Effect of competing self-structure on triplex formation with purine-rich oligodeoxynucleotides containing GA repeats

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ABSTRACT

Competition between triplex formation with double-stranded DNA and oligonucleotide self-association was investigated in 23mer GA and GT oligonucleotides containing d(GA)₅ or d(GT)₅ repeats. Whereas triplex formation with GT oligonucleotides was diminished when temperature increased from 4 to 37°C, triplex formation with GA oligonucleotides was enhanced when temperature increased within the same range due to the presence of competing intermolecular GA oligonucleotide self-structure. This self-structure was determined to be a homoduplex stabilized by the internal GA repeats. UV spectroscopy of these homoduplexes demonstrated a single sharp transition with rapid kinetics ($T_m = 38.5\text{--}43.5^\circ\text{C}$ over strand concentrations of 0.5–4 μM , respectively, with transition enthalpy, $\Delta H = -89 \pm 7$ kcal/mol) in 10 mM MgCl₂, 100 mM NaCl, pH 7.0. Homoduplex formation was strongly stabilized by multivalent cations (spermine > Mg²⁺ = Ca²⁺) and destabilized by low concentrations of monovalent cations (K⁺ = Li⁺ = Na⁺) in the presence of divalent cations. However, unlike GA or GT oligonucleotide-containing triplexes, the homoduplex formed even in the absence of multivalent cations, stabilized by only moderate concentrations of monovalent cations (Li⁺ > Na⁺ > K⁺). Through the development of multiple equilibrium states and the resulting depletion of free oligonucleotide, it was found that the presence of competing self-structure could decrease triplex formation under a variety of experimental conditions.

INTRODUCTION

Alternating d(GA)-d(TC) sequences are abundant in chromosomal DNA, representing $\leq 0.4\%$ of the mammalian genome (1). These tracts are generally located in non-coding regulatory regions of eukaryotic genes and have been implicated in a variety

of biological processes including recombination and transcriptional control. Due to their homopurine/homopyrimidine nature and frequent localization in upstream promoter regions, these tracts also represent potential target sites for transcriptional repression by triple helix (triplex)-forming oligonucleotides.

Short pyrimidine-rich oligonucleotides can bind in the major groove of double-stranded (ds) DNA oriented parallel to the corresponding purine strand of ds polypurine/polypyrimidine DNA (2,3). A second structural triplex motif also demonstrates that a purine-rich third strand composed of either G and A or G and T residues can bind in a multivalent cation-dependent fashion in the major groove of ds polypurine/polypyrimidine DNA (4,5). GA third strands are restricted to a parallel orientation while GT third strands bind in an orientation dictated by the number of GpA and ApG steps within the target sequence (6). An oligonucleotide containing T, C and G has been shown to bind in a parallel orientation with respect to the target purine strand (7).

Whereas successful *in vitro* inhibition of transcription and transcription factor binding has been mediated by both pyrimidine-rich (8–10) and purine-rich oligonucleotides (5,11–17), of the relatively few demonstrations of successful *in vivo* inhibition of transcription, purine-rich oligonucleotides (18–22) have been employed far more often than pyrimidine-rich oligonucleotides (10,23,24). Purine-rich third strands can be designed to form either G.G–C and A.A–T triplets (GA oligonucleotides), or G.G–C and T.A–T triplets (GT oligonucleotides). Both GA and GT oligonucleotides have demonstrated sequence-specific triplex formation *in vitro* as well as inhibition of gene transcription *in vivo*. However, general consensus as to which purine-rich oligonucleotide confers greater binding affinity to a given target sequence and/or greater potential for transcriptional repression has not yet been reached. Previous reports have compared GA and GT oligonucleotide triplex binding at single temperatures and experimental conditions, but results have not demonstrated a clear or consistent trend. Many factors may be responsible for differences between GA and GT oligonucleotide-mediated

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triplex formation including target DNA sequence, experimental conditions and competing oligonucleotide self-association (25).

