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Use of EDTA Derivatization To Characterize Interactions between Oligodeoxyribonucleoside Methylphosphonates and Nucleic Acids†

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ABSTRACT: EDTA-derivatized oligonucleoside methylphosphonates were prepared and used to characterize hybridization between the oligomers and single-stranded DNA or RNA. The melting temperatures of duplexes formed between an oligodeoxyribonucleotide 35-mer and complementary methylphosphonate 12-mers were 4-12 °C higher than those of duplexes formed by oligodeoxyribonucleotide 12-mers as determined by spectrophotometric measurements. Derivatization of the methylphosphonate oligomers with EDTA reduced the melting temperature by 5 °C. Methylphosphonate oligomer-nucleic acid complexes were stabilized by base stacking interactions between the terminal bases of the two oligomers binding to adjacent binding sites on the target. In the presence of Fe^{2+} and DTT, the EDTA-derivatized oligomers produce hydroxyl radicals that cause degradation of the sugar-phosphate backbone of both targeted DNA and RNA. Degradation occurs specifically in the region of the oligomer binding site and is approximately 20-fold more efficient for single-stranded DNA than for RNA. In comparison to the presence of one oligomer, the extent of target degradation was increased considerably by the additions of two oligomers that bind at adjacent sites on the target. For example, the extent of degradation of a single-stranded DNA 35-mer caused by two contiguously binding oligomers, one of which was derivatized by EDTA, was approximately 2 times greater than that caused by the EDTA-derivatized oligomer alone. Although EDTA-derivatized oligomers are stable for long periods of time in aqueous solution, they undergo rapid autodegradation in the presence of Fe^{2+} and DTT with half-lives of approximately 30 min. This autodegradation reaction renders the EDTA-derivatized oligomers unable to cause degradation of their complementary target nucleic acids. It appears that cleavage of the EDTA portion of the molecule by hydroxyl radicals is the major cause of this autodegradation and that the methylphosphonate backbone is resistant to cleavage.

Recently a class of "DNA affinity cleaving" molecules composed of a DNA binding molecule and a "molecular scissors" has been described (Schultz et al., 1982; Bowler et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Doan et al., 1986, 1987; Chen et al., 1987; Sluka et al., 1987). This class of compounds is able to cleave DNA at the binding site of the DNA binding molecule. Such compounds have been used in footprinting experiments in place of the more tradi-

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tional DNase I digestion method. Usually, the molecular scissors mediate DNA strand scission via metal-ion catalysis or via photoactivation. For example, molecular scissors consisting of EDTA, phenanthroline, or porphyrin require metal ions and a reducing agent for activation (Schultz et al., 1982; Chen et al., 1987; Doan et al., 1986), whereas molecular scissors consisting of azidoproflavin, methylene blue, or acridine orange require ultraviolet light for activation (Doan et al., 1987; OhUigin et al., 1987; Bowler et al., 1984). Various types of molecular scissors have been linked to oligodeoxyribonucleotides to bring about site-specific strand scission of complementary single-stranded DNA (Chu & Orgel, 1985; Dreyer & Dervan, 1985; Chen et al., 1987; Doan et al., 1986). We have employed this strategy to characterize the interaction of oligodeoxyribonucleoside methylphosphonates with DNA or RNA.

Antisense oligonucleoside methylphosphonates are able to inhibit the expression of mRNA in living cells (Miller et al., 1981; Jayaraman et al., 1981; Blake et al., 1985; Agris et al., 1986; Smith et al., 1986; Marcus-Sekura et al., 1987). The mechanism of action of these oligonucleotide analogues is believed to involve hybridization of the oligomer to complementary regions of the target mRNA. We have demonstrated the interaction of the methylphosphonate oligomers with DNA and RNA by several methods. For example, deoxyadenosine methylphosphonate oligomers of two, three, or four nucleosides in length were shown to form hydrogen-bonded complexes with poly(rU) or poly(dT) by monitoring their melting temperatures by UV spectroscopy (Miller et al., 1979, 1981) Methylphosphonate oligomers complementary to rabbit globin mRNAs hybridize with mRNA in agarose gels, and the binding sites of the oligomers were characterized by using the oligomer as a primer for reverse transcriptase (Murakami et al., 1985). Recently sequence-specific interaction of psoralen-derivatized methylphosphonate oligomers with single-stranded RNA and DNA was demonstrated (Murakami et al., 1986; Lee et al., 1988; Kean et al., 1988). These derivatized oligomers covalently bind to target RNA or DNA upon irradiation at 365 nm. Although the experiments described above have consistently indicated that the methylphosphonate oligomers interact with nucleic acids in a sequence-specific manner, a more direct method to characterize their interaction is desirable and is introduced in this paper.

Oligodeoxynucleotides have been derivatized with EDTA,[1] which, in the presence of Fe^{2+} and a reducing agent, catalyze the one-electron reduction of oxygen and produce reactive hydroxyl radicals (Dreyer & Dervan, 1985; Chu & Orgel, 1985). These hydroxyl radicals cause cleavage of the sugar–phosphate backbone of DNA. Hybridization of an EDTA-modified oligodeoxynucleotide to its complementary target sequence brings about strand breakage at the oligomer binding site and thus serves to locate the binding site on the targeted nucleic acid. We have used this technique to characterize the binding sites of oligonucleoside methylphosphonates on targeted DNA and RNA molecules. In addition, we have measured the stability of duplexes formed by these oligomers and their targets. The stability of these derivatized methylphosphonate oligomers was also studied.

