Elongating RNA polymerase II blocked by DNA damage in the transcribed DNA strand is thought to initiate the transcription-coupled repair process. The objective of this study is to better understand the sequence of events that occurs during repair from the time RNA polymerase II first encounters the lesion. This study establishes that an immobilized DNA template containing a unique cisplatin lesion can serve as an in vitro substrate for both transcription and DNA repair. RNA polymerase II is quantitatively stalled at the cisplatin lesion during transcription and can be released from the template, along with the nascent transcript, in an ATP-dependent manner. RNA polymerase II stalled at a lesion and containing a dephosphorylated repetitive carboxyl-terminal domain (CTD) appears to be more sensitive toward release. However, a dephosphorylated CTD can become readily phosphorylated in front of the lesion by CTD kinases in the presence of ATP. The observation that RNA polymerase II and transcript release occurs in a TFIIH-deficient repair extract but not in a reconstituted repair system demonstrates that disassembly of the elongation complex can occur independently of the repair process and vice versa. Indeed, the presence of RNA polymerase II at the lesion does not prevent dual incision from occurring. Finally, we also propose that the Cockayne’s syndrome B protein factor, believed to be the mammalian transcription repair coupling factor, is neither involved in transcript release nor required for dual incision in the presence of lesion-stalled RNA polymerase II in vitro. More likely, it prevents RNA polymerase from backing up when it encounters the lesion. The ability to transcribe and repair the same damaged DNA molecule fixed on beads, along with the fact that the reaction conditions can be freely altered, provides a powerful tool to study the fate of RNA polymerase II blocked on the cisplatin lesion.

The genome is constantly subjected to DNA-damaging agents of both environmental and endogenous origin. In the absence of repair, this damage can lead to uncontrolled cell growth or cell death. Well-established repair mechanisms to remove such lesions and maintain genome integrity include nucleotide excision repair (NER), which operates on bulky helix-distorting damage caused by a variety of mutagens including UV light and certain drugs (1). During NER, the damage-containing oligonucleotide is first eliminated from the DNA in the dual incision step. This step requires the concerted action of various NER factors including xeroderma pigmentosum (XP) group C (XPC) protein complex (XPC-HR23B), transcription factor IIH (TFIIH, a general transcription factor for RNA polymerase II), xeroderma pigmentosum group A (XPA), replication protein A (RPA), XPG, and XPF-ERCC1. The gapped DNA structure is then filled by a resynthesis machinery consisting of proliferating cell nuclear antigen, replication factor C, RPA, polymerase δ, and DNA ligase I (2). Mutations in genes encoding NER proteins give rise to three human genetic disorders, thereby demonstrating the importance of this pathway. These disorders include xeroderma pigmentosum (XP), Cockayne’s syndrome, and trichothiodystrophy (3). DNA lesions located in the transcribed DNA strand potentially block the progression of elongating RNA polymerase II (RNAP II) and are therefore deleterious to the cell. Such lesions can be repaired by a specialized NER pathway designated transcription-coupled repair (TCR) (4).

The damage removal by TCR in actively transcribed genes is more efficient than the removal of damage located elsewhere in the genome (5). TCR is conserved from bacteria (6) to humans (5). The mechanism of TCR is relatively well understood in *Escherichia coli*, where a transcription repair coupling factor (TRCF) displaces stalled RNA polymerase and simultaneously recruits the repair machinery to the site of damage (7). Despite 16 years of studies, the mechanism of TCR in human cells is poorly understood. A eukaryotic RNAP II blocked at a site of DNA damage is thought to trigger the recruitment of the repair machinery (8). Cockayne’s syndrome B protein (CSB), the putative mammalian TRCF that was found in ternary complexes containing the lesion-blocked RNAP II (Ref. 9 and this study), does not disrupt these stalled complexes by itself (10). Whether or not CSB acts as a relay between the transcription and the repair machineries is yet to be determined. The fate of RNAP II with respect to its potential removal, displacement, or conformational change before, during, or after repair is not known (11, 12).