We have investigated GA and GT oligonucleotides targeted to an upstream sequence of the human insulin growth factor-1 (IGF-1) gene (26) to examine the effect of ionic strength, temperature and competing oligonucleotide self-structure on triplex formation and binding affinity. This target sequence, located within a cluster of IGF-1 transcriptional start sites 295 bp upstream of the initiation codon, contains a 23 bp polypurine/polypyrimidine tract which includes a series of d(GA)-d(CT) repeats (Fig. 1). We have previously studied binding of pyrimidine-rich oligonucleotides to this 23 bp sequence (27). Here we present triplex studies obtained on this same target, but with 23mer GA and GT oligonucleotides.

Whereas 23mer-GT oligonucleotides demonstrated strong triplex binding at low temperatures with successive decreases in binding with increasing temperature (consistent with all other known reports of triplex interactions), 23mer-GA oligonucleotides demonstrated weak triplex binding at low temperatures with successive increases in binding with increasing temperature. This oppositely-oriented relationship was investigated further and was determined to result from stable competing 23mer-GA homoduplex formation.

Homoduplex structures have been documented for a variety of oligonucleotides and polymers including poly dC (28), alternating d(CT) oligonucleotides (29), and alternating d(CGA) oligonucleotides (30–32). Oligonucleotides containing alternating d(GA) sequences have also been shown to form duplex structures stabilized by both interstrand base-pairing interactions and intra/interstrand base-stacking interactions (33–35). Proposed base-pairing schemes have included alternating $G_{syn} \cdot G_{syn}$ and $A_{anti} \cdot A_{anti}$ (33), alternating $G_{anti} \cdot G_{anti}$ and $A_{anti} \cdot A_{anti}$ (35), and alternating $G_{anti} \cdot A_{syn}$ and $A_{anti} \cdot G_{anti}$ base-pairs (34). Evidence for both parallel and antiparallel strand orientations have also been presented (33,34,36), although the antiparallel orientation of GA homoduplexes may rely on neighboring Watson–Crick base pairs to confer orientational preference. Four-stranded quadruplex structures have also been proposed for d(GA) polymers (37) based upon modeling and X-ray diffraction studies of poly (rI) and poly (rG) (38,39).

This report provides further evidence for the formation of homoduplexes in oligonucleotides containing d(GA) but not d(GT) repeats, and compares and contrasts the range of ionic and temperature conditions in which triplex and homoduplex structures form. This report also illustrates and explains the oppositely-oriented temperature dependences of triplex formation with GA and GT oligonucleotides, and provides evidence through thermodynamic analysis and experimental results, that a tract of d(GA) repeats in a triplex-forming oligonucleotide can result in a substantial decrease in triplex formation due to competing stable oligonucleotide self-association.

MATERIALS AND METHODS

Oligonucleotides

Unmodified oligonucleotides were purchased from Eurogentec (Belgium) and purified by polyacrylamide gel electrophoresis. Concentrations were determined spectrophotometrically at 50°C using molar extinction coefficients at 260 nm calculated from a nearest-neighbor model (40): 42mer-R, $\epsilon = 4.38 \times 10^5$ /M/cm;

42mer-Y, $\epsilon = 3.7 \times 10^5$ /M/cm; 23mer-GA and 23mer-GA_{inv}, $\epsilon = 2.66 \times 10^5$ /M/cm; 23mer-GT, $\epsilon = 2.17 \times 10^5$ /M/cm; 23mer-CT, $\epsilon = 1.76 \times 10^5$ /M/cm; 42mer-R_{inv}, $\epsilon = 4.41 \times 10^5$ /M/cm; 42mer-Y_{inv}, $\epsilon = 3.73 \times 10^5$ /M/cm; 16mer-GA, $\epsilon = 1.85 \times 10^5$ /M/cm; 15mer-GA, $\epsilon = 1.75 \times 10^5$ /M/cm.

