Induction of Stable Transcriptional Blockage Sites by Adriamycin: GpC Specificity of Apparent Adriamycin–DNA Adducts and Dependence on Iron(III) Ions†

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ABSTRACT: Initiated transcription complexes were exposed to adriamycin for up to 48 h. Subsequent elongation of the transcription complex revealed the presence of a series of discrete long-lived blockage sites. The mole fraction of blocked transcripts increased linearly with reaction time, adriamycin concentration, and Fe(III) concentration. Optimal conditions for formation of the blocked transcript were 24-h reaction time, 10 μM adriamycin, and 75 μM Fe(III) ions. Nine high-intensity blocked transcripts were observed, and all correspond to transcription proceeding up to G of GpC sequences of the nontemplate strand. The presence of 75 μM Fe(III) ions enhanced the amount of transcriptional blockages by 12–15-fold. Two blocked transcripts decayed with a half-life of 0.32 and 1.9 h, and one of these exhibited 100% effective delayed termination 6 bp downstream of the original blockage site. All other blockages were unchanged after 3 h of elongation. Bidirectional transcription footprinting was used to define the physical size of the drug-induced blocking moiety as a maximum of 2 bp, and this was observed at all three GpC elements probed by RNA polymerase from both directions. The nature of the apparent covalent adducts has not yet been established but is probably a G-specific adduct deriving from a reduced form of the drug (quinone methide). Although the GpC specificity suggests an interstrand G–drug–G cross-link, these were not detected by heat denaturation and subsequent denaturing gel electrophoresis of the end-labeled promoter fragment.

Since its discovery in the early 1960s, the anthracycline antibiotic adriamycin has been so effective in the treatment of cancer that it remains as one of the most widely used anticancer agents in clinical use today (Arcamone, 1982). Although the mode of action of adriamycin is still debated, there appear to be three major mechanisms: the effects of membrane binding; impairment of enzymes such as topoisomerase II; the effect of products of reductive activation (Myers et al., 1988). Moreover, there is a large body of evidence which suggests that anthracyclines act at the DNA level (Schwartz, 1983; Valentini et al., 1985).

Reduction of adriamycin and other anthracyclines has been achieved both by chemical activation (Sinha & Chignell, 1980; Sinha & Gregory, 1981) and by a variety of enzymatic methods (Pan & Bachur, 1980; Sinha et al., 1984; Fisher et al., 1983, 1985). The one-electron reduction facilitated by these processes results in the formation of a semiquinone intermediate which may be involved further in a number of reactions (Kappus, 1986). Under anaerobic conditions the semiquinone undergoes further reduction accompanied by reductive cleavage of the sugar residue to form the quinone methide (Moore, 1977), which binds covalently to nucleophiles (Ramakrishnan & Fisher, 1986), and is known to form adducts with DNA (Sinha & Chignell, 1979; Sinha, 1980; Sinha & Gregory, 1981).

We have recently reported that incubation of adriamycin with DNA, when in the presence of dithiothreitol, resulted in sequence-specific blockage of transcription of DNA, consistent with the formation of adriamycin adducts with DNA (Phillips et al., 1989). These blockages were dramatically enhanced by the presence of Fe^{2+} or Fe^{3+} ions, but not by other physiologically relevant divalent metal ions (Phillips et al., 1989). Since adriamycin is known to chelate Fe^{3+} ions, forming $Fe^{3+}Ad_3$ (Gelvan & Samuni, 1988), and Fe^{2+} is rapidly oxidized to Fe^{3+}, it is likely that the $Fe^{3+}Ad_3$ species participates as an intermediate in the formation of adriamycin adducts with DNA. The adriamycin-induced blockages were the result of termination of transcription, as shown by the inability of RNA polymerase to read through past these sites (Phillips et al., 1989). This is in contrast to the rapid dissociation of the reversible, intercalated form of adriamycin which cannot be observed by the transcriptional assay at 37 °C but can be quantitated at 4 °C (Skorobogaty et al., 1988a,b).

We have now utilized the technique of bidirectional transcription footprinting (White & Phillips, 1989) to demonstrate that the transcriptional blockages are specific for GpC sequences, being constrained primarily to the 2 bp GpC element, and that the transcriptional blockage element exhibits the characteristics of a covalent adduct. It is important to note that these adducts were formed under in vitro transcription conditions where there was no enzymatic activation of the anthracycline—the possible mechanism of this process will be considered with respect to the physiological ramifications of this process and the possible avenue now offered for the design of a new class of anthracycline derivatives.

Materials and Methods

Materials

Adriamycin was a gift from Farmitalia Carlo Erba, Milan; ultrapure ribonuclease inhibitor (human placenta) and BSA (DNase free and RNase free) were purchased from Pharmacia. [α-^{32}P]UTP and X-ray film were obtained from Am-

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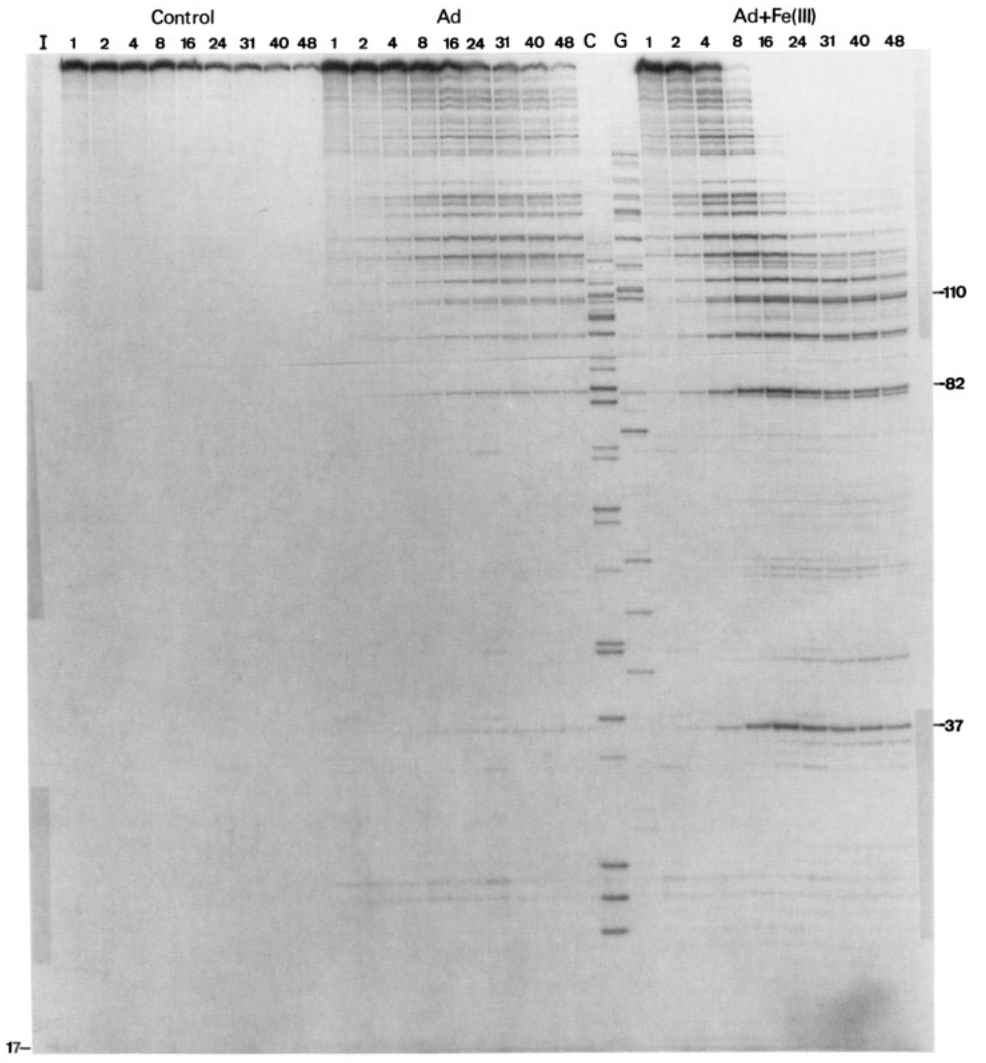

