Stereoselective Interaction with Chiral Phosphorothioates at the Central DNA Kink of the EcoRI Endonuclease-GAATTC Complex*

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We have probed the contacts between EcoRI endonuclease and the central phosphate of its recognition site GAATTC, using synthetic oligonucleotides containing single stereospecific Rp- or Sp-phosphorothioates (Ps). These substitutions produce subtle stereosepecific effects on EcoRI endonuclease binding and cleavage. An Sp-Ps substitution in one strand of the DNA duplex improves binding free energy by -1.5 kcal/mol, whereas the Rp-Ps substitution has an unfavorable effect (+0.3 kcal/mol) on binding free energy. These effects derive principally from changes in the first order rate constants for dissociation of the enzyme-DNA complexes. The first order rate constants for strand scission are also affected, in that a strand containing Sp-Ps substitution is cleaved 2 to 3 times more rapidly than a strand containing a normal prochiral phosphate, whereas a strand containing Rp-Ps substitution is cleaved about 3 times slower than normal. As a result, single-strand substitutions produce pronounced asymmetry in the rates of cleavage of the two DNA strands, and this effect is exaggerated in an Rp-Sp-heteroduplex. Ethylation-interference footprinting indicates that none of the Ps substitutions cause any major change in contacts between endonuclease and DNA phosphates. When an Sp-Ps localizes P=O in the DNA major groove, a hydrogen-bonding interaction with the backbone amide-NH of Gly118 of the endonuclease is improved relative to that with a prochiral phosphate having intermediate P-O bond order and delocalized charge.

Crystallographic images of complexes between DNA and sequence-specific proteins have shown that these proteins form extensive complementary interfaces not only to the DNA bases of the recognition sites, but also to the sugar-phosphate backbone. The tight backbone contacts are mediated by hydrogen bonds from polypeptide main chain amide groups and polar side chains, so as to achieve very precise anchoring and positioning of the base recognition elements (1–4). Our recent studies (5) of the EcoRI endonuclease-DNA interaction have focused on evaluating the energetic consequences of perturbing the complementarity of the interface by modifying the bases or phosphates.

The EcoRI endonuclease appears to be complementary to the DNA backbone over a span of about 12 base pairs (6,7), but ethylation-interference (5) and UV photofootprinting (8) show that only six symmetry-related phosphates (three per strand) are crucial to specificity. These key "clamp" contacts are indispensable to recognition of the canonical base sequence because they anchor and orient the protein recognition helices within the major groove (4) and stabilize the kinked DNA conformation (9) in the complex. Although the "primary clamp" contacts at NpGAATTC are required for any localized binding at GAATTC and closely related sites, the "supplementary clamp" contacts at NpGAATTC show characteristic changes in ethylation-interference when the endonuclease interacts with any site containing 1 incorrect base pair (5). Alteration of the supplementary clamps makes a significant contribution to the energy differential for sequence discrimination (5). A set of weaker phosphate contacts, which includes those at the scissile GpAATTC bonds, contribute nonspecifically to binding free energy.

In ethylation-interference analysis, a bulky ethyl group not only removes a negative charge but may also cause steric hindrance. By contrast, phosphorothioate (Ps)1 substitution produces subtler changes that provide a sharper probe of the interaction with individual DNA phosphates. Whereas a normal prochiral phosphate has P-O bonds of intermediate order and negative charge is delocalized across the two phosphoryl oxygens, the predominant resonance form of a phosphorothioate group is O=PS⁻ (10). Thus, in a phosphorothioate, the bond lengths are different and charge is more localized than in a prochiral phosphate.

Stereospecific phosphorothioate substitutions have been widely used to elucidate the stereochemistry of phosphoryl- and nucleotidyl-transfer reactions (11). For EcoRI endonuclease, it was shown that the Sp-Ps diastereomer at the scissile bond completely inhibits cleavage, whereas the Rp-diastereomer permits cleavage. The Rp-diastereomer was used to demonstrate chemical inversion at the scissile phosphate during hydrolysis (12).

Phosphorothioates can also be used to probe the stereoselective constraints on phosphate contacts that are not directly involved in phosphodiester bond hydrolysis (13–16). For example, whereas Rp-Ps substitution at the scissile bond only partially inhibits cleavage, complete inhibition of cleavage of

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1 The abbreviations used are: Ps, phosphorothioate; nt, nucleotide(s); HPLC, high performance liquid chromatography; HPTLC, high performance TLC; bis-tris, bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane.
the phosphorothioate DNA strand can be achieved when additional Ps substitutions are introduced at other positions within and adjacent to the recognition site (16–18). Preliminary studies (13, 15, 19) suggest that each contact between the endonuclease and an individual DNA phosphate has particular stereospecific requirements.

In this study, we demonstrate an exquisitely subtle modulation of the interaction with the key clamping contact at the central kink in the EcoRI recognition site GAAPpTTTC by a single stereospecific substitution of sulfur for one of the nonbridging phosphoryl oxygens. Whereas the R<sub>p</sub>-Ps substitution has an unfavorable effect (about +0.3 kcal/mol) on binding free energy, the S<sub>p</sub>-Ps-substituted DNA interacts better than the unsubstituted site, improving the binding free energy by about −1.5 kcal/mol. The introduction of this subtle structural asymmetry into the site also leads to energetic distinction between the transition states for chemical cleavage in each of the DNA strands: the S<sub>p</sub>-Ps-substituted strand of a heteroduplex is cleaved about 3 times faster than the strand containing an unmodified prochiral phosphate, whereas an R<sub>p</sub>-Ps-substituted strand in a heteroduplex is cleaved about 3 times slower than the unmodified strand. These data indicate that localization of P=O in the major groove (by S<sub>p</sub>-Ps substitution) permits more favorable hydrogen bonding interaction with the endonuclease than does a prochiral phosphate.

