DNA Sequence Selectivities in the Covalent Bonding of Antibiotic Saframycins Mx1, Mx3, A, and S Deduced from MPE·Fe(II) Footprinting and Exonuclease III Stop Assays†

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ABSTRACT: DNA sequence selectivities in the covalent binding of the antitumor antibiotic saframycins Mx1, Mx3, A, and S have been determined by complementary strand MPE·Fe(II) footprinting and exonuclease III stop assays on two different 545 and 135 base pair long *Hin*dIII/*Rsa*I restriction fragments of pBR322 DNA. Saframycins Mx1, Mx3, A, and S recognize primarily 5′-GGG sequences. All four antibiotics also recognize 5′-GGPy sequences, however a cytosine is preferred over a thymine at the 3′-end of this recognition site in all cases. Saframycins Mx1, Mx3 and S, which possess the OH leaving group, also recognize the 5′-CCG sequence, in contrast to saframycin A, which contains the CN leaving group. In contrast, the OH-containing saframycins also recognize the 5′-CTA sequence. Saframycins Mx2, B and C, which lack the critical CN or OH leaving group, do not show any footprints on the restriction fragments examined in this study. The measured binding site size for all four antibiotics is three base pairs. The exonuclease III stop assay independently confirmed the formation of a covalent bond and the strong preference of the antibiotics for 5′-GGG and 5′-GCC sequences. The latter enzyme assay also suggests that the 5′-terminal or central G of the triad binding site is the base to which reversible covalent attachment of the antibiotic takes place.

The saframycins (Figure 1) are heterocyclic quinonoid antitumor antibiotics (Arai et al., 1977, 1978, 1979, 1980a,b; Yazawa et al., 1982, 1986; Mikami et al., 1988) related structurally to other quinone natural products including the safracins (Ikeda et al., 1983), mimosamycin (Arai et al., 1979), mitomycins (Szybalski & Iyer, 1967), streptonigrin (Rao et al., 1963), renieramycins (McIntyre et al., 1979; Finke & Faulkner, 1982), naphthyridinomycin (Kluepfel et al., 1975), and cyanocycline A (Hayashi et al., 1982). The saframycins A, B, C, R, and S are isolated from the streptothricin-producing strain *Streptomyces lavendulae* (Arai et al., 1977, 1978, 1979, 1980a,b), and, more recently, saframycins Mx1, Mx2, and Mx3 (Figure 1) have been isolated from *Myxococcus xanthus* (Trowitzsch-Kienast et al., 1988).

The saframycins generally exhibit wide-spectrum antibiotic activity especially against Gram-positive bacteria while saframycins A and S [a possible precursor of all saframycins (Arai et al., 1977, 1979, 1980a)] exhibit anticancer activity against Erhlich ascites tumor, B16 melanoma, and murine leukemias P388 and L1210 (Arai et al., 1978, 1980b; Ishiguro et al., 1981).

The saframycins inhibit both DNA and RNA synthesis and a reducing cofactor, e.g. dithiothreitol (DTT), is required in the cytotoxic action as well the DNA covalent-binding properties of saframycins A, S (Ishiguro et al., 1981), Mx1, and Mx3 (vide infra). Our previous mode of action studies with saframycins A and S revealed three types of binding of the saframycins to DNA: (i) a reversible noncovalent interaction, (ii) a reversible covalent binding preferentially at G sites within the minor groove that is acid promoted, and (iii) a major mode consisting of a reversible binding at G-2-NH_2 within the minor groove which is promoted by reducing cofactors (Lown et al., 1982; Rao & Lown, 1990).

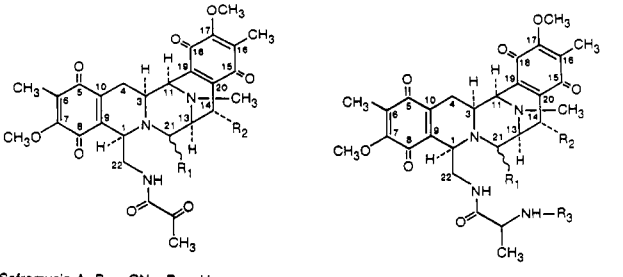

FIGURE 1: Structures of saframycin antitumor antibiotics.

Covalent binding of an intermediate iminium species generated by reduction promoted loss of a leaving group (CN^- for saframycin A; OH in the case of saframycins S, Mx1, and Mx3) forming an aminal link at G-2-NH_2 is the principal mode of binding (Lown et al., 1982).

In addition to these three distinct interactions with DNA, there exists a pathway involving redox cycling of the quinone moieties of the antibiotics leading to the generation of reactive oxygen species which mediate single-strand scission of DNA (Lown et al., 1982). This latter mechanism has been implicated in the antibiotic action of those saframycins (including B and C) that are incapable of covalent binding to DNA (Arai et al., 1977, 1979, 1980a).

