Cooperative Binding Properties of Restriction Endonuclease EcoRII with DNA Recognition Sites*

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EcoRII is a member of the expanding group of type II restriction endonucleases that share the distinguishing feature of requiring cooperativity between two recognition sites in their substrate DNA. To determine the stoichiometry of the active DNA-enzyme complex and the mode of cooperative interaction, we have investigated the dependence of EcoRII cleavage on the concentration of EcoRII dimers. Maximal restriction was observed at dimer/site ratios of 0.25 and 0.5. The molecular weight of the DNA-enzyme complex eluted from a gel filtration column also corresponds to a dimeric enzyme structure bound to two substrate sites. We conclude that one EcoRII dimer is sufficient to interact cooperatively with two DNA recognition sites. A Lac repressor "barrier" bound between two normally reactive EcoRII sites did not inhibit restriction endonuclease activity, indicating that cooperativity between EcoRII sites is achieved by bending or looping of the intervening DNA stretch. Comparative cleavage of linear substrates with differently spaced interacting sites revealed an inverse correlation between cleavage rate and site distance. At the optimal distance of one helical turn, EcoRII cleavage is independent of the orientation of the recognition sequence in the DNA double strand.

Concerning their substrate requirements, certain restriction endonucleases (ENases) that interact at a distance with at least two DNA recognition sites resemble proteins involved in processes like transcription control, DNA recombination, and replication in both prokaryotes and eukaryotes (1–6). In addition to EcoRII, the first ENase for which this special characteristic was described (Ref. 7; for a review see 8), comprehensive studies were also performed on NaeI. Electron microscopy of DNA-NaeI complexes revealed loop structures connecting NaeI sites on the same DNA molecule (9). Another extraordinary type II ENase is SfiI, whose tetrameric enzyme structure bridges two specific recognition sites and cleaves them simultaneously (10, 11). A few other ENases are considered to belong to this particular ENase group (12, 13) termed Ief, characterized by requiring a second DNA (effector) site to perform cleavage (14).

The complex mode of action of these particular ENases appears to limit their efficiency in DNA restriction and may reflect additional biological functions besides the destruction of invading DNA. Other type II ENases (non Ief) like EcoRII and EcoRV use the simpler and more powerful mechanism of linear diffusion along the helical pitch of the DNA that enables them to find every single recognition site and effectively restrict foreign DNA (15, 16). Some experimental results support the idea of an evolutionary relationship of type Ief ENases with other protein families; Jo and Topal (17) discovered a sequence motif shared by ENase NaeI and the active site of human DNA ligase where the essential lysine 43 of the ligase is replaced by leucine in NaeI. A single amino acid substitution, L43K, in NaeI converts the ENase to a DNA topoisomerase with preferred binding to single-stranded and mismatched DNA and an increased sensitivity to intercalating agents (18, 19). Another study revealed the homology between a C-proximal sequence of ENase EcoRII with a motif characterizing the integrase family of recombinases. Site-directed mutagenesis resulting in a Y308F substitution abolished cleavage activity but not specific DNA binding (20). The combination of ligase/topoisomerase or integrase motifs with site-specific cleavage in these enzymes suggests that analyses of the structure-function relation and the unusual reaction mechanism of EcoRII and other type Ief ENases may disclose further evolutionary connections.

