Investigation of the Inhibitory Role of Phosphorothioate Internucleotidic Linkages on the Catalytic Activity of the Restriction Endonuclease *Eco*RV

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ABSTRACT: The inhibitory effect of phosphorothioate residues, located within one strand of double-stranded DNA, on the hydrolytic activity of the restriction endonuclease *Eco*RV was investigated. Specific incorporation of a phosphorothioate group at the site of cleavage yielded the sequence 5'-GATsATC-3'. This modified sequence was cleaved at a relative rate of 0.1 compared to the unmodified substrate. Substrates 5'-GATsAsTC-3' and 5'-GsATsATC-3', both containing one additional phosphorothioate substitution, were linearized at a rate of 0.04 relative to unmodified DNA. However, under the same conditions, fully dAMPS-substituted DNA was found to be virtually resistant to the hydrolytic activity of *Eco*RV. Further experiments showed that double-stranded DNA fragments generated by PCR containing phosphorothioate groups within both strands are potent inhibitors of *Eco*RV catalysis. The inhibition was independent of whether the inhibitor fragment contained an *Eco*RV recognition site. We concluded that substitution of the phosphate group at the site of cleavage by a phosphorothioate residue decreases the rate of *Eco*RV-catalyzed hydrolysis most significantly. Substitution of other phosphate groups within the recognition sequence plays a limited role in enzyme inhibition. The presence of multiple dNMPS residues at regions of the DNA removed from the *Eco*RV recognition site may decrease the amount of enzyme available for catalysis by nonspecific binding to *Eco*RV.

Nucleoside phosphorothioate analogues have been used extensively for the study of protein–DNA interactions (Eckstein, 1985). In many cases, these compounds or the DNA into which they are incorporated exhibit specific inhibitory effects upon hydrolytic enzymes. For example, detailed studies of the 3'–5'-exonuclease activity of the Klenow fragment, snake venom phosphodiesterase, and exonuclease III have shown that these enzymes cleave phosphorothioate linkages of the R_P configuration approximately 100 times more slowly than phosphate linkages (Labeit et al., 1987; Burgers & Eckstein, 1979; Gupta et al., 1984). The inhibition of the latter two enzymes has been exploited for the sequencing of single-stranded DNA (Labeit et al., 1986, 1987) and the direct sequencing of polymerase chain reaction products (Olsen & Eckstein, 1989). Phosphorothioate-containing oligonucleotides have been used for the study of a number of other exonucleases (Spitzer & Eckstein, 1988). Several additional investigations have used phosphorothioate-containing RNA for the analysis of specific binding contacts of the bacteriophage R17 coat protein (Milligan & Uhlenbeck, 1989) and for the study of ribozyme-catalyzed hydrolysis reactions (Waring, 1989; von Tol et al., 1990).

In addition to the applications listed above, the inhibitory effect of phosphorothioate internucleotidic linkages within double-stranded DNA on the activity of a number of restriction endonucleases has been reported (Taylor et al., 1985; Nakamaye & Eckstein, 1986; Sayers et al., 1989). For complete inhibition of all the restriction endonucleases studied to date, the presence of a phosphorothioate at the potential site of cleavage is necessary but not always sufficient. In more detailed studies of the two enzymes *Nci*I and *Ban*II, it was determined that the incorporation of a second specific phosphorothioate residue is critical for the complete protection of the potential substrate from hydrolysis. In the case of *Nci*I, the second phosphorothioate group has to be substituted for the group 5' to the cleavage position (Nakamaye & Eckstein, 1986) whereas for *Ban*II a modified group located on the 3' side of the cleavage site confers full protection of the substrate (Olsen et al., 1990).

In order to obtain a further understanding of how phosphorothioate linkages protect DNA from enzyme-catalyzed hydrolysis, we chose to investigate the effect of several phosphorothioate-substituted DNA substrates on the activity of the restriction endonuclease *Eco*RV.

