Inhibition of Transcription of the Human c-*myc* Protooncogene by Intermolecular Triplex†

Hyung-Gyoon Kim,‡ James F. Reddoch,‡ Charles Mayfield,§ Scot Ebbinghaus,§ Nadarajah Vigneswaran,§ Sheila Thomas,§ David E. Jones, Jr.,§ and Donald M. Miller*,‡,§

Department of Biochemistry and Molecular Genetics and Bolden Laboratory, Department of Medicine, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-0001

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ABSTRACT: Triplex-forming oligonucleotides (TFOs) have been shown to inhibit both transcription in vitro and the expression of target genes in cell culture by binding to polypurine/polypyrimidine sequences in several human gene promoters. The c-*myc* protooncogene is overexpressed in a variety of human cancers and appears to play an important role in the proliferation of these cells. In an attempt to assay the ability of triplex-forming oligonucleotides to inhibit expression of a target gene in vivo, we have developed a cellular system involving transfection of a c-*myc* promoter-driven luciferase reporter plasmid with triplex-forming oligonucleotides targeted to the human c-*myc* protooncogene. To increase the stability of the TFO, we have used modified phosphorothioate oligonucleotides. Triplex formation with a modified phosphorothioate oligonucleotide occurs with approximately equal binding affinity as that seen using a phosphodiester oligonucleotide. Phosphorothioate-modified TFOs targeted to c-*myc* inhibit transcription of the c-*myc* promoter in HeLa cells as demonstrated by a decrease in luciferase expression from a luciferase reporter gene construct. These results suggests that triplex formation may represent a gene-specific means of inhibiting specific protooncogene expression.

The c-*myc* protooncogene performs a crucial function in normal cell proliferation (*1*−*3*) and programmed cell death (*4*−*6*). The rapid induction of c-*myc* mRNA appears to be necessary for the entry of most quiescent cells into the cell cycle (*7, 8*), while terminal differentiation has been linked to the cessation of c-*myc* expression (*9*−*11*). c-*myc* gene expression is shown in a wide variety of neoplasms and result in an increased level of normal c-*myc* protein. Expression of the c-*myc* gene is regulated by a number of mechanisms including transcriptional initiation, elongation, and attenuation, as well as posttranscriptional processes including variations in mRNA stability (*1, 3*). The contribution of each mechanism to determine steady-state levels of c-*myc* expression varies among cell types, and in some instances a combination of mechanisms may be responsible for a steady-state level of c-*myc* mRNA (*8, 12, 13*). The c-*myc* mRNA is encoded by three exons; the major polypeptide coding domain of the gene is contained within the second and third exons, and large portions of the first and third exons constitute the 5′ and 3′ untranslated regions. The 5′ and 3′ untranslated regions are highly conserved and share approximately 75% homology between the mouse and human (*14*). This evolutionary conservation of the untranslated regions suggests that they may play an important role in regulating the differential expression of the c-*myc* gene. Transcription of the human c-*myc* is initiated from two major start sites designated P1 and P2, which are positioned 161 bp[1] apart (*15*). Cis-acting elements residing in the first exon and intron of c-*myc* have been shown to regulate P2 transcription initiation. Two of these regulatory elements, ME1a1 and ME1a2, are positioned between P1 and P2. Deletion of the ME1a1 binding site results in a loss of P2 transcriptional activity (*16*). Recently, a human gene encoding a zinc finger protein named MAZ (*myc*-associated zinc finger protein) has been shown to bind to the ME1a1 site. A sequence element (GGCGGGAAAA) located between ME1a1 and ME1a2 is conserved between the mouse and human and binds the E2F transcription factor (*17, 18*). Since these two sites are essential for transcription of c-*myc* from P2, the inhibition of protein binding by triplex formation represents a method to attenuate specific c-*myc* P2 transcription initiation.

Purine- and pyrimidine-rich oligonucleotides targeted to purine−pyrimidine-rich sequences form pur*pur·pyr and pyr*pur·pyr intermolecular triple helices (*19*−*24*) and inhibit the binding of protein to target DNA (*23*−*28*). The oligonucleotide third strand occupies the major groove of the duplex, forming Hoogsteen hydrogen bonds with the

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* To whom correspondence should be addressed: phone (205) 934-1977; Fax (205) 975-6911.

