In vitro binding assay

T7-tagged proteins were produced in Escherichia coli and partially purified by absorption of T7-tag antibody-phosphocellulose (TNT-1, Promega). In vitro translated proteins were diluted with 0.5 ml of T7-tag binding wash buffer (4.29 mM NaH2PO4, 1.47 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween-20, 0.002% NaN3, pH 7.3) in the presence of 1x ‘Complete EDTA-free’ protease inhibitor cocktail (Boehringer Mannheim). T7-tagged proteins were then added to the corresponding T7-tag antibody-phosphocellulose conjugates (Novagen). T7-tagged proteins were visualized using T7-tag antibody-alkaline phosphatase conjugates (Novagen). Between 0.5 μg and 5 μg of T7-lag-1 or T7–LAG-3 and about 100 ng of each of the S-tagged proteins were used in each assay. To assay the embryonic thickening, T7–LAG-3 and antibody detection was done essentially as described8, except a whole mount freeze message was determined by RT–PCR using lag-3 specific primers and a primer to the coding sequence for LAG-3A and 86 nucleotides of the 3′ untranslated region. To assay the postembryonic lag-3 phenotype, L1 larvae were soaked in 1–3 μg μl−1 of double stranded lag-3 RNA for 4 h at 20 °C or 25 °C in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 85 mM NaCl, 1 mM MgSO4) in the presence of E. coli (Absorbance 600 nm, 0.75–1.0). After soaking, animals were transferred to Petri dishes to continue development. To assay the embryonic lag-3 phenotype, L4 animals were soaked in M9 containing 1 μg μl−1 lag-3 dsRNA overnight, then transferred to plates to lay eggs, using essentially a described method9.

Subcellular localization

Generation of transgenic nematodes carrying HS–Myc–LAG-3, heat shock conditions and antibody detection was done essentially as described9, except a whole mount freeze cracking method was used for fixation of the worms10.

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devised a strategy to determine both the polarity of and the step size for translocation. We first introduced a series of defined-size gaps in one or the other strand of otherwise duplex DNA, and then determined which size single-stranded DNA (ssDNA) gap prevented traversal. We used two types of substrates, each with a double-stranded blunt-end entry site at the 'proximal' 25-mer, an ssDNA gap, a 'tester' oligonucleotide (the 20-mer) distal to the gap and an ssDNA tail downstream of the annealed 20-mer (Fig. 1). The tail limits entry to only the dsDNA end; the gap is used to define the importance of that DNA strand in helicase translocation; and the displacement of the distal, tester oligonucleotide reports translocation and unwinding past the gap. Substrate set A has gaps positioned in the 5'-terminated strand relative to the entry point of the enzyme (the bottom strand of the substrates as shown), and substrate set B has gaps positioned in the 3'-terminated strand relative to the entry point of the enzyme (the top strand as shown). Gaps were initially either 0 (a nick) or 30 nucleotides (nt) in size (Fig. 1). A gap size of 30 nt was selected, as this is 9 nt larger than the contact length of the stationary holoenzyme bound to a dsDNA end.

We reasoned that the translocating enzyme should dissociate from the substrate when it encounters a discontinuity larger than its contact length in a strand of DNA that is critical to translocation. If the enzyme translocates along only one strand of DNA, then it should fail to bypass a gap present in that strand but not the other; if the enzyme requires both strands of DNA for translocation, then it should fail to bypass a gap (larger than its contact length) present in either strand. Failure to bypass a gap would be observed as formation of an intermediate unwinding product, consisting of the 100-mer (or alternatively the 130-mer) with the tester 20-mer distal to the gap, still bound.

When gaps of either 0 or 30 nt are present in the bottom strand, RecBC enzyme unwinds the substrate, displacing both the 25-mer proximal to the gap and the 20-mer downstream (Fig. 1a). This is observed as the disappearance of substrate in a time-dependent manner concomitant with the production of 100-mer ssDNA. Furthermore, displacement of both oligonucleotides is simultaneous (data not shown) with little or no intermediate species produced for either of these substrates, showing that a second RecBC enzyme is not responsible for displacement of the distal oligonucleotide. In the presence of single-stranded DNA binding (SSB) protein, RecBC enzyme cannot unwind substrates with only the internal 20-mers bound to the 100-mer (that is, substrates with a ssDNA tail at each end), showing that the enzyme requires the proximal dsDNA region to mediate unwinding beyond the gap (data not shown). Thus, RecBC enzyme is able to bypass gaps present in the 5'-terminated, or bottom strand, with more than 92% efficiency.

