Influence of GC and AT Specific DNA Minor Groove Binding Drugs on Intermolecular Triplex Formation in the Human c-Ki-ras Promoter†

Nadarajah Vigneswaran,‡ Charles A. Mayfield,§ Brad Rodu,‡ Roger James,‡ H.-G. Kim,§ and Donald M. Miller*,§,||

Department of Oral Pathology, Bolden Laboratory, Department of Medicine, Department of Biochemistry and Molecular Genetics, Comprehensive Cancer Center, University of Alabama at Birmingham, Alabama 35294-3300 and the Birmingham Veterans Affairs Medical Center, Birmingham, Alabama 35294-0001

Received July 10, 1995; Revised Manuscript Received November 20, 1995⊗

ABSTRACT: We have used DNase I footprinting and gel shift assays to characterize the interaction of DNA binding drugs mithramycin, distamycin, and berenil with an intermolecular triplex formed by the human c-Ki-ras promoter. A purine-rich triplex-forming oligonucleotide (ODN) forms a stable intermolecular triple helix (triplex) with a homopurine (PR):homopyrimidine (PY) motif in the human c-Ki-ras promoter which contains a 22bp PR:PY region (−328 to −307). This triplex structure is comprised of 15 G·G:C triplets interspersed with 7 T·A:T triplets. Mithramycin binding sites in the human c-Ki-ras promoter encompass most of the triplex target site and three G-C-rich sequences downstream of this triplex-forming region. Mithramycin binding within the c-Ki-ras promoter completely abrogates triplex formation. Furthermore, the addition of mithramycin to pre-formed triplex by c-Ki-ras promoter displaces the major groove bound ODN. Five prominent distamycin binding sites are noted within the c-Ki-ras promoter including the triplex-forming site as well as A-T-rich regions upstream and downstream of the triplex site. Berenil does not bind within the triplex target sequence, and only one berenil binding sequence downstream of the triplex motif was present within the c-Ki-ras promoter fragment. Neither distamycin nor berenil prevents triplex formation, and, furthermore, the addition of either distamycin or berenil to the pre-formed triplex structure did not displace the major-groove-bound third strand. This study demonstrates that GC-specific and AT-specific minor groove ligands differentially affect the intermolecular pur·pur:pyr triplex. A possible biological significance of mithramycin interaction with intramolecular triplex is discussed.

Homopurine−homopyrimidine sequences occur disproportionately in the regulatory regions of eukaryotic genes and represent approximately 1% of the total genome (Birnboim et al., 1979; Wells et al., 1988). These sequences are DNase hypersensitive, both in chromatin and in superhelical plasmids (Wells et al., 1988). Recent studies suggest that these regions can form H-DNA which represents intramolecular triple-helical DNA (triplex DNA) (Mirkin & Frank-Kamenetskii, 1994). The *in vivo* existence of intramolecular triplex DNA has been suggested in various cell types by the reaction of multiple chromatin sites with an antibody specific for triplex DNA (Agazie et al., 1994). Transient formation of intramolecular triplex by these tandemly repeated homopyrimidine:homopurine tracts may play an important role in regulating gene expression (Wells et al., 1988).

The polypurine:polypyrimidine sequences are often important positive cis-acting elements which are necessary for activated transcription (Firulli et al., 1994; Raghu et al., 1994). In recent years, a number of groups have successfully exploited these regions as targets for the formation of intermolecular triple-helical DNA using synthetic oligonucleotides (ODN)[1] (Beal & Dervan, 1991; Gee et al., 1992; Ebbinghaus et al., 1993; Mayfield et al., 1994a,b). Two types of intermolecular triplex DNA have been described; pyr·pur:pyr triplex and pur·pur:pyr triplex. They differ in their sequence composition and relative orientation of the third strand. In pyr·pur:pyr triplex a pyrimidine-rich ODN binds in a parallel orientation relative to the purine-rich tract of the double-stranded DNA (DS DNA) via the formation of T·A:T and pH-dependent C^+·G:C triplets (Moser & Dervan, 1987). In the pur·pur:pyr type, purine-rich or mixed purine/pyrimidine ODNs bind in an antiparallel orientation to the purine-rich strand of the DS DNA (Moser & Dervan, 1987; Beal & Dervan, 1991. The resulting triplex DNA consists of predominantly of G·G:C triplets interspersed with A·A:T or T·A:T triplets (Moser & Dervan, 1987; Beal & Dervan, 1991). In triple-helical DNA, the pyrimidine- or purine-rich ODN is bound in the major groove of the DS DNA and interacts with specific purine bases of the target DNA by forming either Hoogsteen or reverse Hoogsteen hydrogen bonds, respectively (Moser & Dervan, 1987; Beal & Dervan, 1991). Intermolecular triplex formation by the binding sites of transcription factors has been shown to inhibit the transcription of a number of eukaryotic promoters (Postel et al., 1991; Orsen et al., 1991; Ebbinghaus et al.,

† Supported by Grants CA61964-01A1 (to C.M.), CA42664, and CA42337 (to D.M.M.), from the National Institutes of Health, by the Share Foundation (to D.M.M.), by Veterans Administration Merit Review Grant (to D.M.M.), and by Council for Tobacco Research Scholar Award SA020 (to C.M.).

* To whom correspondence should be addressed, 1824 6th Avenue South, Wallace Tumor Institute, Room 520, Birmingham, AL 35294. Phone: (205) 934-1977. Fax: (205) 975-6911.

‡ Department of Oral Pathology.

§ Bolden Laboratory, Department of Medicine.

|| Department of Biochemistry and Molecular Genetics.

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

[1] Abbrevations: ODN, oligonucleotide; bp, base pair; DS, double strand.

0006-2960/96/0435-1106$12.00/0

1993; Mayfield et al., 1994b). The c-Ki-ras protooncogene is a member of a highly conserved ras gene family whose protein products are important in signal transduction and regulation of cell proliferation (Barbacid, 1987). Although the c-Ki-ras gene is expressed constitutively at basal levels in all cells, its expression is increased transiently during tissue growth and cell division (Muller et al., 1983; Goyette et al., 1984). Because of the pivotal role of c-Ki-ras expression in the control of cell growth and differentiation, it is reasonable to expect that its transcription would be tightly regulated by multiple pathways.

Although regulation of c-Ki-ras transcription in normal and neoplastic cells is not well understood, it is clear that expression of the c-Ki-ras gene is controlled by both proximal and distal elements, generally located in the 5′ upstream region of the gene (Jordano & Perucho, 1986; Yamamoto & Perucho, 1988). As with other housekeeping genes, both the human and mouse c-Ki-ras promoters lack an obvious TATA or CCAAT box but contain four GC-boxes which act as putative binding sites for the transcription factor Sp1 (McGrath et al., 1986; Yamamoto & Perucho, 1988; Hoffman et al., 1987). These GC box sequences are found in a region which corresponds to the essential elements of the c-Ki-ras promoter (Jordano & Perucho, 1986). A homopurine−homopyrimidine region that is sensitive to endogenous nuclease and S1 nuclease in supercoiled plasmids is located in the region between −250 and −400 bp upstream of the exon 0/intron 1 boundary (Jordano & Perucho, 1986). This region contains a 22 bp homopurine:homopyrimidine motif located from −126 to −147 with respect to the the major transcription start site (−307 to −328 from exon 0/intron 1) (Jordano & Perucho, 1986; Mayfield et al., 1994a). Sequence-specific binding of a nuclear protein to this motif has been reported (Mayfield et al., 1994a). This sequence also forms an intermolecular triple helix with a synthetic ODN abrogating the binding of nuclear protein (Mayfield et al., 1994a). A similar region in the mouse c-Ki-ras promoter contains a 27 bp S1 hypersensitive homopurine:homopyrimidine motif which adopts an intramolecular triple-helical H-DNA conformation in mildly acidic conditions (Pestov et al., 1991).

