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Rate enhancements in the DNase I footprinting experiment

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ABSTRACT

Footprinting experiments for DNase I digests of a 139-base-pair segment of pBR-322 DNA in the presence of either netropsin or actinomycin D were carried out. Plots of oligonucleotide concentration as a function of drug concentration were analyzed to study the enhancement in cleavage rates at ~30 sites, accompanying drug binding at other sites. The pattern of enhancements is not consistent with drug-induced DNA structural changes, but agrees with a redistribution mechanism involving DNase I. Since the total number of enzyme molecules per fragment remains unchanged, drug binding at some sites increases the enzyme concentration at other sites, giving rise to increased cleavage. The consequences of the redistribution mechanism for analysis of footprinting experiments are indicated.

INTRODUCTION

Footprinting analysis provides a means of identifying the binding sites of equilibrium binding drugs and proteins on DNA molecules derived from natural sources (1-5). The most frequently used probe in footprinting studies is the endonuclease DNase I. The enzyme not only reports the sites of ligand binding but, since it is sensitive to local changes in DNA structure, it has also been used to determine the sites of ligand-induced structural changes in DNA. These are reflected in enhancements in the DNase I cleavage rate, appearing as sites on the DNA lattice bind ligand (6-10).

The rate of cleavage by DNase I at a phosphodiester linkage of DNA, $(\text{rate})_i$, is governed by rate expression (1), where k_i' is the cleavage rate constant at the site and $[\text{DNaseI}]_i$ is the concentration of enzyme at the

$$(\text{rate})_i = k_i' [\text{DNaseI}]_i \qquad (1)$$

site. As is evident from (1), an increase in $(\text{rate})_i$ (enhancements) can arise from an increase in k_i' caused by a ligand-induced DNA structural change and/or an increase in $[\text{DNaseI}]_i$. The latter could be due to a redistribution of the enzyme on DNA as some sites on the polymer accept ligand. Neither the

rate expression, nor the fact that simple mass action effects due to enzyme redistribution can also give rise to enhancements, are commonly noted in footprinting experiments.

In an effort to uncover the origin of enhancements in drug footprinting experiments, we carried out a series of DNase I digests of an end-labeled 139-base-pair segment of pBR-322 DNA in the presence of various amounts of either the antiviral agent netropsin or the anticancer drug actinomycin D. The former is a minor-groove binding peptide which does not greatly distort the DNA helix (11), while the latter is an intercalating agent which, upon binding, is likely to alter polymer structure (12-14). The autoradiographic data resulting from the experiments were quantitatively analyzed using microdensitometry and ultimately reduced to a series of "footprinting" plots, showing oligonucleotide concentration as a function of drug concentration (15-17). The analysis, presented below, revealed that, while the level of enhancement varied as a function of sequence, the general enhancements could be largely explained by a drug-induced redistribution of the enzyme on DNA and were less likely due to modified DNA structure. The consequences of these observations for detecting DNA structural changes in the footprinting experiment through rate increases, and for quantitative footprinting analysis leading to the evaluation of binding constants from footprinting data, are presented and discussed.

MATERIALS AND METHODS

The preparation of the 139-base-pair restriction fragment obtained by cleavage of pBR-322 DNA using Hind III and Nci I was as previously described (18). The fragment was end-labeled at position 33 (referenced to the standard numbering system of pBR-322 DNA) using $\alpha[^{32}P]$-dATP and reverse transcriptase. Establishment of sequence was earlier described (18). The calf thymus DNA used as carrier DNA (Sigma Chemical Co.) was sonicated and its concentration in base pairs determined optically using $\varepsilon_{260} = 13.2\ mM^{-1}cm^{-1}$. The concentrations of netropsin sulfate and actinomycin-D were determined optically using $\varepsilon_{296} = 20.2$ and $\varepsilon_{440} = 24.45\ mM^{-1}cm^{-1}$ respectively.

