Combinatorial Selection and Edited Combinatorial Selection of Phosphorothioate Aptamers Targeting Human Nuclear Factor-κB RelA/p50 and RelA/RelA†

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ABSTRACT: Nuclear factor-κB (NF-κB) transcription factors are important in regulating the immune response and play critical roles in the pathogenesis of chronic inflammatory diseases and a variety of human cancers. Agents that target specific NF-κB dimers may serve as therapeutic agents for the prevention of pathogenic immune responses. We have selected monothiophosphate-modified aptamers, or "thioaptamers", to the NF-κB p50/RelA heterodimer using combinatorial selection techniques. We also utilized a "double sieve" or editing approach for the generation of thioaptamers with enhanced selectivity to the RelA/RelA homodimer. The thioaptamers from these selections and our previous selections on the p50/p50 and RelA/RelA homodimers all had unique sequences and bound tightly to the recombinant NF-κB dimers against which they were selected. The selected thioaptamers also appear to maintain their selectivity and specificity among other cellular proteins, because they have the ability to bind NF-κB proteins within nuclear extracts from lipopolysaccharide (LPS)-induced macrophages and B cells.

The nuclear factor-κB (NF-κB) transcription factor family mediates the inducible transcriptional activity of a multitude of immune and acute phase response genes in a variety of cell types (*1*, *2*). Several family members have been identified. The amino halves of the proteins include the rel homology region (RHR) where sequences and functional homology have been conserved throughout evolution. The carboxyl halves exhibit more diversity and include the activation domains and ankyrin repeats. The NF-κB family members are divided into two groups based on differences in structure, function, and modes of synthesis. One group, consisting of p50 and p52, lacks the carboxyl transactivation domains and appears to act as transcriptional repressors. The p50 and p52 proteins are derived from inactive precursors, p105 and p100, respectively. The second group of NF-κB family members includes RelA (p65), c-Rel, v-Rel, Rel B, Dorsal, and Dif. Homo- or heterodimers containing at least one member from this group are strong transcriptional activators. Different forms of NF-κB most likely activate different sets of target genes. However, little is known regarding which NF-κB dimers control particular genes. The NF-κB members reside in the cytoplasm in an inactivated state while associated with IκB, an inhibitor protein. IκB masks the nuclear localization signal of NF-κB and prevents translocation to the cell nucleus. A variety of insults, such as UV irradiation or viral infection, activate NF-κB. Upon activation, NF-κB translocates to the nucleus where it influences the transcription of genes that are involved in inflammatory and immune responses.

NF-κB proteins play key roles in the pathogenesis of various chronic inflammatory diseases and in a variety of human cancers (*1*). When NF-κB transcription factors are activated, genes involved in augmenting and perpetuating the immune response and pathogenic responses are coordinately expressed (*3*). Since NF-κB proteins are central in regulating inflammatory responses, agents that specifically target these proteins may serve as therapeutic agents for the prevention of pathogenic immune responses. Glucocorticoids, antioxidants, and naturally occurring NF-κB inhibitors such as gliotoxin serve as current antiinflammatory treatments. However, the selectivity, effectiveness, toxicity, or side effects are problematic (*4*−*6*).

Oligonucleotides are promising therapeutic agents. Aptamers are oligonucleotide agents selected from combinatorial libraries that act by binding directly to target proteins or other molecules and disrupting their activity. Aptamers with high affinity and specificity have successfully been selected recently from random RNA and DNA libraries either through in vitro enzymatic selection methods (*7*−*16*) or from random bead-based aptamer libraries created by chemical split synthesis methods (*17*, *18*). Aptamers with sulfurs partially

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replacing one or both of the nonbridging phosphate oxygens appear to bind to proteins with greater stability, higher selectivity, and enhanced affinity compared to normal backbone aptamers (*17*−*21*). Backbone-modified "thio" aptamers, or "thioaptamers", such as those with monothiophosphate or dithiophosphate backbone substitutions, are promising for use in diagnostics as well as therapeutics. Vitravene is a phosphoromonothioate antisense drug that is commercially available (CIBA Vision, A Novartis Company).

Antisense and siRNA (silencer RNA) oligonucleotides that target NF-κB protein RelA have potential for inhibiting inflammatory bowel disease mimicking human Crohn's disease and may be used for other inflammatory conditions (*22*). However, the disadvantage of using technologies such as antisense or siRNA to target RelA or any other NF-κB monomer is that the elimination of any one member of the NF-κB family will eliminate all the possible dimers of which that protein would be a normal subunit. The five NF-κB proteins can combine to form 15 homo- and heterodimers, each of which performs a specific signaling function by binding to a promoter region of a particular gene. Therefore, the elimination of one NF-κB monomer may broadly disrupt the immune system and pose a problem. The development of thioaptamers to specific NF-κB dimers, rather than to monomers, may allow them to more specifically regulate genes. RNA and DNA double-stranded oligonucleotides have been developed that target NF-κB proteins and influence the expression of NF-κB regulated genes (*23*−*27*; for review, see ref *27*). Some of these decoy aptamers appear to have therapeutic potential. However, global inhibition of NF-κB protein activity is likely to be detrimental. Therefore, we are developing double-stranded DNA thioaptamers or "decoys" that can be used to compete for specific NF-κB dimers binding to the authentic binding elements in cellular DNA.

We have recently developed combinatorial double-stranded DNA aptamer libraries with randomly substituted thiophosphate backbones (*13*, *20*). These thioaptamer libraries are synthesized by amplifying a synthetic DNA library using *Taq* polymerase and a mixture of dATP(αS), dTTP, dGTP, and dCTP in a standard PCR reaction (up to 3 of the 4 dNTPs may be substituted by dNTP(αS), so varying hybrid phosphate/thiophosphate backbone aptamer libraries may be created). These libraries contain potentially 10^{13} different sequences. We previously used combinatorial selection methods to select thioaptamers that tightly and selectively bind to the NF-κB RelA/RelA and p50/p50 homodimers (*13*). In the previous study, the nucleic acid libraries were incubated with the target protein and nonbinding oligonucleotides were separated from those that bind the protein. The bound oligonucleotide fractions were then PCR amplified and subsequently incubated with the target protein for the next round of selection. The iterations were repeated until the library was enhanced for sequences with high affinity for the target protein. The same selection strategy has been used in this study to select for thioaptamers with high affinities for the p50/RelA NF-κB heterodimer. A strategy was also developed to select aptamers for the RelA/RelA homodimer. We used a "double sieve" or editing approach for the generation of thioaptamers to enhance the selectivity toward the RelA/RelA homodimer. In this case, the libraries were first incubated with the p50/p50 dimer. The wash, representing aptamers that do not bind tightly to the p50/p50 homodimer, was used to select sequences that bind more tightly to the RelA/RelA homodimer.

