

Mechanistic insights into type III restriction enzymes

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1. ABSTRACT

Type III restriction-modification (R-M) enzymes need to interact with two separate unmethylated DNA sequences in indirectly repeated, head-to-head orientations for efficient cleavage to occur at a defined location next to only one of the two sites. However, cleavage of sites that are not in head-to-head orientation have been observed to occur under certain reaction conditions *in vitro*. ATP hydrolysis is required for the long-distance communication between the sites prior to cleavage. Type III R-M enzymes comprise two subunits, Res and Mod that form a homodimeric Mod₂ and a heterotetrameric Res₂Mod₂ complex. The Mod subunit in M₂ or R₂M₂ complex recognizes and methylates DNA while the Res subunit in R₂M₂ complex is responsible for ATP hydrolysis, DNA translocation and cleavage. A vast majority of biochemical studies on Type III R-M enzymes have been undertaken using two closely related enzymes, EcoP1I and EcoP15I. Divergent opinions about how the long-distance interaction between the recognition sites exist and at least three mechanistic models based on 1D- diffusion and/or 3D- DNA looping have been proposed.

2. INTRODUCTION

Among the three major classes of Restriction-Modification (R-M) systems, the Type III R-M enzymes have a limited number of enzymes that are biochemically characterized. To date around 140 confirmed and putative Type III R-M systems have been identified by a combination of genetics and bioinformatics (1-2). Among the known Type III restriction enzymes, EcoP15I, EcoP1I, HinfIII and PstII are well characterized that recognize asymmetric sequences, 5'-CAGCAG-3', 5'-AGACC-3', 5'-GGAAT-3' and 5'-CTGATG-3' respectively (3-9). Other enzymes that have been analyzed to a lesser degree include StyLTI, LlaFI, BceSI and PhaBI (Table 1). Mechanistic details of Type III restriction enzymes have been defined primarily based on the characterization of EcoP15I and EcoP1I (3, 6). EcoP15I cleavage properties have been used to develop tools for gene expression analysis. These enzymes are multisubunit and multifunctional proteins possessing endonuclease (ENase), methyltransferase (MTase) and ATPase activities (5-8). The recognition sites of Type III restriction enzymes are non-palindromic 5-6 bp in length (Figure 1A), and DNA is

Table 1. Biochemical properties of Type III restriction-modification enzymes studied so far

Enzyme	Source	Recognition site (head to tail)	Restriction enzyme subunit composition	Cofactor requirements for restriction
EcoP1I	Prophage P1	5' -AG A CC-3' 3' -TCTGG-5'	R ₂ M ₂	Mg ²⁺ , ATP, AdoMet ^a
EcoP15I	Plasmid P15B in <i>E. coli</i> 15T ⁻	5' -CAGC A G-3' 3' -GTCGTC-5'	R ₂ M ₂	Mg ²⁺ , ATP, AdoMet ^a
StyLTI	<i>Salmonella typhimurium</i>	5' -CAG A G-3' 3' -GTCTC-5'	R ₂ M ₂	Mg ²⁺ , ATP
HinfIII	<i>Haemophilus influenzae</i>	5' -CG A AT-3' 3' -GCTTA-5'	R ₂ M ₂	Mg ²⁺ , ATP
PstII	<i>Providencia stuartii</i>	5' -C A TCAG-3' 3' -GTAGTC-5'	R ₂ M ₂	Mg ²⁺ , ATP / GTP / CTP
LlaFI	<i>Lactococcus lactis</i>	ND	ND	Mg ²⁺ , ATP
BceSI	<i>Bacillus cereus</i>	ND	ND	Mg ²⁺ , ATP
PhaBI	<i>Pasteurella haemolytica</i>	ND	ND	ND

A, site of methylation; ND: not determined; ^a, unresolved discrepancy in AdoMet requirement for DNA cleavage (21, 23), variation is the method of enzyme purification

cleaved 25-27 bp downstream of the recognition sequence (3) in presence of ATP and Mg²⁺ cofactors (3, 5-6). These enzymes require a pair of two unmodified recognition sites in head-to-head orientation for efficient DNA cleavage (Figure 1B). The head-to-head orientation of recognition sequence is with respect to the presence of sites on a DNA molecule. For example, site A is 5'CAGCAG3' on one strand if the site B is 5'CTGCTG3' on the other strand of DNA, then the sites are in opposite (head-to-head) orientation (Figure 1). The head of the site is defined as the 3' end of the site and the tail is defined as the 5' end of the site (Figure 1A) (10).

Several host adapted bacterial pathogens (*Pasteurella haemolytica*, *Haemophilus influenzae*, *Helicobacter pylori*) have MTases (*mod* gene) associated with Type III R-M systems which contain single tandem repeats that are prone to phase variation, an adaptive process by which pathogenic bacteria undergo frequent and reversible phenotypic changes in the expression of surface antigens (11-12). The widespread distribution of phase variable Type III R-M systems in host-adapted pathogenic bacteria suggests that this regulated switching of multiple genes may be a commonly used strategy for bacterial pathogens (12). These phase variable Type III R-M systems are currently being studied in order to predict their role in the biology of bacterial pathogens. This review will discuss the biochemical and biophysical properties of the Type III restriction enzymes and highlight their mechanism of action.

2.1. Subunit composition

Type III restriction enzymes comprise two modification (Mod) subunits, each containing a target recognition domain (TRD) to bind to the target sequence and a MTase catalytic domain to monitor the methylation status of an adenine in the target and transfer -CH₃ group to N⁶ of adenine, and two restriction (Res) subunits each containing a DNA helicase and ATP-hydrolysing domain, and an endonuclease domain (6, 8). The Mod subunit of Type III R-M system studied so far exist as dimer (M₂) in solution and is a functional DNA MTase able to recognize

and modify recognition sequence (8). The Mod subunits of EcoP15I and EcoP1I share approximately 85 % amino-acid identity at the N-terminal and C-terminal regions, with the central region having no appreciable similarity. The N-terminal region of the Mod subunits has non-specific DNA binding property and residues forming the catalytic site of adenine MTases (DPPY motif). The amino acids that interact with AdoMet (FxGxG motif) lie towards the carboxyl terminal region. The central region of the Mod subunit has residues responsible for recognition of cognate site on DNA and a C-terminal region is thought to contribute significantly to interaction with the Res subunit (13).

Multiple sequence alignment of all known and putative Res subunits suggests a modular structure (Figure 2). The N-terminal region of Res subunit contains seven amino acid sequence motifs shared by a superfamily 2 of helicases (SF2) which includes the Walker A and B motifs involved in the unwinding of duplex DNA (14) and responsible for ATP hydrolysis, DNA binding and translocation (Figure 3). The C-terminal contains the PD(x)_n...(D/E)xK motif, a characteristic magnesium binding motif, that is present in the catalytic centers of Type II restriction ENases. Thus, Res subunit resembles a fusion between a helicase and a nuclease. The presence of a putative helicase domain in Res correlates with the proposed DNA translocation activity (see below). However, as observed with several proteins having helicase domains (15), Type III restriction enzymes do not exhibit helicase activity in conventional assays. Nevertheless, mutations in the helicase motif abolish DNA cleavage and ATPase activities of EcoP1I, suggesting an important link between the motifs and ATP-dependent DNA translocation and subsequent cleavage (16). Single alanine substitutions in the putative nuclease active site of EcoP1I Res in helicase motif VI abolished both the ATPase and nuclease activities (17).

