Mechanism of open complex and dual incision formation by human nucleotide excision repair factors

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During nucleotide excision repair in human cells, a damaged DNA strand is cleaved by two endonucleases, XPG on the 3' side of the lesion and ERCC1-XPF on the 5' side. These structure-specific enzymes act at junctions between duplex and single-stranded DNA. ATP-dependent formation of an open DNA structure of ~25 nt around the adduct precedes this dual incision. We investigated the mechanism of open complex formation and find that mutations in XPB or XPD, the DNA helicase subunits of the transcription and repair factor TFIIH, can completely prevent opening and dual incision in cell-free extracts. A deficiency in XPC protein also prevents opening. The absence of RPA, XPA or XPG activities leads to an intermediate level of strand separation. In contrast, XPF or ERCC1-defective extracts open normally and generate a 3' incision, but fail to form the 5' incision. This same repair defect was observed in extracts from human xeroderma pigmentosum cells with an alteration in the C-terminal domain of XPB, suggesting that XPB has an additional role in facilitating 5' incision by ERCC1-XPF nuclease. These data support a mechanism in which TFIIH-associated helicase activity and XPC protein catalyze initial formation of the key open intermediate, with full extension to the cleavage sites promoted by the other core nucleotide excision repair factors. Opening is followed by dual incision, with the 3' cleavage made first.

Keywords: cisplatin/DNA repair/nucleases/TFIIH/xeroderma pigmentosum

Introduction

The dual incision reaction during human nucleotide excision repair (NER) involves cleavage 3' and 5' to a lesion in the damaged DNA strand by the XPG protein and ERCC1-XPF protein complex respectively. These are structure-specific endonucleases, cleaving at junctions between duplex and single-stranded DNA with a specific polarity, resulting in excision of 24–32-mer oligonucleotides containing the damage (Mu *et al.*, 1996; Sijbers *et al.*, 1996). Reconstitution of the incision reaction using purified mammalian repair proteins demonstrated that the proteins required include XPA, RPA, XPC-hHR23B, the TFIIH complex (including XPB and XPD) and the XPG

and ERCC1-XPF nucleases (Aboussekhra *et al.*, 1995; Moggs *et al.*, 1996; Mu *et al.*, 1996). Biochemical studies of these proteins have given clues to their roles in the reaction, but the mechanistic steps leading to the formation of 3' and 5' incisions are unclear. For instance, XPA, RPA and XPC-hHR23B have been implicated as candidate damage recognition factors. Alternatively, these proteins may be involved in stabilizing single-stranded intermediates of the reaction, or displacing the damaged oligonucleotide.

TFIIH is a multisubunit factor that is required for basal transcription initiation at RNA polymerase II promoters, as well as in NER as part of the core incision machinery, even during repair of non-transcribed DNA (Svejstrup *et al.*, 1996). The importance of TFIIH during NER is highlighted by the fact that mutations in components of TFIIH can give rise to three inherited human disorders, xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD), with NER defects of different types (Hoeijmakers *et al.*, 1996). XP-B patients are very rare with two cases exhibiting combined XP and CS, while another has TTD symptoms. Patients within the XP-D complementation group are more frequent and exhibit either XP alone, or XP combined with CS or TTD. A distinct complementation group, TTD-A, is associated with an unidentified defect in TFIIH (Vermeulen *et al.*, 1994b).

The isolated XPB and XPD subunits of TFIIH are 3'–5' and 5'–3' DNA helicases respectively (Schaeffer *et al.*, 1993, 1994; Sung *et al.*, 1993; Ma *et al.*, 1994a), but in both transcription and NER they act only as part of TFIIH. In transcription, ATP-dependent TFIIH helicase activity is used to catalyze limited opening of a 10–20 bp region around the promoter which enables priming of the nascent RNA on the template strand (Holstege *et al.*, 1996). During repair, the DNA helicase activities of XPB and XPD have been postulated to catalyze a similar local unwinding around the site of a DNA lesion (Weeda *et al.*, 1990). TFIIH may also have a role in damage recognition, as suggested by the finding that the helicase activity of the XPD homolog in *Saccharomyces cerevisiae* (Rad3) is inhibited by bulky DNA lesions (Naegeli *et al.*, 1993). TFIIH-mediated opening could generate the junctions between duplex and single-stranded DNA which are cleaved by the XPG and ERCC1-XPF nucleases.

