

DNA Supercoiling during ATP-dependent DNA Translocation by the Type I Restriction Enzyme *EcoAI*

Pavel Janscak and Thomas A. Bickle*

Department of Microbiology,
Biozentrum, University of
Basel, Klingelbergstrasse 70,
CH-4056, Basel, Switzerland

Type I restriction enzymes cleave DNA at non-specific sites far from their recognition sequence as a consequence of ATP-dependent DNA translocation past the enzyme. During this reaction, the enzyme remains bound to the recognition sequence and translocates DNA towards itself simultaneously from both directions, generating DNA loops, which appear to be supercoiled when visualised by electron microscopy. To further investigate the mechanism of DNA translocation by type I restriction enzymes, we have probed the reaction intermediates with DNA topoisomerases. A DNA cleavage-deficient mutant of *EcoAI*, which has normal DNA translocation and ATPase activities, was used in these DNA supercoiling assays. In the presence of eubacterial DNA topoisomerase I, which specifically removes negative supercoils, the *EcoAI* mutant introduced positive supercoils into relaxed plasmid DNA substrate in a reaction dependent on ATP hydrolysis. The same DNA supercoiling activity followed by DNA cleavage was observed with the wild-type *EcoAI* endonuclease. Positive supercoils were not seen when eubacterial DNA topoisomerase I was replaced by eukaryotic DNA topoisomerase I, which removes both positive and negative supercoils. Furthermore, addition of eukaryotic DNA topoisomerase I to the product of the supercoiling reaction resulted in its rapid relaxation. These results are consistent with a model in which *EcoAI* translocation along the helical path of closed circular DNA duplex simultaneously generates positive supercoils ahead and negative supercoils behind the moving complex in the contracting and expanding DNA loops, respectively. In addition, we show that the highly positively supercoiled DNA generated by the *EcoAI* mutant is cleaved by *EcoAI* wild-type endonuclease much more slowly than relaxed DNA. This suggests that the topological changes in the DNA substrate associated with DNA translocation by type I restriction enzymes do not appear to be the trigger for DNA cleavage.

© 2000 Academic Press

Keywords: DNA supercoiling; DNA translocation; helicase; topoisomerase; type I restriction enzyme

*Corresponding author

Introduction

Type I restriction enzymes are paradigms for action of proteins at a distance on DNA by active translocation along the DNA duplex. These enzymes recognise a specific non-palindromic DNA sequence (e.g. gagnnnnnngtca for *EcoAI* where n is any nucleotide) but cleave DNA at non-specific sites far away from the recognition

sequence in a reaction coupled to ATP hydrolysis (Yuan, 1981; Bickle, 1993). During this reaction the enzyme remains bound to the recognition sequence and translocates DNA towards itself simultaneously from both directions, which leads to the expansion of DNA loops. DNA cleavage by type I restriction enzymes is dependent on the nature of the DNA substrate. Linear DNA molecules are cleaved effectively only if they contain at least two recognition sites (Murray *et al.*, 1973; Studier & Bandyopadhyay, 1988; Szczelkun *et al.*, 1996; Dreier *et al.*, 1996). The fact that the cleavage of such substrates occurs in the region between the recognition sites with a preference for a position halfway between the sites suggests that DNA clea-

Abbreviations used: AdoMet, S-adenosylmethionine; ADPHNP, β,γ -imidoadenosine 5'-triphosphate; EtBr, ethidium bromide; SSB, single-strand binding protein.

E-mail address of the corresponding author:
bickle@ubaclu.unibas.ch

vage requires collision and co-operative interaction between two translocating enzyme molecules (Studier & Bandyopadhyay, 1988). However, DNA cleavage by type I restriction enzymes occurs also if a translocating enzyme encounters a Holliday junction (Taylor & Smith, 1990; Janscak *et al.*, 1999a) or following a collision of two different type I enzymes of which one is inactive in DNA cleavage because of a mutation at the endonucleolytic active site (Janscak *et al.*, 1999b). Moreover, type I restriction enzymes can efficiently cleave circular DNA molecules with only one recognition site (Rosamond *et al.*, 1979; Janscak *et al.*, 1996). All these findings suggest a general mechanism for cleavage site selection by type I restriction enzymes in which DNA translocation blockage is the only requirement for DNA cleavage to occur (Janscak *et al.*, 1999a).

Based on tests for cross-hybridisation between genes and antibody cross-reactivity, type I restriction enzymes are grouped into the four families IA, IB, IC and ID (Murray *et al.*, 1982; Price *et al.*, 1987; Titheradge *et al.*, 1996). All type I restriction enzymes are composed of three different subunits, HsdS, HsdM and HsdR. The subunit stoichiometry of the functional endonuclease is $R_2M_2S_1$ (Dryden *et al.*, 1997; Janscak *et al.*, 1998). The M_2S_1 complex forms the core of the enzyme, which mediates specific binding to the DNA recognition sequence. It can function as a DNA methyltransferase both in the endonuclease complex and independently, methylating a specific adenine residue in each strand of the recognition sequence (Burckhardt *et al.*, 1981b; Dryden *et al.*, 1993; Taylor *et al.*, 1992; Janscak & Bickle, 1998). The HsdS subunit determines the DNA sequence specificity (Fuller-Pace *et al.*, 1984), while the HsdM subunit contains the binding site for the methyl donor and restriction cofactor S-adenosylmethionine (AdoMet) as well as the catalytic site for the methylation reaction (Willcock *et al.*, 1994). The HsdR subunit is essential for restriction, ATPase and DNA translocation activities of the enzyme. The presence of two HsdR subunits in the enzyme complex is consistent with its ability to translocate and cleave DNA in either direction from the recognition site (Yuan *et al.*, 1980; Studier & Bandyopadhyay, 1988; Janscak *et al.*, 1999a).

HsdR subunits of all type I restriction enzymes appear to have a modular structure. The N-terminal part of HsdR contains a DNA cleavage domain with catalytic residues reminiscent of the PD...(D/E)-X-K catalytic motif of type II restriction enzymes (Davies *et al.*, 1999b; Janscak *et al.*, 1999b). Single amino acid substitutions in the endonuclease motif of EcoKI and EcoAI block DNA cleavage but have no effect on the ATPase or DNA translocation activities of the enzyme (Davies *et al.*, 1999a,b; Janscak *et al.*, 1999b). The central part of the HsdR polypeptide contains a cluster of seven motifs found in the superfamilies I and II of DNA and RNA helicases (the so-called DEAD-box motifs), which are thought to be relevant to the

ATP-dependent DNA translocation activity of type I restriction enzymes (Gorbalenya & Koonin, 1991; Murray *et al.*, 1993). In agreement with this proposal, single amino acid substitutions in any of the DEAD motifs of EcoKI abolish DNA cleavage, ATPase and DNA translocation activities of the enzyme (Webb *et al.*, 1996; Davies *et al.*, 1998, 1999a). The C-terminal part of the HsdR polypeptide appears to be involved in interaction with the methylase, since proteolytic removal of the C-terminal part of EcoKI HsdR abolishes this interaction (Davies *et al.*, 1999b).

Although type I restriction enzymes have been studied for more than three decades, the mechanism of DNA translocation by these enzymes remains largely unclear. Early electron microscopic studies with EcoKI and EcoBI have revealed a formation of twisted DNA loop intermediates (Yuan *et al.*, 1980; Endlich & Linn, 1985). The formation of supercoils has been explained by a mechanism in which the enzyme tracks in a groove of the DNA helix while remaining bound to the recognition site; although a DNA wrapping mechanism has also been considered (Yuan *et al.*, 1980; Endlich & Linn, 1985). A vectorial movement of a protein along the helical path of duplex DNA can necessitate rotation of the DNA helix around its axis, providing that the protein is simultaneously anchored to another site on the DNA, which results in a formation of a negatively supercoiled DNA loop. In the case of closed circular DNA, a negatively supercoiled loop behind the translocating complex and a positively supercoiled loop ahead of it would form simultaneously (Ostrand *et al.*, 1990). Based on some experimental observations, it has been proposed that the initial loop formed by binding of a type I restriction enzyme to DNA is small and it needs to be nicked prior to DNA translocation in order to accommodate reduction in twist resulting from helix-tracking process (Szczelkun *et al.*, 1996). In this model, the build-up of positive supercoils in the contracting DNA loop on circular DNA molecules is thought to be the trigger for DNA cleavage, by causing either a halt or a pause in the translocation process during which the enzyme cuts both DNA strands (Szczelkun *et al.*, 1996).

