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# Type III restriction endonucleases translocate DNA in a reaction driven by recognition site-specific ATP hydrolysis

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**Type III restriction/modification systems recognize short non-palindromic sequences, only one strand of which can be methylated. Replication of type III-modified DNA produces completely unmethylated recognition sites which, according to classical mechanisms of restriction, should be signals for restriction. We have shown previously that suicidal restriction by the type III enzyme *Eco*P15I is prevented if all the unmodified sites are in the same orientation: restriction by *Eco*P15I requires a pair of unmethylated, inversely oriented recognition sites. We have now addressed the molecular mechanism of site orientation-specific DNA restriction. *Eco*P15I is demonstrated to possess an intrinsic ATPase activity, the potential driving force of DNA translocation. The ATPase activity is uniquely recognition site-specific, but *Eco*P15I-modified sites also support the reaction. *Eco*P15I DNA restriction patterns are shown to be predetermined by the enzyme-to-site ratio, in that site-saturating enzyme levels elicit cleavage exclusively between the closest pair of head-to-head oriented sites. DNA restriction is blocked by Lac repressor bound in the intervening sequence between the two *Eco*P15I sites. These results rule out DNA looping and strongly suggest that cleavage is triggered by the close proximity of two convergently tracking *Eco*P15I–DNA complexes.**

*Key words*: DNA-dependent ATPase/DNA strand bias/DNA translocation/*Eco*P15I recognition site-specific ATPase/type III DNA modification and restriction

## Introduction

DNA modification and restriction (M/R) systems fall into three classes, distinguished by the subunit composition of the enzyme species, the temporal regulation of their expression in the cell cycle, their catalytic mechanisms, cofactor requirements and recognition site structure. Type II systems consist of two distinct enzyme species, the methyltransferase and the endonuclease, functionally linked by a common site specificity and the fact that the endonuclease is inhibited by the product of methylation. Type I and type III M/R enzymes are complex molecular species structurally related by the fact that they contain the same target recognition moiety (for recent reviews, see Wilson and Murray, 1991; Bickle and Krüger, 1993; Heitman, 1993). One of the unique characteristics of type III M/R systems is a non-symmetrical recognition sequence which can be methylated on only one strand. Inevitably, during DNA replication, unmethylated recognition sites arise, yet nascent DNA is not suicidally restricted.

The mechanism of restriction evasion was worked out following the observation that phage T7 carrying 36 unmethylated recognition sites for the type III enzyme *Eco*P15I was refractive to restriction, while its close relative T3, with a similar site density, was not. The distinguishing feature of T7 DNA is the unidirectional (strand-biased) orientation of the *Eco*P15I recognition sites, CAGCAG (Schroeder *et al.*, 1986). We subsequently demonstrated that *Eco*P15I does not restrict strand-biased sites and, indeed, all the unmethylated recognition sites generated during replication of an *Eco*P15I-specifically modified genome are strand-biased. The substrate requirement of the restriction endonuclease is satisfied by a pair of inversely oriented non-methylated recognition sites (Meisel *et al.*, 1992).

We have been investigating the mechanism by which such orientation specificity may be accomplished. The ability to discern site orientation implies the discrimination between sites on the same (*cis*) versus sites on another (*trans*) DNA molecule (cf. Krüger *et al.*, 1995). Proteins interacting with spatially separated sites in *cis* achieve this either by DNA looping or by DNA translocation/tracking (reviewed by Wang and Giaever, 1988; Dröge, 1994). The latter is more likely for *Eco*P15I for the following reasons (Meisel, 1993): pairs of inversely oriented sites occur in either of two configurations, head-to-head: →← or tail-to-tail: ←→ (Figure 1a, b). We have found the head-to-head configuration to be the preferred substrate of the endonuclease (Meisel *et al.*, 1992). The orientation of two DNA sites brought into proximity by looping has been shown to be random when their distance exceeds ~500 bp (Hochschild, 1990). However, *Eco*P15I restriction has been observed to occur selectively between the two sites of a head-to-head pair of recognition sequences, irrespective of the length (75 bp–3.5 kbp) of the intervening sequence (Meisel *et al.*, 1992). Based on the original hypothesis for cleavage by type I enzymes (Studier and Bandyopadhyay, 1988), and the published data (Meisel *et al.*, 1992), Murray *et al.* (1993) also suggested that type III enzymes cleave DNA when two translocating enzyme complexes meet.

DNA translocation requires a driving force, usually ATP hydrolysis (Young *et al.*, 1994). While ATP has long been accepted to be an essential cofactor of type III restriction (Haberman, 1974; for a review, see Bickle,

