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*Eco*RII can be activated to cleave refractory DNA recognition sites

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ABSTRACT

EcoRII restriction sites [5'-CC(A/T)GG] in phage T3 and T7 DNA are refractory to cleavage by EcoRII, but become sensitive to cleavage in the presence of DNAs which contain an abundance of EcoRII sensitive sites (e.g. pBR322 or λ DNA). Studies using fragments of pBR322 containing different numbers of EcoRII sites show that the susceptibility to EcoRII cleavage is proportional to the number of sites in the individual fragment.

We postulate that EcoRII is the prototype of restriction endonucleases which require at least 2 simultaneously bound substrate sites for their activation. EcoRII sites are refractory when they occur at relatively low frequency in the DNA. The restriction enzyme can be activated by DNA with a higher frequency of sites.

INTRODUCTION

Certain recognition sites on DNA molecules are refractory to cleavage by the cognate restriction endonucleases. Resistance can be caused by specific DNA methylation or by other less common base modifications (1,2). The effects of individual base substitutions on site recognition (3,4) and the influence of neighbouring bases on the susceptibility of sites (5,6) has been demonstrated.

The "classical" example of restriction endonuclease "site preference" was given by Thomas and Davis (7). They showed that the 5 EcoRI sites of lambda DNA clearly differ in the efficiency with which they are cleaved by EcoRI. Several similar examples of variation or low susceptibility exist, but the molecular causes are largely unknown in these cases.

The DNAs of bacteriophages T3 and T7 are resistant to EcoRII restriction (8). The restriction endonuclease EcoRII interacts with the DNA sequence 5'-CC(A/T)GG-3'; methylation of the central cytosine by the methylases M.EcoRII or M.dcm (the endogenous cytosine methylase of many *Escherichia coli* strains) protects the sequence against EcoRII hydrolysis (9). A number of isoschizomers of EcoRII are not affected by the presence of 5-methylcytosine at the internal cytosine, e.g. BstNI (2,10). We found that the 3 EcoRII sites of T3

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DNA and the unique site of T7 DNA are cleaved normally by BstNI but not by EcoRII (8). Neither 5-methylcytosine, 4-methylcytosine, nor 6-methylaminopurine are present, so specific methylation can be excluded as the cause for the refractivity to EcoRII.

In this paper we show that co-incubation of the initially resistant DNA with heterologous, susceptible DNA activates the cleavage of T3 and T7 DNA by EcoRII. Vovis et al. (11) noted that complete digestion of phage fl RF DNA (2 sites per 6407 bp) by EcoRII could not be obtained; and Hattman et al. (12) found that phage ØX174 RF DNA (2 sites per 5386 bp) could be completely digested only in the presence of unmodified heterologous DNA. Neither group investigated the phenomenon further. Our results suggest that the resistance of these EcoRII sites is related to their low frequency of occurrence in the phage DNAs. We propose that the restriction endonuclease EcoRII requires at least two bound DNA recognition sites for activation, and that recognition sites are in direct competition with non-site DNA for binding. Non-site DNA, when present in relative excess, can occupy one or both of the active sites of the endonuclease and inhibit cleavage.

MATERIALS AND METHODS

DNAs

T3 and T7 DNAs were prepared from CsCl purified preparations of the Berlin strains of phage as well as from the laboratory strain of F.W. Studier (T7/S) by phenol extraction and ethanol precipitation. Plasmid pBR322 Dcm^+ DNA was extracted from E. coli K-12 host cells and purified on ethidium bromide-CsCl gradients according to Maniatis et al. (13). Plasmid pBR322 Dcm^- DNA (prepared from the E. coli GM48 dam dcm strain of M.G. Marinus) was kindly provided by K. Peden. Phage λ Dcm^+ DNA was from N.E. BioLabs and λ Dcm^- DNA was from Pharmacia. A 1 kilobase ladder of DNA size markers was obtained from Bethesda Research Laboratories.

Enzymes

EcoRII endonuclease was obtained from Bethesda Research Laboratories. BstNI, RsaI, and T4 DNA polymerase were from N.E. Biolabs. DNA digestions were run under conditions recommended by the suppliers or in TA buffer (14).

