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DNA cleavage by Type ISP Restriction–Modification enzymes is initially targeted to the 3′-5′ strand

Kara van Aelst, Eva Šišáková and Mark D. Szczelkun*

DNA-Protein Interactions Unit, School of Biochemistry, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK

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ABSTRACT

The mechanism by which a double-stranded DNA break is produced following collision of two translocating Type I Restriction–Modification enzymes is not fully understood. Here, we demonstrate that the related Type ISP Restriction–Modification enzymes LlaGI and LlaBIII can cooperate to cleave DNA following convergent translocation and collision. When one of these enzymes is a mutant protein that lacks endonuclease activity, DNA cleavage of the 3′-5′ strand relative to the wild-type enzyme still occurs, with the same kinetics and at the same collision loci as for a reaction between two wild-type enzymes. The DNA nicking activity of the wild-type enzyme is still activated by a protein variant entirely lacking the Mrr nuclease domain and by a helicase mutant that cannot translocate. However, the helicase mutant cannot cleave the DNA despite the presence of an intact nuclease domain. Cleavage by the wild-type enzyme is not activated by unrelated protein roadblocks. We suggest that the nuclease activity of the Type ISP enzymes is activated following collision with another Type ISP enzyme and requires adenosine triphosphate binding/hydrolysis but, surprisingly, does not require interaction between the nuclease domains. Following the initial rapid endonuclease activity, additional DNA cleavage events then occur more slowly, leading to further processing of the initial double-stranded DNA break.

INTRODUCTION

Nucleases play a wide range of important roles in cellular DNA and RNA metabolism. Yet, they are also inherently dangerous enzymes, capable of inflicting potentially toxic breaks in DNA or RNA. Control of these enzymes is therefore vital to prevent unnecessary genome damage. One large class of enzymes that use nuclease domains is the bacterial Restriction–Modification (RM) enzymes that need to target their nuclease activity to invading foreign DNA but not the host DNA (1,2). In the accompanying article, we examined the DNA cleavage mechanism of the adenosine triphosphate (ATP)-dependent Type ISP class of single polypeptide RM enzyme (3). We demonstrate that ATP-dependent LlaGI and LlaBIII cleave DNA at random non-specific sites located, on average, midway between a pair of head-to-head (HtH)-oriented recognition sites. The process that leads to cleavage of the non-specific sites is a long-range communication driven by ATP hydrolysis, which can occur over many thousands of base pairs (3,4). Enzymes bound specifically at each site start to translocate along the DNA downstream of the site and, on collision of the converging enzymes, a double-stranded DNA (dsDNA) break is introduced. The nuclease domains of the Type ISP enzymes must be under control to prevent promiscuous cleavage of non-specific sites until a collision complex is formed. To understand how this occurs, it is necessary to understand first how a dsDNA break is produced, i.e. how do the nuclease domains cooperate to cleave both DNA strands on collision at a non-specific location distant from the recognition sites?

Three simplified models can be considered for how a dsDNA break is produced on formation of the collision complex and juxtaposition of the nuclease domains:

(i) The interaction of two independent strand-specific nucleases (Figure 1A). In this first model, each nuclease domain in the collision complex is targeted to, and cleaves, one DNA strand. Allosteric activation of the nuclease activity may be due to specific protein–protein contacts between the enzymes or to a strain-dependent mechanism induced by the motor activity. In the latter case, it is possible that collision with an immovable roadblock could also generate nuclease activity, as seen with the multi-subunit Type I enzymes (5). In a slightly modified version of this model, the nucleases are always activated but have slower catalytic rates than the translocase, as in the double-strand break

*To whom correspondence should be addressed. Tel: +44 1173 312158; Fax: +44 1173 312168; Email: mark.szczelkun@bristol.ac.uk

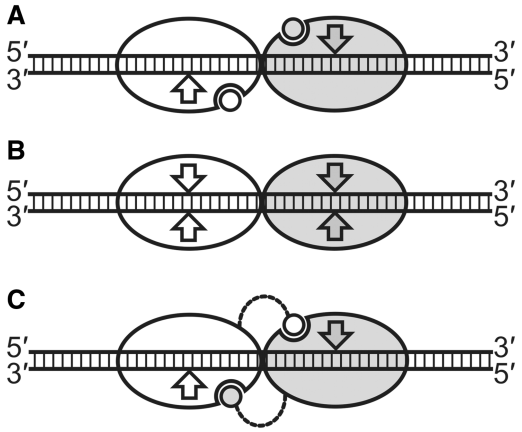

Figure 1. Models for dsDNA cleavage within the collision complex formed by two Type ISP enzymes. DNA is represented as a horizontal ladder, proteins as ovals and strand-specific cleavage by arrows. The circles represent components of the nuclease active site, which are supplied in *cis* or in *trans*. (**A**) Independent strand-specific nucleases. (**B**) Strand promiscuous nuclease(s). (**C**) Domain sharing nucleases. See main text for further explanation.

processing enzymes RecBCD and AddAB (6). Therefore, cleavage will occur when the motor stalls at one location for long enough, as will happen on head-on collision.

(ii) The interaction of two strand promiscuous nucleases (Figure 1B). In this second model, a single nuclease domain may have the potential to cut both DNA strands. We note that for a monomer on anti-parallel DNA, this requires molecular gymnastics to turn the single active site through 180° (7). As drawn in Figure 1B, both enzyme monomers in the dimeric collision complex make a dsDNA break, releasing a short dsDNA fragment. Alternatively, the collision complex may have induced asymmetry, and only one of the two proteins may make the dsDNA break. As in Model (i), both protein–protein and/or mechanical allostery may activate cleavage.

(iii) The sharing of nuclease residues upon collision (Figure 1C). In this third model, the nuclease domains are inactive before collision because they have an incomplete nuclease active site. There are six catalytic residues within the Type ISP Mrr-like nuclease LlaGI (E38, D74, D78, Q92 and K94) (8), and one or more of these residues may be shared across a subunit–subunit interface of the collision complex (Figure 1C). i.e. an enzyme donates a residue to the partner enzyme to complete an active site and vice versa. An example of such domain sharing is seen in the dimeric T7 endonuclease I Holliday junction resolvase (9).

The classical multi-subunit Type I RM enzymes also use a translocation–collision model to activate DNA cleavage (10), and a number of similar models have been considered. By varying the ratios of wild-type and nuclease mutant HsdR subunits, Bickle *et al*. observed variations in the relative levels of DNA nicks versus dsDNA breaks, and they suggested that a dsDNA break requires the collision of two intact nuclease domains (11). They could not, however, rule out the influence of additional HsdR subunits that are recruited from solution to the collision complex, as dsDNA breaks were also produced by unrelated mechanical roadblocks (5). Therefore, clear distinctions between the models in Figure 1 could not be made. What also remains unclear is which strand is targeted by which enzyme on collision.

