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Number and distribution of methylphosphonate linkages in oligodeoxynucleotides affect exo- and endonuclease sensitivity and ability to form RNase H substrates

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ABSTRACT

Oligodeoxynucleotides with different arrangements of methylphosphonate linkages were examined for nuclease sensitivity *in vitro*, stability in tissue culture, and ability to form RNase H-sensitive substrates with complementary RNA. After nuclease treatment, resistance was demonstrated by the ability to alter the electrophoretic mobility of a labeled complementary phosphodiester oligodeoxynucleotide. Both 5′- and 3′-exonuclease activities were retarded by methylphosphonate linkages. Methylphosphonate-containing oligodeoxynucleotides with 1−5 adjacent phosphodiester linkages were tested as substrates for the endonucleases DNase I and DNase II. The results indicated that a span of three or fewer contiguous internal phosphodiester linkages led to the greatest resistance to endonuclease. However, in serum-supplemented culture medium half-lives of these oligodeoxynucleotides were independent of the number of contiguous phosphodiester linkages. Methylphosphonate-containing oligodeoxynucleotides were hybridized to RNA runoff transcripts and tested as substrates for RNase H. The results indicated that a span of three internal phosphodiester linkages in the oligodeoxynucleotide was necessary and sufficient to direct cleavage of the RNA in the duplex.

INTRODUCTION

Oligodeoxynucleotides are of interest as antisense agents that inhibit gene expression by hybrid arrest of translation (1). One approach for achieving high levels of translational inhibition is to chemically alter the oligodeoxynucleotide to increase its half-life and to facilitate its entry into the cell, while maintaining its affinity for the specific mRNA. Oligodeoxynucleotide analogues containing methylphosphonate linkages have been shown to have an antisense effect in various *in vitro* (2) and tissue culture (3−6) systems. The non-ionic linkages appear to enhance entry into cells and the altered chemical structure renders such compounds resistant to various nucleases leading to a an increased half-life in serum-supplemented tissue culture medium and within cells (7).

Bacterial, eukaryotic and retroviral RNase H have all been found to act as endonucleases, destroying RNA in DNA-RNA hybrids (8,9). One mechanism by which phosphodiester oligodeoxynucleotides promote hybrid arrest of translation is through RNase H activity (10−13). On the other hand, DNA-RNA hybrids with fully methylphosphonate-substituted oligodeoxynucleotides failed to serve as RNase H substrates (14). Thus, the antisense effect seen with these compounds in cell-free translation systems was not mediated by RNase H.

To further assess the relevance of chemical modifications on the biochemical properties of oligodeoxynucleotides, we examined the effect of the number and arrangement of methylphosphonate substitutions on nuclease sensitivity, stability in serum-supplemented tissue culture medium, and ability to form RNase H substrates.

TABLE 1: Oligodeoxynucleotides.

Sense		Antisense	
Sequence	**Name**	**Sequence**	**Name**
GGATGCAGCTAAGTCAAG	18–P–1	CTTGACTTAGCTGCATCC	18–P–2
ATGCAGCTAAGTCA	14–P–1	TGACTTAGCTGCAT	14–P–2
ATGCAGCTAAGTCA	14–G–1	TGACTTAGCTGCAT	14–A–2
ATGCAGCTAAGTCA	14–T–1	TGACTTAGCTGCAT	14–C–2
ATGCAGCTAAGTCA	14–C–1	TGACTTAGCTGCAT	14–Me^{5}a–2
ATGCAGCTAAGTCA	14–Me^2–1	TGACTTAGCTGCAT	14–Me^{5}b–2
		TGACTTAGCTGCAT	14–Me^{6}a–2
		TGACTTAGCTGCAT	14–Me^{6}b–2
CGCCATGCAGCCCCAGTC	18–P–3	TGGGGCTGCATG	12–P–4
		TGGGGCTGCATG	12–Me^4–4
		CTGGGGCTGCAT	12–Me^{5}a–4
		TGGGGCTGCATG	12–Me^{5}b–4
		TGGGGCTGCATG	12–Me^{5}c–4
		TGGGGCTGCATG	12–Me^{10}–4

N = Base with a 3' methylphosphonate linkage.

