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DNA sequence-specific adducts of adriamycin and mitomycin C

Don R. Phillips, Robin J. White and Carleen Cullinane

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

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Adriamycin and mitomycin C were reduced by xanthine oxidase/NADH in the presence of a DNA template comprising a stable initiated ternary transcription complex derived from the *lac* UV5 promoter. Subsequent elongation of the transcription complex treated with mitomycin C revealed high levels of terminated transcripts one nucleotide prior to G residues on the coding strand (i.e. at X of XpC sequences of the non-coding strand). Lower levels of termination occurred with adriamycin, and these were also one nucleotide prior to G residues of the coding strand, but with greater sequence specificity since they were observed mainly at G of GpC sequences of the non-coding strand. The same sites were also observed with adriamycin in the absence of reducing conditions and the level of termination at these sites was enhanced up to 10-fold by Fe^{2+} and Fe^{3+}, but not by Cu^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}. These results suggest that an iron-adriamycin complex with DNA is highly sequence-specific and results in adducts, similar to those of mitomycin C, which can terminate the transcription process. Such a mechanism offers new insights into the possible mode of action of anthracyclines.

Adriamycin; Mitomycin C; DNA adduct; Xanthine oxidase; Sequence specificity; Divalent metal ion

1. INTRODUCTION

Adriamycin has been in routine clinical use as an anticancer agent for two decades [1–3]. Full realisation of its antineoplastic potential is limited by an associated cardiotoxicity [4,5] and there have been extensive efforts to develop more active and/or less cardiotoxic derivatives to combat this problem [6,7]. In order to place the development of new derivatives of these anthracyclines (adriamycin and daunomycin) on a more rational basis, there have been widespread and concerted efforts to delineate the mode of action of these drugs. Although membrane binding [8] and impairment of topoisomerase II activity [9] are possible modes of action, there is an emerging body of evidence to show that the biological activity of these drugs correlates with DNA binding [10] or with a range of DNA-related events [2,11].

The role of DNA as an important receptor for anthracyclines may involve the formation of covalent adducts with DNA, and such adducts have been well documented by chemical and enzymic reduction in vitro [12–14]. If these adducts occur to a significant degree in vivo, then it is expected that they would result in termination of transcription, and may therefore comprise an important mode of action of these drugs, particularly in the light that tumour cells contain a more reducing environment than normal cells [15]. Similar adducts with mitomycin C have been well documented [16–18]. We present here an in vitro transcription assay which exhibits blockage of transcription by *E. coli* RNA polymerase at specific DNA sites when mitomycin C is reduced by xanthine oxidase/NADH, or when adriamycin is present with Fe^{2+} or Fe^{3+}.

Correspondence address: D.R. Phillips, Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

2. EXPERIMENTAL

2.1. *Reagents*

Adriamycin was a gift from Farmitalia Carlo Erba (Milan). Mitomycin C was obtained from Sigma. Xanthine oxidase was supplied by Dr A. Wedd (La Trobe University).

