Article No. mb982013 J. Mol. Biol. (1998) **282**, 275–285

JMB

SSB Protein Controls RecBCD Enzyme Nuclease Activity during Unwinding: A New Role for Looped Intermediates

Daniel G. Anderson[1] and Stephen C. Kowalczykowski[1,2]*

[1]Genetics Graduate Group and [2]Sections of Microbiology and of Molecular and Cellular Biology, University of California, Davis, Davis CA 95616-8665, USA

**Corresponding author*

The RecBCD enzyme of *Escherichia coli* initiates homologous recombination by unwinding and simultaneously degrading DNA from a double-stranded DNA end. Single-stranded DNA loops are intermediates of this unwinding process. Here we show that SSB protein reduces the level of DNA degradation by RecBCD enzyme during unwinding, by binding to these ssDNA intermediates. Prior to interaction with the recombination hot spot χ, RecBCD enzyme has both $3' \rightarrow 5'$ exonuclease and a weaker $5' \rightarrow 3'$ exonuclease activity. We show that degradation of the 5′-terminal strand at the entry site is much more extensive in the absence of SSB protein. After interaction with χ, the level of $5' \rightarrow 3'$ exonuclease activity is increased; as expected, degradation of the 5′-strand is also elevated in the absence of SSB protein. Furthermore, we show that, in the absence of SSB protein, the RecBCD enzyme is inhibited by the ssDNA products of unwinding; SSB protein alleviates this inhibition. These results provide insight into the organization of helicase and nuclease domains within the RecBCD enzyme, and also suggest a new level at which the nuclease activity of RecBCD enzyme is controlled. Hence, they offer new insight into the role of SSB protein in the initiation phase of recombination.

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Keywords: helicase; nuclease; RecBCD; recombination; SSB

Introduction

The initiation of homologous recombination in *Escherichia coli* is catalyzed by three enzymes: RecA protein, RecBCD enzyme and single-stranded DNA-binding (SSB) protein (for a review, see Kuzminov, 1996). The importance of RecA protein and RecBCD enzyme in this pathway is illustrated by the severe phenotypes of *recA*, *recB* or *recC* null mutants. Inactivation of the *recA* gene reduces recombination proficiency by up to 10^5-fold (Clark & Margulies, 1965). Similarly, mutations that inactivate the RecBCD enzyme decrease recombination by 10^2 to 10^3-fold (Emmerson, 1968; Howard-Flanders & Theriot, 1966). The effects of *ssb* mutations (which are necessarily non-null mutants, since *ssb* is essential) are less extreme, and vary from 4 to 50-fold, depending on the mutation and the assay (Ennis *et al.*, 1987; Schmellik-Sandage & Tessman, 1990). In addition to these protein components, recombination is stimulated five- to ten-fold by an eight base DNA sequence element known as χ (5′-GCTGGTGG-3′) (Cheng & Smith, 1989; Ennis *et al.*, 1987; Lam *et al.*, 1974; Smith *et al.*, 1981; Stahl *et al.*, 1975, 1980).

The first step in recombination is mediated by the heterotrimeric RecBCD enzyme, which is composed of the products of the *recB*, *recC* and *recD* genes (Amundsen *et al.*, 1986). Upon binding a double-stranded DNA (dsDNA) end, the RecBCD enzyme proceeds to unwind and simultaneously degrade the DNA at rates of up to 1000 base-pairs/second (Roman & Kowalczykowski, 1989). Degradation of the DNA is asymmetric, with the strand that is 3′-terminal at the end that RecBCD enzyme enters being degraded much more vigorously than the 5′-terminal strand (Dixon & Kowalczykowski, 1991, 1993; and see Figure 1). When the RecBCD enzyme reaches a properly oriented χ site, degradation of the 3′-terminal strand is attenuated. Unwinding continues, but the polarity of degradation is switched, with the 5′-terminal strand being degraded (Anderson *et al.*,

Abbreviations used: SSB, single-stranded DNA binding; RPA, replication protein A; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; χ, 5′-GCTGGTGG-3′.

E-mail address of the corresponding author: sckowalczykowski@ucdavis.edu

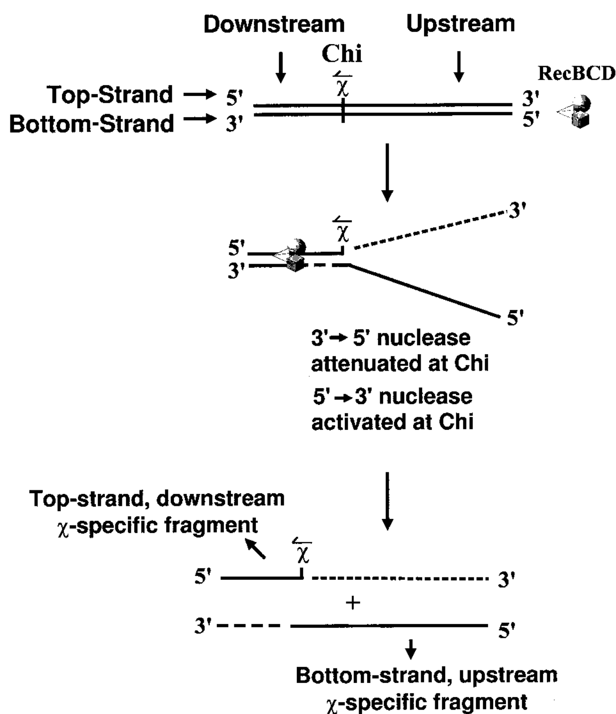

Figure 1. Recognition of χ by the RecBCD enzyme produces two χ-specific fragments. The arrow above the χ site indicates the direction that RecBCD enzyme must travel in order to recognize χ. The region of dsDNA between χ and the entry site of the RecBCD enzyme is the upstream region, and the region between χ and the opposite end is the downstream region. The strand of DNA that terminates 3′ at the entry site for RecBCD enzyme is the top-strand; the opposite strand is the bottom-strand. Unwinding and $3' \rightarrow 5'$ exonuclease activity upstream of χ, followed by a switch in the polarity of exonuclease degradation to $5' \rightarrow 3'$ leads to the production of both a bottom-strand, upstream χ-specific fragment and a top-strand, downstream χ-specific fragment. Processing of dsDNA that does not contain a χ-site leads to the production of full-length ssDNA (not shown). Adapted from Anderson *et al.* (1997).