**MATERIALS AND METHODS**

**General Design of Oligonucleotide Substrates**—All experiments reported here used synthetic duplex 17-nt oligonucleotides (Fig. 1). These substrates carry the GAATTC site off-center so that cleavage in each of the DNA strands gives rise to a distinguishable product. The sequences are the same as those used by Lesser et al. (5). Stereospecific phosphorothioate substitutions were placed in these oligonucleotides by the methods described below.

**Synthesis of 3'-O-DMT-5'-benzoyladenylate-5'-methoxyacetylthymidylyl-O-ethyl Phosphorothioate (d[5'-DMT(A5'Ps(OEt)T-3'-OAc)])—**To a solution of dry tetrazole (1.84 mmol) in a suspension of CH<sub>3</sub>Cl<sub>2</sub> (4 ml), 1.83 mmol of (N,N',N',N'-tetraisopropyl)-O-ethyl phosphorodiamidite (II) was added under dry argon with stirring at room temperature. After 1 h, CH<sub>3</sub>Cl<sub>2</sub> was removed under reduced pressure, the residue was dissolved in 2 ml of dry acetonitrile (3 ml) was added. After 1 h, elemental sulfur (115 mg) was added under dry argon, and stirring was continued overnight.

The reaction mixture was filtered and the filtrate was concentrated in vacuo. The crude product was purified by column chromatography (60 × 60 mm) using 200–400-mesh silica gel (Merck 9385) and elution with CH<sub>3</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH:CH<sub>3</sub>C<sub>2</sub>H<sub>5</sub>OH (81:4). Zones containing diastereomers of product V were cut off from the plate and eluted with CHCl<sub>3</sub>:CH<sub>3</sub>OH:CH<sub>3</sub>C<sub>2</sub>H<sub>5</sub>OH (10:1). The desired diastereomers were pooled and concentrated and then purified by semipreparative HPLC purification was performed on a pBondapak C<sub>18</sub> column (30 cm × 4.6 mm) with an linear gradient of 5–20% CH<sub>3</sub>CN in 0.1 m triethylammonium bicarbonate, pH 7.3 (0.75%/min; flow rate 1.5 ml/min) The diastereomeric purity of the R<sub>p</sub> isomer was >98%, while the purity of the S<sub>p</sub> isomer was 99%.

**In Situ Phosphitylation and Oligonucleotide Synthesis**—Each of the diastereomers of product V (25 mg, approximately 30 pmol) was independently dissolved in 250 µl of dry CH<sub>3</sub>CN and to each of these samples was added tetrazole (50 µmol) and 2'-cyanoethyl(N,N',N',N'-tetraisopropyl)phosphorodiamidite (30 µmol). After 0.5 h, this solution was used as substrate in the appropriate coupling step of automated oligonucleotide synthesis (1 µmol scale) performed on the 380B DNA Synthesizer (Applied Biosystems) using O-β-cyanoethyl phosphoramidite monomers.

After cleavage from the solid support (25% aqueous NH<sub>4</sub>H<sub>2</sub>O, 2 h, 25 °C) and deprotection of bases (25% aqueous NH<sub>3</sub>H<sub>2</sub>O, 24 h, 25 °C), semipreparative HPLC purification was performed on a µBondapak C<sub>18</sub> column (30 cm × 7.8 mm) with an exponential gradient of 5–30% CH<sub>3</sub>COOH in 0.1 m triethylammonium bicarbonate (exponent 0.25; flow 3.5 ml/min; 20 min).

After evaporation of collected fractions containing the desired oligomers, the 5'-O-DMT groups were removed with 20% aqueous acetic acid.

The second HPLC purification was performed on the same column with a linear gradient of 5–20% CH<sub>3</sub>CN in 0.1 m triethylammonium bicarbonate (0.75%/min; flow 3.5 ml/min). Peaks of the desired oligonucleotides (Table I) were collected and evaporated. To remove the ethyl groups from the phosphorothioate moiety, each oligomer was treated with 25% aqueous NH<sub>3</sub>H<sub>2</sub>O for 48 h at 50 °C. HPLC analysis under the conditions described above showed that dealkylation was complete.

![Fig. 1. Design of the duplex oligonucleotide substrates. The recognition hexanucleotide is shown in large letters, and cleavage sites are indicated by arrows. Phosphorothioate-substituted oligonucleotides (Table I) replace the prochiral phosphate between A and T with a chiral phosphorothioate.](image-url)

![Fig. 2. Scheme for the synthesis and purification of the two diastereomers of the dinucleoside monophosphate A<sub>p</sub>T. Details are given under "Materials and Methods."](image-url)
Characterization of the Oligonucleotide Products—The presence and diastereomeric purity of the phosphodiester mixture between A and T in each oligonucleotide were proved by digestion with snake venom phosphodiesterase (EC 3.1.4.1) or with nuclease P1 (EC 3.1.30.1), which are known to be stereoselective (20, 21) toward P-chiral phosphorothioate dinucleotides.

For nuclease P1 digestion, each oligonucleotide was dissolved in 200 μl of 10 mM Tris-HCl, pH 7.2, 1 mM ZnCl₂ and received 1 μg of nuclease P1. For snake venom phosphodiesterase digestion, each oligomer was dissolved in 200 μl of 10 mM Tris-HCl, pH 8.5, 15 mM MgCl₂, and received 10 μg of phosphodiesterase. The digestion mixtures were incubated for 12 h at 37 °C. After additional treatment of each digest with alkaline phosphatase (1 μg of protein, 1 h at 37 °C), HPLC analysis was performed. The dinucleotide (AP₆T) undigested by each enzyme was isolated and compared by HPLC with an authentic sample of this compound with known absolute configuration at phosphorus.

