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Direct Visualization of RecBCD Movement Reveals Cotranslocation of the RecD Motor after χ Recognition

Short Article

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Summary

In *Escherichia coli*, χ (5′-GCTGGTGG-3′) is a recombination hotspot recognized by the RecBCD enzyme. Recognition of χ reduces both nuclease activity and translocation speed of RecBCD and activates RecA-loading ability. RecBCD has two motor subunits, RecB and RecD, which act simultaneously but independently. A longstanding hypothesis to explain the changes elicited by χ interaction has been "ejection" of the RecD motor from the holoenzyme at χ. To test this proposal, we visualized individual RecBCD molecules labeled via RecD with a fluorescent nanoparticle. We could directly see these labeled, single molecules of RecBCD moving at up to 1835 bp/s (0.6 μm/s). Those enzymes translocated to χ, paused, and continued at reduced velocity, without loss of RecD. We conclude that χ interaction induces a conformational change, resulting from binding of χ to RecC, and not from RecD ejection. This change is responsible for alteration of RecBCD function that persists for the duration of DNA translocation.

Introduction

The heterotrimeric RecBCD enzyme is a highly processive DNA helicase and nuclease that is involved in the seemingly conflicting functions of recombinational DNA repair and the destruction of foreign (e.g., phage) DNA (Kowalczykowski et al., 1994; Smith, 2001). However, its biological activities are regulated by the recombination-activating site χ, when χ is recognized during the course of translocation through double-stranded DNA (dsDNA). Interaction with χ not only downregulates the nuclease activity of RecBCD (Dixon and Kowalczykowski, 1991; Dixon and Kowalczykowski, 1993) but also switches the bias of DNA degradation away from the 3′-terminated strand (relative to the dsDNA end entered) to the 5′-terminated strand (Anderson and Kowalczykowski, 1997a). As a result, a 3′ single-stranded DNA (ssDNA) tail is produced onto which the χ-modified RecBCD loads RecA protein for the subsequent step of recombinational repair (Anderson and Kowalczykowski, 1997b). Recognition of χ also reduces the translocation speed of the bipolar RecBCD (Spies et al., 2003), possibly by affecting translocation of one of the two motor subunits, RecB and RecD, that move together within the holoenzyme but at velocities that are independent of one another (Dillingham et al., 2003; Taylor and Smith, 2003). The biochemical regulation by χ has the interesting and curious property that the changes elicited persist for the duration of the translocation event in cis, but the phenomenon is fully reversible and catalytic after dissociation of RecBCD from the χ-containing DNA molecule (Dixon et al., 1994). The molecular basis for this regulation remains unknown. One attractive hypothesis, proposed over a decade ago, is the RecD-ejection model (Koppen et al., 1995; Kuzminov et al., 1994; Myers et al., 1995; Stahl et al., 1990; Thaler et al., 1989). This longstanding hypothesis is based on the many similarities in genetic and biochemical behavior of the χ-modified RecBCD and the RecD-lacking RecBC; however, contravening results exist (Anderson et al., 1997; Lovett et al., 1988; Taylor and Smith, 1992). Although it would appear straightforward to detect loss of a subunit by traditional methods, due to both the rapid translocation of the RecBCD helicase and the transient nature of the proposed RecD-ejection, direct proof is lacking.

Single molecule observation is a powerful way to interrogate an individual enzyme. Recently, using YOYO-1-stained DNA, we showed that RecBCD pauses at χ for about 5 s and, subsequently, continues translocating but with an approximately 2-fold reduced velocity (Spies et al., 2003). These observations could be used to test whether RecD dissociated at χ, provided that RecBCD, specifically RecD, could be visualized directly. However, many single-molecule methods measure interaction indirectly, and direct visualization of rapidly moving molecules is limited by the ability to collect sufficient signal in the time that the molecule is within a given location (see reviews by Allemand et al., 2003; Bockelmann, 2004; Bustamante et al., 2003, and references therein); consequently, direct visualization by fluorescence is typically limited to motor proteins that are moving relatively slowly or requires the use of total internal reflection fluorescence (TIRF) microscopy. An earlier single-molecule study of RecBCD enzyme failed to detect dissociation of the RecD subunit upon encountering a χ sequence (Dohoney and Gelles, 2001), leading those authors to conclude that RecD was not ejected at χ; unfortunately, this conclusion was compromised because neither a χ-dependent pause nor velocity reduction was seen. Experimental limitations required low (≤50 μM) ATP concentrations to slow the enzyme, and recognition of χ apparently did not occur at these conditions. Thus, χ recognition by RecBCD requires high concentrations of ATP and, under these conditions, RecBCD is typically moving at 500 to 1000 bp/s, necessitating a single-molecule procedure that can observe rapidly moving motor proteins. Here, we

