Characterization of Sequence-Specific Oligodeoxyribonucleoside Methylphosphonates and Their Interaction with Rabbit Globin mRNA†

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ABSTRACT: Oligodeoxynucleoside methylphosphonates, nucleic acid analogues that contain nonionic, 3′–5′-linked methylphosphonate internucleotide bonds, can be used to control mRNA function in living cells. In order to use analogues of defined sequence in biochemical and biological experiments, methods have been developed to characterize the chain length and sequence of oligodeoxyribonucleoside methylphosphonates and to study their interaction with mRNA. Methylphosphonate oligomers that terminate at the 5′ end with a 3′–5′ internucleotide phosphodiester bond are readily phosphorylated by polynucleotide kinase. Treatment of these ^{32}P end labeled oligomers with aqueous piperidine randomly hydrolyzes the methylphosphonate linkage and upon gel electrophoresis produces a ladder of oligomers, which allows the chain length of the oligomer to be determined. The sequence of ^{32}P end labeled oligonucleoside methylphosphonates can be determined by a modified chemical sequencing procedure. The interaction of the oligomers with rabbit globin mRNA was studied. The oligomers hybridize with mRNA in agarose gels. The stability of the hybrids increases with increasing chain length of the oligomer. The binding site of the oligomers on mRNA can be determined by using the oligomer as a primer for reverse transcriptase. The length of the resulting transcript is determined by polyacrylamide gel electrophoresis after removal of the methylphosphonate primer by treatment with piperidine. The results indicate that binding and priming ability of the oligonucleoside methylphosphonates are affected by the secondary structure of the mRNA.

In recent papers our laboratory and others have described the preparation of sequence-specific oligodeoxyribonucleoside methylphosphonates on insoluble polymer supports (Miller et al., 1983a,b; Sinha et al., 1983). These unique nucleic acid analogues are able to form complexes with complementary polynucleotides and are capable of entering living cells intact. We have demonstrated that an analogue which is complementary to the Shine Dalgarno sequence of *Escherichia coli* 16S rRNA selectively inhibits bacterial vs. mammalian protein synthesis in cell-free extracts and bacterial cells (Jayaraman et al., 1981). Methylphosphonate oligomers that are complementary to the initiation codon region of globin mRNA inhibit translation in a rabbit reticulocyte lysate and in reticulocytes by a hybridization-arrest mechanism (Blake et al., 1985). We have also found that methylphosphonate oligomers complementary to vesicular stomatitis virus (VSV) mRNA specifically inhibit virus protein synthesis in VSV-infected cells (Miller et al., 1984). Thus, oligonucleoside methylphosphonates show considerable promise for use in controlling expression of nucleic acids inside living cells.

In order to use the methylphosphonate oligomers in biochemical and biological experiments, it is extremely important to have methods for their characterization. Because of the resistance of the methylphosphonate linkage to enzymatic hydrolysis and the novel chemical properties of this backbone, new procedures were required for such characterization. In this paper we describe methods that allow one to confirm the chain length and sequence of an oligonucleoside methylphosphonate. We also describe simple procedures for studying and characterizing the interactions of oligonucleoside methylphosphonates with mRNA.

Experimental Procedures

Oligodeoxyribonucleoside methylphosphonates were synthesized in solution or on polymer supports and were purified as previously described (Miller et al., 1983a,b). Oligodeoxyribonucleotides were synthesized by the phosphotriester/polystyrene support method essentially as described by Ito et al. (1982). The oligomers were purified by preparative ion-exchange high-pressure liquid chromatography (Miller et al., 1980a,b). Dimethyl sulfate and hydrazine were purchased from Aldrich Chemical Co. and were used without further purification. Rabbit globin mRNA, T4 polynucleotide kinase, and avian myeloblastosis virus (AMV) reverse transcriptase were purchased from Bethesda Research Laboratories, Inc. Adenosine [γ-^{32}P]triphosphate and thymidine [α-^{32}P]triphosphate were obtained from New England Nuclear. SEP-PAK C_{18} reversed-phase cartridges were purchased from Waters Associates. All chemicals and reagents used were reagent grade or better. Buffer solutions were prepared in autoclaved water. TBE electrophoresis buffer was prepared according to Maniatis et al. (1982).

