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Sequence specificity of actinomycin D and Netropsin binding to pBR322 DNA analyzed by protection from DNase I

(DNA–drug interaction/DNA sequence analysis)

MICHAEL J. LANE*†, JAMES C. DABROWIAK†‡, AND JOHN N. VOURNAKIS*‡

Departments of *Biology and †Chemistry, Syracuse University, Syracuse, New York 13210

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ABSTRACT **A direct approach to determining the sequence specificities of equilibrium binding drugs by using the DNase protection technique is described. The method utilizes singly end-labeled restriction fragments and partial digestion of the drug·fragment complex with DNase I. Microdensitometry of autoradiograms produced after electrophoretic separation of digestion products allows determination of sequences that are affected by drug binding. The feasibility of the technique for locating small ligands bound to DNA and its eventual use as a quantitative thermodynamic approach to studying ligand binding to heterogeneous DNA as a function of sequence is illustrated by using actinomycin D and Netropsin.**

The antitumor agent actinomycin D, used in the treatment of Wilms tumor (1), and the bactericidal/antiviral (2) compound Netropsin are thought to function, *in vivo*, by binding, in an equilibrium fashion, to cellular DNA. It is of fundamental significance to determine the extent to which such compounds have well-defined nucleotide base sequence specificities on natural heterogeneous-sequence DNA. These agents leave no record of their residence positions on the helix—i.e., they neither cleave the DNA backbone as does bleomycin (3, 4) nor covalently modify the bases as does mitomycin (5). Therefore an accurate determination of the locations of preferential interaction sites has been difficult, relying largely on homopolymer studies.

Another approach to the study of the sequence specificity of drug DNA equilibrium interactions is presented in this paper. The DNase protection technique developed by Galas and Schmitz (6, 7) for determining the binding sites of proteins on a DNA helix has been applied to the problem of locating small equilibrium binders such as actinomycin D and Netropsin. The method derives from the strategy of Maxam and Gilbert (8) for rapid DNA sequence analysis. A specific 142-base-pair (bp) restriction fragment is used as template, and the endonuclease DNase I is the binding site probe. Enzyme digestion conditions are rigorously controlled in order to approximate "single-hit" kinetic criteria and to facilitate quantitative evaluation of the extent of nuclease activity at a site containing the drug. The procedure is further developed by the application of microdensitometric scanning of gel autoradiograms to help visualize band intensity changes and to begin to determine thermodynamic parameters for small ligand binding as a function of base sequence.

MATERIALS AND METHODS

Materials. Calf thymus DNA and DNase I were purchased from Sigma. Actinomycin D and Netropsin were obtained from the National Cancer Institute and J. A. Bush (Bristol–Myers, Syracuse, NY), respectively. Both drugs were used without further purification. Restriction enzymes and phage T4 polynucleotide kinase were purchased from Bethesda Research Laboratories and used in accordance with supplier specifications. [γ-^{32}P]ATP having a specific activity of $\approx$2,500 Ci/mmol (1 Ci = 3.7×10^{10} Bq) was obtained from New England Nuclear. The concentrations of the stock solutions of actinomycin D (ε_{440} = 24,450 $M^{-1}\cdot cm^{-1}$) (9) and Netropsin (ε_{296} = 20,200 $M^{-1}\cdot cm^{-1}$) (10) were determined optically by using a Beckman DK-2A spectrophotometer.

DNA Fragment Isolation/End Labeling/Sequencing. Plasmid pBR322 was prepared according to the procedure of Clewell (11). The plasmid was further purified by three phenol extractions after removal from CsCl gradients to prevent endogenous nuclease activity. A 346-bp fragment was cleaved from the plasmid by simultaneous digestion with *Bam*HI and *Hin*dIII restriction endonucleases. The fragment was separated from the larger DNA by either nondenaturing polyacrylamide gel electrophoresis or precipitation with polyethylene glycol (12). This fragment was used as substrate for T4 polynucleotide kinase labeling.

