Kinetics and Sequence Specificity of Drug–DNA Interactions: An in Vitro Transcription Assay†

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ABSTRACT: An assay has been developed to detect the DNA sequence specificity of drug binding sites, and the kinetics of dissociation of drug from those sites, under conditions involving active transcription of the DNA. Specific transcriptional blockage sites were detected in the presence of actinomycin D and a bis-intercalator, bis(anthracycline); the rate of RNA chain growth past the drug binding sites yields the rate of dissociation of drug from these sites. Rate constants for dissociation from the whole promoter fragment measured by the detergent sequestration method were found to be significantly faster than the rate determined for dissociation from the specific transcriptional blockage site. However, the absence of significant blockage at other drug binding sites implies much more rapid drug dissociation from those sites in the transcriptional complex. We conclude that transcriptional blockage results from a DNA sequence-dependent interaction of the drug–DNA complex with RNA polymerase; the sequences that are effective for blockage appear to be GpC for actinomycin and $(CpA)_3$ for a bis(daunomycin) compound. Transcriptional inhibition may in general show greater sequence specificity than is exhibited by simple binding.

Some of the most important anticancer drugs in current clinical use are intercalators (Wilson & Jones, 1981; Neidle & Waring, 1983), and it is largely for this reason that the mechanism of intercalation into DNA has been studied intensively for over two decades [see, for example, reviews by Berman and Young (1981), Wilson and Jones (1981), and Neidle and Abraham (1984)]. After such extensive investigations, it is unfortunate to acknowledge that the detailed mode of action of intercalators, as anticancer drugs, remains obscure (Brown, 1978; Wilson & Jones, 1981; Siegfried et al., 1983; Ralph et al., 1983). There is a large and convincing body of evidence to show that DNA is indeed a dominant receptor (Wilson & Jones, 1981; Neidle & Waring, 1983), as has been most graphically demonstrated by the correlation between the potency of anthracycline derivatives (spanning 3 orders of magnitude) and a parameter derived from DNA-associated events and cellular uptake (Schwartz, 1983). The major question then is whether effects at the DNA level are primarily related to the thermodynamics or to the kinetics of the intercalator–DNA interaction (Wilson & Jones, 1981). Slow rates of dissociation of actinomycin have been correlated with enhanced in vitro inhibition of RNA polymerase activity (Müller & Crothers, 1968), and there has been an increasing amount of evidence to support the concept that the drug–DNA dissociation event is an important determinant of anticancer activity (Gabbay et al., 1976; Wilson et al., 1976; Aktipis & Panayotatos, 1977; Wakelin & Waring, 1980; Fox & Waring, 1981; Waring & Fox, 1983; Feigon et al., 1984; Denny et al., 1985; Gandecha et al., 1985).

The majority of experimental information on drug–DNA dissociation processes relies upon a SDS-sequestering[1] procedure (Müller & Crothers, 1968). This technique is rapid, simple, and reproducible and requires little sample material. Although some details of the sequestering step remain obscure (Fox & Waring, 1981), the validity of the method has been supported by the agreement between all comparable kinetic parameters determined by the sequestering and T-jump methods for the daunomycin–DNA interaction (Chaires et al., 1985). Given that these drug–DNA dissociation processes are important for drug activity and that the kinetics can be readily measured by the SDS-sequestering procedures, what is the relationship between the dissociation kinetics as measured by this detergent procedure and that existing under more physiological conditions? In order to address this question, we have developed a transcription assay system to enable drug–DNA dissociation kinetics to be measured under conditions where the DNA is being actively transcribed. This in vitro transcription inhibition assay yields not only kinetic information but also sequence specificity of the drug as reflected in its transcriptional blocking ability.

Materials and Methods

Materials. The 203-bp restriction fragment of *lac* DNA was derived from the L8-UV5 double mutant of *Escherichia coli* and contains a mutated CAP binding site, the Z-gene promoter, and the *lac* operator. The UV5 mutation results in an "up" promoter (Silverstone et al., 1970), and the L8 mutation confers CAP independence of the promoter (Ippen et al., 1968). The *lac* fragment was incorporated as 4–5 tandem repeats into plasmid pHW1, which was amplified with chloramphenicol to yield a copy number of 30–40 (Wu & Crothers, 1984). The plasmid was prepared as described by Stefano and Gralla (1979). The DNA was dissolved in 10 mM

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[1] Abbreviations: SDS, sodium dodecyl sulfate; bp, base pair; CAP, chloramphenicol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UTP, uridine 5′-triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; NTP, unspecified nucleoside 5′-triphosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; TBE, tetrabromoethane.

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Tris and 0.5 mM EDTA, pH 8, and the concentration determined by ethidium bromide titration by comparison to a calf thymus DNA standard determined spectrophotometrically (nucleotide concentration) with ϵ_{260} = 6600 M^{-1} cm^{-1}.

For SDS-sequestered dissociation kinetics, calf thymus DNA (Worthington) was used in transcription buffer at 37 °C, and the nucleotide concentration was quantitated with ϵ_{260} = 6600 M^{-1} cm^{-1}.

