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Site and Sequence Specificity of the Daunomycin–DNA Interaction†

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ABSTRACT: The site and sequence specificity of the daunomycin–DNA interaction was examined by equilibrium binding methods, by deoxyribonuclease I footprinting studies, and by examination of the effect of the antibiotic on the cleavage of linearized pBR322 DNA by restriction endonucleases *Pvu*I and *Eco*RI. These three experimental approaches provide mutually consistent results showing that daunomycin indeed recognizes specific sites along the DNA lattice. The affinity of daunomycin toward natural DNA increases with increasing GC content. The quantitative results are most readily explained by binding models in which daunomycin interacts with sites containing two adjacent GC base pairs, possibly occurring as part of a triplet recognition sequence. Deoxyribonuclease I footprinting studies utilizing the 160 base pair (bp) *tyrT* DNA fragment and 61 and 53 bp restriction fragments isolated from pBR322 DNA further define the sequence specificity of daunomycin binding. Specific, reproducible protection patterns were obtained for each DNA fragment at 4 °C. Seven protected sequences, ranging in size from 4 to 14 bp, were identified within the *tyrT* fragment. Relative to the overall *tyrT* sequence, these protected sequences were GC rich and contained a more limited and distinct distribution of di- and trinucleotides. Within all of the protected sequences, a triplet containing adjacent GC base pairs flanked by an AT base pair could be found in one or more copies. Nowhere in the *tyrT* fragment did that triplet occur outside a protected sequence. The same triplet occurred within seven out of nine protected sequences observed in the fragments isolated from pBR322 DNA. In the two remaining cases, three contiguous GC base pairs were found. We conclude that the preferred daunomycin triplet binding site contains adjacent GC base pairs, of variable sequence, flanked by an AT base pair. This conclusion is consistent with the results of a recent theoretical study of daunomycin sequence specificity [Chen, K.-X., Gresh, N., & Pullman, B. (1985) *J. Biomol. Struct. Dyn. 3*, 445–466]. Adriamycin and the β-anomer of adriamycin produce the same qualitative pattern of protection as daunomycin with the *tyrT* fragment. Daunomycin inhibits the rate of digestion of pBR322 DNA by *Pvu*I (recognition sequence 5′-CGATCG-3′) to a greater extent than it does *Eco*RI (recognition sequence 5′-GAATTC-3′), a finding consistent with the conclusions derived from our footprinting studies. Our results, as a whole, are the clearest indication to date that daunomycin recognizes a specific DNA sequence as a preferred binding site.

The sequence specificity of antibiotic–DNA interactions is a topic of intense current interest (Gale et al., 1981; Neidle & Abraham, 1982; Dabrowiak, 1983; Dervan, 1986). The identification and characterization of preferred antibiotic binding sites within DNA sequences are essential for a detailed understanding of the molecular basis of antibiotic action. By understanding what specificity an antibiotic may have, and the molecular determinants of that specificity, it should be possible to develop a rational basis for the design of a new generation of chemotherapeutic agents of enhanced potency.

Daunomycin is an anthracycline antibiotic of clinical importance in cancer chemotherapy. Direct interaction with DNA is thought to be central to the molecular mechanism by which daunomycin acts (Arcamone, 1981). The drug intercalates into DNA and inhibits DNA replication and RNA transcription both in vivo and in vitro. After years of intensive study, daunomycin is now among the best characterized intercalators. The equilibrium (Schutz et al., 1979; Chaires et al., 1982; Graves & Krugh, 1983; Chaires, 1985a) and kinetic (Chaires et al., 1985) properties of the daunomycin–DNA interaction are well characterized. The structure of a daunomycin–oligonucleotide complex is known to atomic resolution (Quigley et al., 1980; Wang et al., 1987). The interaction of daunomycin with nucleosomes (Chaires et al., 1983) and with left-handed Z DNA (Chaires, 1983a, 1985b, 1986) has been explored. These studies show that the drug strongly prefers right-handed B-form DNA as a binding site and will selectively discriminate against alternate DNA conformations.

The possible site or sequence specificity of the daunomycin–DNA interaction remains, however, undefined. In contrast, other DNA binding antibiotics, notably actinomycin and netropsin, show pronounced site specificity (Neidle & Abraham, 1984; Dabrowiak, 1983). Solution studies utilizing synthetic deoxypolynucleotides attempting to elucidate the specificity of the daunomycin binding interaction have shown contradictory results [reviewed in Neidle and Sanderson (1983) and Chaires (1983b)]. Results from this laboratory using purified and well-characterized deoxypolynucleotides dem-

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onstrated, however, that daunomycin preferentially binds to polynucleotides of alternating purine–pyrimidine sequence (Chaires, 1983b). This result is in accord with an earlier suggestion of alternating purine–pyrimidine specificity put forth by Patel, based on NMR studies of a complex of daunomycin with poly(dA-dT)–poly(dA-dT) (Patel et al., 1981). Recent efforts in this laboratory have uncovered the thermodynamic basis of this discrimination. Daunomycin binding to poly(dA-dT) and poly(dG-dC) is driven by a large, negative van't Hoff enthalpy, whereas binding to poly(dA)–poly(dT) and poly(dG)–poly(dC) is characterized by an enthalpy of nearly zero, and is therefore entropically driven (Herrera et al., 1987). The pattern of these thermodynamic results suggests that discrimination between alternating and nonalternating purine–pyrimidine sequences may arise from differing conformations of the polynucleotides. Caution must therefore be exercised in using synthetic polynucleotides as models for base pair specificity.

Conclusions regarding the sequence specificity of the daunomycin–DNA interaction based on theoretical calculations also differ. Newlin et al. (1984) modeled the interaction of daunomycin with tetrameric duplex DNA sequences and predicted preferential intercalation between adjacent AT base pairs. Chen et al. (1985) assumed hexameric duplex DNA sequences for their theoretical calculations and concluded that triplet sequences are required as specific sites for daunomycin, with the optimal site comprised of an AT base pair flanked by adjacent GC base pairs.

Solution studies on natural DNA support the presence of GC base pairs in the optimal daunomycin binding site. Competition dialysis experiments show that daunomycin interacts preferentially with GC-rich DNA (Chaires et al., 1982). Further, the bouyant density of GC-rich satellite DNA in calf thymus is preferentially altered at low binding ratios, suggesting that the drug selectively partitions into these satellite sequences (Chaires et al., 1982). That observation is consistent with an earlier report that showed that the effect of daunomycin on the bouyant density of DNA is dependent on the base composition (Kersten et al., 1966). Daunomycin is intercalated between GC base pairs and is flanked by an AT base pair at the 5′-position in the crystal structure of the drug–oligonucleotide complex determined by X-ray crystallography (Quigley et al., 1980). Even though AT base pairs are available as potential sites in that oligonucleotide, the adjacent GC base pairs must represent the optimal, energetically most favorable, intercalation site in the crystalline form.

