Bidirectional Transcription Footprinting of DNA Binding Ligands†

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ABSTRACT: An in vitro transcription assay has been developed to define the exact location of DNA binding ligands. The method employs two counterdirected *Escherichia coli* promoters separated by approximately 100 bp. Selective transcription from each promoter yields transcripts up to each ligand site. Nonsaturating levels of ligands result in fractional occupancy of ligand at each site, and hence a range of RNA transcript lengths. The bidirectonal promoter system results in a transcription footprint which was derived from transcription from both promoters up to the 5′ side of each occupied ligand site and defines the sequence specificity and binding site size of the DNA-bound ligand. The transcriptional footprint is precise to ±1 bp from the 5′ and 3′ ends of each binding site. Multiple ligand sites can be ranked in terms of relative fractional occupancy at each site, and the ranking is comparable from either transcription direction. The method was compared to classical DNase I footprinting with a series of DNA binding drugs [actinomycin D, echinomycin, bis(thiadaunomycin), mithramycin, nogalamycin, and an acridine–tripyrrole]. In all cases, specific binding sites were resolved more clearly by transcription footprinting than by DNase I footprinting. Because of the nature of the transcription assay, all occupied ligand sites were detected by this method, in contrast to DNase I footprinting where many sequences are not probed, and where ligand sites are often not accurately defined.

Many of the anticancer agents in current clinical use, or being evaluated, are known to bind to DNA with high affinity (Gale et al., 1981; Wilson & Jones, 1981; Neidle & Waring, 1983; Guschlbauer & Saenger, 1986; Chagas & Pullman, 1987; Sarma & Sarma, 1988). While the physiological significance of the DNA binding ability has not yet been resolved (Siegfried et al., 1983; Glisson & Ross, 1987; Lambert & LePecq, 1987), there has been clear documentation that at least some of the mode of action of these drugs is at the DNA level (Manfait et al., 1982; Denny et al., 1983; Schwartz, 1983; Valentini et al., 1985). There has consequently been intense effort in recent years to define the nature of drug binding sites on DNA, particularly those with high affinity for heterogeneous DNA.

Physicochemical studies of drug–DNA interactions have provided information as to the general nature of the DNA receptor site for drugs but lack detail of the stereochemistry and sequence-specific aspects at individual sites of drug–DNA complexes (Denny et al., 1983; Neidle & Waring, 1983; Neidle et al., 1987). Additional detail has come from X-ray diffraction studies of fibers of drug–DNA complexes (Pigram et al., 1972), and more recently, a daunomycin dodecamer crystalline complex has yielded resolution of atomic coordinates to 1.2 Å (Wang et al., 1987). In parallel with solid-state studies of drug–DNA complexes, there has been an increasing level of detail emerging from NMR analyses of these interactions (Neumann et al., 1985; Luagaa et al., 1986), and this information has been supplemented further by a comparable increase in the application of computer molecular modeling of these systems (Neidle et al., 1987). While all three of these approaches continue to yield a wealth of detail concerning the stereochemistry and dynamics of drug–DNA interactions, they generally require the use of short purified DNA fragments, the sequence of which must be decided in advance of the experiment. These methods are therefore not suited to answering the question as to the DNA sequence dependence of these drugs in stretches of heterogeneous DNA under physiological conditions. The technique of footprinting has been widely used in recent years to define preferred binding sequences for a variety of DNA binding ligands (Dabrowiak, 1983; Van Dyke & Dervan, 1983a; Waring, 1987). This technique is used to define those regions on DNA which are protected by ligands from degradation by deoxyribonuclease I (DNase I)[1,2] or hydroxyl radicals, or inhibition of binding by MPE-Fe(II). However, footprinting with DNase I suffers from a major limitation in that protected sites are often broad and not clearly defined (Lon et al., 1986; Waring, 1987). Furthermore, although in all three footprinting procedures the DNA can be titrated with increasing ligand concentration to discern which sites are protected at low drug levels, it is difficult to establish a quantitative ranking of preferred sites. Also, as a result of differential degradation of the DNA by DNase I, some DNA sequences are not probed. We present here an in vitro bidirectional transcription procedure which overcomes these limitations and has been used to rank the relative affinity of different binding sites on DNA, and to define the sequence specificity to ±1 bp on either end of the binding site.

The range of DNA binding ligands used was selected to represent different modes of interaction with DNA, e.g., actinomycin as a GC-specific intercalator (Aivasashvilli & Beabealashvilli, 1983; Neidle & Abraham, 1984; Chen, 1988), nogalamycin as a CA-specific intercalator (Fox & Waring,

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[1] Abbreviations: ApU, adenylyl(3′–5′)uridine; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; UTP, uridine 5′-triphosphate; GTP, guanosine 5′-triphosphate; dGTP, deoxyguanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; CTP, cytosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; TBE, Tris–borate–EDTA; bp, base pair(s); DNase, deoxyribonuclease.

[2] All sequences shown employ the 5′–3′ notation.

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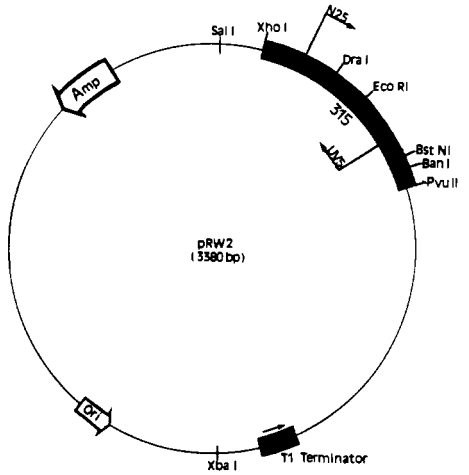


N25: 5′ CATAAATTTGAGAGAGGAGTTTAAA

TAGGGCTGGTTCTCGCAG

UV5: 5′ GGAATTGTGAGCGGATAACAATTTC

FIGURE 1: Structure of the plasmid pRW2. The direction of transcription from the UV5 and N25 promoters is indicated with arrows. Oligonucleotides included in the transcription initiation mixture have been underlined in the DNA sequences shown for initiation from each promoter. The numbering represents the number of nucleotides in RNA chains, and arrows indicate expected stop points during initiation, as defined by the absence of CTP.

1986), bis(daunomycin) as a CACA-specific bisintercalator (Skorobogaty et al., 1988a), echinomycin as a CG-specific bisintercalator (Low et al., 1984; Van Dyke & Dervan, 1984), acridine–tripyrrole as a mixed-function intercalator and minor groove binder (Eliadis et al., 1988), and mithramycin as a G-specific major groove binder (Van Dyke & Dervan, 1983b; Fox & Howarth, 1985).

Materials and Methods

Materials. Actinomycin D was purchased from Calbiochem; mithramycin was purchased from Sigma while echinomycin and nogalamycin were supplied by Dr. L. P. G. Wakelin of the Cancer Institute, Melbourne, Australia. Bis(thiadaunomycin) and acridine–tripyrrole ($m = 2$, $n = 2$) were synthesized in our laboratories (Skorobogaty et al., 1988b; Eliadis et al., 1988). Adriamycin was a gift from Farmitalia Carlo Erba, Milan. *Escherichia coli* RNA polymerase was purchased from New England Biolabs, while Nensorb 20 minicolumns were from New England Nuclear. Ultrapure ribonucleotides, 3′-*O*-methylnucleoside triphosphates, ApU, ribonuclease inhibitor (human placenta), and BSA (RNase/DNase free) were obtained from Pharmacia. [α-^{32}P]UTP, [α-^{32}P]TTP, [α-^{32}P]dGTP, and X-ray film (Hyperfilm-β-Max) were obtained from Amersham. Heparin and DNase I were obtained from Sigma. Urea, agarose, bis(acrylamide), acrylamide, ammonium persulfate, and TEMED were obtained from Bio-Rad as electrophoresis purity reagents. Restriction enzymes and reverse transcriptase were obtained from Boehringer-Mannheim; NA45 DEAE membrane filters were obtained from Schleicher & Schuell. T4 DNA ligase was obtained from Bresatec, Australia. All other chemicals were analytical reagent grade, and all solutions were prepared by using distilled, deionized, and filtered water from a "Milli-Q" four-stage water purification system (Millipore).