End labeling was performed by either the kinase exchange reaction or forward phosphorylation after alkaline phosphatase treatment (13). The 346-bp fragment was rendered singly end labeled by secondary cleavage with *Hae* III. This procedure yielded two end-labeled fragments 76 and 142 bp long after electrophoresis through a nondenaturing 8% polyacrylamide gel. Analytical electrophoresis was performed with 80 × 40 cm 8.3 M urea/8–15% polyacrylamide gels. Sequence analysis was by the chemical modification method of Maxam and Gilbert (8). The sequence determined for the 142-bp fragment was identical to that reported earlier (14).

DNase I Digestion Parameters. The conditions for using DNase I to probe every base pair in a given sequence have been described (6, 7). At least three factors are important in controlling the extent of digestion to which a drug-bound restriction fragment is subjected.

(*i*) In order to compare the effects of a drug on a given sequence at various input drug concentrations the reaction must be reproducible. We have obtained conditions such that the extent of digestion varies by ±5%. This allows a comparison of each nucleotide base with its congener in an adjacent lane because the digestion results in a uniform distribution of high and low molecular weight oligomers in each lane.

(*ii*) An extensive number of DNase I cleavage events on each DNA molecule will lead to a decrease in the available drug binding sites in the reaction mixture. Such a decrease would be expected to perturb the equilibria present in the system.

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Abbreviation: bp, base pair(s).

‡To whom reprint requests should be addressed.

(*iii*) Overdigestion could potentially give rise to artefactual binding phenomena. For example, a DNA molecule that has been excessively nicked on one strand might bind a ligand at the nicked sites. Subsequent cleavages by the nuclease in the region of the initial lesion would then score affected bases as a binding site.

Exact experimental conditions consistent with the constraints discussed above were determined in a series of trial experiments using a 76-bp fragment of pBR322 prepared as described above. The fragment was digested at various enzyme concentrations. The oligonucleotides produced were then separated from the full-length molecules on a denaturing polyacrylamide gel. The full-length ^{32}P-labeled fragment was excised from the gel, and its concentration relative to a full-length undigested control was determined by Čerenkov counting. In the degradation of the DNA, in the absence of the drug, the amount of full-length (uncleaved) DNA remaining after termination of the digest was 70 ± 5%. Assuming that the degradation is governed by a Poisson process, greater than 80% of the products in the digest were the result of a single cleavage in the original full-length DNA fragment.

Experimental Protocol. The singly end-labeled 142-bp *Hin*dIII/*Hae* III restriction fragment was used for all drug binding studies. After end labeling and elution from a nondenaturing 8% polyacrylamide gel, the fragment was dissolved in DNase I digestion buffer (20 mM Tris·HCl/9 mM $MgCl_2$/2 mM $CaCl_2$, pH 7.5) and stored at 4°C until used. After each labeling, the fragment was assayed for nicks or alkali-labile sites by denaturing in the absence and presence of alkali and electrophoresis on a 10% polyacrylamide gel. DNA samples with detectable breaks were rejected from this study. For actual experiments 20,000–30,000 cpm (Čerenkov) of labeled DNA was digested in the presence and absence of the drug in 8 μl as follows: 2 μl of sonicated calf thymus DNA (0.85 μg/μl), 2 μl of labeled DNA, 2 μl of drug (or buffer), and 2 μl of enzyme. The same buffer (20 mM Tris·HCl/9 mM $MgCl_2$/2 mM $CaCl_2$, pH 7.5) was used to dissolve all components. DNA was incubated for 30 min or longer in the presence of drug at 4°C in the dark prior to addition of enzyme. The reaction vessel was incubated for 1 min at 25°C, then, after addition of the enzyme to the drug/DNA mixture, incubated for an additional 60 sec. The reactions were quenched by the addition of 10 μl of a solution containing 10 M urea, 20 mM NaOH, 20 mM Na_2EDTA, 0.15% bromophenol blue, and 0.15% xylene cyanol and were placed on ice. Samples were then heated at 90°C for 2 min to denature the DNA and quickly placed in an ice/water bath prior to loading onto a gel. All lanes on a given gel received the same total amount of labeled fragment. A typical result is shown in Fig. 1.