Experimental Procedures

Materials. *Eco*RV restriction endonuclease (20 units/μL) was purchased from New England Biolabs. T5 exonuclease was kindly provided by J. R. Sayers (Max-Planck-Institut für experimentelle Medizin, Göttingen, FRG). The S_P diastereomers of deoxyadenosine 5'-*O*-(1-thiotriphosphate) (dATPαS),[1] dTTPαS, and dGTPαS were either synthesized as described (Ludwig & Eckstein, 1989) or purchased from Amersham. NuSieve GTG-agarose was obtained from FMC BioProducts. Oligonucleotide primers, AMREV [5'-d(CAGGAAACAGCTATGAC)-3'], AOSEQ2 [5'-d(CATTCGCCATTCAGGCTG)-3'], EXTI [5'-d(TCCCCGGGTACCGAGCTC)-3'], EXTV [5'-d(GGTACCGAGCTCGAT)-3'], M13PCR [5'-d(CACCCTGGCGCCCAATAC)-3'], M13SEQ [5'-d(AGGGTTTTCCCAGTCACG)-3'], and IToV [5'-d(CGAGCTCGATATCGTAAT)-3'], were prepared by the phosphoramidite method using an Applied Biosystems 380 B DNA synthesizer except for the AMREV primer which was kindly provided by R. Jackson (Amersham). The mutant oligonucleotide (IToV) used to create the *Eco*RV site in M13mp18 has the mismatch bases underlined.

Preparation of M13mp18Rv Mutant Using Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using

[1] Abbreviations: dNTPαS, S_P diastereomer of deoxynucleoside 5'-*O*-(1-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; Tris-HCL, tris(hydroxymethyl)aminomethane hydrochloride; RFII, -III, and -IV DNA, open circular, linear, and covalently closed circular double-stranded DNA, respectively; PCR, polymerase chain reaction.

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Table I: Preparation of PCR Fragments

PCR fragment	base pairs	ssDNA[a]	oligomers	polymerized with phosphorothioate
1	452	M13mp18RV	AOSEQ2 M13PCR	none
2	452	M13mp18RV	AOSEQ2 M13PCR	dATPαS
3	452	M13mp18RV	AOSEQ2 M13PCR	dGTPαS
4	452	M13mp18RV	AOSEQ2 M13PCR	dTTPαS
5	124	M13mp18	M13SEQ AMREV	none
6	124	M13mp18RV	M13SEQ AMREV	dATPαS
7	124	M13mp18	M13SEQ AMREV	dATPαS

[a] Wild-type M13mp18 DNA contains no cleavage site for *Eco*RV. The single-stranded DNA M13mp18RV contains a unique *Eco*RV site at position 6233 constructed by site-directed mutagenesis as described under Experimental Procedures.

M13mp18 single-stranded DNA according to the phosphorothioate method of Sayers et al. (1988a). Oligonucleotide IToV was used for a double transversion mutation of the *Eco*RI site found in the polylinker of the DNA creating a unique *Eco*RV site at position 6232. The mutant DNA was named M13mp18RV.

Preparation of Phosphorothioate-Containing RFIV DNA Substrates for Reactions with EcoRV. Polymerization reactions using calf thymus primer were carried out essentially as described by Sayers et al. (1988b). Limited polymerization reactions were carried out by using the Klenow fragment of DNA polymerase I for the extension of the EXTV or EXTI oligonucleotide primers (Nakamaye & Eckstein, 1986). Phosphorothioate-substituted substrates were prepared by limited polymerization of dATPαS alone (100 μM) or in combination with dTTPαS, and two or three unmodified dNTPs. After polymerization at 25° C for 10 min, all four normal dNTPs were added at a concentration of 500 μM to complete the synthesis of the (−) strand. Partially polymerized or unligated DNA species remaining after incubation were digested at 37 °C for 30 min using 2 μg of T5 exonuclease (Sayers & Eckstein, 1990) in the same buffer as the polymerization reaction. The enzyme was then heat-inactivated at 70 °C for 10 min, and the DNA was ethanol-precipitated.

Preparation of PCR Fragments. PCR fragments were prepared by using the buffers and cycle protocol as previously described (Olsen et al., 1990) and purified by ethanol precipitation. Amplification reactions were carried out with 50 ng of wild-type single-stranded M13mp18 DNA or mutant M13mp18RV DNA. Each reaction contained three dNTPs and the corresponding dNTPαS analogue for polymerization as specified in Table I. All PCR products were visualized by ultraviolet light after electrophoresis using 4% NuSieve GTG–agarose run in the presence of 5 mM 2-mercaptoethanol and 0.4 μg/mL ethidium bromide (Potter & Eckstein, 1984). The product from one PCR reaction was quantitated by using standard A_{260} measurement techniques, and the other PCR products were normalized by laser densitometry band comparison after agarose gel electrophoresis.

