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A Molecular Throttle: The Recombination Hotspot χ Controls DNA Translocation by the RecBCD Helicase

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Summary

RecBCD enzyme is a heterotrimeric helicase/nuclease that initiates homologous recombination at double-stranded DNA breaks. Several of its activities are regulated by the DNA sequence χ (5′-GCTGGTGG-3′), which is recognized in *cis* by the translocating enzyme. When RecBCD enzyme encounters χ, the intensity and polarity of its nuclease activity are changed, and the enzyme gains the ability to load RecA protein onto the χ-containing, unwound single-stranded DNA. Here, we show that interaction with χ also affects translocation by RecBCD enzyme. By observing translocation of individual enzymes along single molecules of DNA, we could see RecBCD enzyme pause precisely at χ. Furthermore, and more unexpectedly, after pausing at χ, the enzyme continues translocating but at approximately one-half the initial rate. We propose that interaction with χ results in an enzyme in which one of the two motor subunits, likely the RecD motor, is uncoupled from the holoenzyme to produce the slower translocase.

Introduction

RecBCD helicase/nuclease is a complex molecular machine that processes broken bacterial chromosomes (see Arnold and Kowalczykowski, 1999, for review). The heterotrimeric enzyme is driven by two helicase motors of opposite polarities, RecB and RecD (Dillingham et al., 2003; Taylor and Smith, 2003), and is furnished with the processivity and accessory factors required to carry out a highly coordinated set of biochemical reactions. RecBCD enzyme unwinds, on average, 30,000 bp of dsDNA before dissociation (Bianco et al., 2001; Roman et al., 1992) at a rate of approximately 1000 bp/sec at 37°C (Bianco et al., 2001; Roman and Kowalczykowski, 1989). DNA unwinding is accompanied by degradation of the nascent single-stranded DNA (ssDNA) preferentially on the 3′-terminated strand relative to the entry site of the enzyme (Dixon and Kowalczykowski, 1991, 1993). Central to the function of RecBCD enzyme is the recombination hotspot χ (Lam et al., 1974). In vivo, interaction of RecBCD enzyme with χ results in the stimulation of homologous recombination downstream of χ (Stahl et al., 1975; Stahl and Stahl, 1975). χ is an asymmetric eight-nucleotide sequence that is recognized by the translocating RecBCD enzyme only when the sequence is embedded in the 3′-terminated strand of the double-stranded DNA (dsDNA) molecule on which RecBCD enzyme translocates (Bianco and Kowalczykowski, 1997; Dixon and Kowalczykowski, 1993; Taylor et al., 1985). When χ is recognized, the modified RecBCD enzyme converts broken dsDNA containing a χ sequence into the species active in homologous recombination: ssDNA terminated at χ and coated with RecA protein (Anderson and Kowalczykowski, 1997b; Dixon and Kowalczykowski, 1991). In vitro, χ acts as a molecular switch to reduce degradation of the 3′-terminated strand and to direct this modified nucleolytic activity to the 5′-terminated strand (Anderson and Kowalczykowski, 1997a; Dixon and Kowalczykowski, 1991, 1993). The χ-modified enzyme also gains the ability to load the RecA protein, the DNA-pairing protein that acts in the next step of homologous recombination, onto the χ-terminated ssDNA produced by its helicase/nuclease activity (Anderson and Kowalczykowski, 1997b).

Due to the high translocation rate, the absence of synchronicity, and the wide distribution of translocation rates (Bianco et al., 2001), it has not been possible to detect any effect of χ recognition on the helicase activity of RecBCD enzyme using traditional bulk-phase assays. However, single-molecule analysis overcomes these limitations by permitting direct observation of translocation both before and after χ for individual RecBCD enzymes. We employed the experimental strategy described previously (Bianco et al., 2001). Briefly, individual biotinylated DNA molecules were attached to streptavidin-coated polystyrene beads and were stained with the fluorescent dye YOYO-1. In the absence of ATP, RecBCD enzyme was bound to the end of dsDNA that was not attached to the bead, forming an initiation complex. The optically trapped bead-DNA-RecBCD complex was extended by solution flow and was visualized using fluorescent microscopy. Moving the trapped complex into a flow channel containing ATP initiated RecBCD-mediated translocation and unwinding of the dsDNA, seen as a decrease in length of the dsDNA due to simultaneous DNA strand separation and displacement of the YOYO-1 as the enzyme unwinds and degrades the DNA. Previous work established that in the absence of χ recognition, individual RecBCD enzymes translocate along dsDNA unidirectionally, processively, and at a constant rate with no detectable pauses (Bianco et al., 2001; Dohoney and Gelles, 2001). The mean rate of the RecBCD translocation was similar to that derived from the bulk measurements (Roman and Kowalczykowski, 1989), but the behavior of individual enzymes deviated from the average substantially. Here, we show that χ alters the DNA translocation behavior of RecBCD enzyme, and we suggest that alteration is mediated by uncou-

