Mithramycin Blocks Transcriptional Initiation of the c-myc P1 and P2 Promoters†

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ABSTRACT: The c-myc protooncogene plays an important role in the regulation of cellular proliferation. Mithramycin, a DNA binding antibiotic which binds G-C-rich DNA, inhibits c-myc expression in both differentiating and nondifferentiating cells. The G-C-rich nature of the c-myc promoter suggests that mithramycin may act by directly inhibiting promoter function. The mithramycin binding sites in the c-myc promoter regions were determined by DNase I footprinting. Particularly prominent mithramycin binding is noted in the regions just 5′ of the P1 and P2 promoter TATA boxes. Gel retardation experiments performed in the presence of mithramycin demonstrate that drug binding can prevent the formation of discreet complexes between HeLa cell nuclear proteins and c-myc promoter DNA fragments. Mithramycin also directly blocks the binding of the transcription factor Sp1 to the P1 promoter region. In vitro run-off transcription demonstrates that mithramycin can completely inhibit the in vitro function of both the P1 and P2 promoters. These data suggest that mithramycin inhibits transcription of the c-myc protooncogene by blocking the binding of important regulatory factors, thus preventing formation of the c-myc transcription initiation complex.

Mithramycin is a DNA binding antibiotic which binds specifically to G-C-rich sequences in the presence of Mg^{2+} (Behr & Hartmann, 1965), resulting in the selective inhibition of DNA-dependent RNA synthesis (Ward et al., 1965; Yarbro et al., 1968). Mithramycin inhibits binding of *Escherichia coli* RNA polymerase to a poly(dG-dC) template, but not to a poly(dA-dT) template (Miller et al., 1987). Previous mithramycin binding studies have concentrated on the sequence specificity of binding and mode of binding to DNA (Van Dyke & Dervan, 1983; Fox & Howarth, 1985; Cons & Fox, 1989; Gao & Patel, 1989). We have recently shown that mithramycin prevents Sp1 binding to the SV40 early promoter and inhibits the in vitro transcription of this sequence (Ray et al., 1989a). This suggests that mithramycin inhibits RNA synthesis by binding to G-C-rich sequences in eukaryotic promoters, and preventing the subsequent binding of regulatory proteins, such as Sp1, to these sites.

Mithramycin is an effective differentiating agent of HL-60 promyelocytic leukemia cells, as well as the leukemic cells of certain patients with myeloid blast phase of chronic myelogenous leukemia both in vitro and in vivo (Koller et al., 1985; Koller & Miller, 1986). Treatment of leukemic cells with this agent is accompanied by an early dramatic decrease in c-myc expression which precedes differentiation by 24–48 h. Mithramycin also selectively inhibits expression of the c-myc protooncogene in a numer of differentiating and nondifferentiating cell types (Baker et al., 1988).

The c-myc protooncogene plays an important role in the control of cellular proliferation and differentiation (Cole, 1986). c-myc expression is markedly increased during periods of rapid cell division by both malignant and nonmalignant cells (Kelly & Siebenlist, 1986). Because of the pivotal role of c-myc expression in controlling cell growth and differentiation, c-myc gene regulation and function are of considerable importance.

The c-myc gene is comprised of three exons, with the first exon containing the two principal transcriptional start sites for the P1 and P2 promoters (Battey et al., 1983). The two transcription initiation sites are separated by 165 bp, with TATA boxes located approximately 30 base pairs upstream of each promoter (Watt et al., 1983; Nishikura, 1986). The individual importance of the c-myc P1 and P2 promoters is not clear, but there is evidence for differential regulation of each promoter (Broome et al., 1987).

The level of c-myc mRNA within a cell is regulated by both transcriptional and posttranscriptional mechanisms (Cole, 1986). Transcriptional regulation of c-myc appears to be quite complex with several positive and negative regulatory regions located within 2 kb upstream of the first exon (Siebenlist et al., 1984; Hay et al., 1987; Lipp et al., 1987; Asselin et al., 1989). Downstream regulatory regions have also been identified near the 3′ end of the first exon (Bentley & Groudine, 1986; Kerppola & Kane, 1988; Zajac-Kaye et al., 1988; Wright & Bishop, 1989). Nishikura (1986) has shown that G-C-rich sequences upstream of both the P1 and P2 promoters are essential for transcription of each promoter in microinjected frog oocytes. She suggested that Sp1 may bind to these sequences and regulate c-myc promoter activity.

Sp1 is a sequence-specific DNA binding protein which has been purified and extensively characterized by Tjian and coworkers (Briggs et al., 1986; Kadonaga et al., 1987, 1988; Courey & Tjian, 1988). It binds to a predominantly G-C-rich decanucleotide sequence known as a "G-C box" sequence present within several viral and eukaryotic promoters (Jones & Tjian, 1985; Jones et al., 1986; Ishii et al., 1986; Lee et al., 1987). The 5′ flanking region of the c-myc gene contains a number of control elements including a TATA box and several G-C rich-regions within the promoter regions, but no consensus Sp1 binding sequences. Although the identity of the factors which regulate expression of c-myc is not known, the G-C-rich regions of the c-myc promoter may provide binding sites for regulatory proteins.

