# Inhibition of RNA Polymerase II Transcription in Human Cell Extracts by Cisplatin DNA Damage

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ABSTRACT: The anticancer drug cisplatin induces a spectrum of lesions in DNA. The effect of such DNA damage on transcription by RNA polymerase II (RNA pol II) in human cell extracts was investigated at the level of initiation and elongation. RNA pol II transcription directed from the adenovirus major late promoter was inhibited following treatment of the promoter-containing template with increasing concentrations of cisplatin. Furthermore, transcription from an undamaged promoter fragment was depleted in the presence of increasing amounts of cisplatin DNA damage on an exogenous plasmid, suggesting such damage may hijack an essential factor for transcription initiation. The effect of cisplatin damage on RNA pol II elongation was investigated using site-specifically-placed cisplatin adducts. The GTG adduct was an effective block to RNA pol II elongation, inhibiting the polymerase by 80%. In contrast, RNA pol II completely bypassed the cisplatin GG intrastrand adduct. These studies suggest that the inhibition of RNA pol II transcription observed following the treatment of cells with cisplatin is likely to reflect the combined effects of DNA damage at the level of both transcription initiation and elongation.

*cis*-Diamminedichloroplatinum II (cisplatin)[1] is a chemotherapeutic agent used widely in the treatment of cancers of the testis, ovary, head and neck, and lung (*1*, *2*). The therapeutic efficacy of this agent is suggested to derive from its ability to form complexes with DNA (*3*, *4*). Cisplatin binds the N7 reactive center on purine residues, forming both monofunctional and bifunctional adducts. The 1,2-intrastrand GG cross-link (65%) and the 1,2-intrastrand AG cross-link (25%) are the predominant lesions while minor lesions include the 1,3-intrastrand GNG cross-link (6%), the interstrand GG cross-link (1−3%), monoadducts, and protein−DNA cross-links (*5*).

Many types of helix-distorting DNA lesions, including those induced by cisplatin, are removed from the genome by the nucleotide excision repair (NER) pathway. In hamster cells, UvrABC-sensitive cisplatin intrastrand cross-links in active genes are repaired preferentially with a slight bias for enhanced repair of the transcribed strand (*6*, *7*). In human cells, however, cisplatin damage is efficiently removed from the overall genome, and only a slight bias for repair in coding regions is observed (*8*).

The enhanced repair of certain DNA lesions in the transcribed strand as opposed to the nontranscribed strand suggested a connection between RNA polymerase II (RNA pol II) transcription and DNA repair. A direct link between the two processes was established with the identification of two subunits of the RNA pol II basal transcription factor TFIIH, p89 and p80, as the repair proteins XPB and XPD, respectively (*9*, *10*). The entire transcription factor functions in repair where it is suggested to unwind the DNA around the damage site (*11*).

Transcription from class II promoters is a highly complex process in which a series of general transcription factors direct RNA pol II to a promoter sequence, therefore facilitating initiation and elongation. Five basal transcription factors (TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) are required for accurate initiation from a promoter sequence (*12*). The promoter is initially recognized at the TATA element by TFIID, in particular by the TATA binding protein (TBP). TFIIB directly binds TFIID via TBP and is suggested to stabilize the TBP−TATA box interaction. TFIIF forms a complex with the nonphosphorylated form of RNA pol II, and this preformed complex then binds the promoter via an interaction between TFIIF and TBP. TFIIE subsequently binds RNA pol II, significantly increasing the stability of the assembled protein complex (*13*, *14*).

The final factor to assemble is TFIIH, the largest of the RNA pol II general transcription factors. It contains nine subunits, p89 (XPB), p80 (XPD), p62, p52, p44, p34, MAT1, cyclin H, and cdk7 (*15*, *16*), and is the only transcription factor to possess enzymatic activities. The cyclin-dependent kinase-activating component of TFIIH (Mat 1, cyclin H, and cdk7) is presumed responsible for the phosphorylation of the C-terminal domain of the RNA pol II largest subunit (*17*), resulting in transcription initiation and promoter clearance.

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); RNA pol II, RNA polymerase II; AdMLP, adenovirus major late promoter; TCR, transcription coupled repair; bp, base pair; nt, nucleotide; NER, nucleotide excision repair; TBP, TATA binding protein; RNA pol, RNA polymerase.

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The requirement for TFIIH in transcription initiation and DNA repair raises the possibility of competition between these two processes for the transcription factor in the presence of DNA damage. However, several studies investigating this possibility have yielded conflicting findings (*18*−*20*), and thus the conditions under which competition occurs remain unclear.

Stalling of an actively elongating RNA pol II upon encountering a DNA lesion located in the transcribed strand forms the basis of the current model of transcription coupled repair (TCR). The arrested polymerase complex acts as a signal for the recruitment of the repair machinery to the DNA damage site, thereby facilitating the rapid repair of damage in the transcribed strand (*21, 22*). In an effort to elucidate the role of RNA pol II in TCR, the extent to which specific lesions block RNA pol II elongation has been determined and correlated with the TCR status of the lesion. The cyclobutane pyrimidine dimer, an effective substrate for the TCR pathway, was a complete block to RNA pol II elongation when the lesion was located in the transcribed strand (*23*). Similarly, the acetylaminofluorene adduct also blocked RNA pol II elongation while the less distortive aminofluorene adduct was slowly bypassed by RNA pol II, consistent with the absence of TCR of this lesion (*22*).