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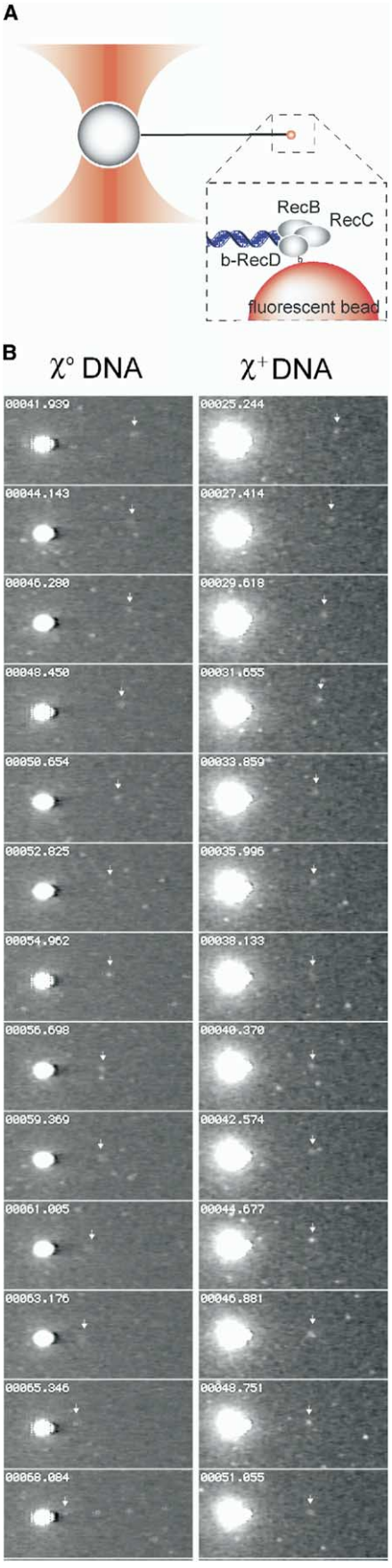

describe a procedure to track translocation by RecBCD enzyme directly, which can be generally applied to other rapidly translocating motor proteins. We show that the RecD motor remains associated with the RecBC subunits throughout translocation, even when χ is encountered.

Results and Discussion

To directly visualize a rapidly moving RecBCD molecule without the constraints of TIRF, we attached a 40 nm streptavidin-coated fluorescent bead (nanoparticle) to RecBCD that had been biotinylated in vivo at a unique site on RecD. The nanoparticle contains several hundred chromophore equivalents, thereby providing a large fluorescent signal that is directly associated with the motor protein. We used the instrument and experimental procedure that we had established previously (Bianco et al., 2001; Spies et al., 2003). In brief, biotinylated DNA was separately bound to a streptavidin-coated 1.0 μm polystyrene bead and then mixed with the RecBCD that had been tagged with the fluorescent bead (Figure 1A); however, in contrast to past procedures, the DNA was not fluorescently labeled. The bead-DNA-RecBCD-nanoparticle complex was captured by an optical trap (Figure 1A) and then moved to the second channel of a flow cell containing ATP to start translocation.

