J. Mol. Biol. (1996) **264**, 722–733

JMB

DNA Cleavage by the Type IC Restriction-Modification Enzyme *Eco*R124II

Jürg Dreier, Maria P. MacWilliams and Thomas A. Bickle*

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

Type I restriction-modification systems bind to non-palindromic, bipartite recognition sequences. Although these enzymes methylate specific adenine residues within their recognition sequences, they cut DNA at sites up to several thousand base-pairs away. We have investigated the mechanism of how *Eco*R124II, a type IC restriction-modification system, selects the cleavage site. Restriction studies with different DNA constructs revealed that circular DNA requires only one non-methylated recognition sequence to be cut, whereas linear DNA needs at least two such sites. Cleavage of linear DNA is independent of site orientation. Further investigations of the linear substrates revealed a mechanism whereby the double-strand break is introduced between two recognition sequences. We propose a model for the selection of restriction sites by type I enzymes where two *Eco*R124II complexes bind to two recognition sequences. Lack of methylation at a site stimulates the enzyme to translocate DNA on both sides of the recognition sequence. Thus the two complexes approach each other and, at the point where they meet, they interact to introduce a double-strand break in the DNA.

© 1996 Academic Press Limited

Keywords: DNA restriction and modification; DNA translocation; protein-DNA interactions; type IC restriction enzymes; type I restriction model

*Corresponding author

Introduction

Type I restriction-modification (R-M) systems are multifunctional complexes composed of three different kinds of subunits: HsdS (S stands for specificity and Hsd for host specificity for DNA), HsdM (methylation) and HsdR (restriction). HsdM and HsdS are sufficient to form a functional DNA methyltransferase and together with HsdR an endonuclease can be assembled. Type I R-M complexes recognise bipartite non-palindromic sequences on DNA. Based on genetic complementation, immunological crossreactivity and DNA hybridisation, type I enzymes can be grouped into three different families named IA, IB and IC (Bickle, 1993; Bickle & Krüger, 1993; Murray *et al.*, 1982; Price *et al.*, 1987a; Wilson & Murray, 1991).

We are working with *Eco*R124II, a type IC R-M system. The genes for *Eco*R124II were originally found on a large conjugative plasmid belonging to the IncFIV incompatibility group (Firman *et al.*, 1983; Hedges & Datta, 1972). This system recognises the sequence 5'-GAA*(N_7)RT*CG-3' where N represents any nucleotide and R represents either purine (Price *et al.*, 1987b). The adenine residue marked with an asterisk is the target for methylation as is the adenine complementary to the marked thymine residue (Taylor *et al.*, 1993). When methylated in both strands the DNA containing the site is not a substrate for the R-M complex. If only one of the adenine residues is methylated (e.g. hemimethylated as is found after DNA replication) the complementary strand will be methylated by *Eco*R124II. When neither adenine residue is methylated the DNA is cut. The cofactors required for these reactions are *S*-adenosylmethionine (AdoMet) for methylation, and Mg^{2+}, AdoMet and ATP for restriction (reviewed by Bickle, 1993; Bickle & Krüger, 1993; Wilson & Murray, 1991). *In vitro* in the absence of ATP, non-methylated DNA is methylated rather than cleaved.

A peculiarity of type I R-M systems is that they cut DNA in a non-specific manner up to several thousand base-pairs away from their recognition sequence. Based on the work with the type IA systems *Eco*BI and *Eco*KI, several models have been

Abbreviations used: R-M, restriction-modification; AdoMet, *S*-adenosylmethionine; LacR, lactose repressor; BSA, bovine serum albumin; EtBr, ethidium bromide.

0022–2836/96/490722–12 $25.00/0 © 1996 Academic Press Limited

proposed for the selection of the cleavage sites by these enzymes. For both *Eco*BI and *Eco*KI, relaxed loop and supercoiled loop DNA intermediates were seen in electron micrographs (Endlich & Linn, 1985; Rosamond *et al*., 1979; Studier & Bandyopadhyay, 1988; Yuan *et al*., 1980). These observations led to the suggestion that DNA is translocated by the enzyme complex. A DNA-dependent ATP hydrolysis measured *in vitro* was proposed to drive such a DNA translocation. For *Eco*BI the relaxed loops were suggested to be derived from the supercoiled ones (Endlich & Linn, 1985). In the case of *Eco*KI the conclusion was that the R-M complex, bound to its unmethylated target sequence, makes a second non-specific contact with the DNA in a diffusion-controlled manner and thus forms relaxed loops. The enzyme then starts to translocate the DNA, resulting in supercoiled, looped DNA molecules (Yuan *et al*., 1980).

Both *Eco*KI and *Eco*BI appear to remain bound to their recognition sequences throughout the restriction reaction, which accords well with the presence of looped intermediates (Rosamond *et al*., 1979; Yuan *et al*., 1980).

*Eco*KI was shown to be able to cut DNA on either side of the recognition sequence, whereas *Eco*BI was found to cut only to the 5' side of its asymmetric target site (Rosamond *et al*., 1979; Yuan *et al*., 1980). In order to cut linear DNA with only one recognition sequence, both *Eco*KI and *Eco*BI require at least ten times more enzyme than is required to cut circular DNA (Murray *et al*., 1973; Rosamond *et al*., 1979; Yuan *et al*., 1980). Studier & Bandyopadhyay (1988) proposed a model for selection of the cleavage site which requires the binding of two enzyme complexes to two separate recognition sequences. Upon binding to the unmethylated recognition sites, each enzyme molecule starts to translocate DNA from both sides. As soon as two neighbouring complexes meet they cut the DNA.

Here we demonstrate that *Eco*R124II cuts circular plasmid DNA which contains at least one recognition site; however, under the same conditions, linear DNA with one such site is not a substrate for restriction. Two or more recognition sequences are required for linear DNA cleavage. In addition, we have several lines of evidence that demonstrate that cleavage of linear DNA with two recognition sequences occurs between the sites. Our results suggest a translocation of DNA until two neighbouring complexes meet and cut DNA. Thus we believe that the model proposed for *Eco*KI by Studier & Bandyopadhyay (1988) is also applicable to *Eco*R124II.

Results

Restriction of DNA by *Eco*R124II: substrate requirements

The cloning vector pBR322 contains one recognition sequence for *Eco*R124II (*Eco*R124II site) at position 1376 (Watson, 1988). When the kinetics of cleavage of circular pBR322 with *Eco*R124II are followed, nicked circular DNA is first formed followed by unit length linear DNA as the final product (Dreier & Bickle, 1996). This reflects a single, double-strand break made per recognition site, presumably in a two-step mechanism. In our experiments Mg^{2+} and ATP are required for restriction whereas AdoMet can be omitted. Apparently AdoMet copurifies with the enzyme since an additional ion-exchange column (MonoQ, Pharmacia) renders the enzyme preparation AdoMet-dependent (data not shown). The phenomenon of copurification of the enzyme with AdoMet has also been reported for *Eco*DXXI, another type IC R-M system (Piekarowicz *et al*., 1985).

If pBR322 DNA is linearised prior to *Eco*R124II treatment, no restriction can be detected under conditions that allow restriction of the circular form (Figure 1; see also Dreier & Bickle, 1996). Figure 1 shows that linear DNA is cut so long as there are at least two *Eco*R124II sites. It is typical for type I DNA restriction that discrete bands are not observed after gel electrophoresis. This reflects the non-specific selection of cutting sites. Smears are also seen when circular DNA containing more than one *Eco*R124II site is cut by *Eco*R124II (data not shown).