Table I: Sequence Specificity of High-Affinity Drug Binding Sites[a]

drug	high-affinity sites	location	preferred sequence
actinomycin	AGCT	37–40	GC
	TGCG	100–103	
acridine–distamycin	GATTAC	49–54	ATT or AATT
	GATTC	56–60	
	GAATTC	65–70	
	GATTTC	77–82	
	TGGATAT	84–90	
bis(thiadaunomycin)	CACA	25–58	CACA
	CACA	27–30	
	ACCA	44–47	
	CACT	60–63	
	CATG	82–85	
echinomycin	CCGG	70–73	CG
	ACGG	53–56	
	GCGG	73–76	
mithramycin	CAGCTA	36–41	GC
	GGCGG	72–76	
nogalamycin	ACAC	26–29	CA
	ACAG	28–31	
	ACAG	35–38	
	TCAT	81–84	
	GCAG	38–41[b]	

[a] Where transcriptional footprints are clearly defined, the longest blocked transcript from the UV5 promoter is shown by a bar above the last transcribed nucleotide, and from the N25 promoter by a bar below the last transcribed nucleotide. The location of high-affinty sites is based on the length of the transcript originating from the UV5 promoter. [b] Numbering is from N25 promoter.

DNA Source. The plasmid pINDU containing the strong N25 early *E. coli* promoter from T5 phage was supplied by Professor H. Bujard (Heidelberg University, West Germany). A 203 bp *Eco*RI restriction fragment of *lac* DNA containing the L8-UV5 double mutant was supplied by Professor D. M. Crothers (Yale University). The 203 bp fragment was digested with *Pvu*II and a 186 bp fragment isolated; the pINDU plasmid was digested with *Pvu*II and *Eco*RI and the remaining vector isolated from the excised fragment. The 186 bp *lac*-containing fragment was then directionally ligated into the vector by using standard methods (Maniatis et al., 1982) and transformed into *E. coli* JM101 cells according to the calcium chloride/rubidium chloride procedure of Hanahan (1983). Screening for the presence of the UV5 fragment and its directional insertion was achieved by restriction enzyme analysis and confirmed by RNA sequence analysis, and the resulting plasmid is shown in Figure 1. The recombinant plasmid was amplified with 170 μg/mL chloramphenicol and isolated by using a modification of the alkaline lysis procedure of Maniatis et al. (1982). The isolated plasmid was routinely digested with *Xho*I and *Pvu*II, and a 315 bp fragment containing the UV5 and N25 promoters was isolated and characterized as previously described (White & Phillips, 1988).

The oligodeoxytrinucleotide dGGA was synthesized on a Model 381A Applied Biosystems DNA synthesizer, analyzed by 5′ end labeling of the detritylated species using [γ-^{32}P]ATP and T4 polynucleotide kinase according to the methods of Maniatis et al. (1982), and used without further purification.

In Vitro Transcription. Transcription conditions employed have been previously described (White & Phillips, 1988). To obtain selective initiation from the UV5 promoter, the 315 bp fragment was digested with *Dra*I, and an initiated ternary

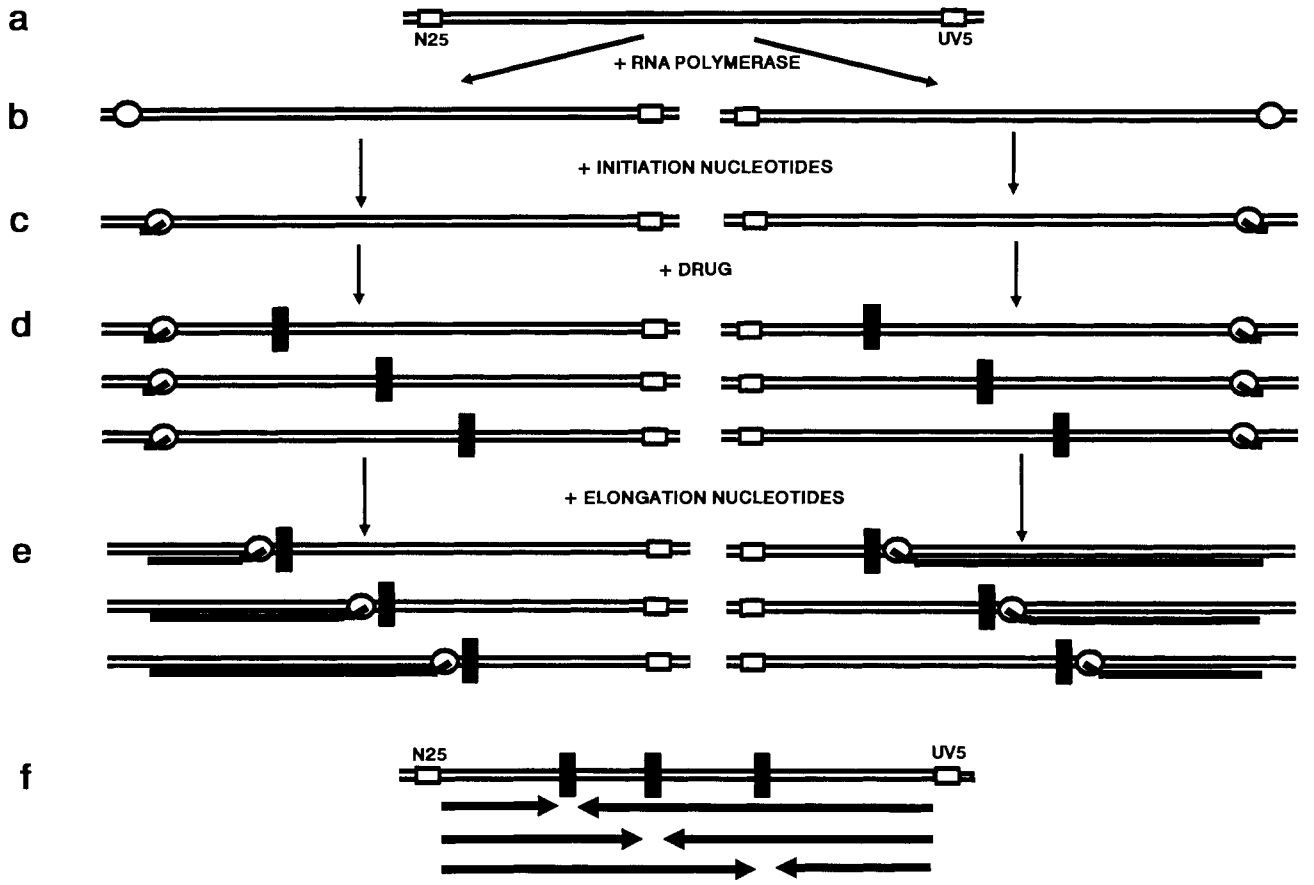


FIGURE 2: Schematic representation of bidirectional transcription footprinting. The DNA fragment containing the counterdirected N25 and UV5 promoters is shown in (a). Selective initiation of either one of the promoters yields the initiated ternary complex (b). The addition of a nonsaturating level of drug results in a range of drug-occupied transcription complexes (d), and upon addition of elongation nucleotides, transcription proceeds up to those drug sites. The RNA transcripts present in (e) are summarized in (f) to show three bidirectional transcription footprints.

complex was formed by using a nucleotide mix containing dGGA (200 μM), 1 μM each of UTP, GTP, and ATP, and [α-^{32}P]UTP. Selective initiation from the N25 promoter was achieved by digesting the 315 bp fragment with *Bst*NI and using ApU (200 μM), 1 μM each of UTP, GTP, and ATP, and [α-^{32}P]UTP.