Transcription complexes stalled at a site of UV DNA damage such as thymine dimers has been widely studied (13–15). Studies presented here focus on another type of lesion repaired by NER/TCR: cisplatin cross-links (16, 17). Cisplatin derivatives are frequently used in chemotherapy and produce different kinds of adducts including: 1,2- and 1,3-intrastrand cross-links and 1

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* The abbreviations used are: NER, nucleotide excision repair; RPA, replication protein A; TFIIH, transcription factor IIH; XP, xeroderma pigmentosum; RNAP II, RNA polymerase II; TCR, transcription coupled repair; TRCF, transcription repair coupling factor; CSB, Cockayne’s syndrome B protein; RIS, reconstituted incision system; WCE, whole cell extract; CTD, carboxyl-terminal domain.

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1 The abbreviations used are: NER, nucleotide excision repair; RPA, replication protein A; TFIIH, transcription factor IIH; XP, xeroderma pigmentosum; RNAP II, RNA polymerase II; TCR, transcription coupled repair; TRCF, transcription repair coupling factor; CSB, Cockayne’s syndrome B protein; RIS, reconstituted incision system; WCE, whole cell extract; CTD, carboxyl-terminal domain.
between adjacent purines, interstrand adducts, and mono-
adducts (18). The present study uses the 1,3-intrastrand adduct that has been shown to be a block to RNAP II transcription (19, 20).

In an attempt to better understand the early steps of the NER process, we developed an in vitro transcription system in which a DNA template, containing a site-specific cisplatin lesion, has been immobilized on paramagnetic beads. This system allows early elongation complexes paused near the transcriptional start site to be chased to the site of the lesion and the fate of RNAP II to be determined (21). This study first shows that an immobilized DNA template containing a unique cisplatin at a specific site can be used in both DNA repair and transcription assays in which elongating RNAP II becomes completely blocked by the lesion. Because of knowing that a cell-free extract can destabilize RNAP II stalled on a thymine dimer (22), it was of interest to establish and to characterize the stability of ternary complexes stalled at a cisplatin lesion. Using a repair-deficient extract containing mutated TFIIH, along with a reconstituted incision system (RIS) consisting of the highly purified XPC-HR23B, TFIIH, XPA, RPA, XPG, and XPF-ERCC1 NER factors, the destabilization of transcription complexes stalled at a cisplatin lesion was shown to be ATP dependent but not repair dependent. Furthermore, use of the RIS confirms that RNAP II stalled on a cisplatin lesion does not inhibit onset of the repair reaction. Moreover, dual incision can occur without the removal of RNAP II from the lesion, whereas the mammalian TRCF, CSB, acts by preventing RNAP II from transcription assays in which elongating RNAP II becomes completely blocked by the lesion. Because of knowing that a transcriptional start site to be chased to the site of the lesion and the presence of 600 mM UTP, dATP, ApC, and 0.6 mM [α-32P]dCTP. Incubation for 30 s at 30 °C resulted in pulse labeled transcripts. Reactions were then stopped by the addition of 0.5 μl of 0.5 M EDTA (pH 8). Beads were sequentially washed with transcription buffer containing 0.1% Triton X-100 and 1% Sarkosyl. The nascent transcripts were then eluted at 30 °C in the presence of 600 mM ATP, UTP, CTP, and GTP. The resulting RNA transcripts were analyzed on a 8% denaturing polyacrylamide gel.

Transcription reactions with [32P]-labeled RNAP II (26) were performed as described above except that HeLa NE was pre-incubated with the radiolabeled RNAP IIA for 10 min on ice before template addition, and [α-32P]dCTP was replaced by cold CTP.

**Dual Incision Reaction**—The removal of the cisplatin damage by dual incision was monitored by the occurrence of the excised damage-containing oligonucleotides (24). Immobilized cisplatinated DNA template (50 ng) was incubated for 40 min at 30 °C in dual incision assay buffer (50 mM Hepes (pH 7.9), 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM ATP, and 20 μM wortmannin) together with 30 μg of a repair-competent HeLa WCE or a repair-deficient WCE from HD2 cells bearing a point mutation in the XPD gene (27). Reactions were stopped by boiling for 5 min after addition of 9 ng of a 32-nucleotide oligonucleotide complementary to the excised damaged DNA patch that contains a 5′ extension of four extra G residues. After annealing the oligonucleotide to the excised DNA fragment, excision products were radiolabeled by extension with 130 milliunits of Sequenase (USB Corporation) and 0.3 μl [α-32P]dCTP for 3 min at 37 °C. Finally, 0.6 μl dATP, dGTP, and dTTP, and 0.1 μl dCTP were added, and the incubation continued for 12 min at 37 °C. DNA was extracted with phenol/chloroform and analyzed on a 12% denaturing polyacrylamide gel.