To address the helix-tracking model, we have employed a topological assay (Figure 1) that was first developed for studies on DNA translocation by RNA polymerase (Tsao *et al.*, 1989). In the presence of eubacterial DNA topoisomerase I, which selectively removes negative supercoils, RNA transcription results in the rapid accumulation of positive supercoils on a circular DNA template. Positive supercoils are not seen when eubacterial topoisomerase I is replaced by eukaryotic topoisomerase I. Using such an assay, DNA helicases such as *Escherichia coli* UvrAB helicase or SV40 large tumor (T) antigen have been shown to induce DNA supercoiling by ATP-dependent translocation along the helical path of the DNA duplex (Koo *et al.*, 1991; Yang *et al.*, 1989). We report here that

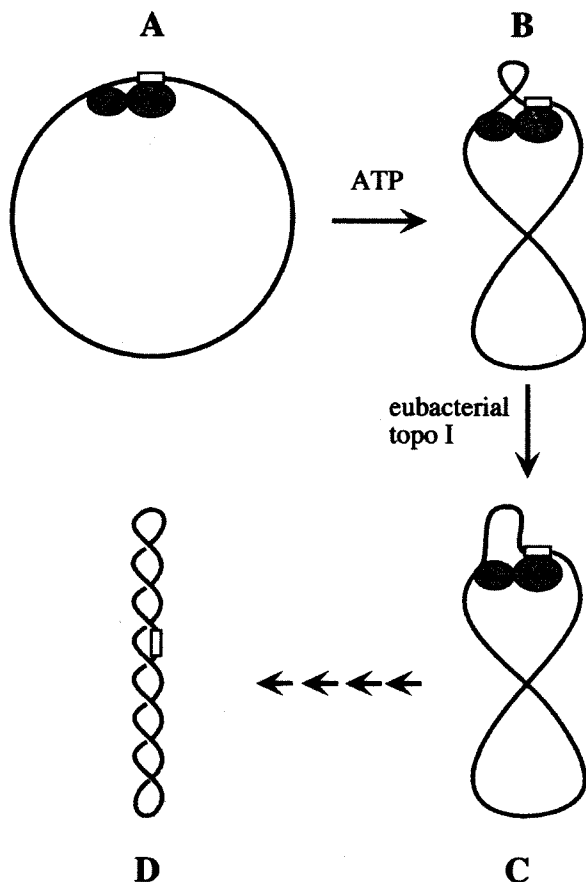


Figure 1. Scheme of the DNA supercoiling assay used to show the helix-tracking activity of *EcoAI*. (a) Relaxed DNA substrate is represented as a circle (black line) with an open rectangle indicating the *EcoAI* recognition sequence. The enzyme is represented as two ovals the larger of which (the methylase M_2S_1) interacts with the recognition sequence, while the smaller (HsdR) interacts with non-specific DNA. For simplicity, only one of the two HsdR subunits is considered in this model. (b) ATP-driven translocation of DNA past the HsdR subunit generates positive supercoils (red line) in the contracting DNA loop and negative supercoils (blue line) in the expanding DNA loop. (c) Relaxation of the negatively supercoiled DNA loop by eubacterial DNA topoisomerase I (topo I) results in accumulation of positive supercoils in the DNA molecule. (d) Repeated action of *EcoAI* in the presence of eubacterial DNA topoisomerase I and ATP produces highly positively supercoiled DNA.

ATP-dependent translocation of closed circular DNA by a cleavage-deficient mutant of *EcoAI*, *EcoAI*_{R-D61A} (Janscak *et al.*, 1999b), indeed produces both positively and negatively supercoiled DNA domains. In addition, we show that the highly positively supercoiled plasmid DNA generated by the *EcoAI* mutant in the presence of eubacterial topoisomerase I is a poor substrate for the *EcoAI* endonuclease compared to relaxed plasmid. This

suggests that the build-up of positive supercoils during translocation on circular substrates is not the trigger for DNA cleavage.

Results

Formation of highly positively supercoiled DNA in the presence of a cleavage-deficient mutant *EcoAI*_{R-D61A} and *E. coli* DNA topoisomerase I

To investigate the mechanism of DNA translocation by *EcoAI*, we have used a DNA cleavage-deficient mutant of this enzyme that has an alanine substitution at the first acidic residue of the HsdR subunit (*EcoAI*_{R-D61A}). This mutant retains the ability to translocate DNA and hydrolyse ATP (Janscak *et al.*, 1999b). The mutant enzyme was assembled by mixing *in vitro* the purified methylase (M_2S_1) and HsdR_{D61A} subunit and used for the DNA supercoiling assay as depicted in Figure 1. If translocation of closed circular duplex DNA by *EcoAI* leads to generation of positively and negatively supercoiled DNA loops, selective relaxation of negative supercoils by *E. coli* DNA topoisomerase I during the translocation process should overall result in the accumulation of positive supercoils in the DNA molecule. Indeed, incubation of relaxed pJP25 DNA (one *EcoAI* recognition site) with *EcoAI*_{R-D61A}, ATP and *E. coli* DNA topoisomerase I for ten minutes at 37°C resulted in the formation of a highly supercoiled DNA species that had a significantly faster electrophoretic mobility than negatively supercoiled DNA molecules isolated from *E. coli* HB101 (Figure 2, top panel, lane 3). The non-hydrolysable ATP analogue ADPNHP could not substitute for ATP in the supercoiling reaction, suggesting that ATP hydrolysis is necessary for this reaction (Figure 2, top panel, lane 4). No supercoiling activity was observed with DNA that did not contain a *EcoAI* recognition site (Figure 2, top panel, lane 5) or when either HsdR_{D61A}, methylase or *E. coli* DNA topoisomerase I were omitted from the reaction (not shown).

To determine the nature of the DNA species formed in the presence of *EcoAI*_{R-D61A} and *E. coli* DNA topoisomerase I, the reaction product was subjected to gel electrophoresis in the presence of chloroquine, an intercalative ligand producing a decrease in the twist of DNA, which is compensated by introduction of positive supercoils (Shure *et al.*, 1977). In the presence of 3.8 μM chloroquine, the *EcoAI*_{R-D61A} product migrated at the position of highly supercoiled DNA, while native negatively supercoiled DNA molecules migrated near the position of relaxed DNA (Figure 2, bottom panel, compare lanes 1 and 3). Compared to the electrophoretic mobilities of the same DNA samples in the absence of chloroquine (Figure 2, top panel), these results indicate that the product of the supercoiling reaction mediated by *EcoAI*_{R-D61A} is positively supercoiled DNA.

