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Pausing of the Restriction Endonuclease *Eco*RI during Linear Diffusion on DNA†

Albert Jeltsch,‡ Jürgen Alves,§ Heiner Wolfes,§ Günter Maass,§ and Alfred Pingoud*,‡

Institut für Biochemie, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany, and Zentrum Biochemie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 30623 Hannover, Germany

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ABSTRACT: Linear diffusion is a mechanism to accelerate association rates beyond their three-dimensional diffusional limit. It is employed by the restriction endonuclease *Eco*RI as well as many other proteins interacting with specific DNA sequences to locate their target sites on the macromolecular substrate. In order to investigate biochemical and biophysical details of the linear diffusion process, we have developed a competitive cleavage assay which allows us to assess with great accuracy the influence of sequence, sequence context, and other structural features on the linear diffusion of *Eco*RI on DNA. We show here that linear diffusion is not a hopping but a sliding movement in which *Eco*RI follows the helical pitch of the DNA, because it does not "overlook" any cleavage site. Linear diffusion is slowed when *Eco*RI encounters sites on the DNA which resemble its recognition site ("star" sites). Pauses of up to 20 s are induced, depending on sequence and orientation of the star site. These data suggest that *Eco*RI can bind to DNA in two binding modes: one tight, specific, and immobile, leading to DNA cleavage, and another one loose and nonspecific, allowing for linear diffusion. Depending on the similarity between the recognition sequence and the DNA sequence being encountered by *Eco*RI, there will be a continuous transition between these binding modes. Other proteins bound to the DNA and irregular DNA structures such as bent DNA or a triple helix constitute a barrier that cannot easily be passed by *Eco*RI.

*Eco*RI is a well-established model system for the study of protein–DNA interactions (Heitman, 1992; Roberts & Halford, 1993). It binds and cleaves DNA containing -GAATTC- with high specificity, which is due to many specific contacts of the enzyme to the bases in the major groove of the recognition sequence, as well as contacts to the phosphodiester backbone of the DNA. Upon complex formation, the DNA is unwound and kinked and the major groove is widened significantly (Rosenberg, 1991). Sequences differing from the recognition sequence by a single base pair ("star" sites) are cleaved orders of magnitude more slowly, because critical contacts cannot be formed (Lesser et al., 1990; Thielking et al., 1990) which are needed for strong binding and for the distortion of the DNA required for catalysis to proceed (Lesser et al., 1993; Jeltsch et al., 1993a). *Eco*RI also binds nonspecifically to DNA. This binding is much weaker than specific binding, but it is a prerequisite for the enzyme to locate its target site by one-dimensional diffusion rather than by a three-dimensional search (Jack et al., 1982; Ehbrecht et al., 1985; Terry et al., 1985).

Linear diffusion is a very effective mechanism to accelerate association rates of proteins to specific sites on macromolecular DNA that contains a large surplus of nonspecific competitor sites (Adam & Delbrück, 1968; Richter & Eigen, 1974). It is a one-dimensional random walk process (Berg & von Hippel, 1985; von Hippel & Berg, 1989), distinct from the ATP-driven directional translocations along the DNA of type I restriction endonucleases (Studier & Bandyopadhyay, 1988).

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* To whom all correspondence should be addressed: Institut für Biochemie, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany. Phone: +49 641 702 5824. Fax: +49 641 702 5821.

‡ Justus-Liebig-Universität.

§ Medizinische Hochschule Hannover.

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The structural basis for the sliding of *Eco*RI on DNA became clear when the *Eco*RI–DNA cocrystal structure was solved (McClarin et al., 1986; Kim et al., 1990), because the protein enwraps the DNA, as found later also for the restriction endonuclease *Eco*RV (Winkler et al., 1993).

