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Analysis of the recognition mechanism involved in the EcoRV catalyzed cleavage of DNA using modified oligodeoxynucleotides

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ABSTRACT

We have prepared a series of undecadeoxynucleotides that contain changes in the functional group pattern present within the EcoRV recognition site - GATATC -. Oligonucleotides were synthesized on solid phase using normal and modified ß-cyanoethylphosphoramidites and analyzed in steady state cleavage experiments with the EcoRV restriction endonuclease. The following groups appear to interact strongly with the enzyme, since their modification or substitution renders the oligonucleotides refractory to cleavage: the exocyclic NH_2-groups of both A residues, the N7 of the first A residue, the exocyclic NH_2-group of the C residue and the CH_3-groups of both T residues. The exocyclic NH-group of the G residue supports effective recognition, since its absence lowers the k_{cat} of the cleavage reaction. The N7 of the second A residue and the C5 position of the C residue apparently are not recognized by EcoRV; their substitution by -CH- or modification with -Br or -CH_3, resp., does not considerably change the rate of cleavage. All oligonucleotides investigated compete with the unmodified substrate for binding to the enzyme. We conclude that EcoRV recognizes its substrate presumably through hydrogen bonds to the exocyclic NH_2-group and the N7 of the first A residue, the exocyclic NH_2-groups of the second A and the C residue, as well as through hydrophobic interactions with both T residues.

INTRODUCTION

The restriction endonuclease EcoRV cleaves double stranded DNA within the sequence - GATATC - (1,2) with high specificity. There is experimental evidence that related sequences are cleaved in the presence of dimethyl sulphoxide or at high pH (3). Under standard buffer conditions cleavage at non-canonical sites is also observed in the presence of high enzyme concentrations (3,4). For example, the site - GAAATC - in pBR322 is cleaved by EcoRV with a k_{cat} of 0.00038 min^{-1}, while the canonical site is cleaved with a k_{cat} of 3 min^{-1} (4). This high specificity must be due to several unique contacts that can only be formed in the specific enzyme substrate complex, not, however, or only in part in non-specific complexes. We address in this paper the question which structural determinants on the DNA are used by EcoRV to discriminate between specific and non-specific DNA. Our experimental approach consists in analyzing the effect of nucleotide substitutions in defined positions of the recognition sequence on the cleavage reaction. This approach has been widely used to probe the DNA determinants required for restriction enzyme recognition in a variety of different systems (5-64). Its success depends on the availability of a large number of suitably modified DNA substrates which carry the modification only in one position of the recognition sequence.

MATERIALS AND METHODS

EcoRV restriction endonuclease purification

The EcoRV restriction endonuclease was purified from an E.coli strain overproducing this protein (65,66). The isolation procedure we used is different from the one previously described (66). Briefly, 50g of frozen cell paste was thawed and suspended in 200 ml buffer A (30 mM K-phosphate pH 7.2, 1 mM 1.4-dithioerythritol (DTE)), containing 1 M NaCl. Cells were broken up by sonication in a MSE MK2 sonifier at 150 W for 30 min with intermittant cooling. Cell debris was removed by centrifugation at 30000 g for 30 min. The supernatant was adjusted to pH 7.2 with a concentrated NH_3 solution, dialyzed against buffer A and loaded onto a 2.5 x 25 cm AG MP-1 (BioRad) high performance liquid chromatography (HPLC) column (67) installed in a Bruker LC 21/31 liquid chromatograph. After rinsing the column with buffer A elution was carried out within 4 hours at a flow rate of 3 ml/min with a gradient from 0 to 2 M NaCl in buffer A. Fractions containing high EcoRV activity were pooled, dialyzed against buffer B (50 mM K-phosphate pH 7.2, 5 mM EDTA, 0.1 mM DTE), containing 0.05 M NaCl and loaded onto a 2 x 7 cm P-cellulose (Whatman) column equilibrated with the same buffer. After rinsing the column with this buffer batch elution was carried out with buffer A containing 0.3 M and 0.5 M NaCl, resp. Fractions obtained with the 0.5 M NaCl batch and which contained high EcoRV activity were pooled, dialyzed against buffer B, containing 5% (v/v) glycerol, and loaded onto a 2.5 x 30 cm hydroxyapatite column equilibrated with the same buffer. After rinsing the column with this buffer gradient elution was carried out with 2 x 2.5 l of 0.0 to 0.95 M K-phosphate in buffer B, containing 5% (v/v) glycerol. Fractions containing high EcoRV activity were pooled, dialyzed against buffer B, containing 0.3 M NaCl, 80% (v/v) glycerol and stored at -20°C. This purification scheme gives within 5 days approximately 20 mg electrophoretically homogeneous EcoRV.

