Triplex Formation by Psoralen-Conjugated Chimeric Oligonucleoside Methylphosphonates†

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ABSTRACT: Interactions between nuclease-resistant, 5′-psoralen-conjugated, chimeric methylphosphonate oligodeoxyribo- or oligo-2′-*O*-methylribo-triplex-forming oligomers (TFOs) and a purine tract found in the envelope gene of HIV proviral DNA (*env*-DNA) were investigated by gel mobility shift assays or by photo-cross-linking experiments. These chimeric TFOs contain mixtures of methylphosphonate and phosphodiester internucleotide bonds. A pyrimidine chimeric TFO composed of thymidine and 5-methyl-2′-deoxycytidine (*C*), d-PS-T*p*C*p*T*p*C*p*T*p*C*p*T*p*T*p*T*p*T*p*T*p*C*p*T*p*C (**1mp**) where PS is trimethylpsoralen and p is methylphosphonate, forms a stable triplex with *env*-DNA whose dissociation constant is 1.3 μM at 22 °C and pH 7.0. The dissociation constant of chimeric TFO **2mp**, d-PS-*U*p*C*p*T*p*C*p*T*p*C*p*T*p*U*p*T*p-*U*p*T*p*U*p*C*p*T*p*C, decreased to 400 nM when four of the thymidines in **1mp** were replaced by 5-propynyl-2′-deoxyuridines (*U*), a result consistent with the increased stacking interactions and hydrophobic nature of 5-propynyl-U. An even greater decrease, 470 −50 nM, was observed for the all-phosphodiester versions of **1mp** and **2mp**. The differences in behavior of the chimeric versus the all-phosphodiester oligomers may be related to differences in the conformations between the propynyl-U-substituted versus the nonsubstituted TFOs. Thus, in the chimeric oligomer, the stabilizing effect of the propynyl-U's may be offset by the reduced ability of the methylphosphonate backbone to assume an A-type conformation, a conformation that appears to be preferred by propynyl-U-containing TFOs. A chimeric oligo-2′-*O*-methylribopyrimidine with the same sequence as **1mp** also formed a stable triplex, $K_d = 1.4$ μM, with *env*-DNA. In contrast to the behavior of the pyrimidine TFOs, antiparallel A/G oligomers and parallel or antiparallel T/G oligomers did not form triplexes with *env*-DNA, even at oligomer concentrations of 10 μM. This lack of binding may be a consequence of the low G content (33%) of the triplex binding site. Irradiation of triplexes formed between the pyrimidine TFOs and *env*-DNA resulted in formation of photoadducts with either the upper-strand C or the lower-strand T at the 5′-CpA-3′ duplex/triplex junction. No interstrand cross-links were observed. The presence of a 5-propynyl-U at the 5′-end of the oligomer caused a reduction in the amount of upper-strand photoadduct but had no effect on photoadduct formation with the lower strand, suggesting that increased stacking interactions caused by the presence of the 5-propynyl-U change the orientation of psoralen with respect to the upper-strand C. The ability of chimeric methylphosphonate TFOs to bind to DNA, combined with their resistance to degradation by serum 3′-exonucleases, suggests that they may have utility in biological experiments.

Triplex-forming oligonucleotides (TFOs)[1] have been proposed as DNA-specific ligands that could be used to control gene expression (*1*−*3*). As used here, the term TFO designates oligonucleotides that are designed to bind to purine bases in the major groove of the target DNA. Several types of TFOs have been identified (*4*). In the pyr·pur-pyr motif, an oligopyrimidine whose backbone orientation is parallel to that of the target purine tract interacts via Hoogsteen hydrogen bonds to form T·A-T and C^+·G-C triads. In the pur·pur-pyr motif, an oligopurine whose backbone orientation is antiparallel to the purine tract interacts via reverse Hoogsteen hydrogen bonds to form A·A-T and G·G-C triads. Additional binding motifs, in which T interacts with A-T base pairs and G interacts with G-C base pairs and the backbone orientation of the TFO is either parallel or antiparallel to the purine tract, have also been identified (*4*).

Most studies on triplex formation have focused on interactions between oligodeoxyribonucleotide TFOs and DNA. Because these TFOs have phosphodiester linkages, they are susceptible to nuclease hydrolysis and thus can be expected to have limited stability in cell culture. There are fewer

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[1] Abbreviations: *A*, 8-oxo-2′-deoxyadenosine; *C*, 5-methyl-2′-deoxycytidine; CPG, controlled pore glass; EMSA, electrophoretic mobility shift assay; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propane sulfonic acid; SAX, strong anion exchange; TBE, tris−borate−ethylenediaminetetracetate; TFO, triplex-forming oligonucleotide; *U*, 5-propynyl-2′-deoxyuridine.

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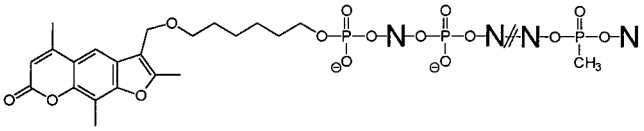
reports on triplex formation by nuclease-resistant oligonucleotide analogues. In general, three different types of modified oligonucleotides have been described which have increased or total nuclease resistance and still retain triplex-forming capabilities. These include oligonucleotides with α-anomeric (*5*−*10*) or 2′-arabino- (*11*), 2′-fluoro- (*12*), 2′-*O*-methyl- (*13*−*18*), or 2′-*O*-aminoethoxy nucleosides (*19*); oligonucleotides containing phosphate-modified internucleotide bonds such as phosphorothioate (*20*−*31*), methylphosphonate (*32*−*36*), or cationic linkages (*37*−*39*); and oligonucleotides containing non-phosphate internucleotide bonds such as carboxamide (*40*), urea (*41*, *42*), or riboacetal linkages (*43*) and peptide nucleic acids (*44*−*51*).

