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Multiple mechanisms may contribute to the cellular anti-adhesive effects of phosphorothioate oligodeoxynucleotides

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ABSTRACT

Phosphorothioate oligodeoxynucleotides complementary to the p65 (Rel A) subunit of the NF-κB nuclear transcriptional regulatory factor have been suggested to be sequence specific blockers of cellular adhesion. We studied the effects of Rel A antisense, Rel A sense and other phosphorothioate oligodeoxynucleotides on cellular adhesion and found that blockade of adhesion was predominately non-sequence specific. Phosphorothioate oligodeoxynucleotides bind to the extracellular matrix (ECM) of NIH 3T3 cells, and to the ECM elements laminin and fibronectin. By use of a gel mobility shift assay, the association of the A subunit of laminin with a probe 12mer phosphodiester oligodeoxynucleotide could be demonstrated. This interaction was described by a single-site binding equation (K_d = 14 μM). Human Rel A antisense and sense oligodeoxynucleotides, and two synthetic persulfated heparin analogs were excellent competitors of the binding of the probe oligodeoxynucleotide to laminin. Taken together, these data indicate that oligodeoxynucleotide binding occurred at or near the heparin-binding site. Competition for 5′ ^{32}P-SdT18 (an 18mer phosphorothioate homopolymer of thymidine) binding to fibronectin with the discrete heparin analogs, as well as with SdC_{28}, was also observed. Phosphorothioate oligodeoxynucleotides (Rel A antisense >> Rel A sense) inhibited the binding of laminin to bovine brain sulfatide, but not to its cell surface receptors on MCF-7 cells. By flow cytometric analysis we have also shown, in contrast to what was observed with laminin, that phosphorothioates non-specifically block the specific binding of fluoresceinated fibronectin to its cell surface receptors on phorbol-12,13-myristate acetate-treated Jurkat cells. Blockade of specific binding occurred in the oligodeoxynucleotide treated cells in the presence or absence of oligomer in the media.

INTRODUCTION

The process of cellular adhesion plays a critical role in tumor metastasis and cell growth. In a series of recent studies, the nuclear transcriptional regulatory factor complex NF-κB has been implicated as a major element that regulates the process of homotypic aggregation. For example, several cell surface species, such as ELAM-1 (1), ICAM-1 and VCAM (2), appear to play an important role in the adhesive response that cells manifest after contacting plastic substrata in culture; these molecules all contain NF-κB binding sites in their promoter regions. It has also recently been shown that 18–24mer phosphorothioate oligodeoxynucleotides targeted to the p65 subunit of the NF-κB nuclear transcriptional regulatory factor (Rel A antisense) may have profound effects upon cellular adhesion to plastic substrata [heterotypic aggregation; (3)]. In addition, stable expression of inducible antisense RNA to p65 also caused inhibition of cell adhesion (4). These observations were attributed to sequence specific down regulation of the expression of NF-κB activity in the cell nucleus.

In our efforts to demonstrate the sequence specificity of the antisense p65 mediated block of cellular adhesion, we synthesized several control phosphorothioate oligodeoxynucleotides of the same chain length as antisense p65 (5). These controls were synthesized because we have previously discovered that more controls (e.g. reverse polarity of the antisense sequence, scrambled sequences) that are synthesized for any given antisense experiment, the more convincingly true antisense efficacy may be demonstrated. Indeed, the synthesis of additional control sequences is virtually obligatory due to the non-sequence specific effects manifested by phosphorothioate oligodeoxynucleotides (6). For example, it has been recently shown that phosphorothioate oligodeoxynucleotides bind in a non-sequence specific manner to basic fibroblast growth factor and to other members of the basic fibroblast growth factor (FGF) family (7); to recombinant soluble CD4 (8); to gp120 (9), and to several protein kinase C isoforms (10).

We found that some of the control phosphorothioate oligodeoxynucleotides had almost the same effect on heterotypic aggregation as the p65 antisense oligo. These data strongly

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suggest that the block of cellular adhesion to plastic substrata might be due to multiple non-sequence specific mechanisms, in addition to the sequence-specific mechanism. We determined that the non-sequence specific mechanisms involve, at least in part, the ability of phosphorothioate oligodeoxynucleotides to bind to elements of extracellular matrix (ECM), including laminin, and to block the binding of laminin to bovine brain sulfatide (galactosylceramide-I^3-sulfate). Furthermore, phosphorothioate oligodeoxynucleotides, in a non-sequence specific manner, are also capable of blocking the binding of fibronectin to its cell surface receptor in phorbol-12,13-myristate acetate (PMA) treated Jurkat cells. Based on these data, we suggest that the block of cellular adhesion by Rel A antisense oligodeoxynucleotide is pleotropic in nature, and potentially augmented by non-sequence specific effects.

MATERIALS AND METHODS

Cells

All cells were obtained from American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852). NIH 3T3 and K-Balb cells were grown and maintained in Dulbecco's modified Eagle's medium (D-MEM) (Gibco-BRL, Grand Island, NY), containing 10% (v/v) calf serum (Gibco-BRL, Grand Island, NY) and 50 μg/ml gentamicin sulfate. DU-145, Jurkat and MCF-7 cells were grown and maintained in RPMI 1640 medium (Gibco-BRL, Grand Island, NY), containing 5% (v/v) fetal calf serum (FCS) (Gibco-BRL, Grand Island, NY), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.

Synthesis of oligodeoxynucleotides

All oligonucleotides were synthesized by standard phosphoramidite chemistry and purified as described by Tonkinson and Stein (11). Concentrations were determined by spectroscopy. When used in native gels (no SDS present), 5′ ^{32}P-labeled phosphorothioate oligodeoxynucleotides were synthesized using T4 polynucleotide kinase by the method of Sambrook *et al.* (12).

Adhesion assays

Transformed cell lines were evaluated for their ability to adhere to plastic substrata as previously described (4). In some experiments, individual wells in a 96-well plate were pre-coated at room temperature for 3 h with continuous agitation with either fibronection (40 μg/ml in PBS; Gibco-BRL, Buffalo, NY) or laminin (80 μg/ml in PBS; Sigma). The protein solutions were then removed by vacuum aspiration. The number of viable cells, both adherent and non-adherent, was evaluated by the 3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay by the method of Maltese *et al.* (13). Where indicated, nuclear extracts were prepared from cells and were analyzed for NF-κB and Sp1 activity by gel shift assay (13).

