Application of Phosphate-Backbone-Modified Oligonucleotides in the Studies on *Eco*RI Endonuclease Mechanism of Action†

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ABSTRACT: Chemical synthesis of oligodeoxyribonucleotides modified at a preselected internucleotide bond by the replacement of one of the two "nonbridging" oxygens by a sulfur atom or an ethoxy group yields model substrates for studies on DNA–protein interactions. Chromatographic (RP-HPLC) separation of the diastereomers of oligonucleotides containing *Eco*RI canonical sequence together with the assignment of the substituent orientation in the DNA molecule allowed study of the stereochemical aspects of DNA–*Eco*RI endonuclease interactions. The DNA segment involved in interactions between *Eco*RI protein and phosphate groups appeared to be larger than its canonical sequence, ...GAATTC..., and was extended to the nonamer. The modification of certain internucleotide bonds within this nonamer caused significant or complete protection against the nucleolytic action of *Eco*RI and, in some cases, manifested the diastereoselectivity of the enzyme. On the basis of the results of *Eco*RI-catalyzed hydrolysis of stereodefined phosphorothioate and phosphotriester substrates, we propose a model to explain this phenomenon at the molecular level.

The specificity of interactions between DNA and proteins depends mainly on the sequence of bases within the recognized fragment of DNA (Seeman et al., 1976). However, several studies have emphasized the participation of internucleotide phosphates in the base-specific interactions between DNA and proteins (Siebenlist & Gilbert, 1980; Siebenlist et al., 1980; Schevitz et al., 1985; Anderson et al., 1987).

Valuable tools for studying the involvement of internucleotide phosphates in DNA–protein interactions appear to be phosphorothioate (Connolly et al., 1984a,b) and phosphotriester (Uznanski et al., 1986) analogues of oligonucleotides. The replacement of one of the two "nonbridging" oxygens at the internucleotide phosphate group by a sulfur atom in phosphorothioates or by an alkoxy (for example, ethoxy) group in phosphotriesters leads to the formation of two diastereomers with different spatial orientations of the substituent at the phosphorus atom. The separation of these diastereomers by HPLC and the assignment of the substituent orientation in the DNA molecule (in chemical terms, the assignment of absolute configuration at the P chiral center) are essential for the preparation of substrates used in the studies on stereochemical aspects of DNA–protein interactions. We have used this approach to study the DNA–*Eco*RI endonuclease interactions.

Although the mechanism of recognition of the duplex GAATTC by *Eco*RI endonuclease has been extensively studied (McClarin et al., 1986; Lesser et al., 1990; King et al., 1989; Wright et al., 1989), some problems concerning the participation of internucleotide phosphates in the recognition/cleavage process remain unsolved. By "ethylation interference" it was demonstrated that four phosphates of the "canonical sequence" are involved in the formation of the DNA–protein complex (Lu et al., 1981). However, ethylation of DNA by ethylnitrosourea produces an inseparable mixture of diastereomers; therefore, the stereochemical aspects of DNA–protein interactions could not be studied by this method. The use of separated diastereomers of phosphorothioates or phosphotriesters as the substrates for *Eco*RI-catalyzed hydrolysis allowed us to find the following: (I) the range of interaction between *Eco*RI and phosphate groups is extended to the nonamer; (II) the modification of a few internucleotide bonds within this nonamer by sulfur or an ethoxy group caused significant or complete protection from nucleolytic action of *Eco*RI; and (III) the inhibitory effect of the modification of certain phosphates within or close to the canonical sequence by sulfur or an ethoxy group manifests the phenomenon of *Eco*RI diastereoselectivity, i.e., enzyme activity toward one of the diastereomers of the substrate.

MATERIALS AND METHODS

1. General. *Eco*RI endonuclease was isolated from an overproducing strain, according to the procedure described by Bickle et al. (1977). Other enzymes were obtained from Boehringer. All solvents and chemicals were either DNA synthesis grade or of the quality necessary in enzyme studies. HPLC was performed using an LDC Milton Roy instrument. T_m values were measured on a Specord M4O (Carl Zeiss Jena).

2. Synthesis and Purification of Phosphorothioate and Phosphotriester Analogues of Oligonucleotides. Oligonucleotides were synthesized as described (Uznanski et al., 1986; Koziolkiewicz et al., 1989). The purification of oligonucleotides and their separation into diastereomers were carried out by RP-HPLC.[1] The phosphorothioate and phosphotriester

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[1] Abbreviations: DMT, dimethoxytrityl; RP-HPLC, high-performance liquid chromatography on reversed phase; svPDE, snake venom phosphodiesterase; dN′, dN″, deoxyribonucleosides; d[N′$_{P(OEt)}$N″], *O*-ethyl phosphotriester analogue of dinucleotide d[N′N″]; d[...N′$_{P(OEt)}$N″...], *O*-ethyl phosphotriester analogue of oligonucleotide; d[N′$_{P(S)}$N″], phosphorothioate analogue of dinucleotide d[N′N″]; d[...N′$_{(PS)}$N″...], phosphorothioate analogue of oligonucleotide; T_m, melting temperature of oligonucleotides.