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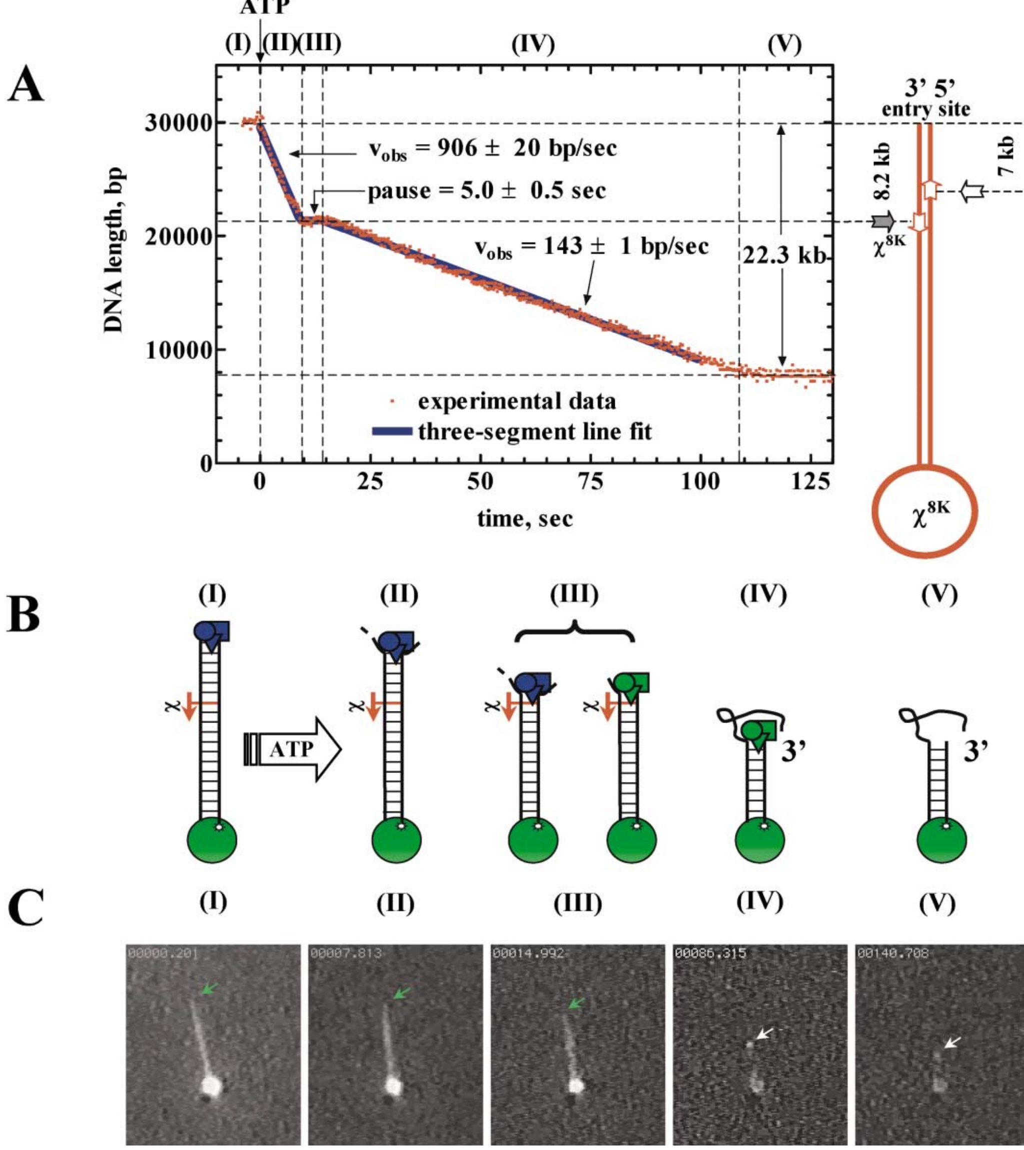


Figure 1. Recognition of χ Results in a Pause, Followed by a Reduction in the Rate of RecBCD Enzyme Translocation

(A) Analysis of a representative video visualizing RecBCD-mediated unwinding of a single DNA molecule (shown schematically at the right of the graph) containing χ positioned 8.2 kb from the DNA end and an additional reverse-oriented χ at 7 kb from the DNA end. The length of the dsDNA molecule was measured for each frame and presented as a time trace (red squares). Zero time corresponds to the moment when the RecBCD-DNA-complex was transferred to the ATP-containing channel. The rates of RecBCD-mediated DNA unwinding and both the duration and the position of the pause were determined by fitting the data corresponding to phases II, III, and IV to a continuous three-segment line (blue line).

(B) Schematic representation of the phases of RecBCD-mediated unwinding of χ-containing dsDNA. (I) RecBCD-DNA-bead complex is trapped in the sample side of the flow cell. (II) dsDNA unwinding by RecBCD enzyme results in a linear decrease in the dsDNA length upon relocation of the bead-DNA-RecBCD complex to the reaction (ATP-containing) side of the flow cell. (III) interaction with χ results in a pause in RecBCD enzyme translocation. (IV) the χ-modified enzyme continues to unwind the DNA, but at significantly reduced rate. (V) RecBCD enzyme dissociates from the DNA.

