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Interactions of oligonucleotide analogs containing methylphosphonate internucleotide linkages and 2′-*O*-methylribonucleosides

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ABSTRACT

The interactions of oligonucleotide analogs, 12-mers, which contain deoxyribo- or 2′-*O*-methylribose sugars and methylphosphonate internucleotide linkages with complementary 12-mer DNA and RNA targets and the effect of chirality of the methylphosphonate linkage on oligomer-target interactions was studied. Oligomers containing a single Rp or Sp methylphosphonate linkage (type 1) or oligomers containing a single phosphodiester linkage at the 5′-end followed by 10 contiguous methylphosphonate linkages of random chirality (type 2) were prepared. The deoxyribo- and 2′-*O*-methylribo- type 1 12-mers formed stable duplexes with both the RNA and DNA as determined by UV melting experiments. The melting temperatures, Tms, of the 2′-*O*-methylribo-12-mer/RNA duplexes (49–53°C) were higher than those of the deoxyribo-12mer/RNA duplexes (31–36°C). The Tms of the duplexes formed by the Rp isomers of these oligomers were approximately 3–5°C higher than those formed by the corresponding Sp isomers. The deoxyribo type 2 12-mer formed a stable duplex, Tm 34°C, with the DNA target and a much less stable duplex with the RNA target, Tm <5°C. In contrast, the 2′-*O*-methylribo type 2 12-mer formed a stable duplex with the RNA target, Tm 20°C, and a duplex of lower stability with the DNA target, Tm <5°C. These results show that the previously observed greater stability of oligo-2′-*O*-methylribonucleotide/RNA duplexes *versus* oligodeoxyribonucleotide/RNA duplexes extends to oligomers containing methylphosphonate linkages and that the configuration of the methylphosphonate linkage strongly influences the stability of the duplexes.

INTRODUCTION

Antisense oligonucleotides and oligonucleotide analogs are generally designed to bind to functional regions of messenger RNA and as a consequence of this binding, inhibit the synthesis of targeted viral or cellular proteins (1–6). Although oligodeoxyribonucleotides are often used for this purpose they suffer from their lack of stability in cell culture. Therefore considerable effort has been expended in the design of modified oligomers which show increased resistance to hydrolysis by nucleases found in cell culture medium or the intracellular environment. Modifications have usually focused on replacement of the phosphodiester internucleotide linkages with nuclease-resistant groups, the simplest being phosphorothioate (5,7–9) or methylphosphonate (10,11).

Oligomers containing phosphorothioate or methylphosphonate linkages have usually been synthesized with deoxyribose sugar units. Results from a number of laboratories have shown that oligonucleotides which contain 2′-*O*-alkyl substituents, such as 2′-*O*-methylribose, form very stable duplexes with complementary RNA target molecules (12–16). The improved binding constants observed with oligo-2′-*O*-methylribonucleotides and the enhanced nuclease resistance of the phosphorothioate or methylphosphonate oligomers could be used to advantage to design effective antisense oligonucleotides. To test this possibility, we have prepared oligonucleotide analogs which contain 2′-*O*-methylribose sugars and methylphosphonate internucleotide linkages (17). In this paper we describe the interactions of such oligomers with complementary DNA and RNA targets and the effect of chirality of the methylphosphonate linkage on oligomer–target interactions.

MATERIALS AND METHODS

Protected deoxyribonucleoside-3′-*O*-(β-cyanoethyl)-*N,N*-diisopropyl)-phosphoramidites and controlled pore glass supports derivatized with either protected deoxyribonucleosides or 2′-*O*-methylribonucleosides were purchased from Glen Research, Sterling, VA. Protected deoxyribonucleoside-3′-*O*-(*N,N*-diisopropyl)methylphosphonamidites were obtained from JBL Scientific, San Luis Obispo, CA. The 3′-*O*-(*N,N*-diisopropyl) methylphosphonamidite derivative of 5′-dimethyoxytrityl-2′-*O*-methyluridine (^{31}P-NMR spectrum, 127 and 124 p.p.m.,

