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Selection of Non-specific DNA Cleavage Sites by the Type IC Restriction Endonuclease *Eco*R124I

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The Type IC restriction endonuclease *Eco*R124I binds specifically to its recognition sequence but subsequently translocates non-specific DNA past the complex in an ATP-dependent mechanism. The enzyme thus has the potential to cleave DNA at loci distant from the recognition site. We have scrutinised the link between translocation and cleavage on linear and circular DNA substrates. On linear DNA carrying two recognition sites, the majority of cleavages at loci distant from the recognition site occurred between the two sites, regardless of the inter-site distance or relative orientations. On circular DNA carrying one site, distant cleavages occurred throughout the DNA but an equivalent linear molecule underwent considerably fewer cleavages at distant loci. These results agree with published models for DNA tracking. However, on every molecule investigated, discrete cleavage sites were also observed within ±250 bp of the recognition sites. The localised cleavages were not confined to particular DNA sequences and were independent of DNA topology. We propose a model to account for both distant and localised cleavage events. The conformation of the DNA loop extruded during tracking may result in two DNA segments being held in proximity to the restriction moiety on the protein, one close to the *Eco*R124I site and another distant from the site: cleavage may occur in either segment. Alternatively, the cutting of DNA close to recognition sites may be the result of multiple nicks being generated in the expanding loop before any extensive translocation.

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Introduction

The presence of a specific recognition sequence for a restriction endonuclease renders a DNA molecule susceptible to digestion by that enzyme. For the type II endonucleases, the scissile phosphodiester bonds are located at fixed positions within or immediately adjacent to the recognition site (Roberts & Halford, 1993). Similarly, the type III endonucleases cleave DNA 25 to 26 bp from their sites (Bickle, 1993). In contrast to such precision, the occupancy of a recognition site by a type I endonuclease results in DNA digestion at manifold random loci (Bickle, 1993). Cleavage is no longer limited to the region surrounding the cognate site and can occur anywhere on the DNA molecule.

The type IC endonuclease *Eco*R124I, is a multi-functional complex composed of the subunits HsdR (restriction), HsdM (methylation) and HsdS (specificity), which recognizes an asymmetric sequence, GAA$nnnnnn$RTCG (where n is any base and R is a purine: Price *et al.*, 1987). It requires both ATP and Mg^{2+} as cofactors for activity but each molecule of *Eco*R124I cleaves only one DNA molecule (Janscak *et al.*, 1996). The connection between recognition and cleavage was characterised on a DNA catenane carrying an *Eco*R124I site on the smaller of two interlinked rings (Szczelkun *et al.*, 1996). Cleavage by *Eco*R124I occurred exclusively in the small ring, which shows that the communication cannot occur between rings but is absolutely constrained to sites *in cis* on the same

DNA molecule by a tracking mechanism. In this type of one-dimensional motion, the enzyme remains bound to a cognate site whilst translocating adjacent non-specific DNA past itself, generating an expanding loop of DNA (Rosamund *et al.*, 1979; Yuan *et al.*, 1980). Since *Eco*R124I never cleaved the large ring of the catenane, the initial contact with non-specific DNA must be at a site close to the recognition site, producing a very small DNA loop. The subsequent transit of DNA past the protein is not a passive event, but is driven processively by the hydrolysis of ATP (Janscak *et al.*, 1996; Dreier & Bickle, 1996).

The activities of type I endonucleases are dependent on the nature of the DNA substrate. Linear molecules with a single site are either refractory to cleavage (Rosamund *et al.*, 1979; Dreier & Bickle, 1996) or only undergo limited cleavage with a large excess of enzyme over DNA (Murray *et al.*, 1973; Szczelkun *et al.*, 1996). However, linear DNA carrying two or more sites is fully digested under similar conditions. This disparity has been justified in terms of a co-operative model, in which the convergence and collision of two tracking enzymes is required for cleavage (Shulman, 1974; Studier & Bandyopadhyay, 1988). The digestion of one-site substrates may need a collision between a complex tracking from the recognition site and a second enzyme bound to non-specific DNA (Janscak *et al.*, 1996). A collision-dependent tracking mechanism has also been proposed for type III restriction enzymes (Meisel *et al.*, 1995).

In contrast to linear substrates, circular DNA carrying at least one recognition site is readily cleaved by type I enzymes, irrespective of the total number of sites present (Rosamund *et al.*, 1979; Janscak *et al.*, 1996; Dreier *et al.*, 1996). This susceptibility is probably a consequence of changes in DNA topology during tracking (Szczelkun *et al.*, 1996). For any protein that binds simultaneously to two separate sites on a circular DNA and then translocates one site past the other, a figure-of-eight structure will be created, with expanding and contracting loops (Gellert *et al.*, 1978). The obligatory rotation of DNA during active translocation produces negative and positive supercoils in the expanding and contracting loops, respectively (Ostrander *et al.*, 1990). Since the binding of *Eco*R124I initially creates a relatively small expanding loop, the DNA in the expanding loop would need to be nicked prior to tracking in order to accommodate the reduction in twist (Szczelkun *et al.*, 1996). However, the corresponding increase in twist in the contracting loop would eventually generate an impassable topological barrier. The barrier will prevent further translocation and the enzyme may then stall for long enough to make a double strand break. This physical constraint would occur equally well on single-site plasmids as on multiple-site plasmids. For tracking on linear DNA, the increase in twist in the contracting domain can dissipate by free rotation of the DNA ends without any topological restraints.

What distribution of random cleavage sites can be expected based on the above models? In common with other type I enzymes (Yuan *et al.*, 1980; Dreier *et al.*, 1996), *Eco*R124I cleaves DNA on either side of its site (Szczelkun *et al.*, 1996). This could arise from either unidirectional motion, with the enzyme translocating ''leftwards'' in some instances and ''rightwards'' in others (Yuan *et al.*, 1980), or from bi-directional motion with the enzyme simultaneously translocating the DNA on both sides of its site, extruding two expanding loops (Studier & Bandyopadhyay, 1988; Dreier *et al.*, 1996). Once translocation has begun, where do the enzymes actually pause and cleave DNA? For the collision model of Studier & Bandyopadyhyay (1988), this should occur most frequently half-way between pairs of sites. But, depending on the synchrony, processivity, and direction of tracking, a whole succession of locations will probably be cleaved. For the topological model on circular DNA (Szczelkun *et al.*, 1996), the distance translocated before pausing will be dictated by the level of supercoiling torque in the contracting loop that forms the topological barrier. Here, we describe the mapping of random cleavage sites produced by *Eco*R124I on linear and circular substrates with either one or two recognition sites, and relate the observed cleavage patterns to the various models for DNA tracking.