Oligodeoxynucleotide synthesis

Oligodeoxynucleotides, if not otherwise stated, were synthesized on a Biosearch model 8600 DNA synthesizer using commercially available ß-cyanoethylphosphoramidites. Protected monomers were from Cruachem and Pharmacia, tetrazole from Cruachem, acetonitrile from Baker, all other chemicals from Merck or Fluka. The yield of the crude product obtained on a 1 µmole scale was about 80 A^{260}units. The 5'-dimethoxytrityl protected oligodeoxynucleotides were purified by preparative HPLC on a 2.5 x 25 cm Lichrosorb RP18 (Merck) column at a flow rate of 4 ml/min using a 90 min gradient from 15% to 50% acetonitrile in 0.1 M triethylammonium acetate (TEAA) pH 7.0. Purified oligodeoxynucleotides were lyophilized and deprotected in 80% (v/v) acetic acid, lyophilized again and extracted with diethyl ether. The purity of the oligodeoxynucleotides was checked by HPLC on a 0.46 x 25 cm C4 wide pore (Baker) column at flow rate of 1 ml/min using a 25 min gradient from 5% to 17.5% acetonitrile in 0.1 M TEAA pH 7.0 (68). The yield of the purified oligodeoxynucleotide was about 10 A^{260} units. The synthesis of the 7-deazadeoxyadenosine containing oligonucleotides followed the protocol given in (59) and will be described elsewhere.

Spectroscopic characterization of synthetic oligodeoxynucleotides

Melting curves of oligodeoxynucleotides were recorded in 1 cm cuvettes in a Zeiss DMR 10 spectrophotometer at a concentration of approx. 0.9 A^{260nm} in the same buffer as used for the cleavage experiments (vide infra). The temperature increase per time was 20°C/hour in all experiments. The circular dichroism (CD) spectra were recorded at ambient temperature in 0.1 cm cuvettes at a sensitivity of 1 x 10^{-6} absorbance units/mm in a Jobin-Yvon Dichrograph R.J. Mark III at a concentration of approx. 1 A^{260nm}.

Cleavage of synthetic oligodeoxynucleotides by the EcoRV restriction endonuclease

All cleavage experiments were performed at 11°C in 20 mM Tris·HCl pH 7.2, 10 mM $MgCl_2$, 50 mM NaCl. Cleavage experiments were carried out with 2 μM dimeric EcoRV and variable amounts of the oligodeoxynucleotides. Oligodeoxynucleotides which were refractory to cleavage under these conditions were analyzed for their ability to inhibit competitively the EcoRV catalyzed cleavage of oligodeoxynucleotides. For product analysis aliquots were withdrawn after defined time intervals and subjected to HPLC on a 0.46 x 25 cm C4-wide pore (Baker) column installed in a Merck Hitachi 655 A-12 liquid chromatograph with a Merck Hitachi 655 A-22 spectrophotometer set at 260 nm. Elution was carried out with a linear gradient from 5% to 15% acetonitrile in 0.1 M TEAA pH 7.0 in 20 min at a flow rate of 1 ml/min. Peaks in the chromatogram were integrated with the Merck Hitachi D 2000 chromato-integrator.

