# Inhibition of *in Vitro* Transcription by a Triplex-Forming Oligonucleotide Targeted to Human c-*myc* P2 Promoter†

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ABSTRACT: Triplex-forming oligonucleotides (TFOs) have been shown to bind in a sequence-specific manner to polypurine/polypyrimidine sequences in several human gene promoters, including the c-*myc* P1 promoter. TFOs have been shown to inhibit transcription *in vitro* and the expression of target genes in cell culture. The human c-*myc* protooncogene contains a 23 base pair purine−pyrimidine-rich motif (−62 to −40) within its predominant promoter, P2, that is a potential target for purine−purine−pyrimidine triplex formation. Using electrophoretic mobility shift analysis (EMSA) and competition experiments, we have demonstrated that a MAZ (myc-associated zinc finger protein) consensus sequence is capable of competing with the purine−pyrimidine motif for the binding of a HeLa nuclear protein. We have shown the formation of an intermolecular triplex using a 23-base purine-rich oligonucleotide antiparallel to the purine-rich target sequence. DNase I footprinting was performed to confirm the exact location of triplex formation. Triplex formation by this oligonucleotide prevents binding of a HeLa nuclear protein (presumably MAZ) to the target site. We have also shown that the P2-targeted TFO is a potent and specific inhibitor of c-*myc* transcription *in vitro*. These data demonstrate that this novel TFO inhibits transcription of the c-*myc* P2 promoter. We propose that the P2-targeted TFO has its effect by blocking the binding of the regulatory factor MAZ.

The c-*myc* protooncogene is the normal cellular homolog of v-*myc*, the viral transforming oncogene from avian myelocytomatosis virus strain MC29 (Colby et al., 1983). It encodes a nuclear phosphoprotein (Persson et al., 1984) whose expression is associated with rapid cellular proliferation in both malignant and nonmalignant cells (Sugiyama et al., 1989). The induced overexpression of c-*myc* appears to be necessary for the rapid proliferation of these cells, while the decreased expression of c-*myc* correlates with a decrease in cellular proliferation. The c-*myc* gene is activated in a wide variety of neoplasms as a consequence of proviral insertion, amplification, and chromosomal translocation (Cole, 1986; Bishop, 1983). Each of these mechanisms increases the expression of normal c-*myc* protein. The c-*myc* gene consists of three exons. The first exon is largely untranslated and contains two promoters with two distinct transcriptional start sites, designated P1 and P2, which are separated by approximately 165 bp[1] (Battey et al., 1983). A third promoter designated P0 is found 550−650 bp upstream of P1 and accounts for only 0−5% of c-*myc* transcription, while P1 generates 10−25% and P2 gives rise to 75−90% c-*myc* mRNA (Bentley et al., 1986).

*Cis*-acting elements residing in the c-*myc* first exon and intron have been shown to regulate P2 transcription initiation. Two of these regulatory elements, ME1a1 and ME1a2, are positioned between P1 and P2. These sequences are major sites for nuclear factor binding and are highly conserved between the human and mouse c-*myc* genes. Deletion of the ME1a1 binding site results in loss of P2 transcriptional activity (Asselin et al., 1989). In addition, Hall et al. (1990) have shown that ME1a1 is required for transcription initiation from P2 in an *in vitro* transcription system. Recently, a human gene encoding a zinc finger protein that binds ME1a1 was named MAZ (myc-associated zinc finger protein). In addition, a sequence element (GGCGGGAAAA) located between ME1a1 and ME1a2 is conserved between mouse and human and binds the E2F transcription factor (Thalmeier et al., 1989; Hiebert et al., 1989). Since these two sites are essential for transcription of c-*myc* from P2, the inhibition of protein binding by triplex formation presents a method to attenuate c-*myc* transcription.

Purine- and pyrimidine-rich oligonucleotides targeted to purine−pyrimidine-rich sequences form pur*pur·pyr and pyr*pur·pyr intermolecular triple helices (Moser & Dervan, 1987; Fedorova et al., 1988; Lyamichev et al., 1988; Praseuth et al., 1988; Hanvey et al., 1989; Maher et al., 1989) and inhibit binding of protein to target DNA (Hanvey et al., 1989; Maher et al., 1989; Grigoriev et al., 1992; Gee et al., 1992; Mayfield et al., 1994; Ebbinghaus et al., 1993). The oligonucleotide third strand occupies the major groove of the duplex, forming Hoogsteen hydrogen bonds with the purine bases of the duplex (Moser & Dervan,1987; Beal & Dervan, 1991) 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

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[1] Abbreviations: bp, base pair; TFOs, triplex-forming oligonucleotides; EMSA, electrophoretic mobility shift analysis; MAZ, myc-associated zinc finger protein; PNK, polynucleotide kinase.

