

Overcoming potassium-mediated triplex inhibition

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ABSTRACT

Sequence-specific duplex DNA recognition by oligonucleotide-directed triple helix formation is a possible approach to *in vivo* gene inhibition. However, triple helix formation involving guanine-rich oligonucleotides is inhibited by physiological ions, particularly K^+ , most likely due to oligonucleotide aggregation via guanine quartets. Three oligodeoxynucleotide (ODN) derivatives were tested for their ability to resist guanine quartet-mediated aggregation, yet form stable triplexes. Electrophoretic mobility shift and dimethyl sulfate footprinting assays were used to analyze the formation of triplexes involving these oligonucleotide derivatives. In the absence of K^+ , all ODNs had similar binding affinities for the duplex target. Triplexes involving a 14mer ODN derivative containing 7-deazaxanthine substituted for three thymine bases or an 18mer ODN containing two additional thymines on both the 5' and 3' termini were abolished by 50 mM K^+ . Remarkably, triplexes involving an ODN derivative containing four 6-thioguanine bases substituted for guanine resisted K^+ inhibition up to 200 mM. We hypothesize that the increased radius and decreased electronegativity of sulfur in the 6-position of guanine destabilize potential guanine quartets. These results improve the prospects for creating ODNs that might serve as specific and efficient gene repressors *in vivo*.

INTRODUCTION

Oligonucleotide-directed triple helix formation is a highly specific approach to duplex DNA recognition that has been proposed as a strategy for designing artificial gene repressors (1-4). DNA recognition occurs by hydrogen bonding between oligonucleotide bases and purine bases in the major groove of duplex DNA. Triple helix formation arises in either of two patterns, termed the pyrimidine motif and the purine motif (4). The pyrimidine motif involves the formation of T•A•T and C+•G•C base triplets by Hoogsteen hydrogen bonding where the third strand is parallel to the purine strand of the duplex (1). The purine motif involves the formation of A•A•T (or T•A•T) and G•G•C base triplets by reverse Hoogsteen hydrogen bonding where the third strand is antiparallel to the purine strand of the duplex (5).

To promote protonation of oligonucleotide cytosines, oligonucleotide binding in the pyrimidine motif often requires a

slightly acidic environment (1,6,7). In contrast, the purine motif is pH-independent, so has been thought to be a more viable approach for *in vivo* applications (2,8-10). However, we and others have previously reported that triplexes involving guanine-rich oligonucleotides are inhibited by physiological ionic conditions, particularly the presence of K^+ (11-13). Triplex inhibition is most likely due to oligonucleotide aggregation stabilized by the formation of guanine quartets (13,14). Such inhibition might severely constrain the use of purine motif oligonucleotides as artificial gene repressors *in vivo*.

Using a test system in which triplex formation had previously been shown to be inhibited by monovalent cations (M^+), we wished to develop an ODN that was resistant to potassium-mediated aggregation, yet was still able to form stable triplexes (13). Toward this end, three derivatives of unmodified ODN 1 were synthesized (Fig. 1B). First, ODN 1S was synthesized by substituting the analog 6-thioguanine (Fig. 1A) in place of certain guanines. Specifically, we reasoned that the bulkier size and decreased electronegativity of sulfur at the 6-position should not affect triplex formation, but might interrupt hydrogen bonding and ion chelation critical for the formation of quartets. Because tetraplex stabilization requires stacked guanine quartets, ODN 1S was synthesized such that no two adjacent guanines were present. Secondly, ODN 1X was synthesized by incorporating the analog 7-deazaxanthine (Fig. 1A) in place of thymines. Incorporation of 7-deazaxanthine into less guanine-rich ODN sequences has previously been shown to stabilize triplexes in the presence of physiological K^+ concentrations (11). Finally, ODN 1T was synthesized with two additional thymines on both the 5' and 3' termini. This design is based on the premise that these terminal thymines might decrease the number of oligonucleotides sequestered into quartets by preventing the formation of aggregates involving ODNs aligned out of register (15).

