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Sequence-specific binding of echinomycin to DNA: evidence for conformational changes affecting flanking sequences

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ABSTRACT

The technique of DNAase I footprinting has been used to investigate preferred binding sites for echinomycin on a 160-base-pair DNA fragment from E. coli containing the tyr T promoter sequence. Six binding sites have been precisely located in the sequence; a seventh has been partially identified. The minimum site-size is six base pairs. All the binding sites contain the dinucleotide sequence CpG but no other regularities can be discerned. When the protected regions on each complementary strand are compared it is evident that they are staggered by 2-3 base-pairs towards the 3' end at each site. Footprinting with DNAase II reports a similar, though less precise, pattern of protection. Cutting by both enzymes is markedly enhanced at AT-rich regions flanking the antibiotic-binding sites. This increased susceptibility to nuclease attack can be attributed to an altered helix conformation in the vicinity of the bis-intercalated echinomycin molecule. It seems that certain sequences, mainly runs of A or runs of T, switch from a nuclease-resistant to a nuclease-sensitive form when echinomycin binds nearby.

INTRODUCTION

The quinoxaline group of antibiotics, of which echinomycin (Figure 1) is the best-known member, are highly active against Gram-positive bacteria, viruses and a variety of experimental tumours (1). Their mode of action is believed to involve interference with the normal enzymatic functions of transcription and replication in susceptible cells, as a result of binding to DNA by a mechanism involving bifunctional intercalation (2,3).

Various techniques have been used to probe the interaction between DNA and quinoxaline antibiotics or their analogues (4-7). The most detailed studies, performed with echinomycin, have provided evidence that the antibiotic displays an unusual pattern of specificity in its interaction with DNA. In general, it tends to bind more tightly to GC-rich DNA species (8). However, kinetic analysis has revealed the existence of several different classes of potential binding sites in natural DNA, the existence of which clearly reflects some subtle sequence-selectivity on the part of the antibiotic (9,10). Experiments with synthetic DNAs have thrown some light on the nature of preferred

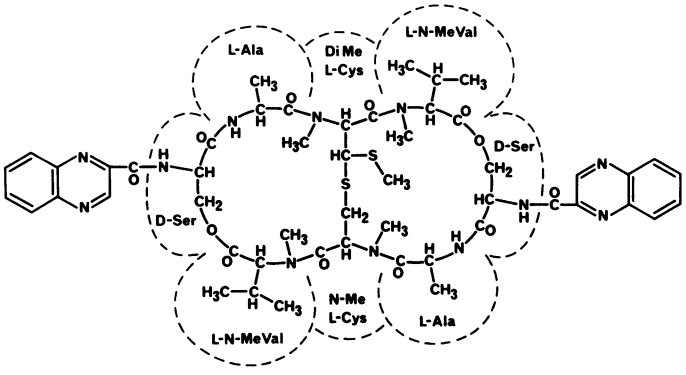

Figure 1. Chemical structure of echinomycin.

sites; for example, the kinetics of association between echinomycin and poly (dG-dC) have suggested a large difference in binding constants for the two potential intercalative sites CpG and GpC (10).

None of the techniques employed hitherto, however, permit an unambiguous determination of the nature and location of preferred binding sites in a natural DNA of mixed nucleotide sequence. This is in a sense because quinoxaline antibiotics, in common with other intercalators, leave no record of their residence positions on the helix, i.e. they neither cleave the DNA backbone nor modify the bases. The purpose of the present study was therefore to apply the novel method of DNA-drug "footprinting" to investigate the precise nature of preferential sites of interaction with a natural DNA.

Footprinting is a technique that combines the enzymatic (or chemical) cleavage of DNA fragments with analysis of the products using Maxam-Gilbert sequencing gels (11,12). The antibiotic-protected sites are visualized at single-bond resolution via gaps in the autoradiograph of a denaturing polyacrylamide gel, revealing the positions and lengths of individual binding sites (11,12). The method relies on the relative lack of selectivity of an endonuclease which can cleave the bonds between all the DNA nucleotides in the region of interest at roughly similar rates, so that inhibition at each of these sites by a DNA-bound ligand may be detected. The extent of enzyme digestion is adjusted so as to produce less than one, randomly-located, cleavage in each strand of the original DNA molecule.

