Volume 245, number 1,2, 141-144 FEB 06919 March 1989

Oligonucleotide duplexes containing CC(A/T)GG stimulate cleavage of refractory DNA by restriction endonuclease *Eco*RII

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Received 21 December 1988; revised version received 20 January 1989

Some DNA species are resistant towards the restriction endonuclease *Eco*RII despite the presence of unmodified recognition sites. We show that 14 base-pair oligonucleotide duplexes containing the *Eco*RII recognition site 5'-CC(A/T)GG are cleaved by this enzyme and are able to stimulate *Eco*RII cleavage of such resistant DNA molecules (e.g. DNA of bacterial virus T3). A direct correlation between the concentration of oligonucleotide duplex molecules and the degree of *Eco*RII digestion of the primarily resistant DNA is observed. This indicates a stoichiometric rather than a catalytic mode of enzyme activation. An excess of DNA devoid of *Eco*RII sites ('non-site' DNA, e.g. *Mva*I-digested T7 DNA) does not interfere with the activity of *Eco*RII.

Restriction endonuclease *Eco*RII; Enzyme activation; Oligonucleotide duplex; DNA restriction; Bacterial virus T3

1. INTRODUCTION

A number of DNA species is partially or totally resistant to certain restriction endonucleases, despite the presence of cognate recognition sequences and the demonstrated absence of impurities (contaminating proteins). Besides protection by specific DNA methylation or unusual bases in the recognition sequences, adjacent nucleotide sequences have been described to influence the susceptibility of individual sites to restriction [1-4].

A new mechanism of resistance was discovered for the restriction endonuclease *Eco*RII which is unable to cleave the DNAs of *E. coli* viruses T3 and T7. C-5 methylation of the internal cytosine by M.*Eco*RII or the *dcm* methylase protects the *Eco*RII recognition sequence 5'-CC(A/T)GG agsinst cleavage [4]; however, the 40 000 bp T3 and T7 DNAs which contain 3 and 1 site, respectively, are not methylated, yet are resistant to *Eco*RII [5].

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Dedicated to Professor H.A. Rosenthal on the occasion of his 65th birthday

The isoschizomeric enzymes *Bst*NI [5,6] and *Mva*I (this paper) cleave these DNAs normally.

The cause for the refractory behavior of T3 and T7 DNA is seen in a unique catalytic mechanism of *Eco*RII which requires at least two recognition sites for its activation [6]. Co-incubation of primarily resistant T3 or T7 DNA with *Eco*RII-sensitive phage or plasmid DNA makes the former susceptible to cleavage [6]. Following its activation on susceptible DNA, *Eco*RII can obviously 'turn over' to attack resistant DNA species.

We now show that even short 14 bp oligonucleotide duplexes containing only a single *Eco*RII recognition site stimulate the cleavage of T3 and T7 DNA. In contrast to longer DNA molecules with only one site [6] these short duplexes are subject to *Eco*RII digestion. This is apparently the precondition for their ability to activate *Eco*RII.

2. MATERIALS AND METHODS

2.1. *DNAs*

T3 and T7 DNAs were prepared from CsCl-purified phage stocks of the Berlin strains by phenol extraction and ethanol precipitation. Plasmid pBR322 (Dcm$^-$) DNA was extracted

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

from *E. coli* B/Berlin host cells and purified on ethidium bromide/CsCl gradients according to Maniatis et al. [7]. A 1 kb ladder of DNA size markers was obtained from Bethesda Research Laboratories.

2.2. *Oligodeoxynucleotides*

(Ia) 5′-GCCAA*CCTGGC*TCT-3′ and (Ib) 3′-CGGTT-*GGACC*GAGA-5′ which form duplex I (*Eco*RII sequence in italics) as well as (IIa) 5′-GATGCTGCCAA*CCGGC*TCTAGC-TTCATAC-3′ and (IIb) 3′-CTACGACGGTT*GGCC*GAGAT-CGAAGTATG-5′ (forming duplex II with truncated recognition sequence) were synthesized by the phosphoroamidite method on a DNA synthesizer (Applied Biosystems 401A) and purified by polyacrylamide gel electrophoresis.

