Effects of the Antitumor Antibiotic Mithramycin on the Structure of Repetitive DNA Regions Adjacent to Its GC-Rich Binding Site†

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ABSTRACT: Regions of $A_n \cdot T_n$, $(GA)_n \cdot (TC)_n$, and $(GT)_n \cdot (AC)_n$ have been cloned into the *Sma*I (CCC/GGG) site of plasmid pUC19. *Hin*dIII–*Eco*RI restriction fragments containing these inserts have been used as substrates for footprinting experiments using DNase I, DNase II, and micrococcal nuclease as probes. These present good mithramycin binding sites (GGG) flanking repetitive regions to which the drug does not bind. In each case, mithramycin footprints are observed at the CCC/GGG sites, which are not affected by the nature of the surrounding sequences. Some weaker binding is detected at TCGA and ACCA sites and at regions of alternating GA. No binding is found to regions of alternating GT. $A_n \cdot T_n$ inserts ($n = 23$ or 69) are normally resistant to cleavage by all these probes; in the presence of mithramycin, a dramatic increase in DNase I cleavage is observed throughout the entire insert and is indicative of an alteration in DNA structure. Similar changes are seen with DNase II and micrococcal nuclease. These changes cannot be explained by invoking changes in the ratio of free substrate to cleavage agent. In contrast, cleavage of $(GA)_n \cdot (CT)_n$ and $(GT)_n \cdot (AC)_n$ inserts is not affected by drug binding. The results are consistent with a model in which mithramycin causes dramatic changes in the width of the DNA minor groove, generating a structure which has some properties of A-DNA, and suggest that this can be propagated into surrounding DNA regions in a sequence-dependent manner. The structural alterations with $A_n \cdot T_n$ are highly cooperative and can be transmitted over at least three turns of the DNA helix.

Mithramycin (Figure 1A) and the related antitumor antibiotics chromomycin and olivomycin act by binding to DNA, thereby inhibiting its activity as a template for DNA-directed RNA polymerase (Kersten et al., 1966; Gause, 1975). The interaction requires stoichiometric amounts of divalent metal ions (magnesium not calcium; Cons & Fox, 1989a) and the presence of guanine bases (Behr et al., 1969). The drug does not bind to poly(dA-dT), though it does inhibit RNA synthesis directed by a synthetic polymer consisting of alternating diaminopurine and thymine residues (Cerami et al., 1967), suggesting that the 2-amino group of guanine is critical for binding. Footprinting studies with methidiumpropyl-EDTA (Van Dyke & Dervan, 1983), DNase I (Fox & Howarth, 1985), and hydroxyl radicals (Cons & Fox, 1989b) have confirmed this selectivity. The first such footprinting experiments, using methidiumpropyl-EDTA-Fe(II) as a cleavage agent, revealed that the binding sites were about three base pairs long and contained at least two contiguous GC base pairs (Van Dyke & Dervan, 1983). The footprinting patterns on a 70 base pair fragment changed as a function of ligand concentration and were (in order of decreasing affinity) 3′-GGG, CGA > CCG, GCC > CGA > CCT > GTG-5′. However, it was not possible unambiguously to determine the preferred binding sequence, since binding to each site was affected by the nature of the flanking sequences. Further high-resolution footprinting studies with hydroxyl radicals (Cons & Fox, 1989b) showed that, while two contiguous GC base pairs could sometimes constitute a good ligand binding site, the best sites contained three contiguous GC base pairs. However, not all such sites were protected from cleavage, indicating that binding is affected by the flanking sequences.

In the present paper, we have studied the effect of mithramycin binding to the sequence GGG (CCC) on the structure of adjacent regions. In all studies to date, this sequence has appeared as one of the best binding sites.

NMR studies with chromomycin (Gao & Patel, 1989a,b; Banville et al., 1990a) and mithramycin (Banville et al., 1990b) have shown that these drugs bind as dimers in the DNA minor groove coordinating a single magnesium ion. Specificity is achieved by the formation of hydrogen bonds from the C8-phenolic proton to the 2-amino group of guanine. The location of such a large complex within the narrow minor groove causes dramatic changes in DNA structure. The minor groove is widened, and the helix more closely resembles an A-type DNA structure, though some characteristics of B-DNA are retained (Gao & Patel, 1989b; Banville et al., 1990b). Despite these large conformational changes, it has long been known that mithramycin does not unwind the DNA helix (Waring, 1970). DNase I footprinting studies with natural DNA fragments have shown that mithramycin causes enhanced cleavage in regions surrounding some of its binding sites (Fox & Howarth, 1985). These enhancements have been attributed to drug-induced changes in local DNA structure rendering it more susceptible to nuclease attack. In contrast, footprinting studies using a variety of chemical cleavage agents on natural DNA fragments, probing the accessibility of DNA base substituents, did not provide any evidence for major conformational changes, though some changes were observed in the reactivity of purines to diethyl pyrocarbonate (Cons & Fox, 1990a).

In order to assess any DNA structural changes caused by mithramycin binding, we have examined its effect on the nuclease susceptibility of repetitive sequences positioned adjacent to its GGG binding site. In a previous study, we showed that adjacent regions of alternating AT, to which mithramycin does not bind, are rendered unusually sensitive to cleavage by DNase II (Cons & Fox, 1990b). DNase I cleavage in these regions is also enhanced, though the alternating cleavage

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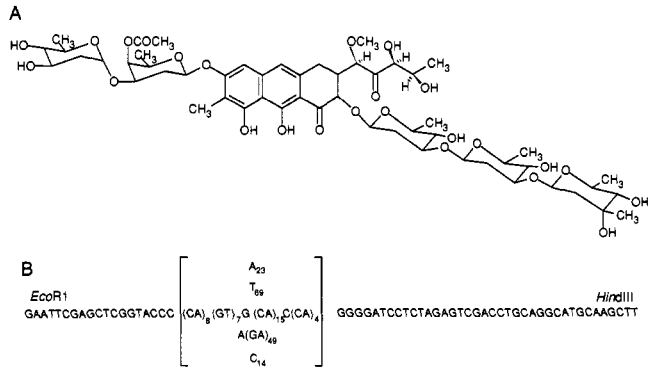