In order to define the mechanism by which mithramycin inhibits expression of the c-myc gene, we have investigated the location and consequences of mithramycin binding within the human c-myc promoter region. We have identified the mithramycin binding sites within the P1 and P2 promoter regions

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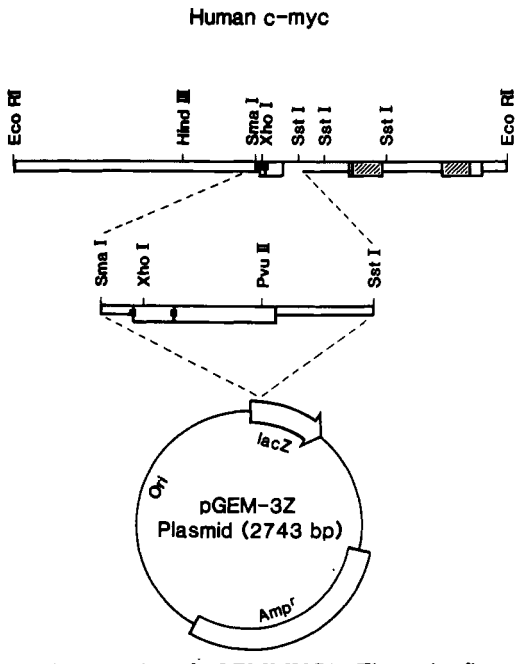

FIGURE 1: Construction of pGEMMYC1. The entire first exon of human c-myc was subcloned as an *Sma*I–*Sst*I fragment (1.04 kb) from a 12.5-kb *Eco*RI genomic fragment containing the human c-myc gene. The first exon was inserted into the *Sma*I and *Sac*I sites of pGEM3Z (Promega). The exons are designated by boxes with the translated regions with the second and third exons hatched. The small black boxes within the first exon designate the P1 and P2 transcription start sites. Various restriction sites are shown for reference.

through DNase I footprinting. Gel retardation experiments in the presence of this drug enabled us to determine the effect of mithramycin binding on nuclear protein binding. We demonstrate that the formation of certain discrete DNA–protein complexes is prevented by mithramycin whereas others are relatively insensitive. Prevention of the formation of these DNA–protein complexes by mithramycin results in the selective inhibition of in vitro promoter function.

Experimental Procedures

Preparation of Labeled DNA Probes. The *Sma*I–*SSt*I fragment containing the entire first exon and 102 bp of 5′ flanking region of c-myc was subcloned into the pGEM 3Z vector to create the pGEMMYC1 plasmid (Figure 1). This insert was derived from a 12.5-kb *Eco*RI fragment of c-myc isolated from a human genomic library (Ray et al., 1989b). Sequence analysis of this subcloned fragment has shown it to have a normal sequence. ^{32}P-Labeled DNA restriction fragments used in the gel retardation assays and footprinting experiments were prepared by digestion of the plasmid pGEMMYC1 at an appropriate unique restriction site and 3′ end labeling the DNA ends by the method of Maniatis et al. (1982), followed by digestion with a second restriction enzyme. The appropriate fragments were purified by gel electrophoresis through nondenaturing polyacrylamide gels.

The restriction fragments A (*Sma*I–*Xho*I) and C (*Xho*I–*Nae*I) were prepared by digestion of pGEMMYC1 with *Xho*I and 3′ end labeling, followed by digestion with *Sma*I and *Nae*I, respectively. Fragment D (*Xho*I–*Sau*3AI) was prepared by digestion of the plasmid with *Xho*I and 3′ end labeling, followed by digestion with *Sau*3AI. Fragment B (*Sal*I–*Dde*I) and *Acc*I–*Dde*I were prepared by digestion of the plasmid with either *Sal*I or *Acc*I, respectively, and 3′ labeling, followed by digestion with *Dde*I. The *Xma*I–*Xho*I fragment was prepared by digestion with *Xma*I, 3′ labeling, and digestion with *Xho*I.

Protein Extract Preparation. The HeLa cell nuclear extracts were prepared by the method of Dignam et al. (1983). The protein concentrations used in the gel retardation experiments were 1–2.5 mg/mL as determined colormetrically using a Coomassie Blue G-250 containing reagent (Pierce Chemical Co.). The Sp1 extract was prepared by the method of Kadonaga et al. (1987) using pSp1-516C and *E. coli* strain BL21.

Electronic Gel Retardation Assay. The reactions were performed by a modification of the method of Fried and Crothers (1981). In reactions containing mithramycin, the DNA was preincubated with the stated concentration of mithramycin in the presence of 1× binding buffer for 20–30 min at 37 °C before the addition of protein extracts, except where otherwise noted. The DNA–HeLa cell nuclear protein binding reactions were carried out in 1× binding buffer [25 mM Tris-HCl (pH 7.5), 6.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM KCl, 10% glycerol, and 0.5 mM $ZnCl_2$] and 2 μg of poly[d(I-C)] (Boehringer Mannheim) for 20–30 min at 22 °C. The Sp1 extract reactions were performed at 0 °C in the same buffer for 20–30 min. Typical reactions were performed with 12–20 μg of HeLa extract or 1–5 μg of Sp1 extract and 20000 cpm (0.3 ng) of end-labeled DNA.

DNase I Footprinting Analysis. The DNase I footprinting reactions were performed by incubating mithramycin or protein extracts with approximately 80000 cpm (1–2 ng) of end-labeled DNA fragments as described for the gel retardation assay experiments. The DNA was then digested with 0.1–1 unit of DNase I (Boehringer Mannheim) for 1 min at room temperature and the reaction terminated by the addition of a formamide loading buffer. The samples were analyzed by electrophoresis on 8 M urea–polyacrylamide gels with Maxam and Gilbert (1980) sequencing reactions used for sequence determination.

