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TITLE: Development of Triplex-Forming Oligonucleotides to
Inhibit Expression of the *c-myc* Oncogene in Breast Cancer
Cells

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Triple helix-forming oligonucleotides (TFOs) directed to regulatory sequences in gene promoters can selectively block transcription. We are investigating TFO-mediated reduction of <i>c-myc</i> oncogene expression as a means of decreasing breast tumor growth. We designed a novel parallel/antiparallel TFO (Myc-GTC) that had high binding affinity <i>in vitro</i> , but required modifications for increased stability in cells. To further optimize TFO activity we investigated effects of conjugation with the anthracycline antibiotic daunomycin (Dnm), which intercalates into double-stranded DNA. This modification increased stability of triple helix formed by parallel and antiparallel short TFOs targeted to each segment of the target sequence. The anti-parallel TFO inhibited expression in breast cancer cells of a luciferase gene under the control of the <i>c-myc</i> promoter. Gel shift assays showed that triple helix formed by this TFO was specifically bound by a protein or proteins in MCF-7, MDA-MB-231 and Hela nuclear extracts. These may be DNA repair-associated proteins, but do not appear to include XPA, a required protein in the nucleotide excision repair mechanism. Efforts to identify the triple helix-binding protein(s) are in progress.			
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Description of training and research accomplishments.

In 2002-2003, research on the proposal has taken some unexpected directions. This has allowed the trainee to explore new fields related to breast cancer, and to extend her knowledge of the potential effects of triple-helix forming oligonucleotides in breast cancer cells.

Introduction

Triple helix formation offers a direct means of selectively manipulating gene expression in cells. TFOs bind with high affinity and specificity to the purine strand in the major groove of homopurine-homopyrimidine sequences in double-stranded DNA. TFOs have proved effective in various gene-targeting strategies in living cells and, recently, in animals. TFOs targeted to regulatory sequences in target genes have been able to selectively reduce gene transcription. This approach has potential application in anti-cancer treatment since it can be used to reduce levels of proteins essential to proliferation of cancer cells.

C-Myc plays a central role in promoting cell growth and proliferation. The *c-myc* gene is expressed at abnormally high levels in many cancers, and its overexpression has been strongly associated with initiation and progression of breast cancer. Reduced levels of c-Myc are associated with decreased breast cancer cell growth. The purpose of our investigation is to assess triplex DNA-mediated reduction of *c-myc* expression as a potential means of decreasing growth and spread of breast tumors.

The aims of our investigation were to overcome certain limitations that had previously restricted the potential therapeutic value of TFOs targeted to the major promoter of *c-myc*. Our progress towards this goal, and concurrent development of some new lines of investigation, is outlined below.

Training and research accomplishments

As described in the attached journal article, (McGuffie *et al*, 2002) we designed a novel TFO that had higher binding affinity than any previously tested molecule. However, we found that this TFO had only modest inhibitory effects on transcription of a *c-myc* promoter-driven luciferase gene in breast cancer cells, although it efficiently blocked transcription factor binding to the *myc* promoter in vitro.

We hypothesized that binding affinity of the TFO had to be even higher to compete with proteins for binding to the promoter in cells. We investigated whether conjugation of TFOs with a DNA intercalating agent would help stabilize triple helix. Binding studies showed that TFOs conjugated to the anthracycline antibiotic daunomycin (Dnm) formed more stable triple helix than their unconjugated counterparts. In shift assays with nuclear extracts Dnm-TFOs inhibited binding of proteins to the target duplex more efficiently than unconjugated TFOs. Furthermore, Dnm-TFOs were efficiently internalized by MCF-7 cells, as determined by fluorescence microscopy, and at nanomolar concentrations, inhibited expression in breast cancer cells of a *c-myc* promoter-driven luciferase gene.

In gel shift assays using MCF-7 nuclear extracts and a probe containing the *c-myc* promoter sequence, we observed a novel band representing a protein or proteins that

bound to triple helix formed by a Dnm-conjugated TFO targeted to part of the promoter sequence. Exhaustive studies were done to confirm the specificity of this binding.

We wondered whether DNA repair proteins might be involved. This was based on reports by other investigators that DNA repair proteins, including XPA and RPA, bound to triple helices formed by psoralen-conjugated TFOs. We tested whether XPA and/or RPA might be involved in our system. These experiments are continuing, but so far the results suggest that XPA and RPA are unlikely to be involved.

This work has led to collaboration with experts in the field of DNA repair, and has been a valuable training experience for the recipient of this award. We are continuing with efforts to identify the proteins contained in the novel band. This will likely be a lengthy process, but may provide new insights into activities and potential uses of TFOs targeted to the *c-myc* gene. A manuscript describing progress of this work is in final preparation.

Techniques and experimental approaches developed by the trainee and mentor during the course of this award have proved valuable in other investigations in the laboratory. The second attached article (Carbone *et al*, 2003), describes design and testing of a highly effective TFO targeted to the *Ets2* gene in prostate cancer cells. This work benefited from experience gained in the *c-myc* project, and allowed the trainee to attain knowledge of genes and processes important in development of prostate cancer.

Key accomplishments.

- A novel *c-myc*-targeted TFO has been designed and tested.
- Uptake and stability of daunomycin-conjugated TFOs in breast cancer cells has been demonstrated.
- Novel triple-helix binding protein(s) and triplex-induced DNA repair mechanisms are under investigation.

Reportable outcomes.

Papers.

McGuffie, EM. and Catapano, CV. Design of a novel triple helix-forming oligodeoxyribonucleotide directed to the major promoter of the *c-myc* gene. 2002, *Nucleic Acids Research*, 30: 2701-2709

Carbone, GM, McGuffie, EM, Collier, A., Catapano, CV. Selective inhibition of the *Ets2* gene in prostate cancer cells by a triplex-forming oligonucleotide. *Nucleic Acids Research*, 2003, 31. 833-843

Catapano, C.V., McGuffie, E.M., Carbone, G.M.R., Flanagan, C.E., Dembech, C., Arcamone, F., and Capobianco, M.L.. Synthesis of daunomycin-conjugated triplex-forming oligonucleotides that act as selective repressors of *c-myc* gene expression. 2002. Manuscript in preparation.

Abstracts

Novel approaches to design of triple helix-forming oligonucleotides to inhibit expression of *c-myc* in breast cancer cells. McGuffie, E.M. Capobianco M.L and Catapano, C.V.C. 2002, *Era of Hope Abstracts*

Appendix

Design of a novel triple helix-forming oligodeoxyribonucleotide directed to the major promoter of the *c-myc* gene

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ABSTRACT

Altered expression of *c-myc* is implicated in pathogenesis and progression of many human cancers. Triple helix-forming oligonucleotides (TFOs) directed to a polypurine/polypyrimidine sequence in a critical regulatory region near the *c-myc* P2 promoter have been shown to inhibit *c-myc* transcription *in vitro* and in cells. However, these guanine-rich TFOs had moderate binding affinity and required high concentrations for activity. The 23 bp *myc* P2 sequence is split equally into AT- and GC-rich tracts. Gel mobility analysis of a series of short TFOs directed in parallel and anti-parallel orientation to the purine strand of each tract showed that only parallel CT and anti-parallel GT TFOs formed stable triplex on the AT- and GC-rich tracts, respectively. A novel full-length GTC TFO was designed to bind simultaneously in parallel and anti-parallel orientation to the polypurine strand. Gel-shift and footprinting assays showed that the new TFO formed a triple helix in physiological conditions with significantly higher affinity than an anti-parallel TFO. Protein-binding assays showed that 1 μ M GTC TFO inhibited binding of nuclear transcription factors to the P2 promoter sequence. The novel TFO can be developed into a potent antigene agent, and its design strategy applied to similar genomic sequences, thus expanding the TFO repertoire.

INTRODUCTION

Triple helix formation offers a direct means of selectively manipulating gene expression in cells. Synthetic triple helix-forming oligonucleotides (TFOs) bind with high affinity and specificity to the purine strand in the major groove of homopurine–homopyrimidine sequences in double-stranded DNA. TFOs have proved effective in various gene-targeting strategies in living cells and, recently, in animals (reviewed in 1). Binding characteristics of TFOs depend on their nucleotide composition. Thus, oligonucleotides composed of pyrimidine bases (C and T) bind through Hoogsteen hydrogen bonds and

are oriented parallel to the purine-rich strand of the target duplex, forming C⁺·G–C and T·A–T triplets. TFOs composed of purine bases (G and A), or of mixed purine/pyrimidine (G and T) bind in anti-parallel orientation through reverse Hoogsteen bonds, forming G·G–C and A·A–T or T·A–T triplets (2). In certain circumstances GT TFOs can bind in parallel orientation (3). Protonation of cytosine is required to form two Hoogsteen bonds in C⁺·G–C triplets, so that binding of CT TFOs is favored by low pH. Triplex formation by GA and GT TFOs is pH independent. Binding in each of these motifs can occur in physiological conditions, although cytosines must be modified to overcome pH dependence of CT TFOs.

Purine-rich tracts are frequently found in gene promoter regions and TFOs directed to these regulatory sites have been shown to selectively reduce transcription of the targeted genes, likely by blocking binding of transcriptional activators and/or formation of initiation complexes (reviewed in 4). Triplex-mediated modulation of transcription has potential application in therapy since it can be used, for example, to reduce levels of proteins thought to be important in disease processes. TFOs can also be useful molecular tools for studying gene expression and function. We are investigating the triplex approach as a means of down-regulating expression of the *c-myc* oncogene in cancer cells. *c-myc* is an attractive target for antigene agents in cancer cells because its expression drives cell proliferation. The importance of *c-myc* in cancer cell growth is emphasized by the findings that it is frequently amplified or involved in chromosomal rearrangements and that its expression is deregulated and augmented through various mechanisms in many cancers (5,6). Functions of the c-Myc protein, in tandem with its obligatory binding partner Max, include transcriptional activation and repression. Both activities appear to promote cell proliferation (7,8). Recent studies demonstrated that reduced expression of inducible *c-myc* was sufficient to cause regression of hematologic tumors in mice, suggesting that that this approach may have therapeutic potential (9).

Several sequences suitable for triplex formation are present in the *c-myc* gene. Of particular interest is a highly conserved polypurine/polypyrimidine tract in the *c-myc* P2 promoter region. P2 is the major *c-myc* promoter and gives rise to 75–90% of transcripts in almost all cells, with P1 contributing most of the remainder. The polypurine sequence lies from –39 to –61 relative to the P2 start site, and includes or overlaps binding

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sites for a number of transcription factors including Sp1 and Sp3 (10), ZF87/MAZ (11), ets (12), E2F (13,14) and Stat 3 (15). The site is required for transcription from P2 in the murine gene (11,16). Mutating the sequence in an episomal vector carrying the human gene severely disrupted transcription from both the P1 and P2 sites (17). A survey of TFOs directed to four different sequences in *c-myc*, including one in the P1 promoter region, showed that a P2-targeted TFO had highest antigene activity (18). Other studies showed that TFOs directed to this sequence had transcriptional inhibitory activity *in vitro* and in cells (19–21). However, all P2-targeted TFOs tested so far required high micromolar concentrations for triplex formation *in vitro*. This implied that high concentrations were required for antigene activity in cells. High oligonucleotide concentrations might be harmful *in vivo*, and cause non-specific effects that could confuse studies of cellular responses to reduced *c-myc* expression.

In the study reported here, we identified target sequence elements contributing to moderate binding affinity of P2-targeted TFOs. Based on our findings we designed a novel TFO incorporating parallel and anti-parallel binding motifs. Gel-shift and footprinting assays showed that the new TFO had significantly higher binding affinity than an anti-parallel TFO directed to the P2 sequence. In assays using nuclear extracts *in vitro*, the TFO at <1 μ M inhibited binding of transcription factors to the targeted region, whereas similar concentrations of the anti-parallel TFO had little effect. The presence of double 5' ends conferred resistance to digestion *in vitro* by nucleases in fetal bovine serum, but did not prevent rapid degradation of the TFO in cells. Intracellular instability was probably responsible for the TFO's modest inhibitory activity in reporter gene assays. Our findings encourage progress towards synthesizing a nuclease-resistant TFO for testing in cells. Also, the strategy used to optimize triplex formation on the *c-myc* target can be applied to similar sequences in the genome, expanding the repertoire of sequences available for oligonucleotide-directed high-affinity triplex formation.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Unmodified oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Gel-purified oligonucleotides with 3'-3' central linkages, double 5' ends and 5-methyl cytosines were purchased from Oligos Etc. (Wilsonville, OR). All oligonucleotides were dissolved in water, and concentrations were determined spectrophotometrically using extinction coefficients for each base as follows: A, 15 400; C, 7300; G, 11 700; T, 8800.