Phosphorylation of Oligodeoxyribonucleoside Methylphosphonates. The reaction mixture contained 0.05 M Tris-HCl[1] (pH 9.6), 0.01 M magnesium chloride, 5 mM dithiothreitol, 20 μM spermidine, 0.1 mM EDTA, 50–330 pmol of oligonucleoside methylphosphonate, 50–330 pmol of adenosine [γ-^{32}P]triphosphate (sp. act. 50–500 Ci/mmol), and 5 units of T4 polynucleotide kinase in a total volume of 10 μL. For kinetic analysis, 1-μL aliquots were removed at appropriate times and added to ice-cold loading buffer that contained 80% formamide and 0.2% bromophenol blue in TBE buffer. The samples were loaded onto a 15% polyacrylamide slab gel containing 7 M urea in TBE buffer and electrophoresed at 800 V for 50 min. The gel was dried and autoradiographed at −80 °C. Regions of the gel corresponding to the phosphorylated

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[1] Abbreviations: d-N*p*N*p*N*p*N, a deoxyribonucleoside methylphosphonate that contains 3′–5′ methylphosphonate linkages; NaOAc, sodium acetate; Me_2SO_4, dimethyl sulfate; EDTA, ethylenediaminetetraacetic acid; SSC, standard sodium citrate buffer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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oligomers were excised and counted in Betafluor.

For preparative experiments, the phosphorylation reactions were carried out for 1.5 h under the same conditions as described above. The wet gel was autoradiographed, and the area corresponding to the phosphorylated oligomer was excised. The gel was crushed and extracted with three 0.5-mL portions of 1 M triethylammonium bicarbonate (pH 7.5) at room temperature. The extract was passed through a SEP-PAK C_{18} cartridge, and the cartridge was washed with two 10-mL portions of 25 mM triethylammonium bicarbonate and two 10-mL portions of 5% acetonitrile in 100 mM triethylammonium bicarbonate. The phosphorylated oligomer was eluted with 3 mL of 30% acetonitrile in 100 mM triethylammonium bicarbonate and lyophilized (Lo et al., 1984).

Partial Hydrolysis of Oligodeoxyribonucleoside Methylphosphonates. A sample of 5′-^{32}P-labeled oligonucleoside methylphosphonate prepared as described above was lyophilized. The residue was dissolved in 5 μL of 1 M aqueous piperidine, and the solution was incubated at 37 °C for 30 min. The sample was lyophilized, and the residue was dissolved in 5 μL of gel loading buffer, which contained 80% formamide and 0.2% bromophenol blue in TBE buffer. The digest was electrophoresed on a 15% polyacrylamide slab gel as described above. The gel was autoradiographed at −80 °C after drying.

Sequencing Oligodeoxyribonucleoside Methylphosphonates. 5′-^{32}P-labeled oligonucleoside methylphosphonates prepared as described above were sequenced under the reaction conditions shown in Table I. This procedure is similar to that of Maxam & Gilbert (1980), except for the following modifications: (1) each base specific cleavage reaction was carried out at 37 °C for 30 min; (2) t-RNA (10 μg/sample) and 85% ethanol were used to precipitate the oligomer fragments; (3) cleavage of the methylphosphonate linkages at the modified base sites was carried out by incubating the precipitates in neutral aqueous solution at room temperature for 30 min for the A+G-, C+T-, and C-specific reactions and at 90 °C for 0.5–2 h for the G-specific reaction; (4) a 20-fold higher concentration of Me_2SO_4 in potassium cacodylate (pH 8) was used for the G-specific reaction; (5) hydrazine acetate (Rubin & Schmid, 1980) was used instead of 5 M sodium chloride for the C-specific reaction. The samples were analyzed on an 18% polyacrylamide slab gel (13.2 × 14 × 0.075 cm).

Hybridization of Oligodeoxyribonucleoside Methylphosphonates to Rabbit Globin mRNA in Agarose Gels. Rabbit globin mRNA was electrophoresed on a 3-mm thick 1.5% agarose gel at 75 V for 1 h at room temperature in TBE buffer that contained 0.5 μg/mL ethidium bromide. The gel was blotted with paper towels under vacuum at room temperature for 15 min and then dried onto filter paper at 60 °C for 30 min with a gel dryer (Bio-Rad, Inc.). The filter paper was removed from the gel by soaking the gel in water for 15 min. The swollen gel, which was approximately 0.11-mm thick, was prehybridized at room temperature for 30 min with buffer containing 1× SSC, 0.2% polyvinylpyrolidone, 0.2% Ficoll, and 0.2% bovine serum albumin. The gel was incubated with 0.3 mL of the same buffer containing 2 × 10^5 cpm/mL 5′-^{32}P-labeled oligonucleoside methylphosphonate (sp act. 32–184 Ci/mmol) in a heat-sealed bag at 45 °C for 30 min and then at 7 °C for 16 h. The gel was washed with 10 mL of ice-cold 1× SSC for 10 min, blotted on filter paper, covered with Saran wrap, and autoradiographed at −80 °C.

