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Nucleotide Excision Repair Driven by the Dissociation of CAK from TFIIH

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SUMMARY

The transcription/DNA repair factor TFIIH is organized into a core that associates with the CDK-activating kinase (CAK) complex. Using chromatin immunoprecipitation, we have followed the composition of TFIIH over time after UV irradiation of repair-proficient or -deficient human cells. We show that TFIIH changes subunit composition in response to DNA damage. The CAK is released from the core during nucleotide excision repair (NER). Using reconstituted in vitro NER assay, we show that XPA catalyzes the detachment of the CAK from the core, together with the arrival of the other NER-specific factors. The release of the CAK from the core TFIIH promotes the incision/excision of the damaged oligonucleotide and thereby the repair of the DNA. Following repair, the CAK reappears with the core TFIIH on the chromatin, together with the resumption of transcription. Our findings demonstrate that the composition of TFIIH is dynamic to adapt its engagement in distinct cellular processes.

INTRODUCTION

The genome of eukaryotes is vulnerable to an array of DNA-damaging agents. Cells possess several DNA repair pathways to avoid the harmful effects of DNA damage on replication and transcription. NER removes a broad variety of DNA lesions, including cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) generated by UV light, through two related subpathways (Lindahl and Wood, 1999). The general global genome repair (GGR) removes DNA damages from the entire genome, and the transcription-coupled repair (TCR) corrects lesions located on actively transcribed genes (Hanawalt, 2002). Three repair-deficient disorders emphasize the importance of NER in genome stability: the xeroderma pigmentosum (XP), the trichothiodystrophy (TTD), and the Cockayne syndrome (CS) (Lehmann, 2003).

The widely accepted model of NER includes the detection of the damage-induced DNA distortion by XPC in GGR and by RNA polymerase II (RNA pol II) in TCR (Hanawalt, 2002). Both subpathways then funnel into a common process, and the DNA is unwound by the XPB and XPD ATPases/helicases of the transcription/repair factor TFIIH (Zurita and Merino, 2003). Such initiation of DNA opening favors the recruitment of XPA and RPA that assist in the expansion of the DNA bubble around the damage (Evans et al., 1997; Riedl et al., 2003). Next, the endonucleases XPG and XPF generate cuts in the 3′ and 5′ sides of the lesion, respectively (O'Donovan et al., 1994; Sijbers et al., 1996), thereby removing the damaged oligonucleotide (Huang et al., 1992; Moggs et al., 1996). Finally, the resynthesis machinery fills the DNA gap (Shivji et al., 1995). In TCR, two additional proteins, CSA and CSB, are required to assemble NER proteins and various TCR-specific factors onto the stalled RNA pol II (Fousteri et al., 2006).

Seminal studies on the TFIIH complex established its function in the transcription of protein-coding genes (Gerard et al., 1991) but provided only a glimpse of its cellular importance. Later, the unexpected finding that TFIIH was involved in NER and in the transcription of ribosomal genes made this factor a remarkable example of a multifunctional cellular complex (Feaver et al., 1993; Schaeffer et al., 1993; Drapkin et al., 1994; Guzder et al., 1994a, 1994b; Iben et al., 2002). Mammalian TFIIH includes a core, containing the seven subunits XPB, XPD, p62, p52, p44, p34, and p8/TTD-A (Giglia-Mari et al., 2004; Ranish et al., 2004; Coin et al., 2006) coupled to the cdk-activating kinase module (CAK) composed of the three subunits Cdk7, cyclin H, and MAT1 (Roy et al., 1994).

The major part of the work carried out on TFIIH relates to the pivotal role and the specific function of each of its subunits in either transcription or DNA repair (Dubaele et al., 2003; Giglia-Mari et al., 2006). Yet one of the most intriguing questions is how a single TFIIH complex faces the task of participating both in transcription and NER. In the present study, we have followed the makeup of TFIIH in vivo over time after a genotoxic attack. Using a chromatin immunoprecipitation (ChIP) approach, we showed that the CAK is released from the core TFIIH following the engagement of the complex in DNA repair. Meanwhile, the core TFIIH associates with NER-specific factors, including XPA, which catalyzes the detachment of the CAK from the core thereby triggering the incision/excision of the damaged oligonucleotide. Following damage removal, the NER factors are released from the complex and the CAK reappears with the core TFIIH on the chromatin, concomitantly with the recovery of the transcription formerly inhibited by UV irradiation.

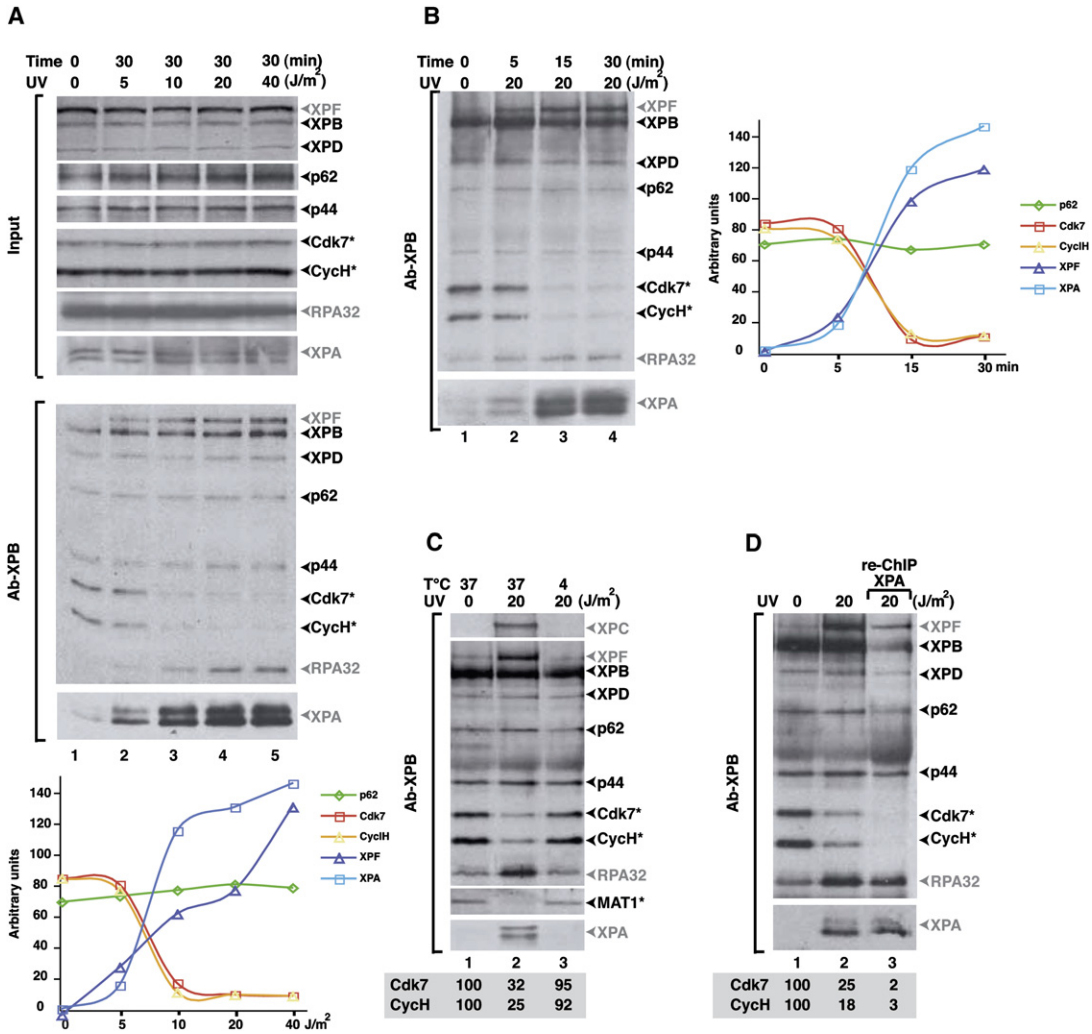

Figure 1. The Repair-Specific TFIIH Complex Is Depleted of the CAK Module

(A) Western-blotting analysis of Ab-XPB ChIP samples from chromatin extracts of untreated (lane 1) or UV-treated (lanes 2–5; 5, 10, 20, or 40 J/m^2) WT MRC5 fibroblasts, fixed 30 min later. The chromatin inputs are indicated. Subunits of TFIIH are in black; NER proteins in gray. Subunits of the CAK subcomplex are marked with an asterisk. Note that two bands are detected for XPA. The WB signals for XPF, XPB, p62, CyclH, Cdk7, and XPA were quantified using Genetool (Syngene) and plotted on the graphs. For each single lane, XPB was used as reference.

