Multiple States of Stalled T7 RNA Polymerase at DNA Lesions
Generated by Platinum Anticancer Agents *

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Transcription inhibition by DNA adducts of cisplatin is considered to be one of the major routes by which this anticancer drug kills cancer cells. Stalled RNA polymerases at platinum-DNA lesions evoke various cellular responses such as nucleotide excision repair, polymerase degradation, and apoptosis. T7 RNA polymerase and site-specifically platinumated DNA templates immobilized on a solid support were used to study stalled transcription elongation complexes. In vitro transcription studies were performed in both a promoter-dependent and -independent manner. An elongation complex is strongly blocked by cisplatin 1,2-intrastrand d(GpG) and 1,3-intrastrand d(GpTpG) cross-links located on the template strand. Polymerase action is inhibited at multiple sites in the vicinity of the platinum lesion, the nature of which can be altered by the choice and concentration of NTPs. The [(R,2R)-diaminocyclohexane]PtCl2 DNA adducts formed by oxaplatin, which carries a stereochemically more demanding spectator ligand than the ammine groups in cisplatin, also strongly block the polymerase with measurable differences compared with cis-(NH3)2Pt2+ lesions. Elongation complexes stopped at sites of platinum damage were isolated and characterized. The stalled polymerase can be dissociated from the DNA by subsequent polymerases initiated from the same template. We also discovered that a polymerase stalled at the platinum-DNA lesion can resume transcription after the platinum adduct is chemically removed from the template.

The anticancer drug cis-DDP, cis-diaminedichloroplatinum(II) (cisplatin)3 and related platinum anticancer agents such as (R,2R)-diaminocyclohexaneoxalatoplatinum(II) (oxaplatin) and cis-di- ammine(1,1’-cyclobutanedicarboxylato)platinum(II) (carboplatin) are used successfully to treat various types of cancers, including those of the head, neck, and testis (1). Much evidence reveals that the cytotoxicity of these platinum compounds is related to their ability to attack cellular DNA. Platinum agents react with the N-7 atoms of the purine bases, forming 1,2-intrastrand and 1,3-intrastrand cross-links as the major adducts. These DNA adducts inhibit essential cellular processes including transcription and trigger cell death. The detailed mechanism by which the platinum drugs selectively kill cancer cells is a subject of continued study in our laboratory and many others. A better understanding of cellular responses to platinum-DNA lesions is being sought to develop more effective cancer therapies through the optimization of platinum-DNA chemistry in the context of biochemical responses to the cytotoxic lesions.

Platinum-DNA adducts are encountered and processed by many cellular proteins, events that determine the ultimate outcome of DNA damage (2–5). Among these proteins, RNA polymerase has become a major focus of study because of its various roles in processing damaged DNA. Several types of DNA lesions, including cisplatin cross-links, inhibit transcription by blocking RNA polymerase (6–15). Arrested RNA polymerase not only functions as a damage recognition factor, eliciting transcription-coupled repair, but also triggers programmed cell death, or apoptosis (16, 17). The dominant consequence of RNA polymerase blockage by platinum-DNA adducts, either damage repair or apoptosis, will strongly bias the fate of cancer cells treated with the drugs.

DNA adducts of cisplatin block a variety of RNA polymerases including those present in Escherichia coli, bacteriophage SP6, T7, and mammals (10, 14, 15, 18, 19). Different kinds of cisplatin-DNA adducts, 1,2-intrastrand (dGpG) and 1,3-intrastrand (dGpTpG) cross-links, provide different levels of transcription inhibition. In vitro transcription by RNA polymerase II in human cells extracts is strongly inhibited by a 1,3-intrastrand but not a 1,2-intrastrand adduct, whereas both types of cross-link efficiently block T7 and SP6 RNA polymerases (10, 19). To date, however, little is known about the properties of stalled RNA polymerase at the site of platinum damage.

In the present study, we employed site-specifically platinumated DNA templates immobilized on a solid support in order to analyze the effects of defined platinum lesions on transcription and to isolate and study the stalled elongation complexes. Cisplatin 1,2-intrastrand (dGpG) and 1,3-intrastrand (dGpTpG) cross-links strongly block T7 RNA polymerase. Promoter-independent transcription on immobilized DNA templates allowed for the investigation of the exact stop sites of RNA polymerase at a platinum lesion under many different conditions. In addition to cisplatin, [Pt(dach)Cl2], which forms the same adducts as oxaplatin on our synthetic DNA probes, was examined to understand the influence of the spectator ligand on transcription inhibition (Fig. 1). We also investigated the transcription system whereby another polymerase trails the polymerase stalled at a site of platinum damage. Finally, the ability of RNA polymerase to resume transcription after being arrested at a platinum-DNA lesion was examined by chemically removing the platinum adduct.
**EXPERIMENTAL PROCEDURES**

