plotted and rates were calculated by linear regression forced through the origin. Data points in Fig. 1c, d are from measurements of 2, 7, 10 and 6 molecules for the loop one-tail structures, and 17, 12, 12 and 10 molecules for the two-tail structures at 20, 30, 45 and 60 s, respectively, with 16 nM $RecB^{K29Q}CD$ enzyme and 0.2 nM λ DNA. Data points in Fig. 3a are means of measurements of 9, 7, 12 and 13 molecules for the 15, 30, 45 and 60 s points with 0.5 nM wild-type enzyme and of 4, 3 and 6 molecules for the 60, 120 and 240 s points with 2 nM $RecBCD^{K177Q}$ enzyme and 0.2 nM λ DNA. Typically, more than 90% of the observed partly unwound structures were interpretable. The topology of 651 of the 862 structures at double-stranded DNA ends seen with RecBCD was similar to the structure in Fig. 1a. The topology of 141 molecules, including 41 with gold at the end of the tail, of the 1,117 structures seen with $RecB^{K29Q}CD$ was similar to the structure in Fig. 1b, which is unique to $RecB^{K29Q}CD$. The topology of 146 of the 258 structures seen with $RecBCD^{K177Q}$ was similar to the structure in Fig. 2b. The remaining structures were predominantly forks (Fig. 2a), which could result either from release of the loop during unwinding or from failure of the glutaraldehyde fixation necessary to preserve partly unwound structures during preparation for microscopy.

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Correspondence and requests for materials should be addressed to G.R.S. (gsmith@fhcrc.org).

RecBCD enzyme is a bipolar DNA helicase

Mark S. Dillingham*†, Maria Spies* & Stephen C. Kowalczykowski*

** Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, University of California, Davis, California 95616, USA*

***Escherichia coli* RecBCD is a heterotrimeric helicase/nuclease that catalyses a complex reaction in which double-strand breaks in DNA are processed for repair by homologous recombination[1]. For some time it has been clear that the RecB subunit possesses a 3′ → 5′ DNA helicase activity[2–4], which was thought to drive DNA translocation and unwinding in the RecBCD holoenzyme. Here we show that purified RecD protein is also a DNA helicase, but one that possesses a 5′ → 3′ polarity. We also show that the RecB and RecD helicases are both active in intact RecBCD, because the enzyme remains capable of processive DNA unwinding when either of these subunits is inactivated by mutation. These findings point to a bipolar translocation model for RecBCD in which the two DNA helicases are complementary, travelling with opposite polarities, but in the same direction, on each strand of the antiparallel DNA duplex. This bipolar motor organization helps to explain various biochemical properties of RecBCD, notably its exceptionally high speed and processivity, and offers a mechanistic insight into aspects of RecBCD function.**

RecBCD enzyme processes DNA breaks for repair by homologous recombination by means of an elaborate reaction involving coordinated and regulated helicase and nuclease activities. After binding specifically to a double-stranded DNA end, this 330-kDa heterotrimer uses the free energy of ATP hydrolysis to translocate into and separate the duplex while preferentially degrading the 3′-terminated nascent single strand[5]. On encountering the recombination hotspot Chi, an octameric DNA sequence that is recognized as single-stranded DNA (ssDNA) by the enzyme approaching from its 3′ side[6], the frequency of cleavage is reduced and its polarity is switched to the 5′-terminated strand[7]. Because translocation and unwinding continue after recognition of Chi, the final product is a duplex DNA with a single-stranded DNA tail terminated at its 3′ end with the Chi sequence. RecBCD is also capable of loading the RecA protein onto this ssDNA tail[8] to form a substrate for DNA-strand invasion, the next step in the general pathway for

† Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

homologous recombination[9]. RecBCD is of particular interest because of its enigmatic biochemical properties. Isolated RecB protein contains helicase motifs and a carboxy-terminal nuclease domain, and displays modular $3' \rightarrow 5'$ helicase and nuclease activities[2,3,10]. It is related, and behaves similarly, to the Rep/UvrD/PcrA family of superfamily I (SF1) DNA helicases, which are poorly processive enzymes that initiate preferentially from a ssDNA region flanking the duplex[2,3,10]. In contrast, RecBCD protein is among the most processive and rapid helicases characterized, unwinding 30,000 base pairs per binding event at 1,000 base pairs s^{-1} (ref. 11), with a strong preference for initiation from a duplex end. The similarity between RecB and the Rep/UvrD/PcrA helicase family implies that the complex biochemistry of RecBCD can be attributed to additional (non-helicase) domains within RecB protein, or to the RecC and RecD subunits. Little is known about the isolated RecC protein, which is devoid of sequence motifs that could provide clues to its role, but the specificity of Chi sequence recognition is altered by mutations in this subunit[1]. RecD contains SF1 helicase motifs and is a ssDNA-dependent ATPase[12] but, until now, helicase activity has not been demonstrated.

We and others[12,13] have experienced difficulty in obtaining native RecD protein, and so a His-tagged RecD protein (^{his}RecD) was purified to near homogeneity by refolding on a Ni^{2+}-affinity column (Fig. 1a). To serve as a control, a mutant ^{his}RecD protein (^{his}RecD^{K177Q}) with a Lys → Gln substitution in the Walker A box (helicase motif I) was also purified. Mutation of the Walker A box lysine drastically decreases ATP hydrolysis in many helicases, including the isolated RecB and RecD subunits[12,14]. With the use of a coupled ATP hydrolysis assay, ^{his}RecD was shown to be an ATPase whose activity is stimulated 70-fold by ssDNA: K_m(poly(dT)) = 9 μM nucleotides; V_{max} = 5 mol ATP per mol ^{his}RecD per second, which represents a minimum limit on the turnover number (k_{cat}) if 100% of the protein is active (Fig. 1b). Linear blunt-ended duplex DNA did not stimulate the ATPase activity (data not shown). To address the question of whether ^{his}RecD protein possesses DNA helicase activity, we tested its ability to separate duplex DNA substrates formed from short oligonucleotides (Table 1). ^{his}RecD was capable of separating the $5' \rightarrow 3'$ substrate: a 40-base-pair duplex flanked by a 5′-terminated ssDNA tail (Fig. 2). Helicase activity was undetectable on the equivalent blunt duplex and was decreased at least 10-fold on the $3' \rightarrow 5'$ substrate: a 40-base-pair duplex flanked by a 3′-terminated ssDNA tail (Fig. 2a, b). Unwinding of the $5' \rightarrow 3'$ substrate was absolutely dependent on ^{his}RecD and ATP hydrolysis (Fig. 2c). Removal of ATP or inclusion of EDTA resulted in no strand displacement activity, which is consistent with the expected requirement for a Mg^{2+}-dependent ATPase activity to support the helicase reaction. The non-hydrolysable ATP analogues

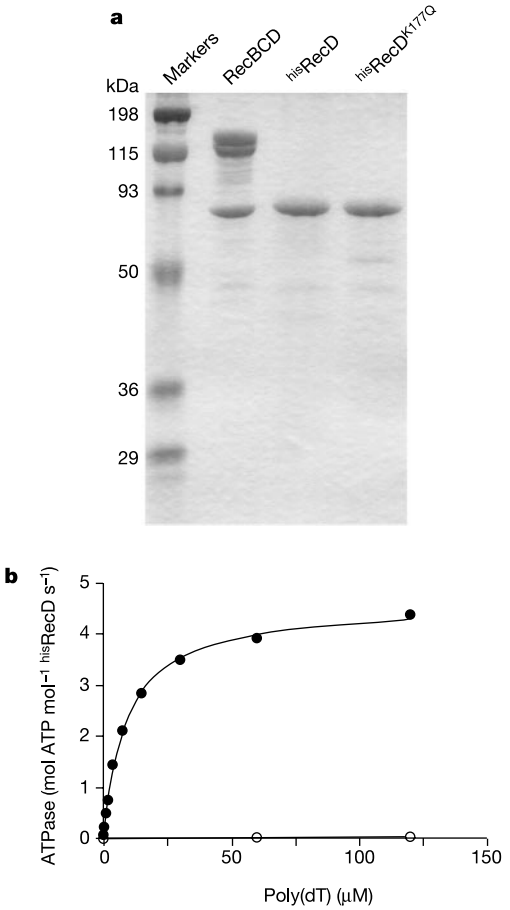

Figure 1 Purified ^{his}RecD protein is a ssDNA-dependent ATPase. **a**, SDS–PAGE gel (12% polyacrylamide) showing molecular mass markers, 7.5 pmol RecBCD, 15 pmol ^{his}RecD and 15 pmol ^{his}RecD^{K177Q}. **b**, Dependence of the ATPase activities of ^{his}RecD (filled circles) and ^{his}RecD^{K177Q} (open circles) on ssDNA.

