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Cleavage of a model DNA replication fork by a Type I restriction endonuclease

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ABSTRACT

Cleavage of a DNA replication fork leads to fork restoration by recombination repair. In prokaryote cells carrying restriction–modification systems, fork passage reduces genome methylation by the modification enzyme and exposes the chromosome to attack by the restriction enzyme. Various observations have suggested a relationship between the fork and Type I restriction enzymes, which cleave DNA at a distance from a recognition sequence. Here, we demonstrate that a Type I restriction enzyme preparation cleaves a model replication fork at its branch. The enzyme probably tracks along the DNA from an unmethylated recognition site on the daughter DNA and cuts the fork upon encountering the branch point. Our finding suggests that these restriction–modification systems contribute to genome maintenance through cell death and indicates that DNA replication fork cleavage represents a critical point in genome maintenance to choose between the restoration pathway and the destruction pathway.

INTRODUCTION

When a replication fork meets damaged DNA, it will be stalled. This leads to replication restart by error-prone polymerase-mediated translesion synthesis or by error-free homologous recombination (1–3). Multiple models have been proposed for the latter pathway, most of which envision restart without fork breakage (4–6). However, previous studies have indicated that a stalled fork can be cleaved by DNA structure-specific endonucleases, *Escherichia coli* RuvC and phage T4 endonuclease VII, after Holliday junction formation through fork reversal *in vivo* (7,8). The reversed fork cleavage by RuvC observed in *recBC* mutant is lethal because of the absence of recombinational repair (3). In contrast, phage T4 endonuclease VII is proposed to be involved in fork reactivation (9). Several lines of evidence support the idea that Mus81-Eme1/Mms4 endonuclease in eukaryotes can cleave a stalled replication fork (10,11), but contribution of the fork cleavage to fork restart is yet to be elucidated (11). Archaeal Hef that cleaves several branched forms of DNA *in vitro* was proposed to be involved in cutting off the arm containing a newly synthesized leading strand from a stalled fork (12).

A replication dependent DNA breakage causes lethality in the absence of RecBCD recombination pathway for repair (13). In mammalian cells, cell death coupled with fork breakage is assumed to occur when a replication fork stalls or collapses by a DNA damage (14). However, it remains unclear how much contribution the stalled fork cleavage has to the cell death.

Movement of a replication fork changes the epigenetic status of the chromosome, for example, *via* DNA methylation. Loss of DNA methylation may occur through replication recovery after fork collapse as previously proposed: processing of a double-strand DNA end with RecBCD enzyme, homologous pairing and D-loop formation with RecA, and establishment of a new replication fork with a primosome (15). Action of restriction–modification systems can be directly associated with this type of epigenetic change. Restriction endonucleases (REases) recognize a specific DNA sequence and cleave DNA when the sequence is not methylated by the cognate methyltransferase (16). They would cleave incoming or foreign DNA lacking proper methylation, but sometimes they attack their host bacterial chromosome, sensing its lack of methylation. For example, several Type II REases cleave chromosomal DNA and lead to cell death after the restriction–modification gene complex is lost from the cell, which contributes to maintenance of the restriction–modification gene complex in the population of viable cells (17–19). A Type I REase EcoKI can cleave bacterial chromosomes when unmethylated recognition sequences are generated through base substitution mutations by 2-aminopurine (2-AP) (20). A replication fork passage through a full-methylated recognition site would generate a hemi-methylated site, and one through a

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hemi-methylated site would generate a hemi-methylated site and an unmethylated site, which is a target for restriction cleavage. Generation of an unmethylated site should be generally rare, but it can occur in certain mutants with replication fork crowding, as described below. Therefore, it is possible that some relationship exists between restriction cleavage and replication fork passage.

Another relationship between Type I restriction and DNA replication is suggested by the restriction alleviation phenomena: the phenotypic decrease of restriction activity on invading DNA, it is induced by several DNA damaging agents or is constitutively seen in some mutants. The underlying mechanism varies among the enzyme subtypes. For EcoKI (Type IA), proteolytic cleavage of the R (restriction) subunit represses the restriction activity (21,22). For EcoR124I (Type IC), R subunit degradation is not involved (23), and subunit assembly control has been proposed to be responsible (24). For the Type IA, restriction alleviation is induced under conditions of recovery from DNA damage through generation of a DNA replication fork, whose movement should generate unmethylated DNA (22,25). In addition, some mutants showing constitutive restriction alleviation [*dam*, *topA, rnhA* and *recG for* EcoKI (Type IA); *rnhA*, *recG*, for EcoR124I (Type IC)] are also those with a potentially increased number of replication forks from abnormal replication initiation or recovery (22,26,27). Therefore, restriction alleviation has been hypothesized to be a mechanism for protection of chromosomes from restriction at a newly generated replication fork, which produces unmethylated sites, though the underlying mechanism is different for each subtype (28). Indeed, the above chromosome breakage by Type IA and Type IC enzymes, accompanied by cell death, is observed in the absence of restriction alleviation (20,23,27).

Coupling of replication and Type I restriction by EcoKI is observed in the bacteriophage T7 genome during its translocation into a cell (29). Such coupling is also suggested by the decrease of restriction by homologous recombination when only a single genome of bacteriophage lambda enters a cell (30). Recombination requires two copies of homologous DNA, therefore this result implies that at least two copies of phage DNA were formed and underwent restriction cleavage followed by recombinational repair. Furthermore, direct interaction between the M subunit of EcoKI, a Type IA restriction enzyme and DnaB, a central component of DNA replication machinery, was reported in a large-scale protein–protein interaction analysis in *E. coli* (31).

In addition to these *in vivo* phenomena that suggest the coupling of replication and Type I restriction, properties of Type I REases revealed *in vitro* raise the possibility that replication forks might serve as their direct targets. Among the three subunits (HsdS, HsdM and HsdR) of a Type I restriction–modification enzyme, HsdS plays the role of specific DNA sequence recognition. The complex of M and S subunit exhibits methyltransferase activity at the recognition site. Joining of HsdR to the complex is essential for the endonuclease activity. After binding to an unmodified recognition sequence, the restriction enzyme complex translocates DNA towards itself from both directions in a reaction coupled to ATP hydrolysis, followed by DNA cutting that is triggered when two REase complexes collide (32). Recently, it was reported that the cleavage can be stimulated by DNA structures such as Holliday junction, single-strand gaps and nicks (33,34). We hypothesize that the translocating enzyme might cleave DNA upon encountering a replication fork structure.

In the present work, we demonstrate that a Type I REase, EcoR124I, indeed cleaves a model DNA replication fork at the branch point *in vitro*. Our finding of the replication fork as a potential direct target of Type I REases is discussed in relation to the biology of restriction–modification and to the interaction of DNA replication, repair, recombination and cell death in genome integrity.

MATERIALS AND METHODS

Plasmids and bacterial strains

Escherichia coli K12 strain JM109 {*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) [F' traD36 proAB*$^+$ *lacI*^{q}*ZΔM15]*} was a gift from Dr Akio Nomoto (University of Tokyo). JM109 (DE3) was constructed using a λDE3 Lysogenization Kit (Novagen) following the manufacturer's protocol. A plasmid for expression of the EcoR124I R subunit, pACR124, and one for its M and S subunit, pJS4M, were kindly provided by Dr Piero Bianco (University of Buffalo). pEU3-NII was purchased from TOYOBO co., Ltd.

To construct pGap1 and pES1, pEU3-NII was digested with EcoRV and BamHI followed by ligation with a double-strand oligo, DNA Gap1, prepared by annealing two single-strand oligos, gap1 and gap1-C, or ligated to Gap1-cis, made from oligos gap1-cis and gap1-cis-C (Supplementary Table S1).

