Syntheses of Alternating Oligo-2′-O-Methylribonucleoside Methylphosphonates and Their Interactions with HIV TAR RNA†

Tomoko Hamma and Paul S. Miller*

Department of Biochemistry and Molecular Biology, School of Hygiene and Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205

Received August 20, 1999

ABSTRACT: Oligonucleotide analogues 15−20 nucleotides in length have been prepared, whose sequences are complementary to nucleotides in the upper hairpin of HIV TAR RNA. These alternating oligonucleoside methylphosphonates, mr-AOMPs, contain 2′-O-methylribonucleosides and alternating methylphosphonate and phosphodiester internucleotide linkages. The methylphosphonate and phosphodiester linkages of these oligomers are highly resistant to hydrolysis by exonuclease activity found in mammalian serum and to endonucleases, such as S1 nuclease. The oligomers were prepared using automated phosphoramidite chemistry and terminate with a 5′-phosphate group, which provides an affinity handle for purification by strong anion exchange HPLC. A 15-mer mr-AOMP, 1676, that is complementary to the 5′-side of the TAR RNA hairpin, including the 3-base bulge and 6-base loop region, forms a 1:1 duplex with a complementary RNA 18-mer, mini-TAR RNA. The T_m of this duplex is 71 °C, which is similar to that of the duplex formed by the corresponding all phosphodiester 15-mer. Introduction of two mismatched bases reduces the T_m by 17 °C. The apparent dissociation constant, K_d, for the 1676/mini-TAR RNA duplex as determined by an electrophoretic mobility shift assay at 37 °C is 0.3 nM. Oligomer 1676 also binds tightly to the full length TAR RNA target under physiological conditions (K_d = 20 nM), whereas no binding was observed by the mismatched oligomer. A 19-mer that is complementary to the entire upper hairpin also binds to TAR RNA with a K_d that is similar to that of 1676, a result that suggests only part of the oligomer binds. When two of the methylphosphonate linkages in the region complementary to the 6-base loop are replaced with phosphodiester linkages, the K_d is reduced by approximately a factor of 10. This result suggests that interactions between TAR RNA and the oligomer occur initially with nucleotides in the 6-base loop, and that these interactions are sensitive to presence and possibly the chirality of the methylphosphonate linkages in the oligomer. The high affinities of mr-AOMPs for TAR RNA and their resistance to nuclease hydrolysis suggests their potential utility as antisense agents in cell culture.

TAR RNA, a 59-nucleotide stem-loop structure found at the 5′-untranslated end of all HIV mRNAs, regulates transcription of HIV proviral DNA. Interaction of TAR with HIV-encoded tat protein and cellular factors results in activation of RNA polymerase II-mediated transcription (*1*−*3*). Interference with tat-TAR transactivation would be expected to cripple viral mRNA synthesis and thus inhibit viral replication (*1*, *4*).

Because of the essential role it plays in viral transcription and replication, TAR RNA has become an attractive target for the design of compounds that could interfere with viral replication. A variety of compounds have been tested for their ability to bind to TAR RNA and as a consequence prevent tat-TAR interaction. These include: aminoglycoside antibiotics (*5*−*9*); a derivative of tetrahydropyrimidine (*10*); the small organic molecules 2,3-dioxo-8-[2-(5-tetrazolyl)]-2,3,4,7,8,9-hexahydro-1*H*-6-nitrocyclopental[*f*]quinoxaline and 2,4,5,6-tetraaminoquinozaline (*9*); acridine−spermidine conjugates (*11*); peptoid/peptide oligomers (*4*); tat-derived oligocarbamates (*12*), oligoureas (*13*) and D-amino acid oligopeptides (*14*); tat peptide−oligonucleotide conjugates (*15*) and antisense oligonucleotides.

Of these compounds, antisense oligonucleotides are perhaps conceptually the easiest to design. However the extensive secondary structure of TAR RNA presents a potential obstacle to obtaining antisense oligonucleotides with high binding affinities. TAR RNA consists of two stems 20- and 4-base pairs in length joined by a 3-base bulge that forms part of the tat protein binding site. The upper, 4-base pair stem is capped with a 6-base loop that is believed to interact with a complex consisting of cellular factors cyclin T (CycT) and cyclin kinase 9 (Cdk9) (*3*, *16*). High-resolution proton NMR and X-ray crystallographic studies suggest that the two stems stack upon each other and that the bases comprising the hairpin loop are available for hydrogen bonding (*17*−*20*).

Despite this extensive secondary structure, a number of studies have demonstrated that antisense oligodeoxyribonucleotides can bind to TAR RNA (*21*−*23*), albeit with low affinities. These and more recent studies have also investigated nuclease resistant oligonucleotide analogues, including oligodeoxyribonucleotide phosphorothioates, α-anomeric oligodeoxyribonucleotides, oligodeoxyribonucleotide meth-

† This research was supported by a grant from the National Institutes of Health (GM 57140).

10.1021/bi991962p CCC: $18.00 © 1999 American Chemical Society
Published on Web 10/29/1999

ylphosphonates, (N3′−P5′)-phosphoramidate-linked oligodeoxyribonucleotides, oligo-2′-O-methylribonucleotides and peptide nucleic acids (*24*). In these latter studies, a 16-mer 2′-O-methylribonucleotide and a 16-mer (N3′−P5′)-phosphoramidate-linked oligomer both showed significant binding interactions with TAR RNA with dissociation constants in the low nM range.

Previous studies from our laboratory have shown that oligo-2′-O-methylribonucleoside methylphosphonates bind with significantly higher affinity to RNA than do oligodeoxyribonucleoside methylphosphonates of the same sequence (*25*, *26*). We and others have found that oligo-2′-O-methylribonucleotides that contain alternating methylphosphonate/phosphodiester linkages, mr-AOMPs,[1] retain their high level of resistance to nuclease hydrolysis and show high affinity for complementary RNA (*27*). In this paper, we describe the syntheses of mr-AOMPs that are complementary to sequences in the upper hairpin portion of TAR RNA and their interactions with TAR RNA. The high affinity of these oligomers for TAR RNA and their resistance to nuclease hydrolysis suggests their potential as antisense agents in cell culture.

