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Functional cooperation between exonucleases and endonucleases—basis for the evolution of restriction enzymes

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ABSTRACT

Many types of restriction enzymes cleave DNA away from their recognition site. Using the type III restriction enzyme, *Eco*P15I, which cleaves DNA 25–27 bp away from its recognition site, we provide evidence to show that an intact recognition site on the cleaved DNA sequesters the restriction enzyme and decreases the effective concentration of the enzyme. *Eco*P15I restriction enzyme is shown here to perform only a single round of DNA cleavage. Significantly, we show that an exonuclease activity is essential for *Eco*P15I restriction enzyme to perform multiple rounds of DNA cleavage. This observation may hold true for all restriction enzymes cleaving DNA sufficiently far away from their recognition site. Our results highlight the importance of functional cooperation in the modulation of enzyme activity. Based on results presented here and other data on well-characterised restriction enzymes, a functional evolutionary hierarchy of restriction enzymes is discussed.

INTRODUCTION

A wide variety of restriction–modification (R-M) systems have been discovered and characterised. They are classified based on their subunit composition, cofactor requirement and mode of DNA cleavage (1). Table 1 summarises the characteristic features of the main R-M systems. Restriction enzymes cleaving DNA in both the type I or type III classes are dependent upon ATP and *S*-adenosyl-L-methionine (AdoMet) (2,3). Type III enzymes require two inversely oriented, asymmetric, unmethylated recognition sites on the same DNA molecule to introduce one double strand break between the two sites. Type I enzymes are able to cleave supercoiled DNA containing only one site but, in general, restrict DNA containing multiple sites more effectively. Type I enzymes cleave many thousands of base pairs away from the recognition site, while type III enzymes cleave 25–27 bp away from the recognition site. ATP-independent restriction enzymes do not perform DNA translocation and include all types other than types I and III. A variety of subtypes belonging to type II have been characterised (4). Type IIE restriction enzymes such as *Nae*I and *Eco*RII interact with two copies of their recognition sequence, one being the target for cleavage and the other serving as an allosteric effector. Type IIF restriction endonucleases such as *Ngo*MIV also interact with two copies of their recognition sequence but cleave both sequences in a concerted reaction. Both types IIE and IIF cleave DNA within the recognition site. Type IIG enzymes cleave 14–16 bp away and type IIS enzymes cleave 9–13 bp away from the recognition site while type IIB enzymes cleave 10–12 bp away on both sides of the recognition site. This contrasts with the orthodox type II restriction enzymes that cleave DNA within the recognition site.

The consequences of the presence of an intact recognition site upon restriction enzyme activity after cleavage of the DNA by the enzyme have not yet been addressed. While essentially all restriction enzymes require the divalent metal ion, Mg^{2+}, for DNA cleavage, types I and III in addition require ATP and AdoMet. It has also been shown that AdoMet stimulates DNA cleavage by type IIB and type IIG restriction endonucleases.

Restriction enzymes bear little sequence similarity, yet all of them contain functionally conserved motifs and structurally conserved domains (4). Lack of sequence similarity hinders sequence based phylogenetic analysis and computational modelling. As more R-M systems are found and characterised, new ones with novel subunit structures, cofactor requirements and cleavage patterns are still being identified. Although several investigators have proposed possible evolutionary links among various types of restriction enzymes based on structural and sequence data (5–7), a clear evolutionary link among the well-characterised types of restriction enzymes has not been established.

*Eco*P15I restriction enzyme (R.*Eco*P15I), a member of the type III R-M system, is composed of two subunits, restriction (Res, R) and modification (Mod, M) and recognises an asymmetric sequence, 5′-CAGCAG-3′ (8). The DNA binding domain and methyl donor AdoMet binding domain are present only in the Mod subunit (75 kDa) (9). The ATPase, helicase and endonuclease domains are present in the Res subunit (106 kDa) (10). Res cannot bind DNA independent of Mod and is degraded when expressed alone (11). The Mod subunit alone can methylate DNA at the N^6 position of the second adenine in the recognition site (12) and is a dimer, Mod_2, in solution (13). Two inversely oriented (→ ←) unmethylated

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Table 1. Characteristic features of R-M systems

	MTase subunits	ENase subunits	Site requirement for cleavage	Site of cleavage[a]
ATP, AdoMet and Mg^{2+}-dependent restriction enzymes				
Type I	S, M	S, M, R	Two	1000–7000 bp
Type III	M	M, R	Two	25–27 bp
AdoMet and Mg^{2+}-dependent restriction enzymes				
Type IIG	M	M + R	One	14–16 bp
Type IIB	A, B	A, B	One	10–12 bp on either side of site
Mg^{2+}-dependent restriction enzymes				
Type IIS	M	R	One	9–13 bp
Type II	M	R	One	Within site

M and R refer to methyltransferse and endonuclease subunits, respectively. A and B refer to A chain and B chain, respectively.
[a]Distance of cleavage site with respect to recognition site is given.

sites are the substrates for cleavage by type III restriction enzymes. The intervening sequence between these sites can be anywhere between 30 and 3000 bp in length (14). Physical interaction of enzyme molecules bound to both sites is essential for DNA cleavage to occur. It is proposed that ATP hydrolysis allows the interaction of the enzyme molecules during the cleavage reaction (15). More recently, the presence of the methyl donor AdoMet has been shown to be mandatory for restriction (16).