2.3. *Enzymes*

*Eco*RII was obtained from Bethesda Research Laboratories; *Mva*I was a kind gift from E.S. Gromova (Moscow State University).

2.4. *DNA digestion and gel electrophoresis*

DNA digestions were run under conditions recommended by the suppliers or in TA buffer [8]. The fragments were separated on 0.7% agarose gels and visualized by ethidium bromide staining. Per slot 180 ng T3 DNA or 200 ng pBR322 Dcm^- DNA were digested with 3 U enzyme.

3. RESULTS AND DISCUSSION

Fig.1. shows that T3 DNA which is originally refractory to *Eco*RII (cf. lanes 5 and 6) becomes susceptible after addition of the 14 bp oligonucleotide duplex I (lane 3), exhibiting the same cleavage pattern as under conditions of *Eco*RII stimulation by pBR322 Dcm^- DNA (lane 2) or following *Mva*I digestion (lane 4). Oligoduplex I itself is cleaved by *Eco*RII [9], in contrast to single-stranded oligonucleotides like Ia and Ib, or to oligoduplex II lacking the central A/T pair of the *Eco*RII recognition site, all three of which are also unable to activate *Eco*RII (not shown). Equivalent results were obtained with T7 DNA instead of T3 DNA as the target.

The difference between the effect of oligoduplex I and II corresponds to that between pBR322 Dcm^- (*Eco*RII-sensitive) and pBR322 Dcm^+ (*Eco*RII-resistant due to methylation) [6]. Only DNA which itself is cleaved is able to stimulate the digestion of originally resistant DNA.

The stimulation of *Eco*RII cleavage of T3 and T7 DNA correlates with the amount of oligoduplex I present in the incubation. We determined the minimal duplex concentration supporting the digestion of T3 DNA (fig.2). A molecular ratio of

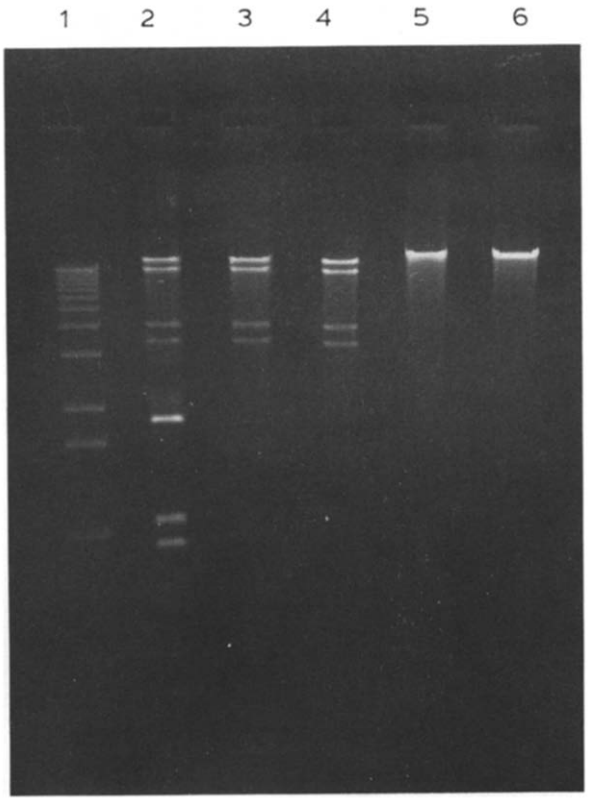

Fig.1. Stimulation of *Eco*RII-cleavage of T3 DNA by oligonucleotide duplex I. Lanes: 1, 1 kb size marker; 2, T3 DNA + pBR322 Dcm^- DNA + *Eco*RII; 3, T3 DNA + oligoduplex I (200 ng) + *Eco*RII; 4, T3 DNA + *Mva*I; 5, T3 DNA + *Eco*RII; 6, T3 DNA.

T3 DNA to oligoduplex I of 1:420 allows the complete digestion of the former. This is equivalent to a ratio of recognition sites of 1:140 and a mass ratio of 1:0.14. On the other hand, complete cleavage of T3 DNA in the presence of pBR322 (Dcm^-) DNA was achieved at a molecular ratio of 1:1, a site ratio of 1:2 and a mass ratio of 1:0.13 [6].