Nitrocellulose filter binding experiments

The complex formation between EcoRV and modified oligodeoxynucleotides which were refractory to cleavage by EcoRV was analyzed by nitrocellulose filter binding experiments. For this purpose oligodeoxynucleotides were radioactively labelled with γ-[^{32}P]-ATP (Amersham-Buchler) using T4 polynucleotide kinase (Boehringer-Mannheim). 3 to 5 μM double stranded oligodeoxynucleotides were mixed with varying amounts of EcoRV (0 to 50 μM dimer) in 20 mM Tris·HCl pH 7.2, 10 mM $MgCl_2$, 50 mM NaCl, 50 μM 1.4-dithioerythritol and 0.1 mg/ml bovine serum albumin at 11°C, 20 μl samples were pipetted onto prewetted nitrocellulose filters and filtered. After washing the filters with 1 ml 20 mM Tris·HCl, 10 mM $MgCl_2$, 50 mM NaCl, they were dried and immersed into a liquid scintillation cocktail. The filter bound radioactivity was determined in a scintillation counter. Binding isotherms were evaluated using a non-linear least-squares fitting procedure.

RESULTS AND DISCUSSION

We have used synthetic oligodeoxynucleotides containing modified bases (hypoxanthine, 6-methyladenine, 7-deazaadenine, uracil, 5-bromouracil, 5-methylcytosine and 5-bromocytosine) at unique positions within the EcoRV recognition sequence in order to study the role of the unmodified bases in the recognition process. It is an essential prerequisite for such studies that the modifications do not change the gross structural features of the modified oligodeoxynucleotide compared with the unmodified oligodeoxynucleotides. In order to verify that the modified

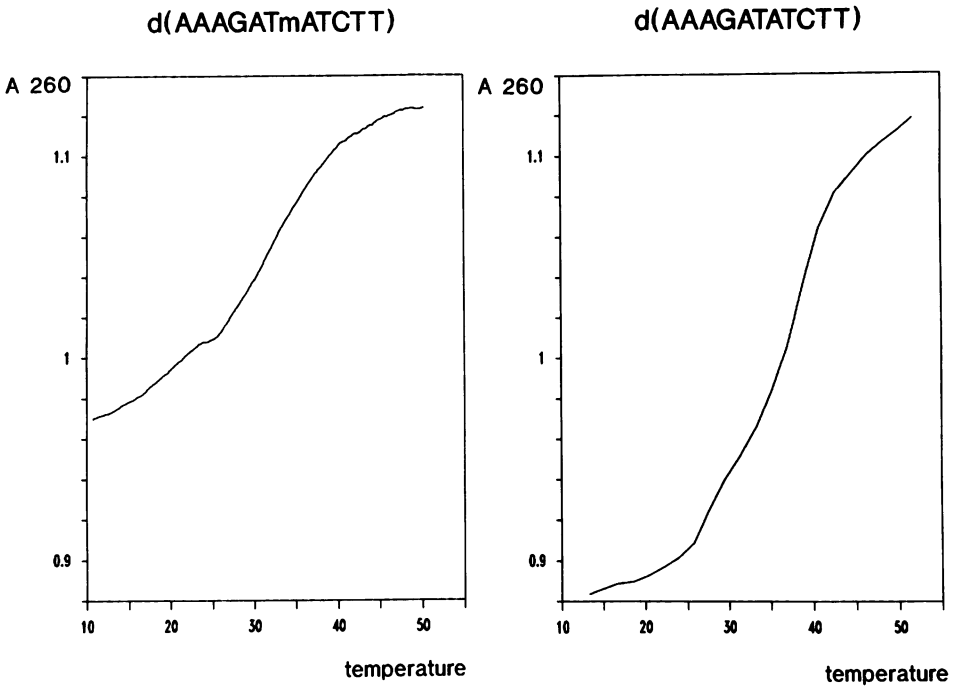

Fig. 1:*Melting curves of d(AAAGATATCTT) and d(AAAGATmeATCTT).*
Melting curves were recorded in cleavage buffer at a concentration of approx. 0.9 A^{260nm}. It should be noted that the hyperchromicity for the melting of d(AAAGATATCTT) is larger than that for d(AAAGATmeATCTT). The apparent T_M for the methylated oligodeoxynucleotide is 31°C and for the unmodified oligodeoxynucleotide 36°C.