Oligodeoxyribonucleoside Methylphosphonates as Primers for Reverse Transcriptase. The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 5 mM magnesium chloride, 10 mM dithiothreitol, 50 μM each of the four deoxyribonucleoside triphosphates, 10–15 μCi of thymidine [α-^{32}P]triphosphate, 0.2 μg of rabbit globin mRNA, 40 μM oligodeoxyribonucleoside methylphosphonate, and 50 units of AMV reverse transcriptase in a total volume of 10 μL. The reactions were incubated at 37 °C for 60 min and then stopped by adding 0.5 μL of 0.5 M EDTA. A 1-μL aliquot was added to 9 μL of gel loading buffer, which contained 30% glycerol, 6 M urea, and 0.2% bromophenol blue in TBE buffer. The reaction mixture was electrophoresed on a 10% polyacrylamide slab gel that contained 7 M urea. The gel was run in TBE buffer at 800 V for 45 min. The gel was then dried and autoradiographed at −80 °C. For preparative runs, the entire reaction mixture was lyophilized, and the residue was dissolved in 10 μL of glycerol gel loading buffer. After electrophoresis, the wet gel was autoradiographed at −80 °C. The region of the gel containing the transcript was excised, and the transcript was extracted and desalted as described above for the isolation of phosphorylated oligodeoxyribonucleoside methylphosphonates. For chain-length determination, the lyophilized transcript was treated with 25 μL of 1 M aqueous piperidine at 60 °C overnight. The hydrolyzed transcript was lyophilized, and the residue was dissolved in 10 μL of glycerol gel loading buffer. The sample was electrophoresed on a 10% polyacrylamide gel under denaturing conditions as described above. Oligomers of known chain lengths were run as standards. The chain length of the hydrolyzed transcript was determined from plots of the log of the chain lengths of the standards vs. the mobility of the oligomers on the gel (Rickwood & Hames, 1982).

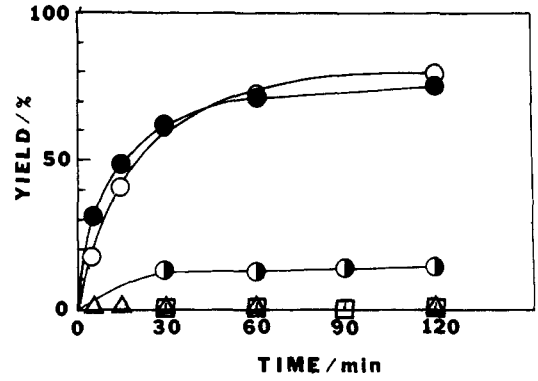

FIGURE 1: Time course of T4 polynucleotide kinase catalyzed phosphorylation of d-A*p*A (◑), d-A*p*A*p*A and d-C*p*C*p*A*p*G*p*G*p*T-*p*A*p*A (□), d-C*p*C*p*A*p*T (△), d-TpG*p*C*p*A*p*C*p*C*p*A*p*T (●), and d-CpCpApApGpCpTpTpGpG (○).

Results and Discussion

The base composition of oligodeoxyribonucleoside methylphosphonates up to four nucleoside units can be determined by degradation of the oligomer with acid or base. The products of these degradative reactions can be characterized and quantitated by analytical reversed-phase HPLC. This procedure is not applicable to longer oligomers because complex mixtures of products are obtained and rather large amounts of oligomer are required for the analysis. A more convenient approach follows the methods used to characterize oligonucleotides, which involves phosphorylation of the 5′-terminal hydroxyl group with ^{32}P-labeled phosphate, base-specific cleavage of the internucleotide bond, and separation and analysis of the products by polyacrylamide gel electrophoresis.

As shown in Figure 1, the 5′-hydroxyl group of the dinucleoside methylphosphonate d-A*p*A, but not of the tri-, tetra-, or octanucleoside methylphosphonates d-A*p*A*p*A, d-C*p*C*p*A*p*T, and d-C*p*C*p*A*p*G*p*G*p*T*p*A*p*A, can be phosphorylated enzymatically with T4 polynucleotide kinase. Similar