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Table I: Isolation of Unmodified Oligonucleotides **1–3** and **9** by RP-HPLC[a]

compd	sequence 5′ → 3′	5′-DMT mode[b] of sepn	5′-DMT ret time (min)	5′-HO mode[c] of sepn	5′-HO ret time (min)
1	d[GGAATTCC]	A	21.50	B	9.00
2	d[GGGAATTCCC]	A	15.50	B	10.00
3	d[GAAGGGAATTCCCTTC]	A	16.00	B	10.20
9a	d[AAGAATTCCC]	A	15.00	B	11.00
9b	d[GGGAATTCTT]	A	16.00	B	11.00

[a] Column: μBondapak C_{18} (300 × 7.8 mm); flow rate, 3.5 mL/min. [b] A: 5–30% CH_3CN–0.1 M triethylammonium bicarbonate (TEAB) in 20 min (exponent 0.25), then isocratic mode. [c] B: linear gradient, 5–30% CH_3CN–0.1 M TEAB, 1.25%/min.

analogues were first isolated as 5′-DMT derivatives and, after detritylation with a 20% (v/v) solution of CH_3COOH, as 5′-HO oligonucleotides. Oligonucleotides **4c** and **4d** were obtained from d[GGA$_{P(S)(OEt)}$ATTCC] and d[GGAA$_{P(S)(OEt)}$TTCC], respectively, which were separated into diastereomers and then dealkylated by NH_4OH (Koziolkiewicz et al., 1989). The HPLC conditions used for the purification of oligonucleotides and for their separation into diastereomers are presented in Tables I–III.

3. Assignment of the Absolute Configuration at the P Chiral Center in Phosphorothioate Analogues of Oligonucleotides. The absolute configuration at the P chiral center in each of two diastereomers of oligonucleotides **4** and **5** was assigned enzymatically using snake venom phosphodiesterase (svPDE, EC 3.1.4.1) and nuclease P1 (P1, EC 3.1.30.1). A 0.25–0.5 A_{260} unit of oligonucleotide **4–5** was digested with 1 μg of nuclease P1 in 200 μL of 100 mM Tris-HCl (pH 7.2) and 1 mM $ZnCl_2$ or with 10 μg of svPDE in 200 μL of 100 mL Tris-HCl (pH 8.5) and 15 mM $MgCl_2$. The digestion mixtures were incubated for 12–16 h at 37 °C. After additional treatment of each digest with alkaline phosphatase (1 μg of the protein for 1 h at 37 °C), the samples were subjected to HPLC analysis. Undigested dinucleoside phosphorothioates d[N′$_{P(S)}$N″] present in the incubation mixtures were compared with genuine samples of d[N′$_{P(S)}$N″] of known absolute configuration by HPLC coinjection (Bryant & Benkovic, 1979; Potter et al., 1983; Stec et al., 1984).

4. Assignment of Absolute Configuration at the Chiral Phosphorus Atom in Phosphotriester Analogues of Oligonucleotides. The absolute configuration at the P chiral center in these oligonucleotides was assigned after treatment with svPDE. Each diastereomer of **7** (0.5 A_{260} unit) was digested with svPDE as described for the phosphorothioate analogues. Dinucleoside *O*-ethyl phosphotriesters are not hydrolyzed by svPDE, and their comparison with genuine samples of d[N′$_{P(OEt)}$N″] of known absolute configuration by HPLC coinjection (Guga et al., 1987) allowed us to assign the absolute configuration of the oligonucleotide substrate.

5. Digestion of Oligonucleotides **1–8** *with EcoRI Endonuclease.* *Eco*RI endonuclease digestions were performed using 0.5 A_{260} unit of oligonucleotides **1–8** (3.62–7.25 μM) in 400 μL of buffer A containing 10 mM Tris-HCl (pH 7.6), 80 mM NaCl, and 20 mM $MgCl_2$ in the presence of 100 units of *Eco*RI endonuclease (0.2 μg of the protein, 3 pmol of the enzyme in its dimer form). One unit of the enzyme cleaves 1.0 μg of λ-DNA in 1 h at 37 °C. Reaction mixtures containing oligonucleotides **1**, **2**, and their analogues were incubated at 16 °C, while those containing oligonucleotide **3** and its analogues were incubated at 25 °C. Aliquots were removed after 3, 6, 9, 12, and 24 h, heat-denatured, and analyzed by HPLC. In all reactions, one of three components was identified: dimer d[GG] for analogues of octanucleotide **1**, trimer d[GGG] for analogues of **2**, and hexamer d[GAAGGG] for analogues of **3**. The identity of the components was confirmed by coinjections with genuine samples of d[GG], d[GGG], or d[GAAGGG].

6. EcoRI-Catalyzed Hydrolysis of Heteroduplex **9a** × **9b**. The hydrolysis of heteroduplex **9a** × **9b** was performed as described above except that the incubation was carried out at 35 °C in order to destabilize the undesired homoduplex (**9a**)$_2$ and (**9b**)$_2$ structures. Since the T_m values of double-stranded homoduplexes are lower than that of the **9a** × **9b** heteroduplex (Table IV), incubation at 35 °C resulted in a decrease in the homoduplex stability. At 35 °C, the double-stranded forms of (**9a**)$_2$, (**9b**)$_2$, and **9a** × **9b** represented 18%, 20%, and 75%, respectively, as determined according to Brennan and Gumport (1985).

7. EcoRI-Catalyzed Hydrolysis of d[GGAATTCC] Performed in the Presence of Phosphotriester Analogues of d[GGGAATTCCC]. Each sample (in 80 μL of buffer A, see paragraph 5) containing 0.2 A_{260} unit of **1** (1.16 nmol, 14.45 μM) was heated to 60 °C. The samples contained modified oligonucleotide **7** [in 80 μL of buffer A, 0.25 or 0.5 A_{260} unit (0.95 or 1.9 nmol, 14.85 or 29.70 μM)] were prepared in the same way. Each sample containing octamer **1** was mixed with one of the samples containing modified oligonucleotide **7**. These mixtures were cooled to 16–18 °C. *Eco*RI endonuclease (40 units) was added to each sample. After 30, 60, 90, and 120 min, 30-μL aliquots of the digestion mixtures were removed, heat-denatured, and analyzed by HPLC. The quantities of d[GG] in these samples were compared to the control sample digested in the absence of modified decamer.