Materials and Methods

Poly(dT), poly(rU), $(dT)_{20}$, $(dT)_{12}$, cytidine 3′,5′-bisphosphate, and nucleotide triphosphates were purchased from Pharmacia Inc. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from Amersham Inc. Cytidine 3′,5′-[^{32}P]bisphosphate, and EN^{3}HANCE spray were obtained from NEN Du Pont Inc. T_4 polynucleotide kinase, T_7 RNA polymerase, and RNA ligase were purchased from United States Biochemical Corp. Cellulose plastic sheets (0.1 mm) for thin-layer chromatography were purchased from E. Merck, Darmstadt, Germany. EDTA (acetic 1-^{14}C- and acetic 2-^{14}C-labeled 4 mCi/mmol) were obtained from ICN Inc. The melting temperature (T_m) was measured by using a Varian Cary 219 spectrophotometer. The concentrations of the oligomers and the buffers used in each experiment are described in Tables I and II. The samples were heated to 80 °C for 15 min, cooled to room temperature, and kept at 4 °C for 16 h. The absorbance of the sample was monitored at 254 nm in a 1-cm masked cuvette as temperature changed from 5 to 60 °C. The temperature was increased at a rate of about 1 °C/min. Anhydrous nitrogen gas was passed continuously through the sample compartment while the temperature was below 20 °C.

Preparation of Oligodeoxyribonucleoside Methylphosphonates. Oligonucleoside methylphosphonates were synthesized on a 1% cross-linked polystyrene support using 5′-(dimethoxytrityl)nucleoside 3′-(methylphosphonic imidazolide) intermediates (Miller et al., 1986) or on a controlled-pore glass support using 5′-(dimethoxytrityl)nucleoside 3′-(*N*,*N*-diisopropylmethylphosphonamidite) intermediates (Jager et al., 1984; Dorman et al., 1984), which were purchased from American Bionetics Inc. A Systec Model 1440 DNA synthesizer was used. The oligomers were deblocked, purified (Miller et al., 1986; Lee et al., 1988), and sequenced (Murakami et al., 1985) as described previously.

Preparation of EDTA-Derivatized Oligonucleoside Methylphosphonates. Oligonucleoside methylphosphonates derivatized with EDTA at the 5′-terminus were prepared from methylphosphonate oligomers (0.2–0.5 μmol) (**1**) that were phosphorylated by using 3 units/μL of T_4 polynucleotide kinase and 1–2.5 μmol of ATP in a reaction volume of 0.2–0.5 mL. After purification, the phosphorylated oligomer **2** was dissolved in 0.2 mL of 0.1 mM imidazole buffer (pH 6) containing 0.15 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. The mixture was stirred at 25 °C for 2 h, and an equal volume of 0.3 M EDA/0.5 M lutidine buffer (pH 7.6) was then added; the reaction was continued for another 2 h to convert the oligomer–imidazolide to the EDA-derivatized oligomer **3**. EDTA–EDA–oligomer **4** was obtained by reaction of 0.1 mM **3** with 1% EDTA anhydride (Hnatowich et al., 1982) in 0.3 M HEPES buffer (pH 7.6) at 25 °C for 1 h. EDA–oligomer **3** and EDTA–EDA–oligomer **4** were each purified by DEAE-cellulose chromatography using a linear gradient of 0–0.5 M NH_4HCO_3. ^{32}P-Labeled **2**, **3**, and **4** were also prepared and were purified by PAGE using 7 M urea–15% polyacrylamide gel (Maniatis et al., 1982) as previously described for oligonucleoside methylphosphonates (Miller et al., 1986; Lee et al., 1988).

Cytidine-derivatized methylphosphonate oligomers were prepared by transamination of the oligomer in a solution containing 0.5 μmol of methylphosphonate oligomer, 200 μL of 1 M $NaHSO_3$/3 M EDA (pH 6.2), and 10 μL of 100 mM hydroquinone–ethanol solution. Both the bisulfite and hydroquinone solutions were freshly prepared. After incubating at 25 °C for 6 h, the reaction was terminated by adding 50

[1] Abbreviations: CM, carboxymethyl; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDA, ethylenediamine; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 5′-EDTA-I or -II, oligodeoxynucleoside methylphosphonate I or II with EDTA derivatized on the 5′-terminal phosphate; C-EDTA-II or -III, oligodeoxynucleoside methylphosphonate II or III with EDTA derivatized on a cytosine residue.

volumes of distilled water. A DEAE-cellulose column in the HCO_3^- form and subsequently a CM-Sephadex column in the NH_4^+ form were used to remove charged components from the transaminated product **5**. Oligomer **5** was converted to the EDTA-derivatized oligomer **6** by using the same reaction conditions as described for preparation of EDTA–EDA–oligomer **4**. Oligomer **6** was purified by DEAE-cellulose chromatography using a linear gradient of 0–0.5 M NH_4HCO_3. For purposes of characterization, a ^{32}P-labeled phosphate group was attached to the 5′-end of **5** and **6** by using T_4 polynucleotide kinase and [γ-^{32}P]ATP, and the products were purified by PAGE.