The aim of the current study was to investigate the effects of DNA damage induced by cisplatin on RNA pol II transcription, at the level of both initiation and elongation. Treatment of adenovirus major late promoter (AdMLP)-containing templates with cisplatin resulted in a drug concentration dependent inhibition of RNA pol II transcription initiation. RNA pol II transcription of nondamaged template was subsequently shown to be significantly inhibited by the addition of exogenously damaged DNA, suggesting that cisplatin damage may sequester an essential transcription factor. Constructs containing site-specifically-placed cisplatin lesions were subsequently utilized to investigate the effect of individual lesions on RNA pol II elongation. Differential sensitivity of the polymerase to cisplatin damage was observed in which elongating RNA pol II was shown to stall at a GTG intrastrand adduct but bypass a GG adduct.

## MATERIALS AND METHODS

*Materials.* The restriction enzymes *Apa*LI, *Stu*I, *Kpn*I, *Sma*I, and *Hin*dII, RNase T1, T4 gene 32 protein, T4 DNA ligase, T4 DNA polymerase, ultrapure nucleotides, and deoxynucleotides were purchased from Boehringer Mannheim. T4 polynucleotide kinase was from U.S. Biochemicals. *Escherichia coli* DH11S cells were from Gibco BRL. RNase inhibitor, T3 RNA polymerase, and Turbo *Nae*I were purchased from Promega, and cis-diamminedichloroplatinum(II) was from Sigma.

*Cell Lines. Xeroderma pigmentosum* complementation group F (GM8437A) fibroblasts were obtained from the Human Genetic Mutant Cell Repository. Cultures were grown in RPMI 1640 medium containing 15% heat-inactivated fetal bovine serum and supplemented with 0.1 mg/mL streptomycin and 100 units/mL penicillin. HeLa cells were grown in RPMI 1640 containing 10% fetal bovine serum, 0.1 mg/mL streptomycin, and 100 units/mL penicillin.

*Cell Extracts.* Whole cell extracts were prepared from GM8437A cells as described by Manley et al. (*24*). Nuclear extracts were prepared from HeLa cells essentially as described by Dignam et al. (*25*). HeLa nuclear extracts and XPF whole cell extracts were used as the source of proteins for the transcription studies as both types of extracts were shown to support efficient RNA pol II transcription (results not shown).

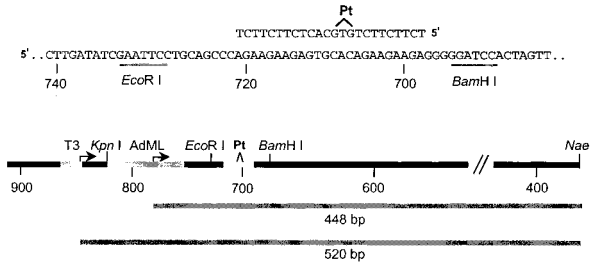


FIGURE 1: Single cisplatin lesion containing substrates. The *Kpn*I−*Hin*dII fragment from pDNAdML containing the AdMLP and a 24 bp sequence containing a central unique hexameric sequence were ligated into the *Kpn*I−*Hin*dII and *Sma*I sites of pBluescript II KS(+), respectively. The (+) strand of the resultant plasmid was isolated and annealed to a complementary 24-mer containing a single cisplatin lesion as shown. The oligonucleotide was extended and ligated to yield a covalently closed plasmid containing a cisplatin lesion in the transcribed strand. The position of the drug lesion in relation to the T3 promoter and AdMLP is shown together with the lengths of the full-length transcript generated from each promoter.

*G-less Cassette Transcription Substrate.* The plasmid pML($C_2$AT), which contains the AdMLP (from positions −400 to +10) fused to a G-less cassette (a synthetic cassette that lacks cytidine residues in the transcribed strand) (*26*), was prepared using a modified lysozyme lysis procedure and was finally purified by cesium chloride/ethidium bromide centrifugation. Ethidium bromide was removed by five successive butanol extractions. The solution was dialyzed against TE overnight and the DNA concentrated by ethanol precipitation.

*Single-Lesion Transcription Substrates.* The plasmid pDNAdML, which contains the AdMLP, was kindly provided by Dr. D. Reines (Emory, GA). pDNAdML was digested with *Kpn*I and *Hin*dII to release a 70 bp promoter-containing fragment. The fragment was isolated and ligated into pBluescript II KS(+) digested with the same two enzymes to yield the promoter-containing vector. This vector was then digested with *Sma*I and one of two different 24 bp oligonucleotide duplexes subsequently ligated into the site. The plasmid was transformed into *E. coli* DH11S for propagation of both its single-strand and its double-strand forms. The orientation of the oligonucleotide insert was determined by sequencing. The plasmid containing the 24 bp GTG sequence, for example, was designated pML-GTG to indicate the presence of the GTG oligonucleotide sequence in the *Sma*I site (Figure 1). pML-GG was also constructed.

Single-strand DNA was generated from pML-GTG by routine methods (*27*). Fifty micrograms of the plus strand of pML-GTG was annealed to a 3-fold molar excess of phosphorylated lesion containing oligonucleotide prepared as described by Yarema et al. (*28*) in 250 $\mu$L of buffer containing 50 mM Tris, pH 8.0, 20 mM KCl, 7 mM $MgCl_2$, and 0.1 mM EDTA. Conversion of the hybrid into closed circular DNA was completed by the addition of dATP, dCTP, dGTP, and dTTP (500 $\mu$M), ATP (0.1 mM), T4 gene 32 protein (100 $\mu$g), T4 DNA polymerase (20 units), and T4 DNA ligase (25 units) and incubation at 37 °C for 90 min.

The closed circular product was purified by cesium chloride/ethidium bromide centrifugation as described above. The construct was designated pML-GTG(Pt) to indicate the presence of the site-specific lesion (Figure 1).

