

Measuring motion on DNA by the type I restriction endonuclease *EcoR124I* using triplex displacement

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The type I restriction enzyme *EcoR124I* cleaves DNA following extensive linear translocation dependent upon ATP hydrolysis. Using protein-directed displacement of a DNA triplex, we have determined the kinetics of one-dimensional motion without the necessity of measuring DNA or ATP hydrolysis. The triplex was pre-formed specifically on linear DNA, 4370 bp from an *EcoR124I* site, and then incubated with endonuclease. Upon ATP addition, a distinct lag phase was observed before the triplex-forming oligonucleotide was displaced with exponential kinetics. As the distance between type I and triplex sites was shortened, the lag time decreased whilst the displacement reaction remained exponential. This is indicative of processive DNA translocation followed by collision with the triplex and oligonucleotide displacement. A linear relationship between lag duration and inter-site distance gives a translocation velocity of 400 ± 32 bp/s at 20°C. Furthermore, the data can only be explained by bi-directional translocation. An endonuclease with only one of the two HsdR subunits responsible for motion could still catalyse translocation. The reaction is less processive, but can ‘reset’ in either direction whenever the DNA is released.

Keywords: DNA translocation/DNA triplex/motor protein/restriction–modification

Introduction

Protein motion along DNA is essential for the repair (Jiricny, 1998), recombination (George *et al.*, 1999), replication (Hiasa and Marians, 1999), transcription (Landick, 1999) and restriction (Bickle, 1993) of genetic material. Enzymes that catalyse directed linear steps along polymers differ from other ‘molecular machines’ in that they generate both motion and force, i.e. they are true motors. This has been investigated most thoroughly for the classical ‘motor proteins’, kinesin and myosin. Although it is clear that DNA translocation also fits these criteria (Szczelkun, 2000), the dynamics of the nucleic acid enzymes remain inadequately characterized. A rigorous evaluation of any DNA-bound motor must first provide answers to some fundamental questions: what is the translocation rate; what is the direction of movement relative to binding-site polarity; and how often does an

enzyme stall on, or release the DNA? In this study, these questions will be addressed with the type I restriction enzyme *EcoR124I*.

Type I restriction endonucleases recognize specific asymmetric DNA sequences (e.g. Figure 1A). However, DNA cleavage is dependent upon the free energy of ATP hydrolysis and occurs at random non-specific loci from 50 to >10 000 bp distant (Horiuchi and Zinder, 1972; Szczelkun *et al.*, 1997). It was first suggested >25 years ago that the link between binding and cleavage may be due to protein motion along DNA (Shulman, 1974). Electron microscopy of endonucleases bound to DNA in the presence of ATP revealed loops of DNA (Rosamund *et al.*, 1979; Yuan *et al.*, 1980), and a general model was proposed in which an enzyme remains bound to its recognition site whilst simultaneously pulling adjacent non-specific DNA past itself (Yuan *et al.*, 1980). Studier and Bandyopadhyay (1988) developed the model by demonstrating that DNA cleavage follows the collision of two converging *EcoKI* enzymes, midway between each pair of sites. Their results suggested that despite the asymmetry of the recognition sequence, movement occurs in both ‘leftward’ and ‘rightward’ directions simultaneously. This was termed bi-directional translocation. More recent experiments using atomic force microscopy have confirmed the presence of the expected multiple DNA loops (Ellis *et al.*, 1999), but more importantly have permitted direct observation of temporal changes in the size of the loops, indicative of translocation. An unambiguous demonstration of linear communication *in vitro* was provided by examining cleavage of DNA catenanes (Szczelkun *et al.*, 1996). Of two singly interlinked DNA circles, only the ring carrying an *EcoR124I* recognition site could be cleaved—the connected ring remained intact despite its physical proximity. This proved that DNA recognition and cleavage must be coupled by one-dimensional translocation. Furthermore, motion generates a decrease in twist behind and an increase in twist ahead of the complex (Szczelkun *et al.*, 1996; Janscak and Bickle, 2000), indicating that translocation follows the helical path of DNA. Molineux and co-workers have analysed DNA translocation elegantly *in vivo* (Davies *et al.*, 1999a; Garcia and Molineux, 1999). Introduction of an *EcoKI* site into the leader sequence of a modified T7 phage genome allowed endonuclease translocation to internalize the DNA into bacteria. By following the progressive methylation of *Sau3AI* restriction sites, an *EcoKI* translocation velocity of ~100 bp/s was estimated (Garcia and Molineux, 1999). An accurate determination of translocation rate is fundamental to the description of molecular motor function. Consequently, a direct assay of linear motion would be an invaluable tool in the study of type I enzymes.

