Interaction of EcoRII restriction and modification enzymes with synthetic DNA fragments. V. Study of single-strand cleavages

A.A.Yolov, E.S.Gromova, E.A.Kubareva, V.K.Potapov and Z.A.Shabarova

Department of Chemistry, Moscow State University, Moscow 119899, USSR

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ABSTRACT

Concatemer DNA duplexes which contain at the EcoRII restriction endonuclease cleavage sites ($\dots\downarrow\text{C-C-A-G-G}\dots$) phosphodiester, phosphoamide or pyrophosphate internucleotide bonds have been synthesized. It has been shown that this enzyme did not cleave the substrate at phosphoamide bond. EcoRII endonuclease catalyzes single-strand cleavages both in dA- and dT-containing strands of the recognition site if the cleavage of the other strand has been blocked by modification of scissile bond or if the other strand has been cleaved. This enzyme interacts with both strands of the DNA recognition site, each of them being cleaved independently on the cleavage of another one. Nucleotide sequences flanking the EcoRII site on both sides are necessary for effective cleavage of the substrate.

INTRODUCTION

An investigation of the cleavage of supercoiled circular DNAs by EcoRI [1-3] and some other restriction endonucleases [1,4-6] revealed that all the enzymes are capable of cleaving both strands of the substrate in a single binding event (double-strand cleavage) and also of hydrolyzing the substrate in one of the two strands (single-strand cleavage). The data on the capacity of restriction endonucleases to catalyze single-strand and double-strand cleavages are essential for understanding the mechanism of action of these enzymes [1-6].

The present work looks into the ability of endonuclease EcoRII (which recognizes the $5'\dots\text{C-C-A-G-G}\dots$ sequence in $3'\dots\text{G-G-T-C-C}\dots$ DNA and cleaves it at the 5'-termini [1,2] to effect single-strand cleavages and also considers the relationship between the scissions of the first and second strand of the substrate. These data enabled us to draw certain conclusions about the

mechanism of substrate cleavage by EcoRII endonuclease and about the structure of the enzyme-substrate complex. In this work single-strand cleavages were analyzed by use of specially designed synthetic DNA duplexes.

As we showed previously [7-9], a concatemer DNA duplex obtained via template-guided polycondensation of an equimolar mixture of the phosphorylated derivatives of nonanucleotides d(C-C-A-G-G-A-G-C-T) (I) and d(C-C-T-G-G-A-A-T-T) (II),

is effectively cleaved by endonuclease EcoRII, EcoRI and AluI at respective recognition sites repeated every 9 or 18 base pairs. Endonuclease EcoRII does not cleave this substrate if the scissile phosphodiester bonds (p) are replaced by pyrophosphate bonds (pp), though this enzyme specifically binds such DNA duplex [9]. Possibly DNA duplexes containing phosphoamide bonds (Np) at EcoRII cleavage sites may also possess this property. To elucidate this question DNA duplex II was synthesized:

Here and below the EcoRII recognition sites are designated by $\overline{\hspace{1.5cm}}$, EcoRI and AluI sites are not designated. Therefore we propose four polymer DNA duplexes as models for studying the cleavage of one of two strands of the EcoRII recognition site:

These duplexes contain repeated EcoRII recognition sites in each of which one of the scissile phosphodiester bonds is replaced by a phosphoamide or pyrophosphate bond. To model single-strand cleavages of the second strand the nonanucleotide dimers,

and their 5'-phosphorylated analogs IXa and Xa were used. Thereby we proceeded from the fact that DNA duplexes IX and X lacked a scissile phosphodiester bond in one of two strands of the EcoRII recognition site. It should be noted that the rotational symmetry of this site being distorted in the center, single-strand cleavages may take place in the dA- or in the dT-containing strand. The substrates V and VI, VII and VIII, as well as dimers IXa and Xa, make it possible to model both varieties of single-strand cleavages.

Important feature of dimers IX and X is that EcoRII recognition sites are not flanked by nucleotides in one of two strands from the 5'-terminus. DNA duplex XI, the product of cleavage of polymer III by EcoRI endonuclease,

has EcoRII sites not flanked in one of two strands from the 3'-terminus. This allowed us to use these substrates for studying the possible interactions of endonuclease EcoRII with the possible interactions of endonuclease EcoRII with the nucleotide sequences outside the recognition site. DNA duplex XII, the AluI-digest of polymer III,

was also used for this purpose.