Footprinting with DNAase I has previously been used to study the interaction with DNA of actinomycin D (13-15), chromomycin A3 and distamycin A (14). DNAase I is not the only available cleaving agent; methidiumpropyl-

EDTA-Fe(II) (MPE-Fe(II)) has also been used to study the sequence-specificity of actinomycin, distamycin, netropsin, chromomycin, mithramycin and olivomycin on heterogeneous DNA (16,17). The DNAase I footprinting method appears to be more sensitive for detecting weakly bound sites, while the MPE-Fe(II) footprinting method has been reported to be more accurate as regards determining the actual size and location of ligand-binding sites (14).

Here we have adopted the DNAase I technique, and have compared the results with those produced by the unrelated enzyme DNAase II which has only been used previously to study the accessibility of DNA in the nucleosome core particle (18). With echinomycin, we find that the correlation between footprints reported by the two enzymes is good. We also report the unexpected finding that DNA strand cleavage is markedly enhanced at AT-rich sequences flanking the sites protected by bound antibiotic molecules.

MATERIALS AND METHODS

Antibiotic Solutions

Echinomycin was a gift from Drs H. Bickel and K. Scheibli of CIBA-Geigy Ltd., Basel, Switzerland. Concentrations were determined spectrophotometrically from the absorbance at 325 nm (ε_{325} = 11,500 M^{-1} cm^{-1})(2). Because of the low aqueous solubility of echinomycin (5 μM) stock solutions for footprinting work were prepared in a methanol-buffer mixture (40/60, v/v) so that the final concentration of methanol present in the reaction mixture was 20%. To verify that the presence of methanol did not interfere with the enzyme action a control experiment was performed in which methanol was added and then removed by evaporation just prior to addition of the nuclease. This produced essentially the same banding pattern as those resulting from reaction mixtures prepared with or without the addition of methanol.

Enzymes

Deoxyribonuclease I (DNAase I) was obtained from Sigma and prepared as a 7200 units/ml stock solution in 0.15M NaCl containing 1 mM $MgCl_2$. It was stored at -20°C and diluted to working concentrations immediately before use. Deoxyribonuclease II (DNAase II) was obtained from P-L Biochemicals and prepared as a 200 units/ml stock solution in 10 mM ammonium acetate, pH 5.6, containing 0.2 mM EDTA and stored at -20°C. This enzyme solution was used undiluted.

Buffers

The antibiotic was dissolved in 10 mM tris-HCl, pH 7.5, containing 10 mM NaCl and 40% methanol for experiments using DNAase I; or 20 mM ammonium

acetate, pH 5.4, containing 1 mM EDTA and 40% methanol for experiments with DNAase II. The digestion buffer used to dilute DNAase I to working concentrations contained 20 mM NaCl, 2 mM $MgCl_2$ and 2 mM $MnCl_2$. Mn^{2+} was used together with Mg^{2+} because it speeds up the cutting rate 50-fold without causing any change in the drug-free DNA digestion pattern (19). Both monovalent and divalent cations affect the DNA-binding affinity of echinomycin (8,20), so it was considered important to keep the salt concentration as low as possible while still maintaining DNA and enzyme stability. A high concentration corresponding to 100 mM NaCl, 10 mM $MgCl_2$ was also explored in some control experiments; it yielded essentially the same footprints as those obtained at low salt concentration.

3'-End-labelling with Reverse Transcriptase

A 160 base-pair duplex DNA fragment from E. coli, containing the tyrosine tRNA promoter together with its adjacent sequences, was isolated as previously described (21). This tyr T duplex has sticky ends of unique sequence which could be filled in by the enzyme reverse transcriptase, so as to give specific 3'-end labelling on one or other of the DNA strands. Incubation with reverse transcriptase, dGTP and α-[^{32}P] dCTP led to selective radioactive labelling of the 3'-end at the Ava I site on the Watson strand (upper sequence in Figure 3). Incubation with reverse transcriptase, dTTP and α-[^{32}P] dATP led to selective labelling of the 3'-end at the EcoR I site on the Crick strand (lower sequence in Figure 3) (21).

DNAase I Footprinting

3 µl of the labelled DNA (9 p moles in base pairs) were incubated with 5 µl of echinomycin solution (4-15 µM) at 37°C for 30 mins, then digested with 2 µl of DNAase I (final concentration, 0.05 units/ml). 3 µl aliquots were removed from the mixture after 1, 5 and 30 mins of digestion. Reactions were stopped by the addition of 2.5 µl of 80% formamide containing 0.1% bromophenol blue and 10 mM EDTA. Samples were heated at 100°C for at least 2 min prior to electrophoresis.

