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Single strand targeted triplex-formation. Destabilization of guanine quadruplex structures by foldback triplex-forming oligonucleotides

Ekambar R. Kandimalla and Sudhir Agrawal*

Hybridon, Inc., One Innovation Drive, Worcester, MA 01605, USA

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ABSTRACT

Oligonucleotides that can hybridize to single-stranded complementary polypurine nucleic acid targets by Watson–Crick base pairing as well as by Hoogsteen base pairing, referred to here as foldback triplex-forming oligonucleotides (FTFOs), have been designed. These oligonucleotides hybridize with target nucleic acid sequences with greater affinity than antisense oligonucleotides, which hybridize to the target sequence only by Watson–Crick hydrogen bonding [Kandimalla, E. R. and Agrawal, S. *Gene* (1994) 149, 115–121 and references cited therein]. FTFOs have been studied for their ability to destabilize quadruplexes formation by RNA or DNA target sequences. The influence of various DNA/RNA compositions of FTFOs on their ability to destabilize RNA and DNA quadruplexes has been examined. The ability of the FTFOs to destabilize quadruplex structures is related to the structurally and thermodynamically stable foldback triplex formed between the FTFO and its target sequence. Antisense oligonucleotides (DNA or RNA) that can form only a Watson–Crick double helix with the target sequence are unable to destabilize quadruplex structures of RNA and DNA target sequences and are therefore limited in their repertoire of target sequences. The quadruplex destabilization ability of FTFOs is dependent on the nature of the cation present in solution. The RNA quadruplex destabilization ability of FTFOs is ~20% higher in the presence of sodium ion than potassium ion. The use of FTFOs, which can destabilize quadruplex structure, opens up new areas for development of oligonucleotide-based therapeutics, specifically, targeting guanine-rich sequences that exist at the ends of pro- and eukaryotic chromosomes and dimerization regions of retroviral RNA.

INTRODUCTION

In recent years, many groups have investigated the therapeutic potential of oligonucleotides, which results from their ability to selectively interact with genetic material (RNA/DNA) and interfere with gene expression (1,2). Antisense oligonucleotides are effective against viral and cancer targets both *in vitro* and *in vivo*, and human clinical trials are currently underway exploring their possible use as therapies in several diseases (3–7). Most of these oligonucleotides are designed to interact with either single-stranded mRNA by Watson–Crick base pairing (antisense approach) or double-stranded DNA by Hoogsteen hydrogen bonding (antigene approach) (1,2). There are few reports on the development of oligonucleotides targeting unusual nucleic acid structures that occur naturally within both RNA and DNA forms (8–10).

Unusual nucleic acid forms, such as multiplex structures, or the unusual triple repeats that are associated with human genetic disorders such as Huntington's disease, Kennedy's disease and myotonic dystrophy might be good targets for rational drug design because of their transient and rare occurrence *in vivo*, and these might allow for highly selective intervention. Telomeres, the ends of eukaryotic chromosomes composed of tandem repeats containing guanine tracts that are often interspersed with short stretches of thymines or adenines, are essential for the stability and duplication of linear chromosomes, since they form guanine quadruplexes (11). These G-rich tracts form self-association complexes involving other than Watson–Crick hydrogen bonding patterns. Both solution (12) and solid state (13) studies have revealed that these sequences can form stable structures with Hoogsteen-type hydrogen bonded guanine tetrads (G4), not only by intermolecular association of four different strands (14), but also by intermolecular association of two hairpins (15) or even by intramolecular folding of a single-strand (16).

RNA quadruplexes also occur in *in vitro* conditions (17). As in the case of telomeric DNA, the putative encapsidation–dimerization regions of the genomic RNA of 40 distinct retroviruses possess purine G-tracts (18). Such sequences occur two to five times in the RNA of all HIV-1 and HIV-2 isolates (18). Experimental evidence suggests that dimerization of two retroviral genomic RNAs is governed by RNA–RNA rather than RNA–protein interactions through the formation of a quadruplex-like structure involving G-rich sequences (10,19,20). Dimerization of genomic RNA is essential for genetic recombination and encapsidation in retroviruses.

* To whom correspondence should be addressed

New designs of antisense oligonucleotides have recently been reported, including circular (21,22), linear (22–25) and ligand-conjugated linear oligonucleotides (26,27), all of which target single-stranded nucleic acid structures through triplex formation. These oligonucleotides contain three distinct domains. The first is a Watson–Crick domain that forms a double helical structure with the complementary homopurine target strand in an anti-parallel mode. The second is a Hoogsteen domain that forms a triple helical structure with the homopurine strand in a parallel fashion in the major groove of the already formed double helix. The third is a short linker or loop that joins the Watson–Crick and Hoogsteen domains. Such oligonucleotides are called foldback triplex-forming oligonucleotides (FTFOs). FTFOs bind to their target sequences with greater affinity and sequence specificity than do antisense or antigene agents (24).