EcoRII recognizes the partially degenerate nucleotide sequence 5’-CCWGG and cleaves it 5’ of the first base. The endonucleolytic activity is inhibited by canonical C-5 methylation of the internal cytosine. Moreover, the enzyme is catalytically inactive on single recognition sites in larger DNA molecules and even on two sites in the same DNA (located “in cis”) when their distance exceeds a critical length. Hydrolysis of these a priori resistant DNA substrates can be achieved by the addition of susceptible EcoRII sites “in trans,” e.g. of synthetic EcoRII recognition site-containing oligonucleotide duplexes. This intermolecular EcoRII enzyme activation depends on the DNA site concentration and is inversely correlated to the length of the activator DNA molecule (for a review, see Ref. 8). Apart from the demonstration of positive cooperativity of EcoRII enzyme activity and the development of a preliminary mechanistic model (8, 21–23), the molecular mechanism of the EcoRII-DNA interaction has not been determined. To this aim, we have now analyzed the native molecular weight of homogeneous NH2-terminally His6-tagged EcoRII alone and complexed with substrate. We then investigated the dependence of EcoRII cleavage on the concentration of EcoRII dimers to calculate the composition of the active complex under optimal cleavage conditions. To discriminate between DNA translocation and looping as modes of cooperative site interaction, we examined the consequences of binding the Lac repressor as a molecular barrier between two normally reactive EcoRII sites on EcoRII cleavage activity. Furthermore, a series of DNA substrates was designed to elucidate the influence of spacing

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and orientation of two cooperating recognition sites on the EcoRII ENase reaction.

**EXPERIMENTAL PROCEDURES**

Oligonucleotides—The oligonucleotides were synthesized by the phosphorodiester method with a DNA synthesizer (Gene Assembler Plus, Amersham Pharmacia Biotech) and purified by 15% PAGE. Where required, the oligonucleotides were 5'-labeled with [γ-32P]ATP (ICN Biomedicals GmbH) using T4 polynucleotide kinase and purified by gel filtration on Probequant columns (Amersham Pharmacia Biotech).

Cloning of the EcoRII ENase Gene into the His-tag Vector pQE-30—The EcoRII endonuclease gene was amplified from pRZ09 using polymerase chain reaction with primers that created a 5'-terminal BamHI site and a 3'-terminal KpnI site (24). After amplification, the reaction mixture was diluted and digested with both enzymes. Due to the internal BamHI site, two fragments resulted, which were gel-purified and cloned in two steps. The 855-bp BamHI–KpnI fragment was cloned into pQE-30 (Qiagen GmbH). This plasmid was then BamHI-cleaved, treated with alkaline phosphatase, and ligated with the 326-bp BamHI fragment.

One-step Purification of EcoRII ENase by Nickel-NTA Affinity Chromatography—A 250-ml culture of E. coli JM109 containing the expression plasmid coding for the EcoRII ENase was incubated at 37 °C for 2 h. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 5 h. The cells were centrifuged, the pellet was resuspended in 4 ml of sonication buffer (50 mM NaH2PO4, pH 7.8, 300 mM NaCl), and the crude lysate was prepared by sonication. The cell debris was centrifuged for 30 min at 38,000 × g, and the supernatant was removed, followed by resuspending and a second centrifugation. The pooled supernatants were loaded on a 2-ml nickel-NTA column. The washing and elution procedures for His-tagged proteins (recommended by the supplier, Qiagen) were optimized for the preparation of EcoRII ENase. The wash stringency was increased by adding minimal imidazole concentrations (10–30 mM) to the wash buffer (50 mM NaH2PO4, pH 6.0, 1 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol). No detectable EcoRII was eluted under these conditions, whereas the amount of contaminating proteins in the final elution fractions could be minimized. For the specific elution of the His-tagged endonuclease, 0.1 mM imidazole was added to the wash buffer and applied as a step gradient. The main peak of EcoRII eluted in the second column wash. EcoRII concentrations were determined from the absorbance at 280 nm using the formula of Beavan and Holiday (25).

**Molecular Weight Determination of the Substrate-Enzyme Complex by Gel Filtration**—The molecular weight of His-tagged EcoRII was determined by analytical gel filtration on a Superdex 200 column using the standard proteins specified in the legend to Fig. 2. Chromatography was carried out at a flow rate of 0.7 ml/min at 4 °C in EcoRII assay buffer containing 0.1 mM EDTA, 0.1 mM DTT, 10 mM Tris-OAc, pH 7.6, 10 mM Mg(OAc)2, 25 mM NaCl, and 10 μg/ml BSA (Stratagene). Cleavage patterns were monitored by electrophoresis on 0.9% agarose gels stained with ethidium bromide.