Work in our laboratory has focused on TFOs that contain methylphosphonate linkages. Oligopyrimidines that consist of thymidine and deoxycytidine and contain only methylphosphonate linkages form triplexes with a DNA target, but only at low pH (*32*). However, recently we reported that an oligodeoxyribopyrimidine that contains alternating methylphosphonate and phosphodiester internucleotide linkages forms a stable triplex at pH 7.0 and low, 2.5 mM, magnesium concentration (*52*). In the present study we examine interactions between psoralen-conjugated, chimeric oligodeoxyribo- or 2′-*O*-methylribonucleotides and a purine tract found in the envelope gene of HIV proviral DNA. These chimeric oligomers contain mixtures of phosphodiester and methylphosphonate internucleotide bonds and are remarkably resistant to the nuclease activity found in mammalian serum. Oligomers that consist of pyrimidine nucleosides form stable triplexes at pH 7.0.

EXPERIMENTAL PROCEDURES

Protected deoxyribonucleoside- and 2′-*O*-methylribonucleoside-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites, nucleoside-derivatized controlled pore glass supports, and 6-[4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen] hexyl-1-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite were purchased from Glen Research, Inc., Sterling, VA. Protected deoxyribonucleoside- and 2′-*O*-methylribonucleoside-3′-*O*-(*N*,*N*-diisopropyl) methylphosphonamidites were obtained from JBL Scientific, San Luis Obispo, CA, and Prime Synthesis, Ashton, PA, respectively. The exocyclic amino group of the 5-methyl-deoxycytidine phosphoramidite was protected with a benzoyl group, whereas the exocyclic amino group of 2′-*O*-methylcytosine phosphoramidite was protected with an acetyl group. Anhydrous acetonitrile was prepared by storing HPLC-grade acetonitrile over calcium hydride. All chemicals used were reagent grade or better. 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 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 at a flow rate of 0.6 mL/min 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. Polyacrylamide gel electrophoresis was carried out on 20 × 20 × 0.075-cm gels.

Synthesis of Oligonucleotides. The sequences of the oligonucleotides and their DNA target are shown in Figure 1. The oligonucleotides were synthesized on controlled pore glass supports using an ABI model 392 DNA/RNA synthesizer. Protected nucleoside phosphoramidites and methylphosphonamidites were dissolved in anhydrous acetonitrile at a concentration of 0.12 or 0.15 M. The nucleoside methylphosphonamidite solutions were stored for 2 h over 4 Å molecular sieves prior to use. The activating agent used to prepare the oligodeoxyribonucleotides was 0.45 M tetrazole in acetonitrile, whereas the 0.25 M dicyanoimidazole in acetonitrile was used to prepare the oligo-2′-*O*-methylribonucleotides (*53*). Capping reagent B was 0.5 M (dimethylamino)pyridine in dry pyridine, and the oxidizer was 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 coupling time was 120 s. The synthesizer was programmed to carry out a capping step, followed by the oxidation step, followed by another capping step after each coupling step.



d-PS-TpCpTpCpTpCpTpTpTpTpTpCpTpC 1
d-PS-TpCpTpCpTpCpTpTpTpTpTpCpTpC 1mp

d-PS-UpCpTpCpTpCpTpUpTpUpTpUpCpTpC 2
d-PS-UpCpTpCpTpCpTpUpTpUpTpUpCpTpC 2mp

d-PS-UpCpTpApTpApTpUpTpUpTpUpCpTpC 3
d-PS-UpCpTpApTpApTpUpTpUpTpUpCpTpC 3mp

mr-PS-UpCpUpCpUpCpUpUpUpUpUpCpUpC 4
mr-PS-UpCpUpCpUpCpUpUpUpUpUpCpUpC 4mp

ApGpApGpApGpApApApApApGpApGpA-PS-d 5
ApGpApGpApGpApApApApApGpApGpA-PS-d 5mp

d-PS-TpGpTpGpTpGpTpTpTpTpTpGpTpGpT 6
d-PS-TpGpTpGpTpGpTpTpTpTpTpGpTpGpT 6mp

TpGpTpGpTpGpTpTpTpTpTpGpTpGpT-PS-d 7
TpGpTpGpTpGpTpTpTpTpTpGpTpGpT-PS-d 7mp

d-T G G T G C A G A G A G A A A A A A G A G A T G T G G
A C C A C G T C T C T C T T T T T T C T C T A C A C C-d

FIGURE 1: General structure of the psoralen-conjugated chimeric-triplex-forming oligomers, the sequences of the oligomers, and the sequence of the *env*-DNA target. Nucleosides, 5-methyldeoxycytidine, 5-propynyldeoxyuridine, and methylphosphonate linkages are represented by N, *C*, *U*, and p, respectively.

Depending upon the nature of the cytosine protecting group, different procedures were used to deprotect the oligomers. Support-bound protected oligomers that contain 5-methylcytosine, oligomers **1**−**3** and **1mp**−**3mp**, were first treated in the synthesis cartridge with 1 mL of a solution containing 0.84 M hydrazine in pyridine/acetic acid (4/1 v/v) for 36 h at room temperature. This step, which removes the *N*-benzoyl protecting group from 5-methylcytosine, was included to prevent transamination during the subsequent ethylenediamine treatment described below. The hydrazine solution was flushed from the cartridge, and the CPG was washed with 10 mL of 95% ethanol followed by 10 mL of acetonitrile. The support was dried under vacuum, transferred to a 4-mL autosampler vial fitted with a Teflon-lined cap, and treated with 400 μL of concentrated ammonium hydroxide for 2.5 h at room temperature. The solution was removed from the CPG, the CPG was washed with four 200-

μL aliquots of 50% aqueous acetonitrile, and the combined supernatant and washings were evaporated to dryness at 37 °C. The residue was then treated with a solution containing 5 μL of water, 22.5 μL of 95% ethanol, 22.5 μL of acetonitrile, and 50 μL of ethylenediamine for 6 h at room temperature. The solution was neutralized by the addition of ice-cold 2 N hydrochloric acid. The crude oligomer was then desalted on a SEP PAK C-18 cartridge. Oligomers **4**−**7** and **4mp**−**7mp** were deprotected by first treating the support-bound oligomer with concentrated ammonium hydroxide, followed by the ethylenediamine treatment as described above.

