

Exogenous AdoMet and its analogue sinefungin differentially influence DNA cleavage by R.EcoP15I—Usefulness in SAGE

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Abstract

While it has been demonstrated that AdoMet is required for DNA cleavage by Type III restriction enzymes, here we show that in the presence of exogenous AdoMet, the head-to-head oriented recognition sites are cleaved only on a supercoiled DNA. On a linear DNA, exogenous AdoMet strongly drives methylation while inhibiting cleavage reaction. Strikingly, AdoMet analogue sinefungin results in cleavage at all recognition sites irrespective of the topology of DNA. The cleavage reaction in the presence of sinefungin is ATP dependent. The site of cleavage is comparable with that in the presence of AdoMet. The use of EcoP15I restriction in presence of sinefungin as an improved tool for serial analysis of gene expression is discussed.

Keywords: AdoMet; Sinefungin; EcoP15I; DNA topology; Site requirement; SAGE

S-Adenosyl-L-methionine (AdoMet) plays a vital role in several biological activities ranging from gene expression to methylation [1]. AdoMet is the major methyl group donor for methylation of a variety of biological molecules across living systems [2,3]. Type I restriction enzymes (RE) show a requirement for AdoMet to cleave DNA [4]. The requirement of AdoMet for DNA cleavage by Type III restriction enzymes has been demonstrated to vary with the monovalent ion used in the assay [5,6]. AdoMet was found to be essential for DNA cleavage reactions in the presence of Na⁺ ions and not required for cleavage in the presence of K⁺ ions. AdoMet has, therefore, been suggested to suppress the promiscuous DNA cleavage of Type III restriction enzymes, achieved at high enzyme concentrations under certain reaction conditions [6]. It has been shown that AdoMet enhances the cleavage activity of some Type IIB and IIG restriction enzymes [7]. Restriction enzymes BplI (Type IIB) and BseMII (Type IIG) can cleave

DNA in the presence of a structural analogue of AdoMet, sinefungin (Sf), or *S*-adenosyl-L-homocysteine (AdoHcy) instead of AdoMet [8,9].

Type III REs are ATP dependent enzymes composed of restriction (Res, R) and modification (Mod, M) subunits. The holoenzyme is a heterotetramer (R₂M₂). To introduce one double strand break 25–27 bp downstream of recognition site, the presence of two oppositely (head-to-head) oriented recognition sites on circular DNA is essential [10]. Recently, we have shown that linear DNA with single recognition site can be cleaved provided the enzyme can interact with DNA end on 3' side of the site [11]. Type III REs perform one round of cleavage reaction *in vitro* and require the presence of exonucleases for multiple turnover reactions [12]. EcoP15I restriction enzyme (R.EcoP15I) belongs to Type III R-M system and recognizes the sequence 5'-CAG-CAG-3'. For R.EcoP15I, head-to-head orientation is the presence of two recognition sites on one 5'–3' strand as CAGCAG and GTCGTC [13]. The R.EcoP15I holoenzyme co-purifies with bound AdoMet, while the related enzyme EcoPII does not, hence in DNA cleavage

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assays addition of exogenous AdoMet is not required for R.EcoP15I but is essential for R.EcoP1I enzyme [5].

Serial analysis of gene expression (SAGE) is a method for comprehensive analysis of the cellular gene expression patterns. The key feature in SAGE is the generation of a short 'sequence tag' containing sufficient information to 'uniquely identify' a mRNA transcript. Quantitation of the number of times a particular tag is observed provides the expression level of the corresponding transcript. Type IIS restriction enzymes such as *BsmFI* generate sequence tags of 13–15 bp, but the length of such tags is not sufficient to unambiguously assign the transcript it is generated from. R.EcoP15I that can yield 26 bp tags has been demonstrated to be useful in SAGE assays owing to the possibility of increased fidelity in mRNA transcript assignment [14]. However, the use of R.EcoP15I can lead to an underestimation of the levels of expressed mRNA owing to its cleavage close to only one of the head-to-head sites. In this study, we demonstrate that addition of exogenous AdoMet influences the methylation and cleavage activity of AdoMet bound R.EcoP15I enzyme depending on the topology of DNA. Our results suggest an additional role to the already multifaceted molecule AdoMet. Strikingly, an AdoMet analogue sinefungin that has inverted charge configuration at the $\delta\text{CHN}^+\text{H}_3$ centre as compared with $\delta\text{S}^+\text{CH}_3$ centre of AdoMet permits cleavage at all the recognition sites irrespective of their orientation. The Type III restriction enzymes had never been demonstrated earlier to perform complete non-promiscuous cleavage at all the recognition sites (independent of their orientation) on the DNA. The potential use of R.EcoP15I cleavage in the presence of sinefungin for SAGE is discussed.

Materials and methods

Chemicals. AdoMet, AdoHcy, sinefungin, BSA, and ATP (sodium salt) were from Sigma Chemical, USA. Restriction enzymes were from New England Biolabs, USA.

Protein purification. M.EcoP15I, Lac rep, and R.EcoP15I were purified as described earlier [11,15]. R.EcoP15I was stored in buffer B containing 50% glycerol at -20°C .

DNA substrates. Plasmids pUC19, pJA24, pJA31, pMDS32, and pSHI180 were purified following alkaline lysis method [16]. The Type IIP restriction enzyme digested pJA31, pJA24, and pUC19 DNA were generated by incubating the DNA with 20 U of, respective, enzymes at 37°C for 3 h.

DNA cleavage assay. DNA cleavage reactions were performed in Lac buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM MgCl_2 , 0.1 mM EDTA, and 0.1 mM dithiothreitol) as described [11]. The final concentration of exogenous AdoMet and sinefungin was $5\ \mu\text{M}$ in the reactions unless mentioned otherwise. Stoichiometric amounts of R.EcoP15I (R_2M_2 molecules) with respect to number of recognition sites ($\sim 200\ \text{nM}$) on DNA were determined [12] and used in all the assays except where stated otherwise. All experiments were repeated at least thrice.