**Cleavage of the pUC18/HaeII Fragment by EcoRII in the Presence of Lac Repressor—**pUC18 DNA was cleaved to completion with HaeII. The 445-bp fragment was isolated according to the instructions of the gel extraction kit Qiaex (Qiagen GmbH). Upon dephosphorylation of its 5'-ends, the fragment was labeled with [γ-32P]ATP (ICN Biomedicals GmbH). Unincorporated triphosphates were removed by microspin column S-300 HR (Amersham Pharmacia Biotech) centrifugation. Binding reactions were carried out in 20 μl containing 0.05 pmol of the DNA fragment with a 50-fold molar excess of purified Lac repressor in 0.5× universal buffer and 0.5× Lac repressor binding buffer (10 mM Tris/HCl, pH 8.0, 10 mM KCl, 10 mM Mg(OAc)2, 0.1 mM EDTA, 0.1 mM DTT, 50 μg/ml BSA) at 37 °C for 30 min. Complex formation was abolished by the addition of 1 nmol of IPTG. For separation of free DNA and DNA-Lac repressor complexes, samples were run on a 5% nondenaturing polyacrylamide gel in 1× TBE at 4 °C; the gel was dried and exposed to an x-ray film overnight at room temperature. To investigate the influence of bound Lac repressor on the cleavage efficiency, 0.05 pmol of EcoRII was added to parallel samples and run on a 5% polyacrylamide gel with 0.1% SDS to dissociate enzyme-DNA complexes.

Quantitative Evaluation of EcoRII Cleavage of Synthetic Substrates with Differently Spaced Sites in Cis—Substrate DNAs containing 0.1 pmol of EcoRII sites were incubated with 0.05 pmol of dimeric EcoRII protein in 1× universal reaction buffer (Stratagene) in 40-μl samples at 37 °C for 30 min, and DNA fragments were separated on 5% and 12% PAGE, respectively, depending on the length of the substrate. After exposure of the dried gel, cleavage was quantified and analyzed with a PhosphorImager and ImageQuant software (Molecular Dynamics). Cleavage rates were calculated as ratios of PhosphorImager units in the complete and/or partial cleavage products and the total radioactivity in all bands of the lane (initial substrate concentration). The time dependence of oligonucleotide duplex cleavage was studied on 0.9 pmol of EcoRII sites incubated with 0.4 pmol of dimeric EcoRII in a 360-μl volume of 1× universal buffer. At 0, 1, 2, 4, 6, 8, 10, 20, and 30 min, samples of 40 μl were withdrawn, and the reaction was stopped with 1 mM EDTA. The 0-min sample was removed before adding the enzyme.

**RESULTS AND DISCUSSION**

One-step Purification of EcoRII by Nickel-NTA Affinity Chromatography—From a 250-ml culture grown for 5 h after IPTG induction, the cell lysate was prepared and loaded on a 2-ml nickel-NTA column. Using the purification scheme specified under “Experimental Procedures,” 7.5 mg of nearly homogeneous protein, representing 600,000 units at a concentration of about 150 units/μl could be purified in just one step, taking about 7 h. The crude lysate as well as the main elution fraction of EcoRII ENase are shown in a 12% polyacrylamide gel in Fig. 1. The enzyme runs at a molecular mass of about 47 kDa as expected for the His-tagged monomer (calculated molecular mass, 46.6 kDa).

**Characterization of the Native DNA-EcoRII Complex by Gel Filtration**—Using the plot of log Mv, log Me (Fig. 2), the molecular mass of the native His-tagged EcoRII ENase was determined to be 85 kDa, which is approximately twice that of the protein monomer (26). This would confirm that the His6-tagged enzyme is a dimer in solution as was previously shown by gel filtration through Superose 12 and 1000 for the native ENase (26).

