Inhibition of Nuclear Protein Binding to Two Sites in the Murine c-*myc* Promoter by Intermolecular Triplex Formation†

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Received November 28, 1994; Revised Manuscript Received February 27, 1995⊗

ABSTRACT: The c-*myc* gene is overexpressed in a variety of tumor types and appears to play an important role in the abnormal growth of a number of cell types. In an effort to determine the ability of sequence- and species-specific triplex-forming oligonucleotides to inhibit expression of a targeted gene in animals, we have identified two novel triplex-forming sites in the murine c-*myc* promoter. One is homologous to the triplex-forming human PuF binding element located upstream of the P1 transcription start site. The other triplex-forming site is found in a region between P1 and P2 that encompasses the ME1a1 binding site and part of the E2F binding site and is highly homologous to the human sequence. Synthetic oligodeoxyribonucleotides designed to target these essential regulatory elements form sequence-specific triple helices as demonstrated by gel mobility shift analysis and DNase I footprinting. Polypurine:polypyrimidine regions in the P1 and P2 promoters form specific protein−DNA complexes upon incubation with a murine YC8 nuclear extract. Preincubation of each of the promoter fragments with its respective triplex-forming oligonucleotide results in the inhibition of nuclear protein binding. Non-triplex-forming oligonucleotides do not significantly affect protein binding. The data presented are a preliminary step toward generating an animal model for the phenotypic effects of triplex formation within the c-*myc* promoter.

The c-*myc* gene product plays an integral role in the cascade of signal transduction events following a mitogenic stimulus (Eisenman, 1989). Overexpression of c-*myc* is important in the tumorigenesis of a number of experimental and naturally occurring systems (Kato & Dang, 1992; Cole, 1986). The mechanisms responsible for c-*myc* overexpression include proviral insertion, gene amplification, and chromosomal translocation (Alt & Zimmerman, 1990; Cory, 1986). Although the protein has been localized to the nucleus (Hann et al., 1983), the precise cellular function of the c-Myc remains somewhat undefined. It is known, however, that Myc forms a heterodimer with Max (Blackwood & Eisenman, 1992; Prendergast et al., 1991) and that dimerization with Max is required for the oncogenic activity of Myc (Amati et al., 1993). The Myc/Max complex binds the first intron of prothymosin α to mediate transcription of that gene, supporting the role of Myc as a transactivator of gene expression (Gaubatz et al., 1994).

Regulation of c-*myc* transcription has been relatively well characterized. The highly conserved human and murine c-*myc* genes have three transcription initiation sites in common. In normal and resting cells, P2 is the dominant promoter in both species, giving rise to 75−90% of cytoplasmic c-*myc* mRNA (Taub et al., 1984; Bentley & Groudine, 1986; Eick & Bornkamm, 1989; Marcu et al., 1992). P1, located 164 bp upstream in mice (Bernard et al., 1983), generates 10−25% of c-*myc* transcripts in normal cells but may predominate in serum-stimulated and malignant cells (Siebenlist et al., 1984). P1 and P2 appear to be independently regulated by a composite of positive- and negative-acting factors binding within a 2.3 kb region upstream of the promoters (Yang et al., 1985; Ruppert et al., 1986; Hay et al., 1987). Recent evidence by Lavenu et al. (1994), however, showing that murine c-*myc* genomic constructs containing 3.5 kb upstream and 1.5 kb downstream are silent in the resultant transgenic mice, indicates that regulation may be even more complex than expected.

It has been suggested that inhibition of transcription initiation could provide an effective method of reducing expression from activated or amplified loci which participate in the malignant cell phenotype (Hélène, 1991). Mithramycin, an antibiotic which binds GC-rich regions of DNA (Van Dyke & Dervan, 1983), inhibits c-*myc* transcription initiation from both P1 and P2 by blocking the interaction of the transcription factor Sp1 with its target sites (Ray et al., 1990; Snyder et al., 1991). Unfortunately, mithramycin also inhibits the transcription of other genes containing similar GC-rich regulatory regions (Ray et al., 1989; Blume et al., 1991). Triplex DNA provides a gene-specific means of inhibiting transcription initiation since a triplex-forming oligonucleotide can interact with the targeted region in a sequence-specific manner. The relative sequence-specificity of triplex formation suggests that the oligonucleotide will not interfere with transcription factor binding to other genes.

Previous studies on intermolecular triplex formation by polypurine:polypyrimidine (pur:pyr) regions in gene promoters demonstrate the ability of a pyrimidine-rich third strand to form Hoogsteen hydrogen bonds with the purine-rich acceptor strand in the major groove of the target duplex (Lyamichev et al., 1988; de los Santos et al., 1989; Maher

† Supported by NIH Grant CA54380, by a research grant from the Share Foundation, and by U.S. Army Grant DAMD 17-93-J-3018.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1995.

0006-2960/95/0434-7659$09.00/0

et al., 1990; Pilch et al., 1990; Plume et al., 1990; Mergny et al., 1992; Lu & Ferl, 1993). The third strand in pyr·pur: pyr triple helices adopts a parallel orientation relative to the polypurine strand of the duplex (Moser & Dervan, 1987; Fedorova et al., 1988; Praseuth et al., 1988). The stability of a pyr·pur:pyr triplex is dependent on nonphysiological acidic conditions required by the C^+·G:C triplets responsible for sequence specificity (Wells et al., 1988; Rajagopal & Feigon, 1989). Recent studies have shown triplex formation by a purine-rich or mixed purine/pyrimidine third strand involving G·G:C and A·A:T or T·A:T triads at physiological pH (Beal & Dervan, 1991; Kolwi-Shigematsu & Kolwi, 1991; Postel et al., 1991; Durland et al., 1991). These triple helices arise through reverse-Hoogsteen bonding, resulting in an antiparallel orientation of the third strand (Beal & Dervan, 1991; Gee et al., 1992; Ebbinghaus et al., 1993; Mayfield et al., 1994a).

Cooney and co-workers (Cooney et al., 1988) have demonstrated triplex formation (pur·pur:pyr) by a nuclease-hypersensitive region upstream of the human c-*myc* P1 transcription start site. This hypersensitive element is coincident with the binding site of several nuclear proteins. Davis et al. (1989) have reported a ribonucleoprotein association that appears to involve RNA:DNA hybridization. Postel and co-workers (Postel et al., 1989, 1993) have demonstrated binding of a protein, PuF, which they later cloned and found that it was Nm23-H2, a putative suppressor of tumor metastasis (Steeg et al., 1988). The nuclear protein NSEP-1, which exhibits overlapping but distinct single- and double-stranded DNA binding specificities, has also been shown to interact with this sequence in the human c-*myc* promoter (Kolluri et al., 1992). Triplex-forming oligonucleotides directed to this region selectively suppress c-*myc* mRNA levels *in vitro* and in HeLa cells (Cooney et al., 1988; Postel et al., 1991).