Electrophoretic mobility shift and dimethyl sulfate footprinting assays were used to test ODN 1 and its derivatives for the ability to form stable triplexes, yet resist guanine quartet-mediated aggregation. We report that in the absence of K^+ , all ODNs had similar binding affinities for the duplex DNA target. Triplex formation involving ODN derivatives 1X and 1T was abolished by 50 mM K^+ , while triplexes involving ODN derivative 1S resisted K^+ inhibition up to 200 mM. This result suggests that the substitution of 6-thioguanine for guanine at some positions within an ODN alleviates the guanine quartet-mediated inhibition of triplexes by physiological ionic conditions.

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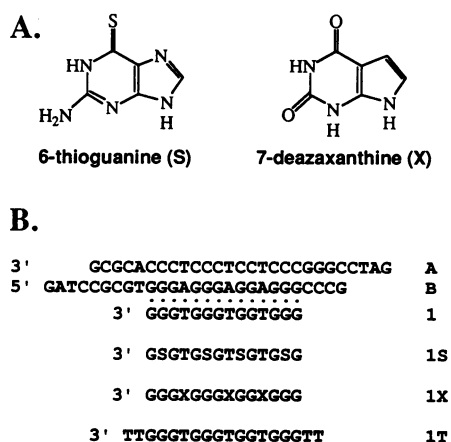


Figure 1. (A) Structures of modified bases 6-thioguanine and 7-deazaxanthine. (B) Experimental design. The duplex DNA target is formed by annealing complementary ODNs A and B. ODNs 1, 1S, 1X and 1T bind in the major groove of the duplex, antiparallel to ODN B. ODNs 1S, 1X and 1T have the same sequence as ODN 1, but with the following changes: ODN 1S contains 6-thioguanine (S) substituted for four guanines, ODN 1X contains 7-deazaxanthine (X) substituted for three thymines, and ODN 1T contains two additional thymines on both the 5' and 3' termini.

MATERIALS AND METHODS

Oligonucleotides

ODN sequences are shown in Figure 1. ODNs A, B, 1, 1S and 1T were synthesized by phosphoramidite chemistry on an ABI Model 380B DNA synthesizer, purified by denaturing polyacrylamide gel electrophoresis, eluted from gel slices and desalted by Sep-Pak C₁₈ cartridge chromatography (Waters). ODN 1S was synthesized using S6-DNP-dG-CE phosphoramidite (Glen Research) at some positions (16). The resulting precursor is converted to 6-thio dG, as described by the supplier, upon deprotection of the oligonucleotide with 10% mercaptoethanol in concentrated ammonium hydroxide for 48 h at room temperature prior to gel purification. The identity of ODN 1S was confirmed by laser desorption mass spectroscopy (17). ODN 1X was synthesized as previously described (11). All oligonucleotides were quantitated by absorbance at 260 nm using molar extinction coefficients ($M^{-1}cm^{-1}$) of 15 400 (dA), 11 700 (dG), 7300 (dC), 8800 (dT), 8820 (6-thio dG) and 8440 (7-deaza dX), assuming no hypochromicity. Oligonucleotides comprising the target duplex were annealed as follows: 500 pmol each of ODNs A and B were mixed with 2 μ l 5 M NaCl and brought to a total volume of 42 μ l with H₂O. The annealing reaction mixture was incubated at 75°C for 12 min and then gradually cooled to 25°C. This procedure results in no detectable depurination. Sixty pmol of the resulting oligonucleotide duplex were radiolabeled using the Klenow fragment of DNA polymerase I and [α -³²P]dATP in the presence of 2 mM dGTP, dTTP and dCTP. The resulting labeled duplex oligonucleotides were purified by precipitation with ethanol in the presence of ammonium acetate, and resuspended in H₂O.

Electrophoretic mobility shift assays

For triplex assays in which [ODN] varied and [M⁺] remained constant, binding reaction mixtures contained (in order of

addition) H₂O, labeled duplex (50 000 c.p.m.; ~0.02 pmol), 1 μ l of 10 \times binding buffer (250 mM Tris-HCl, pH 8.0, 60 mM MgCl₂), 1 μ l of 100 mM NaCl, 100 mM KCl or H₂O (as indicated), 1 μ l yeast tRNA (1 mg/ml) and 1 μ l ODN 1, 1S, 1X or 1T (to yield the indicated final concentration) in a final volume of 10 μ l. Reaction mixtures were incubated at 22°C for 5 h and were then supplemented with 1 μ l of an 80% glycerol solution containing bromophenol blue. Reactions were analyzed by electrophoresis through 20% native polyacrylamide gels (19:1 acrylamide:bisacrylamide) prepared in 1 \times TBE (100 mM Tris-base, 110 mM boric acid, 2 mM EDTA) supplemented with 8 mM MgCl₂. Electrophoresis was performed in this buffer (with recirculation) at 4°C overnight (9 V/cm). The resulting gel was imaged and quantified by storage phosphor technology using a Molecular Dynamics PhosphorImager.

