Sequence- and Strand-Specific Cleavage in Oligodeoxyribonucleotides and DNA Containing 3′-Thiothymidine†

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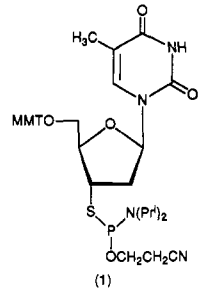
ABSTRACT: Oligonucleotides containing a 3′-thiothymidine residue (T3′s) at the cleavage site for the *Eco*RV restriction endonuclease (between the central T and A residues of the sequence GATATC) have been prepared on an automated DNA synthesizer using 5′-*O*-monomethoxytritylthymidine 3′-*S*-(2-cyanoethyl *N*,*N*-diisopropylphosphorothioamidite). The self-complementary sequence GACGAT3′sATCGTC was completely resistant to cleavage by *Eco*RV, while the heteroduplex composed of 5′-TCTGAT3′sATCCTC and 5′-GAGGATATCAGA (duplex 4) was cleaved only in the unmodified strand (5′-GAGGATATCAGA). In contrast, strands containing a 3′-*S*-phosphorothiolate linkage could be chemically cleaved specifically at this site with Ag^+. A T3′s residue has also been incorporated in the (−) strand of double-stranded closed circular (RF IV) M13mp18 DNA at the cleavage site of a unique *Eco*RV recognition sequence by using 5′-pCGAGCTCGAT3′sATCGTAAT as a primer for polymerization on the template (+) strand of M13mp18 DNA. On treatment of this substrate with *Eco*RV, only one strand was cleaved to produce the RF II or nicked DNA. Taken in conjunction with the cleavage studies on the oligonucleotides, this result demonstrates that the 3′-*S*-phosphorothiolate linkage is resistant to scission by *Eco*RV. Additionally, the phosphorothiolate-containing strand of the M13mp18 DNA could be cleaved specifically at the point of modification using iodine in aqueous pyridine. The combination of enzymatic and chemical techniques provides, for the first time, a demonstrated method for the sequence-specific cleavage of either the (+) or (−) strand.

In previous publications (Cosstick & Vyle, 1989, 1990) we have reported the synthesis of an appropriately protected thymidine 3′-*S*-phosphorothioamidite (**1**) and described its use for the introduction of 3′-thiothymidine (T3′s) into short oligonucleotides, both in solution and on a solid-phase support. These modified oligodeoxyribonucleotides containing a 3′-*S*-phosphorothiolate linkage are of interest since while the phosphorus–sulfur bond is susceptible to chemical cleavage under mild conditions (aqueous solutions of either iodine or silver nitrate) it exhibits resistance to hydrolysis by certain nucleases (Cosstick & Vyle, 1988, 1990). To extend these studies, we investigated the resistance of a 3′-*S*-phosphorothiolate linkage to cleavage by the restriction endonuclease *Eco*RV (cleavage site between the T and A bases in GATATC) with the aim of developing a strategy for site- and strand-specific cleavage of DNA using a combination of chemical and enzymatic techniques.



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Restriction endonucleases act upon a recognition sequence in double-stranded DNA, and the end result of their action is cleavage of both strands of the duplex. With cognate sites and under optimal conditions, two mechanisms are observed. Some restriction endonucleases (e.g., *Eco*RI) (Halford et al., 1979; Halford & Johnson, 1983) cleave duplex DNA in two sequential reactions cutting first one strand, then dissociating from the nicked DNA, and finally rebinding before cleaving the second strand. Other enzymes cut both strands at the same time with no dissociation between individual strand cleavage and no formation of a nicked intermediate. The *Eco*RV endonuclease used in the present studies operates through this latter mechanism (Halford & Goodall, 1988). However, even enzymes that usually cut both strands simultaneously can be made to cleave DNA, under appropriate conditions, in two sequential reactions with a nicked intermediate. These conditions include "star" activity where noncognate sites (usually differing from the true recognition sequence by one base) can be cut under suboptimal conditions (often buffers containing organic solvents, Mn^{2+} rather than Mg^{2+}, and not being of optimal pH or ionic strength) (Polisky et al., 1975; Taylor & Halford, 1989). Similarly performing restriction endonuclease cleavage in the presence of ethidium bromide often leads to a nicked intermediate (Modrich, 1982; Parker et al., 1977; Shortle & Nathans, 1978). Using the RF IV form of M13 DNA containing phosphorothiolate linkages in the (−) strand only, Eckstein and co-workers have shown that many restriction endonucleases given single-strand cleavage resulting in a nicked intermediate (Potter & Eckstein, 1984; Taylor et al., 1985a; Olsen et al., 1990). Importantly, cleavage is directed to the (+) strand, and this method for preparing RF II DNA cut specifically in one strand forms the basis for an elegant procedure for site-specific mutagenesis (Taylor et al., 1985b; Nakamaye & Eckstein, 1986; Sayers et al., 1988).