The pGap4, a version of pGap1 lacking the EcoR124I site, was prepared as follows: pGap1 linearized with PvuII and a region of pGap1 prepared by PCR with primers fr.g1B-F and fr.g1B-R were ligated. The resulting plasmid was cleaved with BamHI and XhoI and ligated with another pGap1 fragment, prepared by PCR with primers fr.g1A-F and fr.g1A-R, to generate pGap4.

The pES2, a version of pES1 lacking the EcoR124I site, was constructed by changing the region of pES1 containing the recognition site to the corresponding region of pGap4 lacking the site, by ligating fragments prepared by BamHI and PvuII digestion of pES1 and pGap4.

To construct pGap1R and pES1R, both with an inverted EcoR124I site, pGap4 or pES2 was cut with XhoI and KpnI. The resulting larger fragment was ligated with a double-strand oligo DNA including an EcoR124I site, R124I (XK), prepared by annealing two complementary single-strand oligo DNAs, r124I (XK) and r124I (XK)-C.

The pES1.20, a plasmid with a deletion between the gap region and EcoR124I site, was constructed by PCR with pES1 and primers and self-ligation of the resulting fragment. Sequences of each of these oligonucleotides are

available as Supplementary Material (Supplementary Table S1).

Enzyme purification

The protocol was modified from published one (35). To express the proteins, 6 l of LB medium, with ampicillin (250 μg/ml) and chloramphenicol (25 μg/ml), was inoculated with a 1:100 dilution of an overnight culture of JM109 (DE3) harboring pACR124 and pJS4M, and incubated at 37°C with vigorous shaking. Induction occurred by adding IPTG to a final concentration of 1 mM when the OD_{600} was 0.4, and the culture was grown for an additional 5 h. The cells were harvested by centrifugation (10 000 r.p.m. for 5 min), and the cell pellet was resuspended in a buffer [50 mM Tris–HCl (pH 7.5) and 25% sucrose] and frozen at −80°C. Thawed cells were lysed as previously described (36). Polynucleotide materials in the lysate were precipitated by streptomycin sulfate [final concentration, 2.4% (w/v)] and removed by centrifugation (34 000 r.p.m. for 90 min) together with the other insoluble materials, and then the proteins in the supernatant were precipitated by addition of ammonium sulfate to 70% saturation. The pellet formed after centrifugation (34 000 r.p.m. for 30 min) was resuspended in buffer B-100 [20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 100 mM NaCl] and dialyzed overnight against the same buffer. This was subjected to Q-sepharose FF and Heparin FF column chromatography (GE Healthcare, Co., Ltd.) as previously reported (35).

This procedure yielded 0.95 mg (6.3 μg/μl, 150 μl). From the intensity of CBB-stained bands in the SDS–PAGE photograph (Supplementary Figure S1), the subunit stoichiometry was estimated as R : M : S = 1 : 2 : 1 as in several previous reports (35,37,38). The purity of the enzyme was estimated to be 91%. The concentration of the protein was analyzed by Lowry assay with a protein assay™ kit (BioRad), using BSA as a standard as described in the manufacturer's manual.

Long-branched DNA

The substrates for the cleavage assay were prepared by annealing two DNA fragments with complementary single-strand regions (Figure 1). The fragments with a gap region were prepared as reported previously with some modifications (34).

For the upper arm fragment arm1tE (Figure 1A and B, right), a region between the primer pair on pGap1 was amplified by PCR with KOD-plus™ polymerase (TOYOBO) and primers, arm1tE-F and EcoT22I-R, listed in supplemental data (Supplementary Table S1). The arm1tE-F had been labeled with ^{32}P at its 5′-end. The amplified fragment was purified with MagExtracter™ (TOYOBO) and subjected to nicking reaction with Nt. BbvCI (New England Biolabs) (Figure 1B, right). Tris–HCl (pH 8.0) and oligo DNA gap1, with sequence complementary to the single-strand DNA on the gap region to be eliminated, were added at a final concentration of 330 mM and 3.3 nM, respectively. This was followed by incubation at 78°C for 10 min and then 37°C for 10 min. The resulting solution was subjected to agarose gel electrophoresis, followed by product isolation and purification. From 100 μl of the PCR reaction, about 4.8 μg of the arm1tE fragments were prepared.

For the bottom arm fragment base1bSP (Figure 1A and B, left), pES1, instead of a PCR fragment, was used as the starting material. The procedure is the same as described above, except for use of nicking enzyme Nb. BbvCI, rather than Nt. BbvCI, SphI and PvuII (New England Biolabs). An oligonucleotide, gap1-cis-C was used to remove single-strand DNA produced by the nicking reaction (Figure 1A and B, left). From 16 μg of the pES1 plasmid DNA, about 2.4 μg of the base1bSP fragments were prepared.

Then, the arm and the base were annealed in the appropriate combination listed in Supplementary Table S2. Equimolar amounts of the two arms were mixed and incubated at 75°C for 10 min, then the switch of the incubator turned off, when the temperature reaches 37°C, that temperature was maintained for 10 min. About 65–95% of the parts were annealed. The resulting solution was directly subjected to each assay.

Cleavage assay

The reaction was performed in 1.3–1.9 nM of the substrates (estimated from the annealing efficiency), 50 mM Tris–HCl (pH 7.5), 2 mM ATP, 10 mM $MgCl_2$, at 37°C, for the indicated time, with or without 120 nM EcoR124I. The total reaction volume was 15 μl. This condition is identical to that used previously except for the $MgCl_2$ concentration (33). Under the same condition, 5.3 nM of a circular DNA with a single EcoR124I site was completely cleaved (data not shown). The molecular concentration was calculated from the analyzed mass concentration described above and the theoretical molecular weight of the complex ($R_1M_2S_1$), 2.82×10^5 g/mol. The reaction was stopped by adding SDS to 0.1% and by placing the tube in ice water. The resulting solutions were subjected to 1% agarose gel electrophoresis, followed by drying of the gel and ^{32}P-signal detection by an imaging analyzer, FLA5100 (Fuji-Film, Japan).

The contrast of presented figures was adjusted as follows. Raw data were imported to Photoshop CS version 8.0 (Adobe), and the image mode was changed to grayscale, then the setting of adjustment curves were changed with an end point coordinate (input, output) = (77, 100) in a linear relationship with the other end point (0, 0) for Figures 2, 3, 4A and 4C; (90, 100), (0, 0) for Figures 4B and 5. These adjustments were independent of the quantification results, those were analyzed by a software Image Gauge version 4.22 (Fuji-Film), and the graphs were prepared by the GraphPad PRISM version 4.03 (GraphPad softwear).

Determination of the cleavage position

The EcoR124I cleavage product from eF12 was subjected to a primer extension experiment. The cleavage reaction was performed as described above followed by 1% agarose gel electrophoresis. The cleaved product (corresponding to the dense band in lane 4 in Figure 3A) was isolated from the gel. A primer DNA (ForkD42 in Supplementary Table S1) illustrated in Figure 6 was

extended with KOD-plus polymerase (TOYOBO) with the recovered DNA as the template. The extension products and dideoxy-sequencing reaction products were subjected to 6% polyacrylamide/8 M urea gel electrophoresis followed by signal detection with an imaging analyzer, FLA5100 (Fuji-Film). The contrast of the presented Figure 6 was adjusted by the photoshop as described above with the following setting (70, 100), (25, 0) in a linear relationship.

Preparation of a hemi-methylated fork

Hemi-methylated DNA was prepared by PCR with a primer including N6-methyl-dA and another primer (Supplementary Table S1, [N6MdA] T22-R124I-R and SphI-F), followed by the gap generation process described above. The methylated oligonucleotide was purchased from Hokkaido System Science Co., Ltd, Japan, who purchased N6-methyl-dA phosphoramidite, the precursor of the methylated residue, from GLEN Research Co., USA.