FIGURE 1: (A) Structure of mithramycin. (B) Sequence of the pUC19 polylinker containing the various inserts. The strand shown is the one labeled at the 3′ end of the *Hin*dIII site. The opposite strand is labeled at the *Eco*RI end.

pattern, in which ApT is cut better than TpA, was retained. Cleavage by micrococcal nuclease was also enhanced. These changes were propagated over at least one turn of the DNA helix and seemed to occur in an all-or-none cooperative fashion. In the present paper, we examine the effect of mithramycin, binding to the sequence CCC (GGG), on adjacent regions of $A_n{\cdot}T_n$, $(GT)_n{\cdot}(AC)_n$, and $(GA)_n{\cdot}(TC)_n$. DNA structural changes have been assessed by footprinting experiments using DNase I, DNase II, and micrococcal nuclease as probes. DNase I introduces single-stranded nicks in double-stranded DNA, cutting from the minor groove (Drew, 1984; Drew & Travers, 1984; Oefner & Suck, 1986). The minor groove is widened by 3 Å and bent by 21° on interaction with the enzyme (Suck et al., 1988). Cleavage is therefore sensitive to the width of this groove and the local DNA flexibility. Homopolymeric runs of A and T are poor substrates, on account of their narrow minor groove width; regions rich in GC residues are also poorly cleaved (Drew & Travers, 1984) even though they possess a wider than average minor groove. Micrococcal nuclease cut exclusively at pA and pT bonds (Flick et al., 1986; Dingwall et al., 1982; Horz & Altenburger, 1981). A crystal structure of its complex with pTp has been determined (Cotton et al., 1979) and shows the DNA binding surface to be a long narrow cleft which is capable of holding only one DNA strand. It is therefore presumed to be sensitive to DNA local breathing motions. The structural requirements for DNase II have been less well-defined; it cleaves best in runs of purines, and may recognize some features of A-DNA (Drew, 1984; Drew & Travers, 1984).

Materials and Methods

Drugs and Enzymes. Mithramycin was a gift from Pfizer and was stored at 4 °C as a 2 mM stock solution in 10 mM Tris-HCl containing 10 mM NaCl. DNase I, DNase II, and micrococcal nuclease were purchased from Sigma and stored as previously described (Low et al., 1984; Fox & Waring, 1987). All synthetic polynucleotides were purchased from Pharmacia.

Plasmids. Plasmids containing repetitive DNA inserts cloned into the *Sma*I site of pUC19 were constructed as previously described (Fox, 1990; Fox & Kentebe, 1990; Cons & Fox, 1990b). Synthetic polynucleotides [poly(dA)·poly(dT), poly(dA-dC)·poly(dT-dG), poly(dG-dA)·poly(dT-dC), and poly(dG)·poly(dC)] were cut to short lengths by lightly digesting with DNase I and were made blunt-ended by filling in with the appropriate nucleotide triphosphates using reverse transcriptase. These were ligated into *Sma*I-cut pUC19 and transformed into *Escherichia coli* TG2. Transformants were picked in the usual way as white colonies from agar plates containing X-gal and IPTG. The sequences of the inserts were determined either by Maxam–Gilbert reactions on ^{32}P-labeled *Hin*dIII–*Eco*RI fragments or by dideoxy sequencing using a Pharmacia T7 sequencing kit. The plasmids used in this work contained the sequences A_{23}, T_{69}, $A(GA)_{49}$, $(CA)_8(GT)_7G$-$(CA)_{15}C(CA)_4$, and C_{14}. In each case, the sequence is described for the strand read by using universal primer, i.e., the strand seen when the *Eco*RI–*Hin*dIII fragment is labeled at the 3′ end of the *Hin*dIII site. The sequences of the *Eco*RI–*Hin*dIII fragments are shown in Figure 1B. Milligram quantities of these plasmids were prepared with Qiagen columns according to the manufacturer's instructions.

DNA Fragments. Radiolabeled DNA fragments containing these inserts were prepared by digesting the plasmids with *Hin*dIII, labeling with [α-^{32}P]dATP using reverse transcriptase, and cutting again with *Eco*RI. These were then purified from 8% polyacrylamide gels. In some instances, the DNA was labeled at the 3′ end of the *Eco*RI site by reversing the order of addition of *Hin*dIII and *Eco*RI.

Footprinting. Footprinting with DNase I, DNase II, and micrococcal nuclease (MNase) was performed as previously described (Low et al., 1984; Fox & Waring, 1984, 1987). Typical reactions contained DNA at a concentration of about 1 μM base pairs with a drug concentration of 0–100 μM. Under these conditions, ligand binding is not determined by the stoichiometry but is limited by the binding constant (about 10^5 M^{-1}; Cons & Fox, 1989a). In each case, 1 mM $MgCl_2$ was included in the reaction mixture since this is required for mithramycin binding (Behr et al., 1969; Cons & Fox, 1989a). The products of enzyme digestion were resolved on 10% (w/v) (*Hin*dIII labeled) or 14% (w/v) (*Eco*RI labeled) polyacrylamide gels containing 8 M urea. These were run at 1500 V for about 2 h before being fixed in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, and dried at 80 °C under vacuum. These were subjected to autoradiography at −70 °C with an intensifying screen. Bands in DNase I digests were assigned by comparison with Maxam–Gilbert sequencing reactions. Since DNase II and MNase cut the O5′–P bond, they generate radiolabeled products which terminate in a 5′-hydroxyl group, in contrast to DNase I and Maxam–Gilbert sequencing which end in a 5′-phosphate. As a result, bands in the digests with DNase II and MNase run more slowly than the corresponding Maxam–Gilbert markers, the difference (about two bonds) being more pronounced for the shorter fragments. Bands in the MNase digestion were easily assigned from the known specificity of this enzyme, cleaving only pA and pT bonds. DNase II digestion products were assigned by comparison with MNase cleavage.

