60.6, 61.5, 64.0, 127.1, 128.4, 131.2, 146.1, 166.9, 171.0, 171.9, 173.0; EIMS m/e 421 (M^+), 348, 219 (base), 202, 159; HRMS calcd for $C_{22}H_{31}NO_7$ 421.2101, found 421.2104. Anal. Calcd for $C_{22}H_{31}NO_7$: C, 62.69; H, 7.41; N, 3.32. Found: C, 62.91; H, 7.27; N, 3.37.

Diethyl *N*-[4-[4-[(2-Amino-6-chloro-5-formylpyrimidin-4-yl)amino]butyl]benzoyl]-L-glutamate (60). Diester **60** was prepared in 71% yield from amine **57** by the procedure described above for the preparation of **53**: mp 113–115 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.22 (t, 3 H, J = 7.1 Hz), 1.30 (t, 3 H, J = 7.1 Hz), 1.56–1.76 (m, 4 H), 2.08–2.21 (m, 1 H), 2.27–2.34 (m, 1 H), 2.40–2.53 (m, 2 H), 2.67 (t, 2 H, J = 6.8 Hz), 3.39–3.48 (m, 2 H), 4.11 (q, 2 H, J = 7.1 Hz), 4.23 (q, 2 H, J = 7.1 Hz), 4.76–4.83 (m, 1 H), 5.80 (br s, 1 H), 7.15–7.23 (m, 3 H), 7.73 (d, 2 H, J = 8 Hz), 9.23 (t, 1 H, J = 5.5 Hz), 10.04 (s, 1 H); ^{13}C NMR ($CDCl_3$, 75.6 MHz) δ 14.0, 27.1, 28.0, 28.3, 30.4, 35.1, 40.1, 52.2, 60.6, 61.5, 102.2, 127.1, 128.4, 131.3, 146.0, 162.1, 162.3, 166.3, 167.1, 172.1, 173.0, 188.5; EIMS m/e 533 (M^+), 505, 460, 331 (base), 302, 185; HRMS calcd for $C_{25}H_{32}ClN_5O_6$ 533.2041, found 533.2017. Anal. Calcd for $C_{25}H_{32}ClN_5O_6$: C, 56.23; H, 6.04; N, 13.11; Cl, 6.64. Found: C, 56.03; H, 5.79; N, 12.88; Cl, 6.77.

***N*-[4-[4-[(2-Amino-1,6-dihydro-5-formyl-6-oxopyrimidin-4-yl)amino]butyl]benzoyl]-L-glutamic Acid (61).** Diacid **61** was prepared in 25% yield from diester **60** by the procedure described above for the preparation of **18**: mp 222–224 °C; ^{1}H NMR (DMSO-d_6, 270 MHz) δ 1.67–1.75 (m, 4 H), 2.03–2.26 (m, 2 H), 2.46–2.52 (m, 2 H), 2.64 (s, 2 H), 2.81 (t, 2 H, J = 6.9 Hz), 3.58 (m, 2 H), 4.49–4.57 (m, 1 H), 7.44 and 7.94 (AA'BB', 4 H), 8.67 (d, 1 H, J = 7.6 Hz), 9.77 (s, 1 H), 9.88 (t, 1 H, J = 5.5 Hz), 10.40 (s, 1 H), 12.52 (br s, 2 H); HRFABMS calcd for $C_{21}H_{26}N_5O_7$ (MH^+) 460.1832, found 460.1834. Anal. Calcd for $C_{21}H_{25}N_5O_7$: C, 54.90; H, 5.48; N, 15.24. Found: C, 55.14; H, 5.40; N, 15.04.

Acknowledgment. We acknowledge with gratitude the expertise of Dr. Dorothy Little in obtaining the electron impact mass spectral data. Elemental analyses and FAB mass spectral analyses were performed by Eli Lilly and Co., Indianapolis, IN, and the biological evaluations were carried out by Dr. G. B. Grindey of Eli Lilly and Co. This work was supported by grants to Princeton University from the National Cancer Institute, NIH (CA42367), and from Eli Lilly & Co.

Supplementary Material Available: ^{1}H NMR spectra for compounds **4**, **10**, **11**, **13–19**, **22**, **24–34**, **38–45**, **49**, **54**, and **57** and IR spectral data for compounds **4–6**, **8–11**, **13**, **14**, **16–19**, **21**, **22**, **24–45**, **47–49**, **51**, **53**, **54**, and **56–61** (42 pages). Ordering information is given on any current masthead page.

Triplex Formation of an Oligonucleotide Containing 2′-*O*-Methylpseudoisocytidine with a DNA Duplex at Neutral pH

Akira Ono, Paul O. P. Ts'o, and Lou-sing Kan*

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

Received June 7, 1991 (Revised Manuscript Received February 3, 1992)

The synthesis of the hexadecanucleotide 5′TTTT1TTTT111111T3′ (**1-16mer**) containing 2-amino-5-(2-*O*-methyl-β-D-ribofuranosyl)-4(1*H*)-pyrimidinone (2′-*O*-methylpseudoisocytidine or **1**) is described. Triplex formation of **1-16mer** with a deoxyribonucleotide duplex 5′d(ACCAAAAGAAAAGGGGGGACCA)3′–5′d-(TGGTCCCCCCTTTTCTTTTGGT)3′ (**duplex-22**) which contains the "polypurine tract" found in the genome of human T-cell leukaemia (lymphotropic) virus (HTLV-III) was studied by thermal denaturation and circular dichroism (CD) spectra in aqueous solution at neutral pH. The "polypurine tract" contains a homoguanine cluster consisting of 6 deoxyguanine residues. The results indicate that **1-16mer** and **duplex-22** formed a triplex at neutral condition (0.01 M Na cacodylate, 0.5 M NaCl, 5 mM $MgCl_2$, pH 7.2. T_m = 20 °C). In contrast, a hexadecadeoxynucleotide, 5′TTTTMTTTTMMMMMMT3′ (**M-16mer**), containing 5-methyl-2′-deoxycytidine (**M**) did not form a stable triplex with **duplex-22** at the same condition (T_m < 0 °C). The CD mixing titration indicated that the triplex was formed with 1:1 (duplex:third strand) molecular stoichiometry.

