

MONOCLONAL ANTIBODIES WITH AFFINITY TO SELF-COMPLEMENTARY LEFT-HANDED DNA CONTAINING CYCLONUCLEOSIDES WITH HIGH ANTI CONFORMATION

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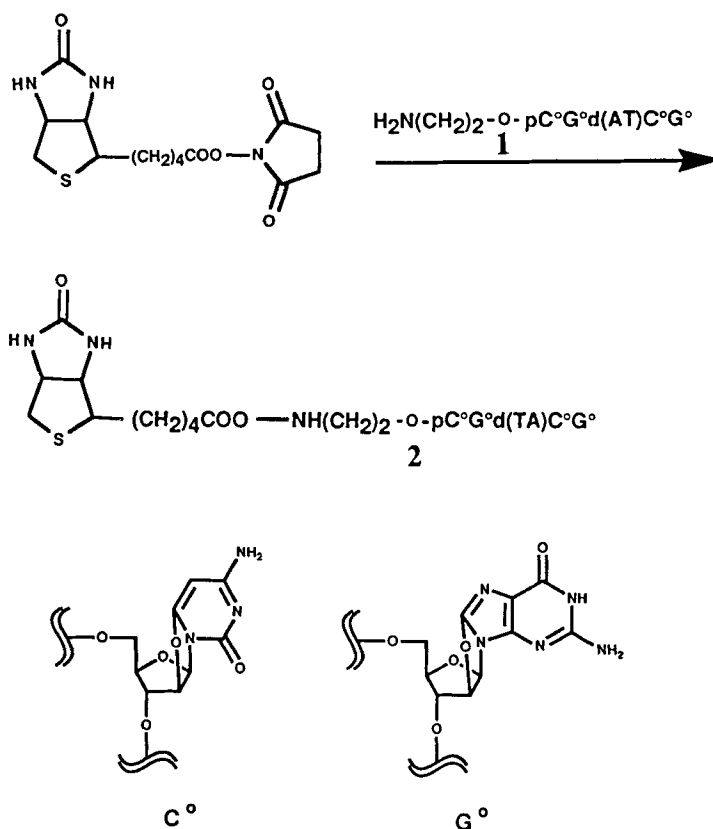
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Abstract. Monoclonal antibodies specific for a self-complementary hexanucleotide with a high-anti conformation were found to bind another hexanucleotide with a high-anti conformation, but with a different sequence. A higher order structure seemed to be recognized in the reaction.

INTRODUCTION

Recognition of the tertiary structure of nucleic acids with proteins can be studied by using a variety of polynucleotide analogs. We have previously synthesized polynucleotides containing cyclonucleosides and reported that they reveal a unique left-handed structure.¹⁻⁵ 8,2'-*S*-Cycloadenosine, for example, had a different torsion angle in an anti range and adopted a high anti conformation ($\chi = 110-120^\circ$ according to Sundaralingam's definition⁶).³ A self-complementary hexamer containing 6,2'-*O*-cyclocytidine (C⁰) and 8,2'-*O*-cycloguanosine (G⁰) (G⁰C⁰dGdCG⁰C⁰) has been studied by UV, CD and ¹HNMR spectroscopy for its left-handed duplex structure in which not only cyclonucleosides but also deoxynucleosides adopted the high anti glycosyl conformation.² Monoclonal antibodies have been raised against this duplex and the antibodies were found to recognize a sequence isomer, C⁰G⁰dCdGC⁰G⁰, but not the

§ This paper is dedicated to the memory of Professor Roland K. Robins.



Scheme 1

natural counterpart, dGCGCGC, indicating that the recognition resulted from the tertiary structure of the oligonucleotide.⁷ In this paper we report that another high-anti hexanucleotide containing cyclonucleosides, 2'-deoxyadenosine and thymidine ($\text{C}^\circ\text{G}^\circ\text{dTdAC}^\circ\text{G}^\circ$)¹ is recognized by one of the antibodies raised against $\text{G}^\circ\text{C}^\circ\text{dGdCG}^\circ\text{C}^\circ$.

