

Antigene and Antiproliferative Effects of a *c-myc*-targeting Phosphorothioate Triple Helix-forming Oligonucleotide in Human Leukemia Cells¹

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ABSTRACT

The *c-myc* gene is frequently deregulated and overexpressed in human cancers, and strategies designed to inhibit *c-myc* expression in cancer cells may have considerable therapeutic value. The purpose of the present work was to characterize the antigene and antiproliferative activity of a triple helix-forming oligonucleotide (TFO) targeted to a homopurine-homopyrimidine sequence in the P2 promoter of the *c-myc* gene. The TFO was synthesized with phosphorothioate (PS) internucleotide linkages to confer resistance to intra- and extracellular nucleases. This property is required of oligonucleotides designed for *in vivo* testing and therapeutic applications. The PS-TFO was found to form triplex DNA with affinity and specificity comparable with that of the corresponding phosphodiester TFO, as shown by gel mobility shift and footprinting assays. Fluorescence microscopy and polyacrylamide gel analysis showed that the fluorescein-labeled PS-TFO accumulated in nuclei of CEM leukemia cells and remained intact for at least 72 h. Incubation of CEM cells with PS-TFO reduced *c-myc* RNA and protein levels. A single exposure of leukemia cells to the PS-TFO was sufficient to induce dose-dependent growth inhibitory effects. Growth inhibition correlated with accumulation of cells in S phase and with induction of cell death by apoptosis. The PS-TFO was also effective in other leukemia and lymphoma cell lines. Control oligonucleotides had minimal effects in all assays. These data indicate that the *c-myc*-targeted PS-TFO is an effective antigene and antiproliferative agent, with potential for testing *in vivo* as a novel approach to cancer therapy.

INTRODUCTION

The *c-myc* gene encodes a nuclear phosphoprotein, which acts as a key regulator of cell growth and differentiation (1). When dimerized with its partner protein Max, c-Myc functions as a transcription factor, capable of both activating and repressing transcription (1). Although the critical target genes of the Myc/Max heterodimer are not yet fully characterized, its primary activity appears to be stimulation of cell cycle progression (1). Expression of *c-myc* is very tightly regulated in normal cells and correlates closely with proliferation. During quiescence, c-Myc is undetectable, but levels rise rapidly in response to growth factor stimulation and then sharply decrease as cells progress through the proliferative cycle (2). In human cancers, deregulation and inappropriate activation of the *c-myc* gene by various mechanisms is frequent. Examples include Burkitt's lymphoma, in which chromosomal translocations place *c-myc* adjacent to immunoglobulin gene elements, causing deregulation of *c-myc* expression (3). Increased expression of *c-myc* as a result of gene amplification is seen in breast, prostate, lung, and other cancers (4). Importantly, elevated expression of *c-myc* is frequently observed in cancer cells with no translocation or amplification of the gene (4). This is likely due to activities of other

oncogenes and transforming proteins, which can directly or indirectly regulate expression of *c-myc* (1).

Given the frequency of *c-myc* deregulation and overexpression in cancer, it is reasonable to suppose that the activity of the protein contributes to the growth and survival of cancer cells. Although the complex molecular pathways of c-Myc activity have not yet been fully elucidated, available evidence strongly supports the idea that high levels of c-Myc can drive cells toward uncontrolled growth, and conversely, that reduced levels promote differentiation and cessation of growth (5). This has led investigators to measure the effects of blocking *c-myc* expression in cancer cells. The approach most commonly taken to date has used AOs³ (3) to reduce translation of *c-myc* mRNA. AOs have been shown to reduce c-Myc protein levels and to inhibit growth of leukemia (6–9) and other cancer cell types (10–13). These studies support the idea that blocking *c-myc* expression can reduce proliferation in a variety of cancer cells and encourage further investigation of oligonucleotide-based strategies for therapeutic applications.

An alternative strategy to antisense uses a triplex DNA-based approach to inhibit gene transcription. Unlike AOs, TFOs act directly on the gene by binding to duplex DNA in a stable, sequence-specific manner. Purine-rich (GA) or mixed purine- and pyrimidine-rich (GT) TFOs bind in antiparallel orientation to the polypurine strand of the target duplex, forming G·G:C and A·A:T or T·A:T base triplets, (conventionally written with the first base in each triplet belonging to the TFO). Binding occurs in the major groove of the double helix, with base pairing to the purine strand of the duplex stabilized by Hoogsteen-type hydrogen bonds (14, 15). Homopurine and homopyrimidine sites are common in gene promoter regions and often overlap binding motifs for transcription-regulatory proteins. Oligonucleotide-directed triplex DNA formation has been shown to inhibit transcription factor binding to purine-rich motifs, and TFOs have been used in this way to block transcription of various genes *in vitro* and in intact cells (16).

The *c-myc* gene contains several sequences suitable for triplex DNA formation. Those studied to date include homopurine tracts located in the two major *c-myc* promoters, P1 and P2. TFOs directed to either of these sites have been found to inhibit transcription of the *c-myc* gene *in vitro* (17, 18) and in cells (19, 20) and to reduce expression of a reporter gene fused to the *c-myc* promoter sequence (21). We have recently compared binding affinities, cellular uptake, and antigene and antiproliferative activities of TFOs targeted to various sequences in the *c-myc* gene, including the P1 and P2 promoter sites. A TFO directed to the P2 site was found to inhibit expression of the endogenous *c-myc* gene and to have the highest antiproliferative activity in leukemia and lymphoma cells (22).

The TFOs used in our earlier studies were composed of DNA having normal PO internucleotide linkages, which are susceptible to rapid cleavage by intra- and extracellular nucleases. This instability would limit the *in vivo* activity and therapeutic potential of the TFO. We therefore considered modifying the structure of the TFO by

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³ The abbreviations used are: AO, antisense oligonucleotide; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PO, phosphodiester; PS, phosphorothioate; TFO, triple helix-forming oligonucleotide.

replacing PO with PS linkages to enhance nuclease resistance. The *in vitro* triplex-forming ability of purine-rich and purine-pyrimidine PS-TFOs has been confirmed against a variety of target sequences (21, 23–25), and *c-myc*-targeted PS-TFOs were found to inhibit expression of a reporter gene cotransfected into HeLa cells (21). However, PS-TFOs have thus far had limited use against chromosomal targets in living cells (24, 25). The goal of the present study was to characterize the antigene and antiproliferative activity of the *c-myc*-directed TFO previously found to be effective and now synthesized with PS linkages to confer nuclease resistance. We found that the PS-TFO was able to form triplex DNA *in vitro* and was efficiently taken up by leukemia cells, where it remained intact for at least 72 h. Examination of the activity of PS-TFO in a lymphocytic leukemia cell line showed that it specifically inhibited endogenous *c-myc* expression, reduced cell proliferation, disrupted cell cycle progression, and induced apoptosis. The antiproliferative effect of the PS-TFO in various leukemia and lymphoma cell lines was significantly increased over that previously measured for the PO-TFO. These results suggest that a nuclease-resistant *c-myc*-targeted TFO can be used as a specific and potent antigene and antiproliferative agent and encourage further development of this approach.

MATERIALS AND METHODS

Oligonucleotides

The sequences of oligonucleotides used in this study and of the double-stranded DNA target on the *c-myc* gene are shown in Fig. 1. Oligonucleotides were obtained from the DNA synthesis facility of the Medical University of South Carolina and were synthesized by the phosphoramidite method with all-PS linkages. Oligonucleotides were deprotected and desalted and then dissolved in water and sterilized by filtration through 0.22- μ m filters. Concentrations were determined by measuring absorbance at 260 nm and using nucleotide extinction coefficients. For use in some of the growth inhibition assays, high-performance liquid chromatography-purified oligonucleotides were purchased from Genset (La Jolla, CA). For uptake studies, a 5' end fluorescein-labeled PS-TFO (F-PSY2T) was purchased from IDT Technologies, Inc., (Coralville, IA) and purified by PAGE. The PO-TFO Myc2T, which is identical in sequence to PSY2T, has been described previously (22). Immediately before addition to binding reactions or to cell cultures, appropriate dilutions of TFOs and control oligonucleotides were heated at 65°C for 10 min and then chilled on ice.