In general, phage DNA after endonuclease restriction is further degraded by bacterial exonucleases (17,18). The role of exonucleases in processes such as DNA replication, recombination and repair has been well documented in various organisms (19). Although restriction by *Eco*KI was observed to be reduced in the absence of the RecBCD exonuclease (20), there has been no study demonstrating essentiality of exonucleases for the activity of restriction enzymes. In the present study, we have explored possible functional cooperation between exonucleases and endonucleases. The effect of intact recognition sites on cleaved DNA on restriction enzyme activity has been investigated. Based on our results and the biochemical, structural and sequence data of well characterised R-M systems, we propose a model by which restriction enzymes might have evolved through domain duplication, deletion and shuffling to acquire the ability to cleave DNA within their recognition site.

MATERIALS AND METHODS

Chemicals used

AdoMet, *S*-adenosyl-L-homocysteine (AdoHcy), sinefungin, bovine serum albumin, adenosine-5′-triphosphate (sodium salt), ampicillin, HEPES, polyethyleneimine, Coomassie Brilliant Blue R-250 and RNase A were from Sigma Chemical Company, USA. Restriction enzymes and exonuclease III were from New England Biolabs, USA. The DNA gel elution kit was from Amersham Pharmacia Biotech, Asia Pacific Ltd, Hong Kong. All other reagents used were of the highest grade.

DNA cleavage assay

DNA was incubated with purified R.*Eco*P15I at 37°C for 60 min in the HEPES reaction buffer [100 mM HEPES (pH 8.0), 0.25 mM EDTA, 6.4 mM $MgCl_2$, 12 mM 2-mercaptoethanol] in the presence of 1 mM ATP, except where indicated otherwise. This was followed by Proteinase K treatment at 56°C for 1 h [20 mg/ml Proteinase K in 20 mM EDTA, 0.5% (w/v) SDS]. The digests were analysed by electrophoresis in TAE at 100 V on 1% (w/v) agarose gels for 1 h. All gels were documented and DNA bands were quantitated using UVI Tech gel documentation system. All assays used a 25 µl reaction volume, except for those with non-specific DNA (Fig. 3C), which used a 60 µl reaction volume and 2 h of electrophoresis.

Purification of *Eco*P15I restriction enzyme

R.*Eco*P15I enzyme was purified according to the protocol described previously (16). Enzyme purity was monitored by SDS–polyacrylamide gel electrophoresis (21) and found to be homogeneous. Purified enzyme was checked for ATP-dependent and AdoMet-independent cleavage of DNA, indicative of enzyme-bound AdoMet. Titration of the enzyme with DNA was accomplished by incubating increasing amounts of the enzyme with supercoiled pUC19 DNA (2 µg/3.37 pmol *Eco*P15I sites) for 1 h at 37°C in the reaction buffer and analysed by electrophoresis. The minimum amount of enzyme yielding complete DNA cleavage was considered to represent a 1:1 ratio of enzyme to *Eco*P15I site on DNA.

DNA preparations

pUC19 DNA was prepared as described (22) and found to be 95% supercoiled. pUC19 DNA linearised by R.*Eco*P15I was prepared by incubating supercoiled pUC19 DNA with R.*Eco*P15I in the presence of 1 mM ATP for 1 h at 37°C in reaction buffer. Linearised DNA was purified using the gel elution kit. R.*Eco*P15I acts as methyltransferase in the absence of ATP and in the presence of AdoMet (23). DNA methylated by R.*Eco*P15I was prepared by incubating the supercoiled pUC19 DNA with excess enzyme in the absence of ATP for 3 h at 37°C. DNA was checked for complete methylation by subjecting it to R.*Eco*P15I restriction digestion in the presence of ATP at 37°C for 1 h. The absence of any linearisation of supercoiled DNA was considered as indicative of complete *Eco*P15I methylation. *Eco*P15I methylated DNA was further linearised with *Pst*I to yield a linear *Eco*P15I methylated pUC19 DNA. Non-specific DNA was prepared by restriction digestion of supercoiled pUC19 DNA with *Hin*fI restriction

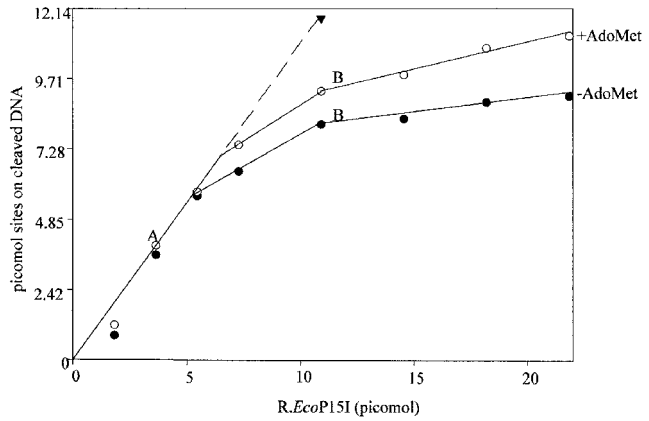

Figure 1. DNA cleavage as a function of R.*Eco*P15I concentration in the absence and presence of exogenous AdoMet. The amount of supercoiled pUC19 DNA (7.2 µg) in each assay corresponds to 12.14 pmol *Eco*P15I sites. The DNA cleavage assays were carried out in the HEPES reaction buffer at 37°C for 1 h as described. The amount of cleaved DNA was quantitated from intensities of the product and substrate bands after staining the agarose gel with ethidium bromide. The linear phase, indicating stoichiometric cleavage by R.*Eco*P15I, is labelled as A, the non-linear phase of cleavage is labelled as B. The dashed line indicates the assay done with supercoiled pUC19 DNA containing 24.28 pmol *Eco*P15I sites as described in the text.

enzyme followed by agarose gel electrophoresis and purification of the 1419 bp pUC19 DNA *Hin*fI fragment. DNA was quantified by absorbance at 260 nm assuming an OD of 1.0 corresponds to 50 µg/ml of double stranded DNA.

