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Electron Microscopic Studies of the Mechanism of Action of the Restriction Endonuclease of *Escherichia coli* B

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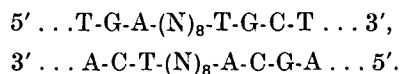
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Reaction intermediates and products formed by the restriction endonuclease of *Escherichia coli* B with fd replicative form DNA substrates containing recognition sites in known positions and orientations have been characterized by electron microscopy. After exposure of these substrates to enzyme, loops of duplex DNA were frequently observed, usually at or near the termini. Analysis of the size and structure of the loops observed with various DNA substrates suggests that the enzyme binds initially to the recognition site then remains bound to the DNA in the region of this site while tracking towards a site of cleavage. Tracking appears to occur only on the 5' side of the asymmetric recognition sequence, 5'...T-G-A-(N)₈-T-G-C-T...3'; however, the location of the cleavage sites appears to be random, at least within certain limits of distance from the recognition site. Enzyme-DNA complexes remain intact even after the double-strand cleavage is completed, and this complex acts as a potent ATPase with no obvious function. This latter reaction might represent an artifactual uncoupling of ATP hydrolysis from the tracking of the enzyme along the DNA; alternatively, it might indicate an *in vivo* function for the enzyme of which we are unaware.

1. Introduction

In contrast to the type II restriction endonucleases (Boyer, 1971), which recognize and cleave the DNA within a specific, usually symmetrical, nucleotide sequence (Roberts, 1976), those enzymes classed as type I are more complex. The restriction endonuclease from *Escherichia coli* B (*EcoB*)‡, for example, recognizes the nucleotide sequence



This sequence has no apparent symmetry and is discontinuous, containing two regions of specific base sequence separated by a region of eight non-specific base-pairs (Lautenberger *et al.*, 1978; Ravetch *et al.*, 1978). After binding to an unmodified

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‡ Abbreviations used: *EcoB* and *EcoK*, the restriction endonucleases from *E. coli* B and *E. coli* K, respectively; *HincII*, *HindII* and *BamH-I* are restriction endonucleases from *Haemophilus influenzae* serotypes c and d, and *Bacillus amyloliquefaciens*, respectively; *sB*, recognition site for *EcoB*; AdoMet, *S*-adenosyl-L-methionine; RF, replicative form.

recognition site, *EcoB* travels 1000 to 5000 nucleotides before cleaving one strand of the DNA at an apparently random site, introducing a gap of about 75 nucleotides by releasing an equivalent amount of acid-soluble oligonucleotide (Horiuchi & Zinder, 1972; Horiuchi *et al.*, 1974; Linn *et al.*, 1974; Kimball & Linn, 1976). Since *EcoB* does not turn over as a DNase, a second enzyme molecule is required to complete the double-strand break (Eskin & Linn, 1972*a*).

EcoB endonuclease activity requires ATP, *S*-adenosyl-L-methionine, and Mg^{2+} as well as duplex DNA containing the appropriate unmodified recognition sites. Although the function of these cofactors is unclear, it has been demonstrated that AdoMet is not degraded during the restriction reaction (Eskin & Linn, 1972*a*), but that ATP is hydrolyzed to ADP and inorganic phosphate (Eskin & Linn, 1972*b*). *EcoB* ATPase activity also requires duplex DNA with unmodified B-specificity sites and AdoMet. However, once initiated, *EcoB* continues to hydrolyze ATP long after DNA degradation has finished. In addition, during this latter stage, the ATPase reaction requires only that the enzyme molecule remain bound to the particular DNA molecule which it has restricted: exogenous unmodified recognition sites and AdoMet are no longer required (Eskin & Linn, 1972*a,b*).

To continue our studies of *EcoB*, we have now used as substrates fd replicative form DNAs that contain *EcoB* recognition sites in known positions and orientations. Using electron microscopy to examine the reaction intermediates and products obtained with these substrates, we have uncovered an unusual scheme by which *EcoB* cleaves DNA.

2. Materials and Methods

(a) Materials

(i) DNA

Unmodified, RF DNAs of phages fd and fd101 were prepared after phage growth on the *E. coli* K *thy* strain, Hfr C6-2 (Goldmark & Linn, 1970, 1972). B-modified RF DNA was prepared as described by Eskin & Linn (1972*a*). All DNA concentrations are expressed as nucleotide equivalents.

(ii) Enzymes

EcoB was purified and assayed as described previously; unless noted otherwise, the sucrose gradient fraction of enzyme was used (Eskin & Linn, 1972*a*). One unit of enzyme is the amount that produces 0.6 pmol of single-strand DNA scissions under standard reaction conditions (Eskin & Linn, 1972*a*). *EcoB* modification methylase was purified and assayed as described by Lautenberger & Linn (1972). *BamH*-1 (> 12,500 units/mg protein) and *Hinc*II (5000 units/mg protein, as a mixture with *Hind*III) were obtained from New England Biolabs. *Hind*III does not cleave fd RF DNA; *Hinc*II (an isoenzyme of *Hind*III) makes one break; *BamH*-1 cleaves fd RF DNA at two sites (Roberts, 1976). One unit of each of these enzymes is the amount required to degrade 1 μ g of phage lambda DNA in 15 min at 37 C in a 50- μ l reaction mixture.

(iii) Other materials

S-adenosyl-[methyl- 3H]methionine and NCS tissue solubilizer were purchased from Amersham. Unlabelled *S*-adenosyl-L-methionine was prepared as described by Schlenk *et al.* (1965). [γ - ^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Bovine serum albumin was purchased from Sigma and acetylated by the method of Epstein & Goldberger (1964) to eliminate contaminating nuclease activity. Omni-fluor was purchased from New England Nuclear.

(i) Restriction endonucleases

Unless noted otherwise, reactions were carried out in 100 μ l of a reaction mixture containing 10 mM-Tris-HCl, pH 7.5, 50 mM-NaCl, 10 mM-MgCl₂, 5 μ g acetylated bovine serum albumin, and 100 μ g DNA. Reactions were carried out at 37°C for 60 min, reaction products were extracted with phenol.

For digestion of DNA with varying amounts of enzyme, reactions were terminated by adding EDTA to a final concentration of 10 mM.

(ii) Agarose gel electrophoresis

DNA samples were prepared in 100 μ l of a mixture (w/v) sodium dodecyl sulphate (SDS) 1%, glycerol 10%, and water 89%. The mixture was incubated at 100°C for 5 min. The mixture was then diluted with 1.4% (w/v) agarose slab gel (Bio-Lab, Studier (1973)). The buffer was 0.05 M Tris, 0.02 M EDTA, and had been adjusted to pH 8.0. The gel was run at 12.5 V/cm, the gel was stained with ethidium bromide, and bands were visualized under short wave UV light.

(iii) Electron microscopy

DNA samples were prepared by the technique described by Iversen & Linn (1972). Samples were rotary shadowed with 40% (v/v) formamide in ethanol. Samples were rotary shadowed on a projected electron microscope. Samples were also prepared for electron microscopy by direct observation of protuberances on a film of polylysine and uranyl acetate.

(a) Location and orientation of recognition sites

Wild-type fd RF DNA was prepared from phages 6329 and 6329 to 6343 in fd101. Phage fd101 has been shown to contain a recognition site (Kühnlein & Arber, 1972). fd101 RF DNA was labelled with [*methyl*- 3H]methionine with *Hinc*II and *BamH*-1. After resolution of the DNA, the label was found only in the recognition site at residues 959-960. This site has been designated *sB*₂ (Kühnlein & Arber, 1972). fd101 has only *sB*₂ at residues 6329 to 6343.

fd101 RF DNA is cleaved by *EcoB*. Wild-type fd RF DNA is cleaved at some unit length mate-
rials support the *EcoB*

(b) *Methods*(i) *Restriction endonuclease digestions*

Unless noted otherwise, RF DNA was digested with *HincII* in 30- μ l reaction mixtures containing 10 mM-Tris·HCl (pH 7.8), 7 mM-MgCl₂, 7 mM-2-mercaptoethanol, 50 mM-NaCl, 5 μ g acetylated bovine serum albumin, 1 nmol DNA and 0.8 unit enzyme. After incubating at 37°C for 60 min, reactions were stopped by cooling to 0°C.

For digestion of DNA with *EcoB*, 50- μ l reaction mixtures containing 1 nmol of DNA and varying amounts of enzyme were as described by Eskin & Linn (1972*a*). Reactions were terminated by adding EDTA to a final concentration of 18 mM.

(ii) *Agarose gel electrophoresis*

DNA samples were prepared for electrophoresis by adding 0.2 vol. of a solution of 5% (w/v) sodium dodecyl sulphate, 50% (v/v) glycerol, 0.015% (w/v) bromophenol blue, then incubating the mixture at 60°C for 5 min. After chilling, the DNA was loaded onto a 1% or 1.4% (w/v) agarose slab gel (0.3 cm thick) in an apparatus similar to that described by Studier (1973). The buffer system contained 40 mM-Tris base, 5 mM-sodium acetate, 1 mM-EDTA, and had been brought to pH 7.9 with glacial acetic acid. After electrophoresis at 12.5 V/cm, the gel was stained in a solution of 1 μ g propidium iodide/ml, then the DNA bands were visualized and photographed under ultraviolet light.