‡ Department of Biochemistry and Molecular Genetics.

§ Bolden Laboratory, Department of Medicine.

[1] Abbreviations: bp, base pair; TFOs, triplex-forming oligonucleotides; EMSA, electrophoretic mobility shift analysis; PNK, polynucleotide kinase; P.S., phosphorothioate.

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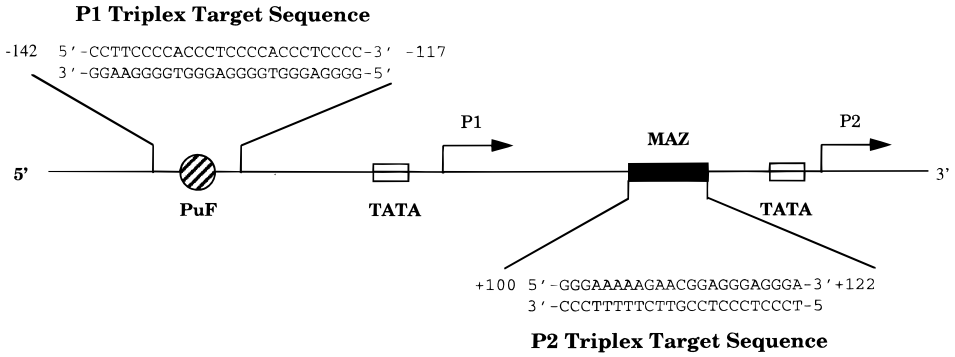

FIGURE 1: Schematic diagram of the human c-*myc* promoter showing binding sites for the transcription factors, PuF and MAZ, important for transcriptional activity. Target sites for triplex formation are indicated relative to the transcription start site of the P1 promoter.

purine bases of the duplex (*19*, *29*). Both pur*pur·pyr and mixed pur/pyr*pur·pyr triplexes can be formed at physiological pH with predominantly G*G·C triplets along with A*A·T and T*A·T triplets interspersed (*29*−*31*). Purine-rich and mixed purine/pyrimidine third strands bind to their target sequences in an antiparallel orientation with respect to the purine-rich strand of duplex target (*29*, *31*). Giovannangeli et al. (*32*) have also demonstrated that an oligonucleotide containing three bases (thymine, cytosine, and guanine) is capable of binding to the target in a parallel orientation with respect to the homopurine sequence of a homopurine/homopyrimidine target of human immunodeficiency virus (HIV) proviral DNA.

It has been previously shown that a discrete 27 base oligonucleotide forms a stable triplex within the P1 promoter region of the human c-*myc* gene −116 to −142 base pairs upstream from P1 transcription start site and is capable of inhibiting c-*myc* transcription in vitro and in HeLa cells (*33*). This region contains the binding site for the transcription factor PuF, which is required for in vitro initiation of mRNA synthesis from the c-*myc* P1 promoter (*30*, *34*, *35*). We have identified the target site for triplex formation in the human P2 promoter +100 to +122 relative to the P1 transcription start site. This region of the c-*myc* P2 promoter is also known to bind to several transcription factors required for P2 promoter activity. We have shown that this TFO is capable of inhibiting in vitro runoff transcription (*36*).

Using purine-rich TFOs targeted either to the region of the c-*myc* P1 promoter containing the PuF binding site or to the c-*myc* P2 promoter encompassing the MAZ/E2F binding sites, we demonstrated the ability of triplex-forming oligonucleotides to inhibit specific transcription of a c-*myc* reporter gene in HeLa cells.

MATERIALS AND METHODS

Oligonucleotides. Unmodified phosphodiester oligonucleotides (sequences shown in Figure 1) were synthesized on a Milligen Cyclone Plus DNA synthesizer using standard phosphoramidite chemistry. All oligonucleotides were purified by OPEC. Phosphorothioate oligonucleotides were purchased from Oligo Etc. The structural integrity and purity of each oligonucleotide was verified by 5′ ^{32}P labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase (PNK) followed by electrophoresis on a polyacrylamide gel. The concentration of oligonucleotides was determined by measurements of UV absorption at 260 nm using the following molar extinction coefficients for each base:15 400 (A), 7300 (C), 11 700 (G) and 8800 cm^{-1} M^{-1} (T).