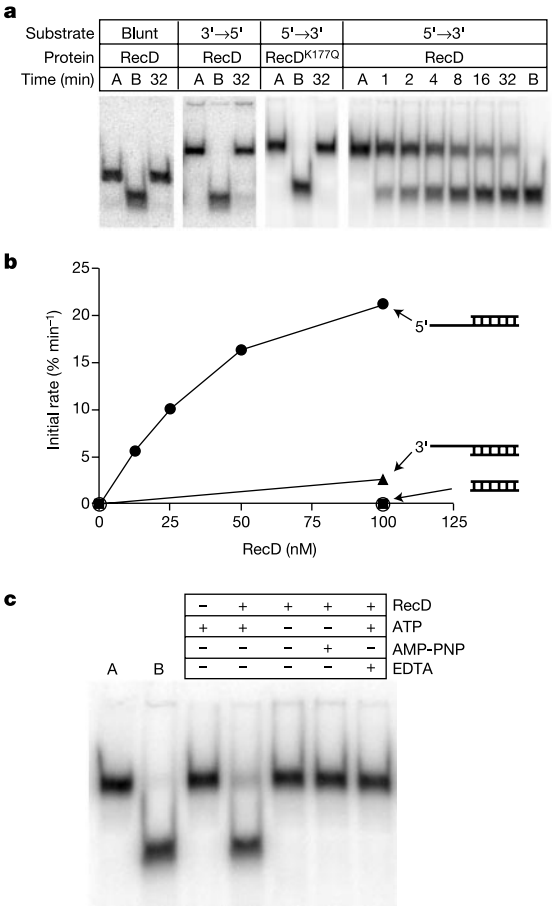

Figure 2 ^{his}RecD is a $5' \rightarrow 3'$ DNA helicase. **a**, Helicase assays in which 100 nM ^{his}RecD or ^{his}RecD^{K177Q} was incubated with 1 nM DNA substrate and 2.5 mM ATP for the times indicated. Lanes A and B are controls for annealed and heat-denatured substrate respectively. Experiments were performed with three different DNA substrates (Table 1) and either wild-type or K177Q mutant ^{his}RecD. **b**, Rates of helicase activity, determined from the initial slopes of helicase time-course assays, for blunt (filled squares), $3' \rightarrow 5'$ (filled triangles), and $5' \rightarrow 3'$ (filled circles) DNA substrates using ^{his}RecD. Unwinding of the $5' \rightarrow 3'$ substrate by ^{his}RecD^{K177Q} (open circles) is very poor. **c**, Controls demonstrating that ATP hydrolysis is required for ^{his}RecD-catalysed helicase activity on the $5' \rightarrow 3'$ DNA substrate. Helicase assays were performed for 30 min with the indicated components.

Table 1 **DNA substrates used for strand displacement helicase assays**

Name	Substrate structure
Blunt	3′- GTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGT-5′ 5′-*CAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCA-3′
3′ → 5′	3′- CTCGCGTCGCTCAGTCACTCGCTCCTTCGCCTTCTCGCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGT -5′ 5′- *CAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCA -3′
5′ → 3′	3′- GTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGT*-5′ 5′- GAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCA -3′

The asterisk indicates the position of the ^{32}P-phosphate radiolabel.

AMP-PNP (Fig. 2c) and ATP-γS (data not shown) did not support helicase activity but did bind to ^{his}RecD as shown by virtue of their ability to inhibit the ATPase activity (Supplementary Information). The ATPase[12] and 5′ → 3′ helicase activities are drastically decreased in the ^{his}RecD^{K177Q} mutant protein (Figs 1b and 2), which indicates that they are intrinsic to the wild-type RecD polypeptide and dependent on the Walker A box lysine residue.