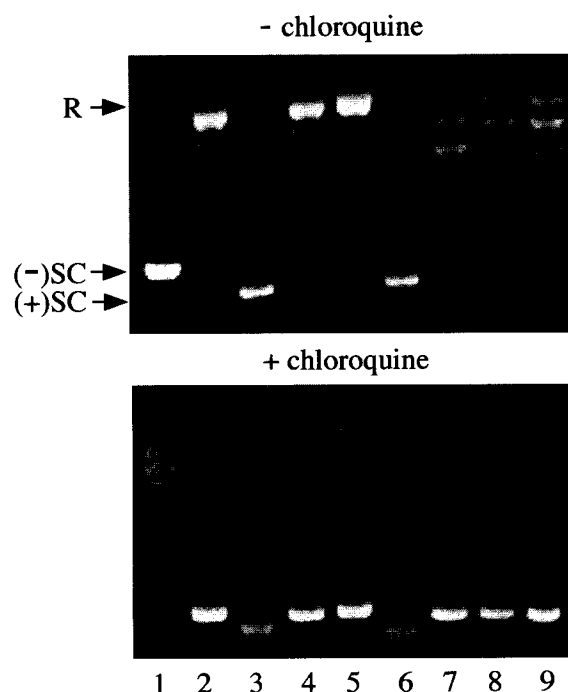


Figure 2. Formation of highly positively supercoiled DNA in the presence of *EcoAI*_{R-D61A} and *E. coli* DNA topoisomerase I. The DNA supercoiling assay was performed as described in Materials and Methods. Reactions were carried out in a volume of 20 μ l for ten minutes and contained 20 nM DNA and 80 nM *EcoAI*_{R-D61A}. Where required, four units of *E. coli* topoisomerase I (EC-topo I) or four units of wheatgerm topoisomerase I (WG-topo I) were added. Reaction products were subjected to agarose gel electrophoresis in the absence (top gel) and in the presence of 3.8 μ M chloroquine (bottom gel). Lane 1, negatively supercoiled pJP25 DNA (one *EcoAI* site); lane 2, pJP25 DNA relaxed by EC-topo I; lane 3, relaxed pJP25 DNA + *EcoAI*_{R-D61A} + EC-topo I + ATP; lane 4, relaxed pJP25 DNA + *EcoAI*_{R-D61A} + EC-topo I + ADPNHP (5 mM); lane 5, relaxed pDRM.1R DNA (no *EcoAI* site) + *EcoAI*_{R-D61A} + EC-topo I + ATP; lane 6, negatively supercoiled pJP25 + *EcoAI*_{R-D61A} + EC-topo I + ATP; lane 7, relaxed pJP25 DNA + *EcoAI*_{R-D61A} + WG-topo I + ATP; lane 8, product of the reaction in lane 3 + WG-topo I; lane 9, relaxed pJP25 DNA + WG-topo I. Electrophoretic mobilities of relaxed (R), negatively supercoiled (-SC) and positively supercoiled (+SC) plasmid DNA molecules in the absence of chloroquine are marked on the left of the gel.

Formation of highly positively supercoiled DNA in the *EcoAI*_{R-D61A} reaction was dependent on the presence of eubacterial DNA topoisomerase I. Substitution of *E. coli* topoisomerase I by wheatgerm DNA topoisomerase I, which has no DNA conformational specificity, did not result in the formation of the fast-migrating species (Figure 2, lane 7). The same result was obtained when *E. coli* topoisomerase I and wheatgerm topoisomerase I were added simultaneously to the *EcoAI*_{R-D61A} reaction (not shown). Furthermore, the addition of wheatgerm

DNA topoisomerase I to the highly positively supercoiled product of the *EcoAI*_{R-D61A} reaction led to the conversion of this DNA species to relaxed DNA (Figure 2, lane 8). Together, these results suggest that positive supercoils generated in the *EcoAI*_{R-D61A}/*E. coli* topoisomerase I reaction are not constrained by protein binding, but are torsionally strained.

Characterisation of the *EcoAI*_{R-D61A}-mediated DNA supercoiling reaction

Kinetics of the reaction

To follow the kinetics of the DNA supercoiling mediated by *EcoAI*_{R-D61A} in the presence of *E. coli* DNA topoisomerase I and ATP, reactions were carried out at 37°C with 10 nM relaxed pJP25 DNA substrate and a fourfold molar excess of the enzyme over DNA. The reaction was started by the addition of ATP. Samples were removed at different time-points and analysed by agarose gel electrophoresis. The *EcoAI* mutant was found to gradually introduce positive supercoils into the relaxed plasmid DNA (Figure 3). The reaction was very fast and it reached the steady state in less than two minutes, giving rise to highly supercoiled species.

Reaction stoichiometry

Type I restriction enzymes do not turn-over in the DNA cleavage reaction, but their ATPase activity continues long after DNA cleavage has been accomplished (Eskin & Linn, 1972; Endlich & Linn, 1985). DNA cleavage data for *EcoR*124I and *EcoAI* endonucleases suggested that cleavage of a circular DNA containing a single recognition site is accomplished by one enzyme molecule (Janscak

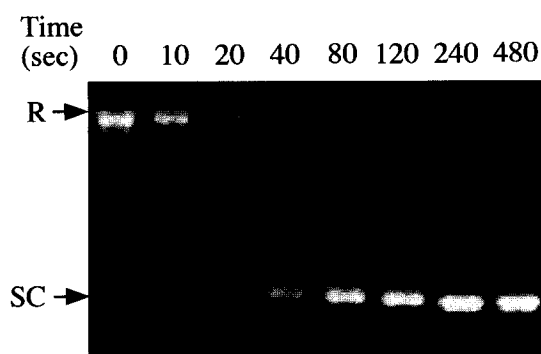


Figure 3. Kinetics of DNA supercoiling by *EcoAI*_{R-D61A}. Reaction (40 μ l) was carried out in buffer C at 37°C and contained 10 nM relaxed pJP25 DNA, 40 nM *EcoAI*_{R-D61A}, two units of *E. coli* topoisomerase I and 5 mM ATP. Aliquots (4 μ l) were removed at the time-points indicated and were analysed as described in Materials and Methods. Electrophoretic mobilities of relaxed (R) and highly positively supercoiled (SC) DNA are marked on the left of the gel.

et al., 1998; Janscak & Bickle, 1998). To investigate the stoichiometry of the *EcoAI*_{R-D61A}-mediated DNA supercoiling reaction on pJP25 DNA (one *EcoAI* site), a protein titration experiment was performed. In this experiment, we incubated 10 nM relaxed pJP25 DNA with increasing amounts of *EcoAI*_{R-D61A} and a fixed amount of *E. coli* DNA topoisomerase I in the presence of ATP for ten minutes, and assayed for DNA supercoiling activity. We have found that the degree of supercoiling in the final product started to gradually increase from an enzyme to DNA ratio of 0.35 and reached the maximum at an enzyme to DNA ratio of approximately 3 (Figure 4). These results suggest that the supercoiling reaction supported by *EcoAI*_{R-D61A} is a stoichiometric rather than catalytic reaction. The minimum of about three enzyme molecules per DNA molecule is unlikely to be the absolute reaction stoichiometry. Instead, it may result from incomplete DNA binding and/or from dissociation of the enzyme complex at low protein concentrations.

Dependence on ATP concentration

The data above indicate that DNA supercoiling mediated by *EcoAI*_{R-D61A} requires ATP hydrolysis. To investigate the effect of ATP concentration on this reaction, reactions were carried out for ten minutes with 10 nM relaxed pJP25 DNA, 40 nM *EcoAI*_{R-D61A} and a range of ATP concentrations from 20 to 5000 μ M (Figure 5). No supercoiling occurred at [ATP] < 50 μ M. The mean degree of supercoiling in the final product gradually increased over the range of [ATP] between 100 and 1000 μ M. In comparison, K_m values for ATP hydrolysis estimated for *EcoKI*, *EcoBI* and *EcoR124-II* are in the range of 10 to 100 μ M (Burckhardt

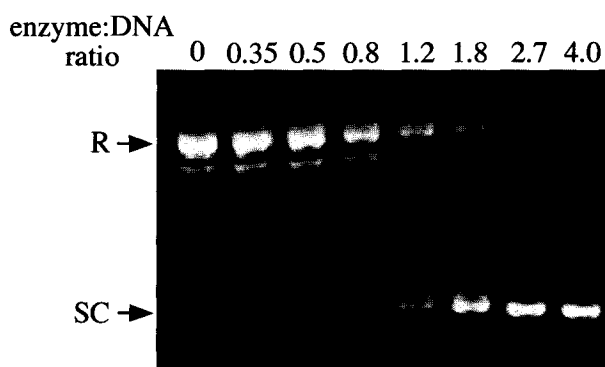


Figure 4. Dependence of *EcoAI*_{R-D61A}-mediated DNA supercoiling on enzyme to DNA ratio. Reactions in buffer C (20 μ l) contained 10 nM relaxed pJP25 DNA, varied concentrations of *EcoAI*_{R-D61A} to give the indicated enzyme to DNA ratios, two units of *E. coli* topoisomerase I and 5 mM ATP. After ten minutes incubation at 37°C, samples were analysed as described in Materials and Methods. Electrophoretic mobilities of relaxed (R) and highly positively supercoiled (SC) DNA are marked on the left of the gel.

