Activation of restriction endonuclease *EcoRII* does not depend on the cleavage of stimulator DNA

Claus-Dietmar Pein, Monika Reuter¹, Andreas Meisel¹, Dieter Cech and Detlev H. Krüger^{1*}

Institute of Bioorganic Chemistry, Humboldt University, Invalidenstraße 42, D-O-1040 Berlin and

¹Institute of Virology, Humboldt University (Charité), Schumannstraße 20–21, D-O-1040 Berlin, FRG

Received August 23, 1991; Accepted September 12, 1991

ABSTRACT

The restriction endonuclease *EcoRII* is unable to cleave DNA molecules when recognition sites are very far apart. The enzyme, however can be activated in the presence of DNA molecules with a high frequency of *EcoRII* sites or by oligonucleotides containing recognition sites: Addition of the activator molecules stimulates cleavage of the refractory substrate. We now show that endonucleolysis of the stimulator molecules is not a necessary prerequisite of enzyme activation. A total *EcoRII* digest of pBR322 DNA or oligonucleotide duplexes with simulated *EcoRII* ends (containing the 5' phosphate group), as well as oligonucleotide duplexes containing modified bases within the *EcoRII* site, making them resistant to cleavage, are all capable of enzyme activation. For activation *EcoRII* requires the interaction with at least two recognition sites. The two sites may be on the same DNA molecule, on different oligonucleotide duplexes, or on one DNA molecule and one oligonucleotide duplex. The efficiency of functional intramolecular cooperation decreases with increasing distance between the sites. Intermolecular site interaction is inversely related to the size of the stimulator oligonucleotide duplex. The data are in agreement with a model whereby *EcoRII* simultaneously interacts with two recognition sites in the active complex, but cleavage of the site serving as an allosteric activator is not necessary.

INTRODUCTION

Restriction endonuclease *EcoRII* recognizes the sequence 5' CC(A/T)GG 3' and cleaves it at the 5' end; the cognate modification consists in a C-5 methylation of the internal cytosine [1, 2]. The molecular weight of the restriction enzyme was predicted from its sequenced gene to be 45,6 kD [3, 4]. An unusual property of this enzyme is its apparent inability to cleave certain DNAs like those of the phages T3 and T7 [5] or M13 [6] even where not protected by methylation. The resistance of such *EcoRII* sites is not caused by flanking base sequences [7], but is probably the consequence of a sub-threshold density of recognition sites in the substrate DNA. Resistance can be

overcome by coinubation with a susceptible DNA of higher site density, like lambda, pBR322 or site-containing oligonucleotide duplexes. We concluded that activation of *EcoRII* requires its coordinated interaction with a minimum of two recognition sites in the substrate DNA [8, 9].

We have also shown that a number of other restriction endonucleases which cleave their substrates incompletely can be activated by the addition of oligonucleotide duplexes, containing cognate recognition sequences [Reuter et al., in preparation]. Although the activation mechanisms were not investigated in these cases, the observations indicate that *EcoRII* is not unique among restriction enzymes. Also other proteins are known to depend on the coordinated presence of two sites in their target molecule. This is true, for example, of enzymes catalysing site-specific, intramolecular recombination. They not only need two sites but are sensitive to their distance apart and, in some cases, relative orientation [10, 11]. The mode of action of *EcoRII* may have implications extending beyond the restriction enzymes. We have, therefore, conducted a comprehensive study of the activation mechanism of *EcoRII*.

MATERIAL AND METHODS

T3 DNA was prepared from CsCl-purified phage by phenol extraction and ethanol precipitation. Plasmid pBR322 (Dcm⁻) DNA was extracted from *E. coli* B/Berlin host cells and purified on ethidium bromide/CsCl gradients according to Maniatis et al. [12]. *EcoRII* and 1 kb ladder were obtained from Bethesda Research Laboratories and *Bst*NI from New England Biolabs. *Mva*I was a kind gift from V. Butkus (Institute of Applied Enzymology, Vilnius, Lithuania).

DNA digestions were run under conditions recommended by the suppliers. The fragments were separated on agarose gels and visualised by ethidium bromide staining or by polyacrylamide gel electrophoresis and autoradiography, respectively.