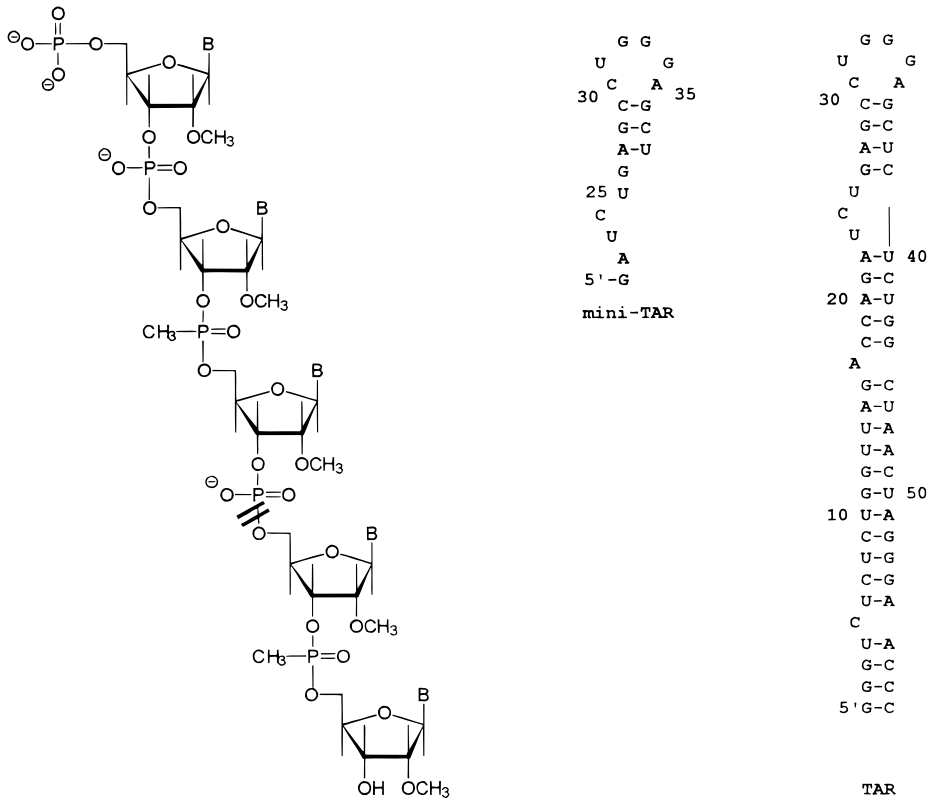
EXPERIMENTAL PROCEDURES

Materials. Protected 2′-O-methylribonucleoside derivatized controlled pore glass supports, protected 2′-O-methylribonucleoside-3′-O-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites, protected ribonucleoside-3′-O-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites, [3-(4,4′-dimethoxytrityloxy)-2,2-dicarboxyethyl]propyl-(β-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite, 4,5-dicyanoimidazole, and 5-ethylthio-1*H*-tetrazole were purchased from Glen Research, Inc., Sterling, VA. In the case of the 2′-O-methylribonucleoside phosphoramidites, the exocyclic amino groups of mr-C and mr-G were protected with acetyl and formamidine protecting groups, respectively. Protected 2′-O-methylribonucleoside-3′-O-(*N*,*N*-diisopropyl)methylphosphonamidites were obtained from Prime Synthesis Inc, Easton, PA. The exocyclic amino groups of the mr-C and mr-G methylphosphonamidites were protected with isobutryl groups. Triethylamine trihydroflouride was purchased from Aldrich Chemical Co. The phosphoramidite and phosphonamidite solutions were prepared using HPLC grade acetonitrile that was dried and stored over calcium hydride. Reversed phase HPLC was carried out using a Microsorb C-18 column (0.46 × 15 cm) purchased from Rainin Instruments Co. The column was eluted with a 20 mL of a linear gradient of acetonitrile in 50 mM sodium phosphate buffer (pH 5.8) at a flow rate of 1.0 mL/min. Strong anion exchange (SAX) HPLC was carried out using a Dynamax II column (0.46 × 25 cm) purchased from Rainin Instruments Co. The column was eluted with 18 mL of a linear gradient of ammonium sulfate in a buffer that contained 1 mM ammonium acetate (pH 6.2) in 20% acetonitrile at a flow rate of 0.6 mL/min. For analytical runs, the columns were monitored at 260 nm and for preparative runs at 290 nm. Unless otherwise noted, polyacrylamide gel electrophoresis was carried out on 20 × 20 × 0.75 cm gels containing 20% acrylamide and 7 M urea. The running buffer was TBE, which contained 89 mM Tris, 89 mM boric acid, and 0.2 mM ethylenediaminetetraacetate buffered at pH 8. The gel loading buffer contained 90% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue.

Syntheses of Anti-TAR Oligonucleotides. Alternating oligo-2′-O-methylribonucleoside methylphosphonates (mr-AOMPs) and oligo-2′-O-methylribonucleotides (mr-ONs) were synthesized on a Applied Biosystems Model 392 DNA/RNA synthesizer. The sequences of the these oligomers are shown in Figure 1. The protected nucleoside methylphosphonamidites and nucleoside β-cyanoethylphosphoramidites were dissolved in anhydrous acetonitrile at a concentration of 0.1 M. In the case of oligomers 1800 and 1801, the concentrations of the G methylphosphonamidites were 0.12 M. The nucleoside methylphosphonamidite solutions were stored over 4 Å molecular sieves 2 h prior to use. The synthesizer was programmed to carry out two capping steps, one prior to the oxidation step and one immediately following the oxidation step. The oxidizing agent consisted of a solution containing 1.27 g of iodine, 37.5 mL of tetrahydrofuran, 12.5 mL of 2,6-lutidine, and 100 μL of water. The capping solutions consisted of Cap A (10% acetic anhydride, 10% pyridine in tetrahydrofuran (v/v)) and Cap B (1.25 g of (dimethylamino)pyridine in 50 mL of anhydrous pyridine). Coupling reactions were carried out for 90 or 120 s, using a 0.25 M solution of 4,5-dicyanoimidazole as the activating agent. For oligomers terminating with a 5′-phosphate, the final coupling step was carried out using a 0.1 M solution of [3-(4,4′-dimethoxytrityloxy)-2,2-dicarboxyethyl]propyl-(β-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite in acetonitrile. The coupling time for this step was 360 s; both capping steps were eliminated and the synthesizer was programmed to leave the dimethoxytrityl (DMT) group on the oligomer. In the cases of mr-ONs 1707 and 1757, the synthesizer removed the DMT group.

The mr-AOMPs were deprotected using the following procedure. The controlled pore glass (CPG) was treated in the synthesis column with 0.5 mL of anhydrous triethylamine for 32 h at room temperature. The solution was flushed from the column, the CPG was washed with two, 10 mL aliquots of acetonitrile, and the support was dried under vacuum. The CPG was then treated with two, 1 mL aliquots of 2% dichloroacetic acid in methylene chloride for 30 s each. The DCA solution was flushed from the support, the support was washed with 10 mL of acetonitrile and dried under vacuum. The dry CPG was transferred to a 4.5 mL autosampler vial and treated with 400 μL of concentrated ammonium hydroxide for 2.5 h at room temperature. The supernatant was removed by a pipet and the CPG was washed with four, 200 μL aliquots of 50% aqueous acetonitrile. The combined supernatant and washings were evaporated to dryness under vacuum at room temperature. The residue was then treated with a solution that contained 5 μL of water, 22.5 μL of acetonitrile, 22.5 μL of 95% ethanol, and 50 μL of ethylenediamine for 6 h at room temperature. The solution was cooled in an ice−water bath and neutralized by addition of 600 μL of ice cold 2 N hydrochloric acid. After neutralization, the solution was diluted into 10 mL of 2% acetonitrile in 50 mM sodium phosphate buffer at pH 5.8 (C-18 buffer A). This solution was loaded onto a C-18 SEP PAK cartridge

[1] Abbreviations: DCA, dichloroacetic acid; DMT, dimethoxytrityl; EDA, ethylenediamine; mr-ON, oligo-2′-O-methylribonucleotide; mr-AOMP, alternating oligo-2′-O-methylribonucleoside methylphosphonate; mr-OMP, oligo-2′-O-methylribonucleoside methylphosphonate; TEA, triethylamine.



1676 mr-pCpU pCpCpCpApGpGpCpUpCpApGpApU
1677 mr-pCpU pCpCpCpGpGpApCpUpCpApGpApU

1707 mr-pCpUpCpCpCpApGpGpCpUpCpApGpApU
1757 mr-pCpUpCpCpCp GpGpApCpUpCpApGpApU

1800 mr-pApGpApGpCpUpCpCpCpApGpGpCpUpCpApGpApC
1801 mr-pApGpApGpCpUpCpCpCpApGpGpCpUpCpApGpApUpC

1770 mr- ApGpApGpCpUpCpCpCpApGpGpCpUpCpApGpApUpC
1798 mr- ApGpApGpCpUpCpCpCp GpGpApCpUpCpApGpApUpC

FIGURE 1: General structure of an alternating oligo-2′-O-methylribonucleoside methylphosphonate. The sequences of the mr-AOMPs, mr-ONs, and TAR RNA targets are also shown.

(Waters Inc.) that had been previously equilibrated with 10 mL of acetonitrile, 10 mL of 50% aqueous acetonitrile, and 10 mL of C-18 buffer A. The SEP PAK was washed with 10 mL of water, and the oligomer was eluted with 3 mL of 50% aqueous acetonitrile.