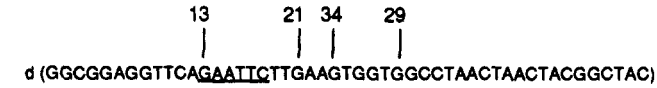
So far, linear diffusion on DNA has been demonstrated for several proteins: lac repressor (Barkley, 1981; Winter & von Hippel, 1981; Winter et al., 1981), RNA polymerase (Singer & Wu, 1987; Ricchetti et al., 1988), λ cro protein (Kim et al., 1987), *Bam*HI and *Hin*dIII endonuclease (Ehbrecht et al., 1985), *Bam*HI methylase (Nardone et al., 1986), UV-endonuclease (Hamilton & Lloyd, 1989), and T4 endonuclease V (Lloyd et al., 1980; Ganesan et al., 1986; Gruskin & Lloyd, 1988). The molecular mechanism of linear diffusion, however, has not yet been analyzed in detail. For example, it can be asked whether the linear diffusion process is more or less effective depending on sequence and conformation of the DNA. Sequences that resemble the recognition sequence of the protein could slow down the diffusion rate, as probing interactions are formed and must be loosened again before the diffusion process can continue. Irregular DNA structures, like curved DNA and triple helices, might similarly constitute a barrier for the protein and thereby interrupt the linear diffusion process. Two other very interesting questions regarding linear diffusion of a protein along the DNA are whether the movement is a continuous sliding or a hopping process and whether the movement follows the pitch of the double helix. To be able to answer such questions, we have developed a very accurate competitive cleavage assay (Jeltsch et al., 1993b) which allows us to analyze the influence of sequence, sequence context, and structural features, as well as the effect of other proteins bound to DNA, on the process of linear diffusion.

MATERIALS AND METHODS

Preparation of Proteins. *Eco*RI and the *Eco*RV mutant D90A were homogeneous preparations obtained from overproducing *Escherichia coli* strains by procedures described previously (Geiger et al., 1989; Fliess et al., 1988). *Eco*RV-D90A is a catalytically inactive *Eco*RV mutant that specifically binds DNA in the presence of $MgCl_2$ (Thielking et al., 1992). Myb protein that binds to -AACNGTT- sequences (Zobel et al., 1991) was a kind gift of Mr. A. Ebneth (Medizinische Hochschule Hannover). Serum response factor (SRF) that specifically binds to the serum response element (SRE) (Schroeter et al., 1990) was kindly provided by Dr. R. Janknecht (Janknecht et al., 1991).

Oligodeoxynucleotides. Oligodeoxynucleotides were synthetized on solid support with a Milligen Cyclone DNA synthesizer using β-cyanoethylphosphoramidites obtained from MWG Biotech (Ebersberg, Germany). Biotin–phosphoramidites were obtained from Glen Research; "amino linker" containing phosphoramidites, from Millipore. Oligodeoxynucleotides containing an amino linker on their 5′ ends were labeled with fluorescein isothiocyanate (Sigma) or digoxygenin-3-*O*-(methylcarbonyl)-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim) as described (Jeltsch et al., 1993b) to produce oligodeoxynucleotides whose 5′ ends were labeled with fluorescein or digoxygenin.

PCR. Several 912-bp long substrates were used to investigate the influence of different test sequences on the efficiency of linear diffusion of *Eco*RI. These substrates were generated by PCR as described (Jeltsch et al., 1993b) using a pUC8 plasmid template, a biotinylated lower primer, and an appropriate upper primer, labeled with fluorescein or digoxygenin. The general sequence of the upper primer was



The different substrates (Table 1) were obtained by PCR using appropriate variants of the upper primer. The length of the upper primers used varied from 39 to 46 nucleotides, depending on the number and positions of mismatches to the template. All upper primers were labeled on their amino linker with fluorescein (see above) prior to the PCR reaction. In these substrates the *Eco*RI cleavage site is flanked by a short arm comprising 12 bp and a long arm comprising 894 bp. In different substrates the long arm contains different test sequences adjacent to the *Eco*RI site (Table 1).

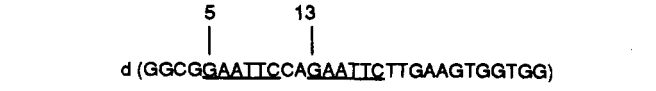
Generation of an Internal Standard. The 39 nucleotide long upper PCR primer used to generate the reference substrate was labeled with digoxygenin (see above) and annealed to a complementary oligodeoxynucleotide, which had been biotinylated on its 5′ end. The resulting 39-bp substrate was used as an internal standard in the competitive cleavage assay.