Cell culture

MCF-7 and MDA-MB-231 breast cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and passaged twice weekly.

Electrophoretic mobility shift assays (EMSA) to detect triplex formation

Either of two protocols was used as previously described (18). Briefly, either the pyrimidine-rich strand of the 23 bp target

duplex, or TFOs were 5'-end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. Increasing concentrations of unlabeled TFO or duplex were added to labeled duplex or TFO, respectively, and reactions were incubated overnight at 37°C, except short CT TFOs, which were incubated at 4°C. Binding buffers contained either 90 mM Tris, 90 mM borate (TBM) pH 8; 50 mM 2-[*N*-morpholino] ethane sulfonic acid (Mes) pH 5.6; or 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) pH 7–7.4. All buffers contained 10 mM MgCl₂. Electrophoresis at 10 or 20°C in native gels was done using the same buffer as in binding reactions. Films were scanned and bands quantified by densitometric analysis using Gel Pro software. Apparent dissociation constant values (K_d) were defined as concentration of TFO required to shift 50% of duplex to triplex DNA.

DMS footprinting

A 339 bp fragment containing the *myc* P2 promoter region was prepared and samples processed as described (21), except that TFO-binding reactions were incubated overnight at 37°C in HEPES–MgCl₂, pH 7.2–7.4. Densitometric analysis of scanned X-ray films was done by determining total optical density units in the TFO target region, and correcting for unequal loading using values obtained from an unprotected region of identical size.

EMSA to examine protein binding

MCF-7 and MDA-MB-231 breast cancer nuclear extracts were prepared using the method of Dignam (22), with minor modifications as described (23). Double-stranded 32 P-labeled probes with *myc* P1 and P2 promoter sequences were prepared by annealing complementary, gel-purified single-stranded oligonucleotides then filling in 5' overhangs using Klenow fragment and [32 P]dCTP (3000 Ci/mmol; Amersham Pharmacia, Piscataway, NJ). Labeled probes were further purified on native gels and eluted as described (24). The sequence of the polypurine strand of each probe was: Myc P1, 5'-GCGCTT-ATGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGGA-GGAGACTCAGCC-3'; Myc P2, 5'-GAGGCTTGGCGGG-AAAAGAACGGAGGGAGGGATCGCGCT-3'. Double-stranded Sp1/Sp3 and control probes used as competitors were purchased from Promega (Madison, WI) or Geneka Biotechnology Inc. (Montreal, Quebec). Antibodies used in supershift assays were from Geneka. To detect nuclear protein binding to labeled probes, nuclear extracts (~5 μ g) were pre-incubated with 1 μ g poly(dI–dC) (Sigma) for 10 min on ice, in 10 mM HEPES pH 7.4, 50 mM NaCl, 4% glycerol, 1 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT. Extracts were then added to ~20 fmol probe (final concentration 1 nM) and incubated for 20 min at 10°C. Competitor oligonucleotides were added to extracts immediately before labeled probes. Antibodies were added to extracts pre-incubated with poly(dI–dC) as above, then incubation was extended for a further 20 min at 10°C before probes were added. To pre-form triplex, probes were pre-incubated with TFOs for 2–3 h at 37°C in HEPES pH 7.2–7.4, 10 mM MgCl₂ before nuclear extract was added. Samples were resolved on 4% polyacrylamide gels (45 mM Tris, 45 mM borate, 0.5 mM EDTA) run at 200 V for ~2 h at 10°C. Gels were dried and exposed at –70°C to X-ray films with intensifying screens.

Assay of oligonucleotide degradation in cell culture medium and in breast cancer cells

Oligonucleotides (200 pmol) were 5'-end-labeled and incubated at 37°C in 20 µl DMEM containing 10% fetal bovine serum which had been heat inactivated at 55°C for 30 min. Reactions were stopped at successive time points by adding 20 µl buffer containing 98% formamide and 10% EDTA, then heating at 90°C for 10 min. Samples were electrophoresed on a 15% polyacrylamide-7 M urea gel, and visualized by exposing the dried gel to X-ray film overnight. To test oligonucleotide stability in cells, MDA-MB-231 cells were plated in 24-well plates and grown for 24 h. Oligonucleotide 5'-end-labeled with ³²P was mixed with unlabeled oligo to give a final concentration of 100 nM (~6 × 10⁶ c.p.m./ml). Cells were transfected for 6 h with labeled oligos using DOTAP reagent (Roche). Samples of transfection mix were retained for subsequent analysis. Transfection medium was removed, cells were washed three times with DMEM, and harvested by scraping into formamide/EDTA buffer, and heating at 90°C for 10 min, or grown for a further 24 or 48 h. Similar washes preceded each harvest. Radioactivity in each sample was determined, and equal numbers of counts loaded on a denaturing gel. The amount of intact TFO in each sample was determined by autoradiography and densitometric analysis.

Luciferase assays

MDA-MB-231 cells were transfected with a plasmid containing the luciferase gene driven by the *c-myc* P2 minimal promoter (pMyc262), and with the Renilla luciferase-expressing plasmid pRLTK, as control for transfection efficiency. TFO and control oligonucleotides were co-transfected with plasmids. Luciferase assays were done according to the manufacturer's protocol (Dual Luciferase System, Promega).

RESULTS

Survey of short TFOs directed to different domains of the *c-myc* P2 target sequence

In an attempt to improve binding affinity of a TFO targeted to a critical regulatory sequence near the *c-myc* P2 transcriptional start site, we first identified the optimal binding motif for TFOs directed to two discrete domains present in the sequence. The 23 bp tract has an almost equal number of AT and GC base pairs. Distribution is uneven, however, with A grouped at the 5', and G at the 3' end of the purine strand. Anti-parallel motif TFOs were found previously to bind the P2 sequence with moderate affinity (19–21). We investigated whether the unbalanced composition of the target might be responsible for triplex instability. Using EMSA, we determined binding of a series of short 11mer PO TFOs targeted to either the A-rich 5' or the G-rich 3' segment of the 23 bp target duplex. In these initial studies, we tested GT TFOs in the parallel and anti-parallel motifs; GA TFOs in the anti-parallel motif, CT TFOs and a parallel GT TFO containing one cytosine (Table 1). This approach allowed us to identify the preferred binding motif for each segment of the target. We ³²P-labeled the TFOs and incubated them with increasing concentrations of unlabeled duplex to detect a shift from single-stranded to triple-stranded DNA. The use of labeled TFOs also allowed us to determine

Table 1. Triplex formation by TFOs directed to *c-myc* P2 sequence

Myc P2 target sequence		Orientation ^b	Triplex ^c
2	1		
5' -GGGAAAAGAACGGAGGGAGGGA-3'	Myc R		
3' -CCCTTTTCTTCCTCCCTCCCT-5'	Myc Y		
TFO ^a			
1-GT	5' -TGGGTGGGTGG-3'	Anti-parallel	Yes
1-GT-P	5' -GGTGGGTGGGT-3'	Parallel	No
1-GA	5' -AGGGAGGGAGG-3'	Anti-parallel	Yes
1-CT	5' -CCTCCCTCCCT-3'	Parallel	No ^d
2-GT	5' -TTGTTTTGGG-3'	Anti-parallel	No
2-GT-P	5' -GGTTTTTTGTT-3'	Parallel	No
2-GA	5' -AAGAAAAGGG-3'	Anti-parallel	No
2-GTC	5' -GGTTTTTCTT-3'	Parallel	No ^d
2-CT	5' -CCCTTTTCTT-3'	Parallel	Yes ^d
Myc-CT	5' -CCCTTTTCTTTCCTCCCTCCCT-3'	Parallel	Yes ^d
Myc-GT	5' -TGGGTGGGTGGTTTTGTTTTGGG-3'	Anti-parallel	Yes
Myc-GTC ^e	5' -CCCTTTTCTTTCCTGGGTGGGT-5'	Parallel + Anti-parallel	Yes
Myc-GTC-C ^e	5' -TGGTTTTTCTG-TTGTGGGCC-5'	NC ^f	No

^aShort TFOs 1- and 2-targeted to regions 1 and 2 of the *myc* P2 sequence, respectively.

^bTFO orientation to the purine-rich strand of the target sequence.

^cTriplex helix formation detected by EMSA as described in Materials and Methods.

^dAssayed at pH 5.6.

^eSynthesized with 5-methyl cytosine (C) and central 3'-3' linkage (-).

^fSequence non-complementary to the target sequence.

whether the TFOs formed alternative structures with themselves or other single-stranded DNA. In comparing TFOs targeted to the G-rich 3' sequence, we determined that an 11mer GT TFO (1-GT) binding in the anti-parallel motif was able to form triple helix on the 23-bp target (Table 1). Triplex was detected at 50 nM added duplex and increased to a maximum level at 0.5–1 µM. Two prominent gel-shifted bands were seen with 1-GT, possibly indicating that binding of this short TFO was partially unstable under gel-running conditions. The shifted bands represented complexes of TFO with duplex DNA since under identical conditions, 1 µM of unlabeled TFO, or of each single strand of the duplex caused no mobility shift (data not shown). This also indicated that the GT TFO had little tendency to associate with itself and other G- or C-rich single-stranded oligonucleotides. The formation of triplex was further confirmed in experiments where the 23-bp target was labeled and incubated with increasing concentrations of 1-GT (data not shown).

An anti-parallel 11mer GA TFO (1-GA) also formed triplex but predominant multiple bands of higher mobility than the double-stranded target were detected at all concentrations of duplex (data not shown). These non-triple-helical structures may represent TFO detached from the target during electrophoresis, or complexes of TFO with unlabeled purine or pyrimidine strands detached from the duplex. We did not characterize this further, since the GA TFO appeared to form triplex less efficiently than its GT counterpart. No other short TFO formed detectable triplex with the G-rich region (Table 1). We then tested a series of TFOs directed to the A-rich segment of the target. A CT TFO (2-CT), binding in parallel orientation and at acidic pH formed triplex that was essentially complete in the

presence of 1 μM added duplex (Table 1). The triplex formed by 2-CT was also unstable and could be detected only when samples were incubated and gels run at low temperatures (4 and 10°C, respectively). No other short TFO formed detectable triplex on this sequence under any conditions, indicating that weak binding to this region probably contributed to the moderate affinity of full-length anti-parallel TFOs.

Design and binding affinity of parallel/anti-parallel GTC TFO

These results suggested that the preferred TFO-binding motif varied within the 23-bp *myc* P2 target sequence. The G-rich 3' segment was most efficiently bound by an anti-parallel GT TFO, while only a parallel CT TFO was able to form triplex on the A-rich 5' segment. The 11mer TFOs, however, could not be proposed as antigene agents since they did not form stable triplexes at physiological temperatures. To initiate design of a TFO with high binding affinity and specificity, we investigated whether the GT and CT short TFOs could be linked to create a molecule capable of binding simultaneously in parallel and anti-parallel orientations to the target purine strand.

A possible pitfall was that intermolecular base-pairing between groups of guanines and cytosines in the TFO could interfere with triplex formation. To investigate this, we incubated 2-CT at 100-fold molar excess with ^{32}P -labeled 1-GT, and used EMSA to detect possible complex formation. No gel-shifted complexes were observed, and 2-CT had no adverse effect on triplex formation by 1-GT, suggesting that the TFOs were not associating in stable base-paired complexes. To test whether the two unlinked short oligos might act cooperatively to form a stable triplex, we used EMSA in which both TFOs together at 10 μM were incubated with labeled duplex at low pH to allow binding by 2-CT as well as 1-GT. No difference in binding affinity was observed between the short TFOs alone or paired, and a full-length CT TFO bound with ~2-fold higher affinity, suggesting that short TFOs did not form a triple helix in a cooperative manner (data not shown). This is consistent with results of a recent study of short TFOs targeting neighboring sequences (25).