Figure 1B shows individual frames from the video recording of two typical experiments. We found that the fluorescent nanoparticles bind nonspecifically to the polystyrene bead, leading to a very bright spot at the focus of the optical trap. Although the DNA is not visible, the labeled nanoparticle could be seen at a distance corresponding to the expected length of the DNA (the direction of flow is to the right in each panel). This tethered particle moved when the complex was introduced to ATP, verifying that the particle was attached to RecBCD (the bright arrows point to the faint nanoparticle). Nonspecific binding of the nanoparticle to DNA, nonbiotinylated RecBCD, or RecBC was negligible (<1%, based on 400–500 randomly trapped beads for each); in contrast, 13% of the trapped DNA-beads (n = 272) had a nanoparticle bound to the DNA end when biotinylated RecBCD was present. Measurement of a nanoparticle's

Figure 1. Direct Visualization of Translocation by a Single RecBCD Molecule via a Fluorescent Nanoparticle Attached to RecD

(A) Illustration of an optically trapped DNA molecule to which a RecBCD nanoparticle is attached at the opposite end (not to scale). The large sphere represents the 1.0 μm bead caught in the optical trap. DNA is attached to this bead and is extended by flow. Biotinylated RecBCD is bound to the free end of the DNA, and a 40 nm streptavidin-coated, fluorescent sphere is attached to the biotinylated RecD subunit ("b-RecD").

(B) Individual frames from a video recording of the RecBCD-nanoparticle translocating along a DNA molecule. The position of the RecBCD-nanoparticle is indicated by the arrow; the entire video is available as Supplemental Data. The distance between the microsphere and the nanoparticle is approximately 12–13 μm in the first frame of each panel. The numbers in each frame represent an arbitrary time stamp in seconds.

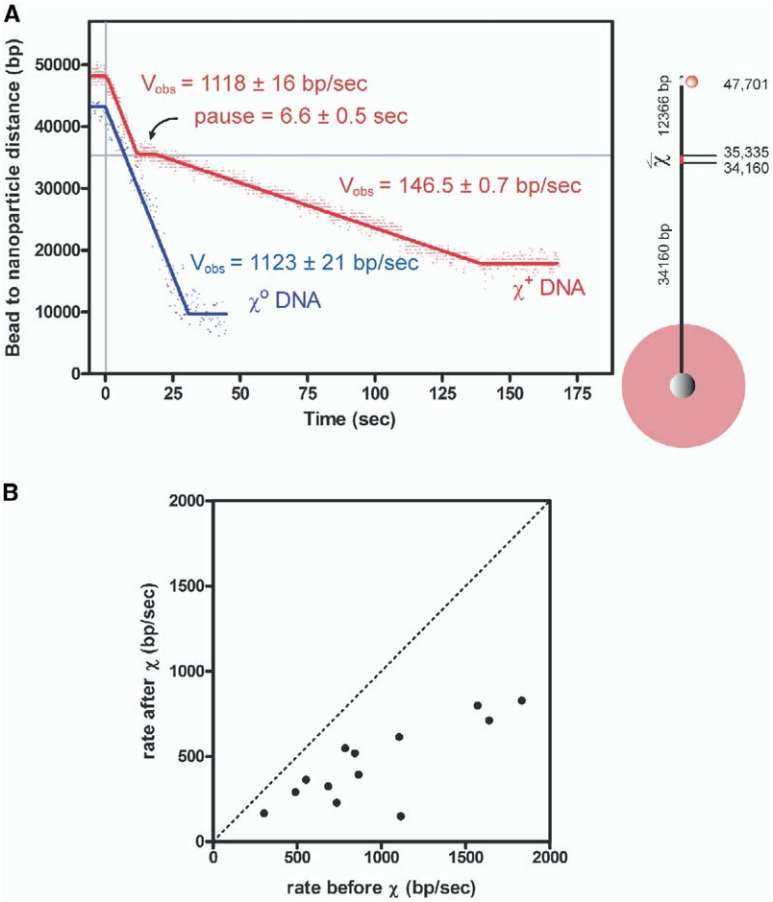

Figure 2. The RecD Motor Remains Associated with the Holoenzyme Both at and after Recognition of χ

(A) Quantitative analysis of the full video data set represented by the frames shown in Figure 1B. The horizontal line indicates the position of χ in the DNA.