observations have been made in other laboratories (Agarwal & Riftina, 1979; Sinha et al., 1983). Longer methylphosphonate oligomers can be phosphorylated if the 5′-terminal nucleoside is linked to the oligomer via a phosphodiester bond (Miller et al., 1983b). The rate of phosphorylation of this type of oligomer is identical with that of an oligodeoxyribonucleoside that contains only phosphodiester linkages. These observations suggest that in addition to the 5′-hydroxyl group the enzyme probably interacts with the negatively charged phosphodiester linkage of longer oligonucleotides. Oligomers of the type d-NpN′(pN″)$_n$, where n is 2–10 and N is any one of the four deoxynucleosides, have been efficiently phosphorylated by this procedure. This suggests that there are no restrictions on the reaction in terms of size or base composition of the methylphosphonate oligomers. As is the case with 5′-phosphorylated oligo- or polynucleotides, the terminal phosphate of d-pNpN′(pN″)$_n$ is easily removed by treatment with alkaline phosphatase to regenerate d-NpN′(pN″)$_n$.

The phosphorylated oligomers are conveniently purified by polyacrylamide gel electrophoresis. Because the charge to mass ratio is low, the methylphosphonate oligomers have much lower mobilities on the gel than do oligodeoxyribonucleotides of the same chain length and sequence. Consequently, gels with high percentages of acrylamide are required to separate methylphosphonate oligomers up to 12 nucleosides in length.

The methylphosphonate oligomers can be recovered from the wet gels by extraction. Initially, we used the ammonium acetate buffer system, which is used to extract oligodeoxyribonucleotides from polyacrylamide gels (Maniatis et al., 1982). However, the recoveries of oligomer from the gel were usually less than 50% even after repeated and prolonged extractions. Almost quantitative recovery is achieved by extracting the gel with 1 M aqueous triethylammonium bicarbonate (Lo et al., 1984). Three 30-min extractions are sufficient to elute 95% or more of the oligomer. The oligomers are then desalted by absorption onto a SEP-PAK C_{18} reversed-phase column followed by elution with 30% acetonitrile in 100 mM triethylammonium bicarbonate. At least 80% of the total counts applied to the column are recovered by this procedure.

The methylphosphonate linkage is stable at neutral and acidic pH. Methylphosphonate oligomers can be stored in 50% aqueous ethanol at 4 °C for at least 1 year or in 30% acetonitrile in 100 mM triethylammonium bicarbonate (pH 7.5) at −20 °C for 1 month without any noticeable degradation. However, the methylphosphonate linkage is readily cleaved by a variety of bases, including aqueous piperidine. This cleavage is random with respect to the 3′ or 5′ side of the linkage and the position of the linkage in the oligomer chain. Partial hydrolysis of d-[^{32}P]pNpN′(pN″)$_n$ in 1 M aqueous piperidine generates two sets of 5′-end-labeled oligomers whose 3′ ends terminate with a methylphosphonate group or with a hydroxyl group. When separated by polyacrylamide gel electrophoresis, a ladder appears in which the oligomers of each set are separated according to their chain lengths. This is shown for the octamer d-[^{32}P]pTpGpCpApCpCpApT and dodecamer d-[^{32}P]pCpApTpTpCpTpGpTpCpTpGpT in Figure 2. Oligomers that terminate with a methylphosphonate group move farther than oligomers of the same chain length that terminate with a 3′-hydroxyl group. The chain length of the original oligomer can be determined by counting the number of bands from each set of oligomers as shown in Figure 2. The shortest oligomers correspond to the two dimers d-pNpN′ and d-pNpN′p because the 5′-terminal phosphodiester linkage is not cleaved by this procedure.

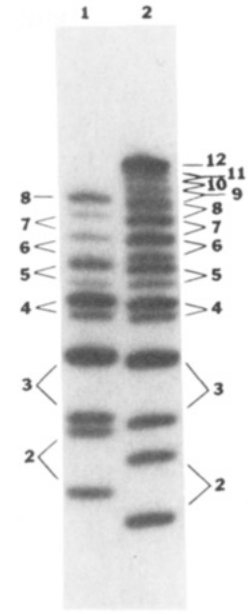

FIGURE 2: Products of partial hydrolysis of d-[^{32}P]-pTpGpCpApCpCpApT (lane 1) and d-[^{32}P]-pCpApTpTpCpTpGpTpCpTpGpT (lane 2) after treatment with 1 M aqueous piperidine at 37 °C for 30 min. The chain length of each oligomer is indicated along the side of the gel.