The corresponding region of the murine *myc* promoter, roughly the area proximal to DNase I hypersensitive site III_1 (Siebenlist et al., 1984; Bentley & Groudine, 1986; Marcu et al., 1992), is highly homologous to the human sequence that binds the aforementioned proteins. Asselin et al. (1989) have demonstrated through deletion analysis that this region is essential for efficient transcription from the murine c-*myc* P1 transcription initiation site. The effect of this deletion on P2 activity was not addressed.

The presence of a nuclear factor binding site in the murine c-*myc* P2 promoter, composed entirely of pyr:pur sequence, in close proximity to DNase I hypersensitive site III_2, has been documented (Asselin et al., 1989). Deletional mutagenesis of this *cis* element showed that it is essential for transcription initiation from the murine start site P2. This region, designated ME1a1, is very highly conserved between humans and mice and has since been found to interact with a zinc-finger protein (termed Maz in humans) (Bossone et al., 1992). Three distinct *cis*-acting elements (5′ to 3′: ME1a2, E2F, and ME1a1) are required for optimal transcription initiation from P2 (Moberg et al., 1992). Hypersensitive site III_2 is situated almost exactly in the center of this positive regulatory element (Marcu et al., 1992). This pyr:pur region around site III_2 has not previously been shown to form triplex DNA.

The observations above suggest that these regions in the murine c-*myc* promoter, likely possessing similar functions as their human homologues, serve as ideal triplex target regions for determining the effect of triplex-forming oligonucleotides on endogenous gene expression in an animal model. We have demonstrated here that two regions in the murine c-*myc* gene promoter corresponding to binding sites for essential nuclear factors in the human c-*myc* promoter bind murine nuclear proteins with a high degree of specificity. We have shown triple helix formation with these murine P1 and P2 promoter sequences by gel mobility shift analysis and DNase I footprinting. The inhibition of nuclear factor binding by triplex formation is established by a decrease in protein−DNA interaction, also shown by gel shift, following preincubation of the target region with triplex-forming oligonucleotides. This is the first report of triplex formation in a murine gene promoter and, as such, is the foundation for a murine model allowing assessment of the effects of triplex-forming oligonucleotides directed to the endogenous c-*myc* gene.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides were synthesized by standard phosphoramidite chemistry on a Milligen Cyclone Plus DNA synthesizer and purified by reverse phase chromatography over a Clontech Oligonucleotide Purification/Elution Cartridge. Concentrations were determined by absorption measurements at 260 nm by using molar extinction coefficients. Oligonucleotide integrity was verified by 5′ end-labeling with [γ-^{32}P]ATP and T_4 polynucleotide kinase followed by gel electrophoresis under denaturing conditions. Radiolabeled duplexes of 27 and 30 bp were prepared by 5′ end-labeling one strand with [γ-^{32}P]ATP and T_4 kinase and annealing the labeled strand with its unlabeled complement.

Protein Binding Assays. For protein shifts, ^{32}P-labeled double-stranded target sequence oligonucleotides representing a section of the murine c-*myc* promoter were incubated with a YC8 nuclear extract (Dignam et al., 1983) for 30 min at 22 °C in a buffer consisting of 20 mM Tris (pH 7.9), 2.5 mM $MgCl_2$, 140 mM KCl, 1 mM DTT, and 8% glycerol. The final total protein concentration was 265 ng/μL. For competition studies, unlabeled murine myc P1 or P2 duplex target (27 or 30 bp, respectively), 22 bp nonspecific duplex DNA, or duplex DNA representing the homologous region in the human c-myc P1 or P2 promoter (27 or 23 bp, respectively) was mixed with the ^{32}P-labeled murine myc duplex target prior to adding extract. Samples were electrophoresed on 5% native polyacrylamide gels at 100 V. Both gel and running buffer contained 90 mM Tris−borate (pH 8.5), 2 mM EDTA. Bands were visualized by autoradiography.

Sequences of the competitors for protein binding are as follows (nucleotide positions are relative to P1):

NS22: 5′ G C A T A T T A C T G G T G C A G G A C C A 3′
3′ C G T A T A A T G A C C A C G T C C T G G T 5′

P1 specific (−177 to −151):

5′ T C C T C C T C C T C T T T C C C C G G C T C C C C A 3′
3′ A G G A G G A G G A G A A A G G G G C C G A G G G G T 5′

P2 specific (+89 to +118):

5′ T T G G C G G G A A A A A G A A G G G A G G G G A G G G A T 3′
3′ A A C C G C C C T T T T T C T T C C C T C C C C T C C C T A 5′

P1 human (−142 to −116):

5′ C C T T C C C C A C C C T C C C C A C C C T C C C C A 3′
3′ G G A A G G G G T G G G A G G G G T G G G A G G G G T 5′

P2 human (+99 to +121):

5′ G G G A A A A A G A A C G G A G G G A G G G A 3′
3′ C C C T T T T T C T T G C C T C C C T C C C T 5′

Gel Mobility Shift Analysis of Triplex Formation. Potential triplex-forming oligonucleotides were heated at 65 °C for 5−10 min to reduce self-aggregation. After quick-cooling, the oligonucleotides were added to their respective radiolabeled double-stranded target in 20 mM Tris (pH 7.9), 2.5 mM $MgCl_2$, 140 mM KCl, 1 mM DTT, and 8% glycerol (except for Figure 4C) and incubated at 37 °C for 2 h. The reactions in Figure 4C were conducted in 20 mM Tris (pH 7.9), 10 mM $MgCl_2$ and incubated at 37 °C for 3 h. Samples were electrophoresed on 16% native polyacrylamide gels at 100 V. Both gel and running buffer contained 90 mM Tris−borate (pH 8.0), 10 mM $MgCl_2$. Bands were visualized by autoradiography.

DNase I Footprinting Analysis. "P1" triplex formation was conducted as follows: oligonucleotides of 61 and 56 nucleotides were synthesized, purified, and quantitated as described above. After annealing, the resulting duplex, representing the mouse *myc* promoter region from −199 to −138 with respect to P1, was Klenow-labeled with [α-^{32}P]-dATP. This labeled fragment was incubated with triplex-forming oligonucleotide (preheated to 65 °C and quick-cooled) or poly[d(I-C)] and incubated overnight at 37 °C in 20 mM Tris (pH 7.9), 10 mM $MgCl_2$.