Electrophoretic Mobility Shift Analysis. For the oligonucleotide titration experiments the synthetic pyrimidine-rich strands of c-*myc* P1 and P2 targets were 5′ labeled with [γ-^{32}P]dATP and T4 PNK. The duplex was formed by heating the labeled strand with a complementary purine-rich strand for 5 min at 95 °C and then cooling slowly to room temperature. After incubation with either P1- or P2-targeted TFO or control oligonucleotides in a buffer consisting of 90 mM Tris, 90 mM borate (pH 7.4), and 10 mM $MgCl_2$ (TBM buffer) for 24 h at 37 °C, products were analyzed on a 16% nondenaturing polyacrylamide gel. To stabilize triplexes, both the running buffer and polyacrylamide gel contained 90 mM Tris, 90 mM borate (pH 7.4), and 10 mM $MgCl_2$.

Plasmid Construction. pMAMneoluc (Clontech) was cut with *Nhe*I and both ends were filled with the Klenow fragment of DNA polymerase I. pSV2Neo-*myc* containing a 12.5 kb fragment of the human c-*myc* gene was cut with *Pvu*II and separated on a 1% low melting point agarose gel. The 862 bp fragment containing the human P1 and P2 promoters was purified and the ends were filled with the Klenow fragment of DNA polymerase I to make the ends blunt. The 862 bp c-*myc* promoter fragment was cloned into the pMAMneoluc vector by blunt-end ligation. The resulting plasmid is designated as pMYCLUCneo.

Cell Line. The HeLa human cervical carcinoma cell line was obtained form the American Type Culture Collection. It was grown in cell culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were cultured at 37 °C with 85% humidity and 5% CO_2.

DOTAP/DOPE Preparation. A liposome vehicle consisting of a 1:1 (w/w) mixture of the cationic lipid DOTAP [1,2-dioleoyloxy-3-(trimethylammonio)propane] and the neutral lipid DOPE (dioleoylphosphatidylethanolamine) (Avanti Polar Lipids) has been shown in our laboratory to display transfection properties similar to Lipofectin (Gibco BRL) but with less toxicity (unpublished observations). DOTAP/DOPE liposomes were prepared by mixing 0.5 mg of DOTAP and 0.5 mg of DOPE and evaporating the chloroform solvent. Following the addition of 500 μL of cyclohexane, the mixture was placed on dry ice and lyophilized. One milliliter of sterile water was added to the powdered lipids and the solution was vortexed every 5 min for 30 min.

Triplex Formation for Transient Transfection. pMYCLUCneo (1 μg) was incubated with a 10 000-fold molar

excess of TFO or control oligonucleotides in 50 μL of 1× TBM for 5 h at room temperature. A Chroma-spin 100 column (Clontech) was equilibrated with 1× TBM, and buffer was removed by spinning at 700*g* for 3 min. After incubation, DNA was put into the Chroma-spin 100, and plasmid was separated from excess oligonucleotide by spinning the column at 700*g* for 5 min.

Transfection. Cells were seeded at 40% confluency in a 24-well plate and allowed to grow overnight. Immediately prior to transfection, cells were washed three times with sterile PBS. To transfect one well, 4 μg of DOTAP/DOPE was mixed with 1 μg of plasmid DNA that had already formed triplex and was incubated at room temperature for 15 min. After removal of excess oligonucleotide by using the Chroma-spin 100, the liposome/DNA complexes were mixed with 500 μL of serum-free medium and added to the well. The plates were incubated for 4 h at 37 °C, and 500 μL of DMEM containing 20% FCS was added. The plates were incubated for 24 h at 37 °C. Each experiment was performed in triplicate.