(iii) *Electron microscopy*

DNA samples were mounted for electron microscopy using a method based on the technique described by Davis *et al.* (1971) as modified by MacKay & Linn (1974), with 40% (v/v) formamide in the spreading solution and 10% formamide in the hypophase. Samples were rotary shadowed with tungsten. Molecular length measurements were performed on a projected 9 \times enlargement of photographic negatives. DNA samples were also prepared for electron microscopy by the method of Williams (1977), which allows the direct observation of protein molecules bound to the DNA. In this system DNA is adsorbed to a film of polylysine and rotary shadowed with tungsten.

3. Results

(a) *Location and orientation of recognition sites in fd replicative form DNA*

Wild-type fd RF DNA contains two *EcoB* recognition sites at residues 959 to 973 and 6329 to 6343 in the orientation shown in Figure 1. The DNA of the mutant phage fd101 has been shown to contain only one of these *EcoB* recognition sites (Kühnlein & Arber, 1972). In order to determine which of the sites had been retained, fd101 RF DNA was labelled specifically at the *EcoB* recognition site using *S*-adenosyl-[methyl-³H]methionine and *EcoB* modification methylase, then digested with *HincII* and *BamH*-1, which produced three fragments, A, B and C (see Fig. 1). After resolution of the fragments by electrophoresis on agarose gels, radioactive label was found only in fragment B (Fig. 2). This fragment contains the *EcoB* recognition site at residues 959 to 973 (Fig. 1). The site remaining in fd101 had previously been designated *sB*₂ (Kühnlein & Arber, 1972) so that we may now summarize that fd101 has only *sB*₂ at residues 959 to 973, whereas wild-type fd also contains *sB*₁ at residues 6329 to 6343.

(b) *EcoB activity with circular DNA*

fd101 RF DNA is cleaved once by *EcoB* to form unit length molecules, while the wild-type fd RF DNA is degraded to produce fragments of 0.3 to 0.8 unit length as well as some unit length material (Fig. 3(a), lanes A to D; Linn *et al.*, 1974). Both DNA substrates support the *EcoB* ATPase activity (Table 1).

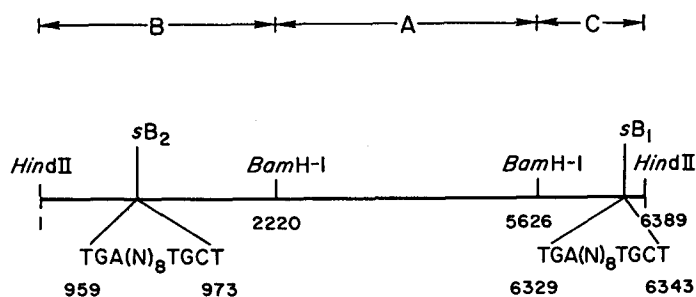


FIG. 1. Location and orientation of B recognition sites in fd RF DNA. The genome of wild-type fd RF is shown after cleavage at the unique *Hind*II site, which serves as the origin for nucleotide numbering. *Hinc*II, the enzyme used in these studies, is an isoschizomer of *Hind*II (Roberts, 1976). The sequences shown for the two *sB* sites are those of the viral strand, and are 5' → 3' from left to right (H. J. Schaller, personal communication). The locations of the two *Bam*H-I cleavage sites, at residues 2220 and 5626, are also shown. The *sB* sites in regions B and C are shown in this paper to be *sB*₂ and *sB*₁, respectively. Hyphens have been omitted for clarity.

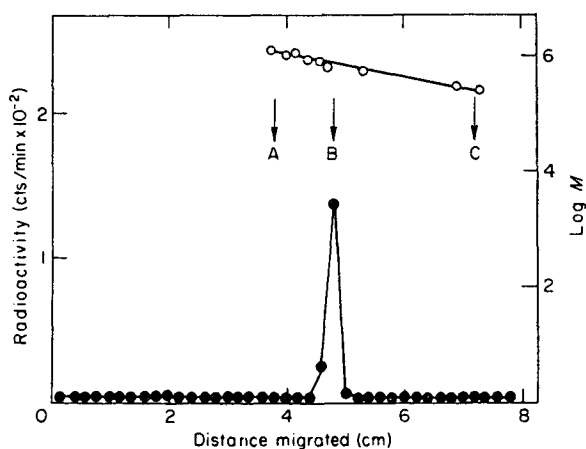


FIG. 2. Determination of the location of the single *sB* in DNA from the phage fd101. RF DNA was methylated in a reaction containing (in 92 μ l): 55 mM-potassium phosphate (pH 6.0), 10 mM-MgCl₂, 0.1 mM-dithiothreitol, 27 pmol *S*-adenosyl-[methyl-³H]methionine, 12.5 nmol fd101 RF DNA and 1.2 μ g *Eco*B modification methylase. After incubating at 37°C for 2 h, methyl group incorporation was verified with a portion of the reaction mixture (Lautenberger & Linn, 1972), while the remainder was dialyzed extensively to remove unincorporated label. The ³H-labelled methylated DNA was then digested with *Hinc*II and *Bam*H-I in an 80- μ l reaction containing 10 mM-Tris-HCl (pH 7.8), 7 mM-MgCl₂, 7 mM-2-mercaptoethanol, 50 mM-NaCl, 8 μ g acetylated bovine serum albumin, 1.5 nmol methylated fd101 RF, 2.5 nmol unlabelled fd101 RF DNA, 2 units *Hinc*II and 6.25 units *Bam*H-I. After incubating at 37°C for 1 h, the reaction was stopped by adding EDTA to a final concentration of 15 mM, the DNA was subjected to electrophoresis and the DNA bands were visualized as described in *Materials and Methods*. The agarose gel was subsequently sliced into 2-mm portions and dissolved in 10 ml NCS-Omnifluor scintillant (15.2 g Omnifluor, 143 ml NCS, 3700 ml toluene) at 30°C for 12 h. After chilling at 0°C for 4 h, the radioactivity in each gel slice was determined (—●—●—). The arrows show the position of the three fragments produced after digestion of fd101 RF DNA with *Hinc*II and *Bam*H-I; these fragments contain 3406 base-pairs (A), 2220 base-pairs (B) and 763 base-pairs (C). The migration of these fragments on electrophoresis was monitored by comparison with the migration of restriction fragments of known molecular weight (*M*) (—○—○—).

† AdoMet was omitted from the reaction mixture in the presence of dependent ATPase.

Reaction mixtures as described in the text, containing 1.4 mM-[γ -³²P]ATP (1500 cpm/pmol) and 1.4 mM-[γ -³²P]ATP (1500 cpm/pmol) were analysed by thin layer chromatography as described (Linn, 1972).

When reaction mixtures were analysed by electron microscopy after digestion (while the reaction was continuing), loops of DNA were visualized (Fig. 4(a)) and linear molecules (Fig. 4(b)). The loops were of unit length (Fig. 4(b)). These loops are likely to be eliminated by extraction.

DNA substrate	Fragmentation
fd RF	Complete
fd101 RF	Complete
<i>Hinc</i> II-treated fd RF	Complete
<i>Hinc</i> II-treated fd101 RF	Complete
<i>Hinc</i> II-treated fd RF-B	Complete
<i>Hinc</i> II-treated fd101 RF	Complete
<i>Hinc</i> II-treated fd RF	Complete
<i>Hinc</i> II-treated fd101 RF	Complete

† Complete reactions were used.

‡ Under the conditions used, the migration of the fragments was to approx. 300 base-pairs, or less.

§ The distribution of loop sizes was determined.

TABLE 1
DNA dependence of *EcoB* ATPase activity

DNA substrate	ATP hydrolyzed (nmol/30 min)
fd RF	44.3
fd101 RF	20.8
<i>HincII</i> -treated fd RF	30.6
<i>HincII</i> -treated fd101 RF	12.3
fd101 RF†	0.3
<i>HincII</i> -treated fd101 RF†	0.2
Calf thymus	17.7
Phage T7	24.3

† AdoMet was omitted from these incubations to control any contaminating AdoMet-independent ATPase.

Reaction mixtures as described by Eskin & Linn (1972a) contained 1 nmol DNA, 0.1 unit *EcoB* and 1.4 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (150 cts/min per nmol). After incubating at 37°C for 30 min, ATP hydrolysis was determined as the amount of radioactivity that did not adsorb to Norit (Goldmark & Linn, 1972).

When reaction mixtures with wild-type RF DNA were examined by electron microscopy after digestion with *EcoB* had terminated (but while ATP hydrolysis was continuing), loops of DNA were observed at the termini of 8 to 13% of the DNA molecules visualized (Fig. 4). The loops varied in size from 0.1 to 0.74 μm , equivalent to 0.05 to 0.35 unit length (Table 2); they were found at the termini of unit length linear molecules (Fig. 4(a)) as well as at the termini of molecules that were less than unit length (Fig. 4(b)). In all cases, the DNA in the loop appeared totally duplex. These loops are likely to represent transiently stable complexes of enzyme and DNA, since their frequency decreased on storage of reaction mixtures, and they were eliminated by extraction with phenol or incubation with formamide.