Gel retardation assay

For triplex gel retardation, either the purine strand of the 42mer duplex or the triplex-forming oligonucleotide was 5' end-labeled with [γ -³²P]ATP (Amersham, Arlington Heights, IL) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Increasing concentrations (10 nM–10 μ M) of either the triplex-forming oligonucleotide or the corresponding 42mer duplex target, were added to 10 nM of the labeled 42mer duplex or triplex-forming oligonucleotide, respectively, in the presence of 1–10 mM MgCl₂ (as indicated), 100 mM NaCl, 10% sucrose, and 0.5 μ g/ μ l tRNA with sample incubation at the indicated temperature (4–37°C) overnight. Electrophoresis was performed on a non-denaturing 10% polyacrylamide gel containing 1–10 mM MgCl₂ and 50 mM Hepes, pH 7.2 at the incubation temperature.

For single-stranded (ss) gel retardation, 10 nM of 5' end-labeled 23mer-GA or GT oligonucleotide was added to increasing concentrations (10 nM–50 μ M) of the corresponding cold oligonucleotide in the presence of 1–10 mM MgCl₂, 100 mM NaCl, 10% sucrose and 0.5 μ g/ μ l tRNA, with sample incubation at the indicated temperature (4–37°C) overnight. Electrophoresis was performed as described for triplex gel retardation. Reference duplexes were made by annealing 10 nM of radiolabeled 23mer-GA to 20 nM of the complementary 23mer-CT strand in 10 mM MgCl₂, 100 mM NaCl.

UV absorption spectroscopy

A Kontron Uvikon 940 spectrophotometer with 1 cm optical path length quartz cuvettes was used to study thermal denaturation and renaturation of oligonucleotide homoduplexes. The cell holder was thermoregulated by an 80% water/20% ethylene glycol circulating liquid as previously described (27). Sample temperature was decreased from 70 to 1°C and increased back to 70°C at 0.2°C/min with absorption readings at 260 and 540 nm taken every 5 min. Samples were maintained at each temperature extreme for an additional 15 min. Experimental conditions were as described in the figure and table legends. All samples contained 10 mM sodium cacodylate, pH 7.0, unless otherwise noted. For melting temperature (T_m) analysis, absorptions at 540 nm were subtracted from those at 260 nm and first derivatives plotted against 1/Temperature (in K⁻¹). T_m was estimated as the value at which dA/dT reached maximum.

Thermodynamic analysis of homoduplex and triplex formation

Thermodynamic parameters of homoduplex formation were calculated from thermal denaturation profiles based upon a two-state bimolecular association model for self-complementary oligonucleotides: 23mer-GA + 23mer-GA $\leftrightarrow$ (23mer-GA)₂. This assumption gives rise to the following equations for deriving enthalpy, entropy and free energy values for the single-strand to homoduplex transition:

As Mg^{2+} concentration was lowered to 5 and 1 mM, the temperature/triplex formation correlations were maintained for the two oligonucleotides, although absolute triplex-binding affinity decreased substantially in accordance with known

Figure 3. Self-association of 23mer-GT and 23mer-GA oligonucleotides. Oligonucleotide self-association was investigated using incubation and electrophoresis conditions described for Figure 2. In each gel, lanes 1–6 correspond to added concentrations of 0, 10, 100 nM, 1, 10 and 50 μ M of cold 23mer-GT (A) or 23mer-GA (B, C and D), respectively, to 10 nM of the corresponding radiolabeled oligonucleotide (*). Lane R refers to the position of the reference 23 bp duplex.

divalent cation dependence on triplex formation (4) (data not shown).

GA oligonucleotides form gel retarded complexes migrating as duplexes

These oppositely-oriented temperature dependences with GA and GT triplex-forming oligonucleotides were investigated further by ss gel retardation assays to explore the possibility of competing higher-order oligonucleotide interactions (Fig. 3). Under identical conditions used in triplex gel retardations, the 23mer-GT oligonucleotide did not support any higher-ordered self-structure (Fig. 3A). In contrast, the 23mer-GA oligonucleotide supported extensive self-structure (Fig. 3B, C and D). This self-structure was destabilized with increasing temperature and was strongly dependent on oligonucleotide strand concentration. The association kinetics were rapid—5 min and 5 day incubations produced the same gel retardations.