Results

Cleavage of two-site linear DNA by *Eco*R124I

The model for DNA tracking by the type IA endonuclease *Eco*KI (Studier & Bandyopadhyay 1988) predicts that linear DNA will be cleaved midway between a pair of sites, following the collision of two tracking enzymes. This model was first tested by identifying the loci for random cleavages on linear DNA carrying two recognition sites for the type IC endonuclease *Eco*R124I. Due to the asymmetry of the *Eco*R124I cognate sequence, pairs of sites can be positioned in either direct (head-to-tail) or inverted (head-to-head) orientations. To investigate both alignments, the plasmids pMDS4a and pMDS4b (Szczelkun *et al.*,1996), were cut with the type II enzyme *Nde*I. This produced the 4.3 kb linear substrates, 4alin and 4blin, with sites in direct and inverted repeat, respectively (Figure 1(a)). Digestion of DNA by *Eco*R124I was carried out using a fixed molar excess of enzyme over DNA. Samples were withdrawn from reactions at sequential time points and the appearance of cleavage products followed by agarose gel electrophoresis. A representative gel for 4alin is shown in Figure 1(b); analogous results were obtained with 4blin (not shown). The sizes of the DNA products were estimated from their electrophoretic mobilities relative to DNA standards of known size.

As the reaction progressed, multiple DNA products were observed as smears on the EtBr-stained

gel (Figure 1(b)). One smear corresponded to a heterogeneous series of products between ~3500 and ~2500 bp in size and another smear to fragments in the range ~1800 to 800 bp. These sets of heterogeneous products match those expected for a succession of DNA species generated by random cleavage between the two *Eco*R124I sites on 4alin. However, emerging in parallel with these random fragments were a number of discrete products, giving rise to distinct bands on the gel (Figure 1(b)). Far from being minor species, these specific fragments were of considerable intensity and were clearly visible from the background smears of random fragments. Neither the nature of the products from 4alin nor the distribution between discrete and random fragments were altered by lengthening the incubation of the DNA with *Eco*R124I, or by changing the molar ratio of enzyme to DNA (not shown). Different buffer conditions and separate preparations of the endonuclease also produced the same results (not shown).

To identify the discrete products, gels for 4alin (Figure 1(b)) and 4blin (not shown) were scanned by densitometry. The bands corresponding to the substrate and the discrete products were clearly resolved as peaks in the densitometer traces (Figure 2(a)). However, the broad smears indicative of random cleavage were not fully resolved from the intense peaks. Nevertheless, the sizes of the individual elements were determined from the positions of each peak on the *x*-axes relative to the DNA markers, by the 2×3 point analysis described by Sealey & Southern (1990) (see Materials and Methods). This allowed for the determination of the positions along the DNA that were cleaved to give rise to the discrete fragment: the loci are illustrated in Figure 2(b), together with the approximate boundaries for the random DNA cleavages. At least some of the distinct bands resulted from DNA cleavages close to the *Eco*R124I recognition sites. The mapping of the cleavage sites located near the recognition sites was confirmed by analysing permuted forms of 4alin and 4blin that were generated by linearising pMDS4a and pMDS4b with other type II restriction enzymes (not shown).

Almost all of the DNA cleavages observed on 4alin were located either close to or in between its two *Eco*R124I recognition sites. Very few of the detectable products resulted from cleavages at distant sites between the ends of the molecule and the recognition sites. The sites for random cleavages had a broad distribution centred around ~1450 bp, midway between the recognition sequences (Figure 2(b)). This conforms to the collision model for DNA cleavage by type I enzymes (Studier & Bandyopadhyay, 1988; Dreier *et al.*, 1996). In contrast, the discrete products observed as distinct bands on the gel (Figure 1(b)) came from DNA cleavages close to the recognition sites. Two such sites were detected to the left of the 1040 bp site as drawn, whilst a third site was detected close to the 1846 bp site (Figure 2(b)). Similar results were

obtained with 4blin (Figure 2(a)): its cleavage sites also mapped to equivalent positions adjacent to the recognition sites and around the midpoint between the sites (Figure 2(b)). The relative orientation of the *Eco*R124I sites thus has negligible
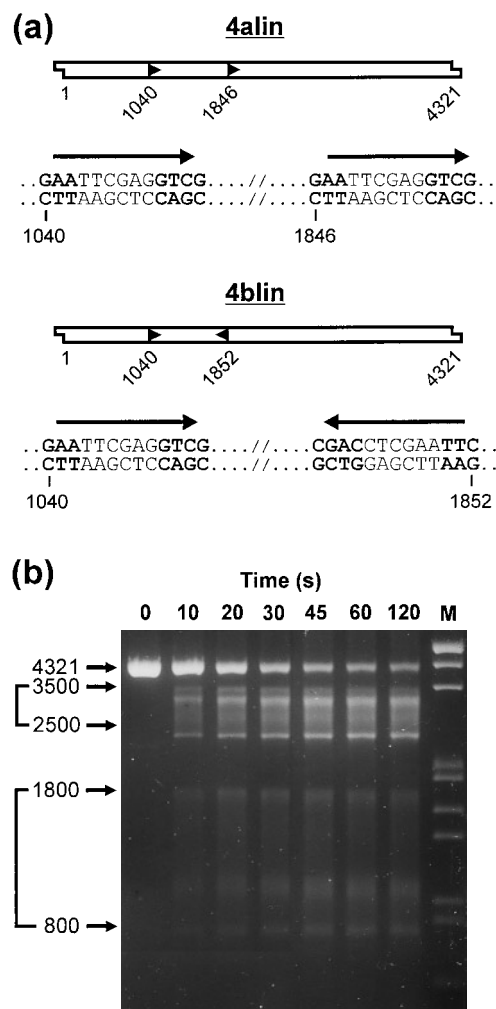