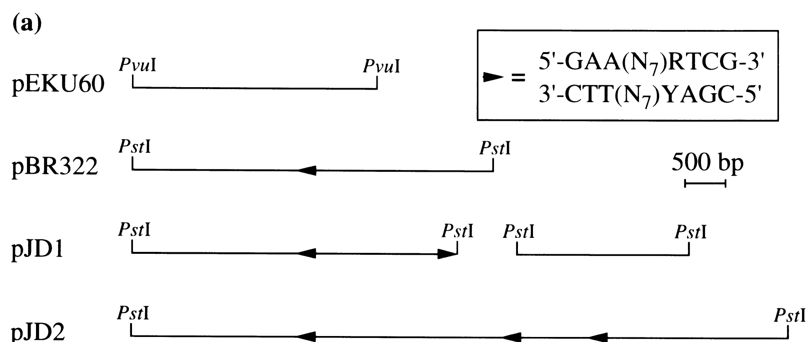
It has been reported that certain restriction enzymes like *Eco*RII can be stimulated by cleavable DNA to cut otherwise non-cleavable substrates which contain a single *Eco*RII site (Reuter *et al*., 1993). We were unable to stimulate cleavage of linear DNA containing only one *Eco*R124II site by addition of double-stranded oligonucleotides containing an *Eco*R124II site (data not shown). On the other hand, ATP hydrolysis is stimulated with these oligonucleotides, which demonstrates that the enzyme interacts with them (Dreier & Bickle, 1996). Thus, *Eco*R124II needs two recognition sites in *cis* to cut linear DNA. Another requirement for restriction both of linear and circular DNA is that the recognition site must not be methylated since methylation prevents restriction *in vivo* (Firman *et al*., 1985; Glover *et al*., 1983) and *in vitro* (data not shown).

To test whether the relative orientation of the recognition sites influences restriction, we constructed the DNA substrates shown in Figure 2. Both substrates have two *Eco*R124II sites very close to the ends. In one case, pJD1s16 linearised with *Sty*I, the sites are both pointing toward the inside (→←) and in the other, pJD16 cut with *Hin*dIII, they are in the inverse orientation (←→; Figure 2(a)) If *Eco*R124II can only cut to one side of its recognition sequence we would expect one of the two constructs to be non-cleavable. As shown in Figure 2(b), both substrates were cut equally well. Thus *Eco*R124II is able to cut DNA on either side of the recognition sequence.

pJD1 served as another example of site orientation independence. The two *Eco*R124II sites are oriented as on the *Sty*I-cut pJD1s16 when pJD1 is linearised with *Sal*I (→←). If pJD1 is cut with *Eco*RI, the recognition sequences are oriented as on the *Hin*dIII-cut pJD16. The recognition sequences on the linear pJD1 species are at least 726 bp away from the DNA ends. Linear DNA with sites oriented as direct repeats (→→) is also cleavable (Figure 1, pJD2 and see Figure 5(b), lane 4). These results indicate that neither the orientation of the *Eco*R124II sites nor their close proximity to the DNA ends interfere with cleavage.

Restriction of linear DNA by *Eco*R124II: influence of the distance between two recognition sequences

To determine the minimum distance between two recognition sequences required for *Eco*R124II cleavage, we decreased the original distance of 1479 bp between the two *Eco*R124II sites on pJD1 by exonuclease III deletions (Figure 3). The plasmids generated contained non-specific spacer DNA between two recognition sequences ranging from 16 bp to 135 bp. Additional plasmids completing the set of differently spaced recognition sites were constructed by inserting an 18 bp double-stranded oligonucleotide (DXXI, see Materials and Methods) into pJD1s16. In these constructs the recognition sequences are oriented head to head (→←): 5'-GAA(N_7)RTCG-spacer-CGAY(N_7)TTC-3' (Y represents either pyrimidine). The length of the spacer is indicated by adding x to the plasmid name, where x is the number of base-pairs between the recognition sequences. In the circular form, all of these plasmids are cut. The circular plasmids with spacer lengths of less than 40 bp yield full-length linear molecules whereas the others are multiply cut and yield smears on gels (data not shown). On linear substrates, we do not observe restriction when the two sites are very close together. These substrates behave as though they contained a single site and we postulate that binding of the enzyme to one site sterically hinders binding to the other. With spacer lengths between 39 and 135 bp, discrete bands appear as the products of restriction



(b)

DNA	SM	pEKU60		pBR322		pJD1		pJD2	
number of recognition sites		0		1		2		3	
*Eco*R124II		-	+	-	+	-	+	-	+

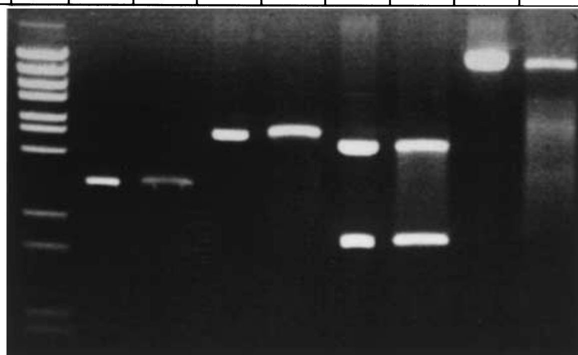


Figure 1. Restriction of linear DNA. Plasmid DNA was linearisd with *Pst*I (except pEKU60 where *Pvu*I was used) and purified as described in Materials and Methods. DNA (8.75×10^{-14} mol) was incubated with 1.3×10^{-12} mol *Eco*R124II in a 10 μl reaction volume. The digestions were run for one hour at 37°C. (a) Schematic drawing of the substrates with arrows indicating the recognition sequences (the positions of the 5'-G residues are in the appropriate position but for illustrative purposes the arrows are oversized). (b) Lanes marked *Eco*R124II – show reactions without enzyme and those marked + show reactions with enzyme. SM is the size marker, a BstEII digest of λ DNA.

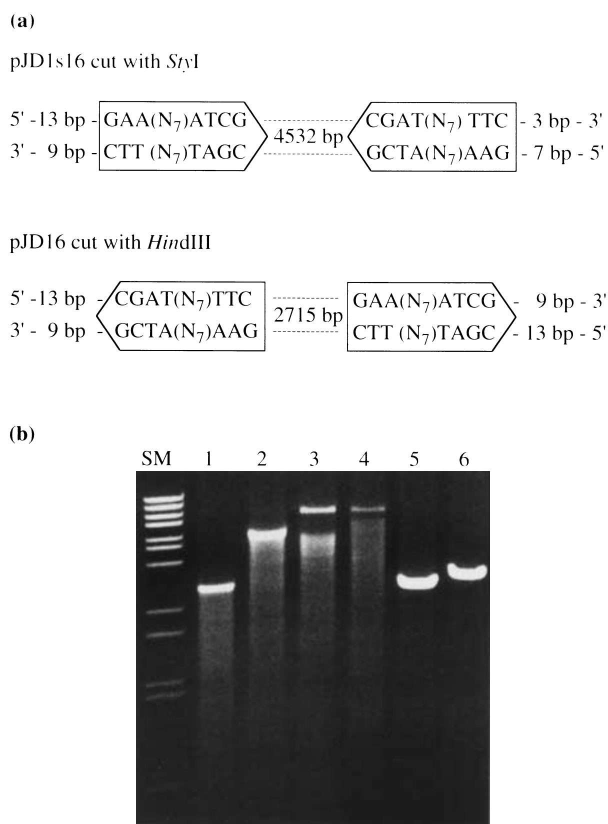


Figure 2. *Eco*R124II can cut DNA on either side of its recognition sequence. (a) Diagrams illustrating the orientations of *Eco*R124II sites on plasmids linearised with the indicated enzymes. (b) Different DNA substrates were digested with 2.9×10^{-12} mol *Eco*R124II in a 10 µl reaction for 30 minutes. pJD1 containing two *Eco*R124II sites (see Figure 1(a)) as well as pEKU60 and pUC19, both lacking *Eco*R124II sites, were used as controls. The substrates in the reactions shown are: lane 1, 8.3×10^{-14} mol pJD16 cut with *Hin*dIII; lane 2, 5×10^{-14} mol pJD1s16 cut with *Sty*I; lane 3, 3.8×10^{-14} mol pJD1 cut with *Eco*RI; lane 4, 3.8×10^{-14} mol pJD1 cut with *Sal*I; lane 5, 8.6×10^{-14} mol pUC19 cut with *Hin*dIII; and lane 6, 7.7×10^{-14} mol pEKU60 cut with *Afl*III.