Methylation assay. Supercoiled pJA31 DNA was subjected to cleavage assay with stoichiometric amounts (1:1 site:enzyme) of R.EcoP15I in the absence or presence of $5\ \mu\text{M}$ exogenous AdoMet or

sinefungin. The cleaved DNA was ethanol precipitated, resuspended in $20\ \mu\text{l}$ methylation assay mix [Lac buffer, $20\ \mu\text{M}$ AdoMet (1:4 tritiated:cold), and M.EcoP15I] and reaction was carried out as described earlier [15]. The amount of tritiated methyl groups transferred to pJA31 DNA not subjected to R.EcoP15I restriction was taken as 100%. The difference between the 100% value and those obtained with DNA subjected to R.EcoP15I cleavage provides the extent of methylation during cleavage assay. The assays were repeated four times.

Results and discussion

Exogenous AdoMet and sinefungin differentially alter DNA cleavage activity of R.EcoP15I

We had shown earlier that AdoMet is essential for DNA cleavage by Type III REs [5], while monovalent ion in the assay was shown later to alter the requirement for AdoMet [6]. The requirement for AdoMet was demonstrated with supercoiled pUC DNA having 'one' head-to-head pair of sites for R.EcoP15I [5]. Several analogues of AdoMet were used to demonstrate that the carboxyl group and any substitution at the epsilon carbon of methionine are essential for DNA cleavage. In the course of our study with DNA having 'more than one pair' of head-to-head R.EcoP15I sites, addition of exogenous AdoMet, to the reaction having AdoMet bound R.EcoP15I, resulted in cleavage of only one pair of head-to-head sites leaving the other head-to-head oriented sites intact. Earlier observations of an incomplete cleavage pattern with R.EcoP15I were attributed to be a consequence of parallel methylation activity of the enzyme blocking its nuclease activity [17]. We demonstrated recently that complete cleavage of DNA, i.e., one double strand break within each head-to-head pair of sites, by R.EcoP15I requires the presence of stoichiometric amounts of enzyme to that of the recognition sites [12]. R.EcoP15I enzyme does not turnover in the absence of exonucleases. The associated methylation activity does not interfere with the nuclease activity of the enzyme and complete cleavage can be observed with stoichiometric amounts of enzyme.

To further analyse the 'strict cleavage of only one of the two head-to-head pair' in the presence of exogenous AdoMet, the DNA cleavage assay was performed in the absence or presence of $20\ \mu\text{M}$ exogenous AdoMet or its analogues using pJA31 DNA. The supercoiled pJA31 DNA has two head-to-head pairs of sites, B/C and A/A' (Fig. 1), and the restriction assay included stoichiometric amounts of R.EcoP15I with respect to number of recognition sites on DNA. As can be seen from Fig. 2A, in the absence of exogenous AdoMet (lane 2), after cleavage at one of the oppositely oriented sites in the origin of replication (*ori*) pair (sites B and C), cleavage close to site A (results in 3.65 and 2.85 kb bands) and cleavage close to site A' (results in 4.85 and 1.65 kb bands) can be seen to have occurred with an

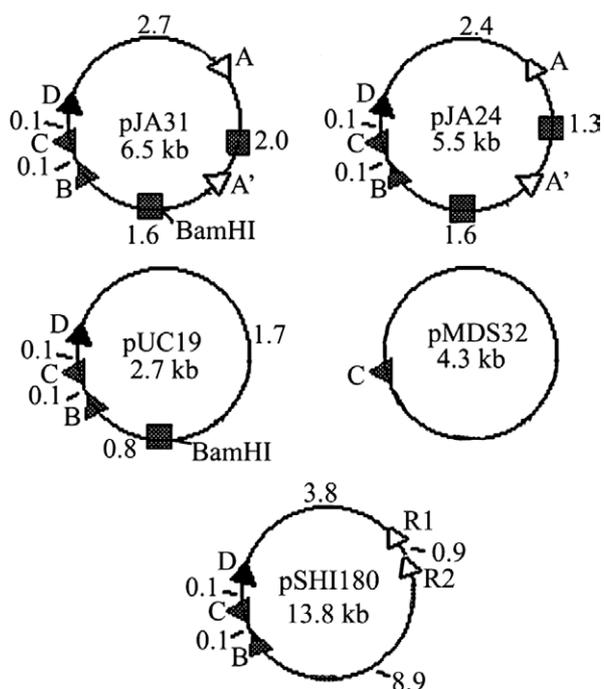


Fig. 1. DNA with single and multiple recognition sites. Schematic representation of DNA substrates. R.EcoP15I sites are shown by triangles (filled: ori sites—B, C, D and empty: sites A and A' or R1 and R2) and Lac operator sites by squares. The base and tip of the triangles indicate 5' and 3' ends of 5'-CAGCAG-3'. Distances between sites are given in kilobases (kb).

equal probability close to sites A and A' (expected cleavage) [11]. Cleavage close to site B or C within the *ori* pair cannot be distinguished from cleavage close to other site in this pair under the electrophoretic conditions used in this investigation, as the sites of cleavage are just 50 bp apart. In the presence of 20 μ M exogenous AdoMet (lane 3), the DNA was only linearised (6.5 kb), indicating cleavage between only one of the two head-to-head pairs, either *ori* pair (B/C) or A/A' pair, and not both.