In dual incision reactions with purified NER factors (RIS), the WCE was replaced by 0.3 μl of TFIIH (HAP fraction (28)), 5 μg of XPG, 15 ng of XPF-ERCC1, 10 ng of XPC-HR23B, 50 ng of RPA, and 25 ng of XPA, which were expressed and purified as described previously (29).

**DNA Resynthesis Assay**—Fifty ng of the immobilized cisplatinated DNA template was incubated with 30 μg of HeLa WCE or CSB-depleted WCE in the dual incision reaction buffer for 60 min at 30 °C. The reactions contained 5 μg of each dATP, dGTP, dTTP, 1 μM dCTP, and 0.8 μM [α-32P]dCTP. After incubation, the DNA was purified by phenol/ chloroform extraction and digested with EcoRI and NdeI, resulting in a 94-nucleotide fragment containing the resynthesized DNA patch. A second phenol/chloroform extraction, DNA was loaded on a 8% denaturing polyacrylamide gel and visualized by autoradiography.

**RNAP II and Transcription Release Assays**—Immobilized, damaged DNA was transcribed as described above. After transcription elongation, the DNA beads were magnetically separated and washed three times with the transcription buffer containing 0.5% Sarkosyl. Washed beads were then incubated for 40 min at 30 °C under dual incision assay conditions in the presence or absence of 2 mM ATP. After incubation, DNA beads were separated from the supernatant, and both fractions were analyzed for radiolabeled RNA transcript (5%) denaturing polyacrylamide gel) and/or the [32P]-labeled RNAP II (6% SDS-PAGE). Bands were visualized by autoradiography. Recombinant CSB used in release assays was purified as described previously (30).

**mRNA Immunoprecipitation**—The cisplatin-damaged DNA template (953-bp fragment) was radioabeled in a Klenow fill-in reaction with [α-32P]dATP and subsequently purified with a NucleoSpin Extract Kit (Macherey-Nagel). Transcription on the radiolabeled template was initiated as described above, and 600 μM nucleotide triphosphates were directly added to the pulse labeling mix. After transcription elongation for 10 min at 30 °C, purified DNA antisense RNA antibody (31), kindly provided by Daniel Reines) was added, and incubation continued for 30 min at room temperature. Immunocomplexes were then absorbed to pre-equilibrated Protein A-Sepharose beads (Pharmacia Biotech) for 60 min at 4 °C with gentle agitation. After several washes with 100 μl of 10 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 50 mM KCl, 20% glycerol, 0.1 mM EDTA,
can be repaired when a transcription elongation complex is blocked at the cisplatin lesion. The immobilized, radiolabeled DNA containing the cisplatin lesion was first transcribed as described above, whereas in control reactions, transcription was inhibited by the addition of α-amanitin. After being extensively washed, the immobilized damaged DNA contained RNAP II, primarily in its hyperphosphorylated (elongating) IIo form, the nascent RNA transcript (see below and Figs. 3 and 4), as well as TFIIF (data not shown). The cisplatinated DNA containing the RNAP II elongation complexes stalled at the lesion was further tested for NER by the dual incision and DNA resynthesis assay using a HeLa extract (Fig. 2B, upper and middle panels). Because RNAP II has initiated transcription on only a minor fraction of template DNA (21), a difference between transcribed and nontranscribed DNA may not be apparent (Fig. 2B, lanes 3 and 4). Thus, to eliminate damaged DNA that does not contain a stalled RNAP II elongation complex, the immobilized DNA was digested with CiaI restriction endonuclease after the washing step and prior to the repair reaction (Fig. 2B, lanes 1 and 2). The CiaI restriction site is located at position +85, 20 nucleotides upstream of the lesion site (Fig. 1). Accordingly, template DNA containing lesion-stalled elongation complexes will be protected from digestion, whereas unprotected DNA will be cut by CiaI. In this later case, the DNA fragment containing the cisplatin lesion will be removed from the immobilized DNA template, and consequently, the remaining immobilized DNA fragment cannot serve as a substrate in a repair reaction (Fig. 2B, middle and upper panels, lane 2). Indeed, CiaI cleavage of the template was nearly complete when transcription was inhibited by α-amanitin (Fig. 2B, lower panel, lane 2). On the contrary, when the DNA template was transcribed before CiaI digestion, a significant portion of the template remained uncleaved (Fig. 2B, lower panel, compare lane 1 with lane 2). The transcribed template digested with CiaI still allows dual incision and resynthesis to occur although, as expected, to a lower extent than with the undigested template. This decrease in activity directly reflects the portion of immobilized DNA that is actively transcribed (−5%) (Fig. 2B, compare lane 1 with lane 3). The fraction of immobilized DNA resistant to CiaI digestion is in agreement

