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Sequence composition effects on the stabilities of triple helix formation by oligonucleotides containing N^7-deoxyguanosine

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ABSTRACT

A nonnatural nucleoside, 7-(2-deoxy-β-D-*erythro*-pento-furanosyl)-guanine (d^7G), mimics protonated cytosine and specifically binds GC base pairs within a pyrimidine·purine·pyrimidine triple helix. The differences in association constants (K_T) determined by quantitative footprint titration experiments at neutral pH reveal dramatic sequence composition effects on the energetics of triple helix formation by oligonucleotides containing d^7G. Purine tracts of sequence composition 5′-d(AAAAAGAGAGAGAGA)-3′ are bound by oligonucleotide 5′-d(TTTTT^{7}GT^{7}GT^{7}GT^{7}GT^{7}GT)-3′ three orders of magnitude less strongly than by 5′-d(TTTTT-^{m}CT^{m}CT^{m}CT^{m}CT^{m}CT)-3′ ($K_T = 1.5 \times 10^6$ M^{-1} and $K_T \geq 3 \times 10^9$ M^{-1} respectively). Conversely, purine tracts of sequence composition 5′-d(AAAAGAAAAGGGGGGA)-3′ are bound by oligonucleotide 5′-d(TTTT^{m}CTT-TT^{7}G^{7}G^{7}G^{7}G^{7}G^{7}GT)-3′ five orders of magnitude more strongly than by 5′-d(TTTT^{m}CTTTT^{m}C^{m}C^{m}C^{m}C^{m}CT)-3′ ($K_T \geq 3 \times 10^9$ M^{-1} and $K_T < 5 \times 10^4$ M^{-1} respectively). The complementary nature of d^7G and ^{m}C expands the repertoire of G-rich sequences which may be targeted by triple helix formation.

INTRODUCTION

The thermodynamic stability of pyrimidine·purine·pyrimidine triple helices decreases with increasing pH due to the requirement of protonating cytosine bases to form C+GC triplets (1–4). Within the context of pyrimidine oligonucleotide-directed recognition of double helical DNA, there are serious sequence composition limitations with regard to targeting contiguous G-rich purine tracts near physiological pH, presumably due to electrostatic repulsion between protonated cytosines in adjacent C+GC triplets. Replacement of cytosine by 5-methylcytosine (^{m}C) increases the stability of pyrimidine·purine·pyrimidine triple helices, but does not alleviate the pH dependence (2–5). Development of oligonucleotides whose energetics of triple helix formation are less sensitive to pH would benefit applications which require near physiologically relevant conditions.

In an attempt to eliminate the necessity for protonation, recent efforts have been directed toward the synthesis of nonnatural nucleosides which display the hydrogen bonding functionality of protonated cytosine (6–17). We previously reported that an N^7-glycosylated purine, 7-(2-deoxy-β-D-*erythro*-pentofuranosyl) guanine (d^7G), when incorporated in a single position within a pyrimidine oligonucleotide, binds with remarkable specificity the Watson–Crick guanine–cytosine (GC) base pair by triple helix formation (16). By attaching the deoxyribose moiety at the N^7-position of a guanine base, the third strand orientation in a G·GC base triplet is reversed and becomes parallel to the purine Watson–Crick strand (Fig. 1) (16).

Although NMR studies do not reveal any major backbone distortion for ^{7}G·GC triplets, the ^{7}G·GC triplet is not isomorphous with adjacent T·AT triplets (18). Envisioning a new parallel-stranded motif comprising wholly N^7 purines for DNA recognition by triple helix formation, we were interested whether triple helices containing multiple d^7G residues are energetically disfavoured relative to ^{m}C at neutral pH.

MATERIALS AND METHODS

All commercially available compounds for organic synthesis were from Aldrich Chemical Co., Milwaukee or Fluka Chemical Corp., St Louis and were used without purification. 5-Methyl-2′-deoxycytidine phosphoramidite was from Biogenex, while thymidine phosphoramidite and chemicals for DNA synthesis were from Glen Research. Restriction endonucleases were from either Boehringer Mannheim or New England Biolabs and used according to the supplier's protocol in the buffer provided. Sequenase (Version 2.0) was from United States Biochemicals. Deoxynucleoside triphosphates (Ultrapure grade), calf thymus DNA (sonicated and phenol extracted), DNase I (FPLCpure) and NAP-10 Sephadex columns were from Pharmacia LKB. Snake venom phosphodiesterase, alkaline phosphatase and glycogen were from Boehringer Mannheim. The radiolabelled triphosphates 5′-[α-^{32}P]dTTP (≥3000 Ci/mmol) and 5′-[γ-^{32}P]ATP (≥6000 Ci/mmol) were from DuPont NEN. Standard molecular biological methods were used, if not mentioned otherwise (18). Silica gel column chromatography was performed on Merck silica gel 60 (230–400 mesh ASTM) according to known protocols using the indicated solvents. NMR spectra were recorded on a QE 300 NMR spectrometer (General