(Figure 3 and Table 1). This is unusual in the sense that type I systems normally cut DNA non-specifically and therefore we would expect to see smears on a gel. A circular plasmid with two sites closer than 40 bp can be the source of either *Eco*R124II cleavable or *Eco*R124II non-cleavable linear DNA. Cleavable linear molecules are obtained if the type II cut is within the 40 bp spacer so that the sites are now at the ends of the DNA molecule (Figure 2(b), lanes 1 and 2). If the type II cut is outside the spacer region, non-cleavable DNA is generated (Figure 3 and Table 1).

We compared the size of the product DNA fragments with the substrates used and found them to be compatible with the idea that the cut occurred in the middle of the spacer between the two recognition sequences. To confirm this hypothesis, pJD1s85 was linearised with different type II restriction enzymes that have unique sites on pJD1s85. *Bsa*I, *Bsa*AI or *Hin*dIII were used and the DNAs created were subsequently treated with *Eco*R124II. In all cases the size of the digestion products could be predicted correctly by assuming a cut between the two *Eco*R124II sites (data not shown).

To see if the orientations of the recognition sequences influenced restriction we constructed plasmids with the head to tail (→←) and tail to tail (←→) site orientations. The results were the same as with the first head to head set of substrates (Table 1), confirming our finding that restriction can occur on either side of the recognition sequence.

If cleavage can only occur between two recognition sequences then cuts will be restricted to these regions of linear DNA molecules. Figure 4 shows a schematic drawing of such a molecule where, according to our model, *Eco*R124II cleavage would occur somewhere between C and D. Cleavage would create two pieces of DNA that can vary in length, namely one in the range of AC to AD and a second from BD to BC. The example drawn in Figure 4 is pJD5 linearised with *Pvu*II and after cleavage with *Eco*R124II the lengths of the products are expected to be between 691 bp (AC) to 1908 bp (AD) and 2783 bp (BD) to 4000 bp (BC). *Eco*R124II cleavage of *Pvu*II-linearised pJD5 did result in a smear composed of two discrete parts (Figure 5(b), lane 4). Using the size marker as a guide we estimate the size of the products to range approximately from 4200 to 3000 bp and from 2000 bp on down. This is in agreement with the products expected from cleavage between the two *Eco*R124II sites. Thus *Eco*R124II cleavage of other linear DNA molecules with two recognition sites should result in smears of predictable size ranges. Other examples of this can be seen in Figure 2(b), lanes 3 and 4 and Figure 3, the lane with linear pJDl DNA. As the prediction explains the results with all of the linear DNAs tested, we take it as another line of evidence that cutting occurs between the two sites.

Restriction of linear DNA by *Eco*R124II: influence of site-specific nicks between two recognition sequences

Since linear DNA is cut between the two *Eco*R124II sites, a nick in this region could possibly interfere with the process. To test this hypothesis, a linear DNA substrate was constructed with a site-specific nick between the two recognition sequences. The *Eco*R124II sites were separated by 1231 bp which normally gives a smear upon *Eco*R124II digestion (Figure 5(b), lane 4). The nicked analogue is faintly smeared (Figure 5(d), lane 2), but in contrast to unnicked DNA, bands are created upon *Eco*R124II treatment. The lengths of these fragments depend on the location of the recognition sites with respect to the DNA ends as well as the location of the nick within the spacer region

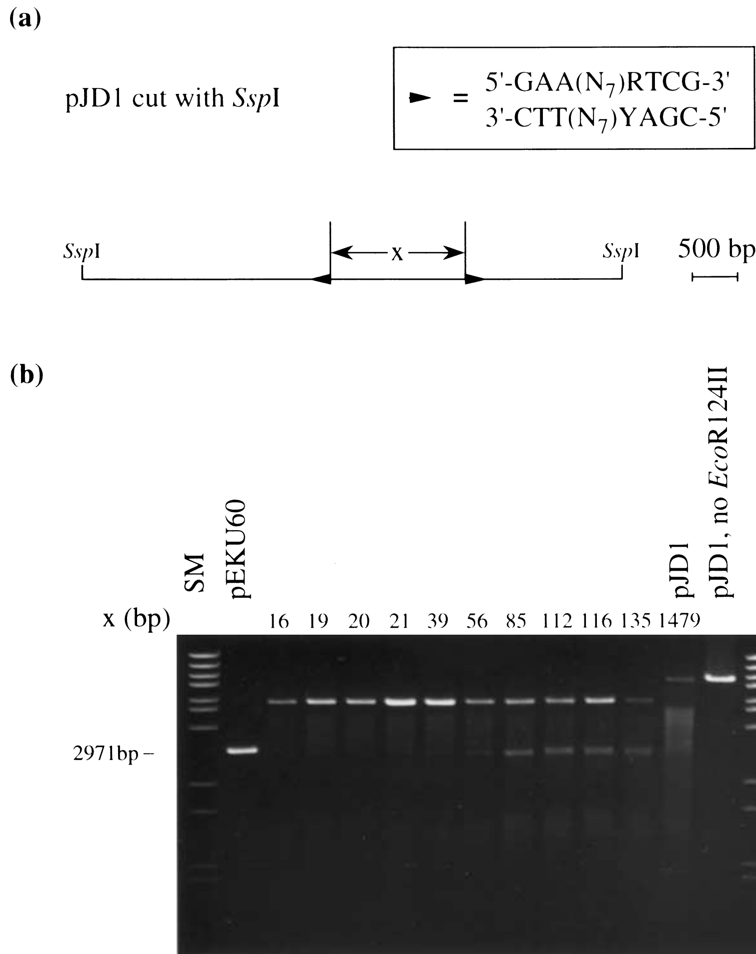


Figure 3. Restriction of linear DNA containing different lengths of spacer DNA between two recognition sites. (a) Structure of the linear substrates using pJDl as an example. The distance between two *Eco*R124II sites is given as x bp. (b) Results of the *Eco*R124II digestions; 1.7×10^{-14} mol pJD1s16 and 1.3×10^{-14} mol pJD1 were incubated with 2.9×10^{-12} mol *Eco*R124II for 30 minutes in a 10 μl reaction volume. pEKU60 has no recognition sites.

(Figure 5(a)). When the sizes of the products were compared to the corresponding substrates, it was clear that *Eco*R124II cleavage was in the region of the nick (Figure 5(c), lane 4 and 5(d), lane 2).

We next asked whether a nick and a single recognition site were sufficient to elicit restriction of linear DNA. The nicked circular DNA was first digested with two type II restriction enzymes to obtain linear DNA with one *Eco*R124II site and a nick. This substrate was not cut by *Eco*R124II. Thus a nick cannot substitute for a second recognition site.

