RNA Polymerase II Blockage by Cisplatin-damaged DNA

STABILITY AND POLYUBIQUITYLLATION OF STALLED POLYMERASE*

Received for publication, September 2, 2005, and in revised form, November 7, 2005 Published, JBC Papers in Press, November 7, 2005, DOI 10.1074/jbc.M509688200

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The consequences of human RNA polymerase II (pol II) arrest at the site of DNA damaged by cisplatin were studied in whole cells and cell extracts, with a particular focus on the stability of stalled pol II and its subsequent ubiquitylation. Site-specifically platinated DNA templates immobilized on a solid support were used to perform in vitro transcription in HeLa nuclear extracts. RNA elongation was completely blocked by a cisplatin intrastrand cross-link. The stalled polymerase was quite stable, remaining on the DNA template in nuclear extracts. The stability of pol II stalled at the site of cisplatin damage was also observed in live cells. A cell fractionation experiment using cisplatin-treated HeLa cells revealed an increased level of chromatin-associated pol II proteins following DNA damage. The stalled polymerase was transcriptionally active and capable of elongating the transcript following chemical removal of platinum from the template. Transcription inhibition by α-amanitin in vitro enhanced pol II ubiquitylation at ubiquitin residues Lys-6, Lys-48, and Lys-63. In live cells expressing epitope-tagged ubiquitin mutants, several ubiquitin lyses also participated in pol II ubiquitylation following DNA damage. Cisplatin treatment triggered ubiquitylation-mediated pol II degradation in HeLa cells, which could be prevented by the proteasomal inhibitor MG132. Fractionation of pol II from cells co-treated with MG132 and cisplatin indicated that the undegraded ubiquitylated polymerase was mostly unbound or only loosely associated with chromatin. These data are consistent with a model in which only a fraction of pol II, ubiquitylated in response to cisplatin damage of DNA, dissociates from the sites of platination. This altered polymerase is rapidly destroyed by proteasomes.

The anti-tumor activity of platinum-based drugs is mediated by their ability to attack DNA. The resulting DNA lesions generate numerous cellular signals, which eventually decide the fate of cells treated by platinum agents (1, 2). Our understanding of this complicated process requires knowledge of the proteins that interact at the site of platinum damage on DNA and how they alter cell biology. Among such proteins, those that arrive first at a platinum-DNA lesion are likely to have the greatest influence (3). The identification of these proteins and comprehension of their functions are therefore important for elucidating the role of these proteins in the DNA damage-response process. Nearly 25% of this enzyme is constantly transcribing cellular DNA (5). Platinum-DNA adducts inhibit transcription by blocking RNA polymerases, as demonstrated in many laboratories, including our own (6–8). Cisplatin 1,2- and 1,3-intrastrand cross-links almost completely block T7 RNA polymerase and human pol II.

The arrest of pol II initiates transcription-coupled repair (9). Although the exact mechanism is unknown, it is generally accepted that stalled pol II must be removed or translocated from the site of damage in order for the repair machinery to assemble (10). In vitro transcription experiments have demonstrated that pol II arrested at cyclobutane pyrimidine dimers (CPDs) on the template strand is fairly stable but dissociates from the damage sites in whole cell extracts, possibly with assistance from human transcription release factor-2 (11, 12). ATP-dependent release from the template strand of pol II stalled at a cisplatin lesion in whole cell extracts has also been observed (8). Despite numerous such in vitro studies, the outcome of pol II blockage and the mechanism of subsequent transcription-coupled repair initiation are not fully understood.

Polyubiquitylation of pol II following DNA damage by UV radiation and cisplatin treatment has been reported (13, 14). This modification does not occur in cells lacking Cockayne syndrome proteins A and B, however, which are essential for transcription-coupled repair. In vitro transcription experiments with damaged DNA have also demonstrated that pol II ubiquitylation is transcription-dependent, further suggesting a possible link between ubiquitylation of the polymerase and DNA repair (15). Polyubiquitin chains linked at their C termini and Lys-48 residues signal proteasomal degradation of a lysine-modified target and are a major form of protein modification. Proteins are less frequently ubiquitylated by chains involving Lys-63 of ubiquitin, an event that triggers pathways distinct from protein degradation (16). Beside Lys-48 and Lys-63, five other lyses are available to form polyubiquitin chains, with many of their roles yet to be determined. Recently, Lys-63-linked ubiquitylation of pol II was reported when the enzyme was inhibited by α-amanitin in nuclear extracts obtained from cells synchronized in S phase (17).

To explore the effects of cisplatin-DNA damage on pol II, site-specifically platinated DNA templates immobilized on a solid support were employed. The properties of stalled pol II and its RNA transcripts in the resulting ternary complexes were analyzed. Changes in the cellular pol II distribution following DNA platination were investigated. Pol II ubiquitylation sites were mapped with the aid of ubiquitin mutants both in vitro and in live cells. The latter experiments were facilitated in HeLa cells by expressing hemagglutinin-tagged ubiquitin mutants both in vitro and in live cells. The latter experiments were facilitated in HeLa cells by expressing hemagglutinin-tagged ubiquitin mutants, several ubiquitin lyses also participated in pol II ubiquitylation following DNA damage. Cisplatin treatment triggered ubiquitylation-mediated pol II degradation in HeLa cells, which could be prevented by the proteasomal inhibitor MG132.
tin (HA)-tagged ubiquitin. We also investigated the stability of ubiquitylated pol II in cisplatin-treated cells.