oligodeoxynucleotides are similar in structure as the unmodified one we have carried out two kinds of experiments. Melting curves were recorded for all oligodeoxynucleotides in order to demonstrate that under the conditions of the cleavage experiments the modified oligodeoxynucleotides are double stranded. Two representative melting curves are shown in fig. 1. In line with published data the results of these experiments (not shown) demonstrate that deoxyinosine (46,60), 6-methyldeoxyadenosine (46,71), 7-deazadeoxyadenosine (38,59), and deoxyuridine (46,73) slightly destabilize, while 5-bromodeoxyuridine (46,62,72), 5-methyldeoxycytidine (46,69,70) and 5-bromodeoxycytidine (46) slightly stabilize the duplex structure. We have also recorded the CD spectra of all oligodeoxynucleotides similarly as described recently (38,60-62) in order to show that the duplex structure has the characteristic features of a regular B-DNA. Within the limits of error all oligodeoxynucleotides show very similar CD spectra, representative examples are shown in fig. 2. These results are in agreement with more detailed NMR (71,73,74) and X-ray crystallography studies (75) which demonstrate that the N-6 methylation of deoxyadenosine (71), the substitution of thymidine by deoxyuridine (73) or 5-bromodeoxyuri-

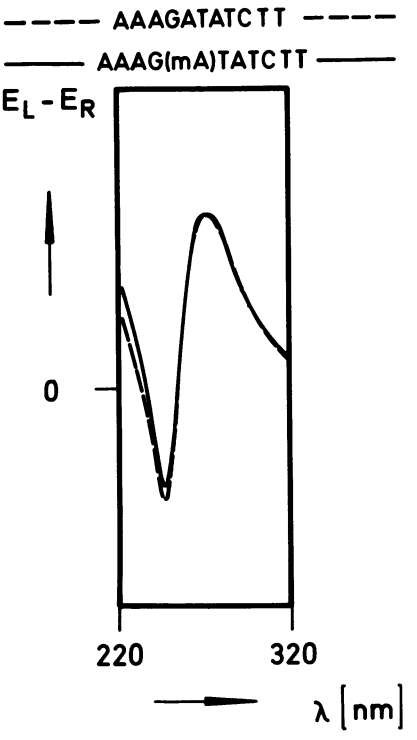

Fig. 2:*Circular dichroism spectra of d(AAAGATATCTT) and d(AAAGmeATATCTT).* CD spectra were recorded in cleavage buffer at 20°C at a concentration of approx. 1 A^{260nm}.

dine (74), as well as the replacement of deoxycytidine by 5-bromodeoxycytidine (75) in double stranded oligodeoxynucleotides does not lead to a major distortion of the B-helix.

The kinetics of the EcoRV catalyzed cleavage of the unmodified and the modified oligodeoxynucleotides were determined at 11°C. Under these conditions all oligodeoxynucleotides form stable duplex structures. The product analysis was carried out by HPLC. A representative chromatogram is shown in fig. 3. Fig. 4 shows the kinetics of cleavage of 14 μM double stranded d(AAAGATATmeCTT) in the presence of 2 μM dimeric EcoRV. In table 1 the relative rates of cleavage (v_{rel}) determined for all modified oligodeoxynucleotides under identical conditions are given. For some oligodeoxynucleotides the dependence of the initial rate of cleavage on the substrate concentration was determined (fig. 5). The Michaelis-Menten parameters derived from these experiments are given in table 1. Oligodeoxynucleotides containing 6-methyldeoxyadenosine in position 2 or 4 of the recogniton sequence, or deoxyuridine in position 3 or 5, as well as 7-deazadeoxyadenosine in position 2, not however in position 4, were refractory to cleavage, even at equimolar concentrations of enzyme and oligodeoxynucleotide. In order to find

