Rotllan, P., & Miras-Portugal, M. T. (1985) *Eur. J. Biochem. 151*, 365–371.
Schnebli, H. P., Hill, D. L., & Bennett, L. L., Jr. (1967) *J. Biol. Chem. 242*, 1997–2004.
Scrutton, M. C., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 3714–3723.
Shimizu, H., Tanaka, S., & Kodama, T. (1972) *J. Neurochem. 19*, 687–698.
Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1–5.
Yamada, Y., Goto, H., & Ogasawara, N. (1980) *Biochim. Biophys. Acta 616*, 199–207.
Yamada, Y., Goto, H., & Ogasawara, N. (1981) *Biochim. Biophys. Acta 660*, 36–43.
Yamada, Y., Goto, H., & Ogasawara, N. (1982) *Comp. Biochem. Physiol., B: Comp. Biochem. 71B*, 367–372.

Effect of Drug–DNA Interactions upon Transcription Initiation at the *lac* Promoter†

David C. Straney‡ and Donald M. Crothers*,‡,§
Department of Molecular Biophysics and Biochemistry and Department of Chemistry, Yale University, New Haven, Connecticut 06511

Received September 19, 1986; Revised Manuscript Received December 4, 1986

ABSTRACT: We have examined the effects of six DNA binding drugs upon initiation at the *lac* UV5 promoter by *Escherichia coli* RNA polymerase. Experiments were directed at determining the influence of added drug on open complex formation, open complex stability, initiation from the open complex, and stability of the resulting initiated complex. The narrow groove binding drugs distamycin and 4′,6-diamidino-2-phenylindole dihydrochloride were more effective in inhibiting initiation through their effect on the first three of these factors than were the intercalators ethidium bromide, daunomycin, and actinomycin. The bisintercalator bis(daunomycin) inhibited open complex formation better than its parent daunomycin. With the possible exception of actinomycin, the drugs tested were not able to disrupt preformed initiated complex, in contrast to their destabilizing effect upon the open complex. Combined with other results, the data suggest that the antitumor activity of daunomycin is unlikely to result from its effect on transcription. We compare the relative effectiveness of the drugs with the known physical properties of the corresponding drug–DNA interactions. The rate of open complex formation seems to be influenced by both the on and off rates of the drug, probably due to the relative slowness of open complex formation. This is in contrast to elongation, a much quicker process, which seems to be limited by the drug off rate alone; these considerations may possibly rationalize the difference in relative effect of particular drugs upon initiation and elongation. All drugs were able actively to disrupt open complex, although to substantially different extents; some possible mechanisms for this disruption, and the insensitivity of the initiated complex, are discussed.

DNA binding drugs have the potential for blocking DNA replication and transcription, which are vital for cell maintenance and proliferation. The effect of drugs upon transcription in vitro has been studied with a wide variety of templates including bulk bacterial and calf thymus DNA and copolymers such as poly(dA-dT) [see reviews by Gale et al. (1972) and Zimmer et al. (1975)]. Although studies on bulk DNA allow an overview on how a drug will affect transcription, this heterogeneous mix of genes or nonpromoter sequences cannot provide details upon the specific target of the drug action in this complex process. Recent developments in molecular genetic technology have encouraged us to reexamine longstanding problems, initially addressed many years ago, related to the specific biochemical targets of action of DNA binding drugs.

The importance of studying the target of drug action is seen in the differential inhibition of transcription from certain genes by a given drug (Bleyman et al., 1969; Pennman et al., 1968); target specificity could therefore be utilized for selective drug action. Knowledge of which sequences a drug will bind [see Niedle & Abraham (1984)], and the thermodynamic and kinetic characteristics of the interaction, provides a basis for predicting and analyzing the drug's effect upon each step of transcription from a given sequence. Several studies have focused upon the stages of transcription affected by drug bindng. Distamycin has been shown to inhibit transcription initiation but not elongation, on the basis of the resistance of transcription to drug added to DNA after polymerase preincubation (Puscendorf et al., 1976; Küpper et al., 1973). Also, actinomycin D has been shown to inhibit elongation rather than initiation (Richardson, 1966; Hyman & Davidson, 1970). The sites of drug-induced blockage of *Escherichia coli* RNA polymerase in the elongation stage have been studied on T7 phage DNA (Aivashahvilli & Beabeahashvilli, 1983) and on *lac* run-off transcripts (Phillips & Crothers, 1986), providing information on both sequence specificity of drug binding and the efficiency of blockage.

In this paper, we compare the effect of six DNA binding drugs upon the steps of transcription initiation at the *lac* UV5 promoter using *E. coli* RNA polymerase. We utilize native polyacrylamide gel electrophoresis of the intermediates in initiation; gel complexes representing open complex and a

† This work was supported by Grants GM 34205 and CA 15583 from the National Institutes of Health. D.C.S. was supported by National Institutes of Health Training Grant GM 07223.
* Address correspondence to this author at the Department of Chemistry, Yale University.
‡ Department of Molecular Biophysics and Biochemistry.
§ Department of Chemistry.

0006-2960/87/0426-1987$01.50/0

minimal initiated complex (containing a nascent 11-mer RNA) allow us to study the drugs' effect upon open complex formation, open complex stability, open complex conversion to the initiated complex, and stability of initiated complex in the presence of the drugs. This analysis of each step separately would not be possible by RNA product analysis alone. These results complement the study of Phillips and Crothers (1986) on the effects of the same drugs upon elongation from the initiated complex using this promoter. The choice of drugs used in this study allows us to compare intercalators (ethidium bromide, daunomycin, and actinomycin) with outside narrow groove binders [distamycin and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)],[1] and a monointercalator with a derivative bisintercalator [daunomycin and bis(daunomycin)]. The kinetic and thermodynamic properties of the drugs show a relationship to their effects upon the various initiation steps.

Materials and Methods

Formation of Complexes. Polymerase–DNA complexes were formed in 100 μL of 100 mM KCl, 10 mM $MgCl_2$, 40 mM Hepes, pH 8.0, 0.1 mM EDTA, and 0.1 mM DTT with 3.4 pmol of 203 bp *Eco*RI DNA fragment and 8.8 pmol of RNA polymerase holoenzyme. The DNA fragment contains the *lac* UV5 promoter, which allows constitutively high transcription without CAP stimulation. One-tenth of the DNA was labeled by filling in the *Eco*RI ends with [α-^{32}P]ATP and Klenow fragment. All incubations were carried out at 37 °C.