Methylphosphonate oligomers that are labeled at their 5′ end can be sequenced by a method similar to the Maxam–Gilbert chemical sequencing procedure used for DNA (Maxam & Gilbert, 1980). In these studies, G-, A+G-, C-, and C+T-specific reactions were followed by precipitation of the labeled oligomer. The detailed reaction conditions are described in Table I. Each reaction was carried out at 37 °C for 30 min. For the G-specific reaction the concentration of dimethyl sulfate was increased 20-fold over that used to sequence DNA. When concentrated sodium chloride and hydrazine were used for the C-specific cleavage reaction, difficulty was encountered in removing the sodium chloride from the oligomers by precipitation. This problem was circumvented by using hydrazine acetate in these reactions (Robin & Schmid, 1980). The oligomer fragments generated by each reaction were precipitated from ethanol. Less than 10% of the total counts was obtained when 75% ethanol was used. This may be due to the low charge and short chain length of the fragments. Addition of large amounts of tRNA (10 μg/reaction) and use of 85% ethanol increased the recovery 50%–70%.

As described previously (Miller et al., 1983b), oligonucleoside methylphosphonates that contain apurinic or apyrimidinic sites are spontaneously hydrolyzed in neutral aqueous solutions to give oligomers that terminate with a 3′-OH group. Thus, in contrast to the procedure required for DNA, it is not necessary to carry out base-catalyzed β-elimination reactions to cleave the chain after removal of the base residues. Following precipitation, the methylphosphonate oligomers were incubated in water, pH 7.0, at room temperature for the A+G-, C+T-, and C-specific reactions and at 90 °C for the G-specific reaction. Each reaction was then electrophoresed on an 18% polyacrylamide slab gel which contained 7 M urea. Figure 3 shows a typical sequencing gel for the octamer d-[^{32}P]pTpGpCpApCpCpApT. The sequence of the methylphosphonate portion of the oligomer is easily read from the gel. The 3′-terminal thymidine that appears in the C+T lane is somewhat difficult to read since the oligomer that has lost this residue migrates close to the original octamer on the gel. The band appears more distinct on the original autoradiogram

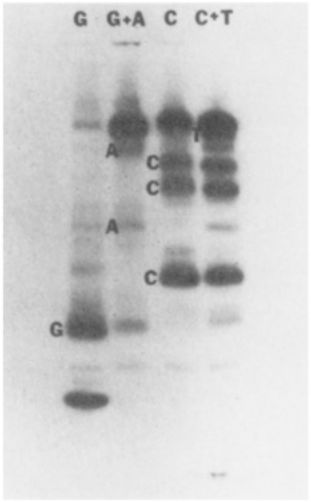

FIGURE 3: Autoradiogram of sequencing gel of d-[^{32}P]-pTpG*p*C*p*A*p*C*p*C*p*A*p*T.

of the gel. The additional faint bands that appear on the gel most likely come from random hydrolysis of the methylphosphonate backbone during the base-specific cleavage reactions. This problem appears to be more serious for the C and C+T reactions, which are carried out with hydrazine at a higher pH.

The 5′-terminal nucleotide unit is not determined by this sequencing method. This residue can be easily identified by treating the nonphosphorylated oligomer with spleen phosphodiesterase. The 5′-nucleotidyl unit is readily hydrolyzed by the enzyme to give the nucleoside 3′-monophosphate, which can be identified by analytical reversed-phase HPLC. Under these conditions, the methylphosphonate linkages of the oligomer are not hydrolyzed.

Oligonucleoside methylphosphonates can form hydrogen-bonded complexes with complementary polynucleotides (Miller et al., 1979). The interaction of sequence-specific oligonucleoside methylphosphonates with RNA can be studied by hybridization procedures. The interaction of three 5′-end-labeled oligodeoxyribonucleoside methylphosphonates, d-[^{32}P]pTpG*p*C*p*A*p*C*p*C*p*A*p*T, d-[^{32}P]pCpA*p*T*p*T*p*C*p*T*p*G-*p*T*p*C*p*T*p*G*p*T, and d-[^{32}P]pTpG*p*A*p*T*p*G*p*T*p*T*p*G*p*G, with rabbit globin mRNA was used as a model system. Two of these oligomers, d-pTpG*p*C*p*A*p*C*p*C*p*A*p*T and d-pCpA*p*T*p*T*p*C*p*T*p*G*p*T*p*C*p*T*p*G*p*T, are complementary to nucleotides in the initiation codon regions of α- and β-globin mRNA while d-pTpG*p*A*p*T*p*G*p*T*p*T*p*G*p*G is complementary to nucleotides 62–70 in the coding region of α-globin mRNA (see Figure 4). Hybridization of the oligomers to rabbit globin mRNA baked onto nitrocellulose filters was not successful. The methylphosphonate oligomers have an extremely high affinity for nitrocellulose membranes, and the resulting high background obscured any binding of the oligomer to the mRNA. The background binding could only be reduced by washing the filters in water at 100 °C. These conditions, of