The mr-ONs were deprotected by treating the CPG with 1 mL of concentrated ammonium hydroxide for 6 h at 60 °C. After cooling, the supernatant was removed and the support was washed with three, 500 μL aliquots of 50% aqueous acetonitrile. The combined supernatant and washings were evaporated to dryness.

The mr-AOMPs and mr-ONs 1707, 1757, and 1798 were purified by SAX HPLC. The crude oligomers, 10−20 A_{260} units, were dissolved in 100 μL of SAX buffer A that contained 1 mM ammonium acetate, pH 6.2, in 20% acetonitrile. The samples were injected onto the SAX column and the column was eluted with a linear gradient of 0−0.125 M or 0−0.2 M ammonium sulfate for mr-AOMPs 1676, 1677, and 1800, 1801, respectively and 0−0.8 M ammonium sulfate for the mr-ONs. Oligomer 1770 was purified by polyacrylamide gel electrophoresis on a 20% gel run under denaturing conditions. The oligomer was located by UV shadowing and was isolated by incubating the gel slice with a solution that contained 1 mM ammonium acetate, pH 6.2, in 20% acetonitrile for 3 h at 65 °C and overnight at 37 °C. All the oligomers were desalted on C-18 SEP PAK cartridges as described above.

The purity of the oligomers was assessed by SAX and C-18 HPLC. The mr-ONs were digested by incubating 0.1 A_{260} unit of the oligomer with a combination of snake venom phosphodiesterase (2 μg) and bacterial alkaline phosphatase (1 unit) in 20 μL of buffer containing 10 mM Tris, pH 8.1, and 2 mM magnesium chloride overnight at 37 °C. The digests were analyzed by C-18 reversed phase HPLC and the products compared to authentic samples. Each mr-ON was completely digested to its component 2′-O-methylribonucleosides, and these nucleosides appeared in the expected ratios. The molecular weights of the mr-AOMPs were verified by MALDI-TOF mass spectrometry. The extinction coefficients of the mr-AOMPs were determined by comparing the absorbencies of a given amount of oligomer before and after sequential digestion with 1 M aqueous piperidine

for 1 h at 37 °C followed by treatment with a combination of 2 μg of snake venom phosphodiesterase and 2.5 μg of spleen phosphodiesterase in 1 mM ammonium acetate, pH 6.2, at 37 °C for 18 h. In the case of the mr-ONs, the oligomers were digested with snake venom phosphodiesterase alone, as previously described (*28*). The ϵ_{260} values were: 1676, 1.3×10^5; 1677, 1.2×10^5; 1707, 9.9×10^4; 1757, 9.9×10^4; 1800, 1.7×10^5; 1801, 1.8×10^5; 1770, 1.6×10^5; 1798, 1.4×10^5.

Synthesis of Mini-TAR RNA. Mini-TAR RNA was synthesized on controlled pore glass derivatized with 1 μmol of protected uridine. The protected nucleoside phosphoramidites were dissolved in anhydrous acetonitrile to a concentration of 0.15 M. Coupling times were 310 s, and 0.65 M ethylthiotetrazole in anhydrous acetonitrile was used as the activating agent. The synthesizer was programmed to carry out a capping step prior to and following the oxidation step. The capping solutions were Cap A (10% acetic anhydride, 10% pyridine in tetrahydrofuran (v/v)) and Cap B (10% methylimidazole in tetrahydrofuran). The oxidizer was 0.1 M iodine in a solution of tetrahydrofuran/pyridine/water. The synthesizer was programmed to remove the 5′-terminal DMT group.

The oligomer was deprotected by treating the CPG with a solution that contained 0.25 mL of 95% ethanol and 0.75 mL concentrated ammonium hydroxide for 4 h at 55 °C. The solution was removed from the support by a pipet, and the support was washed with two, 1 mL portions of 50% aqueous acetonitrile. The combined supernatant and washings were evaporated to dryness and the residue, which totaled 70 A_{260} units, was dissolved in 50% aqueous acetonitrile. An aliquot containing 15 A_{260} units of the oligomer was evaporated, and the residue was treated with 150 μL of triethylamine trihydroflouride at room temperature for 46 h. The solution was quenched by addition of 150 μL of water, and the solution was diluted into 10 mL of C-18 buffer A and loaded onto a SEP PAK preequilibrated as described in the previous section. The SEP PAK was washed with 10 mL of water and the oligomer was eluted with 3 mL of 50% aqueous acetonitrile. The resulting 12.5 A_{260} units of crude oligomer were purified by electrophoresis on a 20% polyacrylamide gel run under denaturing conditions. The oligomer was located by UV shadowing, the gel band was excised, and the gel slice extracted twice by incubation with 1 mL portions of 10% aqueous acetonitrile at 37 °C for 24 h each. The combined extracts contained 3.3 A_{260} units of oligomer that was then desalted on a C-18 SEP PAK cartridge. A portion of the oligomer (0.025 A_{260} units) was phosphorylated using polynucleotide kinase and γ-[^{32}P]-ATP (specific activity, 50 Ci/mmol). Unreacted ATP and buffer were removed using a MicroSpin G-25 Sephadex column. The phosphorylated oligomer was incubated with RNase T_1 or RNase U_2 and digests were electrophoresed on a 20% polyacrylamide gel run under denaturing conditions. The product bands observed were consistent with the sequence of the oligomer.

Synthesis of TAR RNA. TAR RNA was prepared by in vitro transcription of a plasmid carrying a single copy of TAR DNA. The TAR DNA duplex was prepared from two chemically synthesized oligonucleotides. The duplex was inserted into the Eco RI and Hind III sites of the polylinker of pGEM3Z (Promega, Inc.) such that transcription was under control of the SP6 promoter in the plasmid. The sequence and orientation of the insert were verified by sequencing. The plasmid (5 μg) was digested with 36 units of Eco RI in 50 μL of buffer. After purification on a Wizard (Promega Inc) DNA cleanup cartridge, 50 ng of the linearized DNA was transcribed using 6 units of SP6 RNA polymerase in 10 μL of buffer containing 40 mM Tris, pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 10 mM sodium chloride, 100 mM dithiothreitol, and 2.5 mM each of the four nucleoside triphosphates. The reaction mixture was incubated for 60 min at 37 °C and then heated at 65 °C for 2 min. This reaction produced a 73 nucleotide run off transcript, 5′-GAAUACUCAAGCU*GGGUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCUCUGGCUAACUAGGGAACCC*G, that contained the 59-nucleotide TAR RNA sequence in italics. The reaction mixture was then supplemented with 2 μL of water, 1.5 μL of 10× buffer that contained 200 mM Tris, pH 8.0, and 100 mM magnesium chloride, and 1 unit of arctic shrimp alkaline phosphatase and was incubated for 60 min at 37 °C to remove the 5′-terminal triphosphate group from the transcript. The reaction mixture was heated at 65 °C for 15 min to denature the enzyme. The solution was then supplemented with: 0.8 μL of water; 2.2 μL of 10× buffer that contained 700 mM Tris, pH 7.6, 100 mM magnesium chloride, 50 mM dithiothreitol; 1 μL of 100 μM ATP and γ-[^{32}P]-ATP (30 μCi); 1 μL (10 units) of polynucleotide kinase; incubated for 60 min at 37 °C. The 5′-end labeled transcript was purified by electrophoresis on a 8% polyacrylamide gel run under denaturing conditions. The transcript was extracted from the gel by incubating the gel slice with 1 mM ammonium acetate in 20% acetonitrile for 3 h at 65 °C. The transcript was then precipitated with ethanol and stored in 50% aqueous ethanol at −20 °C. The sequence of the transcript was confirmed by digestion with T1, U2, and phyM ribonucleases and analysis of the digests by polyacrylamide gel electrophoresis.