For triplex assays in which [K⁺] varied and [ODN] remained constant, binding reaction mixtures contained (in order of addition) H₂O, labeled duplex (50 000 c.p.m.; ~0.02 pmol), 1 μ l of 10 \times binding buffer, 1 μ l yeast tRNA (1 mg/ml), 1 μ l KCl (to yield the indicated final concentration) and 1 μ l of 10 μ M ODN 1, 1S, 1X or 1T in a final volume of 10 μ l. Reaction mixtures were analyzed as described above.

Analysis of gel mobility shift titrations

The apparent fraction, θ , of target duplex bound by ODN 1, 1S, 1X or 1T was calculated for each gel lane using the definition:

$$\theta = S_{\text{triplex}} / (S_{\text{triplex}} + S_{\text{duplex}}) \quad (1)$$

where S_{triplex} and S_{duplex} represent the storage phosphor signal for triplex and duplex complexes respectively. Values of the apparent triplex dissociation constant, K_d , were obtained by least squares fitting of the data to the binding isotherm:

$$\theta = ([O]^n / K_d^n) / (1 + [O]^n / K_d^n) \quad (2)$$

where [O] is the concentration of ODN 1, 1S, 1X or 1T, and n is the Hill coefficient (18). Relative ODN affinities toward the target DNA duplex were calculated with reference to that observed for ODN 1 binding in the absence of M⁺:

$$\text{Relative affinity} = (K_{d, \text{ODN 1, -M}^+} / K_{d, \text{exp}}) \times 100 \quad (3)$$

where $K_{d, \text{ODN 1, -M}^+}$ is the measured triplex dissociation constant of ODN 1 in the absence of M⁺, and $K_{d, \text{exp}}$ is the measured triplex dissociation constant of the ODN in question under the indicated conditions.

Dimethyl sulfate footprinting

ODNs A and B (500 pmol) were phosphorylated by addition of 2 μ l of 10 \times kinase buffer, 0.6 μ l of 100 mM ATP, 1 μ l T4 polynucleotide kinase (10 U) and H₂O in a total volume of 20 μ l. Reaction mixtures were incubated at 37°C for 45 min. Phosphorylated A and B were combined with 2 μ l of 5 M NaCl and annealed as described above. The resulting duplex was ligated into plasmid pG₅E4T that had been cleaved by BamHI (19). A clone bearing the desired insertion was confirmed by sequencing. A 445 bp PstI-EcoRI restriction fragment was then prepared and uniquely end-labeled using the Klenow fragment of DNA polymerase I. Labeled fragment (50 000 c.p.m.; ~0.1 pmol) was incubated with 1 μ l of 10 \times binding buffer (see above), 1 μ l yeast tRNA (1 mg/ml), 1 μ l ODN 1 or 1S (10 μ M), 1 μ l of NaCl or KCl (to yield the indicated final concentration) or 2 μ l 5 \times nuclear extract buffer

[NEB; 100 mM Hepes, pH 7.4, 25 mM MgCl₂, 500 mM KCl, 50% (v/v) glycerol], and H₂O in a final volume of 10 μ l. Binding reactions were incubated overnight at 22°C. Dimethyl sulfate [1 μ l of a 4% (v/v) aqueous solution] was added to each reaction mixture and allowed to incubate for 30 min at 4°C. Reactions were stopped with 5 μ l of stop mix [1.5 M NaOAc, 7% (v/v) β -mercaptoethanol and 100 μ g/ml yeast tRNA]. Following ethanol precipitation, 10% piperidine (100 μ l) was added, and the samples were incubated for 30 min at 90°C. Piperidine was removed by repeated lyophilization. The DNA was then resuspended in 5 μ l of formamide dye mix, electrophoresed on a 20% polyacrylamide sequencing gel (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in 0.5 \times TBE buffer, and imaged by storage phosphor technology.