The antiproliferative effects of DNA binding drugs are thought to be primarily due to the interaction of these drugs with double-stranded DNA and their resultant inhibition of DNA and RNA synthesis as well as DNA repair. Although DNA bindings drugs may show some degree of sequence preference, the degeneracy of their potential binding sequences is quite high and their binding affects the expression of multiple genes. Mithramycin is a GC-specific DNA binding drug which binds as a dimer within the minor groove, in the presence of Mg^{2+} (Behr & Hartmann, 1965; Sastry & Patel, 1993). Mithramycin selectively inhibits the transcription of genes with GC-rich promoters such as c-myc, H-ras, and dihydrofolate reductase (dhfr) (Snyder et al., 1991; Blume et al., 1991). This effect has been attributed to its interference with Sp1 binding sequences in these promoters (Snyder et al., 1991). On the other hand, distamycin and berenil bind in the minor groove of AT-rich DNA although there are subtle differences in their preferential binding sequences (Portugal & Waring, 1987).

The effect of DNA binding drugs on pur·pur:pyr triplex is not known. A number of recent studies have addressed the ability of nonintercalative minor groove binders to interact with triplex DNA (Chalikian et al., 1994; Park & Breslauer, 1992; Thomas & Thomas, 1993; Pilch & Breslauer, 1994; Durand et al., 1994). These studies have been limited to short pyrimidine·purine:pyrimidine triple helices (less than 20 bp long) comprised of primarily poly[$d(T)_n$·$d(A)_n$:$d(T)_n$] triplex sequences. Most of these studies have also utilized artificial triplex-forming sequences as opposed to naturally occurring gene specific triplex-forming sequences. The specific interaction of DNA binding drugs with naturally occurring triplex target sequences is important for a number of reasons. First, a DNA binding drug might interact preferentially with triplex DNA, as opposed to DS DNA, enhancing the stability of the triplex structure. Secondly, the combined exposure to ODN and "triplex-specific" DNA binding drugs may increase their ability to inhibit specific genes. Finally, the identification of a drug which can cause the disassociation of triplex DNA may have applications in the study of function and structure of intramolecular triplex (H-DNA) *in vivo*.

In this study we have examined the interaction of mithramycin, distamycin, and berenil with the intermolecular pur·pur:pyr triplex structure formed by the human c-Ki-ras promoter. These three DNA minor groove binding ligands demonstrate well characterized differences in their recognition sequences (Sastry & Patel, 1993; Carpenter et al., 1993; Portugal & Waring, 1987). We have utilized the human c-Ki-ras promoter region as a prototypic triplex-forming sequence since this region, comprised of poly(dG-dA) stretches, provides potential binding sites for both GC- and AT-specific minor groove binding compounds (Jordano & Perucho, 1986; Yamamoto & Perucho, 1988; Mayfield et al., 1994a).

EXPERIMENTAL PROCEDURES

Drugs and Enzymes. Mithramycin, distamycin, and berenil (Diminiazine aceturate) were obtained from Sigma Chemical Co. These drugs were stored as a 10^{-2} M stock solution in distilled water at −20 °C. Restriction endonucleases *Eco*R1 and *Bam*H1, T_4 polynucleotide kinase, Klenow fragment of DNA polymerase, and DNase I were purchased from Gibco BRL.

Oligonucleotide Synthesis. Phosphodiester oligonucleotides were synthesized with standard DNA bases on the Milligen Cyclone DNA synthesizer using phosphoramidite chemistry. Oligonucleotides were purified using reverse-phase oligonucleotide purification-elution cartridges (CLONTECH) followed by gel electrophoresis. The structural integrity and purity of these oligonucleotides were confirmed by 5′-^{32}P labeling using [^{32}P]ATP and T_4 polynucleotide kinase, followed by electrophoresis on a denaturing polyacrylamide gel. Oligonucleotide concentrations were determined from absorbance measurements at 260 nm using calculated molar extinction coefficients for each oligonucleotide.

Isolation of c-Ki-ras Promoter Fragment. The 181 bp (−61 to −234) c-Ki-ras2 promoter fragment was amplified from HL-60 genomic DNA using Vent DNA polymerase. The 5′ PCR primer was designed with an *Eco*R1 recognition site and the 3′ primer with a *Bam*H1 recognition site. The amplified fragment was gel purified, digested with *Eco*R1 and *Bam*H1, and then cloned into a plasmid vector pTZ 18R. ^{32}P-labeled promoter fragments used in DNase I footprinting

experiments were prepared by digestion of the plasmid construct with *Eco*R1 followed by 3′ end labeling with [^{32}P]dATP using the Klenow fragment of DNA polymerase. The fragment was then digested with *Bam*H1, and the resulting 181 bp promoter fragment was purified by gel electrophoresis through nondenaturing polyacrylamide gels. Sequence analysis of this fragment by Maxam−Gilbert method confirmed the correct sequence.

Electrophoretic-Gel Mobility Shift Analysis of Drug Binding and Triplex Formation. The DS DNA probe used in this study is a 22 bp synthetic c-Ki-ras triplex target sequence. To prepare the ^{32}P-labeled DS DNA, the synthetic coding strand was 5′-^{32}P-end-labeled with [^{32}P]ATP and T_4 polynucleotide kinase and annealed to its complement. Labeled target duplex and ODN were coincubated overnight in a buffer consisting of 90 mM Tris, 90 mM borate, and 10 mM $MgCl_2$ (TBM, pH 8.0) at 37 °C. Samples were electrophoresed at 100 V approximately for 15 h, through 16% nondenaturing polyacrylamide gel buffered with 90 mM Tris-borate (pH 8)/10 mM $MgCl_2$. Gels were then exposed at −70 °C for autoradiography. For drug interference gel shift studies, the 5′-^{32}P-end-labeled ODN was used to shift the cold duplex probe under the experimental conditions which remained the same as mentioned above. Mithramycin and distamycin were added in appropriate concentrations to the sample containing target DS DNA in three different circumstances: I, 30 min before the addition of ODN; II, simultaneously with addition of the ODN; III, approximately 30 min before loading the pre-formed triplex. Controls included the incubation of individual drugs in appropriate concentration with either labeled DS DNA probe alone or labeled ODN alone.