Results

A_n Inserts. Figure 2 presents DNase I digests of DNA fragments containing A_{23} (labeled at either end) and T_{69} inserts in the presence and absence of various concentrations of mithramycin. In each case, cleavage of the homopolymeric inserts is poor in the controls, with significant cleavage only occurring outside the insert. This is typical of the behavior of A tracts in natural DNA fragments (Drew & Travers, 1984) and consistent with the poor cleavage of poly(dA)·poly(dT) by DNase I. In the presence of mithramycin, dramatic changes in the cleavage patterns are evident. Clear footprints can be seen around the CCC and GGG sequences at either end of the inserts, confirming sequence selectivity of the antibiotic and demonstrating that these repetitive sequences have not restricted the ability of the drug to bind to its preferred site. These footprints are staggered toward the 3′ ends of the binding sites, typical of other results seen with DNase

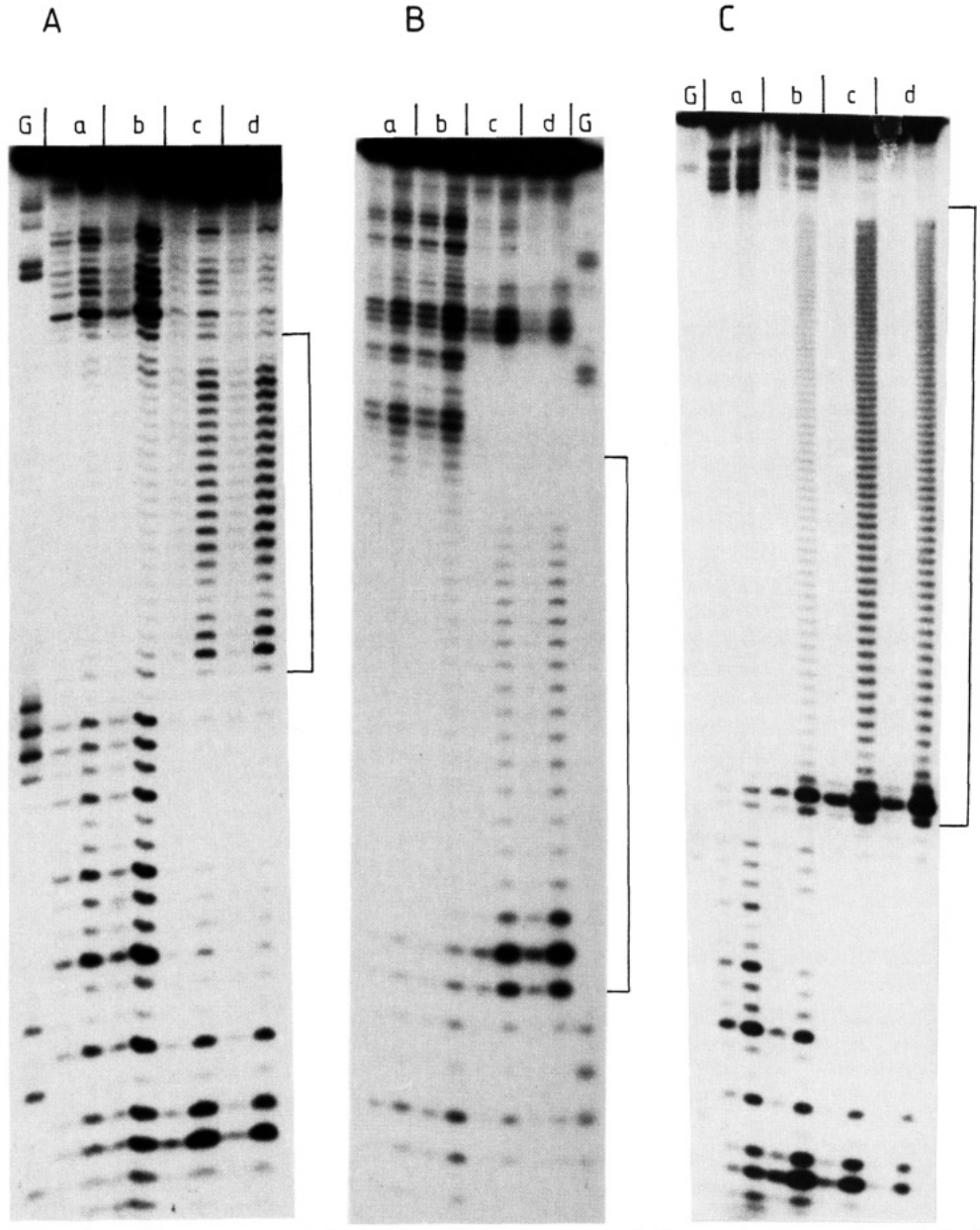

FIGURE 2: DNase I footprints of fragments containing A_n inserts in the presence and absence of mithramycin. (A) A_{23} insert labeled at the 3′ end of the *Hin*dIII site. (B) A_{23} insert labeled at the 3′ end of the *Eco*RI site. (C) T_{69} insert labeled at the 3′ end of the *Hin*dIII site. (B) and (C) correspond to the T-containing strand whereas (A) refers to the A strand. The brackets indicate the positions of the inserts. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. (a) Control, no drug; (b) 10 μM mithramycin; (c) 50 μM mithramycin; (d) 100 μM mithramycin. The tracks labeled "G" are Maxam–Gilbert dimethyl sulfate–piperidine markers specific for guanine.