Melting Experiments. Duplexes were formed by mixing 0.5 mL of a 1 μM solution of 1676, 1677, 1707, or 1757 in a buffer containing 100 mM sodium chloride, 10 mM Tris, pH 7.5, and 10 mM ethylenediaminetetraacetate with 0.5 mL of 1 μM mini-TAR RNA in the same buffer at room temperature. The solutions were stored at 4 °C overnight. Melting experiments were carried out using a Cary 3E UV−vis spectrophotometer equipped with a thermostated sample holder and temperature controller as previously described (*25*). The duplexes were heated from 0 to 90 °C at a rate of 0.5 °C/min and the absorbance at 260 nm was recorded as a function of temperature. Experiments in which the duplex solutions were cooled from 90 to 0 °C gave curves identical to those seen in the melting experiments.

Gel Mobility Shift Experiments. Gel mobility shift experiments were carried out using a procedure adapted from Vickers et al. (*21*). Stock solutions of the oligonucleotides ranging in concentrations from 0.2 nM to100 μM were prepared in a buffer containing 100 mM sodium chloride, 10 mM Tris, pH 7.5, 0.5 mM ethylenediaminetetraacetate. Solutions containing 0.2 nM 5′-end [^{32}P]-labeled mini-TAR RNA (specific activity, 1000 Ci/mmol) or 0.2 nM 5′-[^{32}P]-labeled TAR RNA (specific activity, 1000 Ci/mmol) were prepared in the same buffer. Equal volumes, 5 μL, of oligomer and target were mixed and the solution incubated at 37 °C for 45 min (mini-TAR RNA) or 24 h (TAR RNA).

A 1 μL aliquot of 50% glycerol was added, and the solution was loaded onto a polyacrylamide gel. In the case of the mini-TAR RNA, the complex was separated from free target by electrophoresis on a 20% nondenaturing gel run in TBE buffer at 500 V for 1 h at 37 °C. For the TAR RNA target, electrophoresis was carried out on a 8% nondenaturing gel run in TBE buffer at 200 V for 2 h at 37 °C. The wet gel was then analyzed and quantitated by phosphorimaging. The apparent dissociation constants were determined as the half-maximal point on a plot of percent complex formation vs log of the oligonucleotide concentration. The average apparent dissociation constant for each oligomer was determined from at least three experiments.

Oligonucleotide Stability in Serum. Oligomers 1676 and 1707 were each dephosphorylated by incubating 0.028 A_{260} unit with 1 unit of arctic shrimp alkaline phosphatase in 10 μL of buffer containing 20 mM Tris, pH 8.0, 10 mM magnesium chloride for 60 min at 37 °C. The solution was heated at 65 °C for 15 min to deactivate the enzyme. The oligomers were then phosphorylated by incubation with 20 units of polynucleotide kinase in 15 μL of buffer that contained 6.6 μM γ-^{32}P-ATP, (specific activity, 100 Ci/mmol), 50 mM Tris, pH 7.6, 10 mM magnesium chloride, and 10 mM mercaptoethanol for 60 min at 37 °C. A 14 μL aliquot of the reaction mixture was diluted with 140 μL of 0.1 M imidazole buffer, pH 6.0 and 16 μL of 1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The solution was incubated at room temperature for 4 h after which 6.4 μL of ethylenediamine and 13 μL of 12 N hydrochloric acid were added. The pH of the solution was adjusted to 7.4 by further addition of hydrochloric acid and the solution was incubated overnight at room temperature. The reaction mixture was diluted into 10 mL of C-18 buffer A, and the solution was loaded onto a preequilibrated C-18 SEP PAK cartridge. The SEP PAK was washed with 10 mL of C-18 buffer A, 10 mL of 5% aqueous acetonitrile and 10 mL of water. The oligomer was eluted with 2 mL of 50% aqueous acetonitrile. The 5′-aminoethyl derivatized oligomers were purified by electrophoresis on a 20% polyacrylamide gel run under denaturing conditions. The oligomers were extracted from the gel by incubation of the gel slice with 1 mL of 20% aqueous acetonitrile for 1 h at 65 °C and overnight at 37 °C. The oligomers were then desalted on C-18 SEP PAK cartridges.

The derivatized oligomers (1.1×10^5 cpm) were each dissolved in 10 μL of RPMI medium supplemented with 10% fetal bovine serum. The solutions were incubated at 37 °C. One μL aliquots were removed at various times, diluted into 4 μL of gel loading buffer and analyzed by polyacrylamide gel electrophoresis on a 20% denaturing gel. Products were visualized and quantitated by phosphorimaging the wet gel.

RESULTS AND DISCUSSION

Oligonucleotides and Their Targets. The general structure of the alternating 2′-O-methylribonucleoside methylphosphonates, mr-AOMPs, is shown in Figure 1. These oligomers contain 2′-O-methylribonucleosides joined by a combination of nuclease resistant methylphosphonate linkages, p, and normal phosphodiester linkages, p. The sequences of these oligomers and the corresponding 2′-O-methylribonucleotides, mr-ONs, are shown in Figure 1. Oligomers 1676 and 1707, 15-mers, are complementary to nucleotides 22−36 of HIV TAR RNA, a region that includes the binding sites for tat peptide and the CycT/Cdk9 complex (*3*). Oligomers 1677 and 1757, also 15-mers, are partially complementary to this region and provide two potential mismatches, a G−U mismatch with U 31 and a A−C mismatch with C 29 in the six base loop of TAR RNA. Oligomers 1800 and 1801 are a 19-mer and 20-mer respectively whose sequences are complementary to TAR RNA nucleotides 21−42. Oligomer 1800 is a mr-AOMP, whereas 1801 is a chimeric oligomer that contains alternating methylphosphonate/phosphodiester linkages, except in the region of the oligomer that is complementary to the 6-base loop. Here, the linkages are phosphodiesters. Oligomer 1770, a 20-mer, contains only phosphodiester linkages and is completely complementary to TAR RNA, whereas phosphodiester 20-mer 1798 contains potential G−U31 and A−G32 mismatches with TAR. In addition to the TAR RNA target, a shorter version of this target, mini-TAR RNA, whose sequence corresponds nucleotides 21−38 of TAR RNA, was also prepared.

Syntheses of mr-AOMPs. The mr-AOMPs were prepared on an automated DNA synthesizer using protected 2′-O-methylribonucleoside phosphoramidites and protected 2′-O-methylribonucleoside methylphosphonamidites. The exocyclic aminos of the mr-C and mr-G phosphoramidites were protected with acetyl and formamidine protecting groups, respectively, whereas the exocyclic aminos of the mr-C and mr-G methylphosphonamidites were protected with isobutryl groups. This protection scheme was chosen because these groups can be removed under mild basic conditions as described below.

Coupling reactions involving the methylphosphonamidites are very sensitive to moisture. Consequently, care was required to exclude moisture when preparing acetonitrile solutions of these reagents, particularly when the relative humidity exceeded 40%. Acetonitrile solutions of the 2′-O-methylribonucleoside methylphosphonamidites were stored over molecular sieves to remove traces of water that are apparently present in the solid methylphosphonamidites. We also employed a "low-water oxidizer" that contains 0.2% water was used in place of the standard iodine oxidizer that contains 2% water. Finally, an additional capping step was employed after the oxidation step in an effort to further dry the support before the next coupling cycle.

Even when these precautions were taken, when tetrazole was used as the activating agent, the average coupling yields seldom exceeded 92% even at methylphosphonamidite concentrations of 0.15 M. A significant improvement in the coupling was obtained, however, when 4,5-dicyanoethylimidazole (*29*) was used as the activator. In this case, average coupling yields were 97% even when reactions were carried out using 0.1 M solutions of the methylphosphonamidites.

