

When a helicase is not a helicase: dsDNA tracking by the motor protein *EcoR124I*

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Using a combination of single molecule and bulk solution measurements, we have examined the DNA translocation activity of a helicase, the Type I restriction modification enzyme *EcoR124I*. We find that *EcoR124I* can translocate past covalent interstrand crosslinks, inconsistent with an obligatory unwinding mechanism. Instead, translocation of the intact dsDNA occurs principally via contacts to the sugar-phosphate backbone and bases of the 3'–5' strand; contacts to the 5'–3' strand are not essential for motion but do play a key role in stabilising the motor on the DNA. A model for dsDNA translocation is presented that could be applicable to a wide range of other enzyme complexes that are also labelled as helicases but which do not have actual unwinding activity.

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Introduction

The ATP-dependent translocation of motor proteins along polynucleotides is a fundamental feature of DNA metabolism. One important class of such motors are the helicases (Lohman and Bjornson, 1996). As defined on the basis of characteristic protein motifs, helicases encompass a large enzyme group with diverse genetic roles. For a great many of these proteins, the catalysis of nucleic acid strand separation is key to their translocation mechanism (Soultanas and Wigley, 2001); they are 'helicases' as originally defined (Kuhn *et al.*, 1979). The strand separation activity of these classical helicases is often measured using synthetic polynucleotide

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substrates. However, there are also many enzymes classified as helicases which fail to unwind such substrates. Occasionally this may be because a vital nucleotide or protein component has not been included in the assay. But more often, the lack of unwinding activity reflects the fact that ssDNA or ssRNA production is not required for the cellular role of the enzyme. A more all-encompassing definition of a helicase is that the protein motifs actually form an energy-coupling module based around RecA-folds that converts the binding and hydrolysis of NTPs into mechanical events on DNA or RNA (Singleton and Wigley, 2002).

While the mechanisms of DNA unwinding by helicases have been studied extensively, much less is known about how mechanochemical coupling occurs in other processes catalysed by helicases; for example, the remodelling of polynucleotide structures (e.g., RecG; Singleton *et al.*, 2001) and nucleoprotein complexes (e.g., RSC; Lia *et al.*, 2006). Is nucleic acid strand separation still required for these processes? What mechanistic properties, if any, are shared with the orthodox unwindases? Here, we have addressed these questions using *EcoR124I*, an *Escherichia coli* Type I restriction-modification (RM) enzyme that can be defined on the basis of primary amino-acid sequence as a helicase (McClelland and Szczelkun, 2004) and which has DNA translocation activity (Firman and Szczelkun, 2000; Seidel *et al.*, 2004).

EcoR124I is a multifunctional, hetero-oligomeric enzyme complex comprising two main components: a core methyltransferase (MTase), which undertakes sequence-specific DNA recognition and modification; and two HsdR subunits, which are loaded by the MTase onto the adjacent nonspecific DNA and which carry out ATP hydrolysis, DNA translocation and endonuclease activities (Szczelkun *et al.*, 1996; Firman and Szczelkun, 2000; Seidel *et al.*, 2004, 2005; McClelland *et al.*, 2005). ATP hydrolysis is coupled to DNA translocation by the superfamily 2 (SF2) helicase motifs in HsdR (McClelland and Szczelkun, 2004). Each HsdR motor translocates independently on the DNA away from the core MTase (Szczelkun *et al.*, 1996; Firman and Szczelkun, 2000; Seidel *et al.*, 2004, 2005; McClelland *et al.*, 2005). As the HsdRs remain bound to the MTase, which in turn remains bound to the *EcoR124I* binding site, two DNA loops are extruded (van Noort *et al.*, 2004). The MTase–DNA complex is much longer lived than the MTase–HsdR complex so that termination of a translocation event results in HsdR dissociation from both the DNA and the MTase (Seidel *et al.*, 2005). Another motor event is then initiated by the MTase loading a new HsdR molecule. On viral DNA, motor events will fire repeatedly until cleavage is triggered by the collision of two converging HsdRs. Cleavage of the host genome is prevented by attenuation of productive motor events (Makovets *et al.*, 2004; Seidel *et al.*, 2005).

No evidence of DNA unwinding by Type I enzymes has been reported using classical oligoduplex helicase assays. However, it is not clear whether the substrates utilised were appropriate to the analysis of a heterooligomeric system such as *EcoR124I*. One significant problem is that motor activity

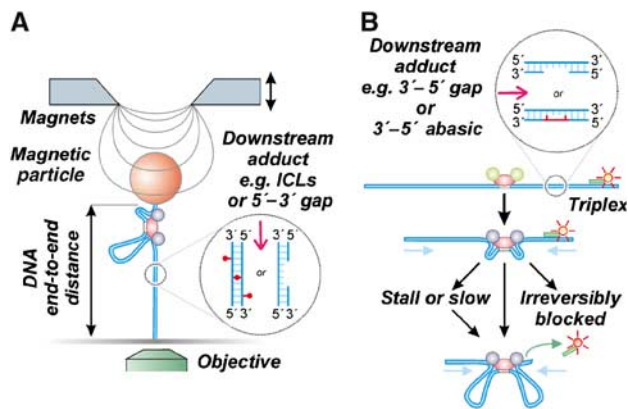


Figure 1 Approaches for examining the translocation of *EcoR124I* on modified DNA. (A) Magnetic tweezers assay. A DNA molecule with a single *EcoR124I* binding site and containing suitable modification(s) is tethered between a magnetic bead and a glass slide. Magnets are used to elongate the DNA by the application of different forces. *EcoR124I* translocation causes loop extrusion that, in turn, shortens the apparent end-to-end length of the DNA, which can be measured using video microscopy. Due to the close location of the *EcoR124I* site to the magnetic bead (275 bp for ICLs, 90 bp for gaps) mainly unidirectional DNA translocation towards the modification(s) is probed. Examples are shown of: ICLs (red circle) and accompanying monoadducts (red lollipop); and, a 4 nucleotide gap in the 5'-3' strand. (B) Triplex displacement assay. A labelled triplex is introduced downstream of an *EcoR124I* binding site, with modifications to the DNA made in the intervening region. Translocation and collision with triplex leads to displacement and an increase in fluorescence, which can be measured using a stopped-flow fluorimeter. Inhibition of translocation can be inferred from changes to the triplex displacement profiles. Examples are shown of: a 4 nucleotide gap in the 3'-5' strand; and two abasic groups in 3'-5' strand introduced by annealing a modified oligonucleotide (red strand) into a gap.

must be measured in the presence of the MTase loading complex, HsdR has no motor activity in isolation (Firman and Szczelkun, 2000). Since HsdR loading requires the MTase to be bound at a specific dsDNA binding site, it is difficult to imagine how strand separation could be observed using a simple dsDNA substrate. Short oligoduplex substrates, as used in most helicase assays, may also fail because their limited size does not support initiation of productive motor events. Our alternative approach has been to investigate the motor kinetics of *EcoR124I* on long (>1 kbp) modified DNA using a combination of single molecule and bulk solution techniques (Figure 1). Our results are consistent with a mechanism in which *EcoR124I* translocates without unwinding along the 3'-5' strand of intact duplex DNA. Contacts to the 5'-3' strand play a supplementary 'clamp' role, stabilising the translocating complex. Since Type I enzymes have provided a model system for other helicases, such as the chromatin remodelling factors (Lia *et al*, 2006), we believe that the dsDNA translocation scheme we present may be generally applicable to other helicase-based motors which do not demonstrate unwinding activity.

