Chromomycin, Mithramycin, and Olivomycin Binding Sites on Heterogeneous Deoxyribonucleic Acid. Footprinting with (Methidiumpropyl-EDTA)iron(II)†

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ABSTRACT: The DNA binding sites for the antitumor, antiviral, antibiotics chromomycin, mithramycin, and olivomycin on 70 base pairs of heterogeneous DNA have been determined by using the (methidiumpropyl-EDTA)iron(II) [MPE·Fe(II)] DNA cleavage inhibition pattern technique. Two DNA restriction fragments 117 and 168 base pairs in length containing the lactose operon promoter-operator region were prepared with complementary strands labeled with ^{32}P at the 3′ end. MPE·Fe(II) was allowed to partially cleave the restriction fragment preequilibrated with either chromomycin, mithramycin, or olivomycin in the presence of Mg^{2+}. The preferred binding sites for chromomycin, mithramycin, and olivomycin in the presence of Mg^{2+} appear to be a minimum of 3 base pairs in size containing at least 2 contiguous dG·dC base pairs. Many binding sites are similar for the three antibiotics; chromomycin and olivomycin binding sites are nearly identical. The number of sites protected from MPE·Fe(II) cleavage increases as the concentration of drug is raised. For chromomycin/Mg^{2+}, the preferred sites on the 70 base pairs of DNA examined are (in decreasing affinity) 3′-GGG, CGA > CCG, GCC > CGA, CCT > CTG-5′. The sequence 3′-CGA-5′ has different affinities, indicating the importance of either flanking sequences or a nearly bound drug.

A class of small molecules important in antibiotic, antiviral, and antitumor chemotherapy bind to double-helical DNA (Gale et al., 1981). The base sequence preferences of drugs binding to DNA have usually been determined from spectrophotometric analyses of the overall affinity and stoichiometry of drug binding on synthetic homopolymer and copolymer DNAs. (Methidiumpropyl-EDTA)iron(II), which contains the DNA intercalator methidium covalently bound by a short hydrocarbon tether to the metal chelator ethylenediaminetetraacetate (EDTA) (Figure 1) in the presence of ferrous ion and oxygen, efficiently produces single-strand breaks in double-helical DNA (Hertzberg & Dervan, 1982). Importantly, MPE·Fe(II) is a relatively non-sequence-specific DNA cleaving agent (Van Dyke et al., 1982; Van Dyke & Dervan, 1982). In effect, MPE·Fe(II) is a small synthetic scissor for DNA that mimics the behavior of the DNA cleaving enzyme DNase I. One useful method for determining protein binding sites on native DNA is the DNA cleavage inhibition pattern technique, which combines DNase I cleavage of protein-protected DNA fragments and Maxam–Gilbert sequence determination methods (Galas & Schmitz, 1978; Schmitz & Galas, 1982). This useful "DNase I footprinting" techniques relies on the relatively low sequence specificity of the enzyme in a partial DNA cleavage reaction and the ability of DNA-bound protein to prevent cleavage of the DNA backbone between the base pairs it covers. The protein-protected DNA sequence is expressed as a gap in the sequencing ladder seen in the autoradiogram of a Maxam–Gilbert gel revealing the position and extent of the protein binding sites. Because MPE·Fe(II) cleaves native DNA with *lower* sequence specificity than DNase I, "MPE·Fe(II) footprinting" is useful for determining the locations, size, and relative importance of multiple binding sites on native DNA for *small molecules* such as DNA binding drugs (Van Dyke et al., 1982; Van Dyke & Dervan, 1982) (Figure 2). Using a combination of MPE·Fe(II) partial cleavage of drug-protected DNA restriction fragments and Maxam–Gilbert sequencing methods, we determined the DNA cleavage inhibition patterns on heterogeneous DNA from pBR322 plasmid for the intercalator actinomycin and the minor groove binders netropsin and distamycin A (Van Dyke et al., 1982). The DNA cleavage inhibition patterns on opposite strands are asymmetric, shifted at least one base pair to the 3′ side of the drug binding site (Van Dyke & Dervan, 1982) (Figure 3).