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interspersed (Beal & Dervan, 1991; Postel et al., 1991; Durland et al., 1991). 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 (Beal & Dervan, 1991; Durland et al., 1991).

It has been previously shown that a discrete 27-base oligonucleotide forms a stable triplex within the P1 promoter of the human c-*myc* gene, −116 to −142 base pairs upstream from P1 transcription start site (Cooney et al., 1988). This region contains binding sites for several cellular factors, at least one of which is required for *in vitro* initiation of mRNA synthesis from the c-*myc* promoter (Boles & Hogan, 1987; Postel et al., 1989; Davis et al., 1989). Consistent with those findings, it was found that oligonucleotide binding to the −116 to −142 bp upstream from the P1 start site is capable of inhibiting c-*myc* transcription *in vitro* (Cooney et al., 1988).

We have designed a purine-rich oligonucleotide targeted to the region of the c-*myc* P2 promoter containing the MAZ and E2F binding sites, both of which are important for P2 activity. We have determined the ability of this oligonucleotide to form a sequence-specific interstrand triplex. We also examine the ability of triplex-forming oligonucleotide to block the binding of nuclear protein to the target sequence. Finally, we examined the effect of triplex formation on *in vitro* transcription of the c-*myc* promoter.

## MATERIALS AND METHODS

*Oligonucleotide Synthesis.* Phosphodiester oligonucleotides were synthesized on a Milligen Cyclone Plus DNA synthesizer using standard phosphoramidite chemistry. All oligonucleotides were purified by OPEC (Clontech). The structural integrity and purity were verified by 5′-$^{32}$P-labeling using [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (PNK) followed by electrophoresis on a polyacrylamide gel. Yields were determined from absorbance measurements at 260 nm using molar extinction coefficients.

*Electrophoretic Mobility Shift Analysis (EMSA) of Triplex Formation.* For triplex formation, the synthetic pyrimidine-rich strand of the 23-base c-*myc* target was 5′-labeled with [$\gamma$-$^{32}$P]ATP and T4 PNK and annealed to its oligonucleotide complement. The potential triplex-forming oligonuclotide was heated at 65 °C for 10 min to reduce self-aggregation of the G-rich oligonucleotides and then quick-chilled on ice. After the oligonucleotides were incubated with the labeled 23-bp target in a buffer consisting of 90 mM Tris, 90 mM borate (pH 7.4), and 10 mM $MgCl_2$ for 1 h at 37 °C, products were analyzed on a 16% nondenaturing polyacrylamide gel in the same buffer at room temperature (duration of the gel, 3 h), at 150 V.

*Promoter Fragment Isolation.* pSV2Neo-*myc* containing 12.5 kb of the human c-*myc* gene was digested with *Pvu*II and separated on 1% low melting point agarose gel. The 864-bp fragment containing the human P1 and P2 promoter was purified and ligated into the *Sma*I site of pGL-2 (Promega). The resulting plasmid pMyc-Luc was then digested with *Xho*I and labeled with [$\alpha$-$^{32}$P]dATP using the Klenow fragment of DNA polymerase I. The fragment was then digested with *Mlu*I and run on 5% preparative native polyacrylamide gel. The resulting 465-bp promoter fragments were cut out, and the gel slice was passed through a 3-mL syringe to crush it. DNA was eluted in 0.5 M $NH_4$-OAc, pH 5.2, for overnight at 37 °C with shaking, filtered to remove polyacrylamide, and precipitated with ethanol.

*DNase I Footprinting.* After being heated at 65 °C for 10 min and then quick-chilled on ice, the oligonucleotides were incubated with the $^{32}$P-labeled promoter fragment in 10 mM Tris (pH 7.4) and 10 mM $MgCl_2$ for 1 h at 37 °C. Samples were precooled on ice and 1 $\mu$g of poly(dI-dC) was added. DNase I digestion was then performed for 1 min on ice with 0.0001 unit of DNase I, and the reactions were stopped by adding 10 mM EDTA in 90% formamide. Samples were extracted once with phenol–chloroform and twice with chloroform and ethanol precipitated. After precipitation, samples were heated at 95 °C for 5 min, quick-chilled on ice, and analyzed by electrophoresis on a 8 M urea–8% polyacrylamide sequencing gel at 42 W. Bands were visualized by autoradiography, and protected sequences were identified from gels containing both Maxam–Gilbert sequence and DNase I footprints.