Because protein prepared by refolding might be inactive, we sought to detect a functional interaction with RecBC by assaying for the production of Chi-specific ssDNA fragments, a biochemical property associated exclusively with the RecBCD holoenzyme (Fig. 3a). As expected, RecBCD enzyme produces Chi-specific fragments and degrades the DNA to an extent typically observed[5], whereas RecBC enzyme (which is largely devoid of nuclease activity) produces very few Chi-specific fragments but is active as a helicase, generating full-length ssDNA product (Fig. 3b). ^{his}RecD protein has no activity on this plasmid-length duplex DNA substrate. However, when RecBC and ^{his}RecD were mixed, a substantial increase in Chi-specific fragments was observed, indicating the reconstitution of functional RecBCD enzyme. Note that ^{his}RecD protein stimulates the rate of substrate use (that is, the helicase activity) by RecBC. This effect was also observed when a dye displacement helicase assay was used (data not shown).

To address the question of whether the RecB and RecD helicases are active within the RecBCD holoenzyme, we purified RecBCD enzymes containing Lys → Gln mutations in the Walker A box (helicase motif I) of either RecB (K29Q) or RecD (K177Q), and examined their DNA-unwinding capacities. As expected[1], wild-type RecBCD rapidly unwound a linearized plasmid DNA lacking Chi sequences, producing full-length ssDNA product and substantially degrading the DNA by means of its nuclease activity (Fig. 4). Remarkably, both the RecB^{K29Q}CD and the RecBCD^{K177Q} enzymes retained potent helicase activity, showing that either motor can support processive DNA translocation, and indicating strongly that RecB and RecD are simultaneously active in the wild-type holoenzyme. As expected, and in contrast to these single-mutant proteins, the RecB^{K29Q}CD^{K177Q} double-mutant protein is inactive both as a ssDNA-dependent ATPase[14] and as a helicase on long duplex DNA substrates[15].

In this study we have demonstrated that RecD protein is a 5′ → 3′ DNA helicase. The most closely related proteins to RecD include the TraI and Dda SF1 helicases, both of which also possess 5′ → 3′ DNA helicase activity[16,17]. Similarly, RecB protein is related to the Rep/UvrD/PcrA class of SF1 helicases, which all possess 3′ → 5′ polarity. It is well established that RecB possesses a 3′ → 5′ DNA helicase activity, as this has been demonstrated for both RecB[2,3], (M.S.D. and S.C.K., unpublished observations) and RecBC[4] proteins. We have

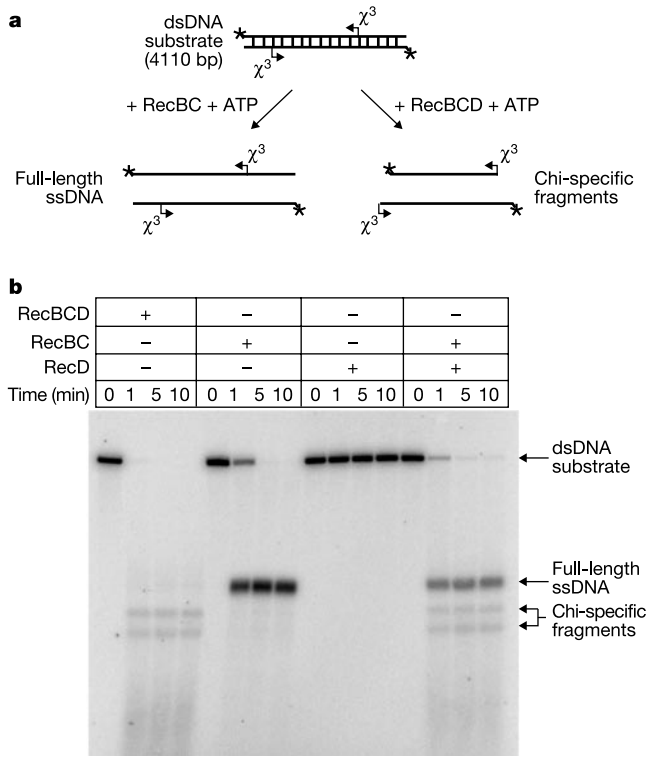

Figure 3 Reconstitution of the Chi-specific cleavage activity of RecBCD from purified RecBC and ^{his}RecD proteins. **a**, A 5′-radiolabelled (asterisks) linearized plasmid containing 'triple Chi' (χ^3) sequences is unwound to full-length ssDNA by RecBC, whereas RecBCD enzyme processes this substrate to Chi-terminated ssDNA fragments. dsDNA, double-stranded DNA. **b**, The substrate was incubated with the enzyme(s) indicated in the presence of ATP as described in Methods.

