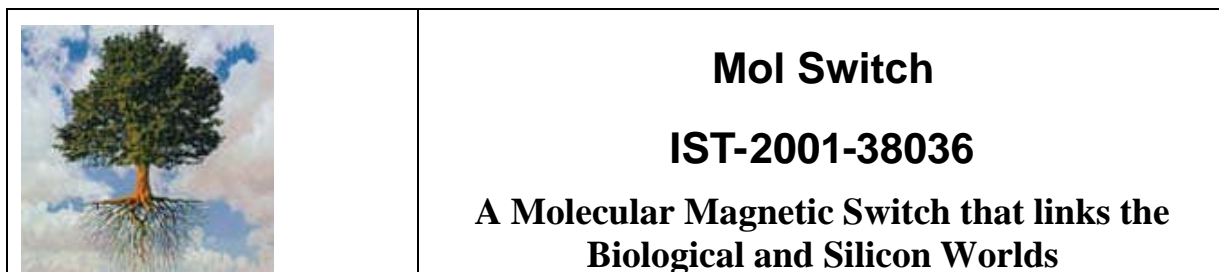


Measurement of Translocation by DNA Translocases using Single-Molecule Techniques

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Summary

Much of the data obtained from the work involved in this Deliverable has been published in highly respectable journals. This work cannot be reproduced without permission of the said journals, therefore, we have included links to the papers involved.



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Background

Type I Restriction-Modification (R-M) enzymes are the most complex of the many R-M systems known (for a recent review see Murray, 2000). They are multisubunit, multifunctional enzymes composed of three separate subunits (HsdR – the restriction/motor subunit, HsdM – the methylation subunit, and HsdS – the DNA binding subunit). The active endonuclease (REase) is composed of all three subunits in a ratio 2:2:1 (HsdR₂:HsdM₂:HsdS₁, or R₂-complex). The R₂-complex also functions as a DNA methyltransferase (MTase), an ATPase, and as a ‘DNA pulling’ molecular motor (Endlich and Linn, 1985; Yuan *et al.*, 1980).

HsdM and HsdS alone are sufficient to assemble an independent MTase with a stoichiometry of M₂S₁ (Taylor *et al.*, 1992). The R-M enzyme EcoR124I can be assembled *in vitro* from the core MTase by addition of the motor subunit HsdR (Janscák *et al.*, 1998). However, the purified EcoR124I REase exists as an equilibrium mixture of two species - R₂M₂S₁ and R₁M₂S₁ of which only the former is able to cleave DNA (Janscák *et al.*, 1996; Janscák *et al.*, 1998); although the R₁-complex is an ATPase and is able to translocate DNA (Firman and Szczelkun, 2000; Janscák *et al.*, 1998; Seidel *et al.*, 2004). The R₂-complex is relatively unstable and can dissociate into free HsdR subunit and the restriction-deficient R₁-complex intermediate, under concentrations expected *in vivo* (Janscák *et al.*, 1998). This situation assists structural analysis of the EcoR124I R-M enzyme because complexes with only one HsdR subunit present (i.e. R₁-complex) can more easily be visualised by AFM.

Unlike other restriction endonucleases, Type I R-M enzymes cut distal to the DNA recognition site to which they bind. DNA cleavage can occur many thousands of basepairs from the recognition site, using a process of DNA movement known as DNA translocation (Yuan *et al.*, 1980). This REase-based motor activity is driven by ATP hydrolysis (Endlich and Linn, 1985), but unlike other DNA-based motors (e.g. DNA polymerase) it does not involve a linear tracking motion along the DNA; instead the motor remains bound at the recognition site and ‘pulls’ the adjacent DNA toward the bound enzyme (Figure 1). A detailed study of DNA translocation by Type I restriction enzymes has recently been published, based on single molecule analysis using Magnetic Tweezers (Seidel *et al.*, 2004). This intriguing motor activity presents the enzyme with an interesting topological problem at the initiation of translocation – how to ‘grasp’ adjacent DNA and produce the first loop of DNA. (Dryden, 2004)