TABLE 2
Summary of loops observed

DNA substrate	Reaction conditions	Total number molecules observed	Number of loops observed	Length of loops† (μm)
fd RF	Complete†	175	12	0.10-0.74
fd101 RF	Complete	150	14	0.20-0.89
<i>HincII</i> -treated fd RF	Complete	450	56	0.09-1.27§
<i>HincII</i> -treated fd101 RF	Complete	300	36	0.28-0.32§
<i>HincII</i> -treated fd RF·B	Complete	850	0	—
<i>HincII</i> -treated fd101 RF	— AdoMet	600	0	—
<i>HincII</i> -treated fd RF	— <i>EcoB</i>	500	0	—
<i>HincII</i> -treated fd101 RF	— <i>EcoB</i>	500	0	—

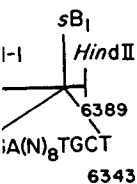
† Complete reactions were as described by Eskin & Linn (1972a).

‡ Under the conditions used to prepare the samples for electron microscopy, 0.1 μm is equivalent to approx. 300 base-pairs, or 5% of the genome.

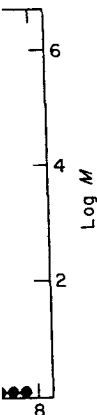
§ The distribution of loop size within this range is shown in Fig. 7.

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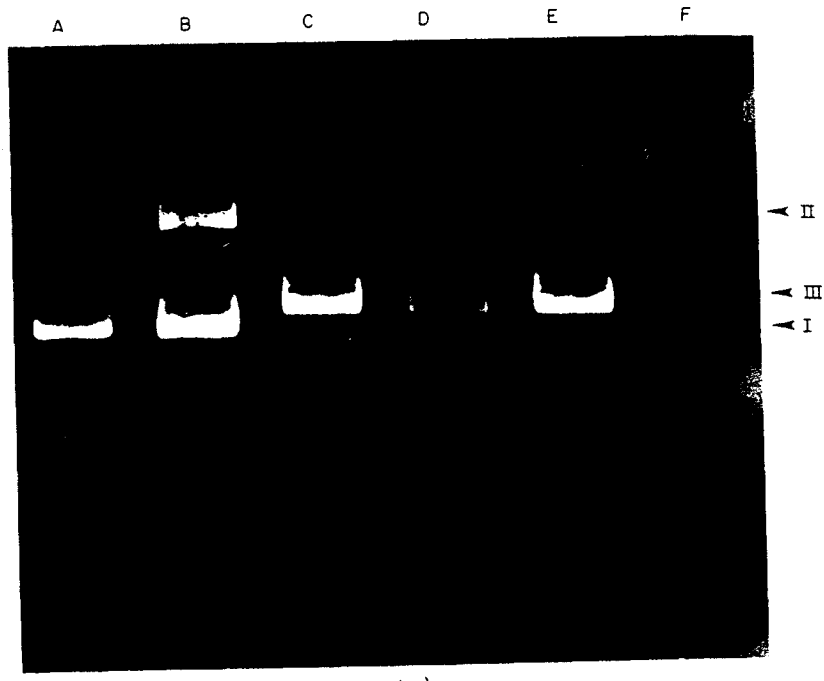
-C-→



A. The genome of wild-type *E. coli* is shown as the origin for nucleotide numbering. The *HindII* site is shown, and are 5' → 3' from left to right. The two *BamHI* cleavage sites B and C are shown in brackets for clarity.



from the phage fd101. RF DNA was digested with *EcoB* in phosphate (pH 6.0), 10 mM Tris-HCl, 12.5 nmol fd101 RF at 37°C for 2 h, methyl group (Lautenberger & Linn, 1972). The ^{32}P -labelled DNA was in an 80- μl reaction containing 50 mM-NaCl, 8 μg acetylated and unlabelled fd101 RF DNA. After 1 h, the reaction was stopped and the DNA was subjected to electrophoresis and the fragments were visualized by autoradiography. The agarose gel was subsampled and analysed for radioactivity. The migration of restriction



(a)



(b)

FIG. 3. (a) Agarose gel electrophoresis of DNA after restriction. Where indicated, wild-type and mutant fd RF DNA were converted to linear molecules with *HincII* as described in *Methods* and incubation was with 0.25 unit *EcoB* for 15 min. Electrophoresis was at 100 V for 2 h. Lane A, untreated fd RF DNA; lane B, *EcoB*-digested fd RF DNA; lane C, *HincII*-treated fd RF DNA; lane D, *HincII*-treated fd RF DNA; lane E, *HincII*-treated wild-type fd RF DNA; lane F, *HincII*-treated mutant fd RF DNA. (b) Agarose gel electrophoresis of DNA after restriction. Lane A, untreated fd RF DNA; lane B, *EcoB*-digested fd RF DNA; lane C, *HincII*-treated wild-type fd RF DNA; lane D, *HincII*-treated mutant fd RF DNA; lane E, *HincII*-treated wild-type fd RF DNA; lane F, *HincII*-treated mutant fd RF DNA; lane G, *HincII*-treated wild-type fd RF DNA; lane H, *HincII*-treated mutant fd RF DNA.

Similar structure product that is for loops appeared when equivalent to 0.09 μm RF DNA product at the limit of digestion. Structures observed in 1 activity has ceased.

The circular DNAs between the loop wild-type and fd10 digestion with *HincII* during subsequent (Fig. 3(a), lane F; was no longer susceptible. *HincII*-treated

When examined DNAs always appeared $2.1 \pm 0.1 \mu\text{m}$ (Fig. 6) DNA were found at addition, in some cases loop (Fig. 6(e)). The tails, the length of $0.32 \mu\text{m}$, equivalent to the distance (Fig. 1), and obviously circular fd101 RF DNAs.

Loops of duplex type fd RF DNA after of approximately covalent DNA terminus (Fig. 8(a)) and fractal variable size, up to 0 length molecules (Fig. 8(b)) in the presence of a small tail equivalent to 60 to 100 nm. This distance is

for 2 h. Lane A, untreated fd RF DNA; lane B, *EcoB*-digested fd RF DNA; lane C, *HincII*-treated fd RF DNA; lane D, *HincII*-treated fd RF DNA; lane E, *HincII*-treated wild-type fd RF DNA; lane F, *HincII*-treated mutant fd RF DNA; lane G, *HincII*-treated wild-type fd RF DNA; lane H, *HincII*-treated mutant fd RF DNA.

(b) Agarose gel electrophoresis of DNA after restriction. Lane A, untreated fd RF DNA; lane B, *EcoB*-digested fd RF DNA; lane C, *HincII*-treated wild-type fd RF DNA; lane D, *HincII*-treated mutant fd RF DNA; lane E, *HincII*-treated wild-type fd RF DNA; lane F, *HincII*-treated mutant fd RF DNA; lane G, *HincII*-treated wild-type fd RF DNA; lane H, *HincII*-treated mutant fd RF DNA.

Similar structures were observed at the termini of the unit length, non-circular product that is formed after treating fd101 RF DNA with *EcoB* (Fig. 5). Again the loops appeared wholly duplex and they varied in length between 0.20 and 0.89 μm , equivalent to 0.09 to 0.42 unit length (Table 2). Since the unit length, linear fd101 RF DNA product and fractional length wild-type RF DNA product occur only after the limit of digestion of the respective circular DNA substrates by *EcoB*, the structures observed in these cases have apparently been maintained after endonuclease activity has ceased.

(c) *EcoB* activity with linear DNAs

The circular DNA substrates do not allow the determination of the relationship between the loop location and that of the *EcoB* recognition sites. Therefore the wild-type and fd101 RF DNAs were converted to unit length linear molecules by digestion with *HincII*. The wild-type DNA treated in this manner was degraded during subsequent incubation with *EcoB* and supported the *EcoB* ATPase activity (Fig. 3(a), lane F; Table 1). However, the fd101 RF DNA pretreated with *HincII* was no longer susceptible to random cleavage by *EcoB* (Fig. 3(a), lane E). Nonetheless, *HincII*-treated fd101 RF DNA supported the *EcoB* ATPase activity (Table 1).

When examined by electron microscopy, *HincII*-treated wild-type and fd101 RF DNAs always appeared as unstructured linear duplex molecules, with a length of $2.1 \pm 0.1 \mu\text{m}$ (Fig. 6(a)). After further treatment with *EcoB*, however, loops of duplex DNA were found at the termini of the *HincII*-treated fd101 RF DNA (Fig. 6(b)). In addition, in some cases, there was a small "tail" of duplex DNA associated with the loop (Fig. 6(c)). The contour length of the loop (or in the case of molecules with short tails, the length of the loop plus tail) was consistently within the range 0.28 to 0.32 μm , equivalent to 850 to 980 base-pairs (Table 2; Fig. 7(a)). This is approximately the distance from sB_2 to the proximal terminus of the DNA molecule (see Fig. 1), and obviously contrasts to the situation found after *EcoB* digestion of circular fd101 RF DNA, where loop size varied widely.