Autoradiography and Microdensitometry. After gel electrophoresis, the gel was autoradiographed with Kodak X-Omat AR x-ray film at −20°C with an intensifying screen for periods between a few days and 2 weeks. The levels of radiation and the exposure time used in the experiments produced bands having optical densities in the range 0.01–1.4. Background of the exposed film was 0.3–0.5 OD; therefore, the maximal OD recorded for the oligonucleotide bands was within the linear response range of the film, 0.15–2.5 OD.

A Jarrel–Ash model 23-100 densitometer with slit dimensions 1.6 nm × 10 μm was used to scan all films. A wavelength of 500 nm was used. All lanes, including those in which the DNA had not been exposed to a drug, were scanned three times at various positions along the long axis of the film. Output from a strip chart recorder in percent transmittance was converted to optical density. For this conversion, optical density was calculated from peak heights by using base lines obtained by connecting minima in the densitometric scans. Peak densities for all scans of a given lane were averaged and were reproducible to ±3%.

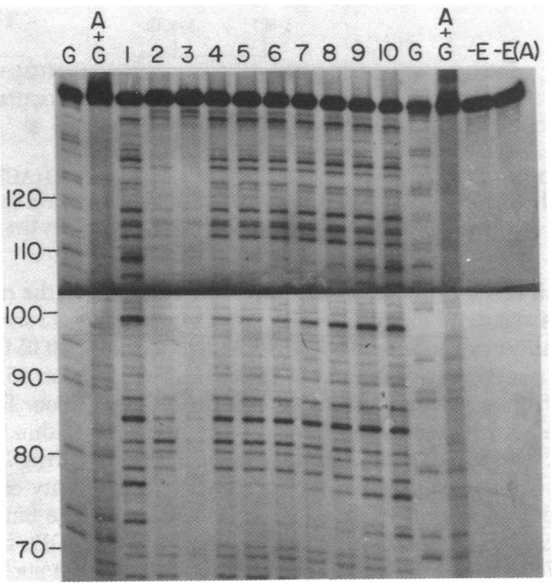

FIG. 1. Autoradiogram of actinomycin D binding experiment with the 142-bp DNA fragment. G and A+G are chemical sequencing lanes. Lanes 1 and 10 contain products of digestion in the absence of the drug. Lanes 2–9 contain products of identical digestions in the presence of (from left to right) actinomycin D at total drug-to-DNA ratios (r_t), in terms of molecules per bp, of 0.178, 0.134, 0.09, 0.071, 0.059, 0.051, 0.044, and 0.022. Lane 3 contains underdigested products. Lanes −E and −E(A) contain products from DNA treated identically but in the absence of enzyme and drug to check for breaks and in the presence of drug alone to check for actinomycin D-induced breaks, respectively. DNase I digestions run in parallel with sequencing reactions show mobility differences for the same length oligonucleotide due to lack of a 3′ phosphate in the enzyme-generated oligonucleotides. Assignment of binding sequences took this mass-to-charge difference into account (15). See Fig. 2 for the sequence. The gel, which is the upper half of an 80-cm gel, shows actinomycin D-dependent protection at bases 72–78 and 102–110.

Data Analysis. Detection of those sequences to which a drug molecule binds preferentially requires that the densitometric scans for a lane generated with drug present are compared with scans for a lane produced in the absence of drug. Actinomycin D binding sites were defined as those sites that exhibited 75% protection from nuclease activity at a ratio (r_t) of input drug molecules to total bp of DNA of 0.09. (All r_t values are expressed as molecules of drug per bp of DNA.) For Netropsin binding sites, the criteria were 75% protection at a r_t of 0.045. The value of protection for each base is given by Eq. 1:

$$\text{Percent protection} = \left(1 - \frac{OD_{Drug\cdot DNA}}{OD_{DNA}}\right) \times 10^2, \quad [1]$$

in which $OD_{Drug\cdot DNA}$ is optical density of a band obtained from digestion in the presence of drug and OD_{DNA} is optical density of the same band obtained from digestion in the absence of drug. The data obtained by using these criteria are summarized, for both actinomycin D and Netropsin, in Fig. 2.