Chromomycin A_3, mithramycin, and olivomycin are a series of anticancer, antiviral, antibiotics discovered in Japan, USA, and USSR, respectively (Figure 4) (Gause, 1967; Kersten & Kersten, 1974; Slavik & Carter, 1975; Remers, 1979). These derivatives of aureolic acid possess similar structural features such as an aglycon moiety with five attached hexopyranoses. Each selectively inhibits DNA-dependent RNA synthesis by forming complexes with DNA in the presence of Mg^{2+} (Ward et al., 1965). The interaction with DNA requires the presence of a guanine base (Ward et al., 1965; Behr et al., 1969). Despite the fact that these drugs have received intensive clincal study, not much else is known about their DNA sequence specificity. Their DNA binding affinities and dissociation kinetics are similar to those of the antibiotic actinomycin (Hartmann et al., 1968; Prasad & Nayak, 1976). However, their mode of binding to DNA is unknown with intercalation considered unlikely (Waring, 1970).

We report the DNA binding sites for chromomycin A_3, mithramycin, and olivomycin on heterogeneous DNA using MPE·Fe(II) footprinting. DNA restriction fragments 117 and 168 base pairs in length containing regions of identical sequence with ^{32}P end labeling on complementary strands were prepared (Figure 5). These were equilibrated with either chromomycin, mithramycin, or olivomycin in the presence of Mg^{2+}. Partial cleavage by MPE·Fe(II) of this antibiotic-protected DNA and Maxam–Gilbert sequence methods (Maxam & Gilbert, 1980) provide opposite-strand DNA cleavage inhibition patterns. This affords the location, minimum binding site size, and order of preference of multiple binding sites on 70 base pairs of heterogeneous DNA for the above antibiotics.

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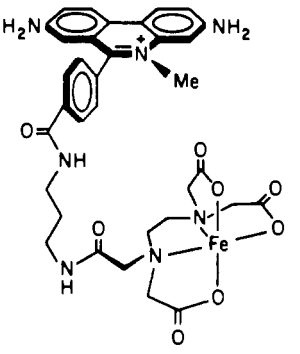

FIGURE 1: (Methidiumpropyl-EDTA)iron(II) structure.

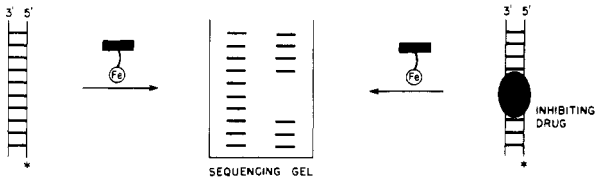

FIGURE 2: Illustration of the MPE·Fe(II) DNA cleavage technique with the Maxam–Gilbert gel on native and drug-protected segments of double-helical DNA.

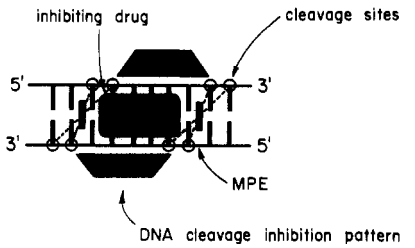

FIGURE 3: Model for asymmetric DNA cleavage inhibition patterns.

Materials and Methods

Antibiotics. Chromomycin A_3 and olivomycin were obtained in powdered form from Calbiochem-Behring. Stock solutions were prepared by dissolving each antibiotic in ethanol and measuring its UV absorbance with dilution to 1 mM. UV maxima used were 282 nm (log = 4.60) and 277 nm (log = 4.67) for chromomycin A_3 and olivomycin, respectively (Miyamoto et al., 1964; Berlin et al., 1964, 1968). Mithramycin was obtained from Sigma, supplied as a mixture of mannitol, sodium phosphate, and 2.5% mithramycin. This was purified by precipitation with 95% ethanol containing 2.5 mM each of $Mg(OAc)_2$ and $CaCl_2$, followed by salt-free ethanolic precipitations until no white solids were observed. The concentrations of the mithramycin-containing supernatant were determined spectrophotometrically by assuming the same spectral characteristics as the chromophore contained in antibiotic chromomycin A_3. After dilution to 1 mM, this served as the mithramycin stock solution. For antibiotic/Mg^{2+} binding studies, 2 equiv of $MgCl_2$ was added. This ethanolic solution was then diluted to 1 mM. All antibiotic stock solutions were stored at −20 °C.

