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Resolution of the EcoRII restriction endonuclease-DNA complex structure in solution using fluorescence spectroscopy

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ABSTRACT

The X-ray structure for the type IIE EcoRII restriction endonuclease has been resolved [X.E. Zhou, Y. Wang, M. Reuter, M. Mucke, D.H. Kruger, E.J. Meehan and L. Chen. Crystal structure of type IIE restriction endonuclease EcoRII reveals an autoinhibition mechanism by a novel effector-binding fold. J. Mol. Biol. 335 (2004) 307–319.], but the structure of the R.EcoRII–DNA complex is still unknown. The aim of this article was to examine the structure of the pre-reactive R.EcoRII–DNA complex in solution by fluorescence spectroscopy. The structure for the R.EcoRII–DNA complex was resolved by determining the fluorescence resonance energy transfer (FRET) between two fluorescent dyes, covalently attached near the EcoRII recognition sites, that were located at opposite ends of a lengthy two-site DNA molecule. Analysis of the FRET data from the two-site DNA revealed a likely model for the arrangement of the two EcoRII recognition sites relative to each other in the R.EcoRII–DNA complex in the presence of Ca^{2+} ions. According to this model, the R.EcoRII binds the two-site DNA and forms a DNA loop in which the EcoRII recognition sites are 20±10 Å distant to each other and situated at an angle of 70±10°.

1. Introduction

Type IIE restriction endonucleases (REases) are attractive models for studying the specific protein–DNA interactions accompanying complex genetic processes in which enzymes simultaneously interact with DNA at several sites. Type IIE REases exhibit the particular property that they have to simultaneously bind two copies of their palindromic DNA recognition sequence for efficient DNA cleavage, where one copy is the target for cleavage and the other serves as an allosteric effector [1].

The EcoRII restriction endonuclease (R.EcoRII) was the first documented example of a Type IIE enzyme [2]. It is a homodimeric protein. R.EcoRII cleaves the ↓CCA/TGG sequence in DNA at both strands at the positions defined by the arrow. The low cleavage efficiency of R.EcoRII on DNA substrates with only a single or a few DNA recognition sites separated by >1000 bp was overcome by adding short duplexes with a single EcoRII recognition site (due to *trans*-interactions) [3]. A study of the cleavage by R.EcoRII of two distinct recognition sites on the same DNA molecule shows that cooperativeness between the EcoRII sites is achieved by bending or looping of the intervening DNA stretch (*cis*-interactions) [4]. Transmission electron microscopy provided direct evidence that R.EcoRII mediates a loop formation [5]. Further studies revealed that the enzyme-substrate active complex includes two subunits of R.EcoRII interacting with two DNA recognition sequences [4,6]. Recently, according to a kinetic study of the R.EcoRII cleavage of plasmids containing a single, two or three recognition sites, it was suggested that R.EcoRII requires simultaneous binding of three rather than two recognition sites *in cis* to achieve concerted DNA cleavage at a single-site [7]. Atomic force microscopy provided direct visualization and characterization of the synaptic protein–DNA complexes involving a two-loop structure with three DNA binding sites [8]. The X-ray structure of R.EcoRII was determined [9]. REase EcoRII contains an N-terminal effector-binding domain and a C-terminal endonuclease-like domain [9,10]. The putative catalytic site of R.EcoRII is located in the C-terminal domain and is spatially blocked by the N-terminal domain. The removal of the N-terminal effector-binding domain of R.EcoRII converts this type IIE enzyme into a very active Type IIP enzyme [10]. DNA binding studies indicate that the isolated C-terminal domain exists as a dimer that binds a single cognate DNA molecule in the presence of the analog cofactor, Ca^{2+} ions, whereas the N-terminal domain exists as a monomer that also binds a single copy of cognate DNA, but in a Ca^{2+}-independent manner [11]. It has been suggested that the full-length R.EcoRII contains three putative DNA binding interfaces: one at the C-terminal domain dimer and two at each of the N-terminal domains [11,12]. The distance between the EcoRII recognition sites and their arrangement relative to

Abbreviations: REase, restriction endonuclease; EMSA, electrophoretic mobility shift assay; ON, oligodeoxynucleotide; **FAM**, 5(6)-carboxyfluorescein; **TAMRA**, 5(6)-carboxytetramethylrhodamine; FRET, fluorescence resonance energy transfer; C_{DNA}, DNA concentration.

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each other in this complex are not known. The structural organization of the pre-reactive R.EcoRII–DNA complex, which is formed in the presence of a cofactor, Mg^{2+} ions, is of a special interest. It was shown that substitution of Ca^{2+} ions for Mg^{2+} ions results in inhibition of DNA cleavage by R.EcoRII, but preserves the ability of R.EcoRII to form the pre-reactive R.EcoRII–DNA complex [13].

The use of fluorescence resonance energy transfer (FRET) can provide information on the distance between a donor and an acceptor dye in the range of 10 to 100 Å. To study the structure of the DNA-protein complexes, 5(6)-carboxyfluorescein, **FAM**, (as the donor) and 5 (6)-carboxytetramethylrhodamine, **TAMRA**, (as the acceptor) were used [14]. Using FRET allowed us to determine the distance between the DNA ends and, as a result, to distinguish the two U-shaped DNA global structures induced by the integration of the host factor upon binding with intact and nicked DNA [15]. The study of energy transfer between **FAM** and **TAMRA**, which were attached near the two operator DNA sites, about 130 bp apart, confirmed and extended the data about the geometry of the loop formed by the binding of the two operators by the LacI repressor [16]. FRET experiments allowed for the investigation of the DNA looping by the NgoMIV restriction endonuclease and the determination of the distance between the ends of the DNA molecule when bound to NgoMIV [17].

To determine the arrangement of the EcoRII recognition sites relative to each other within the R.EcoRII–DNA complex, the data from a FRET analysis of the R.EcoRII–DNA complex were studied using two fluorescent dyes that were covalently attached near the EcoRII recognition sites, which were located at opposite ends of the lengthy two-site DNA molecule. The R.EcoRII–DNA complex was formed in the presence of the Ca^{2+} ions to promote the formation of a pre-reactive R.EcoRII–DNA complex, which is hypothesized to resemble the same REase-DNA complex in the presence of cofactor Mg^{2+} ions prior to a reaction. A suggested model describing the arrangement of the two EcoRII recognition sites relative to each other in the complex of R.EcoRII with a two-site DNA is shown.

