

Formation of a G-tetrad and higher order structures correlates with biological activity of the RelA (NF- κ B p65) 'antisense' oligodeoxynucleotide

Lyuba Benimetskaya, Myriam Berton⁺, Alexander Kolbanovsky¹, Simon Benimetsky¹ and C. A. Stein*

Departments of Medicine and Pharmacology, Columbia University, College of Physicians and Surgeons, 630 West 168 Street, New York, NY 10032, USA and ¹Department of Chemistry, New York University, 31 Washington Place, New York, NY 10003, USA

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ABSTRACT

We have examined the behavior of the phosphorothioate antisense Rel A (NF- κ B p65) oligodeoxynucleotide (oligo) and related molecules. Because of the presence of a G-tetrad near its 5' terminus, this molecule is capable of forming tetraplexes and other higher order structures in a temperature and time dependent manner. The G-tetrad in the phosphodiester congener is protected from methylation by dimethyl-sulfate when the oligomer is 3'-phosphorylated. However, this protection is completely lost when it is 5' phosphorylated, indicating that the formation of at least some higher order structures has been blocked. In addition, we also prevented tetraplex formation by substitution of 7-deazaguanosine (7-DG) for guanosine at several positions within and outside of the tetrad. This substitution retains Watson–Crick base pair hybridization but prevents Hoogsteen base-pair interactions. When murine K-Balb cells were treated with 20 μ M antisense RelA oligo, complete blockade of nuclear translocation of RelA was observed. However, this effect was virtually entirely abrogated in most cases by 7-DG substitution within the tetrad, but retained when the substitution was made 3' to the tetrad. The AS RelA-induced downregulation of Sp-1 activity behaved similarly after 7-DG substitution. Thus, the parent phosphorothioate AS RelA molecule cannot be a Watson–Crick antisense agent. However, these conclusions cannot be extrapolated to other G-tetrad containing oligomers and each must be evaluated individually.

INTRODUCTION

Successful application of antisense oligodeoxynucleotide technology relies on the specificity of the Watson–Crick base pair interaction (1). This concept has been the motive force behind

numerous studies, prominent among them the elegant work of R.Narayanan and co-workers, who examined in detail inhibition of NF- κ B activity in many cell lines (2–4). One of the cell lines which was perhaps the major focus of their work was the K-Balb (K-ras-transformed) murine fibroblast line, to which they targeted a 24mer phosphorothioate oligodeoxynucleotide against the initiation codon region of the RelA (p65) subunit of the murine NF- κ B nuclear transcriptional regulatory factor (2–4). This antisense oligodeoxynucleotide, AS RelA (a phosphodiester or phosphorothioate oligodeoxynucleotide complementary to the mRNA of the p65 subunit of the NF- κ B nuclear transcriptional regulatory factor) was shown to cause a profound block in nuclear translocation of RelA, as well as inhibition of nuclear expression of the Sp-1 transcription factor, a property that was not shared by the sense control (5). However, the 5'-region of this oligomer contains four contiguous guanosine residues. Additional experiments employing single base mutated AS RelA oligomers suggested that the presence of four contiguous guanosines was essential for antisense activity (6). Furthermore, activity was not dependent only on the presence of this motif, but clearly on its position within the molecule as well.

Other investigators (7,8) have noted an enhanced non-sequence-specific activity in phosphorothioate oligodeoxynucleotides that contain four contiguous guanosines. Burgess *et al.* (7) examined inhibition of smooth muscle cell proliferation by a phosphorothioate oligodeoxynucleotide targeted to *c-myb* and noted that most of the activity was a function of this sequence element. Guvakova *et al.* (8) demonstrated increased binding of a phosphorothioate oligomer containing this motif to basic fibroblast growth factor (bFGF). A guanosine-rich phosphodiester oligodeoxynucleotide has recently been demonstrated to be highly nuclease resistant and to be a relatively specific inhibitor of HIV-1 integrase (9). Because this and other examples (10,11) show that G-rich sequences enhance non-sequence specificity of both phosphodiester and phosphorothioate oligomers, we conjectured that the biological activity of AS RelA might not be due to Watson–Crick hybridization, but rather to an effect of the guanosine residues themselves.

*To whom correspondence should be addressed. Tel: +1 212 305 3606; Fax: +1 212 305 7348; Email: stein@cuccfa.ccc.columbia.edu

⁺Present address: University of Geneva, Laboratory of Pharmacy, 30, quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

Guanine-rich regions in oligonucleotides can form tetrads (also known as quartets), in which the four residues cyclize to form an array held together by hydrogen bonds between N¹H and O⁶ and N²H and N⁷. The G-rich region on one strand can combine with that on another to form higher order structures, for example in a bimolecular fashion by association of two strands or in a tetramolecular manner by appropriate positioning of four individual strands (12–17). A wide diversity of structures may result, as in some tetraplexes, for example, the strands may be parallel and in others antiparallel. Furthermore, the rates at which these strands associate and dissociate may also be very sensitive to the sequence context, ionic strength and temperature.

In this work we demonstrate that the non-sequence-specific activity of at least some G-rich oligomers is dependent on tetraplex formation only for short oligomers. We then address the question of higher order structure formation in the antisense RelA oligodeoxynucleotide. We demonstrate that this oligomer forms higher order structures, but that 5'-phosphorylation blocks formation of at least some of these structures. We then show that substitution of 7-deaza-2'-deoxyguanosine (which eliminates Hoogsteen but preserves Watson–Crick base pairing) (18) within, but not outside, the G-tetrad in the AS-RelA phosphorothioate oligodeoxynucleotide drastically alters its biological activity.

MATERIALS AND METHODS

Synthesis of oligodeoxynucleotides

Phosphodiester oligodeoxynucleotides were synthesized by standard phosphoramidite chemistry on an Applied Biosystems (Foster City, CA) 380B synthesizer. Phosphorothioate oligodeoxynucleotides were also synthesized by standard methods (19) and sulfurization was performed using tetraethylthiuram disulfide/acetonitrile (TETD; Applied Biosystems). Following cleavage from the controlled pore glass support, oligodeoxynucleotides were base deblocked in ammonium hydroxide at 60°C for 8 h and purified by reversed phase HPLC [0.1 M triethylammonium bicarbonate (TEAB)/acetonitrile, PRP-1 support]. Oligomers were detritylated in 3% acetic acid and precipitated with 2% lithium perchlorate/acetone, dissolved in sterile water and reprecipitated as the sodium salt from 1 M NaCl/ethanol. Concentrations were determined by UV spectroscopy.