RESULTS

We have analyzed octa-, deca-, and hexadecanucleotides containing the recognition hexamer ...GAATTC... and bearing a single phosphorothioate or phosphotriester group at the preselected position as substrates for *Eco*RI endonuclease. Since analogues of the octanucleotide d[GGAATTCC] used in the first stage of our studies appeared not to cover the DNA binding site of *Eco*RI, we have subsequently synthesized analogues of the decanucleotide d[GGGAATTCCC] and the hexadecanucleotide d[GAAGGGAATTCCCTTC] (Tables I–III).

The assignment of absolute configuration at the P chiral center of phosphorothioate analogues of oligonucleotides was performed by their alternative treatment with two phosphodiesterases, svPDE and nuclease P1, which are known to be diastereoselective (Bryant & Benkovic, 1979; Potter et al., 1983) toward P-chiral phosphorothioate dimers (Figure 1). The absolute configuration at the phosphorus atom of modified internucleotide bonds in *O*-ethyl phosphotriester analogues was assigned using the chemical–enzymatic method (Guga et al., 1987).

Products of *Eco*RI endonuclease-catalyzed digestion of oligonucleotides **1–8** were analyzed by HPLC under conditions allowing the complete separation of the oligonucleotide constituents. The relative extent of hydrolysis of the oligomers is presented in Tables II and III. All experiments measuring *Eco*RI-catalyzed hydrolysis of modified substrates were performed under conditions allowing the complete degradation of the corresponding unmodified standard oligonucleotide (for **4** it was octamer d[GGAATTCC], for **5** and **7** it was decamer d[GGGAATTCCC], and for **6** and **8** it was hexadecamer d[GAAGGGAATTCCCTTC]). On the basis of these results,

Table II: HPLC Isolation and Separation of the Diastereomers of Phosphorothioate Analogues of Oligonucleotides **4–6** and the Results of Their *Eco*RI-Catalyzed Hydrolysis[c]

		5′-DMT		5′-HO			
compd	sequence 5′ → 3′	mode of sepn	ret time (min)	mode of sepn	ret time (min)	abs config[a]	% of digestion[b]
4a	d[$G_{P(S)}$GAATTCC]	A	18.4	B	18.1	R_P	20
			19.8		18.9	S_P	0
4b	d[G$G_{P(S)}$AATTCC]	A	19.2	C	14.7	R_P	15
			21.8		14.7	S_P	0
4c	d[GG$A_{P(S)}$ATTCC][d]					R_P	10
						S_P	0
4d	d[GGA$A_{P(S)}$TTCC][e]					R_P	50
						S_P	100
4e	d[GGAA$T_{P(S)}$TCC]	F	10.9	G	17.4	R_P	100
			10.9		17.9	S_P	100
4f	d[GGAATT$_{P(S)}$CC]	F	11.2	G	19.9	R_P	100
			11.2		17.7	S_P	100
4g	d[GGAATTC$_{P(S)}$C]	F	11.9	G	17.1	R_P	100
			11.9		17.6	S_P	100
5a	d[$G_{P(S)}$GGAATTCCC]	H	23.5	B	17.7	R_P	100
			24.6		18.1	S_P	100
6a	d[$G_{P(S)}$AAGGGAATTCCCTTC]	D	15.1	E	10.2	NS[f]	100
6b	d[GA$_{P(S)}$AGGGAATTCCCTTC]	D	16.4	E	10.4	NS	100
6c	d[GAA$_{P(S)}$GGGAATTCCCTTC]	D	16.5	E	11.2	NS	100

[a] Absolute configuration at chiral phosphorus atom. [b] % of digestion after 24 h under conditions allowing 100% degradation of corresponding unmodified standard. [c] Isolation and separation of oligomers **4–6** were performed on a column with the following gradients (μBondapak C_{18} (300 × 7.8mm)): A, linear gradient of CH_3CN in 0.1 M TEAA (triethylammonium acetate) (pH 7.0), 20–40% CH_3CN–0.1 M TEAA, 0.5%/min, flow rate 5 mL/min; B, 5–20% CH_3CN–0.1 M TEAA, 0.5%/min, 5 mL/min; C, 5–30% CH_3CN–0.1 M TEAA, 1.0%/min, 4 mL/min; D, 5–30% CH_3CN–0.1 M TEAB (exp. 0.25), t = 20 min, 3.5 mL/min; E, 5–20% CH_3CN–0.1 M TEAB, 0.75% min, 3.5 mL/min; F, 20–40% CH_3CN–0.1 M TEAA, 1.0% min, 4 mL/min; G, 5–20% CH_3CN–0.1 M TEAA, 0.5%/min, 4 mL/min; H, 20–40% CH_3CN–0.1 M TEAA, 0.33%/min, 4 mL/min. [d,e] Obtained after the separation and dealkylation of d[GGA$_{P(S)(OEt)}$ATTCC] (*d*) and d[GGAA$_{P(S)(OEt)}$TTCC] (*e*), respectively (Koziolkiewicz et al., 1989). [f] NS, diastereomers not separated.