DNase I Footprinting. The 181 bp ^{32}P-labeled c-Ki-ras promoter fragment was incubated overnight with the oligonucleotide in the presence and absence of the appropriate concentrations of either mithramycin or distamycin or berenil in a buffer consisting of 90 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ at 37 °C. Samples were precooled on ice and then digested with DNase I for 2−3 min, and the reaction was terminated by the addition of 10 mM ethylenediaminetetraacetic acid (EDTA) followed by heating to 95 °C for 5 min. Samples were cooled on ice, and the digested DNA product was extracted once with phenol/chloroform and then ethanol precipitated. The cleaved DNA product was resuspended in 50% formamide containing bromophenol blue and xylene cyanol and denatured by heating at 95 °C for 5 min. The samples were quickly cooled on ice and then analyzed on an 8% polyacrylamide sequencing gel in the presence of 8 M urea, at 42 W for 2 h. The gels were visualized by autoradiography. To identify the drug binding sites, the ^{32}P-labeled promoter fragment was coincubated individually with appropriate concentrations of mithramycin, distamycin, or berenil in a buffer identical to the one used for oligonucleotide binding. Maxam−Gilbert sequencing reactions (A+T) and (G+C) of the promoter fragment were performed and analyzed on the same gel as the DNase I digest for sequence determination (Maxam & Gilbert, 1980). Approximately 15 000 cpm of labeled plasmid fragment was used for all DNase I protection assays resulting the final target concentration of 10 nM.

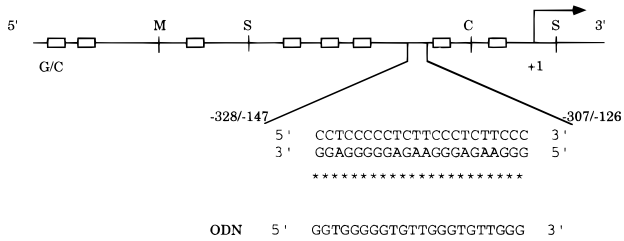

FIGURE 1: Map of the human c-Ki-ras promoter showing the 22 bp triplex target sequence. The alignment and the sequence of the triplex-forming oligodeoxynucleotide (ODN) is also shown.

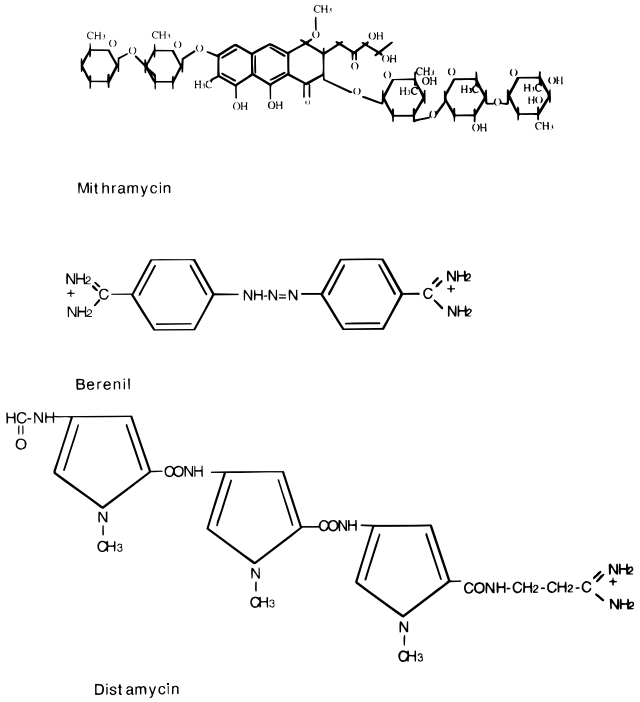

FIGURE 2: Structures of mithramycin, berenil, and distamycin.

RESULTS

The sequence of the target site and its relative position within the c-Ki-ras promoter is shown in Figure 1. The specific alignment and the sequence of the ODN which forms triplex at this site triplex is also shown in Figure 1. Mithramycin, distamycin, and berenil represent DNA binding drugs of different chemical structural families (Figure 2) that bind in the minor groove of the DS DNA.

Electrophoretic Mobility Shift Analysis of Triplex Formation. Triplex formation was initially documented by electrophoretic mobility shift analysis. The synthetic coding strand of the 22 bp c-Ki-ras duplex DNA target was used as a probe for gel shift analysis. The addition of ODN at 1 μM final concentration (1000-fold excess relative to duplex) leads to the complete shift of the 5'-^{32}P- labeled double-stranded DNA target (DS) to a distinct triplex band (Figure 3, lane 5). Alternatively, when the ODN (100 nM) was 5′-end-labeled and added to unlabeled DS target DNA (50 nM) to give a 2-fold excess of ODN, the labeled ODN involved in triplex formation appears as a distinct more slowly migrating band separated from the excess unbound ODN (Figure 4, lane 3).

Specificity of Triplex Formation. As shown in Figure 5, lane 3, DNase I cleavage of the 181 bp human c-Ki-ras promoter fragment in the presence of ODN gives rise to protection of the entire 22 bp polypurine−polypyrimidine

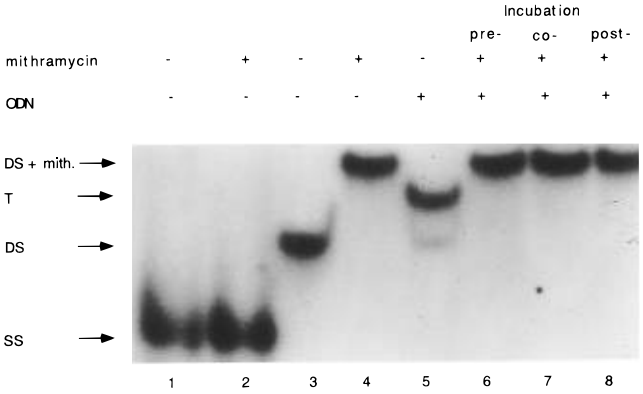

FIGURE 3: Gel mobility shift analysis demonstrating the binding of triplex-forming ODN and mithramycin to triplex target site. The ^{32}P-labeled synthetic target DNA (10 nM) was incubated in the absence or presence of ODN (1 μM) or mithramycin (100 μM) or both. Mithramycin was added prior (pre-), during (co-), and after (post-) the formation triplex. SS = single 22 bp pyrimidine strand; DS = double-stranded target DNA; T = triplex DNA; mith. = mithramycin.

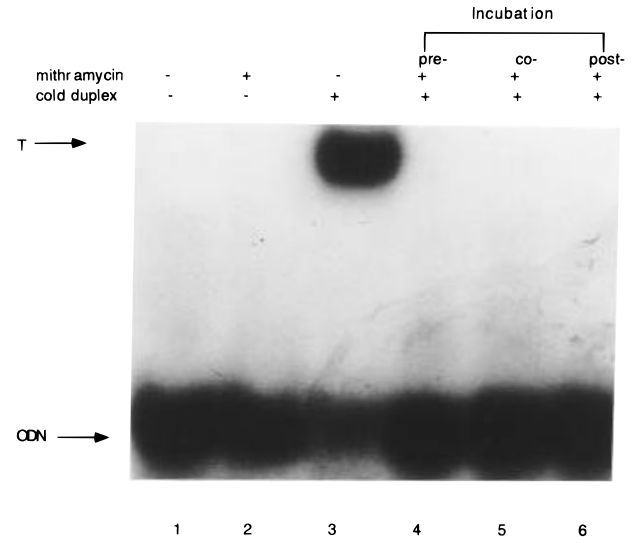

FIGURE 4: Gel mobility shift analysis showing the inhibition of triplex formation by mithramycin. Mithramycin (100 μM) was incubated with unlabeled synthetic target DNA (50 nM) prior to (pre-), during (co-) the addition of ^{32}P-labeled triplex ODN (100 nM), or after overnight triplex formation (post-). ODN = radiolabeled oligodeoxynucleotide; T = triplex DNA.

target sequence which confirms the complete formation of triple-helical DNA under these conditions. The specific sequence of this protection site is determined by alignment of Maxam−Gilbert sequencing of this promoter fragment in conjunction with the DNase I footprints (Figure 5). Complete protection of the target sequence is achieved at 5 μM concentration of ODN (approximately 5000-fold in excess compared to target site concentration) consistent with our previous data (Mayfield et al. 1994a).