To help address these deficiencies, we used the related Type ISP enzymes LlaGI (12) and LlaBIII (3). These proteins recognize distinct DNA sequences but have nearly identical nuclease-helicase domains in terms of amino acid sequence (3,13). We reasoned that on a mixed DNA substrate with one site for each enzyme in an HtH repeat, addition of both enzymes would lead to activation of cleavage and the production of a dsDNA break. Addition of only one or other enzyme would produce, at best, a single-stranded DNA (ssDNA) nick, as a collision complex would not be formed. The models in Figure 1 could then be tested by making point mutations in one or both enzymes or by removing protein domains. Our results point to a model based around that in Figure 1A, in which each protein in the collision complex cuts one specific strand. Activation appears specific to interactions between Type ISP enzymes but does not require protein–protein contacts between the nuclease domains.

MATERIALS AND METHODS

DNA

pG + B was constructed using QuikChange mutagenesis (Stratagene) based on pInvR (3); mutations at C1731A and A1734T were introduced to knock out the LlaBIII site at 1734. The production of the 5′ end-labelled linear substrates and ladders was performed as described in the accompanying article (3). pG + B was digested first using NdeI, labelled with ^{32}P and then digested using either AatII (to leave a labelled 'top' strand) or SphI (to leave a labelled 'bottom' strand). Plasmid DNA for biochemical assays was prepared and ^{3}H-labelled where needed as described previously (14).

Proteins

Wild-type LlaGI, D74A, D78A and K94A were purified as described previously (8,12), and wild-type LlaBIII was purified as in the accompanying article (3). LlaBIII(D74A) and LlaGI(K210A) were produced using QuikChange mutagenesis (Stratagene) of pET28aLlaBIII and purified as for the wild-type enzymes. The cloning, purification and characterization of LlaGIΔN will be described elsewhere (K. van Aelst, E. Sisakova, M.D. Szczelkun and K. Saikrishnan, unpublished data). EcoRI(E111G) and Lac Repressor were purified and supplied by Prof. Peter McGlynn (University of York, UK).

DNA cleavage assays

Cleavage assays contained 2 nM DNA (supercoiled or linear), 4 mM ATP and 200 nM LlaGI and/or LlaBIII in

TMDK buffer [50 mM Tris–Cl (pH 8.0), 10 mM $MgCl_2$, 150 mM KCl and 1 mM DTT]. Reactions were started by adding ATP, and incubated at 25°C for the times indicated. Reactions were stopped with 0.5 volumes of 3X STEB [0.1 M Tris (pH 7.5), 0.2 M EDTA, 40% (w/v) sucrose and 0.4 mg/ml bromophenol blue], and analysed by agarose gel electrophoresis or alkaline denaturing agarose gel electrophoresis. For the native gels, the amount of 3H-labelled DNA in each band was ascertained by scintilation counting (14) and is presented as a percentage relative to the total amount of DNA in each lane. For the denaturing gels, the imaging and analysis of the lanes were performed as described previously (3).

RESULTS

LlaGI and LlaBIII can cooperate in cleaving a DNA substrate with HtH sites for both enzymes

To compare the Type ISP cleavage activity to the models in Figure 1, we first tested that a mixed DNA substrate containing one LlaGI site and one LlaBIII site in HtH repeat (Figure 2A) could be cleaved by the cooperative action of both enzymes. As the absence of potassium ions leads to a non-specific DNA cleavage by LlaBIII (3), all reactions were carried out in TMDK ('Materials and Methods' section). In the presence of saturating ATP and a 100-fold molar excess of enzyme over DNA, the addition of either enzyme individually to pG + B only resulted in DNA nicking, a feature characteristic of reactions on one-site plasmid DNA substrates (Figure 2B) (3,12). However, addition of both enzymes resulted in the production of full-length linear (FLL) DNA, indicating that LlaGI and LlaBIII can cooperate to produce a dsDNA break.

One potential issue in using the TMDK buffer is that DNA cleavage by LlaGI is inhibited in these conditions (3). However, we show later (Figure 5) that the presence of LlaBIII in the collision complex appears sufficient to stabilize the complex and produce reaction profiles more characteristic of LlaBIII alone.

The nuclease domains of LlaGI and LlaBIII are targeted initially to the proximal 3′-5′ strand

A simple way to distinguish the models in Figure 1 is to mutate the catalytic residues in the nuclease domain of one of the two enzymes and to observe the effects on the cleavage products: for Figure 1A, regardless of the residue mutated, DNA cleavage will result in DNA nicking as the partner wild-type enzyme can still cleave its strand; for Figure 1B, regardless of the residue mutated, DNA cleavage will still result in dsDNA breaks; while for Figure 1C, the outcome will be a nicked DNA, but the strand specificity will vary. For example, based on the cartoon enzymes in Figure 1C, if the mutation is in a core residue in the white protein (oval), then the cleavage of the 'bottom' strand will be knocked out. However, if the mutation is in a residue in the donated domain (white circle), then the catalytic site of the grey protein is disrupted and cleavage of the 'top' strand will be knocked out.

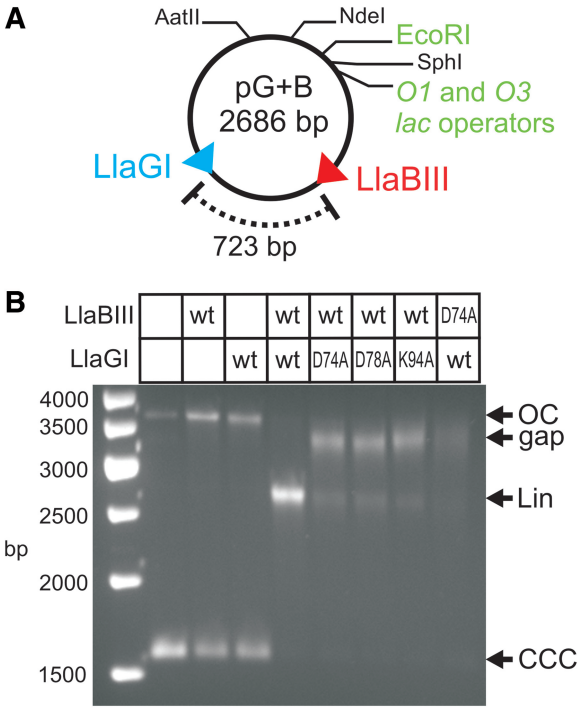

Figure 2. Cooperation between LlaGI and LlaBIII. (**A**) Mixed plasmid substrate used in the subsequent assays. The arrows show the orientation of the LlaGI (5′-CTnGAYG-3′) and LlaBIII (5′-nTnAGCC-3′) sites, where the arrowheads indicate the 3′ ends of the sequences. The locations of the EcoRI and *lac* operator sites used in Figure 4 are also indicated. (**B**) Agarose gel showing the substrate and product bands following incubation with pG + B and the enzymes indicated for 2 min. OC is open circle (i.e. nicked DNA) and CCC is covalently closed circular DNA (i.e. plasmid substrate). Gap represents the putative location of DNA in which a single strand gap has been produced (15).