Oligonucleotides containing the R₆-P₆ substitution were completely digested to mononucleotides by venom phosphodiesterase and yielded R₆-d[AP₆T] upon nuclease P1 digestion. Oligonucleotides containing S₆-P₆ substitution were completely digested to mononucleotides by nuclease P1 and yielded S₆-d[AP₆T] upon digestion with venom phosphodiesterase.

Labeling and Purification of Oligonucleotide Duplexes—An equimolar mixture of complementary single strands was 5'-end-labeled with γ-³²P]ATP and polynucleotide kinase as previously described (22), then purified on NENsorb-20 columns (Du Pont-New England Nuclear) to remove labeled ATP. Complementary single strands were annealed, and the duplexes were purified by electrophoresis on non-denaturing 12.5% polyacrylamide gels. The duplexes were repurified on NENsorb-20 columns, renatured, and verified to be entirely in the duplex form at DNA concentrations as low as 5 pM by non-denaturing gel electrophoresis (12.5% polyacrylamide; 20 mM bis-tris-propane acetate, 50 mM NaCl, 2.5 mM Na₂EDTA, pH 7.0).

Measurements of Equilibrium Binding, Dissociation Kinetics, and Cleavage Kinetics—Equilibrium binding constants were measured by either the direct filter binding method (23) as modified by Jen-Jacobson et al. (25) or the rate-competition method (24) as modified by Lesser et al. (5). The kinetics of dissociation of endonuclease-DNA complexes were measured as described by Jen-Jacobson et al. (25). Detailed conditions are given in the legends to the figures.

Cleavage rate constants were determined essentially as described (5) in 0.1 M bis-tris-propane, pH 7.5, 0.08 M NaCl, 100 μg/ml bovine serum albumin, 5 μM dithiothreitol. Each time point was determined by a separate reaction in an individual tube, in which enzyme (1.2 μM) and DNA (0.6 μM duplex) were pre-equilibrated for 30 min at 25 °C. A drop of MgCl₂ solution sufficient to give a final concentration of 5 mM was placed on the wall of each tube, and the reaction was initiated by placing the tube on a Vortex mixer to mix the MgCl₂ with the enzyme-DNA solution. Each reaction was stopped by the addition of urea (final concentration 8 M) and EDTA (final concentration 0.1 M), and the reaction time was recorded to the nearest 0.1 s. Reaction products were separated and data were analyzed as described by Lesser et al. (5).

Ethylation-interference Footprinting—Footprints were obtained using the detailed protocols described previously (5, 8). Each DNA strand was separately ethylated, then 5'-end-labeled with [γ-³²P] ATP (see above) and annealed to its unlabeled, unethylated complement. Binding reactions were conducted in 0.01 M bis-tris-propane, pH 7.5, 0.08 M NaCl, 50 μg/ml bovine serum albumin, 5 μM dithiothreitol. The DNA concentration was 1 nM for the unmodified GAATTC site and the R₆-P₆ site and 0.5 nM for the S₆-P₆ site; endonuclease was in each case present at 3 times molar excess to DNA.

RESULTS

General Strategy for Synthesis of Phosphorothioate-substituted Oligonucleotides—Previous methods (19, 26, 27) for the preparation of oligonucleotides containing single stereospecific phosphorothioate substitutions have been based upon synthesis of oligonucleotides containing a mixture of R₆-P₆ and S₆-P₆ diastereomers, followed by separation of the diastereomeric oligonucleotides by HPLC. However, the ability to resolve the diastereomers at the oligonucleotide level is dependent upon both chain length and the position of the Ps substitution (26), and, in the case of the present 17-nt products, we could not achieve preparative-scale purification at the oligonucleotide level (Table I).

Accordingly, we adopted a strategy (27) of purification of the diastereomers at the level of dinucleoside monophosphates (Fig. 1). The R₆- and S₆-diastereomers of protected AP₆T were separated as the O-ethyl phosphorothioates (V) by HPTLC, and each of these pure diastereomers was used separately for subsequent oligonucleotide synthesis. We have verified the diastereomeric purity of the products (see "Materials and Methods") both at the dinucleotide level (by HPLC after deprotection) and at the oligonucleotide level by enzymatic digestion.

Stereospecific Effects on Equilibrium Binding—The introduction of an R₆-P₆ into one strand of a duplex oligonucleotide has a small inhibitory effect (+0.3 kcal/mol) on equilibrium binding of EcoRI endonuclease (Table II), relative to the parent (unmodified) duplex. By contrast, introduction of an S₆-P₆ in one strand improves binding (~1.3 to ~1.7 kcal/mol) relative to the unmodified duplex. In both cases, the magnitude of the effect is the same regardless of which DNA strand is modified.

When both strands of a duplex are modified with the S₆-P₆ diastereomer (i.e. S₆/S₆ homoduplex), the effect on binding is not much larger than when only one strand is modified. Thus, the favorable increments of binding free energy (ΔΔG°₂) due to modification of each strand are not additive in a double-substituted homoduplex. In the R₆/R₆ homoduplex, the unfavorable effect on ΔΔG°₂ is larger than for either single-strand modification, but the precision of the data does not permit us to determine whether the effects are strictly additive.

Superimposition of energetic effects becomes more evident in R₆/S₆ heteroduplexes (Table II). Because the favorable effect of an S₆-P₆ is larger than the unfavorable effect of an R₆-P₆, the net effect in the heteroduplexes is an improvement in binding free energy (~0.4 kcal/mol) relative to the unmodified duplex. This net effect on ΔΔG°₂ is smaller than the summed effects of the separate single strand modifications.