Biochemical probes of the sequence specificity of daunomycin have thus far yielded less than satisfying results. Footprinting experiments utilizing either (methidiumpropyl-ethylenediaminetetraacetato)iron(II) [MPE–Fe(II)] (van Dyke et al., 1982) or DNase I (Fox & Waring, 1986b) have not yielded definitive protection patterns for the daunomycin–DNA complex, thus giving no clear indication of possible sequence specificity. In contrast, Robbie and Wilkins (1984) found that adriamycin, a close relative of daunomycin, caused a site-specific inhibition of nick translation in a DNA template identical with that used by van Dyke et al. (1982) for their footprinting studies. Robbie and Wilkins concluded that adriamycin bound specifically to selected GC-rich regions. Daunomycin was shown to inhibit the reactivity of the restriction enzyme *Hpa*I, suggesting a preferential interaction at or near the DNA recognition sequence of that enzyme (Malcolm & Moffatt, 1981).

In order to clarify the base and sequence specificity of the daunomycin–DNA interaction, we have undertaken a multifaceted approach to study the interaction of the drug with natural DNA in solution. Equilibrium binding methods, DNase I footprinting techniques, and an examination of the effect of daunomycin on certain restriction endonucleases have all been employed to provide information about the specificity of daunomycin binding. Our results are mutually consistent and provide the clearest experimental evidence to date that daunomycin indeed recognizes specific DNA binding sites. We find that the preferred site must contain adjacent GC base pairs. Our data further suggest, in accord with the recent theoretical studies of Chen et al. (1985), that the optimal daunomycin binding site may be a triplet sequence, comprised of AT base pairs at the 5′-position, flanked by two contiguous GC base pairs.

Materials and Methods

Materials. Daunomycin and adriamycin were gifts from Dr. F. Arcamone, Farmitalia, Milan, Italy. The β-anomer of adriamycin was a gift of Dr. F. Zunino, Instituto di Tumori, Milan, Italy. Deoxyribonuclease I (DNase I) was purchased from Sigma Chemical Co. (St. Louis, MO) and was stored at −20 °C at a concentration of 7200 units/mL in 150 mM NaCl/1 mM $MgCl_2$. This stock solution was diluted to working concentrations immediately before use.

DNA for Equilibrium Binding Studies. Samples of DNA of varying GC composition were purchased from commercial sources: calf thymus (Boehringer-Mannheim, Indianapolis, IN); *Micrococcus lysodeikticus* (type XI, Sigma, St. Louis); *Clostridium perfringens* (type XII, Sigma, St. Louis); and *Escherichia coli* (type VIII, Sigma, St. Louis). Poly(dG-dC) was obtained from Pharmacia (Piscataway, NJ). Samples were dissolved in 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA), and 0.185 M NaCl, pH 7.0 (henceforth referred to as BPES buffer), dialyzed exhaustively against the same buffer, and fractionated with Sepharose 4B as previously described (Chaires et al., 1982). The DNA samples were characterized by thermal and alkali denaturation. The observed hyperchromicity values in all cases were within 1% of the standard values cited by Mueller and Crothers (1975).

DNA concentrations were determined spectrophotometrically using published values for the molar extinction coefficients at 260 nm (Mueller & Crothers, 1975).

DNA Fragments for DNase I Footprinting Studies. The 160 base pair (bp) duplex DNA fragment containing the tyrosine tRNA promoter (the "*tyrT* fragment") was isolated and labeled as previously described (Drew & Travers, 1984; Low et al., 1984a,b).

Labeled 61 and 53 bp fragments were obtained from pBR322 DNA by the following procedure. A 114 bp fragment corresponding to positions 434–548 was cut from pBR322 DNA by digestion with *Aha*II, and the fragment was isolated by gel electrophoresis using standard procedures (Maniatis et al., 1982). Both strands of the duplex fragment were labeled at their 3′ ends with [α-^{32}P]dCTP, using reverse transcriptase as previously described (Fox & Waring, 1984). The labeled fragment was cleaved with *Hha*I, yielding fragments of 61 and 53 bp, each containing a single label at one 3′ terminus. These fragments were purified by gel electrophoresis on an 8% polyacrylamide gel using standard procedures (Maniatis et al., 1982).

Binding Studies. Absorbance and fluorescence titration methods were used to determine the binding of daunomycin, following the procedures previously described (Chaires et al., 1982; Chaires, 1983b).

Data Analysis. Binding data were analyzed by using the

neighbor exclusion model of McGhee and von Hippel (1974):

$$r/C = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \quad (1)$$

where r is the ratio of bound drug to total DNA base pair concentration, C is the free drug concentration, K is the binding constant for the interaction of drug with an isolated base pair binding site, and n is the exclusion parameter. Experimental data were fit to eq 1 using a nonlinear least-squares fitting routine available through the NIH PROPHET computer resource.

Competition Dialysis. Competition dialysis experiments followed the basic procedure of Mueller and Crothers (1975). Aliquots of *M. lysodeikticus* and *C. perfringens* DNA of identical concentration were dialyzed against a common solution containing daunomycin. Following 48–72 h of equilibration, the amount of daunomycin bound to each DNA was determined by fluorescence measurements following the addition of dimethyl sulfoxide to a final concentration of 50% (v/v) to dissociate the drug–DNA complex (Chaires, 1986).

DNase I Footprinting Experiments. The procedures for DNase footprinting and the electrophoretic analysis of the cleavage products were exactly as previously described (Fox & Waring, 1984, 1986a,b).

Densitometry and Analysis. Autoradiographs were scanned on a Joyce–Loebl microdensitometer, yielding profiles from which the relative intensity of each band was measured. Band intensities were transformed into values for the fractional cleavage as previously described (Low et al., 1984a,b; Fox & Waring, 1984, 1986b). Plots of ln (f_a/f_c) versus band number, where f_a and f_c are the fractional cleavage at a given bond in the presence of antibiotic and in the control (i.e., DNA alone), respectively, were used to represent the differential cleavage at each internucleotide bond.

Effect of Daunomycin on the Rate of Digestion of pBR322 DNA by EcoRI and PvuI. The general procedure described by Malcolm and Moffatt (1981) to analyze the effect of antibiotics on the rate of digestion of individual sites by restriction enzymes was used. The plasmid pBR322 was isolated by cesium chloride density gradient centrifugation (Maniatis et al., 1982) and was linearized with either *Pvu*I or *Eco*RI (both from New England Biolabs, Inc.). Linearization was verified by agarose gel electrophoresis, using the system described by Maniatis et al. (1982). The rate of digestion of *Eco*RI-linearized pBR322 DNA by *Pvu*I, or the rate of digestion of *Pvu*I-linearized pBR322 DNA by *Eco*RI, was determined in a buffer containing 100 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol at 37 °C. A DNA concentration of 50 μM (bp) was used for all experiments, and final enzyme concentrations of 0.2 unit/μL for *Pvu*I or 0.06 unit/μL for *Eco*RI were used. Digestion of linearized pBR322 DNA alone or in the presence of daunomycin was initiated by the addition of the appropriate enzyme. Aliquots were removed at known time intervals, and the digestion was stopped by dilution into 5% glycerol, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.04% bromophenol blue, and 0.04% xylene cyanol. The aliquots were heated for 5 min at 70 °C, and the digestion products were resolved by electrophoresis in a system using 0.7% agarose in 50 mM Tris–borate, 50 mM boric acid, and 0.5 mM EDTA. Typically, 0.08 μg of total DNA was loaded into each lane. Gels were stained with ethidium bromide and photographed. Negatives of the photographs were scanned, and the relative area of each band was determined with a Bio-Rad Model 620 video densitometer equipped with a Model 3392A integrator. The area of the ethidium-stained band was found to be a linear function of the total amount of loaded DNA over the range 0–0.15 μg in a separate control experiment. The amounts of DNA used in our experiments fell within this linear range. The relative amount of uncut, linear pBR322 DNA was expressed as

$$f = A/A_T$$

where A is the area under the band for the linearized form and A_T is the total area under all bands. Linear least-squares fits of ln f versus time allowed for the estimation of the first-order rate constant for cleavage at the restriction site of interest.