Competitive Cleavage Assay of Two Substrates. Cleavage reactions were carried out with a 912-bp substrate and the 39-bp internal standard in competition by using an ELISA-based assay procedure as described (Jeltsch et al., 1993b). Briefly, the assay employs oligodeoxynucleotides that are labeled with different haptens (912-bp substrate, fluorescein; 39-bp standard, digoxigenin) on one strand. A 5′ biotin label of the complementary strand is used to separate products and uncleaved substrates on avidin-coated microtiter plates by washing steps. Cleavage of the long substrate and the 39-bp standard is carried out in competition in one tube; the disappearance of both substrates is detected by two separate series of ELISA reactions. Cleavage reactions were carried out in buffer conditions that were demonstrated to promote linear diffusion of *Eco*RI (Ehbrecht et al., 1985): 20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, and 50 μg/mL BSA at 21 °C. Both substrate concentrations were 50 nM; *Eco*RI concentration was 20 nM.

All long substrates contain different test sequences in the long arm adjacent to the *Eco*RI cleavage site to measure the influence of these test sequences on the linear diffusion of *Eco*RI. A triple helix (Levene et al., 1986) was formed by adding dT_{16} (1 μM) to the reaction mixture with substrate polyA (Table 1, substrate 11) and incubating for 30 min at 21 °C before the reaction was started. The "bend" substrate contains an $A_5N_5A_6$ tract, known to induce an 18° bend in the DNA (Zahn & Blattner, 1987; Koo & Crothers, 1988; Koo et al., 1990; Nelson et al., 1987). Substrates 14–16 contain specific binding sites for *Eco*RV, Myb, or SRF, respectively. *Eco*RI cleavage experiments with these substrates were carried out in the presence of 0.2 μM *Eco*RV-D90A, Myb, or SRF, respectively, after a 30-min preincubation of the reaction mixture containing the DNA-binding protein.

EcoRI Substrates Containing Two EcoRI Sites. Substrate A (30 bp) (Figure 1) containing two *Eco*RI sites was generated by annealing a 5′-^{32}P-labeled oligodeoxynucleotide (oligo A1) containing the sequence -GAATTC- at positions 5 and 13 and a complementary oligodeoxynucleotide.

Oligo A1:



Substrate B (912 bp) was generated by PCR, using the labeled oligo A1 as upper primer and the same lower primer as for

Table 1: Linear Diffusion Efficiency of *Eco*RI on Variants of the 912-bp Reference Substrate, Which Contain Different Test Sequences Adjacent to the *Eco*RI Cleavage Site on the Long Arm of the Substrates

no.	substrate	test sequence[a]	$f = k_{\text{long substrate}}/k_{39\text{bp}}$[b]
1	reference	G^{21}AAGTG	3.9 ± 0.2
2	*4A	G^{21}AAATC	3.4 ± 0.05
3	*6G	G^{21}AATTG	2.9 ± 0.1
4	*4G	G^{21}AAGTC	1.7 ± 0.1
5	*6T ($^1/_4$ turn)	G^{21}AATTT	2.6 ± 0.1
6	*6T ($^1/_2$ turn)	G^{24}AATTT	2.7 ± 0.1
7	*6T (1 turn)	G^{29}AATTT	2.7 ± 0.2
8	*1A	A^{21}AATTC	2.3 ± 0.05
9	*1C	C^{21}AATTC	2.5 ± 0.1
10	**6T	G^{21}AATTTGTGAATTT	1.4 ± 0.1
11	poly A	$G^{21}A_{17}CG$	3.9 ± 0.2
12	triple helix	poly A + dT_{16}	1.1 ± 0.1
13	bend	$G^{21}A_5GTGGCA_6TC$	2.3 ± 0.1
14	*Eco*RV	G^{21}ATATC	1.2 ± 0.1
15	Myb	G^{21}AACTGTTGG	1.5 ± 0.2
16	SRF	G^{21}TCCATATTAGGAC	1.2 ± 0.1

[a] The test sequence was inserted at position 21, 24, or 29, respectively, of the upper primer used in the PCR to obtain variants of the 912-bp reference substrate (cf. Materials and Methods). [b] Cleavage reactions were measured with the competitive cleavage assay in 20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, and 50 μg/mL BSA at 21 °C. The ELISA analyses were done in duplicate; all experiments were repeated twice.

all other PCR reactions. Cleavage reactions were carried out with both substrates separately in 20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, and 50 μg/mL BSA at 21 °C. At appropriate times, aliquots were withdrawn, spotted on DEAE thin-layer chromatography plates (Macherey & Nagel), and subjected to homochromatography as described (Thielking et al., 1990). Subsequently, the radioactivities of the spots representing the substrate and the two labeled products were counted.