2.2. *Transcription*

A 497-bp *Puv*II/*Sal*I restriction fragment of a modified

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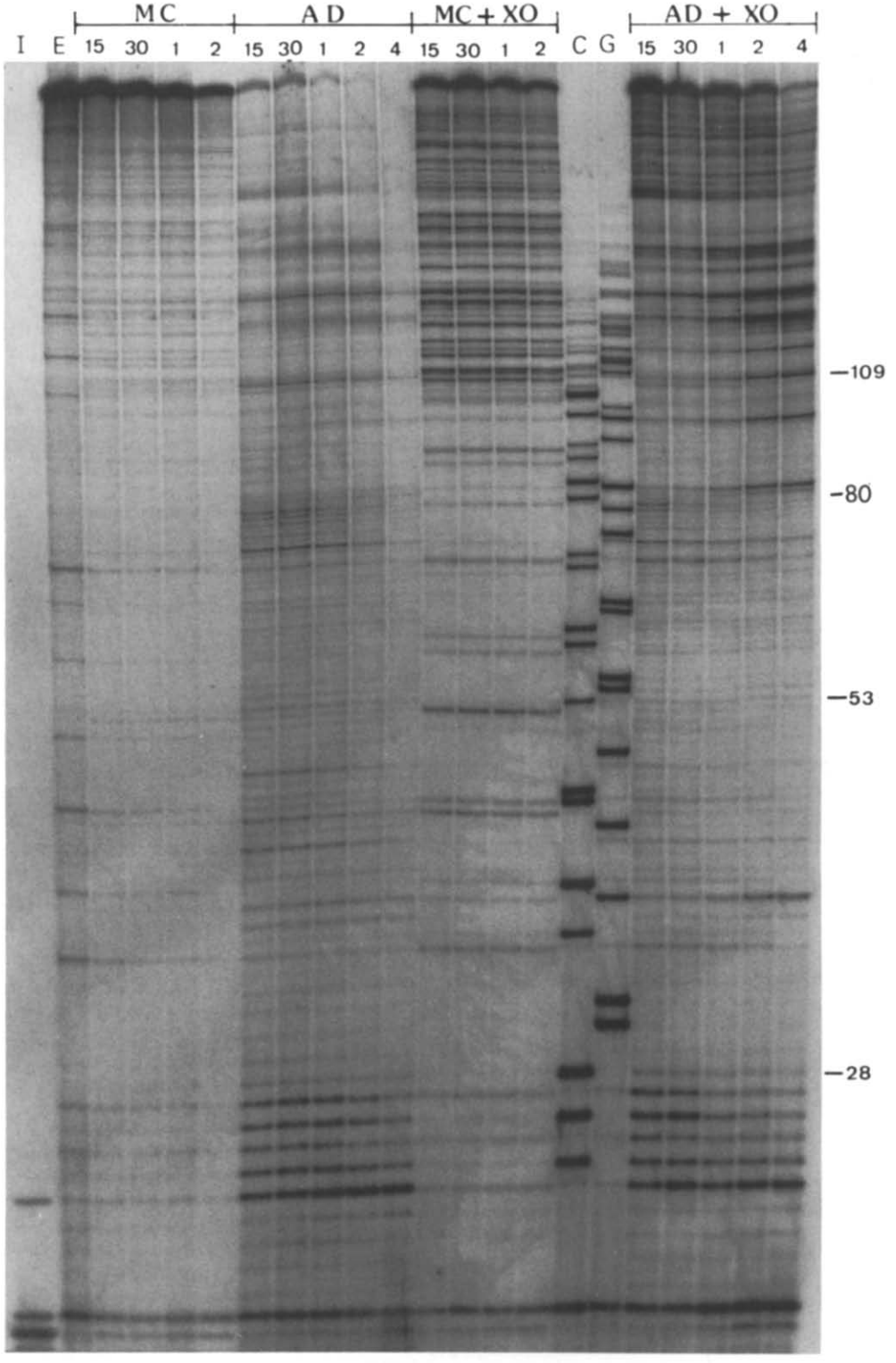
MC
AD
MC + XO
AD + XO
I E 15 30 1 2 15 30 1 2 4 15 30 1 2 C G 15 30 1 2 4
-109
-80
-53
-28

Fig.1. Autoradiogram of RNA transcripts detected following elongation (5 min) of the initiated complex treated with 1 mM mitomycin C (MC) or adriamycin (AD) 15 min, 30 min, 1, 2 or 4 h (denoted 15, 30, 1, 2 and 4, respectively) at 37°C, and also under reducing conditions in the presence of xanthine oxidase (XO) and NADH. The initiated complex is shown in lane I, and the resulting transcript after 5 min of elongation in lane E. Sequence lanes are denoted as C (termination by methoxy-CTP) and G (termination by methoxy-GTP).

pBR322 vector contained the *lac* UV5 promoter and was utilized for in vitro transcription with *E. coli* RNA polymerase [19]. Transcription was initiated to form a stable ternary complex as in [19,20]. Adriamycin was added to defined concentrations. Metal ions were then added to a concentration of 150 μM while the reducing system was formed by the addition of NADH (2 mM) and 1.5 U xanthine oxidase [21]. The reaction mixtures were incubated at 37°C for various times before the transcript was elongated and then terminated as described [19,20].