The crude oligomers were purified by SAX HPLC. Samples containing 10−20 A_{260} units were injected onto the column, and the column was eluted at a flow rate of 0.6 mL/min with 18 mL of a linear gradient of 0−0.8 M (phosphodiesters) or 0−0.2 M (methylphosphonates) ammonium sulfate in 20% acetonitrile buffered with 1 mM ammonium acetate, pH 6.2. The purified oligomers were desalted on a SEP PAK C-18 cartridge and stored in 50% aqueous acetonitrile at 4 °C.

A 0.1-A_{260}-unit sample of each oligomer was treated with a combination of 2 μg of snake venom phosphodiesterase and 5 units of calf intestinal phosphatase in 20 μL of buffer containing 10 mM Tris, pH 8.1, and 2 mM magnesium chloride for 18 h at 37 °C. The digests were analyzed by C-18 reversed-phase chromatography using a 20 mL linear gradient of either 2−20% or 2−50% acetonitrile in 50 mM sodium phosphate, pH 5.8, at a flow rate of 1.0 mL/min. The all-phosphodiester oligomers were completely digested to their component nucleosides in the expected ratios. In the case of oligomers **1**−**3**, no transamination products, that is, N^4-aminoethyl-5-methyl-2′-deoxycytidine, were observed. The chimeric methylphosphonate oligomers were digested to their expected component nucleosides and dinucleoside methylphosphonates, d- or mr-NpN. The extinction coefficients of the oligomers were determined as previously described (*54*).

Interactions of Oligonucleotides with DNA. Gel mobility shift experiments were adapted from Verma and Miller (*55*). Stock solutions of the TFOs ranging in concentrations from 0.002 to 200 μM were prepared. TFOs **5**,−**7** were prepared in a 10 mM magnesium chloride/100 mM MOPS buffer (pH 7.0). All other TFOs were prepared in a 2.5 mM magnesium chloride/100 mM MOPS buffer (pH 7.0). Solutions containing 20 nM 5′-end ^{32}P-labeled *env*-DNA were prepared in the same buffers, respectively. Equal volumes of TFO and target (5 μL of each) were mixed and the solution was incubated at room temperature overnight. A 1-μL aliquot of 50% aqueous glycerol solution was added just before the samples were loaded onto the gels. Gel mobility shift experiments were performed on 15% polyacrylamide gels under nondenaturing conditions using 100 mM MOPS (pH 7.0) as the running buffer. The gels were run at a constant voltage of 400 V and at a constant temperature of 22° C. The wet gels were analyzed and quantitated by phosphorimaging. Dissociation constants were determined as the TFO concentration that gave half-maximal binding on a plot of log of TFO concentration versus percent triplex formation. The results of at least three separate experiments were averaged when determining the dissociation constants. The gels and running buffer used in these assays did not contain magnesium. In a separate experiment, binding of TFO **2mp** to *env*-DNA was assayed using a 15% gel and running buffer that contained 10 mM magnesium chloride. The results obtained under these conditions were the same as those obtained when the gel was run in the absence of magnesium.

Photoadduct Formation by TFOs. Solutions containing 10 nM *env*-DNA target, in which the purine-rich strand was 5′-end-labeled with a ^{32}P-phosphate; 10 μM TFO in 10 μL of buffer containing 100 mM MOPS, pH 7.0; and 2.5 mM magnesium chloride were incubated overnight at 22 °C. The solutions were transferred to a 10 × 75-mm borosilicate tube and incubated for 5 min at 0 °C. The solutions were then irradiated with 365-nm UV light for 10 min at 0 °C as previously described (*56*). A 1-μL aliquot of 50% aqueous glycerol was added to the sample just before the samples were loaded onto the gel. The solution was electrophoresed on a denaturing 20% polyacrylamide gel that contained 7 M urea. The gel was run at 800 V using TBE running buffer that contained 89 mM Tris, 89 mM boric acid, and 0.2 mM ethylenediaminetetracetate buffered at pH 8. The wet gel was wrapped in Saran wrap and imaged by using a FujiX BAS 1000 phosphorimager. Phosphorimages were analyzed and quantitated using the Fuji software package.

Stability in Mammalian Serum. TFOs **2** and **2mp** (both lacking psoralen) were phosphorylated by incubation for 1 h at 37° C with 5 units of polynucleotide kinase in 10 μL of buffer that also contained 260 μM [γ-^{32}P]ATP (specific activity, 10 Ci/mmol), 50 mM Tris (pH 7.6), 10 mM magnesium chloride, and 10 mM mercaptoethanol.

A 9-μL aliquot of each of the radioactively labeled TFOs was added to 140 μL of 0.1 M imidazole buffer (pH 6.0), along with 16 μL of 1 M 1-ethyl-3- [3-(dimethylamino)-propyl]carbodiimide. This solution was incubated at room temperature for 4 h. Afterward, 6.4 μL of ethylenediamine and 13 μL of 12 N hydrochloric acid were added, the pH of this solution was adjusted to 7.4, and this reaction mixture was incubated overnight at room temperature. The mixture was diluted into 10 mL of buffer A that consisted of 2% acetonitrile in 50 mM phosphate buffer (pH 5.8), and the solution was loaded onto a preequilibrated C-18 SEP PAK cartridge. The cartridge was washed with 10 mL of buffer A, followed by 10 mL of 5% aqueous acetonitrile/50 mM sodium phosphate (pH 5.8) and finally with 10 mL distilled water. The oligomers were eluted with 2 mL of 50% aqueous acetonitrile. The 5′-aminoethyl derivatized oligomers were purified by electrophoresis on a 20% polyacrylamide denaturing gel. The TFOs were extracted from the gel by incubation of each gel slice with 1 mL of 20% aqueous acetonitrile for 1 h at 65° C, then overnight at 37 °C, then once again at 65 °C for 3 h, using fresh solution. The TFOs were desalted on C-18 SEP PAK cartridges.