Only one species of DNA-EcoRII complex with an apparent molecular mass of 197 kDa at different enzyme/substrate ratios and oligonucleotide duplex concentrations of about 10 μM (ratios 1:4 and 1:1) as well as 2.5 μM (ratio 4:1) was detected. This would correspond to a complex of the dimeric enzyme molecule (85 kDa) bound to the recognition sites of two oligonucleotide
duplexes (2 × 45 kDa). The presence of Mg$^{2+}$ in the assay buffer did not affect the size of the complex. The only other composition agreeing with the size of about 197 kDa would be an enzyme tetramer (2 × 85 kDa) bound to just one oligonucleotide duplex. In this case, one would expect that with an increase of available substrate sites, the second DNA-binding site of the tetramer should be occupied. Since we never detected any larger complex even at a 4-fold substrate excess, it seems unlikely that the dimeric enzyme molecule should form a tetramer to bind a single oligonucleotide duplex.

**Dependence of EcoRII Cleavage on the Ratio of Enzyme Molecules to DNA Recognition Sites**—The stoichiometry of an active EcoRII-DNA complex was determined by titrating the enzyme dimers against a constant substrate site number. pBR322 containing one 14-bp oligonucleotide duplex. In this case, one would expect that with an increase of available substrate sites, the second DNA-binding site of the tetramer should be occupied. Since we never detected any larger complex even at a 4-fold substrate excess, it seems unlikely that the dimeric enzyme molecule should form a tetramer to bind a single oligonucleotide duplex.

**Fig. 2.** Gel filtration of substrate-bound and free ENase EcoRII on Superdex 200. The standard curve of $v_0/v_0$ versus log $M_r$ was derived from the elution profiles of the following protein standards, with $v_0$ representing the peak elution volume of the protein and $v$ the void volume of the column determined with blue dextran 2000, ferritin (440.0 kDa), catalase (232.0 kDa), aldolase (158.0 kDa), transferrin (81.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), ribonuclease A (13.7 kDa). The apparent molecular masses of the 30-bp oligonucleotide duplex, the purified EcoRII ENase and the complex formed by them were calculated from the standard curve. The apparent molecular mass of the 30-bp oligonucleotide duplex substantially exceeds its actual molecular mass of 19.5 kDa due to its inflexible rod structure, in comparison with the globular shape of the standard proteins.

**Fig. 3.** Titrating EcoRII dimers against a constant concentration of EcoRII recognition sites. Varying amounts of EcoRII endonuclease and 0.035 pmol of pBR322 (Dcm-I) were incubated for 30 min in a 20-μl reaction volume containing 1 × universal buffer (100 mM KOAc, 25 mM Tris-OAc, pH 7.6, 10 mM Mg(OAc)$_2$, 0.5 mM β-mercaptoethanol, 10 μg/ml BSA, Stratagene) at 37 °C. Cleavage patterns were monitored by electrophoresis on 0.5% agarose gels stained with ethidium bromide. Lanes 1 and 14, 1-kilobase pair ladder (Life Technologies, Inc.); lane 2, pBR322 plasmid DNA without enzyme; lanes 3–12, 0.035 pmol of pBR322 and varying concentrations of EcoRII resulting in the indicated enzyme/site ratios; lane 13, enzyme concentration as in lane 12, enzyme/site ratio as in lane 8. The sizes of the three largest complete pBR322/EcoRII digestion products in bp are indicated on the right.

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We therefore conclude that 0.25–0.5 EcoRII dimers/recognition site are necessary for the formation of an active DNA-EcoRII complex. Higher ratios of enzyme dimers to recognition sites result in the formation of inactive DNA-enzyme complexes, since the enzyme dimers cannot bind two target sites simultaneously. The cooperative interaction of the ENase EcoRII with two recognition sites can thus be realized by one EcoRII dimer. In demonstrating reversible inhibition by high EcoRII concentrations, these results extend those of Petrauskene et al. (27), who investigated the cleavage of a 14-bp oligonucleotide duplex containing one EcoRII site as well as of pBR322 DNA in the narrower range of dimer/site ratios between 0.02 and 2.7.