*Protein Binding Assay.* Oligonucleotides were added to the labeled duplex target and incubated in a protein binding buffer consisting of 25 mM Hepes, 12.5 mM $MgCl_2$, 70 mM KCl, 1 $\mu$M $ZnSO_4$, 1 mM DTT, 0.1% NP-40, and 10% glycerol (v/v) for 1 h at 37 °C. The samples were cooled on ice; then HeLa nuclear extract and 1 $\mu$g of poly(dI-dC) were added and allowed to bind for 30 min on ice. Samples were analyzed by electrophoresis on 5% native polyacrylamide gels at 150 V in 90 mM Tris–borate (pH 8.5) and 2 mM EDTA followed by autoradiography.

*In Vitro Transcription. In vitro* transcription was performed using a HeLa nuclear extract *in vitro* transcription system (Promega). The template DNA used was generated from *Hin*dIII digestion of the plasmid pMyc-Luc which contains the 864-bp c-*myc* promoter. The concentration of *Hin*dIII-digested DNA was quantitated by UV spectrophotometry. The optimization of transcription reaction was performed with varying concentration of $Mg^{2+}$ and templates. It was found that 3 mM $MgCl_2$ and 1 $\mu$g of linearized template gave the most *in vitro* transcription products (data not shown). The template DNA (1 $\mu$g) was incubated with varying concentrations of either the TFO or a purine-rich control oligonucleotide in a buffer identical to the one used for triplex gel shift for 12 h at 37 °C. As a control, the cytomegalovirus early promoter (100 ng) yielding a 363-base transcript was treated identically to the c-*myc* promoter template. After incubation with TFO, reactions were cooled to room temperature, and transcription was initiated by the addition of HeLa nuclear extract (79.9 $\mu$g of protein, 8 standardized transcription units) to a final volume of 25 $\mu$L, containing final concentration of 3 mM $MgCl_2$, 17.6 mM Tris (pH 7.4), 15.6 mM KCl, 31.2 $\mu$M EDTA, 78 $\mu$M dithiothreitol, 8.8% glycerol, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 16 $\mu$M GTP, and 0.4 mCi/mL [$\alpha$-$^{32}$P]GTP (3000 Ci/mmol). Transcription was allowed to proceed for 1 h at 30 °C then terminated by the addition of a stop solution containing 0.3 M Tris-HCl (pH 7.4), 0.3 M NaOAc, 0.5% SDS, 2 mM EDTA, and 3 $\mu$g/mL yeast tRNA. Run-off transcripts were extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and ethanol precipitated. RNA pellets were resuspended in equal volumes of nuclease-free $H_2O$ and loading solution containing 98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue. The run-off transcription products were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel containing 8 M

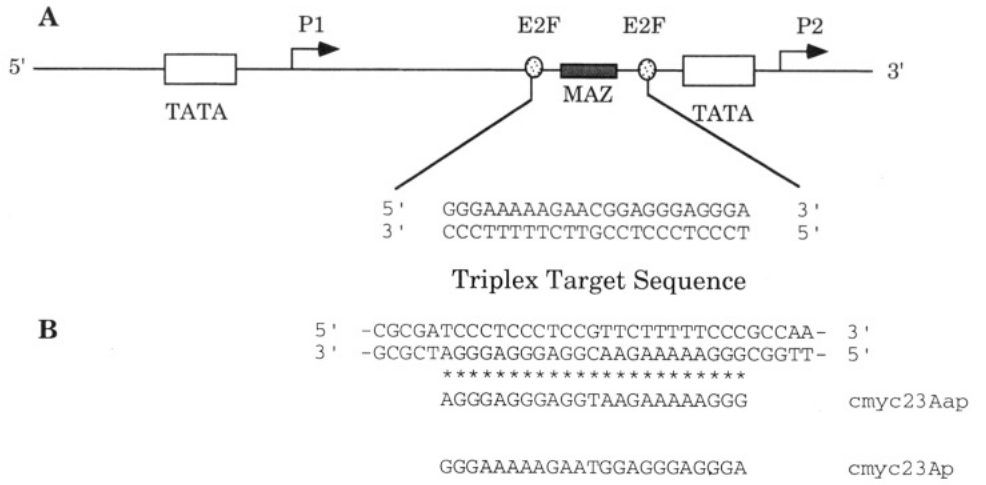


FIGURE 1: (A) Map of the human c-*myc* gene showing two major promoters, P1 and P2, the 23-bp purine−pyrimidine-rich motif, and the triplex target sequence relative to the transcription start site P2 (−62 to −42). Transcription factor, MAZ and E2F, binding sites are also indicated. (B) Oligonucleotide sequences and their alignment with the duplex target. The p and ap nomenclature indicates parallel or antiparallel orientation of the oligonucleotide relative to the purine-rich target strand, respectively.

urea at 42 W for 4 h followed by autoradiography of the gel at −80 °C.