This result cannot be a consequence of parallel methylation by the enzyme blocking its nuclease activity as one would expect a mixture of uncleaved, linearised, and fragmented DNA under such conditions. Hence, in the presence of exogenous AdoMet, following cleavage within one of the head-to-head pairs that gives rise to the intermediate product of the cleavage reaction, a linear form of DNA, the cleavage within another head-to-head-to-head pair is largely suppressed. Surprisingly, in the presence of 20 μ M sinefungin (lane 4), cleavage occurred close to all the R.EcoP15I sites (A and A' and *ori* pair: sites B and C) resulting in 2.85 kb (combined cleavage at site A and site C or D), 2.0 kb (cleavage at both sites A and A'), and 1.65 kb (cleavage at both sites A' and B) fragments. This is the first observation of any Type III restriction enzyme cleaving close to all the recognition sites on DNA, irrespective of the orientation of the site, using stoichiometric amounts of

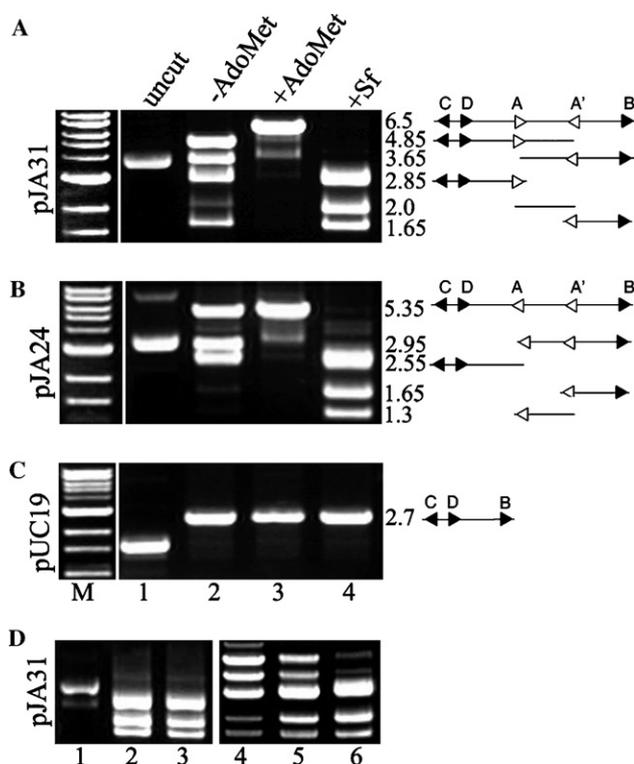


Fig. 2. Effect of exogenous AdoMet and sinefungin. (A–C) Assay with supercoiled pJA31, pJA24, and pUC19 DNA, respectively. In (A–C), lane 1, uncut supercoiled DNA; lane 2, R.EcoP15I restriction assay in absence of exogenous AdoMet; lane 3, in presence of exogenous AdoMet; lane 4, in the presence of sinefungin (Sf). Lane M, marker DNA fragments of size 10, 8, 6, 5, 4, 3, 2, 1.5, 1, and 0.5 kb from top to bottom. The sizes (kb) given on right side of gel in all figures correspond to that of cleaved DNA fragments. (D) Monovalent metal ion and enzyme concentration effect. Lane 1, uncleaved supercoiled DNA, lanes 2 and 3, assay in the presence of Na⁺ and K⁺ ions; lanes 4–6, assay containing 1:0.4, 1:0.7, and 1:1 of site:R.EcoP15I amounts, respectively. Schematic representation of cleavage products with the recognition sites they contain is shown to the right of the gels in the same order of their appearance on the gel.

DNA. All the DNA fragments observed in the presence of sinefungin correspond to cleavage close to the recognition sites and do not represent any promiscuous cleavage by the enzyme.

The above assays were repeated with supercoiled pJA24 having two head-to-head pairs of sites, B/C and A/D (11) and pUC19 DNA having one head-to-head pair of sites, B/C (Fig. 1). In pJA24, after cleavage at one of the sites in the *ori* pair (sites B and C), cleavage close to site D results in 5.35 and 0.15 kb DNA bands and cleavage close to site A results in 2.95 and 2.55 kb bands. As seen with pJA31, in a reaction having pJA24 DNA in absence of exogenous AdoMet (Fig. 2B, lane 2), cleavage close to site D and site A can be seen to have occurred with an equal probability close to sites A and D (expected cleavage) [11]. Only linearisation (5.5 kb) is observed in the presence of 20 μ M exogenous AdoMet (Fig. 2B, lane 3), again indicating

that cleavage has occurred only within one of the two head-to-head pairs (either *ori* pair or A/D pair) and is suppressed within other pair. As can be seen in Fig. 2B, lane 4, cleavage close to all the R.EcoP15I sites is observed in presence of sinefungin, yielding 2.55 kb (cleavage at site A and site C or D), 1.65 kb (cleavage at both A' and B), and 1.3 kb (cleavage at both sites A and A') DNA fragments.

In case of pUC19 DNA having only one pair of head-to-head sites (*ori* sites), only linearisation (2.7 kb) under the three conditions is seen (Fig. 2C). The cleavage at both sites C and D occurring in the presence of sinefungin could not be distinguished under the electrophoretic conditions used in this investigation. Nonetheless, from cleavage at both head-to-head oriented sites A and A' of pJA31 and pJA24 it can be considered that cleavage has occurred at site D. It has to be noted that in both pJA31 and pJA24 (Figs. 2A and B) the DNA topology on which the second head-to-head pair of sites exist changes from being a supercoiled one on uncleaved DNA to a linear one on the intermediate form following cleavage within one pair of sites. It is the head-to-head pair on such a linear intermediate form DNA that the effect of exogenous AdoMet is observed (lane 3).

Potassium ions were shown earlier to alter R.EcoP15I restriction activity in an assay having high enzyme:site ratio [6]. Substituting Na⁺ ions instead of K⁺ ions in the Lac assay buffer did not alter the sinefungin effect on R.EcoP15I cleavage activity observed here. Fig. 2D shows the cleavage of pJA31 DNA in the presence of sinefungin and either Na⁺ (lane 2) or K⁺ (lane 3) ions. The presence of only 2.85, 2.0, and 1.65 kb fragments under both conditions clearly indicates that cleavage occurred at all the R.EcoP15I recognition sites.