Introduction

The formation of triple-stranded nucleic acid helices (triplex) has been studied for more than three decades.[1-3] In recent years, studies of sequence-specific triplex formation of short synthetic oligonucleotides (or their analogues) are of prime interest because the approach is applicable to biological and biochemical studies such as site specific cleavage of DNA,[4-7] inhibition of DNA–protein binding,[8-10] and inhibition of gene expression.[11]

The most common motif is that each case triad of a triplex consists of two pyrimidine residues from two homopyrimidine strands and a purine residue from a homopurine strand. The third strand (i.e., the second pyrimidine strand) is located in the major groove of a duplex consisting of Watson–Crick base pairing, and its sugar–phosphate backbone polarity is parallel to that of the purine strand.[4,12-16] Thymines or cytosines in the third

(1) Felsenfeld, G.; Davies, D. R.; Rich, A. *J. Am. Chem. Soc.* **1957**, *79*, 2023–2024.
(2) Felsenfeld, G.; Miles, T. *Annu. Rev. Biochem.* **1967**, *36*, 407–448.
(3) Michelson, A. M.; Massoulie, J.; Guschlbauer, W. *Prog. Nucleic Acid Res. Mol. Biol.* **1967**, *6*, 83–141.
(4) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645–650.
(5) Francois, J.-C.; Saison-Behmoaras, T.; Barbier, C.; Chassignol, M.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9702–9706.
(6) Beal, P. A.; Dervan, P. B. *Science* **1991**, *251*, 1360–1363.
(7) Strobel, S. A.; Dervan, P. B. *Nature* **1991**, *350*, 172–174.
(8) Maher, L. J. III; Wold, B.; Dervan, P. B. *Science* **1989**, *245*, 725–730.
(9) Francois, J.-C.; Saison-Behmoaras, T.; Thoung, N. T.; Helene, C. *Biochemistry* **1989**, *28*, 9617–9619.
(10) Hanvey, J. C.; Shimizu, M.; Wells, R. D. *Nucleic Acids Res.* **1990**, *18*, 157–161.
(11) Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E. *Science* **1988**, *241*, 456–459.
(12) Arnott, S.; Bond, P. J.; Selsing, E.; Smith, P. J. C. *Nucleic Acids Res.* **1976**, *3*, 2459–2470.
(13) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(14) Santos, C.; Rosen, M.; Patel, D. *Biochemistry* **1989**, *28*, 7282–7289.
(15) Rajagopal, P.; Feigon, J. *Nature* **1989**, *339*, 637–640.

0022-3263/92/1957-3225$03.00/0

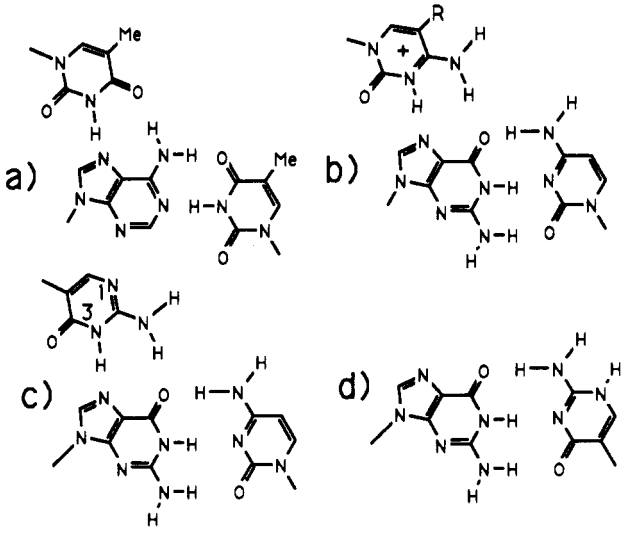

Figure 1. The hydrogen bonding schemes of triplexes of TAT (a), C^+GC (R = H) or M^+GC (R = Me) (b), 1GC (c), and G1 in Watson–Crick type base pair (d).

strand form hydrogen bonds with adenines or guanines, respectively, in a scheme of Hoogsteen type (Figures 1a,b). Since protonation of cytosine bases is essential in order to provide the second hydrogen bonding between the N-3 of cytosine to N-7 of guanine to form a Hoogsteen-like base pair in the triad, this C^+-G-C triad is stable in acidic conditions but is not stable in neutral conditions.[17-19] This requirement prevents the formation of triplex in living cells since the cellar pH is usually above 7.0.

In recent reports, the substituion of 2′-deoxycytidine by 5-methyl-2′-deoxycytidine (**M**) can cause the formation of triplex above neutral pH.[20-22] However, Lee et al. reported that poly(dG) and poly(**M**) did not form a triplex at neutral condition.[21] They interpreted this phenomenon to be the result of charge repulsions between cations on neighboring protonated 5-methylcytosine bases which destabilize $(M^+)_n$-$(dG)_n$-$(M)_n$ type of triplexes even in neutral conditions.[20] This phenomenon limits the application of an oligonucleotide containing 5-methyl-2′-deoxycytidine for triplex formation with a duplex containing a $(dG)_n$-$(dC)_n$ sequence.

To overcome these limitations, we designed and synthesized an oligonucleotide containing 2-amino-5-(2-*O*-methyl-β-D-ribofuranosyl)-4(1*H*)-pyrimidinone (2′-*O*-methylpseudoisocytidine, 1) which may form both Hoogsteen type and Watson–Crick type base pairings through hydrogen bondings with guanine in neutral and slightly basic conditions (Figures 1c,d).[23] As indicated in this figure, 1 can contain a hydrogen at the N-3 position for hydrogen bonding with N-7 of guanine. Therefore, a 1-dG-dC triad can be formed in neutral conditions. Also, since base residue of 1 in the triad of 1-dC-dG is uncharged or unprotonated (Figure 1c), an oligonucleotide containing

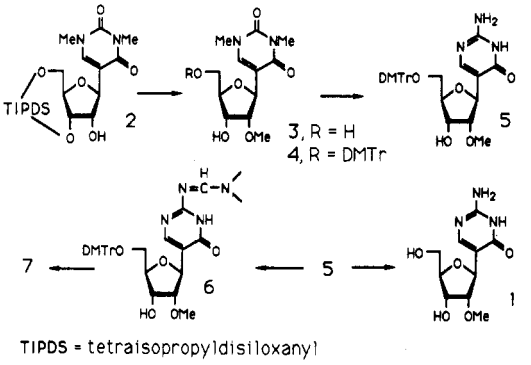

Figure 2. Scheme for a synthesis of 1 and the corresponding amidite, 7.

1 will be able to form a triplex with a DNA duplex containing a 2′-deoxyguanine cluster. Also, since 1 can form two major tautomeric forms, 1 may form a Watson–Crick type base pair with guanine (Figure 1d). Furthermore, triplexes formed by oligonucleotides containing 2′-*O*-methylpyrimidine nucleosides are more stable than those formed by corresponding natural deoxyribooligonucleotides (data not shown).