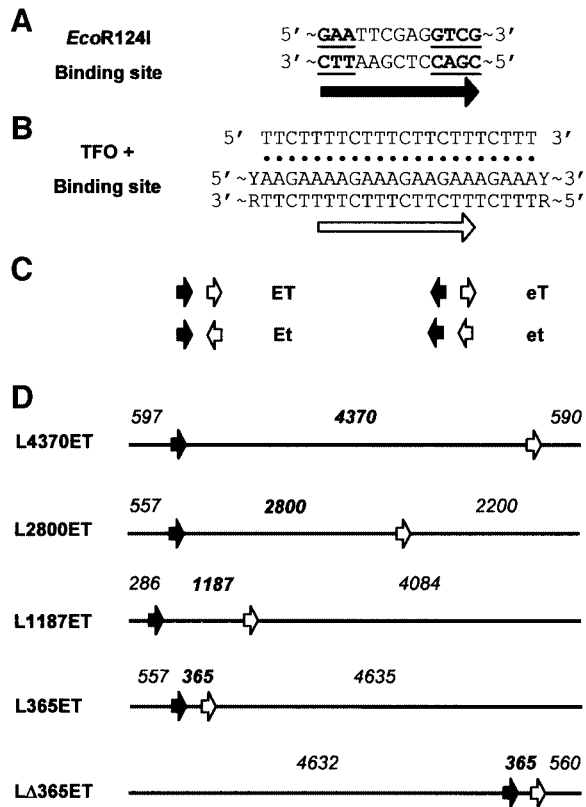


Fig. 1. Triple helix substrates. (A) *EcoR124I* recognition sequence. Conserved nucleotides are underlined. (B) TFO and TFO-binding site sequences. Hoogsteen base pairs between the TFO and purine-rich strand of the duplex are indicated (filled circles). Arbitrary site orientations are defined by the black (*EcoR124I*) and white (triplex) arrows. (C) Substrates are designated a two-letter code depending on the relative orientations of the *EcoR124I* (E or e) and TFO (T or t) sites. Upper case characters indicate that the site runs left to right on the substrate as drawn, lower case characters indicate the reverse. (D) Examples of linear substrates (5557 bp) used in the TFO displacement assay. DNA is drawn as a solid line with arrows as in (C). Numbers indicate base pairs from a DNA end to the G of GAA in the *EcoR124I* site, to the T-AT triplet at the 5' end of the triplex, and to the other DNA end. Substrates are named L (for linear), followed by inter-site distance and relative site orientation (from C). Cleavage of pMDS21 with *NcoI* or *ScaI* produced L365ET or LA365ET, respectively; cleavage of pMDS22 with *NcoI* or *ScaI* produced L4370ET or L1187ET, respectively; and cleavage of pMDS28 with *ScaI* produced L2800ET.

The type I endonucleases consist of three subunits: HsdS (DNA recognition), HsdM (DNA methylation) and HsdR (DNA and ATP hydrolysis), with the stoichiometry $R_2M_2S_1$ (Dryden *et al.*, 1997). Which part of the complex acts as the motor? The HsdR amino acid sequences contain motifs previously identified in helicases, the so-called DEAD-box motifs (Gorbalenya and Koonin, 1991), and also have predicted secondary structure homology to the PcrA and Rep helicases (Davies *et al.*, 1999a). Mutations in these motifs affect ATPase activity (Davies *et al.*, 1998), impair DNA restriction (Webb *et al.*, 1996) and prevent internalization of phage DNA (Davies *et al.*, 1999b), suggesting a DNA translocation mechanism akin to that suggested for the DNA helicases (Soultanas and Wigley, 2000). Changes in DNA topology during translocation are also indicative of strand separation (Janscak and Bickle, 2000), but a helicase-like DNA unwinding

activity has yet to be demonstrated directly for type I enzymes (Szczelkun, 2000). Moreover, it has yet to be established whether HsdR subunits contact DNA with a defined polarity (i.e. 5'-3' or 3'-5') or even if the strands need to be separated at all. A full understanding of DNA translocation by *EcoR214I* would not only pertain to type I enzymes, but would generate valuable information about DEAD-box proteins in general, as well as other DNA-based motors such as the methyl-directed mismatch repair enzymes for which an equivalent bi-directional translocation model has been proposed (Allen *et al.*, 1997).

To evaluate protein motion on DNA, the displacement of a DNA triplex was assayed. Triplexes are potential therapeutic gene regulators (Vasquez and Wilson, 1998), produced when a triplex-forming oligonucleotide (TFO) binds specifically to duplex DNA (Gowers and Fox, 1999). Triplex formation within transcription initiation regions is highly effective at blocking RNA polymerases (Kim and Miller, 1995). However, the effect on transcription elongation of a TFO binding downstream of a promoter depends on triplex stability. Both phage and *Escherichia coli* polymerases can read-through a weakly associated TFO (Skoog and Maher, 1993). However, augmenting the binding efficiency using phosphorothioates or covalently attached intercalating moieties increases the chance of inhibiting motion (Xodo *et al.*, 1994; Giovannangeli *et al.*, 1996). In contrast, DNA polymerase elongation is inhibited readily by triplexes (Hacia *et al.*, 1994). The most effective unmodified TFOs are pyrimidine rich (CT), occupy the major groove parallel to a purine-rich strand and utilize Hoogsteen hydrogen bonds to form T-AT and C⁺-GC triplets (Gowers and Fox, 1999). Sub-nanomolar equilibrium dissociation constants are attainable (Maher *et al.*, 1990; Plum *et al.*, 1990), but protonation of the N3 position of cytosine demands a low pH. However, Kopel *et al.* (1996) demonstrated that a CT-rich TFO bound to a GA sequence in acid conditions and subsequently diluted into neutral buffer remained associated indefinitely. Using this strategy, we have evaluated the *EcoR124I*-directed displacement of a stable triplex *in vitro* under 'standard' reaction conditions.

A triplex was formed on either leftward or rightward sides of an *EcoR124I* recognition site. DNA translocation resulted in a lag phase followed by TFO displacement. By measuring lag time as a function of distance translocated, a translocation speed was calculated. The data also indicate that translocation is both processive and bi-directional. Using conditions that produce endonucleases with a single HsdR subunit, unidirectional motion independent of site asymmetry could still be recorded. The reaction was less processive than with a full complement of HsdR and correspondingly slower. However, the enzyme could 'reset' and resume translocation in either direction if it released the DNA during motion or at a DNA end.

Results and discussion

Triplex formation and dissociation

The aim of this study was to investigate the effect of a DNA triplex 'roadblock' on translocation by the type I restriction endonuclease *EcoR124I*. Linear DNA substrates were generated containing both *EcoR124I*- and TFO-binding sites (Figure 1A-D). Since bimolecular