Either the open complex was formed from DNA preincubated with drug for 15 min (polymerase on-rate studies) or, alternatively, the open complex was allowed to form for 5 min, followed by addition of the drug and heparin to 80 μg/mL (polymerase off-rate studies). Time samples (12 μL) were removed from the reactions and mixed with sucrose loading buffer (10×: 60% sucrose–xylene cyanol) and heparin (to 80 μg/mL). The samples were loaded onto a 4% polyacrylamide gel [1:40 bis(acrylamide):acrylamide] as described by Straney and Crothers (1985), which was run at 150 V in TBE buffer. Use of a constant-temperature gel box maintained the gel temperature during electrophoresis at 37 °C. After the xylene cyanol dye was run to 10 cm from the top, the gels were autoradiographed and the bands cut out and measured by Cerenkov counting. Exponential curves were fit to the data points, and the initial slope at $t = 0$, the final plateau value, and the $t_{1/2}$ of dissociation were derived analytically from these curves.

Initiated complex was produced by adding ribonucleotides to preformed open complex at a final concentration of 200 μM GpA, 10 μM ATP, GTP, and UTP, and 20 μM 3′-*O*-methyl-CTP. KCl was added to each time point sample (final concentration of 350 mM) to dissociate open complex. After 2–4 min of high-salt treatment, the initiated complex was run as described above except that the temperature of gel electrophoresis was maintained at 22 °C since the complex is not stable on a 37 °C gel. Heparin and drug were present during initiated complex formation (open complex initiation studies) or added after forming the initiated complex first for 20 min (initiated complex stability studies). Analysis of the RNA produced during initiation was performed by adding 20 μCi of [α-^{32}P]UTP to the above ribonucleotides; the time samples were added to 1 volume of 7 M urea, 6% sucrose, 15 mM EDTA, and bromophenol blue dye. These samples were heated to 90 °C, quenched on ice, and run on a 20% polyacrylamide gel [1:20 bis(acrylamide):acrylamide] until the bromophenol blue had migrated 23 cm.

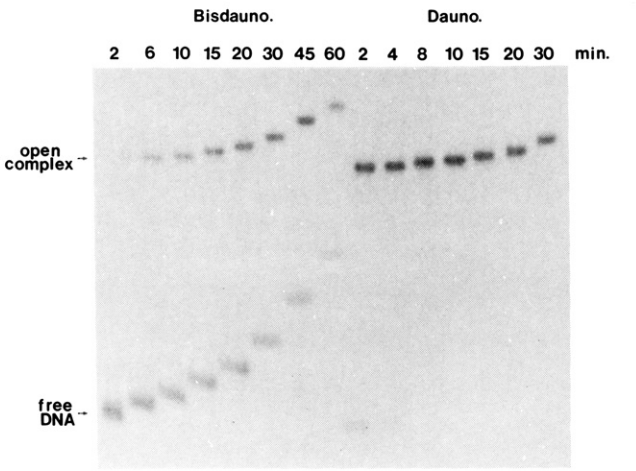

FIGURE 1: Open complex formation assayed on polyacrylamide gel. The open complex and free DNA are separated on a 4% polyacrylamide gel to assay the conversion of free DNA into open complex. The left lanes represent the time course with bis(daunomycin) and the right lanes with daunomycin. Samples were taken and loaded at the times stated at the top of the lanes. Gel loading over a period of time produces the higher position of successive time points; however, bis(daunomycin) binding causes a decreased gel mobility in both the free DNA and open complex.

The drugs were added to the above reactions to a final concentration of 0.7 μM, producing a drug:DNA base pair ratio of 1:10. The concentrations of the drug, DNA, and polymerase stock solutions were determined spectrophotometrically. The molar exctinction coefficients used for the drugs are as follows: ethidium bromide (Sigma), $\epsilon^{480} = 5850$ M^{-1} cm^{-1}; DAPI (kindly provided by Dr. Georgio Palu and Dr. Manlio Palumbo, University of Padova), $\epsilon^{342} = 23000$ M^{-1} cm^{-1}; the same coefficients used by Phillips and Crothers (1986) for the remaining drugs.

To measure the rate of open complex formation while the drug is simultaneously sequestered by addition of excess unlabeled DNA, a 0.34-pmol sample of labeled DNA was preincubated with drug at 0.7 μM drug in a 10-μL volume for 10 min. Then 90 μL of buffer with 3.06 pmol of unlabeled DNA was added, followed by 8.8 pmol of RNA polymerase. These conditions allow direct comparison with the above experiments since the concentrations of total DNA and polymerase are unchanged. The amount of open complex is then measured as a function of time after polymerase addition as above.

Results

Effect of Drugs upon On Rate of Open Complex Formation. The rate of open complex formation was measured by using promoter DNA which was preincubated with the drug; the control reaction used untreated DNA. Heparin, added immediately before each time aliquot was loaded on the gel, binds free polymerase and so removes weakly bound complexes from the promoter DNA. Therefore, the open gel complexes quantitated represent only stably bound complexes with an off rate slower than the approximately 1 min required to load the reaction onto the gel.

A sample gel is shown in Figure 1. The fraction of total DNA (complex plus free DNA bands) contained in the open complex is plotted in Figure 2 against the time of sampling after polymerase addition. The exponential curves fitted to the experimental points for each drug were used to express

[1] Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; bp, base pair(s).

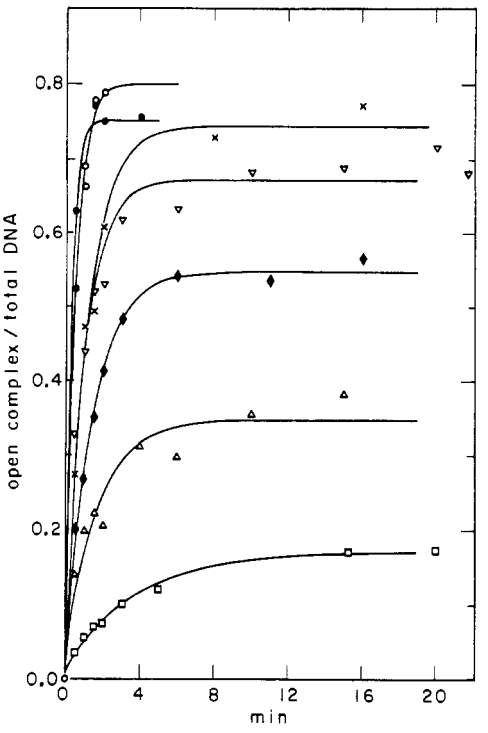

FIGURE 2: Time course of the formation of open complex. The fraction of open complex over total DNA (open and free DNA) is plotted over time of sampling after polymerase addition. Exponential curves fitted to the points are used to determine the initial rate (slope at $t = 0$) and the equilibrium level of open complex (plateau) in Table I. The drugs present during the open complex formation are (●) control, (○) ethidium bromide, (×) daunomycin, (▽) actinomycin, (♦) DAPI, (△) bis(daunomycin), and (□) distamycin.