Table III: HPLC Separation of the Diastereomers of *O*-Ethyl Phosphotriester Analogues of Oligonucleotides (**7** and **8**) and the Results of Their *Eco*RI-Catalyzed Hydrolysis.

compd	sequence 5′ → 3′	5′-DMT ret time[a] (min)	5′-OH ret time[b] (min)	abs config[c]	% of digestion[d]	T_m[e] (°C)
7a	d[$G_{P(OEt)}$GGAATTCCC]	19.00	11.00	R_P	0	43.0
		21.00	11.00	S_P	0	43.0
7b	d[GG$_{P(OEt)}$GAATTCCC]	19.50	10.50	R_P	0	46.5
		21.00	10.50	S_P	0	47.5
7c	d[GGG$_{P(OEt)}$AATTCCC]	20.00	10.75	R_P	0	45.0
		21.00	10.50	S_P	0	46.0
7d	d[GGGA$_{P(OEt)}$ATTCCC]	18.50	11.00	R_P	0	39.5
		20.00	10.50	S_P	0	46.5
7e	d[GGGAA$_{P(OEt)}$TTCCC]	19.00	11.50	R_P	0	40.0
		22.00	11.00	S_P	0	47.5
7f	d[GGGAAT$_{P(OEt)}$TCCC]	16.00	11.50	R_P	10	38.5
		18.00	11.30	S_P	100	47.5
7g	d[GGGAATT$_{P(OEt)}$CCC]	16.00	11.00	R_P	20	42.0
		18.00	11.25	S_P	10	48.0
7h	d[GGGAATTC$_{P(OEt)}$CC]	16.00	11.00	R_P	20	43.5
		18.00	11.00	S_P	15	47.0
7i	d[GGGAATTCC$_{P(OEt)}$C]	19.00	10.70	R_P	100	ND
		20.00	10.70	S_P	100	ND
8a	d[$G_{P(OEt)}$AAGGGAATTCCCTTC]	16.20	11.15	NS[f]	100	ND[f]
8b	d[GA$_{P(OEt)}$AGGGAATTCCCTTC]	16.25	11.15	NS	100	ND
8c	d[GAA$_{P(OEt)}$GGGAATTCCCTTC]	16.50	11.40	NS	100	ND

[a] Mode of separation: μBondapak C_{18} (300 × 7.8 mm) column, flow rate 3.5 mL/min. Gradient 5–30% CH_3CN–0.1 M TEAB, t = 20 min (exponent 0.25), between 20 and 25 min. Isocratic mode: 30% CH_3CN–0.1 M TEAB. [b] Same column, gradient 5–30% CH_3CN–0.1 M TEAB, 1.25%/min. [c] Absolute configuration at chiral phosphorus atom. [d] % of digestion after 24 h under conditions allowing 100% degradation of corresponding unmodified standard. [e] T_m values were measured in buffer A: 10 mM Tris-HCl (pH 7.6), 80 mM NaCl, and 20 mM $MgCl_2$; T_m value for unmodified decamer d[GGGAATTCCC] = 47.0 °C. [f] ND, no determined; NS, diastereomers not separated.

Table IV: T_m Values for Decanucleotides **9**

compd	sequence	T_m (°C)
9a	(d[AAGAATTCCC])$_2$	27.0
9b	(d[GGGAATTCTT])$_2$	28.0
	9a × **9b**	42.0

we have attempted to identify the internucleotide bonds involved in the formation of the DNA–*Eco*RI complex.

It has been previously shown that *Eco*RI exhibits diastereoselectivity toward the R_P isomer of the octanucleotide d[GG$_{P(S)}$AATTCC] (Connolly et al., 1984b). In order to study the role of other internucleotide bonds involved in the recognition and cleavage of canonical sequence by *Eco*RI, we have used octanucleotides bearing a phosphorothioate function in the positions flanking the "scissile bond", namely, d[$G_{P(S)}$GAATTCC] (**4a**), d[GGA$_{(P)S}$ATTCC] (**4c**), and d[GGAA$_{(P)S}$TTCC] (**4d**), as substrates for the enzyme. The presence of the phosphorothioate function between two guanosine or two adenosine residues caused the S_P isomers of these octanucleotides to be resistant to *Eco*RI endonuclease.

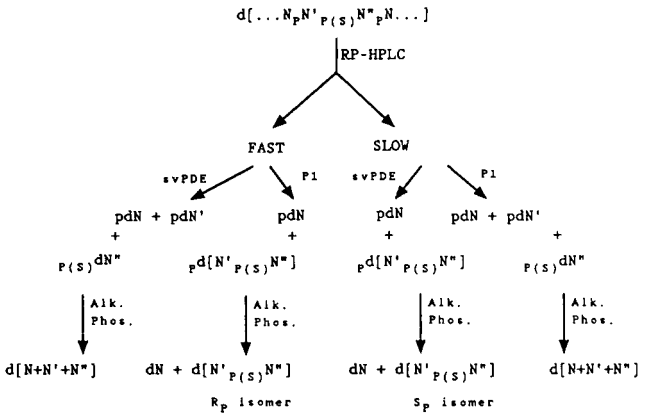


FIGURE 1: Assignment of absolute configuration at the phosphorus atom in the modified internucleotide bond of the oligonucleotide d[...NpN'P(S)N"pN...]. FAST and SLOW = description of the diastereomers with different retention times during RP-HPLC separation of the modified oligonucleotide.