Results

Is DNA unwinding a prerequisite of DNA translocation by Type I RM enzymes?

DNA unwinding by an orthodox helicase is facilitated by ssDNA translocation (Lohman and Bjornson, 1996; Soultanas

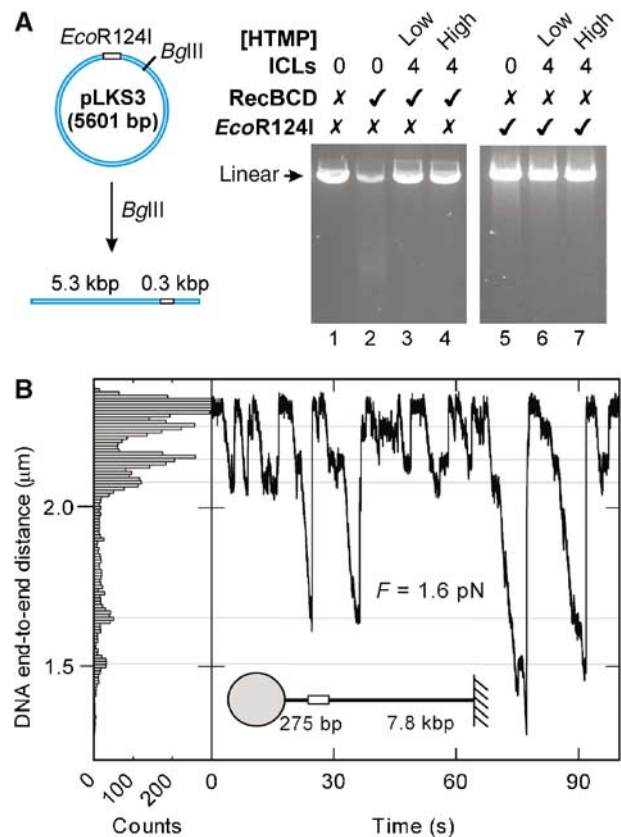


Figure 2 The effect of DNA ICLs on *EcoR124I* and RecBCD activity. (A) pLKS3 was linearised with *BglIII* to produce a linear DNA substrate. ~4 ICLs per DNA were introduced at two HTMP concentrations: either <0.033 HTMP/dT (low) or >1 HTMP/dT (high). RecBCD conditions were chosen so that DNA unwinding would occur without DNA cleavage. *EcoR124I* reactions conditions were chosen that would allow cleavage of circular DNA. DNA substrates and products were separated by gel electrophoresis. (B) Time trace (right panel) recorded in the magnetic tweezers at 20 nM MTase and 160 nM HsdR using a DNA substrate as shown with ICLs introduced at ~1 ICL/kbp using a low concentration of HTMP (<0.033 HTMP/dT). Multiple motor events are observed over a 90 s window, showing multiple stall events occurring at the same sites on the DNA. (In comparison, on unmodified DNA there is only one stall event corresponding to collision with the bead.) In the left panel is the occupancy of different DNA lengths (all detected lengths of a time trace binned at 10 nm intervals). Peaks in the counts correspond to the full length DNA at ~2.3 µm (i.e., when the enzyme is not translocating) and correlated stalls at ICLs/adducts (grey lines) and the bead during translocation. If stalling were independent of sequence then the former peaks would not be observed.

and Wigley, 2001); intact dsDNA cannot be accommodated by the motor. Accordingly, if interstrand crosslinks (ICLs) are introduced into duplex DNA, an obligate unwindase cannot progress (e.g., DNA unwinding by RecBCD is completely blocked on crosslinked substrates; Figure 2A; Karu and Linn, 1972). But what is the situation for a dsDNA translocase? For one such enzyme, *E. coli* RuvB, bypass of an ICL has been observed, suggesting that ssDNA does not play an extensive role in translocation (George *et al*, 2000). It has also been reported that Type I RM enzymes can still cleave crosslinked DNA (Karu and Linn, 1972; Endlich and Linn, 1985), although experimental data were never presented. It was therefore concluded that ICLs have no effect on translocation. However, it has been shown subsequently that Type I

enzymes cleave DNA when translocation is impeded (Janscak *et al*, 1999; also, see below). Therefore, ICLs may actually induce endonuclease activity through inhibition of translocation. To resolve these issues, we analysed *EcoR124I* on DNA in which ICLs were randomly introduced by treatment with 4'-hydroxymethyl-4,5',8'-trimethylpsoralen (HTMP) and UV light (Karu and Linn, 1972; Hearst, 1981), using conditions which resulted in either a low or high density of corresponding HTMP monoadducts (see Materials and methods).

We first investigated if ICLs could induce cleavage by analysing linear DNA carrying a single *EcoR124I* site (Figure 2A). Such substrates are normally not cleaved under the reaction conditions used here (Szczelkun *et al*, 1996). At an average crosslinking density of ~ 1 ICL per 1.4 kbp, no cleavage was observed following extensive incubation with *EcoR124I* (Figure 2A, lanes 5–7). Therefore, neither ICLs nor the accompanying HTMP monoadducts activate *EcoR124I* endonuclease activity. We next tested circular substrates with a single *EcoR124I* site that, in contrast to linear DNA, can be cut under the conditions used here (Szczelkun *et al*, 1996). As has been suggested previously (Karu and Linn, 1972; Endlich and Linn, 1985), ICLs had no effect on the rate or amplitude of DNA cleavage, although elevated monoadduct levels did block *EcoR124I* binding (Supplementary Figure 1). We also examined the distribution of cleavage sites on circular DNA as a function of increased crosslinking; no significant differences were observed with between 0 and 8 ICLs per DNA (Supplementary Figure 1). Therefore, during cleavage of crosslinked circular DNA, the same collision/cleavage events must be occurring irrespective of multiple ICLs/adducts.

To observe directly the effects of ICLs on translocation, we measured motor activity of single *EcoR124I* molecules using magnetic tweezers (Figure 1A; Seidel *et al*, 2004, 2005). This setup allows us to monitor the end-to-end distance of a single DNA molecule in real time, with *EcoR124I* translocation seen as characteristic saw tooth-shaped DNA shortening events (Seidel *et al*, 2004). We introduced ~ 6 ICLs into an 8 kbp linear DNA substrate (Figure 2B) under conditions that produced a low monoadduct density (see Supplementary Methods). While typical profiles on non-crosslinked DNA exhibit translocation events with stalling only observed at the DNA bead (Seidel *et al*, 2004, 2005), profiles on cross-linked DNA exhibited multiple short temporary stalls within a single translocation event (Figure 2B). There was a strong correlation in stall location between successive events, suggesting that they are dependent upon substrate- and site-specific modifications. In many instances, translocation continued after stalling, showing that the modifications could be bypassed. Given our crosslinking conditions, both randomly distributed ICLs and/or monoadducts could be responsible for these events. Nonetheless, for the longest motor runs observed (representing translocation of ≥ 1 kbp), at least one ICL will have been overcome on average. We also examined the ATPase activity of *EcoR124I* on modified and unmodified linear DNA; no differences were observed (data not shown).