The initiated complex was then added to by the appropriate drug (to a concentration between 5 and 20 μM) and incubated at 37 or 15 °C for 30 min. An elongation nucleotide mix was then added to give a final nucleotide concentration of 2.5 mM. Incubation was continued at 37 or 15 °C, aliquots were removed at appropriate times, and transcription was terminated by the addition of an equal volume of loading/terminating buffer (10 M urea, 10% sucrose, 40 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue in 2 × TBE) and placed on ice. Sequencing, electrophoresis, and quantitation of RNA transcripts were carried out as previously described (White & Phillips, 1988).

DNase I Footprinting. DNase I footprinting was performed as in Skorobagaty et al. (1988a). To footprint the strand equivalent to UV5 transcripts, the *Xho*I 5′-overhanging end of the 315 bp fragment was labeled with [α-^{32}P]TTP using reverse transcriptase. The strand equivalent to N25 transcripts was labeled by digesting the 315 bp fragment with *Ban*I, the resulting 242 bp fragment was isolated, and the *Ban*I 5′-overhanging end was labeled with [α-^{32}P]dGTP using reverse transcriptase. The labeled fragments were separated from unincorporated label by using a Nensorb-20 minicolumn, lyophilized, and then taken up in a DNase I buffer containing 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM NaCl, and 400 μM (bp) sonicated calf thymus DNA.

Degradation of the labeled fragment was statistically confined to a "one-hit" limit (Goodisman & Dabrowiak, 1985). Typically, 6 μL of the labeled fragment/calf thymus DNA mixture had 2 μL of either additional DNase I buffer or drug solution added and equilibrated at 37 or 15 °C for 30 min. Following equilibration, 2 μL of DNase I (final activity of 5.5 units/mL) was added and allowed to digest for 2 min at 37 or 15 °C; then the reaction was terminated by addition of an equal volume of loading buffer. Calibration of the DNA sequence was performed by G-specific degradation (Maxam & Gilbert, 1980). Electrophoresis and densitometry of the degraded DNA were performed as previously described (White & Phillips, 1988).

Analysis of DNase I footprints involved expressing bands in the drug lanes as a percentage of the area under the comparable control lane. Identification of the size of degraded DNA fragments and location of drug binding sites was achieved by direct comparison to the G sequencing lane.

RESULTS

Overview of Bidirectional Transcription Footprinting. In order to facilitate understanding of the following results, an outline of the steps and processes involved in bidirectional transcription footprinting is summarized in Figure 2. Of the two counterdirected promoters, only one is activated at a time with RNA polymerase to yield a stable initiated ternary complex (Phillips & Crothers, 1986; White & Phillips, 1988). The initiated complex is then equilibrated with subsaturating

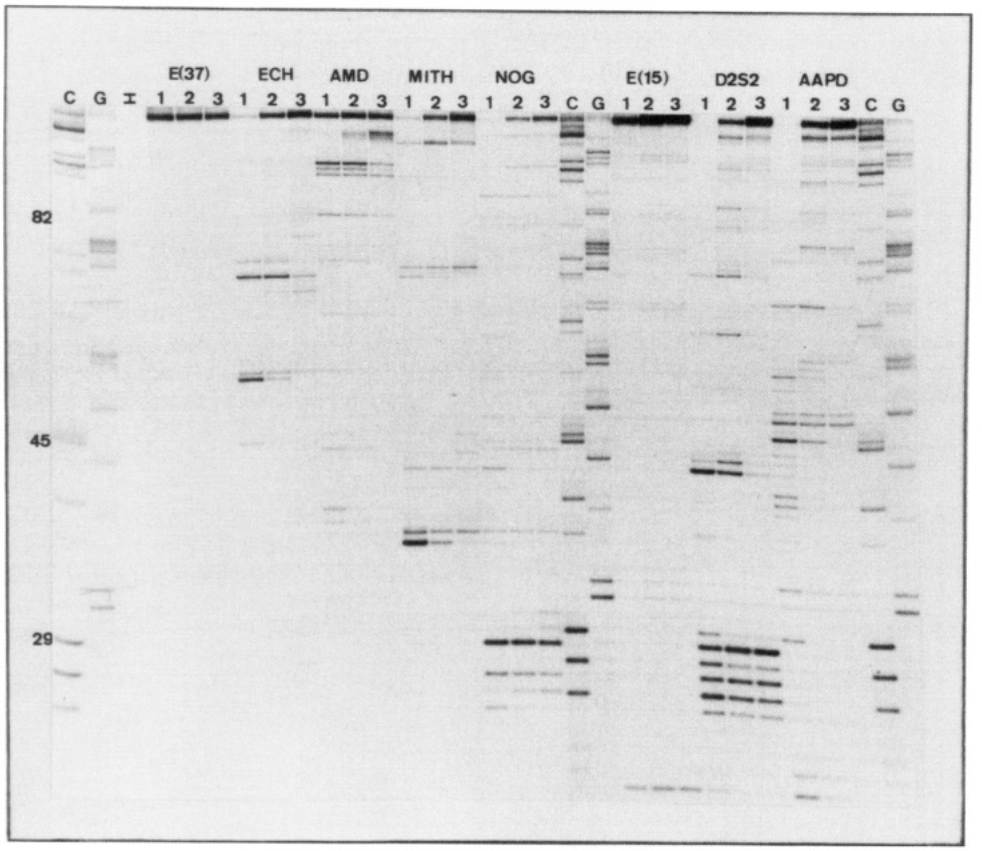


FIGURE 3: Drug-induced blockage of transcription from the UV5 promoter. The autoradiograms shows RNA transcripts from the initiated complex (I) at 37 °C, and control elongation lanes (E) at 15 and 37 °C. Elongation of the initiated complexes for 1, 7, and 20 min (1, 2, and 3) in the presence of 12 μM echinomycin (ECH), 6 μM actinomycin (AMD), 6 μM mithramycin (MITH), and 3 μM nogalamycin (NOG) was carried out at 37 °C, while 3 μM bis(thiadaunomycin) (D2S2) and 12 μM acridine–tripyrrole (AAPD) were elongated at 15 °C. Sequencing lanes were obtained by elongating the initiated complex in the presence of 3′-methoxy-CTP (C) or 3′-methoxy GTP (G).

amounts of drug (or DNA binding ligand) to yield a population of DNA molecules containing drug at only a few potential drug binding sites. The ideal drug concentration employed yields a small fractional drug occupancy at each site (i.e., $\ll 1$). For this reason, when the initiated complex is elongated, the polymerase will have a finite probability of being able to read directly to each occupied site. The elongation process is terminated after a short period of time (to minimize "read-through" past each drug accompanying dissociation of the drug) and results in a series of RNA transcripts of differing length, with each transcript corresponding to a drug-induced block site. This process is then repeated with the second promoter. A combination of the two sets of transcripts reveals a series of nonoverlapping regions, and these are the transcription footprints (Figure 2f).

Transcription. The 315 bp fragment of pRW2 (*Xho*I to *Pvu*II, Figure 1) was used for all in vitro transcription studies in this work and contained two counterdirected promoters for *E. coli* RNA polymerase (N25 and UV5). In order to obtain transcripts arising from only one promoter at a time, the desired promoter was selectively initiated, while the other promoter was deactivated. For transcription from N25, this was achieved by cutting with *Bst*NI, while UV5 activity was selected for by cutting with *Dra*I (Figure 1).

Initiation of the UV5 promoter with dGGA, ATP, UTP, and GTP yielded a nascent RNA 11′-mer, with lesser amounts of 18- and 24′-mers, as observed previously (Phillips & Crothers, 1986; White & Phillips, 1988). Elongation of the initiated complex resulted in a full-length transcript of 119 nucleotides, with a negligible amount of natural pausing (Figure 3). When different drugs were equilibrated with the initiated complex, prior to elongation, specific drug-induced blockages were observed for all drugs (Figure 3), with "read-through" past most sites with increasing elongation time (Phillips & Crothers, 1986; White & Phillips, 1988). When the N25 promoter was initiated with ApU, ATP, UTP, and GTP, the nascent RNA was mainly a 29′-mer, and some 35- and 37′-mers similar to drug-induced blocked transcripts were observed (Figure 4).