RESULTS

Experimental design

The model system reported here is based on guanine-rich ODN 1, which binds site-specifically to a homopurine sequence in the target DNA duplex (ODNs A + B) according to the purine triple helix motif. We have previously reported that ODN 1 binding to duplex DNA is inhibited by physiological concentrations of monovalent cations (particularly K⁺), most likely due to aggregation of ODN 1 into complexes involving guanine quartets (13). Therefore, ODNs 1S, 1X and 1T were designed as analogs of ODN 1 that might be resistant to guanine quartet-mediated aggregation, yet still be capable of forming stable triplexes. Electrophoretic mobility shift and dimethyl sulfate footprinting assays were used to analyze the formation of triple helices involving ODNs 1, 1S, 1X and 1T.

Triplex stabilities in the absence of monovalent cations

Equilibrium dissociation constants (K_d s) were first measured for triplexes in the absence of inhibitory monovalent cations. Figure 2A and B presents examples of such experiments, in which labeled duplex A+B was incubated in the presence of increasing concentrations of ODNs 1, 1S, 1X or 1T in binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM MgCl₂). Figure 2C depicts quantitative results from these experiments. Values of K_d were calculated as described in Materials and Methods and are listed in Table 1. The values of K_d measured for triplexes involving ODNs 1, 1S, 1X and 1T are all $\sim 2 \times 10^{-7}$ M, with the largest difference being 3-fold between ODNs 1X and 1T.

Table 1. Triplex K_d values and relative affinities^a

ODN	10 mM M ⁺	K_d (M)	Relative affinity
1	–	1.4×10^{-7}	100
1	Na ⁺	2.1×10^{-7}	67
1	K ⁺	5.3×10^{-6}	3
1S	–	2.2×10^{-7}	64
1S	Na ⁺	2.0×10^{-7}	70
1S	K ⁺	1.9×10^{-7}	74
1X	–	1.2×10^{-7}	117
1X	Na ⁺	3.0×10^{-7}	47
1X	K ⁺	1.9×10^{-6}	7
1T	–	3.6×10^{-7}	39

^aValues of K_d were calculated from equation 2 and relative affinities calculated from equation 3 as described in Materials and Methods.