Radioactive labeling of DNA

RsaI fragments of pBR322 Dcm^- DNA were obtained and 3'-end labeled as follows. The digestion reaction mixture (50μl) contained 1μg of pBR322 DNA in TA buffer (33mM Tris-acetate, pH7.6, 66mM potassium acetate, 10mM magnesium acetate, 1mM dithiothreitol, 50μg/ml bovine serum albumin) and 5

units of RsaI. After 60 min at 37°C, one unit of T4 DNA polymerase was added, and incubation was continued at 37°C for 10 min to produce 3'- terminal gaps. These were then filled in by addition of 5µl of [α^{32}P]dCTP (1mCi/ml, 3000Ci/mmole, NEN), and 2µl of a mixture of dATP, dTTP, and dGTP (5mM each). After 15 min at 37°C the DNA was recovered by phenol extraction, chloroform extraction, ethanol precipitation, and redissolving in 100µl of TA buffer.

Agarose gel electrophoresis of DNA

DNA fragments were separated by 0.7% or 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining (15). To visualize radioactive DNA, gels were dried onto Whatman DE-81 paper and autoradiographed using X-Omat XAR-5 film (Kodak). Individual pBR322 Dcm$^-$ RsaI DNA fragments were purified by agarose gel electrophoresis onto SERVACEL-DEAE ion exchange paper (Serva, Heidelberg) as described (16).

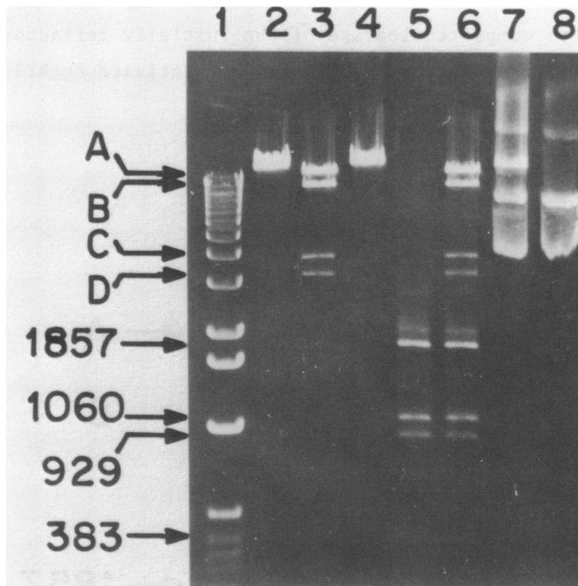

Figure 1. EcoRII cleavage of T3 DNA (40 Kbp) in the presence of pBR322 Dcm$^-$ DNA. From left to right are shown: 1 kb ladder (lane 1), T3 DNA untreated (lane 2), T3 DNA treated with BstNI (lane 3) or EcoRII (lane 4), pBR322 Dcm$^-$ DNA treated with EcoRII (lane 5), mixture of T3 DNA and pBR322 Dcm$^-$ DNA treated with EcoRII (lane 6), mixture of T3 DNA and pBR322 Dcm$^+$ DNA treated with EcoRII (lane 7), pBR322 Dcm$^+$ DNA treated with EcoRII (lane 8). T3 DNA cleavage products are labeled A,B,C,D. pBR322 DNA cleavage products are given in basepairs. 3-5 units of restriction enzyme per assay (400-500 ng DNA) were used. The fragments were separated in a 0.7% agarose gel.

RESULTS

The results presented in Fig. 1 and Fig. 2 show that the 3 EcoRII sites in T3 DNA and the unique EcoRII site in T7 DNA are cleaved by the isoschizomeric BstNI (lane 3) but not by EcoRII (lane 4). We investigated different means to overcome the refractory behavior of these recognition sites. First, we tried adding spermine or spermidine to the incubation buffer, since polyamines have been shown to stimulate DNA cleavage by EcoRI, PstI and BamHI (17). This failed. An increase of enzyme concentration up to 25 units per μg phage DNA also had no effect (data not shown).

