Initiation of DNA repair mediated by a stalled RNA polymerase IIO

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The transcription-coupled repair (TCR) pathway preferentially repairs DNA damage located in the transcribed strand of an active gene. To gain insight into the coupling mechanism between transcription and repair, we have set up an *in vitro* system in which we isolate an elongating RNA pol IIO, which is stalled in front of a cisplatin adduct. This immobilized RNA pol IIO is used as 'bait' to sequentially recruit TFIIF, XPA, RPA, XPG and XPF repair factors in an ATP-dependent manner. This RNA pol IIO/repair complex allows the ATP-dependent removal of the lesion only in the presence of CSB, while the latter does not promote dual incision in an XPC-dependent nucleotide excision repair reaction. In parallel to the dual incision, the repair factors also allow the partial release of RNA pol IIO. In this 'minimal TCR system', the RNA pol IIO can effectively act as a loading point for all the repair factors required to eliminate a transcription-blocking lesion.

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Introduction

The different mechanisms involved in deciphering the genetic information leading from a gene to a protein are highly regulated and any circumstances that impede them can have severe consequences. Unfortunately, the integrity of cellular DNA is constantly jeopardized by UV radiation, antitumoral drugs (cisplatin) or environmental agents that distort the DNA helix (Hoeijmakers, 2001). Those lesions pose a serious threat to cellular processes such as transcription, as they can obstruct an elongating RNA polymerase II (RNA pol II) and lead to an apoptotic response if left unrepaired (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996). Moreover, persistent lesions can also be bound by proteins such as TBP or HMGA/B, which could indirectly lead to an inhibition of transcription and repair (Vichi *et al*, 1997; Reeves and Adair, 2005). Keeping the gene sequence intact to protect the genetic information then is one of the cell's priorities and is carried out by a transcription-dependent mechanism called transcription-coupled repair (TCR).

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TCR and global-genome nucleotide excision repair (GG-NER) are subpathways of NER. While GG-NER deals with the removal of lesions present in the overall genome, TCR is specific for removing lesions present on the transcribed strand of active genes (Bohr *et al*, 1985; Mellon *et al*, 1987).

At the molecular level, TCR and GG-NER can be distinguished by their damage recognition factor. In GG-NER, the lesion is recognized by the complex XPC-HR23B in an ATP-independent manner, and will further allow for the binding of TFIIF and the subsequent NER factors (Sugasawa *et al*, 1998; Riedl *et al*, 2003). In TCR, an elongating RNA pol IIO, likely helped by additional specific factors, is thought to be the switch for engaging the repair mechanism (Christians and Hanawalt, 1992; Sweder and Hanawalt, 1992; Svejstrup, 2003). In *Escherichia coli*, the stalled RNA polymerase triggers the recruitment of the mutation frequency decline (*mfd*) protein repair, allowing for the release of RNA polymerase and further recruitment of the repair factors (Selby and Sancar, 1993). In mammals, CSA and CSB (rad28 and rad26 in yeast, respectively) are thought to play this function. Indeed, the trademark characteristic of TCR-deficient Cockayne syndrome (CS) cells is a defect in the mRNA recovery synthesis after UV irradiation (Wade and Chu, 1979; Troelstra *et al*, 1992; van der Horst *et al*, 1997). CSB was found to be connected to both the transcription and the repair machineries: (1) CSB, as a member of the SWI/SNF family of proteins, behaves as a chromatin remodeling factor most likely through its DNA-dependent ATPase activity (Citterio *et al*, 2000); (2) CSB/rad26 stimulates RNA synthesis both *in vivo* and *in vitro* (Balajee *et al*, 1997; Selby and Sancar, 1997b; Lee *et al*, 2001); (3) CSB interacts with transcriptional complexes containing TFIIF, TFIIE, p53, RNA pol II and I, as well as with repair factors such as XPG, XPA and XAB2 (Iyer *et al*, 1996; Tantin *et al*, 1997; Selby and Sancar, 1997b; van Gool *et al*, 1997; Nakatsu *et al*, 2000; Bradsher *et al*, 2002). Therefore, it is not surprising that inherited mutations in proteins specifically involved in the TCR pathway give rise to the rare autosomal recessive disorder CS (Venema *et al*, 1990; van Hoffen *et al*, 1993). While the majority of CS cases are caused by mutations in the CSA and CSB genes (Troelstra *et al*, 1992; Henning *et al*, 1995), CS phenotypes can also be associated with the xeroderma pigmentosum (XP) arising from mutations in *XPB/XPD* (TFIIF) and *XPG* genes. CS patients are characterized by progressive neurodegeneration and developmental defects (Nance and Berry, 1992). They also display sun sensitivity manifested as a severe rash.

We hypothesized that a stalled RNA pol IIO would trigger the TCR reaction. We then set up an *in vitro* system in which a DNA fragment containing a single site-specific lesion located downstream from a promoter is immobilized on magnetic beads, allowing for both transcription and repair. We showed that an isolated elongating RNA pol IIO stalled at the lesion is able to sequentially recruit the repair factors in the absence of XPC-HR23B factor. Furthermore, we demonstrate

that this RNA pol II-associated complex initiates and/or mediates an ATP-dependent incision of the damaged DNA in the presence of CSB.