The major reductant-promoted reversible covalent binding of saframycins to both native and synthetic DNAs showed a distinct preference for binding at GC base sequences. MPE·Fe(II) [methidium-propyl-EDTA·Fe(II)] complementary strand footprinting of saframycins A and S on an *Eco*RI/*Hin*dIII restriction fragment of pBR322 DNA revealed binding locations common to saframycins A and S to be GGGG, CCCC, GCC, and ACC (underlined bases are

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shared by two adjacent binding sites) (Rao & Lown, 1990). Some differences in binding affinity were apparent for the two antibiotics at individual sites. Because of the possible relationship between binding site preference and cytotoxic action of these agents, we now report an examination of the DNA sequence specificities of saframycins Mx1, Mx2, Mx3, A, and S by MPE·Fe(II) complementary strand footprinting on different restriction fragments but also employing an exonuclease III stop assay.

MATERIALS AND METHODS

Saframycins A and S were kindly supplied by Professor T. Arai of Chiba University, Chiba, Japan, and saframycins Mx1, Mx2, and Mx3 were kindly furnished by Professor G. Hofle, Braunschweig, Germany.

Biochemicals. The pBR322 and sonicated calf thymus (ct) DNAs and restriction enzymes *Hin*dIII/*Eco*RI were obtained from Pharmacia P.L. Biochemicals. All were used without further purification. The T4 polynucleotide kinase, Klenow fragment (large fragment of DNA polymerase I), and urea were from Bethesda Research Labs. Dithiothreitol (DTT) and calf intestine phosphatase (CAP) were obtained from Calbiochem. Acrylamide, bromophenol blue, and xylene cyanol were from Serva. [γ-^{32}P]ATP and [α-^{32}P]dATP were purchased from New England Nuclear. All other reagents were of analytical grade. MPE was a gift from Professor P. B. Dervan (California Institute of Technology). Exonuclease III was purchased from New England Biolabs.

Footprinting Experiments. pBR322 DNA was cut with *Hin*dIII and labeled either at the 5′-end using [^{32}P]ATP and T4 polynucleotide kinase or at the 3′-end using [^{32}P]dATP and the Klenow fragment. Then the labeled DNA was further digested with *Rsa*I. The fragments were separated by preparative nondenaturating polyacrylamide gel electrophoresis. The 545 and 135 base pair long labeled fragments were recovered and used in footprinting and exonuclease stop assays.

Footprinting Procedures. The footprinting reactions were performed in the presence of sonicated calf thymus DNA, either 5′- or 3′-labeled DNA, DTT, and the saframycin (not present in control) in 1 mM Tris and 20 mM NCl buffer, pH 7.4. After equilibrating the antibiotic–DNA reaction mixtures in the presence of DTT for 40 min at 37 °C, freshly made MPE·Fe(II) was added to each reaction tube. The reaction mixtures contained 100 μM DNA, 10 mM Tris, 20 mM NaCl, 10 μM MPE·Fe(II), 9.5 mM DTT, and 8, 16, or 78 μM of the antibiotic. Reactions were run at room temperature for 15 min and then stopped by freezing at −70 °C. The solutions were then lyophilized.

Exonuclease III Stop Assay. Exonuclease stop assay was performed in 20 mM Tris·HCl, pH 7.4, 10 mM KCl, 0.1 mM EDTA, and 6 mM $MgCl_2$ buffer. DNA and antibiotic concentrations were maintained as in the case of footprinting assay. After incubating the 5′-end-labeled DNA with antibiotic for 40 min at 37 °C, 2 units of exonuclease III was added to each tube and further incubated for 15 min at 37 °C. Then the reaction mixtures were extracted with phenol and chloroform and precipitated with ethanol and dried. The dry DNA samples from either of the above procedures were suspended in formamide loading buffer for gel electrophoresis. Electrophoresis was performed on 0.4 mm thick, 55 cm long, 6% polyacrylamide and 7 M urea denaturing gels at 55 °C and 1900 V. The gel was transferred on to a filter paper, dried on a Bio-Rad Model 483 slab dryer, and autoradiographed at −70 °C using Kodak X-Omat AR film. The resulting autoradiograms were scanned on a LKB Ultroscan XL laser densitometer.

The densitometric data were corrected for the background absorbance of the film (<0.4 absorbance). In order to compensate for possible variations in extent of reaction, all band intensities within a lane were normalized to a band which is unaffected in the presence of compound in that lane. The extent of protection from cleavage for each base was determined by

$$\% \text{ protection} = [1 - (A_{\text{ligand+DNA}}/A_{\text{DNA}})] \times 100$$

where $A_{\text{ligand+DNA}}$ is the absorbance of a band obtained from cleavage in the presence of added ligand and A_{DNA} is the absorbance of the same band in the absence of ligand (control lane). Negative values for protection correspond to cleavage enhancement in the presence of ligand.

