Methidiumpropyl-EDTA·Fe(II) and DNase I footprinting report different small molecule binding site sizes on DNA

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ABSTRACT

　　　DNase I and MPE·Fe(II) footprinting both employ partial cleavage of ligand-protected DNA restriction fragments and Maxam-Gilbert sequencing gel methods of analysis. One method utilizes the enzyme, DNase I, as the DNA cleaving agent while the other employs the synthetic molecule, methidium-propyl-EDTA (MPE). For actinomycin D, chromomycin A$_3$ and distamycin A, DNase I footprinting reports larger binding site sizes than MPE·Fe(II). DNase I footprinting appears more sensitive for weakly bound sites. MPE·Fe(II) footprinting appears more accurate in determining the actual size and location of the binding sites for small molecules on DNA, especially in cases where several small molecules are closely spaced on the DNA. MPE·Fe(II) and DNase I report the same sequence and binding site size for lac repressor protein on operator DNA.

INTRODUCTION

　　　A rapid, direct method for determining the location and size of the binding sites of proteins on heterogeneous DNA is DNase I footprinting, which combines DNase I cleavage of protein-protected DNA fragments and Maxam-Gilbert sequencing gel method of analysis.(1,2) This useful DNA cleavage inhibition pattern technique relies on the relatively low sequence specificity of the enzyme DNase I in a partial DNA cleavage reaction and the ability of the 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 sequencing gel revealing the position and extent of the protein binding site.(1,2)

　　　Many small molecules, such as drugs useful in antibiotic, antiviral, and antitumor chemotherapy bind double helical nucleic acid in a sequence specific fashion at sites typically two to four base pairs in size.(3) Some small molecules, such as bleomycin, chemically modify DNA which allows identification of specific binding sites on heterogenous DNA from DNA cleavage patterns on Maxam-Gilbert sequencing gels (4-6). However, many DNA binding molecules do not modify nucleic acids and our understanding of their sequence

preferences has been limited to comparison of binding isotherms obtained by spectrophotometric analyses of drug binding to homopolymer and copolymer nucleic acids.(3) One direct method reported for determining the binding sites of small molecules with binding site sizes in the range of three to four base pairs on heterogeneous DNA is MPE·Fe(II) footprinting.(7-9)

Methidiumpropyl-EDTA (MPE), which contains the DNA intercalator, methidium, covalently bound by a short hydrocarbon tether to the metal chelator, EDTA, efficiently produces single strand breaks in double helical DNA in the presence of Fe(II) and O_2. (Figure 1) (10) Importantly, MPE·Fe(II) is a relatively non-sequence specific DNA cleaving agent with lower sequence specificity than DNase I.(7-9) Using MPE·Fe(II) footprinting, the binding sites of actinomycin, netropsin, distamycin, chromomycin, mithramycin, and olivomycin over a range of binding densities on some DNA restriction fragments have been determined.(7-9) The DNA cleavage inhibition patterns (footprints) on opposite strands are asymmetric, shifted at least one base pair to the 3' side of the presumed drug binding site.(8,9)

Recently, DNase I has been shown capable of generating DNA cleavage inhibition patterns with actinomycin and distamycin at very low binding densities.(11) The question arises whether MPE·Fe(II) and DNase I footprinting report the same information. It is not obvious whether the synthetic MPE·Fe(II) and the enzyme DNase I are equally sensitive to the same phenomena. They are common because they are both DNA cleaving reagents. However, they differ in size, mechanism of cleavage, and level of sequence neutrality. We report here a comparison between MPE·Fe(II) and DNase I footprinting patterns generated on DNA fragments protected by actinomycin D, chromomycin A_3 and distamycin A.(Figure 2) Actinomycin and chromomycin bind DNA preferentially at guanine rich sequences with binding site sizes of 3-5 base pairs.(7-9,13-21) Distamycin binds DNA preferentially at A+T rich
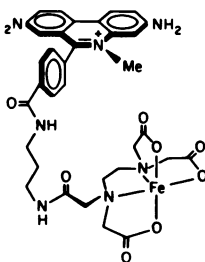

Figure 1: Methidiumpropyl-EDTA·Fe(II).

sequences with a binding site size of 4-5 base pairs.(7,8,22-24) DNase I treatment of a DNA restriction fragment containing the _lac_ operator sequence protected by _lac_ repressor protein has been shown to afford a footprint 23 base pairs in size.(1) For comparison an MPE·Fe(II) footprint of the protein _lac_ repressor on operator DNA is included.