The fluctuation in extent of digestion (±5%) can affect the band intensities significantly and uniformly along each lane. Because of this fluctuation, precise quantitation of the percent protection requires that all band intensities within a given lane be normalized to bands within that lane that show no intensity dependence on input drug concentration. A 25-bp segment, bases 63–88, containing the site 2-ActD (Fig. 2) was analyzed

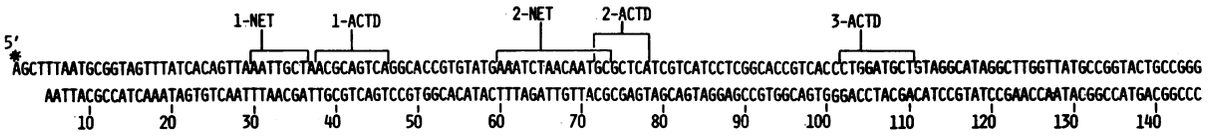

FIG. 2. Summary of Netropsin (NET) and actinomycin D (ACTD) binding sites found in the central region (bases 25–115) of the 142-bp *Hin*dIII/*Hae* III restriction fragment from pBR322. See text for criteria used for binding site assignment. Bases that gave a low band intensity within a site were presumed to belong to that site. The asterisk indicates the position of the label (^{32}P).

in this fashion for actinomycin D, with values of r_t in the range $0.004 \leq r_t \leq 0.178$ (Fig. 3). Of the 25 bases examined, 13 showed intensity great enough to be measured reproducibly (≥0.05 OD). Our analysis shows that the densities recorded at bases 65, 66, and 68 vary randomly as a function of drug concentration. These signals were used to standardize band intensities within each lane. The corrected optical density for a band in a drug·DNA lane is then compared to the corrected optical density of the same band in a minus drug lane by use of Eq. 1. The binding site 2-ActD shows three signals of intensity ≥0.05 OD, bases 77, 74, and 73, which are protected substantially from nuclease cleavage. A plot of percent protection by actinomycin D as a function of $-\log r_t$ (Fig. 4) constructed with corrected peak intensities shows the behavior of peak 73 (C) (arrow 1 in Fig. 3). The other bases (77 and 74) within this region respond identically (data not shown), implying that the entire sequence acts as a unit in the presence of actinomycin D. Ascertaining the free drug concentrations at low r_t will allow calculation of apparent equilibrium constants for such sequences.

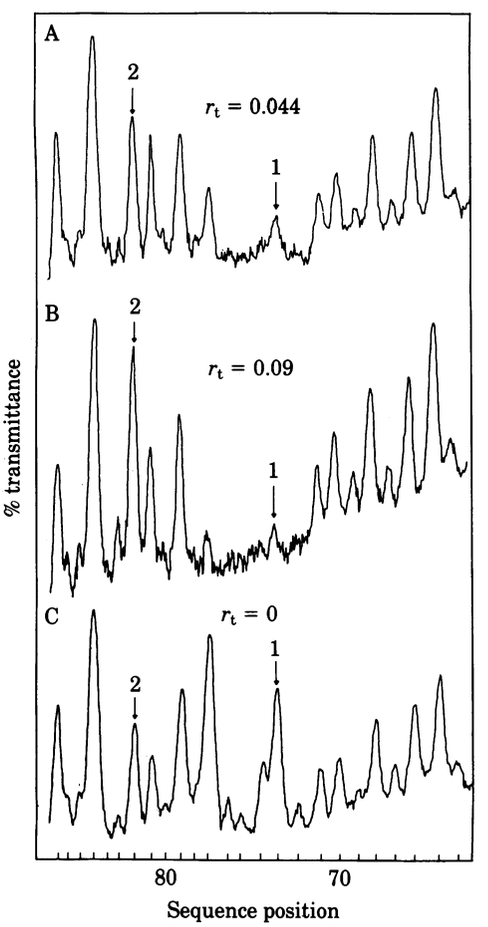

FIG. 3. Densitometric scans of a DNase I partial digest, showing the effect of lower actinomycin D drug-to-DNA ratio (r_t) for a 25-bp segment of the 142-bp fragment that includes 2-ActD (Fig. 2). Peak 1 is base 73 (C) (intensity reduction); peak 2 is base 82 (T) (enhancement).