FIGURE 1: Dependence on reaction time. The autoradiogram shows the dependence of transcriptional blockages on the reaction time (37 °C) of adriamycin (10 μM) with the 497 bp *lac* UV5 containing DNA fragment in the absence (central nine lanes entitled "Ad") and presence of 75 μM Fe(III) [right-hand nine lanes entitled "Ad + Fe(III)"], for reaction times of 1–48 h. Control reactions in the absence of both adriamycin and Fe(III) ions were also left for the corresponding times of 1–48 h. Lane "I" is the initiated complex prior to elongation. All other samples were subjected to elongation conditions for 5 min. Lanes C and G are sequencing lanes using 3′-*O*-methoxy-CTP and 3′-*O*-methoxy-GTP, respectively. The length of some of the major blocked transcripts is shown on the right-hand side of the autoradiogram.

ersham. Acrylamide and ammonium persulfate were obtained from Bio-Rad, urea, bis(acrylamide), and TEMED were from IBI, New Haven, and heparin was from Sigma. Restriction enzymes were purchased from Boehringer Mannheim. NA45 paper was purchased from Schleicher and Schuell.

All other chemicals used were of analytical grade while all solutions were made up by using type I water from a Milli-Q four-stage purification system (Millipore, MA).

Methods

DNA Source. A 203 bp[1] *Eco*RI restriction fragment containing the L8UV5 double mutant of the *lac* promoter was ligated into pBR322 and the resulting plasmid isolated as described previously (White & Phillips, 1988). Digestion of the plasmid with *Pvu*II and *Sal*I yielded a 497 bp fragment containing the UV5 promoter, which was subsequently isolated by preparative agarose gel electrophoresis (White & Phillips, 1989).

The plasmid pINDU containing the strong N25 early *Escherichia coli* promoter from T5 phage, supplied by Professor H. Bujard, Heidelberg University (West Germany), was used in the construction of a second vector consisting of two counter-directed promoters (UV5 and N25) separated by 139 bases (White & Phillips, 1989). A 186 bp *Pvu*II fragment from the 203 bp sequence was directionally ligated into pINDU by routine methods (Maniatis et al., 1982) and then transformed into JM101 cells (Hanahan, 1983). The recombinant plasmid was amplified with chloramphenicol and isolated by using a modified alkaline lysis procedure (Maniatis et al., 1982). Digestion of the plasmid with *Xho*I and *Pvu*II yielded a 315 bp fragment which contained both the N25 and UV5 promoters. Isolation and characterization of the fragment were as described previously (White & Phillips, 1988).

End Labeling of 315 bp DNA. The *Xho*I 5′ overhanging end of the 315 fragment was filled in by using reverse transcriptase and [α-^{32}P]dTTP and was subsequently separated from unincorporated label as described previously (White & Phillips, 1989). The fragment was redissolved in TE buffer and used for testing for single-stranded nicks and interstrand cross-links.

In Vitro Transcription. In vitro transcription conditions using the 497 bp UV5 fragment contained 10 mM DTT and were as described previously (White & Phillips, 1988). An

[1] Abbreviations: bp, base pair(s); TE, Tris–EDTA; TBE, Tris–borate–EDTA; DTT, dithiothreitol.

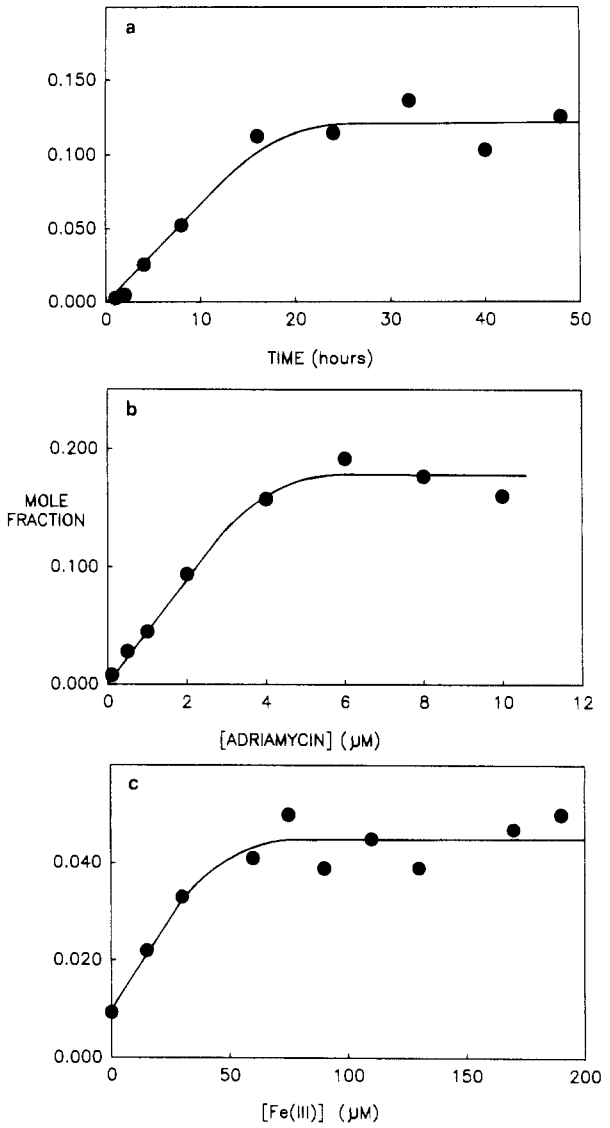

FIGURE 2: Time, adriamycin, and Fe(III) dependence of formation of 37-mer. The mole fraction of 37-mer is shown as a function of (a) reaction time of 10 μM adriamycin in the presence of 75 μM Fe(III); (b) adriamycin concentration in the presence of 75 μM Fe(III), reaction time of 24 h, 5-min elongation time; (c) Fe(III) concentration, in the presence of 10 μM adriamycin, reaction time of 24 h, 5-min elongation time.