Preparation of ^{32}P-Labeled Target Oligodeoxynucleotides and Oligoribonucleotide. Oligodeoxyribonucleotides were synthesized by the β-cyanoethylphosphoramidite method (Sinha et al., 1983) on controlled-pore glass supports using a Systec Model 1440 DNA synthesizer and were sequenced as described by Chandrasegaran et al. (1985). DNA_{35}, which was used as a target for the EDTA-derivatized methylphosphonate oligomers, was phosphorylated by T_4 polynucleotide kinase and purified by PAGE. Poly(dT), $(dT)_{20}$, and $(dT)_{12}$ were 5′-end-labeled by the same method, but were used in the degradation assays without further purification. RNA_{33} was synthesized by in vitro transcription of the duplex formed between d-$^{5'}$TAATACGACTCACTATAG$^{3'}$ and d-$^{3'}$ATTATGCTGAGTGATATCCGACTGATTAAAAAAAATAATACGTCTCCGG$^{5'}$ using T_7 RNA polymerase. The conditions for in vitro transcription are as follows: 3.4 units/μL T_7 RNA polymerase, 0.5 μM promoter and template DNA, 1 mM NTP, 10 mM $MgCl_2$, 25 mM NaCl, 5 mM DTT, and 40 mM Tris-HCl (pH 7.6) in a total volume of 100 μL were incubated at 37 °C for 1 h. 5′-End-labeled RNA_{33} was obtained by including [γ-^{32}P]GTP in the reaction, whereas 3′-end-labeled RNA_{33} was obtained by ligation of the transcript with [^{32}P]pCp using RNA ligase. EDTA was added to a final concentration of 10 mM to stop the enzyme reactions, and the RNA was desalted on a SEP-PAK C_{18} cartridge (Lo et al., 1984). The RNA was then purified by PAGE. The chain length of the end-labeled RNA was characterized by partial hydrolysis with 0.5 M $NaHCO_3$–Na_2CO_3, pH 9.3, at 95 °C for 3–5 min. The position of the guanines in the RNA was determined by ribonuclease T_1 digestion under denaturing conditions using 1–10 units/μL of enzyme, in a buffer containing 6 M urea and 20 mM sodium citrate, pH 5, incubated at 55 °C for 5–7 min (D'Alessio, 1982). The RNAs from both the base hydrolysis and RNase T_1 digestion were analyzed by PAGE using a 15% polyacrylamide gel containing 7 M urea, 30 cm in length.

Assays of Target Nucleic Acid Degradation and Autodegradation. Each degradation assay solution contained 5 μM EDTA-derivatized oligomer, 100 nM 5′-^{32}P-labeled target DNA/RNA (>20000 cpm), 10 μM $FeSO_4$, 5 mM DTT, and 50 mM Tris-HCl (pH 7.6) in a total volume of 10 μL. Autodegradation of the EDTA-derivatized oligomers was carried out under the same conditions but in the absence of target. A ^{32}P-labeled oligomer at a concentration of 10 nM was used. Both reactions were initiated by adding DTT to form a complete assay mixture and quenched by adding 1 mM EDTA solution to a final concentration of 0.1 mM. The reaction mixtures were then evaporated to dryness and directly analyzed by PAGE.

Characterization of EDTA–Oligomer Autodegradation Product. The product of 5′-EDTA-I autodegradation was purified by PAGE and then treated with 0.1 N HCl at 37 °C for 20 min or treated with 1 M piperidine at 37 °C for 30 min to cleave the phosphoramidate linkage or methylphosphonate oligomer (Murakami et al., 1985), respectively. The products of these reactions were analyzed by PAGE.

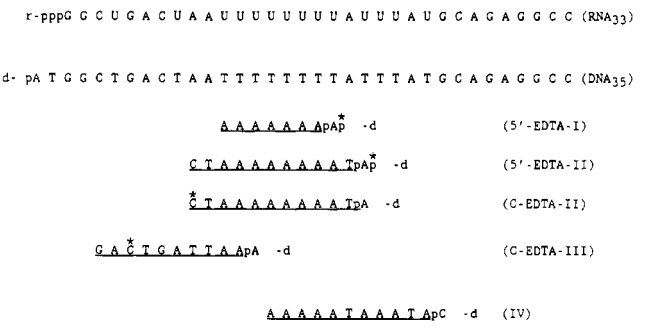

FIGURE 1: Nucleotide sequences of DNA_{35}, RNA_{33}, and the EDTA-derivatized oligonucleoside methylphosphonates. The asterisk indicates the position of the EDTA group, and the underline indicates the position of methylphosphonate linkages.

Stability of [^{14}C]EDTA in the Presence of Fe^{2+} and DTT. A solution containing 150 μM EDTA (60 μCi), 200 μM $FeSO_4$, 50 mM Tris-HCl (pH 7.6), and 10 mM DTT in a total volume of 100 μL was incubated at 25 °C for 2 h. A 10-μL aliquot was then removed and applied to a cellulose TLC plate. The plate was eluted with $EtOH/NH_4OH/H_2O$ (20:1:40), air-dried, sprayed with EN^{3}HANCE spray, and then exposed to Kodak SB X-ray film at −80 °C for 24 h.

Results and Discussion

A DNA 35-mer and an RNA 33-mer, whose sequences are shown in Figure 1, were used as targets for EDTA-derivatized methylphosphonate oligomers. For ease of reference, the targets are designated DNA_{35} and RNA_{33}. The sequences of the methylphosphonate oligomers are designated by a Roman numeral prefixed with the type of EDTA derivative as shown in Figure 1. The sequence of DNA_{35} corresponds to nucleotides 6–40 of the origin of replication of SV-40 DNA, and the sequence of RNA_{33} corresponds to the 33 nucleotides at the 3′-end of DNA_{35}. The interaction of psoralen-derivatized methylphosphonate oligmers with DNA_{35} has been previously described (Lee et al., 1988).

The EDTA moiety is attached to either the 5′-terminal phosphate or the C-4 of a deoxycytidine residue of the methylphosphonate oligomer as shown in Figure 2, compounds **4** and **6**. In order to study oligomer derivatized with only one EDTA group, methylphosphonate sequences were chosen that have only one complementary cytidine residue or that have a non-hydrogen-bonding cytidine at the 3′-end of the oligomer as in the case of C-EDTA-II.

Measurements of the melting temperatures of duplexes formed between DNA_{35} and the methylphosphonate oligomers were used to ascertain (1) the stability of the complexes formed by oligonucleoside methylphosphonates in comparison to the complexes formed by oligodeoxynucleotides with DNA_{35}, (2) the effect of EDTA derivatization on the stability of oligonucleoside methylphosphonate–DNA complexes, and (3) the cooperativity and mutual stabilization between oligomers that hybridize to adjacent sites on DNA_{35}.