There is no indication from the single molecule profiles that bypass of DNA damage occurs by DNA release followed by long-range re-association. The same conclusion can be made from the mapping studies (Supplementary Figure 1), where significant changes in motor protein distribution would have been revealed as changes in cleavage site

distribution. We confirmed that long-range 'hopping' by *EcoR124I* is highly unlikely by testing translocation on a DNA catenane (Supplementary Figure 2). Although these data do not completely exclude short-range hopping, this mechanism of bypass seems unlikely for a Type I enzyme as release of the topologically strained expanding loop(s) would most likely result in a rapid diffusion of the HsdR and DNA away from one another, making rebinding unlikely. This is particularly true in the tweezers where separation of the HsdR and loop would be strongly supported by the applied force (1.6 pN in Figure 2B), making any hopping involving a transient disengagement practically impossible. An alternative mechanism of bypass would be for the motor to 'step over' the adduct (see Discussion for detailed definitions of protein step sizes). Given that HTMP crosslinking of thymine residues across a dTA dinucleotide produces a bulky major groove adduct, a protein would most likely need a step size of 3 bp or more to reliably overcome the damage. Since we present evidence in the discussion that *EcoR124I* steps along DNA in increments of 2 bp or less, we also disfavour this mechanism of bypass.

Is there a strand polarity of DNA translocation by Type I RM enzymes?

From the data above, strand separation during translocation is highly unlikely and *EcoR124I* can be classified as a true dsDNA translocase. But how do the HsdRs contact DNA? For the orthodox helicases, the binding and hydrolysis of NTPs has been suggested to drive the opening and closing of a cleft between RecA-like motor domains, which in turn drives motion of ssDNA in one of two polarities—either 5'-3' or 3'-5'—across the domains (the 'inchworm mechanism'; Soutanas and Wigley, 2001). Thus, helicases can be defined as having a distinct strand polarity (Lohman and Bjornson, 1996). In a similar manner, a dsDNA motor could contact and directionally convey just one strand, the other strand being passively transported. Alternatively, a dsDNA motor could make contacts with both strands.

To investigate these alternatives, we introduced ssDNA gaps downstream of an *EcoR124I* site and measured the effects on DNA translocation using complementary single molecule and bulk solution approaches (Figure 1). For both sets of DNA substrates, the gaps were introduced by modifications of a published method (see Wang and Hays, 2001; Materials and methods; Supplementary Figure 3). One consequence of this method is that the relative locations of the gaps differ from substrate-to-substrate (Supplementary Tables 1–3). However, we show later by the analysis of polyethylene glycol (PEG) spacers that the relative location of an adduct does not affect the outcome (Figure 6A).

Translocation on gapped DNA measured using magnetic tweezers

EcoR124I motor events on DNA containing a 20 nt gap in one or other strand are shown in Figure 3A. A clear difference between the substrates emerged. (1) On the 3'-5' modified DNA, only short-range motor events were observed (upper panel). The maximum distance translocated was 1210 ± 20 bp, corresponding to the 1180 bp distance between the *EcoR124I* site and the gap, with the majority of events terminating almost exactly at this point (Supplementary Figure 4). In comparison, on unmodified DNA, a significant

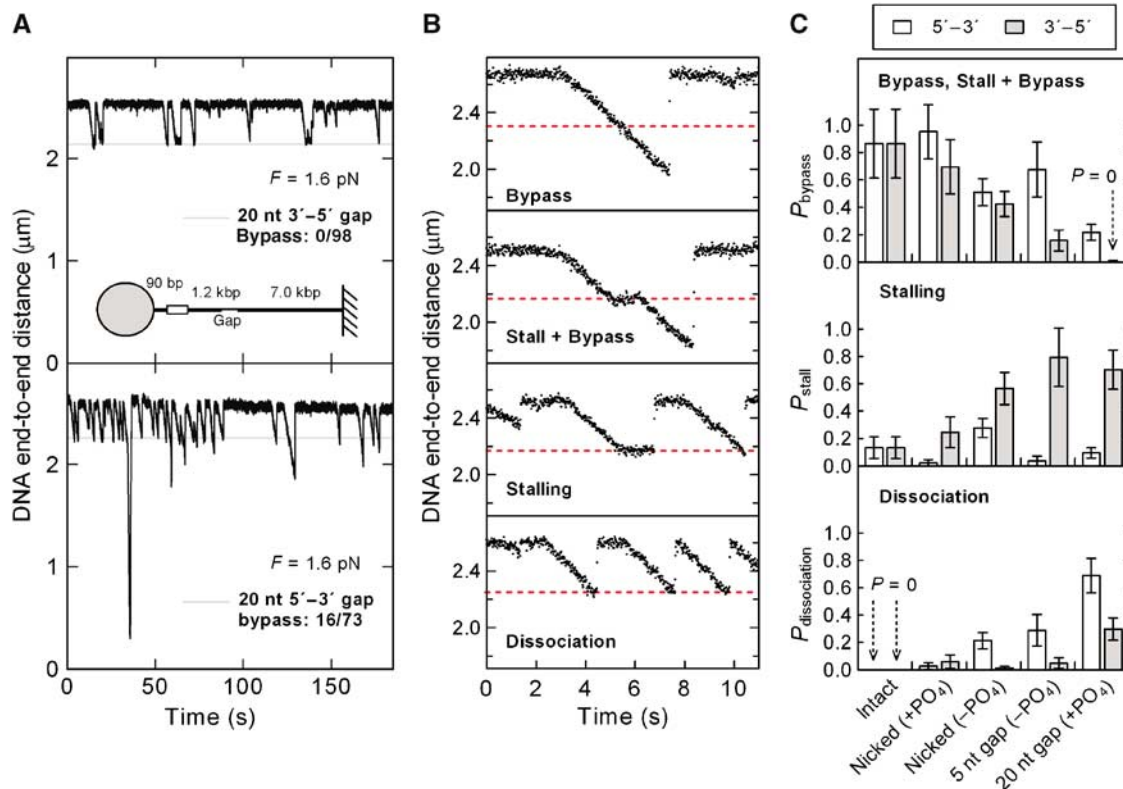


Figure 3 Single molecule measurements of DNA translocation on gapped DNA. **(A)** Two time traces recorded in the magnetic tweezers at 20 nM MTase and 160 nM HsdR using the DNA substrate as shown with a 20 nt gap in one or other strand. *EcoR124I* translocation towards the bead is lost in the thermal noise so the only events observed directly are those towards the gap. The grey lines represent the approximate locations of the gaps. **(B)** Classification of different motor events as observed at the gap. Broken line represents location of the gap. **(C)** Statistics of different motors events on each strand as a function of gap length (see Supplementary Methods for details of statistical analysis). *Bypass* and *stall + bypass* events were scored together.

number of translocation events are observed beyond this distance (Supplementary Figure 4). The simplest explanation is that *EcoR124I* can translocate as far as the 3'-5' gap but then cannot continue. (2) On the 5'-3' modified DNA, a significant proportion of events also terminated upon reaching the gap (lower panel). However, in $22 \pm 6\%$ of events bypass was observed, with termination occurring downstream of the gap (Supplementary Figure 4). This suggests that *EcoR124I* can only bypass a 20 nt gap when it is on the 5'-3' strand. We note that DNA cleavage at the gaps was not observed for either substrate.