Results

A template for transcription and DNA repair

To investigate the connection between transcription and DNA repair, we have set up an assay in which the DNA template can be used for both reactions. A plasmid, containing a promoter and cisplatin DNA adduct (at position 105 on the transcribed strand), was cut by two restriction enzymes and biotinylated at the extremity, leading to a damage-containing DNA fragment of 709 base pairs (bp), further immobilized on streptavidin magnetic beads (Cax-Pt, Figure 1A).

The ability of the Cax-Pt and the undamaged Cax templates to be transcribed was analyzed by using either a reconstituted transcription system (RTS), containing, in addition to RNA pol II, the basal transcription factors TBP, TFIIB, TFIIE, TFIIF and TFIIH (Figure 1B, upper panel), or an XPC-deficient cell extract (WCE/XPC). Run-off transcription on Cax-Pt resulted in a 105-nucleotide (nt)-long RNA transcript, suggesting that the cisplatin lesion presents a strong impediment to the progression of RNA pol II (Figure 1C, lanes 5 and 7). On the contrary, transcription on the undamaged Cax

template allowed the synthesis of a full 328 nt length of RNA (lanes 1 and 3). The addition of α -amanitin, a specific RNA pol II transcription inhibitor, prevented RNA synthesis (lanes 2, 4, 6 and 8).

We also monitored a dual incision assay on Cax-Pt using either a reconstituted incision system (RIS), containing recombinant XPC-HR23B, TFIIH, XPA, RPA, XPG and XPF/ERCC1 (Figure 1B, lower panel), a HeLa whole-cell extract (WCE/HeLa) or WCE/XPC (Figure 1D). Both the RIS and WCE/HeLa (lanes 1 and 3) were able to release the damaged 26–34 nt dual incision products contrary to WCE/XPC and RIS lacking XPC (RISAXPC) (lanes 2 and 4). The lack of repair activity of WCE/XPC could be overcome by adding recombinant XPC (lane 5).

Recruitment of NER factors onto the stalled RNA pol II

Having established a system in which both transcription and repair could be carried out on the same substrate, we next wished to isolate a single RNA pol II stalled at the lesion. The Cax-Pt template was first preincubated with RTS for 15 min at 24°C, and then incubated either in the absence of (Figure 2A, lane 1) or in the presence of nucleotide triphosphates for 45 min (lanes 2 and 3). After being washed at different salt concentration, the supernatant was discarded and the remaining proteins bound to the immobilized DNA were

Figure 1 A template for both transcription and DNA repair reactions. **(A)** The transcription/repair template (Cax-Pt) contains a single cisplatin adduct (GTG, Pt) at position +105 nt in the transcribed strand downstream from the adenovirus major late promoter. The TATA box is represented by a triangle and the start site +1 by a bent arrow. The positions of the restriction enzymes sites are indicated. **(B)** Coomassie staining of highly purified transcription and repair factors. **(C)** Transcription on Cax (lanes 1–5) and Cax-Pt (lanes 6–10) was performed using either RTS or WCE/XPC as indicated at the top of the panel. Full length (328 nt) or prematured stopped transcripts (105 nt) were resolved on 8% urea/PAGE. The addition of α -amanitin is indicated. **(D)** Dual incision on Cax-Pt was carried out with RIS in either the presence of (lane 1) or the absence of XPC (RISAXPC; lane 2), WCE/HeLa (lane 3) or WCE/XPC (lane 4) supplemented with XPC (lane 5). Dual incision is indicated by the occurrence of a 26–34-nucleotides excision products.

Figure 2 Recruitment of NER factors onto the stalled RNA pol IIO. (A) Western blots of transcription complexes on immobilized DNA before (PIC, lane 1) and after (EC, lanes 2 and 3) the addition of nucleotide triphosphates. Immobilized protein complexes were washed either at 50 mM KCl (PIC-50 and EC-50) or at 400 mM KCl (EC-400). (B) Dual incision reaction on the immobilized Cax-Pt transcribed or not, and cut by *ClaI*. (C) Transcription reactions on Cax-Pt (lane 1) were further incubated with TFIIS or CSB (lanes 2 and 3). Arrows indicate the different lengths of RNA transcripts. (D) Immobilized *ClaI*-cut Cax-Pt (lane 1) as well as *ClaI*-cut EC-400 complex (lanes 2 and 3) were incubated with XPC. Following a second wash, these templates were tested in a dual incision reaction in which XPC is omitted. Fgt-Pt competitor damaged DNA (lane 2) is added where indicated. Complete NER (lane 4) is used as a control. (E) Immobilized Cax-Pt (lanes 1 and 4) or *ClaI*-cut EC-400 containing the stalled RNA pol IIO (lanes 2 and 3, 5–8) were pretreated with CIP (as indicated), and further incubated with either WCE/XPC or WCE/XPCΔCSB in the presence of ATP and CSB as indicated. Proteins remaining bound to the immobilized Cax-Pt template were next analyzed by Western blots. (F) Immobilized Cax-Pt (lanes 1) or *ClaI*-cut EC-400 (lanes 2–6) were treated as indicated at the top of the panel similarly to (E); the RNA pol IIO and CSB remaining on the immobilized Cax-Pt template were next analyzed by Western blots. (G) *ClaI*-cut Cax-Pt (lane 1) or *ClaI*-cut EC-400 complex (lane 2) were incubated with RISΔXPC, the dual incision system in which XPC is lacking. Following washes at 50 mM KCl, the remaining bound proteins were analyzed by Western blot.