(B) ChIP was performed with an Ab-XPB on the chromatin of WT MRC5 fibroblasts, incubated over time (5, 15, and 30 min) after UV (20 J/m^2). A dose of 20 J/m^2 generates four photolesions per 10 KB of genomic DNA (van Hoffen et al., 1995). With DNA fragments of 200–400 bp (data not shown), we estimate that ~20% of the DNA fragments in our experiment contain a UV-induced lesion after sonication.

(C) ChIP was performed with an Ab-XPB on the chromatin of nontreated (lane 1) or treated WT MRC5 fibroblasts (20 J/m^2) incubated for 30 min after UV at 37°C (lane 2) or 4°C (lane 3).

(D) ChIP was performed as in (C), lanes 1 and 2, then pulled-down fractions from irradiated cells were eluted and re-ChIPed with an Ab-XPA (lane 3).

RESULTS

UV Irradiation Elicits a Change in the Composition of TFIIH

An antibody directed against XPB (Ab-XPB) was used to isolate TFIIH from the chromatin of wild-type (WT) fibroblasts 30 min after UV treatment. Western blotting (WB) revealed that though Cdk7 and Cyclin H of CAK were equally present in the inputs (Figure 1A, upper panel), their amount progressively decreased in the ChIP fractions on increasing UV dose (Figure 1A, lower panel). Meanwhile, the amount of XPD, p62, and p44 subunits of the core TFIIH remained constant. Together with the progressive diminution of CAK, the NER factors, including XPA, RPA, and XPF, assembled with the core TFIIH on the damaged chromatin (Figure 1A, lower panel; see also the graph). Similar findings were obtained with an antibody directed against p62 subunit of TFIIH (see Figure S1 available online). To investigate further the release of CAK, proteasome inhibitors were used and showed no effect on its disappearance from TFIIH, suggesting that it is detached from the core and not degraded (data not shown). The UV-induced change in TFIIH was also investigated at different time points and temperatures of incubation. When

cells were irradiated at 20 J/m^2, the changes were optimum within 15 min (Figure 1B, WB and graph) and did not occur at 4°C (Figure 1C, lanes 2 and 3), suggesting that the release of CAK was an active process. Next, the ChIP fractions isolated after irradiation were eluted and reimmunoprecipitated with an XPA antibody (re-ChIP). The ChIP re-ChIP assay demonstrated that the core TFIIH (XPB, XPD, p62, and p44) was present in a repair-specific complex containing XPA, XPF, and RPA, without CAK (Figure 1D, lanes 2 and 3). Eighty percent of TFIIH present on the chromatin did not contain CAK (Figure 1D) 15 min after irradiation. Together, these findings pinpoint that genotoxic attack induces a change in the composition of TFIIH with a release of the CAK from the core TFIIH, together with the arrival of the NER factors.

Next we checked TFIIH in the soluble fractions of noncrosslinked irradiated cells. The cells were irradiated and lysed in RIPA buffer, and immunoprecipitations were performed with Ab-p44 antibody followed by elution with an excess of competitor peptide (Coin et al., 1999). Western blotting, kinase assay (measuring Cdk7 activity; Tirode et al., 1999), and silver staining demonstrated that most of the CAK was released from the core TFIIH after irradiation (Figure S2). However, the dissociation was observed only after irradiation at 100 J/m^2, and no repair factors were found associated with the core TFIIH. These data indicate that soluble "repairosomes" are unstable in human and can be detected only on the chromatin, after fixation.

Release of the CAK Takes Place during NER

To assess whether the change in TFIIH was part of the NER reaction, we investigated the amount of CAK and repair factors associated to the core TFIIH in various repair-deficient cell lines by ChIP. First, we analyzed the composition of TFIIH in GGR defective XP-C versus TCR-defective CS-B cells. The Ab-XPB immunoprecipitated TFIIH pattern in XP-C cells was markedly different from the WT after UV: CAK was not released and none of the NER factors accumulated to TFIIH (Figure 2A, lanes 1–4). In CS-B cells, the TFIIH pattern after UV was similar to WT cells (cf. lanes 1 and 2 with 11 and 12). Then, ChIP was performed on XP-A cells. Under our experimental conditions, the release of the CAK (less than 20%, compared with 80% in WT cells) from the core and the recruitment of RPA were barely detectable after UV, and association of XPF with TFIIH was not observed (cf. lanes 1 and 2 with 5 and 6). In both UV-treated XP-G and XP-F cells, up to 80% of CAK was released together with the recruitment of XPA and RPA to the core TFIIH (cf. lanes 1 and 2 with lanes 7 and 8, and 9 and 10). Notably, XPF associated with TFIIH after UV in XP-G cells (lanes 7 and 8; Volker et al., 2001). To demonstrate the role of XPA in the CAK release, WT cells were treated with siRNA toward XPA (siXPA). The amount of XPA strongly decreased after siXPA transfection (Figure 2B, upper panel, lanes 1–3). ChIP using Ab-XPB showed that RPA was not recruited to the core TFIIH in the absence of XPA (Figure 2B, lower panel, lanes 2 and 3). Similar to what was observed in XP-A cells, the CAK (visualized by CyclinH) was barely released in siXPA-treated WT cells (cf. lane 1 with 3).

Our findings suggest that the recruitment of XPA to the preincision complex, which is composed of XPC and TFIIH, may promote the release of CAK. Therefore, we carried out ChIP analysis with an antibody against XPC in both the WT and XP-A cells. The association of the core TFIIH (XPB, XPD, and p62) with XPC took place both in WT and XP-A cells, after UV (Figure 2C, cf. lanes 1 and 2 with 3 and 4). However, the subunits of the CAK complex were found together with the core TFIIH and XPC in XP-A but not in WT cells (lanes 2 and 4).

In addition, we investigated whether mutations in the XPD helicase would modify the composition of TFIIH. In UV-treated XP-D cells, neither a release of the CAK from the core nor a recruitment of XPA and RPA to the TFIIH were observed (Figure 2D, lanes 1–4).

Overall, our findings suggest that the release of the CAK from the core TFIIH takes place during GGR, after the recruitment of XPA.

XPA and ATP Promote Release of CAK

To further dissect the change in TFIIH composition when engaged in the NER process, we set up an in vitro assay that allows us to analyze the composition of functionally active intermediate NER complexes. A linear DNA substrate containing a single 1,3-intrastrand d(GpTpG) cisplatin lesion was immobilized on magnetic beads and incubated with a repair-competent nuclear extract (NE) at 4°C for 15 min to allow the binding of XPC and TFIIH to the damaged DNA (Riedl et al., 2003). The samples were subsequently incubated at 30°C over time. Then, the proteins bound to the immobilized DNA were analyzed by WB. Without ATP, the composition of TFIIH was unchanged (Figure 3A, WB, lanes 1–4). ATP promoted a time-dependent release of CAK (85% of CAK is released after 10 min of incubation), together with the arrival of XPA, XPF, and RPA (Figure 3A,WB, lanes 5–8 and upper graph) and the removal of the damaged oligonucleotides (Figure 3A, NER, lanes 5–8). Note that the dissociation of the CAK was observed only in the presence of damaged DNA (data not shown).