MATERIALS AND METHODS

Drugs: Actinomycin D was obtained from Merck, Sharp and Dohme. Chromomycin A_3 was obtained from Calbiochem. Distamycin A was obtained from Boehringer Mannheim. MPE was synthesized and purified as described by Hertzberg and Dervan.(10) Purities were determined by thin layer chromatography. Concentrations were determined spectroscopically.

Proteins: _Lac_ repressor, a gift from R. E. Dickerson (UCLA), was supplied as a 9.35 mg protein/ml solution in a K_2PO_4 (pH 7.4)/glucose buffer and stored at -70^oC until immediately before use. Deoxyribonuclease 1 (DNase I), isolated from bovine pancreas, was obtained from Worthington (code: DPFF).
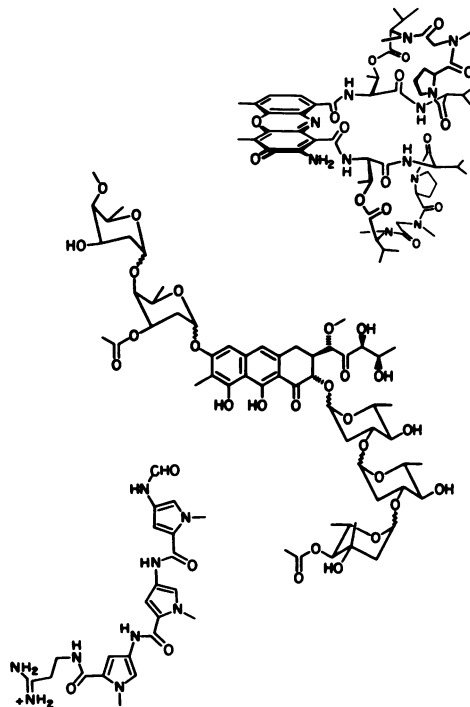

Figure 2: Top to Bottom: actinomycin, chromomycin, distamycin.

DNase I was prepared as a 1 mg/ml stock solution in 0.15 M NaCl, stored at −20°C and diluted to working concentrations immediately before use. Restriction endonucleases and the Klenow fragment of DNA polymerase 1 were from New England Biolabs. Bacterial alkaline phosphatase and T4 kinase were from Bethesda Research Laboratories.

Buffers: MPE cleavage reactions were done in a buffer (TN) containing 10 mM Tris, pH 7.4 and 50 mM NaCl. DNase I digestions were done in a buffer (TKMC) containing 10 mM Tris pH 7.9, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

DNA Fragments: A 381 base pair DNA restriction fragment was isolated from pBR322. Superhelical pBR322 plasmids were first digested with the restriction endonuclease Bam H1 and then 3' end labeled with α−^{32}P dATP and the Klenow fragment of DNA polymerase 1. Similarly, the Bam H1 restriction fragment was treated with bacterial alkaline phosphatase and then 5' end labeled with γ−^{32}P dATP and T4 kinase. A second enzymatic digest with the restriction endonuclease Eco RI yielded either the 3' (or 5') end labeled 381 bp DNA fragment which was isolated and eluted from polyacrylamide gels by known procedures.[12] A 117 base pair DNA fragment containing a sequenced segment of the lactose operon in E. coli was isolated from the plasmid pLJ3.[25] Superhelical pLJ3 plasmids were first digested with Eco RI, then 3' end labeled with α−^{32}P and the Klenow fragment of DNA polymerase 1. Digestion of this material with Hae III yielded the singularly 3' end labeled 117 base pair fragment used for lac repressor binding.

MPE Footprinting: To 8 µl of a solution containing 1.25 x TN buffer, 250 µM base pairs DNA (primarily deproteinized calf thymus with sufficient 3' ^{32}P end-labeled fragment for autoradiography), and inhibiting drug (either actinomycin D, chromomycin A_3/$MgCl_2$, distamycin A; see figures 3 and 4 for concentrations) was added 1 µl of a 100 µM MPE·200 µM Fe$(NH_4)_2(SO_4)_2$ solution. This was allowed to equilibrate at 37°C for 30 mins. Addition of 1 µl of 40 mM dithiothreitol (DTT) initiated the cleavage reaction which was allowed to continue at 37°C for 15 min before stopping by freezing in dry ice. Samples were then lyophilized and resuspended in a formamide loading buffer. In the case of the 5' ^{32}P end labeled fragment the final DNA concentration was 400 µM in base pairs. For the lac repressor binding, the final DNA concentration was 100 µM base pairs and equilibration was at room temperature for 10 mins.