**Materials**—Histidine-tagged T7 RNA polymerase was expressed in *E. coli* BL21 cells carrying plasmid pBH161 kindly provided by W. T. McGhee and purified as described (20). Gliplatin was obtained from Johnson-Matthey. The [Pt(dach)Cl]2 compound was prepared as previously described (21). T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs, and Dynabeads M280 streptavidin was from DYNAL. MagneHis nickel particles and RNAsin (RNase inhibitor) were obtained from Promega.

**Preparation Specifically Phosphorylated DNA Templates—**Phosphorylated DNA fragments containing cisplatin and [Pt(dach)Cl]2 intrastrand cross-links were synthesized as reported previously (21). A 145-bp DNA template (T7 145) containing a T7 promoter and a site-specific platinum lesion was prepared by enzymatic ligation of five synthetic oligonucleotide fragments and a sixth containing a platinum intrastrand cross-link (Fig. 1B). 5′-Phosphorylated DNA fragments were annealed, ligated, and gel-purified as described previously (22). The platinum damage site was located in the template strand of the duplex DNA probe, and a biotin moiety was placed at the 3′-end of fragment T7 T50. For promoter-independent transcription, an 86-bp DNA probe with a 9-nucleotide (nt) 3′-overhang carrying a platinum intrastrand cross-link was prepared by ligating four oligonucleotides (Fig. 1C). RNAs was purchased from Dharmacon. Complete sequences of all DNA and RNA oligonucleotides are supplied as supplemental material.

**Promoter-dependent in Vitro Transcription**—The biotin-labeled DNA templates (T7 145; Fig. 1B) were immobilized onto Dynabeads following the protocol provided by the manufacturer. Immobilized DNA templates were pre-equilibrated with the transcription buffer (40 mM Tris, pH 7.9, 6 mM MgCl2, 2 mM spermidine, and 10 mM NaCl). Transcription by T7 RNA polymerase was carried out by incubating ~5 μM DNA template with an equimolar amount of T7 RNA polymerase, 1 unit/μl of the RNase inhibitor RNasin, 10 mM dithiothreitol, 0.12 mM ATP, 0.12 mM GTP, 5 mM UTP, and 1 μM [α-32P]UTP in transcription buffer at room temperature for 5 min. This transcription elongation complex was washed three times after the synthesis of a 14-nt RNA transcript (EC14; Fig. 1B) due to the lack of CTP in the reaction solution. Magnetic beads containing transcription complexes were separated from the solution by placing reaction tubes in a magnetic rack (DYNAL). The supernatant solution was removed by aspiration with a pipette. The transcription elongation complex was washed with cold transcription buffer three times and used for the next transcription steps. Single round transcription assays were performed without magnetic separation of elongation complexes from the reaction solution. NTPs (1 mM final concentration) and 2 μg of heparin were added into the transcription solution containing the elongation complex comprising 14 residues of transcribed RNA (EC14) formed by 0.12 mM ATP, 0.12 mM GTP, 5 mM UTP, and 1 μM [α-32P]UTP. Transcribed RNAs were ethanol-precipitated and analyzed on 6% urea polyacrylamide gels. The gels were dried and visualized by autoradiography.

**Promoter-independent in Vitro Transcription**—A 5′-phosphorylated PI T50 deoxyoligonucleotide was annealed with RNAs, labeled with 32P and purified as described (30). Gliplatin was obtained from Johnson-Matthey. The [Pt(dach)Cl]2 compound was prepared as previously described (21). T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs, and Dynabeads M280 streptavidin was from DYNAL. MagneHis nickel particles and RNAsin (RNase inhibitor) were obtained from Promega.