Table I: Summary of Binding Constants and Exclusion Parameters Estimated for the Interaction of Daunomycin with DNAs of Varying GC Content[a]

DNA	% GC	K ($\times 10^5$ M^{-1})	n (bp)
poly(dG-dC)	100	37.0	2.8
M. lysodeikticus	72	15.0	3.1
E. coli	50	7.1	3.3
calf thymus	42	6.6	3.6
C. perfringens	31	3.9	3.0

[a] Binding parameters were estimated from data obtained in fluorescence and absorbance titration experiments and analyzed according to the neighbor exclusion model of McGhee and von Hippel (1974) (eq 1). The high affinity of daunomycin toward some of these DNAs, particularly those rich in GC, made it difficult to cover a wide range of r values in our binding studies. For example, in the case of *M. lysodeikticus* DNA, only a range in r of 0.2–0.3 was accessible by the optical methods used. For this reason, we assign a rather liberal error estimate of 15–20% to the values of K. Values for the exclusion parameter are more narrowly constrained, probably to within 10%.

Results and Analysis

Equilibrium Binding Studies. Table I summarizes the results obtained from a series of equilibrium binding experiments which examined the interaction of daunomycin with DNA of varying GC content. Values for the apparent equilibrium constant, K, are seen in Table I to increase with increases in the overall GC content of the DNA. The neighbor exclusion parameter, n, does not vary in any systematic way. The qualitative result is clear and suggests that daunomycin interacts preferentially with GC base pairs in some fashion. The ratio of binding constants for the interaction of daunomycin with DNA from *M. lysodeikticus* and *C. perfringens* may, using the values from Table I, be defined as

$$\alpha = K_{Ml}/K_{Cp} = 3.9$$

The parameter α is a quantitative measure of base specificity and, as will be discussed, provides a means of discerning the base composition of the preferred drug binding site (Mueller & Crothers, 1975; Dougherty & Pigram, 1983).

The competition dialysis method of Mueller and Crothers (1975) provides an alternate experimental method for the determination of α. Data using the DNA pair *M. lysodeikticus* and *C. perfringens* were obtained by this method over a range of r values of 0.17–0.3. Defining $\alpha' = r_{Ml}/r_{Cp}$, where r_{Ml} and r_{Cp} denote the ratio of bound daunomycin per base pair for *M. lysodeikticus* and *C. perfringens* DNA, respectively, the results of our experiments are described in the linear equation

$$\alpha' = 4.15 - 10.74r$$

The intercept value of 4.15 defines α and may be compared directly with the estimate of α obtained above from the ratio of apparent binding constants estimated for the same DNA pair. The two estimates of α agree to within 10%, and we will conservatively use a value of $\alpha = 4.0 \pm 0.5$ in our subsequent analysis.

Table II: Predicted Values of α for Various Assumed Models of Base-Specific Binding[a]

type of site	probability	α
(1) one side, GC	f	2.3
(2) one side, AT	$1-f$	0.4
(3) GA, AG, or GG	$2f-f^2$	1.75
(4) adjacent GC	f^2	5.4
(5) three GC's	f^3	12.5
(6) AGG, GAG, or GGA	$3f^2-3f^3$	2.2
(7) AGG, GGA, or GGG	$2f^2-f^3$	4.1

[a] The value of α is the ratio of intrinsic antibiotic binding constants for two DNAs of varying GC content, calculated here for the DNA pair *M. lysodeikticus* (72% GC) and *C. perfringens* (31% GC). The method of calculating α using concepts of conditional probabilities has been outlined in detail (Mueller & Crothers, 1975; Dougherty & Pigram, 1982). Briefly, the probability of finding a particular type of antibiotic binding site is determined by the fractional GC content of the DNA, f, as listed in the second column. The value of α is then essentially the ratio of the probability of a given type of site for two types of DNA, i.e., $\alpha = P_{Ml}/P_{Cp}$ (where the subscripts denote *M. lysideikticus* and *C. perfringens* DNA, respectively).

The numerical value of α is a reflection of the type of base pair specificity governing the drug–DNA interaction (Mueller & Crothers, 1975; Dougherty & Pigram, 1983). Table II lists the predicted values of α for several assumed models that might describe the binding specificity of daunomycin binding to DNA. The calculation of α values using simple conditional probability concepts is straightforward and has been described for several models involving one and two base pair sites (Mueller & Crothers, 1975; Dougherty & Pigram, 1983). We have used the same approach to calculate α values for more complex models involving three base pairs, shown as the last three entries in Table II.

Inspection of Table II indicates that simple models of base specificity involving one base pair cannot account for the experimentally observed value of $\alpha = 4.0$. More complex models are therefore required to explain the observed value. Two models predict values of α near 4.0. Model 4, assuming preferential binding to adjacent GC base pairs, predicts $\alpha = 5.4$, while model 7, assuming binding to the triplet sites AGG, GGA, or GGG, predicts 4.1. Within the error of our determination of α, we cannot rationally distinguish between these two possibilities, although the latter model of drug binding to triplet sequences predicts an α value closest to the experimental value. The important consequence of this analysis is that several possible models are eliminated and several properties of the preferred site are defined. We can state with confidence that daunomycin preferentially interacts with a site containing GC base pairs and that adjacent GC base pairs are a particular feature of the preferred site. The preferred site is possibly a triplet sequence, in which adjacent GC base pairs are flanked by either an additional GC base pair or an AT base pair.