Reaction of RFIV DNA with EcoRV. RFIV DNA (1–2 μg) prepared by using oligonucleotide primers was cleaved by *Eco*RV in 90-μL reactions containing 10 mM Tris-HCl, pH 8, 100 mM NaCl, 7.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 9.5 units of enzyme. Aliquots of 14 μL were removed at various time intervals and added to 6 μL of a 50% glycerol solution containing 100 mM EDTA and 0.1% (w/v) bromophenol blue for gel electrophoretic analysis.

Reaction of 452 Base Pair PCR Fragment with EcoRV. All reactions contained 10 mM Tris-HCl, pH 8, 100 mM NaCl, 7.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and approximately 400 ng of one of the 452 base pair PCR fragments (Table I) prepared as described above. Reactions were carried out in a volume of 43 μL at 37 °C and were initiated by the addition of 2–40 units of *Eco*RV. Aliquots (14 μL) were removed for agarose gel electrophoresis analysis. The extent of cleavage was determined by scanning laser densitometry.

*Inhibition Studies with PCR Fragments. Eco*RV restriction endonuclease (2 units) was added to a solution (total volume 43 μL) containing 10 mM Tris-HCl, pH 8, 100 mM NaCl, 7.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 400 ng of PCR fragment 1 obtained by polymerization with dNTPs. The above reaction was also performed in the presence of 100 ng of (1) PCR fragment 6 containing dAMPS linkages and an *Eco*RV site, (2) PCR fragment 7 containing dAMPS linkages but no *Eco*RV site, or (3) PCR fragment 5 which contained neither phosphorothioate linkages nor any *Eco*RV site. Aliquots of 14 μL were removed at 10, 30, and 60 min after addition of the enzyme and added to 6 μL of a 50% glycerol solution containing 100 mM EDTA and 0.1% xylene cyanol FF marker dye. The 239 and 213 base pair cleavage products were easily separated from the 452 base pair substrate and 124 base pair inhibitor DNA by electrophoresis (120-min running time, 3 V cm^{-1}) using a 4% NuSieve GTG–agarose gel containing 5 mM 2-mercaptoethanol and ethidium bromide (0.4 μg/mL). The extent of cleavage by the enzyme was monitored by scanning laser densitometry.

Gel Scanning. Agarose gels were transilluminated by ultraviolet light and photographed. The negatives were scanned with an LKB 2222-020 UltroScan XL laser densitometer (Pharmacia, Sweden).

Results

In this study, we examined the effect of various phosphorothioate-substituted substrates on the catalytic activity of *Eco*RV. Initially, various DNAs were prepared by polymerization of either four normal dNTPs or three dNTPs and dATPαS substituted for dATP using M13mp18RV single-stranded DNA as template, calf thymus DNA primer, and DNA polymerase I. The 5′–3′-exonuclease activity of this enzyme degrades the primer so that dAMPS residues will be present throughout the (−) strand when dATP is replaced by dATPαS in the polymerization reaction. The two dAMP residues located in the recognition site of the *Eco*RV are among those replaced by the analogue. It is known from previous studies (Sayers et al., 1989) that the (−) strand of DNAs produced in this way is resistant to the hydrolytic activity of this enzyme. This result is confirmed here. Figure 1 clearly shows that reaction of *Eco*Rv with dAMPS-substituted DNA generated nicked DNA in a time-dependent manner resulting from cleavage of the unmodified (+) strand and resistance of the (−) strand to cleavage. By contrast, the unmodified DNA was readily linearized by the enzyme (Figure 2).

In order to localize the inhibitory effect of the modified groups to specific positions, we carried out limited extensions of an oligonucleotide primer which hybridized adjacent to the *Eco*RV site located in M13mp18RV. By omitting a fourth nucleotide in the first phase of the reaction, the incorporation of phosphorothioates is limited to the region of interest (Figure 3). For example, to place a phosphorothioate exclusively at the site of cleavage, the EXTV oligonucleotide was used in