Each of the derivatized TFOs (1.1×10^5 cpm) was dissolved in 10 μL of RPMI medium containing 10% fetal bovine serum. The solutions were incubated at 37° C. Aliquots (1 μL) were taken initially and then at 0.5, 1, 1.5, 2.0, and 20 h. These aliquots were diluted into 4 μL of gel loading buffer and were analyzed by polyacrylamide gel electrophoresis on a 20% denaturing gel. Radioactive bands were visualized and quantitated by phosphorimaging the wet gels.

RESULTS AND DISCUSSION

Triplex-Forming Oligonucleotides. The generic structure of the triplex-forming oligonucleotides and their sequences are shown in Figure 1. Each oligomer is conjugated with a trimethylpsoralen group tethered through a six-carbon linker to a 5′-terminal phosphate group of the oligomer. Oligomers with two types of chimeric backbones were studied: those with partially alternating methylphosphonate/phosphodiester linkages, oligomers **1mp**−**4mp**, and those with alternating methylphosphonate/phosphodiester linkages, oligomers **5mp**−**7mp**. Control oligomers, **1**−**7**, contain an all-phosphodiester backbone.

The oligomers are targeted to the underlined purine tract of the duplex shown in Figure 1. This target, denoted *env*-DNA, corresponds to part of the coding region of the envelope gene of HIV-1 proviral DNA. The polarities of oligomers **1(mp)**−**4(mp)** are the same as that of the purine target tract, and these oligomers are designed to bind by the pyr•pur-pyr motif. Oligomers **5** and **5mp** are oriented antiparallel to the purine tract and are designed to bind by the pur•pur-pyr motif. The backbone polarities of T/G oligomers **6(mp)** and **7(mp)** are parallel and antiparallel, respectively, to the purine tract. With the exception of oligomers **4** and **4mp**, which consist of 2′-*O*-methylribonucleosides, all the oligomers are composed of deoxyribonucleosides.

The chimeric methylphosphonate oligomers were prepared as previously described (*52*, *53*). To improve coupling efficiencies, the coupling time was extended to 2 min and an iodine oxidizer containing 0.2% water was used instead of the standard oxidizer that contains 2% water. In addition, a solution of 0.5 M (dimethylamino)pyridine in pyridine was used as the catalyst in the capping step in place of methylimidazole. The tetrazole activator normally used to synthesize oligonucleotides was replaced by dicyanoimidazole when preparing the 2′-*O*-methylribonucleoside-containing oligomers.

The oligomers were derivatized with trimethylpsoralen on the support using the commercially available phosphoramidite. The oligomers were deprotected by sequential treatment with ammonium hydroxide, followed by incubation with ethylenediamine (*57*). In the case of the oligomers that contained 5-methylcytosine, **1(mp)**−**3(mp)**, the oligomers were incubated with hydrazine hydrate (*58*) prior to treatment with ammonium hydroxide and ethylenediamine. The hydrazine treatment removes the *N*-benzoyl protecting groups from the 5-methylcytosine residues and thus eliminates the possibility of transamination during subsequent treatment with ethylenediamine. Thus digestion of oligomers **1**, **2,** or **3** with a combination of snake venom phosphodiester and calf intestinal phosphatase gave 5-methyldeoxycytidine as the sole cytosine-containing product with no evidence of transaminated products. The deprotected oligomers were purified by SAX HPLC.

Interactions between TFOs and env-DNA. Interactions between the oligomers and the *env*-DNA target duplex were examined using an electrophoretic mobility-shift assay on nondenaturing polyacrylamide gels. This method is more reliable than UV-melting experiments for assessing binding by the chimeric oligomers because we previously found that oligomers with alternating methylphosphonate/phosphodiester linkages give melting curves that have very broad transitions and small hypochromicity changes (*52*). The target, whose upper strand was 5′-end-labeled with ^{32}P-phosphate, was incubated at 22 °C overnight prior to electrophoresis with increasing concentrations of oligomer in pH 7.0 buffer containing 2.5 mM magnesium chloride. A typical experiment is shown in Figure 2. The labeled *env*-DNA target migrates as a single band on the gel. Triplex formation is signaled by the presence of a new band of slower mobility whose amount increases with increasing TFO concentration. The gels were analyzed by quantitative phosphorimaging, and the data were used to generate binding isotherms as shown in Figure 2C. Dissociation constants were determined as the oligomer concentration at which half-maximal binding occurred; these are tabulated in Table 1.