R.EcoP15I does not perform multiple rounds of catalysis *in vitro* and the cleavage within all head-to-head pairs requires stoichiometric R.EcoP15I amounts [12]. Similarly, cleavage at all recognition sites in the presence of sinefungin occurs at stoichiometric enzyme concentration, indicating no catalytic turnover by the enzyme (Fig. 2D). Higher concentrations of 4.85 and 3.65 kb cleaved products in lane 4 compared to that in lane 6 and that of 2.0 and 1.65 kb products in lane 6 compared to that in lane 4 clearly indicate that cleavage has occurred close to only one of the head-to-head oriented sites (lane 4) and both the sites (lane 6), respectively. The extent of cleavage (in Fig. 2D) proportionate to the amount of enzyme also indicates that the activity to be enzymatic and not that of sinefungin or a contaminant in it. Incubation of DNA with sinefungin in absence of R.EcoP15I does not result in DNA cleavage (data not shown). Enzyme concentrations above stoichiometric amounts (1:2 and 1:3) resulted in cleavage similar to that with stoichiometric amounts (data not shown). The effect of sinefungin was evident even in the presence of exogenous AdoMet and cleavage oc-

curred at all the recognition sites (data not shown), probably reflecting higher affinity of enzyme for sinefungin than AdoMet. The cleavage at all sites by R.EcoP15I is specific to sinefungin alone, as addition of 20 μM of seven other AdoMet analogues [5], did not alter the expected cleavage at one of the head-to-head sites (data not shown).

Exogenous AdoMet suppresses cleavage of head-to-head oriented sites on a linear DNA

AdoMet was earlier suggested to suppress the promiscuous or secondary cleavage by Type III enzyme R.EcoP1I under different buffer conditions and high enzyme concentrations [6], but not the primary cleavage occurring at one of the sites within the head-to-head pair of sites. In the assays described above (Fig. 2), the absence of further fragmentation (following linearisation) with supercoiled pJA31 and pJA24 in the presence of exogenous AdoMet prompted us to analyse, if exogenous AdoMet would suppress cleavage of both the head-to-head pair of sites on a linear DNA. Cleavage assays were performed with BamHI linearised pJA31.

BamHI digested pJA31 (Fig. 3A) has both pairs of head-to-head sites (*ori* and A/A'). The BamHI site is present 0.8 kb away from site B on DNA between sites B and A'. Following cleavage within the *ori* pair, cleavage at site A or A' results in appearance of 2.85, 0.85 kb or 0.75, 0.85 kb bands, respectively (rather than 3.65

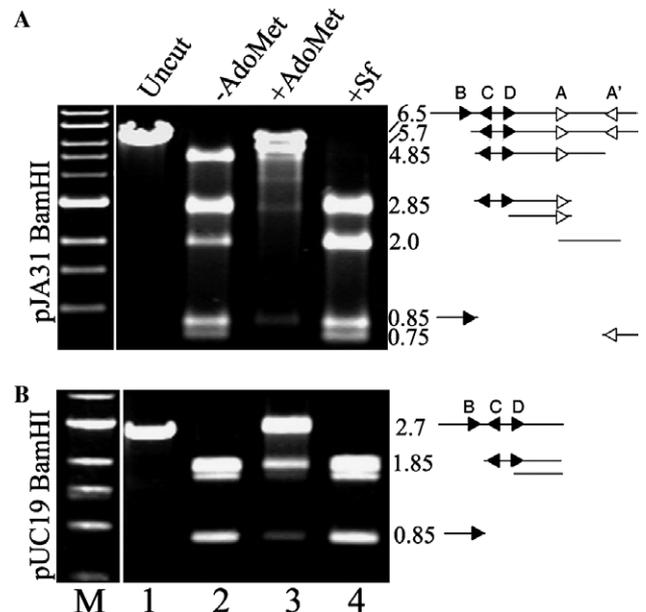


Fig. 3. Exogenous AdoMet effect on linear DNA cleavage. (A) BamHI digested pJA31. (B) BamHI digested pUC19 DNA. Lanes 1–4 in both panels and lane M of (A) same as in Fig. 2A; lane M of (B) sizes of DNA marker fragments are 4, 3, 2, 1.5, 1, and 0.5 kb from top to bottom of gel. Schematic representation corresponding to the cleavage fragments is shown to the right of the gels as in Fig. 2.

and 1.65 kb fragments observed in Fig. 2A, lane 2). Hence 4.85, 2.85, 0.85, and 0.75 kb bands represent the expected cleavage. The restriction assays were done in the presence of 5 μ M exogenous AdoMet or sinefungin or none. In the absence of exogenous AdoMet, expected cleavage close to one of the head-to-head oriented sites was observed (Fig. 3A, lane 2). The 2.0 kb band representing cleavage at both sites A and A' of A/A' pair can also be observed [11]. In presence of sinefungin, cleavage occurred at all R.EcoP15I recognition sites (Fig. 3A, lane 4). The complete cleavage close to both sites A and A', combined with cleavage within *ori* pair, resulted in the appearance of intense 2.85 and 2.0 kb fragments and absence of 4.85 kb fragment as compared to that of lane 2. As can be seen in Fig. 3A, lane 3, the presence of uncleaved 6.5 kb fragment and absence of 4.85, 2.85, 2.0, 0.85, and 0.75 kb cleavage products indicate that cleavage has not occurred within both A/A' and *ori* pairs. There is however a detectable amount of cleavage only within the *ori* pair and none in A/A' pair under these conditions (5.7 kb). These results indicate that exogenous AdoMet is capable of greatly suppressing cleavage at both the head-to-head pairs (A and A', and *ori* pair) on a linear DNA and not a supercoiled DNA (Fig. 2A, lane 3).

The suppressive effect of exogenous AdoMet on cleavage within head-to-head pair on linear DNA was also observed in the assays using BamHI linearised pUC19 DNA. BamHI digested pUC19 (Fig. 3B) has one head-to-head pair in *ori* region (*ori* pair, sites B and C). The BamHI site is present 0.8 kb downstream of site B as in pJA31 DNA. The cleavage within the *ori* pair thus results in fragmentation of the 2.7 kb fragment into 0.85 and 1.85 kb fragments. Both in the absence of exogenous AdoMet and in the presence of sinefungin, complete cleavage within the *ori* pair giving rise to 1.85 and 0.85 kb fragments can be seen (Fig. 3B, lanes 2 and 4). The doublet at 1.85 kb DNA fragment with pUC19 DNA reflects alternate cleavage close to either site B or C and D of the *ori* sites. The presence of >95% of the BamHI linearised 2.7 kb input DNA fragment (with negligible amount of cleavage within *ori* pair—1.85 and 0.85 kb fragments) in the reaction carried out in the presence of exogenous AdoMet (Fig. 3B, lane 3) shows that exogenous AdoMet was able to largely suppress cleavage within the head-to-head sites on a linear DNA. These results described in Figs. 2A and B clearly demonstrate that exogenous AdoMet influences cleavage of head-to-head sites by R.EcoP15I based on the topology of DNA.