RESULTS

Synthesis of oligonucleotides containing cyclonucleosides and biotin Self-complementary oligonucleotides containing cyclonucleosides, thymidine and deoxyadenosine ($\text{C}^\circ\text{G}^\circ\text{dTdAC}^\circ\text{G}^\circ$) have been shown to adopt a left-handed double-helical structure resulting from the high anti glycosyl conformation.¹ To facilitate the immunobinding assay the oligonucleotides were converted to biotinylated derivatives as

shown in Scheme 1. The amino-linked oligonucleotide (1) was prepared by the phosphoramidite method, except monomethoxytritylaminoethyl H-phosphonate was used as the unit for the last condensation. Controlled pore glass was linked to G⁰ as the support. The oligonucleotides were purified by high performance liquid chromatography (HPLC). The biotinylated product (2) was obtained by treatment of 1 with a biotinyl-*N*-hydroxysuccinimide ester.

Binding activities of monoclonal antibodies (a, A56) and (b, B331) with hexanucleotides. 5'-Biotinylated C⁰G⁰dTdAC⁰G⁰, closed circles; 5'-Biotinylated dCGTACG, open circles.

Binding of monoclonal antibodies to oligonucleotides

The self-complementary high-anti oligonucleotide (2) was tested for its ability to bind to the previously obtained two monoclonal antibodies, which recognize the high anti oligonucleotides (G⁰C⁰dGdCG⁰C⁰ and its sequence isomer, C⁰G⁰dCdGC⁰G⁰).⁷ As shown in FIG. 1a, one of the antibodies (A56) binds to the biotin-linked C⁰G⁰dTdAC⁰G⁰ (2) at an order of magnitude lower concentration as compared to the natural counterpart (dCGTACG). The other monoclonal antibody (B331) recognized these oligonucleotides to almost the same extent (FIG. 1b). These results suggest that antibody A56 recognizes a higher structure in the oligonucleotides or it is specific for a high anti conformation of 2. In the previous competition experiment, binding of A56 to G⁰C⁰dGdCG⁰C⁰ was strongly inhibited by the addition of C⁰G⁰dCdGC⁰G⁰, but not by natural hexanucleotides.⁷ On the other hand, binding of antibody B331 to the high anti hexanucleotide was also inhibited by dGCGCGC. The reactivities of these two antibodies were confirmed by the present study in which a more direct binding reaction, using the biotin-linked oligonucleotide was employed.

Experimental

Assay with monoclonal antibodies

Monoclonal antibodies A56 (IgM, ascitic fluid) and B331 (IgM, ascitic fluid) were prepared as described.⁷ A conjugate of streptavidin and horseradish peroxidase (HRP) was obtained from Vector, Inc. and *o*-phenylenediamine (OPD) was obtained from Tokyo Kasei. The assay was performed by the procedure described previously.⁷ EIA plates (96 wells, MaxiSorp) were coated with 100 µl of ascitic fluids diluted 1 : 100 with saline at room temperature overnight, covered with 300 µl of 0.1% BSA, then kept at 4°C. The blocking solution was discarded and each oligonucleotide (50 µl, two-fold serially diluted) was added. The plates were incubated for 1 hr at room temperature and washed

