Triplex formation with α anomers of purine-rich and pyrimidine-rich oligodeoxynucleotides

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Received July 24, 1995; Revised and Accepted September 8, 1995

ABSTRACT

Nuclease-resistant α anomers of pyrimidine-rich CT- and purine-rich GA- and GT-containing oligonucleotides were investigated for their triplex-forming potential and compared with their corresponding nuclease-sensitive β anomers. Both 23mer CT- α and 23mer CT- β had quite similar triplex binding affinities. Synthetic 23mer GT- α oligonucleotides were capable of triplex formation with binding affinities slightly lower than corresponding 23mer GT- β oligonucleotides. The orientation of third strand GT- α binding was parallel to the purine strand of the duplex DNA target, whereas the orientation of third strand GT- β binding was found to be antiparallel. Triplex formation with both GT oligonucleotides showed the typical dependence on magnesium and temperature. In contrast, 23mer GA- α oligonucleotides did not support triplex formation in either orientation under a variety of experimental conditions, whereas the corresponding 23mer GA- β oligonucleotides demonstrated strong triplex formation in the antiparallel orientation. GA- α oligonucleotides covalently conjugated to acridine were similarly unable to demonstrate triplex formation. GA- α oligonucleotides, in contrast to GT- α oligonucleotides, were capable of self-association, detectable by gel retardation and UV spectroscopy, but competing self-association could not fully account for the lack of triplex formation. Thus for *in vivo* triplex gene regulation strategies using GT oligonucleotides the non-natural α anomer may be a feasible alternative to the natural β anomer, allowing for a comparable degree of triplex formation without rapid cellular degradation. However, α anomeric inversion does not appear to be a feasible alternative in applications involving GA oligonucleotides.

INTRODUCTION

Triple helix formation on polypyrimidine/polypurine sequences of double-stranded DNA with pyrimidine-rich or purine-rich

oligonucleotides may represent a means through which gene-specific repression can be achieved (1). DNA binding affinity, accessibility of the target site, pharmacokinetic behavior and rapid extracellular and intracellular nuclease-mediated degradation may limit the therapeutic potential of unmodified natural triplex-forming oligonucleotides. To augment oligonucleotide stability several non-natural oligonucleotide analogs have been synthesized with modified backbone structures, such as peptide nucleic acids, 2'-O-methyl oligoribonucleotides, oligomethylphosphonates and oligophosphorothioates. These backbone modifications can have different effects on triplex binding affinity (2). Peptide nucleic acids and 2'-O-methyl oligoribonucleotides form triple helix complexes with higher binding affinities than their corresponding unmodified oligonucleotides (3–6). In contrast, triple helices formed by oligonucleotides that contain methylphosphonate (7,8) or phosphorothioate linkages (9–13) have in most cases lower binding affinities than those obtained with their corresponding unmodified oligonucleotides. Oligonucleotides have also been synthesized with 3'-terminus modifications to offer increased protection against ubiquitous cellular 3' exonucleases (14). Another technique used to augment oligonucleotide stability has been to synthesize an oligonucleotide in which the anomeric configuration of the nucleosides are changed from the natural β form to the non-natural α form (15–18).

α anomers have been shown to be nuclease-resistant in both *in vitro* (18,19) and *ex vivo* (20) systems, though recent studies indicate sequence specificity may exist (21). These properties led to their earlier development as antisense oligonucleotides which were able to bind tightly to complementary β sequences. However, the inability of RNase H to act on α/β hybrid duplexes has limited their utility (22). Binding of α/β hybrid duplexes was found to be parallel in orientation, as opposed to the standard antiparallel orientation of β/β duplexes (23,24).

Subsequently, pyrimidine-rich α oligonucleotides composed of either strictly thymine residues, strictly cytosine residues or mixed cytosine and thymine residues were shown to be capable of triplex formation with corresponding polypurine/polypyrimidine DNA sequences (25–29). While triplex binding affinity with α oligonucleotides was found to be slightly lower than that found with corresponding β oligonucleotides, affinity was substantially increased by the addition of a DNA intercalating agent on the

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3'-terminus (25). Third strand orientation was shown to be parallel for strictly thymine-containing α oligonucleotides and antiparallel for both strictly cytosine-containing α oligonucleotides and mixed thymine and cytosine-containing α oligonucleotides (25,26,28,30). This finding stands in contrast to the uniformly parallel orientation found with β anomeric triplex-forming oligopyrimidines.