These findings prompted design of a novel TFO in which 1-GT and 2-CT were synthesized contiguously to form equal domains with opposite polarity (Table 1). The novel TFO had two 5' ends with a central 3'-3' phosphodiester linkage. Cytosines were methylated at the C5 position to promote binding of the CT portion at physiological pH (26). The central cytosine present in the purine-rich target strand was matched with a thymine in the parallel portion of the TFO. Thymine, which can form a single Hoogsteen bond with cytosine, is recognized as the best natural base to target this pyrimidine inversion (27).

EMSA using labeled target duplex incubated with increasing concentrations of unlabeled TFO confirmed that binding by the GTC TFO at pH 5.6 and 7.2 was similar, with 50% binding observed at concentrations between 100 and 250 nM in both conditions (Fig. 1). This represented a 50–125-fold molar excess of TFO over target. Triplex formation was complete at a TFO molar excess of 250–500-fold. A full-length CT TFO without modified cytosines bound with comparable affinity to the GTC TFO at pH 5.6. Stability of the full-length TFO must have been conferred by binding to the A-rich region, since no binding to the G-rich portion of the target was detected with

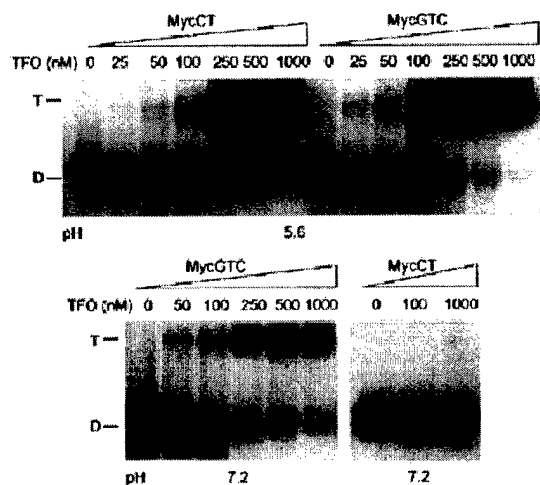


Figure 1. Triplex formation by 23mer TFOs on 23 bp *c-myc* P2 sequence. The pyrimidine-rich strand of the target duplex was labeled with ^{32}P and annealed to the complementary purine-rich strand. Indicated concentrations of TFOs Myc CT or Myc GTC were incubated with duplex (2 nM) overnight at 37°C and pH 5.6 or 7.2, except Myc CT at pH 7.2, where incubation was at 4°C. Complexes were resolved on non-denaturing gels at the same pH and at 20°C. T, triplex DNA; D, duplex DNA.

the short CT TFO at pH 5.6. The full-length TFO did not form detectable triplex at pH 7.2.

We then used DMS footprinting to examine binding affinity and specificity of the parallel/anti-parallel TFO. This assay also allowed direct comparison between the GTC TFO and a full-length anti-parallel GT TFO Myc-GT. We previously drew attention to the difficulty of using EMSA to examine binding affinity of the GT TFO due to the small mobility shift observed (21). Binding reactions for footprinting were carried out at pH 7.2–7.4. Figure 2A shows that the GTC TFO formed triple helix with equally high specificity and significantly improved affinity compared with an anti-parallel GT TFO. Densitometric analysis showed that the GTC TFO at 0.01, 0.1 and 1 μM reduced cleavage in the target sequence to ~72, ~25 and ~17%, respectively, of a control sample without oligonucleotide, whereas 10 μM GT TFO caused ~55% reduction. The effect of Myc-GT was consistent with previous DMS footprinting experiments (21). Footprinting also showed that the single guanine at the 3' end of the A-rich sequence was protected by 1 μM GTC but not by 20 μM anti-parallel TFO, indicating that only the former was able to bind this region of the target (Fig. 2B). The three guanines at the 5' end of the target were not protected, which may reflect unstable binding by the adjacent methyl cytosines in the GTC TFO. The pH-dependent CT TFO caused no footprint at pH 7.2, confirming that triplex formation was required to protect from cleavage.

Inhibition of nuclear protein binding by parallel/anti-parallel TFO

Since the GTC TFO displayed improved affinity without loss of target specificity, we investigated its ability to block binding of nuclear proteins to the *c-myc* promoter sequence. A 40 bp probe including 5' and 3' *c-myc* sequences flanking the TFO target site was used in gel-shift assays with breast cancer cell nuclear extracts. To ensure that proteins known to bind the

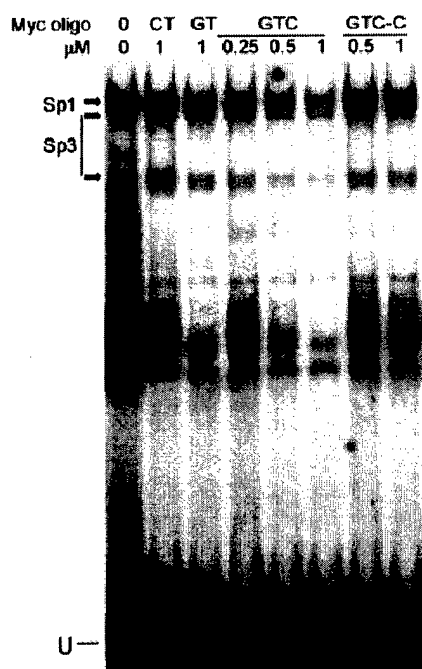


Figure 4. TFO-mediated inhibition of nuclear protein binding to *myc* P2 sequence. Myc P2 probe was pre-incubated alone (0) or with indicated concentrations of TFOs and control oligos in HEPES-MgCl₂ for 2 h at 37°C. MCF-7 nuclear extracts were added, mixtures incubated, and complexes resolved on a polyacrylamide gel as described in Figure 3. Sp1 and Sp3 complexes are indicated. U, unbound DNA probe.

were similarly decreased by the TFOs, but not by control oligos. These results showed that triplex formation was required to block protein binding and that the GTC TFO blocked more efficiently than the GT TFO.

We then tested target specificity of the GTC TFO by examining its effect on a polypurine/polypyrimidine sequence in the *c-myc* P1 promoter region, which is also recognized by Sp1/Sp3 (28). Pre-incubation with an antibody against Sp1, done as described for the P2 probe, specifically removed two major complexes, confirming Sp1 binding (Fig. 5A). The Sp3 antibody also removed smaller bands and, in this case, caused a supershift (data not shown). Pre-incubation of the P1 probe with 1 μM GTC TFO did not affect binding of any proteins, whereas the Sp1 complex on the P2 probe was again reduced by ~50% compared with the control chimeric oligo (Fig. 5B). This series of protein binding experiments clearly showed that the GTC TFO selectively blocked transcription factor binding to the P2 promoter sequence with increased efficiency compared with a conventional TFO. Similar results were obtained in experiments where nuclear extracts from MDA-MB-231 cells were used, showing that the TFO was effective in a different cell background.

Assay of nuclease resistance and activity in cells of parallel/anti-parallel TFO

Unmodified phosphodiester oligos are rapidly degraded by exo- and endonucleases. Since the GTC TFO lacks a 3' end, we investigated whether double 5' ends would increase resistance to 3' exonuclease activity. We compared *in vitro* stability of the

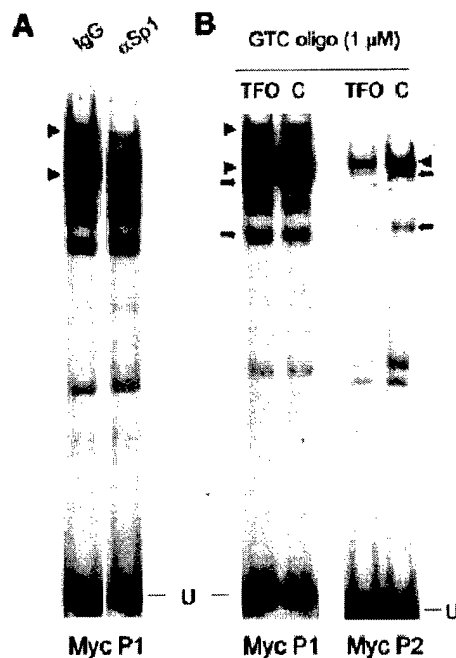


Figure 5. Specificity of GTC TFO inhibition of Sp1 and Sp3 binding to *myc* P2 probe. Gel-shift assays used double-stranded probes containing either the TFO target and flanking sequence (Myc P2) or a polypurine/polypyrimidine tract and flanking sequence from the *c-myc* P1 promoter region (Myc P1). (A) Antibody pre-incubation with the P1 probe was done as described in Figure 3. (B) Pre-incubation of P1 (left) or P2 (right) probes with 1 μM Myc GTC or GTC-C and subsequent protein-binding assays and electrophoresis were done as described in Figure 6. Arrowheads and arrows indicate Sp1 and Sp3 protein complexes, respectively. U, unbound probe.

GTC and the GT TFOs in cell culture medium that contained 10% fetal bovine serum. Nuclease resistance of modified and unmodified oligos has previously been assessed by this method (29,30). We found that the GTC TFO was stable in serum-containing medium for at least 24 h while the unmodified GT TFO was significantly degraded within 2 h (Fig. 6A). We next tested GTC TFO resistance to intracellular nucleases by transfecting MDA-MB-231 breast cancer cells with 5'-radio-labeled TFO. Slight degradation of the TFO was already detectable at 6 h, and at 24 h only ~35% of TFO present in the transfection mix remained intact (Fig. 6B). The half-life in cells of unmodified phosphodiester oligos has been estimated at 15–30 min, with both exonuclease and endonuclease activity causing degradation (31). Our results indicated that the presence of double 5' ends prolonged partially the intracellular half-life of the GTC TFO, but did not ensure long-term stability in cells.

Lack of long-term stability was reflected in limited activity of Myc-GTC in reporter gene assays in breast cancer cells. We assayed activity of the TFO in cells by transfecting MDA-MB-231 cells with Myc-GTC or Myc-GTC-C along with a plasmid containing a Myc promoter-driven luciferase reporter gene. The TFO had only limited inhibitory effect (~20% inhibition of luciferase activity at concentrations up to 1 μM in the transfection medium, data not shown). Since *in vitro* studies strongly suggest that the GTC TFO is the best approach to targeting the *c-myc* major promoter, future studies will focus on selecting optimal, practicable modifications to enhance

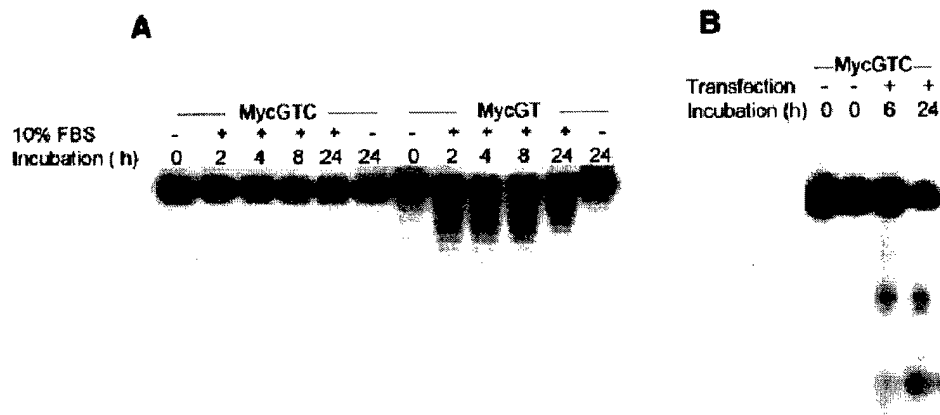


Figure 6. Myc GTC resistance to degradation by nuclease activity in fetal bovine serum, and partial resistance in cells. Unmodified phosphodiester TFOs Myc GTC and Myc GT were labeled using [32 P]ATP and T4 kinase. (A) Labeled oligos were incubated for the indicated times at 37°C in cell culture medium alone (-) or with 10% fetal bovine serum (+). Formamide loading buffer was added and samples resolved on a 15% polyacrylamide-7 M urea denaturing gel. (B) Labeled oligo was left unprocessed (lane 1), or mixed with transfection reagent and cell culture medium (lanes 2-4). Cells were transfected for 6 h, then washed and lysed in formamide sample buffer (lane 3) or grown for a further 24 h (lane 4) before lysis. Equal amounts of radioactivity were loaded in each lane of a 7 M urea denaturing gel. Dried gels were exposed to X-ray films.

nuclease resistance, allowing further examination of antigene effects in cells.