Methylation in the presence of AdoMet and sinefungin

R.EcoP15I has both methyltransferase and endonuclease activities [13]. A probable cause for the absence of cleavage on linear DNA in the presence of exogenous

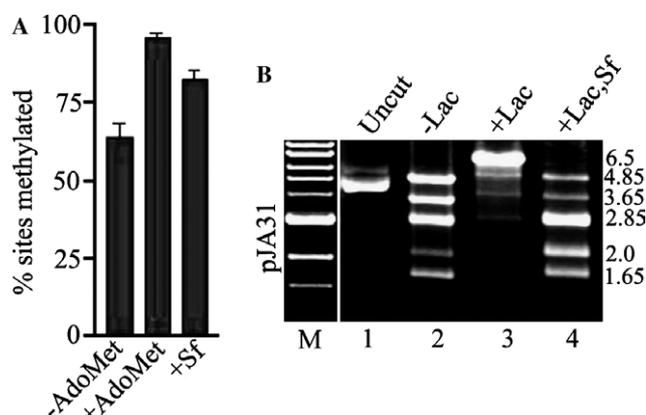


Fig. 4. Methylation activity and Lac repressor effect. (A) Percentage of recognition sites methylated in restriction assay carried out, as described under Materials and methods, in the absence and presence of exogenous AdoMet or sinefungin (Sf). (B) Effect of Lac repressor on supercoiled pJA31 DNA cleavage. Lane 1, DNA incubated without restriction enzyme; lane 2, restriction assay in absence of Lac repressor; lane 3, assay performed in presence of Lac repressor; lane 4, assay in the presence of both Lac repressor and sinefungin (Sf). Lane M, marker fragments as in Fig. 2A.

AdoMet is preferential methylation of sites (over restriction) by R.EcoP15I. To check this, the methylation status of sites on R.EcoP15I cleaved pJA31 DNA fragments (Fig. 2A) was analysed. The percentages of sites methylated were ~60% in the absence of exogenous AdoMet, ~95% in the presence of exogenous AdoMet, and ~80% in the presence of sinefungin (Fig. 4A). The methylation of only ~60% sites by AdoMet bound R.EcoP15I (in absence of exogenous AdoMet) in a reaction with complete expected cleavage (Fig. 2A, lane 2) probably reflects the association of endonucleolytic complex with only one of the head-to-head sites during cleavage event [11].

The absence of head-to-head site cleavage on linear DNA in conjunction with methylation of ~95% sites indicates that exogenous AdoMet drives preferential methylation of head-to-head sites. It can, therefore, be suggested that exogenous AdoMet acts as a sort of 'DNA topology sensor' for R.EcoP15I by modulating the methylation and cleavage activities of the enzyme based on the topology of DNA. Although methylation (~80%) in the presence of sinefungin indicates that it does not displace bound AdoMet, it probably binds the Res subunit (through its adenine moiety) to bring about the observed restriction activity. However, the increase in methylation compared to that in the absence of sinefungin needs further investigation.

DNA cleavage in the presence of Lac repressor and sinefungin

The cleavage at all sites in the presence of sinefungin (Figs. 2 and 3) indicates that the sinefungin based

cleavage is independent of the orientation of the recognition sites. Most importantly, such cleavage might not require interaction of two enzyme molecules (basis for cleavage of one of the two head-to-head sites). To test whether sinefungin based cleavage involves two enzyme molecules for cleavage close to each of the recognition sites, restriction assays were performed with supercoiled pJA31 in the presence of Lac repressor (rep) and sinefungin. A Lac rep bound to DNA between head-to-head sites avoids interaction of R.EcoP15I molecules [18], as well as obstructs translocation of R.EcoP15I on DNA. We had shown earlier that a Lac repressor binding to the Lac operator site present between sites A and A' completely avoids the physical interaction of the enzyme molecules from these sites and hence the expected cleavage within this pair does not occur [11].

As can be seen in Fig. 4B, lane 2, expected cleavage of pJA31 DNA was observed in the absence of Lac rep (4.85, 3.65, 2.85, and 1.65 kb cleaved DNA products). However, in the presence of Lac repressor (lane 3), only linearised DNA (6.5 kb) was seen. The cleavage involving sites A and A' is avoided by the Lac repressor bound on the intervening DNA and the linearisation is the consequence of cleavage within *ori* pair (sites B and C). In the presence of both Lac rep and sinefungin (lane 4) the cleavage was complete (>95%) at all the recognition sites (2.85, 2.0, and 1.65 kb cleaved DNA products). These results demonstrate that complete cleavage close to both sites A and A' has occurred in spite of the presence of a Lac repressor on the intervening DNA (Fig. 4B, lane 4) comparable to that in the absence of Lac repressor (Fig. 2A, lane 4). Hence the interaction between two R.EcoP15I molecules from sites A and A' is not essential for cleavage close to both the sites in the presence of sinefungin.

Cleavage of circular DNA containing a single site

The results described in Fig. 4B indicate that a single R.EcoP15I enzyme molecule might perform a double strand break in the vicinity of its recognition site without interacting with other enzyme molecule bound to an oppositely oriented site on the same DNA. It was reported earlier that R.EcoP15I cannot cleave both the DNA strands, but can introduce a nick on a circular DNA having a single recognition site [10]. On the other hand, a linear DNA with single recognition site and an accessible DNA end for the enzyme was demonstrated to be cleaved close to R.EcoP15I and such cleavage involves interaction of two enzyme molecules [11].