DNase I Footprinting: A 9 µl solution containing 1.11 x TKMC buffer, 222 µM base pairs DNA, and inhibiting drug is allowed to equilibrate for 30 mins at 37°C. Cleavage is initiated by the addition of 1 µl of 50 µg/ml DNase

I and 1 mM DTT solution and allowed to react for 30 sec at room temperature before quenching with 2.5 μl of a DNase stop solution (3 M NH₄OAc and 0.25 M EDTA). This was then precipitated with ethanol, lyophilized, and resuspended in a formamide loading buffer. For lac repressor binding, DNase I reactions followed the conditions described by Galas and Schmitz.(1)

Sequencing Gels: Resolution of inhibition patterns was achieved by electrophoresis on 0.4 mm thick, 40 cm long, 8% polyacrylamide, 1:20 crosslinked sequencing gels containing 50% urea. Electrophoresis was carried out at 1000V for 3.5 hours to sequence 100 nucleotides, beginning 20 nucleotides from the 3' (or 5') end-labeled end. Autoradiography was carried out at -50°C without the use of an intensification screen.

Densitometry: An 8x10" copy of the original autoradiogram was scanned at 485 nm with the incident beam collimated to a width of 0.2 mm on a Cary 219 spectrophotometer. The data was recorded as absorbance relative to the film base density and analyzed using an Apple microcomputer.

RESULTS

MPE·Fe(II) Cleaves DNA with Lower Sequence Specificity than DNase I

MPE·Fe(II) cleavage of a 381 base pair DNA restriction fragment labeled at either the 3' or 5' end with ^{32}P affords a relatively uniform DNA cleavage pattern on a Maxam-Gilbert sequencing gel. (Figures 3 and 4, lane 2) Densitometry reveals that the variation in average peak height is modest throughout the entire scan. Although MPE·Fe(II) cleavage of DNA is relatively non-specific, the reagent is not sequence neutral. DNase I exhibits a higher sequence specificity than MPE·Fe(II) as seen in both autoradiograms (Figures 3 and 4, lane 3). DNase I cleavage specificity over the span of a few base pairs can range one order of magnitude in absorbance intensity in densitometer scans.