RESULTS

Long-branched DNA

Short-branched DNAs made of oligonucleotides are widely used in the analysis of biochemical reactions involving the DNA replication fork. However, in order to examine whether Type I REase EcoR124I cleaves a branched DNA imitating a DNA replication fork, we prepared branched DNAs with arms of several hundred base pairs (Figure 1C). We thought it essential to use such long-branched DNAs in consideration of the following properties of Type I REases: (i) their nucleolytic reaction is coupled with their translocation along DNA (39); (ii) when the EcoR124I REase complex binds to DNA, it occupies at least 18 bp including the recognition site (40), which suggests the necessity of a scaffold DNA longer than 20 bp for translocation; and (iii) they translocate DNA forming DNA loops (41,42).

For preparation of the long-branched DNAs we devised a method employing nicking endonucleases, which can introduce a single-strand break in a duplex DNA (Figure 1A and B). They can expose a single-strand region duplex DNA after dissociation of a single-strand DNA. These single-strand regions can serve in annealing of two DNA parts to form the branched DNA (Materials and methods section). When one of the primers carries a ^{32}P label at its 5′-end, the resulting branched DNA carries the label in the stem (open circle in Figure 1).

Our branched DNAs have 304 bp between the recognition sequence [5′CGATGCTGTATTC, drawn as a leftward arrow, residues with an underline indicate specific recognition sequence (43)] and the branch point (Figure 1). We chose this distance because, in the cases of linear DNAs with two recognition sites and circular DNAs with two recognition sites, the enzyme can cleave DNA approximately 300 bp from the recognition sequence (44).

EcoR124I-mediated cleavage

A long-branched DNA with an EcoR124I recognition sequence on each arm (eF1) and with a ^{32}P label (Figure 2, left top) was incubated with purified EcoR124I under standard conditions for restriction digestion (see Materials and methods section). EcoR124I we used was purified in the $R_1M_2S_1$ form (37,38) as described in Materials and methods section. The reaction mixture contained ATP for translocation but no S-adenosylmethionine (SAM) for methylation; EcoR124I does not require SAM addition for DNA cleavage (37). The resulting solution was subjected to agarose gel electrophoresis followed by detection of the radioisotope by imaging (Figure 2). We found two major products. From their size and ^{32}P label, the larger species was likely produced by losing the upper arm from the substrate, while the smaller one was produced by losing the lower arm. How we assigned structure to these bands is detailed in a Supplementary description and Supplementary Figure S2.

Time course results indicated that they were the final products. In other words, we did not detect any signal corresponding to cleavage of both arms (drawn in the parentheses in Figure 2). This suggests that the cleavage at the branch point occurred only once on one of the two arms. There is a third, smaller band visible on the gel image (Figure 2) and this is discussed later.

Cleavage at an arm with a recognition sequence

To examine the dependence of cleavage on the recognition sequence, we prepared long-branched substrates with a single recognition sequence on the lower arm (eF12) or on the upper arm (eF13) or no recognition sequence (eF14) (Figure 3A). EcoR124I cleavage was observed only when the substrates have the recognition sequence(s), indicating the cleavage is dependent on EcoR124I sequence specificity. When the substrate with a single recognition sequence in the lower arm (eF12) was cleaved, only the product that has lost the lower arm was detected. Likewise, with the substrate carrying a single recognition sequence on the top arm, only the product that has lost the top arm was detected. In other words, the enzyme cleaves the arm carrying the recognition sequence but does not cleave the other arm lacking the recognition sequence (Figure 3A).

The recognition sequence of EcoR124I is not palindromic but has a direction: 5′CGAYNNNNNNTTC (43) drawn in Figure 3A as a leftward arrow, therefore we next tested if the orientation of the recognition sequence affects the cleavage reaction. We prepared derivatives of the above long-branched DNAs with the recognition sequence in an inverted orientation (Figure 3B, top). The cleavage results were indistinguishable from the previous results (Figure 3B, bottom). We concluded that the orientation of the recognition sequence does not affect the cleavage. This is consistent with the reported bidirectional DNA translocation of Type I restriction enzymes from a recognition sequence (32,45).

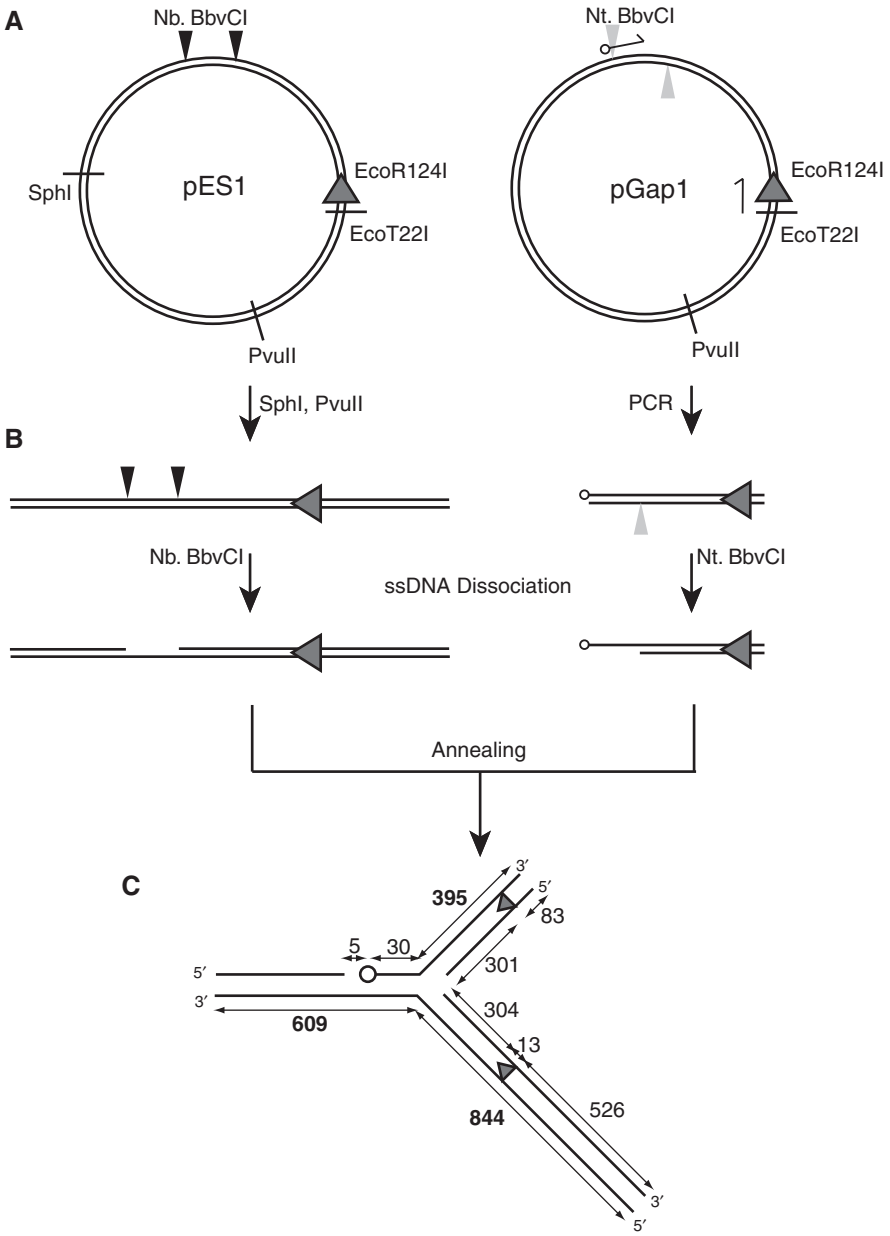


Figure 1. Preparation of a long branched DNA (eF1). (**A**) pES1 was treated with SphI, PvuII and nicking endonuclease Nb.BbvCI. A partial fragment from pGap1 was prepared by PCR with indicated primers, followed by nicking with Nt.BbvCI. (**B**) Single strands between the nicks introduced in the previous step were dissociated by heating with removal by complementary oligo DNA from the fragment containing a gap structure. (**C**) Resulting DNAs with the gap were annealed to form a branched structure (Materials and methods section). A leftward triangle indicates an EcoR124I site, 5′CGATGCTGTATTC. An open circle indicates ^{32}P for 5′-end labeling.