Electrophoretic Mobility Shift Assays

Oligonucleotides corresponding to the purine- and pyrimidine-rich strands of the duplex target were synthesized. The pyrimidine-rich strand was end labeled with [γ - 32 P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) and T4 polynucleotide kinase and then annealed to the complementary purine strand. TFOs and control oligonucleotide were heated at 65°C for 10 min to reduce self-aggregation and then chilled on ice. Oligonucleotides were incubated with duplex DNA (1 nM) for 24 h at 37°C in a buffer containing 90 mM Tris, 90 mM borate (pH 8) and 10 mM MgCl₂ (TBM). Products were resolved by PAGE in TBM under nondenaturing conditions and analyzed after exposure to X-ray film.

DMS Footprinting

Preparation of DNA Target. An *Xba*I fragment comprising 2005 bp of the *c-myc* gene exon 1 was cloned into pGEM-3Z (Promega, Madison, WI). This plasmid was digested with *Pst*I and *Ngo*M IV, a 339-bp fragment containing the TFO target site was excised from an agarose gel, and then DNA was extracted using the Qiaex II kit (Qiagen, Valencia, CA). The fragment was 3' end labeled using the Klenow fragment of DNA polymerase in the presence of [α - 32 P]dCTP (3000Ci/mmol; Amersham). Using this approach, only the strand containing the target sequence (lower strand) was labeled.

Triplex Formation and Probing with DMS. Oligonucleotides were incubated with end-labeled DNA for 24 h at 37°C in TBM. Salmon sperm DNA (2

μ g) and DMS (final concentration 0.5%) were added to binding mixtures and incubated at room temperature for 3 min. Reactions were stopped with 10 μ l of stop solution (1.5 M sodium acetate, pH 7, 1 M β -mercaptoethanol, and 100 μ g/ml yeast tRNA), and then DNA was precipitated with ethanol, washed twice with 70% ethanol, and treated with 10% piperidine at 95°C for 20 min. Piperidine was removed by three rounds of lyophilization, and then DNA was resuspended in formamide dye mix and loaded on a 10% polyacrylamide, 7.5 M urea sequencing gel. Gels were dried and exposed to X-ray film, and results were analyzed by densitometric analysis.

Cell Culture

The following cell lines were used in this study: CEM and Molt-4 T-cell acute lymphoid leukemia, KG-1 and HL60 acute myeloid leukemia, and Raji B-cell lymphoma. CEM cells were cultured in Eagle's minimal essential medium with 10% fetal bovine serum. All other cells were grown in RPMI 1640 with 10% fetal bovine serum. All tissue culture reagents were obtained from Life Technologies (Rockville, MD).

Cell Uptake of Fluorescein-labeled Oligonucleotide

The TFO F-PSY2T, 5' end labeled with fluorescein, was added at a final concentration of 1 μ M to CEM cells (2 ml at 2×10^5 cells/ml) and incubated for 24 h. Cells to be examined after 72 h were plated at a density of 1×10^5 cells/ml in 2 ml of medium, and F-PSY2T was added at a final concentration of 0.5 μ M. Cells were washed twice and then resuspended in 1 ml of PBS containing 10 μ g/ml Hoechst 33258 (Sigma, St. Louis, MO) to stain nuclei. After 10 min of staining, 100 μ l of the cell suspension were transferred to glass slides by centrifugation at 500 rpm for 2 min on a Cytospin 3 (Shandon, Pittsburgh, PA). To help prevent cell lysis during centrifugation, slides were precoated by centrifugation with 100 μ l of 1% BSA in PBS (26). Centrifuged cells were mounted under coverslips in Vecta-Shield antifade medium (Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy. Digital images were captured using Image Pro software. To determine the integrity of TFO associated with cells, aliquots containing 1.6×10^5 cells were removed from samples before Hoechst staining. Cell aliquots were centrifuged, and the pellet was resuspended in 10 μ l of gel-loading buffer containing 95% formamide. Samples were stored at -70°C before electrophoresis on a 15% polyacrylamide, 7 M urea denaturing gel. Bands were detected by fluorimaging (Fluorimager; Molecular Dynamics, Sunnyvale, CA). Mobility and approximate amounts of TFO present in cell lysates were determined by comparison with serially diluted samples of unprocessed TFO, run in adjacent lanes of the gel. Cell number and volume were determined using the Coulter Electronics (Hialeah, FL) Multisizer II.

RNA Analysis

CEM cells (2 ml at 2×10^5 cells/ml) were treated for 24 h with oligonucleotides, and then total RNA was extracted using the guanidine isothiocyanate method (27). For analysis of *c-myc* expression, 7 μ g of RNA were applied to a denaturing agarose gel containing formamide, electrophoresed, and transferred to a nylon membrane. DNA probes were labeled by the random primers system (Life Technologies) using as template a 1.4-kb cDNA fragment encompassing the third exon of *c-myc* (Oncor, Gaithersburg, MD). Probes were hybridized to the blot overnight at 50°C, and then the blot was washed and exposed to X-ray film as described previously (28). The blot was subsequently rehybridized with a probe for GAPDH prepared from cDNA (American Type Culture Collection, Manassas, VA) as described for *c-myc*.

Western Blot Analysis

Oligonucleotides at indicated concentrations were added to CEM cells (2 ml at 2×10^5 cells/ml) and incubated for 24 h, and then cells were analyzed for c-Myc protein levels by Western blotting. Cells (0.5 – 1×10^6) were lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 144 mM NaCl, 0.5% NP-40, 0.5% SDS, 0.1% aprotinin, 10 μ g/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride and then sonicated twice for 4 s. Protein concentrations were measured using the BCA assay (Pierce, Rockford, Ill), and then 20–30- μ g aliquots of protein were separated on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with monoclonal c-Myc antibody 9E10

(Santa Cruz Biotechnology, Santa Cruz, CA). Levels of c-Myc were detected using peroxidase-conjugated sheep antimouse secondary antibody, and the enhanced chemiluminescence (ECL) system (Amersham). Membranes were reprobbed without stripping, using a monoclonal antibody against actin (Oncogene Research Products). X-ray films were scanned, and c-Myc levels were quantified and normalized to actin levels by densitometric analysis using Gel-Pro Analyzer software (Media Cybernetics).

Cell Cycle Analysis

TFO and control oligonucleotide were added at final concentrations of 5 and 10 μM to CEM cells (2 ml at $2 \times 10^5/\text{ml}$) and incubated for 24, 48, and 72 h. For analysis of cell cycle distribution, cells were washed, fixed with ethanol, and stained with propidium iodide, and DNA content was analyzed by flow cytometry as described previously (29). Data were analyzed using ModFitLT (Verity Software House, Inc.).

Nuclear Staining for Detection of Apoptosis

CEM cells (2 ml at $2 \times 10^5/\text{ml}$) were incubated with TFO and control oligonucleotide at 10 μM for 72 h. Approximately 2×10^5 cells were harvested, washed once with PBS, and then stained for 10 min with 10 $\mu\text{g}/\text{ml}$ Hoechst 33258 in 50 μl of PBS. A drop of cell suspension was transferred to glass slides, coverslipped, and examined by fluorescence microscopy.

