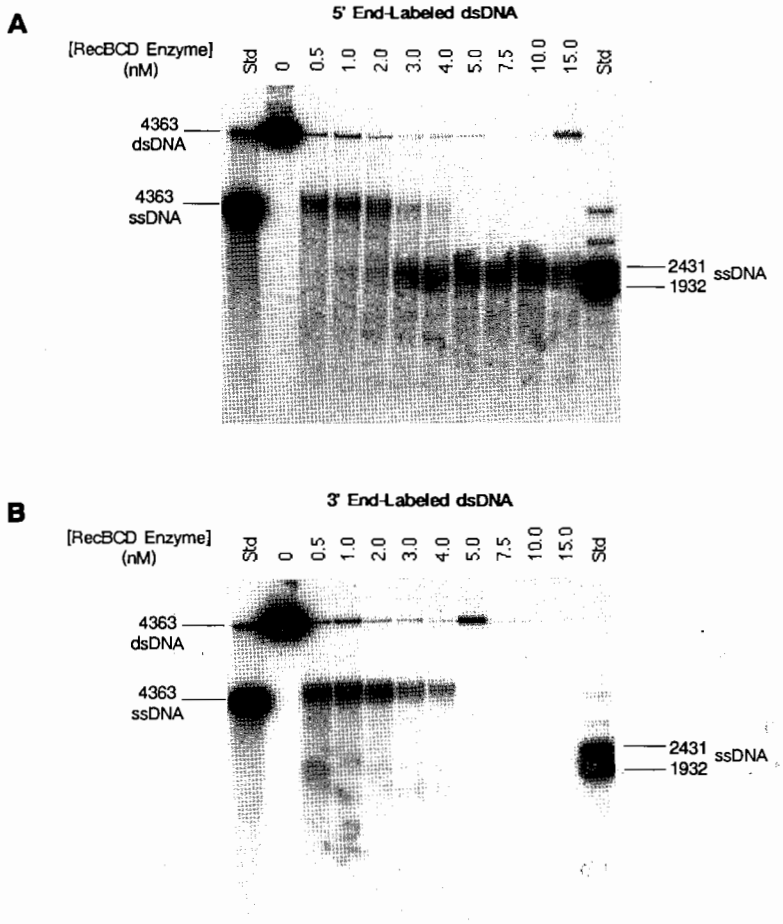

Figure 2. The Unwinding and Degradation of dsDNA at Various Concentrations of RecBCD Enzyme

Unwinding reactions were carried out under standard conditions using pBR322 dsDNA (4363 bp) linearized with the restriction enzyme EcoRI and radioactively labeled at either the 5′ (A) or 3′ (B) end. The concentration of RecBCD enzyme (nM RecBCD enzyme) used in each reaction is indicated, and the length of each unwinding reaction after the addition of ATP is 0–0.5 nM RecBCD enzyme, 4 min; 1–4 nM RecBCD enzyme, 2 min; and 5–15 nM RecBCD enzyme, 1 min. The reactions were stopped and analyzed on a 1.2% agarose gel that was dried and visualized by autoradiography. The main ssDNA species produced at subsaturating concentrations (<3 nM RecBCD enzyme) has a size similar to that of a full-length strand ssDNA standard (Std) of 4363 nt (4363 ssDNA [A] and [B]), whereas at saturating RecBCD enzyme concentrations (>3 nM RecBCD enzyme) the size of the major ssDNA fragment produced is approximately one-half the length of a DNA strand as compared with the ssDNA standards of 2431 and 1932 nt (2431 and 1932 ssDNA [A]). The cause of the resulting ssDNA band doublets is presumed to be dissimilar conformations of the two DNA strands resulting in different mobility under these electrophoresis conditions. The initial amount of linear dsDNA used in each reaction is indicated in the 0 nM RecBCD enzyme reaction (4363 dsDNA).

ing by RecBCD enzyme (>30 kb, Roman et al., 1992), if an unwinding reaction is performed at subsaturating RecBCD enzyme concentrations with respect to dsDNA ends (i.e., <1 functional RecBCD enzyme molecule per dsDNA end), then each DNA molecule is fully unwound from only one end. The ssDNA products of such a reaction should consist of a full-length ssDNA strand containing both the 5′ and the 3′ ends (derived from the strand 5′ terminal at the entry site), along with oligomeric sized fragments that contain the 5′ and 3′ ends derived from the degraded DNA strand (derived from the strand 3′ terminal at the entry site) (Figure 1A); under these subsaturating enzyme reaction conditions, similar results will be obtained using dsDNA that is radioactively labeled at either the 5′ or 3′ end. However, if the unwinding of dsDNA is conducted at saturating RecBCD enzyme concentrations (i.e., ≥1 functional RecBCD enzyme molecule per dsDNA end), then an unwinding reaction using 5′ end–labeled dsDNA will result in an ssDNA fragment that is one-half as long as the original dsDNA substrate and contains the 5′ end label, provided that DNA strand degradation displays the bias illustrated in Figure 1. If, instead, 3′ end–labeled linear dsDNA is unwound from both ends, then the radioactive label should be found only in oligomeric ssDNA fragments, and no half-length ssDNA will be observed. These distinctive patterns of 5′ or 3′ terminal ssDNA fragment production would result if the unwound DNA strands are degraded with the bias displayed; alternatively, if the bias is opposite to that shown in Figure 1, then the opposite labeling pattern would be observed.