There are comparatively few reports on the use of mild chemical reagents for the specific cleavage of oligodeoxy-

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ribonucleotides or DNA containing modified sugars or phosphodiester linkages. The alkylation of phosphorothioate-containing DNA with 2-iodoethanol leads largely to desulfurization but is accompanied by a very small extent of chain cleavage. While this procedure is unsuitable as a means of sequence-specific DNA cleavage, it can be used for DNA sequencing providing the DNA is either radioactively or fluorescently labeled (Gish & Eckstein, 1988). Oligodeoxyribonucleotides containing 5′-*N*-phosphoramidate linkages can be prepared chemically using standard methods of DNA synthesis (Bannwarth, 1988), and the linkage is susceptible to specific hydrolysis with mild aqueous acid (Bannwarth, 1988; Gryaznov & Sokolova, 1990; Hata et al., 1975). Cleavage under these conditions results in one oligonucleotide fragment bearing a 5′-amino function while the other terminates with a 3′-phosphate group.

In this work we examine the *Eco*RV endonuclease catalyzed scission of oligodeoxyribonucleotide duplexes containing a 3′-*S*-phosphorothiolate linkage at the cleavage site and the lability of this linkage toward Ag^+. Additionally, we demonstrate that RF IV M13 DNA containing this modification in the (−) strand at a unique *Eco*RV site can be nicked in the (+) strand by the *Eco*RV endonuclease or the (−) strand in the presence of aqueous iodine. To our knowledge, this is the first report of a method which allows sequence-specific cleavage of either the (+) or (−) strand.

Experimental Procedures

Reagents and Materials. 5′-*O*-Monomethoxytritylthymidine 3′-*S*-(2-cyanoethyl *N*,*N*-diisopropylphosphorothioamidite) (**1**) (Cosstick & Vyle, 1990), tetra-*n*-butylammonium periodate (Santaniello et al., 1980), and 5-(4-nitrophenyl)tetrazole (Froehler & Matteucci, 1983; Finnegan et al., 1958) were prepared as previously described. T4 polynucleotide kinase, T7 exonuclease, T4 DNA ligase, and the Klenow fragment of DNA polymerase were obtained as previously reported (Sayers et al., 1988). The *Eco*RV restriction endonuclease was either isolated according to the published procedure (D'Arcy et al., 1985; Newman et al., 1990) (for studies on the oligonucleotides) or purchased from New England Biolabs (for studies on phosphorothiolate DNA). Alkaline phosphatase (special molecular biology grade) and all other enzymes were purchased from Boehringer Mannheim.

Instrumentation. HPLC was performed on a Varian 5000 liquid chromatograph equipped with a Varian UV 50 variable-wavelength detector. Separations were carried out on analytical columns (25 × 0.45 cm) packed with either Bio-Rad RSil (Bio-Rad) or Apex-1 (Jones Chromatography, Llanbradach, U.K.) octadecylsilyl silica (C-18) reverse-phase silica. Elution gradients were prepared from 0.1 M triethylammonium acetate (pH 6.5) containing 5% acetonitrile and 0.1 M triethylammonium acetate (pH 6.5) containing 65% acetonitrile with a flow rate of 1 mL/min. Columns were heated in a water bath at 50 °C for the separation of oligonucleotides. Thermal melting (T_m) profiles and circular dichroism spectra were recorded as previously described (Connolly & Newman, 1989). Agarose gel electrophoresis was performed on 1% agarose slab gels containing 0.5 μg/mL ethidium bromide and 5 mM 2-mercaptoethanol (Potter & Eckstein, 1984).

Oligonucleotide Synthesis. All oligonucleotides were prepared using an Applied Biosystems 381A automatic DNA synthesizer on a 1-μmol scale. Unmodified oligonucleotides were synthesized with the standard 1-μmol cyanoethyl phosphoramidite cycle using reagents purchased from Applied Biosystems. Oligonucleotides containing a 3′-*S*-phosphorothiolate linkage were also prepared using a standard reaction cycle, but the oxidation step was performed using a solution of tetra-*n*-butylammonium periodate (0.43 M) in dry acetonitrile:2,6-lutidine (95:5) which had previously been filtered through a PTFE filter (0.5 μm). The reaction time for this step was the same as that used with the standard iodine oxidation procedure (20 s). In order to introduce the 3′-thiothymidine residue, the synthesis cycle was stopped immediately prior to the coupling step and the reaction column was removed from the machine. A solution of the thymidine 3′-*S*-phosphorothioamidite (**1**) (40 mg) in acetonitrile (0.6 mL) saturated with 5-(4-nitrophenyl)tetrazole was injected into the column through a PTFE filter (0.5 μm) over a period of 8 min. The coupling step was repeated with half-quantities of the reagents over 4 min, and the column was washed with dry acetonitrile (2 mL) and flushed with argon. Oxidation was performed immediately by the passage of a solution of tetra-*n*-butylammonium periodate (0.5 M) in acetonitrile (0.8 mL) through the column over a period of 2 min. The column was washed with dry acetonitrile, flushed with argon, and replaced on the synthesizer. The reaction cycle was continued with the start of the capping step, the subsequent oxidation step was omitted, and the program was continued uninterrupted from the start of detritylation of the modified nucleoside residue. The coupling yield for the introduction of the 3′-thiothymidine was about 30–40% as determined by the release of the dimethoxytrityl cation following the coupling and detritylation of the subsequent residue in the sequence. Cleavage from the support, deprotection, and purification of the oligodeoxyribonucleotides were performed as previously described (Newman et al., 1990; Connolly & Newman, 1989), and the finally obtained detritylated oligodeoxyribonucleotides were stored at −20 °C as frozen solutions in H_2O. Purified yields for the oligonucleotides were generally 50% for the unmodified sequences and 15–20% for those containing 3′-thiothymidine.

