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Model for how type I restriction enzymes select cleavage sites in DNA

(*Eco*B/*Eco*K/ATPase/bidirectional translocation/primary and secondary cleavages)

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ABSTRACT **Under appropriate conditions, digestion of phage T7 DNA by the type I restriction enzyme *Eco*K produces an orderly progression of discrete DNA fragments. All details of the fragmentation pattern can be explained on the basis of the known properties of type I enzymes, together with two further assumptions: (*i*) in the ATP-stimulated translocation reaction, the enzyme bound at the recognition sequence translocates DNA toward itself from both directions simultaneously; and (*ii*) when translocation causes neighboring enzymes to meet, they cut the DNA between them. The kinetics of digestion at 37°C indicates that the rate of translocation of DNA from each side of a bound enzyme is about 200 base pairs per second, and the cuts are completed within 15–25 sec of the time neighboring enzymes meet. The resulting DNA fragments each contain a single recognition site with an enzyme (or subunit) remaining bound to it. At high enzyme concentrations, such fragments can be further degraded, apparently by cooperation between the specifically bound and excess enzymes. This model is consistent with a substantial body of previous work on the nuclease activity of *Eco*B and *Eco*K, and it explains in a simple way how cleavage sites are selected.**

The type I restriction enzymes *Eco*B and *Eco*K have a complex mode of action (reviewed in refs. 1–3). They act only on double-stranded DNA that contains a unique recognition sequence, $TGAN_8TGCT$ for *Eco*B and $AACN_6GTGC$ for *Eco*K (N = any nucleotide). Specific binding to these sites requires *S*-adenosylmethionine, and further reactions require (or, in the case of methylation, are stimulated by) ATP. In the presence of ATP, the state of methylation of the recognition sequence determines the course of the reaction: if both strands are methylated, the enzyme falls off the DNA; if one strand is methylated, the enzyme rapidly methylates the second strand; if neither strand is methylated, the enzyme hydrolyzes large amounts of ATP, translocates considerable lengths of DNA, and cuts the DNA at seemingly random sites far from the recognition sequence. In the nucleolytic mode, the enzyme is used up in the reaction, apparently remaining bound at its recognition site. The effect of this complex set of reactions is to maintain resident DNA intact but to degrade unmethylated foreign DNA.

One puzzling aspect of the nuclease activity of *Eco*B and *Eco*K has been how cleavage sites are selected in the DNA. We believe we have now discovered how this is done.

MATERIALS AND METHODS

Wild-type phage T7 DNA was prepared by phenol extraction of purified phage particles. T7 DNA contains 39,936 base pairs, its entire nucleotide sequence is known, and position in the molecule is given in terms of T7 units, the genetic left end being 0 and the right end being 100 (4). The calculated molecular weight of the sodium salt of T7 DNA is 26.4×10^6, and 100 ng would contain 2.3×10^9 molecules.

The purified *Eco*K used in these experiments was the gift of R. Yuan and is the third preparation described in ref. 5. This preparation had a protein concentration of about 300 μg/ml and was estimated by gel electrophoresis to be about 10–20% *Eco*K: 1 μl was estimated to contain about 5×10^{10} molecules of active *Eco*K.

Type II restriction endonucleases were obtained from New England Biolabs, and *S*-adenosylmethionine was obtained from Sigma.

RESULTS AND DISCUSSION

***Eco*K Recognition Sites in Phage T7 DNA.** Four *Eco*K recognition sites are found in the nucleotide sequence of phage T7 DNA, at positions 38.0, 66.6, 81.7, and 93.8 (4). The latter three sites have the recognition sequence oriented $AACN_6GTGC$ from left to right in the *l* strand of T7 DNA; the site at position 38.0 has the opposite orientation. These sites are not methylated in T7 DNA because methylation by *Eco*K is prevented by action of the T7 gene *0.3*-encoded protein during infection (5, 6).