The amino acid residues critical for interaction between Mod and Res subunits are not fully defined. The Mod and Res subunits of EcoP15I can heteromerize with the subunits of EcoP1I restriction enzyme reflecting the

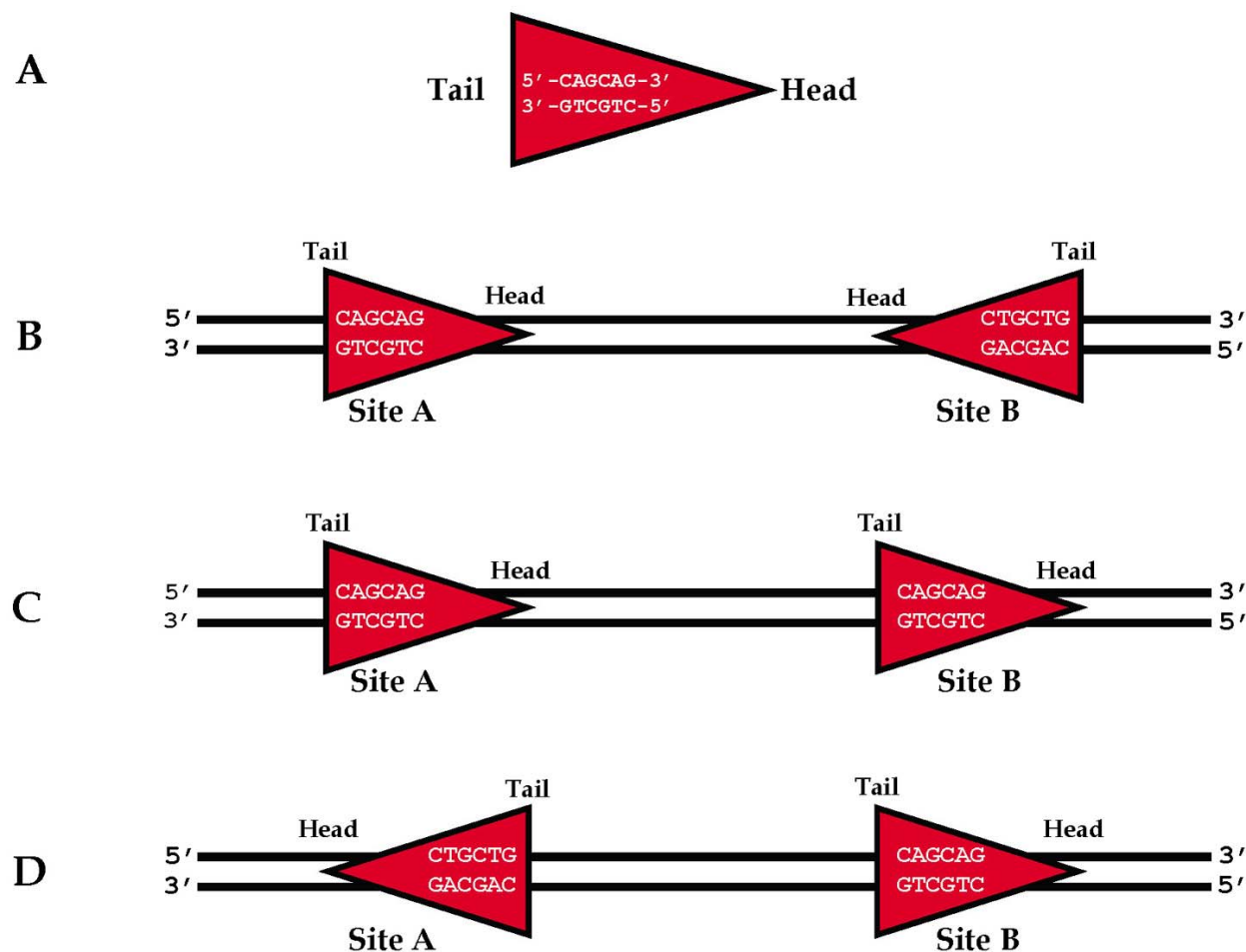


Figure 1. Recognition sequence of EcoP15I. A. The recognition site of the Type III R-M enzymes is represented as a triangle. The base of the triangle (5') is tail and tip (3') of the triangle is head of the recognition sequence. B. Representation of head-to-head oriented recognition sites on a DNA molecule which is a preferred substrate for Type III R-M enzymes. C. Representation of head-to-tail oriented recognition sites. D. Representation of tail-to-tail oriented recognition sites.

amino-acid identities between the subunits (18-19). For most of the Type III R-M enzymes, the Mod subunit alone forms a homodimer (M_2) in solution, while the Mod and Res subunits together form the heterotetramer R_2M_2 complex (17). PstII restriction enzyme, on the other hand, occurs primarily as R_2M_2 but subassemblies (RM_2) in which one Res subunit has dissociated have been observed in solution (20).

2.2. Cofactor Requirements

EcoP15I, EcoP1I, HinfIII and PstII restriction enzymes require ATP hydrolysis and Mg^{2+} for DNA cleavage and possess sequence-specific ATPase activity (3, 9). Interestingly, PstII restriction enzyme has been shown to cleave DNA in presence of GTP and CTP and the relaxed specificity in the nucleotide hydrolysis probably reflects a flexible arrangement of residues in the NTP binding site (9). While AdoMet is an absolute requirement for DNA methylation by Type III R-M enzymes, there is conflicting data on the effects of AdoMet or its analogs on DNA cleavage activity of these restriction enzymes (21-

23). The ENase activity of Type III restriction enzymes, EcoP15I and EcoP1I is influenced by cofactors, ionic conditions, enzyme concentration and particular flanking sequences of the recognition sites. One study showed that purified EcoP15I restriction enzyme has bound AdoMet and that the apoenzyme cleaves DNA with sites in head-to-head orientation only in the presence of exogenously added AdoMet (21). In contrast, another study showed that EcoP15I restriction enzyme neither contains endogenously bound AdoMet nor requires addition of external AdoMet to specifically cleave DNA with sites in head-to-head orientation (23). The reasons for the discrepant findings on cofactor requirements of EcoP15I restriction enzyme remain unclear. Varying buffer conditions and time of dialysis after purification or the host strains used to express the enzymes could influence EcoP15I restriction enzyme characteristics (Table 1). The presence of AdoMet however seems to decrease the promiscuity of DNA cleavage (at sites other than 25-27 bp downstream of recognition site) by Type III R-M enzymes (22), this might decrease the non-specific cleavage of DNA inside a bacterial cell. The