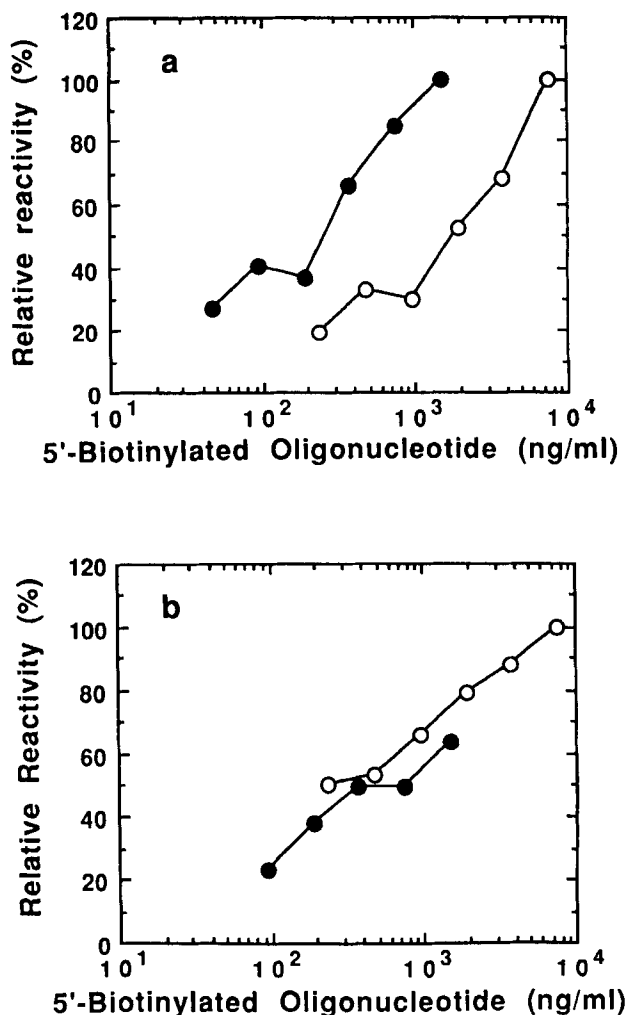


Figure 1

with saline. Diluted streptavidin-HRP conjugate (100 μ l, 1 : 2000) was added, the plate was incubated for 1 hr at room temperature, and then washed. The HRP substrate and OPD (5 mM) were allowed to react with the bound conjugate at room temperature for 30 min. The reaction was terminated by the addition of 100 μ l of 3 N sulfuric acid. Absorbance at 492 nm was measured by an EIA reader (Sanko Junyaku).

General methods for oligonucleotide synthesis

Thin-layer chromatography was performed on silica gel plates (DC-Fertigplatten Kieselgel 60F254, Merck) using a mixture of chloroform and methanol. For purification,

silica gel column chromatography (silica gel 60H, Merck) and alkylated silica gel (C-18, 55-105 μ , Waters) were used under moderate air pressure. HPLC was performed on a column of alkylated silica gel (TSK gel LS-410AK or ODS-120T, Toyo Soda) for reverse phase and a column of *N,N*-diethylaminoethylated silica gel (TSK gel DEAE-2SW, Toyo Soda) for anion exchange. UV spectra were measured on a JASCO UVIDEK-610C or a SHIMADZU UV-2100 spectrometer. CD spectra were measured on a JASCO J-500A spectrometer. Molar ellipticity, $[\theta]$ is presented in terms of per base residue value. ^1H NMR spectra (500 MHz) were measured on a JEOL GX-500 spectrometer. The chemical shifts were determined downfield from internal 2-methyl-2-propanol which has been referenced to DSS.

Derivatives of cyclonucleosides

Cyclonucleosides (G^0 and C^0) were synthesized as previously reported^{8,9} and protected as described.¹ Cyclonucleoside phosphoramidites ($\text{G}^0\text{amidite}$ and $\text{C}^0\text{amidite}$) were prepared from 5'-*O,N*-protected cyclonucleosides, DMTr G^0iBu (0.75 mmol, 490 mg) and DMTr C^0Bz (483 mg) as described.⁹ The yields were 523 mg (0.61 mmol, 81%) and 410 mg (0.48 mmol, 64%), respectively.

Solid support

The G^0resin , a controlled pore glass linked to DMTr G^0iBu

Succinic anhydride (150 mg, 1.5 mmol) and 4-dimethylaminopyridine (DMAP, 183 mg, 1.5 mmol) were added to a solution of DMTr G^0iBu (490 mg, 0.75 mmol) in 4 ml of CH_2Cl_2 and stirred at room temperature for 2 h. The resulting solution was washed with 0.5 M KH_2PO_4 solution (4 ml) and water (4 ml), then the organic layer was evaporated to dryness *in vacuo*. After reverse phase column chromatography, 510 mg (0.68 mmol, 91%) of the product, 3'-succinylated DMTr G^0iBu (G^0suc), was collected. G^0suc (302 mg, 0.40 mmol) was dissolved in 7 ml of CH_2Cl_2 with pentachlorophenol (117 mg, 0.44 mmol) and 1,3-dicyclohexylcarbodiimide (124 mg, 0.60 mmol), and this was kept at room temperature for 12 h. After the dicyclohexylurea was filtered off, pentachlorophenylated G^0suc (G^0pcp) was purified by silica gel column chromatography. The yield was 390 mg (0.39 mmol, 97%). G^0pcp (150 mg, 0.15 mmol) and aminomethylated polystyrene resin (1 g, 30 μmol of NH_2) were mixed in 6 ml of DMF containing 0.2 ml of triethylamine (1.5 mmol), and the mixture was stirred at room temperature for 12 h. The resin was washed with DMF (10 ml, 2 times) and pyridine (10 ml, 2 times), then the capping reaction was carried out with 0.2 ml of acetic anhydride in 2 ml of 0.1 M DMAP/pyridine solution at room temperature for 10 min. The resulting G^0