To obtain a direct proof of one enzyme molecule cleaving both the DNA strands upon interacting with one recognition site, the cleavage assay using a 4.3 kb circular DNA having a single R.EcoP15I site, pMDS32 (a kind gift from Mark Szczelkun [19]), in the absence

and presence of exogenous AdoMet or sinefungin was performed. Using the DNA substrates derived from pMDS32 it was demonstrated that R.EcoP15I does not interact in *trans* with enzyme molecules in the solution to bring about cleavage close to the recognition site [19]. In the absence of exogenous AdoMet, partial nicking of DNA, as described earlier [10], was observed (Fig. 5A, lane 2). Inclusion of 5 μ M exogenous AdoMet in the reaction (lane 3) abolished even the nicking activity of the enzyme and no nicked DNA was seen. The supercoiled DNA was completely linearised in the presence of 5 μ M sinefungin (lane 4), clearly indicating that sinefungin enables each R.EcoP15I molecule to cleave both the DNA strands close to the single recognition site on circular DNA. Few faint additional bands that did not affect our analysis were always observed in the DNA preparations and restriction digestions with the pMDS32 plasmid, probably owing to the mutations introduced in the 'origin of replication' of this plasmid [19].

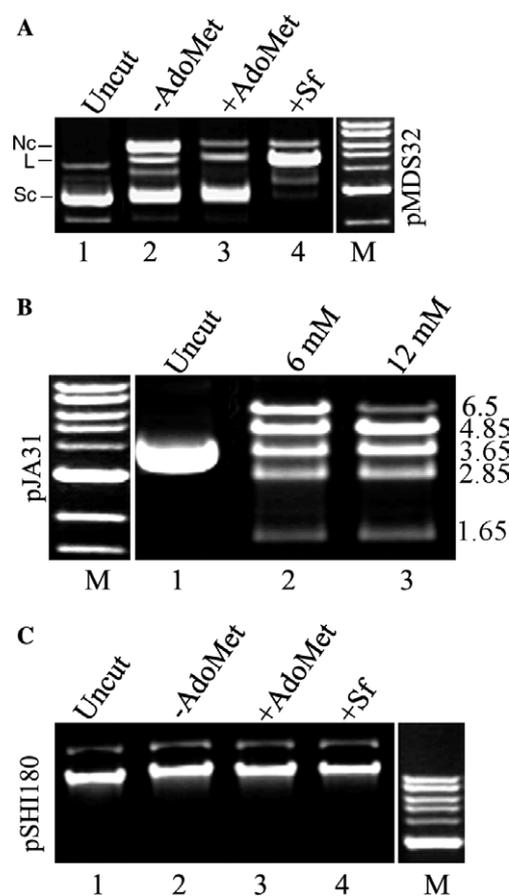


Fig. 5. Site, ATP, and methylation dependence. (A) pMDS32; (B) pJA31; (C) pSHI180 DNA. (A) Sc, L, and Nc represent supercoiled, linear, and nicked forms, respectively. In all panels, lane 1, uncut supercoiled DNA; lane M, marker DNA fragments as in Fig. 2A. Lanes 2-4 of (A) and (C) indicate reactions in the absence of exogenous AdoMet, in the presence of exogenous AdoMet, and in the presence of sinefungin (Sf), respectively. Lanes 2 and 3 of (B), reactions carried out in the presence of 6 and 12 mM AMP-PNP, respectively.

ATP requirement for sinefungin based cleavage

ATP hydrolysis is essential for DNA cleavage by Type III restriction enzymes. ATP hydrolysis drives the enzyme translocation process involved in the cleavage reaction. Two such translocating enzyme molecules from oppositely orientated sites on DNA interact on the intervening DNA and form a single endonucleolytic complex. The endonucleolytic complex translocates to one of the two sites and cleaves DNA 25–27 bp downstream of the site [11]. The cleavage at all sites by R.EcoP15I in the presence of sinefungin, most importantly in the presence of Lac repressor and of the single site circular DNA indicates that enzyme molecule might be capable of cleaving without requiring to perform translocation process. This raises the possibility of DNA cleavage without the requirement of ATP binding or ATP hydrolysis.

To test if sinefungin based DNA cleavage by R.EcoP15I is ATP independent, supercoiled pJA31 DNA cleavage assays were performed in the presence of sinefungin and increasing concentrations of non-hydrolysable analogue of ATP, AMP-PNP (1–12 mM). DNA fragments corresponding to cleavage at site A or A' or *ori* pair, 4.85, 3.65, 2.85, and 1.65 kb, were observed only at 6 mM AMP-PNP (Fig. 5B, lane 2) and above. The presence of 6.5 kb DNA fragment indicates cleavage within one of the two head-to-head pairs. Increasing AMP-PNP concentration to 12 mM increased the amount of cleavage with the disappearance of 6.5 kb fragment and increase in intensity of other fragments (lane 3), but cleavage at all sites as seen in the presence of 1 mM ATP (Fig. 2A, lane 4) could not be achieved. These results imply that in the presence of sinefungin, upon binding to ATP, R.EcoP15I enzyme might be capable of cleaving DNA without requiring to hydrolyse ATP. Difference in cleavage levels obtained with 12 mM AMP-PNP and 1 mM ATP possibly reflects differential binding affinities of, or, conformational change in R.EcoP15I associated with AMP-PNP and ATP. Additionally, ATP hydrolysis might be making the enzyme more cleavage competent. Hence, ATP binding and/or hydrolysis is essential even in the presence of sinefungin, although translocation may not be required.

Methylated site cleavage

It is well established that R.EcoP15I is methylation sensitive enzyme [20]. The cognate methyltransferase methylates the second adenine of the recognition site at the N6 position. The expression of both the Mod and the Res genes from the bicistronic mRNA is regulated by the expression of the Mod gene, permitting methylation of all the recognition sites on the DNA, prior to the expression of the Res gene [13]. The in vitro generated plasmid pSHI180 carries the operon for EcoP15I

restriction-modification system [20] and has been used extensively for over two decades to study the regulation of gene expression and also to purify the restriction enzyme from transformed bacteria. The R.EcoP15I recognition sites on this plasmid isolated from the transformed bacteria are fully methylated.