Mithramycin Binding to Human c-Ki-ras Promoter Region and Its Effect on Intermolecular Triplex Formation. We also determined the pattern of mithramycin binding to the c-Ki-ras promoter by DNase I footprint analysis. The reaction conditions used for mithramycin binding were identical to those described for triplex formation. Typical DNase I digestion patterns of the 181 bp fragment of the c-Ki-ras promoter in the presence and absence of mithramycin are shown in Figure 5, lanes 1 and 2. The DNase I digestion pattern of this fragment is modified by mithramycin binding,

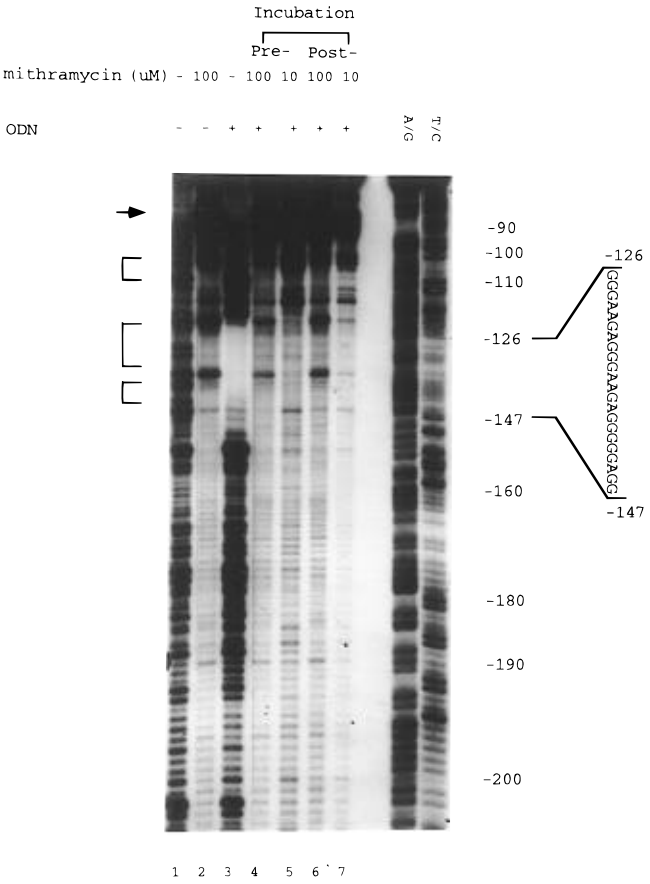

FIGURE 5: DNase I protection assay demonstrating mithramycin binding (lane 2) and triplex formation (lane 3) to the c-Ki-ras promoter. A 181 bp ^{32}P-labeled c-Ki-ras promoter fragment (10 nM) was incubated in the absence or presence of ODN (5 μM), mithramycin (100 and 10 μM), or both. The abrogation of triplex formation by mithramycin binding prior (pre-, lanes 4 and 5) and after (post-, lanes 6 and 7) the formation of triplex is also demonstrated. Maxam−Gilbert sequencing reactions A+G and C+T are seen in the last two lanes.

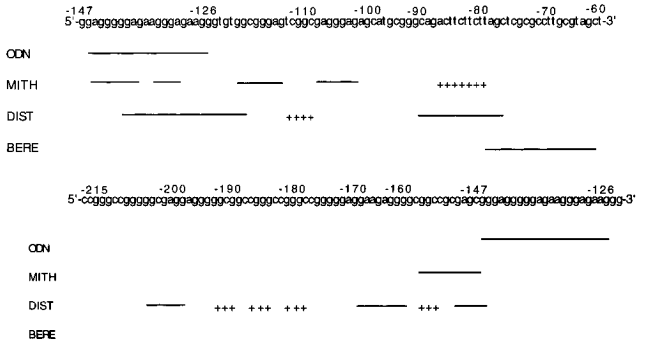

FIGURE 6: DNase I protection map showing the effects of triplex formation, mithramycin, distamycin, and berenil binding to the human c-Ki-ras promoter. Bars indicate regions which are protected from cleavage; + represents the hypersensitive region.

as evidenced by at least three regions (as shown in brackets) of DNase I protection (lane 2) observed only in the presence of mithramycin (Figure 5). In addition, the extent of DNase I cleavage at certain sites (arrows) is strongly enhanced in the presence of mithramycin relative to that in control DNA (Figure 5, lane 2). Alignment of the DNase I digestion patterns with the Maxam−Gilbert sequencing data demonstrates that each of these mithramycin induced protected sites center around G+C-rich regions (Figures 5 and 6). On the other hand, DNase I hypersensitivity sites correspond to the

A+T-rich regions which flank large mithramycin binding sites (Figures 5 and 6). DNase I protection by mithramycin binding is primarily confined to the region between −145 and −90 where −1 represents the 3′ boundary of the major transcription start site of c-Ki-ras (Figures 5 and 6). Specifically, these DNase I protected sites are seen from −115 to −105, −123 to −118, −135 to −128, and −144 to 140 (Figures 5 and 6). Importantly, two of these protected sites are located within the triplex target sequence (Figures 5 and 6).

Furthermore, incubation of mithramycin (100 and 10 μM) with the c-Ki-ras promoter fragment for 30 min prior to the addition of triplex-forming ODN completely abrogates triplex formation as evidenced by the absence of an ODN-induced pattern of DNase I protection in Figure 5, lanes 4 and 5, and the preservation of all mithramycin-induced DNase I protected sites in these lanes. The pattern of protection in the presence of both ODN and mithramycin is identical to that obtained with mithramycin alone indicating that ODN binding is inhibited as a direct result of mithramycin binding to its target sequences (Figure 5). A similar inhibition of triplex formation was noted when the ODN and mithramycin were incubated with the promoter fragment simultaneously (data not shown). Moreover, the addition of mithramycin to preformed triplex (overnight incubation) still gives rise to a mithramycin-induced pattern with the disappearance of ODN-induced protection (Figure 5, lanes 6 and 7). The DNase I digestion pattern of these reactions, shown in lanes 6 and 7, reveals only the mithramycin-induced modifications, but not the ODN-dependent protection site (Figure 5). Perhaps the strongest footprinting evidence that mithramycin does displace the third strand comes from the strong band in the center of the triplex target site which is not affected by mithramycin (Figure 5, lane 2) but is abolished by the ODN (lane 3). When mithramycin and the ODN are present together, this band is still present resembling the binding of mithramycin alone (lanes 4 and 6).