Luciferase Assays. Each well was washed three times with cold PBS, and 100 μL of lysis buffer (Luciferase Assay System, Promega) was added to each well of a 24-well plate. After incubation for 15 min at 37 °C, the lysate and cell debris were collected. Fifty microliters of the lysate was added to 100 μL of luciferase assay substrate (Promega) in a clear polystyrene 12 × 75 mm tube. The tube was then placed immediately in a luminometer (Analytical Luminescence Laboratory, model 2010), and light production was measured for 10 s. Some lysate samples were stored at −70 °C, and luciferase activity could be measured up to 7 days later without loss of signal. To normalize the amount of protein in each sample, total protein concentration was measured by reading absorbance at 620 nm with a microtiter plate reader (Molecular Dynamics).

RESULTS

Triplex Formation with the P1 Target. The human c-*myc* promoter contains a 26-bp purine−pyrimidine-rich sequence located at −142 to −117 from the transcription start site (Figure 1). This region is a binding site for the transcription factor PuF. The sequence of this region is not strictly homopurine/homopyrimidine but contains two A·T interruptions. Sequences of TFO and control parallel oligonucleotide are shown in Figure 2A. Triplex formation was demonstrated by gel mobility shift analysis in Figure 3. Because of its decreased charge density, triplex DNA migrates more slowly than duplex DNA in gel mobility shift analysis. The parallel oligonucleotide was used as a control oligonucleotide that would not form a triplex with the target. Gel shifts were performed by the addition of increasing concentrations of the purine-rich TFO to the target duplex labeled on the pyrimidine-rich strand. Triplex formation occurs at 1.0 μM concentration of TFO, and the shift is complete at 100 μM; however, parallel oligonucleotide failed to demonstrate triplex formation at concentrations up to 100 μM. These data demonstrate that triplex formation occurs with the third strand oriented antiparallel with respect to the purine-rich target strand. Some dissociation of the triplex may occur during the course of electrophoresis, as indicated by the smear between duplex and triplex bands (*37*, *38*).

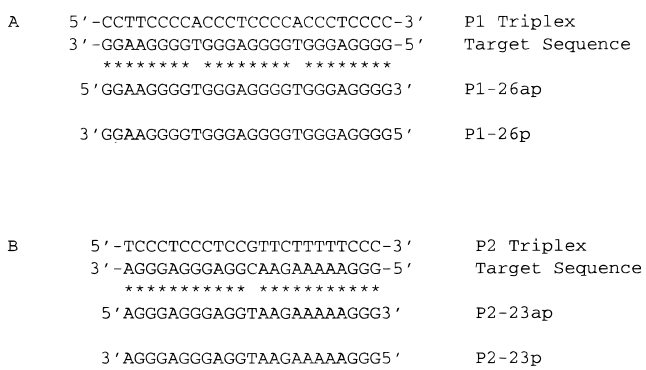

FIGURE 2: (A) Oligonucleotide sequences and their alignments with the 26-bp human c-*myc* P1 target site. (B) Oligonucleotide sequences and their alignments with the 23-bp human c-*myc* P2 target site. The ap and p refer to the orientation (antiparallel and parallel) of the TFO with respect to the purine-rich strand of target, and asterisks show reverse-Hoogsteen hydrogen bonds.

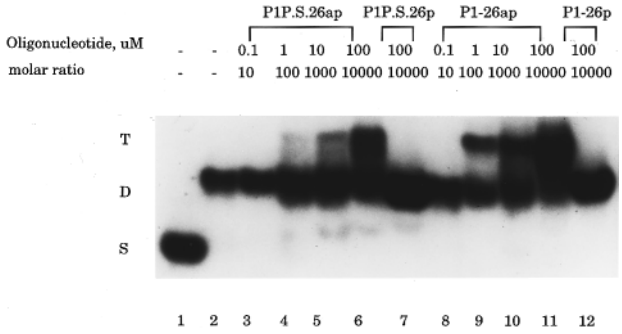

FIGURE 3: EMSA demonstrating oligonucleotide-directed triplex formation in the c-*myc* P1 promoter target. The 26-bp duplex target was incubated with increasing concentrations of triplex-forming oligonucleotides (lanes 3−6 and 8−12) or control oligonucleotides (lanes 7 and 13). Phosphorothioate oligonucleotides were used in lanes 8−13. P.S. stands for phosphorothioate oligonucleotides. The concentration of triplex-forming oligonucleotide or control oligonucleotide added to 10 nM ^{32}P-labeled 23-bp duplex and their molar ratio to the target duplex DNA are indicated above each lane. S = single-strand DNA; D = duplex DNA; T = triplex DNA.