MATERIALS AND METHODS

Oligodeoxynucleotides and plasmids

All oligodeoxynucleotides (Table 1) were synthesized on an Applied Biosystems Model 380B DNA Synthesizer. Phosphodiester linkages were generated by standard phosphoramidite chemistry, and methylphosphonate bonds were introduced by the coupling of methylphosphonamidite monomers (ABN-Fisher). Hydrolysis of base-protecting groups and cleavage from the support for phosphodiester oligodeoxynucleotides was accomplished by NH_4OH treatment, which was followed by ethanol precipitation. Oligodeoxynucleotides containing mixed phosphodiester and methylphosphonate bonds were released and deprotected in ethylenediamine:ethanol (1:1) for 7 hours at room temperature (15). To increase the yield, the support material was also treated in NH_4OH for 2 hours at room temperature (16). These oligodeoxynucleotides were purified by 4 M ammonium acetate elution from NACS Prepac columns (BRL). All oligodeoxynucleotides were 5'-labeled with ^{32}P using T4 polynucleotide kinase, separated by electrophoresis on 20% acrylamide−8 M urea gels, and visualized by autoradiography. For every oligodeoxynucleotide employed, over 90 percent of the radioactivity was observed in the electrophoretic band containing the desired product.

All methylphosphonate-substituted oligodeoxynucleotides were quantitated by the ability to change the electrophoretic mobility of their respective 5'-^{32}P-labeled complementary phosphodiester oligodeoxynucleotides on non-denaturing polyacrylamide gels (17).

pSP65-ALA-D is a derivative of the SP6 cloning vector pSP65 (18) containing the cDNA sequence of human delta-aminolevulinic acid dehydratase (ALA-D), a heme biosynthetic enzyme (19), inserted in the sense orientation in the *PstI* site of the polylinker.

Exo- and endonuclease digests

Nuclease digests of oligodeoxynucleotides (5−10 ng) were carried out in 10 μl as described

below for each enzyme. Appropriate enzyme units and half-times for digestion were determined by titration with phosphodiester oligodeoxynucleotide controls:

(1) One μg bovine pancreas DNase I (3.1.4.5) (BRL): 50 mM sodium acetate (pH 6.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 37°C.

(2) Twenty units bovine spleen DNase II (3.1.4.6) (BRL): 0.8 mM $MgSO_4$, 83.3 mM HOAc (pH 4.6), 25°C.

(3) Thirty mU bovine spleen exonuclease (phosphodiesterase II, 3.1.4.18) (Sigma): 0.1 M sodium citrate (pH 6.0), 5 mM EDTA, 37°C.

(4) Ten μU venom exonuclease from *Crotalus adamanteus* (phosphodiesterase I, 3.1.4.1) (Sigma): 0.2 mM TrisCl (pH 8.9), 37°C. Additional experiments were done with 11.5 mU venom exonuclease from Worthington Biochemical: 0.11 M TrisCl (pH 8.9),0.11 M NaCl, 15 mM $MgCl_2$, 25°C.

DNase I and venom exonuclease reactions were stopped by addition of EDTA to 25 mM and incubation at 70°C for 10 minutes. Spleen exonuclease reactions were stopped by incubation at 70°C for 10 minutes. DNase II was inactivated by addition of EDTA to 21 mM and TrisOH to pH 6.0, followed by incubation at 70°C for 30 minutes. Nucleic acid hybridization was carried out in a minimum of 100 mM NaCl.