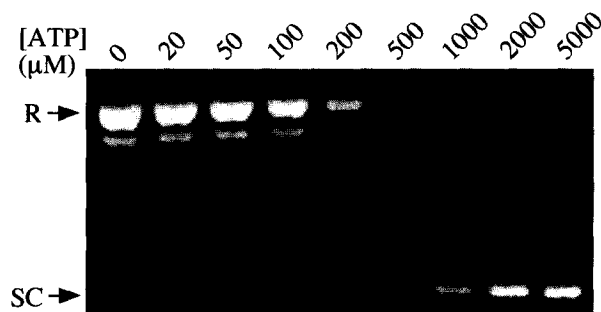


Figure 5. Dependence of *EcoAI*-mediated DNA supercoiling on ATP concentration. Reactions in buffer C (20 μ l) contained 10 nM relaxed pJP25 DNA, 40 nM *EcoAI*_{R-D61A} and two units of *E. coli* topoisomerase I and the indicated ATP concentrations. After ten minutes incubation at 37°C, samples were analysed as described in Materials and Methods. Electrophoretic mobilities of relaxed (R) and highly positively supercoiled (SC) DNA are marked on the left of the gel.

et al., 1981a; Eskin & Linn, 1972; Dreier & Bickle, 1996).

Stability of the supercoiled intermediates

The experiments above suggest that the translocation of closed circular DNA by *EcoAI*_{R-D61A} leads to simultaneous formation of positively and negatively supercoiled DNA loops. To investigate whether the supercoiled species produced by *EcoAI*_{R-D61A} translocation are stable, 20 nM relaxed pJP25 DNA was preincubated with 80 nM enzyme and 5 mM ATP to allow formation of these loops and *E. coli* DNA topoisomerase I was added at different time-points of this reaction, which was followed by incubation for ten minutes to fix changes in DNA topology. This experiment revealed that the degree of positive supercoiling in the final reaction product gradually decreased with time of addition of *E. coli* topoisomerase I, with no supercoiling observed after two minutes of preincubation (Figure 6(a)). This was found to correlate with the level of ATP remaining in the reaction mixture prior to the addition of the topoisomerase I (Figure 6(b)) such that the less ATP, the lower the degree of positive supercoiling. However, when *E. coli* topoisomerase I was added together with fresh ATP, formation of highly positively supercoiled DNA was observed even after a long period of preincubation (Figure 6(b)). These data indicate that the maintenance of the supercoiled loops generated by *EcoAI*_{R-D61A} requires continuous ATP hydrolysis.

DNA supercoiling by the wild-type *EcoAI* enzyme

The *EcoAI*_{R-D61A} mutant was used in our experiments in order to study the effects of DNA trans-

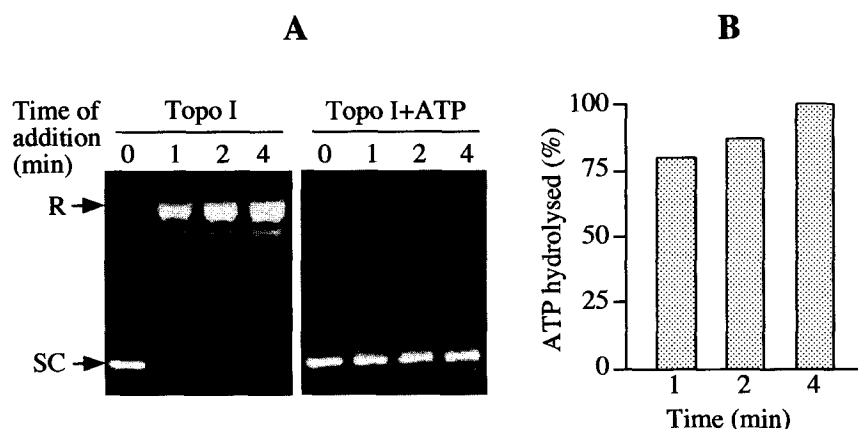


Figure 6. Continuous ATPase activity is required to maintain the supercoiled DNA loops generated by *EcoAI*_{R-D61A}. (a) Effect of time of addition of *E. coli* topoisomerase I into the reaction of *EcoAI*_{R-D61A} with relaxed pJP25 DNA and ATP on the resulting DNA supercoiling activity. DNA (20 nM) was incubated with 80 nM *EcoAI*_{R-D61A} and 5 mM ATP at 37°C. At the indicated times, reaction aliquots (20 µl) were removed and supplemented with either four units of *E. coli* topoisomerase I or four units of *E. coli* topoisomerase I and 5 mM ATP. This was followed by

further incubation at 37°C for ten minutes and subsequent agarose gel electrophoresis. (b) ATPase assay of *EcoAI*_{R-D61A}: 20 nM relaxed pJP25 DNA was incubated with 80 nM *EcoAI*_{R-D61A} and 5 mM ATP in buffer C at 37°C. At the indicated time-points, aliquots were removed and the amount of hydrolysed ATP was measured as described in Materials and Methods.

location in the absence of possible complications due to DNA cleavage. However, the wild-type enzyme also exhibits DNA supercoiling activity, as shown in Figure 7. For the wild-type enzyme, the DNA supercoiling assay was carried out with 40 nM relaxed pJP25 DNA and 40 nM enzyme. Again, the reactions were started by the addition of ATP and incubated at 37°C. Samples were removed at different time-points and analysed by agarose gel electrophoresis. DNA supercoiling by the *EcoAI*_{R-D61A} mutant was monitored as a control. We have found that in the absence of *E. coli* topoisomerase I, the wild-type enzyme rapidly cleaved the relaxed plasmid to its linear form (Figure 7). In the presence of *E. coli* topoisomerase I, however, the DNA cleavage reaction was accompanied by the generation of supercoiled species with a degree of supercoiling similar to that observed in the control reaction with the *EcoAI*_{R-D61A} mutant (Figure 7). It is noteworthy that the rate and the extent of DNA cleavage in this reaction were reduced compared to the reaction in the absence of *E. coli* topoisomerase I.

Positive supercoiling affects DNA cleavage by *EcoAI* endonuclease

It has been proposed that DNA translocation by type I restriction enzymes on circular substrates will stop after some time due to the build up of positive supercoils in the contracting DNA loop and this will trigger DNA cleavage reaction (Szczelkun *et al.*, 1996). According to this model, a positively supercoiled DNA substrate should be cleaved faster than its relaxed form. However, our previous experiments revealed that positively supercoiled plasmid pJP25 DNA, prepared by incubation of relaxed plasmid with the histone-like protein HMfB in the presence of wheatgerm DNA topoisomerase I, was cleaved by *EcoAI*

endonuclease at a slightly lower rate than relaxed DNA molecules, suggesting that the changes in DNA topology induced by translocation are not the trigger for DNA cleavage by type I restriction enzymes (Janscak *et al.*, 1999a).

To further investigate the effect of positive DNA supercoiling on DNA cleavage by *EcoAI* endonuclease, we have used the highly positively supercoiled pJP25 DNA produced in the *EcoAI*_{R-D61A}-mediated supercoiling reaction. The degree of positive DNA supercoiling of this DNA preparation was much higher than that produced by HMfB, as judged from relative mobilities of these species in agarose gel (not shown). We have found that the highly positively supercoiled pJP25 DNA was cleaved much more slowly than the relaxed form of the plasmid under conditions where *EcoAI* was in fourfold molar excess over DNA substrate (Figure 8). Thus, it is evident from our data that positive DNA supercoiling has rather an inhibitory effect on DNA cleavage by *EcoAI* endonuclease.