Table I: Conditions for Sequencing Oligodeoxyribonucleoside Methylphosphonates

G	G+A	C+T	C
oligomer[a]	oligomer[a]	oligomer[a]	oligomer[a]
buffer,[b] 2 μL	H_2O, 2 μL	H_2O, 2 μL	H_2O, 2 μL
Me_2SO_4, 0.2 μL	1 M HCl, 1 μL	hydrazine, 2 μL	hydrazine acetate,[c] 2 μL
37 °C, 30 min	37 °C, 30 min	37 °C, 30 min	37 °C, 30 min
stop in ice	stop in ice	stop in ice	stop in ice
3 M NaOAc, 0.5 μL	1 M NH_4OH, 1 μL	3 M NaOAc, 0.5 μL	3 M NaOAc, 0.5 μL
tRNA,[d] 2 μL	3 M NaOAc, 0.5 μL	tRNA,[d] 2 μL	tRNA,[d] 2 μL
95% EtOH, 27 μL	tRNA,[d] 2 μL	95% EtOH, 37 μL	95% EtOH, 37 μL
chill, at −78 °C	95% EtOH, 37 μL	chill, at −78 °C	chill, at −78 °C
centrifuge[e]	chill, at −78 °C	centrifuge[e]	centrifuge[e]
pellet	centrifuge[e]	pellet	pellet
H_2O, 5 μL	pellet	H_2O, 5 μL	H_2O, 5 μL
95% EtOH, 30 μL	H_2O, 5 μL	95% EtOH, 30 μL	95% EtOH, 30 μL
chill, at −78 °C	95% EtOH, 30 μL	chill, at −78 °C	chill, at −78 °C
centrifuge[e]	chill, at −78 °C	centrifuge[e]	centrifuge[e]
pellet	centrifuge[e]	pellet	pellet
H_2O, 10 μL (adjust to pH 7)	pellet H_2O, 10 μL (adjust to pH 7)	H_2O, 10 μL (adjust to pH 7)	H_2O, 10 μL (adjust to pH 7)
95 °C, 2 h	room temperature, 30 min	room temperature, 30 min	room temperature, 30 min
load on gel	load on gel	load on gel	load on gel

[a] 5′-[^{32}P]Phosphorylated oligodeoxyribonucleoside methylphosphonate (0.8 pmol, sp act. 80 Ci/mmol, lyophilized). [b] Buffer was 50 mM Potassium cacodylate–8 mM EDTA. [c] Hydrazine/acetic acid (2:1 v/v). [d] At 5 mg/mL. [e] At 5 °C for 10 min.

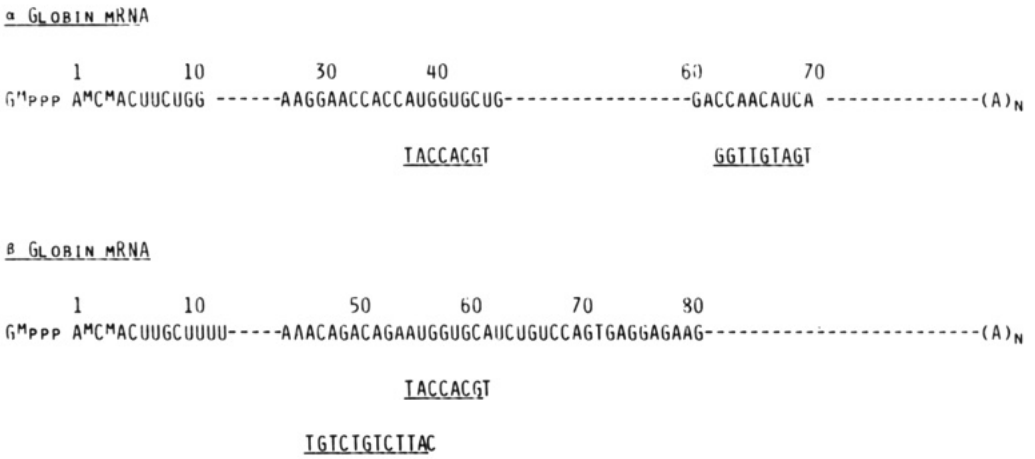

FIGURE 4: Sequences of oligonucleoside methylphosphonates complementary to α- and β-globin mRNA. The sequences of the methylphosphonate oligomers are shown below the mRNA sequences. The portion underlined indicates the methylphosphonate linkages in the oligomer.