Direct evidence for the formation of an unwound open DNA intermediate prior to incision was recently obtained by chemical footprinting around the site of a single cisplatin-DNA adduct (Evans *et al.*, 1997). This reaction required XPA protein and was completely dependent on the presence of ATP. We have now carried out a comprehensive study to determine which core NER factors participate in the formation of this key open intermediate during the sequence of events that leads to dual incision

formation and removal of DNA damage. Of particular importance, we asked whether the TFIIH-associated DNA helicases are involved in this step. TFIIH was indeed found to be involved in opening, in a role that is likely to be similar to its function in initiation of RNA polymerase II transcription. We also uncovered the biochemical defect associated with a human mutation in XPB, which causes repair to be trapped at an unusual intermediate stage where opening and 3' incision can occur, but 5' incision does not take place.

Results

Dissection of open complex formation by the core NER factors

The experimental approach makes use of a 1,3-intrastrand d(GpTpG)-cisplatin crosslink located at a unique site in closed-circular duplex DNA. Mammalian cell extracts form incisions on each side of the adduct and release it in a 24 to 32-mer oligonucleotide segment. The reaction is monitored using a probe that simultaneously detects this dual incision, as well as any uncoupled 3' or 5' incisions that form (Figure 1A). During incubation of the adducted DNA with repair-proficient HeLa cell extract, dual incisions were detectable after 4 min and increased for ~15 min (Figure 1B and C). Uncoupled 3' incisions were detectable throughout the time course but few uncoupled 5' incisions were observed (Figure 1B and C). The coordinate appearance of 3' incisions and dual incisions indicates that the 3' and 5' incisions are nearly synchronous during the normal course of a reaction.

To monitor the formation of an open DNA intermediate around the lesion prior to strand scission, potassium permanganate footprinting is used. Linearized duplex DNA is terminally labelled with ³²P and incubated with cell extracts. Permanganate oxidizes T residues in regions with single-stranded character and results in sensitivity to piperidine cleavage. Analysis on a denaturing polyacrylamide gel yields a pattern of sensitive T residues on the adducted and complementary non-adducted strands. This defines a region of opening produced during the course of a repair reaction, summarized at the top of Figure 2A (Evans *et al.*, 1997). The 1,3-d(GpTpG)-cisplatin lesion itself causes some disruption of the duplex DNA structure, with an induced bend on the order of 25–30°, helical unwinding of about 23°, local denaturation that includes the central T residue and distortion over 4–5 base pairs (Anin and Leng, 1990; Bellon *et al.*, 1991; van Garderen and van Houte, 1994). This causes an intrinsic permanganate sensitivity of residues T[0], T[–2], T[–4], T[–5] and G[+1] which can be observed in the absence of cell extract (Figure 2A, lane 11).

In order to dissect the roles of the core NER factors in open complex formation, extracts defective in each of the factors have now been tested for their ability to promote the sensitivity of T residues surrounding the adduct to oxidation by permanganate. The adducted-strand residues T[+5], T[–2], T[–7], T[–8] and T[–10] are particularly informative in this regard, as they show the most conspicuous increases in sensitivity during DNA repair (Figure 2A).