Oligodeoxyribonucleotide Characterization. Oligodeoxyribonucleotide purity was checked by reverse-phase HPLC on C-18 silica using a gradient of 5–18% acetonitrile in triethylammonium acetate over a period of 20 min. Base composition analysis was performed by digestion of approximately 0.5 A_{260} units of the dodecamer in a reaction mixture containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH (Hepes–KOH), pH 7.4, 10 mM magnesium chloride, snake venom phosphodiesterase (6 μg), and alkaline phosphatase (5 μg). Digestion was complete within 2 h at room temperature, and the resulting nucleoside mixture was analyzed by HPLC on C-18 silica using isocratic elution with 5% acetonitrile for 10 min followed by a gradient of 5–23% acetonitrile in triethylammonium acetate over an additional 10 min. To reaction mixtures derived from the digestion of oligodeoxyribonucleotides containing 3′-thiothymidine were added dithiothreitol (10 μL; 100 mM) and sodium hydrogen carbonate (50 μL, 100 mM), and the mixtures were incubated at room temperatures for 30 min prior to HPLC analysis. The nucleosides present in the digestion mixtures were determined by UV detection at 254 nm, and their relative proportions were calculated by integration of the peak areas using the following extinction coefficients (Fasman, 1975) (M^{-1} cm^{-1}): dA, 14.3 × 10^3; dC, 6 × 10^3 dG, 13.5 × 10^3; dT, 7 × 10^3; 3′-thiothymidine, 7 × 10^3. Oligonucleotide molar extinction coefficients were determined as previously described (Newman et al., 1990).

Oligonucleotide Cleavage with EcoRV. Cleavage reactions containing 50 mM Hepes–KOH, pH 6.8, 55 mM sodium chloride, 25 mM magnesium chloride, 20 μM each oligo-

nucleotide strand, and either 8 μg (0.7 μM of dimeric endonuclease for unmodified oligonucleotide substrates) or 20 μg (1.8 μM) enzyme (for oligonucleotide substrates containing 3′-thiothymidine) in a total volume of 150 μL were incubated at 38 °C. At appropriate times, 15-μL aliquots were withdrawn and added to 5 μL of 0.5 M sodium hydroxide to quench the reaction. Cleavage rates were determined by reverse-phase HPLC analysis of the reactions as previously reported (Newman et al., 1990). Optimum resolution of the cleavage products was obtained using a gradient of 5–20% acetonitrile in triethylammonium acetate over 20 min with a Bio-Rad RSil C18HL column for the modified oligonucleotides and an Apex-1 C-18 column for the unmodified sequences.

Silver-Mediated Cleavage of Oligonucleotides Containing T3′s. A solution of 5′-GACGAT3′sATCGTC (0.5 A_{260} units) and silver nitrate (20 mM) in water (50 μL) was reacted at room temperature for 1 h. Silver was removed by the addition of dithiothreitol (100 mM, 30 μL), followed by centrifugation to remove the insoluble silver–dithiothreitol complex. Aliquots of the supernatant were analyzed by HPLC using a 5–20% gradient of acetonitrile in triethylammonium acetate over 20 min. The 5′-phosphorylated component was identified by HPLC by coinjection with authentic samples prepared from *Eco*RV catalyzed digestion of 5′-GACGATATCGTC. A similar comparison was performed after a 1-h treatment with alkaline phosphatase which removes the 5′-phosphate group. The 3′-thiol-containing product was identified by base composition analysis after purification by HPLC.