However, on co-incubation of T3 or T7 DNA with heterologous, susceptible DNA (pBR322 Dcm^-) complete cleavage of the EcoRII sites was achieved. pBR322 possesses 6 EcoRII/BstNI sites (18,19). Only pBR322 Dcm^- DNA (prepared from dcm host cells) was sensitive to EcoRII while pBR322 DNA from dcm^+ cells was protected against EcoRII endonucleolysis (lanes 5 and 8 of Figs. 1 and 2). While co-incubation of susceptible pBR322 Dcm^- DNA with T3 or T7 DNA leads to complete cleavage of the initially refractory DNA, pBR322 Dcm^+ DNA (which is resistant itself) did not activate EcoRII cleavage of T3 or T7 DNA (lanes 6 and 7 of Figs. 1 and 2).

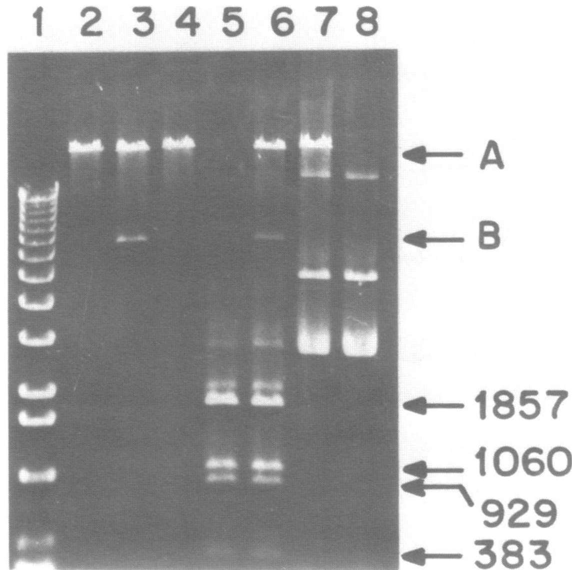

Figure 2. EcoRII cleavage of T7 DNA (40 Kbp) in the presence of pBR322 Dcm^- DNA. See legend to Fig. 1 for explanations. T7 DNA was used instead of T3 DNA. T7 DNA cleavage products are labeled A and B.

We determined the concentration at which pBR322 Dcm$^-$ DNA activated the cleavage of phage DNA. These experiments were done with T3 DNA as the target since this DNA contains more EcoRII sites than T7 DNA and its cleavage pattern was more easily followed in agarose gels. Fig. 3 shows the result of titrating pBR322 Dcm$^-$ DNA. In the presence of an excess of pBR322 molecules over T3 DNA molecules (and therefore, an excess of pBR-located sites over T3-located sites) there was full activation of T3 DNA restriction. Complete EcoRII cleavage of T3 DNA occurred down to a ratio of about 2 pBR322 sites to 1 T3 DNA site. Then, as the ratio was lowered further, the cleavage of T3 DNA declined. Table 1 presents a summary of the results shown in Fig. 3.

Cleavage of T3 or T7 DNA by EcoRII could also be activated by other susceptible DNA species. For example, the presence of λ Dcm$^-$ (71 EcoRII sites) as well as λ Dcm$^+$ DNA enabled complete cleavage of T3 DNA. λ DNA isolated from *dcm*$^+$ host cells is only partially resistant to EcoRII since the capacity of the *dcm*$^+$ methylase is exhausted during phage replication and Dcm$^+$ methylation is, therefore, incomplete (20,21). The number of susceptible sites in λ Dcm$^+$ DNA was sufficient to promote complete cleavage of T3 DNA (data not shown). Interestingly, T3 DNA was not self-activating at higher concentrations. An increase in T3 DNA concentration up to 5μg in a 20μl reaction volume did not improve EcoRII cleavage.

Digestion of pBR322 Dcm$^-$ DNA with RsaI yields 3 DNA fragments A, B, and C which carry 2, 3, or 1 EcoRII sites each (see Fig. 4). This fragment mixture was cleaved normally by EcoRII and supported T3 DNA endonucleolysis with the same efficiency as uncleaved pBR322.