We have designed novel FTFOs that can destabilize RNA and DNA quadruplex structures. A 30 nt long sequence (411–440) (18) was chosen from the gag initiation codon region of HIV-1 as a target for study (Fig. 1). Oligonucleotides **1** and **2** are 16mers that bind to the target sequence through Watson–Crick duplex formation, referred to here as conventional antisense oligonucleotides (Fig. 1). Oligonucleotides **3–6** are FTFOs with different combinations of DNA and 2′-*O*-methyl-RNA content in the Watson–Crick and Hoogsteen domains, that bind to the same sequence on DNA and RNA targets as oligonucleotides **1** and **2** (Fig. 1). We describe here the results of studies on the influence of various DNA/RNA compositions of FTFOs on their ability to destabilize RNA and DNA quadruplexes formed in sodium and potassium ion containing solutions.

Target sequences

DNA 5′-d(TAAGGCC**AGGGGGAAAGAAAAAA**TATAAAT)-3′

RNA 5′-UAAGGCC**AGGGGGAAAGAAAAAA**UAUAAAU-3′

Antisense sequences

1 5′-TTTTTTCTTTCCCCCT-3′

2 5′-UUUUUCUUUCCCCCU-3′

Foldback triplex forming sequences

3
5′-TCCCCCTTTCTTTTTT T C
T
3′-TCCCCCTTTCTTTTTT T C

4
5′-UCCCCCUUUCUUUUUU T C
T
3′-UCCCCCUUUCUUUUUU T C

5
5′-T CCCCCTTT CTTTTTT T C
T
3′-UCCCCCUUUCUUUUUU T C

6
5′-UCCCCCUUUCUUUUUU T C
T
3′-T CCCCCTTT CTTTTTT T C

Figure 1. The oligonucleotide sequences used in the study. The polypurine site for triplex formation is showed in bold. In sequences **1–6** plain and outlined letters represent deoxyribonucleotides and 2′-*O*-methylribonucleotides, respectively.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

The oligonucleotides were synthesized on Milligen 8700 DNA synthesizer (Bedford, MA) using phosphoramidite chemistry. β-cyanoethyl-*N,N*-diisopropyl phosphoramidites and 2′-t-butyldimethylsilyl-3′-β-cyanoethyl-*N,N*-diisopropyl phosphoramidites were purchased from Millipore for DNA and RNA syntheses, respectively. The 2′-*O*-methyl-3′-β-cyanoethyl-*N,N*-diisopropyl phosphoramidites for the synthesis of oligonucleotides containing 2′-*O*-methylribonucleotides were obtained from Glen Research Laboratories (Sterling, VA). After synthesis, deoxyribonucleotides and 2′-*O*-methylribonucleotides were deprotected, purified on reverse phase HPLC (C_{18}), detritylated, and desalted using C_{18} sep-pack cartridges (Waters, Milford, MA). The purity of the oligonucleotides was checked by denaturing polyacrylamide gel electrophoresis (PAGE).

Oligoribonucleotide (RNA) was deprotected with a 3:1 mixture of ammonium hydroxide and ethanol at 55°C for ~15 h and then with tetrabutylammonium fluoride at room temperature for another 15 h. RNA was then purified on 20% denaturing PAGE, eluted from the gel, and desalted using C_{18} sep-pack cartridge (Waters).

The target oligonucleotide RNA or DNA was labeled with ^{32}P at the 5′-end using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham). The unreacted label was removed by passing through a C_{18} sep-pack cartridge (Waters). The reactions were carried out in either 100 mM sodium or potassium acetate, pH 5.0 buffer as described below, unless otherwise specified.

Tetraplex formation

The 5′-end-labeled oligonucleotide (~3000 c.p.m.) was mixed with unlabeled oligonucleotide to bring the concentration to the desired level in 20 µl of buffer containing various concentrations (0–500 mM) of sodium or potassium acetate, pH 5.0 for studying tetraplex formation. The oligonucleotide in buffer was heated to 95°C for 15 min, allowed to cool to room temperature and then stored at 4°C overnight before electrophoresis.

Tetraplex destabilization

The solutions containing labeled target sequence as described above in 100 mM sodium or potassium acetate, pH 5.0 buffer were heated to 95°C for 15 min, allowed to cool to room temperature and then stored at 4°C overnight in order to facilitate quadruplex formation. Then various concentrations of oligonucleotides **1–6** were added to the reaction mixtures and incubated at 37°C for 12–24 h.

The samples were then electrophoresed on 15% non-denaturing polyacrylamide gels using 50 mM Tris–glycine, pH 5.0 buffer containing 10 mM sodium or potassium chloride at room temperature. The gels were exposed to Kodak X-Omat AR film with an intensifying screen on at –70°C. Autoradiograms were scanned with a UMAX UC840 Max Vision scanner and analyzed

using Scan Analysis program from Biosoft, Cambridge, UK on a Macintosh Quadra 650 computer.

