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Research Article

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Mithramycin Blocks Protein Binding and Function of the SV40 Early Promoter

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Abstract

Specific interactions between DNA and transcription factors are necessary for transcription initiation. These interactions provide a potential target for the selective inhibition of eukaryotic gene expression. Mithramycin is a DNA binding antibiotic which, in the presence of Mg^{2+} , binds G-C containing sequences in the minor groove. The SV40 early promoter contains six G-C decanucleotide sequences, which are binding sites for the transcriptional activating factor, Sp1. Each of the six Sp1 binding sites of this promoter is protected from DNase I digestion by mithramycin binding. Mithramycin binding to the G-C rich sequences in the SV40 early promoter prevents subsequent protein binding to these sequences. The gel retardation of the SV40 early promoter fragment incubated with a HeLa cell extract is completely abrogated by pretreatment of the DNA fragment with mithramycin. The functional significance of mithramycin binding is reflected in the ability of mithramycin to block promoter function. Mithramycin inhibits promoter dependent transcription in an in vitro runoff transcription system in a concentration dependent manner. This suggests that mithramycin prevents transcriptional activation of the SV40 early promoter by blocking binding of transcriptional activating proteins to G-C rich promoter regions.

Introduction

Gene expression in higher eukaryotic cells is mediated by systematic control of RNA synthesis. Formation of the transcription initiation complex involves interaction of a number of cellular factors which bind specific DNA sequences located within the promoter regions of eukaryotic genes (1-4). It is likely that both temporal and tissue specific gene expression are controlled, at least in part, by the interaction of these factors with DNA.

The most well characterized transcription factor is Sp1 (5) which was first detected in HeLa cells on the basis of its ability to activate the SV40 early promoter. Tjian and his co-workers have shown that Sp1 recognizes and binds selectively to a GC rich decanucleotide sequence (6, 7). This "G-C box" sequence is present as six tandem copies in SV40 early promoter. Sp1 interacts with the GC box sequences by forming protein-DNA

complexes in the major groove of DNA. Sp1 binding appears to be necessary for transcriptional activation of the SV40 early promoter (6-12).

Sp1 can also activate transcription of other viral and cellular genes which have one or more GC box elements in their 5' flanking promoter sequences. Genes whose promoters contain such sequences include the HSV-TK (herpes simplex virus-thymidine kinase) (13), human metallothionein II_A (14), dihydrofolate reductase (15), and the Ha-ras protooncogene (16). The human *c-myc* gene also has short G-C rich sequences 5' of both the P1 and P2 promoters, but does not contain consensus Sp1 binding sites. The importance of these sequences in the regulation of cellular gene expression is not known.

Mithramycin is a G-C specific DNA binding antibiotic that inhibits RNA synthesis initiation (17, 18). It inhibits binding of *Escherichia coli* RNA polymerase to a poly dG·dC template, but does not inhibit binding to a poly dA·dT template (18). This suggests that mithramycin may bind to G-C-rich sequences in eukaryotic promoters and inhibit RNA synthesis by preventing binding of regulatory proteins, such as Sp1, to these sites.

Mithramycin is an effective differentiating agent of HL60 promyelocytic leukemia cells, as well as the leukemic cells of certain patients with the myeloid blast phase of chronic myelogenous leukemia (19, 20). 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. Expression of the *c-myc* protooncogene is selectively inhibited by mithramycin treatment in a number of differentiating and nondifferentiating cell types (21).

We have used the SV40 early promoter sequence to correlate the specificity and effects of mithramycin binding. The results of these experiments indicate that this compound prevents formation of the transcription initiation complex by binding to G-C-rich promoter regions.