RecBCD Enzyme Degrades dsDNA Asymmetrically

Wild-type plasmid pBR322 is devoid of χ sequences (χ°; Smith et al., 1981a); consequently, linear pBR322 was used to examine DNA strand degradation by RecBCD enzyme in the absence of any influence by χ sites. Figure 2A shows the products of an unwinding reaction as a function of RecBCD enzyme concentration, using linear dsDNA that is radioactively labeled at the 5′ end. When limiting amounts of RecBCD enzyme (relative to dsDNA ends) are used (<3 nM RecBCD enzyme), the prominent ssDNA species is the size of a full-length molecule (4363 nt). A transition in the major ssDNA species produced occurs at approximately 3–4 nM RecBCD enzyme, or approximately 1.3–1.7 RecBCD enzyme molecules per dsDNA end. Beyond this concentration, all the dsDNA ends are bound by enzyme molecules, and the main ssDNA species produced is a fragment approximately one-half as long as the linear ssDNA (~2200 nt), as predicted in Figure 1B. The prominence of this half-length ssDNA fragment and its

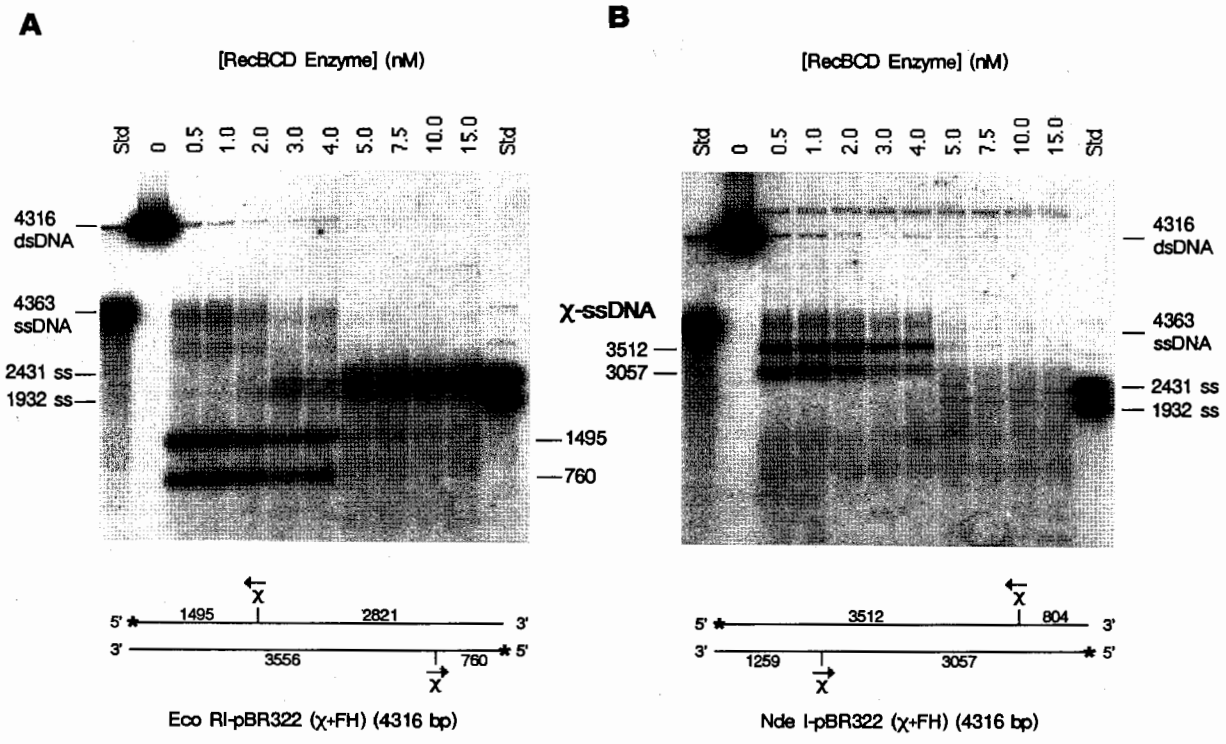

Figure 3. The Unwinding and Degradation of 5′ End–Labeled χ^+ dsDNA at Various Concentrations of RecBCD Enzyme