As shown in Figure 3B, nearly all the 23mer-GA oligonucleotide had slightly reduced mobility relative to a Watson–Crick duplex composed of 23mer-GA and 23mer-CT at 4°C. Bands of slightly reduced mobility relative to standard B-form duplexes have previously been shown to be characteristic of parallel-stranded duplexes (33,44). However, at 20°C (Fig. 3C) and 37°C (Fig. 3D), the mobilities of retarded bands demonstrated a concentration dependence, and only at higher oligonucleotide concentrations (10–50 μ M) did the mobilities correspond with the complexes found at 4°C. Further experimentation with intermediate concentrations between 100 nM and 10 μ M gave rise to bands of intermediate mobility, and experiments with 100 μ M oligonucleotide concentrations did not produce any additional gel retardation (data not shown). These discrete bands with mobilities intermediate between ss and ds species were also noted by Rippe *et al.* (33) within the same range of concentrations and

Figure 4. Ionic effects on 23mer-GA structural transitions. Effects of increasing magnesium (A) and increasing monovalent cations in the presence and absence of magnesium (B) were investigated by UV absorption spectroscopy at 260 nm and 540 nm. Samples in (A) contained 100 mM NaCl and 10 mM sodium cacodylate, pH 7.0 and 2 μ M 23mer-GA oligonucleotide in addition to the indicated magnesium concentrations. Samples in (B) contained 10 mM sodium cacodylate, pH 7.0 and 2 μ M 23mer-GA oligonucleotide in addition to the indicated monovalent cation concentrations in the presence or absence of 10 mM MgCl_2 .

experimental conditions, and were presumed to be due to partially dissociated homoduplex. Another explanation is that the rapid concentration and temperature-dependent kinetics allow for association and dissociation to occur within the gel, leading to a statistical average mobility at each strand concentration. This same phenomenon has been noted in concentration-dependent changes in sedimentation rate when rapidly equilibrating self-associating molecules are subject to ultracentrifugation [reviewed in (45)].

UV absorption spectroscopy on GA self-associated complexes

Thermal profiles of these GA oligonucleotide structures were obtained by UV absorption spectroscopy. The resulting melting curves demonstrated single sharp transitions at temperatures

strongly dependent on cation concentration (Fig. 4; Table 1). In all cases, curves were reversible with no apparent hysteresis. No intermolecular structural transitions could be detected under any of a variety of conditions for the 23mer-GT or the 23mer-GA oligonucleotide in the absence of both mono- and multivalent cations.

Figure 4A illustrates the effect of increasing Mg^{2+} concentration from 0 to 10 mM in the presence of 100 mM NaCl. Even in the absence of divalent cations, a clear transition could be noted with $T_m = 23^\circ C$, and as Mg^{2+} concentration increased to 1, 5 and 10 mM, T_m increased to 30, 37.5 and $40^\circ C$, respectively. A similar trend existed in the absence of multivalent cations and the presence of increasing Na^+ , K^+ or Li^+ monovalent cations (Fig. 4B), demonstrating that multivalent cations are not required for retention of the complex at physiological temperatures. The presence of 10 mM $MgCl_2$ at lower concentrations of monovalent cations served to decrease T_m while at higher monovalent cation concentrations, $MgCl_2$ had little effect on T_m . Interestingly, for both mono- and divalent cations, relative hypochromism correlated with cation concentration. Should the structure be the homoduplex described by Rippe *et al.* (33) and stabilized by the

internal alternating d(GA) repeats, the higher cation concentrations may have led to increased G.G and A.A associations outside the alternating sequence, bringing the strands into closer apposition with greater stacking interactions.