Tissue culture

Suspension cultures of B95-8 cells, an Epstein Barr virus-positive lymphoid cell line, were maintained at concentrations of 4×10^5 to 2×10^6/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum and penicillin and streptomycin (all from Gibco) at 37°C in an humidified 5% CO_2 incubator. For the stability studies 200 μl of either RPMI 1640 only, serum-supplemented medium, or cell suspension was transferred to 96-well tissue culture dishes. Oligodeoxynucleotides were added to the wells to a final concentration of approximately 0.2 μM and were allowed to incubate at 37°C for times ranging up to 24 hours. Twenty-five μl samples were removed, incubated at 70°C for 5−10 min to curtail further degradation and stored at −20°C prior to gel migration analysis.

Gel migration analyses

Marker (14- or 18-mer) phosphodiester oligodeoxynucleotides were 5′-labeled with ^{32}P using T4 polynucleotide kinase and purified using a spun column (20) of Sephadex G-50 in H_2O. Hybridization to equal concentrations of nuclease-treated, complementary oligodeoxynucleotides was carried out at room temperature for 15 minutes prior to electrophoresis. Control hybridizations were carried out in appropriate enzyme buffers. Samples were loaded onto a 20% acrylamide gel in 2.5% Ficoll 400. Gel electrophoresis was carried out at 4°C at 400 volts, 5−15 milliamps in TBE (89 mM TrisCl, 89 mM borate, 1 mM EDTA) buffer for 2−4 hours. For the studies of tissue culture media, appropriate volumes of samples were mixed with the ^{32}P-labeled oligodeoxynucleotide in 100 mM NaCl, 10 mM TrisCl (pH 7.6), and 1 mM EDTA. Hybridization was carried out by heating to 65−70°C for 5 minutes followed by slow cooling to room temperature. These samples were analyzed on 20% polyacrylamide gels by electrophoresis in TBE at a constant current (12 mA) with cold (5−10°C) circulating water. All gels were dried and examined by autoradiography.

In vitro transcription and RNase H analyses

In vitro transcription was carried out using SP6 RNA polymerase (BRL) with *Hin*dIII-, *Nco*I-, or *Pvu*II-digested plasmid pSP65-ALA-D. Approximately 40 ng of RNA transcript was hybridized in 9 μl with 5 to 100 ng (a large molar excess) antisense oligodeoxynucleotide, in 20 mM TrisCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 100 μM

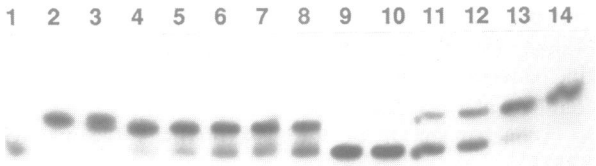

Figure 1. Gel migration analysis of exonuclease digests of 14-P-2. All lanes contain 5′-^{32}P-labeled complementary oligodeoxynucleotide 18-P-1. Lane 1, 18-P-1 only; lane 2, undigested 14-P-2 + 18-P-1 (control duplex); lanes 3–8, spleen exonuclease (5′ >—— > 3′) 5, 10, 15, 20, 25, 30 mU, respectively; lanes 9–14 venom exonuclease (3′ >—— > 5′) 31.2, 15.6, 7.8, 3.9, 1.9, 0.9 μU, respectively.

DTT, and 5% (w/v) sucrose, in the presence of 10 units of RNasin (Promega). After one minute at 60°C, the mixtures were incubated at room temperature (22°C) for 30 minutes. One μl (2 units) of *E. coli* RNase H (3.1.26.4) (BRL) was added, and the reactions were carried out for 60 minutes at 37°C. One μl of 0.1 M EDTA was added to stop the reaction. Electrophoresis was carried out in 6% denaturing (8 M urea) acrylamide gels at 350 volts, 45 mA, for 1 hour at room temperature.

Table 2. Summary of nuclease sensitivities and stability in culture media.