When gaps are positioned in the 3'-terminated strand, however, the results are significantly different (Fig. 1b). For the 0-nt-gap substrate, the enzyme can unwind and displace both the proximal 25-mer and the distal 20-mer; no intermediate is produced and ~65% of the starting substrate is converted to 100-mer in 2 min. In contrast, for the 30-nt-gap substrate the enzyme can displace only the proximal 25-mer; the 20-mer distal to the gap is not displaced efficiently, a significant amount of intermediate species accumulates (~ 55%), and the amount of 100-mer produced is decreased (down from 65% to 16%). Thus, RecBC enzyme is unable efficiently to bypass a 30-nt gap when that gap is present in the 3'-terminated, or top strand.

Identical results were obtained using single-round kinetic experiments in which the enzyme was pre-bound to the dsDNA end, and the reactions were initiated with a mixture of ATP and heparin (data not shown). Heparin prevents re-binding of RecBC enzyme after it has dissociated from a DNA molecule (ref. 5; and data not shown). Thus, the ability of the enzyme to bypass the 30-nt gap in the bottom strand (versus the top strand) is not owing to dissociation of

![Figure 1](image1.png)

**Figure 1** The RecBC helicase translocates in the 3'→5' direction. Time courses of unwinding reactions using substrates with gaps present in the 5'-terminated (bottom) strand (a) and in the 3'-terminated (top) strand (b) are shown. The substrate for each reaction is shown above the gel. RecBC enzyme must translocate from right to left as shown (arrow); displacement of the oligonucleotide distal to the gap (20-mer) requires both displacement of the oligonucleotide proximal to the gap (25-mer) and traversal through the gap. The positions of substrate (filled circles), intermediate (triangles) and 100-mer product (open circles) are indicated to the right of each gel. Lanes A, B and C are standards: A, intermediate for the nicked substrate; B, 100-mer product; C, the intermediate for the 30-nt gap.

![Figure 2](image2.png)

**Figure 2** The observed step size for the translocating RecBC enzyme is 23 (±2) nucleotides. A compilation of the extents of intermediate formation from two-minute time courses for the substrates with increasing gap lengths is shown. Inset shows the substrates, which consist of a 100-mer with a 25-mer annealed to the 5' end, and with various 20-mers annealed to different positions on the 100-mer. Reactions and data analyses were carried out as in Fig. 1.
which the curves shown in dissociation for each substrate set (proximal oligonucleotide length) is the gap length at set (Table 1). Numbers in parentheses indicate the length of the proximal oligonucleotide.

Fit to a sigmoid function (Prism v3.0, GraphPad Software) and were used to obtain the normalized to the average maximum extent of intermediate formation for that set: set IV, done on separate days for each substrate. For each substrate set, the extents were identical fashion to those in Fig. 2 and are arranged according to the length of the mer (or 130-mer) is shown. Thirteen additional substrate sets were constructed in identical fashion to those in Fig. 2 and are arranged according to the length of the oligonucleotide proximal to the gap (right grey box). In each set, the sequences of the 100-mer (or 130-mer, with 30 additional bases at the 3' end) and the set of 20-mers (left grey box) were always the same. The potential gap length generated is indicated (black box).

Data compiled from helicase assays such as those shown in Fig. 2 using substrate sets N–IX. Data are shown for a two-minute time point, averaged from two to seven assays, done on separate days for each substrate. For each substrate set, the extents were normalized to the average maximum extent of intermediate formation for that set: set IV, set V, set VI, set VII, and sets VIII and IX, 50%. Curves represent a fit to a sigmoid function (Prism v3.0, GraphPad Software) and were used to obtain the observed step size. Dashed lines and arrows indicate the observed step size for each data set (Table 1). Numbers in parentheses indicate the length of the proximal oligonucleotide.

