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Unidirectional translocation from recognition site and a necessary interaction with DNA end for cleavage by Type III restriction enzyme

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ABSTRACT

Type III restriction enzymes have been demonstrated to require two unmethylated asymmetric recognition sites oriented head-to-head to elicit double-strand break 25–27 bp downstream of one of the two sites. The proposed DNA cleavage mechanism involves ATP-dependent DNA translocation. The sequence context of the recognition site was suggested to influence the site of DNA cleavage by the enzyme. In this investigation, we demonstrate that the cleavage site of the R.EcoP15I restriction enzyme does not depend on the sequence context of the recognition site. Strikingly, this study demonstrates that the enzyme can cleave linear DNA having either recognition sites in the same orientation or a single recognition site. Cleavage occurs predominantly at a site proximal to the DNA end in the case of multiple site substrates. Such cleavage can be abolished by the binding of Lac repressor downstream (3′ side) but not upstream (5′ side) of the recognition site. Binding of HU protein has also been observed to interfere with R.EcoP15I cleavage activity. In accordance with a mechanism requiring two enzyme molecules cooperating to elicit double-strand break on DNA, our results convincingly demonstrate that the enzyme translocates on DNA in a 5′ to 3′ direction from its recognition site and indicate a switch in the direction of enzyme motion at the DNA ends. This study demonstrates a new facet in the mode of action of these restriction enzymes.

INTRODUCTION

Types I and III restriction-modification systems are multisubunit, multifunctional enzymes. As well as being ATP-dependent restriction enzymes, both the types bear resemblance in requiring two oppositely oriented sites on linear DNA for cleavage, introducing only one double-strand break and having helicase domains in their restriction subunits (1,2). However, Type III restriction enzymes differ from Type I enzymes in several respects. While the recognition sequence of Type III enzymes is asymmetric and continuous, that of Type I enzymes is asymmetric and bipartite. Type III enzymes are composed of two kinds of subunits (Res and Mod), whereas Type I enzymes are composed of three kinds of subunits (HsdS, M and R) (3). The amount of ATP hydrolysed by Type III enzymes is <1% as compared with Type I enzymes under equivalent assay conditions (4). Type III enzymes always cleave at 25–27 bp downstream of one of the head-to-head pair of sites (5), whereas Type I enzymes cleave anywhere on DNA between the sites (6). The presence of blocks on DNA such as a Holliday junction does not prevent cleavage by Type I enzymes (7) while binding of a Lac repressor (Lac rep) on intervening DNA prevents Type III restriction enzyme activity (8). Despite these mechanistic differences, a similar mode of cleavage action involving DNA translocation has been proposed for both Types I and III restriction enzymes (8,9).

According to the DNA tracking and collision model proposed for Type III restriction enzymes (8), two enzyme molecules bound to head-to-head oriented sites perform translocation of the intervening DNA driven by ATP hydrolysis. The enzyme molecules eventually interact to form a collision complex. The complex introduces a double-strand break 25–27 bp downstream of one of the sites. The requirement of head-to-head sites for cleavage was proposed based on different susceptibility of T3 and T7 phage genomes to restriction by the Type III restriction enzyme EcoP15I (R.EcoP15I) (10). DNA translocation by Type III enzymes was proposed based on the prevention of R.EcoP15I cleavage activity by the presence of a Lac rep on intervening DNA between head-to-head oriented sites (8). DNA looping was ruled out as Lac rep would have not prevented interaction of enzyme molecules through looping of intervening DNA.

Several observations reported with Type III restriction enzymes are not in accordance with the proposed DNA tracking collision model. Electron microscopic evidence revealed DNA translocation by Type I enzymes but not by Type III enzymes (11). Importantly, R.EcoP15I shows cleavage of DNA containing non head-to-head oriented sites and single site (5,10,12). The cleavage of inversely oriented sites with no intervening DNA has been reported that cannot be accounted by the proposed DNA translocation model. In addition, the footprint of enzyme molecules bound to the recognition site prior to DNA cleavage, predicted by the DNA translocation model, could not be observed (12).

An interesting yet intriguing feature of Type III restriction enzymes is that cleavage is close to only one of the

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head-to-head oriented sites, while both sites are equally suitable for cleavage. Identifying factors guiding such cleavage activity is expected to throw light on new aspects of DNA–protein interactions in general. The sequence context of recognition sites has been suggested to influence the site of cleavage by Type III restriction enzymes (13). We have analysed the properties of R.EcoP15I independent of the proposed DNA translocation model. Our results highlight an interesting property of the enzyme upon interacting with DNA end. Based on these results, a mechanistic model for Type III restriction enzymes, significantly different from that of Type I enzymes, is proposed.

MATERIALS AND METHODS

Purification of EcoP15 restriction enzyme

DH5α cells harbouring plasmid pSHI180 (1% inoculum) were grown in 500 ml terrific broth supplemented with ampicillin (100 μg/ml) (TB supplemented with Amp) at 37°C for 5 h. Chloramphenicol (17.5 μg/ml) was added to the culture and the cells were grown for an additional 12 h. The cells were harvested and resuspended in 500 ml fresh TB supplemented with Amp and grown for another 12 h. The cells were pelleted and resuspended in lysis buffer (10 mM Tris–HCl, pH 8, 10 mM $MgCl_2$), lysed by sonication and extract clarified by high-speed centrifugation at 26 000 r.p.m. for 2 h at 4°C. Nucleic acids were removed by treating the supernatant with 0.2 M NaCl and 1% w/v polyethylenimine for 30 min, followed by centrifugation at 10 000 r.p.m. for 30 min. To the supernatant, ammonium sulfate was added to 70% saturation and stirred for 1 h. Precipitated proteins were resuspended in buffer A (10 mM potassium phosphate, pH 7.0, 7 mM 2-mercaptoethanol, 100 μM EDTA and 10% (v/v) glycerol) and dialysed for 8 h against the same buffer. Dialysed protein was loaded onto a DEAE Sepharose (Amersham Pharmacia, Sweden) column equilibrated with buffer A. The column was washed with 75 mM NaCl containing buffer A. R.EcoP15I was eluted with a linear gradient of 75–150 mM NaCl in buffer A. The enzyme eluted between 100 and 125 mM NaCl. The peak fractions were pooled and dialysed against buffer B (10 mM Tris–HCl, pH 7.4, 7 mM 2-mercaptoethanol, 100 μM EDTA and 10% (w/v) glycerol) for 6 h. The dialysed protein was loaded onto a heparin Sepharose (Amersham Pharmacia) column equilibrated with buffer B. The column was washed with buffer B containing 200 mM NaCl. The elution was carried out using 600 mM NaCl. Fractions containing homogeneous enzyme, as analysed on SDS–PAGE (14), were pooled and dialysed against fresh buffer B. The enzyme was stored at 4°C.