DNAase II Footprinting

Antibiotic-DNA reaction mixtures were incubated as described above, then digested with 2 µl of DNAase II (final enzyme concentration 40 units/ml). All samples were frozen on dry ice immediately after addition of the stop solution, then heated at 100°C prior to electrophoresis.

Gel Electrophoresis

Product mixtures were analyzed on 8% polyacrylamide gels (0.3 mm thick) containing 7M urea and tris-borate-EDTA buffer, pH 8.3. Electrophoresis was

carried out at 1500V for about 2 hrs. Gels were then soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, dried under vacuum at 70°C, and subjected to autoradiography against an intensifying screen at -70°C.

Densitometry

Autoradiographs were analysed using a Joyce-Loebl scanning microdensitometer to produce profiles from which the relative intensity of each band was measured. Data were calculated in the form of fractional cleavage ($\underline{f}$) = A_i/A_t where A_i is the area under band i and A_t is the sum of the areas under all bands in any gel lane. Control lanes (without enzyme) were run alongside experimental lanes and used to ensure accurate estimation of relative band areas, as well as to verify that cutting was due to added DNAase and not a result of contamination. When comparing different digestion patterns, care was taken to ensure that the extent of digestion in each case was closely similar and limited to 20-40% of the starting material so as to minimize the incidence of multiple cuts in any strand. Data from the analysis of DNAase I cleavage inhibition patterns are presented in the form of $\ln(\underline{f}_{antibiotic}) - \ln(\underline{f}_{control})$ which represents the differential cleavage at each bond relative to that in the control. The results are shown on a logarithmic scale for convenience in plotting. Positive values indicate enhancement, negative values blockage.

RESULTS

DNAase I Footprinting

The 160-base-pair DNA fragment used here for footprinting purposes was studied previously in two contexts: first, as a functional promoter for a major species of tyrosine tRNA in E. coli (22); and secondly, as a model system for studying DNA structural variation in solution (21). Now we are using it for a third purpose, as a substrate for DNA-drug footprinting. If the third purpose ever catches up with the first, then data will be on hand for the design of promoters which may be efficiently regulated - turned on and turned off - by the specific binding of drug molecules.

Patterns of DNAase I digestion for this 160-mer duplex are shown in Figures 2a and 2b, respectively, for its upper (Watson) and lower (Crick) strands. In each case, the digestion procedure was repeated several times in the presence of varying amounts of echinomycin in order to catch intermediate states of antibiotic-induced protection from cleavage. This was to little avail, however, since changes in the pattern proved to be of the "all-or-none" variety, with little or no protection observed below 2 μM antibiotic and no