Preparation of RF IV M13mp18 DNA Containing a 3′-Thiothymidine Residue in the (−) Strand at a Unique EcoRV Site (RF IV M13mp18 EcoRV DNA). A unique *Eco*RV site in M13mp18 DNA was created at position 6232 according to the procedure previously described (Nakamaye & Eckstein, 1986; Sayers et al., 1989). The *Eco*RV primer containing 3′-thiothymidine at the cleavage site (5′-CGAGCTCGAT3′sATCGTAAT) was phosphorylated according to the following protocol. The primer (2 μL of a solution containing 5 A_{260} units/mL) was treated with polynucleotide kinase (5 units) in 100 mM Tris-HCl, pH 8.0, containing 10 mM magnesium chloride, 1 mM ATP, and 7 mM dithiothreitol in a total volume of 30 μL at 37 °C for 15 min. The enzyme was heat-inactivated at 70 °C for 10 min. Annealing of the primer to the template was performed in a reaction mixture containing 10 μg of the *Eco*RV M13mp18 DNA, 125 mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8, 125 mM sodium chloride, and the phosphorylated primer (6 μL of the above reaction mixture, ca. 2 equiv) in a total volume of 32 μL. The mixture was first heated at 70 °C for 5 min, then transferred to a heating block at 37 °C for 20 min, and finally held on ice until required. To effect polymerization and ligation to double-stranded closed circular (RF IV) DNA, the annealing solution was adjusted to 50 mM Tris-HCl, pH 8, 50 mM sodium chloride, 8 mM magnesium chloride, 0.2 mM each deoxynucleotide triphosphate, and 0.8 mM adenosine triphosphate. Klenow enzyme (15 units) and T4 DNA ligase (16 units) were added to give a total volume of 80 μL, and the reaction was incubated in a water bath at 16 °C. After about 30 h, analysis by agarose gel electrophoresis indicated the presence of double-stranded DNA that was about 60–70% RF IV, the remaining being RF II. Polymerization was terminated by heating at 70 °C for 10 min, and the remaining RF II DNA was removed by incubation with T7 exonuclease (50 units) at 37 °C for 15 min. The reaction mixture was extracted with phenol, and the DNA was precipitated and redissolved in distilled and autoclaved water (50 μL). Approximately 50% of the RF IV DNA was recovered, as estimated from agarose gel analysis. RF IV M13mp18 DNA containing the unmodified *Eco*RV site was prepared in an identical manner using the primer 5′-CGAGCTCGATATCGTAAT.

DNA Cleavage Reactions with EcoRV. The RF IV M13mp18 *Eco*RV DNA (1.5 μg) was incubated at 37 °C with *Eco*RV (15 units) in a total volume of 40 μL of 6 mM Tris-HCl, pH 7.9, 6 mM magnesium chloride, 150 mM sodium chloride, and 6 mM 2-mercaptoethanol. Aliquots of 5 μL were removed and analyzed by agarose gel electrophoresis.

Chemical Cleavage of DNA. RF IV M13mp18 *Eco*RV DNA (0.5 μg) was treated with 16 mM iodine in a total volume of 12 μL of aqueous dioxane (water:dioxane 83:17) containing 0.3% pyridine at 70 °C for 10 min. Reactions were then cooled to room temperature and quenched by the addition of 4 μL of 100 mM 2-mercaptoethanol, and 5-μL aliquots were analyzed by agarose gel electrophoresis.

Results and Discussion

Synthesis and Characterization of Oligonucleotides Containing 3′-Thiothymidine. In earlier reports, we have described the incorporation of 3′-thiothymidine into dinucleotides, using solution chemistry (Cosstick & Vyle, 1988, 1990) and into a pentanucleotide (Cosstick & Vyle, 1989) using solid-phase procedures. However, the synthesis of dodecanucleotide sequences required for the present study necessitated the application of an automated procedure. The coupling of the 3′-thiothymidine residue was performed by removing the reaction column from the machine and introducing the reagents through a syringe; yields for this step were generally 30–40%. As the monomethoxytrityl (MMT) cation has a different absorbance maximum and extinction coefficient from the dimethoxytrityl (DMT) cation, the coupling yield for the introduction of T3′s was best evaluated by DMT cation release following coupling of the subsequent residue. The comparatively low yields for the coupling of the 5′-*O*-monomethoxytritylthymidine 3′-*S*-phosphorothioamidite has previously been noted and is known to result from side-reactions that occur on activation of the phosphorothioamidite with 5-(4-nitrophenyl)tetrazole (Cosstick & Vyle, 1989, 1990). A potential source of low coupling efficiency is the use of the MMT group for the 5′ protection of 3′-thiothymidine in place of the routinely used and more acid-labile DMT group. Clearly incomplete removal of the MMT group under the standard conditions used to cleave the DMT function (3% trichloroacetic acid in dichloromethane for 100 s) would reduce the number of hydroxyl groups subsequently available for coupling and be perceived as a reduction in coupling efficiency. However, MMT release is associated with an intense yellow color and can simply be monitored by visual observation of the reaction column; when the color is fully discharged from the column, deblocking is complete. We observed that the standard acidic conditions used for DMT deblocking also completely removed the MMT group. The purified yields of the oligonucleotides were generally 50% for the unmodified sequences (based on starting with 1 μmol of support-bound nucleoside) and 15–20% for those containing 3′-thiothymidine. HPLC analysis of the purified oligonucleotides indicated that they were in excess of 97% pure. All oligonucleotides were shown to have the correct nucleoside composition after hydrolysis to their constituent nucleosides (Table I). Direct analysis of digestion mixtures obtained from the modified oligonucleotides gave low ratios for 3′-thiothymidine because of its partial oxidation to the corresponding disulfide. Satisfactory analyses were only obtained after reduction of the disulfide with dithiothreitol.