\[ \text{DNA Repair} \]

Because RNAP II stalled at the site of a lesion is thought to trigger the TCR process (8), it was of interest to determine whether the immobilized damaged DNA template
Lesion-stalled Elongation Complexes Are Released in an ATP-dependent Manner—We also investigated whether the DNA repair reaction with HeLa WCE might affect elongation complexes stalled at the site of the lesion. The fate of paused complexes was monitored by determining the fraction of radio-labeled RNAP II and transcript bound to and released from the template before and after the repair reaction (Fig. 3).

Transcription on the immobilized damaged DNA was performed as described above, and stalled ternary complexes (RNAP II-RNA-DNA) were extensively washed with buffer containing 0.5%. Sarkosyl before the addition of a repair-competent HeLa WCE as indicated (Fig. 3). Repair was initiated by the addition of ATP. First, complexes stalled at the lesion are stable in that extensive washes of elongation complexes failed to dissociate an appreciable amount of either the nascent transcript or RNAP II from the immobilized DNA (Fig. 3, compare lane 1 with lane 7). As expected, RNAP II stalled at the cisplatin lesion is primarily in the hyperphosphorylated IIO form (Fig. 3, lower panel, lane 7). Second, when stalled ternary complexes are incubated in the presence of ATP, conditions that support repair, most of the RNA transcript (95%) and a substantial amount of the RNAP II (50%), are released from the template (Fig. 3, lanes 2 and 8), indicating destabilization of the elongating complex and suggesting that RNA release might occur independently from RNAP II release. Moreover, we noticed that ~50% of such released RNA transcript is shortened (Fig. 3, upper panel, lanes 2 and 5), indicating that damage-stalled RNAP II is part of a dynamic process that allows it to slide back before being released from the DNA template and/or to give access to RNA-digesting enzymes.

Surprisingly, the release of the nascent transcript and RNAP II was not observed in the absence of ATP (Fig. 3, lanes 3 and 9). However, in the absence of ATP, the shortening of the RNA is enhanced (Fig. 3, upper panel, compare lane 2 with lane 9) but can be reversed by the later addition of ribonucleotides (data not shown). The shortening of the RNA transcript is likely dependent on the activity of TFIIS (Ref. 23 and see “Discussion”).

Taken together, these results demonstrate that initiation of the NER reaction in the presence of HeLa extract and ATP destabilizes RNAP II elongation complexes stalled in front of the damage. Moreover, the observed backsliding of RNAP II and its reversion in the presence of ribonucleotides suggest that a lesion-stalled RNAP II is rather dynamic.

Carboxyl-Terminal Domain (CTD) Dephosphorylation Destabilizes Lesion-stalled RNAP II—In an effort to determine whether the phosphorylation state of the RNAP II CTD influences the disassembly of the ternary complex, the same set of experiments was carried out except that the RNAP IIO contained in U11 complexes was dephosphorylated by treatment with the TFIIF-associating CTD phosphatase 1 (previously designated FCP1) before elongation to the lesion (25). Under these conditions, the stalled ternary complexes in front of the damage contain primarily RNAP IIA with a hypophosphorylated CTD (Fig. 3, lower panel, lane 10).