Influence of a DNA-bound Lac repressor on restriction

A molecular block on the DNA might interfere with the cleavage reaction if *Eco*R124II reaches the cleavage site by tracking along the DNA. We tested the ability of the lactose repressor (LacR) bound to a *lac* operator site to serve as such a block to

Table 1. Restriction of linear DNA with two recognition sites at different distances

← x → (bp)	Restriction	→ x ← (bp)	Restriction	→ x → (bp)	Restriction
16	–	4	–	5	–
19	–	22	–	23	–
20	–	40	+	41	+
21	–	4532	+	1231	+
39	+				
56	+				
85	+				
112	+				
116	+				
135	+				
1479	+				

Orientations of *Eco*R124II sites are indicated by arrows where → represents 5'-GAA(N_7)RTCG-3'.

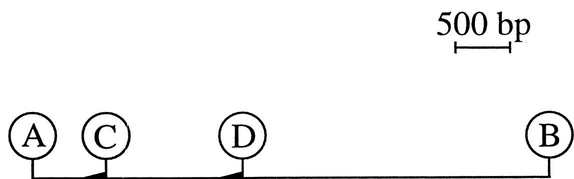


Figure 4. Scheme of a linear DNA molecule containing two specific recognition sequences. A and B represent the ends of the molecule while C and D designate the two *Eco*R124II sites. The drawing shows pJD5 cut with *Pvu*II. Further explanations are given in the text.

type I restriction. To do this, we constructed pMMW105 which has two *Eco*R124II sites with a *lac* operator between them (Figure 6(a)). Binding of the repressor to DNA was optimised by gel shift assays under the conditions used in restriction assays (Figure 6(b)). pBluescript II SK – digested with *Pvu*II and *Ssp*I was used as the DNA substrate. This digest generated a 448 bp fragment with the *lac* operator and two additional DNA fragments, 1920 bp and 553 bp in length, that lack LacR binding sites and thus serve as controls for non-specific protein-DNA interaction. The *lac* operator fragment is identical to the one present in pMMW105. The result shown in Figure 6(b) indicates a specific binding by LacR to operator sites at repressor to DNA ratios of 5.9 to 24.

The *Eco*R124II cleavage products of linear pMMW105 DNA in the presence and absence of LacR is shown in Figure 6(c). Restriction is not blocked by the repressor at concentrations (24:1) where only operator-containing DNA is band shifted. Even at a high LacR:DNA ratio (59:1, not shown) restriction is not blocked. Also no inhibition of restriction was observed when the assay was modified in the following ways: (1) enzyme was added prior to ATP; (2) different incubation times were used (one, two, five, ten, 15 and 30 minutes); (3) the reaction was performed at 37°C or room temperature; (4) the entire reaction mix (including *Eco*R124II) was preincubated on ice; and (5) the LacR concentration. was varied.

Linear DNA with three *Eco*R124II sites offers three possible pairs of recognition sequences that can be used for restriction. Binding of LacR between two sites could render the cleavage between those sites more difficult and result in a preferred selection of other *Eco*R124II site pairs, in which case the cleavage product pattern should be influenced by LacR. A third *Eco*R124II site was cloned into pMMW105 to see if the presence of LacR could bias the *Eco*R124II cleavage reaction so that the two *Eco*R124II sites that are not separated by the *lac* operator would be preferentially used. This was not the case (data not shown).

We tested the cleavability of linear DNA with one *Eco*R124II recognition site and a Lac repressor bound to a *lac* operator. Substrate DNA of that kind is obtained when pMMW105 DNA is cut with *Ssp*I and *Eco*RI or with *Sal*I and *Eco*RI (Figure 6(a)). The results indicate that an operator-bound Lac repressor cannot replace a second *Eco*R124II recognition sequence (Figure 6(c)).

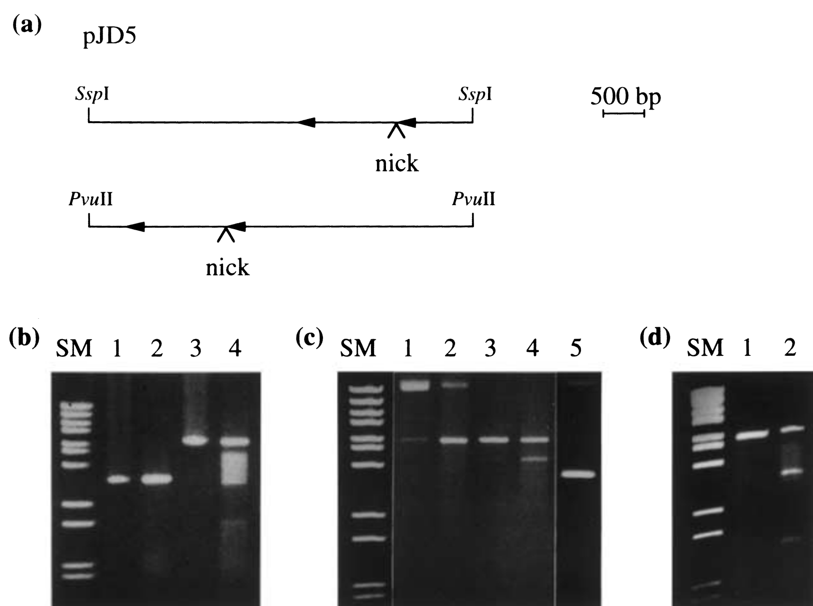


Figure 5. Restriction of DNA containing a site-specific nick. (a) pJD5 linearised by *Ssp*I or *Pvu*II with the nick indicated by an arrowhead. The gels in (b) to (d) show the results of different nicked DNA restriction reactions. (b) Lanes 1 and 2, 3.1×10^{-14} mol pEKU60 linearised with *Pvu*II in a 22 µl restriction reaction with 1.6×10^{-14} mol *Eco*R124II (lane 1) and without *Eco*R124II (lane 2). Lanes 3 and 4, as in 1 and 2 but with 3×10^{-14} mol pJD5 linearised with *Pvu*II. (c) Lanes 1 and 2, 8.3×10^{-15} mol nicked circular pJD5 incubated in a 20 µl reaction without (lane 1) and with 1.2×10^{-11} mol *Eco*R124II (lane 2). Lanes 3 and 4, as in 1 and 2 but with linear (*Ssp*I), nicked pJD5. Lane 5, 7.4×10^{-15} mol untreated, circular pJD5 to indicate the position of supercoiled and nicked circular JD5. The three parts of (c) are derived from the same gel. (d) Lane 1, 2.6×10^{-14} mol linear (*Pvu*II), nicked pJD5. Lane 2, 6.6×10^{-14} mol linear (*Pvu*II), nicked pJD5 was incubated in a 35 µl reaction with 2.9×10^{-11} mol *Eco*R124II after which 17 µl were loaded on the gel. SM = *Bst*EII digest of λ DNA.