**EXPERIMENTAL PROCEDURES**

*Materials—* Cisplatin (cis-diamminedichloroplatinum(II)) was obtained from Johnson Matthey. The proteasomal inhibitor MG132 was purchased from Calbiochem. T4 polynucleotide kinase, T4 DNA ligase, and all restriction enzymes were procured from New England Biolabs Inc. Streptavidin-coupled Dynabeads M-280 were obtained from Dynal Biotech. RNasin (RNase inhibitor) was purchased from Promega Corp. His-tagged ubiquitin expression vectors (pQE30-HisUb, pQE30-HisK48Rub, and pQE30-HisK63Rub) were kindly provided by Dr. K. B. Lee, and the proteins were prepared as described previously (17). His-tagged ubiquitin proteins containing only one lysine residue, with the other six lysines mutated to arginines (His-K6onlyUb, His-K48onlyUb, and His-K63onlyUb), were purchased from Boston Biochem. HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

*Construction of DNA Templates—* Plasmid pG5MLPG380 (a gift from Dr. K. B. Lee) containing an adenovirus major late (AdML) promoter was prepared from a 10-ml LB culture of *Escherichia coli* XL1-Blue cells harboring the plasmid using a Promega midi-prep kit. The plasmid was further purified by 1% agarose gel electrophoresis. The DNA fragment with a site-specific cisplatin lesion and a 4-nucleotide 5'-overhang was constructed. A DNA fragment containing an AdML promoter was prepared by PCR amplification from plasmid pG5MLPG380 (see Fig. 1). A biotin moiety was placed at the 5'-end of the coding strand by performing PCR with a 5'-biotinylated primer. The recognition site (GGTCTC) of restriction enzyme Eco31I, which produces a non-palindromic 4-nucleotide (nt) 5'-overhang, is located near the downstream end of the PCR product. The excess primers, NTPs, and DNA polymerases were removed from the PCR product by passage through Sephadex G-50 spin columns, followed by phenol extraction. The resulting DNA was treated with Eco31I and purified on a 4% native polyacrylamide gel, producing the DNA fragment with a 4-nt 5'-overhang (see Fig. 1, *PCR Promoter*).

Single-stranded DNA containing a cisplatin 1,2- or 1,3-intrastrand cross-link was synthesized as reported previously (18). A 95-bp DNA fragment with a site-specific cisplatin lesion and a 4-nt 5'-overhang was prepared by enzymatic ligation of five pieces of synthetic oligonucleotides (see Fig. 1, 99–95 DNA) following a previously described method (7). The complete sequence of each oligonucleotide piece is given in supplemental Fig. S1. Phosphorylated oligonucleotides were annealed, ligated by T4 DNA ligase, and purified by denaturing polyacrylamide gel electrophoresis. Extracted 95- and 99-nt single-stranded DNAs were reannealed by heating to 90 °C and slowly cooling to room temperature. The resulting 95-bp DNA fragment with a 4-nt 5'-overhang was ligated with the Eco31I-treated PCR product containing an AdML promoter (see Fig. 1). The final DNA templates were purified on a 3.5% native polyacrylamide gel.

*Preparation of HeLa Nuclear Extracts—* HeLa nuclear extracts were obtained from Promega Corp. or prepared from HeLa suspension cells (obtained from American Type Culture Collection) following a previously reported method (19). Hydroxyurea (750 μM)-treated HeLa cells were obtained as described previously (15). Cells were suspended in five times the packed cell volume of low salt buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)) and incubated for 15 min on ice. The cell suspension was centrifuged at 420 × g for 5 min, and the swollen cell pellet was resuspended again in twice the packed cell volume of low salt buffer. Cells were disrupted using a Dounce homogenizer with a type B pestle for 10 strokes. The crude nuclear pellet was obtained by centrifugation at 10,000 × g for 10 min. Two-thirds of the packed cell volume of high salt buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) was added to resuspend the pellet, and the nuclei were homogenized again with the Dounce homogenizer. After incubation with rotation at 4 °C for 30 min, the nuclear extract was obtained by centrifugation at 12,000 × g for 5 min. The resulting nuclear extract was dialyzed against storage buffer (20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and stored at −80 °C.

*In Vitro Transcription Assays in HeLa Nuclear Extract—* Transcription by human pol II was carried out by incubating 300–500 ng of DNA template with ∼50 μg of HeLa nuclear extract, 1 unit/μl of RNasin, 10 mM DTT, 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, and 10 μM [α-32P]UTP in transcription buffer (12 mM HEPES (pH 7.9), 12% glycerol, 60 mM KCl, 8 mM MgCl2, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, and 0.3 mM DTT) in a total volume of 25 μl. The standard incubation was for 60 min at 30 °C. The reaction was stopped by addition of 180 μl of buffer containing 0.3 mM Tris (pH 7.4), 0.3 mM sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 μg/ml tRNA. For solid-phase transcription, the DNA templates were immobilized on magnetic beads coated with streptavidin (Dynal Biotech) following the manufacturer’s instructions and incubated with HeLa extract in transcription buffer for 30 min at 30 °C before adding NTPs. Transcription initiation was initiated with NTPs containing 10 μM [α-32P]UTP and continued for 25 min. Nonradioactive UTP was added to a final concentration of 0.1 mM, and the reaction mixture was incubated for an additional 5 min. Stalled elongation complexes on immobilized templates were isolated with a magnetic separator. Termy complexes were washed with transcription buffer containing the indicated detergents and further incubated under various conditions. Cyanide ion treatment to remove platinum from stalled elongation complexes was performed as described previously (7). A 1 mM sodium cyanide stock solution was prepared in 10 mM Tris (pH 8.2) and stored at −80 °C until used. RNA transcripts were isolated by phenol extraction and ethanol precipitation and analyzed on urea-polyacrylamide gels.