Table 1 illustrates the effects of spermine $\cdot Cl_4$ and $CaCl_2$ on T_m . When compared in isolation, spermine had the greatest stabilizing effect on the complex, followed equally by Mg^{2+} and Ca^{2+} . Thus, in categorizing cation effect on T_m values, spermine $> Mg^{2+} = Ca^{2+} > Li^+ > Na^+ > K^+$. These overall trends are similar to those described in recent reports on the effect of varying monovalent, divalent and multivalent cations on triplex formation (46–48). In addition, Table 1 demonstrates the concentration dependence of T_m , providing further evidence that this structure is intermolecular rather than intramolecular. This structure was also unaffected by protonation of the N1 adenine position as seen by the nearly identical T_m at pH 4.2 and 7.0. Using the equations given above, $\Delta H_{GA} = -89 \pm 7$ kcal/mol and $\Delta S_{GA} = -0.26 \pm 0.02$ kcal/mol for complexes in 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.0. Thus, $\Delta G_{GA} = -8.8 \pm 1$ kcal/mol at $37^\circ C$. All curves analyzed demonstrated a close fit to a two-state model of bimolecular association as judged by the linearity of $\ln(K)$ versus $1/T$ plots.

Table 1. Effects of multivalent cations, sequence, and concentration on T_m

Oligonucleotide	Concentration (μM)	Mg^{2+} (mM)	Ca^{2+} (mM)	Spermine $\cdot 4^+$ (mM)	T_m ($^\circ C$)
23mer-GA/23mer-GA	2	0	0	0	23
	2	1	0	0	30
	2	5	0	0	37.5
	2	10	0	0	40
	2	15	0	0	42.5
	2	20	0	0	43.5
	2	0	5	0	36
	2	0	10	0	40
	2	0	15	0	42
	2	0	0	2.5	42
	2	0	0	5	44
	0.5	10	0	0	38.5
	2	10	0	0	40
	4	10	0	0	43.5
23mer-GA/23mer-GA, pH 4.2	2	10	0	0	38.5
23mer-GAinv/23mer-GAinv	2	10	0	0	40.5
	2	0	10	0	41
	2	0	0	2.5	42.5
23mer-GA/23mer-CT	2	10	0	0	62.5
23mer-GT/23mer-GT	2	10	0	0	NA
15mer-GA/15mer-GA	4	10	0	0	29.5
	10	10	0	0	31.5
16mer-GA/16mer-GA	4	10	0	0	32
	10	10	0	0	36

All samples contained sodium cacodylate, pH 7.0 as a buffer except at pH 4.2 where 20 mM sodium acetate was used. Samples also contained 100 mM sodium chloride. T_m determinations were rounded off to the nearest $0.5^\circ C$ and error is estimated at $\pm 1^\circ C$. NA = no transition detected.

To verify that the complexes seen by gel retardation matched the complexes seen by UV absorption spectroscopy, a ss gel retardation was performed in 1 mM MgCl₂. In good correlation with the Mg²⁺ dependence curve of Figure 4A (T_m = 30°C), nearly all the 23mer-GA migrated as a duplex at 4°C, whereas only slight retardation was detected at 50 μM strand concentration at 37°C (data not shown).

The structure is a homoduplex and stabilized by the d(GA) repeats

In order to determine the sequence elements responsible for the structure formed by 23mer-GA, we investigated the complex formation between differing oligonucleotides. We found that the structure formed with 10 nM of radiolabeled 23mer-GA and increasing concentrations (10 nM–50 μM) of 23mer-GA_{inv}, as well as with 10 nM of radiolabeled 23mer-GA_{inv} and increasing concentrations of 23mer-GA. These hybrid structures gave rise to similar patterns of gel retardation seen in the homo-structures of Figure 3B, C and D (data not shown). This observation is consistent with previous reports stating that the complex is stabilized by the alternating d(GA) sequence and that three to four GA repeats are sufficient for stabilization. By contrast, no duplex formation was detected with 10 nM radiolabeled 23mer-GA or 23mer-GA_{inv} and increasing concentrations of 23mer-GT or 15mer-GA. 15mer-GA, a shorter GA oligonucleotide with similar GA composition but no more than two contiguous GA repeats, did not support self-structure by gel retardation (data not shown), but did exhibit some intermolecular structure by UV spectroscopy (Table 1). The 16mer-GA, a shorter GA oligonucleotide with a d(GA)₅ repeat similar to 23mer-GA did support self-structure (Table 1). Both the 15mer-GA and the 16mer-GA structures are less stable than the self-structure adopted by 23mer-GA, suggesting that the d(GA) repeat and neighboring sequences play a stabilizing role in self-structure formation.