Cell Growth Measurement

Cells ($1 \times 10^4/\text{ml}$ for CEM and $4 \times 10^4/\text{ml}$ for Molt-4, KG-1, HL60, and Raji) were seeded in 96-well plates at 90 $\mu\text{l}/\text{well}$, and then TFOs and control oligonucleotides were added at concentrations of 1.25–10 μM . Cells were allowed to grow for 96 h, and then the number of viable cells was measured by colorimetric assay using MTT tetrazolium salt (Sigma) as described previously (28). In some experiments, cells were plated in 24-well plates and growth was monitored by cell counting using a Coulter counter.

RESULTS

Oligonucleotide Design. Previous results from our laboratory showed that Myc2T, a PO-TFO targeted to a 23-bp sequence upstream of the P2 promoter region, had antigene and antiproliferative activity in leukemia and lymphoma cells (22). P2 is the major *c-myc* promoter, giving rise to 75–90% of mRNA transcripts. Fig. 1A shows a schematic map of the *c-myc* gene and the location of the TFO target site. The target sequence, shown in Fig. 1B, is in a regulatory region required for transcription from P2 and overlaps binding sites for activating factors MAZ, E2F, and *ets* family members (30, 31). The sequence of PSY2T, the PS-TFO used in the present study, is identical to that of Myc2T. Both TFOs were designed to bind in antiparallel orientation to the purine-rich target strand, with G opposite G:C base pairs and T opposite A:T pairs. T was placed opposite the single pyrimidine (C) interruption in the otherwise homopurine sequence. PSY2A is also designed to form a triplex in the antiparallel motif, differing from PSY2T only in having A opposite A:T base pairs. The PO counterpart of PSY2A was found to be extremely inefficient at triplex formation *in vitro* and to be ineffective in inhibiting *c-myc* expression and cell growth (22). PSY2A was included here in cell growth assays as an additional control. PSY2M, a control oligonucleotide with nucleotide content identical to PSY2T, but scrambled sequence, is also shown in Fig. 1.

Triplex Formation at the *c-myc* P2 Promoter Region. Previous studies have demonstrated *in vitro* triplex formation at the P2 site by Myc2T, a PO-TFO having identical nucleotide sequence to PSY2T (22). To confirm that PSY2T could also form a triplex at its target site, we first used EMSA. In these experiments, binding reactions were incubated for 24 h to ensure that the complexes had reached equilibrium. Incubations of 24 h have been used previously to assess PS-TFO binding *in vitro* (21, 23). Fig. 2A shows that PSY2T at concentrations

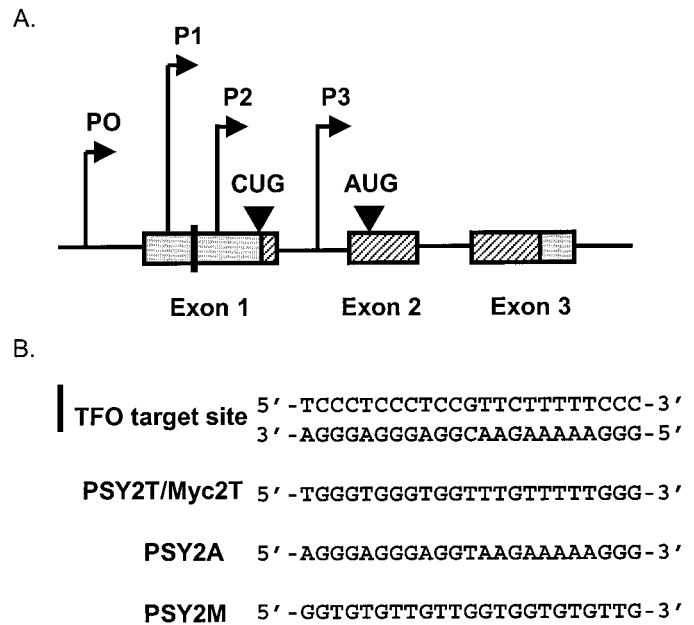


Fig. 1. Location and sequence of the TFO target site on the *c-myc* gene. A, map of the *c-myc* gene. Exons 1–3 are represented by boxes, with the coding region hatched. Translational start sites are indicated by arrowheads, and transcriptional start sites are indicated by arrows. The TFO target site (black rectangle) comprises a 23-bp purine-rich tract located immediately 5' of the P2 transcriptional start site in exon 1. The site includes or overlaps binding sites for proteins regulating transcription from P2. B, sequence of the target site, TFO, and control oligonucleotides. PSY2T is a mixed purine-pyrimidine PS-TFO designed to bind in antiparallel orientation to the purine strand of the target duplex, forming G-G:C and T-A:T triplets, and having T opposite the single pyrimidine (C) in the target sequence. PSY2A is a TFO designed to form G-G:C and A-A:T triplets but exhibits poor binding *in vitro* and is included in tissue culture studies as a control. PSY2M is a control oligonucleotide having nucleotide content identical to PSY2T but scrambled sequence.

of $\geq 5 \mu\text{M}$ was able to retard mobility of the radiolabeled duplex target DNA, indicating triplex DNA formation. Myc2T at 1 μM , also shown in Fig. 2A, caused a similar shift in the mobility of the target duplex. The control oligonucleotide, PSY2M, caused no mobility shift. PSY2T at concentrations of $< 5 \mu\text{M}$ did not appear to alter mobility of target DNA (data not shown), whereas Myc2T caused a complete shift at 1 μM . We have previously addressed the difficulty of detecting triplex formation and estimating the K_D of the P2-targeted TFO due to the small upward shift seen in EMSA experiments (22). Nevertheless, EMSA experiments shown here suggested that PS modification might have decreased binding affinity of the TFO by ~ 5 -fold.

To confirm binding of PSY2T and to examine its sequence specificity, we used DMS footprinting. As shown in Fig. 2B, triplex formation by PSY2T protected the 23-bp target sequence from methylation by DMS, resulting in reduced cleavage by piperidine. No other regions of the 339-bp DNA fragment were protected, and no protection was seen with the control oligonucleotide PSY2M. These results confirm that PSY2T is able to form triplex DNA specifically and selectively at its target site in the *c-myc* P2 promoter region. In this experiment, $\sim 85\%$ protection was achieved with PSY2T at 50 μM , whereas Myc2T gave 100% protection at 35 μM . PSY2T at $\leq 20 \mu\text{M}$ did not prevent cleavage of the target sequence, whereas Myc2T gave complete protection at 20 μM (data not shown). This suggested again that the PS-TFO had reduced binding affinity compared with its PO counterpart, consistent with data from EMSA. Interestingly, the extent of the footprint seen with PS- and PO-TFOs showed that guanines at the extreme 5' end of the target sequence were not protected, suggesting that TFO binding might be less stable at this location.