The clear difference in optimal stimulating concentration for plasmid vs oligoduplex probably relates to their size difference. The larger size of the plasmid not only increases the probability of collision between enzyme and DNA but also the probability of finding a second recognition site. In addition, the stability (thermodynamic and kinetic) of the enzyme-DNA complex may depend on the size of the DNA.

Irrespective of their size, the correlation between

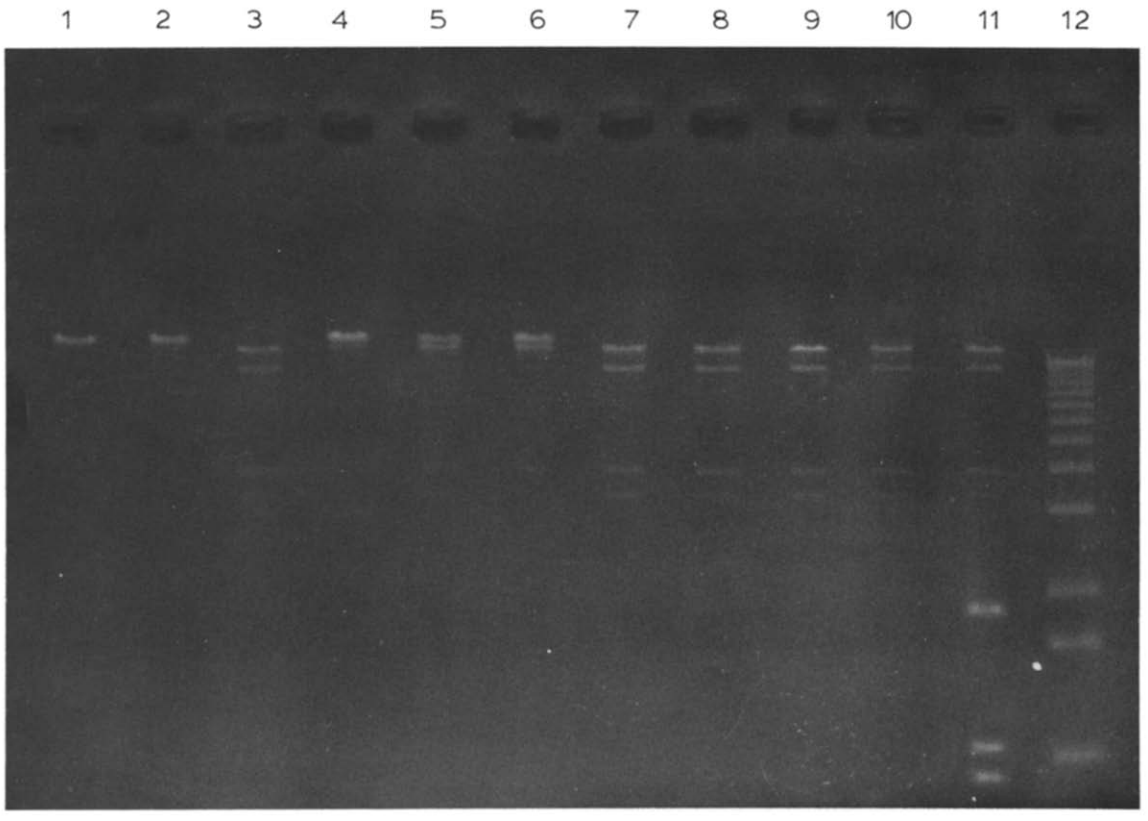

Fig.2. Titration of the stimulation of *Eco*RII activity by oligonucleotide duplex I. Lanes: 1, T3 DNA; 2, T3 DNA + *Eco*RII; 3, T3 DNA + *Mva*I; 4–10, T3 DNA + different amounts of oligonucleotide duplex I (4, 5.6 ng; 5, 11.2 ng; 6, 16.8 ng; 7, 22.4 ng; 8, 28.0 ng; 9, 33.6 ng; 10, 44.8 ng) + *Eco*RII; 11, T3 DNA + pBR322 Dcm$^-$ DNA + *Eco*RII; 12, 1 kb size marker.

the concentration of susceptible DNA molecules and the degree of stimulation indicates a stoichiometric rather than a catalytic mode of activation.