2.3. *Electrophoresis and autoradiography*

Electrophoresis, autoradiography and densitometric analysis were performed using the techniques in [19,20].

3. RESULTS

The effect of the xanthine oxidase/NADH reducing system on mitomycin C and adriamycin is shown in fig.1. Minimal pausing is evident in the control elongation lane compared to the amount of full-length transcript. An extensive amount of specific blockage was obtained with mitomycin C under reducing conditions, while the non-reducing control lanes show essentially only background pausing. A quantitative analysis of the sequence specificity of mitomycin C is presented in fig.2.

In contrast, adriamycin alone resulted in many blocked transcripts after 15 min reaction time, and a low level of full-length transcript which decreased further with reaction time (fig.1). The blockages observed under non-reducing conditions are essentially the same when in the presence of xanthine oxidase/NADH, and some additional bands are also evident (e.g. between RNA lengths of 80 and 109). The enhanced intensity of those bands under reducing conditions is due largely to a greater stability of the ternary complex under these conditions, and this was confirmed by separate experiments in which the addition of xanthine oxidase/NADH (but not with either agent alone) produced much higher yields of full-length transcript (not shown). This effect probably relates to a destabilisation of the ternary complex by

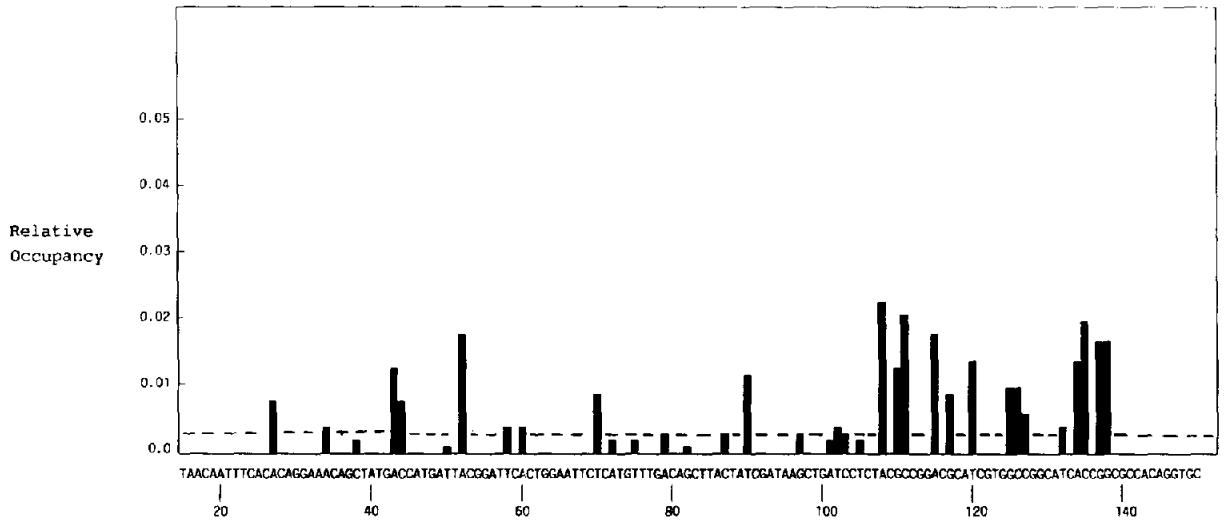

Fig.2. Mitomycin C transcriptional blockage sites induced by reductive activation. The mole fraction of RNA transcripts is shown (2 h incubation lane of fig.1), together with the sequence of the DNA non-coding strand, numbered from G of the initiating GpA dinucleotide. High-occupancy sites (>0.25% mole fraction of RNA) have been defined by a dashed line.

