RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity

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Helicases are molecular motors that move along and unwind double-stranded nucleic acids[1]. RecBCD enzyme is a complex helicase and nuclease, essential for the major pathway of homologous recombination and DNA repair in *Escherichia coli*[2]. It has sets of helicase motifs[1] in both RecB and RecD, two of its three subunits. This rapid, highly processive enzyme unwinds DNA in an unusual manner: the 5′-ended strand forms a long single-stranded tail, whereas the 3′-ended strand forms an ever-growing single-stranded loop and short single-stranded tail. Here we show by electron microscopy of individual molecules that RecD is a fast helicase acting on the 5′-ended strand and RecB is a slow helicase acting on the 3′-ended strand on which the single-stranded loop accumulates. Mutational inactivation of the helicase domain in RecB or in RecD, or removal of the RecD subunit, altered the rates of unwinding or the types of structure produced, or both. This dual-helicase mechanism explains how the looped recombination intermediates are generated and may serve as a general model for highly processive travelling machines with two active motors, such as other helicases and kinesins.

Most helicases form Y-shaped molecules, with two equal-length single-stranded tails, during the unwinding of duplex DNA, but RecBCD enzyme forms structures with a single-stranded loop and two single-stranded tails. This unusual topology was demonstrated by electron microscopy of reaction intermediates[3], as shown in Fig. 1a. Both the extent of unwinding and the size of the loop increase linearly with time for tens of kilobases, at rates of ~370 and ~150 nucleotides s^{-1}, respectively[3] (see also Fig. 3a). The ever-

increasing lengths of both the loop and the short tail during unwinding suggest that the passage of single-stranded DNA from the loop, through the enzyme, to the short tail is a directional, and hence active (ATP-dependent), process.

It has been proposed that some helicases act through two separate activities, the melting of base pairs and the movement of the enzyme along one strand of the resultant single-stranded DNA[4]. Alternating use of these two activities both unwinds the DNA and moves the enzyme along it. In this view, the unwinding of duplex DNA by RecBCD enzyme would be mediated by a fast helicase, whereas the strand movement needed to move single-stranded DNA from the loop to the short tail would be provided by the motor component of a second, slower helicase. The RecB and RecD subunits each have canonical helicase domains and belong to superfamily 1 (SF1) of helicases[1]. Isolated RecB and RecD subunits each have helicase activity and DNA-dependent ATPase activity[5–7]. Mutational alterations ($RecB^{K29Q}$ and $RecD^{K177Q}$) in the Walker A box of the helicase motifs[1] of RecB and RecD decrease ATPase activities of individual subunits or reconstituted holoenzyme at least 50-fold[6–8]. Alteration of the lysine (Lys 29 or Lys 177) in this motif, which is invariant in helicases and many other ATP-binding proteins[9], inactivates the ATPases without affecting the helicases' ability to bind DNA[10], suggesting that this alteration specifically inactivates the motor activity. Heterotrimeric RecBCD enzyme, with one copy of each polypeptide, can unwind DNA[11]. Collectively, this information indicated that RecB and RecD might be two helicases that act in concert to make the loop-tail unwinding structures described previously[3]. Here we used derivatives of RecBCD enzyme, mutated in one or the other helicase activity or lacking one helicase subunit, to test the hypothesis that these unwinding structures result from the combined actions of a fast and a slow helicase motor. Unwinding of double-stranded DNA by $RecB^{K29Q}CD$ enzyme, in which RecD is the only active ATPase, produced a novel unwinding intermediate, with a terminal single-stranded loop accompanied by only one single-stranded tail (Fig. 1b; Supplementary Figs S1 and S2). We term this a 'loop one-tail' structure, to distinguish it from the wild-type 'loop two-tail' structure (Fig. 1a), previously referred to as a 'loop-tails' structure[3]. The loop and the tail of the loop one-tail structures had equivalent lengths (Fig. 1c), indicating that these structures did not result from nucleolytic removal of a second tail.