Figure 1. Cleavage of linear DNA carrying two *Eco*R124I sites. (a) The plasmids pMDS4a and pMDS4b (Materials and Methods) were linearized with *Nde*I to give the substrates 4alin and 4blin, respectively. The full length DNA is represented by a thin rectangle with the 5′ overhang produced by *Nde*I indicated. The *Eco*R124I sites are shown as filled arrowheads. The first bp at one end of the DNA was designated as position 1, such that the first *Eco*R124I site on both 4alin and 4blin are in the same position (1040 bp). The relative alignment and numbering of the *Eco*R124I sites is described below each DNA and is determined by the first G·C base-pair of GAATTCGAGGTCG. (b) Reactions at 37°C contained 7 nM 4alin and 49 nM *Eco*R124I endonuclease. Samples were removed at timed intervals and the products of digestion resolved by agarose gel electrophoresis. Reaction times are given in seconds above each lane. The marker lane (M) is an *Eco*RI/*Hin*dIII digest of λ DNA to give bands of 5148/4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831 and 564 bp. The location of the substrate band (4321 bp) and the approximate boundaries to the smears of DNA products are indicated to the left of the gel.
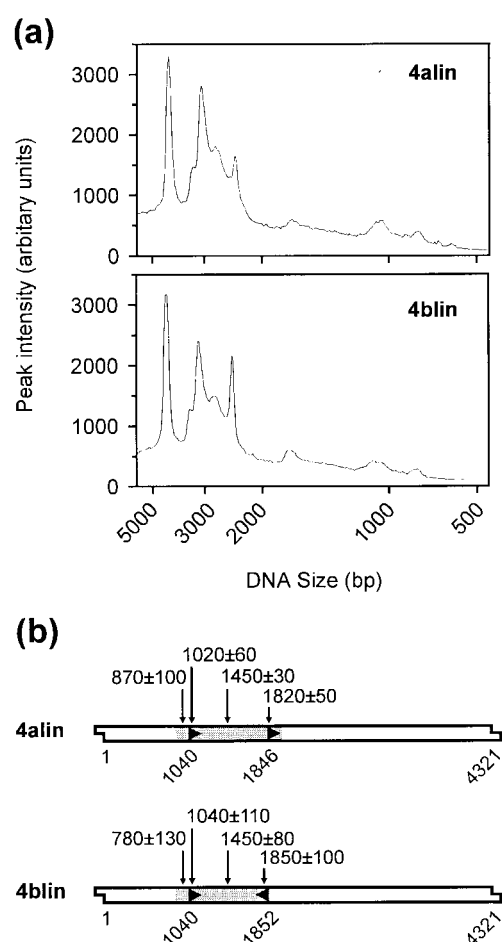
(a)

(b)

Figure 2. Mapping the products from digestion of two-site linear DNA by *Eco*R124I. (a) Reactions on 4alin and 4blin were carried out as in Figure 1(b) for 120 seconds and the DNA subsequently analysed by electrophoresis through agarose. The gels were scanned by densitometry and the densitometric records are shown here. DNA size is indicated on a non-linear scale on the *x*-axis, whilst fluorescent intensity is represented on the y-axis. (b), 4alin and 4blin are represented as in Figure 1(a). Loci for discrete cleavages were calculated from the fragment sizes for the major peaks in the densitometer traces and are indicated on the DNA by vertical arrows. The standard deviations represent the variation in data from at least three repeat experiments. The extent of the smeared DNA was estimated directly from the gels and is represented as a grey region.

effect on the distribution of either random or localised cleavage sites.

Cleavage of one-site linear DNA by *Eco*R124I

Is the cleavage of loci close to a recognition site due to the enzyme bound to that site, or to a second enzyme which collides with the enzyme at the recognition site after tracking along the DNA? Furthermore, if random cleavages on a two-site substrate are confined to the region between the sites (Figure 2(b)), what happens in the presence of

only a single site? We pursued both of these questions by using one-site linear substrates generated by cleaving pMDS3 (Szczelkun *et al.*, 1996), with the type II enzymes *Nde*I (to yield 3linα), *Sca*I (3linβ) or *Bgl*II (3linγ) (Figure 3(a)). The plasmid pMDS3 was the precursor used in the construction of pMDS4a and pMDS4b: the single *Eco*R124I site on each of the 3lin series is equivalent to the 1040 bp site on 4alin and 4blin (Figure 1(a)). In contrast to linear DNA with two sites, linear molecules with one site are poorly cleaved: <10% of the one-site DNA is cleaved under conditions which result in >90% digestion of two-site molecules (Szczelkun *et al.*, 1996). The three one-site substrates were analysed by agarose gel electrophoresis after ten minute digestions with a large excess of *Eco*R124I over DNA (Figure 3(b)). No additional cleavages were observed by altering the reaction conditions (not shown).

In contrast to the two-site substrates, visualisation of the EtBr-stained gels failed to reveal the extensive smears of DNA that would indicate the series of heterogeneous products from random cleavages (Figure 3(b)). Nonetheless, by using DNA labelled with [³H]thymidine (Materials and Methods), a low level of randomly cleaved DNA species were recorded by scintillation counting of sections of the gel apparently devoid of DNA (not shown). However, discrete species were clearly discernible on the gel (Figure 3(b)) and these gave rise to sharply defined peaks in densitometer traces (Figure 3(c)). The different sizes of the discrete products from 3linα, 3linβ and 3linγ allowed for unambiguous mapping of the cleavage sites (Figure 3(a)). For on all three of the one-site linear substrates, two preferred loci were identified; ~50 bp and ~250 bp to the left of the recognition sites as drawn (Figure 3(a)). Two sites in the same relative positions had also been observed adjacent to the 1040 bp recognition site on 4alin and 4blin (Figure 2(b)).

The data from the one-site linear substrates illustrate two points. Firstly, the lack of any predominant smear (Figure 3(b)) suggests that random cleavages are occurring less frequently than on 4alin or 4blin, or are distributed over a longer stretch of DNA. Secondly, the accurate mapping of discrete cleavage sites to loci close to the recognition site (Figure 3(a)) suggests that this class of cleavage cannot be the result of a second enzyme tracking in from elsewhere on the DNA, but is due to an enzyme bound at a cognate site cleaving the DNA adjacent to that site.