1997; Anderson Kowalczykowski, 1997a). The χ-activated RecBCD enzyme also promotes the preferential loading of the homologous pairing protein, RecA, onto the 3′-end of this strand (Anderson & Kowalczykowski, 1997b). The net result of these activities is a 3′-overhang that is coated with RecA protein, an ideal substrate for RecA protein-catalyzed homologous pairing (Konforti & Davis, 1987, 1990; Tracy & Kowalczykowski, 1996). The RecA protein-single-stranded DNA (ssDNA) filament can then catalyze the pairing and exchange of this ssDNA with a homologous dsDNA molecule (for a review, see Kowalczykowski & Eggleston, 1994).

The role of SSB protein in recombination reactions depends on its ability to bind ssDNA. This homotetrameric enzyme binds ssDNA, with a site size that ranges from 35 to 65 depending on conditions (for reviews, see Meyer & Laine, 1990; Lohman & Ferrari, 1994). *In vitro*, SSB protein stimulates RecA protein filament formation by destabilizing secondary structure (Muniyappa *et al.*, 1984; Kowalczykowski *et al.*, 1987; Kowalczykowski & Krupp, 1987). In addition, RecA protein-promoted exchange of DNA strands is stimulated by SSB protein binding to the displaced strand of DNA in the joint molecule (Lavery & Kowalczykowski, 1992).

Electron microscopy studies of RecBCD enzyme during unwinding of dsDNA revealed the formation of ssDNA loop-tail and twin-loop intermediates (Taylor & Smith, 1980a,b; and see Figure 6). Interestingly, unwinding in the presence of SSB protein favors the formation of the loop-tail intermediate. The loop in this structure is the strand of DNA that is 3′-terminal at the point of RecBCD enzyme entry (Braedt & Smith, 1989), and to which RecA protein is loaded after χ recognition (Anderson & Kowalczykowski, 1997b). Initially, loop structures were discovered in the presence of calcium ions, an inhibitor of the nuclease activity of RecBCD enzyme. The formation of twin-loops in the absence of SSB protein was interpreted as a consequence of DNA re-annealing behind the translocating RecBCD enzyme. Telander-Muskavitch & Linn (1982) examined RecBCD enzyme unwinding under conditions that allow some exonuclease activity, and found twin-loop intermediates at either condition.

Here, we show that single-stranded DNA binding proteins control the level of RecBCD enzyme nuclease activity. Both *Escherichia coli* SSB protein and *Saccharomyces cerevisiae* replication protein A (RPA: Brill & Stillman, 1989) reduce the level of $5' \rightarrow 3'$ exonuclease activity before and after χ-recognition. We propose that SSB protein decreases degradation by binding to ssDNA in the loops of unwinding intermediates, thereby disrupting nuclease activity occurring at the second enzyme-DNA contact point at the back of the loop. Furthermore, we show that processing of dsDNA in the absence of SSB protein is gradually inhibited by the accumulation of ssDNA products of unwinding; this inhibition is alleviated by SSB protein. Hence, these results offer new insight into the role of SSB protein in the initiation phase of recombination.

Results

SSB protein controls the nuclease activity of RecBCD enzyme during unwinding

The asymmetric degradation and unwinding of χ-containing, linear dsDNA by RecBCD enzyme generates three discrete-sized ssDNA products, in addition to the non-specific degradation products (Anderson & Kowalczykowski, 1997a). For reference purposes, we designate the strand of DNA that terminates 3′ at the entry point of RecBCD enzyme to be the top-strand (Figure 1) and the

strand of DNA complementary to the top-strand is defined as the bottom-strand. The region of DNA between the entry point of the RecBCD enzyme and the χ site is defined as the upstream region, and that between the χ site and the opposite end is termed the downstream region. Thus, the three major ssDNA molecules produced by RecBCD enzyme using this terminology are: (1) the full-length ssDNA originating from the bottom-strand when χ is not recognized; (2) the top-strand, downstream χ-specific fragment; and (3) the bottom-strand, upstream χ-specific fragment. Since the RecBCD enzyme degrades the top-strand during unwinding much more extensively than the bottom-strand, the asymmetric degradation of dsDNA that does not contain a χ-site, or when χ is not recognized, leads to the production of one ssDNA product: full-length ssDNA originating from the bottom-strand.

Initial examination of dsDNA processing by RecBCD enzyme revealed that much less ssDNA is produced in the absence of SSB protein (MacKay & Linn, 1976; Anderson & Kowalczykowski, 1997a,b). One possibility is that the increased DNA degradation in the absence of SSB protein occurs after the DNA molecule has been unwound, rather than during unwinding, since the RecBCD enzyme possesses ssDNA exonuclease activity. To distinguish between degradation occurring during unwinding from that occurring after unwinding, two complementary reactions were performed. In the first reaction, half of the DNA is linear, 5′ end-labeled double-stranded pBR322 χ° (which has no χ-site), while the other half is linear, single-stranded, heat-denatured pBR322 χ° that is unlabeled (Figure 2(a)). In the second reaction, the same protocol is followed, but now the ssDNA is 5′ end-labeled, and the dsDNA is unlabeled (Figure 2(c)). This DNA mixture is treated with RecBCD enzyme in the presence and absence of SSB protein, and a time-course of unwinding is examined (the concentration of SSB protein is saturating with respect to the total amount of DNA present; see Materials and Methods. The reactions were analyzed using native agarose gel electrophoresis. Analysis of the first reaction (where the dsDNA is labeled; Figure 2(a)) shows that much less full-length, bottom-strand ssDNA is produced by RecBCD enzyme processing of the dsDNA in the absence of SSB protein. When normalized to the amount of dsDNA unwound (because only 70% of the dsDNA is unwound after ten minutes in the absence of SSB protein; see Figure 2(b)), six- to tenfold less full-length ssDNA is produced. Analysis of these reactions using alkaline agarose gel electrophoresis shows that the dsDNA remaining in the absence of SSB protein is not nicked (data not shown). Thus, it is very unlikely that this DNA was processed by the RecBCD enzyme, and then re-annealed. When, instead, the ssDNA is labeled rather than the dsDNA, we observe that there is a low level of degradation of the ssDNA; however, there is no significant differ-