DNA cleavage in the presence of exonuclease III

These reactions were carried out in Lac buffer (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol) instead of HEPES reaction buffer for 3 or 6 h as indicated in the text. Fifty units of exonuclease III (NEB) was added to each reaction at time zero.

RESULTS AND DISCUSSION

DNA cleavage versus R.*Eco*P15I concentration

The amount of DNA cleaved by increasing amounts of R.*Eco*P15I was studied to establish the relationship between DNA cleavage activity and enzyme concentration. Supercoiled pUC19 DNA containing a pair of recognition sites in inverse orientation (→ ←) was the substrate. Increasing amounts of R.*Eco*P15I enzyme (1.82–21.84 pmol) were added to supercoiled pUC19 DNA (7.2 µg/12.14 pmol *Eco*P15I sites) in a standard restriction assay and incubated for 1 h at 37°C. The result is shown in Figure 1 (filled circles). Initially, the amount of DNA cleaved is directly proportional to the enzyme concentrations (Fig. 1, phase A), indicating that the R.*Eco*P15I catalysed reaction is stoichiometric with respect to enzyme concentration and that it performs a single round of catalysis *in vitro*. However, there is a non-linear relationship between the amount of DNA cleaved and higher concentrations of R.*Eco*P15I (Fig. 1, phase B). Phase B shows that at concentrations >6.3 pmol of enzyme, there is sub-stoichiometric cleavage. One possibility for the stoichiometric cleavage observed in phase A as opposed to multiple rounds of cleavage could be limitation of one or other of the reaction component(s). Such a limitation might become more apparent at higher concentrations of the enzyme leading to the observed sub-stoichiometric cleavage seen in phase B. Sub-stoichiometric cleavage could be a consequence of oligomerisation of enzyme at higher concentrations or shortage of ATP or AdoMet.

The concentration of ATP and/or time of incubation could affect the amount of DNA cleaved. Increasing the concentration of ATP in the restriction assay (1–5 mM) or increasing the incubation period up to 3 h, did not seem to have any significant effect on DNA cleavage, at any enzyme concentration used in Figure 1 (data not shown).

Recently, it has been shown that the cofactor AdoMet is required for cleavage by type III restriction enzymes and that R.*Eco*P15I copurifies with bound AdoMet (16). The molecular details of the role of AdoMet in cleavage are not yet clear, but it has been suggested that AdoMet binding causes conformational changes in the restriction enzyme which are essential for cleavage (16). AdoMet copurified with R.*Eco*P15I might be sufficient to support only one round of cleavage (Fig. 1, phase A). Therefore, the above restriction assays were repeated in the presence of 20 µM exogenous AdoMet. The progress of DNA cleavage as a function of R.*Eco*P15I concentration in the presence of 20 µM exogenous AdoMet is shown in Figure 1 (open circles). As there was no detectable increase in DNA cleavage in phase A, it would imply that exogenously added AdoMet did not increase the catalytic activity of the enzyme. These results clearly demonstrate that R.*Eco*P15I does not perform multiple rounds of catalysis *in vitro*. However, a 10% increase in the amount of DNA cleaved was observed in the non-linear phase B in the presence of 20 µM exogenous AdoMet (open circles) relative to assays without exogenous AdoMet (closed circles). This 10% increase in cleavage at all enzyme concentrations in phase B indicates that 10% of the R.*Eco*P15I had lost AdoMet and became re-activated upon the addition of exogenous AdoMet.

We next studied the effect of an increased DNA substrate concentration. Incubation of double the amount of supercoiled pUC19 DNA (24.28 pmol sites) with 11.0 pmol of R.*Eco*P15I for 1 h at 37°C resulted in stoichiometric cleavage of half of the pUC19 DNA (11.8 pmol sites) (closed inverted triangle on dashed line in Fig. 1). This indicates that although excess substrate DNA alleviated the decrease in enzyme activity seen in phase B of Figure 1, there was still only a single round of cleavage by the enzyme. This rules out the possibility of oligomerisation of enzyme at higher concentrations, as the activity of oligomers would not have been affected by increasing substrate DNA concentration.

To study the effect of DNA concentration upon cleavage by R.*Eco*P15I enzyme in phase A of Figure 1, 4.2 pmol of R.*Eco*P15I was incubated with supercoiled pUC19 DNA (4.6–13.9 pmol *Eco*P15I sites) for 1 h at 37°C. When the amount of substrate DNA (4.6 pmol *Eco*P15I sites) was slightly more than that of enzyme (4.2 pmol of R.*Eco*P15I) (Fig. 2A, lane 1), a detectable amount of supercoiled DNA (~8%) was left uncleaved. Even when the total amount of substrate DNA was in great excess over the enzyme, DNA corresponding to only 4.2 pmol *Eco*P15I sites was cleaved (Fig. 2A, lanes 2 and 3). When an assay containing 4.2 pmol of R.*Eco*P15I and supercoiled pUC19 DNA containing 4.6 pmol