DISCUSSION

We addressed the problem of moderate binding affinity demonstrated by anti-parallel design TFOs targeted to a critical regulatory site close to the *c-myc* P2 promoter. We found that two discrete 11 bp domains in the target, one G-rich and the other A-rich, were bound most efficiently by 11mer anti-parallel GT (1-GT) and parallel CT TFOs (1-CT), respectively. Besides the GT and CT TFOs mentioned above, only one other TFO of the series examined was able to bind to either element of the target sequence. This was an anti-parallel GA TFO (1-GA), which formed a triplex on the G-rich sequence, but with low efficiency. Some GA TFOs can bind duplex DNA with very high affinity, while others tend to self-associate in hairpin, homoduplex and quadruplex structures, which can affect their ability to form triplex (32-34). TFOs directed to the *c-myc* P2 site appear to fall into the latter category, perhaps due to the long A-tract in both the target and the TFO. We previously reported that a full-length GA TFO directed to the *myc* P2 site had decreased ability to form triplex compared to its GT counterpart, and formed complexes that migrated more slowly than triplex in gel-shift assays (18). A phosphorothioate TFO with identical sequence was nevertheless able to reduce expression in HeLa cells of a luciferase gene driven by the *c-myc* promoter, but this required incubation of the plasmid with 10 000-fold excess of GA TFO before transfection, again suggesting low efficiency binding (20). Debin *et al.* (35) reported that optimal stability of triplex formation by 13mer GA TFOs depended on a high guanine content (~85%) in the target sequence. A sub-optimal level of guanine in the P2 sequence (72% in the G-rich domain and 52% in the 23 bp tract) may also have contributed to low efficiency binding by short and full-length GA TFOs.

Although the 11mer GT TFO bound to the G-rich target, the triplex was unstable in native gels at temperatures $\geq 25^\circ\text{C}$ (data not shown). We reported a similar result with the full-length

P2-directed GT TFO, suggesting that this TFO may have formed only a partial triplex, likely on the G-rich but not the A-rich domain (21). The failure of the 11mer oligo (2-GT) forming the 3' portion of the long TFO to form triplex, and the partial DMS footprint observed with the 23mer TFO, support this conclusion.

In contrast to 2-CT, the short TFO targeted to the A-rich part of the target sequence, 1-CT was not able to bind the G-rich portion even at low pH and temperature. The latter TFO has a high number of adjacent cytosines, which may have affected triplex formation by causing the TFO to self-associate through interaction of cytosines in the i-motif (36). In addition, adjacent C⁺G-C triplets may be destabilized by electrostatic repulsion between the protonated cytosines (37). The full-length CT TFO formed a triplex quite efficiently at 37°C in low pH, indicating that the T-A-T triplets had a general stabilizing effect. The stability of these triplets was emphasized by our finding that only the short CT TFO was able to form a detectable triplex on the A-rich part of the target. A parallel-binding 11mer GT TFO (2-GTC) with a single cytosine opposite the isolated guanine in the target did not bind to the A-rich sequence. Interestingly, a similar TFO was found to be optimal for binding a 16 bp target sequence with an extremely unbalanced distribution of GC and AT pairs (38). In this case, six guanines, clustered at the 3' end of the purine strand, were matched with guanine in the third strand. One guanine present in a 5' stretch of eight adenines was matched with a third strand cytosine. Clearly, the *c-myc* sequence, although somewhat similar, did not favor this unusual third strand composition.

Detailed examination of the *myc* P2 sequence resulted in design of a novel parallel/anti-parallel TFO with significantly improved binding affinity. To the best of our knowledge, this approach to targeting an unbalanced sequence has not been reported before. Use of TFOs with central 3'-3' linkages has been described previously in the alternate strand binding strategy, where purine tracts adjacent but on opposite strands can be targeted by TFOs that cross over from one strand to the other. These TFOs consist of two domains binding in the same

triplex motif (either parallel or anti-parallel), usually with a linker structure at the central interface (39,40). Continuously synthesized TFOs without linkers, that bind to alternate strands in opposite triplex motifs, have also been developed (41). We reasoned that weak binding of thymine to cytosine at the pyrimidine inversion would offer flexibility at the 3'-3' junction. However, we did not test any alternative linker structures. It is possible that non-nucleotide linkers may improve binding affinity of parallel/anti-parallel TFOs such as Myc-GTC.

The GTC TFO described here, binding with opposite polarity to adjacent sequences on the same strand, showed higher affinity than any previously described TFO targeted to the same critical sequence in *c-myc*. Further improvements in design of this TFO might enhance binding affinity even more, and reduce concentrations required to compete with transcriptional activators in cells. For example, in the CT portion of the TFO, replacement of methyl C with 8-oxo-2' deoxyadenosine would likely improve binding at neutral pH (42). Alternatively, replacing DNA in the pyrimidine portion with 2'-alkyl-modified RNA could improve binding and increase nuclease resistance (43,44). Other base and backbone modifications have been found to improve binding and nuclease resistance of CT TFOs, and may be suitable for the *c-myc* sequence (reviewed in 45,46).

Since we found that double 5'-ended structure was only partially protective against degradation of the TFO by nucleases, modifications to enhance nuclease resistance will be required for the TFO to have extended life in cells. Phosphorothioate (PS) modification, in which sulfur replaces non-bridging oxygens in internucleotide linkages, has been widely used for antisense oligonucleotides, and has little, if any, inhibitory effect on binding of anti-parallel TFOs. Unfortunately, PS linkages severely reduce binding by parallel motif TFOs and cannot be used in this context. Conversely, other backbone modifications, like 2'-alkylated ribose and N3'-P5' phosphoramidate linkages, are advantageous for parallel-binding TFOs but detrimental to anti-parallel binding (46). These limitations, as well as possibilities for improving binding affinity along with nuclease resistance, will help guide the choice of strategy for developing a *c-myc* TFO for use in cells.

Our approach to designing an improved TFO for the unbalanced sequence in *c-myc* may be useful for other genes. Database searches revealed other sequences in the human genome similar to the one in *c-myc*, some associated with known genes. As the genome becomes more fully characterized, and genes important in disease are identified, extending the repertoire of sites available for high-affinity triplex formation will be of increasing interest.

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Selective inhibition of transcription of the Ets2 gene in prostate cancer cells by a triplex-forming oligonucleotide

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ABSTRACT

The transcription factor Ets2 has a role in cancer development and represents an attractive therapeutic target. In this study, we designed a triplex-forming oligonucleotide (TFO) directed to a homopurine:homopyrimidine sequence in the Ets2 promoter. Transcription factors of the Sp family bound to this sequence and mutation of the Sp1 site reduced Ets2 promoter activity. The Ets2-TFO had high binding affinity for the target sequence and inhibited binding of Sp1/Sp3 to the overlapping site. This effect occurred with a high degree of sequence specificity. Mismatched oligonucleotides did not inhibit Sp1/Sp3 binding and mutations in the target sequence that abolished triplex formation prevented inhibition of Sp1/Sp3 binding by the TFO. The Ets2-TFO inhibited Ets2 promoter activity and expression of the endogenous gene in prostate cancer cells at nanomolar concentrations. The TFO did not affect reporter constructs with mutations in the TFO binding site and promoters of non-targeted genes. Expression of non-targeted genes was also not affected in TFO-treated cells. Collectively, these data demonstrated that the anti-transcriptional activity of the Ets2-TFO was sequence- and target-specific, and ruled out alternative, non-triplex mediated mechanisms of action. This anti-transcriptional approach may be useful to examine the effects of selective downregulation of Ets2 expression and may have therapeutic applications.

INTRODUCTION

The human Ets family includes 25 genes that code for positively and negatively acting transcription factors involved in various aspects of cell proliferation and differentiation. Ets factors share a highly conserved DNA binding domain (Ets domain), which binds to DNA elements (Ets binding sites) characterized by the purine-rich core sequence GGAA/T (1).

A large number of genes, including genes for transcription factors, matrix metalloproteinases, cell cycle regulators, extracellular matrix receptors and growth factors, are known to contain Ets binding sites (1). Ets factors are downstream effectors of the Ras-signaling pathway, which undergoes oncogenic activation in many cancers (2–4). The oncogenic potential of various Ets factors, including Ets2, has been demonstrated in several experimental systems (5–9). Recent studies indicate that Ets2 contributes to neoplastic transformation and maintenance of the malignant phenotype in various cancer types, including prostate, breast and thyroid cancers (10–14). The oncogenic effects of Ets2 are likely related to its ability to activate expression of multiple genes that promote cell proliferation and invasion, or prevent apoptotic cell death. Therefore, targeting this transcription factor may be a valid therapeutic strategy for various forms of cancer.

Oligonucleotide-directed triple helix formation offers a means to target specific sequences in DNA and interfere with gene expression at the transcriptional level (15–17). Antigene or triplex-forming oligonucleotides (TFOs) bind to homopurine:homopyrimidine sequences forming a stable, sequence-specific complex with duplex DNA. Recent studies have provided convincing evidence of triplex formation at extra-chromosomal and chromosomal sites in cells by analyzing site-specific mutagenesis and covalent crosslinking of target DNA induced by TFOs (18–22). Purine-rich sequences are frequent in gene regulatory regions and TFOs directed to promoter sequences have been shown to prevent binding of transcription activators and inhibit transcription initiation *in vitro* (15,16). TFOs have also been shown to downregulate expression of targeted genes in cells by blocking transcription initiation or elongation (16,17). This anti-transcriptional approach could complement other gene-targeted strategies, such as antisense, small-interfering RNA, and dominant negative constructs, which interfere with gene expression at post-transcriptional level. However, a clear demonstration of triplex-mediated and sequence-specific inhibition of transcription initiation by TFOs has generally been lacking in most cell culture studies. Oligonucleotides are known to elicit non-specific effects by interacting with proteins or non-target nucleic acids both in sequence and non-sequence specific manners (23–25). These effects may depend on nucleotide

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sequence, chemical composition, and tendency of oligonucleotides to form particular secondary structures (24,26–28). Therefore, one cannot always rule out that the effects of TFOs on transcription initiation observed both in cells and cell-free systems might be due to non-specific and non-triplex mediated effects.

Our goal in the present study was to design a TFO directed to a homopurine:homopyrimidine sequence in the Ets2 promoter with the intent to selectively inhibit transcription of this gene. The sequence selected for triplex-mediated gene targeting was located ~40 bp upstream of the transcription initiation sites in the Ets2 promoter (29–31). Our data show that transcription factors of the Sp family bound to this region and that mutation of the Sp1 site significantly reduced promoter activity. The Ets2-targeting TFO had high affinity and specificity for the target sequence, inhibited binding of Sp1/Sp3 transcription factors to the target site *in vitro* and was effective as repressor of Ets2 transcription in cells. Experiments with mutated oligonucleotide targets and mutated promoter reporter constructs provided evidence that the effects of the TFO both *in vitro* and in cells were sequence- and target-specific. These results demonstrated that the anti-transcriptional activity of the Ets2-TFO was due to triplex formation, and ruled out alternative, non-triplex mediated mechanisms of action. The activity of the TFO combined with a high degree of specificity suggest that this anti-transcriptional approach may be useful to analyze the effects of selective downregulation of Ets2 expression in experimental settings and may have potential therapeutic applications.

MATERIALS AND METHODS

Oligonucleotides

Phosphodiester (PO) and phosphorothioate (PS) oligonucleotides were purchased from Genset (La Jolla, CA). PO and PS oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE) or high-performance liquid chromatography. Stock solutions of oligonucleotides were made in sterile water. Oligonucleotide concentrations were determined with a spectrophotometer using appropriate nucleotide extinction coefficients (32). Immediately before each experiment, oligonucleotide solutions were heated at 65°C for 10 min and then chilled on ice. This heating step was performed to eliminate self-aggregates (e.g. tetraplex and homoduplex) that might have formed during storage of the oligonucleotides at low temperatures. Sequences of the TFO, control oligonucleotides and oligonucleotides corresponding to purine and pyrimidine strands of the target site are shown in Figure 1.