To follow pol II proteins in stalled ternary complexes, solid-phase transcription was performed as described above, except that 0.1 mM UTP (total concentration) was used instead of 10 μM [α-32P]UTP. Polypeptides in the reaction supernatant and wash solutions from templates immobilized on beads were applied to a 7.5% SDS-polyacrylamide gel; blotted onto a polyvinylidene difluoride membrane; and probed with mouse anti-pol II IgM (antibody H14, Covance), which probes the phospho-Ser-5 version of pol II.

*In Vitro Ubiquitylation Assays in HeLa Nuclear Extract—* Ubiquitylation assays with plasmid DNA were performed following a previously reported method (15). A 50-μg portion of HeLa nuclear extract was incubated with ∼1 μg of DNA template in transcription buffer with and without 5 μM α-amanitin for 15 min at 30 °C. To initiate transcription elongation and subsequent ubiquitylation, NTPs (1 mM ATP and 0.2 mM each CTP, GTP, and UTP) and various His-tagged ubiquitin proteins (1 μg) were added to the reaction solution. Following 40 min of additional incubation, ubiquitylated proteins including pol II were separated from the nuclear extract by incubation with 20 μl of nickel-
nitrilotriacetic acid (Ni-NTA)-agarose resin (Novagen) in His-NTA binding buffer (50 mM sodium phosphate (pH 7.9), 0.3 M NaCl, 10 mM imidazole, and 0.05% Tween 20) at 4 °C for 1 h. The resin was washed twice with His-NTA binding buffer containing 30 mM imidazole before eluting ubiquitylated proteins by adding SDS loading buffer (20 mM Tris (pH 6.8), 10% glycerol, 100 mM 2-mercaptoethanol, 1% SDS, and 0.02% bromophenol blue) supplemented with 0.1 M EDTA. The eluted proteins were separated on a 7.5% SDS-polyacrylamide gel and analyzed as described above with mouse anti-pol II antibody H14.

The assay was also carried out with bead-immobilized linear DNA templates. The immobilized template (1 μg) was incubated with HeLa nuclear extract (50 μg) as described above for 30 min at 30 °C before adding NTPs (1 mM ATP and 0.2 mM each CTP, GTP, and UTP). Transcription was inhibited either by adding 5 mM α-amanitin or by using a site-specifically platinated DNA template. Following a 10-min transcription elongation reaction, ubiquitylation was initiated by addition of 1 μg of ubiquitin and continued for 40 min. The beads were separated and washed with detergents as described above. pol II proteins in each sample were examined by Western blot analysis with mouse anti-pol II antibody H14.

Cellular Protein Fractionation—HeLa cells were collected with a cell scraper or by trypsinization with or without cisplatin treatment. The cells obtained were sequentially extracted with various detergents and salts according to a previously reported method (20). All solutions contained a protease inhibitor mixture (Calbiochem). Cells harvested from a 100-mm dish (~5 × 10^6 cells) were lysed with 500 μl of hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl_2, and 0.1% Triton X-100) for 10 min on ice. The nuclear pellets were separated from the supernatant (designated as the S2 fraction containing cytoplasmic proteins) and washed with isotonic sucrose buffer (50 mM Tris (pH 7.4), 0.25 M sucrose, and 5 mM MgCl_2) producing the STM fraction. The washed nuclear pellets were extracted with a series of low salt buffers (10 mM Tris (pH 7.4) and 0.2 mM MgCl_2) supplemented with 1% Triton X-100, 0.5 mM NaCl, 0.3 M NaCl, 0.5 mM NaCl, or 2.0 M NaCl, yielding the TW, LS, 0.3, 0.5, and 2.0 fractions, respectively. Finally, the remaining nuclear residue was solubilized in low salt buffer by sonication (NR fraction). A 10–20-μl portion of each cell fraction was subjected to SDS-PAGE and analyzed by immunoblotting with the following antibodies: mouse anti-pol II antibody H14, mouse anti-actin IgG (Upstate), rabbit anti-HMGB1 IgG (Upstate), mouse anti-histone H3 IgG (Upstate), and rabbit anti-transcription factor (TF) IIH p89 IgG (Santa Cruz Biotechnology, Inc.).

To examine the portion of transcriptionally engaged pol II, cellular proteins were fractionated as described (21) with a minor modification. HeLa cells obtained from a 25-cm^2 culture flask (~2 × 10^6 cells) were suspended in 400 μl of cytoskeleton buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl_2, 0.5% Triton X-100, 1 mM DTT, and 1 mM EDTA) and incubated for 10 min on ice. The supernatant (S fraction) was separated from the nuclei, which were subsequently extracted with 400 μl of TD buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, and 2 mM EGTA) to yield the NE fraction. The remaining nuclear residue was boiled in 100 μl of hot SDS buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1% SDS, 5 mM EDTA, and 2 mM EGTA) and diluted with 300 μl of cold TD buffer. The solution was sonicated to disrupt chromatin further and centrifuged to produce the NR fraction.