Table I: Effect of Drug upon Formation of Open Complex

drug	relative initial rate of formation	open complex[a] equilibrium level	$t_{1/2}$ of drug dissociation (s)	K_{eq}, drug–DNA
control	1.0	0.75		
ethidium bromide	0.62	0.80	0.006[b]	2×10^{4}[b]
daunomycin	0.24	0.75	0.69[c]	2×10^{4}[c]
actinomycin	0.24	0.67	375[d]	2×10^{6}[d]
DAPI	0.14	0.55	*h*	5×10^{6}[e]
bis(daunomycin)	0.069	0.35	38[f]	10^{8}–10^{9}[f]
distamycin	0.015	0.18	*h*	1×10^{6}[g]

[a] Expressed as fraction of total DNA. [b] Bresloff & Crothers (1975). [c] Chaires et al. (1985). [d] Müller & Crothers (1968). [e] Manzini et al. (1983). [f] Phillips & Crothers (1986). [g] Luck et al. (1974). [h] Not known.

the relative initial rate of open complex formation (analytically derived slope of the exponential curve at 0 min) and the final plateau level of binding; these are summarized in Table I. The control reaction demonstrates the rapid on rate of RNA polymerase without drug. The drugs slow down the observed on rate to the following percent of the control: ethidium bromide, 61%; daunomycin and actinomycin, 24%; DAPI, 14%; bis(daunomycin), 6.9%; distamycin, 1.5%. The final plateau value of the fraction of open complex formed in the percentage of control is as follows: ethidium bromide and daunomycin, 100%; actinonmycin, 89%; DAPI, 73%; bis(daunomycin), 46%; distamycin, 23%. Further time points past 50 min with distamycin and bis(daunomycin) demonstrate a slower component of the on rate, plateauing at an open complex fraction of 0.24 with distamycin and 0.46 with bis(daunomycin); this biphasic behavior is presumably due to a slower off rate of a different drug–DNA species from the promoter site. This slower component is not considered in our analysis since it does not reorder the relative effectiveness of the drugs.

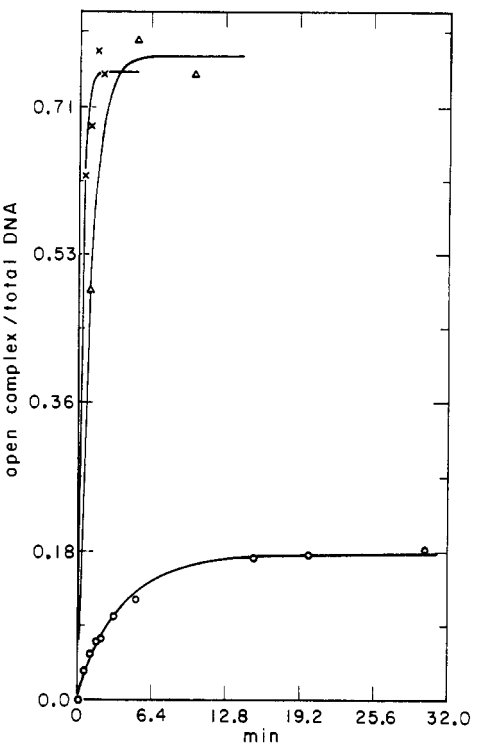

FIGURE 3: Comparison of open complex formation during distamycin equilibration with or dissociation from the DNA. The formation of open complex is followed either while distamycin is allowed to equilibrate with the DNA (○) or while the distamycin is dissociating from the DNA and being sequestered by excess unlabeled DNA (△). The absence of drug (×) allows rapid open complex formation. If distamycin action was limited by its dissociation rate, then the initial rates of open complex formation would be the same in this comparison, although the equilibrium level would be different. Since the initial rate of open complex formation is 2-fold greater for the sequestered drug than the equilibrating drug, the inhibition by distamycin is not soley limited by its dissociation.

In light of the strong inhibition of open complex formation by distamycin, we compared the above rate to that seen when free distamycin is sequestered in the presence of polymerase so that there was solely competition between the drug off rate and the polymerase on rate. Sequestering of free drug was achieved by incubating distamycin with labeled DNA at a drug:base pair ratio (r) of 1:10 as used in the above experiments; however, a 9-fold excess of unlabeled promoter DNA was added to the labeled DNA immediately before the polymerase was added. The excess unlabeled DNA sequesters the distamycin as it dissociates from the labeled DNA until equilibrium is reached at an overall r ratio of 1:100, at a total drug concentration of 0.07 μM. Equivalent behavior under the two conditions would demonstrate that the on rate of the polymerase is mainly governed by the off rate of the drug. However, comparison of these two approaches (Figure 3) shows that the rate of open complex formation is greatly increased, approaching that of the control, if free distamycin is sequestered during the time course in contrast to the case in which it is allowed to equilibrate with open complex. Distamycin and DAPI (the only ones tested due to lack of off-rate data in the literature for these drugs) show a respective 2- and 3-fold increase in the rate of open complex formation when the drug is sesquestered. This demonstrates that these drugs limit the on rate of polymerase mostly because of strong equilibrium binding, not as a consequence of a slow off rate from the DNA.

Table II: Effect of Drugs upon the Dissociation of Polymerase from Open Complex

drug	$t_{1/2}$ of open complex dissociation (min)	rate of formation[a] of drug–DNA complex	reference
control	127		
actinomycin	91	1×10^4 M^{-1} s^{-1} 0.081 s^{-1} [a]	Müller & Crothers (1968)
ethidium bromide	72	1×10^6 M^{-1} s^{-1}	Bresloff & Crothers (1975)
daunomycin	64	7×10^6 M^{-1} s^{-1} 4.2 s^{-1} [a]	Chaires et al. (1985)
bis(daunomycin)	52	*b*	
DAPI	17	*c*	
distamycin	17	*c*	

[a] Slowest step in forming the most stable drug–DNA interaction. [b] Similar to daunomycin but not known. [c] Not known.

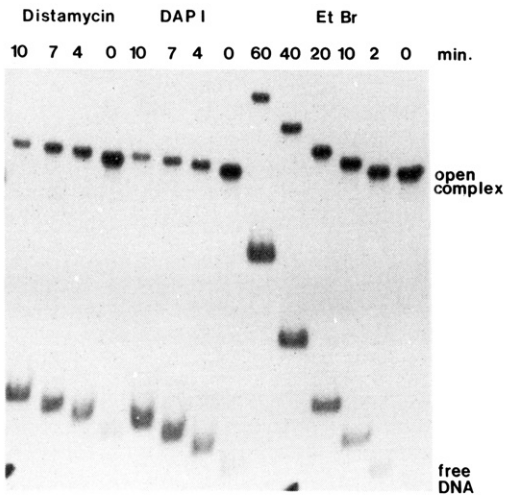

FIGURE 4: Dissociation of open complex followed by gel electrophoresis. The dissociation of open complex into free DNA in the presence of drug and heparin is shown from right to left: ethidium bromide, DAPI, and distamycin. The times of sampling after drug and heparin addition are stated at the top of each lane.