I (Low et al., 1984; Fox & Waring, 1984). Each of these footprints appears to extend over 8–10 bases. This presumably represents the sum of two adjacent mithramycin binding sites: GGGG and CCTC toward the *Hin*dIII end of the fragment and CCC and CGG toward the *Eco*RI end. The footprints are accompanied by changes in the susceptibility of the surrounding regions of A_n to cleavage by the enzyme. In each case, enhancements are found throughout the entire length of the insert. For each fragment, bonds 2–4, closest to the 3′ end (bottom) of the insert, are strongly enhanced; the next two bands are weaker, though still stronger than in the control. Cleavage of the rest of the insert shows an even level of enhancement. No equivalent pattern is seen at the opposite (5′) end, presumably because this overlaps with the drug binding site on account of the 3′ stagger of the probe. The reason for this reduced enhancement at bonds 5–6 will be considered further under Discussion in light of results with other fragments. The pattern of enhancements for the A_{23} fragment is the same when the DNA is labeled at either end (Figure 2A,B).

A simple explanation for these enhancements is that mithramycin has distorted the DNA structure so that the flanking regions of A_n are in a conformation which is more susceptible to cleavage by DNase I. Regions of poly(dA) are normally poor substrates for this enzyme, on account of their unusually narrow minor groove and rigid structure. The increased rate of DNase I attack could arise either from an increase in the DNA minor groove width or from an increased flexibility. In order to investigate these changes further, we have studied the effect of mithramycin on cleavage of these fragments by two other nucleases having very different DNA structural requirements.

Figure 3A presents MNase digests of the A_{23}-containing fragment in the presence and absence of mithramycin. This enzyme is sensitive to local DNA breathing motions since it only cleaves pA and pT bonds (Flick et al., 1986; Dingwall

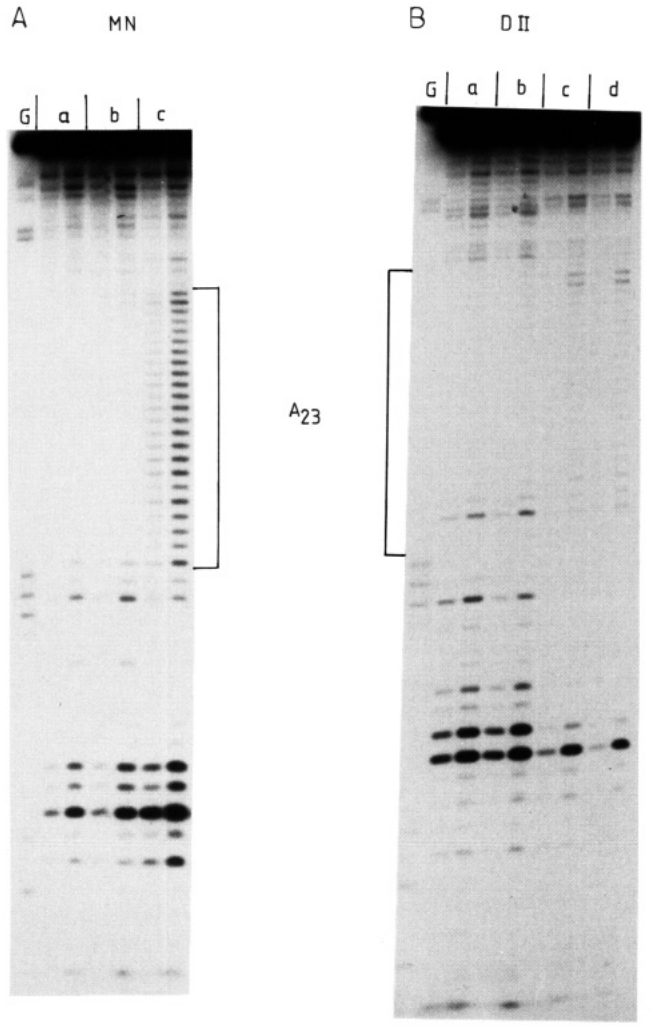

FIGURE 3: Micrococcal nuclease (MN) and DNase II (DII) digestion of fragments containing A_{23} inserts in the presence and absence of mithramycin. The DNA was labeled at the 3′ end of the *Hin*dIII site, thereby showing the adenine-containing strand. The brackets indicate the positions of the insert. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "G" are Maxam–Gilbert dimethyl sulfate–piperidine markers specific for guanine. (a) Control, no drug; (b) 5 μM mithramycin; (c) 50 μM mithramycin; (d) 100 μM mithramycin.

et al., 1982; Horz & Altenburger, 1981). Cleavage of homopolymeric A or T is generally poor (Fox & Waring, 1987), consistent with their higher melting temperature than regions of alternating AT (Riley et al., 1966). The control lanes in Figure 3A confirm the poor cutting of the A_{23} insert. In the presence of mithramycin, cleavage of the entire A tract is much enhanced, though this is still not as strong as other pA and pT bonds evident at the bottom of the gel.

Figure 3B presents the results of DNase II digestion of the A_{23} fragment in the presence and absence of mithramycin. Although the precise structural requirements of this enzyme are not known, it cuts the DNA strands asymmetrically with the best cleavage in runs of purines (especially G or GA); runs of A do not represent good cleavage sites (Drew, 1984; Drew & Travers, 1984). Cleavage of the A_{23} fragment by DNase II in the absence of drug is best around the sequence AGAG, toward the bottom of the gel; no cleavage is apparent in most of the insert, though a few bands close to the 3′ end of the insert can be seen at the junction with the rest of the fragment in the sequence AAGG. In the presence of mithramycin, clear footprints are visible around the GGG sequences, staggered toward the 3′ end (bottom). In addition, some, albeit weak, cleavage is now observed within the insert. This is greatest at the ends, near the drug binding sites, becoming weaker toward the center.

These results are again consistent with the formation of an altered DNA conformation, induced by drug binding, which is more sensitive to DNase II attack. It has been suggested that mithramycin causes a dramatic increase in the width of the DNA minor groove, rendering it more like an A-DNA structure. DNase II is known to efficiently cleave the purine strand of sequences which adopt an A-like structure (Drew, 1984). These results are therefore consistent with a model in which the changes in DNA structure, induced by mithramycin binding, are transmitted into neighboring sequences.