Both the gel mobility of the complex and the ability to fit closely the thermal denaturation profiles to a model of bimolecular association argue strongly that the structure is composed of two 23mer-GA strands. However, to prove that this structure is not a homodimeric quadruplex of the type demonstrated by Scaria *et al.* (49), DMS protection and interference assays were performed to show the accessibility of the N7 guanine position. No DMS protection was noted in the presence or absence of formed self-structure, or with 23 bp Watson–Crick duplexes. To confirm this result, the 23mer-GA oligonucleotide was methylated by DMS, but not followed by subsequent hot piperidine cleavage. Instead, these methylated oligonucleotides were used as substrates for ss gel retardation assays as described above. Aside from overall slightly reduced mobilities due to the added methyl groups (34), the N7 guanine-methylated oligonucleotides demonstrated the same concentration-dependent gel retardation as the 23mer-GA oligonucleotides of Figure 3 (data not shown). Such results argue against the involvement of triplex or quadruplex self-structures, as these would require N7 participation in hydrogen bonding.

The ss-specific mung bean nuclease was also used to probe these 23mer-GA self-structures. A strong internal footprint was noted when 5 nM of radiolabeled 23mer-GA was incubated with 10 μM cold 23mer-GA that was not seen with 10 μM 15mer-GA or with no additional oligonucleotide (data not shown). These results provide further evidence of the involvement of the d(GA) repeats in structure formation and the lack of hairpin-generated ss regions.

In further support, near total protection against DNase I was seen in preformed 23mer-GA self-structures whereas Watson–Crick duplexes were rapidly degraded by this enzyme (data not shown). These results confirm that no ss hairpin regions exist within the structure and suggest that major and minor groove dimensions are altered in this structure so as to inhibit nuclease digestion. DNase I protection has previously been described for parallel-stranded duplex DNA (50).

Taken together, the lack of increase in thermal stability afforded by K⁺, the lack of hairpin structures, the lack of N7 guanine participation in structure formation, the duplex gel mobility, the requirement for alternating GA residues and the close fit between experimental results and theoretical models of bimolecular association (41) leads us to believe that the structure is a homoduplex stabilized by the alternating d(GA) repeats.

Homoduplex formation inhibits triplex formation

Given the presence of stable GA homoduplexes from 4 to 37°C, we determined whether these complexes could compete with triplex formation on the target duplex. As demonstrated in Figure 3D, no homoduplex formation is detected at 10 nM GA oligonucleotide strand concentrations at 37°C in 10 mM MgCl₂, 100 mM NaCl. Therefore, instead of determining apparent triplex K_d by adding increasing 23mer-GA to 10 nM of radiolabeled target duplex, the reverse experiment was performed—increasing amounts of target duplex were added to 10 nM of radiolabeled 23mer-GA. Figure 5A and C illustrates the significant differences in extent of triplex formation between the two experiments.

When 23mer-GA was radiolabeled and in concentrations low enough to remain ss at 37°C, apparent triplex K_d in 10 mM MgCl₂ was 8.8×10^{-8} M ($\Delta G = -10.0$ kcal/mol). However, when the duplex was radiolabeled and 23mer-GA was in concentrations sufficient for homoduplex formation, a near 10-fold loss in extent of triplex formation was noted (Fig. 5A). By contrast, when the same experiment was repeated with 23mer-GT at 37 and 4°C, differences in extent of triplex formation were not significant (Fig. 5B and D).