Effect of Drugs upon Stability of Open Complex. The rates of dissociation of polymerase from the open complex were followed in the presence of drugs to determine their effect upon the stability of the open complex. Drug and heparin were added to preformed open complex, and the dissociation was followed on gels (Figure 4). As plotted in Figure 5, the control decays with the natural off rate of open complex due to sequestration of polymerase by the heparin competitor; destabilization of open complex by the drug is seen as a faster off rate than in the control. The drugs increased the off rate of polymerase from the open complex, as seen in the decrease in the $t_{1/2}$ from control (Table II) to the following extents: actinomycin, 40%; ethidium bromide, 57%; daunomycin, 96%; bis(daunomycin), 140%; distamycin and DAPI, 360%. Distamycin and DAPI were substantially more disruptive of open complex than were the other drugs tested.

Effect of Drugs upon Initiation from Open Complex. The transcription initiation phase we studied is limited to forming a stable initiated complex, which occurs between 8 and 11 bases transcribed; blockage at this stage is achieved by using a modified nucleoside triphosphate (3′-*O*-methyl-CTP) to stop transcription at the 11-mer RNA product corresponding to addition of the first C residue. As described previously (Straney & Crothers, 1985), a short 8-mer transcript is produced as an abortive transcription product while the 11-mer forms a stable gel complex lacking σ subunit. The effect of drug upon initiation of transcription from open complex was measured in two different assays. In the first of these, we assayed the formation of the stable initiated complex, I_u, using native polyacrylamide gels (Figure 6). The second assay relies

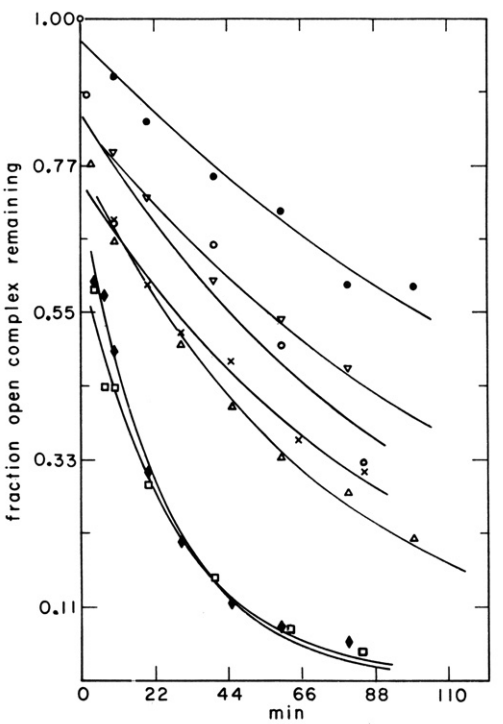

FIGURE 5: Rates of dissociation of open complex in the presence of drug. The dissociation of open complex into free DNA is plotted as the percent of initial open complex remaining at the time of sampling and is fit by an exponential curve. The control (●) represents the natural off rate of polymerase in the presence of the heparin competitor. All drugs actively disrupted the open complex, increasing the rate of dissociation, as shown for (▽) actinomycin, (○) ethidium bromide, (×) daunomycin, (△) bis(daunomycin), (♦) DAPI, and (□) distamycin. A faster component of dissociation seems apparent in the first minute of the time course with these drugs, since the experimental curves do not pass through the initial value of open complex, and probably represents a more sensitive state of open complex; the curves and the values derived from them only describe the slow component measured here.

on determination of the amount of labeled 8-mer-abortive and 11-mer-productive RNAs produced in solution by running the reactions on denaturing polyacrylamide gels (Figure 7).

In both assays, the drug was preincubated with the DNA before polymerase was added to form open complex; only after the amount of open complex had reached equilibrium (3–20 min) were the ribonucleotides added to allow initiation. The amount of initiated products, I_u complex or RNA, is expressed relative to the initial amount of open complex formed with the drug present. This procedure simplifies analysis since the effect of the drug upon open complex stability is minimized, in contrast to having added the drug to preformed open complex and then starting transcription. However, this order of addition limits our analysis of drug effects upon initiation to sites of drug–DNA interaction which do not interfere with the pre-

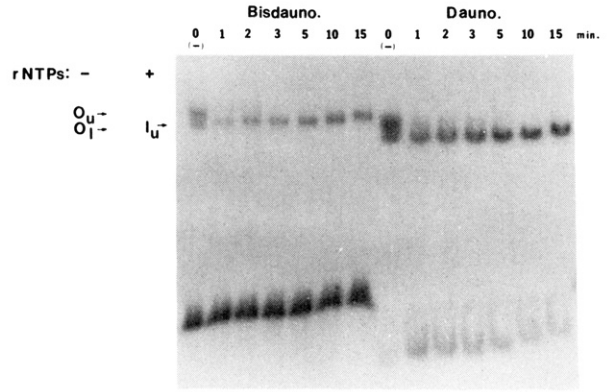

FIGURE 6: Conversion of open complex into initiated complex assayed on gel. The amount of initiated complex (I_u) containing the stopped 11-mer RNA formed from the initial amount of open complex (O_u and O_l; 0-min lanes) is assayed at the stated times after ribonucleotide addition. High-salt treatment of the initiated complex samples removes remaining open complex which would interfere with quantitation of initiated complex. The two open complex bands on this gel are the result of the reduced temperature of gel electrophoresis; only the O_u complex is formed at 37 °C but equilibrates with the faster mobility O_l state at lower temperatures while being loaded onto the cooler gel. The time courses of initiation are shown for bis(daunomycin) (left) and daunomycin (right).

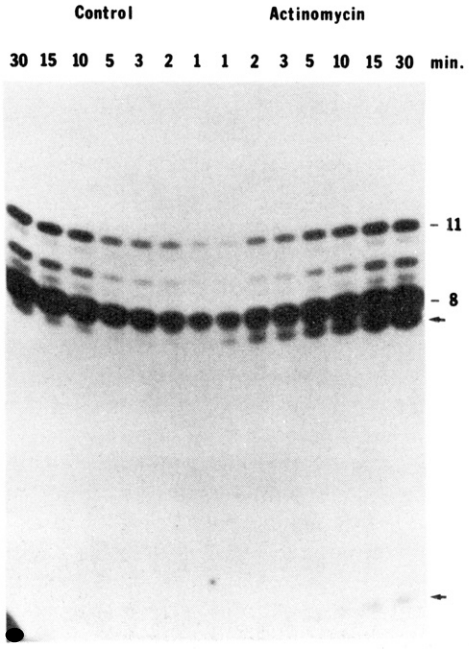

FIGURE 7: Formation of RNA products in the presence of drug. The time course of formation of labeled RNA products is shown on this denaturing polyacrylamide gel. The 11-mer is a productive transcript, stopped by a 3′-*O*-methylcytosine. The 8-mer is an abortive transcript produced by dissociation of this RNA from the complex, allowing the polymerase to return to open complex and reinitiate. Initiation was performed in the absence of drug (left) and in the presence of actinomycin (right). The new short RNA products made only with actinomycin are marked with an arrow.

ceding step of open complex formation.