initiated ternary complex was formed (White & Phillips, 1988; Skorobogaty et al., 1988a) and was divided into three equal aliquots. One aliquot was incubated in the presence of adriamycin and $FeCl_3$, another aliquot with adriamycin alone, and the third in just transcription buffer. These solutions were incubated at 37 °C for 24 h (unless otherwise stated), and then an elongation mixture comprising UTP, ATP, GTP, and CTP (2 mM final concentration) and KCl (400 mM final concentration) was added to all three aliquots. Elongation was continued for 5 min at 37 °C (unless otherwise stated) before the reaction was stopped with the addition of an equal volume of loading/termination buffer (10 M urea, 10% sucrose, 40 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue in 2 × TBE).

Transcriptional studies utilizing the 315 bp UV5/N25 dual promoter fragment required prior treatment to inactivate the competing promoter. In order to selectively form an initiated ternary complex from the UV5 promoter, the 315 bp fragment was digested with *Dra*I and then incubated with GpA (200 μM), ATP, GTP, and [α-^{32}P]UTP as previously described

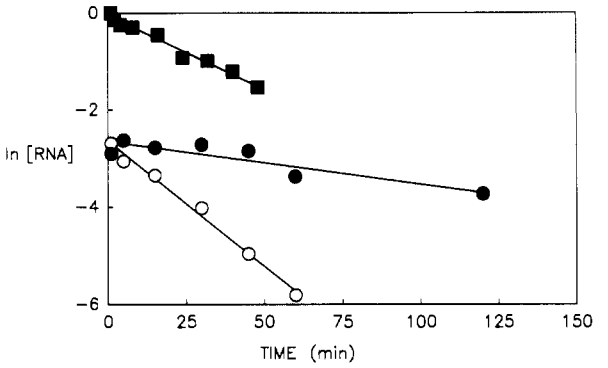

FIGURE 3: Decay of full-length and blocked transcripts. The relative concentration of full-length transcript in the absence of adriamycin (Figure 1) was taken as a measure of the amount of stable initiated complex—the first-order plot yields the rate constant for the decay of the initiated transcription complex (■). Read-through past the 37-mer (○) and 97-mer (●) is shown as first-order processes for the decay of the mole fraction of each blocked transcript.

(White & Phillips, 1989). Selective initiation of the N25 promoter required digestion of the 315 bp fragment with *Bst*N1 and incubation in the presence of ApU (200 μM), ATP, GTP, and UTP (5 μM) and [α-^{32}P]UTP (White & Phillips, 1989).

Sequencing, electrophoresis, and quantitation were as described previously (White & Phillips, 1988). Quantitation relies upon linearity between the amount of radioactivity in a band and the absorbance of that band in the autoradiogram, and this linearity has been demonstrated up to a maximum absorbance of 1.0 (Dabrowiak et al., 1986).

RESULTS

Adriamycin Reaction Time. In order to ascertain the reaction time necessary for maximal formation of irreversibly blocked transcripts, an arbitrary concentration of adriamycin was chosen (10 μM) and the drug allowed to react with the initiated transcription complex for various periods of time (Figure 1). The initiated complex was then elongated with high levels of all four nucleotide triphosphates. In the absence of Fe(III) ions there was a gradual increase of sequence-specific blocked transcripts with reaction time, and the same blockages were greatly enhanced in the presence of Fe(III) ions (75 μM), with a concomitant decrease in the amount of full-length transcript. The extent of enhancement of blocked transcripts induced by Fe(III) ions was quantitated as 15- and 12-fold for relative amounts of the 37-mer and 82-mer, respectively, after 16 h of reaction time.

A maximal amount of the 37-mer and 82-mer was apparent after 16 h of reaction time (Figure 1). However, when assessed in terms of the mole fraction of blocked transcript in each lane, it is clear that the reaction continues for approximately 24 h before the yield is independent of further reaction time (Figure 2a). This apparent discrepancy arises from the first-order decay of 0.030 h^{-1} (half-life of 23 h) of the initiated complex in the absence of drug and Fe(III) ions (Figure 3), as determined from the decreasing amount of full-length transcript observed with increasing incubation time (Figure 1). Therefore, with increasing reaction time, there is less of the active initiated complex available for the subsequent elongation process, resulting in decreasing absolute amounts of blocked transcripts at later reaction times, even though the reaction of adriamycin with the DNA may continue to yield more adducts. For convenience, the reaction time selected for further studies was usually 24 h.

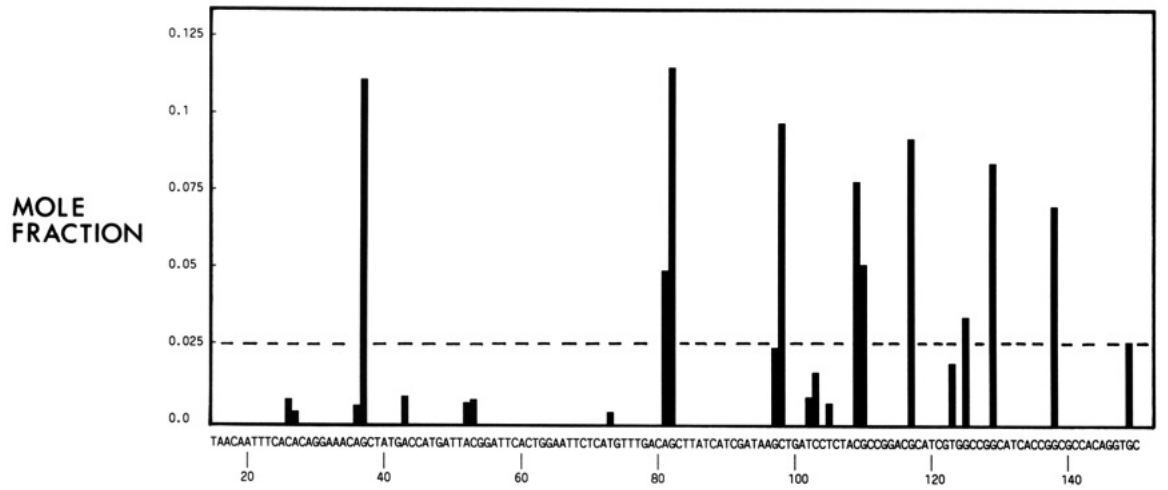

FIGURE 4: Quantitation of blocked transcripts. The mole fraction of each blocked transcript is shown with respect to the length of each transcript detected in the 16-h reaction time lane of Figure 1. The numbering represents the length of the RNA transcript, beginning from G of GpA in each chain (where A is the +1 position for the start of the transcriptional message from the UV5 promoter). The dashed line is an arbitrary level to define high-occupancy sites as above a mole fraction of 0.025.