The footprinting experiments were conducted as previously described (18). Digests in the presence of carrier DNA were carried out in a total volume of 8µl which contained a final DNA concentration of ~194µM in DNA base pairs: 193µM in sonicated carrier DNA and ~1µM labeled fragment. After addition of a specified amount of netropsin or actinomycin-D to a buffered medium containing DNA, the system was allowed to equilibrate for 30 min prior to the addition of

the enzyme. Digests with DNase I were for 10 min at 37°C in a 50mM Tris-Cl, 8mM $MgCl_2$, 2mM $CaCl_2$ pH 7.5 buffer. For each drug, 25 different concentrations were used in the range, 0 to 3.88×10^{-5}M.

The concentration of enzyme used, ~0.2 μM, and the length of the digest time were such that the total amount of fragment cleavage, as measured by the amount of full length DNA remaining, was <20%. This means that only a few percent of the cleaved fragments were the result of multiple cuts by the enzyme. The labeled oligonucleotide products were separated in a 12% denaturing polyacrylamide gel using an in-house developed, thermostated field gradient electrophoresis device.

Analysis of the autoradiographic data was by linear scanning microdensitometry (15,16). From the autoradiographic data, a file consisting of a peak's number, corresponding to the genomic numbering system for pBR-322 DNA, and its cross-sectional area was created. The concentration of an oligomer was plotted as a function of drug concentration for each DNase I cleavage site in the region 48-~110 of the fragment. These "footprinting plots" along with the summed band areas for all oligomers produced in a given digest formed the basis of the analysis. In the region 48-~110 on the fragment, single nucleotide resolution was observed. Differences due to errors in gel loading and digest periods were small, <10% (16).

The concentration of each oligonucleotide was fitted by least squares to a linear function of total drug concentration, using data for the first 14 netropsin concentrations (or 16 actinomycin concentrations). The slopes obtained were divided by the intercepts to give relative slopes which were used in the analysis. Results for cleavage sites showing low correlation coefficients or low concentrations (intensities) were rejected. For actinomycin D the drug concentrations used corresponded to drug binding to only the strong sites (the initial loading events) on the restriction fragment. The footprinting plots showed that, at the highest drug concentration, 7.64 μM, these sites were ~50% occupied. For netropsin the highest drug concentration, 6.49 μM, corresponded to nearly complete occupancy of the strong sites with some binding to the weaker sites on the fragment (actinomycin has a much lower binding constant than netropsin).

RESULTS

Photographs of the autoradiograms used in the analysis are shown in Figures 1 and 2. Typical footprinting plots for various sites on the 139-mer for actinomycin D and netropsin are shown in Figure 3. Strong binding sites