Are these structural changes a peculiar property of surrounding regions of A and T, or do they occur with other repetitive DNA sequences, which may adopt different structures in the absence of ligand? In order to address this question, we have performed similar experiments on other DNA fragments containing regions of $(GA)_n$·$(TC)_n$ and $(GT)_n$·$(AC)_n$ cloned in an identical location.

$(GT)_n$·$(AC)_n$. DNase I footprinting patterns of the fragment containing the insert $(CA)_8(GT)_7G(CA)_{15}C(CA)_4$ are presented in Figure 4A. This fragment (henceforth known as the GT fragment) was chosen because as well as containing good mithramycin binding sites (CCC/GGG) at either end of the insert it contains two potential internal drug sites at the GC and CC steps. Such sequences have previously been shown to yield mithramycin footprints, though these depend on some unknown feature of the surrounding DNA sequence (Van Dyke & Dervan, 1983; Cons & Fox, 1989b). DNase I cleavage of this DNA in the absence of mithramycin reveals an alternating pattern of products, in which GpT is cut better than TpG and ApC better than CpA. This alternation is broken around the AG, GC, and CC steps. The origin of this cleavage preference is not certain, though it may be similar to the observation that this enzyme cuts ApT better than TpA (Suggs & Wagner, 1986; McClellan et al., 1986), an effect which has been related to the unusual alternating B structure of poly(dA-dT), in which stacking at the TpA step is poorer than at ApT (Viswamitra et al., 1978; Klug et al., 1979). DNase I is also known to cleave GpC much better than CpG (Grant et al., 1972). In the presence of 20 μM mithramycin, clear footprints can be seen at either end of the insert in the sequences CCC and GGG; with 50 μM drug, additional footprints can be seen at the GC and CC sites within the insert, demonstrating their ability to form weaker mithramycin binding sites. Cleavage throughout the rest of the insert is unaffected by the presence of the antibiotic. Either mithramycin has not affected the structure of the GT·CA repeats or any changes do not affect cleavage by DNase I. In order to distinguish between these possibilities, we have performed footprinting experiments with MNase as a probe.

Results of MNase digestion of the GT fragment in the presence and absence of mithramycin are presented in Figure 4B. As expected, the only products in the drug-free control correspond to cleavage of pA and pT bonds. In the presence of mithramycin, the cleavage products closest to both the internal and external drug binding sites are absent. Apart from these drug footprints, no changes in the overall cleavage pattern are evident. Similar experiments with DNase II (not shown), which cuts ApC better than CpA and has little cleavage at GpT or TpG, also revealed no drug-induced changes in the cleavage pattern.

$(GA)_n$·$(TC)_n$. Figure 5 presents the results of DNase I and MNase cleavage of a DNA fragment containing an $A(GA)_{49}$

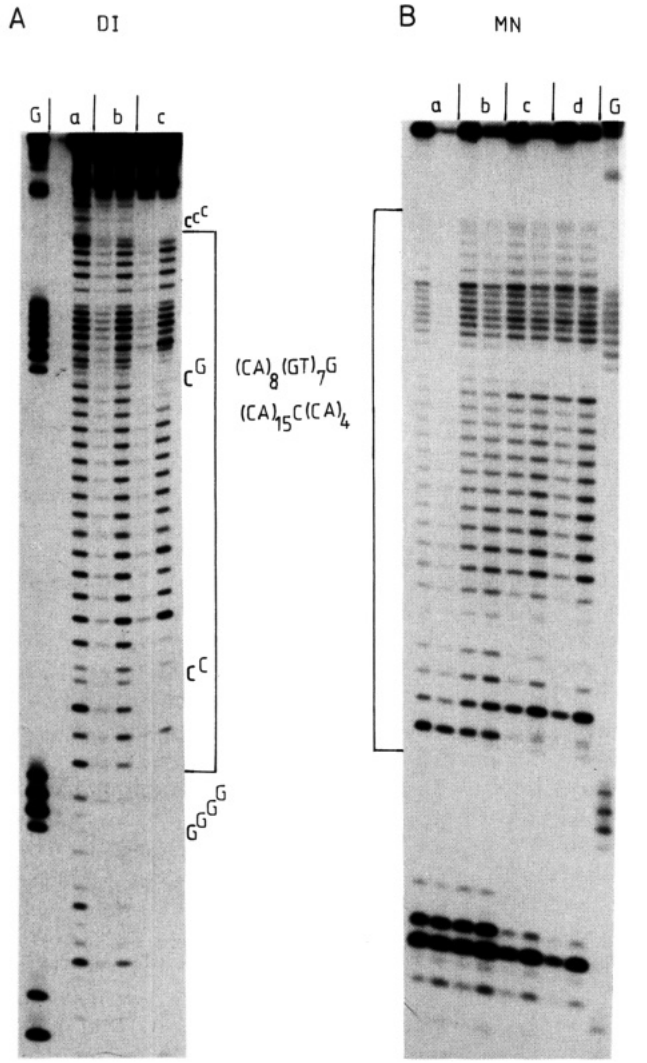

FIGURE 4: Footprinting patterns of a DNA fragment containing the sequence $(CA)_8(GT)_7G(CA)_{15}C(CA)_4$ cloned into the *Sma*I site of the pUC19 polylinker in the presence and absence of mithramycin. The *Eco*RI–*Hin*dIII fragment was labeled at the 3′ end of the *Hin*dIII site, so that the strand bearing the label has the sequence shown. The brackets indicate the positions of the insert. (A) Digestion by DNase I (DI). The sequences of the potential mithramycin sites are indicated. (B) Digestion by micrococcal nuclease (MN). Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "G" are Maxam–Gilbert dimethyl sulfate–piperidine markers specific for guanine. (a) Control, no drug; (b) 20 μM mithramycin; (c) 50 μM mithramycin; (d) 100 μM mithramycin.