RESULTS AND DISCUSSION

The oligonucleotides used in the study are shown in Figure 1. We chose a 30 nt long sequence (411–440) from the gag coding region of HIV-1 genome, which contains a 5′-GGGGGAAAGA sequence, as the target (18). This sequence is necessary for dimerization of the HIV-1 genome through tetraplex formation (18,19). The 16 nt purine-rich core in the middle of the sequence (Fig. 1) was the target for FTFO and antisense oligonucleotides.

Cation dependence of higher order structure formation

The electrophoretic mobility of the DNA target, incubated in 100 mM potassium or sodium acetate, pH 5.0 solution on a non-denaturing polyacrylamide gel, is shown in Figure 2. The appearance of a slow moving band in the presence of potassium or sodium ions on native gels could be the result of the formation of a tetraplex structure (14). The tetraplex band formation was dependent on the nature and concentration of the cation present in the solution (23–25). Only two bands that correspond to single- and four-stranded structures were formed in the presence of sodium (Fig. 2B). In contrast, at least two other bands that are different from the two observed with sodium ions were formed in the presence of potassium (Fig. 2A). With increasing concentrations of potassium ion (from 10 to 200 mM), the intensity of the band that corresponds to single-stranded structure decreased as a new band formed that moved slower than a single-stranded structure but faster than a tetraplex structure. This band is not observed in the presence of sodium ions at the concentration of oligonucleotide used in the experiment (Fig. 2B). At a higher concentration of the oligonucleotide (> 1.1×10^{-7} M), however, a band comparable to this was observed in the presence of sodium ions also (data not shown). Intermolecular duplex structures were proposed as intermediates in the formation of different types of quadruplex structures (28,29). The intermediate band observed in the present experiments could be one of the intermediate duplex structures that leads to the formation of tetraplex structure. Both of the fast moving bands disappeared with the formation of two slow moving bands at 0.5 M potassium ion concentration. The band labeled 'Tetr' corresponds to a quadruplex structure (Fig. 2A). The slowest band could be an eight-stranded structure as described by others (20,29,30). A concentration of 0.5 M potassium is sufficient to force the equilibrium to tetraplex structure formation completely, unlike in the case of sodium (Fig. 2).

Concentration dependence of higher order structure formation

The formation of a higher order structure is dependent on the concentration of the oligonucleotide. A tetraplex band was present even at the lowest concentration of the oligonucleotide (1.53×10^{-10} M) studied. The intensity of the band corresponding to the single strand decreased with increasing concentration of the oligonucleotide, and at 6.1×10^{-7} M concentration of the oligonucleotide no other bands except a slow moving band (tetraplex) was observed (data not shown). In addition, formation of the slow moving band was kinetically slow, and the band was stable thermodynamically once formed (31,32).

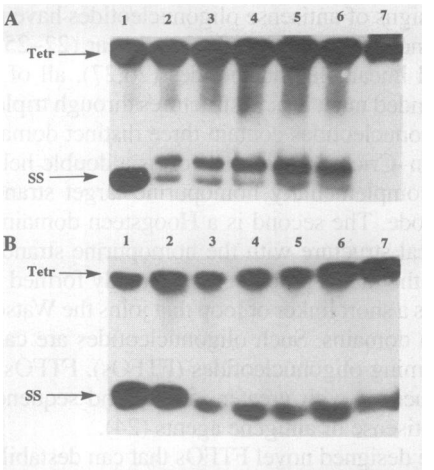

Figure 2. Effect of potassium (**A**) and sodium (**B**) ions on the formation of DNA quadruplex structure on a non-denaturing polyacrylamide gel. The concentration of the DNA target oligonucleotide in each lane is 2.0 μM. Concentration of salt in both the panels is—lane 1: no salt; lane 2: 10 mM; lane 3: 50 mM; lane 4: 100 mM; lane 5: 150 mM; lane 6: 200 mM and lane 7: 500 mM. SS and Tetr stand for single-stranded and tetraplex structures, respectively.

Dependence of quadruplex formation on contiguous guanines

Presence of three to five contiguous Gs is necessary for the formation of tetraplex structure. The target oligonucleotide contains five contiguous Gs that favor tetraplex structure formation and an isolated G flanked by adenines in the 16 nt polypurine target region. Several oligonucleotides were synthesized with one or more guanines or adenines replaced with either C (for G) or T (for A) in the 5′-GGGGGAAAGA region of the DNA target sequence to further establish that the slow moving band observed on gels is a tetraplex structure that requires contiguous Gs. Native polyacrylamide gel electrophoresis of these modified oligonucleotides in 100 mM sodium acetate, pH 5.0 solutions suggests that all five contiguous Gs are involved in and necessary for tetraplex formation, and neither the isolated G flanked by As nor the adenines themselves, adjacent to the G stretch, is involved in tetraplex formation (data not shown).