We mapped the *Eco*K sites in T7 DNA even before its nucleotide sequence was determined, by analyzing *Eco*K digestion of the specific fragments produced by cleavage with type II restriction endonucleases whose cleavage sites were known. This mapping placed each *Eco*K site at the position ultimately found in the nucleotide sequence, within an interval as small as 360 and as large as 800 base pairs in the T7 DNA. Previous work had shown that *Eco*B can be inefficient in degrading small, linear DNAs (7–9), and we observed similar behavior with *Eco*K. However, we found that when *Eco*K reaction mixtures were applied directly to a 3–10% gradient polyacrylamide gel, any fragment that contained an *Eco*K site was missing from its normal position in the electrophoresis pattern, even when the fragment was not degraded, apparently because its mobility was changed by the *Eco*K that remained bound to the fragment. Loss of the fragment was observed even when the *Eco*K site was as close as 2 or 9 base pairs (plus a four-base single-strand extension) from the end of the fragment.

Synchronized Digestion of T7 DNA by *Eco*K. We also analyzed the degradation of intact T7 DNA by *Eco*K. The nuclease action of *Eco*K can be synchronized by first saturating the recognition sites with enzyme in the presence of *S*-adenosylmethionine and then starting the reaction by adding ATP (10–12). When this was done with T7 DNA and the reaction was stopped at intervals by mixing samples of reaction mixture with EDTA and sodium dodecyl sulfate, a striking pattern of degradation was seen (Fig. 1).

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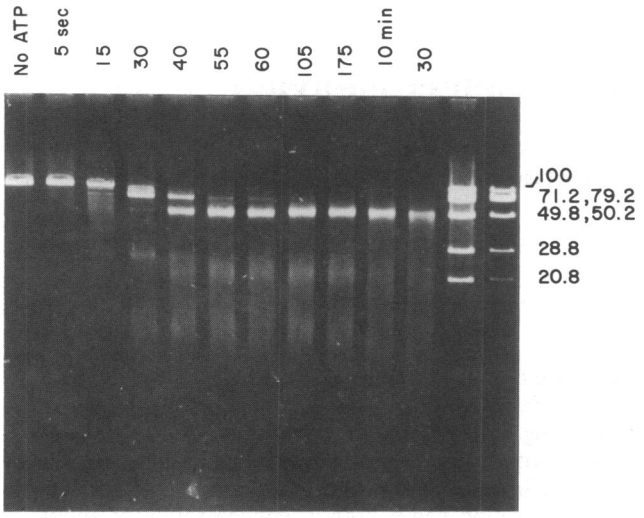

FIG. 1. Synchronized degradation of phage T7 DNA by *Eco*K. A 240-μl reaction mixture containing 800 ng of T7 DNA and 16 μl of *Eco*K (an estimated 11 molecules of *Eco*K per recognition site) was incubated 5 min at room temperature and 5 min at 37°C in 100 mM Tris chloride, pH 8.1/6 mM $MgCl_2$/0.26 mM EDTA/12 mM mercaptoethanol/100 μM *S*-adenosylmethionine/50 μg of bovine serum albumin per ml. ATP was then added to produce a concentration of 1.7 mM. Immediately before, and at intervals after the ATP was added, 15-μl samples were mixed with 4 μl of 0.1 M Na_3EDTA/5% sodium dodecyl sulfate and placed at 65°C for 5 min. The samples were then subjected to electrophoresis for 9 hr at 1.25 V/cm through a 0.5% agarose gel in 40 mM Tris acetate, pH 8.1/2 mM Na_3EDTA. The picture shows the fluorescence after the gel was soaked with ethidium bromide at 0.5 μg/ml. A mixture of full-length T7 DNA and fragments produced from it by *Bcl* I, *Bgl* II, and *Bst*EII provided size markers (4); the lengths are indicated in T7 units at the right of the gel patterns. (The right two lanes show different exposures of the same lane of size markers.) Samples were removed before addition of ATP; at 5, 15, 30, 40, 55, 60, 105, and 175 sec after addition of ATP; and at 10 and 30 min after addition of ATP, as indicated.