Loops of duplex DNA were also observed at the termini of *HincII*-treated wild-type fd RF DNA after incubation with *EcoB*. About 70% of the observed loops were of approximately constant size, equivalent to the distance from sB_2 to the proximal DNA terminus (Fig. 7(b); Table 2). Loops of this size were found on unit length (Fig. 8(a)) and fractional length (Fig. 8(b)) molecules. The remaining loops were of variable size, up to 0.6 unit length (Fig. 7), and were found on both full and fractional-length molecules (Fig. 8(c) and (d)). These latter loops were also characterized by the presence of a small tail of duplex DNA of approximately constant size, about 0.02 μm , equivalent to 60 to 100 base-pairs. The length of this tail of duplex DNA approximates the distance from sB_1 to the nearer DNA terminus (see Fig. 1).

for 2 h. Lane A, untreated fd101 RF DNA; lane B, untreated fd RF DNA which contains some slow migrating form II (nicked, circular) DNA; lane C, *EcoB*-digested fd101 RF DNA; lane D, *EcoB*-digested fd RF DNA; lane E, *HincII*-treated fd101 RF DNA digested with *EcoB*; lane F, *HincII*-treated fd RF DNA digested with *EcoB*. The migration of covalently closed circular DNA (I), nicked circular DNA (II) and unit length linear DNA (III) under these conditions is shown.

(b) Agarose gel electrophoresis after restriction of form I fd101 RF DNA to a limit with limiting amounts of *EcoB*. Reaction mixtures containing 1 nmol DNA (nucleotide) substrate were incubated for 15 min, then subjected to electrophoresis in 1% agarose for 3h. Reaction mixtures contained 0.0, 0.0, 0.02, 0.04, 0.08, 0.15 and 0.20 unit of *EcoB* from left to right, respectively. One unit makes 0.6 pmol of single-strand cleavages, and 15 min is a time by which each enzyme molecule has made a single-strand break and assumed a form no longer active as a DNase (Eskin & Linn, 1972a).

indicated, wild-type and
described in *Materials and*
electrophoresis was in 1% agarose

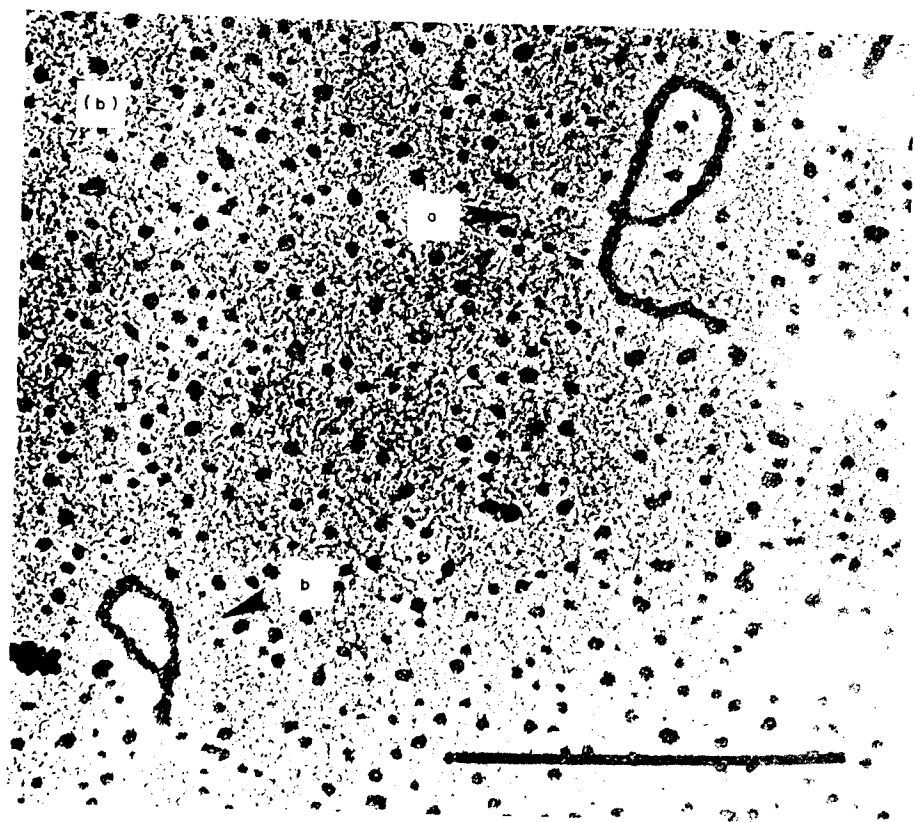
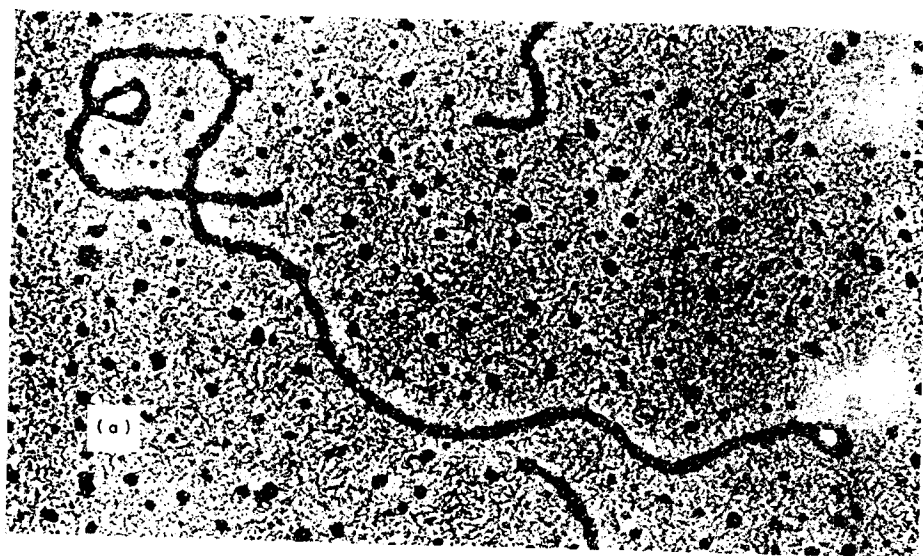


FIG. 4. Examples of electron micrographs of intermediates and products in the reaction of *Eco*B with wild-type ϕ 1 RF DNA. Reaction mixtures containing 0.2 unit of *Eco*B were incubated at 37°C for 2 min. Reactions were stopped with EDTA and the DNA spread for electron microscopy by the formamide technique. (a) Total length 2.17 μ m (equal to unit length); length of loop, 0.11 μ m. (b) a, Total length, 0.80 μ m; length of loop, 0.53 μ m. b, Total length, 0.43 μ m; length of loop, 0.35 μ m. The bar represents 0.5 μ m.

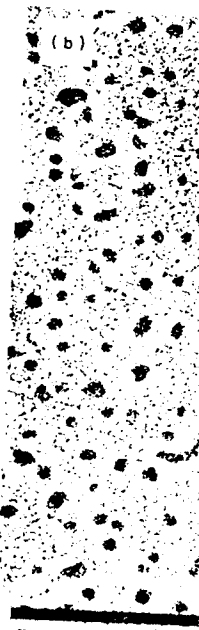
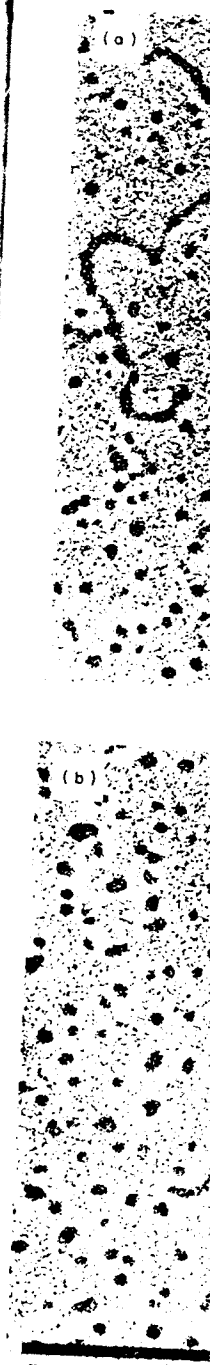
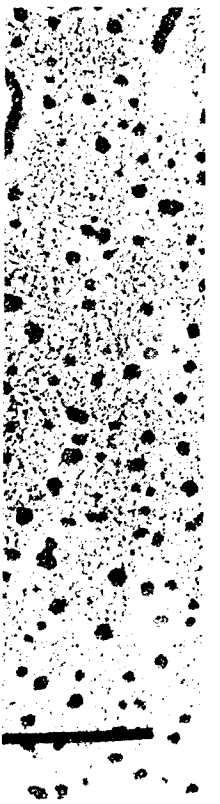
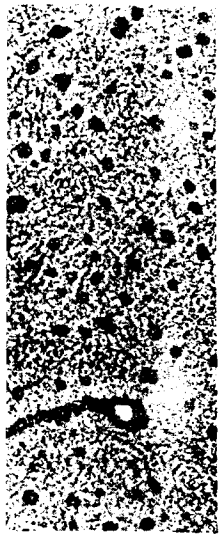