The linear χ-containing dsDNA substrates were created by cutting the plasmid pBR322 χ^+FH (4316 bp), which contains two χ sites, with the restriction enzyme EcoRI (A) or NdeI (B). The arrow above the χ site indicates the direction that RecBCD enzyme must approach for recognition of the χ, and the location of each χ site from the dsDNA end is given in nucleotides. The DNAs were labeled at the 5′ ends with ^{32}P; reactions were performed and analyzed as described in Figure 2. The full- and half-length ssDNA fragments produced by RecBCD enzyme are seen as ssDNA bands with mobilities near the ssDNA standards of 4363 or 2431 and 1932 nt, respectively (labeled 4363 ssDNA; 2431 and 1932 ss). ssDNA fragments resulting from site-specific cleavage events at the χ sites (χ-ssDNA) have the indicated sizes of 1495 or 760 nt (A) and 3512 or 3057 nt (B). The size of the ssDNA fragments that make up the heterogeneous smear when saturating amounts of RecBCD enzyme (>4 nM RecBCD enzyme) are used to unwind NdeI-digested pBR322 χ^+FH ranges from 3000 to 800 nt (B). Eco RI-pBR322 (χ+FH), EcoRI-digested pBR322 χ^+FH; Nde I-pBR322 (χ+FH), NdeI-digested pBR322 χ^+FH.

narrow size distribution demonstrate that the enzymes must be translocating through the DNA from each end in a relatively synchronous manner.

The products of an unwinding reaction in which the linear dsDNA substrate was labeled at the 3′ ends are shown in Figure 2B. At limiting RecBCD enzyme concentrations, the main ssDNA species produced has the size of a full-length molecule (4363 nt). These results are similar to those obtained using dsDNA labeled at the 5′ ends (Figure 2A). However, in contrast with the results obtained in Figure 2A, when saturating enzyme concentrations are used, a half-length ssDNA fragment containing a 3′ end label is not detected (Figure 2B); in fact, no labeled DNA fragments are detected by agarose gel electrophoresis. In agreement, polyacrylamide gel electrophoresis detects ssDNA fragments containing the 3′ DNA end that range in length from 5 to 100 nt (data not shown); 90% of these 3′ end–labeled DNA fragments are soluble in 5% trichloroacetic acid (data not shown), suggesting that they are smaller than 16 nt in length (Cleaver and Boyer, 1972).

These results clearly demonstrate that RecBCD enzyme degrades DNA asymmetrically during the course of dsDNA unwinding, and that the 3′ DNA end is degraded much more aggressively than the 5′ DNA end, consistent with the proposal illustrated in Figure 1.

Degradation of DNA Is Regulated by Interaction with χ Sites

To investigate the effect of χ sites on the DNA unwinding and nucleolytic activities of RecBCD enzyme, experiments identical to those presented in Figure 2 were conducted using linear dsDNA substrates containing χ sites. The plasmid pBR322 χ^+FH contains two χ sites that are positioned so that when the plasmid DNA is linearized with the restriction enzyme EcoRI or NdeI, the resultant linear DNA molecules contain χ sites near each dsDNA end but with the χ sequences in opposite orientations (Figures 3A and 3B, bottom).

Figure 3A shows the ssDNA products produced by the unwinding of 5′ end–labeled EcoRI-digested pBR322 χ^+FH (i.e., the linear dsDNA substrate with χ sequences oriented outward) under subsaturating and saturating RecBCD enzyme concentrations. At limiting RecBCD enzyme concentrations (<4 nM RecBCD enzyme), the enzyme will bind to either the right or left DNA end to initiate unwinding of the DNA. To encounter a properly oriented