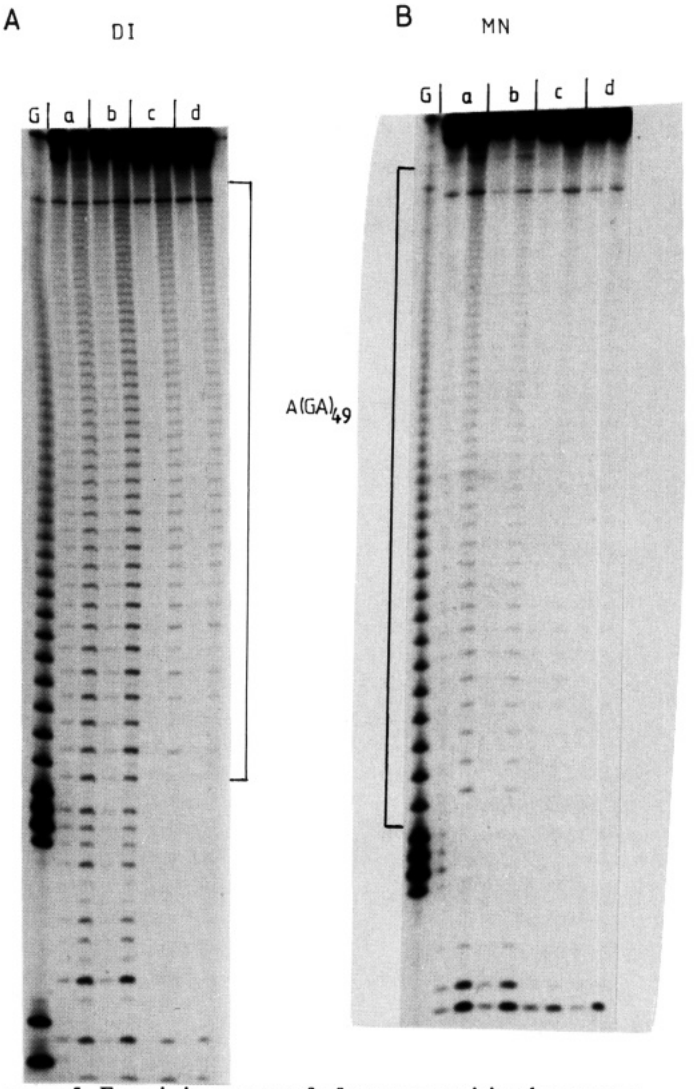

FIGURE 5: Footprinting patterns of a fragment containing the sequence $A(GA)_{49}$ cloned into the *Sma*I site of the pUC19 polylinker in the presence and absence of mithramycin. The *Eco*RI–*Hin*dIII fragment was labeled at the 3′ end of the *Hin*dIII site, so that the purine-containing strand bears the label. The brackets indicate the position of the insert. (A) Digestion by DNase I (DI). (B) Digestion by micrococcal nuclease (MN). Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "G" are Maxam–Gilbert dimethyl sulfate–piperidine markers specific for guanine. (a) Control, no drug; (b) 10 μM mithramycin; (c) 50 μM mithramycin; (d) 100 μM mithramycin.

insert. DNase I cleavage of the control exhibits a preference for cutting ApG over GpA. In the presence of mithramycin, a clear footprint is visible at the lower GGG site (a similar site at the opposite end of the insert is not resolved). Within the insert, the preference for cutting ApG is maintained in the presence of mithramycin, indicating that no long-range structural changes have occurred. The generally weaker band intensity at higher drug concentrations may indicate that the drug is interacting, albeit more weakly, with these regions of alternating GA. It is interesting to note the unusual cleavage pattern at the 3′ end (bottom) of the insert in the presence of mithramycin. The last ApG bond is absent, due presumably to overlap with the drug binding site, and cleavage of the next ApG step is still evident, while the band corresponding to cutting of the third ApG bond is missing. This is very similar to the result seen with the A_n inserts and will be considered further under Discussion. MNase digestion of this fragment shows the expected cleavage at the GpA steps. In the presence of mithramycin, the lowermost bands are missing, due to steric occlusion by the drug. The cleavage pattern for the rest of the fragment is unaltered by the presence of the antibiotic, though all the bands have a much weaker intensity, consistent with the suggestion that regions of alternating GA represent weaker mithramycin binding sites.

C_n. Figure 6 presents the results of DNase I digestion of a fragment containing a C_{14} insert within the *Sma*I site of the *Eco*RI–*Hin*dIII polylinker in the presence and absence of mithramycin. This fragment contains the sequence $C_{17}G_4$, which should correspond to a series of overlapping mithramycin binding sites. Cleavage of this insert is poor in the drug-free control. In the presence of mithramycin, the whole stretch presents a single long footprint. Within the range of concentrations tested, and the resolution of DNase I footprints, mithramycin does not seem to distinguish between the C_n portion and the CCG and CGG steps.