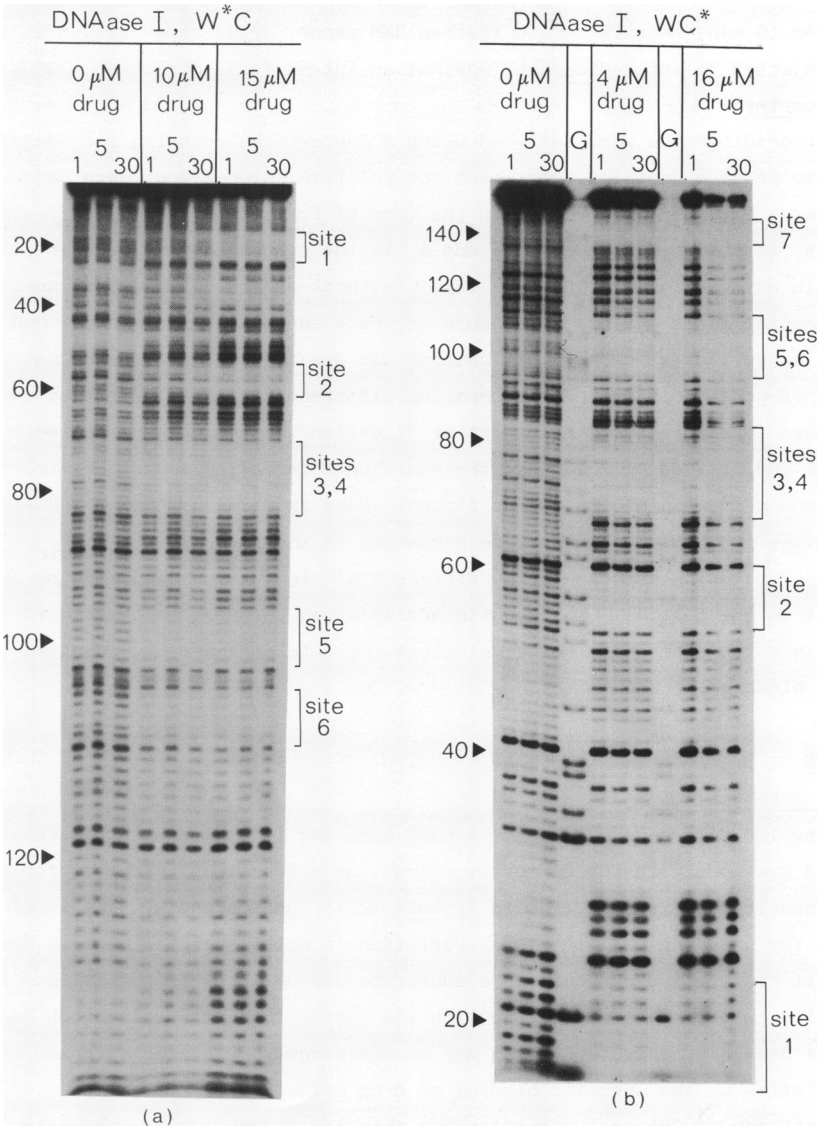

Figure 2. DNAase I footprinting of echinomycin on a 160-base-pair DNA fragment from E. coli. Symbols W*C in Figure 2(a) and WC* in Figure 2(b) indicate which of the two strands (Watson or Crick) bears a radioactive 3'-end label. Time in minutes (1, 5, 30) after the addition of enzyme is shown at the top of each gel lane. Tracks labelled "G" are dimethyl sulfate-piperidine markers specific for guanine. Numbers on the left refer to the numbering scheme shown in Figure 3, while sites of protection from DNAase I digestion are identified on the right.

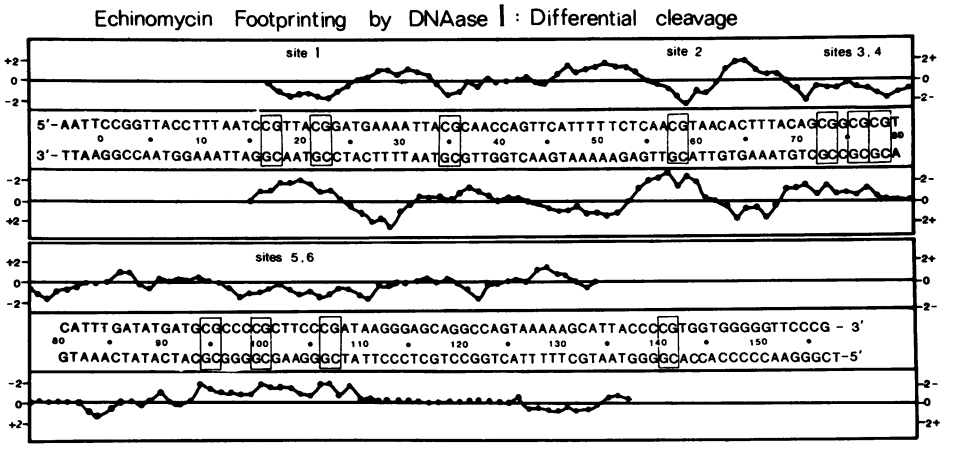

Figure 3. Echinomycin-induced differences in the susceptibility to DNAase I digestion. The upper Watson strand reads 5' to 3' left-to-right, while the lower Crick strand reads 5' to 3' right-to-left. Vertical scales on both sides are in units of $\ln(f_a)-\ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of 15 μM antibiotic and f_c is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion (approx. 30% of the starting material in both cases). Positive values indicate enhancement, negative values blockage. Sites of protection are labelled as in Figure 2.

further protection observed beyond 8 μM. Each gel lane contains about 120 reasonably-resolved bands, and these were converted through careful densitometric measurements into the differential cleavage plot shown in Figure 3. In this diagram, dips in the curve toward the lettered sequence indicate sites of antibiotic-induced protection from DNAase I cleavage, whereas peaks in the curve indicate regions of antibiotic-induced enhancement. At first glance, variations in gel band intensity appear somewhat more pronounced than corresponding variations in the densitometrically-derived curve, but this is simply because the curve has been drawn on a logarithmic scale for the sake of convenience.