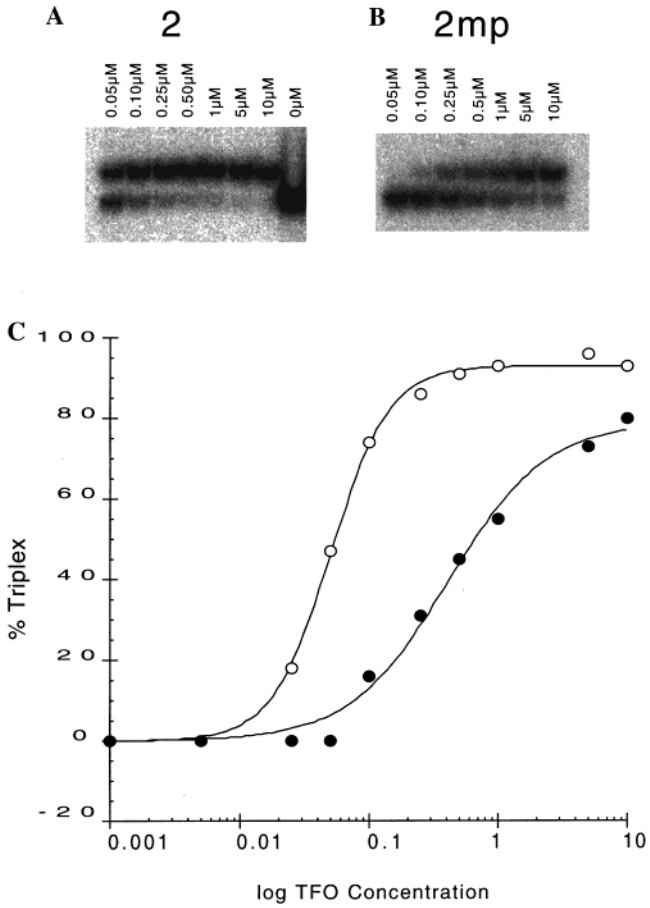


FIGURE 2: Gel mobility shift assays of the interaction between *env*-DNA and phosphodiester oligomer **2** (A) or chimeric oligomer **2mp** (B). The e*nv*-DNA was incubated with various concentrations of the oligomers in a buffer containing 100 mM MOPS, pH 7.0, and 2.5 mM magnesium chloride for 24 h at 22 °C. The reaction mixtures were subjected to electrophoresis on a native 20% polyacrylamide gel. The resulting binding isotherms derived from analysis of these gels are shown in panel C.

Oligomers designed to bind to *env*-DNA by the parallel, pyr•pur-pyr motif were examined first. The purine tract in this target site consists of 10 A-T base pairs and 5 G-C base pairs. All phosphodiester oligomer **1** and chimeric oligomer **1mp** whose backbone is made up of partially alternating methylphosphonate/phosphodiester linkages form triple-stranded complexes with the target. The dissociation constant of TFO **1** was 470 nM, and that of chimeric TFO **1mp** was 1300 nM for these triplexes when the binding experiment

Table 1. Dissociation Constants of Triplexes Formed with *env*-DNA Duplex

oligomer[a]	K_d (nM)[b]
d-PS-TpCpTpCpTpCpTpTpTpTpTpCpTpC (**1**)	470
d-PS-TpCpTpCpTpCpTpTpTpTpTpCpTpC (**1mp**)	1300
d-PS-UpCpTpCpTpCpTpUpTpUpTpUpCpTpC (**2**)	50
d-PS-UpCpTpCpTpCpTpUpTpUpTpUpCpTpC (**2mp**)	400
d-PS-UpCpTpApTpApTpUpTpUpTpUpCpTpC (**3**)	400
d-PS-UpCpTpApTpApTpUpTpUpTpUpCpTpC (**3mp**)	>10000
mr-PS-UpCpUpCpUpCpUpUpUpUpUpCpUpC (**4**)	140
mr-PS-UpCpUpUpUpCpUpUpUpUpUpCpUpC (**4mp**)	1500

[a] The symbols used to denote the oligonucleotides are: *C*, 5-methyldeoxycytidine; *A*, 8-oxo-deoxyadenosine; *U*, 5-propynyldeoxyuridine; p, methylphosphonate linkage; and PS, trimethylpsoralen. [b] Dissociation constants were determined in a buffer containing 100 mM MOPS, pH 7.0, and 2.5 mM magnesium chloride at 20 °C by EMSA as described in Experimental Procedures.

was carried out at pH 7.0 in the presence of 2.5 mM magnesium chloride. Previous studies with a chimeric methylphosphonate TFO **1443**, d-PS-TpTpTpTpCpTpTpTp-TpCpTpTpCpTpT, showed that, unlike the all-phosphodiester TFO of the same sequence, maximal binding occurs when the magnesium concentration reaches 2.5 mM (*52*). This reduced dependence upon magnesium ions is attributed to the reduced charge repulsion between the partially charged chimeric TFO and its DNA target.

The binding affinity of **1mp** for *env*-DNA is comparable to that of **1443** for its DNA target (*52*). Similar to the case with **1** and **1mp**, the affinity of this oligomer for its target is approximately 7 times less than that of its all-phosphodiester counterpart. The methylphosphonate linkages in these chimeric oligomers consist of a mixture of (*R*)p and (*S*)p diastereoisomers. Preliminary studies on a TFO that contains a single methylphosphonate linkage suggest that the (*S*)p diastereoisomer reduces the T_m by approximately 2 °C relative to the corresponding all-phosphodiester TFO (*59*). This reduction is in agreement with theoretical calculations (*60*) and with results seen with triplex-forming oligodeoxyribonucleoside methylphonamidates (*35*). Thus, the reduced binding affinity of the chimeric TFOs may in part be a consequence of the racemic nature of the backbone of these oligomers.

Triplex formation by pyrimidine TFOs requires protonation of their 5-methylcytosine bases in order to provide the two hydrogen bonds required to form each C^+·G-C triad (*61*−*66*). We and others have shown that when targeted to purine tracts containing multiple contiguous G-C base pairs, pyrimidine TFOs are capable of forming stable triplexes only at low pH (*36, 65, 67, 68*). The situation appears to be different for TFOs **1** and **1mp**. Despite the presence of five G-C base pairs in the target, stable triplex formation by both oligomers is observed at pH 7. In the present case, the G-C base pairs are separated from each other by one or more A-T base pairs. This arrangement may allow for more facile cytosine protonation than is the case for a tract of contiguous cytosines where interbase charge repulsion lowers the apparent pK_a of cytosine residues (*69, 70*).

The stability of triplexes formed by pyrimidine TFOs can be significantly enhanced by replacing TFO thymidine residues with 5-propynyldeoxyuridine (*71, 72*). Propynyl-U residues stack well with neighboring thymine and 5-methylcytosine bases in the TFO, and this stacking, in combination with the increased hydrophobicity of the propynyl group, appears to contribute to the increased stability of the triplexes (*73*). To see if similar stabilization would occur in a TFO with a chimeric methylphosphonate backbone, four of the thymidine residues of **1mp** were replaced by 5-propynyl-U to give **2mp**. In this oligomer three of the propynyl-U's are linked through their 5′-oxygens to the methylphosphonate groups of neighboring thymidines and the remaining propynyl U resides at the 5′-end of the oligomer. A similarly substituted all-phosphodiester TFO, **2**, was also prepared.