As expected, the endonuclease reaction proceeds very rapidly and is essentially complete within 1 min, although slow changes continue for 3–30 min. Contrary to what has been reported previously for type I enzymes, DNA fragments of discrete sizes were observed: fragments with lengths approximately 88%, 74%, and 26% of that of intact T7 DNA are intermediates in the degradation, appearing in the 15- to 40-sec samples; these in turn were converted to a fragment with a length about 52% of that of intact T7 DNA and to more heterogeneous smaller DNAs with lengths distributed around 22% and 12–14%. Clearly, digestion of T7 DNA by *Eco*K in the early stages of a synchronized reaction is an orderly and specific process.

To determine which parts of the T7 DNA molecule are represented in the 52% and 74% pieces, a synchronized reaction was stopped 37 sec after adding ATP, when these two fragments were the major components of the reaction mixture. The DNAs were separated by gel electrophoresis, eluted individually from the gel, cut with *Hpa* I, and analyzed by gel electrophoresis. Both the 52% and 74% fragments contained a set of *Hpa* I fragments that began at the left end of T7 DNA and extended a distance appropriate to the length of the fragment (not shown). The 74% fragment appeared to contain a less than equimolar amount of the *Hpa* I fragment that occupies position 67.5–74.1 in T7 DNA, indicating that some but not all of the fragments in the population extended past position 74.1.

Clearly, no cuts were made in the left half of the T7 DNA molecule. Therefore, the degradation intermediate whose length is 88% of that of T7 DNA must also extend to the left end of T7 DNA, and *Eco*K cleavages must be localized near positions 52, 74, and 88 in T7 DNA. Furthermore, these are the only cleavages needed to account for all of the fragments observed in Fig. 1 (see maps in the lower part of Fig. 3). The degradation intermediates whose length are 74% and 26% of that of T7 DNA must arise from a cleavage near position 74 before the cleavages at positions 52 or 88 are made, and the fragment whose length is 88% of that of T7 DNA must arise from a cleavage near position 88 before the cleavages at positions 52 or 74 are made. The fragments produced after all three cleavages have been made would have lengths about 52%, 22%, 14%, and 12% of that of T7 DNA, the distribution observed from 1 min onward in Fig. 1.

The different fragments produced by *Eco*K degradation of T7 DNA gave bands of different sharpness in the gel electrophoresis patterns (Fig. 1). These differences are easily explained if, for each of the three *Eco*K cleavage sites, the cuts are made at somewhat different positions in different molecules. Since the ends of T7 DNA are homogenous, fragments that retain an intact end of T7 DNA (the 88%, 74%, 52%, 26%, and 12% fragments) will have one homogenous and one heterogeneous end. If the variability in cleavage position is similar at the three *Eco*K cleavage sites, the longer fragments will have a narrower fractional size distribution than shorter fragments and will give sharper bands upon gel electrophoresis—the result that in fact was observed. The internal fragments (the 22% and 14% fragments) will have a more diffuse distribution because both ends will be heterogeneous, again consistent with the pattern observed.

Model To Explain How *Eco*K Selects Cleavage Sites. As expected from previous work, the primary cleavage sites for *Eco*K in T7 DNA are not located at the recognition sites. However, a remarkable correlation is apparent: the positions of the cleavage sites cluster about the midpoints between adjacent recognition sites. The *Eco*K recognition sites in T7 DNA are at positions 38.0, 66.6, 81.7, and 93.8, and the midpoints between adjacent sites are at positions 52.3, 74.1, and 87.7—exactly the positions of the cleavage sites we have mapped. How are these cleavage sites selected, and what determines the order of cleavage?