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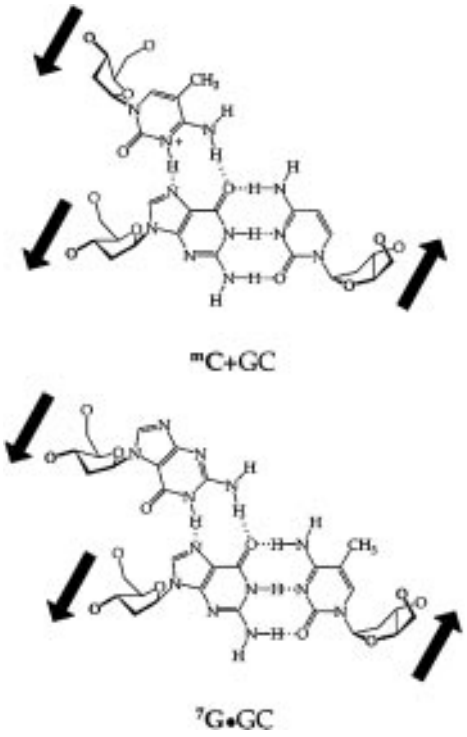

Figure 1. Schematic representation of ^{m}C G · C and ^{7}G · GC (right). The relative backbone orientations are indicated by arrows.

Electric) with the solvent indicated. Chemical shifts are reported in p.p.m. relative to residual undeuterated solvent.

Synthesis of the d^{7}G building block

Two convenient routes for the synthesis of the d^{7}G phosphoramidite are available (16,20). The one described here has four steps, but requires more extensive chromatography than the other, which has eight steps (Fig. 2). The last two steps in both procedures are identical and analytical data obtained for nucleosides **C** and **D** match the previously reported data. Both routes are shorter than the more complicated eight step synthesis of the previously described protonated cytosine mimic P (11).

7-(2′-Deoxy-3′,5′-di-*O*-benzoyl-β-D-*erythro*-pentofuranosyl)-*N*^{2}-isobutyryl-guanine (A)

To a suspension of methyl 3,5-di-*O*-benzoyl-2-deoxy-α,β-D-*erythro*-pentofuranoside (21) (25.70 g, 72.1 mmol) and *N*^{2}-isobutyryl-guanine hydrate (22) (11.50 g, 48.1 mmol) in CH_3CN (250 ml) was added under argon *N*,*O*-bis(trimethylsilyl) acetamide (58.9 ml, 241.4 mmol). After stirring for 8 h at room temperature a clear solution was formed and $SnCl_4$ (16.9 ml, 144.0 mmol) was added dropwise within 20 min. Stirring was continued for 12 h at room temperature. Then the reaction mixture was poured into $CHCl_3$ (800 ml) and washed with water (800 ml) and saturated aqueous $NaHCO_3$ (2 × 800 ml). The aqueous layers were re-extracted with $CHCl_3$ (400 ml). The combined organic phases were dried (Na_2SO_4) and evaporated. The resulting mixture was analyzed by ^{1}H NMR which revealed a glycosylation yield of 68% (48% d^{7}G [1:1, α:β mixture] and 20% d^{9}G [1:1, α:β mixture]. Multiple silica gel column chromatography using toluene–acetone (3:1, v/v) as eluent afforded the title compound as a white foam. Yield 2.64 g (10%). Tlc: EtOAc (0.27). δ_H(CD_3OD) 1.21 (3 H, d, 6.8), 1.23 (3 H, d, 6.8), 2.22–2.36 (1 H, m), 2.85 (1 H, m), 2.99–3.05 (2 H, m), 4.75–4.78 (1 H, m), 4.69–4.71 (1 H, m), 6.77–6.79 (1 H, m), 7.17–7.63 (6 H, m), 7.99–8.10 (4 H, m), 8.19 (1 H, s), 10.65 (1 H, br s), 12.44 (1 H, br s).