We report the synthesis of 1 and the hexadecanucleotide 5′TTTT1TTTT111111T3′ (**1-16mer**). Triplex formation of **1-16mer** or a control hexadecanucleotide 5′TTTTMTTTTMMMMMMT3′ (**M-16mer**) with a deoxyribonucleotide-duplex, 5′d(ACCAAAAGAAAAGGGGGGACCA)3′–5′d(TGGTCCCCCCTTTTCTTTTGGT)3′ (**duplex-22**), was studied by UV spectroscopy, circular dichroism (CD), and gel electrophoresis. The **duplex-22** contains a sequence of "polypurine tract" found in the genome of human T-cell leukaemia (lymphotropic) virus (HTLV-III).[24] The "polypurine tract" contains a homoguanine cluster consisting of six deoxyguanine residues. Also duplex formation of **1-16mer** and **M-16mer** with **Pu-16mer** (5′d(AGGGGGGAAAAGAAAA)3′) was studied by thermal denaturation.

Materials and Methods

General Procedures. 5-β-D-Ribofuranosyluracil (pseudouridine) was obtained from Kyowa Hakko USA Inc. The 5-methylcytosine phosphoramidite monomer was purchased from Glen Research, Inc., and used without further modification as a synthon for synthesis of the **M-16mer** oligonucleotide. TLC was performed on silica gel $60F_{254}$ plates and column chromatography on silica gel G60 (70–230 mesh). Melting points were determined and elemental analyses were performed by Galbraith Laboratoies, Inc. NMR spectra were recorded on a Bruker WM-300 spectrometer with tetramethylsilane or 85% phosphoric acid as reference for chemical shift (δ). The oligodeoxyribonucleotides were synthesized on a DNA synthesizer. HPLC was performed on a Varian Model 5000 with a partisil ODS-3 column. Polynucleotide kinase was purchased from Bethesda Research Laboratories. Snake venom phosphodiesterase I and alkaline phosphatase was purchased from P-L Biochemicals.

Synthesis of 1 and the Corresponding Amidite (7) (Figure 2). 1,3-Dimethyl-5-(2-*O*-methyl-β-D-ribofranosyl)uracil (1,3,2′-*O*-Trimethylpseudouridine, 3). Ag_2O (10 g) and CH_3I (20 mL) were added to the solution of 1,3-dimethyl-3′,5′-*O*-(tetraisopropyldisiloxanyl)pseudouridine (2) (4.1 g, 8.2 mmol)[25] in benzene (10 mL).[26] The mixture was stirred at room temperature

(16) Rajagopol, P.; Feigon, J. *Biochemistry* **1989**, *28*, 7859–7870.
(17) Lipsett, M. N. *Biochem. Biophys. Res. Commun.* **1963**, *11*, 224–228.
(18) Howard, F. B.; Frazier, J.; Lipsett, M. N.; Miles, H. T. *Biochem. Biophys. Res. Commun.* **1964**, *17*, 93–102.
(19) Thiele, D.; Guschlbauer, W. *Biopolymer* **1971**, *10*, 143–157.
(20) Lee, J. S.; Woodsworth, M. L.; Latimer, L. J. P.; Morgan, A. R. *Nucleic Acids Res.* **1984**, *12*, 6603–6614.
(21) Lee, J. S.; Woodsworth, M. L.; Latimer, L. J. P. *Biochemistry* **1984**, *23*, 3277–3281.
(22) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059–3061.
(23) Ono, A.; T'so, P. O. P.; Kan, L.-S. *J. Am. Chem. Soc.* **1991**, *112*, 4032–4033.
(24) Ratner, L.; Haseltine, W.; Patarca, R.; Livak, K. J.; Starcich, B.; Josephs, S. F.; Doran, E. R.; Rafalski, J. A.; Whitehorn, E. A.; Baumeister, K.; Ivanhoff, L.; Petteway, S. R., Jr.; Pearson, M. L.; Lautenberger, J. A.; Papas, T. S.; Ghrayeb, J.; Chang, N. T.; Gallo, R. C.; Wong-Staal, F. *Nature* **1985**, *313*, 277–284.
(25) Pankiewicz, K.; Matsuda, A.; Watanabe, K. A. *J. Org. Chem.* **1982**, *47*, 485–488.
(26) Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miura, K.; Ohtsuka, E. *Nucleic Acids Res.* **1987**, *15*, 6131–6148.

for 7 days. Solids were filtered, and the solution was concentrated in vacuo. The residue was dissolved in a mixture of dioxane (50 mL), ethanol (50 mL), and 0.1 N HCl (10 mL). The solution was kept at room temperature for 24 h and then neutralized by Dowex 1-X8 ($HCOO^-$ form). The resin was filtered and washed by 50% ethanol (500 mL). The solution was concentrated, the residue was dissolved in ethanol, and the solution was concentrated again. The residue was dissolved in water, and the solution was washed by diethyl ether. The water layer was concentrated, and the residue was crystallized from ethanol to give a white powder, yield 2.0 g (7.3 mmol, 89%). Mp: 178 °C. NMR (D_2O): δ 7.74 (1 H, singlet, H-6), 4.79 (1 H, doublet, H-1', $J_{1'2'}$ = 4.6 Hz), 4.23 (1 H, double doublet, H-3', $J_{2'3'}$ = 5.3 Hz, $J_{3'4'}$ = 6.3 Hz), 3.99–3.93 (2 H, multiplet, H-2' and H-4'), 3.91 (1 H, quartet, H5', $J_{4'5'}$ = 3.0, $J_{5'5''}$ = 12.5), 3.65 (1 H, quartet, H-5'', $J_{4'5''}$ = 4.6), 3.46 (3 H, singlet, 3-Me), 3.39 (3 H, singlet, 1-Me), 3.27 (3 H, singlet, 2'-OMe). UV: λ_{max} (MeOH) 269 (ϵ 9200), 208 nm (10 300); λ_{min} 237 nm (1800). MS: m/z 287 ($M^+ + 1$). Anal. Calcd for $C_{12}H_{18}N_2O_6$: C, 50.34; H, 6.34; N, 9.70. Found: C, 50.41; H, 6.47; N, 9.71.