HA-tagged Ubiquitin Expression in HeLa Cells—Plasmid pMT127 (named MTHA_Ub×1 in this study; shown in supplemental Fig. S2), comprising a cytomegalovirus promoter and a HA-tagged ubiquitin gene, was obtained from Dr. D. Bohmann (22). Site-directed mutagenesis was performed to mutate the indicated lysine residues of ubiquitin to arginines. The expression vectors (MTHA_Ub×n) (supplemental Fig. S2), which produce mRNAs encoding precursor proteins consisting of multimeric ubiquitins and ubiquitin mutants (n = number of ubiquitins in a multimer), were constructed as shown in supplemental Fig. S2. MTHA_Ub×7 (containing a heptameric ubiquitin gene) was used in this study.

HeLa cells were grown to 90% confluence on a 6-well plate. Approximately 2 μg of plasmid DNA was transfected with 10 μl of Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s protocol. After a 24-h incubation with transfection complexes, the medium was replaced with standard Dulbecco’s modified Eagle’s medium. Cells were washed with cold phosphate-buffered saline and harvested by trypsinization. HA-tagged ubiquitin expression was determined by Western blotting with rabbit anti-ubiquitin IgG (Calbiochem) and anti-HA tag IgG (Rockland Inc.).

Immunoprecipitation—HeLa cells (90% confluent) from a 25-cm^2 culture flask (~2 × 10^6 cells) were transfected with 12 μg of HA-tagged ubiquitin expression vector using Lipofectamine 2000 reagent. Following 24 h of protein expression, cells were treated with cisplatin and collected by trypsinization. Total cell extracts were prepared by boiling the cells in 100 μl of hot SDS buffer and diluting in 600 μl of cold TD buffer as described previously (23). The solution was sonicated and cleared by centrifugation. The cell extracts obtained were incubated with anti-HA antibody for 3 h at 4 °C, and proteins were captured on protein G Plus-agarose beads (Calbiochem). The beads were washed five times with 1.6 hot SDS buffer/TD buffer. Proteins were eluted from the beads with SDS loading buffer and analyzed by Western blotting with mouse anti-pol II antibody H14. Immunoprecipitations of cell fractions with anti-HA antibody were carried out by diluting each fraction solution in 6× TD buffer, adding antibody, and capturing proteins as described above.

RESULTS

Stability of pol II Stalled at a Platinum-DNA Lesion—A linear DNA template containing an AdML promoter and a cisplatin-DNA lesion with a biotin moiety was prepared (Fig. 1). In vitro transcription assays were carried out with the probe. The undamaged probe (no platinum) produced a runoff transcript of 185 nt. On the other hand, RNA transcripts ~114 nt in length were generated when a cisplatin lesion stopped transcription elongation, as illustrated in Fig. 1. Transcription assays were also performed with DNA templates immobilized on streptavidin-coated magnetic beads. Preinitiation complexes were formed on the promoter site by incubating HeLa extract with the immobilized templates. Transcription elongation was started by adding NTPs to preinitiation complexes. Elongation complexes on the immobilized DNA templates were separated from the reaction solution at a different time point. RNA transcripts on beads or in solution were isolated and analyzed. As indicated above, a cisplatin L13-intrastrand cross-link strongly blocked the polymerase and yielded RNA transcripts ~114 nt in length. Interestingly, most 114-nt transcripts were found on the beads (supplemental Fig. S3B), indicating that pol II stalled at a cisplatin lesion is fairly stable under these conditions. Stalled elongation complexes appeared to remain on DNA templates for at least 2 h (data not shown).

Detergent such as Sarkosyl and heparin have been successfully used in in vitro transcription assays to remove transcription initiation and unstable elongation complexes (24). Elongation complexes stalled at the site of cisplatin damage on the immobilized template were washed with transcription buffer containing 0.1% Triton X-100, followed by washing.
under more stringent conditions with solutions containing either 1% Sarkosyl or 100 μg/ml heparin. RNA transcripts in stalled ternary complexes were not eluted with these washes (supplemental Fig. S3C), indicating that the complexes are stable under these conditions.

Ternary complexes stalled at the damage sites on immobilized DNA templates were also tracked by probing the pol II protein. After solid-phase in vitro transcription, the beads containing transcription complexes were separated from the reaction solution and sequentially washed with buffer solutions containing 0.1% Triton X-100, 1% Sarkosyl or 100 μg/ml heparin. RNA transcripts in stalled ternary complexes were not eluted with these washes (supplemental Fig. S3C), indicating that the complexes are stable under these conditions.