Purification of Lac rep

Lac rep was purified using a modified protocol of Kumar *et al.* (15). Briefly, an overnight culture (500 ml) of pTrc99 transformed DH5α bacterial cells grown in TB supplemented with Amp at 37°C was used as a starting material for purification. The cell pellet was resuspended in ice-cold cell suspension buffer (10 mM Tris–HCl, pH 7.2, 0.2 M KCl, 10 mM $MgCl_2$, 5% glycerol, 0.3 mM DTT), lysed by sonication and cell lysate centrifuged at 26 000 r.p.m. for 2 h at 4°C. Nucleic acids were removed by treating supernatant with 0.2 M NaCl and 1% w/v polyethylenimine for 30 min, followed by centrifugation at 10 000 r.p.m. for 30 min. To the supernatant, ammonium sulfate was added to 33% saturation and stirred for 1 h. The precipitated proteins were resuspended in 0.075 M potassium phosphate glucose (KPG) buffer (0.075 M potassium phosphate, pH 7.2, 5% glucose, 0.3 mM DTT, 0.1 mM EDTA) and dialysed for 8 h against same buffer. The dialysed protein was loaded onto a 0.075 M KPG pre-equilibrated DEAE Sepharose (Amersham Pharmacia) column. Lac rep was eluted with a linear gradient of 0–300 mM NaCl in 0.075 M KPG buffer. The purified Lac rep eluted between 50 and 75 mM NaCl concentration. The purest fractions, as analysed on SDS–PAGE (14), were pooled and dialysed against storage buffer. The protein was stored at 4°C. Lac rep was dialysed for 3 h against buffer B before use in restriction assays.

DNA preparations

Plasmid DNA was prepared as described previously (16). In order to generate sites A and A′ in substrates pJA31/pJA24 (Figure 1A), a 1.2 kb fragment having a single R.EcoP15I recognition site was cloned in opposite or tandem orientation, respectively, into the multiple cloning site of pBlueScript SK+ vector. A DNA fragment containing a Lac rep binding sequence from pET21b vector was cloned between sites A and A′ in both pJA31 and pJA24. Substrate pJA31 contains an additional 1 kb fragment having no R.EcoP15I site between Lac rep binding site and site A. There is an additional Lac rep binding site in the multiple cloning site of pBlueScript SK+ vector. The non head-to-head pair substrates (Figures 2 and 3) were generated from plasmid pJA24 by digesting with 20 U of the required restriction enzymes as indicated for 6 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, 0.1 mM DTT) containing BSA (0.1 mg/ml) at 37°C for 30 min in a reaction volume of 30 μl. All experiments were repeated at least three times.

DNA cleavage in presence of Lac rep, IPTG

The appropriate amount of Lac rep (8) was added to the reaction mixture and kept at room temperature for 5 min. The reaction was started by adding R.EcoP15I and incubated at 37°C for 30 min. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM) was added along with Lac rep, where indicated and the reaction carried out as described above.

DNA cleavage in presence of HU

pJA31 DNA was incubated in 60 μl reaction volume with HU protein in Lac buffer at 37°C for 10 min. The reaction mixture was then split. One part was incubated at 37°C for a further 30 min and analysed on a TBE gel. To the other aliquot, R.EcoP15I was added and incubated at 37°C for 30 min. R.EcoP15I digestions were followed by Proteinase K treatment [20 μg/ml Proteinase K in 20 mM EDTA, 0.5% (w/v) SDS] at 56°C for 1 h, and products were analysed on a 1% TBE (0.5×) agarose gel. In the absence of Proteinase K treatment, DNA bands showed mobility shift on agarose gel.