We also investigated whether the core TFIIH engaged in DNA repair maintained its transcriptional activity. NER complex formation was allowed to proceed over the time in the presence of ATP. At the indicated times, the immobilized protein/DNA complexes were further incubated in a reconstituted transcription assay containing all the general transcription factors (except TFIIH) and the AdMLP template (Gerard et al., 1991). In this challenge experiment, we observed a progressive decrease of the TFIIH transcriptional activity over time, which correlates with the progressive increase of the elimination of the damaged oligonucleotide observed during NER (Figure 3A, Tx and NER panels; see also bottom graph). Addition of CAK restores the TFIIH transcription activity (Figure 3A, Tx; cf. lanes 5 and 6).

We further examined the role of XPA in the release of the CAK. An XP-A cell NE (that does not express the XPA protein; Koberle et al., 2006) was incubated with the immobilized damaged DNA at 30°C, in the presence of ATP. CAK (vizualized by Cyclin H) was not released from TFIIH over the time (Figure 3B, lanes 1–4). Addition of recombinant XPA WT catalyzed not only the release of CAK but also the recruitment of the repair factors on the DNA (lanes 5–8).

In another set of experiments, highly purified recombinant XPC and TFIIH were assembled on the damaged DNA. The immobilized DNA was subsequently washed to remove nonspecifically bound proteins, and the reactions were supplemented with a combination of repair factors, as indicated, in the presence of ATP. Addition of XPA-WT to the DNA/XPC/TFIIH complex was

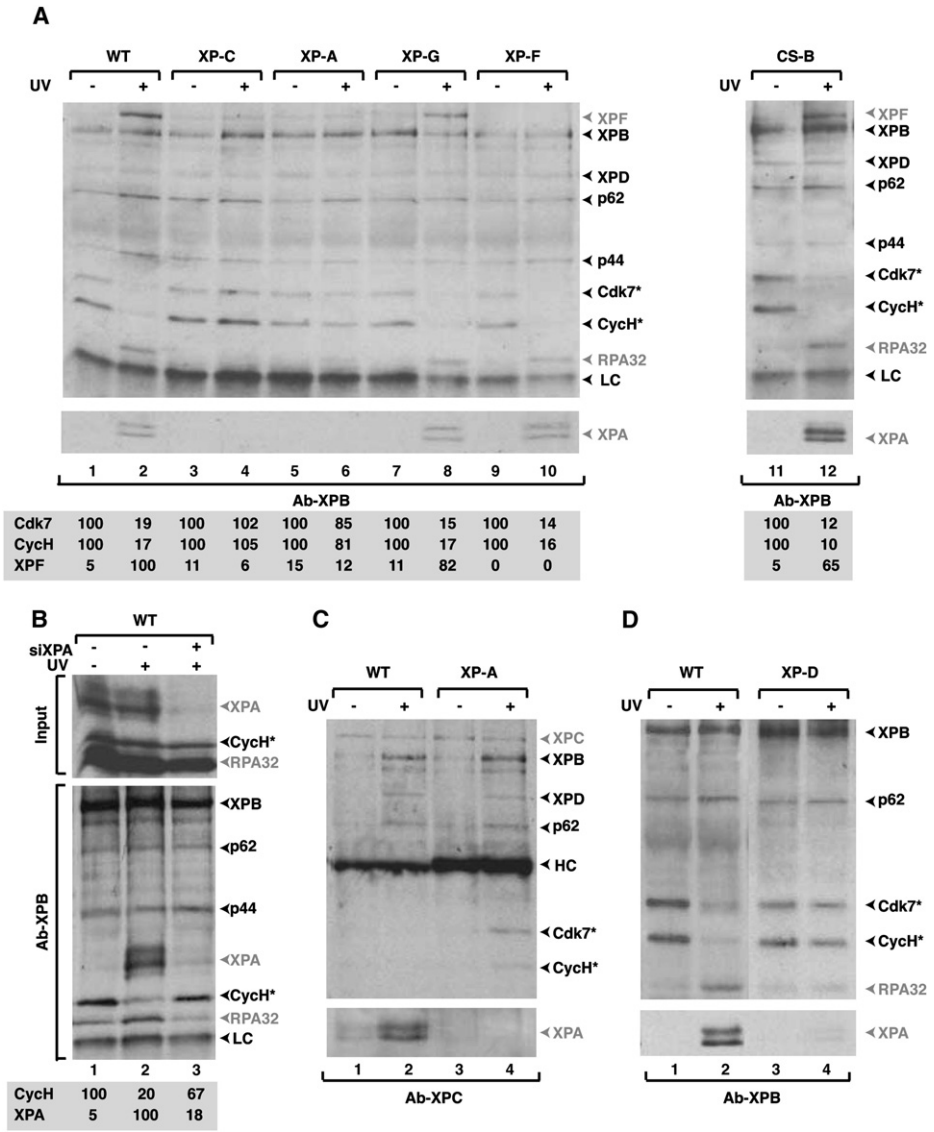

Figure 2. Analysis of the Release of the CAK Module in XP and CS Patient Cell Lines

(A) ChIP were performed with an Ab-XPB, 30 min after irradiation (20 J/m^2) of either WT (lanes 1 and 2), XP-C (GM14867; lanes 3 and 4), XP-A (XP12ROSV; lanes 5 and 6), XP-G (XPCS1RO; lanes 7 and 8), XP-F (GM08437; lanes 9 and 10), or CS-B (CS1ANSV; lanes 11 and 12) patient cell lines. LC indicates the light chain of the antibody. The percentage of CAK components that remain associated to TFIIH is shown; the amount of CAK in nonirradiated cells is considered 100%. For the quantification of XPF, the amount of XPF in wild-type irradiated cells was considered 100%.

(B) WT cells were transfected with siRNA toward XPA (siXPA). The chromatin inputs were analyzed by WB. ChIP were performed as in (A). For quantification, the amount of cyclinH in untreated cells was considered 100%, the amount of XPA in UV-treated cell was considered 100%. XPB was used as reference.

(C) ChIP was performed as in panel (A) with an Ab-XPC in the chromatin of either WT (lanes 1 and 2) or XP-A (lanes 3 and 4) cells.

(D) ChIP were performed as in (A) with chromatin of either WT (HeLa; lanes 1 and 2) or XP-D (HD2; lanes 3 and 4) cells.

sufficient to induce a time-dependent release of CAK (Figure 3C, cf. lanes 1 and 2 with 3 and 4) that was not observed following addition of RPA, XPG, and XPF (cf. lanes 1 and 2 with 5 and 6). Our in vitro data confirm that XPA catalyzes the dissociation of CAK from TFIIH during NER.

Release of CAK Drives NER

We then sought to identify which domain of XPA mediated the release of CAK from the core TFIIH. We designed constructs expressing XPA-WT and XPA(1-228) polypeptides (Figure 4A), knowing that TFIIH interacts with the carboxy-terminal part of XPA (Park et al., 1995; Nocentini et al., 1997). Equal amounts of purified XPA-WT and XPA(1-228) were incubated over time, with an XP-A NE and the damaged DNA. Contrary to XPA-WT, the truncated XPA(1-228) was unable either to catalyze the release of CAK (visualized by Cyclin H) from the core TFIIH or to join the preincision repair complex (Figure 4B).

XPA-WT and XPA(1-228) were next incubated in a dual incision assay containing either rIIH7 (the core TFIIH) or rIIH10 (the entire TFIIH, including CAK; Figure 4C, left panel). In the presence of XPA-WT, a similar dual incision activity was observed with either rIIH7 or rIIH10 (lanes 2 and 5). Dual incision was barely detectable with XPA(1-228) in the presence of rIIH10 but was clearly observed with rIIH7 (Figure 4C, right panel, lanes 3 and 6), although to a lower extent compared with XPA-WT.

Together, our data indicate that the detachment of the CAK from the core of TFIIH, mediated by the C-terminal part of XPA, drives dual incision.