Monte Carlo Simulation of the Linear Diffusion of EcoRI. Monte Carlo simulations were carried out to estimate the average number of associations to the star site (located at position 21 on most of the 912-bp substrates) before the enzyme hits the canonical site (position 13) by using the program LINDIF (Jeltsch, 1994). This program simulates a one-dimensional random walk process of an enzyme on a DNA substrate providing 912 binding sites. After a random association to the DNA, the protein molecule starts a stepwise random walk along the linear lattice. In this simulation the probability of linear diffusion vs the probability of dissociation of the enzyme from the DNA was taken to be 10^6 as determined by Ehbrecht et al. (1985). The number of hits to position 21 (position of the star site in substrates 2–5, 8, and 9) is counted before the enzyme reaches position 13 (position of the cleavage site). LINDIF assumes that the probability to leave the star site in each direction by linear diffusion is 0.5.

RESULTS AND DISCUSSION

To investigate the molecular details of linear diffusion of *Eco*RI on DNA, we determined the rate of DNA cleavage by *Eco*RI using several different substrates which are derived from a 912-bp PCR product. This 912 bp long DNA is cleaved 3.9 times faster than a 39-bp oligodeoxynucleotide used as internal standard (Jeltsch et al., 1993b). The 39-bp standard is identical in sequence to one end of the 912-bp DNA. Both substrates contain one *Eco*RI cleavage site 13 bp from one end. The 912-bp substrate has an 894-bp stretch of nonspecific DNA on the other side of the cleavage site, along which *Eco*RI can diffuse. As the flanking sequences of the cleavage sites

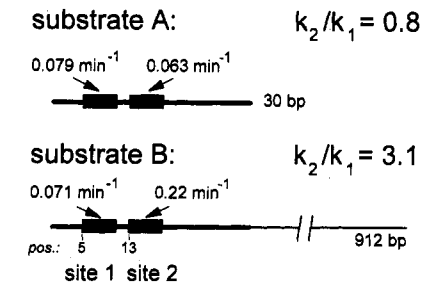


FIGURE 1: Cleavage of substrates containing two *Eco*RI cleavage sites. Substrate concentrations were 50 nM; *Eco*RI concentration was 20 nM. Cleavage reactions were carried out in 20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, and 50 μg/mL BSA at 21 °C. Each experiment was done twice; the errors of k_2/k_1 are estimated to be ±10%. The cleavage rate of site 2 in substrate B is enhanced 3.9-fold relative to that in substrate A (3.1/0.8).

in both substrates are identical and both substrates are cleaved in competition in one reaction tube, the higher cleavage rate of the long substrate can only be due to the fact that *Eco*RI more efficiently locates its target site on the long substrate by linear diffusion. Our experimental strategy was to introduce several test sequences adjacent to the cleavage site in the "long arm" of the 912-bp substrate and measure changes in cleavage rates and thereby determine the efficiency of linear diffusion in order to investigate the influence of the test sequence on linear diffusion.

In order to clarify first whether during linear diffusion *Eco*RI "overlooks" any recognition site on its way, two substrates were used. Substrates A (30 bp) and B (912 bp) each contain two *Eco*RI sites: site 1 at position 5 and site 2 at position 13. As shown in Figure 1 with the long substrate B, DNA cleavage rate enhancement by linear diffusion is only observed at site 2 adjacent to the long arm of the substrate, and not, however, at site 1 adjacent to the short arm. Rate enhancement was found to be 3.9-fold, in close agreement to a measurement with the reference substrate (Jeltsch et al., 1993b). This result clearly demonstrates that *Eco*RI cleaves the site it meets first, which means that it does not overlook any recognition site while diffusing along the DNA and, hence, that linear diffusion by *Eco*RI must be a continuous sliding rather than a hopping process.