Effects of sequence and distance between *Eco*R124I sites

It is possible that the discrete DNA cleavages noted above may be due to *Eco*R124I acting essentially at random but with a preference for certain DNA sequences over others, as is the case with DNaseI (Doherty *et al.*, 1995). In other words, each
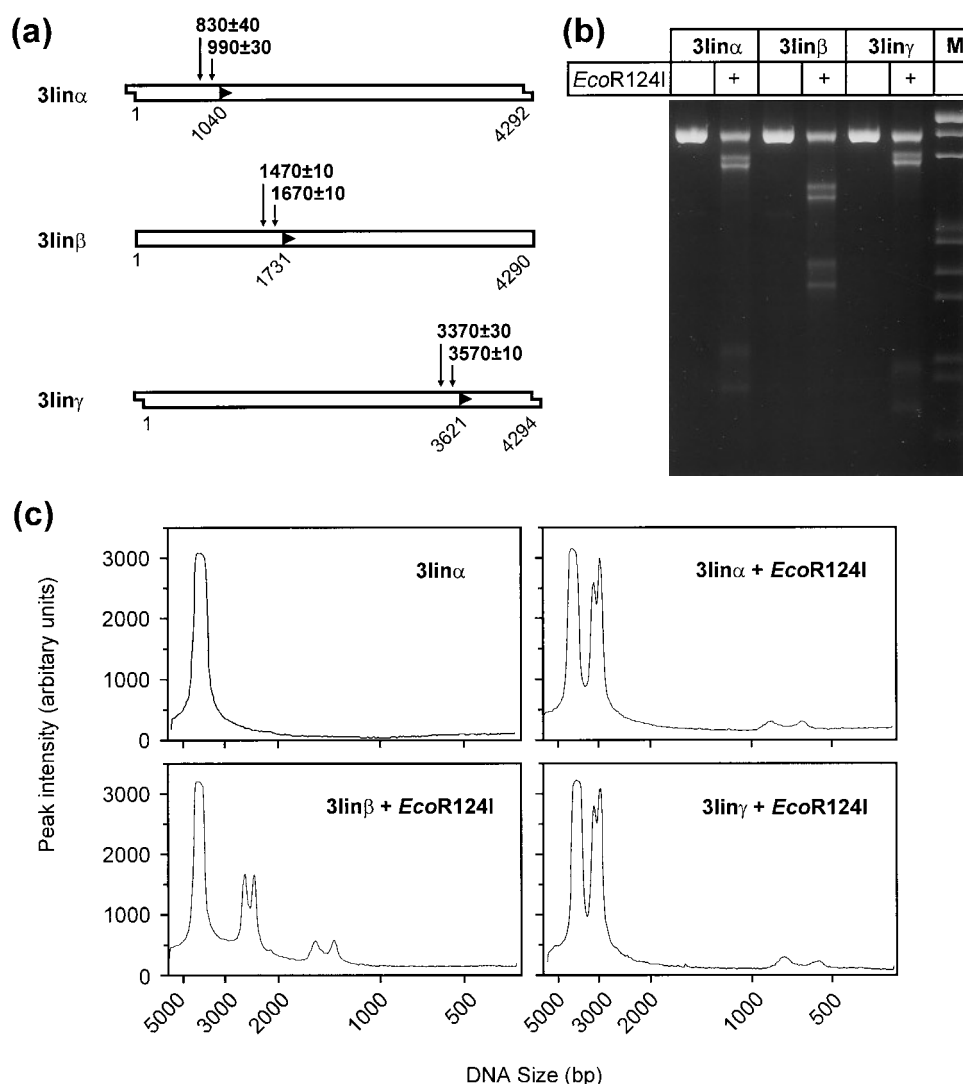
Figure 3. Mapping the products from digestion of one-site linear DNA by EcoR124I. (a) The plasmid pMDS3 was cleaved with NdeI, ScaI and BglII to produce 3linα, 3linβ, and 3linγ, respectively. DNA representations, site numbering and orientations are equivalent to those in Figure 1(a). (b) Reactions for ten minutes at 37°C contained 7 nM DNA and, where marked (+), 112 nM EcoR124I endonuclease. Products were separated on the agarose gel shown here. The marker lane (M) is as described in Figure 1(b). (c) Densitometric scans of the gel shown in (b). DNA sizes are indicated on a non-linear scale on the *x*-axis, whilst fluorescent intensity is represented on the *y*-axis. (The broad peak widths for the substrate bands are due to the large amounts of intact DNA still present at the end of these incubations, resulting in saturation of EtBr fluorescence with respect to the camera sensitivity.) The resulting cleavage sites determined from the peaks are illustrated in (a) by vertical arrows. The standard deviations represent variations in data from at least four repeats of each experiment.

member in the family of substrates used above may simply possess DNA sequences close to the EcoR124I recognition sites that are particularly susceptible to this endonuclease. To test this possibility, we examined a series of two-site linear substrates derived from the plasmid pDRM-2R[450] (Janscak *et al.*, 1996) and a derivative of this molecule, pDRM-2R[1000], produced by inserting a 0.5 kb fragment between the sites (Materials and Methods). These substrates carry the same recognition sequence as 4alin (Figure 1(a)) but have different DNA flanking the sites (not shown). pDRM-2R[450] was cleaved with the type II en-

zymes ScaI (to yield 2Rlinα) or AflIII (2Rlinβ), while pDRM-2R[1000] was cleaved by ScaI (2Rlinγ), AflIII (2Rlinδ) or HindIII (2Rlinε) (Figure 4(a)). The resulting series of substrates have sites separated by 441 bp (2Rlinα, 2Rlinβ), 972 bp (2Rlinγ, 2Rlinδ) or 2476 bp (2Rlinε). In every case, the sites are orientated in direct repeat (Janscak *et al.*, 1996). Reactions with EcoR124I were carried out as described above for 4alin and 4blin.

For each of the substrates, a combination of smeared and discrete products were observed (Figure 4(b)), similar to those found for 4alin (Figure 1(b)). The gels were analysed by densito-
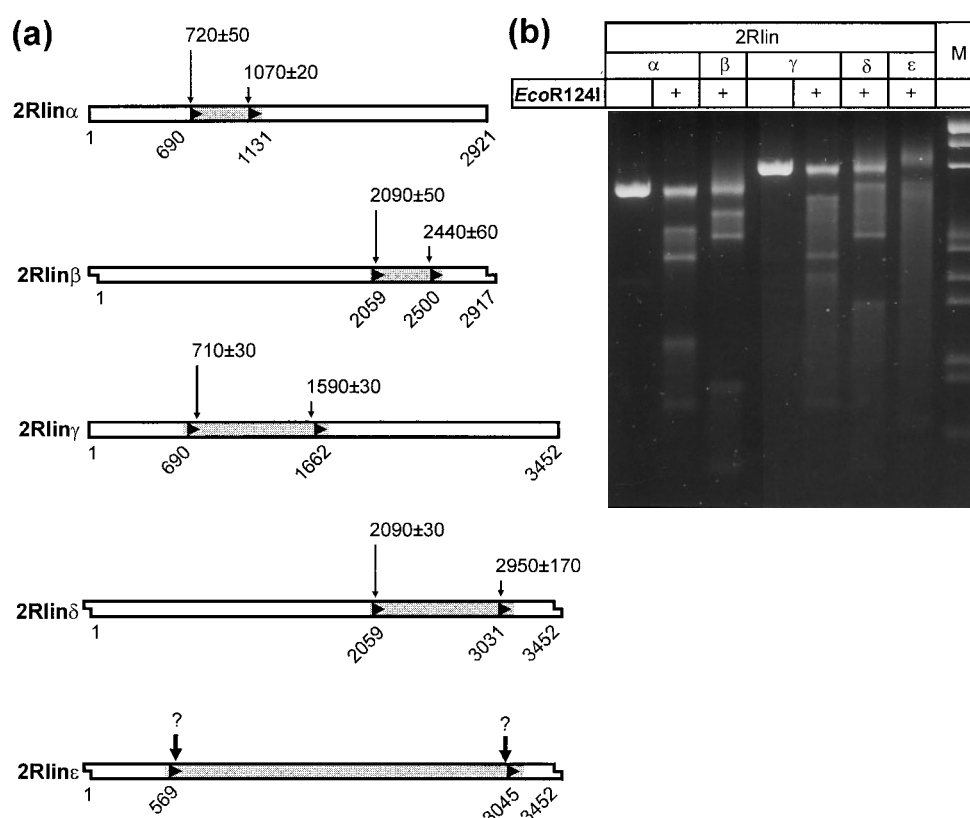
Figure 4. Mapping the cleavage between two *Eco*R124I sites as a function of distance between the sites. (a) Plasmid pDRM-2R(450) was cleaved with *Sca*I or *Afl*III to produce 2Rlinα and 2Rlinβ, whilst pDRM-2R(1000) was cleaved with *Sca*I, *Afl*III or *Hin*dIII to produce 2Rlinγ, 2Rlinδ and 2Rlinε. DNA representations, site numbering and orientations are equivalent to those in Figure 1(a). (b) Reactions for two minutes at 37°C contained 7 nM DNA and, where marked (+), 49 nM *Eco*R124I endonuclease. Products were separated on the agarose gel shown here. The marker lane (M) is as described in Figure 1(b). The loci for discrete cleavages on each of these substrates were mapped from densitometric traces of the gel (not shown): the positions are marked by vertical arrows on the representations of each DNA in (a). (The discrete loci on 2Rlinε could not be determined precisely (see the text) and are shown as question marks.) Standard deviations represent the variation in data from at least three experiments. The extent of the smeared DNA was estimated directly from the gel and is represented as grey regions.