MATERIALS AND METHODS

Oligonucleotides. Nonanucleotides I, II and their 3'-phosphorylated analogs Ib and IIb were synthesized by T.S. Oretskaya, E.A. Romanova and V.P. Veiko [7,8]. Nonanucleotides with a 3'-terminal 3'-amino group d(C-C-A-G-G-A-G-C-T_{NH₂}) (XIII) and d(C-C-T-G-G-A-A-T-T_{NH₂}) (XIV) were synthesized jointly with A.F. Gorkun [10].

Enzymes. EcoRII restriction endonuclease was isolated in an electrophoretically homogeneous state according to Kosykh et al. [11]. T4 polynucleotide kinase, T4 DNA ligase, restriction endonuclease EcoRI and AluI, terminal deoxynucleotidyl transferase (DNTPase) were from NIKTI BAV (Novosibirsk, USSR).

Introduction of 5'-terminal phosphate groups. 5'-phosphorylated nonanucleotides Ia, IIa, XIIIa and XIVa were obtained from I, II, XIII and XIV respectively using T4 polynucleotide kinase with ATP as described earlier [12]. The same method was used for 5'-phosphorylation of nonanucleotide dimers.

Chemical ligation of nonanucleotides. To synthesize polymer IV an equimolar mixture of XIIIa and XIVa (the concentration per nucleotide residue, C_N, was equal to 1 mM) was incubated in 50 mM potassium 2-(N-morpholino)ethanesulphonate buffer, pH 6.0, 20 mM MgCl₂ in the presence of 20 mM hydrochloride of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide ("Merck") at 0°C for 2-3 days. Dimers IX, X and their modified analogs XV-XVIII [13]:

and

were obtained by the same method from the mixtures of Ib and II, IIb and I, XIII and IIa, Ib and IIa, XIV and Ia, IIb and Ia respectively.

Enzymatic ligation. DNA duplexes V, VI, VII and VIII were synthesized by incubating 0.05 OD₂₆₀ units (350 pmol) of products of 5'-phosphorylation of dimers XV, XVII, XVI and XVIII respectively in 20 μl of 50 mM tris-HCl buffer, pH 7.5, 10 mM

MgCl₂, 5 mM dithiothreitol, 1 mM spermidine and 1 mM ATP at 8–10°C for 15–18 h. The same procedure was used for obtaining polymer III from Ia and IIa, and dimers IX or X from I and IIa or II and Ia, respectively [7].

DNA duplexes XI and XII were obtained by cleaving substrate III by endonucleases EcoRI and AluI respectively [8].

³²P-labeling of DNA-duplexes III–XII was performed in the course of their synthesis during 5'-phosphorylation of nonanucleotides or their dimers by using [γ -³²P ATP] (V/O "Isotope", USSR) instead of ATP. The substrates thus obtained contained ³²P-labeled phosphate, phosphoamide or pyrophosphate groups at the joints of nonanucleotide blocks I and II.

Synthesis of dimer XIX (see "Results and Discussion"). 0.02 OD₂₆₀ units (138 pmol) of dimer X was incubated in 10 μ l of 0.1 M K-cacodylate, 25 mM tris-HCl buffer, pH 7.0, 1 mM CoCl₂, 2 mM dithiothreitol, containing 2 μ M of the mixture of dATP ("Sigma") and [α -³²P]dATP ("Amersham") with 10 act. units of DNTase for 1 h at 37°C. The reaction was stopped by addition of 1 μ l of 0.1 M EDTA and heating the mixture for 5 min at 80°C, then oligonucleotide was precipitated with ethanol, redissolved and 5'-phosphorylated with T4 polynucleotide kinase and ATP. The labeled 19-membered product was isolated by electrophoresis in a polyacrylamide gel and added to the required quantity of a "cold" dimer Xa to obtain the dimer XIX preparation.