Mithramycin binding to the c-Ki-ras triplex forming sequence and the inhibition of triplex formation by its binding was also confirmed by electrophroetic gel mobility shift assay. As shown in Figure 3, the binding of mithramycin (100 μM) to the radiolabeled 22 bp target DS DNA fragment (10 nM) resulted in retardation of this fragment's gel mobility (lane 4). Importantly, the gel mobility retardation of this fragment induced by mithramycin binding is slightly greater than that of triplex formation (Figure 3, lanes 4 and 5). Figure 3, lane 6, shows that the gel shift seen when the radiolabeled DS target DNA fragment is incubated with mithramycin followed by triplex formation is identical to the gel shift seen with mithramycin binding alone. Similar results were obtained when mithramycin was added to the target DNA simultaneously with the ODN or after overnight triplex formation (Figure 3, lanes 7 and 8). Since the migration of triplex DNA is only slightly greater than the mithramycin-bound DS c-Ki-ras target DNA, it is difficult to determine whether the gel retardation of the target DS DNA was caused by mithramycin binding alone or by both mithramycin and ODN binding. This problem was solved by forming triplex with radiolabeled ODN to shift the cold DS DNA as a way of demonstrating triplex formation. Figure 4 shows that the addition of mithramycin prevents triplex formation by the labeled ODN whether it was added prior to the ODN binding (lane 4), or simultaneously (lane 5), or to the pre-formed

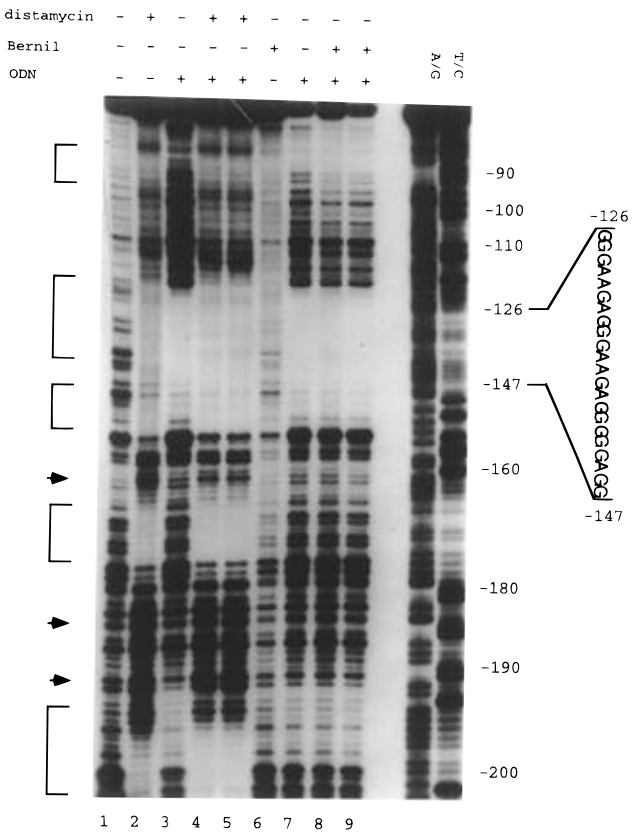

FIGURE 7: DNase I protection assay demonstrating the binding of distamycin (100 μM; lane 2), berenil (100 μM; lane 6), and triplex-forming ODN (5 μM; lane 3) to the c-Ki-ras promoter. A 181 bp ^{32}P-labeled c-Ki-ras promoter fragment was incubated with either distamycin or berenil before (lanes 4 and 8) and after (lanes 5 and 9) the formation of triplex. Maxam−Gilbert sequencing reactions A+G and C+T are seen in the last two lanes.

triplex DNA (lane 6). Importantly, no interaction between mithramycin and labeled ODN alone was noted (Figure 4, lane 2).

The concentration dependence of mithramycin inhibition on triplex formation was also tested. c-Ki-ras target DNA (10 nM) was pre-treated with 100, 10, and 1 μM concentration of mithramycin, and triplex formation was tested by DNAse I footprint and gel retardation assays (data not shown). These experiments showed significant inhibition of triplex formation at 100 and 10 μM mithramycin concentrations but not in the presence of 1 μM concentration of mithramycin.

Distamycin and Berenil Binding to Human c-Ki-ras Promoter Region and Their Effects on Intermolecular Triplex Formation. We also characterized the DNase I digestion patterns of the 181 bp human c-Ki-ras promoter fragment in the presence and absence of distamycin and berenil which are shown in Figure 7. The DNase I digestion patterns in the presence of 100 and 10 μM (data not shown) concentrations of either distamycin (lane 2) or berenil (lane 6) were altered when compared to control digest in lane 1 (Figure 7). Distamycin binding to the c-Ki-ras promoter fragment produces at least five regions of DNase I protection distributed within the whole length of this fragment (Figure 7, lane 2). On the other hand, berenil produces relatively small changes in the DNase I digestion pattern of this promoter fragment (Figure 7, lane 6). A single DNase I protection site was attributable to berenil binding to the c-Ki-ras fragment extending from −92 to −65 bp (Figure 7, lane

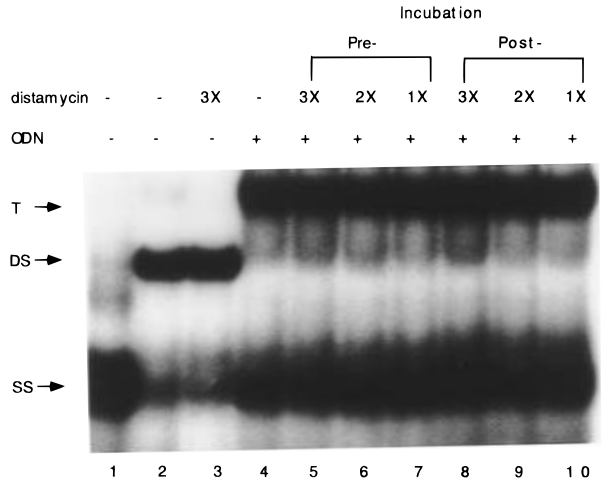

FIGURE 8: Gel mobility shift analysis demonstrating the binding of triplex-forming ODN and distamycin to the triplex target site. The ^{32}P-labeled synthetic target DNA (10 nM) was incubated with in the absence or presence of ODN (1 μM), distamycin (100 μM = 3×; 50 μM = 2×; 10 μM = 1×), or both. SS = single 22bp pyrimidine strand; DS = double-stranded target DNA; T = triplex DNA.

6). This sequence contains two alternating A/T sequences (TTGCGTA, −70 to −64 and TTCTTAGCT, −89 to −82) which are known to be preferred binding sequences for berenil (Portugal & Waring, 1987; Barceló & Portugal, 1993). Berenil does not bind within the triplex target site which does not contain its preferred binding sequence.