Propynyl-U substitution enhanced the binding affinity of both **2** and **2mp** for the *env*-DNA target. The dissociation constant for binding between all-phosphodiester TFO **2** and *env*-DNA was reduced approximately 1 order of magnitude relative to the K_d for the nonsubstituted oligomer **1**. A similar reduction in the K_d of chimeric oligomer **2mp** relative to that of **1mp** was also seen, although the amount of this decrease, 3.3 times, was less than that observed for the all-phosphodiester oligomer. Thus, in this system, propynyl-U substitution has a greater stabilizing effect on the all-phosphodiester TFO than is the case for the chimeric methylphosphonate TFO. The source of this difference may be related to the difference in conformation between the propynyl-U-substituted TFO and the nonsubstituted TFO when bound to the DNA target. High-resolution NMR studies have been carried out on an intramolecular triplex in which propynyl-U's were substituted for thymidines in the Hoogsteen region (*73*). These studies showed that the propynyl-U-substituted Hoogsteen region adopts an A-like conformation,whereas the T-substituted Hoogsteen region adopts a B-like conformation. Previous studies on duplex formation between oligodeoxyribonucleoside methylphosphonates and complementary single-stranded DNA or RNA oligomers suggest that duplexes in which the methylphosphonate oligomer can adopt a B-like conformation are energetically more favorable than those in which the methylphosphonate oligomer is required to adopt an A-like conformation (*74*). Thus, in the case of the triplex formed by **2mp**, the stabilizing effect of the propynyl-U may be offset somewhat by the conformational preference of the deoxyribonucleoside methylphosphonate backbone.

Replacement of cytosine or 5-methylcytosine residues in TFOs by 8-oxo-adenine reduces the pH dependence of triplex formation by these pyrimidine-motif TFOs (*36, 75*−*79*). This *syn* purine analogue exists in the keto-tautomeric form and can provide two hydrogen bonds to the guanine of a G-C base pair. Previous experiments have shown that all-phosphodiester, psoralen-conjugated TFOs that contain 8-oxo-A alone or in combination with 5-methylcytosine form stable triplexes at pH 7 (*36*). To test the effect of 8-oxo-A substitution on triplex formation by chimeric methylphosphonate oligomers, two of the 5-methylcytosines in **2mp** were replaced by 8-oxo-A to give chimeric TFO **3mp**.

As shown in Table 1, no binding was observed between **3mp** and *env*-DNA, even at an oligomer concentration of 10 μM. This behavior is in contrast to that of the "parent" oligomer, **2mp,** whose K_d is 400 nM. A similar, although less dramatic, decrease in binding affinity was also observed for all-phosphodiester TFO **3**. The K_d of this oligomer is approximately 8 times less than that of TFO **2**. The decrease in affinity of TFO **3** was not expected, on the basis of previous results with 8-oxo-A containing oligomers. In these

studies, 15-mers that contained seven 8-oxo-A's, d-PS-TpApApApTpApApApApTpTpTpTpTpTpApT, or a combination of four 8-oxo-A's and three 5-methylcytosines, d-PS-TpApApCpTpApApCpTpTpTpTpTpCpT, formed stable duplexes at neutral pH, whereas the corresponding oligomer containing seven 5-methylcytosines, d-PS-TpCpCpCpTp-CpCpCpTpTpTpTpTpCpT, did not form a triplex (*36*). The loss of binding of the latter TFO is most likely due to the contiguous 5-methylcytosines, whose protonation is not favored at neutral pH. Oligomer **2** contains isolated 5-methylcytosines, an arrangement that supports triplex formation at neutral pH. Replacement of positively charged 5-methylcytosines in the triplex formed by **2** by neutral 8-oxo-A's in the triplex formed by **3** could result in increased charge repulsion between the TFO and the target, thus leading to reduced binding.

Alternatively or additionally, the reduced binding affinity of **3** relative to **2** may be a consequence of the presence of the 5-propynyl-U residues in TFO **2**. As discussed above, the Hoogsteen strand of propynyl-U-substituted triplexes appears to prefer an A-type conformation. Although the conformational preference of the 8-oxo-deoxyadenosines in TFOs is not known, if they prefer a B-conformation, this could serve to destabilize the triplex relative to the triplex formed by TFO **2**. Restrictions on overall backbone conformation could be even more serious when multiple methylphosphonate linkages are introduced, as in chimeric TFO **3mp**. However, it appears unlikely that the presence of the methylphosphonate linkage itself prevents binding. Thus, preliminary studies of a pyrimidine TFO containing a single TpA- methylphosphonate linkage showed that this oligomer forms a triplex whose T_m was 3 °C higher than that of the corresponding all-phosphodiester oligomer (*59*).

Oligo-2′-*O*-methylribopyrimidines form stable triplexes with duplex DNA, and the stabilities of these triplexes are often greater than those of triplexes formed by the corresponding oligodeoxyribopyrimidine (*13, 80, 81*). Chimeric TFO **4mp** was prepared in order to examine the effect of methylphosphonate linkages on triplex formation by an oligo-2′-*O*-methylribonucleotide. This oligomer and its all-phosphodiester counterpart, **4**, contain cytosines instead of the 5-methylcytosines used in the deoxyribo-TFOs, because previous studies have shown that 5-methylcytosines in ribo- or 2′-*O*-methylribo-TFOs destabilize triplex formation (*14, 82*).

Chimeric TFO **4mp** binds with approximately the same affinity as its deoxyribo counterpart, **1mp**. Thus, the chimeric methylphosphonate backbone supports triplex formation by the 2′-*O*-methylribo-TFO. Oligo-2′-*O*-methylribonucleotides with alternating methylphosphonate/phosphodiester backbones have been found to be extraordinarily resistant to both exo- and endonuclease hydrolysis (*53, 83*). This property may be advantageous when using such oligomers in cell culture experiments.