Triplex Formation with the P2 Target. Figure 2B illustrates the 23-bp P2 target and the TFO that is used for the formation of triplex. Again, the addition of increasing concentrations of the antiparallel oligonucleotide, P2-23ap, relative to target results in a gradual shift from duplex (D) to triplex (T), as indicated by a distinct migrating band. The concentration-dependent shift of the c-*myc* target from duplex to triplex begins at 0.1 μM oligonucleotide, corresponding to a 10-fold molar excess of oligonucleotide to duplex. At 100 μM (10 000-fold excess) most of the duplex is shifted to triplex (Figure 4, lane 6). With the parallel oligonucleotide, P2-23p, there is no evidence of triplex formation, even at 100 μM, which indicates that the parallel oligonucleotide does not form triplex under these conditions of 10 000-fold molar excess relative to target (Figure 4, lane 7).

Binding of Phosphorothioate Oligonucleotide Relative to Phosphodiester Oligonucleotide. The comparison of triplex formation by unmodified phosphodiester oligonucleotides to that of phosphorothioate oligonucleotides is shown in Figures 3 and 4. In the case of P2-targeted TFO, the extent of triplex formation with phosphodiester oligonucleotides (Figure 4, lanes 3−6) is comparable to that with phosphorothioate oligonucleotides at identical concentrations (Figure 4, lanes 8−11). The concentration-dependent shift of the c-*myc* P2

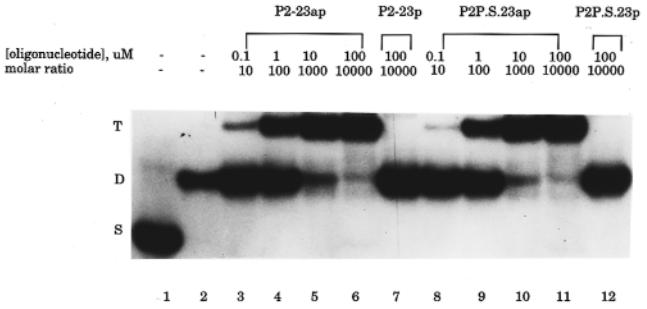

FIGURE 4: EMSA demonstrating oligonucleotide-directed triplex formation in the c-*myc* P2 promoter target. The 23-bp duplex target was incubated with increasing concentrations of triplex-forming oligonucleotides (lanes 3−6 and 8−12) or control oligonucleotides (lanes 7 and 13). Phosphorothioate oligonucleotides were used in lanes 8−13. P.S. stands for phosphorothioate oligonucleotides. The concentrations of oligonucleotides, as well as their molar ratio to the target duplex DNA, are indicated above each lane. S = single-strand DNA; D = duplex DNA; T = triplex DNA.

target from duplex to triplex begins at 0.1 μM oligonucleotide, which corresponds to a 10:1 molar ratio of oligonucleotide to duplex target. At 100 μM (10 000-fold excess) concentrations of either phosphodiester or phosphorothioate oligonucleotides, all labeled duplex target sequences are shifted to triplex (Figure 4, lanes 6 and 11). Again, there was no triplex formation with control oligonucleotides even at 100 μM (10 000-fold excess), which indicates that the parallel oligonucleotide does not form triplex under these conditions (Figure 4, lanes 7 and 12). The only difference in triplex-forming ability between P2-23ap and P2P.S.23ap is the slight difference in the relative amounts of duplex and triplex species in the presence of 0.01 μM concentrations of each oligonucleotide. P1-targeted phosphodiester oligonucleotide shows better triplex-forming ability than phosphorothioate oligonucleotide as shown by a relatively high amount of triplex to compared to duplex at every concentration range (Figure 3). This may be due to the number of interruptions in the target. In other words, there are two interruptions in the P1 target and only one interruption in the P2 target. This additional interruption may have an unstablizing effect on triplex-forming potential.