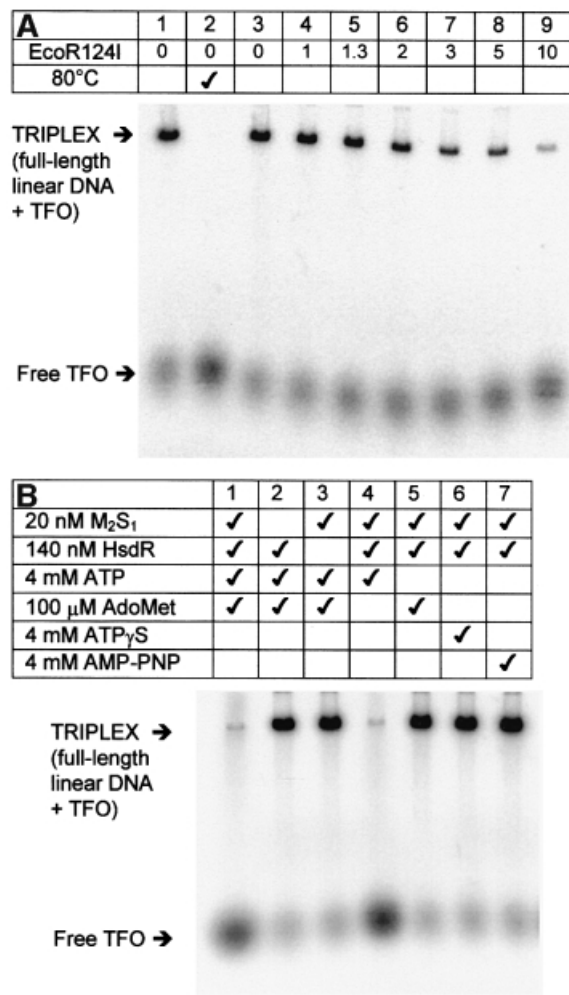


Fig. 2. TFO binding and displacement. Reactions were examined by agarose gel electrophoresis of the ³²P-labelled TFO (Materials and methods). Bound and free TFO are indicated. (A) TFO (50 nM) and linear DNA were incubated at pH 5.5 overnight. Lane 1, 5 nM L365ET was incubated in buffer R (Materials and methods) for 25°C for 1 h; lane 2, 5 nM L365ET was heated to 80°C for 10 min in buffer R, cooled to 20°C and then incubated for a further hour; lanes 3–9, 5 nM L365ET was incubated with varying nanomolar concentrations of *EcoR124I* in buffer R as indicated, ATP added to 4 mM and the reaction incubated for a further hour at 20°C. (B) L365ET (5 nM) was pre-incubated with MTase (M₂S₁), HsdR and/or AdoMet in buffer R as shown. Where indicated ATP, ATPγS or AMP-PNP were also added to 4 mM. Reactions were allowed to proceed for 1 h at 20°C.

association rate constants for CT-rich TFOs are ~1000/M/s (Maher *et al.*, 1990; Paes and Fox, 1997), the ³²P-labelled TFO was incubated with its target DNA overnight. Triplex formation was assessed by agarose gel electrophoresis (Materials and methods). After binding at pH 5.5, the triplex could be diluted into *EcoR124I* reaction buffer R (pH 8.0; Materials and methods) without the labelled TFO dissociating (Figure 2A, lane 1). The triplex was stable for at least 12 h at 20°C (data not shown). However, once displaced by heating to 80°C, the TFO did not re-associate when cooled to 20°C (lane 2). Similarly, removing Mg²⁺ ions from the buffer completely dissociated the triplex (not shown). Neither ATP nor the methyl donor *S*-adenosyl methionine (AdoMet) affected the binding. These results confirm the observations of Kopel *et al.* (1996) that a CT-

based triplex is stable close to neutral pH if it is pre-bound in acid conditions. The simplest explanation is that the protonated form of cytosine in a C⁺-GC triplet is stabilized at neutral pH by Mg²⁺ ions bound to the DNA (Singleton and Dervan, 1993). Consequently, the off-rate at pH 8.0 remains as slow as it would be in acid conditions (Maher *et al.*, 1990; Paes and Fox, 1997). Once dissociated, however, the cytosines are deprotonated rapidly, C⁺-GC triplets cannot form and the on-rate becomes so slow that rebinding is negligible. These distinct non-equilibrium 'on' and 'off' states make this triplex an ideal probe for protein–DNA interactions.

TFO displacement by *EcoR124I* motion

The effect of *EcoR124I* on triplex stability was assayed by varying the concentration of endonuclease and analysing the products 1 h after addition of 4 mM ATP (Figure 2A, lanes 3–9). Unlike equivalent observations with RNA and DNA polymerases, *EcoR124I* readily displaced the tightly bound TFO. Under the conditions used, no rebinding of the free TFO was observed. As the concentration of *EcoR124I* was increased, the percentage of free TFO also increased, until at a 2-fold molar excess of enzyme over full-length linear DNA, >95% free TFO had been generated (average value quantified from three repeat experiments; see Materials and methods). The relative orientation of the TFO had no effect on this process (not shown). The requirement for a 2-fold molar excess of enzyme over DNA for maximum activity does not represent an absolute stoichiometry, but probably results from dissociation of the enzyme complex at nanomolar protein concentrations (Janscak *et al.*, 1998). Data from DNA cleavage reactions indicate that type I enzymes cannot turn over (Bickle, 1993). Similarly, our data show that endonuclease–DNA complexes do not dissociate appreciably once translocation has started; TFO displacement did not increase if the reactions were incubated for longer periods (not shown). TFO displacement was not seen with methyltransferase (MTase) or HsdR alone (Figure 2B, lanes 1–3), in the absence of ATP (lane 5) or with non-hydrolysable ATP analogues ATPγS or AMP-PNP (lanes 6 and 7). Excluding AdoMet from the reaction had no apparent effect (lane 4). Similarly, DNA cleavage by *EcoR124I* is unaffected by the absence of AdoMet (Szczelkun *et al.*, 1996, 1997). Although AdoMet is unstable in aqueous solution, it may be stable indefinitely when bound tightly to a protein. Since we cannot rule out co-purification of AdoMet with *EcoR124I*, the effect of cofactor binding on translocation could not be assessed. Overall, these results suggest that DNA translocation must be responsible for TFO displacement.