We next examined whether the susceptibility of pBR322 DNA itself depends on the number of sites per fragment or on the activation by sites in another

Table 1
Titration of activating pBR322 Dcm$^-$ DNA molecules

Slot no. in Fig. 3	DNA amounts (ng) T3	DNA amounts (ng) pBR	Relative No. of molecules T3	Relative No. of molecules pBR	Relative No. of EcoRII sites T3	Relative No. of EcoRII sites pBR	Activation of T3 DNA cleavage
2	400	500	1	11.25	1	22.5	++++
3	400	100	1	2.25	1	4.5	++++
4	400	50	1	1.13	1	2.3	++++
5	400	10	1	0.23	1	0.5	++
6	400	5	1	0.11	1	0.2	+

The DNA length is 40 kbp for T3 and 4.36 kbp for pBR322, the number of EcoRII sites per molecule is 3 for T3 (ref. 8) and 6 for pBR322 (ref. 18). Total cleavage, ++++, partial cleavage, ++, bulk of T3 DNA molecules uncleaved, +.

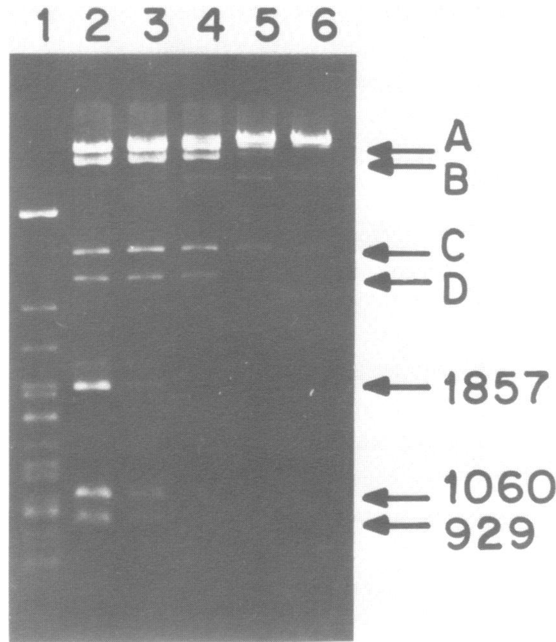

Figure 3. Titration of stimulating pBR322 Dcm⁻ DNA. Decreasing amounts of pBR322 Dcm⁻ DNA were mixed with 400ng T3 DNA and treated with 3 units EcoRII in a final volume of 20µl. After electrophoresis through a 0.7% agarose gel the following restriction analyses are shown: λ DNA treated with BstNI (molecular mass standard, lane 1), T3 DNA treated with EcoRII in the presence of 500ng (lane 2), 100ng (lane 3), 50ng (lane 4), 10ng (lane 5), or 5ng pBR322 Dcm⁻ DNA (lane 6). T3 DNA cleavage products are labeled A,B,C,D. pBR322 DNA cleavage products are given in basepairs.

fragment. Following RsaI cleavage of pBR322 Dcm⁻ DNA, the fragments were labeled at their 3' termini with [^{32}P] to enable autoradiographic visualization of very small amounts of DNA. The labeled fragments were employed in two kinds of experiments: (i) time-dependent EcoRII cleavage of the pBR322 Dcm⁻ DNA fragments and (ii) prevention of interaction between the fragments by step-wise dilution, followed by addition of a constant amount of EcoRII.

Fig. 5 shows the reaction kinetics of EcoRII on a mixture of the three RsaI fragments of pBR322. Under the specified experimental conditions, fragment A (2 sites) and B (3 sites) are almost completely cleaved after 10 min (lane 5) while fragment C (1 site) is cleaved no more than 50%. EcoRII has an obvious preference for A and B. The digestion of fragment C in this experiment is only explained via activation by the other two fragments which con-

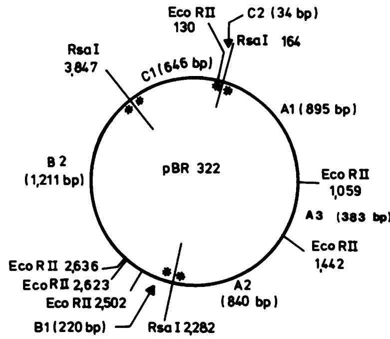

Figure 4. Location of RsaI and EcoRII sites in the pBR322 DNA derived from a computer search of the published 4,363 bp sequence (18,19). Position of terminal 3'-radioactive labeling of RsaI fragments are indicated by asterisks. The nucleotide position and orientation of EcoRII sites are as follows: 130 (CCTGG); 1,059 (CCTGG); 2,502 (CCAGG); 2,623 (CCAGG); 2,636 (CCTGG).