The results from this study reveal that (i) the thioaptamers selected to the various NF-κB targets all had unique sequences and bound the respectively targeted NF-κB dimers, (ii) enhanced specificity for the RelA/RelA homodimer was observed using a "double sieve" or editing combinatorial selection methodology, and (iii) the selected thioaptamers specifically bind to NF-κB proteins within nuclear extracts from lipopolysaccharide (LPS)-induced macrophages and B cells.

MATERIALS AND METHODS

NF-κB Expression and Purification. Human NF-κB proteins were purified as described previously (*13*). Briefly, NF-κB−GST fusion proteins were expressed in *Escherichia coli* BL21 using the pGEX vector (Pharmacia). The NF-κB RelA construct contained the sequences for residues 12−317. The p50 construct contained sequences for residues 11−400. Bacteria were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to express these proteins. Cell lysates were obtained, and the soluble fraction was loaded onto a glutathione-Sepharose affinity column. Thrombin was used to cleave and elute the proteins, which were then loaded onto a Mono Q anion-exchange column. This purification scheme results in soluble and active protein that is greater than 99% pure, as measured by SDS−PAGE. The heterodimer was formed by mixing equimolar amounts of the purified p50 and RelA proteins together in 30% glycerol and incubating at 25 °C for 1 h. Freshly prepared heterodimer was used for each round of selection.

Combinatorial Selection of p50/RelA Thioaptamers. The methods for combinatorial selection of thioaptamers targeting RelA and p50 have been described previously (*13*, *20*). The methods for combinatorial selection of p50/RelA thioaptamers are essentially the same. Briefly, a 66-mer single-stranded library was chemically synthesized (Midland Certified Reagents). This library contained a 22-base random or variable region flanked by primer regions. The sequence of this library was 5′-CAGTCTTGCGGAAGAGTGTCCAC-N_{22}-CGATTATCGAGCATCCGAGCG-3′. The single-stranded library was replicated in a standard Klenow reaction. The library template and reverse primer were annealed by preparing a 100-μL reaction mix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.9 μM library DNA, and 3.9 μM reverse primer (5′-CGCTCGGATGCTCGATAATCG-3′). The annealing reaction was boiled for 5 min and then cooled slowly to 25 °C. A 100-μL Klenow reaction mix containing 10 μL of the annealing reaction, Klenow buffer, 0.8 mM dNTP mix, and 5 U of Klenow (Fisher) was prepared. The reaction mix was incubated for 5 h at 37 °C and subsequently amplified using PCR. A 100-μL reaction PCR mix containing *Taq* PCR buffer (Applied Biosystems), 80 μM each dTTP, dGTP, and dCTP, 160 μM dATP(αS), 500 μM $MgCl_2$, 39 nM 66-mer random template, 400 nM each primer, and 0.5 unit of *Taq* polymerase was prepared. The forward and reverse primers were 5′-CAGTCTTGCGGAAGAGTGTCCAC-3′ and 5′-CGCTCGGATGCTCGATAATCG-3′, respectively. The PCR reactions were performed using the following scheme for amplification: 94

°C for 5 min (1 cycle); 94 °C for 2 min, 55 °C for 2 min, 72 °C for 2 min (35 cycles); and 72 °C for 7 min (1 cycle). The resulting library that was amplified contained R_p configured monothiophosphate modification 5′ to every dA residue.

Thioaptamers were selected to purified recombinant p50/RelA NF-κB proteins using a modified filter binding method until the library converged with 15 iterations (*28*, *29*). Equimolar amounts of purified recombinant p50 and RelA NF-κB proteins were mixed in 30% glycerol and incubated for 1 h at 25 °C to form the heterodimer immediately prior to performing the binding reaction. The PCR-amplified random phosphorothioate library was prefiltered to deplete the population that bound nonspecifically to the filter. The resulting library was incubated with NF-κB p50/RelA in a binding reaction mix for 1 h at 25 °C. The 50 μL reaction mix contained 0.5 μM library, 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.125−0.5 μM p50/RelA, and 25−350 mM KCl. The stringency of the selection was heightened at each round of selection by increasing the KCl concentration and decreasing the protein concentration. The binding reaction mix was incubated on a Micropure EZ filter unit (Millipore) for 10 min to retain the DNA−protein complexes on the filter. The filter was then washed 4−6 times with 250 μL of the binding reaction buffer to remove the unbound DNA. The protein-bound DNA was then eluted with 100 μL of 8.0 M urea for 10 min at 25 °C. A negative control without protein was performed simultaneously to monitor nonspecific binding of the library to the filter. The DNA was ethanol-precipitated and again PCR-amplified in the presence of dATP(αS) for the next round of selection. At various stages of the selection process, the libraries were amplified in a normal phosphoryl PCR, and the products were cloned into bacterial plasmids using the TOPO-TA Cloning Kit (Invitrogen). The sequences from individual colonies were analyzed.

Edited Combinatorial Selection of RelA/RelA Thioaptamers. A different 66-mer single-stranded library was chemically synthesized (Midland Certified Reagents). This library contained a 22-base random or variable region flanked by primer regions. The sequence of this library was 5′-CAG-TGCGGATCTAGATCCGTGAC-N_{22}-CGAAGCATCTC-GTTACGAGCG-3′. The single-stranded library was replicated in a standard Klenow reaction and subsequently amplified by PCR exactly as described above. The forward and reverse primers were 5′-CAGTGCGGATCTAGATC-CGTGAC-3′ and 5′-CGCTCGTAACGAGATGCTTCG-3′, respectively.

Thioaptamers were selected using an editing method until the library converged with 14 iterations. The phosphorothioate random library was prefiltered, and 1.0 μM was incubated with 2.0 μM p50 in a 100-μL reaction mix containing 10 mM Tris-Cl, pH 7.5, 1 mM DTT, and 25−250 mM KCl. The reaction mix was incubated for 1 h at 25 °C. The protein−DNA complexes were retained on the filter, and the flow-through, representing aptamers that do not bind tightly to the p50 homodimer, was used to select sequences that bind more tightly to the RelA/RelA homodimer. The flow-through was then incubated with RelA in the same binding buffer for 1 h at 25 °C. The protein−DNA complexes were filtered, and the unbound DNA was washed away as described above. The protein-bound DNA was then eluted with 100 μL of 8.0 M urea for 10 min at 25 °C. A negative control without protein was performed simultaneously to monitor nonspecific binding of the library to the filter. The DNA was ethanol-precipitated and again PCR-amplified in the presence of dATP(αS) for the next round of selection.

Enzymatic Synthesis of 5′-Biotin-Labeled Thioaptamers. Chiral phosphorothioates were PCR synthesized in the presence of dATP(αS) as previously described (*13*, *20*). Briefly, the aptamers have a monothiophosphate modification 5′ to each dA residue in the 22 base pair random region. The thioaptamers were amplified by PCR using limiting amounts of plasmid DNA and a 5′ biotinylated PCR primer (Midland Certified Reagents) for each construct. The PCR products were purified using a Micropure EZ spin column (Millipore) to remove the *Taq* polymerase.

Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA). The EMSA conditions have been described previously (*13*). Briefly, the ability of the aptamers to bind the NF-κB proteins was qualitatively assessed using the electrophoretic mobility shift assay (EMSA). Native polyacrylamide minigels were prepared with 0.25× Tris−borate−EDTA (TBE) buffer (Bio-Rad), 8% acrylamide, 0.075% bisacrylamide, 2.5% glycerol, 0.0675% ammonium persulfate, and 0.075% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The conditions of the binding reactions were 10 mM KH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 40 mM DTT, 6% glycerol, and 1 mg/mL bovine serum albumin at pH 7.0. Enzymatically synthesized 5′-biotin-labeled thioaptamers were used in the commercially available LightShift chemiluminescent EMSA kit (Pierce). The thioaptamer/NF-κB binding reactions were incubated for 1 h at 25 °C and then run on a native gel at 100 V for 45 min. The reaction products were electrophoretically transferred from the gel to a Biodyne B modified nylon membrane (Pierce) in 0.25× TBE for 1 h at 100 V. The membranes were developed in a buffer containing avidin-linked horseradish peroxidase. The chemiluminescence was detected using a Fluor Chem 8800 (Alpha Innotech).

Klenow Enzymatic Synthesis of the Random Region of Chiral Phosphorothioate Aptamers. To prepare larger quantities of shorter length chiral monothiophosphate DNA thioaptamers without the primer sequences, we developed a Klenow DNA polymerase enzymatic synthesis, modifying an approach by Zimmer and Crothers (*30*). Briefly, hairpin oligonucleotides that terminated with a 3′ ribonucleotide and contained three consecutive biotins at the 5′ end were chemically synthesized (Midland). The oligonucleotides were dissolved in nuclease-free water. Hairpins were formed in a solution of 0.1 mM oligonucleotide, 10 mM Tris-Cl (pH 8.0), and 10 mM NaCl. The mixture was heated at 90 °C for 2 min and then iced immediately. A duplex product containing the thioaptamer sequence was generated from the hairpin oligonucleotides by filling in the overhangs with Klenow DNA polymerase in the presence of dATP(αS). The Klenow reaction mix contained 6.7 μM of hairpin DNA template, the manufacturer's buffer, 80 μM each dCTP, dGTP, and dTTP, 160 μM dATP(αS) (Pharmacia), and 75 units of Klenow DNA polymerase (3′→ 5′ exo$^-$, New England Biolabs). The reaction mix was incubated at 37 °C for 5−16 h. The Klenow DNA polymerase was then heat inactivated at 75 °C for 20 min. The thioaptamer sequence was released from the duplex by alkaline hydrolysis at the 3′ ribonucleo-

Table 1: Variable Region Sequences (a) from Rounds 13 and 15 of the p50/RelA Combinatorial Selection and (b) ClustalW Multiple Sequence Alignment[a] Results

a)

Round 13		
Clone number	**Sequence**	**Number of Clones[b]**
13-1	5′-T.GGGGCT.GT$_s$AC$_s$AGGGT.GC$_s$AC$_s$AC-3′ 3′-A$_s$CCCCGA$_s$CA$_s$TG.TCCCA$_s$CG.TG.TG-5′	6/9
13-4	5′-GGCT.GT$_s$AC$_s$ACGGT.GGGT.GGGGG-3′ 3′-CCGA$_s$CA$_s$TG.TGCCA$_s$CCCA$_s$CCCCC-5′	1/9
13-5	5′-CGGGGCG$_s$AC$_s$ACC$_s$AT$_s$AT.GG$_s$ACGC-3′ 3′-GCCCCGC.TG.TGG.TA$_s$TA$_s$CC.TGCG-5′	1/9
13-12	5′-GGCGGT.GGGCGGT.GC$_s$AGT.GT$_s$AG-3′ 3′-CCGCCA$_s$CCCGCCA$_s$CG.TCA$_s$CA$_s$TC-5′	1/9
Round 15		
15-2 (13-1)	5′-T.GGGGCT.GT$_s$AC$_s$AGGGT.GC$_s$AC$_s$AC-3′ 3′-A$_s$CCCCGA$_s$CA$_s$TG.TCCCA$_s$CG.TG.TG-5′	11/14
15-5	5′-CGGCGCGGGG$_s$ACC$_s$AGG$_s$ACGGC$_s$A-3′ 3′-GCCGCGCCCC.TGG.TCC.TGCCG.T-5′	1/14
15-11	5′-CCGCCGT.GT$_s$AC$_s$AGGT.C$_s$AC$_s$ACCG-3′ 3′-GGCGGCA$_s$CA$_s$TG.TCCA$_s$G.TG.TGGC-5′	1/14
15-12	5′-CGGCGT.G$_s$AGGCG$_s$AC$_s$A$_s$AC$_s$AC$_s$A$_s$AG-3′ 3′-GCCGCA$_s$C.TCCGC.TG.T.TG.TG.T.TC-5′	1/14