We chose to examine three principal catalytic residues (D74, D78 and K94), where single alanine substitutions resulted in 100% inactivity of the Mrr nuclease (8). We first examined the reactions at a fixed time point on plasmid DNA (Figure 2B). Where one protein was wild-type and one was a nuclease mutant, and regardless of the mutation or the identity of the mutated enzyme, the main product was a DNA band with an electrophoretic mobility in-between that of nicked and linear DNA. A smaller proportion of FLL DNA was produced. We have noted previously that gapped DNA runs faster than the corresponding nicked DNA (15), and we interpret the intermediate band seen here as being a gapped circular DNA molecule. We suggest that the first product produced is a strand-specific DNA nick, which is further processed close to the nick site to produce a DNA gap. We discuss the additional nucleolytic DNA processing activity of the Type ISP enzymes in more detail later (Figure 5).

To refine our analysis, we needed to map the DNA cleavage to particular DNA strands. To do this, we produced two linear substrates from pG + B in which either one or other strand was labelled with ^{32}P. Following cleavage with combinations of LlaGI and/or LlaBIII at a fixed time point, the samples were separated by alkaline denaturing agarose gel electrophoresis.

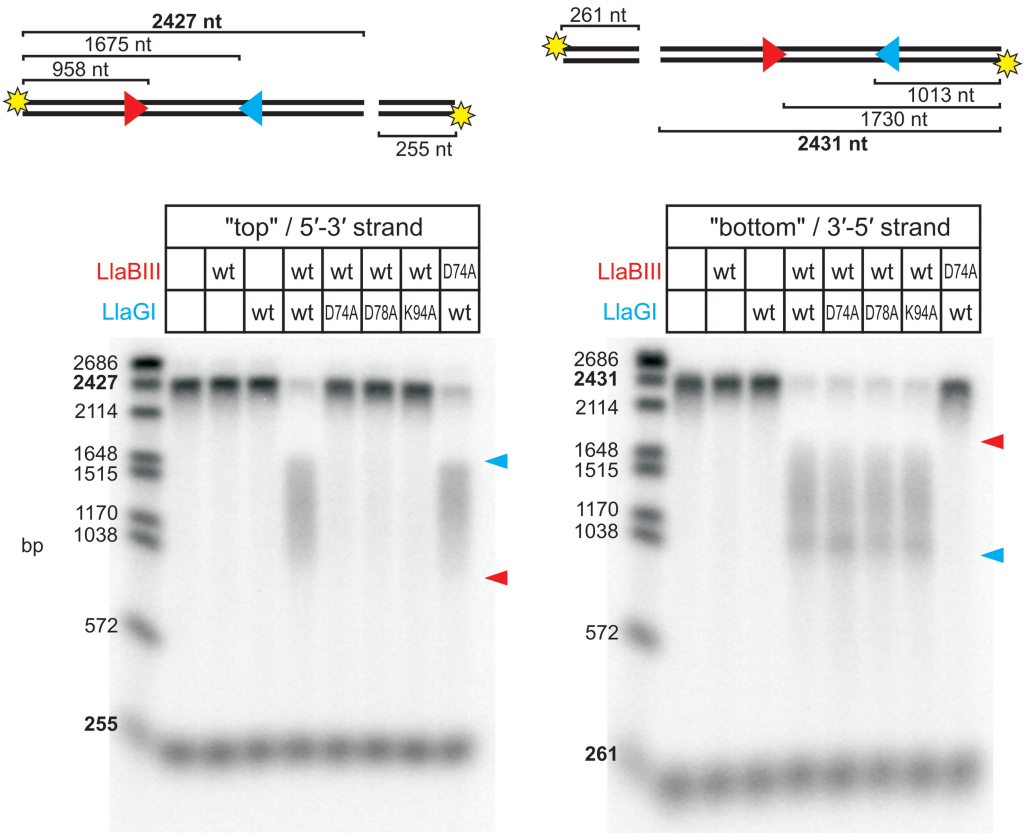

Figure 3. Mapping of the Type ISP cleavage sites to individual strand loci. Cartoons of the linear DNA substrates with sun symbols to indicate the ^{32}P-labelled strands. The smaller 255/261-bp fragments were side products of substrate production. The linear DNA was incubated with the Type ISP proteins as indicated for 2 min, and the substrates/products were separated by alkaline denaturing agarose gel electrophoresis. The definition of the strand orientation is arbitrary and is based on the position of the LlaBIII site.

By comparing the two substrates, we could determine whether DNA nicking was targeted to a particular strand and locus. Because the recognition sites are in an inverted repeat, we arbitrarily defined the strand orientation according to the leftward LlaBIII sequence as drawn (red triangle in Figure 3).

In the presence of one or another wild-type enzyme, we noted that significant levels of DNA cleavage did not occur on either strand. This contrasts with the DNA nicking clearly seen on the plasmid substrate in Figure 2B. This suggests that the DNA nicking is a consequence of either: (i) topology of the circular DNA; or (ii) translocation around the full length of the circular DNA followed by collision with a recognition site or a protein at the site. On linear DNA, the translocating domain would instead exit the DNA track at the free end without meeting another site.

In the presence of both wild-type enzymes, we observed almost complete substrate DNA cleavage and a smear of DNA fragments on both the 'top' and 'bottom' strands. This is consistent with the production of predominantly dsDNA breaks. The distribution of break sites is considered in more detail later, but it was immediately clear that the locations mapped to the DNA between the two sites and was most reminiscent of that seen using LlaBIII with its cognate HtH substrate (3).