Szczelkun and Halford (11) obtained comparable results for SfiI by titrating circular plasmid DNA carrying two SfiI sites with increasing concentrations of tetrameric SfiI ENase. In reactions with enzyme in excess of DNA sites, each site becomes loaded with one SfiI tetramer, thus preventing the protein from simultaneously binding the two sites essential for activity. Since the optimum of SfiI activity was observed as its concentration was raised from 1 to 2 mol/mol of DNA a single tetramer of SfiI is sufficient to form the active cleavage complex with two SfiI recognition sites.

**Influence of Bound Lac Repressor on Cleavage of Two Distinct Recognition Sites on the Same DNA Molecule by EcoRII**—There are several possible mechanisms to explain how restriction enzyme molecules cover the distance between neighboring sites: ATP-dependent DNA translocation/tracking or ATP-independent sliding or looping (3, 28). Since type II ENases are not ATP-dependent in their enzymatic activity, a sliding or
looping process is much more likely for the interaction of EcoRII with two distant sites. A key experiment to discriminate between the looping and the sliding model is to place a DNA-binding protein between two cooperating sites. The linear 445-bp pUC18/HaeIII fragment containing two EcoRII sites at a distance of 191 bp and a lac operator located in between (Fig. 4A) was chosen as substrate. The Lac repressor masks about 24 nucleotides on the DNA (29) and is released by the inducer IPTG.

Having demonstrated in earlier experiments that DNA fragments containing two EcoRII sites can be cleaved by intramolecular ("cis") as well as intermolecular ("trans") interactions in a substrate concentration-dependent manner (22), it was necessary to ensure an exclusively intramolecular EcoRII interaction with the two DNA sites so that the bound Lac repressor could not be circumvented by EcoRII via intermolecular substrate site cooperation. Therefore, we investigated EcoRII cleavage at very low DNA site concentrations of 2.5–5 nM, largely excluding the trans interaction, as shown earlier using a 111-bp oligonucleotide duplex possessing one recognition site (22).

We determined buffer conditions that supported both Lac repressor binding to the lac operator and the EcoRII cleavage reaction. Fig. 4B shows that a 50-fold molar excess of Lac repressor molecules over substrate molecules was able to shift the DNA fragment in a complex (lane 3). The Lac repressor was removed from the operator sequence in the presence of IPTG (lane 4). At the defined substrate site concentration for the reaction in cis, densitometry of the bands in Fig. 4C revealed no significant difference in EcoRII cleavage, regardless of whether the Lac repressor was bound (data not shown).

The Lac repressor placed between the two EcoRII sites does not present a barrier for the ENase. This is in contrast to findings for the type III ENase EcoP15, where the essential cooperativity of two inversely oriented recognition sites is completely abolished by an intervening bound Lac repressor protein. The mechanism by which the EcoP15 enzyme-substrate complexes communicate is an ATP-dependent translocation process (30). A sliding mechanism is equally unlikely for EcoRII, since Jeltsch et al. (15) showed that ENases that move by linear diffusion along the DNA (like EcoRII) are stopped by bound proteins as well as by irregular DNA structures. Our results suggest that the essential cooperativity between EcoRII sites at the investigated distance of approximately 190 bp is achieved by a process involving DNA looping rather than “tracking” of the intervening DNA stretch.