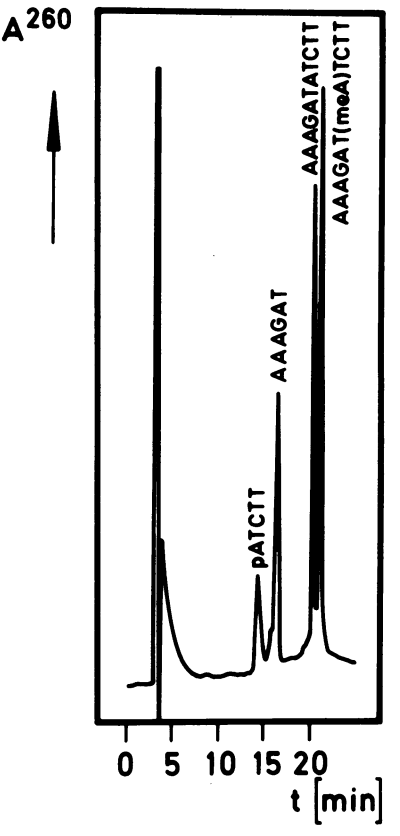

Fig. 3:*Analytical reversed-phase high performance liquid chromatography of oligodeoxynucleotides after incubation with EcoRV.*
10 μM d(AAAGATATCTT) and 5 μM d(AAAGATmeATCTT) were incubated with 1 μM EcoRV for 2 min at 11°C. Reaction products were analyzed by HPLC as described in MATERIALS AND METHODS. d(AAAGATATCTT) is cleaved by EcoRV to give d(AAAGAT) and d(pATCTT), while d(AAAGATmeATCTT) is not a substrate for EcoRV. The small shoulder on the peak for d(AAAGAT) represents a chromatographic artefact. The numerical integration of this chromatogram yields: d(AAAGATmeATCTT) = 35, d(AAAGATATCTT) = 29, d(AAAGAT) = 22, d(pATCTT) = 14% of total area.

out, whether these oligodeoxynucleotides are bound at all by EcoRV, we have carried out nitrocellulose filter binding experiments (fig. 6). Since these oligodeoxynucleotides are not cleaved by EcoRV, these experiments could be carried out in the presence of Mg^{2+}. The results of the binding experiments (table 1) show that all non-cleavable oligodeoxynucleotides are bound by EcoRV with equilibrium constants in the order of 10^{-5} to 10^{-6} M. For some of these oligodeoxynucleotides we have demonstrated that they inhibit the cleavage of the unmodified substrate in a competitive manner (fig. 7). These data demonstrate that the inactivity of some

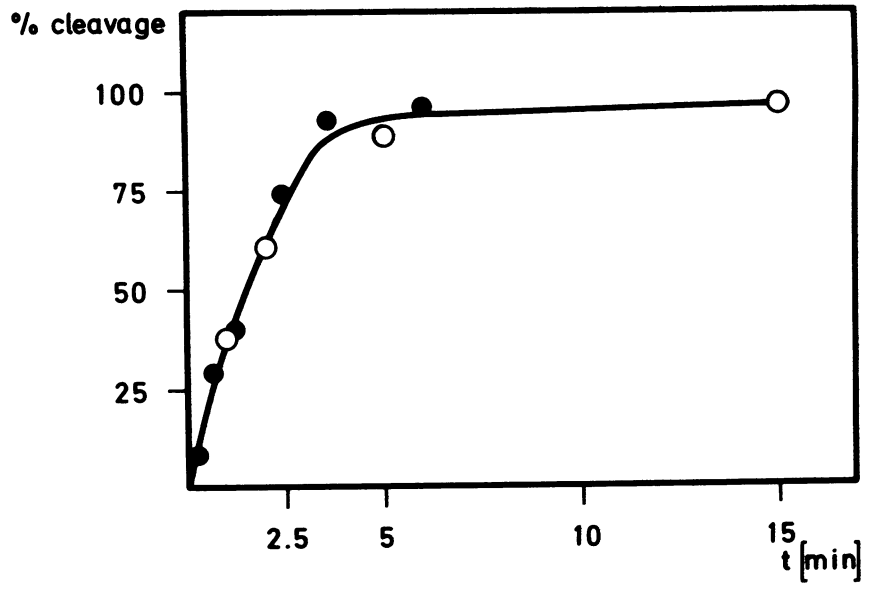

Fig. 4:*Kinetics of the EcoRV catalyzed cleavage of d(AAAGATATmeCTT)*
14 μM d(AAAGATATmeCTT) was incubated with 2 μM EcoRV at 11°C. Reaction products were analyzed by HPLC as shown in fig. 3. Open and filled circles represent the data of two different experiments.

modified oligodeoxynucleotides to function as substrates for EcoRV is not due to a major defect in binding. We conclude, therefore, that it is mainly the cleavage, i.e. the propensity to form an enzyme stabilized transition state, that is being affected by the chemical modification of individual bases within the recognition sequence of EcoRV.