(C) Representative frames from the movie analyzed in (A). The green arrow in frames I–III indicates the end of the dsDNA molecule, where RecBCD enzyme acts, while the white arrow in frames IV–V points to the putative ssDNA.

pling one of the motor subunits of the holoenzyme. Thus, χ not only regulates the nuclease and RecA-loading capabilities of RecBCD enzyme, but it also regulates its DNA translocation velocity to perhaps coordinate the loading of RecA protein onto ssDNA and the subsequent DNA pairing step of homologous recombination.

Results

Recognition of χ Causes RecBCD Enzyme to Both Pause and Reduce Its Translocation Rate

The unwinding by a single RecBCD enzyme of a DNA molecule containing a χ locus located approximately

Table 1. RecBCD-Mediated Unwinding of Various dsDNA Substrates

Substrate (Length in bp; Number of Molecules Analyzed)	Rate before χ (bp/s)	Pause at χ (s)	Rate after χ (bp/s)	Number of Molecules Paused at χ	Position of the Pause (bp from End) [Actual Position of the χ Locus]
χ^{8K} (30010; 11)	540 ± 260	5.4 ± 3.0	203 ± 50	9	8280 ± 300 [8229]
$\chi^{7K(8K)}$(30010; 11)	623 ± 210	4.5 ± 4.1	289 ± 105	10 (upstream χ) 1 (downstream χ)	6809 ± 520 [7042] 8468 ± 274* [8229]
χ^{3K}(30095; 13)	611 ± 150	2.0 ± 0.9	346 ± 60	11	3540 ± 280 [3262]
χ^{rev}(30010; 9)	539 ± 130	–	N/A	0	–
χ^{0} (30885; 9)	502 ± 240	N/A	N/A	N/A	–

DNA substrates are shown schematically attached to the microspheres. Solid arrows represent correctly oriented χ sequences, whereas reverse-oriented χ sequences shown by dotted arrows. Values for the rates and both the position and duration of the pauses are given as an average value for all assayed molecules ± one standard deviation.

*Since only one molecule paused at the χ^{8K}, the position of the pause in this case calculated from the average length of the DNA molecule measured for each video frame during the duration of the pause ± one standard deviation for the DNA lengths measured during this stage. N/A = not applicable.

8.2 kb from the free DNA end is shown in Figure 1. The substrate, designated as χ^{8K}, is represented graphically on the right of the plot. (A movie showing unwinding of this molecule is provided as Supplemental Data online at http://www.cell.com/cgi/content/full/114/5/647/DC1). The length of the dsDNA was measured from captured video frames and plotted as a function of time in Figure 1A. The time trace can be divided into five distinctive phases, which are represented schematically in Figure 1B, and for which typical movie frames are shown in Figure 1C. Prior to the initiation of unwinding, the bead-DNA-RecBCD complex is held in the sample side of the flow cell in the absence of ATP (phase I), where the DNA length is constant. When the complex is moved to the ATP-containing side of the flow cell to initiate unwinding (phase II), a rapid shortening of the dsDNA is observed. This phase corresponds to the translocation of RecBCD enzyme along, and the consequent unwinding and degradation of, both strands of the dsDNA prior to χ, resulting in displacement of YOYO-1 from the dsDNA. This particular RecBCD enzyme traveled at 906 ± 20 bp/s, a velocity that is faster than average but within the range typically observed in this study. The average rate of unwinding (± one standard deviation) for 11 enzymes was 540 ± 260 bp/s at 23°C (Table 1, "rate before χ"), which is similar to that previously established (Bianco et al., 2001).

Because χ was reported to have no effect on DNA translocation by RecBCD enzyme (Dohoney and Gelles, 2001), we were surprised to observe a 5.0 ± 0.5 s pause in translocation at the expected position of χ^{8K}, which is indicated by the second horizontal dashed line (Figure 1A, phase III). For eleven χ^{8K} DNA molecules, a pause was observed in nine of the ten molecules whose unwinding did not terminate before χ (for details, see Supplemental Data). The average position (8280 ± 300 bp) of the observed pause was, within experimental error, at χ (which was 8229 bp from the end) (Table 1, "position of the pause"). The average length of the pause for this substrate was 5.4 ± 3.0 s (Table 1, "pause at χ").

RecBCD enzyme continued unwinding the DNA molecule after the pause but, quite unexpectedly, at a slower rate (phase IV). The molecule depicted in Figure 1 translocated after χ at a constant rate of 143 ± 1 bp/s for over 95 s. The average rate for the nine χ^{8K} molecules that recognized χ was 203 ± 50 bp/s (Table 1, "rate after χ"). Furthermore, during this phase of DNA unwinding, a clearly discernable fluorescent "spot" appeared at the end of the DNA molecule (Figure 1C, panel IV, and video in Supplemental Data). This fluorescent ellipsoid both translocated with the enzyme and grew in intensity (but was also subject to time-dependent photobleaching; Figure 1C, white arrow in panel V). Although YOYO-1 is commonly assumed to be a dsDNA-specific intercalating dye, it intercalates into regions of DNA secondary structure within ssDNA, and it also displays an alternative external binding mode that allows it to interact with both ds- and ssDNA (Larsson et al., 1994). These and other criteria (our unpublished data) lead us to suspect that fluorescent ellipsoid is the expected χ-containing ssDNA that is generated after χ recognition by the attenuated-nuclease form of the enzyme; however, the physical and functional characteristics of this unwinding product are still being analyzed. Finally, RecBCD en-