The full-length linear DNA remained intact during these reactions (not shown), confirming that DNA cleavage is not a prerequisite of translocation (Davies *et al.*, 1999a,b; Janscak *et al.*, 1999b). However, a feature of type I enzyme activity is that DNA is cleaved wherever an endonuclease stalls, e.g. upon collision of converging enzymes (Studier and Bandyopadhyay, 1988) or upon collision of a single enzyme with an immobile Holliday junction (Janscak *et al.*, 1999a). The same is not true upon collision with a triplex. Is a simple pause in translocation sufficient to generate DNA cleavage or is a significant build-up in mechanical load required? Morris and Raney

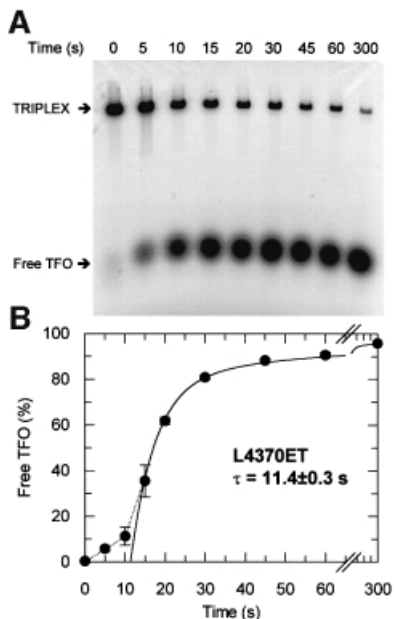


Fig. 3. Kinetics of TFO displacement on L4370ET. (A) L4370ET (5 nM) was pre-incubated in buffer R with 20 nM *EcoR124I* for 5 min, and the reaction initiated with ATP. Aliquots were removed and the reaction quenched at the time points shown, and analysed as described (Materials and methods). Triplex and free TFO are indicated. (B) TFO displacement in terms of the percentage of free TFO liberated at each time point (filled circles). Error bars represent the standard deviation from the mean of three independent experiments. The solid line is a least-squares fit to equation (3) (Materials and methods). The estimated lag time (τ) is taken from the intercept with the abscissa. A dotted line emphasizes the lag phase.

(1999) have shown that DNA helicases can displace streptavidin from biotin-labelled oligonucleotides. The force required to disrupt this interaction has been estimated at ~ 250 pN, far more energy than that available to a motor protein. However, the force required to break a non-covalent bond correlates with activation enthalpy and not overall free energy. Consequently, the application of a force, even a weak one, results in a shift in thermal fluctuations in free energy to the extent that the dissociation rate of the bond is increased considerably (Morris and Raney, 1999). Therefore, the increase in force associated with TFO displacement may not be as great as that required for DNA cleavage. Similarly, displacement of Lac repressor by *EcoR124II* did not result in cleavage at the operator site (Dreier *et al.*, 1996). Moreover, if type I enzymes unwind DNA as they translocate (Davies *et al.*, 1999a; Janscak and Bickle, 2000), then displacement of a bound ligand may be accelerated by disruption of the underlying duplex DNA.

Kinetics of DNA translocation by *EcoR124I*

To analyse the kinetics of TFO displacement, samples were withdrawn from reactions containing fixed concentrations of *EcoR124I* at sequential time points after addition of ATP. These were analysed by agarose gel electrophoresis (Figure 3A) and the percentage of free TFO ascertained by densitometry (Materials and methods). The kinetic profile for L4370ET is shown (Figure 3B). Initially, there is little dissociation of the triplex, but after 10 s there is a rapid rise in free TFO,

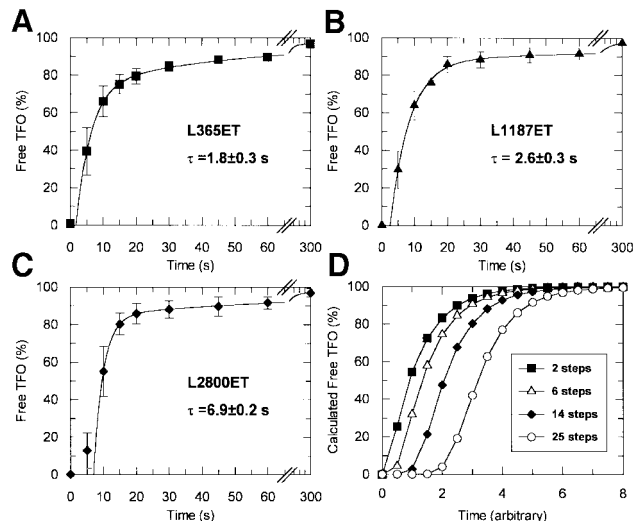


Fig. 4. Kinetics of TFO displacement as a function of distance. (A–C) L365ET, L1187ET or L2800ET (5 nM) were pre-incubated in buffer R with 20 nM *EcoR124I* for 5 min, and the reactions initiated with ATP. Aliquots were removed and the reactions quenched at the time points shown, and analysed as in Figure 3. TFO displacement is shown in terms of the percentage of free TFO liberated at each time point. Error bars represent the standard deviation from the mean of at least three independent experiments. The solid lines are least-squares fits to equation (3) (Materials and methods). The resulting lag times (τ) are shown. (D) Theoretical TFO displacement profiles. The percentage of free TFO was calculated by numerical integration using equation (1) (Materials and methods). In each case, k_2 was an order of magnitude slower than k_1 (absolute values are not defined; see main text). The percentage of displaced TFO is plotted for reactions where $y = 2, 6, 14$ or 25 .

reaching $\sim 90\%$ by 1 min, followed by a 10- to 20-fold slower phase reaching $>95\%$ by 5 min. To analyse the kinetics further, we measured TFO displacement on a series of linear substrates with varying distances between the *EcoR124I* and triplex sites (Figure 4A–C). As the distance between the sites was lengthened, the lag phase increased whilst the rates of the exponential phases remained equivalent.