XPG endonuclease and open complex formation

Permanganate sensitivity indicating opening around the cisplatin adduct was detected with an extract from XPG-defective XPG83 cells (Evans *et al.*, 1997). The causative mutation in this line is an amino acid change at a conserved residue in the presumed active site of XPG (Nouspikel and Clarkson, 1994). This suggested that the nuclease activity of XPG is not needed for opening up of the DNA prior to incision. In order to test the generality of this observation, we analyzed opening in cell extracts from other independent XP-G defective cell lines (Figure 2A and Figure 3) and all four tested gave similar results. In Figure 2A, DNA containing the cisplatin adduct (lanes 1–11), or non-adducted (lanes 12–15) was radiolabeled at the 3' end and reactions treated with KMnO₄ as indicated. On adducted DNA, each mutant extract displayed a pattern of KMnO₄-sensitive bands that included the sites T[0], T[–2], T[–4] and T[–5] which show intrinsic sensitivity (Figure 2A, lane 11). Active opening by the extracts also took place, as indicated by the hypersensitivity of T[–2], T[–7], T[–8] and T[–10] towards the 5' side of the lesion, and sensitivity of T[+5] towards the 3' side (lanes 3, 6 and 9). These extracts catalyze little or no 3' uncoupled incisions or dual incisions (not shown). In each case, the sensitivity of 5' T residues was slightly enhanced when XPG protein was included in the reaction to restore dual incision formation (Figure 2A, compare lanes 3 and 4, 6 and 7, 9 and 10). This is presented graphically in Figure 3 for the severely defective XP-G cell line AG08802 (Moriwaki *et al.*, 1996). Although the exact pattern varies somewhat between different mutant lines, it appears that significant opening takes place in the absence of XPG nuclease activity, but that addition of active XPG protein modulates the pre-incised DNA structure on the 5' side of damage. A small amount of full-length mutant XPG exists in some of these cells, leaving open the possibility that the mutant XPG may participate structurally but non-catalytically in formation of pre-incision complexes.

XPC and XPA participate in open complex formation

The XPC protein normally associates with hHR23B protein in human cells and is required for the repair of some DNA lesions *in vitro*, including major UV photoproducts. The repair of certain other lesions does not require XPC *in vitro* (Mu and Sancar, 1997), nor is this factor needed during transcription-coupled repair of UV lesions *in vivo* (van Hoften *et al.*, 1995). These observations have suggested that XPC may have a role in changing the DNA structure around lesions at an early stage of NER. Since XPC is needed for dual incision of the cisplatin adduct *in vitro* (Moggs *et al.*, 1996), we asked whether XPC participated in the opening reaction.

Figure 2B compares the pattern of permanganate-sensitive T residues formed by extracts from a repair deficient XP group C cell line (GM2249) and an XPA-defective line (GM2345; see Evans *et al.*, 1997). On damaged DNA, the pattern of KMnO₄-sensitive bands with both extracts (Figure 2B, lanes 4 and 6) included three sites (T[0], T[–4], T[–5]) that show sensitivity in the absence of extract (lane 8). The XP-C extract gave no detectable sensitivity of the flanking residues T[+5], T[–7], T[–8]

Fig. 1. The 3' and 5' incisions are tightly coupled (A) Schematic of hybridization assay to detect uncoupled and dual incisions. The products of dual incisions are detected as 24 to 32-mer platinated oligonucleotides. Uncoupled incisions at the 8th and 9th phosphodiester bonds 3' to the lesion are detected as platinated 48 and 49-mers. Incubation in extracts can lead to 3' to 5' degradation of these fragments up to the Pt crosslink, resulting in a band migrating at ~42 nt. Non-incised and fully repaired DNAs are detected as 84-mers. Uncoupled 5' incisions would result in bands of 61, 64 and 65 nt. (B) Time course of incision formation. Pt-GTG DNA was incubated with HeLa extract protein for the times indicated, and the DNA analyzed as in panel A. A phosphorimage of the gel is shown. The sizes of DNA fragments corresponding to uncoupled and dual incisions are indicated at the left. Lane M contains a 5' phosphorylated 24-mer containing a single 1,3 intrastrand d(GpTpG)-cisplatin crosslink. (C) Quantification of B. Values for the each incision product are displayed as a percentage of the total signal in each lane.