The requirement for acidic pH in order to hemiprotonate cytosines within the pyrimidine-rich C.G*C⁺, T.A*T triplex binding motif has led to the development of a second triplex binding motif based upon C.G*G and T.A*T (GT oligonucleotides) or C.G*G and T.A*A triplets (GA oligonucleotides) (31). This purine-rich binding motif has been shown to be pH independent, stabilized by multivalent cations and able to mediate triplex binding with affinities comparable with those obtained with pyrimidine-rich third strands at acidic pH. Both GA- β and GT- β oligonucleotides have demonstrated their ability to block transcription factor binding and transcription *in vitro* (32–34), as well as mRNA transcription initiation *ex vivo* (35–37). A given target sequence can exhibit binding preference for either GA- β or GT- β oligonucleotides, but an adequate explanation for this sequence specificity has not yet been elucidated. Thus choosing between GA- β and GT- β oligonucleotides for *ex vivo* and *in vivo* applications is often aided by prior *in vitro* studies. In addition, self-association of G-rich oligonucleotides (to form G-quartets; 38), of alternating G.A sequences (to form parallel-stranded duplexes; 39,40) and of C-rich oligonucleotides (to form a four-stranded i-DNA structure; 41) can lead to competition with triplex formation.

This report uses standard *in vitro* experimental techniques to determine whether the utility of α anomeric triplex oligonucleotides can be extended from the CT pyrimidine-rich binding motif to also include the GA and GT purine-rich binding motif. Our results comparing both α and β GA and GT oligonucleotides with a common DNA target indicate that the use of α anomeric triplex-forming oligonucleotides can be extended to include the GT binding motif, but cannot be extended to include the GA binding motif, at least for the sequences investigated in the present study. Consistent with previous reports on opposite binding orientations of CT- α and CT- β oligonucleotides, GT- α oligonucleotides bound in a parallel orientation, whereas GT- β oligonucleotides demonstrated a strong preference for an antiparallel orientation with the investigated sequence. Binding affinity was slightly lower in triplexes containing GT- α oligonucleotides than in those containing GT- β oligonucleotides.

While the inability of GA- α oligonucleotides to mediate triplex formation on a duplex DNA target may not necessarily extend to all sequences, we explored the possibility that for this sequence interfering 23mer GA- α oligonucleotide self-association was fully or partially responsible for the difference in GA- α and GT- α triplex binding capabilities. Consistent with this hypothesis is the presence of a series of alternating d(GA) repeats within the sequence, which have recently been shown to facilitate formation of parallel-stranded homoduplexes with G.G and A.A base pairs (39). We have also previously demonstrated that the 23mer GA- β sequence formed a self-structure which could compete with triplex formation in a temperature-dependent fashion (40). Using both gel retardation assays and UV absorption spectroscopy we show that, indeed, the GA- α oligonucleotide can stably self-associate in a manner dependent upon strand concentration, magnesium concentration and temperature. However, both

thermodynamic analyses of melting curves and gel retardations at various temperatures suggest that the presence of this self-structure is not sufficient to fully explain the complete lack of triplex formation at temperatures ranging from 4 to 37°C. Furthermore, another GA- α oligonucleotide covalently linked to an intercalating agent (Acr-15mer GA- α) was synthesized which could neither self-associate nor support triplex formation, in contrast to its β -counterpart (15mer GA- β -Acr), which could both self-associate and support triplex formation. Thus other factors must account for the dramatic differences in triplex forming potential seen with GA- α and GA- β oligonucleotides and not seen with GT- α and GT- β oligonucleotides.

This report adds to the growing database of non-natural oligonucleotides and their differing capacities for triplex formation. In addition, our results suggest that for target sequences where GT- β oligonucleotides have already demonstrated high triplex binding affinity (32,33,35), use of GT- α oligonucleotides may present unique advantages by conserving triplex binding capability while conferring oligonucleotide stability against cellular nucleases.