Nucleotides which were protected from DNase I cleavage due to distamycin binding included −92 to −79, −139 to −123, −153 to −149, −176 to −165, and −201 to −198 (Figures 6 and 7). Moreover, distamycin also produces changes in the digestion efficiency of the DNA sequences flanking its binding sites. For example, enhanced cleavage is noted in the vicinity of bases −110, −160, −180, and −190 which generally correspond to longer stretches of alternating G/C sequences flanking its binding sites (Figures 6 and 7). Figure 7 demonstrates that preincubation of the c-Ki-ras promoter fragment with either distamycin (lane 4) or berenil (lane 8) prior to the addition of TFO did not prevent triplex formation. The DNase I digestion pattern shown in Figure 7, lane 4, demonstrates that the observed protection sites are the result of both distamycin and ODN binding to their respective target sites. Although berenil does not bind within the triplex target sequence, its binding proximal to the triplex target sequence does not affect the formation of triplex as seen by the presence of both berenil- and ODN-induced footprints simultaneously in Figure 7, lane 8. Addition of either distamycin or berenil to the promoter fragment following triplex formation results in the digestion patterns seen in lane 5 (distamycin) and lane 9 (berenil) (Figure 7). DNase I digestion patterns seen in lane 5 and lane 9 are identical to those seen in lane 4 and 8, respectively, and suggest that these modifications are the result of simultaneous binding of drug molecules (distamycin or berenil) and triplex-forming ODN to their respective binding sites (Figure 7).

Data obtained by gel mobility shifts confirm the DNase I footprinting studies of the effect of berenil and distamycin binding on triplex formation (Figure 8). Figure 8 shows that the presence of distamycin in concentrations of 100, 50, and 10 μM has no effect on triplex formation. Unlike mithramycin, distamycin binding to the duplex target DNA does not retard its mobility in 16% native gel as demonstrated by the identical gel mobility of bands in lane 2 and 3 (Figure 8). This may be due to rapid dissociation of duplex (or triplex) bound distamycin during the electrophoresis, in contrast to mithramycin which is known to dissociate slowly from DNA. Thus gel retardation of the DS DNA can only be due to triplex formation. Again, in agreement with DNase I protection data, the presence of berenil in either 100, 50, or 10 μM concentrations did not alter the binding of ODN to its 22 bp duplex target fragment as evidenced by a complete shift of the labeled duplex probe to a distinct triplex band (data not shown). No changes were noted in the gel mobility of either ODN or duplex target fragment when they were preincubated with 100 μM berenil (data not shown). These findings confirm the observation that berenil does not bind within the triplex-forming target sequence and thus neither prevents the triplex formation nor destabilizes preformed triple-helical DNA in the c-Ki-ras promoter. In Figure 8, lanes 5, 6, and 7, the target DS DNA was preincubated with distamycin for 30 min prior to the addition of ODN. These experiments confirm that distamycin binding to the triplex target sequence does not interfere with triple-helical DNA formation. Similarly, addition of distamycin to preformed triplex DNA (Figure 8 lanes 8, 9, and 10) has no effect on the stability of triplex.

DISCUSSION

Our data demonstrate that mithramycin binds within the triplex target site of the c-Ki-ras promoter and strongly inhibits the formation of triplex DNA. Moreover, binding of mithramycin in the minor groove results in complete displacement of a preformed the major groove-bound triplex ODN. In comparison, AT-specific minor groove binders distamycin and berenil interact with the c-Ki-ras promoter without disrupting the triplex. Furthermore, occupancy of the major groove by a third strand did not prevent the binding of distamycin. In agreement with previous studies, distamycin and berenil demonstrate marked differences with regard to their binding sites within the c-Ki-ras promoter sequence (Portugal & Waring, 1987; Barceló & Portugal, 1993). The c-Ki-ras triplex forming motif provides binding sites for distamycin but not for berenil.

The results presented here confirm a previous report that a 22 bp homopurine:homopyrimidine motif within the human c-Ki-ras promoter forms intermolecular triple helix (Mayfield et al., 1994a). The binding affinity of oligonucleotides which form T·A:T triplets is slightly greater than the affinity of an oligonucleotide which forms A·A:T triplets (Mayfield et al., 1994a), so the ODN used in this study was designed to bind antiparallel to the purine-rich strand of the duplex forming G·G:C and T·A:T triplets. The triplex formed at physiological pH in the presence of 10 mM Mg^{2+} at 37 °C, and the effect of drug binding was characterized both before and after the formation of triplex. The resulting data provide a detailed comparison of the effects of GC-specific and AT-specific minor groove binding drugs on a gene-specific, intermolecular purine·purine:pyrimidine triplex structure. These findings may aid in important practical understanding of how these DNA binding drugs affect intramolecular triplex DNA (H-DNA) structures *in vivo*.

The minor groove in G+C-rich regions of double-stranded DNA is wider and shallower than in A+T-rich regions and

contains the amino group of guanine which makes it less favorable for groove binding ligands. Thus, most of non-intercalating groove binders prefer the more narrow and deep minor groove of the A+T-rich region. The exception is mithramycin, which interacts specifically with the 2-amino group of the guanine in GC-rich areas of DS DNA (Cons & Fox, 1989; Carpenter et al., 1993). It is evident from previous studies that incubation in the presence of 10 mM Mg^{2+} for 30 min is adequate to attain >95% saturation of mithramycin binding to the target sites in the DNA (Sarker & Chen, 1989). Under these experimental conditions, mithramycin binds to at least four well defined sites within the c-Ki-ras promoter region which lie between −60 to −215 relative to the major transcription initiation site. As expected, all of these binding sites cluster around regions of high GC content (Figure 6). Close examination of each of these regions reveals that mithramycin binding requires a minimum of two contiguous GG or GC dinucleotides with a binding site of 3 bp (Figure 6), which is in agreement with previous studies (Van Dyke & Dervan, 1983; Con & Fox, 1989). It is also apparent from the DNase I protection pattern that mithramycin does not bind to all GC-rich regions of the c-Ki-ras promoter fragment (Figure 6). This may be due to the fact that mithramycin's ability to recognize a particular sequence is influenced by the structural environment in which the sequence is located (Carpenter et al., 1993). The presence of adjacent GC base pairs is not mandatory for mithramycin binding since it is able to bind as a dimer with any of the following sequences: TCCC, GGGG, CCGC, GGCA, and CCCT (Carpenter et al., 1993). In fact, most of the mithramycin binding regions in the c-Ki-ras promoter are restricted to the long stretches of $A(G)_n{\cdot}(C)_nT$ sequences (Figure 6). Consequently, mithramycin binding to the c-Ki-ras promoter encompasses most of the triplex target site and sequences downstream of this site (Figure 6).

The DNA adjacent to mithramycin binding sites is distorted and exhibits sequence-dependent variations in the helical twist and rise parameters (Sastry & Patel, 1993). Furthermore, mithramycin-induced alterations involving local DNA structure render the adjacent $(AT)_n$ and $A_n{\cdot}T_n$ regions hypersensitive to DNase I (Cons & Fox, 1991; Carpenter et al., 1993; Fox & Cons, 1993). This is reflected by the enhanced DNase I cleavage of an AT-rich cluster, at −80 to −90 of the c-Ki-ras promoter fragment (Figure 6). These findings lend credence to the argument that mithramycin binding within and near the triplex-forming region of the c-Ki-ras promoter distorts the local DNA structure, making it more A-DNA-like without unwinding or lengthening the DNA helix as reported in previous studies (Fox & Cons, 1993). Thus, mithramycin's inhibitory effect on triple-helical assembly is very likely attributable to conformational changes of duplex target DNA and not to other structural changes. Although it was initially suggested that the triplex DNA has an A-form conformation (Arnott & Selsing, 1974), recent reports dispute this concept and indicate that the underlying DS DNA remains B-form with a sugar pucker near C2′-endogeometry (Howard et al., 1992). Our data agree with this finding, since mithramycin-induced structural changes in the triplex-forming region (B-form to A-form) do not enhance the triplex formation but inhibit its formation. Similar inhibitory effects of mithramycin on pyrimidine·purine:pyrimidine triplex formation have been reported recently (Stonehouse & Fox, 1994). In contrast to mithramycin, most AT-specific minor groove binders have no influence on DNA conformation and no consequential inhibitory effects on triplex formation. The DNA binding ability of mithramycin is not affected by spermine or spermidine bound in the major groove (Sarker & Chen, 1989). Our study demonstrates that mithramycin binds its target in pre-formed triplex DNA. Indeed, the major groove bound ODN is displaced from its target site by mithramycin.