Four different events could be classified as occurring at a gap (Figure 3B): bypass without visible stalling (*bypass*); stalling followed by bypass (*stall + bypass*); stalling followed by dissociation (*stalling*); and dissociation without visible stalling (*dissociation*). The statistics of these events were calculated for gaps in each strand, and for variations in gap size (Figure 3C). *Bypass* and *stall + bypass* events were scored together. *Bypass* events were always more efficient when the gap was in the 5'-3' strand, even when the modification was only a break in the phosphodiester backbone (top panel). This suggests that the principal motor contacts are made to the 3'-5' strand. This conclusion is reinforced by the *stalling* probability, which was much greater with gaps on the 3'-5' strand (middle panel)—a break in the translocated strand would necessarily stall a motor, whereas a break in the non-translocated strand could be overcome by translocation across the intact strand. In con-

trast, *dissociation* events were more likely when the gap was in the 5'-3' strand (lower panel). This suggests that contacts to this strand are more important in stabilising a translocating complex. Given that even a dephosphorylated nick can have a significant effect on the translocation kinetics, it also seems likely that *EcoR124I* moves in small steps (see below and Discussion).

An alternative view of the data is that translocation along the 5'-3' strand would also lead to an increase in dissociation at 5'-3' gaps as the motor would dissociate from the end of the DNA. However, in most cases helicase motors tend to be stabilised at DNA ends (e.g., Dillingham *et al*, 2000), as there are still sufficient contacts between the DNA and the rear of the motor. Data on modified DNA presented below (Figure 6) supports the notion that the 3'-5' strand plays a more important role than the 5'-3' strand. Therefore, the overall conclusion we draw from our data is that *EcoR124I* translocates dsDNA principally by contacts to the 3'-5' strand, with additional stability coming from contacts to the 5'-3' strand. We have also obtained further support for 3'-5' translocation by measuring the directionality of *EcoR124I* motion at DNA branch points (Louise K Stanley and Mark D Szczelkun, unpublished observations).

Is applied force critical to the probability of gap bypass?

A prerequisite of measuring translocation by a Type I enzyme in the tweezers assay is that the DNA substrate must be extended by the application of force parallel to the chain

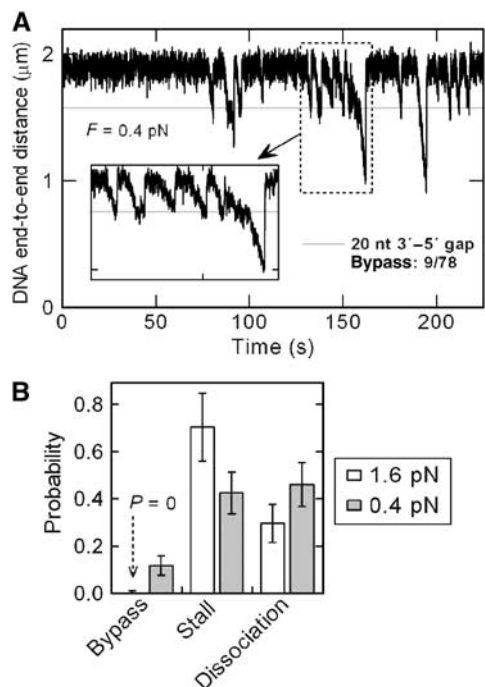


Figure 4 Force dependence of gap bypass on the 3'-5' strand. (A) Time trace recorded in the magnetic tweezers at 0.4 pN using the DNA substrate as shown in Figure 3A with a 20 nt gap in the 3'-5' strand. Enzyme concentrations were 20 nM MTase and 80 nM HsdR. *EcoR124I* translocation towards the bead is lost in the thermal noise so the only events observed directly are those towards the gap. The grey line represents the estimated location of the gap (1190 ± 40 bp, Supplementary Figure 4). Note that compared to Figure 3A, at the lower force there is an increase in noise and a decrease in apparent distances due to the reduced DNA stretching. (Inset) Detail from the full time trace (dotted box) showing stall events at the gap region and one bypass event. (B) Statistics of 3'-5' gap bypass over 20 nt at different forces. The 1.6 pN data is taken from Figure 3C. Calculation of statistics was carried out as in Figure 3. At least some of the reduction in P_{stall} at 0.4 pN comes from the increased probability of bypass. There is also an increase in $P_{\text{dissociation}}$, which might reflect complexes that terminate during bypass of 20 nt ssDNA loops.

(1.6 pN in Figure 3A; Seidel *et al*, 2004). This is critical to our interpretation of the above data as applied force could effect the motor reaction itself; for *EcoR124I*, initiation rates and processivity are force dependent (Seidel *et al*, 2004, 2005). Additionally, applied force could alter the conformational dynamics of the DNA (Blumberg *et al*, 2005; Sankararaman and Marko, 2005; Yan *et al*, 2005), which might influence the efficiency of gap bridging.

To investigate if applied tension affected gap bypass, we re-examined *EcoR124I* translocation on the 20 nt 3'-5' gap substrate at a reduced force ($F=0.4$ pN, Figure 4A). In contrast to the complete inhibition at 1.6 pN (Figure 3A), gap bypass was now observed in $\sim 12\%$ of events (Figure 4B). Therefore, DNA tension seems to inhibit gap bypass. How these observations tally with possible translocation mechanisms is discussed below.

Translocation on gapped DNA measured using triplex displacement

To provide supporting data for the interpretations of the single molecule experiments, we also measured translocation on gapped DNA using triplex displacement (Figure 1B;