The data in table 1 allow us to draw conclusions as to the involvement of particular functional groups on the DNA in the recognition by the EcoRV endonuclease. These conclusions are drawn on the basis that the chemical modifications do not alter the conformation of the oligodeoxynucleotides. While this is true for the gross conformation as shown by the CD experiments (vide supra), small but nevertheless critical conformational changes cannot be excluded. Consequently, the effect of a modification can be direct, i.e. not involving a conformational change or indirect, i.e. becoming manifest as a consequence of an induced conformational change. Only in the first case is it reasonable to conclude that the site of modification is a site of contact for the enzyme.

I. The 2-NH_2 group of the guanine in position 1 of the recognition sequence is not essential but beneficial for the cleavage reaction.

II. The 6-NH_2 group of the adenine in position 2 of the recognition sequence is essential. This is

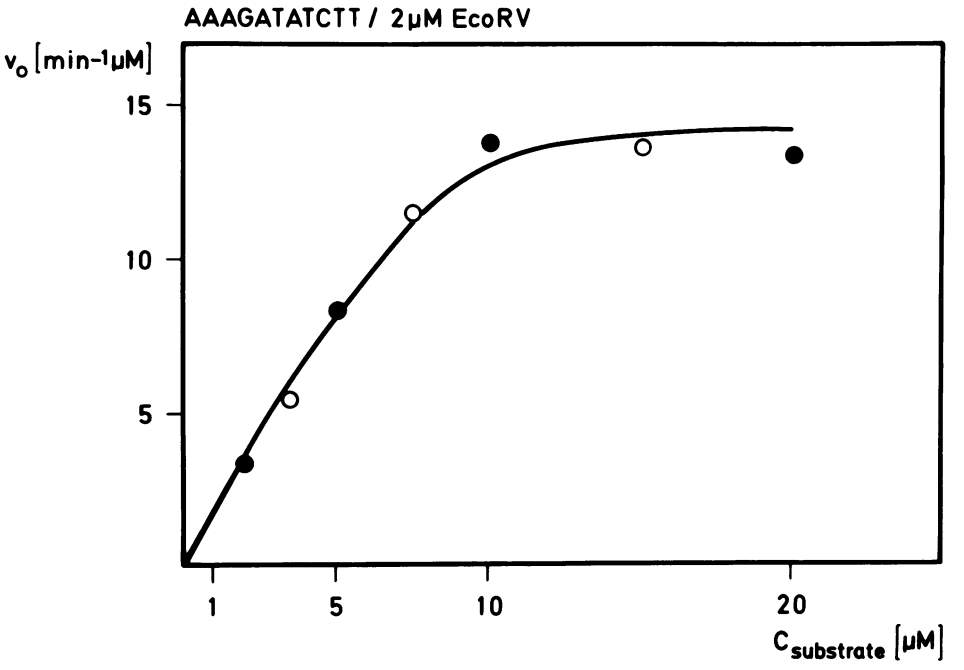

Fig. 5:*Michaelis-Menten analysis of the EcoRV catalyzed cleavage of d(AAAGATATCTT).* d(AAAGATATCTT) was incubated with 2 µM EcoRV at 11°C. The initial rate of cleavage (v_0) was determined from graphs as shown in fig. 4. Open and filled circles represent the data of two data sets obtained with two different enzyme preparations.

in agreement with the finding that this group is methylated by the EcoRV methylase (76,77) and thereby prevents cleavage of DNA by EcoRV. The N7 moiety of this adenine also is essential for cleavage.