Footprinting at Low Drug Binding Densities

Actinomycin D, chromomycin A₃ and distamycin A were equilibrated at low concentrations with the 381 base pair DNA restriction fragment (0.06 drug/DNA base pairs) followed by partial cleavage with MPE·Fe(II) or DNase I. The autoradiograms of the DNA cleavage inhibition patterns are shown in Figures 3 and 4. From densitometric analyses, the footprints on 70 base pairs of the 381 bp DNA fragment are shown in Figure 5. For actinomycin D, DNase I affords a footprint 6 to 12 base pairs in size while MPE·Fe(II) does not detect a strongly bound drug site. For chromomycin, DNase I reports three footprints while MPE·Fe(II) reports five which are smaller in size. For
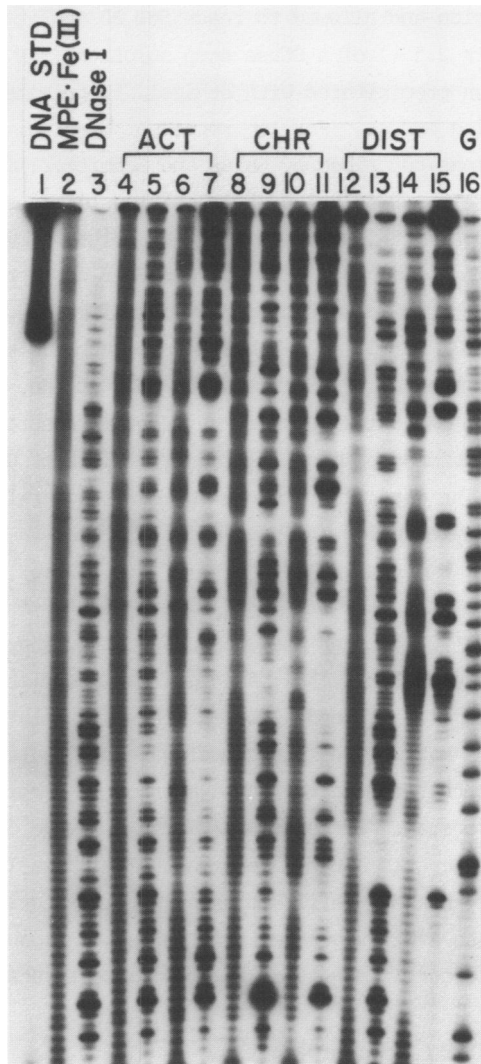
Figure 3: Autoradiogram of 3' end labeled 381 DNA fragment: Lane 1 contains the intact DNA. Lane 16 is the Maxam-Gilbert G lane. All other even-numbered lanes (2,4,6,8,10,12,14) contained 10 mM Tris pH 7.4, 50 mM NaCl, 200 μM base pairs DNA, 4 mM DTT and 10 μM MPE·Fe(II) with final concentrations in a 10 μl reaction volume. All other odd-numbered lanes (3,5,7,9,11,13,15) contained 10 mM Tris pH 7.9, 10 mM KCl, 10 mM MgCl$_2$, 5 mM CaCl$_2$, 200 μM base pairs DNA, 0.1 mM DTT, and 50 pg DNase I final concentrations in a 10 μl reaction volume. Inhibiting drugs in these reactions were: lanes 4 and 5, 12.5 μM actinomycin D; lanes 6 and 7, 50 μM actinomycin D; lanes 8 and 9, 12.5 μM chromomycin A$_3$ and 25 μM MgCl$_2$; lanes 10 and 11, 50 μM chromomycin A$_3$ and 100 μM MgCl$_2$; lanes 12 and 13, 12.5 μM distamycin A; lanes 14 and 15, 50 μM distamycin A.
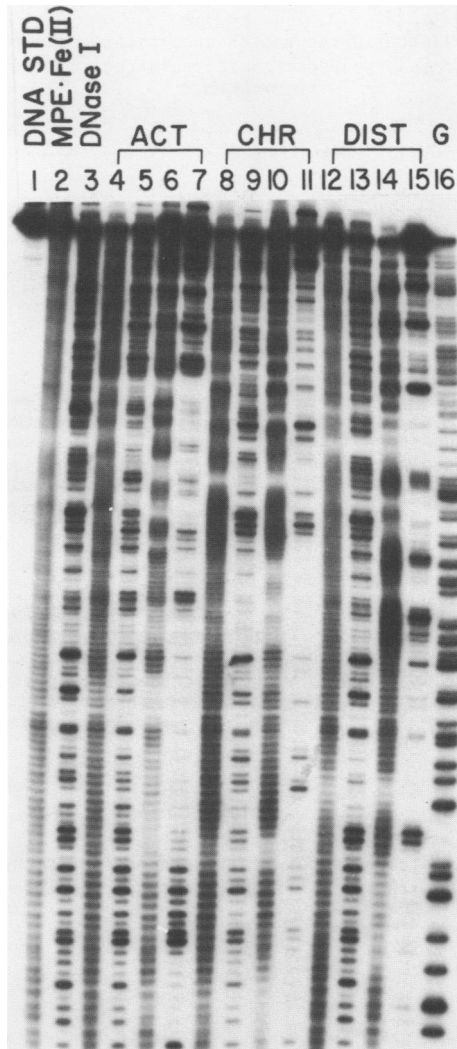
Figure 4: Autoradiogram of 5' end labeled 381 DNA fragment: Lane 1 contains
the intact DNA. Lane 16 is the Maxam-Gilbert G lane. All other even-numbered
lanes (2,4,6,8,10,12,14) contained 10 mM Tris pH 7.4, 50 mM NaCl, 400 µM base
pairs DNA, 4 mM DTT and 10 µM MPE·Fe(II) with final concentrations in a 10 µl
reaction volume. All other odd-numbered lanes (3,5,7,9,11,13,15) contained 10
mM Tris pH 7.9, 10 mM KCl, 10 mM MgCl₂, 5 mM CaCl₂, 400 µM base pairs DNA, 0.1
mM DTT, and 50 pg DNase I final concentrations in a 10 µl reaction volume.
Inhibiting drugs in these reactions were: lanes 4 and 5, 25 µM actinomycin D;
lanes 6 and 7, 100 µM actinomycin D; lanes 8 and 9, 25 µM chromomycin A₃ and
50 µM MgCl₂; lanes 10 and 11, 100 µM chromomycin A₃ and 200 µM MgCl₂; lanes 12
and 13, 25 µM distamycin A; lanes 14 and 15, 100 µM distamycin A.