1,3-Dimethyl-5-[2-*O*-methyl-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]uracil (1,3,2'-*O*-Trimethyl-5'-*O*-(dimethoxytrityl)pseudouridine, 4). Dimethoxytrityl chloride (2.17 g, 6.4 mmol, 1.05 equiv) was added to the solution of **3** (1.66 g, 6.1 mmol) in pyridine (10 mL), the mixture was kept at room temperature for 18 h, and then ethanol (1 mL) was added to the solution. After 10 min, solvents were evaporated in vacuo. The residue was dissolved in $CHCl_3$, and the solution was washed by 5% $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was chromatographed over a column of silica gel (i.d. 3.8 × 30 cm) with $CHCl_3$–methanol (30:1) as a eluent to give **4** as a foam, yield 3.4 g (5.9 mmol, 97%). NMR (DMSO-d_6): δ 7.57 (1 H, singlet, H-6), 7.50–6.95 (13 H, multiplet, aromatic protons of DMTr group), 4.90 (1 H, doublet, 3'-OH, J = 7.4 Hz), 4.81 (1 H, singlet, H-1'), 4.11 (1 H, multiplet, H-3'), 3.94 (1 H, multiplet, H-4'), 3.73–3.67 (3 H, multiplet, H-2' and H-5',5''), 3.50 (3 H, singlet, 3-Me), 3.24 (3 H, singlet, 1-Me), 3.04 (3 H, singlet, 2'-OMe). MS: m/z 588 ($M^+ + 1$), 303, 287.

2-Amino-5-[2-*O*-methyl-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]-4(1*H*)-pyrimidinone (2'-*O*-Methyl-5'-*O*-(dimethoxytrityl)pseudoisocytidine, 5). Guanidine hydrochloride (0.4 mmol, 100 equiv) was added to a sodium ethoxide solution (0.4 mol in 400 mL of ethanol).[27] The mixture was stirred at room temperature for 20 min, and then the solids were filtered. The solution was added to **4** (2.3 g, 4.0 mmol), and the mixture was concentrated in vacuo. The residue was refluxed under N_2 for 30 min. Then, the mixture was cooled on ice bath, and water (300 mL) was added to the mixture. The solution was neutralized by adding acetic acid, and then **5** was precipitated as a gum. Ethyl acetate was added to the mixture, and the organic layer was separated and dried as described previously. The residue was crystallized from ethyl acetate to give white powder, yield 1.4 g (2.5 mmol, 62%). Mp: 179 °C dec. NMR (DMSO-d_6): δ 10.84 (1 H, singlet, imino proton), 7.62 (1 H, singlet, H-6), 7.42–6.84 (13 H, multiplet, aromatic protons of DMTr group), 6.49 (2 H, broad singlet, NH_2), 4.73 (1 H, doublet, 3'-OH, J = 7.2 Hz), 4.69 (1 H, doublet, H-1', $J_{1'2'}$ = 3.5 Hz), 3.84 (2 H, multiplet, H-3' and H-4'), 3.72 (6 H, singlet, OMe groups of DMTr), 3.56 (1 H, triplex, H-2', $J_{2'3'}$ = 4.2 Hz), 3.35 (3 H, singlet, 2'-OMe), 3.10 (2 H, multiplet, H-5' and H-5''). MS: m/z 560 ($M^+ + 1$), 303, 258. UV: λ_{max} (in MeOH) 282 (ϵ 8800), 230 nm (28 100); λ_{min} 258 (4500), 220 nm (12 500). Anal. Calcd for $C_{31}H_{33}N_3O_7$: C, 66.54; H, 5.94; N, 7.51. Found: C, 66.29; H, 6.0; N, 7.48.

2-[[(Dimethylamino)methylene]amino]-5-[2-*O*-methyl-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]-4(1*H*)-pyrimidinone [N^2-[(Dimethylamino)methylene]-2'-*O*-methyl-5'-*O*-(dimethoxytrityl)pseudoisocytidine, 6]. *N*,*N*-Dimethyformamide dimethyl acetal (1 mL) and **5** (250 mg, 0.45 mmol) were dissolved in DMF (1 mL), and the solution was kept at room temperature for 5 h.[28] Ethanol (1 mL) was added to the solution, and the mixture was concentrated in vacuo. The residue was chromatographed over a column of silica gel (i.d. 1.5 × 10 cm) with $CHCl_3$–methanol (30:1) as a eluent to give **6** as a gum, yield 280 mg (0.45 mmol, 100%). NMR (DMSO-d_6): δ 11.59 (1 H, singlet, imino proton), 8.58 (1 H, singlet, N=CHN(Me)$_2$), 7.74 (1 H, singlet, H-6), 7.43–6.85 (13 H, multiplet, aromatic protons of DMTr group), 4.76 (2 H, multiplet, H-1' and 3'-OH), 3.87 (2 H, multiplet, H-3' and H-4'), 3.72 (6 H, singlet, OMe groups of DMTr), 3.58 (1 H, triplet, H-2', $J_{1'2'}$ = 2.6 Hz, $J_{2'3'}$ = 4.1 Hz), 3.39 (3 H, singlet, 2'-OMe), 3.32–3.31 (2 H, multiplet, H-5' and H-5''), 3.11 (3 H, singlet, NCH_3a), 3.00 (3 H, singlet, NCH_3b). MS: m/z 615 ($M^+ + 1$), 559, 303. UV λ_{max} (in MeOH) 320–290 (broad shoulder), 282, 234 nm; λ_{min} 256, 223 nm.

2-[[(Dimethylamino)methylene]amino]-5-[2-*O*-methyl-3-*O*-[(cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl]-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]-4(1*H*)-pyrimidinone [N^2-[(Dimethylamino)methylene]-2-*O*-methyl-5'-*O*-(dimethoxytrityl)pseudoisocytidine 3'-*N*,*N*-diisopropylphosphoramidite, 7]. **6** (250 mg, 0.41 mmol) was dissolved in pyridine, and the solvent was concentrated in vacuo. This procedure was repeated twice, and then the residue was dissolved in CH_2Cl_2 (5 mL). 2-Cyanoethyl *N*,*N*-diisopropylphosphoramidous chloride (280 μL, 3 equiv)[29] and diisopropylethylamine (286 μL, 4 equiv) were added to the solution. The whole was kept at room temperature for 40 min, and then $CHCl_3$ and 5% $NaHCO_3$ were added to the reaction. The organic layer was separated and dried as described previously. The residue was chromatographed over a column of silica gel (i.d. 1.2 × 5 cm) with ethyl acetate as a eluent to give **7** as a gum, yield 70%. The ^{31}P NMR resonances are 146.8 and 147.6 ppm (area ratio = 1 to 1.1).