Methods

DNase I footprinting. Plasmid PL1 (22) containing the SV40 early promoter was first digested with NcoI and 3' labeled with [³²P]dCTP and the Klenow fragment of DNA polymerase. Digestion with NsiI yielded the labeled 89-bp fragment containing the SV40 early promoter sequences shown in Fig. 1 A. This fragment was isolated by electrophoresis on a 10% polyacrylamide gel. 3' labeled DNA (35,000 cpm) was incubated with or without mithramycin at 37°C for 20 min in presence of 3 mM MgCl₂. 1 U of DNase I was added and after 1 min the reaction was terminated with formamide dye. Guanine and cytosine specific chemical sequencing reactions of 3' labeled SV40 early promoter fragment were used as a marker (23). The samples were analyzed on a 12% urea-acrylamide sequencing gel.

Gel binding analysis. The nuclear extract was prepared from HeLa cells as described by Dignam et al. (24). The DNA-protein binding reaction was carried out in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2.5 μg poly d(I-C)

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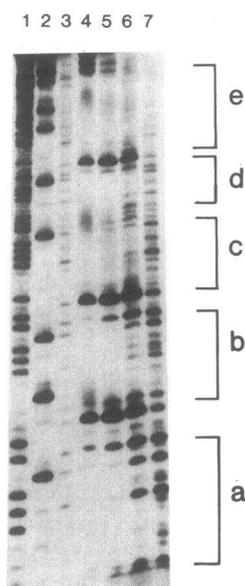
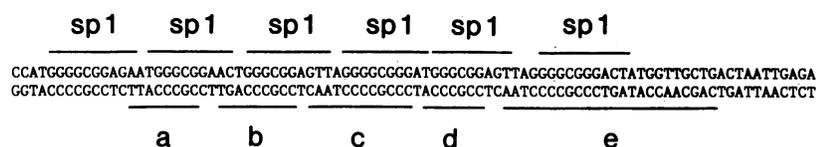
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for 20–40 min at room temperature (25). Each reaction contained 20,000 cpm of 3' labeled DNA, 8 μ g of nuclear extract, and varying concentrations of mithramycin in a final volume 15 μ l. The binding mixture was analyzed by electrophoresis on a 5% polyacrylamide gel containing 40 mM Tris-acetate, 1 mM EDTA.

In vitro transcription with whole cell extracts. The polymerase-II rich whole cell extract (9 mg/ml) prepared from HeLa cells according to Manley (26). A standard 25- μ l reaction mixture consisting of 17 μ l of whole cell extract; 1.5 μ g of DNA template, 50 mM of CTP, ATP, GTP, and UTP, 4 mM creatine phosphate, 10 μ Ci [32 P]GTP or UTP (410 Ci/mmol; Amersham Corp., Arlington Heights, IL) was incubated for 60 min at 30°C. The reaction was terminated by the addition of 100 μ l of termination buffer (1% sarkosyl, 100 mM NaCl, 100 mM Tris-HCl, pH 8.0 100 mM EDTA) and extracted twice with phenol followed by chloroform, and ethanol precipitation. The reaction mix was analyzed by electrophoresis on a 6% polyacrylamide-urea gel.

Results

The mithramycin binding sites on the SV40 early promoter were determined by DNase I protection assays. Fig. 1 shows the binding of mithramycin to the SV40 promoter region as detected by the DNase I protection. An 89-bp fragment of the SV40 promoter region (Fig. 1 A), which contains the six "G-C box" recognition sites for Sp1, was incubated with varying concentrations of mithramycin before DNase I digestion. As shown in Fig. 1 B, mithramycin binds selectively to the G-C box regions of the SV40 promoter, in a fashion nearly identical to binding of Sp1 (10). It also binds to shorter G-C containing sequences in this DNA fragment, although to a lesser degree. The footprinting pattern is mithramycin concentration dependent (data not shown) and maximal binding occurs at DNA/mithramycin ratios that result in RNA synthesis inhibi-