Removal of substrates lacking site-specific damage was performed by digestion of pML-GTG (Pt) with *Apa*LI. The DNA was subsequently resolved on a 1% agarose gel. A 1780 bp fragment (containing an *Apa*LI sequence resistant to cleavage due to the presence of the adduct) was cut from the gel and the DNA eluted from the gel slice by using a Biotrap electroeluter (Schleicher & Schuell, Germany). The eluted sample was extracted once with phenol and once with chloroform, ethanol precipitated, and resuspended in TE buffer. The promoter-containing fragment was finally digested with *Nae*I to yield a full-length transcription product from the AdMLP and T3 promoter of 448 and 520 nt, respectively.

pML-GG (Pt) was digested with *Stu*I prior to cesium chloride/ethidium bromide purification to enrich for lesion-containing plasmids. The remaining covalently closed single-lesion plasmids were subsequently purified by cesium chloride centrifugation. The substrate was subsequently linearized with *Nae*I.

*Cisplatin Treatment of Transcription Templates.* One microgram of pML($C_2$AT) (150 $\mu$M bp) was allowed to react with 0−1 $\mu$M cisplatin in 10 mM $Na_2HPO_4$, pH 7.4, and 30 mM NaCl in the dark at 37 °C overnight. The DNA was subsequently ethanol precipitated, resuspended in TE, and used directly in transcription reactions.

*RNA Pol II Transcription from G-less Cassette Templates.* Approximately 1 $\mu$g of pML($C_2$AT) DNA was incubated in buffer comprising 37.5 mM Hepes−KOH, pH 7.9, 1.5 mM DTT, 0.5 mM EDTA, 8.5% glycerol, 8.5 mM $MgCl_2$, 50 mM KCl, 20 units of RNase inhibitor, 500 $\mu$M each of ATP and CTP, 5 $\mu$M UTP, 10 $\mu$Ci of [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol), and cell extracts as indicated in a final reaction volume of 25 $\mu$L. The samples were subsequently incubated at 30 °C for 60 min. The reaction was terminated by the addition of 200 $\mu$L of buffer containing 10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA, and 200 units of RNase T1 and incubated at 30 °C for 10 min. SDS and proteinase K were added to final concentrations of 0.5% and 0.25 mg/mL, respectively, and incubated at 30 °C for 20 min. The transcripts were precipitated, and the pellet was washed, dried, and resuspended in a loading buffer containing 90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were electrophoresed through a 6% denaturing polyacrylamide gel. The gel was fixed, dried, and subsequently visualized using a Model 400B PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

*RNA Pol II Transcription on Single-Lesion Templates.* The single-lesion-containing templates (50 nM promoter) were incubated in buffer containing 37.5 mM Hepes−KOH, pH 7.9, 70 mM KCl, 8.5 mM $MgCl_2$, 1.5 mM DTT, 0.5 mM EDTA, 8.5% glycerol, 0.4 unit/$\mu$L RNase inhibitor, 500 $\mu$M each of ATP, CTP, and GTP, 10 $\mu$M UTP, 10 $\mu$Ci of [$\alpha$-$^{32}$P]-UTP, and 55 $\mu$g of GM8397A whole cell extract. The samples were incubated at 30 °C for up to 90 min. Aliquots were removed at various time intervals, and SDS and proteinase K were added as described above and incubated at 30 °C for 20 min. The samples were subsequently extracted once with phenol and once with chloroform and then ethanol precipitated. The pellets were washed, dried, and resuspended in a formamide loading dye. The samples were denatured and resolved on a 6% sequencing gel as described above.

*T3 RNA Polymerase Transcription on Single-Lesion Templates.* Single-lesion-containing templates were incubated in 20 $\mu$L of buffer containing 40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each of ATP, CTP, and GTP, 50 $\mu$M UTP, and 10 $\mu$Ci of [$\alpha$-$^{32}$P]UTP. The reaction was initiated by the addition of 5 units of T3 RNA polymerase. At various time intervals, aliquots were removed and the reactions terminated by the addition of an equal volume of formamide loading dye. Due to the high efficiency of the viral RNA polymerase transcription, reaction times up to 15 min were sufficient for the detection of nascent transcripts.

RNA sequencing lanes were generated following initiation of the L8UV5 mutant of the *lac* promoter by *E. coli* RNA polymerase, and elongation in the presence of 3′-*O*-methyl nucleotides as described previously (*29*).

## RESULTS

*RNA Polymerase II Transcription Inhibition of Globally Damaged Templates.* The G-less cassette containing the AdMLP was chosen as the template for this study as RNA pol II transcription has been well characterized using this construct (*26*). Furthermore, as the study sought to investigate the effect of cisplatin intrastrand adducts on transcription, the absence of guanine residues in the nontranscribed strand within the region of interest precluded the formation of DNA interstrand cross-links within the 392 bp region of interest.

In our previous studies investigating the effect of cisplatin damage on *E. coli* RNA polymerase, DNA templates were randomly damaged with cisplatin (*30*). Using an analogous procedure, the RNA pol II transcription template was incubated with increasing concentrations of cisplatin up to 1 $\mu$M. RNA pol II run-off transcripts were generated from the AdMLP of the drug-damaged templates and digested with RNase TI to remove any nonspecific, guanine-containing transcripts. The RNase T1 resistant 392 nt full-length transcript (generated from AdML promoter directed transcription through the G-less cassette) was subsequently detected by gel electrophoresis. As seen in Figure 2A, in the absence of drug, a high level of full-length transcript was observed. In contrast, increased cisplatin damage to the template prior to transcription resulted in a corresponding decrease in the amount of full-length transcript generated.

The relative amount of full-length transcript generated from each drug-treated sample was quantitated and is expressed in Figure 2B as a function of cisplatin concentration. Reaction of the transcription template with 0.25 $\mu$M cisplatin (drug:bp ratio $= 1.7 \times 10^{-3}$) was sufficient to reduce the level of full-length transcript to 50% of that of the undamaged control. These results indicate that under the reaction conditions employed, RNA pol II transcription is highly sensitive to low levels of cisplatin-induced DNA damage.