The gels and difference map together reveal six major sites of antibiotic-induced protection, located near positions 20, 58, 75, 80, 100 and 108 on both strands. These are segments of double helix where the DNAase I cutting rate has been reduced so far as to leave a blank space or "gap" in an otherwise continuous ladder of bands. A seventh site may be discerned from the gap in the gel near position 140 at the top of Figure 2b, which lies beyond the range of accurate densitometric analysis but is clearly visible in the photograph. The narrowest of these protected zones is "site 2", which extends for

only six bonds on each strand; while the broadest is "sites 5, 6", which extends for 15. But this very long site may be resolved into two shorter 6-bond blockages when examined closely (Figure 2a), so it is more probably a combination of two closely-spaced sites. The same may hold true for the rather broad "sites 3, 4" near position 75, although the data provide no direct evidence in this regard.

All seven of these drug-protected regions seem to be associated with one or more steps of the kind CpG, a dinucleotide arrangement found at positions 17, 22, 35, 58, 73, 76, 78, 95, 100, 107 and 141 of the primary sequence (Figure 3). The match is a good one: "site 1" with steps 17, 22; "site 2" with step 58; "sites 3, 4" with steps 73, 76, 78; "sites 5, 6" with steps 95, 100, 107; and "site 7" with step 141. Not every CpG produces this effect, however, as evidenced by the weak protection near step 35; so the sequence CpG might better be regarded as a prerequisite for protection from DNAase I, rather than as its sole determinant.

Adjoining many of these protected sites are regions where the DNAase I cutting rate has been increased substantially relative to its value in the antibiotic-free control. Adjoining "site 2", for example, the sequences ATTTTTCTC and ACACTTTA are cut approximately 10 times faster in the presence of the antibiotic (Figures 2a, 2b, 3). One can find regions of enhanced cleavage surrounding almost all of the protected sites, the notable exception being "sites 5, 6" which are flanked on both sides by G/C base pairs. The other five sites are flanked on at least one side by A/T base pairs, and all of these AT-rich flanking regions exhibit enhanced rates of cleavage.

The ability to measure patterns of protection and enhancement on both strands, and then to centre in on the determining sequences, contributes greatly to the accuracy of our observations. Rates of DNAase I attack are strongly correlated between the two strands of the double helix, with a slight stagger of 2-3 bonds toward 3'-ends. Hence, in Figure 3, the curve for the lower Crick strand may be superimposed on its Watson counterpart by a slight left-to-right shift in the 5'-direction. At "site 2", for example, protection on the Crick strand extends between positions 54 and 60; the same area of protection extends between positions 56 and 62 on the Watson strand, with step CpG in the middle at position 58.

In accordance with previous studies (13-17), we interpret the antibiotic-induced protection of certain sites as evidence for binding at or near the protected locality. The presence of a bound echinomycin molecule could inhibit DNAase I action through either of two mechanisms: localized change

in DNA structure or a reduction in DNA accessibility. We are less certain as to the meaning of the antibiotic-induced enhancements, but think it likely that they come about primarily through a consequential change in DNA helix structure. Digestion experiments with a second enzyme, DNAase II, seem to support this view.

DNAase II Footprinting

Patterns of DNAase II digestion in the presence and absence of echinomycin are shown in Figures 4a and 4b; a densitometric analysis of these data is presented in Figure 5.

Without a prior knowledge of the DNAase I results, it would be difficult if not impossible to define accurately limits of antibiotic-induced protection from these gels. DNAase II does not leave a sufficiently uniform distribution of cleavage products to make it generally useful as a footprinting reagent. On the Watson strand in Figure 4a, for example, the only well-defined positions of cleavage lie near positions 115 and 130 in runs of purine nucleotides. Upon careful inspection, antibiotic-induced zones of protection from DNAase II attack can be observed in many of the same places as for DNAase I (e.g. near position 75 in Figure 4a, or near position 100 in Figure 4b), but the actual extent and degree of protection can be mapped with only moderate accuracy.

Regions of antibiotic-induced enhancement are more readily evident. Three regions in particular (Figure 4) are hardly cut at all in the control, but begin to be cut quite well in the presence of echinomycin. These map at position 130 on the Watson strand, where the sequence is TAAAAAGCA, and at positions 50 and 130 on the Crick strand, where sequences are TTGAGAAAAAT and TGCTTTTTACT respectively. Several less prominent zones of enhancement can be detected near position 26 on both strands (ATGAAAA), and near position 68 on the Crick strand (GTAAAG). All of these DNAase II-enhanced regions lie in locations where enhancements were observed with DNAase I; thus, it seems likely that they monitor some drug-induced alteration of helix structure, propagated to either side of the drug binding site through a series of A/T base pairs.