FIG. 5. Examples of electron micrographs of intermediates and products in the reaction of *Eco*B with ϕ 101 RF DNA. Reactions were stopped with EDTA and the DNA spread for electron microscopy by the formamide technique. (a) Total length, 0.89 μ m; length of loop, 0.35 μ m. (b) Total length, 0.89 μ m; length of loop, 0.35 μ m. The bar represents 0.5 μ m.



s in the reaction of *EcoB*
EcoB were incubated at
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 length); length of loop,
 length, 0.43 μm ; length of

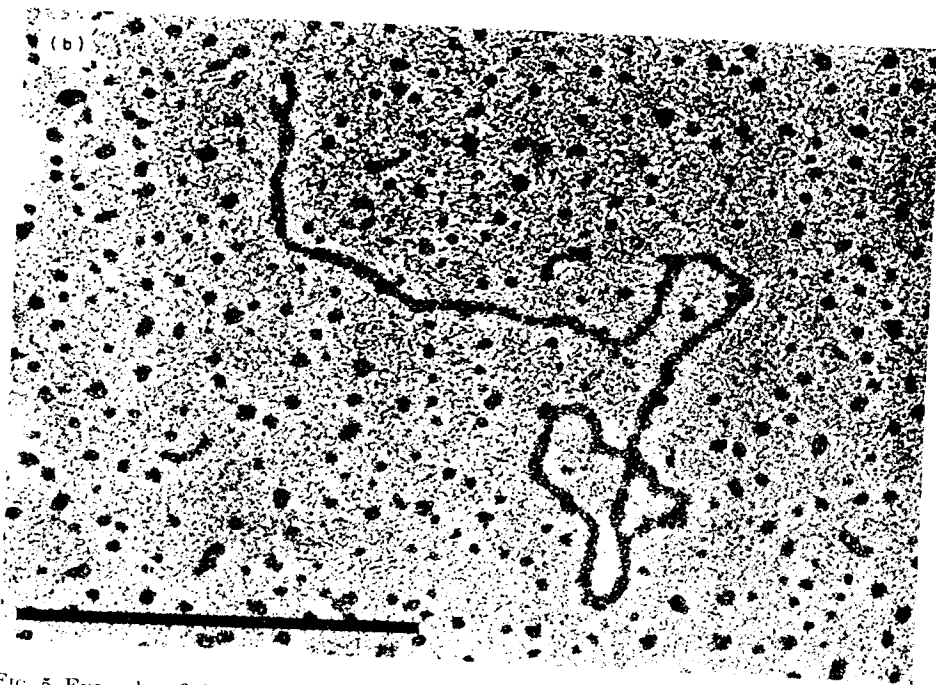
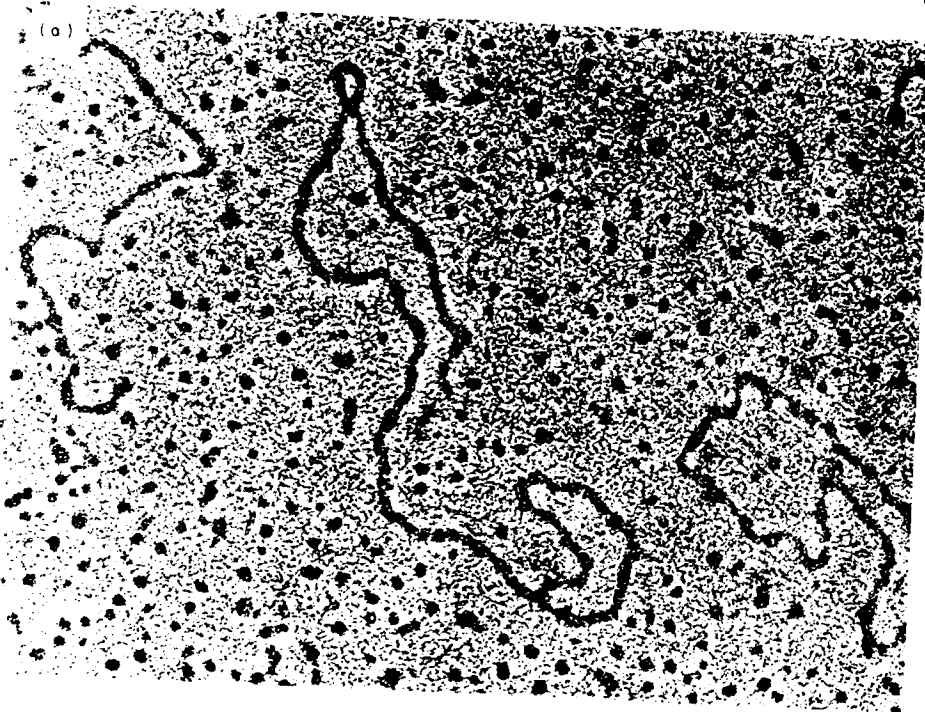


FIG. 5. Examples of electron micrographs of intermediates and products in the reaction of *EcoB* with ϕ (1101) RF DNA. Enzyme reactions and electron microscopy were as described in the legend to Fig. 4. The length of the molecules shown are 2.17 μm (a) and 2.18 μm (b); the loops are 0.69 μm (a) and 0.89 μm (b). The bar represents 0.5 μm .

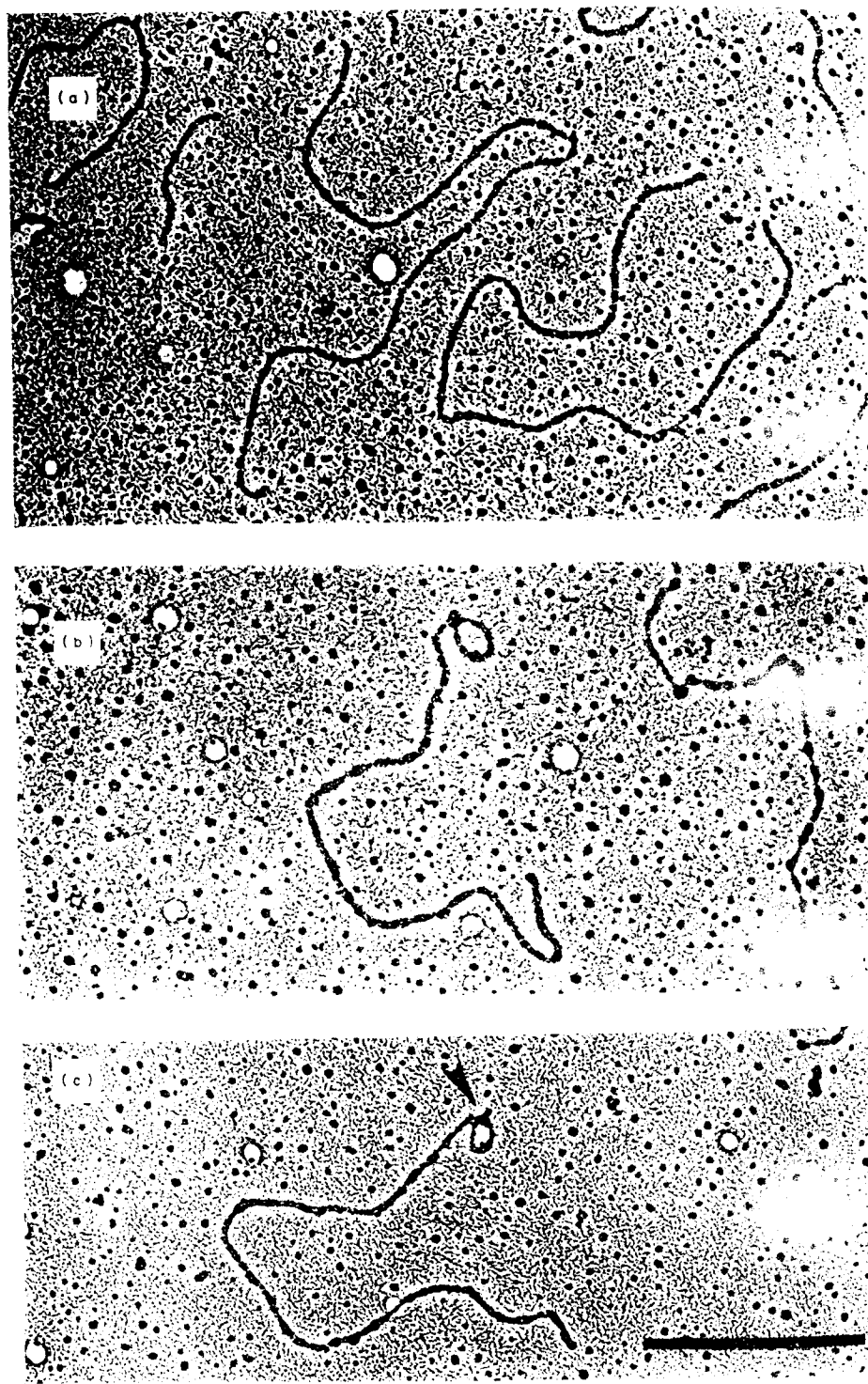


FIG. 6. Examples of electron micrographs of *HincII*-treated fd101 RF DNA after incubation with *EcoB*. fd101 RF DNA was converted to the linear form by digestion with *HincII*, incubated with *EcoB* for 5 min and mounted for electron microscopy by the formamide technique. (a) *HincII*-treated fd101 RF DNA, not subsequently treated. (b) *HincII*-treated fd101 RF DNA, not subsequently treated, subsequent incubation with *EcoB*; total length of molecule, 2.03 μm ; length of loop, 0.31 μm . (c) *HincII*-treated fd101 RF DNA, not subsequently treated, subsequent incubation with *EcoB*; total length of molecule, 2.07 μm ; length of loop plus tail, about 0.29 μm . The bar represents 0.5 μm .