resin was washed with pyridine (10 ml, 2 times), CH_2Cl_2 (10 ml, 2 times), diethyl ether (10 ml, 2 times) and dried. A quantitative analysis of the coupled DMTrG^oiBu was carried out based on the determination of the amount of dimethoxytrityl residue in a 3:2 HClO_4 /ethanol solution by measuring the absorbance at 500 nm ($\epsilon=7.1734$). 1 g of G^o resin contained 41.7 μmol of DMTrG^oiBu.

5'-Aminoethylating reagent (MTrNHEtOH H-phosphonate)

Monomethanolamine (0.45 ml, 7.5 mmol), diisopropylethylamine (1.3 ml, 7.5 mmol), and methoxytritylchloride (1.55 g, 5 mmol) were dissolved in 20 ml of CH_2Cl_2 and stirred at room temperature for 1 h. CH_2Cl_2 (20 ml) was added and the solution was washed with 40 ml of 50 mM triethylammonium bicarbonate buffer (TEAB, pH8.5). The organic layer was concentrated by evaporation and the residue was applied to a column of silica gel. The eluted product, *N*-methoxytritylmonomethanolamine (MTrNHEtOH), was evaporated *in vacuo* to give a gum. The yield was 1.97 g (4.5 mmol, 91%) as a triethylammonium salt (MTrNHEtOTEA). *N*-Methylmorpholine (5.6 ml, 50 mmol) and PCl_3 (436 μl , 5.0 mmol) were added to 20 ml of CH_2Cl_2 suspended 1,2,4-triazole (1.16 g, 17 mmol). After 15min stirring at room temperature, the solution was transferred to an ice bath, MTrNHEtOTEA (434 mg, 1 mmol) in 10 ml of CH_2Cl_2 was added dropwise, and the resultant mixture was stirred at 0°C for 15 min. The reaction was terminated by the addition of 1 M TEAB (30 ml) and the product was extracted with 50 ml of CH_2Cl_2 . The CH_2Cl_2 layer was washed with 1 M TEAB (30 ml, 3 times) and evaporated *in vacuo*. After purification by silica gel column chromatography, the yield of the yellow gum product was 324 mg (0.75 mmol, 75%).

Amino linked oligonucleotides

d(CGTACG) and C^oG^od(TA)C^oG^o were prepared using the cyanoethyl diisopropylphosphoramidite method¹¹ with 5.0 μmol of G- or G^o resin and 40 μmol (8.0 eq.) of the amidite in each step. The introduction of aminoethylphosphate to the 5' end was carried out by 40 μmol of MTrNHEtOTEA H-phosphonate with pivaloyl chloride as activating agent.¹² The cleavage of these oligonucleotides from the support and the deprotection were carried out with 5 ml of 1:4 pyridine/ NH_4OH solution for 3 h at 55°C. These aminolinked oligonucleotides were isolated with yields of 12% (34 A₂₆₀ units) and 4.7% (14 A₂₆₀ units), respectively, after purification by reverse phase and ion exchange HPLC. The UV, CD and ¹H NMR spectra of these oligonucleotides corresponded to those of the 5'-free oligonucleotides.^{1,2}

Biotinylation of oligonucleotides

Biotinyl-*N*-hydroxysuccinimide ester (BNHS) was prepared from biotin and *N*-hydroxysuccinimide by coupling with dicyclohexylcarbodiimide as described previously.¹³ The amino-linked oligonucleotide (74 A₂₆₀ units, 1.2 μmol) was dissolved in a solution containing BNHS (40 mg, 120 μmol) in 1 ml of 1:1 pyridine/H₂O, and the solution was incubated at 35°C for 24 h. The biotinylated oligonucleotide was isolated with a yield of 72% by gel filtration (G-10), reverse phase column chromatography, and ion exchange HPLC.

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