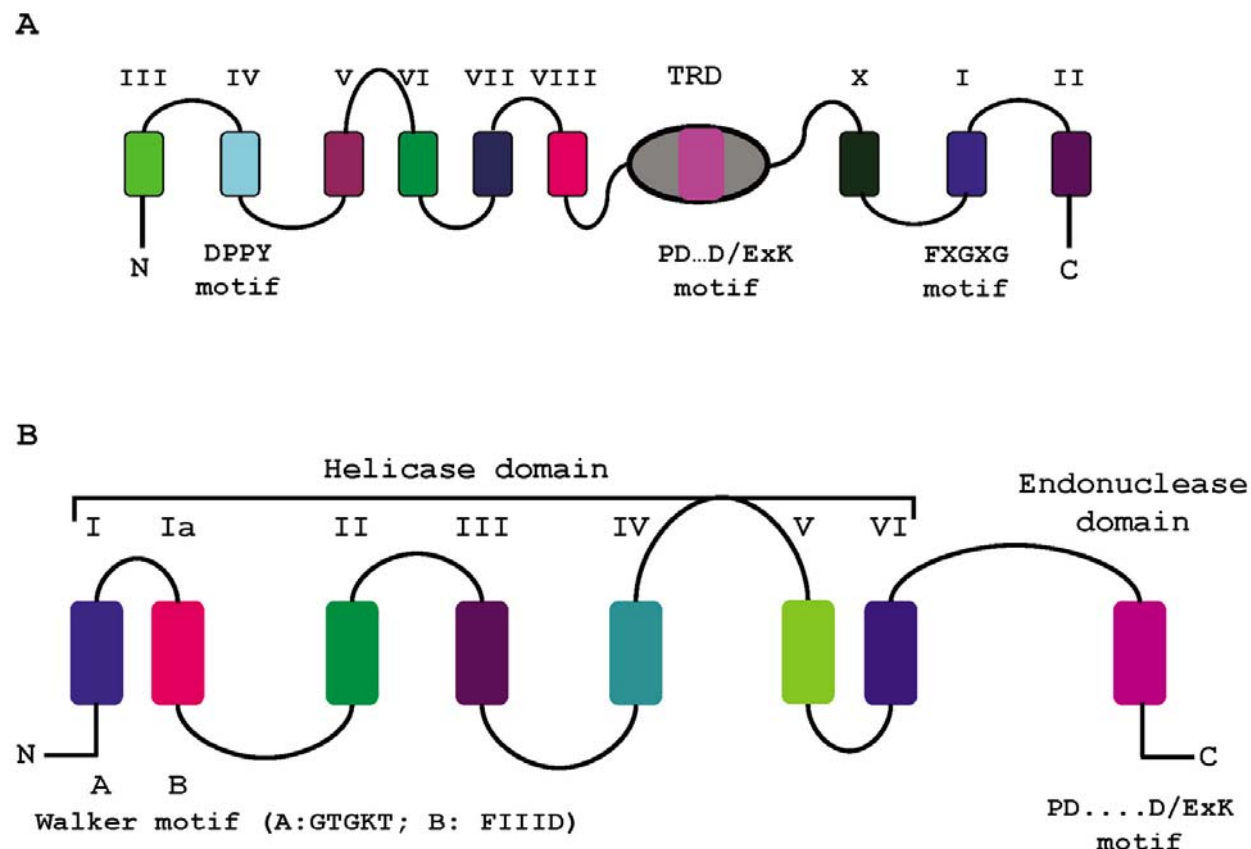


Figure 2. Motif organization of Type III restriction-modification enzyme subunits. A. Arrangement of motifs characteristic of N⁶-adenine MTases in the Mod subunit. Catalytic motif (DPPY) and AdoMet binding motif (FxGxG) are present towards amino- (N') and carboxyl (C') terminus of the subunit, respectively. Endonuclease (PD_n....D/ExK) motif characterized in Mod subunit of EcoP15I is shown within target recognition domain (TRD) present in the central region of subunit. B. Res subunit contains seven well-defined motifs of helicase domain at the N' terminus (numbered I through VI) and signature motif of endonuclease catalytic motif, PD....D/ExK, at C' terminus. Motifs are represented as colored boxes and connecting residues between motifs shown as lines.

presence of AdoMet supports methylation of the sites by restriction enzyme (R₂M₂) following cleavage reaction.

Studies with HinfIII restriction enzyme showed that restriction reaction does not strictly require AdoMet but is stimulated by it. HinfIII restriction enzyme has been reported to exist as AdoMet-bound form (HinfIII*) and found to gradually convert to an AdoMet-free form upon storage (24). AdoMet has no effect on cleavage by BceS1 while LlaFI is stimulated by it. At least two clear plaque mutants of phage P1, c2-134 and c2-440 characterized as point mutations in Mod subunit were shown to be modification deficient. In addition, the c2-134 mutant (T298I) EcoP1I restriction enzyme was not restriction proficient, while the c2-440 mutant (T316I) enzyme was partially restriction proficient (25-26). The inability or partial ability of these mutant enzymes to cleave DNA was attributed to the fact that they are either unable to bind AdoMet or that they bind very weakly (21).

Sinefungin, a structural analog of AdoMet has been shown to stimulate the ENase activity of a few

restriction enzymes, such as BcgI, a Type IIB RM system. Addition of AdoMet analogue sinefungin to the EcoP15I restriction enzyme cleavage reaction has been observed to result in cleavage downstream of all EcoP15I recognition sites on DNA (the two site requirement is not a strict prerequisite in presence of sinefungin) as well as cleavage of single site on linear or circular DNA (27). However, the cleavage efficiency seems to vary with the DNA substrate used, with some substrates being cleaved at all sites (27) and others showing marginally enhanced cleavage (23). Interestingly, S-adenosyl-L-homocysteine (AdoHcy), the product of all AdoMet-dependent methylation reactions, has been shown to stimulate promiscuous nuclease activity of the EcoP1I restriction enzyme under certain permissive conditions. The promiscuous nuclease activity is where EcoP1I restriction enzyme cleaves DNA nonspecifically in the presence of AdoHcy after first cleavage near the recognition site. As in the case of sinefungin, AdoHcy supports and promotes DNA cleavage without the requirement for two sites in head-to-head orientation (28).