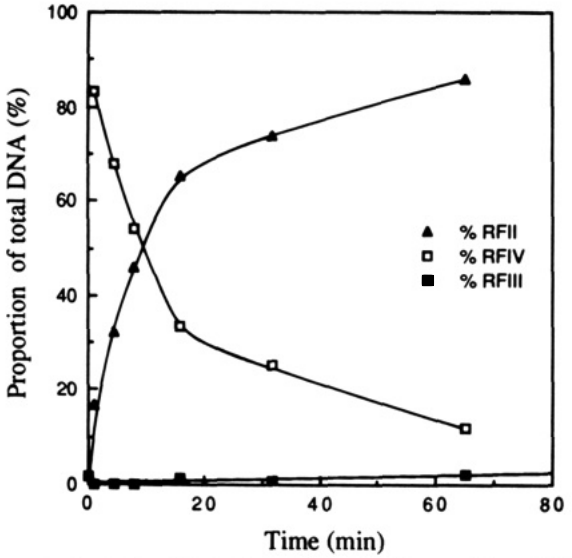

FIGURE 1: Analysis of *Eco*RV reaction of DNA containing dAMPS residues incorporated by polymerization with DNA polymerase I. The reaction contained 30 units of restriction endonuclease and 2 μg of RFIV DNA. Aliquots of 14 μL taken at the times indicated were quenched by the addition of stop mix (see Experimental Procedures), and the percentages of covalently closed circular (□), open circular (△), and linear (■) DNA were determined by laser densitometry after agarose gel electrophoresis.

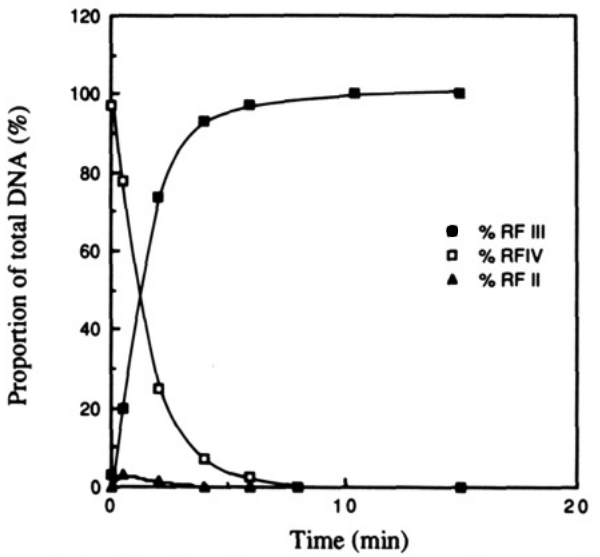

FIGURE 2: Analysis of *Eco*RV reaction of DNA containing unmodified phosphate residues incorporated by polymerization with DNA polymerase I. The reaction contained 10 units of restriction endonuclease and 2 μg of RFIV DNA. Samples were removed and treated as described under Figure 1. Percentages of covalently closed circular (□), open circular (△), and linear (■) DNA were determined after agarose gel electrophoresis by laser densitometry.

combination with dATPαS along with dCTP and dTTP. The synthesis of the (−) strand was completed by carrying out the polymerization reaction by addition of all four normal dNTPs.

These polymerization reactions very often yield significant quantities of unligated DNA which complicates the analysis of the *Eco*RV-catalyzed reactions. This DNA was therefore destroyed by incubation of the reaction mixture with T5 exonuclease (Sayers & Eckstein, 1990), resulting in very clean preparations of RFIV DNA (Figure 4).

The effect of the specific substitutions is shown in Figure 5. The incorporation of one dAMPS at the site of cleavage reduced the rate of linearization by approximately 10-fold. The presence of an additional dAMPS, yielding the *Eco*RV

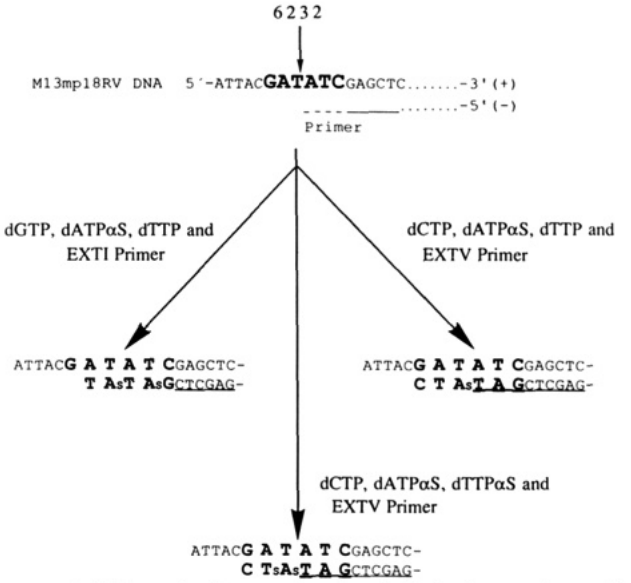

FIGURE 3: Schematic diagram showing the limited extension methodology used for the incorporation of specific phosphorothioate groups within the recognition sequence of *Eco*RV. The sequences contributed by the oligonucleotide primers are underlined, and the positions of phosphorothioate groups are represented by an s.