Dependence on the fork structure

To further reveal the preference of EcoR124I for replication fork as a potential physiological substrate, various structurally modified long-branched DNAs were prepared and examined (Figure 4).

We first changed the length of the arm on the opposite side from the arm containing the recognition sequence (Figure 4A). A substrate with a shorter arm of 30 bp (eF125) gave discrete cleavage signal but with slightly decreased intensity compared to the case of eF12 that has a 395-bp long opposite arm. This suggests that EcoR124I prefers a long arm on the opposite side (Figure 4A, lane 2 and 4). Furthermore, the substrate without the branch but carrying a nick (eF126) gave only a very faint signal of the cleavage product. This may correspond to the cleavage at a nick or gap reported previously (34) (Figure 4A, lane 6). These results suggest that EcoR124I has structure preference to branched DNA stronger than nicks or gaps, and that the enzyme prefers branched DNAs with long arms.

We then changed the DNA length on both sides of the recognition sequence (Figure 4B). When the recognition sequence is only 20 bp away from the branch point, no

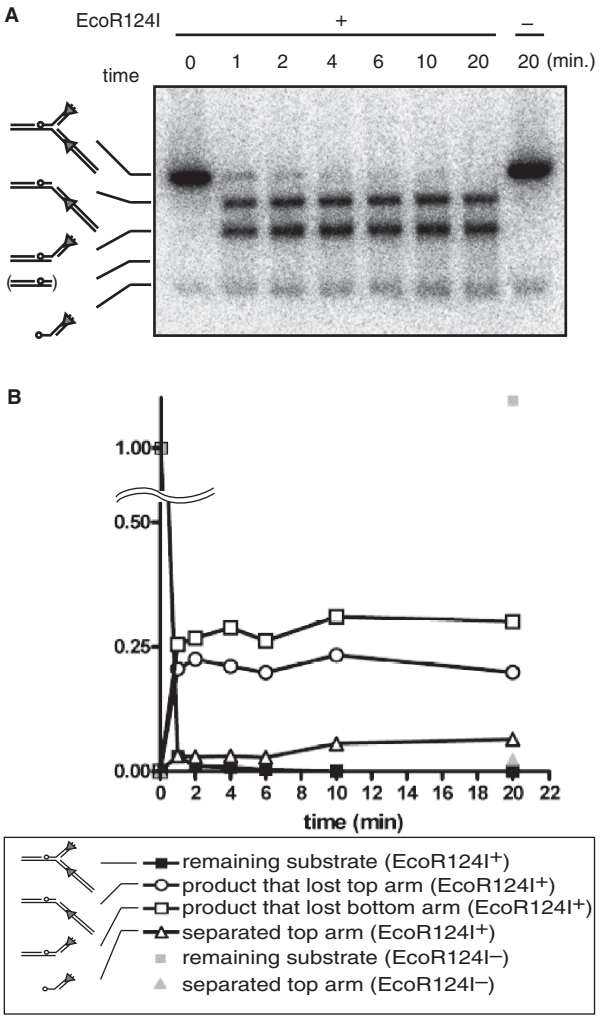


Figure 2. EcoR124I cleaves the long branched DNA. (**A**) Time-course of cleavage. (**B**) Quantification. The fork substrate eF1 was cleaved with EcoR124I, leaving linear DNA products that lost the top or bottom arm. ^{32}P-labeled eF1 DNA was incubated with EcoR124I at 37°C for indicated time. The resulting solution was subjected to 1% agarose gel electrophoresis, followed by ^{32}P signal detection by an imaging analyzer. The graph shows intensity of each band relative to that of the starting substrate (average from two experiments). A leftward triangle indicates an EcoR124I site, while an open circle indicates ^{32}P for 5′-end labeling.

detectable cleavage was observed (Figure 4B, lane 6 and 8). This indicates that the cleavage requires a recognition sequence distant from the branch point, possibly because of a requirement for the translocation for the cleavage reaction (39). Shortening the length of the branch distal to the recognition sequence to 83 bp did not affect the cleavage (Figure 4B, lane 2 and 4). This suggests that translocations in the two opposite directions are not required to be the same distance for successful cleavage.

Cleavage at an immobile branch point

In these substrates, the two arms have identical sequences around the branch point. This identity might allow branch migration to form a Holliday structure, which is known to be a substrate of RuvC of *E. coli* (46,47) and a Type I REase (33). There is a possibility that the real substrate of our cleavage reaction is a Holliday structure generated by branch migration of the fork.

In order to test this possibility, we prepared an *immobile* DNA fork in which the upper arm and the lower arm carry different sequences (eF127 in Figure 4C) and cannot experience branch migration to form a Holliday structure. As shown in Figure 4C, this substrate was indistinguishable from the DNA with identical arm sequence (eF125) in the cleavage reaction. We concluded that the cleavage reaction on a DNA fork is distinct from that observed on Holliday structures.

Product of DNA unwinding

In our reaction, we noticed a third, minor product, which is represented by the lowest band in Figure 2. This product is identical to one of the two parts used for preparation of the branched DNA in size (Figure 1B, right bottom). These unreacted molecules are present before the cleavage reaction, as seen at time zero or for incubation without the enzyme (Figure 2). However, this species increases during incubation with the enzyme (Figure 2). More of this species is produced when the upper arm carries the recognition sequence (eF13 in Figure 3A, eF13R in Figure 3B). Therefore, this species is likely to be produced by the EcoR124I reaction on the branched DNA.

Unwinding of the 30-bp long duplex DNA in the stem is necessary for generation of this species although there has been no report of such helicase-like activity for Type I restriction enzymes to date (34). This unwinding activity was observed by using substrates with a recognition site on the top arm. The features of the unwinding activity will be reported in a future publication.

Quantification of the results of Figure 3 suggests an inter-arm communication for the cleavage. Cleavage of the top arm of substrates with a single recognition site on the top arm (Figure 3, eF13 and eF13R) was decreased from that of substrates with two recognition sites (Figure 3, eF1 and eF1R). Instead, generation of the unwound products was increased to the same extent. These results suggest that cleavage of the top arm is, at least partially, dependent on the recognition site on the bottom arm through some inter-arm interaction dependent on the recognition sequence and that the unwinding reaction is enhanced by loss of the inter-arm interaction. We do not know yet whether molecular nature of the inter-arm interaction is like a collision of an REase molecule on the top arm with another REase molecule from the bottom arm. Such a change in the product population was not observed in substrates with a single site on the bottom arm (Figure 3, eF12 and eF12R).