(B) Translocation speed before and after interaction with χ, showing that each molecule is slowed after the interaction.

position as a function of time yielded the data in Figure 2A. For a χ^0 DNA molecule, the change in position is linear with time, revealing that the translocation speed of this particular RecBCD-nanoparticle complex is 1123 ± 21 bp/s at 37°C. As observed previously (Bianco et al., 2001; Spies et al., 2003), the translocation rate of any given RecBCD molecule was always constant to within a few percent, and there were no detectable pauses or changes in velocity (Figure 2A; Movie 1). However, as seen before, the variation within the population of molecules examined was large, ranging from 388 (±38) bp/s to 1835 (±293) bp/s. Due either to uncertainty in defining the precise center of the intensely bright 1.0 μm polystyrene bead or to breakage of some DNA molecules during preparation of the complexes, some DNA lengths appeared shorter than full length (e.g., Figure 2A, the χ^0 DNA). This uncertainty had no effect on the rate measurements, and the average rate of unwinding for 28 fluorescently tagged RecBCD molecules (which includes 13 molecules prior to their encounter with χ; see below) was 830 ± 700 bp/s at 37°C and is consistent with our earlier measurements (Bianco et al., 2001; Eggleston and Kowalczykowski, 1993; Roman and Kowalczykowski, 1989; Spies et al., 2003). In addition, the processivity of translocation remained high, with the average distances traveled on χ^0 DNA being 21,300 ± 8500 bp (~7.2 μm); this value is the same, within experimental error, as the processivity measured previously (Bianco et al., 2001; Roman et al., 1992).

To determine the fate of RecD at and after interaction of RecBCD with χ, DNA containing χ was used (Spies et al., 2003). As before, the lambda DNA contained two χ loci (three χ sequences at each locus), both in the active orientation, located 12,366 and 13,541 bp from the entry site. Figures 1B and 2A show that prior to the χ site, a fluorescently tagged RecBCD can be seen translocating on the DNA at a speed of 1118 ± 16 bp/s (Movie 2); for the 13 molecules that recognized χ (see below), the average speed prior to χ was 960 ± 470 bp/s. Importantly, at χ, the enzyme in Figures 1B and 2A is seen to pause for 6.6 ± 0.5 s. Only 2 of the 13 molecules visualized failed to pause (but they did change their translocation speed; see below); the duration of the observed pauses ranged from 0–15 s and averaged 4.9 s. The fact that the nanoparticle remained attached to RecBCD for the same length of time as observed in our previous, DNA signal-based study (Spies et al., 2003) shows that RecD remained bound to the holoenzyme for the duration of the pause at χ.

More significantly, after pausing, the nanoparticle moved away from the χ site at a reduced rate of 147 ± 0.7 bp/s. The collection of molecules observed here reduced their rate of translocation by ~2-fold, on average, moving at 450 ± 230 bp/s after χ, paralleling previous results exactly (Spies et al., 2003). For each enzyme, the translocation velocity after χ was constant and always lower than the velocity prior to χ (Figure 2B). This behavior demonstrates directly and unequivo-