Number of loops



FIG. 7. Summary of loop formation. Full length molecules are 2.0 μm . (a) fd101, (b) the wild type.

We have observed loops of fd101 DNA after incubation with *EcoB* in the presence of *HincII* nuclease activity. In control experiments, in the absence of *EcoB*, fd101 DNA was incubated with DNase I and no loops were observed. *EcoB* endonuclease activity prevents *HincII* from cleaving the DNA at the loop-tail junction. The loops observed are normal intermolecular loops. Further evidence in the form of loops is observed under conditions that allow the formation of loops (Williams, 1977). With *HincII* treatment, molecules that were maintained in the looped form were measured the length of the loop to be 920 to 980 base-pairs. The tail of the molecule is visible at the loop-tail junction. The loops observed tend to form a biased size distribution.

(a) A model for the formation of loops.

HincII-treated fd101 RF DNA. The loop formed from the end was not at all typical. It is possible that the enzyme can cleave the DNA at the loop-tail junction. This would then be consistent with the observation that the loops are shorter than roughly 1000 base-pairs.

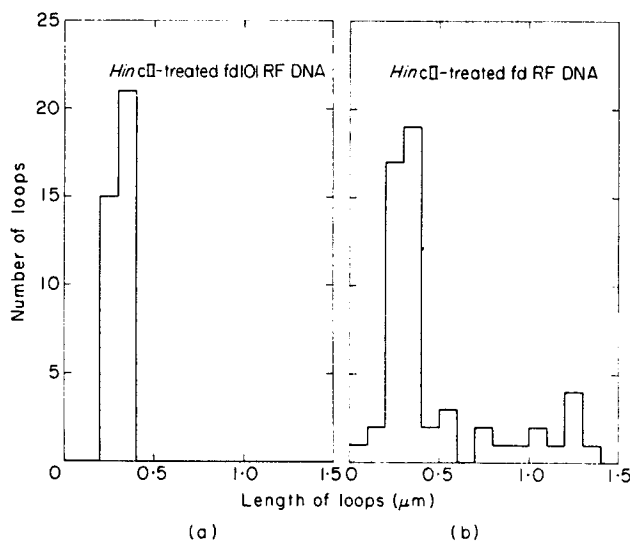


FIG. 7. Summary of loop sizes observed with fd replicative form DNAs treated with *HincII*. Full length molecules are $2.1 \pm 0.1 \mu\text{m}$. The total number of molecules measured was 36 and 56 for the fd101 (a) and the wild-type fd DNAs (b), respectively.

(d) Requirements for loop formation

We have observed loops at the termini of 5 to 13% of the DNA molecules visualized after incubating *EcoB* with DNA under conditions which allow *EcoB* endonuclease activity. In contrast, loops have not been observed after incubating DNA in the absence of *EcoB*, when AdoMet was omitted from the reaction or when *EcoB* was incubated with DNA that was B-modified (Table 2). All of these latter conditions prevent *EcoB* endonuclease activity, implying that the structures that we have observed are normal intermediates and/or products of the *EcoB* endonuclease reaction.

Further evidence in this regard has been obtained by electron microscopy under conditions that allow the direct visualization of enzyme molecules bound to DNA (Williams, 1977). With *HincII*-treated fd101 RF DNA, we again found DNA molecules that were maintained in a looped structure; furthermore, for ten molecules measured the length of the loop plus tail was between 0.30 and 0.32 μm , equivalent to 920 to 980 base-pairs. In addition, what appeared to be enzyme molecules were visible at the loop-tail junctions of each measured molecule (Fig. 9). All molecules in obvious loops appeared to have these enzyme-like structures, although such molecules tend to form a biased sample.

4. Discussion

(a) A model to explain the formation of the duplex loops

HincII-treated fd101 RF DNA, with a recognition site less than 1000 base-pairs from the end was not at all obviously cleaved by *EcoB* (Fig. 3(a), lane E). If we assume that the enzyme can cleave only to the left of the sequence, this observation would then be consistent with previous reports that the enzyme does not cleave molecules shorter than roughly 1000 base-pairs and does not seem to form products smaller

DNA after incubating with *HincII*, incubated technique. (a) *HincII*-treated fd101 RF DNA after subsequent incubation with *EcoB*. (b) shows the approximate length of loop plus tail, about

Fig. 8. Examples of electron micrographs of *HindIII*-treated wild-type (a, b) and mutant (c, d) DNA after incubating with *EcoRI*. DNA was prepared for electron microscopy by the formamide technique. (a) Total length of molecule, 2.17 μm ; length of loop, 0.29 μm . (b) Total length of molecule, 1.14 μm ; length of loop, 0.32 μm . (c) Total length of molecule, 2.16 μm ; length of loop, 1.29 μm . (d) Total length of molecule, 1.28 μm ; length of tail (arrow), 0.02 μm . The bar represents 0.25 μm .

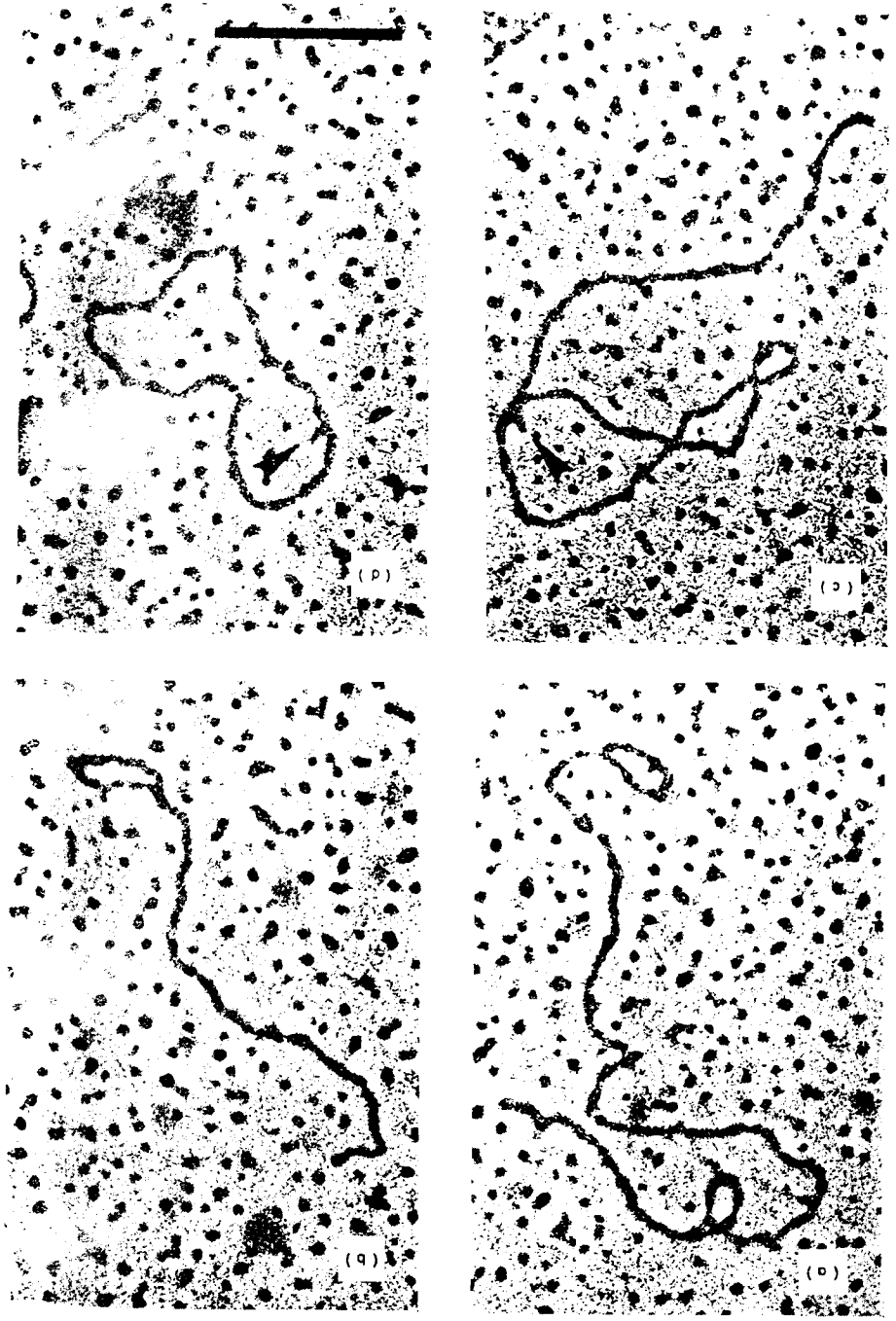
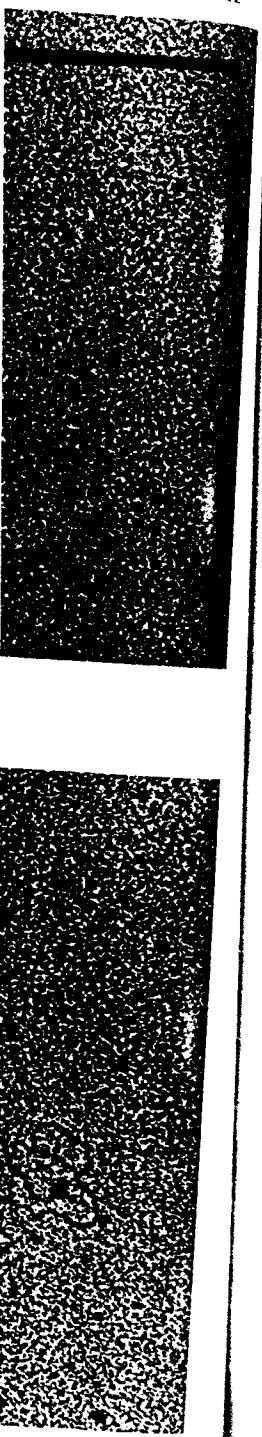