The G-less cassette transcription template was allowed to react with 0.4 $\mu$M cisplatin (drug:bp ratio $= 3 \times 10^{-3}$) at 37 °C overnight. The damaged template was subsequently incubated under optimal RNA pol II transcription conditions.

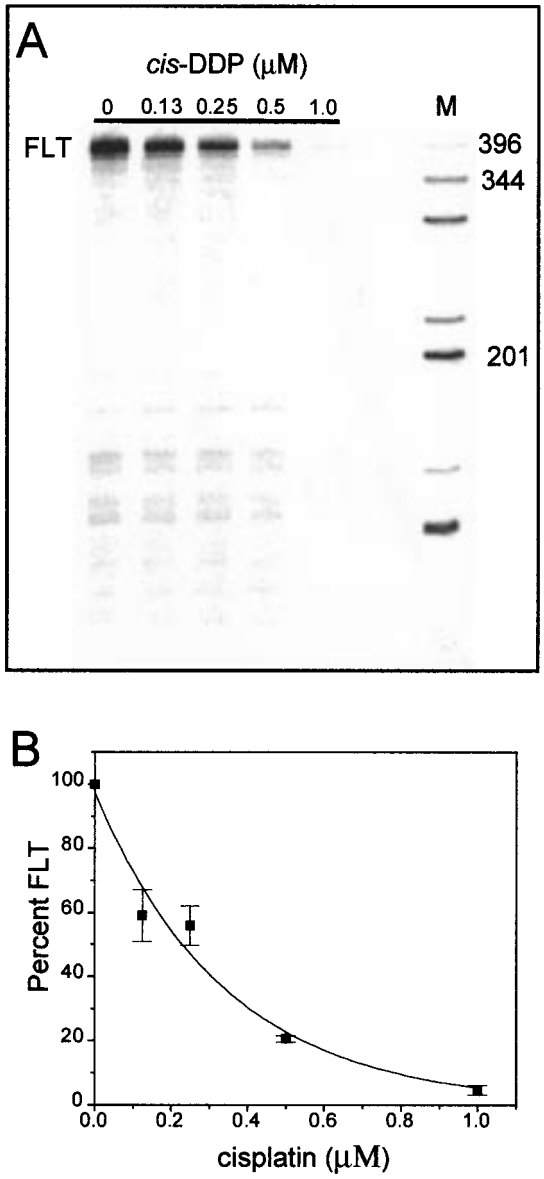


FIGURE 2: Cisplatin concentration dependent inhibition of RNA pol II transcription. (A) pML($C_2$AT) was incubated overnight at 37 °C with 0−1 $\mu$M cisplatin (as shown) in 10 mM $NaH_2PO_4$, pH 7.4, and 30 mM NaCl. The DNA was ethanol precipitated, and transcripts were generated from the AdMLP in the presence of 30 $\mu$g of HeLa nuclear extract. The 392 nt RNase T1 resistant transcripts were visualized using a PhosphorImager following resolution on a 4% denaturing polyacrylamide gel. Lane M represents a DNA marker lane, and the length of several DNA fragments is indicated. (B) The amount of 392 nt RNase T1 resistant full-length transcript generated at each drug concentration in panel A was quantitated and calculated as the percentage of that generated by the control, undamaged template and is expressed as the mean ± SEM of two independent experiments.

Aliquots were removed at various time intervals and were analyzed for the amount of full-length transcript generated. The results are shown in Figure 3A. Quantitation of the rate of formation of the full-length transcript (Figure 3B) demonstrated that in the absence of cisplatin damage, the transcript reached maximal levels within 60 min. In contrast, the absolute amount of transcript generated by the damaged template was greatly reduced but continued to increase over the 90 min reaction time course.

*Inhibition of RNA Pol II Transcription by Exogenous DNA Damage.* The drug concentration dependent reduction in

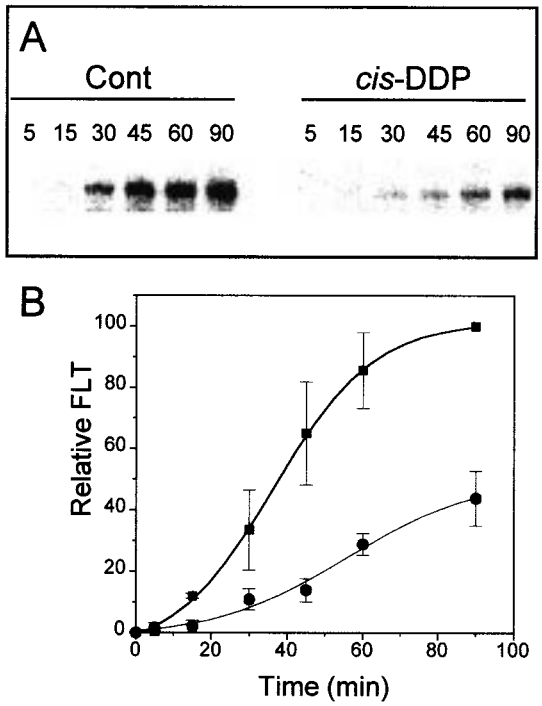


FIGURE 3: Time dependence of transcript formation. (A) pML-($C_2$AT) was incubated in the absence (Cont) or presence (*cis*-DDP) of 0.4 $\mu$M cisplatin at 37 °C overnight. The DNA was precipitated and transcription performed in the presence of 70 $\mu$g of GM8437A whole cell extract. The reactions were incubated at 30 °C, and aliquots were removed at time intervals up to 90 min as shown. Transcripts were treated with RNase T1 and resolved on a 4% denaturing gel. (B) The amount of FLT generated from the control (■) and damaged (●) plasmid was quantitated from two independent experiments and is expressed as the mean percentage (±SEM) of that generated in the 90 min lane of the control reaction.