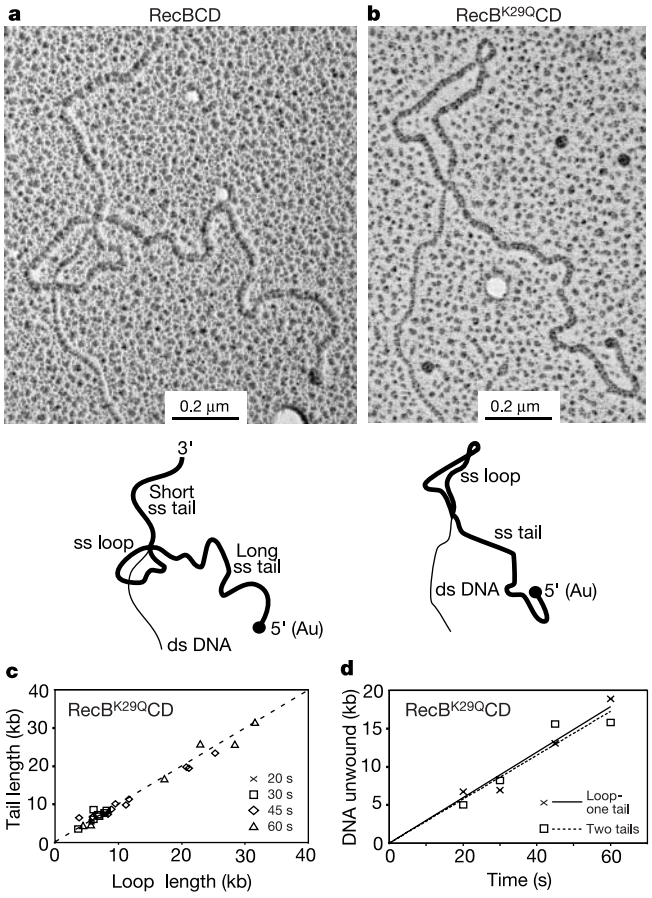

Figure 1 $RecB^{K29Q}CD$ enzyme, with an inactive RecB helicase, unwinds DNA by means of a loop and a 5′-terminated tail. **a**, **b**, Electron micrographs of unwinding intermediates, produced by reaction of 0.7 nM 5′-biotinylated double-stranded DNA with 4 nM RecBCD enzyme or $RecB^{K29Q}CD$ enzyme for 60 s. As identified below the micrographs, the thin sinuous lines are double-stranded DNA, the thicker ones are SSB-coated single-stranded DNA, and the electron-dense particles are anti-biotin antibody-conjugated gold beads that identify the 5′ termini of the unwound DNA. **a**, A wild-type loop two-tail unwinding intermediate produced by incubation with RecBCD enzyme. **b**, A loop one-tail unwinding intermediate produced by incubation with $RecB^{K29Q}CD$ enzyme. ds, double-stranded; ss, single-stranded. **c**, The single-stranded loop and the tail made by $RecB^{K29Q}CD$ enzyme are of equal lengths. All the data used in **d** are plotted. The dashed line depicts equality. kb, kilobases. **d**, Travel rate of loop one-tail and two-tail structures produced by $RecB^{K29Q}CD$ enzyme: rates are 300 nucleotides s^{-1} (loop one-tail) and 290 nucleotides s^{-1} (two-tails).