The EDTA-derivatized methylphosphonate oligomers were used to study oligomer binding specificity by monitoring the degradation of DNA_{35} and RNA_{33}. C-EDTA-III and oligomer IV, which have adjacent binding sites on DNA_{35}, were used to study the effects of end-to-end base stacking interactions on hybridization of the oligomers to DNA_{35}.

Synthesis of EDTA-Derivatized Oligonucleoside Methylphosphonates and Their Targets. The oligonucleoside me-

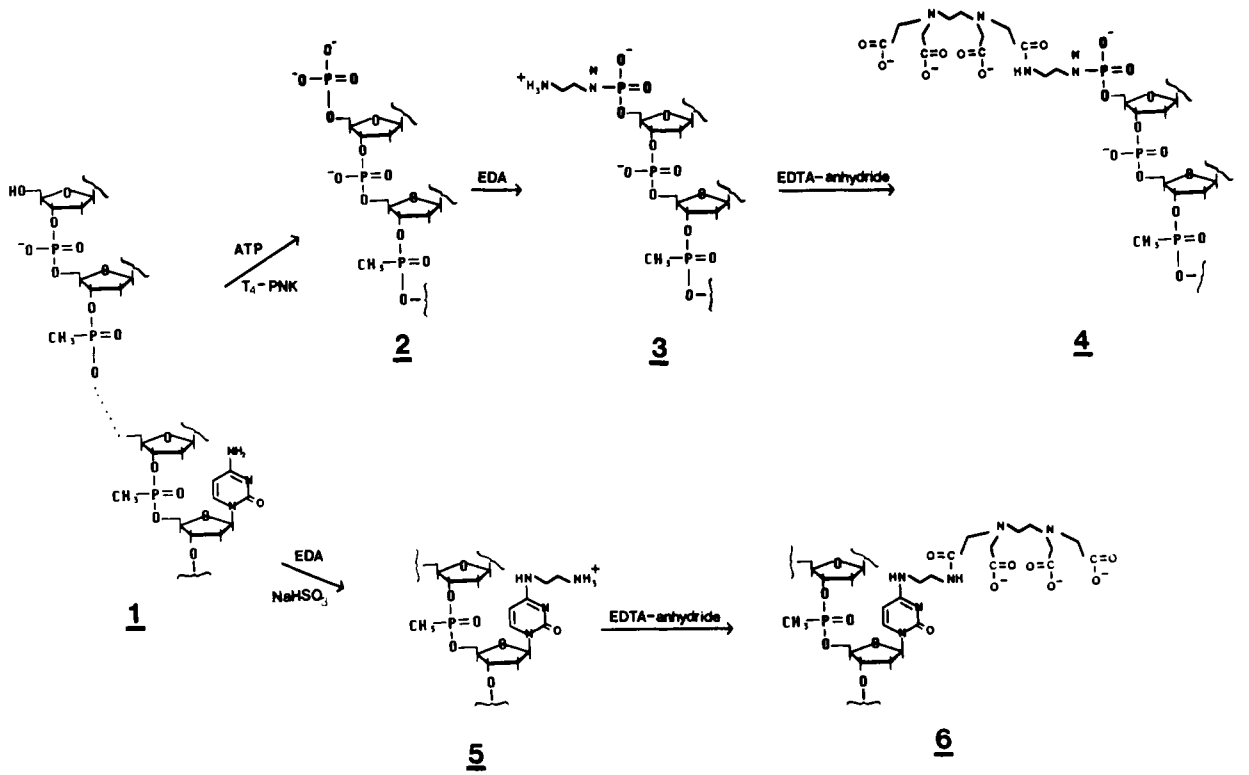

FIGURE 2: Synthesis of EDTA-derivatized oligodeoxyribonucleoside methylphosphonates.

thylphosphonates were derivatized with EDTA either at their 5′-end or through the C-4 position of a deoxycytidine in the oligomer. Derivatization at the 5′-position proceeded in higher yield and gave purer product than derivatization of deoxycytidine. For the preparation of the 5′-derivatized oligomers, the use of a 5–10-fold excess of ATP in the polynucleotide kinase catalyzed phosphorylation reaction generally resulted in an 80–90% isolated yield of phosphorylated oligomer. Derivatization of this oligomer with EDA proceeded in 90–95% overall yield. A single product was produced, and the methylphosphonate linkage was stable throughout the reaction. The EDA-derivatized oligomers were quantitatively converted to their EDTA adducts by reaction with EDTA anhydride. Oligomers **1**, **2**, **3**, and **4**, which have net charges of −1, −3, −1, and −5, respectively, were eluted with 0.19, 0.26, 0.18, and 0.34 M NH_4HCO_3 on a DEAE-cellulose column using a linear gradient of 0–0.5 M NH_4HCO_3. A minor product of lower negative charge was usually observed during the purification of **4**. This may correspond to oligomer in which the EDTA is complexed with trace metal ions from water. This material was shown to be capable of directing DNA cleavage in the presence of Fe^{2+} and DTT, although it had lower activity than **4** in the presence of added Fe^{2+} and DTT. This lower activity may be due to competition between Fe^{2+} and other metal ions, such as Mg^{2+} and Ca^{2+}, which are unable to undergo one-electron oxidoreduction reaction. A similar product was also observed with the cytidine-derivatized oligomer.