MPE. MPE was synthesized and purified by procedures already described (Hertzberg & Dervan, 1982). An orange aqueous solution was made whose concentration was determined by using a visible maximum of 487 nm (log = 3.7). To this was added 2 equiv of freshly prepared $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ solution (Baker, reagent grade), and a pink precipitate resulted. This was then diluted to 0.1 mM in MPE, stored at −20 °C, and served as the MPE stock solution.

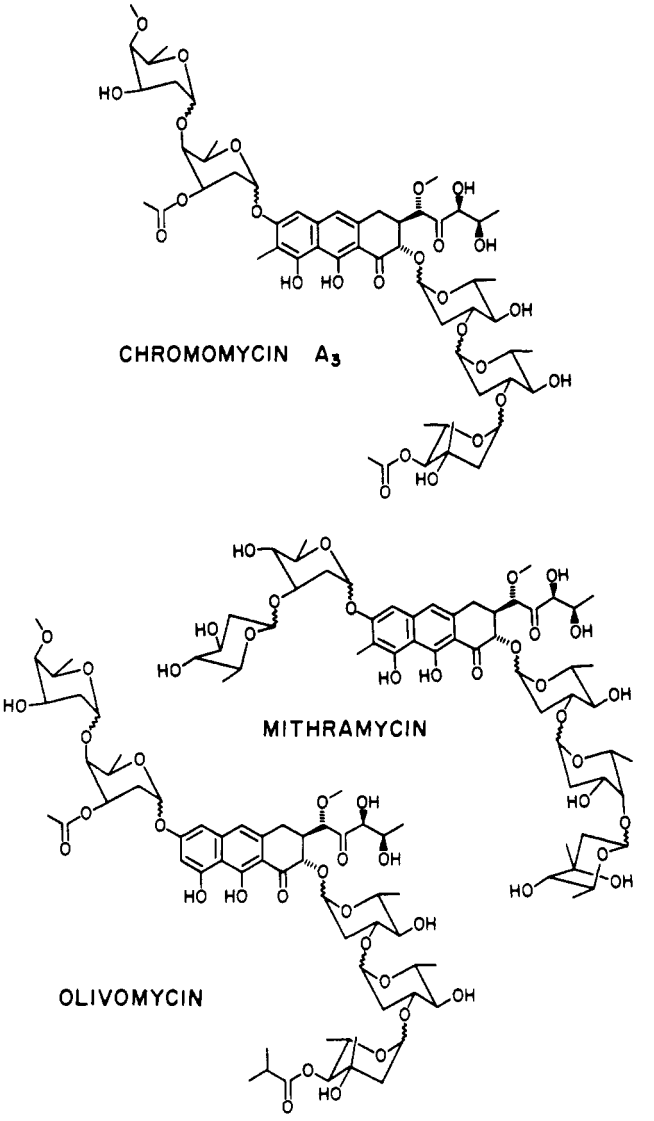

FIGURE 4: Structures of chromomycin, mithramycin, and olivomycin.

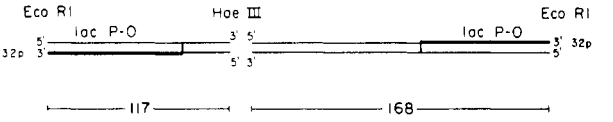

FIGURE 5: Diagram of 117 and 168 base pair fragments derived from cleavage of the 285 base pair 3′ end-labeled fragment with the restriction enzyme *Hae*III. The 117 and 168 base pair fragments each contain one copy of the lactose operon promoter-operator region with complementary strand 3′ end labeling (Haseltine et al., 1980).