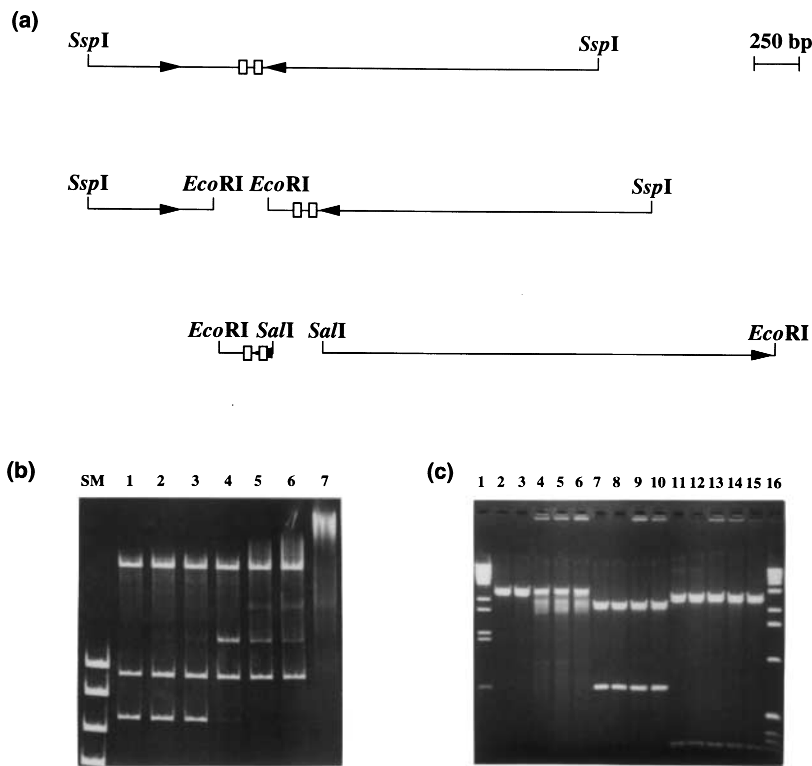


Figure 6. Influence of DNA-bound Lac repressor on *Eco*R124II restriction. (a) Schematic drawing of linear pMMW105 DNA molecules. Open squares represent lac operator sites and black arrows represent *Eco*R124II sites. For illustrative reasons the *Eco*R124II site on the smaller *Eco*RI-*Sal*I fragment is drawn to overlap a *lac* operator site which is not the case (see the text). (b) LacR gel mobility shift assay; 1×10^{-13} mol pBluescript II SK – digested with *Pvu*II and *Ssp*I was used as the DNA substrate in 20 μl reactions (for more details see the text). Lane 1, no LacR; lane 2, 1.2×10^{-13} mol LacR; lane 3, 2.4×10^{-13} mol LacR; lane 4, 5.9×10^{-13} mol LacR; lane 5, 1.2×10^{-12} mol LacR; lane 6, 2.4×10^{-12} mol LacR; lane 7, 5.9×10^{-12} mol LacR; SM = *Msp*I digest of pBR322 DNA. (c) Restriction assay; 5×10^{-14} mol linear pMMW105 DNA was cut with 8×10^{-12} mol *Eco*R124II in the presence or in the absence of 1.2×10^{-12} mol Lac repressor. Lane 1, *Bst*EII digest of λ DNA; lane 2, *Ssp*I linearised pMMW105 DNA; lane 3, as lane 2, + LacR; lane 4 as lane 2, + *Eco*R124II; lane 5, as lane 2, + LacR + *Eco*R124II, 30 minutes incubation; lane 6, as lane 5, five minutes incubation; lane 7, *Ssp*I- and *Eco*RI digested pMMW105 DNA; lane 8, as lane 7, + LacR; lane 9, as lane 7, + *Eco*R124II; lane 10, as lane 7, + LacR + *Eco*R124II; lane 11, *Sal*I- and *Eco*RI-digested pMMW105 DNA; lane 12, as lane 11, + LacR; lane 13, as lane 11, + *Eco*R124II; lane 14, as lane 11, + LacR + *Eco*R124II, 30 minutes incubation; lane 15, as lane 14, five minutes incubation; lane 16, 1 kb ladder molecular mass marker.

Two enzyme complexes, which need not be identical, are required to cleave linear DNA

So far it had shown that linear DNA requires at least two recognition sequences to be cut by *Eco*R124II. Furthermore it is not possible to substitute one of the recognition sequences by either a site-specific nick or by Lac repressor bound to a *lac* operator. These results suggest that cleavage requires not just a physical block but that more specific protein-protein contacts are needed. To see whether two heterologous type IC enzymes could interact to elicit cleavage, pA-CYC184 (a plasmid that contains a single recognition site each for *Eco*R124II and *Eco*DXXI) was used.

Both *Eco*R124II and *Eco*DXXI can cut circular pACYC184 DNA (Figure 7, lanes 3 and 4) but neither can cleave it if it is first linearised with a type II restriction enzyme (Figure 7, lanes 6 and 7). However, if both enzymes are added, cleavage of the linear DNA is observed (Figure 7, lanes 8 and 9). Because this DNA has one recognition sequence for each enzyme, two enzyme complexes can be formed on the DNA and it is clear that the two enzymes can complement each other *in vitro*.

Discussion

Restriction of DNA with one recognition site

Circular DNA molecules with single sites for type IC enzymes are good substrates. The product of restriction is unit length linear DNA showing that they are cut only once, and the position of cleavage is non-specific. The appearance of the linear product is preceded by the formation of nicked circular DNA, suggesting a two-step mechanism for the double-strand cut (Dreier & Bickle, 1996).

Linear DNA with one recognition site is not a substrate for *Eco*R124II. For *Eco*BI, a type IA system, the same phenomenon has been reported (Rosamond *et al.*, 1979). *Eco*KI, another type IA system, was found to require an excess of enzyme to allow cutting of DNA containing a single recognition sequence (Murray *et al.*, 1973). Several type II and type IIs restriction enzymes can be stimulated by the addition of cleavable DNA or oligonucleotides to cut otherwise non-cleavable substrates containing few recognition sites. It is thought that these enzymes have two binding sites for substrate DNA, both of which need to be occupied before cleavage can occur (Reuter *et al.*, 1993). We tried to stimulate

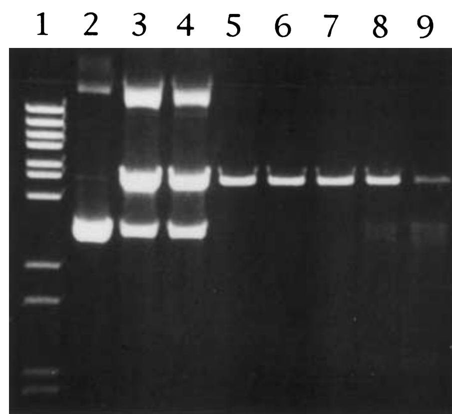


Figure 7. Interaction of *Eco*R124II and *Eco*DXXI to cut linear DNA. Circular as well as linear pACYC184 DNA was cut with *Eco*R124II and *Eco*DXXI in 20 µl restriction reactions. All reactions were incubated at 37°C for 30 minutes. Lane 1, size marker (*Bst*EII digest of λ DNA); lane 2, 2.6×10^{-14} mol circular pACYC184, no enzymes; lane 3, as lane 2, with 5.1×10^{-14} mol *Eco*R124II; lane 4, as lane 2, with 5.1×10^{-14} mol *Eco*DXXI; lane 5, 4.8×10^{-15} mol *Eco*RV-linearised pACYC184, no enzymes; lane 6, as lane 5, with 5.1×10^{-14} mol *Eco*R124II; lane 7, as lane 5, with 5.1×10^{-14} mol *Eco*DXXI; lane 8, as lane 5, with 2.6×10^{-14} mol *Eco*R124II and 2.6×10^{-14} mol *Eco*DXXI; lane 9, as lane 5, with 5.1×10^{-14} mol *Eco*R124II and 5.1×10^{-14} mol *Eco*DXXI.

the cleavage of linear DNA containing one recognition site by providing an excess of double-stranded oligonucleotides containing the *Eco*R124II recognition sequence, but no stimulation of cleavage was observed. Type I restriction enzymes show a DNA-dependent ATP hydrolysis and the concentration of added oligonucleotide was sufficient to elicit a high level of ATP hydrolysis (Dreier & Bickle, 1996), suggesting that the enzyme can bind to the oligonucleotides and that the failure to stimulate restriction is not due to the lack of oligonucleotide-protein interactions.