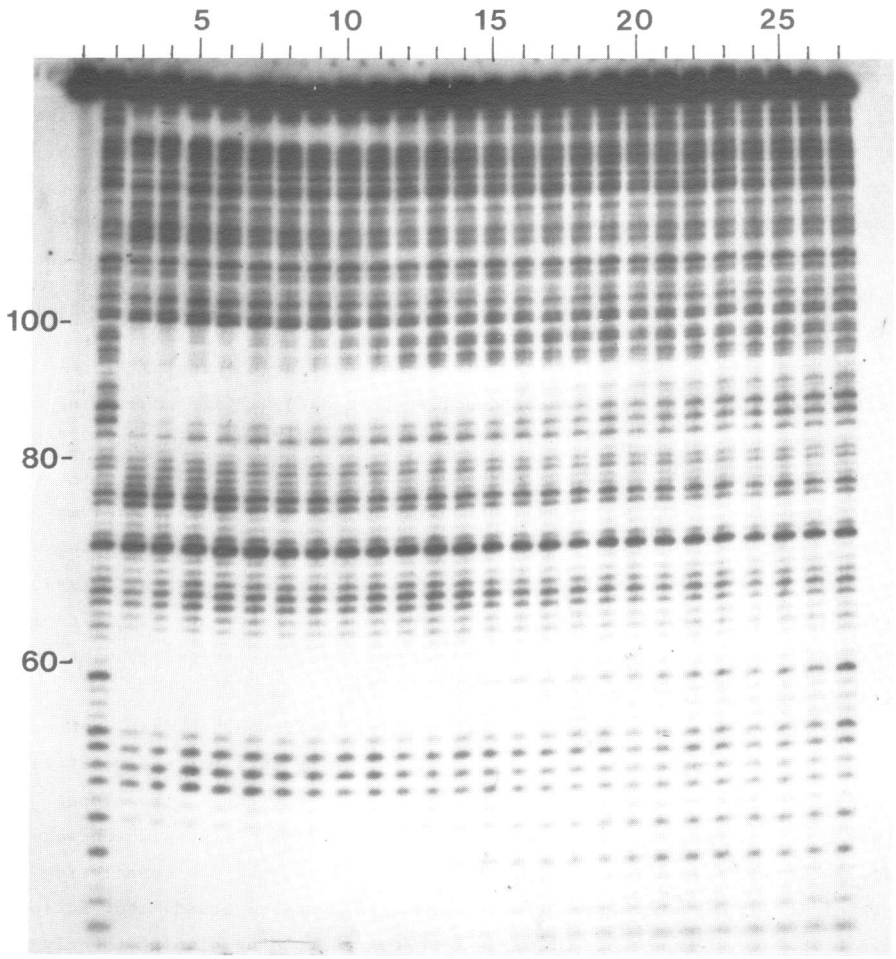

Figure 1. A photograph of the netropsin autoradiographic data. Lane 1 is the no enzyme control while lanes 2 and 27 are DNase I digests of the 139-base-pair fragment in the absence of netropsin. The lane number and drug concentration (μM) for digests involving drug were: 3, 38.8; 4, 33.0; 5, 28.0; 6, 23.8; 7, 20.3; 8, 17.2; 9, 14.6; 10, 12.4; 11, 10.57; 12, 9.99; 13, 7.64; 14, 6.49; 15, 5.52, 16, 4.69; 17, 3.99; 18, 3.39, 19, 2.88; 20, 2.45; 21, 2.08; 22, 1.77; 23, 1.50; 24, 1.28; 25, 1.09; 26, 0.92. The numbering system pertaining to sequence is the genomic number system of pBR-322 DNA.

for either drug appear as inhibitions in the rate of cleavage while weak binding sites exhibit an initial rate increase followed by inhibition. For sites located between binding sites, both drugs cause rate enhancements, e.g. site 75 for netropsin and 57 for actinomycin D. The slopes, calculated as