transcript production (Figure 2) and the altered kinetics of RNA pol II transcription of the damaged template (Figure 3) may result from drug damage at the promoter, leading to promoter inactivation. These effects, however, were observed at drug levels well below that expected to induce such damage. An alternative possibility may be that a factor essential for RNA pol II initiation may also bind cisplatin-damaged DNA. Therefore, in the presence of a sufficient level of DNA damage, this factor may become limiting, resulting in an inhibition of transcription initiation due to the factor being sequestered to DNA damage sites. Earlier studies have established the feasibility of this model for RNA pol II (*19*) and RNA pol I (*31*, *32*) driven transcription. To investigate the possibility that an element of the transcriptional complex was 'hijacked', a competition assay was performed. RNA pol II run-off transcription from undamaged pML($C_2$AT) was performed in the presence of increasing levels of a second, exogenous plasmid containing multiple cisplatin lesions. As shown in Figure 4A, the initial addition of control, undamaged plasmid resulted in an overall increase in the level of transcript generated by pML($C_2$AT) which remained constant upon the further addition of plasmid. This increase in transcription efficiency may result from an increase in macromolecular crowding induced by the presence of higher levels of DNA. Macromolecular crowding has been shown to significantly increase the activity of specific macromolecules in vitro (*33*). Furthermore, macromolecular crowding agents have been used recently in a

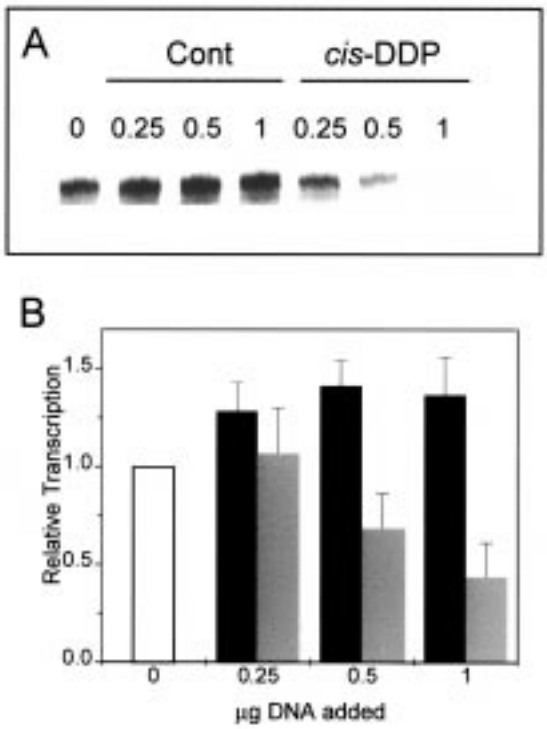


FIGURE 4: Inhibition of RNA pol II transcription by exogenous DNA damage. (A) Exogenous DNA substrates were prepared by incubation of 50 $\mu$g of pUC19 (150 $\mu$M bp) in the presence or absence of 15 $\mu$M cisplatin. The reacted DNA was subsequently purified by cesium chloride gradient centrifugation and was resuspended in TE buffer. p($C_2$AT) was incubated with 30 $\mu$g of HeLa nuclear extract under optimal RNA pol II transcription conditions in the presence of 0.25−1 $\mu$g of the exogenous undamaged (Cont) or cisplatin-damaged (*cis*-DDP) plasmid. RNase T1 resistant transcripts were then resolved on a 4% denaturing polyacrylamide gel. Lane 0 represents a control lane in which transcription was performed in the absence of any exogenously added DNA. (B) The amount of full-length transcript in each lane of panel A was quantitated from two independent experiments and is expressed as the mean fraction (±SEM) of that generated in the absence of exogenously added DNA (lane 0)(white bar). The black bars represent transcription in the presence of control, undamaged DNA and the gray bars, transcription in the presence of cisplatin-damaged DNA.

similar study to investigate sequestering of TFIIH by AAAF damage in yeast cells (*20*).

In contrast, RNA pol II transcription from pML($C_2$AT) was significantly reduced by the addition of increasing amounts of cisplatin-damaged plasmid. The relative transcript levels generated were quantitated and are summarized in Figure 4B. Under the transcription conditions employed, the presence of cisplatin damage (on nontranscribed DNA) was sufficient to reduce transcription to 40% of that detected in the presence of the same amount of control, undamaged DNA.

*Effect of Specific Cisplatin Lesions on RNA Pol II Transcription.* To investigate the effect of the specific cisplatin lesions on RNA pol II transcription, a series of plasmids were constructed to contain a single cisplatin lesion in the transcribed strand, approximately 70 bp downstream of the AdMLP (Figure 1). The constructs used in these studies contained either the 1,2-intrastrand d(GG) cross-link or the 1,3-intrastrand d(GTG) cross-link. The presence of the drug lesions and the purity of the transcription templates were confirmed by run-off transcription assays from the T3 promoter which is located 72 bp upstream of the AdMLP +1 site (Figure 1). Figure 5A shows that the GG construct control template is efficiently transcribed by T3 RNA polymerase to yield high levels of a 520 nt transcript. In contrast, transcription of the single GG lesion-containing template resulted in a high level of shorter length transcripts arising from blockage of the T3 RNA polymerase by this lesion. A very low level of full-length transcript was also observed, most likely as a result of the presence of a small number of templates free of the drug lesion. Sequence analysis revealed the T3 RNA polymerase was blocked both one and two bases prior to the adduct site (Figure 6A). T3 run-off transcription from the GTG construct templates yielded similar results to those of the GG template (Figure 5B). The GTG lesion was also a strong block to the progression to the RNA polymerase, displaying a blockage pattern identical to that of the GG adduct (Figure 6B). From the intensity of the T3 RNA polymerase blockage sites relative to the intensity of the full-length transcript, it can be calculated (taking into account the relative number of radiolabeled nucleotides incorporated into the RNA) that there was 83% and 84% blockage at GTG and GG sites, respectively.