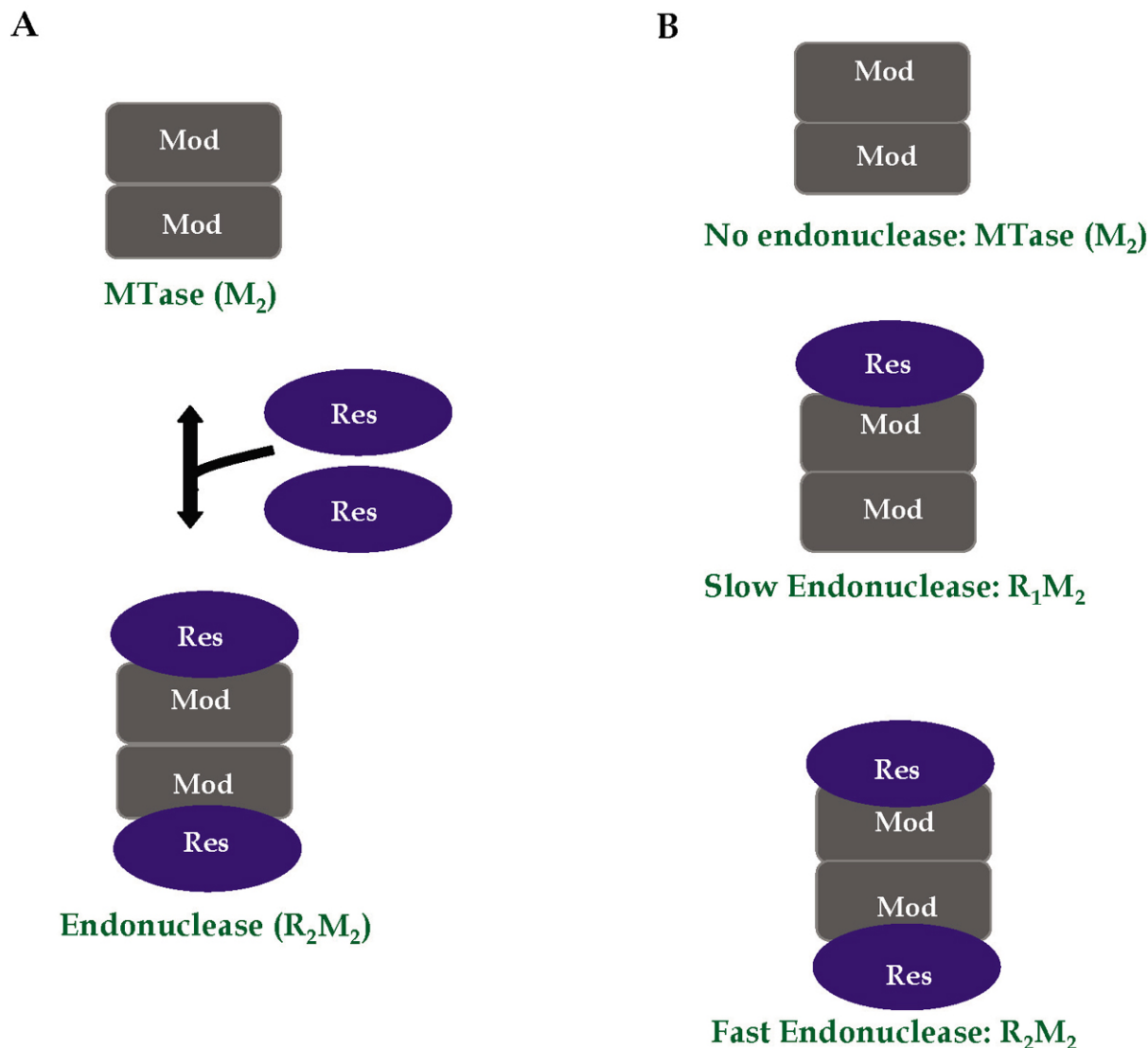


Figure 3. Model for Type III restriction-modification enzymes assembly and its effect on enzyme activity. A. Methyltransferase of Type III restriction-modification enzymes is composed of homodimer of Mod subunits (M_2). Mod subunit is represented as a filled rectangle in gray color. Association of M_2 with two Res subunits (shown as filled ovals in blue color) results in formation of heterodimeric endonuclease enzyme (R_2M_2) that exhibits both DNA methylation and cleavage properties based on substrate. B. Endonucleolytic ability of various oligomeric forms of Type III restriction enzymes. Homodimeric Mod subunits (M_2) have no DNA cleavage activity. Formation of a heterodimer with two Mod and one Res subunits results in formation of an endonuclease with slow cleavage activity (eg., PstII). Heterodimeric enzyme having two Res and two Mod subunits (R_2M_2) exhibits faster endonuclease activity.

It has been reported that EcoP1I restriction enzyme catalyzed DNA cleavage activity is influenced by cationic and anionic buffer components, in particular the identity of the cation and enzyme concentration. In presence of Na^+ ions, even at high enzyme to site ratios (more than 15:1) cleavage was observed only on substrates with two sites in inverted repeat, while with K^+ ions, at an enzyme to site ratio of over 3:1 a DNA molecule with two sites in direct repeat and a DNA molecule with a single site was susceptible to cleavage (22). In contrast, a recent study probed the influence of different combinations of cationic and anionic species on the

ENase activity of EcoP15I restriction enzyme and found that buffer components did not have any effect (23). The activity of the enzymes in the cellular milieu can be influenced by the changes in concentration of ions and AdoMet that occur during bacteriophage infection to benefit the bacterial host (29), however, experimental evidence for such a regulation is still awaited. A systematic and thorough investigation of buffer-dependence of Type III restriction enzymes will be required to reveal whether activity of other AdoMet-dependent ENases can be modulated specifically by monovalent ions.

2.3. Regulation at protein synthesis and stability

The regulation of DNA cleavage by the enzyme occurs at the level of subunit stability as Mod subunit can be expressed in the absence of Res subunit, while Res subunit is quickly degraded in the absence of Mod subunit (30). Thus Mod subunit serves as a molecular chaperone and also provides specificity to Res subunit activity by recognizing the cognate DNA. The cellular mechanism to regulate the Type III restriction enzymes activity also occurs at the translational level as both Mod and Res subunits are coded by a bicistronic mRNA. The Mod subunit is translated from the first cistron of the mRNA, while the Res subunit is synthesized upon ribosomal realignment at the stop codon of Mod subunit facilitating translation from the second cistron (30). This provides the bacterial cell encoding the Type III restriction enzymes with more Mod subunit to protect the genomic DNA by methylation of cognate sites prior to synthesis of active ENase. It is unclear how a bacteriophage would be preferentially restricted than modified under such conditions. It can be speculated that the bacterial genome is protected from the activity of the ENase by being decorated by proteins such as bacterial histone-like protein Hu, while DNA of the incoming bacteriophage would be more exposed. One of the Type III restriction enzyme that has evolved to allow modulation of its activities through subunit assembly is PstII (20). While in many respects the enzyme activity of EcoP15I and EcoP1I are very similar to PstII restriction enzyme, unlike the former, the association of Mod and Res subunits of PstII is dynamic. Disassembly of PstII restriction enzyme by loss of a Res subunit has been shown to act to control relative modification/restriction activities (Figure 3). It has been proposed that the intact R_2M_2 complex favored at high PstII concentrations is a fast ENase / slow MTase while the various subassemblies which coexist at lower concentrations are fast MTase. This dynamic association of Res and Mod subunits could play a key role in the control of PstII restriction enzyme activity *in vivo*.

3. ENZYME ACTIVITIES ASSOCIATED WITH TYPE III RESTRICTION ENZYMES

The Type III restriction enzymes have MTase, ATPase and ENase activities that are well characterized. In addition they have the DNA helicase domains with no observable DNA helicase activity. Depending on the presence of cognate recognition sequence, the methylation status and orientation of the sites, Type III restriction enzymes exhibit various enzymatic activities. Understanding how these enzymes distinguish between an unmodified and a modified recognition site and either cleave downstream of only one of the two unmodified sites or modify sites that are not amenable to cleavage could hold and answer to regulation of activities by multiprotein complexes acting on DNA.