If motion on DNA is considered as a series of reactions governed by single macroscopic rate constants, then translocation will generate a succession of highly populated intermediate states (Ali and Lohman, 1997). If the rate constants are equal, then the culmination of such reactions will be a lag phase, the length of which is diagnostic of the number of steps (Gutfreund, 1995). The unwinding of short DNA duplexes by UvrD helicase has been analysed in this manner (Ali and Lohman, 1997). In our experiments, however, TFO displacement is not part of the translocation mechanism and is rate limiting with respect to the preceding steps. Consequently, the same fitting procedure could not be applied. Instead, we evaluated the effect of altering the number of steps prior to TFO displacement by modelling theoretical reactions using equation (1) (Materials and methods). This model considers translocation as a series of unimolecular reactions as described above, except that the final step, TFO displacement, is slower than the preceding translocation steps. Although motion is only considered in one direction, bi-directional motion can still be accommodated (see below). The resulting series of kinetic profiles (Figure 4D) illustrate two important features of the model. First, as the

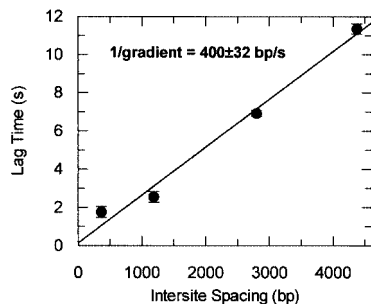


Fig. 5. Velocity of DNA translocation. Lag times from Figures 3 and 4 are plotted as a function of distance between *EcoR124I* and triplex sites (from Figure 1D). Error bars are the standard deviation from the mean of the intercepts. The solid line represents a least-squares linear fit to the data (Materials and methods). The velocity (reciprocal gradient) is shown as bp/s.

number of steps is increased from two to 25, a linearly increasing lag phase is observed. Secondly, the ensuing TFO displacement follows an exponential function, the rate of which is independent of the number of steps. Because TFO displacement is rate limiting, the model cannot distinguish between a large number of fast steps and a small number of slow steps (not shown). Furthermore, each ‘step’ in reality comprises multiple reactions associated with ATP binding and hydrolysis, physical protein motion along DNA and release of ADP and inorganic phosphate. Nevertheless, the pattern of kinetic profiles in Figures 3 and 4A–C matches the predictions of the model.

If our data can be represented by equation (1) (albeit with a larger number of steps), there should be a linear relationship between lag time and inter-site distance, where the gradient gives the average lag time per base pair (or bp/s in reciprocal form). To evaluate the lag times, the exponential phases of each reaction were fitted to equation (3) (Figures 3B and 4A–C). The rates and relative proportions of the two exponential phases did not vary significantly with distance (not shown). Variations in the profiles would be indicative of an increased frequency of events that slow translocation. Therefore, stalling (Uptain *et al.*, 1997), back-stepping (Komissarova and Kashlev, 1997) or protein–DNA dissociation events during DNA translocation must be rare. In other words, translocation by *EcoR124I* has a high processivity; thousands of base pairs can be transported before an enzyme dissociates or stalls. The slower exponential phase most probably results from a subset of the enzyme population that moves less efficiently. This heterogeneity may be due to a fraction of $R_1M_2S_1$ species being present in the $R_2M_2S_1$ reactions (see below). Figure 5 shows the combined lag times from Figures 3 and 4. A linear relationship between lag time and inter-site distance is observed, justifying the contraction of multiple kinetic events to a single unimolecular rate constant. The increase in free TFO must be due to DNA translocation via a discrete number of rapid steps, collision with the triplex and finally rate-limiting TFO displacement.

The reciprocal gradient of the best-fit line in Figure 5 gives a translocation velocity of 400 ± 32 bp/s at 20°C. It should be noted, however, that the inter-site distances defined in Figure 1D are not the exact lengths of DNA

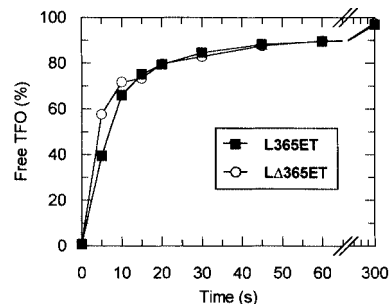


Fig. 6. Uni- and bi-directional translocation by *EcoR124I*. The percentage of TFO liberated as a function of time is shown for L365ET and LΔ365ET. Reactions were performed and analysed as in Figures 3 and 4.

translocated, as the true starting position is unknown. DNA fingerprinting of *EcoR124I* has not revealed where HsdR binds (Mernagh *et al.*, 1998), but it almost certainly occurs outside the recognition sequence (Powell *et al.*, 1998). Although we can discount long-range random DNA looping events (Szczelkun *et al.*, 1996), we cannot discount local DNA wrapping around the protein such that translocation starts at an appreciable distance from the site (i.e. tens of base pairs). If this were the case, then the x -axis intercept of the line in Figure 5 would specify this distance. Because of errors in evaluation of lag time, however, we cannot state precisely where translocation starts. Note that the difference in rate that this uncertainty generates is less than the observed standard error.

Bi-directional translocation by *EcoR124I*

According to the bi-directional model of Studier and Bandyopadhyay (1988), the relative orientation of an *EcoR124I* site should have little or no effect on TFO displacement kinetics. Conversely, a sequence-dependent unidirectional model as described for *EcoBI* (Rosamund *et al.*, 1979) would result in motion in one direction exclusively. However, similar kinetic profiles to that for L4370ET were obtained with the substrates L4370Et and L4370eT (data not shown), in which the relative orientations of *EcoR124I* and triplex sites were inverted. Similarly, DNA cleavage shows no dependence on site orientation (Szczelkun *et al.*, 1996, 1997). Therefore, translocation cannot be constrained rigidly by site asymmetry. However, a unidirectional model could still be accommodated by our results if DNA binding resulted in, on average, 50% of enzymes going leftward and 50% going rightward, and if, when an enzyme reached a DNA end, it could dissociate, randomly rebind and restart translocation. We term this last process ‘resetting’. This alternative unidirectional scheme is described by equation (2) (Materials and methods). Enzymes travel down either a leftward (L) or rightward (R) pathway (because motion is highly processive, we will assume that the dissociation rate, k_2 , is zero). Any enzyme moving down the L pathway eventually will reach the end of the DNA and ‘step off’. Translocation can then reset, governed by a unimolecular rate constant k_5 . There is no memory of the antecedent pathway, e.g. an enzyme that starts down the L pathway will dissociate, reset and restart down either L or R pathways with equal probability. Given that TFO displacement rapidly approaches 95% (Figures 3 and 4), the