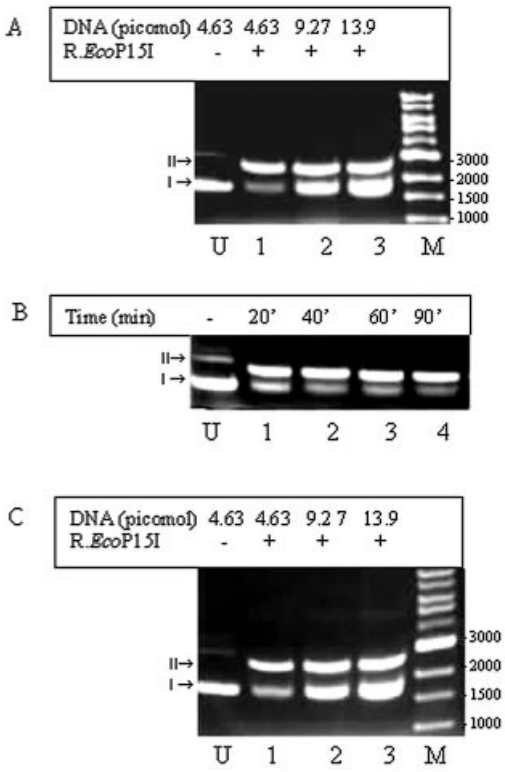

Figure 2. DNA cleavage by R.*Eco*P15I as a function of supercoiled pUC19 DNA concentration. (**A**) In the absence of exogenous AdoMet. Increasing amounts of supercoiled pUC19 DNA were incubated with R.*Eco*P15I (4.2 pmol) and DNA cleavage assays were carried out as described. Lane 1, 4.63 pmol *Eco*P15I sites; lane 2, 9.27 pmol *Eco*P15I sites and lane 3, 13.9 pmol *Eco*P15I sites. (**B**) Time course of cleavage. Supercoiled pUC19 DNA (4.63 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (4.2 pmol) in the absence of exogenous AdoMet for different periods of time and the assay performed as described. Lane 1, 20 min; lane 2, 40 min; lane 3, 60 min and lane 4, 90 min. (**C**) In the presence of exogenous AdoMet (20 μM). Increasing amounts of supercoiled pUC19 DNA were incubated with R.*Eco*P15I (4.2 pmol) and DNA cleavage assays performed as described. Lane 1, 4.63 pmol *Eco*P15I sites; lane 2, 9.27 pmol *Eco*P15I sites and lane 3, 13.9 pmol *Eco*P15I sites. Lanes U and M, uncut supercoiled pUC19 DNA and marker DNA fragments (sizes given to the right in base pairs). I and II represent supercoiled and linearised pUC19 DNA, respectively.

of *Eco*P15I sites was carried out, the kinetics of cleavage showed that most of the DNA was cleaved within 20 min (Fig. 2B, lane 1). The effect of exogenous AdoMet was tested by repeating the above assays in the presence of 20 μM exogenous AdoMet. As can be seen in Figure 2C, the extent of cleavage was not significantly different compared with that in Figure 2A, indicating that addition of exogenous AdoMet had no appreciable effect on the extent of DNA cleavage.

Sequestration of the enzyme

R.*Eco*P15I has been shown to hydrolyse ATP in the presence of a DNA recognition site (15). Similar observations were obtained with the closely related type III enzyme R.*Eco*PI (24), whose Res subunit is almost identical to that of R.*Eco*P15I (25). Mutations in the helicase motifs of R.*Eco*PI affecting ATP hydrolysis abolished DNA cleaving ability (26,27). DNA translocation during the cleavage reaction catalysed by these enzymes has been proposed to be assisted by ATP hydrolysis. ATP hydrolysis by R.*Eco*PI was shown to continue even after DNA cleavage (S.Saha and D.N.Rao, unpublished results). It has been reported that R.*Eco*P15I binds both methylated and unmethylated recognition sites and hydrolyses comparable amounts of ATP in a manner independent of DNA length and restriction (15). One interpretation of these findings could be that continued ATP hydrolysis after cleavage leads to a low dissociation or 'off' rate of the enzyme from the cleaved DNA. The lack of increase in DNA cleavage in our assays carried out over 3 h supports this interpretation. In addition, binding of any free restriction enzyme to cleaved DNA, thus decreasing the concentration of free enzyme in the assay, could elicit site-specific ATPase activity and a low off rate of enzyme from DNA.

To test if cleaved DNA was responsible for the decrease in R.*Eco*P15I concentration and thus its restriction activity, supercoiled pUC19 substrate DNA (4.6 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I enzyme (4.2 pmol) in the presence of either increasing amounts of R.*Eco*P15I cleaved pUC19 DNA or R.*Eco*P15I methylated pUC19 DNA or non-specific DNA (1419 bp *Hin*fI fragment of pUC19). DNA cleavage by R.*Eco*P15I decreased (as evidenced by the increase in uncleaved supercoiled DNA) in a dose-dependent manner in the presence of R.*Eco*P15I cleaved DNA (Fig. 3A) or R.*Eco*P15I methylated DNA (Fig. 3B) but not in the presence of non-specific DNA (Fig. 3C). Hence, the sequestration of enzyme by DNA is dependent upon there being a recognition site on the DNA. These results demonstrate that binding of enzyme molecules to cleaved DNA or methylated DNA decreases the restriction activity of the enzyme. As demonstrated previously with methylated and unmethylated DNA (15), DNA cleaved by *Eco*P15I restriction enzyme and methylated DNA (Fig. 3A and B) seem to elicit ATPase activity of enzyme and result in a low off rate of enzyme from DNA. Sequestration of enzyme by cleaved DNA thus leads to a decrease in the effective number of enzyme molecules.