In Vitro Run-Off Transcription. HeLa whole cell extract (13 mg/mL) was prepared according to Manley (1984). The template used in these reactions was *Pvu*II-linearized pGEMMYC1. The template was first incubated in the presence or absence of mithramycin and 3 mM $MgCl_2$ for 30 min at 37 °C. A standard 25-μL reaction containing 15 μL of whole cell extract, 1.5 μg of template, 50 mM each of CTP, ATP, GTP, and UTP, 4 mM creatine phosphate, and 10 μCi of [^{32}P]GTP, approximately 400 Ci/mmol (Amersham), was incubated at 30 °C for 2 h. The reactions were terminated by the addition of 25 μL of termination buffer [1% sarkosyl, 100 mM NaCl, 100 mM Tris-HCl (pH 8.0), and 100 mM EDTA]. The reactions were extracted twice each with equal volumes of phenol and chloroform and ethanol precipitated. The samples were then dried, resuspended in formamide loading buffer, and analyzed by electrophoresis on 6% polyacrylamide sequencing gels.

Results

Mithramycin Binding Sites. This work was initiated by cloning the entire first exon of the human c-myc gene into the pGEM3Z vector to create the pGEMMYC1 plasmid (Figure 1). The promoter region of the human c-myc gene along with restriction fragments used for this work is illustrated in Figure 2. To further characterize the mechanism by which c-myc expression is inhibited by mithramycin, we determined the mithramycin binding sites within the P1 and P2 promoter regions by DNase I footprinting of promoter fragments A and C (Figure 2). Representative autoradiographs of mithramycin footprinting experiments are shown in Figure 3A–C. In each of the experiments, concentrations of 100 and 10 μM mithramycin demonstrate drug binding to G-C-rich DNA, whereas 1 μM reactions are essentially identical with control

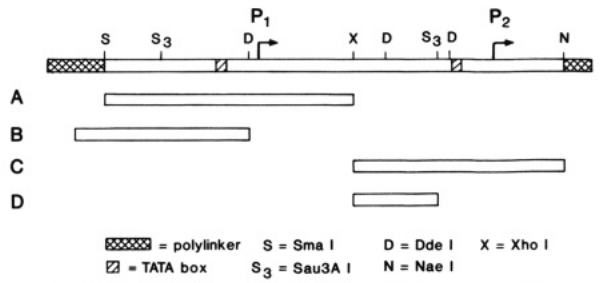

FIGURE 2: Summary of human c-myc P1 and P2 promoter fragments used for these experiments. The upper bar represents the promoter region of human c-myc with various restriction sites shown. The P1 and P2 transcription start sites are designated by arrows. The TATA boxes are represented by boxes within the bar in each promoter. The different restriction fragments used are designated A through D, and the bars to the right of each letter depict the location of each fragment within the promoter region.

reactions with no mithramycin.

Particularly prominent mithramycin binding is observed in the regions just upstream of both TATA boxes. Specifically, these strongly protected regions are from −47 to −30 upstream of the P1 promoter (Figure 3A) and from 110 to 128 upstream of the P2 promoter (Figure 3C). This suggest that these regions may be the most functionally important binding sites. Significant protection is also observed in the regions from 52 to 65 (Figure 3B) and from 89 to 100 (Figure 3C) though this is not as pronounced. Smaller protected regions are also observed from −49 to −57 and from −59 to −65 (Figure 3A) as well as from 38 to 42 (Figure 3B). Since mithramycin has been determined to recognize a 3 base pair sequence, the large footprints indicate that several drug molecules are bound adjacently to one another. The absolute concentration of mithramycin required to give evidence of binding is dependant on the concentration of "template" DNA. A more detailed summary of the mithramycin footprinting data is given in Figure 8 including the coding strand of fragment C which is not shown in Figure 3.

Several regions of DNase I hypersensitivity are also observed in the presence of 100 or 10 μM mithramycin. Prominent hypersensitive sites are present at −29 and −14 in Figure 3A (lanes 3 and 4) and of 86, 103–107, and 131–138 in Figure 3C (lanes 3 and 4). In all cases, these hypersensitive sites are A-T base pairs which flank large mithramycin binding sites. The DNase I hypersensitivity presumably arises due to structural changes in these A-T-rich regions induced by the drug binding in the adjacent G-C-rich regions.

Nuclear Protein Binding. To determine the mechanism of transcriptional inhibition of c-myc expression by mithramycin, we first determined which c-myc promoter fragments were bound by proteins present in a HeLa cell nuclear extract. A series of gel retardation experiments were performed with the various overlapping restriction fragments shown in Figure 2 and HeLa cell nuclear extracts or partially purified recombinant Sp1. These gel retardation experiments were performed in the presence of poly[d(I-C)] to prevent nonspecific protein binding (Singh et al., 1986). The results of these experiments enabled us to localize the binding of HeLa cell nuclear proteins to small regions of the P1 and P2 promoters of c-myc. It also allowed us to characterize Sp1 binding to the P1 promoter fragment.

Nuclear Protein Binding in the Presence of Mithramycin. Once we had determined the c-myc promoter fragments which were bound by HeLa nuclear factors and identified the mithramycin binding sites with both promoter regions, we used gel retardation experiments to determine if mithramycin