Daunomycin hydrochloride was a gift from Farmitalia Carlo-Erba. Bis(daunomycin) (daunomycinglycylsuccinylglycyldaunomycin) was prepared and purified as described by Reiss et al. (1984) and Scourides et al. (1984). Actinomycin D and distamycin A were supplied by Boehringer. The concentration of these drugs was determined spectrophotometrically in transcription buffer with extinction coefficients of 11 500 M^{-1} cm^{-1} at 480 nm for daunomycin (Chaires et al., 1982), 19 800 M^{-1} cm^{-1} for bis(daunomycin) (Phillips et al., unpublished results), 5850 M^{-1} cm^{-1} at 488 nm for actinomycin D (Bresloff & Crothers, 1975), and 37 000 M^{-1} cm^{-1} at 303 nm for distamycin A (Van Dyke, 1984). [α-^{32}P]UTP with specific activity >5000 Ci/mmol was supplied by New England Nuclear and used within 5 days. The solution was dried and redissolved in transcription buffer immediately before use. *E. coli* RNA polymerase holoenzyme (EC 2.7.7.6), 1000 units/mL, was supplied by New England Biolabs and the concentration determined with ϵ_{280} = 6.5. Heparin and nucleoside triphosphates were purchased from Sigma. BSA was acetylated as described by Gonzalez et al. (1977), and solutions were stored at −20 °C. GpA was obtained from Pharmacia and P-L Biochemicals.

Transcription-Initiated Complex. The routine procedure for formation of an initiated complex for single copy transcription, a modification of the procedures of Reisbig and Hearst (1981) and Morgan et al. (1983), required 100 μL of 2× transcription buffer (75 mM Tris, pH 7.9, 0.25 mM EDTA, 0.2 M KCl, 0.5 mM DTT, and 6 mM $MgCl_2$), 50 μL of H_2O, 30 μL of 203-bp *lac* DNA fragment (75 μg/mL), 10 μL of acetylated BSA (10 mg/mL), and 10 μL of *E. coli* RNA polymerase, with the mixture incubated at 37 °C for 10 min. Heparin (40 μL, 1 mg/mL), preincubated in transcription buffer, was then added, and the mixture was incubated for 1 min at 37 °C to displace nonspecifically bound RNA polymerase and to prevent reinitiation at the promoter site. Preincubated NTP's (135 μL) in transcription buffer were then added [50 μL of 50 μM ATP, 50 μM GTP, 50 μM UTP, and 2 mM GpA, together with 85 μL (250 μCi) of [α-^{32}P]UTP], and the final mixture (375-μL total volume) was incubated at 37 °C for a further 10 min. This procedure resulted in high yields of the *lac* DNA being converted from the open complex (with RNA polymerase associated only at the promoter site) to an initiated complex with a decanucleotide nascent RNA transcript. This results from initiation at the −1 site with GpA as primer (A being the first nucleotide of the normal transcript), with synthesis up to the +9 position, where it is halted because of the absence of CTP, required for the +10 base incorporation (Maizels, 1973). GpA was used at high concentrations to eliminate multiple initiation sites of the UV5 *lac* promoter [29% and 55% for the −1 and +1 sites, respectively (Carpousis et al., 1982)]. Use of the dinucleotide therefore resulted in considerable simplification of the transcript electrophoretic profiles and has been used previously for this reason (Maizels, 1973; Reisbig & Hearst, 1981).

Transcription–Elongation. After the stable initiated complex had been formed, it was divided into two 170-μL aliquots and two 15-μL control samples. Preincubated transcription buffer (30 μL) or an equal volume of drug in transcription buffer (to yield approximately 0.1 unit of drug/bp) was then added to each aliquot, and the sample was incubated at 37 °C for 10 min. The elongation process was commenced by the addition of 22 μL of 100 μM CTP to each aliquot of the initiated complex.

Electrophoresis of RNA Transcripts. At appropriate time intervals, 20-μL samples were removed, and the transcription reaction was terminated by adding them to 5 μL of 0.1 M EDTA on ice. An equal volume of electrophoresis loading buffer was then added (4.5 mM Tris–borate, pH 8.3, 8 M urea, 0.05% xylene cyanol, and 0.05% bromophenol blue), and 15-μL samples were subjected to denaturing 12% polyacrylamide–7 M urea gel electrophoresis in TBE buffer, as described by Maniatis et al. (1982). The gels were 0.8 mm thick and 40 cm long and were usually run at 700–800 V until all free [α-^{32}P]UTP had been removed from the gel (5–6 h). A thick metal block was clamped to the back of the glass plates to minimize "smiling" effects.

Quantitation of RNA Transcripts. Autoradiography was carried out overnight at room temperature, using X-ray film, and processed in Kodak C-ray developer and rapid fixer. The autoradiogram was scanned with a microdensitometer, coupled to an integrator. The autoradiograph was exposed for differing times when necessary to ensure that the maximum absorbance of any band was less than 1.0. This ensured that the calculated area was directly proportional to the amount of label associated with that band, since it has been shown that ^{32}P counts are linear with absorbance in the absorbance range 0–1.0 (Dabrowiak et al., 1986).

Sequencing RNA Transcripts. Sequencing of the transcript was carried out in three ways. Varying concentrations of 3′-*O*-methyl-CTP were incorporated together with CTP during the transcription process (Axelrod et al., 1978; Reisbig & Hearst, 1981) to provide information concerning the location of cytosine residues in the sequence. The complete sequence was obtained by using limiting amounts (0.05 μM in the elongation mode) of one NTP, while maintaining the other three NTP's at 5 μM. This procedure yielded transcript lengths up to, but not including, the limiting NTP in each case. A third source of sequence information derives from the known natural pausing sites detected by Maizels (1973) for this transcription system. All three sources of RNA transcript sequence information were consistent with the *lac* mRNA sequence (Maizels, 1973).