Binding of oligodeoxynucleotides to extracellular matrix

Six-well plates coated by the ECM of NIH 3T3 cells were produced by the method of Haimovitz-Friedman *et al.* (14), and stored at 4°C. Then, 0.5 ml PBS and 10 μM of the stated 5′ ^{32}P-labeled phosphorothioate oligodeoxynucleotide were added and allowed to incubate at 37°C for 5 min. The supernatant was removed, and the plates washed three times with cold PBS. NaOH (10 N) was then added to solubilize the ECM, and bound oligodeoxynucleotide quantitated by β-counting. Protein content of each ECM-coated well was quantified by use of the Bradford reagent. As a control, six-well plates were treated identically in the absence of ECM. To eliminate the possibility of enzymatic cleavage of the 5′ ^{32}P-label from the phosphorothioate oligodeoxynucleotide, solubilized ECM was subjected to electrophoresis on a 7 M urea–20% polyacrylamide gel. No free ^{32}P was observed.

Binding of fibronectin to phosphorothioate oligodeoxynucleotides

This was accomplished by a electrophoretic mobility shift assay in native 6% polyacrylamide gels. Fibronectin (5 μM) in 10 μl 0.1 M Tris–HCl, pH 7.4 was treated with the stated concentrations of phosphorothioate oligodeoxynucleotide for 1 h at 37°C. To this was added 10 μl 0.625 M Tris–HCl, pH 6.8, 30% glycerol and 0.2% bromophenol blue. In some experiments, 2-mercaptoethanol (1%) was also added to reduce the fibronectin (M_r = 440 kDa) to its two M_r = 220 kDa subunits. After electrophoresis, the gel was dried, and allowed to expose Kodak X-ray film at –70°C. The data were quantitated by excision of the gel bands and β-counting.

Synthesis of alkylating, radioactive phosphodiester oligodeoxynucleotide 5′-*N*-methyl-*N*-(2-chloroethyl)amino-benzylamine-^{32}P-OdT18 or -OdT12 (RClNH^{32}P-OdT18 or RClNH^{32}P-OdT12)

These compounds were synthesized by a modification of the method of Knorre *et al.* (15). *N*-methyl-*N*-(2-chloroethyl)amino-benzylamine was coupled to the 5′ terminal ^{32}P by reaction with triphenylphosphine/dipyridyl disulfide. The final product was stored at –70°C.

Modification of laminin by ClRNH^{32}P-OdT18 or ClRNH^{32}P-OdT12

This was done by the method of Yakubov *et al.* (8). The laminin concentration was 0.25 μM. In some experiments, a putative competitor (e.g. phosphorothioate oligodeoxynucleotide) of the binding of the modifying oligomer to laminin was also added, as indicated in the figure legends. We also used persulfated discrete, synthetic heparin mimetics as competitors, which were kindly donated by R. Tressler (Glycomed, Inc., Alameda, CA). 1306 is a persulfated maltotetraose containing 14 sulfates/mol; 1474 is a persulfated maltoheptaose containing 23 sulfates/mol. After SDS–PAGE (6% acrylamide) the gels were dried and allowed to expose Kodak X-ray film until bands were visualized. Band densities were quantitated by laser scanning densitometry.

Binding of laminin to sulfatide

Inhibition of the binding of ^{3}H-laminin (Amersham) to bovine brain sulfatide by phosphorothioate oligodeoxynucleotides was evaluated in triplicate wells by the method of Zabrenetzky *et al.* (16). The carrier lipids were cholesterol and phosphatidylcholine; oligodeoxynucleotide binding to these lipids in the absence of sulfatide was subtracted from the total to give the specific laminin binding.

Inhibition of binding of ^{3}H-laminin to cell surface of K-Balb cells

K-Balb cells (4 x 10^4) were seeded into triplicate wells in 96-well plates and incubated until adherent and spread. Phosphorothioate oligodeoxynucleotides (20 μM) were then added at 4°C for 15 min. The media was removed and replaced with PBS + 0.25% BSA + 500 μM $CaCl_2$. After 0.5 h, the cells were washed with PBS + 500 μM $CaCl_2$ and the ^{3}H-laminin (14.6 nM, 2.5 mCi/ml; DuPont, Boston, MA) added. After 1 h at 4°C the wells were washed three times with cold PBS. Bound c.p.m. were determined by β-counting. Specificity of binding was established by addition of 50-fold excess of unlabeled laminin (Gibco), and was ≥60–70% of the total binding. The standard deviation among triplicate wells was <15%.

Synthesis of fluoresceinated fibronectin

A solution of fibronectin (1 ml, 0.3 mg/ml) in 100 mM Na_2CO_3, pH 9.5, was placed in a plastic tube and 1 mg fluorescein isothiocyanate (dissolved in 10 μl dimethyl sulfoxide) added to it. The mixture was kept at 4°C for 16 h, and then dialyzed (m.w. cutoff 3500 Da) first against 0.5× PBS for 24 h, and then against 0.1 mM Tris–HCl, pH 7.4 for 24 h. The volume was reduced and the sample passed through Sephadex G-25 pre-equilibrated in 0.01 mM Tris–HCl, pH 7.4. The fluoresceinated fibronectin ran as a single band with the correct migration rate as assessed by 8% polyacrylamide gel electrophoresis.

Flow cytometric analysis of fluorescent fibronectin binding to Jurkat cells

Jurkat cells (5×10^5 cells/ml) in complete media were incubated at 37°C with phorbol-12,13-myristate acetate (5 nM) for 24 h. Then, the cells were washed, and 10^5 cells per 200 μl serum free media were plated at 4°C in 96-well plates. The cells were then incubated for 15 min with 5 μg/ml fluorescein fibronectin (F-Fn). In some experiments, putative phosphorothioate oligodeoxynucleotide competitors of binding were added simultaneously with F-Fn at the concentrations stated in the figure legends. In other experiments, the phosphorothioate oligodeoxynucleotides were added at the stated concentrations to the cells for 15 min (4°C). The cells were then washed twice in cold PBS, resuspended in cold serum-free media, and incubated with 5 μg/ml F-Fn. Subsequently, cells were washed twice with PBS/BSA and resuspended in 300 μl PBS/BSA containing 0.3 μg/ml propidium iodide (PI). Relative fluorescence intensities were determined on a Becton-Dickinson FACStar Plus dual-laser flow cytometer using DESK software, as previously described (11). The mean fluorescence intensity for a population of 10 000 cells was determined. Specific binding was assessed by competition of F-Fn binding with the peptapeptide G-R-G-D-S (0.5 mg/ml). Using this technique, ~60–70% of the total F-Fn binding to the cell surface of phorbol-12,13-myristate acetate (PMA)-induced Jurkat cells was reproducibly specific.