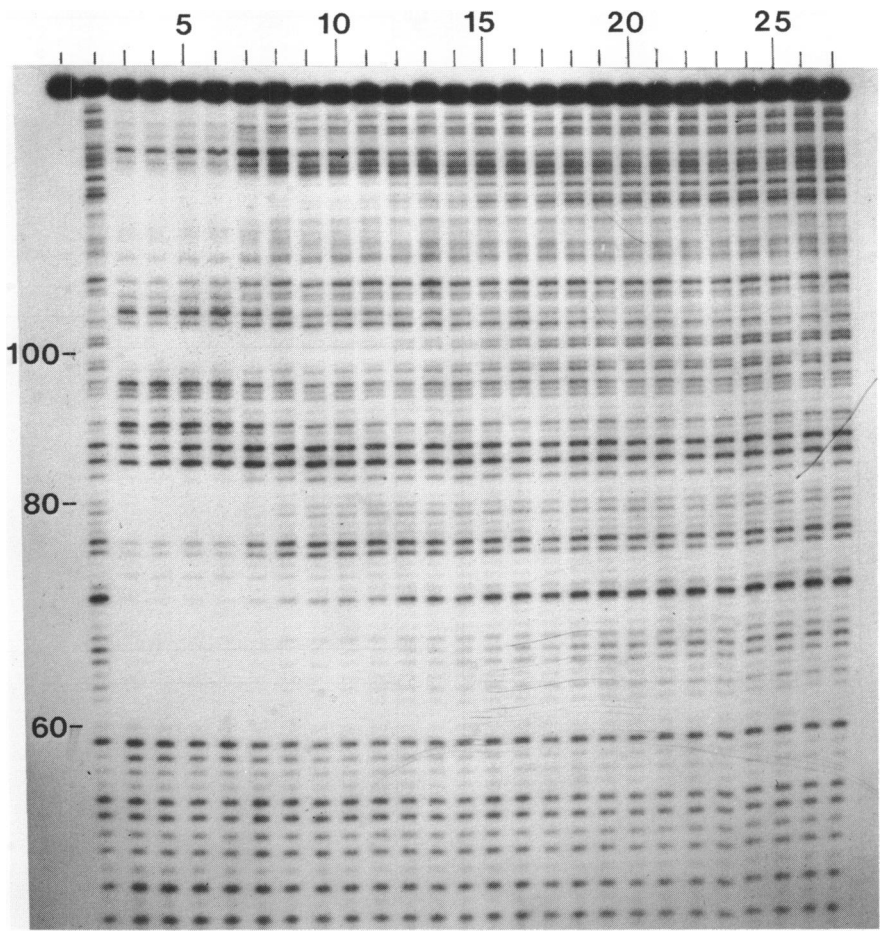

Figure 2. A photograph of the actinomycin-D autoradiographic data. Lane 1 is the no-enzyme control while lane 2 is a DNase 1 digest of the 139-base pair fragment in the absence of drug. The lane numbers and drug concentrations for lanes 3-26 are as indicated in the caption to Figure 1. The drug concentration for lane 27 was 0.78 μM.

outlined in the Materials and Methods Section, are negative for binding sites and positive for enhancements. They are given in Table I, with standard deviations as a measure of reliability, and are indicated graphically on the sequence of the fragment in Figure 4.

Except for anomalously high values for sites adjacent to binding sites, the enhancements, as measured by values of positive slopes, are relatively constant along the polymer. For netropsin the average slope is 0.95×10^5