The oligonucleotides containing cytosine bases require acidic pH conditions for triplex formation. We studied the effect of pH on tetraplex formation to confirm that at pH 5.0 conditions tetraplex formation was not effected. Results of experiments performed in solutions ranging from pH 5.0 to 8.0 revealed no influence of pH on tetraplex formation (data not shown). All further experiments were, therefore, carried out in pH 5.0 solutions, unless otherwise specified, as the tetraplex formation was not dependent on pH, but that a low pH was necessary for triplex formation.

RNA/DNA quadruplex destabilization ability of FTFOs containing different deoxyribonucleotide/2′-*O*-methylribonucleotide combinations

We studied the ability of the FTFOs to destabilize quadruplex structures formed by either an RNA or a DNA target by using oligonucleotides **3–6** in both sodium and potassium ion containing solutions.

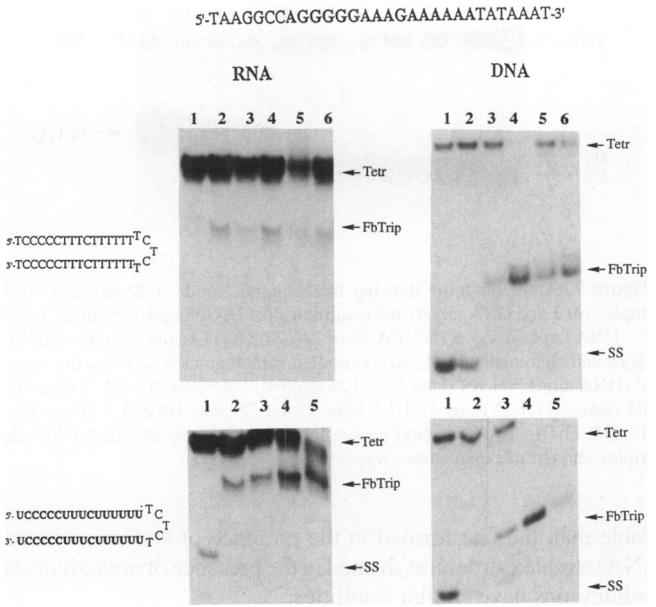

Figure 3. Autoradiograms showing foldback triplex-forming oligonucleotides **3** and **4** destabilizing quadruplex structures formed by the RNA and DNA target sequences in sodium ion containing solutions. Top left (RNA target) and right (DNA target) with oligonucleotide **3** at 1:0.1 (lane 1); 1:0.2 (lane 2); 1:0.5 (lane 3); 1:1 (lane 4); 1:5 (lane 5) and 1:10 (lane 6). Bottom left (RNA target) and right (DNA target) with oligonucleotide **4** at 1:0.1 (lane 1); 1:0.2 (lane 2); 1:0.5 (lane 3); 1:1 (lane 4) and 1:2.5 (lane 5). The concentration of RNA and DNA targets in each lane is 30.5 μM. SS, FbTrip and Tetr stand for single-stranded, foldback triplex and tetraplex structures, respectively.

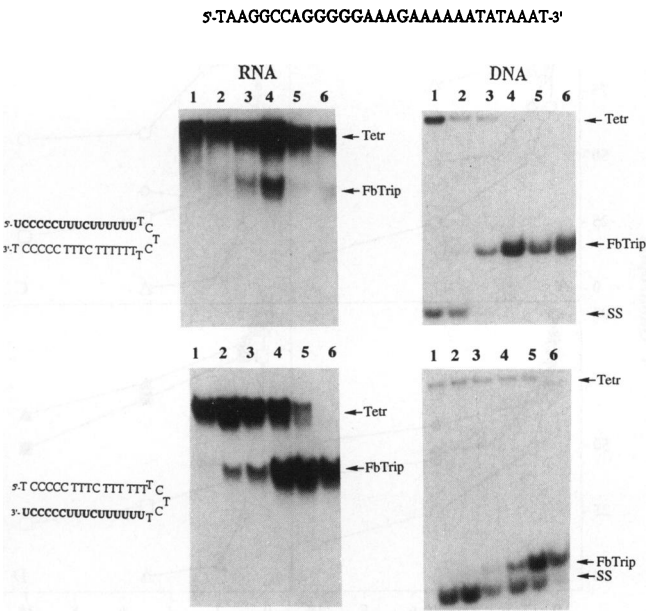

Figure 4. Autoradiograms showing foldback triplex-forming oligonucleotides **5** and **6** destabilizing quadruplex structures formed by the RNA and DNA target sequences in sodium ion containing solutions. Top left (RNA target) and right (DNA target) with oligonucleotide **6** at 1:0.1 (lane 1); 1:0.2 (lane 2); 1:0.5 (lane 3); 1:1 (lane 4); 1:2 (lane 5) and 1:5 (lane 6). Bottom left (RNA target) and right (DNA target) with oligonucleotide **5** at 1:0.1 (lane 1); 1:0.2 (lane 2); 1:0.5 (lane 3); 1:1 (lane 4); 1:5 (lane 5) and 1:10 (lane 6). The concentration of RNA and DNA targets in each lane is 30.5 μM. SS, FbTrip and Tetr stand for single-stranded, foldback triplex and tetraplex structures, respectively.