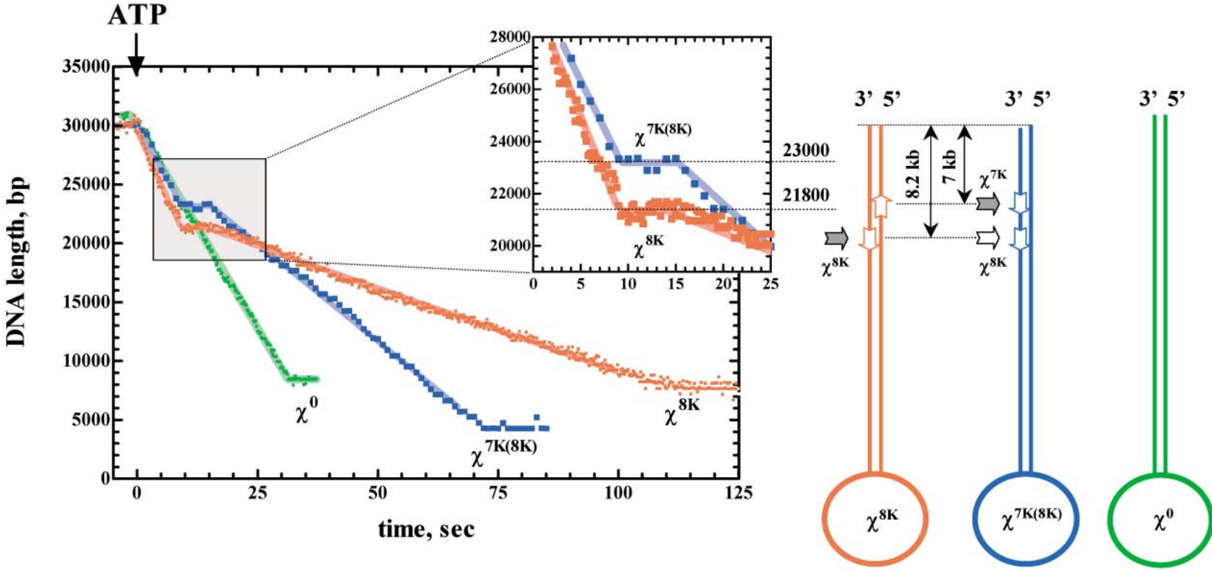


Figure 2. RecBCD Enzyme Pauses Precisely at χ

Representative time courses for RecBCD-mediated unwinding of dsDNA molecules with different positions and orientations of χ. The substrates are shown schematically on the right. Black arrows point to the correctly oriented χ sequences. Movies showing the unwinding of these substrates are provided in the Supplemental Data.

zyme stops translocating, DNA shortening ceases (phase V), and, as presumed based on bulk-phase observations, the enzyme dissociates. For the enzyme molecule depicted in Figure 1, dissociation occurred after unwinding 22.3 kb of DNA.

Translocation rates and both the position and duration of the pause were determined by fitting the experimental data corresponding to phases II, III, and IV (red squares in Figure 1A) to a contiguous three-segment line (blue line in Figure 1A). The slopes of the first and the third segments provided the rates of RecBCD translocation before and after interaction with χ, while the duration

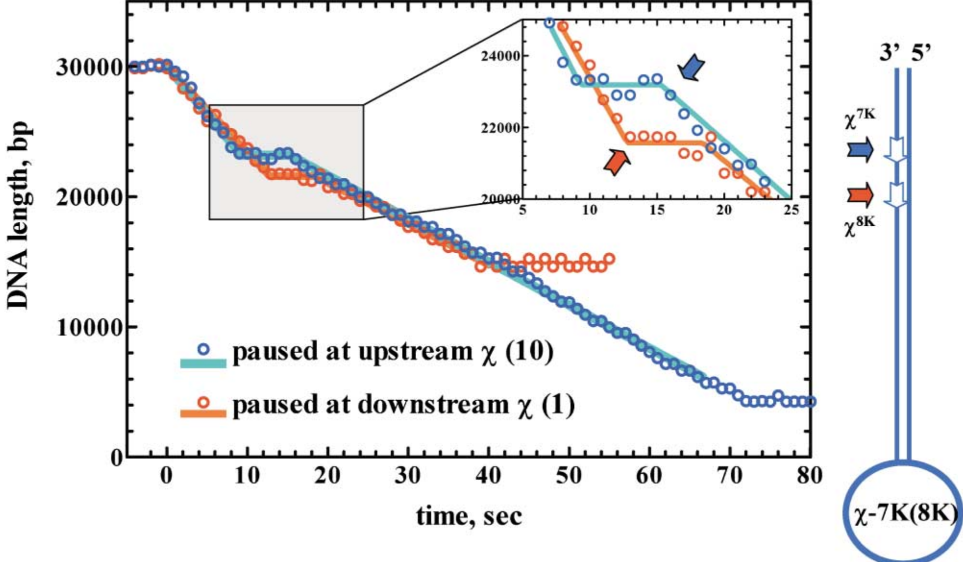
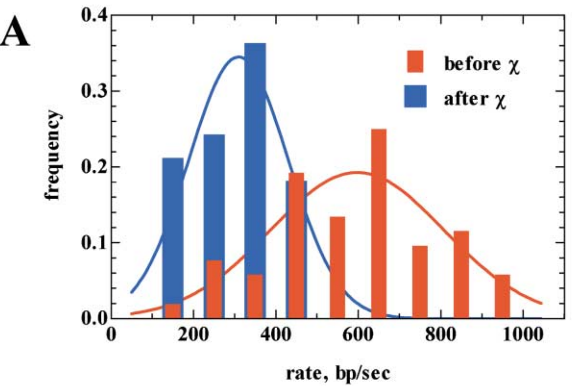