b)

```
13-1        ----TGGGGCTGTACAGGGTGCACAC---
15-2        ....TGGGGCTGTACAGGGTGCACAC...
15-11       ......G...TGTACAGG...CACAC...
13-5        .....GGGGC.a.AC.a.aTG.ACgC...
15-12       ......GG..TG.g..Ga..aCACA....
13-4        .......GGCTGTACA.GGTG.g.g....
15-5        .....G.G...G.AC.aGG...gCA....
13-12       ......GGGC.GTgCAG.GTa........
consensus   ----tggggctgtacagggtgcacac---
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[a] The sequence similarities are shown from the alignments using Boxshade 3.21. [b] Number of clones with identical sequences/number of clones analyzed.

tide in a final concentration of 100 mM NaOH at 55 °C for 16 h. Following the hydrolysis reaction, the solution was neutralized with 10% acetic acid. The thioaptamer sequence was then purified from the starting biotinylated oligonucleotides using magnetic porous glass−streptavidin beads (MPG−streptavidin, CPG Inc.). Briefly, the hairpin duplex product was attached to MPG−streptavidin beads (500 μL) with gentle rocking for 30 min at room temperature in B/W buffer (2.0 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The supernatant was removed, and the unbound DNA was washed from the beads 2 times with 1 mL of B/W buffer. A melting solution of 0.1 M NaOH (250 μL) was added to the beads for 10 min at room temperature to denature the duplex. A magnetic particle separator (CPG Inc.) was used to separate the biotinylated strand, attached to the magnetic beads, from the unbiotinylated single-stranded thioaptamer in the supernatant. The supernatant was collected, and the beads were washed 1 time with 500 μL of melting solution. The supernatant, containing the single-stranded thioaptamers, was neutralized with 10% acetic acid, and the complementary strands were annealed to produce the double-stranded chiral thioaptamers. Mass spectrometry and analysis on a denaturing polyacrylamide gel were used to confirm the identify and purity of the chiral thioaptamer.

Nuclear Extract Preparation. Nuclear extracts were prepared from the murine macrophage-like cell line P388D1 and from the murine preB cell line 70Z/3. Cells were treated with LPS (10 μg/mL) for 2 h prior to extraction. Nuclear extracts were prepared following standard procedures (*31*).

Electrophoretic Mobility Shift Assay (EMSA) for Nuclear Extracts. For EMSA reactions, 1−5 μg of nuclear extract was incubated with 0.1 pmol of radiolabeled oligonucleotide in a 15 μL volume under standard reaction conditions (20 mM Hepes, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM DTT, 20 mM EDTA, 50 μg/mL poly(dIC), 10% glycerol, 0.1 mg/mL BSA). For competition experiments, a 35-fold

Table 2: Variable Region Sequences (a) from Rounds 10, 12, and 14 of the RelA "Double Sieve" Selection and (b) ClustalW Multiple Sequence Alignment[a] Results

a)

Round 10		
Clone number	**Sequence**	**Number of Clones[b]**
10-1	5′-CGCGCGT$_s$AT$_s$AT.GG$_s$ACGT.GC$_s$AC$_s$A-3′ 3′-GCGCGCA$_s$TA$_s$TA$_s$CC.TGCA$_s$CG.TG.T-5′	7/9
10-7	5′-CGGGCGGGCA$_s$CA$_s$GGT.GT.A$_s$CA$_s$GC-3′ 3′-GCCCGCCCGT.GT.CCA$_s$CA$_s$T.GT.CG-5′	1/9
10-8	5′-CGCGGG$_s$AT.GG$_s$A$_s$ACT.GT.GGGCGCC-3′ 3′-GCGCCC.TA$_s$CC.T.TGA$_s$CA$_s$CCCGCGG-5′	1/9
Round 12		
12-1 (10-1)	5′-CGCGCGT$_s$AT$_s$AT.GG$_s$ACGT.GC$_s$AC$_s$A-3′ 3′-GCGCGCA$_s$TA$_s$TA$_s$CC.TGCA$_s$CG.TG.T-5′	16/18
12-6	5′-C$_s$ACGCGT$_s$AT$_s$AT.GG$_s$ACGT.GC$_s$AC$_s$A-3′ 3′-G.TGCGCA$_s$TA$_s$TA$_s$CC.TGCA$_s$CG.TG.T-5′	1/18
12-17	5′-CGT.GC$_s$AC$_s$AC$_s$AGT.GG$_s$ACT.GC$_s$AC$_s$A-3′ 3′-GCA$_s$CG.TG.TG.TCA$_s$CC.TGA$_s$CG.TG.T-5′	1/18
Round 14		
14-1 (10-1)	5′-CGCGCGT$_s$AT$_s$AT.GG$_s$ACGT.GC$_s$AC$_s$A-3′ 3′-GCGCGCA$_s$TA$_s$TA$_s$CC.TGCA$_s$CG.TG.T-5′	16/17
14-10	5′-CGCGT.GT$_s$AC$_s$ACGGT.T.GG$_s$ACGGC-3′ 3′-GCGCA$_s$CA$_s$TG.TGCCA$_s$A$_s$CC.TGCCG-5′	1/17

b)

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14-1        CGCGCGTATA-TGGAC-GTGCACA--
10-1        CGCGCGTATA.TGGAC.GTGCACA..
12-1        CGCGCGTATA.TGGAC.GTGCACA..
12-6        CaCGCGTATA.TGGAC.GTGCACA..
12-17       CG.GCa.A.A.TGGAC..TGCACA..
10-7        CG.GCG.g.A....A..GTG.ACA..
14-10       CGCG.GTA.A..G.....TG.ACg..
10-8        CGCG.G.ATg..GaAC.GTG.gCg..
consensus   CgcGcgtata-tggac-gTGcaCa--
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[a] The sequence similarities are shown from the alignments using Boxshade 3.21. [b] Number of clones with identical sequences/number of clones analyzed.

excess of unlabeled oligonucleotide was also added. After 15 min, the reaction was loaded onto a standard 6% nondenaturing polyacrylamide gel in 0.25× TBE. Following electrophoresis, the gel was dried and quantified using a Packard InstantImager. Images for figures were derived from exposure to XAR-5 film. For supershift experiments, nuclear extracts were incubated with antibody to p50 (Santa Cruz Biotechnology) or RelA (Abcam) for 30 min in a 15 μL volume under standard reaction conditions prior to the addition of the radiolabeled oligonucleotide. The following oligonucleotides were used in EMSA reactions: standard NF-κB, AGTTGAGGGGACTTTCCCAGGC; mutant NF-κB, AGTTGAGGCGACTTTCCCAGG.

RESULTS