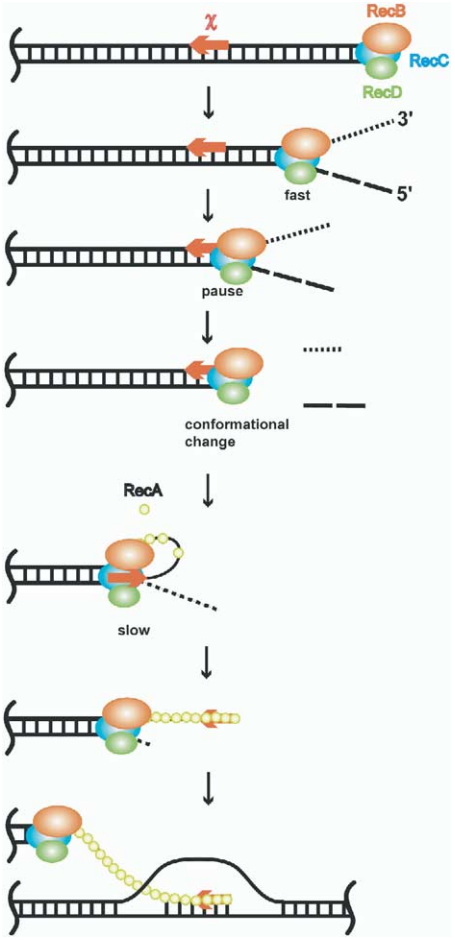

Figure 3. Molecular Mechanism for the Initial Steps of Homologous Recombination Mediated by RecBCD and χ

RecBCD translocates along dsDNA with the RecB motor translocating 3′→5′ and the RecD motor moving 5′→3′ direction on the complementary strand (Dillingham et al., 2003; Taylor and Smith, 2003). Before χ, RecBCD cleaves the 3′-terminated ssDNA frequently. RecBCD recognizes χ, pauses, and changes its conformation, because χ is bound to the enzyme (Spies et al., 2003), likely via RecC (Arnold et al., 2000). Consequently, continued threading of the 3′-terminated strand into the nucleolytic site is halted, blocking further degradation but permitting occasional cleavage of the 5′-terminated ssDNA (Singleton et al., 2004). Conformational modification, but not dissociation, of a motor subunit (likely RecD) due to χ binding results in the reduced translocation speed. The conformational change also reveals the RecA-loading site of RecB (Churchill and Kowalczykowski, 2000) and RecA protein is loaded onto the χ-containing 3′-terminated ssDNA. RecA protein promotes invasion of homologous dsDNA to commence the subsequent steps of recombination and DNA repair.

cally that the RecD subunit does not dissociate at or after χ. Thus, the RecD-ejection hypothesis is not confirmed. Although one can formally propose that RecD dissociates from RecBC and then translocates on its own, since it does have a 5′→3′ helicase activity (Dillingham et al., 2003), this possibility is eliminated because on its own, RecD has a low processivity and can unwind no more than approximately 55 bp before dissociation (M.S. Dillingham and S.C.K, unpublished data). In contrast, here we saw that the nanoparticle-labeled, χ-modified enzymes traveled an average total distance of 26,800 ± 10,000 bp (ranging from 12,388–45,572 bp; data not shown), which is an average distance of 13,250 bp beyond χ. Thus, all indications support the simplest model that the heterotrimeric enzyme persists throughout the span of travel through χ-containing DNA.

We have established a generally applicable strategy for visualizing the rapid movement of individual motor proteins. Furthermore, by attaching a nanoparticle to the RecD subunit of the bipolar helicase RecBCD, we established that this motor remains part of the molecular machine that repairs DNA by recombination. It has proven difficult, if not impossible, to prove or disprove this longstanding model by bulk phase methods (Churchill, 1999), but our single-molecule results are clear. The failure to see RecD ejection does, however, raise the question of how RecBCD is regulated by χ at the molecular level. Using a complementary single-molecule approach (Spies et al., 2003) and ensemble studies with a RecBCD-homolog (F.S. Chédin, N.H., and S.C.K., unpublished data), we suggested that the χ-containing ssDNA remains bound to RecBCD after χ. Consequently, we propose that regulation by χ is due to binding of the χ-containing ssDNA to RecC (Arnold et al., 2000) (Figure 3). This binding must affect translocation by one of the motor subunits, and recent crystallographic solution of the enzyme's structure reveals that such a proposed binding event would, at the very least, limit entry of the 3′-tailed ssDNA into the nucleolytic site of the enzyme and likely affect translocation (Singleton et al., 2004). Upon dissociation from the DNA, RecBCD may disassemble (Dixon et al., 1994; Taylor and Smith, 1999), allowing release of χ from the holoenzyme and thereby permitting renewed catalytic action after reassembly of the subunits. This hypothesis, namely, the regulation of RecBCD function directly by the binding of χ in cis, explains most, if not all, of the χ-regulated phenomena.