Figure 3. After Recognition of a χ Sequence, RecBCD Enzyme Does Not Alter Its Translocation Behavior at the Downstream χ

Time courses for RecBCD-mediated unwinding of the $\chi^{7K(8K)}$ dsDNA. The $\chi^{7K(8K)}$ dsDNA contains two correctly oriented χ loci, which are indicated by solid arrows in the schematic representation of the substrate molecule. The unwinding of a representative molecule in which a pause occurs at the upstream χ (blue arrow) is represented by blue circles and a light blue line. The red circles and an orange line represent the unwinding of DNA where the downstream (red arrow), but not the upstream, χ was recognized. The nearly identical rates of unwinding are purely coincidental.



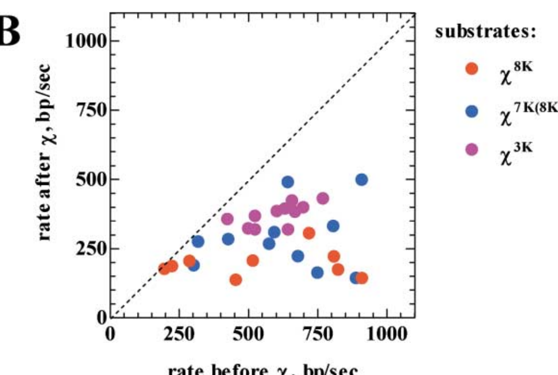


Figure 4. The Rates of RecBCD Enzyme Translocation before and after χ Recognition Are Independent of One Another

(A) Distribution of RecBCD enzyme translocation rates assigned to 100 bp/s intervals. Rates before χ (red bars) are summarized for all substrates (see Table 1), whereas rates after χ (blue bars) are only for molecules that displayed a pause at χ. The data were fit to Gaussian distribution curves with the following parameters: mean rate = 598 ± 34 bp/s, standard deviation = 210 ± 35 bp/s before χ, and mean rate = 309 ± 14 bp/s, standard deviation = 121 ± 14 bp/sec after χ.

(B) Scatter plot to illustrate the absence of correlation between rates before and after χ recognition.

and position of the pause were extracted from the second segment. This analysis was used for all of the data that are summarized in Table 1 and that are also presented in detail as Supplemental Data.

RecBCD Enzyme Pauses Precisely at χ

To confirm that the observed pause was indeed χ dependent, we designed DNA substrates essentially identical to the χ^{8K} DNA but with χ positioned at different distances from the DNA end. These substrates were designated as $\chi^{7K(8K)}$, χ^{3K}, χ^{rev}, and χ^{0}, reflecting the position and orientation of χ in these substrates (all substrates are presented schematically in Table 1): $\chi^{7K(8K)}$ DNA contains χ in the correct orientation at two locations, approximately 7 and 8.2 kb from the DNA end; χ^{3K} DNA contains χ at approximately 3.3 kb and also a reverse χ at 2.1 kb from the DNA end; χ^{rev} DNA has χ at 7 and 8.2 kb, both in the reverse orientation; and χ^{0} DNA is devoid of χ.

Unwinding of the χ^{8K} DNA molecule that was shown in Figure 1A is compared in Figure 2 to a $\chi^{7K(8K)}$ and to a χ^{0} DNA molecule (parameters for the unwinding of all individual molecules and movies showing the unwinding of three different molecules depicted in Figure 2 are presented as Supplemental Data, Tables S1–S3 and Movies S1–S3). The χ^{0} DNA shows a monotonic decrease in length, with no evidence of a pause, as reported previously (Bianco et al., 2001). However, a pause is evident in the $\chi^{7K(8K)}$, as was seen for the χ^{8K} substrate. The pause in unwinding of $\chi^{7K(8K)}$ DNA occurred approximately 7 kb from the DNA end, at the position of the first χ. The 1.2 kb difference in the position of the pause for χ^{8K} and $\chi^{7K(8K)}$ substrates was easily discerned (Figure 2, inset). Moreover, when χ was placed at a distance of 3.3 kb from the end, RecBCD enzyme paused exactly at 3.3 kb, as expected (Table 1, "position of the pause").

RecBCD enzyme spent, on average, 5.4 ± 3.0 s at χ when it was positioned 8.2 kb from the enzyme entry point, 4.5 ± 4.1 s when it was positioned 7 kb from the end, and 2.0 ± 0.9 s when χ was positioned 3.3 kb from the DNA end (Table 1, "pause at χ"). Considerable variation was observed in the pauses of individual enzymes: the longest observed pause was 15.6 s, while the shortest was 0.8 s (see Supplemental Tables S1–S3 for details).