Restriction of DNA with two or more recognition sequences

Linear DNA containing two or more *Eco*R124II sites is cleaved by the enzyme provided the sites are sufficiently far apart. A smearing of the DNA products on the gel reflects the non-specific selection of cutting sites. Plasmids with sites closer than 40 bp are linearised whereas longer distances separating the sites lead to multiple cuts. This suggests that for the very short spacings only one cut is made on circular DNA, even though two recognition sequences are present. The most straightforward explanation for this is that one bound enzyme complex sterically prevents the binding of a second one.

When plasmids with sites closer than 40 bp are linearised by cutting between the *Eco*R124II recognition sequences, they can be cleaved by *Eco*R124II to result in smears. When the same plasmids are linearised by cleavage elsewhere, such that the *Eco*R124II sites retain their spacing, they are resistant to cleavage, that is, they are functionally single-site molecules. This supports the idea of steric exclusion derived from the digestion of the circular DNA.

Linearised plasmids are cut if the two recognition sequences are separated by more than 40 bp. However, with a 40 to 135 bp spacing, we observed discrete bands instead of a smear as the result of restriction. The sizes of the bands suggested that cleavage had occurred between the two recognition sequences. For larger spacings, *Eco*R124II digestion results in heterogeneous products generated by cleavage between the two sites.

The results can be summarised as follows: linear DNA is cut if two *Eco*R124II complexes bind to two unmethylated recognition sequences. The relative orientation of the asymmetric recognition sites is unimportant, that is, for cleavage they are functionally symmetrical. Cleavage occurs between the two recognition sequences. If the sites are close together, there is only a limited region in which cleavage can take place and therefore bands are visible upon electrophoresis. For larger distances there is a bigger choice of places where cleavage can occur which is the reason for the smears observed.

These results imply that two *Eco*R124II complexes must interact in order to cut linear DNA. When a nick at a fixed position between two recognition sequences is present on linear DNA, the DNA is cut in the region of the nick. The fact that a nick influences DNA cleavage suggests that DNA is translocated by the enzyme after binding to the recognition sequence. Linear DNA with one recognition sequence and a nick is not cleaved by *Eco*R124II, indicating that a nick itself is not sufficient to elicit DNA cutting by the enzyme. We think that a DNA-translocating enzyme complex is stalled by the nick and as soon as a second DNA translocating complex meets the stalled one they interact to introduce a double-strand break.

Strong evidence for DNA translocation by the type III enzyme *Eco*P15I, which also requires two sites in its substrate for cleavage, was reported recently (Meisel *et al*., 1995). In that system a molecular block in the form of a Lac repressor bound to DNA is able to prevent translocation and inhibit restriction. However, for *Eco*R124II, Lac repressor binding between sites did not block cleavage. Both type I and type III enzyme classes are thought to drive DNA translocation by ATP hydrolysis. A difference between the two classes is that the ATPase activity of type III enzymes is only about 1% of that found in type I systems. Type I enzymes hydrolyse large amounts of ATP during restriction and may be able to use this energy to displace a bound Lac repressor, whereas type III enzymes, hydrolysing small amounts of ATP, are blocked.

The plasmid pACYC184 contains one *Eco*R124II

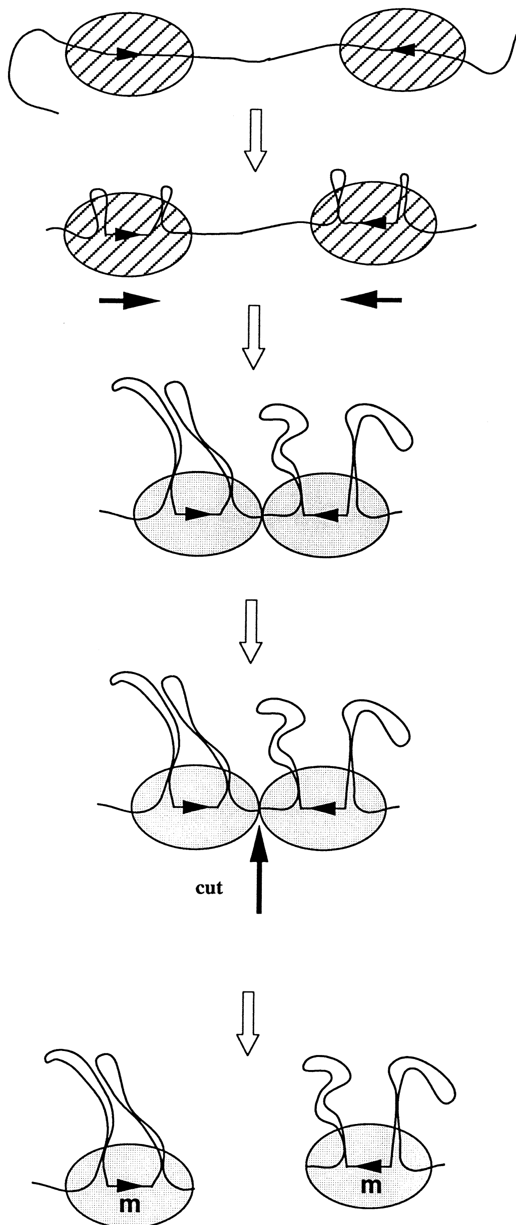


Figure 8. Mechanistic model of DNA restriction by type I R-M enzymes. Two recognition sequences (arrowheads) are occupied by one restriction and modification complex each (hatched ovals). This binding elicits a DNA translocation on both sides of the recognition sequence by the enzyme complexes. In this way the two complexes which remain bound to the recognition sequences approach each other, as indicated by the black arrows. When they meet a conformational change occurs (grey ovals) activating them for restriction. DNA is eventually cut at the meeting point and the products become methylated. The drawings are purely schematic and do not propose any structures of the reaction intermediates.

site and one site for another type IC enzyme, *Eco*DXXI. Either enzyme can cut the circular form of this DNA and, as expected, neither can cut if the DNA is first linearised. However, if both enzymes are present, linear pACYC184 DNA is cut. This result shows that the specific recognition site does not influence DNA cleavage once the enzyme has bound. The amino acid residue sequences of the HsdM and HsdR subunits of *Eco*R124II and *Eco*DXXI are very similar. The two enzymes mainly differ in the sequence of the HsdS subunit, which is responsible for specific DNA sequence recognition. Thus, if specific protein-protein interactions between either the HsdM or HsdR subunits of the two colliding enzyme complexes are required for cleavage, they could take place in this heterologous system. It would be interesting to see whether this kind of *in vitro* complementation could occur between type I enzymes of different families.

Our results agree with the model proposed by Studier & Bandyopadhyay (1988). After *Eco*R124II has bound to a non-methylated recognition sequence it starts to translocate DNA on both sides of the recognition sequence. As soon as it meets a second *Eco*R124II complex, the DNA is cut (Figure 8). As shown earlier, the cleavage products are subsequently methylated (Dreier & Bickle, 1996).

In the case of circular plasmids, DNA may be translocated until physical constraints, such as extensive supercoiling, lead to restriction of the DNA. RNA polymerases and DNA helicases have been found to alter the template supercoiling by tracking processes (reviewed by Dröge, 1994). *In vitro* as well as *in vivo* data from these systems suggest models where DNA structures participate in the regulation of the enzyme actions. The superhelicity of DNA possibly also influences restriction by type I R-M complexes.