## RESULTS

*Oligonucleotide Design.* The human c-*myc* P2 promoter contains a 23-bp purine−pyrimidine-rich sequence located at −62 to −40 from the P2 transcription start site (Figure 1A). This region is a binding site for MAZ (*myc*-associated zinc finger protein) and E2F, both of which are essential for P2 transcription. The sequence in this region is not strictly homopurine−homopyrimidine but contains only a single C·G interruption. A potential triplex-forming oligonucleotide targeted to the human c-myc P2 pur·pyr motif was designed in parallel and antiparallel orientation with respect to the purine-rich strand, containing guanine to recognize GC (G*GC triplets) and adenine to recognize AT (A*AT triplets) (Figure 1B). The parallel oligonucleotide was used as a control oligonucleotide that would not form triplex with target sequence.

*Triplex Formation by the c-myc P2 Promoter.* Triplex formation was demonstrated by EMSA and DNase I footprinting. Triplex DNA, because of its decreased charge density, migrates more slowly than duplex DNA in gel mobility shift assay. As shown in Figure 2, the addition of increasing concentrations of the antiparallel oligonucleotide cmyc23Aap relative to target results in a gradual shift from duplex (D) to a distinct migrating band (T), indicating the formation of triplex DNA (Cooney et al., 1988; Durland et al., 1990, 1991). Some dissociation of the triplex may occur during the course of electrophoresis as indicated by the smear between duplex and triplex bands (Orson et al., 1991; McShan et al., 1992). The concentration-dependent shift of the c-*myc* target duplex to triplex begins at 1 $\mu$M oligonucleotide, corresponding to a 100-fold molar excess oligonucleotide to duplex. At 100 $\mu$M (10 000-fold excess) most of the duplex is shifted to triplex (Figure 2, lane 6). With the parallel oligonucleotide cmyc23Ap, there is no evidence of triplex formation, even at 100 $\mu$M, indicating that the parallel oligonucleotide does not form triplex under these conditions (Figure 2, lane 7).

The data from DNase I footprinting experiments are consistent with those obtained from gel mobility shifts and

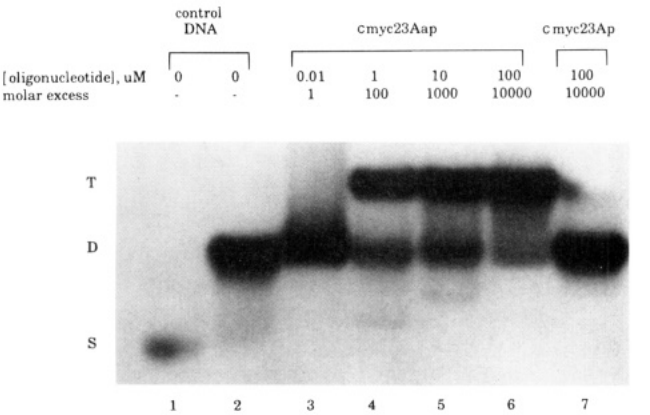


FIGURE 2: EMSA of oligonucleotide-directed triplex formation in the c-*myc* P2 promoter target. The pyrimidine-rich strand of the 23-mer target was labeled (lane 1) and annealed with the unlabeled purine-rich strand (lane 2). The 23-bp duplex target was then incubated with increasing concentrations of triplex-forming oligonucleotide $\pi$ (lanes 3−6) or control oligonucleotide (lane 7). The concentration of triplex-forming oligonucleotide or control oligonucleotide added to the 10 nM $^{32}$P-labeled 23-bp duplex and their molar ratio to the target duplex DNA are indicated above each lane. Abbreviations: S = single-strand DNA; D = duplex DNA; T = triplex DNA.

confirm that triplex formation occurs in a sequence-specific manner when the oligonucleotide third strand is oriented antiparallel relative to the purine-rich strand of target duplex. Protection of the target sequence by the triplex-forming oligonucleotide is concentration-dependent, in a manner consistent with the gel mobility shift analysis. Figure 3 shows that, at an oligonucleotide concentration of 30 $\mu$M (3000-fold molar excess with respect to the 465-bp promoter fragment), the antiparallel cmyc23Aap shows complete protection (lane 6) from DNase I digestion. The footprint obtained corresponds to protection of the 23 base pair target sequence (defined by the brackets). These data demonstrate that, within the −62 to −40 region of c-*myc* P2 promoter, cmyc23Aap binds in a sequence-specific manner to its target site. On the other hand, the parallel oligonucleotide cmyc23Ap, at a concentration of 30 $\mu$M, shows little protection from digestion by DNase I (lane 7). This may be due to less DNase I digestion or the amount of labeled fragments is less than those of other lanes. These data further