RESULTS

Elimination of anti-adhesive effects of phosphorothioate oligodeoxynucleotides by laminin and fibronectin

We have focused our studies primarily on the Rel A sense and antisense phosphorothioate oligodeoxynucleotides. These (Rel A antisense; Rel A sense; oligomer #2; see sequences in Table 1, all

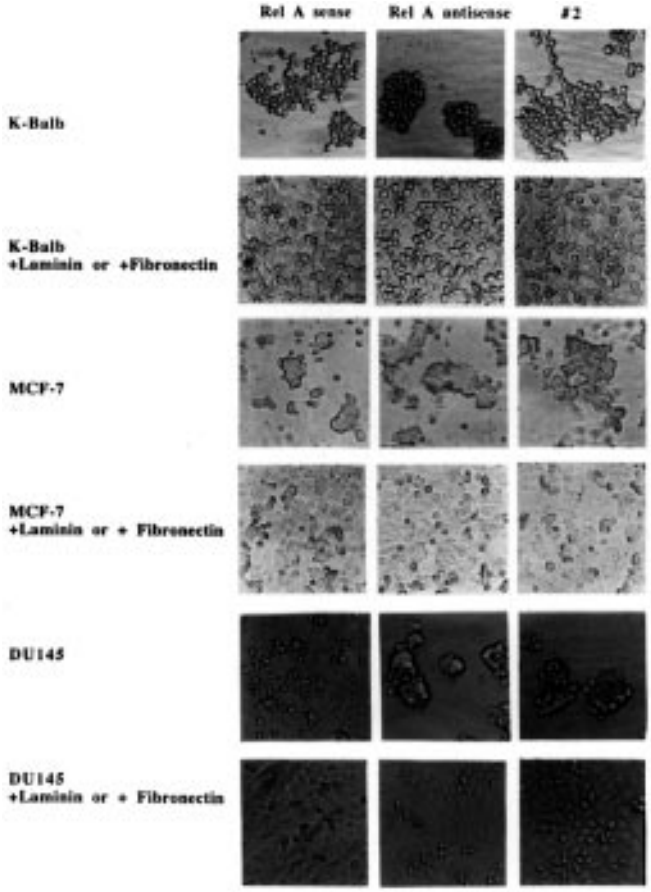


Figure 1. Phosphorothioate oligodeoxynucleotides block cellular adhesion to plastic substrata. 2–2.5 × 10^4 cells per well were trypsinized and incubated at 37°C with the stated phosphorothioate oligodeoxynucleotides (sense and antisense Rel A, #2, [10 μM]) for 20 h, then photographed. For the K-Balb murine fibroblasts, murine sense and antisense Rel A oligomers were used (see Table 1). For the MCF-7 and DU145 cells, human sense and antisense Rel A oligomers were used. Treated cells which were plated in wells pre-coated with either fibronectin or laminin as described in the text are also shown. They appear microscopically identical to each other, and are also identical to control wells (no added oligodeoxynucleotide; data not shown).

[10 μM]) efficiently blocked adhesion and spreading of K-Balb, MCF-7 and DU-145 cells on polyethylene plates. At 10 μM concentration, however, control oligomers, including SdT18, SdC18 and rat antisense c-myb oligodeoxynucleotide (which, like Rel A antisense contains a G-quartet) did not block adhesion and spreading. The non-adherent cells remained ~80% viable, as judged by the MTT assay. However, when the polyethylene plates were pre-coated with fibronectin (40 μg/ml) or laminin (80 μg/ml), but not collagen IV or vitronectin (data not shown), the anti-adhesive effects of the Rel A sense, antisense and all other phosphorothioate oligodeoxynucleotides were virtually entirely eliminated. Representative experiments are shown in Figure 1. Interestingly, when commercially available (Collaborative Biomedical Products, Bedford, MA) fibronectin-coated plates were used, the oligodeoxynucleotide-mediated block of adhesion was also completely abrogated, except for that induced in K-Balb cells by the mouse Rel A antisense oligomer. Here, the anti-adhesive character of the antisense Rel A was similar to that obtained with uncoated dishes. However, the concentration of fibronectin used to coat these surfaces is unknown.

Table 1. Sequences of phosphorothioate oligodeoxynucleotides

Sequence	Comments	IC_{50} (μM)[a]
Rel A antisense 5′-GGG GAA CAG TTC GTC CAT GGC-3′	human, dG = 4	0.1
Rel A sense 5′-GCC ATG GAC GAA CTG TTC CCC-3′	human	4
5′-CCG GGG TCT CCC GGC CAT-3′ (codons 1–6)	rat antisense c-myb	N/D
#1 5′-GGG GCC TCT CCG GGC CAT-3′	dG = 4	0.12
#2 5′-GGG GGG GCT CCG GGC CAT-3′	dG = 7	0.01
#3 5′-GGG GGG GGG CCG GGC CAT-3′	dG = 9	0.01
Rel A antisense 5′-GAG GGG AAA CAG ATC GTC CAT GGT-3′	mouse; dG = 4	N/D
Rel A sense 5′-ACC ATG GAC GAT CTG TTT CCC CTC-3′	mouse	N/D
#4 5′-GAT GGG AAA CAG ATC GTC CAT GGT-3′	dG = 3	0.2
#5 5′-GAG TGG AAA CAG ATC GTC CAT GGT-3′	–	N/D
#6 5′-GAG GTG AAA CAG ATC GTC CAT GGT-3′	–	0.52
#7 5′-GAT TTT AAA CAG ATC GTC CAT GGT-3′	–	1
#8 5-GAG GGG AAA CAG GGG GGG GGG GGG-3′	dG = 4, dG = 12	N/D

[a]Blockade of binding of laminin to sulfatide. See Figure 4.

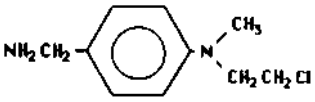
An antisense oligodeoxynucleotide targeted to Rel A caused inhibition of nuclear NF-κB activity in a dose dependent manner, in agreement with earlier observations. This diminution directly correlated with inhibition of the Sp1 transcription factor (13,17). Inhibition of nuclear localization of both transcription factors by the Rel A antisense oligomer was independent of whether the K-Balb cells treated were adherent or non-adherent (not shown).