Materials and Methods

DNA preparations

Plasmid DNA was purified using the Qiagen system (Qiagen). pACYC184 (Chang & Cohen, 1978) was isolated from the *Escherichia coli* strain HB101 (Boyer & Roulland-Dussoix, 1969). All other DNA preparations were from DH5α (Woodcock *et al*., 1989). pEKU60 is a pBR322 derivative from which the *Eco*R124II site has been excised (Kulik & Bickle, 1996). Phage λ DNA (New England Biolabs) was cut with *Eco*RV and the fragments from 21,271 to 22,950 and from 42,233 to 45,828 were inserted into pBR322 at the *Eco*RV site to result in pJD1 and pJD2, respectively. pJD4 is similar to pJD1 but has the λ insert in the opposite orientation. These constructions were confirmed by restriction fragment analysis and site-specific methylation of the *Eco*R124II sites as described previously (Gubler & Bickle, 1991). Linear DNA was prepared by digestions with the enzymes indicated in the text followed by extraction with phenol and precipitation with ethanol (Maniatis *et al*., 1982) or by purification from a 0.8% (w/v) agarose gel using the Gene Clean procedure (Bio101). DNA used in the assay shown in Figure 1 was purified by extraction with phenol (Maniatis *et al*., 1982) and gel filtration over a 1 ml Sepharose CL-6B (Pharmacia) spin column. The sequences of the annealed oligonucleotides used in this

work with the *Eco*R124II sites shown in bold type are:

annealed oligos HH (head to head; Dreier & Bickle, 1996):

5'-TCGAC**GAACGGCCGTATCG**ATAT**CGATACGCGTGTTC**-3'
3'-G**CTTGCCGGCATAGC**TATA**GCTATGCGCACAAG**AGCT-5'

annealed oligos HT (head to tail):

5'-TCGAGCCGCG**GAACGGCCGTATCG**ATATC**GAATACGCGTGTCG**-3'
3'-CGGCGC**CTTGCCGGCATAGC**TATAG**CTTATGCGCACAGC**AGCT-5'

oligo DXXI (self-complementary, no *Eco*R1 24II sites; Gubler *et al*., 1992):

5'-GAATTCAAGCTTGAATTC-3'
3'-CTTAAGTTCGAACTTAAG-5'

The plasmids pJD1s16, pJD1s85, pJD1s112, pJD1s116 and pJD1s135 were products of double-strand nested deletion with exonuclease III and nuclease S_1 starting at the *Sal*I site of pJD1 (Pharmacia kit). pJD1s19, pJD1s20, pJD1s21, pJD1s39 and pJD1s56 were constructed as follows: pJD1s16 was cut with *Sty*I, the sticky ends filled in using T4 DNA polymerase and then ligated. Prior to the ligation reaction annealed DXXI oligonucleotides were added. The structures of these plasmids were determined by DNA sequencing (Pharmacia T7 DNA polymerase kit). pJD10 and pJD20 were constructed by cloning annealed oligonucleotides HH and HT, respectively, into the *Sal*I site of pUC19 (Yanisch-Perron *et al*., 1985). These oligo pairs each contain an *Eco*RI site between the *Eco*R124II sites. One or two annealed DXXI oligonucleotides were inserted into the pJD10 and pJD20 *Eco*RV sites to obtain pJD12 (→ 22 bp ←), pJD13 (→ 40 bp ←), pJD22 (→ 23 bp →) and pJD23 (→ 41 bp →) which were sequenced to confirm the constructions.

Nicked DNA was made by cutting pJD4 with *Bam*HI and isolating the 4775 bp fragment. This fragment was treated with calf intestinal alkaline phosphatase to remove the 5' phosphate groups. It was then purified by extraction with phenol (Sambrook *et al*., 1989) and gel filtration over a 1 ml Sepharose CL-6B (Pharmacia) spin column or with Gene Clean. The next steps were to cut the DNA with *Bgl*II to create a sticky end compatible with the dephosphorylated *Bam*HI end and to ligate the ends with T4 DNA ligase at 16°C overnight. This resulted in a circular DNA molecule with a site-specific nick. The ligase was heat inactivated (65°C for ten minutes) before the DNA was used for further work. The presence of the nick was demonstrated in two ways: (1) an aliquot of the dephosphorylated pJD4 *Bam*HI fragment was tested by ligation with T4 ligase at 16°C overnight and subsequent analysis on a 0.8% agarose gel. The same was done after the *Bgl*II cut and the final ligation. The products were seen to have formed circular DNA in contrast to the dephosphorylated species. (2) The S_1 nuclease treatment of linearised nicked DNA resulted in bands expected for a cut at the position of the nick. By omitting the phosphatase step a similar construction but without the nick was made (pJD5).

pJD16 was constructed as follows: pUC19 (Yanisch-Perron *et al*., 1985) was cut with *Hin*dIII, blunt ended with mung bean nuclease (New England Biolabs), and religated to yield pJD15. Cleavage with *Sca*I and *Pst*I excised a 944 bp piece from pJD15 and the corresponding 999 bp *Sca*I-*Pst*I fragment from pJD12 (containing two *Eco*R124II sites head to head with a spacer length of 22 bp) was substituted to create pJD16. pJD16, like pJD12 contains a *Hin*dIII site in the spacer DNA between the two *Eco*R124II sites but, in contrast to pJD12, none in the polylinker.

pMMW105 was constructed as follows: pJD10 was digested with *Sma*I and partially digested with *Pvu*II to remove 106 bp from the *Pvu*II site in *lacZ* to the *Sma*I site in the polylinker which resulted in pJD10ΔZ. pJD10ΔZ was cut with *Hin*dIII, blunt ended with Klenow fragment, cut with *Pvu*II and religated. The 448 bp fragment of *Pvu*II-digested pBluescript II SK – DNA (contains O1 and O3 of the wild-type *lac* operator) was inserted into this plasmid at the *Eco*RV site to result in pMMW105.

The1 kb DNA ladder molecular mass standard was purchased from Gibco BRL. *BstE*II-cut λ DNA and *Msp*I-digested pBR322 were from New England Biolabs.

Protein preparations

*Eco*R124II and *Eco*DXXI were purified as described by Ramsden & Dreier (1996). *Eco*DXXI was isolated from *E. coli* WA921/pMMW68 (MacWilliams & Bickle, 1996; Wood, 1966) and *Eco*R124II from *E. coli* DH5 α/pUNG30 (Firman *et al*., 1985; Woodcock *et al*., 1989). Both enzymes were over 90% pure as judged from Coomassie-blue-stained, 12% (w/v) polyacrylamide gels. The stoichiometry of the restriction enzymes is not unambiguously determined.

The Lac repressor (LacR) was purified from strain BMB8117/pWB 1000 (Fickert & Müller-Hill, 1992) according to Müller-Hill *et al*. (1971) as modified by Andrew Barker (20 to 33% ammonium sulphate cut, personal communication). An additional Superose 6 column (Pharmacia) was used to remove residual non-specific DNase activity from the preparation. The purified protein was stored in 1 × TMS buffer (10 mM Tris-HCl (pH 7.4), 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 6 mM 2-mercaptoethanol; Müller-Hill *et al*., 1971) and diluted prior to use in binding buffer (BB; 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 3 mM magnesium acetate, 0.1 mM EDTA, 50 μg/ml bovine serum albumin (BSA), 0.1 mM dithiothreitol; Riggs *et al*., 1970).

Type II restriction enzymes, T4 DNA ligase, mung bean nuclease, T4 DNA polymerase and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Promega.