RESULTS

Saframycins Mx1, Mx2, Mx3, A, and S have been studied for their sequence-selective bonding to DNA on two 545 and 135 base pair long *Hin*dIII/*Rsa*I restriction fragments of pBR322 DNA. The MPE·Fe(II) footprinting technique is utilized to locate the binding regions of the antibiotics and the exonuclease III stop assay used to locate the probable reactive base in the binding site on DNA.

MPE·Fe(II) Footprinting. MPE·Fe(II) footprinting studies were carried out on two restriction fragments of 545 and 135 base pairs long, obtained upon digestion with *Hin*dIII/*Rsa*I from pBR322 DNA to identify the preferred binding sites of saframycin antibiotics. The experimental conditions to evoke the reaction between saframycins and DNA were reported recently (Rao & Lown, 1990).

The footprints were obtained in the range of r = 0.08–0.8. The autoradiograms of the MPE·Fe(II) footprinting assay on both 3′- and 5′-end-labeled strands of 545 mer are shown in Figures 2 and 3. About 120 base pairs from 4360 to 4240 [numbering corresponds to pBR322 sequence (Peden, 1983)] are analyzed. Decreased intensity regions in the saframycins- or distamycin-containing lanes relative to the control lane, without ligand, are the result of protection of DNA from MPE·Fe(II) cleavage due to binding of the ligands to DNA. Densitometric analysis of the patterns of each lane permitted estimation of the location, approximate binding site size, and relative strength of the binding of the antibiotics to DNA. The data are presented in the form of a histogram in Figure 4.

It is evident from the autoradiograms that the saframycins bind in different locations than the known AT sequence recognizing distamycin A. This study further confirms the minor differences in the sequence recognition of saframycins A and S. The new saframycins Mx1 and Mx3, which possess the hydroxyl active group like saframycin S, give similar sequence-recognition patterns as saframycin S. In contrast, saframycin Mx2, in which OH group is acetylated, does not show binding to DNA.

Saframycins Mx1, Mx3, and S show footprints mainly in the five regions at 4247–4261 (all sequences from 5′ to 3′) (ATAGGGGTTCCGCGC), 4266–4274 (TACCCCGAA), 4277–4287 (GTGCCACCTGA), 4330–4336 (TAGGCGT), and 4342–4349 (GAGGCCCT). These compounds also have secondary or weak binding sites in three locations at 4289–4294 (GTCTAA), 4297–4301 (AACCA), and 4318–4322 (AACCT). Saframycin A, with a different active group CN, also shared all the strong and weak binding sites of the former saframycins with the exclusion of a secondary site at 4289–

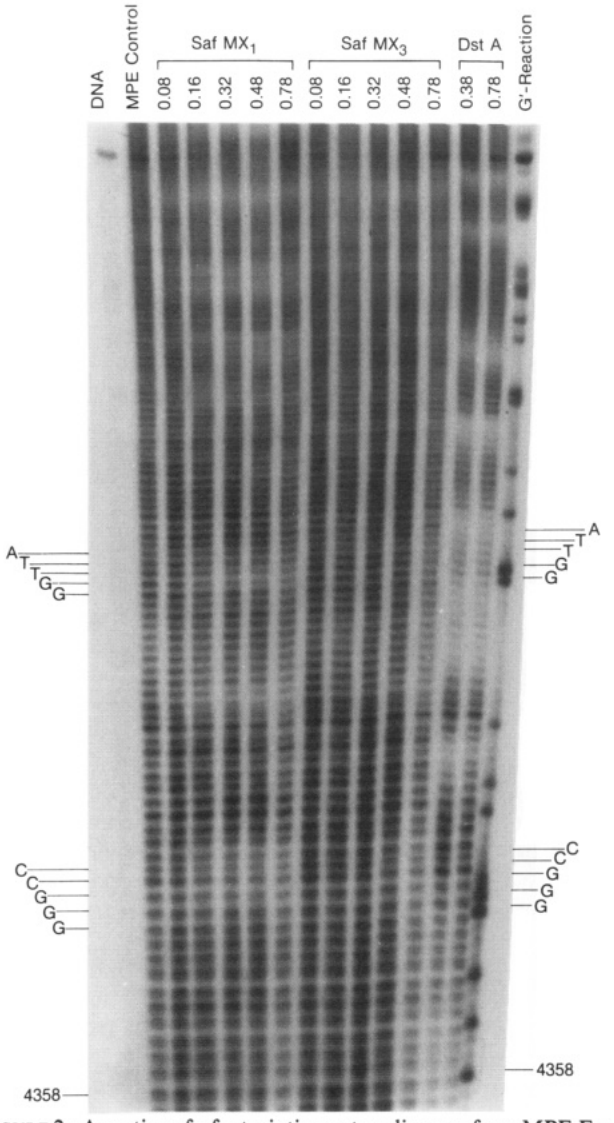

FIGURE 2: A portion of a footprinting autoradiogram from MPE·Fe(II) cleavage of 5′-^{32}P end-labeled *Hin*dIII/*Rsa*I restriction fragment. The lane labeled DNA contains intact DNA fragment. Control lane is MPE·Fe(II) cleavage in the absence of antibiotics. The other lanes contain the antibiotics as labeled and the numbers indicate antibiotic to DNA base pair ratio. G′-rxn lane is the Maxam–Gilbert guanine-specific sequencing reaction.