Scheme I

x - phosphates which are affected by phosphorothioate modification

^ - phosphates which are affected by O-ethyl phosphortriester modification

The R_P counterparts were hydrolyzed by *Eco*RI, but the extent of hydrolysis was reduced to 20% and 10%, respectively, as compared to that of d[GGAATTCC]. While these experiments revealed the diastereoselectivity of *Eco*RI toward R_P isomers of **4a** and **4c**, the d[GGAA$_{(P)S}$TTCC] (**4d**) substrate produced a different result. *Eco*RI endonuclease catalyzed the hydrolysis of both isomers of **4d**, but with different extents of cleavage. Under conditions where 100% hydrolysis was observed for standard d[GGAATTCC], 100% of (S_P)-d[GGAA$_{(P)S}$TTCC] was cleaved, compared to 50% for (R_P)-d[GGAA$_{(P)S}$TTCC]. Similar results for the *Eco*RI-catalyzed hydrolysis of these substrates were also reported by Gallo et al. (1986). However, since the HPLC separation of the diastereoisomers of **4d** appeared not to be effective, these researchers used a mixture of R_P and S_P isomers as the substrate for *Eco*RI.

The isomers of octanucleotides d[GGAAT$_{P(S)}$TCC] (**4e**), d[GGAATT$_{P(S)}$CC] (**4f**), and d[GGAATC$_{P(S)}$C] (**4g**) were hydrolyzed to the same extent as the unmodified octamer d[GGAATTCC], independent of the absolute configuration at the P chiral center.

O-Ethyl phosphotriester analogues of decamer d[GGGAATTCCC] and hexadecamer d[GAAGGGAATTCCCTTC] were synthesized as described previously (Uznanski et al., 1986; Koziolkiewicz et al., 1989). Purification and separation into diastereomers were performed by HPLC under conditions presented in Table III. *Eco*RI-catalyzed hydrolysis of separated isomers of decanucleotides demonstrated that ethylation of phosphates located at positions –II, –I, +I, +II, and +III (see Scheme I) effectively hampered the cleavage of the internucleotide phosphate between G and A. The ethylation of positions +V and +VI caused a dramatic decrease in the extent of hydrolysis, independent of the absolute configuration at the phosphorus atom in these positions. *Eco*RI endonuclease appeared to be diastereoselective only toward the isomers of decanucleotide **7f** containing a phosphotriester function located at position +IV. Cleavage of the S_P isomer was equivalent to that of the unmodified substrate (100%), while only 10% of the R_P isomer was hydrolyzed under the same conditions.

The diastereoselectivity of *Eco*RI endonuclease toward the isomers of **7f** containing an *O*-ethyl function away from the scissile bond prompted us to carry out additional experiments. We decided to construct a double-stranded substrate containing an *O*-ethyl function in only one strand; therefore, we undertook the synthesis of decamers d[AAGAATTCCC] (**9a**), d[GGGAATTCTT] (**9b**), and d[AAGAAT$_{P(OEt)}$TCCC] (**9c**). Unfortunately, the separation of the diastereomers of **9c** by HPLC was unsuccessful, and in further experiments we were forced to use their mixture. Heteroduplexes d[AAGAATTCCC] × d[GGGAATTCTT] (**9a** × **9b**) and d[AAGAAT$_{P(OEt)}$TCCC] × d[GGGAATTCTT] (**9c** × **9b**) were incubated with *Eco*RI endonuclease at 35 °C, and the reaction mixtures were analyzed by HPLC. Because of the different nucleotide composition of the flanking sequences (Alves et al., 1984) in the decamers forming duplex **9a** × **9b**, the extent of hydrolysis was different. After 1 h of incubation, d[AAGAATTCCC] was 95% hydrolyzed while d[GGGAATCTT] was only 45% digested. Under the same conditions, d[AAGAAT$_{P(OEt)}$TCCC] (**9c**) was 76% hydrolyzed, while the opposite strand in duplex **9b** × **9c** (i.e., d[GGGAATTCTT]) was 25% hydrolyzed.

To examine the stability of the duplex form of *O*-ethyl phosphotriester analogues, T_m measurements were performed (Table III). The T_m values for R_P isomers of **7d–g**, although apparently lower than the T_m for decamer **2**, were still much higher than the temperature of *Eco*RI digestion (16 °C). Thus, under standard reaction conditions, the modified *Eco*RI substrates **7a–h** were present in the duplex forms.

The modification of specific internucleotide bonds could interfere with either binding or hydrolysis of the modified substrates. We have indirectly tested the binding of the enzyme to the modified substrates by measuring the hydrolysis of the unmodified oligomer in the presence of the modified competitor. None of the modified phosphotriester analogues used at 14.85 or 29.7 μM affected the hydrolysis of the d[GGAATTCC] octamer at a concentration of 14.45 μM. Apparently, the modified substrates do not bind to the enzyme.

DISCUSSION

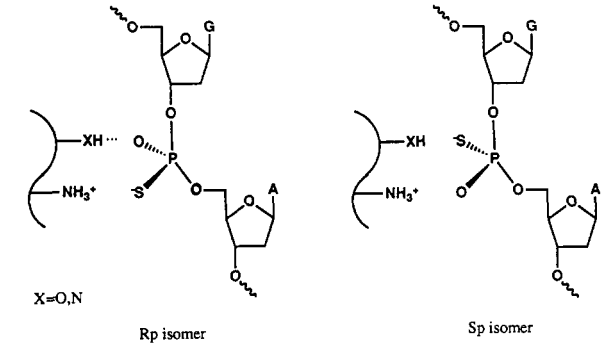
The importance of internucleotide phosphates in sequence-specific interactions between DNA and proteins has been well documented only recently (McClarin et al., 1986; Anderson et al., 1987; Becker et al., 1988; Lesser et al., 1990; Otwinowski et al., 1988). Until now, phosphate contacts between DNA and proteins were identified by the "ethylation interference" method (Siebenlist & Gilbert, 1980; Siebenlist et al., 1980; Lu et al., 1981). However, this approach precluded studies on the role of the spatial orientation of replaced nonbridging oxygen atoms. We decided to study this problem using phosphorothioate and *O*-ethyl phosphotriester analogues of oligonucleotides bearing the *Eco*RI canonical sequence. As a prerequisite step, it was necessary to assign the spatial orientation of the function modifying the internucleotide bond. We have performed these assignments using enzymatic and chemical–enzymatic methods for phosphorothioates and *O*-ethyl phosphotriesters, respectively (Gallo et al., 1986; Guga et al., 1987). The assignment of the absolute configuration as R_P indicates that the sulfur atom or the ethoxy group is oriented into the major groove of the DNA molecule ("inward" orientation). In the S_P configuration these substituents are oriented "outward" of the DNA molecule (Summers et al., 1986).