The complex molecular motor-function makes these enzymes particularly interesting. The restriction subunit (HsdR) is responsible for this motor activity and contains a series of conserved amino acid motifs (DEAD box motifs) including a Walker-type ATP binding site, which are associated with helicase-like activities (Gorbalenya and Koonin, 1991). They belong to a large superfamily (SF-II) of helicase-like enzymes (Flaus and Owen-Hughes, 2001) that also include Type III R-M enzymes, chromatin remodelling factors and a few chimeric enzymes. It has been suggested that chromatin-remodelling factors also make use of DNA translocation, in a similar mode as Type I restriction enzymes, which stresses the significance of a detailed analysis of the translocation process of the SF-II superfamily (Flaus and Owen-Hughes, 2001; Mahdi *et al.*, 2003).

ATP is an allosteric effector for the REase and is closely involved in the initial switch between methylation activity and translocation activity which leads to DNA cleavage (Yuan *et al.*, 1975). *In vivo*, the normal function of these enzymes is that of a maintenance methylase – following DNA replication, the enzyme will methylate specific adenines on the newly synthesised strand of the recognition sequence. However, invasion of the bacterial cell by ‘foreign’ DNA (usually bacteriophage DNA) elicits an ATP-dependent switch in behaviour of the Type I R-M enzyme, and the enzyme becomes an endonuclease. To accomplish this switch in activity the enzyme must read and compare the two adenines of the recognition sequence (one on



each strand) and *if* they are *both* unmethylated the enzyme will undergo a conformational change resulting in motor and endonuclease activity. The switch in function was shown to be an ATP-dependent event by Yuan et al. (1975) who ‘trapped’ the initial complex in filter binding studies. The non-hydrolysable ATP-derivative ATP γ S can also be used to trap this initial complex by preventing translocation and allows studies of the initiation of translocation.

It has been shown that the REase only ‘grasps’ the DNA *in cis* (Szczelkun *et al.*, 1996) suggesting a loop would form adjacent to the DNA-bound REase. However, DNA footprinting studies (Mernagh and Kneale, 1996; Mernagh *et al.*, 1998) have shown little difference between the footprints of the MTase and that of the REase suggesting the enzyme ‘grasps’ the DNA remote to the sequence recognition site. To initiate translocation, the enzyme would need to produce a loop that is smaller than the enzyme, and far shorter than the persistence length of DNA, making this mechanism very unlikely (Figure 1d). Another possibility is that the DNA initially wraps around the motor subunit, forming multiple DNA-protein interactions along the loop (Figure 1e). This would not have been detected in the DNA footprinting studies mentioned above because of the limited length of the oligoduplexes used (56 bp). Alternatively, the endonuclease may induce a distortion of the usual ds-DNA structure (such as a region of non-duplexed DNA) to allow the enzyme to ‘grasp’ adjacent DNA over short distances (Figure 1f).

In this paper, we have used Atomic Force Microscopy (AFM) on single DNA-restriction enzyme complexes to observe the conformational changes associated with the initial switch from recognition to translocation. We have used ATP γ S to trap these translocation initiation complexes. Using the R₁-complex produced by mixing equimolar ratios of MTase and HsdR, we could observe a small bulge extending from the protein-DNA complex that was only formed when ATP γ S was added. This bulge was accompanied by a shortening of the contour length of the DNA molecule. As expected, the bulge was more difficult to delineate in the R₂-complex, but additional shortening of the DNA contour length was observed supporting the idea of a two-bulge complex. Thus we obtained a detailed picture of the important first-step of initial loop formation that may be generic for all superfamily II enzymes that act as DNA-based molecular motors.

Aims of Deliverable

Measurement of speed and force for translocation by the EcoR124I molecular motor using AFM.

TuDelft and CNRS/ENS will provide reports describing progress toward the measurement of the forces generated by the molecular motors under study. These initial measurements will be produced using pre-existing set-ups and the data can be compared to data from the AFM studies above. In addition, these reports will provide information for the design of customised magnetic tweezer set-ups that will be developed.

To determine the ability of the EcoR124I molecular motor to function at low concentrations and on different surfaces.