The low isolated yield of cytidine-derivatized oligomers was attributed to the transamination reaction that resulted in cleavage of the methylphosphonate backbone. Transamination of DNA/RNA has been widely used to place an alkylamine group at N4 of cytidine in order to attach nonisotopic labels (e.g., biotin, fluorescent molecules) (Draper et al., 1980; Viscidi et al., 1986; Gebeyehu et al., 1987). Deamination of cytosine to form uracil has been shown to occur during the transamination reaction (Shapiro et al., 1970). The reaction conditions used by Draper et al. (1980) were reported to give higher transamination yields and less deamination. Hydroquinone was added to scavenge radicals generated from bisulfite that might otherwise cause DNA/RNA backbone cleavage. However, we found that the methylphosphonate oligomers were hydrolyzed during transamination. Possibly bisulfite participates in a nucleophilic attack on the methylphosphonate linkages, leading to oligomer backbone cleavage. The transamination product, zero net charged oligomer **5**, was not retained by either DEAE-cellulose or CM-Sephadex ion-exchange columns, whereas oligomers with either unreacted cytidine or the deamination product uridine, which have a net charge of −1, were retained on the DEAE column. Other charged contaminants were also removed by these two columns. Oligomer **5** was quantitatively converted to **6** by reaction with EDTA anhydride. The product **6** was purified by DEAE-cellulose chromatography using a gradient of 0–0.5 M NH_4HCO_3 and was eluted at 0.30 M. As in the case of the 5′-derivatized oligomers, **6** appeared to form complexes with metal ions in the water.

The 5′-derivatized oligomers were characterized by treatment with dilute acid, which cleaves the phosphoramidate linkage to yield the original 5′-phosphorylated oligomer (Lee et al., 1988), or by treatment with piperidine, which cleaves the methylphosphonate linkages (Murakami et al., 1985). S_1 nuclease did not cleave the phosphodiester linkage of the derivatized oligomer even after prolonged treatment with the enzyme. This behavior is in contrast to that of psoralen-derivatized oligomers in which the phosphodiester linkage is slowly hydrolyzed (Lee et al., 1988). Possibly the negatively charged EDTA group, which is adjacent to the phosphodiester bond, prevents the enzyme from hydrolyzing this linkage.

The single-stranded DNA_{35} target was synthesized via β-cyanoethylphosphoramidate intermediates (Sinha et al., 1983), and its sequence was verified by a modification of the Maxam/Gilbert sequencing procedure (Chandrasegran et al., 1985). RNA_{33} was prepared by transcription of the synthetic DNA containing a T_7 RNA polymerase promoter. The chain

Table I: Melting Temperatures of Complexes Formed between DNA_{35} and Oligonucleoside Methylphosphonates or Oligodeoxynucleotides

oligomer	T_m[a] (°C) oligonucleoside methylphosphonate	T_m[a] (°C) oligodeoxynucleotide
III	30	26
IV	33.5	21
C-EDTA-III	25	
III + IV	35.5	28
C-EDTA-III + IV	35	

[a] Melting experiments were carried out on a 1:1 mixture of the oligomer and DNA_{35} at a total strand concentration of 1 μM in a solution containing 50 mM Tris-HCl, pH 7.6, and 140 mM sodium chloride.

length of RNA_{33} was characterized by base hydrolysis, and the sequence was characterized by digestion with RNase T_1. These results showed that two major transcripts 32 and 33 nucleotides in length were produced. It appears that the DNA template can form a hairpin structure containing 9 base-pairs in its stem. The stem region contains a bulged thymidine corresponding to the fifth nucleotide of RNA_{33}. Our results suggest that this bulged nucleotide can be skipped by the polymerase during transcription, resulting in formation of the 32-mer transcript (Lin, 1988).

Stability of Complexes Formed between EDTA-Derivatized Oligonucleoside Methylphosphonates and Their Targets. As shown in Table I and Figure 3 the the T_ms of duplexes formed by oligodeoxynucleotides with DNA_{35} are lower than those of the duplexes formed by the corresponding methylphosphonate oligomers. Besides the difference in T_m, the absorbance of a solution of the methylphosphonate/DNA_{35} duplex increases 30–50% when the temperature is changed from 4 to 45 °C. Under the same conditions, the absorbance of the oligodeoxynucleotide/DNA_{35} duplex increased only 10–13% (Figure 3). These results suggest that duplexes formed by nonionic methylphosphonate oligomers (with one negative charge per molecule) have increased base stacking interactions and are more stable than duplexes formed by the fully charged oligodeoxynucleotides.

The effect of EDTA derivatization on the stability of duplexes formed between oligomer III and the DNA_{35} is shown in Table I and Figure 3. Derivatization results in a 5 °C decrease in the T_m of the duplex compared to that of the underivatized oligomer. This decrease in stability can be attributed to the increased charge repulsion and/or increased steric hindrance created by the negatively charged EDTA group. In the present case it appears that the decrease is due mainly to steric factors because the T_m measurement was carried out in the presence of Fe^{3+}, which should greatly diminish the charge repulsion caused by the EDTA group. The steric hindrance can be caused by substitution of EDTA on the internally located cytosine base.

The effect of EDTA derivatization on interaction of oligomer I with poly(dT) was also determined, and the results are shown in Table II. The stoichiometry of the complex is 2T:1A as determined by the mixing curve (Lin, 1988), which is consistent with the dramatic increase in T_m with increasing ionic strength. The transition profiles of these complexes are much steeper than those presented in Figure 3, with the breadth of the total transition less than 7 °C (Lin, 1988). Derivatization of I with EDTA lowers the T_ms of the triplexes by 11 °C in 50 mM Tris-HCl buffer or by 14.5 °C in 50 mM Tris-HCl and 0.14 M NaCl (Table II). It is unlikely that the negative charge of the EDTA group is the cause of the decrease in the T_m because adding Fe^{3+} to the triplex increases