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$CDCl_3$) was prepared from 5'-dimethoxytrityl-2'-*O*-methyluridine in a manner similar to that described below for the syntheses of the methylphosphonamidite derivatives of 2'-*O*-methylguanosine and 2'-*O*-methyladenosine. This synthon was a gift from GENTA, Inc., San Diego, CA. 2'-*O*-methyladenosine (18) and 2'-*O*-methylguanosine (19) were prepared by published procedures. Analytical and preparative high performance liquid chromatography (HPLC) was carried out on Microsorb C-18 reversed phase columns, 4.6 mm×15 cm, analytical, and 10 mm×25 cm, preparative. The columns were eluted at a rate of 1.0 ml/min, analytical column, or 2.0 ml/min, preparative, with linear gradients of acetonitrile in 50 mM sodium phosphate buffered at pH 5.8. Polyacrylamide gel electrophoresis was carried out on 14 cm×16 cm×0.75 mm gels using a gel buffer which contained 0.089 M Tris, 0.089 M boric acid and 0.1 mM EDTA buffered at pH 8.0. The extinction coefficients of the oligonucleotides were determined after digestion with snake venom phosphodiesterase as previously described (20). The extinction coefficients of the oligonucleoside methylphosphonates were determined in a similar manner after digestion with 1 M aqueous piperidine overnight at 37°C.

Preparation of the methylphosphonamidite derivatives of 2'-*O*-methylguanosine and 2'-*O*-methyladenosine

Methylene chloride was distilled from phosphorous pentoxide, and diisopropylamine and diisopropylethylamine were both distilled from calcium hydride prior to use. The reaction vessel was dried in an oven at 150°C and flushed with dry argon. All transfers were carried out using argon flushed syringes. Three millimoles of diisopropylamine were added dropwise over a period of 15 min to a solution containing 1.5 ml methyldichlorophosphine in 1 ml of methylene chloride. The reaction mixture was stirred for an additional 15 min. A solution of 0.52 mmol of 5'-*O*-dimethoxytrityl-2'-*O*-methyl-N^2-isobutyrylguanosine (19) in 1 ml of methylene chloride and 0.58 ml of diisopropylethylamine was added dropwise simultaneously to the reaction mixture with stirring. The reaction mixture was stirred for 2 h at room temperature after which time the reaction was determined to be complete as judged by silica gel tlc. The reaction mixture was treated with 0.25 ml of methanol for 15 min and then extracted with 5 ml of 5% sodium bicarbonate solution. The organic layer was isolated and the solvents evaporated. The residue was dried under vacuum overnight. The residue was dissolved in 2 ml of methylene chloride, and the solution was added dropwise with stirring to 30 ml of hexane. The precipitate was isolated and dried under vacuum to give 0.49 mmol of product. The FAB mass spectrum (MH^+ = 815) and the ^{31}P-NMR spectrum (125 and 118 p.p.m., $CDCl_3$) were consistent with the structure of the product. The 3'-*O*-methylphosphonate derivative of 5'-*O*-dimethoxytrityl-2'-*O*-methyl-N^6-benzoyladenosine was prepared in a similar manner from the protected nucleoside. The FAB meass spectrum (MH^+ = 888) and the ^{31}P-NMR spectrum (125 and 123 p.p.m., $CDCl_3$) were consistent with the stucture of the product.

Preparation of oligonucleotides and oligonucleotides which contain methylphosphonate linkages

The RNA target oligomer, r-GACAAAUCCUAU, was obtained from GENTA, Inc. DNA oligomers, d-GACAAATCCTAT and **1** (see Table 1), and 2'-*O*-methylribo-oligomer **3** were prepared on controlled glass supports derivatized with a protected deoxyribonucleoside or 2'-*O*-methylribonucleoside using deoxyribonucleoside phosphoramidite or 2'-*O*-methylribonucleoside phosphoramidite synthons, respectively. The syntheses were carried out on 1 μmol scales using a Biosearch 8700 DNA synthesizer. The protected oligomers were cleaved from the support and deprotected by treatment with 1 ml of 50% concentrated ammonium hydroxide in pyridine for 5 h at 55°C. The oligomers were purified by preparative C-18 reversed phase HPLC using linear gradients of 2−20% or 2−30% acetonitrile in 50 mM sodium phosphate. The oligomers were desalted using SEP PAK C-18 cartridges (Waters Associates) and were eluted from the SEP PAK with 50% aqueous acetonitrile. The purified oligomers migrated as single bands on 20% polyacrylamide gels containing 7 M urea after phosphorylation with γ-[^{32}P]-ATP in the presence of polynucleotide kinase.