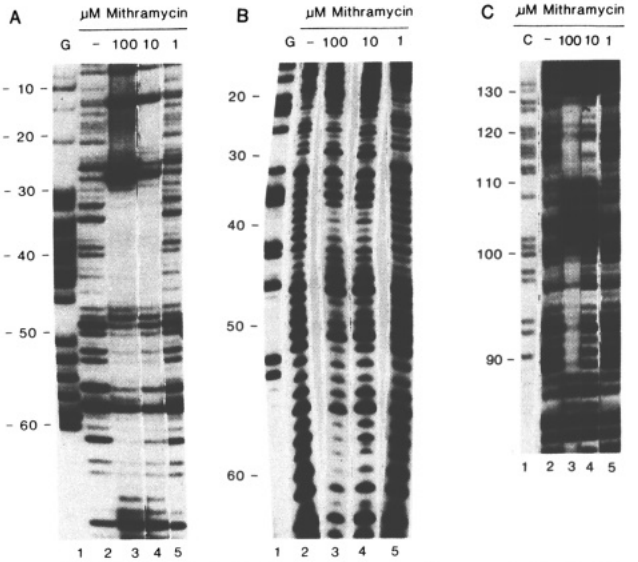

FIGURE 3: DNase I footprinting analysis of mithramycin binding to the human c-myc promoter region. (A) Autoradiograph of an 8% polyacrylamide sequencing gel used to analyze the partial DNase I digests of the end-labeled P1 promoter noncoding strand DNA of fragment A. Lane 1, Maxam–Gilbert guanosine-specific reaction. Lane 2, control DNase I digestion of the end-labeled fragment in the absence of mithramycin. Lanes 3–5, DNase I digestions of the end-labeled fragment in the presence of 100, 10, and 1.0 μM mithramycin, respectively. (B) Autoradiograph of a 12% polyacrylamide sequencing gel used to analyze the partial DNase I digests of the end-labeled P1 promoter coding strand of fragment A. Lane 1, Maxam–Gilbert guanosine-specific reaction. Lane 2, DNase I control digestion. lanes 3–5, DNase I digestion of the end-labeled fragment in the presence of 100, 10, and 1.0 μM mithramycin, respectively. (C) Autoradiograph of an 8% polyacrylamide sequencing gel used to analyze the partial DNase I digests of the end-labeled P2 noncoding strand of fragment C. Lane 1, Maxam–Gilbert cytosine-specific reaction. Lane 2, DNase I control digestion. Lanes 3–5, DNase I digestion of the end-labeled fragment in the presence of 100, 10, and 1.0 μM mithramycin, respectively.

binding prevented the formation of DNA–protein complexes. Using various subfragments of the c-myc P1 and P2 promoters, we used assays in the presence or absence of mithramycin to identify protein complexes which were sensitive to mithramycin. A gel retardation analysis of P1 promoter fragment A is shown in Figure 4. Two DNA–protein complexes can be observed when fragment A is incubated with a HeLa cell nuclear extract (lane 5) compared to radiolabeled fragment A alone (lane 7). Formation of both DNA–protein complexes is inhibited by mithramycin binding to the DNA (lanes 1–4 and lane 6). In the presence of decreasing concentrations of mithramycin (lanes 1–4), the formation of both of the DNA–protein complexes is more prominent, indicating that each is sensitive to mithramycin in a concentration-dependent manner. The more rapidly migrating complex, however, is more sensitive to mithramycin. Lane 6 demonstrates that the addition of protein and mithramycin simultaneously to the DNA also results in inhibition of DNA–protein complex formation.

A similar result is observed with fragment D which contains a portion of the P2 promoter region of human c-myc (Figure 5). When this fragment was incubated with HeLa nuclear extract, we observed three DNA–protein complexes which were differentially sensitive to mithramycin. All three complexes are observed in the DNA protein control (lane 4) and absent in the DNA control (lane 1). The most rapidly migrating complex, designated C_I, is extremely sensitive to mithramycin; C_{II} is relatively insensitive; and C_{III} has an intermediate sensitivity to mithramycin (lanes 2 and 3). This indicates that there are at least two different nuclear proteins

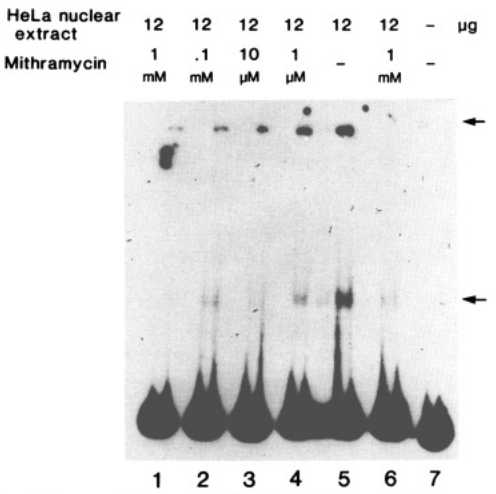

FIGURE 4: Gel retardation analysis with a HeLa nuclear extract and P1 promoter fragment A. Gel-purified, radiolabeled fragment A was incubated with a HeLa cell nuclear extract, and gel retardation assay experiments was performed as described under Experimental Procedures. Autoradiograph of a 6% nondenaturing acrylamide gel is shown. Lanes 1–4, HeLa nuclear extract reactions in the presence of decreasing amounts of mithramycin. Lane 5, HeLa extract with no mithramycin. Lane 6, nuclear extract and mithramycin added simultaneously. Lane 7, DNA control.

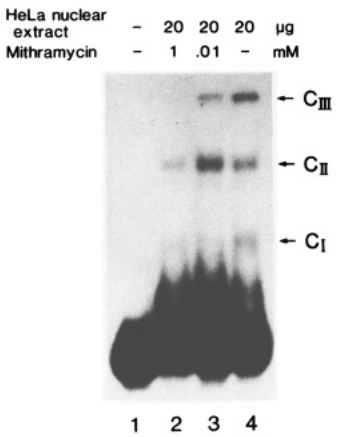

FIGURE 5: Gel retardation analysis of a HeLa nuclear extract and P2 promoter fragment D. Autoradiograph of a 6% nondenaturing acrylamide gel. Lane 1, DNA control. Lanes 2 and 3, nuclear factor binding in the presence of a high and a low concentration of mithramycin, respectively. Lane 4, nuclear factor binding control.

which can bind specifically to this small subfragment of the P2 promoter region. The DNA–protein complex C_{III} may represent a complex with both of these proteins bound to the DNA or perhaps a third protein with intermediate sensitivity. Our data in Figure 5 suggest that C_{III} is formed when both of the proteins present in C_I and C_{II} bind to the DNA. When C_I is prevented from binding at 0.01 mM mithramycin, less C_{III} is formed, and more C_{II} is formed than when no mithramycin is present (compare lanes 3 and 4). When the formation of both C_I and C_{II} is inhibited by 1 mM mithramycin, no C_{III} is observed (lane 2). Competition experiments were also performed with P1 and P2 promoter fragments which revealed that all protein complexes formed were specific for the c-myc promoter fragment used (data not shown).