"P2" triplex formation was conducted as follows: a 775-bp fragment of the murine c-*myc* gene was PCR-amplified, using *Taq* polymerase, from the plasmid pMMTV-Stu (American Type Culture Collection) which harbors 9.3 kb of the murine myc locus. The 5′ PCR primer was designed with a *Kpn*I restriction endonuclease recognition sequence and the 3′ primer with a *Bgl*II recognition sequence. The PCR-amplified fragment was purified from a 1% low melting point agarose gel and ligated into the vector pCR (Invitrogen). This plasmid was digested with *Kpn*I and *Bgl*II, and the liberated fragment was again gel-purified and subcloned into pGLII (Promega). The resulting plasmid yields four fragments upon digestion with *Sca*I and *Hin*dIII. The 343 bp fragment, with an upstream *Sca*I blunt end and a downstream *Hin*dIII sticky end, was resolved and excised from a 3% low melting point agarose gel. This fragment was end-labeled with [α-^{32}P]dCTP using the Klenow fragment and incubated overnight with oligonucleotide (preheated to 65 °C and cooled) in 20 mM Tris (pH 7.9), 10 mM $MgCl_2$ at 37 °C. The sequence of the target purine-rich region was confirmed by chemical cleavage sequencing (Maxam & Gilbert, 1980).

After triplex formation, samples were allowed to cool to room temperature and then were digested with DNase I on ice (20 s for the P1 fragment, 30 s for the P2 fragment). Digestion was terminated by the addition of 20 mM EDTA in 90% formamide followed by heating to 95 °C for 5 min. Samples were electrophoresed on 8 M urea/polyacrylamide gels at 42 W.

Analysis of Inhibition of Protein Binding. Potential triplex-forming oligonucleotides were heated at 65 °C for 5−10 min, quick-cooled, and then added to their respective labeled double-stranded target oligonucleotides in 20 mM Tris (pH 7.9), 2.5 mM $MgCl_2$, 140 mM KCl, 1 mM DTT, and 8% glycerol. Incubation at 37 °C proceeded for 2 h. Samples were then allowed to cool to room temperature, at which point YC8 nuclear extract was added to achieve a final concentration of 265 ng of protein/μL. Incubation at 22 °C proceeded for 30 min. Samples were then electrophoresed on 5% native polyacrylamide gels at 100 V. Both gel and running buffer contained 90 mM Tris−borate (pH 8.5), 2 mM EDTA. Bands were visualized by autoradiography.

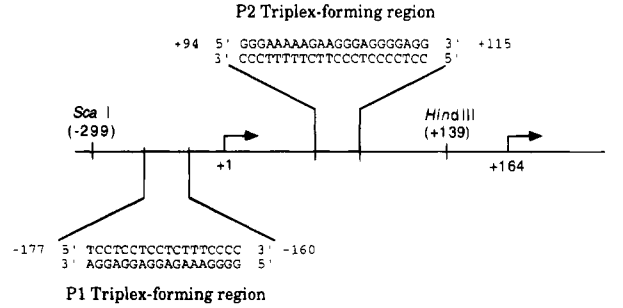

FIGURE 1: Map of the murine c-*myc* promoter showing the P1 and P2 targets for protein binding and triplex formation. The P1 transcription initiation site is designated "+1". The P2 initiation site is located 164 bp downstream. Restriction endonucleases *Sca*I and *Hin*dIII (cutting site positions relative to the P1 start site) were used to prepare a DNase I footprinting template.

RESULTS

Nuclear Protein Binding. The c-*myc* locus contains two major transcription initiation sites, P1 and P2 (Figure 1). Purine-rich elements upstream of each of these sites have been shown to bind factors that are essential for efficient transcription initiation. Synthetic duplexes representing c-*myc* promoter pur:pyr motifs are illustrated "P1 Triplex-forming region" (27 bp) and "P2 Triplex-forming region" (30 bp) in Figure 1. Protein binding was demonstrated by gel mobility shift experiments (Figure 2). The murine T lymphoma cell line YC8 (Ghanta et al., 1993) was chosen as a source of nuclear proteins based on its high level of c-*myc* expression (data not shown). Incubation of the 27 bp P1 probe (panel A) with a YC8 nuclear extract results in the formation of a highly retarded major band indicating the formation of a protein−DNA complex (arrow). The specificity of this interaction is demonstrated by the fact that the 27 bp P1 duplex, in its unlabeled form, competes for binding. This specific competition is shown by the significant decrease in the amount of labeled DNA that shifts, reflecting the binding of the protein of interest to this unlabeled DNA of the same sequence. Competition of the unlabeled P1 sequence is accompanied by the increasing presence of free probe in lanes 3, 4, and 5. Whereas a 50-fold excess of specific competitor results in decreased nuclear protein−DNA interaction, the same concentration of nonspecific competitor probe is unable to compete, as evidenced by the persistence of a shifted protein−DNA complex in lane 8 of the same intensity as that in lane 2. The results of the specific and nonspecific competitions demonstrate that nuclear factor binding to this P1 region involves a sequence-specific interaction. When a 27 bp duplex representing the corresponding human P1 pur:pyr motif is used as a competitor (lanes 9, 10, and 11), the pattern of protein displacement closely resembles that observed when the unlabeled probe itself is used as a competitor (lanes 3, 4, and 5). These results suggest that there is adequate homology between the two duplexes to allow binding of the same protein, and that the human sequence possesses similar affinity for this nuclear proteins.

Panel B of Figure 2 illustrates similar specificity of nuclear factor binding to the 30 bp probe representing the polypyrimidine:polypurine region upstream of P2. As shown in lane 2, a specific protein−DNA complex (arrow) is formed