Inhibition by recognition site methylation

Methylation of the recognition sequence by the modification activity of Type I enzymes protects DNA from the cognate restriction cleavage (16). Methyltransferase of EcoR124I is suggested to generate the following methylation pattern (48):

5′CG $_{m6}$AYNNNNNN TTC3′
3′GC TRNNNNNN $_{m6}$AAG5′

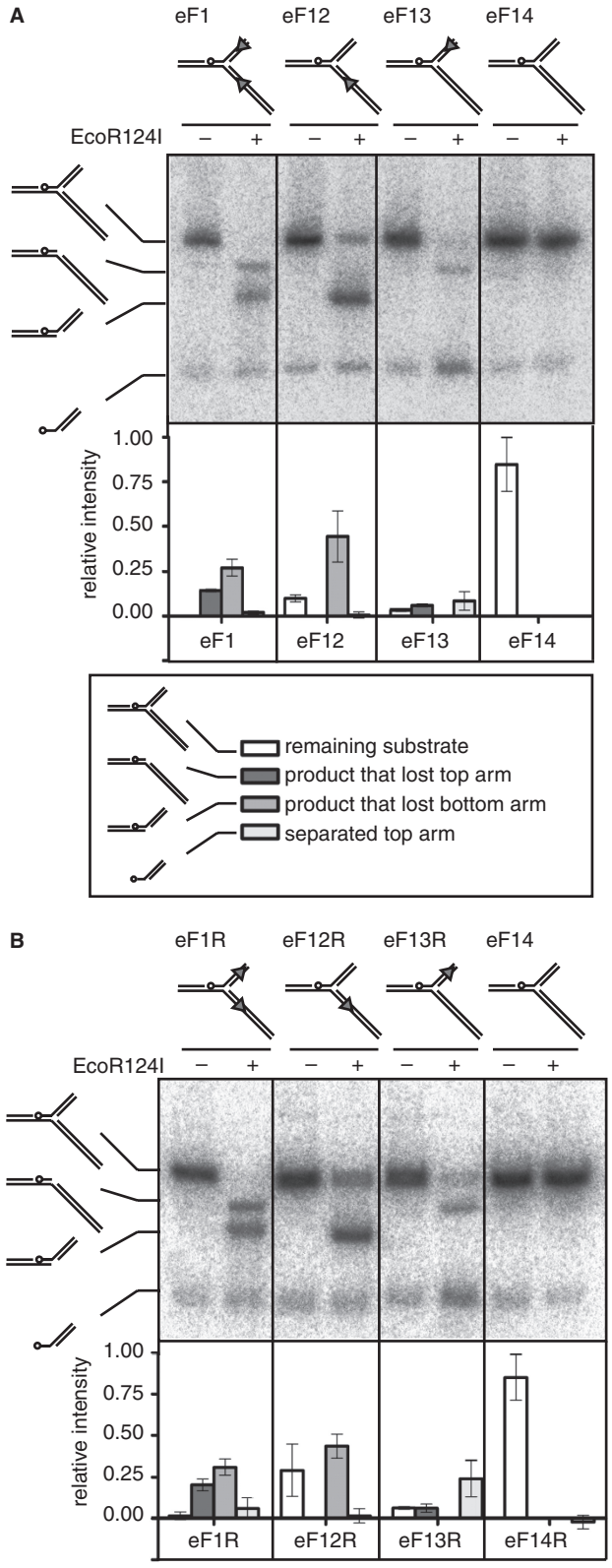


Figure 3. Dependence on the recognition sequence. (**A**) One orientation. (**B**) The other orientation. ^{32}P-labeled long branched DNAs were incubated with or without EcoR124I at 37°C for 10 min. After gel electrophoresis, the ^{32}P signal was detected. From the density of each band, the density of the corresponding region from the reaction without the enzyme was subtracted and divided by the intensity of the starting substrate (long branched DNA). The data are represented as mean ± SD from three measurements. A triangle indicates an EcoR124I site, while an open circle indicates ^{32}P for 5′-end labeling.

although no experiment has been performed to measure the effect of methylation on EcoR124I restriction cleavage. Replication of a fully methylated sequence generates two hemi-methylated daughter duplex DNAs. Replication of a hemi-methylated sequence will generate a hemi-methylated duplex DNA and an unmethylated duplex DNA. A hemi-methylated site will be methylated on the opposite strand by the enzyme to form a fully methylated sequence (48).

To test the effect of methylation on the cleavage activity, we prepared two long-branched DNA substrates with a hemi-methylated recognition site on the lower arm (MeF5 and MeF52 in Figure 5) starting from a synthetic methylated DNA (see Materials and methods section).

5′CGATGCTGTA TTC3′
3′GCTACGACAT $_{m6}$AAG5′

The bottom arm cleavage was severely inhibited by the hemi-methylation; for eF5 and MeF5, 0.21 ± 0.01 to 0.086 ± 0.004 for eF5 and MeF52, 0.67 ± 0.06 to 0.080 ± 0.006 in the relative intensity of three experiments (Figure 5). The cleavage of the methylated substrate was further decreased by addition of SAM (for MeF5, to 0.043 ± 0.002; for MeF52, to 0.045 ± 0.005 in three experiments, data not shown)

Mapping of the cleavage sites

In order to determine the precise position of the cleavage, we performed primer extension on the cleavage products (Figure 6). We detected strong signals corresponding to 5–8 bp from the branch point to the recognition sequence proximal side (Figure 6). To our knowledge, this is the first report of the precise position of the Type I restriction cleavage at a barrier structure on DNA.

DISCUSSION

Replication fork cleavage by a Type I restriction endonuclease

We discovered that a Type I REase, EcoR124I, cleaves DNAs with long branches that mimic a DNA replication fork structure. This cleavage requires the branched structure (Figure 4A) and a single unmethylated recognition site on one of the arms (Figures 3A and 5). Cleavage occurs at the arm carrying the recognition site (Figures 3A and 6). The cleavage requires some distance between the recognition sequence and the branch point but is independent of the orientation of the recognition sequence (Figures 3 and 4B).

These observations combined with the known properties of this and the other Type I restriction enzymes led us to propose the reaction model illustrated in Figure 7A. The enzyme binds to DNA at the recognition sequence and starts tracking along the DNA. When it encounters