Kinetic Stability of the Complexes—The changes in binding affinities described above may reflect changes in either the dissociation rate constants (k₆) of the endonuclease-DNA complexes, the association rate constants (k₆₈) for complex formation, or both. We therefore measured dissociation kinetics directly; Fig. 3 shows representative data at 0.08 M

| Oligonucleotide | 5'-DMT | 5'-OH | 5'-OH |
|----------------|--------|-------|-------|
|                | EO - P = S |       | O = P - S' |
| R₆-TCCGGCAGAATTTCTGCC | 15.5 | 11.2 | 10.40 |
| S₆-TCCGGCAGAATTTCTGCC | 15.7 | 11.3 | 10.45 |
| R₆-TCCGGCAGAATTTCTGCC | 16.0 | 11.2 | 10.50 |
| S₆-TCCGGCAGAATTTCTGCC | 16.0 | 11.0 | 10.40 |

TABLE I

HPLC characteristics of protected and deprotected oligonucleotides

Retention times for each oligonucleotide derivative are given in minutes. Conditions for HPLC purification are described in the text.
the S,-Ps/P heteroduplex complexes are 8-9 times smaller than the parent (unmodified) complex, whereas the complex with the S,-Ps/P heteroduplex is stable than with the R,-Ps/P counterpart. Differences of rates. At 0.08 M NaCl, the complexes with Rp-Ps/P were removed at the indicated times and filtered through nitrocellulose. The upper strand of each duplex is written with the 5'-end on the left. The recognition site GAATTC in each is double-underlined.

Table II

| Sequence*                   | $K_a$ | $\Delta G^0$s | Observed$^{a}$ | Calculated$^{b}$ |
|-----------------------------|-------|--------------|----------------|------------------|
|                            | m$^{-1}$ | kcal/mol | (s$^{-1}) \times 10^6$ | (m$^{-1}$ s$^{-1}$) |
| TCGGCAGAaTTCTCGCC           | 2.9 (± 0.4) x 10$^{11}$ | 0 | 4.8 ± 0.5 | 1.4 (± 0.2) x 10$^{10}$ |
| GCCGCCTTTpaAGACGCT          | 4.9 (± 1.6) x 10$^{11}$ | -1.7 ± 0.2 | 0.58 ± 0.02 | 2.8 (± 0.9) x 10$^{10}$ |
| TCGGCAGAaTTCTCGCC (Sp)      | 2.8 (± 0.8) x 10$^{11}$ | -1.3 ± 0.2 | 0.53 ± 0.02 | 1.5 (± 0.4) x 10$^{10}$ |
| GCCGCCTTTpaAGACGCT          | 6.1 (± 1.6) x 10$^{11}$ | -1.8 ± 0.2 | 0.30 ± 0.02 | 1.8 (± 0.5) x 10$^{10}$ |
| TCGGCAGAaTTCTCGCC (Rp)      | 1.8 (± 0.3) x 10$^{10}$ | +0.3 ± 0.1 | 7.1 ± 1.0 | 1.3 (± 0.3) x 10$^{10}$ |
| GCCGCCTTTpaAGACGCT          | 1.8 (± 0.3) x 10$^{10}$ | +0.3 ± 0.1 | 9.8 ± 2.4 | 1.8 (± 0.5) x 10$^{10}$ |
| TCGGCAGAaTTCTCGCC (Sp)      | 6.3 (± 2.1) x 10$^{10}$ | +0.9 ± 0.3 | ND$^c$ | ND$^c$ |
| GCCGCCTTTpaAGACGCT          | 6.4 (± 1.1) x 10$^{10}$ | -0.5 ± 0.2 | 2.0 ± 0.2 | 1.3 (± 0.3) x 10$^{10}$ |
| TCGGCAGAaTTCTCGCC (Rp)      | 5.5 (± 0.2) x 10$^{10}$ | -0.4 ± 0.1 | 1.7 ± 0.2 | 0.9 (± 0.1) x 10$^{10}$ |

* The upper strand of each duplex is written with the 5'-end on the left. The recognition site GAATTC in each is double-underlined.

* Equilibrium association constants were determined as described under "Materials and Methods." Solution conditions were 10 mM bis-tris-propane, pH 7.5, 25°C; 100 μg/ml bovine serum albumin; 5 mM dithiothreitol.

* The difference in standard binding free energy between the unmodified duplex and each duplex shown, calculated as $\Delta G^0_s = -RT \ln (K_a/K_a^{mod})$.

* Dissociation rate constants were determined as described in the legend to Fig. 3, but these data were obtained at 0.12 M NaCl. Solution conditions for these data were the same as those for the measurement of equilibrium association constants $K_a$.

* Apparent association rate constants ($k_a$) were calculated from the relation $k_a = K_a \times k_d$.

* ND, not determined.

Fig. 3. Representative kinetics of dissociation of endonuclease-DNA complexes. Complexes between EcoRI endonuclease (4 nM) and each of the radiolabeled oligonucleotides (6 nM) were formed in a solution containing 0.01 M bis-tris-propane, pH 7.5, 0.08 M NaCl, 100 μg/ml bovine serum albumin, 5 mM dithiothreitol. After an equilibrium of 30 min at 25°C, a 50-fold molar excess of unlabeled DNA (200 nM) was added to each reaction mixture. Aliquots were removed at the indicated times and filtered through nitrocellulose membranes (Millipore type HA). The amount of complex retained on each filter was determined by liquid scintillation counting. The lines shown were fitted by linear regression.

That is, the calculated values of the association rate constant $k_a$ (Table II) are about the same for the unmodified complex and all Ps-substituted complexes. (A minor discrepancy for the $S_p/P$ heteroduplex in one orientation is likely not significant.) It should be borne in mind that the observed $k_d$ and calculated $k_a$ may themselves be composites of rate constants for elementary steps involved in complex dissociation or association.