Combinatorial Selection of Thioaptamers. The variable region sequences from the p50/RelA and p50-edited RelA-selected "double sieve" combinatorial selections are shown in Tables 1 and 2, respectively. Since only dATP(αS) was used in the enzymatic reactions, the only thioate linkages in these sequences are chiral and 5′ to the adenosine bases. In the p50/RelA selection and the RelA double sieve selection, the predominant sequence had emerged by round 13 and 10, respectively. Additional rounds of amplification produced different minor sequences, but the predominant sequence remained unaltered.

The 22 base pair random region is potentially large enough to bind to two dimers of NF-κB. NF-κB proteins bind to specific sites resembling the 10 base pair consensus sequence GGGRNNT(Y)CC that are found in promoters and enhancers of a variety of genes such as those involved in the immune response, viruses, growth control proteins, and NF-κB proteins (*32*−*36*). NF-κB-like binding motifs were observed in the consensus sequences from the NF-κB combinatorial

Table 3: Comparison of Sequences from the Various NF-κB Dimer Selections

Selection	Sequence[a]
RelA/RelA (SB65)	5′-C**GGGG**T.GT.T.GT.CCT.GT.GCT.CT.CC-3′ 3′-GCCCCA$_s$CA$_s$A$_s$CA$_s$GGA$_s$CA$_s$CGA$_s$GA$_s$GG-5′
p50/p50 (SB50)	5′-**GGGG**T.T.CC$_s$ACCT.T.C$_s$ACT.GGGCG-3′ 3′-CCCCA$_s$A$_s$GG.TGGA$_s$A$_s$G.TGA$_s$CCCGC-5′
P50/RelA (SBHet)	5′-T.**GGGG**CT.GT$_s$AC$_s$AGG**GT**.**GC**$_s$**AC**$_s$**A**C-3′ 3′-A$_s$CCCCGA$_s$CA$_s$TG.TCCCA$_s$CG.TG.TG-5′
RelA/RelA-edited, or "double sieve"	5′-CGC**GGG**$_s$AT.GG$_s$A$_s$ACT.GT.GGGCGCC-3′ 3′-GCGCCC.TA$_s$CC.T.TGA$_s$CA$_s$CCCGCGG-5′
NF-κB consensus sequence	**GGG**RNNYYCC

[a] Predominant sequence or sequence with best binding ability from each selection.

selections, though they did not match the NF-κB consensus exactly. Although NF-κB-like binding motifs were observed from the various selections, the sequences all differed slightly (Table 3). The variation in the sequences may reflect the ability of the thioaptamers to selectively bind to the different NF-κB dimers. From previous studies, we know that the p50/p50 and RelA/RelA clones specifically bind to their respective recombinant proteins (*13*). Several clones from the later rounds of the p50/RelA and the "double sieve" edited RelA/RelA selection were screened for the ability to bind recombinant NF-κB proteins.

Qualitative Analysis of Enzymatically Synthesized Chiral Thioaptamer Binding to NF-κB Dimers. Qualitative analysis of thioaptamer binding was performed using a chemiluminescent EMSA. Several clones from rounds 13 and 15 of the p50/RelA selection were tested for the ability to bind to recombinant heterodimer proteins (Figure 1a). The DNA and protein concentrations used were 2 and 120 nM, respectively. Three of the clones (15-2, 13-1, and 13-4) analyzed bound the NF-κB proteins, including the heterodimer, with high affinity. Various concentrations (0−80 nM) of the heterodimer protein were titrated with 2 nM of thioaptamer 13-1 DNA, and 55% was bound with 40 nM of protein (Figure 1b). Although the DNA−protein complex shifted up in the gel, indicating DNA binding, we were not able to determine from these experiments whether the DNA was binding to p50/p50, RelA/RelA, or the p50/RelA heterodimer because of the presence of multiple bands. The sulfur modification enhances the ability of aptamer 13-1 to bind to NF-κB. When aptamer 13-1 was PCR synthesized with a nucleotide mix containing dATP rather than dATP(αS), there was little binding of the normal phosphoryl backbone aptamer (lanes 6−8) in comparison to the monothiophosphate-modified aptamer (lanes 2−4; Figure 2).

Several clones from the p50-edited RelA-selected combinatorial selection were also qualitatively analyzed for the ability to bind to RelA/RelA (data not shown). Thioaptamer 10-8 was amplified and incubated with decreasing concentrations of RelA/RelA and p50/p50 (Figure 3). This thioaptamer bound RelA/RelA more tightly than p50/p50, indicating that the affinity for the RelA/RelA homodimer was enhanced using this editing selection strategy (Figure 3).

For further evaluation of selected clone sequences, the variable regions from the thioaptamer sequences were enzymatically synthesized using the protocol outlined in Figure 4 and the hairpin oligonucleotide sequences in Table 4. This allowed the purification of the chiral 22 base long aptamer without the presence of the flanking sequences used

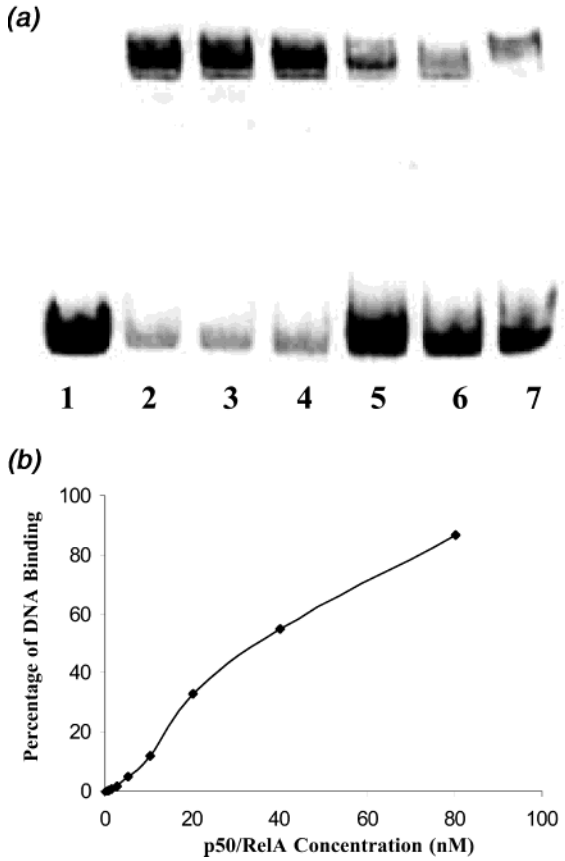

FIGURE 1: Panel a shows that several p50/RelA heterodimer-selected sequences bind to NF-κB proteins. Thioaptamers from round 13 and 15 of the p50/RelA selection were enzymatically synthesized with a 5′ biotin label by amplification from plasmids using PCR. The thioaptamers (2 nM) were incubated with 120 nM of p50/RelA. Lane 1 is DNA without protein from thioaptamer 15-2, the predominant sequence. Lane 2 is thioaptamer 15-2 with p50/RelA. Lanes 3−7 are thioaptamers 13-1, 13-4, 15-5, 15-11, and 15-12 with p50/RelA. Panel b shows titration of p50/RelA with thioaptamer 13-1. Thioaptamer 13-1 (predominant sequence) was enzymatically synthesized with a 5′ biotin label by amplification from plasmids using PCR. The thioaptamer (2 nM) was incubated with 0−80 nM of p50/RelA.

in the previous PCR-amplified 66-mer thioaptamer duplexes. The aptamers enzymatically synthesized in this manner were the sequences from clone 13-1 (SBhet-e (enzymatic)), as well as the p50-selected sequence (SB50-e) and the RelA-selected sequence (SB65-e). The same sequences were also chemically synthesized with phosphorothioate linkages in the corresponding positions for 22-mer duplex thioaptamers SBhet-c (chemical), and SB50-c. This allowed a comparison