Tasks scheduled:

1. Determine the speed of translocation using the AFM.
2. Setup Magnetic Tweezers at TUDelft, for analysis of EcoR124I molecular motor.
3. Setup Magnetic Tweezer at ENS/CNRS for analysis of other molecular motors that translocate DNA.
4. Determine motor forces and translocation rates for motors of interest to the design of the Mol Switch Device.



Methodology Used:

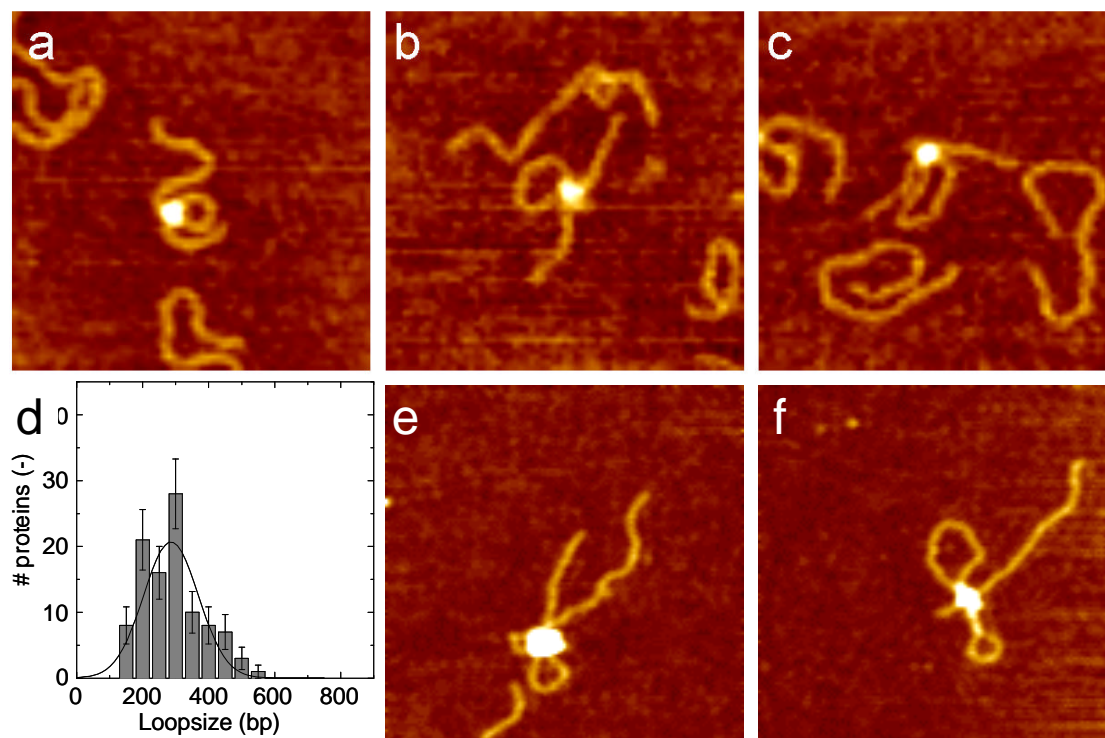
The main methods used were single-molecule techniques such as magnetic tweezers and AFM.

Results achieved:

Translocation measurements using AFM

R_1 -complex of the EcoR124I R-M enzyme was produced by mixing equimolar ratios of MTase and HsdR (Janscák *et al.*, 1998). Analysis of these complexes, bound to DNA, using the AFM showed a uniform collection of similar-sized complexes, with very few R_2 -complexes (larger) observed (data not shown). That these complexes were indeed functional R_1 -complexes was confirmed by demonstrating unidirectional translocation (single loop formation) as shown in Figure 1.

Figure 1 AFM images of DNA translocation



The amount of translocated DNA was determined by tracing the loop size for more than 100 complexes. The resulting distribution for 10s incubation time, is shown in Figure 1d. Though the average loop size increased with longer incubation times, as expected, the apparent translocation velocity is an order of magnitude smaller than previously reported values (Firman and Szczelkun, 2000; Seidel *et al.*, 2004). However, though AFM experiments are valuable for qualitative insights into structural changes of DNA-enzyme complexes, quantitative information on the timing can be trusted less due to artefacts related to surface immobilisation of the complexes (Kasas *et al.*, 1997; van Noort *et al.*, 1998).