A. Exonucleases:	**Spleen (5′→3′)**	**Venom (3′→5′)**	
	Relative Half-Life		
14-P-2*	1	1	
14-P-1*	1	1	
14-C-2	>200	>500	
14-G-1	>200	100	
14-A-2	>200	>500	
14-T-1	>200	>500	
14-Me^{6}a-2	>200	>500	
B. Endonucleases:	**DNaseI**	**DNaseII**	
	Relative Half-Life		
14-P-2*	1	1	
14-A-2	1	>5	
14-C-2	6	2	
14-Me^{5}b-2	12	>5	
14-Me^{5}a-2	300	>5	
14-Me^{6}b-2	>600	>5	
14-Me^{6}a-2	>600	>5	
C. Culture media:	**RPMI**	**+ Serum**	**+ Serum & Cells**
	Relative Half-Life		
14-P-2	Stable	1	1
14-Me^{5}b-2	Stable	30	30
14-Me^{5}a-2	Stable	30	30
14-Me^{6}b-2	Stable	30	30
14-Me^{6}a-2	Stable	30	30
12-Me^{10}-4	Stable	Stable	Stable

*Control phosphodiester oligodeoxynucleotides. Half-lives of modified oligodeoxynucleotides are compared to the half-lives of control oligodeoxynucleotides with the same sequence.

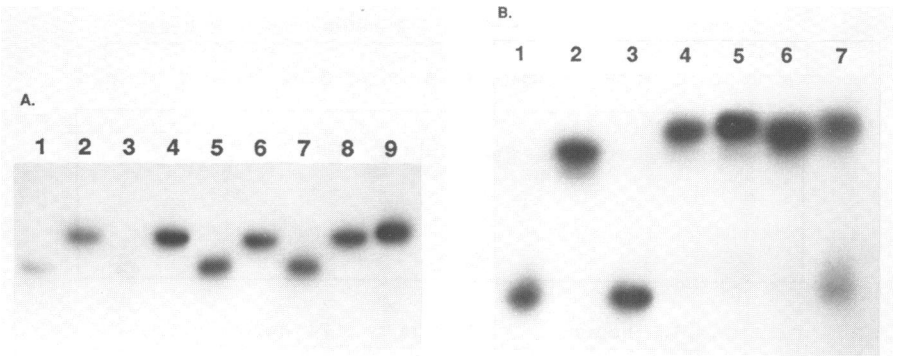

Figure 2. Gel migration analysis of DNase I digests of oligodeoxynucleotides.
(A) All lanes contain 5′-^{32}P-labeled complementary oligodeoxynucleotide 18-P-1. Lanes 2,4,6,8: undigested. Lanes 3,5,7,9: DNase I digests. Lane 1, 18-P-1 only; lanes 2−3, 14-P-2; lanes 4−5, 14-A-2; lanes 6−7, 14-C-2; lanes 8−9, 14-Me^{6}a-2.
(B) All lanes contain 5′-^{32}P-labeled complementary oligodeoxynucleotide 14-P-1. Lanes 2,4,6: undigested. Lanes 3,5,7: DNase I digests. Lane 1, 14-P-1 only; lanes 2−3, 14-P-2; lanes 4−5, 14-Me^{6}b-2; lanes 6−7, 14-Me^{5}a-2.

RESULTS

Analysis of nuclease sensitivity was based on the ability of an oligodeoxynucleotide to form a duplex with and retard the electrophoretic mobility of a ^{32}P-labeled complementary oligodeoxynucleotide. The oligodeoxynucleotides used in these studies are presented in Table 1, grouped into complementary sets designated as sense and antisense. The oligodeoxynucleotides are named by length-descriptor-number where an even or odd number is used to denote sense or antisense, respectively. Nuclease action on substituted oligodeoxynucleotides was compared to results with equivalent quantities of control phosphodiester oligodeoxynucleotides. An example of a gel migration assay may be seen in Figure 1. Labeled 18-P-1 (P for phosphodiester), which was the marker oligodeoxynucleotide (lane 1), had a lower mobility when hybridized to 14-P-2 (lane 2). Failure to decrease the mobility of (form a duplex with) the labeled complement indicates a sensitivity to a tested nuclease.