Uptake and Stability of the *c-myc* TFO in CEM Cells. To investigate whether PSY2T could be used to target the *c-myc* P2

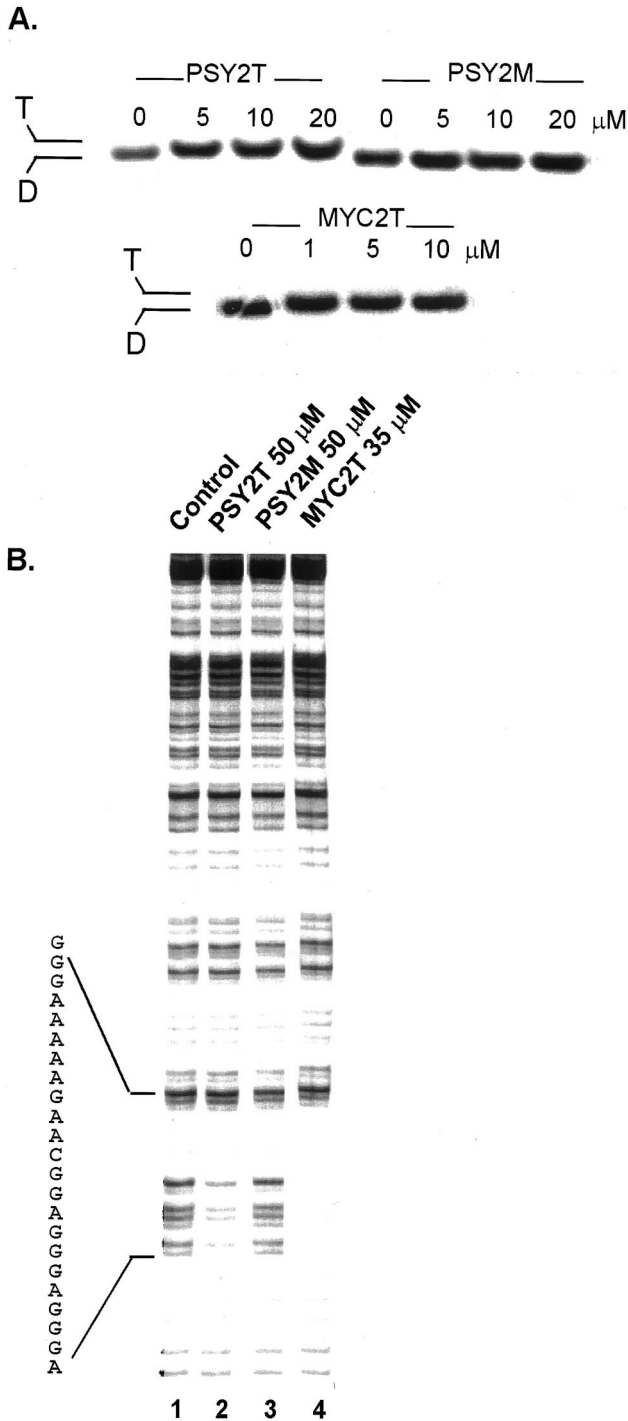


Fig. 2. Specificity of TFO binding to duplex target shown by EMSA and DMS footprinting. **A**, EMSA. The oligonucleotide corresponding to the pyrimidine strand of the duplex target was 5' end labeled with ^{32}P and annealed to the complementary purine strand. Duplex DNA was incubated with TFOs (PSY2T and Myc2T) or control oligonucleotide (PSY2M) at the indicated concentrations for 24 h at 37°C in TBM buffer. Samples were resolved on a nondenaturing polyacrylamide gel. *D* and *T*, double- and triple-stranded DNA, respectively. **B**, DMS footprinting. The 339-bp duplex fragment labeled with ^{32}P on the strand containing the polypurine sequence was incubated alone (control) or with the indicated TFOs or control oligonucleotide for 24 h at 37°C in TBM buffer. After 3 min of exposure to 0.5% DMS, the reaction was stopped, and DNA was recovered and treated with piperidine at 95°C to cleave at methylated guanines. DNA was vacuum dried, dissolved in formamide, and run on a sequencing gel. The position and sequence of the protected polypurine target are shown on the left. PSY2T and PSY2M were present in binding reactions at a concentration of 50 μM, and Myc2T was present at 35 μM. PSY2T and Myc2T have identical sequence but are composed of PS and phosphodiester internucleotide linkages, respectively. The control oligonucleotide PSY2M has phosphorothioate linkages and scrambled nucleotide sequence. Lane 1, control; Lane 2, PSY2T; Lane 3, PSY2M; Lane 4, Myc2T.

promoter in living cells, we assessed the ability of the TFO to be taken up by cells and to resist degradation by nucleases during extended incubation with cells. To assess uptake, PSY2T was 5' end labeled with fluorescein and incubated with CEM leukemia cells for 24 and 72 h. Examination using fluorescence microscopy showed that the majority of cells at both 24 and 72 h exhibited fluorescent staining, predominantly in a punctate pattern, and localized within or peripheral to Hoechst-stained cell nuclei (Fig. 3A). To confirm that the fluorescent labeling detected in CEM cells represented intact TFO, we compared electrophoretic mobility of cell-associated fluorescein-labeled TFO with samples of intact F-PSY2T not incubated with cells. As shown in Fig. 3B, a single band comigrating with intact control TFO was detected in cell lysates after 24 h of incubation, indicating that only full-length TFO contributed to the observed intracellular staining. At 72 h, faint minor bands were also present below the full-length one, suggesting that the TFO had undergone some degradation, likely by progressive loss of bases from the 3' end. The apparently lower amount of TFO present at 72 compared with 24 h reflects the lower initial concentration of TFO added to the medium (0.5 and 1 μM at 72 and 24 h, respectively). By comparing intensities of bands in experimental lanes with the serially diluted control samples shown in Fig. 3B, left, we attempted to estimate intracellular concentrations of TFO at 24 and 72 h. Cell-associated fluorescence represented ~3 and 1.5 pmol of TFO at 24 and 72 h, respectively. Using a mean volume of 1500 fl/cell, mean intracellular concentrations of labeled TFO were calculated to be ~12 and 6 μM at 24 and 72 h, respectively. Although this may not be an accurate measure of intracellular concentration, these calculations suggested that the TFO was able to accumulate in cells, reaching levels ~10 times higher than the concentration in the extracellular medium. These findings are consistent with those reported previously for uptake of PS oligonucleotides in leukemia cells (32, 33) and of PO-TFOs in the same cell line (22).

TFO-mediated Reduction of Endogenous *c-myc* Expression.

The results of footprinting and cell uptake studies indicated that PSY2T could specifically bind the P2 promoter site of the *c-myc* gene *in vitro*, was efficiently internalized by CEM cells, and remained stable in cells for up to 72 h at concentrations potentially sufficient for antigene activity. This indicated that PSY2T could be used to target the *c-myc* gene in living cells. We measured *c-myc* mRNA and protein levels in CEM cells incubated with PSY2T to investigate whether the TFO might affect expression of the *c-myc* gene. Northern blot analysis showed a reduction of ~50% in the level of *c-myc* mRNA in cells incubated for 24 h with 10 μM PSY2T compared with untreated cells (Fig. 4A). The control oligonucleotide PSY2M had no effect on *c-myc* transcription, and levels of GAPDH mRNA were similar in all samples, indicating that the effects of the TFO were specific, and selective for the *c-myc* gene. To confirm that reduction of *c-myc* transcription would result in lower levels of c-Myc protein, we performed Western blot analysis of cells treated with TFO and control oligonucleotide as described for Northern blotting. Two c-Myc proteins were translated from a single transcript, initiated from the alternative codons shown in Fig. 1. The major 64-kDa protein (Myc2) results from initiation at the AUG codon, and the minor 67-kDa species (Myc1) results from the upstream CUG. Fig. 4B shows that the levels of both proteins were reduced by ~50% in cells exposed for 24 h to 10 μM TFO. As was seen in Northern blotting, the control oligonucleotide at 10 μM had no effect on c-Myc protein expression, and the level of actin protein was similar in all samples.