metry (not shown), and the locations of preferred cleavage sites and the distribution of random cleavage sites were determined (Figure 4(a)). As with 4alin and 4blin, the smear of random fragments again corresponded to cleavages between the *Eco*R124I sites. As the distance between the sites was extended from 0.44 kb to 2.5 kb, the boundaries of the smeared DNA increased correspondingly (Figure 4(a)). This indicates that, within the limits tested here, neither the distance between two recognition sites nor the sequence of the intervening DNA affect the nature of the random cleavage events. In addition, the loci for discrete cleavages on these alternative substrates still mapped closely to the *Eco*R124I sites, typically ∼30 bp to the right of the first site and ∼70 bp to the left of the second site as drawn (Figure 4(a)). (The exact positions of the discrete sites on 2Rlinε could not be determined accurately: the distances between either *Eco*R124I site and the nearest end of the DNA were too small and too similar to allow for complete resolution of the products on agarose gels.) Nonetheless, the presence of preferred cleavage loci on all

substrates tested suggests that these reaction products are universal and are not dependent on particular sequences flanking the *Eco*R124I sites.

Cleavage of one-site circular DNA by *Eco*R124I

All of the results described above were obtained on linear DNA substrates. However, reactions on circular DNA do not require collisions between tracking enzymes (Szczelkun *et al.*, 1996) and a single recognition site is sufficient for rapid and complete cleavage of the DNA (Janscak *et al.*, 1996). Therefore, how do the patterns of digestion observed on linear substrates compare to those on circular molecules? To address this question, we employed the plasmid pDRM-1R (Janscak *et al.*, 1996): this DNA was used in the construction of pDRM-2R[450] and, as such, the 1098 bp site on the plasmid (Figure 5(a)) is equivalent to the 1131 bp site on 2Rlinα (Figure 4(a)). Cleavage of pDRM-1R was followed by agarose gel electrophoresis after reactions with a 1:1 molar ratio of enzyme and DNA. This ratio is considerably lower than
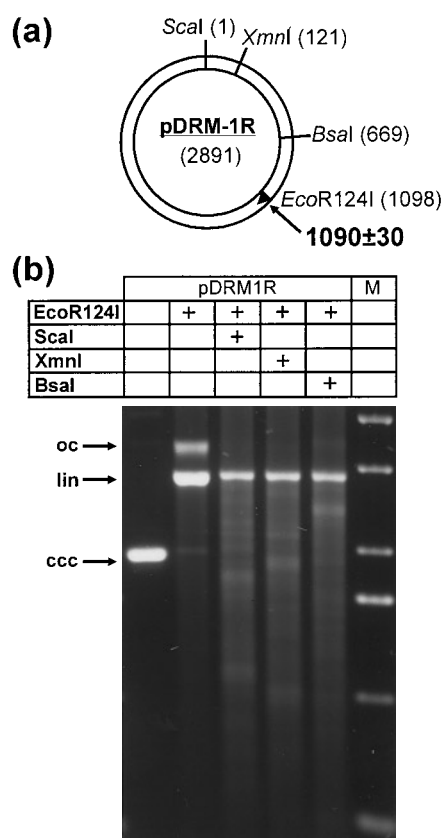
(a)

(b)

Figure 5. Mapping of the DNA products from digestion of one-site plasmid DNA by *Eco*R124I. (a), pDRM-1R is represented as a circle, with the *Eco*R124I site as a filled arrowhead oriented as in Figure 1(a). The *Sca*I site was arbitrarily designated as position 1. (b) Reactions contained pDRM-1R (50 nM) and, where indicated (+), *Eco*R124I (50 nM), *Sca*I (ten units), *Xmn*I (ten units) and *Bsa*I (10 units). Reactions with *Eco*R124I alone were incubated at 37°C for five minutes. When both type I and type II enzymes were used, the DNA was first digested with *Eco*R124I followed by the type II enzyme for a further 30 minutes. Samples were analysed on an agarose gel. The DNA marker (M) was a 1 kb ladder (Gibco-BRL, MD, USA), with visible fragments of 4072, 3054, 2036, 1636, 1018, 517 bp. The mobilities of the supercoiled (covalently closed circles: ccc), open circle (oc) and linear (lin) forms of the DNA are marked on the left of the gel. The sizes of the discrete fragments produced by digestions with both type I and type II enzymes were determined by densitometry and the estimated loci for *Eco*R124I cleavages illustrated by arrows in (a). Standard deviations are given as the variation in data from three experiments.

that needed for the assays on the linear substrates described above, but it was sufficient for complete cleavage of the circular DNA. Under the electrophoresis conditions used here, the linear product was clearly resolved from both the supercoiled substrate and the nicked open-circle intermediate (Figure 5(b)). To determine the positions of the double strand breaks, the DNA that had been linearised by a single *Eco*R124I cut was further

digested with *Sca*I, *Xmn*I or *Bsa*I (Figure 5(a)). If the circular DNA is cleaved by *Eco*R124I at random positions, then the subsequent digest with the type II enzyme should produce a uniform smear of DNA on the agarose gel. However, if the linearisation by *Eco*R124I occurs at preferred sites, the type II digest will generate discrete fragments and the preferred loci for *Eco*R124I can be mapped accordingly.