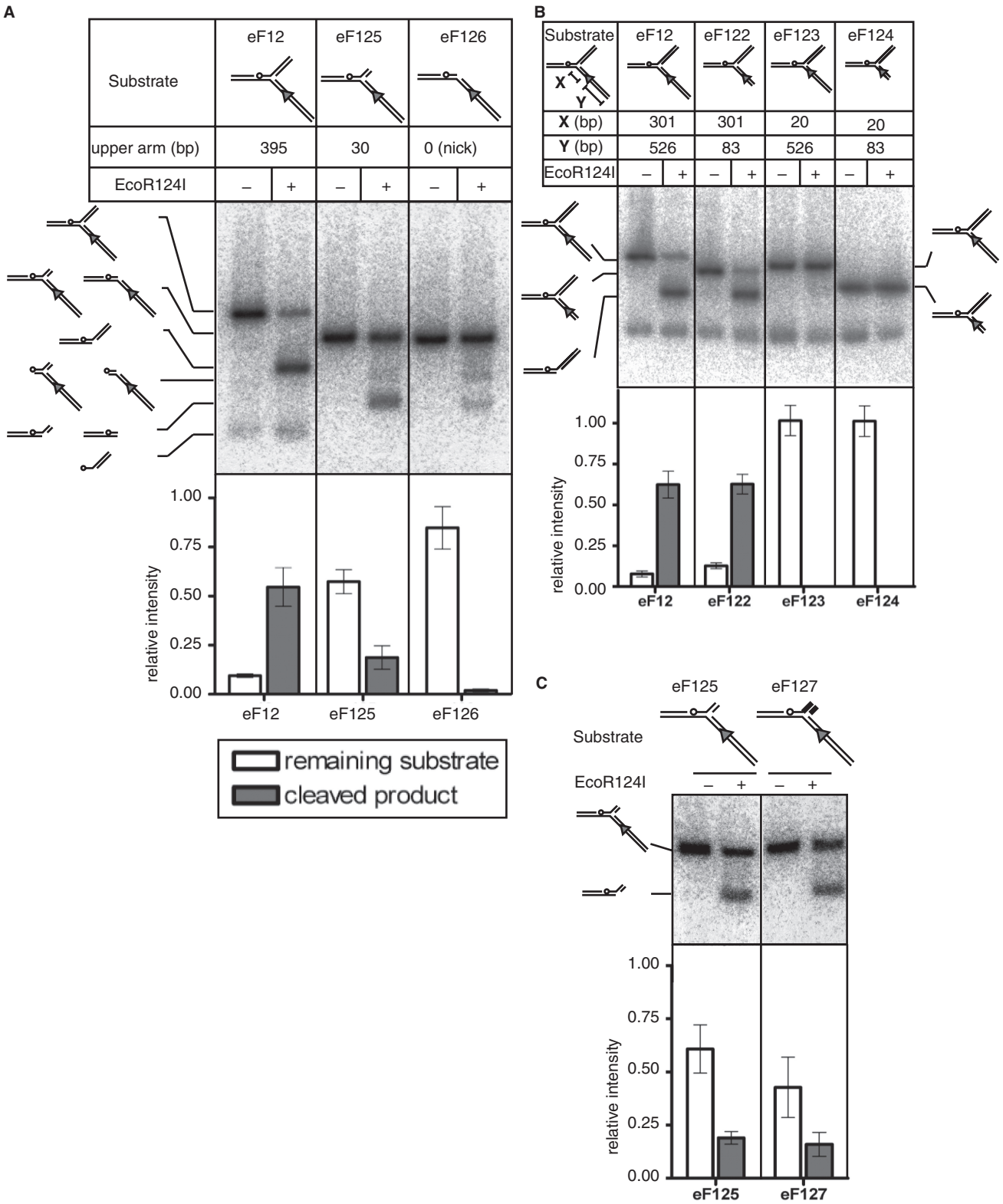


Figure 4. Dependence on the fork structure. (**A**) Dependence on the other arm. The upper arm lacking an EcoR124I site was shortened from 395 to 30 bp or 0 bp. (**B**) Dependence on distance between the recognition site and the branch point. Based on eF12 structure, the branch distal region from the EcoR124I recognition site was shortened from 526 to 83 bp (eF122). The branch proximal region from the EcoR124I site was shortened from 301 to 20 bp (eF123). Both of the regions were shortened as in eF122 and eF123 (eF124). (**C**) Immobile fork. In eF125, the sequence of the upper arm is identical to that of the lower arm. In eF127, the sequence of the upper arm was changed to be non-identical to the bottom arm. Graphs show relative intensity of each band to the starting substrate. The data are represented as mean ± SD from three measurements. A triangle indicates an EcoR124I site, while an open circle indicates ^{32}P for 5′-end labeling.

a branch point, it cleaves the DNA there [the M_2S complex would remain bound to the recognition site (24)].

A fork with two arms of an identical sequence is expected to experience branch migration generating a Holliday junction (49). (Replication forks usually do not reverse to generate Holliday junctions, but when the replisome malfunctions in a particular way, they are suspected to do so.) However, the cleavage reported here does not require mobility of the branch point (Figure 4C). Our major products are products of cleavage at the branch point (Figures 2–6). These results indicate that our reaction is distinct from the cleavage of Holliday junctions by a Type I REase EcoR124II (33).

The results in Figure 4B (lane 5, 6), showing the inability of a recognition sequence near the branch point to stimulate cleavage, cannot be ascribed to complete destruction of the duplex recognition sequence by such branch migration, because spontaneous branch migration is inhibited by a single base-pair mismatch (50) and should be inhibited by the following sequence lacking similarity around the recognition sequence: 5′... CCAT**GACCAAA GGCGATGCTGTATTCTT** ... on the bottom arm with a recognition sequence and 5′... CCAT**ATATAGGGCCCG GGTTATAATTACC** ... on the upper arm of the eF123, in which the bold characters indicate the dissimilar region and the underlines indicate the recognition sequence. No activity that promoted branch migration has been detected so far for Type I restriction enzymes (51). Interaction of branch migration and Type I restriction is an interesting subject of future research (see below).

Restriction enzyme EcoR124I can be prepared in either of $R_2M_2S_1$ form or of $R_1M_2S_1$ form (37,38). These two forms show different activities (38,45,52,53). The present preparation is of $R_1M_2S_1$ form (37,38) (see Materials and methods section). The effect of subunit stoichiometry on the fork cleavage reaction is yet to be examined.

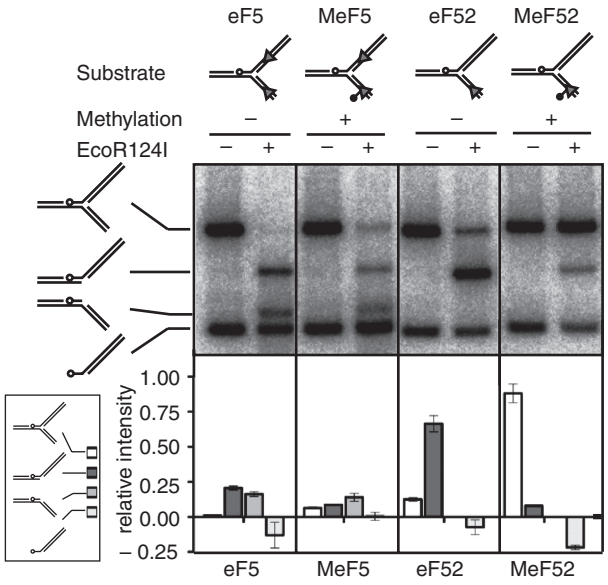


Figure 5. Inhibition by methylation of the recognition site. The fork with unmethylated or hemi-methylated Eco124I recognition sequence was treated with EcoR124I. Graphs show relative intensity of each band to the starting substrate as in Figure 3. The data are represented as mean ± SD from three measurements. A closed circle with a bar indicates hemi-methylation at the position, 3′GCTACGACAT $_{m6}$AAG5′ on the longer strand of the bottom arm. An open circle indicates ^{32}P for 5′-end labeling.

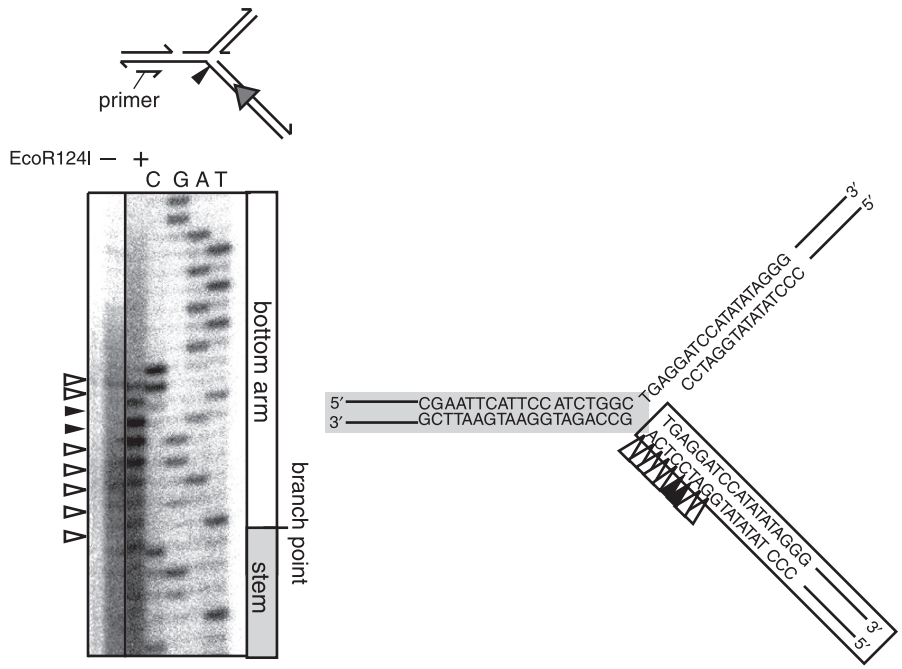


Figure 6. Cleavage sites as determined by primer extension. Left: eF12 was incubated with EcoR124I. After gel electrophoresis, the products containing the primer region were recovered and subjected to a primer extension reaction. The products were subjected to 6% polyacrylamide/8 M urea gel electrophoresis together with a dideoxy-sequencing reaction of the products, followed by signal detection by an imaging analyzer. Black triangle: strong cleavage position; white triangle: weak cleavage position. Right: the cleavage sites mapped on the fork.