Previous studies have shown that mithramycin inhibits the transcription of genes with GC-rich regulatory sequences, such as the c-myc, dhfr, and collagen 1 genes. The drug prevents binding of the transcription factor Sp1 (Snyder et al., 1991; Blume et al., 1991; Nehls et al., 1993), which forms protein−DNA complexes in the major groove of the DNA. Although Sp1 binding sites in c-Ki-ras regulatory regions are not well characterized, the triplex-forming site of this gene also binds one or more nuclear proteins (Mayfield et al., 1994a). Our evidence that mithramycin binding sites in c-Ki-ras promoter overlap triplex-forming and protein-binding sequences suggests that the triplex site represents an essential element for transcriptional initiation. Although mithramycin blocks Sp1 binding to the major groove by altering local DNA structure, it does not displace previously bound Sp1 (Snyder et al., 1991). In contrast mithramycin binding results in complete displacement of a triplex-forming ODN from the major groove.

We found that recognition sequences of distamycin and berenil within the c-Ki-ras promoter are distinctly different which is in agreement with a previous study comparing their binding sites in the double stranded tyrT DNA fragment (Portugal & Waring, 1987). Two binding sites for distamycin are found in the triplex forming region of the c-Ki-ras promoter, with additional sites present in upstream and downstream sequences. On the basis of this footprint analysis, distamycin appears to bind to longer sequences, including several lying outside the presumed A+T-rich primary binding sites (Figure 6). This finding is in agreement with a previous study which reported that distamycin can bind to sequences containing clusters of G/C interruptions, particularly when they are present at the ends of the binding sequences (Portugal & Waring, 1987). It appears that distamycin binds to poly-dA:poly-dT sequences with isolated G:C interruptions. It has been suggested that the absence of a cationic terminus on distamycin allows this molecule to unravel from the bottom of the minor groove, in compensation for the intrusion of a guanine's 2-amino group (Kopka et al., 1985). This may further explain the ability of distamycin to bind to the minor groove of the triplex region without any destabilizing effect. Our results agree with NMR data which suggest that the interaction of distamycin did not change the chemical shift of a triplex composed of $d(T)_6{\cdot}d(T)_6{:}d(A)_6$, except for some broadening effect in the imino protons of the triplex structure (Umemoto et al., 1990). Netropsin and Hoechst 33258 dye, which are AT-specific minor groove binding drugs similar to distamycin, have been shown to interact with short sequences of $d(T)_n{\cdot}d(T)_n{:}d(A)_n$ triple helix without displacing the major-groove-bound third strand (Park & Breslauer, 1992; Durland et al., 1994). However, distamycin binding to the DS DNA target reduces the affinity of ODN to this site. Furthermore, it has been shown that distamycin binds within the minor groove of the DS DNA only in the B-form and not in the A-form (Zimmer & Wahnert, 1986). Distamycin binding

to the minor groove of the triplex DNA in the c-Ki-ras promoter supports the view that this triplex structure has a B-form conformation.

There is only one berenil binding sequence downstream of the triplex forming region of the c-Ki-ras promoter. Unlike distamycin, berenil preferentially binds to alternating AT sequences (TpA or ApT steps) which do not contain G:C interruptions (Barceló & Portugal, 1993). Berenil produces relatively small changes in DNA structure in regions distal from the binding site. This explains why berenil has no effect on c-Ki-ras triplex DNA. It is noteworthy that berenil can induce the formation of unusual triple helical structures [DNA (pyrimidine)·RNA (purine)·DNA (pyrimidine); RNA (purine)·RNA (purine)·DNA (pyrimidine)] that otherwise would not form (Pilch & Breslauer, 1994).

The affinity and specificity of various DNA binding drugs with DS DNA and pyr·pur:pyr triplex DNA have been studied extensively in recent years. Ethidium, an intercalator, binds more strongly to the T·A:T triplets than to duplex A:T, whereas it destabilizes triplex DNA containing predominantly C^+·G:C sequences (Scaria & Shafer, 1991). A derivative of benzo[e]pyridoindole consisting of four planar rings binds preferentially with and stabilizes the pyrimidine·purine:pyrimidine triplex DNA that contains both T·A:T and C^+·G:C triplets (Mergny et al., 1992). Another DNA intercalating drug, coralyne, has also been reported to show higher affinity for triple helix composed of T·A:T and C^+·G:C over DS DNA (Lee et al., 1993).

The influence of minor groove binders on triplex formation by the naturally occurring c-Ki-ras triplex forming motif may have important implications for the proposed transcriptional regulatory role of intramolecular triplex DNA formation by this promoter (Hoffman et al., 1990; Pestov et al., 1991; Raghu et al., 1994). As yet, intramolecular triplex formation in the human c-Ki-ras regulatory regions has not been demonstrated, although sequence motifs involved in H-DNA formation by the murine Ki-ras promoter are conserved in the human c-Ki-ras gene. The highly conserved human and murine Ki-ras genes share 82% nucleotide homology (McGrath et al., 1983). The 22 bp homopurine.homopyrimidine triplex motif in the human c-Ki-ras promoter consists of 10 bp overlapping repeats of CCCTCTTCCC (or 7 bp tandem repeats of CCCTTCT) and another 10 bp mirror repeat of CCTTCTCCCC immediately upstream of this motif (Jordano & Perucho, 1986; Yamamoto & Perucho, 1988; Mayfield et al., 1994a). In a similar location (−290 to −318 from exon 0/intron 1), the murine Ki-ras promoter contains a 27 bp homopurine·homopyrimidine mirror repeat which shows strong S1 hypersensitivity and forms an intramolecular triplex DNA (H-DNA) in a supercoiled plasmid (Hoffman et al., 1990; Pestov et al., 1991). Moreover, this motif demonstrates considerable sequence homology with the human sequence, forms intermolecular triplex DNA, and competes for binding of a HeLa nuclear factor with the purine:pyrimidine motif in the human promoter (Mayfield et al., 1994a). A recent study has shown that this motif is responsible for approximately half of the murine Ki-ras promoter activity (Hoffman et al., 1990; Raghu et al., 1994). In addition, it was noted that H-DNA forming ability of this motif as well as its sequence integrity are essential for the promoter activity (Raghu et al., 1994). There is a similar polypurine·polypyrimidine region in the c-myc promoter which has been proposed to form an intramolecular triple helix which is transcriptionally important (Firulli et al. 1994). Interestingly, a common protein called NSEP-1 binds to the H-motifs of both c-Ki-ras and c-myc genes (Kolluri et al., 1992). This suggests that interaction of this protein with promoter DNA may be regulated by intramolecular triplex DNA formation.