Cell number, assessed with a cell counter at 24 h, was not significantly decreased by incubation with 10 μM concentrations of either the TFO or the control oligonucleotide (88 ± 8 and 96 ± 8% of control, respectively). This indicated that reduced levels of c-Myc at

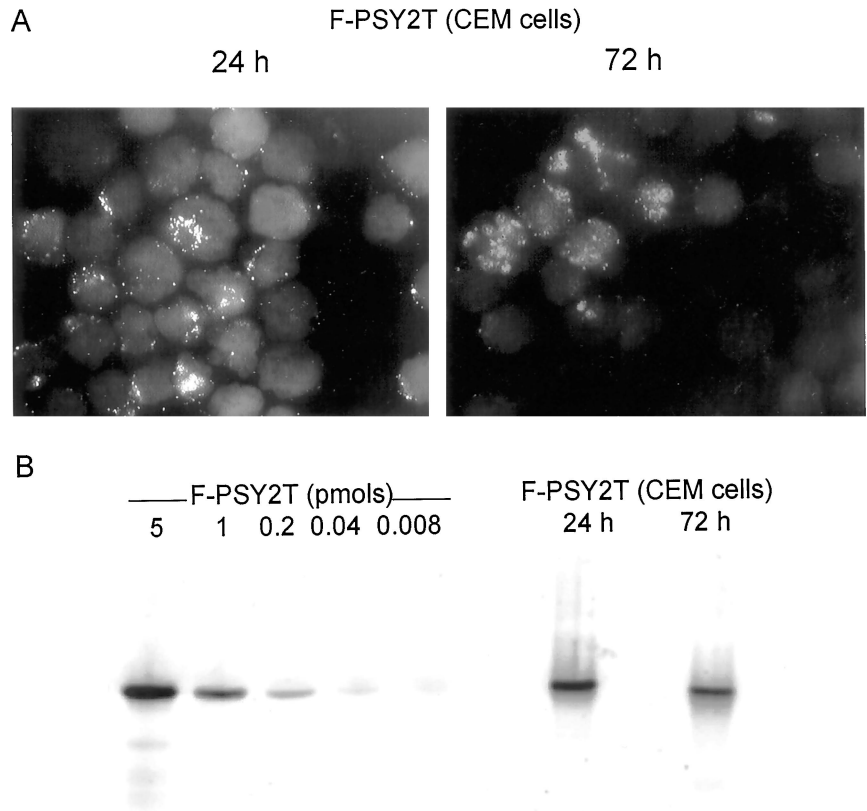


Fig. 3. Uptake and stability of fluorescein-labeled PS-TFO in CEM leukemia cells. CEM cells were incubated for 24 or 72 h with 1 and 0.5 μM fluorescein-labeled PSY2T (*F-PSY2T*), respectively, and then intracellular accumulation of labeled TFO was examined. A, nuclei of live cells were stained with Hoechst, and then cells were transferred to glass slides by centrifugation and analyzed using fluorescent microscopy (magnification, $\times 100$). Fluorescein staining is shown to be associated with the majority of cells at 24 and 72 h, appearing to localize within or peripheral to nuclei. B, Cell lysates (1.6×10^5 cells) were subjected to gel electrophoresis, and cell-associated fluorescein-labeled TFO was visualized by fluorimaging. Integrity of cell-associated TFO was verified by comparing its mobility with samples of intact, labeled TFO in adjacent lanes, shown on the left.

24 h did not correlate with a sharp decrease in cell proliferation. At 48 h, however, PSY2T caused a significant reduction in cell growth compared with PSY2M (54 ± 1 and $79 \pm 6\%$ of control, respectively;

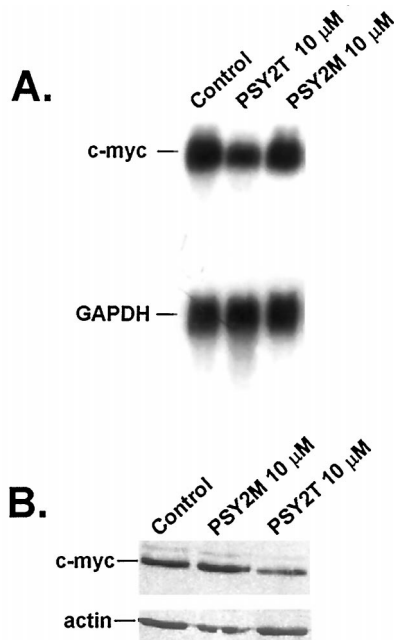


Fig. 4. Reduction of *c-myc* expression in CEM cells by PS-TFO. CEM cells were either untreated (*Control*) or incubated with TFO (*PSY2T*) or control oligonucleotide (*PSY2M*) for 24 h, and then gene expression was analyzed by Northern (A) and Western (B) blotting. Blots were reprobated, and levels of unrelated gene products (GAPDH mRNA and actin protein) were ascertained in each case, to control for nonspecific effects on gene expression and for possible unequal sample loading.

$P < 0.001$, *t* test). This suggested a cause-and-effect relationship between TFO-mediated inhibition of *c-myc* expression and reduced cell proliferation. Western blotting performed at earlier time points (4–10 h) showed no reduction of *c-Myc* protein levels in TFO-treated cells, whereas those done later (48 h) showed continued inhibition of *c-Myc* expression compared with controls (data not shown). Taken together, these findings support the idea that PSY2T can reduce expression of the endogenous *c-myc* gene by forming a triplex at the P2 site. They also indicate that PSY2T has an antiproliferative effect, associated with its ability to reduce *c-myc* expression and clearly distinguishable from the effects seen with the control oligonucleotide. The slight reduction in cell growth induced by the control oligonucleotide may be due to non-sequence-dependent effects previously reported to be associated with PS-oligonucleotides in antisense experiments (34).

During immunoblotting experiments using *c-Myc* monoclonal antibody 9E10, we detected a novel protein band with an apparent molecular mass of ~ 48 kDa, which appeared to be induced in a dose-dependent manner by incubation of CEM cells with PSY2T but not with Myc2T or any other PO-oligonucleotide (data not shown). The protein was visible as early as 6 h after exposure to PSY2T and continued to be expressed at relatively steady levels for at least 72 h. The control oligonucleotide PSY2M also induced expression of the protein but at barely detectable levels. Because recognition of the protein by 9E10 appeared to be highly specific and extended to immunoprecipitation reactions, we wondered whether the protein might be a truncated form of *c-Myc*. Short forms of *c-Myc*, termed MycS, have been detected previously in avian, murine, and human cells. These proteins have apparent molecular masses ranging from 45 to 50 kDa and lack ~ 100 amino acids of the NH_2 -terminal domain, but retain the COOH -terminal domain required for protein dimeriza-