5'-³²P-Labeled oligodeoxynucleotides were synthesized using T4 polynucleotide kinase and [γ -³²P]ATP by the method of Sambrook *et al.* (20). 3'-End-labeling was performed using terminal deoxynucleotidyltransferase and [α -³²P]dideoxyATP (20).

Gel electrophoresis

Higher order structure formation of deoxynucleotides was analyzed in native and 20% polyacrylamide gels (19:1 polyacrylamide/bisacrylamide) after electrophoresis in TBE buffer at room temperature. Gels were run at 10–16 V/cm and then subjected to autoradiography or stained with Stains-All (Sigma).

³²P-Labeled oligodeoxynucleotides, after chemical modification with dimethylsulfate (DMS) and cleavage with piperidine, were analyzed by electrophoresis in 20% polyacrylamide–7 M urea sequencing gels and autoradiographed.

Nucleotide base protection assays

DMS and diethylpyrocarbonate (DEPC) cleavage was performed at 25°C for the times shown in the figure legends. 3'- or 5'-³²P-labeled oligodeoxynucleotides were dissolved in 5 μ l 90 mM Tris–borate, pH 8.3, 1 mM EDTA (TBE), 25 mM KCl and an equal volume of 1% (v/v) DMS (Aldrich) or 10% (v/v) DEPC (Aldrich) in the same buffer. The reaction with DEPC was allowed to proceed for 30 min or 1 h, with vortex mixing at 5–10 min intervals throughout. Reactions were stopped by addition of 1.5 M sodium acetate, pH 7.0, 1 M 2-mercaptoethanol and cooling at 4°C for 5 min. The oligodeoxynucleotides were precipitated with 2% lithium perchlorate/acetone. Chemically modified oligodeoxynucleotides were cleaved by reaction with 20 μ l 10% piperidine at 90°C for 45 min. Traces of piperidine were removed by evaporation. Samples were then dissolved in 5 μ l formamide loading buffer, heated for 5 min at 90°C, cooled to 4°C and analyzed by electrophoresis in 20% polyacrylamide–7 M urea sequencing gels.

Cells

K-Balb cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown and maintained at 37°C in 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% (v/v) fetal calf serum (Gibco BRL), 1 mM L-glutamine, 100 U/ml penicillin G and 100 μ g/ml streptomycin.

Electrophoretic mobility-shift assays (EMSA)

K-Balb cells (25 \times 10⁴) were seeded into 100 \times 20 mm tissue culture dishes and incubated until adherent and spread. Phosphorothioate oligodeoxynucleotides (20 μ M) were then added and cells incubated for 48 h at 37°C. Nuclear extracts were isolated and EMSA were performed as described (3). Protein concentrations were determined using the Bradford protein assay (BioRad, Richmond, CA). The NF- κ B HIV-1 consensus probe sequence was 5'-GTA GGG GAC TTT CCG AGC TCG AGA TCC TAT G-3'. The Sp-1 and CK-1 consensus binding sequences were respectively 5'-T CGA ATT CGA TCG GGG CGG GGC GAG C-3' (5) and 5'-TGA TAA GGG CCA GGA GAT TCC ACA GTT CAG GTA GTT-3' (4). The probes were labeled with [³²P]dNTP as described (5). Nuclear extracts (7–10 μ g) and ³²P-labeled probes were co-incubated and complexes were resolved by electrophoresis in a non-denaturing 4% polyacrylamide gel and autoradiographed. Unlabeled probe at 100 \times concentration was used as a competitive inhibitor to establish gel shift specificity.

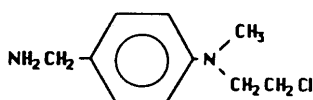
Western blot analysis

Aliquots of cell extracts containing 10–15 μ g protein were resolved by 12% SDS–PAGE (polyacrylamide gel electrophoresis) and transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL). Filters were incubated at 25°C for 1 h in Blotto A [5% non-fat milk powder in Tris–buffered saline (TBS); 10 mM Tris–HCl, pH 8.0, 150 mM NaCl] plus 0.05% Tween-20 and then incubated at 25°C for 1 h in Blotto A containing 1:1000 NF- κ B p65 rabbit polyclonal IgG mAb (Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBS-T (TBS + 0.05% Tween-20), filters were incubated for 1 h at 25°C in TBS-T containing a 1:10 000 dilution of peroxidase-conjugated goat

anti-rabbit secondary mAb (Amersham). After washing, ECL was performed according to the manufacturer's instructions (Amersham).

Synthesis of alkylating, radioactive phosphodiester oligodeoxynucleotide 5'-N-methyl-N-(2-chloroethyl)amino-benzylamine [³²P]OdT₁₂ (CIRNH[³²P]OdT₁₂)

This compound, a dodecathymidylate phosphodiester oligodeoxynucleotide derivative with an alkylator moiety:



Scheme 1.

coupled to the 5' radioactive phosphate through a phosphoramidate bond, was synthesized by the method of Knorre *et al.* (21). The final product was stored at -70°C. Note that OdT₁₂ indicates the 12mer phosphodiester of thymidine.

Competitive inhibition of binding of CIRNH[³²P]OdT₁₂ to bFGF by tetraplex-forming oligodeoxynucleotides

This was performed by the method of Yakubov *et al.* (22). Individual G-rich and control oligodeoxynucleotides (Table 1) were used at the stated concentrations as competitors of binding of the probe, a ³²P-labeled oligodeoxynucleotide to bFGF. The IC₅₀ of competition was determined by visual inspection and values of K_c were calculated by the Cheng-Prusoff equation (23):

$$K_c = IC_{50} / (1 + [CIRNH[^{32}P]OdT_{12}] / K_d) \quad 1$$

where the K_d value is 0.5 μM (8).

Table 1. Competition for binding of probe oligodeoxynucleotide CIRNH[³²P]OdT₁₂^a by G₆T_x phosphorothioate oligodeoxynucleotides

Competitor	IC ₅₀ (μM)	K _c (μM)
SdG ₆ T ₂	0.2	0.02
SdT ₈	27	3.8
SdG ₆ T ₄	0.22	0.03
SdT ₁₀	8	1.1
SdG ₆ T ₆	0.3	0.05
SdT ₁₂	7.1	1.0
SdG ₆ T ₈	0.3	0.05
SdT ₁₄	0.8	0.11
SdG ₆ T ₁₀	0.1	0.02
SdT ₁₆	0.4	0.06
SdG ₆ T ₁₂	0.19	0.03
SdT ₁₈	0.3	0.05
SdGGGGGGT ₄	4.5	0.64
SdGGGGGGT ₄	1.8	0.25
SdGGGGGGT ₄	4.2	0.60

^a3 μM CIRNH[³²P]OdT₁₂; K_d = 0.5 μM. **G**, 7-deazaguanine substitution.