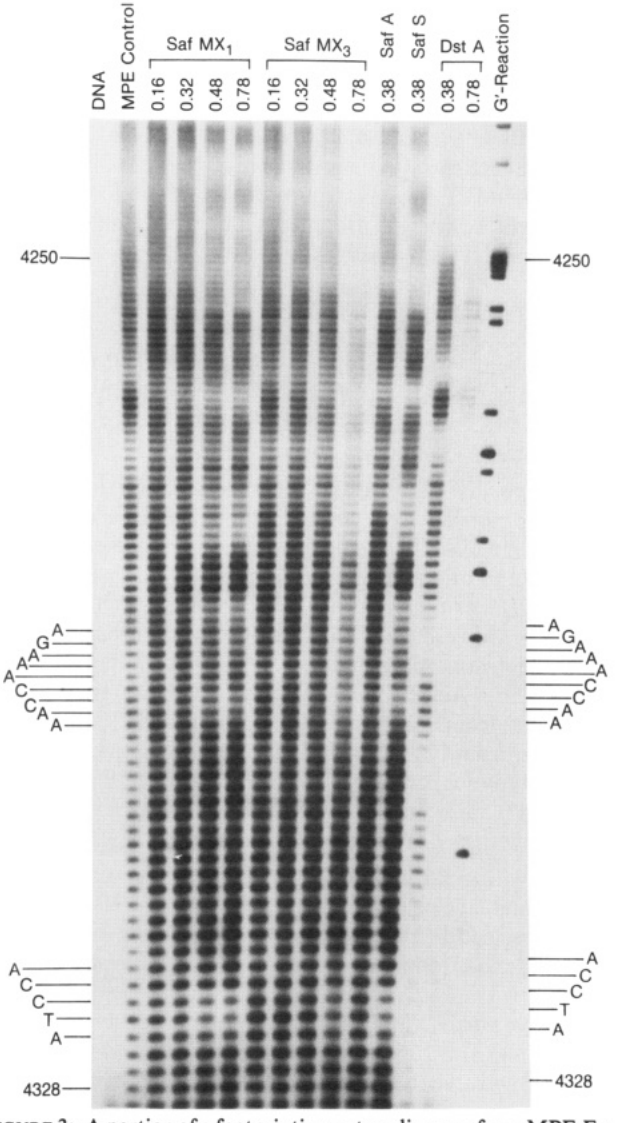

FIGURE 3: A portion of a footprinting autoradiogram from MPE·Fe(II) cleavage of 3′-^{32}P end-labeled *Hin*dII/*Rsa*I restriction fragment. The lane labeled DNA contains intact DNA fragment. Control lane is MPE·Fe(II) cleavage in the absence of antibiotics. The other lanes contain the antibiotics as labeled and the numbers indicate antibiotic to DNA base pair ratio. G′-rxn lane is the Maxam–Gilbert guanine-specific sequencing reaction.

4294. The minor groove binding ligand distamycin A binds to entirely different sequences compared with the saframycin antibiotics. The binding regions of distamycin A are 4264–4368 (ATTTC), 4272–4277 (GAAAAG), 4291–4298 (CTAAGAAA), 4302–4309 (TTATTATC), 4312–4333 (GACATTAACCTATAAAAATAGG), and 4335–4339 (GTATC). The binding site size and location are determined on the basis of the position of maxima of the asymmetric inhibition patterns of the antibiotics on complementary strands (Harshman & Dervan, 1985).

MPE·Fe(II) footprinting on 135 mer of pBR322 DNA obtained upon digestion with *Hin*dIII/*Rsa*I does not show many binding sites (figure not shown) for these antibiotics except a moderately strong binding sequence at bases 73–85 (TCAGGCACCGTGT) and a strong footprint at bases 116–134 (CTCGGCACCGTCACCCTGG). The probable binding sites are represented in histograms in Figure 5. Though several consecutive GC base pairs are available on both the fragments, none of these sites are recognized by these antibiotics.

