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The DNA Restriction Endonuclease of *Escherichia coli* B

II. FURTHER STUDIES OF THE STRUCTURE OF DNA INTERMEDIATES AND PRODUCTS*

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The DNA intermediates and final products formed by the Type I restriction endonuclease, *Eco*B, were further characterized. DNA cleaved on only one strand (hemi-restricted DNA) contains gaps of approximately 70–100 nucleotides, while the fully restricted products contain 3′-single-stranded tails averaging approximately 70–100 nucleotides for each strand cleaved. The gaps and tails are formed with the release of an equal number of nucleotides as small oligonucleotides that are soluble in acid. After purification, neither the hemi-restricted nor the fully restricted DNAs are cleaved again by *Eco*B. There is no apparent specificity for which strand of a duplex is initially cleaved by *Eco*B, nor is there specificity with respect to the composition of the 3′-terminal nucleotide formed on the DNA or the 3′- or 5′-terminal nucleotides of the acid-soluble oligonucleotides released during DNA cleavage. The structure formed at the 5′ terminus of the DNA product which blocks phosphorylation by T4 polynucleotide kinase remains unknown, but its removal with phage λ exonuclease allows at least some reutilization of recognition sites by *Eco*B as well as phosphorylation of the newly formed 5′ termini. To explain the complex mechanism of this enzyme, it is suggested that the unidentified 5′-tails prevent wasteful rerestriction from occurring, whereas the 3′-single-stranded tails create DNA which, when nonhomologous to chromosomal DNA, cannot be rescued because such tails are not substrate for DNA polymerases. However, when homologous chromosomal DNA exists, the randomly cleaved large fragments with these tails can easily be assimilated by *rec*A-mediated genetic recombination, thus stimulating DNA exchange between related organisms.

In the presence of *S*-adenosylmethionine (AdoMet[1]) and ATP, a single *Eco*B enzyme cleaves a single strand of unmodified DNA several thousand nucleotides to one side of the recognition site (1, 2). Since *Eco*B catalyzes only one strand cleavage, cleavage of the opposite strand is then catalyzed by a second enzyme molecule (3). A strand cleavage is accompanied by the release of approximately 70–100 nucleotide as small, acid-soluble oligonucleotides (4). Fully restricted DNA is known to be susceptible to degradation by nucleases which degrade DNA containing 3′-hydroxyl and 5′-phosphoryl termini (3); however, the termini of these products are refractory to $^{32}PO_4$ labeling by polynucleotide kinase even following phosphatase treatment (3). In this paper, the DNA strand cleavage event, the resulting products, and the nature of the termini formed by *Eco*B are studied further and a comprehensive model for *Eco*B action based on our knowledge to date is presented.

EXPERIMENTAL PROCEDURES

Materials

Enzymes—Exonuclease I was the hydroxylapatite fraction of Lehman and Nussbaum (5); 1 unit renders 10 nmol of single-stranded DNA acid-soluble in 30 min at 37 °C. *rec*BC DNase (*Escherichia coli* exonuclease V) was the DNA-cellulose fraction (6); 1 unit hydrolyzes 1 nmol of duplex *E. coli* DNA in 30 min at 37 °C and pH 7 in the presence of 60 μM ATP. Polynucleotide kinase and calf intestinal alkaline phosphatase were from P-L Biochemicals. Phage λ exonuclease was purchased from Boehringer Mannheim, and spleen exonuclease and micrococcal nuclease were from Sigma; units are those of the manufacturer. Bacterial alkaline phosphatase, pancreatic DNase I, and snake venom phosphodiesterase were purchased from Worthington. The alkaline phosphatase was further purified on DEAE-cellulose, according to Weiss *et al.* (7). Terminal deoxynucleotidyl transferase was a gift of Dr. R. Ratliff, Los Alamos Scientific Laboratories. *Neurospora crassa* nuclease was the BioRex 70 fraction purified according to the procedure of Linn (8). Novikoff hepatoma DNA polymerase β was fraction IV (9); 1 unit incorporates 10 nmol of nucleotide into activated salmon sperm DNA in 30 min at 37 °C.

DNAs—^{3}H-, ^{14}C-, and ^{32}P-labeled unmodified phage DNAs were prepared according to Goldmark and Linn (10) with the *E. coli* K strain HfrC6-2. Unlabeled calf thymus and salmon sperm DNAs were purchased from Sigma and dialyzed extensively prior to use.

Other Materials—*S*-[*Ade*-^{3}H]Adenosylmethionine (6400 cpm/nmol) was a gift of Dr. H. Hogenkamp, University of Minnesota, and was equally as efficient as the unlabeled compound as an *Eco*B ATPase cofactor. [α-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]dTTP, [α-^{32}P]dGTP, and [γ-^{32}P]ATP were purchased from Amersham Corp. Other materials were described previously (2, 3, 10, 11).

Methods

Assays of *Eco*B were described in the previous paper (1).

The transfer of DNA fragments to nitrocellulose was accomplished by the method of Southern (12) with 0.9 M NaCl, 0.09 M sodium citrate used as the transferring solvent. Hybridization of DNA probes was carried out according to the protocol of Wahl *et al.* (13) with the following modifications: a sheet of nitrocellulose was used in place of DBM (diazobenzyloxymethyl) paper and the acid-alkali treatment was omitted. Hybridizations were carried out at 55 °C for 48 h.

Sucrose gradient sedimentation of DNA was at 4 °C through 5–20% sucrose gradients which contained 0.25 M NaOH, 5 mM EDTA or 0.25 M NaCl, 0.02 M Tris·HCl, 5 mM EDTA, pH 8.2, for alkaline or neutral pH sedimentation, respectively. Alkaline samples were neutralized before liquid scintillation counting. Determination of DNA rendered acid-soluble and liquid scintillation counting were as described by Goldmark and Linn (10). Other methods were described previously (1-3, 10, 11).

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[1] The abbreviations used are: AdoMet, *S*-adenosylmethionine; RF, replicative form.

RESULTS

[I]nvestigation of the Hemi-restricted DNA Product

[Pre]sence of a Gap in Hemi-restricted DNA—The observa[tion t]hat acid-soluble material is released during the restric[tion p]rocess (4) suggests that the release of oligonucleotides [may] be an inherent part of a strand cleavage event. If this [is s]o, one would expect to find a gap in the hemi-restricted [DNA] intermediate, the single-stranded DNA segment of [which] would be expected to be susceptible to the single-[stran]d-specific endonuclease from *N. crassa*, which cleaves [single]-stranded regions within DNA duplexes. Therefore, [hemi-]restricted Form II fd 101 RF DNA was separated by [agaro]se gel electrophoresis from unrestricted Form I DNA [and f]ully restricted Form III DNA, and then exposed to the [*N. cr*]*assa* enzyme. Indeed, the nuclease converted hemi-re[strict]ed DNA to Form III (Fig. 1), implying that this DNA [conta]ined a gap rather than a simple nick.

[To] estimate the length of the putative gap, the incorpora[tion o]f ^{32}P-labeled nucleotides into the hemi-retricted DNA [by N]ovikoff hepatoma DNA polymerase β was monitored. [As a] polymerase control substrate, fd 101 RF Form I DNA [was t]reated with *N. crassa* nuclease, which puts one small gap [into] a supercoiled molecule (14). Under the conditions used, [the β]-polymerase preparation did not contain detectable 3′-[or 5′]-exonuclease activities and did not catalyze significant [stran]d displacement synthesis (9). With the hemi-restricted [DNA], 60–70 nucleotides were incorporated when either [α-[^{32}P]d]TTP or [α-^{32}P]dGTP were utilized to monitor incorpo[ratio]n; conversely, roughly five nucleotides were incorporated [into] the DNA treated with the *N. crassa* nuclease (Fig. 2). [The] former number is in good agreement with the estimation [that] approximately 70 nucleotides are released as acid-soluble [mate]rial for each *Eco*B strand scission (4) and with the finding [(see] below) that restricted DNA contains 3′-tails of approxi[mate]ly 60–80 nucleotides. In conclusion, a gap of roughly 70 nucleotides does appear to be present in the hemi-restricted intermediate.

[F]IG. 1. **Action of *N. crassa* nuclease on hemi-restricted [DN]A.** After treatment of fd 101 RF DNA with a limited amount of [Eco]B, the Form II DNA product was isolated by electrophoresis on [a]garose gel, electroelution into a dialysis membrane, and extensive [dial]ysis against 5 mM Tris·HCl, pH 7.5. Reactions with *N. crassa* [nuc]lease (40 μl) contained 1 nmol of hemi-restricted DNA, nuclease [as n]oted, 90 mM Tris·HCl, pH 7.5, and 10 mM $MgCl_2$. After 30 min [at 3]7 °C, reactions were terminated by adding EDTA to 20 mM and [chil]ling to 0 °C. Samples were analyzed by electrophoresis on a 1% [aga]rose gel. *Lanes 1–4*, hemi-restricted DNA treated with 0.0, 0.1, [0.2,] or 0.5 unit of *N. crassa* nuclease, respectively; *Lane 5*, 1 nmol of [fd 1]01 RF DNA incubated with 0.2 unit of *Eco*B to provide Form II [and] III markers; *Lane 6*, the untreated Form I DNA substrate.