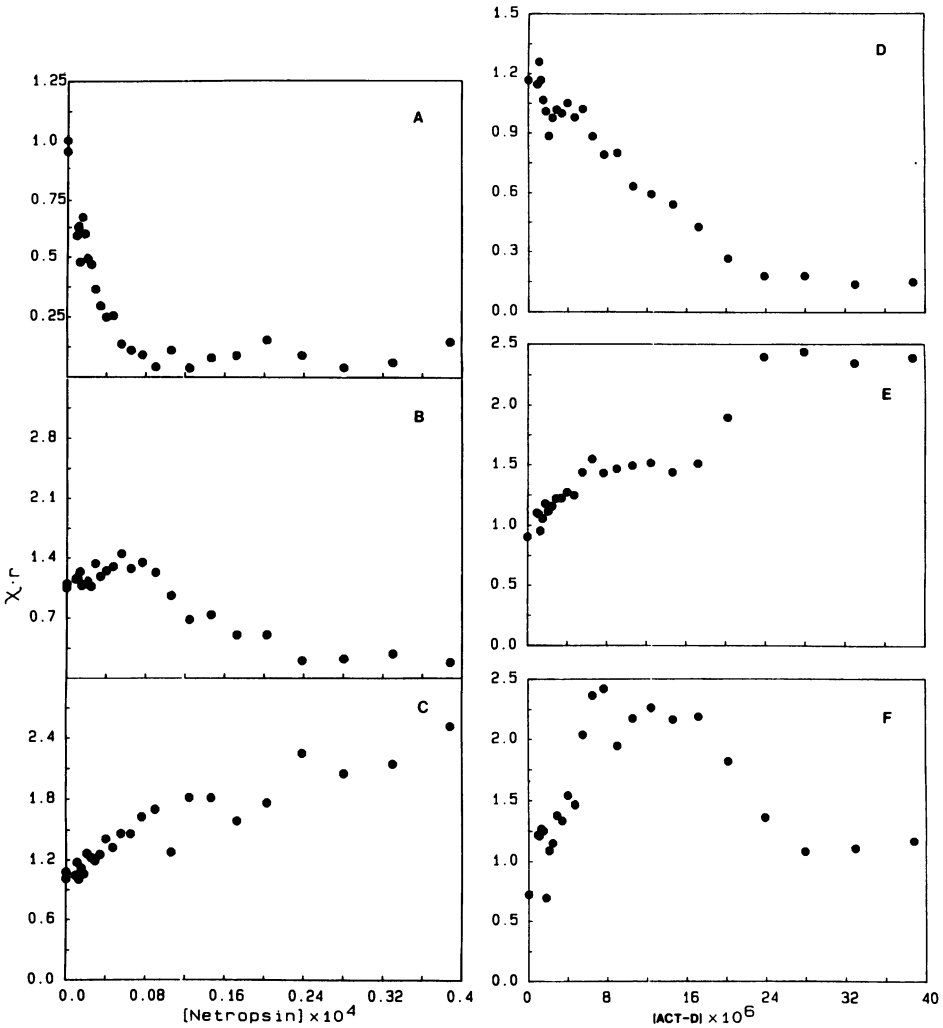

Figure 3. Selected footprinting plots obtained from digests involving netropsin (A, base 59, thymine; B, base 98, thymine; C, base 75, thymine) and actinomycin-D (D, base 67, thymine; E, base 57, adenine; F, base 74, guanine). See figure 4 for the sequence of the fragment. The relative change in oligomer concentrations, $\chi \cdot r$, is as defined in reference 16.

M^{-1}. Eliminating temporarily the high values for sites 53 and 64, we have an average slope of 0.84 x $10^5 M^{-1}$ with a mean-square deviation of 0.30. The average slope for actinomycin D is 0.48 x 10^5 M^{-1}. Dropping the high slopes for sites 58 and 73, we have an average slope of 0.35 x $10^5 M^{-1}$ with a rms deviation of 0.22.

Table I

Slopes and Standard Deviations[a]

Site	Netropsin		Actinomycin	
	Slope	Standard Deviation	Slope	Standard Deviation
45	-0.45	0.21		
48	-0.76	0.12	0.72	0.11
50	-0.80	-0.10	0.65	0.10
51			0.85	0.11
52	1.35	0.14	0.35	0.06
53	2.06	0.21	0.32	0.07
54	0.93	0.17	0.31	0.06
55			0.36	0.05
56			0.42	0.07
57			0.57	0.07
58	-1.31	0.24	1.50	0.11
59	-1.51	0.16	-0.06	0.05
62			-0.49	0.06
63			-0.48	0.05
64	1.49	0.15	-0.33	0.06
65	1.13	0.13	-0.61	0.05
66	1.03	0.14	-0.81	0.05
67	1.12	0.08	-0.36	0.06
68	1.33	0.16	-0.47	0.05
69			-0.50	0.06
71	0.91	0.13	-0.45	0.05
72			-0.54	0.09
73			1.61	0.22
75	0.77	0.09	0.51	0.09
76	0.63	0.07	0.20	0.06
79	0.55	0.13		
80	0.48	0.16		
81			0.46	0.09
83	0.73	0.11	0.20	0.05
85	-0.74	0.07	0.29	0.05
87	-1.34	0.07		
89			0.18	0.05
90	-1.40	0.12	0.17	0.04
92			0.04	0.07
94			0.14	0.04
95			0.00	0.05
96	0.46	0.12	0.27	0.05
98	0.39	0.10	-0.30	0.08
99			-0.70	0.07
102			-0.73	0.07
103			-0.77	0.07
106	0.76	0.21		
108			0.97	0.10

a · Times 10^{-5} M

For both drugs, the enhancements immediately adjacent to binding sites are 2-3 times greater than those associated with sites more remote from binding. There also seems to be a decrease of slope in going from lower to