Previous work on *Eco*B and *Eco*K has been interpreted to mean that the enzymes remain bound at their recognition site throughout the reaction and translocate DNA past themselves for long distances to reach their cleavage sites, forming simple or supertwisted loops in the process (9, 12–14). It was further concluded that *Eco*B can translocate DNA from only one side of the asymmetric recognition sequence (the 5′ side, as usually written) (9) and that *Eco*K is capable of translocating DNA from either side (13); but the mechanism for selecting cleavage sites remained a mystery. We have not been able to make a satisfactory interpretation of the results shown in Fig. 1 by any mechanisms involving translocation from only one side of a recognition site, whether the direction of translocation is fixed or random relative to the asymmetric recognition sequence. However, all of our observations are easily explained on the basis of two simple assumptions: (*i*) in the presence of ATP, a molecule of *Eco*K that is specifically bound at its recognition site translocates DNA toward itself from both directions simultaneously: and (*ii*) when this translocation causes neighboring enzymes to meet, they cut the DNA between them. In this context, simultaneous is meant to imply only that a single enzyme translocates equivalent amounts of DNA toward itself from both sides; the mechanism could be concerted or alternating. This model is illustrated in Figs. 2 and 3.

Translocation of DNA from both sides of each enzyme bound at a recognition site would eventually cause all of the neighboring enzymes to meet each other in the DNA. If rates of translocation are similar throughout the DNA, the en-

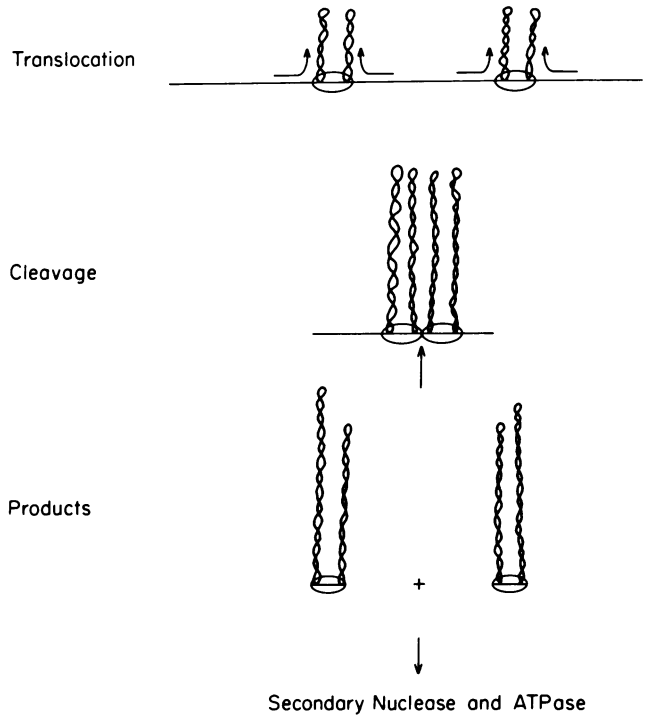

FIG. 2. Model for the nuclease action of *Eco*B and *Eco*K. In the presence of ATP, the enzyme bound at its recognition site is assumed to translocate DNA toward itself simultaneously from both directions at approximately equal rates. If the enzyme remains bound to its recognition site, such translocation should produce double loops (as indicated in the figure), which have been observed occasionally in electron micrographs (13); if the enzyme remains bound only at the translocation points, translocation should produce single loops, the predominant type observed in electron micrographs (9, 13, 14). When translocation brings neighboring enzyme molecules together, they make a double-strand cut in the DNA between them. In the secondary phase of the reaction, excess enzymes apparently cooperate with specifically bound enzymes to make further cuts and to hydrolyze large amounts of ATP.

zymes should meet at the midpoints between adjacent recognition sites after time intervals that reflect the different lengths of DNA between sites. The observed heterogeneity of cleavage positions presumably reflects differences in the time it takes for translocation to begin upon addition of ATP, fluctuations in rates of translocation, or both.