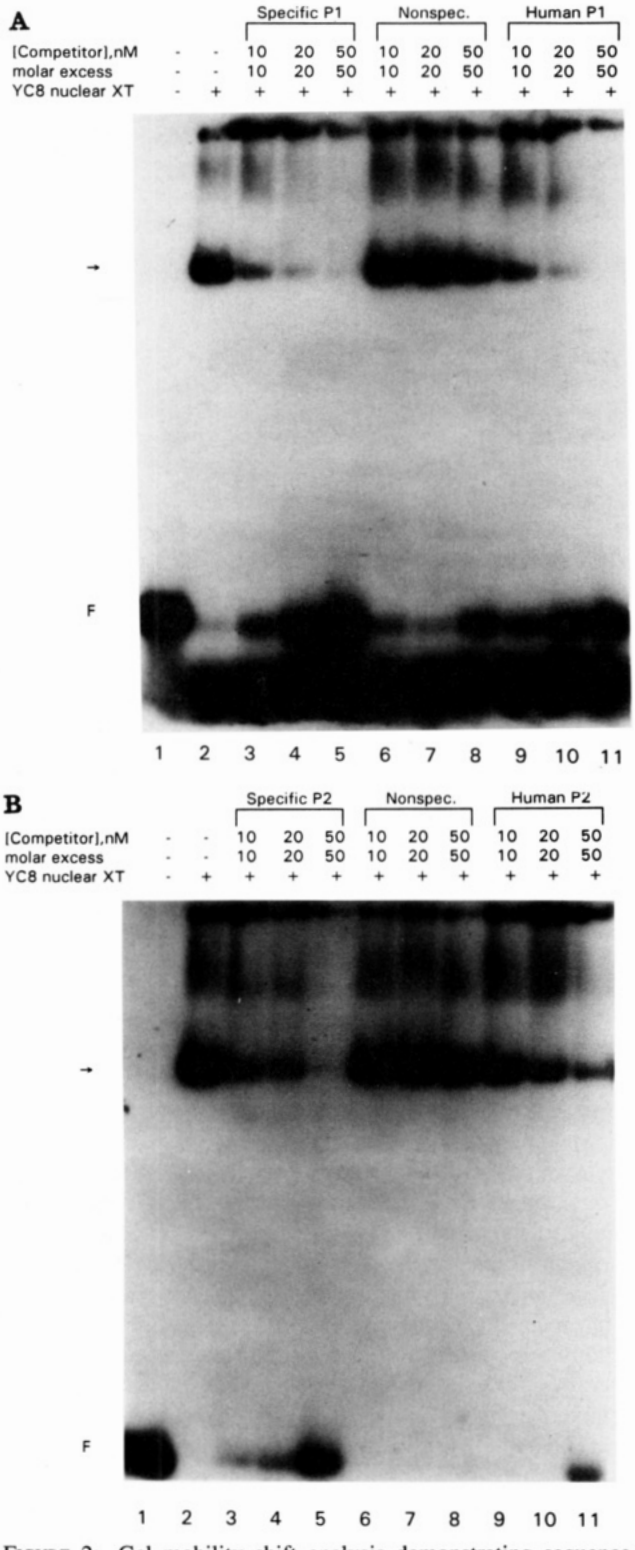

FIGURE 2: Gel mobility shift analysis demonstrating sequence-specific nuclear protein binding to the P1 purine:pyrimidine and the P2 pyrimidine:purine motifs in the murine c-*myc* promoter. Except for lanes 1 and 2 in each panel, synthetic ^{32}P-kinase-labeled 27 bp (A) and 30 bp (B) murine c-*myc* fragments were incubated with competitor duplexes and YC8 nuclear extract as described. "Molar excess" refers to the stoichiometric quantity of competitor DNA with respect to labeled probe. Lane 1 (both panels) does not contain either competitor DNA or extract. Lane 2 (both panels) does not contain competitor DNA. Arrows indicate specific protein−DNA complexes. "F" indicates free probe.

following incubation of the labeled probe with YC8 nuclear extract. Upon addition of increasing amounts of unlabeled specific murine competitors (lanes 3, 4, and 5), the labeled DNA−protein complexes disappear accompanied by a corresponding increase in the amount of the faster-migrating free probe. The unlabeled duplex representing the analogous region of the human promoter (lanes 9, 10, and 11) also competes, suggesting, as with the P1 sequence, a functional conservation of this *cis*-acting element between mice and humans. The nonspecific competitor does not affect the formation of protein complexes with the labeled probe (lanes 6, 7, and 8). These data demonstrate that the nuclear factor binding to this P2 pyr:pur motif, as with the P1 pur:pyr motif, involves a sequence-specific interaction.

Oligonucleotide Design and Triplex Formation with the P1 Target. Figure 3A illustrates the 27 bp pur:pyr P1 target duplex and the oligonucleotides that were assessed for their ability to form triplex with this sequence. Oligonucleotide sequences were designed to allow guanine recognition of G:C base pairs in the target, thus forming G•G:C triads, and thymine recognition of A:T base pairs, forming T•A:T triads. Potential triplex-forming oligonucleotides targeted to this pur:pyr motif were designed with identical sequences, but in both parallel (P1−18p) and antiparallel (P1−18ap) orientation with respect to the polypurine strand (i.e., the strand to which the reverse Hoogsteen hydrogen bonds form) of the target duplex. Because the target sequence is highly asymmetrical, the parallel oligonucleotide can bind only in the parallel orientation (improbable under these conditions), and the antiparallel oligonucleotide can bind only in the antiparallel fashion in order to form the defined triads.

The relative ability of each oligonucleotide to form triplex with the target duplex is determined by gel mobility shift analysis and confirmed by DNase I footprinting. In order to approximate more closely the ionic conditions *in vivo*, the gel shift reactions were conducted in the presence of 140 mM K^+, a concentration which has previously been shown to inhibit purine third-strand intermolecular triple helix formation (Milligan et al., 1993; Cheng & Van Dyke, 1993; Olivas & Maher, 1995). As illustrated in the gel shift in panel B, the P1−18ap oligonucleotide causes a shift of duplex (D) to a distinct, slower migrating complex (T) indicating triplex formation (Durland et al., 1990). A 1 μM concentration (a 10^3-fold molar excess) of this antiparallel oligonucleotide is sufficient to shift approximately half of the labeled target to the triple-helical form (lane 3). A 10^4-fold molar excess of the antiparallel oligonucleotide reveals a near-complete shift to triplex (lane 4). Lane 5, in contrast, features a 10^4-fold excess of parallel oligonucleotide (P1−18p) and demonstrates this oligonucleotide's failure to form triplex as all of the labeled target continues to migrate as a duplex. The use of oligonucleotides of the same sequence but opposite orientation is designed to illustrate the high degree of sequence-specificity with which the third strand binds its duplex ligand.