Sp1 Binding in the Presence of Mithramycin. Using a small DNA fragment (B) which contains from −6 to −101 bp upstream of the P1 transcription start site in our gel retardation assay, we observed two DNA–protein complexes, the intensity of which was dependent upon the amount of Sp1 extract used (Figure 6, lanes 1–4). Both of these DNA–protein complexes were sensitive to increasing concentrations of mithramycin

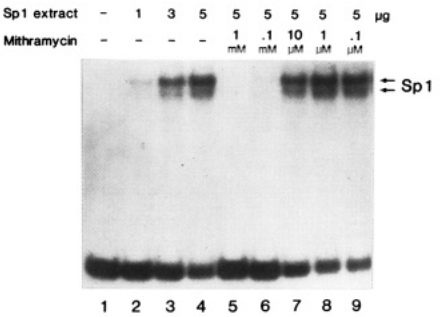

FIGURE 6: Gel retardation analysis of interactions between partially purified recombinant Sp1 and P1 promoter fragment B. Lane 1, DNA control. Lanes 2–4, reactions with increasing amounts of Sp1 extract. Lanes 5–9, Sp1 binding in the presence of decreasing concentrations of mithramycin.

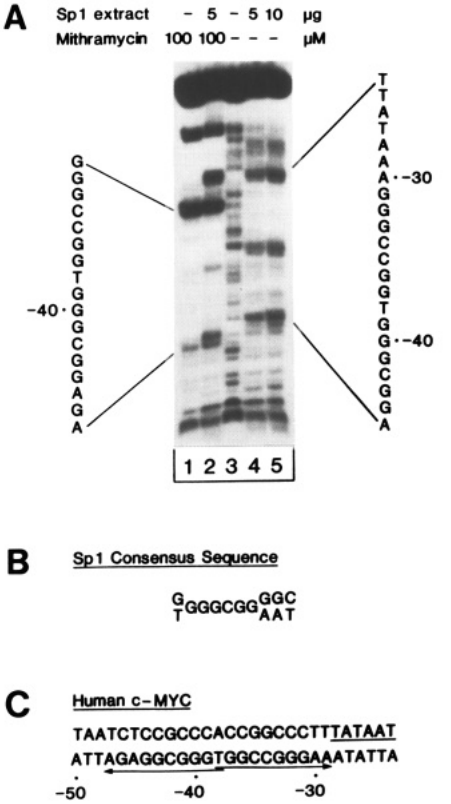

FIGURE 7: DNase I footprinting analysis of Sp1 and mithramycin binding to fragment B. (A) Autoradiograph of a 6% 8 M urea–acrylamide gel. Lane 1, DNase I reaction with 100 µM mithramycin. Lane 2, DNase I reaction with Sp1 extract and mithramycin. Lane 3, DNase I control. Lanes 4 and 5, DNase I reactions with Sp1 extract. The sequences corresponding to the mithramycin and Sp1 footprints are given at the sides of the figure. (B) The consensus sequence for Sp1 binding as reported by Kadonaga et al. (1986). (C) The sequences of the human c-myc gene upstream of the P1 TATA box. The TATA box is underlined. The numbering under the sequence is relative to the P1 transcriptional start site. The two sequences with eight or nine bases of homology with the consensus sequence are underlined with arrows.

(lanes 5–9). Other experiments performed with fragments labeled to a higher specific activity showed significant inhibition of Sp1 binding at a mithramycin concentration of 0.01 mM. Similar gel retardation experiments with the P2 promoter revealed that Sp1 does not bind specifically to the P2 promoter although very low affinity binding was detected (data not shown).

Overlapping Binding Sites of Sp1 and Mithramycin. The specific site of Sp1 binding to the c-myc promoter was determined by DNase I footprint analysis of Sp1 binding to fragment B. This fragment was demonstrated by gel retar-