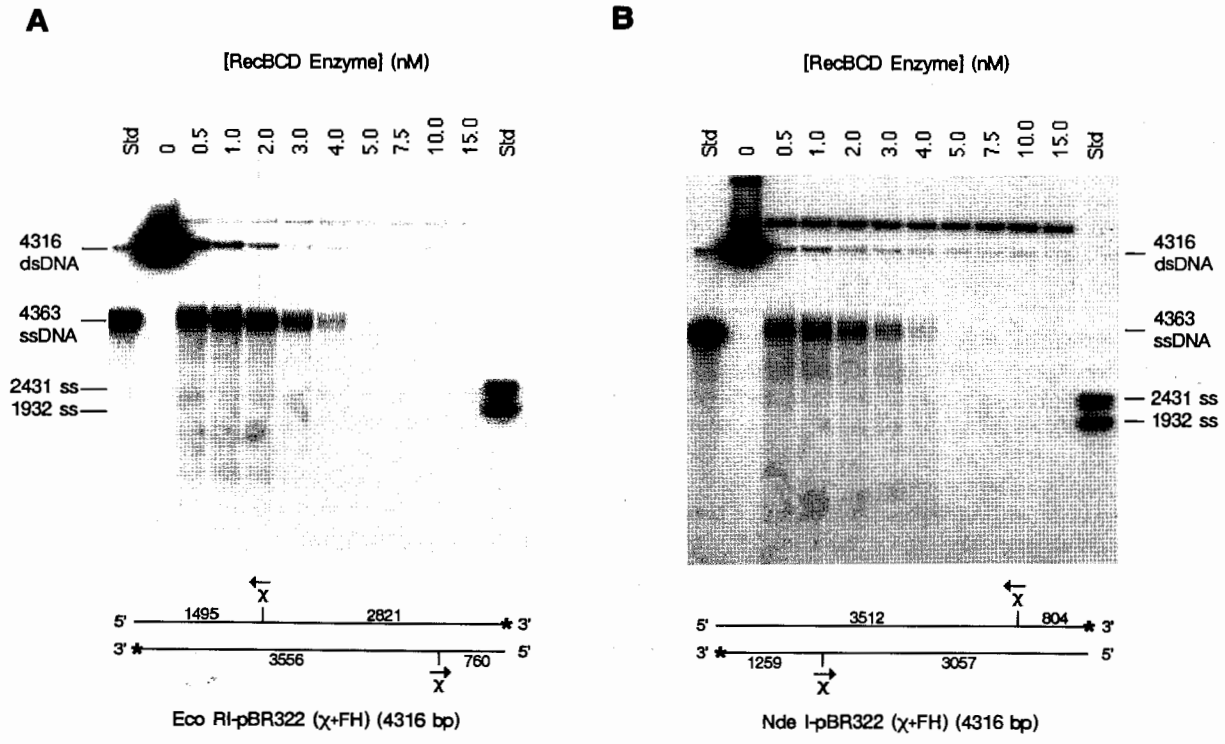

Figure 4. The Unwinding and Degradation of 3′ End–Labeled χ^+ dsDNA at Various Concentrations of RecBCD Enzyme

The linear dsDNA χ-containing substrates shown (A and B) were labeled at the 3′ ends with ^{32}P. Reactions were performed and analyzed as described in Figures 2 and 3. Eco RI-pBR322 (χ+FH), EcoRI-digested pBR322 χ^+FH; Nde I-pBR322 (χ+FH), NdeI-digested pBR322 χ^+FH.

χ sequence, the enzyme must bypass the proximal incorrectly oriented site and translocate to the distal properly oriented site. Upon encountering this properly oriented χ site, the RecBCD enzyme nicks the DNA at the χ site; continued unwinding of the DNA results in the production of a downstream χ–dependent ssDNA fragment (Ponticelli et al., 1985; Taylor et al., 1985; Dixon and Kowalczykowski, 1991). Figure 3A demonstrates the appearance of two distinct ssDNA fragments whose sizes are near the calculated lengths of the downstream ssDNA fragments produced from cutting at the properly oriented χ sites (1495 and 760 nt) in the linear dsDNA substrate EcoRI-digested pBR322 χ^+FH. When the RecBCD enzyme concentration exceeds the concentration of dsDNA ends (>4 nM RecBCD enzyme), the predominant ssDNA species produced is one-half the length of the original dsDNA substrate (~2200 nt), and no χ-specific fragments are formed. These results, which agree with those seen in Figure 2A, demonstrate that at saturating protein concentrations the RecBCD enzyme molecules unwinding from each end of the dsDNA meet in the center of the DNA molecule; consequently, half-length ssDNA fragments containing the 5′ terminal DNA end are produced, since the χ sites at each end have been unwound before an enzyme encounters them in the correct orientation, and, thus, no effect of χ is observed.

When the linear NdeI-digested pBR322 χ^+FH dsDNA substrate (i.e., linear dsDNA with χ sequences oriented inward) is unwound using subsaturating concentrations of RecBCD enzyme, ssDNA fragments are produced whose sizes coincide with those derived from the downstream sides of the χ sites (3512 and 3057 nt; Figure 3B). Upon increasing the RecBCD enzyme concentration to a point where the linear dsDNA substrate is unwound from both ends, discretely sized χ-specific ssDNA fragments are no longer created; instead, ssDNA fragments ranging in length from approximately 3000 to 800 nt are produced. These results contrast with those seen in Figure 3A, where saturating amounts of RecBCD enzyme produce a distinct half-length ssDNA fragment. The production of ssDNA fragments with a size of greater than half length (~2200 nt) at saturating enzyme concentrations demonstrates the χ-dependent attenuation of the nuclease activity of the RecBCD enzyme, yet the production of fragments with sizes of less than half length at subsaturating enzyme concentrations implies that the attenuation of the nuclease activity is not absolute. Furthermore, these findings suggest that the interaction with χ disrupts the synchronized translocation of each RecBCD enzyme; this follows from the observation that the point in the DNA molecule where the two opposing, and now dephased, RecBCD enzymes meet and introduce an endonucleolytic cleavage event is random, thus producing ssDNA fragments whose sizes are both greater than and less than half length.