The endonuclease DNase I is less effective in cleaving the A form of DNA, which accompanies triplex formation (Arnott & Selsing, 1974), than the B form (Rhodes & Klug, 1986). A DNase I footprint experiment was conducted to ensure that the oligonucleotide binds the region to which it is directed and affords protection from DNase cleavage to the entire 18 bp sequence, thus conclusively proving antiparallel triplex formation (Figure 3C). Lane 4 shows digestion of the Klenow-labeled target after incubation with an excess of the P1−18ap. Triplex formation protects the polypurine stretch (displayed in lane 2) from cleavage, demonstrating the localization of the oligonucleotide to the correct target sequence. P1−18p (lane 5), in the same

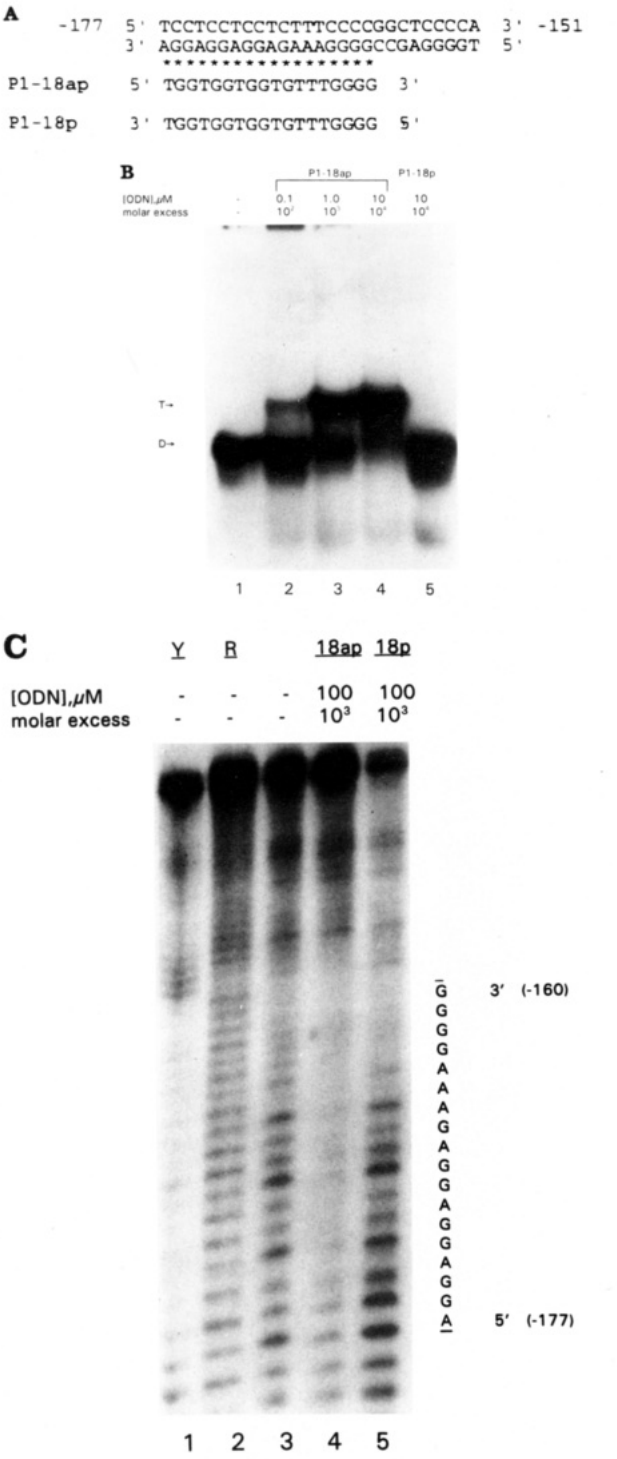

FIGURE 3: (A) Oligonucleotide sequences and their alignment with the 27 bp murine c-*myc* P1 target site. The "ap" and "p" notations refer to the orientation (antiparallel and parallel, respectively) of the oligonucleotide with respect to the purine-rich target strand. Asterisks denote reverse-Hoogsteen hydrogen bonds. (B) Gel mobility shift analysis demonstrating oligonucleotide-directed triplex formation in the P1 promoter target. The concentration of oligonucleotide (ODN), as well as the excess of oligonucleotide, is indicated above each lane. The buffer for triplex formation is identical to that used in protein binding. Lane 1 is a control migration of the labeled target without oligonucleotide. "T" and "D" indicate the electrophoretic positions of triplex and duplex DNA, respectively. (C) DNase I footprinting analysis of triplex formation in the P1 promoter target. Lanes 1 and 2 are Maxam–Gilbert sequencing reactions (pyrimidine-specific and purine-specific, respectively) conducted with the synthetic ^{32}P-Klenow-labeled 61 bp P1 target duplex. This duplex was incubated in the absence (lane 3) or presence (lanes 4 and 5) of oligonucleotides. The polypurine target sequence is shown vertically at left.

concentration, does not afford protection as demontrated by a cleavage pattern similar to that in the lane 3 control digestion. The data in panel C are consistent with those obtained from gel mobility shift analysis (panel B), further demonstrating that triplex formation occurs with the third strand in an antiparallel orientation relative to the polypurine target strand.

Oligonucleotide Design and Triplex Formation with the P2 Target. Figure 4A shows the P2 target polypyrimidine: polypurine duplex and the oligonucleotides assessed for their ability to form triplex. Panel B is a gel mobility shift analysis used to follow the change in mobility following triplex formation. Again, incubation of oligonucleotides with the target duplex was conducted in the presence of 140 mM K^+. The antiparallel oligonucleotide, P2−22ap, shifts nearly all of the labeled duplex to triplex at a 10^4 molar excess (lane 5). Lane 6 exhibits the inability of P1−18p, which bears sequence similarities to P2−22ap, to shift the labeled target to the triple-helical form, thus underscoring the specificity of the interaction between P2−22ap and the target. The parallel oligonucleotide, P2−22p, is capable of forming triplex with this particular target (panel C). The results in panel C were achieved in TM buffer (see Materials and Methods), but were entirely reproducible in the physiological approximation buffer used above (data not shown). The unique sequence of P2−22p suggests that the orientation of the third strand is antiparallel to the target purine strand in triplex formation. Panel D shows the 12 consecutive hydrogen bonds that would result from an antiparallel, reverse-Hoogsteen interaction of P2−22p with the P2 target duplex.

A DNase I footprint analysis illustrates the sequence-specificity of triplex formation by the P2 promoter (Figure 4, panel E). The sequence lanes (1 and 2) highlight the distinctive stretch of all pyrimidines in the coding strand and all purines in the noncoding strand. It is to this unique motif that the P2 oligonucleotide is directed. Lane 3 is a control for DNase I digestion in the absence of oligonucleotide. Lanes 4, 5, and 6 show the protection afforded by increasing concentrations of P2−22ap oligonucleotide, demonstrating triplex formation within the target region. The cleavage pattern following incubation of the target with the P1−18ap oligonucleotide displays fragments resulting from cleavage within the target region of a greater intensity than those in the P2−22ap-treated reactions, indicating the failure of the P1−18ap oligonucleotide to form triplex within this region. Because of uneven levels of total radioactivity in each lane, the extent of cleavage in the target region must be judged in a non-triplex-forming region context. The lanes with the triplex-forming oligonucleotide show a greater non-target region:target region band intensity ratio than that of the no-oligo and non-triplex-forming controls. Taken together, the gel mobility shift analysis and the footprint experiment conclusively demonstrate the specificity of the interaction of the P2−22ap oligonucleotide with its polypyrimidine: polypurine target.