We compared the strand polarity of the unwinding structures made by $RecB^{K29Q}CD$ enzyme with that of structures made by the wild-type enzyme, using DNA with biotinyl moieties at the 3′ or the 5′ end and anti-biotin antibody conjugated to gold particles. In structures made by the wild-type enzyme the long tail had a 5′ end (Fig. 1a; Supplementary Fig. S3), as reported previously[12]; similar loop two-tail structures with the same strand polarity were made under a variety of reaction conditions by using 3′ end-labelled substrates (Supplementary Fig. S4). In structures made by the $RecB^{K29Q}CD$ mutant enzyme the single tail had a 5′ end (Fig. 1b and Supplementary Fig. S2), showing the absence of 5′ end degradation as inferred from the length measurements noted above. The nearly wild-type rate of unwinding by $RecB^{K29Q}CD$ enzyme (Fig. 1d; see also Fig. 3a) shows that the RecD helicase can by itself provide the motive power to rapidly move the enzyme and unwind DNA. We infer that the inactive $RecB^{K29Q}$ subunit remained bound to the 3′ end that it initially bound, producing a loop but no second tail.

We addressed the contribution of the RecB helicase motor to the unwinding activity in two ways, with RecBC enzyme lacking the RecD subunit but retaining helicase activity[13], and with $RecBCD^{K177Q}$ enzyme lacking the RecD ATPase[6]. The reaction of double-stranded DNA with RecBC enzyme produced only Y-shaped molecules with two equal-length single-stranded tails (~80%; Fig. 2a) or molecules that could be derived from them by mechanical shear (~20%). Because RecC protein lacks the set of helicase motifs[1] and has no detectable ATPase activity[14], we infer that the single RecB helicase of RecBC enzyme unwinds DNA but without loop formation. In an assay that couples a single-strand-specific exonuclease to the helicase activity, RecBC unwinds DNA only about 20% as rapidly as RecBCD[13]; our electron microscopy measurements agree with this estimate (data not shown). Thus, RecD is necessary for fast unwinding; RecB, complexed with RecC, is a slower helicase.

The contribution of the RecB motor was also addressed by using $RecBCD^{K177Q}$ enzyme, which lacks an active RecD motor. This mutant enzyme unwound DNA at ~73 nucleotides s^{-1} (Fig. 3a), about 20% of the rate of the wild-type enzyme (~370 nucleotides s^{-1}); these relative rates of the wild-type and $RecBCD^{K177Q}$ enzymes are similar to those found with the exonuclease-coupled assay[13] and by gel-electrophoresis assays of unwinding (data not shown). Thus, either removal of RecD or inactivation of its ATPase resulted in an unwinding rate about 20% of that of the wild-type enzyme, which is consistent with the conclusion above that RecB is a slower helicase than RecD.

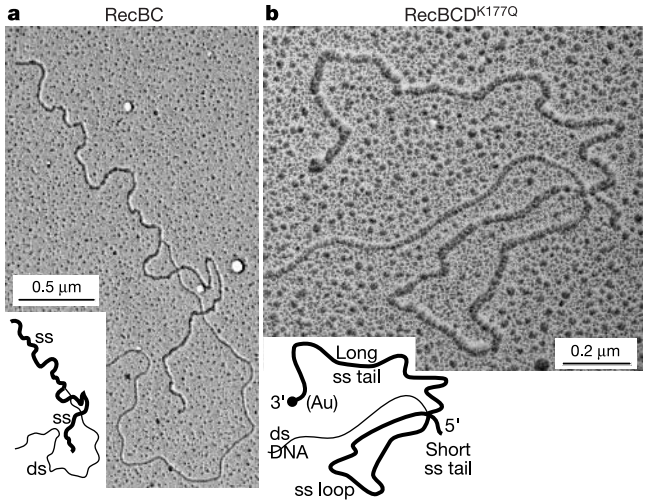

Figure 2 Enzymes without RecD or with mutant RecD (RecBCD^{K177Q}) unwind DNA slowly and with different topologies. **a**, Double-stranded λ DNA (0.2 nM) after reaction for 30 s with 80 nM RecBC enzyme, lacking the RecD subunit. No looped structures were observed among 80 partly unwound molecules. **b**, Loop two-tail structure produced by reaction of 1 nM 3′-biotinylated double-stranded DNA for 60 s with 2 nM RecBCD^{K177Q} enzyme: the gold particle on the 3′ terminus shows that the strand polarity is opposite to that of the wild-type enzyme (Fig. 1a). ds, double-stranded; ss, single-stranded.