SDS-Sequestered Kinetics. Drug–DNA dissociation kinetics were studied with the SDS-sequestering procedure of Müller and Crothers (1968). In order to overcome the low solubility of SDS in transcription buffer, 0.1 M KCl was replaced with 0.1 M NaCl. Drug–DNA samples (2 mL) in the modified transcription buffer at 37 °C were mixed rapidly with 0.5 mL of 10% SDS in the same buffer, and the reaction was monitored at 480 nm in a Cary 118 spectrophotometer. The data were recorded and averaged to yield some 30 time points, and initial parameters were estimated by "peeling" the data; these values were subsequently evaluated more rigorously with a kinetic simulation package, CONSAM (Boston et al., 1981; Foster & Boston, 1983).

RESULTS

Absence of Drug. When NTP's in the initiation reaction were limited to ATP, UTP, GTP, and high levels of GpA, initiated products contained predominantly the decaribonucleotide GAATTGTGAG. Upon addition of CTP, elongation commenced from the initiated complexes. The range of RNA transcript lengths is shown in the autoradiogram in

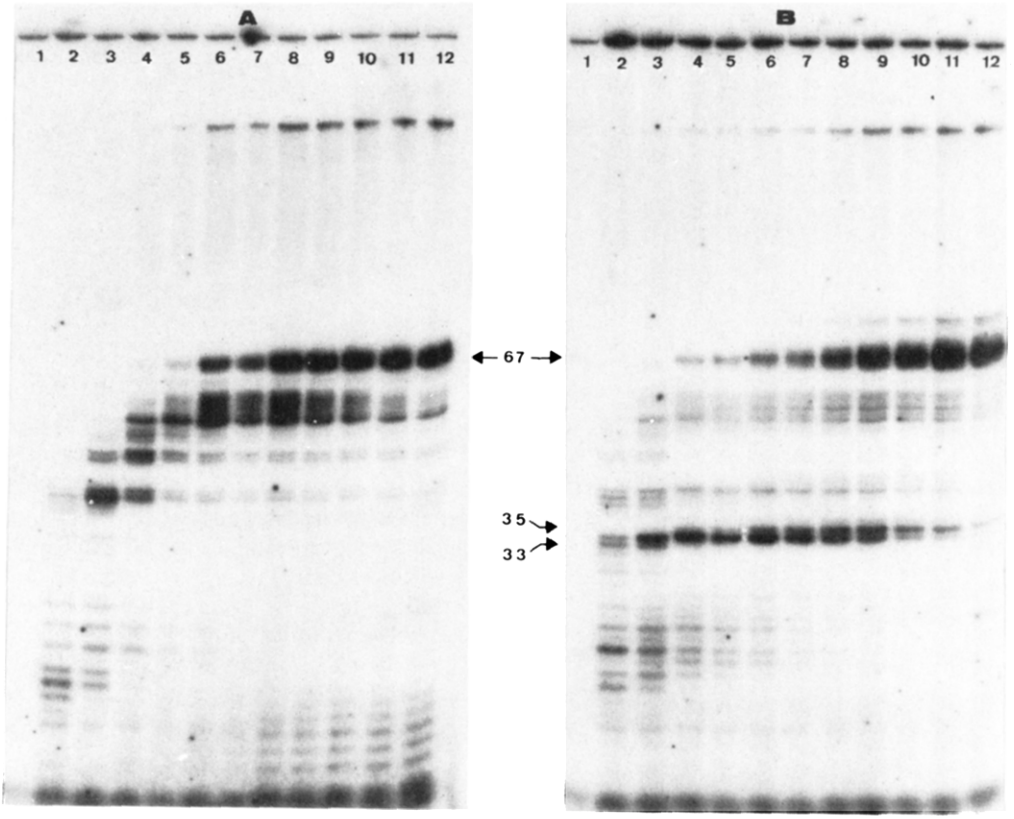

FIGURE 1: Sequencing gel of actinomycin D blocked transcripts. Lanes 2–12 showed a time course (0.25, 0.5, 1, 1.5, 3, 5, 8, 12, 20, 30, and 50 min, respectively) for elongation of the transcripts subsequent to addition of CTP, in the absence (A) and presence (B) of actinomycin D after the initiated complex had been formed. Lane 1 is a control that lacked CTP. Several time points were rerun in a sequencing gel to assign sequence lengths.

Figure 1A, at various time intervals after elongation was started with CTP. Natural pausing is evident for a variety of transcripts, all of which correspond to those sites previously observed by Maizels (1973). Under the conditions employed, a slow elongation rate is seen, with substantial amounts of full transcript (up to +67) appearing only after 1 min. This rate is slow compared to maximal possible rates of 20–30 nucleotides/s (von Hippel et al., 1984) but is consistent with the low NTP concentrations used in the elongation phase (5 μM). The breadth of the band corresponding to full transcript (+67) is probably due to multiple initiation sites [7% and 9%, respectively, from the +2 and +5 sites for UV5 *lac* DNA (Carpousis et al., 1982)] yielding shorter "full-length" transcripts that migrate "anomalously" since their first nucleotide is a tetraphosphate.

Small amounts of longer RNA transcripts are observed and may be due to improperly initiated nearly end-to-end transcripts of the restriction fragment (Stefano & Gralla, 1979) or to read-through from one DNA fragment to a second DNA molecule. The source of small amounts of radioactivity remaining associated with the sample wells remains obscure but may be due to degradation of the label, as suggested by others (Reisbig & Hearst, 1981).