Fig. 9. Electron micrographs of DNA molecules at the junction of the loop plus the length of the loop plus the length of the tail (arrow) after incubating with *EcoRI*. DNA was prepared for electron microscopy by the formamide technique. (a) Total length of molecule, 1.14 μm ; length of loop, 0.32 μm . (b) Total length of molecule, 1.28 μm ; length of tail (arrow), 0.02 μm . The bar represents 0.25 μm .





fd101 RF DNA after iminoformamide treatment. Length of molecule, $1.14 \mu\text{m}$; length of tail (arrow), about $0.02 \mu\text{m}$.

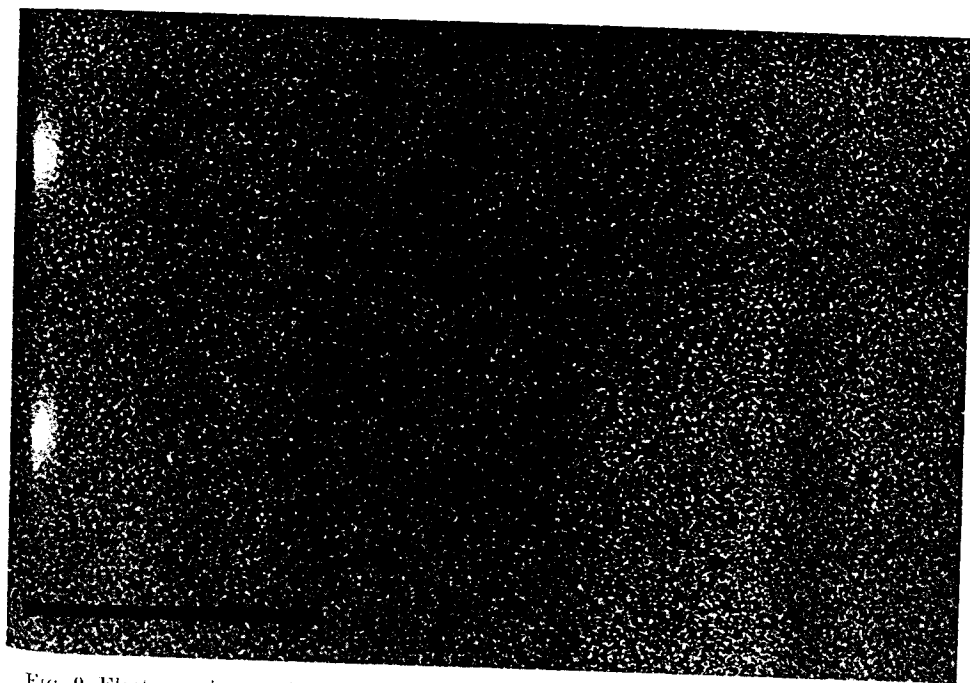
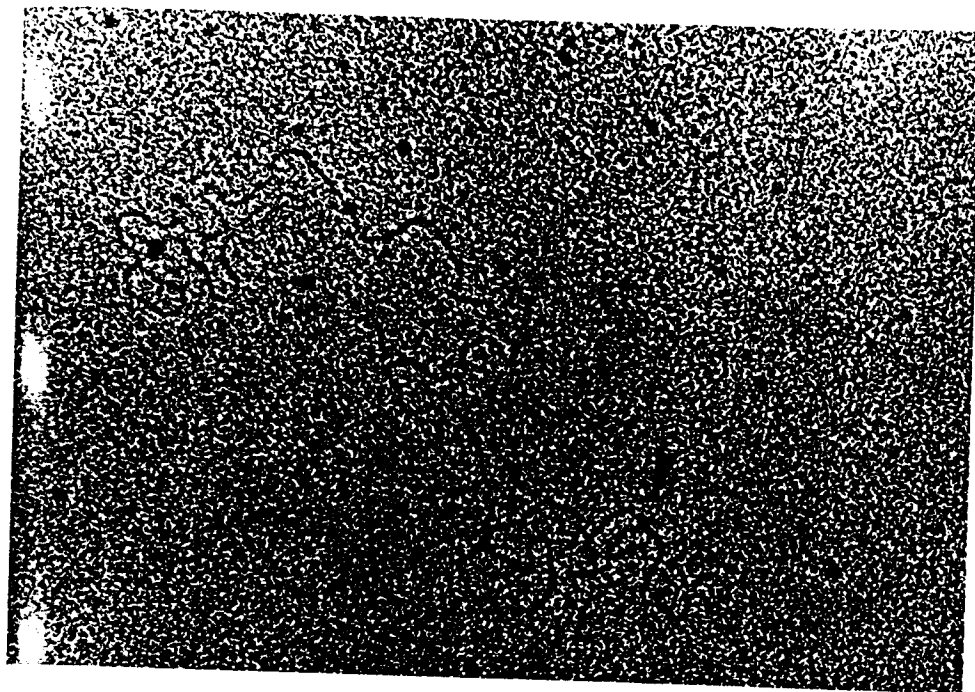


Fig. 9. Electron micrograph of *HincII*-treated fd101 RF DNA after treatment with *EcoB* and spread for electron microscopy under conditions which allow visualization of enzyme molecules bound to the DNA. fd101 RF DNA was treated with *HincII* and subsequently incubated with 0.2 unit of *EcoB* for 1 min. The reaction was stopped with EDTA and the DNA was spread for electron microscopy by the polylysine method. In the molecules shown, the arrows indicate the enzyme molecules at the junction of a loop and tail. The total length of the molecules is $2.13 \mu\text{m}$; the length of the loop plus tail is $0.31 \mu\text{m}$ in each case. The bar represents $0.25 \mu\text{m}$.

than that size (Horiuchi *et al.*, 1974; Linn *et al.*, 1974). We therefore hypothesize that *EcoB* travels along the linear fd101 RF DNA only from one side of the recognition site, sB_2 , specifically on the 5' side of the sequence, 5' . . . T-G-A-(N)₈-T-G-C-T . . . 3' (see Fig. 1). Therefore, in this case the enzyme would not be able to travel the necessary distance prior to cleavage, and thus does not cleave the DNA.

Generalizing both this sequence-dependent directional constraint and the minimum travel distance requirement to each of the substrates studied in this paper, we can predict the restriction potential of each recognition site as diagrammed in Figure 10. These various restriction potentials are totally consistent with the observed restriction patterns (Fig. 3). Moreover, the formation of the duplex loops that were observed by electron microscopy as well as their length distribution (see Table 2) and structure, can be explained in each case if we make one further assumption: that *EcoB* remains bound to a part of the recognition site while it tracks from the recognition site to a site of DNA strand cleavage in the direction defined by the recognition site orientation, as noted in Figure 10. Thus for the circular DNA substrates, the variable sizes of the observed loops would reflect the non-unique location of the cleavage sites with respect to the recognition sites. With the linear DNA substrates, the population of loops measuring 850 to 980 base-pairs (Fig. 7) would arise by *EcoB* binding to sB_2 and tracking toward the proximal terminus of the molecule (while remaining bound to sB_2). In the case of the linear wild-type DNA, the appearance of this class of loops at the termini of unit length molecules (Fig. 8(a)) as opposed to fractional length molecules (Fig. 8(b)) would depend on whether restriction had also occurred from sB_1 (cf. Fig. 10). Finally, the variable-sized loops uniquely observed with the linear wild-type DNA (Fig. 7) would occur as a result of restriction by *EcoB* after tracking

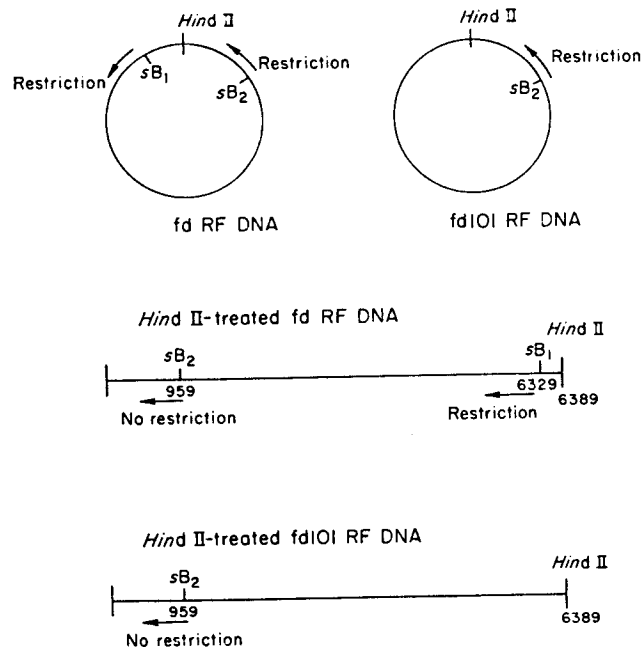


FIG. 10. Schematic representation of the DNA substrates used. The locations of the sB sites are given relative to the unique *Hind*II site. The direction of travel of *EcoB* is shown in all cases as being from the right to left of the sequence 5' . . . T-G-A-(N)₈-T-G-C-T . . . 3'. Restriction is shown to occur only when the enzyme is able to travel more than 1000 base-pairs.