Compilation of data from helicase assays using substrate sets I–XV. The gap causing dissociation for each substrate set (proximal oligonucleotide length) is the gap length at which the curves shown in begin to decrease. Data for substrate sets I–III and X–XIV are not shown. Error bars represent the minimum and maximum value of the extrapolated step size. Grey boxes indicate the number of translocation steps taken by the helicase. the enzyme at the gap followed by rapid re-binding with continued translocation. Furthermore, the inability to bypass gaps is not owing to a failure of RecBC enzyme to displace SSB protein bound in the gap, as similar results were obtained using the bacteriophage T4 gene 32 protein (data not shown). Collectively, these data show that, although it initiates by binding to dsDNA, RecBC enzyme requires only one strand of dsDNA for translocation (the strand 3’ terminated at the entry end). Thus, RecBC enzyme translocates on this strand with 3’→5’ polarity.

Because the enzyme dissociates from the substrate when the strand on which it translocates has a gap of 30 nt, but not when the gap is a nick, this suggested a strategy to define the translocation step size of the enzyme. We reasoned that by varying the length of the gap in the 3’-terminated strand, we could determine the smallest gap size that the enzyme fails to bypass during translocation, and that this should correspond to the ‘step size’ for the enzyme. We therefore constructed a family of substrates with ssDNA gaps ranging from a nick to 35 nt (Fig. 2, inset).

Reactions and analyses identical to those in Fig. 1 were carried out for the substrates in Fig. 2. RecBC enzyme can bypass gaps up to 18 nt in size as determined by the low extent of intermediate formation. Above this gap size, the accumulation of intermediate increased ninefold to an average of 36% for gaps of 25 nt or bigger. Some RecBC enzymes may be able to traverse these larger gaps because of the inherent flexibility of the ssDNA regions in these gapped substrates; that is, the ssDNA in these gaps may ‘loop out’ bringing the two flanking dsDNA regions of the substrate into close proximity, thereby facilitating enzyme bypass. In any case, the sharp transition at 20–25 nt shows that translocation through such gaps is seriously impaired. Thus, these data show that when RecBC enzyme encounters gaps smaller than 22–23 nt, it can ‘step across’ them as though they were dsDNA, suggesting that the step size for the translocating enzyme is ~23 nt. In addition, these results imply the disposition of the DNA unwinding domain relative to the footprint of the enzyme: this domain must be positioned at or near the rear of the helicase for gap bypass to occur. If it were positioned at the front, then a gap in the translocating strand as small as 1 nt would function as a suicide substrate by causing the helicase to dissociate as a complex with the ssDNA that it had produced and released by DNA unwinding.

Such a large step size was unanticipated, as the largest unwinding step size reported to our knowledge for a DNA helicase is 4–5 nt (ref. 6). Furthermore, this translocation step size is much larger than the amount of DNA that can be unwound from the free energy derived from the hydrolysis of one molecule of ATP2,3. To ensure that the failure of RecBC enzyme efficiently to bypass gaps of 23 (±2) nt or greater was not a consequence of the oligonucleotides used to construct the gap (because, for example, the sequence of the 20-mers following the gap is always different), additional substrate sets were constructed. For each set, the same 100-mer and family of distal 20-mers were used as for the substrates in Fig. 2, but the length of the oligonucleotide proximal to the gap was modified (Fig. 3a, substrate sets IV–IX). Thus, a gap of the same size would be present in each substrate set but the sequence of the oligonucleotides flanking that gap would be different. We expected that gap bypass should be the same in each of the six substrate sets, that is, ~23 nt.

Reactions and analyses were carried out using sets IV–IX; these data were converted to a ‘relative efficiency of gap traversal’ (Fig. 3b). Rather than being identical, each substrate set displays a distinctive curve. Unexpectedly, the gap-bypass efficiency decreases rapidly at a gap size that changes monotonically with each substrate set. Thus, the observed step size for the translocating RecBC enzyme appears to be dependent on the length of the proximal oligonucleotide (Table 1). This unexpected finding shows that the ability of RecBC helicase to traverse gaps as large as ~23 nt cannot be explained by proposing that the enzyme, with a static footprint of ~22 nt, simply positions itself across the gap so that the leading end
Table 1 Effects of proximal oligonucleotide length on gap bypass

<table>
<thead>
<tr>
<th>Step cycle</th>
<th>Substrate set</th>
<th>Proximal oligo length (nt)</th>
<th>Observed step size (nt)</th>
<th>Total (nt)§</th>
<th>Inferred step size (nt) (mean = 23)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>15</td>
<td>8</td>
<td>23</td>
<td></td>
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<td>XIV</td>
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<td>9</td>
<td>69</td>
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</table>