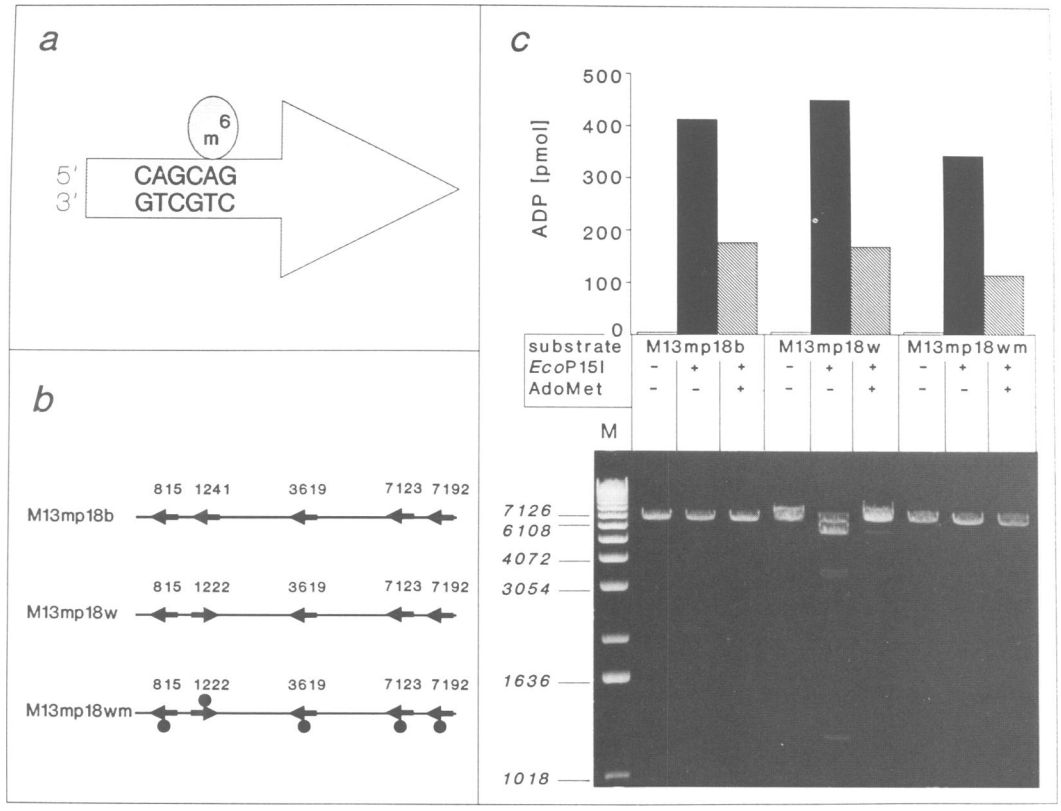


**Fig. 1.** ATPase activity of *Eco*P15I. (**a**) Recognition sequence of *Eco*P15I. The arrow represents the orientation of this non-palindromic sequence; the arrowhead indicates the location of the cleavage site, 25–27 bp 3′ of the A strand of the recognition sequence; $m^6$ corresponds to the methylation position. (**b**) Substrates used in the ATPase assay. An *Eco*P15I site-containing oligonucleotide duplex was cloned into the *Pst*I site of M13mp18. The two resulting constructs differ in the direction of the inserted *Eco*P15I site (the second site from the left). In M13mp18b the inserted *Eco*P15I site has the same orientation as the four *Eco*P15I sites of M13mp18, in M13mp18w it has the opposite orientation. M13mp18wm is *in vivo Eco*P15I-modified M13mp18w (cf. Materials and methods). Arrows indicate the orientations of *Eco*P15I sites, filled circles designate modified recognition sites, and numbers represent map positions of cleavage sites. (**c**) ATPase activity in correlation to the restriction activity of *Eco*P15I. M13mp18b, M13mp18w and M13mp18wm were incubated with and without *Eco*P15I in the presence or absence of AdoMet. Lane M, DNA marker size (bp) indicated at the left.

1993) an intrinsic ATPase activity was ruled out, as activity was found to be <1% of that of a type I enzyme assayed under equivalent reaction conditions (Reiser and Yuan, 1977) and because a non-hydrolyzable ATP analog allowed marginal DNA restriction (Yuan *et al.*, 1980b). The availability of homogeneous and sufficiently active *Eco*P15I restriction endonuclease has now enabled a re-assessment of the enzyme's ATPase activity. Here we characterize an intrinsic, recognition site-specific *Eco*P15I ATPase activity, define a functional hierarchy of the three enzyme activities and integrate the observations in a mechanistic model.

## Results

### *EcoP15I ATPase activity: substrate requirements, recognition site specificity and the influence of methylation status*

By using recognition site-saturating *Eco*P15I concentrations, enzyme levels 30-times higher than in previous studies (Yuan *et al.*, 1980b), we were able to demonstrate *Eco*P15I-specific ATPase activity (Figure 1). These are also the conditions necessary for complete DNA digestion (see below). ATP hydrolysis did not differ significantly during incubation with M13mp18b DNA containing strand-biased, or M13mp18w DNA containing inversely oriented *Eco*P15I sites. When the essential cofactor for modification, the methyl donor AdoMet, is available, modification and restriction are competing reactions (Reiser and Yuan, 1977). ATP hydrolysis triggered by modified M13mp18wm DNA was not significantly lower than on unmodified DNA, but AdoMet reduced ATP hydrolysis by ~50%. In accord with previous results (Meisel *et al.*, 1992), the concomitant restriction activities of *Eco*P15I differed widely: M13mp18w with a head-to-head pair of sites was fully susceptible to cleavage which was suppressed in the presence of AdoMet, while unmodified M13mp18b and modified M13mp18wm were refractive to cleavage (Figure 1c).

As Figure 1 shows, the ATPase activity of *Eco*P15I was constrained neither by the orientation nor by the methylation of recognition sites and is therefore independent of restriction. Figure 2 compiles data on the substrate requirements and site specificity of *Eco*P15I-associated ATPase activity for DNA (or RNA) molecules (Figure 2a) and oligonucleotides (Figure 2b and c). The activity was dependent on the presence of double-stranded DNA substrates (linear or circular), was reduced to ~10% on single-stranded DNA and was virtually absent on RNA. DNA devoid of recognition sites for *Eco*P15I did not support ATPase activity (Figure 2a).

The dependence of the ATPase activity on DNA size

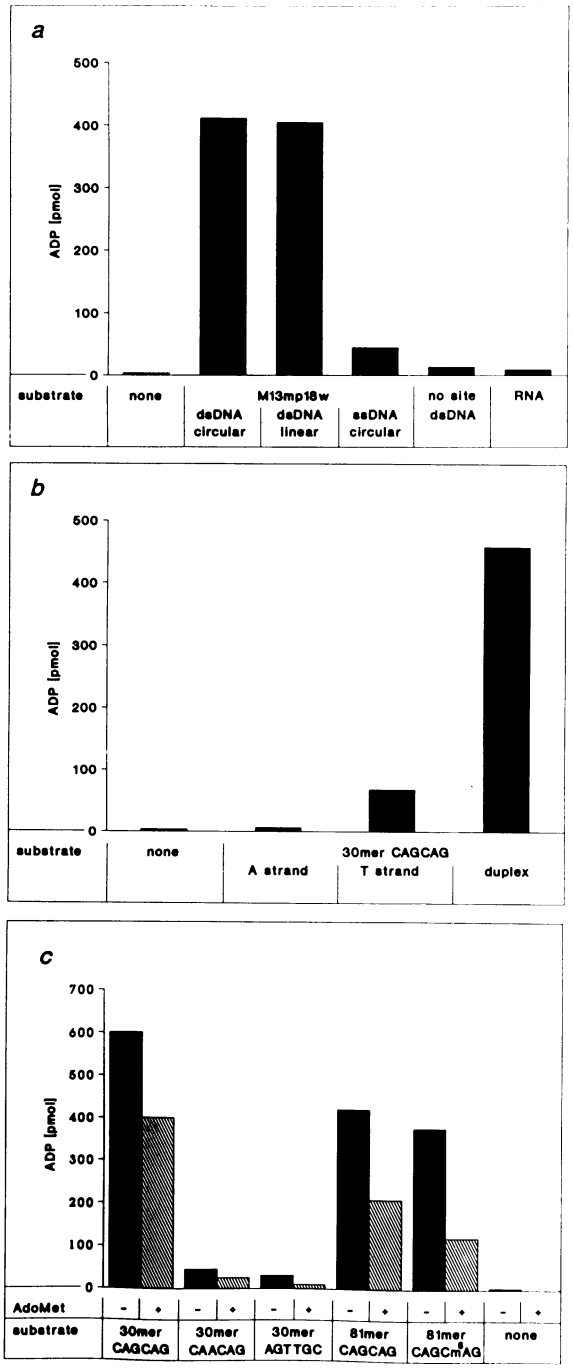


**Fig. 2.** Dependence of *Eco*P15I ATPase activity on recognition site structure. (**a**) Dependence of ATPase activity on the structure of macromolecular substrates. 0.5 pmol enzyme was incubated with 500 ng nucleic acids (0.5 pmol *Eco*P15I sites in M13mp18w); (**b**) activity on 30mer oligonucleotides containing the A strand and/or the T strand of a single *Eco*P15I recognition site; (**c**) effect on ATPase activity of single or multiple base substitutions, and of *Eco*P15I-specific modification of the single *Eco*P15I site on an 81mer oligonucleotide duplex, in the presence or absence of AdoMet. 0.5 pmol enzyme was incubated with 2 pmol oligonucleotides. Oligonucleotides and the ATPase assay are described in Materials and methods.