Circular dichroism (CD) spectroscopy

CD spectra were recorded at room temperature using an AVIV Model 62A DS CD spectropolarimeter (Aviv Associates, Lakewood, NJ). Solutions of oligodeoxynucleotides (1 OD U/ml) were prepared in 0.1 M Tris-HCl, pH 7.6, to which, in some experiments, 140 mM KCl was added. The spectra were recorded at room temperature.

RESULTS

Binding of SdG₆T_x to bFGF is a function of oligomer length

We initially wanted to determine the effect of tetraplex formation on the non-sequence-specific binding to protein of phosphorothioate oligodeoxynucleotides. To do this, we synthesized the model series of tetraplex-forming 5'-SdG₆T_x-3' oligomers listed in Table 1. Electrophoresis of this series under non-denaturing conditions (with or without 25 mM KCl) demonstrated that the shorter oligomers existed predominately as discrete tetraplexes, as evidenced by their appropriately slow rate of migration (Fig. 1). However, for dT ≥ 8, only smears can be visualized. Moreover, the presence of 100 mM KCl as against 100 mM LiCl did not markedly stabilize the discrete tetraplex form versus the smear. The presence of the smear, which may in part represent 'bleeding out' of lower order from higher order structures, may imply that the rate of dissociation of the tetraplexes to the putative dimers and monomers under the electrophoretic conditions is relatively rapid.

We next examined the ability of tetraplex-forming G-rich phosphorothioate oligodeoxynucleotides to compete binding of the probe, radioactive phosphodiester oligodeoxynucleotide CIRNH[³²P]OdT₁₂ to a model heparin-binding protein, bFGF (Table 1 and Fig. 2). The values of K_c, as calculated by equation 1, were compared with those obtained with homopolymers of thymidine, which do not form higher order structures. There was a marked decrease in K_c due to formation of tetraplexes, nevertheless, this decrease, relative to what was seen with thymidine homopolymers of identical length, diminishes with increasing number of dT residues. Indeed, by dT ≥ 8 the values of K_c are essentially identical for the G-rich and non-G-rich oligomers. However, as observed on the sizing gels, this may be a reflection of decreased tetraplex concentration at greater oligomer length. Nevertheless, it is also true that although K_c for the dT homopolymers falls markedly as a function of length, it does not fall proportionately for the SdG₆T_x oligomers.

In order to eliminate G-tetrad formation, we then substituted 7-deaza-2'-deoxyguanosine (7-DG) at several positions within the G-tetrad (Table 1). Values of K_c lie between those found for non-7-DG-substituted SdG-rich and SdT homopolymeric oligomers, but are much closer to the latter.

Formation of higher order structures by the phosphodiester and phosphorothioate antisense RelA oligodeoxynucleotides

We examined the ability of these oligomers to form higher order structures by native gel electrophoresis. As shown in Figure 3, at least three distinct bands can be observed. The slowest migrating band (top of the gel) is a tetraplex: we demonstrated this by the method of Wyatt *et al.* (10), by boiling the phosphodiester and phosphorothioate AS RelA oligomer with an equal concentration of a phosphodiester oligomer of half length (5'-GAG GGG AAA

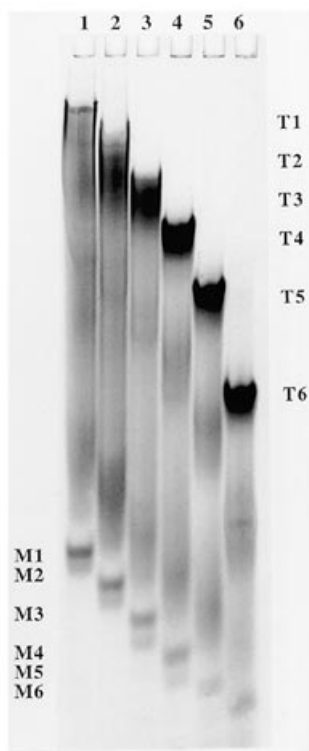


Figure 1. Native gel electrophoresis of SdG₆T_x phosphorothioate oligodeoxynucleotides. 0.5 OD units of oligomer in 0.1 M Tris-HCl, pH 7.5, and 25 mM KCl were removed from a -20°C freezer 20 min prior to dissolving in the loading buffer containing TBE and electrophoresis at room temperature on a 20% polyacrylamide gel. The gel was then stained with Stains-All. Lane 1, SdG₆T₁₂; lane 2, SdG₆T₁₀; lane 3, SdG₆T₈; lane 4, SdG₆T₆; lane 5, SdG₆T₄; lane 6, SdG₆T₂. T1-T6, area of tetraplex migration for oligomers in lanes 1-6 respectively; M1-M6, area of migration of monomers for oligomers in lanes 1-6 respectively. A small amount of $n - 1$ oligomer migrates faster than the M1-M6 monomers. Areas of oligomer migration between M and T, which appear as smears, may contain dimers, although this is not certain.

CAG-3'). Native gel electrophoresis showed five bands with approximate tetraplex mobility, as would be expected by the number of terms in the binomial expansion (not shown).

Monomer is seen at the bottom of the Figure 3 gel, along with fainter, intermediate species. It is possible, but by no means certain, that these represent dimers, which increase in concentration in 25 mM K⁺. However, the 5'-phosphorylated phosphodiester AS RelA did not form tetraplexes at 25°C (with or without 25 mM K⁺), although the intermediate bands still remained (data not shown). Far less tetraplex was commonly observed for the phosphorothioate AS RelA under identical electrophoresis conditions (Fig. 3, lanes 3 and 4), consistent with the observations of Wyatt *et al.* (11) that the association rates are slower and dissociation rates are faster for the phosphorothioate tetraplexes. However, the absolute amount of phosphorothioate AS RelA tetraplex could vary significantly over the course of multiple experiments, for reasons that have yet to be discerned.