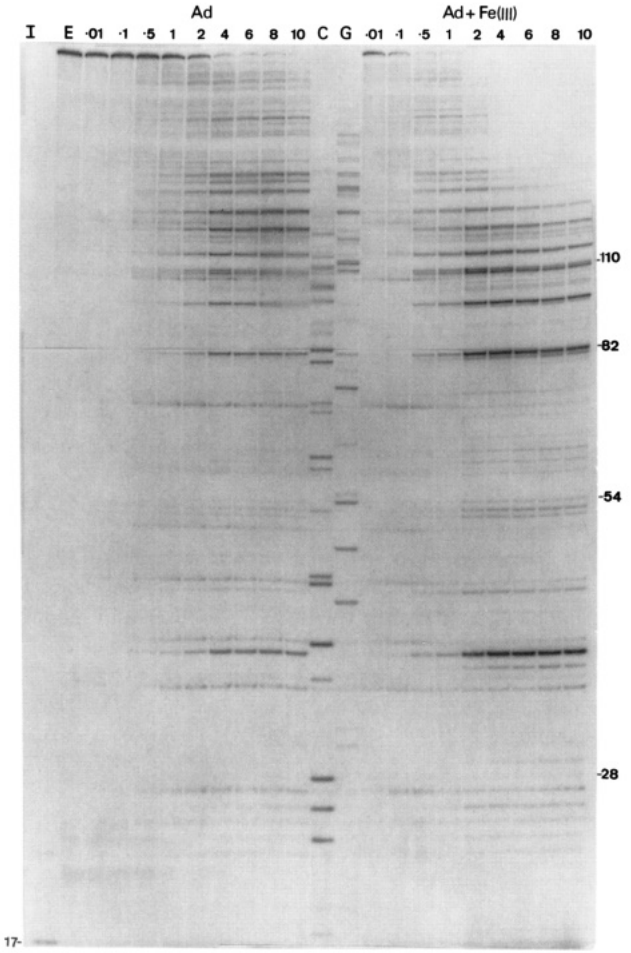

FIGURE 5: Dependence on adriamycin concentration. The initiated 497 bp UV5 promoter complex was reacted with 0.01–10 μM adriamycin (Ad) for 24 h in the absence and presence of 75 μM Fe(III) ions. The transcription complex was subsequently elongated for 5 min. Lanes I and E represent control lanes of the initiated complex and elongation of this complex to the full-length transcript.

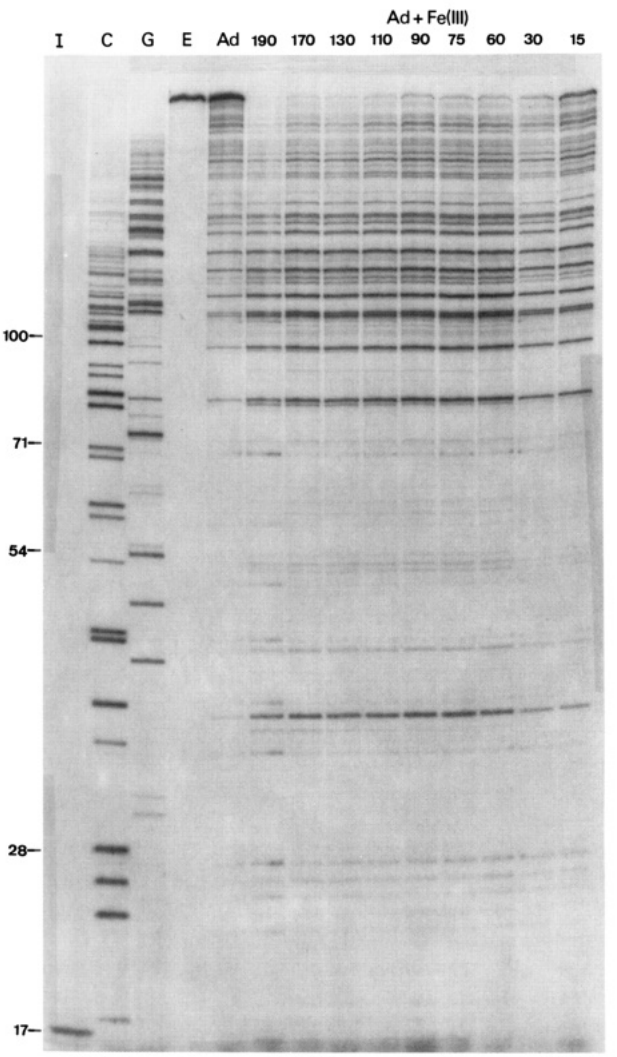

FIGURE 6: Dependence on Fe(III) concentration. The initiated transcription complex was reacted with 10 μM adriamycin and 15–190 μM Fe(III) for 24 h, followed by elongation for 5 min. Lanes I and E represented the initiated and full-length transcript, respectively. C and G are sequencing lanes with termination by 3′-*O*-methoxy-CTP and 3′-*O*-methoxy-GTP, respectively.

The mole fraction of each blocked transcript in the 16-h lane (Figure 1) was quantitated by densitometry, and the sequence specificity of these blockages is summarized in Figure 4. All major blockages occur at the G of 5′-GpC sequences, and some (e.g., 82 and 97) exhibit an additional blocked transcript one nucleotide less in length (Figure 4), and these were not observed in the absence of Fe(III) ions (Figure 1).