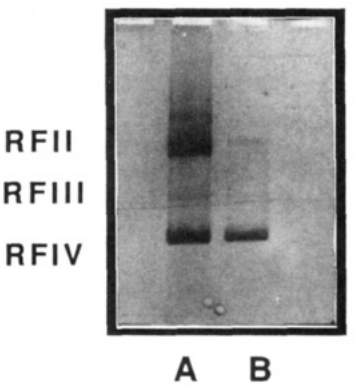

FIGURE 4: Agarose gel analysis (1%) of products obtained after limited polymerization. Lane A, polymerization using EXTV oligonucleotide and dATPαS as described under Experimental Procedures. Lane B contains a sample of T5 exonuclease treated DNA from lane A (see Experimental Procedures).

recognition sequence 5′-GsATsATC-3′, decreased the rate of cleavage by 25-fold. The same decrease in the rate of linearization was observed when the DNA contained a phosphorothioate at the position of cleavage in combination with one immediately 3′ to the cleavage site (5′-GATsAsTC-3′).

In order to carry out further *Eco*RV inhibition studies, small DNA fragments were synthesized by the PCR methodology. The resulting fragments contain the phosphorothioate groups in the R_P configuration in both strands when synthesized in the presence of dNTPαS analogues. Thus, they differ from the RFIV DNA preparations used in the above reactions which carry these groups only in the (−) strand. The use of PCR fragments for this study has the advantage that the well-defined fragments produced are easily separated by agarose gel electrophoresis. This facilitates quantitation of the extent of hydrolysis by densitometry.

Four separate 452 base pair PCR fragments comprising the multiple cloning site of M13mp18RV were produced by polymerization using either four unmodified dNTPs or three dNTPs and one dNTPαS analogue (Table I, fragments 1–4). These were subsequently reacted with *Eco*RV. Three time points were taken for each reaction, and the extent of cleavage was determined by scanning densitometry after separation of

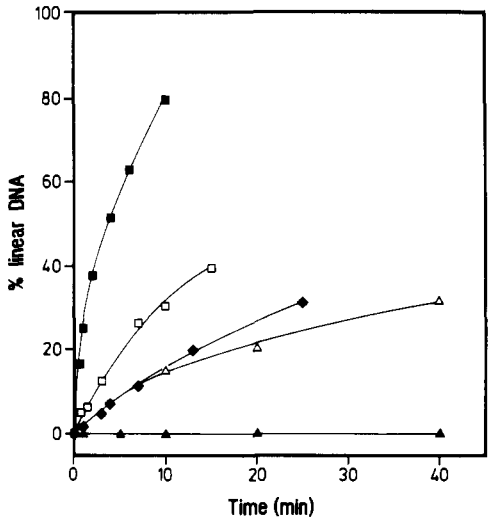

FIGURE 5: Comparison of the rate of *Eco*RV restriction endonuclease linearization of various phosphorothioate-substituted DNA substrates. Reactions were carried out as described under Experimental Procedures. Samples of 14 μL were removed at the times indicated, and the reaction was quenched by the addition of stop mix. After agarose gel electrophoresis, the percentage of linear DNA was determined by scanning laser densitometry. DNA substrates containing specific incorporation of phosphorothioate groups, GATATC (■), GATsATC (□), GATsAsTC (♦), and GsATsATC (△), were prepared by limited extension of an oligonucleotide primer as described under Experimental Procedures. One DNA substrate [GsATsATC (▲)] was prepared by replacing dATP with dATPαS in a normal polymerization reaction using DNA polymerase I and calf thymus DNA as primer for described under Experimental Procedures.