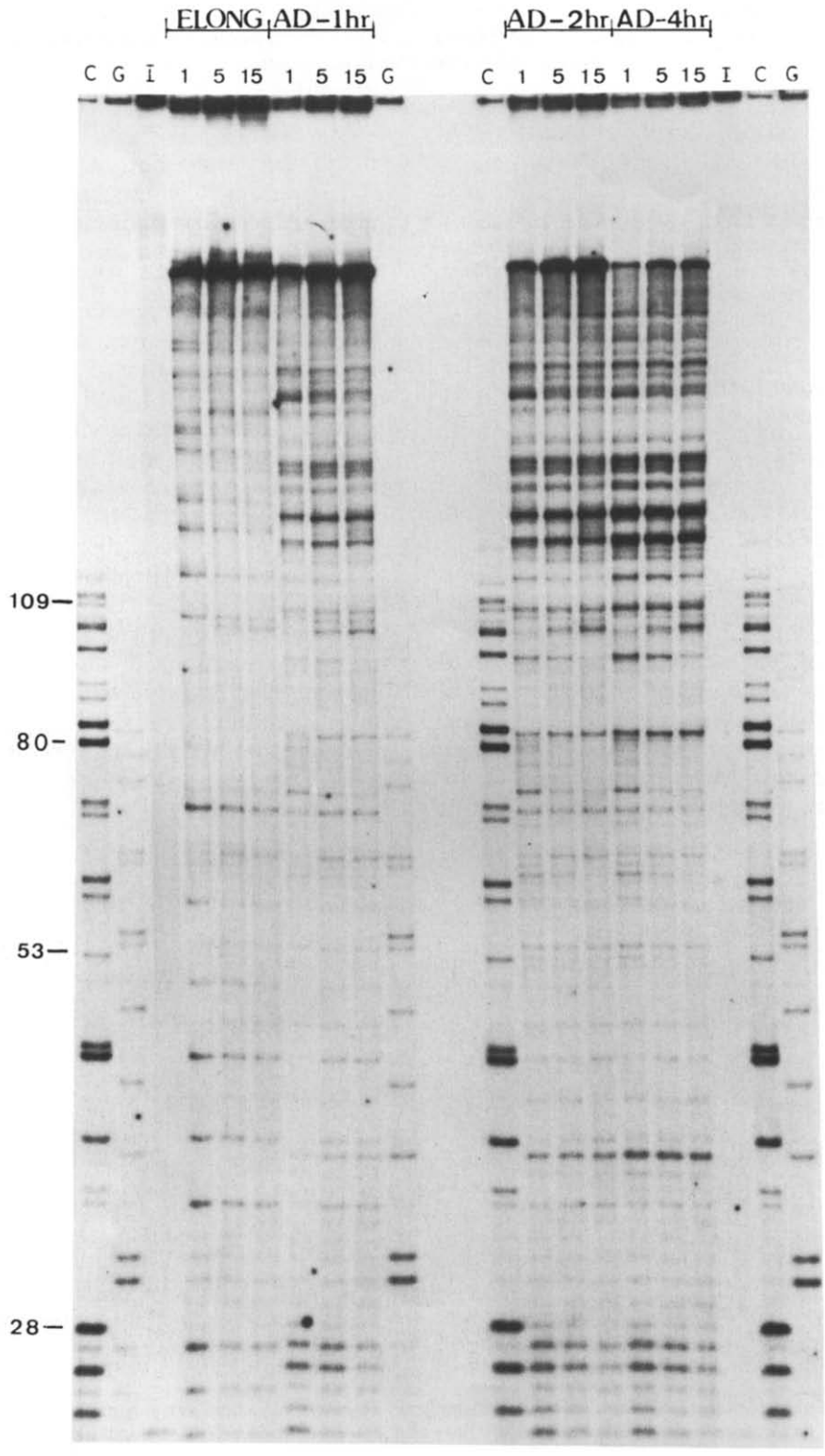
ELONG
AD-1hr
AD-2hr
AD-4hr
C G I 1 5 15 1 5 15 G
C 1 5 15 1 5 15 I C G
109
80
53
28

Fig.3. Autoradiogram of adriamycin-induced transcriptional blockage sites following 1, 5 and 15 min of elongation after 1, 2 or 4 h of exposure of the initiated complex to adriamycin (50 μM) at 37°C. The initiated complex is shown in lane C and methoxy-CTP and methoxy-GTP sequencing lanes in C and G.

adriamycin, but was diminished when the drug was bound to the xanthine oxidase-NADH complex.