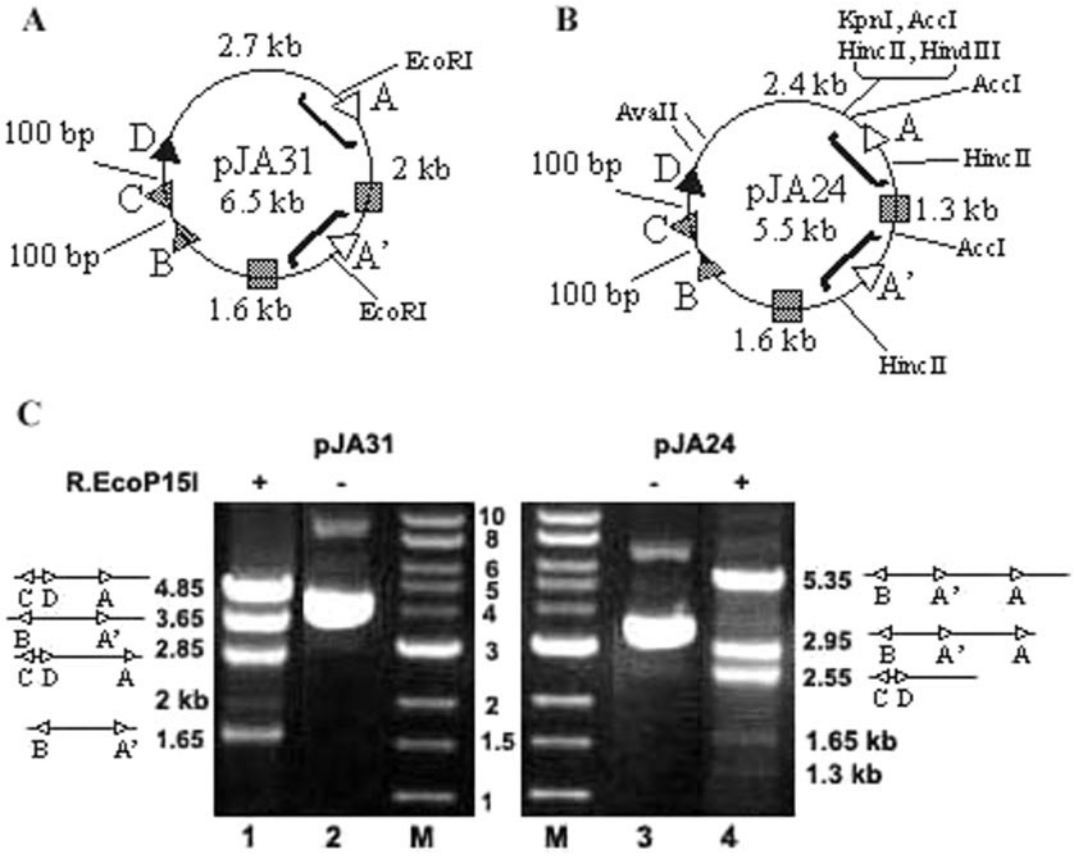

Figure 1. Identical and non-identical sequence context of recognition sites and R.EcoP15I activity. (**A**) Schematic representation of pJA31 substrate. Head-to-head oriented recognition sites A and A′in identical sequence context. (**B**) Representation of pJA24. Recognition sites A and D in non-identical sequence context. In both (A) and (B), the distances between consecutive sites are given in kb or bp. Recognition sites are symbolized by a black triangle representing 5′-CAGCAG-3′. Base of the triangle is 5′ and tip of triangle is 3′ end of recognition site. Lac rep binding site is represented by a hatched square. The 1.2 kb fragment described in the text is marked by a bracket. (**C**) R.EcoP15I cleavage of circular pJA31 and pJA24. Lanes 1 and 4, digestions of pJA31 and pJA24 with R.EcoP15I. Lanes 2 and 3, undigested pJA31 and pJA24 substrates, respectively. Lane M, DNA marker bands, sizes are given in kb between gel pictures. Sizes of R.EcoP15I digested bands are given in kb next to respective gel pictures.

RESULTS AND DISCUSSION

Site of DNA cleavage is not influenced by sequence context

The mechanism of action for Type III restriction enzymes predicts that enzyme complexes are bound to recognition sites (5′-CAGCAG-3′) when cleavage occurs downstream of one of the sites. The selection of site for cleavage was proposed to be stochastic (8). The local sequences at the sites of cleavage were suggested to produce different cleavage rates at different sites, leading to selection of one cleavage site over the other in a head-to-head pair. The absence of cleavage close to both the recognition sites was explained to be a consequence of the collision complex becoming unstable after one double-strand cut (13). The basis for the loss of collision complex stability has never been investigated. We, therefore, initiated a comparative study using substrates having identical sequences at the two potential sites of cleavage in a head-to-head pair. For R.EcoP15I, head-to-head orientation is the presence of two recognition sites on one 5′–3′ strand as CAGCAG and GTCGTC. The 5′ and 3′ side of recognition site refer to 5′ and 3′ side of the sequence CAGCAG on DNA, respectively.

We generated substrates pJA31 and pJA24 (Figure 1A and B) having head-to-head oriented recognition sites in identical and non-identical sequence contexts, respectively, and analysed percent cleavage by R.EcoP15I close to each site under identical assay conditions. Both substrates contain two pairs of head-to-head oriented sites. One pair (sites B and C) lies in the *ori* region of the plasmids (referred to as *ori* pair), and such a pair of sites is present in both substrates. Cleavage in the *ori* pair acts as control to demonstrate normal activity of R.EcoP15I in all the assays performed under different conditions in this study. Cleavage close to site B or C cannot be distinguished from cleavage close to the other site in this pair under the electrophoretic conditions used in this investigation, as the sites of cleavage are just 50 bp apart.

Sites A and A′ form the second head-to-head pair in pJA31 substrate (Figure 1A). Since sites A and A′ are generated from the same 1.2 kb fragment DNA (see Materials and Methods), the 0.5 kb sequence on 3′ side and 0.7 kb sequence on 5′ side of these sites are identical. Hence, the sites of cleavage 25–27 bp downstream of these recognition sites are in an identical sequence context. After cleavage in the *ori* pair (sites B and C), cleavage close to site A (results in