To simplify oligomer purification, the mr-AOMPs were synthesized with a 5′-terminal phosphate group. This was achieved by using [3-(4,4′-dimethoxytrityloxy)-2,2-dicarboxy-ethyl]propyl-(β-cyanoethyl)−(*N*,*N*-diisopropyl)phosphoramidite (*30*) in the final coupling step. The dimethoxytrityl group was retained at the end of the synthesis to give support-bound oligomers of the type shown in Figure 2.

The presence of the masked 5′-phosphate group required modification of the "one pot" deprotection scheme previously used for oligodeoxyribonucleoside- and oligo-2′-O-methy-

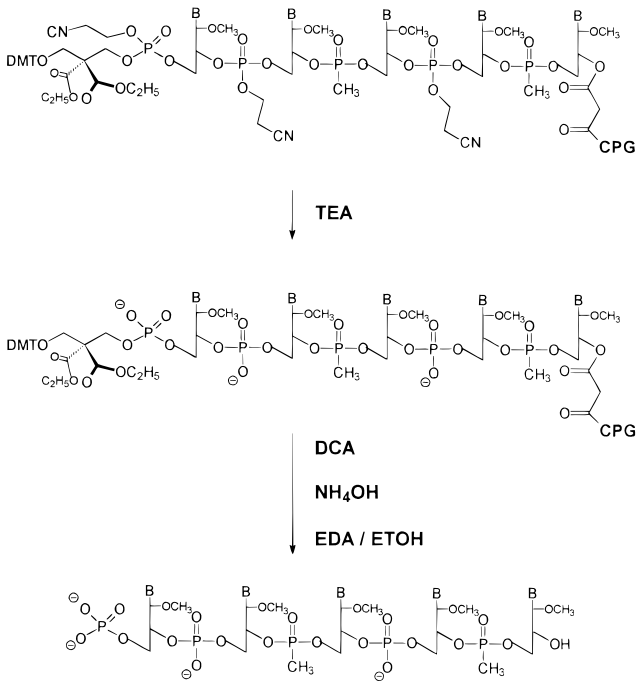


FIGURE 2: Deprotection of support-bound mr-AOMP.

ribonucleoside methylphosphonates (*25*, *31*). Thus, it was necessary to first remove the β-cyanoethyl phosphate protecting groups in order to prevent subsequent generation of a 5′-terminal β-cyanoethyl phosphate group. Although 5′-β-cyanoethyl phosphate can be converted to the 5′-phosphate, the strong basic conditions required to affect this transformation lead to cleavage of the methylphosphonate linkages. Removal of the β-cyanoethyl groups was accomplished by treating the support with anhydrous triethylamine. This treatment did not cleave the succinate ester linkage that attaches the oligomer to the controlled pore glass or result in cleavage of the methylphosphonate linkages.

After removal of the dimethoxytrityl group with 2% dichloroacetic acid, the oligomer was cleaved from the support by reaction with concentrated ammonium hydroxide for 2.5 h at room temperature. Control experiments showed that the methylphosphonate linkages were completely stable to these relatively mild basic conditions. The ammonium hydroxide treatment also hydrolyzed the carboxyethyl groups, resulting in subsequent cleavage of the 5′-phosphate protecting group and of the mr-C−N4-acetyl and mr-G−N2-isobutryl and formamidine protecting groups. The remaining N6-benzoyl protecting groups on mr-A were removed by treatment with ethylenediamine.

The resulting 5′-phosphorylated oligomers were purified by strong anion exchange HPLC. The two extra negative charges of the 5′-phosphate group, which is attached only to the full length oligomer, increases the retention time of the oligomer relative to the nonphosphorylated oligomer by approximately 5 min. Thus, the 5′-phosphate group in essence serves as an "affinity handle" on the SAX column, in much the same manner as the dimethoxytrityl group can be used to affinity purify oligonucleotides on reversed phase columns.

The mr-AOMPs prepared in this manner were shown to be pure as judged by SAX and C-18 reversed phase HPLC. The 5′-terminal phosphate groups were removed by treatment with artic shrimp alkaline phosphatase, and after heat

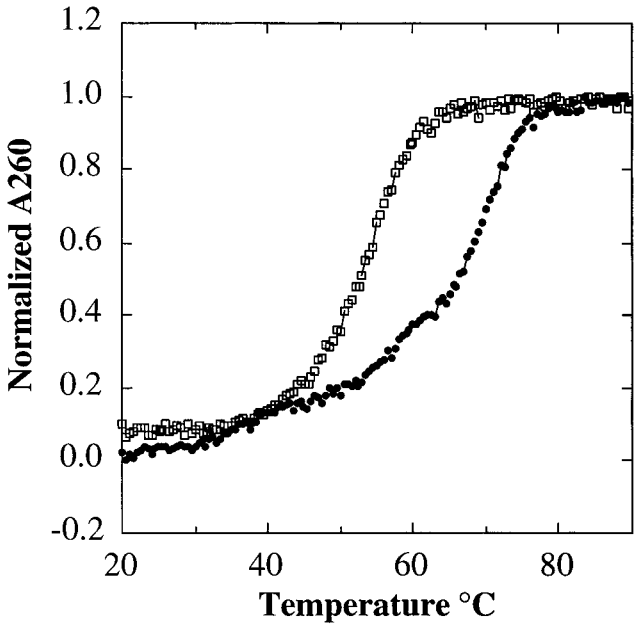


FIGURE 3: Absorbance vs temperature profiles for duplexes formed between mr-AOMP 1676 (●) or 1677 (□) and mini-TAR RNA. The melting experiments were carried out in buffer that contained 100 mM sodium chloride, 10 mM Tris, pH 7.5, 0.5 mM ethylenediaminetetraacetate at a total strand concentration of 2 μM. The solutions were heated at 0.5 °C/min.

denaturation of the phosphatase, the resulting dephosphorylated oligomers were 5′-end labeled by polynucleotide kinase and γ-[^{32}P]-ATP. The end-labeled oligomers migrated as single bands on polyacrylamide gels. In addition, mass spectral analysis gave molecular weights consistent with the structure of the oligomer and showed that the protecting groups had been completely removed.

Interaction of mr-AOMPs with Mini-TAR RNA. TAR RNA contains extensive secondary structure that could potentially interfere with oligonucleotide binding. To evaluate the ability of mr-AOMPs to bind to their complementary single-stranded sequence, mini-TAR RNA, whose sequence is shown in Figure 1, was synthesized. This 18-mer comprises the loop and bulge sequences of TAR RNA. Although the oligomer could potentially form a 3 base-pair helix at its 3′-end, UV thermal denaturation experiments provided no evidence for such formation (data not shown). Thus, it is likely that mini-TAR RNA exists primarily in a random coil conformation.

Thermal denaturation experiments were first carried out on 1:1 mixtures of mini-TAR RNA and the mr-AOMP 1676 or 1677. Typical absorbance vs temperature profiles obtained at pH 7.5 in buffer that contained 0.1 M sodium chloride are shown in Figure 3. The melting profiles are consistent with duplex formation between the oligomers and the RNA target. The duplex formed by fully complementary 1676 melts at 71 °C. Introduction of two base mismatches reduces the melting temperature of the duplex formed by 1677 by 17 °C.

As shown in Table 1, the T_m of the 1676/RNA duplex is similar to that of the duplex formed by phosphodiester 15-mer 1707. These results suggest that introduction of seven alternating methylphosphonate linkages into the oligomer backbone does not significantly perturb the ability of the oligomer to bind to RNA. Similar results were observed by Reynolds et al. (*27*) in their studies on the binding of a

Table 1: Melting Temperatures of Duplexes Formed Between Anti-TAR Oligonucleotides and Mini-TAR RNA

oligomer	T_m °C[a]
1676	71
1677	54
1707	78
1757	65

[a] Melting temperatures were measured in a buffer containing 100 mM sodium chloride, 10 mM Tris pH 7.5, 0.5 mM ethylenediaminetetraacetate at a total strand concentration of 2 μM.

pyrimidine-rich mr-AOMP, mr-(C*p*Up)$_7$A, to a complementary 15-mer RNA target. In this case, the all diester version of this oligomer formed a duplex whose T_m was 60.8 °C, whereas the mr-AOMP/RNA duplex melted at 59.0 °C.