When the experiment was repeated with 23mer-GA at 5 mM MgCl₂, the discrepancy between the extent of triplex formation was even greater, whereas again, the extent of triplex formation with 23mer-GT in the two experiments remained reasonably consistent. At 1 mM MgCl₂, no triplex formation could be detected when 23mer-GA was added in increasing concentrations, but when the target duplex was added in increasing concentrations, weak triplex interactions at 10 μM target duplex concentrations could still be detected (data not shown). These results, and those of Figure 4A, demonstrate that the effect of magnesium is to stabilize the triplex structure relative to the homoduplex structure.

Figure 5C demonstrates an even greater disparity in triplex formation at 4°C where triplex formation is barely detectable at 10 μM concentrations of added 23mer-GA, but nearly 100% formed at 10 μM added duplex. In addition, this experiment illustrates clearly the three structural species involving 23mer-GA: free 23mer-GA, homoduplex 23mer-GA and triplex-bound 23mer-GA. As the concentration of added duplex to radiolabeled 23mer-GA increases, the free 23mer-GA is driven to the triplex state. However, as the concentration of added 23mer-GA to radiolabeled duplex increases, the 23mer-GA is driven to the homoduplex state, successfully outcompeting triplex formation.

In contrast to homoduplex and triplex structures, no competition is observed between homoduplex and Watson–Crick duplex

Figure 5. Triplex formation with single-stranded versus double-stranded targets. (A) Triplexes were formed at 37°C either with increasing concentrations of duplex target (lanes 1–5) or increasing 23mer-GA (lanes 6–10). (B) Triplexes were formed at 37°C either with increasing concentrations of duplex target (lanes 1–5) or increasing 23mer-GT (lanes 6–10) in 10 mM MgCl₂. (C) Triplexes were formed at 4°C either with increasing concentrations of duplex target (lanes 1–5) or increasing 23mer-GA (lanes 6–10). (D) Triplexes were formed at 4°C either with increasing concentrations of duplex target (lanes 1–5) or increasing 23mer-GT (lanes 6–10). In all gels, lanes 1–5 and lanes 6–10 correspond to added nucleic acid concentrations of 0, 10, 100 nM, 1 and 10 µM of cold duplex or triplex-forming oligonucleotide at 10 mM MgCl₂, and (*) denotes radiolabeled species.

structures when 23mer-GA is added to the complementary 23mer-CT at 4 or 37°C—only Watson–Crick duplexes are formed. These observations can be explained by the ~2-fold greater ΔG of Watson–Crick duplex formation over homoduplex formation, both at 37°C (–19.1 versus –8.8 kcal/mol) and at 4°C (–31.9 versus –17.0 kcal/mol). Thermodynamic values for the Watson–Crick 23 bp duplex were derived from T_m curves obtained under identical conditions using a model of bimolecular association of complementary oligonucleotide strands (41).

DISCUSSION

This report describes the effect of competing oligonucleotide self-association on triplex formation with oligonucleotides containing d(GA) and d(GT) repeats. While the ability of guanine-

rich nucleic acids to self-associate has long been recognized, the nature of this self-association: the stoichiometries, binding motifs, strand orientations, ionic constraints and thermodynamics are only beginning to be understood. Given the therapeutic potential of triplex formation with guanine-rich oligonucleotides on genomic DNA, a better understanding of competing oligonucleotide interactions will aid in choosing optimal target sequences, as well as in designing more effective triplex-forming oligonucleotides.