The area of each drug-induced blocked transcript was determined by densitometry and expressed as a fractional amount of the total area of all bands in that lane. This fraction therefore represents the mole fraction of RNA transcripts with a particular length, since all transcripts contain the same amount of radioactive label in the initiated region, and there is minimal incorporation of label during subsequent elongation of the RNA.

The mole fraction of RNA observed at each drug-induced block site has been corrected to accommodate the fact that downstream drug sites are exposed to less RNA polymerase than upstream sites because of blockage of polymerase by upstream drug sites. The observed mole fraction of all downstream-blocked transcripts is therefore underestimated, and can be corrected to allow for the probability that they exist behind upstream sites. The corrected relative mole fraction (A_i) is defined by

$$A'_i = A_i/(1 - \sum_{1}^{i-1} A_i)$$

where A_i is the measured mole fraction of each blocked transcript. No allowance was made for read-through from preceding drug sites.

The corrected mole fractions of drug-induced blocked transcripts have been shown (as a percentage) at each site of

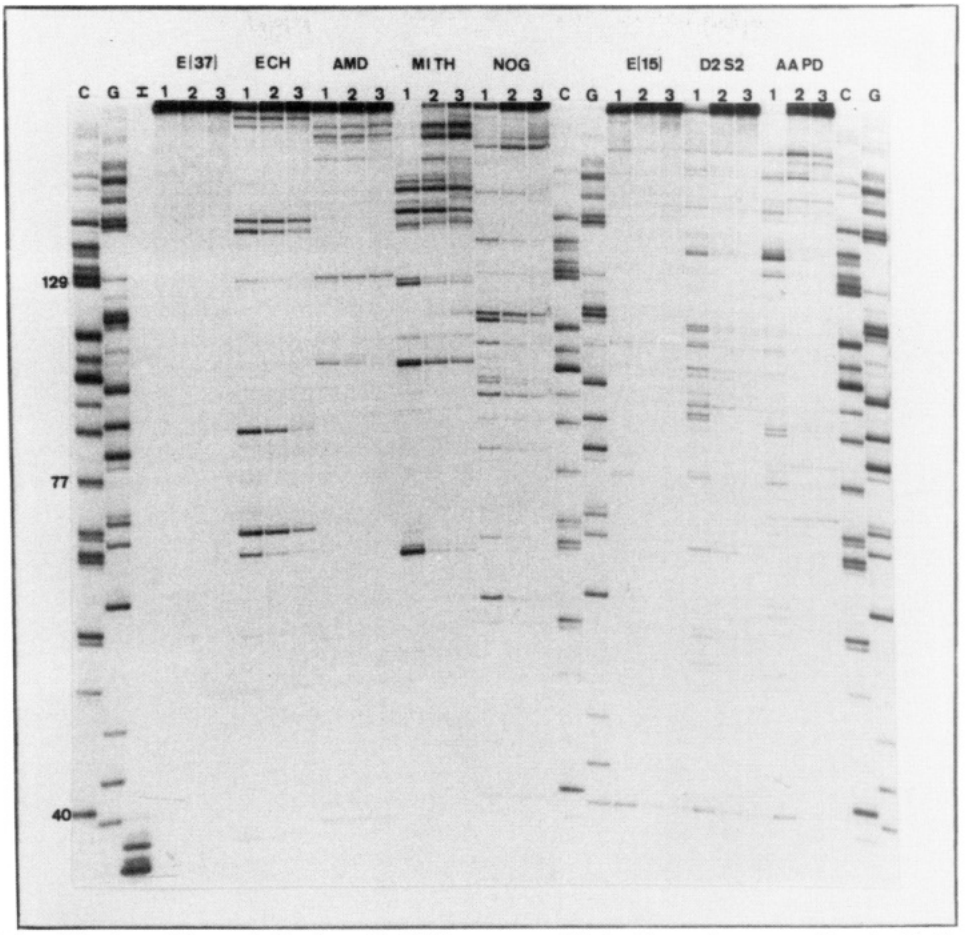


FIGURE 4: Drug-induced blockage of transcription from the N25 promoter. All details are the same as described in the legend to Figure 3 except that mithramycin concentration was 8 μM and nogalamycin concentration was 5 μM.

the transcribed DNA sequences, for transcription (1-min elongation) from both the UV5 and N25 promoters. Since the direction of transcription from these two promoters is toward each other, they transcribe the same DNA fragment, and only the drug-induced effects in the overlapping region are shown in Figure 7.

DNase I Footprinting. The 315 bp *Xho*I–*Pvu*II fragment was end-labeled at the *Xho*I site with reverse transcriptase and [α-^{32}P]TTP and then subjected to conventional DNase I footprinting in the presence of each of the six drugs employed in bidirectional transcription studies. These results are shown in Figure 5, where the sequence probed corresponds to the noncoding strand of the UV5-directed template, but the sequence numbering now begins at the 3′-labeled end of the fragment. The noncoding strand corresponding to the N25-directed template was end-labeled at the *Ban*I site by reverse transcriptase and [α-^{32}P]dGTP and was footprinted in a similar manner with the same six drugs (Figure 6).

Each drug was examined at a range of drug loadings, but only two representative drug levels have been shown in order to accommodate all drug effects on the one sequencing gel. It should be noted that the lower drug concentration was the minimum required in order to observe drug footprints. The higher drug loading lanes have been included primarily to emphasize drug footprints, and also to illustrate that additional lower affinity sites can be detected with increasing drug concentration. All six drugs elicited footprints on both DNA strands at the lower drug loading, and these have been summarized in Figure 7 as a percentage inhibition of DNase I cleavage at each base pair compared to the control in the absence of any drug. Sites where enhanced DNase I cleavage was observed have been summarized as 1, 2, or 3 (Figure 7), and regions of insignificant DNase I activity have also been indicated.

Discussion

Bidirectional Transcription Sequence Specificity. The relative mole fractions for drug-induced blockage of transcription can be interpreted as a relative drug occupancy at each drug site (White & Phillips, 1988). The bidirectional transcription assay therefore defines not only the DNA sequence specificity of the drug but also the relative affinity of drugs for that site. All high-affinity sites, which were clearly defined by blocked transcripts from both promoters, have been summarized in Table I, and the expected sequence specificity was confirmed for all six drugs, i.e., GC for actinomycin (Neidle & Abraham, 1984), $(A{\cdot}T)_n$ for acridine–distamycin (Eliadis et al., 1988), CACA, CAXY, and XYCA for bis(daunomycin) (Skorobogaty et al., 1988a), CG for echinomycin (Low et al., 1984), G for mithramycin (Fox & Howarth, 1985), and CA for nogalamycin (Fox & Waring, 1986).

The transcriptional footprint for actinomycin is very clear and shows drug occupancy at AGCT (37–40) and TGCA (100–104) as substantially greater than GGCG (72–75), and this is consistent with the general model for high-affinity actinomycin sites of XGCY (X $\neq$ G, Y $\neq$ C) (Aivasashvilli & Beabealashvilli, 1983). A weaker AC site is also observed, consistent with earlier findings (White & Phillips, 1988).