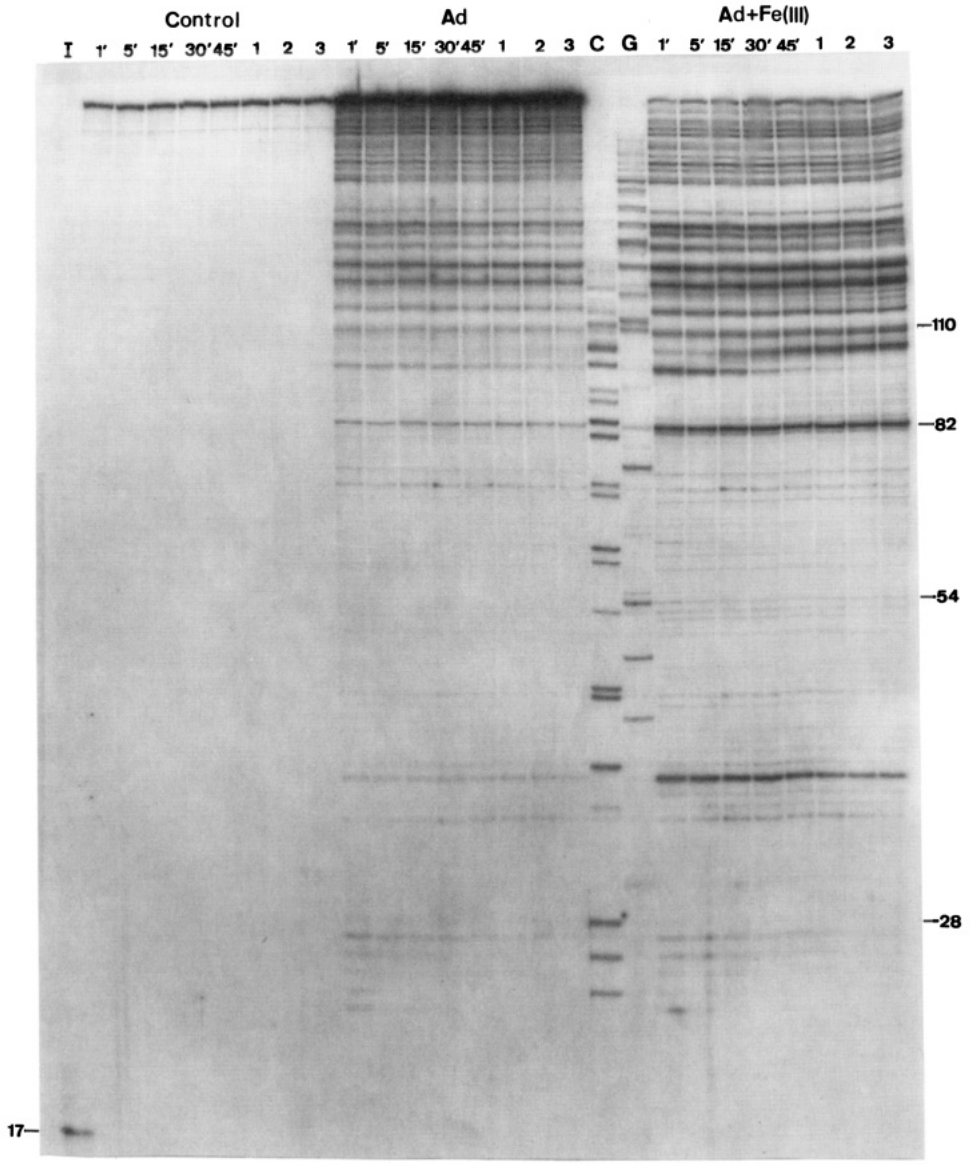

FIGURE 7: Permanence of blockages. Adriamycin (10 μM) was incubated with the initiated 497 bp transcription complex for 4 h in the absence and presence of 75 μM Fe(III). The initiated complex was then subsequently elongated for times ranging from 1 min to 3 h. The control lanes represent the initiated complex, which was allowed to stand for 4 h before elongation was commenced for the designated times.

Adriamycin Concentration Dependence. To establish the optimal concentration of adriamycin required to achieve maximal formation of irreversible blocked transcripts, various concentrations of adriamycin were reacted with the initiated transcription complex for 24 h, in both the absence and presence of Fe(III) ions (Figure 5). The increasing amount of blocked transcript with drug loading is apparent (Figure 5), and quantitation of the mole fraction of 37-mer reveals a linear dependence up to 4 μM of adriamycin, with saturation evident at 10 μM of drug (Figure 2b).

Iron Concentration Dependence. An increasing amount of sequence-specific termination of transcription was observed with increasing Fe(III) concentration (Figure 6) and leads to a decrease of full-length transcript at higher iron levels. The maximal mole fraction of 37-mer was achieved at iron levels of $\geq$75 μM (Figure 2c), and this level (75 μM) was used for all subsequent studies.

Stability of Transcriptional Blockages. Optimal levels of adriamycin (10 μM) and Fe(III) ions (75 μM) were reacted with an initiated transcription complex for the optimal reaction time of 24 h. The initiated complex was then elongated for various periods of time up to 3 h. A range of sequence-specific blockages was again observed (Figure 7). Apart from the 37-mer, and transcripts in the (97–104)-mer range, all other sequence-specific blockages were independent of elongation

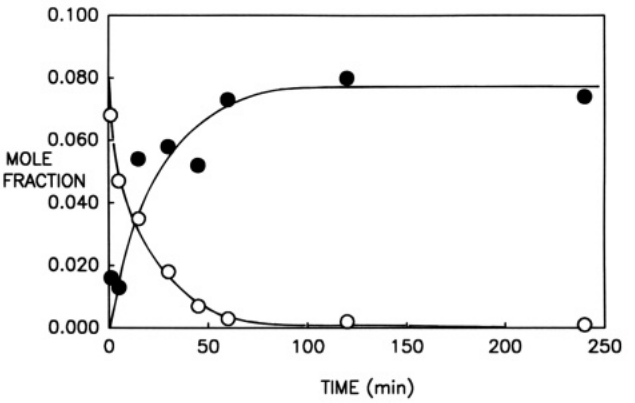

FIGURE 8: Delayed termination. The decay of the mole fraction of 97-mer (○) in Figure 7 [adriamycin plus Fe(III) lanes] is shown together with the buildup of the corresponding 103- and 104-mer transcripts (●).

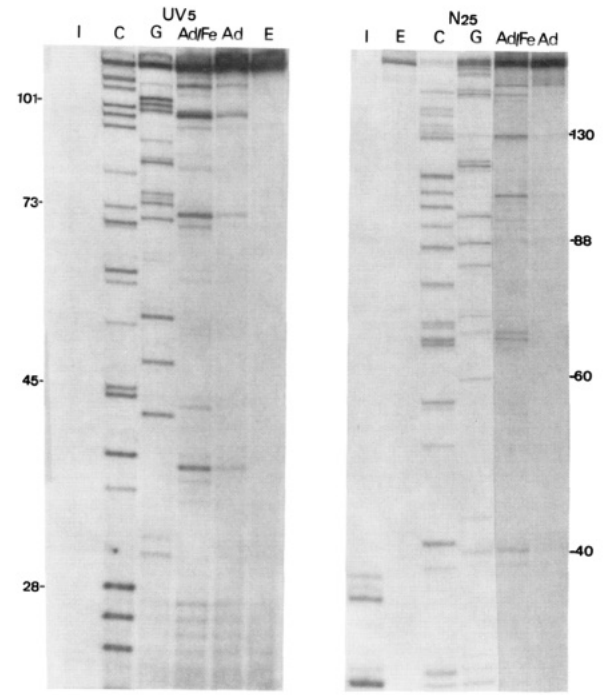

FIGURE 9: Bidirectional transcription footprinting. Each promoter of the 315 bp DNA fragment containing both the N25 and UV5 *E. coli* promoters was selectively initiated and then reacted for 24 h with 10 μM adriamycin, in the absence and presence of 75 μM Fe(III), followed by elongation of the transcription complex for 5 min. Transcriptional blockages are shown following reaction with the initiated UV5 promoter, and with the initiated N25 promoter. Lane I is the initiated complex, and E is the full-length transcript after elongation of the initiated complex for 5 min. Lanes C and G are sequencing lanes derived by elongation of the initiated complex in the presence of 3′-*O*-methoxy-CTP and 3′-*O*-methoxy-GTP, respectively.

time (Figure 7), consistent with the presence of an essentially irreversible blockage on the DNA. Read-through of RNA polymerase past the 37-mer and 97-mer sites were first-order processes, with rate constants of 0.32 and 1.9 h, respectively (Figure 3).