Type III restriction enzymes do not have detectable ATPase activity in the absence of DNA with cognate recognition site, implying that the cell has evolved to conserve the energy source in absence of invading bacteriophage (31). It should be noted that the ATPase

activity of EcoP15I and EcoP1I restriction enzyme were found to be only 1 % compared to that of EcoKI, a Type I R-M enzyme (3, 5). The ability to hydrolyze ATP only upon interaction with cognate recognition site also ensures that the enzyme does not exhibit non-specific DNA cleavage activity. ATPase-driven activities of Type III restriction enzymes are due to the presence of the helicase motifs. Motifs I and II, called Walker A and B, are most characteristic for ATP-hydrolyzing enzymes and defined by highly conserved sequences GxGKS/T and DExx (e.g. DEAH or DEPH), respectively (16). The lysine residue in motif I is essential as it contacts the phosphates of Mg-ATP/Mg-ADP, whilst the following serine / threonine and aspartic acid from motif II coordinate the Mg ion, which is essential for DNA cleavage. Both these motifs are crucial for the function of Type III restriction enzymes and single amino acid substitutions therein lead to decrease in ATPase and DNA cleavage activities by EcoP1I and EcoP15I restriction enzymes (16-17). It has been shown that the ATPase activity of EcoP15I and EcoP1I restriction enzymes are uniquely recognition-site specific, but EcoP15I and EcoP1I-modified sites also support the ATPase activity (32).

A third enzymatic activity associated with Type III restriction enzymes is methylation of DNA. A homodimer of Mod subunit (M_2) of Type III restriction enzymes functions as a DNA MTase which recognizes the specific recognition sequence and methylates the adenine residue (5, 18, 33). Both EcoP15I and EcoP1I restriction enzymes (R_2M_2) are also efficient MTases as they modify the DNA following cleavage reaction. The well studied EcoP15I and EcoP1I MTases (M_2) belong to the beta-group of amino MTases and like all N^6 -adenine MTases, contain nine characteristic motifs, including two highly conserved sequences, FxGxG (motif I) involved in AdoMet binding and DPPY (motif IV) involved in catalysis (34). An interesting aspect of EcoP15I restriction enzyme is that methylation of the 5'-CAGCAG-3' sequence by both MTases (M_2) and restriction enzymes (R_2M_2) requires magnesium ions (18, 35). It has been shown that the PD(x)_n-(D/E)xK-like motif, characteristic of the majority of restriction enzymes, present in EcoP15I MTase (M_2) is involved in magnesium ion binding. When the M.EcoP15I bound to DNA containing 2-aminopurine substitutions within cognate sequence, an 8- to 10-fold fluorescent enhancement resulting from enzymatic flipping of the target adenine base was observed (36). Target base flipping has been postulated for other N^6 -adenine MTases and supported by biochemical and biophysical evidences and computer modeling with the X-ray data (37). Both 2-aminopurine and chemical probing were used to demonstrate in EcoP15I DNA MTase (36). A number of reviews have described various aspects of DNA MTases (37-40). For equivalent amounts of restriction enzyme (R_2M_2) and MTase (M_2), the kinetics of methylation by the latter is different from those for the restriction enzymes. Although methylation by the restriction enzyme (R_2M_2) was stimulated by ATP on a DNA with sites that are not amenable to cleavage, the activity of the MTases (M_2) is not affected by ATP (5-6, 18). It has been suggested that the stimulation of methylation activity of EcoP15I and

Type III restriction-modification enzymes

Substrate	Cleavage	Substrate	Cleavage	Substrate	Cleavage
<u>Single site substrate</u>		<u>Head-to-head orientation</u>		<u>Head-to-tail orientation</u>	
A	+ (17, 23) - (9, 41)	H	+ (1, 3, 7, 43)	P	- (7, 38, 40, 43)
B	+ (36)	I	+ (7, 26)	Q	- (37, 38) + (36)
C	- (36)	J	+ (19, 38)	R	- (36)
D	+ (36)	K	+ (24, 37)	S	- (37)
E	+ (37)	<u>Tail-to-tail orientation</u>		<u>Hybrid substrate</u>	
<u>Catenated substrate</u>		L	+ (1, 3, 43)	T	+ (14, 46)
F	- (35)	M	+ (36-38) - (7)		
G	+ (35)	N	+ (37)		
		O	+ (19, 38)		

Figure 4. Orientation of recognition sites and cleavage ability of Type III restriction enzymes. DNA is shown as black circle or a straight line for closed circular or linear forms. Recognition site is represented as filled red triangle with base and head of triangle being 5' and 3' ends of site. Observed cleavage and lack of cleavage are shown as '+' and '-'. For hybrid substrate, P15 and P1 stand for recognition site of R.EcoP15I and R.EcoP1I and P15 and P1 sites are susceptible for cleavage.

EcoP1I restriction enzyme (R_2M_2) in the presence of ATP could be a consequence of ATP hydrolysis decreasing the off-rate of enzyme from DNA thus transforming modification reaction from a distributive to a processive mode. Distributive mode is where enzyme binding to DNA and dissociating from DNA is a continuous event till it reaches the recognition site. However, in processive mode, enzyme scans for recognition site on a DNA molecule without dissociating from it in a single binding. Interestingly, modification by Type III enzymes which occurs on only one strand is adequate to protect DNA from cleavage by the cognate ENase.

4. DNA CLEAVAGE

Type III restriction enzymes cleave DNA having two recognition sites in opposite orientation on the same DNA. The two sites have to be unmethylated and bound by two enzyme molecules. The cleavage has an absolute requirement for ATP hydrolysis and Mg^{2+} . The distance between the two sites on DNA can range from tens to thousands of base pairs (bp) apart. Although efficient cleavage requires two sites on the same DNA (Figure 4; substrates: H and I), phosphodiester bond cleavage occurs close to only one of the two sites (Figure 5). The double strand cleavage is outside the recognition site and is

approximately 25 bp on the top strand and 26-27 bp on the bottom strand downstream of the site (10, 17, 32). Type III restriction enzymes have an interesting DNA cleavage mechanism with amino acid residues required for nucleotide sequence recognition in Mod subunit and those for phosphodiester bond hydrolysis (nuclease) activity in Res subunit. With the Type III restriction enzymes being a hetero-oligomer of Mod and Res subunits (R_2M_2), the sequence of events leading to the cleavage of DNA poses a competition between the protective DNA methylation activity and the restrictive cleavage activity. The DNA cleavage requires ATP hydrolysis by the enzyme and both unmodified and modified sites elicit the ATPase activity of the enzyme. How are these enzymes fine tuned to distinguish between an unmodified and a modified recognition site and result in cleavage downstream of only one of the two unmodified sites in head-to-head orientation is intriguing as well as challenging to study.