The G specificity of mithramycin has recently been defined as two contiguous G·C bp, with a preference for GG or GGG sequences (Fox & Howarth, 1985; Van Dyke & Dervan,

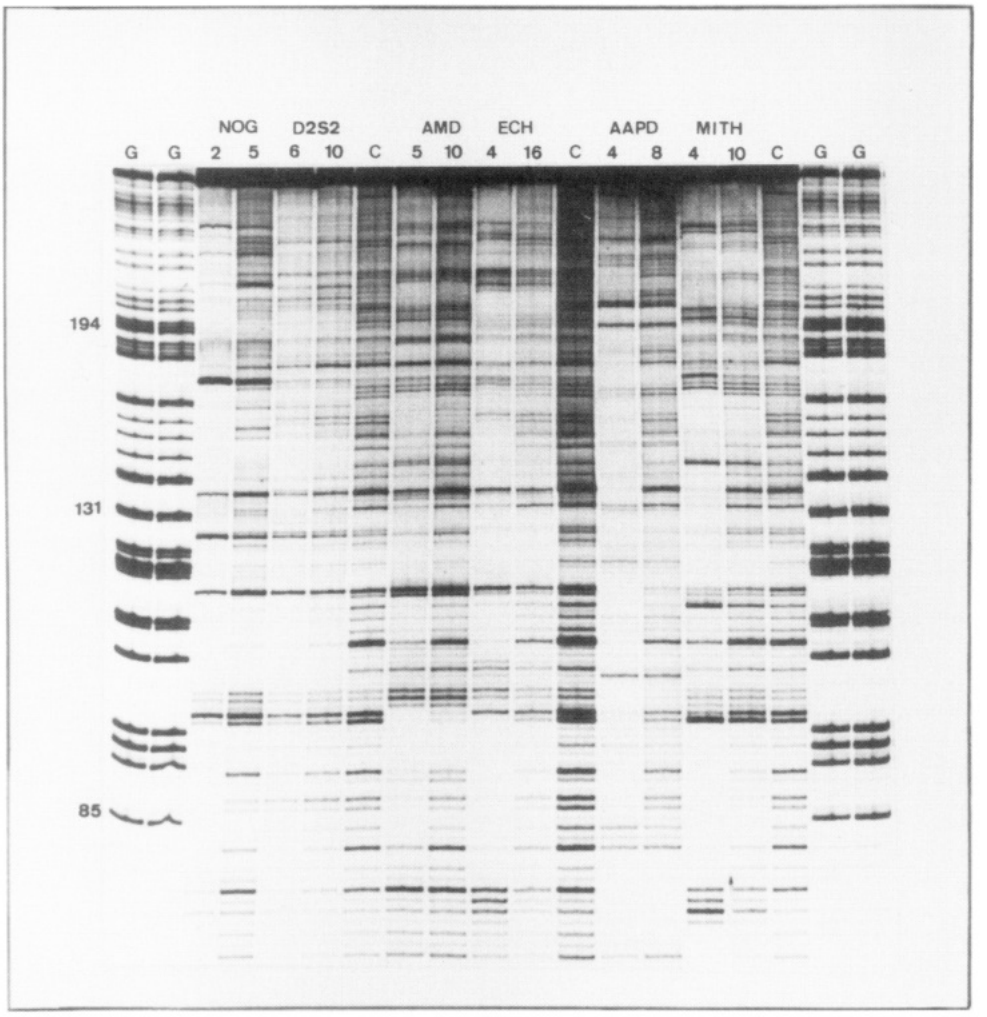


FIGURE 5: Footprinting of the UV5 noncoding DNA strand. The autoradiogram shows sequencing lanes of the 3′-radiolabeled 315 bp fragment probed with DNase I at 37 °C. Lane C is a control in the absence of drug, and G is a Maxam–Gilbert G-specific sequencing lane. All other lanes represent the presence of drugs (see legend to Figure 3) at different bp/drug ratios.

1983). The transcription results confirm the requirement for contiguous G·C bp, but also unambiguously define the preferred binding site as GC (Figure 7). In contrast, there was no detectable binding at GG or GGG sequences which were distributed over the DNA template.

Relative Affinity. The relative affinity for each binding site can be assessed from either promoter. This is best illustrated by the relatively simple bidirectional transcriptional profiles obtained with echinomycin where the relative occupancy at the four sites is the same irrespective of which promoter was activated. The same relative ranking of site occupancy is also observed with mithramycin and actinomycin, but is not so clear when a greater number of binding sites are present (e.g., nogalamycin) where greater errors arise from corrections required for "hidden" downstream sites, and from "read-through" from upstream sites. Errors arising from read-through are readily illustrated by the fact that the first drug site detected from one promoter experiences no contribution from read-through from upstream sites whereas it is the last site detected by transcription from the other promoter and therefore experiences some degree of read-through from previous blocked sites. This problem is minimal for ligands with long residence times and is further minimized by quantitating blocked transcripts after as short an elongation time as possible (1 min in this work).

Sensitivity. The bidirectional transcription assay provides a means of defining a footprint of drug binding sites. How reliable is this new procedure in defining the sequence specificity of drug binding sites, and with what sensitivity and sequence resolution? These questions can be answered from the information summarized in Figure 7 and Table I.

Of the six drugs analyzed in the present study, actinomycin, echinomycin, and nogalamycin have been the most characterized with respect to their DNA sequence specificity, and therefore provide a reference point to answer the question of the reliability of the transcription assay. For actinomycin, 3 GC sequences occur in the transcribed region, and all 3 are detected from both promoters; for echinomycin, 4 CG sites exist, and 3 are seen from both directions; for nogalamycin, there are 13 CA sites, of which 6 are seen from both directions and a further 6 from one direction. The reason that the detection efficiency decreases with an increasing number of sites is because the total radioactivity contained within blocked transcripts is then spread over more locations, resulting in a lower percentage of label at each site. Given the conservative approach used in defining the presence of a drug-induced blockage (at least 15% above that of the control background, which may derive from natural pausing), then some actual drug-induced blockages will be discarded, although they may actually be observed at low levels. Under the experimental conditions employed, we conclude that any ligand with a specificity of less than one site per 20 bp will be detected from both directions with essentially 100% certainty. This conclusion presupposes that the ligand residence time will be

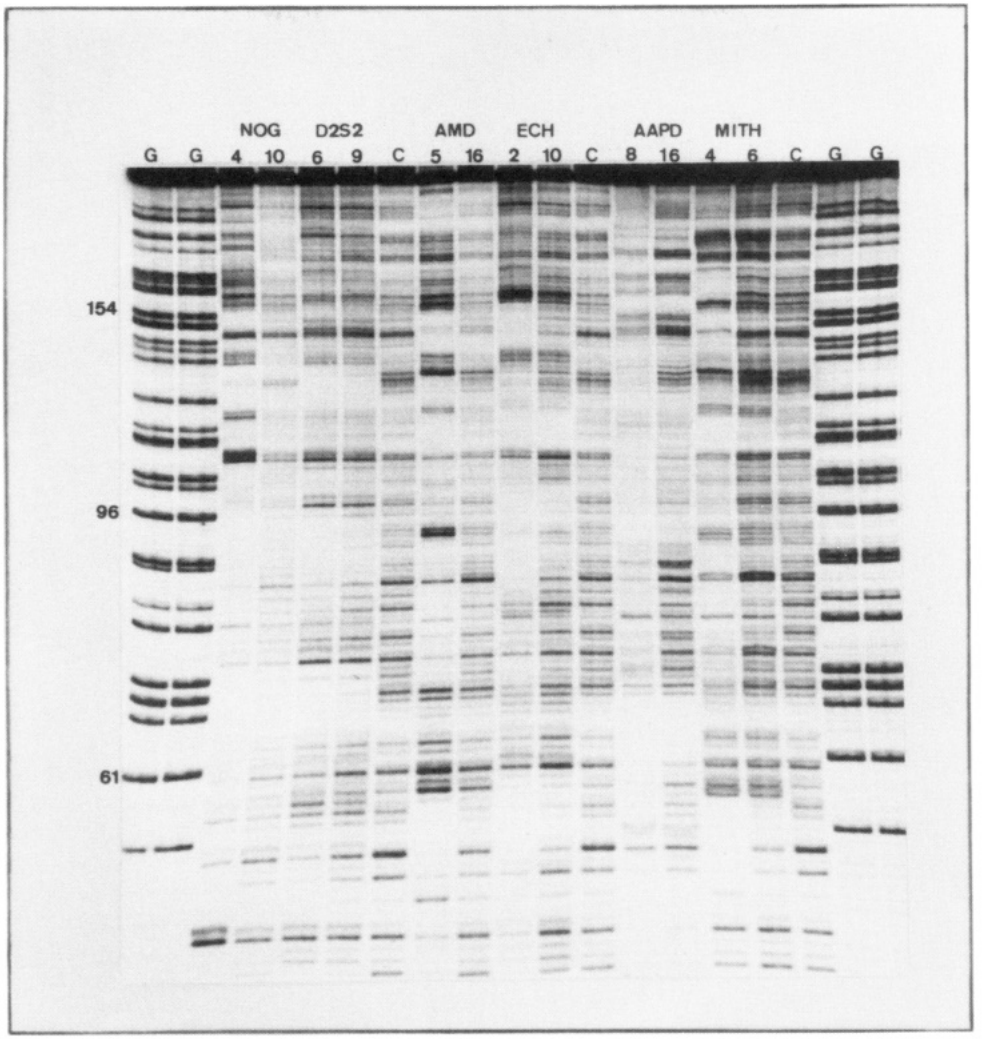


FIGURE 6: Footprinting of the N25 noncoding DNA strand. All details are the same as described in the legend to Figure 5.