Oligodeoxyribonucleotide **2** and oligo-2'-*O*-methylribonucleotides **4**, **5** and **6** were prepared on controlled pore glass supports using the appropriate protected nucleoside phosphoramidite and methylphosphonamidite synthons. Syntheses were carried out on 1 μmol scales. The oligomers were deprotected and cleaved from the support by sequential treatment with ammonium hydroxide and ethylenediamine (23). The oligomers were then purified by preparative C-18 reversed phase HPLC using linear gradients of 8−12% acetonitrile in 50 mM sodium phosphate. The diastereoisomers of oligomers **2**, **4**, and **5** were each resolved into two peaks of approximately equal area and each diastereoisomer was collected separately. After phosphorylation with γ-[^{32}P]-ATP in the presence of polynucleotide kinase, the diastereoisomers each migrated as single bands with identical mobilities on 20% denaturing polyacrylamide gels containing 7 M urea. These individual diastereoisomers as well as the mixture of diastereoisomers of oligomer **6**, were each digested with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase at 37°C in 10 mM Tris, 2 mM magnesium chloride, buffered at pH 8.2. In order to ensure complete digestion of each oligomer to nucleosides and dimer, it was necessary to add a total of 4 units of bacterial alkaline phosphatase (United States Biochemical Corp.) and 12 μg snake venom phosphodiesterase (Boehringer Mannheim Corp.) in small aliquots over a period of 24 h. These enzymatic digestions as well as the Rp and Sp diastereoisomers of d-ApT were analyzed by analytical C-18 HPLC using a 12 min linear gradient of 2−3% acetonitrile in 50 mM sodium phosphate followed by an 8 min linear gradient of 3−30% acetonitrile in 50 mM sodium phosphate.

Oligodeoxyribonucleoside methylphosphonate **7** (Table 2) and 2'-*O*-methylribonucleoside methylphosphonate **8** were prepared on controlled glass supports using methylphosphonamidite synthons according to published procedures (21,22). The oligomers were deprotected by brief treatment with ammonium hydroxide followed by treatment with ethylenediamine for 6 h as described by Hogrefe *et al.* (23). The oligomers were purified by sequential chromatography on DEAE cellulose and C-18 reversed phase HPLC. The purified oligomers migrated as single peaks on an analytical C-18 reversed phase column and after phosphorylation with γ-[^{32}P]-ATP in the presence of polynucleotide kinase migrated as single bands on 20% polyacrylamide gels containing 7 M urea.

Melting experiments

Melting experiments were carried out in either 10 mM HEPES, 2 mM EDTA, pH 8.0; or in 0.1 M NaCl, 10 mM HEPES, 2 mM EDTA, pH 8.0. Duplexes containing a final strand

concentration of 1.5 μM or 3.0 μM of each oligomer were formed, respectively, by mixing 0.5 ml of 3.0 μM or 6.0 μM solutions of the oligomers and incubating overnight at 4°C. The melting transitions were measured using a Cary 3E UV/vis spectrophotometer fitted with a thermostatted cell block and temperature controller. At low temperature the cell block was continually purged with dry nitrogen to prevent condensation. The A_{260} of the solution was recorded as a function of temperature as the solutions were heated from 0 to 70°C at a rate of 0.5°C/min.

Gel mobility shift experiments

Solutions containing 0.2 μM gel-purified, 5′-[^{32}P]-end-labeled d-GACAAATCCTAT or r-GACAAAUCCUAU, and 0–100 μM oligomer in 5 μl of 0.1 M sodium chloride, 50 mM Tris, pH 7.6, were incubated for 5 min at 37°C, cooled for 20 min at 20°C, and then cooled for 20 min on ice. After addition of 5 μl of ice-cold glycerol/H_2O (1:1 v/v), the samples were subjected to gel electrophoresis at 4°C on a 20% non-denaturing polyacrylamide gel for 90 min at 500 V. After drying, the gels were autoradiographed at −80°C.

RESULTS AND DISCUSSION

Preparation of oligonucleotides containing methylphosphonate linkages

Oligodeoxyribonucleotides and oligo-2′-*O*-methylribonucleotides containing methylphosphonate internucleotide linkages and whose sequences are complementary to a 12-mer DNA target, d-GACAAATCCTAT, or the corresponding RNA target, r-GACAAAUCCUAU. The RNA target corresponds to nucleotides 165–176 found in the coding region of vesicular stomatitis virus M-protein mRNA (24). These oligomers were synthesized on controlled glass supports using the appropriately protected nucleoside methylphosphonamidite synthons.