MATERIALS AND METHODS

Oligonucleotides

Purified unmodified oligodeoxynucleotides were purchased from Eurogentec (Belgium). Their absorbances were measured at 50°C to determine concentrations using the following molar extinction coefficients at 260 nm (42): 42mer-R1, $\epsilon = 4.38 \times 10^5$ /M/cm; 42mer-Y1, $\epsilon = 3.7 \times 10^5$ /M/cm; 23mer CT- β , $\epsilon = 1.76 \times 10^5$ /M/cm; 23mer GA- β , $\epsilon = 2.66 \times 10^5$ /M/cm; 23mer GT- β , $\epsilon = 2.17 \times 10^5$ /M/cm; 42mer-R1inv, $\epsilon = 4.41 \times 10^5$ /M/cm; 42mer-Y1inv, $\epsilon = 3.73 \times 10^5$ /M/cm; 42mer-R2, $\epsilon = 3.85 \times 10^5$ /M/cm; 42mer-Y2, $\epsilon = 4.11 \times 10^5$ /M/cm; 15mer GA- β , $\epsilon = 1.75 \times 10^5$ /M/cm. Purified α oligonucleotides and acridine-oligonucleotide conjugates were obtained from Appligene (France). The acridine derivative 2-methoxy-6-chloro-9-amino-acridine was linked either to the 3'-end (15mer GA- β -Acr) or to the 5'-end (Acr-15mer GA- α) of the oligomer via a pentamethylene bridge. The molar extinction coefficients of α oligomers were assumed to be identical to those of β oligonucleotides with a reverse orientation. The concentrations of acridine-containing oligomers 15mer GA- β -Acr and Acr- α -15mer GA were determined at 425 nm using a molar extinction coefficient $\epsilon = 8500$ /M/cm (43).

Gel retardation assay

For triplex gel retardations the purine strand of the 42mer duplex was 5'-end-labeled with [γ -³²P]ATP (Amersham, Arlington Heights, IL) with T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Increasing concentrations (10 nM–50 μ M) of the triplex-forming oligonucleotide were added to 10 nM labeled 42mer duplex in the presence of 10 mM MgCl₂, 100 mM NaCl, 10% sucrose, 50 mM Hepes, pH 7.2, and 0.5 μ g/ μ l tRNA, with sample incubation at the indicated temperature (4–37°C) overnight. Electrophoresis (7 W constant power) was performed on a non-denaturing 10% polyacrylamide gel containing 10 mM MgCl₂ and 50 mM Hepes, pH 7.2, at the incubation temperature in a thermostated gel electrophoresis unit.

For self-association studies using gel electrophoresis 10 nM 5'-end-labeled 23mer GA- α or 23mer GT- α oligonucleotide was

added to increasing concentrations (10 nM–10 μ M) of the corresponding unlabeled α oligonucleotide in the presence of 10 mM MgCl_2 , 100 mM NaCl, 10% sucrose and 0.5 $\mu\text{g}/\mu\text{l}$ tRNA, with sample incubation at the indicated temperature (4–37°C) overnight. Electrophoresis was performed as described above for triplex gel retardations.

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 the oligonucleotide homoduplexes. The cell holder was thermoregulated by a circulating liquid composed of 80% water/20% ethylene glycol as previously described (6). Sample temperature was decreased from 69 to 1°C and increased back to 69°C at a rate of 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. Cation and oligonucleotide strand concentrations and buffer were as described in the figure and table legends. For melting temperature analysis absorption readings at 540 nm were subtracted from readings at 260 nm and the first derivatives of these values were plotted against the reciprocal of the temperature (in K). Melting temperature, T_m , was estimated as the value at which dA/dT reached a maximum. In all cases heating and cooling curves were superimposeable, with no apparent hysteresis noted.

Degradation kinetics of α and β oligonucleotides in tissue culture media

Oligonucleotide degradation was assessed by incubating 10 nM ^{32}P 5'-end-labeled and 1 μM unlabeled α or β oligonucleotides in 1 ml Ham's F-10 culture medium containing 10% fetal calf serum (heat inactivated for 30 min at 60°C) at 37°C. At successive time points 100 μl of each sample were phenol extracted and electrophoresed on 7 M urea–20% polyacrylamide gels. The extent of degradation of the full-length oligonucleotide (n) and of the first 3' exonuclease degradation product ($n - 1$) was determined by quantitative analysis on a Molecular Dynamics phosphorimager.