Figures 3 and 4 show the ability of the four possible RNA/DNA combinations of FTFOs (oligonucleotides **3–6**) to destabilize the RNA/DNA quartets in 100 mM sodium acetate, pH 5.0 buffer. Up to a 10-fold excess of oligonucleotide **3** had no effect on RNA quartet structure (Fig. 3, top left). DNA quartet structure, however, was destabilized at 1:1 ratio of DNA target sequence and oligonucleotide **3** (lane 4, Fig. 3, top right). We attribute the inability of oligonucleotide **3** to destabilize the RNA quartet structure to its formation of a less stable triple helix with RNA target. Recent studies by others (33–39) and in our laboratory (manuscript in preparation) on the stabilities of triplexes containing different RNA/DNA compositions revealed that a triplex of DNA–RNA–DNA is not stable (24) because of structural reasons. In solutions containing potassium (100 mM potassium acetate, pH 5.0 buffer), oligonucleotide **3** was less effective in destabilizing DNA quadruplex structure (Fig. 6D). This effect is related to the higher stability of the K^+ ion coordinated DNA tetraplex structure (29–32).

Oligonucleotide **4** containing 2′-*O*-methylribonucleotides in both the Watson–Crick and Hoogsteen domains more effectively destabilized the DNA quartet than the RNA quartet structure in sodium ion-containing solutions (Fig. 3, bottom). These data do show that oligonucleotide **4** can destabilize RNA quartet compared to oligonucleotide **3**, although at higher concentrations (lane 5, Fig. 3, bottom left). This result further supports that triplex formation is more favored with a purine DNA target strand than with an RNA strand. In the presence of potassium, oligonucleotide **4** destabilized the RNA quartet structure similar to that observed in the presence of sodium (Figs 5A and 6B)

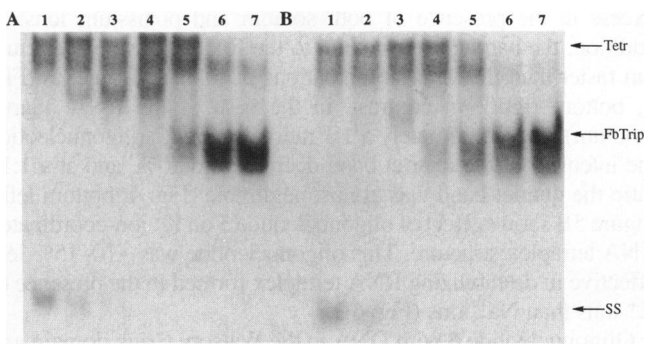

Figure 5. Autoradiograms showing foldback triplex-forming oligonucleotides **4** (**A**) and **5** (**B**) destabilizing the RNA quadruplex structure formed in the presence of potassium ions. The ratios of the target sequence and oligonucleotides **4** (A) or **5** (B) is—RNA target alone (lane 1); 1:0.1 (lane 2); 1:0.2 (lane 3); 1:0.5 (lane 4); 1:1 (lane 5); 1:5 (lane 6) and 1:10 (lane 6) in both the panels. The concentration of the RNA target sequence in each lane is 30.5 μM. SS, FbTrip and Tetr stand for single-stranded, foldback triplex and tetraplex structures, respectively.

suggesting that both sodium and potassium ion-coordinated RNA tetraplex structures have similar stabilities, unlike the DNA tetraplex structures. A 10-fold excess of oligonucleotide **4**, however, was needed to destabilize up to 50% of the DNA tetraplex formed in the presence of potassium ions.

Oligonucleotide **5** with DNA in the Hoogsteen domain and 2′-*O*-methyl-RNA content in the Watson–Crick domain showed