Table II: Cleavage of 452 Base Pair PCR Fragments with *Eco*RV

PCR fragment[b]	dNTPαS incorporated	% cleavage[a] 5 min	10 min	30 min	units/ reaction
1	none	26	47	91	2
2	dATPαS	0	0	0	2
2	dATPαS	0	0	10	40
3	dGTPαS	0	6	26	2
4	dTTPαS	0	9	28	2

[a] Separation of reaction products (239 and 213 bp) from substrate was accomplished by 4% agarose gel electrophoresis. The degree of cleavage was determined by laser densitometric scanning as described under Experimental Procedures. [b] See Table I.

the fragments by gel electrophoresis (Table II). The unmodified fragment (fragment 1) was cleaved more than 90% in 30 min. By contrast, no cleavage of the fragment containing dAMPS internucleotidic linkages (fragment 2) was observed after 30 min using 2 units of enzyme. When the concentration of the enzyme was increased by a factor of 20, a small amount of fragment 2 cleavage product was observed. Two other substrates containing either dGMPS (fragment 3) or dTMPS (fragment 4) residues were also linearized by using only 2 units of enzyme, but to a lower rate than the cleavage of fragment 1.

Additional experiments utilized three different 124 base pair fragments (Table I, fragments 5–7) as inhibitors of *EcoRV*-catalyzed hydrolysis. In each reaction, a 452 base pair PCR product, also derived from M13mp18RV, was used as a substrate (Table I, fragment 1). One inhibitor contained an *Eco*RV site and was amplified in the presence of dATPαS (fragment 6). The other two inhibitors were identical with fragment 6 in sequence and length; however, the *Eco*RV site, 5′-GATATC-3′, was destroyed by changing two bases to 5′-GAATTC-3′. This fragment was amplified either with four dNTPs (fragment 5) or with dATPαS (fragment 7).

Table III: Effect of Three Different Inhibitor PCR Fragments on the *Eco*RV-Catalyzed Hydrolysis of PCR Fragment 1[a]

inhibitor PCR fragment[a] added	containing an *Eco*RV site	dNTPαS incorporated	% cleavage 5 min	10 min	30 min
			41	61	93
5	no	none	42	65	91
6	yes	dATPαS	0	0	4
7	no	dATPαS	0	11	13

[a] See Table I.

The cleavage of fragment 1 in the absence of any potential inhibitor fragment went to 93% completion in 30 min. Table III shows that there is no significant decrease in the rate of cleavage in the presence of inhibitor PCR fragment 5 which contains neither an *Eco*RV site nor phosphorothioate groups. However, a significant decrease in cleavage was observed when the added fragment contained dAMPS groups, regardless of whether this fragment contained an *Eco*RV site (fragment 6) or not (fragment 7).

Discussion

The restriction endonuclease *Eco*RV recognizes the sequence

(+) 5′-GAT▼ATC
(−) 3′-CTA▲TAG

and cleaves the DNA at the points designated by the triangles. This enzyme has a pH optimum between 7.5 and 8 and essentially cleaves both strands of the DNA in a concerted reaction when used under these conditions (Halford & Goodall, 1988). The object of our study was to gain an understanding of the inhibitory role of phosphorothioate groups incorporated into DNA by determining their effect on the catalytic activity of *Eco*RV.

From the data presented in Figures 1 and 2, it is clear that the presence of dAMPS groups in the (−) strand of M13mp18RV greatly affects the catalytic activity of the enzyme. Whereas unmodified DNA is readily linearized, very little cleavage of the DNA strand which carries the phosphorothioate group is observed. The inhibitory effect could conceivably be caused by the presence of either one particular phosphorothioate or a combination of several groups. It is known from the study of over 30 other restriction endonucleases that complete inhibition of enzyme activity is only observed when a modified group is present at the site of cleavage (Taylor et al., 1985; Sayers, et al., 1989). Therefore, if the incorporation of a single phosphorothioate was responsible for the observed inhibition of *Eco*RV, it is natural to assume that this phosphorothioate is located at the position which normally undergoes hydrolysis.

To investigate this, we prepared a series of DNAs in which single or multiple substitutions of dNPS were made in the hexanucleotide recognition site. This methodology provided inter alia a substrate with the recognition site, 5′-GATsATC-3′, possessing only one phosphorothioate group at the site of cleavage (Figure 3). Reaction with *Eco*RV showed that this substrate was cleaved 10 times slower than a substrate prepared in the same manner but without the incorporation of modified groups (Figure 5). Therefore, complete protection of DNA requires more than the presence of the dAMPS at the cleavable position.