RESULTS AND DISCUSSION

A 42 bp DNA fragment (duplex 1) containing a 23 bp polypurine/polypyrimidine sequence was chosen as the target sequence to investigate triplex formation with α and β anomers of 23mer GA and 23mer GT oligonucleotides. This target sequence corresponds to a region of the human insulin growth factor I (IGF-1) gene located within a cluster of transcriptional start sites 295 bp upstream from the initiation codon (44). A second 42 bp DNA fragment was synthesized in the reverse orientation in order to assess third strand binding orientation (invduplex 1). A third 42 bp DNA fragment (duplex 2) containing a 15 bp polypurine/polypyrimidine sequence was chosen as the target for 15mer GA- β , 15mer GA- β -Acr and Acr-15mer GA- α oligonucleotides (Fig. 1).

Triplex formation with CT- α and GT- α , but not GA- α , oligonucleotides

Figure 2 illustrates the triplex binding affinity, parallel strand orientation and temperature dependence of 23mer GT- β oligo-

nucleotides. A comparison of Figure 2E and F with G indicates that the third strand orientation of the 23mer GT- α oligonucleotide is parallel with respect to the purine strand of the duplex target, whereas the third strand orientation of the 23mer GT- β oligonucleotide is antiparallel with respect to the purine strand. The binding orientation of the 23mer GT- β oligonucleotide is in accord with previous results demonstrating that when target sequences contain numerous GpA/ApG steps (as in the duplex 1 sequence) an antiparallel orientation is favored (45). The reverse binding orientation of 23mer GT- α oligonucleotides is analogous to previous results demonstrating the antiparallel orientation of CT- α third strands (as opposed to the parallel orientation on CT- β third strands), as well as the parallel orientation of α/β duplexes (as opposed to the antiparallel orientation of β/β duplexes). These opposing orientational preferences between α and β forms are due to the inversion about the glycosidic bond upon synthesizing the two anomeric configurations.

For the duplex 1 sequence triplex formation with 23mer GT- α oligonucleotides was slightly lower than with 23mer GT- β oligonucleotides, similar to a previous report comparing CT- α and CT- β triplex formation with another DNA sequence (25). The apparent association constants (K_a) obtained at pH 7 and 4°C were found to be ~10-fold lower for GT- α oligonucleotides than for GT- β oligonucleotides ($\sim 3 \times 10^5 \text{ M}$ versus $\sim 5 \times 10^4 \text{ M}$). However, at pH 6 and 37°C the K_a values for CT- α and CT- β triplex formation (Fig. 3) were nearly identical ($\sim 2.8 \times 10^6 \text{ M}$) and substantially greater than for the GT- α oligonucleotides at 4 or 37°C. In support of previous findings (25), CT- α was bound in an antiparallel orientation, while CT- β was bound in a parallel orientation.

In contrast to CT and GT oligonucleotides, triplex formation was noted in 23mer GA- β oligonucleotides within the temperature range 4–37°C, but could not be detected with 23mer GA- α oligonucleotides (Fig. 4) in either orientation within this temperature range. A variety of incubation and electrophoresis conditions were then tested, including varying magnesium concentrations between 0 and 20 mM, replacing magnesium with other bi- and multivalent cations (calcium and spermine) and extending incubation times to 5 days, but none were compatible with triplex formation. These negative results were then verified by analogous experiments wherein 10 nM labeled 23mer GA- α was treated with increasing concentrations of unlabeled duplex 1 or invduplex 1 (data not shown).

Triplex formation with GA- β , but not GA- α oligonucleotides, covalently linked to an intercalating agent

It was previously shown that the covalent linkage of an intercalating agent, such as an acridine derivative, to the 5'- or 3'-end of oligonucleotides could increase their affinity toward either single-stranded or double-stranded targets (18,25,28,43). Stabilization is thought to occur by intercalation of the acridine ring in the double helix when the target is single-stranded DNA or at the triplex/duplex junction when the target is double-stranded DNA.

In order to determine whether the covalent linkage of acridine to a GA- α oligonucleotide could induce and stabilize triplex formation, 15mer GA- β -Acr and Acr-15mer GA- α were synthesized and tested for triplex binding potential. Acr-15mer GA- α was synthesized to bind in a parallel orientation, whereas 15mer GA- β -Acr was synthesized to bind in an antiparallel

42 base-pair double-stranded target (duplex 1):

5' TAACTTTGCC**AGAAGAGGGAGAGAGAGAGAAGG**CAAATGTTC 3' 42mer-R1
 3' ATTGAACGGT**TCTCTCCCTCTCTCTCTCTCTCCG**TTTACAAG 5' 42mer-Y1