FIG. 2. **Ability of hemi-restricted DNA to serve as a DNA polymerase-β substrate.** Twenty nmol of fd 101 RF [^{3}H]DNA (3240 cpm/nmol) was digested with 2 units of *Eco*B for 15 min. After chilling and adding EDTA to 20 mM, the Form II DNA was isolated as in Fig. 1. fd 101 RD DNA containing one single-strand scission was also produced by incubation of 10 nmol of DNA with 2 units of *N. crassa* nuclease in 90 mM Tris·HCl, pH 7.5, 19 mM $MgCl_2$ for 15 min at 37 °C. To measure nucleotide incorporation, 100-μl reactions containing 1 nmol of hemi-restricted or control DNA, 25 mM Tris·HCl, pH 8.4, 10 mM $MgCl_2$, 40 μM of each unlabeled dNTP, either 15 μm [α-^{32}P]dTTP (420 cpm/pmol) or 15 μM [α-^{32}P]dGTP (1430 cpm/pmol), and DNA polymerase β as indicated were incubated for 30 min at 37 °C. Reactions were terminated by the addition of 100 μl of 0.1 M $Na_4P_2O_7$, 1 mg/ml bovine serum albumin, and then precipitated with 0.5 ml of 10% trichloroacetic acid. After 20 min at 0 °C, acid-precipitable material was collected onto GF/C glass fiber filters and extensively washed with 0.1 M $Na_4P_2O_7$, 1 N HCl. The filters were dried with a heat lamp and counted in a scintillation counter. ●, hemi-restricted DNA; ○, DNA treated with *N. crassa* nuclease.

Selection of the Strand Initially Cleaved—Since each *Eco*B molecule catalyzes the cleavage of only one strand and, since the DNA is translocated by the enzyme only from the left side of the asymmetric sequence 5′-TGA(N$_8$)TGCT-3′ (2, 15, 16), there could exist a preference or specificity for which strand is initially cleaved. To test for such a preference, Form I fd 101 RF DNA was partially digested with *Eco*B and the Form II product was purified. This DNA was then denatured to yield its single-stranded linear and circular components; then these were separated by agarose gel electrophoresis and transferred to a sheet of nitrocellulose by the Southern trans-

fer technique (12) and probed with fd viral (plus) DNA. If the plus strand of the RF DNA molecule were cleaved preferentially, this strand would be manifested as a linear strand which would not hybridize to the plus strand probe. Instead, hybridization to the circular minus strand would be seen. The opposite pattern would be seen if the minus strand of the RF DNA would have been preferentially cleaved. If there were no preference, the bands representing the circular and linear molecules would each contain minus strands and would therefore each hybridize to the plus strand probe. In fact, both bands hybridized efficiently to the probe (Fig. 3) with no quantitative preference (Table I). Therefore, there is no apparent strand selectivity by *Eco*B in generating the initial

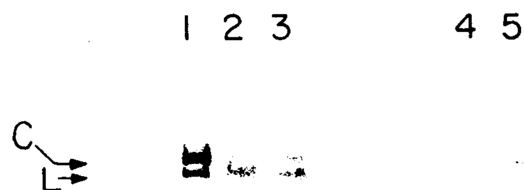

FIG. 3. **Specificity of initial strand cleavage.** Twenty nmol of ^{3}H-labeled fd 101 RF DNA was partially digested with 1 unit of *Eco*B for 15 min; then the Form II DNA was isolated as in Fig. 1 and concentrated under vacuum. Two nmol of the DNA in 20 μl was denatured by the addition of 4 μl of 1.0 N NaOH, incubated for 5 min at 25 °C, neutralized with 1.0 N HCl, and quick-chilled. A sample of Form III DNA produced by *Hinc*II digestion of fd 101 RF DNA was also denatured by the same procedure to serve as a marker for linear single strands and as a hybridization control. Two samples of the denatured, *Eco*B-treated DNA, and one each of the denatured *Hinc*II-DNA and the ^{14}C-labeled phage DNA probe containing predominantly circular molecules were run on a 1% agarose gel. The DNA was transferred over 2 days to a nitrocellulose sheet by the Southern transfer method (12) and the nitrocellulose sheet was cut to separate the duplicated DNA samples. One set of samples was hybridized to a ^{14}C-labeled fd DNA probe (5100 cpm/nmol) and the other to a ^{32}P-labeled fd DNA probe as described under "Methods." Each hybridization mixture contained approximately 5×10^5 cpm of probe DNA. The hybridized samples were extensively rinsed in 0.1% sodium dodecyl sulfate plus 2 × SSC and then successively with rinses while decreasing the SSC to a final concentration of 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). The rinsed nitrocellulose sheets were dried overnight and autoradiography was carried out at −80 °C for 1 week with Kodak XR-5 film. *Lane 1*, ^{14}C-labeled fd DNA probe; *Lane 2*, denatured Form III DNA generated with *Hinc*II and hybridized to the [^{14}C]DNA probe; *Lane 3*, denatured Form II DNA generated by *Eco*B and hybridized to the [^{14}C]DNA probe; *Lanes 4* and *5* are the same as *Lanes 2* and *3*, respectively, except with the [^{32}P]DNA probe. *C* and *L* designate the positions of the single-stranded circular and linear DNA molecules, respectively.

TABLE I

Specificity of initial strand cleavage

The bands of *Lane 3* of the nitrocellulose sheet shown in Fig. 3 were cut out and counted by liquid scintillation. The ^{3}H-labeled fd 101 RF DNA had 3500 cpm/nmol; the ^{14}C-labeled fd DNA probe, 5100 cpm/nmol.

DNA band	^{3}H-labeled sample DNA	^{14}C-labeled probe DNA
	nmol	
Circular	0.52	0.25
Linear	0.50	0.23

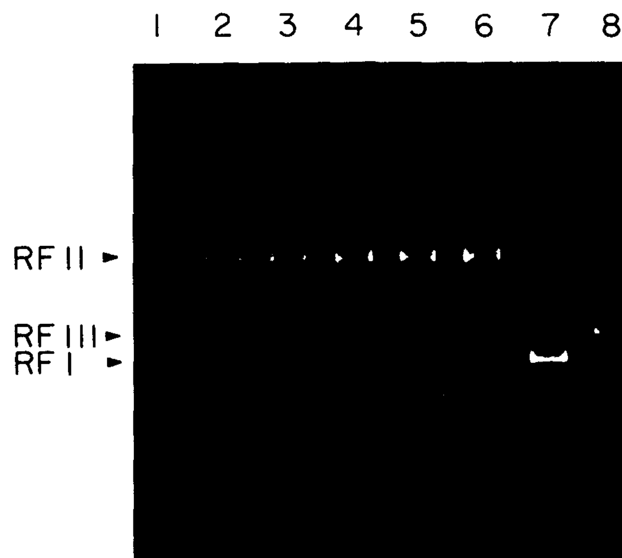

FIG. 4. **Effect of *Eco*B upon purified, hemi-restricted DNA.** fd 101 RF DNA (10 nmol) was incubated with 1 unit of *Eco*B for 15 min at 37 °C; then the reaction was terminated by the addition of ⅕ volume of 1% sodium dodecyl sulfate and heating for 30 min at 65 °C. The Form II DNA was then purified as described in Fig. 1 and concentrated by evaporation under a stream of nitrogen gas. Aliquots (0.5 nmol) were then digested with *Eco*B as indicated in 25-μl reaction mixtures for 15 min at 37 °C. For comparison, 0.5 nmol of untreated fd 101 RF DNA was simultaneously digested with *Eco*B. *Lanes 1–6*, purified, hemi-restricted fd 101 RF II DNA treated a second time with 0.0, 0.01, 0.02, 0.04, 0.06, and 0.10 unit of *Eco*B, respectively; *Lanes 7* and *8*, untreated fd 101 RF DNA treated with 0.0 and 0.10 unit of *Eco*B, respectively.

cleavage, in spite of the asymmetric translocation.