The most striking effect of competition between oligonucleotide self-association and oligonucleotide-mediated triplex formation was the seemingly inversely-oriented temperature dependence of triplex formation with GA oligonucleotides between 4 and 37°C. This phenomenon is most likely the result of stable intermolecular homoduplex formation with a ΔH of formation more negative than found for triplex formation. Thus, at lower temperatures, homoduplex formation is favored, serving to deplete the free fraction of ss GA oligonucleotide available for triplex formation. However, as temperature is raised, the homoduplex state is destabilized relative to the triplex state, giving rise to greater concentrations of free ss GA oligonucleotide available for triplex formation. The net result leads to the unanticipated increase in triplex formation with increasing temperature from 4 to 37°C.

The ionic conditions which augment GA homoduplex formation are very similar to those previously described for triplex formation by both purine-rich as well as pyrimidine-rich oligonucleotides (6,46–48). However, in notable contradiction to triplex formation with GA and GT oligonucleotides which are strongly dependent on the presence of multivalent cations, homoduplex formation with GA oligonucleotides can take place at moderate levels of only monovalent cations. The elimination of this multivalent cation constraint widens the range of environments in which this homoduplex structure would be formed over a triplex structure. Within the nuclear environment of eukaryotic cells are monovalent cations such as Na⁺ and K⁺, divalent cations such as Mg²⁺ and Ca²⁺, as well as multivalent cations such as spermine and other polyamines. Thus, a structure which is least constrained by the ionic environment has distinct advantages in a milieu which may be fluctuating.

Interestingly, despite the presence of competing homoduplex formation at 37°C, the 23mer-GA oligonucleotide investigated in the present study was found to be far more effective at promoting triplex formation than its 23mer-GT oligonucleotide counterpart which did not self-associate. However, at 20°C, the differences are less pronounced, and at 4°C, the 23mer-GT oligonucleotide was more effective at promoting triplex formation. These observations have several implications. First, for the IGF-1 DNA target sequence, factors other than competing oligonucleotide structure must be responsible for the destabilization of 23mer-GT relative to 23mer-GA at 37°C. Possible explanations include differences in intrastrand base-stacking, differences in oligonucleotide flexibility in the major groove, or differences in backbone distortions caused by the non-isomorphism of base triplets.

Secondly, in order to make estimates of the relative *in vivo* binding potential of GA versus GT oligonucleotides, one must perform the *in vitro* experiments at physiological temperatures. Previous reports have compared GA and GT oligonucleotide-mediated triplex formation to a common target (14,21,46), but these experiments were performed all or in part at lower temperatures. More comparative data on triplex formation with

a variety GA and GT oligonucleotides is needed to explain the sequence-specificity of binding, as well as to define the general rules governing the choice of GA versus GT oligonucleotides to target a given sequence.

The effect of d(GA)-mediated homoduplex formation also has implications for the use of GA or GT triplex-forming oligonucleotides targeted against *in vivo* genomic polypurine/polypyrimidine sequences containing more than three to four d(GA)-d(TC) repeats. Given that triplex-forming oligonucleotides would most likely be added to cell cultures in concentrations ranging from 1 to 10 μ M, it is reasonable that GA, but not GT, oligonucleotides may be able to form homoduplexes within the extracellular media. Thus, the cellular uptake of ss GT oligonucleotides may have different characteristics than the cellular uptake of GA homoduplexes. Furthermore, the susceptibility of GA homoduplexes to intracellular and extracellular nuclease degradation may differ from ss GT oligonucleotides. Once intracellular, competing GA homoduplex formation may serve to reduce triplex formation by depleting the free fraction of ss oligonucleotide. The rapid kinetics of association and the negative free energy of formation of homoduplex formation suggest that this structure may dominate initially. However, despite the slower kinetics of association, thermodynamic analysis as well as experimental results suggest that triplex formation may be favored given the physiological conditions approximated in our *in vitro* experiments. In fact, the initial intracellular presence of homoduplexes may theoretically act as a reservoir for the GA oligonucleotide and prolong the bioavailability and the duration of triplex-mediated gene inhibition. Studies involving cellular uptake and stability, as well as efficacy of transcriptional repression *in vivo* are needed to test these hypotheses and to compare further GA and GT triplex-forming oligonucleotides.

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