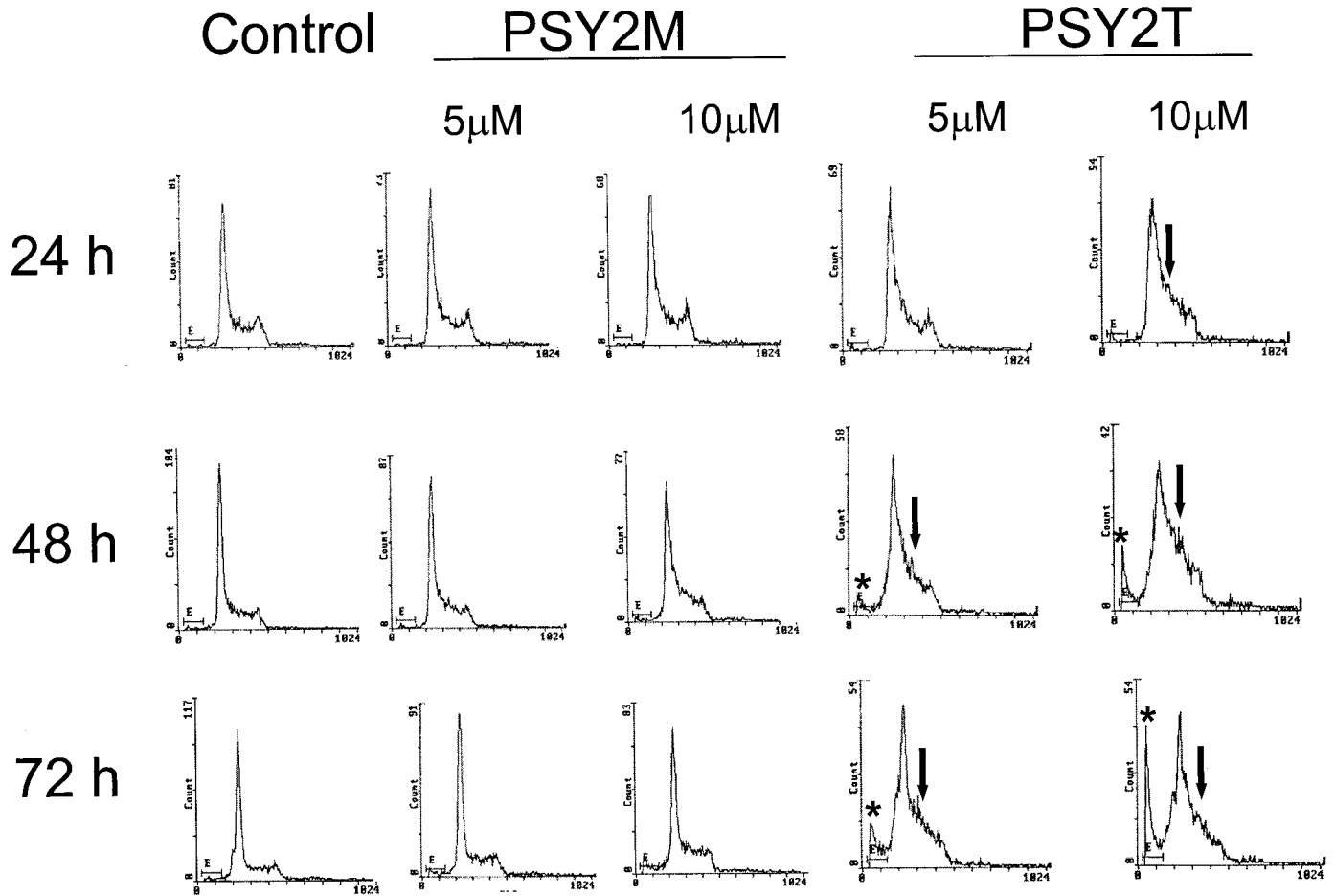


Fig. 5. Induction of S phase arrest and apoptosis by PS-TFO. CEM cells were untreated (*Control*) or incubated with TFO (*PSY2T*) or control oligonucleotide (*PSY2M*) for the indicated times. Propidium iodide-stained nuclei were scored for DNA content by flow cytometric analysis. Equal numbers of events were recorded for each sample (*Y axis*), and increasing DNA content is shown from *left to right* on the *X axis*. In the *right panels*, peaks representing S phase-arrested and apoptotic cells are indicated with *arrows* and *asterisks*, respectively.

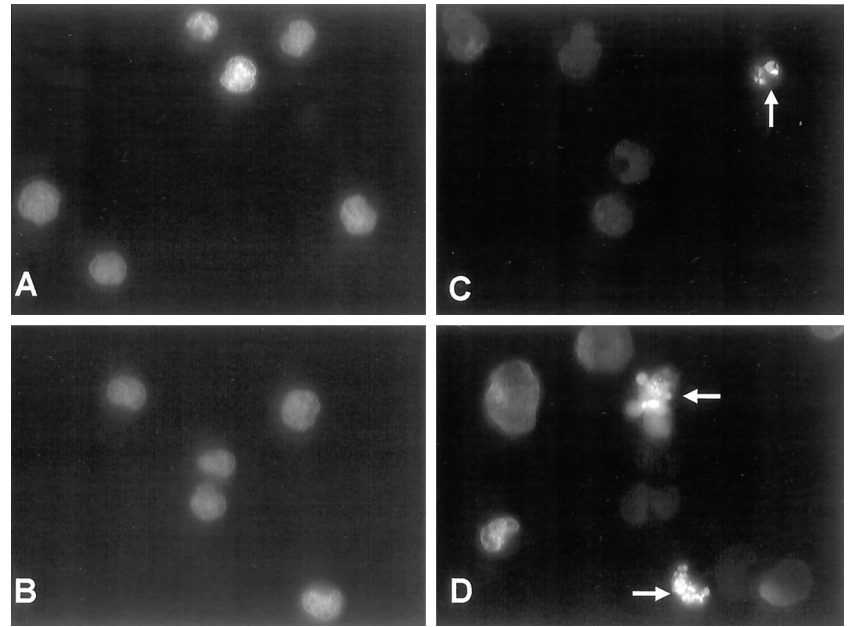
tion and can form heterodimers with Max (35). However, it seemed unlikely that the protein detected in our experiments was MycS or any form of c-Myc. It was not detected in immunoblots either by a polyclonal antibody against a COOH-terminal sequence (Santa Cruz) or by anti-mycf1 and anti-MycN100, recognizing the full-length protein and the NH₂-terminal 100 amino acids, respectively (kindly provided by Dr Stephen R. Hann, Vanderbilt University School of Medicine, Nashville, TN). In addition, we were unable to coimmunoprecipitate the protein using an antibody against Max, under conditions in which full-length c-Myc was coimmunoprecipitated from CEM cell lysates. We subsequently found that the protein was strongly induced by various other PS-oligonucleotides, which were unable to form a triplex but which contained groups of three or more contiguous guanines. None of these oligonucleotides had growth-inhibitory activity equal to PSY2T. The identity as well as the causes of production of this protein remain unknown.

Delayed Cell Cycle Progression and Apoptosis in TFO-treated CEM Cells. Reduced levels of *c-myc* expression have been associated with lengthening of the cell cycle (2), and our results indicated that cell growth was inhibited by exposure to *c-myc* TFOs. We therefore examined cell cycle distribution of CEM cells treated with PSY2T to identify possible effects on cell cycle progression. Flow cytometric analysis, shown in Fig. 5, revealed that PSY2T caused accumulation of cells in S phase beginning at 24 h and continuing through 72 h of incubation. This effect was less marked in cells

exposed to 5 μM compared with 10 μM TFO, indicating dose dependence. S phase cells increased to 60% at 24 h and 68% at 48 h in samples incubated with 10 μM PSY2T, representing increases of 18 and 22%, respectively, compared with control samples. The control oligonucleotide had little effect on cell cycle distribution, with the highest concentration causing only a slight increase in S phase cells at 48 h, compared with control samples. No increase in the G₀-G₁ population was detected in cells treated with any concentration of PSY2T or control oligonucleotide. In addition to S phase arrest, PSY2T appeared to induce apoptosis, as evidenced by the presence of a sub-G₁ peak at 48 and 72 h. At 72 h, apoptotic cells accounted for 10.7 and 18% of the cell population treated with 5 and 10 μM PSY2T, respectively. Few apoptotic cells were detected in untreated or control oligonucleotide-treated cell samples (<3% in all except at PSY2M at 10 μM , 72 h, in which 6.3% of the cells were found in the sub-G₁ population). To confirm induction of apoptosis by PSY2T, we examined morphology of stained nuclei at 72 h. Fig. 6 shows that approximately one in five cells exposed to the TFO had condensed chromatin and nuclear fragmentation characteristic of apoptosis. This number correlated with the percent of total cells identified by flow cytometry as having sub-G₁ DNA content at 72 h. Fewer than 5% of control and PSY2M-treated cells had apoptotic nuclei.