Action of EcoB on Hemi-restricted DNA—If DNA were to be hemi-restricted by *Eco*B and then purified free of the endonuclease, would fresh *Eco*B cleave this substrate efficiently? Specifically, would it recognize the gap introduced by the first *Eco*B molecule and act at this site, would it introduce another random single-strand cleavage, or would it not act at all? To answer this question, fd 101 RF DNA was partially digested with *Eco*B and then exposed to sodium dodecyl sulfate (SDS) at 65 °C. The hemi-restricted Form II product was purified by agarose gel electrophoresis and incubated once more with *Eco*B. No conversion to linear molecules was observed with levels of *Eco*B which gave substantial or total conversion of untreated supercoiled fd 101 RF DNA to Form III (Fig. 4). Similar results were found with phenol-extracted hemi-restricted DNA.[2] Since Form II DNA produced by *N. crassa* nuclease is an efficient substrate for digestion by *Eco*B (see below), the resistance of purified hemi-restricted DNA is not a consequence of its lack of supercoiling. Moreover, DNA digested with *Eco*B is still an efficient substrate for the *Eco*B methylase (17), suggesting that the recognition site should be accessible to *Eco*B restriction enzyme molecules. On the other hand, fd 101 RF DNA which had been restricted by *Eco*B to linear molecules and phenol-extracted is also a poor substrate for *Eco*B restriction (see below). Presumably, some property of the purified restricted DNA inhibits reutilization of a restriction site by the nuclease.

Effect of a Pre-existing Strand Scission on the Site of the EcoB Cleavage—An unsolved question about the Type I restriction process is what factors determine where the enzyme chooses to cleave. There is no obvious nucleotide sequence

[2] B. Endlich and S. Linn, unpublished data.

specificity at the points of cleavage (see below), but other structures might have an effect. To test the effect of a pre-existing strand scission, single-stranded scissions were randomly introduced into supercoiled fd 101 RF DNA with *N. crassa* nuclease, this DNA was incubated with *Eco*B, and then the products were analyzed by alkaline sucrose gradient sedimentation. Since fd 101 RF DNA is small enough (~6400 base pairs) so that *Eco*B can translocate nearly the entire molecule (2), there is a high probability that an *Eco*B molecule will encounter the pre-existing strand cleavage. If *Eco*B were to cleave directly opposite the scission, the product would contain predominantly full-length linear strands. If, however, the cleavage introduced by *Eco*B were not affected by the pre-existing scission, a large number of molecules shorter than unit length would be formed. Indeed, smaller DNA fragments do appear after treatment with preparations of *Eco*B that form only unit-length linear molecules from the analogous un-incised substrate (Fig. 5). By this criterion, a strand cleavage in the DNA substrate does not detectably affect the site at which *Eco*B acts.

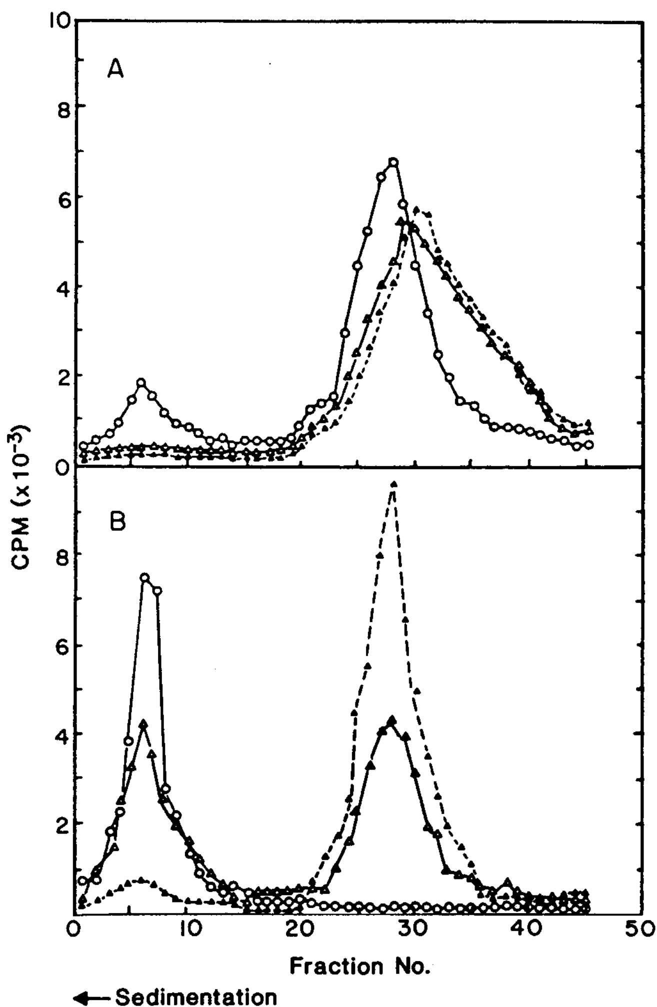

FIG. 5. **Action of *Eco*B on DNA containing a pre-existing strand scission.** Supercoiled fd 101 RF DNA was treated to produce Form II DNA containing one strand scission by digestion with *N. crassa* nuclease, and 2 nmol of this DNA were then incubated with *Eco*B as indicated in 100-μl reactions for 15 min. Control reactions were simultaneously carried out with un-nicked fd 101 RF DNA. Reactions were terminated by chilling and adding EDTA to 20 mM; then samples were layered onto 5.2-ml, 5–20% alkaline sucrose gradients and sedimented at 50,000 rpm for 210 min at 4 °C in an SW 50.1 rotor. Fractions were collected from tube bottoms, neutralized, and counted by a liquid scintillation. *Panel A*, DNA substrate incised with *N. crassa* nuclease; *Panel B*, untreated DNA substrate. O, 0.0 units; Δ, 0.1 units; ▲, 0.2 units of *Eco*B, respectively.

Characterization of Fully Restricted DNA

In the previous paper (1), some properties of restricted DNA that still remained complexed to *Eco*B were presented. This section examines the properties of such DNA after disruption of the protein-DNA complexes.

Presence of Single-stranded Tails—The presence of gaps of 70–100 nucleotides in hemi-restricted DNA and the fact that, on the average, 70–100 nucleotides are released for each strand cleavage of fully restricted DNA could be taken to predict several alternatives for the presence of single-stranded regions in purified, fully restricted DNA. To distinguish among these, T7 [^{3}H]DNA was restricted with *Eco*B and then exposed to each of several single-strand-specific nucleases: the *N. crassa* endonuclease, which will digest single-stranded regions of DNA whether they be tails or internal; *E. coli* exonuclease I, which will degrade only 3′-single-stranded tails containing a 3′-hydroxyl terminus; and *E. coli* exonuclease VII, which will degrade DNA tails with 3′-hydroxyl, 5′-hydroxyl, or 5′-phosphoryl termini. In this experiment, the *Eco*B alone released an average in four separate determinations of 93 ± 20 nucleotides/single-strand scission (Table II). In the reactions in which *Eco*B digestion was followed by nuclease treatment, exonuclease I released an additional 60 ± 30 nucleotides/single-strand scission, *N. crassa* nuclease released 90 ± 10 nucleotides, and exonuclease VII released 90 ± 20 nucleotides, in agreement with the *N. crassa* enzyme result.

Conceivably, the acid-soluble material produced by the single-strand nucleases originated not from the restricted DNA molecule, but from large, acid-insoluble oligonucleotides liberated during the restriction reaction. To rule out this possibility, restricted DNA was purified on neutral sucrose gradients and then treated with the nucleases. The results of this experiment (Table III) are in close agreement with those

TABLE II

Treatment of restricted DNA with single-strand-specific nucleases

Reactions (100 μl) contained 2 nmol of T7 [^{3}H]DNA (6190 cpm/nmol) and 0.2 unit of *Eco*B in the standard mixture, except that the dithiothreitol concentration was reduced from 0.5 to 0.1 mM and AdoMet was omitted where indicated. After 15 min at 37 °C, reactions were adjusted to 90 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$ in a final volume of 300 μl and then 0.5 unit of *N. crassa* nuclease, 0.2 unit of *E. coli* exonuclease I, or 0.5 unit of *E. coli* exonuclease VII was added as indicated and incubation was continued for 30 min at 37 °C. The level used for each of these enzymes would completely digest this quantity of DNA had it been totally single-stranded. The amount of acid-soluble material formed was determined by precipitating the samples with 200 μl of 10 mg/ml bovine serum albumin and 500 μl of 7% trichloroacetic acid, incubating for 15 min at 0 °C, and centrifuging for 10 min at 10,000 × *g*. An aliquot of the supernatant was counted by liquid scintillation. Blank reactions for each nuclease were run identically on an equivalent amount of unrestricted T7 DNA. The values of these nuclease blanks were approximately 10% of the total nucleotides released from restricted DNA for each case and the data in the last column has been corrected for these values. Each value represents an average of four determinations.