23mer triplex-forming oligonucleotides:

5' **TCTTCTCCCTCTCTCTCTCTTCC** 3' 23mer CT-beta (β)
 3' **AGAAGAGGGAGAGAGAGAGAAGG** 5' 23mer GA- β
 3' **TGTTGTGGGTGTGTGTGTGTGG** 5' 23mer GT- β

 3' **TCTTCTCCCTCTCTCTCTCTTCC** 5' 23mer CT-alpha (α)
 5' **AGAAGAGGGAGAGAGAGAGAAGG** 3' 23mer GA- α
 5' **TGTTGTGGGTGTGTGTGTGTGG** 3' 23mer GT- α

42 base-pair double-stranded inverse target (invduplex 1):

5' CTTGTAAAC**GGAAGAGAGAGAGAGGGAGAAG**ACCGTTTCAAT 3' 42mer-R1inv
 3' GAACATTG**CCTTCTCTCTCTCTCCCTCTTCT**GGCAAAGTTA 5' 42mer-Y1inv

42 base-pair double-stranded target (duplex 2):

5' CTTCAACGGCAGGGGA**TCTCCCTCTCCTTTT**ATGGGCGTAG 3' 42mer-R2
 3' GAAGTTGCCGTCCCT**TAGAGGGAGAGGAAAA**TACCCGCATC 5' 42mer-Y2

15mer triplex-forming oligonucleotides:

5' **AGAGGGAGAGGAAAA** 3' 15mer GA- β
 5' **AGAGGGAGAGGAAAA-ACR** 3' 15mer GA- β -Acr
 3' **AGAGGGAGAGGAAAA-ACR** 5' Acr-15mer GA- α

Figure 1. Duplex targets and triplex-forming oligonucleotides. The duplex 1 fragment corresponds to a 42 bp upstream sequence of the IGF-1 gene with the polypurine/polypyrimidine region displayed in bold. The 23mer CT- β was designed to bind in a parallel orientation relative to the purine strand (42mer-R1) of duplex 1. Both the 23mer GA- β and 23mer GT- β were designed to bind in an antiparallel orientation. All alpha oligonucleotides, 23mer CT- α , 23mer GA- α and 23mer GT- α , were designed to bind in reverse orientation to the 42mer duplex 1 with respect to their counterparts 23mer CT- β , 23mer GA- β and 23mer GT- β . The 42 bp invduplex 1 was synthesized to test triplex binding orientation. The 42 bp duplex 2 was the target sequence for triplex formation with 15mer GA- β , 15mer GA- β -Acr and Acr-15mer GA- α .

orientation (Fig. 1). As shown in Figure 5, 15mer GA- β and 15mer GA- β -Acr bound to duplex 2 with similar affinities. However, as was observed with the 23mer GA- α oligonucleotide, Acr-15mer-GA- α was unable to form a triplex with duplex 2 within the temperature range 4–37°C.

Existence of self-structure in GA- α , but not GT- α , oligonucleotides

The target duplex 1 contains a series of d(GA)-d(CT) repeats, a sequence over-represented in mammalian genomes and often found in non-coding regulatory regions of genes (46). This alternating GA sequence in β oligonucleotides has previously been shown to facilitate self-structure in the form of homoduplexes (39,40). Evidence has suggested that the formation of this self-structure in GA- β oligonucleotides can compete with and inhibit triplex formation on the duplex 1 target by depleting the free fraction of single-stranded oligonucleotide (40). We thus set out to determine whether similar self-structure was present in the 23mer GA- α oligonucleotide and to assess whether this self-structure could fully account for the lack of triplex formation.

Figure 6 illustrates the presence of self-structure in 23mer GA- α , but not 23mer GT- α , oligonucleotides by both UV absorption spectroscopy and gel electrophoresis. Gel retardation assays at 4°C (Fig. 6A) show 23mer GA- α containing molecular species migrating with a mobility between that of a single-strand and that of a duplex at low oligonucleotide concentrations and with a mobility of a duplex at high oligonucleotide concentrations. This concentration-dependent gel retardation has previously been observed for 23mer GA- β containing homoduplexes (Fig. 6B) (40). We propose that intermediate mobilities arise from rapid kinetics which allow association and dissociation within the gel during electrophoresis. By 20°C this 23mer GA- α self-structure was no longer apparent (data not shown). In contrast, the self-structure of 23mer GA- β still existed at 37°C at high (10 μ M) oligonucleotide concentration (40).