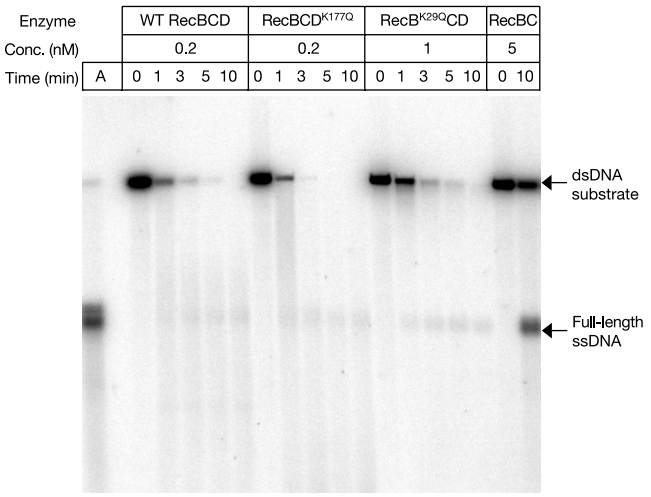

Figure 4 The RecB and RecD helicase subunits are both active in the RecBCD holoenzyme. Unwinding of a 5′-radiolabelled linearized plasmid (lacking Chi sequences) by wild-type RecBCD, wild-type RecBC and mutant holoenzymes containing a substitution in the Walker A motif of either the RecB or RecD subunit (see the text). Lane A contained a heat-denatured sample of the substrate to mark the position of full-length ssDNA. The DNA substrate was incubated with the enzyme indicated in the presence of ATP as described in Methods. dsDNA, double-stranded DNA; wt, wild-type.

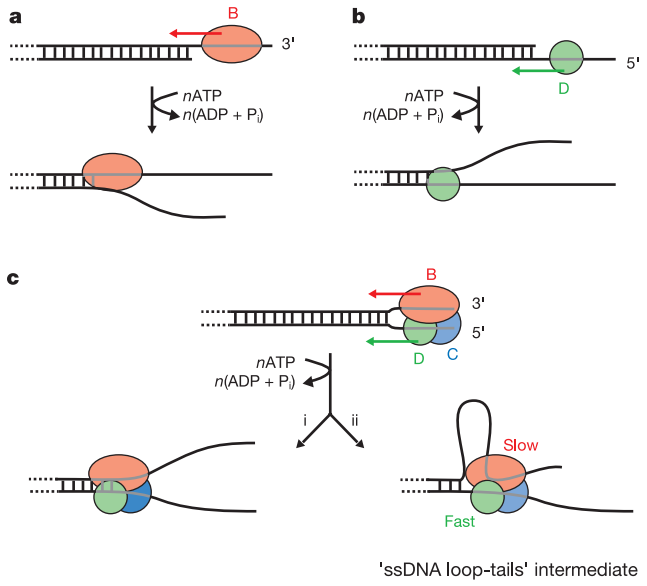

Figure 5 A bipolar DNA helicase translocation model. **a**, RecB is a 3′ → 5′ helicase; **b**, RecD is a 5′ → 3′ DNA helicase. Experiments with other helicases (see the text) indicate that helicase polarity relates to a unidirectional ssDNA translocation activity as illustrated. **c**, Because RecB and RecD helicases are of opposite polarity relative to ssDNA, they complement the antiparallel DNA structure, producing cooperative movement that is unidirectional relative to the duplex. Before translocation, the RecB and RecD proteins are positioned on the 3′- and 5′-terminated single strands respectively, in accordance with crosslinking data[23] and their observed polarities. The position of the RecC protein is arbitrary. Pathway i: on ATP hydrolysis the two helicases move uniformly along the DNA, unwinding the duplex as they progress. Pathway ii: alternatively, if the motors move independently and at unequal speeds, then a bipolar translocation mechanism would explain the generation of the 'ssDNA loop-tails' unwinding intermediates that have been observed by electron microscopy[25] (see the text). The relative rates of the RecB and RecD helicases are not determined in this work. This assignment of fast and slow activities is based on ref. 15, in which it was shown that RecD subunit is the faster motor under the conditions used.