The strand polarity of the unwinding structures made by RecBCD^{K177Q} enzyme was opposite to that of the structures made by wild-type enzyme noted above (Fig. 1a). In 24 of 25 loop two-tail structures made by RecBCD^{K177Q} and bound to a gold particle, the longer tail had a 3′ terminus, indicating that the loop and shorter tail were on the 5′-ended strand (Fig. 2b and Supplementary Fig. S3). The mean rate of growth of the 5′ tail was 15 nucleotides s^{-1} by RecBCD^{K177Q} and 370 nucleotides s^{-1} by RecBCD (Fig. 3a). We infer that in the structures made by RecBCD^{K177Q} enzyme the inactive RecD subunit moves very slowly along the 5′-terminated strand, about 5% of its rate in the wild-type enzyme. This very slow movement of the RecD^{K177Q} subunit might result from residual, weak ATPase activity in that subunit or from force generated by the wild-type RecB subunit, perhaps by an allosteric conformational change of RecD upon ATP hydrolysis by RecB. Travel of the mutant RecD subunit requires an active RecB subunit, as the double-mutant enzyme RecB^{K29Q}CD^{K177Q} had no detectable unwinding or ATPase activity (Supplementary Table S1). Finally, we infer from the data in Fig. 3a that the active RecB motor in RecBCD^{K177Q} enzyme unwinds the DNA, producing the longer single-stranded tail with a 3′ end, at about the same rate (73 base pairs s^{-1}) that the RecB motor unwinds DNA in RecBC enzyme; this is about one-third of the rate at which we infer that RecB translocates along the 3′-ended single-stranded DNA strand in the RecBCD holoenzyme.

We examined the degree of coupling of the fast RecD and slow RecB motors in wild-type enzyme by comparing the distance travelled by the fast motor with that travelled by the slow motor in individual molecules (Fig. 3b). Whereas the mean lengths of both tails increased linearly with time (Fig. 3a), much variation in the relative rates of the two can be seen in individual molecules. There is therefore no tight coupling between the two motors, despite a constant rate of the two for the average behaviour of molecules. Figures 1c and 3b also illustrate an approximately twofold variation in unwinding rate of individual molecules, which has been seen by other techniques with the wild-type RecBCD enzyme[15,16].

How do the RecB and RecD helicases interact with DNA to produce the loop two-tail intermediates? In a simple model (Fig. 4), each of the two helicase motors travels along (translocates on) one or the other strand of the DNA; the faster helicase actively unwinds the DNA, and a single-stranded loop accumulates on the strand with the slower helicase. Other helicases of the SF1 and SF2 families, such as PcrA and PriA, can translocate along single-stranded DNA; that is, without actual unwinding[17,18]. The strand polarities of the unwinding structures agree with the ultraviolet-dependent cross-linking of RecB to the 3′-ended strand and RecD to the 5′-ended strand in the RecBCD–DNA initiation complex[19] (Fig. 4a) and with the inferred helicase polarities of isolated RecB and RecD[5,7]. That RecD is a faster helicase than RecB is indicated by the slower unwinding of DNA by RecBCD^{K177Q} and RecBC enzymes than by wild-type RecBCD enzyme, and the similar fast rates by RecB^{K29Q}CD and RecBCD enzymes (Figs 1 and 3).