Formation of higher order structures for the phosphodiester AS RelA (3'-labeled with ddA) was dependent on temperature and time. The quantity of intermediate forms (putative dimers?) was reduced at room temperature (0.1 M Tris, pH 7.5) compared with 4°C, while formation of tetraplex was unaffected. However, if the

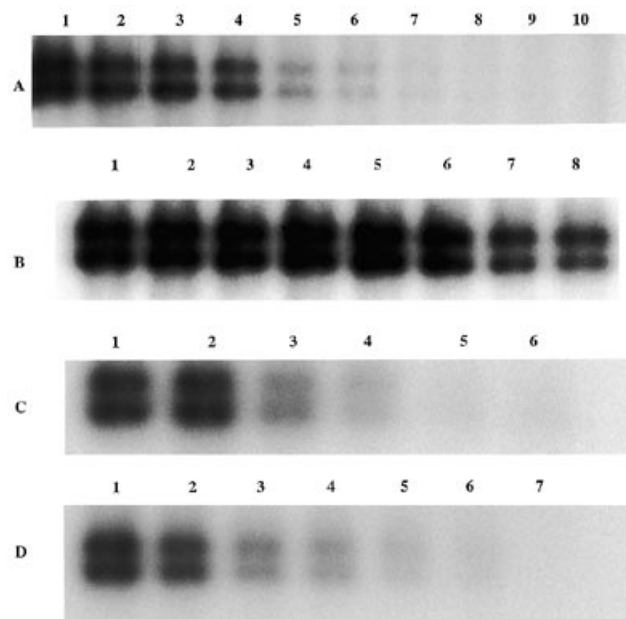


Figure 2. Competition by SdG₆T_x and SdT_{6+x} oligodeoxynucleotides for binding of CIRNH[³²P]OdT₁₂ to bFGF. SdG₆T_x and SdT_{6+x} were used as competitors of CIRNH[³²P]OdT₁₂ (3 μM) binding to bFGF (0.3 μM) as described in the text (6% PAGE). Bands represent the 17.5 kDa bFGF modified by the oligodeoxynucleotide. The concentration of SdG₆T₂ (A) was (lanes 1-10 respectively) 0, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5 and 10 μM. The concentration of SdT₈ (B) was (lanes 1-8 respectively) 0, 2.5, 5, 7.5, 10, 15, 25 and 50 μM. The concentration of SdG₆T₁₀ (C) was (lanes 1-6 respectively) 0, 0.1, 0.25, 0.5, 0.75 and 1 μM. The concentration of SdT₁₆ (D) was (lanes 1-7 respectively) 0, 0.5, 0.75, 1, 2.5, 5 and 10 μM. Gel bands were quantitated by β counting, as described in the text.

oligomer was kept in water for 3 days in the presence of 140 mM NaCl, virtually complete dissociation of the tetraplex and intermediate forms to monomer occurred.

The G-tetrad in the RelA phosphodiester oligodeoxynucleotide is protected from DMS methylation

Because 5'-phosphorylated AS RelA does not appear to form tetraplexes as easily as non-5'-phosphorylated AS RelA and because of our earlier unpublished observations that certain 5'-fluorescein-labeled 5'-G-rich oligomers are observed largely as monomers on electrophoresis in native gels, we examined the extent of protection at guanosine N⁷. This was done by alkylation with DMS and piperidine cleavage. The radioactive label was located at the 5'-terminus in some experiments and at the 3'-terminus in others (25 mM K⁺, 10-15 min reaction time). These conditions produce single hit methylation and the products of piperidine cleavage of the methylated oligomers were resolved on sizing gels. As is seen in Figure 4, when the ³²P label (as ddA) is at the 5'-terminus, G₂-G₅ are clearly observed and the bands are roughly equal in intensity to those of G₅-G₉. In contrast, when the oligomer is 3'-phosphorylated, the G₂-G₅ bands are highly under-represented, indicating that the N⁷ of these guanines are protected from methylation by Hoogsteen base pair formation. Weak protection of G₆-G₇ was also observed. If the 3'-labeled sample was boiled before DMS treatment, G₂-G₅ were visible and, with the exception of G₂, equal in intensity to the other spots.

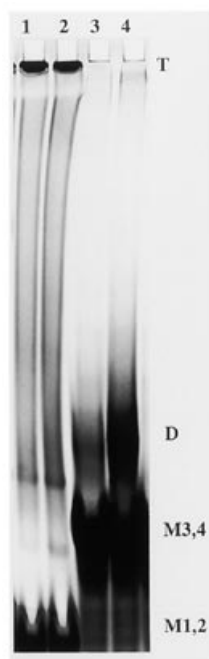


Figure 3. Formation of higher order structures by AS RelA. Shown is a native 20% polyacrylamide gel. 1 OD unit of oligomer in 0.1 M Tris-HCl, pH 7.5, was removed from a -20°C freezer 20 min prior to dissolving in the loading buffer containing TBE and electrophoresis at room temperature on a 20% polyacrylamide gel. The gel was then stained with Stains-All. Lane 1, phosphodiester AS RelA, no added K^+ ; lane 2, + 25 mM KCl; lane 3, phosphorothioate AS RelA, no added K^+ ; lane 4, + 25 mM KCl. T, area of migration of tetraplexes in lanes 1–4; M1–M4, area of migration of monomers in lanes 1–4 respectively; D, area of migration of putative dimeric structures.

Thus, on the basis of these data, we conclude that the parent AS RelA exists in solution predominately as a relatively labile tetraplex, whose formation can be substantially prevented by 5'-phosphorylation. As a control, we alkylated the adenosine residues with DEPC. Both the 3'- and 5'- ^{32}P -labeled phosphodiester oligodeoxynucleotides were treated in 25 mM KCl as in Materials and Methods at 25°C , cleaved with piperidine and electrophoresed. All adenine moieties in both oligomers were equally reactive to DEPC/piperidine, indicating that these bases do not play a significant role in formation of higher order structures.

Biological effects of the modified AS rela phosphorothioate oligodeoxynucleotides

Because of the formation of G-tetrads with the AS RelA oligomer and the subsequent formation of higher order structures, we substituted 7-DG at several positions both inside and outside the G-tetrad (Table 2). However, as is shown in Figure 5, only substitution at certain positions will block formation of higher order structures. For example, under the conditions of electrophoresis, no tetraplexes were observed for the molecule with a 7-DG substitution at G_1 (lane 1) and G_2 (lane 2), although the G_1 -substituted molecule should still be able to form a tetraplex. Substitution of 7-DG at G_5 (lane 4), however, did not eliminate formation of higher order structures. Murchie and Lilley (18) demonstrated that the AGGG motif is also capable of forming tetrads and tetraplexes. Interestingly, it was observed by CD (Fig.