Assay of EcoRII restriction endonuclease cleavage. The reaction was performed at 37°C in 10 μ l of 40 mM tris-HCl buffer, pH 7.5, 5 mM MgCl₂, 50 mM NaCl, 5 mM dithiothreitol, 4% v/v glycerol, containing 7–8 act. units of EcoRII, C_N 6.4 or 12.8 μ M, the time of incubation being variable. One unit of EcoRII endonuclease is defined as the minimum amount of enzyme able to digest completely 1 μ g of pBR 322 plasmid (from E.coli B834/pBR 322) in 10 μ l of this buffer in 1 h at 37°C. The reactions were stopped by adding 1 μ l of 0.1 M EDTA and the resultant products were analysed by electrophoresis in a denaturing 20% polyacrylamide gel containing 7 M urea, 50 mM tris-borate, pH 8.3, and 1 mM EDTA. The positions of the intact substrate and reaction products were localized by

Fig. 1. Electrophoresis of the products of polymers IV (1,3) and III (4,6) cleavage by 15% acetic acid for 5 min at 90°C (1,6) and by endonuclease EcoRII (C_N 6.4 μ M, time of reaction 30 min) (3,4). 2 and 5 are the initial polymers IV and III respectively. Here and in Fig. 2,3 and 5 XC and BPB indicate the positions of xylene cyanol and bromophenol blue respectively, figures on the right - the lengths of the products (the numbers of nucleotide residues).

autoradiography with subsequent determination of 32 P-content of respective gel slices by Cherenkov counting. These data were further used for computing the amount of cleaved internucleotide bonds as described previously [14].

RESULTS AND DISCUSSION

1. Synthesis and properties of the substrates

Polymer IV was synthesized by chemical ligation of XIIIa and XIVa at conditions similar to those described earlier for polymer III synthesis from Ib and Iib [8] and other concatemer duplexes [12]. Water-soluble carbodiimide induced polycondensation of XIIIa and XIVa proceeded with good efficiency. In two days the conversion of the initial nonanucleotides into 18-360-membered and longer polynucleotides was at least 90%. The presence of phosphoamide bonds in the reaction products was verified by 15% acetic acid hydrolysis (Fig. 1). The cleavage of polymer IV was practically complete, with nonanucleotides and their oligomers being the products. Polymer III containing phosphodiester bonds only is not cleaved at the same conditions.

To obtain DNA duplexes V-VIII dimers XV, XVII, XVI and XVIII respectively were 5'-phosphorylated and then treated with T4 DNA ligase. The latter reaction was also performed for the non-modified dimers IXa and Xa. In all the cases 180-membered and longer products were formed. The conversion of the initial dimers into the reaction products was almost quantitative. Dimers XV-XVIII were obtained by chemical ligation of respective nonanucleotides as described previously [13].

DNA duplexes IXa and Xa have four-membered cohesive ends and thus they can further associate into more extended complementary complexes. It is essential to consider the possible effect of such association on the EcoRII cleavage. For this purpose dimer XIX was synthesized. In this substrate, unlike the dimer Xa, part of 3'-termini is deoxyadenylated, only the additional dA-residues (designated by ^{*}pA) being ³²P-labeled:

Because 3'-terminal AGCTA-sequences are not self-complementary the dimer XIX is not capable of associating at these termini. Additional ³²P-labeled dA-residues were introduced to dimer Xa using DNTFase with [α -³²P]dATP. To obtain products containing only one additional deoxynucleotide residue (Fig. 2), a 5-10-fold excess of the oligonucleotide over dATP was used.

2. EcoRII cleavage of DNA duplexes IV-VIII

To study the EcoRII restriction endonuclease cleavage of the substrates obtained the reaction mixtures were assayed after different times of the incubation. EcoRII endonuclease does not cleave polymer IV containing phosphoamide bonds at EcoRII cleavage sites (Fig. 1), but it forms a specific complex with this DNA duplex which is retained on nitrocellulose filters [15]. Similar data were obtained previously for another DNA duplex - analog of polymer III, containing pyrophosphate bonds at the EcoRII cleavage sites [9]. Consequently, replacement of EcoRII endonuclease-scissile phosphodiester bonds with phosphoamide or pyrophosphate bonds converts the substrate into its non-hydrolyzable analog, which can be sequence-specifically bound by the enzyme, but cannot be cleaved.

Fig. 2. Electrophoresis of the products of 3'-deoxyadenylation of dimer X (C_N 250 μ M) by DNTFase in the presence of 2 μ M [α - 32 P]dATP and subsequent 5'-phosphorylation (3). 2 - the products of similar treatment of nonanucleotide I, 1 and 4 - Ia and dimer Xa respectively.