Thus, in this model the loop and short tail are predicted to be on the 3′-ended strand in DNA unwound by RecBCD enzyme, as observed (Fig. 1a, Supplementary Fig. S3 and ref. 12). Complete mutational inactivation of the RecB or RecD subunit would result in the mutant (inactive) subunit's remaining bound at or near its initial terminus, forming a loop on that strand and an equal-length single-stranded tail on the other strand (Fig. 4c, d), as was observed for RecB^{K29Q}CD (Fig. 1b). The very short 5′-terminated tails observed with RecBCD^{K177Q} (Fig. 2b) might result, as noted above, either from slight residual activity in the mutated ATPase or from interaction between active and inactive ATPases in that mutant. RecBC enzyme, having only one helicase, would make only forks (Fig. 4e), as observed (Fig. 2a). For RecBCD to form a loop two-tail structure (Figs 1a and 4b), the two motors (RecB and RecD)

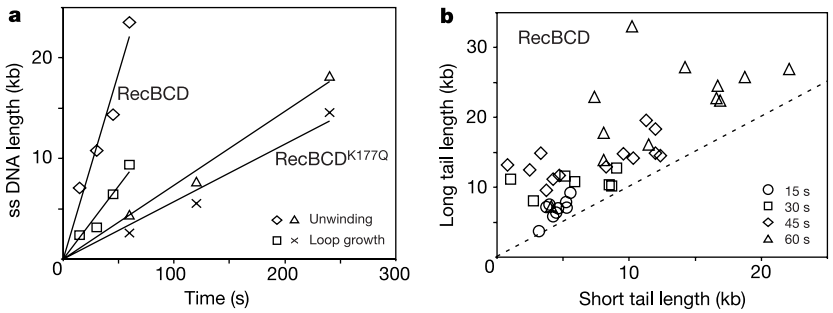

Figure 3 Analysis of DNA unwinding by RecBCD and RecBCD^{K177Q}. **a**, Slow unwinding and loop growth by RecBCD^{K177Q} enzyme. Mean unwinding and loop-growth rates are 370 and 150 nucleotides s^{-1} for RecBCD enzyme and 73 and 57 nucleotides s^{-1} for RecBCD^{K177Q} enzyme. ss, single-stranded. **b**, Lack of tight coupling between unwinding and single-stranded translocation by RecBCD enzyme. For each of the wild-type molecules measured in **a**, the length of the long tail (representing unwinding) is plotted against that of the short tail (representing strand passage from loop to tail). The dashed line indicates equality; by definition of the short and long tails, the data points must be on or above this line.

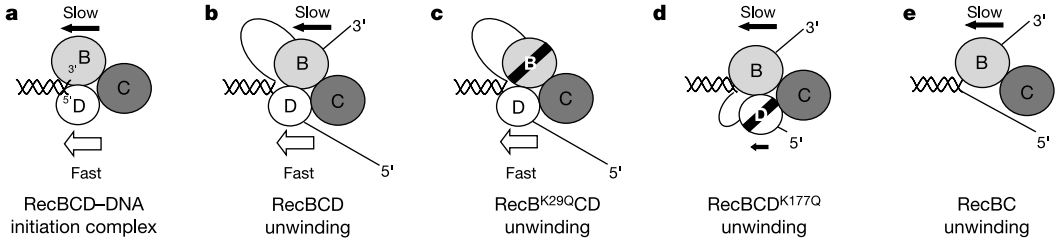

Figure 4 Model for unwinding by RecBCD enzyme. Arrows represent the active helicases in RecB and RecD; bars through circles depict mutant helicases (RecB^{K29Q}, RecD^{K177Q}) devoid of ATPase activity. **a**, Complex of RecBCD bound to a double-stranded DNA end in the absence of ATP. Irradiation with ultraviolet light crosslinks RecB to the 3′-ended strand, and RecC and RecD to the 5′-ended strand[19], which is consistent with the inferred polarities of the RecB and RecD helicases[5,7]. **b**, Wild-type holoenzyme travels at the speed of RecD, the faster helicase; a loop of single-stranded DNA accumulates ahead of RecB, the slower helicase. **c**, The inactive RecB^{K29Q} subunit remains bound to the 3′ end of the unwound strand, producing a loop one-tail intermediate. **d**, **e**, The lower velocity of the RecB helicase results in slow unwinding by RecBCD^{K177Q} (**d**) and RecBC (**e**) enzymes, the latter without any looped intermediates.

must be held together during unwinding. Although we know of no direct evidence for a direct RecB–RecD contact, the lack of nuclease activity in RecBC enzyme[20], which harbours the RecB nuclease domain but lacks RecD[21], implies that RecB and RecD must interact to form the active nuclease, perhaps by direct contact.