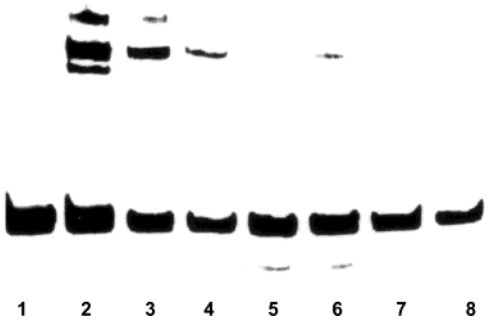

FIGURE 2: Binding of aptamer 13-1 to p50/RelA is influenced by the presence of monothiophosphate modification. Lanes 1 and 5 represent 2 nM aptamer without protein. Lanes 2−4 and lanes 6−8 represent aptamer 13-1 with and without the monothiophosphate modification, respectively. Decreasing concentrations of thioaptamer 13-1 (2, 1, and 0.5 nM) were incubated with 40 nM of p50/RelA.

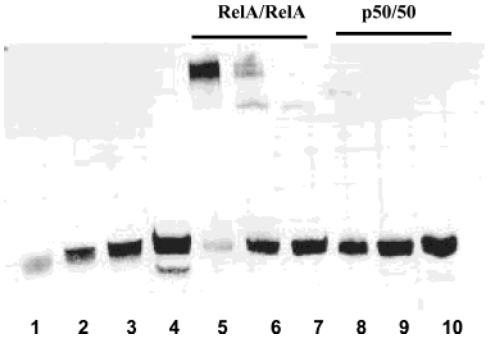

FIGURE 3: A RelA "double sieve" selected sequence binds more selectively to RelA/RelA than to p50/50. Thioaptamer 10-8 was amplified from a clone from the 10th round of selection. The thioaptamer was incubated with decreasing amounts of RelA/65 and p50/50. Lanes 5, 6, and 7 represent thioaptamer 10-8 with 800, 400, and 200 nM RelA/RelA, respectively. Lanes 8, 9, and 10 represent thioaptamer 10-8 with 800, 400, and 200 nM p50/50, respectively. Lane 1 is an initial library thioaptamer negative for RelA/RelA binding. Lane 2 is an initial library thioaptamer negative for p50/50 binding. Lane 3 is the initial library thioaptamer without protein. Lane 4 is the 10-8 thioaptamer clone without protein. Similar results were observed with the clone 10-1 thioaptamer.

of the chiral and diastereomerical mixture of 22-mer thioaptamers. In addition, aptamers were synthesized without any phosphorothioate linkages (SBhet-p (phosphate) and SB50-p.)

When these 22-mer aptamers were mixed with recombinant p50, RelA, or the heterodimer p50/RelA, all showed protein bound to the oligonucleotide and a band shift (data not shown). Differences in the affinity of the oligonucleotides became more apparent when we used the aptamers at 50 times excess to compete with binding of a standard NF-κB oligonucleotide to the recombinant proteins (lanes 2−9; Figure 5). Both the chemically and enzymatically synthesized p50-selected 22-mer thioaptamers (SB50-c and SB50-e) successfully bound and competed for p50 homodimers but were much less effective in competition using RelA homodimers or p50/RelA heterodimers. The phosphorothioate substitutions in SB50-e and SB50-c were required for this binding, since the unmodified aptamer (SB50-p) was inactive. In both the chemically synthesized and the enzymatically synthesized versions of the oligonucleotide, only the linkages adjacent to the adenosine bases are modified. Because the chemical synthesis is nonstereospecific, SB50-c is a diastereomeric mixture of the two R_p and S_p chiral forms (at phosphorus) of each modified monothioate linkage (2^N diastereomers for N monothioates), while the SB50-e contains only the single enantiomerically pure stereoisomer supported by the enzymatic reaction.

The phosphorothioate modification was also required for the binding of the SBhet aptamer to RelA homodimers, though in this case the chemically synthesized thioaptamer with mixed diastereomers was also significantly less effective. Though the SBhet oligo had been selected by binding to the p50/RelA heterodimer, it is still much less effective than the "standard" NF-κB oligonucleotide at binding the heterodimer. The enzymatically synthesized SB65-e was somewhat effective at competing for binding with each of the dimers with the strongest competition shown with the RelA dimer for which it was selected.

Binding of Thioaptamers in Nuclear Extracts. To help determine their potential for binding and inhibiting NF-κB dimers within a cell, additional binding studies with the NF-κB-selected thioaptamers were performed with nuclear extracts. Oligonucleotides that contain phosphorothioate linkages exclusively often fail to bind appropriately in cell extracts. The "stickiness" of phosphorothioate oligonucleotides often causes them to bind to numerous nuclear proteins, obscuring the binding to the specific targeted NF-κB complexes (*19*). The selected thioaptamers, however, have thioate linkages only 5′ to the adenosine bases. Therefore only 7 (SB50) or 8 (SBhet and SB65) of the 42 linkages are modified (see Table 3).

Two different nuclear extracts were used to evaluate the binding: one from the murine preB cell line 70Z/3 stimulated with LPS and one from the murine macrophage line P388D1.

Table 4: Hairpin Sequences for the Enzymatic Synthesis of the Random Regions of the Phosphorothioate Aptamers

Thioaptamer	Sequence[b]
RelA/RelA[a]	5′-(B)$_3$-CGGGGTGTTGTCCTGTGCTCTCCAGATCCGAAAGGATCU-3′
p50/p50	5′-(B)$_3$-CGCCCAGTGAAGGTGGAACCCCAGATCCGAAAGGATCU-3′
	5′-(B)$_3$-GGGGTTCCACCTTCACTGGGCGAGATCCGAAAGGATCU-3′
P50/RelA	5′-(B)$_3$-TGGGGCTGTACAGGGTGCACACAGATCCGAAAGGATCU-3′
	5′-(B)$_3$-GTGTGCACCCTGTACAGCCCCAAGATCCGAAAGGATCU-3′
RelA/RelA-edited	5′-(B)$_3$-GGCGCCCACAGTTCCATCCCGCGAGATCCGAAAGGATCU-3′
	5′-(B)$_3$-CGCGGGATGGAACTGTGGGCGCCAGATCCGAAAGGATCU-3′

[a] Only one hairpin was needed since one strand of the thioaptamer did not contain adenines. The strand without adenines was chemically synthesized.
[b] (B)$_3$ indicates three consecutive biotins.

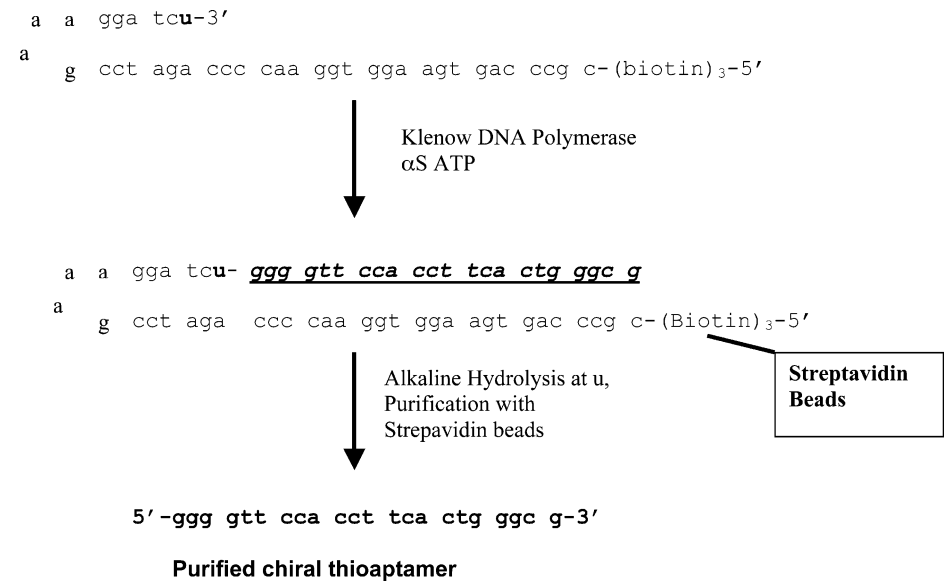

FIGURE 4: Enzymatic synthesis of the random region of phosphorothioate aptamers.

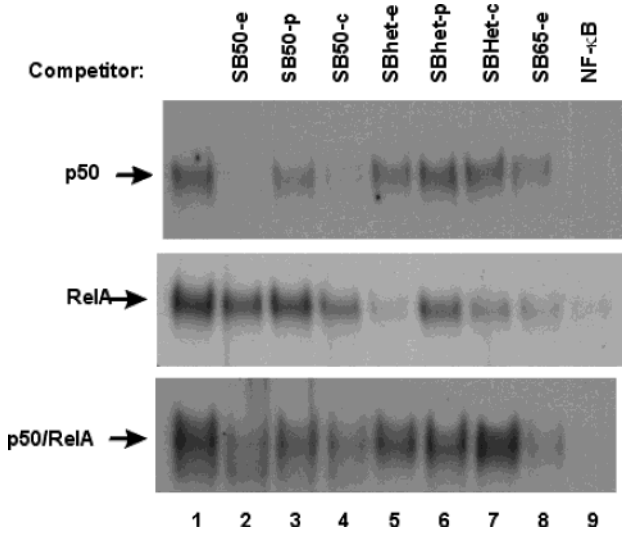