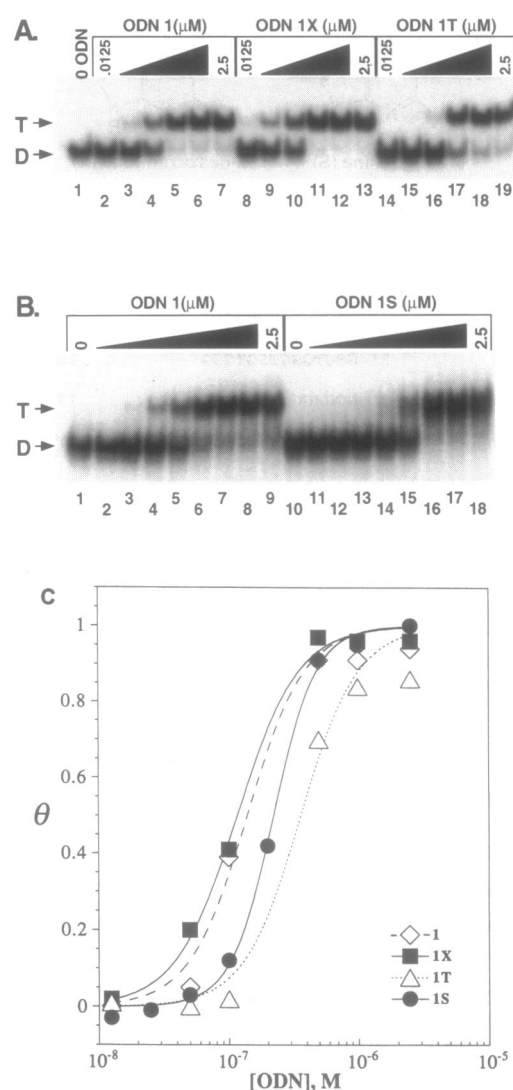


Figure 2. Determination of triplex K_d in the absence of M⁺. (A) Electrophoretic mobility shift assay. Increasing micromolar concentrations (0.0125, 0.05, 0.1, 0.5, 1, 2.5) of ODN 1 (lanes 2–7), 1X (lanes 8–13), 1T (lanes 14–19), or no ODN (lane 1) were added to labeled target duplex A+B in binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM MgCl₂). Mobilities of free duplex (D) and triplex (T) are indicated. (B) Comparison of ODNs 1 and 1S. Increasing micromolar concentrations (0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2.5) of ODN 1 (lanes 1–9) or 1S (lane 10–18) were added to the labeled target duplex in binding buffer. (C) Binding curves for ODNs 1 (\diamond), 1S (\bullet), 1X (\blacksquare) and 1T (\triangle) with the target duplex were fitted using data from electrophoretic mobility shift titrations. The fraction of duplex in triplex form (θ) was calculated from equation 1 and fitted to equation 2 as described in Materials and Methods to provide estimates of K_d (Table 1).

Triplex stabilities in the presence of monovalent cations

Having determined that all ODNs promoted stable triplexes in the absence of monovalent cations, triplexes involving derivatives of ODN 1 were tested for resistance to monovalent cation inhibition. Figure 3 depicts the results of such an experiment. When the ratios of duplex and triplex species were quantitated, the order of resistance to triplex inhibition was found to be ODN 1S > ODN 1X > ODN 1T > ODN 1. Specifically, triplexes involving ODN

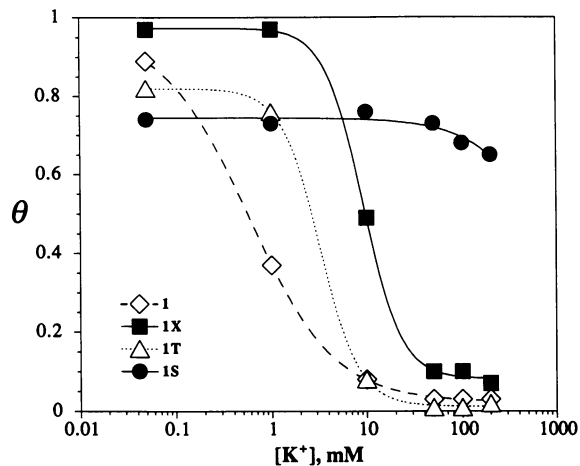


Figure 3. Triplex inhibition by K^+ . Triplex inhibition curves for 1 μ M ODN 1 (\diamond), 1S (\bullet), 1X (\blacksquare) and 1T (Δ) binding the target duplex in binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$) and increasing millimolar concentrations (0.05, 1, 10, 50, 100, 200) of K^+ were fitted using data from electrophoretic mobility shift titrations. The fraction of duplex in triplex form (θ) was calculated from equation 1 as described in Materials and Methods.

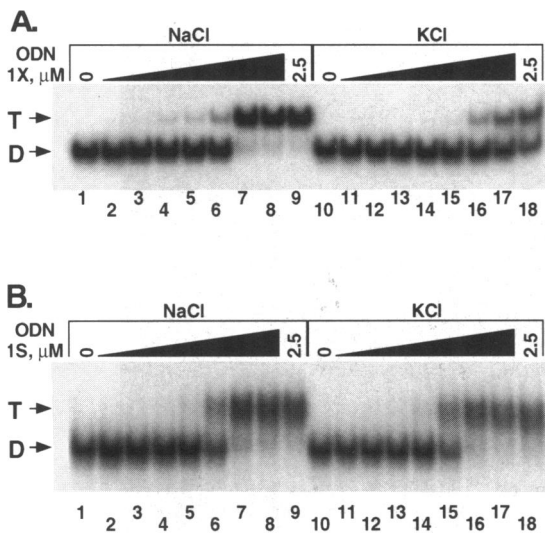


Figure 4. Determination of triplex K_d in the presence of Na^+ or K^+ . Increasing micromolar concentrations (0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2.5) of ODN 1X (A), or 1S (B) were added to the labeled target duplex and binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$) in the presence of 10 mM Na^+ (lanes 1-9) or K^+ (lanes 10-18). Mobilities of free duplex (D) and triplex (T) are indicated.

1S remained almost completely resistant to K^+ inhibition up to 200 mM K^+ , while triplexes involving ODNs 1X, 1T and 1 were abolished by 50 mM K^+ .

