Interaction of *Eco*RII restriction and modification enzymes with synthetic DNA fragments

*Eco*RII endonuclease cleavage of substrates with repeated natural and modified recognition sites

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Interaction of *Eco*RII restriction endonuclease with a set of synthetic concatemer DNA duplexes with natural and modified sites for this enzyme has been studied. DNA duplexes with repeated natural sites are cleaved by *Eco*RII. Substitution of central AT-pair in the recognition site for a non-complementary TT- or AA-pair reduces the rate of cleavage, this effect being much more pronounced in the last case. Absence of site flanking in one strand from the 5'-terminus also results in very slow cleavage. The results obtained testify to the interaction of *Eco*RII with both strands of the substrate.

*Eco*RII *cleavage* *DNA duplexes with repeats* *Modified recognition site* *Non-complementary pair* *Site flanking*

1. INTRODUCTION

Restriction endonuclease *Eco*RII recognizes in DNA the

5'↓C-C-A-G-G-
3'-G-G-T-C-C↑

sequence and cleaves it as is indicated by the arrows [1]. A way to elucidate the mechanism of this process is to use synthetic concatemer DNA duplexes with regularly repeated restriction endonuclease recognition sites [2,3].

Here interaction of *Eco*RII restriction enzyme with a set of concatemer DNA duplexes obtained from two nona-nucleotides, d(C-C-T-G-G-A-A-T-T) (I) and d(C-C-A-G-G-A-G-C-T) (II), has been studied.

Internucleotide phosphate groups which carry ^{32}P and are situated at the 'joints' of nona-nucleotides are indicated by asterisks. In these duplexes natural and modified *Eco*RII sites are repeated every 9 base pairs. Polymer III contains *Eco*RII sites with alternating orientation of the central AT-pair (-C-C-A-G-G- is followed by -C-C-T-G-G- in the same strand, etc). In the IV and V polymers this pair is replaced by a non-complementary TT or AA pair. Nona-nucleotide dimers VI and VII as well as their 5'-phosphorylated analogues VIa and VIIa contain two *Eco*RII sites which are not flanked in one of the chains from the 5'-terminus. Moreover there is no 5'-terminal phosphate group outside of the site in dimers VI and VII. The study of the *Eco*RII cleavage of these duplexes has permitted us to obtain primary information about the mechanism of substrate recognition by this enzyme.

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Accompanying paper is [3]

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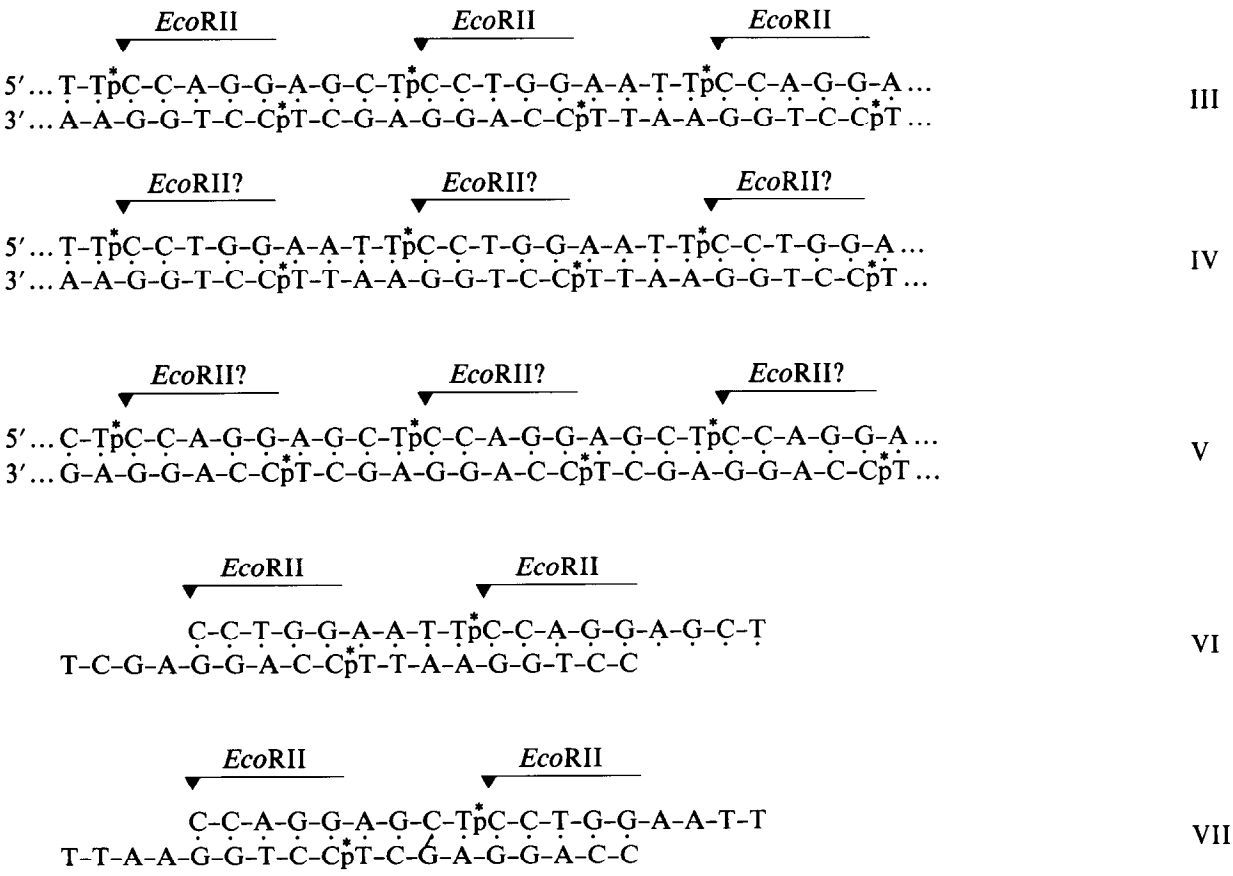