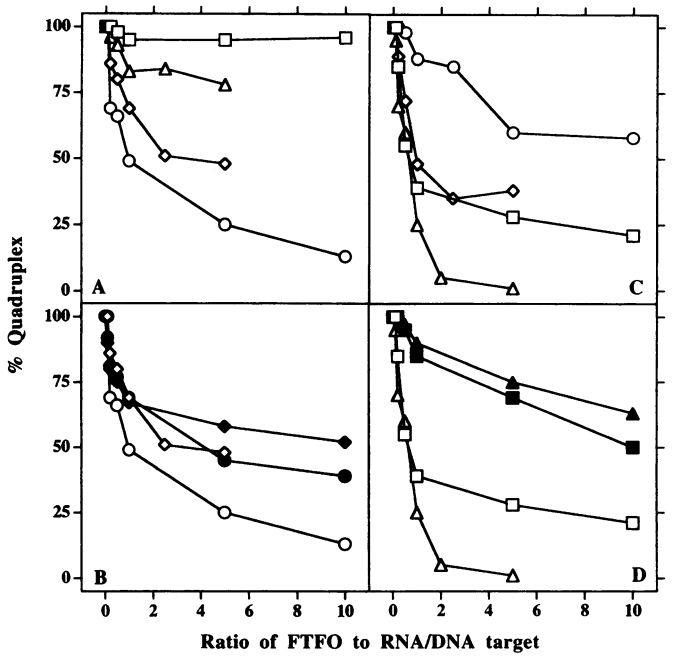

Figure 6. RNA (plots **A** and **B**) and DNA (plots **C** and **D**) tetraplex destabilizing ability of foldback triplex-forming oligonucleotides (FTFOs) **3** (□—□), **4** (◇—◇), **5** (O—O) and **6** (Δ—Δ) in the presence of sodium (open symbols) and potassium (filled symbols) salts.

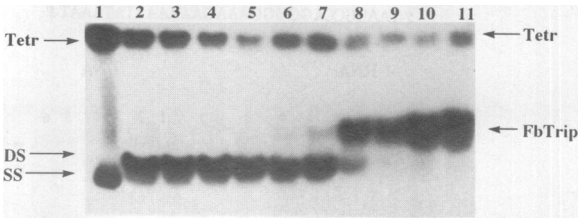

Figure 7. Autoradiogram showing that oligonucleotide **3** destabilizes both duplex (of **1** and DNA target) and quadruplex (of DNA target) structures. Lane 1—DNA target alone at 15.3 μM. Lane 2—1:1.2 equilibrium mixture of DNA target and oligonucleotide **1** and was titrated with oligonucleotide **3** at the ratios of (DNA target:3) 1:0.1 (lane 3); 1:0.25 (lane 4); 1:0.5 (lane 5); 1:0.75 (lane 6); 1:1 (lane 7); 1:1.25 (lane 8); 1:1.5 (lane 9); 1:1.75 (lane 10) and 1:2 (lane 11). SS, DS, FbTrip, and Tetr stand for single-stranded, double-stranded, foldback triplex and tetraplex structures, respectively.

significant differences in destabilizing the RNA and DNA quartets in the presence of sodium ions (Fig. 4, bottom). This oligonucleotide had no effect on the DNA quartet structure up to a 10-fold excess in the presence of both sodium and potassium ions. In addition, the band corresponding to the foldback triplex structure ran faster than the other combinations of foldback triplexes (Fig. 4, bottom right). In contrast, in the case of the RNA quartet (sodium), at approximately a 1:1 ratio of target to oligonucleotide the intensity of the quartet band decreased to 50%, and at a 1:10 ratio the quartet band was almost negligible (Fig. 4, bottom left). Figure 5B shows effect of oligonucleotide **5** on K^+ ion-coordinated RNA tetraplex structure. This oligonucleotide was ~10–15% less effective in destabilizing RNA tetraplex formed in the presence of K^+ ions than Na^+ ions (Fig. 6B).

Oligonucleotide **6** with DNA in the Watson–Crick domain and 2′-*O*-methyl-RNA component in the Hoogsteen domain, had the opposite effect of oligonucleotide **5**. This oligonucleotide completely inhibited or destabilized the DNA quartet structure at a 1:1 ratio of target sequence and oligonucleotide **6** in solutions containing sodium ions (Fig. 4, top right). In contrast, it was completely ineffective against the RNA quartet structure formed in the presence of sodium and potassium ions up to a 10-fold excess as demonstrated by the titration experiments (Fig. 4, top left). Similar results to that of oligonucleotide **3** were obtained with this oligonucleotide also on DNA quadruplex in the presence of potassium (Fig. 6D).

These results suggest that the destabilizing efficiency of FTFOs is determined by the differences in relative stabilities of the foldback triplexes formed with RNA and DNA target sequences and the stabilities of the tetraplex structures of RNA and DNA formed in the presence of potassium and sodium salts. Apparently, the DNA tetraplex formed in the presence of potassium is more stable than the one formed in the presence of sodium, whereas RNA tetraplex structures formed in the presence of potassium and sodium ions have similar stabilities.

Destabilization of tetraplex as well as duplex structures by FTFOs

We examined the ability of oligonucleotide **3** to destabilize both duplex and quadruplex structures. The equilibrium mixtures of DNA target sequence and oligonucleotide **1** (1:1.2 ratio) in 100 mM sodium, pH 5.0 buffer were titrated with increasing amounts of oligonucleotide **3** at room temperature and analyzed on a non-denaturing gel. The autoradiogram shown in Figure 7 demonstrates that increasing the concentration of oligonucleotide **3** destabilizes both duplex and quadruplex structures simultaneously.