The Correct Orientation of χ Is Required for Alteration of Translocation by RecBCD Helicase

In addition to the correctly oriented χ that is 8.2 kb from the free DNA end, the χ^{8K} molecules contained an additional χ positioned approximately 7 kb from the DNA end but in the opposite, or reverse, orientation (indicated by the open arrow in the schematic representation of χ^{8K} in Figure 1A). Since χ modifies the nuclease activity of RecBCD enzyme only when the enzyme approaches χ from the correct direction (its 3′ side), this reversed χ locus should not be recognized (Taylor et al., 1985). As expected, no alteration of RecBCD translocation at 7 kb was observed, indicating that both the pause and reduction in the translocation rate were specific to a correctly oriented χ sequence. Additionally, no pause at 2.1 kb was observed when the χ^{3K} substrate was used (Supplemental Table S3), and the behavior of RecBCD enzyme on DNA containing reverse-oriented χ (substrate χ^{rev}) was indistinguishable from that on χ^{0} DNA (Table 1).

When the Helicase Activity of RecBCD Enzyme Is Modified at One χ, No Additional Modification Is Observed at a Downstream χ

Both genetic (Myers et al., 1995) and biochemical (Taylor and Smith, 1992) studies have shown that when modified by its interaction with χ, RecBCD does not respond to a second χ sequence in *cis*. These studies examined recombination frequency and nuclease activity, respectively, but helicase activity was not tested.

To determine whether recognition of χ alters subsequent recognition of a downstream χ, the DNA substrate that contained two correctly oriented χ loci separated by 1.2 kb ($\chi^{7K(8K)}$) was examined. We observed that 10 of 11 RecBCD enzymes translocating through $\chi^{7K(8K)}$ paused at χ^{7K} (Table S2). Notably, none of the ten enzymes that

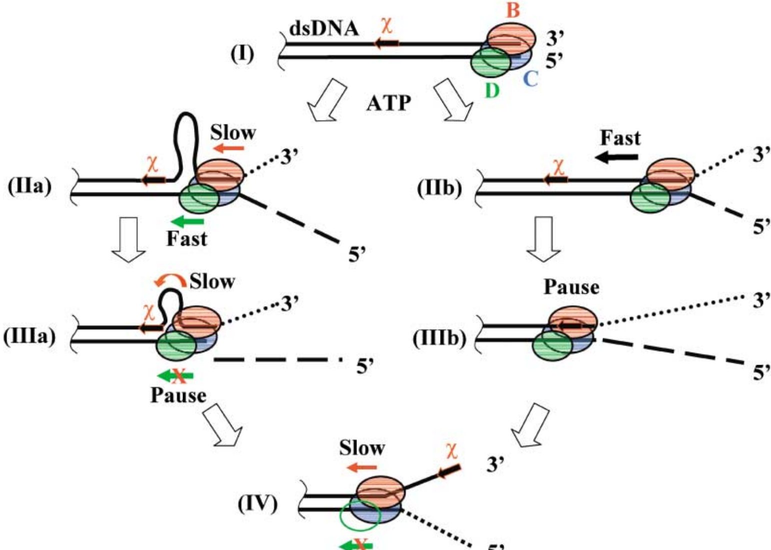


Figure 5. Model for the Control of the DNA Translocation Behavior of RecBCD Enzyme by χ

RecBCD enzyme is shown as a bipolar helicase with its two motor subunits translocating on the opposite strands of the DNA substrate molecule. In panels IIa–IIIa, RecD subunit is assumed to be the leading motor subunit prior to χ recognition. RecD is proposed to be inactivated at χ, and the pause reflects the time required for the slower (RecB) motor subunit to catch up with RecD and RecC stalled at χ. In panels IIb–IIIb, translocation of both motor subunits is assumed to occur at the same velocity. Pausing at χ in this model reflects the time required for the conformational change within RecBCD enzyme required to inactivate the RecD subunit. After χ recognition, the modified RecBCD enzyme is proposed to lack RecD function and, hence, translocates slowly. See text for more details.

paused at the first χ^{7K} locus paused at the second χ^{8K} locus (Figure 3, blue circles and light blue line). Conversely, the enzyme that failed to recognize χ^{7K} did recognize the second χ^{8K}, demonstrating that the downstream χ can indeed modify the behavior of RecBCD enzyme (Figure 3, red circles and orange line). These and the previously published findings (Myers et al., 1995; Taylor and Smith, 1992) show that interaction with a single χ is sufficient to modify RecBCD enzyme. This modified enzyme is now incapable of responding to a second χ in *cis*, a conclusion that now can be extended to include translocation behavior.