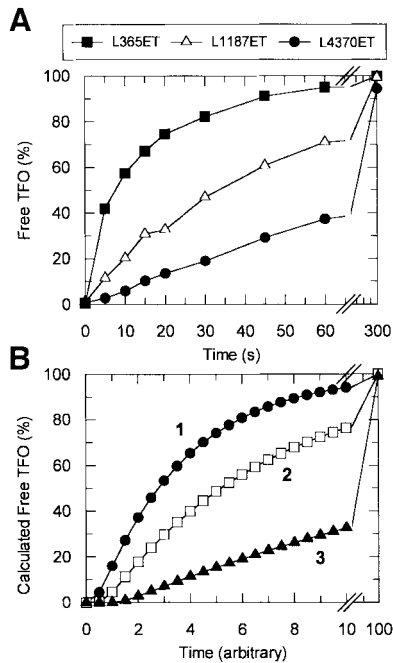


Fig. 7. DNA translocation by *EcoR124I* with one HsdR subunit. (A) DNA (5 nM; as indicated) was pre-incubated with 20 nM M_2S_1 and 20 nM HsdR in buffer R at 20°C. Reactions started with ATP were analysed as in Figures 3 and 4. The percentage of liberated TFO is indicated as a function of time. (B) The theoretical percentage of TFO_{free} was calculated by numerical integration using equation (2) (Materials and methods). Simulation 1 (filled circles), $x = 4$, $y = 4$; simulation 2 (open squares), $x = 4$, $y = 8$; simulation 3 (filled triangles), $x = 4$, $y = 16$. For each simulation, $k_5 \gg k_1 = k_4 > k_2 > k_3$ (absolute values are not defined; see main text).

resetting process cannot be rate limiting. However, if resetting was fast, then equation (2) could produce kinetic profiles indistinguishable from those illustrated in Figure 4 (not shown).

To differentiate between uni- and bi-directional models, we compared TFO displacement kinetics on L365ET and Δ L365ET (Figure 6). The distance between *EcoR124I*- and triplex-binding sites is the same on each substrate (365 bp), but their context is different (Figure 1D). Enzymes moving leftward as drawn on Δ L365ET would have to travel 4632 bp before they could reset, compared with only 557 bp on L365ET (Figure 1D). Even if resetting was fast, the kinetic profile on Δ L365ET would be markedly biphasic compared with that on L365ET. However, little difference can be seen between the profiles (Figure 6). Furthermore, TFO displacement on circular DNA shows a similar displacement profile to that on linear DNA (M.D.Szczelkun, unpublished data). Collectively, the data indicate that *EcoR124I* is capable of translocating DNA from both sides of its site simultaneously, independently of recognition sequence asymmetry. Therefore, it should also be noted that the translocation rate refers to motion in one direction only—twice the amount of DNA is actually transported per second by each enzyme.

Unidirectional translocation and resetting by *EcoR124I* with a single HsdR subunit

Since *EcoR124I* can translocate DNA in two directions, how do the HsdR subunits generate motion? Do the

subunits act independently, a single HsdR translocating DNA on each side of the site, or do they communicate directly to provide added processivity and/or synchronicity? To assess these possibilities, we examined TFO displacement kinetics as a function of endonuclease stoichiometry. Since the equilibrium dissociation constant for the first HsdR subunit of $R_2M_2S_1$ is >1000-fold lower than the second (Janscak *et al.*, 1998), mixing equal nanomolar concentrations of HsdR and MTase produces the $R_1M_2S_1$ form exclusively (<0.01% $R_2M_2S_1$ will be present assuming equivalent sites). This form of the enzyme cannot cleave linear or plasmid DNA (not shown), whereas a DNA-activated ATP hydrolysis rate approximately one-quarter that of $R_2M_2S_1$ has been measured (Janscak *et al.*, 1998). TFO displacement by $R_1M_2S_1$ was observed on L365ET, L1187ET and L4370ET (Figure 7A), and on L365eT (not shown). Despite there being only one HsdR subunit, in each case >95% free TFO was generated in 5 min, independently of the relative site orientations. However, as the inter-site distance was increased (Figure 7A), the shapes of the resulting profiles varied markedly compared with those expected from equation (1) (Figure 4D). The exponential phases became more extended with distance, indicating an increasing frequency of events that slow translocation. To explain these observations, we will consider a random unidirectional model, introducing dissociation events during motion. Although a bi-directional model can also accommodate these results, there is no evidence from studies of DNA helicases that a monomeric enzyme can bind and transport two DNA duplexes simultaneously (Soultanas and Wigley, 2000).

To evaluate a unidirectional model, we simulated equation (2) (Materials and methods), varying the number of R steps and keeping the number of L steps equal (Figure 7B). Forward motion (k_1) was defined as 10 times faster than the dissociation rate (k_2) and 10 times slower than the resetting rate (k_5). If dissociation were more frequent, then translocation would be less processive, to the extent that free TFO would not approach 100% over a reasonable time scale (not shown). A slower resetting rate would have the same result. Conversely, if there were no dissociation events and resetting only occurred at DNA ends, then the exponential reactions would be less extended than observed. As the number of R steps is increased, the cumulative occurrence of dissociation events results in a corresponding decrease in the apparent translocation rate (Figure 7B). Nevertheless, due to resetting, free TFO reaches >95% in each case. Thus, the distribution of kinetic profiles is graphically equivalent to that in Figure 7A. However, the model can neither determine the number of L or R steps directly nor estimate the relative values of k_1 , k_2 or k_5 . Furthermore, the profiles illustrated assume that upon reaching a DNA end, an enzyme steps off at the same rate as any previous forward step (Figure 7B). An alternative model, in which enzymes stall at ends and dissociate more slowly, results in an equivalent distribution of curves (not shown). Nevertheless, the $R_1M_2S_1$ data (Figure 7A) can only be justified by a model that incorporates both dissociation during motion and resetting.