and T[−10], indicating a strong defect in opening around the lesion. Interestingly, T[−2], which is only weakly sensitive in the absence of extract, also did not become hypersensitive in the presence of GM2249 extract. This

is in contrast to extracts from repair proficient cells, or cells from most other XP groups (compare Figure 2B lanes 4, 6, 8 and the densitometric traces in Figure 3). This same pattern was observed in another XPC-defective

cell line, GM2246 (data not shown). Neither of these lines contain detectable XPC protein, suggesting that XPC protein is required for generating hypersensitivity at T[−2] in the vicinity of the platinum lesion. Addition of purified XPC-hHR23B to complement either cell extract restored dual incision and generated the full pattern of sensitive sites on the damaged strand extending from T[+5] to T[−10] (Figure 2B lane 5; Figure 3). As previously observed, XPA-defective extracts were also

defective in opening, although T[−2] remained hypersensitive and some residual sensitivity at T[−7], T[−8] and T[−10] occurred in the absence of complementation (compare the pattern of bands obtained with GM2345 extract to the BSA control in Figure 3). Thus, structural alteration of DNA immediately around the lesion is strongly dependent on functional XPC protein, and further opening extending toward the incision sites depends on the presence of both XPA and XPC.

Fig. 2. Opening of DNA around a Pt-GTG crosslink during repair depends on core NER factors. **(A)** KMnO₄ modification in XPG-defective extracts. Pt-GTG DNA (lanes 1–11) or non-adducted DNA (lanes 12–15) was 3' end-labeled and incubated with 100 µg HeLa cell extract protein (H), 200 µg XP-G extract protein or 100 µg BSA (B) as indicated. Reactions in lanes 4, 7, 10 and 14 also contained 50 ng (7.7 nM) purified XPG protein. After 15 min, reactions were treated with KMnO₄ where indicated. An autoradiograph of the gel is shown with G+A and C+T ladders of 3'-end-labeled DNA as markers. The sequence of the relevant portion of Pt-GTG DNA is displayed at the top with the position of the adduct indicated (Pt). Residues are numbered with the central thymine of the crosslinked GTG sequence designated T[0]. Adducted strand residues to the 3' of T[0] are denoted by positive integers [+N] and those to the 5' side by negative integers [−N]. Thymine sensitive to oxidation in the absence of extract are boxed. Residue G[+1] is also sensitive. Thymine that become sensitive as described in the text are circled. These residues define the limits of an open complex formed during NER (Evans *et al.*, 1997). **(B)** KMnO₄ modification reactions in XPA- and XPC-defective whole cell extracts. Pt-GTG DNA (lanes 1–8) or non-adducted DNA (lanes 9–12) were incubated with 100 µg HeLa extract protein (H), 200 µg XP extract protein or 100 µg BSA as indicated. Lanes 5 and 11 were complemented with ~12 ng XPC-hHR23B protein. Lane 7 was complemented with 80 ng (52 nM) purified XPA protein. Reactions were treated with KMnO₄ where indicated and analyzed as in (A). **(C)** KMnO₄ modification in RPA depleted HeLa cell extracts. Pt-GTG DNA was 3' labeled and incubated with 100 µg HeLa whole cell extract protein WCE, 60 µg HeLa CFII protein or 100 µg BSA as indicated. Five hundred ng (90 nM) purified recombinant human RPA was added for lane 2. Reactions were treated with KMnO₄ and analyzed as in (A).

B **C**

Fig. 3. Graphic representation of the pattern of permanganate sensitivity in repair-proficient and deficient extracts. Phosphorimages of KMnO_4 footprinting experiments were traced using NIH Image software. For comparison, normalized traces from two related reaction conditions are superimposed, with one trace displayed as a solid line and the other as a shaded area.