2. Experimental

2.1. Chemicals and enzymes

(γ-^{32}P)-ATP (1000 Ci/mmol) was from Izotop (Russia). R.EcoRII was overexpressed as an N-terminally His_6-tagged protein and purified by chromatography on a nickel chelate column as previously described [18]. The R.EcoRII dimer concentration was determined by Bradford assay (2.3 μM). T4 polynucleotide kinase was from MBI Fermentas (Lithuania). The buffers A–D, prepared with Milli-Q water, were compositions of the following solutions: A — 40 mM Tris–HCl at pH 7.6 and 50 mM NaCl; B — buffer A containing 5 mM $MgCl_2$ and 7 mM DTT; C — buffer A containing 5 mM $CaCl_2$ and 7 mM DTT; D — buffer A containing 7 mM DTT and 0.2 mM EDTA and E — 60 mM Tris–HCl at pH 8.5, 25 mM KCl, 10 mM 2-mercaptoethanol and 0.1% Triton X-100.

Oligonucleotides (ONs) were from Syntol (Russia) (Table 1). The fluorescent labels **FAM** and **TAMRA** were introduced to the 5′-end or 3′-end of the ONs via an aminoalkyl linker containing six methylene groups. ^{32}P-5′-phosphorylation of the ONs was carried out using T4-polynucleotide kinase and [γ-^{32}P]-ATP. ON concentrations were determined spectrophotometrically. Extinction coefficients (ε_{260}) of unmodified ONs at 260 nm were calculated according to a published protocol [19]. For the modified ONs, ε_{260} were calculated as the sum of ε_{260} of unmodified ON and ε_{260} for 5′-FAM (20,960 M^{-1} cm^{-1}), 3′-FAM (21,000 M^{-1} cm^{-1}) or 5′-TAMRA (29,100 M^{-1} cm^{-1}) (www.idtdna.com).

2.2. Preparation of fluorescence-labeled DNA segments

The 327–339 bp linear DNAs (Table 2) were obtained by PCR amplification. The plasmid pCAL7/nH was used as a DNA template. The 5′-**TAMRA**-$(CH_2)_6$-CAT CTA CCT G**CC TGG** ACA G, 5′-**TAMRA**-$(CH_2)_6$-ACC TG**C CTG G**AC AGC ATG G, 5′-ACC TG**C CTG G**AC AGC ATG G and 5′-**TAMRA**-$(CH_2)_6$-CTG **CCT GG**A CAG CAT GGC were used as forward primers. The 5′-**FAM**-$(CH_2)_6$-ACG TGG CTG G**CC TGG** TTC, 5′-**FAM**-$(CH_2)_6$-GCT GG**C CTG G**TT CAC CAC and 5′-GCT GG**C CTG G**TT CAC CAC were used as reverse primers. PCR amplification was carried out in 80 μl of buffer E containing 10 U of DNA-polymerase Taq, 1.4 μg of DNA template, 150 pmol of primers and a 2 mM mixture of the four dNTPs (0.5 mM of each). (α-^{32}P)-ATP was added to introduce a ^{32}P-label onto the DNAs. All PCR products migrated as individual bands in 0.5% agarose gels. PCR products were purified using a "Promega" kit (#A7170, Wizard PCR Preps DNA purification system).

2.3. DNA cleavage by R.EcoRII

Cleavage of **14^A/14^T** and **14^A/5′-FAM-14^T** duplexes (Table 1) was determined spectrophotometrically [20]. The experiment was repeated three times.

2.4. FRET measurements

Fluorescence measurements were performed with a "PTI technology" spectrofluorometer or with a spectrofluorometer "FluoroMax™" (SPEX^R) equipped with a thermostated cell holder in 150–3000 μl of buffer C. Aliquots of R.EcoRII ($C_{R.EcoRII}$ 0–500 nM) were added to DNA (C_{DNA} is

Table 1
Oligodeoxynucleotide sequences

14^A	5′-AGAG**CCAGG**TTGGC
14^T	5′-GCCAA**CCTGG**CTCT
5′-FAM-14^T	5′-**FAM**-GCCAA**CCTGG**CTCT
18^A	5′-TCAGAG**CCAGG**TTGGCTC
18^T	5′-AGTCTC**GGTCC**AACCGAG
5′-FAM-18^A	5′-**FAM**-TCAGAG**CCAGG**TTGGCTC
5′-TAMRA-18^A	5′-**TAMRA**-TCAGAG**CCAGG**TTGGCTC
5′-TAMRA-18^T	5′-**TAMRA**-GAGCCAA**CCTGG**CTCTGA

FAM – donor – 5(6)-carboxyfluorescein; **TAMRA** – acceptor – 5(6)-carboxytetramethylrhodamine.

Table 2
Linear DNAs containing two EcoRII recognition sites and fluorescent dyes

$DNA^{FAM10}_{TAMRA10}$*	5′-FAM-ACGTGGCTGGCCTGG$(N)_{309}$CCAGGAGGTAGATGA ATGCACCGACC**GGACC**$(N)_{309}$**GGTCC**GTCCATCTAC-**TAMRA**-5′
DNA^{FAM10}_{TAMRA5}	5′-**FAM**-ACGTGGCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTA ATGCACCGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA-**TAMRA**-5′
DNA^{FAM10}_{TAMRA3}	5′-**FAM**-ACGTGGCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGA ATGCACCGACC**GGACC**$(N)_{309}$**GGTCC**GTC-**TAMRA**-5′
$DNA^{FAM5}_{TAMRA10}$	5′-**FAM**-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTAGATGA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTCCAT CTAC-**TAMRA**-5′
DNA^{FAM5}_{TAMRA5}	5′-**FAM**-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA-**TAMRA**-5′
DNA^{FAM5}_{TAMRA3}	5′-**FAM**-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTC-**TAMRA**-5′
DNA^{FAM5}_{5}	5′-**FAM**-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA-5′
DNA^{FAM10}_{5}	5′-**FAM**-ACGTGGCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTA ATGCACCGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA-5′
$DNA^{5}_{TAMRA10}$	5′-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTAGATGA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA TCTAC-**TAMRA**-5′
DNA^{FAM5}_{5}	5′-**FAM**-GCTGG**CCTGG**$(N)_{309}$**CCAGG**CAGGTA ACGACC**GGACC**$(N)_{309}$**GGTCC**GTCCA-5′

The numbers of nucleotide residues between a dye and the nearest EcoRII recognition site is indicated.

denoted in the figure legends and table notes) and allowed to equilibrate for 1.5 or 5 min at room temperature. The emission spectra were recorded from 500 to 650 nm. The excitation wavelengths were 495 nm for the **FAM** or 560 nm for the **TAMRA** fluorescence. Slits were 1.0 and 1.0 nm. The titrations were repeated three to four times with independent samples. In all the sets of experiments, titration of the DNA containing only **FAM** or **TAMRA** with R.EcoRII were performed to take into account the influence of the R.EcoRII–DNA binding on **FAM** or **TAMRA** fluorescence.