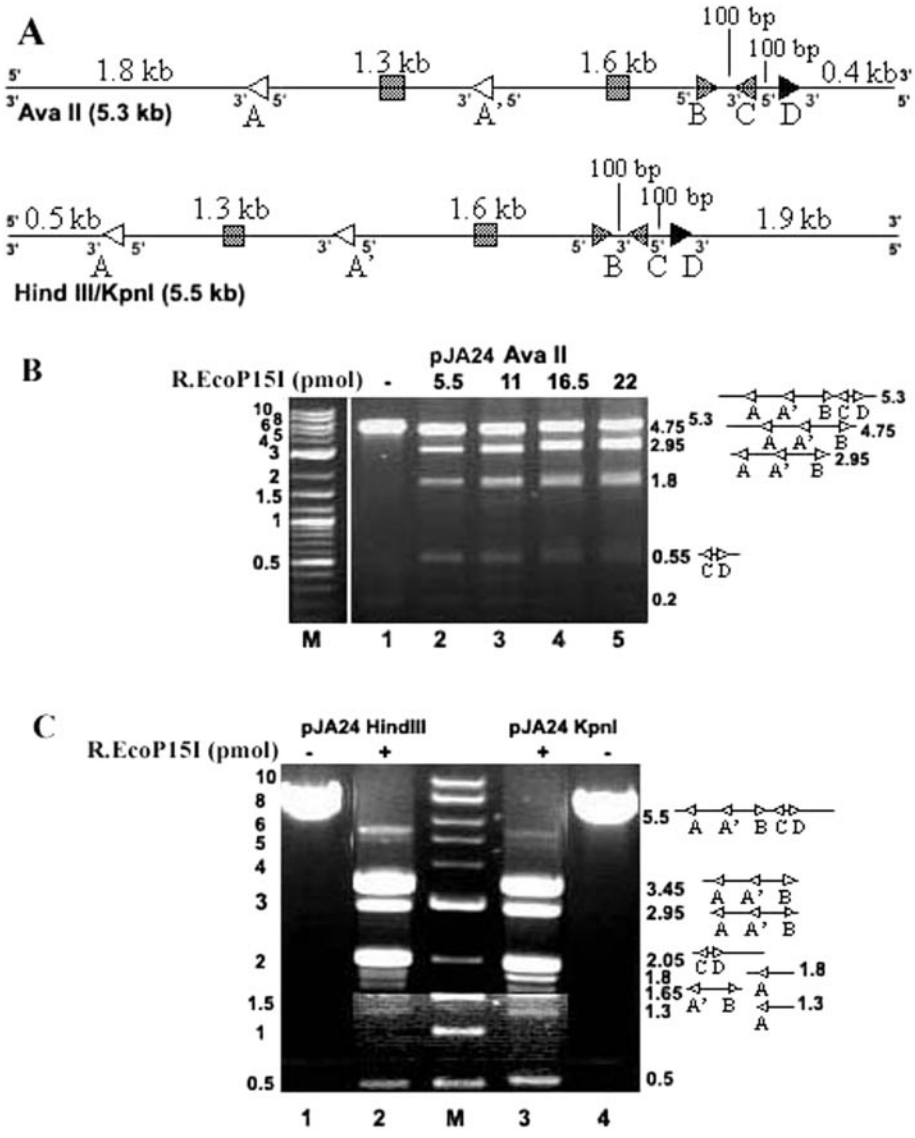

Figure 2. Cleavage of sites proximal to DNA end. (**A**) Substrates having non head-to-head oriented sites. Symbols as in Figure 1. Sites A and D have the 3′ side of the recognition site towards the free DNA end. (**B**) R.EcoP15I cleavage pattern of AvaII-digested pJA24 DNA. Lane 1, AvaII-digested pJA24 DNA; lanes 2–5, AvaII-digested pJA24 DNA incubated with increasing amounts of R.EcoP15I, respectively. (**C**) R.EcoP15I cleavage of HindIII- or KpnI-digested pJA24 DNA. Lanes 1 and 4, pJA24 DNA digested with HindIII and KpnI, respectively; lanes 2 and 3, HindIII- or KpnI-digested pJA24 DNA incubated with R.EcoP15I enzyme. The lower portion (<1.6 kb) of this gel picture has been intensified and realigned with rest of the picture to increase clarity. In (B) and (C), lane M, DNA molecular weight markers.

3.65 and 2.85 kb bands) can be clearly distinguished from cleavage close to site A′ (results in 4.85 and 1.65 kb bands).

The second head-to-head pair in the pJA24 substrate is constituted by site D and site A (Figure 1B). These two sites are in non-identical sequence contexts, and hence the site of cleavage close to these sites is in non-identical context. In this substrate, sites A and A′are generated from same 1.2 kb DNA fragment as used in pJA31 substrate but are in a head-to-tail orientation. As with pJA31, after cleavage in the *ori* pair, cleavage close to site D (results in 5.35 and 0.15 kb DNA bands) can be distinguished from cleavage close to site A (results in 2.95 and 2.55 kb bands in pJA24). The number of times cleavage occurs close to site A and site A′ (pJA31) or site D (pJA24) has been expressed as a percentage derived from the combined intensities of specific bands in each case.

Substrates pJA31 (195 nM/5.85 pmol EcoP15I sites) or pJA24 (230 nM/6.9 pmol EcoP15I sites) were incubated with stoichiometric amounts of R.EcoP15I enzyme (197 nM/5.9 pmol and 230 nM/6.9 pmol R_2M_2, respectively) and the cleavage assay performed as described in Materials and Methods. After the assay, digestion patterns were analysed on 1% agarose gels and DNA bands quantitated by densitometric analysis. Figure 1C shows results of such an assay. The combined intensities of 4.85 and 1.65 kb and 3.65 and 2.85 kb DNA bands involving pJA31 (lane 1) were 42.7 and 57.3%, representing the number of times cleavage occurred close to site A and A′, respectively. Similarly, intensity of the 5.35 kb