Full open complex formation depends on RPA protein

RPA is required for dual incision formation and has been implicated, together with XPA, in preferential binding to

DNA damage. To explore the participation of RPA in open complex formation, KMnO_4 sensitivity was monitored in a HeLa extract that had been fractionated to deplete it of RPA and PCNA proteins (Shivji *et al.*, 1992). The resulting

extract (designated CFII) showed only barely detectable dual incision of the platinum adduct, and repair could be restored by adding purified RPA (data not shown). Addition of HeLa CFII caused a marked sensitivity of T[−2] to oxidation and a modest sensitivity of T residues [−7], [−8] and [−10] flanking the lesion to the 5′ side (Figure 2C, compare lane 1 with 4). Adding neutralizing levels of an antibody against the p34 subunit of RPA to the CFII did not reduce the sensitivity of these residues (data not shown). Complementation of the HeLa CFII with RPA significantly enhanced the sensitivity of T residues [−7], [−8] and [−10], indicating a role for RPA in formation of the full open complex (Figure 2C, lane 2). A strong band also appeared at the position of the 3′ incision. Dual incision intermediates accumulate in this case because the absence of the DNA polymerase cofactor PCNA from the reaction mixture prevents repair synthesis.

Mutations that inactivate the 5′ endonuclease still allow full opening

Extracts from ERCC1 and XPF-defective rodent cells are defective in the 5′ cleavage reaction, but can still form 3′ incisions (Sijbers *et al.*, 1996). We tested their ability to open the DNA, which is presumed to be a prerequisite for 3′ incision formation by XPG. Adducted DNA was incubated with extracts of XPF-defective human (GM8437, Figures 4A and 3) or rodent cells (UV41, data not shown), or ERCC1-defective cell extract (CHO 43–3B, Figure 4A) and treated with KMnO₄ as indicated. With each mutant extract, a complete pattern of permanganate sensitivities appeared on the adducted strand T residues [−10], [−8], [−7], [−2] and [+5], as well as a band corresponding to the position of 3′ incisions (Figure 4A, lanes 2 and 4). This pattern was indistinguishable from that seen in the corresponding complemented reaction (Figure 4A, lanes 3 and 5) in which dual incision formation is restored (Figure 4B and data not shown). Like the XPF-defective rodent lines, human XP-F extracts were defective in 5′ and dual incision formation but formed uncoupled 3′ incisions (Figure 4B, lane 1). Complementing with ERCC1-XPF proteins resulted in 5′ cleavage and gave products of dual incision (Figure 4B, lanes 2 and 3). Thus, extracts specifically defective in 5′ nuclease activity can still open the DNA normally.

ATP-hydrolysis and open complex formation

Open complex formation was inhibited when ATP was omitted from the reaction (Evans *et al.*, 1997), suggesting that it is needed for opening up of the duplex DNA, most likely as a cofactor for one or both of the DNA helicase subunits of TFIIH. We tested the effect of substituting non-hydrolyzable analogs during a reaction with HeLa extract (Figure 5). Adducted DNA was incubated with extract in the absence or presence of ATP, ATPγS or AMP-PNP, and treated with KMnO₄ after 15 min. T[−2] became distinctly more sensitive to oxidation even without added nucleotide (lane 1) or in the presence an analog (lanes 3 and 4), compared with the reaction without extract (lane 5). A modest sensitivity of T residues on the 5′ side of the lesion was seen in the presence of ATPγS or AMP-PNP, but this sensitivity was much less than that seen in the presence of ATP (lane 2). This suggests that most opening requires ATP hydrolysis.