The FRET efficiency (E^{FRET}_{FAM}) was determined from quenching of the donor emission intensity in the presence of an acceptor according to the following previously published equation [15]:

$$E^{FRET}_{FAM} = \frac{\left(I^{495}_{FAM/R.EcoRI} - I^{495}_{FAM/TAMRA/R.EcoRII}\right)}{I^{495}_{FAM/R.EcoRII}} \cdot 100\%, \quad (1)$$

where $I^{495}_{FAM/R.EcoRI}$ and $I^{495}_{FAM/TAMRA/R.EcoRII}$ are emission intensities of the **FAM** at 518 nm (λ_{ex} 495 nm) in the complex with R.EcoRII in the absence and in the presence of **TAMRA**, respectively.

Also, the FRET efficiency E^{FRET}_{FAM} was determined from the sensitized emission of the acceptor normalized to the fluorescence of the acceptor using the following previously published equation [21,22]:

$$E^{FRET}_{TAMRA} = \frac{\varepsilon_{TAMRA}(560)}{f_{FAM} \cdot \varepsilon_{FAM}(495)} \cdot \left[\text{ratio}_A - \frac{\varepsilon_{TAMRA}(495)}{\varepsilon_{TAMRA}(560)}\right], \quad (2)$$

where $\varepsilon_{TAMRA}(\lambda)$ and $\varepsilon_{FAM}(\lambda)$ are extinction coefficients of the acceptor and donor, respectively, $(\text{ratio})_A = \frac{[F_{DA}(\lambda_{em},495) - a \cdot F_D(\lambda_{em},495)]}{F_{DA}(\lambda_{em},560)}$, where $F_{DA}(\lambda_{em}, 495)$, $F_D(\lambda_{em}, 495)$ and $F_{DA}(\lambda_{em}, 560)$ are fluorescence spectra of the donor in the presence of an acceptor (λ_{ex} 495 nm), donor (λ_{ex} 495 nm) and acceptor in the presence of a donor (λ_{ex} 560 nm) in the complex with R.EcoRII, respectively. f_{FAM} is the fraction of **FAM** labeled DNA molecules, calculated from the absorption spectrum of the FAM labeled ON. a is the fitted weighting factor of the $F_D(\lambda_{em}, 495)$ spectral component.

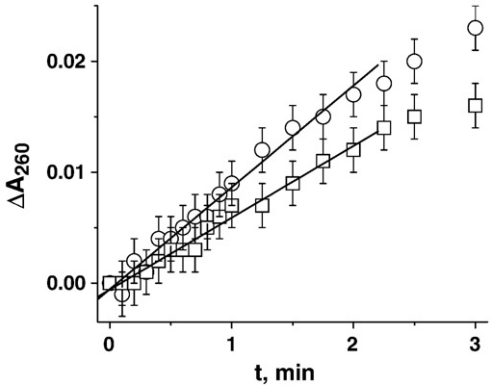

Fig. 1. Kinetic curves of the change in the UV-absorbance of $\mathbf{14^A/14^T}$ (○) and $\mathbf{14^A/5'\text{-}FAM\text{-}14^T}$ (□) duplexes at 260 nm (ΔA_{260}) as a result of their cleavage by R.EcoRII. C_{DNA} 5 μM, $C_{R.EcoRII}$ 20 nM, buffer B at 37 °C. Initial velocities of the DNA cleavage, V_0, were determined as a slope ratio of the linear part of the kinetic curve over a duration of 0–2 min.

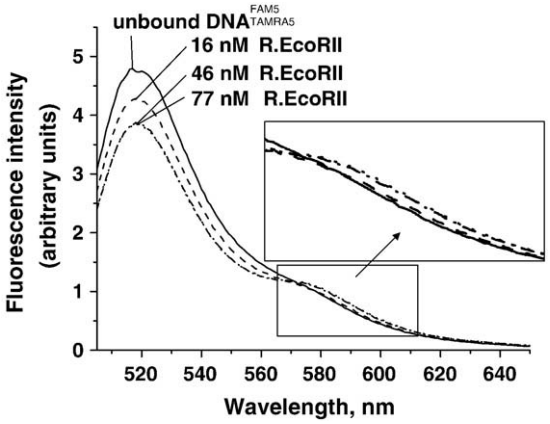

Fig. 2. Fluorescence spectra for unbound DNA^{FAM5}_{TAMRA5} and complexed with increasing concentrations of R.EcoRII. λ_{ex} 495 nm, C_{DNA} 50 nM, buffer C at 20 °C. Insert: increase of the acceptor fluorescence at 565–612 nm.

2.5. Calculation of geometry of DNA loop induced by R.EcoRII

The FRET efficiency ($E^{FRET}_{X,Y}$) between the **FAM** and **TAMRA** located at Y and X bp apart from the EcoRII site (Fig. 5A), respectively, can be converted to a donor–acceptor distance R_{XY} (in Angstrom) using the Förster equation

$$E^{FRET}_{X,Y} = \frac{R_0^6}{\left(R_0^6 + R_{X,Y}^6\right)}, \tag{3}$$

where R_0-Förster is the distance at which the energy transfer efficiency is 50%.

The ratio of FRET efficiencies is equal to

$$\frac{E^{FRET}_{X',Y'}}{E^{FRET}_{X'',Y''}} = \frac{R_0^6 + R_{X'',Y''}^6}{R_0^6 + R_{X',Y'}^6}, \tag{4}$$

where $E_{X',Y'}$ ($E_{X'',Y''}$) and $R_{X',Y'}$ ($R_{X'',Y''}$) are FRET efficiencies and distances (in Angstrom) for the first (and the second) DNA molecules; X' (X'') and Y' (Y'') are varied.

The calculated average position of the **FAM** attached with a six-carbon linker to the 5′-end of a DNA is 10 Å from the 5′-termini in a virtual elongation of the DNA backbone, whereas **TAMRA** stacks on top of the DNA helix end [14]. Accordingly, the distance between **FAM** and the center of EcoRII site is [(*Y*+2.5)*3.4+10] Å and the distance between **TAMRA** and the center of EcoRII site is [(*X*+2.5)*3.4] Å (Fig. 5C). The distance, $R_{X,Y}$ (in Angstrom), between **FAM** and **TAMRA** distant from the EcoRII site at *Y* and *X* bp, respectively, can be calculated by using the following geometric rules:

$$R^2_{X,Y} = [(2.5+Y)\cdot 3.4+10]^2 + [(2.5+X)\cdot 3.4]^2 - 2\cdot[(2.5+Y)\cdot 3.4+10]\cdot[(2.5+X)\cdot 3.4]\cdot\cos\alpha + h^2, \tag{5}$$

where h is the distance between the centers of the two EcoRII sites and α is the angle between the two EcoRII sites projected on the same plane.