Gel mobility shift assays

Oligonucleotides corresponding to the pyrimidine-rich strand of the wild type or mutated targets were 5'-end labeled with [γ -³²P]ATP and T4 polynucleotide kinase and annealed to complementary oligonucleotides as previously described (33). Samples containing 1 nM duplex DNA and increasing concentrations of either TFO or control oligonucleotides were incubated for 24 h at 37°C in a buffer containing 90 mM Tris, 90 mM borate (pH 8) and 10 mM MgCl₂ (TBM buffer). To examine triplex DNA formation, samples were resolved by PAGE under non-denaturing conditions (33). Apparent

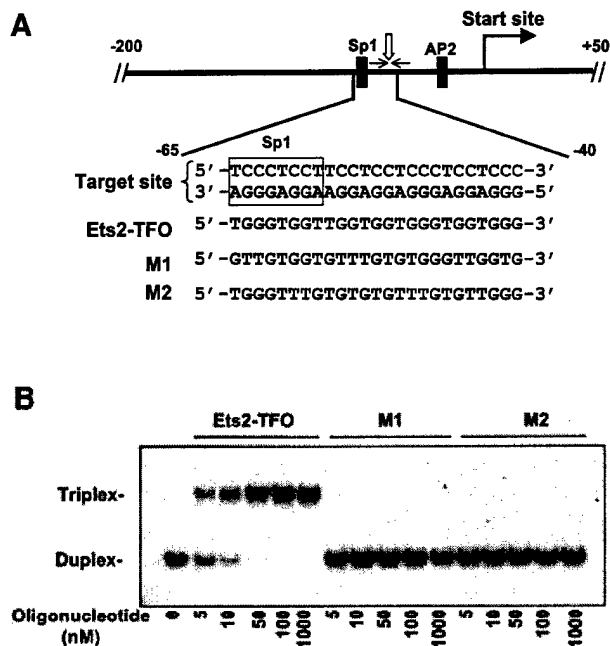


Figure 1. Position of the target site in the Ets2 promoter and triplex DNA formation by the Ets2-TFO. (A) The 25-bp homopurine:homopyrimidine target sequence is located ~40 bp upstream of the transcription start site in the Ets2 promoter. Putative binding sites for Sp1 and AP2 (black boxes), inverted repeat sequence (small arrows) and nuclease hypersensitive site (vertical arrow) adjacent to the target site are indicated. Sequences of the target site, TFO and control oligonucleotides are shown below the promoter map. The Sp1 consensus sequence within the TFO target site is boxed. (B) The oligonucleotide corresponding to the pyrimidine strand of the target sequence was 5'-end labeled with [γ -³²P]ATP and annealed to the complementary purine-rich oligonucleotide. Duplex DNA (1 nM) was incubated with the indicated concentrations of TFO and control oligonucleotides M1 and M2 for 24 h at 37°C. Samples were resolved on a 12% polyacrylamide gel under non-denaturing conditions. Positions of double- and triple-stranded DNA are indicated.

dissociation constants (K_d) of TFOs were estimated as previously described (33). To examine nuclear protein binding to the Ets2 promoter site, nuclear extracts were prepared from breast cancer cells as described previously (32). Nuclear extracts were incubated with 1 nM of duplex DNA, which had been pre-incubated with or without TFOs overnight in TBM buffer. Binding reactions were incubated for 20 min at 10°C and then were resolved on 4% polyacrylamide gels (32). Sources of antibodies for supershift assays and double-stranded oligonucleotide competitors and related experimental conditions have been reported previously (32).

Promoter reporter constructs

To generate pGL3-Ets2, a pBluescript plasmid containing a 3.6 kb fragment of the human Ets2 promoter was digested with *Sac*II (29). After filling-in with T4 DNA polymerase (Promega), the *Sac*II fragment was subcloned into the *Sma*I site of the pGEM-3Z vector (Promega). A pGEM-3Z-Ets2 clone with the Ets2 promoter insert in the 5'–3' orientation was expanded, digested with *Kpn*I and *Hind*III, and the resulting fragment was subcloned into the pGL3-basic (Promega, Madison, WI). Promoter reporter constructs containing either

three or five mutations in the TFO target sequence were produced from pGL3-Ets2 using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Ets2-Mut3 oligonucleotides were used as primers in the PCR to generate the pGL3-Ets2-Mut3. The pGL3-Ets2-Mut5 was then produced using pGL3-Ets2-Mut3 as template and Ets2-Mut5 oligonucleotides as primers. The pGL3-Ets2-mSp1, which contained a mutated Sp1 site in the TFO target sequence, was produced by changing the wild type sequence 5'-TCCC-TCCT-3' to 5'-AGACTCCT-3' using the QuickChange Site-Directed Mutagenesis kit. Incorporation of the desired mutations in the isolated clones was confirmed by sequencing. The pGL3-Myc-P1 plasmid was generated by subcloning a *KpnI-XhoI* fragment of 1126 bp of the c-myc promoter into pGL3-basic. The pGL3-Src was obtained by subcloning a *KpnI-XbaI* fragment of the c-src promoter into pGL3-basic (34).

Transfection with reporter plasmid and luciferase assay

Human prostate cancer cells (DU145 and PC3) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were transfected with reporter vectors in the absence or presence of oligonucleotides using either DOTAP (Roche, Indianapolis, IN) or Oligofectamine (Invitrogen) as transfection reagent. For transfection with DOTAP, cells were plated in 96-well plates at a density of 8×10^3 cells/well. After 24 h, 100 ng of pGL3-Ets2 was mixed with TFO or control oligonucleotide in 20 mM HEPES and incubated with DOTAP at a mass ratio of 5:1 (DOTAP:DNA) for 15 min at room temperature. Either pRL-SV40 (5 ng) or pRL-TK (50 ng) control vector was added to each sample to monitor transfection efficiency. The mixture was then diluted in 100 μ l of serum-containing medium and added to the cells. After 6 h, medium was removed and replaced with fresh medium. When Oligofectamine was used, cells were plated in 48-well plates at a density of 1×10^4 cells/well. Reporter vectors (100 ng), control vector (5 ng), oligonucleotides and Oligofectamine (0.8 μ l) were diluted in 20 μ l of Opti-MEM, incubated for 15 min at room temperature and then added to the cells seeded in 80 μ l of Opti-MEM. After 4 h, growth medium containing 10% serum was added. Cells were lysed 24 h later and luciferase activity was measured using dual-luciferase assay system (Promega). Each experiment was repeated at least three times to ensure reproducibility of the data. In the co-transfection experiments, oligonucleotides represented a significant amount (100–200 ng) of the total DNA to be transfected and preliminary experiments indicated that the efficiency of transfection depended greatly on the amount of DNA and transfection reagent used. Thus, reporter activity of TFO-treated cells was compared with the reporter activity of cells transfected with an equal amount of control oligonucleotide rather than reporter plasmid alone, therefore keeping total amount of DNA (plasmid plus oligonucleotide) and transfection reagent constant. Hence, similar transfection efficiencies were achieved in the different experimental groups.

RNA analysis

DU145 cells were seeded at 7×10^4 cells/well in 6-well plates and transfected 24 h later with TFO or control oligonucleotide using Oligofectamine as described above. Total RNA was

extracted from control and TFO-treated cells using Trizol (Invitrogen). RNA concentrations were determined by spectrophotometry. RT-PCR was performed using the SuperScript One Step RT-PCR system (Invitrogen) and gene-specific primers. Forward and reverse primers for Ets2 were 5'-TCA-GCTCTGAGCAGGAGTTTCAGA-3' and 5'-GGTTGGCTT-ATTGAGGCAGAGAGA-3', respectively, which amplified a fragment of 296 bp. Sequences of the GAPDH primers have been published previously (33). Sequences of forward and reverse primers for Ets1 were 5'-TGCTATCAAACAAG-AAGTCGTCAC-3' and 5'-GACAGGAGATGGCTGGGAA-TTCA. RT-PCR was performed using 100 ng of total RNA. Each reaction contained 0.2 μ M forward and reverse gene-specific primers in addition to reagents present in the SuperScript PCR buffer. RNA was reverse-transcribed for 30 min at 50°C and then subjected to 26 cycles of PCR (94°C, 15 s; 55°C, 30 s; 72°C, 15 s). Samples were analyzed on 2% agarose gels. Following staining with ethidium bromide, PCR products were visualized using ChemiImager 4400 AlphaInnotech (San Leandro, CA). Densitometric analysis was performed using AlphaEase software (AlphaInnotech). The amount of RNA (100 ng) and number of PCR cycles (26 cycles) were optimized in preliminary experiments to ensure that PCR was performed in the exponential phase of amplification and there was a linear relationship between the amount of RNA template and yield of amplified products. Three individual RNA preparations were used with identical results and RT-PCR was performed at least two times with each individual preparation to ensure data reproducibility. Northern blot analysis was done as previously described using 3 μ g of total RNA and a radio-labeled Ets2 cDNA fragment as hybridization probe (11,33).

RESULTS

Selection of the target site and design of the TFO

The Ets2 gene is transcribed from multiple initiation sites directed by a promoter lacking typical TATA and CAAT elements (29,30). To target this gene using the triplex DNA-based approach, we selected a 25-bp homopurine:homopyrimidine sequence located ~40 bp upstream of the cluster of transcription initiation sites and overlapping a putative Sp1 binding site (Fig. 1). The selected sequence is located within a 160-bp region that has been previously shown to be essential for maximal promoter activity (29,30). This region includes multiple putative transcription factor binding sites, an inverted repeat and a long polypyrimidine (CT) tract, which is able to stimulate transcriptional activity of heterologous promoters (29,30). Nuclease hypersensitive sites, which are generally found in the promoter of actively transcribed genes and indicate the presence of an open chromatin structure, have been mapped in this region of the promoter in Ets2-expressing cells (29). In particular, a nuclease hypersensitive site co-localizes with the inverted repeat within the target sequence.

We designed a GT-rich oligonucleotide to bind to this sequence. As shown in Figure 1, the Ets2-targeting TFO had G residues opposite G:C base pairs, and T residues opposite A:T base pairs. The TFO was not identical or complementary to either strand of the target duplex and was expected to bind to the purine-rich strand of the duplex in antiparallel orientation.

Similar antiparallel GT-rich TFOs have been shown to bind with high affinity to target DNA at physiological pH (33,35–38). Two mismatched control oligonucleotides, designed as M1 and M2, which had nucleotide composition similar to the Ets2-TFO but scrambled sequences, were used in the study (Fig. 1A). Both control oligonucleotides conserved potentially critical sequence elements (e.g. short G₂ and G₃ runs) present in the TFO. M2 was identical to the TFO at the 5' and 3' ends and had extensive sequence homology to the TFO in the remaining part. TFO and control oligonucleotides were synthesized as both PO and PS oligonucleotides. All the data presented herein were obtained with oligonucleotides synthesized with a fully modified PS backbone to increase resistance to nuclease degradation.

Triplex DNA formation by the Ets2-targeting TFO

To examine binding of the Ets2-TFO, a radio-labeled duplex DNA corresponding to the target sequence was incubated with increasing concentrations of Ets2-TFO or control oligonucleotides. Binding was then assessed by gel mobility shift assay. In these experiments, samples were incubated for 24 h to ensure that binding was evaluated under equilibrium conditions as done in previous studies (38–40). In addition, potassium ions, which have been often shown to reduce the ability of PO and PS oligonucleotides to form triplex DNA at physiological intracellular concentration (15,16), were not included in the binding reaction buffer. As shown in Figure 1B, the PS-modified Ets2-TFO exhibited high affinity for the target sequence under these conditions. Its apparent K_d , estimated by gel mobility shift assay, was ~5 nM. Complete formation of triplex DNA was observed at a concentration of 50–100 nM of TFO, which corresponded to a 50- to 100-fold molar excess of TFO compared with the target DNA. PS-modified TFOs have shown reduced binding affinity compared with the PO counterpart in previous studies (38–40). However, the PS modification did not appear to affect binding of the Ets2-TFO to its target, since similar results were observed with both PS and PO TFO (data not shown). As shown in Figure 1B, EMSA was performed also with the control oligonucleotides M1 and M2. Both control oligonucleotides did not form triplex DNA at concentrations as high as 1 μ M, confirming the sequence specificity of the interaction of the Ets2-TFO with its target sequence.