As the 912-bp reference substrate did not contain *Eco*RI star sites, we were able to measure the influence of deliberately introduced star sites on linear diffusion of *Eco*RI. As expected, a star site on the "short arm" of the 912-bp substrate has no measurable influence on the cleavage rate (data not shown). However, cleavage rates are clearly reduced by star sites located on the long arm of the 912-bp substrate (Figure 2; Table 1). The preference of cleavage of the 912-bp substrates over the 39-bp standard ($f = k_{\text{long substrate}}/k_{39\text{bp}}$) drops from 3.9 (reference) to 1.7 for *4G containing a -GAAGTC- site in the long arm adjacent to the cleavage site. This effect can be attributed to a reduced efficiency of linear diffusion on the long substrates containing the star sequences, because all test sequences were introduced without altering the immediate flanking sequences of the recognition site, and the DNA cleavage rates by *Eco*RI are diffusionally limited under the reaction conditions employed here. Therefore, it takes more time for the enzyme to discriminate a canonical site from closely related ones (i.e., star sites) than from any nonspecific site. The results obtained with **6T and the three different *6T substrates (substrates 5–7 in Table 1) show that the inhibitory effect on linear diffusion of one star site can be amplified by a second star site nearby. The data given in Table 1 for substrates 2–5 can be used to estimate the pausing

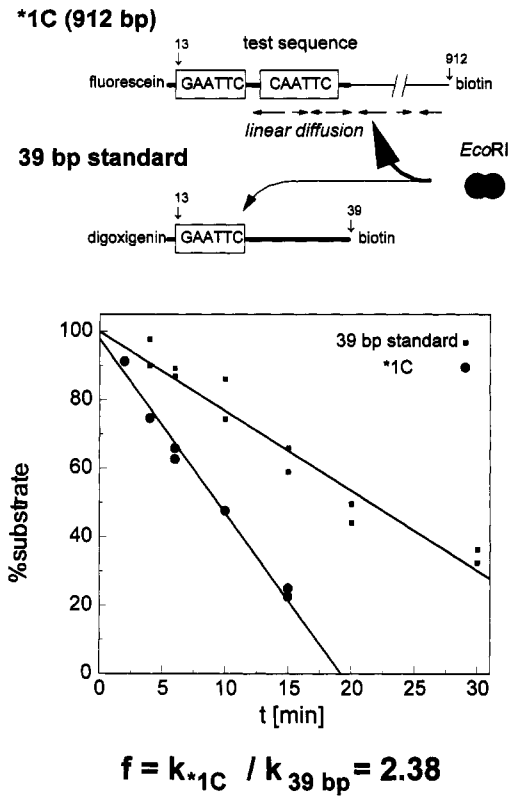


FIGURE 2: Competitive cleavage of substrate *1C and the 39-bp standard. Concentrations of both substrates were 50 nM; *Eco*RI concentration was 20 nM. Reaction conditions were the same as given in the caption to Figure 1.

time of *Eco*RI on the individual star site. Pausing time is defined as

$$t_{pause} = (1/k - 1/k_{reference})/n_{events} \quad (1)$$

$k = fk_{39bp}$ represents the cleavage rate of each substrate. k_{39bp} and $k_{reference}$ were determined to be 0.065 ± 0.03 and 0.251 ± 0.07 min^{-1}, respectively. n_{events} corrects for the fact that, due to the random nature of linear diffusion, on average an enzyme will hit the star site several times before it reaches the cleavage site. This value was determined by a Monte Carlo simulation to be 16 for a star site at position 21.

Using eq 1 and the values given in Table 1, pausing times can be calculated to be 2.2 (*4A), 5.2 (*6G), 7.5 (*6T), and 19.3 s (*4G). These pausing times are intermediate between the half-life of a canonical *Eco*RI–DNA complex, which is about 15 min (Jack et al., 1982), and that of a nonspecific *Eco*RI–DNA complex, which is approximately 0.1 s (Ehbrecht et al., 1985).

Interestingly, those star sites that are readily attacked (e.g., -GAAGTC- as contained in *4G) inhibit linear diffusion more than star sites that are only cleaved very slowly (e.g., -GAAATC- as contained in *4A) (Thielking et al., 1990). Obviously, the greater the probability of cleavage of the star site, the more difficult it is for *Eco*RI to move across the site and proceed with linear diffusion. Nevertheless, moving on is still much preferred over cleavage at a star site.