Dissociation constants for ODNs 1, 1S and 1X were determined in the presence of 10 mM Na^+ or K^+ in addition to binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$). Figure 4A and B presents examples of such experiments, in which labeled duplex A+B was incubated in the presence of increasing concentrations of ODN 1S or 1X. The resulting estimates of K_d are listed in Table 1. The presence of 10 mM Na^+ had minor effects on

ODN 1, 1 μ M	+	+	+	+	+	+						
ODN 1S, 1 μ M							+	+	+	+	+	+
Na^+ (mM)		10	100					10	100			
K^+ (mM)				10	100					10	100	
NEB							+					+
DMS	+	+	+	+	+	+	+	+	+	+	+	+

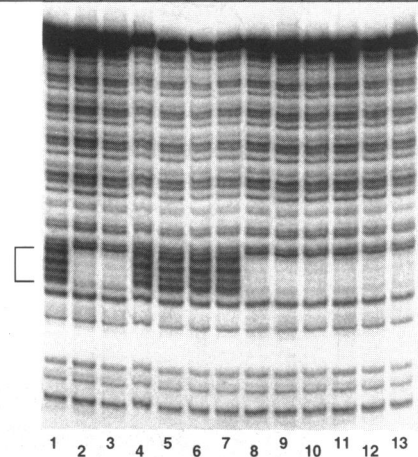


Figure 5. Dimethyl sulfate footprinting assay. The target duplex was subcloned into a recombinant plasmid that was then linearized, labeled and subjected to DMS modification in the presence of 1 μ M ODN 1 (lanes 2-7), ODN 1S (lanes 8-13) or no ODN (lane 1), and the indicated M^+ in binding buffer (25 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$) or nuclear extraction buffer (NEB). The target site for triplex formation is bracketed.

observed triplex dissociation constants. These data are presented in Table 1 as relative affinities (compared with the binding of ODN 1 in the absence of monovalent cations). The relative affinities of ODNs 1 and 1X decreased by only 1.5- and 2.5-fold, respectively, compared with the relative affinities of these ODNs in the absence of M^+ . The relative affinity of ODN 1S was unaffected by Na^+ (Table 1). In contrast, the presence of 10 mM K^+ severely inhibited triplexes involving ODNs 1 and 1X, with relative affinities decreasing by 33- and 17-fold, respectively, compared with the relative affinities of these ODNs in the absence of M^+ . Remarkably, the relative affinity of ODN 1S was not affected by the presence of 10 mM K^+ (Table 1). In addition, incubation of 1 μ M labeled ODN 1S with 200 mM K^+ did not promote the formation of aggregates with reduced electrophoretic mobilities (data not shown).

Confirmation of ODN specificity and affinity in the presence of monovalent cations

To verify the binding specificity and affinity of ODNs 1 and 1S for the target duplex DNA sequence in the presence and absence of physiological M^+ , a DMS footprinting analysis was performed. Protection of guanine N7 from DMS modification is conferred by triple helix formation. It should be emphasized that a DMS footprint reflects oligonucleotide binding to the purine strand of the radiolabeled duplex DNA. Quadruplex formation is not measured in this assay. When analyzed after cloning into plasmids, targeted guanines in the purine strand of the DNA duplex were specifically protected by ODN 1 (1 μ M) in the absence of M^+ or in the presence of 10 mM Na^+ (Fig. 5, lanes 2 and 3, respectively). However, no protection was observed by