further shown that both the RecB and RecD subunits are capable of driving the unwinding of DNA in the RecBCD holoenzyme. Why might RecBCD enzyme possess two DNA helicase subunits of opposite polarity? Crystallographic[18] and biochemical[19–21] data show that the SF1 helicases PcrA and Dda contain an autonomous unidirectional ssDNA translocation activity that is consistent with the polarity of their helicase activities. The structural work suggests that this ssDNA translocation motor is formed by the 'helicase' motifs. Consequently, SF1 helicase motifs are suggested to be a blueprint for a ssDNA motor: a modular structure capable of providing the translocation function in a DNA helicase or other DNA-processing protein[22]. At first glance, the observation of two helicase activities of opposite polarity contained within a single protein complex might seem unusual. However, the notion that the two proteins function as ssDNA motors suggests an elegant mechanism by which they might cooperate to translocate along a DNA duplex. The antiparallel nature of the duplex would allow the two proteins to bind to opposite strands at the DNA break, yet to translocate in the same direction relative to the duplex (Fig. 5). This simple bipolar helicase model helps to explain many of the enigmatic properties of RecBCD enzyme. First, the blunt-end loading of the enzyme might reflect the positioning of two ssDNA motors side by side on each strand of the duplex. This suggestion is consistent with crosslinking data that place the RecB and RecD subunits on the 3′- and 5′-terminated strands, respectively[23]. Second, a bipolar helicase organization can explain the exceptional processivity of RecBCD: the two motors would have to dissociate simultaneously to release the holoenzyme from its DNA track. Indeed, RecBCD is more processive and faster than RecBC enzyme[11,24] (Fig. 3), which might indicate that the RecD motor is faster than RecB, or that RecD augments that activity of RecB. Last, our model can explain the 'ssDNA loop-tails' unwinding intermediates observed by electron microscopy[25]. If two linked motors were to travel on opposite strands at unequal speeds, the faster motor would be associated with a long ssDNA tail, whereas the slower motor would be associated with a ssDNA loop ahead of its itself and a trailing ssDNA tail (Fig. 5c, pathway ii). In this model, the leading motor is a genuine helicase whereas the slower, second motor acts simply as a ssDNA translocase. However, our data with the mutant holoenzymes (Fig. 4) suggest that either motor subunit can function as the leading helicase, which is consistent with general models for helicase activity in which a single ssDNA motor is the vital component. Recent electron microscopy data[15] support these ideas, showing that mutation of helicase motif I in either the RecB or the RecD subunit alters the looped intermediates formed by RecBCD in a manner that suggests a 'dual-helicase' model. Our proposal of a bipolar motor organization raises several questions about the RecBCD mechanism. Can RecBCD bypass DNA damage or gaps in both strands as it progresses along the duplex[4]? Importantly, because the RecD subunit is required for the production of Chi-specific fragments by RecBCD (Fig. 3), and given the prevailing models of RecD subunit inactivation in response to recombination hotspot recognition[1], does this translocation mechanism provide an opportunity for the differential DNA processing observed before and after the recognition of Chi? Last, it will be of interest to see how bipolar helicase motors might be employed in other macromolecular assemblies that process DNA structure, such as the TFIIH complex: a basal transcription and nucleotide excision repair factor containing the 3′ → 5′ XPB and 5′ → 3′ XPD helicases[26]. □