In the absence of reducing conditions the formation of adriamycin block sites increased slowly with time over 4 h (fig.3). There was no evidence of elongation past most of these sites over 15 min (fig.3) and this demonstrates that the drug association at these sites is much more permanent than for

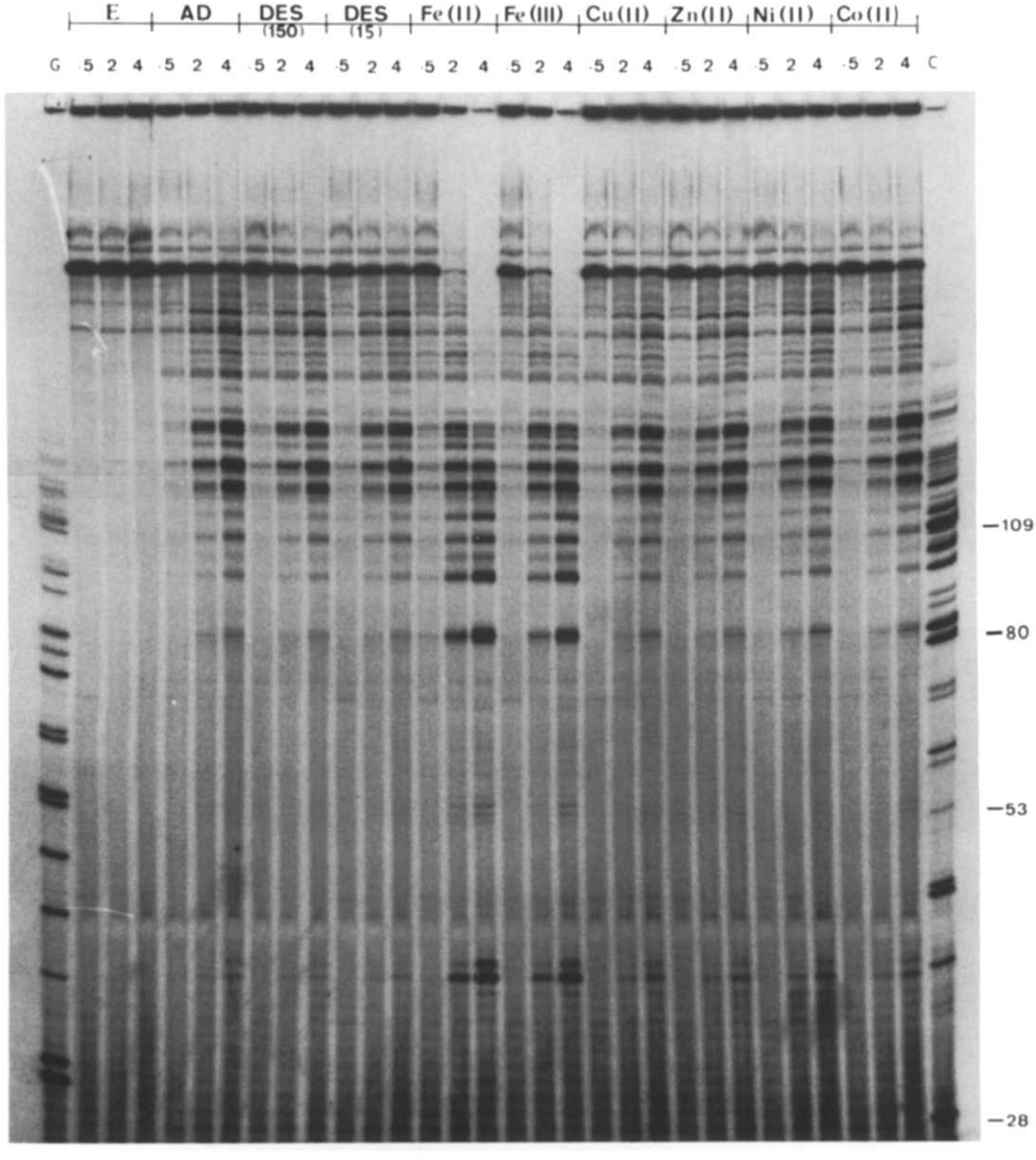

Fig.4. Autoradiogram of adriamycin (50 μM) induced transcriptional blockage sites after treatment of the initiated complex for 0.5, 2 or 4 h at 37°C in the presence of divalent metal ions (150 μM) or desferrioxamine (DES) at 15 or 150 μM. The ternary transcription complexes were elongated for 2 min for all samples.