Functional NER in CAK Knockdown Cells

The question remained as to whether CAK plays a role in NER before it is released from the preincision complex. Specific siRNA duplexes (siCdk7) were used to reduce endogenous Cdk7 expression in WT cells. Confocal microscopy revealed that siCdk7 transfected cells had a marked reduction in the amount of Cdk7 and Cyclin H as compared with either the untransfected or control siRNA (siCt)-treated cells (Figures 5Ab and 5Ae). In contrast, the amount of either XPB (Figures 5Aa and 5Ad) or p62 (data not shown) was not affected. Immunoprecipitation experiments performed with Ab-XPB showed the presence and the stability of the core TFIIH even in the absence of CAK (Figure 5B). To check for the efficiency of Cdk7 knockdown, we investigated the transcriptional response of UV-irradiated cells. Quantitative RT-PCR showed that the transactivation of the three UV-inducible genes *p21*, *Mdm2*, and *ATF3* (Fan et al., 2002) was significantly reduced in siCdk7-treated cells compared with siCt-treated cells (Table 1).

Next, the amount of 6-4PP lesions was measured in vivo using confocal microscopy (Mori et al., 1991), 4 hr after irradiation of either siCdk7- or siCt-treated cells. SiCdk7-treated cells (blue fluorescent latex beads) were fully capable of removing the 6-4PP lesions (Figures 5Cb and 5Ce), compared with either the repair-proficient siCt transfected (no beads) or repair-deficient XP-A (green fluorescent latex beads) cells (Figures 5Ca and 5Cd). Using an immuno-dot blot assay (Riou et al., 2004), we measured the amount of 6-4PP lesions remaining in the DNA at different time points after UV and observed identical repair activity in siCdk7- and siCt-treated cells (Figure 5D). Our data suggests that 6-4PP lesions are repaired regardless of the presence of CAK in vivo.

Return of CAK after DNA Repair

We also investigated the fate of TFIIH after DNA repair. ChIP assay using Ab-XPB established that the level of CAK in WT cells was shifted back to that of nonirradiated cells within 8 hr after UV (Figure 6A, upper panel). A progressive release of the NER factors was also observed, together with the recovery of a whole TFIIH. In parallel, we investigated RNA synthesis recovery by [^{3}H]uridine incorporation performed after UV. The kinetics of inhibition of transcription followed both the leaving of CAK from TFIIH and the coming of the NER factors. The resumption of transcription began at 4 hr and was complete at 8 hr, when both the CAK reassociated with TFIIH and the NER factors were released (Figure 6A, lower panel). The recovery of transcription started after the complete removal of 6-4PP lesions measured in Figure 5D.

Hereafter, we focused on CS-B, XP-G, and XP-F cell lines for which a release of CAK had been observed (Figure 2). In TCR-deficient CS-B cells, a whole TFIIH complex is present in the chromatin 8 hr after UV. Concomitantly, XPF, XPA, and RPA dissociated from TFIIH in a time course similar to that observed in WT cells (Figure 6B, WB and bottom graph). These data indicate that the restoration of TFIIH pattern is CSB independent. As previously observed (Mayne and Lehmann, 1982), RNA synthesis was not restored in CS-B cells because of the involvement of CSB in transcription (Proietti-De-Santis et al., 2006). In XP-G cells, the XPA, RPA, and XPF factors were dissociated from the core TFIIH in a time course similar to that of WT or CS-B cells (Figure 6C). However, we observed neither the return of a whole TFIIH complex on the chromatin nor the recovery of the transcription. A slightly different situation was obtained in XP-F cells, where XPA and RPA repair factors remained associated with the core TFIIH over time after treatment (Figure 6D). Collectively, these data suggest that the return of a whole TFIIH to the chromatin occurs once the removal of the DNA lesions is complete.

DISCUSSION

Dynamic State of the Human TFIIH Complex

Considerable efforts have been invested in the dissection of the molecular events that lead to the elimination of DNA lesions by the NER pathway. However, detailed information on the composition of the various complexes that participate in the repair process has been lacking. We were interested in understanding how the 10 subunits that constitute the TFIIH complex participate in the NER and/or transcription processes. In the present study, we have found that the composition of TFIIH changes according to various cellular responses. We have observed a dynamic dissociation/reassociation of the CAK complex, a crucial transcriptional module (Tirode et al., 1999), onto the core TFIIH that coincided with the recruitment/release of the NER factors during the DNA repair reaction. Thus, our findings dispel the broadly accepted idea that TFIIH is a stable complex without large-scale alterations in composition when switching between different cellular processes (Hoogstraten et al., 2002).

The release of the CAK from TFIIH was also observed when classical fractionations of the soluble fractions were performed in the absence of formaldehyde crosslinking. However, the dose of UV required to dissociate the CAK in this condition (100 J/m^2) was higher than the dose needed to detect such dissociation by ChIP (10 J/m^2). Also, though repair factors accumulate on the core TFIIH by ChIP, no accumulation was observed on the TFIIH in the soluble fraction after UV. These findings pinpoint the fragility of the repairosome complex in human cells and the difficulties in isolating this complex by traditional methods. They also explain why such dissociation was not observed in high eukaryotes until now.

The accumulation of NER factors with the core TFIIH depends on both the UV dose and the recovery time after UV exposure. It reached a maximum 15 min after UV treatment at 20 J/m^2 and occurred when cells were incubated at 37°C and not when they were kept at 4°C. In these conditions, we determined that