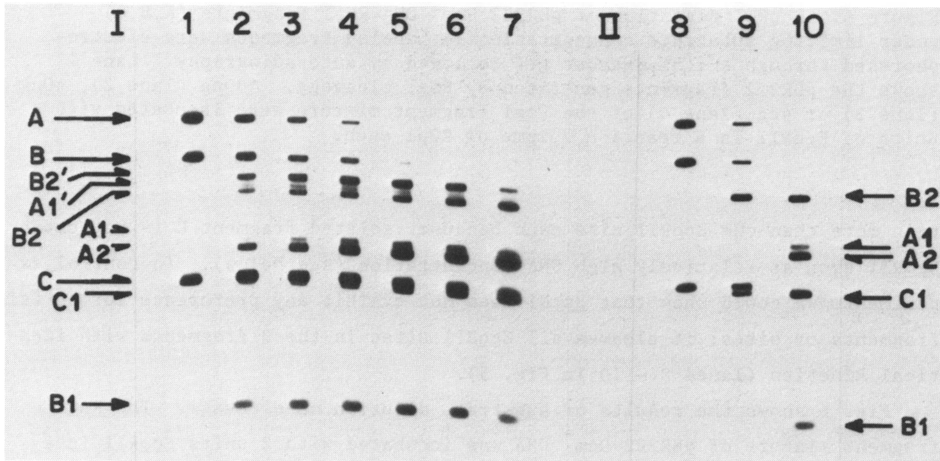

Figure 5. Panel I shows the kinetics of EcoRII digestion of pBR322 Dcm⁻ DNA RsaI fragments. Radiolabeled fragments were electrophoresed through a 1.5% agarose gel and then subjected to autoradiography. 1.5 µg DNA were treated with 6 units EcoRII in a total volume of 100µl. Samples of 10µl (150ng DNA) were withdrawn at time 0 (without enzyme, lane 1) or 1 min (lane 2), 2 min (lane 3), 5 min (lane 4), 10 min (lane 5), 20 min (lane 6) and 60 min (lane 7) after EcoRII addition and the reaction was stopped by heat inactivation in the presence of 10mM EDTA. Panel II shows the analogous experiment using BstNI (1.5µg DNA, 15 units BstNI in 100µl reaction volume). 10 µl samples were taken at times 0 (lane 8), 2 min (lane 9) and 5 min (lane 10). pBR322 RsaI fragments are labeled A,B,C. EcoRII or BstNI cleavage products of the RsaI fragments are labeled A1,A1',A2,B2,B2',C1. Fragment sizes are given in Fig. 4. Note that EcoRII cleavage yielded specific partial digestion products A1' and B2' which migrated immediately above fragment B2.

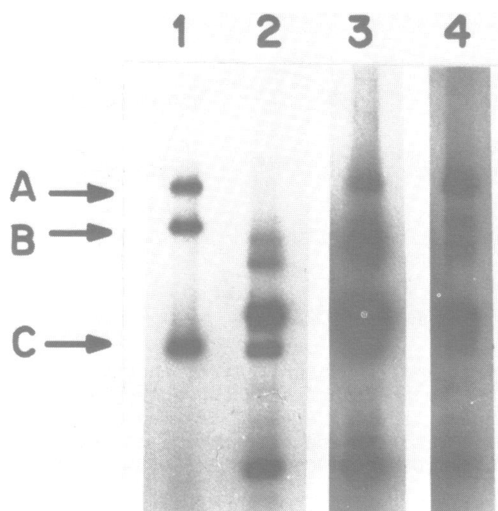

Figure 6. EcoRII digestion of pBR322 Dcm⁻ DNA RsaI fragments (A,B,C) under limiting substrate concentrations. Labeled fragments were electrophoresed through a 1.5% agarose gel followed by autoradiography. Lane 1 shows the pBR322 fragments generated by RsaI cleavage. 400ng (lane 2), 40ng (lane 3) or 4ng (lane 4) of the RsaI fragment mixture were incubated with 2 units of EcoRII in a reaction volume of 20µl each.

tain more than one EcoRII site each because isolated fragment C is not cut by EcoRII even at relatively high DNA concentration (see below). In control experiments we could show that BstNI does not exhibit any preference for certain fragments or sites: it cleaves all EcoRII sites in the 3 fragments with identical kinetics (lanes 8 - 10 in Fig. 5).