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**Promoter-independent in Vitro Transcription**—A 5′-phosphorylated PI T50 deoxyoligonucleotide was annealed with RNAs, labeled with 32P at the 5′-end, by heating at 50 °C and cooling slowly to room temperature (Fig. 1C). An 8-μmol portion of this annealed probe was mixed with an equimolar quantity of T7 RNA polymerase in a total volume of 20 μl of transcription buffer at 37 °C. RNA transcription was resumed for each of the complexes by the addition of 100 μM NTPs. The relative abilities of the individual adducts to inhibit T7 RNA polymerase, however, are unchanged by the two different experimental procedures. GTG dach, with a sterically larger

**RESULTS**

**Promoter-dependent in Vitro Transcription on Phosphorylated Templates**—An immobilized 145-bp DNA probe containing a biotin moiety at the 3′-end of the template strand, a T7 promoter, and a platinum intrastrand cross-link was constructed as indicated in Fig. 1. T7 RNA polymerase was employed to investigate minimal transcription in vitro with the immobilized DNA probe. Fig. 2 shows that T7 RNA polymerase is capable of generating RNA transcripts following initiation on the immobilized DNA templates. In the absence of CTP, a transcription elongation complex comprising 14 residues of transcribed RNA transcript (5′-GAAUU AUGC GGAU-3′) was expected to form (Fig. 1). An RNA transcript of 14 residues is produced from this reaction (Fig. 2). In addition, many short RNA transcripts containing fewer than 10 residues are produced by abortive transcription initiation. Nonspecific RNA transcripts longer than the expected 14 residues are also observed. CTP contamination in [α-32P]UTP and false transcription initiation before the start sites are the most likely sources of these longer transcripts. Isolation of the transcription elongation complex on streptavidin-coated magnetic beads, however, removes all of these abortive initiation products and diminishes the amount of nonspecific transcripts (compare lane 1 with lane 2). Transcription elongation is resumed by adding 0.5 mM of NTPs (lanes 3, 6, and 9), which extends the 14-residue RNA in an elongation complex to a 125-nt RNA run-off transcript along the undamaged DNA probe (lane 3). Elongation stops at both cisplatin damage sites, GG Pt and GTG Pt (lanes 6 and 9, respectively), however. T7 RNA polymerase appears to stall at several positions in the vicinity of the platinum adducts (lanes 6 and 9).

**Transcription Bypass through Platinum Binding Sites**—Single-round transcription was performed by heparin treatment without magnetic separation of the elongation complexes (EC14). These elongation complexes, as well as EC14 products isolated by the magnetic bead methodology for comparison, were subjected to a second round of elongation to investigate bypass through the platinum lesions. In these two different transcription systems, DNA templates containing [Pt(dach)Cl]2 1,2- and 1,3-intrastrand cross-links were employed together with probes containing the analogous cisplatin cross-links. As revealed in Fig. 3, the bands for run-off transcripts in single-round transcription (lanes S) are more intense compared with those for transcription from isolated EC14 complexes (lanes B). The relative abilities of the individual adducts to inhibit T7 RNA polymerase, however, are unchanged by the two different experimental procedures. GTG dach, with a sterically larger
spectator ligand, most strongly blocks T7 RNA polymerase. The polymerase bypasses GG dach adducts most efficiently, however, despite the bulky ligand. Overall, platinum 1,3-d(GpTpG) adducts block the polymerase more efficiently than 1,2-d(GpG) adducts.

Promoter-independent In Vitro Transcription—In vitro transcription on the immobilized DNA template allows us to use a “transcription walking” method to provide the exact positioning of the polymerase along the DNA probe. The promoter-dependent transcription methodology, however, generates several populations of RNA transcripts, which most likely originate from nonspecific transcription initiation around the start site (data not shown and see above). Multiple stop sites in the vicinity of the platinum adduct, such as those observed in Fig. 2, might therefore derive from such false initiation.

In order to circumvent this problem, we employed a promoter-independent T7 RNA polymerase transcription assay by assembling an elongation complex to study the exact stop sites of the stalled polymerase (Fig. 1C). The construction of similar elongation complexes of yeast RNA polymerase II and T7 RNA polymerase using RNA:DNA hybrids has been reported previously (24, 25). RNA transcripts corresponding to RNA48 and RNA62 were produced from “transcription walking” along the undamaged DNA probe (Fig. 4A, lines 1 and 3). In this experiment, the 5’-end of the T-95 DNA fragment was also labeled with $^{32}$P in order to confirm the ligation between an assembled EC and the 86-bp DNA probe (Fig. 1C). 145-mer DNA probes (PI 145) and unligated 95-mer DNA fragments (T-95) are visible in Fig. 4A. It is noteworthy that 133-nt run-off RNA transcripts are not detectable in this experiment, indicating that most T7 RNA polymerases stall at the GG Pt and GTG Pt lesions on the template strand. At a low concentration of NTP, the polymerase stops before the cisplatin 1,2-intrastrand d(GpG) cross-link, producing 62 residues of RNA transcript (Fig. 4, B and C). High NTP concentration drives the polymerase to the damaged d(GpG) site. At a cisplatin 1,3-intrastrand d(GpTpG) cross-link, most of the T7 RNA polymerase reaches the first G residue of d(GpG) and d(GpTpG) cross-links (Fig. 5, A and B).