In the presence of one wild-type enzyme and one nuclease mutant, strand-specific cleavage was observed consistent with the nicked/gapped DNA observed in Figure 2B. For the LlaGI nuclease mutants, in all three cases, the 'top' strand remained intact while the 'bottom' strand was cleaved, i.e. wild-type LlaBIII must cleave its proximal 3′-5′ strand. When we repeated the experiment using wild-type LlaGI and a LlaBIII nuclease mutant, we observed that the 'bottom' strand remained intact while the 'top' strand was cleaved, i.e. wild-type LlaGI must also cleave *its* proximal 3′-5′ strand. We therefore conclude that on collision with a nuclease mutant, the 3′-5′ strand is cut relative to the wild-type enzyme while the complementary strand remains intact. In all cases, the distribution of 3′-5′ cleavage sites observed was similar to that seen using both wild-type enzymes (see later and Figures 5 and 6).

The results in Figure 3 are not consistent with the domain sharing model (Figure 1C). In such a model, we would have expected the strand specificity to switch with different mutants. Although we cannot rule out a domain swap for other conserved amino acids within the active site, these additional residues were not critical for DNA cleavage activity (8). The results are also inconsistent with the independent nuclease model (Figure 1B). In such a model, we would have expected the appearance of more dsDNA breaks. Instead, we suggest that the Mrr nuclease

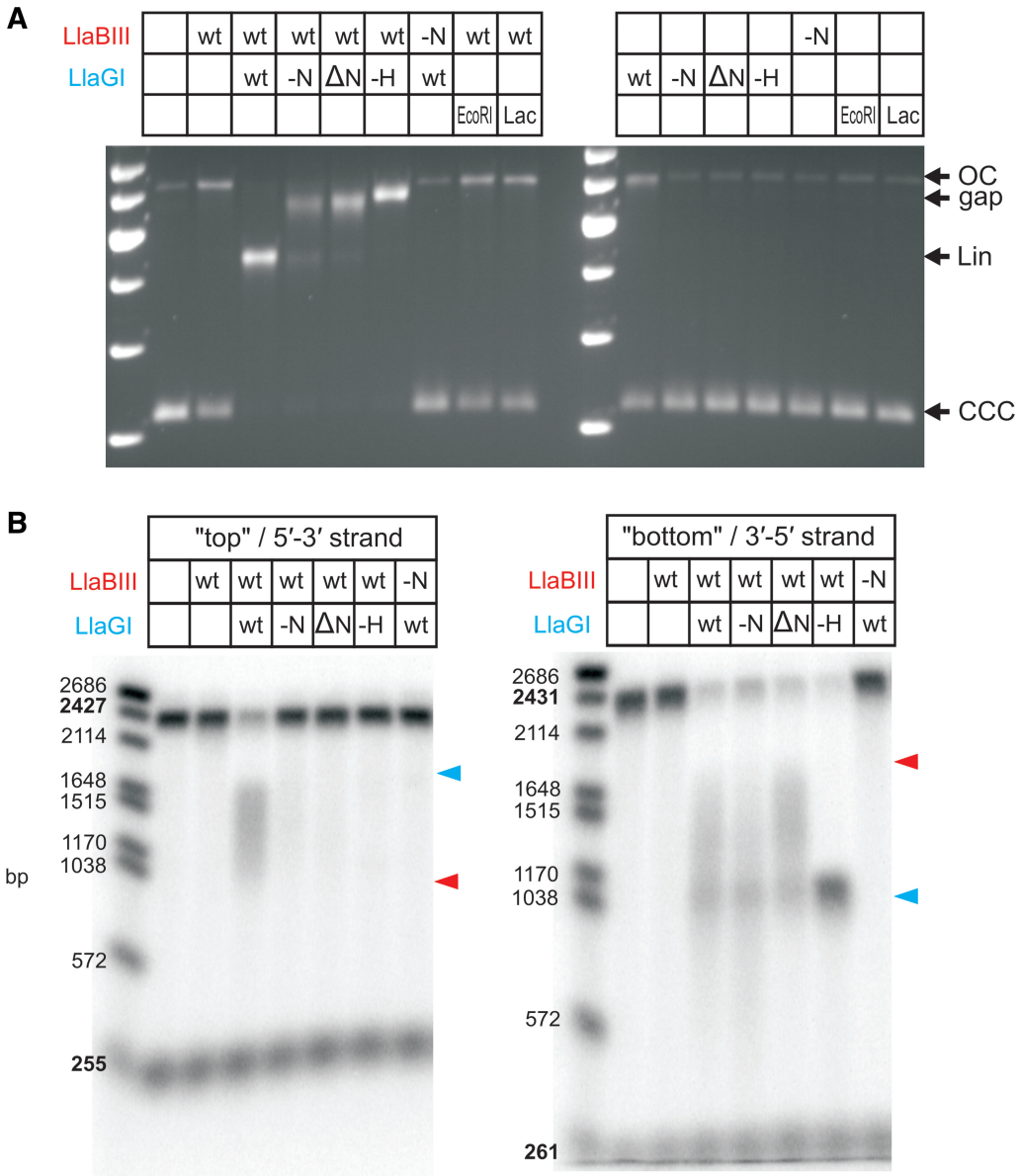

Figure 4. The protein determinants of endonuclease activation within the collision complex. The plasmid pG + B (Figure 2A) or the linear DNA substrates (Figure 3) were incubated with the Type ISP proteins as indicated for 2 min. Substrate/product DNA was separated by electrophoresis on agarose gels (plasmid reactions, (**A**) or alkaline denaturing agarose gels (linear DNA reactions, (**B**). Gels labelled as in Figures 2 and 3.

domains are independent catalytic modules that are initially targeted to a specific DNA strand, i.e. cleavage is most likely to occur as shown in Figure 1A. However, we should also note that further DNA processing can occur with increased incubation times, and this is discussed in the final 'Results' section.

Protein determinants of endonuclease activation within the collision complex

As proposed in the 'Introduction' section, once a collision complex is formed, the activation of the endonuclease activity could be the result of protein–protein interactions and/or mechanical allostery. To further test this, we repeated the single time point nuclease activity experiments on pG + B (Figure 2A) and the two labelled linear DNA (Figure 3), using additional combinations of wild-type and mutant enzymes (Figure 4). Each gel contains control lanes with either a single wild-type enzyme, both wild-type enzymes or a wild-type and nuclease mutant enzyme that give the same qualitative results as Figure 3. The right hand lanes in Figure 4A also show controls where each of the enzyme types are reacted with the DNA in isolation, to show that only a wild-type enzyme produces the single site-specific nicking activity.