Non-linearity in the amount of DNA cleaved at high concentrations of R.*Eco*P15I (Fig. 1, phase B) could, therefore, be a consequence of the accumulation of cleaved DNA sequestering free enzyme molecules. This would require that the recognition site be exposed on the accumulating cleaved DNA to allow site-specific sequestration of free enzyme molecules. In the absence of multiple rounds of catalysis, as evident in Figure 1 (phase A), it can be postutated that the enzyme molecules once they cleave stay bound to DNA but not necessarily at the recognition site. Absence of turnover by R.*Eco*P15I enzyme, demonstrated here, combined with an earlier observation of a weak footprint in the presence of both ATP and AdoMet (28) support this interpretation. Doubling the amount of supercoiled pUC19 DNA in an assay would decrease the proportion of accumulating cleaved DNA and also enhance the binding of enzyme molecules to supercoiled DNA. As expected, stoichiometric cleavage was restored in the assay when double the amount of supercoiled DNA was used (Fig. 1, closed inverted triangle on dashed line). Sequestration of the enzyme by cleaved DNA resulting in a single round of cleavage (Fig. 3A and Fig. 1, phase A) with R.*Eco*P15I may also explain the single round of cleavage observed with the functionally related type I restriction enzymes (29). It is possible that other types of restriction enzymes that cleave DNA sufficiently far away from their

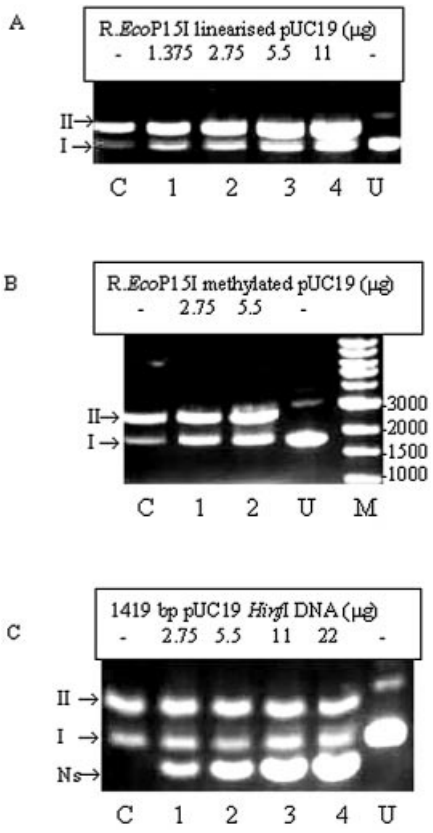

Figure 3. Sequestration of *Eco*P15I restriction enzyme. (**A**) Supercoiled pUC19 DNA (4.63 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (4.2 pmol) in the presence of increasing amounts of R.*Eco*P15I cleaved pUC19 DNA. Lane 1, 1.375 μg; lane 2, 2.75 μg; lane 3, 5.5 μg and lane 4, 11 μg. (**B**) Supercoiled pUC19 DNA (4.63 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (4.2 pmol) in the presence of increasing amounts of R.*Eco*P15I methylated pUC19 DNA. Lane 1, 2.75 μg and lane 2, 5.5 μg. (**C**) Supercoiled pUC19 DNA (4.63 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (4.2 pmol) in the presence of increasing quantities of non-specific DNA. Lane 1, 2.75 μg; lane 2, 5.5 μg; lane 3, 11 μg and lane 4, 22 μg. I, II and Ns represent supercoiled pUC19 DNA, linearised pUC19 DNA and 1419 bp pUC19 DNA *Hinf*I fragment, respectively. Lane C in all three panels: supercoiled pUC 19 DNA (4.63 pmol *Eco*P15I sites) incubated with R.*Eco*P15I (4.2 pmol). Lanes U and M, uncut supercoiled pUC19 DNA and marker DNA fragments, respectively.

recognition site are also sequestered by cleaved DNA and may exhibit slower cleavage kinetics.

Phage restriction is not generally absolute and phage genomes that do escape cleavage normally become modified. Progeny of such modified phages are protected against restriction by bacteria with R-M system of the same specificity (30). The molecular basis of this phenomenon has so far remained obscure. The sequestration phenomenon described here provides a clue to understanding how non-resistant phages acquire resistance to a given R-M system. Infection of a bacterium with an 'overwhelming number' of non-resistant phages might result in a scenario where all the intracellular restriction enzyme is involved in stoichiometric cleavage of infecting phage and gets sequestered by the cleaved phage DNA. Such a situation provides the remaining phages with an oppurtunity to be completely methylated by the cognate methyltransferase (MTase) before the endonuclease acts on them. The phages with methylated recognition sites are resistant to cleavage by cognate restriction enzymes. Sequestration of restriction enzyme by cleaved DNA *per se* provides the phage with a restriction alleviation mechanism.