Calf Thymus DNA. To 1.2 mL of 2% NaOAc solution at 50 °C was added 5 mg of highly polymerized calf thymus DNA (type I, Na^+ salt) obtained from Sigma. After being cooled, this solution was extracted with a 25:24:1 mixture of phenol (pH 7.4), chloroform, and isoamyl alcohol to remove unwanted protein. Adding a 2-fold excess of ethanol to the aqueous phase, cooling in dry ice, and spinning for 15 min at 15 000 rpm in an Eppendorf microcentrifuge precipitated the DNA. This pellet was resuspended in 1 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 5 mM NaCl, and 0.1 mM EDTA and then heated at 70 °C for 30 min. The concentration was measured by assuming an extinction coefficient at 260 nm of 11 800 per mole of base pairs.

Preparation of Radiolabeled DNA Fragments. DNA for this investigation was a gift from D. Galas (University of Southern California) and consisted of a sequence segment of

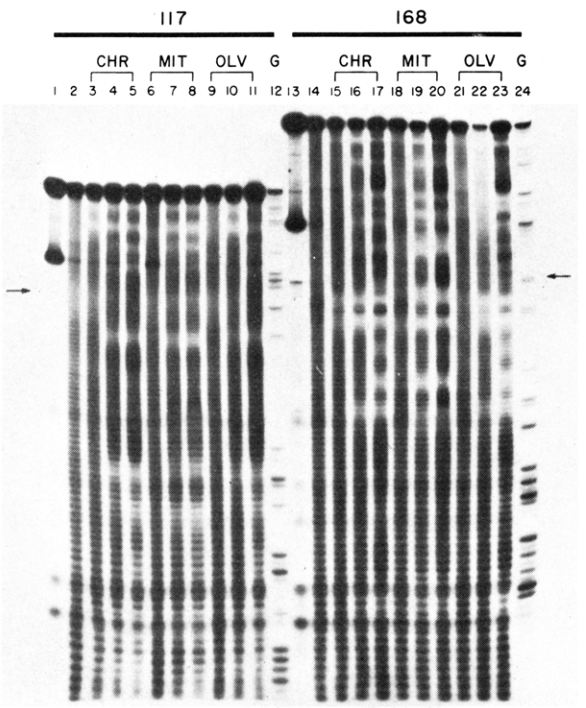

FIGURE 6: Autoradiogram of DNA cleavage inhibition patterns for chromomycin, mithramycin, and olivomycin on 117 and 168 base pair fragments. Lanes 1–11 (117 base pair fragment) and 13–23 (168 base pair fragment) had a 10-μL final volume, each containing 10 mM Tris, pH 7.4, 50 mM NaCl, 0.1 mM DNA base pairs, 4.0 mM DTT, 1.1 mM NH_4OAc, and 0.18 mM EDTA. Lanes 2–11 and 14–23 also contained 10 μM MPE·Fe(II). Lanes 12 and 24 were the Maxam–Gilbert G reaction on the 117 and 168 base pair restriction fragments, respectively. Lanes 1 and 13, intact DNA restriction fragments; lanes 2 and 14, MPE·Fe(II) cleavage of DNA restriction fragments; lanes 3 and 15, MPE·Fe(II), 6.3 μM chromomycin, and 13 μM Mg^{2+}; lanes 4 and 16, MPE·Fe(II), 25 μM chromomycin, and 50 μM Mg^{2+}; lanes 5 and 17, MPE·Fe(II), 100 μM chromomycin, and 200 μM Mg^{2+}; lanes 6 and 18, MPE·Fe(II), 6.3 μM mithramycin, and 13 μM Mg^{2+}; lanes 7 and 19, MPE·Fe(II), 25 μM mithramycin, and 50 μM Mg^{2+}; lanes 8 and 20, MPE·Fe(II), 100 μM mithramycin, and 200 μM Mg^{2+}; lanes 9 and 21, MPE·Fe(II), 6.3 μM olivomycin, and 13 μM Mg^{2+}; lanes 10 and 22, MPE·Fe(II), 25 μM olivomycin, and 50 μM Mg^{2+}; lanes 11 and 23, MPE·Fe(II), 100 μM olivomycin, and 200 μM Mg^{2+}.