sufficiently long for this method to be applicable, and this is of the order of 1 s at 20 °C (Phillips et al., 1988; Skorobogaty et al., 1988b). In the case of echinomycin, the absence of blocked transcripts at CG (102, 103) implies that the GCGA site is an inherently lower affinity site than the other four sites detected.

Resolution and Binding Site Size. The location of blocked transcripts closest to the drug site is summarized in Table I for transcription from both directions. In general, transcription is seen to proceed up to the bp of intercalating sites, as observed previously (Phillips & Crothers, 1986; White & Phillips, 1988). There are, however, several instances where the longest RNA transcribed was from 1 bp prior to the bp comprising the intercalation site (e.g., sites TGCG and GGCG of actinomycin and sites ACAC and GCAG of nogalamycin; Table I and Figure 7). The dominant characteristic in the case of echinomycin is for the blocked transcripts to be 1 bp back from the drug site, with a smaller amount of transcription proceeding one nucleotide further into the intercalation site. This characteristic is even more pronounced with actinomycin, where most transcripts were halted 2 bp back from the AGCT (37–40) site, with a decreasing amount of transcription proceeding up to the intercalation site (Figure 7). In summary, transcription can be viewed as proceeding up to drug sites, or 1 bp upstream of the site, and there may be some additional drug-dependent distribution of transcripts one or two nucleotides shorter than the maximum length drug-induced transcript observed.

The variation of transcript lengths presumably derives from several sources, including the stereochemistry and flexibility of the bound drug as well as the equilibrium state and dynamics of altered DNA conformations in the 5′ region flanking the drug site. While the latter effect would be expected to be more pronounced for those drugs which exhibit a large unwinding angle, there is insufficient data currently available to test this notion.

The most clearly defined stereochemical factor is whether the ligand protrudes into the minor or major grove. For example, the bisintercalator echinomycin is known to have its linker group in the minor grove, and since RNA polymerase tracks in the major grove (White & Phillips, 1988), transcripts from both promoters are observed up to the center of the bisintercalation site, and this is one nucleotide longer (because of DNA helicity) in the 3′ direction than expected from the known CG sequence straddled by echinomycin (Waring, 1987). In contrast, mithramycin, a major groove binder (Keniry et al., 1987), exhibits transcripts which appear to be blocked one nucleotide prior to the apparent GC dinucleotide binding site, and this is consistent with the notion of RNA polymerase tracking in the major groove up to ligands residing in the major groove.

Comparison with DNase I and MPE-Fe(II) Footprinting. DNase I footprints, detected at the lowest possible drug loading, have been included in Figure 7 to enable a direct comparison with bidirectional transcription footprinting of the same six drugs on the same DNA sequence. The most dra-

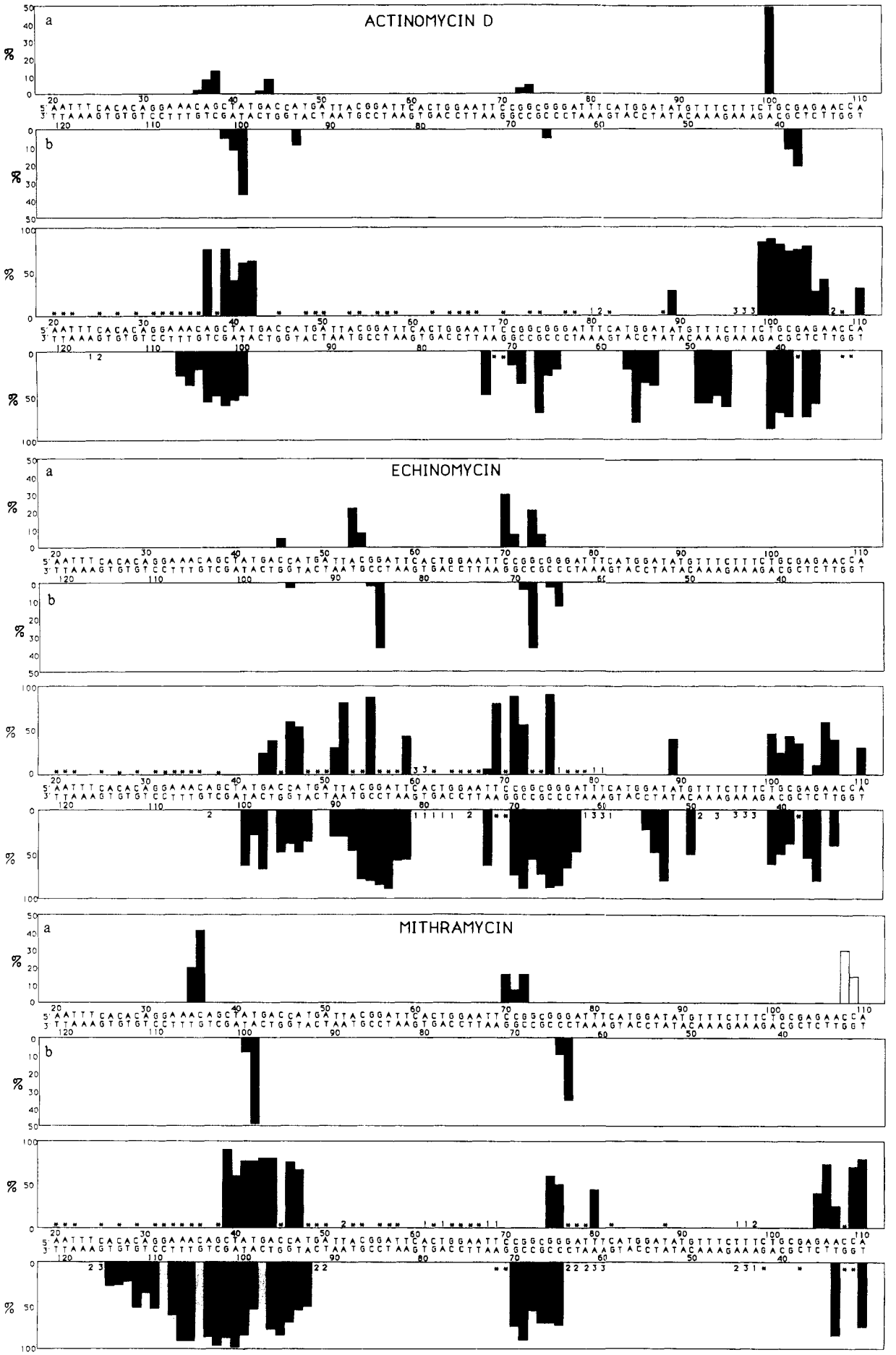
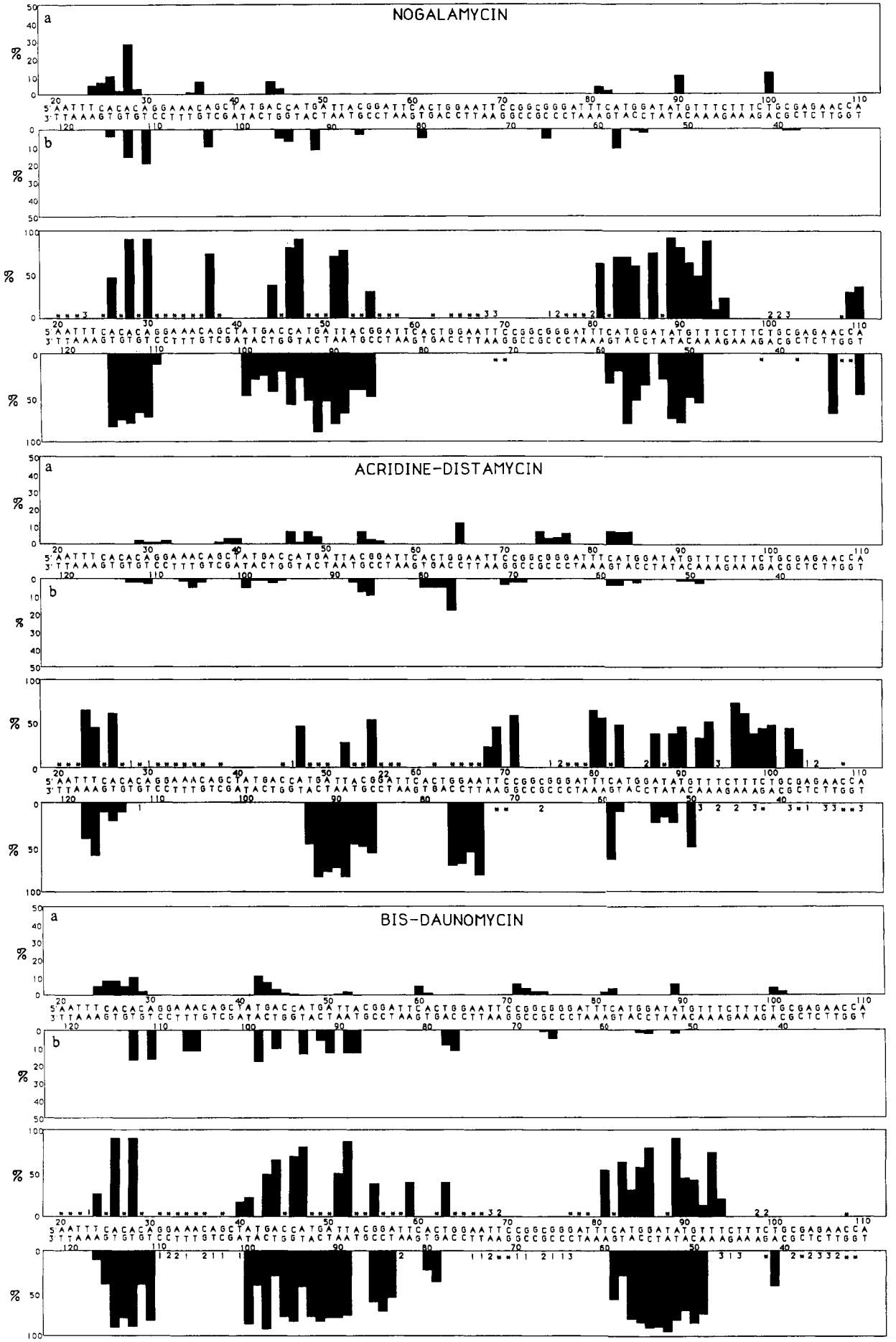