Substantial MPE·Fe(II) cleavage enhancement is observed on both the strands adjacent to the binding sites as a result of binding of the antibiotics to DNA. The cleavage enhancement regions are shown in Figures 4 and 5. Typical asymmetric shifts of the footprints with 3′-skewing are obtained with all the antibiotics studied. This is probably an indication of location of the ligands in the minor groove of the DNA and/or the nature of the MPE·Fe(II) cleaving agent.

Exonuclease III Stop Assay. Exonuclease III is a processive enzyme that cleaves DNA from the 3′-end until the molecule is digested to the mononucleotide level. However, when DNA is modified by covalently reacting agents at one of the bases, the enzyme digestion is stalled three to six bases 3′ to the modified site. Employing this procedure allowed the base and sequence specificity of some DNA-modifying agents or antibiotics including anthramycin (Walter et al., 1988), CC-1065, and cis- and trans-DPP (Tullius & Lippard, 1980; Royer-Pokodo et al., 1981) to be reported. Exonuclease III stop assay alone does not definitively identify the modified base since the enzyme digestion is always stalled three to six base pairs away from the modified base on the 3′-side. Thus

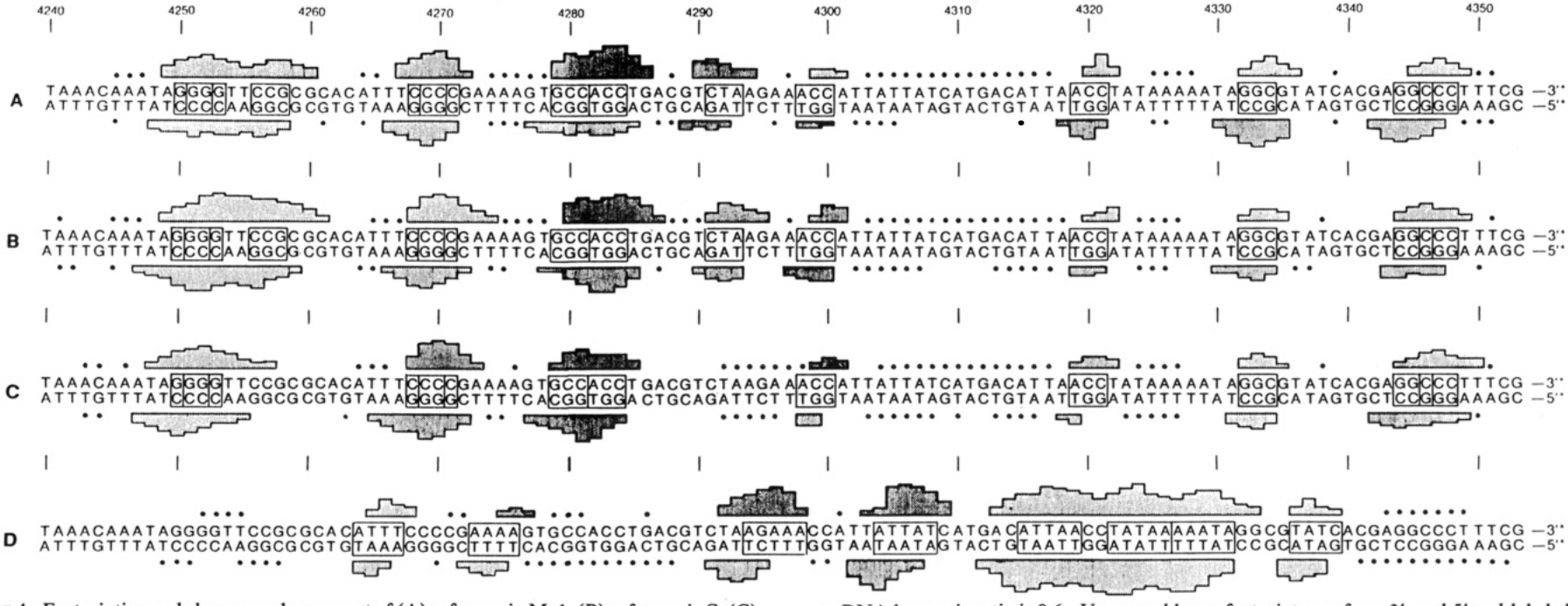

FIGURE 4: Footprinting and cleavage enhancement of (A) saframycin Mx1, (B) saframycin S, (C) saframycin A, and (D) distamycin. Histogram height is proportional to the protection from the cleavage at each base pair relative to unprotected DNA (in the absence of antibiotics). Saframycins to DNA base pair ratio is 0.6. Upper and lower footprints are from 3′- and 5′-end-labeled DNA, respectively. Boxes indicate proposed binding sites. Dots indicate enhanced cleavage.