Effect of exonucleases

Figure 1 depicts the gel migration assay of the digestion of control 14-P-2 by titrated quantities of spleen (lanes 3−8) and venom (lanes 9−14) exonucleases. The results of the studies of the action of these enzymes on 14-P-2, 14-P-1 and modified oligodeoxynucleotides are summarized in Table 2A. The half-time for spleen exonuclease digestion of the control oligodeoxynucleotides was about 30 minutes for the enzyme concentration used. All of the partially modified oligodeoxynucleotides tested with spleen exonuclease were >200 times more resistant than their respective controls based on absence of detectable cleavage following overnight digestion. The oligodeoxynucleotides 14-C-2, 14-G-1, and 14-T-1 were apparently shortened, as evidenced by a slight increase in the electrophoretic mobility of the hybrid (data not shown). The half-time for venom exonuclease digestion of the control oligodeoxynucleotides was about 12 minutes for the enzyme (Sigma) concentration used. The oligodeoxynucleotide with the shortest relative half-life (14-G-1) had its first methylphosphonate bond as the third linkage in from the 3′ end, while the

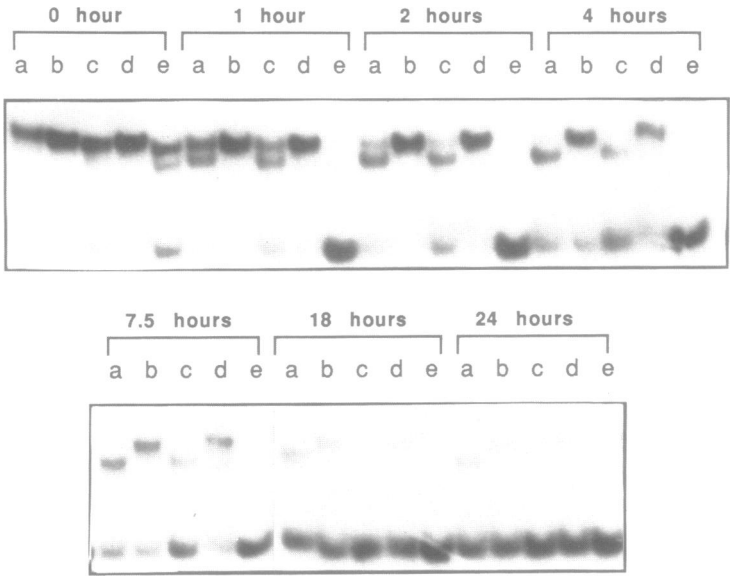

Figure 3. Stability of oligodeoxynucleotides in tissue culture medium. Oligodeoxy-nucleotides were incubated in suspensions of B95-8 cells in serum-supplemented medium. Samples were taken at the indicated times and were treated as described in Materials and Methods. Tested oligodeoxynucleotides: lanes a) 14-Me^{6}a-2, b) 14-Me^{6}b-2, c) 14-Me^{5}a-2, d) 14-Me^{5}b-2, and e) 14-P-2. The ^{32}P-labeled complementary oligodeoxynucleotide was 14-P-1.

other oligodeoxynucleotides tested had a methylphosphonate as the first or second linkage from the 3′ end. In another study, using Worthington venom exonuclease, the presence of two consecutive methylphosphonate linkages at the 3′ end (14-Me^2-1) yielded approximately a two-fold increase in the half-life of the oligodeoxynucleotide, as compared to 14-C-1, which has only one methylphosphonate linkage at the 3′ end (data not shown).