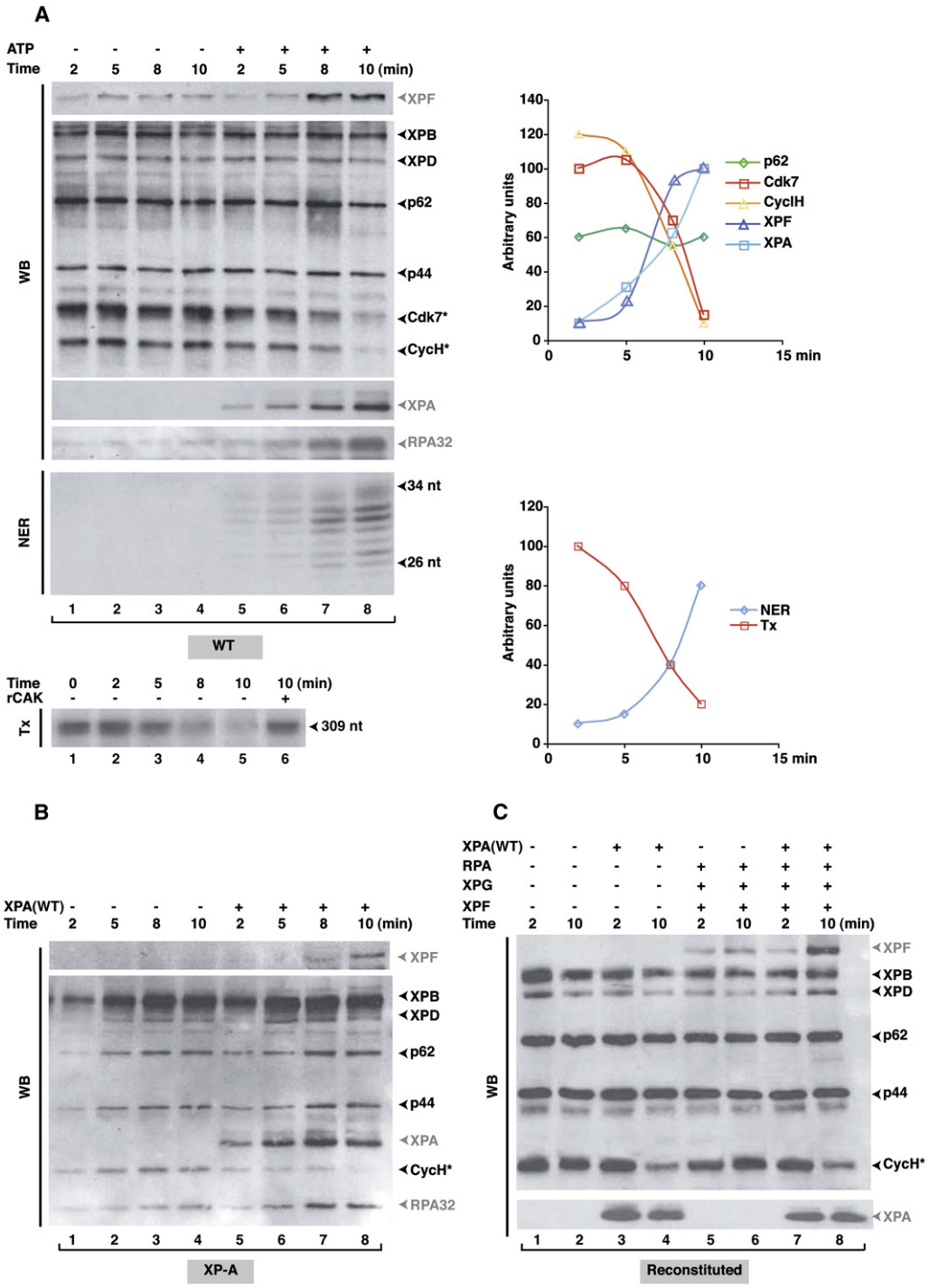

Figure 3. Progress of the CAK Release In Vitro

(A) HeLa cell extract (wild-type) was incubated 15 min at 4°C with damaged DNA coupled to magnetic beads. Reactions were then supplemented with ATP (lanes 6–9) and incubated at 30°C over the time indicated. After washes, aliquots were analyzed either by western blotting (WB) or in a dual-incision assay (NER). For transcription (Tx), reactions were allowed to proceed as described, and following washes at different times points as indicated, the magnetic beads were incubated in a reconstituted transcription assay containing an adenovirus major late promoter (AdMLP) template and the RNA pol II, TFIIA, TFIIB, TBP, TFIIE, TFIIF transcription factors (Gerard et al., 1991). mRNA products (309 nt) are visualized by the incorporation of radioactive nucleotides. In lane 6, 10 ng of recombinant CAK was added to the reaction. Signals for WB or NER and Tx were quantified and spotted on graphs. For WB, XPB was used as reference.

(B) XP-A cell extract (XP12ROSV) was used as in panel (A) with ATP and 50 ng of recombinant XPA-WT protein when indicated. After washes, aliquots were analyzed by western blotting.

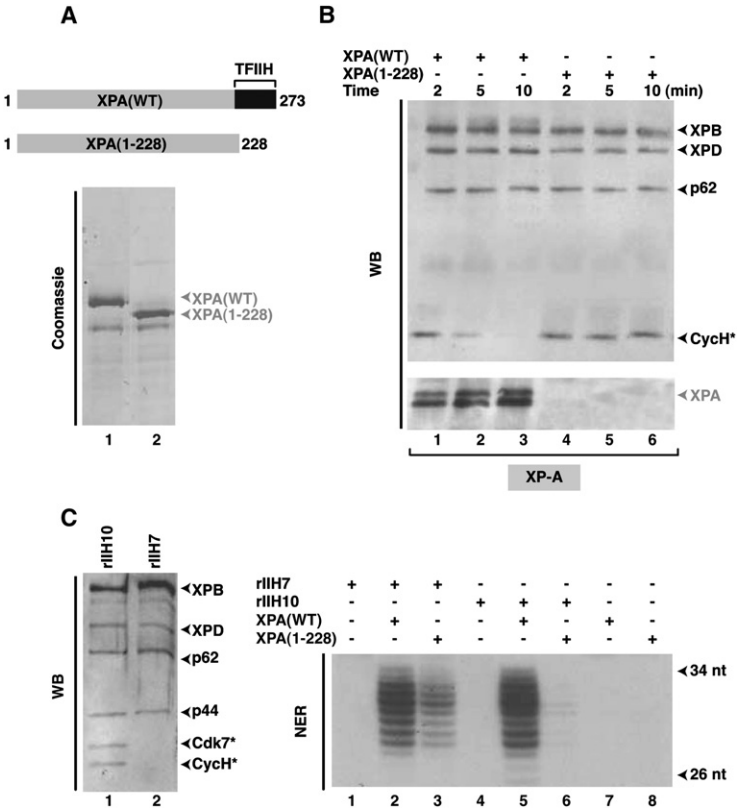

Figure 4. Release of the CAK Initiates DNA Repair

(A) Upper panel: Schematic representation of XPA-WT and XPA (1-228). The dark gray box indicates the TFIIH interacting domain. Lower panel: Coomassie staining of XPA-WT and XPA(1-228).

(B) XP-A cell extract was incubated as in Figure 3A, in the presence of ATP and 50 ng of recombinant XPA-WT or (1-228) polypeptides when indicated. After washes, aliquots were analyzed by western blotting (WB).

(C) Left panel: Purified recombinant TFIIH complexes were expressed in baculovirus infected cells with (rIIH10) or without (rIIH7) the CAK subcomplex and purified as described (Tirode et al., 1999). Complexes were resolved by SDS-PAGE and western blotted with TFIIH antibodies. rIIH7 (lanes 1–3) and rIIH10 (4–6) were added in a dual-incision assay containing XPC, RPA, XPG, and XPF, in the presence of either XPA-WT (lanes 2, 5, and 7) or XPA(1-228; lanes 3, 6, and 8).

~70% of the TFIIH complexes are missing the CAK and are involved in repair. That the remodeling of the complex is temperature dependent indicates that it is elicited by a cellular response to UV damage and not by a crosslinking effect of UV or by damaging the TFIIH complex itself. In addition, the synchronization between the release of the CAK from the core TFIIH and the arrival of the NER factors suggests that the change in TFIIH subunit composition is due to its participation in the removal of 6-4PP lesions that are repaired in the first hours after UV, and not in the removal of CPD lesions that takes place later (van Hoffen et al., 1995). This theory is strengthened by our finding that the release of CAK was impaired in XP-C cells in which TFIIH is not recruited to the damaged DNA (Volker et al., 2001). That the CAK is released and NER factors accumulate with the core TFIIH in CS-B but not in XP-C cells strongly suggests that the modifications we observed take place during GGR. In accordance with these findings, the release of the CAK was observed when cells were treated with actinomycin D or H8, two inhibitors of transcription (data not shown). It suggests that under our experimental condition, the TCR of UV-induced lesions attracts insufficient numbers of NER proteins to allow visualization of the incision complexes in XP-C cells, but it does not rule out the possibility that the CAK is detached from the core TFIIH during TCR. The reproducibility of our findings with the siRNA or XP cell lines validates the ChIP/western technology and its use in analyzing the composition of TFIIH after a cellular perturbation.