Treatment	Nucleotides released per single-strand *Eco*B site	Approximate number of nucleotides released by second nuclease
*Eco*B (AdoMet omitted)	10	
*Eco*B alone	93 ± 20	
*Eco*B + exonuclease I	157 ± 42	60 ± 30
*Eco*B + *N. crassa* nuclease	179 ± 14	90 ± 10
*Eco*B + exonuclease VII	182 ± 15	90 ± 20

TABLE III

Treatment of purified restricted DNA with single-strand-specific nucleases

Twenty nmol of T7 [^{3}H]DNA (6190 cpm/nmol) were restricted with 6 units of *Eco*B under standard reaction conditions in a total volume of 200 μl for 15 min at 37 °C. The reaction was stopped by the addition of EDTA to 20 mM and the material was layered onto a 5-ml, 5–20% neutral sucrose gradient and centrifuged at 50,000 rpm for 180 min at 4 °C in a Beckman SW 50.1 rotor. Fractions of approximately 200 μl were collected from the tube bottom. Unrestricted DNA was also carried through the procedure and the peak fraction corresponding to full-length molecules was collected. For calculating the amount of restriction, the number average molecular weight of each fraction was determined by the formula $s_{20,w} = 0.0882M^{0.346}$, where $s_{20,w}$ is the sedimentation coefficient and M is molecular weight (18). (T7 DNA has a molecular weight of 25.2×10^6 and a corresponding $s_{20,w}$ value of 34.2; fully restricted T7 DNA has an average molecular weight of 5.4×10^6, which corresponds to an $s_{20,w}$ of 18.8 (19).) The DNA of this restricted sample was found to have an average $s_{20,w}$ of 28.8, corresponding to approximately 37% restriction. Approximately 15 nmol of DNA was recovered from each gradient and dialyzed against 5 mM Tris·HCl, pH 8.2. Two-nmol aliquots of restricted or nonrestricted DNA were then treated with 0.2 unit of *E. coli* exonuclease I, 0.5 unit of *N. crassa* nuclease, or 0.5 unit of *E. coli* exonuclease VII in 90 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$ for 30 min at 37 °C. These levels of the nucleases had been determined to be saturating by testing their capacity to hydrolyze to a limit an equivalent amount of heat-denatured T7 DNA. The amount of material released was determined as described in Table II. In each case, the values for the nuclease treatment of the unrestricted DNA has been subtracted.

Nuclease treatment	Nucleotides released by second nuclease/ single-strand *Eco*B site
Exonuclease I	76
N. crassa nuclease	100
Exonuclease VII	120

described for Table II. Therefore, *Eco*B does not appear to release a substantial number of oligonucleotides of a size which would be insoluble in acid.

The general similarity of values obtained with the exo- and endonucleases support the alternative that fully restricted DNA contains single-stranded tails. Moreover, most or all of these putative tails appear to have 3′-hydroxyl termini as judged by their susceptibility to exonuclease I. Unfortunately, the data do not distinguish whether some 5′-tails might also be present. However, DNA polymerase β could be used to assay directly for 5′-tails, since this enzyme will act only from a 3′-hydroxyl terminus that lies opposite a 5′-tail (or a gap). Therefore, purified unit-length linear fd 101 RF DNA that had been formed by *Eco*B or, as a control, unit-length linear RF III DNA with blunt ends, produced with *Hinc*II, were exposed to a DNA polymerase β preparation from Novikoff hepatoma cells which contained no detectable 3′- or 5′-exonuclease activity (9). In separate experiments, either [α-^{32}P]dTTP or [α-^{32}P]dGTP and the remaining 3 unlabeled triphosphates were utilized (Fig. 6). Incorporation of [α-^{32}P]dTTP was limited to approximately 0.91 dTTP residues/terminus for the *Hinc*II-generated RF III control DNA and to about 2 dTTP residues/terminus for the *Eco*B-restricted DNA (Fig. 6). Likewise, the incorporation of [α-^{32}P]dGTP resulted in 0.8 dGTP residues/terminus in the control and 1.6 dGTP residues/terminus in the *Eco*B-restricted DNA. Within the limits of this type of experiment, it appears that no significant level of incorporation occurs at the termini of *Eco*B-restricted DNA molecules, strongly suggesting that the fully restricted DNA product has 3′-, but no 5′-, tails.

3′-Terminal Nucleotides of Restricted Duplex DNA—Previous observations with a variety of exonucleases implied that the termini formed by *Eco*B contain 3′-hydroxyl and 5′-phosphoryl groups (3). However, the resistance of such DNA to polynucleotide kinase has precluded characterization of the 5′-nucleotides at break sites of Type I restriction enzymes. To examine the base composition of the 3′-terminal nucleotides of the *Eco*B product, we have labeled the DNA with a ribonucleoside [α-^{32}P]triphosphate using terminal deoxynucleotidyl transferase by a modification of the procedure of Roychoudhury *et al.* (20), in which the enzyme incorporates up to two ribonucleotides onto each 3′ terminus of duplex DNA. Treatment of this material with alkali then leaves a single ^{32}PrN^{32}P/terminus. Treatment with alkaline phosphatase then removes the 3′-phosphomonoester, leaving the internal $^{32}PO_4$, and digestion of this DNA to 3′-mononucleotides with micrococcal nuclease and spleen phosphodiesterase results in the ^{32}P label being transferred to the 3′-terminal deoxynucleotide. When the above regime was followed, chromatographic analysis of the ^{32}P-labeled nucleotides showed that the 3′ termini formed by *Eco*B were apparently random, reflecting simply the nucleotide composition of the substrate DNA (Table IV).

3′-Termini of Acid-soluble Oligonucleotides—To determine the 3′-terminal nucleotides of the oligonucleotides formed by *Eco*B during the restriction reaction, a nearest neighbor analysis patterned after that used for the 3′ termini of duplex DNA product was done, except that phenol extraction and dialysis were omitted and, prior to the addition of the ^{32}P-labeled ribotriphosphate, alkaline phosphatase was used to degrade the ATP and ADP present from the *Eco*B reaction. Although the acid-soluble oligonucleotides were not separated from the restricted duplex DNA, the number of 3′ termini present in the acid-soluble material should have been roughly

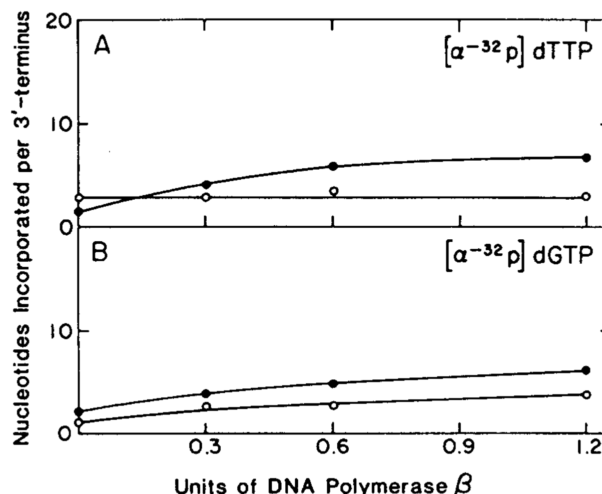

FIG. 6. **Incorporation of dNTPs into restricted DNA by DNA polymerase β.** Twenty nmol of fd 101 RF [^{3}H]DNA (3500 cpm/nmol) were incubated with 8 units of *Eco*B for 15 min. As a control, 20 nmol of the DNA was digested with 5 units of *Hinc*II in 10 mM Tris·HCl, pH 7.9, 60 mM $NaCl_2$, 1 mM dithiothreitol for 1 h at 37 °C. The linear DNA products were purified as described in Fig. 1, and then 1-nmol aliquots were incubated with DNA polymerase β in 100 μl of 25 mM Tris·HCl, pH 8.4, 12 mM $MgCl_2$, 40 μM of each unlabeled dNTP, and either 15 μM [α-^{32}P]dTTP, 890 cpm/pmol (*Panel A*) or 15 μM [α-^{32}P]dGTP, 890 cpm/pmol (*Panel B*). After 1 h at 37 °C, 100 μl of 0.1 M sodium pyrophosphate, 1 mg/ml bovine serum albumin, and then 500 μl of 10% trichloroacetate were added. After 15 min at 0 °C, the precipitated material was collected on GF/C glass fiber filters, dried, and counted by liquid scintillation. Simultaneous reactions with "activated" salmon sperm DNA confirmed the activity of the polymerase preparation. ○, *Hinc*II-digested DNA; ●, *Eco*B-digested DNA.