Effect of endonucleases

Figures 2A and B depict gel migration assays of the sensitivity of several oligodeoxynucleotides to overnight DNase I digestion. Digests of shorter duration were carried out to determine the half-times for survival of those oligodeoxynucleotides which were completely degraded by the overnight treatment. Table 2B presents the relative half-times for digestion of oligodeoxynucleotides by the endonucleases DNase I and DNase II. The half-time for digestion of control 14-P-2 by DNase I was approximately 10 minutes. 14-A-2, which has an maximum internal span of five phosphodiester linkages, was as sensitive to DNase I as the control 14-P-2. 14-Me^{6}a-2 has alternating phosphodiester and methylphosphonate linkages and was over 600 times more resistant to DNase I than 14-P-2. In general, as the phosphodiester span was decreased, the oligodeoxynucleotide became more resistant to endonuclease activity. Specifically, the greatest increase in stability was seen upon decreasing the span of contiguous phosphodiester linkages from four to three. Given that the DNase II reactions were much slower than the DNase I reactions, with a half-time for 14-P-2 digestion of 10 hours, the most resistant methylphosphonate oligodeoxynucleotides could only be designated as >5 times more stable than the control.

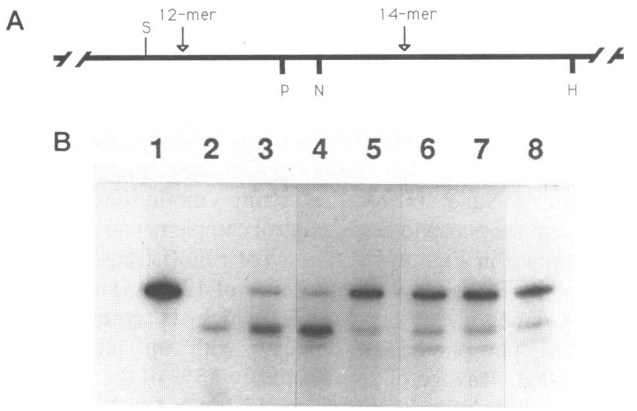

Figure 4. RNase H assay. (A) Map of pSP65-ALA-D. Arrows indicate oligodeoxynucleotide binding sites. Restriction endonuclease sites for runoff transcription: P = *Pst*I, N = *Nco*I, H = *Hin*dIII. S = start of transcription. (B) All lanes, *Nco*I RNA runoff transcript (501 nt); Lane 1, transcript only; lanes 2−8, transcript + oligodeoxynucleotide treated with RNase H, lane 2, 12-P-4, (control, 386 nt cleavage product); lanes 3−4, 12-Me^4-4; lanes 5−6, 12-Me^{5}b-4; lanes 7−8, 12-Me^{5}c-4. Lanes 4, 6, 8 contained 4-fold more oligodeoxynucleotide than lanes 3, 5, 7.

Stability of oligodeoxynucleotides in tissue culture medium

Table 2C summarizes the results of gel migration assays carried out to determine the stability of various oligodeoxynucleotides to incubation in tissue culture medium, serum-supplemented medium, and serum-supplemented medium conditioned by 24 hours of cell growth. All of the oligodeoxynucleotides tested were completely stable to 37°C incubation for 24 hours in RPMI 1640 alone. In contrast, all of the oligodeoxynucleotides were degraded with identical kinetics after incubation in either serum-supplemented medium or in conditioned medium. The degradation kinetics indicated that the enzymatic activity leading to degradation was associated with the serum.

Figure 3 presents the results of stability testing in conditioned medium. The phosphodiester oligodeoxynucleotide 14-P-2 had a half-life of less than 60 minutes. Results from similar experiments in serum-supplemented or conditioned medium using shorter time increments suggested a half-life of approximately 15 minutes reported in Table 2A. The oligodeoxynucleotides with methylphosphonate substitutions were significantly more stable than their phosphodiester analogue. Unexpectedly, they all displayed relatively similar half-lives of approximately 7.5 hours, independent of the arrangement of the methylphosphonate linkages. A 3′-exonucleolytic activity was evidenced in Figure 3 by single stepwise cleavage of oligodeoxynucleotides 14-Me^{6}a-2 and 14-Me^{5}a-2, both of which contain 3′-phosphodiester-linked thymidine residues, and the absence of cleavage of oligodeoxynucleotides 14-Me^{6}b-2 and 14-Me^{5}b-2, which contain 3′-methylphosphonate-linked thymidine residues. In contrast to the partially methylphosphonate-substituted oligodeoxynucleotides, a fully-substituted oligodeoxynucleotide (12-Me^{10}-4) was stable for 24 hours with no apparent diminution of hybridizable material (Table 2C).