Dynamic State of Stalled pol II: Backtracking and Transcription Resumption—To study further the properties of stalled pol II under different conditions, ternary complexes on immobilized templates were isolated and incubated with fresh nuclear extract. As shown in Fig. 3A, most RNA transcripts remained associated with the immobilized templates on the beads, showing good stability of stalled pol II under these incubation conditions. In the absence of NTPs, freshly added nuclear extract triggered RNA transcript cleavage (Fig. 3A, lane 10). These data are consistent with previously reported polymerase backtracking and transcript cleavage induced by TFIIS (6, 8). In the presence of both NTPs and nuclear extract, only ~114-nt RNA transcripts were obtained (Fig. 3A, lane 8). These results suggest that, following polymerase backtracking and RNA cleavage, pol II is still transcriptionally active and will resume elongation with NTPs until re-encountering the platinum-DNA lesion.

Because stalled pol II showed great stability at the site of cisplatin damage under our various experimental conditions, it was of interest to study whether stalled pol II would be able to resume transcription beyond the DNA adduct after the damage is removed. In our previous study, cyanide ion was successfully used to remove platinum adducts from immobilized DNA in the presence of T7 RNA polymerase (7). DNA templates containing cisplatin 1,3-intrastrand cross-links were incubated with 0.1 mM NaCN for 30 min at 30 °C and used for in vitro transcription. As shown in Fig. 3B, a low level of 185-nt runoff transcripts was produced with damaged DNA templates when the probe was pretreated with cyanide ion (lanes 3 and 4 versus lanes 5 and 6), indicating that a small portion of cisplatin 1,3-intrastrand cross-links was removed. Stalled ternary complexes were isolated and allowed to react with cyanide ion in the presence of 1 mM NTPs. The treatment with 0.1 mM NaCN facilitated the release of more transcripts (~114 nt) in stalled elongation complexes from the templates (Fig. 3B, lanes 7 and 8).
versus lanes 9 and 10). However, the cyanide reaction also provoked the generation of runoff transcripts as shown in Fig. 3B (lane 7 versus lane 9). Taken together, our results indicate that pol II proteins stalled at platinum lesions are transcriptionally active in nuclear extracts and capable of resuming transcription after the damage site when platinum is removed.

Polyubiquitylation of Stalled pol II: in Vitro Ubiquitylation—Polyubiquitylation of pol II upon transcription inhibition has been reported in an in vitro system in which nuclear extracts were obtained from synchronized cells in S phase (15). Following this work, Lys-63-linked polyubiquitylation of pol II by α-amanitin inhibition was also demonstrated (17). To characterize further the nature of polyubiquitin chains on inhibited pol II, we first investigated the ubiquitylation process by performing previously established in vitro assays with additional ubiquitin mutants. Nuclear extracts prepared from HeLa cells synchronized in S phase by hydroxyurea previously effected α-amanitin-induced ubiquitylation of pol II (15). Similarly prepared HeLa nuclear extracts were incubated with plasmid DNA in the presence of α-amanitin and His-tagged ubiquitin proteins. Transcription inhibition by α-amanitin was verified in a transcription assay (data not shown). After the ubiquitylated proteins were collected using Ni-NTA resin, the level of pol II ubiquitylation was analyzed by Western blotting employing antibody H14. In reactions with wild-type ubiquitin and the K48R and K63R mutants, α-amanitin-induced transcription inhibition clearly stimulated pol II ubiquitylation (Fig. 4A, IIo-Ub in lane 3 versus lane 4 (wild-type), lane 5 versus lane 6 (K48R), and lane 7 versus lane 8 (K63R)). Overall ubiquitylation of nuclear proteins by each mutant was examined by Western blotting of the His tag epitopes (supplemental Fig. S4). In comparison with wild-type ubiquitin and the K63R mutant, K48R afforded considerably less ubiquitylated protein. These data validate the conclusion that ubiquitylation of most proteins occurs via Lys-48 in our assays, which is consistent with previously reported results (17). His-tagged ubiquitin mutants containing only a single lysine residue (Lys-48, Lys-63, or Lys-6) were also examined. As shown in Fig. 4A, all mutants displayed increased levels of pol II ubiquitylation upon treatment with α-amanitin. Our data indicate that pol II transcriptionally inhibited by α-amanitin can be polyubiquitylated by lysines other than Lys-48 in vitro under the present experimental conditions.

Plasmid DNA was also globally damaged by cisplatin and examined for in vitro ubiquitylation. Although transcription was successfully inhibited by the resulting cisplatin lesions, stimulation of pol II ubiquitylation by the cisplatin-damaged plasmid was less apparent than in the experiment with α-amanitin (data not shown). To study different properties of pol II inhibited by α-amanitin and cisplatin adducts, the ubiquitylation assay was carried out with immobilized linear DNA templates, and transcription was inhibited either by α-amanitin or by using...
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The ubiquitylation assay with immobilized linear DNA was also performed with various His-tagged ubiquitin mutants, and transcription was inhibited by α-amanitin. Because ubiquitylated pol II found in the reaction supernatant was unrelated to damage-specific transcription inhibition as discussed above (Fig. 4B), the immobilized DNA templates were separated from the reaction solution. pol II ubiquitylated by α-amanitin-induced transcription inhibition most likely remains on the templates and can be eluted with a 1% Sarkosyl wash based on the data presented above (Fig. 4B). pol II proteins eluted with 1% Sarkosyl were incubated with Ni-NTA resin to enrich the ubiquitylated protein fraction. As shown in Fig. 4C, ubiquitylation of pol II stalled by α-amanitin occurred with wild-type ubiquitin as well as with all three ubiquitin mutants containing a single lysine residue (Lys-6, Lys-48, and Lys-63).