Discussion

Here we describe the DNA supercoiling activity of a DNA cleavage-deficient mutant of the *EcoAI* restriction enzyme, *EcoAI*_{R-D61A}, which still retains the ability to hydrolyse ATP and translocate DNA (Janscak *et al.*, 1999b). We demonstrate that highly positively supercoiled DNA is produced upon incubation of relaxed plasmid DNA with the *EcoAI*_{R-D61A} mutant and ATP in the presence of eubacterial DNA topoisomerase I, which selectively removes negative supercoils. DNA supercoiling activity has also been detected during the DNA cleavage reaction of the wild-type *EcoAI* enzyme, indicating that this activity is not only a characteristic of the mutant. Positive supercoils were not seen when *E. coli* topoisomerase I was replaced by eukaryotic topoisomerase I or when

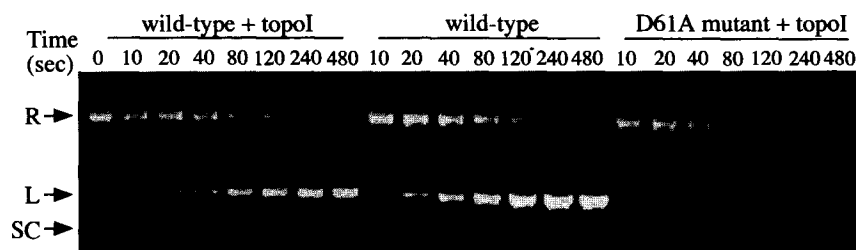


Figure 7. DNA supercoiling assay of the wild-type *EcoAI* endonuclease. The reaction (40 μ l) was carried out in buffer C at 37°C and contained 40 nM relaxed pJP25 DNA, 40 nM *EcoAI* endonuclease (MTase/R = 1:6), eight units of *E. coli* topoisomerase I and 5 mM ATP. In control reactions, either no topoisomerase I was added or *EcoAI* endonuclease was replaced

by the *EcoAI*_{R-D61A} mutant (MTase/R = 1:6). Aliquots (4 μ l) were removed at the time-points indicated and were analysed as described in Materials and Methods. Electrophoretic mobilities of relaxed (R), linear (L) and highly positively supercoiled (SC) plasmid DNA are marked on the left of the gel.

both topoisomerases were present in the reaction. This suggests that the supercoils generated by *EcoAI* are torsionally strained rather than constrained by protein binding. Our data are most consistent with a model in which the enzyme anchored at its recognition site tracks along the helical path of the DNA duplex and forces it to rotate around the helical axis to produce negative supercoils in the expanding DNA loop and positive supercoils in the contracting DNA loop as the translocation process proceeds. On a linear DNA substrate, where any increase in DNA twist ahead of the tracking enzyme can be relieved by rotation of free DNA ends, only negative supercoils would be introduced into the expanding DNA loop. A similar model was proposed for a chimera of the yeast GAL4 protein and T7 RNA polymerase (Ostrander *et al.*, 1990) or for the *E. coli* UvrAB helicase involved in nucleotide excision repair of damaged DNA (Koo *et al.*, 1991). Considering the fact that electron microscopic studies on DNA translocation by *EcoKI* and *EcoBI* endonucleases revealed the formation of twisted DNA species, it is highly likely that the DNA supercoiling activity observed with *EcoAI* is a general characteristic of all type I restriction enzymes.

Our data exclude the model proposed by Szczelkun *et al.* (1996) according to which a type I restriction enzyme introduces a nick prior to translocation so that a relatively small loop initially formed by enzyme binding to the specific and non-specific sites on DNA can accommodate the reduction in twist resulting from the translocation process. This model was based on the observation with some type I restriction enzymes, such as *EcoBI* or *EcoR124I*, that nicking of negatively supercoiled substrates occurs prior to double-strand cleavage by these enzymes (Adler & Nathans, 1973; Janscak *et al.*, 1996). However, no accumulation of nicked circular intermediate is seen in the early stages of the *EcoAI* cleavage reaction (Janscak *et al.*, 1999a). Therefore, it appears that the observed nicking activity reflects rather sequential cleavage of the two DNA strands at the site of cleavage, and that the breakage of the two strands by *EcoAI* occurs in a concerted fashion (Janscak *et al.*, 1999a). Recently, a nicking activity of some *EcoKI*

DEAD-box mutants (defective in ATP hydrolysis and DNA cleavage) has been reported (Davies *et al.*, 1998). This activity was also considered to be a prerequisite for the DNA translocation activity of the enzyme (Davies *et al.*, 1998). However, it is likely that the nicking by the DEAD-box mutants may simply reflect residual activity of the DNA cleavage domain (Janscak *et al.*, 1999b). Our experiments with the *EcoAI*_{R-D61A} mutant clearly demonstrate that the enzyme can translocate closed circular DNA without nicking it.

Type I restriction enzymes, in general, cleave closed circular DNA substrates at variable distances from the enzyme's DNA recognition site (Yuan, 1981; Szczelkun *et al.*, 1997; Janscak *et al.*, 1999a). Moreover, the *EcoR124II* endonuclease has been shown to cleave a closed circular DNA molecule linked by a Holliday junction by introducing two double-strand breaks, one on either side of the junction (Janscak *et al.*, 1999a). All these findings suggest that type I restriction enzymes can translocate long stretches of closed circular DNA, and that DNA cleavage occurs when bi-directional DNA translocation past the enzyme is prevented following translocation of the entire DNA circle (Janscak *et al.*, 1999a). In the case of the helix-tracking mechanism, which may be expected to generate one superhelical turn per 10-11 bp of translocated DNA, the enzyme could not translocate long stretches of closed circular DNA without being capable of relieving the topological constraints resulting from this process. Some evidence that the type I restriction enzymes have such an ability may come from the electron microscopic studies on DNA translocation by *EcoKI* and *EcoBI*, which revealed a formation of relaxed loops on both linear and circular substrates as well as highly twisted species (Rosamond *et al.*, 1979; Yuan *et al.*, 1980; Endlich & Linn, 1985). One mechanism that would allow the enzyme to translocate long stretches of closed circular DNA may be that the HsdR-methylase complex acts as a swivel to relieve superhelical tension produced by the helix-tracking process. It has also been proposed that type I restriction enzyme may possess an intrinsic topoisomerase activity (Yuan *et al.*, 1980). However, no such activity is seen with our *EcoAI*_{R-D61A} mutant.

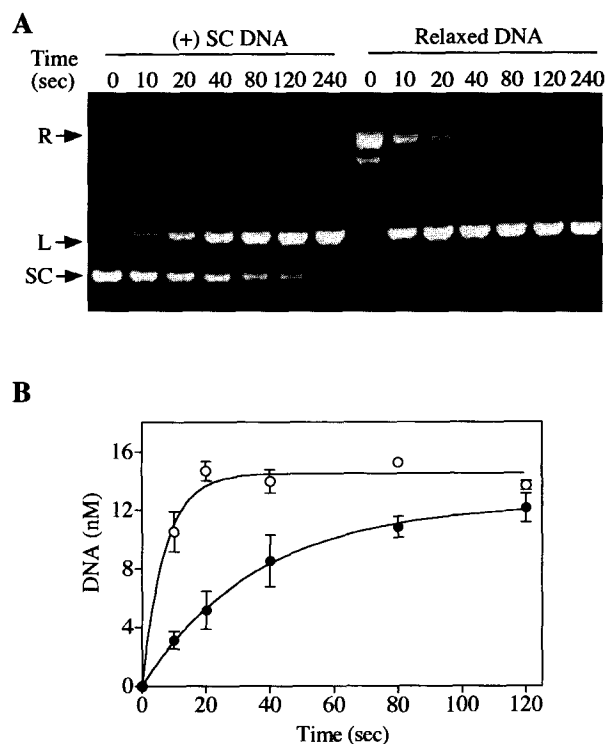


Figure 8. Kinetics of DNA cleavage by *EcoAI* endonuclease on relaxed and highly positively supercoiled substrates. (a) Typical *EcoAI* restriction assay. Highly positively supercoiled pJP25 DNA was generated in *EcoAI*_{R-D61A}-mediated supercoiling reaction. Relaxed pJP25 DNA substrate was prepared using *E. coli* topoisomerase I. Cleavage reactions were carried out in buffer C at 37°C and contained 20 nM DNA, 80 nM *EcoAI* and 5 mM ATP. Aliquots (4 µl) were removed at the indicated time-points and analysed by agarose gel electrophoresis as described in Materials and Methods. Electrophoretic mobilities of relaxed (R), linear (L) and highly positively supercoiled (SC) plasmid DNA are marked on the left of the gel. (b) Plot of the concentration of linear DNA product against time for cleavage of relaxed (○) and positively supercoiled (●) substrates. Concentration of reaction product was determined by gel scanning as described in Materials and Methods. The average values from three independent measurements are plotted in the graph.