From a mechanistic standpoint, we assume that the CAK is detached from the core and not degraded because proteasome inhibitors did not alter the dynamic composition of TFIIH upon irradiation in vivo. Moreover, the disappearance of CAK from the core TFIIH engaged in NER was reconstituted in vitro, in a system that does not support active protein degradation. Unexpectedly, we observed that the NER factors are already present in the chromatin of untreated cells and are sequentially reallocated to the site of damage after irradiation to form intermediate repair complexes during the NER reaction (Figure 7). Notably, ChIP assays have shown several protein complexes containing TFIIH at different time points after UV. Before treatment, we observed a complex with TFIIH alone (or presumably present in heterogeneous transcription preinitiation complexes). Then, just after irradiation, a complex containing TFIIH and XPC was detected. Thirty minutes after irradiation, a complex containing only the core TFIIH in addition to XPA, RPA, XPF, and probably XPG was found on the chromatin. Finally, 8 hr after irradiation, when most of the 6-4PP lesions are removed, Ab-XPB captures a TFIIH complex that contains the CAK but that lacks all the NER factors. In a broader perspective, the findings presented here fully support the scenario of a sequential assembly of the repair factors to the damaged DNA, guided at each step by numerous protein-protein interactions (Riedl et al., 2003). However, contradictory findings remain about which factor initiates the subsequent recruitment of all NER proteins to the preincision complex, as both protein complexes of XPC-hHR23B and XPA-RPA have been proposed to play a key role in the first step of DNA damage recognition (Kesseler et al., 2007). Our ChIP experiments show that UV irradiation initiates the formation of a XPC/TFIIH complex in the chromatin in the absence of XPA, whereas a TFIIH/XPA complex is not detected without XPC. These findings suggest that XPC initiates the assembly of the preincision complex,

(C) Recombinant XPC and TFIIH were incubated 15 min at 4°C with damaged DNA coupled to magnetic beads. After washes, highly purified recombinant NER factors were added to the reaction mixture as indicated in the presence of ATP, and the reactions were allowed to proceed for 2 min or 10 min at 30°C. After a second wash, aliquots were analyzed by western blotting.

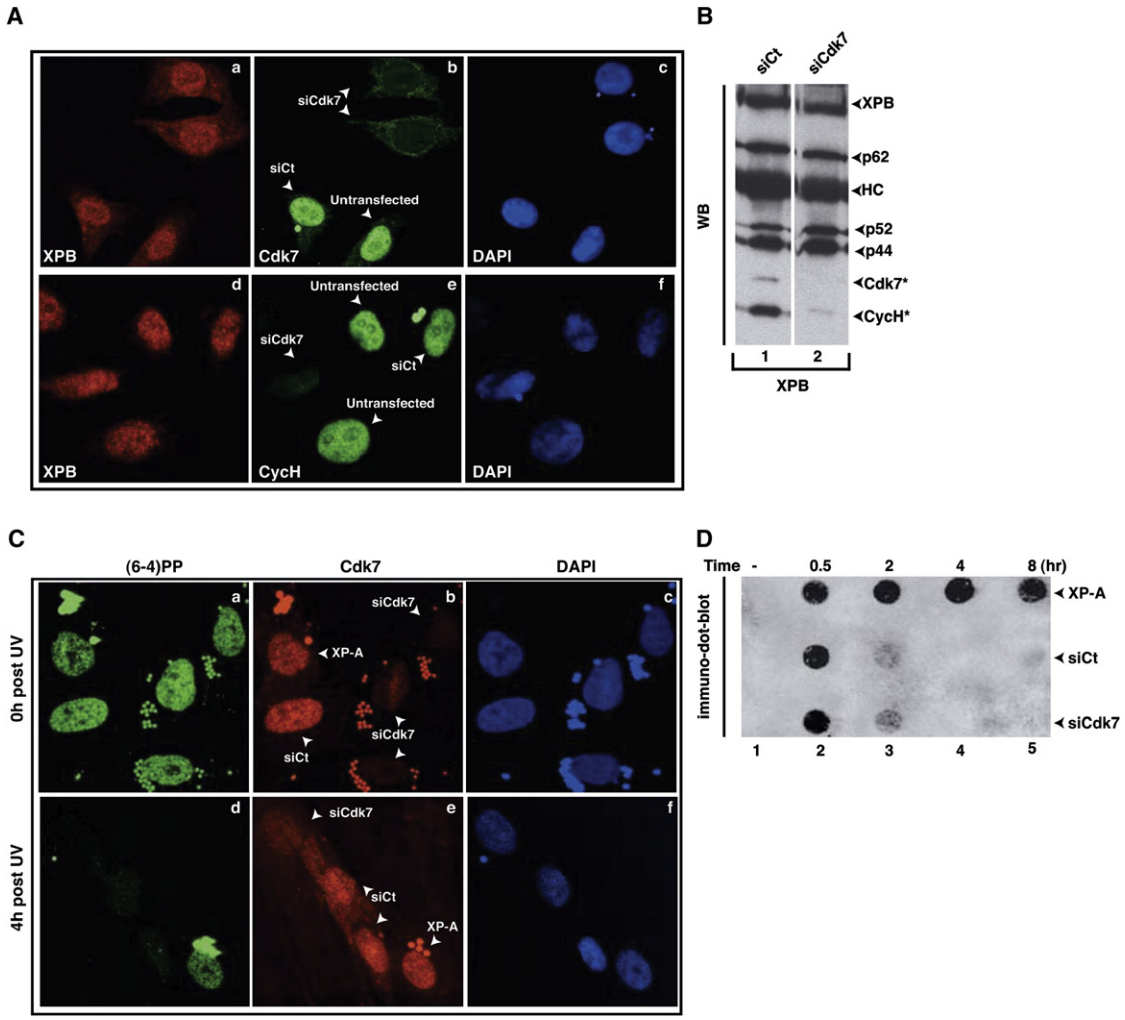

Figure 5. NER Takes Place without CAK Backup In Vivo

(A) siCdk7-treated WT cells prelabeled with small blue fluorescent latex beads, siCt-treated WT cells prelabeled with large green fluorescent latex beads, and untransfected WT cells (no beads) were plated on the same slide and analyzed by confocal microscopy using Ab-XPB (Aa and Ad), Ab-Cdk7 (Ab), or Ab-Cyclin H antibodies (Ae).

(B) WT cells were transfected either with a pool of siRNA oligonucleotides against Cdk7 (siCdk7) or with control siRNA (siCt). TFIIH from 200 μg of total extracts was immunoprecipitated with Ab-XPB and resolved by SDS-PAGE followed by western blotting. HC indicates heavy chain of the Ab.

(C) Blue fluorescent-labeled WT cells were transfected with siCdk7, and WT with no beads were transfected with siCt. Twenty-four hours later, these cells were plated on the same slide with green fluorescent-labeled XP-A cells. Forty-eight hours posttransfection, cells were irradiated with 10 J/m^2, and repair was measured after 4 hr of recovery by confocal microscopy using Ab-(6-4)PP (directed against the 6-4PP lesions; Ca and Cd) and Ab-Cdk7 (Cb and Ce).

(D) Immuno-dot blot experiments were carried out using Ab-(6-4)PP antibody with 500 ng of genomic DNA samples of siCdk7, siCt, or XP-A cells irradiated at 10 J/m^2. Post-UV recovery time is indicated. Lane 1 contains 500 ng of nonirradiated genomic DNA.

thereby supporting the conclusion that this protein is the earliest known NER factor to join the lesion.

Recruitment of XPA: A Checkpoint in NER

The question remains as to how and when the CAK is released from the core TFIIH. ChIP assays and the in vitro reconstituted incision system provide evidence that the detachment of the CAK from the core TFIIH is catalyzed by XPA, in the presence of ATP. This statement is based on the observations that mutations in either XPC or TFIIH, two repair factors that are recruited to the damaged DNA before XPA (Volker et al., 2001; Riedl et al., 2003), hinder the CAK release. The release of the CAK in an XP-A cell that does not express the XPA protein was barely observable. In contrast, the release of the CAK occurred in cell lines mutated in either XPG or XPF, two proteins that are recruited after XPA. Additionally, the detachment of the CAK from the core TFIIH was hardly detectable in a reconstituted in vitro system containing XPC, TFIIH, and a damaged DNA unless XPA and ATP were added to the reaction. In vitro studies have further revealed that the C-terminal region of XPA that interacts with TFIIH (Park et al., 1995) is required for the release of the CAK. The consequence of the detachment of the CAK

Table 1. Impaired UV-Dependent Transactivation of *p21*, *Mdm2*, and *ATF3* Genes in siCdk7-Treated Cells

Gene	siCt	siCdk7
p21	16.65 ± 1.0	5.61 ± 0.5
Mdm2	8.02 ± 0.5	2.30 ± 0.6
ATF3	22.29 ± 3.6	3.33 ± 1.0

RT-PCR analysis was performed 12 hr after UV irradiation (30 J/m^2) of siCt- or siCdk7-treated cells. The findings are expressed as fold induction (mRNA UV[+]/mRNA UV[−]) and are the mean of three independent experiments. *Mdm2*, murine double minute2; *ATF3*, activating transcription factor 3.

from the core TFIIH is the initiation of the incision/excision step of NER. The C-terminal truncated XPA, which was almost inactive in the presence of a whole TFIIH, catalyzed dual incision in the presence of a TFIIH lacking CAK. We thus propose that the accurate recruitment of XPA constitutes a major checkpoint in NER that will accelerate, in the appropriate situation, the removal of the damaged DNA by separating the CAK from the core TFIIH. This pinpoints one of the key roles of XPA, a scaffold protein that has no enzymatic activity on its own but is nevertheless indispensable for DNA incision.