To investigate the effect of the individual cisplatin adducts on RNA pol II transcription, whole cell extracts were used to generate run-off transcripts from the AdMLP of the single-lesion templates. On the basis of the efficiency of RNA pol II activity observed in previous experiments (Figures 2 and 3), reaction times up to 90 min were measured. As seen in Figure 7A, RNA pol II efficiently transcribed the undamaged GG construct template to yield a full-length transcript of 448 nt with the transcript levels increasing over the 90 min reaction time course. Similarly, the single cisplatin GG lesion-containing template was efficiently transcribed to a full-length product with no RNA pol II blockage observed about the position of the DNA lesion (indicated by the arrow in Figure 7A). This result is in sharp contrast to the lesion-induced blockage of T3 RNA polymerase on the same template (Figure 5A) and suggests that the cisplatin GG lesion is completely bypassed by RNA pol II under these conditions.

The undamaged GTG construct template was also transcribed efficiently to yield high levels of the full-length product (Figure 7B). The presence of the cisplatin GTG lesion within the transcription template, however, was sufficient to block the elongation of RNA pol II. A low level of full-length transcript was evident, suggesting that RNA pol II is able to bypass the lesion to some degree. From the relative intensities of the truncated and full-length transcripts and the number of incorporated radiolabeled nucleotides in each band, taken together with the known purity of the template (84% from above), the bypass frequency was calculated as 20%. The position of the blockages was determined (Figure 6) and confirmed that RNA pol II was blocked one or two nucleotides prior to the GTG lesion, and hence yielded an identical blockage pattern to that observed with T3 RNA polymerase.

## DISCUSSION

In this report we have utilized two in vitro transcription assays to investigate the effects of cisplatin-induced DNA

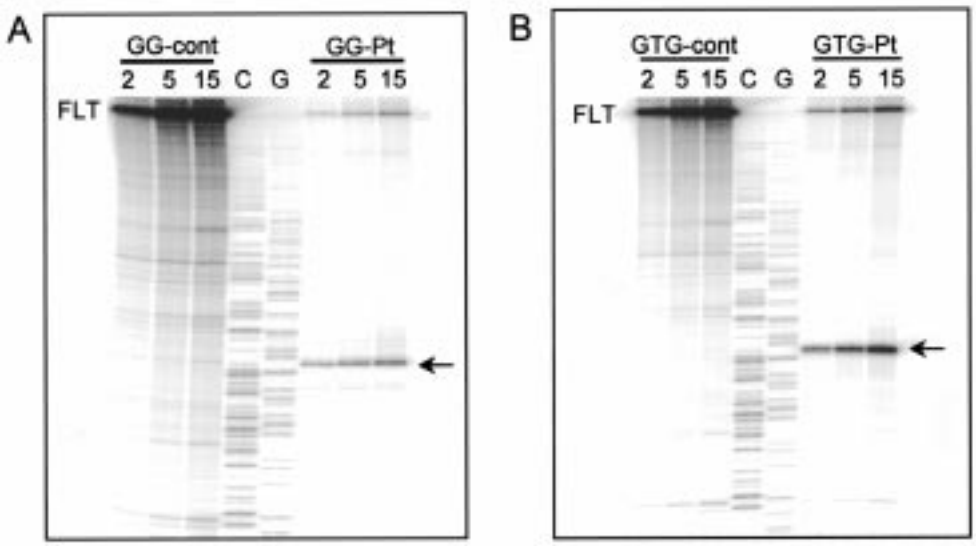


FIGURE 5: T3 RNA polymerase transcription of single lesion containing templates. The control (cont) and single lesion containing (GG-Pt, GTG-Pt) templates of pML(GG) (panel A) and pML(GTG) (panel B) were transcribed using T3 RNA polymerase. Aliquots were removed at 2, 5, and 15 min as indicated, and the transcription reaction was terminated by the addition of a formamide loading buffer. The transcripts were then resolved on an 8% denaturing gel. C and G sequencing lanes were performed as described under Materials and Methods. The position of the drug-induced blockages is indicated by the arrow.

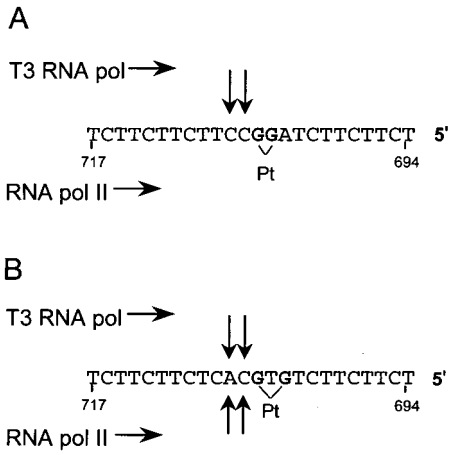


FIGURE 6: Schematic diagram illustrating sites of T3 RNA polymerase and RNA pol II transcription inhibition by site-specifically-placed cisplatin adducts. The 24 bp oligonucleotide-derived template sequence of pML(GG)-Pt (A) and pML(GTG)-Pt (B) is shown. Sequence numbering is as in Figure 1. The arrows show the positions of RNA polymerase blockage, and the direction of transcription is indicated.

damage on RNA pol II transcription. In our initial studies, it was observed that increasing cisplatin damage to a RNA pol II transcription template significantly reduced its transcription capacity. Indeed, RNA pol II transcription of a cisplatin-damaged template revealed a concentration-dependent decrease in full-length transcript levels (Figure 2), suggesting that at very low levels of cisplatin damage transcription is strongly inhibited at the level of initiation. DNA damage may cause numerous effects at the level of RNA pol II transcription initiation. DNA lesions located within the recognition sequence of DNA binding transcription factors (*34*), including TATA binding protein (*35*, *36*), directly inhibit the binding of such factors. The low level of drug damage used in the present study, however, was unlikely to have induced significant damage in the promoter region. This notion is further supported by the observation that RNA pol II transcription on an undamaged template can be specifically inhibited in trans by the addition of a second plasmid containing multiple cisplatin-damaged sites.