Table I: Extinction Coefficients, T_m Values, and Nucleoside Compositions for the Oligonucleotides Studied

oligonucleotide	extinction coefficient at 254 nm (M^{-1} cm^{-1} per single strand)	T_m (°C)	nucleoside composition				
			dA	dG	dC	dT	dT3's
GACGATATCGTC[a]	83000	53	2.8	3.0	2.8	2.7	0
GACGAT3'sATCGTC	82900	54	2.7	3.0	3.1	1.7	1.2
TCTGATATCCTC	82100	nd	1.8	0.96	4.4	5.0	0
GAGGATATCAGA	115500	nd	4.7	4.3	1.3	2.1	0
TCTGAT3'sATCCTC	82000	nd	1.9	1.0	4.2	4.2	1.3
GAGGAT3'sATCAGA	116000	nd	4.7	4.1	1.2	1.1	1.2

[a] Data for this oligonucleotide is taken from Newman et al. (1990). nd = not determined.

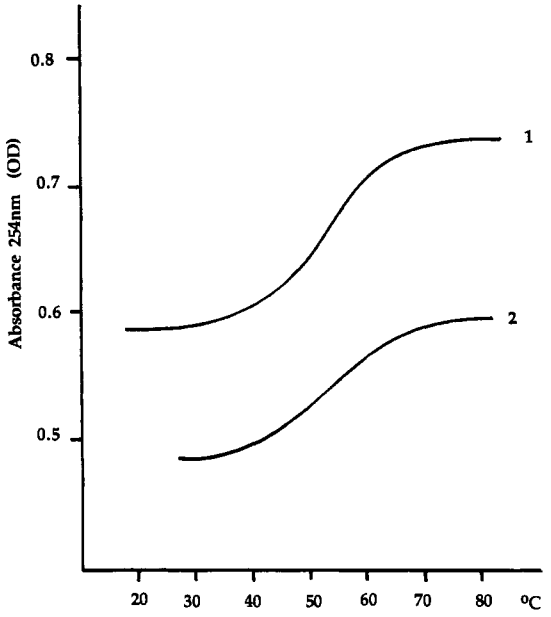

FIGURE 1: Melting curves for GACGATATCGTC (1) and GACGAT3'sATCGTC (2) recorded in a solution containing 50 mM Hepes–KOH, pH 7.5, 100 mM sodium chloride, and 10 mM magnesium chloride. The melting temperatures (Table I) were obtained from differential plots of the above data.

Table II: Cleavage Rates of Oligonucleotide Duplexes with *Eco*RV (See Experimental Procedures for Details)

oligonucleotide duplex	duplex no.	rate of cleavage (nmol of single strand cut min^{-1} mg^{-1})
5'-GACGATATCGTC 3'-CTGCTATAGCAG	1	162[a]
5'-GACGAT3'sATCGTC 3'-CTGCTA3'sTAGCAG	2	0
5'-TCTGATATCCTC 3'-AGACTATAGGAG	3	47.5[a]
5'-TCTGAT3'sATCCTC 3'-AGACTA TAGGAG	4	8.0[b]
5'-GAGGAT3'sATCAGA 3'-CTCCTA TAGTCT	5	6.5[b]

[a] Both strands were cleaved at an equal rate. [b] Only the unmodified strand was cleaved.

However, this remedy was not completely satisfactory since dithiothreitol and its oxidation product introduced additional peaks into the HPLC chromatograms.

Further characterization of GACGAT3'sATCGTC was provided by quantitative cleavage of the phosphorothiolate linkage using 20 mM silver nitrate at room temperature for 1 h. Following treatment with dithiothreitol and alkaline phosphatase, HPLC analysis revealed the presence of two products (retention times 15.4 and 18.2 min, respectively) and the complete disappearance of the original dodecamer (retention time 16.9 min). The more rapidly eluting oligonucleotide was shown to be ATCGTC by coelution with an authentic sample, while isolation of the more slowly eluting component and its digestion to constituent nucleosides established its composition as dC(1), dG(2), dA(2) and T3's(1), which is correct for GACGAT3's. Thus, T3's-containing oligonucleotides can be cut cleanly and specifically at the phosphorothiolate linkage using the mild reagent Ag^+. As previously reported, cleavage can also be accomplished using iodine in aqueous pyridine, but these reagents interfere with the HPLC analysis (Cosstick & Vyle, 1990).

In order to establish that the phosphorothiolate linkage did not have a destabilizing effect on double-helix formation, the T_m of the self-complementary GACGAT3'sATCGTC sequence was determined. The T_m value for the modified oligonucleotide (54 °C) was very close to that previously measured for the analogous unmodified sequence (53 °C) (Figure 1 and Table I). This result confirmed our initial assumption that, given the inherent flexibility of the double helix, the replacement of the 3'-oxygen atom by sulfur would not distort the structure sufficiently to induce duplex destabilization. It has been assumed that the T3's does not destabilize the other oligonucleotide sequences used in this study. This appears to be a valid assumption on the basis that all the modified oligonucleotides studied have the same central GAT3'sATC sequence and therefore sequence and positional effects resulting from T3's incorporation should be negligible.

The circular dichroism (CD) spectrum for GACGAT3'sATCGTC was essentially indistinguishable from that obtained for the unmodified sequence, showing an absorption curve typical of that observed for B-type DNA with almost equally sized maxima and minima at about 280 and 247 nm, respectively.