submitted to Western blotting. We observed that the 50 mM washed preinitiation complex (PIC-50, lane 1) contained most of the basal transcription factors in addition to the hypophosphorylated RNA pol IIA. After adding the nucleotide triphosphates and further washes at 50 mM KCl, all the transcription factors, in addition to both the RNA pol IIA associated with the transcription initiation complex and the hyperphosphorylated elongating RNA pol IIO (EC-50, lane 2), were present on the immobilized DNA. Following additional washes of EC-50 at 400 mM KCl plus 0.1% sarkosyl, we observed that only RNA pol IIO remained tightly bound to Cax-Pt (EC-400, lane 3), ridding the promoter of the basal transcription factors and RNA pol IIA as well. However, only a small percentage (5–10%) of PIC efficiently promotes transcription; therefore, most of the damaged region of the substrate is not protected by RNA pol IIO (data not shown). To eliminate the unprotected lesion, EC-400 was then incubated with *ClaI* restriction enzyme, which cuts at position +86 (Figure 1A). When the *ClaI*-cut EC-400 immobilized DNA was incubated with RIS, no dual incision repair signal could be observed as compared to an uncut Cax-Pt (Figure 2B, lanes 3 and 1, respectively). As a control, untranscribed Cax-Pt cut by *ClaI* did not exhibit a dual incision signal when incubated with RIS (lane 2). *ClaI*-cut EC-400 represents

therefore a single lesion-stalled RNA pol IIO on the damaged DNA fragment, whose unprotected remaining cisplatin lesions have been removed.

The dynamics of the stalled RNA pol IIO was studied by incubating the transcription elongation factor TFIIS, which stimulates the nascent RNA cleavage activity intrinsic of RNA pol II (Reines *et al.*, 1993); its addition leads to a 97 nt transcript (Figure 2C, lane 2). Moreover, the addition of CSB helps RNA pol IIO to move forward (Selby and Sancar, 1997a) and to resume transcription from position 97 nt to position 105 nt (lane 3).

We also checked whether XPC was able to target the DNA structure induced by the cisplatin damage when RNA pol IIO was stalled in front. Recombinant XPC was incubated with the *ClaI*-cut EC-400 complex (see Figure 2A, lane 3), for 30 min at 30°C, and the immobilized template was subsequently rinsed with a buffer containing 50 mM KCl and then further incubated with RISΔXPC. In these conditions, we did not observe the removal of damaged oligonucleotides, suggesting that XPC did not displace RNA pol IIO (Figure 2D, lane 3). Nor did we observe a repair signal when we added a challenge-damaged DNA fragment (Fgt-Pt) to RISΔXPC and the immobilized EC-400 complex (lane 2). However, when a nontranscribed damaged DNA was first incubated

with XPC before addition of RISΔXPC, we observed dual incision activity (lane 4); this was not the case when a *Clal*-cut DNA fragment was used instead (lane 1). Altogether, our data suggest that XPC neither binds to nor displaces a RNA pol II stalled in front of a DNA damage.

We next investigated whether the stalled RNA pol II could be specifically recognized by NER factors. *Clal*-cut EC-400 was then incubated with WCE/XPC in the presence of or in the absence of ATP. Most of the DNA repair factors including XPG, TFIIH, XPA, XPF/ERCC1 and RPA were recruited onto RNA pol II in the presence of ATP (Figure 2E, compare lanes 1 and 2). Interestingly, when ATP was omitted from the incubation, we observed a decrease in the recruitment of XPG, TFIIH, XPA and XPF/ERCC1 (lane 3), while RNA pol II was still bound to the template (Figure 2F, lanes 2 and 3). We also tested the influence of CSB in recruiting NER factors on RNA pol II. CSB has been suggested to be part of a complex containing RNA pol II, TFIIH and XPG (Iyer *et al*, 1996; Bradsher *et al*, 2002), and to allow for TFIIH recruitment onto the RNA pol II (Tantin *et al*, 1998). However, adding recombinant CSB to WCE/XPCΔCSB (WCE/XPC immunodepleted for CSB; Figure 2F, lane 5 and 6), as well as ATP on *Clal*-cut EC-400, did not modify the recruitment of the NER factors (Figure 2E, lanes 7 and 8). In parallel, the amount of RNA pol II on the immobilized DNA remained constant (Figure 2F, lanes 5 and 6).