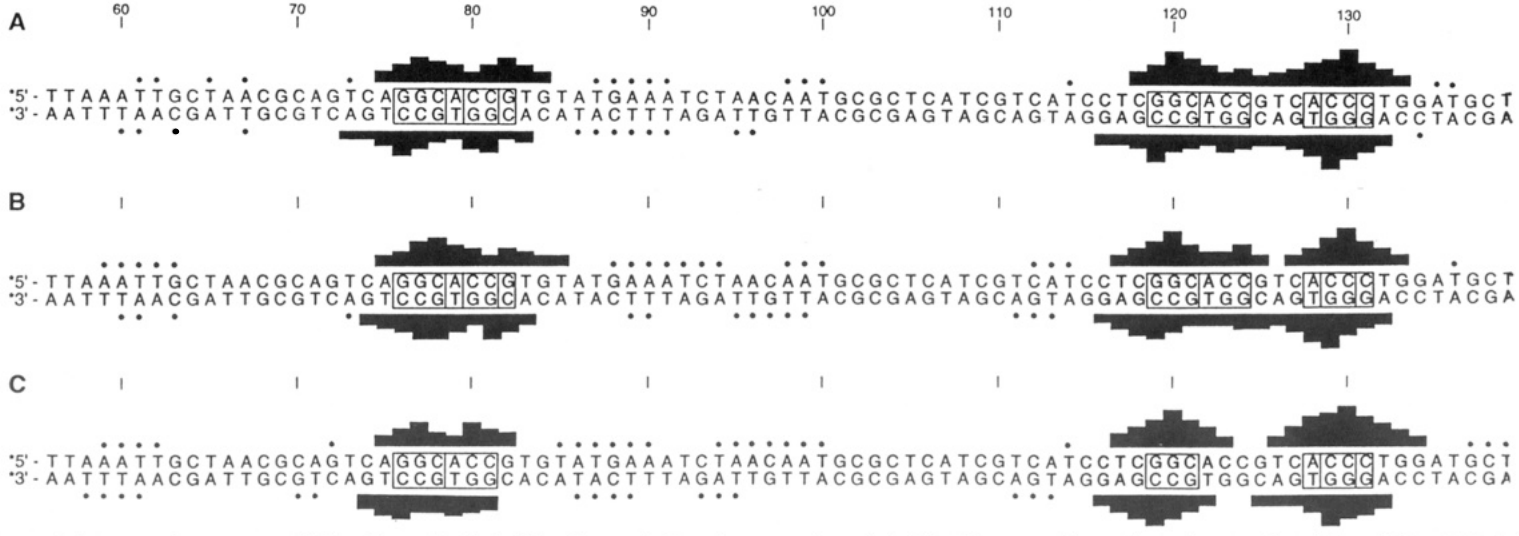

FIGURE 5: Footprinting and cleavage enhancement of (A) saframycin Mx1, (B) saframycin S, and (C) saframycin A. Histogram height is proportional to the protection from the cleavage at each base pair relative to unprotected DNA (in the absence of antibiotics). Saframycins to DNA base pair ratio is 0.6. Upper and lower footprints are from 5′- and 3′-end-labeled DNA, respectively. Boxes indicate proposed binding sites. Dots indicate enhanced cleavage.

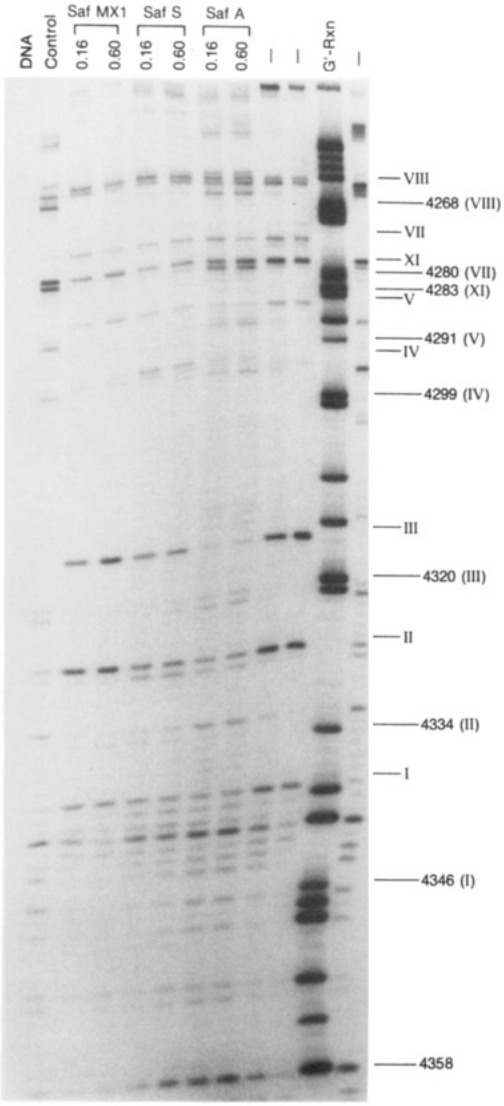

FIGURE 6: A portion of the stop assay autoradiogram from exonuclease III digestion of 5′-^{32}P end-labeled *Hin*dIII/*Rsa*I restriction fragment. The lane labeled DNA contains intact DNA fragment. Control lane is exonuclease III cleavage in the absence of antibiotics. The other lanes contain the antibiotics as labeled and the numbers indicate antibiotic to DNA base pair ratio. G′-rxn lane is the Maxam–Gilbert guanine-specific sequencing reaction.