In agreement with previous studies, the K_d of the all-phosphodiester 2′-*O*-methylribo-TFO, **4**, is approximately 3 times lower than that of the corresponding deoxyribo-TFO, **1**. The difference in stability between the triplexes formed by **4mp** and **4** may again be due to the racemic nature of the chimeric methylphosphonate backbone of **4mp**. There is currently no data available on the effect of configuration on binding of methylphosphonate-substituted 2′-*O*-methylribo-TFOs to duplex DNA. However, studies on duplex formation by oligo-2′-*O*-methylribonucleotides show that (*R*)p methylphosphonate linkages favor duplex stability over (*S*)p linkages (*74*).

An A/G oligonucleotide designed to bind via the antiparallel pur·pur-pyr motif, **5**, and parallel and antiparallel T/G oligonucleotides **6** and **7** were also tested for their abilities to form triplexes with *env*-DNA. In contrast to the behavior of the pyrimidine oligonucleotides, triplexes were not formed by any of these oligonucleotides, even at concentrations as high as 10 μM. Because the G residues are dispersed in the TFO sequences, and because their A_{260}-versus-temperature profiles show only a slight linear increase over the temperature range 0 °−70 °C (data not shown), it is unlikely that the lack of binding is due to aggregation of the G residues in either the A/G or T/G oligomers (*84*). In the case of A/G TFO **5**, lack of binding may be due to the low G-content of the purine tract. Previous studies have suggested that a G-content of greater than 50% is required for high-affinity binding by A/G- and G/T-type TFOs (*85*). A similar explanation may also apply to T/G TFOs **6** and **7**. Studies on triplex-forming oligomers that employ this motif suggest that binding is highly dependent upon both the sequence of the oligomer and its polarity relative to the purine binding site (*84*). Thus, sites that contain clusters of A's favor binding by parallel T/G oligomers, whereas sites that contain contiguous G's favor binding by antiparallel T/G oligomers (*86*). In the case of oligomers **6** and **7**, neither backbone orientation supported stable triplex formation.

Photoadduct Formation by Pyrimidine TFOs. Upon oligomer binding, the psoralen group of psoralen-conjugated TFOs intercalates between the duplex base pair and triplex base triad at the duplex/triplex junction (*87, 88*). This interaction enhances the stability of a triplex formed by a psoralen-derivatized TFO versus that formed by a nonderivatized TFO (*36*). In addition, irradiation by long-wavelength UV light can result in the formation of 2+2 cycloaddition products between the double bonds of the furan and/or pyrone rings of psoralen and the 5,6-double bonds of pyrimidines in the DNA target. If psoralen intercalates between a 5′-TpA-3′ sequence at the duplex/triplex junction, an interstrand cross-link forms, which in effect covalently links the TFO to both strands of the target duplex. It appears that psoralen prefers 5′-TpA-3′ sites, although photoadducts can form when other sequences are present at the junction (*88*).

The sequence at the duplex/triplex junction of the TFO/*env*-DNA triplex is 5′-CpA-3′. Photoreactions between the pyrimidine TFOs and *env*-DNA were investigated using targets that were 5′-^{32}P-labeled in either the upper, purine-rich, or lower, pyrimidine-rich, strand. Solutions containing 10 μM TFO and 10 nM *env*-DNA were incubated overnight at 22 °C and irradiated for 10 min at 0 °C, and the products of the reaction were analyzed on a denaturing polyacrylamide gel. Photoproducts with mobilities consistent with the formation of a monoadduct with C-6 of the upper strand or T-21 of the lower strand were observed. No interstrand cross-links were detected. As shown in Table 2, irradiation of the triplex formed by TFO **1** and *env*-DNA gave a total of 80% photoproducts. Reaction occurred preferentially with the upper strand of the target and gave approximately twice as much photoadduct as with the lower strand.

Table 2. Effects of 5′-Terminal Base on Photoadduct Formation

oligomer[a]	% photoadduct[b] upper	lower
d-PS-TpCpTpCpTpCpTpTpTpTpTpCpTpC (**1**)	54	26
d-PS-UpCpTpCpTpCpTpUpTpUpTpUpCpTpC (**2mp**)	18	26
d-PS-UpCpTpCpTpCpTpTpTpTpTpCpTpC (**1-U**)	18	20
d-PS-TpCpTpCpTpCpTpUpTpUpTpUpCpTpC (**2mp-T**)	49	23

[a] The symbols used to denote the oligonucleotides are: *C*, 5-methyldeoxycytidine; *U*, 5-propynyldeoxyuridine; p, methylphosphonate linkage; and PS, trimethylpsoralen. [b] The target duplex was irradiated in the presence of 10 μM oligomer in buffer containing 100 mM MOPS and 2.5 mM magnesium chloride for 10 min at 0 °C.

These results are consistent with previous studies from our laboratory on photoreactions of d-PS-TpApApApTpAp-ApApTpTpTpTpTpTpApT. This TFO reacts with the C and T bases of the 5′-CpA-3′ junction sequence of its target DNA (*36*). As in the case of the *env*-DNA system, only monoadducts were formed with the C of the purine-rich and the T of the pyrimidine-rich strands of the target, and no interstrand cross-link was detected.

Similar photoadduct formation was also observed when the triplex formed by chimeric TFO **2mp** and *env*-DNA was irradiated. However, in this case only 44% photoadduct formation occurred. The amount of photoproduct formed with the lower strand was the same as that seen with TFO **1**, whereas the amount formed with the upper strand decreased to 18%. It seemed unlikely that reduced product formation resulted from reduced triplex formation. Under the conditions of the experiment, 10 μM TFO should convert almost all of the *env*-DNA to triplex, and any reduction in the amount of triplex formed would be expected to decrease photoadduct formation with both strands of the target.