To verify that the recruitment was dependent on the presence of the RNA pol II on the DNA, we treated RNA pol II with a phosphatase as dephosphorylation of a stalled RNA pol II CTD destabilizes the RNA pol II from the template when incubated next with a cellular extract (Tremereau-Bravard *et al*, 2004) (Figure 2F, compare lane 4 with lanes 2, 5 and 6). In these circumstances, we observed a drop in the presence of NER factors (Figure 2E, lanes 5 and 6) and CSB (Figure 2F, lane 4) associated with the loss of RNA pol II.

Similarly, we showed that when the incubation was carried out in the presence of the five recombinant repair proteins and ATP, an increased recruitment of each protein onto RNA pol II could be detected compared to the nontranscribed template (Figure 2G). This further suggests that their recruitment does not necessarily require intermediate proteins.

Altogether, our results show the ability of the lesion-stalled RNA pol II to specifically recruit DNA repair factors whose recruitment is stimulated by ATP. Furthermore, while CSB appears to be part of the complex, it does not influence the recruitment of the NER factors.

CSB together with the stalled RNA pol II promote dual incision

Having shown that RNA pol II can recruit repair factors, we next wondered whether this complex could mediate the removal of the lesion. Knowing that the lengths of the repair patches were similar for TC-NER and for GG-NER (Bowman *et al*, 1997), we used our *in vitro* DNA repair system to check for the release of a damaged single-strand fragment (Aboussekhra *et al*, 1995). When EC-400 was first incubated with RISΔXPC, no incision signal was detected. However, when increasing amounts of CSB were also added (Figure 3A), we detected a low but significant increased amount of released damaged oligonucleotides (lanes 3–5) characteristic of a repair signal. Nevertheless, to check whether

such a dual incision pattern would have been nonspecifically generated by XPG and XPF, we incubated EC-400 and CSB with varying combinations of four of the five NER factors. The omission of TFIIH, XPA, RPA, XPG, or XPF completely abolished the repair reaction, suggesting that the removal of the damaged oligonucleotide is highly specific and not promoted by unspecific endonuclease cuts (Figure 3B, lanes 3–7).

The RNA pol II-mediated dual incision is rather weak compared to an XPC-mediated dual incision and might reflect either the low efficiency of the reaction (and the absence of putative additional stimulatory repair factors) and/or a different incision pattern, which cannot be fully detected by our repair system due to the design of our probe. Therefore, instead of using a probe, the DNA fragments generated from the 3'-incision were amplified by primer extension using a radiolabeled oligonucleotide annealed 100 bp upstream from the site of the lesion. Increasing amounts of CSB together with the recruited factors on RNA pol II stimulated the 3'-incision activity (Figure 3C, compare lanes 3 and 4 to lane 2). We observed a particular increase in the intensity of the hypersensitive sites at positions +9, +12, +14, +16, +17 and +20 (Figure 3C, lanes 4 and 9), also depicted in the histogram in which the intensity of the bands were quantified and normalized to the background bands located at positions +8 and +24 found in every samples. Dephosphorylation of RNA pol II, which leads to its partial destabilization, resulted in a decreased recruitment of the NER factors (Figure 2E) and in a decreased of the 3'-incision pattern (Figure 3C, lanes 5 and 6). The 3'-incision positions mediated on the one hand by RNA pol II and CSB and on the other hand by XPC are rather similar (lanes 13 and 9). However, we reproducibly observed in the RNA pol II-mediated repair a more sensitive site at position C+11 and less-sensitive sites at positions T+17 and A+20 compared to GG-NER (Figure 3C, right scans). Furthermore, to evaluate the specificity of the CSB-dependent incision reaction, we tested several mutated CSB (provided by A Lehmann): (CSBΔ440, whose 440–446 glycine residues were deleted; CSBΔ378, which lacks seven glutamine residues (from position 378 to position 384); and CSBR670W, found within a CS patient, in which the arginine located in the conserved motif III of the ATPase domain was changed into a tryptophane). We observed a slight decrease incision activity with CSBΔ440, and an inhibition with CSBΔ378 and CSBR670W (Figure 3C, lanes 10–12).

To further investigate the specificity of CSB for the RNA pol II-mediated incision, we tested the influence of CSB on an XPC-mediated repair reaction, and more specifically its effect on TFIIH and XPG as CSB interacts with these two factors (Iyer *et al*, 1996) (Figure 3D). Cax-Pt and increasing amounts of CSB were then incubated with RIS (lanes 6–8), RISΔXPC (lanes 12 and 13) or RIS containing limited amounts of either TFIIH (lanes 3–5) or XPG (lanes 9–11). CSB did not affect the efficiency of the repair reaction by stimulating TFIIH or XPG, nor did it stimulate the overall rate of the reaction. Moreover, when CSB alone was incubated with RISΔXPC (lanes 12 and 13), we did not observe any repair signal, suggesting that CSB did not promote the dual incision and that the CSB fraction was not contaminated by XPC.

Altogether, these results strongly suggest that the RNA pol II-mediated incision reaction is CSB and NER factor specific, as well as XPC-independent.