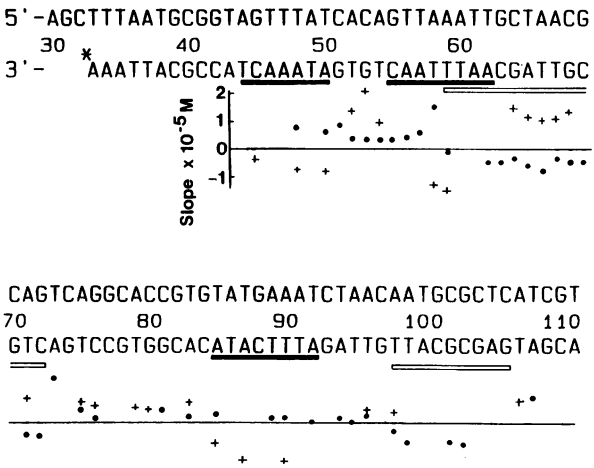

Figure 4. The slopes, as defined in the text, for netropsin (+) and actinomycin-D (●). The region of inhibition for netropsin and actinomycin D are indicated as (▬) and (▭) respectively.

higher site numbers. Since cleavage at any site is measurable only if there is no cutting between the site and the labeled end of the fragment, multiple cuts on a fragment would lead to lower intensities at higher site numbers. However, since we are in the "single-hit" regime, multiple cuts are unimportant.

The ratio of drug-blocked sites to free sites on the restriction fragment, associated with the initial loading events, was 0.27 for netropsin and 0.39 for actinomycin D. The sum of the concentrations of all oligomers produced in a given digest, as determined by microdensitometry, was constant over the concentration ranges studied for both drugs.

DISCUSSION

In the drug-DNA footprinting experiment, the drug may influence the cleavage rate by blocking the enzyme from cleaving at certain sites (16,17), altering DNA structure away from the sites of binding (19,20), or causing redistribution of the enzyme to regions where no drug binding is occurring. Alteration of DNA structure can either increase or decrease the cleavage rate constant at a site, k_i', so both inhibitions and enhancements in rates can be expected. Furthermore, the changes should differ markedly from one site to the next, and not always extend over the length of DNA expected for a drug binding site.

The redistribution of enzyme should lead to increases in the rate of cleavage associated with all sites not involved in binding. If the total amount of cleavage on the fragment remains constant as drug loading occurs, as is the case for both the netropsin and actinomycin D footprinting experiments reported here, the amount of enzyme on the fragment is also constant. Thus, drug binding shifts enzyme to unblocked sites, increasing $[DNase\ I]_f$ at these sites. This mechanism predicts that all unblocked sites will exhibit the same fractional enhancement, the magnitude of which is related to the fraction of the total number of enzyme cleavage sites which are blocked by drug.

The footprinting results for both netropsin and actinomycin D show no significant decreases in cleavage rates at sites where no drug binding is occurring. Furthermore, the enhancements are relatively constant along the sequence, the exceptions being for sites immediately adjacent to drug-binding sites, Figure 4. This suggests that most of the enhancements are not due to drug-induced structural changes, but rather are caused by a redistribution of enzyme over the fragment as sites load with drug. A check on this is provided by the magnitude of the enhancements.