2. MATERIALS AND METHODS

*Eco*RII endonuclease was purified as in [4]. I and II were synthesized as in [5,6]. Oligonucleotides were 5′-phosphorylated by T4 polynucleotide kinase in the presence of ATP or [γ-^{32}P]ATP. Polymers III–V were obtained by ligation with T4 DNA ligase of either mixture or each of d(pC-C-T-G-G-A-A-T-T) (Ia) and d(pC-C-A-G-G-A-G-C-T) (IIa). Ligation of I and IIa or II and Ia resulted in dimers VI and VII, respectively. Analogous compounds obtained by 'chemical ligation' were described in [3].

Cleavage of the ^{32}P-labeled substrates was performed by incubating DNA duplexes (nucleotide concentration per monomer, C_N, was 4–16 μM) with 3–4 act. units of *Eco*RII in 10 μl of 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 5 mM dithiothreitol, 4% (v/v) glycerol at 20 or 37°C. Reaction mixtures were analyzed by electrophoresis of denaturating 20% polyacrylamide gel. The ^{32}P-content of the gel slices that corresponded to the initial polymer and the reaction products was determined by Cherenkov counting. On the basis of these data and the initial amount of the substrate, the amount of cleaved phosphodiester bonds was calculated.

3. RESULTS AND DISCUSSION

Polymers III–V which have been used as substrates for *Eco*RII endonuclease are extended (more than 200-membered) polynucleotides (fig.1). All 3 polymers [3], as well as dimers VI, VIa, VII and VIIa are stable duplexes in ionic solutions. In conditions of *Eco*RII cleavage the melting temperature of dimer VIa is 58°C, that of dimer VIIa is 63°C. Because of cohesive ends, some of these duplexes may associate to form more extended complementary complexes.

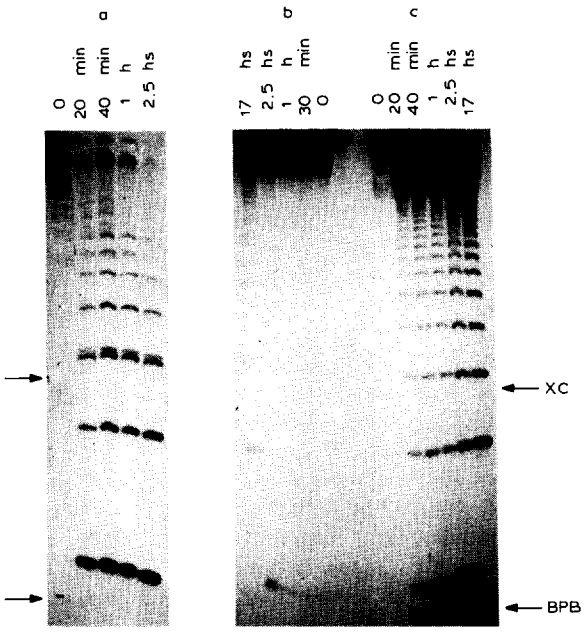

Fig.1. Cleavage of polymers III (a), V (b) and IV (c) by *Eco*RII endonuclease. Electrophoresis of reaction mixtures in 20% polyacrylamide gel containing 7 M urea is shown. XC and BPB are the positions of xylene cyanol and bromophenol blue. Reaction was performed at 37°C, C_N 4 μM. Time of reaction (h, min) is indicated above the gel columns.