UTP-specific Incorporation by T7 RNA Polymerase at the Site of a Cisplatin 1,2-Intrastrand d(GpG) Cross-link—We further studied nucleotide incorporation by T7 RNAP at the site of a cisplatin 1,2-intrastrand d(GpG) cross-link.

Fig. 1. Construction of DNA templates. A, structures of cisplatin, oxaliplatin, and [Pt(dach)Cl$_2$]. B, scheme illustrating the preparation of DNA templates for promoter-dependent transcription, indicating the locations of the biotin moiety, T7 promoter, and platinum adduct. Sequences of the template strand between the T7 promoter and platinum site are depicted together with the expected RNA products initiated from the T7 promoter. The complete sequence is given in supplemental material. C, schematic representation of promoter-independent transcription. DNAs and RNAs are indicated by solid and dotted lines, respectively. Each fragment is named according to its length on the template (T) or coding (C) strand.
platinum damage under conditions of high NTP concentration, with the aim of determining whether a specific nucleotide might be responsible for this effect. T7 RNAP elongation complexes, stalled before the damage site at low NTP concentration (10 μM) and containing 62-nt RNA, were subsequently treated with 1 mM ATP, UTP, GTP, CTP, or all four NTPs. As shown in Fig. 6A, T7 RNAP inserts two additional nucleotides at the site of a cisplatin 1,2-(GpG) intrastrand cross-link only when 1 mM UTP is present in addition to 10 μM NTP, forming 64-nt RNA transcripts. Interestingly, the elongation complex is not able to transcribe into the damage site at a high concentration (1 mM) of ATP, GTP, or even CTP, the correct nucleotide for the d(GpG) template. A fraction of T7 RNAP, stalled at the first G site of the cisplatin 1,3-(GpTpG) cross-link, incorporates a nucleotide opposite T in the template strand of this lesion after addition of 1 mM NTP (Fig. 6A). These RNA products are observed only at 10 μM NTP with 1 mM ATP or UTP.

In a parallel experiment, we removed the 10 μM NTP from the reaction solution after forming the elongation complexes stalled at the site of a cisplatin 1,2-(GpG) cross-link under the conditions (10 μM NTP) described above. Upon the addition of 1 mM ATP, UTP, GTP, CTP, or all four NTPs, we observed that only 1 mM UTP could drive T7 RNAP transcription into the d(GpG) site (Fig. 6B). The data also indicate that T7 RNAP readily incorporates the incorrect nucleotide (UTP), not the correct one (CTP), at the site of the cisplatin d(GpG) cross-link.

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Fig. 2. Gel electrophoresis analysis of promoter-dependent in vitro transcription on immobilized DNA templates containing cisplatin intrastrand cross-links. Transcription by T7 RNA polymerase was performed as described under "Experimental Procedures" with an undamaged DNA template (No Pt; lanes 1–3) and templates containing cisplatin 1,2-intrastrand d(GpG) (GG Pt; lanes 4–6) and 1,3-intrastrand d(GpTpG) cross-links (GTG Pt; lanes 7–9). Elongation complexes containing 14 nt of transcripts were formed under CTP deprivation conditions (lanes 1, 4, and 7). Immobilized DNA templates containing elongation complexes were separated from the reaction solutions (lanes 2, 5, and 8) and subsequently treated with 0.5 mM NTP (lanes 3, 6, and 9). RNA transcripts were analyzed on a 6% urea polyacrylamide gel. The sizes of the RNA transcripts were determined by "transcription walking" experiments (data not shown).

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Fig. 3. Gel electrophoresis analysis of transcription bypass through cisplatin- and oxaliplatin-DNA intrastrand cross-links. Elongation complexes containing 14-residue transcripts (EC14) were treated with 1 mM NTP and heparin to afford a single round of transcription (lanes marked S). In a separate experiment, EC14s were separated from the solution as described under "Experimental Procedures" and treated with 1 mM NTP (lanes marked B, for beads). RNA transcripts were analyzed on a 6% urea polyacrylamide gel. The percentages of run-off to total RNA products through GG Pt, GTG Pt, GG dach, and GTG dach adducts are given at the bottom of each lane in the gel.

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Fig. 6B also demonstrates the presence of RNA products shorter than 62 nt only when ATP, GTP, or CTP is added to stalled polymerase. This result would appear to indicate intrinsic cleavage of RNA transcripts by stalled T7 RNAP.