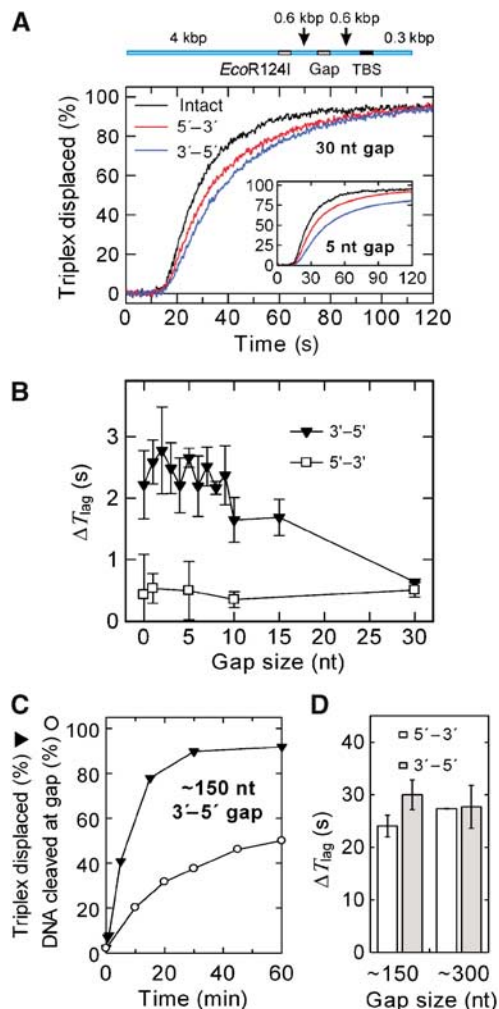


Figure 5 Stopped flow measurements of DNA translocation on gapped DNA. (A) Triplex displacement profiles from the stopped flow experiments. The final solution contains 1 nM DNA (0.5 nM triplex), 30 nM MTase, 300 nM HsdR, and 25 μ M ATP. (B) Changes in the triplex displacement profiles with different gap sizes, with $\Delta T_{\text{lag}} = T_{\text{lag}}^{\text{modified}} - T_{\text{lag}}^{\text{unmodified}}$, where $\langle T_{\text{lag}}^{\text{unmodified}} \rangle = 16.28 \pm 0.14$ s (the average of T_{lag} from intact and nicked DNA, which were identical within error, data not shown). A gap size of zero represents a dephosphorylated nick. Due to the method used to make the gaps (Materials and methods, Supplementary Figure 3 and Table 2), all spacings shown are dephosphorylated except for the 30 nt spacing. To within experimental error, phosphorylated gaps gave identical results (data not shown). (C) Comparison of DNA translocation and gap cleavage using a substrate with a 150 nt 3'-5' gap. A triplex displacement profile from a hand-mixed radioactive triplex assay (5 nM DNA (2.5 nM triplex), 30 nM MTase and 300 nM HsdR) is compared to the appearance of product DNA cleaved at a gap from an agarose gel assay using ^3H -labelled DNA (5 nM DNA, 30 nM MTase and 90 nM HsdR). Similar traces were obtained for the 5'-3' gap and the 300 nt spacings (data not shown). (D) ΔT_{lag} values calculated for long gaps (all phosphorylated). All error bars in the figure represent the standard error of the mean calculated from ≥ 2 independent experiments.

Firman and Szczelkun, 2000; McClelland *et al*, 2005; Seidel *et al*, 2005). We generated 5.6 kbp linear DNA substrates containing an *EcoR124I* site, a gap site 0.6 kbp downstream, followed by a triplex binding site a further 0.6 kbp downstream (Figure 5A; Supplementary Figure 3). Displacement of a fluorescent triplex by a translocating enzyme can be measured and is preceded by a lag phase, the size of which

reflects initiation and translocation events (McClelland *et al*, 2005).

Complete triplex displacement was observed on all substrates, irrespective of gap size or strand polarity (e.g., 30 and 5 nt gaps in Figure 5A). We did not observe irreversible inhibition at longer 3'-5' spacings as in Figure 3A. Since P_{bypass} is critically sensitive to DNA tension (Figure 4), the higher efficiency in the triplex assay most likely reflects the conformation of the DNA under the zero force regime in bulk solution (also, see Discussion). Nonetheless, we observed in nearly every case that the modifications altered the lag phase preceding triplex displacement. Unlike our most recent study (Seidel *et al*, 2005), we did not use full kinetic models to extract translocation rate constants as simulations showed that, due to model complexity, indistinguishable profiles were returned using completely different schemes. Instead, we estimated the lag time (T_{lag}) for each substrate using an established exponential fit method (Firman and Szczelkun, 2000; McClelland *et al*, 2005) and plotted the difference in T_{lag} to the unmodified substrate as $\Delta T_{\text{lag}} = T_{\text{lag}}^{\text{modified}} - T_{\text{lag}}^{\text{unmodified}}$ (Figure 5B).

While very little difference was observed between intact and nicked DNA ($\Delta T_{\text{lag}} \approx 0$), all other modifications, including a single missing phosphate, showed positive ΔT_{lag} values (Figure 5B). This reflects slower translocation events at the gap. Note, however, that since dissociation does not alter $T_{\text{lag}}^{\text{modified}}$ (McClelland *et al*, 2005), changes in $P_{\text{dissociation}}$ will not alter ΔT_{lag} . If stalling is more frequent at 3'-5' gaps, as suggested by the tweezers measurements (Figure 3C), then $\Delta T_{\text{lag}}^{3'-5'}$ should increase relative to $\Delta T_{\text{lag}}^{5'-3'}$. Indeed, at short spacings (<10 nt), $\Delta T_{\text{lag}}^{3'-5'}$ was four- to five-fold higher than $\Delta T_{\text{lag}}^{5'-3'}$, consistent with the single-molecule experiments. However, as the spacings were increased (≥ 10 nt), $\Delta T_{\text{lag}}^{3'-5'}$ decreased, until at the 30 nt there was little difference between the strands. This trend appears to be the opposite of that expected given the data in Figure 3. While $\Delta T_{\text{lag}}^{3'-5'}$ varied with gap size, $\Delta T_{\text{lag}}^{5'-3'}$ was largely invariant ($\langle \Delta T_{\text{lag}}^{5'-3'} \rangle = \sim 0.5$ s). Note that although the relative positions of the gaps are not identical (Supplementary Table 2), there is no evidence for position-dependent effects with *EcoR124I* (Figure 6A). We could also show that the kinetics were unaffected by 5' phosphorylation of the gap (data not shown). A small amount of gap cleavage was observed (<10% after 1 h incubation), independent of strand polarity or gap length (data not shown).

Bypass of long gaps *in preference* to short gaps seemed surprising, so we lengthened the gaps by exonuclease III treatment (Materials and methods). This produces gaps that are distributive in size and represent a range rather than an absolute length. Complete triplex displacement was still observed with ssDNA gaps in either strand of ~ 150 and ~ 300 nt, although the kinetics were considerably slower (Figure 5C and D, other data not shown). There was a small, strand-dependent difference in ΔT_{lag} at 150 nt, but not at 300 nt. Significant cleavage at the gaps was also observed, but on timescales significantly slower than translocation (Figure 5C); in other words, bypass of a large gap could occur multiple times before it were cleaved. We suggest that *EcoR124I* remains at the gap for long periods of time during which slow cleavage could occur (Janscak *et al*, 1999). This might represent long-lived stall states or slow ssDNA translocation.