The EMBO Journal VOL 25 | NO 2 | 2006 **391**

The coming of the TCR components

We further investigated whether the recruitment of each of the repair proteins was interconnected. The EC-400 complex (Figure 4B, upper panel) was incubated with different combinations of the five NER factors (in which the omitted factor is referred to by Δ as indicated at the top of each lane), and the remaining RNA pol II-associated proteins were next detected by Western blot. Incubation of TFIIH, XPA, RPA, XPG and XPF resulted in their recruitment on the RNA pol II (Figure 4B, lane 1; see also Figure 2). We next found that TFIIH, revealed by the p62 subunit (Figure 4B, panel 2), could still be detected in the absence of RPA (lane 4), XPG (lane 5), and XPF (lane 6). In the latter case, we observed a slight decrease of TFIIH. Similarly, XPA (panel 3) could also be detected in Δ RPA (lane 4), Δ XPG (lane 5) and Δ XPF (lane 6). Interestingly, we observed an interdependence between TFIIH and XPA in which, in the absence of one, the other is less present on the damaged complex. We found that if TFIIH was almost completely absent in Δ XPA (lane 2), there was a significant drop of XPA recruitment in Δ TFIIH (lane 2). Since the omission of RPA, XPG or XPF did not

prevent them from binding to the RNA pol II (panels 2 and 3), we conclude that TFIIH and XPA might be recruited first onto the RNA pol II. The level of RPA (panel 4) remained the same in the absence of Δ TFIIH (lane 2), Δ XPA (lane 3) or Δ XPG (lane 5), and only decreased in Δ XPF (lane 6), suggesting that the RPA is able to load independently from the rest of the NER factors onto the complex. As previously observed in GG-NER (Riedl *et al.*, 2003), XPG (panel 5) is no longer recruited onto the RNA pol II in Δ TFIIH (lane 2) or Δ XPA (lane 3), and strongly decreases in Δ RPA (lane 4). However, XPG is still present when XPF is omitted (lane 6), suggesting that XPG is recruited late on the RNA pol II and likely independently of, or before, XPF. Regarding the recruitment of XPF (panel 6), its presence was strongly diminished when either TFIIH or XPA was omitted (lanes 2 and 3, respectively). Neither the omission of RPA nor the absence of XPG affected the presence of XPF onto the RNA pol II (lane 4). Therefore, XPF might be recruited late onto the RNA pol II and positioned in relation with XPG.

The presence of an NER factor onto the RNA pol II following the first incubation can be further investigated for

Figure 4 Sequential assembly of NER factors onto RNA pol II. **(A)** Scheme depicting the reaction. Cax-Pt was transcribed and then cut by *Clal* to form the *Clal*-cut EC-400, which was further incubated with different combinations of four repair factors (the omitted factor is referred to by Δ in each lane), or with different combinations of indicated NER factors. After soft washes (50 mM KCl), the recruited proteins were analyzed by Western blots **(B)** and **(F)** or by a functional dual incision complementation assay (see text for details). **(C–E)** Either RNA pol II (B) or RNA synthesis levels (C), (D) and (E) are used as a loading control.

its requirement in the dual incision reaction in which the factor of interest is omitted (Riedl *et al.*, 2003). In our experimental conditions, RNA synthesis, and consequently the amount of the elongating RNA pol IIO, was similar in every sample (Figure 4C–E). Similar to what we observed by Western blot, omission of either TFIID or XPA in the first incubation strongly prevented the recruitment of XPG and XPF (Figure 4D and E, lanes 4 and 5, respectively), underlying the requirement of TFIID and XPA for the further recruitment of NER factors on RNA pol IIO. Furthermore, we also observed a slight decrease of XPF when XPG was omitted (Figure 4E, lane 7). The recruitment of XPF slightly modified the concentration of TFIID on the damaged DNA (Figure 4C) as it was observed in GG-NER, where the release of TFIID from the damage DNA is concomitant with the arrival of XPF (Riedl *et al.*, 2003). We also noticed that the presence of XPF did enhance the recruitment of RPA (Figure 4F, lane 5), a factor that would be later involved in DNA resynthesis.

NER factor-mediated partial release of RNA pol II

Next, questions arose regarding what becomes of RNA pol IIO during the reaction. EC-400 was incubated with the NER factors for 30 min at 30°C. The supernatant was then separated from the beads and analyzed by Western blot for the presence of RNA pol IIO. We also checked for the presence of remaining RNA pol IIO on the immobilized DNA. However, because of a very low percentage of RNA pol IIO released from the immobilized DNA (5–10%), the remaining RNA pol IIO on the immobilized DNA was in saturating amounts. The

addition of each NER factor separately did not significantly promote the release of RNA pol IIO, even in the presence of ATP (Figure 5A, compare lane 1 to lanes 2–6). On the contrary, the sequential addition and recruitment of NER factors led to an increased release of RNA pol IIO from the template (Figure 5A, lanes 7–10). A maximum was obtained when all the NER factors were incubated together (lane 10). We also noticed that when incubated either alone or together with the NER factors, CSB does not help release RNA pol IIO from the DNA (Selby and Sancar, 1997b) (Figure 5B). Furthermore, while RNA pol IIO is ‘destabilized’ by the five NER factors in the absence of ATP, its removal from the template is stimulated by the addition of increasing amounts of ATP (Figure 5C, lanes 3–6). Both TFIID and CSB exhibit a DNA-dependent ATPase activity; however, because CSB is not implicated in the release of RNA pol IIO, TFIID is the most likely to use ATP to unwind the DNA. This hypothesis was confirmed by incubating RNA pol IIO with the repair factors either in the absence of TFIID or in the presence of TFIID/XPB-f99s, whose mutation is detrimental for TFIID DNA-opening activity (Coin *et al.*, 1999). In both cases the RNA pol IIO release is much weaker than in the presence of wild-type TFIID (Figure 5D and E), likely due to the absence of the ‘TFIID helicase functioning’.