In contrast to experiments with RNAP IIO, the incubation of stalled RNAP IIA complexes with WCE in the absence of ATP
resulted in a partial release of RNAP IIA (~40%) and its associated nascent transcript from the immobilized template (Fig. 3, compare lane 6 with lane 12). It thus appears that under these conditions, RNAP II stalled at the site of the lesion is destabilized by dephosphorylation of the CTD (Fig. 3, compare lanes 6 and 12 with lanes 3 and 9). However, when ATP was provided, i.e. under repair conditions, most of the CTD was readily phosphorylated by a CTD kinase(s) present in the WCE, thereby regenerating RNAP IIo (Fig. 3, lower panel, compare lane 10 with lane 11). Such RNAP IIo and its associated RNA transcript were then released from the template at a rate comparable to that of RNAP IIo that encountered the lesion in an already phosphorylated state (Fig. 3, compare lanes 2 and 8 with lanes 5 and 11). Under these conditions, virtually all of the dephosphorylated RNAP IIA was released from the immobilized template, whereas only RNAP IIo remained associated with the damaged DNA. The observation that the addition of ATP alone to RNAP IIA-containing complexes stalled at the lesion does not result in phosphorylation of the CTD (data not shown) demonstrates that the ternary complex either does not contain an associated CTD kinase such as P-TEFb or TFIIH, and/or that an associated CTD kinase is
unable to phosphorylate damage-stalled RNAP IIA unless it has been pre-phosphorylated by another kinase such as TFIIH (32).

**CSB Counteracts RNAP II Backtracking But Does Not Promote Ternary Complex Release**—As shown above, initiation of NER upon ATP addition promotes a partial release of RNAP II, together with the nascent RNA transcript. Because the prokaryotic TRCF acts in TCR to release RNAP stalled at the damage site in an ATP-dependent manner and recruit the repair factor machinery (7), it was of interest to determine whether CSB, its human homolog, might contribute to RNAP II and transcript release in our HeLa extract system (Fig. 3).

Results presented in Fig. 4A establish that washed complex stalled in front of the damage contain not only RNAP IIO but also a significant amount of CSB protein. The association of CSB and RNAP IIO with the immobilized damaged DNA is dependent on transcription, because neither CSB nor RNAP IIO were detected on the DNA beads when transcription was inhibited by the addition of α-amanitin (Fig. 4A, lane 2). To further study the effect of CSB on transcript release, a HeLa WCE immunodepleted of CSB was used (Fig. 4B, left panel). This CSB-immunodepleted extract was then used for repair reactions of the immobilized damaged DNA template where the RNAP II had been stalled on the lesion (Fig. 4C). The extract in Fig. 4B, left panel, lane 2 was treated with a control antibody, whereas lane 3 was treated with antibody directed against CSB. Results presented in the right panel of Fig. 4B demonstrate that depletion of CSB from the WCE did not affect either transcription or DNA repair. Furthermore, the absence of CSB did not affect the efficiency of transcript release in the presence of ATP (Fig. 4C, compare lanes 2 and 3 with lanes 4 and 5). In the absence of CSB, a substantial portion of the released RNA was shortened relative to repair in the presence of the wild-type extract (Fig. 4C, compare lane 3 with lane 5). Addition of recombinant CSB to the immunodepleted extract restored the pattern of RNA shortening observed in the wild-type WCE (Fig. 4C, compare lane 3 with lane 7).

Taken together, these results show that the CSB protein stably associates with the elongating RNAP II stalled in front of the damage. Moreover, CSB stabilizes the lesion-stalled RNAP II complex by means of limiting backtracking and shortening of the RNA transcript. Importantly, CSB is not required for the ATP-dependent release of RNA transcripts from ternary complexes stalled at the site of damage.

**Release of the Nascent Transcript and RNAP II Is Independent of Dual Incision**—To further investigate whether the ATP-dependent release of the RNAP II and the nascent transcript from the damaged DNA template is causally related to repair of the lesion, we used a TFIIH-deficient WCE derived from HD2 cells (WCEExpd). This cell line bears a mutation in the XPD gene (27). XPD helicase is required for the dual incision step of both global genome repair and TCR pathways (33). Results presented in Fig. 5A confirm that dual incision activity is minimal in this extract, (Fig 5A, compare lane 1 with lane 3). The observation that the addition of purified TFIIH to the HD2 WCE restores partial NER activity (Fig. 5A, compare lane 3 with lane 4) establishes that the ability of the extract to support DNA repair is dependent on active TFIIH.