Effect of Triplex Formation on c-myc Transcription in HeLa Cells. We have used a luciferase reporter plasmid for measuring the in vivo effects of triplex-forming oligonucleotides on c-*myc* transcription. The plasmid, pMYCLUCneo, contains the cDNA encoding a firefly luciferase gene under the control of an 862-bp human c-*myc* promoter. Incubation of P1-targeted phosphorothioate TFO (P1P.S.26ap) results in a 60% decrease in luciferase activity; P2-targeted phosphorothioate TFO (P2P.S.23ap) causes a 40% decrease in luciferase activity (Figure 5). After HeLa cells were cotransfected with both P1P.S.26ap and P2P.S.23ap, a more significant decrease in luciferase activity (down to 10% of that without oligonucleotide) was observed, but the non-triplex-forming oligonucleotides, P1P.S.26p and P2P.S.23p, had very little effect on transcription at the same concentration.

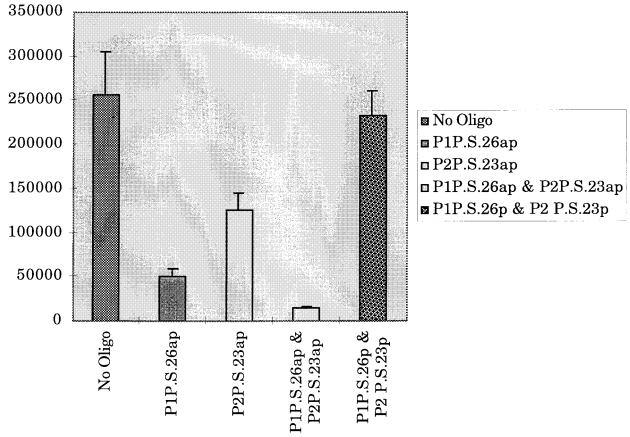

FIGURE 5: Inhibition of luciferase reporter gene expression with triplex-forming oligonucleotides targeted to the human c-*myc* P1 and P2 promoters. The pMYCLUCneo reporter construct was preincubated with either P1-, P2-, or both P1- and P2-targeted TFOs or control oligonucleotides and cotransfected into HeLa cells. The total amount of DNA was kept constant by the addition of various amounts of pBluescript plasmid. Cells were lysed at 24 h post-transfection and luciferase activity was assayed. These data are representative of three independent experiments. Each experiment was performed in triplicate. Vertical bars represent the standard error of the mean for the three samples.

DISCUSSION

The ability of triplex-forming oligonucleotides to compete with site-specific DNA binding proteins for binding to target sites was shown by Maher et al. (*24*). They showed that the binding of *Ava*I, *Taq*I, and the transcription factor Sp1 to artificial recognition sites was inhibited by triplex formation. Gee et al. (*26*) and Mayfield et al. (*39*) have also shown that TFOs targeted to the Sp1 binding sites of human dihydrofolate reductase (DHFR) and Ha-*ras* prevent Sp1 binding. Several reports have demonstrated that triplex-forming oligonucleotides targeted to positive regulatory factor binding sites inhibit transcription in cells. McShan et al. (*38*) have demonstrated that a TFO targeted to the Sp1 binding sites in the long terminal repeats of the human immunodeficiency virus inhibits viral transcription in infected cells.

It has been first demonstrated that an oligonucleotide targeted to a nuclease-sensitive region upstream of the human c-*myc* P1 promoter was able to inhibit transcription in a cell-free system (*33*). This region of the promoter interacts with the transcription factor PuF, which is important for the transcription of not only the P1 but also the P2 promoter. The second important region of the c-*myc* promoter was known to be the multiple protein-binding sites within the P2 promoter of c-*myc*. Proteins binding to this region were designated MAZ (*40*) and E2F (*17*). Deletion studies have shown that MAZ and E2F are required for maximal P2 transcription. We have identified the target site for triplex formation in the c-*myc* P2 promoter and have shown that triplex formation in the c-*myc* P2 promoter inhibits the binding of a HeLa nuclear protein, presumably MAZ, and blocks in vitro transcription of the c-*myc* gene (*36*).