7-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)-*N*^{2}-isobutyryl-guanine (B)

A solution of nucleoside **A** (2.64 g, 4.84 mmol) in THF/MeOH/water 5:4:1 (193 ml) was cooled to 0°C. Then a solution of 2 M aqueous NaOH (19.3 ml) was added. After stirring for 25 min at 0°C the reaction was quenched by addition of ammonium chloride (2.49 g, 46.6 mmol) and stirring was continued for 15 min. Then the solution was evaporated. Silica gel column chromatography using CH_2Cl_2–MeOH (6:1, v/v) as eluent afforded the title compound as a white foam. Yield 1.35 g (83%). Tlc: CH_2Cl_2–MeOH (6:1,

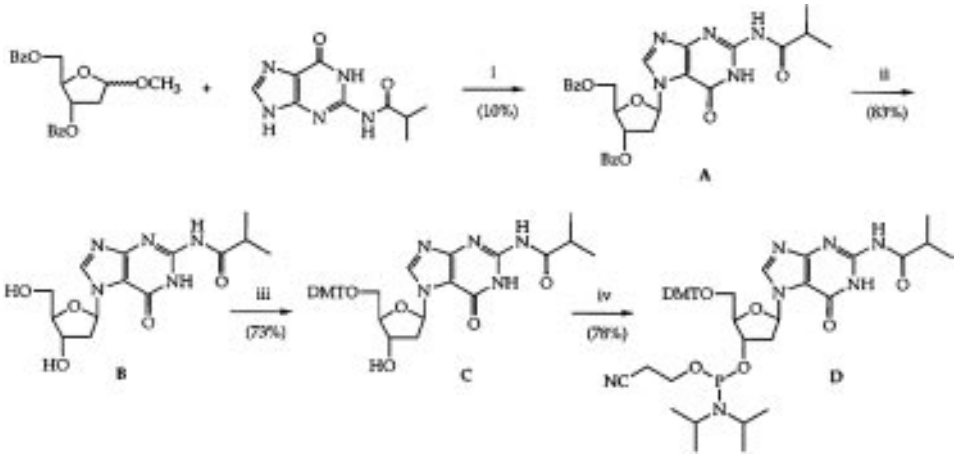

Figure 2. Synthesis of the DMT protected phosphoramidite of d^{7}G: (i) *N*,*O*-bis(trimethylsilyl)acetamide, $SnCl_4$, CH_3CN, room temperature, 2 h, multiple column chromatography; (ii) NaOH, THF, MeOH, H_2O, 0°C, 25 min; (iii) DMTCl, pyridine, room temperature, 4 h; (iv) *N*,*N*-diisopropylethylamine, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, THF, room temperature, 1 h.

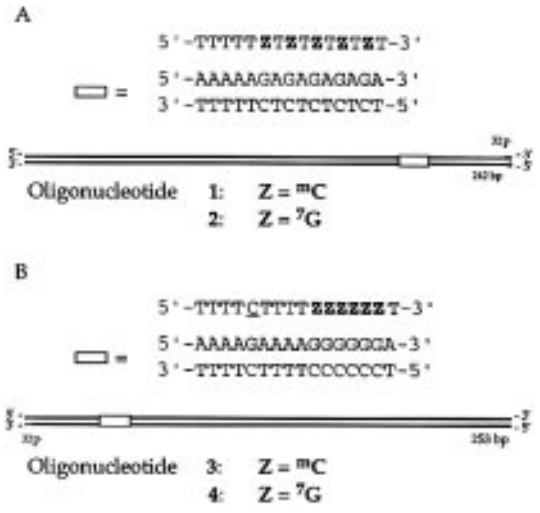

Figure 3. Sequence composition experiments. (**A**) Sequences of oligonucleotides **1** and **2** and the $(GA)_5$ target site. The target site is located within the 242 bp *Eco*RI/*Pvu*II restriction fragment of pGCBGC. (**B**) Sequences of oligonucleotides **3** and **4** and the G_6 target site. The target site is located within the 253 bp *Eco*RI/*Pvu*II restriction fragment of pSPHIV (C indicates 5-methyl-2′-deoxycytidine).