Discussion

Depending on the type of DNA damage, the elongating RNA pol IIO can stall and therefore face a situation in which it might trigger factors to allow for either the bypass

Figure 5 NER factors-mediated release of RNA pol IIO. (A–E) EC-400 were incubated for 30 min at 30°C, either alone or together with NER factors and CSB as indicated at the top of each panel. The removal of RNA pol IIO from the template in the supernatant was further analyzed by Western blots. (B) EC-400 was incubated with indicated factors in the absence or the presence of increasing amounts of ATP. IIH/XPB-f99s was also used (E). Quantification was made as described in Materials and methods. A graphic at the bottom of each panel depicts the relative intensity of each signal.

or the removal of the DNA-blocking lesion. To understand the TCR pathway in which DNA repair is coupled to transcription, we have set up an *in vitro* system: first, a 3'-end immobilized DNA is used as a template by RNA pol II and its highly purified transcription factors; second, to further repair the damaged DNA, the stalled RNA pol IIO recruits the NER factors upon encountering a cisplatin lesion.

RNA pol IIO, the target

TCR requires five of the seven factors already involved in global genome nucleotide excision repair (GG-NER), explaining at least partially some similarities in the sequential recruitment of the NER factors between both mechanisms. In TCR, the stalled RNA pol II supplant for XPC in its role as a DNA damage recognition factor in addition to providing an open DNA structure, which can be targeted by structure-specific factors. In the absence of ATP, RNA pol IIO efficiently allows for the arrival of TFIIH, XPA and RPA proteins. Moreover, the addition of ATP not only increases their proper recruitment, but also attracts and/or stabilizes the two other factors, XPG and XPF/ERCC1. It is possible that ATP is used by both the XPB and the XPD helicases of TFIIH to unwind the DNA, thus offering and/or improving an accurate DNA/protein structure for the forthcoming factors. Similar to what was observed in GG-NER, TFIIH and XPA are likely the first factors recruited in TCR, since the omission of either of them affects the binding of XPG and XPF to RNA pol IIO, and *vice versa*. Furthermore, it seems that TFIIH and XPA may be recruited together and stabilize each other onto the RNA pol IIO. The omission of RPA, XPG or XPF does not affect TFIIH or XPA recruitment. Surprisingly, the level of RPA remained constant. The recruitment of RPA, therefore, is more likely due to the single-strand DNA region created by the elongating RNA pol IIO, rather than the presence of the first NER factors. While this would make RPA one of the first proteins to be present around RNA pol IIO, we do not exclude the role of XPA in further stabilizing and possibly positioning RPA, a situation that was previously described in GG-NER (Asahina *et al*, 1994; Wakasugi and Sancar, 1999; Missura *et al*, 2001; Reardon and Sancar, 2002). As observed in GG-NER, XPG and XPF join the complex formed around RNA pol IIO late. The recruitment of XPG might first be mediated through its interaction with TFIIH, and then further stabilized by RPA, as they physically interact with each other (de Laat *et al*, 1998b). We also observed that the recruitment of XPF is directed not only by the presence of the ssDNA/dsDNA junction on the 5' side within the transcription bubble already formed by the stalled RNA pol IIO (de Laat *et al*, 1998a; Tapias *et al*, 2004), but also upon XPG binding, underlining the connection between both endonucleases.

Next, concerns might arise regarding the specificity of the recruitment of NER factors. However, we have shown that RNA pol IIO recruits the NER factors in an XPC-independent manner, as RNA pol IIO cannot be substituted by XPC. In addition, as dephosphorylation of RNA pol IIO partially destabilizes it from the template (Treméau-Bravard *et al*, 2004), it led to a decrease in the recruitment of the NER factors. We also noticed the additional role of ATP in increasing such a recruitment by probably inducing the unwinding of the DNA template via TFIIH rather than modifying the phosphorylation state of RNA pol IIO, which remains stable.

RNA pol IIO/CSB-mediated incision activity

The RNA pol IIO/NER factor complex was not able to initiate the removal of the oligonucleotide-containing lesion, except in the presence of the TCR-specific factor CSB. To that matter, we observed a specific recruitment of CSB onto RNA pol IIO, which is not surprising since CSB-containing RNA pol II complexes have been previously described *in vivo* as well as *in vitro* (Tantin *et al*, 1997; Bradsher *et al*, 2002; van den Boom *et al*, 2004). We detected a weak but significant signal of dual incision in the presence of CSB. We have also shown that RNA pol IIO dephosphorylation resulted in a loss of the 3'-incision signal, strongly suggesting that the reaction relied on RNA pol IIO. These reactions occur in a CSB-dependent manner, since the absence of or the use of mutated CSB does not allow dual incision. However, the role (if any) of ATP hydrolysis generated by CSB and its putative helicase activity could be to rearrange the interface between the DNA and the NER factors.