and structure was addressed in the experiment shown in Figure 2b. Even a single recognition sequence on a 30mer oligonucleotide duplex too short to accommodate a cleavage site (25–27 bp downstream of a recognition site; Hadi *et al.*, 1979) was sufficient to support the activity. Of the two strands forming the 30mer duplex, the T strand, but not the methylatable A strand, supports an ATPase activity of 20% of the activity on the duplex (Figure 2b). This may account for the relatively high residual ATPase activity on the M13mp18w viral single-stranded DNA which contains four T sites and one A site (Figure 2a, cf. Figure 1b).

Subtle changes of the recognition sequence, i.e. base substitutions and adenine methylation, and the influence of AdoMet were investigated in the experiment shown in Figure 2c. A single substitution within the recognition site decreased ATPase activity >10-fold, nearly as much as exchanges at all six positions (Figure 2c). Given that there is no independent assay (i.e. other than an *Eco*P15I restriction digest, Figure 1c) of the completeness of *Eco*P15I-specific methylation, the effect of DNA recognition site methylation on the *Eco*P15I-dependent ATPase activity was determined on a synthetic 100% modified substrate. An oligonucleotide duplex *Eco*P15I, modified by inserting 6-methylaminopurine at the appropriate position (Meisel *et al.*, 1991) of the recognition site, supported ATPase activity to a similar degree as a non-modified substrate (Figure 2c). AdoMet at 1 μM, that is 2.5 times the $K_m$ of the *Eco*P15I methyltransferase (Reiser and Yuan, 1977), inhibited ATPase activity by 30–50%, apparently independently of the substrate size (Figures 1c and 2c), recognition site structure and methylation status (Figure 2c).

### *Kinetics and stoichiometry of ATPase activity*

The kinetics of *Eco*P15I ATPase activity were assayed on a single site-containing 81mer oligonucleotide duplex at 0.2 mM ATP, a saturating concentration with respect to the $K_m$ of 7 μM determined for the stimulating effect of ATP on DNA endonuclease activity (Reiser and Yuan, 1977), and AdoMet (1 μM) under different pre-incubation conditions (Figure 3). Initial rate conditions prevailed for up to 5 min, after which the rate declined gradually as the product ADP accumulated. When the enzyme was pre-incubated with AdoMet and ATP and the reaction started with DNA, the initial rate was reduced by 50% (Figure 3a). However, when the enzyme was pre-incubated with DNA to allow binding and the reaction initiated with a mixture of AdoMet and ATP, no inhibition was observed (Figure 3b).

The stoichiometry of *Eco*P15I ATPase activity with respect to recognition sites was investigated by complementary titrations of enzyme versus recognition site concentration (Figure 4), in order to determine the saturation level and the slope of the regression line for the linear part of the plot. At sub-saturating enzyme-to-site ratios, the rate of ATP hydrolysis was proportional to the enzyme concentration (slope = 1) and saturation was reached at an enzyme-to-site ratio of 2 (Figure 4a). In the reverse titration beginning at site-to-enzyme ratios $<<1$, the slope of the regression line was 0.9 and saturation was reached at a ratio between one and two. The calculation is based on an *Eco*P15I subunit composition of 2 Mod:2 Res subunits but is hardly changed assuming the alternative composition of 3:1, as proposed by Hadi *et al.* (1983). It cannot be excluded that the saturation level of two enzyme

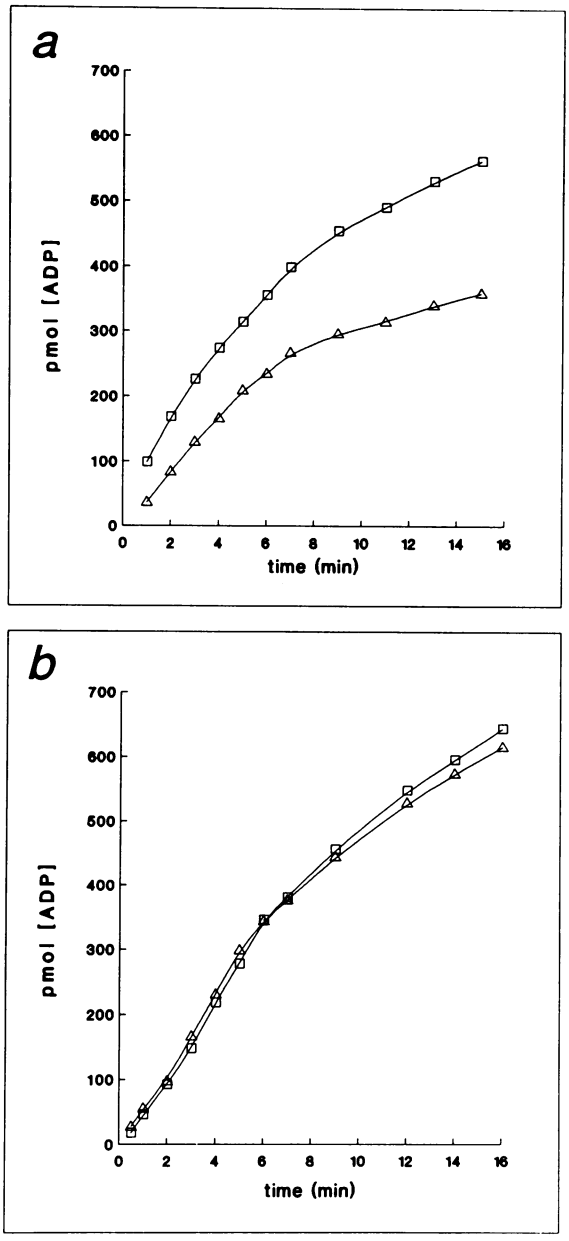


**Fig. 3.** Time dependence of *Eco*P15I ATPase activity and effect of pre-incubation of enzyme with AdoMet. (**a**) Pre-incubation in a volume of 9 µl of *Eco*P15I (0.5 pmol ) with AdoMet (10 pmol) and ATP (2 nmol) for 5 min and start of the reaction by addition of 1 µl single-site 81mer oligonucleotide duplex (0.5 pmol). (**b**) Pre-incubation of 9 µl *Eco*P15I (0.5 pmol) with DNA (0.5 pmol) and start by addition of 1 µl AdoMet (10 pmol) and/or ATP (2 nmol). □, without AdoMet; △, with AdoMet.

molecules per site (Figure 4a) reflects the presence of inactive enzyme molecules.