The assumption that a tracking type I restriction enzyme has the ability to relieve the ensuing superhelical tension may also explain how the initially small DNA loop formed by the enzyme binding to DNA can accommodate the reduction in DNA twist produced by the translocation process.

Continuous ATP hydrolysis is necessary to maintain the twin-supercoiled-domain structure generated by *EcoAI*_{R-D61A} translocation (Figure 6). To explain this observation, one can imagine a scenario in which the enzyme tracking along the DNA helix involves cycles of accumulation and relief of supercoils in the expanding and contracting DNA domains. When the enzyme has translo-

cated the entire DNA circle it stalls and the loop structure subsequently dissociates, but the enzyme can restart DNA translocation from the recognition site providing that ATP is available. Once all ATP has been hydrolysed, DNA translocation stops and the superhelical tension is relieved. A similar cycling model was proposed for the post-nuclease action of the *EcoBI* endonuclease (Endlich & Linn, 1985).

Recent crystal structures of different complexes of PcrA helicase with a DNA substrate and analogues of ATP or the products of its hydrolysis have brought progress in understanding of the mechanism of DNA translocation by helicases (Velankar *et al.*, 1999). These structures suggested an inchworm mechanism in which the enzyme tracks along one strand of the melted DNA duplex *via* a series of conformational changes in the protein induced by ATP binding and hydrolysis. The DNA translocation by PcrA is coupled to duplex binding and destabilization by different domains of the same polypeptide (Velankar *et al.*, 1999). It has been proposed that a similar model could apply for the ATP-dependent DNA translocation mechanism of other DEAD box proteins, including type I restriction enzymes (Velankar *et al.*, 1999). The crystal structures of PcrA and Rep helicases revealed that the DEAD box motifs are clustered around a cleft between two domains, called 1A and 2A. These domains are responsible for coupling ATP hydrolysis to DNA translocation and may represent the basic structural unit of all helicases (Bird *et al.*, 1998). Data from limited proteolysis of the *EcoKI* HsdR subunit combined with secondary structure prediction studies suggested that HsdR subunits contain domains equivalent to the domains 1A and 2A (Davies *et al.*, 1999b).

It has been proposed that the helix-tracking activity of the UvrAB helicase and SV40 large T antigen defined by the same supercoiling assay as that used for *EcoAI* may be related to the helicase activity of these enzymes (Koo *et al.*, 1991; Yang *et al.*, 1989). Although it has been shown that the helicase signature motifs in the HsdR subunit of *EcoKI* are required for restriction, ATPase and DNA translocation activities of the enzyme (Webb *et al.*, 1996; Davies *et al.*, 1998, 1999a), our preliminary attempts to demonstrate helicase activity for any type I restriction enzyme by the conventional strand displacement assay failed (our unpublished data). The SV40 T antigen has been shown to catalyse an extensive unwinding of closed circular duplex DNA, as revealed by introduction of negative supercoils in the presence of eukaryotic DNA topoisomerase I and *E. coli* single-strand DNA binding protein (Wold *et al.*, 1987). However, no DNA duplex unwinding activity was detected when the *EcoAI*_{R-D61A} mutant was subjected to the same unwinding assay (our unpublished data). Nevertheless, it may still be possible that the HsdR subunit can enter the duplex DNA only following the interaction of the methylase core with the recognition site and a series of conformational

changes in the enzyme induced by ATP binding (Bickle *et al.*, 1978; Powell *et al.*, 1998); the enzyme would then track along the DNA helix by a mechanism that involves the transient unwinding and rewinding of double-stranded DNA as proposed for Ruv(A)B helicase (Parsons *et al.*, 1995; Adams & West, 1995). Such transient unwinding could not be detected by the topological assay using eukaryotic topoisomerase I and SSB. For Ruv(A)B, unwinding of circular duplex DNA by the enzyme could be observed only when the transient intermediate was stabilised by the simultaneous presence of ATP and ATP γ S (Adams & West, 1995). Furthermore, many Ruv(A)B complexes are bound to one DNA molecule, which increases the degree of DNA unwinding in the presence of ATP γ S (Mitchell & West, 1994; Adams & West, 1995). In this view it would be interesting to perform the topological assay of putative EcoAI unwinding activity using a DNA substrate containing multiple EcoAI sites.

Here, we show that the highly positively supercoiled pJP25 DNA generated by the EcoAI_{R-D61A} mutant was cleaved by the wild-type EcoAI endonuclease much more slowly than relaxed DNA (Figure 8). This is not in agreement with the model proposed by Szczelkun *et al.* (1996), in which the build-up of positive supercoils on circular DNA molecules induced by enzyme translocation is considered to be the trigger for DNA cleavage, by causing either halt or pause in the translocation process. According to this model, positively supercoiled DNA would be expected to be cleaved faster than relaxed DNA. Previously, we have shown that positively supercoiled DNA molecules, which had a lower degree of supercoiling than the species generated by EcoAI_{R-D61A}, were cleaved by EcoAI slightly more slowly than relaxed DNA (Janscak *et al.*, 1999a). Thus it appears that the higher the degree of positive supercoiling, the lower the rate of DNA cleavage by EcoAI. Previously it has been demonstrated that DNA cleavage activity of EcoR124I endonuclease on a circular substrate decreased with increased negative supercoiling in the DNA substrate (Janscak *et al.*, 1996). Similarly, the EcoAI endonuclease cleaves native negatively supercoiled DNA at a significantly slower rate than relaxed DNA (Janscak *et al.*, 1999a). Taken together, all these observations show that both negative and positive supercoils affect DNA cleavage by type I restriction enzymes, perhaps by presenting difficulties for the DNA translocation process (Janscak *et al.*, 1999a). The inhibitory effect of DNA supercoiling could also provide an explanation for our observation that the rate of cleavage of relaxed pJP25 DNA by EcoAI in the presence of *E. coli* topoisomerase I was lower than in its absence (Figure 8). Because of the continuous removal of negative supercoils by *E. coli* topoisomerase I, the tracking enzyme could not relieve topological constraints and accumulating positive supercoils reduced the DNA cleavage rate in the

same manner as observed with the highly positively supercoiled DNA substrate.