4.1. Site requirement

DNA having two unmodified recognition sites in opposite (head-to-head) orientation is a substrate for cleavage by Type III restriction enzymes (Figure 4; substrates: G, H-K and T). Cleavage of various substrates other than a two-site head-to-head DNA has been observed. It is important to mention here that enzyme concentration

Type III restriction-modification enzymes

and the nature of the monovalent cations of the reaction buffer seem to affect the cleavage patterns by Type III restriction enzymes as reflected in a discrepancy over cleavage observed with certain substrates (A, M and Q). Substrates with sites in the same orientation on a DNA molecule (substrates: P-S) or single site on close circular (substrates: A and F) or catenated substrates with one site per catenane ring (substrate F) are not cleaved (10, 17, 32, 41). Unmodified sites on different DNA molecules do not co-operate to result in cleavage (substrate F), demonstrating that the sites *in trans* are not substrates for cleavage. Type III restriction enzymes have been demonstrated to cleave DNA with single recognition site on a linear DNA (substrates: B-E) albeit less efficiently compared to that of the DNA with two sites in head-to-head orientation (42-43). Blocking of the ends by streptavidin on a linear DNA having sites in head-to-head orientation (substrate K) increased the cleavage events suggesting the enzyme molecules loaded on the DNA might need a free end to increase the off-rate. Linear DNA with sites in tail-to-tail orientation with ends capped by streptavidin (substrate N) promotes cleavage of DNA close to one of the two sites (43). The blocking of DNA ends on a linear substrate with sites in head-to-head or tail-to-tail orientation was suggested to prevent the release of enzyme at the end and consequently enhance translocation of the enzyme backwards and continue the back and forth movement till it interacts with other enzyme molecule for cleavage (43). Less efficient cleavage of DNA with sites in the same orientation (substrate Q) can be observed on a linear DNA (42). Binding of Lac repressor between recognition site and DNA end on 3' of site was shown to inhibit cleavage of a linear DNA with single site (substrate C) or two sites in head-to-tail orientation (substrate R), indicating that DNA end might play a role in cleavage mechanism (42). The study concluded that Type III restriction enzymes have unidirectional translocation from the asymmetric recognition site and DNA end reverses the direction of enzyme translocation mimicking an 180° rotation of the molecule and facilitating interaction with other enzyme molecule translocating towards the DNA end. However, no biophysical evidence has been obtained in either the AFM or single molecule studies to support or disprove this interpretation. These observations warrant further investigation to understand how reversal of translocation or backward movement of enzyme is achieved and the process is linked to DNA end. How the cleavage activity on linear DNA with sites that are not in head-to-head orientation might be important for restricting the bacteriophage needs to be understood.

4.2. Interaction of enzymes for cleavage

The distance between the two recognition sites in opposite orientation in the DNA substrate can be a minimum of 50 bp to more than 5,000 bp. Physical interaction between the enzyme molecules bound to the sites in opposite orientation is suggested to be assisted by ATP hydrolysis by Res subunits. Considering the DNA binding domain is in the Mod subunit, the interaction between the two enzyme molecules is possibly via the Mod subunits that bind to the recognition site (17). The Res subunit also has non-specific DNA binding property and

interaction between the two enzyme molecules on DNA can be achieved between Res subunits. Experimental evidence for the individual Mod and Res subunits of each enzyme molecule participating in the interaction with other enzyme molecule are challenging to obtain. Interestingly, a DNA molecule with one recognition site for EcoP15I restriction enzyme and one for EcoP1I restriction enzyme can be cleaved when bound by respective enzymes. The co-operation between EcoP15I and EcoP1I restriction enzymes for cleaving DNA having the recognition sites demonstrates similarity between the two enzymes (44). However, PstII and EcoP15I restriction enzymes cannot interact and cleave a DNA substrate (9) suggesting that Type III restriction enzymes must make specific protein-protein contacts to activate ENase activity.

Cleavage by inactive mutants of the EcoP1I and EcoP15I restriction enzymes were used on a DNA having one recognition site for each enzyme in opposite orientation to provide more insight into the role of each subunit for DNA cleavage. Recognition of one site by a wild type enzyme and other by an ENase mutant capable of ATP hydrolysis results in nicking and not double strand break. The strand on which nick is observed was dependent on which of the two enzymes was cleavage efficient and which is not. The cleavage of top strand seems to be carried out by enzyme binding to the site downstream of which cleavage is occurring with the bottom strand being cleaved by enzyme recognizing the site in opposite orientation (17, Figure 5A). The results obtained indicate that Res subunits of both the enzymes are involved in interaction between the two molecules and provide active site residues for double strand phosphodiester bond cleavage. The ENase activity of the enzyme molecules recognizing both unmodified sites on DNA is required for cleavage with two strands of DNA being cleaved one by each of the enzymes. The gap between the cleavage on two strands is usually two nucleotides and the cleaved ends have 5' overhang. Although involvement of four Res subunits and four Mod subunits adds to the complexity in DNA cleavage by Type III restriction enzymes, it might provide the host cell expressing the ENase with an opportunity to regulate the enzyme as activity of both Mod and Res subunits of two enzyme molecules is required for cleaving both strands of DNA.

The presence of a protein molecule such as Lac repressor tightly bound to repressor binding site between two recognition sites for Type III restriction enzymes in opposite orientation can block DNA cleavage, indicating that the restriction enzymes bound to two sites have to physically interact for restriction activity. Whether such a block to enzymatic activity on the host genomic DNA decorated by other proteins operates in the bacterial cell is not known.

4.3. Mode of communication

Binding of a restriction enzyme to its recognition site (unmodified or modified) leads to hydrolysis of ATP by Res subunit, however it is unclear how the energy released from ATP is used for DNA cleavage. In accordance to the similarity with Type I restriction enzymes, the ATP hydrolysis is suggested to drive

Type III restriction-modification enzymes

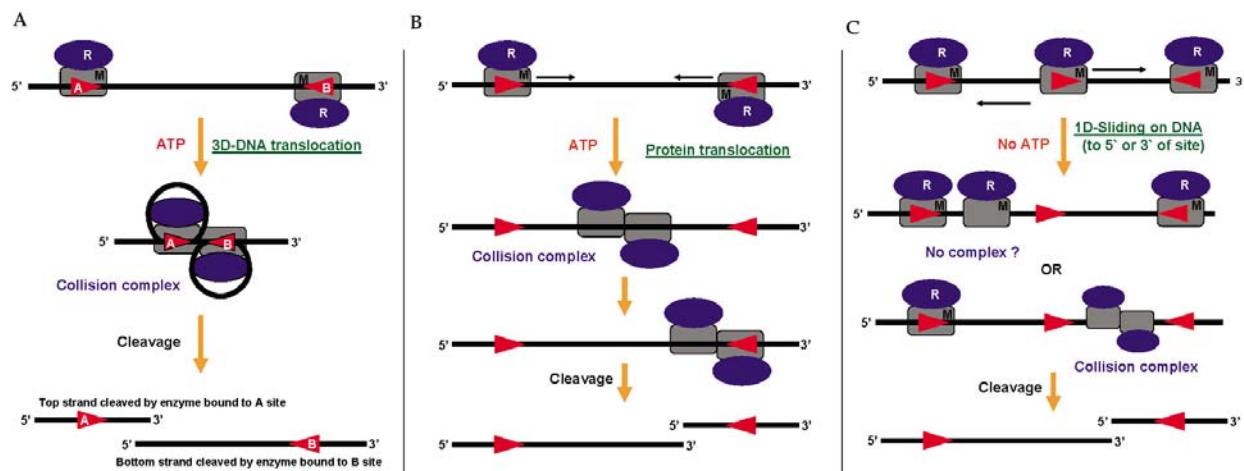


Figure 5. Schematic diagram showing the models proposed for DNA cleavage by Type III restriction enzymes. A. DNA loop and translocation. B. Protein translocation. C. DNA sliding. The recognition sites in panel A are labeled as 'A' and 'B' for ease of explanation, are otherwise sites in opposite orientation. One study (38) shows that site A can be that of EcoP15I and site B that of EcoP1I and the DNA can be cleaved only in presence of both enzymes.