In their previous study, Janscak *et al.* (1998) could not distinguish between an endonuclease assembly model with

two non-equivalent HsdR-binding sites, which would result in only one type of $R_1M_2S_1$ complex, and an assembly model with two equivalent HsdR sites, which would result in two distinct $R_1M_2S_1$ complexes. Since our data indicate no preference for recognition site asymmetry, binding of the first HsdR to the MTase must result in an equal distribution between leftward and rightward translocation states. As the alignment of HsdS is fixed on DNA by its TRD sequences (Powell *et al.*, 1998), the HsdR-binding sites must therefore be equivalent. The weaker binding of the second HsdR subunit could be due to physical occlusion by the first subunit or a conformational change resulting in negative cooperativity (Janscak *et al.*, 1998).

Conclusions

Although a linear relationship between specific binding and non-specific cleavage of DNA by the type I restriction enzymes has been investigated thoroughly (see Szczelkun, 2000), a direct *in vitro* assay of protein motion had not been described. In this study, *EcoR124I*-directed TFO displacement allowed direct measurement of translocation velocity. Furthermore, the kinetics discriminated clearly between uni- and bi-directional translocation schemes, depending upon endonuclease stoichiometry. The $R_2M_2S_1$ species translocates DNA bi-directionally, each side of its site moving at a speed of 400 ± 32 bp/s at 20°C. Unidirectional models can be discounted. Additional events that could slow translocation, such as sequence-dependent pauses or dissociation from the DNA, occur infrequently. Consequently, DNA translocation by *EcoR124I* is highly processive. This explains how DNA >1000 bp from the recognition site can be cleaved efficiently (Janscak *et al.*, 1996; Szczelkun *et al.*, 1997). However, we have yet to determine the exact number of steps that give rise to the kinetics observed. As a result, we cannot define a step size, i.e. the number of base pairs translocated per physical step. The $R_1M_2S_1$ species cannot cleave DNA but still generated translocation, independently of site asymmetry. However, the reaction was less processive than that catalysed by the $R_2M_2S_1$ form, as dissociation events during motion are more frequent. Therefore, the presence of two HsdR subunits must stabilize the translocating complex such that dissociation is rare. A similar increase in processivity with subunit complexity has been observed for the PcrA helicase (Soultanas *et al.*, 1999). Nevertheless, the HsdR subunits appear to act independently. Alongside the subunit assembly of the type I enzymes (Dryden *et al.*, 1997; Janscak *et al.*, 1998), and the pseudo-symmetrical domain organization (Dryden *et al.*, 1995; Powell *et al.*, 1998), this suggests that translocation on each 'side' of the recognition site is undertaken by a single HsdR.

Despite dissociating from the DNA during motion, the $R_1M_2S_1$ complex could restart translocation via resetting. This process must allow for a change in the direction of motion. One possibility is that HsdR completely dissociates from both the MTase and DNA. The free subunit could then rebind to the DNA-bound MTase at either position. However, the concentration of free HsdR would be low in this case and binding would be correspondingly slow (even if it occurred with a bi-molecular association rate constant close to the diffusion limit; Gutfreund, 1995).

Alternatively, the HsdR subunit may release the MTase but not DNA. Translocation could not continue, but the local concentration of the proteins would remain artificially high and reassociation could be faster. A third scenario is that HsdR releases the DNA but remains part of an $R_1M_2S_1$ complex; the HsdR subunit would then have to redistribute between the equal affinity binding sites. This would only be feasible if the sites were on the same 'face' of the MTase. Conversely, we do not believe that resetting plays a role in DNA translocation by the $R_2M_2S_1$ species. There is a possibility that when one of the two HsdR subunits reaches a DNA end it could dissociate from both the DNA and enzyme. An $R_1M_2S_1$ complex would result, and translocation in the opposite direction would become noticeably less processive. However, the kinetics do not support this (Figures 3, 4 and 5). The added stability of the $R_2M_2S_1$ complex may therefore result in stalling at DNA ends, as described for the PcrA DNA helicase (Dillingham *et al.*, 2000).

A comparison of the translocation velocity determined in this study with rates determined in other studies is dependent upon the different reaction conditions used. The experiments of Garcia and Molineux (1999) suggest an *in vivo* *EcoKI* translocation rate of ~100 bp/s. This may differ from our rate not only because of ionic factors, but because their internalization assay relies on pulling DNA through a bacterial pore out of a phage capsid; this may represent an increased load that slows translocation. Comparisons with other DNA motor proteins reveal a range of velocities *in vitro*: elongation rates vary dramatically among RNA polymerases and correlate with subunit complexity (Uptain *et al.*, 1997): single subunit bacteriophage RNA polymerases translocate at 200–400 nucleotides per second (nt/s); bacterial RNA polymerases translocate at an intermediate rate of 10–40 nt/s; and yeast RNA polymerase II and III elongate at 20–30 nt/s. For DNA helicases, unwinding rates over long distances have only been reported for a few enzymes. For instance, RecBCD unwinds DNA at 470 ± 80 bp/s at 25°C and at ~900 bp/s at 37°C (Roman and Kowalczykowski, 1989). On the other hand, single strand translocation rates for PriA (Lee and Marians, 1990) and PcrA (Dillingham *et al.*, 2000) are 90 and 50 nt/s, respectively. The rate of DNA translocation by *EcoR124I* is thus one of the fastest measured so far.

Materials and methods

Proteins

EcoR124I MTase was purified as described previously (Taylor *et al.*, 1992). Purified HsdR was generously provided by Pavel Janscak (Biozentrum, Switzerland). Protein concentrations were calculated from absorbance at 280 nm using molar extinction coefficients derived from amino acid sequences (MTase, 160 400/M/cm; HsdR, 91 900/M/cm). Unless otherwise stated, endonuclease was formed by mixing M_2S_1 with a 7 molar excess of hsdR in buffer R (50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1.0 mM dithiothreitol, 0.2 mM AdoMet). The final concentration of endonuclease was taken as the input concentration of M_2S_1 . The reconstituted enzyme had DNA cleavage activity equivalent to that previously published (Szczelkun *et al.*, 1997). For experiments with one HsdR, endonuclease was prepared with equimolar concentrations of M_2S_1 and HsdR. All other enzymes were from New England Biolabs (Massachusetts, USA).