FIGURE 7: Bidirectional transcription footprints and DNase I footprints. The upper panel for each drug shows the mole fraction (as a percentage) of blocked transcripts arising from the UV5 promoter (a) and N25 promoter (b). The lower panel for each drug shows the decreased DNase I cutting efficiency at each site, as a percentage of the control bands. Asterisks show sites which were not cut by DNase I and did not yield



a corresponding band in the control lane. Sites where DNase I activity was enhanced are denoted as 1, 2, or 3, corresponding to increases in area of individual sequence bands of 0–75%, 75–150%, and >150%, respectively. The numbering is from the −2-position of the UV5 promoter (upper strand) and from the +1-position of the N25 promoter (lower strand), as shown in Figure 1.

matic difference is that DNase I footprints are much broader than the precisely defined transcription footprints, and this is best illustrated in Figure 7 by actinomycin (34–42 and 99–106 for DNase I, compared to 38–39 and 100–102 by transcription footprinting), echinomycin (51–59, compared to 54–55), and mithramycin (25–48 and 71–80, compared to 36–41 and 72–76). This lack of definition of the drug site by DNase I footprinting is further illustrated by the fact that the five drug sites referred to above yielded a total of 61 bp of footprint, compared to only 22 bp defined by bidirectional transcription footprinting, and also by the fact that binding sites which are close together (e.g., echinomycin at 71 and 74; Figure 7) are readily resolved by transcriptional footprinting, but not by DNase I footprinting. In general, the resolution of footprint size obtained by transcriptional footprinting is ±1 bp, comparable to that achieved with MPE-Fe(II) footprinting of chromomycin (3 bp), echinomycin (4 bp), and actinomycin (4 bp) (Van Dyke & Dervan, 1983b, 1984; Harshman & Dervan, 1985).

Two additional problems experienced by DNase I footprinting are that not all sequences are probed, and the resulting autoradiogram and subsequent quantitation can be influenced by enhanced cleavage rates adjacent to drug sites (Fox & Waring, 1984). While these factors are less of a problem with other footprinting agents such as MPE-Fe(II) (Van Dyke & Dervan, 1983a) and hydroxy radicals (Tullius, 1987), they do not occur at all with transcription footprinting, where the only problem of "background" arises from natural pausing, and this has been virtually eliminated (White & Phillips, 1988). However, it should be noted that DNase I footprinting is often employed in preference to MPE-Fe(II) because of the greater sensitivity of DNase I (Van Dyke & Dervan, 1983a). The transcription assay also has the capacity to yield ligand dissociation kinetics at individual sites where the number of time points is increased, as described previously (White & Phillips, 1988), and to assess drug-induced effects such as termination of transcription that occurs at some individual drug binding sites (White & Phillips, 1988).

Since RNA polymerase appears to track in the major groove (White & Phillips, 1988), and DNase I in the minor groove (Suck & Oefner, 1986), these probes may therefore yield somewhat different footprints. It is not possible to predict such differences with any detail since they will depend upon a subtle interplay of many parameters, including the groove into which any drug protrudes and the direction (5′ or 3′) of any such protrusion together with its physical size and electrostatic composition. These factors define an excluded physical volume and an excluded electrostatic volume, both of which will be sensed differently by the two different probes.

Conclusion

A new method has been presented for defining the binding site of ligands on DNA. This bidirectional transcription footprinting procedure probes all sites on the DNA and defines both ends of the ligand site to a resolution of ±1 bp. It is therefore much more exact in defining DNA ligand sequence specificity than DNase I footprinting but is comparable to the resolution obtained with MPE-Fe(II) footprinting. Ranking of the relative order of affinity for different sites is readily achieved since the mole fraction of blocked transcript is a direct measure of the relative drug occupancy. Any desired DNA sequence can be inserted in the unique *Eco*RI site between the two promoters, and the method can therefore be applied to protein–DNA interactions.

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Registry No. Actinomycin, 50-76-0; acridine–distamycin, 118970-57-3; bis(thiadaunomycin), 116978-91-7; echinomycin, 512-64-1; mithramycin, 18378-89-7; nogalamycin, 1404-15-5; DNase, 9003-98-9.

References

Aivasashvilli, V. A., & Beabealashvilli, R. Sh. (1983) *FEBS Lett. 160*, 124–128.

Chagas, C., & Pullman, B. (1987) *Molecular Mechanisms of Carcinogenic and Antitumor Activity*, Adenine Press, New York.

Chen, F. (1988) *Biochemistry 27*, 1843–1848.

Dabrowiak, J. C. (1983) *Life Sci. 32*, 2915–2931.