Cleavage of Linear DNA Substrates with Differently Spaced EcoRII Recognition Sites: Cooperativity and Distance—As in the Lac repressor experiment, the exclusion of the trans reaction is a prerequisite for investigating the influence of site distance on EcoRII cleavage. To find appropriate conditions, we Cleaved a 71-mer containing two EcoRII recognition sites at a distance of n = 10 bp, where n is the number of base pairs between the 5'-cytosines of the two recognition sites, in parallel with a single-site substrate derived from the former by elimination of the second site by a 1-bp substitution. Fig. 5 presents evidence that within the site concentration range of 2.5 nM used throughout these experiments, the single-site 71-mer is not hydrolyzed at all by EcoRII (lane 2). At the same concentration, the two-site oligonucleotide duplex is efficiently cleaved (lane 5), which can only be due to the presence of the second site in cis. The correct location of both substrate sites on the oligonucleotide duplexes was proven by control cleavage with the iso-
The synthetic oligonucleotide duplexes used for comparative digestion studies were designed by inserting different numbers of nucleotides (x) between two inversely oriented EcoRII sites that are arranged as a direct inverted repeat in the smallest substrate (66-mer; x = 0) and are separated by 68 bp in the largest one (134-mer; x = 68). The functionally relevant site distance (n) is between the 5'-cytosines in the two EcoRII recognition sequences. All oligonucleotide duplexes have the same flanking sequences and differ only in the DNA stretch between the two EcoRII sites. The common construction principle, the distances between EcoRII sites, and the total lengths of the synthetic substrates are shown in Fig. 6A. In addition to this set of synthetic substrates, we amplified a 445-bp fragment from pUC18 as well as a 1160-bp M13 fragment by polymerase chain reaction; they provide EcoRII site distances of n = 191 and 952 bp, respectively, which could not be created using oligonucleotide duplexes. It was important to include these longer distances in the study, since our earlier observations suggested that 952 bp between the two EcoRII sites in M13 phage DNA might approximate the distance limit for two sites in cis to be bridged by EcoRII (31).

Comparative digestions of substrates with two differently spaced EcoRII sites were performed, and the efficiency of partial and complete cleavage was quantified (Fig. 6B). The differently shaded horizontal bars above the diagram indicate the probability of a suggested EcoRII-DNA interaction at the respective site distance. The smallest substrate (66-mer) with two EcoRII sites directly in head-to-head configuration (x = 0) was cleaved to approximately 26%, indicating that the enzyme still recognized them as distinct sites. By introduction of 5 bp, the 71-mer was designed; cleavage rose to nearly 84%, representing the most efficient intramolecular hydrolysis we observed between two EcoRII recognition sequences. In this substrate, the site distance n represents approximately one helical turn, allowing the two EcoRII enzyme subunits to approach the two DNA sites from the same face of the helix, which apparently constitutes the best-fit position. In contrast, in the 66-mer, where the two sites have a distance (n) of only one-half turn, EcoRII has to contact them on opposite sides of the DNA helix, which probably yields a complex under considerable torsional stress (compare Fig. 8, top diagram). A doubling or tripling of the optimal site distance (n) of 10 bp is inversely correlated to cleavage efficiency. EcoRII ENase can only bridge the increased distance between the two cooperating substrate sites by bending the intervening DNA, which might also involve distortion of the enzyme dimer, leading to a significant decrease of substrate cleavage to 30–40%. Above a site distance (n) of 31 bp, up to at least 191 bp, cleavage efficiency exhibits a plateau at about 20–25%. These distances can now be overcome by the formation of DNA loops. The smallest loops were observed at site distances of six helical turns (4). Our earlier results with circular and prelinearized M13 DNA have already shown that the two EcoRII sites (distance, n = 952 bp) in this DNA molecule are nearly resistant to cleavage and are completely hydrolyzed only by the addition of susceptible sites in trans (31). Therefore, we assumed a distance of about 1000 bp to be critical for loop formation by EcoRII. As expected, EcoRII cleavage decreased to about 6% at a spacing of 952 bp, demonstrating that the probability of one site occupied by an EcoRII dimer finding the second DNA site decreases with in-

**Fig. 5.** Cleavage is exclusively in cis at high substrate dilution. 71-mers (32P-labeled in one strand) with one (71/1) or two (71/2) EcoRII sites were digested with 1.25 EcoRII dimer at 2.5 μm DNA site concentration for 30 min at 37 °C, and samples were separated by 12% PAGE with 0.1% SDS. Substrate digested with the isoschizomer BstNI served as a positive control. The sizes of digestion products are indicated on the right in bp.