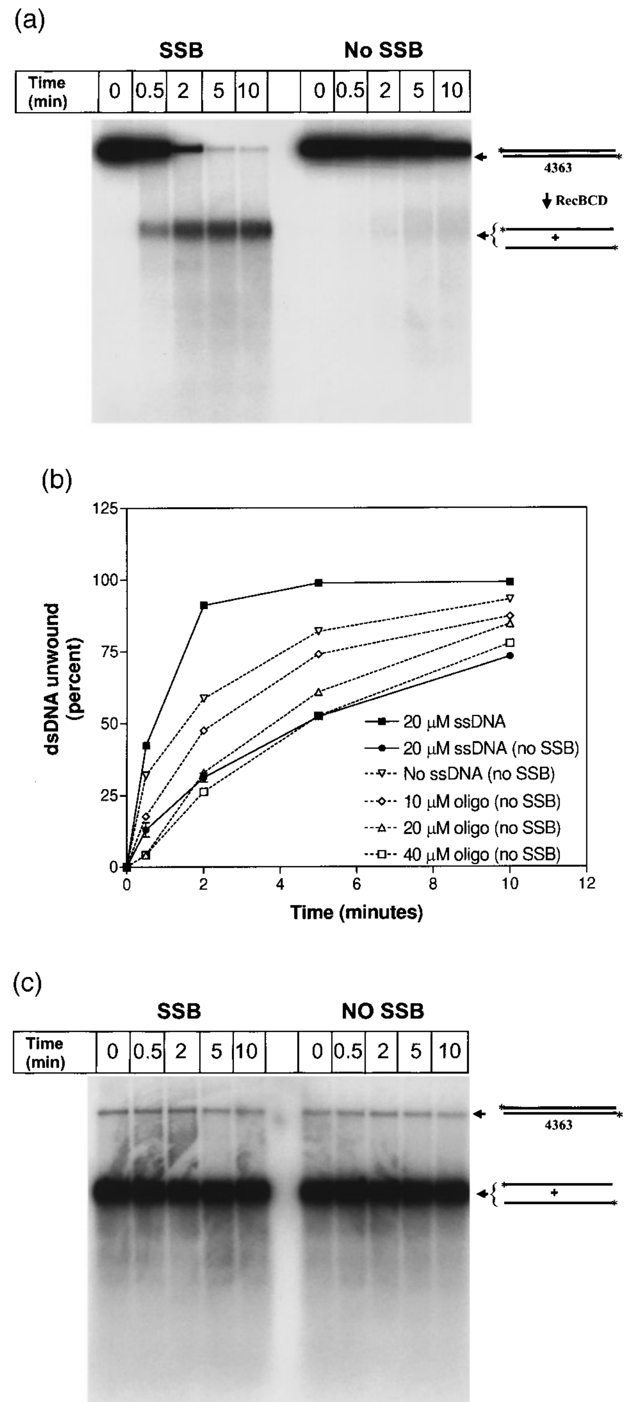

Figure 2. SSB protein controls RecBCD enzyme bottom-strand nuclease activity. The linear dsDNA substrate was created by restriction of pBR322 χ° with *Nde*I and labeled at the 5′-end with ^{32}P. This DNA does not contain a χ site. (a) The DNA (20 μM) was treated with RecBCD enzyme in the presence of 20 μM (nucleotides) heat-denatured, unlabeled, ssDNA, and 0 μM or 8 μM (saturating) SSB protein. The reaction was also performed in the absence of heat-denatured ssDNA and SSB protein, but with varying amounts of oligonucleotides. Quantification of unwinding, as measured by loss of dsDNA, in these and the previous reactions is shown in (b). (c) To measure post-unwinding degradation, the reactions in A were repeated; however, the dsDNA was unlabeled and the heat-denatured ssDNA was 5′-end labeled with ^{32}P.

ence in the degradation of the existing ssDNA in the presence or absence of SSB protein. This indicates that there is little post-unwinding degradation by the RecBCD enzyme (Figure 2(c)). Furthermore, there is no detectable re-annealing of ssDNA during the reaction. Thus, the decreased yield of full-length, bottom-strand ssDNA in the absence of SSB protein must be due to increased degradation during unwinding and simultaneous with translocation.

ssDNA is an inhibitor of the RecBCD enzyme in the absence of SSB protein

In the absence of SSB protein, the apparent rate of RecBCD enzyme-catalyzed unwinding decreases over the course of the reaction. Quantification of dsDNA unwinding in Figure 2(a) is shown in Figure 2(b). Unwinding in the presence of SSB protein appears to occur at a constant rate, while the rate of unwinding in the absence of SSB protein slows over the course of the reaction. This behavior suggests that an inhibitor of the RecBCD enzyme is generated in the absence of SSB protein. One candidate for an inhibitor is ssDNA, since it is generated over the course of the reaction and is tightly bound by SSB protein. To test whether ssDNA is indeed the inhibitor, the reactions in Figure 2(a) were performed in the absence of SSB protein or ssDNA, and the effects of adding an oligonucleotide were tested, since short DNA fragments are a major product of RecBCD enzyme processing of dsDNA. As can be seen in Figure 2(b), the initial rate of unwinding without the oligonucleotide is significantly faster relative to unwinding in the presence of ssDNA. This initial rate decreases with increasing oligonucleotide concentration. Thus, both full-length ssDNA and oligonucleotides are capable of inhibiting RecBCD enzyme-catalyzed unwinding. These results suggest that ssDNA produced by RecBCD enzyme processing of the dsDNA acts as an inhibitor of DNA unwinding in the absence of SSB protein.