Reduced Growth in Leukemia and Lymphoma Cells Exposed to *c-myc* TFO. Results of cell cycle analysis indicated that decreased cell number at 48 h correlated with S phase arrest and apoptosis. To

Fig. 6. Morphological evidence of apoptosis in TFO-treated CEM cells. CEM cells were untreated or incubated with TFO and control oligonucleotide ($10 \mu\text{M}$) for 72 h. Live cells were Hoechst stained and transferred in suspension to glass slides for fluorescent microscopic analysis. Typical fields are shown. A, untreated control cells; B, PSY2M-treated cells; C and D, PSY2T-treated cells at $\times 40$ and $\times 100$ magnification, respectively. Apoptotic cells with condensed chromatin and fragmented nuclei are indicated by arrows.



further assess effects of the TFO on the growth of CEM cells, we measured cell viability after 96 h of incubation after a single addition of PSY2T and control oligonucleotides. As shown in Fig. 7, a dose-dependent reduction in growth compared with untreated controls was measured in cells exposed to PSY2T. At the highest concentrations of 5 and $10 \mu\text{M}$, growth was inhibited by 75 and 90%, respectively. A slight growth-inhibitory effect was also measured in cells incubated with the control oligonucleotide PSY2M (25% at $5 \mu\text{M}$ and 35% at $10 \mu\text{M}$), suggesting again that slight non-sequence-specific effects were associated with exposure of cells to high concentrations of PS oligonucleotides. The TFO PSY2A was included in growth assays as an additional control. The PO counterpart of this TFO was previously found to have poor triplex-forming ability *in vitro* and minimal inhibitory effect on *c-myc* gene expression and cell growth (22). It remained possible that PS modification could enhance the ability of

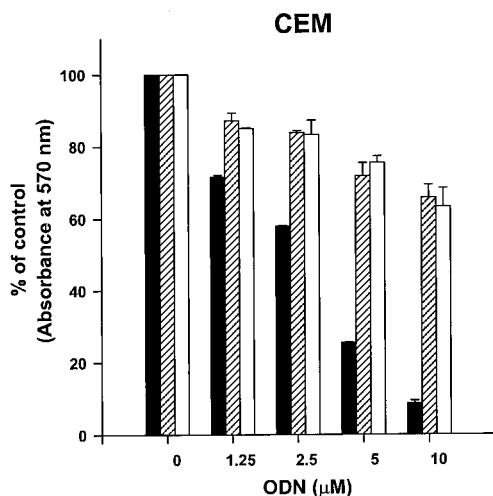


Fig. 7. Inhibition of CEM cell growth by PS-TFO. CEM leukemia cells were treated at the time of seeding with PSY2T (black bars), PSY2A (white bars), or PSY2M (hatched bars). After a 96-h incubation, the number of viable cells was determined by the MTT assay. Absorbance at 570 nm was measured, and the results are expressed as percentage of absorbance in untreated samples. Triplicate samples were analyzed in each of three experiments, and mean \pm SD of the results of a representative experiment are shown.

the TFO to bind its target DNA in cells; however, PSY2A was no more active in cells than PSY2M (Fig. 7).

We then extended our studies of antiproliferative activity of the TFO to include other leukemia cell lines, Molt-4, KG-1, and HL60. HL60 contains ~ 20 copies of the *c-myc* gene (4, 36). Also included were Raji B-cell lymphoma cells, with a t(8;14) chromosomal translocation involving the *c-myc* gene and the immunoglobulin locus. The translocation break point in Raji cells is upstream of the first *c-myc* exon (37). Fig. 8 shows that in all cell lines tested, PSY2T inhibited growth in a dose-dependent manner and with efficacy ($>80\%$ at $10 \mu\text{M}$) similar to that measured in CEM cells, whereas control oligonucleotides had only minimal effects. These results support the conclusion that the antiproliferative activity of the TFO is specific, reproducible, and effective in the context of different cell types and genetic abnormalities.

DISCUSSION

In this study, a PS-TFO targeted to a site in the P2 promoter was found to inhibit *c-myc* expression and to significantly reduce growth of leukemia and lymphoma cells. We previously showed that the PO counterpart of this TFO had the highest antigenic and antiproliferative activity of a panel of *c-myc*-targeted PO-TFOs (22). The present work further explored the potential of the P2-targeted TFO for *in vivo* studies by asking whether replacement of PO with PS internucleotide linkages would enhance, or adversely affect, the activity of the TFO. Increased nuclease resistance, conferred by backbone modification, is essential for survival of oligonucleotides when administered to animals (38) and is therefore a prerequisite of any TFO proposed for *in vivo* testing. Our results indicated that PS modification improved activity of the TFO in cells, encouraging further study of its possible therapeutic applications.

Our examination of *in vitro* binding characteristics of the PS-TFO showed that it was able to bind specifically to its target duplex but with slightly reduced affinity compared with the PO-TFO. This finding is consistent with other comparative studies (23, 25, 39), and we have observed slightly reduced affinity of PS- compared with PO-

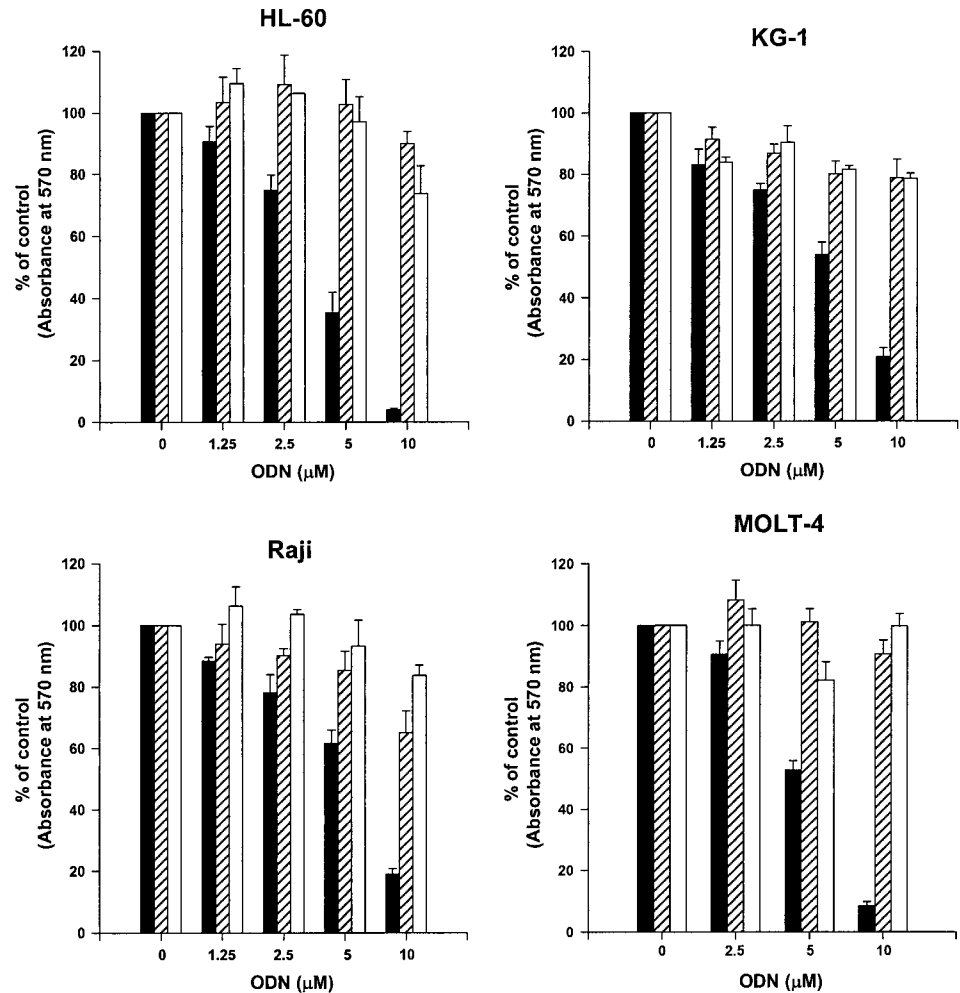


Fig. 8. Inhibitory effects of PS-TFO on growth of various leukemia and lymphoma cells. HL60 myeloid leukemia, KG-1 myeloid leukemia, Raji B-cell lymphoma, and Molt-4 T-cell leukemia cells were treated with the indicated concentrations of PSY2T (black bars), PSY2A (white bars), or PSY2M (hatched bars). Cell numbers were measured by MTT assay at 96 h as described in Fig. 7. Absorbance at 570 nm is expressed as a percentage of that obtained from untreated control cells. Data shown are mean \pm SD of triplicate samples in a representative experiment. Similar results were obtained in at least two separate experiments.