### *Kinetics and stoichiometry of the cleavage reaction*

The existence of a site-specific ATPase activity is consistent with a tracking mode of *Eco*P15I–DNA interaction, which was also implicated by the pronounced preference of the head-to-head configuration of recognition sites (Meisel *et al.*, 1992). The following experiment was designed to discriminate between different modes of enzyme–DNA recognition site interaction. On variation

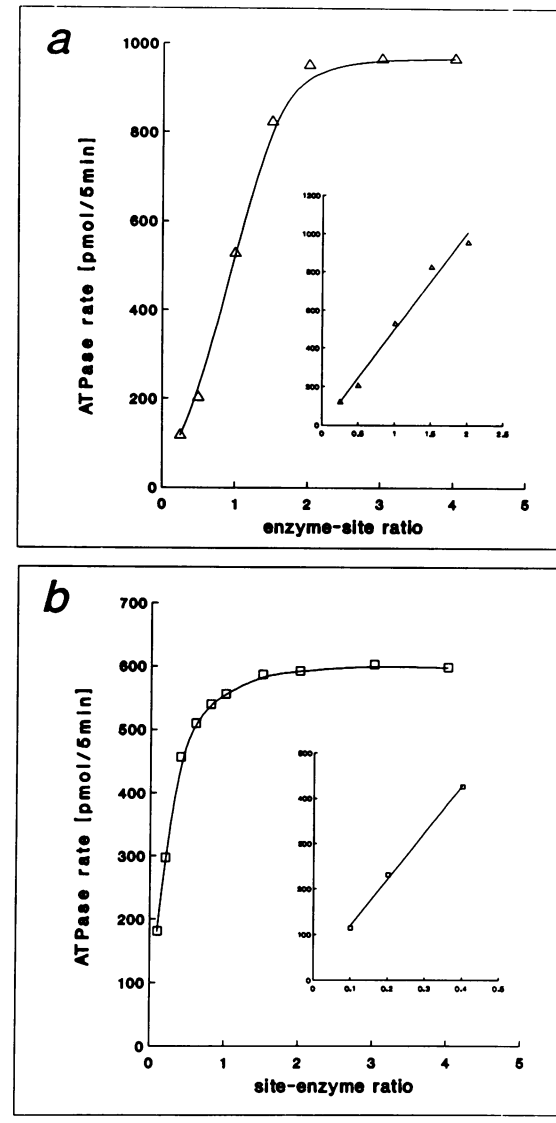


**Fig. 4.** Stoichiometry of ATPase activity: complementary titrations of *Eco*P15I and *Eco*P15I recognition sites. (**a**) Variation of enzyme concentration at fixed site concentration (0.5 pmol in 10 µl) and (**b**) variation of site concentration at fixed enzyme concentration (0.5 pmol in 10 µl). Enzyme and 81mer oligonucleotide duplex were pre-incubated in 9 µl and reactions started with 1 µl ATP (2 nmol). The insets show the regression lines through the linear part of the relationships between the reaction rate and the enzyme-to-site ratio.

of the enzyme-to-site ratio, different cleavage patterns would result from tracking or looping. The experiment also answers the question of whether restriction requires both sites of a head-to-head pair to be occupied by an enzyme molecule in order to interact productively or whether initial occupation of one of the sites is sufficient and interaction with the second site is brought about by tracking. We used a DNA substrate containing two head-to-head pairs of recognition sequences which share a central site (site number 3 in Figure 5a) and restriction patterns were recorded as a function of time (Figure 5).

Incubation with a saturating enzyme concentration initially elicited restriction between the head-to-head pair (2/3), the most closely spaced recognition sequences, consistent with the tracking hypothesis predicting that translocating enzyme complexes bound to sites 2 and 3