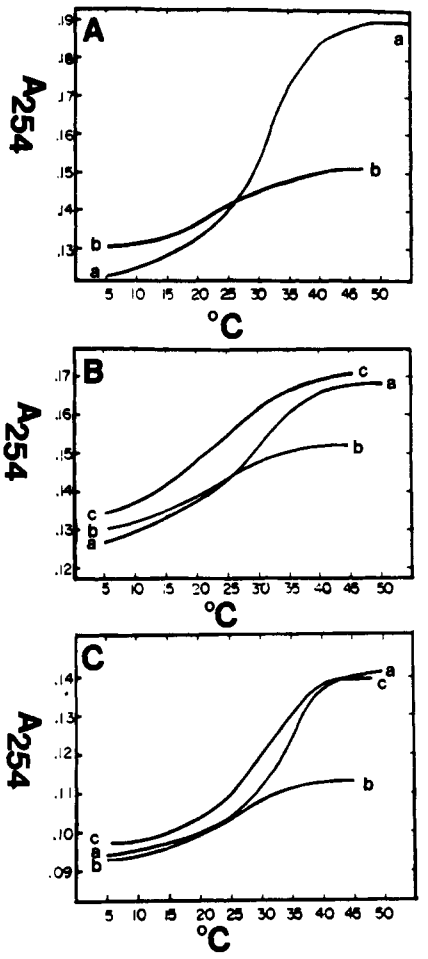

FIGURE 3: Melting profiles of duplexes formed between DNA_{35} and oligodeoxyribonucleoside methylphosphonates or oligodeoxyribonucleotides. Complexes formed between DNA_{35} and the following oligomers: (A) (a) oligonucleoside methylphosphonate IV and (b) oligodeoxyribonucleotide IV; (B) (a) oligonucleoside methylphosphonate III, (b) oligodeoxyribonucleotide III, and (c) oligonucleoside methylphosphonate C-EDTA-III; (C) (a) oligonucleoside methylphosphonates III + IV, (b) oligodeoxyribonucleotides III + IV, and (c) oligonucleoside methylphosphonates C-EDTA-III + IV. The salt conditions and oligomer concentrations are described in Table I.

Table II: Melting Temperatures of Complexes Formed between Poly(dT) or Poly(rU) and Oligomer I or 5′-EDTA-I

complex	T_m[a] (°C) 50 mM Tris (pH 7.6)	T_m[a] (°C) 50 mM Tris, 140 mM NaCl (pH 7.6)
I–poly(dT)	42.5	52.5
5′-EDTA-I–poly(dT)	28	41.5
I–poly(rU)	34	44
5′-EDTA-I–poly(rU)	25	37

[a] The solutions contained equal molar concentrations of poly(dT) and methylphosphonate oligomer at a total nucleotide concentration of 3×10^{-5} M.

the T_m by only 2 °C. On the other hand, steric hindrance from the bulky EDTA group may exert the largest effect by reducing the cooperative interaction of the adjacent oligomers along the poly(dT) strands.

Cooperative interactions were also observed for oligomers having adjacent binding sites on the DNA_{35}. As shown in Table I and Figure 3, the T_m of the duplex formed between oligomers III and IV and DNA_{35} is higher than that of the duplex formed by III and DNA_{35} or by IV and DNA_{35}. A similar increase in T_m is observed for the duplex formed by

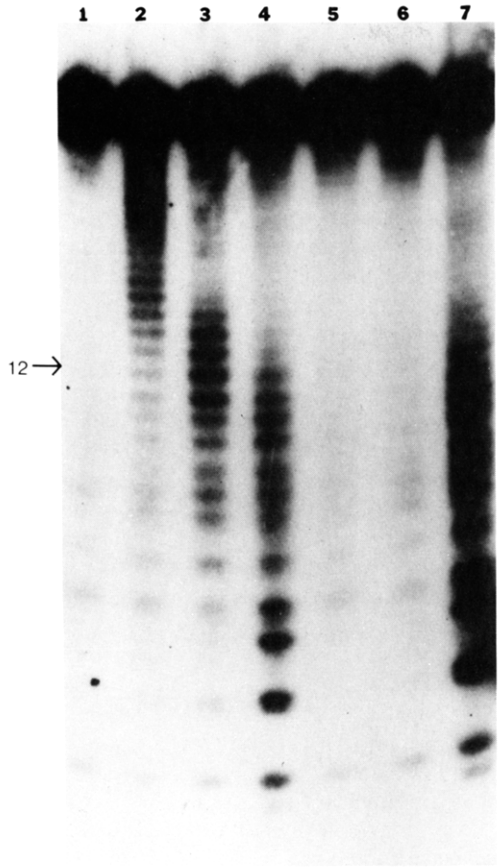

FIGURE 4: Sequence-specific degradation of the 5′-[^{32}P]DNA_{35} by EDTA-derivatized oligonucleoside methylphosphonates. The reactions were carried out in 50 mM Tris-HCl buffer, pH 7.6, and contained DNA_{35} alone in lane 1 and 0.1 μM DNA_{35} and 10 μM $FeSO_4$ in lanes 2–7. In addition, lanes contained 5 μM 5′-EDTA-II (lane 2), 5 μM C-EDTA-II (lane 3), 5 μM C-EDTA-III (lane 4), 5 μM EDTA (lane 5), and 5 μM C-EDTA-III + 5 μM IV (lane 7). There was no addition in lane 6. The reactions were initiated by addition of 5 mM DTT, incubated at 25 °C for 2 h, and subjected to PAGE on a 15% polyacrylamide gel containing 7 M urea. The position of an oligodeoxynucleotide 12-mer is indicated by the arrow.

the corresponding oligodeoxyribonucleotides. Derivatization of the cytosine residue of III with EDTA does not diminish this cooperative interaction as shown by the *Tm* measurements. These results suggest that the cleaving effectiveness of EDTA-derivatized oligomers could be enhanced by the addition of an adjacently binding oligomer to increase the overall stability of the duplex.