Cleavage of the Oligonucleotides with EcoRV. The absolute rates of oligonucleotide cleavage are reported in nanomoles of single strand cut per minute per milligram of endonuclease and are shown in Table II. The self-complementary sequence GACGATATCGTC (duplex 1) was hydrolyzed most rapidly (162 nmol min^{-1} mg^{-1}) while essentially no cleavage could be detected with the analogous sequence containing the phosphorothiolate linkage at the cleavage site (duplex 2), even on extended incubation with large quantities of enzyme (rate of cleavage less than 0.01% of the unmodified oligonucleotide). Additionally, no cleavage was observed when Mg^{2+} in the

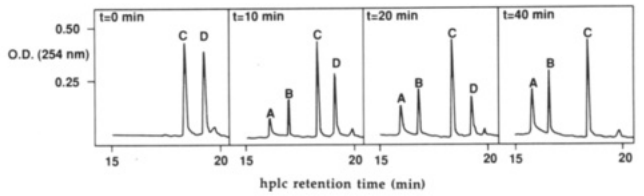

FIGURE 2: The hydrolysis of 5′-GAGGAT3′sATCAGA (C)/5′-TCTGATATCCTC (D) by the *Eco*RV endonuclease (see Experimental Procedures section for details) after 0, 10, 20, and 40 min of reaction time. Only 5′-TCTGATATCCTC (D) is hydrolyzed to give the two products pATCCTC (A) and TCTGAT (B). As can be clearly seen, 5′-GAGGAT3′sATCGTC is refractory to cleavage by *Eco*RV. All substrates and products eluted between 15 and 20 min, and only this region of the HPLC traces is shown.

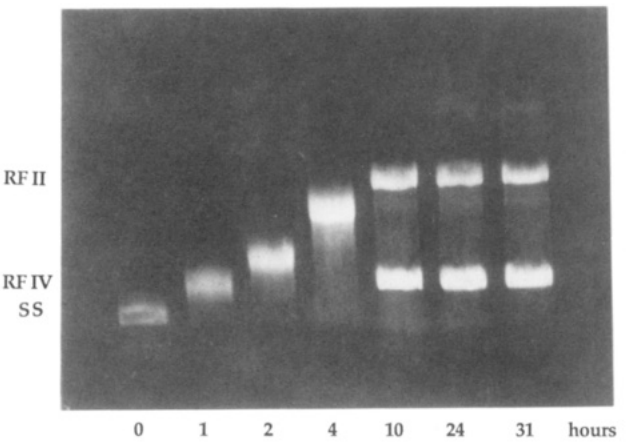

FIGURE 3: Agarose gel electrophoresis showing time course of polymerization and ligation on M13mp18 DNA using 5′-pCGAGCTCGAT3′sATCGTAAT as a primer (see Experimental Procedures section for details).

reaction buffer was replaced by Mn^{2+} or Co^{2+}. However, since we have no evidence that the endonuclease was able to bind duplex 2, this experiment does not demonstrate that the phosphorothiolate group per se is resistant to cleavage. In order to establish that a phosphorothiolate duplex can bind to the enzyme, we investigated the cleavage of a non-self-complementary heteroduplex in which only one strand contained a T3′s residue at the scissile linkage. As a model system, the duplex composed of 5′-TCTGATATCCTC/5′-GAGGATATCAGA (duplex 3) was chosen. This substrate was cleaved at a rate of 47.5 nmol min^{-1} mg^{-1}, with both strands being cleaved at an equal rate. In contrast, 5′-TCTGAT3′sATCCTC/5′-GAGGATATCAGA (duplex 4) was cleaved only in the unmodified strand at a rate of 8.0 nmol min^{-1} mg^{-1}. The products from this reaction were isolated and identified by base composition analysis as 5′-GAGGAT and 5′-pATCAGA, thereby confirming that cleavage of the unmodified strand was occurring at the usual site between the central AT residues. As a control experiment, we examined the ability of the single strand 5′-GAGGATATCAGA to serve as a substrate for the endonuclease. While this sequence is not fully self-complementary, the central hexamer is obviously palindromic and the formation of a duplex structure over this region of the oligonucleotide is possible. However, the rate of cleavage of 5′-GAGGATATCAGA alone was less than 5% of that observed for cleavage of the same sequence in duplex 4. This result demonstrates that cleavage of the unmodified strand does not arise from self-association of 5′-GAGGATATCAGA. Similar experiments have been performed with 5′-TCTGATATCCTC/5′-GAGGAT3′sATCAGA (duplex 5) in which the phosphorothiolate linkage is in the opposite strand as compared to duplex 4. Once again, cleavage occurred only in the unmodified strand (Figure 2) at a rate of 6.5 nmol min^{-1} mg^{-1}. Composition analysis of the products established cutting at the correct site, and control experiments demonstrated that the single strand 5′-TCTGATATCCTC was cleaved at a rate of less than 5% of that observed when it was present in the duplex.