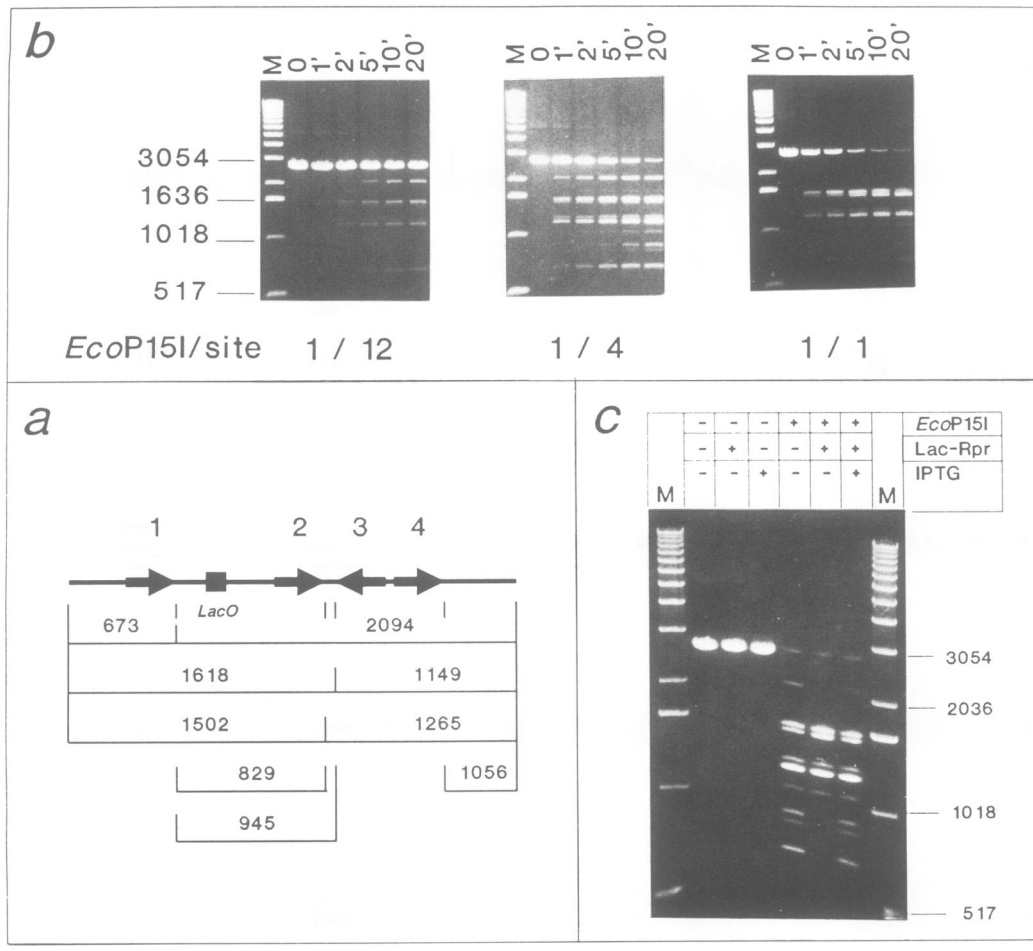


**Fig. 5.** *Eco*P15I cleavage depends on the enzyme–site ratio and is blocked by Lac repressor bound between sites. (**a**) Site constellation in the DNA of the *Ssp*I-linearized pUC19w substrate. The arrows indicate the orientations of the *Eco*P15I sites. The lengths of the resulting DNA fragments (in bp) are indicated. The box represents the binding site of the Lac repressor. (**b**) Dependence of *Eco*P15I restriction on enzyme-to-site ratio. *Ssp*I-linearized pUC19w DNA was treated with increasing amounts of *Eco*P15I. Numbers above lanes: incubation time (min). The molar ratio of *Eco*P15I enzyme to recognition sites is indicated below each gel. Lanes M, DNA marker sizes (bp) given to the left. (**c**) Lac repressor binding blocks the interaction of two *Eco*P15I–DNA complexes. *Ssp*I-linearized pUC19w DNA incubated with *Eco*P15I, Lac repressor and IPTG alone and in combination. Lanes M, marker DNA fragments as in b.

would be the first ones to meet. If pairs of sites interacted by looping, cleavage between sites 1 and 3 would also be observed at a saturating enzyme-to-site ratio. [The cleavage products of (2/3) appear as doublets because cleavage occurs 25–27 bp 3′ of the A strand of *either* site (Hadi *et al.*, 1979)]. Furthermore, the fact that enzyme complexes bound to sites 1 and 3 failed to interact also indicates that an *Eco*P15I complex bound to site 1 cannot track past site 2 if the latter is also occupied by an enzyme molecule.

An enzyme-to-site ratio of 1:4, i.e. one enzyme molecule per DNA molecule, produced qualitatively different kinetics. Again there was a fast initial reaction but now *both* head-to-head pairs were equally susceptible. Cleavage at site 1 of the pair (1/3) produced a DNA fragment containing the head-to-head pair (2/3) which was subsequently digested, generating additional products. According to the Poisson distribution, about one third of the DNA molecules carry two or more enzyme molecules, but the simultaneous occupation by *Eco*P15I of sites 1, 2 and 3 in one DNA molecule becomes rare. Therefore, the use of either substrate (2/3) or (1/3) is equally probable.

Reducing the enzyme-to-site ratio to 1:12, or one enzyme molecule per three DNA molecules, greatly retarded the reaction in which both head-to-head pairs (1/3) and (2/3) served as equivalent substrates. The fact that traces of cleavage products only began to appear after 2 min, when, at an enzyme-to-site ratio of 1:4, ~20% of the substrate was already digested (Figure 5b), supports a restriction mechanism depending on the simultaneous occupancy of both sites of a head-to-head pair. This would enable convergent DNA tracking of the two enzyme–site complexes. The kinetics of restriction indicate a stoichiometric rather than a catalytic type of reaction, in the sense that dissociation or post-cleavage turnover of enzyme molecules followed by re-occupation of recognition sites are considerably slower than a reaction sequence involving initial binding to recognition sites, DNA tracking and productive interaction of enzyme complexes leading to substrate cleavage.

### *Lac repressor binding between sites inhibits cleavage*

A crucial test of DNA translocation versus DNA looping was to place a molecular block between two normally reactive sites. We achieved this by exploiting the *lac* operator located between the two head-to-head sites (1/3)

in pUC19 DNA (Figure 5a). Figure 5c shows *Eco*P15I restriction of this DNA at an enzyme-to-site ratio of 1:4 on co-incubation with Lac repressor. Cleavage by *Eco*P15I at (1/3) was blocked by Lac repressor bound between sites 1 and 3 and restored by addition of the inducer IPTG. Restriction at (2/3) was not influenced, providing an internal control of the enzyme activity in the presence of the Lac repressor. The relatively small Lac repressor molecule which covers 24 bp (Kolkhof *et al.*, 1992) and is located acentrically with respect to the sites of pair (1/3) at a distance of 73 bp from the cleavage site 1 (Figure 3a) would hardly obstruct the formation of a DNA loop. The most straightforward explanation of this result is that the Lac repressor bound between two potentially interactive sites prevents the convergence of the two tracking *Eco*P15I–recognition site complexes.

## Discussion

DNA restriction by type III systems cannot proceed in the absence of ATP (Reiser and Yuan, 1977). ATP was believed to be a cofactor, not a substrate (Yuan *et al.*, 1980b), because the ATPase activity of *Eco*P15I was found to be only 1% compared to that of the type I enzyme *Eco*K assayed in parallel, i.e. at the limit of detection (Reiser and Yuan, 1977; cf. Bickle, 1982). We now confirm this relationship by showing that 1 pmol of *Eco*P15I hydrolyzes 100 pmol ATP/min (calculated from data in Figure 3), while 1 pmol of *Eco*K hydrolyzes 10 000 pmol ATP/min (Reiser and Yuan, 1977). Another reason for initially favoring a cofactor role of ATP was the observation that a non-hydrolyzable ATP analog allowed marginal restriction. A re-analysis of the restriction patterns of SV40 and pBR322 DNAs produced in the presence of the ATP analog (Yuan *et al.*, 1980b) indicated exclusive cleavage at pairs of head-to-head recognition sites so closely clustered as to conceivably allow the rare, occasional formation of a restriction complex without DNA translocation.

We have characterized the *Eco*P15I ATPase activity to be totally recognition site dependent but not influenced by cognate DNA methylation. This is concordant with the observation that of the two single strands of a one-site substrate, the non-methylatable T strand supports a basal ATPase activity. In view of the acentric location of CAGCAG within the 30mer oligonucleotide used and the lack of intrastrand homology (cf. Materials and methods), it is unlikely that the T strands re-associated to form a duplex with a double-T strand 'pseudosite'. The most straightforward interpretation of the properties of the ATPase activity (Figures 2 and 4), in conjunction with the strict site-orientation dependence of the restriction process (Figure 5), is that restriction requires ATP-driven DNA translocation. ATP may play an additional role in recognition site discrimination (Ahmad and Rao, 1994).

A mechanistic model of the function of *Eco*P15I that integrates these findings and makes additional, testable hypotheses follows. It comprises all steps of the enzyme–DNA interaction, beginning with DNA–enzyme association and terminating with dissociation. *Eco*P15I is represented by {[R][MS]}, [R] indicating the Res subunit and [MS] or [SM] the Mod (target recognition) subunit. The model makes no predictions of subunit composition or the structures of reaction intermediates.