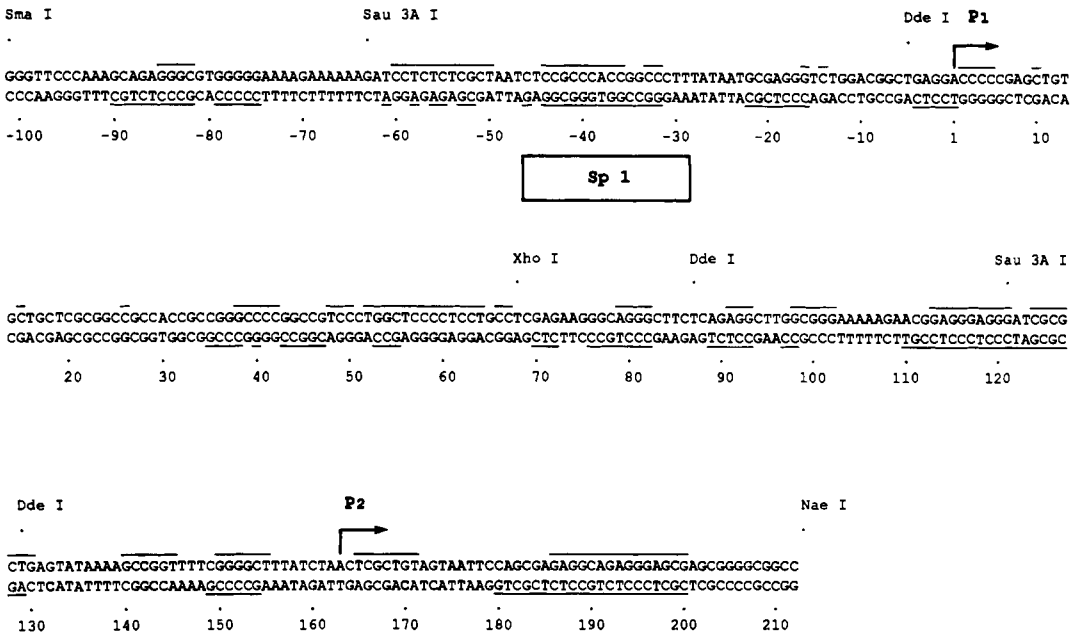

FIGURE 8: Summary of Sp1 and mithramycin binding to the human c-myc P1 and P2 promoters. The promoter sequence from −101 to 212 is shown with various restriction sites given above the sequence. The mithramycin binding sites determined by DNase I footprinting are indicated by lines above and below the sequence. The Sp1 binding sites are indicated by a box below the P1 promoter sequence. Sequencing was performed by the method of Maxam and Gilbert (1980).

dation experiments to bind Sp1 (Figure 6). The results of the DNase I footprint analysis of Sp1 are shown Figure 7. The reactions containing Sp1 demonstrate protection of two prominent sites in the −45 to −24 region of the P1 promoter (lanes 4 and 5) as compared to the control (lane 3). Lesser protection is also observed in the −50 to −55 region. DNase I hypersensitive sites are also present in the DNA flanking the sites protected by Sp1. The 100 μM mithramycin reaction gives a large footprint in this region extending from −47 to −31 (lane 1) overlapping the protein footprints by 15 base pairs. The reaction of fragment B with both Sp1 and mithramycin at a concentration shown previously to block protein binding (see Figure 6, lane 6) results in a footprint characteristic of mithramycin (lane 2). These data indicate that mithramycin blocks Sp1 binding to this region of the c-myc P1 promoter directly by binding to the same sequences, thereby preventing Sp1 binding. The facts that mithramycin binds extensively to the same site as Sp1 and that Sp1 is quite sensitive to mithramycin suggest that extensive overlap of the binding sites may be required to effectively block protein binding.

The reaction with both mithramycin and Sp1 extract also gave intensified bands at −23 and −22 characteristic of the Sp1 reactions (compare lane 2 with lanes 4 and 5). The fact that the DNase I hypersensitive sites at −22 and −23 are also present in lane 2 where Sp1 binding is blocked by mithramycin suggests that the apparent footprint of the TATA box is due to another component within the Sp1 extract and is not due to Sp1 itself. Gel retardation experiments performed with fragment B gave a band identical in both mobility and intensity in both *E. coli* extracts with and without the Sp1 recombinant plasmid, indicating that a bacterial component was also binding to this region of the P1 promoter (data not shown). The consensus sequence of the Sp1 binding site is shown in Figure 7B, and the two c-myc sequences in this region with 8 or 9 base pairs of homology with the consensus sequence are underlined with arrows in Figure 7C. The sequences to which Sp1 binds are designated by a box under the sequence in Figure 8.

A summary of the mithramycin binding sites within the human c-myc promoter region is shown in Figure 8 including some regions not shown in Figure 3. It is apparent that mithramycin has a strong preference for G-C-rich DNA. Using DNase I footprinting, however, we determined that not all G-C-rich regions were bound by mithramycin. It appears that regions containing long stretches of pyrimidines (predominantly C's) on one strand and long stretches of purines (predominantly G's) on the complimentary strand are preferentially bound. In general, the protection of the C-rich strand was more complete. Alternating sequences of G's and C's, containing less than three consecutive G's or C's, such as the sequence in the +10 to +33 region, are not significantly bound by mithramycin (Figure 8).

In Vitro Transcription in the Presence of Mithramycin. The functional importance of mithramycin's ability to block formation of various DNA–protein complexes was determined by in vitro run-off transcription using the c-myc first exon as a template. The result of a representative experiment is shown in Figure 9A. Both the P1 and P2 transcripts are clearly visible in the control (template) reaction with 80–90% of the transcription beginning at the P2 start site (lane 2). In the presence of 10 μM mithramycin, however, transcription from both promoters is clearly inhibited (lane 3). Lower concentrations of mithramycin, 1 μM (lane 4) and 0.1 μM (lane 5), inhibited transcription from both promoters to a lesser degree. These data indicate that the DNA–protein complexes whose formation are inhibited by mithramycin at a concentration of 10 μM are necessary for in vitro transcription to occur from both the P1 and P2 promoters of the human c-myc gene. This suggests that mithramycin blocks c-myc transcription by preventing putative transcription factors from binding and thus blocking the formation of the transcription initiation complex. Figure 9B shows the *Pvu*II-linearized c-myc plasmid template which was used for these experiments, as well as the P1 and P2 transcript sizes resulting from transcription of this template.