III. The 5-methyl group of thymine in position 3 of the recognition sequence is required for cleavage. This has been shown earlier for EcoRV in a less detailed analysis (48) and supports the notion (78) that thymine methyl groups are as important in the recognition of specific DNA sequences by proteins as the more widely recognized hydrogen bonding sites of bases in the major groove (79). The thymine methyl group can be substituted without affecting the cleavage very much by a bromine residue (48) which is only slightly larger than the methyl group and induces a similar hydrophobicity (31).

IV. The 6-NH_2 group of the adenine in position 4 of the recognition sequence is also essential, not, however, the N7 moiety, indicating that the two adenine residues in the sequence - GATATC - interact with the EcoRV endonuclease in a qualitatively different manner.

V. The 5-methyl group of thymine in position 5 of the recognition sequence is essential for cleavage, its substitution by bromine does not prevent cleavage (48). It seems that the two thymine

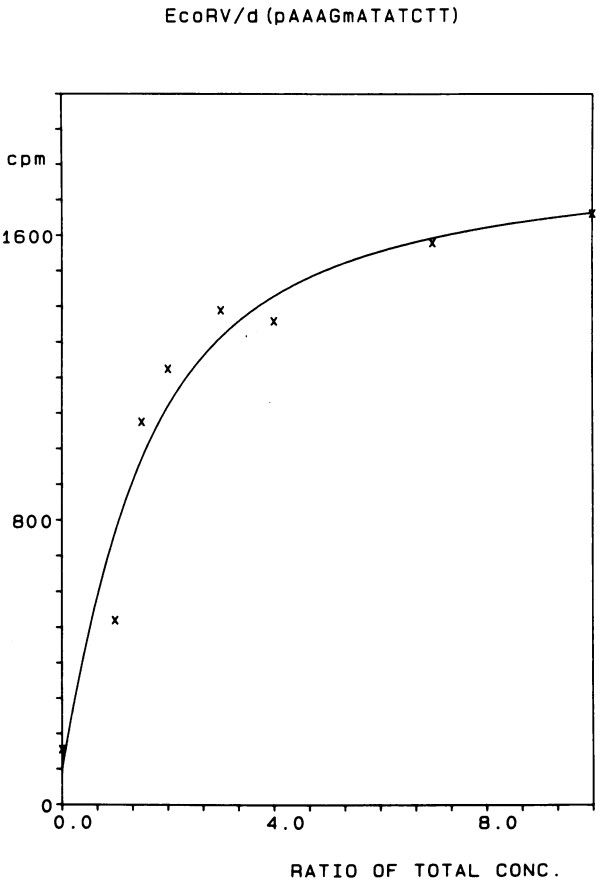

Fig. 6:*Nitrocellulose filter binding experiments with EcoRV and d(pAAAGmeATATCTT)*
5 μM d(pAAAGmeATATCTT) were mixed with varying amounts of EcoRV in cleavage buffer at 11°C. Complex formation was analyzed as described in MATERIALS AND METHODS. The filter bound radioactivity as a measure of complex formation is shown as a function of the ratio of total concentrations of dimeric enzyme and double stranded oligodeoxynucleotide. The drawn out curve is the theoretical binding isotherm with n = 1, K_{Diss} = 5 μM.

residues in the sequence - GATATC - are recognized by the EcoRV endonuclease in a similar manner. Since an oligodeoxynucleotide carrying a C·A mismatch base pair at this position is also cleaved by EcoRV, albeit by an order of magnitude more slowly than the oligodeoxynucleotide with the T·A base pair (80), it can be concluded that the O4 moiety of thymine in position 5 of the recognition sequence is not essential, but beneficial for cleavage.

VI. The 4-NH_2 group of cytosine in position 6 of the recognition sequence is essential for cleavage, while the H5 moiety is not in contact with the enzyme, since it can be replaced by a methyl group or bromine with no major effect on the rate of cleavage.