Materials and Methods

Proteins and DNA

The EcoAI endonuclease was reconstituted *in vitro* by mixing the purified EcoAI methylase and the HsdR subunit produced by the plasmids pJP21 and pJP22, respectively (Janscak & Bickle, 1998). For EcoAI_{R-D61A} mutant reconstitution, the wild-type HsdR subunit was replaced by HsdR_{D61A} produced from the plasmid pJP41D61A (Janscak *et al.*, 1999b). Typically, the methylase and HsdR (HsdR_{D61A}) were mixed in a molar ratio of 1:12 directly in reaction mixtures. The large excess of HsdR_{D61A} subunit over the methylase was used to ensure the assembly of the weak R₂M₂S₁ complex of EcoAI (Suri *et al.*, 1984; Janscak & Bickle, 1998). The concentration of the reconstituted enzyme is expressed as the input molar concentration of the methylase. *E. coli* DNA topoisomerase I was produced in *E. coli* DH5 α (Woodcock *et al.*, 1989) from the plasmid pJW312-SalI (a generous gift from Professor James C. Wang of Harvard University) and purified as described (Lynn & Wang, 1989). Wheatgerm DNA topoisomerase I was purchased from Promega. For both topoisomerases, one enzyme unit is defined as the amount of the enzyme required to convert 1 μ g of supercoiled pJP25 DNA to relaxed form in 30 minutes at 37°C in 20 μ l of buffer C (see below). One unit (ca 100 ng) of *E. coli* topoisomerase I preparation was sufficient to relax 0.38 μ g of pJP25 DNA in 20 μ l of buffer C (10 nM DNA) in one minute at 37°C.

The following derivatives of the plasmid pTZ18R (Pharmacia) were used as DNA substrates: the plasmid pJP25 (2870 bp) containing a single EcoAI site (Janscak & Bickle, 1998) and the plasmid pDRM.1R (2891 bp), which has no EcoAI site (Janscak *et al.*, 1996). Plasmid DNAs were isolated from *E. coli* HB101 (Sambrook *et al.*, 1989) using the Qiagen Maxiprep kit (Qiagen). Relaxed plasmid DNA substrates were prepared by treatment of native negatively supercoiled DNA molecules with *E. coli* DNA topoisomerase I, which was followed by DNA purification on a QIAquick column (Qiagen).

DNA supercoiling assay

Reactions were carried out at 37°C in buffer C (50 mM Tris (pH 8), 25 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.2 mM AdoMet). Relaxed plasmid DNA, EcoAI_{R-D61A} mutant (wild-type EcoAI) and topoisomerases were added in the concentrations given in each Figure legend. Reactions were started by the addition of 5 mM ATP and incubated for ten minutes unless otherwise stated. Reactions were terminated by adding 0.5 vol. of the stop solution (150 mM EDTA, 3% (w/v) SDS, 60 μ g/ml proteinase K) followed by incubation at 37°C for 15 minutes. Agarose gel electrophoresis was used to visualise the reaction products (see below).

DNA cleavage and ATPase assays

DNA cleavage reactions with the wild-type EcoAI endonuclease were performed at 37°C in buffer C. For the measurement of the rate of cleavage of highly positively supercoiled pJP25 DNA by EcoAI, the product of the EcoAI_{R-D61A}-mediated supercoiling reaction (see above) was purified on a QIAquick column (Qiagen).

The relaxed DNA substrate, used in control reactions, was treated under the same conditions including the incubation with the EcoAI_{R-D61A} mutant, but in the absence of *E. coli* DNA topoisomerase I. Cleavage reactions contained 20 nM DNA substrate and 80 nM EcoAI endonuclease and were started by the addition of ATP to a concentration of 5 mM. During the reaction, aliquots were removed and mixed with 0.5 vol. of the stop solution. Reaction products were analysed by agarose gel electrophoresis (see below). The concentration of the EcoAI DNA cleavage product, full-size linear pJP25 plasmid, was determined by densitometric scanning of ethidium bromide-stained agarose gels calibrated with a series of known concentration of the same plasmid DNA linearized by HindIII. NIH Image 1.62 software was used for quantitative analysis.

For ATPase assay, reactions with EcoAI_{R-D61A} (see above) were terminated by adding one volume of 0.1 mM EDTA (pH 8) and concentration of inorganic phosphate released by ATP hydrolysis was estimated as described (Janscak *et al.*, 1996).

Gel electrophoresis

DNA samples were analysed by electrophoresis through 1% (w/v) agarose gels in 45 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA at 5 V/cm for three hours. DNA was visualised by ethidium bromide staining. Where it was required, gels were run in the presence of 3.8 µM chloroquine diphosphate (Sigma).

Acknowledgements

We thank Professor James C. Wang (Harvard University, Cambridge, MA) for the generous gift of the plasmid pJW312-SalI and Professor V. Nagaraja (Indian Institute of Science, Bangalore) for helpful discussions. We thank Daniel Panne for comments on the manuscript and Ursula Sandmeier for technical assistance. This work was supported by grants from the Swiss National Science Foundation.

References

- Adams, D. E. & West, S. C. (1995). Unwinding of closed circular DNA by the *Escherichia coli* RuvA and RuvB recombination/repair proteins. *J. Mol. Biol.* **247**, 404-417.
- Adler, S. P. & Nathans, D. (1973). Studies of SV40 DNA. V. Conversion of circular to linear SV40 DNA by restriction endonuclease from *Escherichia coli* B. *Biochim. Biophys. Acta*, **199**, 177-188.
- Bickle, T. A. (1993). The ATP-dependent restriction enzymes. In *Nucleases* (Linn, S. M., Lloyd, R. S. & Roberts, R. J., eds), pp. 89-109, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Bickle, T. A., Brack, C. & Yuan, R. (1978). ATP-induced conformational changes in the restriction endonuclease from *Escherichia coli* K 12. *Proc. Natl Acad. Sci. USA*, **75**, 3099-3103.
- Bird, L. E., Subramanya, H. S. & Wigley, D. B. (1998). Helicases: a unifying structural theme? *Curr. Opin. Struct. Biol.* **8**, 14-18.
- Burckhardt, J., Weisemann, J., Hamilton, D. L. & Yuan, R. (1981a). Complexes formed between the restriction endonuclease EcoK and heteroduplex DNA. *J. Mol. Biol.* **153**, 425-440.
- Burckhardt, J., Weisemann, J. & Yuan, R. (1981b). Characterization of the DNA methylase activity of the restriction enzyme from *Escherichia coli* K. *J. Biol. Chem.* **256**, 4024-4032.
- Davies, G. P., Powell, L. M., Webb, J. L., Cooper, L. P. & Murray, N. E. (1998). EcoKI with an amino acid substitution in any one of seven DEAD-box motifs has impaired ATPase and endonuclease activities. *Nucl. Acids Res.* **26**, 4828-4836.
- Davies, G. P., Kemp, P., Molineux I. J. & Murray, N. E. (1999a). The DNA translocation and ATPase activities of restriction-deficient mutants of EcoKI. *J. Mol. Biol.* **292**, 787-796.
- Davies, G. P., Martin, I., Sturrock, S. S., Cronshaw, A., Murray, N. E. & Dryden, D. T. (1999b). On the structure and operation of type I DNA restriction enzymes. *J. Mol. Biol.* **290**, 565-579.
- Dreier, J. & Bickle, T. A. (1996). ATPase activity of the type IC restriction-modification system EcoR124II. *J. Mol. Biol.* **257**, 960-969.
- Dreier, J., MacWilliams, M. P. & Bickle, T. A. (1996). DNA cleavage by the type IC restriction-modification enzyme EcoR124II. *J. Mol. Biol.* **264**, 722-733.
- Dryden, D. T. F., Cooper, L. P. & Murray, N. E. (1993). Purification and characterization of the methyltransferase from the type I restriction and modification system of *Escherichia coli* K12. *J. Biol. Chem.* **268**, 13228-13236.
- Dryden, D. T. F., Cooper, L. P., Thorpe, P. H. & Byron, O. (1997). The *in vitro* assembly of the EcoKI type I DNA restriction/modification enzyme and its *in vivo* implications. *Biochemistry*, **36**, 1065-1076.
- Endlich, B. & Linn, S. (1985). The DNA restriction endonuclease of *Escherichia coli* B. I. Studies of the DNA translocation and the ATPase activities. *J. Biol. Chem.* **260**, 5720-5728.
- Eskin, B. & Linn, S. (1972). The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. III. Studies of the restriction adenosine triphosphatase. *J. Biol. Chem.* **247**, 6192-6196.
- Fuller-Pace, F. V., Bullas, L. R., Delius, H. & Murray, N. E. (1984). Genetic recombination can generate altered restriction specificity. *Proc. Natl Acad. Sci. USA*, **81**, 6095-6099.
- Gorbalenya, A. E. & Koonin, E. V. (1991). Endonuclease (R) subunits of type-I and type-III restriction-modification enzymes contain a helicase-like domain. *FEBS Letters*, **291**, 277-281.
- Janscak, P. & Bickle, T. A. (1998). The DNA recognition subunit of the type IB restriction-modification enzyme EcoAI tolerates circular permutations of its polypeptide chain. *J. Mol. Biol.* **284**, 937-948.
- Janscak, P., Abadjieva, A. & Firman, K. (1996). The type I restriction endonuclease R. EcoR124I: over-production and biochemical properties. *J. Mol. Biol.* **257**, 977-991.
- Janscak, P., Dryden, D. T. F. & Firman, K. (1998). Analysis of the subunit assembly of the type IC restriction-modification enzyme EcoR124I. *Nucl. Acids Res.* **26**, 4439-4445.
- Janscak, P., MacWilliams, M. P., Sandmeier, U., Nagaraja, V. & Bickle, T. A. (1999a). DNA translocation blockage, a general mechanism of cleavage site selection by type I restriction enzymes. *EMBO J.* **18**, 2638-2647.
- Janscak, P., Sandmeier, U. & Bickle, T. A. (1999b). Single amino acid substitutions in the HsdR subunit of the