Figure 1. DNase I footprinting analysis of mithramycin binding to the SV40 promoter region. An 89-bp fragment of the SV40 promoter (Fig. 1 A) containing six G-C boxes was used for DNase I footprinting reactions. The footprint result (B) shows that most of the G and C residues were protected by mithramycin. The sequence of protected G-C region corresponds to the "G-C" box regions of the early promoter of SV40. (Lane 1) Cytosine-specific reaction using Maxam Gilbert method; (lane 2) guanine specific reaction using Maxam Gilbert method; (lane 3) DNase I treated, 3' end labeled 89 bp SV40 early promoter fragment; (lane 4) DNase I digestion of the 3' labeled fragment in the presence of 100 μ M mithramycin; (lane 5) 10 μ M mithramycin; (lane 6) 1 μ M mithramycin; (lane 7) 0.1 μ M mithramycin. The areas of the gel identified as a-e represent the G-C-rich regions which are selectively protected by mithramycin binding.

tion. Mithramycin appears to require at least three consecutive G or C residues for effective binding. This is consistent with the previous observations regarding mithramycin binding to the lac operon (27).

Binding of the partially purified recombinant Sp1 product of the Sp1-516c plasmid and mithramycin were compared as shown in Fig. 2. The binding patterns of mithramycin and the recombinant Sp1 to the G-C-rich portion of the SV40 early promoter were similar, but not identical. Mithramycin appears to recognize all of the sequences bound by Sp1, but also binds to additional G-C-rich sequences. This most likely reflects the smaller size of the drug compared to the recombinant Sp1.

Gel retardation studies were used to determine whether the binding of mithramycin to the G-C box regions of the SV40 early promoter region may prevent sequence-specific protein binding. The effect of mithramycin binding on the specific changes in gel mobility of the SV40 early promoter fragment, induced by a partially purified HeLa cell extract, enriched in Sp1 were examined. As shown in Fig. 3 A, binding of mithramycin blocks specific binding of proteins to the 89-bp fragment of the SV40 promoter, as reflected by the loss of gel mobility retardation. Treatment of the DNA fragment with the HeLa cell extract, but not mithramycin alone, resulted in retardation of the fragment containing the SV40 promoter region. Three retarded bands were seen in the nuclear extract treated larger DNA fragment, most likely representing binding by different proteins or combinations of proteins to this portion of the SV40 promoter sequence.

Higher mithramycin concentrations prevented the gel mobility shift of all three bands binding to the 89-bp fragment, whereas the binding of the more specific band was restored at

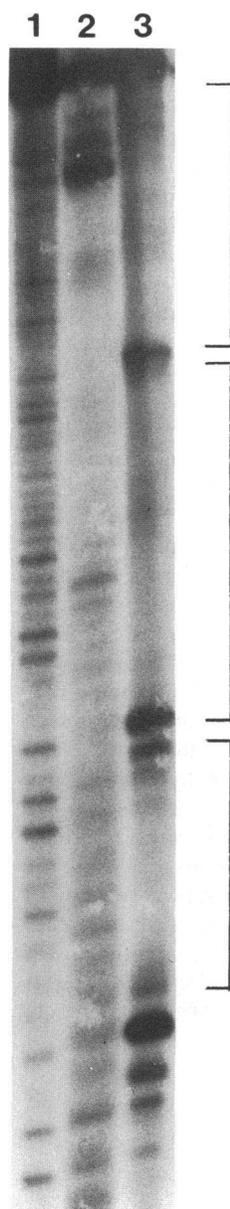


Figure 2. DNase I footprinting of the SV40 early promoter with recombinant Sp1. The plasmid pSp1-516c was obtained from J. Kadonaga and R. Tjian. A crude extract was made according to Kadonaga et al. (28). Mithramycin-DNA binding and the DNase I reaction was performed as described in Methods. Sp1-516c protein and DNA binding was performed at 0°C for 30 min. The Sp1 footprint is indicated by the bracket. (Lane 1) Labeled DNA treated with DNase I; (lane 2) Labeled DNA treated with DNase I in the presence of 1 mM mithramycin; (lane 3) Labeled DNA preincubated with Sp1-516c (10 µg protein) followed by DNase I digestion.