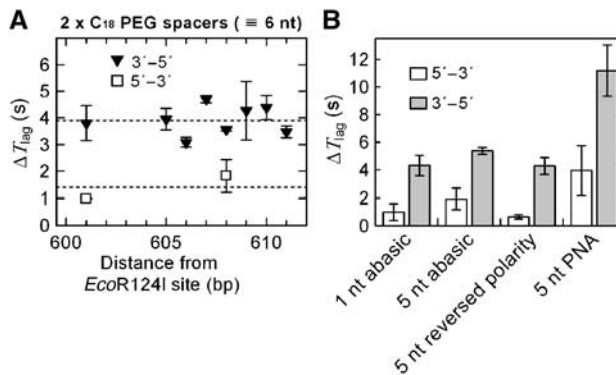


Figure 6 Stopped flow measurements of DNA translocation over DNA adducts. Triplex displacement profiles were recorded using DNA substrates containing the modifications indicated (see Supplementary Table 3). Experimental conditions were as given in Figure 5. (A) ΔT_{lag} values measured on DNA containing two C18 PEG spacers (replacing 6 nt of DNA) in one or other strand. The relative location of the spacers was varied from 601–611 bp downstream of the *EcoR124I* binding site. The dashed lines represent $\langle \Delta T_{\text{lag}} \rangle$ for each strand. (B) ΔT_{lag} values measured on DNA containing the modifications indicated. All error bars in the figure represent the standard error of the mean calculated from ≥ 2 independent experiments.

Overall, the triplex data confirm increased stalling (or hindered translocation) at the 3'-5' gap relative to the 5'-3' gap and support the proposition above that the principal *EcoR124I* motor contacts are made with the 3'-5' strand (although both strands are clearly important). Also, in line with the results above, a dephosphorylated nick produced significant increases in ΔT_{lag} , indicative of a small step size. However, it seems that *EcoR124I* can, *under conditions of zero force*, translocate over considerable gaps in either strand.

What nucleotide contacts are made during DNA translocation by Type I RM enzymes?

For the classical monomeric helicases, there are two possible modes of binding ssDNA: for SF1 enzymes the principal contacts come from DNA bases, which flip into binding pockets where hydrophobic stacking interactions are made with aromatic amino acids (the 'Mexican Wave' mechanism; Soutanas and Wigley, 2001), although electrostatic interactions with the phosphodiester backbone are not completely superfluous (Eoff *et al*, 2005); in contrast, for SF2 enzymes the principal contacts appear to be with the backbone (Kim *et al*, 1998; Kawaoka *et al*, 2004). It has been suggested that backbone tracking has allowed adaptation to translocation on either ss- or dsDNA (Kawaoka *et al*, 2004). A recently determined structure of the catalytic core of a Rad54-homologue bound to DNA has revealed an even more complex picture for a dsDNA translocase (Dürr *et al*, 2005), with evidence for base and backbone contacts in both strands.

Here, we used triplex displacement to measure the effect of backbone and base adducts on *EcoR124I* translocation. This was achieved by rescuing our gapped substrates with synthetic oligonucleotides containing different adducts (see Materials and methods, Supplementary Table 3). We first replaced a 6 nt stretch with an equivalent length of PEG spacer (thus removing completely the nucleic acid contacts), with the position of the spacer relative to the *EcoR124I* site varied within an 11 bp window (Figure 6A). As observed with gapped DNA, modifications in either polarity were overcome,

with those on the 3'-5' strand slowing translocation to a greater degree than those on the 5'-3' strand ($|\Delta T_{\text{lag}}^{3'-5'}| > |\Delta T_{\text{lag}}^{5'-3'}|$). Within experimental error, the relative location of the spacer did not significantly alter the strand-dependent differences in ΔT_{lag} ; bypass of 3'-5' adducts never became more efficient than 5'-3' adducts. At first, it may seem contradictory that a motor can bypass a region of the translocated strand devoid of any nucleotide contacts. However, we believe that because of the conformational flexibility of the PEG spacer, bypass of this adduct occurs in a similar manner as for gapped DNA (see Discussion).

We next investigated stretches of stable abasic DNA (1 or 5 nt of tetrahydrofuran), reversed backbone polarity (5 nt of 3'-5' linkage within the 5'-3' chain, or *vice versa*) or peptide nucleic acid (5 nt of PNA). For each adduct, the B-form DNA conformation is also likely to be altered (Tackett *et al*, 2001; Eoff *et al*, 2005). Nonetheless, none of the substrates irreversibly blocked *EcoR124I* translocation (data not shown). In every case, $\Delta T_{\text{lag}}^{3'-5'}$ was greater than $\Delta T_{\text{lag}}^{5'-3'}$, supporting strongly a defined translocation strand polarity (Figure 6B). The $\Delta T_{\text{lag}}^{3'-5'}$ values obtained with these substrates are somewhat higher than those obtained on the DNA with short gaps, although not as high as those obtained on long gaps (Figure 5). The range of ΔT_{lag} values obtained most likely reflects underlying differences in the mechanisms of bypass. In contrast to some other helicase motors (e.g., see Kawaoka *et al*, 2004), we did not observe a striking difference in kinetics between sugar-phosphate backbone and base analogues. Where $\Delta T_{\text{lag}}^{3'-5'}$ did increase noticeably with the PNA modification (Figure 6B), $\Delta T_{\text{lag}}^{5'-3'}$ also increased proportionally, suggesting that this adduct has a greater influence on overall DNA structure (Tackett *et al*, 2001). It therefore appears that, as suggested for Rad54 (Dürr *et al*, 2005), *EcoR124I* must utilise contacts to the backbone and bases of both strands during translocation.

Discussion

When is a helicase not a helicase?

We have investigated the motor activity of an SF2 helicase, the Type I RM enzyme *EcoR124I*, using both single molecule

and bulk solution measurements. Overall we find excellent consistency between our approaches. Where we have observed incongruity, this can be explained in terms of quantifiable technical differences (e.g., the application of force in the tweezers setup). In fact, the discrepancies have highlighted important mechanistic features that would otherwise have been overlooked, as discussed below. Key features of the *EcoR124I* translocation mechanism can be highlighted and incorporated into a view of dsDNA motor activity (Figure 7A):

Translocation of intact dsDNA leads to changes in DNA twist. The HsdR motor domain interacts tightly with dsDNA and, in an ATP-dependent reaction, pumps DNA downstream of the complex into an expanding DNA loop (Figure 7A). Although there is evidence that non-duplex DNA is formed transiently in the initial expanding loop to compensate for induced strain (van Noort *et al*, 2004), ICLs do not inhibit translocation during the subsequent loop expansion (Figure 2; Supplementary Figure 1). Therefore, despite the presence of characteristic helicase motifs in HsdR, *EcoR124I* is very unlikely to be an obligate unwindase.