knowledge of the sequence locations of the DNA binding agent obtained from the footprinting technique is always necessary and useful in interpreting exonuclease III stop bands. However exonuclease III occasionally gives some stop bands on the gel that do not correspond to modified sites which complicates the analysis. It appears that this latter kind of stop at unmodified sites is base and sequence dependent. Exonuclease III action has been reported to proceed at different rates on different DNA sequences and to stall at only certain sequences (Tullius & Lippard, 1980; Linxweil & Harz, 1982; Walter et al., 1988). Despite these pitfalls, the exonuclease III stop assay can be carried out conveniently by comparing the modified DNA with control, unmodified DNA, and the data provided are informative if interpreted with caution.

Figures 5–7 show the exonuclease III stop patterns on both modified and unmodified DNA. For saframycin A-modified DNA, seven exonuclease III stop sites are identified in the readable portion of the autoradiogram. The stop sites are labeled in Figure 6 as I–VII. Saframycin Mx1- and S-modified DNA showed eight exonuclease III stop bands. These bands are either completely absent or very light in intensity in the

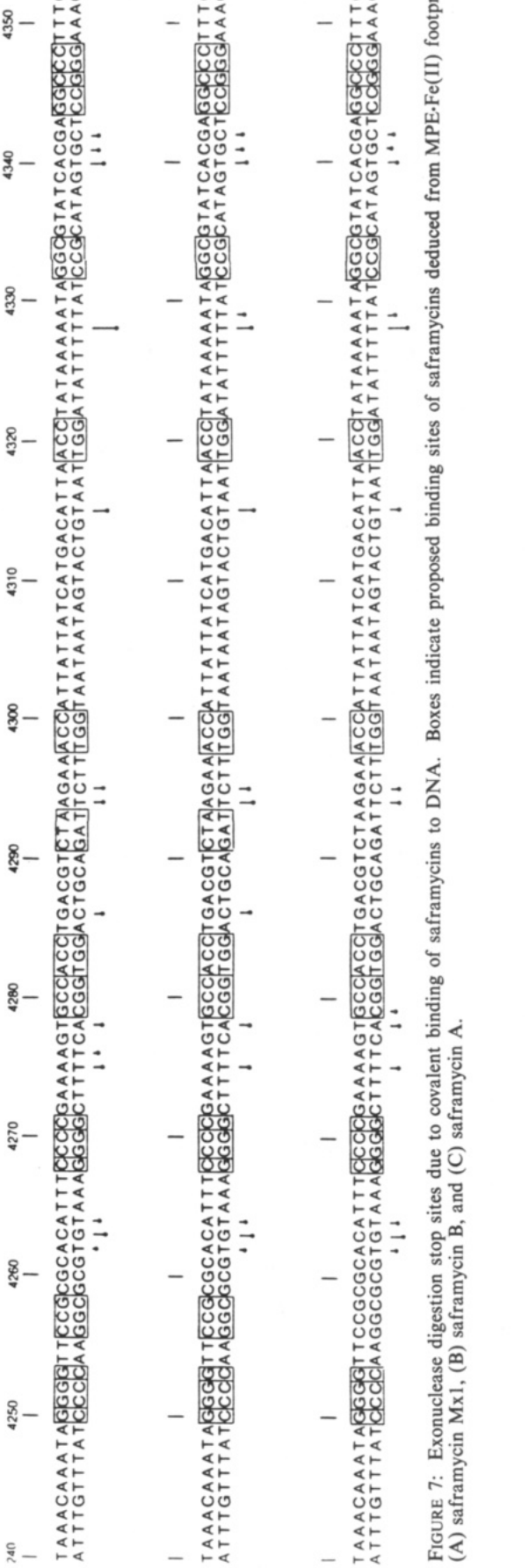

FIGURE 7: Exonuclease digestion stop sites due to covalent binding of saframycins to DNA. Boxes indicate proposed binding sites of saframycins deduced from MPE-Fe(II) footprinting: (A) saframycin Mx1, (B) saframycin B, and (C) saframycin A.