These results clearly demonstrate that, in a heteroduplex containing a normal phosphodiester linkage opposite a phosphorothiolate linkage at the *Eco*RV cleavage site, cutting by the enzyme occurs only in the natural strand. In contrast, treatment with Ag^+ has the opposite effect, producing cleavage only in the modified strand. This provides a system for DNA manipulation in which it is possible to cut either strand of heteroduplex which contains a phosphorothiolate linkage. The difference in *Eco*RV catalyzed hydrolysis rates observed with duplexes 1 and 3 (which both contain solely phosphate groups) is most probably due to the different sequences outside the GATATC recognition sequence. It has previously been observed that restriction endonuclease cleavage rates can be modulated by flanking sequences (Halford et al., 1980; Halford & Johnson, 1980). Interestingly, cleavage of a particular phosphate-containing strand is much faster when paired with another all-phosphate-containing oligonucleotide. This can be seen from duplexes 3, 4, and 5, where a hydrolysis rate of 47.5 nmol min^{-1} mg^{-1} is observed for a duplex consisting of two all-phosphate-containing oligonucleotides, but the rate drops to 6.5 or 8.0 nmol min^{-1} mg^{-1} when one of the strands contains a phosphorothiolate linkage. This effect is probably due to a small difference in overall duplex conformation when phosphate/phosphorothiolate strands are paired which will necessarily exert an effect on the *Eco*RV catalyzed cleavage rates.

Synthesis of M13mp18 DNA Containing a Single Phosphorothiolate Residue in the (−) Strand. Our cleavage studies on DNA substrates required double-stranded closed circular (RF IV) DNA in which one strand contained a single T3′s residue at the cleavage position of a unique *Eco*RV site. Reactions on this substrate that cleaved just one of the DNA strands would produce a nicked species (RF II) which could be readily distinguished from the RF IV DNA or linear (RF III) DNA by agarose gel electrophoresis in the presence of ethidium bromide (Potter & Eckstein, 1984). It was evident that these features could be readily introduced into M13 DNA which had been engineered to convert the unique *Eco*RI site into an *Eco*RV site. Thus, the template (+) strand of M13mp18 DNA (SS DNA) containing a unique *Eco*RV site at position 6232 was annealed to the modified primer 5′-pCGAGCTCGAT3′sATCGTAAT (*Eco*RV sequence underlined) or the natural sequence 5′-pCGAGCTCGATATCGTAAT. Synthesis and ligation of the (−) strand was effected using the Klenow enzyme and the 4 deoxynucleotide triphosphates in the presence of T4 DNA ligase. Assaying the reaction over a period of 30 h showed that after about 10 h the amount of RF IV DNA remained constant and represented 60–70% of the total DNA, the remainder being the RF II form (Figure 3). In particular, it was noted that high yields of RF IV DNA required the correct balance of Klenow and ligase activity; too much Klenow enzyme caused the polymerization to overrun and displace the primer, while too little of this enzyme resulted in premature termination of the polymerization. The RF II DNA was removed by a short treatment with T7 exonuclease which is known to hydrolyze the nicked strand of RF II DNA in a progressive manner to give a mixture of RF IV DNA and what is essentially the original M13 (+) strand (Sayers et al., 1988;

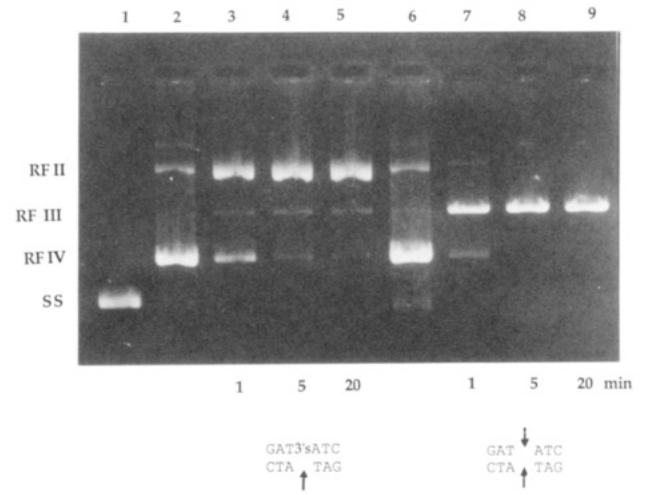

FIGURE 4: Agarose gel analysis of *Eco*RV cleavage experiments performed on M13mp18 RF IV DNA containing the *Eco*RV site. Reaction conditions are described in the Experimental Procedures section. Lane 1, marker of single-stranded (+) M13mp18 DNA; lane 2, RF IV DNA containing T3′s at the *Eco*RV cleavage site; lanes 3, 4, and 5, as lane 2, but after treatment with *Eco*RV (10 units of enzyme/μg of DNA) for 1, 5, and 20 min, respectively; lane 6, RF IV DNA containing all phosphodiester linkages; lanes 7, 8, and 9, as lane 6, but after treatment with *Eco*RV (10 units of enzyme/μg of DNA) for 1, 5, and 20 min, respectively.