To examine the effect of chirality on binding interactions, oligomers were prepared which contain a single methylphosphonate linkage. Their sequences are shown in Table 1. Deoxyribose oligomer **2** contains a single methylphosphonate linkage between the sixth and seventh nucleosides. The methylphosphonate linkage of 2′-*O*-methylribose oligomer **4** is similarly positioned, whereas the methylphosphonate linkages of oligomers **5** and **6** are positioned at the 5′- and 3′-ends, respectively, of the oligomers. The presence of the single

Table 1. Thermal stabilities of duplexes formed by oligonucleotides containing a single methylphosphonate linkage

Oligomer[d]		Tm°C[a] DNA target[b] 0 M NaCl	0.1 M NaCl	RNA target[c] 0 M NaCl	0.1 M NaCl
d-ATAGGATTTGTC	1	21	40	17	36
d-ATAGGApTTTGTC	2				
Rp-isomer		21	38	19	35
Sp-isomer		17	34	16	31
mr-AUAGGAUUUGUC	3	20	38	36	53
mr-AUAGGApUUUGUC	4				
isomer 1		18	37	38	53
isomer 2		16	32	34	49
mr-ApUAGGAUUUGUC	5				
isomer 1		22	38	39	54
isomer 2		19	38	37	53
mr-AUAGGAUUUGUpC	6	18	32	33	49

[a]Experiments were carried out in a buffer containing 10 mM HEPES, pH 7.0, 2 mM EDTA, 0 M or 0.1 M sodium chloride at an oligomer strand concentration of 1.5 μM or 3 μM per strand.
[b]The DNA target is d-GACAAATCCTAT.
[c]The RNA target is r-GACAAAUCCUAU.
[d]Sequences preceeded by d- are oligodeoxyribonucleotides and those preceeded by mr- are oligo-2′-*O*-methylribonucleotides. The symbol p indicates a methylphosphonate linkage.

Table 2. Thermal stabilities of duplexes formed by oligonucleoside methylphosphonates

Oligomer[d]		Tm°C[a] DNA target[b] 0 M NaCl	0.1 M NaCl	RNA target[c] 0 M NaCl	0.1M NaCl
d-ATAGGATTTGTC	1	21	40	17	36
d-ApTAGGATTTGTC	7	32	34	<5	<5
mr-AUAGGAUUUGUC	3	20	38	36	53
mr-ApUAGGAUUUGUC	8	<5	<5	17	20

[a]Experiments were carried out in a buffer containing 10 mM HEPES, pH 7.0, 2 mM EDTA, 0 M or 0.1 M sodium chloride at an oligomer strand concentration of 1.5 μM or 3 μM per strand.
[b]The DNA target is d-GACAAATCCTAT.
[c]The RNA target is r-GACAAAUCCUAU.
[d]Sequences preceeded by d- are oligodeoxyribonucleotides and those preceeded by mr- are oligo-2′-*O*-methylribonucleotides. The symbol p indicates a phosphodiester linkage and the underline indicates the position of the methylphosphonate linkages.

methylphosphonate linkage required that the oligomers be deprotected using ethylenediamine rather than prolonged treatment with ammonium hydroxide, the method usually employed when deprotecting oligodeoxyribonucleotides. This was carried out using the procedure of Hogrefe *et al.* (23) which involves a brief, 30 min, exposure to ammonium hydroxide in ethanol/acetonitrile solution, followed by a 6 h treatment with ethylenediamine. This procedure satisfactorily removed the base protecting groups and all of the phosphate β-cyanoethyl protecting groups.

Oligomers **2**, **4** and **5** appeared as two partially resolved peaks of approximately equal area on analytical C-18 reversed phase chromatography. These peaks could be resolved on a preparative C-18 column and were collected separately. The oligomers from each peak, after phosphorylation by polynucleotide kinase, migrated as single bands with identical mobility on a denaturing polyacrylamide gel. The chromatographic and electrophoretic behavior of the oligomers in the two peaks is consistent with the resolution of the two diastereoisomers of the oligomer. In the case of oligomer **2**, this was confirmed by enzymatic digestion of the oligomers from each peak with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase. In addition to the expected nucleosides, a peak whose mobility corresponded to one of the diastereoisomers of the dimer, d-ApT, was also observed. The dimer obtained from the first peak corresponded to authentic Rp-d-ApT, whereas the dimer obtained from the second peak corresponded to authentic Sp-d-ApT (25–27). The retention times of these two diastereomers differed by 0.22 min on the C-18 column using a linear gradient of 2–3% acetonitrile for the first 12 min of elution followed by a linear gradient of 3–30% acetonitrile for the next 8 min. Based on these results, the two peaks are assigned as the Rp-isomer of **2** and the Sp-isomer of **2**.