(*i*) *Formation of a Stable Initiated Complex.* The time course of formation of stable initiated complex from open complex is shown in Figure 6 and quantitated in Figure 8. In the control reaction (no drug), 70% of the open complex is converted to stable initiated complex. In comparison to control, daunomycin, ethidium bromide, and bis(daunomycin) demonstrated little effect, to within 90% of control. Distamycin and DAPI inhibited the conversion from open into initiated complex, to 50% and 70%, respectively, of that seen in the control.

In the presence of actinomycin, I_u complex formation rose initially but peaked prematurely followed by a slower decay of the complex already formed. Considering the normal amount of RNA produced in this reaction (see below), this behavior is consistent with formation of an unstable I_u complex in the presence of actinomycin under these conditions.

(*ii*) *Formation of RNA Products.* The time course of formation of the productive 11-mer transcript is shown in Figure 7 and quantitated in Figure 8. The intercalating drugs show no significant effect upon formation of 11-mer transcript in comparison to control; however, there was minor inhibition by daunomycin and minor stimulation by bis(daunomycin) as in the initiated complex assay. The lack of inhibition by actinomycin indicates that the early halt in I_u formation in the above assay with this drug is most likely due to the instability of the initiated complex formed in the presence of the drug, rather than through an inhibition of initiation.

As in the initiated complex assay, distamycin and DAPI produced the greatest inhibition of initiation. Formation of 11-mer was reduced to 57% and 22% of control with DAPI and distamycin, respectively. In order to determine if this inhibition was an artifact resulting from the relatively rapid disruption of open complex by these drugs, we performed the same experiment without addition of heparin. In the absence of heparin, the equilibrium level of open complex should be maintained through reinitiation since the polymerase on rate ($t_{1/2}$ = 2.8 min) is 6-fold faster than its off rate in the presence of distamycin. Significantly, inhibition of transcription by these two drugs was not greatly changed when heparin was not included.

The 8-mer abortive RNA produced during the formation of the 11-mer in the above experiments was quantitated since it is also a measure of initiation, albeit not representing a productive initiation event. As with the 11-mer, the intercalators allowed production of the expected amount of 8-mer, but distamycin and DAPI induced a change in the 8:11-mer ratios; distamycin induced a 50% reduction in this ratio whereas DAPI increased it by 36% above control. In the presence of actinomycin, short RNAs approximately five and seven bases long were also made which would represent stopping at the thymidines in the sequence TpGpTpG. These short RNAs probably dissociate to allow reinitiation since the final amount of productive transcript was not reduced by actinomycin.

Effect of Drug upon Initiated Complex Stability. The off rate of polymerase from the productive initiated complex, containing the 11-mer RNA, was measured in the presence of drug to test for destabilization of this complex. Drug was added to preformed initiated complexes, and these reactions were sampled over time. Before each time point was loaded on the gel, its salt concentration was raised from 100 to 350 mM KCl to dissociate remaining open complex, leaving only the stable initiated complex. Consequently, this assay not only tested for dissociation of I_u complex but also tested for weakening of the complex in its characteristic stability to high salt. Of all six drugs tested, none exhibited any deviation from the control (absence of drug) off rate. Although the off rate of initiated complex is slower than that of open complex, any effect of drug upon the stability of initiated complex would have been observed in our time course spanning the initial 80 min.

This relative stability of initiated complex to drugs, in contrast to the open complex's susceptibility, was greatly enhanced at higher concentrations of drug. At ethidium bromide:DNA base pair ratios of 1:1 and 70:1, we observed a rapid