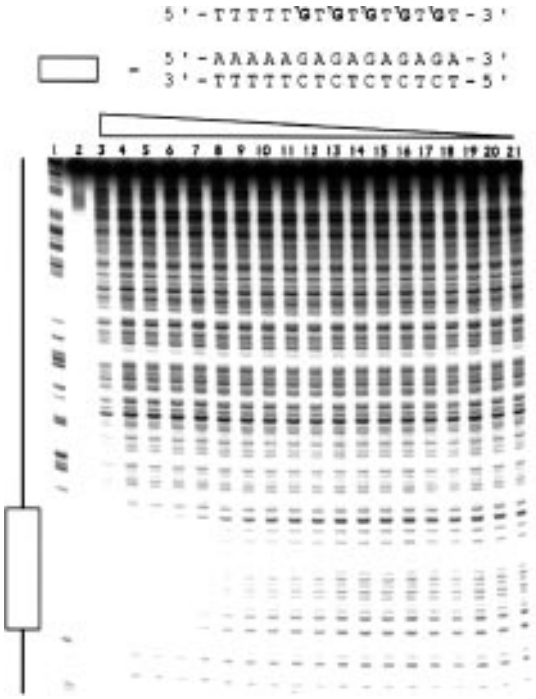

Figure 4. (Top) Sequences of oligonucleotide **2** and the $(GA)_5$ target site. (Bottom) Autoradiogram of an 8% denaturing polyacrylamide gel used to separate the cleavage products generated by DNase I digestion during a quantitative DNase footprint experiment at neutral pH. The bar drawn to the left of the autoradiogram indicates the position of the 15mer binding site within the 242 bp restriction fragment. Lane 1, products of a guanine-specific sequencing reaction; lane 2, intact 5′ labelled duplex obtained after incubation in the absence of a third strand oligonucleotide; lanes 3–20, DNA cleavage products produced by oligonucleotide **2** at various concentrations (40 µM, lane 3; 20 µM, lane 4; 8 µM, lane 5; 4 µM, lane 6; 2 µM, lane 7; 800 nM, lane 8; 400 nM, lane 9; 200 nM, lane 10; 80 nM, lane 11; 40 nM, lane 12; 20 nM, lane 13; 8 nM, lane 14; 4 nM, lane 15; 2 nM, lane 16; 800 pM, lane 17; 400 pM, lane 18; 200 pM, lane 19; 80 pM, lane 20); and lane 21, DNA cleavage products produced in the absence of a third strand oligonucleotide.

v/v, 0.29). ^1H and ^{13}C NMR data are identical with the data reported previously (20).

Synthesis of oligonucleotides

Oligonucleotides containing nonnatural nucleosides were synthesized on an Applied Biosystems Model 380B DNA synthesizer using standard solid-phase β-cyanoethyl phosphoramidite chemistry on an 1 µmol scale. The d^7G phosphoramidite was coupled at 0.15 M concentration using extended coupling times (6 min). Coupling efficiencies were ≥96%. 5′-OH deprotected oligonucleotides were treated with concentrated ammonia at 55°C for 48 h. The solutions were lyophilized and purified twice, initially by ion exchange FPLC on a MonoQ HR 10/10 column (Pharmacia) using a linear gradient of 0.1–1.0 M NaCl in 0.01 M bis Tris–HCl, pH 7.0, then a second time by reversed phase FPLC on a ProRPC HR 10/10 column (Pharmacia) using a linear gradient of 0.1 M TEAA and 40% CH_3CN in TEAA (0.1 M, pH 7.0). The purified oligonucleotides were desalted on NAP-10 columns and the concentrations of all oligonucleotides determined by UV measurement at 260 nm using the following molar extinction coefficients: 4000 (d^7G); 5700 (^mC) and 8800 (T) $cm^{-1}\,M^{-1}$. The oligonucleotide solutions were divided into aliquots, lyophilized to dryness, and stored at –78°C.