To quantify further the effects of ssDNA on RecBCD enzyme-catalyzed unwinding, dye-displacement helicase assays were performed (Eggleston *et al.*, 1996). In this assay, displacement of the dsDNA-binding dye, Hoechst 33258, is measured spectrofluorophotometrically. Processing of the DNA by the RecBCD enzyme leads to displacement of Hoechst 33258 from the dsDNA, and a simultaneous decrease in fluorescence, thereby providing a continuous measure of dsDNA unwinding. Unwinding of linearized pBR322 in the presence and absence of SSB protein using this assay is shown in Figure 3. These reactions were the same as those in Figure 2, except that there was no ssDNA present, the amount of dsDNA was doubled (to 40 μM), and the RecBCD enzyme was increased (to one functional RecBCD enzyme molecule per 16 linear dsDNA ends). As expected, the rate of unwinding in the presence of SSB protein (as measured by the slope) is constant during the

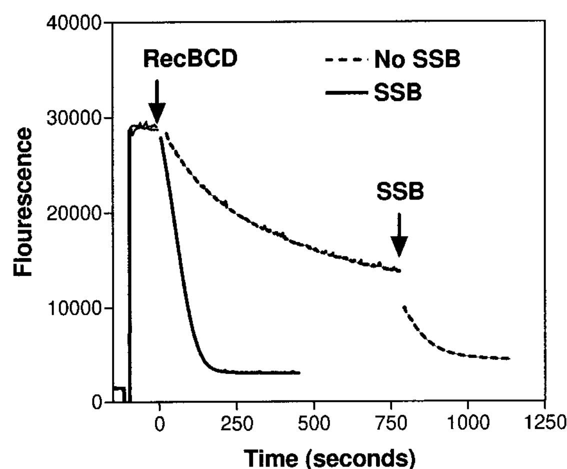

Figure 3. Unwinding in the absence of SSB protein is reversibly inhibited by ssDNA accumulation. pBR322 χ° linearized with *Nde*I (40 μM) was treated with RecBCD enzyme in the presence of 1 μM Hoechst 33258 dye, and 0 or 8 μM SSB protein. The rate of unwinding in the presence of SSB protein is constant, while unwinding in the absence of SSB protein is gradually inhibited. Addition of SSB protein late in the reaction relieves most of the inhibition.

majority of the reaction. In contrast, the apparent rate of unwinding in the absence of SSB protein decelerates over the course of the reaction. Furthermore, addition of SSB protein late in the reaction causes an increase in the unwinding rate, an observation consistent with the idea that ssDNA is the inhibitor in this reaction. Addition of SSB protein also causes a sudden drop in fluorescence, due to ejection of some Hoechst 33258 from secondary structure within the ssDNA (Figure 3; Eggleston *et al.*, 1996; unpublished results). The subsequent rate of unwinding is slightly slower than the initial rate seen in the absence of SSB protein. Collectively, these data show that processing of dsDNA by RecBCD enzyme, in the absence of SSB protein, is inhibited by the ssDNA products of unwinding and degradation.

SSB protein controls χ-activated nuclease activity

As previously mentioned, recognition of χ by the translocating RecBCD enzyme leads to an activation of bottom-strand nuclease activity. The observation that bottom-strand nuclease activity is controlled by SSB protein before RecBCD enzyme reaches χ leads to the question of whether SSB protein also controls RecBCD enzyme's χ-activated nuclease activity. Eggleston & Kowalczykowski (1993) showed that RecBCD enzyme's frequency of DNA cleavage per distance traveled is sensitive to the free magnesium ion concentration. In the presence of high concentrations of magnesium ion, the frequency of cleavage is much higher than at low concentrations. This sensitivity to the concentration

of free magnesium ion also affects the location of the last cleavage on the top-strand after the RecBCD enzyme encounters χ (Taylor & Smith, 1995; unpublished results), and the first cleavage on the bottom after χ-activation of nuclease (Anderson *et al*., 1997; Taylor & Smith, 1995). In the presence of high levels of free magnesium ion, the first cut on the bottom-strand after χ is very close to the χ sequence. As the magnesium ion concentration is decreased, the frequency of χ-activated nuclease cleavage is also reduced, resulting in a first cleavage on the bottom-strand that is downstream of the χ sequence. Since SSB protein, like low concentrations of free magnesium ion, reduces the frequency of cleavage on the bottom-strand (Figure 2), we would predict that the location of the first cleavage on the bottom-strand after χ would be shifted further downstream in the presence of SSB protein.

To examine the effect of SSB protein on the RecBCD enzyme's χ-activated nuclease, we repeated the experiments in Figure 2, but used *Nde*I-linearized pBR322 χ^{+}F; a χ-containing DNA (Figure 4). Again, half of the dsDNA was 5′ end-labeled and the other half, unlabeled, was heat-denatured to create ssDNA. These were treated