Although the slower RecB helicase might contribute only modestly to the very high rate of unwinding catalysed by RecBCD enzyme, it is clearly crucial for many activities of the enzyme. *E. coli* cells harbouring RecB^{K29Q}CD enzyme are profoundly recombination deficient (conjugal recombination is decreased ~100-fold, as in *recB*-null or *recC*-null mutants), and modestly sensitive to ultraviolet radiation, whereas those harbouring RecBCD^{K177Q} enzyme are resistant to radiation and are only slightly impaired for conjugal recombination (S. K. Amundsen and G.R.S., unpublished observations). Similarly, RecBCD^{K177Q} enzyme is only slightly impaired in double-stranded and single-stranded DNA exonuclease[22], but RecB^{K29Q}CD enzyme shows complete loss of double-stranded DNA exonuclease activity, although it retains single-stranded DNA exonuclease[23]. A nuclease determinant is located within the carboxy-terminal domain of RecB[21]. The lack of double-stranded DNA exonuclease in RecB^{K29Q}CD enzyme is therefore consistent with the 3′-terminated tail, the strand more vigorously degraded by the wild-type enzyme[24], being translocated past that nuclease motif during its exit from the enzyme. However, the nuclease activity of RecBCD is not itself needed for recombination: cells bearing the (nuclease-deficient) RecBC enzyme are recombination proficient[20]. The recombination deficiency of cells harbouring RecB^{K29Q}CD enzyme might therefore reflect the importance of the 3′-terminated single strand, uniquely lacking in this mutant, to homologous recombination. This is the strand on which the Chi hotspot sequence is recognized[25] and onto which RecA protein is loaded by RecBCD enzyme[26].

We have shown here that the two helicases of RecBCD enzyme, specified by the helicase domains in RecB and RecD, are both required for full activity of the enzyme. The helicase in RecD provides the fast unwinding activity. RecB, which has very weak helicase activity in isolation[5], is a genuine but slow helicase when complexed with RecC alone (Figs 2a and 4e), but seems only to translocate single-stranded DNA from the loop to the 3′-terminated tail in RecBCD holoenzyme (Figs 1a and 4b). One model of helicase action[4], with separate melting and translocation steps, fits well with this separation of RecB functions. RecBCD is highly processive: it can unwind more than 20 kilobases of DNA without dissociation[3,16]. However, the isolated subunits have very low processivity, being able to unwind only about 50 base pairs[5,7]. The exceptionally high processivity of RecBCD might stem at least in part from its having two motors: the probability that both would simultaneously dissociate from the DNA would be much less than that of one motor acting on its own. Other processive enzymes also have more than one motor: some helicases have two or six identical subunits[1] and kinesins typically have two[27]. Recent reports show that a monomeric helicase and a monomeric kinesin become active or more processive when converted to the dimeric form[27,28]. Thus, RecBCD enzyme, like the human TFIIH factor involved in transcription and DNA repair[29], fits this model but has the unusual feature of having two non-identical motors with opposite DNA-strand polarities. This asymmetric feature might impart RecBCD enzyme's asymmetry in other aspects of its promotion of genetic recombination[2]. □