Read-through past the 97-mer site resulted in a buildup of transcripts of 103 and 104 nucleotides in length, effectively trapping all of the RNA polymerase released from the 97-mer site (Figure 8). From the constant intensity of the 110-mer, there was no evidence of RNA polymerase from the 97-mer site reaching the 110-mer site (Figure 7).

Bidirectional Transcription Footprinting. In order to understand the nature of the adriamycin-induced irreversible transcriptional blockages, it is necessary to establish the physical size of this moiety on the DNA. This was achieved by using bidirectional transcription footprinting in which two counter-directed promoters (UV5 and N25) were separated by 139 bp (White & Phillips, 1989). Each promoter was selectively activated to the initiated transcription complex and then exposed to adriamycin (10 μM) and Fe(III) ions (75 μM) for 24 h to ensure maximal yields of adduct. Sequence-specific blockages were observed when these initiated complexes were elongated, with enhanced blockages resulting when Fe(III) ions were present (Figure 9). These blockages were all irreversible, as defined by the lack of any detectable read-through of RNA polymerase past any of the blockage sites (Figure 10).

The mole fraction of blocked transcripts is a measure of the relative occupancy of adduct at each blockage site (Phillips & Crothers, 1986; White & Phillips, 1988), and the blockages shown in Figure 9 have been summarized in Figure 11 for transcription proceeding from both the UV5 and N25 promoters. The striking feature of this result is that only five high-occupancy sites are apparent, and all five show transcriptional blockage at G of 5′-GpC sequences. Three of these sites were probed by RNA polymerase from both promoters, while the fourth site in each case was observed from only one direction of transcription, since the initiated complex from the alternate promoter protected that region prior to reaction with adriamycin. In all cases transcription proceeded to G of the GpC sequence, or one nucleotide prior to the G.

For all three sites probed by RNA polymerase from both directions (sites 1–3 from the UV5 promoter) the major blockage observed was at G of the nontemplate strand for transcription proceeding from either promoter. This transcriptional footprint therefore defines the physical size of the entity which causes termination of transcription at these three sites as occupying a maximal region of 2 bp.

The 4′th site is not observed from the counter-directed promoter because the RNA polymerase extends at least as far as the halted initiation complex (i.e, the majority of polymerases proceed up to the first C residue to be transcribed) (White & Phillips, 1988), and this region is shown in Figure 11. Furthermore, from DNase I footprinting studies, the region of DNA protected by RNA polymerase is known to extend approximately 5 nucleotides downstream of the *E. coli* RNA polymerase catalytic site (Carpousis & Gralla, 1985). The region of DNA that is protected prior to the addition of adriamycin therefore completely overlaps the region of the 4′th high-occupancy site.

Test for Single-Strand Nicks and Interstrand Cross-Links. An initiated UV5 transcription complex was formed by using the end-labeled 315 bp fragment. The complex was treated with adriamycin (10 μM) and Fe(III) ions (75 μM) for 19 h, and these solutions were divided into two aliquots. One fraction was analyzed for blocked transcripts, and these were as observed previously (i.e., comparable to the UV5 strand of Figure 11). The other half of the reaction was denatured (90 °C for 5 min in a loading buffer containing 90% formamide) and then subjected to denaturing gel electrophoresis. A single band was seen which migrated with the control lane [incubated in the absence of adriamycin and Fe(III)]. No additional bands were observed, consistent with the absence of single-stranded nicks on the 315 bp fragment. There was no altered migration of the 315 bp nucleotide band, consistent with the absence of interstrand cross-links.

Discussion

Reaction Conditions. In the region transcribed from the initiated 11-mer to the end of the 497 bp fragment (a total of 370 bp), there were approximately 22 high-intensity block sites discernible after 40 h of reaction time in the presence of adriamycin and Fe(III) ions. This corresponds to an estimated total of approximately 30 such sites in the 497 bp DNA fragment. From this estimate of the 30 sites per promoter, and the approximately 50 nM of promoter fragment used, it can be calculated that there are approximately 1.5 μM of potential adduct sites in the reaction mixture. If each site was fully occupied by an adriamycin adduct, saturation would require a minimum of a 1.5 μM solution of adriamycin. However, at saturating levels of adriamycin a fractional occupancy of 0.18 was observed (Figure 2b), implying that approximately 0.3–0.6 μM of adriamycin would be required to fully saturate all potential sites to this level of occupancy. Since saturation was only observed at adriamycin levels >4