TFIIH subunits XPB and XPD are needed for open DNA complex formation

The requirement for ATP hydrolysis for full DNA opening around the lesion strongly implicates the DNA-dependent ATPase/DNA helicase activities of the XPB and/or XPD subunits of TFIIH in this reaction. To test this directly, extracts were used from two XP-D lines (GM2485 and GM2253) and three XP-B lines (GM2252, XPCS1BA and CHO 27-1). The XP-D cell extracts and two of the XP-B extracts (CHO 27-1, XPCS1BA) were completely defective in opening the DNA surrounding the platinum adduct (Figure 6A, lanes 5, 7, 9 and Table I). This defect was corrected by the addition of TFIIH (lanes 6, 8 and 10). Uniquely, GM2252 (XP-B) cell extract reproducibly exhibited a complete pattern of thymine hypersensitivity on the adducted strand (lane 3), similar to that seen with HeLa cell extract (lane 2). Furthermore, fragments corresponding to 3′ incisions were also produced by GM2252 extract (lane 3). This pattern of permanganate sensitivity was unaltered by adding functional TFIIH (lane 4).

One XPB mutant is specifically defective in 5′ incision formation

The ability of the TFIIH-defective cell extracts to form 3′ and 5′ incisions was examined. Both XP-D and two of the XP-B cell extracts (XPCS1BA and CHO 27–1) were completely defective in both 3′ and 5′ cleavage reactions (Figure 6B, lanes 1, 3, 5 and 7; Table I). Dual incision activity was restored by adding TFIIH (Figure 6B, lanes 2, 4, 6 and 8). We also tested the single cell line in the TTD-A group (TTD1BR) which has been proposed to harbor an unidentified mutation in TFIIH (Vermeulen *et al.*, 1994b). TTD1BR cell extract could form low levels of dual incisions (Figure 6B, lane 11) which were stimulated ~3-fold upon addition of TFIIH (lane 12).

In marked contrast to the cases above, the XPB-defective cell extract GM2252 was specifically defective in 5′ incision formation. The extract produced bands migrating in the position of uncoupled 3′ incisions, but no uncoupled 5′ incisions and only a trace amount of dual incision products (Figure 6B, lane 9). Complementing with TFIIH restored 5′ incision function, leading to dual incisions (lane 10). To check that the 3′ uncoupled incisions were authentic, a primer extension assay (Moggs *et al.*, 1996) was used and confirmed that the positions of the uncoupled 3′ incisions in GM2252 cell extract were predominantly at the 9th phosphodiester bond 3′ to the cisplatin-DNA lesion (data not shown). Furthermore, neutralizing anti-XPG antiserum inhibited the formation of uncoupled 3′ incisions by GM2252 extract, and this inhibition was reversed by adding excess XPG protein (not shown). The uncoupled 3′ incisions depended on ATP hydrolysis (Figure 6C). In repair-proficient extracts, neither dual nor uncoupled 3′ incisions occurred in the absence of ATP, nor did they form in XPB-defective GM2252 or XPF-defective GM8437 extracts. ATPγS did not substitute for ATP, indicating that ATP hydrolysis rather than just binding is necessary for 3′ cleavage of the damaged DNA strand (Figure 6C).

These results show that the XPB mutation in GM2252 cells does not interfere with the opening of the DNA or capacity of XPG to form a 3′ incision, yet despite the

Fig. 5. Full opening depends on ATP hydrolysis. (A) The effect of ATP analogs on permanganate sensitivity during repair. Pt-GTG DNA incubated with 100 μ g HeLa cell extract protein (lanes 1–4) or 100 μ g BSA (lane 5) in repair buffer lacking ATP, dNTPs and the ATP regenerating system. ATP or the non-hydrolyzable analogs ATP γ S or AMP-PNP were added to 2 mM. Each reaction was treated with KMnO₄ and analyzed as described in Figure 2A. (B) Graphic representation of (A).

does function during damage removal. Interestingly, the Rad3 K48R strain can carry out limited incision of UV damaged DNA, and a low level of nicking of UV-irradiated DNA without damage release was observed in a reconstituted incision reaction when Rad3 was replaced with a K48R mutant protein (Sung *et al.*, 1988, 1996). It is possible that uncoupled incisions are formed in this mutant, implying that Rad3/XPD helicase might perform a catalytic function after the formation of one or both incisions. Deletion of the *RAD3* gene is lethal, indicating that the protein does have an essential role, probably as a structural component of TFIIH.