DISCUSSION

Sequence Selectivity. The results presented in this paper

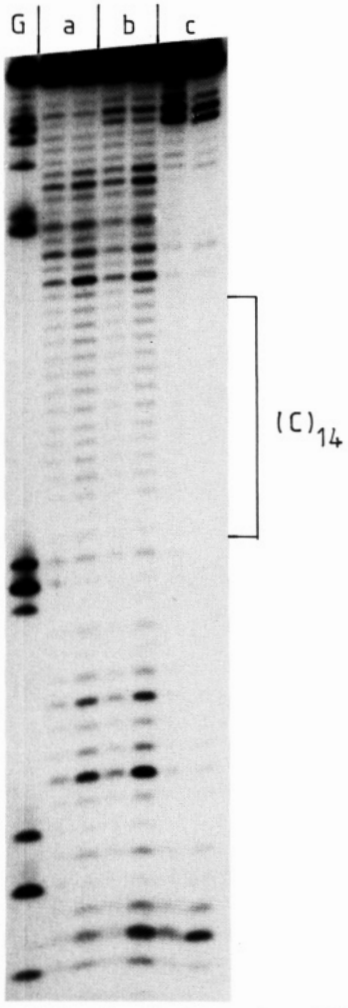

FIGURE 6: DNase I digestion of a fragment containing a C_{14} insert in the presence and absence of mithramycin. The *Eco*RI–*Hin*dIII fragment was labeled at the 3′ end of the *Hin*dIII site so that the cytosine-containing strand bears the radioactive label. The bracket indicates the position of the insert. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "G" are Maxam–Gilbert dimethyl sulfate–piperidine markers specific for guanine. (a) Control, no drug; (b) 20 μM mithramycin; (c) 50 μM mithramycin.

confirm the selectivity of mithramycin for at least two contiguous GC base pairs. The ability to bind to the trinucleotide GGG (CCC) was not affected by any of the surrounding repetitive sequences studied. In addition, results with the GT fragment revealed selective, but weaker, binding to regions with only two GC base pairs in the sequences TGCA and ACCA. In a previous study, using several natural DNA fragments, we demonstrated that mithramycin produced hydroxyl radical footprints at some, but not all, sites containing only two GC base pairs and that certain GC trinucleotides did not yield footprints (Cons & Fox, 1989b). Several of those regions to which the drug did not bind were close to blocks of A residues. It seems that the presence of an adjacent narrow minor groove may be one factor which limits drug binding, though this alone is not a sufficient criterion for prohibiting binding. Since mithramycin causes large changes in DNA structure, the drug will only bind to those sequences which possess a wide minor groove or which can be readily distorted to such a structure. The mithramycin footprints at two contiguous GC base pairs, presented in this paper, are flanked by regions of alternating GT·AC, and should possess a relatively wide minor groove, accessible to drug binding. The effects of DNA structure and sequence on the binding of chromomycin have been demonstrated by NMR studies which reveal that the drug binds well to ATGCAT but not ATCGAT (Banville et al., 1990a). Since none of the fragments studied in this paper contain the dinucleotide CG, we cannot comment further on its ability to serve as a potential mithramycin binding site.

Sarker and Chen (1989) have presented data suggesting that mithramycin binds reasonably well to poly(dA-dG)·poly(dC-dT), though not poly(dA-dC)·poly(dG-dT). We find no clear footprints for mithramycin at either of these sequences. This discrepancy most probably arises from differences in the kinetics of dissociation, which may be faster for those sequences which do not possess adjacent GC base pairs. Since DNase I footprinting will only detect sites with a slow dissociation rate, we cannot rule out the existence of other secondary sites with a faster exchange rate. Some interaction with $(GA)_n$ but not $(GT)_n$ sequences may be inferred from the experiments with micrococcal nuclease; MNase cleavage of the GT insert is unaffected by the drug while cleavage at GA is abolished by higher concentrations of the ligand. This probe is known to be more sensitive to weaker, secondary, drug binding than DNase I (Fox & Waring, 1987).

The results with the GT fragment confirm that regions of alternating GT (AC) do not represent good mithramycin binding sites (Sarker & Chen, 1989). Previous footprinting studies have shown a good binding site in the sequence AGTGTA at position 65 of the *tyrT* DNA fragment (Cons & Fox, 1989b). It is possible that this anomalous protection is similar to the reduced cleavage seen four to five bases 5′ of the drug binding site for the A_n and GT fragments. This effect is unusual and is unlikely to arise from direct steric blockage of enzyme access by bound mithramycin. We are not aware of any other reports of "shadowing" in drug footprinting experiments. It may be significant that the additional protection is found about half a helical turn from the true drug binding site. More probable are explanations in terms of the effect of the drug on DNA structure. NMR studies have shown that mithramycin binding makes the minor groove much wider and deeper. This could have some effect on the width of the adjacent major groove (located half a helical turn away), either narrowing it or causing it to bend toward the minor groove, thereby occluding enzyme access.

Effects on DNA Structure. The results presented in this paper demonstrate clearly that mithramycin can cause changes in the susceptibility of certain flanking sequences to nuclease attack. The simplest explanation of these changes is that mithramycin has caused changes in the DNA structure at its binding site and that these have been propagated into neighboring regions, rendering them more susceptible to nuclease attack. Before considering what the actual structural changes may be, and why they are not found in all surrounding sequences, it is worth considering whether any other explanations can account for the drug-induced enhancements. Ward et al. (1988a,b) have used quantitative footprinting to demonstrate that, for natural DNA fragments of random sequence, some apparent enhancements can be accounted for by changes in the ratio of free DNA to enzyme. According to this model, drug binding displaces the nuclease from the DNA helix so that the amount of enzyme available, relative to the amount of unbound DNA, is increased. They explain how some sequences appear to display a relatively unchanged rate of cleavage while others are enhanced by invoking secondary, weaker drug binding sites which were occupied for proportionately less time, but which do not generate footprints. Can this explanation account for the changes that we have observed with the A_n fragments? We think not, for the following

reasons. Mithramycin shows no interaction with regions of poly(dA). In the A_{69} fragment, the insert constitutes 57% of the DNA fragment. Even if all the rest of the DNA was protected from cleavage (which it is not), then this would only result in a 75% increase in cutting of the insert. The effects that we see are much greater than this; indeed, they are hard to accurately quantify since cleavage of these regions is almost negligible in the control. We estimate that DNase I cleavage of the A_n inserts is increased by at least 10-fold by the binding of mithramycin.