Relative Molar Yields of Transcript. Each lane of the autoradiogram was scanned with a densitometer, and typical profiles are shown in Figure 2. The area under each band is proportional to the number of labeled phosphates incorporated into that particular length of RNA. To allow for the differing numbers of [α-^{32}P]UMP's incorporated into each transcript, the area of every peak was divided by the number of uridine residues in the RNA fragment. By this procedure, the corrected area therefore represented the relative number of transcripts in each band.

To eliminate errors due to differential loading volumes, radioactivity in the wells and in the other larger products, the corrected areas were summed, and each band was expressed as a fraction of the total corrected area. This procedure therefore yields the fractional number of moles of RNA transcript corresponding to each band. These normalized mole fractions are shown in Figure 3 at various times after elongation of the initiated complex had been started by the addition of CTP.

Influence of Actinomycin D. When actinomycin D was incubated with the initiated complex immediately prior to elongation of the RNA transcript, an additional drug-induced blockage was detected at RNA lengths of +32 to +35, as shown in Figures 1B and 2. The delay of read-through of the DNA past the +33 to +35 region is paralleled by a delay in the buildup of substantial amounts of the full transcript (Figures 1 and 3). The buildup of +33 to +35 transcripts and their subsequent decay (Figure 1B) is shown quantitatively in Figure 3 in terms of a time course of the mole fraction of those transcripts.

If the decay is attributed to dissociation of the drug from DNA (i.e., a first-order kinetic process), then the decay of the transcript concentration at that site should also be a first-order process. Such a plot is shown in Figure 4 and exhibits a time constant of 1500 s.

Bis(daunomycin). Autoradiograms obtained for elongation of the initiated *lac* DNA are shown in Figure 5 in the absence and presence of the bis(daunomycin) derivative. Blockage of elongation is observed at the +19 to +23 region. The time course of the mole fraction of these transcripts and the first-order analysis of that decay are shown in Figures 6 and 4, respectively. The time constant for the dissociation process is 190 s.

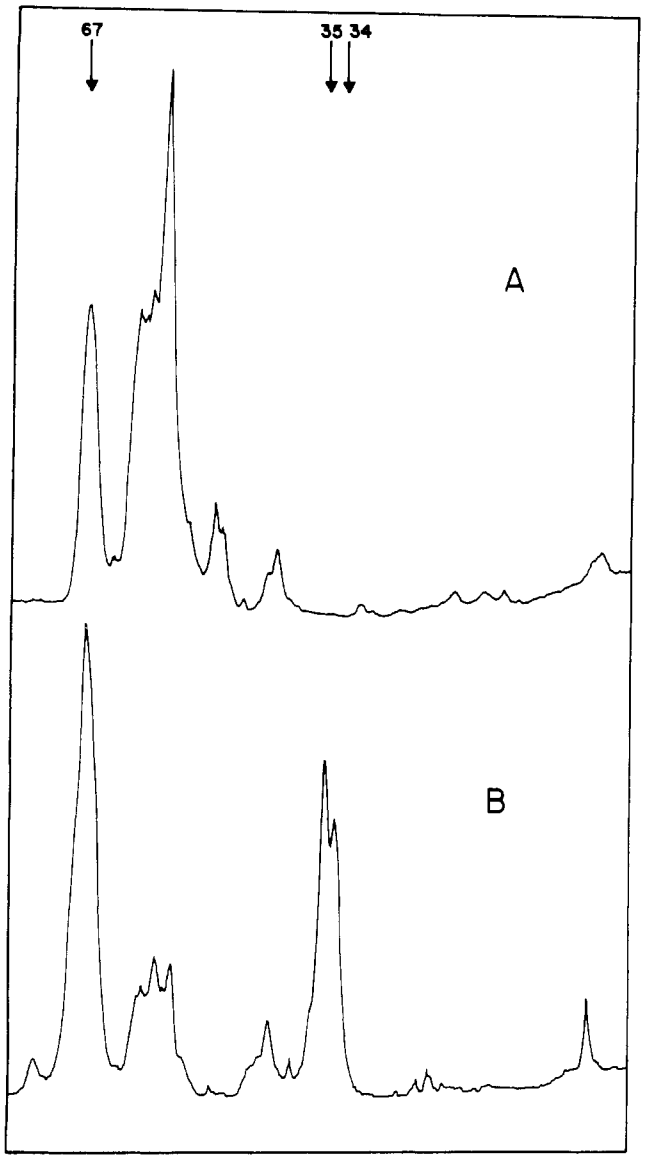

FIGURE 2: Quantitation of transcript lengths. A densitometer trace of the autoradiography bands obtained in the absence (A) and presence (B) of actinomycin D after 3 min of elongation (lane 6 of Figure 1).

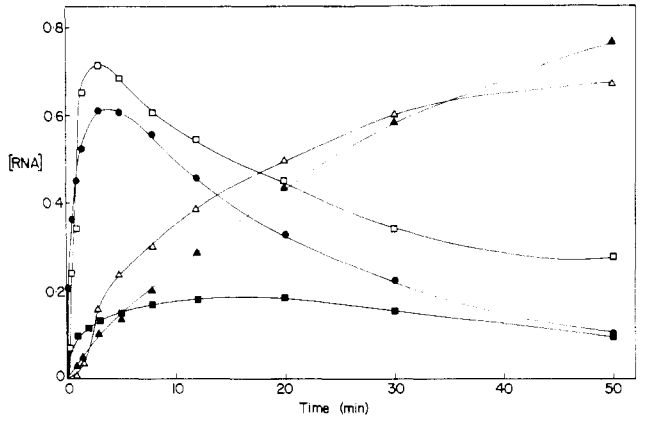

FIGURE 3: Kinetic profile of transcript formation. The kinetics of the elongation process are shown as the mole fraction of each discrete transcript, as a function of elongation time in the absence (open symbols) and presence (filled symbols) of actinomycin D. Transcript lengths are +33 to +35 (○), +52 to +57 (□), and full-length transcripts (△).