Oligonucleotide duplexes used in this study are listed in Figure 1. Oligonucleotides of duplexes I, IIa,b and VI–VIII were synthesized by the phosphoramidite method on a DNA synthesizer (Pharmacia Gene Assembler Plus) and purified by HPLC. Oligonucleotides of duplexes III–V were synthesized by the same method on a synthesizer Viktoria-4M (Novosibirsk,

* To whom correspondence should be addressed

USSR). Phosphorylated oligonucleotides of duplexes I Ib were obtained by phosphorylation of synthetic oligonucleotides IIa with T4 polynucleotide kinase (Boehringer Mannheim GmbH).

For enzymic digestion 3 pmol of the oligonucleotide duplex were incubated with 2 units of restriction endonuclease in 20 μ l for 1.5 h at 37°C. The digestion of oligonucleotide duplexes VII and VIII was stimulated by incubation with 150 pmol oligonucleotide duplex VI under identical reaction conditions.

RESULTS

Noncleavable recognition sites as activators

Recently we have shown that *Eco*RII is stimulated by incubation with digestible DNA containing unmodified target sequences but not by DNA that is modified or that lacks target sequences [8, 9].

In order to investigate whether cleavage of the activator DNA is a necessary prerequisite of stimulation of *Eco*RII, pBR322 was first exhaustively digested by *Eco*RII after which T3 DNA was added to the incubation mixture. As Figure 2 (lane 3) shows, the digestion of T3 DNA was stimulated by this pre-digested DNA. Stimulation could be effected either by a form of the enzyme activated during cleavage of pBR322 or by the fragments of pBR322 which no longer contain any intact *Eco*RII recognition sites. This question was examined by digesting pBR322 DNA with *Eco*RII and then removing the enzyme by phenol treatment and ethanol precipitation of the DNA fragments. To check for complete removal of enzyme, the fragment mixture was co-incubated with T3 DNA. In this case there is no cleavage of T3 DNA (lane 4) but after addition of new *Eco*RII, T3 DNA is digested (lane 5). Obviously, the enzyme is activated by the pre-digested pBR322 DNA, i.e. by the product of previous cleavage reaction. The enzyme stimulation cannot be caused by possibly uncleaved pBR322 molecules which could remain after *Eco*RII digestion: In our experiments we used 50 ng pBR322 DNA which

was shown to be the minimal amount of intact plasmid DNA able to stimulate the complete cleavage of 400 ng T3 DNA by *Eco*RII [8].

As in other cases [8, 9], stimulation of *Eco*RII by pre-digested pBR322 depends on the concentration of activator DNA (data not shown).

Fig. 2. Stimulation of *Eco*RII cleavage of T3 DNA by pBR322 DNA (Dcm^-) predigested by *Eco*RII. lane 1: 1 kb ladder; lane 2: pBR322 DNA + *Eco*RII; lane 3: pBR322 DNA + *Eco*RII, after 1 h incubation T3 DNA was added; lane 4: predigested pBR322 DNA (phenol extracted) + T3 DNA; lane 5: predigested pBR322 DNA (phenol extracted) + T3 DNA + *Eco*RII; lane 6: pBR322 DNA (uncleaved) + T3 DNA + *Eco*RII; lane 7: T3 DNA + *Bst*NI; lane 8: T3 DNA + *Eco*RII; lane 9: T3 DNA; lane 10: 1 kb ladder. 400 ng T3 DNA, 50 ng of the respective pBR322 DNA as well as 2 units *Eco*RII or 2 units *Bst*NI were incubated for 2 h in buffer recommended by the supplier.