2-Amino-5-(2-*O*-methyl-β-D-ribofuranosyl)-4(1*H*)-pyrimidinone (2'-*O*-Methylpseudoisocytidine, 1). A solution of **5** (100 mg, 0.18 mmol) in 80% acetic acid (5 mL) was kept at room temperature. After 24 h, the mixture was concentrated in vacuo. The residue was dissolved in water, and the solution was washed by diethyl ether. The water layer was evaporated in vacuo, and the residue was crystallized from water–ethanol to give a white powder, yield 40 mg (0.14 mmol, 78%). Mp: 210 °C dec. NMR (D_2O): δ 7.73 (1 H, singlet, H-6), 4.73 (1 H, doublet, H-1', $J_{1'2'}$ = 5.7 Hz), 4.29 (1 H, triplet, H-3', $J_{2'3'}$ = 5.2 Hz, $J_{3'4'}$ = 5.1 Hz), 4.04 (1 H, triplet, H-2'), 4.03 (1 H, multiplet, H-4'), 3.80 (1 H, double doublet, H-5', $J_{4'5'}$ = 2.9 Hz, $J_{5'5''}$ = 12.6 Hz), 3.70 (1 H, double doublet, H-5'', $J_{4'5''}$ = 4.4 Hz), 3.45 (3 H, singlet, 2'-OMe). MS: m/z 258 ($M^+ + 1$). UV λ_{max} (pH 7.2) 288 (ϵ 5700), 204 nm (14 100); λ_{min} 248 nm (2500); λ_{max} (pH 2.2) 261 (7400), 219 nm (9900); λ_{min} 242 nm (4700); λ_{max} (pH 12.0) 288 (4500), 231 nm (9000); λ_{min} 252 nm (2900). Anal. Calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33. Found: C, 46.23; H, 6.04; N, 16.69.

Synthesis and Characterization of the 5'TTTT1TTTT111111T3' (1-16mer). Synthesis. The hexadecanucleotide, 5'TTTT1TTTT111111T3' (**1-16mer**) containing **1** was synthesized on a Applied Biosystem DNA synthesizer using the solid-phase phosphoramidite method,[30] beginning with 5'-DMTr-T (1 μmol) bound to a controlled glass support. A coupling time for amidites of 2'-O-methylated nucleosides was 5 times longer than that for amidites of 2'-deoxynucleosides. Synthesis was monitored by the spectrophotometric quantitation of released dimethoxytrityl cation at 500 nm on each addition. An average yield for couplings of amidites was 97%. After being cleaved from the solid support, the oligomer was treated with concd NH_4OH at room temperature for 36 h and then concentrated (45 o.d. units at 254 nm). Half of the oligomer protected with the DMTr group was stored at −20 °C and the other part of the sample was purified by Sep-Pak (Waters, Milford, MA) according to the method of Lo et al.[31] Fractions containing **1-16mer** were treated by 80% acetic acid solution at room temperature for 30 min. The mixture was concentrated and then dissolved in water (1 mL). The solution was washed by diethyl ether and the water layer was concentrated. **1-16mer** (13 o.d. units) of this preparation showed a single peak by HPLC analysis (data not shown). After labeling by ^{32}P at 5'-end, **1-16mer** of this preparation showed one spot by 20%

(27) Chu, C. K.; Wempen, I.; Watanabe, K. A.; Fox, J. J. *J. Org. Chem.* **1976**, *41*, 2793–2797.

(28) Zemlicka, J.; Holy, A. *Collect. Czech. Chem. Commun.* **1967**, *32*, 3159.

(29) Sinha, N. D.; Biernat, J.; Koster, H. *Tetrahedron Lett.* **1983**, *24*, 5843–5846.

(30) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859–1862.

(31) Lo, K.-M.; Jones, S. S.; Hackett, N. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2285–2289.

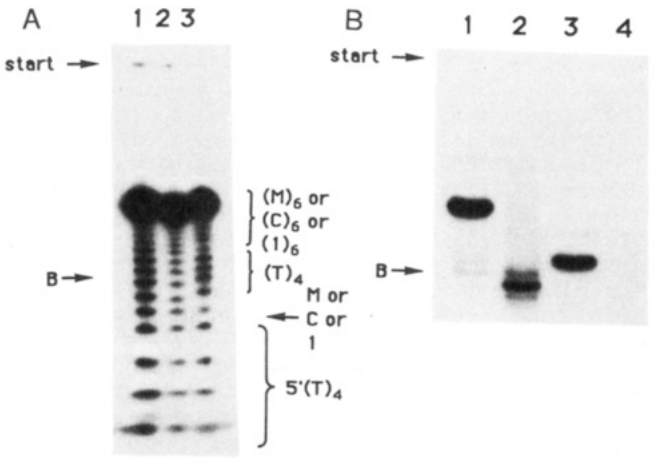

Figure 3. (A) Autoradiogram of the polyacrylamide (20%) gel electrophoresis of oligonucleotides under denaturing condition.[32] Lanes 1, 2, and 3 are **M-16mer**, **N-16mer**, and **1-16mer**, respectively, treated by chemical modification (see Materials and Methods). (B) Lanes 1 and 3 are the undigested **1-16mer** and 5′TT1TT1TT1TT3′ as markers. Lanes 2 and 4 are **1-16mer** and **M-16mer** which are incubated with phosphodiesterase and alkalinephosphatase enzymes (see Materials and Methods). B indicates the position of bromophenol blue marker dye.

polyacrylamide electrophoresis under denaturing condition[32] (data not shown).

Characterizations. Chemical Modification. The synthesized oligomer showed a single peak of an analytical HPLC analysis (datum not shown).

The sequence of **1-16mer** was confirmed by chemical modification (Figure 3A) according to the reported methods.[33,34] Water (5 μL), hydrazine monohydride (30 μL), and glacial acid (15 μL) were added to the oligomers 5′TTTTCTTTTCCCCCCT3′ (**N-16mer**), **M-16mer**, and **1-16mer** which were labeled by ^{32}P at 5′-ends. The solutions were incubated at 37 °C for 30 min, purified by Sep-pak (Waters, Milford, MA), and treated with 1 M piperidine at 90 °C for 30 min. The reactions were analyzed by polyacrylamide gel electrophoresis under denaturing condition.[32]

Enzymatic Hydrolysis. **1-16mer** and **M-16mer** (0.25 o.d. units at 254 nm) were incubated with 10 ng of venom phosphodiesterase I, 0.4 units of alkaline phosphatase in 25 mM Tris-HCl, 5 mM $MgCl_2$ at pH 8.0 (100 μL) at 37 °C for 72 h. The mixtures were heated at 90 °C for 10 min and cooled, and 200 μL of ethanol was added to the mixtures. The mixtures were kept at −20 °C overnight and then centrifuged at 12000 rpm for 20 min. Supernatants were separated and concentrated. Each residue was dissolved in 0.1 M Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.6 (10 μL), and then γ-^{32}P ATP and polynucleotide kinase (5 units) were added to the solution and the whole was incubated at 37 °C for 24 h. The reactions were analyzed by 20% polyacrylamide gel electrophoresis under denaturing condition,[33] followed by autoradiography of the gel (Figure 3B).