Other Drugs. The effect of distamycin A and daunomycin on the elongation process was examined by an identical procedure with that outlined for actinomycin D and bis(daunomycin). Distamycin A had no discernible effect. In the presence of daunomycin, no discrete blockage sites were observed but formation of comparable amounts of full-length and near-full-length full transcripts were delayed by approximately 30 s (Figure 7). This is well outside experimental error as each time point was accurate to ±3 s.

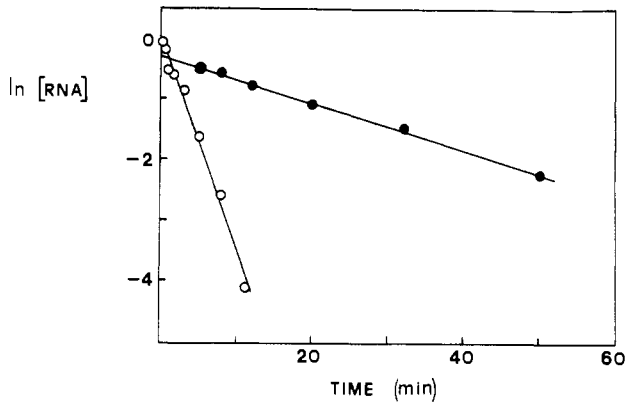

FIGURE 4: Kinetics of drug-induced blockage site. First-order kinetic analysis of blocked transcripts (mole fraction) induced by actinomycin D (+33 to +35 sites, ●) and bis(daunomycin) (+19 to +23 sites, ○).

Discussion

Sequence Specificity. The relationship between the *lac* DNA sequence and the RNA lengths where drug-induced blockage is observed is shown diagrammatically in Figure 8. From the DNA binding specificity of actinomycin D [see reviews by Dabrowiak (1983) and Neidle (1983)], it is expected that transcription would proceed to near a G·C base pair site and therefore produce RNA transcript lengths corresponding to the upstream side of the binding site. This is indeed observed, as a 5′-GpC-3′site exists at +36/+37, and transcription blockage there is consistent with all footprinting (Van Dyke & Dervan, 1983; Lane et al., 1983; Fox & Waring, 1984), nick translation (Wilkins, 1982; Robbie & Wilkins, 1984), and transcription (Aivashahvilli & Beabeahashvilli, 1983) detection systems of actinomycin D binding sites on DNA. However, as shown in Figure 8, transcription proceeds dominantly up to 1 bp prior to the intercalation site, with only a slight amount of +35 transcript found (Figures 1B and 8). This differs slightly from the observation by Aivashahvilli and Beabeahashvilli (1983), who used a transcriptional assay similar to ours, although they did not analyze it kinetically. They found actinomycin D induced blockage immediately adjacent to the GpC intercalation site, with the A1 promoter region of T7-phage DNA at low (<0.05) drug:DNA (bp) ratios. The reason for this discrepancy is not clear. It is surprising that transcription continues even to 1 bp upstram from the drug site, since RNA polymerase is known to unwind DNA by approximately 12 bp (Von Hippel et al., 1984). Given the small separation between binding and blockage sites, it seems likely that unwinding does not extend more than 1 or 2 bp ahead of the site of RNA synthesis.

An additional 5′-GpC-3′ site is also present on the transcribed DNA at position +9/+10. However, one expects drug binding at that position to be inhibited by prior formation of the open complex, since RNA polymerase strongly inhibits DNase I cleavage in that region (Straney & Crothers, 1985). Furthermore, it was not possible to quantitate reliably drug-induced blockage associated with this site because of the large amount of background observed for all lanes (in both the presence and absence of drug) corresponding to RNA lengths of approximately +8 to +11.