The fixed complexes, imaged by AFM, reveal exciting structural features. Figures 1e and f, show two examples of R_2 -complexes after incubation with DNA and ATP. As expected for the fully functional EcoR124I enzyme, *two* DNA loops are formed. Interestingly, contrary to the R_1 -DNA complexes, some of the loops formed by R_2 -complexes show evidence of supercoiling. The loop sizes within single R_2 -complexes appear to be distinctly different. Assuming the same translocation velocity for both HsdR subunits, this implies that the switch from

recognition to translocation occurs independent in both subunits and forms a rate-limiting step for initial DNA translocation (c.f. the data presented by Seidel et al. Seidel *et al.*, 2004).

Full details of these results are available at:

<http://nar.oxfordjournals.org/cgi/content/full/32/22/6540?ijkey=VVq1t3of.rAL.&keytype=ref&siteid=nar>

Magnetic Tweezer Setup and measurements of translocation



A magnetic Tweezer Set-up was established at ENS/CNRS to study motors other than EcoR124I as a backup in case EcoR124I did not function as expected, or in case single-molecule measurements failed.

Single-molecule measurements of the manipulation of three different DNA motor proteins are reviewed. Despite some

differences in the structure and mechanisms of the proteins, there are consistent phenomenological themes that relate them. Each of the experiments described represents a significant advance in the understanding of the mechanisms of DNA transport.

Full details of these results are available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15884063

It was obvious that the most likely motor for use in the proposed Mol Switch Device was FtsK, which was also found to be one of the fastest motors known.

In addition, another Magnetic Tweezer Set-up was established at TUDelft. This machine was dedicated to studies of the EcoR124I molecular motor in collaboration with Ports.

FtsK:

Escherichia coli FtsK is an essential cell division protein, which is thought to pump chromosomal DNA through the closing septum in an oriented manner by following DNA sequence polarity. Here, we perform single-molecule measurements of translocation by FtsK_{50C}, a derivative that functions as a DNA translocase *in vitro*. FtsK_{50C} translocation follows Michaelis–Menten kinetics, with a maximum speed of ~6.7 kbp/s. We present results on the effect of applied force on the speed, distance translocated, and the mean times during and between protein activity. Surprisingly, we observe that FtsK_{50C} can spontaneously reverse its translocation direction on a fragment of *E. coli* chromosomal DNA, indicating that DNA sequence is not the sole determinant of translocation direction. We conclude that *in vivo* polarization of FtsK translocation could require the presence of cofactors; alternatively, we propose a model in which tension in the DNA directs FtsK translocation.

Full details of these results are available at:

<http://www.nature.com/emboj/journal/v23/n12/full/7600242a.html>

FtsK is a bacterial protein that translocates DNA in order to transport chromosomes within the cell. During translocation, DNA's double-helical structure might cause a relative rotation between FtsK and the DNA. We used a single-molecule technique to quantify this rotation by observing the supercoils induced into the DNA during translocation of an FtsK