translocation of DNA with the enzyme remaining bound to the recognition site. DNA translocation by the enzyme molecules bound to sites in opposite orientation would facilitate to bring the enzymes into close proximity for the interaction among the subunits of the enzymes to occur and result in double strand cleavage (10, 32). During DNA translocation, each of the enzyme binds DNA at two different sites, one is the recognition site of the enzyme and the other being non-specific DNA that is translocated past the enzyme. Consequently, loops of DNA that increase in size with more translocation would be formed until no further DNA can be pulled by the enzyme due to block by another enzyme or a protein such as Lac repressor that is tightly bound to DNA (Figure 5A). A simple block to DNA translocation by the enzyme does not trigger cleavage as Lac repressor bound to DNA between two sites in opposite orientation blocks cleavage but not ATP hydrolysis by the enzyme (32). The direct interaction of the two enzyme molecules is essential for cleavage of DNA having unmodified sites. DNA translocation by the enzyme would ensure that once an unmodified site is bound by the enzyme another molecule of enzyme would not bind to the same site and hydrolyze ATP, and this way the host cell does not lose the energy source because of redundant activity.

The evidence obtained with Lac repressor blocking the interaction between restriction enzymes binding sites in opposite orientation does not exclude the possibility that the enzymes might translocate along the DNA till they interact. The movement of enzyme molecules along the DNA in an ATP dependent fashion is defined as protein translocation (Figure 5B) and differs from the DNA translocation (41-42 and Figure 5A). A R_2M_2 enzyme molecule interacts with DNA at one site rather than two sites during the process and does not result in DNA loop formation. Each enzyme molecule (R_2M_2) can effectively translocate either towards the 5' side or towards the 3' side of recognition site. But cleavage of only head-to-head as opposed to head-to-tail on circular DNA indicates

that the enzyme always translocates towards the 3' side of a site to form a collision complex. The enzyme molecules translocating on DNA towards the 3' side of a recognition site would interact with each other between the sites and form a single endonucleolytically active complex (R_4M_4). DNase I footprinting experiments provide evidence for protection of both the recognition site and non-specific site on the spacer DNA between two sites in opposite orientation supporting the view that enzyme translocates DNA (45). However, the possibility that binding of a second enzyme molecule to the same recognition site once the first molecule starts to translocate along the DNA cannot be excluded. Type III restriction enzymes cleave DNA with single recognition site on linear DNA albeit less efficiently compared to that of the DNA with two sites in opposite orientation (42). Such cleavage activity has been used to lead support for the protein translocation by the enzyme verses translocation of the DNA, as cleavage of single sites on closed circular DNA is not achieved under similar conditions of reaction. Type I enzymes that involve DNA translocation for cleavage can act on single site circular DNA where cleavage is trigger upon stalling of translocation on account of enzyme having looped out all the DNA (46). Protein translocation facilitates binding of single recognition site by more than one enzyme molecule and interaction of these molecules on the DNA to form single entity (R_4M_4) capable of cleaving DNA, while DNA translocation precludes binding of second enzyme molecule and consequently that of R_4M_4 complex (42). On a circular DNA with single site, any enzyme molecule loading on to a recognition site and translocating towards 3' of site would fail to form a complex with another enzyme loading later on to site and also translocating to 3' of site. However, on a linear DNA with single site the reversal of direction by enzyme upon reaching the end of DNA has been implied to mimic an enzyme translocating from a site in opposite orientation and hence facilitating formation of collision complex (42).

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Obtaining physical evidence for mode of communication between enzyme molecules recognizing sites in opposite orientation with spacer DNA of thousands of base pairs has remained an area of intense study and interest. Scanning force microscopy (SFM) study using EcoP151 restriction enzyme revealed 1 to 10% of total DNA with loop structures at one of the two recognition site, arguing DNA translocation to be responsible for interaction between the enzymes. This study highlighted the challenge involved in capturing the cleavage reaction intermediates such as those with loops at both recognition sites, as the reaction is ATP driven and expected to occur rapidly upon binding of enzyme to recognition site (47).

Atomic Force Microscopy (AFM) studies using a linear DNA with one recognition site (48) and two sites in same or opposite orientation (49) concluded that Type III restriction enzymes decrease the distance between the two sites by initial diffusive DNA looping (ATP independent) followed by DNA translocation driven by ATP hydrolysis. The initial diffusive looping has been postulated to provide interaction between enzymes even in the presence of a Lac repressor that is bound half way on the spacer DNA. These AFM studies suggest that the looping phenomenon observed under the conditions where DNA is confined to two-dimensional (2D) geometry are not correlated to cleavage activity of the enzymes, although the loop formation is site and ATP-dependent, implying DNA loops are formed independent of the cleavage pathway of the enzymes. Whilst evidence from several independent AFM studies is consistent with loop formation, other studies have failed to find DNA looping, changes in topology or stepwise motion on DNA. The energy utilized by Type III enzymes is minimal compared to that by Type I R-M enzymes which have been demonstrated to actively loop out the intervening DNA between the sites. Thus, it appears that Type III restriction enzymes have found an optimal strategy for their function by utilizing minimum energy for communication prior to cleavage. Communication between enzyme molecules recognizing sites that are tens of hundreds of base pairs apart without a need to loop out the intervening DNA might be an evolutionary step to conserve the energy source of the cell. A multiplex magnetic tweezers assay was developed to follow both DNA cleavage and looping simultaneously as a function of tensional and torsional force. DNA cleavage was shown to be force-independent and was never preceded by active DNA looping (43, 50). The single molecule study provided evidence that both passive and active-DNA looping are not on pathway to cleavage and that communication occurs bidirectionally in one-dimension along the DNA contour-motion and occurs by thermally-driven one-dimensional diffusion i.e. DNA sliding (Figure 5C) which differs from ATP-driven translocation of the enzyme on DNA termed as protein translocation in not utilizing energy from ATP (Figure 5B). However, the DNA sliding model requires the conversion of ATP to ADP by the enzyme upon interaction with recognition site. Such ATP to ADP conversion serves as a molecular switch for changing the enzyme from site-searching mode to one capable of forming an endonucleolytically active collision complex. Two different single molecule techniques, AFM/SFM and MT lead to

conclusions that are in disagreement (51-53). It is important to note that DNA looping and movement of enzyme along a DNA contour are both fast events occurring on the millisecond timescale, much faster than the time resolution of the assays used. It is obvious that future experiments on Type III restriction enzymes should include a direct visualization of fluorescently-labeled enzymes diffusing on stretched DNA (54-56).