TABLE IV

Characterization of the 3′-terminal nucleotides of restricted DNA

Twenty nmol of fd 101 RF [^{3}H]DNA were restricted with 7.5 units of *Eco*B for 15 min at 37 °C; then samples were chilled and EDTA was added to 20 mM. A control incubation omitted *Eco*B. Analysis of 0.5-nmol aliquots of each reaction by electrophoresis on a 1% agarose gel revealed that the undigested DNA was approximately 95% Form I, whereas the restricted DNA was completely digested to unit-length linear molecules. The DNA samples were diluted to 1 ml and extracted twice with equal volumes of phenol and dialyzed extensively. Approximately 10 nmol of each DNA was incubated with 2 units of terminal deoxynucleotidyl transferase in 120 mM potassium cacodylate, pH 7.6, 10 mM $CaCl_2$, 0.1 mM dithiothreitol, and 4.5 nmol of [α-^{32}P]CTP (7.5×10^4 cpm/pmol) for 1 h at 37 °C. Incorporation was monitored with a 0.5-nmol aliquot, treated as described in Fig. 6. This analysis showed that 1.2 [α-^{32}P]CMP residues were incorporated per 3′ terminus. Blanks containing unincubated, restricted DNA or incubated, unrestricted DNA showed less than 0.02 and 0.25 residues incorporated per 3′ terminus, respectively. The sample was then adjusted to 0.4 M KOH, incubated for 18 h at 25 °C, neutralized with 1.0 N HCl, and adjusted to 50 mM Tris·HCl, pH 8.2. Alkaline phosphatase, 0.4 units, was added and the reaction was incubated for 2 h at 45 °C. The sample was chilled, diluted to 1 ml, and extracted with phenol and then anhydrous diethyl ether. Residual ether was evaporated off under a stream of nitrogen gas and the DNA, in approximately 500 µl, was mixed with 0.2 mg of unlabeled salmon sperm DNA and 500 µl of 1 N perchloric acid, incubated for 5 min at 0 °C, diluted to 2 ml with H_2O, and centrifuged at 12,000 × *g* for 10 min. The precipitated sample was resuspended in 0.2 N NaOH, and then reprecipitated three more times with 1 N perchloric acid. Following the final precipitation, it was resuspended in 0.1 N NaOH and dialyzed against 4 mM Tris·HCl, pH 8.6, 2 mM $CaCl_2$. To this sample (volume, approximately 500 µl), was added 10 µg of micrococcal nuclease, 29,000 units/mg. After 3 h at 37 °C, an aliquot was taken to verify complete digestion to acid-soluble material. The reactions were adjusted to 28 mM potassium phosphate, pH 6.8, 2.4 mM EDTA, 0.03% Tween 80, and then 10 µg of spleen phosphodiesterase, 19 units/mg, was added. After 12 h at 37 °C, an aliquot was developed by descending paper chromatography on Whatman No. 1 paper using saturated ammonium sulfate, 1 M sodium acetate, isopropyl alcohol, 80:18:2. To each aliquot to be chromatographed, 0.05 µmol of each 3′-dNMP standard was added to aid in the visualization and identification of the nucleotides. The chromatogram was dried, cut into strips and ^{32}P was counted on a gas flow counter. The final recovery of material, as determined from the ^{3}H-label in the dTMP spot, was approximately 20%; essentially all of the ^{32}P migrated with the mononucleotide markers.

Base	Frequency at 3′ terminus	Reported fd RF base composition (21)
	%	
A	34	29
G	19	21
T	27	29
C	19	21

10 termini/single-strand scission, based on the fact that approximately 70 nucleotides are released as oligonucleotides of an average length of 7/single-strand scission (4). This expected number of 3′ termini was verified by testing an aliquot of the reaction for ^{32}P incorporation. When the samples were finally digested to 3′-mononucleotides and characterized by chromatography, no significant base preference was found (Table V).

5′-Termini of Acid-soluble Oligonucleotides—T7 DNA was restricted with *Eco*B and the unfractionated products labeled with [γ-^{32}P]ATP and polynucleotide kinase following phosphomonoesterase treatment. Although the 5′ termini formed on the duplex DNA by *Eco*B are resistant to labeling by this procedure, it appears that labeling of the oligonucleotides was possible. For reasons stated above, approximately 10 5′ termini would be expected in the oligonucleotides for every terminus formed in the duplex DNA and it was seen that the acid-soluble oligonucleotides were labeled to this extent (Table V). Samples were then treated with *E. coli* exonuclease V (*rec*BC DNase) which both partially hydrolyzed the DNA samples and degraded the unreacted [γ-^{32}P]ATP to $^{32}P_i$ and ADP. When the samples were finally digested totally to 5′-mononucleotides, freed of $^{32}P_i$, and separated by electrophoresis, no significant preference for label was found among the nucleotides (Table V).

Absence of Incorporation of Components of the AdoMet Molecule into Restricted DNA—It was previously shown with appropriately radiolabeled preparations of *S*-adenosylmethionine that neither the methyl group nor the homoserine group (as determined by label in the methionine-2 position) are found in the restricted DNA after purification on CsCl density gradients (23). (Likewise, neither the γ-phosphate, the adenine base, nor the ribose segments of the ATP cofactor are incorporated (3).) In order to rule out the possible incorporation of atoms from the adenosine moiety of *S*-adenosylmethionine, *S*-[*Ade*-^{3}H]adenosylmethionine was included in restriction reactions with unlabeled calf thymus DNA. When acid-precipitable material from the reactions was collected onto glass fiber filters and monitored for incorporated ^{3}H, none was found (Table VI). Therefore, no portion of the *S*-adenosylmethionine or the ATP cofactors appears to be incorporated into restricted DNA; in particular, such incorporation does not account for the resistance of fully restricted DNA to polynucleotide kinase or further cleavage by *Eco*B (see below).

Fully Restricted DNA Is Rerestricted Only upon Removal of Its 5′-Termini—Even with excess *Eco*B, no more than one double-strand cleavage is introduced per recognition site (3); however, the restricted DNA is a substrate for the *Eco*B methylase (11), suggesting that part or all of the recognition sequence remains intact. Conceivably, the looped structures formed by *Eco*B remaining bound to product (1, 2) could account for the lack of reutilization of recognition sites when excess enzyme is present. To rule out this possibility, fd 101 RF DNA was digested to the limit of unit-length linear molecules with *Eco*B, phenol-extracted, and dialyzed. Since *Eco*B cuts this DNA at sites at random distances from the recognition site, this population of molecules contains the recognition site randomly positioned relative to the ends. When such material was exposed again to *Eco*B and then analyzed by alkaline sucrose gradient sedimentation, no further degradation was in evidence (Fig. 7). Appropriate controls verified that the phenol-extraction procedure neither affected the DNA sedimentation rate nor prevented *Eco*B from acting upon DNA containing unused recognition sites. Moreover, linear wild type fd RF molecules, which contain two recognition sites, are easily cleaved to a limit of two double-strand cleavages/molecule (2, 17), so there appears to be no size limitation for two cleavages being introduced into fd RF molecules. Finally, the possibility that the block to reutilization was due to a complex in which *Eco*B molecules are still attached to the recognition sequence is unlikely, as the purification treatment disrupts all looped structures and removes all known *Eco*B subunits from the DNA. (The latter was shown by the absence of the density change expected for the covalent attachment of the smallest (55,000-dalton) subunit to the restriction product (24).

An alternative block to recognition site reutilization might be the unknown structure at the 5′ terminus that suppresses polynucleotide kinase action. Therefore, purified, restricted DNA was treated with phage λ exonuclease to remove roughly 110 nucleotides from each 5′ terminus and then reexposed to *Eco*B (Fig. 8). Indeed, this DNA was rerestricted to a large

TABLE V

Characterization of 3′- and 5′-terminal nucleotides of oligonucleotides formed by ecoB