Experimental Procedures

Proteins and DNA substrates

Biotinylated RecBCD was purified from an *E. coli* strain harboring four plasmids, pMS421 (Heath and Weinstock, 1991) (*lacI*^{q} spc), pPB700 (Boehmer and Emmerson, 1991) (*recB*$^+$ amp), pPB520 (Boehmer and Emmerson, 1991) (*recC*$^+$ cml), and pWKS6 (*biotin-recD* kan; a derivative of pWKS130 [Wang and Kushner, 1991] produced as described below). The resulting heterotrimeric RecBCD enzyme contains RecD with an N-terminal hexahistidine tag, followed by a 16 amino acid sequence containing the uniquely biotinylated lysine residue.

The vector initially used for expression of the biotinylated protein construct was pTrcHisB (Invitrogen). The region between the NheI and BamHI restriction enzyme sites, encoding a enterokinase cleavage site, was removed and replaced with complementary annealed oligonucleotides (5′-CTAGCCTGGCTGTCATTTTCGAAGCT CAGAAAATGGAATGGCACTCTAGAGCGAAG-3′ and 3′-GGACCA CAGTAAAAGCTTCGAGTCTTTTACCTTACCGTGAGATCTCGCTTCC TAG-5′) that encode for a 16 amino acid peptide (LAVIFEAQ**K** MEWHSRA), which included the lysine residue (bold) critical for biotinylation. The sequence of the peptide encoded by these oligonucleotides was obtained from the previously defined consensus sequence (Schatz, 1993). The resulting vector, pTrc-biotin-HisB, maintained the NheI and BamHI sites embedded within the recognition sequence for BirA protein, biotin synthetase. The *RecD*

gene was then cloned in frame into the BamHI site of the multiple cloning site. The resulting plasmid was pTrc-biotin-HisB-RecD. Because expression from this vector was too high, resulting in diminished levels of RecB and RecC subunit expression, the entire coding region of pTrc-biotin-HisB-RecD from the ApaI site to the EcoRI site was subcloned into the multiple cloning site of pWKS130 (Wang and Kushner, 1991), which is a low copy number vector compatible with the plasmids used for RecB and RecC subunit coexpression. The resulting vector was then transformed into strain V186 (Chaudhury and Smith, 1984). This construct also contained a hexahistidine tag on RecD that does not interfere with function (Chen et al., 1997), which was included as an aid in protein purification if needed. The cells were grown in L broth with selective antibiotics and biotin (113 μM). After addition of 1 mM IPTG at an OD_{600} of 0.5, cells were grown for 3 hr and then harvested. RecBCD holoenzyme was purified as described (Bianco and Kowalczykowski, 1997) up to the Q-sepharose chromatography step. After dialysis against 0.1 M phosphate (pH 7.2) and 0.15 M NaCl, the pool from the Q-sepharose column (Amersham-Pharmacia) was applied to an UltraLink Immobilized Monomeric Avidin column (Pierce). Biotinylated RecBCD was eluted with 2 mM D-biotin (Sigma), dialyzed to remove biotin, and then stored in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, and 50% glycerol. The yield was 11.7 mg of holoenzyme from 2 liters of culture. The resulting protein contained each of the three subunits in a 1:1:1 ratio and was approximately 98% pure, with the contaminant likely being GroEL. The purified RecBCD enzyme contained only one subunit, the biotinylated RecD subunit, that gave a positive signal in a biotin-blot assay; in this assay, RecBCD was subjected to SDS-PAGE and then transferred to nitrocellulose membranes by using standard Western blotting techniques. Instead of probing the membranes with antibodies, however, streptavidin conjugated to alkaline phosphatase (Bio-Rad) was used to probe for the presence of biotin; detection was with alkaline phosphatase color reagents that were used according to the instructions supplied with the supplier. This biotinylated enzyme displayed fully wild-type behavior in all in vitro assays: ATPase, helicase, nuclease, χ recognition and response, and RecA loading (data not shown). Helicase assays showed that saturation of activity occurred at approximately one holoenzyme per dsDNA end, corresponding to a protein preparation that is 100% active.