*Translocation step as shown in Fig. 3c.
†The length of the oligonucleotide proximal to the gap.
‡The observed step size corresponds to the gap at which the relative efficiency of gap traversal decreases below 100%.
§The total is the sum of the length of the oligonucleotide proximal to the gap and the observed step size.

minimum at a proximal oligonucleotide length of ~46 nt, and so on. The results of helicase assays using these substrates clearly show a cyclic pattern (Fig. 3c), in which the gap-size traversed approaches a minimum, increases to 24 nt, decreases to a new extrapolated minimum at 46 nt, and then is followed by yet another sharp increase and another (partial) linear decline; that is, the cycle resets approximately every 23 nt. Thus, the step size of ~23 nt is constant for at least three cycles of translocation.

Our finding that RecBC enzyme can traverse ssDNA gaps smaller than 23 nt in length requires a mechanism to explain how a helicase can translocate with a step size of 23 despite being limited, thermodynamically, to no more than ~5 bp of unwinding per mole of ATP hydrolysed. To explain this potential dilemma, we propose a mechanism that we have termed the ‘quantum inchworm’ (Fig. 4), which is similar to a model proposed for E. coli RNA polymerase. In the quantum inchworm model, translocation and DNA unwinding are two separate, consecutive events: first, translocation occurs by a 23-bp ‘step’; second, unwinding at the trailing domain occurs by several, smaller events of 2–5 bp for each ATP molecule hydrolysed. Consistent with the latter proposal, kinetic measurement of the DNA unwinding step size for RecBCD enzyme shows that it unwinds dsDNA in increments of 4–5 bp (A. Vindigni, personal communication). We propose that RecBC enzyme possesses two, non-equivalent DNA-binding sites: the first DNA-binding site is the leading domain of the enzyme that binds to one strand (the 3’-terminated strand) of dsDNA and functions to anchor the enzyme during DNA unwinding. The second DNA-binding site is the trailing domain that functions as the helicase domain of the enzyme and is responsible for separating the strands of DNA; this second domain must move in several smaller steps relative to the leading domain. We propose that one complete cycle of translocation and DNA unwinding is achieved by the expansion and contraction of the enzyme that includes the hydrolysis of at least 5 to 12 ATP molecules.

The cycle (Fig. 4) begins with the enzyme bound to a dsDNA end (stage 1). The trailing domain is bound at the end and, consequently, the leading edge is positioned 23 nt from this end (23 bp in B-form dsDNA corresponds to ~7.8 nm). The leading domain anchors the enzyme in place by binding to only one strand of the DNA duplex (indicated by the hands wrapping around one strand of the duplex). The trailing domain unwinds the dsDNA behind the enzyme translocates with a step size of 46 nt, or that the step size is 23 nt and the enzyme fails to bypass the gap and dissociation occurs during the second step. The first interpretation is unlikely because the footprint of the holoenzyme enzyme is only 19 (±3) base pairs (bp) (ref. 4) and it functions as a single multimeric complex containing only one ATPase subunit. Furthermore, if the step size were 46 nt, then for all substrates where the sum of the proximal and distal oligonucleotides and the gap was less, neither of the oligonucleotides would be displaced, because in the first step the enzyme would step into a gap beyond the bound oligonucleotides and dissociate. Clearly, this is not the case. The second interpretation is more likely, which argues that the step size for RecBC enzyme is 23 nt (Table 1, IV–IX, right column).