In a reverse experiment, the equilibrium mixtures of DNA target sequence and oligonucleotide **3** (1:1.1 ratio) under the same experimental conditions as above were titrated with oligonucleotide **1** to study whether oligonucleotide **1** can destabilize the foldback triplex. The results suggest that up to a 15-fold excess oligonucleotide **1** is unable to destabilize the foldback triplex of DNA target sequence and oligonucleotide **3** (data not shown).

Destabilization of quadruplex structure by antisense oligonucleotides

Oligonucleotides **1** and **2** are complementary to the DNA and RNA target sequences. They hybridize to the target sequences at polypurine site by Watson–Crick base pairing. Oligonucleotide **1** is composed of deoxyribonucleotides and **2** is a 2′-*O*-methyl-ribonucleotide analog of **1**. We chose to use the 2′-*O*-methyl analog of RNA because it is easy to handle, is stable against nucleases compared to RNAs (40), and can bind to the target sequences by duplex as well as triplex formation (41).

Figure 6 shows the ability of oligonucleotides **1** and **2** to destabilize the quadruplex structures. The RNA and DNA target sequences were titrated with either oligonucleotide **1** or **2** in 100 mM sodium acetate, pH 5.0 buffer up to 20-fold excess over the target sequence concentration. Each of the oligonucleotides was unable to destabilize the DNA super-structure (Fig. 8A and B), and only formed a duplex with the single-stranded species of the DNA target. The slower mobility of the duplex band at higher ratios (over a 1:5 ratio) compared with the lower ratios could be

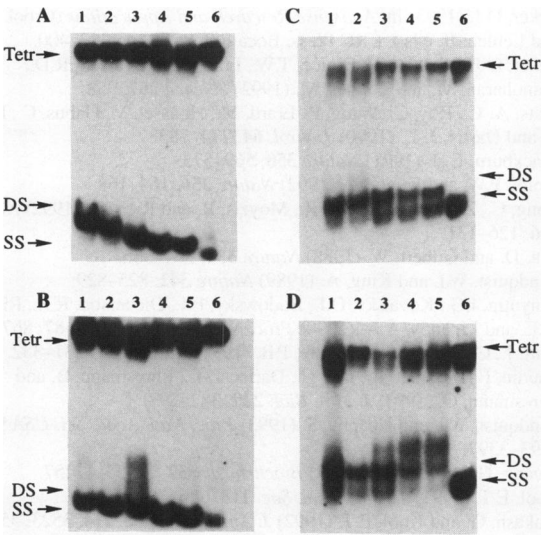

Figure 8. Autoradiograms showing the ability of oligonucleotides **1** (A and C) and **2** (B and D) to destabilize DNA (A and B) or RNA quadruplex structures (C and D). (**A**) DNA target alone (lane 6); and in the presence of oligonucleotide **1** at 1:0.5 (lane 5); 1:1 (lane 4); 1:5 (lane 3) 1:10 (lane 2) and 1:20 (lane 1) ratios. (**B**) DNA target alone (lane 6); and in the presence of oligonucleotide **2** at 1:0.5 (lane 5); 1:1 (lane 4); 1:5 (lane 3) 1:10 (lane 2) and 1:20 (lane 1) ratios. (**C**) RNA target alone (lane 6); and in the presence of oligonucleotide **1** at 1:0.5 (lane 5); 1:1 (lane 4); 1:5 (lane 3) 1:10 (lane 2) and 1:20 (lane 1) ratios. (**D**) RNA target alone (lane 6); and in the presence of oligonucleotide **2** at 1:0.5 (lane 5); 1:1 (lane 4); 1:5 (lane 3) 1:10 (lane 2) and 1:20 (lane 1) ratios. The concentration of the RNA or DNA target in each lane is 15.25 μM. SS, DS and Tetr stand for single-stranded, double-stranded and tetraplex structures, respectively.

the result of formation of a triplex structure in the presence of excess antisense oligonucleotide.

As in the case of the DNA target, both oligonucleotides **1** and **2** formed double-stranded structures with the RNA target (Fig. 8C and D). Up to a 20-fold excess of oligonucleotide **1** was not able to effectively destabilize the RNA quartet structure (Fig. 8C). RNA quartet band intensity decreased in the presence of oligonucleotide **2** at higher concentrations, although it was not completely diminished, suggesting that oligonucleotide **2** might be better than oligonucleotide **1** at destabilizing the RNA quartet structure (Fig. 8D).

We examined whether the destabilization ability of FTFOs results from binding of both the Watson–Crick and Hoogsteen domains or only the Watson–Crick domain of the FTFO to DNA/RNA target sequences. Experiments in pH 7.2 solutions, under which conditions the third strand (Hoogsteen domain of FTFOs) cannot bind firmly because the cytosines are not protonated, revealed that up to a 5-fold excess of oligonucleotide **3** was unable to destabilize DNA quartet structure (data not shown). Results of this experiment, coupled with the results obtained with oligonucleotides **1** and **2** (that bind to the target sequence only by Watson–Crick duplex formation) lead to conclude that quadruplex destabilization is the result of foldback triplex formation but not just by the formation of a duplex.