In our experiments and those of Reynolds et al., each mr-AOMP methylphosphonate linkage is racemic, resulting in a mixture that contains 128 diastereoisomers. Previous studies have shown that 2′-O-methylribo-oligomers with a single Rp methylphosphonate linkage bind with a T_m approximately 2 °C higher than corresponding oligomer with a single Sp linkage (*25*). Thus, one would predict that the all Rp version of 1676 should have a higher T_m than the racemic version of this oligomer. An increase in stability was in fact observed for the Rp version of mr-(C*p*Up)$_7$A that formed a duplex whose T_m was 70.3 °C (*27*). Chiral alternating methylphosphonate oligomers can be synthesized from protected Rp or Sp methylphosphonate dimers (*27*, *32*). In the case of oligomers that contain repeating dimer sequences such as mr-(C*p*Up)$_7$A, synthesis requires preparation of only a single protected Rp dimer phosphoramidite. An oligomer such as 1676 on the other hand would require preparation of five different chiral dimer intermediates, which represents a considerable increase in synthetic effort.

Duplexes formed between oligo-2′-O-methylribonucleotides RNA are significantly more stable than those formed by oligodeoxyribonucleotides (*25*, *33*−*35*). Although the reasons for this increased stability are not completely understood, it may arise in part from the propensity of the 2′-O-methylribo-oligomer to preorganize in a conformation favorable for forming A-type RNA duplexes (*35*−*37*). In addition, the 2′-O-methyl groups may participate in stability enhancing intraresidue stacking interactions in the minor groove of the duplex (*35*). Previous studies on oligo-2′-O-methylribonucleoside methylphosphonates (mr-OMPs) showed that these oligomers, which contain contiguous methylphosphonate linkages, do indeed form more stable duplexes with RNA than do the corresponding oligodeoxyribonucleoside methylphosphonates (*25*). However, the difference in stability between the mr-OMP/RNA duplexes and the corresponding mr-ON/RNA duplex was considerably greater than the corresponding difference observed with the mr-AOMP/RNA duplexes seen in the present study. This effect could result if the adjacent methylphosphonate linkages perturb the ability of the mr-OMP to adopt an oligomer conformation favorable to RNA binding as a consequence of steric interactions or changes in oligomer solvation resulting from the presence of the nonionic methylphosphonate linkages.

Interaction of 1676 with mini-TAR RNA was also studied by electrophoretic mobility shift assays. Mini-TAR RNA labeled with a 5′-terminal-[^{32}P]-phosphate was incubated with increasing concentrations of oligomer at 37 °C in a buffer containing 0.1 M sodium chloride. Duplex was separated from target RNA by electrophoresis on a nondenaturing gel run at 37 °C and the amount of duplex formed was quantitated by phosphorimaging. A typical binding isotherm is shown in Figure 4. The apparent dissociation constant, K_d, was taken as the inflection point on the binding isotherm, which represents half-maximal binding under the conditions of the experiment.

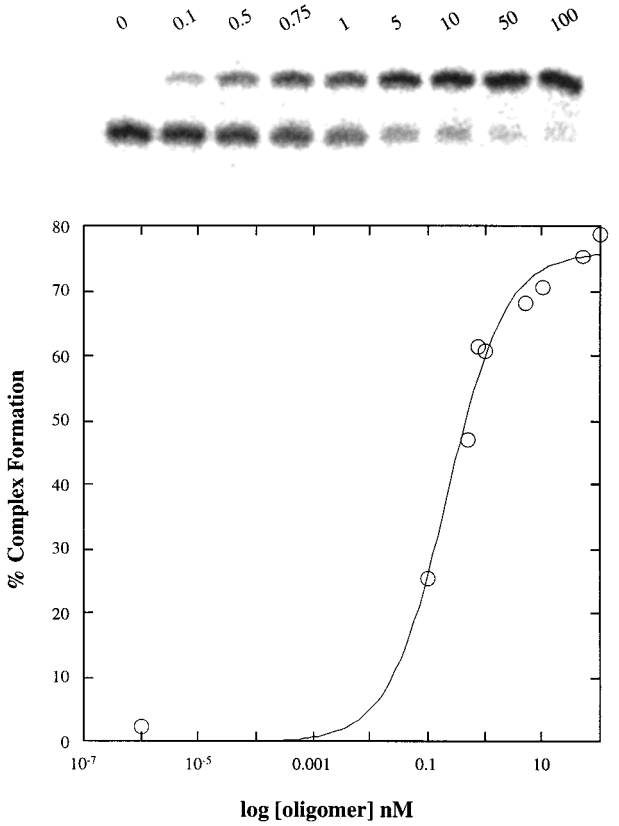


FIGURE 4: Electrophoretic mobility shift assay of the interaction between mr-AOMP 1676 and mini-TAR RNA at 37 °C in buffer containing 100 mM sodium chloride, 10 mM Tris, pH 7.5, 0.5 mM ethylenediaminetetraacetate. A phosphorimage of the gel is shown in at the top and the binding isotherm derived from quantitation of the phosphorimage is shown at the bottom.

Table 2: Apparent Dissociation Constants of Duplexes Formed Between Anti-TAR Oligonucleotides and Mini-TAR RNA or TAR RNA

		K_d (nM) [(a)]	
	oligomer	mini-TAR	TAR
15-mers	1676	0.33	20
	1677	308	>50 000
	1707	0.40	2.4
	1757	1.9	>50 000
20-mers	1800	n.d.	14.8
	1801	n.d.	2.0
	1770	0.76	4.7
	1798	n.d.	31

[a] Dissociation constants were determined at 37 °C in a buffer containing 100 mM sodium chloride, 10 mM Tris pH 7.5, 0.5 mM ethylenediaminetetraacetate.

Table 2 shows the apparent K_d's for the mr-AOMPs and the corresponding phosphodiester oligomers. Both the complementary alternating methylphosphonate, 1676, and the phosphodiester oligomer, 1707, formed duplexes whose apparent dissociation constants were less than 1 nM at 37 °C. In the

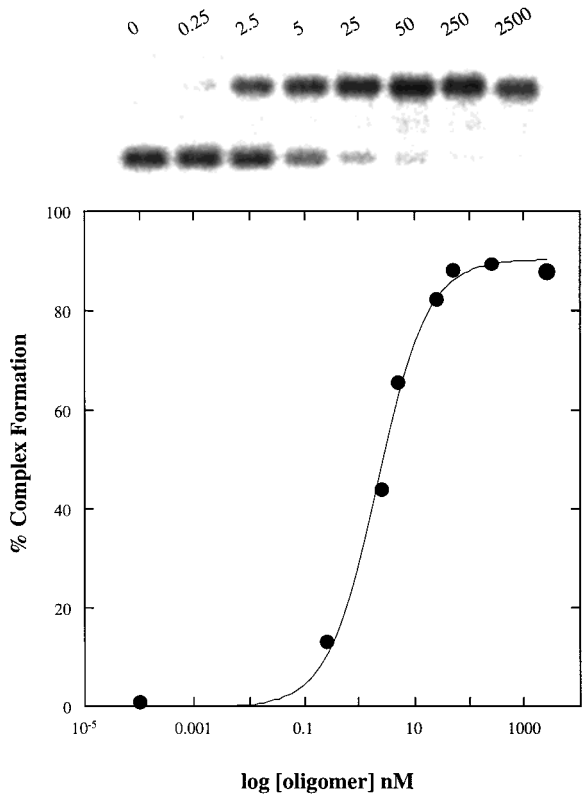


FIGURE 5: Electrophoretic mobility shift assay of the interaction between mr-AOMP 1801 and TAR RNA at 37 °C in buffer containing 100 mM sodium chloride, 10 mM Tris, pH 7.5, 0.5 mM ethylenediaminetetraacetate. A phosphorimage of the gel is shown in at the top and the binding isotherm derived from quantitation of the phosphorimage is shown at the bottom.

case of mr-AOMP 1677, introduction of two mismatched bases, increased the dissociation constant by approximately 3 orders of magnitude. In contrast, the presence of two mismatches in the phosphodiester oligomer 1757 increased the dissociation constant only by a factor of 5. This result appears to be consistent with the approximately 11 °C higher T_m of the duplex formed by 1757 compared to that formed by 1677. The presence of nonionic methylphosphonate linkages in the mr-AOMPs should result in less charge repulsion between the backbones of the oligomer and its target compared to the same duplex formed by mr-ONs. Despite this reduced charge repulsion, the results suggest that the fidelity of binding is maintained by the mr-AOMPs.