Consistent with our gel retardation data are thermal denaturation and renaturation curves, which demonstrate a broad transition for 23mer GA- α oligonucleotides (but not 23mer GT- α oligonucleotides), with T_m ranging from 11 to 18°C over 23mer GA- α strand concentrations of 0.5–4 μ M (Fig. 6C and Table 1). (Transition T_m values are consistent with the temperatures needed

Figure 2. Gel retardation assays comparing triplex binding of 23mer GT- β and 23mer GT- α oligonucleotides to the 42 bp duplex 1 or the 42 bp invduplex 1 targets. Triplex formation was investigated at 37, 20 and 4°C using 10 nM radiolabeled target duplex (*) and increasing concentrations of 23mer GT- β (A, C and E) or 23mer GT- α (B, D and F) oligonucleotides. The targets used were either duplex 1 (A–F) or invduplex 1 (G). Samples were incubated at the indicated temperatures overnight in 10 mM MgCl₂, 100 mM NaCl, 0.5 μ g/ μ l tRNA, 50 mM HEPES, pH 7.2 and 10% sucrose. In each gel lanes 1–6 correspond to added concentrations of 0, 10 and 100 nM and 1, 10 and 50 μ M oligonucleotides, respectively. Electrophoresis was performed using gels and buffers containing 10 mM MgCl₂ and 50 mM Hepes, pH 7.2, at the indicated temperature.

Figure 3. Gel retardation assays comparing triplex binding of 23mer CT- β and 23mer CT- α oligonucleotides to the 42 bp duplex 1 at pH 6 and 37°C. Radiolabeled target duplex (10 nM, *) was incubated overnight with increasing concentrations of 23mer CT- β and 23mer CT- α oligonucleotides in a buffer containing 50 mM MES buffer, pH 6, 5 mM MgCl₂, 0.5 μ g/ μ l tRNA and 100 mM NaCl.

to visualize the structure by gel assay.) Kinetics of association and dissociation were rapid on the time scale of the melting experiments, as denaturation and renaturation curves were superimposable and no hysteresis could be detected upon cooling and reheating at a rate of 0.2°C/min with 15 min pauses at each temperature extreme (1 and 69°C). In accord with previous results on GA- β homoduplexes (39,40), the structure demonstrated salt dependence and neither thermal transitions nor gel retarded species could be seen in the absence of salt (data not shown).

We have previously shown that 15mer GA- β exhibited a self-structure far less stable than that of 23mer GA- β (40). To

Figure 4. Gel retardation assays comparing triplex binding of 23mer GA- β and 23mer GA- α oligonucleotides to the 42 bp duplex 1 (A–D) or the 42 bp invduplex 1 (E) targets. Triplex formation was investigated at 37 and 4°C using 10 nM radiolabeled target duplex (*) and increasing concentrations of 23mer GA- β (A, C and E) and 23mer GA- α (B, D and E) oligonucleotides. Sample incubation and gel analysis were performed as described in Figure 2.

Figure 5. Gel retardation assay comparing triplex binding of 15mer GA- β , 15mer GA- β -Acr and Acr-15mer GA- α oligonucleotides to the 42 bp duplex 2 target. Triplex formation was investigated at 4°C using 10 nM radiolabeled target duplex (*) and increasing concentrations of third strand oligonucleotides as indicated. Samples were incubated overnight in 10 mM MgCl₂, 0.5 μ g/ μ l tRNA and 50 mM Hepes, pH 7.2. Electrophoresis was performed at 4°C using gels and buffers containing 10 mM MgCl₂ and 50 mM HEPES, pH 7.2.

observe the homoduplex transition of 15mer GA- β it was necessary to perform melting experiments at substantially higher oligonucleotide concentrations ($\geq 6 \mu$ M). In performing similar melting experiments with 15mer GA- β -Acr we found that the covalent linkage of the acridine derivative significantly increased the stability of the homoduplex formed by the 15mer-GA sequence, as shown in Table 1, even at lower oligonucleotide concentrations. Thus it is possible that by stabilizing the self-structure of the oligonucleotide the expected beneficial effect of the intercalating agent on stabilizing the triplex structure was offset. This interpretation may explain why the enhancement of triplex stability obtained with addition of acridine to 15mer GA- β was far smaller than expected. Correspondingly, at higher temperatures (37°C) differences in triplex binding affinities obtained with 15mer GA- β and 15mer GA- β -Acr on duplex 2 were more pronounced. Such findings are most likely the result of destabilization of the 15mer-GA- β -Acr self-structure relative to the triplex structure (data not shown). In contrast, no evidence of self-structure was observed with Acr-15mer GA- α oligonucleotides by either UV absorption spectroscopy or gel retardation assays, demonstrating the importance of alternating GA repeats and anomeric configuration on the formation of self-structure.