Another important feature of the model in Figure 7A is that the motor follows the helical twist of the DNA (Seidel *et al*, 2004). As the HsdR unit is fixed to the MTase, this alters the topology of the up- and downstream DNA (Figure 7A; Halford *et al*, 2004). Every motor that moves on DNA will do so in incremental steps along the polynucleotide; this is defined as the motor step size (d bp). If the stepping motion also results in DNA twisting, then we can also define a *twisting step size* (Halford *et al*, 2004); this is the number of base pairs tracked before the DNA is twisted by 360° ($Step_{\text{Tw}}$ bp). For *EcoR124I*, $Step_{\text{Tw}}$ has been measured as ~ 11 bp (Seidel *et al*, 2004). For a protein to rotate the DNA 360° in 11 bp, it must track the DNA helix, which in turn can only occur if the motor has a relatively small translocation step size ($d \ll 11$ bp). We suggest here for *EcoR124I* that d is only 1–2 bp. This is consistent with our observations that loss of even a single phosphate or base can have an appreciable effect on translocation (Figures 3C and 5B). Moreover, our kinetics were insensitive to incremental changes in the relative position of a PEG spacer

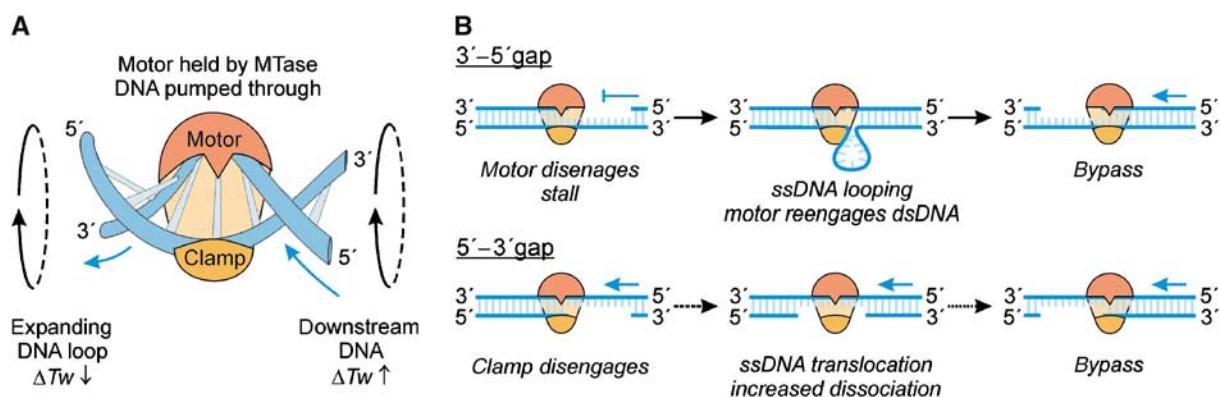


Figure 7 Model for strand-specific dsDNA translocation by a Type I RM motor. (A) Schematic of the motor contacts and DNA transitions. As a function of ATP binding and hydrolysis, downstream dsDNA is pumped via the 3'-5' strand into the expanding loop. Secondary clamp contacts to the 5'-3' strand from the HsdR subunit and/or other parts of the enzyme complex stabilise the dsDNA translocation and maintain processivity. DNA strand separation is not necessary during steady-state translocation but may play a role in initiation. The DNA is twisted as it passes the motor, 360° for every ~ 11 bp translocated (Seidel *et al*, 2004). Accordingly, DNA in the expanding loop is undertwisted and becomes negatively supercoiled. (B) Schematic mechanisms for gap bypass by ssDNA looping (upper panel) or ssDNA translocation (lower panel). See main text for more details.

(Figure 6A); a larger step size might have produced a more conspicuous periodic relationship (Bianco and Kowalczykowski, 2000). Further support for a small d value comes from noise analysis of single molecule traces (Ralf Seidel, Joost GP Bloom, Cees Dekker and Mark D Szczelkun, manuscript in preparation).

Principal motor contacts occur via the 3′–5′ strand. For every substrate examined here, discontinuities in and modifications on the 3′–5′ strand had a more significant effect on translocation than those on the 5′–3′ strand (Figures 3, 5 and 6). The simplest explanation of our data is that HsdR steps along dsDNA via contacts to the 3′–5′ strand (Figure 7A). Accordingly, 3′–5′ gaps are more likely to result in stalling (Figure 3C), as the leading edge of the motor disconnects from the tracked strand (Figure 7B, upper panel). Stalling will be rarer at 5′–3′ gaps as the motor can continue unimpeded on the intact 3′–5′ track (Figure 7B, lower panel). This model necessitates that *EcoR124I* can translocate both ds- and ssDNA. If ssDNA translocation were >10-fold slower than dsDNA translocation (~580 bp/s at 25°C; Seidel *et al*, 2004), then this would have been resolved in the tweezers assay as a reduced translocation rate during gap bypass and in the triplex assay as distance-dependent changes in $\Delta T_{\text{lag}}^{5′-3′}$. That neither were observed suggests that translocation is about as fast over short ssDNA stretches as on dsDNA. The significantly slower $\Delta T_{\text{lag}}^{5′-3′}$ values on longer gaps (Figure 5D) could be due to a slower translocation rate over long distances, but could also be due to conformational anisotropy in the track (e.g., hairpin structures).

Supplementary ‘Clamp’ contacts occur via the 5′–3′ strand. We observed that while stalling events at 5′–3′ gaps were infrequent, the probability of dissociation was elevated (Figure 3C). We interpret these data as showing that *EcoR124I* makes ‘clamp’ contacts to the 5′–3′ strand that do not have a direct role in DNA transport, but which ensure that the motor remains in tight contact with the tracked 3′–5′ strand (Figure 7A). Accordingly, during bypass of a 5′–3′ gap via ssDNA translocation of the 3′–5′ strand, the loss of available clamp contacts will increase the overall dissociation rate (Figure 7B, lower panel). Modifications to the 5′–3′ strand also had a lesser effect on translocation bypass than those on the tracked strand (Figure 6).

Contacts to both backbone and bases play a role in translocation. We observed that both backbone and base modifications affected translocation, with the greatest effects seen on the 3′–5′ strand (Figure 6). We therefore suggest that, like other related dsDNA motors (Dürr *et al*, 2005), *EcoR124I* utilises the entire dsDNA structure for its motor activity.

A model for the bypass of gapped DNA via ssDNA looping

For some DNA motors, translocation can be completely blocked by discontinuities in the tracked strand (Bertram *et al*, 2002; Whitehouse *et al*, 2003; Kawaoka *et al*, 2004). For others, as here, modifications can be overcome relatively efficiently (Amaratunga and Lohman, 1993; Bianco and Kowalczykowski, 2000; Tackett *et al*, 2001; Eoff *et al*, 2005). One explanation for bypass is that if a motor has a large step size, its leading protein domain can ‘step over’ an adduct,

grabbing the downstream DNA (Amaratunga and Lohman, 1993; Lohman and Bjornson, 1996). It has also been suggested that some helicases move on DNA using two coordinated but distinct step sizes; the quantum inchworm model of Bianco and Kowalczykowski (2000). In this mechanism, a leading domain makes a large forward step (≥ 1 bp) and stabilises the motor on the downstream dsDNA while a secondary domain unwinds the captured upstream DNA segment in smaller substeps (<5 bp). However, it is difficult to reconcile either of these bypass mechanisms with translocation by *EcoR124I* over gaps of ~300 nt, particularly as the maximum step that this enzyme makes on DNA is ≤ 2 bp.