Effect of Triplex Formation on Nuclear Protein Binding. The effect of triplex formation within the P1 and P2 target regions on nuclear protein binding was determined by gel mobility shift analysis. Figure 5A indicates a concentration-dependent inhibition of protein binding to the P1 probe following incubation of the probe with the triplex-forming oligonucleotide, P1−18ap. A 10 μM P1−18ap concentration (lane 5), sufficient to shift nearly all of the probe to the triplex

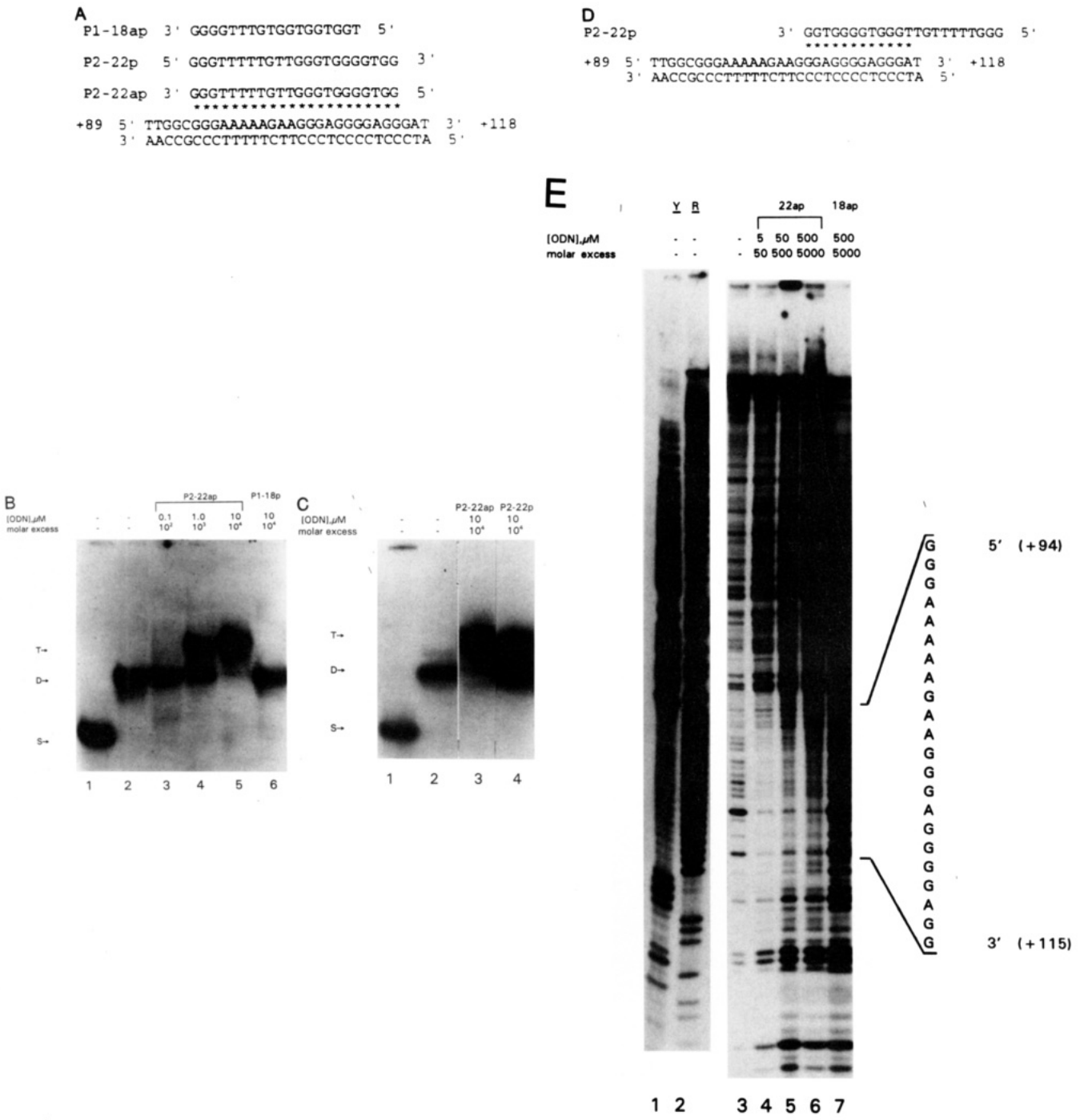

FIGURE 4: (A) Oligonucleotide sequences and their alignment with the 30 bp murine c-*myc* P2 target site. The "ap" and "p" notations refer to the orientation (antiparallel and parallel, respectively) of the oligonucleotide with respect to the purine-rich target strand. Asterisks denote reverse-Hoogsteen hydrogen bonds. (B) Gel mobility shift analysis demonstrating oligonucleotide-directed triplex formation in the P2 promoter target. The concentration of oligonucleotide (ODN), as well as the excess of oligonucleotide, is indicated above each lane. The buffer for triplex formation is identical to that used in protein binding. Lane 1 is a control for the migration of the labeled polypurine strand component of the target duplex. Lane 2 is a control for the migration of the target duplex without oligonucleotide. "T" = triplex; "D" = duplex; "S" = single-stranded DNA. (C) Gel mobility shift analysis demonstrating the ability of parallel oligonucleotide to form triplex with the P2 promoter target. The concentration of oligonucleotide (ODN), as well as the excess of oligonucleotide, is indicated above each lane. This experiment was conducted in TM buffer. Lane 1 is a control for the migration of the labeled polypurine strand component of the target duplex. Lane 2 is a control for the migration of the target duplex without oligonucleotide. "T" = triplex; "D" = duplex; "S" = single-stranded DNA. (D) Sequence of the parallel P2 oligonucleotide and the reverse-Hoogsteen hydrogen binding pattern (asterisks) it would assume in forming antiparallel triplex. (E) DNase I footprinting analysis of triplex formation in the P2 promoter target. Lanes 1 and 2 are Maxam−Gilbert sequencing reactions (pyrimidine-specific and purine-specific, respectively) conducted with the ^{32}P-Klenow-labeled 343 bp P2 target duplex. This duplex was incubated in the absence (lane 3) or presence (lanes 4−7) of oligonucleotides. The polypurine target sequence is shown vertically at left.

form in Figure 3B, results in a near-complete inhibition of protein binding. Protein binding to the labeled probe is not inhibited by the same concentration of P1−18p, shown to be unable to form triplex with this target. The difference in protein bound to the labeled target in lane 2 (binding in the absence of oligonucleotide) and lane 5 is sufficient to illustrate the inhibitory effect of triplex formation within this P1 pur:pyr motif on nuclear factor binding.