The combination of Eqs. (4) and (5) gives the following expression:

$$\frac{E^{FRET}_{X',Y'}}{E^{FRET}_{X'',Y''}} = \frac{R_0^6 + \left([(2.5+Y'')\cdot 3.4+10]^2 + [(2.5+X'')\cdot 3.4]^2 - 2\cdot[(2.5+Y'')\cdot 3.4+10]\cdot[(2.5+X'')\cdot 3.4]\cdot\cos\alpha + h^2\right)^3}{R_0^6 + \left([(2.5+Y')\cdot 3.4+10]^2 + [(2.5+X')\cdot 3.4]^2 - 2\cdot[(2.5+Y')\cdot 3.4+10]\cdot[(2.5+X')\cdot 3.4]\cdot\cos\alpha + h^2\right)^3} \tag{6}$$

The R_0 for the **FAM**–**TAMRA** pair is 55 Å, calculated from $R_0 = 9790\cdot\left(J\cdot k^2\cdot\phi_D\cdot n^{-4}\right)^{1/6}$ Å, where J is the spectral overlap integral of the dyes, ϕ_D is the quantum yield of the donor in absence of the acceptor, n the refraction index of the medium and k^2 the orientation of the transition dipole

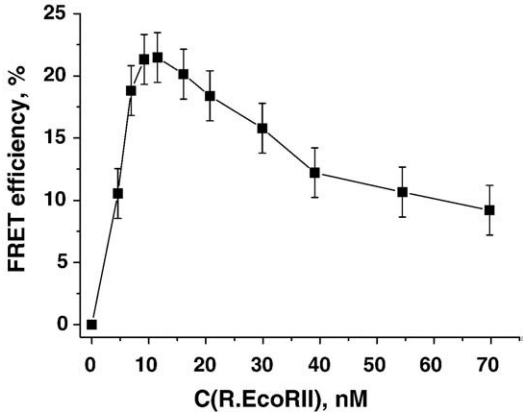

Fig. 3. Dependence of FRET efficiency (registered from an increasing of acceptor fluorescence) between **FAM** and **TAMRA** of the two-site DNA^{FAM5}_{TAMRA5} on the concentration of R.EcoRII. C_{DNA} 10 nM, buffer C at 20 °C.

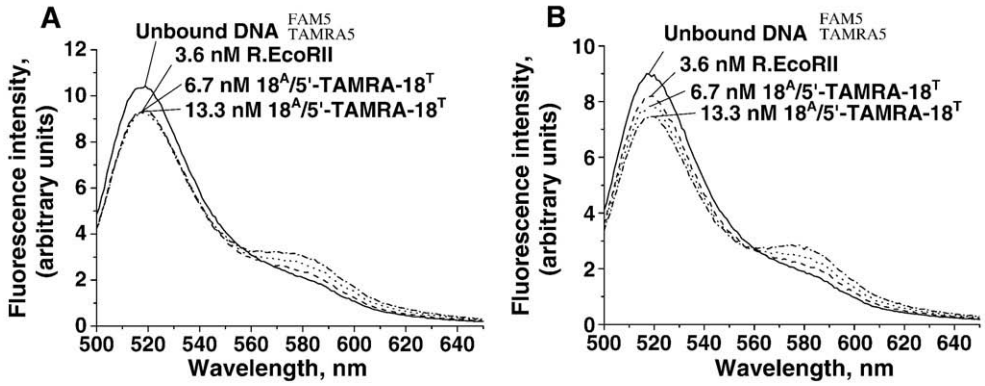

Fig. 4. Change of fluorescence spectra for DNA^{FAM5}_{TAMRA5} as a result of an R.EcoRII–DNA^{FAM5}_{TAMRA5} complex formation followed by an addition of varying concentrations of **18^{A}**/5′**-TAMRA-18^{T}** in the absence (A, buffer D) or in the presence (B, buffer C) of Ca^{2+} ions. λ_{ex} 495 nm. $C(DNA^{FAM5}_{TAMRA5})$ 2.5 nM.

moments [15,16]. The value, k^2, was equal to 2/3 in supposition of rapid randomization of the relative orientation between **FAM** and **TAMRA** [14,15]. The FRET efficiencies $E^{FRET}_{X,Y}$ were determined experimentally (Fig. 5B, ■ and ▲). The ratios of all possible combinations of FRET efficiencies $\left(\frac{E^{FRET}_{X,Y}}{E^{FRET}_{X^*Y^*}}\right)$ were calculated (Fig. 6, top insert). The distance, h, was varied in the range of 10–70 Å. For each defined distance h, the values of angle α were calculated according to Eq. (6). The values of angle α were plotted vs. h (Fig. 6). The normal deviations for the values of angle α were plotted vs. h (Fig. 6, bottom insert). The minimal deviation for the values of angle α was obtained and corresponded to h equal to 20 ± 10 Å and the value of the α angle was equal to 70 ± 10°.

3. Results

3.1. FRET studies

To determine the arrangement of the EcoRII recognition sites relative to each other in the R.EcoRII–DNA complex, the FRET between fluorescent labels located at the ends of the two-site DNA molecule (EcoRII sites *in cis*) (Table 2) was studied in the presence of the cofactor analog, Ca^{2+} ions.

FAM and **TAMRA** were covalently attached to the 5′-ends of DNA molecules containing two EcoRII sites (Table 2). The distances between the EcoRII sites were 314 bp in accordance with several observations. The stable loop formation by R.EcoRII on a linear DNA containing two EcoRII sites 21–500 bp apart was visualized by electron microscopy [5] or by measurement of the tension of single DNA molecule [23]. When two EcoRII sites were located on a plasmid at a distance of 362 bp, their effective cleavage by R.EcoRII was observed [7]. The distances between the dyes and EcoRII sites were varied (Table 2).

The ability of EcoRII endonuclease to bind to two-site DNAs was examined (Fig. 1S). At a 2.5 nM concentration of DNA^{FAM5}_{5}, several types of R.EcoRII–DNA complexes were observed when separated on a non-denaturing gel. The formation of a complex of R.EcoRII with the two-site DNA was evident by virtue of the observation of a decreasing of amount of unbound DNA at increasing enzyme concentrations. At a DNA:R.EcoRII molar ratio between 1:1 and 1:1.4, all the DNA was bound by R.EcoRII.