Inhibition of Sp1/Sp3 protein binding to the Ets2 promoter by the Ets2-TFO

The TFO target site directly overlaps a Sp1 consensus sequence (Fig. 1). Sp1 sites are thought to be involved in determining the position of the initiation site in absence of a classical TATA box and be critical for activity of genes with TATA-less promoters, like Ets2 (41). However, the identity of the proteins binding to this region and their role in the activation of Ets2 transcription were not known. To examine nuclear protein binding, a double-stranded oligonucleotide probe, Ets2-WT, which included the TFO target sequence and the putative Sp1 consensus site was prepared. Gel mobility shift assay with nuclear extracts and the Ets2-WT probe showed formation of three major protein/DNA complexes, which were identified as Sp1 and Sp3 complexes (Fig. 2). Formation of these complexes was inhibited by the addition of a competitor oligonucleotide containing a Sp1 consensus

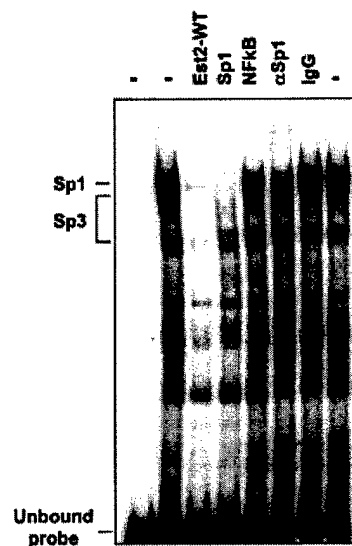


Figure 2. Nuclear protein binding to the Ets2 promoter site. Radiolabeled double-stranded oligonucleotide corresponding to the Ets2 target sequence (Ets2-WT) was incubated with nuclear extracts, with the exception of the first lane. To identify proteins binding to the site in the Ets2 promoter, binding reactions contained either unlabeled Ets2 target (Ets2-WT), Sp1 and NFkB consensus oligonucleotides (Sp1 and NFkB), anti-Sp1 antibody (α Sp1), and normal rabbit IgG (IgG). Samples were electrophoresed on a polyacrylamide gel under non-denaturing conditions. Positions of unbound probe and Sp1/Sp3 complexes are indicated.

sequence and antibodies against Sp1 and Sp3 (Fig. 2, and data not shown). Next, we determined whether formation of the Sp1/Sp3 complexes with the duplex DNA could be blocked by the Ets2-TFO. Incubation of the Ets2-WT probe with the TFO under conditions that allowed triplex formation resulted in a clear reduction of Sp1/Sp3 complex formation (Fig. 3). A >50% reduction of Sp1/Sp3 binding was already observed at 10 nM of TFO, i.e. only 10-fold molar excess compared with target DNA. These data were consistent with the high binding affinity of the Ets2-TFO estimated by gel mobility shift assay. Both control oligonucleotides M1 and M2 did not affect Sp1/Sp3 binding, indicating sequence specificity of the TFO effects. These data suggested that the TFO formed a stable complex that was able to block binding of Sp factors to the Ets2 promoter. This could lead to transcription inhibition by preventing assembly of an active initiation complex.

Inhibition of Sp1/Sp3 binding by the Ets2-TFO is mediated by triplex formation

The TFO-induced inhibition of Sp1/Sp3 binding to the Ets2 promoter could be due to a sequence-specific but non-triplex mediated effect (e.g. a protein decoy mechanism). To rule out this possibility, we used double-stranded oligonucleotide probes, Ets2-Mut3 and Ets2-Mut5, which contained mutations in the TFO target sequence (Fig. 4A). These mutations were specifically designed to disrupt the ability of the TFO to form triplex DNA but not binding of Sp1/Sp3 factors. Gel mobility shift assays showed that the Ets2-TFO was unable to bind to the mutated probes, while it was clearly able to form triplex DNA with the probe containing the wild type sequence (Fig. 4B). Next, the mutated probes were incubated with

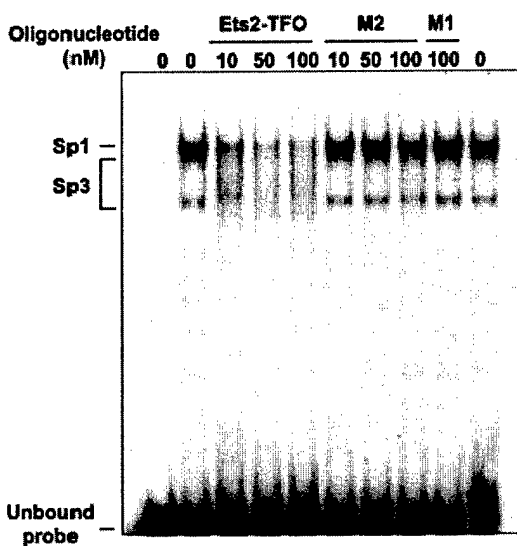


Figure 3. TFO-induced inhibition of Sp1/Sp3 protein binding to the Ets2 promoter. Double-stranded oligonucleotide Ets2-WT was radiolabeled and incubated without or with the indicated concentrations of Ets2-TFO or control oligonucleotides for 24 h at 37°C. Nuclear extracts were added to the binding reactions, with the exception of the first lane, incubated for 20 min and then samples were analyzed by gel electrophoresis as described in the legend to Figure 2.

nuclear extract to examine nuclear protein binding. Gel mobility shift assays with the Ets2-Mut3 probes showed formation of three major protein/DNA complexes similar to those formed with the wild type probe (Fig. 4C). These complexes were identified as Sp1/Sp3 complexes by oligonucleotide competition and antibody supershift assays (Fig. 4C). When the Ets2-Mut3 probe was incubated with the Ets2-TFO or control oligonucleotide, the TFO was unable to prevent binding of Sp1 and Sp3, under conditions that inhibited binding of these proteins to the wild type target (Fig. 4C). Formation of Sp1/Sp3 complexes on the Ets2-Mut5 probe was also not affected by pre-incubation with the Ets2-TFO (data not shown). Taken together, these data confirmed the sequence specificity of the interaction between the TFO and the Ets2 target site. Moreover, these results demonstrated that inhibition of Sp1/Sp3 binding by the Ets2-TFO required triplex formation with the target DNA and was not due to a direct interaction of the TFO with the transcription factors or other proteins in the nuclear extract.

Inhibition of Ets2 promoter activity by the Ets2-TFO in prostate cancer cells

To evaluate the biological activity of the Ets2-TFO, we determined whether it had any effect on the Ets2 promoter in prostate cancer cells known to express high levels of this gene. The pGL3-Ets2 plasmid contained an 880-bp fragment of the Ets2 promoter, including the transcription start site and TFO target sequence, cloned upstream of the firefly luciferase gene. Hence, the level of luciferase activity in transfected cells was a direct measurement of transcription initiation within the Ets2 promoter. DU145 and PC3 cells were transfected with the pGL3-Ets2 plasmid along with either pRL-TK or pRL-SV40 control vectors. After 24 h, the activity of both firefly and Renilla luciferase were measured in cell extracts. Upon

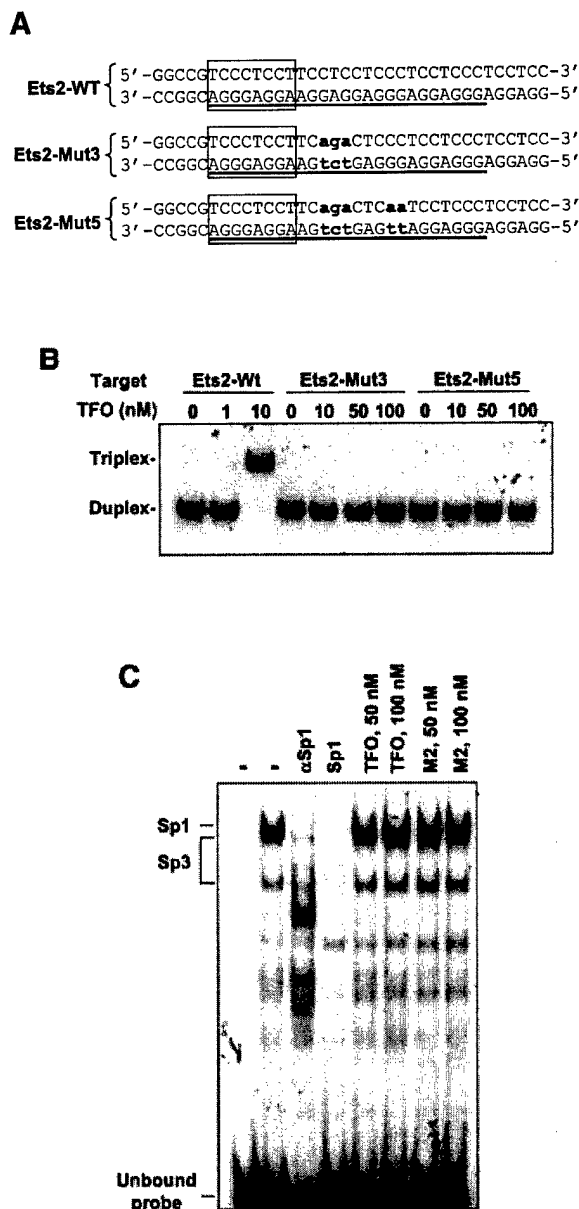


Figure 4. TFO-induced inhibition of nuclear protein binding to the Ets2 promoter is sequence specific and triplex mediated. (A) Sequence of wild type and mutated oligonucleotides. Sp1 sites in the Ets2-WT, Ets2-Mut3 and Ets2-Mut5 oligonucleotides are boxed. TFO target sequence is underlined. Mutated bases in the Ets2-Mut3 and Ets2-Mut5 oligonucleotides are shown in small bold letters. (B) Binding of the Ets2-TFO to wild type and mutated targets. Ets2-WT, Ets2-Mut3 and Ets2-Mut5 oligonucleotides were radiolabeled and incubated with the indicated concentrations of Ets2-TFO. Binding and electrophoresis were performed as described in the legend of Figure 1. (C) Nuclear protein binding to the mutated Ets2 target and effects of the TFO. Ets2-Mut3 was radiolabeled and incubated either in the absence or presence of the indicated concentrations of Ets2-TFO or control oligonucleotide M2. Nuclear extracts were added to the binding reactions with the exception of the first lane. Where indicated, anti-Sp1 antibody (α Sp1) and a Sp1 consensus oligonucleotide (Sp1) were added to the samples to confirm presence of Sp1 factors in the protein/DNA complexes. Samples were separated on a polyacrylamide gel as described in the legend to Figure 2.

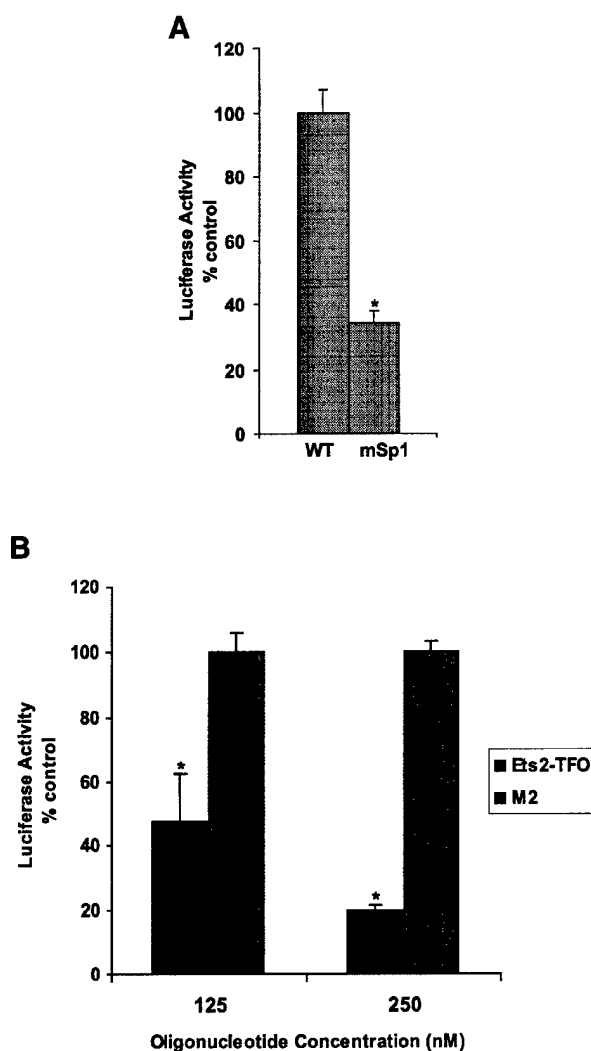


Figure 5. Inhibition of Ets2 promoter activity by the Ets2-TFO. (A) pGL3-Ets2 and pGL3-Ets2mSp1 were transfected into DU145 cells along with a pRL-SV40 control vector using DOTAP. Luciferase activity was measured after 24 h from transfection. Renilla luciferase activity was used to control for transfection efficiency. Asterisk, $P < 0.005$ compared with wild type reporter. (B) The pGL3-Ets2 plasmid was transfected into DU145 cells along with Ets2-TFO or control oligonucleotide M2 and pRL-SV40 control vector using DOTAP. Firefly and Renilla luciferase activities were measured after 24 h from transfection. Firefly luciferase activity was normalized to the Renilla luciferase activity and data are presented as percent of luciferase activity compared with control transfected cells. Asterisks, $P < 0.05$ and $P < 0.0005$ at 125 and 250 nM of Ets2-TFO compared with mismatched-treated cells.

transfection of the Ets2 promoter construct, luciferase activity was induced ~100-fold in DU145 and PC3 cells compared with cells transfected with pGL3 basic vector, indicating that the Ets2 promoter effectively activated luciferase expression in these cells. To determine the functional relevance of the Sp1 site overlapping the TFO target sequence, we mutated the corresponding sequence in the pGL3-Ets2 reporter construct. As shown in Figure 5A, Ets2 promoter activity

was significantly reduced in the pGL3-Ets2-mSp1 compared with wild type reporter.