lower mithramycin concentrations. Fig. 3 *B* shows that gel mobility experiments using a smaller 70-bp fragment demonstrate only one retarded band, which is absent when the fragment is preincubated in the presence of mithramycin. This indicates that specific binding of mithramycin to the G-C-rich areas of this promoter sequence is able to prevent subsequent protein binding to the G-C box regions of the SV40 promoter. Mithramycin does not appear to be capable of displacing proteins that have already bound to the DNA. The HeLa cell extract used for these experiments contains relatively high amounts of the Sp1 regulatory protein, which is most likely responsible for the single retarded band shown in Fig. 3 *B*.

The gel mobility shift analysis of recombinant Sp1 (Sp1-516c) binding to the SV40 early promoter in the presence or absence of mithramycin is shown in Fig. 4. This experiment demonstrates that recombinant Sp1 is unable to bind to the SV40 early promoter when the DNA has been preincubated with mithramycin (lane 2). However, in the absence of

mithramycin, Sp1 binds to the DNA (lane 1). This suggests that mithramycin and Sp1 recognize similar sequences, and that competitive binding of Sp1 binding sites by mithramycin prevents Sp1 binding.

To determine the functional effect of mithramycin binding to the SV40 promoter, we also measured the effect of mithramycin on the cell-free runoff transcription of this sequence by a whole cell HeLa cell extract (29). We determined the ability of the whole cell extract to transcribe the PL1 plasmid linearized with Eco RI (22). This plasmid contains both the SV40 early and late promoter region (shown in Fig. 5 *A*) but RNA polymerase II transcribes only from the early promoter. As shown in Fig. 5 *B*, the HeLa cell extract faithfully transcribes this sequence, yielding a RNA fragment of 200 bp (lane 1). This transcription is inhibited by alpha-amanitin (0.5 µg/ml), indicating that RNA polymerase 2 activity is responsible for