Additionally, the influence of introduction of dyes on interaction of R.EcoRII with substrate DNA was examined using short 14, and 18 bp DNA duplexes composed of oligonucleotides listed in Table 1. The **FAM** and **TAMRA** dyes were covalently attached to the 5′- or 3′-end of one

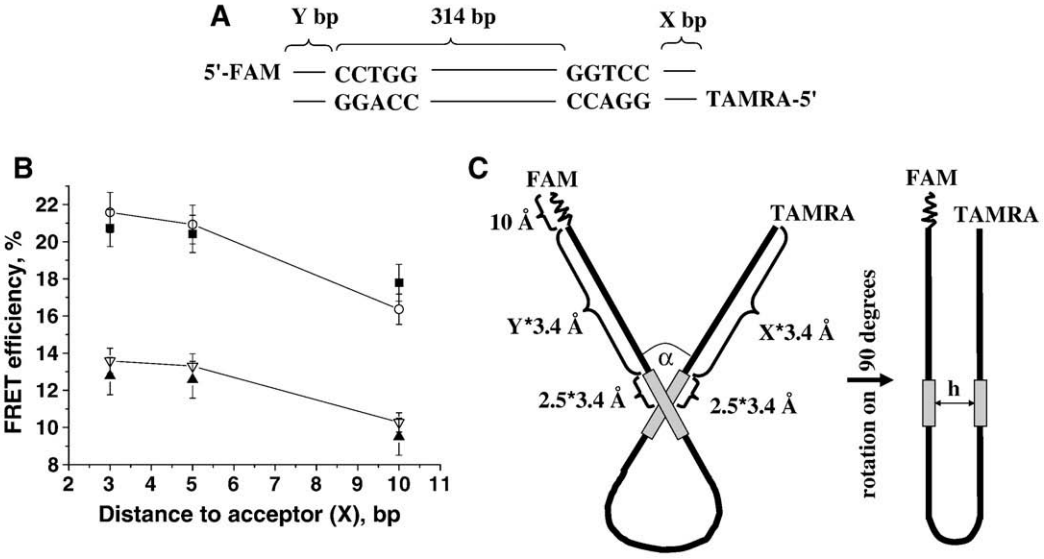

Fig. 5. (A) Two-site DNA containing donor (**FAM**) and acceptor (**TAMRA**) located Y and X bp apart from the nearest EcoRII sites, respectively. (B) Dependence of FRET efficiencies between the dyes within the R.EcoRII–DNA complex on the distance X bp, when Y = 5 (■) and 10 (▲) bp. $E^{FRET}_{X,Y}$ were registered from the quenching of the donor fluorescence. C_{DNA} 50 nM, $C_{R.EcoRII}$ 50 nM, buffer C at 20 °C. For comparison, theoretical FRET efficiencies for Y = 5 (-○-) and 10 (-▽-) bp are shown. They were calculated using the Eq. (6) and the proposed model of a DNA loop on the assumption that 26% of the DNA within the R.EcoRII–DNA complex is in a DNA loop (i.e. reduced 4.2 times). (C) Proposed model of the DNA loop induced by R.EcoRII, where α is equal to 70° and h is equal to 20 Å, ▭ - EcoRII site.

of the strands of the DNA duplexes. The binding of the 5′-**FAM-18^{A}/18^{T}**, 5′-**TAMRA-18^{A}/18^{T}** and **18^{A}/18^{T}** to the R.EcoRII enzyme was studied by EMSA at a DNA:R.EcoRII molar ratio of around 2:1. Under these conditions, the dye-containing 5′-**FAM-18^{A}/18^{T}** and 5′-**TAMRA-18^{A}/18^{T}** duplexes were bound by R.EcoRII with the same affinity as the **18^{A}/18^{T}** duplex (Fig. 2S). The initial velocity of cleavage of **14^{A}**/5′-**FAM-14^{T}** (V_0=0.0065 A_{260}/min) by R.EcoRII was 1.4 times lower compared to that of **14^{A}/14^{T}** (0.0092 A_{260}/min) (Fig. 1). Hence, introduction of the dyes near the EcoRII site does not significantly influence on the substrate properties of the DNA.

Titration of the two-site DNA, listed in Table 2, with R.EcoRII in the buffer containing Ca^{2+} ions resulted in both quenching of the donor fluorescence and an increase in the acceptor fluorescence due to the FRET between **FAM** and **TAMRA** (Fig. 2). This suggests that in each case, there was loop formation by the two-site DNA molecules within the DNA-enzyme complexes. The FRET efficiencies did not depend on the two-site DNA concentration in the range of 2.5–250 nM (Fig. 3S). This fact is an argument in favor of a rapprochement of the two EcoRII recognition sites of the two-site DNA molecule as a consequence of intramolecular (*cis*)-interactions, which do not depend on DNA concentration. If *trans*-interactions were present, FRET efficiency would depend on DNA concentration.

A typical dependence of FRET efficiencies on the R.EcoRII concentration in the presence of Ca^{2+} ions is shown in Fig. 3. The maximal FRET values were observed when one two-site DNA molecule binds one R.EcoRII dimer. This is consistent with the interaction of the two-site DNA molecules listed in Table 2 with two of three putative DNA binding sites for R.EcoRII. The addition of the **18^{A}**/5′-**TAMRA-18^{T}** duplex containing an acceptor to the preformed R.EcoRII–DNA^{FAM5}_{TAMRA5} complex in the presence of Ca^{2+} ions results in an increase of FRET efficiency, since it is evident from the additional donor quenching (Fig. 4B). This effect was comparable with an increase of FRET efficiency upon R.EcoRII–DNA^{FAM5}_{TAMRA5} complex formation. These data are consistent with the presence of the third DNA