The use of phosphorothioates and phosphotriesters as the substrates for *Eco*RI endonuclease indicated that modification of internucleotide bonds within or near the *Eco*RI canonical sequence strongly affected the nucleolytic activity of the enzyme.

Phosphorothioate analogues of octamer d[GGAATTCC] allowed the identification of four internucleotide bonds involved in the DNA–*Eco*RI interaction (–I, +I, +II, +III; Scheme I). In further studies, decanucleotide d[$G_{P(S)}$GGAATTCCC] (**5a**) and three hexadecanucleotides **6a–c** were used to identify the role of internucleotide bonds: –II, –III, –IV, and –V. Both isomers of decamer **5a** were hydrolyzed to the same extent as the unmodified decamer. Similarly, the presence of a phosphorothioate function near the 5′ end of hexadecanucleotides **6a–c** did not affect the extent of hydrolysis. These results confirmed that only four internucleotide bonds (–I, +I, +II, +III) were involved in the DNA–*Eco*RI interaction. It should be pointed out that, contrary to our conclusions, Eckstein et al. observed only a minor inhibitory effect on the rate of *Eco*RI hydrolysis using DNA with phosphorothioates in only one strand as the substrate (Taylor et al., 1985; Eckstein, 1986). However, in a more recent report these authors suggest that phosphorothioate modifications of phosphates adjacent to the scissile bond may protect DNA from the *Eco*RI cleavage (Eckstein & Gish, 1989).

While the replacement of phosphate oxygen by sulfur influences the charge distribution in the internucleotide bond (Frey & Sammons, 1985), esterification eliminates the charge and introduces an important additional factor, namely, steric hindrance. Four internucleotide bonds involved in the DNA–*Eco*RI recognition (–I, –II, +I, +III) (see Scheme I) were identified using esterification of internucleotide bonds by ethylnitrosourea (Lu et al., 1981). Since our earlier results using separated isomers of phosphorothioate analogues of DNA indicated the importance of the stereochemical orientation of nonbridging oxygen atoms, we undertook similar studies using separated isomers of *O*-ethyl phosphotriester analogues. The sequences of oligonucleotides containing *O*-ethyl phosphotriester and phosphorothioate functions were identical; thus, it was possible to compare the biological effects of these modifications. The nucleolytic activity of the enzyme was strongly inhibited when the oxygen atom was esterified by an ethyl group in one of eight positions (from –II to +IV). Most likely, the effects of phosphorothioate and phosphotriester modifications on *Eco*RI specificity differ due to steric differences between these DNA analogues, because ester modification introduces a profound increase in "steric bulk" of modified oligonucleotide. It should be noted that the presence of the rather bulky ethyl group in the internucleotide bond may prevent the formation of the DNA–enzyme complex by steric hindrance or may change the conformation of oligonucleotide. The change of conformation is more possible in the case of R_P isomers due to the inward orientation of the ethyl group. The conformation of S_P isomers containing an ethyl group oriented outward is expected to be the same as that of unmodified oligonucleotides. The analysis of T_m values assigned for R_P and S_P isomers of **7** clearly indicated that the stability of homoduplexes strongly depends on the orientation of the ethyl group. S_P isomers possess T_m parameters similar to that of unmodified decamer **2**, while R_P isomers have significantly lowered T_m values. Although the conformation of the duplex formed by S_P isomers of **7** may be unchanged, outward-oriented ethyl groups may be responsible for shielding internucleotide bonds from contact groups at the protein. Also, ethyl groups oriented into the major groove of DNA (inward orientation) may prevent the formation of the DNA–enzyme complex, since the majority of DNA–protein contacts is assumed to be at the major groove. Thus, although the modified bond may not directly interact with the protein, it may indirectly affect the DNA–protein binding.

Scheme II



The comparison of *Eco*RI-catalyzed hydrolysis of phosphorothioates and phosphotriesters prompts us to formulate a plausible model describing the contacts between *Eco*RI and DNA phosphates. In general, this model proposes that both nonbridging heterotopic oxygen atoms of the phosphate moiety can act as a hydrogen-bond acceptor or a salt-link partner. The symmetry of charge distribution at the internucleotide phosphate allows localization of negative charge on *pro-R* or *pro-S* oxygens according to the requirements of charge distribution at the surface of the approaching *Eco*RI. Elemental replacement of either of two oxygens by sulfur disturbs the flexible functionality of the phosphate, since preferable charge localization at the sulfur atom (Frey & Sammons, 1985) and the hydrogen-bond-acceptor function of the "neutral" oxygen fit only one spatial arrangement of functional groups at the protein. In this arrangement the sulfur interacts with the positively charged side chain of the corresponding amino acid, and the oxygen is involved in a hydrogen-bond-type interaction. Such an arrangement may improve binding free energy, while the opposite absolute configuration at phosphorus would require charge localization at oxygen and the involvement of neutral sulfur in hydrogen-bond interactions, which apparently should have an unfavorable effect on binding. Although there are indications of bidentate chemical reactivity of phosphorothioate anions (Mastryukova, 1976), undoubtedly the ability of the phosphorothioate sulfur to act as a hydrogen-bond acceptor is remarkably lower than that of oxygen (Sherry & Purcell, 1972). Thus, only one phosphorus configuration of the internucleotide phosphorothioate mimics the functionality of the natural phosphate.