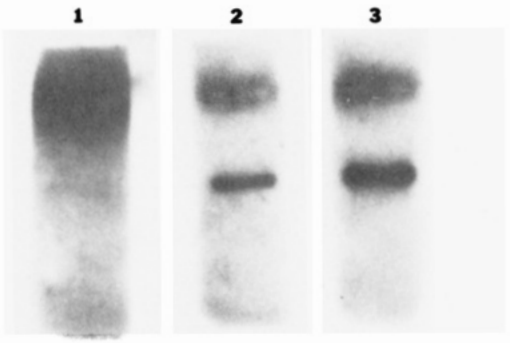

FIGURE 5: Hybridization of rabbit globin mRNA with (1) d-[^{32}P]-pTpG*p*C*p*A*p*C*p*C*p*A*p*T, (2) d-[^{32}P]pTpG*p*A*p*T*p*G*p*T*p*T*p*G*p*G, and (3) d-[^{32}P]pCpA*p*T*p*T*p*C*p*T*p*G*p*T*p*C*p*T*p*G*p*T in agarose gels at 7 °C. Gel 1 was exposed at −80 °C for 6 h whereas gels 2 and 3 were exposed at −80 °C for 1 h.

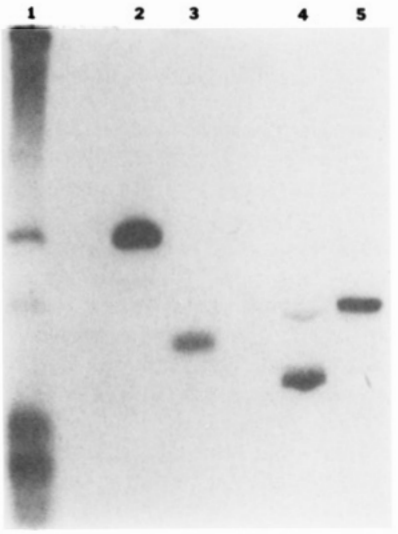

FIGURE 6: Reverse transcriptase catalyzed copying of rabbit globin mRNA using d-TpG*p*C*p*A*p*C*p*C*p*A*p*T as a primer: (lane 1) products of the reaction; (lane 2) β mRNA transcript isolated from the gel; (lane 3) β mRNA transcript hydrolyzed with 1 M piperidine; (lanes 4 and 5) oligonucleotide markers 39 and 56 nucleotides in length.

course, also dissociate the oligomer/mRNA complex. Similar problems were also encountered when other supports such as cellulose, PEI–cellulose, DEAE-cellulose, and glass-fiber filters were used.

Sufficiently low backgrounds were obtained when the hybridization reactions were carried out in agarose gels. This method was originally used by Shinnick et al. (1975) for screening DNA fragments by hybridization with diester probes. The rabbit globin mRNA is electrophoresed into the gel matrix and then fixed by drying the gel at 60 °C for 30 min under vacuum. The gel, which is now very thin and mechanically strong, is then incubated with labeled oligomer in hybridization buffer at 7 °C. As shown in Figure 5, all three labeled oligodeoxyribonucleoside methylphosphonates hybridized with the mRNA. The diffuse band at the top of the gel may result from mRNA that did not enter the gel during electrophoresis. It is possible to remove the oligomers by extensive washing and to reuse the gel for other hybridization experiments. The order of binding based on the intensities of the bands observed in the gels is dodecamer methylphosphonate > nonamer methylphosphonate > octamer methylphosphonate. This behavior is consistent with the expected increase in stability of the complexes with increasing chain length of the oligomer. It appears that the oligodeoxyribonucleotide d-[^{32}P]-pCpApTpTpCpTpGpTpCpTpGpT has a higher binding constant than the corresponding methylphosphonate oligomer (data not shown). The apparent greater binding of the dodecamer phosphodiester vs. the dodecamer methylphosphonate may arise because the various diastereoisomers of the dodecamer methylphosphonate form complexes of different stabilities with mRNA. Previous studies on the interactions of di- and oligonucleoside methylphosphonates of fixed backbone configuration with complementary polynucleotides have shown that the configuration of the methylphosphonate linkages affects the stability of the oligomer/polynucleotide complex (Miller et al., 1979, 1980a,b). The dodecamer methylphosphonate should consist of 2^{10} (1024) diastereoisomers. If a portion of these isomers have low binding ability, then the apparent binding constant of the dodecamer methylphosphonate will be less than that of the dodecamer phosphodiester when equal concentrations of the two oligomers are used in the hybridization experiments.