RESULTS AND DISCUSSION

DNase I protection has been used to determine the binding at specific sequences of several DNA binding proteins, including *lac* repressor (6, 7), topoisomerase (16), and a RNA polymerase III transcription factor (17). These data are consistent with the interpretation that cleavage inhibition occurs at a given base due to steric blockage of the nuclease probe by the protein from that site. Our results with actinomycin D and Netropsin are consistent with this observation.

Actinomycin D Binding. Systematic variation of the total actinomycin D concentration at constant DNA levels shows that while actinomycin D is capable of binding to many sequences at high drug concentration, $r_t > 0.1$ (data not shown), three sites exhibit higher affinity than others (Fig. 2). As r_t is decreased, the bands at each site increase in intensity until, at $r_t = 0$, they are equal to the intensity of bands in the minus drug lanes. The effect of lowering r_t is shown in Fig. 3 at 2-ActD. Each of the sequences showing high affinity for actinomycin D is 7–9 bp in length and ≈32 bp apart. All three sites are rich in G+C and contain the dimer 5′-G-C-3′. This observation is in accord with earlier studies (18–23). It is interesting to note that other G-C sites are present between the observed binding sites but they are not occupied by actinomycin D. The significance of the 32-bp binding-site periodicity observed is unclear and requires further investigation.

Close examination of Fig. 3 indicates that intensity changes occur away from the 2-ActD binding site, the most notable being an enhancement in cleavage at base 82 (T). Our interpretation of this enhancement is that it is due to a conformational change in the DNA molecule induced by actinomycin D binding. This view is consistent with data generated from NMR investiga-

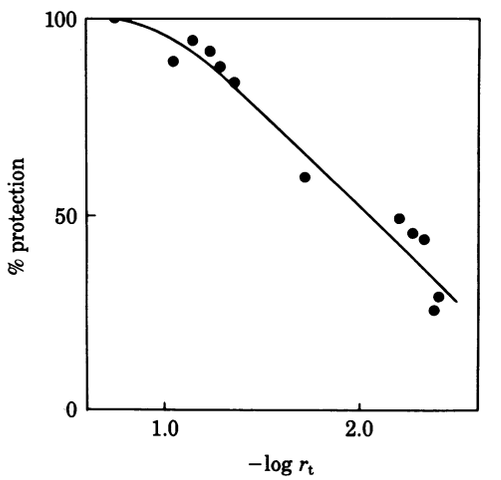

FIG. 4. Percent protection from nuclease cleavage by actinomycin D binding at base 73 (C). Data are generated from 12 separate experiments. $0.004 \leq r_t \leq 0.178$.

tions (20, 21) and block copolymer studies (24), which support the argument that the binding of actinomycin D can affect DNA structure away from the primary binding sequence.

Netropsin Binding. In the presence of Netropsin, DNase I cleavage rate is significantly reduced at two specific locations on the 142-bp fragment (Fig. 2). Both of these sites contain greater than 70% A+T. This result is in accordance with the proposed base preference of the drug (10, 20, 21). Whereas the sites for actinomycin D binding are relatively uniform in length, the two Netropsin sites have significantly different lengths (7 and 14 bp). It is probable that these length differences are a result of different numbers of Netropsin molecules at each site. A more detailed analysis is required to verify this possibility.

Actinomycin D and Netropsin Simultaneous Binding. Reports that actinomycin D and Netropsin can simultaneously bind to the adjacent sites on a dodecamer (20, 21) led us to examine the interaction of actinomycin D and Netropsin in the overlapping regions 2-ActD and 2-Net. Both Netropsin binding (Fig. 5*B*) and actinomycin D binding (Figs. 3 and 5*C*) are clearly visible when the two drugs are added simultaneously (Fig. 5*A*). The nuclease inhibition pattern is similar to that which would be expected by summing the results of the two drug inhibition patterns alone (Fig. 5 *B* and *C*). In the presence of Netropsin, actinomycin D appears to be binding in the same manner as in its absence, as judged by the intensity reduction at base 77 and the enhancement at base 82. The determination of the degree to which the two molecules exert an effect on one another will have to await more detailed analysis. It should be noted that the pattern of nuclease inhibition is independent of the order of addition of the drugs.