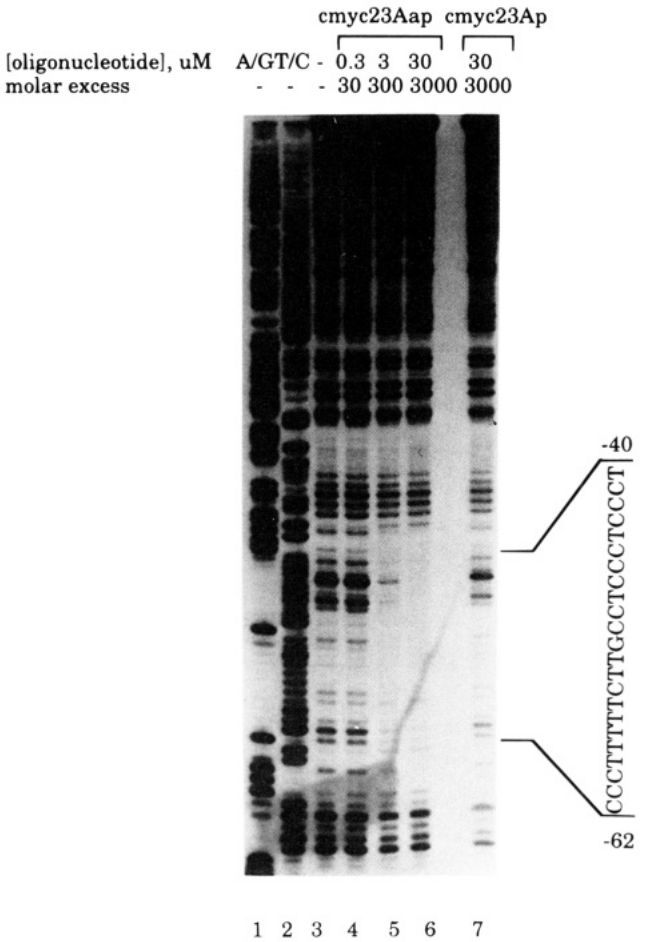


FIGURE 3: DNase I footprinting analysis demonstrating sequence-specific binding of triplex-forming oligonucleotide to the c-*myc* P2 promoter target. Lanes 1 and 2 show Maxam−Gilbert sequencing reactions performed with the same labeled promoter fragments. Lane 3 is the control DNase I digest with no oligonucleotide added. Oligonucleotides were incubated at the concentration indicated above each lane with 100 nM 465-bp $^{32}$P-Klenow-labeled promoter fragment followed by limited DNase I digestion. The c-*myc* target sequence indicated by brackets was determined from Maxam−Gilbert sequencing.

suggest that, under these conditions, triplex formation occurs exclusively with the third strand oriented antiparallel to the purine-rich strand of the c-*myc* P2 promoter target.

*Effect of Triplex Formation on Nuclear Protein Binding.* The effect of triplex formation within the c-*myc* P2 promoter on HeLa nuclear protein binding to the 23-bp promoter target was demonstrated by electrophoretic mobility shift analysis (Figure 4). Lane 2 of Figure 4 shows that the labeled 23-bp duplex target is bound to nuclear proteins as evidenced by retardation of the target upon incubation with HeLa nuclear extract. Incubation of the 23-bp c-*myc* P2 target region with HeLa nuclear extract results in the formation of several shifted bands representing different protein−DNA complexes. Incubation of the target with cmyc23Aap at 10 $\mu$M (4000-fold molar excess) followed by incubation with HeLa nuclear extract results in complete abrogation of the lowest retarded band (lane 6), demonstrating that the TFO inhibits nuclear protein binding to the target sequence. The control oligonucleotide cmyc23Ap of identical sequence, but opposite orientation to the target, appears to have little if any effect on protein binding at 10 $\mu$M (4000-fold excess) (lane 7).