the lactose operon in *Escherichia coli*. This is isolated from the plasmid pLJ3 (Johnsrud, 1978; Haseltine et al., 1980) grown in *E. coli* strain MM294. Part of this plasmid is a 285 base pair insert containing two copies of the lactose operon promoter-operator sequence in the same orientation (Figure 5). Milligram quantities of the plasmid were isolated by procedures similar to those of Tanaka & Weisblum (1974). Superhelical pLJ3 plasmids were first digested with the restriction endonuclease *Eco*RI and then labeled at the 3′ end with [^{32}P]dATP and the Klenow fragment of DNA polymerase I. A second enzymatic digestion with restriction endonuclease *Hae*III yielded the two end-labeled fragments 117 and 168 base pairs in length. These were isolated by gel electrophoresis on a 5% polyacrylamide, 1:30 cross-linked, 4-mm-thick preparatory gel. Further recovery and purification followed procedures similar to those of Maxam–Gilbert. These fragments were stored in a 60 mM NH_4OAc/5 mM EDTA solution at −20 °C.

DNA Cleavage Reactions with MPE. Each reaction initially consists of a 2-μL solution of 50 mM Tris (pH 7.4), 250 mM NaCl, and 0.5 mM DNA base pairs composed of 3′-^{32}P end-labeled restriction fragment and carrier calf thymus DNA. To this is added 1 μL of a solution of antibiotic in ethanol. This was incubated for 30 min at room temperature. The

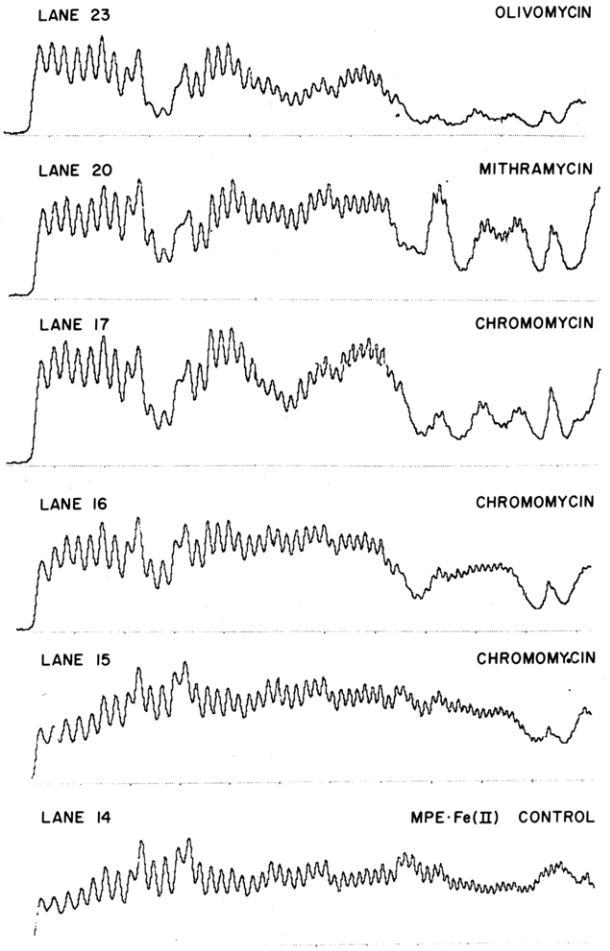

FIGURE 7: Densitometer scan. Left to right corresponds to the bottom to the arrow of the gel autoradiogram shown in Figure 6 (lanes 14–17, 20, 23). Valleys are drug-protected regions from MPE·Fe(II) cleavage.

cleavage reaction was initiated by the addition of 5 μL of 20 μM MPE·Fe(II) (freshly diluted from stock) and 2 μL of freshly prepared 20 mM dithiothreitol solution (Calbiochem-Behring). Final concentrations in the 10-μL reaction volume are 10 mM Tris (pH 7.4), 50 mM NaCl, 4 mM dithiothreitol, 3.6 mM NH_4OAc, 0.3 mM EDTA, 100 μM DNA base pairs, and 10 μM MPE·Fe(II). Antibiotic concentrations (6–100 μM) were adjusted as specified in the figure legends. Each reaction was allowed to run at 37 °C for 15 min, stopped by freezing in dry ice, and then lyophilized and resuspended on a formamide loading buffer for gel electrophoresis.