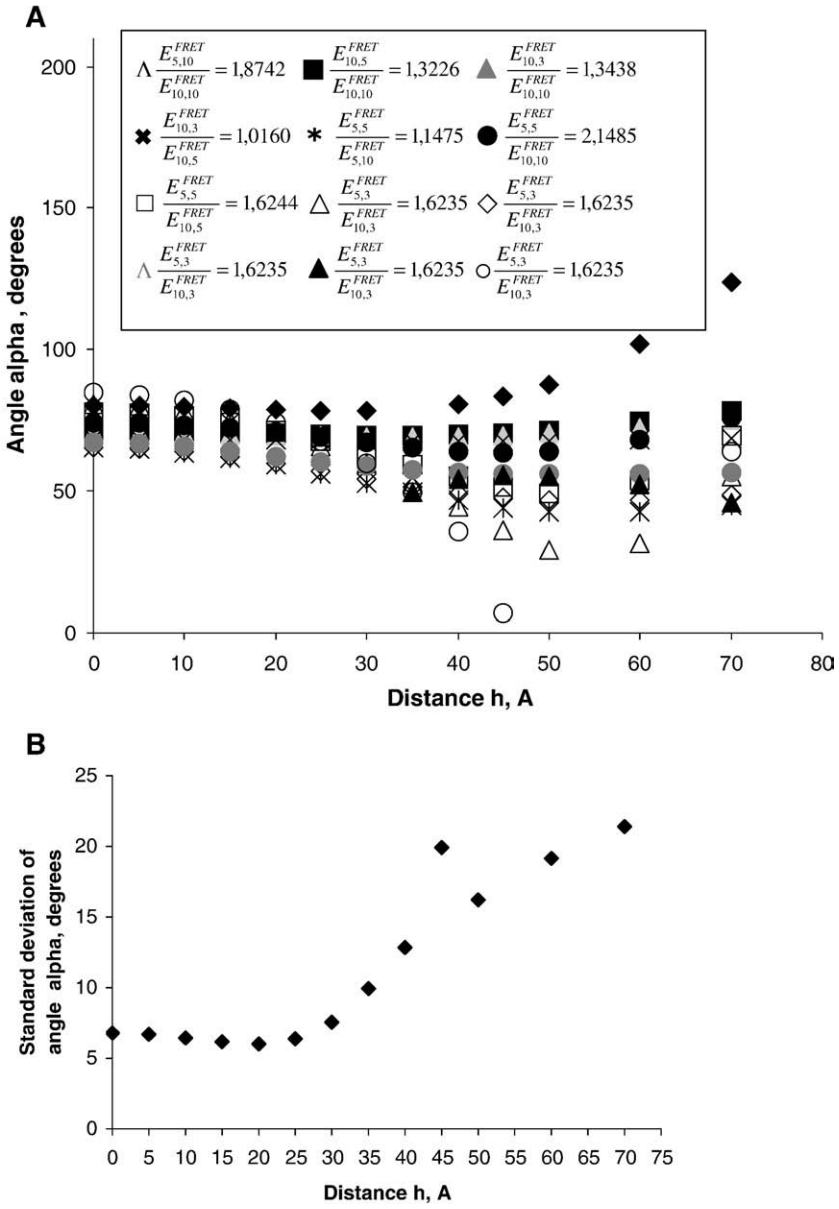

Fig. 6. (A) The dependence of angle α calculated according to Eq. (6) (Experimental) vs. distance (h) between the centers of the EcoRII sites in the DNA loop shown in Fig. 5C. Point designations correspond to designations on the top insert. Insert: the ratios of the experimental FRET efficiencies, $(E^{FRET}_{X,Y}/E^{FRET}_{X',Y'})_{exp}$, for the different combinations of X and Y. (B) The normal deviations of angle α values vs. distance h.

biding site in R.EcoRII. In the absence of Ca^{2+} ions, the addition of **18^{A}/5′-TAMRA-18^{T}** to the R.EcoRII–DNA$^{FAM5}_{TAMRA5}$ complex did not influence the FRET efficiency (Fig. 4A). One can suggest that three binding sites are formed in the presence of Ca^{2+} ions when the pre-reactive R.EcoRII–DNA complex exists. When the concentration of R.EcoRII is equal to that of the substrate DNA, about 85% of the two-site plasmid was cleaved by R.EcoRII within 2 min, predominantly at one strand yielding the nicked DNA [7]. Since R.EcoRII C-terminal domains are responsible for DNA cleavage [10], one can suggest that the two EcoRII recognition sites of the studied two-site DNA predominantly occupy the N- and C-terminal domains rather than the N- and N-terminal domains. We will use this supposition further in our calculations of the DNA loop geometry within the R.EcoRII–DNA complex.

Determination of the FRET efficiency between the donor and acceptor dyes of the two-site DNA enables one to calculate the distance (R) between the DNA ends. However, this is insufficient for the determination of the arrangement of the two EcoRII recognition sites within the R.EcoRII–DNA complex. This is why we have determined the FRET efficiencies for several two-site DNA molecules that differ in the number of base pairs between the **FAM** and the EcoRII site (Y=5 or 10 bp) and between the **TAMRA** and the EcoRII site (X=3, 5 or 10 bp) (Fig. 5A and B). Since it is not known what part of the DNA within the R.EcoRII–DNA complex forms the loop, the $E^{FRET}_{X,Y}$ obtained (Fig. 5B, ■ and ▲) are not absolute values and could not be used for R determinations. Instead, we calculated the ratios of experimental FRET efficiencies for different combinations X and Y, $(E^{FRET}_{X',Y'}/E^{FRET}_{X'',Y''})_{exp}$ (Fig. 6, top insert), which did not depend on the percent of DNA looping. Then, the theoretical ratios, $(E^{FRET}_{X',Y'}/E^{FRET}_{X'',Y''})_{theor}$ were calculated for the different orientations of the two EcoRII recognition sites relative to each other in the R.EcoRII–DNA complex by virtue of a variation of the angle (α) and the distance between the centers of the EcoRII sites (h) in the range of 0 to 180°and 0 to 70 Å, respectively (Eq. (6), Experimental). The ratios $(E^{FRET}_{X',Y'}/E^{FRET}_{X'',Y''})_{theor}$ calculated for the model, depicted in Fig. 5C, most closely correspond to the experimentally determined ratios. According to this model, h is equal to 20±10 Å and α is equal to 70±10°.

The absolute $(E^{FRET}_{X,Y})_{theor}$ were calculated according to Eq. (6) and the h and α values were obtained as above-mentioned. They are 4.2 times greater than the experimentally determined FRET efficiencies (Fig. 5B). According to EMSA (Fig. 1S), all the two-site DNA molecules are bound by R.EcoRII quantitatively in our conditions. Hence, only 26±3% of the DNA bound by R.EcoRII enzyme form loops. The $(E^{FRET}_{X,Y})_{theor}$ values, reduced by a factor of 4.2, coincide well with the experimentally determined $E^{FRET}_{X,Y}$ (Fig. 5B). Previously, it was visualized by transmission electron microscopy that the efficiency of the loop formation by R.EcoRII for the two-site DNA substrate was approximately 20% [5] and was consistent with the cleavage efficiency of 23.8% for a DNA substrate with two sites separated by the same distance of 191 bp [4].

4. Discussion

To cleave DNA, R.EcoRII requires two or three copies of a recognition sequence [7,24,25]. In the absence of an R.EcoRII–DNA co-crystal structure, the mutual orientation of the EcoRII recognition sites within the DNA-enzyme complex is unknown. To gain information about the structural organization of the R.EcoRII–DNA complex in solution, the DNA was labeled with two fluorescent dyes, **FAM** and **TAMRA**, and the energy transfer between these dyes was studied. The experiments were performed in the presence of the cofactor analog, Ca^{2+} ions, to characterize the structure of the pre-reactive R.EcoRII–DNA complex. DNA cleavage by type II REases is blocked in the presence of Ca^{2+} ions [26]. As shown by crystal structure analysis and biochemical data, the Ca^{2+} ions promote formation of a pre-reactive REase-DNA complex that is supposed to resemble the specific REase-DNA complex with cofactor Mg^{2+} ions prior to a reaction [26–28].