Multiround in Vitro Transcription—Recently, the ability of a trailing RNA polymerase molecule to alleviate an arrested RNAP was reported when more than one RNA polymerase transcribes along the same DNA template (26). Multiround transcription was performed as described under "Experimental Procedures" to study the effect of a trailing RNA polymerase on polymerase stalled at a platinum cross-link. Upon treatment with additional RNA polymerase, the RNA transcripts in stalled elongation complexes are dissociated from the immobilized DNA template, whereas the transcripts remain at damage sites on the DNA templates without such treatment (Fig. 7, lanes 5 and 6 versus lanes 7 and 8; lanes 9 and 10 versus lanes 11 and 12). T7 RNA polymerase that initiates from the same T7 promoter and follows the stalled elongation complex is responsible for the dissociation of the stalled polymerase from immobilized DNA, since added polymerases have no effect on the stalled polymerase in the absence of NTPs (Fig. 7, lanes 1 and 2 versus lanes 3 and 4).

Restarting Transcription following Platinum Removal by Cyanide Ion Treatment—Cyanide ion has been successfully used to remove platinum adducts from DNA as the [Pt(CN)₄]²⁻ complex (23, 27). This method was used to dissociate platinum from immobilized DNA in the presence of T7 RNA polymerase stalled at the damage site. Platinum adducts were abstracted most efficiently from the [Pt(dach)Cl]₂ intrastrand cross-link under our cyanide ion treatment conditions (data not shown). As indicated in Fig. 8, most elongation complexes stay on the DNA templates after exposure to NaCN. Upon treatment with cyanide ion, more than 90% of the early elongation complexes EC14 are still active (lanes 2 and 4). On the other hand, 40% of EC40s are unable to resume transcription after 0.2 mM NaCN treatment, and 0.1 mM NaCN inactivates 30% of these complexes (lanes 6 and 8). A similar level of elongation complex inactivation occurred upon 0.2 mM NaCl treatment without any platinum abstraction (data not shown). These results are presumably the consequence of increased ionic strength. In the absence of cyanide ion, 16% of T7 RNAP bypasses the GG

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Approximately 77 and 95% of active elongation complexes generate run-off RNA transcripts beyond the site of the platinum lesion after 0.1 and 0.2 mM NaCN treatment, respectively (lanes 2, 4, 6, and 8), indicating successful removal of the GG dach adduct. Similar levels of run-off transcripts are observed following re-elongation of stalled elongation complexes compared with EC40s following platinum complexation by NaCN. This result indicates that T7 RNA polymerase stalled at a platinum lesion is able to resume transcription after the damage is removed.

**DISCUSSION**

**Promoter-dependent and -independent Transcription Inhibition at Platinum Cross-links**—The transcription system in which platinum-DNA adducts are immobilized on a solid support provides a powerful tool to investigate the molecular mechanism of transcription inhibition by, and the properties of RNA polymerases stalled at, the major cross-links formed by cisplatin, carboplatin, and oxaliplatin. The DNA template attached to a solid phase provides a simple way to separate the transcription elongation complex from the reaction solution,
allowing us to manipulate the position of the polymerase and detect the release of RNA transcripts from the complex. The activity of the protein Mfd on stalled E. coli RNA polymerase was studied by using an immobilized DNA template in a similar strategy (28).

The present results indicate that bacteriophage T7 RNA polymerase, a 100-kDa monomer, transcribes RNA from a biotinylated DNA template attached to streptavidin-coated magnetic beads. Early elongation complexes (EC14; Fig. 1), formed under CTP deprivation conditions, are fully capable of performing further transcription by the subsequent addition of all four NTPs. The 14-residue RNA transcript in EC14 is labeled with $^{[32P]}$UTP (1 mM), and incorporation of additional $^{[32P]}$UTP is prevented by isolating EC14 from the reaction solution on the beads and washing the immobilized elongation complex. Comparison of the relative amount of radioactivity in run-off (125-nt) versus platinum-aborted (54-nt) transcripts, therefore, allows us to quantify the extent of bypass compared with the amount of stalled T7 RNAP (Fig. 1A).

We also performed single round transcription assays without isolation of EC14 from the reaction solution. In these experiments, EC14 formed in the presence of ATP, GTP, and $^{[32P]}$UTP (1 mM) is elongated by adding 1 mM NTPs such that further incorporation of $^{[32P]}$UTP is prevented due to the excess of unlabeled UTP in the reaction solution. As shown in Fig. 3, these conditions afford more intense bands corresponding to run-off transcripts than observed for transcription from EC14 isolated on the magnetic beads. One possible explanation for this difference is that $^{[32P]}$UTP (1 mM) might be incorporated into RNA transcripts even in the presence of excess cold UTP (1 mM), which would produce a more intense signal for the longer (125-nt) run-off RNA products compared with the shorter (54-nt) transcripts. Another possibility is that elongation complexes isolated by separation on magnetic beads might behave differently from those that are not separated in this manner from the reaction solution.