The application of the type II enzymes to the DNA that had been linearised by *Eco*R124I produced a large distribution of fragments, including both continuous smears and discrete bands (Figure 5(b)). In each case, bands were resolved from the background smear by densitometry (not shown). The most intense peaks corresponded to the fragments resulting from cleavage by EcoR124I at or near the recognition site (Figure 5(a)). Hence, the discrete cleavage sites for *Eco*R124I near its recognition sites are common to both circular and linear DNA. The smeared DNA fragments from the *Eco*R124I reactions on the circular substrate (Figure 5(a)) correlated to random cleavage sites throughout the entire plasmid. Equivalent results were also observed on another plasmid with one *Eco*R124I site, pMDS3 (not shown), thus ruling out any sequence-specific effects. Attempts to map cleavage sites on two-site plasmids, such as pMDS4a and pDRM-2R[450] (not shown), were hampered by these substrates undergoing multiple cleavages by *Eco*R124I alone (Szczelkun *et al.*, 1996).

Discussion

Random and localised cleavages by *Eco*R124I

Although type I restriction endonucleases specifically recognise their target sequences, the subsequent cleavage of the DNA generally occurs at random sites, often located many kb away from the cognate site (Bickle, 1993). The link between these two spatially distinct events is achieved by the linear motion of the protein along the DNA in an ATP-dependent tracking mechanism (Rosamund *et al.*, 1979; Yuan *et al.*, 1980). The current models for tracking make some predictions about the link between translocation and the ensuing DNA cleavage sites. On linear substrates carrying multiple recognition sites, cleavage may be facilitated by the collision of two translocating complexes halfway between a pair of sites (Shulman, 1974; Studier & Bandyopadhyay, 1988). Equivalent loci will not be observed on one-site linear DNA, as cleavage is then dependent on the collision between a tracking enzyme and a second non-specifically bound protein. However, on circular DNA, there is no requirement for tracking by two enzymes (Janscak *et al.* 1996; Dreier *et al.*, 1996). In these cases, cleavage may result from the topological tangling of the DNA concurrent with translocation by a single protein: indeed, it may be impossible for two enzymes tracking on circular

DNA to ever collide on account of the severe tangling of the intervening DNA (Szczelkun *et al.*, 1996). To test these concepts, DNA cleavage by the type IC restriction endonuclease *Eco*R124I was examined on a series of linear and circular substrates. The sizes of the ensuing DNA products were determined and the appropriate cleavage loci mapped. Our data reveal that *Eco*R124I cleaves DNA at both random and at localised sites.

For every substrate investigated, random cleavages were observed at distant loci well separated from the recognition site. On linear DNA carrying two sites, the random cleavages occurred predominantly between the sites, with the maximum frequency at the mid-point (Figure 1 and 2). Cleavage of distant loci between the cognate sites and the proximal ends of the DNA were rarely detected. When the distance between a pair of sites was increased from 0.4 to 2.4 kb, the distribution of random cleavages between the sites expanded accordingly (Figure 4). Random cleavage has been observed between pairs of sites separated by as little as 100 bp and as much as 7 kb (Adler & Nathans, 1973; Bickle *et al.*, 1978; Rosamund *et al.*, 1979; Yuan *et al.*, 1980; Dreier *et al.*, 1996; Dryden *et al.*, 1997). Overall, our data for random cleavages on linear DNA concur with the collision model of Studier & Bandyopadhyay (1988). The low level of distant cleavages on the one-site linear molecules (Figure 3) is probably a consequence of the rarity of collisions between tracking enzymes and nonspecifically bound proteins. The distribution of sites for random cleavage was independent of the relative orientation of the recognition sequences (Figure 2(b)), suggesting that *Eco*R124I can track in both directions from its site. This is inconsistent with models involving unidirectional motion in only one orientation (Rosamund *et al.*, 1979). However, we cannot distinguish between unidirectional motion with both leftwards and rightwards directions being allowed (Yuan *et al.*, 1980) and bi-directional motion, in which both sides of the DNA are tracked simultaneously to give two expanding loops (Studier & Bandyopadhyay, 1988).

On circular DNA, tracking will generate a figure-of-eight structure in which DNA passes from a contracting loop into an expanding loop (Ostrander *et al.*, 1990). After translocating a given length of DNA, a type I enzyme will have introduced a specific amount of twist into the contracting loop (Szczelkun *et al.*, 1996). Consequently, if the barrier to tracking is conferred by a precise level of twist and writhe, then cleavage should occur when the enzyme has covered a precise distance from the recognition site. However, the one-site plasmids underwent random cleavages throughout the DNA (Figure 5). Nevertheless, a topological model can still be justified for a number of reasons. Firstly, a plasmid from *Escherichia coli* possseses numerous topoisomers, each with a different linking deficiency (Bates & Maxwell, 1993). Each topoisomer will require a different change in twist to reach the same endpoint and

therefore tracking could continue for a different distance on each topoisomer before cleavage occurs. Secondly, we do not know how the effects of DNA rotation are propagated along the rest of the molecule; this could occur by local changes in twist at the bp level or by the relative rotation of complete segments of DNA. Furthermore, these changes could either be uniformly distributed throughout the contracting loop, or they could build up as regions of plectonemic or toroidal writhe immediately ahead of the translocating enzyme. Thirdly, no information is available on the processivity of the enzymes on circular DNA. Any combination of these effects could result in each DNA-protein complex needing a different level of twist and writhe to block further tracking. This could account for the random cleavages throughout the DNA.

Unexpectedly, the agarose gels used to analyse the products from *Eco*R124I reactions revealed not only the smear of heterogeneous DNA fragments from random cleavages, but also a series of discrete bands that stood out clearly from the background smear of DNA. The latter were due to DNA cleavages at loci close to the *Eco*R124I recognition sites (within ~250 bp). Although this localised cleavage was observed with every substrate analysed, the number and positions of the susceptible loci varied slightly. This may be due to the different flanking sequences around the *Eco*R124I sites but is more likely a reflection of the inconsistencies in determining the sizes of DNA fragment by electrophoresis (Sealey & Southern, 1990), which results in some imprecision in mapping the sites. Nonetheless, cleavage of these sites is a general phenomenon and is not specific to one particular substrate.