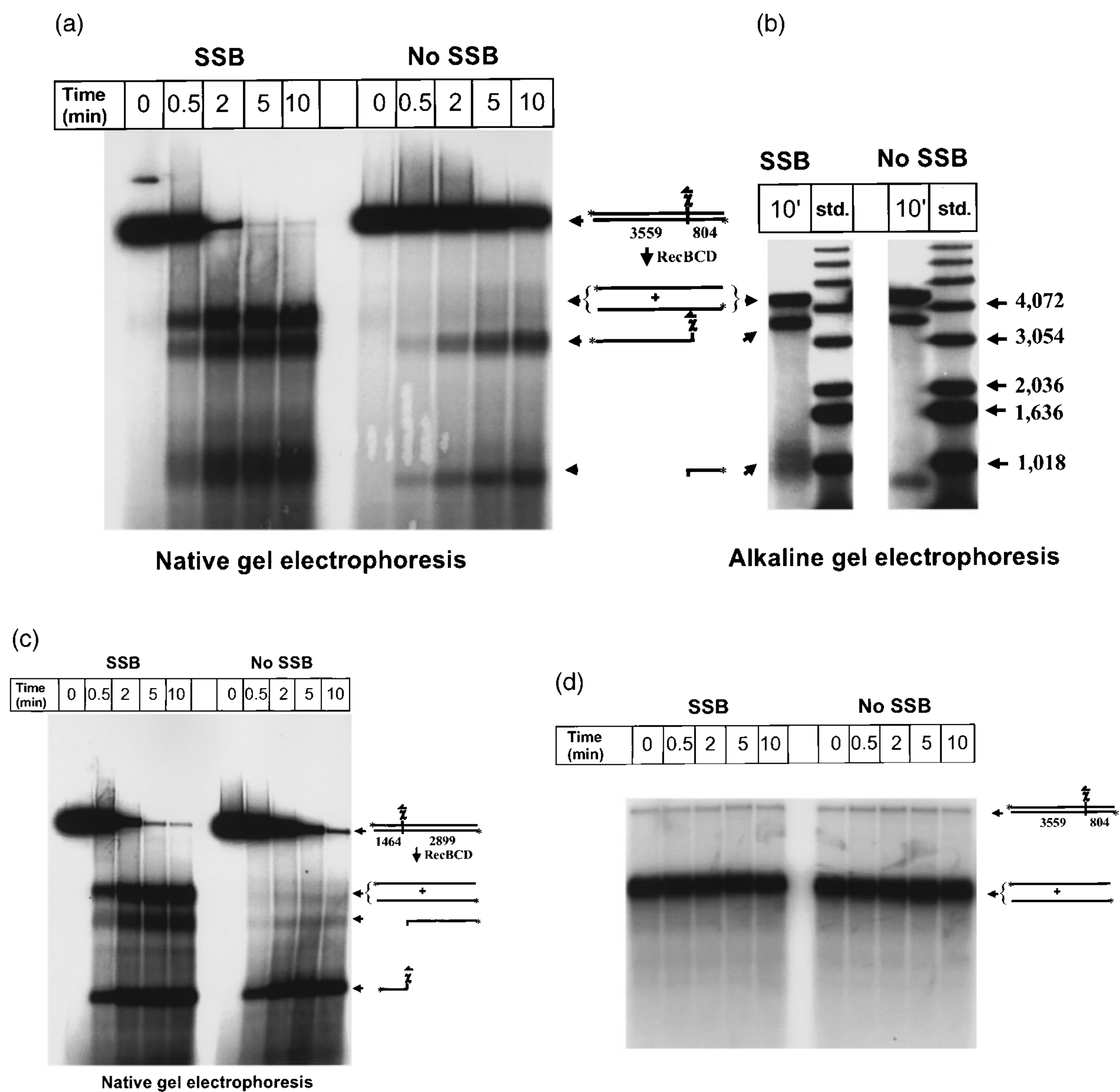

Figure 4. SSB protein changes the location of the first cleavage on the bottom-strand after χ. (a), (b) and (d) *Nde*I or (c) *Hin*dIII-linearized pBR322 χ^{+}F (20 μM) was 5′-end labeled with ^{32}P, and treated with RecBCD enzyme in the presence of heat-denatured, unlabeled, ssDNA (20 μM) and 0 or 8 μM SSB protein. These reactions were analyzed using either (a), (c) and (d) native or (b) alkaline gel electrophoresis. The 5′-end labeled 1kb ladder was used as a size standard on the alkaline gel. Processing of χ-containing dsDNA in the presence of SSB protein shifts the location of the first cleavage on the bottom-strand after χ approximately 100 to 400 nucleotides downstream. (d) These reactions were also repeated with the dsDNA unlabeled, and the heat-denatured ssDNA 5′-end labeled with ^{32}P.

with RecBCD enzyme under standard conditions, and a time-course was examined (Figure 4). The products of unwinding were analyzed using native (Figure 4(a)) and alkaline (Figure 4(b)) agarose gel electrophoresis. In the absence of SSB protein, the χ-specific bottom-strand cleavage occurs 0 to 100 nucleotides downstream of the χ sequence (Figure 4(b)). As predicted, the presence of SSB protein produces a broader distribution of χ-specific fragments, and causes the location of the bottom-strand χ-specific fragment to be shifted 100 to 400 nucleotides downstream from the χ sequence (Figure 4(b)). Reduction of the SSB protein concentration by 50% does not significantly change the results (data not shown).

Since bottom-strand degradation is occurring during translocation, the further the RecBCD enzyme travels before it reaches χ, the more likely a cleavage event will occur upstream of χ. Thus, the yield of longer bottom-strand χ-specific fragments in the absence of SSB protein should be less than the yield of shorter ones. Due to the short length of the bottom-strand χ-specific fragment obtained using *Nde*I-linearized pBR322 χ^{+}F, only 35% less bottom-strand fragment is produced in the absence of SSB protein (when normalized for the extent of unwinding). In contrast, 20% less of the longer top-strand downstream χ-fragment was produced in the absence of SSB protein. To examine the effect of fragment length, these reactions were repeated using pBR322 χ^{+}F linearized with *Hin*dIII, which has a χ-site located further downstream (Figure 4(c)). After five minutes, the yield of bottom-strand χ-specific fragment was three- to fivefold lower in the absence of SSB protein, but there was no significant difference in the yield of top-strand χ-specific fragment. Thus, the yield of the top-strand χ-fragment in the absence of SSB protein is relatively insensitive to its size, while the yield of bottom-strand is a function of its length.

Analysis of the complementary reaction, where the dsDNA is unlabeled but the ssDNA is 5′ end-labeled (Figure 4(d)), shows that there is no significant difference in the amount of post-unwinding ssDNA degradation in the presence or absence of SSB protein. In addition, there is no evidence for either re-annealing or χ-fragment production. These results show that SSB protein controls DNA degradation before and after χ-recognition on the bottom-strand.

Control of RecBCD enzyme nuclease activity is not specific to the *E. coli* SSB protein

SSB protein can affect the nuclease activity of the RecBCD enzyme by either binding to the ssDNA or by binding to and affecting the RecBCD enzyme. The presence of ssDNA loops as intermediates of unwinding provides an opportunity for ssDNA binding proteins to affect the RecBCD enzyme during translocation. To investigate whether SSB protein acts *via* the ssDNA, we tested the effects of another ssDNA binding protein, the *S. cerevisiae* replication protein-A (RPA), on RecBCD enzyme-promoted DNA degradation (Figure 5). The reaction conditions were exactly the same as those in Figure 4(a), but RPA was used instead of SSB protein. In the presence of saturating amounts of RPA, processing of χ-containing DNA by the RecBCD enzyme produces three- to fourfold more full-length ssDNA than when no ssDNA binding protein is present. In addition, RPA causes the location of the first cleavage on the bottom-strand after χ-activation of nuclease to shift downstream in a manner similar to that induced by SSB protein (Figure 5; compare RPA to SSB).