DNA

Unless stated otherwise, all DNA manipulations were carried out using standard procedures (Sambrook *et al.*, 1989). Six oligonucleotides were supplied desalted by Cruachem (Glasgow, UK) and annealed as below to produce duplexes possessing either an *EcoR124I* site (single underlined) or a TFO-binding site (double underlined):



Plasmid pMDS20 was created by introducing an *EcoR124I* site in the *Bam*HI of pNEO (Pharmacia, Sweden) as outlined previously (Szczelkun *et al.*, 1996), whilst pMDS21 was created by cloning duplex 1 into the *Hind*III site of pNEO. The *EcoR124I*-binding site has the same spacer and flanking sequences as used previously (Szczelkun *et al.*, 1996, 1997). Cloning of duplex 2 into the *Bgl*II site of pMDS20 or pMDS21 produced pMDS22 and pMDS23, respectively. Cloning duplex 3 into the *Bsp*EI site of pMDS20 produced pMDS28. Alternative orientations of the *EcoR124I* and TFO sites were selected by sequencing (L.Hall, Bristol). Plasmids were either used directly as supercoiled substrates or were first digested with an appropriate type II restriction endonuclease, which cleaved the DNA once to produce full-length linear substrates (Figure 1D). The 22 bp CT-rich TFO sequence (Figure 1B) was designed according to observations by Gowers and Fox (1997, 1999). It was supplied desalted by Cruachem, 5' end-labelled with ³²P using T4 polynucleotide kinase and purified by ultrafiltration (Bio-Spin, Bio-Rad).

Triplex formation and analysis

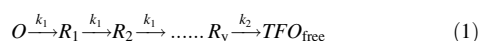
Equimolar concentrations (50 nM) of linear DNA and ³²P-labelled TFO were mixed in buffer MM (25 mM MES, pH 5.5, 10 mM MgCl₂) at 57°C for 15 min and left to cool to room temperature overnight. The resulting triplex was diluted 1/10 into buffer R before use. To analyse the proportion of bound and free TFO, reactions were quenched using GSMB buffer [15% (w/v) glucose, 3% (w/v) SDS, 250 mM MOPS pH 5.5, 0.4 mg/ml bromophenol blue] and analysed in 1% (w/v) agarose gels (40 mM Tris-acetate, 5.0 mM sodium acetate, 1.0 mM MgCl₂ pH 5.5) at 10 V/cm for 2 h at 4°C. Gels were fixed in 5% (v/v) acetic acid, 50% (v/v) methanol for at least 1 h and air-dried overnight between sheets of cellophane. Dry gels were scanned in a Molecular Dynamics PhosphorImager, and the image analysed with ImageQuant software (MD) to determine the volume of each 'band', taking into account background readings. The fraction of free TFO in each sample was calculated as volume_{free TFO}/(volume_{free TFO} + volume_{triplex}). Triplexes were not observed at or above pH 6.0 at 20°C using nanomolar concentrations of linear DNA and TFO (not shown). However, full occupancy at this concentration range and temperature was observed at pH 5.5 (see main text). Attempts to ascertain an equilibrium dissociation constant for the triplex were hampered because at the detection limit of the assay the TFO was still tightly bound, indicating a sub-nanomolar *K*_d value.

EcoR124I translocation reactions

For a standard reaction, 5 nM triplex (see above) was pre-incubated with the appropriate concentration of *EcoR124I* at 20°C in buffer R for 5 min. Unless otherwise stated, reactions were started by the addition of ATP to a final concentration of 4 mM, and aliquots quenched by the addition of 0.25 vols of GSMB buffer. Optimal reaction times were determined empirically (not shown). Resulting samples were analysed as above. DNA cleavage was analysed as described elsewhere (Szczelkun *et al.*, 1996).

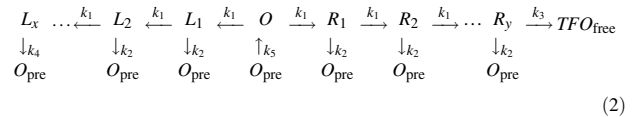
Kinetic models for DNA translocation

Theoretical TFO displacement kinetics were simulated by numerical integration in Scientist (MicroMath Software, Salt Lake City, UT). Models were constructed from a series of consecutive reactions governed by unimolecular rate constants as described by Ali and Lohman (1997). Equation (1) describes one branch of a bi-directional model, where only those molecules that travel from the origin (O) towards the triplex are considered.



Each step (*R*₁, *R*₂, etc.) is governed by the same unimolecular rate

constant, *k*₁. Subsequent TFO displacement is governed by *k*₂. Equation (2) describes a two-branch scheme for unidirectional motion.



This scheme has a number of features different from equation (1): (i) motion begins at an origin (O), but then proceeds down either the L (leftward) or R (rightward) pathway; (ii) each step can be governed by two unimolecular rate constants—a forward translocation rate (*k*₁) and a dissociation rate (*k*₂); (iii) only those molecules that travel down the R pathway can reach the triplex, generating free TFO with the rate constant *k*₃; (iv) those molecules that travel down the L pathway eventually reach a DNA end (*L*_x): at this stage, the enzyme can dissociate (*k*₄); and (v) any molecule that releases the DNA, either at an end or during motion, produces *O*_{pre}. This form of the enzyme can then restart translocation at the origin, governed by a unimolecular rate constant, *k*₅. For both models, the number of steps and the rate constants were varied as indicated in the main text. The percentage of final product was analysed in GraFit (Erithacus Software, Slough, UK).

Kinetic data analysis

Kinetic data were fitted to linear or exponential functions in GraFit. Residual data analysis of single exponential fits of TFO displacement showed non-random errors indicative of a higher order exponential relationship (data not shown). Consequently, displacement data were fitted using a double exponential increase with offset function:

$$TFO_{\text{free}} = A_1[1 - e^{-k_1(t - \text{offset})}] + A_2[1 - e^{-k_2(t - \text{offset})}] \quad (3)$$

where *TFO*_{free} is the percentage of liberated oligonucleotide, *A*₁ and *A*₂ are the amplitudes of the first and second phases, respectively, *k*₁ and *k*₂ are the rates of the first and second phases, respectively, and offset is the *x*-axis intercept (or lag time).

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