Restriction assays

Assays were performed by mixing AdoMet (4.75 μM final concentration) and ATP (1 mM final concentration) with DNA in restriction buffer (100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 250 μM EDTA, 14 mM 2-mercaptoethanol). The reaction volumes are indicated in the Figure legends. This mix was preincubated for five minutes at 37°C and the restriction reaction was started by the addition of enzyme. At the indicated times the reaction was stopped by the addition of 10 × sample buffer (40% (w/v) Ficoll 70, 7.2 mM bromophenol blue, 0.2% (w/v) SDS, 50 mM EDTA) to a 1 × final concentration and the sample was then put on ice. The reactions were heated to 65°C for two minutes and analysed on 0.8% agarose gels run in 0.5 × TBE buffer (Sambrook *et al*., 1989) supplemented with 0.5 μg/ml ethidium bromide (EtBr). In the presence of LacR the 20 μl restriction reactions consisted of 1 × Lac buffer (10 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50 μg/ml BSA, 0.1 mM dithiothreitol), 1 mM ATP, 4.75 μM AdoMet, 2.5×10^{-9} M pMMW105 DNA, 4×10^{-7} M *Eco*R124II and 6×10^{-8} M LacR. Reactions were first set up on ice with all the components except LacR and *Eco*R124II. The LacR (or binding buffer)

was then added and the mixture was incubated at 37°C for ten minutes. This was followed by the addition of *Eco*R124II (or buffer B + 50 μg/ml BSA) and the 37°C incubation was continued for 30 minutes. The reactions were stopped by the addition of 5 μl of 10 × sample buffer (see above) and placing the tubes on ice. To ensure the complete dissociation of the protein from the DNA, the tubes were heated at 70°C for ten minutes. The samples were then loaded on a 1.2% agarose gel in 0.5 × TBE + 0.5 μg/ml EtBr.

Lac repressor gel mobility shift assay

The gel mobility shift assays employed the same buffer conditions as the restriction assays performed in the presence of LacR. The reaction components were mixed on ice with LacR being the last component added. The samples were then incubated at room temperature for 15 minutes. Before loading on a 5% polyacrylamide gel, each sample was combined with 5 μl of load buffer (binding buffer + 50% (v/v) glycerol). The gel was electrophoresed at 12 V/cm for two to three hours. After electrophoresis, the gel was incubated in EtBr to visualise the DNA bands.

Acknowledgements

We thank Eva Kulik for providing pEKU60. We are grateful to Andrew Barker and Benno Müller-Hill for plasmids, strains and help with the LacR purification. This work was supported by the Swiss National Science Foundation.

References

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. & Krüger, D. H. (1993). Biology of DNA restriction. *Microbiol. Rev.* **57**, 434–450.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* **41**, 459–472.

Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**, 1141–1156.

Dreier, J. & Bickle, T. A. (1996). ATPase activity of the type IC restriction-modification system *Eco*R124II. *J. Mol. Biol.* **257**, 960–969.

Dröge, P. (1994). Protein tracking-induced supercoiling of DNA: a tool to regulate DNA transactions *in vivo*? *BioEssays*, **16**, 91–99.

Endlich, B. & Linn, S. (1985). The DNA restriction endonuclease of *Escherichia coli* B. 1. Studies of the DNA translocation and the ATPase activities. *J. Biol. Chem.* **260**, 5720–5728.

Fickert, R. & Müller-Hill, B. (1992). How Lac repressor finds *lac* operator *in vitro*. *J. Mol. Biol.* **21**, 59–68.

Firman, K., Creasey, W. A., Watson, G., Price, C. & Glover, S. W. (1983). Genetic and physical studies of restriction-deficient mutants of the Inc FIV plasmids R124 and R124/3. *Mol. Gen. Genet.* **191**, 145–153.

Firman, K., Price, C. & Glover, S. W. (1985). The *Eco*R124 and *Eco*R124/3 restriction and modification systems: cloning the genes. *Plasmid*, **14**, 224–234.

Glover, S. W., Firman, K., Watson, G., Price, C. & Donaldson, S. (1983). The alternate expression of two restriction and modification systems. *Mol. Gen. Genet.* **190**, 65–69.

Gubler, M. & B ickle, T. A. (1991). Increased protein flexibility leads to promiscuous protein-DNA interactions in type IC restriction-modification systems. *EMBO J.* **10**, 951–957.

Gubler, M., Braguglia, D., Meyer, J., Piekarowicz, A. & Bickle, T. A. (1992). Recombination of constant and variable modules alters DNA sequence recognition by type IC restriction-modification enzymes. *EMBO J.* **11**, 233–240.

Hedges, R. W. & Datta, N. (1972). R124, an fi + R-factor of a new compatibility class. *J. Gen. Microbiol.* **71**, 403–405.

Kulik, E. M. & Bickle, T. A. (1996). Regulation of the activity of the type IC *Eco*R124I restriction enzyme. *J. Mol. Biol*. In the press.

MacWilliams, M. P. & B ickle, T. A. (1996). Generation of new DNA binding specificity by truncation of the type IC *Eco*DXXI *hsdS* gene. *EMBO J.* **15**, 4775–4783.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Meisel, A., Mackeldanz, P., Bickle, T. A., Krüger, D. H. & Schroeder, C. (1995). Type-III restriction endonucleases translocate DNA in a reaction driven by recognition site-specific ATP hydrolysis. *EMBO J.* **14**, 2958–2966.

Müller-Hill, B., Beyreuther, K. & Gilbert, W. (1971). Lac repressor from *E. coli*. *Methods Enzymol.* **21**, 483–487.

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.

Piekarowicz, A., Gougen, J. D. & Skrzypek, E. (1985). The *Eco*DXXI restriction and modification system of *Escherichia coli* ET7. Purification, subunit structure and properties of the restriction endonuclease. *Eur. J. Biochem.* **152**, 387–393.

Price, C., Pripfl, T. & Bickle, T. A. (1987a). *Eco*R124 and *Eco*R124/3: the first members of a new family of type I restriction and modification systems. *Eur. J. Biochem.* **167**, 111–115.

Price, C., Shepherd, J. C. W. & Bickle, T. A. (1987b). DNA recognition by a new family of type I restriction enzymes: a unique relationship between two different DNA specificities. *EMBO J.* **6**, 1493–1497.

Ramsden, J. J. & Dreier, J. (1996). Kinetics of the interaction between DNA and the type IC restriction enzyme *Eco*R124II. *Biochemistry*, **35**, 3746–3753.

Reuter, M., Kupper, D., Pein, C. D., Petrusyte, M., Siksnys, V., Frey, B. & Krüger, D. H. (1993). Use of specific oligonucleotide duplexes to stimulate cleavage of refractory DNA sites by restriction endonucleases. *Anal. Biochem.* **209**, 232–237.

Riggs, A. D., Suzuki, H. & Bourgeois, S. (1970). *lac* repressor-operator interaction. *J. Mol. Biol.* **48**, 67–83.

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*. 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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.

Taylor, I., Watts, D. & Kneale, G. (1993). Substrate recognition and selectivity in the type IC DNA modification methylase M.*Eco*R124I. *Nucl. Acids Res.* **21**, 4929–4935.

Watson, N. (1988). A new revision of the sequence of plasmid pBR322. *Gene*, **70**, 399–403.

Wilson, G. G. & Murray, N. E. (1991). Restriction and modification systems. *Annu. Rev. Genet*. **25**, 585–627.

Wood, W. B. (1966). Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. *J. Mol. Biol.* **16**, 118–133.

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.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

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 20 August 1996; received in revised form 23 September 1996; accepted 24 September 1996)