Polyubiquitylation of Stalled pol II in HeLa Cells—Previously, a ubiquitin expression vector producing a precursor protein consisting of eight ubiquitin molecules was successfully used to express recombinant ubiquitin in HeLa cells (22). The precursor protein is endogenously processed into active ubiquitin proteins. To express HA-tagged ubiquitin and ubiquitin mutants in mammalian cells, plasmid vectors containing multimeric ubiquitin genes were constructed. Precursor expression and processing into HA-tagged ubiquitin were examined in HeLa cells transfected with the resulting plasmids. Following the transfection, the expression of ubiquitin and ubiquitylated proteins was determined by immunoblotting with anti-HA epitope and anti-ubiquitin antibodies as illustrated in Fig. 5A. HA-tagged ubiquitin and ubiquitin mutants were successfully expressed in HeLa cells. The expressed protein level of HA-tagged ubiquitin was, however, relatively low compared with that of endogenous ubiquitin as examined using anti-ubiquitin antibody (Fig. 5A, lanes 1 and 2).

To determine the nature of polyubiquitin chains conjugated on pol II inhibited by DNA damage in cells, HeLa cells were transfected with the plasmid vectors expressing a series of HA-tagged ubiquitin mutants and treated with cisplatin. Cellular proteins modified by HA-tagged ubiquitin were immunoprecipitated using anti-HA tag antibody. As shown in Fig. 5B, immunoprecipitation amplified the population of ubiquitylated pol II (Ilo-Ub in lane 1 versus lane 3). Cisplatin treatment was responsible for the pol II ubiquitylation because polyubiquitylated pol II proteins were not detected in the absence of DNA damage (Fig. 5B, Ilo-Ub in lane 3 versus lane 4). Three HA-tagged ubiquitin mutants (K48R, K63R, and K48R/K63R) were expressed in HeLa cells. Following cisplatin treatment and immunoprecipitation with anti-HA tag antibody, similar levels of pol II ubiquitylated by HA-ubiquitin were observed in cells expressing all three mutants compared with those in HA-labeled wild-type ubiquitin-expressing cells (Fig. 5B, lane 3 versus lanes 5–7). These results suggest that lysines other than Lys-48 might be involved in the polyubiquitin chain formation on pol II following DNA damage. To provide additional evidence for this idea, we tested two other ubiquitin mutants: K48only, containing only Lys-48 available for the chain formation, and NoK, containing no lysines for the process. As shown in Fig. 5C, low levels of polyubiquitylated pol II were detected upon cisplatin treatment in cells with K48only and NoK HA-ubiquitin mutants compared with cells with other mutants (Ilo-Ub in lanes 1–3 versus lanes 4 and 5).

The HeLa cells used in this study were treated with cisplatin in the absence or presence of the proteasomal inhibitor MG132. The amount of the phospho-Ser-5 version of pol II clearly decreased following DNA damage (supplemental Fig. S5). Upon addition of MG132, however, the
pol II level was maintained over 10 h after DNA damage, and even more ubiquitylated pol II proteins were observed. These data are consistent with previous work reporting that DNA damage induces down-regulation of pol II following ubiquitylation of the protein (14).

Several studies have demonstrated that DNA damage-induced ubiquitylation occurs predominantly on the hyperphosphorylated form of pol II, which is transcriptionally engaged (25, 27). However, our understanding of the fate of ubiquitylated pol II is very limited. Here, the stability of ubiquitylated pol II was investigated in HeLa cells by observing only the modified form of the protein. HeLa cells were transfected with the HA-ubiquitin expression vector. Following protein expression and cisplatin treatment, cells were lysed with 0.5% Triton X-100 and centrifuged to afford cytoplasmic and soluble nuclear proteins (S fraction) (21). The pellet was subsequently treated with 0.5% Triton X-100 and 0.5% sodium deoxycholate to extract nuclear proteins loosely bound to chromatin (NE fraction). Based on our previous experiment (Fig. 2B), Western blot analysis of TFIIH validated successful cell fractionation (Fig. 6A, lower panel). Approximately 40% of the phospho-Ser-5 versions of pol II proteins were extracted by two detergent extractions (Fig. 6A, upper panel, lanes 1 and 2 versus lanes 3 and 5 versus lane 6). However, the ubiquitylated form of pol II was observed mostly in NR fractions, suggesting that ubiquitylated polymerases are strongly bound to chromatin.

The proteasomal inhibitor MG132 prevented pol II degradation following ubiquitylation upon DNA damage, resulting in accumulation of the ubiquitylated form of pol II. HeLa cells were transfected and incubated with MG132 before cisplatin treatment. pol II proteins were again fractionated, and the ubiquitylated proteins were immunoprecipitated with anti-HA tag antibody. Similar levels of ubiquitylated pol II were observed in NR fractions regardless of MG132 treatment as shown in Fig. 6B (Ilo-Ub in lanes 3 and 6). In S and NE fractions, however, significantly more ubiquitylated pol II proteins were detected when the cells were treated with MG132 (Fig. 6B, lanes 1 and 2 versus lanes 4 and 5). These results indicate that, in the absence of protein degradation, the accumulation of ubiquitylated pol II occurs in the S and NE fractions.
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FIGURE 5. Western blot analysis of ubiquitylation of pol II in HeLa cells. A, total cell extracts were obtained from HeLa cells transfected with control plasmid (lanes 1), MTHA_Ub×7 (lanes 2), MTHA_K48RUB×7 (lanes 3), MTHA_K63RUB×7 (lanes 4), MTHA_K48+63RUB×7 (lanes 5), and no plasmid (lanes 6). Proteins were subjected to 4–20% SDS-PAGE and immunoblotted with anti-HA tag and anti-ubiquitin antibodies. WB, Western blot. B and C, HeLa cells were transfected with plasmid vectors expressing the indicated HA-anti-HA tag and anti-ubiquitin antibodies. Follow-}