Commercially available λ phage DNA was used as the χ^0 DNA substrate (New England Biolabs), and the χ-containing λ DNA was described previously (Spies et al., 2003).

Bead-DNA-Biotinylated RecBCD-Nanoparticle Complex Preparation

A complementary 12 nucleotide 3′-biotinylated oligonucleotide was annealed and ligated to the *cosL* end of λ DNA. This biotinylated λ DNA was attached to 1.0 μm streptavidin-coated polystyrene beads (Bangs Laboratories, Inc.) by incubation of 4 μl of 100 mM $NaHCO_3$ (pH 8.2), 4 μl of beads (~30 pM), and 16 μl of biotinylated lambda DNA (~100 pM) for 1 hr at 37°C. The biotinylated RecBCD enzyme (5.9 pmol) was separately incubated with 0.054 pmol of 40 nm streptavidin-coated fluorescent beads (TransFluoSpheres; excitation 488 nm; emission 645 nm; Molecular Probes) for 10 min at 37°C in 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, and 0.02% (v/v) Tween 20 by mixing 4.8 μl of biotinylated RecBCD (1.22 μM) and 3 μl of nanoparticles (~18 nM); an excess of RecBCD enzyme over the bead concentration was used due to observed nonspecific loss of enzyme throughout the subsequent procedures. Next, 1.5 μl of 20 mM $Mg(OAc)_2$ and 6 μl of the λ DNA-bead complex was added to 7.8 μl of the biotinylated RecBCD-nanoparticle complex, and the mixture was further incubated for 2 min. Finally, the solution (15.3 μl) was transferred into 400 μl of degassed sample buffer containing 45 mM $NaHCO_3$ (pH 8.2), 20% (w/v) sucrose, 50 mM dithiothreitol, 2 mM magnesium acetate, and 0.5% (v/v) blocking solution (Molecular Probes). The resulting bead-DNA-RecBCD-nanoparticle complex was then transferred into a sample syringe. Under these conditions, when visualized by YOYO-1 staining, approximately 80% of the 1.0 μm beads have a single DNA molecule bound, despite the use of excess DNA over bead, due to both nonspecific loss of DNA and less than perfect ligation and binding. The reaction solution contained 1 mM ATP in sample buffer.

Optical Trapping and Fluorescence Microscopy

The instrument used was previously described (Bianco et al., 2001). The changes were that the fluorescent nanoparticle-RecBCD complex was excited with an appropriate filter set (Ethidium Bromide set 41006; Chroma Technology Corp.) and the images were captured in real time by an electron bombardment CCD camera (EB-CCD C7190-23; Hamamatsu Photonics, Hamamatsu, Japan). The sample syringe contained bead-DNA-RecBCD-nanoparticle complexes in the buffer without ATP. When the 1.0 μm bead was captured by the optical trap, depending on the preparation, 5%–25% of the beads were also were associated with a smaller fluorescence signal at the expected position of the DNA end. For the flow rate used (30 μL/hr [a linear flow rate of 80 μm/s]), DNA is not completely extended to its contour length; therefore, we measured the observed extension of λ DNA experimentally and determined that a 1 nm length of DNA corresponds to 3.8 ± 0.1 bp. All reactions were performed at 37°C. Videos were analyzed using Scion Image Software. The position of the nanoparticle relative to the microsphere was determined in individual video frames and then plotted using Prism (GraphPad) software. For the χ^0 DNA, the rate of translocation was determined by fitting the experimental data to a line; for the χ-containing DNA, the rates before and after χ, as well as position and duration of the pause, were determined by fitting the data to a contiguous three-segment line (Spies et al., 2003). The reported error for each individual parameter is the standard deviation obtained from the fit. In cases where a population average is given, the uncertainly represents the standard deviation for that group of molecules.

Supplemental Data

Supplemental Data include two movies and are available with this article online at http://www.molecule.org/cgi/content/full/17/5/745/DC1/.

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