Thermal Denaturation Study. UV absorbance was measured on Varian DMS 100 and Varian 219 spectrophotometers. Oligomers were dissolved at 3 μM each in 0.01 M Na cacodylate, 0.5 M NaCl, 5 mM $MgCl_2$, pH 7.2. Thermally induced transition of each mixture of the oligomers was monitored by the Varian 219 instrument with a thermoregulated sample compartment. Sample temperature was controlled by fluid circulating from a temperature-regulated bath monitored with a calibrated thermistor probe inserted in a "dummy" cuvette.

CD Spectra Study. CD spectra were obtained using a JASCO 500A CD spectrometer. Total concentration of the oligomers was 4.1 μM in the same buffer system used for the thermal denaturation studies. Sample temperature was controlled by using a circulating water bath.

(32) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular cloning*; Cold Spring Harbor Laboratory: New York, 1982.
(33) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560–564.
(34) Chandrasegaran, S.; Lin, S.-B.; Kan, L.-S. *BioTechniques* **1985**, 6–8.

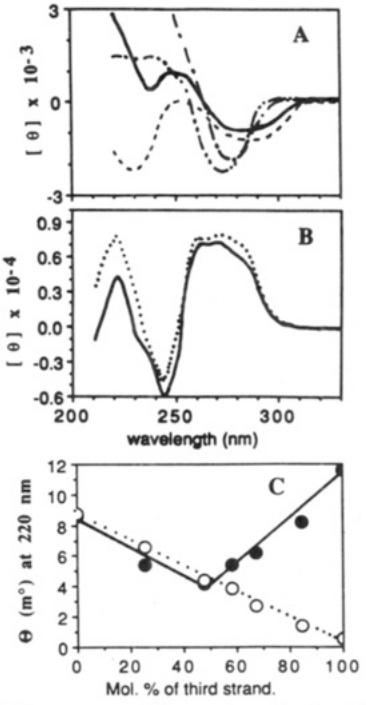

Figure 4. (A) CD spectra of the nucleosides in 0.01 M sodium phosphate, pH 7.2. (—) **1**, (- - -) pseudoisocytidine, (-·-) **3**, (-··-) pseudouridine. (B) CD spectra of the 1:1 mixture of the oligomers in 0.5 M NaCl, 5 mM $MgCl_2$, 0.01 M sodium phosphate, pH 7.5 at 13 °C. (—) The mixture of **duplex-22** and **1-16mer** (···) a calculated spectrum derived from the summation of a spectrum of **1-16mer** itself and that of **duplex-22** itself. Molar ellipticity is shown per base residue. (C) CD mixing curve in 0.5 M NaCl, 5 mM $MgCl_2$, 0.01 M sodium phosphate, pH 7.5 at 13 °C (concentration of **duplex-22** + concentration of the third strand = 4.1 μM). Filled circles are mixing of **duplex-22** and **1-16mer**, and open circles are mixing of **duplex-22** and **M-16mer**.

Results

The Synthesis of the 5′TTTT1TTTT111111T3′ (1-16mer). A scheme for the synthesis of 1 and its phosphoramidite derivative **7** is shown in Figure 2. 5-β-D-Ribofuranosyluracil (pseudouridine) was converted to 1,3-dimethyl-3′,5′-*O*-(tetraisopropyldisiloxanyl)pseudouridine (**2**) according to a reported method.[25] The 2′-hydroxyl group of **2** was methylated according to the method of Inoue et al.[26] Deprotection of the silyl group gave 1,3-dimethyl-5-(2′-*O*-methyl-β-D-ribofuranosyl)uracil (**3**) in a good yield. Protection of 5′-hydroxy group of **3** with dimethoxytrityl chloride gave 5′-dimethoxytrityl derivative **4** which was treated with guanidine according to the method of Chu et al.[27] After neutralization of the reaction, one major product, 2-amino-5-[2-*O*-methyl-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]-4(1*H*)-pyrimidinone (**5**), and traces of several minor products which showed higher mobilities than **5** were detected on TLC (MeOH:$CHCl_3$ = 1:10). No spots which correspond to another diastereomer of **5** were detected. A crystallization from ethyl acetate gave pure **5**.

5 was converted to 2-amino-5-(2-*O*-methyl-β-D-ribofuranosyl)-4(1*H*)-pyrimidinone (**1**) to determine the configuration around the glycosidic bond. Chemical shifts (in D_2O) of H-6 (7.73 ppm) and H-1′ (4.73 ppm) are similar to those of 2-amino-5-β-D-ribofuranosyl-4(1*H*)-pyrimidinone (β-pseudoisocytidine) (7.75 and 4.72 ppm)[27] but were different from those of α-pseudoisocytidine (7.65 and 5.04 ppm).[27] Also, coupling constants (in D_2O) of protons of the sugar moiety of **1** ($J_{1'2'}$ = 5.7 Hz and $J_{2'3'}$ = 5.2 Hz and $J_{3'4'}$ = 5.1 Hz) were similar to those of β-pseudouridine (5.0, 5.0, 5.2 Hz)[35,36] but were different from those of α-pseu-

douridine (3.3, 4.2, 7.9 Hz).[35,36] Furthermore, the CD spectra of 1 was similar to that of 3 and β-pseudoisocytidine (Figure 4A). From these results, we concluded that 5 and 1 were β anomers.

Protection of 2-amino group of 5 by the (*N*,*N*-dimethylamino)methylene group[28] gave 6 in excellent yield. Phosphitylation of 3′-hydroxy group[29] of 6 gave phosphoramidite derivative 7 in good yield.

1-16mer was synthesized on a DNA synthesizer (Applied Biosystem, Foster City, CA) using solid-phase phosphoramidite method.[30] An average yield for couplings of amidites was 97%.