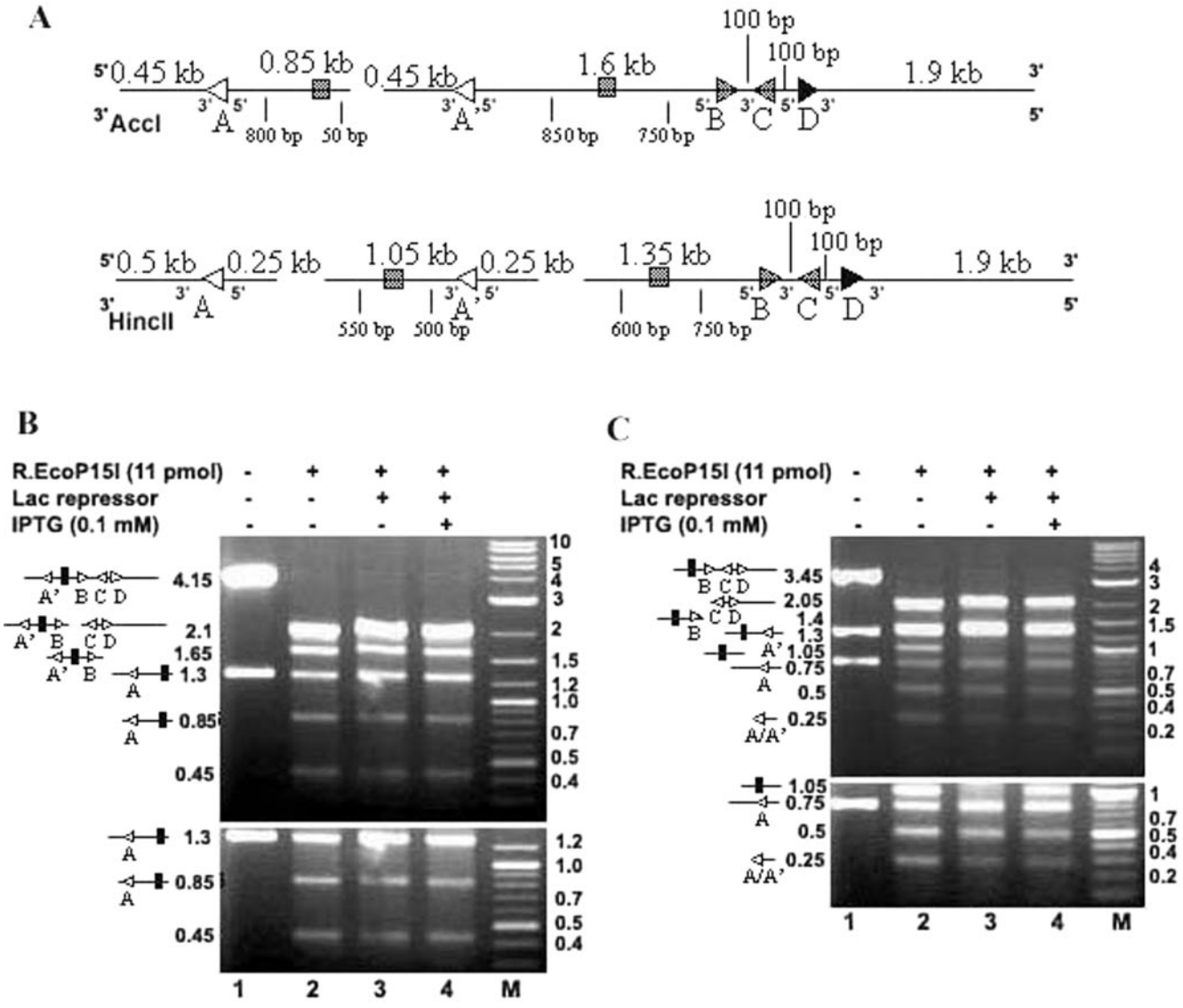

Figure 3. R.EcoP15I activity on DNA containing a single recognition site in the absence or in the presence of Lac rep. (**A**) Single-site DNA substrates generated by digesting pJA24 with AccI (top panel) and HincII (bottom panel). (**B**) Blocking access to the DNA end on the 5′ side of site A. Lane 1, AccI-digested pJA24 DNA; lane 2, AccI-digested pJA24 DNA incubated with R.EcoP15I; lane 3, same as in lane 2 in the presence of Lac rep; lane 4, same as in lane 3 in the presence of IPTG (0.1 mM). The lower portion (<1.5 kb) of this gel picture has been intensified and shown below. (**C**) Blocking access to the DNA end on the 3′ side of site A′. Lane 1, HincII-digested pJA24 DNA; lane 2, HincII-digested pJA24 DNA incubated with R.EcoP15I; lane 3, same as in lane 2 in the presence of Lac rep; lane 4, same as in lane 3 in the presence of IPTG (0.1 mM). The lower portion (<1.25 kb) of this gel picture has been intensified and shown below. In (B) and (C), lane M, DNA molecular weight markers.

band and the combined intensities of the 2.95 and 2.55 kb DNA bands from digestion of pJA24 (lane 4) were 42.8 and 57.2%, representing the number of times cleavage occurred close to sites D and A, respectively. These results clearly demonstrate that cleavage by R.EcoP15I occurs with equal probability close to both the head-to-head oriented sites irrespective of the sequence context at the site of cleavage. Hence, the selection of cleavage site is independent of local sequence. More importantly, this result indicates that factors influencing the site of cleavage might not lie in the DNA but rather be present in the endonucleolytic (collision) complex itself. Earlier work with R.EcoP15I and R.EcoP1I also indicated equal probability of cleavage close to one or the other of both putative sites (12,17). However, the effect of sequence context like the one described here was not carried out. The inclusion of Lac rep in these experiments prevents cleavage between sites A and A′ in pJA31, while there is no effect on cleavage between sites D and A in pJA24 under these conditions (data not shown).

Cleavage of non head-to-head sites on linear DNA

In the digestions with R.EcoP15I shown in Figure 1C, a faint but clear 2 kb band with pJA31 and 1.65 and 1.3 kb bands with pJA24 DNA substrate were always observed. The distance between sites A and A′ in pJA31 is 2 kb and cleavage

close to both these sites releases a 2 kb DNA fragment. The predominant cleavage close to one of these sites (either at site A or A′) leaves the other cleavage site intact on the linear DNA fragment (4.85 and 3.65 kb bands), but with no recognition site in the opposite orientation to form a head-to-head pair.

Similarly, in pJA24, the distance between sites A and A′ is 1.3 kb and that between site A′ and B is 1.6 kb. Under the stoichiometric enzyme concentrations used in the above assay, cleavage occurs at either site D or site A in the D:A pair. Cleavage close to recognition site D results in a linear fragment (5.35 kb band) having an intact cleavage site close to recognition sites A and A′. Both sites A and A′on this linear fragment do not have a recognition site in the opposite orientation. Cleavage close to site A in the 5.35 kb fragment results in 2.35 and 3 kb bands, while cleavage close to site A′ results in 3.7 and 1.65 kb bands. Cleavage close to site A in the D:A pair leaves cleavage site close to recognition sites D (2.55 kb band) and A′ (2.95 kb band) intact, with no site in the opposite orientation. A subsequent cleavage close to site A′ in the 2.95 kb fragment will result in the appearance of a 1.3 kb band (fragment between A and A′) and a 1.65 kb band (fragment between A′ and *ori* pair). Cleavage close to site D in the 2.55 kb fragment cannot be scored, as it is only 100 bp away from *ori* pair and will result in a 2.45 kb band.

Previous work (8) reported cleavage close to both recognition sites, when sharing a common site in opposite orientation, to be possible with sub-saturating enzyme concentrations and not with stoichiometric enzyme amounts. In addition, cleavage close to both sites in a head-to-head pair was reported under conditions of no cofactor but with high amounts of enzyme to site ratio (8:1) (18). The consistent appearance of a 2 kb band with pJA31 and 1.65 and 1.3 kb bands with pJA24 with stoichiometric amounts of enzyme prompted us to investigate the nature of the cleavage mechanism the enzyme follows to cleave at both sites A and A′.

We generated substrates having sites A and A′ in a non head-to-head context by digesting substrate pJA24 with either AvaII, HindIII or KpnI restriction enzymes. Plasmid pJA24 has two AvaII, one HindIII and one KpnI site. The schematic representation of the resulting DNA fragments is shown in Figure 2A. The 0.2 kb AvaII fragment having no R.EcoP15I recognition site is not shown. AvaII and HindIII provide 5′ protruding termini, while KpnI results in 3′ protruding end. The distance between site A and the DNA end on the 3′ side of site A in AvaII-, HindIII- and KpnI-digested pJA24 is 1.8, 0.5 and 0.5 kb, respectively.