DNA-RNA hybrids formed with partially methylphosphonate-substituted oligodeoxynucleotides can serve as RNase H substrates

^{32}P-labeled runoff transcripts were made from pSP65-ALA-D (Figure 4A) and incubated with various oligodeoxynucleotides in the presence of RNase H. Using a *Hin*dIII runoff

transcript, hybrids with methylphosphonate-substituted oligodeoxynucleotides 14-A-2 and 14-C-2 served as RNase H substrates, while 14-Me^{6}a-2 did not, even up to a concentration of 10 μg/ml (data not shown). The 14-mer binding site appeared to be a poor site for RNase H digestion since the use of the control phosphodiester oligodeoxynucleotide 14-P-2 only led to cleavage of approximately half of the original transcript, perhaps due to secondary structure of the RNA in this region. Thus, further studies were carried out at a different site.

The results of the RNase H studies using methylphosphonate-substituted oligodeoxynucleotides and the phosphodiester control complementary to the 12-mer binding site (Figure 4A) are shown in Figure 4B. The *Nco*I runoff transcript (501 nt) (lane 1) was completely cleaved by RNase H in the presence of 12-P-4 (lane 2). Lane 1 contains more transcript than lanes 2−8. 12-Me^4-4, which is composed of alternating methylphosphonate and phosphodiester linkages with an internal span of three phosphodiesters, permitted cleavage of approximately 85% of the RNA (lanes 3 and 4). Less cleavage occurred with hybrids containing 12-Me^{5}b-4, which has a span of two phosphodiesters, or 12-Me^{5}c-4, which has alternating linkages throughout (lanes 5 through 8). A four-fold increase in oligodeoxynucleotide concentration, which was already in excess over complementary RNA, did not significantly increase the RNA cleavage. Thus, sufficient oligodeoxynucleotide was present to maximize formation of the RNA-DNA hybrids. The degree of cleavage by RNase H reflects the ability of each of these RNA-DNA hybrids to function as an RNase H substrate. On the other hand, the extent of cleavage of all of the RNA-DNA hybrids was found to be linearly dependent on the concentration of RNase H in the concentration range of enzyme employed for these studies (data not shown).

The relative rates of cleavage of the various RNA-DNA hybrids by RNase H were determined by quantitative autoradiography. Consider the cleavage rate for the hybrid with 12-Me^4-4 (three contiguous internal phosphodiester linkages) to be a standard. The rate of hybrid cleavage using the control, all phosphodiester, oligodeoxynucleotide was approximately 10-fold greater. Such a difference in cleavage rates is expected on a statistical basis where the RNase H may associate with any short span of nucleotides within the all phosphodiester oligodeoxynucleotide. On the other hand, the cleavage rates of hybrids with 12-Me^{5}b-4 or 12-Me^{5}c-4 (two contiguous internal phosphodiester linkages or alternating linkages, respectively) were more than 10-fold lower, indicating that they were poor RNase H substrates.

12-Me^{5}a-4, which has an internal span of two phosphodiesters and two phosphodiesters at the 5′ end, formed an RNase H-sensitive substrate (data not shown). However, the 5′ end of 12-Me^{5}a-4 contains three adjacent nucleotides prior to the first methylphosphonate linkage. The greatest increase in ability to form an RNase H-sensitive substrate occurred upon increasing the span of continuous phosphodiesters from two to three. Recalling that the greatest increase in resistance to DNase I occurs upon decreasing the span of contiguous phosphodiester linkages from four to three, a structure with three contiguous phosphodiester linkages is compatible with both endonuclease resistance and ability to form RNase H-sensitive substrates.