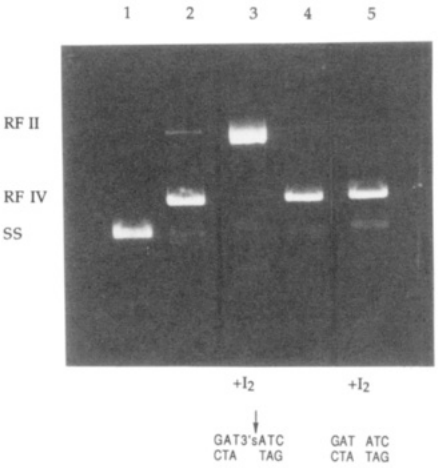

FIGURE 5: Agarose gel analysis of iodine cleavage reactions on the M13mp18 RF IV DNA. Reaction conditions are as described in the Experimental Procedures section. Lane 1, marker of single-stranded (+) M13mp19 DNA; lane 2, RF IV DNA containing a single T3′s residue; lane 3, as lane 2, but after treatment with iodine in water:dioxane (83:17) containing 0.3% pyridine; lane 4, RF IV DNA containing all phosphodiester linkages; lane 5, as lane 4, but treated under conditions described for lane 3.

Kerr & Sadowski, 1972). Since the remaining (+) strand does not interfere with subsequent reactions and was not readily visible on gel electrophoresis, its removal by filtration through nitrocellulose was not attempted.

Cleavage of the Phosphorothiolate-Containing DNA with EcoRV. Cleavage reactions with *Eco*RV were performed with an excess of this enzyme under optimal conditions of salt and pH. Figure 4 (lanes 2–5) shows that the DNA with a T3′s in the (−) strand is converted to about 80% RF II DNA within 1 min and is greater than 95% nicked after 20 min. A faint band corresponding to linearized DNA is also discernible, but the intensity of this band does not noticeably increase with time and indicates that if the RF II form is being converted to the linear form by the enzyme, the rate for the second cleavage event is negligible. In contrast, the unmodified DNA (lanes 6–9) is, as expected, rapidly converted to linear DNA with the reaction being about 90% complete within 1 min and total conversion to RF III DNA achieved within 5 min.

Chemical Cleavage of the Phosphorothiolate-Containing DNA. Our previous studies (Cosstick & Vyle, 1988, 1989, 1990) had shown that the phosphorothiolate linkage could be efficiently cleaved using an aqueous iodine solution and the reaction is catalyzed by pyridine presumably due to the formation of a phosphorylpyridinium intermediate (Cummins & Potter, 1985; Richard & Frey, 1983; Mikolajczyk, 1966). Thus, the phosphorothiolate DNA was heated at 70 °C for 10 min in a 16 mM solution of iodine in aqueous dioxane (water:dioxane 83:17) containing 0.3% pyridine, and after cooling the reaction was quenched by the addition of 2-mercaptoethanol. Analysis of the reaction mixture by agarose gel electrophoresis (Figure 5, lane 3) revealed a clean and essentially quantitative conversion to RF II DNA while, in contrast, the unmodified DNA was unchanged by this treatment (lane 5). Thus, the only logical interpretation of these data is that the RF II DNA has been generated by the specific cleavage of the phosphorothiolate linkage as we had observed in the oligonucleotides. It should be noted that iodine solutions similar to those used here, but of much higher iodine concentration, are routinely used in the oxidative step of oligonucleotide synthesis and to our knowledge these oxidative conditions have not been reported to have any deleterious effect on either phosphodiester or the more sensitive phosphotriester linkages.

Unfortunately, cleavage with 20 mM aqueous silver nitrate could not be investigated since these conditions were incompatible with the analysis of large molecular weight DNA by agarose gel electrophoresis and resulted in weak, smeared bands that were consistent with silver complexation by the DNA (Jensen et al., 1966; Arya & Yang, 1985).

CONCLUSIONS

The results from the studies on both the oligonucleotides and DNA unequivocally demonstrate that for substrates that contain a 3′-phosphorothiolate linkage in one strand *Eco*RV cleaves only the unmodified strand. Additionally, it is shown that the phosphorothiolate linkage can be cleaved specifically and in essentially quantitative yield using aqueous iodine and that this reaction is compatible with biologically relevant DNA. It is apparent that the 3′-phosphorothiolate group is a very versatile analogue of the natural phosphodiester linkage which may be of potential use in the cleavage and manipulation of DNA. The interaction of other restriction endonucleases with phosphorothiolate-containing oligonucleotides and the synthesis of oligonucleotides containing multiple phosphorothiolate linkages is currently under investigation.

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Registry No. *Eco*RV, 83589-02-0; GACGAT3′SATCGTC, 126884-15-9; TCTGAT3′sATCCTC, 138666-19-0; GAGGAT3′SATCAGA, 138666-20-3; GACGATATCGTC, 99131-03-0; GACGAT3′SATCGTC, 126884-15-9; TCTGA-

TATCCTC/GAGGATATCAGA, 138630-90-7; TCTGAT3′sATCCTC/GAGGATATCAGA, 138666-21-4; GAGGAT3′SATCAGA/TCTGATATCCTC, 138666-22-5; Ag, 7440-22-4; I_2, 7553-56-2.

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