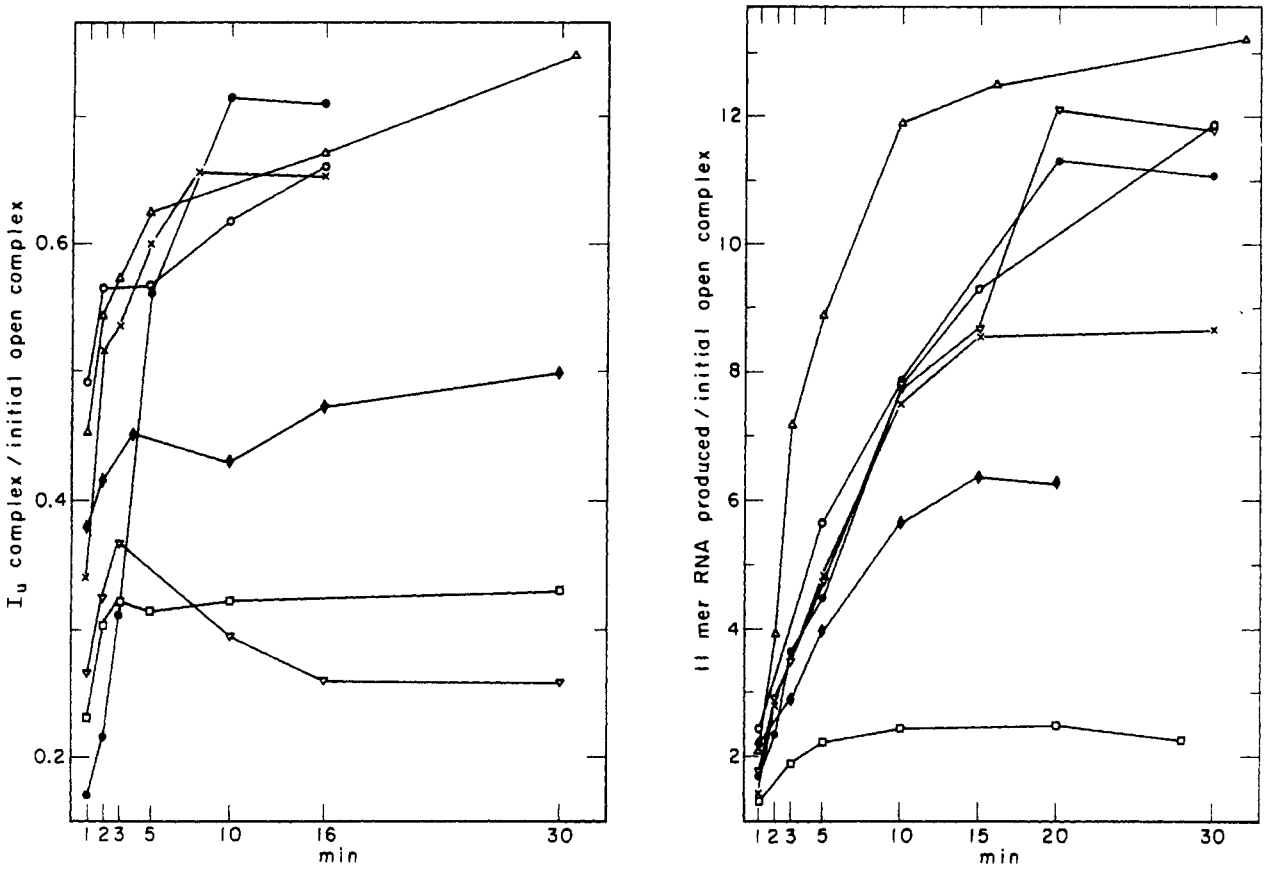

FIGURE 8: Quantitation of initiation from open complex in the presence of drugs. The effect of drug upon the ability of open complex to initiate transcription is assayed by formation of stable initiated gel complex (left) and formation of RNA (right). The level of I_u complex is represented as the fraction of the initial oepn complex present with each drug at the time after ribonucleotide addition; the relative amount of RNA produced is similarly normalized to the initial amount of open complex. The final amount of initiation in the presence of ethidium bromide (○), daunomycin (×), and bis(daunomycin) (△) is similar to control (●). Actinomycin (▽) allows RNA production but limits I_u formation. Distamycin (□) and DAPI (♦) inhibit initiation in both assays.

dissociation of all open complex within 5 min of drug addition whereas similar treatment produced no dissociation of initiated complex.

The initiated complex displayed greater stability to actinomycin in this assay than in the above experiment in which I_u complex was formed in the presence of actinomycin. This difference may result from the GpC sequence, a preferred site of actinomycin binding, between +9- and 10-positions. Actinomycin would be bound to this site in the latter assay where the drug is added before polymerase and so could destabilize the subsequently formed initiated complex which has transcribed up to this position. The drug was added to the preformed initiated complex in the former assay and so presumably could not enter this site to produce destabilization. Actinomycin was the only drug which displayed this differential type of behavior, the other drugs showing no effect upon I_u complex stability.

Discussion

We have tested the effect of six DNA binding drugs upon steps in initiation from the *lac* UV5 promoter. A simple diagram of initiation (Chamberlin, 1974) is

$$R + P \underset{}{\overset{K_1}{\rightleftharpoons}} RP_{closed} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_{open} + rNTPs \xrightarrow{k_3} \text{initiated}$$

where R represents the polymerase and P is the promoter DNA. The conversion from closed to open complex must include base pair unwinding in the promoter.

The steps in initiation that were tested in the presence of drug are formation of the stable open complex (K_1 and k_2), stability of open complex (k_{-2}), conversion of open complex into an initiated complex (k_3), and stability of the initiated complex. These were assayed by measuring the on rate, off rate, and final level of open or initiated complexes isolated on native polyacrylamide gels.

All six drugs slowed the on rate of polymerase in forming open complex and destabilized preformed open complex. Of these two processes, inhibition of open complex formation would most likely have the largest effect upon gene expression; the disruption of open complex by most of the drugs tested was slower than initiation from open complex and so would have a diminished effect upon overall initiation in the presence of ribonucleotides. However, disruption of open complex could play a major role in inhibition of other promoters which may remain in the drug-sensitive open complex longer [as in slow-start promoters described by Carpousis and Gralla (1985)] before forming a stable initiated complex. This could provide some promoter selectivity of drug action. A greater effect upon both the on and off rates of open complex was seen with the two drugs which bind outside the DNA in the minor groove, distamycin and DAPI (Zimmer, 1975; Manzini et al., 1983), in comparison to the monointercalators. The bisintercalator bis(daunomycin) displayed a greater inhibition of open complex formation than its monointercalator parent daunomycin; the difference between these mono- and bisintercalators was not as great in destabilization of open complex.

Only the two outside binders, distamycin and DAPI, inhibited RNA transcription initiation from open complex formed in the presence of drug; the intercalators produced no inhibition beyond a small inhibition by daunomycin. Formation of the stable initiated complex from open complex

involves minimal movement downstream from the promoter [4 bp additional DNase I protection on the top strand at +24 and +26 (Straney & Crothers, 1985)]. We therefore suspect that this inhibition is due to a drug-induced conformation in the transcribed region which is unsuitable for transcription initiation, rather than due to a physical block of polymerase movement. This would explain why the intercalators fail to inhibit this step, even though there are binding sites for them in the transcribed region; the neighboring DNA conformation induced by intercalators in unwinding the DNA may be more compatible with transcription (involving DNA unwinding and formation of an RNA–DNA A-form duplex) than that induced by the minor groove binders in clamping the DNA in the B form. Drugs which inhibit productive transcription from open complex have the potential of preferentially inhibiting slow start promoters, which are relatively slow at this step (Carpousis & Gralla, 1985). This differential effect would be most evident for promoters in which this step is already rate limiting for productive transcription, such as *lac* UV5 (Stefano & Gralla, 1979; S. B. Straney, unpublished results).

No drug tested destabilized a preformed productive initiated complex. This stability is in contrast to the sensitivity of the open complex to the drug treatment. Although the initiated complex is normally more stable than open complex, having a longer off rate and higher salt stability, this difference in drug sensitivity is significant. Factors allowing for the observed differential resistance may include the following: (1) filling in the unwound region present in open complex with an RNA–DNA duplex in the initiated complex, making it less susceptible to forced DNA conformational changes. Such differential resistance to unwinding has been reported in response to decreased temperature (Gamper & Hearst, 1982). (2) Compaction of the protein–DNA contacts in the initiated complex (DNase I protection of approximately 80 bp in open complex contracts to about 45 bp in I_u) may prevent entry of a drug between two binding regions on the DNA which could dephase the binding regions by intercalation.

The stability of the 11-mer initiated complex is also significant in assessing the effect of drugs upon elongation. Since these drugs do not disrupt a preformed initiated complex, inhibition of elongation by these drugs must occur only from blockage of chain synthesis by the drug, as assumed in previous studies on elongation (Phillips & Crothers, 1986; Aivashahvilli & Beabeahashvilli, 1983), and not by a general disruption of elongation polymerase similar to that seen with open complex. Actinomycin seems to be able to destabilize the I_u complex if the drug is present while the complex is synthesized, but not if the drug is added to preformed complex. Although this effect was seen here with the polymerase artificially stopped at the actinomycin binding site and assayed for high salt stability, a similar destabilization may possibly play a role in enhancing actinomycin's inhibition of elongation by actually terminating elongation where polymerase has paused at a template-bound actinomycin.