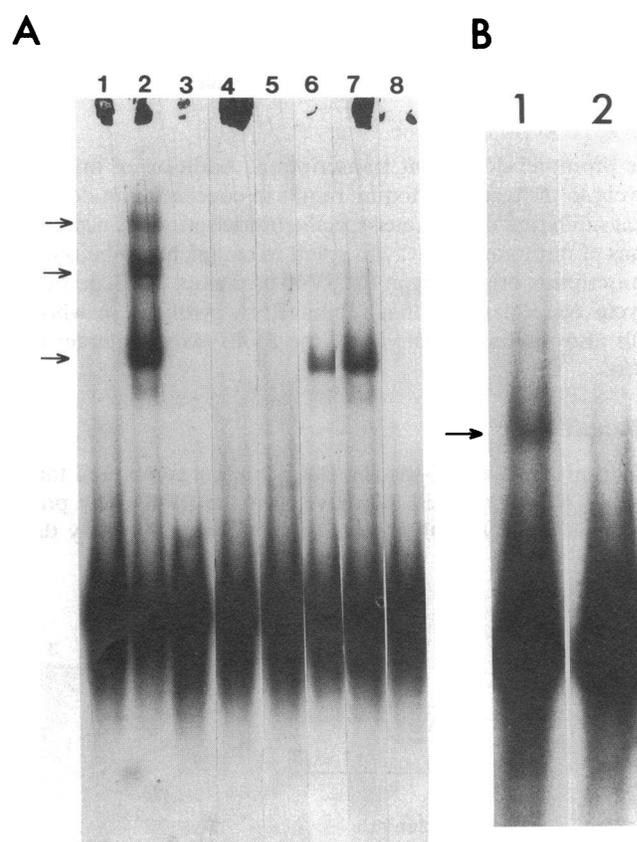


Figure 3. Gel mobility shift analysis of the SV40 early promoter with HeLa cell nuclear extract in the presence or absence of mithramycin. (A) 3' labeled 89-bp fragment of the SV40 early promoter (described in Fig. 1) after incubation with the HeLa cell nuclear extract in the presence or absence of mithramycin. (Lane 1) DNA fragment only; (lane 2) DNA with HeLa cell extract (8 µg protein); (lane 3) DNA preincubated with mithramycin (1 mM) followed by the addition of HeLa cell nuclear extract (8 µg protein); (lane 4) Same as lane 3, but with 100 µM mithramycin; (lane 5) 10 µM mithramycin; (lane 6) 1 µM mithramycin; (lane 7) 0.1 µM mithramycin; (lane 8) 1 mM mithramycin, preincubated with HeLa cell extract (8 µg protein). (B) 3' labeled SphI-Hind III fragment of plasmid PL1 (70 bp) incubated with the HeLa cell nuclear extract (8 µg) lane 1; SphI-Hind III fragment incubated with nuclear extract alone; (lane 2) SphI-Hind III fragment preincubated with mithramycin (1 mM) followed by nuclear extract. Arrow indicates formation of protein DNA complex.

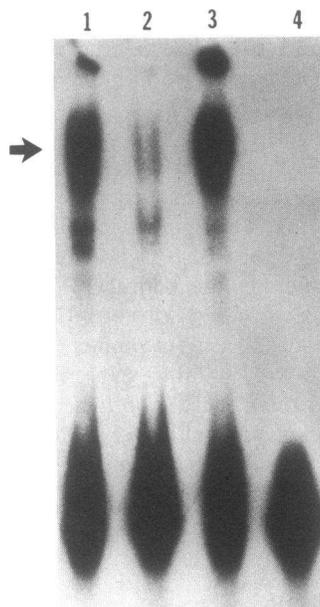


Figure 4. Gel retardation assay of SV40 early promoter with cloned Sp1 in the presence or absence of mithramycin. (Lane 1) DNA with Sp1-516c (10 μ g protein); (lane 2) DNA preincubated with 1 mM mithramycin, followed by the addition of Sp1-516c; (lane 3) DNA preincubated with 1 μ M mithramycin; (lane 4) labeled DNA. The binding conditions were those described in Methods and Fig. 2. Arrow indicates formation of protein DNA complex.

the promoter-dependent transcription. Addition of mithramycin to the reaction mixture results in concentration-dependent inhibition of promoter-specific transcription. Concentrations of mithramycin $> 1 \mu$ M result in complete inhibition of transcription originating at the SV40 promoter. Thus, mithramycin concentrations that inhibit RNA synthesis in whole cells also inhibit the activity of the SV40 early promoter *in vitro*.

Discussion

Mithramycin is a G-C-specific DNA binding compound that inhibits RNA synthesis. We have used the SV40 early promoter as prototype DNA sequence with which to study the

specific functional effects of mithramycin binding. These studies have demonstrated that specific binding of mithramycin to G-C-rich regions of the SV40 early promoter results in a subsequent inability of proteins to bind to these sequences. This is accompanied by inhibition of promoter function. Our data suggest that mithramycin binds to important regulatory sequences within the SV40 early promoter, blocking binding of regulatory proteins to these sequences resulting in inhibition of transcription initiation complex formation, and decreased promoter function. The relative insensitivity of nuclear runoff transcription to mithramycin (18) indicates that transcription initiation is the site of mithramycin action and provides further support for this model.

Previous chemical footprinting studies of mithramycin and the related drugs, chromomycin and olivomycin (26) have demonstrated that at least three consecutive guanosine or cytosine nucleotides are necessary for mithramycin binding. More recent studies with echinomycin have suggested that chromatin structure may play a role in determining the binding ability and specific binding sites of these drugs (30). It is likely that both chromatin structure and sequence specificity determine the cellular effect of DNA binding drugs.