Similar results were obtained when the individual chromatographic peaks of oligomers **4** or **5** were digested with snake venom phosphodiesterase and bacterial alkaline phosphatase. Each oligomer yielded a peak corresponding to mr-ApU. The retention time of mr-ApU obtained from the digest of the first peak of oligomer **4** or **5** was 0.21 min greater than that of mr-ApU obtained from the digest of the second peak of oligomer **4** or **5** using the gradient described above. Based on the similarity to d-ApT and the observation by others that the Rp methylphosphonate diastereoisomers have shorter retention times than the Sp diastereoisomers (25–33), we have tentatively assigned the configuration of isomer 1 of oligomers **4** and **5** as Rp and the configuration of isomer 2 of oligomers **4** and **5** as Sp. Confirmation of these assignments awaits further physical characterization of the dimers by NMR spectroscopy (27).

Repeated attempts to resolve the diastereomers of oligomer **6** were unsuccessful. However, digestion of **6** with snake venom phosphodiesterase and bacterial alkaline phosphatase gave the expected nucleosides and two additional peaks, which corresponded to the two diastereoisomers of mr-UpC.

Oligomers with contiguous methylphosphonate linkages were also prepared. As shown in Table 2, oligomers **7** and **8** contain methylphosphonate linkages throughout with the exception of a single phosphodiester linkage at the 5′-terminal. These oligomers each contain 10 methylphosphonate linkages and would therefore be expected to consist of a mixture of 1024 diastereoisomers. Both oligomers were prepared on a 1 μmol scale. In the case of the deoxyribose oligomer, the coupling yields averaged 97% per step. These yields are somewhat lower than those encountered when preparing normal oligodeoxyribonucleotides which are usually 99% and above. This may reflect the somewhat lower reactivity of the deoxyribonucleoside methylphosphonamidite synthons *versus* the deoxyribonucleoside β-cyanoethyl-phosphoramidite synthons and their greater sensitivity to the presence of traces of water in the coupling reaction. Considerably lower coupling yields were encountered for the synthesis of oligomer **8** which contains 2′-*O*-methylribose sugars, even when the length of coupling time was extended to 5 min. Coupling yields varied between 85 and 90% per step. This apparent reduced activity may be a consequence of steric hindrance by the 2′-*O*-methoxy group.

Oligomers **7** and **8** were both purified by ion exchange chromatography on DEAE cellulose followed by reversed phase HPLC. As was the case for the deoxyribose oligomer, the 5′-hydroxyl group of oligomer **8** could be phosphorylated enzymatically by polynucleotide kinase. The resulting phosphorylated oligomer migrated as a single band on 15% polyacrylamide gel run under denaturing conditions and had a mobility slightly greater than that of the 5′-phosphorylated **7**.

Interaction of oligonucleotide analogs with complementary DNA and RNA targets

Interactions between the methylphosphonate oligomers and complementary 12-mer DNA and RNA targets were investigated by measuring the absorbance *versus* temperature profiles of 1:1 mixtures of the oligomer and the target molecule. Samples were incubated overnight at 4°C prior to melting. The rate of heating was 0.5°C/min. Essentially the same profile was obtained when the sample were cooled from 70 to 0°C at 0.5°C/min (data not shown).

As shown in Table 1, oligodeoxyribonucleotide **1** formed stable duplexes with both the DNA and RNA target. The melting temperature, Tm, of the duplex formed with the DNA target, **1/DNA**, was 4°C higher than that of the duplex formed with the RNA target, **1/RNA**, and the Tms recorded in buffer containing