The release of nascent transcripts from damage-stalled ternary complexes in the presence of ATP and either HeLa WCE, WCEExpd, or WCEExpd substituted with purified TFIIH did not differ significantly (Fig. 5B, upper panel). Furthermore, the release of RNAP II from the immobilized DNA was comparable for each of the above extracts (Fig. 5B, lower panel). Similar to results presented in Fig. 3, all RNA transcripts and a significant portion (~40%) of RNAP II were released from the immobilized DNA. This suggests that in the presence of WCE, NER and in particular dual incision is not required for the disassembly of elongation complexes stalled at the lesion.

**Dual Incision Can Occur While Stalled RNAP II Remains Associated at the Lesion**—Results presented above indicate that in the presence of WCE, the stalled RNAP II and its associated transcript can be released from the DNA template in an ATP-dependent manner (Figs. 3 and 5). Moreover, in the presence of an XPD-deficient extract and ATP, release takes place in a dual incision-independent manner (Fig. 5). Because these reactions were carried out in the presence of a crude extract, it is not possible to establish a direct link between disassembly of the elongation complex and the repair reaction. Accordingly, the following series of experiments were carried out in a defined, reconstituted, dual incision system (RIS) composed of six highly purified NER factors (XPC-HR23B, TFIIH, XPA, RPA, XPG, and XPF-ERCC1).

The observation that the reconstituted system in the presence of ATP did not stimulate the release of nascent transcript indicates that elongation complexes stalled at the lesion are stable in this system (Fig. 6A, compare lanes 2 and 3 with lanes 4 and 5). These data strongly suggest that, contrary to the WCE, highly purified NER factors are not sufficient to disassemble a ternary complex at the lesion site.

Because RNAP II remains bound to DNA during incubation with the reconstituted dual incision system (RIS), it was of interest to know whether the presence of RNAP II influences
the dual incision reaction. In this series of experiments, free (i.e., not immobilized) radiolabeled damaged DNA was first transcribed in the presence of radiolabeled CTP. Resulting ternary complexes were then immunoprecipitated by a head-bound antibody reactive toward RNA and extensively washed with buffer containing 50 mM KCl and 0.5% Sarkosyl. The RNA immunoprecipitation resulted in the immobilization on the beads of ~40% of the total transcripts generated (Fig. 6B, upper panel, lanes 1 and 2). The co-precipitated tRNAs serve as an internal control for the immunoprecipitation efficiency (Fig 6B, lower panel). Analysis of the immunoprecipitated, radiolabeled DNA revealed that in addition to some unspecifically co-precipitated DNA template (Fig. 6C, lower panel, lane 2), a certain amount of damaged DNA was specifically co-immunoprecipitated because of its association with the RNA transcript contained in stalled ternary transcription complexes (Fig. 6C, lower panel, compare lane 1 with lane 2).

When the washed immunoprecipitated complexes were subjected to a dual incision reaction with the highly purified NER factors (RIS) under the same conditions as described above, a strong correlation was observed between the amount of immunoprecipitated, damaged DNA and the amount of excised DNA (Fig. 6C, compare lane 1 with lane 2). Importantly, the signal intensity also correlated with that of a purified, damaged standard DNA (Fig. 6C, lane 3). Taken together, these results indicate that: (a) stalled RNAP II does not inhibit the dual incision reaction; and (b) stalled ternary complexes are not destabilized by the dual incision reaction. In particular, this demonstrates that the ATP-dependent helicases of TFIIH may unwind the DNA to further allow dual incision by the two XPG and XPF endonucleases without destabilizing the stalled RNAP II.

Moreover, our data show for the first time that RNAP II remains stably associated with the DNA template already incised by XPG and XPF endonucleases and lacks a DNA fragment spanning around 24 to 32 nucleotides around the lesion (34).

**DISCUSSION**

Various DNA lesions induced by either endogenous or environmental mutagens can block the progression of elongating RNAP II. To allow the resumption of gene expression in the presence of such DNA lesions, the TCR pathway is an important mechanism to rescue lesion-stalled RNAP II (4). Although lesion-stalled RNAP II is thought to trigger TCR (8), the exact mechanism of eukaryotic TCR has yet to be elucidated (11, 12). To provide insights in the early steps of TCR, we focused our attention on the fate of RNAP II when it encounters cisplatin-DNA cross-links. Such cross-links result from chemotherapy with platinum derivatives. For the present study, we used an immobilized template containing the adenovirus major late promoter upstream of a unique cisplatin lesion on the transcribed strand. This template supports both the initiation of transcription as well as repair of the cisplatin damage by NER in vitro. Using this template in a combined approach, we analyzed the RNAP II elongation complex as it encounters the lesion and during the repair process.