The yield of χ-specific ssDNA fragment generated at subsaturating levels of RecBCD enzyme (from 0.5 to 2.0

nM RecBCD enzyme; Figure 3A) is 12% ± 2% for both the 1495 and 760 nt fragments of the input dsDNA substrate EcoRI-digested pBR322 χ^+FH and 12% ± 1% for the 3512 nt fragment and 13% ± 3% for the 3057 nt fragment of NdeI-digested pBR322 χ^+FH dsDNA (Figure 3B). Similar amounts of χ-specific fragments are detected when longer dsDNA substrates that produce χ-dependent ssDNA fragments of approximately 9600 nt in length are used, demonstrating the attenuation of strand degradation activity of RecBCD enzyme after an encounter with χ for up to 10 kb downstream of χ (D. A. D. and S. C. K., unpublished data). Under these conditions the ssDNA upstream of χ is primarily degraded to fragments smaller than 20 nt; therefore, we estimate that χ-dependent attenuation must be greater than 500-fold. Since each DNA molecule is unwound from only one end under these conditions, the maximum probability of cutting at a given χ sequence is only 50%; consequently, the observed cutting frequency implies that, under these conditions, cleavage occurs for 25% of the encounters with the χ sequence. These results are consistent with a previous estimate of the maximal χ-dependent cleavage frequency as 40% of the RecBCD enzyme–χ encounters (Taylor and Smith, 1992).

Unwinding reactions using 3′ end–labeled χ-containing dsDNA are shown in Figure 4. For both of the DNA substrates, the main ssDNA species produced at subsaturating RecBCD enzyme concentrations corresponds to full-length ssDNA; χ-specific fragments are not observed. As suggested previously, this full-length ssDNA derives from the ssDNA strand that is 5′ terminal at the RecBCD enzyme entry site. At saturating concentrations of RecBCD enzyme, no half-length ssDNA molecules are detected; instead, under these conditions, degradation of the 3′ terminal strand occurs at both ends of the DNA, resulting in the complete loss of 3′ end–labeled ssDNA (Figure 4). The results shown in Figures 3 and 4 substantiate the asymmetric nature of DNA degradation by RecBCD enzyme as depicted in Figure 1 and demonstrate that the vigorous degradation of the 3′ terminal DNA strand is dramatically attenuated upon encountering a χ site.

Discussion

The specific nature of the interaction between the RecBCD enzyme and the recombination hotspot χ that results in an increased frequency of crossing over is incompletely understood. In addition to the discovery by Smith and colleagues that RecBCD enzyme introduces a specific nick in the vicinity of χ (Ponticelli et al., 1985; Taylor et al., 1985), we have uncovered a feature of the RecBCD enzyme–χ interaction. We previously concluded (Dixon and Kowalczykowski, 1991) and, here, directly demonstrate that the nonspecific nuclease activity of RecBCD enzyme is attenuated upon interaction with the χ sequence. In addition, we have shown that the dsDNA nuclease activity of RecBCD enzyme is asymmetric, with the DNA strand that is 3′ terminal at the DNA entry site being degraded much more vigorously than the strand that is 5′ terminal at the entry site. Our results conclusively demonstrate that the 3′ terminal strand degradation is attenuated at least 500-fold upon interaction with χ but do not permit a firm conclusion regarding attenuation of 5′ terminal strand degradation.

Our observations and conclusions are consistent with both in vivo and in vitro results. Genetic studies established the orientation dependence of χ activity and demonstrated that the directionality of χ stimulation of recombination is almost exclusively downstream of χ (Faulds et al., 1979; Kobayashi et al., 1982; Cheng and Smith, 1989). In vitro reconstitution studies directly demonstrated the formation of χ-specific homologously paired joint molecules whose formation required the presence of RecA, RecBCD, and SSB proteins and a properly oriented χ sequence (Dixon and Kowalczykowski, 1991); those studies strongly argued for the attenuation of nuclease activity at χ that is directly demonstrated here. The results presented in Figures 3 and 4 demonstrate the production of χ-dependent ssDNA fragments with lengths corresponding to the region downstream of the χ site, yet the upstream ssDNA fragment is completely degraded. These observations are most simply explained by the proposal that χ is a unique regulatory switch that regulates the nuclease activity of RecBCD enzyme.