The G-C specificity of DNA binding by mithramycin suggests that expression of genes regulated by G-C binding proteins may be selectively sensitive to this agent. This is supported by the observation that expression of the *c-myc*, *c-Ha-ras*, and *c-abl* genes is selectively inhibited by mithramycin (21). These genes have G-C-rich promoter regions and the *c-Ha-ras* gene contains consensus Sp1 binding sites. The binding specificity of mithramycin for G-C residues may allow the selective inhibition of expression of a group of genes with G-C-rich promoters. Although the number of eukaryotic genes whose promoter regions contain G-C box sequences (or closely related sequences) is not known, a number of Sp1 binding sites of varying affinity have been identified (31). Mithramycin may inhibit expression of all of these genes to a varying degree.

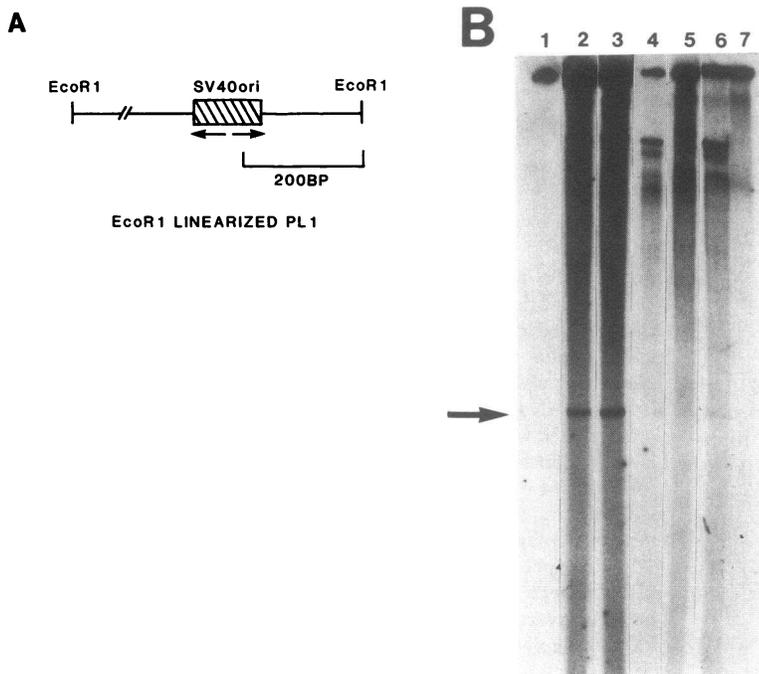


Figure 5. Effect of mithramycin on the *in vitro* runoff transcription of the SV40 early promoter. (A) The PL1 plasmid template containing SV40 early promoter was linearized by Eco RI and the runoff transcription product was expected to be ~ 200 bp. (B) (Lane 1) DNA only; lanes 2 and 3 show the transcript from DNA with whole cell extract using [32 P]GTP or [32 P]UTP, respectively. (Lane 4) DNA fragment incubated with 4 mM mithramycin at 37°C for 20 min, followed by transcription assay; (lane 5) 40 μ M mithramycin; (lane 6) 0.4 μ M mithramycin; (lane 7) 0.04 μ M mithramycin.

The cellular effects of mithramycin may be dictated by its selective inhibition of expression of genes with G-C-rich promoters. Mithramycin induces myeloid differentiation of leukemic cells in certain patients with chronic granulocytic leukemia (18, 19) which is accompanied by an early decrease in *c-myc* expression. The differentiation of leukemic cells in which *c-myc* expression is specifically inhibited by the presence of antisense DNA sequences (32, 33) suggests that decreased *c-myc* expression alone may be adequate to induce differentiation of these cells. Our data suggest that mithramycin may selectively block formation of the transcription initiation complex of genes with G-C-rich promoter regions, including the *c-myc* gene.

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