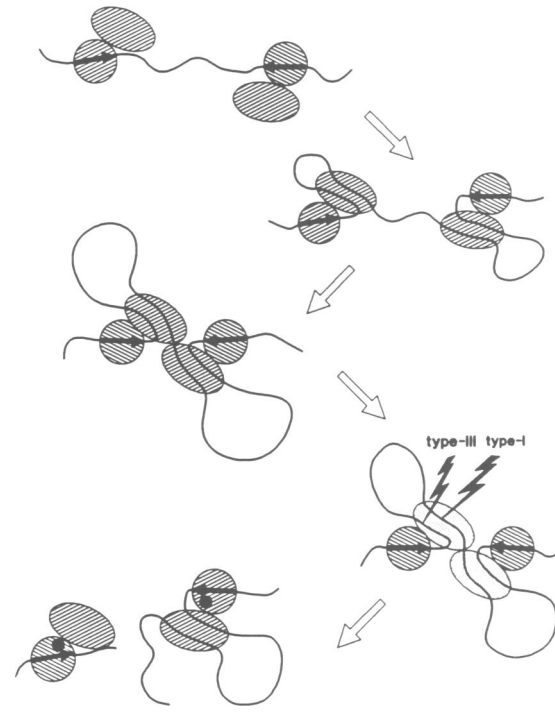


**Fig. 6.** Mechanistic model of DNA restriction by type III modification/restriction enzymes. A pair of head-to-head oriented recognition sites (→) is occupied by one enzyme molecule each. The modification (Mod) subunit is represented by a hatched circle, the restriction subunit (Res) by a hatched oval. Both enzyme–site complexes utilize ATP to translocate DNA, represented by a loop of increasing size, and convergently track until collision. Collision elicits a conformational change of the enzyme molecules (Res subunit white oval) activating them for cleavage. The difference in cleavage position for type III and type I enzymes is highlighted. Cleavage by type III enzymes produces two fragments of different lengths containing the original recognition sites, both of which are subject to modification (methyl groups are represented by small filled circles). The drawing is purely schematic and makes no assumptions on the architecture of reaction intermediates.

### *Association with DNA and linear diffusion (sliding)*

In the absence of ATP this is the only type of enzyme motion. Dissociation and re-binding of the enzyme are frequent.

### *Binding to recognition site (→) and DNA tracking*

$$\{[R][MS]\} + \rightarrow \Rightarrow \{[R][MS]\rightarrow\}$$

Binding to the recognition site in the presence of ATP initiates a sequence of events concluding either in restriction or modification. Site binding elicits ATPase activity as a result of functional coupling of the [MS] and the [R] subunits and this drives DNA tracking in the direction determined by site orientation, i.e. from 5′ to 3′ with respect to the A strand of the recognition sequence (cf. Figure 1). DNA tracking is illustrated schematically in Figure 6.

### *Modification mode (default)*

While modification by *Eco*P15I is also possible in the absence of ATP, it becomes 5- or 20-times as efficient in its presence (Reiser and Yuan, 1977; Hadi *et al.*, 1983). We suggest this is because DNA tracking transforms modification from a *distributive* to a *processive* reaction.

Methylation of → to →* takes place within a time interval which depends on the AdoMet concentration (Meisel, 1993).

$$\{[R][MS]\rightarrow\} \Rightarrow \{[R][MS]\rightarrow^*\}$$

AdoMet inhibits the ATPase activity by 50%, but only if the enzyme is *pre*-loaded with AdoMet before binding DNA. This alteration is not transient, since the rate of ATP hydrolysis remains at the lower level throughout the observation period (Figure 3a) and confirms the observation of Yuan and Reiser (1978) that recognition complexes formed in the presence of AdoMet behave differently to those formed in its absence. An increased AdoMet concentration during DNA replication would favor complete modification of nascent self DNA, while a baseline AdoMet concentration during the rest of the cell cycle would facilitate the restriction of foreign DNA. A decrease in ATPase activity may cause a reduction in tracking speed, allowing more time for recognition site modification.

The [MS] subunit alone is also capable of DNA modification but it is not stimulated by ATP (Hadi *et al.*, 1983), and AdoMet just above the $K_m$ actually decreases the methylation rate, apparently by inhibiting the dissociation of *Eco*P15I from DNA (Rao *et al.*, 1989). While impeding the distributively acting [MS] methylase, the same effect should improve the processivity of the holoenzyme.

Modification should alter enzyme conformation, disabling the formation of a restriction complex. In fact, a head-to-head pair of sites one of which is methylated is not a substrate for *Eco*P15I restriction (Meisel, 1993).

$$\{[R][MS]\rightarrow^*\} \Rightarrow \{[ms]\rightarrow\}$$

If the conformationally altered enzyme has a somewhat higher affinity for non-methylated than methylated sites it may switch to the first non-methylated site encountered during tracking and return to default state.

$$\{[ms]\rightarrow^*\} + \rightarrow \Rightarrow \rightarrow^* + \{[R][MS]\rightarrow\}$$

It is mechanistically unlikely that the enzyme would be capable of recognizing an inversely oriented non-modified site in its track and invert the direction of translocation.

The fact that ATPase activity is independent of the modification status of recognition sites enables the *Eco*P15I molecule to continuously patrol and modify the DNA until it collides with an enzyme patrolling in the opposite direction. It is noteworthy that, as illustrated in Figure 6, the head-to-head orientation of the recognition sites defines the direction of convergent translocation as well as the enzyme subunit orientation in the productive cleavage complex. Moreover, Figure 7 shows that all tracking *Eco*P15I molecules bound to unmodified sites of nascent DNA being copied from an *Eco*P15-specifically modified template will move in the same direction as the replication fork (5′ to 3′ with respect to the A strands of the unmodified sites, or 3′ to 5′ with respect to the template, both on the leading and the lagging strand). Thus, the properties of *Eco*P15I enable modification to be coordinated to DNA replication.

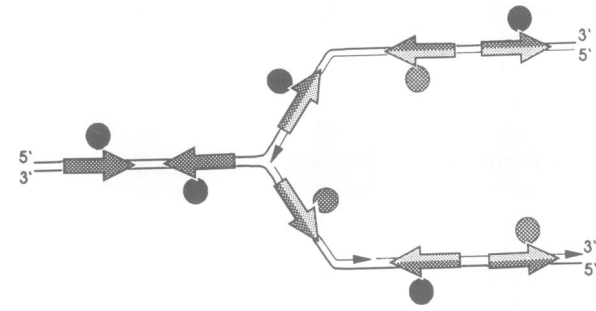


**Fig. 7.** Coordination of type III-specific DNA modification with the movement of the DNA replication complex. A replication fork of a type III-specifically modified genome is shown. The recognition sites on parental DNA are represented by dark grey arrows (cf. Figure 1a), carrying methyl groups (filled circles). Nascent DNA is represented by thin black lines, uninterrupted for the leading strand and interrupted for the lagging strand, with arrows indicating the direction of synthesis. Recognition sites on replicated DNA consist of a parental strand (shaded) and a daughter strand (pale). All unmodified recognition sites on daughter DNA molecules consist of nascent A strands and parental T strands and therefore have the same orientation. The type III enzyme translocates DNA in a direction (symbolized by the arrow-shaped recognition site) 3′ with respect to the A strand of the recognition site on nascent DNA, which is identical to the direction of DNA synthesis. This would be a prerequisite for temporal and spatial coordination of DNA modification (new methyl group = hatched circle) and replication which can be envisaged as the type III M/R enzyme trailing behind the replication machinery.