Fractional standard deviations are generally smaller in dissociation rate measurements than in $K_a$ measurements, so dissociation kinetics provide more sensitive and reliable quantitation of relatively minor perturbations in the protein-DNA complex, like that caused by the $R_p$-Ps substitution. For example, at 0.12 M NaCl, the half-lives of the complexes are 144 s for the unmodified complex and 70-97 s for the $R_p$-Ps complexes. At 0.08 M NaCl, the corresponding half-lives are 39 min for the unmodified complex and 14-15 min for the $R_p$-Ps complexes. Since the perturbation in $K_a$ was small (+0.3 kcal/mol), the changes in dissociation rate constants provide an independent estimate of the magnitudes of the changes.

Cleavage of Phosphorothioate-substituted DNA—To assess stereospecific effects of phosphorothioates on the cleavage reactions, we measured first order rate constants after addition of Mg$^{2+}$ to preformed endonuclease-DNA complexes, at high concentrations of endonuclease and DNA and with endonuclease in excess. Under these conditions, the formation of the distinguishable oligonucleotide products resulting from cleavage in each DNA strand is a parallel-sequential process (5). With the unmodified duplex as substrate, the endonuclease forms a fully symmetrical complex in which the two DNA strands are cleaved at equal rates. The cleavage rates of the nicked intermediates are virtually the same as for the intact duplex, such that the 6-n tail products appear at equal rates (5).

The introduction of a phosphorothioate into one DNA strand produces a subtle asymmetry in the complex, with the
result that cleavage rates in the two strands become unequal. This is demonstrated visually in Fig. 4, which shows that the $S_p$-Ps strand of a heteroduplex is cleaved more rapidly than the unmodified strand and that the $R_p$-Ps strand of a heteroduplex is cleaved more slowly than the unmodified strand. In both cases, the effect depends only upon the modification and not upon whether the “top” or “bottom” DNA strand is modified.

We computed the first order cleavage rate constants for each strand of each substrate from the entire time course of appearance of each product, including for each experiment 7 to 9 intermediate time points not shown in Fig. 4. The integrated rate equations and method of calculation are given in Lesser et al. (5).

As shown in Table III, the introduction of $R_p$-Ps into one strand of a heteroduplex affects the rate constants ($k_{\text{cleave}}$) for cleavage of both strands, but in opposite directions. Relative to the rate constants for the unmodified duplex, $k_{\text{cleave}}$ for the $R_p$-Ps strand is decreased about 2-fold, whereas $k_{\text{cleave}}$ for the unmodified strand is increased by about 50%, with the net result that the modified strand of an $R_p$-Ps/P heteroduplex is cleaved about 3 times slower than the unmodified strand. Conversely, in the $S_p$-Ps/P heteroduplex, the modified strand is cleaved faster and the unmodified strand is cleaved slower than in the parental unmodified duplex, with the result that the modified strand is cleaved about 3 times faster than the unmodified strand. In both cases, the effect is independent of which strand is modified.

This inequality of cleavage rates is exaggerated in $R_p/S_p$ heteroduplexes (Table III). The $k_{\text{cleave}}$ for the $S_p$-Ps strand is about the same as with single $S_p$-Ps substitution, but $k_{\text{cleave}}$ for the $R_p$-Ps strand decreases further. The net effect is such that the $S_p$-Ps strand is cleaved about 7 times faster than the $R_p$-Ps strand.

In $S_p/S_p$ or $R_p/R_p$ homoduplexes, there is no longer an asymmetric modification in the DNA. In these cases, the two strands are cleaved at equal rates, in both cases about 2-fold slower than the parental unmodified duplex.

_Ethylation-interference Footprinting—_We have shown previously (5, 8) that the endonuclease-GAATTC complex contains symmetrical contacts with DNA phosphates. Six phosphates (the primary and supplementary clamps at CpAp-GAACGCT) showed strong ethylation-interference and six more (at GpAATpTpC) showed weak interference.

As shown in Fig. 5, the pattern of strong and weak contacts
on an S<sub>P</sub>-Ps/P heteroduplex is essentially identical with that on the unmodified DNA, with the single interesting exception that ethylation of the phosphate at the scissile bond (GpAA<sub>5</sub>TTC) actually produces a slight enhancement of endonuclease binding. This may be contrasted to the reproducible 2- to 3-fold interference at this position in the parent unmodified duplex and the unmodified strand of the S<sub>P</sub>-Ps/P heteroduplex. There is thus a correlation between loss of interference at the scissile GpA bond and an enhanced rate of cleavage of this bond. Similarly, in the R<sub>P</sub>-Ps/P heteroduplex where the unmodified DNA strand is cleaved faster, we observed a loss of interference at the GpA bond in the unmodified strand. In both cases, the observed loss of interference upon ethylation may be interpreted to mean that the bound enzyme can more readily accommodate an ethyl group on the scissile phosphate.

These correlations suggest that close contact (as detected by ethylation-interference) of the endonuclease with the GpA bond in the enzyme-substrate complex may be detrimental to formation of the transition state for the strand scission reaction. We have previously reported (5) that the introduction of an incorrect base pair anywhere in the recognition site greatly reduces the first order rate constants (k<sub>cleave</sub>) for cleavage of both DNA strands, and that this is invariably associated with a pronounced increase in ethylation-interference at the GpA bonds on both DNA strands, as well as with other more profound changes in protein interactions with the supplementary clamp phosphates. Furthermore, we have found that some substitutions of single base analogues produce reduced cleavage rate constants and increased ethylation-interference at the GpA bonds, whereas others have no effect on k<sub>cleave</sub> and no effect on the degree of ethylation-interference at GpA.