Interaction of mr-AOMPs with TAR RNA. Gel mobility shift analysis was used to study the interactions of mr-AOMPs with TAR RNA. As in the case with mini-TAR RNA, these experiments were carried out at 37 °C. A typical binding isotherm is shown in Figure 5 and the apparent dissociation constants are shown in Table 2. Methylphosphonate oligomer 1676 bound with a dissociation constant of approximately 20 nM that is approximately 10 times higher than that observed for the corresponding phosphodiester oligomer 1707. These results are similar to those recently published by Boulmé et al. (*24*), who found that a 16-mer 2′-O-methylribonucleotide whose sequence is similar to that of mr-ON 1707, bound to TAR RNA with a K_d of approximately 20 nM at 22 °C.

Increasing the length of the antisense oligomer did not alter the dissociation constants significantly. Thus, the K_d for the mr-AOMP 19-mer 1800, 14.8 nM, was similar to that of 15-mer 1676, and likewise the K_d for phosphodiester 20-mer 1770, 4.7 nM, was similar to that of 15-mer 1707. The longer oligomers are complementary to both sides of the upper stem as well as the 6-base loop and 3-base bulge of TAR RNA. These results are consistent with those of Ecker et al. (*38*) who found that antisense oligonucleotides that are complementary to either the 5′- or 3′-side of the TAR RNA hairpin bind through the formation of pseudo-half-knot structures in which the oligomer/RNA duplex stacks on the lower stem of TAR RNA. Because disruption of the lower stem would be energetically unfavorable, longer antisense oligomers tend to bind in a partial manner and thus maintain this pseudo-half-knot structure.

Previous studies have shown that 26- and 28-mer oligodeoxyribonucleotides and oligodeoxyribonucleotide phosphorothioates bind to TAR RNA at 22 °C with apparent dissociation constants in the range 300−1500 nM (*21*). Essentially no complex formation was observed between TAR RNA and oligodeoxyribonucleotides or oligodeoxyribonucleotide phosphorothioates whose chain lengths were less than 14 nucleotides (*21*, *38*). The ability of relatively short oligonucleotides, containing 2′-O-methylribose sugars to form stable complexes with TAR RNA is consistent with the increased stability of 2′-O-methylribo-oligomer/RNA duplexes vs those of deoxyribo-oligomer/RNA duplexes and suggest that the 2′-O-methylribose backbone can be used effectively to create oligonucleotide ligands with high affinities for highly structured RNA targets.

Unlike the behavior observed with mini-TAR, introduction of mismatched bases essentially eliminates binding between 15-mer mr-AOMP 1677 or 15-mer mr-ON 1757 and TAR RNA. Because TAR RNA is a highly structured target and most of its nucleotides are sequestered in stable base pairs, it is likely that there are a limited number of initial interactions between the target and oligomer that could lead to productive binding. Potential initial binding sites for the 15-mers are the 6-base loop or 3-base bulge in the upper hairpin of TAR. If binding initiates with nucleotides in the 6-base loop, then interaction with 1677 or 1757 would entail formation of a G−U base pair with U 31 of the loop and further propagation of binding into the 5′-side of the upper stem would create a A−C mismatch with C 29 of the base pair adjacent to the loop. These mismatch interactions may be sufficiently destabilizing as to prevent duplex formation.

When mismatches were introduced into the phosphodiester 20-mer 1798, binding to TAR RNA was reduced, but not eliminated. In this case, if initial interaction between the oligomer and the RNA occurs with the 6-base loop, then helix formation can propagate into the upper stem in the 3′-direction to form a pseudo-half-knot with nucleotides 32−40. Pseudo-half-knot formation with the 3′-side of the stem would avoid formation of an A−C mismatch that would occur if binding occurs on the 5′-side of the stem.

Phosphodiester oligomer 1770 binds to TAR RNA with a K_d of 4.7 nM, whereas the dissociation constant of the alternating methylphosphonate oligomer 1800 is 14.8 nM. Replacement of two methylphosphonate linkages in the region of the oligomer that is complementary to the 6-base loop of TAR RNA with phosphodiester groups to give chimeric oligomer 1801, decreases the K_d to 2 nM. This enhanced binding might be expected if initial binding

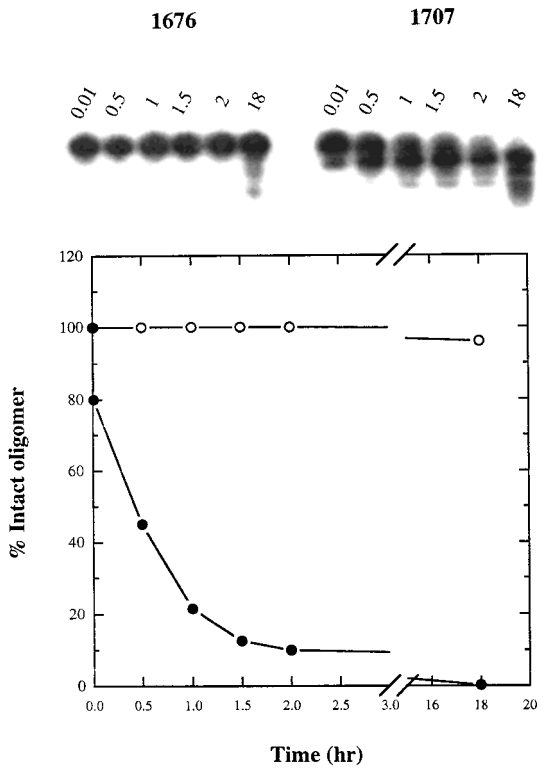


FIGURE 6: Stabilities of mr-AOMP 1676 (○) and mr-ON 1707 (●) in RPMI medium containing 10% fetal calf serum. The oligomers were incubated in medium for the indicated lengths of time indicated and then subjected to electrophoresis on a 20% polyacrylamide gel run under denaturing conditions.

interactions occur between the 6-base loop of the target and complementary bases of the oligomer. Although the structure of the 6-base loop is not known with certainty, it seems possible that conformational constraints imposed by loop formation may make binding to the loop particularly sensitive to methylphosphonate configuration. This restriction could be eliminated or reduced either by replacing the methylphosphonate linkages with phosphodiester linkages or by using Rp methylphosphonate linkages at this position. Experiments to test this possibility are currently in progress.

Stability in Serum. Both the methylphosphonate and phosphodiester linkages of mr-AOMPs are stable to hydrolysis by the 3′-exonuclease activity found in mammalian serum. Figure 6 shows the effects of incubating mr-AOMP 1676 or mr-ON 1707 in culture medium that contains 10% fetal calf serum. In these experiments, the 5′-end of the oligonucleotide was labeled with a [^{32}P]-phosphate group that in turn was converted to its aminoethyl phosphoramidate derivative. This derivatization protects the phosphate from removal by a phosphatase activity that is also present in the serum (*39*). As shown in Figure 6, mr-AOMP is completely resistant to hydrolysis over the 18 h period of the experiment, whereas the phosphodiester linkages of 1707 are rapidly hydrolyzed.