Figure 6. Self-association of purine-rich oligonucleotides analyzed by gel retardation and UV spectroscopy. (A and B) Oligonucleotide self-association was investigated using the incubation and electrophoresis conditions described in Figure 2. In (A) lanes 1–4 and 5–8 correspond to added concentrations of 0, 0.1, 1 and 10 μM unlabeled 23mer GA- α or 23mer GT- α , respectively, to 10 nM of the corresponding radiolabeled oligonucleotide (*). Gel (A) was run at 4°C. In (B) lanes 1–6 and 8–13 correspond to added concentrations of 0, 0.1, 1, 10 and 50 μM unlabeled 23mer GA- β or 23mer GT- β , respectively, to 10 nM corresponding radiolabeled oligonucleotide (*). R (lane 7) refers to the position of a reference 23 bp Watson–Crick duplex formed by 23mer GA- β and the complementary sequence 23mer CT- β . The gels were run at 20°C. At 4°C the 23mer GA- β was self-associated even at the lowest concentration investigated (10 nM). (C) Structures of 23mer GA- α and 23mer GA- β oligonucleotides analyzed by UV absorption spectroscopy. Samples contained 10 mM MgCl_2 , 100 mM NaCl and 10 mM sodium cacodylate, pH 7.0, with 2 μM 23mer GA- β , 23mer GA- α or 23mer GT- α oligonucleotides.

At low temperature no triplex formation with 23mer GA- α was observed, in spite of the presence of oligonucleotide self-association. As no triplex was found with Acr-15mer GA- α , which does not self-associate at temperatures ranging from 4 to 37°C, others factors than the presence of self-association of the triplex-forming oligonucleotides (e.g. steric hindrances, triplet isomorphisms, etc.) must account for the different triplex forming capabilities observed with GA- α and GA- β oligonucleotides.

Table 1. Melting temperatures of self-structures formed by GA- β -, GA- α -, GT- β - and GT- α -containing oligonucleotides

Oligonucleotide	Concentration (μM)	T_m (°C)
23mer GA- β /23mer GA- β	2	40
23mer GA- α /23mer GA- α	2	15
23mer GT- β /23mer GT- β	2	NA
23mer GT- α /23mer GT- α	2	NA
23mer GA- β /23mer CT- β	2	62.5
15mer GA- β /15mer GA- β	2	NA
15mer GA- β /15mer GA- β	6	25
15mer GA- β -Acr/15mer GA- β -Acr	6	40
Acr-15mer GA- α /Acr-15mer GA- α	6	NA

All samples contained 10 mM sodium cacodylate, pH 7.0, 100 mM NaCl and 10 mM MgCl_2 . T_m determination error is estimated at $\pm 1^\circ\text{C}$ for sharp transitions (23mer GA- β and 15mer GA- β -Acr) and at $\pm 2^\circ\text{C}$ for broader transition (23mer GA- α and Acr-15mer GA- α). NA, no transition detected.

Stability of α -oligonucleotides in serum-containing media

The α oligonucleotides are generally accepted to be more nuclease-resistant than β oligonucleotides (21). To confirm these findings α and β GT and CT oligonucleotides were incubated at 37°C in serum-containing media and their degradation products analyzed on denaturing polyacrylamide gels. Half-lives ($t_{1/2}$) for the full-length oligonucleotides (length $n = 23$) and for the first degradation products (length $n - 1 = 22$) are given in Table 2. Similar $t_{1/2}$ values were found for the n and $n - 1$ β oligonucleotides, but differing and much longer $t_{1/2}$ values were found for the n and $n - 1$ α oligonucleotides. The CT- α $n - 1$ oligonucleotide was still intact after 50 h, whereas the GT- α $n - 1$ oligonucleotide was half degraded after 40 h. These results are in good agreement with recent results on the effect of sequence on stability with α oligonucleotides (21). Having a terminal 3' cytosine nucleotide appeared to enhance the stability of an α oligonucleotide, as was found on CT- α $n - 1$. However, a terminal 3' cytosine did not affect the stability of β oligonucleotides.