These results suggest that both TFIIH helicase activities do function during NER, and our results implicate one or both of them in DNA opening, since functional XPB and XPD, as well as ATP hydrolysis, are necessary for formation of a normal open intermediate. Because the helicase activities associated with the mutant proteins in the opening-defective XP-B and XP-D cell extracts are not yet known, the precise catalytic and/or structural roles of the subunits in each step of the reaction remain to be dissected.

Pinpointing the biochemical repair defect in cells from an individual with the disorders XP and CS

Although the XPB and XPD subunits of TFIIH are required for open complex formation during NER, they

may also function during subsequent steps. Our results with extracts from GM2252 mutant cells (patient XP11BE) suggest a further role for XPB in the formation of 5' incisions. The C to A transversion mutation in XP11BE cells causes a splicing defect that changes the C-terminal 41 amino acids of the XPB polypeptide (Weeda *et al.*, 1990). This alteration of the C-terminus does not inhibit open complex formation or 3' incisions but prevents formation of 5' incisions (Figure 6). Mutation of the C-terminus of XPB does not disrupt the conserved helicase motifs in the primary sequence (Figure 7A). A truncation of the C-terminus of the yeast homolog Rad25 is also outside these motifs and leads to impaired repair but not transcription (Guzder *et al.*, 1994). Moreover, biochemical characterisation of TFIIH purified from XP11BE cells and of recombinant XPB with an identical mutation shows that the mutant XPB protein retains appreciable 3'–5' DNA helicase activity, ~40% of normal (Hwang *et al.*, 1996). The reduced DNA helicase activity may result from a conformational change in XPB caused by the mutated C-terminus. However, the helicase activity remaining in XP11BE cells appears to be sufficient to form a full open complex of ~25 nt and uncoupled 3' incisions.

The occurrence of inactivating mutations in the C-termini of XPB and Rad25 has led to the suggestion that this domain may interact with other NER factors

(Park *et al.*, 1992; Ma *et al.*, 1994b; Sweder and Hanawalt, 1994; van Vuuren *et al.*, 1994; Hwang *et al.*, 1996). The alteration of XPB protein in XP11BE cells does not appear to influence targeting or cleavage by the 3' endonuclease XPG, since normally positioned 3' incisions are still formed in XP11BE cell extract. Consistent with this, although XPG is known to interact with several TFIIH subunits including XPB, XPG does not interact with the C-terminus of XPB (Iyer *et al.*, 1996). The very similar repair defects in XP11BE, XP-F and ERCC1-defective cells specifically suggest that the C-terminus of XPB interacts with an NER factor involved in the formation of 5' incisions. XPB might recruit ERCC1-XPF to the 5' incision site or may interact with another NER factor which facilitates the 5' cleavage reaction. We attempted to overcome the defect in XP11BE cell extract by adding a large excess of purified ERCC1-XPF and RPA proteins, but were unable to induce 5' incision (data not shown).

In contrast to XP11BE, the mutation in XPCS1BA cells

is an F99S amino acid substitution (Vermeulen *et al.*, 1994a) close to or within the domain of XPB which interacts with XPG and XPD (Figure 7A; Iyer *et al.*, 1996). Consistent with either type of disruption, we observed neither open complex formation nor incision with XPCS1BA extract.

Other factors facilitating open complex formation

In addition to TFIIH, other NER proteins participate in reaction steps preceding and coincident with open complex formation. One important question is whether the nucleases themselves participate in creation of the open complex.