Allosteric activation of the nuclease activity in the collision complex might arise from a specific protein–protein contact between the enzymes. The most obvious candidate would be an interaction between the nuclease domains themselves. We therefore tested the outcome of a

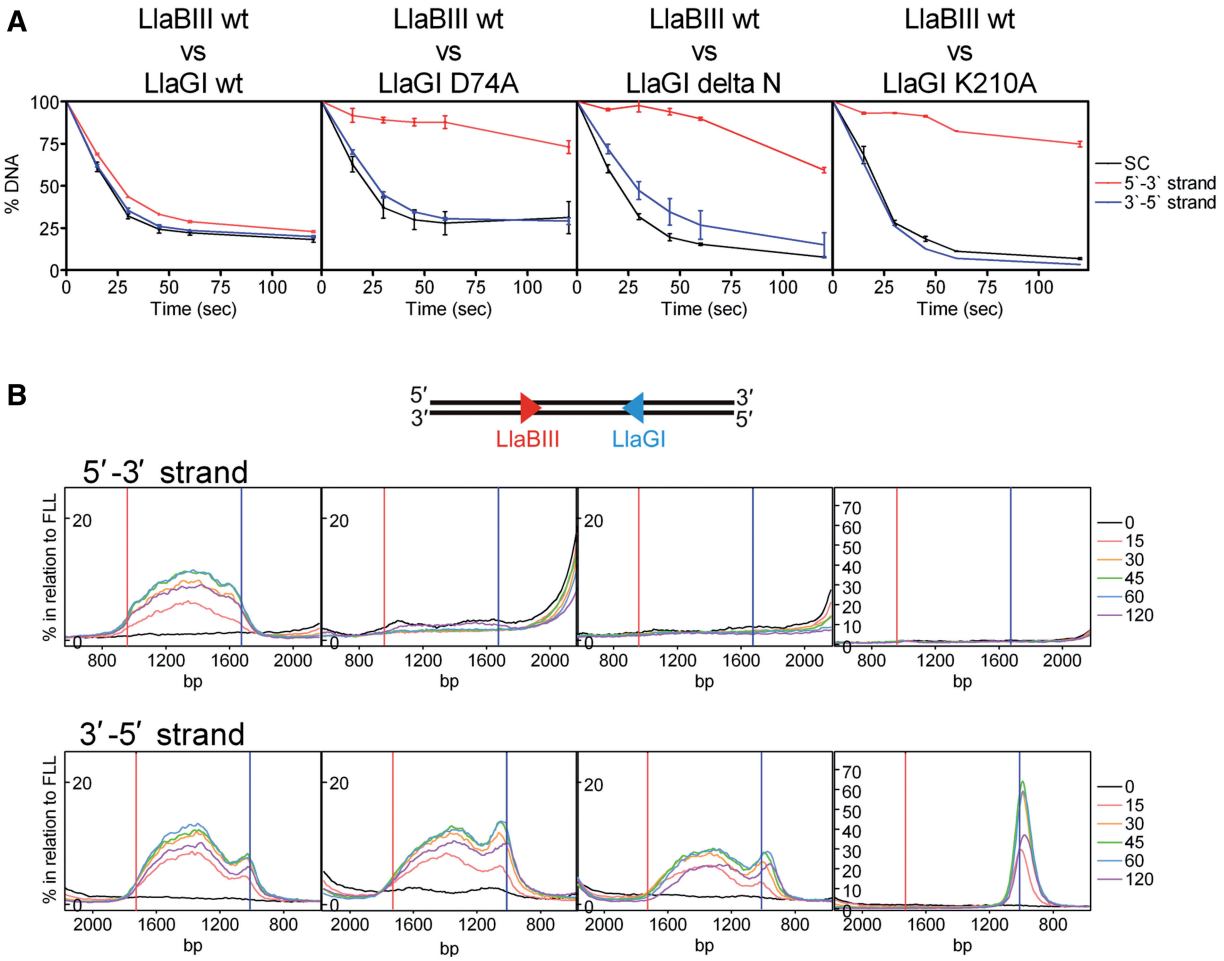

Figure 5. Spatial and temporal analysis of DNA cleavage by LlaBIII and LlaGI. (**A**) Rates of DNA cleavage using plasmid (SC) or each linear DNA were determined using the combinations of LlaGI and LlaBIII indicated ('Materials and Methods' section). Points are averages with error bars as standard deviation for at least two repeat reactions. Points are joined with straight lines to guide the eye. (**B**) For the linear DNA substrates, gel images at each time point were quantified using ImageQuant TL. DNA marker lanes were used to calibrate and correct the pixel positions to DNA lengths. The *y*-axis intensity values were calculated as a percentage relative to the uncut FLL bands.

collision reaction between wild-type LlaBIII and a variant of LlaGI in which the nuclease domain was removed. LlaGIΔN has had the N-terminal 165 amino acids removed, comprising the entire nuclease domain (the truncation being in a putative loop region before the Q-tip motif of the helicase domain) (van Aelst, Sisakova, Saikrishnan and Szczelkun, unpublished data). LlaGIΔN cannot support DNA cleavage of a cognate LlaGI HtH substrate but has wild-type DNA translocation properties. The reaction between LlaBIII and LlaGIΔN resulted in DNA products (gapped and linear) similar to those seen with LlaBIII and the LlaGI nuclease mutants. On the linear DNA, cleavage by LlaBIII was observed exclusively on the 3′-5′ strand with a similar distribution to that seen with the LlaGI nuclease mutants. These data suggest that activation of DNA cleavage does not require the protein–protein interactions between the nuclease domains in the partner enzymes.

Alternative explanations are that either another domain or domains within the monomeric enzyme supplies the protein–protein interaction surface, or that non-specific mechanical strain between the motors is the allosteric activator. It is difficult to remove other domains in the Type ISP enzymes without disrupting the structure completely and preventing either site-specific binding or translocation. To test if the collision complex requires two active motors, we mixed wild-type LlaBIII with a LlaGI mutant in which the Walker A helicase motif is disrupted (K210A). Using the plasmid substrate, the major DNA product was a band with mobility similar to nicked DNA. There was also less linear DNA produced than with the nuclease mutants (see later). Using the linear DNA, only cleavage of the 3′-5′ strand was observed. The distribution was distinct to that seen with the nuclease mutants, with a band characteristic of a single species (or a tightly distributed set of fragments) located close to the LlaGI site. This is consistent with translocation of LlaBIII and subsequent collision with the immobile LlaGI at its site. Therefore, DNA cleavage does not require two active motor complexes.