Table I: Sequences Preferred in Covalent Binding of Saframycins A, S, Mx1, and Mx3

saframycin	binding sites	
	primary	secondary
A	GGGG (4250–4253)	ACC (4282–4284, 4298–4300, 4319–4321)
	CCC (4268–4271)	
	GCC (4279–4281)	
	GGC (4331–4333)	
	GGCCC (4344–4348)	
S, Mx1, and Mx3	GGGG (4250–4253)	ACC (4282–4284, 4298–4300, 4319–4321)
	CCG (4257–4259)	
	CCCC (4268–4271)	CTA (4291–4293)
	GCC (4279–4281)	
	GGC (4331–4333)	
	GGCCC (4344–4348)	

unmodified DNA lane. All these bands are shifted four to six base pairs toward the 3′-side of the modified site that corresponds to one of the Gs in the binding site of these saframycins as identified by MPE·Fe(II) footprinting.

DISCUSSION

Using MPE·Fe(II) footprinting and exonuclease III stop assays, we are able to identify the binding and modification sites on DNA due to the saframycin antibiotics.

On the *Hin*dIII/*Rsa*I 545 mer the saframycins S, Mx1, and Mx3 appear to recognize eight distinct GC-rich sites. However, only seven of these eight sites are recognized by saframycin A. These preferred sequences are given in Table I. Several consecutive GC sites are available on this fragment, but none of them are recognized. From the table and Figure 4 it is evident that the saframycin antibiotics have preferences for GGG or GGC sequences. The triplet binding sites at 4279–4281, 4334–4332 (GCC), 4282–4284, 4298–4300, 4319–4321 (ACC) suggest that these antibiotics also have preference for GGPy sequences.

On the shorter fragment, the assigned probable binding sites are GGC (78–78), ACCG (79–82), GGC (119–121), and ACCC (128–131). These proposed binding sites are consistent with the triplet sites observed on the longer fragment. The only triplet site observed on this shorter fragment, but not on the longer fragment, is CCG. Similarly, though CTA triplet sites are available on the shorter fragment, no measurable binding is seen at these sites. These results clearly demonstrate that the mere occurrence of a binding site on heterogeneous DNA is not sufficient for recognition by the antibiotics, but the flanking sequences also play an important role in sequence selectivity or specific recognition of ligands. However, from the relative intensities of the MPE·Fe(II) footprints of the compounds to the two possible sequences GGC and GGT, it can be concluded that a cytosine is preferred at the 3′-end over a thymine base.

Saframycin S, Mx1, and Mx3 recognize two unique sequences, CCG and CTA, that are not binding sites for saframycin A. Although the principal difference between saframycin A and the others is only in the nature of the active functional group (see Figure 1), the intermediate unstable iminium species is common for all four saframycins studied. However, the slight observed differences in recognition of sequences by CN- and OH-possessing saframycins are real and are plausibly due to the subtly different molecular recognition requirements of the individual antibiotics.

The exonuclease III stop assay confirms the binding location of the antibiotics determined by MPE·Fe(II) footprinting. All the binding sites identified by MPE footprinting are also revealed by the exonuclease III stop assay. In all cases exonuclease III stop bands are identified four to six base pairs toward the 3′-side of the binding site. Possibly either 5′- or 3′-terminal guanine in the triplet binding site is the site of covalent interaction for saframycins. However, it is difficult to comment on the orientation of binding of the antibiotic to the DNA. The exonuclease III assay does not identify a CTA binding site for saframycin A, while the other saframycins indicate binding at this site. The *Hin*dIII/*Rsa*I short fragment was found to be unsuitable for exonuclease assay.

The evidence suggests that the intermediate electrophile iminium ion or α-carbinolamine generated by reductive metabolism of the antibiotics binds to 2-NH_2 of guanine by forming an aminal linkage as proposed earlier (Lown et al., 1982; Rao & Lown, 1990). The exonuclease III stop assay supports the formation of such a covalent linkage.

CONCLUSIONS

The saframycins Mx1, Mx3, A, and S recognize primarily 5′-GGG sequences. All four antibiotics also recognize 5′-GGPy sequences; however, C is preferred to T in the last position in all cases. Saframycins Mx1, Mx3, and S, which possess the OH leaving group, also recognize the 5′-CCG sequence, in contrast to saframycin A, which contains a CN leaving group. On the other hand the OH-containing saframycins also recognize the 5′-CTA sequence. As in the case of saframycins B and C, which lack the critical CN or OH leaving group, saframycin Mx2 also does not show any footprints on the restriction fragments examined in this study. The measured binding site size for all four antibiotics is three base pairs. The exonuclease III stop assay independently confirms the formation of a covalent bond and the strong preference of the antibiotics for 5′-GGG and 5′-GGC sequences. This latter enzyme assay also suggests that the 5′-terminal or central G of the triad binding site is the base to which reversible covalent attachment takes place. Structural analysis of a 1:1 complex of saframycin A bound covalently to an appropriate duplex oligodeoxyribonucleotide by high-field NMR is in progress. The results of this analysis together with molecular mechanics calculations will be reported in due course.

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