These data suggest that *Eco*RI can bind to DNA in two extreme binding modes: a nonspecific and loose binding mode, which is dominated by nondirectional interactions, like electrostatic attraction, and allows for linear diffusion, and a tight, specific binding mode, which eventually may lead to DNA cleavage and which is dominated by many specific contacts between *Eco*RI and the DNA. In the latter binding mode the frictional coefficient for linear diffusion is high, and it, therefore, does not permit linear diffusion. There might be a continuous transition between these two binding modes. More and more specific contacts between a DNA site and *Eco*RI decrease the ability of *Eco*RI for linear diffusion but increase the probability that the binding site occupied is cleaved.

Substrates 5–7 (see Table 1) contain the same star site at different distances from the cleavage site, namely, 1/4, 1/2, and 1 turn of the DNA. Their *f*-values are identical within the limits of error, demonstrating that phasing of the cleavage site and the star site is not important. This finding, combined with the result that *Eco*RI does not overlook any recognition site while diffusing along the DNA, can only be rationalized if *Eco*RI follows the helical pitch of the DNA during linear diffusion. This allows during linear diffusion for a continuous scanning of the major groove of the DNA by the enzyme.

Comparison of the results obtained with the substrate pairs *6T/*1A and *6G/*1C (see Table 1), which both contain the same star site in a different orientation with respect to the cleavage site nearby, shows that depending on their orientation the same star sites slow down linear diffusion to a different extent. Possibly, this difference is due to a preferential leaving direction of *Eco*RI from the star site. If leaving is preferred in the direction toward the cleavage site, statistically fewer encounters of linearly diffusing *Eco*RI and the star site will occur as compared to the situation where the other direction is preferred. Our data suggest that the enzyme tends to leave both star sites in the direction of the canonical -GAA- half-site. Alternatively, the effect of a star site (which is asymmetric) on linear diffusion may be modulated by the asymmetry of its flanking sequences. A substrate containing a star site with the canonical -GAA- half-site next to a particular flanking sequence may be more strongly bound by *Eco*RI than a substrate containing the same star site in the opposite orientation, where the -GAA- half-site is located next to another flanking sequence.

While star sites interfere with linear diffusion of *Eco*RI along the DNA by keeping the protein bound, unusual DNA structures may constitute barriers across which *Eco*RI cannot easily move. The former effect is best characterized by a pausing time; the latter, by a transmission probability (P_t):

$$P_t = (k - k_{39bp})/(k_{reference} - k_{39bp}) = (f - 1)/(f_{reference} - 1) \quad (2)$$

Using eq 2 and the data given in Table 1, P_t can be calculated to be 1.0 for a polyA stretch, 0.45 for a bend, and 0.03 for a triple helix. Therefore, a polyA stretch does not interfere with linear diffusion, whereas a bend significantly reduces the ability of *Eco*RI to pass over this region, and *Eco*RI is hardly able to pass over a triple helix region. These results suggest that during linear diffusion *Eco*RI is continuously probing the structural features of the DNA and scanning the major groove for specific contact points. Regions with largely altered conformational properties (e.g., a bend) or with an inaccessible major groove (e.g., due to triple helix formation) do not support linear diffusion of *Eco*RI on the DNA. Triple helix forming oligodeoxynucleotides in this respect resemble proteins firmly bound to the DNA which also prevent linear diffusion, as shown here for the specific DNA-binding proteins *Eco*RV-D90A, Myb, and SRF (Table 1) and previously for nonspecific DNA-binding proteins (Ehbrecht et al., 1985). One may question the biological significance of linear diffusion by *Eco*RI, because it is strongly inhibited by other proteins bound to the DNA. It is, however, reasonable to assume that

a phage DNA is relatively free of bound proteins immediately after penetration of the cell. The biological target of *Eco*RI, therefore, presumably permits linear diffusion.

CONCLUSIONS

By investigating the effect of various DNA sequences, structures, and ligands bound to the DNA on the linear diffusion of *Eco*RI, we could demonstrate that linear diffusion is a punctuated process in which *Eco*RI slides along the DNA following the helical path, pausing at sites that resemble its recognition site and being blocked by irregular DNA structures or ligands bound to the DNA. With appropriate modifications, the methodology we have developed can be used to study details of linear diffusion of other enzymes interacting with DNA.

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