For netropsin, the fractional enhancement, at 6.49 μM drug, for a relative slope of $0.84 \times 10^5\ M^{-1}$ is $(0.84 \times 10^5 M^{-1})\ (6.49 \times 10^{-6} M)$ or 0.54. The level of enhancement expected if all of the strong sites on the fragment are totally occupied by drug can be calculated from the ratio of blocked to free sites, i.e. $(1-0.27)^{-1}=1.37$, giving 37% enhancement. The discrepancy is due to loading of secondary sites, which is indicated by the leveling off of the footprinting plot for site 98 (see Fig. 3). To eliminate this, one can determine the level of enhancement at lower netropsin concentration where only the primary sites (no secondary sites) have bound drug. For example, at 2.88 μM netropsin, the strong drug sites are ~70% occupied, so the predicted relative intensity is $(1- 0.7 \times 0.27)^{-1}=1.23$ and the enhancement 0.23; the actual enhancement is $(0.84 \times 10^5 M^{-1})\ (2.88 \times 10^{-6} M) = 0.24$.

For actinomycin D, the highest drug concentration used was 7.64μM making the observed enhancement $(0.35 \times 10^5 M^{-1})(7.64 \times 10^{-6} M)=0.27$ or 27%. The enhancement calculated from the strong sites which are only half occupied is $(1-0.5 \times 0.39)^{-1}$ or 1.24, ie 24% enhancement. Thus, it appears that simple mass action effects associated with the enzyme can explain the general level of enhancement observed in the footprinting experiments involving both drugs.

The enhancements at positions 58, 73, and 108 for actinomycin D, and 53 and 64 for netropsin, are significantly above the level of enhancement which can be explained by a redistribution mechanism. Since all are adjacent to

drug binding sites they may be due to altered DNA structure and/or a "sticking" mechanism involving DNase I interacting with bound drug. Since the enzyme possesses hydrogen bond donor and acceptor sites as well as charged groups it could hydrogen bond and electrostatically interact with netropsin and actinomycin D while they are bound to DNA. This would result in local increases in $[DNase\ I]_i$ adjacent to drug sites, causing increased cleavage in these regions.

The higher-than-average enhancements in cleavage rates at 48 and 51 and lower-than-average enhancements at 92 and 95 for actinomycin D may be due to structural alteration induced by drug binding. However, they may also be outlying statistical fluctuations around a constant value. Additional footprinting studies involving other cleaving agents will be necessary in order to establish the presence of altered structure at these sites.

The redistribution mechanism has important consequences for quantitative footprinting experiments leading to the evaluation of binding constants from footprinting data. The footprinting plots associated with a binding site must be corrected for the redistribution effect in order to obtain a valid isotherm for the site. If the ratio of blocked to free cutting sites is small, the correction will be negligible and the resulting footprinting plots can be directly used to determine the occupancy of the site. This situation most readily obtains in DNA-protein footprinting experiments, where, due to high binding specificity, only a small fraction of the total DNA present may be blocked from cleavage (21,22). However, if the fragment bearing the protein binding site is short, the fraction of protein bound sites to free sites will be significant. In this case accurate quantitative studies will require correction for redistribution effects. On the other hand, since drugs and other small ligands exhibit low binding specifities the ratio of blocked to free sites is generally large. In this case, the footprinting plots must be corrected for redistribution in order to obtain valid isotherms for the various binding sites present in the system.

Although the netropsin-DNA interaction has been analyzed using quantitative footprinting methods, and drug binding constants as a function of sequence determined (16,17), actinomycin D has not been studied in this way. However, from the slope data presented in Table I and shown in Figure 4, the strong binding sites for the drug are the sequences: 5'→3', TGCT (62-65); CGCA (68-71); TGCG (100-103) and CGCT (102-105), all of which contains the dinucleotide sequence GC. This result is consistent with earlier footprinting studies (3-5,7) and the large amount of physicochemical data collected on the

antibiotic (12-14) indicating that the preferred site is GC. Interestingly, the sequence, GGCA (76-79), which also contains the dinucleotide GC, is only a weak binding site, Figures 2 and 3. Although further study will be necessary, the stacking energy associated with the purine-purine sequence, GG, which precedes the intercalation site, may be the reason why binding at the adjacent GC site is discouraged.

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