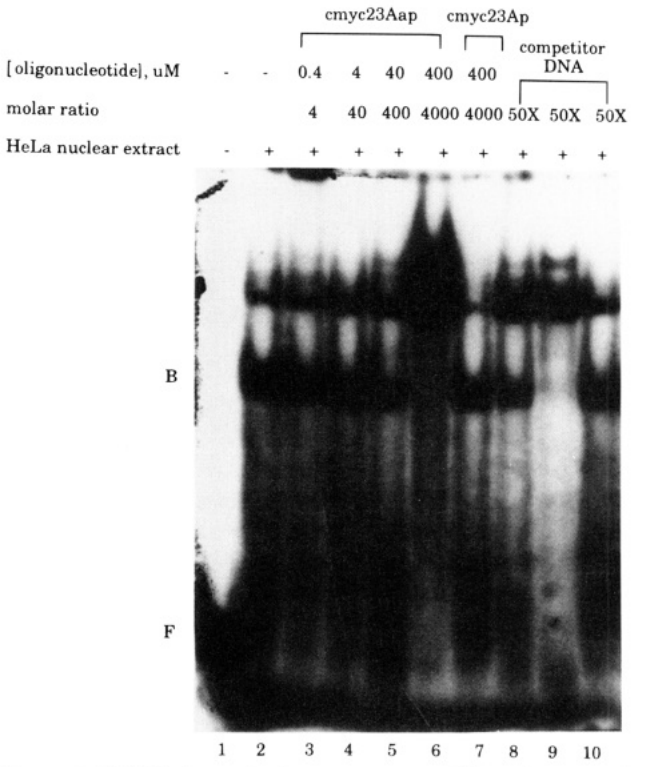


FIGURE 4: EMSA demonstrating sequence-specific nuclear protein binding to the human c-*myc* duplex target and its inhibition by triplex-forming oligonucleotide. $^{32}$P-Labeled 23-bp human c-*myc* fragment was incubated with competitor duplex or oligonucleotides as described, and then HeLa nuclear extract was added. Nonspecific competitor, MAZ consensus, and E2F consensus DNA were added to lanes 8−10, respectively. Nonspecific competitor sequence: 5′-AAAGATCCTCTCTCGCTAATCTC-3′. MAZ consensus sequence: 5′-GGGGGAGGGGG-3′. E2F consensus sequence: 5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′. Abbreviations: B = protein−DNA complex; F = unbound DNA probe.

The sequence specificity of this protein−DNA interaction was examined by competition binding assay. As shown in Figure 4, a 50-fold excess of unlabeled MAZ consensus sequence (Ashfield et al., 1994) effectively competes with the labeled sequence for the binding of protein that disappeared in the presence of triplex-forming oligonucleotide (lane 9), while the same excess of an unlabeled 23-bp nonspecific DNA or E2F consensus sequence (Helin et al., 1992) has no effect on protein binding to the target (lanes 8 and 10). Therefore, this protein−DNA complex is presumably derived from MAZ. These data demonstrate that the nuclear factor binding to this c-*myc* P2 promoter region involves a sequence-specific interaction. Because a low percentage gel (5%) was used to analyze protein binding, the migration of the triplex was indistinguishable from that of duplex and was not detectable in Figure 4. However, when 16% TBM gel was used, it was possible to demonstrate triplex formation by cmyc23Aap but not by cmyc23Ap (Figure 5). These data suggest that significant inhibition of protein binding to the c-*myc* P2 target is a direct result of triplex formation.

*Effect of Triplex Formation on c-myc Transcription.* The effect of triplex formation on c-*myc* P2 transcription was examined in an *in vitro* "run-off" transcription assay using a HeLa nuclear extract transcription system (Promega). In this assay, the human c-*myc* promoter is incubated with HeLa nuclear extract in the presence of nucleotide triphosphates;

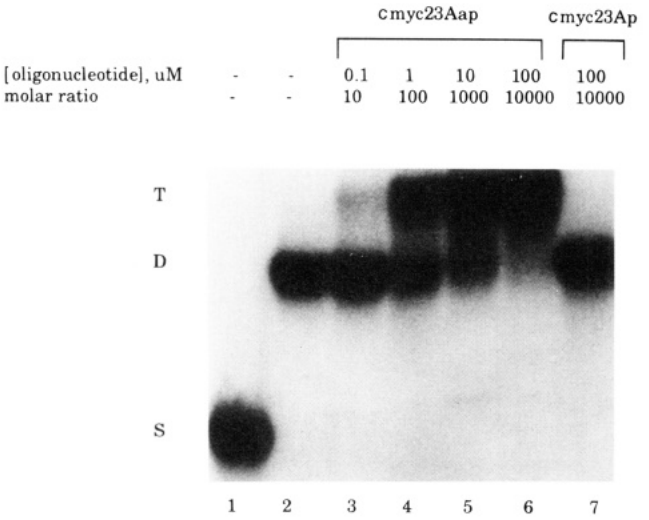


FIGURE 5: Gel mobility shift analysis showing triplex formation with the 23-bp c-*myc* promoter under protein binding conditions. The 23-bp target was incubated with oligonucleotide at the concentrations indicated under the protein binding conditions described in Materials and Methods.

transcripts are labeled by the incorporation of [α-$^{32}$P]GTP and correspond in size to the length of template DNA. The *Hin*dIII digest of the plasmid, pMyc-Luc containing 864 bp of the c-*myc* promoter, linearized the plasmid with 412 bp downstream of the transcription start site. After incubation of the promoter with the triplex-forming cmyc23Aap at 20 μM (1000-fold molar excess with respect to template), significant inhibition of transcription was observed, while the non-triplex-forming cmyc23Ap had little or no effect at the same concentration (Figure 6A). There are some longer bands seen in the gel. These may be due to nonspecific initiation of transcription from the upstream vector sequence in plasmids.