Methods

Proteins and DNA

RecBCD enzyme was purified from a highly overexpressing strain[30] by chromatography on Amersham Biosciences Hi-Trap Heparin and BioRad Agarose A1.5m columns. RecB^{K29Q}CD and RecBCD^{K177Q} enzymes[22,23] were prepared from strain V330 [Δ(*recC-argA*)*234* λ$^-$ F$^-$] containing derivatives of plasmid pACYC184 bearing an 18.5-kilobase *Bam*HI fragment of *E. coli* carrying the mutant *recBCD* region (a gift from D. Julin). Enzymes were purified by chromatography on Amersham Biosciences HiTrap Q Sepharose, HiPrep Sephacryl S-300 HR and HiTrap Heparin columns. RecB and RecC were purified separately[30] with HiTrap Q Sepharose, BioRad CHT-II and HiTrap Heparin columns. RecBC enzyme was made by mixing RecB and RecC, with subsequent purification on a HiPrep Sephacryl S-300 HR column. *E. coli* single-strand DNA-binding protein (SSB) was from Promega. Bacteriophage λ was induced from lysogens, purified by two CsCl-gradient centrifugations, and the DNA (48 kilobases, with 12-nucleotide 5′ overhangs) was extracted with phenol. Plasmid pDWS2 DNA (23 kilobases) was linearized with *Eco*RI and labelled at its 3′ ends with bio-16-dUTP[12] (Roche), producing a blunt-ended double-stranded DNA substrate. DNA with 5′-biotin labels was produced by ligation of annealed oligonucleotides (5′-biotin-tetra-ethylene glycol-GGGCGGCGACC TAGCT-3′ and 5′-pAGGTCGCCG-3′; IDT Inc.) to pDWS2 DNA linearized with *SacI*, followed by sucrose-density-gradient sedimentation to remove unincorporated oligonucleotides. φX174 RFII and viral DNA were from New England Biolabs.

Reactions

RecBCD, RecB^{K29Q}CD or RecBCD^{K177Q} enzyme was incubated with 20 mM MOPS-KOH pH 7.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 3 μM SSB and DNA at 37 °C for 15 min, to allow binding. Reaction was started by adding ATP to 5 mM and stopped by adding EDTA to 40 mM; goat anti-biotin gold conjugate (10 nm; Ted Pella Inc.) was added where appropriate, and the reactions were fixed by incubation at 37 °C for 20 min with 0.2% glutaraldehyde. RecBC enzyme was incubated with 50 mM MOPS-KOH pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 3 μM SSB and 0.2 nM λ DNA at 37 °C for 15 min, to allow binding. Reaction was started by adding ATP to 5 mM and stopped by adding EDTA to 100 mM. The products were diluted 1:4, fixed as above and dialysed overnight against 20 mM MOPS-KOH pH 7, 0.1 mM EDTA.

Microscopy and data analysis

Samples were spread for microscopy[3] and examined in a JEOL 1010 transmission electron microscope. Images were acquired with a Gatan digital camera and DNA contour lengths measured by manual tracing using NIH Image software. Contour lengths were converted to nucleotides by reference to standard molecules, namely λ or pDWS2 double-stranded DNA and relaxed φX174 double-stranded circles or SSB-coated φX174 single-stranded circles. Loop one-tail or two-tail structures were identified as the junction of a double-stranded DNA segment with three or four SSB-coated single-stranded DNA arms, two of which were connected to form a loop. Y structures were identified as the junction of a double-stranded DNA segment and two linear SSB-coated single-stranded DNA arms. For each molecule the distance unwound was defined as the length of the longer continuous single strand: loop plus short tail versus long tail for RecBCD or RecBCD^{K177Q} enzymes, and loop versus tail for RecB^{K29Q}CD enzyme. Mean values for each time point were

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.

Received 7 November 2002; accepted 14 April 2003; doi:10.1038/nature01674.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank D. Julin for advice on protein purification; M. Dillingham, M. Spies and S. Kowalczykowski for sharing their unpublished information[7]; S. Amundsen for permission to cite unpublished results; J. Cooper, M. Gellert, N. Maizels, R. Strong and our colleagues for comments on the manuscript; and L. Caldwell and staff for help with the electron microscopy. This research was supported by grants from the NIH.

Competing interests statement The authors declare that they have no competing financial interests.

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