![EcoRII cleavage diagram](image)

**Fig. 6.** EcoRII cleavage of linear substrates with two differently spaced recognition sites. A, six synthetic oligonucleotide duplexes with two EcoRII sites separated by a spacer of increasing length were synthesized. The general substrate structure is shown in the diagram, where x is the number of bp inserted between the two inverted EcoRII recognition sequences and n is the site distance between the 5'-cytosines in the two recognition sequences. The table in panel A lists the parameters of all investigated substrate oligonucleotide duplexes. Spacings of 191 and 952 bp were achieved by use of the 445-bp pUC18 and the 1160-bp M13 fragments. B, cleavage with EcoRII was performed as described in the legend to Fig. 5. Partial (circles) and total (squares) cleavage percentages were calculated after quantification of the respective DNA bands on a PhosphorImager and are mean values of three independent experiments.
compared. 2.5 nM EcoRII sites were incubated with 1.25 nM EcoRII dimers in a volume of 360 μl, and 40-μl aliquots were taken at the indicated time intervals. Following PAGE, digestion was quantified by PhosphorImager analysis. Two independent experiments were performed with each substrate; calculated mean values for 71/10i, 71/10u, and 134 are indicated by triangles, circles, and squares, respectively.

The cleavage of two 71-mers with a distance of 10 bp, as well as the 134-mer (site distance, 73 bp) were compared. The initial rate of cleavage at a site distance of 10 bp is approximately 10 times faster than that with n = 73 bp. These different cleavage rates were reproducible after cloning both oligonucleotide duplexes in pUC19 plasmid DNA and amplifying the appropriate sequences in polymerase chain reaction products of about 260 bp, which have a greater resemblance to native DNA molecules (data not shown). This increase in reaction rate under best-fit conditions results from simultaneous cleavage (Fig. 6B).

A comparison of the time dependence of the cleavage of synthetic oligonucleotide duplexes with n = 10 bp (71-mer) and 73 bp (134-mer) in Fig. 7 showed that EcoRII cleavage of the substrate containing the 10 bp-spaced EcoRII sites had an initial rate approximately 10 times faster than that with n = 73 bp. These different cleavage rates were reproduced after cloning both oligonucleotide duplexes in pUC19 plasmid DNA and amplifying the appropriate sequences in polymerase chain reaction products of about 260 bp, which have a greater resemblance to native DNA molecules (data not shown). This increase in reaction rate under best-fit conditions results from simultaneous cleavage (Fig. 6B).

**Influence of the Orientation of Cooperating EcoRII Sites within the DNA Double Strand**—We have previously shown that EcoRII site orientation had no significant effect on EcoRII activity either in complex circular DNA molecules like M13mp18 with seven EcoRII recognition sites or in defined short oligonucleotide duplexes with two sites. This may be explained by the flexibility of DNA loops, which increases with intersite distance (4). It was of interest to reinvestigate the issue of site orientation in the context of the optimal configuration of the enzyme-site complex at a distance of n = 10 bp. The cleavage of two 71-mers with a distance of n = 10 bp, differing only by the orientation of the central A/T pair in the second recognition site, was compared. The initial rate of cleavage of the two substrates and the progression of the reaction were identical, supporting an identical reaction mechanism for both orientations (Fig. 7).

The EcoRII dimer, therefore, does not require a specific disposition of the central A/T pairs of the two communicating recognition sites, although it does discriminate efficiently against a central C/G pair (33). There are two possible explanations for this behavior. Either each enzyme monomer within the dimer accepts A and T as synonymous and assumes an arbitrary orientation relative to its recognition site, or each monomer establishes nonsymmetrical specific contacts to the central A/T pair, but the interaction between the two monomers is not affected. The former explanation is favored by the observation that EcoRII recognition sites with a central T/T or A/A mismatch still present substrates for the EcoRII ENase (34).