We have made a reporter plasmid carrying a luciferase gene driven by an 862-bp *Pvu*II digest fragment of the human c-*myc* promoter that contains both P1 and P2 and shows maximal transcriptional activity. It has also been demonstrated by S1 nuclease mapping that P2 is a stronger promoter than P1 during transient expression in BHK cells (*41*). This appears to reflect the situation, at least in vivo, in which the P2 promoter is used more efficiently than P1 for the

transcription of c-*myc* gene in normally proliferating cells (*42*).

We decided to target both P1 and P2 promoters on the basis of the work by Postel et al. (*30*). They have demonstrated specific triplex formation to the −148 to −122 promoter region of P1 at physiologic temperature and pH. They have also shown that TFO targeted to the same region of the human c-*myc* P1 promoter caused a 10-fold reduction in P1-initiated *myc* mRNA in HeLa cells and that P2 transcription was much less affected by the same TFO. Therefore, a triplex-forming oligonucleotide targeted to either P1 or P2 is unlikely to inhibit c-*myc* expression completely.

We formed triplex in vitro by incubating supercoil plasmid with oligonucleotides, and then the entire DNA complex was transfected into HeLa cells. As we expected, neither P1- nor P2-targeted TFO alone was able to decrease luciferase significantly. However, when both P1- and P2-targeted TFOs were used together, 90% of luciferase activity was inhibited. Inhibition was sequence-specific since both control parallel oligonucleotides, P1P.S.26p and P2P.S.23p, inhibit transcription from the reporter constructs minimally. Furthermore, luciferase activity was also inhibited by the triplex-forming oligonucleotide in a dose-dependent manner (data not shown).

We also observed some nonspecific inhibition with the control oligonucleotide unless excess oligonucleotide was removed after incubation with plasmid. This nonspecific inhibition of transcription is not surprising since it has been documented that oligonucleotides tend to exhibit a nonspecific effect and can inhibit both reverse transcriptase and DNA polymerases (*43*). Interestingly, we found that P1-targeted TFO decreased luciferase activity more than TFO targeted to the P2 promoter. Asselin and Marcu (*16*) have shown that deletion of the ME1a1 binding site affected transcription by reducing P2 promoter usage and shifting the P1:P2 initiation ratio in favor of P1. When P2-targeted oligonucleotide is used it may increase P1 promoter usage. Therefore, it is possible that, once the RNA polymerase binds to the P1 promoter and initiates transcription, the presence of the TFO in the downstream P2 promoter may have little or no effect. It is also possible that the 862-bp c-*myc* promoter fragment does not contain negative sequence elements that may exist in the endogenous c-*myc* locus and repress P1 transcription.

It has been shown by Maher et al. (*24*) that triple-helical complexes assembled on the promoter inhibit in vitro transcription primarily by blocking assembly of the initiation complexes rather than by occluding the positive regulatory factor. It is likely that TFO targeted to both the P1 and P2 promoters of the c-*myc* gene exert their effects on formation of the transcription initiation complex either by direct occlusion of important transcription factors or by altering DNA flexibility.

Since polypurine/pyrimidine tracts occur relatively often in the promoters of eukaryotic genes, inhibition of transcription through intermolecular triplex formation is very attractive. Triplex-forming oligonucleotides could be potential therapeutic agents because of their specificity, because current DNA-binding agents are not very sequence-specific. We have used oligonucleotides targeted to the human c-*myc* promoters and demonstrated that these TFOs are capable of inhibiting c-*myc* transcription in HeLa cells in a sequence-specific manner. This is the first example of using multiple distinct triplex-forming oligonucleotides targeted to regions in the promoter of a single gene. The data presented in this report suggest a potential future application of this oligonucleotide on the specific modulation of c-*myc* expression in vivo.

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