A somewhat similar kind of sequestration phenomenon has been reported when bacteriophage T7 infects *Escherichia coli* and bacteriophages PBS-1 and -2 infect *Bacillus subtilis*. The gene 0.3 protein of phage T7, also known as ocr (overcome classical restriction) (31) competitively inhibits type I DNA restriction enzymes by preventing them from binding to their DNA target (32). Biochemical observations that ocr is a competitive inhibitor of DNA binding (33) and that it contains a large excess of negatively charged amino acids, prompted the suggestion that ocr was not only a polyanionic inhibitor but that it could also be a mimic of an extended DNA structure (34). The atomic structure of ocr reveals remarkable molecular mimicry of B-form DNA (35). Ugi (UDG inhibitor) is an early gene product of *B.subtilis* bacteriophage PBS-1 and -2 and a protein mimic of DNA. These phages have genomes containing uracil and UGI protects the genome by forming an extremely specific and physiologically irreversible complex with the host uracil DNA glycosylase (UDG) (36). Clearly, bacteriophages have a number of ways of regulating the activity of cellular nucleases.

Exonuclease activity is essential for multiple rounds of DNA cleavage by *Eco*P15I restriction enzyme

Type I restriction enzymes have been shown to perform only one round of cleavage reaction *in vitro*, i.e. each enzyme molecule cuts the DNA once only. No turnover of the enzyme can be measured with respect to its cleavage function or with respect to its capacity to bind unmodified DNA to nitrocellulose membrane (29). Therefore, it has been an interesting question, whether one is justified calling these proteins 'enzymes' (37). The results presented in Figures 1 and 2 indicate that type III restriction enzymes also show single turnover kinetics *in vitro*. The ability of R.*Eco*P15I to perform multiple rounds of catalysis was tested as follows. When the restriction assay is performed in the presence of exonuclease III, DNA cleaved by R.*Eco*P15I would be further digested by the exonuclease. The removal of cleaved DNA should release R.*Eco*P15I to perform a further round of catalysis.

Supercoiled pUC19 DNA (4.5 μg/7.6 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (3.8 pmol) and 50 U of exonuclease III (where indicated) for 3 h at 37°C. Under stoichiometric conditions, R.*Eco*P15I should cleave only 2.25 μg (3.8 pmol *Eco*P15I sites) of supercoiled pUC19 DNA. However, the cleavage of 4.5 μg of DNA (7.6 pmol *Eco*P15I sites) would indicate a second round of catalysis by the enzyme. In this assay, linearisation of 2.25 μg DNA was observed both in the absence and presence of 20 μM AdoMet and in the absence of exonuclease III (Fig. 4A, lanes 1 and 2). In the assays performed in the presence of exonuclease III, DNA linearised by R.*Eco*P15I will be subjected to complete degradation by exonuclease III, hence the amount of supercoiled pUC19 DNA left at the end of the assay would indicate the amount of DNA cleaved by R.*Eco*P15I. Complete disappearance of DNA would indicate cleavage of 4.5 μg supercoiled pUC19 DNA by 3.8 pmol of R.*Eco*P15I. As can be seen from Figure 4A (lane 3) the enzyme showed complete cleavage of 4.5 μg supercoiled pUC19 DNA only in the presence of exonuclease III. As a control, we show that the

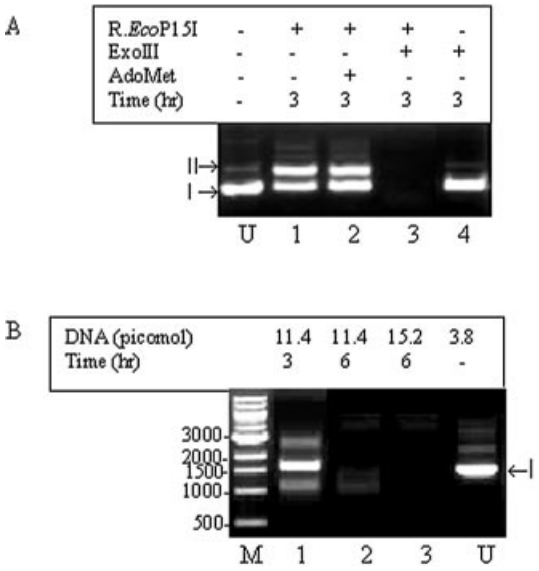

Figure 4. Multiple rounds of DNA cleavage by *Eco*P15I restriction enzyme in the presence of exonuclease III. (**A**) Supercoiled pUC19 DNA (7.6 pmol *Eco*P15I sites) was incubated with *Eco*P15I restriction enzyme (3.8 pmol) in the absence or presence of 20 μM exogenous AdoMet or exonuclease III or both as indicated on top of each lane. (**B**) Supercoiled pUC19 DNA was incubated with R.*Eco*P15I (3.8 pmol) for 3 or 6 h at 37°C in the presence of exonuclease III. Lane 1, 11.4 pmol *Eco*P15I sites; lane 2, 11.4 pmol *Eco*P15I sites; lane 3, 15.2 pmol *Eco*P15I sites; lanes U and M, uncut supercoiled pUC19 DNA and marker DNA fragments, respectively.

supercoiled DNA was not affected by exonuclease III in the absence of *Eco*P15I restriction enzyme (Fig. 4A, lane 4). This demonstrated that removal of DNA, once cleaved by R.*Eco*P15I, allowed a second round of catalysis by the restriction enzyme.

To check for multiple rounds of catalysis, supercoiled pUC19 DNA (11.4 pmol *Eco*P15I sites) was incubated with R.*Eco*P15I (3.8 pmol) and 50 U of exonuclease III for 3 h at 37°C. As evident from Figure 4B (lane 1) 2.25 μg supercoiled DNA was left uncleaved, indicating only two rounds of cleavage by the enzyme. The above assay was repeated by increasing the incubation period from 3 to 6 h at 37°C. Disappearance of the entire supercoiled DNA in Figure 4B (lane 2) clearly shows that a third round of DNA cleavage is possible. A fourth round of catalysis was also demonstrated by incubating supercoiled pUC19 DNA (15.2 pmol *Eco*P15I sites) for 6 h at 37°C with 3.8 pmol of R.*Eco*P15I in the presence of exonuclease III (Fig. 4B, lane 3).