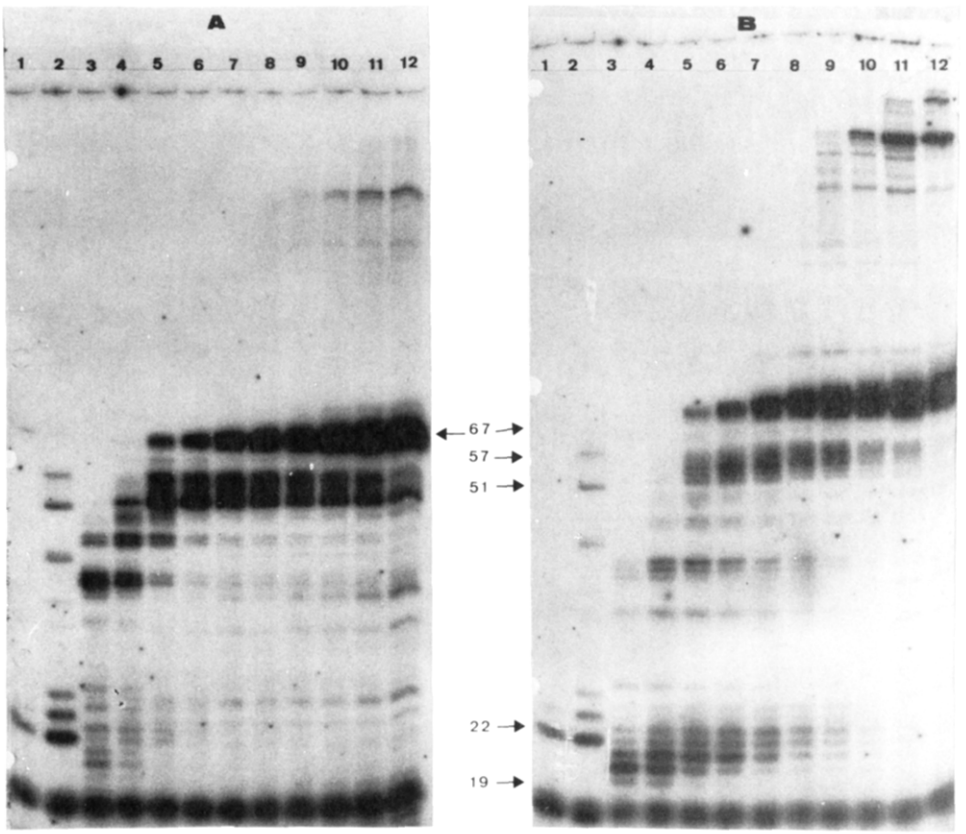

FIGURE 5: Sequencing gel of bis(daunomycin)-blocked transcripts. Lanes 3–12 show a time course (0.25, 0.5, 1, 1.5, 3, 5, 8, 13, 20, and 35 min, respectively) in the absence (A) and presence (B) of bis(daunomycin). Lanes 1 and 2 are respectively a control in the absence of CTP and a sequencing lane (10 min, incubation with 0.02 μM CTP).

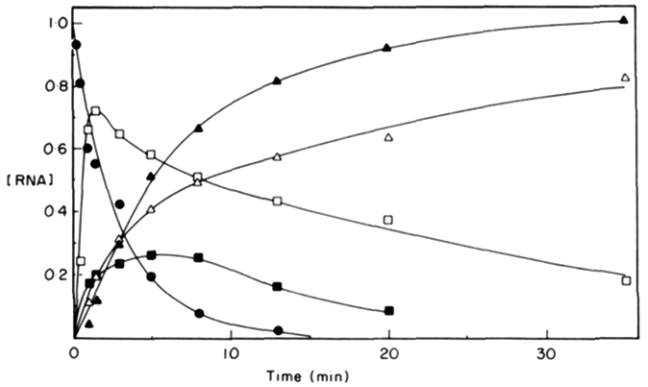

FIGURE 6: Kinetic profile of transcripts. The mole fraction of transcripts is shown in the absence (open symbols) and presence of bis(daunomycin) (fillted symbols) at times after addition of CTP. Transcript lengths are +19 to +23 (○), +52 to +61 (□), and full-length transcripts (△).

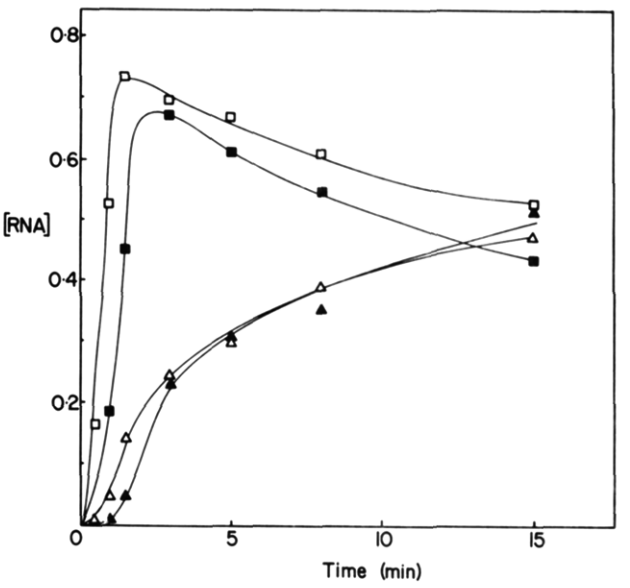

FIGURE 7: Effect of daunomycin on transcription kinetics. The mole fractions of +52 to +61 (□) and full-length (△) transcripts are shown for the absence (open symbols) and presence (filled symbols) of daunomycin.

For the bis(daunomycin) derivative, specific binding appears to be associated with the 5′-CACACA-3′ sequence immediately downstream from the drug-induced blocked transcripts (Figure 8). As the bis(daunomycin) derivative is a bis-intercalator with a flexible link between the intercalating chromophores (Reiss et al., 1984; Scourides et al., 1984), it might be expected to bind to a repeating structural unit of DNA. This suggests that the binding site is ACAC or CACA. The broad range of terminating RNA transcript lengths observed for this drug (ranging over five nucleotides) suggests that overlapping binding sites may exist and supports the notion of two overlapping CACA sites at 23–26 and 25–28. This suggestion receives support from the recent gas-phase energy calculations of Newlin et al. (1984), who showed that 5′-CA-3′ and 5′-TA-3′ sequences were energetically favored for intercalation by daunomycin. Such repeating units do not exist elsewhere on the *lac* DNA fragment transcribed in this work.