If cleavages do not occur until neighboring enzymes meet in the DNA, we can estimate the minimum translocation rates, using the distances that must be translocated to bring neighboring enzymes together and the approximate times at which the first cuts are made at each cleavage site. Each of the neighboring enzymes must translocate 2400 base pairs of DNA to meet at position 88 in T7 DNA, 3000 base pairs to meet at position 74, and 5700 bp to meet at position 52; and the first cleavages at these sites are made shortly before 15, 15, and 30 sec, respectively. These figures give minimum translocation rates of 160, 200, and 190 base pairs per second. Thus, the rate of translocation of T7 DNA from each side of an *Eco*K molecule bound at its recognition site appears to be approximately 200 base pairs per second.

The remarkably good agreement in translocation rates calculated for translocation distances that differ by more than a factor of 2 suggests that the time it takes to begin translocation after ATP is added and the minimum time it takes to cut the DNA after neighboring enzymes meet must both be less than a few seconds. However, it takes 15–25 sec for all of the molecules to be cut at any individual site. Presumably, most of this variability is in the time it takes for

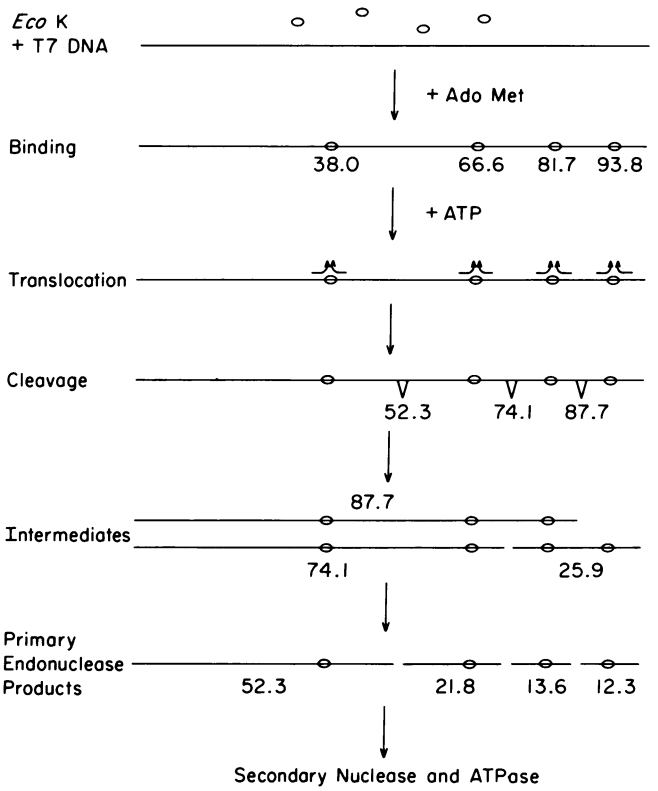

FIG. 3. Primary cleavage of phage T7 DNA by *Eco*K. Details of the reaction are given in the legend to Fig. 2 and in the text. The lines represent T7 DNA, and the ellipses represent *Eco*K molecules. The numbers given for the binding reaction are the positions of the *Eco*K recognition sites in T7 DNA; the numbers given for the cleavage reaction are the mean positions of the cleavages in T7 DNA; the numbers given for the intermediates and products are the mean sizes of the DNA fragments in T7 units.

enzymes to cut the DNA after they have met because such a large variation in lag time before translocation begins, or in the translocation rate, would broaden the distribution of cleavage sites to such an extent that discrete DNA fragments would be difficult to detect.