In the aforementioned reaction, the collision complex has two intact nuclease domains present—one from

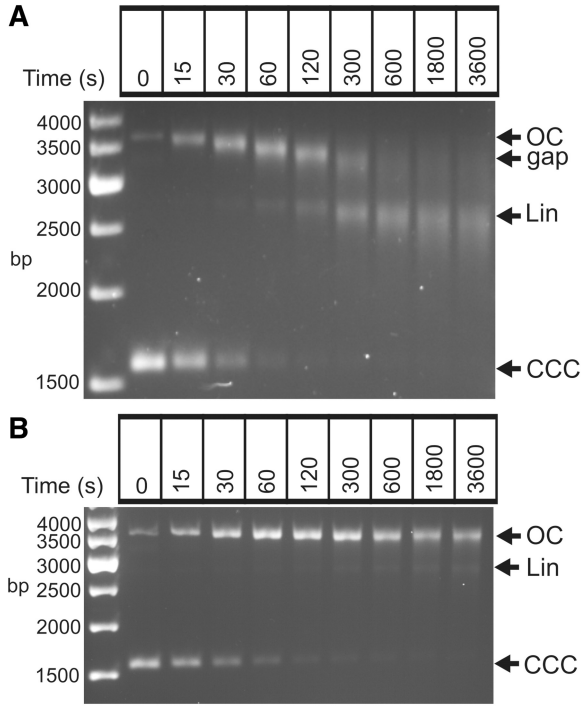

Figure 6. Additional DNA processing by Type ISP RM enzymes requires two active helicase motors in the collision complex. pG + B was incubated with wild-type LlaBIII and either LlaGI(D74A) (**A**) or LlaGI(K210A) (**B**); aliquots were removed at the time points indicated and the substrate/products separated by agarose gel electrophoresis. Gel labelled as in Figure 2.

wild-type LlaBIII and one from the LlaGI helicase mutant—and yet, DNA cleavage occurs on only one strand. Given the strand specificity suggested earlier, this would be consistent with wild-type LlaBIII cutting the 3′-5′ strand and nuclease domain of the helicase mutant remaining inactive. To further test this, we substituted the wild-type LlaBIII for the D74A nuclease mutant. DNA cleavage was not observed on either circular or linear DNA with this combination. This confirms that the DNA nicking seen earlier is exclusively because of wild-type LlaBIII, and that the nuclease domain of the helicase mutant cannot be activated.

To test if any mechanically stable nucleoprotein complex can activate DNA cleavage, we combined wild-type LlaBIII with either the nuclease mutant EcoRI(E111G) or Lac repressor, both of which are characterized as binding tightly to their respective recognition sites (16–18). Binding sites for both proteins are present downstream of the LlaBIII site on pG + B (Figure 1A). If the LlaGI helicase mutant used earlier is acting simply as a static roadblock, we would expect to see similar elevated levels of DNA nicking with EcoRI(E111G) or Lac repressor. However, the amount of nicking observed in the presence of either protein was no more than that seen in the absence of the roadblocks (Figure 4, plasmid gel). Although we cannot rule out that LlaBIII displaces both EcoRI and Lac repressor, the LlaGI helicase mutant can also be considered as a similar non-motor roadblock that should also be displaced. Instead, we suggest our results are most consistent with the necessity for an as yet undefined protein–protein interaction between the helicase-methyltransferase domains.

Spatial and temporal analysis of DNA cleavage by combinations of LlaBIII and LlaGI

To provide further validation of the aforementioned results, we repeated the experiments on the linear and plasmid DNA but sampled the reactions at different time points (Figure 5). We examined wild-type LlaBIII versus: wild-type LlaGI, nuclease mutant LlaGI(D74A), nuclease truncation LlaGIΔN and helicase mutant LlaGI(K210A). The rates of DNA cleavage were assessed by measuring the disappearance of substrate DNA (either plasmid or top/bottom strand linear DNA) (Figure 5A). The distribution of cleavage loci at each time point was assessed using the linear DNA substrates (Figure 5B).

For reactions between the two wild-type enzymes, the rate of DNA cleavage assessed from the disappearance of the plasmid DNA appears, within error, to be the same as for the cleavage of either the 3′-5′ or 5′-3′ strand (Figure 5A). Given that the disappearance of plasmid DNA can be because of the first cleavage event on either strand, one might have expected the plasmid cleavage rate to be twice as fast as for the individual strands. However, if the microscopic cleavage rates are fast relative to other events that lead to the formation of a cleavage competent complex (i.e. the translocation process, formation of the collision complex, etc), then the observed rates can be identical.

The distribution of cleavage species on the 5′–3′ strand for reactions between the two wild-type enzymes is symmetrically distributed between the sites, with a peak at approximately midway (Figure 5B). A similar peaked distribution is also observed on the 3′–5′ strand, but there is also a relatively significant peak of cleavage at the LlaGI site. This suggests that some cleavage events result from LlaBIII translocating the full length of the intervening DNA and colliding with a LlaGI enzyme that has not initiated from its site, resulting in nicking of the 3′-5′ strand by LlaBIII close to the LlaGI site. LlaGI however is not activated.

For reactions between wild-type LlaBIII and the LlaGI nuclease mutant, the rate of cleavage of the 3′–5′ strand by LlaBIII matches the disappearance of the plasmid DNA, suggesting that the kinetics are unaffected by the loss of one nuclease active site. This further reinforces the idea that there is no direct crosstalk between the active sites. There is a slow cleavage of the 5′-3′ strand that gives rise to the small percentage of linear DNA seen in Figures 2B and 4A. The distribution of cleavage on the 3′-5′ strand is qualitatively similar to that seen when both enzymes are wild type. This is to be expected, as the wild-type and nuclease mutant enzymes translocate at the same rate (8). However, there is an increased preference for nicking adjacent to the LlaGI site. This suggests that the LlaGI nuclease mutant fails to initiate more often than its

wild-type parent, resulting in a higher frequency of LlaBIII collisions and nicking events at the LlaGI site.

For reactions between wild-type LlaBIII and LlaGIΔN, the rate profiles and the distributions of cleavage match closely to what was observed with the LlaGI nuclease mutant, previously. This suggests that the nuclease domain does not play a role in collision complex stability, in activation of the partner enzyme in a collision complex or in control of the cleavage rates.