DNase I Footprinting Studies. An example of the DNase I digestion pattern of the 160 bp *tyrT* fragment alone and in the presence of increasing concentrations of daunomycin is shown in Figure 1. Data were obtained at 4 °C. Over 100 bands are satisfactorily resolved in each gel lane, providing the primary data for analysis. The presence of daunomycin clearly alters the digestion pattern compared to that of the control lane containing DNA alone. For example, regions of lower intensity compared to the control lanes, indicative of sites protected from digestion, are readily apparent near positions 40, 60, and 75. Regions of higher intensity, indicative of enhanced cleavage, are apparent near positions 37 and 55. One additional notable feature of the data of Figure 1 is the dependence of the intensity of certain bands on the total antibiotic concentration, especially near positions 40 and 60. A similar

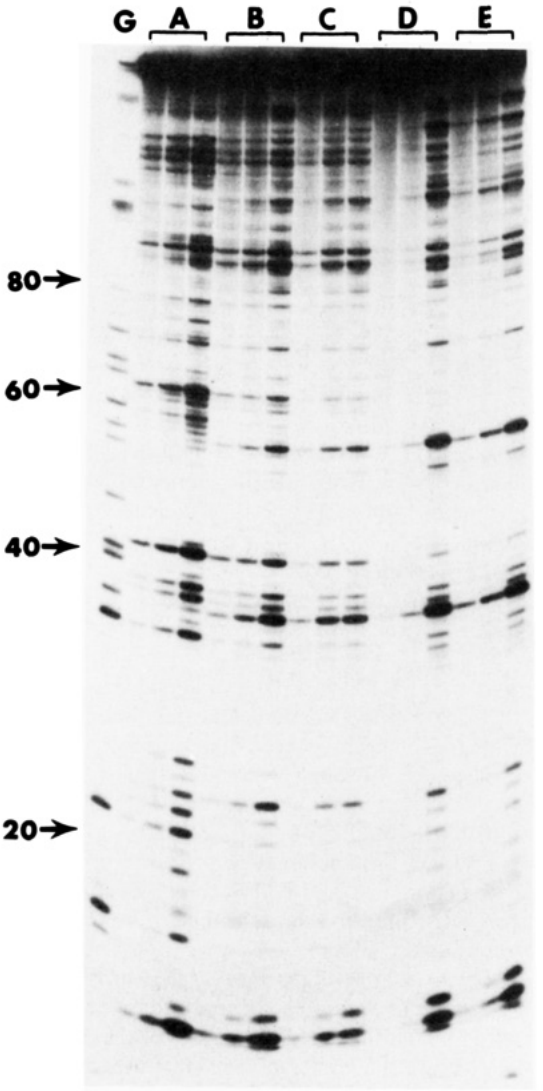

FIGURE 1: DNase I footprinting of daunomycin bound to the 160 bp duplex DNA fragment from *E. coli* containing the tyrosine tRNA promoter region. The sequence of the fragment is shown in Figure 2. In this experiment, the 3′ end of the lower "Crick" strand of the fragment has been labeled with [^{32}P]dCTP. Digestion with DNase was carried out at 4 °C, and aliquots for analysis were removed at 1, 5, and 30 min. These time points for each sample are presented (from left to right) in three adjacent lanes in the autoradiograph. The control (A) contained no daunomycin. Increasing total concentrations of daunomycin are shown in the remaining lanes: (B) 1 μM; (C) 2 μM; (D) 4 μM; (E) 8 μM. The lane labeled "G" shows the products resulting from the Maxam–Gilbert dimethyl sulfate–piperidine reaction and indicate the location of guanine residues within the sequence.

concentration dependence was observed for nogalamycin (Fox & Waring, 1986a) but was not observed for actinomycin or distamycin (Fox & Waring, 1984). DNase I digestion patterns in the presence of daunomycin obtained at 37, 25, and 4 °C were found to be qualitatively similar, but with improved resolution at the lower temperatures (data not shown). This undoubtedly arises from the increase in the lifetime of the bound ligand at the lower temperatures. From our previous kinetic data (Chaires et al., 1985), we estimate that a decrease in temperature from 37 to 4 °C would result in an approximately 20-fold increase in the lifetime of the bound antibiotic.

Data such as shown in Figure 1 may be more rigorously analyzed by scanning the autoradiogram with a microdensitometer in order to quantify the intensity of each band and then casting the data into the form of a differential cleavage plot as described under Materials and Methods. A differential cleavage plot showing the protection of both strands of the *tyrT*

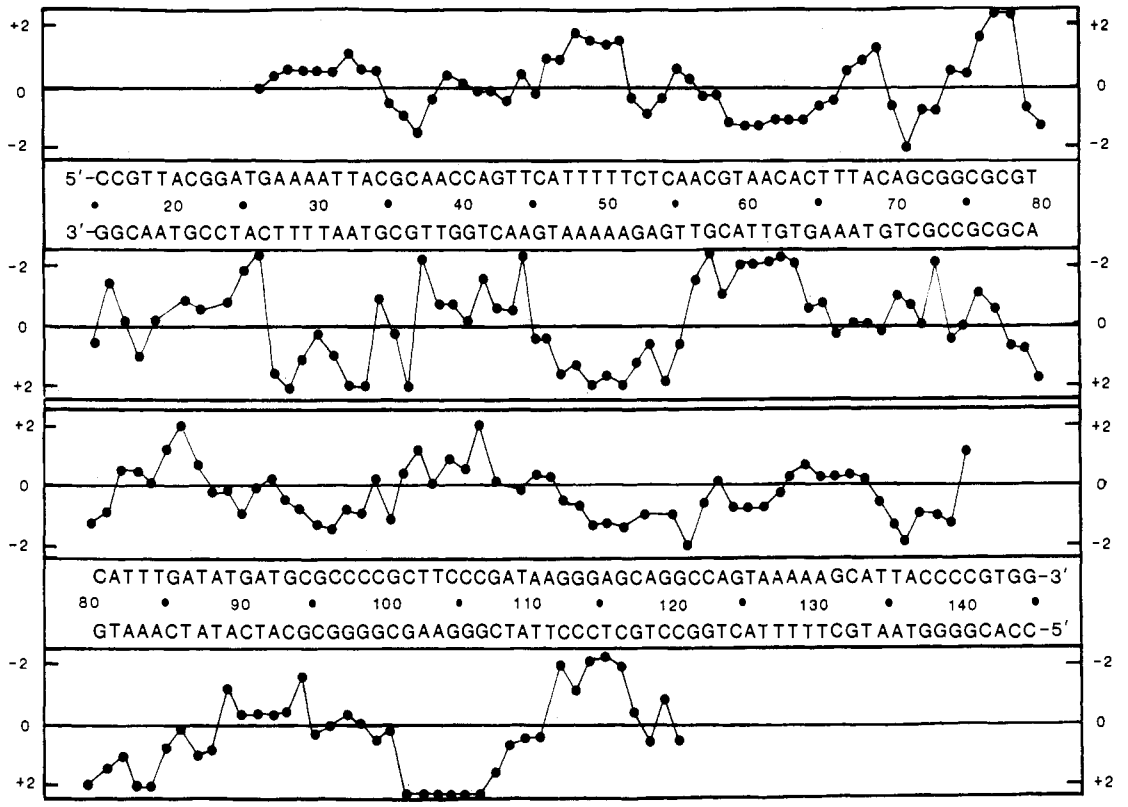

FIGURE 2: Differential cleavage plot for the difference in susceptibility of the *tyrT* fragment to DNase I attack in the presence of 4 μM daunomycin. The sequence of the *tyrT* fragment is shown along the abscissa. The ordinate scales are the dimensionless difference ($\ln f_a - \ln f_c$) where f_a is the fractional cleavage of the same bond in the absence of antibiotic, calculated as previously described (Fox & Waring, 1984). The upper panel shows the differential cleavage of the upper "Watson" strand, while the lower panel shows the differential cleavage of the lower "Crick" strand, observed in each case by the selective labeling of their respective 3' ends. Negative values on the ordinate indicate protection by the bound drug, while positive values indicate enhanced cleavage. Note that the ordinate scales for the two strands are inverted, such that deviation of experimental points *toward* the lettered sequence corresponds to an antibiotic protected site and deviation *away* represents enhanced cleavage.