Relationship between RecBCD Translocation before and after χ

Before χ recognition, the RecBCD enzyme translocation rate was, on average, similar to that determined from both bulk-phase studies (Roman and Kowalczykowski, 1989) and single-molecule experiments using χ^0 λ DNA (Bianco et al., 2001). However, as shown previously (Bianco et al., 2001), the translocation rates of individual enzymes deviated significantly from the mean. The rates observed after χ also showed wide deviations from the mean. A histogram of translocation rates for 31 RecBCD enzymes (Figure 4A) shows that rates before and after χ are distributed normally around the mean. The mean translocation velocity for these enzymes was 598 ± 34 bp/s prior to χ recognition, and it decreased to 309 ± 14 bp/sec after interaction with χ. This represents a 2-fold change in translocation velocity that is attributable to the χ-induced modification. Interestingly, when the behavior of individual enzymes was compared (Figure 4B), no correlation between the two rates was observed: some of the enzymes that translocated relatively fast before χ proceeded after the pause more slowly than the mean, whereas some slower enzymes translocated after the pause with a faster than average rate. However, in all cases where χ was recognized, the translocation rate after χ was always slower than the rate prior to interacting with χ (Figure 4B), indicating that χ recognition results either in uncoupling of a factor responsible for the fast translocation or in loss of one of the two motor subunits that are involved in the translocation before χ.

Discussion

Our direct observation of the translocation by individual RecBCD enzymes along single dsDNA molecules literally shows that interaction with χ alters the translocation properties of RecBCD enzyme. We discovered that RecBCD enzyme pauses in response to a χ sequence recognition. The pause occurred precisely at χ, and only when χ was encountered in the correct orientation. After the pause, RecBCD enzyme continued to translocate and unwind DNA, but at a reduced rate. Thus, interaction with χ not only directs RecBCD enzyme to change its nucleolytic behavior and to deliver RecA protein to the resultant ssDNA, but now we see that it also causes the enzyme to both pause and change its translocation behavior.

Most models for the molecular basis of the modification in RecBCD enzyme that is induced by χ propose that the RecD subunit of the RecBCD enzyme is modified, ejected, or inactivated at χ (Anderson et al., 1997; Dixon et al., 1994; Myers et al., 1995; Taylor and Smith, 1999). Reduction in the RecBCD enzyme translocation rate after χ recognition provides another piece of evidence supporting this hypothesis. The mean rate of DNA unwinding after χ was approximately one-half of the initial rate (Table 1). Interestingly, while the unwinding rate of RecBC enzyme has never been determined accurately, perhaps it is not coincidental that it is approximately 2-fold lower than that of the RecBCD holoenzyme (Korangy and Julin, 1994; Rinken et al., 1992; and our unpublished data).

A persistent change in the translocation velocity of a DNA motor protein in response to signal embedded in the DNA is without precedent. Here, we present a model with two possibilities that offer a molecular explanation for this phenomenon (Figure 5). RecBCD holoenzyme is known to be a bipolar helicase driven by two motors,

the RecB and RecD subunits, each of which is capable of being the motor of the holoenzyme that drives DNA unwinding (Dillingham et al., 2003; Taylor and Smith, 2003). Upon binding to the dsDNA end (I), RecBCD enzyme translocates through and unwinds dsDNA (IIa and IIb). For the first possibility, we consider the case that the translocation rates of RecB and RecD subunits are unequal (IIa). In this case, only the leading motor acts as a bona fide helicase, and it is this translocation that we are detecting. The slower subunit simply translocates along ssDNA, allowing a ssDNA loop (Taylor and Smith, 1980) to form between the leading and the lagging subunits. While it is uncertain which subunit recognizes χ, the existence of *recC* mutants that enable RecBCD enzyme to recognize an altered χ sequence suggests that the RecC subunit recognizes χ (Arnold et al., 1998). Since the RecC subunit is not a motor protein itself, we envision that it is delivered to χ by the leading motor. Recognition of χ by RecC protein is likely manifest as tighter binding of this subunit to the χ sequence, which can stall the leading motor subunit (IIIa) until the slower motor subunit catches up with the stalled one. In this case, the observed pause could be explained as the time required for the slower motor to reach χ (IV). Our finding that the pause at χ is proportional to the distance from χ to the free DNA end (Table 1) apparently supports this model, but the high experimental uncertainty of those data precludes their use as unambiguous verification of this view. However, this model would also explain why no pausing was observed in the tethered particle motion experiment (Dohoney and Gelles, 2001), because a short DNA substrate was used, and the resultant pause might have been too brief to detect; in addition, the required use of low concentrations of ATP ($<<K_m$) in those experiments may have contributed to the failure to detect a pause. In our model then, once the slower motor reaches χ, unwinding beyond χ ensues, but at the lower rate of the slower motor. Because the RecD motor is proposed to be nonfunctional beyond χ, in this scenario the RecB subunit would need to be the slower motor, a view that would also be consistent with the conclusion of Taylor and Smith (2003). The second possibility is that both motors move at the same rate (IIb). In this case, χ recognition would represent the same stalling of the enzyme as described above, during which time the RecD motor subunit is inactivated or dissociated. After exiting from the pause (IIIb), the enzyme is now slowed due to the proposed loss of RecD motor function. Hence, the translocation rate after χ recognition is always slower than that before χ (IV).