For the 3′ termini, 10 nmol of T7 [^{3}H]DNA were digested with 1.6 unit of *EcoB* in 100 μl under standard reaction conditions, except that the dithiothreitol was 0.1 mM, rather than 0.5 mM. Incubation was for 15 min at 37 °C and the completeness of digestion was verified by analyzing a 0.5-nmol aliquot by electrophoresis on a 1% agarose gel. Bacterial alkaline phosphatase, 3.5 units, was then added. Incubation was for 2 h at 37 °C. (A Norit nonabsorbable assay of a pilot reaction containing an equivalent amount of [γ-^{32}P]ATP demonstrated that all residual ATP would have been hydrolyzed.) The reaction was stopped by the addition of potassium phosphate, pH 7.5, to a final concentration of 2 mM and then adjusted to 120 mM potassium cacodylate buffer, pH 7.6, 10 mM $CoCl_2$, 1 mM dithiothreitol. Two nmol of [α-^{32}P]CTP (5.5×10^4 cpm/pmol) and 0.2 units of terminal deoxynucleotidyl transferase were added in a total volume of 150 μl, and the reaction was incubated for 1 h at 37 °C, then adjusted to 0.5 N KOH with 10 N KOH, and incubated for 16 h at 37 °C. One N HCl was added to pH 8.5 and the sample was diluted to a total phosphate concentration of less than 1 mM. Alkaline phosphatase (3.5 units) was added and the samples incubated for 5 h at 37 °C. To test incorporation efficiency into the duplex DNA product, an aliquot of approximately 1 nmol of DNA was precipitated with bovine serum albumin and trichloroacetic acid and collected on a GF/C filter. The filter was counted by liquid scintillation and the amount of precipitated DNA was determined by its tritium label. An incorporation of 0.104 pmol of ^{32}P was found for the 0.139 pmol of DNA 3′ termini (1.11 nmol of DNA nucleotide). To determine the incorporation into the acid-soluble oligonucleotides plus the DNA, a similar aliquot was absorbed onto Norit (6) and incorporation of ^{32}P was detected by Cerenkov radiation: 0.97 pmol of ^{32}P were present for an estimated 1.25 pmol of total 3′ termini. The phosphate concentration was increased to 3 mM with potassium phosphate, pH 6.7, and 100 μg of unlabeled salmon sperm DNA was added. The sample was adjusted to 50 mM Tris·HCl, pH 8, 2 mM $CaCl_2$ and 290 units of micrococcal nuclease were added. After 4 h at 37 °C, the sample was adjusted to 28 mM potassium phosphate, pH 6.8, 2.4 mM EDTA and 0.15 units of spleen phosphodiesterase were added. After 18 h at 37 °C, the sample was desalted on a 20-ml Sephadex G-10 column equilibrated in 5 mM Tris·HCl, pH 8. The final sample volume was approximately 500 μl. One hundred μl of each sample was spotted onto Whatman No. 1 paper, along with 0.1 μmol of each 3′-dNMP and the chromatogram was developed with saturated ammonium sulfate, 1 M ammonium acetate, isopropyl alcohol, 80:18:2. Nucleotide spots were visualized with a UV mineral lamp and the lanes were cut into 1-cm slices for scintillation counting. ^{32}P was monitored by Cerenkov radiation. Recovery of material was estimated to be approximately 20% by monitoring the amount of tritium in the 3′-dTMP spot.

To monitor the 5′ termini, 5 nmol of T7 [^{3}H]DNA were digested with 2 units of *EcoB* in 100 μl for 15 min at 37 °C. Completeness of digestion was verified by electrophoresis of a 0.5-nmol aliquot on a 1% agarose gel. Alkaline phosphatase (0.4 unit) was added to hydrolyze residual ATP and remove 5′-phosphomonoester groups, and, after 2 h at 37 °C, potassium phosphate, pH 6.7, was added to a final concentration of 2 mM. The sample was then adjusted to 100 mM Tris·HCl, pH 8.2, 10 mM $MgCl_2$ and 2 nmol of [γ-^{32}P]ATP (6.3×10^3 cpm/pmol) was added to a final ATP concentration of 34 μM. Polynucleotide kinase (8.5 units) was added and the incubation was for 30 min at 37 °C. An aliquot containing approximately 0.4 nmol of DNA nucleotide was tested for total incorporation of ^{32}P. The 10-μl aliquot was boiled in 100 μl of 1 N HCl for 5 min to hydrolyze residual ATP, chilled, precipitated with bovine serum albumin, adsorbed onto Norit, collected on a planchet, and counted in a gas flow counter. Assuming that the number of 5′ termini present in acid-soluble material should have been roughly 10 termini/single-strand scission, 0.5 pmol of ^{32}P was expected to be present; 0.47 pmol was found. The sample was then adjusted to 50 mM glycylglycine, pH 7.0, 10 mM $MgCl_2$, 0.66 mM dithiothreitol, 1 mg/ml acetylated bovine serum albumin, and then 4 nmol of unlabeled calf thymus DNA were added. *recBC* DNase (8 units) was added to hydrolyze unincorporated ATP, and, after 30 min at 37 °C, the sample was adjusted to 5 mM Tris·HCl, pH 8.6, and 0.25 μg of pancreatic DNase was added. After 2 h at 37 °C, 5 μg of venom phosphodiesterase and β-mercaptoethanol to 5 mM were added and incubation was for 12 h at 37 °C. Contaminating $^{32}P_i$ was removed by adsorption of the nucleotides to Norit by a modification of the procedure of Mandeles and Kammen (21) and by elution with ethanol/ammonia. The eluted nucleotides were concentrated under a stream of nitrogen gas, resuspended in 100 μl of 10 mM Tris·HCl, pH 8.2; then 50 μl was mixed with 0.1 μmol of each dNMP and analyzed by electrophoresis on 3MM paper utilizing 50 mM sodium citrate buffer, pH 3.5. The nucleotide spots were visualized with the aid of a UV lamp and cut into 1-cm strips for counting by Cerenkov radiation.

Base	Frequency at 3′ terminus	Frequency at 5′ terminus	Reported T7 DNA base composition (22)
		%	
A	24	26	26
G	22	21	24
T	28	24	27
C	26	29	23

TABLE VI

Test of S-[Ade-^{3}H]adenosylmethionine incorporation into restricted DNA

Reactions contained 50 nmol of calf thymus DNA, 1.4 nmol of 14 μM *S*-[*Ade*-^{3}H]adenosylmethionine (6400 cpm/nmol), and *EcoB* as indicated, in 100 μl. After 15 min at 37 °C, reactions were chilled and adjusted to 20 mM EDTA. Then 200 μl of 0.1 M sodium pyrophosphate, 100 μl of 2.5 mg/ml bovine serum albumin, and 0.6 ml of cold 10% trichloroacetic acid were added. After 20 min at 0 °C, acid-precipitable material was collected onto GF/C glass fiber filters and ^{3}H-incorporation was monitored by liquid scintillation counting. Calf thymus DNA contains 0.16 pmol of *EcoB* sites/50 nmol of DNA nucleotide. Thirteen and 26 units of *EcoB* would introduce 7.8 and 15.6 pmol of strand cleavages, respectively, so that 50 and 100 cpm, respectively, would be expected if one AdoMet fragment were incorporated per single-strand scission.

Units of *EcoB*	Counts/min on filter
0	33.1
13	33.4
26	34.5
26 (DNA omitted)	33.5

degree as evidenced by the conversion of a substantial amount of unit-length linear DNA to smaller material. The pattern obtained is consistent with that expected for one cleavage/molecule that is relatively random with regard to the ends. Moreover, some unit-length DNA remained, presumably from those molecules in which the first cleavage had left the restriction site too near to the terminus to give detectable recleavage.

In a related experiment, fd 101 RF DNA was restricted with *EcoB*, treated with λ exonuclease to remove roughly 320 nucleotides/5′ terminus, treated with phosphomonoesterase, and then exposed to polynucleotide kinase in the presence of [γ-^{32}P]ATP (Table VII). Now the 5′ termini were efficiently phosphorylated, demonstrating that it is the 5′ termini left by *EcoB* treatment that obviate phosphorylation by the kinase.

In summary, some structure formed at the 5′ terminus of DNA by *EcoB* precludes not only polynucleotide kinase action, but also reutilization of recognition sites by the restriction enzyme. Presumably, a fresh *EcoB* enzyme molecule binds to purified, restricted DNA and travels to this 5′-end where it is induced to release the DNA before cleaving it. In the case of hemi-restricted DNA, complete restriction is possible only when the recognition site and the first *EcoB* molecule are still bound in a looped complex. Once this complex is dismantled, hemi-restricted DNA is no longer cleavable. A corollary to the above scheme is that *EcoB* scans DNA molecules several times before cleaving. Otherwise, in those instances where a recognition site is relatively far from a re-