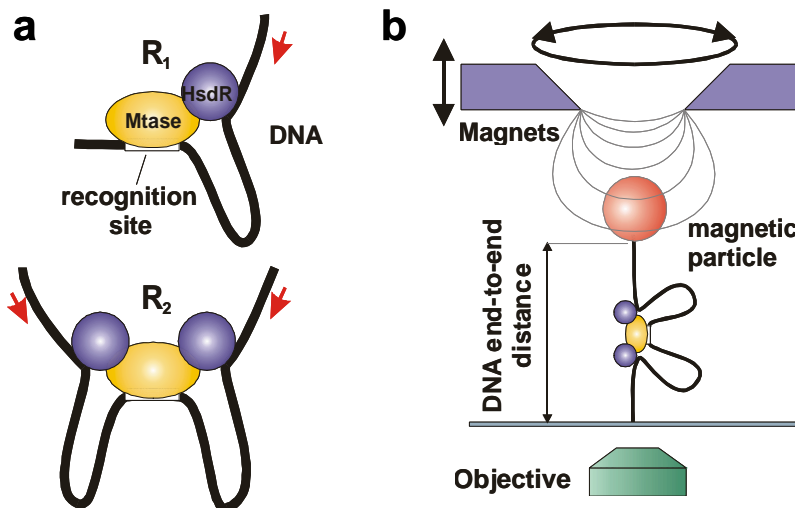
complex. We find that FtsK induces approximately 0.07 supercoils per DNA helical pitch traveled. This rate indicates that FtsK does not track along DNA's groove, but it is consistent with our previous estimate of FtsK's step size. We show that this rate of supercoil induction is markedly near to the ideal value that would minimize in vivo disturbance to the chromosomal supercoil density, suggesting an origin for the unusual rotational behaviour of FtsK.

Full details of these results are available at:

<http://www.nature.com/nsmb/journal/v12/n5/abs/nsmb926.html>

EcoR124I Molecular Motor

Figure 2 Schematic drawing of a Type I restriction enzyme translocating DNA



Type I restriction enzymes bind sequence-specifically to unmodified DNA and subsequently pull the adjacent DNA toward themselves. Cleavage then occurs remotely from the recognition site. The mechanism by which these members of the superfamily 2 (SF2) of helicases translocate DNA is largely unknown. We report the first single-molecule study of DNA translocation by the type I restriction enzyme

EcoR124I. Mechanochemical parameters such as the translocation rate and processivity, and their dependence on force and ATP concentration, are presented. We show that the two motor subunits of *EcoR124I* work independently. By using torsionally constrained DNA molecules, we found that the enzyme tracks along the helical pitch of the DNA molecule. This assay may be directly applicable to investigating the tracking of other DNA-translocating motors along their DNA templates.

Full details of these results are available at:

<http://www.nature.com/nsmb/journal/v11/n9/abs/nsmb816.html>

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.

Full details of these results are available at:

<http://www.nature.com/emboj/journal/v25/n10/full/7601104a.html>

Influence of [ATP] on translocation

Type I restriction enzymes use two motors to translocate DNA before carrying out DNA cleavage. The motor function is accomplished by amino-acid motifs typical for



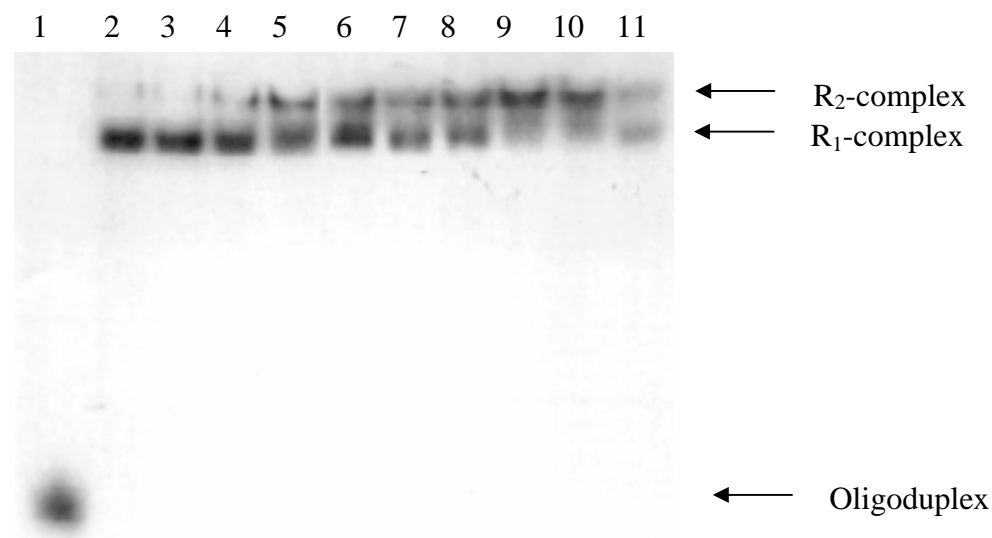
Project funded by the European Community under the "Information Society Technologies" Programme