R.EcoP15I restriction assay was carried out as described in Materials and Methods with AvaII-digested pJA24 DNA (184 nM/5.52 pmol sites) and increasing amounts of R.EcoP15I enzyme (183–732 nM R_2M_2). As is evident from Figure 2B (lanes 2–5), the appearance of 0.55 and 4.75 kb bands and the complete disappearance of the 5.3 kb band, at all concentrations of enzyme used, clearly indicates that 183 nM/5.5 pmol of enzyme is sufficient to bring about 100% cleavage at the *ori* pair. Cleavage close to site A results in the appearance of 2.95 and 1.8 kb bands, while cleavage close to site A′ results in 3.1 and 1.65 kb bands. As can be seen from Figure 2B (lane 2), a stoichiometric amount of enzyme resulted in cleavage close to non head-to-head oriented sites (A or A′) as evident from the appearance of 2.95 and 1.8 kb bands. This suggests the absence of promiscuous or secondary cleavage proposed for Type III enzymes based on different buffer conditions and high enzyme concentrations (18). Figure 2B (lanes 3–5) shows that increasing amounts of enzyme (11–22 pmol) leads to increased cleavage close to site A or A′ as evident from the increase in intensities of the 2.95 and 1.8 kb bands.

The results of HindIII and KpnI-linearized pJA24 DNA (184 nM/5.52 pmol sites) incubated with 549 nM/16.5 pmol of R.EcoP15I enzyme are shown in Figure 2C (lanes 2 and 3). Cleavage in the *ori* pair resulted in 3.45 and 2.05 kb DNA fragments as expected. Cleavage of 3.45 kb DNA at site A results in 2.95 and 0.5 kb bands, whereas cleavage close to site A′ gives 1.8 and 1.65 kb bands. Cleavage close to both sites A and A′ results in 0.5, 1.3 and 1.65 kb bands. Thus, 2.95, 1.8 and 1.3 kb bands serve as unique bands as a result of cleavage close to site A, A′and at both A and A′, respectively. A very prominent 2.95 kb band compared with the 1.8 kb band can be seen in these assays (Figure 2C, lanes 2 and 3) demonstrating that cleavage occurred predominantly at site A than at site A′. The appearance of a faint 1.3 kb band (Figure 2C, lanes 2 and 3) suggests that cleavage has occurred close to both sites A and A′. The substitution of Na^+ ions instead of K^+ ions in the assay buffer did not alter these cleavage patterns (data not shown).

Clearly, these results demonstrate that R.EcoP15I can cleave non head-to-head oriented sites on linear DNA. Such a cleavage activity of the enzyme appears to be independent of the type of DNA termini (5′ or 3′ protruding) and the distance of the site from a DNA end (0.5 or 1.8 kb). Strikingly, site A appears to be more susceptible to cleavage than the internal site A′ which is also in the same orientation and present in an identical sequence context (Figure 2C). Note that the distance of site A′ in HindIII- or KpnI-digested pJA24 from DNA end is 1.8 kb which is the same as the distance of site A from the DNA end in AvaII-digested pJA24. This might possibly indicate that the first site from the DNA end is highly susceptible to cleavage irrespective of its distance in these substrates. On closer examination of the findings of Meisel *et al*. (10), who proposed earlier that head-to-head oriented sites are required for cleavage by Type III restriction enzymes, it can be seen that linear DNA with sites in the same orientation are indeed cleaved by R.EcoP15I.

Binding of Lac rep on 3′ side of recognition site prevents DNA cleavage

In order to address why there is preferential cleavage close to site A in the linear substrates used above (Figure 2A) and to analyse whether R.EcoP15I would cleave at site A′, provided it is the first recognition site from a DNA end, we generated linear substrates having single R.EcoP15I recognition sites (A or A′) and a Lac rep binding site either 5′ or 3′ side of the recognition site(s). This was achieved by digesting substrate pJA24 with either AccI or HincII restriction enzymes. Figure 3A shows a schematic representation of the fragments generated from such digestions.

In the case of pJA24 digested with AccI enzyme, two fragments of sizes 4.15 and 1.3 kb are obtained (Figure 3B, lane 1). The 1.3 kb fragment has site A and a Lac rep site at a distance of 800 bp to the 5′ side of site A. Cleavage close to site A on this 1.3 kb fragment releases 0.85 and 0.45 kb bands. The unique DNA fragments released by R.EcoP15I cleavage in *ori* pair, close to site A′ and site A are 2.1, 1.65 and

0.85 kb, respectively. The 4.15 kb fragment has the *ori* pair, sites A′ and D.

AccI-digested pJA24 DNA (184 nM/5.52 pmol sites) was incubated with 366 nM/11 pmol (2:1 enzyme:recognition site) of R.EcoP15I enzyme under three different assay conditions (in the absence or in the presence of Lac rep or in the presence of both Lac rep and IPTG), and the restriction assays were performed as described in Materials and Methods. The complete disappearance of the 4.15 kb band and appearance of 2.1 and 2.05 kb DNA fragments representing cleavage in the *ori* pair can be observed under all three assay conditions (Figure 3B, lanes 2–4). Cleavage close to site A′ is evident from a unique 1.65 kb DNA band under all three conditions, demonstrating cleavability at site A′ by R.EcoP15I enzyme provided it is the first site from a DNA end. This result clearly indicates a requirement for a free DNA end on the 3′ side of the single recognition site on linear DNA for R.EcoP15I cleavage activity. The 1.3 kb linear DNA containing site A is cleaved irrespective of the presence or absence of Lac rep and IPTG (0.1 mM), releasing 0.45 kb and unique 0.85 kb fragments. More importantly, cleavage of site A with Lac rep bound on the DNA to the 5′ side of the site (Figure 3B, lane 3) implies that the DNA end on the 5′ side of the recognition site plays no role in cleavage close to such sites.

pJA24 DNA when digested with HincII enzyme releases three fragments of sizes 3.45, 1.3 and 0.75 kb (Figure 3C, lane 1). There is a Lac rep site in the 3.45 and 1.3 kb fragments but not in the 0.75 kb fragment. Site A is present on the 0.75 kb fragment at a distance of 0.5 kb from the DNA end on the 3′ side and 0.25 kb on the 5′ side of the site. The 1.3 kb fragment has site A′ with a Lac rep binding site at a distance of 0.5 kb on the 3′ side of the site. The 3.45 kb has the *ori* pair and site D. The unique bands for cleavage in *ori* pair, close to site A and site A′, are 2.05, 0.5 and 1.05 kb, respectively.