### *Restriction mode*

Cleavage is triggered by the collision of two convergently tracking enzyme–recognition site complexes.

$$\{[R][MS]\rightarrow\} + \{\leftarrow[SM][R]\} \Rightarrow \{[MS]\rightarrow [R][R]\leftarrow[SM]\}$$

Type I R/M enzymes also translocate DNA while hydrolyzing ATP (Rosamund *et al.*, 1979; Yuan *et al.*, 1980a; Studier and Bandyopadhyay, 1988). Collision of tracking enzyme complexes was first suggested to occur for type I M/R enzymes by Studier and Bandyopadhyay (1988), and was later also proposed for type III enzymes (Meisel, 1993; Murray *et al.*, 1993; Krüger *et al.*, 1995). It is therefore pertinent to discuss the differences and similarities between the two types of M/R systems here.

Collision converts the enzymes into 'restriction mode', probably involving a re-structuring of the whole enzyme–DNA complex which is resolved by cleavage (shown by // below).

$$\{[MS]\rightarrow[R][R]\leftarrow[SM]\} \Rightarrow \{[\mathit{MS}]\rightarrow[\mathit{RR}]\leftarrow[\mathit{SM}]\} \Rightarrow \{[R][MS]\rightarrow//\leftarrow[SM][R]\} \Rightarrow \{[R][MS]\rightarrow/\} + \{/\leftarrow[SM][R]\}$$

One of the differences between type I and type III enzymes is the site of cleavage. Type III enzymes cleave at the recognition site-proximal end of the tracking loop, whereas type I enzymes cleave distally, as illustrated in Figure 6. The point of cleavage would be exactly equidistant from two recognition sites if both type I molecules initiated tracking simultaneously, but it could be anywhere between them under non-synchronous tracking conditions (Studier

and Bandhyopadhyay, 1988). In contrast, the proximal cleavage position of type III enzymes is independent of the length of the tracking loop.

Which of the two recognition sites of the head-to-head pair is selected for cleavage by *Eco*P15I appears to be stochastic, at least at first approximation (cf. Figure 5a, b). After performing cleavage, the enzyme returns to modification mode (Meisel *et al.*, 1992). Both cleavage products contain the original recognition sites, allowing the enzyme to remain bound and continue tracking. Figure 6 shows only the asymmetric products generated by type III cleavage.

### *Dissociation*

Following cleavage, type I and type III enzymes behave differently. *Eco*P15I turns over (Reiser, 1975) and must therefore dissociate from its recognition site under certain conditions. The gradual increase in cleavage products at an enzyme-to-site ratio of 1:12 (Figure 5b) is the consequence of turnover. In contrast, type I enzymes remain bound to their cleavage products and catalyze massive ATP hydrolysis. This could either be a laboratory artifact or represent an 'altruistic' biological function to eliminate phage-infected cells from the population (discussed by Bickle, 1982).

The conditions for release of *Eco*P15I from the DNA substrate have not been studied systematically. AdoMet inhibits dissociation (Yuan and Reiser, 1978). Since *Eco*P15I is capable of binding to all recognition sites, as restriction progresses, more and more enzyme molecules will be bound to cleavage products which, in the presence of AdoMet, are continuously modified (Meisel *et al.*, 1992). This explains why at a low enzyme-to-site ratio (Figure 5b), even in the absence of AdoMet, restriction is not completed. If *Eco*P15I did tend to dissociate irreversibly from the cleavage products, the enzyme would eventually saturate the remaining head-to-head substrate sites and cleave to completion at an ever increasing rate.

It is proposed that dissociation of *Eco*P15I is facilitated by collision of translocating complexes when one or both complexes are disabled for restriction (see above, Modification mode). In that case, the enzyme may complete modification before being released. We suggest that a reversal of tracking direction must be preceded by a dissociation of the *Eco*P15I–recognition site complex and binding to an inversely oriented recognition site.

The mechanistic model makes predictions of the kinetic properties of the three enzyme activities. At low enzyme-to-site ratios the rate of ATP hydrolysis is proportional to enzyme concentration (Figure 4a). The model of *Eco*P15I–DNA interaction suggests that the same relationship should hold for the methylation rate. However, the rate of DNA restriction should be proportional to the square of enzyme concentration, and at saturating enzyme-to-site ratio it should be inversely proportional to the distance between the closest head-to-head pair of sites. Quantitative predictions can also be made of the relation of the restriction rate to the total number of recognition sites of either orientation per DNA molecule. The rate of DNA translocation can be deduced from the cleavage rate at saturating enzyme-to-site ratios, as well as from the rate of unidirectional progression of DNA methylation.

The three intrinsic enzymatic activities of *Eco*P15I differ in the stringency of their recognition site requirements. In being recognition site-dependent but independent of site methylation, the ATPase activity exhibits the least stringency. Non-methylated recognition sites are required by the modification activity, while the restriction activity is dependent on a head-to-head pair of inverted unmodified recognition sites, i.e. a palindrome with variable spacer length (Meisel *et al.*, 1992). This order of stringency is consistent with the ATPase activity being a prerequisite for restriction and for processive methylation.

In defining a hierarchy and interdependence of enzyme activities, it is clear that target site recognition by the Mod subunit is the prerequisite of ATPase activity. Distributive DNA methylation is exhibited by *Eco*P15I in the absence of ATP, or by the Mod subunit alone independently of ATP. The ATPase activity most probably resides in the Res subunit which, like the Res subunits of type I enzymes, possesses several sequence motifs common to ATP-dependent helicases (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993; Murray *et al.*, 1993). ATPase activity has also been identified recently in the type III enzyme *Eco*P1 (D.N.Rao, personal communication).

The proposed model is compatible with the biological function of type III M/R systems. Estimating that the *Escherichia coli* genome contains ~2000 *Eco*P15I recognition sites, and calculating the number of enzyme molecules per cell to be ~200, one enzyme molecule should be patrolling a 20 kb stretch of DNA containing ~10 recognition sites. The processivity of DNA methylation and its potential coupling to replication makes this an efficient modification system. The postulated ability to switch to unmethylated recognition sites would allow the enzyme to preferentially attack and digest non-self DNA, provided it presented at least one pair of inverted sites in head-to-head configuration.

## Material and methods

### *DNA preparations*

The constructs M13mp18b and M13mp18w were generated by cloning an oligonucleotide duplex consisting of two complementary 81mers with terminal *Pst*I sites, into the *Pst*I locus of M13mp18. The plasmid pUC19w was constructed by cloning the same 81mer oligonucleotide duplex into the *Pst*I locus of pUC19. All constructs were confirmed by sequencing using T7 DNA polymerase (Tabor and Richardson, 1989). The single-stranded DNA for sequencing and the double-stranded DNA for the cloning and ATPase experiments were purified according to standard methods (Sambrook *et al.*, 1989).