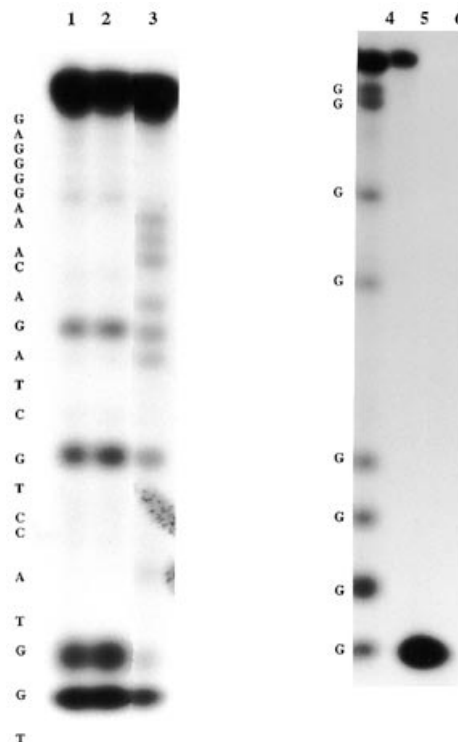


Figure 4. N^7 methylation by DMS or DEPC and piperidine cleavage of phosphodiester AS RelA. The oligomer was treated as described in the text and the products separated on a 20% polyacrylamide-7 M urea gel. Lanes 1–3, oligomer was labeled on the 3'-terminus with ddA. Lane 1, DMS, 10 min; lane 2, DMS, 15 min; lane 3, DEPC, 60 min. Lanes 4–6, oligomer was labeled on the 5'-terminus with ^{32}P . The position of the guanosine residues is noted. Lane 4, DMS, 15 min; lane 5, no DMS; lane 6, ATP. G_1 is not observed on this gel.

6) that the strong positive Cotton effect at 270–280 nm for this molecule (Fig. 6b) was shifted to a shorter wavelength in the presence of K^+ . The shift was also observed for the parental AS RelA molecule (Fig. 6a), but not for molecules with substitutions at G_3 (not shown) or G_4 or for SdT_{24} , none of which can form tetrads (Fig. 6c and d).

Table 2. Sequence of AS RelA and positions of 7-deazaguanosine substitutions

AS RelA	5'-G ₁ AG ₂ G ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
1	5'- <u>G</u> ₁ AG ₂ G ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
2	5'-G ₁ A <u>G</u> ₂ G ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
3	5'-G ₁ AG ₂ <u>G</u> ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
4	5'-G ₁ AG ₂ G ₃ <u>G</u> ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
5	5'-G ₁ AG ₂ G ₃ G ₄ <u>G</u> ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
6	5'-G ₁ AG ₂ <u>G</u> ₃ <u>G</u> ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
7	5'-G ₁ AG ₂ G ₃ G ₄ G ₅ AAA CAG ₆ ATC <u>G</u> ₇ TC CAT G ₈ G ₉ T-3'
8	5'-G ₁ AG ₂ G ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT <u>G</u> ₈ G ₉ T-3'
9	5'- <u>G</u> ₁ AG ₂ G ₃ <u>G</u> ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'
10	5'- <u>G</u> ₁ AG ₂ <u>G</u> ₃ G ₄ G ₅ AAA CAG ₆ ATC G ₇ TC CAT G ₈ G ₉ T-3'

G, 7-deazaguanosine substitution.

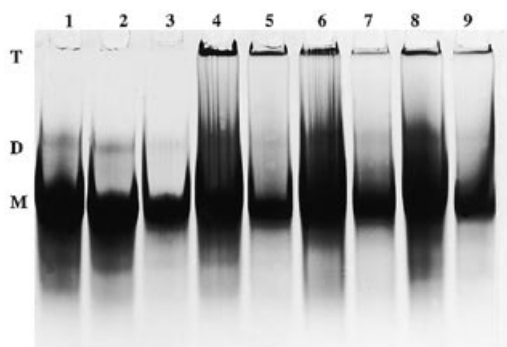


Figure 5. Formation of higher order structures in 7-DG-substituted phosphorothioate AS RelA. Shown is a native 20% polyacrylamide gel. 0.85 OD units of oligomer in 0.1 M Tris-HCl, pH 7.5, were removed from a -20°C freezer 20 min prior to dissolving in the loading buffer containing TBE and electrophoresis at room temperature on a 20% polyacrylamide gel. Position of 7-DG substitution is noted in the description of the contents of each lane, as referred to in the text, along with the oligomer number, as in Table 2. Lane 1, G_1 (1); lane 2, G_2 (2); lane 3, G_2 (2; 0.25 OD); lane 4, G_5 (5); lane 5, G_5 (5; 0.25 OD); lane 6, G_7 (7); lane 7, G_7 (7; 0.25 OD); lane 8, G_8 (8); lane 9, G_8 (8; 0.25 OD). T, D and M are areas of migration of tetraplex, putative dimeric and monomeric forms respectively.

We then investigated whether 7-DG-substituted AS RelA oligomers blocked nuclear translocation of NF- κ B in K-Balb cells in experiments identical to those performed by Narayanan and co-workers (2-4). Log growth phase cells were treated with phosphorothioate oligomers (20 μM , 2 days) because the phosphodiester oligomers are not active in this assay. As shown in Figure 7, top, the parent phosphorothioate AS RelA eliminated RelA nuclear translocation as demonstrated by EMSA, with relative sparing of p50 translocation. [The assignment of the subunits to EMSA bands in this cell line has previously been extensively evaluated by mAb supershift experiments (2-4). Nevertheless, it should be noted that relatively low levels of c-rel, p105 and other members of the NF- κ B family may also be present in the bands]. However, 7-DG substitution at $G_1 + G_3$, G_2 , $G_1 + G_4$ and G_5 , all of which have substitutions within the G-tetrad, greatly diminished the inhibitory effect of the oligomer. Interestingly, more RelA heterodimers (with NF- κ B1 or c-rel or other members of the NF- κ B family) were found in cells treated with the 7-DG-substituted oligomers as opposed to what was seen in the control, in which RelA homodimers seemed to predominate. Nevertheless, by Western blotting (Fig. 7, bottom), oligomers substituted with 7-DG at G_5 , G_4 and G_3 , all within the G-tetrad, lost virtually all ability to down-regulate total expression of RelA. In sharp contrast, 7-DG substitution at G_7 , which lies outside the G-tetrad and which, by the N^7 -methylation experiments described