Next, prostate cancer cells were co-transfected with pGL3-Ets2 and either Ets2-TFO or control oligonucleotide at concentrations of 125 and 250 nM. When DU145 cells were incubated with the Ets2-TFO, the activity of the reporter gene was significantly reduced compared with cells incubated with identical concentrations of control oligonucleotide (Fig. 5B). Inhibition of Ets2 promoter activity by the TFO was dose dependent with ~50 and 75% reduction at 125 and 250 nM, respectively. A similar effect of the TFO on Ets2 promoter activity was observed in PC3 cells (data not shown). Significantly, the extent of inhibition of Ets2 promoter activity by the Ets2-TFO was similar to that observed by mutating the Sp1 site overlapping the TFO target sequence. This finding suggested that inhibition of Sp1/Sp3 binding at the target site was a likely mechanism of transcription inhibition by the TFO. Furthermore, the significant inhibition of promoter activity by the Ets2-TFO and the lack of effect of the non-triplex forming oligonucleotide suggested that the inhibition was likely due to triplex DNA formation at the TFO target site. Since the short incubation prior to transfection should not permit triplex formation, these data suggested that the TFO was able to interact with the target DNA once inside the cells and to form a stable complex that inhibited promoter activity.

Triplex formation is required for inhibition of promoter activity by the Ets2-TFO

To examine the mechanism of inhibition of Ets2 promoter activity by the TFO, we generated promoter reporter constructs that contained mutations in the TFO target sequence identical to those tested in gel mobility shift assays. The pGL3-Ets2-Mut3 and pGL3-Ets2-Mut5 constructs were derived from the pGL3-Ets2 plasmid and differed only in the presence of either three or five mutations in the TFO target sequence (Fig. 4A). In both constructs, the Sp1 site overlapping the target sequence was not affected by the mutations, and gel shift mobility assays showed binding of Sp1/Sp3 proteins to oligonucleotide probes containing identical base changes (Fig. 4B). In luciferase reporter assays, activity of the mutated promoters was decreased ~25% compared with the wild type reporter (Fig. 6A). This reduction in promoter activity was probably due to the partial disruption of the inverted repeated adjacent to the Sp1 site. When DU145 cells were transfected with the TFO or control oligonucleotide along with reporter constructs, the Ets2-TFO was unable to affect the activity of mutated promoters under conditions that resulted in significant inhibition of the wild type promoter (Fig. 6B). Thus, the presence of the intact target sequence, which was required for triplex formation and inhibition of transcription factor binding *in vitro*, was also essential for inhibition of promoter activity by the TFO in cells. These data strongly support the conclusion that inhibition of transcription initiation by the Ets2-TFO was due to triplex formation and rule out that alternative mechanisms, such as a decoy-like mechanism, were responsible for these effects.

Inhibition of promoter activity by the Ets2-TFO is target specific

To further prove the selectivity of the Ets2-TFO for the targeted gene promoter, we evaluated its effects on promoter

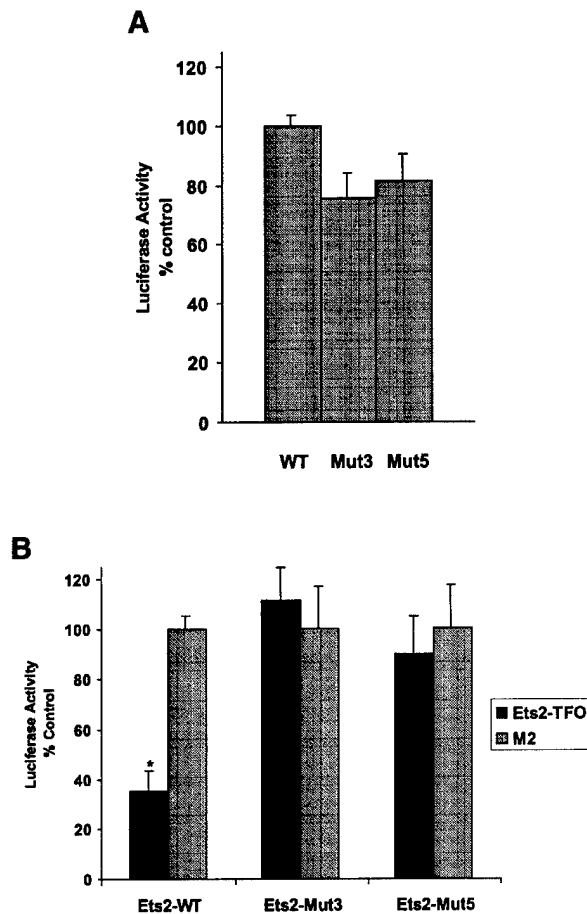


Figure 6. TFO-induced inhibition of Ets2 promoter activity requires triplex formation. (A) DU145 cells were transfected with pGL3-Ets2, pGL3-Ets2-Mut3 or pGL3-Ets2-Mut5 along with pRL-SV40. Firefly and Renilla luciferase activities were measured 24 h later. (B) DU145 cells were transfected with pGL3-Ets2, pGL3-Ets2-Mut3 or pGL3-Ets2-Mut5 along with 200 nM of Ets2-TFO or control oligonucleotide M2 using Oligofectamine. The pRL-SV40 control vector was included to monitor transfection efficiency. Luciferase activity was measured after 24 h. Data are presented as described in the legend to Figure 5. Asterisk, $P < 0.0005$ compared with control treated cells.

reporter constructs with significant structural and functional similarity to the Ets2 promoter. Both the c-myc and c-src promoter contained purine-rich sequences similar to that targeted by the Ets2-TFO and multiple Sp1 binding sites (32–34,42). The c-src promoter contained a long polypyrimidine region located upstream of the transcription initiation site known to be critical for promoter activity (42). A 25-bp sequence within this polypyrimidine region had only four mismatches compared with the Ets2 target sequence. The c-myc P1 promoter construct used in this study also contained a polypyrimidine tract (CT element) upstream of the P1 promoter (33). To prevent potential secondary effects due to Ets2 downregulation by the TFO, however, the Ets binding site near the c-myc P2 promoter had been deleted (43). Gel mobility shift assays confirmed that Sp1/Sp3 proteins bound to sites proximal to the purine-rich sequences in both c-myc and c-src promoters (32,42). We also determined that binding of

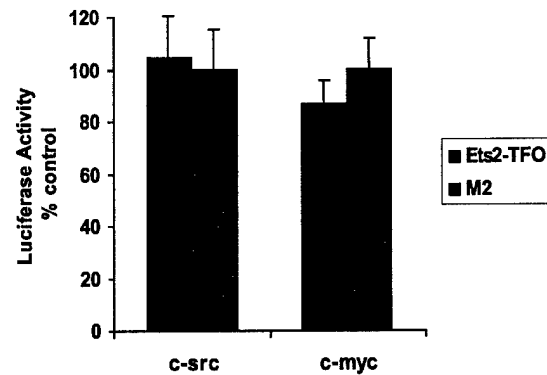


Figure 7. Target specificity of the effects of the Ets2-TFO on promoter reporter activity. DU-145 cells were transfected using DOTAP with the reporter constructs pGL3-c-myc-P1 and pGL3-c-src along with 250 nM of TFO or control oligonucleotide M2 and the pRL-SV40 control vector. Luciferase activity was measured as described in the legend to Figure 5.

Sp1/Sp3 to oligonucleotides containing the myc P1 promoter sequence was not affected by the Ets2-TFO (data not shown). As shown in Figure 7, the Ets2-TFO did not inhibit the activity of the c-myc and c-src promoter constructs at concentrations that induced significant inhibition of Ets2 promoter. Thus, these data indicated that the effects of the TFO depended strictly on the presence of the complementary target sequence and were specific for the targeted gene promoter. The TFO was unable to affect activity of promoters with similar purine-rich sequences and Sp1 sites, suggesting that the inhibition of Ets2 promoter by the TFO was not due to non-specific interactions of the TFO with transcription factors or other components of the transcription machinery.

TFO-mediated inhibition of expression of the endogenous Ets2 gene

The results described above indicated that the Ets2-TFO was able to bind to the target sequence *in vitro* and inhibit Ets2 promoter activity in cells in a sequence- and target-specific manner. These data suggested that the Ets2-TFO could be used to selectively downregulate expression of the endogenous Ets2 gene. To determine the effect of the TFO on Ets2 expression, DU145 cells were transfected with 200 nM of TFO or control oligonucleotide and total RNA was isolated after 24 and 48 h. The level of Ets2 RNA was determined by RT-PCR under conditions that ensured that the amount of amplified product was proportional to the initial amount of RNA template as described in Materials and Methods (Fig. 8A). Ets2 RNA was reduced by ~40 and 50% at 24 and 48 h, respectively, in TFO-treated cells compared with untreated and control oligonucleotide-treated cells (Fig. 8B and C). The level of GAPDH RNA was similar in TFO- and control-treated cells. In addition, we measured RNA levels of another transcription factor, Ets1, which is highly homologous to Ets2 and is expressed in prostate cancer cells. Ets1 expression was unaffected by the Ets2-targeting TFO (Fig. 8B). We also investigated whether increasing the concentration of TFO would result in greater inhibition of Ets2 transcription. As shown in Figure 8D and E, Ets2 RNA was reduced by ~50 and 65% in cells incubated for 48 h with 200 and 400 nM of TFO compared with untreated control cells, indicating a dose-dependent effect of the TFO on Ets2 gene expression. The