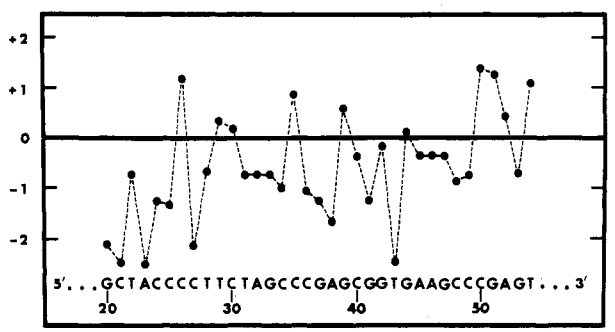

FIGURE 3: Differential cleavage plot for a 61 bp duplex fragment from pBR322 in the presence of 4 μM daunomycin. Data for a single strand are shown. The form of the presentation is described in the caption to Figure 2.

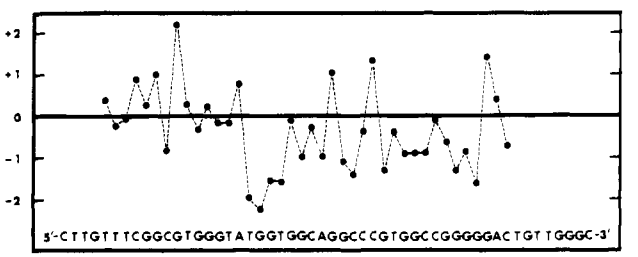

FIGURE 4: Differential cleavage plot for a 53 bp duplex fragment from pBR322 in the presence of daunomycin. Data for a single strand are shown.

fragment by daunomycin is shown in Figure 2. Several regions protected by the antibiotic are evident, indicated by negative ordinate values. Enhanced cleavage, indicated by positive ordinate values, is evident at several other locations.

The pattern of protection of two additional DNA fragments by daunomycin was also examined. Figure 3 shows the differential cleavage plot for one strand of a 61 bp duplex fragment from pBR322 DNA. Figure 4 shows a differential cleavage plot for one strand of a 53 bp duplex fragment from pBR322 DNA. Both of these fragments are largely protected by daunomycin, but strikingly enhanced cleavage is evident at certain positions.

Table III provides a catalog of the sequences protected from DNase I digestion by daunomycin for the three DNA fragments studied. The size of protected regions ranges from a minimum of 3 bp, a value near that of the exclusion parameter of daunomycin (Chaires et al., 1982), to 14 bp, a value corresponding to more than one turn of the B-DNA helix. For the *tyrT* fragment, where data were measured for both strands of the duplex fragment, it is apparent that protected bonds are not always in strict register and that in certain regions protection is staggered across the two strands in an often complex way.

In order to infer the possible base sequence selectivity of daunomycin from the data of Table III with as little bias as possible, the properties of the protected sequences found in the *tyrT* fragment were analyzed by using the algorithm of Pustell and Kafatos (1984). Table IV shows a comparison of the base composition properties of the complete *tyrT* sequence shown in Figure 2 and the protected *tyrT* sequences catalogued in Table III. While the *tyrT* sequence is 49% GC, the overall base composition of the daunomycin-protected *tyrT* sequences is 61% GC. The most frequent dinucleotide in the protected sequences is GpC, instead of TpT as seen in the unprotected sequence. In the *tyrT* sequence, 57 of the possible 64 trinucleotides occur at least once. The most frequently occurring trinucleotides are AAA, TTT, and CCC. In the

Table III: Catalog of Sequences Protected by Bound Daunomycin Inferred from Differential Cleavage Plots[a]

A. tyrT		B. 61-mer from pBR322
1. (19-26)	-------	1. 5' GCTACC
	3' ATGCCTA	
		2. 5' CCT
2. (34-44)	5' CGCAA	
	3' GCGTTGGTCCA	3. 5' TAGC
3. (56-65)	5' ACGTAACACT	4. 5' CGA
	3' TGCATTGTGA	
		5. 5' CGGTGAAGCC
4. (70-74)	5' AGCG	
	3' TCGC	
		C. 53-mer from pBR322
5. (89-99)	5' TGCGCCCC	
	3' ACTACGC	1. 5' TGGTGGCA
6. (112-126)	5' GGGAGCAGGCCAGT	2. 5' GCC
	3' CCCTCGT	
		3. 5' GTGGC
7. (134-139)	5' TTACCC	
	------	4. 5' GGG

[a] For the *tyrT* fragment, the position of the sequence, with reference to the numbering scheme in Figure 2, is indicated in parentheses. Sequence data for both strands are shown where possible. For the upper "Watson" strand, single bond resolution on the sequencing gel was obtained from positions 60–140. For the lower "Crick" strand, single bond resolution was obtained for positions 15–120. The apparent skewing of the protected sites on each strand may be valid only over these regions where single bond resolution is obtained. For the 61 and 53 bp fragments from pBR322 DNA, protection data were obtained for only one strand.

Table IV: Base Composition Properties of the *tyrT* Fragment and *tyrT* Sequences Protected by Bound Daunomycin[a]

	tyrT	protected
no. of bases	130	72
GC content (%)	49	61
most frequent dinucleotides	TT (0.101)	GC (0.128)
	AA (0.085)	CC (0.128)
	CC (0.085)	CG (0.106)
	CG (0.085)	AC (0.106)
	GC (0.078)	GG (0.085)
	CA (0.078)	CA (0.085)
		AG (0.085)
most frequent trinucleotides	AAA (0.039)	CCC (0.075)
	TTT (0.039)	ACG (0.05)
	CCC (0.039)	AGC (0.05)
	TTA (0.031)	GCA (0.05)
	ATT (0.031)	GGA (0.05)
	CCG (0.031)	GCG (0.05)
	GCG (0.031)	GGC (0.05)
	TAC (0.031)	GCC (0.05)
	CAC (0.031)	TAC (0.05)
	GAT (0.031)	CAG (0.05)
	CGT (0.031)	
	CGC (0.031)	

[a] The column labeled "*tyrT*" shows the base composition statistics for the complete *tyrT* DNA sequence shown in Figure 2. The values shown in parentheses are the actual frequencies of each di- or trinucleotide listed within the entire unprotected fragment. The column labeled "protected" shows the base composition statistics for the *tyrT* sequences protected by daunomycin as shown in Table III, part A. The frequencies listed in parentheses are for the di- and trinucleotides within these protected sequences.

protected *tyrT* sequences, only 29 of the possible 64 trinucleotides occur at least once. The most frequent trinucleotide is CCC. Of the nine next most frequent trinucleotides, seven contain adjacent GC base pairs. This analysis shows that daunomycin selectively protects GC-rich sequences and that adjacent GC base pairs are a particular feature of protected sequences.