4.4. Deciding the site of cleavage

The puzzling aspect of cleavage by Type III restriction enzymes is how does the enzyme decide which one of the two unmodified sites is preferred for cleavage as cleavage occurs downstream of one of the two unmodified sites in opposite orientation. Cleavage always occurs at fixed distance downstream of recognition site and a block by another protein such as Lac repressor does not trigger the cleavage. DNA translocation by the enzyme while remaining bound to the recognition site makes both the recognition sites identical with respect to susceptibility for cleavage. For two Type III restriction enzyme molecules translocating DNA, the enzyme has to position the site of cleavage within the active site of Res subunit while translocating the remaining spacer DNA past the bound enzyme. Any mechanism to trigger cleavage activity downstream of one of the two sites requires accessibility of strands to be cleaved by both enzyme molecules. A stochastic model predicts that there is equal probability for cleavage of any of the two sites and hence cleavage downstream of each site would occur half of the time. Conclusive evidence for the influence of sequence of nucleotides at the recognition site or site of cleavage is lacking although it has been suggested that the presence of a track of adenines 3' of the site increases the probability of cleavage close to the site. The length of the adenine stretch on the two sides seems to affect the efficiency of cleavage (22). DNA cleavage by Type III restriction enzymes involving protein translocation might explain the cleavage properties observed with these enzymes realistically. Interaction of two enzyme molecules on the spacer DNA between the sites and formation of a complex comprised of both molecules as a single entity (R_4M_4) is possible. Such an entity can translocate towards one of the two sites and cleave DNA upon reaching the recognition site explaining why one but not both recognition sites are susceptible to cleavage simultaneously (42). Yet why the entity fails to translocate towards other site after the cleavage event and result in cleavage of other site is not clear. Probably, the entity dissociates into individual enzyme molecules once cleavage occurs at a recognition site.

4.5. Following cleavage reaction

Unlike the Type II restriction enzymes that cleave DNA within the recognition site and effectively destroying the recognition site, cleavage by Type III restriction enzymes occurs outside the recognition site. The DNA cleaved by Type III restriction enzymes has an intact recognition site and enzyme binding to such site can hydrolyze ATP and effectively perform DNA or protein translocation. Binding of enzyme to intact recognition sites (close to or away from the cleavage site) on cleaved DNA and hydrolysis of ATP decreases the off-rate of the enzyme

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from the products of cleavage reaction. Type III restriction enzymes have been determined to have single turnover with increasing cleaved DNA in the reaction decreasing the turnover to less than one. Sequestration of enzyme by intact recognition site on the cleaved DNA would be ineffective for the host cell during infection by a bacteriophage (57). However, degradation of the cleaved DNA by cellular exonuclease seems to result in restoration of turnover by the enzyme (57-58), explaining the ability of Type III restriction enzymes to degrade incoming bacteriophage DNA despite cleavage outside recognition site. A functional cooperation between EcoP15I restriction enzyme and an exonuclease is essential for multiple rounds of DNA cleavage by the enzyme (57). Extensive exonuclease-like processing of DNA in presence of AdoHcy by EcoP1I restriction enzyme after initial double-strand break is yet another example of processive shortening of linear DNA (28). Since DNA cleavage by EcoP1I is followed by methylation of the recognition, the methyl donor AdoMet is converted to reaction by-product AdoHcy and the AdoHcy bound enzyme could trigger exonuclease activity of the restriction enzyme. Given that the central role of restriction enzymes is to destroy invading foreign DNA, additional end-specific processing following a double-strand break could be seen as advantageous.

4.6 Applications

The distance of 24-28 bp between the recognition and cleavage sites in the DNA molecule by a Type III restriction enzyme is the longest defined distance known so far for any restriction enzyme. Therefore, EcoP15I restriction enzyme is used as the tagging enzyme in Serial Analysis of Gene Expression (SAGE) method of transcriptome analysis. As SuperSAGE is based on the extraction of DNA fragments of defined length, it would be great benefit to direct cleavage by EcoP15I restriction enzyme to exactly that recognition site located in the sequence tags. In the absence of AdoMet and with enzyme in molar excess over recognition sites, minor cleavage at two communicating DNA sites simultaneously has been observed. These results could be potentially exploited in the high-throughput quantitative transcriptome analysis method SuperSAGE (23). The cleavage activity of Type III restriction enzyme EcoP15I in presence of sinefungin has provided a powerful tool for generation of 25 bp long cDNA tags during SAGE assays (27) and using this principle a commercial kit called SOLid SAGE Kit has been developed by Applied Biosystems, USA.

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by a CAG trinucleotide repeat expansion located in the first exon of the HD gene. HD exon 1 DNA is a substrate of EcoP15I ENase and because of the overlapping recognition sites within the CAG repeat, EcoP15I ENase digestion of DNA fragments bearing HD exon 1 generates a ladder of restriction fragments, whose number corresponds to the number of CAG repeats. EcoP15I ENase digestion and subsequent analysis of the restriction fragment pattern by electrophoresis through non-denaturing polyacrylamide gels using the ALFexpress DNA analysis system has been used to determine the number of CAG repeats in the HD

gene. CAG repeat numbers in normal (30-35 repeats) as well as in the pathological range (81 repeats) could be determined using this assay (59).

5. FUTURE PERSPECTIVES

The glaring problem we now need to address is precisely how the Type III R-M enzymes couple relatively low ATPase rates to communication events between distant DNA sites. Unfortunately, we currently do not have any structural information on Type III R-M enzymes and answers to several questions await structural information.

The past few years have marked a turning point in our understanding how Type III R-M enzymes recognize, modify, and cleave DNA. Type III R-M enzymes promise to be a rich source of information about protein-DNA, and protein-protein interactions. These enzymes have proven attractive for study of mechanisms by which proteins accurately and efficiently recognize unique DNA sequences. The novel features of the structural elements and mechanisms of these enzymes pose exciting questions for the enzymologist. Lack of three dimensional structures of the Mod and Res subunits of these enzymes are primarily because of the size of M_2 and R_2M_2 complexes being enormous and laborious and low-yielding purification protocols (60). Computational prediction of the structures is hindered by absence of sequence similarity between the subunits of Type III R-M enzymes and that of the crystal structures in RCSB Protein Data Bank (www.rcsb.org/pdb). Availability of the crystal structures of the Type III R-M enzymes will provide insights into the complex mechanistic details of DNA recognition, modification and cleavage.

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Abbreviations: AdoHcy: S-adenosyl-L-homocysteine; AdoMet: S-adenosyl-L-methionine; AFM: atomic force microscopy; ENase: endonuclease; Mod: modification; MT: magnetic tweezers; MTase: methyltransferase; R-M: restriction-modification; Res: restriction; SFM: scanning force microscopy; TRD: target recognition domain

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