If we assume that the enhancements seen in the A_n inserts result from drug-induced alterations in DNA structure, what is the nature of the changes? NMR studies have demonstrated that mithramycin binds as a dimer within the DNA minor groove. The presence of such a large complex within the minor groove induces a structure more akin to an A-DNA helix. It is also known that poly(dA)·poly(dT) adopts an unusual structure with 10.0 base pairs per turn, in which the bases are highly propeller-twisted, and which is resistant to nuclease attack and intercalative drug binding (Bresloff & Crothers, 1981; Arnott et al., 1983; Nelson et al., 1987; Aymani et al., 1989). This structure is stabilized by the formation of an additional bifurcated hydrogen bond (Nelson et al., 1987) and possesses an unusually narrow minor groove. It seems reasonable to suppose that the increase in groove width caused by mithramycin will not be restricted to its actual binding site, especially if the surrounding sequence has an unusually narrow minor groove. The surrounding DNA structure will change so as to avoid the formation of an abrupt junction between two types of DNA conformation. NMR studies have suggested that the DNA structure formed by mithramycin binding has characteristics of both A- and B-DNA (Gao & Patel, 1989a,b; Banville et al., 1990a,b). This is consistent with the nuclease digestion patterns observed in the presence of the drug. DNase II cuts DNA best in regions which resemble A-DNA (Drew, 1984) and does not normally cleave poly(dA) tracts. The enhancements seen with this enzyme would tend to support the view that A_n inserts have been forced to adopt a structure which is similar to A-DNA. In contrast, DNase I cuts B-DNA much better than A-DNA, yet it too cuts the A tracts better in the presence of the drug.

Although micrococcal nuclease cuts almost exclusively at pA and pT bonds, regions of poly(dA)·poly(dT) are normally poor substrates. This is explained by the rigid structure formed by blocks of A, caused by the presence of the extra bifurcated hydrogen bonds and the improved base stacking, preventing DNA breathing motions. It is because of this that poly(dA)·poly(dT) has a higher melting temperature than poly(dA-dT). The observation that mithramycin increases the rate of MNase digestion of surrounding blocks of A_n suggests that the DNA has become more mobile and that at least some of the improved base stacking has been sacrificed in order to form the novel structure. However, the altered structure is clearly not identical with the "average" B-DNA conformation, since the insert is still less sensitive to MNase than other bands in the rest of the DNA fragment.

All three enzymic probes used confirm that mithramycin alters the structure of A_n inserts in a highly cooperative fashion. The enhancements do not fade out at positions remote from the drug binding site. The simplest explanation for this is that such sequences exist in multiple polymorphic states with similar energies and that mithramycin binding shifts the equilibrium of the entire insert in favor of an alternative structural form.

The formation of novel DNA structures by the binding of mithramycin is not a general effect but appears to be limited to A_n and $(AT)_n$ sequences. Cleavage of flanking regions of $(GA)_n$·$(TC)_n$ and $(GT)_n$·$(AC)_n$ is not affected by drug binding as assessed with any of the enzymic probes. Why should this be? Several explanations are worth considering. First, it is possible, though unlikely, that mithramycin does induce conformational changes in these sequences but that they are not detected by any of the probes used. More probably, mithramycin does not induce any radical changes in these DNA structures. Either these sequences adopt a natural conformation which is compatible with the mithramycin binding site, or they form a rigid block through which it is not possible to transmit conformational changes. The former seems likely for alternating GA sequences since runs of purines more easily adopt a structure resembling A-DNA. The situation is less clear for surrounding regions of alternating GT. It has previously been shown that mithramycin affects nuclease cleavage of surrounding regions of alternating AT (Cons & Fox, 1990b), causing a dramatic change in the pattern of DNase II cleavage, and an increased cutting by DNase I and MNase. It is also worth noting that enzymic enhancements should only be possible for those sequences which adopt a configuration which is not optimal for cleavage. For sequences which present excellent enzyme recognition sites, any drug-induced structural changes either will have no effect or will serve to cause a reduction in cleavage efficiency.

Comparison with Other Drugs. It appears that a common feature of sequence-selective ligands is an ability to alter the conformation of sequences surrounding their binding sites. These changes are sequence-dependent and are characteristic of the ligand in question. The only other ligand for which similar studies on the nuclease susceptibility of surrounding A_n tracts have been investigated is actinomycin D (Lane et al., 1987; Huang et al., 1988; Waterloh & Fox, 1991). Studies with short oligonucleotides revealed no changes in the structure of the A_n tracts (Huang et al., 1988), whereas when these are cloned into longer DNA fragments there is a dramatic change in cleavage by DNase I (Waterloh & Fox, 1991). The enhancements produced by actinomycin fade out further from the ligand binding site; with T_9GCA_9, they are found on both sides of the ligand binding site whereas for A_9GCT_9 enhancements only occur on the 5′ side. These differences between actinomycin and mithramycin must arise from their different modes of binding. Enhancements induced by actinomycin must be the result of its ability to unwind the DNA helix and the local flattening of the DNA base pairs caused by insertion of its phenoxazone chromophore. In contrast, mithramycin does not unwind DNA (Waring, 1970) or intercalate into the helix. Any structural changes induced by this ligand must derive from the large changes in minor groove width caused by its binding. Although unwinding, flattening of propeller twist, and increases in minor groove width are interrelated events, they have different molecular origins. If the enhancements produced by actinomycin are primarily due to flattening of the propeller twist, then we envision that this effect will diminish further from the drug binding site. It may be that the increased groove width generated by mithramycin is less easily diluted out with distance. If this is correct, then long-range structural changes may be a general property of groove binding ligands such as mithramycin, whereas intercalators may generate effects over a shorter distance. In this regard, it will be interesting to examine the effects of distamycin, a ligand which binds well to a narrow minor groove on the structure of surrounding regions of G_n and $(GC)_n$. Previous footprinting studies with natural DNA fragments revealed enhancements of DNase I cleavage in GC-rich regions

adjacent to is AT binding sites (Fox & Waring, 1984).

Registry No. Mithramycin, 18378-89-7; guanine, 73-40-5; cytosine, 71-30-7.

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