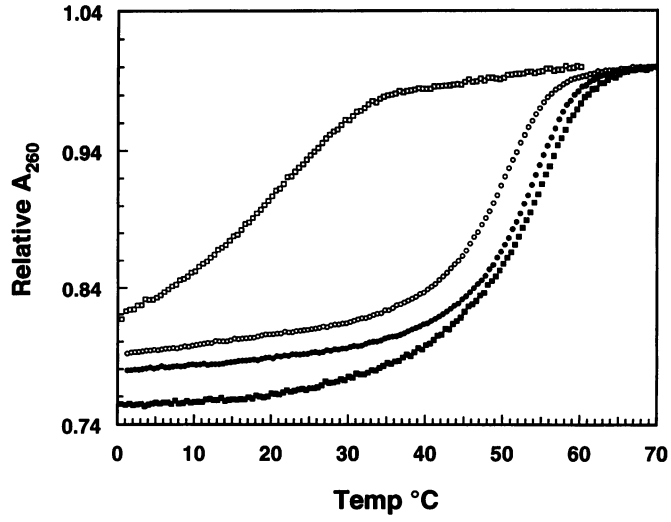

Figure 1. Absorbance vs. temperature profiles for **3/RNA** (■), **4(isomer 1)/RNA** (●), **4(isomer 2)/RNA** (○), and **8/RNA** (□) in 0.1 M sodium chloride, 10 mM HEPES, 2 mM EDTA at pH 8.0. The oligomer strand concentrations are 1.5 μM per strand for **3/RNA** and **8/RNA**, and 3 μM per strand for **4(isomer 1)/RNA** and **4(isomer 2)/RNA**.

0.1 M sodium chloride were 19°C higher than those recorded in buffer containing no sodium chloride. Opposite results were obtained with the oligo-2′-*O*-methylribonucleotide **3**. This oligomer forms a duplex with RNA, **3/RNA**, whose Tm is approximately 16°C higher than that of the DNA duplex, **3/DNA**. These results are consistent with those of Inuoe *et al.* (12) who found that a nona-2′-*O*-methylribonucleotide formed more stable duplexes with complementary RNA than did the corresponding nonadeoxyribonucleotide.

Introduction of a single methylphosphonate linkage into the backbone of oligomer **1** or **3** affected the thermal stability of duplexes formed with either the DNA or RNA target. The magnitude of the effect depended upon the configuration of the methylphosphonate linkage and its position within the oligomers. As shown in Table 1, the Rp isomer of deoxyribo oligomer **2** forms duplexes with both the DNA and RNA target whose stabilities are similar to those formed by the unmodified oligomer, **1**. Duplexes formed by the Sp isomer show a significant reduction in Tm of 5 and 6°C for the RNA and DNA targets, respectively, in buffer containing 0.1 M sodium chloride. These results are consistent with those of others who have found that the Rp isomers of oligodeoxyribonucleotides containing methylphosphonate linkages form more stable duplexes with complementary DNA targets than do the corresponding Sp isomers (28–31,34). The Tms of the duplexes formed with the Sp isomer are also lower than duplexes with the Rp isomer in buffer lacking sodium chloride, although the reduction is somewhat less relative to that observed in 0.1 M sodium chloride buffer. This behavior may result from the slight reduction in charge repulsion between the backbones of the targets and oligomer **2**, which contains 10 negative charges, *versus* that of oligomer **1** which contains 11 negative charges.

Similar results were obtained with 2′-*O*-methylribo oligomer **4**. In this case the Tms of duplexes formed by isomer 1, whose methylphosphonate linkage is tentatively assigned as Rp, were similar to those of the parent oligomer **3**, whereas the Tms of duplexes formed by isomer 2, whose methylphosphonate linkage is tentatively assigned as Sp, were reduced 4–6°C in 0.1 M sodium chloride buffer. As shown in Figure 1, the melting curves for isomer 1 and isomer 2 of duplex **4/RNA** were similar in shape and hypochromicity to that observed for duplex **3/RNA**.

The position of the methylphosphonate linkage also plays a role in determining the stability of the duplex. Thus, duplexes formed by oligomer **5** in which the methylphosphonate linkage is placed at the 5′-end of the oligomer, have stabilities almost identical to those formed by parent oligomer **3** regardless of the configuration of the methylphosphonate linkage. On the other hand, placement of the linkage at the 3′-end of the oligomer as in oligomer **6** results in destabilization of both the RNA and DNA duplexes. Unfortunately, it was not possible to separate the two diastereoisomers of this oligomer, and thus, it is unclear if the oligomer with the Sp methylphosphonate configuration is the primary contributor to the reduced Tm.

The effect of multiple methylphosphonate linkages on duplex stability was also investigated. As shown in Table 2, oligomers **7** and **8**, each of which contains 10 contiguous methylphosphonate linkages, behave in a manner distinct from that of the oligonucleotide phosphodiesters or the oligomers with single methylphosphonate linkages. Deoxyoligomer **7** forms stable duplexes with the DNA target at both low and high ionic strength. Thus, it appears that the introduction of contiguous methylphosphonate linkages of random configuration into the backbone of the oligodeoxyribonucleotide does not seriously perturb its ability to bind to the DNA target. In fact in buffer lacking sodium chloride, the Tm of this duplex is 11°C higher than that of **1/DNA**. This increased stability is attributed to the reduced charge repulsion between the methylphosphonate backbone of **7** and the diester backbone of the DNA target, *versus* the greater charge repulsion experienced by **1/DNA** which contains two fully charged sugar-phosphate backbones (34).