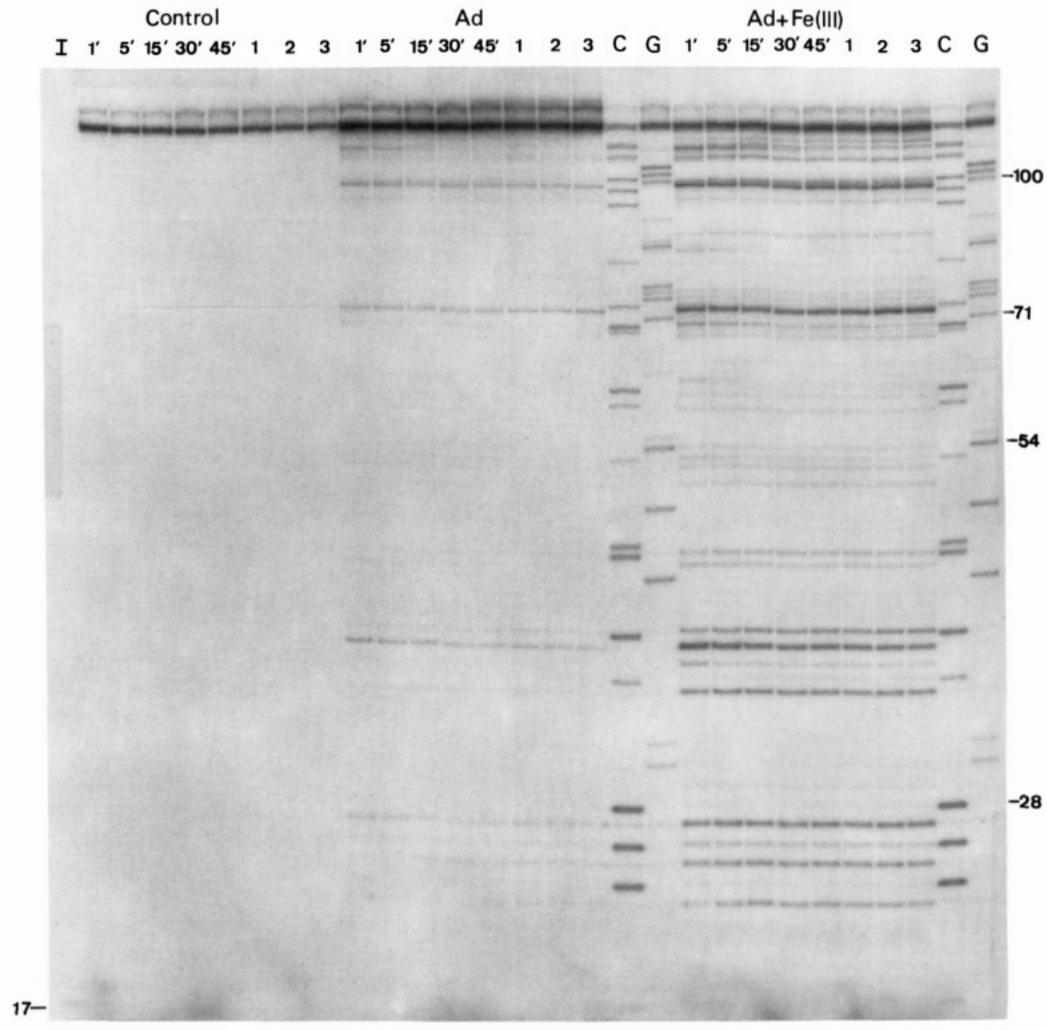

FIGURE 10: Permanence of bidirectional blockages. The UV5 promoter of the dual promoter 315 bp DNA fragment was initiated and then reacted for 24 h with 10 μM adriamycin, in the absence and presence of 75 μM Fe(III). The initiated complex was then elongated for various times, from 1 min to 3 h. Lane I is the initiated complex, and C and G are sequencing lanes. The control lanes represent the initiated complex, elongated after 24 h for the indicated times.

μM, only 20% or so of the drug ultimately results in adducts on the DNA fragment under these conditions. The reason that the mole fraction of blocked transcript does not approach unity is probably due to the complexity of reactions involved—if the reactive intermediate is a reactive species such as a quinone methide, which is known to act as either a nucleophile or electrophile (Fisher et al., 1985; Ramakrishnan & Fisher, 1986), a wide range of chemical behavior will result (Fisher et al., 1985). It has been well documented that the quinone methide can react with other nucleophiles such as thiols (Ramakrishnan & Fisher, 1986) present at high levels in the transcription buffer (e.g., BSA, 0.125 mg/mL; DTT, 10 mM) or with water (Fisher et al., 1982).

The high apparent Fe:adriamycin ratio reflected by the concentration of each species required for the production of maximal blockages (75 and 10 μM, respectively) should be interpreted with caution. Much of the Fe(III) ions will be bound to multiple sites on the BSA (present at 7 μM), as well as to multiple sites on the DNA (Eichhorn & Shin, 1968). It is not therefore possible to elucidate from the present experiments the stoichiometry of a putative Fe–adriamycin complex which may be involved in formation of the transcriptional blockages.

The 22 block sites identified in Figure 1 in the 370 transcribed base pairs (from the 11-mer to full-length transcript) represent a binding ratio of one site per 17 bp, comparable to the binding ratio of 15–20 observed for covalent adducts with calf thymus DNA formed by chemical activation of adriamycin with $NaBH_4$ (Sinha, 1980). It is therefore likely that the adducts deriving from these two processes may be similar.

Sequence Specificity. All major transcriptional blockages observed with adriamycin (after extensive exposure to the initiated transcriptional complex) have been at G of GpC sequences on the nontemplate strand (Figures 4 and 11) and comprise a total of 17 such blockages. Previous transcriptional studies with intercalators have shown that transcription proceeds up to the base pairs comprising the intercalation site (Phillips & Crothers, 1986; White & Phillips, 1988, 1989). On this basis it is likely that the adduct is on the G of GpC residues. In this context it is interesting to note that Sinha and Gregory (1981) observed that adriamycin activated with $NaBH_4$ exhibited a strong preference for the formation of adducts at G residues.

The bidirectional footprinting result showing that the size of the physical blocking unit is limited to the 2 bp element of GpC (Figure 11), with minimal blockage at isolated G residues, provides direct evidence that the adduct must comprise a relatively small moiety capable of binding strongly (presumably covalently to at least one residue within the GpC domain). Although the bidirectional transcriptional footprinting results suggest a possible G–adduct–G cross-link,