Fig. 6 shows the results of substrate dilution on cleavage. The RsaI fragment mixture of pBR322 Dcm^- DNA was incubated with 2 units EcoRII in a reaction volume of 20µl. Under these conditions essentially complete cleavage of all 3 fragments was observed. When the DNA was serially diluted and EcoRII held constant at 2 units, cleavage of fragments A, B, and C was roughly proportional to the number of EcoRII sites contained.

In order to prevent any interaction between the 3 RsaI fragments of pBR322, we isolated them and treated them separately with EcoRII. Fig. 7 shows that the isolated fragments A and B are cleaved by EcoRII with a preference proportional to the number of sites each contains, while the isolated C fragment is highly resistant to cleavage.

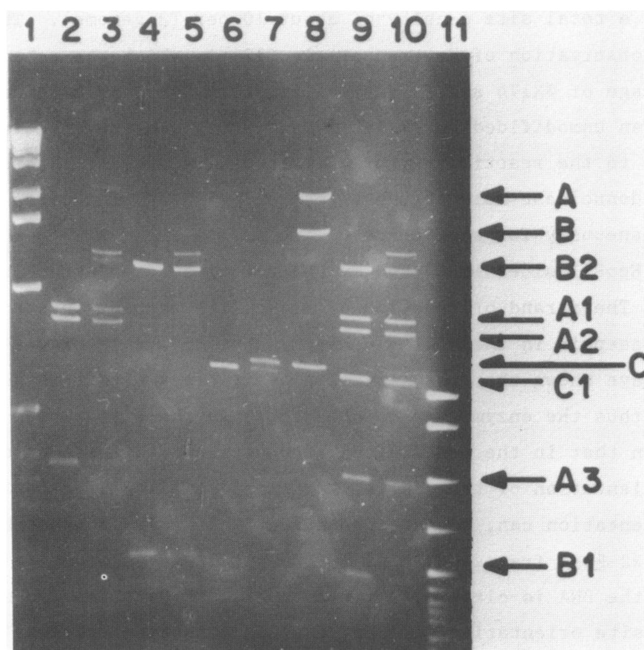

Figure 7. EcoRII cleavage of the isolated RsaI fragments of pBR322 Dcm⁻ DNA. 400ng of each fragment DNA was used per restriction assay, from left to right: 1 kb ladder (lane 1), RsaI A fragment treated with BstNI (lane 2) or EcoRII (lane 3), RsaI B fragment treated with BstNI (lane 4) or EcoRII (lane 5), RsaI C fragment treated with BstNI (lane 6) or EcoRII (lane 7), mixture of RsaI fragments A, B, and C untreated (lane 8), mixture of RsaI fragments A, B and C treated with BstNI (lane 9) or EcoRII (lane 10), pBR322 treated with HpaII (DNA molecular mass standard, lane 11). The DNAs were separated on a 1.5% agarose gel. DNA fragments are labeled as in Fig. 5.

DISCUSSION

Refractory DNA recognition sites can, in some cases, be made susceptible to cleavage by addition of heterologous DNA containing an abundance of susceptible sites. While EcoRII is unable to cut its 3 recognition sites in T3 DNA and its unique site in T7 DNA, co-incubation of these DNAs with heterologous susceptible DNA (pBR322 Dcm⁻, λ Dcm⁻) elicits complete EcoRII digestion of the phage DNAs. Apparently the restriction endonuclease can be activated to cleave initially resistant sites by adding another DNA species. Enough heterologous susceptible DNA must be added to the reaction mixture to bring the ratio of site to non-site DNA to its critical cleavage value. The data in Table 1 show that in order to achieve complete cleavage of phage T3 DNA by EcoRII, the minimum relative number of pBR322-contributed sites per T3 site

was 2.3 (for a total site density of about 10 per T3 genome). These data explain the observation of Hattman et al. (12) in which the enhancement of EcoRII cleavage of ØX174 and f1 replicative form DNAs was obtained in the presence of an unmodifided "stimulator DNA" which was contributing susceptible EcoRII sites to the reaction mixture. Our results suggest that an EcoRII restriction endonuclease molecule must interact with two (or more) recognition sites simultaneously for activation.