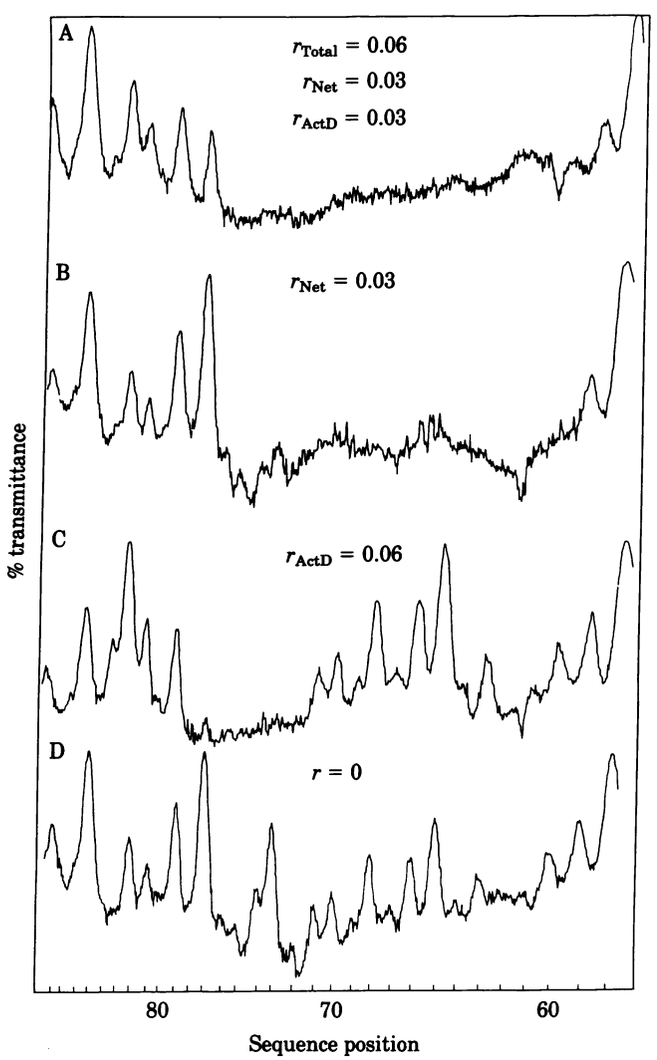

FIG. 5. Densitometric scans showing the effect of actinomycin D and Netropsin around 2-ActD/2-Net (see Fig. 2). Scan A, Netropsin + actinomycin D; scan B, Netropsin; scan C, actinomycin D; scan D, minus drug lane.

Criteria for Protection Analysis. In principle the protection method can be used to study any ligand-induced nuclease inhibition allowing the determination of the sequences at which the ligand binds. The observation of ligand binding sites is limited by the ability of the experimental approach to detect differences that result from digestions in the presence and in the absence of the ligand. The differences in turn depend on the residence time of the ligand at a binding site and on the cleavage rate of the nuclease. Because small ligands possess a diversity of DNA "off" rates, probes that rapidly cleave DNA hold the highest potential for studying drug–DNA interactions. It is clear that for actinomycin D and Netropsin with DNase I as a probe the conditions necessary for observation of nuclease protection have been met.

CONCLUSIONS

In this report we show that DNase I can be used as a probe to study drugs that bind in an equilibrium fashion to natural DNAs. Through careful control of the digestion conditions and with the help of microdensitometry it is possible to quantitatively evaluate drug dependent phenomena at each base site along the DNA helix. The approach is useful for assigning the relative binding affinities of the various drug binding sites and for studying drug-induced structural changes in DNA. With the full development of the technique, it should be possible to evaluate the binding constant for a drug at each of the base positions within a binding site.

While this manuscript was in preparation, Van Dyke *et al.* (25) reported on a similar study using methidiumpropyl-EDTA·iron(II) to probe drug binding sites on ^{32}P-labeled restriction fragments.

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