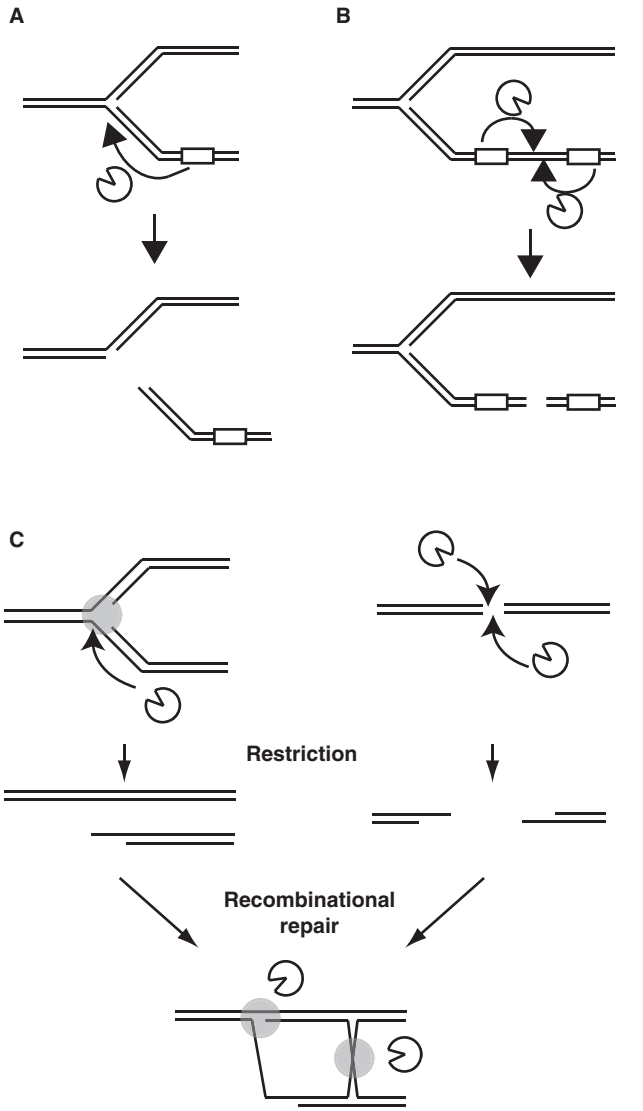


Figure 7. Models for Type I restriction coupled with DNA replication. (**A**) Single-site model. From a single unmethylated recognition site, the restriction enzyme translocates along the DNA and cleaves it at the branch point. The replication fork might not be in progress, but stalled or just established (see Discussion section). (**B**) Two-site model. The cleavage occurs by two endonuclease molecules from different unmethylated sites (adapted from ref. 20). An open square indicates an unmethylated recognition site. (**C**) A hypothesis of a secondary attack by a Type I restriction enzyme on the DNA. When a DNA replication fork arrests, a Type I restriction enzyme cleaves the fork. Two restriction enzyme molecules will generate a double-strand break. Attempts at recombination repair of these breaks will generate another branched structure such as a replication fork or a Holliday structure. A Type I restriction enzyme will cleave them to abort repair.

Replication and recombination repair versus restriction

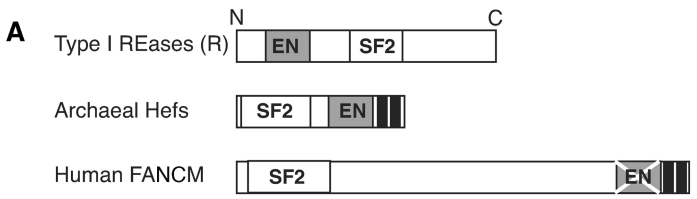
Our finding *in vitro* suggests the possibility that the DNA replication fork can be a direct target for Type I REases. It has been hypothesized that replication-coupled cleavage by a Type I enzyme occurs by collision of two enzyme molecules moving from two unmethylated sites on a daughter duplex DNA generated through replication fork passage (Figure 7B) (20). Our findings raise the possibility that breakage by a Type I REase takes place at the replication fork through the above mechanism (Figure 7A).

Our fork cleavage reaction may reflect interaction of a Type I restriction enzyme with an arrested fork (or a re-established fork through recombinational repair as discussed below). The speed of translocation of an $R_1M_2S_1$ form of EcoR124I in one direction is ~500 bp/s and that of its $R_2M_2S_1$ form in two directions is twice as large (52). These numbers are comparable to that of the speed of the *E. coli* replication fork *in vivo*, 600–900 bp/s (53,54). A translocating restriction enzyme may be able to catch up a moving replication fork to cleave it. The race to catch up with the replication fork could be a venue for several kinds of interaction between a genome of a phage or a bacteria and a Type I restriction–modification system.

Cleavage at a replication fork provides an explanation for the recombination repair of restriction damage observed after the single infection of a phage genome (30). An incoming genome will start replication. The restriction enzyme would recognize a site on a daughter chromosome and start tracking the DNA to catch up the moving replication fork. By the time the enzyme finally reaches the fork and cleaves the DNA, the replicated daughter chromosome would be long enough to participate with phage-mediated homologous recombination with the other daughter chromosomes. Indeed, such phage-mediated homologous recombination can repair DNA breaks made by REases (55).

The homologous recombination process to repair the breaks will generate branched intermediates, Holliday junctions and replication forks. These will be attacked again by the Type I restriction enzyme (Figure 7C) [(33), the present work]. It is possible that there is an endless fight between the restriction–modification system and the replication/recombination/repair machinery of a genome (either phage or bacteria). This may take place on incoming, unmethylated DNAs. For bacterial chromosomes, the cleavage at the branched forms may take place in situations where unmethylated DNA is generated, for example, replication fork progression in the presence of 2-AP, replication recovery *via* recombinational repair, and constitutive stable DNA replication, if the activity control is absent (15,27,28,56). Movement of a newly generated replication fork may create unmethylated recognition sites and expose the fork to the danger of restriction attack.

With the further disturbance of homologous recombination and replication (Figure 7C), the structure-specific activities of Type I REases would contribute to cell death. This disturbance might differentiate their fork cleavage from those mediated by other enzymes regarded as parts of recombinational repair machinery (Figure 7C). Illegitimate recombination that requires interaction between two homologous DNAs and is mediated by a Type I restriction system (57) could represent another consequence of such interaction between the Type I restriction and the recombination repair machinery: a branched intermediate of homologous recombination is presumably cleaved by a Type I REase to generate a recombinogenic DNA end.