These results indicate that the type III restriction enzyme R.*Eco*P15I, in the presence of an exonuclease, can perform multiple rounds of DNA cleavage. This is the first report demonstrating functional cooperation between a restriction endonuclease and an exonuclease and this may reflect the situation *in vivo*. Such functional cooperation between DNA transaction proteins has been reported recently (38). The unexpected functional cooperation between an exonuclease and an endonuclease provides a highly efficient mechanism for an increase in the efficiency of the R.*Eco*P15I restriction enzyme. Our observations make it attractive to suggest that exonucleases might play a similar role in the *in vivo* activity of other restriction enzymes, such as type I restriction enzymes, that cleave DNA sufficiently far outside their recognition site. An earlier report demonstrating reduction in restriction by *Eco*KI in the absence of the RecBCD exonuclease supports this suggestion (20). Assistance of multiple rounds of catalysis by the restriction enzyme in the presence of exonuclease is characterised by a time lag between DNA cleavage and dissociation of the restriction enzyme from the cleaved DNA. The time lag provides an opportunity for the infecting phage to acquire methylation by the cognate methyltransferase before the restriction enzyme acts on them under situations discussed in the previous section.

Implications for the evolution of R-M enzymes

Sequence comparisons and biochemical experiments in conjunction with site-directed mutagenesis studies have established the fact that the Mod subunit of type III R-M systems contains the target recognition domain (TRD) (9,13,23). The Res subunit contains the characteristic endonuclease motif (PD…D/E-X-K) involved in phosphodiester bond cleavage (27), and the helicase motifs responsible for DNA translocation (driven by ATP hydrolysis) (26). Consequently, the amino acid residues involved in target recognition and the active site residues involved in DNA cleavage are likely to be spatially distant in the holoenzyme and thus lead to cleavage some distance from the recognition site. This is probably true for other restriction enzymes cleaving DNA outside their recognition site (39). In contrast, these sequence recognition and cleavage residues are in close proximity in the tertiary structures of orthodox type II enzymes, allowing cleavage to occur within the recognition site (40).

Since the amino acid sequences of R-M enzymes do not share significant similarity and are not easily amendable to standard alignment procedures, it has been difficult to find an evolutionary link among R-M systems through sequence analysis alone. Despite limited sequence similarities, from the crystal structures of restriction enzymes and methyltransferases and predicted secondary structures of other R-M enzymes, the presence of common folds responsible for enzyme activity has been shown (41–47). It has been suggested that spatial conservation of side-chain locations plays a dominant role and that the primary sequence of conserved active site residues is less important when searching for similarities among restriction enzymes (5). This would suggest that an evolutionary link among these enzymes could be deduced from their catalytic efficiency (as they share the same folds) in conjunction with sequence analysis. Based on the results described here, which indicate that the efficiency of restriction enzymes increases with decreasing affinity for cleaved DNA, we propose a functional evolutionary hierarchy for R-M systems illustrated schematically in Figure 5. Our scheme suggests that restriction enzymes are under pressure to evolve the ability to cut within their recognition site rather than cutting away from the site and, as a consequence, having to rely upon other enzymes to release them from the DNA for further catalytic cycles.

Restriction enzymes have structural homology with enzymes involved in DNA repair and recombination which also perform phosphodiester bond cleavage (5,46,48–51). Type I R-M systems (e.g. R.*Eco*KI) have high structural complexities and can be considered as the most suitable forms for the evolution of new types of R-M systems through domain

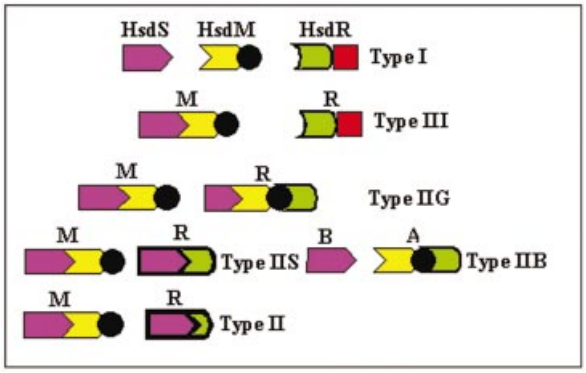

Figure 5. Schematic representation of a functional evolutionary hierarchy of R-M systems. Different colours indicate different domains. All domains in single polypeptide are represented as fused domains. (Purple, target recognition domain; green, endonuclease domain; yellow, AdoMet binding domain; red, helicase domain; black, methyltransferase catalytic domain.)

shuffling, deletions, insertions and duplication (2,52). The HsdS subunit determines DNA specificity and it is composed of two separate DNA binding domains, each recognising one specific part of a non-palindromic sequence. The HsdM subunit contains the AdoMet binding site and the catalytic site for DNA methylation (53). The HsdR subunit is essential for restriction and contains a set of seven amino acid sequence motifs typical of the superfamily of helicases, the so-called DEAD box proteins, involved in ATP binding and ATP-dependent DNA translocation (54). Fusion of one DNA binding domain of a type I HsdS subunit, recognising one specific part of a non-palindromic sequence, with a type I HsdM subunit, would, in conjunction with rearrangements of the tertiary structure, result in a subunit similar to the Mod subunits of type III R-M enzymes. This would be a step towards increasing the efficiency of the enzyme with now only one subunit performing the functions of two subunits, HsdS and HsdM. However, both types I and III restriction enzymes perform ATP and AdoMet-dependent DNA cleavage and cleave outside the recognition sequence (Table 1). In type III R-M enzymes, ATP hydrolysis and DNA translocation are essential for oligomerisation of enzyme molecules involved in DNA cleavage. Although type I restriction enzymes possess ATPase activity and have been shown to translocate DNA, there has been evidence from atomic force microscopy indicating that type I R-M complexes interact and oligomerise even in the absence of ATP-hydrolysis (54).