How can the CAK be an obstacle to DNA repair? Strikingly, CAK was shown to negatively regulate the XPD DNA unwinding activity (Sandrock and Egly, 2001). It has also been established that the recruitment of XPA to the XPC/TFIIH intermediate preincision complex led to a full opening of the damaged DNA (Tapias et al., 2004; Andressoo et al., 2006). These observations suggest that the detachment of the CAK from the core may stimulate TFIIH helicase/ATPase activities, thereby accelerating the enlargement and the stabilization of the DNA-opened structure. Supporting this model, XPA has been shown to stimulate the ATPase activity of TFIIH specifically in the presence of damaged DNA (Winkler et al., 2001). Alternatively, it has been shown that CAK can negatively regulate NER by phosphorylation of one or more components of the reaction (Araujo et al., 2000). Even if the target(s) of Cdk7 in NER is

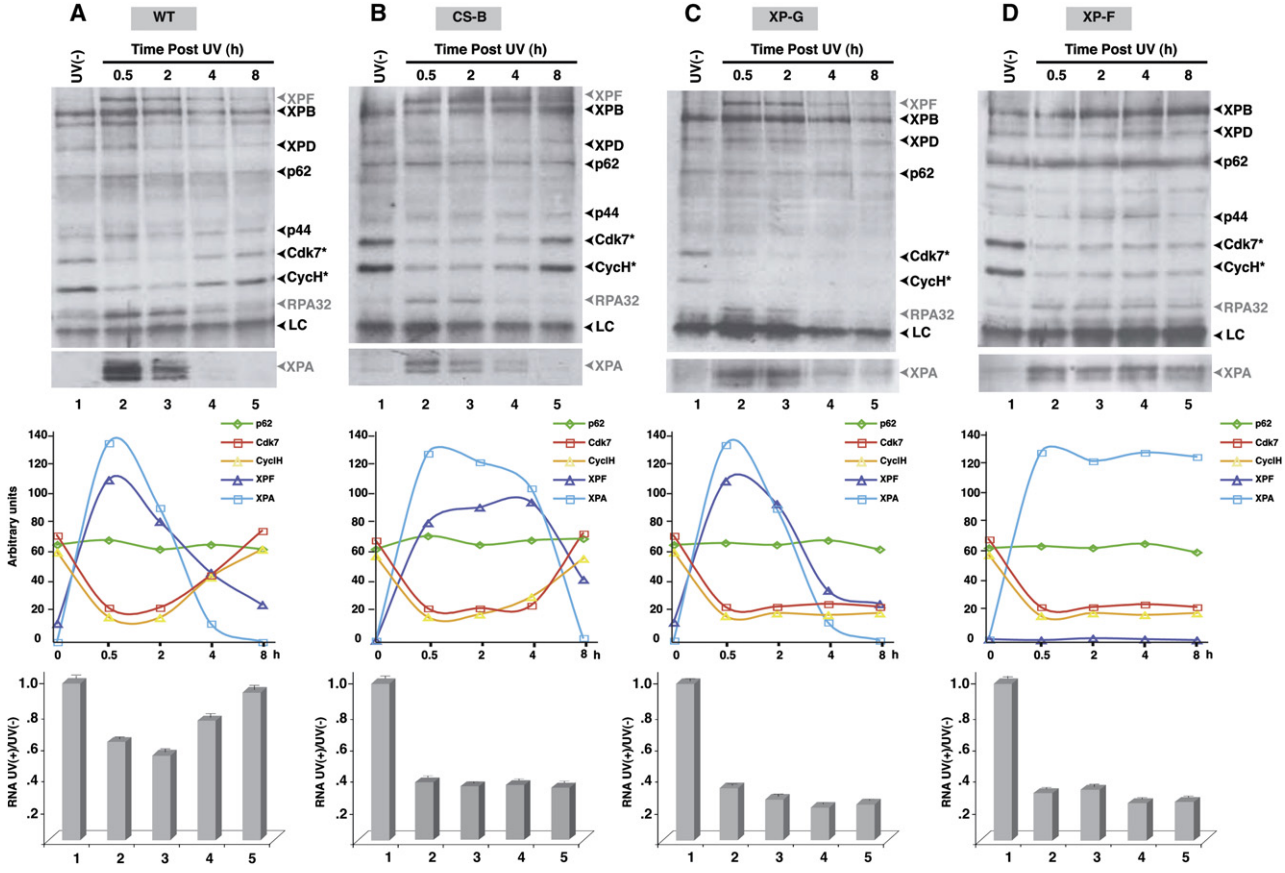

Figure 6. Return of the CAK in the TFIIH Complex

ChIP over the time of repair: ChIP with Ab-XPB was performed on (A) wild-type, (B) CS-B, (C) XP-G, and (D) XP-F cells incubated for different times after UV (20 J/m^2) as indicated. RNA synthesis recovery was also measured in each cell line. After prelabeling with [^{14}C]thymidine (0.02 μCi/ml) for 2 days, nonirradiated or UV (20 J/m^2)-irradiated cells were pulse labeled for 30 min with [3H] uridine at different time points after irradiation, and radioactivity was determined. The findings represent the relative RNA synthesis measured at different time points after UV compared with nonirradiated cells. The WB signals for XPF, XPB, p62, cyclH, Cdk7, and XPA were quantified using Genetool (Syngene) and plotted on the graphs. For each single lane, XPB was used as reference. Mean value and standard deviation of three independent experiments are shown.

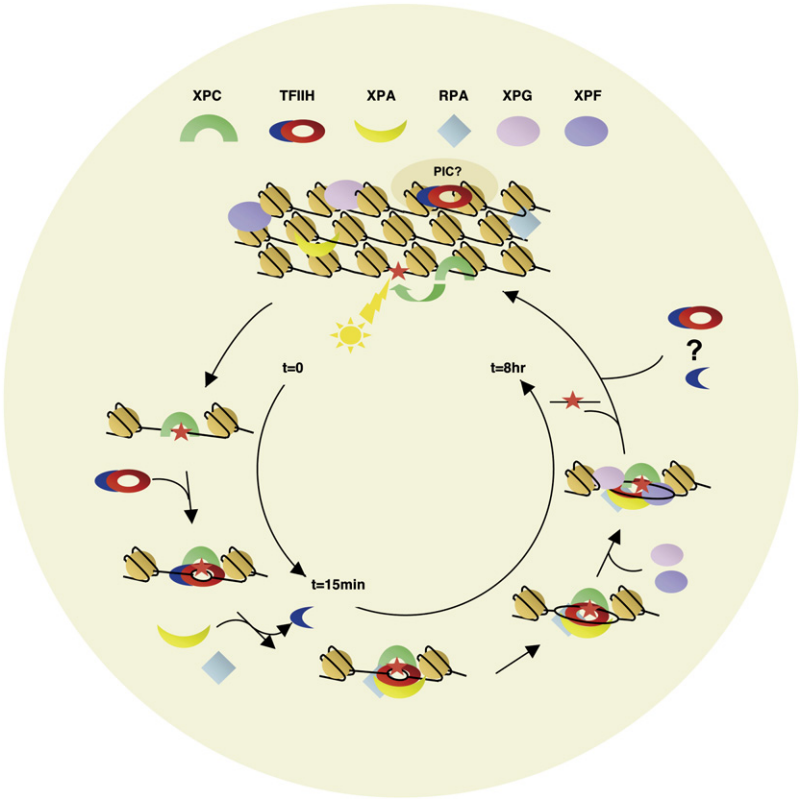

Figure 7. Model for the Assembly of the Human NER Incision Complex on the Chromatin