- type IB restriction enzyme EcoAI uncouple the DNA translocation and DNA cleavage activities of the enzyme. *Nucl. Acids Res.* **27**, 2638-2643.
- Koo, H. S., Claassen, L., Grossman, L. & Liu, L. F. (1991). ATP-dependent partitioning of the DNA template into supercoiled domains by *Escherichia coli* UvrAB. *Proc. Natl Acad. Sci. USA*, **88**, 1212-1216.
- Lynn, R. M. & Wang, J. C. (1989). Peptide sequencing and site-directed mutagenesis identify tyrosine-319 as the active site tyrosine of *Escherichia coli* DNA topoisomerase I. *Proteins: Struct. Funct. Genet.* **6**, 231-239.
- Mitchell, A. H. & West, S. C. (1994). Hexameric rings of *Escherichia coli* RuvB protein. Cooperative assembly, processivity and ATPase activity. *J. Mol. Biol.* **243**, 208-215.
- Murray, N. E., Batten, P. L. & Murray, K. (1973). Restriction of bacteriophage lambda by *Escherichia coli* K. *J. Mol. Biol.* **81**, 395-407.
- Murray, N. E., Gough, J. A., Suri, B. & Bickle, T. A. (1982). Structural homologies among type I restriction-modification systems. *EMBO J.* **1**, 535-539.
- Murray, N. E., Daniel, A. S., Cowan, G. M. & Sharp, P. M. (1993). Conservation of motifs within the unusually variable polypeptide sequences of type I restriction and modification enzymes. *Mol. Microbiol.* **9**, 133-143.
- Ostrander, E. A., Benedetti, P. & Wang, J. C. (1990). Template supercoiling by a chimera of yeast GAL4 protein and phage T7 RNA polymerase. *Science*, **249**, 1261-1265.
- Parsons, C. A., Stasiak, A. & West, S. C. (1995). The *E. coli* RuvAB proteins branch migrate Holliday junctions through heterologous DNA sequences in a reaction facilitated by SSB. *EMBO J.* **14**, 5736-5744.
- Powell, L. M., Dryden, D. T. F. & Murray, N. E. (1998). Sequence-specific DNA binding by EcoKI, a type IA DNA restriction enzyme. *J. Mol. Biol.* **283**, 963-976.
- Price, C., Pripfl, T. & Bickle, T. A. (1987). EcoR124 and EcoR124/3: the first members of a new family of type I restriction and modification systems. *Eur. J. Biochem.* **167**, 111-115.
- Rosamond, J., Endlich, B. & Linn, S. (1979). Electron microscopic studies of the mechanism of action of the restriction enzyme of *Escherichia coli* B. *J. Mol. Biol.* **129**, 619-635.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shure, M., Pulleyblank, D. E. & Vinograd, J. (1977). The problems of eukaryotic and prokaryotic DNA packaging and in vivo conformation posed by superhelix density heterogeneity. *Nucl. Acids Res.* **4**, 1183-1205.
- Studier, F. W. & Bandyopadhyay, P. K. (1988). Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl Acad. Sci. USA*, **85**, 4677-4681.
- Suri, B., Shepherd, J. C. W. & Bickle, T. A. (1984). The EcoA restriction and modification system of *Escherichia coli* 15T-: enzyme structure and DNA recognition sequence. *EMBO J.* **3**, 575-579.
- Szczelkun, M. D., Dillingham, M. S., Janscak, P., Firman, K. & Halford, S. E. (1996). Repercussions of DNA tracking by the type IC restriction endonuclease EcoR124I on linear, circular and catenated substrates. *EMBO J.* **15**, 6335-6347.
- Szczelkun, M. D., Janscak, P., Firman, K. & Halford, S. E. (1997). Selection of non-specific DNA cleavage sites by the type IC restriction endonuclease EcoR124I. *J. Mol. Biol.* **271**, 112-123.
- Taylor, A. F. & Smith, G. R. (1990). Action of RecBCD enzyme on cruciform DNA. *J. Mol. Biol.* **211**, 117-134.
- Taylor, I., Patel, J., Firman, K. & Kneale, G. (1992). Purification and biochemical characterisation of the EcoR124 type I modification methylase. *Nucl. Acids Res.* **20**, 179-186.
- Titheradge, A. J. B., Ternent, D. & Murray, N. E. (1996). A third family of allelic hsd genes in *Salmonella enterica*: sequence comparisons with related proteins identify conserved regions implicated in restriction of DNA. *Mol. Microbiol.* **22**, 437-447.
- Tsao, Y. P., Wu, H. Y. & Liu, L. F. (1989). Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. *Cell*, **56**, 111-118.
- Velankar, S. S., Soutanas, P., Dillingham, M. S., Subramanya, H. S. & Wigley, D. B. (1999). Crystal structures of complexes of PcrA DNA helicase with a DNA substrate indicate an inchworm mechanism. *Cell*, **97**, 75-84.
- Webb, J. L., King, G., Ternent, D., Titheradge, A. J. & Murray, N. E. (1996). Restriction by EcoKI is enhanced by co-operative interactions between target sequences and is dependent on DEAD box motifs. *EMBO J.* **15**, 2003-2009.
- Willcock, D. F., Dryden, D. T. F. & Murray, N. E. (1994). A mutational analysis of the two motifs common to adenine methyltransferases. *EMBO J.* **13**, 3902-3908.
- Wold, M. S., Li, J. J. & Kelly, T. J. (1987). Initiation of simian virus 40 DNA replication *in vitro*: large-tumor- antigen- and origin-dependent unwinding of the template. *Proc. Natl Acad. Sci. USA*, **84**, 3643-3647.
- Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S. S., Michael, M. Z. & Graham, M. W. (1989). Quantitative evaluation of *Escherichia coli* strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucl. Acids Res.* **17**, 3469-3478.
- Yang, L., Jessee, C. B., Lau, K., Zhang, H. & Liu, L. F. (1989). Template supercoiling during ATP-dependent DNA helix tracking: studies with simian virus 40 large tumor antigen. *Proc. Natl Acad. Sci. USA*, **86**, 6121-6125.
- Yuan, R. (1981). Structure and mechanism of multifunctional restriction endonucleases. *Annu. Rev. Biochem.* **50**, 285-315.
- Yuan, R., Hamilton, D. L. & Burckhardt, J. (1980). DNA translocation by the restriction enzyme from *E. coli* K. *Cell*, **20**, 237-244.

Edited by J. Karn

(Received 1 October 1999; received in revised form 26 November 1999; accepted 29 November 1999)