The ability of platinum intrastrand cross-links to block transcription was also investigated in a promoter-independent assay. Here, elongation complexes are assembled by the addition of T7 RNA polymerase to a DNA:RNA hybrid. Almost no detectable run-off transcripts are observed for any of the four kinds of platinum adducts employed in this study (Fig. 4). Thus, the ability of T7 RNA polymerase to bypass platinum-DNA adducts following transcription from elongation com-

Fig. 6. Analysis of nucleotide incorporation by T7 RNA polymerase at the site of platinum cross-links. Promoter-independent transcription on DNA templates containing GG Pt or GTG Pt was examined as described under “Experimental Procedures.” EC RNA14 complexes on templates containing a platinum lesion were treated with 10 μM NTP at room temperature, A, polymerases stalled at platinum damage sites at 10 μM NTP were additionally treated with 1 mM ATP, UTP, GTP, CTP, or all four NTPs. RNA62 and RNA64 markers were prepared by “transcription walking” through undamaged template containing no platinum. B, after 10 μM NTP was removed from the reaction solution, 1 mM ATP, UTP, GTP, CTP, or all four NTPs was added into polymerases stalled at platinum damage sites. The resulting RNA transcripts were analyzed in 8% urea polyacrylamide gels. C, schematic representation of nucleotide incorporation by T7 RNA polymerase at the site of each platinum lesion.
plexes varies according to the methodology employed to investigate them. While this manuscript was in preparation, a report appeared that also demonstrated T7 RNA polymerase blockage at a single cisplatin adduct, with 70 and 90% efficiency of transcription inhibition by cisplatin 1,3- and 1,2-intrastrand cross-links, respectively (15).

The various *in vitro* transcription systems applied to study transcription inhibition by platinum lesions (10, 14, 15), including the present one, clearly demonstrate that the major adducts of the anticancer drugs can significantly affect the process. The ability of different RNA polymerases to bypass a lesion is manifest, for example, by *in vitro* RNA polymerase II transcription initiated in human cell extracts, which proceeds efficiently through a cisplatin 1,2-intrastrand cross-link (10), compared with transcription by rat liver RNA polymerase II with purified transcription factors, which is strongly inhibited by the same adduct (15). Further work is required to determine the degree to which these findings reflect transcription inhibition by platinum-DNA adducts formed by the anticancer drugs in the context of human cancer.

A Closer Look at Polymerase Blockage by Platinum Adducts—T7 RNA polymerase obstruction by platinum adducts was investigated at the level of single nucleotide resolution by using the promoter-independent transcription system. T7 RNA polymerase stops just before a *cis*-diammineplatinum 1,2-intrastrand d(GpG) cross-link (Fig. 4). At a high concentration of NTPs, however, the polymerase can add additional nucleotides. RNA polymerases exist in multiple conformational states during transcription elongation (29). Several lines of evidence indicate that there are two active elongation states, fast and slow, and that the equilibrium between these two states is altered by the NTP concentration (30). RNA polymerase in the fast elongation state at high NTP concentrations might be able to drive RNA transcription up to the site of the platinum atom. The effect of NTP concentration on polymerase blockage by the analogous platinum 1,3-intrastrand d(GpTpG) cross-link is quite similar (Fig. 4).

Although many bulky DNA lesions impede RNA polymerases, it is presently unclear exactly how the polymerases are stalled at the sites of damage. No structural information about an RNA polymerase arrested at a DNA adduct is yet available. A recent crystal structure determination of a T7 RNA polymerase elongation complex reveals the conformation of undamaged DNA at the active site (31, 32). As depicted in Fig. 9A, the nucleotide (+1) serving as template for the incoming NTP is severely distorted from the rest of the template strand. Upon incorporation of the nascent NTP at position +1, further elongation requires distortion of the +2 nucleotide from the +3 nucleotide (Fig. 9A). Covalent cross-linking of the nucleotides at +2 and +3 by cisplatin, however, would prevent this distortion (Fig. 9B). This information explains why the T7 RNA polymerase stops before the cisplatin 1,2-d(GpG) cross-link. Highly active polymerases can proceed to the +3-position, however, and different damage-induced overall conformations of the template strand at the active site will affect this process. In the case of transcription inhibition by a cisplatin 1,3-intrastrand d(GpTpG) cross-link (Fig. 9, +1 and +3 nucleotides), T7 RNA polymerase is able to incorporate an NTP at the first G site of the adduct, even at low NTP concentrations (Figs. 4 and 5).