**ATP-dependent Disassembly of Lesion-stalled RNAP II Complexes**—After initiating transcription on the immobilized DNA, we observed that the cisplatin lesion completely blocked elongating RNAP II (19, 20), resulting in a stalled ternary complex consisting of template DNA, RNAP II, and the RNA transcript. The RNAP II complex stalled at the lesion remained stably associated with the immobilized DNA during several washes and incubation with repair assay buffer. However, when the washed ternary complexes were incubated with a repair-competent HeLa extract along with ATP, we observed a dissociation of the cisplatin-stalled RNAP II transcription complex. This observation is similar to the finding of Selby et al. (22) that a cell-free extract can displace a RNAP II stalled on a thymine dimer. Furthermore, we demonstrate that this disassembly of the RNAP II complex is strictly ATP-dependent. The factor or activity responsible for an ATP-dependent RNAP II release was neither stably associated with the washed RNAP II complex nor part of the core-NER (dual incision) machinery because repair reactions using highly purified NER factors (RIS) did not disrupt the ternary complexes. This finding excludes the participation of CSB and TFIIH helicases in such a reaction, both being either associated with the lesion-stalled elongation complex (Fig. 4A) or part of the RIS, respectively. The ATP-dependent HU2 factor shown to release ternary complexes stalled on a UV-induced lesion in vitro is a candidate for this release activity (13, 35).
It is important to note that during incubations with repair extract in the absence of ATP, the nascent transcript is not released but is shortened, indicating that a non-ATP-dependent factor could backtrack lesion-stalled RNAP II and/or hydrolyze the nascent transcript from its 3’ end. Such a reaction could be promoted by TFIIS because it is known to allow the cleavage of RNA contained in complexes arrested by a pyrimidine dimer and a cisplatin lesion (14, 20, 36). Because backtracking of RNAP II occurs significantly only under conditions where no NER takes place (in the absence of ATP), it is not obvious that this process contributes to TCR as proposed earlier (14, 20, 36).

**Effect of CTD Phosphorylation on the Release of RNAP II and Nascent Transcript**—In an effort to determine the requirements for the ATP-dependent RNAP II release, we first analyzed the effects of CTD phosphorylation on ternary complex stability (Fig. 3). Because lesion-stalled RNAP II is primarily in the hyperphosphorylate IIO form, we used CTD phosphatase 1 to dephosphorylate RNAP II once transcription initiation had occurred (25). RNAP IIA was then elongated to the lesion, and its stability relative to that of RNAP IIO was determined. In the presence of repair extract but in the absence of ATP, hyperphosphorylated RNAP IIA is much more sensitive to release than is RNAP IIO. This increased release of RNAP IIA could be promoted by factors such as DSIF and NELF, present in the extract that destabilizes elongation complexes containing RNAP IIA (37). However, in the presence of extract and ATP, which is required for NER, the lesion-stalled RNAP IIA is rapidly rephosphorylated, thereby converting RNAP IIA to IIO and leading to a stabilization of the stalled elongation complex. Interestingly, our finding that the addition of ATP alone to washed elongation complexes does not result in CTD (re-)phosphorylation suggests that the CTD kinase(s) required for such a CTD rephosphorylation was not stably associated with the elongation complex. Alternatively, additional co-factors or prephosphorylation of the CTD by another kinase, such as TFIHH, might be required for the phosphorylation of damage-stalled RNAP IIA by an associated CTD kinase as proposed for CTD phosphorylation by P-TefB (32).

The sensitivity of RNAP IIO, paused at a cisplatin lesion, to dephosphorylation by CTD phosphatase 1 was also examined. Although RNAP IIO in an elongation complex is generally more resistant to dephosphorylation, relative to free RNAP IIO (25), the sensitivity of RNAP IIO paused at a lesion does not differ significantly from that of RNAP IIO stalled by depletion of nucleotides (data not shown). This suggests that the CTD of RNAP II does not undergo a major conformational change when RNAP II encounters a lesion.