DNA binding drugs, including mithramycin, inhibit the binding of the transcription factors to their target sequences by either overlapping their binding sites or inducing conformational changes in their binding regions. If the effects of mithramycin on intermolecular triplex can be extended to intramolecular triplex, it suggests another possible mechanism for inhibition of transcriptional initiation by mithramycin of genes containing G+C-rich regulatory regions. It is possible that interference of mithramycin with intramolecular triplex assembly may block the interactions of proteins which would bind to the intramolecular-triplex structure in a transcriptionally active locus. However, our initial results require further investigation on the effect of mithramycin on supercoil dependent intramolecular triplex formation in order to substantiate this novel mechanism of transcriptional inhibition.

REFERENCES

Agazie, Y. M., Lee, J. S., & Burkholder, G. D. (1994) *J. Biol. Chem. 269*, 7019−7023.

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509−521.

Barbacid, M. (1987) *Annu. Rev. Biochem. 56*, 779−827.

Barceló, F., & Portugal, J. (1993) *Biophys. Chem. 47*, 251−260.

Beal, P. A., & Dervan, P. B. (1991) *Science 251*, 1360−1363.

Behr, W., & Hartmann, G. (1965) *Biochem. Z. 343*, 519−527.

Birnboim, H. C., Sederoff, R. R., & Patterson, M. C. (1979) *Eur. J. Biochem. 98*, 301−307.

Blume, S. W., Snyder, R. C., Ray, R., Thomas, S., Koller, C. A., & Miller, D. M. (1991) *J. Clin. Invest. 88*, 1613−1621.

Carpenter, M. L., Marks, J. N., & Fox, K. R. (1993) *Eur. J. Biochem. 215*, 561−566.

Chalikian, T. V., Plum, G. E., Sarvazyan, A. P., & Breslauer, K. J. (1994) *Biochemistry 33*, 8629−8640.

Cons, B. M. G., & Fox, K. R. (1989) *Nucleic Acids Res. 17*, 5447−5459.

Cons, B. M. G., & Fox, K. R. (1991) *Biochemistry 30*, 6314−6321.

Durand, M., Thuong, N. T., & Maurizot, J. C. (1994) *Biochimie 76*, 181−186.

Ebbinghaus, S. W., Gee, J. E., Rodu, B., Mayfeld, C. A., Sanders, G., & Miller, D. M. (1993) *J. Clin. Invest. 92*, 2433−2439

Firulli, A. B., Maibenco, D. C., & Kinniburgh, A. J. (1994) *Arch. Biochem. Biophys. 310*, 236−276.

Fox, K. R., & Cons, B. M. G. (1993) *Biochemistry 32*, 7162−7171.

Gee, J. E., Blume, S., Snyder, R. C., Ray, R., & Miller, D. M. (1992) *J. Biol. Chem. 267*, 11163−11167.

Goyette, M., Petropoulos, C. J., Shank, P. S., & Fausto, N. (1984) *Mol. Cell. Biol. 4*, 1493−1498.

Hoffman, E. K., Trusko, S. P., Freeman, N. A., & George, D. L. (1987) *Mol. Cell. Biol. 7*, 2592−2596.

Hoffman, E. K., Trusko, S. P., Murphy, M., & George, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2705−2709.

Howard, F. B., Miles, H. T., Liu, K., Frazier, J., Raghunathan, G., & Sasisekharan, V. (1992) *Biochemistry 31*, 10671−10677.

Jordano, J., & Perucho, M. (1986) *Nucleic Acids Res. 14*, 7361−7378.

Kolluri, R., Torrey, T. A., & Kinniburgh, A. J. (1992) *Nucleic Acids Res. 20*, 111−116.

Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376−1380.

Lee, J. S., Latimer, L. J. P., & Hampel, K. J. (1993) *Biochemistry 32*, 5591−5597.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499−500.
Mayfield, C., Squibb, M., & Miller, D. (1994a) *Biochemistry 33*, 3358−3363.
Mayfield, C., Ebbinghaus, S., Gee, J., Jones, D., Rodu, B., Squibb, M., & Miller, D. (1994b) *J. Biol. Chem. 269*, 18232−18238.
McGrath, J. P., Capon, D. J., Smith, D. H., Chen, E. Y., Seeburg, P. H., Goeddel, D. V., & Levinson, A. D. (1983) *Nature 304*, 501−506.
Mergny, J. L., Duval-Valentin, G., Nguyen, C. H., Perrouault, L., Faucon, B., Rougée, M., Montenay-Garesteier, T. M., Bisagani, E., & Helene, C. (1992) *Science 256*, 1681−1684.
Mirkin, S. M., & Frank-Kamenetskii, M. D. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 541−576.
Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645−650.
Muller, R., Slamon, D. J., Adamson, E. D., Tremblay, J. M., Muller, D., Clina, M. J., & Verma, I. M. (1983) *Mol. Cell. Biol. 3*, 1062−1069.
Nehls, M. C., Brenner, D. A., Gruss, H.-G., Dierbach, H., Mertelsmann, R., & Hermann, F. (1993) *J. Clin. Invest. 92*, 2916−2921.
Orsen, F. M., Thomas, D. W., McShan, W. M., Kessler, D. J., & Hogan, M. E. (1991) *Nucleic Acids Res. 19*, 3435−3441.
Park, Y. W., & Breslauer, K. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6653−6657.
Pestov, D. G., Dayan, A., Siyanova, E. Y., George, D. L., & Mirkin, S. M. (1991) *Nucleic Acids Res. 19*, 6527−6532.
Pilch, D. S., & Breslauer, K. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9332−9336.
Portugal, J., & Waring, M. J. (1987) *Eur. J. Biochem. 167*, 281−289.
Postel, E. H., Flint, S. J., Kessler, D. J., & Hogan, M. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8227−8231.
Raghu, G., Tevosian, S., Shrikant, A., Subramanian, K. N., George, D. L., & Mirkin, S. M. (1994) *Nucleic Acids Res. 22*, 3271−3279.
Sarker, M., & Chen, F. M. (1989) *Biochemistry 28*, 6651−6657.
Sastry, M., & Patel, D. J. (1993) *Biochemistry 32*, 6588−6604.
Scaria, P. V., & Shafer, R. H. (1991) *J. Biol. Chem. 266*, 5417−5423.
Snyder, R. C., Ray, R., Blume, S., & Miller, D. M. (1991) *Biochemistry 30*, 4290−4297.
Stonehouse, T. J., & Fox, K. R. (1994) *Biochim. Biophys. Acta. 1218*, 322−330.
Thomas, T., & Thomas, T. J. (1993) *Biochemistry 32*, 14068−14074.
Umemoto, K., Sarma, M. H., Gupta, G., Luo, J., & Sarma, R. (1990) *J. Am. Chem. Soc. 112*, 4539−4545.
Van Dyke, M. W., & Dervan, P. B. (1983) *Biochemistry 30*, 4290−4297.
Wells, R. D., Collier, D. A., Hanvey, J. C., Shimizu, M., & Wohlrab, F. (1988) *FASEB J. 2*, 2939−2949.
Yamamoto, F., & Perucho, M. (1988) *Oncogene Res. 3*, 125−138.
Zimmer, Ch., & Wahnert, U. (1986) *Prog. Biophys. Mol. Biol. 41*, 31−112.

BI951562B