The substitution of the *pro-S* oxygen atom at the internucleotide phosphate between G and A (scissile bond) with sulfur makes this bond resistant to *Eco*RI cleavage, while similar substitution of the *pro-R* oxygen only decreases the extent of hydrolysis (Connolly et al., 1984b). According to the above model, the *pro-R* oxygen of natural phosphate may form an ionic interaction with a basic amino acid, while the *pro-S* oxygen atom serving as proton acceptor may be engaged in H-bond formation. (R_P)-d[$GG_{P(S)}$AATTCC] is hydrolyzed by *Eco*RI because these two requirements are fulfilled. In contrast, the S_P isomer of d[$GG_{P(S)}$AATTCC] is not hydrolyzed by the enzyme because opposite orientations of the sulfur atom and the uncharged oxygen atom are not favorable for contacts with the protein and/or prevent kinetically competent interaction (Scheme II). Due to the semiquantitative char-

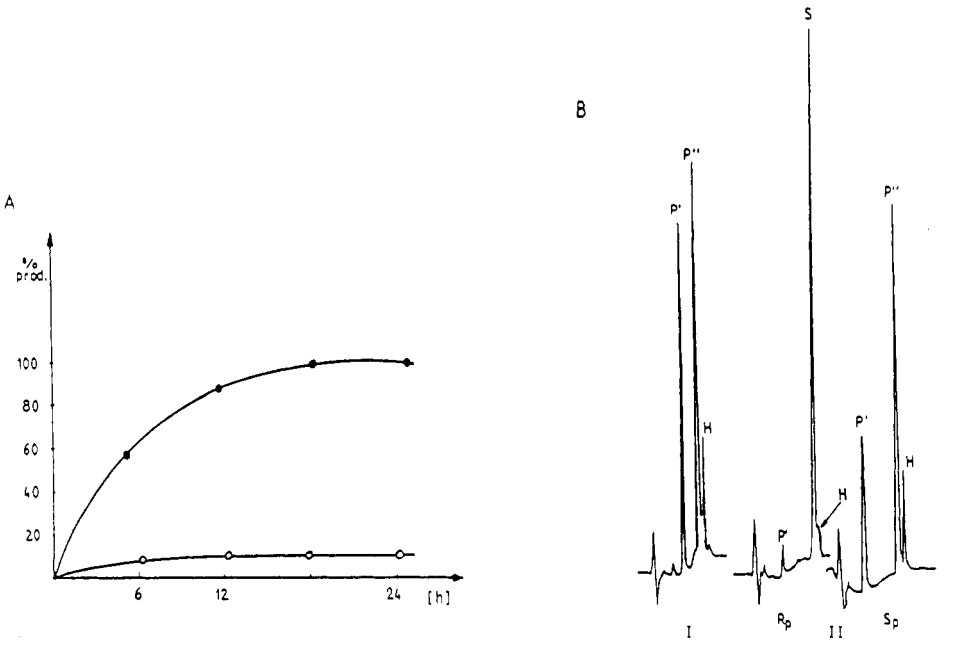


FIGURE 2: (A) Extent of *Eco*RI-catalyzed hydrolysis of R_P (O) and S_P (●) diastereomers of d[GGGAAT$_{P(OEt)}$TCCC] (**7f**). (B) HPLC profiles from the cleavage of d[GGGAATTCCC] (I) and the R_P and S_P isomers of d[GGGAAT$_{P(OEt)}$TCCC] (II) by the *Eco*RI endonuclease under the conditions described in Materials and Methods: P′, d[GGG], P″, pd[AATTCCC] in I or pd[AAT$_{P(OEt)}$TCCC] in II, H, d[AATTCCC] or d[AAT$_{P(OEt)}$TCCC], obtained as the products of the action of phosphatase contaminating the *Eco*RI sample, S, d[GGGAAT$_{P(OEt)}$TCCC].

acter of the data presented, this model should be considered only as a hypothesis explaining the *Eco*RI diastereoselectivity observed for phosphorothioate analogues. However, in our opinion, it seems to give reasonable elucidation of this phenomenon. This model can be applicable not only for the scissile phosphate but also for other internucleotide bonds engaged in the interaction with *Eco*RI protein. It is postulated that "contact" phosphates at –I, +I, +II, and +III are each involved in "directional-two-point" interactions with *Eco*RI protein (see below). We suggest that, in interactions with proteins, the simultaneous involvement of both heterotopic oxygen atoms of the contact phosphate group is a critical factor responsible for the diastereoselectivity of several, perhaps all, phosphonucleases toward phosphorothioate analogues (Gerlt et al., 1983).

Although the internucleotide bonds –I and –II are localized outside of the *Eco*RI canonical sequence, they play an important role in the process of the *Eco*RI–DNA complex formation (Lu et al., 1981; Becker et al., 1988; Lesser et al., 1990). We have observed that the R_P isomer of d[G$_{P(S)}$GAATTCC] (**4a**) containing a phosphorothioate function at position –I was hydrolyzed by *Eco*RI (Stec et al., 1984), while its S_P counterpart, as well as the R_P and S_P isomers of d[GG$_{P(OEt)}$GAATTCCC], were resistant to *Eco*RI digestion.