HincII-digested pJA24 DNA (184 nM/5.52 pmol sites) was incubated with 366 nM/11 pmol (2:1 enzyme:recognition site) of R.EcoP15I enzyme, and the restriction assays were performed as described above. The complete disappearance of the 3.45 kb band and appearance of 2.05 and 1.4 kb DNA fragments representing 100% cleavage in the *ori* pair can be observed under all three assay conditions (Figure 3C, lanes 2–4). Cleavage close to site A is evident from the presence of a unique 0.5 kb band (Figure 3C, lanes 2–4). The presence of Lac rep without (lane 3) or with IPTG (lane 4) evidently does not affect R.EcoP15I cleavage close to site A highlighting that Lac rep does not non-specifically interfere with the enzyme activity. Cleavage close to site A′ releases a 1.05 kb band in the absence of Lac rep (lane 2). In the presence of Lac rep, which binds specifically at a distance of 0.5 kb on the 3′ side of site A′, there is a clear prevention of R.EcoP15I cleavage close to site A′ (lane 3, 1.05 kb band is absent). Inclusion of 0.1 mM IPTG along with Lac rep in the assay (lane 4) leads to the appearance of the 1.05 kb band, demonstrating specificity of Lac rep preventing R.EcoP15I activity.

These results demonstrate that Lac rep can specifically block the required interaction of R.EcoP15I with a DNA end on the 3′ side of a recognition site (Figure 3C, lane 3) essential for cleaving non head-to-head sites on linear DNA. It was observed earlier that R.EcoP15I restriction enzyme could not cleave circular DNA having a single recognition site or two sites in the same orientation (13).

R.EcoP15I activity in the presence of HU protein

The prevention of R.EcoP15I activity by the binding of Lac rep on DNA (Figure 3) indicated that DNA-binding proteins could play a role in regulating Type III restriction enzyme activity *in vivo*. Hence, R.EcoP15I restriction assays were performed in the presence of the bacterial architectural nuclear protein HU (18). The binding of HU protein to DNA was monitored through mobility shift assays. The *in vivo* ratio of R.EcoP15I enzyme to recognition sites on genomic DNA has been estimated to be sub-stoichiometric (8). To reflect this fact, we have used sub-stoichiometric amounts of R.EcoP15I enzyme (1:2 enzyme:site ratio) in restriction assays performed in the presence of HU protein.

Supercoiled pJA31 DNA (156 nM/9.4 pmol sites) was incubated with increasing amounts of HU protein (0–4 μg), and the assay was carried out as described in Materials and Methods. As can be seen in Figure 4A, increasing amounts of HU protein in the reaction led to an increase in mobility shift of pJA31 DNA, demonstrating increased binding of HU to DNA. A further shift in the DNA band was observed with increased HU protein, and the complex remains in the wells with 8 μg HU (data not shown).

R.EcoP15I digestion of pJA31 DNA in the presence of increasing amounts of HU protein can be seen in Figure 4B. As mentioned above, sub-stoichiometric amounts of R.EcoP15I enzyme have been used in this assay. The 4.85, 3.65, 2.85 and 1.6 kb fragments are a consequence of cleavage at the *ori* pair and A/A′ pair (Figure 1C, lane1). The appearance of a linearized 6.5 kb band reflects incomplete digestion owing to sub-stoichiometric amounts of R.EcoP15I enzyme in the assay. A decrease in R.EcoP15I enzyme activity in the presence of HU protein can be monitored by a decrease in intensity of bands <6.5 kb size with concomitant increase in 6.5 kb pJA31 linearized band. In the presence of 4 μg HU protein,

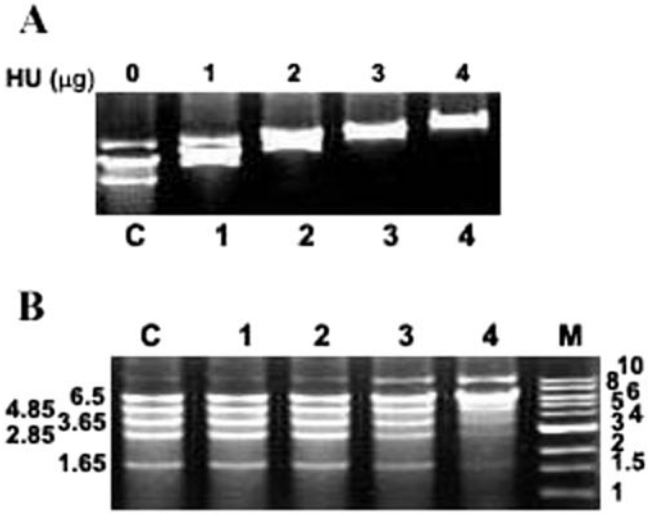

Figure 4. R.EcoP15I activity in the presence of HU protein. (**A**) Binding of HU protein to pJA31 DNA. DNA mobility decreases with increasing HU concentration. (**B**) R.EcoP15I activity in the presence of increasing HU concentration. Lane C, assay in the absence of HU protein; lanes 1, 2, 3 and 4 correspond to reactions having 1, 2, 3 and 4 μg of HU protein, respectively. Lane M, DNA molecular weight markers.

R.EcoP15I activity is drastically reduced as evident from the prominent 6.5 kb band and faint bands <6.5 kb size (Figure 4B, lane 4). DNA cleavage by R.EcoP15I in the presence of HU protein (Figure 4B, lane 4) indicates that HU protein was able to prevent cleavage by R.EcoP15I. Addition of HU may have two effects, physical blocking of R.EcoP15I translocation and/or alteration of DNA conformation by bending/distortion that might interfere with binding of R.EcoP15I to DNA.

The cleavage of single-site DNA by R.EcoP15I (Figure 3) implies that (i) bacterial genomic DNA having double-strand breaks and unmethylated sites can be cleaved and (ii) refractiveness of the T7 phage linear genome to cleavage cannot be satisfactorily explained only on the basis of the unidirectional orientation of R.EcoP15I sites (10). Alternate mechanisms, such as interference by HU protein demonstrated above, might exist in the bacterial cells, harbouring Type III restriction enzymes as well as in T7 phage, to protect their genomic DNA.