Since sinefungin permits cleavage at all the unmethylated sites by R.EcoP15I, it was of interest to see if even the methylated sites could be cleaved. To assess this, EcoP15I methylated supercoiled plasmid pSHI180 (13.8 kb) [20] was used. There are five R.EcoP15I sites on pSHI180 forming two head-to-head oriented pairs. There are three R.EcoP15I sites in the *ori* (B, C, and D) and two in the Res gene of the operon (R1 and R2). Any cleavage within the *ori* pair (B/C) results in the linearisation of the DNA giving a 13.8 kb DNA fragment. Further cleavage at one or both of the R1 and R2 recognition sites in the Res gene shall result in appearance of additional DNA fragments (9.85, 8.95, 4.85, 3.95, and 0.9 kb). The cleavage assay was performed in the absence or presence of exogenous AdoMet or sinefungin. As expected, no nicking or linearisation or fragmentation of DNA was observed in the absence of exogenous AdoMet (Fig. 5C, lane 2). There was no cleavage pattern in the presence of either exogenous AdoMet or sinefungin and the uncleaved supercoiled DNA remained intact (lanes 3 and 4). These results demonstrate that although sinefungin supports cleavage of individual recognition sites, without interfering with the associated methylation activity of the enzyme (Fig. 4A), it does not support cleavage of methylated recognition sites, demonstrating the high specificity of R.EcoP15I enzyme catalytic activity on DNA.

Site of cleavage in the presence of sinefungin

The results described above clearly demonstrate the change in site requirement for cleavage by R.EcoP15I enzyme from that of head-to-head oriented sites to a single site in the presence of sinefungin. The site of cleavage with respect to the recognition site is 25 bp on the CAG-CAG strand and 27 bp on the CTGCTG strand, both occurring downstream of the recognition site when two R.EcoP15I molecules are involved in cleaving two strands of DNA [10]. The possibility of an alteration in the sites of cleavage with respect to the recognition site in the presence of sinefungin exists owing to the involvement of one enzyme molecule per double strand cleavage.

To map the site of sinefungin based cleavage with respect to the recognition site, the single site circular pMDS32 DNA linearised by R.EcoP15I in the presence of sinefungin (Fig. 5A, lane 4) was sequenced using primers on either side of the recognition site. The site of cleavage was mapped to be at 28th nt on the CAG-CAG strand and at 26th nt on the CTGCTG strand,

both occurring downstream of the recognition site. The double strand cleavage in the presence of sinefungin might involve either one or both Res subunits of one R.EcoP15I molecule (R_2M_2). Comparable position of cleavage site with respect to the recognition site in the absence, 25–27 bp [10], and presence of sinefungin, 28–26 bp, indicates a possibility of one of the two R.EcoP15I molecules cleaving both DNA strands in the absence of sinefungin. Alternatively, the generation of a 2 bp 5' extension in the absence of sinefungin and a 2 bp 3' extension in the presence of sinefungin might reflect the differences at the catalytic sites under these conditions.

Implication for serial analysis of gene expression

R.EcoP15I cleaves close to one of the two head-to-head oriented sites. In case of serial analysis of gene expression [21], R.EcoP15I sites are provided in the linkers. The cleavage with R.EcoP15I results in half of the products being longer than that required for 'tag' size (~69 bp), and such tags are not included for the next step in the analysis [14]. This can lead to an under representation of expressed levels or in non-representation of some genes. Additionally, the presence of an R.EcoP15I site(s) within mRNA could contribute to the underestimation of expressed levels. This study provides an improved condition to use R.EcoP15I, advantageously, in SAGE assays and counting CAG repeats in Huntington disease [22], whereby, cleavage can be obtained at all the R.EcoP15I sites by including sinefungin in the reaction having stoichiometric amounts of R.EcoP15I. In addition, providing one R.EcoP15I site on one of the linkers would be sufficient to generate tags for SAGE. Since footprinting experiments demonstrate that R.EcoP15I protects around 13 bp on either side of the site [23], the linkers used in SAGE assays must provide sufficient overhang for efficient R.EcoP15I cleavage.

We demonstrated earlier that, unlike Type IIP REs, R.EcoP15I does not turnover at least in *in vitro* reactions [12]. This would imply that 1 U of R.EcoP15I would cleave only 1 μ g of DNA substrate for any length of incubation and not 1 μ g DNA for every 1 h incubation. This highlights the importance of amount of R.EcoP15I to be used in SAGE assays to achieve complete cleavage at all the desired sites. Only stoichiometric amounts (site: enzyme 1:1) of R.EcoP15I result in one double strand break between all head-to-head oriented sites or at all sites in the presence of sinefungin (Fig. 2D). If excess enzyme (over R.EcoP15I sites) is used, it would necessitate proteinase K treatment following R.EcoP15I digestion, to avoid DNA mobility shift on PAGE (unpublished observations). R.EcoP15I can bind strongly to DNA at high concentrations.

Conclusions

Taken together, this study demonstrates that exogenous AdoMet and its analogue sinefungin strongly influence DNA cleavage by R.EcoP15I. The methyl group donor AdoMet alters enzymatic activities, methylation and restriction, based on the topology of substrate DNA. Exogenous AdoMet favours methylation of head-to-head oriented sites, over cleavage, on linear DNA, but not on supercoiled DNA. DNA cleavage in the presence of sinefungin is site orientation independent, enzyme concentration dependent, and methylation status sensitive. The sinefungin supported cleavage is ATP hydrolysis and translocation independent, although ATP binding is essential and hydrolysis seems to increase the cleavage efficiency. In brief, sinefungin makes R.EcoP15I comparable to a Type IIG restriction enzyme with respect to DNA cleavage activity. While the mechanistic basis for the observed differential effects of AdoMet and sinefungin on R.EcoP15I activity needs to be addressed, R.EcoP15I enzyme in the presence of sinefungin can serve as an efficient tool to generate unambiguous 'tags' in SAGE assays.