Proceeding from these results we have constructed DNA duplexes V-VIII containing EcoRII recognition sites in which the cleavage of one of two strands is blocked by such modification. As seen from Fig. 3, endonuclease EcoRII cleaves polymers VI and VIII with the formation of 18-membered products

Fig. 3. Electrophoresis of EcoRII endonuclease cleavage products of polymers VIII (a), VI (b) and III (c). C_N 6.4 μ M, time of reaction (min) is indicated over gel columns.

Fig. 4. Comparison of the rates of polymers VII (●), VIII (○), V (■), VI (□) and III (Δ) cleavage by EcoRII endonuclease. C_N 6.4 μ M.

and polynucleotides, the length of which is multiple to 18. Similar results were obtained for polymers V and VII. As was mentioned (see Section 1), these four substrates were obtained by DNA ligase induced polycondensation of dimers XV-XVIII containing modified internucleotide bonds at the joints of nonanucleotides. So in polymers V-VIII phosphodiester bonds to be cleaved by EcoRII enzyme are at a distance of 18 nucleotides both from one another and from the polynucleotide ends. Taking into account such structure, it is to be concluded that endonuclease EcoRII cleaves these DNA duplexes at the phosphodiester bonds localized in only one of the strands of the repeated EcoRII recognition sites, i.e. single-strand cleavage takes place. In a parallel experiment polymer I was cleaved and yielded nonanucleotides and products, the length of which is multiple to 9.

The rates of cleavage of DNA duplexes V-VIII and III are compared in Fig. 4. It should be taken into account that III

Fig. 5. Electrophoresis of the products of dimers X (a), Xa (b) and IXa (c), DNA duplexes XI (d) and XII (e) cleavage by endonuclease EcoRII. C_N 12.8 μ M. Time (hours) and the number of cleaved bonds (picomoles) are indicated above and below gel columns respectively.

contains twice as many internucleotide bonds which can be cleaved by EcoRII enzyme than the modified DNA duplexes V-VIII do. One can see that the hydrolysis of phosphodiester bonds in V and VI is only slightly less effective than in III, while in VII and in VIII it is significantly inhibited.

Single-strand cleavages may occur both in the dA- and in the dT-containing strand of the EcoRII recognition sites. As seen at Fig. 4, the dA-containing strand in V is cleaved by EcoRII endonuclease with the similar efficiency as the dT-containing strand in VI. At the same time cleavage of the dT-containing strand in VIII proceeds appreciably faster than the cleavage of dA-containing strand in VII.

3. EcoRII cleavage of nonanucleotide dimers

Fig. 5 shows that dimers Xa and IXa are cleaved slowly by EcoRII endonuclease into nonanucleotides. This enzyme does not cleave dimers IX and X at all. As noted in the previous paper [7], slow cleavage of dimers IXa and Xa may occur due to either splitting the substrate at the recognition site flanked

in one strand by the phosphate group only or the dimer association by cohesive ends which leads to "non-covalent" flanking the recognition sites. Experimental data support the latter supposition. The cleavage of dimer Xa is completely blocked if dA-residue is added to its 3'-terminus (dimer XIX). As noted above, such a substrate cannot associate by 3'-terminal AGCTA-fragments. Only extremely slow, if any, digestion by EcoRII endonuclease can be observed in the case of incapable of association DNA duplex XII (Fig. 5), although it contains short nucleotide sequences flanking the EcoRII sites from both sides.

These data suggest that a specific cleavage of dimers IXa and Xa actually proceeds at a recognition sites of the following structure:

It is this structure that a possible intermediate DNA cleavage product in which only one of two strands of recognition site is cleaved by a restriction enzyme could have [1-6]. Consequently the digestion of dimers IXa and Xa by endonuclease EcoRII models the cleavage of the second strand (indicated by arrow) in such product. This cleavage, as well as that considered in section 2, takes place both in dA- and dT-containing strands of the recognition site. The rate of the cleavage of dA-containing strand in Xa is noticeably faster than that of the dT-containing strand in IXa (Fig. 5). One possible reason is that the dimer IXa with AATT-cohesive ends associates less than the dimer Xa with AGCT-cohesive ends.