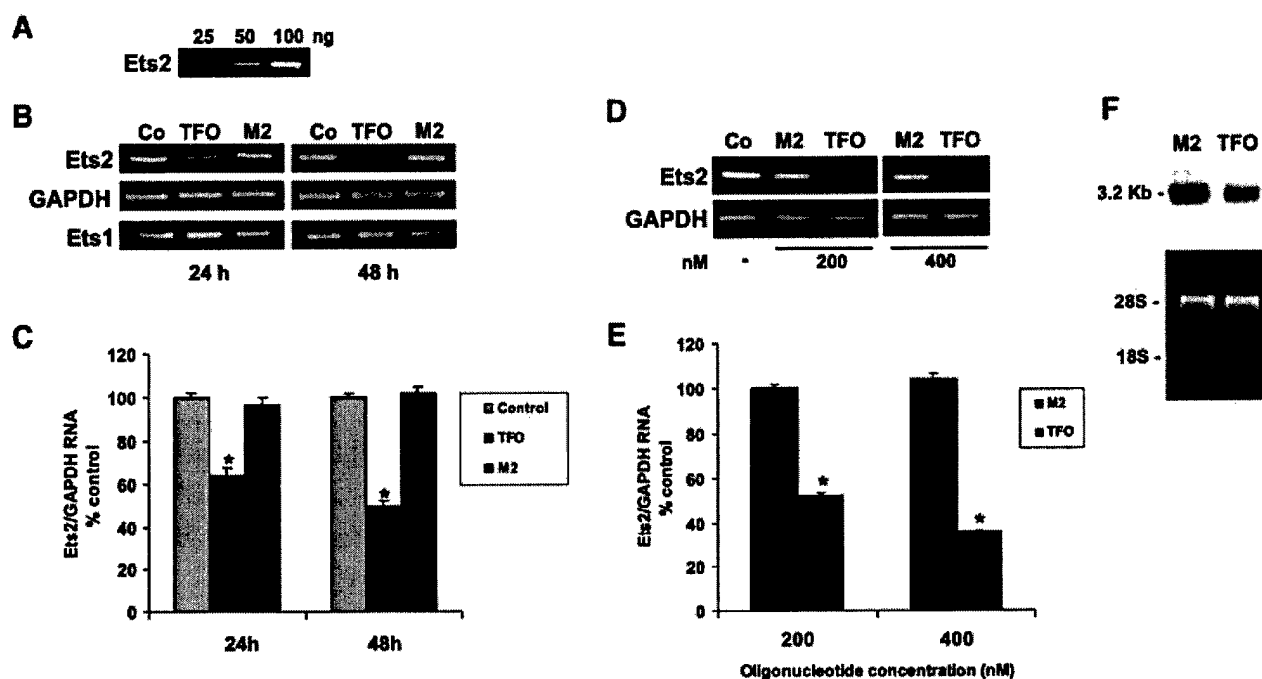


Figure 8. Downregulation of endogenous Ets2 gene expression by the Ets2-TFO. (A) Increased amounts of total RNA from DU145 cells were reverse-transcribed and amplified with Ets2-specific primers. PCR products were separated on 2% agarose gels, stained with ethidium bromide, visualized and quantified using a Chemilmager. (B) DU145 cells were transfected with 200 nM Ets2-TFO or control oligonucleotide M2 using Oligofectamine. After 24 and 48 h total RNA from untreated control (Co), TFO-treated (TFO) and mismatched oligonucleotide-treated (M2) cells was extracted and RT-PCR performed. (C) Densitometric analysis of Ets2 RNA levels at 24 and 48 h after transfection. Data are mean \pm SD from three separate experiments. Asterisks, $P < 0.0005$ at 24 h and $P < 0.0005$ at 48 h compared with untreated control and M2-treated cells. (D) DU145 cells were transfected with 200 and 400 nM of TFO or control oligonucleotide M2. RNA was extracted after 48 h and analyzed by RT-PCR. (E) Densitometric analysis of Ets2 RNA levels in cells incubated with increasing concentrations of TFO and control oligonucleotide. Data are mean \pm SD from two experiments. Asterisks, $P < 0.005$ compared with untreated control and M2-treated cells. (F) RNA from cells transfected with 400 nM of TFO and control oligonucleotide and incubated for 48 h after transfection was subjected to northern blot analysis using an Ets2 cDNA probe. Ethidium bromide-stained gel is shown to confirm integrity and equal loading of RNA.

control oligonucleotide did not affect Ets2 RNA level at these concentrations. We did not detect a significant effect of the TFO on Ets2 gene expression at concentrations < 200 nM (data not shown). Analysis of total RNA from cells incubated with 400 nM TFO or control oligonucleotide by northern blot analysis also showed a reduced level of Ets2 RNA in TFO-treated cells compared with control-treated cells, confirming the data obtained by RT-PCR (Fig. 8F). Similar amounts of ribosomal RNA were detected in the ethidium bromide stained gel indicating equal RNA loading. Collectively, these data indicated that the TFO was able to inhibit transcription of the endogenous Ets2 gene and this effect was specific for the targeted gene.

DISCUSSION

Several studies have established a link between various members of the Ets family of transcription factors and neoplastic transformation (1,9). Increased expression of Ets2 has been associated with initiation and progression of various cancer types (11–14). Inhibition of Ets2 by antisense or dominant negative constructs has been shown to reduce anchorage-independent growth of prostate, breast and thyroid cancer cells (11,12,14). Even partial reduction of Ets2 expression, such as that obtained by disruption of a single

Ets2 allele, limited growth of breast tumors in transgenic mice (13). Thus, a selective inhibitor of this transcription factor would be useful to study its role in cancer development and might have therapeutic applications as an anticancer agent. Our goal in this study was to design an inhibitor of Ets2 using the triplex DNA-based approach. Recent studies have shown successful applications of this approach to induce site-specific mutagenesis, recombination and transcriptional repression in various experimental systems (17). Convincing evidence of triplex formation on chromosomal targets has also been presented (18–22). However, whether the anti-transcriptional effects of TFOs, particularly of those targeting regulatory elements in gene promoters and blocking transcription initiation, are indeed due to a triplex DNA-based mechanism is still controversial. Oligonucleotides could interact in a sequence or non-sequence-specific manner with transcription factors and other components of the transcription apparatus, and inhibit transcription initiation regardless of triplex formation (44–46). In this study, we selected a 25-bp homopurine:homopyrimidine sequence in the Ets2 promoter that appeared optimal for triplex-mediated gene targeting. The sequence was immediately upstream of the transcription start site and overlapped a putative Sp1 binding site. We demonstrated binding of Sp1/Sp3 transcription factors to this sequence by gel mobility shift assay and its relevance for

Ets2 transcription by promoter reporter assays. A PS-modified GT-TFO, directed to this sequence, was able to inhibit nuclear protein binding, promoter activity and expression of the endogenous Ets2 gene. Several lines of evidence support the conclusion that the effects of the TFO were sequence- and target-specific, and that formation of a triplex DNA structure at the target site was required for the anti-transcriptional activity of the TFO. Our results indicate that this TFO can act as a selective transcriptional repressor and suggest that it can be used to antagonize the effects of Ets2 over-expression in cancer cells.

Analysis of the binding properties of the Ets2-TFO showed that it bound to the target DNA with very high affinity. This was consistent with previous reports showing that antiparallel GT-rich oligonucleotides were able to form stable triplex DNA at low concentrations and physiological pH and temperature (33,35–38). The high binding affinity of the Ets2-TFO was probably due to the perfect homopurine: homopyrimidine composition of the target sequence. Any pyrimidine interruption in the purine strand would decrease stability of the triple helix and lower TFO binding affinity (15). Furthermore, the PS modification, which was required to increase nuclease resistance, did not affect binding of the Ets2-TFO. In fact, the K_d value of the PS Ets2-TFO, estimated by gel mobility shift assays, was very similar to that of the corresponding PO TFO. Despite its high affinity, binding of the Ets2-TFO required exact pairing with the target sequence according to the triplex DNA code. Control oligonucleotides with similar length and base composition but a number of mismatched bases relative to the target sequence did not form triplex DNA. Furthermore, the Ets2-TFO did not recognize targets containing even a limited number of mutations compared with the wild type target. It is important to note that we measured binding of the Ets2-TFO in the absence of physiological concentrations of potassium ions, which have been shown to inhibit triplex DNA formation *in vitro* (15,16). However, the relevance of potassium-mediated inhibition of triplex DNA formation by PO and PS oligonucleotides in cells is unclear. Indeed, a number of studies have now provided both direct and indirect evidence of oligonucleotide-mediated triplex DNA formation in cells (18–22). Our present data showing triplex-mediated effects of a PS TFO on promoter activity and endogenous gene expression also support the conclusion that the intracellular concentration of potassium ions may not be a major limiting factor for activity of TFOs in cells.

The sequence targeted by the Ets2-TFO was immediately upstream to the transcription initiation site in the Ets2 gene. The region comprising the target sequence contained multiple putative transcription factor binding sites (29,30). However, the identity of proteins binding to this region and their role in the transcriptional activation was not known. Using gel mobility shift assays, we determined that transcription factors of the Sp1 family bound to the site overlapping the TFO target sequence. The Ets2-TFO inhibited binding of these proteins to the Ets2 promoter in a dose- and triplex-dependent manner. Control oligonucleotides with similar length and base composition that did not bind to the target DNA did not inhibit Sp1/Sp3 binding. Moreover, the introduction of mutations in the target sequence adjacent to the Sp1 site abolished triplex formation and any effect of the TFO on Sp1/Sp3 binding.

These data confirmed that the activity of the TFO depended strictly on the formation of a triple helical complex with the target DNA. These findings also ruled out that the inhibition of Sp1/Sp3 binding was mediated by a direct interaction of the TFO with transcription factors or other proteins in the nuclear extract. Inhibition of Sp1/Sp3 binding, demonstrated in the gel mobility shift assays, could be an important mechanism of TFO-induced inhibition of transcription initiation in cells. In this regard, it is significant that the TFO inhibited Ets2 promoter activity to a degree similar to that achieved by mutating the Sp1 site overlapping the TFO target sequence. It is also relevant that mutations adjacent to the Sp1 site that did not affect Sp1/Sp3 binding induced only a minimal reduction of the Ets2 promoter activity. Sp1 sites may be involved in positioning the transcription start site and may be critical for promoter activity of genes, like Ets2, which lack a TATA box (41). Sp factors can also enhance transcription by facilitating DNA loop formation via protein-protein interaction (41). Thus, by binding to its target sequence, the Ets2-TFO may prevent binding of these trans-activating factors and inhibit formation of an active transcription complex.

In addition to its activity in the cell-free system, the Ets2-TFO was able to inhibit transcription in promoter reporter assays and expression of the endogenous gene in cells at similarly low concentrations. In these experiments a mismatched oligonucleotide, which was unable to form triplex DNA at the Ets2 site, did not inhibit promoter activity and Ets2 expression. The lack of effects of the Ets2-TFO on mutated reporters was also consistent with a triplex-mediated mechanism and indicated that alternative mechanisms of inhibition of transcription initiation by the Ets2-TFO were unlikely. Furthermore, the Ets2-TFO did not affect the activity of promoters of non-targeted genes, although they had significant structural similarity to the Ets2 promoter. Expression of non-targeted genes, such as Ets1 and GAPDH, was also not affected by the Ets2-TFO. Together, these data suggest that binding of the TFO to non-targeted DNA sites might be an unlikely event in the absence of a perfectly matching sequence. The nuclease resistance and high binding affinity of the PS-modified TFO was certainly important in these cellular studies. Intracellular accumulation of PS oligonucleotides is greatly enhanced compared with PO oligonucleotides. As a result of the increased stability, intact TFO would be present in cells for a sustained period, thus favoring triplex formation. However, the Ets2-TFO appeared to be less active on the endogenous gene than on the promoter reporter construct when tested at similar concentrations and under similar conditions. This difference is not surprising and may be due to various factors. Accessibility of a chromosomal target site may be restricted and depend on the transcriptional state of the gene. Faria *et al.* (22) reported significant variability in gene expression levels in single cells over time and markedly different temporal responses to a TFO among individual cells in an unsynchronized cell population. Indeed, the endogenous Ets2 promoter may be accessible to both regulatory proteins and TFO only in cells that are actively transcribing the gene. Thus, restricted accessibility of the endogenous gene promoter combined with heterogeneity of cellular and nuclear uptake of the TFO among cells may have contributed to the apparently reduced efficacy of the Ets2-TFO in inhibiting the endogenous gene.

In the present study, we have shown that transcription inhibition by a TFO targeting the Ets2 promoter was sequence specific and triplex mediated. A concern that we had carrying out these studies was that PS-modified oligonucleotides were known to cause non-specific effects, particularly when used at high concentration (28). An additional concern was the tendency of G-rich oligonucleotides to form self-aggregates (15). Formation of homoduplex and tetraplex structures has been suggested as a possible source of non-antisense or non-triplex mediated effects of G-rich oligonucleotides (24,26,27). Therefore, in this study we used multiple experimental controls to demonstrate the specificity of the TFO effects. These included: (i) mismatched oligonucleotides that conserved nucleotide content and critical sequence elements of the TFO; (ii) oligonucleotide targets and promoter reporter constructs with mutations that prevented binding of the TFO; and (iii) promoter reporter constructs from non-targeted genes that had similar purine-rich tracts and transcription factor binding sites. Control mismatched oligonucleotides ruled out non-sequence-dependent effects that might be due to chemical or nucleotide composition of the TFO. The other experimental controls were important to exclude sequence-dependent, but non triplex-mediated effects of the TFO. Using this combined approach we have shown triplex-mediated inhibition of transcription initiation by a PS-modified GT-rich TFO both in a cell-free system and in cells. Our data suggest that high-affinity and nuclease-resistant TFOs can be used as sequence-specific DNA ligands and gene-specific transcriptional repressors in cells.

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