It should be emphasized that for both actinomycin and bis(daunomycin), other binding sites must exist in addition to those detected by transcription blockage. The drugs were added at a molar ratio of 1/10 bp, at DNA concentrations substantially in excess of the drug dissociation constant, so one expects on average about six drugs bound in the region covered

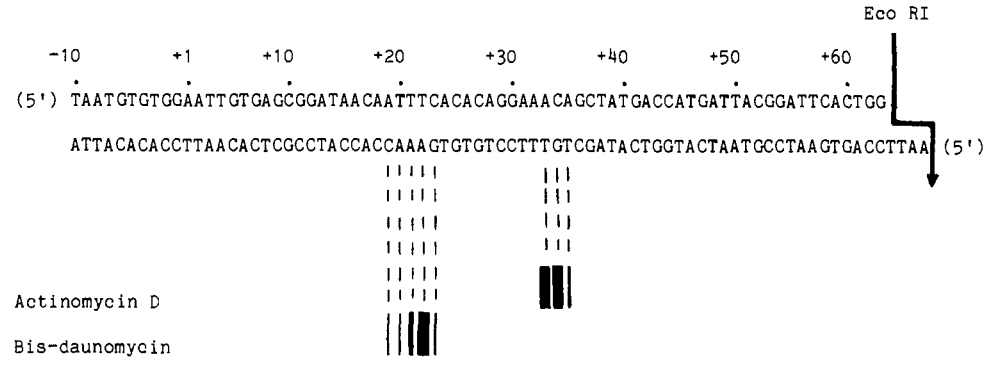

FIGURE 8: Drug-induced blockage sites on L8-UV5 *lac* DNA. Diagrammatic representation of the drug-induced transcription blockage sites (at defined times after commencement of elongation with CTP) in the transcribed region of the 203-bp fragment of *lac* DNA. The *lac* DNA sequence has been established by Dickson et al. (1975) and the *lac* mRNA sequence by Maizels (1973); the UV5 point mutations at −8 and −9 have been established by Stefano and Gralla (1980).

Table I: *Time Constants for Dissociation of Drug–DNA Complexes* (Transcription Buffer, 37 °C)

drug	transcription assay (s)	SDS assay (s)
actinomycin D	1500	300
bis(daunomycin)	190	38

by transcription from the initiated complex (+10) to the runoff product (+67). Failure to observe any other blockage sites implies that the drug is able to escape relatively rapidly from the transcriptional complex at the alternative binding sequences. Transcriptional blockage clearly exhibits greater sequence specificity than is observed for binding.

Dissociation Kinetics. The first-order processes shown in Figure 4 for drug-induced blockage of transcription by actinomycin D and bis(daunomycin), respectively, reflect, in a strict interpretation, the rate of elongation of RNA past the drug blockage site. However, the decay of the blocked transcript (i.e., the "read-through" process) must also measure the dissociation of drug from that site, since the only other process involved is the elongation mode, which is rapid under the conditions employed (see control lanes in Figures 1a and 5a) and therefore not a rate-limiting step.

To assess the utility of this procedure for the study of drug–DNA dissociation kinetics, we measured the same dissociation processes with SDS-sequestering conditions (Müller & Crothers, 1968). Although the mechanism of SDS-sequestered dissociation has not yet been unequivocally proven, the validity of the method has been demonstrated, with essentially identical results being obtained for the DNA–daunomycin system by T-jump and SDS-sequestering procedures (Chaires et al., 1985). Only the slowest step of the SDS-sequestered dissociation is included in Table I for comparison with the transcription inhibition constants, since it is assumed that the slowest process is dominant in retarding transcription.

For both drugs, the dissociation time constants measured by the two assays differ by approximately 5-fold, with the transcription-detected dissociation exhibiting a substantially slower rate in both cases. This is a somewhat surprising result because, in a mechanistic sense, it might be anticipated that, with RNA polymerase present and the DNA unwound for some 12 bp, the result would be a destabilizing effect on drug bound to DNA in the transcription system. This would be expected to manifest itself as a less stable, more rapidly dissociating species than in a system (such as the SDS-sequestered procedure) where such a potentially destabilizing factor is not present. It may be for this reason that other binding sites are not detected by the transcriptional blockage assay; clearly, it is of interest to discover the special features of the drug complex at the sites that produce strong inhibition of RNA synthesis. In this context, it should be noted that the long half-life observed for actinomycin D at a GpC site by the transcription method is consistent with previous observations of unusually slow dissociation from poly(dG-dC) (Krugh et al., 1980), although the influence of the homopolymeric context on the properties of the drug complex remains an open question.

The differences we observe between drug dissociation rates measured by transcriptional blockage and SDS sequestration are unlikely to be attributable to altered ionic strength resulting from addition of SDS, since it has been shown that dissociation of the DNA–daunomycin complex is independent of ionic strength in the range 0.01–0.1 (Phillips and Boston, unpublished results) and this independence is likely to be sustained also by bis(daunomycin). The major difference between the two techniques is that, in the transcription process, only dissociation from a discrete site is measured, whereas an averaged dissociation from all binding sites is detected by the SDS-sequestering process. For this reason, the values measured by the two procedures cannot be expected to be identical, but rather, they would be anticipated to be of a similar order of magnitude. Additional experiments currently in progress with DNA fragments of known sequence and under differing dissociation conditions will be required in order to resolve these issues.