Methods

Protein expression and purification

^{his}RecD and ^{his}RecD^{K177Q} proteins were expressed in the form of inclusion bodies by using the pET15b vector system (Novagen), which introduces an amino-terminal histidine tag on a 20-amino-acid leader sequence. Purification was performed under denaturing conditions with Ni^{2+}-chelating Sepharose resin, followed by an on-column refolding technique (Supplementary Information). The integrity of the cloned *recD* and *recD*^{K177Q} genes was confirmed by DNA sequencing (at the DBS sequencing facility, University of California, Davis, California). The *recD* nucleotide sequence was identical to that found in the *E. coli* K12 complete genome (GenBank accession number NC_000913). Because of the nature of the method used to purify the RecD protein, it is possible that the preparation was not fully active. RecBC protein was expressed with the pPB520 and pPB700 plasmids[13] in a Δ*recBCD* background (V330) containing a plasmid expressing LacI^q, and purified with a RecBCD protocol as described[6]. RecBCD protein was expressed and purified as described[6]. Expression strains for the RecB^{K29Q}CD and RecBCD^{K177Q} proteins[27,28] in a Δ*recBCD* background were a gift from D. A. Julin (University of Maryland, College Park, Maryland, USA). The mutant proteins were expressed as described[27,28] and purified with the protocol for the wild-type enzyme[6].

ATP hydrolysis assays

ATP hydrolysis was measured spectrophotometrically by coupling it to NADH oxidation[29]. Assays were performed with 80 nM ^{his}RecD or 240 nM ^{his}RecD^{K177Q} at room temperature (22 °C) in a buffer containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 4 mM dithiothreitol, 35 U ml^{-1} pyruvate kinase, 20 U ml^{-1} lactate dehydrogenase, 2 mM phosphoenolpyruvate, 0.08 mg ml^{-1} NADH, 2 mM (saturating) ATP, and the indicated concentration (in nucleotides) of poly(dT) ssDNA. Data were fitted directly to the Michaelis–Menten equation with the program GraphPad Prism 3.02 (GraphPad Software).

Strand-displacement DNA helicase assays

^{32}P-labelled helicase substrates were prepared by annealing the oligonucleotides shown in Table 1, which had been purified by polyacrylamide-gel electrophoresis. Labelled oligonucleotide (3 pmol) was annealed to its partner (5 pmol) to form the final substrate, which was purified with a G25 column (AP-Biotech). Assays were performed at room temperature in a buffer containing 20 mM Tris-HCl pH 7.5, 17.5 mM NaCl, 2 mM $MgCl_2$, 4 mM dithiothreitol, 2.5 mM ATP and 1 nM (in molecules) DNA substrate. Where indicated, 2.5 mM AMP-PNP or 10 mM EDTA was included in the reaction. Reactions were initiated with RecD (100 nM or as indicated) and terminated with 0.4% w/v SDS, 40 mM EDTA, 8% v/v glycerol, 0.1% w/v bromophenol blue and 200 nM unlabelled

oligonucleotide as a trap to prevent the reannealing of the labelled strand. However, the presence of trap DNA in the stop buffer made no significant difference to our results. The oligonucleotide reannealing rate was shown empirically to be insignificant over the time course of our assays. Reaction products were separated on native 15% polyacrylamide gels, which were dried on DEAE paper and quantified with a STORM PhosphoImager and ImageQuant software (Molecular Dynamics).

Plasmid DNA unwinding and Chi-specific fragment production assays

Assays were performed with slight modifications to a previously described method[30]. *Nde*I-linearized, ^{32}P-labelled pBR322 $\chi^{3F,3H}$ plasmid containing two sets of 'triple-Chi' sequences was used as the substrate in reactions (initiated with 2 mM ATP after a 2-min preincubation of all other components) and performed at room temperature with either 0.5 nM RecBCD, 5 nM RecBC or 50 nM ^{his}RecD protein, as indicated, in a buffer containing 25 mM Tris-acetate pH 7.5, 6 mM magnesium acetate, 1 mM dithiothreitol, 20 μM (nucleotides) DNA and 2 μM *E. coli* single-stranded DNA-binding protein. Assays with DNA lacking Chi sequences were performed with the same method, but with *Nde*I-linearized, ^{32}P-labelled pBR322 plasmid as the substrate, and using the enzyme concentrations indicated.

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Correspondence and requests for materials should be addressed to S.C.K. (sckowalczykowski@ucdavis.edu).