DISCUSSION

The antisense effects previously reported with fully methylphosphonate-substituted oligodeoxynucleotides required relatively high concentrations (3−6). Since RNase H will not cleave the RNA in a duplex with fully-substituted molecules (14), the antisense effects seen were probably due to a physical block of the translational machinery, and this

mechanism alone may not be very effective. The high concentration requirement might also reflect the helix-destabilizing effect of each chiral methylphosphonate linkage (17,21). In order to provide a logical framework for the design of methylphosphonate-containing antisense oligodeoxynucleotides, we have compared partially methylphosphonate-substituted oligodeoxynucleotides to phosphodiester analogues for sensitivity to specific nucleases, stability in a tissue culture environment, and ability to form RNase H substrates. Gel migration analysis for assessing oligodeoxynucleotide stability is particularly appropriate for selection of candidate oligodeoxynucleotides for antisense agents, since the assay directly tests the ability of an oligodeoxynucleotide to hybridize with complementary sequences.

The exonuclease experiments indicate that the incorporation of a methylphosphonate linkage in a phosphodiester oligodeoxynucleotide converts a good substrate into a poor substrate. This result suggests that exonucleolytic attack on methylphosphonate-substituted oligodeoxynucleotides may be similar to that found for venom exonuclease digestion of DNA-containing thymidine dimers, where the enzyme slowly breaks the internal bond of the base beyond each encountered dimer (22). In a study using HPLC analysis of digest products (16), two consecutive methylphosphonate linkages were found to cause an increase in half-times for digestion by both venom and spleen exonucleases of two orders of magnitude. The gel migration analyses demonstrated that the presence of just one methylphosphonate linkage near the 5′ and 3′ ends of an oligodeoxynucleotide causes an increase in digestion half-times of more than two orders of magnitude. In fact, the presence of one methylphosphonate linkage at the 3′ end provided almost the same protection as two consecutive methylphosphonate linkages.

The gel migration analyses of DNase I-sensitivity of partially methylphosphonate-modified oligodeoxynucleotides indicate that decreasing the span of contiguous phosphodiester linkages leads to greater resistance. As described above, the greatest increase in stability is seen upon decreasing the span of contiguous phosphodiester linkages from four to three.

Methylphosphonate oligodeoxynucleotides with internal contiguous phosphodiester linkages ranging from one to four have relatively uniform half-lives of 7.5 hours when incubated in either serum-supplemented medium or conditioned medium. Wickstrom (23) found that phosphodiester oligodeoxynucleotides were completely stable to incubation for up to 2 hours in medium with 5% fetal calf serum. The discrepancy between these two studies may reflect differences between fetal calf serum preparations. The degradation pattern observed in this study is most consistent with the action of an exonuclease. However, the nature of the activity cannot be determined because the initial 3′-exonucleolytic cleavage seen with two of the oligodeoxynucleotides was not followed by detectable stepwise degradation.

Fully-substituted, antisense methylphosphonate oligodeoxynucleotides presumably function by creating a physical block to the protein translational machinery. They are unable to form hybrids with RNA that are recognized as substrates by RNase H (14). The results presented in this work demonstrate that partially methylphosphonate-substituted oligodeoxynucleotides with three or more contiguous phosphodiester linkages will hybridize with complementary RNA and efficiently promote cleavage of the RNA by RNase H. Although the efficiency of cleavage was not reported, Inoue and coworkers (24), in a search for reagents for site specific cleavage of RNA, observed that a span as small as three contiguous phosphodiester linkages flanked by 2′-O-methyl-nucleotides was capable of forming an RNase H-sensitive substrate. Oligodeoxynucleotides with one or more regions containing three contiguous phosphodiester linkages are most compatible with highly

nuclease-resistant arrangements of methylphosphonate and phosphodiester linkages. Thus, it is possible to synthesize an antisense oligodeoxynucleotide with methylphosphonate and phosphodiester linkages that will have increased stability over a phosphodiester oligodeoxynucleotide in tissue culture, and perhaps *in vivo*, as well as have the ability to promote cleavage of target RNA.

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