Denny, W. A., Baguley, B. C., Cain, B. F., & Waring, M. J. (1983) in *Molecular Aspects of Anti-cancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 1–34, MacMillan Press Ltd., London.

Eliadis, A., Phillips, D. R., Reiss, J. A., & Skorobogaty, A. (1988) *J. Chem. Soc., Chem. Commun.*, 1049–1051.

Fox, K. R., & Howarth, N. R. (1985) *Nucleic Acids Res. 13*, 8695–8714.

Fox, K. R., & Waring, M. J. (1986) *Biochemistry 25*, 4349–4356.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, & Waring, M. J. (1981) *The Molecular Basis of Antibiotic Action*, 2nd ed., Wiley, London.

Glisson, B. S., & Ross, W. E. (1987) *Pharmacol. Ther. 32*, 89–106.

Goodisman, J., & Dabrowiak, J. C. (1985) *J. Biomol. Struct. Dyn. 2*, 967–979.

Guschlbauer, W., & Saenger, W. (1986) *DNA–Ligand Interactions. From Drugs to Proteins*, Plenum Press, New York.

Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.

Keniry, M. A., Brown, S. C., Berman, E., & Shafer, R. H. (1987) *Biochemistry 26*, 1058–1067.

Lambert, B., & LePecq, J. B. (1987) in *DNA-Ligand Interactions. From Drugs to Proteins* (Guschlbauer, W., & Saenger, W., Eds.) pp 141–157, Plenum Press, New York.

Laugaa, P., Delepierre, M., Leon, P., Garby-Jaureguiberry, C., Markovits, J., LePecq, J. B., & Roques, B. P. (1986) in *The Molecular Mechanisms of Carcinogenic and Antitumor Activity* (Chagas, D. & Pullman, B., Eds.) Chapter 10, Adenine Press, New York.

Low, M. L., Drew, H. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 4865–4879.

Lown, J. W., Krowicki, K., Bhat, U. G. Skorobogaty, A., Ward, B. W., & Dabrowiak, J. C. (1986) *Biochemistry 25*, 7408–7416.

Manfait, M., Alix, A. J. P., Jeannesson, P., Jardillier, J. C., & Theophanides, T. (1982) *Nucleic Acids Res. 10*, 3803–3816.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Neidle, S., & Waring, M. J. (1983) *Molecular Aspects of Anti-cancer Drug Action*, MacMillan Press Ltd., London.

Neidle, S., & Abraham, Z. (1984) *CRC Crit. Rev. Biochem. 17*, 73–121.

Neidle, S., Pearl, L. H., & Skelly, J. V. (1987) *Biochem. J. 243*, 1–13.
Neumann, J. M., Cavailles, J. A., Herve, M., Tran-Dinh, S., Langlois d'Estaintot, B., Huynh-Dinh, T., & Igolen, J. (1985) *FEBS Lett. 182*, 360–364.
Phillips, D. R., Greif, P., & Boston, R. C. (1988) *Biochem. Parmacol. 33*, 225–230.
Pigram, W. J., Fuller, W., & Hamilton, L. O. (1972) *Nature (London), New Biol. 235*, 17–19.
Sarma, M. H., & Sarma, R. H. (1988) *DNA and its Drug Complexes*, Adenine Press, New York.
Schwartz, H. S. (1983) in *Molecular Aspect of Anti-cancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 93–126, MacMillan Press Ltd., London.
Siegfried, J. M., Sartorelli, A. C., & Tritton, T. R. (1983) *Cancer Biochem. Biophys. 6*, 137–142.
Skorobogaty, A., White, R. J., Phillips, D. R., & Reiss, J. A. (1988a) *Drug Design Delivery 3*, 125–152.
Skorobogaty, A., Brownlee, R. T. C., Chandler, C. J., Kyratzis, I., Phillips, D. R., Reiss, J. A., & Trist, H. (1988b) *Anti-Cancer Drug Design 3*, 41–56.
Such, D., & Oefner, C. (1986) *Nature 321*, 620–625.
Tullius, T. D. (1987) *Trends Biochem. Sci. 12*, 297–300.
Valentini, L., Nicolella, V., Vannnini, E., Menozzi, M., Penco, S., & Arcamone, S. (1985) *Farmaco, Ed. Sci. 40*, 377–389.
Van Dyke, M. W., & Dervan, P. B. (1983a) *Nucleic Acids Res. 11*, 5555–5567.
Van Dyke, M. W., & Dervan, P. B. (1983b) *Biochemistry 22*, 2373–2377.
Van Dyke, M. M., & Dervan, P. B. (1984) *Science 225*, 1122–1127.
Wang, A. H., Ughetto, G., Quigley, G. J., & Rich, A. (1987) *Biochemistry 26*, 1152–1163.
Waring, M. (1987) in *DNA–Ligand Interactions. From Drugs to Proteins* (Guschlbauer, W., & Saenger, W., Eds.) pp 113–126, Plenum Press, New York.
White, R. J., & Phillips, D. R. (1988) *Biochemistry 27*, 9122–9132.
Wilson, W. D., & Jones, R. L. (1981) *Adv. Pharmacol. Chemother. 18*, 176–222.

Orientation, Accessibility, and Mobility of Equilenin Bound to the Active Site of Steroid Isomerase†

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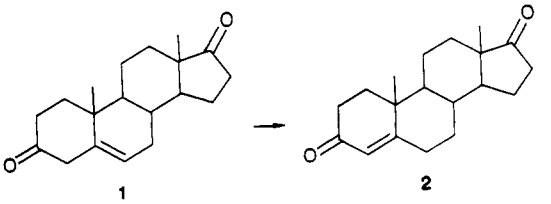
ABSTRACT: The fluorescent aromatic steroid equilenin, which contains a β-naphthol moiety, is bound by 3-oxo-Δ^5-steroid isomerase. The excitation and emission fluorescence spectra of equilenin when bound to the enzyme, as well as the fluorescence decay time, are indicative of ground-state ionization. In view of the high efficiency of tyrosine quenching, which approaches 100%, the β-naphthol moiety of equilenin must be in proximity to all three tyrosines of steroid isomerase to account for the observed efficiency of radiationless energy transfer. From the observed response to an external quencher, it appears that enzyme-bound equilenin is largely shielded from solvent. Fluorescence anisotropy measurements indicate a high degree of immobilization of the bound ligand. These models are consistent with proposed models of the enzyme–substrate complex.

The binding of steroids to proteins is an integral part of the action of steroid-metabolizing enzymes, as well as many hormone receptors and transport proteins (Duax et al., 1983). These steroids mediate a variety of physiological functions, such as sexual differentiation, protein synthesis, calcium uptake, electrolyte balance, and the maintenance of secondary sexual characteristics (Duax et al., 1983). It has also been suggested that steroids may be the natural substrates for hepatatic cytochrome P-450 (Waxman et al., 1983). In many cases, the binding site is relatively tolerant of structural changes in the steroid (Schwarzel et al., 1973; Weintraub et al., 1977; Adams & McDonald, 1981; Rousseau et al., 1981), and several proteins have been found to bind steroids in more than one orientation (Sweet & Samant, 1980; Strickler et al., 1980; Adams & McDonald, 1981; Waxman et al., 1983; Kashino et al., 1987). Ross et al. (1982) have reported that the fluorescent steroid equilenin is bound by human sex steroid binding protein with high affinity.

Probably the most thoroughly characterized protein of this type is the steroid isomerase from *Pseudomonas testosteroni*. This enzyme catalyzes the interconversion of 3-oxo-Δ^5-steroids to their Δ^4-isomers by the transfer of a proton from C-4β to C-6β with the concomitant migration of the double bond from C-5 to C-4 (eq 1). The isomerase is a dimer with identical



1 2 (1)

subunits, each having 125 amino acid residues of known sequence (MW 13394), and 1 binding site per monomer (Benson et al., 1972, 1975; Tivol et al., 1975; Batzold et al., 1976). The

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