Enzymatic oligonucleotide degradation and HPLC analysis

The purified oligodeoxyribonucleotides (10 nmol) were digested with 3 U snake venom phosphodiesterase and 0.01 U calf intestine alkaline phosphatase in 50 µl of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 8.0. The reaction mixture was incubated at 37°C for 3 h and then analyzed for nucleoside content by HPLC. Analytical HPLC analysis (Hewlett-Packard 1090 liquid chromatograph) was performed on a C18 reversed phase column (Rainin, Microsorb-MV™, 5 micron, 4.6 × 250 mm), using a linear gradient of 0–40% CH_3CN in 20 mM ammonium acetate pH 5.0 (1.0 ml/min, 120 bar, 37°C) and detection at 260 nm. Coinjection with standard solutions of d^7G, ^mC and T confirmed the identity of the oligodeoxyribonucleotides and integration of peak areas confirmed the base composition. The exact mass of the oligonucleotides was confirmed by MALDI TOF mass spectrometry, performed at the Protein/Peptide Micro Analytical Facility at the California Institute of Technology.

DNA labelling

The pSPHIV plasmid DNA was digested with *Eco*RI, 3′-end-labelled with [α-^{32}P]dATP and [α-^{32}P]TTP using Sequenase™ (version 2.0), and then digested with *Pvu*II. The pGCBGC plasmid DNA was digested with *Eco*RI, then treated with calf alkaline phosphatase, 5′-end-labelled with [γ-^{32}P]ATP using T4 kinase and digested again with *Pvu*II. Unincorporated radiolabelled nucleotide triphosphates were removed on a gel filtration column (Microspin S-200 HR, Pharmacia) and both labelled fragments purified by 5% nondenaturing polyacrylamide gel electrophoresis. The desired gel bands were visualized by autoradiography, excised, crushed and eluted overnight at 37°C with extraction

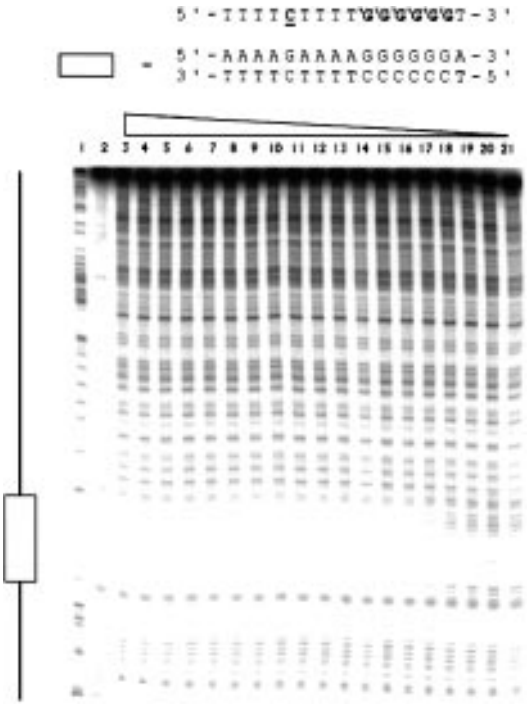

Figure 5. (**Top**) Sequences of oligonucleotide **4** and the G_6 target site. (**Bottom**) Autoradiogram of an 8% denaturing polyacrylamide gel used to separate the cleavage products generated by DNase I digestion during a quantitative DNase footprint experiment at neutral pH. The bar drawn to the left of the autoradiogram indicates the position of the 16mer binding site within the 253 bp restriction fragment. Lane 1, products of a guanine-specific sequencing reaction; lane 2, intact 3′ labelled duplex obtained after incubation in the absence of a third strand oligonucleotide; lanes 3–20, DNA cleavage products produced by oligonucleotide **4** at various concentrations (40 µM, lane 3; 20 µM, lane 4; 8 µM, lane 5; 4 µM, lane 6; 2 µM, lane 7; 800 nM, lane 8; 400 nM, lane 9; 200 nM, lane 10; 80 nM, lane 11; 40 nM, lane 12; 20 nM, lane 13; 8 nM, lane 14; 4 nM, lane 15; 2 nM, lane 16; 800 pM, lane 17; 400 pM, lane 18; 200 pM, lane 19; 80 pM, lane 20); and lane 21, DNA cleavage products produced in the absence of a third strand oligonucleotide.

buffer (25 mM Tris–HCl, 250 mM NaCl, 1 mM EDTA, pH 8.0). This solution was filtered through a 0.45 µm Centrex filter, the restriction fragment precipitated with isopropyl alcohol, resuspended in TE (pH 7.5), phenol extracted and reprecipitated with EtOH. The pellet was resuspended in TE (pH 7.5) to achieve a final activity of ~30 000 c.p.m./µl and stored at –20°C.