We are aware of the low yield (around 1–2%) of our *in vitro* 'TCR reaction', keeping in mind that only 5–10% of DNA template is transcribed *in vitro*. This results in a very low percentage of NER factors/CSB using RNA pol IIO as a repair-initiating factor. We do not exclude that some additional factors are missing, such as CSA, XAB2 and MMS19 (Lauder *et al*, 1996; Lombaerts *et al*, 1997; Nakatsu *et al*, 2000; Groisman *et al*, 2003). Nevertheless, at first glance, the CSB-dependent RNA pol IIO-mediated incision pattern looks rather similar to a XPC-mediated incision pattern, as repair patches from both GG-NER and TCR have been shown to be similar *in vivo* (Bowman *et al*, 1997). The discrepancies in the incision sites could reflect a different bending of the DNA by the RNA pol IIO compared to XPC, or might be associated with a different positioning of the repair factors in the two systems. Interestingly, while our system includes all the putative known repair proteins involved in TCR, a recent study has also shown an incision activity around the RNA pol IIO mediated by XPG and TFIIH (Sarker *et al*, 2005).

Past and future of the RNA pol IIO/CSB couple

How and when CSB joins the RNA pol II, knowing its ability to bind to either the initiating or the elongating RNA pol II. At this stage of our study, we have not yet determined whether CSB travels along with RNA pol II or transiently binds to both forms of RNA pol II when needed. However, only a small percentage of CSB-containing complexes were found to be immobilized in a transcription-dependent fashion (van den Boom *et al*, 2004), arguing in favor of a mechanism in which CSB monitors progression of RNA pol II by regularly probing its elongation. Therefore, when RNA pol IIO encounters DNA damage, CSB becomes more tightly recruited (van den Boom *et al*, 2004) to participate in remodeling the DNA/RNA pol IIO interface (Troelstra *et al*, 1992; Citterio *et al*, 2000). Depending on the type of damage, this would result in either a bypass (our unpublished results and Lee *et al*, 2002) and/or a stabilization of RNA pol IIO to allow for the recruitment of the repair factors. Such reorganization of the DNA repair machinery might then play a central role in the release of the stalled RNA pol IIO, as it occurs concomitantly with the arrival of XPF, the last factor to join the complex. This is associated with an increase recruitment of RPA, which is probably needed to stabilize the open DNA structure. CSB, which does not participate in the release of RNA pol IIO, must

therefore be recruited before the formation of the entire complex: CSB interacts with TFIIH, XPA and XPG, and could be recruited before the arrival of XPF.

In addition, while we cannot exclude that the release was a consequence of the steric hindrance from all the NER factors, part of it was driven by an ATP-dependent reaction. TFIIH is likely to be partially responsible for this phenomenon, since the release of RNA pol II is less efficient in either the absence of TFIIH or in the presence of a mutated TFIIH complex. Whether the release of RNA pol II is associated with its recycling by a FCP1 phosphatase or with its degradation directed by CSA upon ubiquitylation (Groisman *et al*, 2003) has to be further investigated.

Materials and methods

Cell culture and extract preparation

Lymphoblastoid XP-C cells (GM2246D) are cultured in suspension with Dulbecco's modified Eagle medium high glucose with 2 mM L-glutamine supplemented with 10% fetal bovine serum. The whole cell extract is prepared as described previously (Sugasawa *et al*, 2001).

Damaged DNA template

The covalently closed circular DNA-Pt containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink is prepared as described previously (Shivji *et al*, 1995), based on the 105.TS plasmid (Frit *et al*, 2002). The immobilized damaged DNA template is generated by digesting the DNA-Pt plasmid with *FokI* and *Asel*, resulting in a 722-bp fragment containing the AdML promoter, with the cisplatin adduct located at position 105 bp from the start site (Cax-Pt). The damaged strand is biotinylated at the *FokI* site by Klenow fill-in reaction with Bio-dUTP (Roche) and purified after separation on agarose gel with a QIAEX gel extraction kit (Quiagen). Undamaged DNA is prepared following the same procedure. For each sample, 50 ng of purified biotinylated Cax-Pt is then bound to 10 mg magnetic streptavidin beads (Dynabeads M-280 Streptavidin, Dynal) and equilibrated with a dual incision assay buffer prior to use.

Immunoblotting

Proteins were separated on 6 or 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The following mouse monoclonal (Mab) and rabbit polyclonal (Pab) antibodies are used as primary antibodies: p62: Mab 3C9; XPA: Mab 1E11 raised against peptide aa 242–261; RPA 70/32: Pab N2.2 (Henricksen *et al*, 94); RPA32 Mab 1 E9 XPG: Mab 1B5 raised against peptide aa 1167–1186; XPF: Mab Ab-5 (Neomarkers); RNA pol II: Mab 7C2 raised against the Ctd domain and recognizing both forms of RNA pol II and CSB: Pab H300 (Santa Cruz Biotechnology).