Given that ssDNA gaps are intrinsically flexible (Mills *et al*, 1994, 1999; Rippe, 2001; Mills and Hagerman, 2004; Du *et al*, 2005), an alternative explanation for bypass is that during the lifetime of a stall event at a gap, looping of the ssDNA linker allows the broken ends of the translocated strand to juxtapose and form a pseudo-continuous track (Figure 7B, upper panel). As long as the protein complex can accommodate the ssDNA loop, a motor with a relatively small step size could bypass a large gap by stepping across a distance similar to a nick or relatively short gap. It has been suggested that RNA polymerases, motors that explicitly make steps of only 1 nt, can bypass gaps in their template strands in just such a manner (e.g., Zhou *et al*, 1995). Moreover, equivalent ssDNA looping events allows T4 DNA ligase to ligate across gaps without recourse to any protein stepping at all (e.g., Nilsson and Magnusson, 1982). We further suggest that DNA rearrangements can explain bypass of other flexible adducts; for example, by looping of both ssDNA and PEG.

In a looping model, bypass will be sensitive to linker flexibility, and efficiency will peak at a length which is a compromise between energetic suppression of bending at short distances and entropic suppression of looping at long distances (Rippe, 2001). The persistence length of ssDNA (p_{ss}) has been estimated as anywhere between 2–20 nt dependent upon conditions and nucleotide sequence (e.g., Mills *et al*, 1999; Murphy *et al*, 2004; Du *et al*, 2005). Therefore, the probability of end juxtaposition will almost certainly be reduced for gaps <10 nt, as in Figure 5B, due to energetically unfavourable ssDNA bending. We also observed that small changes in the backbone or bases had significant effects (Figures 3 and 5). The DNA around a de-phosphorylated nick is likely to have the same rigid properties as intact or nicked DNA (Mills *et al*, 1994, 1999; Mills and Hagerman, 2004), so *EcoR124I* may be lacking key interactions with the missing phosphate. Once a 1 nt gap is generated, the ssDNA is more flexible (Mills *et al*, 1994, 1999; Mills and Hagerman, 2004), but may be anisotropically bent (Guo and Tullius, 2003). This could reduce bypass efficiency in addition to bend stiffness. For gaps ≥ 30 nt, the probability of end juxtaposition should decrease, as in Figure 5D, due to the lower relative concentration of the ends (although hairpin formation might also play a compensatory role by shortening the apparent end-to-end length of the ssDNA linker).

If increasing gap length above p_{ss} increases flexibility, why does $P_{\text{bypass}}^{3′-5′}$ decrease in the tweezers experiments? The critical factor is the DNA tension necessary for single molecule measurements (1.6 pN in Figure 3). At this force, looping will be suppressed at least 1000-fold, as estimated from calculations on dsDNA (Yan *et al*, 2005). As the ssDNA loop geometry may be critical to allow *EcoR124I* to re-engage

the leading DNA (e.g., the dsDNA ends might need to approach to within ~ 0.4 nm), force could suppress gap bypass to an even greater degree (Blumberg *et al*, 2005; Sankararaman and Marko, 2005; Yan *et al*, 2005). The force dependence observed here (Figure 4), coupled with an ability to efficiently bypass very large gaps at zero force (Figure 5C), are simply not consistent with a model in which *EcoR124I* makes large steps over supposedly straight ssDNA regions (see also Supplementary Section). Clearly, *EcoR124I* is sufficiently stable during translocation (Seidel *et al*, 2004), which potentially slow and complex DNA rearrangements can occur. Our work also highlights a very important consideration when analysing DNA or RNA motors—where single molecule and bulk solutions studies disagree, the disparity may reflect real differences (i.e., only one of the studies is actually correct) or a conditional difference (i.e., measurement conditions, enzyme preparations, etc.) If the measurements require the application of tension, then the consequences of the applied force on the motor kinetics must be carefully considered both in terms of the effects on the enzyme *and* on the polynucleotide.

A clear result from our analysis of *EcoR124I* is that dsDNA translocation requires close contacts with both strands of intact duplex DNA (Figure 7). This interpretation is obviously different from that expected for a classical DNA helicase, which will translocate along just one single strand (Lohman and Bjornson, 1996; Soultanas and Wigley, 2001). Instead *EcoR124I* can be said to be a prototypical member of a new class of dsDNA translocases, which also includes enzymes involved in chromatin remodelling (Whitehouse *et al*, 2003; Dürr *et al*, 2005; Lia *et al*, 2006) and DNA recombination (Singleton *et al*, 2001). The relationship to orthodox helicases is simply the presence of ATP binding motifs that couple nucleotide binding energy to protein domain motions (Singleton and Wigley, 2002). It is clear that the current tendency to classify helicases as ‘unwindases until proven otherwise’ is unsatisfactory, a new motor taxonomy is required with a more transparent nomenclature.

Materials and methods

Proteins

EcoR124I was purified, reconstituted and tested as previously described (Seidel *et al*, 2005). BbvCI and its nicking mutants were supplied by Geoff Wilson (New England Biolabs). *E. coli* RecBCD was supplied by Mark Dillingham (University of Bristol).

DNA constructs

Gapped and modified DNA were generated from suitable plasmid substrates using modifications of the methods described by Wang

and Hays (2001). For a full description of these DNA constructs, as well as those used for the crosslinking studies, please refer to the Supplementary data.

DNA crosslinking

HTMP (Sigma) was dissolved in absolute ethanol to a final concentration of 710 $\mu\text{g}/\text{ml}$. DNA substrates (18–26 μM dT equivalents) were incubated with either < 0.033 HTMP/dT or > 1 HTMP/dT (as indicated in the figure legends) in 10 mM Tris–Cl, 20 mM NaCl, 1 mM EDTA (pH 8.0), and then irradiated at 4°C with 0–76 kJ m^{-2} of 365 nm light. Irradiation conditions were chosen dependent on the HTMP concentration and the number of crosslinks required, and were determined empirically (Supplementary Methods). Irradiated samples were dialysed extensively against ddH_2O using nitrocellulose membranes (0.025 μm , Millipore).

Magnetic tweezers measurements

Magnetic tweezers experiments were carried out as described previously (Seidel *et al*, 2004, 2005) in reaction Buffer R (50 mM Tris–HCl pH 8.0, 10 mM MgCl_2 , 1 mM dithiothreitol) supplemented with 4 mM ATP. All measurements were performed at 25°C.

Triplex displacement measurements

Triplex displacement measurements were carried out at 20°C as described (Firman and Szczelkun, 2000; McClelland *et al*, 2005; Seidel *et al*, 2005) with the final reactions conditions being either 1 nM DNA (0.5 nM tetramethylrhodamine triplex) for the stopped flow assays (McClelland *et al*, 2005) or 5 nM DNA (2.5 nM ^{32}P -labelled triplex) for the gel assays (Firman and Szczelkun, 2000), and, in both cases, 30 nM MTase, 300 nM HsdR, 1.5 U/ml creatine phosphokinase, 14.5 mM phosphocreatine, 100 μM S-adenosyl methionine and 25 μM ATP in Buffer R.

DNA unwinding and cleavage measurements

Conditions for DNA unwinding by RecBCD or DNA cleavage by *EcoR124I* are described in the Supplementary Methods.

Data analysis

Previously described methods were used for the analysis of triplex displacement profiles (McClelland *et al*, 2005) and time traces from the magnetic tweezers (Seidel *et al*, 2004, 2005). Bypass, stalling and dissociation probabilities were calculated as described in Supplementary Methods.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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