This model for the nuclease action of *Eco*K explains every detail of the rapid phase of degradation of T7 DNA, which is complete by about 1 min after addition of ATP. The immediate products of digestion will be DNA fragments containing a single recognition site with an *Eco*K enzyme (or subunit) remaining bound to it (Figs. 2 and 3). These bound enzymes may be able to continue translocating DNA, which could perhaps account in part for the ATPase activity that can continue long after the primary nuclease activity is over (8, 15, 16), but they would not meet another translocating enzyme in the DNA and, therefore, would not be expected to cut the DNA further. However, a slow secondary degradation continues after the primary cuts have been made (particularly noticeable in the 10- and 30-min samples in Fig. 1). Murray *et al.* (17) found that degradation of linear molecules having only a single recognition site does not occur to any significant extent unless a substantial excess of enzyme is present, suggesting that the excess enzymes cooperate with the enzyme that is bound at the recognition site to make the secondary cuts. The reaction analyzed in Fig. 1 was estimated to have 11 *Eco*K molecules per recognition site in T7 DNA. Digestion of linear DNAs having a single recognition site does require specifically bound enzyme because DNAs that have no recognition site are not degraded even when present in the same reaction mixture (results from our mapping of *Eco*K recognition sites in T7 DNA, not shown).

Comprehensive Model and Relationship to Previous Results. Our model provides a simple and consistent way to interpret a large amount of previous work on *Eco*B and *Eco*K. Most *in vitro* experiments on these two enzymes have used small circular DNAs having one or two recognition sites, linear derivatives produced by cutting with either type I or type II restriction enzymes, or phage λ or T7 DNA.The predicted results depend on the molar ratio of enzyme to DNA, the number of recognition sites per DNA molecule, and whether the DNA is linear or circular.

Consider linear DNA molecules. At a ratio of one enzyme per recognition site, all of the enzymes will become bound at recognition sites, and every recognition site will eventually have an enzyme bound to it. DNA molecules having only one recognition site will not be cut because translocating enzymes cannot meet in the DNA; molecules having more than one recognition site will receive a double-strand cut between recognition sites, where the translocating molecules meet in the DNA (referred to as primary cleavages). At least in circular phage λ DNA, the two strands are usually not cut simultaneously (18). After all of the primary cleavages have been made, the remaining DNA fragments will have only a single recognition site per molecule and, therefore, will not be further cut. However, at enzyme-to-DNA ratios considerably higher than one enzyme per recognition site, even DNAs having only one recognition site will be cut in the secondary reaction that involves excess enzymes (ref. 17 and our results).

Circular DNA molecules that have more than one recognition site are expected to be cut in much the same way as linear molecules. Circular molecules might present topological problems for the translocation reaction, but if translocating enzymes meet in the DNA, they should make a primary cut. After the first double-strand cut has been made, the DNA will be equivalent to any other linear molecule that is undergoing translocation and cleavage. The eventual number of primary cuts will equal the number of recognition sites in a circular DNA, whereas it will be one less than the number of recognition sites in a linear DNA.

Circular molecules that have only a single recognition site are a special case. At enzyme-to-DNA molar ratios of 1 or less, the enzyme will bind to its recognition site and translocate DNA, but it can neither meet another enzyme nor reach an end of the molecule. Translocation will eventually stall, either against a topological barrier or when the entire circle has been translocated. Such an enzyme apparently cuts one strand of the DNA (9, 19, 20). At higher molar ratios of enzyme to DNA, a second enzyme molecule cooperates with the stalled enzyme to cut the second strand of the DNA, a reaction that can happen efficiently at molar ratios low enough that few if any secondary cuts are made in the resulting linear DNAs (7, 9, 19–24). As expected, isogenic circular molecules that have two recognition sites are cut twice under the same conditions (21, 23).