DISCUSSION

pol II Stalled at a Platinum-DNA Lesion Is Stable—Previous work in whole cell extracts demonstrated ATP-dependent release from the template of pol II stalled at a cisplatin or a CPD lesion (8, 11). In these studies, early elongation complexes were first allowed to form using cell extracts or purified transcription factors. These isolated pol II complexes were further elongated by addition of NTPs to encounter the DNA adducts and subsequently incubated with cell extracts. In our experiments, transcription elongation and subsequent polymerase blockage were performed in nuclear extracts without first isolating early elongation complexes. Under these conditions, the stalled pol II proteins remained mostly bound to the DNA templates even in the presence of NTPs and nuclear extracts (supplemental Fig. S3). As can be seen, there are clear differences in the incubation conditions of stalled pol II in the present and prior experiments. A possible explanation for the disparity between the present results concerning pol II release from the damage site and those in previous reports (8, 11) would be that there is a different amount of release factor proteins, including human transcription release factor-2 (12), available for processing the stalled polymerase. When cell extracts are added to isolated elongation complexes, release factors supplied by this procedure will primarily work on these complexes. In the present transcription reaction, which did not employ isolated pol II complexes, fewer release factors would be available to process pol II stalled at the damage site because they would have to attend to other transcription complexes. Moreover, when stalled pol II proteins were separated from the transcription reaction solution and reincubated with nuclear extracts in the presence of NTP (Fig. 4), the extent of pol II release varied from batch to batch of added nuclear extract (data not shown). Some nuclear extracts displayed more pol II release than others, probably because of different levels of release factors. Furthermore, cell fractionation experiments showed an increased level of chromatin-associated pol II proteins following DNA damage (Fig. 2B). These data suggest that at least some population of polymerases remains on the damage sites without being removed by release factors in living cells. At present, how cells decide to release stalled pol II is unknown. The nature of the lesion, overall DNA damage level, sequence context, and gene identity at the site of inhibition; the stage of the cell cycle; and the local chromatin structure are some of the possibilities that must be considered to address this question.

Dynamic State of Stalled pol II: Backtracking and Transcription Resumption—Ternary complexes stalled at damage sites, such as cisplatin and CPD lesions, are targets for TFIIIS-mediated transcript cleavage (6, 8, 28). Under our experimental conditions, most stalled pol II proteins remained on the DNA template with ~114 nt RNA transcripts in the presence of nuclear proteins and NTPs as discussed above. All RNA transcripts of these ternary complexes were cleaved, however, and became shorter when they were incubated with nuclear proteins in the absence of NTPs (Fig. 3A). Stalled pol II that is not removed from the damage site by release proteins seems to be in a dynamic state, whereby the polymerase constantly backtracks, cleaves its RNA transcript with the help of TFIIIS, and resumes elongation only to encounter the DNA lesion again. Platinum removal experiments on stalled ternary com-
plexes further support the dynamic behavior of stalled pol II (Fig. 3B). Previously, CPD adducts were removed from the DNA template using photolyase and light (28). In that study, some of the transcripts in ternary complexes stalled at CPD sites were elongated beyond the lesion following photolyase treatment. In our study, 0.1 mM NaCN (pH 8.2) was used to remove platinum adducts as the [Pt(CN)4]2− complex; higher concentrations of cyanide ion significantly destabilized stalled pol II complexes (data not shown). Although the reaction removed only a small fraction of 1,3-intrastrand cross-links on DNA templates, pol II proteins stalled at the platinum lesions resumed elongation past the damage sites as soon as the adducts were removed (Fig. 3B). Previous in vitro studies performed with cisplatin- and UV light-damaged DNA (8, 11) demonstrated that the presence of pol II stalled at the damage site does not affect dual incision of the lesion by nucleotide excision repair factors. These results can be explained by the model discussed above, whereby stalled polymerases are able to backtrack from the damage sites without leaving the DNA templates, thus exposing DNA damage sites for dual incision by repair enzymes.

Polyubiquitylation of Stalled pol II Involves Several Ubiquitin Lysines—In response to DNA damage such as UV radiation and platinum anticancer agents, pol II is ubiquitylated. Several studies in eukaryotic systems have suggested a link between this event and DNA repair (13, 29). The reason why pol II becomes ubiquitylated upon DNA damage in human cells is not, however, fully understood. In our in vitro study, transcription inhibition by α-amanitin stimulated pol II ubiquitylation in nuclear extracts obtained from hydroxyurea-treated HeLa cells, as reported previously (15). We also performed ubiquitylation assays on immobilized templates to demonstrate that pol II stalled by α-amanitin is eluted with a 1% Sarkosyl wash, whereas polymerase stalled by a cisplatin lesion is much more stable (Fig. 4B). These data clearly reveal different stabilities of pol II proteins when arrested by different agents. The ubiquitylated form of pol II was observed only when the polymerases were halted by α-amanitin and not by cisplatin adducts on the immobilized linear DNA probes. It is possible that the fraction of pol II stalled at the sites of cisplatin cross-links was too low for ubiquitin ligases to modify, whereas more pol II proteins were inhibited by preincubation with α-amanitin.