In contrast to this behavior and in contrast to the behavior observed with oligomer **1**, stable duplex formation between oligomer **7** and the RNA target was not detected under the conditions of the melting experiment and only the upper portion of the melting curves was observed. Although these melting experiments suggest that oligomer **7** does not form duplexes with the RNA target, experiments in our laboratory indicate that this oligomer can interact effectively with complementary RNA. Thus, we have shown that the psoralen-derivative of **7** cross-links effectively with an RNA 19-mer, r-AAUUGACAAAUC-CUAUUUU even at 20°C (35). Under the conditions of this experiment, oligomer **7** is in 50-fold excess of the target. Therefore, oligomers with backbone configurations which favor stable duplex formation, even if present as a relatively small portion of the total diastereomer population, will still be present in amounts sufficient to provide significant binding and cross-linking. In the melting experiments, however, the oligomer and the target are present in stoichiometric amounts. Under these conditions contributions from subpopulations of diastereoisomers with high affinity for the target to the overall hypochromicity of the sample will be difficult to detect. Given these considerations, it is perhaps not surprising that we did not observe stable duplex formation between oligomer **7** and the RNA target by this method. Behavior complementary to that of deoxyoligomer **7** was observed with 2′-*O*-methyloligomer **8**. This oligomer forms stable duplexes with the RNA target but does not appear to form stable duplexes with the DNA target under the conditions of the melting experiments. Duplexes formed between the RNA target and oligomer **8** had lower Tms than the corresponding duplexes formed by phosphodiester oligomer **3** under both ionic strength conditions. However, the difference in Tms between duplex **3/RNA** and duplex **8/RNA** in the absence

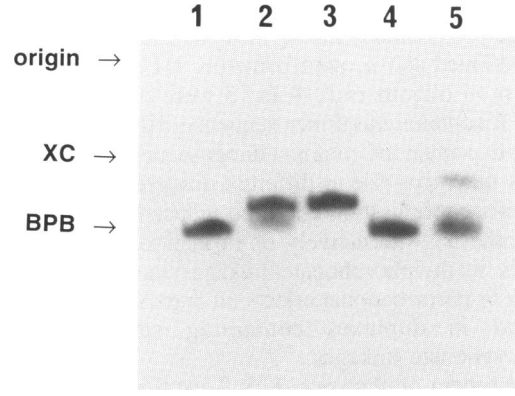

Figure 2. Polyacrylamide gel electrophoresis under non-denaturing conditions of 0.2 μM [^{32}P]-**RNA** (lane 1), 0.2 μM [^{32}P]-**RNA** and 50 μM oligomer **1** (lane 2), 0.2 μM [^{32}P]-**RNA** and 50 μM oligomer **3** (lane 3), 0.2 μM [^{32}P]-**RNA** and 50 μM oligomer **7** (lane 4), and 0.2 μM [^{32}P]-**RNA** and 50 μM oligomer **8** (lane 5). The arrows on the left side of the autoradiogram show the mobilities of xylene cyanol (XC) and bromophenol blue (BPB).

of sodium chloride, 19°C, was significantly less than that observed in the presence of 0.1 M sodium chloride, 33°C. As was the case with the oligodeoxyribonucleoside methylphosphonate **2**, this behavior is consistent with an overall reduction in charge repulsion between the methylphosphonate backbone of the oligomer and the phosphodiester backbone of the target.

There are interesting differences in the manner in which the oligodeoxyribonucleoside methylphosphonate **7** interacts with the DNA target compared with the way the oligo-2′-*O*-methylribonucleoside methylphosphonate **8** interacts with the RNA target. As shown in Figure 1, the melting transition of duplex **8/RNA** in 0.1 M sodium chloride buffer is quite broad and the duplex appears to melt over a range of almost 30°C as contrasted to the narrow range of 15°C that is observed for duplex **3/RNA**. This very broad transition most likely reflects differences in the stabilities of the various duplexes formed by different populations of the diastereoisomers of oligomer **8**. The deoxyoligomer duplex **7/DNA**, on the other hand, melts over a range of approximately 15°C which is similar to the melting transitions observed for the diester duplexes **1/DNA** (data not shown). Although oligomer **7** also consists of a mixture of diastereoisomers, it appears there is less difference in the stabilities of the duplexes formed by these diastereomers than is the case in duplexes formed between the RNA target and the diastereomers of oligomer **8**. This behavior is in contrast to that observed for oligomers with single linkages where the Sp isomer appears to destabilize duplex formation by approximately the same amount for both DNA and RNA duplexes formed by either deoxyoligomer **7** or 2′-*O*-methylribo oligomer **8**.