Degradation of Target Nucleic Acids by EDTA-Derivatized Oligonucleoside Methylphosphonates. As shown in Figure 4, both 5′- and cytidine-derivatized EDTA–oligonucleoside methylphosphonates effectively degrade the target DNA_{35}. DNA_{35} is cleaved by 5′-EDTA-II, C-EDTA-II, and C-EDTA-III in a site specific manner. Binding of the EDTA–oligomer to its target positions the EDTA group for site-specific cleavage of the target. For example, C-EDTA-II whose binding site is nucleotides 12–22 on the DNA_{35} caused hydrolysis of DNA_{35} in the region around nucleotide 11, whereas C-EDTA-III whose binding site is nucleotides 5–15 caused hydrolysis in the vicinity of nucleotide 7 (Figure 4). The specificity of cleavage is further exemplified by comparing the position of hydrolysis caused by 5′-EDTA-II and C-EDTA-II. The DNA_{35} binding site of both of these oligomers is identical; however, the EDTA group is positioned at the 5′-end of 5′-EDTA-II and at the 3′-end of C-EDTA-II. The observed cleavage pattern, nucleotide 22 for 5′-EDTA-II and nucleotide 11 for C-EDTA-II, is consistent with different positions of the EDTA groups in these oligomers. These results directly demonstrate that the oligonucleoside methylphosphonates bind specifically to their complementary binding site on the target DNA.

The pattern of cleavage of DNA_{35} is similar for each oligomer and occurs over a range of 8–12 nucleotides, with the maximum amount of cleavage occurring at the nucleotide position in DNA_{35} opposite the EDTA group. This pattern of degradation is consistent with a mechanism in which diffusible hydroxyl radicals are generated by the EDTA–Fe^{2+} group that then lead to hydrolysis of the sugar–phosphate backbone. Because the concentration of the hydroxyl radical decreases with distance from its point of formation (Sies, 1987), the decrease in the level of cleavage with increasing distance from the position of the EDTA group is also consistent with this mechanism.

The observed pattern of DNA_{35} degradation by the EDTA-derivatized methylphosphonate oligomers is somewhat different from that exhibited by oligodeoxynucleotides derivatized with EDTA at the 5′-position (Chu & Orgel, 1985) or the C-5 of a thymidine residue (Dreyer & Dervan, 1985). In the case of these oligomers, a bimodal distribution of target degradation was observed, with the maximum extent of cleavage occurring at the fourth nucleotide on either side of the position opposite the EDTA group. The range of cleavage extended over four to five nucleotides, and little or no cleavage occurred in the region opposite the EDTA group. Protection of this region by the tether of the EDTA has been discussed (Dreyer & Dervan, 1985). However, in other experiments using thymidine-derivatized EDTA–oligodeoxynucleotides to cleave double-stranded DNA, single-modal cleavage pattern of target DNA was also observed (Moser & Dervan, 1987). The reason for the differences in these cleavage patterns is not apparent.

The extent of degradation of DNA_{35} was 15–23% in the presence of either the 5′- or the cytidine-derivatized EDTA-methylphosphonate oligomers. The observed extents of cleavage varied somewhat with different batches of oligomer or target and may be related to variations in the content of trace metal ions present in the different preparations. In contrast to this behavior, the extent of degradation of RNA_{33} by the 5′-EDTA-II, C-EDTA-II, or C-EDTA-III was less than 1%. We also observed less than 10% degradation of poly(rU) by 5′-EDTA-I after a 2-h reaction period. It appears that the ribose–phosphate backbone is susceptible to cleavage by hydroxyl radicals because methidiumpropyl-EDTA does cause significant levels of degradation of tRNA (Vary et al., 1982) and ribosomal RNA (Kean et al., 1985). However, the relative extents of degradation of double-stranded DNA versus double-stranded RNA produced by this compound have not been determined. It is possible that the difference in the level of degradation of the DNA versus the RNA target may be partly attributable to the different conformation of the duplex formed between the oligomer and the target. Thus, the sugar–phosphate backbone may be more accessible to the hydroxyl radicals generated by the EDTA group when the oligomer is bound to DNA in a B-form duplex as opposed to when it is bound to RNA in an A-form duplex. Alternatively, a relatively lower stability of the duplex formed between the oligonucleoside methylphosphonate and the target RNA could explain these results. As shown in Table II, the T_m of the complex formed by oligomer I and poly(rU) is 8.5 °C lower than that of the complex of oligomer I with poly(dT).

The extent of degradation of DNA_{35} caused by C-EDTA-III can be almost doubled by adding oligomer IV as shown in lane

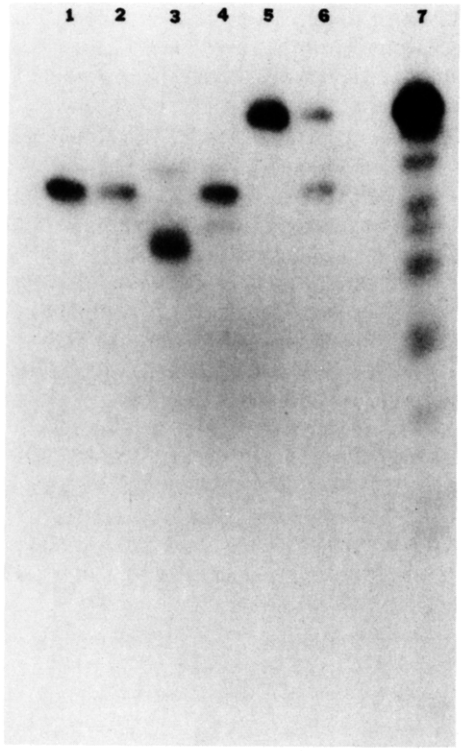

FIGURE 5: Characterization of autodegradation product of 5′-EDTA-I with ^{32}P-labeling between the EDA linker and the oligomer (see Materials and Methods). Oligomer I, untreated (lane 1) and HCl treated (lane 2); 5′-EDTA-I, untreated (lane 3) and HCl treated (lane 4); autodegradation product, untreated (lane 5), HCl treated (lane 6), and piperidine treated (lane 7).