Our results, therefore, demonstrate a new role for the recombination hotspot χ. This octameric DNA sequence was previously found to be a molecular switch that both switched and reduced the enzyme's nuclease activity (Dixon and Kowalczykowski, 1991, 1993). Here, we establish that χ also plays the role of a "molecular throttle" that directs RecBCD enzyme to momentarily stop at χ and then to attenuate its translocation velocity. We imagine that response to the translocation throttle is as important for the recombination function of RecBCD enzyme as is the response to the nuclease switch, perhaps to coordinate loading of RecA by slowing RecBCD enzyme and thereby enabling contiguous nucleoprotein filament formation by the slowly associating RecA protein.

Experimental Procedures

Proteins and DNA Substrates

RecBCD and SSB proteins were purified using published protocols (LeBowitz, 1985; Roman and Kowalczykowski, 1989).

DNA substrates, 30 kb in length, were designed to allow visualization of RecBCD-mediated translocation both before and after χ sequences. The limited probability (30%–40%) of χ recognition (Arnold et al., 1998; Dixon and Kowalczykowski, 1995; Taylor et al., 1985) results in a substantial number of RecBCD enzyme molecules that fail to recognize χ. However, previous studies established that the net probability of recognition in *cis* can be increased by using consecutive copies of χ sequence (Dixon and Kowalczykowski, 1993; Kuzminov et al., 1994). Therefore, to increase the fraction of assayed molecules in which χ would be recognized, we used DNA with three consecutive χ sequences spaced ten nucleotides apart at each recognition locus. Because our instrument cannot resolve χ sequences 10 bp apart, these tandem triple χ sequences effectively behave as a single, high-efficiency χ locus (~80%–90% recognition). All of the DNA substrates used in this paper contain this triple χ construct, which for simplicity, is referred to as χ. These χ-containing DNA substrates were produced by amplification of 30 kb regions using χ-containing λ DNA as a substrate. The χ sequences were introduced into λ DNA by ligation of EcoRI-linearized pBR-3F3H (Anderson et al., 1999) and pSNH33 (constructed by self-ligating the SspI-PvuII restriction fragments of the pBR-3F3H in the reverse orientation) plasmids, with digested λgt10 vector (a generous gift from Dr. Ichizo Kobayashi, Tokyo University). The recombinant λ DNA was recovered by in vitro packaging using the Package-Lambda DNA Packaging System (Promega) and then plating the phage lysate on *E. coli* C600 *hflA* (also a generous gift from Dr. Ichizo Kobayashi, University of Tokyo). The direction of the inserts was determined by restriction digestion with EcoRI and HindIII enzymes. Biotinylated dsDNA substrates for the single-molecule experiments were produced by amplification of a 30 kb region of the χ-containing λ DNA purified using the Lambda Purification Kit (QIAGEN). The Expand 20 kb^{PLUS} PCR system (ROCHE) and a biotinylated and a nonbiotinylated primer were used for the PCR reaction. Upon completion of the reaction, excess primers were removed using MicroSpin S400 spin columns (Amersham Biosciences). Biotinylated 5′-TAACGTCATGTCAGAGCAGAAAAAG-3′ primer and nonbiotinylated 5′-TCCCGTCTTTATCATCAACGTATTT-3′ primer were used to amplify the χ^{8K}, $\chi^{7K(8K)}$, and χ^{rev} substrates, while biotinylated 5′-AGTATCGGTAAGGCGGTGAC-3′ primer and nonbiotinylated 5′-GCCCATGACAGGAAGTTGTT -3′ primer were used to amplify the χ^{3K} and χ^{0} substrates.

DNA Bead Preparation

DNA was attached to beads using modification of a procedure used previously (Bianco et al., 2001). The biotinylated DNA (50 ng) was incubated with 20 μl of 1 μm ProActive streptavidin-coated microspheres (Bangs Laboratories) for 1 hr at 37°C in 80 mM $NaHCO_3$ (pH 8.2). Bead-DNA complexes were transferred into 1.5 ml of degassed sample solution containing 45 mM $NaHCO_3$ (pH 8.2), 20% (w/v) sucrose, 50 mM DTT, and 100 nM YOYO-1 dye (Molecular Probes). DNA was incubated with the dye for at least 1 hr in the dark at room temperature. Immediately before transfer to the sample syringe, 2 mM magnesium acetate and 100 nM RecBCD enzyme were added to the sample mixture. The reaction solution contained 45 mM $NaHCO_3$ (pH 8.2), 20% (w/v) sucrose, 50 mM DTT, 1 mM ATP, 2 mM magnesium acetate, and 20 nM YOYO-1 dye. Under these conditions, prior to χ, both DNA strands are degraded by RecBCD enzyme; after χ recognition, the expected χ-containing ssDNA fragment is produced (data not shown).

Optical Trapping and Fluorescence Microscopy

DNA helicase reactions were performed as described (Bianco et al., 2001). All reactions were carried out at room temperature, at a linear flow rate between 150 and 170 μm/s. Videos of the enzyme translocation were recorded at 5 frames/s and analyzed using Scion Image

Software. The length of the DNA molecule in each frame was measured as described previously (Bianco et al., 2001) and analyzed using Prism (GraphPad) software. The rates before and after χ, as well as position and duration of the pause, were determined by fitting experimental data to the contiguous three-segment line.

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