duplex I	5' ACCACCACCAGGTAGGTAGGTA 3'	3' TGGTGGTGGTGGTCCATCCATCCAT 5'
duplex IIa	5' ACCACCACCA CCAGGTAGGTAGGTA 3'	3' TGGTGGTGGTGGTCC ATCCATCCAT 5'
duplex I Ib	5' ACCACCACCA PCCAGGTAGGTAGGTA 3'	3' TGGTGGTGGTGGTCCp ATCCATCCAT 5'
duplex I Ib-1	5' ACCACCACCA 3'	3' TGGTGGTGGTGGTCCp 5'
duplex I Ib-2	5' 3'	3' PCCAGGTAGGTAGGTA 3' ATCCATCCAT 5'
duplex III	5' ACCTACC TGGTGGT 3'	3' TGGATGGm ⁶ ACCACCA 5'
duplex IV	5' ACCTACCTTGTGGT 3'	3' TGGATGGACACCA 5'
duplex V	5' ACCTAm ⁴ CCTGGTGGT 3'	3' TGGAT GGACACCA 5'
duplex VI (14 mer)	5' GCCAACCTGGCTCT 3'	3' CGTTGGACCGAGA 5'
duplex VII (30 mer)	5' TCGATGCTGCCAACCTGGCTCTAGCTTCAT 3'	3' AGCTACGACGGTTGGACCGAGATCGAAGTA 5'
duplex VIII (71 mer)	5' TAGCGGATCCTGTACATCGATGCTGCCAACCTGGCTCTAGCTTCAT- 3'	3' ATCGCCTAGGACATGTAGCTACGACGGTTGGACCGAGATCGAAGTA- 3'
	-TGCTTAAGCCGGAGATCTTGCTATC 5'	
	-ACGAATTCCGGCTCTAGAACGATAG 3'	

m⁶A - 6-methyl-2'-deoxyadenosine, I - 2'-deoxyinosine,
m⁴C - N⁴-methyl-2'-deoxycytidine

*Eco*RII recognition site in bold letters

Fig. 1. Structure of oligonucleotide duplexes used.

Fig. 3. Stimulation of *Eco*RII cleavage of T3 DNA by synthetic oligonucleotide duplexes. 230 ng T3 DNA were digested with 4 units *Eco*RII in the presence of 140 ng of the respective oligonucleotide duplex for 1.5 h in a reaction volume of 20 μ l. lane 1: 1 kb ladder; lane 2: T3 DNA; lane 3: T3 DNA + *Eco*RII; lane 4: T3 DNA + oligoduplex I + *Eco*RII; lane 5: T3 DNA + oligoduplex I Ib + *Eco*RII; lane 6: T3 DNA + oligoduplex I Ib-1 + *Eco*RII; lane 7: T3 DNA + oligoduplex I Ib-2 + *Eco*RII.

In contrast pBR322 DNA cleaved by the *EcoRII* isoschizomers *MvaI* or *BstNI* is unable to stimulate the digestion of T3 DNA (data not shown). Cleavage by these enzymes generates fragments with only 1 base extension while *EcoRII* fragments have a 5-base overhang [1, 2].

Other DNA species besides *EcoRII*-cleaved pBR322 are equally suitable as activators. Not only oligonucleotide duplex I (Figure 1) containing an *EcoRII* site stimulates cleavage of T3 (Figure 3, lane 4) but also synthetic oligonucleotide duplexes with 5' phosphate group, mimicking the products of *EcoRII* cleavage (duplex IIb, Figure 3, lane 5). The same effect can even be brought about by the individual components of the duplex mixture IIb (IIb-1 and IIb-2) which are of course unable to form an intact *EcoRII* recognition site (Figure 3, lanes 6 and 7).

However, attempts to stimulate *EcoRII* with the oligonucleotide duplex mixture IIa were unsuccessful probably due to the lack of the 5' terminal phosphate group. On the other hand, certain oligonucleotide duplexes displaying intact recognition sequences with one modified base (duplexes III, IV, V) are still capable of activating the enzyme without being cleaved themselves. While duplexes III and IV stimulate a complete restriction of primarily resistant T3 DNA, duplex V only supports a partial digestion of the phage DNA by *EcoRII* (data not shown).

The results show that activation of *EcoRII* restriction endonuclease does not occur during the cleavage reaction of the

activator DNA, since *EcoRII* digestion products of pBR322 as well as different oligonucleotide duplexes which are not substrates of the *EcoRII* restriction endonuclease can act as activator molecules.

Influence of fragment length and number of restriction sites in a DNA molecule on its susceptibility to the restriction endonuclease *EcoRII*

The activity of *EcoRII* is influenced by the length of the substrate and its nucleotide sequence, as demonstrated previously on short synthetic oligonucleotides [13]. We investigated these relations in more detail using various natural and synthetic substrates.