The phosphate at position –II is also involved in the interaction with *Eco*RI protein (Lu et al., 1981; Becker et al., 1988; Lesser et al., 1990). While the substitution of *pro-R* and *pro-S* oxygens with sulfur does not affect the *Eco*RI-catalyzed hydrolysis, esterification of these oxygens makes both isomers of **7a** completely resistant to *Eco*RI action. Photofootprinting studies (Becker et al., 1988) have suggested that contacts between *Eco*RI and positions –I and –II strongly constrain the rotational mobility of the two upstream base pairs on each strand. These phosphate contacts act as clamps to stabilize the kinked DNA conformation in the *Eco*RI–DNA complex (McClarin et al., 1986; Lesser et al., 1990). The substitution of sulfur for the oxygen atoms (at position –I) or their ethylation (at positions –I and –II) may interfere with the binding of the protein. However, the lack of diastereoselectivity toward (R_P)- and (S_P)-**5a** may indicate that at position –II a hydrogen-bond-type interaction with protein does not take place and that this particular phosphate may interact with protein only via a salt-link-type contact, preventing ethylation by ethylnitrosourea. While **5a** may interact with the enzyme due to allowed flexibility of the cationic residue of protein at this particular position, esterified phosphate in both diastereoisomers of oligonucleotide **7a** prevents contact with the enzyme.

Phosphorothioate or phosphotriester modification of the internucleotide bond between the two adenosine residues (position +II) also affected DNA–*Eco*RI interactions. The (R_P)-phosphorothioate analogue of **4c** was hydrolyzed by *Eco*RI endonuclease, but the extent of hydrolysis was lower than that observed for the unmodified decamer (Table II). In contrast, the S_P isomer of **4c**, as well as both isomers of d[GGGA$_{P(OEt)}$ATTCCC] containing an *O*-ethyl function at the +II phosphate, was completely resistant to *Eco*RI action. Ethylation of oligomers containing the *Eco*RI canonical sequence revealed that modification at this position has no effect on enzyme binding (Lu et al., 1981; Lesser et al., 1990; Becker et al., 1988), but strongly inhibits the cleavage of the scissile bond. The most significant difference between the DNA–*Eco*RI binding and cleavage is the absence and presence of magnesium cations, respectively. It is possible that coordination of magnesium ion to the phosphate between the two adenosine residues may be required for enzyme-catalyzed hydrolysis. Alternatively, the enzyme conformation may be affected by magnesium ions resulting in "allosteric activation" (McClarin et al., 1986; Needels et al., 1989; Wright et al., 1989). It is possible that such an activated form of enzyme requires an additional phosphate contact at position +II for the cleavage.

The phosphate at the position +III is also strongly affected by phosphorothioate or phosphotriester modification. Although both phosphorothioate isomers of **4d** were hydrolyzed by *Eco*RI endonuclease, the enzyme was more selective toward

(S_P)-d[GGAA$_{P(S)}$TTCC]. As indicated in separate report, the strand containing (S_P)-phosphorothioate between A and T is cleaved 2–3 times more rapidly than a strand containing a normal prochiral phosphate, whereas a strand containing (R_P)-phosphorothioate is cleaved about 3 times slower than normal.[2] This result may indicate that the "double-bonded" oxygen of phosphorothioate in the S_P isomer, localized in the DNA major groove, is involved in hydrogen bonding with an amide NH in the polypeptide backbone of the endonuclease, while negatively charged sulfur interacts with the positively charged residue of another amino acid. Better localized charge in this phosphorothioate improves its "fitness", compared to that with the prochiral phosphate having intermediate P–O bond order and delocalized charge. In this case directional-two-point contact is reversed with regard to that postulated in Scheme II. The ethylation of nonbridging oxygen atoms of phosphate +III generates modified substrates completely resistant to *Eco*RI hydrolysis. It is important to note that position +III coincides with "neo-kink I" (McClarin et al., 1986) and is strongly engaged in the stabilization of the *Eco*RI–DNA complex (Lesser et al., 1990).

The presence of a phosphorothioate function at positions +IV, +V, or +VI had no effect on the extent of hydrolysis of modified oligonucleotides **4e–g**. This suggests that these phosphates are not involved in DNA–*Eco*RI interactions. However, ethylation of one of the nonbridging oxygen atoms at the same positions strongly affected the hydrolysis of oligonucleotides **7f–h**.

The results of hydrolysis of (R_P)-d[GGGAAT$_{P(OEt)}$TCCC] (**7f**) and its S_P counterpart are particularly interesting. The S_P isomer was cleaved by *Eco*RI to an extent comparable to that observed for the unmodified decamer. For the R_P isomer, the extent of digestion was only 10% under the same conditions (Figure 2). It has been proposed that phosphate +IV is engaged in the formation of a salt bridge with the side chain of the basic amino acid (Jen-Jacobson et al., 1986). The ethylation interference studies performed by Lesser et al. (1990) indicate that ethylation of this phosphate weakly interferes with binding of the enzyme. Since the phosphate between the two residues of thymidine is located almost directly across the scissile bond on the complementary strand, it is possible that its esterification could influence the cleavage of the scissile bond on the opposite strand rather than on the modified strand.

In order to verify this assumption, we synthesized decamers d[AAGAATTCCC] (**9a**), d[GGGAATTCTT] (**9b**), and d[AAGAAT$_{P(OEt)}$TCCC] (**9c**). The comparison of hydrolysis of heteroduplexes **9a** × **9b** and **9b** × **9c** indicated that the presence of an *O*-ethyl function between the two residues of thymidine in **9c** inhibited cleavage of the opposite unmodified strand slightly more effectively than cleavage of the modified one. This result is consistent with the assumption that the esterification at position +IV affects the cleavage on the opposite strand.

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