Krüger et al. [6] showed that *Eco*RII requires at least two sites in the same DNA molecule for its activation. To explain the resistance of the 40 kbp T3 DNA which contains 3 *Eco*RII sites, the authors discussed 3 possibilities: (i) the relation of 'site' to 'non-site' DNA in the genome may be unfavourable because non-site DNA could compete with specific recognition sites for enzyme binding; (ii) the distance between any two sites may not be optimal for their simultaneous interaction with the enzyme; (iii) the orientation of the central, non-symmetric A/T pair could be important.

The results presented in fig.3 make unspecific binding of non-site DNA to *Eco*RII seem unlikely. We investigated this possibility by co-incubating 200 ng pBR322 Dcm$^-$ DNA with a total *Mva*I digest of T7 DNA as the non-site DNA, using the minimal amount of *Eco*RII sufficient for complete digestion of the plasmid. An up to seven-fold molar excess of *Mva*I digest does not inhibit the cleavage of the susceptible pBR322 DNA. The amount of nonspecific DNA per *Eco*RII site is larger here (47 000 bp) than in the refractory T7 and T3 DNAs (40 000 and 13 000 bp, resp.).

Increasing the incubation time or the enzyme concentration did not influence the susceptibility of T3 and T7 DNA to *Eco*RII. Both would be expected to promote cleavage if the refractivity was caused by a shift in the binding equilibrium to non-site DNA. Possibility (i), therefore, appears to be unlikely.

It is already known that enzymes promoting site-specific recombination require the coordinated presence of 2 DNA sequences. By interaction with the protein, the 2 DNA sites can 'sense' each others

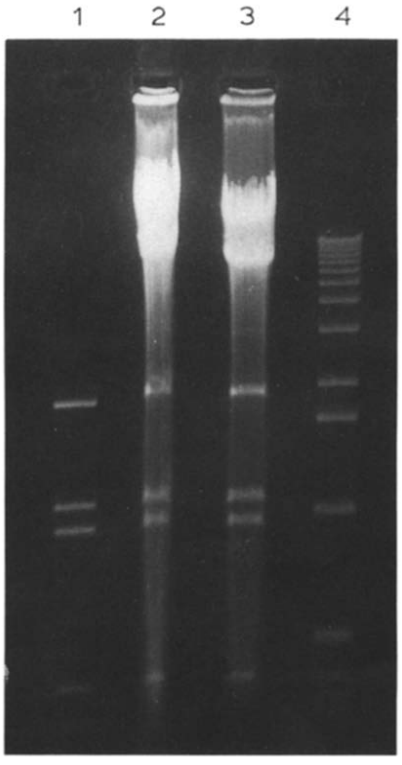

Fig.3. Attempt to inhibit *Eco*RII activity by adding non-site DNA. Lanes: 1, pBR322 Dcm⁻ DNA + *Eco*RII; 2, pBR322 Dcm⁻ DNA + 1.83 μg T7 DNA (*Mva*I digested) + *Eco*RII; 3, pBR322 Dcm⁻ DNA + 3.66 μg T7 DNA (*Mva*I digested) + *Eco*RII; 4, 1 kb size marker.

presence and, in some cases, even their mutual orientation [10,11]. The activation of *Eco*RII endonuclease also requires 2 regognition sites on the same DNA molecule [6]. An activation by simultaneous interaction of the enzyme with different DNA molecules had not yet been observed. In this paper we show that short oligonucleotide duplexes containing only one site can perform this function. Apparently, the *Eco*RII enzyme which consists of 2 subunits [12] is able to bind two oligoduplexes simultaneously as if they were in fact neighbouring sites on the same DNA molecule.

It would be interesting to see whether other restriction endonucleases which cleave certain DNA species incompletely have similar requirements for their substrate DNAs as *Eco*RII and are also activated by suitable oligonucleotide duplexes.

Acknowledgements: We thank Dr C. Schroeder for help in preparing the manuscript, S. Hansen for isolating the phage DNAs, Dr F. Schubert for synthesizing the oligonucleotides and Dr E.S. Gromova for the gift of *Mva*I. The presented data are the subject of a Patent Application.

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