The data obtained also indicate that the hydrolysis of DNA duplex by EcoRII endonuclease requires flanking the recognition site by nucleotides both from 5'- and 3'-termini. The cleavage of DNA duplex XI (Fig. 5) as well as IXa and Xa, is significantly retarded as compared with polymer III (Fig. 4). These results, as well as above noted requirement of dimers IXa and Xa association for their cleavage by endonuclease EcoRII, suggest that the enzyme interacts with DNA fragments flanking the recognition site on both sides. Very slow cleavage of DNA duplex XII enables us to conclude that rather extended DNA frag-

ment is necessary for normal cleavage of the substrate. According to our earlier estimation [14], EcoRII endonuclease binds to the DNA fragment of 23-32 base pairs long.

4. Conclusions

As it follows from the data obtained, endonuclease EcoRII can catalyse single-strand breaks in any of two strands of its recognition site if the other strand is already cleaved or if its cleavage is blocked by modification of scissile internucleotide bond. The pronounced dependence of the efficiency of such cleavages on the nature of modification of the other scissile bond (Figs 3-5) indicates that endonuclease EcoRII interacts with both strands of its recognition site. At the same time the high efficiency of single-strand breaks in the case of polymers V and VI shows that each of two strands of EcoRII recognition site is cleaved independently on the cleavage of another one. These findings allow one to suppose that the hydrolysis of two DNA strands by endonuclease EcoRII is catalysed by two identical hydrolytical active centers operating independently. It is consistent with the "symmetric model" of the enzyme-substrate complex [16,17] taken into consideration for EcoRII endonuclease [14]. This model implies, in particular, binding of the dimeric restriction enzyme to both strands of DNA recognition site and a symmetrical arrangement of two hydrolytical active centers near two scissile bonds. We would like to note that given this structure of enzyme-substrate complex, endonuclease EcoRII could catalyze both single- and double-strand cleavages.

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REFERENCES

- 1 Modrich P. (1982) CRC Crit. Rev. in Biochem. 13, 287-323.
- 2 Modrich P. (1979) Quarterly Rev. in Biophys. 12, 315-369.
- 3 Rubin R.A. and Modrich P. (1978) Nucleic Acids Res. 5, 2991-2997.

- 4 Maxwell A. and Halford S.E. (1982) *Biochem. J.* 203, 85-92.
- 5 Halford S.E., Johnson N.P. and Grinstead J. (1979) *Biochem. J.* 179, 353-365.
- 6 Halford S.E. (1983) *Trends in Biochem. Sci.* 8, 455-460.
- 7 Yolov A.A., Gromova E.S., Romanova E.A., Oretskaya T.S., Oganov A.A., Buryanov Ya.I. and Shabarova Z.A. (1984) *FEBS Lett.* 167, 147-150.
- 8 Gromova E.S., Vinogradova M.N., Yolov A.A., Veiko V.P., Dolinnaya N.G., Drutsa V.L., Metelev V.G., Oretskaya T.S. and Shabarova Z.A. (1984) *Molekularnaya biologiya* 18, 370-381.
- 9 Purmal A.A., Vinogradova M.N., Yolov A.A., Gromova E.S., Drutsa V.L., Metelev V.G., Kholodkov O.A., Buryanov Ya.I. Shabarova Z.A. and Prokofiev M.A. (1984) *Doklady AN SSSR* 276, 992-995.
- 10 Gorkun A.F., Veiko V.P., Potapov V.K., Shabarova Z.A. and Prokofiev M.A. (1984) *Doklady AN SSSR* 275, 94-97.
- 11 Kosykh V.G., Puntezhis S.A., Buryanov Ya.I. and Bayev A.A. (1982) *Biokhimiya* 47, 619-625.
- 12 Shabarova Z.A., Dolinnaya N.G., Drutsa V.L., Melnikova N.P. and Purmal A.A. (1981) *Nucleic Acids Res.* 9, 5747-5761.
- 13 Ivanovskaya M.G., Purmal A.A., Dolinnaya N.G., Metelev V.G., Gorkun A.F., Veiko V.P., Potapov V.K. and Shabarova Z.A. (1983) Manuscript dep. VINITI No. 6782, 20 pages.
- 14 Yolov A.A., Gromova E.S. and Shabarova Z.A. (1985) *Molec. Biol. Rep.* 10, 173-176.
- 15 Vinogradova M.N. (1985) in VI Republican Conference of the Young Scientists-chemists. Abstracts of reports, p. 164. Tallinn.
- 16 Kelly T.J. and Smith H.O. (1970) *J. Mol. Biol.* 51, 393-409.
- 17 Smith H.O. (1979) *Science* 205, 455-462.