The slowest SDS-sequestered dissociation step of daunomycin from DNA has a rate constant of 10–50 s^{-1} at 37 °C, depending on the ionic strength and DNA sequence (Grant & Phillips, 1979; Chaires et al., 1985). The half-life for this step is therefore less than 0.1 s. The small delay in production of the full-length transcript (~30 s) is therefore consistent with a cumulative effect of daunomycin at the five to six binding sites in the 58 nucleotides transcribed (assuming maximal nonspecific drug binding at 0.1 drug/bp). Such small time delays do not warrant more detailed analysis at this stage (given the experimental errors involved), but nevertheless clearly demonstrate that the mode of action of daunomycin cannot be a simple kinetic delay of the elongation process by merely the drug alone, unless the critical binding sequence happens to be missing from the DNA fragment under study. Furthermore, we cannot exclude the possibility of a kinetic contribution from daunomycin in some undefined modified state.

Relative Occupancy. Extrapolation of the first-order decay process of the blocked transcript to zero time yields the fractional number of RNA transcripts subjected to drug blockage at that particular site. This number is a measure of relative drug–DNA affinity for that site, compared to the

nonblocking binding sites, assuming the concentrations are high enough that most of the drug is DNA-bound. For the drugs employed in this study, the bis(daunomycin) has an estimated association constant [physiological ionic strength of 10^8–10^9 M^{-1} (Phillips et al., unpublished results)] and has a higher fractional occupancy at its specific blockage site than does actinomycin D (association constant 10^5–10^6 M^{-1} under similar conditions; Wilson & Jones, 1981b). We interpret this to mean that the bis(daunomycin) derivative has greater binding specificity for its transcriptional blockage site than does actinomycin. At lower drug/bp ratios, this analysis offers a novel method for ranking relative drug–DNA affinity for the special blockage sites, especially for high affinity bis-intercalators that are notoriously difficult to quantitate by direct means (Gaugain et al., 1978; Capelle et al., 1979; Becker & Dervan, 1979).

Drug-Induced Release of Natural Pausing. The buildup of blocked transcripts in the presence of drug is accompanied by a dramatic decrease of natural pausing of the +52 to +60 transcripts (Figures 1, 3, 5, and 6). This is due in part to the direct effect of drug blockage in decreasing the fraction of longer transcripts. However, the effects appear too pronounced to be due to that cause alone but must reflect the influence of drug binding at the alternative sites. This is most clearly manifested by an increase of the mole fraction of full-length transcripts at later elongation times when drug is present (Figures 3 and 6), which appears to be due to less natural pausing immediately prior to the full-length transcript. A plausible explanation for this phenomenon invokes drug stabilization of the DNA duplex in the copied region behind RNA polymerase, by binding there in preference to the RNA–DNA hybrid helix, whose stability is likely to be a contributing factor to natural pausing. Clearly this explanation requires substantial reequilibration over the alternative drug binding sites during the course of the transcription experiment, but according to the SDS-sequestration kinetics, drug dissociation from the alternative sites is unlikely to be a rate-limiting factor. Alternatively, drug binding at lower affinity sites downstream of the natural transcription pause site could influence the DNA or DNA·RNA hybrid stability in the region of natural pausing.

Drug Design and Medical Implications. From this work, it is clear that the time scale of dissociation of drugs from specific blockage sites during active transcription of the DNA differs, but by less than an order of magnitude, from the slowest process detected by the simpler SDS-sequestered dissociation method. For structure–activity aspects of anticancer drug screening programs, it is necessary to have some measurement of drug–DNA dissociation kinetics. It is now apparent that the labor-intensive transcription assay system may not be necessary at the present stage of understanding of the role of drug–DNA dissociation kinetics in the mechanism of action of anticancer drugs and that simple SDS-sequestered kinetics will suffice to a good approximation. Exceptions to this generalization include cases in which one wishes to determine the DNA sequence at the specific sites where transcriptional blockage occurs or in which the objective is design of agents intended to block transcription at specific sequences.

It has previously been observed that ethidium bromide interferes with initiation and elongation of RNA chains (Richardson, 1973). However, if drug–DNA dissociation kinetics are a critical aspect of the mode of action of anticancer drugs, it is likely that this event is more important during the elongation phase of transcription, where many drug sites will be encountered (and accumulated delay may allow other disruptive events to occur), rather than at single drug sites that may exist at the promoter or in the initiation region. Experiments are currently in progress to explore this model, utilizing known specific drug binding sites in the *lac* promoter region.

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Topoisomerase from *Ustilago maydis* Forms a Covalent Complex with Single-Stranded DNA through a Phosphodiester Bond to Tyrosine†

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ABSTRACT: Highly purified topoisomerase from *Ustilago* breaks single-stranded DNA, forming a complex with protein covalently bound to the DNA. Methods used to detect the complexes include a nitrocellulose filter assay, electrophoresis of the DNA–protein complex in agarose gels containing alkali, and isolation of the complex after removal of all but a small oligonucleotide fragment bound to the protein. The linkage of the *Ustilago* topoisomerase is to the 3′ end of the broken strand of DNA. The DNA–protein complex formed is through a phosphodiester bond to tyrosine.

Topological interconversions of DNA require transient breakage and resealing of DNA strands. Topoisomerases are the enzymes that interconvert topological isomers of DNA. Relaxation of negatively supercoiled DNA by topoisomerases was first observed a number of years ago to occur by repeated breakage and rejoining of DNA with no added cofactor providing energy for rejoining (Wang, 1971; Champoux & Dulbecco, 1972). Wang suggested that the energy of the phosphodiester bond was conserved in a reaction intermediate,

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