superfamily 2 helicases, although DNA unwinding is not observed. Using a combination of extensive single-molecule magnetic tweezers and stopped-flow bulk measurements, we fully characterized the (re)initiation of DNA translocation by EcoR124I. We found that the methyltransferase core unit of the enzyme loads the motor subunits onto adjacent DNA by allowing them to bind and initiate translocation. Termination of translocation occurs owing to dissociation of the motors from the core unit. Reinitiation of translocation requires binding of new motors from solution. The identification and quantification of further initiation steps - ATP binding and extrusion of an initial DNA loop - allowed us to deduce a complete kinetic reinitiation scheme. The dissociation/reassociation of motors during translocation allows dynamic control of the restriction process by the availability of motors. Direct evidence that this control mechanism is relevant *in vivo* is provided.

<http://www.nature.com/emboj/journal/v24/n23/full/7600881a.html>

The cofactor ATP is known to be important in the discrimination of methylation status of DNA and must be important for DNA binding (Burckhardt *et al.*, 1981). However, to date analysis of the stoichiometry of the EcoR124I R-M enzyme has been carried out in the absence of cofactors. It has been shown that the DNA binding characteristics of the EcoKI R-M enzyme are influenced by the presence of the cofactors *S*-adenosyl-L-methionine (AdoMet) and/or ATP (Powell *et al.*, 1993; Powell and Murray, 1995). Therefore, we investigated the effect of these cofactors on the DNA-binding properties of the wt EcoR124I enzyme using gel retardation. Mixing MTase and oligoduplex with HsdR at molar ratios between zero and five allowed us to study the stability of the R₁-complex in the presence, or absence of these cofactors. It has been previously shown that when the molar ratio of HsdR:MTase is above two the R₂-complex is formed (Janscák *et al.*, 1998; Lisle *et al.*, 2000; Weiserova *et al.*, 2000). Figure 11 shows that the presence of ATP favours formation of the MTase as well the R₁-complex, but AdoMet has only a small effect on the presence of R₁-complex. Release of the MTase in the presence of ATP was an unexpected phenomenon. In addition, over the wider range of HsdR concentrations, we can see that the presence of ATP also favours the formation of the R₁-complex. Therefore, it appears that ATP shifts the equilibrium of the R-M enzyme in favour of formation of the MTase and R₁-complex. This was further confirmed by an analysis of the dissociation of these complexes (following dilution) in the presence of ATP and/or AdoMet in a manner analogous to that described by Janscák *et al.* (1998).

Figure 3 Dissociation of the R₂-complex in the presence of ATP



Conclusion:

The use of AFM to show translocation is possible and relatively simple to do, and this technology has been transferred from TUDelft, to NPLML and onto Ports.

However, speed measurement is not reliable and should not be determined using this technology.

The Calibrated AFM is described above. The complexity of using the system in liquids, the difficulty associated with attaching DNA between a surface and a tip and the problems associated with cantilever stiffness have all prevented any measurement of motor force using this system.

Magnetic Tweezer Setups were used to characterise both FtsK – one of the fastest molecular motors ever characterised – and EcoR124I.

Both motors are suitable for use within the proposed Mol Switch Device.

At saturating ATP the rate-limiting step in the initiation is the formation of an initial DNA loop for which a rate in the absence of force $k_{\text{ini,R1}} = 2.0 \pm 0.8 \text{ s}^{-1}$ is found for the R₁-complex. Initial loop formation is associated with a large DNA contraction step of 8 nm (van Noort et al, 2004).

The effect of ATP, in promoting dissociation of the R₂-complex may be an artefact of the way in which gel retardation is performed, in particular, because of the use of oligoduplex DNA substrates.

Therefore, the single molecule measurements have provided an extensive and unprecedented characterization of the EcoR124I motor. A complete set of biochemical rates was measured, speeds were accurately determined for the two main motors used.

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