Quantitative footprinting titrations (17,23,24)

In a typical quantitative footprinting titration experiment, a stock solution containing labelled target DNA in equilibration buffer was prepared by combining 189 µl of a 5× stock equilibration buffer (50 mM bis Tris–HCl, 500 mM NaCl and 1.2 mM spermine hydrochloride, pH 7.0 or 7.5), 94.5 µl calf thymus DNA (50 µM bp), 12.6 µl labelled DNA (30 000 c.p.m./µl) and 271 µl H_2O. The stock solution was then distributed in 27 µl aliquots among 20 labelled 1.7 ml microcentrifuge tubes. A dried pellet of the oligonucleotide (10 nmol) was dissolved in H_2O to produce a 100 µM solution which was diluted serially to afford 18 dilutions from 40 µM to 80 pM. To each of the first 18 equilibration reaction tubes was added 18 µl of the appropriate oligonucleotide dilution along with 18 µl H_2O to the 19th and 20th tube and the mixtures were allowed to equilibrate for 72 h at 24°C. The footprinting degradation reactions were initiated by adding 5 µl of a DNase I (0.5 mU/µl) footprinting solution to each equilibration reaction tube with the exception of the 20th tube. The DNase I footprinting solution was prepared immediately before addition as follows: 2 µl DNase I (10 mU/µl) was diluted in an enzyme dilution buffer composed of 20 mM bis Tris–HCl, 50 mM $MgCl_2$, 50 mM $CaCl_2$ and 5% glycerol at pH 7.0 or 7.5 to afford a 0.5 mU/µl solution of DNase I. The digest reactions were allowed to proceed for 6 min at 24°C and were then quenched with the addition of 8.3 µl of a stop solution to each tube. The stop solution was prepared from 175 µl of 2 M NaCl, 4.4 µl glycogen (20 mg/ml) and 70 µl of 0.5 M EDTA, pH 8.0. The reaction mixtures were ethanol precipitated, washed with 75% ethanol and lyophilized to dryness from 20 µl H_2O. The pellets in each tube were resuspended in 5 µl formamide loading buffer containing 1× TBE and assayed for specific activity by scintillation counting. The DNA was denatured at 85°C for 10 min, chilled on ice and loaded onto an 8% denaturing polyacrylamide gel (19:1; monomer/bis). The specific activities of the empty tubes were assayed by scintillation counting to determine the activities of the loaded samples. The gels were then dried and quantified by storage phosphor autoradiography using ImageQuant software (Molecular Dynamics) (24).

RESULTS

Sequence composition effects on the energetics of triple helix formation by oligonucleotides containing multiple d^7G moieties were examined for two different purine target sites within 242 and 253 bp restriction fragments at pH 7.0 and 7.5. The sequence of one site is the purine tract 5′-d(AAAAAGAGAGAGAGA)-3′ in a 242 bp restriction fragment [referred to as the $(GA)_5$ site] (Fig. 3A). The other purine site is derived from the LTR region of the HIV genome and has the sequence 5′-d(AAAAGAAAAG-GGGGGA)-3′ in a 253 bp restriction fragment (referred to as the G_6 site) (Fig. 3B).

The energetics of association of oligonucleotides **1** and **2** of sequence composition 5′-d(TTTTT^{m}CT^{m}CT^{m}CT^{m}CT^{m}CT)-3′ and 5′-d(TTTTT^{7}GT^{7}GT^{7}GT^{7}GT^{7}GT)-3′ respectively, allow comparison of the ability of ^{m}C and d^7G to bind multiple GC base pairs in a target sequence composed of alternating G and A nucleotides [the $(GA)_5$ site] (Table 1). Similarly, the energetics of binding oligonucleotides **3** and **4** of sequence composition 5′-d(TTTT^{m}CTTTT-^{m}C^{m}C^{m}C^{m}C^{m}CT)-3′ and 5′-d(TTTT^{m}CTTTT^{7}G^{7}G^{7}G^{7}G^{7}G^7-GT)-3′ allow this comparison for a target sequence containing contiguous GC base pairs (the G_6 site) (Table 2).

Table 1. Equilibrium association constants for triple helix formation at the $(GA)_5$ site[a]

Oligo	pH	K_T (M^{-1})
1	7.0	$\geq \mathbf{3 \times 10^9}$
	7.5	9.4×10^7
2	7.0	1.5×10^6
	7.5	1.1×10^6

[a]Each reported K_T value is the mean of three independent measurements which were performed in 100 mM NaCl, 10 mM bis Tris–HCl, 250 µM spermine at the indicated pH and 22°C.