Discussion

We have defined the mechanism by which the DNA binding drug mithramycin inhibits the transcription of the human c-myc gene. Our data indicate that HeLa cell nuclear proteins bind to distinct sites upstream of the TATA boxes of both

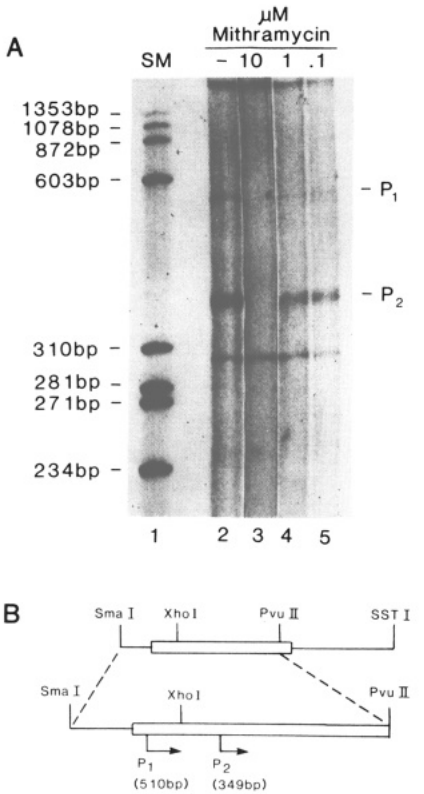

FIGURE 9: Effect of mithramycin on in vitro run-off transcription of the human c-myc P1 and P2 promoters. (A) Autoradiograph of a 6% denaturing acrylamide gel used to visualize in vitro run-off transcription. Lane 1, 5′ end-labeled *Hae*III-digested ϕX174 DNA used as a size standard. Lane 2, in vitro run-off transcription reaction. Lane 3, in vitro run-off transcription performed in the presence of 10 μM mithramycin. Lanes 4 and 5, transcription in the presence of 1 and 0.1 μM mithramycin, respectively. (B) Diagram of the *Sma*I–*Sst*I insert contained in the pGEMMYC1 plasmid and the transcripts resulting from in vitro run-off transcription. Transcripts of 510 bp (P1) and 349 bp (P2) are expected when the plasmid is linearized with *Pvu*II.

c-myc promoters. Mithramycin competition experiments demonstrate that the binding of mithramycin to G-C-rich sequences can prevent the formation of several of these DNA–protein complexes. Gel retardation and DNase I footprinting experiments with partially purified recombinant Sp1 demonstrate that Sp1 binds to the region of the c-myc promoter just upstream of the P1 TATA box but does not bind to the P2 promoter. DNase I footprinting with Sp1 and mithramycin indicates that the binding sites of Sp1 and mithramycin strongly overlap and that mithramycin binding to the DNA prevents Sp1 from binding. Using in vitro run-off transcription, we have demonstrated that mithramycin can block transcription from both promoters, indicating the formation of certain DNA–protein complexes is required for the promoter function of both the P1 and P2 promoters.

DNase I footprinting analysis indicates that mithramycin binds to several G-C-rich regions within the human c-myc P1 and P2 promoter regions. Mithramycin binding to G-C-rich DNA has been well documented. DNase I, methidiumpropyl-EDTA·iron(II), and hydroxyl radical footprinting all have confirmed that mithramycin binds to G-C-rich sequences (Fox & Howarth, 1985; Van Dyke & Dervan, 1983; Cons & Fox, 1989). Methidiumpropyl-EDTA·iron(II) and hydroxyl radical footprinting revealed that the drug binds to a three base pair G-C-rich sequence (Van Dyke & Dervan, 1983; Cons & Fox, 1989). These studies also revealed that the local structure of the DNA was just as important as the G-C richness in determining where mithramycin binds. These data are in complete agreement with our results indicating that the G-C-rich region from 10 to 34 within the c-myc promoter is not significantly bound by mithramycin (see Figure 8). This lack of mithramycin binding cannot be explained by lack of DNase I cleavage within the sequence as nearly every base is cleaved in this region. This suggests that the local structure of this particular region does not favor mithramycin binding.

Several pieces of evidence indicate that mithramycin binds in the minor groove of the DNA. DNA cleavage by DNase I, which is thought to require an interaction with the minor groove in order to bind the DNA (Suck & Oefner, 1986; 1988), is inhibited by mithramycin, suggesting this drug interacts with the minor groove. The hydroxyl radical footprint pattern of mithramycin appears to be most consistent with binding in the minor groove as well (Cons & Fox, 1989). Mithramycin binding fails to affect DNA chemical modification by reagents which react in the major groove (Cons & Fox, 1990a). Additionally, the binding of netropsin, a known minor groove binding drug, interferes with the mithramycin binding to DNA, whereas DNA binding drugs which do not bind within the minor groove have little effect on mithramycin binding (Sarker & Chen, 1989). Recent NMR data of chromomycin, which is structurally very similar to mithramycin, complexed with DNA in the presence of Mg^{2+} indicate it bind in the minor groove as a dimer (Gao & Patel, 1989).

The binding of mithramycin to DNA also appears to induce local structural changes within the DNA helix. A-T-rich DNase I hypersensitive sites often flank drug binding sites, indicating the drug binding alters the DNA structurally in these regions to make it more sensitive to DNase I. Prominent examples of this change are seen within the c-myc P1 and P2 promoters at the sequences from −30 to −25 (Figure 3A) as well as 103–107 and 131–138 (Figure 3C). This structural alteration within A-T-rich sequences has also been further studied and found to be consistent with a local widening of the minor groove induced by mithramycin binding (Cons & Fox, 1990b).