DISCUSSION

A. Protected Sites

The antibiotic-induced patterns of protection observed here for two different enzymes, DNAase I and DNAase II, correlate well (Figure 6). All sites of DNAase I protection lie in the close vicinity of CpG sequences, and all but two sites of DNAase II protection coincide with those observed for

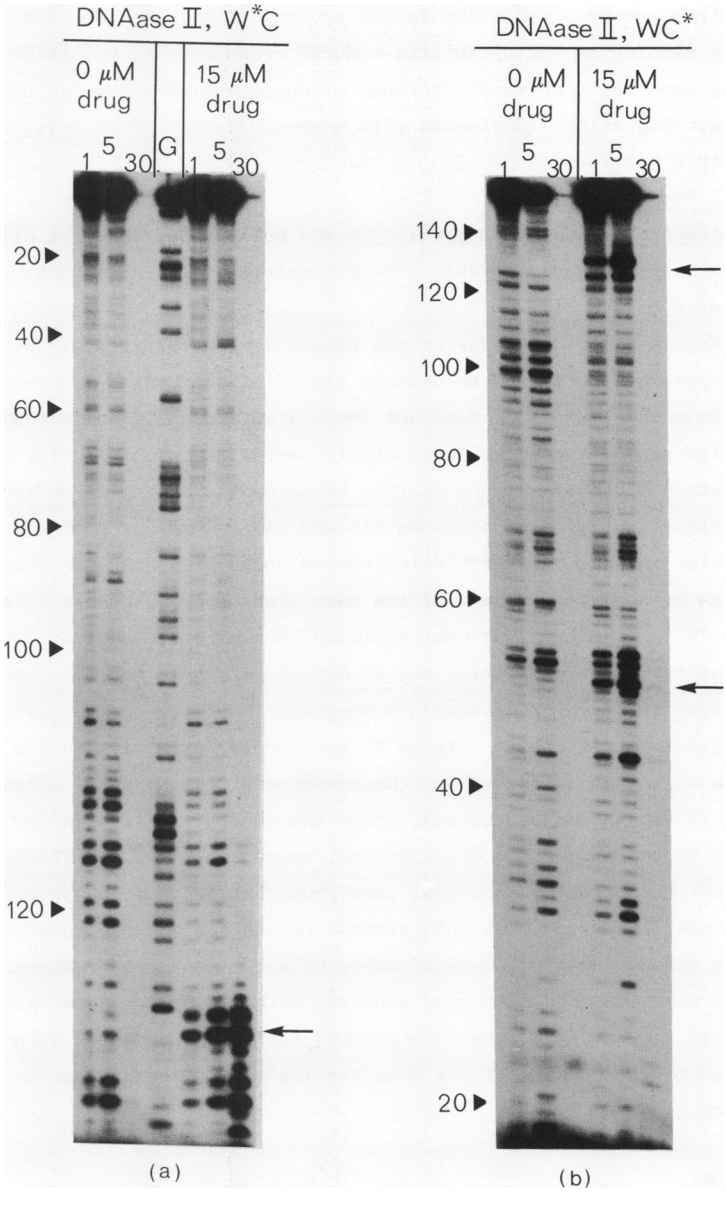

Figure 4. DNAase II footprinting of echinomycin on the 160-base-pair DNA fragment. Lettering as in Figure 2 above. Arrows at the right indicate prominent regions of enhanced cleavage in the presence of antibiotic. After 5 min. digestion not more than 20% of the starting material had been cut; the material in the 30 min tracks was rather over-digested, so quantitative analysis was restricted to the 5 min digested samples.