Comparison of the inhibition of initiation by these drugs with their effect upon elongation (Phillips & Crothers, 1986) shows that there is a difference in target site in transcription among them. Actinomycin, which specifically inhibits elongation most, showed only modest inhibition of open complex formation. Conversely, distamycin does not slow elongation but inhibited open complex formation, destabilized open complex, and inhibited initiation from open complex to the greatest extent seen among the drugs tested. Daunomycin exhibited such modest inhibition of transcription initiation and elongation that it seems unlikely that its primary mode of action is at the level of RNA synthesis; DNA cleavage by alteration of topoisomerase action offers a more attractive model for the antitumor activity (Tewey et al., 1984; Pommier et al., 1985). Bis(daunomycin) produced significant inhibition in both elongation (producing a defined stop) and initiation (inhibiting open complex formation and destabilizing open complex), unlike its parent monointercalator. This specificity does not seem to arise from sequence preference of binding [ethidium bromide and daunomycin, little specificity; bis(daunomycin) CACA and CATA; actinomycin G and GpC; distamycin and DAPI, A·T clusters], since on a gross level preferred sites are evenly distributed throughout the promoter and transcribed region. The physical properties of the drug–DNA interaction are more likely the source of the target specificity.

Comparison of Effectiveness of Drugs with Physical Properties of the Drug–DNA Complex. (i) Effect upon Formation of Open Complex. The results show a correlation between the final amount of open complex formed in the presence of each drug and the equilibrium constant of that drug–DNA interaction. The overall equilbrium constant for the open complex is approximately 10^{10} (Malan et al., 1984). Accordingly, ethidium bromide and daunomycin, with equilibrium binding constants of approximately 10^4, demonstrate little effect upon the final level of open complex at ~ 1 μM drug concentration. Greater reduction in this level was observed in the order of actinomycin ($K_B = 2 \times 10^6$), DAPI ($K_B = 5 \times 10^6$), and bis(daunomycin) ($K_B = 10^8$–10^9). Competition between drug and polymerase for promoter would indeed be expected to reduce open complex levels at a minimum drug–DNA binding constant of 10^6, as seen in these results. Distamycin was unusual in inhibiting open complex formation more than would be expected from its DNA binding constant ($K_B = 1 \times 10^6$) which is similar to that of the less effective DAPI. In light of the similar sequence specificity of these two drugs, it would seem that distamycin is more than a simple competitor of polymerase binding; distamycin may be able to interfere with open complex formation from binding sites beyond the promoter site and so compete with polymerase in altering DNA conformation (see open complex destabilization below). Previous studies (Zimmer et al., 1971; Wartell et al., 1974) indicate that direct distamycin–polymerase interactions do not have a role in inhibition by distamycin.

Previous studies with various DNA binding drugs support the concept that the drug–DNA dissociation rate is a major determinant in antibiotic activity [see references in Phillips & Crothers (1986)]. In testing this concept in regard to initiation in this system, we compared the relative inhibition of open complex formation rate to the known dissociation rate of the drug–DNA complexes (Table I). The results demonstrate this effect in comparison of the intercalators ethidium bromide, daunomycin, and bis(daunomycin), which have a decreasing off rate from the DNA and an increasing inhibition of open complex formation rate in this order. Increasing the off rate of daunomycin by forming the bis derivative produced a slower on rate of polymerase, as predicted. However, data on the other drugs tested do not fit this simple relationship but instead lead to the conclusion that both the off rate and on rate of the drug determine the relative inhibition of open complex formation. Actinomycin allowed open complex formation at the same rate as seen for daunomycin, but actinomycin has an off rate that is 10^3 slower than that of daunomycin. In order for actinomycin to fit the simple competition model, it is necessary to assume that the actinomycin off rate is increased in the presence of polymerase (possible if polymerase weakens the interactions of the peptide arms of

actinomycin with DNA, making the actinomycin off rate similar to that of the simple intercalator daunomycin). Further unexpected behavior was seen with distamycin and DAPI, which seem to inhibit open complex formation more through equilibrium binding than simply by their off rate from DNA. This was demonstrated by the greater inhibition of the initial rate of open complex formation when the drug was allowed to equilibrate with the DNA than when the drug was sequestered as it dissociated from the DNA.

The observed dependence beyond simply the off rate of the drugs may be explained by the relatively slow process of open complex formation in comparison to the on rate of most drugs. Due to this difference in rate, the drug would be equilibrating with the promoter during the formation of open complex—the faster the drug could bind, the greater the inhibition of open complex formation would be above that expected due to the off rate alone. Clearly, this would explain the dependence upon equilibration of distamycin and DAPI with the promoter for maximum inhibition of the rate. Also, actinomycin would be expected, as observed, to demonstrate less inhibition than the other intercalators since its on rate is much slower ($t_{1/2}$ = 375 s, slowest step) than open complex formation ($t_{1/2}$ = 12 s) in contrast to the much more rapid binding of the other intercalators ($t_{1/2}$ = 0.2 s, slowest step for daunomycin; Chaires et al., 1985).

Blockage of elongation would be expected to be more simply dependent on drug dissociation since elongation past a potential drug binding site ($t_{1/2}$ = 0.04 s per base; Chamberlin et al., 1979) is rapid in comparison to the above rates. This may play an important role in the design of drugs which seem to have a greater role in inhibition of initiation than elongation (e.g., distamycin) and in explaining why actinomycin, with a slow dissociation rate from DNA, is not as effective an inhibitor of initiation as it is of elongation.

Another possible factor in the inhibition of open complex formation by equilibrium drug binding is blockage of polymerase sliding in the initial promoter search. The magnitude of this effect would depend upon the processivity of polymerase sliding. It is, however, unlikely to become rate limiting over the slow isomerization (k_2) step.

(*ii*) *Disruption of Open Complex.* The active disruption of open complex by the drugs indicates more than a simple steric competition for binding between drug and polymerase. Two mechanisms for disruption are envisioned which could work separately or together.

(1) Drug could enter the open complex, binding to unprotected DNA, and distort the DNA to a conformation incompatible with polymerase binding. DNase I footprints of the open complex (Schmidt & Galas, 1979) contain several internal sites unprotected from the nuclease which have preferred binding sequences for the drugs. Intercalators could bind in these regions and unwind the DNA, dephasing the flanking protein–DNA contacts on either side and so disrupt the complex. The narrow groove binders DAPI and distamycin could compete for DNA conformation within the open complex by their property of stabilizing a B-DNA structure which may be incompatible with the promoter unwinding in open complex.

(2) Drug could sterically compete for polymerase binding in a binding domain of open complex which is only transiently bound. Such transiently bound domains are seen in this open complex when probed by exo III digestion (Straney & Crothers, 1987), consisting of regions between −44 to −35 and −34 to −24 through which exo III can penetrate but leave the open complex still bound. Entry of drug into these regions of the promoter while the polymerase is transiently unbound may prevent rebinding of that domain and consequently partially weaken the open complex.