Binding of phosphorothioate oligodeoxynucleotides to extracellular matrix (ECM) and to ECM elements

As shown in Figure 2, phosphorothioate oligodeoxynucleotides bind to the ECM of NIH 3T3 cells. The binding was non-sequence specific, and concentration dependent for the human Rel A antisense and Rel A sense constructs. Control binding of the 5′ ^{32}P-labeled phosphorothioate oligodeoxynucleotides to wells not containing ECM was <5% of the binding to the wells in the presence of ECM.

The ECM consists of perhaps as many as 100 different proteins. The fact that two of these, laminin and fibronectin, were capable of vitiating the anti-adhesive effect of phosphorothioate oligodeoxynucleotides, as shown above, suggested that the oligomers might interact with one or both of these proteins. In addition, both laminin and fibronectin are heparin-binding proteins, and we have previously demonstrated that phosphorothioates bind avidly to many heparin-binding proteins (7).

Thus, we initially studied the binding of the ^{32}P-labeled, alkylating oligodeoxynucleotides ClRNH^{32}P-OdT12 and -OdT18 to laminin. (ClRNH^{32}P-OdT12 is a dodecathymidylate phosphodiester oligodeoxynucleotide derivative with an alkylator moiety (below) coupled to the 5′ radioactive phosphate through a phosphoramide bond.)



When these were co-incubated in 0.1 M Tris–HCl (pH 7.4) in the absence of Ca^{2+}, and the reaction products then subjected to electrophoresis in a 6% SDS–polyacrylamide gel, a single band was observed on the exposed X-ray film (see Fig. 3). This band corresponded to the product of chemical modification of the protein by the alkylating group covalently linked to the oligomer, as described above. This alkylation, as described previously (8), occurs subsequent to oligonucleotide binding to the protein.

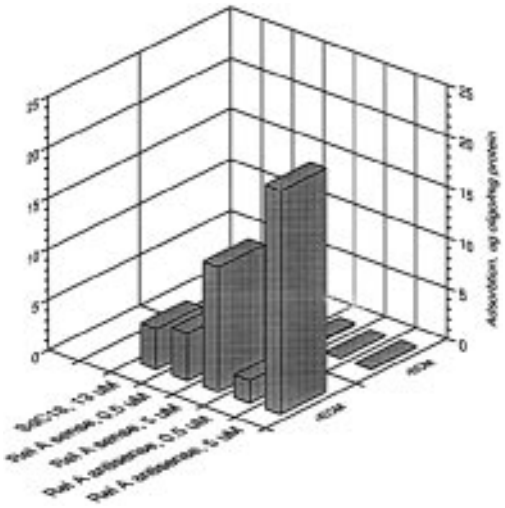


Figure 2. Adsorption of oligodeoxynucleotides by the extracellular matrix of NIH 3T3 cells. The extracellular matrix of NIH 3T3 cells was prepared in tissue culture plates as described in the text. ECM was treated with the sequences and concentrations of phosphorothioate oligodeoxynucleotides, as designated. Control tissue culture plates lacked ECM. Plotted is mean value (from duplicate experiments; 15% difference between the duplicates) of adsorption of oligodeoxynucleotide (in μg/oligomer/mg protein) versus oligodeoxynucleotide concentration.

Laminin (M_r = 850 kDa) is composed of three subunits; the A (or α1) subunit has M_r = 400 kDa. The others, B1 and B2, have M_r = 215 and 205 kDa, respectively (18). In order to determine which subunit was being modified by the probe alkylating oligodeoxynucleotide, we dissociated the subunits by disulfide bond cleavage using 2-mercaptoethanol and boiling. In the

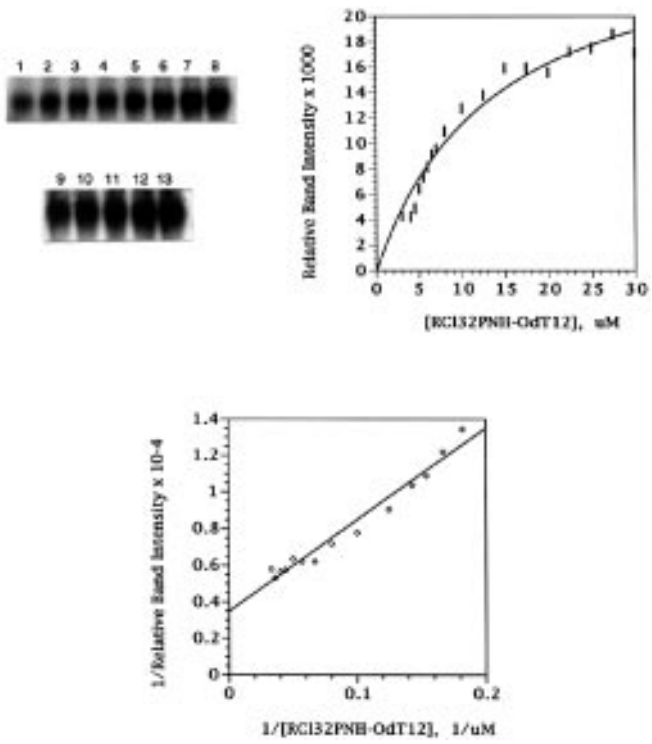


Figure 3. (**Top left**) Modification of the 400 kDa (A) subunit of laminin by the alkylating oligodeoxynucleotide, ClRNH^{32}P-OdT12. Laminin (0.25 µM) was incubated in 0.1 M Tris–HCl (pH 7.5) with ClRNH^{32}P-OdT12 at the concentrations given below for 1 h at 37°C. The mixture was then treated with 2-mercaptoethanol, boiled and subjected to 6% polyacrylamide gel electrophoresis. The concentration of ClRNH^{32}P-OdT12 was as follows (lanes 1–13, respectively): 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 10, 20, 22.5, 25, 27.5 and 30 µM. Unreacted oligomer ran off the bottom of the gel and is not shown. Some data points plotted below are not shown in this figure due to artifacts in the process of photographically transferring the data from X-ray film to prints. (**Top right**) Concentration dependence of modification of the A subunit of laminin by differing concentrations of ClRNH^{32}P-OdT12. The gel bands in Figure 4, top left , were quantitated by laser scanning densitometry. Shown is a plot of relative band intensity versus reactive oligodeoxynucleotide concentration (micromolar). (**Bottom**) Double-reciprocal plot of the data in the top right panel. The value of apparent K_d (–1/x intercept) = 14 µM. The four lowest concentration points in Figure 4 (top right) have been deleted from this analysis because of inappropriate data weighting.

concentration range tested, binding of the modifying oligodeoxynucleotide to laminin occurred on the A (M_r = 400 kDa) subunit, as judged by an electrophoretic gel mobility shift assay. Binding to the other, low molecular weight subunits appeared to be minimal or absent (not shown).