Equilibrium binding data presented above suggest that daunomycin may recognize a triplet sequence composed of adjacent GC base pairs flanked at the 5′ end by either an AT base pair or an additional GC base pair and the theoretical study of Chen et al. (1985) suggested that daunomycin preferentially binds to the triplet sequence 5′-TGC. If we examine the data of Tables III and IV with the bias that the preferred site is a triplet sequence, the DNase I footprinting data are entirely consistent with that proposal. The di- and trinucleotide frequencies presented in Table IV are consistent with such a preferred site. In all of the protected *tyrT* sequences, a triplet containing adjacent GC base pairs flanked at the 5′ end by an AT base pair may be found. Within that general framework, however, several possible sequences may be found, although there is a distinctly higher tendency for the adjacent GC base pairs to be in an alternating purine–pyrimidine arrangement. Nowhere along the length of the *tyrT* sequence accessible for analysis does the putative triplet recognition sequence occur in a region where it is not protected by daunomycin. Of the nine protected regions found in the 61 and 53 bp fragments from pBR322, seven contain adjacent GC base pairs flanked by a 5′-AT base pair. The remaining two protected sequences are comprised of three contiguous GC base pairs. We conclude that the preferred daunomycin binding site consists of adjacent GC base pairs flanked at the 5′ end by an AT base pair. The requirement for a 5′-AT base pair is not strict, however, and a triplet of GC base pairs also provides an acceptable site. The precise sequence of the adjacent GC base pairs within the triplet is also flexible. While there appears to be some preference for an alternating purine–pyrimidine arrangement (GpC or CpG), nonalternating sequences (GpG) may be found.

Other Cleavage Reagents. Daunomycin was previously shown to have no effect on the guanine-specific reactivity of methylene blue toward the *tyrT* fragment (Fox & Waring, 1986). We found further (data not shown) that the presence of daunomycin does not alter the guanine-specific dimethyl sulfate–piperidine cleavage of the *tyrT* fragment at 24 °C. The latter reaction is particularly sensitive to ligand blockage in the major groove, while daunomycin intercalates through the minor groove (Quigley et al., 1983; Yen et al., 1983). Lack of protection by daunomycin in this case is, therefore, not inconsistent with our DNase I footprinting results. Daunomycin does alter the micrococcal nuclease digestion pattern of the *tyrT* fragment at 37 °C (data not shown). While there are no clear regions of protection in that case, bands showing enhanced cleavage are prominent at positions 50, 84, and 125–129 in the *tyrT* fragment. These coincide with regions showing enhanced susceptibility to DNase I digestion as seen in Figure 2.

Comparison of Daunomycin, Adriamycin, and the β-Anomer of Adriamycin. The sequence specificity of the related anthracycline antibiotic adriamycin, and the β-anomer of adriamycin, was compared to that found for daunomycin using DNase I footprinting. An example of the protection found for adriamycin is shown in Figure 5. There is no apparent difference in the protection pattern seen for daunomycin and that observed for adriamycin. Lower concentrations of adriamycin were required, however, to achieve a similar degree of protection compared with daunomycin. This undoubtedly reflects the higher affinity of adriamycin for DNA and the longer lifetime of the bound antibiotic (Britt et al., 1986). A differential cleavage plot comparing the protection of the upper "Watson" strand of the *tyrT* fragment by daunomycin and adriamycin is shown in Figure 6. These data emphasize that there is no major difference in the protection pattern between the two drugs.

The β-anomer of adriamycin is a stereoisomer in which the orientation of the amino sugar moiety is altered. It binds to

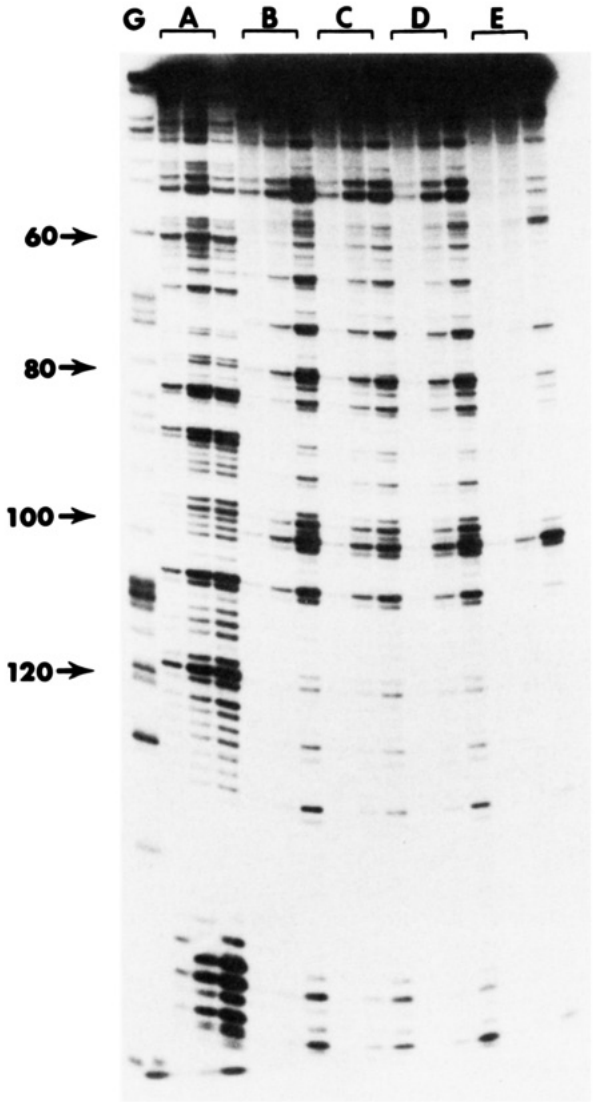

FIGURE 5: Comparison of the DNase I footprinting pattern of daunomycin and adriamycin bound to the 160 bp *tyrT* fragment. In this experiment, the 3′ end of the upper "Watson" strand has been labeled with [α-^{32}P]dATP. The protocol and presentation are identical with those described in Figure 1. (A) Control, no drug added. (B) 4 μM daunomycin. The remaining lanes contain increasing concentrations of adriamycin: (C) 1 μM; (D) 2 μM; (E) 4 μM. The products of the Maxam–Gilbert dimethyl sulfate–piperidine reaction are shown in the lane labeled "G".

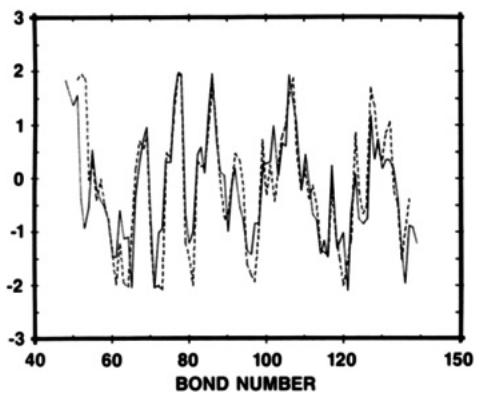

FIGURE 6: Comparative differential cleavage plot for daunomycin (—) and adriamycin (---) bound to the *tyrT* fragment. The form of the plot is described in the legend to Figure 2. The abscissa is compressed relative to that in Figure 2, and the sequence has been replaced by the corresponding bond number. The same qualitative protection pattern is seen for both daunomycin and adriamycin, suggesting that the sequence specificity is the same for the two drugs.