In addition to RPA, saturating RecA protein was tested, since it too is a ssDNA-binding protein. The yield of full-length ssDNA in the presence of RecA protein is approximately twofold more than the amount produced with only the RecBCD enzyme. However, RecA protein does not induce a significant shift in the location of the first cleavage on the bottom-strand. Interestingly, neither of these proteins was able to prevent inhibition of RecBCD enzyme unwinding by ssDNA as effectively as SSB protein, as judged from the lower rate and extent of processing of dsDNA (compare to Figure 4(a)), presumably because they bind these fragments less tightly. Doubling of the RecA protein concentration does not significantly change the results (data not shown).

The observation that other ssDNA binding proteins can control the nuclease activity of the RecBCD enzyme strongly suggests that this effect is mediated by binding to the ssDNA, rather than by direct protein-protein interaction with the RecBCD enzyme. This conclusion is further supported by the fact that saturating amounts of SSB protein (with respect to DNA) are needed to fully control RecBCD enzyme nuclease activity (data not shown). Thus, we conclude that SSB protein controls the 5′ → 3′ nuclease activity of the RecBCD

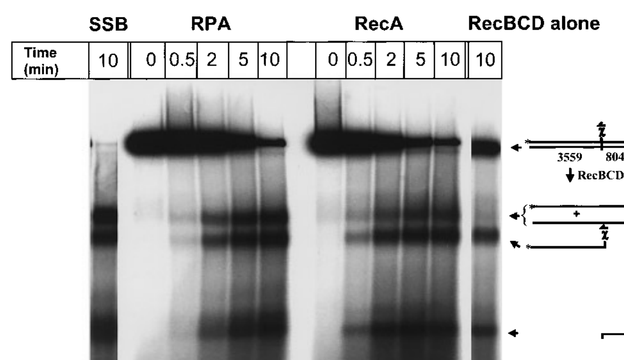

Figure 5. The ssDNA binding proteins RPA and RecA protein modulate 5′ → 3′ DNA degradation by the RecBCD enzyme. *Nde*I-linearzied pBR322 χ^{+}F (20 μM) was 5′-end labeled with ^{32}P, and treated with RecBCD enzyme in the presence of heat-denatured, unlabeled, ssDNA (20 μM) and 3 μM RPA, or 20 μM RecA protein. Processing of dsDNA in the presence of RPA is very similar to processing in the presence of SSB protein (see Figure 4(a)).

enzyme during enzymatic translocation, by binding to ssDNA.

Discussion

In *E. coli*, regulation of the potent nuclease activity of the RecBCD enzyme is essential for successful DNA recombination and repair (Clark & Sandler, 1994). The primary level of regulation is provided by the recombination hot-spot, χ, which switches the RecBCD enzyme from an anti-recombinogenic nuclease into the primary initiator of recombination. Here, we show that *E. coli* SSB protein provides a secondary level of regulation. Using *in vitro* unwinding and nuclease assays, we show that SSB protein controls the $5' \rightarrow 3'$ nuclease activity of the RecBCD enzyme (Figure 2). This effect is exerted at the level of the translocating RecBCD enzyme and not at the post-unwinding level. Furthermore, this regulation also affects the χ-activated $5' \rightarrow 3'$ nuclease activity (Figure 4). The observation that the RecBCD enzyme nuclease activity can be controlled by the functionally analogous, but structurally different, RPA (Figure 5), and that saturating amounts of SSB protein with respect to DNA are required, indicates that this effect is mediated by binding to the ssDNA.

For SSB protein to affect the nuclease activity of the RecBCD enzyme during unwinding, it must bind the ssDNA before contact with the subunit of RecBCD enzyme that is responsible for nucleolytic action. As previously mentioned, ssDNA loop-tail and twin-loop structures are intermediates of RecBCD enzyme unwinding (Braedt & Smith, 1989; Taylor & Smith, 1980a,b; Telander-Muskavitch & Linn, 1982). The formation of these ssDNA loops provides a potential substrate for ssDNA binding proteins. Since SSB protein binds only ssDNA, control of the RecBCD enzyme nuclease activity must be mediated by binding to the ssDNA of the looped intermediates. In addition, it is unlikely that binding to the top-strand ssDNA could effect nucleolytic cleavage on the bottom-strand. Thus, we propose that SSB protein binds to the ssDNA in the bottom-strand loop, and moderates the RecBCD enzyme nuclease activity by disrupting the RecBCD enzyme-ssDNA contact at the back of the loop for this strand (Figure 6). This hypothesis is consistent with the observation that (i) SSB protein shifts RecBCD enzyme unwinding intermediates from twin-loop structures to loop-tail structures (Taylor & Smith, 1980a,b) and (ii) it is the loop of DNA on the bottom-strand that is disrupted by SSB protein (Braedt & Smith, 1989).

These data provide a clue to the organization of the subunits within the RecBCD enzyme during translocation through DNA. Ganesan & Smith (1993) showed that when the RecBCD enzyme is statically bound to the end of DNA, the RecB subunit cross-links to the top-strand, while the RecC and RecD subunits crosslink to the bottom-strand. Thus far, only the RecB subunit, which has limited helicase and nuclease activity, has been shown to possess enzymatic activity without the other subunits (Boehmer & Emmerson, 1992; Yu *et al.*, 1998). The observation that the RecBC enzyme (without the RecD subunit) is an active helicase with little nuclease activity (Anderson *et al.*, 1997; Korangy & Julin, 1993; Masterson *et al.*, 1992; Palas & Kushner, 1990), and the recent discovery that the C terminus of the RecB subunit is required for all nuclease activities of the holoenzyme (Yu *et al.*, 1998), suggests that the nuclease domain(s) of the RecBCD holoenzyme are contained within the RecB and/or the RecD subunits. This hypothesis is further supported by sequence analysis of the *recD* gene, which has revealed homology to a number of $5' \rightarrow 3'$ ssDNA-specific exonucleases (S. T. Lovett, personal communication). The discovery of *recC* mutants that change the DNA sequence requirements for χ-recognition suggests that χ is recognized by the RecC subunit (Handa *et al.*, 1997; Arnold *et al.*, 1998 and unpublished results). Recently, Bianco & Kowalczykowski (1997) showed that the translocating RecBCD enzyme can recognize χ in a single-stranded form, implying that unwinding precedes recognition of χ in its single-stranded form. Finally, our results suggest that degradation of the bottom-strand follows unwinding. Based on these data, we have developed a model that illustrates the subunit organization of RecBCD enzyme during unwinding (Figure 7). In this model, the DNA is unwound at the front of the complex by the RecB subunit. The ssDNA of the top-strand is then contacted by the RecC subunit, which scans the DNA for χ sites, and then by the RecD subunit and the C terminus of the RecB subunit, which are responsible for DNA degradation. The bottom-strand DNA is looped-out, in the absence of SSB protein, contacting the RecD and RecB subunits, which are respon-