In addition to the differences observed between the breaths of the melting transitions of **7/DNA** and **8/RNA**, the difference between the Tm of **7/DNA** and that of the unmodified duplex **1/DNA**, $\Delta Tm = -6°C$, in 0.1 M sodium chloride buffer is much less than the corresponding difference in the Tm of **8/RNA** and that of **3/RNA**, $\Delta Tm = -33°C$. The ΔTm of **7/DNA** *versus* **1/DNA** is less than and the ΔTm of **8/RNA** *versus* **3/DNA** is greater than that predicted if one assumes, based on the behavior of oligomers containing a single methylphosphonate linkage, each methylphosphonate linkage lowers the Tm by approximately 2–2.5°C. These differences could arise if the populations of diastereomers of **7** and **8** are enriched in Rp and Sp methylphosphonate configurations, respectively. For this to occur would require that linkages of the Rp or Sp configuration be synthesized preferentially during the synthesis of oligomers **7** and **8**. As mentioned above, approximately 1:1 ratio of the Rp and Sp isomers of oligomers **2, 4** and **5** were obtained. However, oligomer **2** contains the dimer sequence d-ApT and oligomers **4** and **5** both contain the common dimer sequence mr-ApU. Other sequences may give rise to different diastereomer populations, or a specific configuration could be induced during the course of the synthesis. Alternatively or in addition, the presence of contiguous methylphosphonate linkages may exert additional stabilizing or perturbational effects on duplex formation; effects not found in duplexes containing single or isolated methylphosphonate linkages.

The interactions of oligomers **1, 3, 7** and **8** with the RNA target was also examined by polyacrylamide gel electrophoresis under non-denaturing conditions. In these experiments 0.2 μM ^{32}P-labeled RNA was mixed with 50 μM oligonucleotide, and the mixture was electrophoresed at 4°C for 90 min. The results are shown in Figure 2. Both the oligodeoxyribonucleotide **1** and the oligo-2′-*O*-methylribonucleotide **3** cause some of the labeled RNA to migrate at a reduced rate on the gel, which indicates formation of a duplex. Oligodeoxyribonucleoside methylphosphonate **7** did not give evidence of complex formation under the conditions of the experiment, whereas oligo-2′-*O*-methylribonucleo-side methylphosphonate **8** showed evidence of duplex formation. The latter result is remarkable given the conditions of the experiment. In the case of the reactions involving the RNA targets and the oligonucleotides **1** or **3**, the RNA target and the oligomer are expected to comigrate and thus the two oligomers resulting from dissociation of the duplex can requilibrate as the mixture migrates down the gel. The situation is quite different for mixtures containing the RNA target and the methylphosphonate oligomers. Because the RNA target contains 13 negative charges whereas the methylphosphonate oligomers contains only one negative charge, the duplex will rapidly migrate away from the methylphosphonate oligomer as will any RNA produced as a result of dissociation of the duplex. Thus, once the duplex dissociates, the component oligomers cannot reassociate. Thus, it appears that the duplex formed between 2′-*O*-methylribo oligomer **8** and the RNA target has a long half-life under the conditions of the experiment. The amount of **8/RNA** duplex formed under these conditions increases as the input concentration of oligomer **8** increases. Approximately 40% of the RNA is detected as duplex when the concentration of **8** is 100 μM (data not shown). This observation suggests that a subpopulation of diastereoisomers of **8** can form very stable duplexes with the RNA target.

The results of our experiments are consistent with previous observations that oligonucleotides containing 2′-*O*-methylribose sugars form more stable duplexes with complementary RNA targets than do oligonucleotides containing 2′-deoxyribose sugars. This general order of stability appears to extend to oligonucleotide analogs which contain methylphosphonate internucleotide linkages. Not surprisingly, the stability of the methylphosphonate-linked oligomer/RNA duplexes is dependent upon the configuration of the methylphosphonate linkage and it appears that the Rp methylphosphonate configuration provides duplexes of higher stability than does the Sp methylphosphonate configuration. This combined with the nuclease resistance of methylphosphonate oligomers suggests that oligo-2′-*O*-methylribonucleoside methylphosphonates with linkages of defined configuration should be effective antisense agents.

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