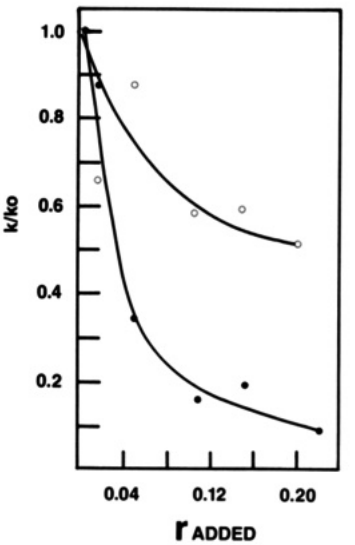

FIGURE 7: Comparison of the relative rate of digestion of linearized pBR322 DNA by *Eco*RI and *Pvu*I as a function of added daunomycin. The relative rate of digestion, k/k_0, determined as described under Materials and Methods, is shown as a function of total daunomycin for *Eco*RI (○) and *Pvu*I (●). Each restriction enzyme has a single recognition site on pBR322. *Eco*RI recognizes the sequence 5′-GAATTC-3′, and k_0 was determined to be 0.03 s^{-1} for the cleavage of its site in pBR322 DNA. *Pvu*I recognizes the sequence 5′-CGATCG-3′, and k_0 = 0.09 s^{-1} was determined for the cleavage of its site in pBR322 DNA. The data show that 50% inhibition of the cleavage reaction occurs at a 5-fold lower concentration for *Pvu*I relative to *Eco*RI.

DNA with an order of magnitude less affinity than does adriamycin and dissociates 20 times more rapidly from DNA than does the parent compound (Britt et al., 1986). Interaction of the β-anomer with the *tyrT* fragment results in only weak protection against DNase I attack, even at concentrations 10-fold higher than used for daunomycin to produce protection (data not shown). Such weak protection as was apparent, however, followed the same pattern as observed for daunomycin and adriamycin.

Inhibition of the EcoRI and PvuI Cleavage of pBR322 DNA by Daunomycin. Observation of differential reactivity at restriction enzyme sites is an established probe for the specific binding of ligands to DNA (Malcolm & Moffatt, 1981). As an alternate biochemical probe of daunomycin sequence selectivity, we examined the effect of the antibiotic on the rate of digestion of pBR322 DNA by the restriction enzymes *Pvu*I and *Eco*RI. Each of these enzymes recognizes a single site on pBR322 DNA. *Eco*RI recognizes the sequence 5′-GAATTC-3′ while *Pvu*I recognizes the sequence 5′-CGATCG-3′. The recognition site of *Pvu*I thus contains the putative triplet recognition sequence for daunomycin inferred from equilibrium binding and DNase footprinting studies. Figure 7 shows the effect of daunomycin on the relative rate of cleavage of pBR322 DNA by the two enzymes. The *Pvu*I reaction is 50% inhibited at a total antibiotic: bp ratio of about 0.04, compared to a ratio of nearly 0.2 required to inhibit the *Eco*RI reaction by half. This observation is consistent with the DNase footprinting results presented above. Closer inspection of the sequences flanking the restriction enzyme recognition sequences in pBR322 DNA shows that the putative daunomycin binding triplet sequence occurs 8 times within 10 bp of the *Pvu*I site, in addition to the recognition site itself. In contrast, that triplet sequence occurs only twice within 10 bp of the *Eco*RI site. The stronger inhibition of the *Pvu*I reaction by daunomycin compared to the *Eco*RI reaction seen in Figure 7 is consistent with the preferential interaction of

the drug at or near the *Pvu*I recognition site.

Discussion

The results presented here provide the clearest experimental evidence to date that the anticancer drug daunomycin recognizes a specific DNA sequence as its optimal binding site. Results from three experimental approaches are presented that are mutually consistent and supportive. Equilibrium binding results indicate that the preferred daunomycin binding site must contain adjacent GC base pairs and further suggest that the drug may recognize a triplet sequence. DNase I footprinting experiments are fully consistent with the presence of adjacent GC base pairs at the daunomycin binding site and further reveal a common triplet sequence within protected regions in which adjacent GC base pairs, most commonly in an alternating purine–pyrimidine arrangement, are flanked at their 5′ side by an AT base pair. Digestion of linearized pBR322 DNA by *Pvu*I (recognition sequence 5′-CGATCG-3′) is inhibited by daunomycin to a greater extent than is digestion by *Eco*RI (recognition sequence 5′-GAATTC-3′), a finding consistent with the general nature of the preferred daunomycin binding site inferred from equilibrium binding and DNase I footprinting studies.

Comparison with Theoretical Studies. The experimental results we report agree with the general conclusions of a recent theoretical study of the sequence specificity of the daunomycin–DNA interaction. Chen et al. (1985) performed computations that modeled the interaction of daunomycin with six duplex hexanucleotides of varying sequence. They inferred from their calculations that the energetically most favorable binding sites for the antibiotic were the triplet sequences 5′-TCG and 5′-ACG. Our experimental results show, however, that the requirement for A or T at the 5′ end is not absolute and that three contiguous GC base pairs form an acceptable, although possibly weaker, binding site. Indeed, from the value of α determined in our equilibrium binding studies, we conclude that an AT base pair cannot be absolutely required within the preferred site. Strict interpretation of the model of Chen et al. (1985) with preferred binding only to 5′-TCG or 5′-ACG predicts $\alpha = 2.2$, a value clearly distinguishable from the value of near 4.0 we observe. Within the restriction fragments of pBR322 DNA we have studied, we observe protected sites comprised of three adjacent GC base pairs (Table III). These conceivably could be lower affinity sites, a point which may be resolved by a closer examination of the antibiotic concentration dependence of protection from DNase I attack. Such studies are currently under way.

Comparison with Other Antibiotics. Preferred binding sites on the *tyrT* fragment have now been mapped for numerous antibiotics (Fox & Waring, 1984, 1986a,b; Low et al., 1984a,b, 1986; Fox & Howarth, 1985). Figure 8 compares the binding sites found here for daunomycin with those previously determined for several other antibiotics of interest. The protection pattern found for daunomycin is unique and clearly differs from that produced by actinomycin, distamycin, and the bis-intercalator TANDEM. The overall extent of protection of the *tyrT* fragment by daunomycin appears to be greater than that observed for these antibiotics. The protection patterns of daunomycin and nogalamycin, a related anthracycline antibiotic, are similar, but not identical.

Footprinting results utilizing DNase I or methidium-propyl-EDTA–Fe(II) [MPE–Fe(II)] have been reported for a variety of other antibiotics (Van Dyke et al., 1982; Van Dyke & Dervan, 1983a,b,c, 1984; Harshman & Dervan, 1985; Scamrov & Beabealashvilli, 1983; Lane et al., 1983). Among these reports, the observed specificity of daunomycin most closely resembles the pattern reported for the nonintercalating compounds mithramycin, chromomycin, and olivomycin. Van Dyke and Dervan (1983) found the preferred binding sites for these antibiotics to consist of three base pairs, containing contiguous GC base pairs. For chromomycin, the most preferred sites, as judged from the dependence of the footprinting pattern on the total antibiotic concentration, were the triplets 5′-AGG and 5′-GGG. Daunomycin behaves similarly in its requirement for a triplet sequence containing adjacent GC base pairs. However, the preferred mithramycin binding sites on the *tyrT* fragment, as determined by Fox & Howarth (1984), are not identical with those of daunomycin (Figure 8). This may reflect subtle differences in the influence of nearby sequences on the specificity of the two compounds, or in the preferred arrangement of the contiguous GC base pairs.