Acknowledgments

We thank Mark Szczelkun for pMDS32 DNA, N.K.R. thanks Madhusoodhanan for experimental help and University Grants Commission for Senior Research Fellowship. Financial support from CSIR, Government of India, is gratefully acknowledged.

References

- [1] M. Fontecave, M. Atta, E. Mulliez, *S*-Adenosylmethionine: nothing goes to waste, *Trends Biochem. Sci.* 29 (2004) 243–249.
- [2] X. Cheng, R.J. Roberts, AdoMet-dependent methylation, DNA methyltransferases and base flipping, *Nucleic Acids Res.* 29 (2001) 3784–3795.
- [3] H.L. Schubert, R.M. Blumenthal, X. Cheng, Many paths to methyltransfer: a chronicle of convergence, *Trends Biochem. Sci.* 28 (2003) 329–335.
- [4] H.W. Boyer, DNA restriction and modification mechanisms in bacteria, *Annu. Rev. Microbiol.* 25 (1971) 153–176.
- [5] P. Bist, S. Sistla, V. Krishnamurthy, A. Acharya, B. Chandrakala, D.N. Rao, *S*-Adenosyl-L-methionine is required for DNA cleavage by type III restriction enzymes, *J. Mol. Biol.* 310 (2001) 93–109.
- [6] L.J. Peakman, M. Antognozzi, T.A. Bickle, P. Janscak, M.D. Szczelkun, *S*-Adenosyl methionine prevents promiscuous DNA cleavage by the EcoPII type III restriction enzyme, *J. Mol. Biol.* 333 (2003) 321–335.
- [7] S. Sistla, D.N. Rao, *S*-Adenosyl-L-methionine-dependent restriction enzymes, *Crit. Rev. Biochem. Mol. Biol.* 39 (2004) 1–19.
- [8] J. Vitkute, Z. Maneliene, M. Petrusyte, A. Janulaitis, *BpII*, a new *BcgI*-like restriction endonuclease, which recognizes a symmetric sequence, *Nucleic Acids Res.* 25 (1997) 4444–4446.

- [9] S. Jurenaite-Urbanaviciene, R. Kazlauskene, V. Urbelyte, Z. Maneliene, M. Petrusyte, A. Lubys, A. Janulaitis, Characterization of *Bse*MII, a new type IV restriction-modification system, which recognizes the pentanucleotide sequence 5'-CTCAG(N)(10/8), *Nucleic Acids Res.* 29 (2001) 895–903.
- [10] P. Janscak, U. Sandmeier, M.D. Szczelkun, T.A. Bickle, Subunit assembly and mode of DNA cleavage of the type III restriction endonucleases EcoP1I and EcoP15I, *J. Mol. Biol.* 306 (2001) 417–431.
- [11] N.K. Raghavendra, D.N. Rao, Unidirectional translocation from recognition site and a necessary interaction with DNA end for cleavage by Type III restriction enzyme, *Nucleic Acids Res.* 32 (2004) 5703–5711.
- [12] N.K. Raghavendra, D.N. Rao, Functional cooperation between exonucleases and endonucleases—basis for the evolution of restriction enzymes, *Nucleic Acids Res.* 31 (2003) 1888–1896.
- [13] D.N. Rao, S. Saha, V. Krishnamurthy, ATP-dependent restriction enzymes, *Prog. Nucleic Acid Res. Mol. Biol.* 64 (2000) 1–63.
- [14] H. Matsumura, S. Reich, A. Ito, H. Saitoh, S. Kamoun, P. Winter, G. Kahl, M. Reuter, D.H. Krüger, R. Terauchi, Gene expression analysis of plant host-pathogen interactions by Super-SAGE, *Proc. Natl. Acad. Sci. USA* 100 (2003) 15718–15723.
- [15] P. Bist, D.N. Rao, Identification and mutational analysis of Mg²⁺ binding site in EcoP15I DNA methyltransferase: involvement in target base eversion, *J. Biol. Chem.* 278 (2003) 41837–41848.
- [16] J. Sambrook, D.W. Russell, *Molecular Cloning. A Laboratory Manual*, third ed., Cold Spring Harbor Laboratory Press, New York, NY, 2001.
- [17] A. Meisel, T.A. Bickle, D.H. Krüger, C. Schroeder, Type III restriction enzymes need two inversely oriented recognition sites for DNA cleavage, *Nature (London)* 355 (1992) 467–469.
- [18] A. Meisel, P. Mackeldanz, T.A. Bickle, D.H. Krüger, C. Schroeder, Type III restriction endonucleases translocate DNA in a reaction driven by recognition site-specific ATP hydrolysis, *EMBO J.* 14 (1995) 2958–2966.
- [19] L.J. Peakman, M.D. Szczelkun, DNA communications by Type III restriction endonucleases—confirmation of 1D translocation over 3D looping, *Nucleic Acids Res.* 32 (2004) 4166–4174.
- [20] S.M. Hadi, B. Bachi, J.C. Shepherd, R. Yuan, K. Ineichen, T.A. Bickle, DNA recognition and cleavage by the EcoP15 restriction endonuclease, *J. Mol. Biol.* 134 (1979) 655–666.
- [21] J. Powell, SAGE. The serial analysis of gene expression, *Methods Mol. Biol.* 99 (2000) 297–319.
- [22] E. Moncke-Buchner, S. Reich, M. Mucke, M. Reuter, W. Messer, E.E. Wanker, D.H. Krüger, Counting CAG repeats in the Huntington's disease gene by restriction endonuclease EcoP15I cleavage, *Nucleic Acids Res.* 30 (2002) e83.
- [23] M. Mücke, S. Reich, E. Moncke-Buchner, M. Reuter, D.H. Krüger, DNA cleavage by type III restriction-modification enzyme EcoP15I is independent of spacer distance between two head to head oriented recognition sites, *J. Mol. Biol.* 312 (2001) 687–698.