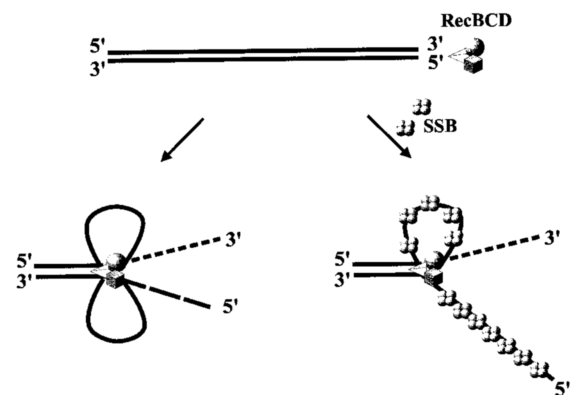

Figure 6. Model for the molecular basis of SSB protein-mediated control of RecBCD enzyme nuclease activity. Details are discussed in the text. SSB protein binds to ssDNA in the bottom-strand loop, and disrupts the contact at the back of the loop. Disruption of the bottom-strand loop results in a decrease in nuclease activity on that strand.

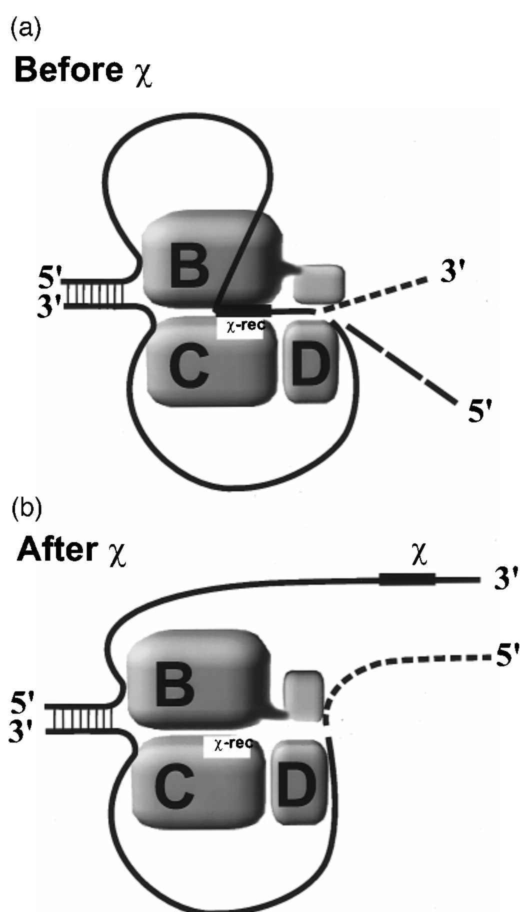

Figure 7. Model for organization of subunits within the RecBCD enzyme during dsDNA processing. (a) Before χ-recognition. DNA is unwound at the front of the complex by the RecB subunit. The ssDNA of the top-strand is then contacted by the RecC subunit, which monitors the DNA for χ sites and then by the C terminus of the RecB subunit and the RecD subunit, which degrade the top-strand DNA. In the absence of SSB protein, the bottom-strand DNA is looped out and contacts the RecB and RecD subunits, which are responsible for bottom-strand degradation, and the RecC subunit, as indicated by cross-linking. (b) After χ-recognition. The DNA of the top-strand is released from the loop, and the conformation of the RecB subunit changes, now focusing degradation onto the bottom-strand. RecA protein loading is catalyzed at the front of the loop by the RecB subunit.

sible for bottom-strand degradation, and the RecC subunit, as indicated by cross-linking. After recognition of the χ site (Figure 7(b)), the DNA in the top-strand is released, and the conformation of the C terminus of the RecB subunit changes, swinging the nuclease domain into a new position (Yu *et al.*, 1998), now focusing degradation onto the bottom-strand. In this model, the loading of RecA protein is catalyzed at the front of the loop by the RecB subunit, and then subsequent cooperative binding extends the RecA protein filament to the back (3′-end) of the loop (Anderson & Kowalczykowski, 1997b). The precise organization of the subunits within the translocating RecBCD enzyme and their respective activities remains to be determined.

We have shown that the ssDNA products of unwinding inhibit the RecBCD enzyme, and that this inhibition can be alleviated by SSB protein (Figures 2 and 3). Interestingly, RPA and RecA protein do not alleviate inhibition as much as SSB protein (Figure 5). This probably reflects differences in the ssDNA binding properties of these proteins. The relatively poor level of nuclease control exerted by RecA protein most likely reflects its slow initial binding to ssDNA (Chabbert *et al.*, 1987).

Our data suggest that SSB protein has multiple roles in recombination *in vivo*. Since recognition of χ leads to a switch in nuclease polarity, it is doubtful that the increased bottom-strand degradation in the absence of SSB protein would significantly affect the structure of χ-containing DNA processed by RecBCD enzyme; processing would still result in the formation of 3′-overhangs, since the top-strand downstream DNA is unaffected by SSB protein (Figure 4). However, degradation of DNA that does not contain χ would be more complete in the absence of SSB protein; both the top- and bottom-strands would be degraded. In *E. coli,* χ is the one of the most over-represented octamers in the genome (Tracy *et al.*, 1997); however, χ is notably absent in a number of *E. coli* phage. Thus, degradation of some phage DNA may be more effected by the absence of SSB protein than genomic DNA. Since bacteriophage infection and DNA damage have the potential to increase the concentration of cellular ssDNA, the presence of either could potentially titrate the cellular SSB protein, thereby inducing a change in RecBCD enzyme activity.