Sequencing Gels. Resolution of inhibition patterns was achieved by electrophoresis on 0.4 mm thick, 40 cm long, 8% polyacrylamide, 1:20 cross-linked sequencing gels containing 50% urea. Electrophoresis was carried out at 1000 V for 3.5 h to sequence 100 nucleotides, beginning 20 nucleotides from the 3′-labeled end. Autoradiography was carried out at −50 °C without the use of intensification screen.

Densitometry. An 8 × 10 in. copy of the original autoradiogram was scanned at 485 nm with the incident beam collimated to a width of 0.05 mm on a Cary 219 spectrophotometer. The data were recorded as the absorbance relative to the film base density and analyzed by using an Apple microcomputer.

Results and Discussion

For an investigation of the DNA cleavage inhibition patterns produced by partial digestion of DNA restriction fragments protected by chromomycin, mithramycin, and olivomycin, two

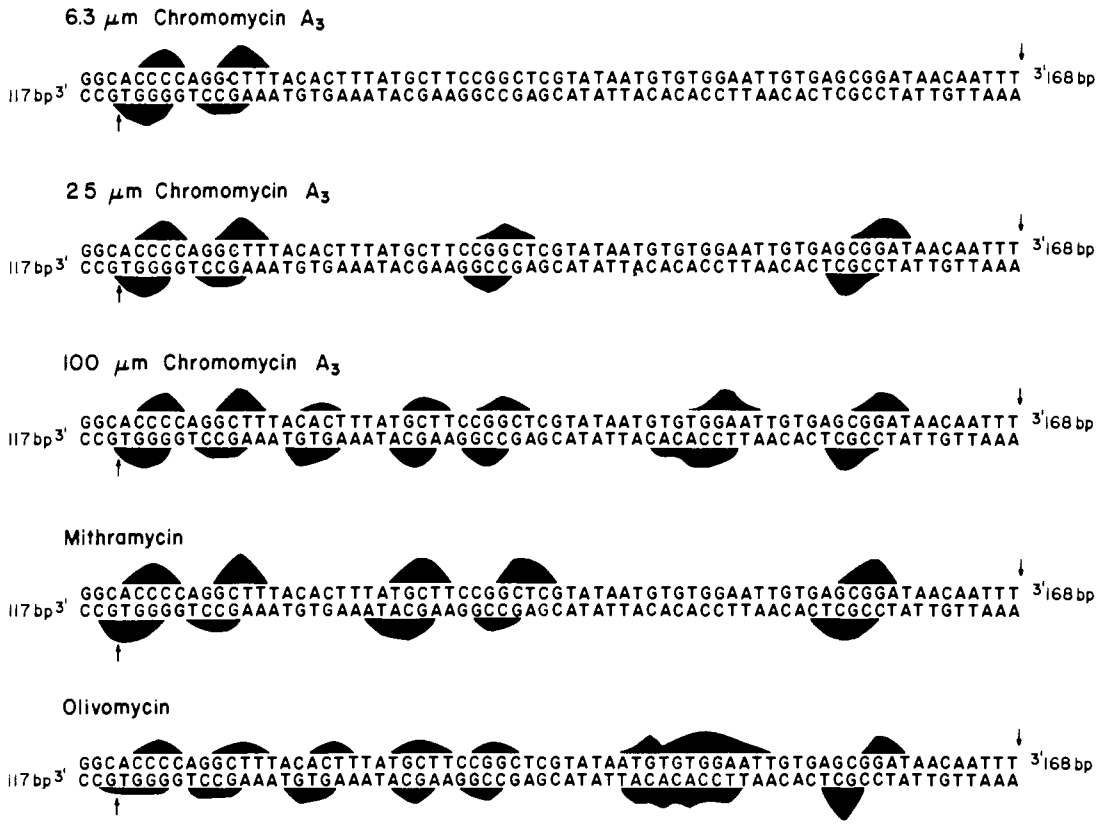

FIGURE 8: Illustrations of drug-protected regions (black areas) from MPE·Fe(II) cleavage on 70 base pairs of the complementary strands containing the lactose operon promotor-operator region. Arrows indicate the bottom of the gel autoradiogram for each strand (Figure 6).