The position of oligomer binding to mRNA may be determined via the use of reverse transcriptase. We have found that oligodeoxyribonucleoside methylphosphonates will serve as primers for this enzyme on RNA templates. The efficiency of the priming reaction is between 5-fold and 10-fold less than that of an oligodeoxyribonucleotide of the same sequence. We found in previous experiments that oligonucleoside methylphosphonates would not serve as primers for *E. coli* DNA polymerase I or calf thymus DNA polymerase α, unless the oligomer terminated at the 3′ end with at least two phosphodiester linkages (Miller et al., 1982). Similar results were obtained with deoxynucleotidyl terminal transferase. The present observations show that, in contrast to these enzymes, reverse transcriptase does not require a phosphodiester linkage at the 3′-terminal nucleotide position in order for productive interaction with the primer. It is possible, however, that the configuration of the 3′-terminal methylphosphonate linkage affects the efficiency of the priming reaction, and this may account, in part, for the overall lower efficiency of the priming reaction vs. that of oligodeoxyribonucleotides.

Lane 1 of Figure 6 shows the results obtained when d-TpG*p*C*p*A*p*C*p*C*p*A*p*T is used as a primer for the reverse transcriptase reaction in the presence of rabbit globin mRNA. The two bands in the middle of the gel represent the products of the primer-dependent reaction. The bands that appear at the bottom and top of the gel also occur in reactions that contain no primer. The former material appears to come from the thymidine 5′-[^{32}P]triphosphate since its intensity varies with different batches of this nucleotide. The latter material may represent some type of primer-independent background synthesis. The two transcripts result from priming at oligomer binding sites on α- and β-globin mRNA (see Figure 4). The slower moving band corresponds to the longer transcript from β-globin mRNA. The difference in the intensities of the two bands may reflect the effects of mRNA secondary structure on oligomer binding and the subsequent polymerization reaction. Recent studies by Pavlakis et al. (1980) show that the binding site for d-TpG*p*C*p*A*p*C*p*C*p*A*p*T on β-globin mRNA occurs in a single-stranded loop region while the corresponding site on α-globin mRNA occurs, in part, in a hydrogen-bonded stem region. Thus, oligomer interaction, and consequently priming ability, would be expected to be more efficient on the β-globin mRNA template.

Because the transcripts terminate with a nonionic methylphosphonate oligomer at the 5′ end, the charge to mass ratio and thus the mobility of the transcripts on the gel are less than those of a corresponding transcript containing only phosphodiester bonds. The chain length of the transcript can be determined however by removing the methylphosphonate

portion of the primer by hydrolysis with 1 M aqueous piperidine. As shown in Figure 6, lanes 2 and 3, this treatment results in the formation of a shorter transcript that contains only phosphodiester linkages. The chain length of this oligomer as estimated from its mobility on the gel (Rickwood & Hanes, 1982) is 51 nucleotides. Thus, the original length of the transcript is 58 nucleotides, which is in agreement with the chain length expected for the transcript from β mRNA.

Sequence-specific oligodeoxyribonucleoside methylphosphonates are able to enter mammalian cells and affect the expression of cellular or viral nucleic acids by interacting with complementary regions of these nucleic acids. Although oligodeoxyribonucleoside methylphosphonates have unusual chemical and biochemical properties compared to those of oligodeoxyribonucleotides, our previous studies and the results presented here show that these nucleic acid analogues may be readily synthesized and characterized. This ability combined with the ability to study their interaction with cellular nucleic acids such as mRNA forms the basis for using oligodeoxyribonucleoside methylphosphonates in various biochemical and biological experiments.

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Registry No. d-A*p*A, 76741-00-9; d-A*p*A*p*A, 76708-99-1; d-C*p*C*p*A*p*G*p*G*p*T*p*A*p*A, 96760-52-0; d-C*p*C*p*A*p*T, 89000-51-1; d-TpG*p*C*p*A*p*C*p*C*p*A*p*T, 96791-04-7; d-CpCpApApGpCpTpTpGpG, 63734-84-9; d-pTpG*p*C*p*A*p*C*p*C*p*A*p*T, 96760-53-1; d-pCpA*p*T*p*T*p*C*p*T*p*G*p*T*p*C*p*T*p*G*p*T, 96791-05-8; d-pTpG*p*A*p*T*p*G*p*T*p*T*p*G*p*G, 96791-06-9; $MeP(O)(OH)_2$, 993-13-5; reverse transcriptase, 9068-38-6; polynucleotide kinase, 37211-65-7.

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