**DISCUSSION**

The Endonuclease Contact at the Central Phosphate—Crystalllographic evidence (28) indicates that even multiple substitutions of chiral phosphorothioates for prochiral phosphates have little or no effect on the conformation of the DNA backbone. Since we have used only one Ps in each DNA strand, the backbone conformation is unlikely to have been affected by the Ps substitution. Thus, for any particular phosphate that is not directly involved in the catalytic mechanism, single Ps substitutions can be used to probe direct protein contacts with that phosphate. When one Ps-diastereomer is introduced into an oligonucleotide, the negative charge (on the sulfur atom) is placed in a defined orientation rather than being delocalized across two phosphoryl oxygens. Bond lengths are also slightly different, in that the P=S bond is longer and the P=O bond is shorter than the analogous intermediate-order P-O bonds in an unmodified prochiral phosphate.

The effect of each Ps-diastereomer on protein binding will depend upon the nature and precise orientation of the protein group that interacts at that position. For example, a hydrogen-bonding interaction with the polypeptide backbone may be highly constrained with respect to distance and bond angle and therefore highly sensitive to Ps substitution, whereas a salt-link with a long, flexible lysine or arginine side chain may be more tolerant. Thus, for phosphates that are not directly involved in the catalytic mechanism, we postulate the following possibilities for phosphorothioate effects and their stereoselectivities.

1. At phosphates that are not contacted by protein, or which form only salt-links to flexible protein side chains, there will be little or no effect of Ps-substitution. Such a situation has been observed at 17 of 21 phosphates of the R17 coat protein binding site on bacteriophage R17 RNA (29).

2. At phosphates where the protein forms constrained (e.g. hydrogen-bonding) interactions to both phosphoryl oxygens, both Ps-diastereomers may be inhibitory. This is the case for the primary clamp phosphate contacts at gpAATTTC, where either S<sub>P</sub>-Ps or R<sub>P</sub>-Ps substitution (in one strand) inhibits binding by about +0.7 kcal/mol.

3. At phosphates where the protein makes a tight hydrogen-bonding interaction to only one of the phosphoryl oxygens, one Ps-diastereomer will improve binding (relative to the unmodified DNA) and the other will be inhibitory.

The data in this paper indicate that phosphorothioates at the central phosphate (GAA<sub>5</sub>TTC) of the recognition site have stereoselective effects on EcoRI endonuclease binding consistent with the third category above. The R<sub>P</sub>-Ps diastereomer has a subtle inhibitory effect (about +0.3 kcal/mol), whereas the S<sub>P</sub>-Ps diastereomer improves binding by about −1.5 kcal/mol. (The total energetic contribution of a single phosphate contact has been previously estimated at 1.3 kcal/mol (25, 30).) These two derivatives should represent, respectively, the least favorable and most favorable ways of resolving a prochiral phosphate at this position in the site into dominant resonance forms with P=O in fixed orientation.

In the favored S<sub>P</sub>-GAA<sub>5</sub>TTC site, the P=O is oriented to project into the major groove of the DNA (28, 31). The current crystallographic model (4, 7) of the EcoRI endonuclease-DNA complex indicates that the polypeptide backbone near Gly<sub>116</sub> is closely apposed to the phosphate at GAA<sub>5</sub>TTC. As can be seen in Fig. 6, the amide-NH of Gly<sub>116</sub> is in position (N → O distance of 2.7 Å; the precision of the current model is ±0.3 Å) to donate a hydrogen bond to the phosphoryl oxygen projecting toward the major groove, whereas the other phosphorothioate in the recognition site is not involved in direct hydrogen bonding to the DNA phosphate.

3 M. Kurpiewski, M. Kozielkiewicz, A. Grajkowski, W. Stec, and L. Jen-Jacobson, unpublished data.

4 Amino acid residues are numbered according to the gene sequence.

The N-terminal methionine is absent from the mature protein.

J. M. Rosenberg, personal communication.

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2 D. Lesser, M. Kurpiewski, T. Waters, B. Connolly, and L. Jen-Jacobson, manuscript in preparation.

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**FIG. 5.** Ethylation interference footprints made by EcoRI endonuclease on the unmodified cognate oligonucleotide (A) and the analogous oligonucleotide containing an S<sub>P</sub>-Ps substitution in one strand (B). The interference axis (ordinate) is the ratio of free to bound ethylated DNA and is positive in both directions. A value >1 indicates that ethylation interferes with binding to the endonuclease; a value <1 indicates that ethylation increases binding. Phosphates outside the region shown exhibited no interference. The diagonally striped bars in B indicate the positions of the phosphodiester bonds susceptible to hydrolysis by EcoRI endonuclease. Histograms derived by densitometric scanning of autoradiograms (5) show means ± S.D. of 6 to 8 independent experiments for each oligonucleotide. Experimental details are given under "Materials and Methods."
Stereoselective Interaction with Chiral Phosphorothioates

Fig. 6. Stereoscopic view of the structure of the endonuclease-DNA complex in the region of the central ApT bond. The DNA chain (blue) shows the sequence AAT (left to right) in the recognition site. At the central ApT, one phosphoryl oxygen projects toward the polypeptide backbone (yellow, proceeding N-terminal to C-terminal left to right) and receives a hydrogen bond (dotted line) from the amide-NH of Gly116. This oxygen is replaced by sulfur in the Rp-Ps derivative. The side chain of Lys117 is visible at the right. Note that the other phosphoryl oxygen at ApT projects back from the plane of this figure, away from the polypeptide chain. (This oxygen is replaced by sulfur in the Sp-Ps derivative.) No other regions of the polypeptide backbone or amino acid side chains are in position to contact this oxygen. This graphic was kindly provided by J. M. Rosenberg, from a refinement of the crystal structure reported by Kim et al. (7).

phoryl oxygen projects away from the bound protein and makes no interaction with it. An Sp-Ps substitution both shortens the phosphorus-to-oxygen bond length in the major groove (by an expected 0.06 Å (10)) and removes a fractional charge from this oxygen. If both the amide nitrogen of Gly116 and the phosphorus atom remained in fixed positions, this would produce a near-ideal (32) N → O hydrogen-bonding distance of ~2.8 Å. On the other hand, the Rp-Ps substitution replaces a P-O bond of intermediate order with a P-S- bond, thus both increasing the bond length (by an expected 0.6 Å (10)) and localizing negative charge in this region. If both the amide nitrogen of Gly116 and the phosphorus atom remained in fixed positions, this would make the N → S distance significantly smaller than the original N → O distance in the prochiral phosphate. The observed effect on binding is subtly unfavorable. Both favorable and unfavorable effects of Rp-Ps substitution have been observed at different phosphate positions of the R17 coat protein binding site on bacteriophage R17 RNA (29).