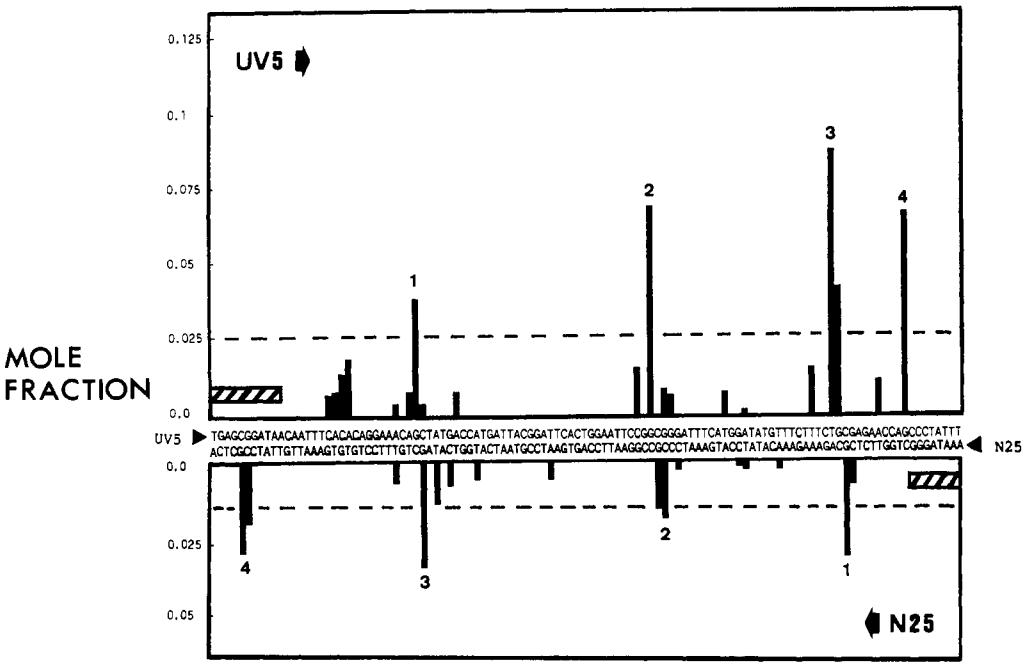

FIGURE 11: Relative intensity of bidirectional blockages. The mole fraction of blocked transcripts shown in Figure 9 was quantitated by densitometry for reaction of the initiated complexes for 24 h with 10 μM adriamycin and 75 μM Fe(III). The mole fraction of each blocked transcript is shown with respect to the length of the transcript, as shown by the nontemplate DNA strand for transcription proceeding from each promoter. The dashed lines at arbitrary levels define high-occupancy sites as above a mole fraction of 0.025 (UV5 promoter) or 0.0125 (N25 promoter). The hatched areas indicate the region occupied by each RNA polymerase, as defined by the length of RNA in the initiated transcription complex (White & Phillips, 1988).

based on the apparent specificity for G residues, there is no evidence from these transcriptional studies to confirm this notion. It should be noted however that Konopa (1980) observed apparent DNA cross-links in the presence of adriamycin in tissue culture, and adducts have also been reported in vivo by Sinha and Sik (1980). These enzymatically generated adducts may well be similar to the adducts detected transcriptionally in the present work.

The minor transcriptional blockages shown in Figures 4 and 11 all occur at (or one nucleotide prior to) a G residue on either the template or nontemplate strand. Since the relative occupancy reflects the relative affinity for these sites, the minor blockages reflect a range of lower affinity sites and may reflect a mono adduct, compared to some form of cross-linking between opposing G residues at high-affinity GpC sites. It should be noted that these suggestions are totally speculative at this stage.

Delayed Termination of Transcription. The complexity of the nature of species comprising long-lived transcriptional blockages induced by adriamycin is highlighted by a delayed termination accompanying the 97-mer blockage site (Figure 8). All read-through past the 97-mer was completely "trapped" 6 or 7 bp further downstream (103 and 104) of the initial blockage site. Since there was little blockage evident at that position (103- and 104-mers) after 5 min of elongation, it appears that it is not an intrinsic site for formation of an adduct. Rather, it probably represents an example of delayed termination of transcription, where the initial delay of RNA polymerase at position 97 provides sufficient time for formation of hairpin helices in the RNA, and as the adduct dissociates from the GpC sequence (97-mer), RNA polymerase is able to read-through past that site but is then destabilized by the presence of the hairpin helix immediately adjacent to the transcription complex. This interpretation is based on the virtually identical behavior observed for the dissociation of actinomycin D from the same GpC sequence, where a transcription blockage was also seen as a 97-mer and 100% efficient delayed termination observed as (104–106)-mers (White & Phillips, 1988). An alternative, less likely explanation is that reversibly bound (intercalated) adriamycin, which normally has a dissociation rate constant of approximately 1 s from DNA (Phillips et al., 1986), may be affected by the presence of RNA polymerase under some specific situation. This seems unlikely given the difference of 3 orders of magnitude between that observed for the dissociation of adriamycin from DNA and the first-order process observed for the 97-mer (Figure 3), and also because there has been no previous indication of such an effect by RNA polymerase (Skorobogaty et al., 1988a,b).

Nature of the Adduct. The most likely model for the formation of adriamycin-dependent irreversible transcriptional blockages involves the reduction of adriamycin to a quinone methide (Sinha, 1981). Since the amount of blockages is dependent on the level of Fe(III) ions (Figure 2c), and adriamycin exhibits a high affinity for Fe(III) ions, it appears that an adriamycin–Fe(III) complex is involved. Reduction of the drug therefore probably occurs by DTT, mediated by Fe(III).

It may be argued that, for this process to occur, only trace amounts of Fe(III) would be necessary for its cyclic role as a catalyst. Indeed, trace amounts of Fe(III) ions in the buffer may well account for the background amount of blocked transcripts observed in the absence of added Fe(III) (Figure 1). However, most added Fe(III) ions are likely to be complexed to the high level of BSA (0.125 mg/mL) present in the transcription buffer, and not able to participate in the above catalytic process.

The N-7 of guanine is the most nucleophilic center in DNA and would therefore be the most likely site for alkylation by a quinone methide (Pullman & Pullman, 1981)—any such adducts would therefore result in transcription blockages preferentially at G residues, as observed. The reason for the observed specificity of transcriptional blockages at GpC residues is not yet clear. The simplest scenario would suggest

a G–drug–G interstrand cross-link. However, we have not been able to observe such a heat-resistant cross-link. Furthermore, while it is possible to define the nature of one covalent link (methide C-7–guanine N-7), the nature of any such second covalent bond from the same aglycon is not obvious. One possibility is that it could be a pseudo-cross-link formed by one quinone methide at each G residue of a GpC duplex, with the two aglycon chromophores providing a stable hydrophobic central core. Such a complex has several attractive features. First, it would be consistent with the instability of such adriamycin-induced cross-links to heat and alkali (Konopa, 1980) and the apparent lack of fully covalent cross-links from the present work. Second, since the transcriptional blockages are much more pronounced at GpC sequences than at isolated G residues, it suggests that there may be an additional stability at GpC sites, and this could arise from aglycon–aglycon stacking interactions. Furthermore, if the adduct at isolated G residues were less stable, then they would exhibit a slow rate of transcriptional read-through past three sites, and that is indeed observed in Figure 7, where the minor background of blocked transcripts decays slowly (e.g., transcripts 34–36 and 52–63). If the apparent adducts on isolated G residues have a slow rate of release from DNA under transcription conditions (pH 8.0), then it might be expected that at GpC sites there will be a range of extent of stacking of the putative two aglycon moieties, and those with little overlap will be stabilized less and therefore also exhibit a slow dissociation rate. The observed decay of blocked transcripts past two GpC sites (37 and 97) is thus consistent with this preliminary model of the GpC sequence specificity of apparent adriamycin adducts.

Conclusions. The nature of adriamycin-induced apparent adducts is complex—a range of different long-lived species was observed, including essentially irreversible blockages at most GpC sequences, slowly dissociating products at two GpC sequences and isolated G residues, and delayed termination 6 bp downstream of one GpC site.

Although the features of the proposed model for the nature of the adducts are consistent with the experimental data, there is no *direct* evidence for these structures. The model must therefore be considered as speculative at this stage. Because of the potential medical significance of such adducts, attempts are continuing in this laboratory to further define the nature of such adducts, both by in vitro transcription assays and by direct chemical isolation and characterization.

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