We examined the concentration dependence of the modification of the A subunit of laminin by ClRNH^{32}P-OdT12 (Fig. 3, left) . These results are also depicted in Figure 3, top right, where the concentration of modifying oligodeoxynucleotide is plotted as a function of gel band intensity. The association of the A subunit of laminin with the modifying oligodeoxynucleotide exhibits approximate saturation binding, and can be described by a single-site binding equation of the Michaelis–Menton type. Figure 3 (bottom) depicts the double-reciprocal replot of the data in Figure 3 (top right). These data are linear (r^2 = 0.97), and the line intersects the abscissa corresponding to an apparent K_d value of 14 µM. A similar experiment was performed with ClRNH^{32}P-OdT18. Saturation binding was also observed and the apparent K_d was 8 µM.

In order to demonstrate that the binding of the modifying oligodeoxynucleotide to laminin was a function of the oligodeoxynucleotide, and not of the alkylator, we performed a series of experiments using other polyanions as competitors of probe binding. All polyanionic oligomers tested, including Rel A antisense and Rel A sense and the control oligomers SdT18 and OdT18, were concentration-dependent competitors of the binding of ClRNH^{32}P-OdT12 [8 µM] to the A subunit of laminin. Similar data were obtained for competition of binding of ClRNH^{32}P-OdT18 to laminin. Furthermore, suramin, a compound known to bind to laminin (16), as well as a sulfonated distamycin derivative (19), were also competitors of the binding of the modifying oligodeoxynucleotides to the A subunit of laminin.

We have, in greater detail, examined the ability of the human Rel A antisense and Rel A sense oligomers to competitively inhibit binding of the modifying oligodeoxynucleotide to the A subunit of laminin. The value of K_c for a competitor of the binding of the modifying oligodeoxynucleotide was calculated from equation **1** (20): we have previously used this method to determine the values of K_c for competitors of modifying oligodeoxynucleotide binding to rsCD4 (8) and to basic fibroblast growth factor (bFGF) (7).

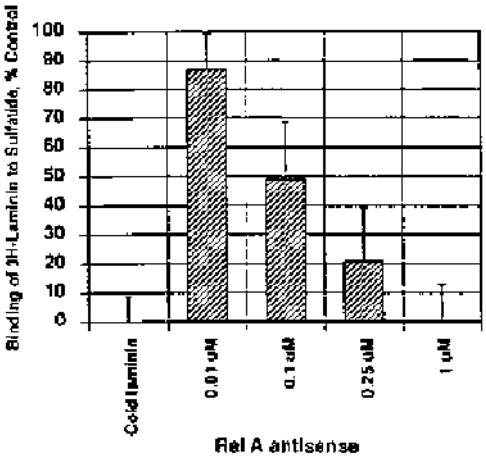
$$K_c = IC_{50}/(1+[ClRNH^{32}P\text{-}OdT12]/K_d) \quad \mathbf{1}$$

The Rel A sense oligodeoxynucleotide is an excellent competitor of the binding of the modifying oligodeoxynucleotide binding to laminin. The value of K_c, as determined by equation **1**, is 0.8 µM. The values of K_c for other polyanionic competitors, determined in an identical manner, are given in Table 2.

The heparin binding site on laminin is located on the E3 fragment of the A subunit (21). We determined that two discrete, synthetic, persulfated heparin mimetics, denoted 1474 (m.w. 3500) and 1306 (m.w. 2095) were also excellent competitors of the binding of the alkylating probe oligo to the A subunit of laminin (see bottom of Fig. 5). This suggested that binding of the modifying oligodeoxynucleotide (and by inference, of its oligodeoxynucleotide competitors of binding), occurs at or near the heparin binding site.

Table 2. Values of K_c for competition of binding of ClRNH^{32}P-OdT12 to the A subunit of laminin. Probe = ClRNH^{32}P-OdT12 (8 µM); K_d = 14 µM

Competitor	K_c (from equation **1**) (µM)
Human Rel A sense	0.8
Human Rel A antisense	1.2
#2	4.4
SdT18	1.0
OdT18	11.3
Suramin	9.8
Sulfonated distamycin A derivative (Ciomei *et al*., 1994)	41



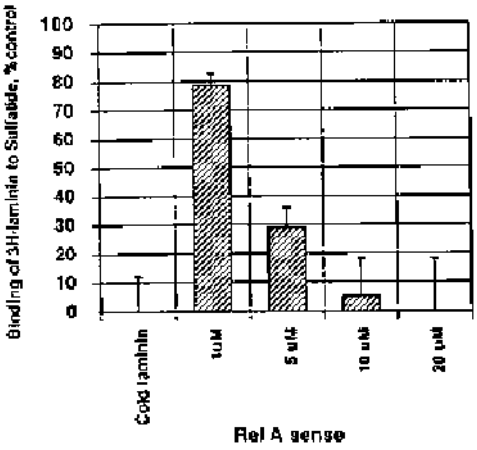


Figure 4. Inhibition of binding of ^{3}H-laminin to sulfatide by human Rel A sense (**top**) and human Rel A antisense (**bottom**). Inhibition of the binding of ^{3}H-laminin to bovine brain sulfatide by Rel A sense (top) and Rel A antisense (bottom) oligodeoxynucleotides as evaluated in triplicate wells as described in the text. Oligodeoxynucleotide binding to these lipids in the absence of sulfatide was subtracted from the total to give the specific laminin binding. Plotted are the mean experimental values ± standard deviations.