Hydrolysis of ATP is probably required for the translocation process (9, 13, 14), but extensive ATPase activity can also continue for long periods after the primary cuts have been made (8, 15, 16). This continued ATPase activity may reflect continued translocation by the enzymes (or subunits) remaining bound at recognition sites after the primary cuts have been made. However, Horiuchi *et al.* (8) found that the ATPase activity stimulated by circular molecules that have a single recognition site continued at a high rate for long periods at low enzyme-to-DNA ratios, whereas continued ATPase activity on linear DNA required much higher levels of enzyme. This finding suggests that the continuing ATPase activity stimulated by circular DNA is associated with stalled translocating enzymes, whereas most of the activity stimulated by linear DNA is associated with secondary cleavages. Furthermore, Linn *et al.* (23) found that, when a small molar ratio of *Eco*B was allowed to react with circular DNA that had a single recognition site, continuing ATPase activity was associated with DNA molecules that sedimented faster than supercoiled DNA, where stalled translocating complexes might be expected to sediment.

Convincing support for the idea that primary cleavage sites occur between recognition sites *in vivo* comes from studies of Brammar *et al.* (25), who analyzed the effects of *Eco*K digestion on expression of *trp* genes carried by phage λ. In a *recBC* mutant, which greatly reduced further degradation of the primary products of restriction enzyme digestion, a single recognition site, even when located within the *trp* operon, had essentially no effect on expression of the *trp* genes. The effect of pairs of recognition sites depended on their location: expression was essentially unaffected when one site lay just downstream of the promoter and the other lay a considerable distance upstream; but expression was reduced by a factor of about six when the sites were located so that a cleavage between them would separate the operon from its promoter.

How can previous results on translocation of DNA by *Eco*B and *Eco*K (9, 13, 14) be reconciled with our model? If the enzyme remains at its recognition site throughout the reaction, bidirectional translocation should produce double loops (Fig. 2). A few such double loops were observed in electron micrographs of reaction mixtures examined by Yuan *et al.* (13), although none were reported by Rosamond *et al.* (9) or Endlich and Linn (14). As noted by Endlich and Linn (14), the different procedures used to prepare samples for electron microscopy gave significantly different results among these three studies; perhaps none of the procedures preserved a high frequency of the double loops predicted by our model. An alternative explanation for the preponderance of single loops might be that the enzyme remains bound to DNA at both translocation points but not at its recognition site. This seems less likely, however, because both *Eco*K and *Eco*B appear to remain bound at the recognition site after reacting (in the presence of ATP) with linear DNAs that have a single recognition site (12, 14).

The conclusions that *Eco*B could translocate DNA from only the 5′ side of its recognition sequence (9) and that *Eco*K could translocate DNA from either side of its recognition sequence (13) were based on the cutting, or lack of cutting, of linear DNAs having only a single recognition site. The assumption was that cleavage would occur only if the enzyme had translocated a relatively long distance (more than 1000 base pairs). We conclude instead that the cleavage, or lack thereof, depended on the enzyme-to-DNA ratio (ref. 17 and our results) and did not provide information about the direction of translocation. The need for a relatively high level of enzyme to cut linear DNAs that have only one recognition site may also explain other results that have been interpreted in more elaborate ways.

Predictions of the Model. Our model gives a rationale for predicting the location and timing of cleavages by *Eco*B and *Eco*K in synchronous reactions with any linear DNAs where the locations of the recognition sites are known. Tests of whether the translocation rate is affected by different base compositions or sequences should be possible by looking for shifts in the positions of cleavage sites relative to the midpoint between adjacent recognition sites in DNAs of known nucleotide sequence. If topological constraints in circular DNAs interfere with uniform rates of translocation, tight clustering of primary cleavage sites might not be observed in these molecules.

*Eco*B and *Eco*K are genetically homologous (26), and subunits of one can complement the other (27). Therefore, we would expect these two enzymes to be able to cooperate to make a primary cleavage. If so, linear DNA that contains a single recognition site for each enzyme should be cut efficiently when the amounts of each enzyme are sufficient to

occupy both sites, but should be resistant to cleavage by equivalent amounts of either enzyme individually (although ATPase should be stimulated). Any differences in the relative rates of translocation by the two enzymes should be revealed (in a synchronous reaction) as a shift in cleavage position relative to the midpoint between the two recognition sites.

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