Our evidence that the formation of certain nuclear protein–DNA complexes is inhibited by mithramycin indicates that these proteins recognize sequence-specific DNA motifs within the c-myc promoters which are also recognized by mithramycin. We have previously shown mithramycin blocks protein binding to the SV40 early promoter. The binding of mithramycin either prevents these proteins from gaining access to their recognition sequence or alters the structure of the DNA in such a way that it is no longer recognizable to the protein. The evidence that mithramycin binds within the minor groove suggests that proteins which recognize or bind to elements within the minor groove in G-C-rich sequences would be most sensitive to mithramycin. Proteins which recognize major groove elements or recognize A-T-rich sequences unaffected by mithramycin would presumably be less sensitive to inhibition. This may explain why some of the nuclear proteins binding with the c-myc promoters are quite sensitive to mithramycin binding while others are relatively insensitive.

The overlapping binding sites of mithramycin and Sp1 in the P1 promoter substantiate the gel retardation data showing that mithramycin blocks Sp1 binding to this sequence. This correlation has also been shown in other systems as well (Ray et al., 1989a). The recombinant Sp1 used in these experiments binds specifically to consensus Sp1 binding sites despite truncation of the N terminus (Kadonaga et al., 1988). We have shown that the Sp1 in our extract binds to previously defined Sp1 binding sites within the SV40 early promoter (Ray et al., 1989a) as well as DHFR (unpublished data). The

mithramycin:DNA ratio required to prevent Sp1 binding to the P1 promoter site is equivalent to that which blocked transcription from the P1 promoter in vitro (Figure 9). This suggests that Sp1 binding is necessary for P1 transcription, in the absence of other upstream regulatory elements such as those identified by Hay et al. (1987) which also regulate P1 transcription.

The location of the nuclear protein binding sites has not been defined within the human c-myc promoter except for our finding that Sp1 binds just upstream of the P1 TATA box. However, HeLa cell nuclear proteins have been shown to bind to murine c-myc promoter sequences which are highly conserved between mouse and human. Marcu and co-workers have identified nuclear protein binding sites within the murine c-myc promoters corresponding to the human sequences from −93 to −75, from −52 to −30, from 62 to 82, and from 104 to 124 (Asselin et al., 1989) referred to here as sites A, B, C, and D, respectively. The locations of these binding sites agree perfectly with our gel retardation data with P1 and P2 promoter fragments. The human Sp1 binding site corresponds to site B, which they also demonstrated bound purified Sp1. Deletion analyses of the c-myc promoter region have indicated that removal of site A has little effect on P1 transcription whereas, removal of site B results in no P1 transcription. Deletion analyses of the P2 promoter revealed that site D was required for P2 transcription while site C was not (Nishikura, 1986; Asselin et al., 1989). This suggests that nuclear factor binding to sites B and D is required for transcription from the P1 and P2 promoters, respectively. These two regions are precisely where the strongest mithramycin binding and structural alterations of the DNA occur within each promoter.

It has been reported that mithramycin binding can block T7 polymerase elongation while *E. coli* polymerase can read through mithramycin binding sites after pausing (White & Phillips, 1989a,b). We have no evidence of premature termination or pausing induced by mithramycin in our in vitro run-off transcription system. Previous work using a nuclear run-on assay indicated that a 1 mM concentration of mithramycin was required to inhibit elongation by only 2.5-fold (Miller et al., 1987). This strongly suggests that the transcriptional inhibition of the c-myc by 10 μM mithramycin (Figure 9) is the result of the inhibition of initiation and not elongation.

It has also been reported that the c-myc gene encodes superimposed promoters for RNA polymerases II and III (Chung et al., 1987). It is possible that our in vitro run-off transcription of the c-myc gene (Figure 9) is the result of polymerase III transcription rather than pol II or perhaps a combination of both. Previously, we have demonstrated that mithramycin blocks the α-amanitin-sensitive transcription of the SV40 early promoter (Ray et al., 1989a), indicating that mithramycin can block the pol II transcription of this promoter. This result has also been observed with the DHFR gene (unpublished results). Our data in Figures 6 and 7 demonstrating that mithramycin blocks the binding of Sp1, a well-characterized transcription factor which modulates pol II transcription of many genes, suggest that mithramycin is inhibiting pol II transcription of the c-myc gene as well. Since pol III transcription also requires the binding of additional factors to the DNA near the start site prior to pol III binding (Kassavetis et al., 1990), mithramycin could conceivably prevent the binding of these other factors and thus block pol III transcription initiation as well.

We have demonstrated that mithramycin prevents HeLa nuclear protein bindiong within the human c-myc promoter region and directly blocks Sp1 binding to a sequence just upstream of the P1 TATA box. Mithramycin completely inhibits in vitro transcription from both the P1 and P2 promoters at the concentration which inhibits the formation of discreet DNA–protein complexes. These data strongly suggest that mithramycin prevents c-myc transcription by blocking the formation of the transcription initiation complex. Since c-myc transcription decreases dramatically in response to mithramycin both in vitro and in vivo, we feel this mode of action may also accurately reflect the in vivo function of this drug. The inhibition of transcription initiation may be a general mechanism of DNA groove binding drugs. Mithramycin, chromomycin A3, and olivomycin all are very similar and bind to G-C-rich sequences within the minor groove. The G-C specificity of these drugs suggests that they would affect the expression of genes which contain G-C-rich regulatory regions. This hypothesis has been strengthened by our observation that mithramycin can prevent transcriptional initiation of c-myc and the SV40 early promoter (Ray et al., 1989a). Recent data indicate that the inhibition of c-myc expression is not peculiar to mithramycin but has also been demonstrated with chromomycin and olivomycin (Ray et al., 1990). The A-T-specific DNA binding drug Hoecht 33258, on the other hand, has no effect on normal c-myc expression in vivo (unpublished data). Other DNA binding drugs of different sequence specificity may be able to affect the regulation of other genes in this manner as well. The synthesis of new DNA binding compounds with a greater sequence specificity may eventually permit drug modulation of specific genes.

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