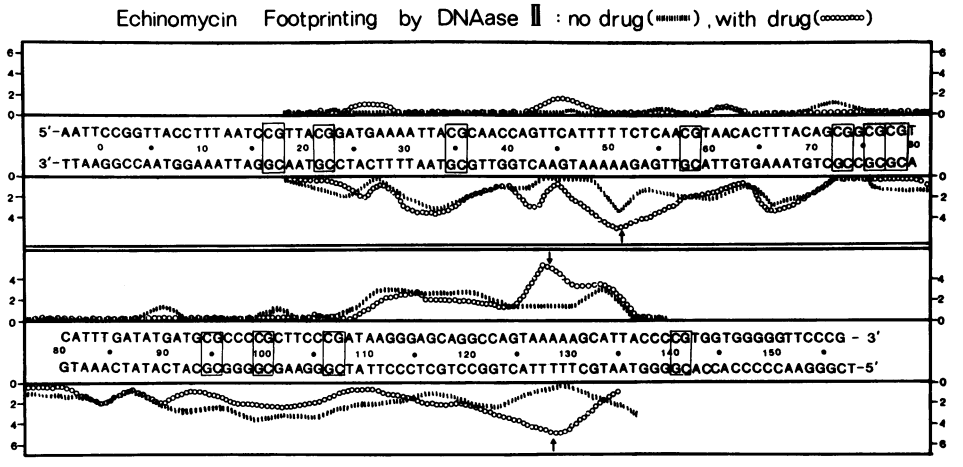

Figure 5. Relative susceptibility to DNAase II digestion in the absence (|||||) or presence (ooooo) of 15 M echinomycin. Vertical scales on both sides are in units of ln(f), where f is the fractional cleavage at any bond in the absence or presence of antibiotic, given closely similar extents of overall digestion (approx. 20% of the starting material in both cases). Each line represents the 3-bond running average of individual data points, calculated by averaging the value of ln(f) at any bond with those of its two nearest neighbours; i.e. ln(f) at bond 100 is averaged with values of ln(f) at bonds 99 and 101 to produce a point on the line at position 100. Arrows indicate regions of strongly enhanced cleavage.

DNAase I. The apparent exceptions occur at positions 90 and 118 on the upper Watson strand, where sequences TATGA and GCAGG are marginally protected from DNAase II attack while DNAase I rates remain unaffected; but these changes may be no more than an indirect consequence of echinomycin binding at adjoining sequences. As will be discussed shortly, rates of DNAase II digestion seem to be sensitive indicators of DNA helix structure, and they appear especially sensitive to drug-induced alterations such as intercalation and unwinding.

Not every CpG step binds echinomycin with equal affinity. At position 35, for example, there is a sequence ACGC which is only weakly protected in gels. This cannot easily be explained in terms of local sequence considerations alone, for there is another sequence ACGC at position 78 which seems to be strongly protected from nuclease attack, and there is a related sequence ACGT at position 58 which also seems to be protected very strongly and selectively. There must be something about the long-range character of sequence environment near the CpG at position 35 which accounts for its unusual behaviour. Perhaps it is the long AT-rich region AAAATTA, to which this CpG step

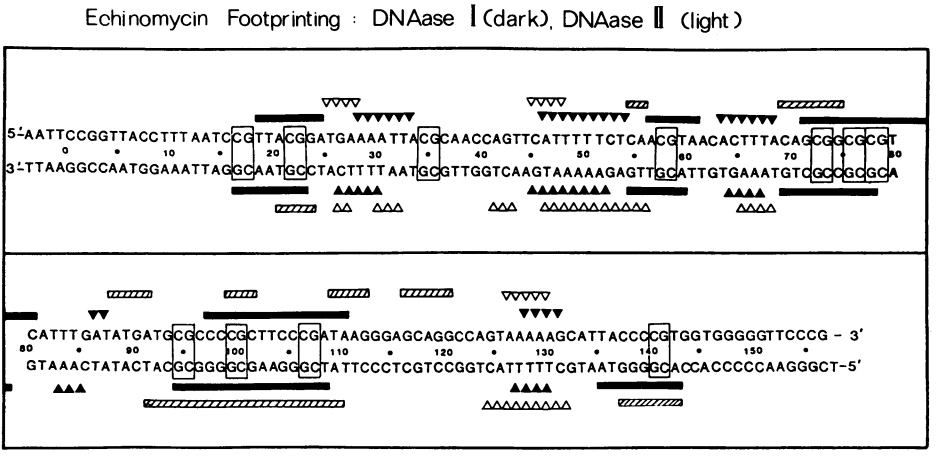

Figure 6. Summary of footprinting results obtained by digestion with DNAase I (dark bars, filled triangles) and DNAase II (light bars, open triangles). Bars indicate protection while triangles show positions of enhancement. Only the clearest, most reliable observations have been included. These were compiled from visual inspection of numerous gels as well as from densitometric tracings, and may be considered as a set of averaged values.

is attached, that hinders its interaction with the antibiotic.