CONCLUSION

In this study we have shown that nuclease-resistant GT- α -containing oligonucleotides were capable of triplex formation, but that the resulting complexes were less stable than those formed by GT- β -containing oligonucleotides. In contrast, the binding of the CT- α -containing oligonucleotide was as strong as the corresponding CT- β -containing oligomer. In both cases the orientation of the α oligonucleotide was opposite to that of the β oligomer (Fig. 7). We have also demonstrated that two different GA- α -containing oligonucleotide sequences were not capable of triplex formation (Figs 4, 5 and 7), despite strong binding with the corresponding GA- β oligonucleotides. In addition, we have presented a series of results on the presence of self-structure with GA-containing oligonucleotides in both anomeric configurations. We have demonstrated that the GT- α and CT- α oligonucleotides are more resistant to degradation than their GT- β and CT- β counterparts. We have also confirmed previous results suggesting that the presence of an α -cytosine at the 3'-end of the oligonucleotide confers an extremely high resistance to nuclease-mediated degradation (21).

Figure 7. Third strand orientation in triple helices formed by α and β oligodeoxynucleotides with duplex DNA. The 23mer CT- β and the 23mer CT- α oligonucleotides bind in a parallel and in an antiparallel orientation, respectively, with respect to the purine-rich strand of the duplex. The 23mer GA- β and the 23mer GT- β bind to the target with an antiparallel orientation. Only the 23mer GT- α oligonucleotide binds to the target in a parallel orientation. No triple helix is observed with the 23mer GA- α and the duplex 1 or invduplex 1 targets.

Table 2. Degradation kinetics of triplex-forming oligonucleotides and their first degradation product in culture media containing 10% heat-inactivated fetal calf serum

Oligonucleotide (n)	Sequence 5'→3'	$t_{1/2}$ (n) (h)	$t_{1/2}$ (n - 1) (h)
23mer CT- β	TCTTCTCCCTCTCTCTCTTCC	1.9	4.4
23mer CT2- β	CCTTCTCTCTCTCTCCCTCTTCT	1.2	3.3
23mer CT- α	CCTTCTCTCTCTCTCCCTCTTCT	3	>50 ^a
23mer GT2- β	TGTTGTGGGTGTGTGTGTGG	1.8	3
23mer GT- α	TGTTGTGGGTGTGTGTGTGG	5	41

^aThe n - 1 product of 23mer CT- α was still intact even after 50 h. Thus a true $t_{1/2}$ could not be determined, but it is expected to be considerably greater than 50 h.

Despite the presence of self-structure in 23mer GA- α , but not 23mer GT- α , oligonucleotides at low temperatures (<20°C), other factors must account for the inability of 23mer GA- α , but not 23mer GT- α , to form triplexes at higher temperatures (20–37°C). In the case of self-structure versus triplex formation with 23mer GA- β the effect of raising the temperature was to destabilize the homoduplex relative to the triplex, thus allowing for a greater extent of GA- β -mediated triplex formation at the higher temperatures (40).

To conclude, this report illustrates the feasibility of using α anomers of CT-rich oligonucleotides in antiparallel orientation and GT-rich oligonucleotides in parallel orientation (with respect to the purine strand of the duplex target) to confer added oligonucleotide stability against nuclease degradation while still preserving triplex-forming capability. No competing oligonucleotide self-structure was detected in our sequence containing multiple d(GT) repeats, although other GT- α -containing oligonucleotides of differing sequence or composition may form other higher ordered structures, such as quadruplexes, as has previously been found with GT- β oligonucleotides (38). As with β anomers of purine-rich GA and GT triplex-forming oligonucleotides, binding affinity is expected to be a function of sequence and composition.

ACKNOWLEDGEMENTS

We are gratefully acknowledge Dr G. Silver, Dr J. L. Mergny, B. Faucon and L. Perrouault for their helpful assistance. This work has been supported in part by Rhone Poulenc Rorer and by a grant and a post-doctoral fellowship to JL from the Ligue Nationale contre le Cancer and also by NIH grant 5-T32-GM08155 to SBN.

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