7 of Figure 4. As described above, oligomers III and IV bind to DNA_{35} in a cooperative manner with a consequent stabilization of duplex formed. This stabilization leads to a greater extent of degradation of the target and thus provides a strategy for enhancing the effectiveness of degrading single-stranded DNAs. A similar increase in the extent of degradation as a result of cooperative binding of 5′-EDTA-I was also observed. Thus, 5′-EDTA-I degraded DNA_{35} and $(dT)_{12}$ to the extent of approximately 3%, whereas it completely degraded poly(dT) within 15 min and degraded $(dT)_{20}$ to the extent of 40% in 2 h. In these cases 5′-EDTA-I can occupy multiple, adjacent binding sites on both poly(dT) and $(dT)_{20}$, leading to a higher degree of effectiveness in degradation.

Although the products of DNA cleavage by these EDTA–oligomers were not characterized, they are speculated to be similar to those formed by methidiumpropyl-EDTA-Fe(II) (Hertzberg & Dervan 1984). In this case, the DNA termini at the cleavage site are 5′-phosphate and 3′-phosphate or 3′-phosphoglycolic acid. A free base is released as a result of each cleavage event.

Autodegradation of EDTA-Derivatized Methylphosphonate Oligomers. Aqueous ethanol solutions of 5′-EDTA-I or 5′-EDTA-II have been stored at −20 °C for over a year and then used in DNA degradation experiments without noticable loss of activity. However, oligomers that have been preincubated with Fe^{2+} and DTT prior to the degradation assay show no activity. We have also observed that the degradation of targets by these oligomers levels off after 2 h and can be restored by adding fresh EDTA–oligomer and Fe^{2+}. These results suggest that the EDTA-derivatized oligomers are inactivated in the presence of Fe^{2+} and DTT and that this inactivation competes with the degradation of the target by the oligomer.

The effect of Fe^{2+} and DTT on 5′- or cytidine-derivatized EDTA–methylphosphonate oligomers was monitored by PAGE. Oligomers derivatized at the 5′-position appeared to be completely converted to a single product within 2 h, whereas cytidine-derivatized EDTA–oligomers were converted to two or more new products. The product obtained from the 5′-EDTA–oligomer was isolated from the gel and further characterized. Treatment of this compound with acid yielded 5′-phosphorylated oligomer, and treatment with piperidine gave a "ladder" of oligomers corresponding to random cleavage of the methylphosphonate linkages of the oligomer. These results showed that the phosphoramidate linkage and the methylphosphonate oligomer remained intact in the modified product as shown in Figure 5 and that the methylphosphonate linkages, the phosphodiester linkage, and the phosphoramidate linkage of the oligomer are not degraded by hydroxyl radicals produced by the EDTA group of the oligomer. It appears therefore that the new product results from modification of either the EDA linker arm or the EDTA portion of the oligomer, possibly as a result of attack of hydroxyl radicals on these groups. In support of this hypothesis, we have shown that [^{14}C]EDTA was converted to several new products upon incubation with Fe^{2+} and DTT under conditions that result in modification of the EDTA-derivatized oligomers. Thus, it appears that inactivation of the EDTA-derivatized oligomers is a result of autodegradation of the EDTA portion. Similar inactivation of EDTA-derivatized oligodeoxynucleotides has been reported (Dreyer & Dervan, 1985), although the products of the reaction were not characterized.

Although autodegradation limits the extent to which EDTA-derivatized oligomers degrade their targets, this extent is still sufficient to allow the binding site of the oligomer to be monitored when the target is single-stranded DNA. The rate at which autodegradation occurs may be a more serious problem when the target nucleic acid is single-stranded RNA. In this case it would appear that the rate of autodegradation exceeds that of cleavage of the backbone of the RNA by the oligomer, thus leading to very low levels of RNA degradation. It may be possible to remedy this situation by employing other linkers to tether the EDTA group to the methylphosphonate oligomer and thus bring the EDTA group into closer proximity with the RNA sugar–phosphate backbone.

The results of our experiments suggest that the use of EDTA to monitor the binding sites of oligonucleoside methylphosphonates is a useful technique for characterizing oligomer/target interactions. It seems less likely that this modification will be useful for developing antisense oligonucleotide reagents for specifically controlling gene expression in cells. In addition to the problem of autodegradation that inherently limits the extent of target degradation, the EDTA oligomers are multiply charged, a feature that may militate against facile uptake of the oligomer by cells.

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Registry No. 5′-EDTA-I, 117873-63-9; 5′-EDTA-II, 117895-97-3; C-EDTA-II, 117895-98-4; C-EDTA-III, 117873-64-0; IV, 117873-65-1; DTT, 3483-12-3; RNA_{33}, 117896-05-6; DNA_{35}, 117896-06-7; DNA_{35}·d-AAATTAGTCAG, 117982-76-0; DNA_{35}·d-CATAAATAAAAA, 117982-75-9; DNA_{35}·C-EDTA-III, 117982-74-8; DNA_{35}·IV, 117982-73-7; DNA_{35}·d-AATTAGTCAG, 117982-72-6; DNA_{35}·d-AATTAGTCAG·IV, 118016-99-2; DNA_{35}·d-AAATTAGTCAG·d-CATAAATAAAAA, 118016-94-7; DNA_{35}·C-EDTA-III·IV, 118017-00-8; poly(dT)·AAAAAAA, 117873-70-8; poly(dT)·RNA_{33}, 117982-77-1; poly(rU)·AAAAAAA, 117873-71-9;

RNA_{33}·poly(rU), 117982-78-2; Fe, 7439-89-6; hydroxyl radical, 3352-57-6.

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