FIGURE 5: Phosphorothioate modification affects the aptamer binding and competition to recombinant NF-κB proteins. EMSA gels with aptamers used in 50× excess to compete with the binding of the radiolabed NF-κB oligonucleotide to purified recombinant NF-κB proteins. Aptamers were either enzymatically (lanes 2, 5, and 7) or chemically synthesized (lanes 4 and 7) with phosphorothioate linkages or chemically synthesized unmodified (lanes 3 and 6).

Both cells should have increased amounts of the NF-κB proteins resulting from LPS stimulation, but they vary in the NF-κB dimer composition and the non-NF-κB proteins. NF-κB-specific complexes were identified as those competed by an NF-κB-specific oligonucleotide. This is a standard NF-κB oligonucleotide that binds all the known NF-κB dimers. The mutant NF-κB oligonucleotide is mutated within the NF-κB site and fails to compete for NF-κB dimers. The results for the 70Z/3 cells (data not shown) and the P388D1 cells (Figures 6) were similar. Analysis was conducted using radiolabeled oligonucleotides in standard EMSA reactions. In both cell lysates, SB50-e bound in two NF-κB complexes, SBhet-e bound predominantly in one complex, and SB65-e did not specifically bind detectably to any NF-κB complexes. Our preliminary designation of the heterodimers was based on previous studies and confirmed using NF-κB specific antibodies in supershifts (Figure 6B). SB50-e bound to both the p50/RelA and the p50/p50 dimers, while SBhet-e bound preferentially to the p50/RelA heterodimer. These cells have little detectable RelA/RelA homodimer to which the thioaptamers could bind. SB65-e, as well as the thioaptamer from the RelA/RelA double sieve edited selection (not shown), bound in several bands that failed to compete with the standard NF-κB oligonucleotide and are, therefore, not NF-κB proteins but other unidentified cellular proteins to which they have a higher affinity. It is possible that a fraction of the SB65-e binding is to NF-κB proteins since there is a slight competition by the NF-κB oligonucleotide. However, this is obscured by the remaining binding to the non-NF-κB bands. It is not possible to easily identify the SB65-e binding proteins within the cell.

DISCUSSION

Recently, aptamers derived from random oligonucleotide libraries have been shown to bind to other molecules with high affinity and specificity (*7*−*13*, *17*−*21*, *23*−*27*). Antisense, aptamer, decoy, and siRNA oligonucleotides have shown increasing promise as diagnostic and therapeutic agents. Aptamers are attractive because they can provide the level of selectivity and affinities often comparable to monoclonal antibodies. Oligonucleotides with monothiophosphate or dithiophosphate internucleotide linkages offer many advantages, such as enhanced nuclease resistance, and appear to bind to proteins with higher affinities than do those with normal phosphate ester backbones (*19*, *20*). Aptamers have previously been developed targeting NF-κB, and they influence the expression of NF-κB regulated genes (*23*−*27*; for review, see ref *27*). Some of these decoy aptamers appear to have therapeutic potential in models of tumor metastasis, cytokine-mediated apoptosis, and immune-mediated neural and cardiac damage. However, global inhibition of NF-κB protein activity is likely to be detrimental. NF-κB proteins are one of the central regulators of the inflammatory response and are therefore very important for host survival, although excessive or inappropriate activation can lead to host injury. Decoy aptamer technology should permit the therapeutic manipulation of the host immune response by modulating

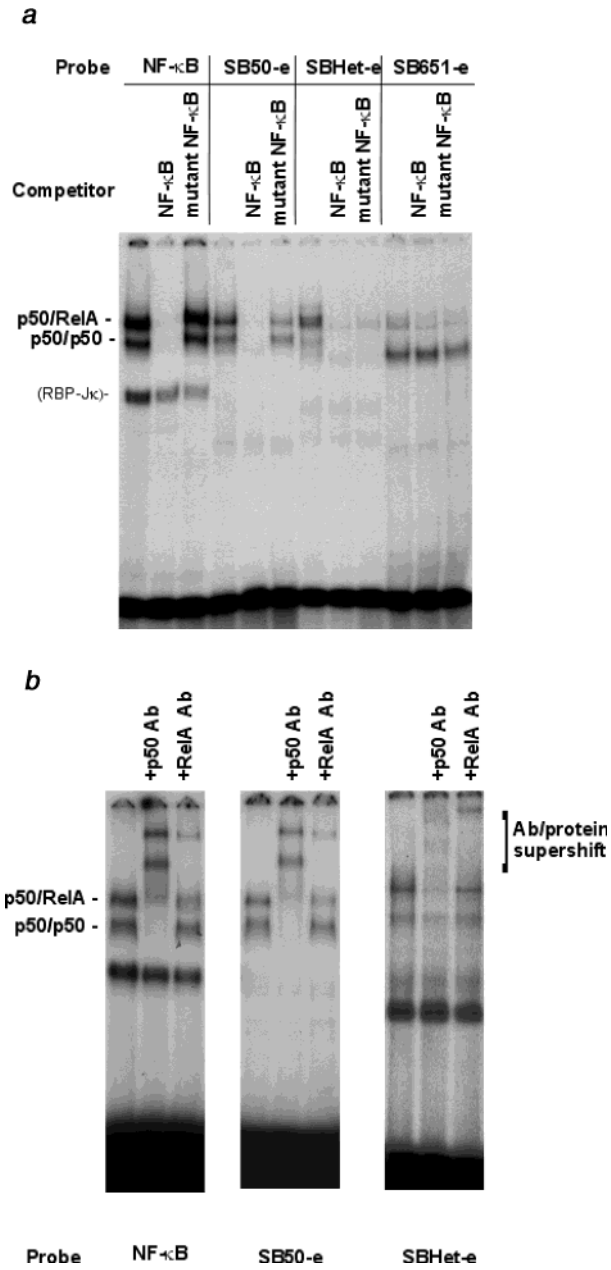

FIGURE 6: Selected thio aptamer oligonucleotides bind to NF-κB proteins in nuclear extracts. EMSA of nuclear extracts from P388D1 cells with radiolabeled thioaptamers and NF-κB standard oligonucleotide as probes. In panel a, NF-κB or mutant NF-κB oligonucleotides were added in 50× excess to indicated lanes. Asterisk marks nonspecific (ns) bands. In panel b, antibody to NF-κB p50 or RelA was added in the indicated lanes.

several effector pathways without singling out a particular pathway for elimination. The development of NF-κB dimer-specific decoy aptamers should permit this modulation without a reduction in the overall immune response capability. Therefore, we sought to develop double-stranded DNA thioaptamers or "decoys" that compete for the binding of specific NF-κB dimers to their authentic binding elements in cellular DNA.

Previously, we observed that the sulfur substitution in the aptamers enhances the binding relative to the same sequence with a normal phosphate backbone (*13*). We have suggested that one explanation for the higher affinity of the thiosubstituted oligodeoxynucleotides is the poor cation coordination of the polyanionic backbone (*13*); sulfur, being a soft anion, does not coordinate as well to hard cations, such as Na^+, unlike the hard phosphate oxyanion. The thiosubstituted phosphate esters then act as "bare" anions, and since energy is not required to strip the cations from the backbone, these agents can in principle bind even more tightly to proteins. However, aptamers with complete thiophosphate backbone substitutions lose much of their specificity (*19*) because of this enhanced "stickiness". The advantage of using combinatorial selection techniques is that the optimal backbone substitutions (phosphate, monothiophosphates, or dithiophosphates) and sequences can be simultaneously identified throughout the selection process of either enzymatically derived in vitro combinatorial libraries (*20*) or chemically synthesized bead-based thioaptamer libraries (*17*, *18*).