Why are EcoRII sites in T3 and T7 DNA not cleaved under normal reaction conditions? The strand orientation of sites with respect to the central asymmetric A/T basepair in the DNA molecule might conceivably play a role. Yolov et al. (4) have shown that the central AT pair plays a role in sequence recognition, and thus the enzyme may be able to sense the orientation of the site. This may mean that in the coordinated recognition of 2 sites on one DNA molecule the orientation of the central A/T pairs could be important. The importance of orientation can, however, be effectively ruled out because in the case of pBR322 RsaI fragments A and B, the sites are in opposite orientation (18,19) and the DNA is cleaved, while for ØX174 RF DNA, the two sites are also in opposite orientation (22) but the DNA cannot be completely cleaved.

Two other factors that we believe to be important are the ratio of "site" to "non-site" DNA and the distance between sites. Regarding the first factor, if EcoRII requires two bound substrates for activation, then the probability of this event will decrease as the ratio of competing non-site DNA increases. In effect the non-site DNA can act as a classical competitive inhibitor, and its effectiveness depends on how well it binds relative to site DNA. In support of this, Vinogradova et al. (23) found that non-site DNA polymers bind to EcoRII only 100-fold less than site-containing DNA polymers.

Secondly, there may be an optimal distance between two sites to allow functional interaction. Two distant sites are essentially independent of each other, whereas two very closely spaced sites may be too constrained to bind simultaneously to the enzyme due to the inherent stiffness of DNA. A spacing somewhere in between these two extremes could allow optimal binding of both sites to the same enzyme molecule. T7/S has 2 identically orientated sites at positions of 2,366 and 8,188 (i.e., at a distance of about 5.8 kbp) of the 39,936 bp genome (24) and is also refractory to EcoRII cleavage (data not shown). T7/Berlin has only 1 EcoRII site (8). Only one of the 3 EcoRII sites in T3 DNA is in a sequenced region: it is located 3,965 bp from the right end of the genome (25), the cleavage of which generates the T3-C fragment. The minimal distance between two sites in the T3 DNA is represented by

the 3.3 kbp long T3-D fragment which is located at the left side of T3-C (Krüger, unpublished).

It is interesting that, during the evolution of T3 and T7 phage, CC(A/T)GG sites apparently were under strong selective pressure. On the basis of the frequencies of the subsequences CC(A/T)G and C(A/T)GG in T7 DNA, CC(A/T)GG is expected to occur 28 times in the genome but it actually occurs only once (T7/Berlin) or twice (T7/S) (26).

Another example of refractory sites is shown by certain class III restriction endonucleases recognizing non-symmetric sites. Here site frequency and polarity may play a role in the cleavage of DNA. Piekarowicz (27) showed that HinfIII (5'-CGAAT) prefers certain recognition sites over others in the target DNAs, moreover, the endonucleolytic activity strongly depends on a minimal number of recognition sites in the DNA. Cleavage is only observed when at least 3 HinfIII sites are present. In the case of EcoP15, there is some evidence that the absolute "strand bias" of the 36 EcoP15 sites (5'-CAGCAG) in the T7 DNA may be the reason for their resistance to EcoP15 cleavage (Schroeder et al. (26), and unpublished results).

We recently cloned a 217 bp fragment of T7 DNA containing the unique EcoRII site in a pUC18 vector (itself having 5 EcoRII sites in alternating A/T orientation; cp. ref. 28). In this environment, the T7 EcoRII site becomes completely sensitive to EcoRII cleavage (Krüger, unpublished). Thus, special properties of the sequences immediately surrounding the T7 EcoRII site cannot be crucial for its refractivity in situ. Still, the appearance of specific partial digestion products, B2' and A1' (Figs. 5 & 7) after EcoRII cleavage of the pBR322 RsaI fragments signifies different intrinsic sensitivities of the individual EcoRII sites.

EcoRII could be the prototype of a newly defined group of restriction endonucleases which require the coordinated presence of at least two recognition sites for activity. Refractory sites can be cleaved by co-incubation with susceptible DNA. In this way originally uncleavable DNA sites can be made accessible to genetic engineering.

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