To create a binding model of the two recognition sites with R.EcoRII, we studied the FRET data between the two dyes located at different ends of the same two-site DNA molecule (EcoRII sites *in cis*) within the R.EcoRII–DNA complex. The major peculiarity of the interaction of R. EcoRII with the two-site DNA, listed in Table 2, is that we can exclude the interaction of the enzyme with a third recognition site on the condition that interactions are absent in *trans*. The FRET did not depend on the concentration of the two-site DNA (Fig. 3S). This observation suggests that *trans*-interactions were absent. These data are in agreement with the poor cleavage of lengthy one-site DNA, which can be cleaved only if *trans*-interactions are possible. Cleavage of the one-site 71-mer substrates by R.EcoRII at the range of 2.5–50 nM was practically absent [4,29]. The efficiency of single-site substrate cleavage by R.EcoRII decreases with increasing length of the sequences flanking the EcoRII site [30]. DNA duplexes of more than 215 bp were not cleaved by R.EcoRII at an EcoRII recognition site concentration of 350 nM [30]. Hence, in the FRET experiments concerning *cis*-localization of EcoRII recognition sites, the *cis*-interactions are present and the *trans*-interactions are absent. *cis*-interactions are more favorable than *trans*-interactions for several reasons [31,32].

Complexes of R.EcoRII with two-site DNAs penetrated into the polyacrylamide gel very poorly, as have been found by EMSA technique. This phenomenon is hypothesized to correspond to the formation of large aggregates of the R.EcoRII and DNA molecules due to *trans*-interactions. We propose that the *trans*-interactions result from a cage effect during the EMSA experiment. Similarly, the formation of similarly large complexes (also called *trans*-complexes) was previously proposed for the SfiI and NgoMIV endonucleases [17,33].

There were two difficulties in the determination of the geometry of the DNA loop induced by R.EcoRII. Firstly, the percentage of DNA (within R.EcoRII–DNA complex) that participates in the formation of the DNA loop is not known. To overcome the determination of the efficiency of DNA looping, we analyzed ratios of FRET efficiencies, which did not depend on the efficiency of DNA loop formation. Secondly, the R.EcoRII contains three potential DNA binding sites: two from the two N-terminal domains of R.EcoRII and one from the two C-terminal domains. We propose that, within the R.EcoRII–DNA complex, the DNA predominantly occupies one N- and two C-domains, but that the third binding site, the N-domain, remains unoccupied. In this supposition, the dependence of the FRET efficiency between the two dyes within the R.EcoRII–DNA complex on the remoteness of the dyes from the EcoRII recognition sites (Fig. 5B) supports the depictured model where a DNA loop is induced by R.EcoRII (Fig. 5C). Thus, in the presence of Ca^{2+} ions, the R.EcoRII binds the two-site DNA and forms a DNA loop in which the EcoRII recognition sites are 20±10 Å distant to each other and situated at an angle of 70±10°. According to the crystal structure data of complexes of another type IIE REase, R.NaeI, and a type IIF REase, R.NgoMIV, with two ON duplexes, recognition sites are located at an angle of 90 and 60°, respectively, and 31 and 55 distant to each other, respectively [34,35]. It is possible that such an arrangement of the DNA sites in the enzyme-substrate complexes provides for a minimal repulsion of the polyanion DNA molecules from each other.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2008.09.002.

References

[1] R.J. Roberts, M. Belfort, T. Bestor, A.S. Bhagwat, T.A. Bickle, J. Bitinaite, R.M. Blumenthal, S. Degtyarev, D.T. Dryden, K. Dybvig, K. Firman, E.S. Gromova, R.I. Gumport, S.E. Halford, S. Hattman, J. Heitman, D.P. Hornby, A. Janulaitis, A. Jeltsch, J. Josephsen, A. Kiss, T.R. Klaenhammer, I. Kobayashi, H. Kong, D.H. Kruger, S. Lacks, M.G. Marinus, M. Miyahara, R.D. Morgan, N.E. Murray, V. Nagaraja, A. Piekarowicz, A. Pingoud, E. Raleigh, D.N. Rao, N. Reich, V.E. Repin, E.U. Selker, P.C. Shaw, D.C. Stein, B.L. Stoddard, W. Szybalski, T.A. Trautner, J.L. Van Etten, J.M. Vitor, G.G. Wilson, S.Y. Xu, A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes, Nucleic Acids Res. 31 (2003) 1805–1812.

[2] D.H. Kruger, G.J. Barcak, M. Reuter, H.O. Smith, EcoRII can be activated to cleave refractory DNA recognition sites, Nucleic Acids Res. 16 (1988) 3997–4008.

[3] C.D. Pein, M. Reuter, D. Cech, D.H. Kruger, Oligonucleotide duplexes containing CC (A/T)GG stimulate cleavage of refractory DNA by restriction endonuclease EcoRII, FEBS Lett. 245 (1989) 141–144.

[4] M. Reuter, D. Kupper, A. Meisel, C. Schroeder, D.H. Kruger, Cooperative binding properties of restriction endonuclease EcoRII with DNA recognition sites, J. Biol. Chem. 273 (1998) 8294–8300.

[5] M. Mucke, R. Lurz, P. Mackeldanz, J. Behlke, D.H. Kruger, M. Reuter, Imaging DNA loops induced by restriction endonuclease EcoRII. A single amino acid substitution uncouples target recognition from cooperative DNA interaction and cleavage, J. Biol. Chem. 275 (2000) 30631–30637.

[6] O.V. Petrauskene, E.A. Karpova, E.S. Gromova, W. Guschlbauer, Two subunits of EcoRII restriction endonuclease interact with two DNA recognition sites, Biochem. Biophys. Res.Commun. 198 (1994) 885–890.

[7] G. Tamulaitis, G. Sasnauskas, M. Mucke, V. Siksnys, Simultaneous binding of three recognition sites is necessary for a concerted plasmid DNA cleavage by EcoRII restriction endonuclease, J. Mol. Biol. 358 (2006) 406–419.

[8] L.S. Shlyakhtenko, J. Gilmore, A. Portillo, G. Tamulaitis, V. Siksnys, Y.L. Lyubchenko, Direct visualization of the EcoRII-DNA triple synaptic complex by atomic force microscopy, Biochemistry 46 (2007) 11128–11136.