The phosphodiester linkages of mr-AOMPs are completely resistant to S1 nuclease and partially resistant to an endonuclease activity found in snake venom phosphodiesterase (data not shown). Digestion of the mr-AOMPs to their component monomers can be achieved by sequential treatment of the oligomer with 1 M aqueous piperidine followed by snake venom phosphodiesterase. This treatment produces a mixture of 2′-O-methylribonucleosides and their 3′- and 5′-methylphosphonate derivatives that can be separated by C-18 reversed phase HPLC. This digestion is useful for determining base ratios and extinction coefficients of the mr-AOMPs.

CONCLUSIONS

Oligo-2′-O-methylribonucleotides that contain alternating methylphosphonate and phosphodiester internucleotide linkages can be prepared on automated DNA synthesizers using commercially available reagents. Despite the presence of the phosphodiester linkages, these oligonucleotide analogues are remarkably resistant to hydrolysis by both exo- and endonucleases. The mr-AOMPs have very high affinities for complementary RNA targets and are able to bind to TAR RNA with dissociation constants in the low nM range under physiological conditions. These properties suggest that mr-AOMPs may be effective antisense compounds in cell culture. The biological activity of anti-TAR mr-AOMPs will be reported in a future publication.

ACKNOWLEDGMENT

The authors wish to thank Dr. Masad Damha for his advice on synthesizing mini-TAR RNA and Dr. David Noll for helpful discussions on various aspects of this work.

REFERENCES

1. Jones, K. A., and Peterlin, B. M. (1994) *Annu. Rev. Biochem. 63*, 717−743.
2. Mann, D. A. (1996) in *The Molecular Biology of HIV/AIDS* (Lever, A. M. L., Ed.), John Wiley & Sons, New York.
3. Emerman, M., and Malim, M. H. (1998) *Science 280*, 1880−184.
4. Hamy, F., Felder, E. R., Heizmann, G., Lazdins, J., Aboulela, F., Varani, G., Karu, J., and Klimkait, T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3548−3553.
5. Zapp, M., Stern, S., and Green, M. R. (1993) *Cell 74*, 969−978.
6. Mei, H.-Y., Galan, A. A., Halim, N. S., Mack, D. P., Moreland, D. W., Sander, K. B., Truong, H. H., and Czarnik, A. W. (1995) *Bioorg. Med. Chem. Lett. 5*, 2755−2759.
7. Hendrix, M., Priestley, E. J., Joyce, G. F., and Wong, C.-H. (1997) *J. Am. Chem. Soc. 119*, 3641−3648.
8. Wang, S., Huber, P. W., Cui, M., Czarnik, A. W., and Mei, H.-Y. (1998) *Biochemistry 37*, 5549−5557.
9. Mei, H.-Y., Cui, M., Heldsinger, A., Lemrow, S. M., Loo, J. A., Sannes-Lowery, K. A., Sharmeen, L., and Czarnik, A. W. (1998) *Biochemistry 37*, 14204−14212.
10. Lapidot, A., Ben-Asher, E., and Eisenstein, M. (1995) *FEBS Lett. 367*, 33−38.
11. Hamy, F., Brondani, V., Florsheimer, A., Stark, W., Blommers, M. J. J., and Klimkait, T. (1998) *Biochemistry 15*, 5086−5095.
12. Wang, X., Huq, I., and Rana, T. M. (1997) *J. Am. Chem. Soc. 119*, 6444−6445.
13. Tamilarasu, N., Huq, I., and Rana, T. M. (1999) *J Am Chem. Soc 121*, 1597−1598.
14. Huq, I., Ping, Y.-H., Tamilarasu, N., and Rana, T. M. (1999) *Biochemistry 38*, 5172−5177.
15. Tung, C. H., Wang, J., Leibowitz, M. J., and Stein, S. (1995) *Bioconjugate Chem. 6*, 292−295.
16. Wei, P., Garber, M. E., Fang, S.-M., Fischer, W. H., and Jones, K. A. (1998) *Cell 92*, 451−462.
17. Puglisi, J. D., Chen, L., Frankel, A. D., and Williamson, J. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3680−3684.
18. Puglisi, J. D., Chen, L., Blanchard, S., and Frankel, A. D. (1995) *Science 270*, 1200−1203.
19. Aboul-ela, F., Karn, J., and Varani, G. (1995) *J. Mol. Biol. 253*, 313−332.

20. Ippolito, J. A., and Steitz, T. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9819−9824.
21. Vickers, T., Baker, B. F., Cook, P. D., Zounes, M., Buckheit, R. W., Germany, J., and Ecker, D. J. (1991) *Nucleic Acids Res. 19*, 3359−3368.
22. Bordier, B., Helene, C., Litvak, S., and Sarih-Cottin, L. (1992) *Nucleic Acids Res. 20*, 5999−6006.
23. Boulmé, F., Perala-Heape, M., Sarih-Cottin, L., and Litvak, S. (1997) *Biochim. Biophys. Acta 1351*, 249−255.
24. Boulmé, F., Freund, F., Moreau, S., Nielsen, P. E., Gryaznov, S., Toulmé, J.-J., and Litvak, S. (1998) *Nucleic Acids Res. 26*, 5492−5500.
25. Kean, J. M., Cushman, C. D., Kang, H. M., Leonard, T. E., and Miller, P. S. (1994) *Nucleic Acids Res. 22*, 4497−4503.
26. Kean, J. M., S. A., K., Miller, P. S., Kulka, M., and Aurelian, L. (1995) *Biochemistry 34*, 14617−14620.
27. Reynolds, M. A., Hogrefe, R. I., Jaeger, J. A., Schwartz, D. A., Riley, T. A., Marvin, W. B., Daily, W. J., Vaghefi, M. M., Beck, T. A., Knowles, S. K., Klem, R. E., and Arnold, L. J. (1996) *Nucleic Acids Res. 24*, 4584−4591.
28. Miller, P. S., and Cushman, C. D. (1993) *Biochemistry 32*, 2999−3004.
29. Vargeese, C., Carter, J., Yegge, J., Krivjansky, S., Settle, A., Kropp, E., Peterson, K., and Pieken, W. (1998) *Nucleic Acids Res. 26*, 1046−1050.
30. Guzaev, A., Salo, H., Azhayev, A., and Lonnberg, H. (1995) *Tetrahedron 51*, 9375−9384.
31. Hogrefe, R. I., Vaghefi, M. M., Reynolds, M. A., Young, K. M., and Arnold, L. J. (1993) *Nucleic Acids Res. 21*, 2031−2038.
32. Miller, P. S., Dreon, N., Pulford, S. M., and McParland, K. B. (1980) *J. Biol. Chem. 255*, 9659−9665.
33. Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987) *Nucleic Acids Res. 15*, 6131−6149.
34. Lamond, A. I., and Sproat, B. S. (1993) *FEBS Lett. 325*, 123−127.
35. Lesnik, E. A., and Freier, S. M. (1998) *Biochemistry 37*, 6991−6997.
36. Egli, M. (1996) *Angew. Chem., Int. Ed. Engl. 35*, 1894−1909.
37. Tereshko, V., Portmann, S., Tay, E. C., Martin, P., Natt, F., Altmann, K.-H., and Egli, M. (1998) *Biochemistry 37*, 10626−10634.
38. Ecker, D. J., Vickers, T. A., Bruice, T. W., Freier, S. M., Jenison, R. D., Manoharan, M., and Zounes, M. (1992) *Science 257*, 958−961.
39. Levis, J. T., and Miller, P. S. (1994) *Antisense Res. Dev. 4*, 231−241.

BI991962P