Preferential cleavage of DNA close to a recognition site has not been reported previously for a type I enzyme. It has yet to be determined whether the ability of *Eco*R124I to cleave DNA at both distant loci and at loci close to the recognition site is unique to this particular type IC enzyme. Other type IA and IB enzymes might still share this property though perhaps without generating such high yields of the discrete products. The activity of *Eco*R124I at loci close to its recognition site is reminiscent of the reaction catalysed by type III endonucleases (Bickle, 1993). The type III enzymes have an absolute requirement for a pair of sites in inverted repeat (Meisel *et al.*, 1992). The collision between two translocating type III enzymes results in DNA cleavages 25 to 26 bp from the sites (Meisel *et al.*, 1995). However, *Eco*R124I has no preference for sites in a particular orientation, nor are a pair of sites absolutely required (Janscak *et al.*, 1996). The question arises as to whether the random and localised cleavages by *Eco*R124I can result from the same tracking event or from two unrelated events?
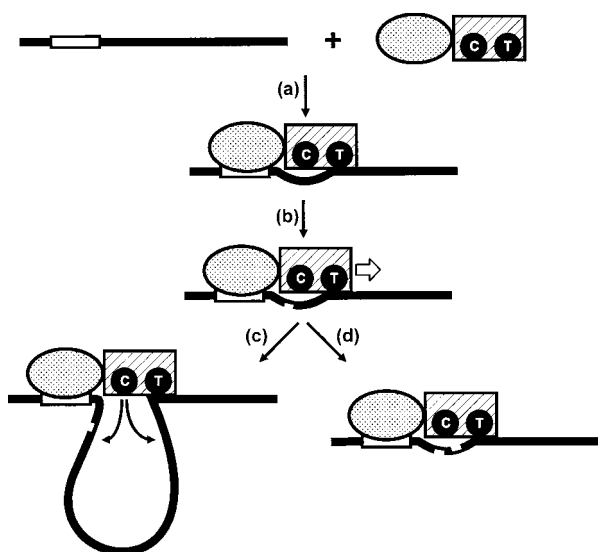
Figure 6. Model of nicking and cleavage of expanding DNA loops by *Eco*R124I. A region of a long DNA is represented as a continuous line, with an *Eco*R124I site as a open box. DNA nicks are shown as indentations in the continuous line. The complex of the HsdS and HsdM subunits of *Eco*R124I are represented as a stippled oval. The HsdR subunit is represented as a hatched box, with catalytic centres for DNA cleavage and translocation shown as black circles marked C and T, respectively. (a) *Eco*R124I binds DNA; the HsdS/HsdM complex to the recognition site and the T unit of HsdR to a proximal non-specific DNA, trapping a small loop of DNA. (b) DNA from the right is translocated past the T moiety on HsdR whilst HsdS remains bound to the cognate site. The complex thus moves in the direction indicated by the white arrow, extruding the expanding loop. However, the expanding loop must be nicked by R to allow for the rotation of DNA as it passes T. (c) Whenever tracking stalls, C can cleave either the DNA adjacent to the recognition site, located on the constant side of the expanding loop, or distant loci on the DNA molecule, on the variable side of the loop. (d) Alternatively, if tracking does not initiate immediately after the first nick (b), then C could re-bind to the DNA and cut the intact strand near the position of the first nick, thus making a double-strand break close to the recognition site without extensive tracking.

Nicking and cleavage of DNA loops during tracking by *Eco*R124I

The activities of *Eco*R124I in cutting DNA both close to and distant from its recognition site cannot be reconciled to the current models for DNA tracking by type I enzymes. However, a general model for both type I and type III endonucleases (Meisel *et al.*, 1995) can be adapted to account for this behaviour (Figure 6). Our model starts with the *Eco*R124I enzyme making two contacts to the DNA (Figure 6(a)). Firstly, the HsdS subunit binds specifically to the recognition sequence (Kusiak *et al.*, 1992). The second contact is made by the HsdR subunit binding to the DNA that is immedi-

ately adjacent to the recognition site (Szczelkun *et al.*, 1996). It will be assumed that, despite any asymmetry in the binding of HsdS to its cognate site (Mernagh & Kneale, 1996), the resulting domain organisation still provides a pseudo-dyad symmetry for the assembly of the HsdM and HsdR subunits (Kneale, 1994; Dryden *et al.*, 1995, 1997). For simplicity, the model is illustrated with a single HsdR subunit associating with the HsdS/HsdM complex, to generate unidirectional translocation (Figure 6); the direction is arbitrary. The model can readily be extended to accommodate multiply associated HsdR subunits (Dryden *et al.*, 1997; P.J & K.F., unpublished), or bi-directional transloca-tion *via* a twin loop model (Studier & Bandyopadhyay, 1988; Dreier *et al.* 1996). HsdR is marked with two functional units, a catalytic centre (C) for the DNA scission and a translocation centre (T) for the ATP-dependent motion of the DNA past the protein: T rather than C makes the initial contact to the DNA adjacent to the recog-nition site though C still lies close to the DNA at this stage (Figure 6a). The justification of assigning the translocation activity of the protein to HsdR comes from the ATPase activity of the isolated HsdR subunit (Zinkevich *et al.*, 1997). Furthermore, a series of conserved sequence motifs found in most HsdR subunits (Webb *et al.*, 1996) form the ATP-binding cleft in the *Pcr*A helicase from *Bacillus stearothermophilus* (Subramanya *et al.*, 1996).

The proximity of the DNA contacts made by HsdS and HsdR produces a relatively small ex-panding loop (Figure 6(a)). This close link absol-utely constrains the endonuclease to act on DNA sites *in cis* (Szczelkun *et al.*, 1996). Since tracking will probably require rotation of the DNA as it moves past the protein, any accompanying decrease in twist in the expanding loop could not be accommodated without an excessive build up of supercoiling torque. To overcome this energetic barrier, the loop needs to be nicked before (or soon after) tracking begins (Figure 6(b): Szczelkun *et al.*, 1996). Most type I endonucleases nick DNA in one strand and cleave the second strand in a later reac-tion (Bickle, 1993). Furthermore, relaxed expanding loops have been observed directly by electron microscopy (Endlich & Linn, 1985). Once the ex-panding loop has been nicked by transiently enga-ging the C moiety of HsdR, translocation can proceed. As ATP is hydrolysed, DNA will be pulled past the enzyme at T whilst HsdS/HsdM remains tightly associated with the cognate site. The DNA ahead of the translocating complex in the contracting domain thus passes into the ex-panding loop and the latter increases in size (Rosamund *et al.*, 1979; Yuan *et al.*, 1980). Even-tually, tracking will stop, either due to a collision with a second protein on linear DNA or by the build up of topological strain on circular mol-ecules. The halt in translocation might allow the C moiety in HsdR to re-engage the DNA but, at this stage, the expanding loop of DNA in the stalled complex presents two alternative cleavage sites

close to C (Figure 6(c)). One is the DNA adjacent to the *Eco*R124I site, whose position remains constant as the loop expands, and the other is the variable DNA on the "other side" of the loop that can potentially come from anywhere along the length of the DNA molecule. If there exists some flexibility in the positioning of C against the DNA, cleavage could be equally divided between these constant and variable regions (Figure 6(c)). Over a large population of stalled species, perhaps half would produce cleavages close to the *Eco*R124I site, irrespective of the final loop size. A precedent for this flexibility is the monomeric type IIs endonuclease *Fok*I, which utilises a mobile catalytic domain to cleave both strands of the DNA downstream of its recognition site (Waugh & Sauer, 1993).