Schematic representation depicting the changes in the composition of TFIIH after a genotoxic attack. The transcription factor TFIIH (the core in red and the CAK in blue) is probably included in preinitiation complexes (PIC) containing either RNA pol I or II. Following irradiation and the formation of a lesion in the DNA (red star), the NER factors reallocate to the site of damage in sequential order. XPC and a whole TFIIH are recruited to the lesion, and a preopening of the damaged DNA is initiated. The arrival of XPA drives the release of the CAK within 15 min after the irradiation and promotes a full opening of the DNA around the damage to favor the binding of XPG and XPF. Following removal of the damage, a whole TFIIH is found in the chromatin, but whether it is a free CAK that reassociates with a core TFIIH formerly involved in NER, or a new whole TFIIH complex that is recruited to the chromatin, remains to be established. In our experimental conditions, the process of dissociation/reassociation of CAK to TFIIH takes 8 hr.

not known, the release of the CAK from the core TFIIH is a possible mechanism to eliminate the kinase from the preincision complex, thereby separating physically the enzyme and its substrate.

TFIIH: Two Sides of the Same Coin

Finally, our study reconciles several divergent observations obtained on the composition of TFIIH from studies in yeast and human models. In yeast extracts, two TFIIH-containing complexes with different principal tasks were characterized; the first form contains the core TFIIH and TFIIK (the homolog of CAK) and works mainly in transcription, whereas the second one contains only the core and is specifically involved in NER (Svejstrup et al., 1995). However, total extracts from human cells do not contain any free core TFIIH (unpublished data; Araujo et al., 2001). In light of our findings, an intriguing picture emerges that differentiates the yeast and human TFIIH complexes; in human, the free core TFIIH is transient and exists only during the short period of DNA repair. This repair-specific core TFIIH disappears when lesions are removed and transcription is ready to resume. We observed that the departure of the repair factors and the return of the CAK to the TFIIH complex are concomitant with the recovery of transcription in WT cells. It cannot be ruled out that the difference between yeast and human also indicates that yeast repair factors have a stronger intrinsic affinity for each other and for the core TFIIH than they do in man. In mammals, it remains to be established whether it is a free CAK that comes back to a core TFIIH formerly involved in NER, or if it is a new TFIIH complex that is recruited to the chromatin (Figure 7). Mutations in NER proteins cause an accumulation of intermediate repair complexes that can persist for several hours on the chromatin. Whether the persistence of these intermediate "poised" complexes are responsible for some of the phenotypes displayed by patients merits further investigation.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation

Chromatin was prepared as described in Fousteri et al. (2006). The chromatin suspension was sonicated on ice (3 × 15 min) on buffer S (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS) using a Diagenode (Liège, Belgium) Bioruptor (power setting 5) in 10 s pulse followed by 20 s of cooling. Samples were spun down (13,200 rpm, 15 min), and the supernatant that contained the crosslinked chromatin was stored at −80°C.

In each assay, 400 μg of protein from crosslinked chromatin was immunoprecipitated with 1 μg of antibody in buffer S, 2 hr at 4°C. The immunocomplexes were collected by adsorption to protein G Sepharose beads (Upstate; Billerica, MA) overnight at 4°C. The beads were next washed three times with 5 vol of buffer S and resuspended in 1× Laemli SDS buffer. Samples were incubated at 95°C for 90 min for decrosslinking prior electrophoresis.

In re-ChIP assay, 1.2 mg of protein from crosslinked chromatin extracted was immunoprecipitated with 6 μg of specific antibody as described, and fractions were eluted with 10 mM dithiothreitol at 37°C for 30 min. After centrifugation, the supernatant was diluted 10 times with buffer S and subjected to another round of immunoprecipitation.

RNA Interference

A pool of four RNA oligonucleotides (Dharmacon; Waltham, MA) forming a 19-base duplex core, specifically designed to target Cdk7 or XPA mRNA, was transfected in cells at the concentration of 50 nM. A pool of RNA oligonucleotides, without any target mRNA, was used as control. RNA transfection was performed by using Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Specific target reduction was analyzed 48 hr posttransfection by western blotting.

Protein Binding Studies on Immobilized DNA

Dynabeads M-280 Streptavidin (Dynal; Carlsbad, CA) coupled to DNA were incubated in blocking buffer (10 mM HEPES, 100 mM glutamate, 10 mM MgOAc, 5 mM EGTA, 3.5% glycerol, 60 mg/ml casein, 5 mg/ml PVP, and 2.5 mM DTT), 15 min at room temperature to limit unspecific binding of proteins (Ranish et al., 2004). Immobilized DNA was then incubated in incision buffer (50 mM HEPES-OH [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) with recombinant XPC-hHR23B and HeLa purified or recombinant TFIIH at 4°C, 15 min. Upon incubation, beads were collected on a magnetic particle concentrator (Dynal MPC) and supernatants removed. Beads were then washed five times in 4 vol of cold incision buffer and resuspended in the same buffer with XPA, RPA, XPG, and ERCC1-XPF with 2 mM ATP as indicated. Following incubation at different times, beads were washed five times in 4 vol of cold incision buffer and resuspended in 1× Laemli SDS buffer for electrophoresis.

Determination of RNA Synthesis after UV Irradiation

Cells in exponential phase were grown in the presence of [^{14}C]thymidine (0.02mCi/ml) for 2 days to uniformly label the DNA. The UV-irradiated cells (10 J/m^2) were pulse labeled with 5 μCi/ml of [^{3}H]uridine for 30 min at different times. The cells were collected and washed once with ice-cold PBS and lysed in buffer containing 0.5% SDS and 100 μg/ml proteinase K for 2 hr at 37°C. After trichloroacetic acid precipitation (10% TCA), the samples were spotted onto glass fiber discs (Whatman; Maidstone, Kent, UK); the filters were next sequentially washed in 5% TCA, 70% ethanol/acetone, and counted for their radioactivity. The ^{3}H/^{14}C ratio was taken as a measure of RNA synthesis.

Dual Incision and Transcription Assays

Dual incision and transcription assays were carried out as described (Coin et al., 2006).

Protein Purification

Recombinant TFIIH complexes were purified as described (Tirode et al., 1999). XPA-WT and (1-228) were expressed in *E. coli* and purified using a N-terminal GST tag.

Reverse Transcription and Real-Time Quantitative PCR

cDNA synthesis was performed by using random hexanucleotides and AMV reverse transcriptase (Sigma; St. Louis, MO). Real-time quantitative PCR was done with the FastStart DNA Master SYBR Green kit and the Lightcycler apparatus (Roche Diagnostic; Basel, Switzerland). Primer sequences are available upon request.

Antibodies

Primary antibodies used in ChIP were rabbit IgG polyclonal anti-XPB (S-19), rabbit IgG polyclonal anti-XPA (FL-273, Santa Cruz Biotechnology; Santa Cruz, CA), and rabbit IgG polyclonal anti-XPC (Riedl et al., 2003). Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labeling were mouse IgG monoclonal anti-6-4 (64M2; 1:2000) (MBL International Corp.) and mouse IgG monoclonal anti-Cdk7 (2F8) (Coin et al., 2006). Secondary antibodies used in this study were Alexa 488 anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG.

SUPPLEMENTAL DATA

Supplemental Data include two figures and are available at http://www.molecule.org/cgi/content/full/31/1/9/DC1/.

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