TFOs targeted to other sites.⁴ However, PS-TFOs have been found to form triplex DNA more efficiently than their PO counterparts in the presence of intracellular concentrations of potassium ions (24), suggesting that PS modification may be advantageous for binding *in vivo*. Furthermore, the increased stability and endurance in the cellular environment might allow the PS-TFO to reach and maintain intracellular concentrations sufficient to ensure adequate binding to DNA in the nucleus. This was supported by our finding that the PS-TFO was efficiently taken up by CEM cells and remained stable for at least 72 h at concentrations significantly higher than those present in the extracellular medium. Interestingly, preferential uptake of oligonucleotides by leukemic cells has been demonstrated *in vivo* (40) and *in vitro* (41), leading to speculation that oligonucleotide-based therapies might be particularly effective against leukemia (42).

The PS-TFO significantly reduced levels of *c-myc* mRNA and protein in CEM cells without affecting levels of GAPDH or actin, suggesting that inhibition of *c-myc* expression was specific. We have previously discussed evidence supporting the idea that inhibition of *c-myc* expression by the PO counterpart of PSY2T was due to triplex formation (22). Similar considerations lead us to believe that the effects of PSY2T are also triplex mediated. First, the effect of the PO-TFO was highly sequence and target specific, because none of a large panel of related oligonucleotides had comparable activity (22). Second, a similar TFO targeted to the site upstream of P2 was able *in*

vitro to block transcription from the P2 start site (18). The PS counterpart of this TFO reduced *c-myc* promoter-dependent expression of a reporter gene when cotransfected into cells (21). Finally, the sequence of Myc2T and PSY2T being neither identical nor complementary to either strand of the target duplex reduces the likelihood that other possible gene-specific inhibitory mechanisms, such as antisense activity, or competition with DNA binding proteins contribute to the observed effect. Protein binding has previously been invoked as a possible explanation for oligonucleotide-mediated inhibition of *c-myc* expression (43, 44). In this case, the sequence and orientation of the GA-containing oligonucleotide were identical to those of a purine-rich site upstream of the P1 promoter start site (17, 20). It was later found that this oligonucleotide could bind in a sequence-specific manner to a transactivating factor required for *c-myc* expression (43). In addition, the oligonucleotide could hybridize to the complementary strand of the target duplex and prevent binding of another transactivating factor to the pyrimidine-rich sequence (45). Previously, we found that a GA-containing TFO (Myc2A) directed to the P2 promoter site had minimal inhibitory effects on *c-myc* expression and cell growth (22). In the present study we ascertained that the GA-containing PS-TFO PSY2A was similarly ineffective in inhibiting cell proliferation. PSY2A, like its PO counterpart, has sequence almost identical to that of the purine strand of the target duplex, with only one base mismatched, and would therefore be more likely to exert any possible non-triplex-specific effects due to an antisense or decoy-like mechanism. It is notable that the majority of the groups who reported

⁴C. V. Catapano and G. M. R. Carbone, unpublished results.

inhibition of endogenous gene expression used GT- rather than GA-containing TFOs (19, 22, 24, 46–51). This suggests that the GA variation of the purine-rich motif may be less suited to *in vivo* applications, probably because of reduced efficiency of triplex DNA formation.

Caution is appropriate, however, when examining growth-inhibitory effects of GT-TFOs. Recent studies have shown that PO-oligonucleotides composed of these bases can exert growth-inhibitory effects in cancer cells due to recognition by specific cellular proteins of oligonucleotide sequence (52) or three-dimensional structure (53). PS modification was subsequently shown to abolish sequence-related protein binding and cytotoxic activity of a series of non-triplex-forming GT-oligonucleotides in CEM cells (54). However, this does not rule out the possibility that the biological effects of PSY2T might result from activities other than triplex DNA formation. Demonstration of oligonucleotide-directed triplex formation in cells would be helpful in this respect. Genomic footprinting offers a means to do this, but difficulties have been reported in using this technique to detect the triple helix (55), and to the best of our knowledge, it has not yet been applied successfully in this regard. Oligonucleotide-directed triple helix formation on chromosomal DNA, however, has been demonstrated by other means (56, 57), lending impetus to further development of *in vivo* detection techniques.

The antigene activity of the *c-myc*-targeted TFO was accompanied by apparent delay in S phase, with concurrent induction of apoptosis. In normal cells, down-regulation of *c-myc* expression is associated with accumulation in G₁ (2). This was also seen when c-Myc activity in colon cancer cells was reduced by exogenous expression of mutant Max (58) or by AOs (11). However, accumulation in S phase has accompanied antisense-mediated reduction of *c-myc* expression in melanoma (59) and chronic myeloid leukemia (9) cells, suggesting that cell cycle-related activity of c-Myc might vary between cancer cell types. In accordance with our findings, induction of apoptosis was associated with reduced c-Myc and S phase delay in both studies cited above. The role of c-Myc in apoptosis is also complex, because under certain circumstances, overexpression of the gene can induce apoptosis in normal cells (60). However, this does not appear to be a dominant effect in cancer cells, where high levels of c-Myc are compatible with vigorous growth, and reduced levels can lead to growth arrest and cell death.

It seemed possible that added nuclease resistance of the PS backbone would enhance the antiproliferative effects of a TFO already moderately active as a PO. The PS-TFO was indeed found to inhibit cell growth more strongly and at ~5-fold lower concentrations than the PO-TFO (22). The observed increase in activity probably was largely due to stability against degradation, which extended the life of the TFO in the intra- and extracellular environment, so that high intracellular concentrations were maintained for several days. An additional source of increased activity may derive from improved ability of PS-TFOs to form triplex DNA in the presence of monovalent cations (24). To the best of our knowledge, there are few previous reports of PS-TFOs being used to reduce expression of an endogenous gene in intact cells (24, 25). Other investigators have used unmodified PO-TFOs (19, 20, 46, 61), or PO-TFOs with 3'-terminal modifications, which confer partial nuclease resistance (22, 47–51). The lack of use of PS-TFOs is in contrast to the antisense field, where PS-oligonucleotides have been extensively used and have progressed from tissue culture experiments to toxicology testing in animals and to evaluation in clinical trials (38, 62). One caveat to the use of all PS-oligonucleotides is their known propensity, at high concentrations, to cause nonspecific cellular and systemic effects (63). In this regard, we noted induction of expression of an unknown protein in CEM cells by a variety of G-rich PS-oligonucleotides, and we consistently mea-

sured a slight growth-inhibitory effect associated with high concentrations of non-triplex-forming control oligonucleotides. However, much progress has been made in the antisense field using PS-oligonucleotides and, later, partially thioated oligonucleotides. Advances in triplex technology may follow a similar course. It is worth noting that antisense activity usually requires multiple doses, whereas we have found that a PS-TFO exhibits strong antigene and antiproliferative activity after a single dose. This may help avoid accumulation of toxic concentrations of PS-TFOs in the intra- and extracellular environment.

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