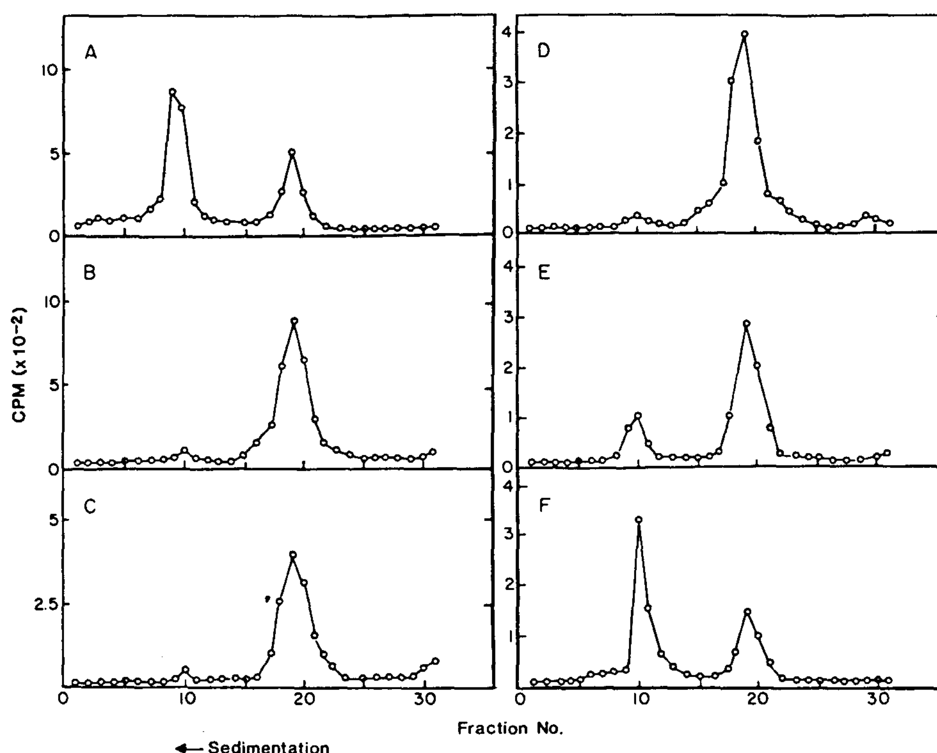

FIG. 7. **Effect of *Eco*B upon purified, fully restricted DNA.** Two nmol of fd 101 RF [^{3}H]DNA (4750 cpm/nmol) were restricted with 0.1 unit of *Eco*B in 100 μl for 15 min at 37 °C. EDTA was added to 20 mM and the sample was diluted to 500 μl with 50 mM Tris·HCl, pH 8.2, extracted twice with phenol, and then dialyzed extensively. The sample was concentrated to 200 μl by evaporation under a stream of nitrogen gas and dialyzed against 5 mM Tris·HCl, pH 8.2. One-half of the sample was incubated with an additional 0.1 unit of *Eco*B as above, while the other half received no further treatment. The samples were then layered onto 5-ml, 5–20% alkaline sucrose gradients and sedimented for 210 min at 4 °C at 50,000 rpm in a Beckman SW 50.1 rotor. Fractions were counted with 10 ml of aqueous fluor by liquid scintillation. Two nmol of untreated fd 101 RF [^{3}H]DNA were also phenol-extracted and dialyzed as described above and then divided into two aliquots. The first aliquot was treated with *Eco*B as above, while the second received no further treatment. *Panel A*, totally untreated DNA; *Panel B*, restricted, but not phenol-extracted DNA; *Panel C*, restricted, phenol-extracted DNA incubated with *Eco*B a second time; *Panel D*, restricted, phenol-extracted DNA, no further treatment; *Panel E*, untreated DNA that was phenol-extracted and then incubated with *Eco*B; *Panel F*, phenol-extracted, untreated DNA.

stricted end, cleavage would have occurred on the purified, restricted DNA.

A Comprehensive Model for EcoB Action

Distinguishing features of Type I restriction reactions include (i) the random nature of cleavage which occurs at sites from 1000 to 5000 base pairs from the recognition sequence, (ii) the limit of a single-strand scission made per enzyme molecule, and (iii) theATPase activity that continues for at least several hours. A sequence for the reaction is proposed in Fig. 9 which is based on these and other observations reported with the *Eco*B and *Eco*K enzymes.

The initial step in the restriction process is defined as recognition of, and binding to, an unmodified recognition site. *S*-Adenosylmethionine, but not ATP, is required to form, and is part of, a recognition complex (25). A more direct role for *S*-adenosylmethionine is not known—it may serve simply as an allosteric effector whose absence in times of methionine deprivation blocks restriction of endogenous DNA. *Eco*K recognition complexes have been trapped on nitrocellulose filters (25), but we have been unable to demonstrate such binding for *Eco*B, though putative recognition complexes have been visualized by electron microscopy (1, 2). As in the case of *Eco*K (26), *Eco*B enzyme molecules bound to the recognition site appear by electron microscopy to have an altered configuration, suggesting an allosteric conversion or the loss of an enzyme subunit (2, 26, 27).

The recognition sequence of *Eco*B contains a trimeric and a tetrameric region separated by eight random base pairs (15, 16), suggesting that there might be two sites for binding to the DNA duplex (2, 26). Two binding sites could explain the translocation loops observed for restriction intermediates of *Eco*B (2) and *Eco*K (26) if the enzyme were to use one of them to remain fixed to the recognition region, while using the other for DNA translocation (see Fig. 9). Assuming such a translocation process which requires, and is fueled by, ATP hydrolysis, the enzyme scans the DNA in order to search for previously formed restriction termini (see below). Should no such termini be encountered, it eventually incises one strand somewhere between 1000 and 5000 base pairs downstream from the recognition site and releases approximately 70–100 nucleotides as acid-soluble oligonucleotides (4), leaving a gap of the same length. The endonuclease molecule, whole or in part, now remains bound to the gapped DNA substrate to maintain a looped structure, and ATP hydrolyis continues. What influences the enzyme to select the site of cleavage and what ultimately triggers cleavage is not known. However, the inhibition by the 5′ termini formed by *Eco*B of rerestriction implies that the enzyme translocates past the ultimate cleavage site at least once before cleaving the DNA.

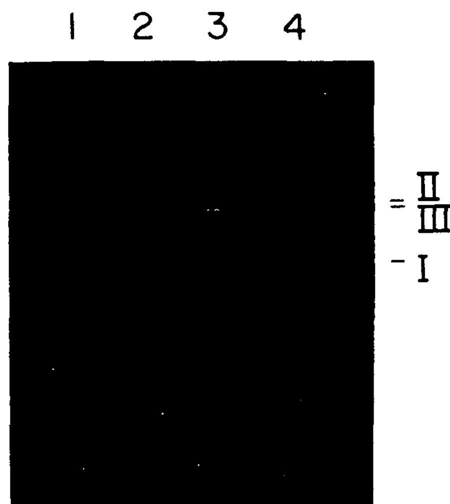

FIG. 8. **Restriction of restricted DNA after treatment with phage λ exonuclease.** Five nmol of fd 101 RF [^{3}H]DNA (1830 cpm/nmol) was treated with 1.5 units of *Eco*B for 15 min at 37 °C in 130 μl, precipitated with ethanol, and resuspended in 60 μl of distilled water. A 10-μl aliquot of this DNA was analyzed on an agarose gel to verify that the DNA had been converted totally to unit-length linear (Form III) molecules. A 33-μl aliquot was then treated with 25 units of λ exonuclease in a 37-μl reaction containing 67 mM glycine·NaOH, pH 9.3, 3 mM $MgCl_2$, and 3 mM β-mercaptoethanol for 3 min at 37 °C. An aliquot was removed and precipitated with 0.7 mg/ml bovine serum albumin and 7% trichloroacetic acid; the soluble counts indicated that an average of roughly 110 nucleotides had been removed from each 5′ terminus. The remaining material was extracted with phenol, precipitated with acid, and resuspended in 25 μl of distilled water. Twenty μl of that material was reexposed to 0.2 unit of *Eco*B in a 35-μl reaction mixture for 15 min at 37 °C. Aliquots of each incubation were then analyzed on a 0.7% agarose gel; the amounts of DNA in each track were determined from the ^{3}H-counts applied. *Lane 1*, untreated DNA; *Lane 2*, 0.37 nmol of the DNA treated once with *Eco*B only; *Lane 3*, 0.42 nmol of the DNA treated with *Eco*B, then with λ exonuclease; *Lane 4*, 0.40 nmol of the DNA treated with *Eco*B, λ exonuclease, and then again with *Eco*B.