FIGURE 9: Preferred chromomycin binding sites, 1 > 2 > 3. The binding site size of three base pairs is a minimum value.

DNA substrates containing regions of identical sequence were studied. Two DNA restriction fragments, 117 and 168 base pairs in length, each containing the lactose operon promoter-operator region were prepared with complementary strands labeled with ^{32}P at the 3′ end. MPE·Fe(II) was allowed to partially cleave the 117 and 168 base pair fragments alone and the 117 and 168 base pair fragments preequilibrated with chromomycin, mithramycin, and olivomycin, respectively. Partial cleavage by MPE·Fe(II) was stopped after 15 min by freezing, lyophilization, and resuspension in formamide buffer. The ^{32}P end-labeled DNA products were analyzed by 8% polyacrylamide/50% urea denaturing gel electrophoresis capable of resolving DNA fragments differing in length by one nucleotide. The autoradiogram data are shown in Figure 6.

Controls. Experiments in the absence of inhibiting drugs were carried out. Lanes 1 and 13 (Figure 6) are the buffered intact 117 and 168 base pair DNA fragments (100 μM in base pairs), respectively. The two bands at the top of control lanes 1 and 13 presumably arise from incomplete denaturation. Lanes 2 (117 base pair DNA fragment) and 14 (168 base pair DNA fragment) are controls containing 100 μM DNA, 10 μM MPE·Fe(II), and 4 mM dithiothreitol (DTT), demonstrating the relatively uniform cleavage pattern generated by MPE·Fe(II). Lanes 2 and 14 provide the base line by which other DNA cleavage patterns in the presence of drugs are compared. In controls not shown, a range of concentrations of Mg^{2+} (1 μM–1 mM) was found not to effect the low sequence-specific cleavage of DNA by MPE·Fe(II). Lanes 12 and 24 are the products of the Maxam–Gilbert sequencing reactions for G and are used as markers for base identification.

Chromomycin, Mithramycin, and Olivomycin (Figure 6). Different concentrations of chromomycin (6.25, 25, and 100 μM) in the presence of a 2-fold excess of Mg^{2+} (13, 50, and 200 μM, respectively) were allowed to equilibrate with the 117 and 168 base pair DNA fragments (100 μM in base pairs). To this were added at a final concentration 10 μM MPE·Fe(II) and 4 mM dithiothreitol. From the DNA cleavage inhibition patterns observed in the gel autoradiogram (Figure 6, lanes 3–5, 15–17), the preferred binding sites for chromomycin/Mg^{2+} appear to be a minimum of three base pairs in size containing at least two contiguous dG·dC base pairs. Similar results were found with mithramycin/Mg^{2+} and olivomycin/Mg^{2+}. We found that at least 1 equiv of Mg^{2+} was required for a well-defined DNA cleavage inhibition pattern (unpublished results). The densitometer trace of the DNA cleavage inhibition pattern on the autoradiogram for the three drugs at the highest concentration (lanes 5, 8, and 11 and 17, 20, and 23) indicates that many but not all binding sites are identical for the three antibiotics (Figure 7). Most striking is the near identity of the chromomycin and olivomycin densitometer traces. The structural feature common to chromomycin and olivomycin is the five sugar moieties. They differ by a methyl group in the aglycon. Mithramycin bears different hexopyranoses. An illustration of the opposite strand footprints for chromomycin, mithramycin, and olivomycin is shown in Figure 8.

The number of sites protected from MPE·Fe(II) cleavage increases as the concentration of drugs with Mg^{2+} is raised.

This allows a determination of the relative binding affinities for multiple sites on the heterogeneous DNA. The chromomycin footprints as a function of increasing concentration are shown in Figure 8. For chromomycin/Mg^{2+}, the preferred sites on the 70 base pairs of DNA examined are (in decreasing affinity) 3′-GGG, CGA ≫ CCG, GCC ≫ CGA, CCT > GTG-5′ (Figure 9). The sequence 3′-CGA-5′ appears twice and has different affinities, indicating the importance of either flanking sequences or a nearby bound drug. Despite the similarity of both the locations and number of preferred binding sites by chromomycin, mithramycin, and olivomycin at high concentration (100 μM), the different order of appearance of DNA binding sites upon increasing drug concentration indicates that the relative binding affinities for each drug differ (unpublished results).