Even these subtle changes at phosphate produce observable adaptations in the complex (see below). These adaptations may also have energetic consequences, so one should not assume that changes in observed binding energies derive solely from differences in the local hydrogen-bonding interaction at ApT.

In contrast to the relatively small effects of phosphorothioates, ethylation of the phosphate at GAAPpTTC strongly inhibits binding (5, 8; cf. Fig. 5). Ethylation of a phosphodiest- ter with ethylnitrosourea produces a mixture of Sp- and Rp-ethylphosphotriester (POEt) diastereomers which are not separated prior to the binding assay; strong interference with binding can only result if both diastereomers are strongly inhibitory. In the Rp-GAAPoEt/TTC derivative, the bulky ethyl group projects into the major groove in the direction of the main chain Gly116-NH so binding is strongly inhibited by steric hindrance, as expected. Although both Sp-Ps and Sp- POEt orient P=O into the major groove, the former stimulates binding and the latter is strongly inhibitory (13, 15), which implies that the ethyl group is bulky enough to produce steric hindrance in this orientation as well. It is also noteworthy that there is no ethylation interference at one phosphate in the site (GAAPA'TTC) where protein side chains approach but do not form a constrained functional contact (5). These examples show that ethylation-interference is useful for identifying individual phosphates that are closely approached by protein in the complex, but that it is a relatively blunt tool for distinguishing the nature of the interactions with phosphorothioyl oxygens. The introduction of stereospecific phosphorothioates permits more subtle and precise geometric probing of individual phosphate contacts.

Our data also show that it is necessary to use single phosphorothioate substitutions in only one DNA strand to obtain maximally useful information about the effects on binding free energy. Oligonucleotides with a second phosphorothioate (of the same or opposite configuration) in the complementary DNA strand show a total effect on binding free energy that is less than the sum of the effects of single Rp-Ps or Sp-Ps substitutions (Table II). This “nonadditivity” is probably related to subtle structural adaptations in the modified DNA-protein complexes (see below).

In general, multiple Ps substitutions may produce energetic effects that are either additive or less than than the sum of the separate effects of single substitutions, depending on the nature of the adaptations in the complex. At present, we see no way of forming an a priori expectations about the additivity of binding energy differentials for modification of any given pair of phosphate positions in a DNA site. It is certainly not legitimate to evaluate the quantitative roles of single phosphate contacts from measurements with substrates containing more than one modification.

Subtle Adaptations in the Complex—Both the crystal structure (6, 7) and ethylation-interference footprints (5, 8) show that the canonical complex is completely symmetrical, so that it cleaves the two DNA strands at equal rates (5). The ethylation-interference footprint of the Sp-GAAAsTTC complex (Fig. 5) shows no major rearrangements of contacts to the DNA backbone. The only indication of an adaptive change in the complex is the disappearance of the weak ethylation-interference at the scissile GpA bond on the modified DNA strand, which implies that the protein bound to the Sp-GAAAsTTC site has assumed a slightly different position that better accommodates an ethyl group at GpAAsTTC.

We believe that this relatively subtle change is nevertheless significant because it correlates with changes in the rates of
cleavage of the two DNA strands, and because this correlation holds true for several classes of modified substrates. The S$_r$-Ps substitution introduces a subtler asymmetry into the complex, so that the S$_r$-Ps strand is cleaved slightly faster than normal and the unmodified strand slightly slower than normal (Table III). Conversely, the R$_p$-GAATTC/TTC both inhibits cleavage of the modified strand and stimulates cleavage of the unmodified strand. The inequality of cleavage rates is further exaggerated in R$_p$/S$_r$ heteroduplexes (Table III). Taken together, these observations imply that subtle rearrangements to favor interaction with one DNA strand necessarily compromise interaction with the opposite DNA strand.

This concept of compensating adaptations explains why the effects of multiple Ps-substitutions are nonadditive with respect to binding free energy. The endonuclease cannot simultaneously adapt to improve interactions with both strands of the S$_r$/S$_r$ homoduplex, so a second full increment of binding free energy cannot be attained in the enzyme-substrate complex.

**Differential Access to Two Distinct Transition States**—Because we designed these oligonucleotides with the cleavage sites off-center, we were able to measure the separate rates of cleavage of each DNA strand. This design does not by itself influence strand preference in cleavage rates, since the unmodified GAATTC site is cleaved at precisely equal rates in both strands. Furthermore, with the Ps-substituted sites we found that strand-cleavage preferences (and the quantitative degree of preference) were strictly determined by the phosphorothioate. In heteroduplexes, an S$_p$-Ps strand was always cleaved faster and an R$_p$-Ps strand slower, regardless of whether the particular substitution was made in the “top” or the “bottom” DNA strand.

When the endonuclease forms a complex with a substrate containing asymmetric Ps substitution (i.e., S$_r$-Ps, R$_p$-Ps, or R$_p$/S$_r$ heteroduplex), the complex has unequal probabilities of reaching each of the two distinct transition states corresponding to cleavage in each DNA strand. The ratio of these probabilities (for any given DNA substrate) is measured by the ratio k$_p$/k$_r$ (Table III) which is on the order of 3 for the R$_p$-Ps/P and S$_r$-Ps/P heteroduplexes.