*A Dual Role for an Initiation Factor.* RNA pol II transcription may be inhibited as a consequence of the sequestering of either upstream or basal transcription factors by DNA damage (*19*, *37*). The high mobility group box-containing transcription factors HMG1, HMG2, and hUBF have been observed to bind strongly to cisplatin damage in vitro, and some evidence exists to support the notion that these proteins are also hijacked in vivo (*31*, *32*, *38*, *39*). Sequestering of such proteins by cisplatin damage may therefore inhibit the expression of genes transcribed by both RNA pol I and RNA pol II. Indeed, sequestering of the upstream transcription factor, Sp1, by DNA lesions induced by benzo[*a*]pyrene diol epoxide results in reduced transcription in a Sp1-responsive gene construct (*37*). However, as transcription from the AdMLP requires only the basal transcription factors, the ability of increasing levels of cisplatin damage to compete with RNA pol II transcription observed in the current study is consistent with an RNA pol II basal transcription factor becoming limited in the presence of DNA damage.

The multisubunit complex TFIIH is essential for both RNA pol II transcription initiation and NER. In RNA pol II transcription, TFIIH is suggested to melt the DNA about the promoter, phosphorylate the C-terminal domain of RNA pol II, and function in the subsequent initiation and clearance of the polymerase from the promoter (*13*). The entire TFIIH complex, which contains the repair helicases, XPB and XPD (*9*, *10*), also functions in NER where it is suggested to be involved in unwinding of the DNA about the lesion site (*11*). In NER, TFIIH shows no affinity for binding damaged DNA. Rather, DNA damage is recognized and bound by the XPC−HR23B complex and XPA which subsequently recruit TFIIH to the damaged site (*40*, *41*). It was therefore suggested that TFIIH may be sequestered from its role in transcription by DNA damage (*42*). This proposal, however, remains controversial. Using a cell-free assay in which RNA pol II

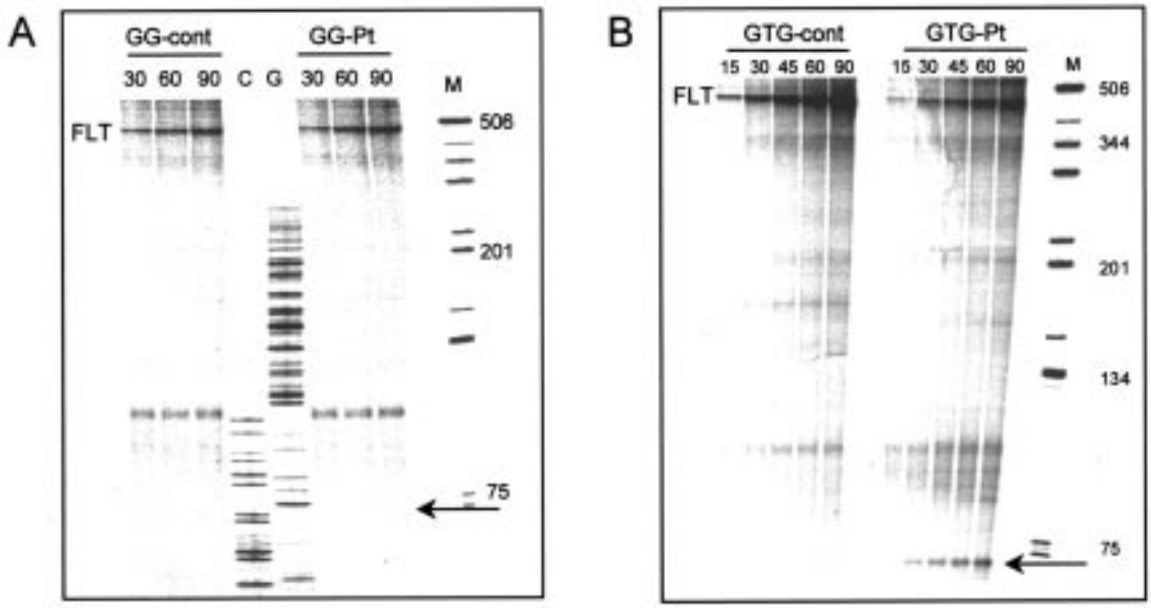


FIGURE 7: RNA pol II transcription of single-lesion templates. The control (cont) and single lesion containing (GG-Pt, GTG-Pt) templates of pML(GG) (panel A) and pML(GTG) (panel B) were transcribed from the AdMLP by RNA pol II in the presence of 55 $\mu$g of GM8437A whole cell extract. Transcript levels were assayed at various transcription time intervals up to 90 min as shown. The (expected) position of drug-induced blockages is indicated by the arrow.