Characterization of 1-16mer. The sequence of **1-16mer** was confirmed by chemical modification method (Figure 3A).[33,34] **M-16 mer** (lane 1), **N-16mer** (lane 2), and **1-16mer** (lane 3) were treated by hydrazine monohydride and then by 1 M piperidine at 90 °C for 30 min. There is no significant difference in these three lanes. Thus, as clearly shown in Figure 3A, all three 16mers have the designed sequences and chain lengths.

In addition, the $(1)_6$ core of **1-16mer** is resistant to venom phosphodiesterase I as shown in Figure 3B. Spots for intact **1-16mer** and 5′TT1TT1TT1T3′[23] are shown in lanes 1 and 3 as markers. Since **M-16mer** was completely hydrolyzed by venom phosphodiesterase I, any spots corresponding to oligonucleotides are not detected in the lane 4 (Figure 3B). In contrast, the $(1)_6$ core and neighboring T residues are resistant for the phosphodiesterase, a spot corresponding to the $(1)_6$ core and weak neighboring spots were detected in the lane 2 (Figure 3B). The single spot of lane 1 in Figure 3B and a single peak of HPLC (not shown) are evidences for the purity of **1-16mer**.

CD Spectra. A CD spectrum of the mixture of **1-16mer** and **duplex-22** and a calculated spectrum derived from the summation of CD spectra of **duplex-22** itself and **1-16mer** itself are shown in Figure 4B. Molar ellipticity at 210–225 nm of the spectrum of the triplex is smaller than that of the calculated spectrum.[23,37-39] A mixing study measured by CD at 13 °C of **duplex-22** itself and **1-16mer** at 220 nm showed that the association of the duplex and the third strand has 1:1 stoichiometry, whereas a mixing of **duplex-22** and **M-16mer** did not shown any end point (Figure 4C). This conclusion based on the mixing study of **duplex-22** and **1-16mer** is supported by the UV thermal transition study discussed below.

Thermal Denaturation of the Mixtures of the Oligomers. Duplex formation of **1-16mer** and **M-16mer** with **Pu-16mer** and triplex formation of **1-16mer** and **M-16mer** with **duplex-22** were studied by thermal denaturation of the mixture of the oligomers at neutral condition (Figure 5A). T_m of the duplex of **1-16mer**:**Pu-16mer** (45 °C) was lower than that of the duplex of **M-16mer**:**Pu-16mer** (69 °C). The 1:1 mixture of **1-16mer** and **duplex-22** showed two transitions at pH 7. The transition at higher temperature (T_m = 72 °C) was identical to the melting of **duplex-22**, and the transition at lower temperature (T_m = 20 °C) is the dissociation of **1-16mer** from **duplex-22** as supported by the mixing study measured by CD at 13 °C. In contrast, the mixture of **M-16mer** and **duplex-22** showed a partial transition

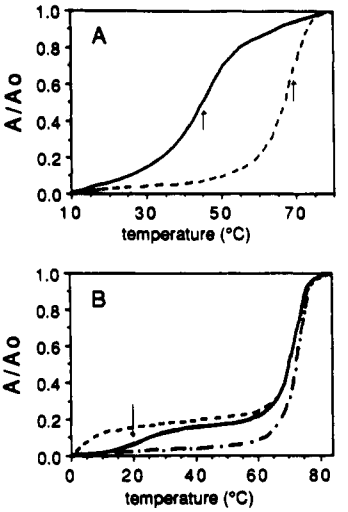

Figure 5. (A) Relative absorbance, $A/A_o = [(A_{t°C} - A_{10°C})/(A_{80°C} - A_{10°C})]$ at 260 nm vs temperature. (—) The mixture of **1-16mer** and **Pu-16mer**, (- - -) the mixture of **M-16mer** and **Pu-16mer**. (B) Relative absorbance, $A/A_o = [(A_{t°C} - A_{0°C})/(A_{84°C} - A_{0°C})]$ at 260 nm vs temperature. (-·-) **duplex-22** alone, (—) the mixture of **duplex-22** and **1-16mer**, (- - -) **duplex-22** and **M-16mer**. All solutions contain 3 μM of each oligomer in 0.5 M NaCl, 5 mM $MgCl_2$, 0.01 M Na cacodylate, pH 7.2. T_m's are indicated by arrows.

corresponding to the dissociation of the third strand at much lower temperature (T_m below 0 °C).

Discussion

Synthesis and Characterization of 1 and 1-16mer. Owing to the presence of a proton on N-1, pyrimidine *C*-nucleosides (pseudouridine or pseudoisocytidine derivatives, etc.) undergo α,β-anomerization in basic and acidic conditions.[40,41] These type of anomerization reactions were always associated with conversions of pseudouridine derivatives to pseudoisocytidine derivatives by reactions with guanidine.[25,26] However, we found that 5′-*O*-(dimethoxytrityl)-β-1,3-dimethylpseudouridine was converted to the β-anomer of 5′-*O*-(dimethoxytrityl)pseudoisocytidine in good yield. Only a trace of product which could be attributed to the α-anomer was detected in the reaction by TLC check (data not shown). In the same condition, unprotected derivatives give mixtures of α- and β-isomers.[25,26] Similarly, 4 was converted to the β-anomer of 5 which was easily extracted from the reaction mixture owing to the hydrophobic DMTr group. The large, hydrophobic group at the 5′-position seems to stabilize the β-anomers. 5 was converted to 1, the configuration of which was confirmed by NMR and CD spectra. The nucleoside 1 was stable against a temperature by concd NH_4OH at room temperature for 24 h or at 50 °C for 5 h. Therefore, any epimerization reaction of 1 will not be expected in the deprotection condition of the protected oligonucleotide.