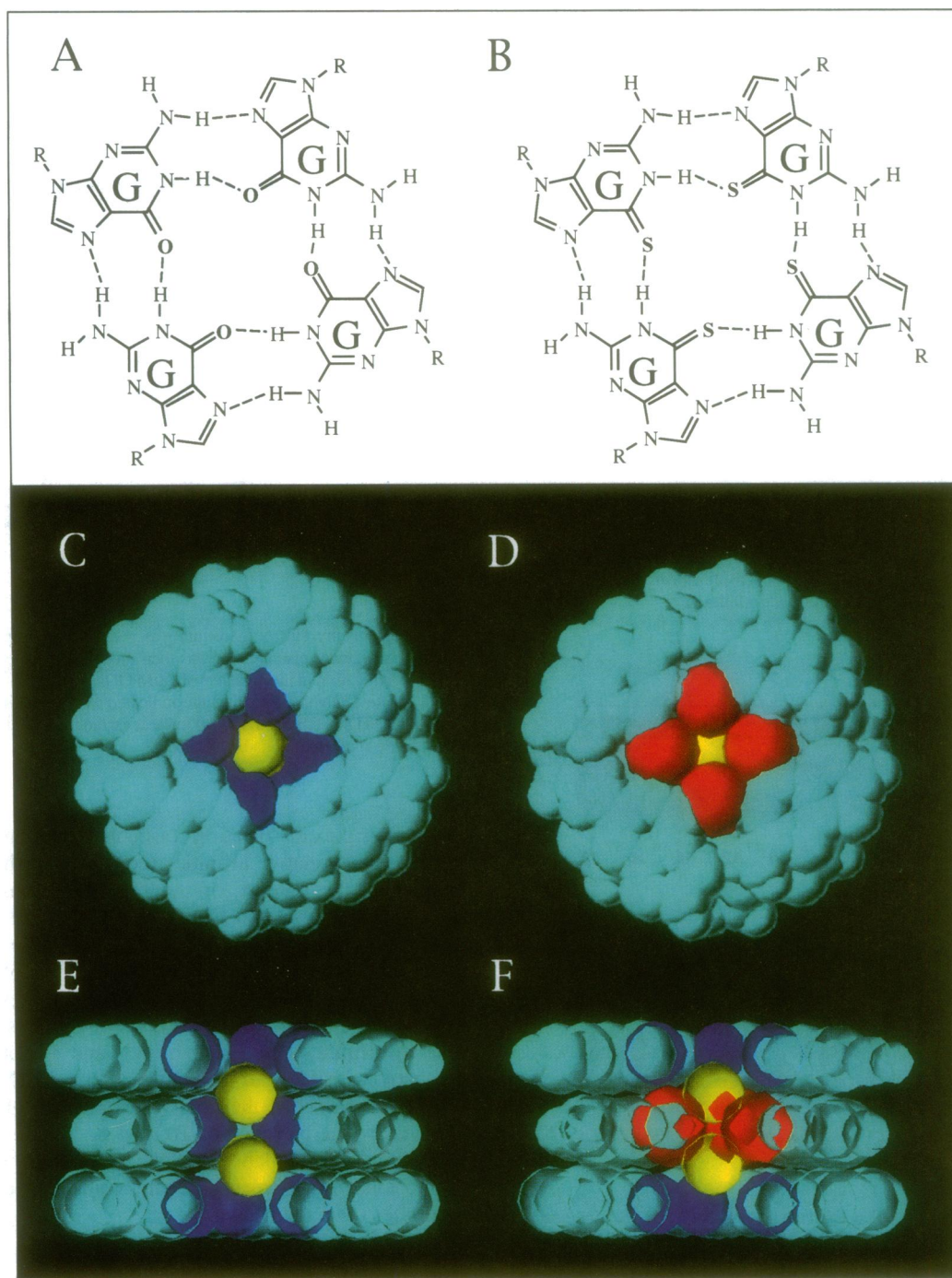


Figure 6. Models of ion chelation in stacked quartets. Hydrogen bonding arrangements are shown for quartets of guanine (A) or 6-thioguanine (B). (C–F) are based on the atomic coordinates in reference 24 and indicate changes in the chelation environment upon 6-thioguanine substitution. Top views (C) and (D) depict three stacked quartets composed of guanine exclusively (C) or 6-thioguanine substituted in the top quartet (D). Side views of three stacked quartets composed of guanine exclusively (E) or 6-thioguanine substituted in the central quartet (F) have been cut away to show the center of the quartets. Color coding: blue, guanine O⁶; red, 6-thioguanine S⁶; yellow, chelated K⁺.

ODN 1 in the presence of physiological monovalent cations (Fig. 5, lanes 4–7). In contrast, all targeted guanines in the purine strand of the DNA duplex were specifically protected by ODN 1S (1 μ M) under all conditions tested (Fig. 5, lanes 8–13). These conditions included 100 mM Na⁺ (lane 10), 100 mM K⁺ (lane 12),

and a typical nuclear extract buffer containing 100 mM K⁺ (lane 13). These data confirm results from electrophoretic mobility shift assays, showing that alternation of guanine and 6-thioguanine in ODN synthesis creates an ODN analog uniquely resistant to triplex inhibition by monovalent cations.