TABLE VII

Phosphorylation of restricted DNA with polynucleotide kinase after treatment with λ exonuclease

Ten nmol of fd 101 RF [^{3}H]DNA (2800 cpm/nmol) was digested with 2 units of *Eco*B in 95 μl for 15 min at 37 °C, diluted to 200 μl, extracted with phenol, then diethyl ether, precipitated with ethanol, and resuspended in 20 μl of distilled water. A 1-μl aliquot was analyzed on a 0.7% agarose gel to verify that complete digestion to Form III had occurred. The remaining material was incubated with 50 units of λ exonuclease in a total volume of 56 μl containing 67 mM glycine·NaOH, pH 9.3, 3 mM $MgCl_2$, and 3 mM β-mercaptoethanol for 30 min at 4 °C and then for 5 min at 37 °C. A 5-μl aliquot was precipitated with acid as described in Fig. 8 in order to determine that an average of 320 nucleotides had been released from each 5′ terminus. The remaining DNA was phenol-extracted, ether-extracted, ethanol-precipitated, and resuspended in 10 mM Tris·HCl, pH 8.0. One nmol of this DNA was treated with 20 units of calf alkaline intestinal phosphatase in 41 μl of 50 mM Tris·HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine for 15 min at 37 °C and then for 15 min at 55 °C. Twenty additional units of phosphatase were added in 1 μl and the incubations were repeated. The enzyme was then inactivated by incubation at 68 °C for 20 min, 4 μl of 3 M sodium acetate was added, and the DNA was precipitated with ethanol and suspended in 5 μl of Tris·HCl, pH 8. A 2.5-μl aliquot was then brought to 24.5 μl of 20 mM Tris·HCl, pH 9.5, 0.1 mM EDTA, 1 mM spermidine, heated to 70 °C, and rapidly chilled. Two μl of 0.5 M Tris·HCl, pH 9.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol, and 50% glycerol were added along with 5 μl of [γ-^{32}P]ATP (25 pmol, 5.7 × 10^4 cpm/pmol) and 10 units of polynucleotide kinase in 10 μl. After 30 min at 37 °C, the ^{32}P incorporated was determined as previously described (6). A parallel protocol was run with fd 101 RF which had initially been converted to unit-length linear molecules with *Hinc*II but not treated with λ exonuclease. Also, a determination of the ^{3}H that was precipitated onto the filters verified that the RF III had been quantitatively recovered by the procedure. Values shown are corrected for ^{32}P blanks (no polynucleotide kinase) which were less than 10% of the experimental values.

Nuclease used	$^{32}PO_4$ incorporated	$^{32}PO_4$ incorporated per 5′ terminus
	pmol	
*Hinc*II	0.072	0.92
EcoB + λ exonuclease	0.059	0.76

An implication of the enzyme remaining bound to the gapped product so as to maintain a looped structure is that the first endonuclease molecule, by being anchored both to the recognition sequence and to the site of single-strand cleavage, might direct a second endonuclease molecule simultaneously to verify the unmodified nature of the recognition site and to execute a second cleavage at the proper site. Support for this idea is provided by the fact that, once freed of protein, these gapped intermediates are no longer substrate for the second *Eco*B scission. Evidently, in this case the second *Eco*B molecule can recognize the unmodified recognition site, but dissociates upon encountering the *Eco*B-mediated 5′ terminus present at the gap.

Since the second strand cleavage also releases roughly 70–100 nucleotides and, since it results in the formation of 3′-tails but not 5′-tails or gaps, we hypothesize it to occur by excision of nucleotides from the intact strand at a position opposite the 3′ terminus of the gapped strand and extending roughly 70–100 nucleotides away from the gap. In this way, both cleavage events would be similar, and two 3′-tails of 70–100 nucleotides (rather than one of 140–200 nucleotides) would be formed.

A looped, hemi-restricted DNA complex in which the recognition site is still occupied could also explain why the site is not used by another endonuclease molecule present in the same reaction mixture to translocate DNA and introduce a cleavage within the area spanned by the first molecule (*i.e.* within the loop). These loops could also serve a similar role with fully restricted products. However, once the loops are disrupted, it appears that the unknown structure at the 5′-terminus formed by *Eco*B prevents additional cleavage. As mentioned in the previous paper (1), purified restricted DNA serves as an ATPase cofactor at about 3% of the efficiency of its equivalent unrestricted counterpart and does not prevent the enzyme from acting on unrestricted DNA that is also present. Therefore, these termini are efficient at terminating the scanning reaction of fresh enzyme without inactivating its endonuclease activity.

Following cleavage, the enzyme enters a phase of continued ATP hydrolysis (long-term ATPase) which is represented in Fig. 9 as the recycling or backtracking phenomenon discussed in the previous paper (1). This phase presumably reflects the initial scanning reaction—*i.e.* the enzyme is continuing to carry out its initial scanning/translocation functions, but is no longer able to cleave DNA. *In vivo*, other nucleases presumably rapidly degrade the restricted DNA and terminate this ATP hydrolysis.

DISCUSSION

The most elusive biochemical aspect of *Eco*B is the structure of the 5′ termini which it forms. No portion of the *Eco*B cofactors, AdoMet or ATP, appears to be present, nor is a known subunit of the enzyme bound to these termini. Perhaps *Eco*B contains an unknown small subunit or cofactor. Covalent linkages exist between the 5′-end of polio virus RNA and its replication protein (28). Also, there exists a protein cova-

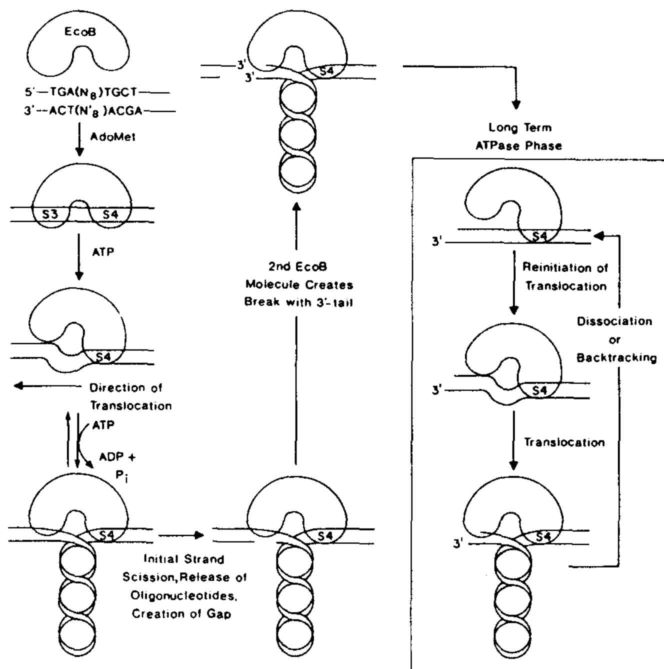

FIG. 9. **A comprehensive model for the degradation of DNA by *EcoB*.** The nucleotide sequence of the recognition site is given for the first step and thereafter it is indicated by the designations *S3* and *S4* for the two specific domains of the sequence (15, 16). The reaction sequence commences with the binding of AdoMet to the *EcoB* molecule, enabling it to bind to the recognition sequence. AdoMet remains bound throughout this entire process (23), but for simplicity of illustration, it is not so shown. ATP hydrolysis is hypothesized to enable the DNA translocation process to commence. Details of the model are discussed in the text.

lently attached to the 5′ termini of adenovirus DNA through an *O*-phosphoserine ester linkage to the terminal cytosine nucleotide (29, 30) and one similarly linked to the 5′-dAMP terminus in phage ϕ29 DNA (31). However, unlike the *EcoB* restriction termini, *O*-phosphoserine ester linkages are labile to NaOH. Therefore, should the 5′ termini of the *EcoB* products be covalently bound to a peptide, other, novel linkages would be necessitated.

Other important questions about the Type I host-specified restriction and modification systems include: Why is the cleavage site so distant from the recognition sequence and why do these complicated Type I (and Type III) systems persist in nature when the much simpler and more prevalent Type II systems appear to be able to efficiently degrade foreign DNA? One might speculate that the Type I enzymes serve functions which are yet to be described. For example, an additional role in some form of nonreciprocal recombination might be imagined because of the ability of the nucleases both to aggregate (3) and to recognize and remain bound to a particular sequence, while cleaving the DNA several thousand nucleotides away. Alternatively, the systems might recently have evolved from some other function and might be in the process of becoming more efficient. Strand-specific mismatch repair which utilizes the *dam*-methylation system of *E. coli*, for example, has many similarities to the *EcoB* and K nucleases (32).

However, we favor the hypothesis that the Type I systems have in fact evolved precisely so as to be able to form, as efficiently as possible, the products which we observe. The 3′-tails might serve two functions. Since they cannot be copied by DNA polymerases, up to 200 base pairs of genetic information is lost per cleavage of foreign DNA, so that inactivation of infectious DNA is assured. However, should the restricted DNA be homologous to chromosomal DNA, it would be an ideal substrate for *recA*-mediated homologous recombination which very efficiently assimilates DNA with unpaired 3′ termini (33). Moreover, by cleaving randomly and far from the restriction recognition sites, the Type I enzymes would assure that each gene would have an equal chance of being rescued, at least among a population of bacteria. Finally, the unknown structures at the 5′-ends of the molecule presumably serve to prevent the unnecessary reutilization of restricted DNA as substrate. Thus, by forming large, randomly cleaved fragments with 3′-tails, the Type I systems might increase the efficiency of destruction of totally foreign genetic information, while allowing for or even promoting the exchange of genetic information with closely related organisms.

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