**RNAP II Release Occurs Independently from Dual Incision**—We next examined whether the observed release of RNAP II is linked to the NER and in particular to the TCR reaction. We thus first incubated the lesion-stalled RNAP II complex with a WCE-deficient for TFIHH XPD helicase activity, an essential activity for the dual incision reaction. Under these conditions, the ATP-dependent RNAP II release was unaffected compared with a repair-competent extract or the XPD-deficient extract substituted with TFIHH.

To further analyze whether a step of TCR before TFIHH action might be required for RNAP II release, the role of the human TCR coupling factor, CSB, was addressed using a CSB-depleted extract. CSB is thought to act prior to TFIHH (9) in a very early stage of the TCR reaction, and its prokaryotic counterpart, the TRCF protein, can displace lesion-stalled RNAP II (7). However, previous work with a minimal system of highly purified proteins suggested that this latter feature might not be conserved in the eukaryotic CSB protein (10). After having demonstrated that CSB is indeed associated with our isolated lesion-stalled RNAP II complexes, we found that CSB is not involved in the ATP-dependent release of the RNA transcript associated with RNAP II in lesion-stalled ternary complexes, as observed in the crude extract system. Because CSB is not involved in the release reaction, it might rather function in maintaining a TCR-competent complex by stabilizing RNAP II (as well as RNAP I) elongation complexes (30). Indeed, CSB prevented the backsliding of RNAP II blocked on the lesion under DNA repair conditions. Because TFIHH is the best candidate for this effect, CSB could act by inhibiting or counteracting TFIHH as proposed for highly purified systems by Selby and Sancar (38). More recently, it has been shown that the prokaryotic CSB homolog TRCF acts by promoting forward translocation and thus pushing stalled RNAP II toward the stall site to rescue arrested RNAP elongation complexes (39, 40). Whether a similar activity of CSB critically contributes to the TCR mechanism, for example by properly positioning RNAP II during TCR, remains to be established.

Taken together, our data suggest that the ATP requirement for the release of lesion-stalled RNAP II is not dependent on action of the ATP-dependent TFIHH and CSB factors. Moreover, because RNAP II release can occur in the absence of DNA repair, it might be released from sites of DNA damage before TCR.

**The Presence of Damage-blocked RNAP II Does Not Affect Dual Incision**—Although lesion-stalled elongation complexes prevent DNA digestion by the CiaI restriction endonuclease (Fig. 2), we demonstrated that the observed ATP-dependent RNAP II release is not a prerequisite for the dual incision reaction on the damaged DNA strand involving incisions by the XPG and XPF endonucleases. Similar to some work performed with UV-damaged DNA (22), we first noticed that our reconstituted dual incision system (RIS) is capable of excising the damaged DNA fragment in the presence of the stalled RNAP IIO elongation complex. More importantly, after having demonstrated that our RIS is free of any RNAP II or RNA release activity, we found that the RNAP II elongation complex remained stably attached with the incised, previously damaged DNA after the dual incision reaction. This finding strongly contributes to the long-held discussion of whether RNAP II might remain associated with the template DNA during TCR to immediately resume transcription once the repair reaction has been completed. Thus, it now remains to be addressed whether the two remaining steps in TCR, DNA resynthesis and ligation, might also take place without displacing stalled RNAP II from the former lesion site. Alternatively, DNA resynthesis and ligation (but not dual incision) might involve disassembly or displacement (breathing and/or backtracking) of the elongation complex. Notably, because of the relatively low efficiency of transcription complex formation, ~5%, which is comparable to estimates based on the amount of transcript produced in single-round transcription experiments (21), we were not able to quantify any stimulation of dual incision or DNA resynthesis in either the RIS or extract system.

The present study suggests a model in which the presence of elongating RNAP II blocked at sites of DNA damage allows the accurate assembly and function of a dual incision complex. Thus, the TCR reaction might not necessarily involve the removal of blocked RNAP II from the damage site. However, alternatively to TCR, damage-blocked RNAP II can be removed from the lesion in an ATP-dependent, but repair-independent, mechanism to clear the template. Finally, it is important to remember that TCR occurs in the context of a native chromatin structure. Models to explain the molecular details of TCR must take this into account (4, 11, 41).
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