The electrophoretic mobility measurements of the RNA and DNA sequences, single-stranded, double-stranded or triple-stranded structures suggest that RNA sequences and the complexes containing RNA sequences run slower than DNA sequences on native polyacrylamide gels (33). A notable feature here, however, was that the RNA quartet structure ran faster than the DNA quartet structure on the same percentage of native polyacrylamide gel. This anomalous behavior of the RNA tetrad structure could be related to the hydration and compactness of the RNA quartet structure.

Prats *et al.* reported that a 29mer antisense oligonucleotide complementary to the dimerization–encapsidation region of MoMuLV inhibits the dimerization process *in vitro* (10). A 50–100-fold excess of the antisense oligonucleotide was needed, however, to abolish the dimerization process to near completion. Recently, DNA antisense oligonucleotides have been used to study the sequences involved in the dimerization process of HIV, but also at a large excess of oligonucleotide (20). Our results with oligonucleotides **1** and **2** confirm that antisense oligonucleotides that bind to the target only by the Watson–Crick duplex formation do not effectively destabilize tetraplex structures.

The differential ability of FTFOs to destabilize the quadruplex structure of DNA and RNA could be related to the differences in the energetics and structures of DNA and RNA triple helices formed (34–37). These studies suggest that the triple helical structures favor a conformation that is distinct from both canonical A- (C3′-endo sugar puckering) and B-form (C2′-endo puckering) helices. Thermodynamic studies (33,38,39), including our own results on foldback triplexes (manuscript in preparation), show a preference of triple helix structures for a DNA homopurine strand and an RNA Hoogsteen hydrogen bonding third strand consistent with structural evidences. In light of these observations, we propose that the quadruplex destabilization by FTFOs is directly related to their ability to form energetically and structurally stable triplexes. Formation of a foldback triplex is kinetically favored over formation of quadruplex and conventional triplex structures. The initial duplex formed via the Watson–Crick domain of the FTFO places the Hoogsteen domain in a favorable position for triplex formation. This results in preorganization and increased local concentration of the third strand at binding site, which contributes to the formation of a stable foldback triplex structure.

We presume that FTFOs pre-associate initially at the non-quartet forming polypurine site (underlined) towards 3′-end of the target sequence 5′-AGGGGG<u>AAAGAAAAAA</u>-3′ through foldback triplex formation and subsequently destabilize and invade quadruplex structure. However, it is difficult to comment whether FTFOs can actively compete and destabilize quartet structures if non-quartet forming polypurine site adjacent to quadruplex forming sequence is absent in the target sequence or the minimum length of non-quartet forming polypurine sequence required for FTFOs to pre-associate and destabilize quartet structure.

Currently, there is compelling evidence for the formation of unusual nucleic acid structures such as tetraplexes that involve guanine repeats in both RNA and DNA sequences, at least under *in vitro* conditions. These structures might play a key role in biological macromolecular functions such as recombination, transcription, encapsidation, etc. Design and development of agents that can selectively intervene with those sequences to prevent formation of such rare but vital structures would lead to novel chemotherapeutic agents.

CONCLUSIONS

We have shown that the sequence 5′-GGGGGAAAG (RNA or DNA) forms a stable tetraplex-like structure that is dependent on the nature and concentration of the cation in solution and the

concentration of the nucleic acid strand itself. Oligonucleotides (RNA or DNA) that can bind to the target through Watson–Crick duplex formation are unable to destabilize such higher order structures formed by the target sequence. FTFOs that can form both Watson–Crick and Hoogsteen base pairing simultaneously with a homopurine target sequence can interfere with and prevent four stranded structure formation by the target, although to different degrees depending on the chemical nature of the target, the chemical composition of the FTFO, and the nature of the cation involved in the tetraplex formation. The data suggest that an FTFO containing deoxyribonucleotides in the Watson–Crick domain and 2′-*O*-methylribonucleotides in the Hoogsteen domain is suitable for targeting DNA sequences that can form tetraplex structures. Similarly, an FTFO containing 2′-*O*-methylribonucleotide content in the Watson–Crick domain and deoxyribonucleotide content in the Hoogsteen domain might be suitable for targeting RNA sequences that are involved in tetraplex structure formation.

The FTFOs (oligonucleotide **3**) also disrupt the Watson–Crick DNA double helix and form foldback triplexes (Fig. 7). Probably the FTFOs pre-associate with the duplex in the major groove via Hoogsteen hydrogen bonding and form a foldback triplex by strand displacement resulting in a D-loop formation (our unpublished results) as in the case of peptide nucleic acids (42). This new design of antisense oligonucleotides might allow development of tumor specific chemotherapeutic agents in which cellular pH goes down to 5.2 (43), either by using synthetic or endogenously expressed FTFOs.

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