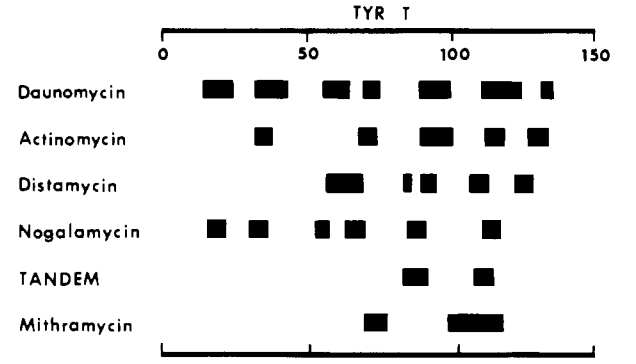

FIGURE 8: Schematic diagram showing regions of the *tyrT* fragment protected from DNase I attack by various antibiotics. The *tyrT* fragment is represented as a line at the top and bottom of the diagram, with tick marks at 50 bp intervals. Protected regions for each antibiotic are indicated by the shaded boxes.

Previous Biochemical Studies of Daunomycin Specificity. Van Dyke et al. (1982) attempted to map specific daunomycin binding sites using the MPE–Fe(II) footprinting method but found no discernible footprinting pattern, even at drug concentrations up to 150 μM. There are several possible reasons for their failure to observe specific sites for daunomycin. First, their experiments were conducted at 37 °C, conditions under which the lifetime of the daunomycin–DNA complex would be short. We found it necessary to conduct our DNase I footprinting experiments at 4 °C to obtain the most discrete protection pattern, even though we were able to see evidence of protection at higher temperatures. DNase I has been shown to be more sensitive than MPE–Fe(II) for mapping binding sites of less stably bound ligands (Van Dyke & Dervan, 1983b). A second, more serious, possibility is that the solution conditions required for MPE–Fe(II) footprinting may damage, or alter, the reactivity of daunomycin. The MPE–Fe(II) method requires 4 mM dithiothreitol and Fe(II). Daunomycin is a strong chelator of iron (Beraldo et al., 1985), and there is evidence for thiol-dependent, nonspecific DNA cleavage produced by daunomycin in the presence of iron (Eliot et al., 1984; Muindi et al., 1985; Mariam & Glover, 1986). The requisite conditions for the MPE–Fe(II) assay may, then, not be suitable for the study of daunomycin specificity. Notably, Robbie and Wilkins (1984) found that adriamycin inhibited the nick translation of a DNA template identical with that used by Van Dyke et al. (1982) at specific GC sites. Their nick translation assay was at 20 °C, a temperature which would result in an increased lifetime of the antibiotic–DNA complex.

Malcom and Moffatt (1981) obtained evidence in restriction enzyme experiments that daunomycin was a more effective inhibitor of *Hpa*I (recognition site 5′-GTTAAC-3′) restriction

in φX174 DNA than was actinomycin, suggesting a possible AT specificity. We note, however, that the sequence 5′-ACC is immediately adjacent to *Hpa*I site 1292 in φX174 DNA and the sequence 5′-TGC is immediately adjacent to site 5022. Thus, a putative daunomycin recognition sequence is near both *Hpa*I sites, and the results reported by Malcom and Moffatt are not inconsistent with the findings we report here.

Possible Structural Basis of Daunomycin Sequence Specificity. The structure of a daunomycin–oligonucleotide complex has been solved to atomic resolution (Quigley et al., 1980; Wang et al., 1987), providing some insight into the possible structural basis of daunomycin binding specificity. In that structure, two daunomycin molecules are bound to the helical form of the self-complementary hexamer d(CGTACG). The drug molecules are intercalated between the d(CpG) steps at either end of the helix and thus are bound to a sequence of the type that we identify here as being a preferred site. The aglycon chromophore of daunomycin is seen in the structure to be oriented at a right angle to the long axis of the DNA base pairs. The cyclohexane A ring and the daunosamine moieties of daunomycin rest in the minor groove of the helix. The complex is stabilized by several hydrogen bonds between daunomycin and DNA bases above and below the bound intercalator. In the major groove, a hydrated sodium ion is coordinated to N7 of the guanine above the intercalator and to O4 and O5 of the bound daunomycin. In the minor groove, the C13 carbonyl oxygen of daunomycin appears to be hydrogen bonded to a water molecule, which in turn is hydrogen bonded to the cytosine above the intercalator. The hydroxyl group on daunomycin C9 participates in two hydrogen bonds with N2 and N3 of the gaunine below the intercalator. Were adenine substituted at this position only, a single hydrogen bond would appear to be possible. These interactions may contribute to the apparent preference for daunomycin intercalation at adjacent GC base pairs.

The theoretical calculations of Chen et al. (1985) provide further insight into the structural basis of daunomycin sequence selectivity. Two key determinants of sequence selectivity were identified by their calculations. The hydroxyl on C9 of daunomycin shows energetically favorable interactions with guanine below the intercalation site, in accord with the interactions observed in the crystal structure. The daunosamine moiety plays an unexpected role in the determination of sequence selectivity. In the crystal structure, the daunosamine is seen to extend in the minor groove to cover a base pair adjacent to the contiguous GC base pairs at the intercalation site. The bound drug thus physically covers three base pairs, in accord with a value of 3 determined for the exclusion parameter from equilibrium binding studies (Chaires et al., 1982a). Chen et al. (1980) calculated that an AT pair is energetically favored at the third position in the binding site, due to repulsive forces that arise between the daunosamine and the 2-amino group of guanine if a GC pair occupies this position.

Possible Biological Implications. Daunomycin is an effective inhibitor of DNA replication and transcription both in vivo and in vitro. How might the apparent sequence specificity of daunomycin affect these processes? No definitive answer to the question is yet possible, but some interesting observations can be made. Brendel et al. (1986) have recently found that within naturally occurring DNA sequences, a certain small set of triplet sequences are either over- or underutilized compared to their expected, random frequency and might, therefore, be important "words" in the language of the gene. Common triplets among this set are sequences to which daunomycin would bind preferentially. A preferred daunomycin binding triplet may be found in the consensus sequence for the CAAT box within promoters (Lewin, 1983), and preferred daunomycin binding sites also abound within the core sequence of enhancer elements (Khoury & Gruss, 1983). Further, two well-characterized sites within the enhancer region of the SV40 genome have been shown to adopt the left-handed Z conformation under torsional stress (Nordheim & Rich, 1983; Hagen et al., 1985). These have the repeating tetrameric sequence $(ATGC)_2$, which contains a high-affinity daunomycin site. The intriguing, but unproven, possibility is that the sequence specificity of daunomycin guides the drug into these special control regions of the genome, where it may selectively interfere with replicative events.

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