Our results have also uncovered an additional unanticipated feature of the RecBCD enzyme–χ interaction. All of our data demonstrate that, in the absence of any interaction with χ, the translocation of RecBCD enzyme molecules from each DNA end is remarkably well phased. This is manifest at saturating RecBCD enzyme concentrations (relative to dsDNA ends) by the formation of nearly precise half-length ssDNA molecules, owing to the nearly exact meeting of opposing and synchronously translocating RecBCD enzymes at the center of the DNA molecule. However, this precise phasing is changed when a χ site is present near an entry site (see Figure 3B). Instead, ssDNA products ranging in length from 3000 to 800 nt are formed. Thus, interaction with χ must randomize the translocational phasing of the two RecBCD enzyme molecules. One possible explanation for this phenomenon is that, upon interaction with χ, each RecBCD enzyme pauses at the χ sequence, with exit from the χ sequence being governed by a first order dissociation event. The consequence of this pause would be that RecBCD enzyme molecules are released randomly from χ to continue unwinding, resulting in a complete loss of phased translocation. Under these conditions, RecBCD enzyme translocates at about 1000 bp/s (Roman and Kowalczykowski, 1989); thus, a differential pause of just 1 s would result in a meeting point for the two RecBCD enzymes that differs by 1000 bp.

The potential for the existence of a pause at a χ sequence may provide a molecular explanation for the well-documented nick introduced 4–6 nt upstream of the χ sequence (Taylor et al., 1985). The accepted view of the dsDNA exonuclease activity of RecBCD enzyme is that cleavage occurs randomly on the ssDNA that is produced coincidental with unwinding and that the cleavage frequency is independent of the rate of translocation: when the rate of translocation is high (e.g., at elevated ATP concentrations), long (~1 kb) ssDNA fragments are produced; when the rate of translocation is low (e.g., at low ATP concentrations), shorter (10–100 nt) oligonucleotides

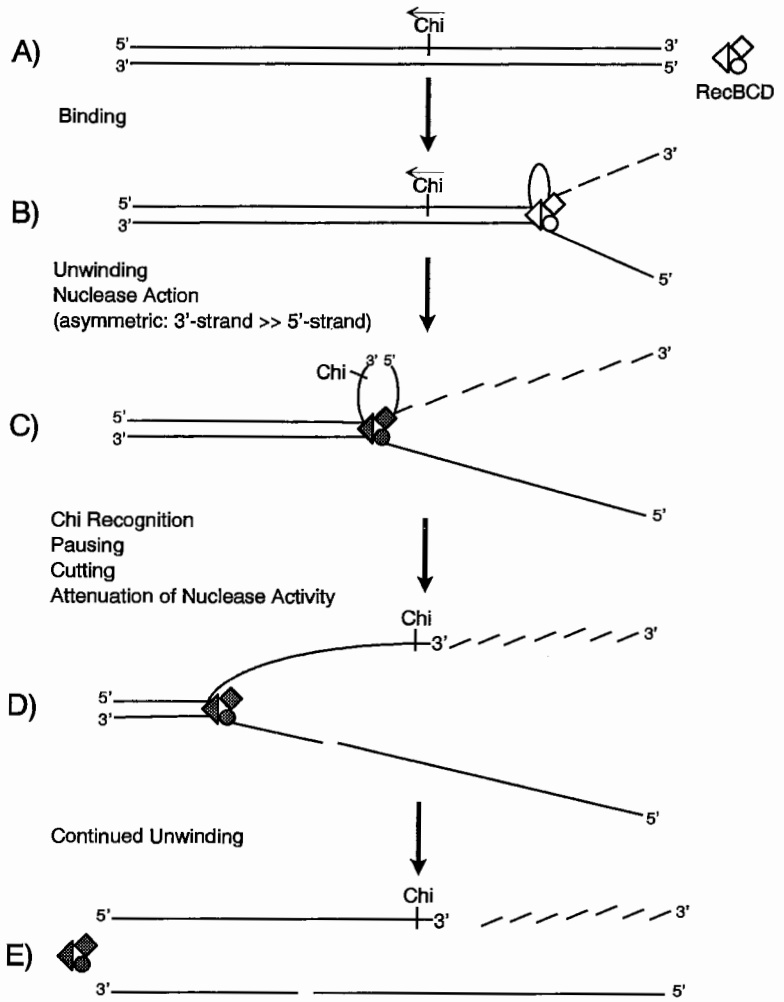

Figure 5. Model for the Unwinding and Degradation of dsDNA by RecBCD Enzyme and the Effects of Its Interaction with χ

Details are discussed in the text. The arrow on the χ site indicates the direction that RecBCD enzyme must approach for recognition of the χ. RecBCD enzyme is represented by the diamond–triangle–circle. Prior to the interaction with the χ site, the asymmetric nuclease activity of RecBCD enzyme is high, resulting in extensive degradation of the 3′ terminal strand; upon encountering a χ site, RecBCD enzyme pauses and becomes functionally altered (stippled symbol) so that its nuclease activity is attenuated. SSB protein is bound to the ssDNA but is not shown.