In addition to the effects that structurally different cisplatin 1,2- and 1,3-intrastrand cross-links have on T7 RNA polymerase inhibition, our study of transcription using DNA containing [Pt(dach)Cl₂] adducts reveals that the nature of the spectator ligands can also influence the process. T7 RNA polymerase, even in its highly active state, reaches only the first G residues of d(GpG) and d(GpTpG) adducts generated by [Pt(dach)Cl₂] (Fig. 5). Besides preventing the structural rearrangement of the template strand required for transcription by covalent bonding, the bulky dach ligand might physically block further translocation of T7 RNA polymerase.

At a low concentration of NTPs, the T7 RNAP elongation complex stalls just before the d(GpG) site and generates 62 residues of RNA, with U at its 3’-end. Theoretically, C is the next correct nucleobase to be added opposite the G site of the d(GpG)
lesion (Fig. 6C). A recent kinetic study revealed an allosteric effect of template-specific NTP binding to E. coli RNA polymerase, which shifts the equilibrium in favor of the fast elongation state (33). Based on this result, we anticipated that a high concentration of CTP might facilitate NTP incorporation by T7 RNAP at the template GG site of a cisplatin 1,2-d(GpG) cross-link. Unexpectedly, however, the elongation complex adds nucleotides at the damage site only when 1 mM UTP is employed (Fig. 6). None of the other three nucleotides can affect such transcription elongation. The single-component T7 RNAP thus appears to be activated at high NTP concentrations in a different manner than the multicomponent E. coli RNA polymerase. It is not clear at this point how a high concentration of UTP would activate the stalled T7 RNAP. It is likely, however, that the severely distorted GG template at a cisplatin 1,2-d(GpG) cross-link will not provide the geometrically correct template site for an incoming CTP (Fig. 9). Thus, even large amounts of CTP cannot activate the polymerase stalled at the damage site. UTP incorporation at a cisplatin 1,2-d(GpG) lesion inserts the incorrect nucleotide into the transcript when T7 RNA polymerase bypasses the damage site. Several biological consequences, such as abortive or mutant transcripts, will ensue.

Many RNA polymerases in both prokaryotes and eukaryotes are able to cleave their transcripts (34–36). Although such an activity has also been reported for T7 RNAP, little is known about the process (37). Such intrinsic transcript cleavage by T7 RNAP stalled at a damage site is observed in the present study (Fig. 6B). Transcripts shortened by one or two nucleotides are generated by T7 RNAP, and this cleavage is not detected in the absence of NTP. Such nuclelease activity requires a nucleotide that cannot be incorporated into the cisplatin (d(GpG)) damage site, namely ATP, GTP, or CTP. These nucleotides are not needed to provide energy to drive the cleavage reaction, since no cleavage occurs in the presence of 1 mM dATP (data not shown). We suggest that the activity we observe might reflect proofreading of transcription by this single-component polymerase. More detailed study of nontemplated NTP-specific transcript cleavage by T7 RNAP is clearly warranted.

The Effect of Multiple Polymerases at the Site of a Platinum-DNA Cross-link—The recent discovery of cooperative transcription elongation by multiple RNA polymerases suggests a possible explanation for the observation of rapid RNA synthesis in vivo compared with single round in vitro transcription (26). When more than one RNA polymerase molecule transcribes along the same DNA strand, the trailing polymerases appear to push forward and rescue the leading polymerases, which become active for transcription, from natural blocks such as pauses and arrests. In a follow-up study, it was reported that the trailing polymerases also rescue a polymerase from the roadblock created by DNA-binding proteins (38). Unlike these natural pause and arrest sites, it is difficult to predict a priori whether the strong physical block of a platinum cross-link would be circumvented by cooperative transcription elongation of multiple polymerases. Nonetheless, in the multiround transcription assay performed here, the trailing T7 RNA polymerases readily removed a polymerase stalled at a platinum lesion (Fig. 7), allowing transcription to be continued. Although T7 RNA polymerase was used in this study, we expect similar cooperative elongation at a platinum lesion to occur with other types of RNA polymerases because of the conserved mechanical action of these enzymes. The E. coli transcription repair coupling factor, Mfd protein, pushes forward and dissociates a stalled E. coli RNA polymerase from the DNA template in the absence of NTPs (28, 39).