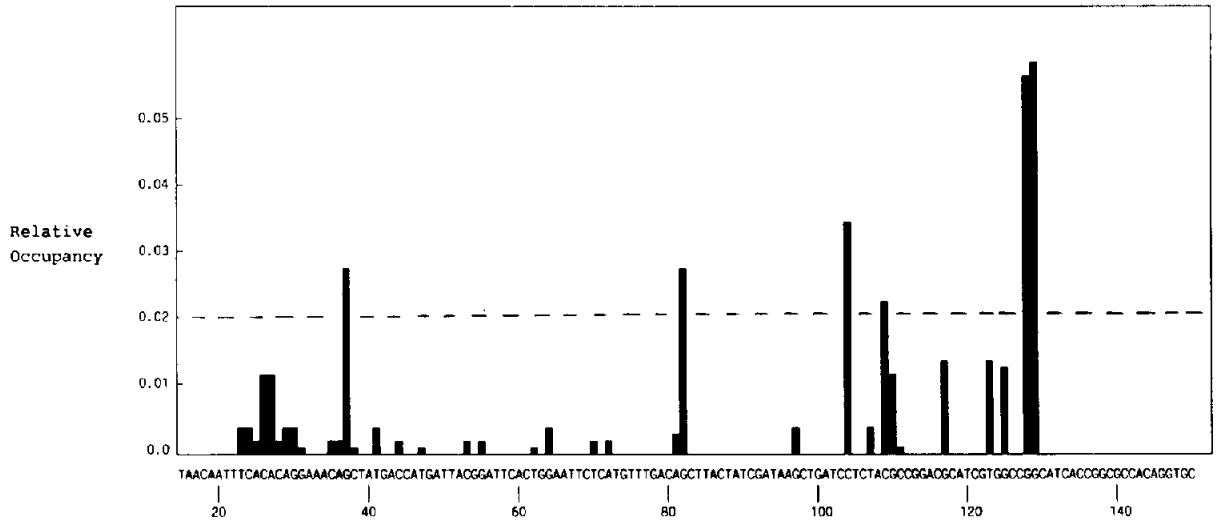

Fig.5. Adriamycin-induced transcriptional blockage sites under non-reductive conditions. The mole fraction of each transcript is shown after 4 h of exposure of the initiated complex to adriamycin, and subsequently 15 min of elongation (fig.3). The mole fraction of transcript has been corrected to allow statistically for undetected adducts downstream of existing blocked sites [31]. High-occupancy sites (mole fraction >2%) have been defined by a dashed line.

reversibly bound adriamycin which dissociates rapidly from DNA and is not detected by the transcription assay at 37°C, but can be seen at 10°C [22].