In pathway c in Figure 6, the initiation of tracking as soon as the expanding loop has been nicked prevents C from cleaving the uncut strand at this stage. The re-binding of C to the DNA perhaps occurs only when tracking has stopped. However, an alternative mechanism to account for DNA cleavages close to the *Eco*R124I recognition sites is that, if the time between the introduction of the first nick and the initiation of tracking is sufficiently long, C could re-bind the small expanding loop and introduce a nick in the second strand before any extensive translocation had taken place (Figure 6(d)). It might have been thought that extensive translocation was a prerequisite for DNA cleavage by a type I endonuclease, since these enzymes hydrolyse large amounts of ATP during and after their DNA cleavage reactions (Eskin & Linn, 1972; Reiser & Yuan, 1977; Endlich & Linn, 1985; Dreier & Bickle, 1996). While the DNA cleavage activity requires ATP, the large amounts of ATP utilised by these enzymes suggests some degree of uncoupling, with not all of the ATP being used for DNA motion.

In their general model for both type I and type III endonucleases, Meisel *et al.* (1995) proposed that type III enzymes cleave DNA close to their recognition sites by attacking the constant side of the expanding loop while type I enzymes randomly cleave DNA at distant loci by attacking the variable side of the expanding loop. The observation that a type IC endonuclease, *Eco*R124I, can make both localised and distant cleavages suggests that one enzyme can attack either side of the loop (Figure 6(c)). Alternatively, all of the localised cleavages could come from double-strand breaks prior to extensive tracking (Figure 6(d)). It is interesting to note that, on most substrates, both localised and distant cleavage are more or less equally represented (Figures 1, 2 and 4), which is consistent with pathway c in Figure 6. However, on the one-site linear DNA, the majority of the DNA cleavage reactions occurred close to the recognition site (Figure 3). This preference could be due to the relative positions of the constant and variable domains in the expanding loop, such that cleavage within the constant region is favoured, but the one-site

linear DNA may be cleaved mainly by pathway d. Further analysis of this model will require structural information on the positioning of the DNA at both ends of the loop relative to the catalytic centre in HsdR, and on the precise nature of the DNA cleavage reactions that give rise to the single-strand and double-strand breaks.

Materials and Methods

Proteins

The *Eco*R124I endonuclease was reconstituted from separate preparations of the methyltransferase (Taylor *et al.*, 1992) and HsdR (Zinkevich *et al.*, 1997), each purified as described previously. Protein concentrations were calculated from the absorbance at 280 nm using molar extinction coefficients derived from amino acid sequences (methyltransferase; 160 400 M^{-1} cm^{-1}; HsdR 91 900 M^{-1} cm^{-1}). The endonuclease was reconstituted immediately before use by mixing 6 moles of HsdR per mole of methyltransferase in twofold diluted buffer R (buffer R is 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM AdoMet). The final concentration of endonuclease was taken as the input concentration of the methyltransferase. The reconstituted enzyme had a maximal DNA cleavage activity at 1 mole endonuclease per mole of circular DNA (not shown), as compared to 5:1 for the endonuclease purified in a single preparation (Szczelkun *et al.*, 1996): the latter had a deficit of HsdR subunits (Janscak *et al.*, 1996). No residual methyltransferase activity was observed as judged by DNA cleavage going to 100% completion (not shown). All other enzymes were purchase from New England Biolabs (MA, USA) and used as directed.

DNA

Unless stated otherwise elsewhere, all DNA manipulations were carried out according to standard procedures (Sambrook *et al.*, 1989). The construction of pMDS3, pMDS4a, pMDS4b, and pDRM-1R and pDRM-2R[450] (previously pDRM-2R), are all described elsewhere (Szczelkun *et al.*, 1996; Janscak *et al.*, 1996). pDRM-2R[1000] was cloned by cleaving pDRM-2R[450] in the short arc between its *Eco*R124I sites with *Hin*dIII and *Sph*I, and ligating in the 533 bp *Hin*dIII / *Sph*I fragment from pACYC184 (Chang & Cohen, 1978). This changed the spacing between the *Eco*R124I sites from 441 bp to 972 bp. The plasmids were used to transform *E. coli* HB101 (Sambrook *et al.*, 1989), and grown in M9 minimal medium with 1 mCi/l [^{3}H-*methyl*]thymidine. The covalently closed form of the each DNA was purified by density centrifugation in CsCl / EtBr (Vipond *et al.*, 1995). Plasmid DNA was used directly, whilst linear DNA was produced by pre-digesting the DNA with the requisite type II endonuclease.

*Eco*R124I restriction reactions

For the mapping reactions, either 7 nM DNA (linear substrates) or 50 nM DNA (plasmid substrates) was digested with the necessary concentration of *Eco*R124I at 37°C in buffer R. Reactions were started by the addition of 2 mM ATP and aliquots quenched by the addition of 0.5 volumes of stop solution (0.1 M EDTA, 0.1 M Tris-HCl (pH 8.0), 40% (w/v) sucrose). Optimal reaction times were determined empirically (not shown). DNA

species were separated by electrophoresis on 1.2% (w/v) agarose gels at 2 V/cm, for ~15 hours at 4°C in TAE (40 mM Tris-acetate, 1 mM EDTA) with 0.5 μg/ml EtBr. A threefold dilution of stop solution plus 0.4 mg/ml bromophenol blue was run in all empty wells. Gels were further stained in TAE with EtBr for one hour at 4°C prior to imaging with a CCD camera and a Gel Documentation System (UVP, Cambridge, UK).

Estimation of fragment size

The size of unknown DNA fragments was estimated from their mobility, as measured on an expanded density profile of each gel lane generated by ImageQuant software (v3.3, Molecular Dynamics Inc., CA, USA). Sizes were calculated by comparison with the relative mobilities of the marker fragments using a 2×3 point analysis (Sealey & Southern, 1990). This method requires four size-standards relative to the unknown band; two of lower mobility and two of higher mobility. The size of any fragment which fell outside this range was approximated from a plot of DNA size against ln(mobility) which, under our electrophoresis conditions, showed a linear relationship for fragments <5000 bp (not shown). Wherever possible, all the fragments resulting from a single reaction by *Eco*R124I were estimated. However, fragments of <500 bp were not adequately resolved by densitometry due to their weak fluorescence with EtBr and the distribution of the band on the agarose gel. In these cases, only the larger fragments were used to calculate cleavage loci.

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