Triple helix formation with the P2 target has a similar effect (Figure 5, panel B). Again, a 10 μM concentration

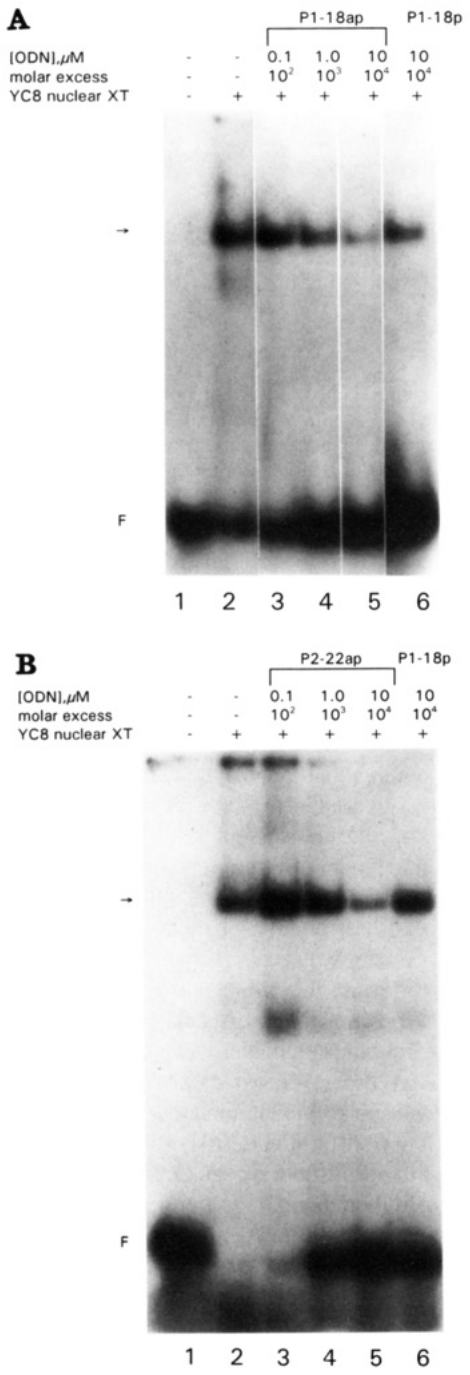

FIGURE 5: Gel mobility shift analysis demonstrating inhibition of nuclear protein binding to the murine c-*myc* P1 and P2 promoters by triplex formation. The 27bp P1 target (A) and the 30 bp P2 target (B) were incubated with their respective third-strand oligonucleotides and then with YC8 nuclear extract as described. Arrows indicate protein−DNA complexes. "F" indicates free probe. Labeled probe which has adopted the triplex confomer cannot be distinguished from duplex labeled probe as a result of the low percentage (5%) of acrylamide in these gels as opposed to the gels used to demonstrate triplex formation.

of the triplex-forming oligonucleotide, P2−22ap in this case, is sufficient to block nearly all protein−DNA complex formation (lane 5). This concentration of oligonucleotide was instrumental in shifting roughly 95% of the probe to the triple-helical form in Figure 4B. At the same concentration, P1−18p, which does not form triplex, has little effect upon protein binding (lane 6). Although triplex formation with the probe cannot be resolved on this gel due to the low polyacrylamide percentage (5%), it is presumed that the triplex is stable throughout the protein binding period since the buffers for triplex formation and protein binding are identical. Therefore, panel B in Figures 3 and 4 is a suitable reference as to the degree of triplex formation in each of the protein binding reactions. As expected, a near-complete shift of the probe to the triple-helical form is required to prohibit the binding of protein to the probe. The data above suggest that triplex formation is directly responsible for the inhibition of nuclear factor binding to duplexes representing both the P1 and P2 polypurine- and polypyrimidine- rich regions of the murine c-*myc* promoter.

DISCUSSION

The ability of triplex formation to inhibit transcription of a target gene was first demonstrated with an oligodeoxynucleotide targeted to a polypurine:polypyrimidine nuclease-hypersensitive region upstream of the P1 start site in the human c-*myc* oncogene in a cell-free system (Cooney et al., 1988). Subsequent work has shown that c-*myc* transcription is repressed in human cervical carcinoma (HeLa) cells by treatment with the triplex-forming oligonucleotide directed to the nuclease-hypersensitive site (Postel et al., 1991). Helm et al. (1993) demonstrated that a triplex-forming oligonucleotide targeted to this same region of the c-*myc* promoter inhibits the growth of ovarian carcinoma (SKOV-3) cells and HeLa cells in culture, in keeping with the necessity for c-*myc* in proliferation (Kelly et al., 1983; Luscher & Eisenman, 1990). Although the mechanism for transcription inhibition has not been elucidated, it has been suggested that triplex formation prevents binding of essential regulatory factors (Maher et al., 1989; Gee & Miller, 1992). In the case of human c-*myc*, the triplex target is the PuF/Nm23 binding site (Postel et al., 1989; 1993). Similarly, a triplex-forming oligonucleotide known to block NFκB binding to an enhancer element (Grigoriev et al., 1992) represses transcription of the interleukin-2 receptor α-subunit gene in cultured lymphocytes (Orson et al., 1991). Triplex-induced inhibition of nuclear protein binding also inhibits transcription from the HER-2/*neu* promoter *in vitro* (Ebbinghaus et al., 1993). Blocking Sp1 binding by the formation of triplex DNA in the human Ha-*ras* promoter (Mayfield et al., 1994b; Mayfield & Miller, 1994) is sufficient to inhibit *in vitro* transcription initiation. These studies demonstrate *in vitro* and *in vivo* gene-specific transcription repression accompanying triplex targeted to nuclear protein binding sites.

The results presented here are the first to demonstrate triplex formation by the murine c-*myc* promoter. Two important regulatory regions in the *myc* promoter have been the object of our attention. The first is the polypurine: polypyrimidine region upstream of the P1 transcription initiation site that exhibits nuclease hypersensitivity. The *cis*-acting importance of this region in transcription from both the human P1 and P2 c-*myc* promoter is well documented (Lipp et al., 1987; Postel et al., 1989). A recent model proposes that it is the ability of this peculiar DNA sequence to form *intra*molecular triple helices that lends it transcriptional significance (Firulli et al., 1994). We demonstrate here that a stable antiparallel intermolecular triple helix is formed upon addition of a mixed purine/pyrimidine third strand to a duplex representing the murine P1 pur:pyr nuclease hypersensitive element.