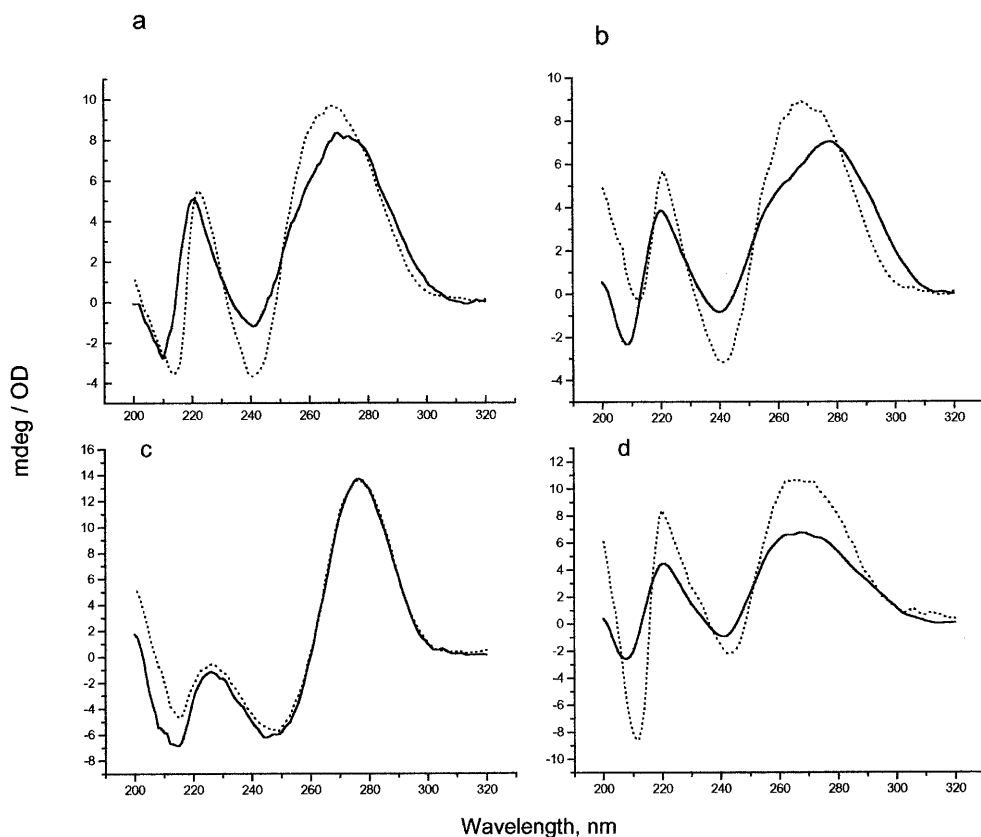


Figure 6. CD Spectra of oligodeoxynucleotides. The spectra were obtained as described in the text. Solid lines, without K^+ ; dotted lines, with 140 mM K^+ . Position of 7-DG substitution in AS RelA is noted in the description of the contents of each lane, as referred to in the text, along with the oligomer number, as in Table 2. (a) Phosphorothioate AS RelA. (b) G_5 (5). (c) SdT₂₄. (d) G_4 (4). The positive Cotton effect band at 270-280 nm in oligomers that form tetrads is shifted to shorter wavelength after addition of K^+ .

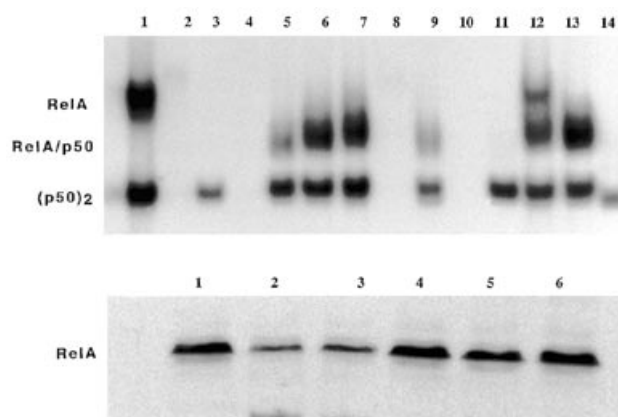


Figure 7. 7-DG substitution alters the biological activity of phosphorothioate AS RelA. (Top) K-Balb cells were treated with 7-DG-substituted oligomers (20 μ M) and nuclear extracts isolated as described in the text. Shown is a representative EMSA ($n = 3$) of binding of the extracts to the NF- κ B HIV-1 consensus binding sequence. The top band represents predominately (RelA)₂ and the bottom band predominately p(50)₂. Intermediate bands, as seen in lanes 5–7 and 12–13, may represent heterodimers. Other members of the NK- κ B family, e.g. c-rel and p105, are also present. Position of 7-DG substitution is noted in the description of the contents of each lane, as referred to in the text, along with the oligomer number, as in Table 2. Lane 1, untreated K-Balb cells; lane 2, + 100 \times cold competitor; lane 3, unsubstituted AS RelA; lane 4, + 100 \times cold competitor; lane 5, G₃ + G₄ (6); lane 6, G₁ + G₃ (10); lane 7, G₃ (3); lane 8, (3) + 100 \times cold competitor; lane 9, G₁ (1); lane 10, (1) + 100 \times cold competitor; lane 11, G₇ (7); lane 12, G₅ (5); lane 13, G₁ + G₄ (9); lane 14, rabbit reticulocyte lysate [p(50)₂ only]. The concentration of all oligomers was 20 μ M. (Bottom) Representative Western blot ($n = 3$) of Rel A (p65) after treatment with 7-DG-substituted oligomers. The Western blot was performed as described in the text and developed by ECL. Lane 1, untreated K-Balb cells; lane 2, unsubstituted AS RelA; lane 3, G₇ (7); lane 4, G₅ (5); lane 5, G₄ (4); lane 6, G₃ (3).

above, plays little if any role in higher order structure formation, behaves identically, by EMSA (Fig. 7, top, lane 11) and Western blotting (Fig. 7, bottom, lane 3), to the parent AS RelA compound. Interestingly, 7-DG substitution at G₁, which in principle preserves the G-tetrad intact (although we did not observe tetraplexes on native gels), produces a molecule which is only slightly less effective than the parent AS RelA by EMSA (Fig. 7, top, lane 9).

In addition, we examined the behavior of the 5'-thiophosphate AS RelA molecule. The 5'-thiophosphate, as opposed to the 5'-phosphate, was synthesized in order to suppress 5'-dephosphorylation by cellular alkaline phosphatases. In EMSA this molecule did not block nuclear translocation of NF- κ B (Fig. 8A, compare lane 5 with lane 1 or 2), a fact which is consistent with our previous data indicating that 5'-phosphorylation blocks formation of at least some higher order structures in the AS RelA molecule.