An alternative explanation is the possibility that the presence of the 5′-terminal 5-propynyl-U residue of **2mp** affects the photoreaction. To test this, all-phosphodiester TFO **1-U** was prepared. This oligomer has the same base composition as **1,** except that the 5′-terminal thymidine is replaced by 5-propynyl-U. As shown in Table 2, irradiation of the **1-U**/*env*-DNA triplex reduced total photoadduct formation to 38%. As was the case with **2mp**, reduced adduct formation occurred almost exclusively with the upper strand of the target.

As a further check on the effect of the 5′-5-propynyl-U on photoadduct formation, chimeric TFO **2mp-T** was prepared. In this oligomer, the 5′-terminal thymidine of **2mp** is replaced by 5-propynyl-U. As expected, and consistent with the results for the all-phosphodiester TFOs, this substitution increased adduct formation with the upper strand of the target but had essentially no effect on adduct formation with the lower strand.

The presence of the 5′-terminal 5-propynyl-U apparently interferes with formation of photoadducts with the upper strand of the duplex. Such perturbation may be a consequence of the hydrophobic nature of this base and its ability to stack strongly with neighboring bases. Increased stacking interactions caused by the presence of the 5-propynyl-U in the 5-terminal U·A-T triad may change the orientation of the psoralen group with respect to C-6. This in turn could affect the efficiency of the photoreaction.

Because the affinity of psoralen for double-stranded DNA is rather low, the extent of photoadduct formation should be directly related to the amount of triplex that is formed when the TFO interacts with its target. Thus, no photoadducts were formed when solutions containing the A/G oligomers **5** or **5mp**, or T/G oligomers **6/6mp** or **7/7mp,** and *env*-DNA were irradiated. This result is consistent with the inability of these oligomers to form triplexes with the target in the EMSA experiments. When the triplexes formed by all-phosphodiester TFO **1** were irradiated at 22 °C, the amount of photoadduct increased with increasing oligomer concentration. The TFO concentration at which half-maximal photoadduct formation was observed, 630 nM, is similar to the dissociation constant of the **1**/*env*-DNA triplex.

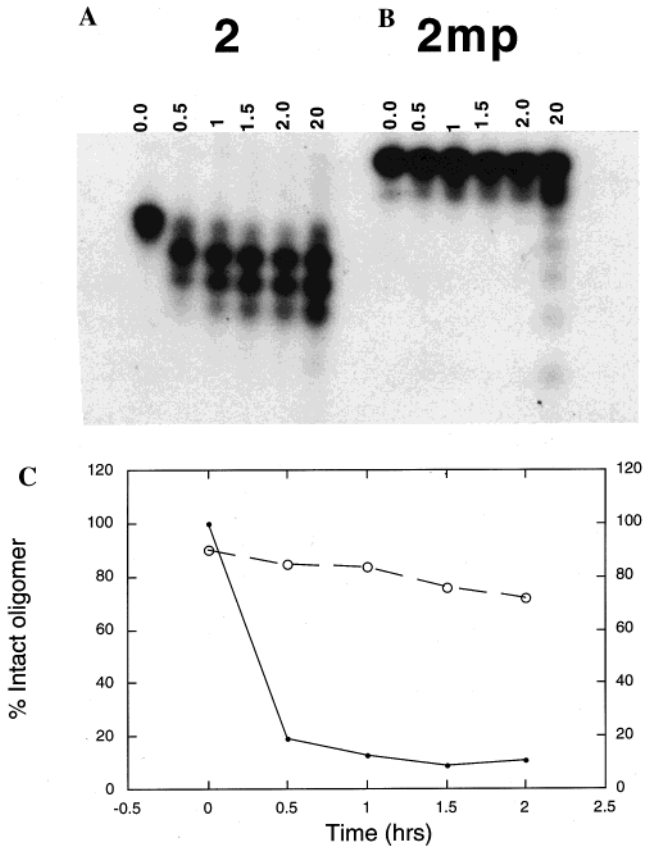


FIGURE 3: Stabilities of phosphodiester oligomer **2** (A) and chimeric oligomer **2mp** in serum containing medium. The 5′-[^{32}P]aminoethylphosphoramidate derivatives of the oligomers were incubated in medium containing 10% fetal bovine serum for the times indicated, and the reaction mixtures were subjected to electrophoresis on a denaturing 20% polyacrylamide gel. The percentage of intact oligomer **2** (●) and oligomer **2mp** (○) versus time is shown in panel C.

Stability of Chimeric TFOs. Chimeric TFO **2mp** and its all-phosphodiester counterpart, **2**, lacking the psoralen group were synthesized and subsequently converted to their 5′-^{32}P-end-labeled derivatives. To protect the 5′-phosphate group from removal by phosphatase activity that is present in serum, the phosphorylated oligomers were transformed to their aminoethyl phosphoramidate derivatives by reaction with ethylenediamine in the presence of a water-soluble carbodiimide (*53, 89*). The oligomers were incubated in cell culture medium supplemented with 10% bovine calf serum and the products of the reaction analyzed by polyacrylamide gel electrophoresis. As shown in Figure 3, chimeric TFO **2mp** remains essentially intact, even after 20 h of incubation. In contrast, the half-life of the all-phosphodiester oligomer

is less than 30 min under the same conditions. The chimeric oligomer terminates with a 3′-methylphosphonate linkage. Despite the presence of two adjacent phosphodiester linkages near the 3′-end, this single methylphosphonate is sufficient to prevent hydrolysis of the oligomer by the 3′-exonuclease activity commonly found in mammalian serum (*90*).

CONCLUSIONS

Pyrimidine TFOs that contain chimeric methylphosphonate linkages form stable triplexes with *env*-DNA. This chimeric backbone is compatible with the introduction of other modifications, such as 5-propynyl-U or 2′-*O*-methylribose sugars, that can be used to enhance triplex stability. Although the stabilities of the triplexes formed by the chimeric TFOs are less than those of comparable triplexes formed by all phosphodiester oligonucleotides, the enhanced nuclease resistance of the chimeric backbone may prove to be advantageous for their use in biological experiments.

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