The synthetic oligonucleotide duplexes (Figure 1) contain one recognition site for *EcoRII*, the larger duplexes encompassing the sequences of the shorter ones, e.g. VI represents the central sequence of VII, and VII the central sequence of VIII. Table I shows that oligonucleotide duplexes VI to VIII are digested with decreasing efficiency. By coincubating the longer oligonucleotide duplexes VII and VIII with the short substrate VI their cleavage efficiency can be enhanced. The isoschizomer *MvaI* does not show this preference and cleaves all 3 substrates almost completely.

In a further series of experiments natural DNA fragments obtained from pBR322 by digestion with other restriction enzymes and purified by agarose electrophoresis, electroelution and phenol extraction were treated with *EcoRII*. Figure 4 summarizes the results. All fragments containing only 1 recognition site are highly resistant to *EcoRII* but are fully digestible by *BstNI* (fragments A–D). Coincubation with oligonucleotide duplex VI renders these fragments susceptible to *EcoRII*, however, digestion was not always complete (data not shown). DNA fragments with more than one site (E–I) are *EcoRII*-sensitive in all cases. Thus, fragment E is cleaved though it contains two sites which are by themselves not susceptible when presented as singular sites in the fragments C and D (Figure 5, lane 4c and 5c). Fragment E is, however, only incompletely digested (Figure 5, lane 3c). Fragments I as well as F and the complete plasmid pBR322 (K) exhibit partial digestion products, the latter two to a lesser extent than the former. In fragments E and I the first site (base number 130) is especially refractive to cleavage. This site is also the least likely to be cleaved in the intact plasmid and it displays the greatest distance from any other site.

Digestion of M13RF DNA by *EcoRII* confirmed the data obtained for the pBR322 fragments. Complete cleavage of the

Table I. Digestion rates of oligonucleotide duplexes VI–VIII by *EcoRII* and *MvaI* restriction endonucleases

	base pairs of oligo duplex	% cleavage by <i>EcoRII</i>	% cleavage by <i>EcoRII</i> in the presence of Duplex VI	% cleavage by <i>MvaI</i>
duplex VI	14	55	—	85
duplex VII	30	37	52	89
duplex VIII	71	28	42	81

For experimental details see Materials and Methods.

Fig. 4. Schematic representation of digesting fragments of pBR322 (Dcm^-) by *EcoRII*. The fragments were generated from pBR322 (Dcm^-) by *EcoRI*, *RsaI*, *SalI* and *SmaI* cleavage. They were isolated on a 1% agarose gel by electroelution, phenol extraction and ethanol precipitation. Recognition sites for *EcoRII* are indicated by arrows. Please note that fragments B, I, and K (the intact circular plasmid) are not cleaved at the *EcoRII* site, as indicated by dashes. The first T of the unique *EcoRII* site was defined as base 1 and numbering is from Tc to Ap.

Fig. 5. Digestion of fragments of pBR322 (Dcm^-) by *EcoRII*. Approximately 300 ng of the appropriate fragment (compare Figure 4) was incubated with 4 units *EcoRII* (lane c) or 4 units *BstNI* (lane b) or without enzyme (lane a) for 1.5 h at 37°C. lanes 1, 7: 1 kb ladder; lanes 2a–c: pBR322 circular; lanes 3a–c: fragment E; lanes 4a–c: fragment C; lanes 5a–c: fragment D; lanes 6a–c: fragment I.

two *EcoRII* recognition sites, separated by 952 bp, is achieved only after stimulation of the enzyme by oligonucleotide duplexes [6]. Cleavage by *EcoRII* alone resulted in partial linearization of the molecule, indicating that only one *EcoRII* site was cleaved. There was no preference for one of the two sites; either can be cleaved but the remaining site is refractory to *EcoRII*.

DISCUSSION

The results confirm our previously postulated hypothesis that *EcoRII* requires the coordinated interaction with two recognition sites for its enzymatic activity and allow a better understanding of the actual mechanisms involved. This follows from experiments with different types of DNA fragments carrying one or more digestion sites. The possibility of stimulating *EcoRII* digestion of T3 DNA by already digested pBR322 DNA, by oligonucleotide duplexes resembling products of restriction and by modified oligonucleotide duplexes themselves refractory to *EcoRII*, supports the notion that the enzyme is simultaneously interacting with 2 sites.