The effect of the c-*myc* P2-targeted triplex-forming oligonucleotide on the transcription process from a promoter unrelated to that of c-*myc* was examined by incubation of oligonucleotide with template DNA containing the cytomegalovirus immediately early promoter followed by *in vitro* transcription. Run-off transcription from the cytomegalovirus promoter yields a 363-nucleotide RNA transcript. As shown in Figure 6B, at the same concentration which inhibits c-*myc* transcription, triplex-forming oligonucleotide, cmyc23Aap has little effect on transcription from the cytomegalovirus promoter under the same conditions. These results suggest that inhibition of c-*myc* transcription by cmyc23Aap is due to sequence-specific triplex formation by the c-*myc* promoter.

## DISCUSSION

Several reports have demonstrated that triplex-forming oligonucleotides targeted to positive regulatory factor binding sites inhibit transcription in cells. McShan et al. have demonstrated that a TFO targeted to the Sp1 binding sites in the human immunodeficiency virus long-terminal repeat inhibits viral transcription in infected cells. Grigoriev et al. have shown that a triplex-forming oligonucleotide targeted to the $NF_kB$ site in the interleukin-2 receptor α regulatory sequence inhibits $NF_kB$-dependent transcription.

The human c-*myc* protooncogene P2 promoter is the predominant promoter for c-*myc* transcription. A detailed

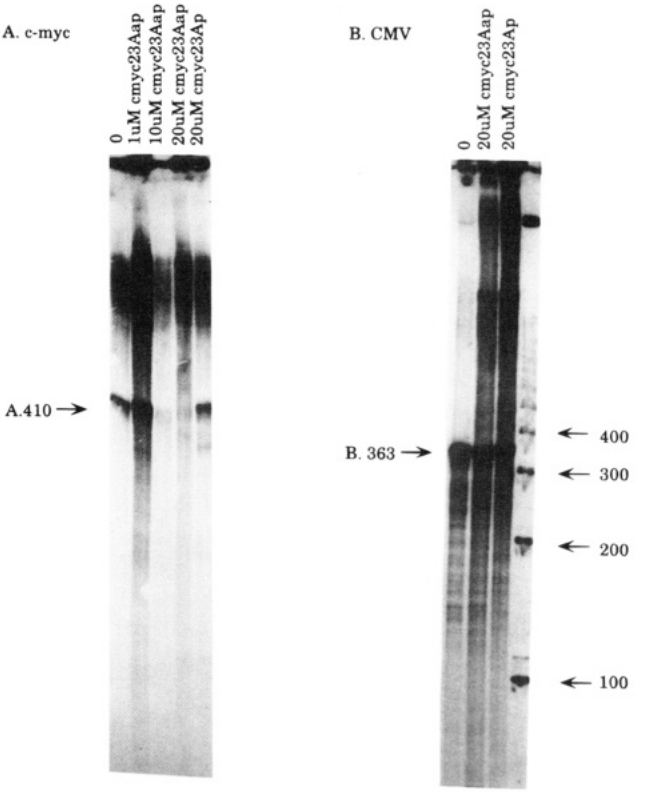


FIGURE 6: *In vitro* transcription assay demonstrating specific inhibition of c-*myc* transcription: (A) *in vitro* transcription from a plasmid containing the c-*myc* promoter region; (B) *in vitro* transcription from a purified CMV early promoter region. Oligonucleotides were added to the template DNA as described at concentrations indicated above each lane. *In vitro* transcription of the plasmid containing c-*myc* promoter yields a 412-base RNA transcript indicated by arrow A. The transcription is inhibited by triplex-forming oligonucleotide whereas the purine-rich control oligonucleotide has no effect on *in vitro* transcription. *In vitro* transcription using CMV promoter template yields a single 363-base transcript indicated by arrow B. Neither the c-*myc* TFO nor the control oligonucleotide has any effect on transcription from the CMV promoter. The 100-bp ladder was end-labeled with T4 PNK and used as the molecular weight marker.

analysis of the c-*myc* promoter regulatory elements have revealed multiple protein-binding sites within the P2 promoter of c-*myc*. Proteins binding to this region were designated MBP-1 (c-*myc* promoter binding protein) (Ray & Miller, 1991), MAZ (Bossene et al., 1992), and E2F (Thalmeier et al., 1989). Deletion studies have shown that MAZ is required for maximal P2 transcription. Deletion of the MAZ binding site increases P1 promoter usage (Asselin et al., 1989). The nuclear factor E2F is also involved in the transcriptional regulation of c-*myc*. This protein binds to two sequence elements within the P2 promoter in a region that is critical for promoter activity. Hiebert et al. (1989) have demonstrated in transient transfection assays that expression of adenovirus E1A gene products induces the transcription of c-*myc* gene and this E1A-dependent transactivation of the c-*myc* is mediated through E2F.