[9] X.E. Zhou, Y. Wang, M. Reuter, M. Mucke, D.H. Kruger, E.J. Meehan, L. Chen, Crystal structure of type IIE restriction endonuclease EcoRII reveals an autoinhibition mechanism by a novel effector-binding fold, J. Mol. Biol. 335 (2004) 307–319.

[10] M. Mucke, G. Grelle, J. Behlke, R. Kraft, D.H. Kruger, M. Reuter, EcoRII: a restriction enzyme evolving recombination functions? EMBO J. 21 (2002) 5262–5268.

[11] G. Tamulaitis, M. Mucke, V. Siksnys, Biochemical and mutational analysis of EcoRII functional domains reveals evolutionary links between restriction enzymes, FEBS Lett. 580 (2006) 1665–1671.

[12] S. Takahashi, H. Matsuno, H. Furusawa, Y. Okahata, Direct monitoring of allosteric recognition of type IIE restriction endonuclease EcoRII, J. Biol. Chem. 283 (2008) 15023–15030.

[13] O.V. Babkina, C.A. Chutko, A.A. Shashkov, M.S. Dzhidzhoev, R.I. Eritja, E.S. Gromova, Iodouracil-mediated photocrosslinking of DNA to EcoRII restriction endonuclease in catalytic conditions, Photochem. Photobiol. Sci. 1 (2002) 636–640.

[14] A. Hillisch, M. Lorenz, S. Diekmann, Recent advances in FRET: distance determination in protein–DNA complexes, Curr. Opin. Struct. Biol. 11 (2001) 201–207.

[15] M. Lorenz, A. Hillisch, S.D. Goodman, S. Diekmann, Global structure similarities of intact and nicked DNA complexed with IHF measured in solution by fluorescence resonance energy transfer, Nucleic Acids Res. 27 (1999) 4619–4625.

[16] L.M. Edelman, R. Cheong, J.D. Kahn, Fluorescence resonance energy transfer over approximately 130 basepairs in hyperstable lac repressor-DNA loops, Biophys. J. 84 (2003) 1131–1145.

[17] Z. Katiliene, E. Katilius, N.W. Woodbury, Single molecule detection of DNA looping by NgoMIV restriction endonuclease, Biophys. J. 84 (2003) 4053–4061.

[18] O.V. Babkina, A.G. Evstaf'eva, N.V. Chichkova, A.B. Vartapetian, S. Muller, V.B. Baskunov, O.V. Petrauskene, S.N. Kochetokov, E.S. Gromova, Recombinant components of the EcoRII restriction-modification system. Ability of restriction endonucleases to interact with DNA–RNA duplexes, Mol. Biol. (Mosk) 34 (2000) 1065–1073.

[19] C.R. Cantor, M.M. Warshaw, H. Shapiro, Oligonucleotide interactions. 3. Circular dichroism studies of the conformation of deoxyoligonucleotides, Biopolymers 9 (1970) 1059–1077.

[20] O.V. Petrauskene, N.F. Krynetskaya, V.N. Tashlitsky, V.M. Belkov, E.A. Kubareva, E.S. Gromova, W. Guschlbauer, Z.A. Shabarova, Use of UV spectroscopy for the study of nucleic acid cleavage by *E. coli* RNase H and restriction endonucleases, Biochem. Mol. Biol. Int. 37 (1995) 1127–1135.

[21] C. Gohlke, A.I. Murchie, D.M. Lilley, R.M. Clegg, Kinking of DNA and RNA helices by bulged nucleotides observed by fluorescence resonance energy transfer, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 11660–11664.

[22] R.M. Clegg, A.I. Murchie, A. Zechel, D.M. Lilley, Observing the helical geometry of double-stranded DNA in solution by fluorescence resonance energy transfer, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 2994–2998.

[23] G.J. Gemmen, R. Millin, D.E. Smith, DNA looping by two-site restriction endonucleases: heterogeneous probability distributions for loop size and unbinding force, Nucleic Acids Res. 34 (2006) 2864–2877.

[24] M. Mucke, D.H. Kruger, M. Reuter, Diversity of type II restriction endonucleases that require two DNA recognition sites, Nucleic Acids Res. 31 (2003) 6079–6084.

[25] D.H. Kruger, D. Kupper, A. Meisel, M. Reuter, C. Schroeder, The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genome, FEMS Microbiol. Rev. 17 (1995) 177–184.

[26] A. Pingoud, M. Fuxreiter, V. Pingoud, W. Wende, Type II restriction endonucleases: structure and mechanism, Cell. Mol. Life Sci. 62 (2005) 685–707.

[27] A. Pingoud, A. Jeltsch, Structure and function of type II restriction endonucleases, Nucleic Acids Res. 29 (2001) 3705–3727.

[28] D.A. Hiller, J.M. Fogg, A.M. Martin, J.M. Beechem, N.O. Reich, J.J. Perona, Simultaneous DNA binding and bending by EcoRV endonuclease observed by real-time fluorescence, Biochemistry 42 (2003) 14375–14385.

[29] O.V. Petrauskene, O.V. Babkina, V.N. Tashlitsky, G.M. Kazankov, E.S. Gromova, EcoRII endonuclease has two identical DNA-binding sites and cleaves one of two co-ordinated recognition sites in one catalytic event, FEBS Lett. 425 (1998) 29–34.

[30] O.V. Petrauskene, E.A. Kubareva, E.S. Gromova, Z.A. Shabarova, Mechanism of the interaction of EcoRII restriction endonuclease with two recognition sites. Probing of modified DNA duplexes as activators of the enzyme, Eur. J. Biochem. 208 (1992) 617–622.

[31] R. Schlief, DNA looping, Ann. Rev. Biochem. 61 (1992) 199–223.

[32] A.J. Bath, S.E. Halford, D.J. Scott, Analysis of Type II restriction endonucleases that interact with two recognition sites, in: A. Pingoud (Ed.), Restriction Endonucleases, Springer-Verlag, Berlin, 2004, pp. 297–317.

[33] M.A. Watson, D.M. Gowers, S.E. Halford, Alternative geometries of DNA looping: an analysis using the SfiI endonuclease, J. Mol. Biol. 298 (2000) 461–475.

[34] Q. Huai, J.D. Colandene, M.D. Topal, H. Ke, Structure of NaeI-DNA complex reveals dual-mode DNA recognition and complete dimer rearrangement, Nat. Struct. Biol. 8 (2001) 665–669.

[35] M. Deibert, S. Grazulis, G. Sasnauskas, V. Siksnys, R. Huber, Structure of the tetrameric restriction endonuclease NgoMIV in complex with cleaved DNA, Nat. Struct. Biol. 7 (2000) 792–799.