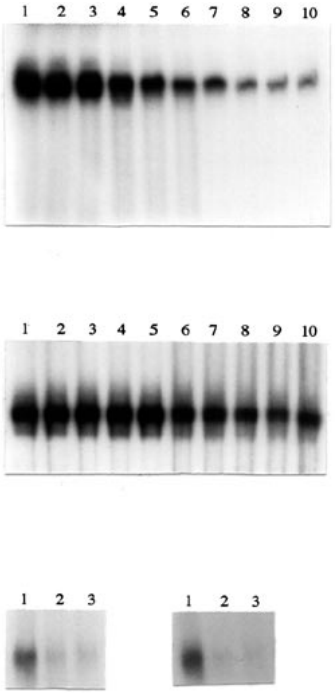


Figure 5. (**Top**) Competition by SdC28 for binding of 5′ ^{32}P-SdT18 to fibronectin. SdC28 was used as a competitor of 5′ ^{32}P-SdT18 (3 µM) binding to fibronectin (5 µM) as described in the text (6% PAGE). The concentration of SdC28 was (lanes 1–10, respectively) 0, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 75 and 100 µM. (**Middle**) Competition by 1306 for binding of 5′ ^{32}P-SdT18 to fibronectin. 1306 was used as a competitor of 5′ ^{32}P-SdT18 (3 µM) binding to fibronectin (5 µM) as described in the text (6% PAGE). The concentration of 1306 was (lanes 1–10, respectively) 0, 0.58, 1.0, 2.5, 5.0, 10, 25, 50, 75 and 100 µM. (**Bottom**) Competition by 1306 (left) and 1474 (right) for binding of 5′ClRNH^{32}P-OdT12 to laminin. 1306 was used as a competitor of 5′ alkylating oligodeoxynucleotide (3 µM) binding to laminin (0.25 µM) as described in the text (6% PAGE). Binding occurs on the A subunit of laminin. The concentration of 1306 and 1474 were (lanes 1–3 respectively) 0, 25 and 50 µM.

Phosphorothioate oligodeoxynucleotides inhibit the binding of laminin to sulfatide but not to its cell surface receptors on MCF-7 cells

We next evaluated the ability of the human Rel A antisense and Rel A sense oligodeoxynucleotides to inhibit the binding of ^{3}H-laminin to one of its ligands, bovine brain galactosyl-I^{3}-ceramide sulfate (sulfatide). For the Rel A antisense oligomer, the value of IC_{50} was ~0.1 µM. In contrast, the value of IC_{50} for the Rel A sense oligomer, under identical conditions, was >1 µM (Fig. 4). As a control [not shown; (16)], we demonstrated that suramin could also inhibit the binding of ^{3}H-laminin to sulfatide (IC_{50} ~100 µM). As can be seen in Table 1, the sequence of the Rel A antisense oligomer contains four contiguous deoxyguanosine (dG) residues, a motif which has been associated with increased avidity for protein (7,22). In order to further probe this sequence effect on the inhibition of binding of ^{3}H-laminin to sulfatide, we examined the ability of mutated versions of the Rel A antisense oligomer to inhibit the binding of ^{3}H-laminin to sulfatide. These mutations (see Table 1) were designed to interrupt the G-quartet motif: in the case of #7, the mutated version contains four contiguous thymidine, as opposed to guanosine, residues. As is shown in Table 1, despite the fact that oligomers #4, #6 and #7 are three residues longer than Rel A antisense, none are as capable at blocking the binding of ^{3}H-laminin to sulfatide. Indeed for #7, in which the entire G-quartet is replaced with four contiguous thymidine residues, the IC_{50} is >1 µM.

We also examined a series of phosphorothioate 18mer oligodeoxynucleotides which were mutated versions of the rat antisense c-myb construct (codons 1–6). These data (oligomers #1, #2, #3; see Table 1) demonstrate that ordering of the values of IC_{50} depends on the number of contiguous guanosines. The value of IC_{50} declines in the order #3 (9dG) < #2 (7dG) < #1 (4dG); for the #3 (9dG) oligomer, the value of IC_{50} is 0.01 µM. This is in contrast to a control of the same length, SdT18, where IC_{50} = 25 µM. These data correlate well with data obtained in the adhesion assay, as SdT18 [20 µM] is also a very poor inhibitor of cellular adhesion to plastic plates. On the other hand, the

oligomers with more than four contiguous guanosines are excellent inhibitors of adhesion (>90% at [10 μM]).

Phosphorothioate oligodeoxynucleotides [10 μM] did not, in the presence of 500 μM Ca^{2+} in PBS + 0.25% BSA, inhibit the specific binding of ^{3}H-laminin to its cell surface receptors on K-Balb cells. In fact, when 20 μM of either Rel A antisense, SdC28 or oligomer #2 were incubated with these cells and ^{3}H laminin for 1 h (4°C), an average doubling of specific ^{3}H-laminin binding to the cell surface was observed (data not shown). This increase in binding was not observed, however with either the Rel A sense oligomer, or with the rat antisense c-myb phosphorothioate oligodeoxynucleotide (see Table 1).

Interaction of fibronectin with oligodeoxynucleotides

Fibronectin contains two heparin binding sites per polypeptide chain and two chains per molecule (21). Initially, we attempted to study the binding of the probe, alkylating oligodeoxynucleotides ClRNH^{32}P-OdT12 and -OdT18 to fibronectin by the methods described for laminin. However, the affinity of fibronectin for the alkylating substituent was sufficiently low (K_d > 35 μM) so that alkylation of the protein occurring preferentially to oligodeoxynucleotide binding. This was demonstrated, in contrast to what was seen with laminin, by relative lack of competition of modifying oligomer binding by polyanionic competitors. Thus, we employed 5′ ^{32}P-labeled phosphorothioate oligodeoxynucleotide probes without alkylating activity, and evaluated their binding to fibronectin in native gels.

^{32}P-SdT18 interacts with 5 μM fibronectin in 0.1 M Tris–HCl, pH 7.4 (data not shown). In contrast to what was observed with laminin, this binding is complex and not saturable at the highest concentrations employed, and is not describable by a single-site binding formulation. The binding is independent of Mg^{2+}, Mn^{2+} and Ca^{2+} [up to 10 mM; (23)]. Interestingly, dissociation of the fibronectin subunits by addition of 2-mercaptoethanol before gel electrophoresis led to an increased number of counts bound to fibronectin. This occurred without a change in the shape of the complex binding curve. These data suggest that perhaps one or more of the heparin binding sites on fibronectin is cryptic, and that it may be exposed after disulfide bond cleavage by the mercaptoethanol. Competition for probe binding to fibronectin was also observed with the discrete heparin mimetics 1474 (IC_{50} = 1.1 μM; [5′-^{32}P-SdT18] = 3 μM; [fibronectin] = 5 μM) and 1306 (IC_{50} = 6.2 μM; Fig. 5, middle). These data suggest that here, too, binding of the probe phosphorothioate oligodeoxynucleotide occurs at or near the heparin-binding site.