Actinomycin D

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5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Actinomycin D

```
5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Chromomycin A₃

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5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Chromomycin A₃

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5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Distamycin A

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5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Distamycin A

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5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
        340·                        320·                          300·                     280·
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA
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Figure 5: MPE·Fe(II) and DNase I footprints on both strands of 70 nucleotides of the 381 bp DNA fragment corresponding to bottom to middle of autoradiograms in Figs. 3 and 4. The DNase I footprints are shown as light and dark bars due to partial and complete cleavage inhibition, respectively. The MPE·Fe(II) footprints are shown as histograms. Bottom strand footprints are from Figure 3. Top strand footprints are from Figure 4. Two binding densities are shown for each inhibiting drug; top is 0.06 drug/base pair, bottom is 0.25 drug/base pair.
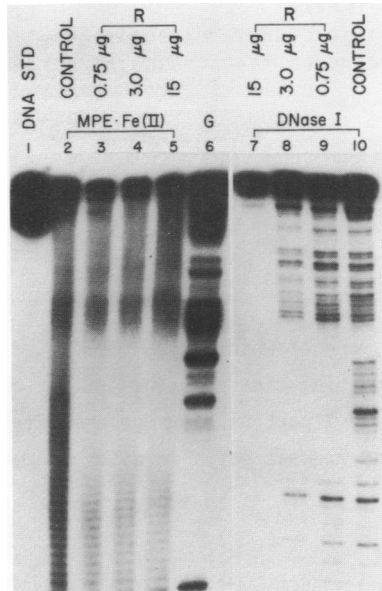
Figure 6: Autoradiogram of lac repressor-operator footprint. Lane 1
contained the intact 117 bp DNA fragment. Lane 6 is the Maxam-Gilbert G lane.
Lanes 2-5 contained 10 mM Tris Ph 7.4,50 mM NaCl, 100 μM base pairs DNA, 4 mM
DTT, and 10 μM MPE·Fe(II) at final concentration in 10 μl reaction volumes.
Lanes 7-10 contained 10 mM Na cacodylate pH 8, 10 mM MgCl$_2$ 5 mM Cacl$_2$, 10 μM
base pairs DNA, 0.1 mM DTT, and 10 pg DNase I final concentrations in 100 μl
reaction volumes. The amount of lac repressor present in each reaction was
none (lanes 2 and 10), 0.75 μg (lanes 3 and 9), 3 μg (lanes 4 and 8), and
15 μg (lanes 5 and 7).

distamycin A, the single binding site detected by DNase I is 9 base pairs in
size whereas the MPE·Fe(II) footprint is 5 base pairs in size (Figure 8).
Footprinting at High Drug Binding Densities
        The three drugs were allowed to equilibrate with the same DNA
restriction fragment at higher concentrations (0.25 drug/DNA base pairs)
followed by partial cleavage with MPE·Fe(II) or DNase I. The autoradiograms
of the corresponding footprints are shown in Figures 3 and 4. From
densitometric analyses, the footprints on 70 base pairs of the 381 bp DNA
fragment are shown in Figure 5. For actinomycin D, MPE·Fe(II) partial
cleavage reveals six footprints 2-5 base pairs in size. DNase I partial
cleavage exhibits three footprints, two 5-6 base pairs and one 36 base pairs
in size which encompasses three of the discrete MPE·Fe(II) footprints. For
chromomycin A$_3$, MPE·Fe(II) partial cleavage reveals seven footprints. DNase I

0.75 µg lac repressor

5' ATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAG 3'
3' TATTACACACCTTAACACTCGCCTATTGTTAAAGTGTGTC 5'

Figure 7: MPE·Fe(II) and DNase I footprint of lac repressor (7.5 µg/ml) on 40 nucleotides of the 117 bp DNA fragment (Figure 6, lanes 3 and 9). The DNase I footprints are shown as a dark bar. The MPE·Fe(II) footprints are shown as histograms.

reports four footprints, one of which is 36 base pairs in size. For distamycin, MPE·Fe(II) partial cleavage reveals four discrete footprints 5-6 base pairs in size. DNase I partial cleavage exhibits three footprints, one 7 base pairs and two 16 and 25 base pairs in size, respectively.(Figure 8)

Actinomycin

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

Chromomycin

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

Distamycin

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

5'CGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGT
3'GCGCATCAGCTATCACCGAGGTTCATCGCTTCGCTCGTCCTGACCCGCCGCCGGTTTCGCCAGCCTGTCA