Fusion of the Mod and Res subunits of type III enzymes, accompanied by deletion of the helicase domains, would probably result in type IIG enzymes (e.g. *Eco*57I) (53) that cleave DNA independent of ATP. Type IIG enzymes would thus have four domains, namely TRD, AdoMet-binding domain, MTase catalytic domain and an endonuclease (ENase) domain in a single polypeptide chain. Type IIG enzymes also cleave DNA in an AdoMet-dependent manner (55). Interestingly, association of endonuclease domains with MTase catalytic domains (type I, type III, type IIG, type IIB) leads to requirement for AdoMet in DNA cleavage (3,16,56).

A type IIG restriction enzyme could give rise to a number of variants. Deletion of one of the four domains with the fusion of remaining three domains can result in four different forms of enzymes. One such form would be a type IIB R-M enzyme. Type IIB enzymes (e.g. *Bcg*I) have two polypeptides, one containing only TRDs, the other containing both MTase and ENase domains (56). The polypeptide with TRDs associates with the polypeptide having both ENase and MTase domains to form a functional type IIB restriction enzyme that can also methylate DNA. Alternatively, duplication of a domain such as TRD in a type IIG enzyme is probable. A polypeptide with a duplicated TRD allows simultaneous segregation of the two TRDs. Segregation of one TRD with MTase domains would produce a protein resembling the MTase of type IIS R-M enzymes, while segregation of other TRD with the ENase domain would produce a protein resembling a type IIS restriction enzyme. Based on the biochemical properties of the prototype type IIG enzyme, *Eco*57I, it was suggested previously that the enzyme might be regarded as an intermediate type reflecting evolutionary link between the enzymes of types III and IIS (55). Later it was argued that type IIG R-M systems might have evolved from a progenitor formed by the fusion of a single strand-specific MTase of type IIS with a DNA endonuclease (57). However, acquisition of MTase domains, which are not related to restriction activity, by an endonuclease to form the predecessor of type IIG seems rather unlikely. Type IIS enzymes (*Fok*I) have DNA binding domain in both the modification and the restriction subunits. A type IIS system has two MTases, one for each strand, which could also be a consequence of these genetic rearrangements. An endonuclease capable of recognising DNA without requiring association with an MTase allows DNA cleavage independent of AdoMet. However, both types IIB and IIS cleave outside their recognition sequence. *Fok*I endonuclease supplemented with a DNA binding-deficient mutant of *Fok*I endonuclease containing an active catalytic domain was shown to increase the rate of DNA cleavage 10–20-fold relative to the wild-type *Fok*I alone (58). This increased cleavage is possibly a consequence of an increased off rate from cleaved DNA for the heterodimer (wild-type + mutant) compared with the homodimer (wild-type). Earlier quantitative evaluation of sequence and structure compatibility has shown that cleavage domain of homologues of *Fok*I (type IIS) have a higher degree of similarity to type I and type III R-M systems than to orthodox type II enzymes such as *Eco*RV and *Pvu*II. Furthermore, the cleavage domain of *Fok*I is apparently evolutionarily older than type II restriction enzymes (51).

Amino acid residues involved in site recognition and phosphodiester bond cleavage are spatially separated in type IIS enzymes. Any change(s) bringing these residues into close proximity (in tertiary structure) might have resulted in orthodox type II enzymes. Orthodox type II restriction enzymes have amino acid residues involved in recognition and cleavage in close proximity in tertiary structure and cleave DNA within the recognition site (4). These are the most efficient restriction enzymes with respect to DNA cleavage. Dissociation of these enzymes from cleaved DNA can be expected to be faster than that of restriction enzymes, which cleave DNA away from the recognition site. The evolutionary hierarchy described here reflects the fact that although the enzymes need to maintain high affinity for their substrates, they should have low affinity for their products to be functionally efficient.

It must be mentioned that the above model does not take into consideration a lot of variants that might have appeared between and within each step of the functional evolutionary hierarchy. Identification and characterisation of new restriction enzymes such as *Hae*IV (59) and *Alo*I (60) provide enzymes that share properties with more than one existing type. As new R-M systems are characterised, it is likely that the biochemical properties of the various types of enzymes will eventually represent a continuum.

Collectively, our data demonstrate that the type III *Eco*P15I restriction enzyme is sequestered by the intact recognition site remaining after DNA cleavage. The presence of cleaved DNA with an intact recognition site decreases the effective concentration of the restriction enzyme—an observation that has implications for the evolution of type III R-M systems and also for all restriction enzymes which cleave at a distance from their recognition site. The type III restriction enzymes depend on exonucleases to perform multiple rounds of catalysis and to act as efficient endonucleases. The functional evolutionary hierarchy proposed here suggests that product release after DNA cleavage, independent of exonuclease, has been the driving force for the evolution of efficient restriction enzymes.

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