In vitro transcription assay

Runoff transcription assays are performed in transcription buffer 50 (20 mM Tris-HCl, pH 7.8, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 50 mM KCl), as described previously (Gerard *et al*, 1991), but on immobilized damaged Cax-Pt.

Dual incision assay

Reconstituted dual incision reactions (RIS, 25 μ l) are carried out in a buffer containing 20 mM HEPES-KOH, pH 7.6, 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 10% glycerol, 0.02% NP40 and 2 mM ATP. NER factors are purified as described (Araujo *et al*, 2000) and used in saturating amounts: XPC-HR23B (10 ng), TFIIH (50 ng) (Gerard *et al*, 1991), XPA (30 ng), RPA (200 ng), XPG (50 ng) and XPF-ERCC1 (10 ng) per reaction. The NER factors are incubated with 50 ng of either free or immobilized Cax-Pt, for 90 min at 30°C. Reactions are stopped by boiling for 5 min after the addition of 9 ng of a 32-nt-long oligonucleotide complementary to the excised DNA fragment with a 5'-extension of four extra G residues (Shivji *et al*, 1995). After annealing the oligonucleotide to the excised DNA fragment, excision products are radiolabeled by extension with 0.15 U of sequenase version 2.0 polymerase (USB) and 2 μ Ci of (α -³²P)dCTP (3000 Ci/mmol).

Labeled excision products are separated on a denaturing 14% polyacrylamide gel and visualized by autoradiography.

Dual incision reactions with HeLa WCE and XPC-WCE (30 μ g) (Frit *et al*, 2002) are performed in the presence of 15 μ M wortmannin, 60 μ M aphidicolin and 2% DMSO, which are preincubated all together for 10 min at 30°C before DNA substrate addition. DNA substrate is then added and the reactions are incubated at 30°C for 40 min.

Quantification was made after scanning the autoradiographies of three independent experiments using the Genetool software (Syngene). Background bands were used to normalize the values obtained for each experiments.

Protein binding studies on immobilized DNA

Reaction mixtures of 50 ng each of previously transcribed (when indicated) immobilized Cax-Pt are washed twice in transcription buffer 400 (20 mM Tris-HCl, pH 7.8, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 400 mM KCl), supplemented with 0.1% sarkosyl, and twice in transcription buffer 50. Cax-Pt was then cut by *Clal* (New England Biolabs) in a reaction volume of 40 μ l.

The transcribed and *Clal*-cut immobilized Cax-Pt is washed again in transcription buffer 400 and 50, before incubation with either purified NER factors or XPC-WCE under dual incision assay condition for 30 min at 30°C. Washed beads in transcription buffer 50 are further analyzed for bound proteins either functionally or by Western blot. Functional protein-binding studies are carried out as described previously (Riedl *et al*, 2003) by omission of the factor of interest in the subsequent dual incision reaction, with the equivalent of one dual incision reaction volume. Western blot analyses were conducted by re-suspending the washed beads in 25 μ l of protein loading buffer 1 $\times$ and loading them on SDS-PAGE protein gel. The amount of proteins (equivalent to five dual incision reactions) is loaded for each condition. Dephosphorylation of the RNA pol II is carried out after the cut by *Clal*, by incubating 1 U of calf intestinal phosphatase (CIP) at 37°C for 1 h. CIP is washed off the DNA and the immobilized Cax-Pt is further treated as described above.

Release of RNA pol II from immobilized DNA

The immobilized Cax-Pt is first transcribed (where indicated) and cut by *Clal* as described above, and then incubated with NER factors as described in the dual incision assay. The reactions were then incubated at 30°C for 30 min. The supernatant is then collected, supplemented with 5 μ l of 6 $\times$ protein loading dye and further analyzed on a 6% SDS-PAGE protein gel. Gels are scanned with the Genetool software (Syngene) and the amount of RNA pol II released in supernatant was calculated after subtraction of the negative background and correlated to the total amount of RNA pol II.

RNA pol II-mediated incision assay

Reaction mixtures of 50 ng each of previously transcribed (when indicated) immobilized DNA are washed twice in transcription buffer 400 and twice in transcription buffer 50. NER factors and others (see figure legends) are incubated as described in the dual incision assay at 30°C for 90 min. Reactions are then stopped by adding 100 μ l of phenol and boiled for 5 min. In all, 100 μ l of chloroform and 200 μ l of stop solution (1% SDS, 300 mM sodium acetate and 25 μ g/ μ l glycogen) are added next and DNA fragments are further ethanol precipitated. The pellet is subjected to 30 cycles (95°C 30 s, 55°C 30 s, 72°C 1 min) of primer extension using a 22-nt-long end-radiolabeled primer, which binds to the transcribed strand 105 nt downstream from the lesion. After phenol-chloroform extraction, ethanol precipitation and wash, the samples are loaded onto a 6% sequencing gel. The gel is dried and autoradiographed. Gels are scanned with the Genetool software (Syngene) and experiments were quantified following the same protocol described in dual incision assay.

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