The present results may be particularly valuable for understanding transcription inhibition on highly active genes, such as those for rRNA. Numerous RNA polymerase I molecules are transcriptionally active on the rRNA gene, whereas only one RNA polymerase II is generally elongating on most genes in a human cell (40–42). Preferential inhibition of rRNA synthesis by cisplatin occurs in vivo (43). For this highly active gene, the large number of bound RNA polymerase I molecules will ensure that cisplatin adducts are continuously encountered. A leading RNA polymerase I stalled at a cisplatin cross-link will be displaced, ultimately affording its rescue or the abortion of RNA synthesis. An abundant nuclear protein, HuF2 (human transcription release factor 2), dissociates efficiently both RNA polymerases I and II stalled at a cyclobutane thymine dimer (44). The relative ability to displace RNA polymerases stalled at DNA lesions by nuclear proteins such as HuF2 or trailing polymerases must be addressed to understand fully how the cell responds to transcription blocked by damaged DNA.

Resumption of Transcription of T7 RNA Polymerase Stalled at a Platinum Binding Site—Like the two states of active elongation complexes, stalled RNA polymerases also exist in multiple conformational states. A portion of the polymerases is backtracked, and some of them are completely inactivated (29). In our study, ~40% of T7 RNA polymerase elongation complexes, stalled either by NTP deprivation or at a platinum lesion, were unable to resume transcription after the addition of 0.2 M NaCN (Fig. 8). Higher salt treatment and longer incubation of stalled T7 RNA polymerases generally inacti-
vated the enzyme (data not shown). The ability of cyanide ion to remove a [Pt(dach)Cl₂] 1,2-intrastrand cross-link from stalled elongation complexes at short incubation times resulted in a high population of active ECs. A T7 RNA polymerase stalled by NTP deprivation is similar to one arrested at a platinum lesion with respect to inactivation by NaCN treatment. Under this experimental condition, active elongation complexes of T7 RNA polymerase resume transcription through sites from which the damage has been removed. This result suggests that a platinum-DNA cross-link does not irreversibly alter the transcriptional activity of T7 RNAP. The ability of mammalian RNA polymerase II to resume transcription through a site of repaired damage, however, would depend upon its post-translational activity of T7 RNAP. The ability of mammalian RNA polymerase II and its ability to bypass a repaired site of damage has been removed. This result suggests that a platinum-DNA cross-link does not irreversibly alter the transcriptional activity of T7 RNAP. The ability of mammalian RNA polymerase II to resume transcription through a site of repaired damage, however, would depend upon its post-translational modification state, including phosphorylation and ubiquitination. Recently, ubiquitination of human RNA polymerase II, induced by transcription inhibition following cisplatin treatment, was reported both in vivo and in vitro (45, 46). Cyanide ion treatment, such as that used in the present study, would allow the investigation of the effect of chemical removal of platinum adducts on transcription by RNA polymerase II. Any correlation between the post-translational modification state of RNA polymerase II and its ability to bypass a repaired site of damage would provide valuable information about transcription-coupled repair.

Conclusions—Platinum 1,2- and 1,3-intrastrand cross-links strongly inhibit transcription by T7 RNA polymerase. The present study reveals that the elongation activity of T7 RNAP, which varies with the choice and concentration of NTPs, determines the stop site of the polymerase at a platinum cross-link. A highly active elongation complex is able to transcribe into the site of the platinum lesion, where the incorrect nucleotide UTP, rather than the correct nucleotide CTP, is readily incorporated at a cisplatin 1,2-d(GpG) cross-link. The severely distorted GG template of a platinum d(GpG) adduct does not appear to be appropriately positioned to form a base pair with the correct nucleotide CTP. Our discovery of UTP incorporation suggests that mRNA and subsequent protein mutations might occur in a cell when the polymerase bypasses cisplatin damage sites, which could contribute to the cytotoxic effects of the drug. Cleavage by T7 RNAP of the nascent transcript reported here under conditions where the added NTP is not incorporated suggests a novel proofreading function for the enzyme. One or two nucleotides are removed from the 3′-end of transcript when the polymerase pauses at a site of damage. Our results with DNA adducts of oxaliplatin reveal that, in addition to limiting template DNA flexibility by covalent cross-linking two bases, the nature of the spectator ligand can further affect polymerase blockage. Our finding that trailing polymerases displace RNA polymerase stalled at a platinum lesion demonstrates one possible consequence of transcription activity at highly active genes damaged by platinum anticancer agents. Finally, platinum removal by cyanide ion indicates that the adduct does not irreversibly modify the transcriptional activity of T7 RNAP stalled at a site of damage.

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