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We would like *EcoB* tracking and results (Eskin & I summarize a hypothesis (Fig. 11). Initially, complex at that site (1975) we presume molecule might have specific nucleotide are separated by remaining on the of distortion of the D Several observations the recognition site enzyme does not track by electron microscopy their presence at the (pairs) appeared to molecules bound to complex was isolated (1974). A plausible

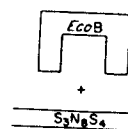


FIG. 11. Schematic representation of modified DNA. S_3 and S_4 represents the intervening scheme is presented in the

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from *sB*₁, but never *sB*₂ (cf. Fig. 10). In this case, the small tail of 60 to 100 nucleotides observed on these molecules (Fig. 8(c) and (d)) would be the region of the linear molecule between *sB*₁ and the proximal DNA terminus (Fig. 10).

(b) *Scheme for EcoB endonuclease activity*

We would like to combine the model for loop formation, the directionality of *EcoB* tracking and the minimum travel requirement with some of our previous results (Eskin & Linn, 1972a,b; Linn *et al.*, 1974; Kimball & Linn, 1976) in order to summarize a hypothetical sequence of events by which *EcoB* might cleave DNA (Fig. 11). Initially, the enzyme binds to the recognition site and forms a specific complex at that site. (By analogy with the similar enzyme from *E. coli* K (Yuan *et al.*, 1975) we presume that this process requires AdoMet.) We suggest that the enzyme molecule might have two DNA binding sites, perhaps each binding to one of the two specific nucleotide sequences of the recognition site. Since these nucleotide sequences are separated by about one turn of the DNA helix, the enzyme could bind while remaining on the outside of the helix, hence alleviating any requirement for structural distortion of the DNA.

Several observations might be taken to suggest that, after forming this complex at the recognition site, the enzyme undergoes some sort of transformation. Firstly, the enzyme does not turn over as a DNase (Eskin & Linn, 1972a); secondly, when viewed by electron microscopy, enzyme molecules in the process of tracking (as judged by their presence at the junction of a loop and tail with a combined length of 950 base-pairs) appeared to be about 20% smaller in diameter than unattached molecules or molecules bound randomly to the DNA; finally, when the *EcoB*-restricted DNA complex was isolated, it did not contain all of the enzyme subunits (Linn *et al.*, 1974). A plausible idea for this transformation would be that, after forming the

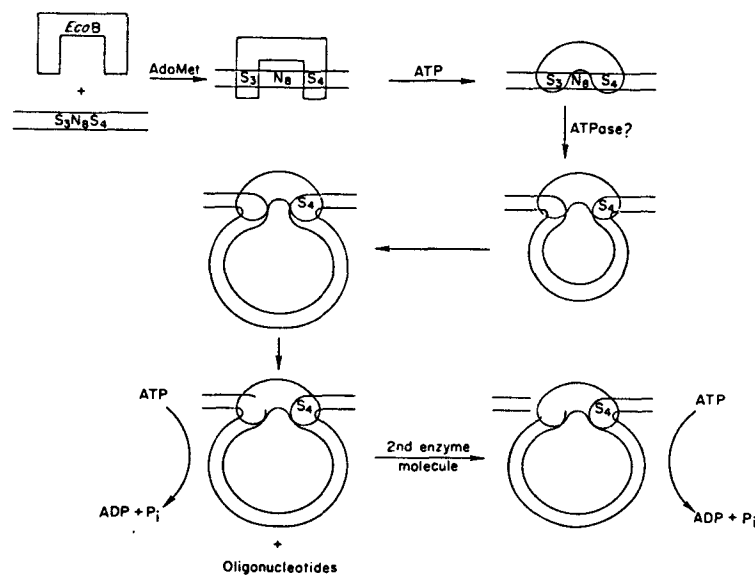


FIG. 11. Schematic representation of a hypothetical sequence by which *EcoB* cleaves unmodified DNA. *S*₃ and *S*₄ represent the specific trimeric and tetrameric regions of *sB*, while *N*₈ represents the intervening 8 non-specific base-pairs. A more detailed description of this reaction scheme is presented in the text.

specific complex at the recognition site, the enzyme loses one or more subunits. It is likely that this alteration of the enzyme would occur only at an unmodified recognition site, and studies with *EcoK* suggest that it may also require the presence of ATP (Bickle *et al.*, 1978).

We propose that the alteration in the subunit arrangement of *EcoB* has two effects. In this new form, the binding of enzyme to the specific trimeric portion of the recognition site is weakened, and therefore becomes more flexible. Also, the enzyme can now act as an ATPase and, initially at least, uses the energy derived from ATP hydrolysis to track from the recognition site towards a nucleolytic site†. The direction in which the enzyme tracks is determined by the orientation of the recognition site. During tracking, the enzyme would remain bound to the specific tetrameric portion of the recognition site, looping out regions of duplex DNA.

Endonuclease activity appears only to occur after the enzyme has travelled more than about 1000 base-pairs (Fig. 3; Horiuchi *et al.*, 1974; Linn *et al.*, 1974). The site of cleavage is apparently random, both with respect to nucleotide sequence and (within a constraint of roughly 1000 to 6000 base-pairs) to the distance from the recognition site. Although we do not know what triggers *EcoB* nuclease activity, one possibility is that factors such as local distortions of the DNA helix might induce DNA cleavage by acting to prevent the formation of the duplex loop structure.

EcoB cleaves one strand of the DNA, introducing a gap of about 75 nucleotides with the release of acid-soluble oligonucleotide (Kimball & Linn, 1976). Thus, when enzyme is not in excess, form II DNA accumulates as a limit product after treatment of form I DNA with *EcoB* (Fig. 3(b)). Subsequently, the enzyme continues to hydrolyze ATP, while remaining bound to the DNA in a complex that also contains AdoMet (Linn *et al.*, 1974). We propose that by remaining bound to the DNA in this way, the enzyme maintains the recognition and cleavage sites in close approximation, thereby providing a structure that can be recognized by a second enzyme molecule which subsequently can act to complete the double-strand break. The continuation of ATPase even after this event may be an *in vitro* artifact, reflecting the absence of regulatory factors such as the enzymes responsible for the further degradation of restricted DNA (Simmon & Lederberg, 1972). Alternatively, the continued ATP hydrolysis after completion of endonuclease activity might reflect an additional *in vivo* function for the enzyme of which we are presently unaware.

(c) Speculations on the function of *EcoB*

The integration of some transposable elements, such as *Tn10*, occurs preferentially within certain regions of the bacterial chromosome, but the precise site of integration within that region is apparently random (Kleckner, 1977). Furthermore, the integrative recombination of phage λ DNA *in vitro* can be completed without the action of *E. coli* NAD-dependent polynucleotide ligase (Mizuuchi *et al.*, 1978). The proteins involved in these recombination events have not been identified; however, it is intriguing that *EcoB* can make a single endonucleolytic cleavage at varying distances in a particular direction from a defined site and that the active ATPase complex of *EcoB* can form large, relatively stable aggregates containing several molecules each

† Although most ATP hydrolysis *in vitro* occurs after DNA cleavage (Eskin & Linn, 1972b), the observation that *HincII*-treated fd101 RF DNA is not randomly cleaved by *EcoB* (Fig. 3), but does support *EcoB* ATPase activity (Table 1) suggests that ATP may be hydrolyzed prior to nucleolytic action.

of enzyme and DNA (but not absolutely). DNA ligase activity. This putative ligase property could be in a form of non-hor-

We are grateful to electron microscopy, phage fd DNA prior to the National Institute. One of us (J. R.) is a

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of enzyme and DNA (Linn *et al.*, 1974). Moreover, we have found that highly purified (but not absolutely homogeneous) preparations of *EcoB* contain an NAD-independent DNA ligase activity (B. Endlich, J. Rosamond & S. Linn, unpublished observations). This putative ligase activity, together with the peculiar nuclease activity and aggregation property could imply a role for *EcoB* (or an evolutionary precursor of *EcoB*) in a form of non-homologous recombination.

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