Summary. We have found that the antibiotic, antiviral, antitumor drugs chromomycin, mithramycin, and olivomycin afford DNA cleavage inhibition patterns in the presence of MPE·Fe(II). The preferred binding sites for all three drugs had a minimum requirement of two guanines with a minimum binding stoichiometry of three base pairs. The relative affinities of the three base pair sites could be ordered, and the order of the sites was different for all three drugs. Identical three base pair binding sites in nonidentical locations had different affinities for chromomycin. At the higher concentrations, the number and location of sites bound by the three drugs were similar but not identical. Chromomycin and olivomycin which share common hexopyranoses were most similar.

MPE·Fe(II) footprinting is a rapid technique for assaying hundreds of potential DNA binding sites for antibiotics on one gel. This direct method should prove useful for identifying the relative affinities of multiple binding sites of other small molecules on the native nucleic acid template which will be necessary for any understanding of the molecular basis of drug action for DNA binding molecules.

Registry No. Chromomycin A_3, 7059-24-7; mithramycin, 18378-89-7; olivomycin, 6988-58-5.

References

Behr, W., Honikel, K., & Hartmann, G. (1969) *Eur. J. Biochem. 9*, 82.

Berlin, Y. A., Esipov, S. E., Kolosov, M. N., Shemyakin, M. M., & Brazhnikova, M. G. (1964) *Tetrahedron Lett. 21*, 1323.

Berlin, Y. A., Kiseleva, O. A., Kolosov, M. N., Shemyakin, M. M., Soifer, V. S., Vasina, I. V., Yartseva, I. V., & Kuznetsov, V. D. (1968) *Nature (London) 218*, 193.

Galas, D. J., & Schmitz, A. (1978) *Nucleic Acids Res. 5*, 3157.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., & Waring, W. J. (1981) *The Molecular Basis of Antibiotic Action*, p 285, Wiley, New York.

Gause, G. F. (1967) *Antibiotics* (Gottlieb, D., & Shaw, P. D., Eds.) Vol. 1, p 246, Springer-Verlag, New York.

Hartmann, G., Behr, W., Beissner, K.-A., Honikel, K., & Sippel, A. (1968) *Angew. Chem., Int. Ed. Engl. 7*, 693.

Haseltine, W. A., Lindan, C. P., D'Andrea, A. D., & Johnsrud, L. (1980) *Methods Enzymol. 65*, 235.

Hertzberg, R. P., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 313.

Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5314.

Kersten, H., & Kersten, W. (1974) *Mol. Biol., Biochem. Biophys. 18*, 86.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499.

Miyamoto, M., Morita, K., Kawamatsu, Y., Noguchi, S., Marumoto, R., Tanaka, K., Tatsuoka, S., Nakanishi, K., Nakadaira, Y., & Bhacca, N. S. (1964) *Tetrahedron Lett. 34*, 2355.

Prasad, K. S., & Nayak, R. (1976) *FEBS Lett. 71*, 171.

Remers, W. A. (1979) *The Chemistry of Antitumor Antibiotics*, Vol. 1, p 133, Wiley, New York.

Schmitz, A., & Galas, D. J. (1982) *Methods in DNA and RNA Sequencing* (Weismann, S., Ed.) Plenum Press, New York (in press).

Slavik, M., & Carter, S. K. (1975) *Adv. Pharmacol. Chemother. 12*, 1.

Tanaka, T., & Weisblum, B. (1974) *J. Bacteriol. 121*, 354.

Van Dyke, M. W., & Dervan, P. B. (1982) *Cold Spring Harbor Symp. Quant. Biol.* (in press).

Van Dyke, M. W., Hertzberg, R. P., & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5470.

Ward, D. C., Reich, E., & Goldberg, I. H. (1965) *Science (Washington, D.C.) 149*, 1259.

Waring, M. J. (1970) *J. Mol. Biol. 54*, 247.