A recent in vitro study reported pol II ubiquitylation via Lys-63 upon transcription inhibition by α-amanitin in nuclear extracts prepared from synchronized cells in S phase (17). Lys-63-linked polyubiquitylation of pol II raises the intriguing possibility that the process not only triggers pol II degradation but also sends additional, non-degradative signals in response to DNA damage. In our study, ubiquitylation assays with both plasmid DNA and immobilized linear DNA templates indicated that stimulated ubiquitylation of pol II induced by α-amanitin occurs in vitro via Lys-6, Lys-63, and Lys-48 of the ubiquitin protein (Fig. 4, A and C). Differently prepared nuclear extracts may contain different levels of proteins required for pol II ubiquitylation and de-ubiquitylation, processes responsible for the observed form of the modification. The nature of polyubiquitin chains formed on pol II following cisplatin treatment was investigated in HeLa cells. Epitope-tagged ubiquitin and ubiquitin mutants were previously used to study the ubiquitylation of various target proteins (30). Consistent with our in vitro results, these data indicate that lysines other than Lys-48 are also involved in ubiquitylation of pol II in response to DNA damage by cisplatin. Although the ubiquitin ligases responsible for ubiquitylation of stalled pol II have not been identified, two proteins, BRCA1/BARD1 (27, 31), and the von Hippel-Lindau protein (26), have been reported to ubiquitylate pol II in vitro as well as in cells in a DNA damage-dependent manner. Both proteins specifically interact and target the hyperphosphorylated form of pol II.

Several studies have suggested that BRCA1/BARD1 mediates ubiquitylation through lysine residues other than Lys-48 (32, 33). In one case, there was Lys-6-linked ubiquitylation by BRCA1/BARD1, which did not trigger degradation but rather affected the stability and activity of the target protein (34). It has been suggested that there is more than one ubiquitin ligase that targets pol II upon DNA damage in vivo (27, 31). In response to DNA damage, pol II proteins may be ubiquitylated by various ligases with a series of different polyubiquitin chains depending on the environment of stalled pol II. A task for the future is to understand the consequences of these pol II modifications.

Ubiquitylation of Stalled pol II: Effects on pol II—UV light-induced ubiquitylation and subsequent proteasomal degradation of pol II have been reported previously (14). Our study has also demonstrated that cisplatin treatment facilitates degradation following ubiquitylation because the proteasomal inhibitor MG132 allowed the accumulation of the ubiquitylated forms of pol II. It is plausible that pol II degradation is one of the main cellular consequences of cisplatin-DNA damage. However, the reason for such pol II degradation is unclear. One hypothesis is that pol II degradation removes the polymerase from the DNA lesions so that the repair machinery can assemble. The present (Fig. 6A) and related (14, 35) studies reveal, however, that a significant fraction of ubiquitylated pol II is tightly bound to chromosomal DNA, suggesting that even this ubiquitylated form is transcriptionally engaged. As discussed above, inhibition of proteolysis by MG132 increased the accumulation of ubiquitylated pol II. We have shown here that this additional portion of ubiquitylated pol II is exclusively unchanged or at best loosely bound to DNA (Fig. 6B). The levels of chromatin-associated ubiquitylated pol II are unchanged by MG132 treatment.

Under our experimental conditions (in which cells were treated with lethal concentrations of cisplatin for a short period of time), we are most
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likely observing direct responses of pol II to cisplatin DNA damage. These results suggest that a fraction of ubiquitylated pol II is dissociated from the damage sites and rapidly degraded by proteasomes. These data also show that more ubiquitylated pol II proteins remain tightly associated with chromatin than are dissociated following ubiquitylation in the presence of MG132 (Fig. 6B). It remains to be determined how cells regulate the release of ubiquitylated pol II from the damage sites, leading to prompt degradation of the protein.

Conclusion—The consequences of pol II blockage by cisplatin lesions were studied in extracts as well as in living cells. Solid-phase in vitro transcription assays and cell fractionation experiments all indicate that a considerable fraction of stalled pol II proteins remains strongly associated with damaged DNA following pol II arrest. Moreover, these polymerases backtrack from the damage sites, cleave the transcripts, and re-elongate. Stalled pol II can also be ubiquitylated by numerous ubiquitin ligases, conjugating polyubiquitin chains on the polymerase through Lys-6, Lys-48, Lys-63, and possibly other lysines of ubiquitin. Only some portion of ubiquitylated pol II is released from DNA and rapidly degraded by proteasomes, whereas other portions remain on DNA and may trigger non-degradative signals or affect the properties of stalled pol II. This study has provided valuable information for understanding the biological consequences when pol II encounters platinum-DNA damage. Based on our data and previous reports (8, 11), we summarize these events in Fig. 7.

Acknowledgments—We thank Professor P. A. Sharp for experimental guidance and helpful discussions. We also thank Cindy Yuan for assistance in constructing DNA templates.

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