Furthermore it can be concluded that stimulation does not result from the hydrolysis of the activator molecules but that recognition and binding are sufficient. The stimulators are probably incorporated into the enzyme-substrate complex as allosteric activators. While the activator site does not need to be cleaved itself, we have identified the following requirements for its configuration:

- a) the site must be recognized and bound by the enzyme (non-site or Dcm modified DNA do not stimulate)
- b) for intermolecular interaction the stimulator molecule must be sufficiently small in order to exclude steric hindrance
- c) for intramolecular interaction the distance between the sites must not exceed a certain limit.

DNA species with several recognition sites are only cleaved when the distance between them does not exceed the limit of about 1000 bp, the efficiency of cleavage decreasing with the distance. At greater distance between sites digestion can only be achieved by adding a stimulator molecule. Thus, M13 is only linearized by *EcoRII*. Possibly the enzyme dissociates from the substrate after cleaving the first site and is then unable to attack the second one which is now presented as singular site. The long M13 molecules obviously do not allow an intermolecular interaction. These results are independent of the topological state of the M13 substrate, i.e. whether it is linear or circular and they agree with the results obtained with another kind of substrate, DNA fragments of pBR322 (Figure 4, 5): In this case too, some fragments carrying more than one recognition site are incompletely digested, probably for the same reasons.

The novel data on the structure of stimulator molecules for the activation of the enzyme show that not only does the length of the oligonucleotide duplexes play a decisive role but that even cleavage products are capable of stimulation. These can be *EcoRII* cleaved pBR322 molecules or even synthetic oligonucleotide duplexes resembling cleavage products (duplexes IIb). Only the absence of the 5' phosphate groups (duplexes IIa) prevents stimulation by the simulated product. Since the synthetic products IIb-1 and IIb-2 retain the stimulatory function, it is probable that recognition by the enzyme requires the phosphate groups [cp. 14].

Other authors have shown that in the case of *NaeI* the reaction products do not act as stimulators [15]. We propose that this difference is based on the different structures of the reaction products. This interpretation is supported by the observation that the short (1 base) *BstNI* ends likewise are unable to stimulate

EcoRII. While *NaeI* generates blunt ends less likely to reassociate, *EcoRII* produces ends with 5 bases overhang competent to reassociate, thus enabling interaction of *EcoRII* with the reformed site. Even synthetic oligonucleotide duplexes IIb-1 (and IIb-2, resp.) can be ligated to form homopolymers although this necessitates a mismatched base pairing (our unpublished data).

Data obtained with modified oligonucleotides support the conclusion that the ability to recognize a molecule is crucial for its stimulator function. While non-site and Dcm methylated DNA are inactive, m⁶dA or dI-containing sites (oligonucleotide duplexes III and IV) are efficient stimulators, and N⁴meC produces an intermediate result. The stimulatory power depends on the number and position of modifications. However, there are modifications, like a pyrophosphate bond at the cleavage point, which abrogate stimulatory activity (Petrauskienė et al., in preparation) without decreasing binding. These substrates in fact have binding affinities which are much stronger than those of natural substrates [16]. Therefore, they become competitive inhibitors of the enzyme.

The data can be accommodated by a model in which the restriction endonuclease *EcoRII* simultaneously interacts with two recognition sites in the active complex. One of the sites functions as an allosteric activator and as such need not be cleaved. *EcoRII* is apparently able to recruit allosteric sites on the same or another DNA molecule than the site to be cut. For intermolecular stimulation the size of the DNA molecule is critical, so that at least one of the partners must be small (i.e. an oligonucleotide duplex). The distance between the sites limits intramolecular cooperation.

ACKNOWLEDGEMENTS

We thank Dr. Cornelia Schroeder for critical discussions and her help in preparing the manuscript. We are greatly indebted to Prof. Z.A. Shabarova and Dr. E.S. Gromova (Moscow State University) for the gift of oligonucleotide duplexes III–V.

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