SdC28 (Fig. 5, top) is an excellent competitor for the binding (pH 7.4) of the probe 5′-^{32}P-SdC18 (3 μM) to fibronectin (5 μM); the IC_{50} is 7 μM. As we observed with laminin, the binding of oligomer to protein are most easily visible when 2-mercaptoethanol is included in the loading buffer. Shorter competitor oligos than SdC28 have values of IC_{50} that are correspondingly higher. For example, when SdT18 (5′-^{32}P-SdC18 probe) was the competitor, the IC_{50} of competition is 39.6 μM.

Phosphorothioate oligodeoxynucleotides block the binding of fluoresceinated fibronectin to its cell surface receptors on phorbol-ester induced Jurkat T-cells

When Jurkat-T cells are stimulated with phorbol-12,13-myristate acetate (5 nM, 24 h) the cell surface receptor for fibronectin,

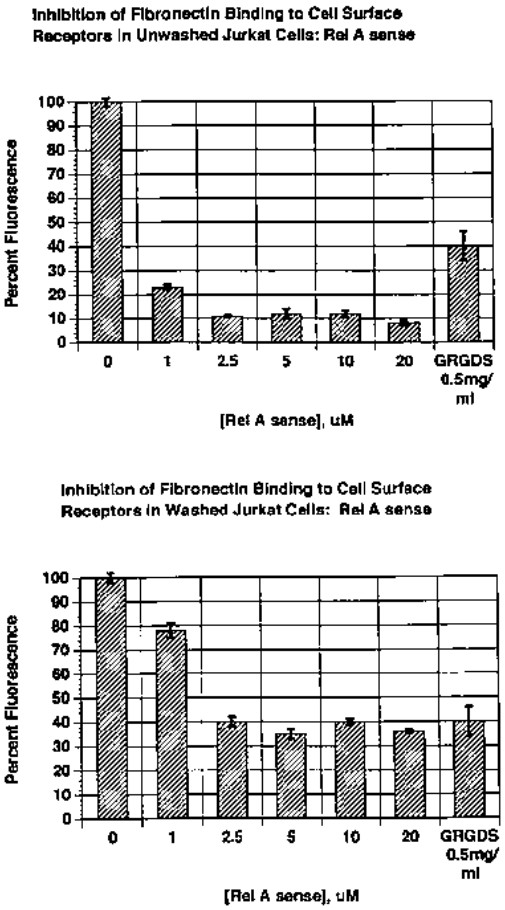


Figure 6. Inhibition of fluorescein isothiocyanate-conjugated fibronectin binding to the cell surface of washed (**top**) and unwashed (**bottom**) Jurkat cells. Jurkat cells were treated with fluorescent fibronectin as described in the text, and analyzed by flow cytometry. Shown is a plot of relative fluorescence (no added Rel A sense oligomer = 100%) versus concentration of the Rel A sense oligomer. Experiments were conducted in triplicate, and data bars represent mean values ± standard deviation. Specificity of binding was demonstrated by the specific competitor of fibronectin binding, the pentapeptide GRGDS at the stated concentration.

which is the α5β1 integrin, is upregulated (24). In the wild-type Jurkat cells used in these experiments, treatment with phorbol ester under the identical conditions also dramatically increased the binding of F-Fn to the cell surface as compared to untreated cells. Thus, all experiments examining binding of F-Fn to the cell surface of Jurkat cells were performed after phorbol ester induction. In each case, specificity of the binding of F-Fn was demonstrated by competition by the pentapeptide G-R-G-D-S. As shown in Figure 6, the IC_{50} of inhibition of binding of F-Fn by SdC19 was ~2–5 μM. When either the Rel A sense or antisense constructs were used as competitors of F-Fn binding, the value of IC_{50} was <1 μM. However, neither these oligodeoxynucleotide, nor SdC28 (10 μM) could inhibit the binding of a β1-specific mAb (Becton Dickinson) to the induced Jurkat cells.

We then treated Jurkat cells for 15 min with Rel A sense and antisense phosphorothioate oligodeoxynucleotides at the stated concentrations (Fig. 6), and then pelleted and washed the cells at 4°C twice with PBS. This reduced the phosphorothioate oligodeoxynucleotide concentration in the media to a negligible

amount. Despite this, pre-treatment of the Jurkat cells with the phosphorothioate oligodeoxynucleotides was sufficient to block the binding of F-Fn to its cell surface receptors. However, the blockade was not as complete relative to what was observed with the unpretreated cells. Similar results were observed for the phosphorothioate oligodeoxynucleotide #2.

DISCUSSION

It has recently been shown (3,14) that non-sequence specific phosphorothioate oligodeoxynucleotides can block cellular adhesion to plastic substrata in a variety of neoplastic cell lines. The presence of the G-quartet motif was felt to be necessary, but not sufficient, for blockade of adhesion. However, in shorter sequences (e.g. 12mer; [20 μM]), the presence of the G-quartet did not produce an anti-adhesive effect. Similar length-dependent effects were observed by Lallier and Bronner-Fraser (25), who examined the inhibition of attachment of quail neural crest cells to fibronectin and laminin substrata in the presence of phosphorothioate oligomers. Watson *et al.* (26) also noted inhibition of attachment of MCF-7 cells to plastic substrata in the presence of an anti-c-myc (G-quartet motif-containing) phosphorothioate oligomer. Similar to what we and Chavany *et al.* (27) observed, adhesion was not blocked on either laminin or fibronectin coated plates. In addition, it has recently been shown (4) that the ECM from a feeder layer of 3T3 fibroblasts will also abrogate the anti-adhesive effects of both the Rel A antisense and sense oligomers. Taken together, all the data consistently demonstrate that the effects of phosphorothioate oligomers on heterotypic cellular aggregation are predominately non-sequence specific, and related, at least in significant part, to an extracellular interaction with ECM proteins. Furthermore, the anti-adhesive effects of the phosphorothioate oligomers evaluated in this work are also related in part to sequence and length: in general, G-rich constructs (#1–#3, Rel A antisense) tended to be better anti-adhesives than other, non-G containing homopolymers (e.g. SdT18, SdC18) or than Rel A sense. On the other hand, SdC28 (10 μM), in K-Balb cells, blocked heterotypic adhesion almost to the same extent as Rel A antisense. However, the rat antisense c-myb oligomer, which contained a G-quartet motif, was a poor anti-adhesive at a 10 μM concentration. Thus, it is also likely that the presence of the G-quartet motif alone is insufficient for maximal anti-adhesiveness: More probably, it must be located in the midst of a set of appropriate flanking sequences (13). The nature of these optimal flanking sequences are as yet unknown in this system.