In all of the aforementioned reactions, the rates of DNA cleavage are ~2.5-fold slower than seen for LlaBIII on its cognate HtH plasmid substrate in TMDK but ~15-fold faster than with LlaGI on its cognate plasmid substrate in TMDK (3). Notwithstanding the nicking events observed at the LlaGI sites, the distributions of cleavage are also more similar to those seen with LlaBIII alone on its cognate DNA than with LlaGI alone on its cognate DNA. It therefore appears that the inhibitory effect of TMDK on LlaGI is somewhat alleviated by the presence of LlaBIII in the collision complex. This is consistent with the effect of TMDK on LlaGI occurring after collision. For the purposes of this study, we take these observations as reassurance that the results we obtained are likely to be the same as would be observed for, say, the interaction of two identical LlaBIII enzymes.

For reactions between wild-type LlaBIII and the LlaGI helicase mutant, the rates of cleavage of the 3′-5′ strand matched that seen in all other cases mentioned earlier. These data show that ATP binding/hydrolysis does not actively affect the rate of cleavage of the partner translocated enzyme in a collision complex. However, in contrast to other combinations, the distribution of cleavage mapped exclusively to the LlaGI site at all time points. From this result, and the observation above of collisions at the site resulting in nicking by the translocated enzyme alone, it appears that activation of a nuclease domain requires ATP binding and hydrolysis by the covalently attached helicase.

Additional DNA processing of DNA breaks by Type ISP RM enzymes following extended incubation times

We noted earlier that following the initial DNA cleavage event, further incubation of the Type ISP RM enzyme with the DNA products leads to further DNA processing. We illustrate this here by following DNA cleavage of pG + B for an extended time course using wild-type LlaBIII and either a LlaGI nuclease mutant (Figure 6A) or the LlaGI Walker A helicase mutant (Figure 6B).

With the LlaGI nuclease mutant, the reaction initially resulted in a DNA nick (on the 3′-5′ strand by LlaBIII) (Figure 5). As the time course progresses, this nicked species increases in electrophoretic mobility and becomes more diffuse/smeared. We interpret this as being the steady increase in the size of an ssDNA gap on the 3′-5′ strand. Concurrent with the gap formation, a band appears that corresponds to the FLL DNA resulting from a dsDNA break. This must be produced by wild-type LlaBIII being targeted to the opposite strand, as the LlaGI mutant cannot cut the DNA. The gapped DNA is by ~600 s almost completely converted into linear product. Meanwhile, the FLL DNA becomes more diffuse/smeared, consistent with further DNA processing of the dsDNA break. When the time course was repeated with the LlaGI helicase mutant, far less smearing of the nicked species, if any, was observed, suggesting that DNA gap formation was occurring to a lesser extent. Additionally, less full-length product was observed, which may indicate that a gap needs to be formed to allow cleavage of the opposite strand under these conditions.

We interpret the steady increase in the size of a ssDNA gap seen in Figure 5B as arising by either: (i) a Type ISP exonuclease activity that initiates at the nick site; (ii) by short-range movement of the collision complex and re-cleavage at a site close to the nick; or (iii) by repeated re-initiation of translocation, re-collision and cleavage. Unfortunately, we currently cannot distinguish between these alternatives.

DISCUSSION

DNA cleavage by the Type ISP RM enzymes requires long-range communication between a pair of translocating enzymes, resulting in collisions and cleavage at distant non-specific locations. We can now define that each enzyme in the collision complex is initially targeted to cleave just one strand, the proximal 3′–5′ strand, as illustrated in Figure 1A. Assuming that the helicase domains of the Type ISP enzymes have the same activity as the related HsdR helicase motors of the multi-subunit Type I enzymes (15), the nuclease activity is therefore targeted to the translocated strand of the associated dsDNA translocase motor. As the Type ISP enzymes in the collision complex are related by rotational symmetry, the two ssDNA cleavage events are on opposite strands and therefore could result in a dsDNA break. The Mrr nuclease domains are catalytically distinct and act independently; there is no domain sharing of catalytic residues, and the rate of cleavage of one strand does not appear to influence the other. In fact, the rate and location of cleavage is the same even when the nuclease domain is completely absent from one enzyme in the collision complex.

What our current data cannot address is the exact relative position of the cleavage sites at an individual collision event. Previous analysis of the multi-subunit Type I RM enzymes showed that cleavage produced a diverse set of DNA ends, with many short 5′ or 3′ overhangs (1–7 nucleotides), but also a relatively high proportion of 3′ overhangs of 10–50 nt (even longer overhangs could not be ruled out but were excluded from the analysis) (11). Our data would be most consistent with the production of 3′ overhangs. Earlier studies also suggested that DNA cleavage results in the release of oligonucleotides that are tens of base pairs in length (19), consistent with distantly located break sites. However, complicating the interpretation of the data is the observation of additional DNA processing events that are linked to a continued post-cleavage adenosine triphosphatase (ATPase) activity (20–25). Here, we also observed that following the initial cleavages, further processing of the DNA occurs that appears to be targeted to the broken DNA ends.

The extent of processing appears related to the extent of translocation activity within the enzymes. This may play a role in the cell in ensuring that cleavage of bacteriophage DNA results in catastrophic DNA damage. A similar model of enzyme recruitment from solution has also been suggested for the multi-subunit Type I enzymes (11). Nonetheless, further analysis of the Type ISP break sites is required to fully define the architecture of the collision complex.

Translocation on linear DNA of a single Type ISP enzyme from its site to a DNA end does not result in cleavage of either strand (Figure 3). Therefore, the nuclease activity is neither activated by nor required for the translocation process. Only on collision with a Type ISP enzyme is the nuclease activated; other enzymes and proteins bound in the path do not appear to activate cleavage suggesting that simple stalling or strain is unlikely to be the allosteric activator of nuclease activity. A partner Type ISP enzyme can be either translocating or specifically bound at its site. We interpret our data as showing that either a specific protein–protein interaction is required, or that the collision complex produces a specific DNA structure that is targeted by the nuclease. Although we can rule out protein–protein contacts between the nuclease domains as being important, our current data cannot further refine the nature of this activation step.