In addition, we also examined nuclear translocation of NF- κ B using the CK-1 probe, which is more specific for RelA as against NF- κ B1 than the HIV-1 κ B probe, although it will also detect heterodimers of RelA with c-rel. As shown in Figure 8B, lane 2, the parent AS RelA oligomer (20 μ M, 2 days) down-regulated NF- κ B nuclear translocation. However, substitution of 7-DG at G₄ (lane 3) blocked this down-regulation. In fact, the results obtained with the CK-1 probe are almost identical to what was obtained with the HIV-1 κ B probe. Substitution of 7-DG at G₁ + G₄, G₁ + G₃ and G₂ led to an increase in mobility of the CK-1

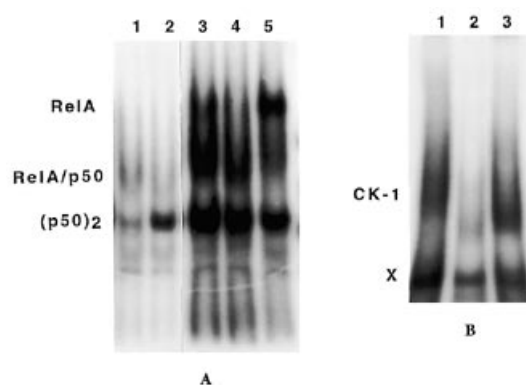


Figure 8. (A) 5'-Thiophosphate blocks down-regulation of nuclear NF- κ B. K-Balb cells were treated with 7-DG-substituted oligomers (20 μ M) and nuclear extracts isolated as described in the text. Shown is a composite picture (but same gel) EMSA of binding of the extracts to the NF- κ B HIV-1 consensus binding sequence. The top band represents predominately (RelA)₂ and the bottom band predominately p(50)₂, although other members of the NF- κ B family are also present. Intermediate bands represent predominately heterodimers. Position of 7-DG substitution is noted in the description of the contents of each lane, as referred to in the text, along with the oligomer number, as in Table 2. Lane 1, AS RelA; lane 2, G₈ (8); lane 3, G₂ (2); lane 4, G₁ + G₃ (10); lane 5, 5'-thiophosphate AS RelA. (B) 7-DG substitution alters the binding of NF- κ B to the CK-1 consensus sequence. K-Balb cells were treated with 7-DG-substituted oligomers (20 μ M) and nuclear extracts isolated as described in the text. Shown is binding of the extracts to the CK-1 consensus binding sequence. X represents a CK-1 recognizable element that has not yet been characterized (4). Lane 1, untreated K-Balb cells; lane 2, AS RelA; lane 3, G₄ (4).

band by EMSA, but little decrease in band intensity. In contrast, 7-DG substitution at G₁ or G₇, which are outside the G-tetrad, led to virtual complete elimination of CK-1 binding, similar to what was seen with the parent AS RelA. Also as previously, substitution by 7-DG at G₃ + G₄, which produces a molecule that cannot form intermolecular tetrads, also led to CK-1 down-regulation. 7-DG substitution at G₅, which permits formation of the AGGG tetrad and thus of tetraplexes, did not, however, cause extensive CK-1 down-regulation.

We then examined the effects of 7-DG-substituted phosphorothioate AS RelA oligomers on nuclear Sp-1 levels. It has previously been shown that these levels decrease in tandem with NF- κ B nuclear translocation in AS RelA-treated cells (5). As shown in Figure 9, lane 2, we have verified this. However, 7-DG substitution at G₃ + G₄, G₁ + G₃, G₁ + G₄, G₂ and G₅, all within the G-tetrad, completely blocked the induced down-regulation of Sp-1, just as they blocked oligodeoxynucleotide-induced down-regulation of nuclear NF- κ B translocation. In contrast, 7-DG substitution at G₇, outside the G-tetrad, produced an oligomer that still caused marked down-regulation of Sp-1 levels (lane 8), similar to the parent AS RelA. Interestingly, substitution at G₁, also outside the G-tetrad, did not produce Sp-1 down-regulation (lane 7), although it did effectively down-regulate NF- κ B nuclear translocation. Thus, this molecule provides an example of decoupling of simultaneous AS RelA-induced down-regulation of NF- κ B translocation (see Fig. 7, lane 9) and Sp-1 activity.

DISCUSSION

Phosphorothioate oligodeoxynucleotides form tetrads and tetraplexes in the same manner as do phosphodiester. However, as demonstrated by Wyatt *et al.* (11), isosequential phosphorothioates

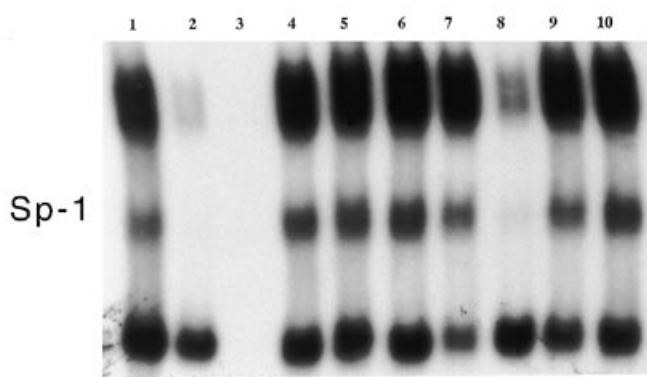


Figure 9. Effects of 7-DG substitution on Sp-1 nuclear activity. K-Balb cells were treated with 7-DG-substituted oligomers (20 μ M) and nuclear extracts isolated as described in the text. Shown is binding of the extracts to the Sp-1 consensus binding sequence. Lane 1, untreated K-Balb cells; lane 2, unsubstituted AS ReLA; lane 3, +100 \times cold competitor; lane 4, G₃ + G₄ (6); lane 5, G₁ + G₃ (10); lane 6, G₂ (2); lane 7, G₁ (1); lane 8, G₇ (7); lane 9, G₅ (5); lane 10, G₁ + G₄ (9).

have less tendency than phosphodiester to exist in solution as tetraplexes, because of slower association and faster dissociation rates. In addition, as we have shown in a model series of phosphorothioate dG₆T_x oligomers, the tendency to form tetraplexes is greatly diminished with increasing oligomer length. This can be seen by the presence of smears rather than discrete tetraplex bands on native gels, where the observed smears represent, at least in part, continuous dissociation in the gel of higher order to intermediate (putative dimeric?) and monomeric species. This increased dissociation rate is probably caused by increased charge repulsion as the strands increase in length and the phosphate groups increase in number. Interestingly, the phosphorothioate dG₆T_x tetraplexes are only minimally stabilized by K⁺ as against Li⁺. This is not generally the case for phosphodiester tetraplexes, which are stabilized by K⁺.