Table 1: Steady state parameters for the EcoRV catalyzed cleavage of synthetic oligodeoxynucleotides containing modified nucleobases and equilibrium dissociation constants for the binding of the non-cleavable oligodeoxynucleotides to EcoRV.

	v_{rel}	K_M [μM]	k_{cat} [min^{-1}]	K_{Diss} [μM]
1 2 3 4 5 6				
A A A G A T A T C T T	100*	4	7	-
A A A I A T A T C T T	7	1	0.2	-
A A A G mA T A T C T T	0	-	-	5**
A A A G cA T A T C T T	0	-	-	10**
A A A G A BU A T C T T	36*	2	2	-
A A A G A U A T C T T	0*	-	-	20**
A A A G A T mA T C T T	0	-	-	10**
A A A G A T cA T C T T T	120	2	2.5	-
A A A G A T A BU C T T	37*	n.d.	n.d.	-
A A A G A T A U C T T	0*	-	-	3**
A A A G A T A T mC T T	60	1	3	-
A A A G A T A T BC T T	123	n.d.	n.d.	-
A A A G A T A T U T T	0	-	-	7**

I = Deoxyinosine
mA = 6-Methyldeoxyadenosine
BU = 5-Bromodeoxyuridine
U = Deoxyuridine
mC = 5-Methyldeoxycytidine
BC = 5-Bromodeoxycytidine
cA = 7-Deazadeoxyadenosine

* The relative rates of the EcoRV catalyzed cleavage of these oligodeoxynucleotides, albeit in the phosphorylated form, had been determined previously under slightly different conditions. Essentially similar results were obtained (Ref. 48).

** These values have been determined for the phosphorylated oligodeoxynucleotides.

CONCLUSIONS

We have identified several functional groups within the recognition sequence -GATATC - as essential for DNA cleavage by the EcoRV endonuclease. This enzyme recognizes its substrate presumably through hydrogen bonds to the exocyclic NH_2 group and the N7 of the adenine residue in position 2, the exocyclic NH_2 groups of the adenine residue in position 4 and the cytosine residue in position 6, as well as through hydrophobic interactions with both thymidine residues. These groups are all located in the major groove. Since, furthermore, the 2-NH_2 group of the guanine in position 1 is not essential for cleavage, we conclude that EcoRV interacts with its substrate essentially via the major groove.

It is noteworthy that in contrast to the EcoRI endonuclease, the so far best studied restriction

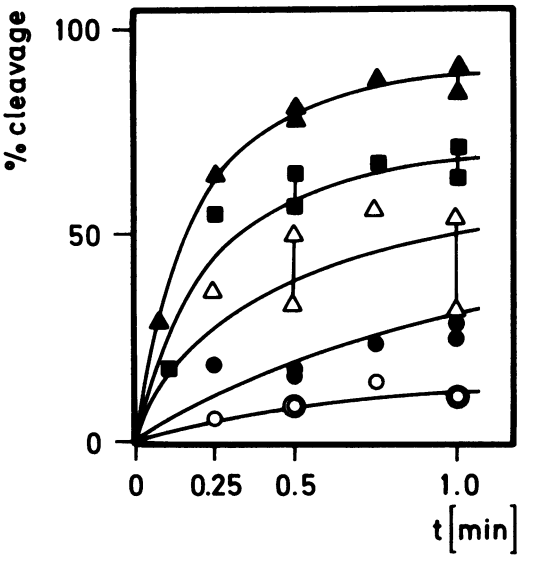

Fig. 7:*Competitive inhibition of the EcoRV catalyzed cleavage of d(AAAGATATCTT) by d(AAAGAUATCTT)*
5 μM d(AAAGATATCTT) was incubated with 1 μM EcoRV at 11°C in the presence of 0 (▲), 0.5 (■), 1 (△), 2 (●) and 5 (○) μM d(AAAGAUATCTT). The kinetics of cleavage of d(AAAGATATCTT) is shown.

enzyme, which recognizes its substrate mainly by forming hydrogen bonds to the purine residues of its recognition sequence - GAATTC - (22,46,53,59,81), the EcoRV endonuclease seems to interact with its substrate by making contacts both to the purine and pyrimidine bases via hydrogen bonds and through hydrophobic interactions with the thymine methyl groups.

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