We have selected thioaptamers to various NF-κB homodimers (p50/p50 and RelA/RelA) and to the p50/RelA heterodimer (*13*). The thioaptamers have unique sequences (Table 3) and bind specifically to the recombinant proteins against which they were targeted in qualitative EMSA experiments (Figures 1 and 3). The sequences from all of the NF-κB selections contain the four-base polypurine tract common to normal phosphoryl NF-κB binding sites. The thioaptamer from the RelA/RelA "double sieve" or edited selection did not appear to bind as tightly to the target relative to the other thioaptamers. The weaker binding may be attributed to the depletion of p50-binding sequences during the editing step.

Several thioaptamer sequences that bound recombinant proteins were subsequently analyzed to determine whether they could specifically bind the NF-κB dimers within nuclear extracts in the presence of many other cellular proteins. Both the p50/p50-selected thioaptamer and the p50/RelA-selected thioaptamer were able to bind to their targets within the cell, although the p50/p50-selected thioaptamer also bound to the heterodimer. It is interesting to note that the limited number of thiophosphate backbone substitutions in the SB50 oligonucleotide increased its NF-κB binding ability without leading to the substantial nonspecific binding normally seen when all linkages are replaced. The increase in nonspecific binding normally prevents fully substituted oligonucleotides from binding properly within a cell lysate, even if they bind the purified target proteins. In this case, the partially modified oligonucleotides still retained specificity, as seen by their ability to bind cleanly in an EMSA gel using nuclear lysates.

The selection for specific NF-κB dimers was only partially successful. Though the SB50 oligoncleotide showed specificity using recombinant proteins, it bound to both the p50/p50 and p50/RelA dimers in nuclear extracts. This may be due to the use of truncated recombinant proteins or perhaps to the presence of phosphorylation or other modifications to the proteins within the nuclear lysate. The SBhet oligonucleotide showed binding to recombinant RelA/RelA, though p50/RelA was the predominant dimer bound using extracts. *As far as we are aware, this is the first report of a sequence that preferentially binds the heterodimer in a nuclear lysate.* Unfortunately, we were unable to determine its ability to bind naturally occurring RelA/RelA homodimers, since this relatively rare dimer is not present in large amounts in our cell lines. The "double-sieve" edited selection allowed us to select sequences that preferentially bound to p50/RelA without binding to p50/p50, but the affinity of binding was too low and not specific enough to be effective in nuclear

extracts. The results indicate that combinatorial selection may be used to specifically select NF-κB dimers. Since there are five different NF-κB monomers, there are potentially 15 different homo- and heterodimers. The selection of specific thioaptamers that bind to specific NF-κB dimers within cell extracts is promising.

We have demonstrated a new method to enzymatically synthesize larger quantities of the chiral thioaptamers utilizing a Klenow extension of a hairpin template. The thioaptamers can now be assessed for their effectiveness in cell culture and animal studies. Chemically synthesized thioaptamers are simple to obtain in large quantities for these studies. However, the disadvantage is that the chemically synthesized thioaptamers consist of a mixture of diastereomers. Since the monothioate modification introduces a new chiral center, both R_p and S_p configured thioaptamers will be present. For example, if a thioaptamer contains eight monothiophosphates, 2^8 diastereomers will exist in the mixture. Thioaptamers with incorrect monothiophosphate configurations may bind with lower affinity to the target and decrease the overall binding affinity. Previously, we observed that PCR-synthesized thioaptamers, which consist of a single stereoisomer, bound with higher affinity to NF-κB proteins than the chemically synthesized diasterereomeric mixture of thioaptamers (*13*). Therefore, the Klenow hairpin template enzymatic synthesis of stereochemically pure thioaptamers is critical in future biological and animal studies.

To conclude, we have selected thioaptamers to various NF-κB dimers, including the heterodimer and the RelA homodimer using a "double sieve" editing selection strategy in this study. The thioaptamers from the various NF-κB selections all had unique sequences and bound to the recombinant NF-κB proteins against which they were selected. The thioaptamers also appear to maintain their selectivity and specificity among other cellular proteins, because they have the ability to selectively bind NF-κB proteins within nuclear extracts.

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