Fig. 8 depicts a model of EcoRII-DNA-interaction that interprets the results of our binding and cleavage studies. Independent experimental data indicate that the EcoRII monomer possesses two DNA-binding regions (shown in light and dark gray). The size of the complete DNA binding site of EcoRII was estimated by Vinogradova et al. (32) to be 21 ± 1 bp, on the basis of dissociation kinetics of the enzyme bound to synthetic DNA concatamers containing different numbers of repetitive EcoRII recognition sites. The highly efficient cleavage at a site distance of 10 bp may therefore indicate that the EcoRII homodimer occupies the available space between the substrate sites and an additional 3 bp on each side of the recognition sequences without the need of forcing sites together by bending the DNA and without conformational strain on the protein (Fig. 8, second diagram). Increasing the distance between the sites causes the enzyme to bend/distort (third diagram) or loop the DNA (fourth diagram), which becomes less probable with increasing space, leading to site resistance when the distance reaches about 1000 bp.

**Comparison of EcoRII with Other Type IIe Enzymes**—For NaeI and SfiI, comparable quantitative data on the correlation between site distance and cleavage efficiency are not available, and, in particular, the optimal and the limiting site distances of the intramolecular interaction have not been established. It was demonstrated that the dimeric NaeI enzyme induces loop

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2 M. Reuter, unpublished data.

3 M. Reuter, manuscript in preparation.
formation between site distances of approximately 160 and 370 bp (9), and the tetrameric ENase SfiI can productively span a distance as great as 6000 bp, albeit with the characteristics of an intermolecular interaction (35). We have previously shown that EcoRII is strictly unable to cleave comparably distant recognition sites either intra- or intermolecularly (for a review, see Ref. 8).

It is probable that in all three cases (EcoRII, NaeI, and SfiI), DNA loops are formed by the interaction of a preformed enzyme oligomer with recognition sites. As reviewed by Hochschild (4), the efficiency of this type of interaction declines rapidly above a site distance of 700 bp, in good agreement with the quantitative data for EcoRII (Fig. 6). It depends crucially on the stability of the bridging protein oligomer species. The only stable EcoRII oligomer observed by us, also in the presence of DNA, is the dimer. A putative tetramer, to be formed by a weak dimer-dimer interaction would not support loop formation above 100 bp (4).

The ability of the SfiI tetramer to interact with sites at unlimited distance, possibly aided by superhelicity, as well as to catalyze an intermolecular interaction between individual sites on large DNA molecules requires additional mechanisms (10, 11) and is adapted to the relative paucity of the SfiI recognition sequence 5′-GGCCN5GGCC, which should occur at an average frequency of once in 6561 bp (4^5 × 0.75^5) in a genome of about 75% G plus C content (36). This contrasts with the more limited catalytic propensity of EcoRII, which is, however, sufficient to cleave at its pentanucleotide recognition sites, expected to occur once in 512 bp (2 × 4^5) in the E. coli genome with 50% G plus C. One potential advantage of this reaction mechanism is the ability to spare the occasional unmodified recognition site in the host genome; a requirement for two unmodified sites for cleavage makes it less likely that the enzyme will suicidally attack the rare unmodified sites in cellular DNA that may arise by DNA repair or incomplete methylation (23).

Our results support the idea that the complex manner by which EcoRII and other type II ENases simultaneously interact with sites at a distance reflects some evolutionary link to other prokaryotic and eukaryotic families of cooperatively DNA-binding proteins like repressors, recombination enzymes, or transcription factors. While the cleavage efficiency of these ENases appears to be moderate due to the limitation on substrates fulfilling the requirement of site cooperativity, the high sensitivity to changes in recognition site concentration could present an advantage. Positive cooperativity is a device to sharpen the response of a system to the changes in a stimulus, e.g. a small alteration in a ligand concentration of an enzyme (approximately 4-fold) may increase its activity from 10 to 90% (37). In the case of the EcoRII ENase, the increase in recognition site concentration by invading foreign DNA may be the stimulus for the enzyme to intensify its activity, taking advantage of its additional intermolecular reaction mechanism.

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