The ability of guanosine-rich phosphorothioate oligodeoxynucleotides to interact with proteins (7,28) probably is best represented as a continuum with respect to dG motif length. It is possible that stacking interactions among the contiguous dG residues cause a diminution in the degree of rotational freedom of the oligomer: this may occur despite the fact that molecules such as Rel A antisense has the electrophoretic mobility of a typical monomer in 7M urea polyacrylamide gels (Stein,C.A., Khaled,Z. and Narayanan,R. unpublished results). This diminution, in turn, may result in a diminished entropic component of the binding of a dG-rich oligomer to protein. Unusual protein avidity may first become observable at dG = 4, but may not be so pronounced as to render irrelevant the sequences flanking the dG motif (13). At dG > 4, other non-specific factors may supervene, chief among them being the tendency of these molecules (e.g. #1–#3) to form quadruple helices (22,29; Khaled,Z. and Stein,C.A. unpublished observations). However, the ability of quadruple helical phosphorothioate oligodeoxynucleotides to interact with proteins has not been extensively studied. An exception this has been recent work on the augmented ability of S-$dT_2G_4T_2$ [relative to S-$d(GT)_4$] to bind to the v3 loop of gp120 (22). The interaction of other dG-rich oligonucleotides with gp120 has also been examined (30,31). At this time, however, it does not appear possible to predict, based on sequence considerations alone, which dG-rich phosphorothioate oligomers will form quadruple helices.

Oligodeoxynucleotide binding occurs on the A (α1) subunit of laminin. This binding, based on competition by the discrete synthetic heparin mimetics 1306 and 1472, thus appears to occur at or near the heparin-binding site. We have recently demonstrated (7,8,10) the extensive similarities in protein binding behavior between both phosphodiester and phosphorothioate oligodeoxynucleotides and heparin. This finding is not altogether unexpected in light of the fact that these molecules are representatives of classes of molecule, i.e. DNA and glycosaminoglycans, respectively, which are polyanions (6). The binding of the phosphorothioate oligomers to protein, as opposed to phosphodiesters, is more readily observed because the values of K_d for the phosphorothioates tend to be one to three orders of magnitude lower than for the phosphodiesters of identical sequence and length (6).

The consequence of phosphorothioate oligodeoxynucleotide binding to laminin is loss of the ability of laminin to bind to sulfatide, a substance on which cells can superficially spread. Curiously, it is in this assay that the largest sequence-dependent differences between Rel A antisense and Rel A sense oligomers were observed. These sequence related differences in IC_{50} of the inhibition of laminin binding to sulfatide were also particularly pronounced with increasing contiguous dG content (data not shown), perhaps for the reasons given above. However, in the presence of phosphorothioate oligodeoxynucleotides, laminin was still capable of binding to its cell surface receptors on MCF-7 cells. These observations are similar to those of Zabrenetzky *et al.* (16), who examined the inhibition of the cell surface receptor binding of laminin by suramin. This compound is a hexaanion, and has many biological properties similar to those of longer phosphorothioate oligodeoxynucleotides (10,32). In the experiments with suramin, however, the value of IC_{50} was much greater than that for phosphorothioate oligodeoxynucleotides.

The binding of oligodeoxynucleotides to fibronectin, in contrast to laminin, was quite complex, presumably due to the fact that each of the two polypeptide chains in fibronectin contains a heparin binding site (21). In further contrast to the interaction of phosphorothioates with laminin, the interaction of these oligomers with fluoresceinated fibronectin efficiently blocks its binding to the α5β1 integrin receptor on PMA-induced Jurkat cells. Interestingly, evidence exists to indicate that the binding of fibronectin to its cell surface receptor may in itself be an inducer of NF-κB activity. Indeed, Qwarnstrom *et al.* (33) have recently shown that the blockade of fibronectin binding to its integrin receptor results in a concentration-dependent reduction in the fibronectin-induced p50/p65 heterodimeric NF-κB activity to ~25% of the control value. This reduction, it has been proposed, may further decrease the tendency of human gingival fibroblasts and smooth muscle cells to adhere and spread, possibly by reducing the interaction of the ankyrin-like repeats of NF-κB subunits with cytoskeletal structural elements (34). Thus, it is

possible that merely inhibiting the binding of fibronectin to its cell surface receptor is alone sufficient to block PMA-induced upregulation of NF-κB activity subsequent to attachment. This mechanism may thus be a major contributor to the anti-adhesive properties of phosphorothioate oligodeoxynucleotides. Militating against this hypothesis, however, is that PMA induction of NF-κB nuclear activity is, in fact, readily observed (Fig. 2) in both attached and suspension K-Balb cells which have been treated with Rel A antisense oligodeoxynucleotide. At the concentrations employed, this oligodeoxynucleotide effectively blocks the binding of F-Fn to cell surface receptors.

The blockade of the binding of F-Fn to its cell surface receptors on PMA-induced Jurkat cells is a complex event. Because oligomer-treated, washed cells still fail to bind F-Fn, direct interaction of the oligomer with the receptor complex must still be considered. This is true despite lack of oligomer inhibition of binding of an anti-β1 mAb and especially in light of the fact that it is believed that the off-rates of phosphorothioate oligomers complexed to protein tend to be slow (6). However, the precise binding site is unknown.

Finally, these data once again point out the difficulty in determining if a given biological endpoint, evaluated by an antisense experiment employing phosphorothioate oligodeoxynucleotides, is being produced by an antisense mechanism (5). Furthermore, the multiple pathways, demonstrated here, by which these oligomers may affect cellular heterotypic aggregation make it quite difficult to generate appropriate control oligomers for these types of experiments. Appropriate care must be taken to ensure that observed biological endpoints do not represent an intricate agglomeration of non-sequence specificity instead of the oft-desired antisense effect.

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