1 was introduced into the oligonucleotide (**1-16mer**) with excellent yield, the same as for the introduction of 2′-deoxynucleosides. The $(1)_6$ cluster core of **1-16mer** was resistant for hydrolysis by venom phosphodiesterase[42,43] at conditions in which **M-16mer** was hydrolyzed completely (Figure 3B). Interestingly, the 5′TTTT1TTTT3′

(35) Deslauriers, R.; Smith, I. C. *Can. J. Biochem.* **1972**, *50*, 766–774.
(36) Chu, C. K.; El-Kabb, F. M.; Thompson, B. B. *Nucleosides Nucleotides* **1984**, *3*, 1–31.
(37) Lee, J. S.; Johnson, D. A.; Morgan, A. R. *Nucleic Acids Res.* **1979**, *6*, 3073–3091.
(38) Callahan, D. E.; Trapane, T. L.; Miller, P. S.; Ts'o, P. O. P.; Kan, L.-S. *Biochemistry* **1991**, *30*, 1650–1655.
(39) Pilch, D. S.; Levenson, C.; Shafer, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1942–1946.
(40) Chambers, R. W.; Kurkov, V.; Shapiro, R. *Biochemistry* **1963**, *2*, 1192–1203.
(41) Matsuda, A.; Chu, C. K.; Reichman, U.; Pankiewicz, K. P.; Watanabe, K. A.; Fox, J. J. *J. Org. Chem.* **1981**, *46*, 3603–3609.
(42) Janion, C.; Zmudzka, B.; Shugar, D. *Acta Biochim. Polon.* **1970**, *17*, 13.
(43) Zumdzka, B.; Shugar, D. *Acta Biochim. Polon.* **1971**, *18*, 321.

region of **1-16mer** and 5′TT1TT1TT3′[23] were hydrolyzed completely to nucleosides. Incorporation of 1 into the oligomers was confirmed by HPLC analysis of the nucleosides (data not shown). The sequence of **1-16mer** was confirmed by chemical modification reaction (Figure 3A).[33,34]

Duplex Formation of 1-16mer and M-16mer with Pu-16mer. The duplex formed by **1-16mer** and **Pu-16mer** (T_m = 45 °C) was much less stable that by **M-16mer** and **Pu-16mer** (T_m = 69 °C) at pH 7.0. **1-16mer** and **M-16mer** differ in four aspects in regard to the cytosine nucleoside moieties. 1 is a 2′-*O*-methylribosyl nucleoside, consisting of a C–C linkage between the sugar and the pseudoisocytosine, and this base in 1 has to tautomeric forms in which the hydrogen can be located either at N_1 or at N_3. In comparison, **M** is a 2′-deoxyribosyl nucleoside, consisting of a C–N linkage between the sugar and the 5-methylcytosine. This base in **M** does not possess the type of tautomerism discussed above but does not contain an extra methyl group at the 5-position as compared to cytosine. The influence of each of these four factors and the influences produced by the mutual interaction of each of these factors in the stability of the duplex cannot be clearly delineated. However, the tautomerism in pseudoisocytosine described above should have a negative impact on the Watson–Crick base pairing. Also, 5-methyl substitution in thymine bases as compared to uracil bases usually increases the thermal stabilities (T_m's) of duplexes such as rA-rT (~65 °C) versus rA-rU (~57 °C) or dA-dT (~68 °C) versus dA-dU (~55 °C).[44] Thus, these two factors are likely to be involved in the observed difference of T_m's for the two duplexes (Figure 5). As for the influence of sugar moieties and the C–C versus C–N nucleoside linkage, the understanding is less clear,[44,45] awaiting further investigation.

Triplex Formation of 1-16mer and M-16mer with Duplex-22. In the original study of the triple helix formation involving **M**,[22] the pH dependence of the triplex formation is clearly evident. Under the most favorable conditions, such as large excess (over 1000-fold) of oligomer to target ratio, in the presence of spermine (1 mM), low-temperature incubation (initially at 0 °C), and with an irreversible reaction (cleavage), no complex formation can be detected above pH 7.4. In our first communication,[23] the triplex formation at a 1:1 (third strand to duplex) ratio can be detected at pH 8.0 with a T_m of about 12 °C. In that study, as well as in this investigation, no pH dependence near or above neutrality can be found.

In this study, the T_m of **1-16mer:duplex-22** is higher than that T_m of **M-16mer:duplex-22** by at least 22 °C at pH 7.5 under identical conditions. It should be noted that the four structural differences between **1** and **M** discussed in relationship to the duplex stability are also applicable here. However, in the triplex formation, the presence of a hydrogen at N_3 of **1** (Figure 1c) may become the dominant influence on triplex stability since oligomers containing 5-methylcytosine (**M**) still require protonation for triplex formation.[20] We further reason that for a duplex containing clusters of guanine residues, such as **duplex-22** which contains a $d(G)_6$ segment, the third strand correspondingly will have to contain a cluster of cytosine or 5-methylcytosine residues for the triplex formation. In such a situation, the formation of triplex may be further adversely affected by the positive charge to positive charge repulsion along the third strand which occurs as an obligatory consequence of protonation at cytosine/5-methylcytosine in the process. This issue was first raised by Lee et al.[21] This current investigation does not critically test this reasoning, since there are four aforementioned structural differences between **1** and **M**, but it does show the substantially higher stability of oligomers containing **1** instead of **M** in the triplex formation.

It should be noted that many homopurine clusters found in mammalian or virus genomes such as human *c-myc* gene[46] or HSV-I,[47] as well as this portion of the HTLV-III genome studied here, do contain $d(G)_n$ sequences.[48] The application of **1** (2′-*O*-methylpseudoisocytidine) together with oligonucleotide analogues containing a nonionic, neutral backbone (such as methylphosphonate) may allow us to attempt the formation of triplex[38] with genomic DNA inside living cells.

Acknowledgment. The work was supported in part by Department of Energy Grant no. DE-FG02-88 ER60636 and in part by National Cancer Institute Grant no. CA-42762-04A1. The NMR spectroscopy was performed in the NMR Center established by National Institutes of Health Grant no. GM-27512. The JASCO 500A CD spectrometer was kindly provided by Dr. S. K. Yang at the Uniformed Services University of The Health Sciences. We would also like to thank Ms. Tina L. Trapane for her excellent editorial assistance.

Supplementary Material Available: ^1H NMR spectra of **4** and **6** and ^{31}P NMR spectrum of **7** (4 pages). Ordering information is given on any current masthead page.

(44) Janik, B. *Physicochemical Characteristics of Oligonucleotides and Polynucleotides*; Plenum: New York, 1971.
(45) Inoue, H.; Hayase, Y.; Imura, S.; Iwai, S.; Miura, K.; Ohtuska, E. *Nucleic Acids Res.* **1987**, *15*, 1631–6148.
(46) Boles, T. C.; Hogan, M. *Biochemistry* **1987**, *26*, 367–376.
(47) Wohlrab, F.; Mclean, M. J.; Wells, R. D. *J. Biol. Chem.* **1987**, *262*, 6407–6416.
(48) Wells, R. D.; Collier, D. A.; Hanvey, J. C.; Shimizu, M.; Wohlrab, F. *FASEB J.* **1988**, *2*, 2939–2949.