As was expected, polymer III is cleaved by the *Eco*RII enzyme to nona-nucleotides (fig.1). Besides this product, one can see the products of non-complete cleavage, the length of which is multiple to 9. Polymers IV and V are cleaved by *Eco*RII into oligonucleotides of the same length as in case of polymer III (fig.1). However, the number of phosphodiester bonds cleaved by *Eco*RII in the same conditions decreases as polymer III > polymer IV ≫ polymer V (fig.2). Thus the enzyme can cleave DNA duplexes at

5′-CCTGG
3′-GGTCC

and, to a much lesser degree, at

5′-CCAGG
3′-GGACC

sequences. These findings suggest that replacement of the central AT pair in the *Eco*RII site by non-complementary AA or TT pair allows recognition.

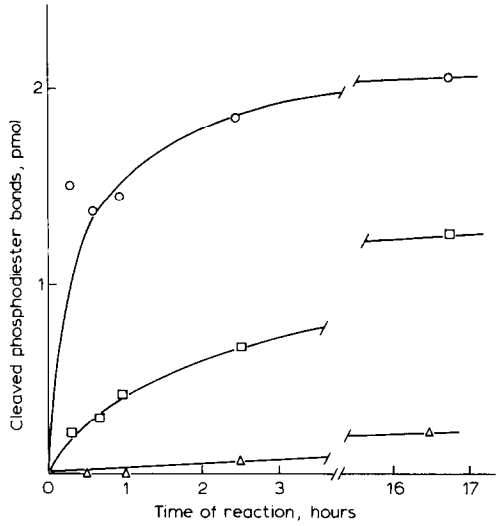

Fig.2. Rate of cleavage of polymers III (○), IV (□) and V (△) by *Eco*RII endonuclease. For reaction conditions, see fig.1.

The reduced rates of cleavage of polymers IV and especially V could result from the local distortion of the helical configuration of the site by non-complementary base pair, this effect being much more pronounced in the region of contact of two bulky adenine residues.

As seen in fig.3, 5′-phosphorylated dimers VIa and VIIa are cleaved by *Eco*RII into nona-nucleotides, although the number of cleaved phosphodiester bonds is an order less than for polymer III hydrolysis in the identical conditions. Dimers VI and VII are not cleaved by endonuclease *Eco*RII at all. It thus follows that specific cleavage of DNA duplexes by *Eco*RII endonuclease requires nucleotide sequences flanking the recognition site from the 5′-termini. The importance of flanking the site was reported for a number of other restriction endonucleases [1]. The possibility of cleavage of dimers VIa and VIIa may occur as a result of both the dimer association by cohesive ends which leads to 'non-covalent flanking' *Eco*RII sites and the ability of the enzyme to digest recognition site which is surrounded in one of the strands only by a phosphate.

Both *Eco*RII sequences in dimers VIa and VIIa are cleaved only at one of the strands because there is no phosphodiester bond which could be cleaved by the enzyme in the second strand. This cleavage may be regarded as a single-strand one. It is

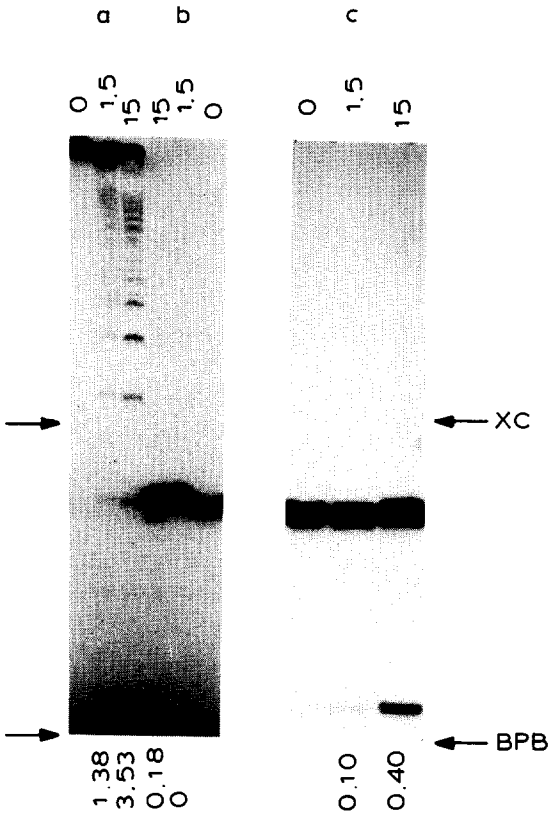

Fig.3. Cleavage of polymer III (a), dimers VIIa (b) and VIa (c) by *Eco*RII endonuclease. For electrophoresis conditions, see fig.1. Reaction was performed at 20°C, C_N 16.3 μM. Time of reaction (h) and the number of cleaved phosphodiester bonds (pmol) are indicated above and under the gel columns, respectively.

blocked completely if the second strand does not possess 5′-terminal phosphate flanking the site. It thus follows that interaction of the enzyme with this phosphate influences the cleavage of the other chain, which testifies to the interaction of the *Eco*RII endonuclease with both strands of the duplex.

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