are produced (Wright et al., 1971; Goldmark and Linn, 1972; Eichler and Lehman, 1977; Telander-Muskavitch and Linn, 1981; Taylor, 1988). From this view, it follows that the consequence of any pause by RecBCD enzyme is an increased probability of a nucleolytic event at the site of pausing. Thus, an interpretation of the observed cleavage at χ sites, in keeping with the pausing interpretation, is that the recognition of the χ sequence by RecBCD enzyme results in a pause; this pause, in turn, results in a high probability of nicking the DNA in the vicinity of χ. Therefore, the "specific" cleavage at χ can be viewed as a manifestation of the nonspecific nuclease activity of RecBCD enzyme that results from the interaction with and the specific pause at χ. Since the nuclease activity is attenuated after exiting the χ site, the cleavage at χ represents the last cleavage event made by the enzyme prior to attenuation. We also propose that sometime during the pause at χ, the as yet unknown molecular event that is responsible for the attenuation of the nuclease activity of RecBCD enzyme occurs.

A model incorporating the present results and previous findings is shown in Figure 5. The RecBCD enzyme initiates events by binding to the terminus of a linear dsDNA molecule (Figure 5A). Utilizing the energy of ATP hydrolysis, the helicase activity of the enzyme begins unwinding the dsDNA, forming a loop on the strand 3′ terminal at the entry site (Braedt and Smith, 1989); immediate binding of the SSB protein (and RecA protein, when present) to the unwound DNA strands (Kowalczykowski and Roman, 1990; Roman et al., 1991) results in the formation of a loop–tail DNA structure (Taylor and Smith, 1980; Telander-Muskavitch and Linn, 1981; Figure 5B). The nonspecific nuclease activity of RecBCD enzyme degrades the DNA asymmetrically as it unwinds; the 3′ terminal strand is cut often, thereby producing short oligonucleotide fragments, while the 5′ terminal strand is cut much less frequently (Figure 5B). χ recognition occurs only if RecBCD enzyme binds the dsDNA end where, upon translocation through the DNA, it will encounter χ in the proper orientation (Taylor et al., 1985). This recognition results in both the pausing of the unwinding activity and an attenuation of the 3′ strand-specific nuclease activity of RecBCD enzyme (stippled RecBCD enzyme), resulting in a nick in the DNA strand containing the χ sequence (5′-GCTGGTGG-3′) (Figure 5C). Owing to the vigorous degradation of the upstream 3′ strand, the downstream χ fragment is the predominant

product. Continued unwinding of the dsDNA produces an ssDNA fragment whose length is dependent upon the location of the χ site in the linear dsDNA and that is preferentially utilized by the RecA protein in DNA strand exchange (Dixon and Kowalczykowski, 1991; Figures 5D and 5E). These biochemical steps are likely to represent the initial in vivo events crucial to the formation of intact ssDNA needed by RecA protein to promote the homologous pairing that is essential to the recombination process.

The molecular basis for the attenuation of the nuclease activity of RecBCD enzyme upon the interaction with χ is unknown. However, to explain the genetic properties of the *recBCD*‡ class of mutations that exhibit a hyperrecombination phenotype (in λ phage crosses) in the absence of χ and a loss of nuclease activity in vivo (Chaudhury and Smith, 1984; Amundsen et al., 1986; Lovett et al., 1988; D. A. D. and S. C. K., unpublished data), Thaler et al. (1988, 1989) suggested that the activity of RecBCD enzyme is altered upon interaction with χ, perhaps by a loss of the RecD subunit, to yield a χ-activated enzyme. Previous results and the findings presented here predict that the alteration of activity manifested by mutant RecBCD‡ enzymes may be the constitutive attenuation of the 3′ terminal strand–specific nuclease activity that normally requires interaction with χ.

Smith and colleagues demonstrated that the RecBCD enzyme will introduce a nick in the vicinity of the χ sequence (Ponticelli et al., 1985; Taylor et al., 1985). Here, we have demonstrated an additional feature of the RecBCD enzyme–χ interaction: attenuation of the nucleolytic activity of the RecBCD enzyme upon recognition of χ. Though we imagine both events to be essential to the recombination process, we suggest that the primary event associated with χ recognition is the attenuation of nuclease activity; the nick at χ is a consequence of this recognition, the pause that occurs at χ, and is the last nucleolytic event before attenuation of nuclease activity. This economical view is consistent with and reconciles many features of RecBCD enzyme behavior; e.g., the paradox that RecBCD enzyme is both a destructive nuclease and a constructive recombination enzyme. This view rationalizes the observations that *recBCD*‡ mutations are recombination proficient (Chaudhury and Smith, 1984) but possess no nuclease activity and only modest helicase activity (Palas and Kushner, 1990; D. A. D. and S. C. K., unpublished data). It explains the observation that, if RecBCD enzyme cuts at a χ site, it does not detectably cut at a second properly oriented χ site on the same molecule (Taylor and Smith, 1992). This view is also consistent with the observations that the *recC** and class I mutations, which fail to recognize χ, also display reduced levels of recombination (Schultz et al., 1983; Amundsen et al. 1990); in vitro, the mutant class I RecB^{2109}CD enzyme fails both to recognize χ and to attenuate its nuclease activity, resulting in a recombination-deficient enzyme with constitutive nuclease activity (Eggleston and Kowalczykowski, 1993a, 1993b). Finally, two independent lines of in vivo evidence suggest that the χ sequence protects DNA downstream, but not upstream, of itself against degradation by the RecBCD enzyme (Stahl et al., 1990; Dabert et al., 1992). All of these observations are readily explained by the nuclease attenuation hypothesis (Figure 5).