We have shown that ssDNA inhibits dsDNA processing by the RecBCD enzyme. SSB protein facilitates a second round of initiation by binding ssDNA that could potentially inhibit RecBCD enzyme function. The observation that the products of RecBCD enzyme unwinding can inhibit re-initiation suggests a feedback mechanism that controls initiation of the RecBCD enzyme. It is possible that when DNA damage leads to a significant amount of free ssDNA, conditions that inhibit RecA protein pairing function (Mazin & Kowalczykowski, 1996), inhibition of the RecBCD enzyme prevents non-productive, and potentially deleterious, initiation events. When the chromosome has been repaired enough such that there is free SSB and RecA protein, RecBCD enzyme activity, and initiation of repair, would be restored. Whether or not this type of feedback inhibition actually occurs *in vivo* remains to be determined.

Materials and Methods

Enzymes

RecBCD enzyme was purified as described (Roman & Kowalczykowski, 1989) with further purification by

FPLC using a Mono-Q column (Pharmacia). Protein concentration was determined using an extinction coefficient of $4.0 \times 10^5\ M^{-1}\ cm^{-1}$ at 280 nm (Roman & Kowalczykowski, 1989). The enzyme was determined to be 55% functional by helicase assays (Roman & Kowalczykowski, 1989). 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 & Wetmur, 1975).

RecA protein was purified using a procedure based on precipitation with spermidine (Griffith & Shores, 1985; S.C.K., unpublished data). Protein concentration was determined using an extinction coefficient of $2.7 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm. RPA was purified by T. Sugiyama (Sugiyama *et al.*, 1997). The concentration was determined using an extinction coefficient of $8.8 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm.

All restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs, Bethesda Research Laboratories or United States Biochemical. The enzymes were used according to Sambrook *et al.* (1989) as indicated by the specific vendor.

DNA substrates

The plasmids pBR322 χ° (wild-type) and pBR322 χ^+F225 (Smith *et al.*, 1981) 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 the dsDNA in nucleotides was determined using an extinction coefficient of $6290\ M^{-1}\ cm^{-1}$ at 260 nm. Plasmid DNA was linearized with either *Hin*dIII or *Nde*I and radioactively labeled at the 5′ end by sequential reactions with shrimp alkaline phosphatase followed by phage T4 polynucleotide kinase and [γ-^{32}P]ATP (NEN), using methods given by the vendor or Sambrook *et al.* (1989). The DNA was further purified by passage through an S-200 MicroSpin column (Pharmacia Biotech). ssDNA was prepared by heating at 95°C for five minutes followed by chilling on ice. The 17-mer oligonucleotide (5′-ACCTCTTATCAAAAGGA-3′) was purchased from Operon Technologies, Inc.

Reaction conditions

The standard RecBCD reaction mixture consists of 25 mM Tris-acetate (pH 7.5), 4 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 4 units/ml pyruvate kinase, 20 μM (nucleotides) linear dsDNA, 20 μM (nucleotides) heat-denatured ssDNA, 0 or 8 μM SSB protein, 0.418 nM total RecBCD enzyme (0.23 nM functional RecBCD enzyme, corresponding to 0.05 functional RecBCD enzyme molecule per linear dsDNA end). Assays were performed at 37°C, and initiated by the addition of RecBCD enzyme after pre-incubation of all other standard components for 2 minutes. When present, the concentration of RecA protein or RPA was 20 μM or 3 μM, respectively. The site sizes of SSB, RecA and RPA are approximately 16, 3 and 25 respectively (Lohman & Ferrari, 1994; Kowalczykowski *et al.*, 1994; Sugiyama *et al.*, 1997). Thus in all cases, the concentration of protein was saturating with respect to ssDNA.

Helicase assay reactions (350 μl volume) were performed as the standard reaction, but with 40 μM (nucleotides) dsDNA, no ssDNA, and 1 μM Hoechst 33258 dye (Eggleston *et al.*, 1996). These were initiated with 1.11 nM total RecBCD enzyme (0.576 nM functional RecBCD enzyme, corresponding to 0.0625 functional RecBCD enzyme molecule per linear dsDNA end).

Analysis of reaction products

Aliquots of the reaction mixture (30 μl) were taken at the indicated times and added to 35 μl of stop buffer (0.15 M EDTA, 3% (w/v) SDS, 10% (v/v) Ficoll, 0.125% (w/v) bromophenol blue, 0.125% (w/v) xylene cylanol) to halt the reaction and to deproteinize the sample. This was followed by the addition of 2 μl of 600 units/ml Proteinase K and incubation at 37°C for ten minutes. Samples were electrophoresed in 1% (w/v) agarose gels for approximately 15 hours at 1.4 V/cm in TAE buffer (40 mM Tris-acetate (pH 8.0), 2 mM EDTA). Samples (30 μl) analyzed by denaturing alkaline agarose electrophoresis were stopped by the addition of stop buffer as described above, followed by 14 μl of alkaline loading buffer (300 mM NaOH, 6 mM EDTA, 18% Ficoll, 0.15% (w/v) bromocresol green, 0.25% xylene cyanol). The samples were mixed and then subject to electrophoresis through 1% alkaline agarose gels for 15 hours at 1.4 V/cm in alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA). The gels were dried onto DEAE paper and analyzed on a Molecular Dynamics Storm 840 phosphorimager using Image-QuaNT software. Data were graphed using GraphPad Prism version 2.0 software. All comparisons of ssDNA fragment generation between different reactions were normalized to the amount of DNA unwound.

Helicase assays were performed as described by Eggleston *et al.* (1996). The excitation and emission wavelengths used were 552 and 596 nm, respectively.

Acknowledgments

We are grateful to both Frank Stahl and Andrei Kuzminov for their comments on this work and to the members of this laboratory for their critical reading of this material: Piero Bianco, Joel Brockman, Frederic Chedin, Jason Churchill, Frank Harmon, Alex Mazin, Jim New and Bob Tracy. This work was supported by funds from the National Institutes of Health grant GM-41347.

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Edited by M. Gottesman

(Received 19 February 1998; received in revised form 10 June 1998; accepted 18 June 1998)