The degree of distinction between the two possible transition states is in general much smaller for Ps-substituted sites than for sites with more pronounced structural asymmetries. In sites with one incorrect base pair, the contacts to the DNA backbone become very asymmetric (5), and there are much larger unfavorable changes in binding free energies, indicating large scale structural adaptations in the enzyme-substrate complexes. As a result of these asymmetric adaptations, complexes containing an incorrect base pair show k$_p$/k$_r$, ranging from about 5.6 for AAATTC/TTTAAG to infinite for sites such as GTATTC/CATAAG (where no cleavage occurs in the GpT bond occurs under near-physiological conditions).

The behavior of the Ps-substituted complexes more closely resembles that of complexes containing a single base analogue (5), where the strandwise inequalities of cleavage rate constants are relatively moderate (k$_p$/k$_r$ ~ 3). The Ps-substituted complexes, like the base-analogue complexes (5), show no large scale rearrangements of backbone contacts (Fig. 5) and can therefore be described as “isosteric” with the unmodified GAATTC complex.

**Role of the ApT Phosphate Contacts in the Transition State**—Contacts between the endonuclease and the phosphates at ApT in the enzyme-substrate complex have been observed crystallographically (4, 7), by footprinting (5, 8), and by the phosphorothioate effects on $\Delta G^r$ reported here (Table II). We now consider the following questions: (a) are the ApT contacts also realized in the transition state(s)? (b) are both ApT contacts realized simultaneously in each transition state? (c) can the adaptations induced by two similar or dissimilar Ps-substitutions be accommodated simultaneously in one complex?

The effects of Ps substitutions on the transition states are best measured by the transition-state interaction free energy $\Delta G^t$, the standard free energy change in forming a transition-state complex from free enzyme and DNA (5). As previously noted (5), $\Delta G^t$ measures the free energy change with reference to well-defined transition states that require extraordinarily precise alignment of those enzyme and substrate elements that directly participate in a catalytic event. Any “adaptive” changes in the enzyme-substrate complexes may influence binding ($\Delta G^b$), and cleavage rate ($\Delta G^f$), but these influences cancel out in the quantity $\Delta G^t$. Table III shows these values for each of the two distinct transition states of each substrate, calculated relative to that for the unsubstituted site ($\Delta G^t$).

The data for S$_p$/P heteroduplexes show that S$_p$-Ps substitution affects $\Delta G^t$ for both of the distinct transition states, albeit to unequal extents, such that the two transition states are separated by about 0.6 kcal/mol. In R$_p$/P heteroduplexes, the R$_p$-Ps substitution destabilizes the transition state for cleavage of the strand containing the R$_p$-Ps substitution by about 0.6 kcal/mol, but has almost no effect ($\Delta G^t$ ≈ 0) on the transition state for cleavage of the strand containing unsubstituted phosphate, as a result of compensating effects on $\Delta G^b$ and $\Delta G^f$. These observations imply that contacts between the endonuclease and the central ApT are realized in the transition states, as they are in the enzyme-substrate complex (Fig. 6).

A complete explanation of these effects is not yet possible because we do not know the detailed structure of the transition state for EcoRI endonuclease. However, we note that the hydrogen bond between Gly$^{116}$ and the central phosphate anchors one end of the β3 element (4) (residues 103–115) that also includes the active-site residue Glu$^{131}$ (4, 33). Thus, subtle changes in position of Gly$^{116}$ in Ps-substituted sites could affect the precise positioning of Glu$^{131}$ (and possibly other active-site residues) to participate in a catalytic event and thereby affect cleavage rate constants.

Examining the effects of Ps-substitution on $\Delta G^t$ for the R$_p$/S$_r$ heteroduplexes, it is evident that the energetic effects are approximately additive in the transition states such that the two distinct transition states are separated by 1.2 kcal/mol. This may be understood as follows. An S$_p$-Ps substitution separates the two transition states by 0.6 kcal/mol (average of the two orientations in the heteroduplexes), favoring the cleavage in the substituted strand. The R$_p$-Ps substitution separates the two transition states by 0.6 kcal/mol, favoring the cleavage in the unsubstituted strand. In the R$_p$/S$_r$ heteroduplex, these effects are approximately additive, so that the difference between the two transition states is exaggerated. This additivity implies that both ApT contacts are realized simultaneously in each of the distinct transition states for cleavage. This is consistent with the simultaneous formation of both ApT contacts and the subunit interdigitation observed crystallographically in the enzyme-substrate complex (4, 7).

The S$_p$/S$_r$ and R$_p$/R$_p$ homoduplexes allow us to ask whether the adaptation produced by one Ps-diastereomer can be accommodated twice in one transition state complex. (Note that in these cases the two transition states of each substrate are not distinguished energetically.) This is possible in the R$_p$/R$_p$ heteroduplex, where $\Delta G^t$ (+1.4 kcal/mol) shows approximately twice the effect of a single R$_p$-Ps substitution (+0.6 kcal/mol).
kcal/mol). This implies that the complex forms a less favorable interaction at both $A_{V307}$ simultaneously. By contrast, $\Delta\Delta\sigma^p$ for the $S_p/S_p$ homoduplex ($\sim 1.5$ kcal/mol) is essentially the same as that for the $S_p/P$ heteroduplex, implying that the complex cannot adapt to improve interaction with both $A_{V307}$ simultaneously.

At present, single phosphorothioate substitutions provide the most subtle perturbations known for probing backbone contacts in sequence-specific protein-DNA interactions, yielding detailed information about the interactions, in both the enzyme-substrate complex and the transition state complexes, that cannot be obtained by any other experimental approach. We are currently carrying out analogous studies to probe the roles of other phosphate contacts in the EcoRI endonuclease-DNA complex.

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