To determine whether the step size remains constant during translocation, we constructed two additional groups of substrates. The first group used the shortest proximal oligonucleotides that could be reliably bound to the 100-mer: these were 15, 17 and 20 nt (I–III, Fig. 3a). The second group used longer proximal oligonucleotides (42, 44, 45, 50 or 60 nt; X–XIV) and required a 130-mer (I–III, Fig. 3a). The second group used longer proximal oligonucleotides and by 3 for sets XII–XIV (see text).
leading edge, using the energy derived from ATP binding/hydration both to separate the DNA strands and to advance with 4–5 bp steps toward the leading domain (stages 2–4). As the lagging domain translocates up to the leading domain (stage 4), a signal (yet to be determined) is transmitted from the lagging to the leading domain causing the leading domain to dissociate and rebind ~23 nt ahead of the bound lagging end (stage 5). The enzyme has now returned to the starting point and the cycle repeats itself until the DNA molecule is completely unwound or the enzyme dissociates. As the lagging domain approaches the anchored leading domain, torsional stress will accumulate in the intervening dsDNA. Because the trailing end of the enzyme must transiently disengage to achieve, any accumulated torsional stress in the DNA could be released at this point, and, provided that the trailing end rapidly engages before the strands fully re-anneal, helicase function will remain processive as long as the leading end remains attached. One might expect to see an occasional uncoupling of unwinding and translocation (for example, the DNA strands partially re-anneal before the trailing end engages; this would result in an ‘inefficiency’ in ATP utilization. In fact, such an inefficiency is observed: for RecBC enzyme, as many as 1.4 ATP molecules are hydrolysed per base pair unwound12, whereas for RecBCD enzyme 2–3 ATP molecules are hydrolysed per base pair unwound13; this is between 3–15 fold more hydrosis than the minimum required for 0.2 molecules ATP molecules hydrolysed per base pair unwound. Therefore, at most, only 1 of 3 hydrolytic events results in DNA unwinding, and the value may be as few as 1 of 15 events being productive. Thus, anchoring of the helicase to DNA by the leading domain gives the trailing unwinding domain multiple opportunities to act on its substrate without macroscopic dissociation.

The large conformational changes proposed are not without precedent. The crystal structures of the Bacillus steaothermophilus PcrA helicase13,14 and the E. coli Rep helicase15 have been determined. PcrA helicase was proposed to function as an inchworm16, as was the related UvrD helicase17. Our mechanism has parallels to those proposed for these helicases and, thus, we conclude that a similar underlying mechanism may be used by at least a subset of the linear motor proteins to perform mechanical work.

Methods

Substrate construction

Oligonucleotides used for substrate construction were purified using denaturing polyacrylamide gels. The sequence of the 130-mer is 5'-TGGCGTCGACAGCGGGCATCCCG ATGCGCCGAGCCACGAGAATCATATAATGGGGAAGGCCACCAGCCTCGCGTGCCG TGAATGGGCCTGCAACGCGGGCATCCCG CACCGAGCCAGCCACGAGAATCATATAATGGGGAAGGCCACCAGCCTCGCGTGCCG CCGCGAACGCCAGCAAGACGTAGCCCAGCGCGTCGGCCGCCATGCCGGCGATCG

Annealing was performed in T4 polynucleotide kinase buffer plus 10 mM magnesium acetate containing the 100- (or 130-), 25- and 20-mers at a ratio of 100-mer:25-mer:20-mer:1:1:1.2.

Assay system

Helicase assays were conducted at room temperature and contained 25 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 8 mM magnesium acetate, 1 mM ATP, 1 μM SSB, 10 mM DNA (in mol of 100-mers) and 1.03 mM active RecBC enzyme. SSB protein ensures entry from the blunt end of the DNA, and prevents re-annealing of unwound strands. For single-round experiments, RecBC helicase was bound to DNA for 2 min and reactions were initiated by the addition of an ATP-heparin mix (1 mM and 10 mg/ml final). Reactions were stopped by an equal volume of ficol gel loading dye mix, containing SDS (1%, final), EDTA (50 mM) and proteinase K (0.4 mg/ml). After 5 min, aliquots were loaded onto polyacrylamide gels (1:15 bisacrylamide in TBE buffer). After electrophoresis, the gels were analysed using a Molecular Dynamics Storm 840 and ImageQuant software.

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The crystal structure of the photoprotein aequorin at 2.3 Å resolution

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Aequorin is a calcium-sensitive photoprotein originally obtained from the jellyfish Aequorea aequorea1. Because it has a high sensitivity to calcium ions and is biologically harmless, aequorin is widely used as a probe to monitor intracellular levels of free calcium. The aequorin molecule contains four helix–loop–helix ‘EF-hand’ domains, of which three can bind calcium2. The molecule also contains coelenterazine as its chromophoric ligand3. When calcium is added, the protein complex decomposes into apoaequorin, coelenteramide and CO2, accompanied by the emission of light4. Aequorin can be regenerated into active aequorin in the absence of calcium by incubation with coelenterazine, oxygen and a thiol agent5. Cloning and expression of the com-