It has been suggested from NMR experiments (23, 24) and molecular model-building (25, 26) that the space between the chromophores in the echinomycin molecule is just sufficient to accommodate two "sandwiched" DNA base pairs. Our footprinting evidence strongly suggests that the optimal site of interaction must contain two C/G base pairs in opposite orientation, but it does not necessarily follow that these should be the "sandwiched" pairs. If we accept the concept of a four-base-pair bis-intercalative binding site deduced from previous work (3, 8, 9, 25), we can envisage that the preferred sequence could be any of XCGY, XYCG or CGXY. The quasi-perfect rotational symmetry of echinomycin, implied by its structural formula (Figure 1) and borne out in its three-dimensional conformation (23-26), suggests that simultaneous intercalation of both quinoxaline chromophores in a symmetry-related fashion would be favoured. This would imply a preference for a symmetrical or palindromic site-sequence such as XCGY above. We have searched our footprinting data for hints of additional symmetry preference but have failed to detect anything positive. We also considered possible recognition sites consisting of three or five base pairs including the sequence CpG, but found no consistent pattern. Thus, the concept deduced from association kinetics (10) that the antibiotic

must greatly prefer one of the two sequences CpG or GpC is upheld, and the preferred sequence is now firmly identified as CpG.

B. Enhancement of Enzymatic Cleavage

It came as something of a surprise to discover that echinomycin should stimulate the rate of digestion at sequences adjacent to its binding site. As summarized in Figure 6, regions of enhanced cleavage occur predominantly in runs of A or T which lie within 5-10 base pairs of a CpG sequence. The DNAase I enhancements are always observed to the same extent on both strands of the double helix, generally with a stagger between strands of about 2 bonds in the 3'-direction. DNAase II enhancements tend to be more variable, sometimes appearing on both strands (e.g. position 130) or else just on one strand (e.g. position 67).

How can we account for these observations? One might initially suppose that both proteins, DNAase I and DNAase II, could exhibit some affinity for the antibiotic molecule: being attracted to the bound ligand, they would then cut more rapidly at sequences adjacent to its binding sites. This model seems unlikely, however, for two reasons: firstly, the conformation of the antibiotic is such as to present few donor-acceptor groups for hydrogen bonding with the enzyme (3, 35); and secondly, only AT-rich regions are affected. The binding sites at positions 95-110, for example, are flanked by GC-rich sequences within which no enhancements are observed.

A better model would suggest that the enhanced cleavage might be due to a conformational change in the DNA brought about by the binding of echinomycin to its preferred sites. It has long been known that echinomycin unwinds DNA by an average of 48° per bound drug molecule (25, 27). This large degree of unwinding would tend to convert the double helix into a kind of weakly-twisted ladder at the intercalation site, and might well have the effect of opening up major and minor grooves at nearby sequences. But why should AT-rich sequences be affected more than others?

Perhaps it is because these sequences exist in a form more susceptible to drug-induced conformational change. Runs of A and runs of T seem to possess a peculiar helical structure not easily recognized by most nucleases (21); they may exhibit an unusually narrow minor groove (28); they twist at a mean rate of 36° per step as compared to 34° for most other sequences (29); and they may have different nucleotide conformations in each of the two strands (30). The echinomycin molecule, presumably by opening both major and minor grooves in the vicinity of its intercalation site, converts these AT-rich sequences into a form more resembling mixed-sequence DNA as regards its

recognition properties towards nucleases.

It is interesting to speculate whether some of the measured 48° unwinding might not be due to helical unwinding in flanking sequences. Supposing that each bound echinomycin molecule were to convert an average of 5 (dA).(dT) steps from their usual 36° twist to a lesser 34° mixed-sequence geometry, then 10° of the total observed unwinding would be accounted for by effects transmitted to neighbouring regions of the helix, leaving 38° as the actual angle of unwinding at the bis-intercalation site. This is about the value reported for a number of synthetic bis-intercalators (31-33).

We are at a loss to explain why the very striking patterns of protection and enhancement seen here were not described by earlier workers (13-17). Possibly their DNA substrates did not contain sufficiently long runs of A and T to observe the effect; or possibly the enhancements were not of primary concern. At all events, we have now observed pronounced enhancements of a similar nature in regions flanking the binding sites of two very different intercalators: actinomycin D and nogalamycin (K.R. Fox, personal communication). What we have reported here, therefore, appears to be a rather general property of sequence-specific intercalating antibiotics.

Footprinting results on two related quinoxaline derivatives, triostin A and its des-N-tetramethyl analogue TANDEM, will be presented elsewhere.

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