*Eco*P15I-specific modification of M13mp18w was performed *in vivo* using TG1(P1-15hyb2cIts) cells (Meisel *et al.*, 1992).

The sequences of the oligonucleotide duplexes used in the experiments described in Figures 2, 3 and 4 with the *Eco*P15I sites or their derivatives shown in bold type are:

30mer/CAGCAG

5′-ATGAGTCCCA **CAGCAG**CTGA AGACTGGCAT-3′

3′-TACTCAGGGT **GTCGTC**GACT TCTGACCGTA-5′

30mer/CAACAG

5′-ATGAGTCCCA **CAACAG**CTGA AGACTGGCAT-3′

3′-TACTCAGGGT **GTTGTC**GACT TCTGACCGTA-5′

30mer/AGTTGC

5′-ATGAGTCCCA **AGTTGC**CTGA AGACTGGCAT-3′

3′-TACTCAGGGT **TCAACG**GACT TCTGACCGTA-5′

81mer/CAGCAG

5′-GCCGTGATCC TCCAGGATGA GTCCCA**CAGC AG**CTGAAGAC TGGCATCGAA TCTGTTTGGG TGGAGACCAG GACGCCC-TGCA-3′

3′-CGGCACTAGG AGGTCCTACT CAGGGT**GTCG TC**GACTTCTG ACCGTAGCTT AGACAAACCC ACCTCTGGTC CTGCGGGACGT-5′

81mer/CAGCm$^6$AG

5′-GCCGTGATCC TCCAGGATGA GTCCCA**CAGC AG**CTGAAGAC TGGCATCGAA TCTGTTTGGG TGGAGACCAG GACGCCCTGC A-3′

3′-CGGCACTAGG AGGTCCTACT CAGGGT**GTCG TC**GACTTCTG ACCGTAGCTT AGACAAACCC ACCTCTGGTC CTGCGGGACG T-5′

### *Purification of EcoP15I*

*Eco*P15I was purified from 10 g batches of *E.coli* TG1(pMT15) to apparent homogeneity by a modification of the protocol of Hadi *et al.* (1983). The cell pellet was sonicated in buffer A (50 mM potassium phosphate, pH 7.6, 10 mM $MgCl_2$, 0.01% Triton X-100, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM $NaN_3$ and 25 mg PMSF/ml) and the extract cleared by successive centrifugation at 6000 (20 min) and 35 000 r.p.m. (2 h) at 4°C. The supernatant was made 0.2 M in NaCl and treated with polyethyleneimine (PEI; final concentration 1%) to precipitate DNA which was removed by 30 min centrifugation at 13 000 r.p.m.. This supernatant was made 70% of saturation in $(NH_4)_2SO_4$ and the precipitate collected for 30 min at 15 000 r.p.m.. The pellet was resuspended in buffer B (50 mM potassium phosphate, pH 7.6, 1 mM EDTA, 7 mM 2-mercaptoethanol and 0.01% Triton X-100) and desalted through Sephadex G-75 superfine (Pharmacia). The desalted fractions were pooled and applied to Q-Sepharose (Pharmacia). Protein was eluted with a 0–1 M gradient of NaCl in buffer B. The purest Q-Sepharose fractions were pooled, re-equilibrated using Centriprep 30 (Amicon) into buffer C (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 7 mM 2-mercaptoethanol), applied to heparin agarose (Pharmacia) and eluted with a 0–1 M NaCl gradient in buffer C. The purified enzyme was desalted using Microcon 30 (Amicon). The average yield of homogeneous *Eco*P15I from 10 g cells was 100 μg.

Endonuclease activity was assayed on *Ssp*I-linearized pUC19 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, 50 μg/ml bovine serum albumin) containing 1 mM ATP, followed by agarose gel electrophoresis. Enzyme purity was monitored by SDS–polyacrylamide gel electrophoresis. The molarity of homogeneous enzyme was calculated from the absorbance at 280 nm, using the formula of Beaven and Holiday (1952), and assuming with Hadi *et al.* (1983) that the enzyme molecule is composed of either two Res and two Mod subunits or one Res and three Mod subunits (which only changes the result by 7%).

### *EcoP15I ATPase assays*

*Eco*P15I (0.5 pmol, 160 ng) was incubated in a total volume of 10 μl, containing *Eco*P15I buffer (Hadi *et al.*, 1983) or Lac buffer (see above), 100 or 200 μM ATP and 33 nM [α-$^{32}$P]ATP, in the absence or presence of 1 μM AdoMet, with or without DNA or oligonucleotide substrate for 5 min at 37°C. In the experiments of Figures 1, 2 and 3a the enzyme was pre-incubated at 37°C with ATP plus/minus AdoMet and the reaction was started with DNA. Alternatively (Figures 3b and 4), enzyme and DNA were pre-incubated and the reaction was started with ATP plus/minus AdoMet. Substrates were either 0.5–2 pmol oligodeoxynucleotide duplexes or 500 ng nucleic acids (0.5 pmol *Eco*P15I sites in M13 derivatives). The reaction was terminated by addition of EDTA to a final concentration of 20 mM. Reaction products were analyzed by thin-layer chromatography. One μl of the reaction mixture was spotted onto PEI cellulose sheets (Merck) and developed by ascending chromatography in 0.375 M potassium phosphate (pH 3.5). The sheets were dried and the ATPase activity was quantified by determining the conversion of ATP to ADP using a PhosphorImager (Molecular Dynamics).

### *EcoP15I cleavage assays*

Assays were performed on linearized DNA substrates. The three circular 7330 bp M13mp18 derivatives, b, w and wm, were linearized with *Msc*I, pUC19w DNA was linearized with *Ssp*I. *Eco*P15I (8, 2 or 0.67 pmol) and 3.7 μg pUC19w DNA (8 pmol *Eco*P15I sites) were incubated in pre-warmed Lac-buffer (see above) for 5 min at 37°C in a total volume of 100 μl. The reactions were started by addition of ATP to a final concentration of 1 mM. Aliquots of 7 μl were removed at the indicated time intervals. Reactions were stopped by the addition of EDTA to a final concentration of 20 mM. To study the influence of the Lac repressor, 370 ng *Ssp*I-linearized pUC19w DNA (0.2 pmol lac operator sites, 0.8 pmol *Eco*P15I sites) were co-incubated in Lac buffer and 1 mM ATP in the absence or presence of 12 pmol Lac repressor, 4 nmol IPTG and 0.2 pmol *Eco*P15I for 30 min or 1 h at 37°C in a total volume of 20 μl. Reactions were stopped by the addition of EDTA to a final concentration of 20 mM. Fragments were separated by electrophoresis through a 0.8% agarose gel.

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