We also observed that on collision between a translocating enzyme and an enzyme that either had yet to initiate translocation or could not initiate translocation (Figure 5), only the translocating enzyme cleaved the DNA. We therefore suggest that the Type ISP enzymes are subject to a two-stage control of the nuclease activity: (i) an ATPase-dependent switch that converts the nuclease into a -ready state'; and (ii) a collision switch that occurs when two Type ISP enzymes meet, which engages the 'go state' for domains that are in the ready state. This double check may be important, as the Type ISP nuclease are targeted to non-specific DNA sites. What the cell must avoid is the introduction of dsDNA breaks into its own genomic DNA. This is principally prevented by methylation of the recognition sites (1). However, in the absence of the two-step controls suggested here, it would be possible that the nuclease domains would occasionally associate with random DNA and cause strand breaks. The recruitment of further enzymes may then result in dsDNA breaks. The chance of this can be significantly reduced by requiring an ATPase activity (which requires an unmethylated site) followed by formation a head-on collision complex (which in turn requires two unmethylated sites in HtH repeat). The requirement for collision between two Type ISP enzymes also prevents unwanted DNA cleavage from occurring when a Type ISP enzyme initiates translocation from an unmethylated site following replication, where collisions with non-specific roadblocks will occur with high frequency. One question is why did the Type I enzymes evolve to use ATP hydrolysis when other nuclease do not require this, and the ATP consumption is an additional burden on the cell? The apparent added complexity may be offset by the additional controls on unwanted DNA damage.

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REFERENCES

1. Wilson,G.G. and Murray,N.E. (1991) Restriction and modification systems. *Annu. Rev. Genet.*, **25**, 585–627.
2. Labrie,S.J., Samson,J.E. and Moineau,S. (2010) Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.*, **8**, 317–327.
3. Šišáková,E., van Aelst,K., Diffin,F.M. and Szczelkun,M.D. (2012) The type ISP restriction-modification enzymes LlaBIII and LlaGI use a translocation-collision mechanism to cleave non-specific DNA distant from their recognition sites. *Nucleic Acids Res.*, **41**, 1071–1080.
4. Smith,R.M., Josephsen,J. and Szczelkun,M.D. (2009) The single polypeptide restriction-modification enzyme LlaGI is a self-contained molecular motor that translocates DNA loops. *Nucleic Acids Res.*, **37**, 7219–7230.
5. Janscak,P., MacWilliams,M.P., Sandmeier,U., Nagaraja,V. and Bickle,T.A. (1999) DNA translocation blockage, a general mechanism of cleavage site selection by type I restriction enzymes. *EMBO J.*, **18**, 2638–2647.
6. Dillingham,M.S. and Kowalczykowski,S.C. (2008) RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.*, **72**, 642–671, Table of Contents.
7. Sasnauskas,G., Kostiuk,G., Tamulaitis,G. and Siksnys,V. (2011) Target site cleavage by the monomeric restriction enzyme BcnI requires translocation to a random DNA sequence and a switch in enzyme orientation. *Nucleic Acids Res.*, **39**, 8844–8856.
8. Smith,R.M., Josephsen,J. and Szczelkun,M.D. (2009) An Mrr-family nuclease motif in the single polypeptide restriction-modification enzyme LlaGI. *Nucleic Acids Res.*, **37**, 7231–7238.
9. Hadden,J.M., Déclais,A.C., Phillips,S.E. and Lilley,D.M. (2002) Metal ions bound at the active site of the junction-resolving enzyme T7 endonuclease I. *EMBO J.*, **21**, 3505–3515.
10. Studier,F.W. and Bandyopadhyay,P.K. (1988) Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl Acad. Sci. USA*, **85**, 4677–4681.
11. Jindrova,E., Schmid-Nuoffer,S., Hamburger,F., Janscak,P. and Bickle,T.A. (2005) On the DNA cleavage mechanism of Type I restriction enzymes. *Nucleic Acids Res.*, **33**, 1760–1766.
12. Smith,R.M., Diffin,F.M., Savery,N.J., Josephsen,J. and Szczelkun,M.D. (2009) DNA cleavage and methylation specificity of the single polypeptide restriction-modification enzyme LlaGI. *Nucleic Acids Res.*, **37**, 7206–7218.
13. Madsen,A. and Josephsen,J. (2001) The LlaGI restriction and modification system of Lactococcus lactis W10 consists of only one single polypeptide. *FEMS Microbiol. Lett.*, **200**, 91–96.
14. Vipond,I.B., Baldwin,G.S., Oram,M., Erskine,S.G., Wentzell,L.M., Szczelkun,M.D., Nobbs,J. and Halford,S.E. (1995) A general assay for restriction endonucleases and other DNA-modifying enzymes with plasmid substrates. *Mol. Biotechnol.*, **4**, 259–268.
15. Stanley,L.K., Seidel,R., van der Scheer,C., Dekker,N.H., Szczelkun,M.D. and Dekker,C. (2006) When a helicase is not a helicase: dsDNA tracking by the motor protein EcoR124I. *EMBO J.*, **25**, 2230–2239.

16. King,K., Benkovic,S.J. and Modrich,P. (1989) Glu-111 is required for activation of the DNA cleavage center of EcoRI endonuclease. *J. Biol. Chem.*, **264**, 11807–11815.
17. Wright,D.J., King,K. and Modrich,P. (1989) The negative charge of Glu-111 is required to activate the cleavage center of EcoRI endonuclease. *J. Biol. Chem.*, **264**, 11816–11821.
18. Riggs,A.D., Suzuki,H. and Bourgeois,S. (1970) Lac repressor-operator interaction. I. Equilibrium studies. *J. Mol. Biol.*, **48**, 67–83.
19. Endlich,B. and Linn,S. (1985) The DNA restriction endonuclease of *Escherichia coli* B. II. Further studies of the structure of DNA intermediates and products. *J. Biol. Chem.*, **260**, 5729–5738.
20. Eskin,B. and Linn,S. (1972) The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. *J. Biol. Chem.*, **247**, 6192–6196.
21. Eskin,B. and Linn,S. (1972) The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. II. Purification, subunit structure, and catalytic properties of the restriction endonuclease. *J. Biol. Chem.*, **247**, 6183–6191.
22. Endlich,B. and Linn,S. (1985) The DNA restriction endonuclease of *Escherichia coli* B. I. Studies of the DNA translocation and the ATPase activities. *J. Biol. Chem.*, **260**, 5720–5728.
23. Yuan,R., Heywood,J. and Meselson,M. (1972) ATP hydrolysis by restriction endonuclease from *E. coli* K. *Nat. New Biol.*, **240**, 42–43.
24. Szczelkun,M.D., Dillingham,M.S., Janscak,P., Firman,K. and Halford,S.E. (1996) Repercussions of DNA tracking by the type IC restriction endonuclease EcoR124I on linear, circular and catenated substrates. *EMBO J.*, **15**, 6335–6347.
25. Szczelkun,M.D., Janscak,P., Firman,K. and Halford,S.E. (1997) Selection of non-specific DNA cleavage sites by the type IC restriction endonuclease EcoR124I. *J. Mol. Biol.*, **271**, 112–123.