DISCUSSION

Triple helix formation involving guanine-rich ODNs is inhibited by physiological ionic conditions most likely due to guanine quartet aggregation (13). Such inhibition would seem to severely limit the use of guanine-rich ODNs as artificial gene repressors. We have tested three guanine-rich ODN analogs (ODNs 1S, 1X and 1T) for their ability to form stable triplexes, yet resist guanine quartet-mediated aggregation.

Triplexes involving ODN analogs 1X and 1T were inhibited by monovalent cations to about the same extent as unmodified ODN 1. The ineffectiveness of ODN 1X above 50 mM K⁺ (Fig. 3) was somewhat surprising in light of a previous report (11). These authors described the ability of triplexes involving a 15mer ODN containing 7-deazaxanthine substituted in place of six thymine residues to resist inhibition by up to 140 mM K⁺. One interpretation of our result is that 7-deazaxanthine substitution is most effective for sequences with more balanced G:T ratios than ODN 1. An even simpler approach to destabilizing inhibitory quartets was suggested by previous studies where terminal thymidine residues blocked the formation of misaligned aggregates (15). As shown in Figure 3, ODN 1T is somewhat more resistant than ODN 1 to inhibition by low concentrations of K⁺, but this effect becomes insignificant above 10 mM K⁺.

In marked contrast to ODNs 1, 1X and 1T, ODN 1S bound specifically and with high affinity to the duplex DNA target in the presence of up to 200 mM K⁺. We hypothesize that ODN 1S is resistant to guanine quartet aggregation for three reasons. First, extensive hydrogen bonding (14,20) undoubtedly stabilizes quartets composed of guanines or 6-thioguanines as seen in Figure 6 (A and B, respectively). It is possible that 6-thioguanine quartets are not stable because of weaker hydrogen bonds involving S⁶. Secondly, stabilization of guanine quartets requires the eight-coordinate chelation of a monovalent cation, particularly K⁺, by the O⁶ atoms of two stacked quartets (21). The substitution of sulfur at these positions may interrupt such chelation due to lower intrinsic affinity of the sulfur for K⁺. Thirdly, the presence of sulfur may sterically hinder quartet formation due to its larger Van der Waals radius relative to oxygen. Comparison of top views of three stacked quartets composed of guanine exclusively (Fig. 6C) versus 6-thioguanine substituted in the top quartet (Fig. 6D) demonstrates the central congestion caused by the larger sulfur atoms. Comparison of interior views of three stacked quartets composed of guanine exclusively (Fig. 6E) versus 6-thioguanine substituted in the middle quartet (Fig. 6F) also reveals that sulfur atoms may not be sterically compatible with chelation of potassium ions.

After completion of this study, a report appeared describing similar results (22). Rao *et al.* described an alternative synthesis of 2'-deoxy-6-thioguanosine phosphoramidites and characterized the resulting oligonucleotides containing 6-thioguanine. These authors determined that such derivatized oligonucleotides were stable for ≥1 year. Though supporting similar conclusions, these studies offer different perspectives. The oligonucleotides designed for triple helix formation by Rao *et al.* were significantly longer (26- and 36mers) than those studied in this report. Moreover, multiple 6-thioguanine substitutions destabilized triplexes (2.5-fold reduced affinity with three substitutions) more than was observed in the present study (1.5-fold reduced affinity with four substitutions). While we observed that oligonucleotide 1S (four 6-thioguanine substitutions) acquired complete resistance to inhibition by up to

100 mM K⁺, the oligonucleotides described by Rao *et al.* required 11–12 substitutions for resistance. Finally, the previous report employed a DMS protection assay to demonstrate a modest change in tetraplex structures involving labeled oligonucleotides. Our work uses DMS footprinting of duplex DNA to clearly document the specificity and affinity of the modified oligonucleotides under a range of solution conditions up to 100 mM K⁺.

Our results suggest that the incorporation of 6-thioguanine into a limited number of positions of purine motif ODNs may alleviate the guanine quartet-mediated inhibition of triplexes by physiological ionic conditions. This improvement represents a significant step toward creating ODNs that might serve as specific and efficient gene repressors *in vivo*. Other improvements may include the use of nuclease-resistant backbones to sustain the effect of repressor ODNs and prevent the release of 6-thioguanine as a cytotoxic agent (23).

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