Since apparent adducts of adriamycin were observed under non-reducing conditions, one possibility was that these adducts derived from reduction via thiol groups (e.g. serum albumin) as suggested by Favaudon [23], with the electron flow mediated by Fe^{2+} [24]. For this reason we examined the dependence of the apparent adducts on the presence of Fe^{2+}. During this process we established that both Fe^{2+} and Fe^{3+} enhanced the formation of transcriptional blockages. A further examination of the metal ion dependence has now shown that the termination of transcription is metal-ion-dependent, with only Fe^{2+} and Fe^{3+} resulting in enhanced sequence specific termination of transcription (fig.4). A quantitative analysis of the sequence specificity of adriamycin is shown in fig.5.

4. DISCUSSION

4.1. *Mitomycin C*

The transcriptional blockages observed following reductive activation of mitomycin C confirm the well-documented observation of DNA adducts with this compound [16–18]. The fact that these blockages are independent of elongation time is consistent with such covalent adducts. The sequence specificity shows that blockages occur at X of XpC sequences and this suggests that adducts are exclusively on the G residue of the coding strand, since drug-induced transcriptional blockages have routinely been detected immediately adjacent to drug sites [19,20]. All of the 21 high-occupancy blockage sites in the transcribed sequence occur at X of XpC sequences and confirms the previous observations of G-specific adducts (non-coding strand) with mitomycin C. Adducts on the non-coding strand were apparently not detected and this is consistent with previous observations with psoralen adducts [25].

4.2. *Adriamycin*

Reductive activation of adriamycin did not yield any transcriptional blockages in addition to those observed under non-reducing conditions, even though the adriamycin was clearly being reduced, as shown by an increasing amount of adriamycinone precipitating from the reaction [26]. The reason that adducts previously observed by others under reducing conditions [12,13] were not observed in the transcriptional analysis is presumably due to the 1000-fold lower DNA con-

centration employed in the present work which is based on nanomolar levels of promoter-containing DNA fragments. The implications then are that reduced adriamycin species are considerably less reactive than the corresponding mitomycin C reactants and that the gradual development of blocked transcripts of adriamycin under reducing conditions is only a background effect, identical to that observed in the absence of a reducing environment.

A much more fundamental question which must be addressed is the nature of termination of transcription induced by adriamycin under non-reducing conditions. This phenomenon is drug-dependent and also specific for Fe^{2+}/Fe^{2+}, suggesting a DNA-adriamycin-Fe complex. Since this complex is independent of elongation time, it has the characteristics of a covalent adduct, but could in principle also be described as a long-lived reversible complex. Irrespective of the exact nature of the complex, the net effect of virtually abolishing the transcription process is dramatic. The DNA sequence specificity of these adducts was GpC (non-coding strand) with blockage at the G residue being due to an adduct or complex on G of the coding strand. All seven GpC sequences in the transcribed region are associated with a blockage at the G site, and 5 of the 6 highest occupancy sites are at GpC sequences (fig.5). By analogy with the XpC site associated with mitomycin C, adriamycin shows a greater degree of specificity, and implies that both of the (G·C) base-pairs are involved in the binding site. This may relate to the conformation of DNA around GpC sites which are known to have a smaller distance between the two guanines, viz. 6.5 Å, compared to 9.1 Å in CpG sequences [27]. The lack of effect of desferrioxamine (fig.4) may be due to the fact that the iron may not be accessible to this complexing agent when bound to drug and/or DNA.

The 1:3 complex of iron and adriamycin is a stable compound [28,29] with high affinity for DNA. It is therefore probable that the ternary DNA-adriamycin-Fe complex involves such a compound with the specificity derived from (G·C) contacts with both drug and iron moieties of the complex.

4.3. *Pharmacological implications*

Any process that results in the complete termination of transcription in vitro, as distinct from merely a delay, as in the case of reversibly binding drugs, must be considered as an important potential mode of action in vivo. The mechanism of action of the anthracyclines has been investigated for two decades, and to the many probable contenders should now be added the possibility of a drug-Fe^{2+}/Fe^{3+}-mediated termination of transcription. If this proves to be a major mode of action, the selectivity for tumour tissues may well be associated with the GpC specificity of the ternary complex possibly affecting the B-Z transition of $(GC)_n$ sequences which are thought to act as regulatory elements in eukaryotes [30].

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