transcription and NER could be studied under a single set of reaction conditions (*18*), no competition between transcription and repair was observed. The failure to observe diminished transcription was suggested to be due to the presence of sufficiently high levels of TFIIH (as high levels of cellular extract were used) to support both processes (*18*). More recently, a reconstituted RNA pol II transcription assay, utilizing low-salt conditions, exhibited sequestering of TBP/TFIID by either cisplatin or UV damage (*19*). DNA damage-dependent inhibition of transcription was alleviated by the addition of purified TBP, and, to a much lesser extent, TFIIH and TFIIB (*19*), suggesting that, under low-salt conditions, DNA damages binding by TBP rather than TFIIH limits transcription. In contrast to these findings, in a yeast system supporting both efficient transcription and NER, TFIIH is diverted from its role in transcription to DNA repair processes (*20*). Indeed, in human WCE, the binding of TFIIH to DNA lesions, rather than TBP, predominates in higher salt buffers (*43*). The results of the current study demonstrate for the first time a significant, but not irreversible, inhibition of RNA pol II transcription in human cell extracts by DNA damage under reaction conditions compatible with repair. This inhibition is most likely the consequence of the hijacking of a basal transcription factor, namely, TFIIH, by the drug lesions. A similar proposal has been made for the disruption of the RNA pol I mediated transcription of the rRNA genes (*31*, *32*). The relevant basal transcription factor, hUBF, binds to the major cisplatin GG cross-link with the strikingly low $K_d$ of 60 pM (*32*).

*Effect of Cisplatin Adducts on RNA Pol II Elongation.* The effect of cisplatin damage on RNA pol II elongation was also investigated. As cisplatin induces a spectrum of DNA lesions (*5*), single-lesion constructs were used to investigate the effect of individual cisplatin lesions on the progression of the polymerase. As expected, the bacteriophage RNA polymerase was highly sensitive to the drug adducts. Both the GG and GTG intrastrand adducts were efficient blocks to T3 RNA polymerase elongation (Figure 5), and the site of transcript termination is similar to that of *E. coli* RNA polymerase, where the major blockage was observed one base prior to the lesion site (*30*).

While the effect of specific lesions on elongation of viral and bacterial RNA polymerases has been studied extensively for a wide spectrum of DNA-damaging agents (*30*, *44*−*47*), understanding of the effect of DNA lesions on RNA pol II elongation remains very limited. Using a similar approach to that undertaken in the current study, a UV-induced cyclobutane pyrimidine dimer has been shown to block RNA pol II elongation completely when the lesion is located in the transcribed strand, but not when located in the nontranscribed strand (*23*). A similar inhibition is observed with the guanine $N^2$-acetylaminofluorene adduct while a smaller and less distortive guanine $N^2$-aminofluorene lesion is slowly bypassed by RNA pol II (*22*).

While previous in vitro studies have demonstrated that the cisplatin GG lesion was blocked by RNA pol II (*48*, *49*), it is important to note that these results were observed using purified wheat germ RNA pol II and a synthetic, promoterless DNA template (in contrast to the current study which used a nuclear extract and a RNA pol II promoter containing template), and therefore may not accurately reflect the physiological response of an elongating RNA pol II molecule to a specific cisplatin adduct. Using reporter vectors containing multiple cisplatin lesions in a host cell reactivation assay, Mello et al. (*50*) have estimated that only 16% of cisplatin damage is bypassed by RNA pol II. Since the major lesion induced by cisplatin is the GG adduct, these results suggest only a small amount of bypass of this adduct in cells. The RNA pol II bypass of the GG adduct observed in the current study may reflect the response of the polymerase to the adduct in a specific-sequence context. It is unknown whether context affects the structure of cisplatin adducts, but it is clear that a HMG domain shows a strikingly varied affinity for the major GG cross-link that is dependent upon the

flanking nucleotide context (*38*). It is therefore likely that polymerases are particularly inhibited in some contexts while allowing read-through in others. Differential DNA polymerase-mediated bypass dependent on context has been seen for several toxic lesions, including the oxidative lesion, *cis*-5,6-dihydro-5,6-dihydroxythymine (thymine glycol) (*51*). With *E. coli* RNA polymerase, it has been shown previously that the sequences flanking a drug site directly influence the ability of the polymerase to elongate through a drug lesion (*47*). Furthermore, upon arrest of RNA pol II elongation at a blockage site, the elongation factor SII has been shown to facilitate the shortening of the nascent transcript. Such transcript cleavage allows the polymerase a second attempt to elongate through the drug adduct (*52*). The sequence context in which the lesion is located may therefore facilitate SII-assisted RNA pol II bypass of DNA damage.

Although bypass of the GG lesion by RNA pol II has not been observed previously, calf thymus DNA polymerase $\beta$ efficiently bypasses such a lesion (*53*) while several DNA polymerases, including T7 DNA polymerase and *E. coli* DNA polymerase I (Klenow fragment) (*54*), bypass such damage to a low level. Replicative bypass of the same lesion has also been detected in bacterial (*28*, *55*) and mammalian cells (*56*). These findings clearly indicate that despite the significant distortion of the helix induced by this lesion, polymerase bypass does occur.

*Transcription-Coupled Repair and Cisplatin.* Activation of the TCR pathway requires the stalling of an actively elongating RNA pol II molecule at a drug lesion. The results of the current study clearly indicate that RNA pol II transcription is severely perturbed by cisplatin damage. However, in contrast to the UV-induced cyclobutane pyrimidine dimer, the repair of cisplatin intrastrand adducts is not strongly coupled to transcription. In CHO cells, in which the overall genome repair is absent, only a moderate strand bias of repair is observed (*7*), while in human cells the rate of removal of cisplatin lesions is similar from both transcriptionally active and inactive genes (*8*). Therefore, the lack of TCR of cisplatin damage, despite the ability of the GTG adduct to block the elongating polymerase, may reflect the inhibition of transcription at the level of initiation and hence, by default, TCR. The observed repair of cisplatin damage is therefore likely to reflect that of repair by the overall genome pathway.

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