On the basis of these data, it would be predicted that the phosphorothioate AS ReLA oligomer would form relatively few tetraplexes at physiological saline concentration, a temperature of 37°C and an oligomer concentration of 20 μ M. In fact, this is what indeed is observed, in spite of the fact that intermediate forms can still be seen. However, as demonstrated by N⁷ methylation protection experiments with the phosphodiester congener, the AS ReLA oligomer, despite what is seen on the native gels, seems to exist in solution predominately as a tetraplex. This contradiction is more apparent than real, because if the tetraplexes are relatively labile they may dissociate under the electrophoretic conditions, as the more mobile monomers migrate in the electric current. However, the tetrad will not form in the presence of a 5'-phosphate group. This is supported by the lack of protection of G₂-G₅ after N⁷ methylation with DMS and piperidine cleavage in the 5'-phosphorylated phosphodiester AS ReLA oligomer, as compared with extensive protection when the oligomer was 3'-phosphorylated. Furthermore, there is a relative lack of 'antisense' activity (inhibition of NF- κ B nuclear translocation) in K-Balb cells treated with the phosphorothioate 5'-thiophosphate AS ReLA oligomer.

Intermediate forms can still be observed with the 5'-phosphorylated phosphodiester AS ReLA in native gel electrophoresis: it is

possible, but not certain, that these forms are linear homodimers. Tetraplexes form with difficulty, probably due to mutual charge repulsion of the phosphate groups.

Substitution of 7-DG at G₅ does not block formation of tetraplexes, as is shown in Figure 5, most likely because a tetrad can still be formed by the AGGG motif. This correlates with the shift, in 140 mM K⁺, of the positive Cotton effect in the CD spectrum from 280 to 265 nm, a peak which is seen in molecules that can form all-G-tetrads (11,24). However, neither phosphorothioate oligomers with 7-DG substitution at G₄ or G₃ nor SdT24, which may not be able to form intermolecular G tetrads, shift the positive Cotton effect band to shorter wavelength in 140 mM K⁺. Interestingly, the substitution of 7-DG at G₅ produces a molecule that is almost incapable of blocking nuclear translocation of NF- κ B in treated K-Balb cells, in contrast to what is seen with the parent all-G-tetrad AS ReLA at equimolar doses. Thus, it appears that the cells can directly recognize the difference between AGGG and GGGG tetraplexes.

The biological activity of the phosphorothioate ReLA AS oligomers is drastically altered based on the position of 7-DG substitution. This alteration is observed with both the HIV-1 κ B and CK-1 probes and is also seen on examination of Sp-1 activity. When 7-DG substitution is located in the G-tetrad, intermolecular tetrads (except in the case of G₅ substitution, as described above) cannot form and 'antisense' activity is partially or totally lost, as assessed by EMSA and Western blotting. However, if 7-DG substitution is made outside the G-tetrad, e.g. at G₁ or G₇, the intermolecular G-tetrad can, in principle, still form and 'antisense' activity is preserved. However, we consider it extremely unlikely that 7-DG single base substitution at any position will affect the value of T_m with complementary mRNA by >0.5°C. (Of interest in this respect is that 'antisense' activity is almost completely preserved in the G₃ + G₄ disubstituted molecule.) Furthermore, it is equally unlikely that cellular uptake will be affected by single base 7-DG substitution. The cellular uptake of phosphorothioate oligodeoxynucleotides is dependent on the processes of fluid phase endocytosis and adsorptive endocytosis (19). The former process will not be influenced by the concentration of higher order structures. The latter process will depend, in part, on the K_d of binding of the oligomers to cell surface heparin binding proteins (8). However, as shown above for the phosphorothioate dG₆T_x model system, the decrease in K_c of protein binding to oligomer may occur only for short tetraplexes (<14mers).

Thus, on the basis of all of our data, we must conclude that the parent phosphorothioate AS ReLA, despite its ability to reduce NF- κ B nuclear translocation in treated K-Balb cells in an apparently sequence-specific manner, cannot be acting solely, if indeed at all, as a Watson-Crick antisense agent. Instead, it appears to exert its remarkable antisense-mimicking effects through a combination of mechanisms, including the following. (i) An aptameric effect of the intermolecular GGGG tetraplex, which as described is different from that of an AGGG tetraplex. The all-G-tetraplex may exert its effect either intracellularly or at the level of the cell membrane. (ii) An intrinsic non-sequence-specific effect of the phosphorothioate oligomer related to its ability to bind to heparin-binding proteins (8,22). For example, Khaled *et al.* (24) have demonstrated that phosphorothioate AS ReLA binds to fibronectin and laminin (on the α 1 chain) and can block binding of laminin to bovine brain sulfatide. This blockade is highly enhanced for the AS ReLA versus the sense ReLA (the sense sequence with respect to the NF- κ B mRNA) oligomer.

Interestingly, the phosphorothioate oligomer that contains 7-DG disubstitution at G₃ + G₄, which blocks formation of any tetraplexes and higher order structures, is almost as active as the parental AS RelA at inhibition of NF-κB nuclear translocation. It also does not, like the parent AS RelA molecule, suppress Sp-1 activity. It is entirely possible that this species represents the Holy Grail for this system, a true antisense effector molecule. If so, the strategy of 7-DG substitution provides an effective, though costly, way of circumventing the G-tetrad problem.

However, some caution should be taken not to generalize these results to other well-studied oligodeoxynucleotides that contain a G-tetrad (e.g. the antisense phosphorothioate *c-myc* codons 2–7 or 2–9 oligomers; 25). It cannot be determined *a priori*, based only on our analysis, whether biological effects observed in other systems with these oligomers are or are not due to an antisense mechanism. Indeed, our preliminary data indicate that the antisense *c-myc* codons 2–9 oligomer does not form tetraplexes even in the presence of high K⁺ and at low temperature.

The induction of biological effects by G-rich phosphorothioate oligomers is a highly complex process. Each G-rich oligodeoxynucleotide must be evaluated individually. However, on the basis of our data it appears that 7-DG substitution is not only an appropriate way to approach the problem, but is also one that can provide some reasonably conclusive answers about mechanism.

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Note on nomenclature: we have preferred to use the term tetrad instead of quartet (though the two appear to be used interchangeably in the literature) as it seems more logical and orthographically esthetic to call the building block of a tetraplex a tetrad, rather than a quartet

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