The results presented here cannot eliminate either model. Comparison of the intercalators shows that the drugs which bind DNA more quickly produce a faster disruption of open complex, as seen in a smaller $t_{1/2}$ of polymerase dissociation (Table II). This would seem to indicate a time-dependent factor in the disruption process as envisioned in the second model since a drug which binds quicker could enter the transient domain more efficiently. However, other properties of the drug–DNA interaction may have created this effect. The unwinding angle of the DNA caused by drug binding is less with daunomycin (11°) than ethidium bromide and actinomycin (26°), yet daunomycin produces quicker disruption of open complex. This is incompatible with the first model unless the competition between polymerase and drug-induced DNA conformation produces too high an activation energy for binding the higher unwinding angle drugs; the bis(daunomycin), with a final unwinding angle twice that of daunomycin, would presumably have benefited by a two-stage intercalation in overcoming this activation energy barrier and so acts similarly to daunomycin.

Distamycin and DAPI, narrow groove binders, produced much faster disruption of open complex than did the intercalators. Although no information concerning their on rates is available in the literature to evaluate the second model, distamycin is known to produce DNA conformational change compatible with the first model. Distamycin induces conversion of DNA from other forms to B [reviewed in Zimmer & Wähnert (1986)]. This ability of distamycin to force a different DNA conformation is also seen in distamycin changing the abnormally slow mobility of bent kineteoplast DNA to the normal mobility of B DNA on polyacrylamide gels (Wu, 1982). Furthermore, propagation of the DNA conformation into neighboring DNA is seen by electric dichroism and NMR (Dattagupta et al., 1980; Patel et al., 1981). These suggest that distamycin could destabilize the open complex by forcing the promoter into a B-DNA conformation, thereby removing the unwinding and stability of open complex. This could provide greater destabilization of the open complex in comparison to the intercalators by being able to act from outside the open complex, possibly from multiple sites, by invasive propagation of the B-form conformation.

Use in Probing Other Systems. Schröter et al. (1985) have recently reported specific dissociation of HMG14 and HMG17 from chromatin using ethidium bromide. Although they assume that drug-induced changes in supercoiling cause dissociation, these proteins may dissociate in a manner similar to that which we observe for the open complex from linear DNA where no gross DNA supercoiling is possible. Our results showing the resistance of one polymerase complex (initiated) in contrast to the sensitivity of another (open complex) suggest that the use of drugs to extract proteins from chromatin may be biased by other factors in the protein–DNA complex. The choice of drug may further affect the specificity of dissociation in light of the different effects of the drug which we observed and the sequence specificity of the drugs. An understanding of the factors affecting the drug sensitivity of a complex may allow the use of these drugs as a specific probe by selective dissociation or reconstitution of certain classes or states of a protein in chromatin.

ACKNOWLEDGMENTS

We thank Dr. Georgio Palu and Dr. Manlio Palumbo for DAPI, Dr. Don Phillips for bis(daunomycin), and all three

for helpful discussions.

REFERENCES

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

Bleyman, M., Kondo, M., Hecht, N., & Woes, C. (1969) *J. Bacteriol. 99*, 535–543.

Bresloff, J. L., & Crothers, D. M. (1975) *J. Mol. Biol. 95*, 103–123.

Carpousis, A. J., & Gralla, J. D. (1985) *J. Mol. Biol. 183*, 165–177.

Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1985) *Biochemistry 24*, 260–267.

Chamberlin, M. J. (1974) *Annu. Rev. Biochem. 43*, 721–775.

Chamberlin, M. J., Nierman, W. C., Wiggs, J., & Neff, N. (1979) *J. Biol. Chem. 254*, 10061–10069.

Dattagupta, N., Hogan, M., & Crothers, D. M. (1980) *Biochemistry 19*, 5998–6005.

Gale, E. F., Reynolds, P. E., Cundliffe, E., Richmond, R. H., & Waring, M. J. (1972) in *The Molecular Basis of Antibiotic Action*, pp 187–241, Wiley, New York.

Gamper, H. B., & Hearst, J. E. (1982) *Cell (Cambridge, Mass.) 29*, 81–90.

Hyman, R. W., & Davidson, N. (1970) *J. Mol. Biol. 50*, 421–438.

Küpper, H. A., McAllister, W. T., & Bautz, E. K. F. (1973) *Eur. J. Biochem. 38*, 581–586.

Luck, G., Triebel, H., Waring, M., & Zimmer, Ch. (1974) *Nucleic Acids Res. 1*, 503–530.

Malan, T. P., Kolb, A., Buc, H., & McClure, W. R. (1984) *J. Mol. Biol. 180*, 881–909.

Manzini, G., Barcellona, M. L., Avitabile, M., & Quadrifoglio, F. (1983) *Nucleic Acids Res. 11*, 8861–8875.

Müller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.

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

Patel, D. J., Kozkowski, S. A., Rice, J. A., Broka, C., & Itakura, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7281–7284.

Pennman, S., Vesco, C., & Pennman, M. (1968) *Science (Washington, D.C.) 154*, 786–789.

Phillips, D. R., & Crothers, D. M. (1986) *Biochemistry 25*, 7355–7362.

Pommier, Y., Schwartz, R. E., Zwelling, L. A., & Kohn, K. (1985) *Biochemistry 24*, 6406–6410.

Puschendorf, B., Becker, H., Bohland, D., & Grunicke, H. (1976) *Eur. J. Biochem. 49*, 531–537.

Schmitz, A., & Galas, D. J. (1978) *Nucleic Acids Res. 6*, 111–137.

Schröter, H., Maier, G., Ponstingl, H., & Nordheim, A. (1985) *EMBO J. 4*, 3867–3872.

Stefano, J. E., & Gralla, J. D. (1979) *Biochemistry 18*, 1063–1067.

Straney, D. C., & Crothers, D. M. (1985) *Cell (Cambridge, Mass.) 43*, 449–459.

Straney, D. C., & Crothers, D. M. (1987) *J. Mol. Biol. 193*, 267–278.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984) *Science (Washington, D.C.) 226*, 466–468.

Wartell, R., Larson, J. E., & Wells, R. D. (1974) *J. Biol. Chem. 249*, 6719–6731.

Wu, H.-M. (1982) Ph.D. Thesis, Yale Unversity, New Haven, CT.

Zimmer, Ch. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 285–320.

Zimmer, Ch., & Wänert, U. (1986) *Prog. Biophys. Mol. Biol. 47*, 31–99.

Zimmer, Ch., Puschendorf, B., Grunicke, H., Chandra, P., & Venner, H. (1971) *Eur. J. Biochem. 21*, 269–278.