We reasoned that the replacment of the second dAMP group within the recognition sequence for the enzyme by dAMPS might be responsible for the disruption of the catalytic activity of *Eco*RV. To test this, two additional substrates were prepared by using the same strategy of limited extension as

described above. A phosphorothioate group was placed at this second dAMP position, as well as at the site of cleavage, yielding 5′-GsATsATC-3′ in the (−) strand of the DNA. Although the incorporation of the second phosphorothioate did not render the DNA completely resistant to hydrolysis, it did decrease the rate of cleavage over the singly substituted substrate by a factor of 2.5, thus making it slower by a factor of 25 in comparison to the unmodified DNA. The rate of cleavage of a substrate with the substitution pattern 5′-GATsAsTc-3′ was also decreased by a factor of 2.5 relative to the linearization of 5′-GATsATC-3′. Therefore, the presence of additional phosphorothioate groups located within the recognition sequence only decreases the rate of hydrolysis by a small factor.

These experiments show that the substitution of the two dAMP groups in the recognition sequence alone does not account for the almost complete lack of cleavage observed for the fully dAMPS-substituted DNA. This is very different from results obtained from a previous study with the restriction enzyme *Nci*I where the presence of a second phosphorothioate in the recognition sequence, in addition to that at the site of cleavage, is sufficient to render such DNA resistant to the action of the enzyme (Nakamaye & Eckstein, 1986). A similar pattern of inhibition was also found for the restriction endonuclease *Ban*II (Olsen et al., 1990). This indicates that the influence of phosphorothioate groups on the catalytic activity of these restriction enzymes differs. Apparently for *EcoRV*, dAMPS groups outside the recognition sequence must contribute to the resistance of DNA against enzyme-catalyzed hydrolysis. To evaluate this, the more general inhibitory effects of such dAMPS groups were investigated by experiments using phosphorothioate-containing PCR fragments.

As expected, the results presented in Table II show that a PCR fragment containing dAMPS residues (fragment 2) is resistant to cleavage by *Eco*RV when reacted under the same conditions which produce greater than 90% cleavage of an unmodified fragment (fragment 1). This result is consistent with the data obtained from the cleavage experiments using phosphorothioate-substituted M13mp18RV RFIV DNA where dAMPS is only present in the (−) strand. However, we did observe that with high concentrations of enzyme the PCR fragment was hydrolyzed to a small extent after 30 min (Table II). This enabled us to estimate that the rate of cleavage of the fully dAMPS-substituted PCR fragment is approximately 300 times lower than that of the unmodified fragment.

The inhibitory effect of phosphorothioate groups other than dAMPS was determined by the reaction of *Eco*RV with PCR fragments prepared by polymerization using dGTPαS or dTTPαS substituted for the unmodified dNTPs. Polymerization with dGTPαS (fragment 3) puts no phosphorothioates into the recognition sequence but, of course, throughout the rest of the DNA. Polymerization with dTTPαS (fragment 4), on the other hand, puts two such groups into the recognition sequence in addition to those in the rest of the DNA. The results obtained with these two fragments (Table II) revealed that the rate of cleavage of both is reduced by a factor of approximately 6 in comparison with the unmodified fragment 1. This result shows that phosphorothioate groups outside the recognition sequence have an inhibitory effect on cleavage. The large difference seen for cleavage of fragment 3 and 4 and that of fragment 2 confirms the importance of having a phosphorothioate group at the site of cleavage in order to obtain a nonproductive enzyme–DNA complex.

To gain some insight into the inhibition caused by the phosphorothioate groups outside the recognition sequence, two forms of inhibition were considered. The enzyme may bind very weakly to phosphorothioate-containing DNA. Alternatively, the enzyme might bind tightly to the modified substrate. Tight binding could decrease catalysis by hindering the diffusion of the enzyme along the DNA strand (von Hippel & Berg, 1989) or by lowering the rate of product release.

In order to distinguish between tight and weak binding of *Eco*RV to phosphorothioate-containing DNA, inhibition experiments were performed using various PCR fragments as inhibitors of the enzyme (Table III). The rate of hydrolysis of fragment I was not appreciably reduced by the presence of a similar fragment which lacked an *Eco*RV site. This result emphasizes the importance of such a site to binding of the enzyme. In contrast, however, phosphorothioate-containing PCR fragments, either with or without an *EcoRV* site (fragments 6 and 7), are strongly inhibitory. We interpret this to mean that unspecific binding of the enzyme to phosphorothioate-containing DNA is strong.