In an effort to provide sequence-specific DNA binding agents which may inhibit c-*myc* transcription, we have designed a triplex-forming oligonucleotide targeted to this region of c-*myc* P2 promoter. The target sequence for triplex formation in this region is not strictly homopurine−homopyrimidine but contains only a single C·G interruption. Previously it was shown by Beal and Dervan that G*GC, A*AT,

and T*AT triplets stabilize a triple helix to a greater extent than the other 13 natural triplets. They have also compared the relative cleaving ability of 15-mer oligonucleotides differing in sequence at a single position and are equipped with thymine−EDTA at each 3′ end so that binding could be monitored by the affinity cleaving method. Their results indicated that while oligonucleotides containing adenosine, cytosine, and guanine opposite a single C·G base pair did not provide cleavage, thymidine-substituted oligonucleotide gave weak but better cleavage than other oligonucleotides. On the basis of these facts, a potential triplex-forming oligonucleotide targeted to the human c-myc P2 pur·pyr motif was designed in parallel and antiparallel orientation with respect to the purine-rich strand, containing guanine to recognize GC (G*GC triplets), adenine to recognize AT (A*AT triplets), and thymine to recognize CG (T*CG triplets) (Figure 1B). The parallel oligonucleotide was used as a control oligonucleotide that would not form triplex with the target sequence. The oligonucleotide cmyc23Aap, which is oriented antiparallel to the purine-rich duplex, was shown to bind in a sequence-specific manner to the target site. A control oligonucleotide, cmyc23Ap, containing a sequence identical to cmyc23Aap but in a parallel orientation, does not form triplex. These results are consistent with those from studies by Beal and Dervan (1991) and Durland et al. (1991), which show that the third strand of the pur/pyr*pur·pyr triplex binds in an antiparallel orientation with respect to the purine-rich strand of the duplex target.

Giovannangeli et al. (1992) have also demonstrated that 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.

We 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. 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. (1989). 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. (1992) and Mayfield et al. (1993) have also shown that TFOs targeted to the Sp1 binding sites of human dihyrofolate reductase (DHFR) and Ha-Ras prevent Sp1 binding. It has also been demonstrated that TFOs targeted to the Ki-Ras and Her-2/Neu inhibit the binding of a protein in HeLa nuclear extract (Mayfield et al., 1994; Ebbinghaus et al., 1993). Postel et al. (1989) have fractionated HeLa cell nuclear extracts into components which support the transcription of c-*myc in vitro* and partially purified a HeLa cell transcription factor that is required for accurate c-*myc* transcription from the P2 promoter. This factor, named PuF (purine factor), binds to the −142 to −115 region upstream from the P1 start site by making contact with a GGGTGGG sequence motif. This group has also shown that a TFO targeted to the PuF binding site in the P1 promoter forms a triplex in HeLa cells and inhibits *in vivo* P1 transcription. Inhibition of P1 is approximately 10-fold greater than the inhibition of P2 under the same conditions (Postel et al., 1991). As mentioned above, P2 is the major promoter of c-*myc* expression and generates more than 75% of c-*myc* mRNA. Therefore, a TFO targeted to P1 promoter is unlikely to block c-*myc* expression completely. It has been shown by Maher et al. (1992) that triple-helical complexes assembled on the promoter inhibit *in vitro* transcription primarily by blocking assembly of the initiation complexes rather than occluding the positive regulatory factor. It is likely that cmyc23Aap, which interacts with a region close to the P2 TATA box, exerts its effect on formation of transcription initiation complex either by direct occlusion or by altering DNA flexibility. Therefore, it is plausible that inhibition of c-*myc* transcription by cmyc23Aap may result from direct blocking of MAZ and/or blocking the assembly of the preinitiation complex.

The identification and characterization of genes that are involved in human disease have provided important targets for modulation of gene expression. Because of their specificity to selectively inhibit transcription of their target genes in intact cells, triplex-forming oligonucleotides could be potential therapeutic agents. We have identified an oligonucleotide targeted to the human c-*myc* P2 promoter and demonstrated that the TFO binds to its target in a sequence-specific manner and inhibits nuclear protein binding and *in vitro* transcription. This is the first example of multiple distinct triplex-forming regions in the promoter of a single gene. The data presented in this report suggest the potential future application of this oligonucleotide on the specific modulation of c-*myc* expression *in vivo*.

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