Experimental Procedures

Enzymes

RecBCD enzyme was purified as described (Roman and Kowalczykowski, 1989). Protein concentration was determined using an extinction coefficient of $4.0 \times 10^5\ M^{-1}\ cm^{-1}$ at 280 nm (Roman and Kowalczykowski, 1989); the specific activity of the enzyme preparation was 5.4×10^4 U of nuclease per mg of protein or 1.1×10^4 U of helicase per mg of protein. Nuclease units and helicase units were measured as described by Eichler and Lehman (1977) and Roman and Kowalczykowski (1989), respectively.

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

All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs, Pharmacia LKB, or Bethesda Research Laboratories. The enzymes were used according to Sambrook et al. (1989) or as indicated by the specific vendor.

DNA Substrates

The plasmid pBR322 χ$^+$FH was constructed by inserting a dsDNA oligomer containing the χ sequence (5′-GCTGGTGG-3′) into the χ-containing plasmid pBR322 χ$^+$F as described for the construction of pBR322 χ$^+$H (Dixon and Kowalczykowski, 1991). E. coli strain JM83 (*ara*, Δ*lac–pro*, *strA*, *thi*, *Φ80d lacZΔM13*) was transformed with the plasmid pBR322 χ$^+$FH; this strain was called SKDD004. Restriction enzyme mapping of plasmid pBR322 χ$^+$FH and χ cutting experiments described by Ponticelli et al. (1985) and Dixon and Kowalczykowski (1991) were conducted to ensure the presence and location of the χ sequences at positions 1493–1500 and 3549–3556 in the plasmid.

The plasmids pBR322 χ° (wild type) and pBR322 χ$^+$F225 (Smith et al., 1981a) were prepared from strains S819 and S818, respectively, provided by G. R. Smith and A. F. Taylor. All plasmid DNAs were purified by cesium chloride density gradient centrifugation (Sambrook et al., 1989). The molar concentration of dsDNA in nucleotides was determined by using an extinction coefficient of 6290 $M^{-1}\ cm^{-1}$ at 260 nm. Specific plasmid DNA and DNA molecular weight standards were linearized with appropriate restriction enzymes and radioactively labeled either at the 3′ end using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (ICN) or at the 5′ end by sequential reactions with calf intestinal phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP (ICN), using methods given by the vendor or Sambrook et al. (1989). The DNA was further purified by passage over an Elutip-d column (Schleicher & Schuell) and ethanol precipitation.

Reaction Conditions

The standard unwinding reaction mixture consisted of 25 mM Tris–acetate (pH 7.5), 8 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, ~4 U/ml pyruvate kinase, 10 μM (in nucleotides) linear dsDNA (2.3 nM dsDNA ends), 2 μM SSB protein, and RecBCD enzyme. The SSB protein facilitates the unwinding reaction by trapping the unwound DNA strands and inhibits the postunwinding ssDNA degradation by RecBCD enzyme (MacKay and Linn, 1976; Roman and Kowalczykowski, 1989). Control experiments confirmed that the amount of postunwinding ssDNA degradation is <2% over the reaction times examined here (data not shown). Assays were performed at 37°C and were begun with the addition of ATP and pyruvate kinase, after preincubation of all other components for 2 min.

Analysis of Reaction Products

At the completion of each unwinding reaction (40 μl) 10 μl of stop buffer (0.1 M EDTA, 2.5% SDS, 40% glycerol, 0.125% bromophenol blue, and 0.125% xylene cyanol) was added to halt the reaction and to deproteinize the sample. Reaction samples were electrophoresed on 1.2% agarose gels for 15 hr at 1.7 V/cm in TAE (40 mM Tris–acetate [pH 8.0], 2 mM EDTA). The gels were dried and autoradiographed at −20°C with Kodak XAR-5 film and an intensifying screen. Quantitative analysis of ssDNA production was accomplished by analysis of the autoradiogram using a Bio Image (Millipore).

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