The observation of tight binding of proteins to phosphorothioate-containing nucleic acids is not unprecedented in the literature. HIV reverse transcriptase exhibits high-affinity binding to a 28-mer dC phosphorothioate oligodeoxynucleotide (Majumdar et al., 1989). This oligomer was found to inhibit polymerization with a K_i approximately 200-fold lower than the K_i for an oxygen-containing inhibitor of the same chain length and base composition. In addition, phosphorothioate groups have been used for probing critical RNA–protein contact points as described by Milligan and Uhlenbeck (1989). In their study, they found that several different substitutions decreased the binding of bacteriophage R17 coat protein. However, one substitution increased K_a by a factor of 10. Therefore, the tight binding of *Eco*RV to phosphorothioate-containing DNA may be a more general phenomenon responsible for the inhibition of the catalytic activity not only of restriction endonucleases[2] but also of a number of other enzymes as well.

In summary, we have demonstrated that the presence of various phosphorothioate internucleotidic linkages alters the rate of hydrolysis of DNA by *Eco*RV. This inhibition can be considered the result of a number of effects. A single phosphorothioate group substituted at the cleavage site of the DNA in the (−) strand only reduces the rate of hydrolysis by a factor of 10. Additional phosphorothioates located at positions other than the site of cleavage, but within the recognition site, are less important in disrupting the formation of a productive enzyme–substrate complex. Finally, the enzyme binds strongly to phosphorothioate groups located in regions of the DNA other than those involved in enzyme recognition. All these factors combined result in the resistance of dAMPS-containing DNA against *Eco*RV-catalyzed hydrolysis.

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Torsional Stress Stabilizes Extended Base Unpairing in Suppressor Sites Flanking Immunoglobulin Heavy Chain Enhancer†

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ABSTRACT: DNA sequences surrounding the immunoglobulin heavy chain (IgH) enhancer contain negative regulatory elements which are important for the tissue specificity of the enhancer. We have shown that sequences located both 5′ and 3′ of the enhancer, corresponding to the negative regulatory elements, become stably and uniformly unpaired over an extended length when subjected to torsional stress. These DNA sequences are also included within matrix association regions. The ability of the sequences to assume a stably unpaired conformation was shown by reactivity with chloroacetaldehyde which is specific for unpaired DNA bases, as well as two-dimensional gel electrophoresis of topoisomers. The sequences located 3′ of the enhancer induce base unpairing in the direction of the enhancer. This unpaired region progressively expands to include as much as 200 base pairs as the ionic concentration decreases or superhelical density increases. When an ATATAT motif within a negative regulatory element located 3′ of the enhancer was mutated, the extensive base-unpairing property was abolished. This base-unpairing property of DNA may be important for negative regulation of gene expression and attachement to the nuclear matrix.

The IgH enhancer sequence is located within the intron between the joining segments (J_H) and the constant regions ($C\mu$) of the murine $C\mu$ locus (Banerji et al., 1983; Gillies et al., 1983; Mercola et al., 1983; Neuberger, 1983). This enhancer is active in B cells but not in fibroblasts and was the first genetic element shown to confer cell-type specificity to a cellular gene (Banerji et al., 1983; Gillies et al., 1983). Evidence for negative control of this cell-type specificity was reported (Kadesch et al., 1986). Imler et al. (1987) have identified, within the surrounding the IgH enhancer, cis-acting negative regulatory elements which repress IgH enhancer activity in fibroblast but not in myeloma cells. Recently, a developmental-specific factor, NF-μNR, that binds to these elements has been reported (Scheuermann & Chen, 1989). Deletion of segments bound by NF-μNR from the enhancer results in an activation of the enhancer function in non-B cells.

Nuclear matrix association regions (MARs) or scaffold-attached regions (SARs) are often found in close vicinity to known enhancer sequences (Cockerill & Garrard, 1986; Gasser & Laemmli, 1986). These regions contain topoisomerase II cleavage consensus sequences and are thought to punctuate chromosomal DNA into functional units of topologically constrained loop domains. The J_H-$C\mu$ intron region has also been found to contain sites that are associated with the nuclear matrix. The MARs for this region were located around the IgH enhancer which contains these negative regulatory elements (Cockerill et al., 1987).

In this paper we report an unusual property of DNA sequences that correspond to the negative regulatory elements and the MAR sequences of the IgH enhancer. These sequences become entirely base-unpaired over an extended length even at room temperature when subjected to the torsional stress of negative supercoiling. This is not due to a high rate of DNA breathing, but instead they are stably base-unpaired. These sequences are A+T rich. However, in addition to being A+T

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