The model mentioned above (Firulli et al., 1994), designating the triplex-forming ability of the human c-*myc* nuclease sensitive element as a positive indicator of promoter

strength, seems to contradict the prevailing notion that triplex mediates an inhibitory effect on transcription by blocking the interaction of regulatory proteins. The transcriptionally active stable triplexes in that study involve C^+•G:C and T•A:T triplets in an intramolecular complex that includes a single-stranded portion of approximately 25 bases and a parallel orientation of the polypyrimidine triplex-forming strand. The authors suggest that one or more of the proteins known to bind this nuclease hypersensitive element could function to stabilize this structure at neutral pH. The likelihood of this type of triplex occurring is not incompatible with the potential down-regulatory role of our triplex. The antiparallel triplex-forming oligonucleotide may function in skewing the equilibrium of intramolecular triplex formation such that intermolecular triplex prevails. Its repressive effect might arise by not allowing the interaction of proteins that would bind to the intramolecular triplex-induced structure in a transcriptionally-active locus. If this hypothesis is correct, the task of achieving and maintaining large molar excesses of oligonucleotide in the nucleus becomes that much more critical in down-regulating c-*myc* expression. The scenario is made exponentially more complicated by the presumed association of various proteins able to bind single- and double-stranded nucleic acids. The fact that a ribonucleoprotein binds this region of the human *myc* promoter suggests that intermolecular interactions might exist that involve the recruitment of regulatory nucleic acids by proteins to a particular subnuclear locale. As of yet, intramolecular triple helix formation has not been demonstrated in the murine c-*myc* promoter, although sequence motifs that are involved in triplex formation in the Firulli model are conserved between human and mouse.

The second important regulatory region in the c-*myc* promoter with which we have formed triplex DNA is coincident with the binding site for several activating proteins. Roussel et al. (1994) have postulated that *ets* family members and E2F-1 may independently regulate c-*myc* expression through the same binding site within this region at different times following a mitogenic stimulus. The adjacent ME1a1 element has been shown to increase initiation of c-*myc* transcription *in vitro* (Hall, 1990) and to be essential for initiation of P2 *in vivo* (Asselin et al., 1989). A zinc finger protein, termed Maz in humans, was shown to bind this site (Pyrc et al., 1992; Bossone et al., 1992). Moberg et al. (1992) named the E2F and Maz binding sites as two of the three distinct elements within the murine c-*myc* promoter that are required for transcription. Our novel triplex-forming site encompasses half of the E2F and all of the ME1a1 canonical *cis* elements. We demonstrate here that nuclear protein−DNA interaction, which we have shown through DNA competitions to be sequence-specific, is inhibited upon triplex formation between the P2 target duplex and the antiparallel oligonucleotide.

Our decision to target both the P1 and P2 promoters was based on the *in vivo* work of Postel and co-workers (Postel et al., 1991). In that study, they achieved an impressive oligonucleotide concentration of 0.35 μM in the nucleus, demonstrated decreased DNase I cleavage at the appropriate hypersensitive site, and documented a decrease in c-*myc* RNA levels. While the level of transcripts originating from P1 decreased by over 80%, the number of P2 transcripts dropped by only about 40%, in keeping with the positive regulatory role of the region from −101 to −293 in P1 transcription (Hay et al., 1987). The disparity between P1 and P2 repression levels prompted our targeting both P1 and P2 promoters, as a triplex-forming oligonucleotide directed to the P1 promoter may be sufficient in inhibiting a majority of initiation events from the P1 start site, but repression of transcription from the entire locus may additionally require triplex formation between P1 and P2.

The apparent ability of a parallel oligonucleotide to form triplex is not without precedent. Durland et al. (1990) noted that a lack of asymmetry in their target duplex allowed a 37-mer parallel oligonucleotide to reverse its orientation and thereby form triplex in an antiparallel fashion. Eosin cleavage data indicated that only 27 bp of the target were protected and that the 3′ 10 bases of the triplex-forming oligonucleotide remained unbound. Our P2 parallel oligonucleotide is highly asymmetrical and is therefore not likely to reverse its orientation and form triplex with the same bases as that of the antiparallel oligonucleotide. However, the 12 potential triplets of canonical reverse-Hoogsteen hydrogen bonding suggest that it might indeed reverse its orientation, albeit to adopt a tailed triple-helical structure similar to that described above. Further evidence supporting a reverse-Hoogsteen structure is the fact that the P2 parallel oligonucleotide featuring adenines substituted for thymines also forms triplex (data not shown). Giovannangeli et al. (1992) have demonstrated that A•A:T triplets adopt only the reverse-Hoogsteen bonding pattern, which corresponds to an antiparallel orientation in the case of a third strand with bases in the *anti* conformation. Our result differs from the Durland et al. (1990) study in that their parallel triplex with a tail displayed reduced mobility relative to that of the antiparallel triplex while our P2 parallel triplex shows slightly increased migration in comparison to the P2 antiparallel triplex.

Both the P1 and P2 murine c-*myc* targets for triplex formation are highly homologous to their equivalent regions in the human *myc* promoter. This sequence homology suggests a conserved role for these elements. Our data demonstrate the ability of the analogous human sequences (both P1 and P2) to compete for murine nuclear protein binding. That the binding is specific is shown by the inability of a nonspecific fragment of DNA to compete. The fact that the human sequences compete suggests that there are proteins with similar DNA binding specificities in the human *myc* transcription system and enhances the potential of a whole animal murine model in that it may more accurately predict human responses to triplex-forming oligonucleotides targeted to human c-*myc*.

Ultimately, we seek to generate a model for the efficacy of triplex-forming oligonucleotides to alter c-*myc* expression. One possible alternative approach might have been a transgenic mouse expressing human c-*myc* for determining the effect of human *myc*-directed triplex-forming oligonucleotides on human *myc* expression in a mouse. We chose instead to construct a whole animal model to examine the effect of targeting a native gene that is instrumental in normal cell proliferation as well as oncogenesis. Another version of this latter approach might be to test human c-*myc* oligonucleotides in a whole animal context. However, the specificity of triplex formation lessens the possibility that human *myc*-targeted oligonucleotides will form triplex with the murine *myc* promoter and turn off the endogenous gene. It is necessary, therefore, to use oligonucleotides with proven triplex-forming ability with murine DNA as well as the ability to block murine nuclear factors from binding their specific targets. Most important, though, is to assess the

effect of triplex-forming oligonucleotides on murine c-*myc* expression within a murine whole animal context.

ACKNOWLEDGMENT

We are grateful to K. Shrestha for preparation of YC8 nuclear extracts. We thank C. A. Mayfield and H. G. Kim for technical assistance and helpful discussion. We also thank B. Hughes for computer time and assistance.

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BI942740O