Figure 8: Illustration of MPE·Fe(II) footprints (boxes) and DNase I footprints (brackets) from Figure 5.

MPE·Fe(II) Footprint of the lac Repressor

        For comparison the characterized lac repressor-operator system was
examined by both footprinting methods.(Figure 6) A 3' ^{32}P end labeled 117
base pair DNA restriction fragment containing one copy of the UV-5 lac operon
mutation, preequilibrated with several concentrations of the lac repressor
protein (0.75 to 15 µg), were subjected to either MPE·Fe(II) or DNase I
partial cleavage. Identical footprinting patterns are observed by both
methods at the low lac repressor binding levels (0.75 and 3 µg) as seen in
Figure 7. At 15 µg lac repressor DNase I cleavage is sufficiently inhibited
such that a discrete footprint is no longer visible (Figure 6, lane 7).
MPE·Fe(II) footprints lac repressor throughout the concentration range tested.

DISCUSSION

        There are significant differences between MPE·Fe(II) and DNase I as
DNA cleaving agents for determining the sequence specific binding of small
molecules to native DNA. In the case of actinomycin at low binding density,
DNase I footprinting appears more sensitive. The sensitivity of DNase I for
weakly bound sites may be due to differences in the binding affinities of
DNase I and MPE·Fe(II) to DNA. However, MPE·Fe(II) footprinting appears more
accurate in determining the actual size and location of binding sites for
small molecules on DNA, especially in cases where several drugs are closely
spaced on DNA. This is implied by the consistently smaller and discrete
footprints observed with MPE·Fe(II) cleavage which more closely resemble the
expected locations and binding site sizes for actinomycin D, chromomycin A_3
and distamycin A from equilibrium binding studies.(3) Direct evidence
supporting this is obtained from DNA cleavage patterns generated from cleavage
of ^{32}P labeled DNA restriction fragments with distamycin-EDTA·Fe(II) and EDTA-
distamycin·Fe(II) which indicate that the binding site size of distamycin is
4 base pairs.(26) In addition, MPE·Fe(II) footprinting of distamycin on these
same DNA fragments reveals DNA cleavage inhibition patterns at identical sites
of similar size.(27) With multiple bound drugs that are closely spaced on
DNA, DNase I footprinting affords large regions of cleavage inhibition making
accurate site and size determinations impossible.
        The difference in the size of the footprints for drug binding sites
generated by DNase I and MPE·Fe(II) may be a reflection of the differences in
size of the DNA cleaving agents. MPE, an intercalator, is significantly
smaller than DNase I, a high molecular weight protein. One might imagine that
the catalytic site on the enzyme might not be accessible to the unprotected

base pairs immediately flanking the bound drug in the minor groove of DNA affording a slightly larger footprint. MPE·Fe(II) presumably binds the unprotected DNA sites by intercalation. The cleavage event, an oxidative degradation of the deoxyribose ring, appears to be mediated by a localized concentration of a short lived diffusible active oxygen species near the DNA backbone.(10,26,28) Therefore, MPE·Fe(II) footprints might simply represent regions of the DNA where a bound molecule directly inhibits intercalation by MPE. Assignment of the drug binding site size from MPE·Fe(II) footprinting is based on a model where the DNA cleavage inhibition pattern is shifted 1-2 base pairs on the 3' side and 1 base pair underprotected on the 5' side of the DNA.(8,9)

DNase I is known to be sensitive to DNA structure.(29) An alternative explanation for the larger binding-site sizes for DNase I footprinting is that DNase I cleavage is inhibited (or enhanced) by altered DNA structure.(11) If this is true and assuming that MPE·Fe(II) footprints represent regions of direct inhibition, the coupled uses of MPE·Fe(II) and DNase I footprinting may provide a method for determining the extent and sequence dependence of altered DNA structure induced by small molecules at specific sites on DNA.

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