	Fork cleavage	dsDNA translocation	unwinding activity	Recognition sequence
Type I REases (R)	**Yes**	**Yes**	**Yes**	Yes
Archaeal Hefs	**Yes**	Not tested	**Yes**	No
Human FANCM	No	**Yes**	No	No

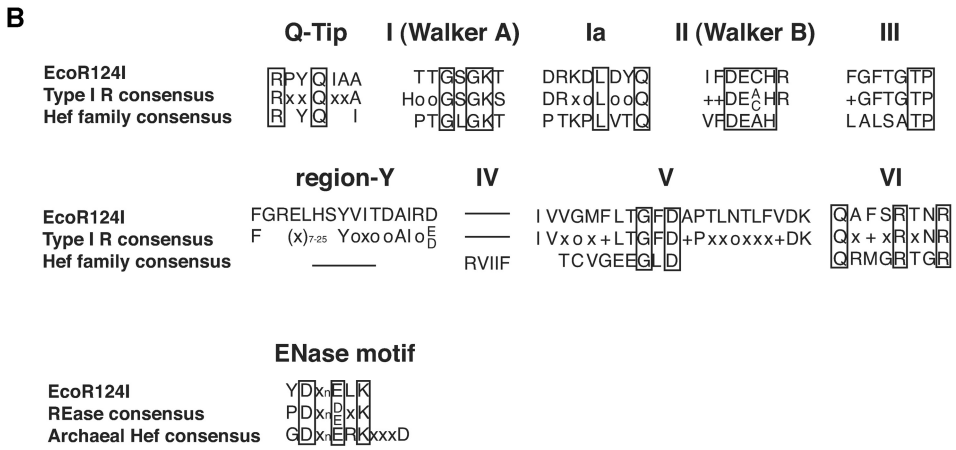


Figure 8. Comparison of Type I restriction enzymes (R subunit) and Hef family enzymes in structure and function. (**A**) Upper, the primary structure; lower, biochemical activities. (**B**) Motifs compared. EN, endonuclease motif, white crossed lines indicate loss of the activity. Filled square, helix-hairpin-helix domain; SF2, superfamily 2 helicase motif. o, hydrophilic residue; +, hydrophobic residue; x, any residue. The architecture and motifs of Type I restriction enzyme R subunit were adapted from an earlier work (67). Those of the Hef family were adapted from previous reports (61,64,65).

A fork cleavage capacity we found can explain why restriction alleviation, that is the chromosome protection against Type I restriction enzymes, needs to be induced under conditions where replication initiation is increased (22,26,27). If the replication forks are direct targets of the Type I restriction cleavage *in vivo*, the restriction activity should be tightly regulated under these conditions.

A replication fork active in DNA synthesis is a complex of branched DNA and a replisome that consists of, at least, DNA polymerase, DNA helicase and the primase (58), which may be hardly accessed by enzymes out of the process *in vivo*. However, it is known that noncovalently bound protein does not block the translocation of EcoR124II that is composed of the same R and M subunits as EcoR124I and with a different S subunit (59), suggesting the possibility that EcoR124I might remove the replisome and reach the branch for its cleavage. On the other hand, if the replisome is so stable that it can block translocation of EcoR124I, it would make a barrier to cleave there. The replication fork that has stalled at some DNA damage and is undergoing replisome re-assembly for replication restart might be more accessible than the running replication fork (58). The potential interactions between other proteins interacting with a replication fork and Type I restriction enzyme are to be examined.

The recognition site has to be at some distance from the branch point for cleavage (Figure 4B), presumably because the cleavage needs translocation of the enzyme (39). If the fork cleavage capacity is shared by EcoKI, a Type IA enzyme, this property might help fork protection through the proteolytic digestion of the enzyme in the restriction alleviation phenomenon. Without such a property, an unmethylated sequence generated at the replication fork would be immediately attacked by the Type I restriction endonuclease before its proteolytic cleavage. This hypothesis is supported by the observation that the proteolysis is directed to translocating enzymes as opposed to free enzymes (22,28).

Our finding suggests the possibility that the fork cleavage reaction leads to cell death in situations where replication fork formation is increased by DNA damage or

other factors. This would contribute to the genome integrity of the population by eliminating cells with an unstable genome copy. In other words, the generation of a new replication fork presumably has the role of providing a point of choice between restoration and destruction, two pathways for genome maintenance, in the presence of the Type I restriction–modification systems. Programmed cell death is induced by a high level of DNA damage in mammalian cells (14), and possible involvement of the destructive fork cleavage in this form of genome maintenance is to be examined. To our knowledge, restriction–modification systems have not been identified in eukaryotes so far, though they have been found in Chlorella viruses (60). DNA structure-specific endonucleases such as XPF, Mus81, GEN1 might be involved in such a process (61,62).

Comparison with other DNases cleaving replication forks

In this study, we discovered that a Type I REase cuts off one arm of a model replication. This reaction is similar to that by an archaeal enzyme, Hef (63). In addition, we found it had an activity of unwinding duplex DNA at the stem of the fork-structured DNA, which is also similar to Hef's (12). FANCM, a human Hef homolog, shows translocation activity on duplex DNA (64). Their structure and function are compared in Figure 8.

There is similarity in the enzyme architecture between Hef family proteins and the R subunits of Type I restriction enzymes, as pointed out previously (65). They all have a superfamily 2 (SF2) helicase motif and an endonuclease domain, which is similar to the PDXn(D/E)XK domain found in most restriction enzymes, although their relative positions are different (Figure 8A) (28,61,65–67). The nuclease domains are similar in three-dimensional structure (65). However, the two families are distant in phylogeny because they are unique in amino acid sequence in these domains (Figure 8B).

The endonuclease domain of Hef family belongs to the ERCC4 endonuclease domain family, whose ortholog has not yet been found in eubacteria (61,68). The ERCC4 nuclease domain has a conserved motif GDX_nERKX_3D, which is similar to, but different from, that of REases including Type I enzymes, $PDX_n(E/D)XK$ (65). Furthermore, only the Hef family have HhH DNA binding motifs in the C-terminal side of the nuclease domain (61,65,68).

The SF2 helicase motifs of the two families can be also distinguished. Our multiple amino acid sequence alignment of selected members of the two families revealed several amino acid conservation in Q-tip, motif I (Walker A), Ia, II (Walker B), III, V and VI between the two families, but a Type I enzyme family-specific helicase motif, region Y (alternative to motif IV), did not fit well into the Hef family helicase domains (Figure 8 and Supplementary Figure S3) (67).

These two families of enzymes also appear different in biochemical properties.

(i) *Structure specificity*. Hef family directly recognize the DNA branch (63,64,69). However, a Type I restriction enzyme recognizes a branched DNA structure as a barrier to its translocation (Figure 4B). It is not known whether the translocation activity contributes to the structure recognition by the FANCM (64,69,70).

(ii) *Specific unmethylated sequence at a distance*. An unmethylated recognition sequence distant from the branch is required for the Type I REase to cleave the fork (Figures 3 and 6). Requirement for a long branch or any sequence preference has not been reported for Hef family (12,63,64,69–73).

(iii) *Cleavage position*. EcoR124I can cleave either of the two arms (Figure 2 and 3), while Hef introduces a nick at the leading strand side of the stem region (corresponding to the top strand in Figure 1C) (63).

(iv) *Quaternary structure*. Type I restriction enzymes work as a complex of R, M, and S subunits (28). Hef is active as a homo dimer (12,65). FANCM forms a hetero dimer with an ERCC4 family member that has lost its nuclease activity (74).

(v) *Branch migration activity*. Though EcoR124I does not have branch migration activity (51), Hef family enzymes have it (70,72,73).

Acquisition of these distinct properties by these enzymes may have been driven by the nature and role of the processes in which they are involved. Likely because of their distinct roles, they are also subject to different types of control: for example, the proteolytic and subunit assembly control for the Type I restriction enzymes (21,22,24), as compared to the cell cycle and signal transduction-dependent activation for Hef family (75).

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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