Importance of DNA ends for R.EcoP15I cleavage activity

According to the DNA translocation model, a possible reason for cleavage close to sites A and A′ as described in Figures 2 and 3 could be due to passing of the DNA end through the enzyme molecule and the triggering of the enzyme cleavage activity. Such a mechanism will be expected to result in complete cleavage at these sites (A and A′) with stoichiometric amounts of enzyme, which has not been observed in this study or in earlier work (10). In addition, it suggests that cleavage can occur close to both sites in a head-to-head pair. However, in this study, we have observed that the predominant cleavage occurs at only one of the two sites (Figure 1). The experiments described in this study clearly demonstrate that cleavage at sites A and A′ (Figures 2 and 3) is not due to *trans* effects or artifacts of assay conditions. The specific requirement for a free DNA end on the 3′ side of recognition site is evident in these assays (Figure 3B). Recently, Reich *et al*. (19) using scanning force microscopy concluded that R.EcoP15I translocates DNA by observing 1–10% of DNA bound R.EcoP15I enzyme molecules with loop structures, while the majority (90–99%) showed no DNA loop-like structures. More recently, Peakman and Szczelkun (20) have reported the absence of any evidence for the formation of extruded DNA loops during translocation by Type III enzymes akin to those observed with Type I enzymes. In view of the results obtained in this study and the earlier observations that are not in accordance with DNA tracking collision model, we considered protein translocation as mode of interaction for R.EcoP15I on DNA.

A probable mechanistic model that can satisfactorily explain the results obtained with Type III restriction enzymes to date is depicted in Figure 5. 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 sites on circular DNA indicates that enzyme always translocates towards the 3′ side of a site to form a collision complex. Probably, the Mod (M_2) subunit binding to the 5′-CAGCAG-3′ sequence dictates activation of the Res subunit ATPase activity and hence the direction of enzyme translocation on DNA. As a result, at any given

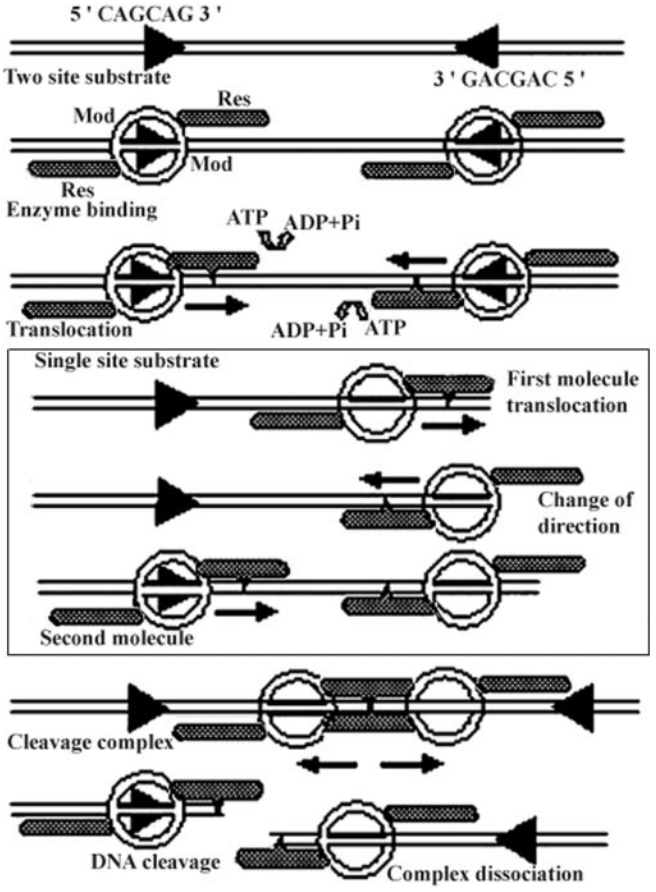

Figure 5. Mechanism of action of Type III restriction enzymes. Interaction of the enzyme with a DNA end. R.EcoP15I recognition site symbolized by '▶'. The Mod subunit is represented by '' and the Res subunit by ''. R_2M_2 is shown as '', '' indicates cleavage complex. The arrows indicate the direction of R.EcoP15I/cleavage complex translocation. The box shows events on single-site DNA leading to cleavage complex formation.

point of time it is probably only one of the two Res subunits that will hydrolyse ATP. 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.

The enzyme translocating from a single site on linear substrates (as shown in Figure 3) will reach a DNA end on the 3′ side of the recognition site. The ability of Lac rep to prevent cleavage when bound to DNA downstream of recognition sites provides evidence for interaction of the enzyme with a DNA end on the 3′ side and subsequent events required for DNA cleavage. The interaction can trigger the enzyme molecule to translocate back into DNA, rather than fall off the DNA. Recently, it was shown that DNA termini generated by cleaving a canonical site for the Type II restriction enzyme SgrAI assisted in the cleavage of secondary sites (21). Two modes of change in translocation direction are possible with the EcoP15I enzyme molecule: (i) it can activate ATP hydrolysis of other Res subunit, or (ii) it can make a 180° rotation at the DNA end. The absence of effect of different DNA termini (Figures 2 and 3) suggests activation of the second Res subunit for reverse translocation. In either case, the enzyme molecule would, therefore, resemble an enzyme molecule translocating from the opposite site, whose interaction with another

molecule translocating towards the 3′ side of a single site results in the formation of a collision complex. Our earlier work indicated that more than one enzyme molecule could bind to a recognition site sequentially and hydrolyse ATP in a recognition site specific manner (22).

The complex $[(R_2M_2)_2]$ can translocate with equal probability in either direction from the point of interaction and cleave DNA upon reaching a recognition site. The equal percent of cleavage at sites in a head-to-head pair demonstrated in Figure 1C with different substrates supports this prediction. Accordingly, for single-site DNA, the probability of cleavage is only 50% for every collision complex formed.

The problem of cleavage site selection does not arise with the protein translocation model as the complex $[(R_2M_2)_2]$ can reach only one of the two head-to-head oriented sites. DNA cleavage occurring at the interface of two enzyme molecules facilitates the dissociation of complex into individual enzyme molecules (R_2M_2). This explains why cleavage is not observed close to both the recognition sites. The ability of a second enzyme molecule translocating from a single site on DNA to form a collision complex would indicate that recognition sites are probably not methylated before DNA cleavage occurs.

Taken together, our results clearly demonstrate that selection of cleavage sites by Type III restriction enzymes is not influenced by the sequence context of the cleavage site. These enzymes can cleave DNA with non head-to-head oriented sites having an accessible DNA end. Our results suggest a necessary interaction of R.EcoP15I with the DNA end on the 3′ side of a recognition site before the cleavage reaction. DNA-binding proteins such as HU can interfere with Type III restriction enzyme activity *in vivo*. Our results show that Type III restriction enzymes might translocate on DNA as opposed to translocation of DNA by Type I enzymes. It is possible that detailed structural and sequence analysis of Type I and III enzymes (having similar motifs) will reveal factors dictating DNA versus protein translocation by DNA-interacting proteins.

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