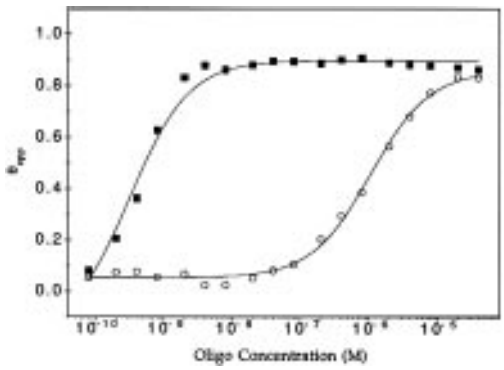

Figure 6. (θ_{app}, $[O]_{tot}$) data derived from the DNase footprinting gels shown in Figures 4 and 5 for binding of oligonucleotide **2** (°) and **4** (n) to the $(GA)_5$ and G_6 target sites respectively.

Table 2. Equilibrium association constants for triple helix formation at the G_6 site[a]

Oligo	pH	K_T (M^{-1})
3	7.0	$<5 \times 10^4$
	7.5	$<10^4$
4	7.0	$\geq \mathbf{3 \times 10^9}$
	7.5	$\geq 2 \times 10^9$

[a]Each reported K_T value is the mean of three independent measurements which were performed in 100 mM NaCl, 10 mM bis Tris–HCl, 250 µM spermine at the indicated pH and 22°C.

Consistent with previous data which has shown that triple helix formation by oligonucleotides containing cytosine or ^{m}C is pH-dependent (2–5,17), we have reported recently that oligonucleotide **1**, containing ^{m}C and T, binds tightly to the $(GA)_5$ site ($K_T \geq 3 \times 10^9\ M^{-1}$) at pH 7.0, and that this affinity drops by a factor of ≥30 as the pH is increased to 7.5 (17). At pH 7.0, oligonucleotide **2**, containing d^7G in place of ^{m}C, binds three orders of magnitude weaker than oligonucleotide **1**, albeit pH independently over the range studied (Figs 4 and 6). The relative affinities are reversed for the G_6 target sequence containing contiguous GC base pairs. Oligonucleotide **3**, which contains $(^mC)_6$, binds the G_6 site very weakly ($K_T < 5 \times 10^4\ M^{-1}$) at pH 7.0. In the case of oligonucleotide **4**, which contains $(d^7G)_6$, the association constant at pH 7.0 is very high ($K_T \geq 3 \times 10^9\ M^{-1}$) (Figs 5 and 6). Notably, the high affinity of oligonucleotide **4** decreases by a factor of <2 at pH 7.5.

DISCUSSION

The $(GA)_5$ site contains ten 5′-AG-3′ or 5′-GA-3′ junctions, while the G_6 site contains only two such junctions in the region of interest. The three orders of magnitude difference in the affinity of d^7G containing oligonucleotides for these two sites is probably due to the lack of structural isomorphism in the ^{7}G · GC and T · AT triplets. The location of the third strand deoxyribose-phosphate backbone is not identical when the two triplets are overlaid. Thus, the 5′-AG-3′ and 5′-GA-3′ junctions could generate energetically unfavourable distortions in the backbone in triple helical complexes relative to contiguous A or G sequences. In the case of ^{m}C containing oligonucleotides the large difference in affinity for the two sites is probably due to electrostatic repulsion between adjacent protonated ^{m}C bases (17).

A quantitative study of the energetics of triple helix formation for different purine tracts has revealed that ^{m}C and d^7G provide complementary solutions to the recognition of GC base pairs by triple helix formation. At neutral pH, ^{m}C binds isolated GC base pairs with higher affinity than d^7G, while d^7G binds contiguous GC base pairs with higher affinity than ^{m}C. Remarkably, both types of G-rich tracts can be targeted by choice of the appropriate oligonucleotide composition.

In conclusion, we found that within the measured pH range the stabilities of d^7G containing triple helical structures were strongly dependent on the sequence context and independent from the pH. A third strand oligonucleotide composed of contiguous d^7G nucleosides provides a conveniently accessible solution for targeting contiguous guanosine and hence broadens the sequence repertoire available by oligonucleotide directed triple helix formation.

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