ERCC1-XPF and XPG nucleases are considered in turn. We tested cell extracts that were defective in either of the two subunits of the 5' incision nuclease, and each was able to open the DNA to the same extent as repair proficient extracts. Each mutant extract could make 3' incisions, but could not catalyze 5' incisions, showing that the nuclease activity of ERCC1-XPF is not necessary

Fig. 6. The role of TFIIH in open complex formation and dual incision. (A) The effect of mutations in XPB and XPD on DNA opening during repair. Pt-GTG DNA was incubated with 100 µg HeLa cell extract protein, 200 µg protein from XP-B or XP-D extracts or BSA as indicated. Reactions in lanes 4, 6, 8 and 10 were complemented by adding TFIIH. Reactions in lanes 2–11 were treated with KMnO₄ and analyzed as in Figure 2A. (B) The effect of mutations in TFIIH on 3' and 5' incision formation. Pt-GTG DNA was incubated with 200 µg protein from extracts of XP-B, XP-D extracts or TTD-A defective TTD1BR. Purified TFIIH was included for lanes 2, 4, 6, 8, 10 and 12. DNA was purified and analyzed as indicated in Figure 1A. (C) The effect of ATP analogs on incision formation. Pt-GTG DNA was incubated in repair buffer lacking ATP, dNTPs and the ATP regenerating system, with 100 µg repair proficient SW48 cell extract protein (lane 7–9), 200 µg extract protein from GM2252 (lanes 1 and 4), GM8437 (lanes 2 and 5) or a mixture of the two (lanes 3 and 6). Reactions were supplemented with 2 mM ATP or 2 mM ATPγS as indicated. DNA was purified and analyzed as shown in Figure 1A. Degradation of 3' uncoupled incision fragments, resulting in a band at ~42 nt, is particularly pronounced in the absence of dNTPs.

for opening. But is the presence of ERCC1-XPF necessary as a structural component? CHO 43-3B cells produce a greatly reduced amount of ERCC1 protein containing a single amino acid change and a small but detectable amount of XPF protein. In GM8437 cells, only a minute amount of mutated XPF protein is detectable by immunoblotting, but a small amount of ERCC1 is readily detectable (Biggerstaff *et al.*, 1993; Yagi *et al.*, 1997). Taken together, the data leave open the possibility that ERCC1 might have a structural role in open complex formation, even if its catalytic function is inactivated. Indeed, ERCC1 may aid the damage recognition step (Nagai *et al.*, 1995).

Further delineation of any role for ERCC1-XPF in the opening reaction requires experiments with defined mutant forms of ERCC1-XPF nuclease in a reconstituted system.

Since opening was also observed using XPG83 cell extract (Evans *et al.*, 1997), it appears that neither endonuclease activity is required to initiate the opening reaction. However, the presence of nuclease-defective XPG protein in a reconstituted NER reaction can permit uncoupled 5' incisions to be made by ERCC1-XPF, suggesting that XPG protein may play a structural role in the repair complex distinct from its nuclease activity (Wakasugi *et al.*, 1997). Similarly, inactivation of the 3' nuclease in

Table I. Repair defects in extracts from mutant cell lines

Cell line (patient derivation)	Mutated NER protein	Open complex formation	Uncoupled 3' incision	Uncoupled 5' incision	Dual incision
HeLa	—	+++	+	±	+++
HeLa CFII	RPA depleted	+	n.d.	n.d.	—
SW48	—	n.d.	+	±	+++
GM2345 (XP20S)	XPA	+	—	—	—
GM2252A (XP11BE)	XPB	+++	+	—	—
XPCS1BA	XPB	—	—	—	—
CHO 27-1	XPB	—	—	—	—
GM2246 (XP1BE)	XPC	—	—	—	—
GM2249 (XP8BE)	XPC	—	—	—	—
GM2485 (XP7BE)	XPB	—	—	—	—
GM2253 (XP17BE)	XPB	—	—	—	—
CHO UV41	XPF	+++	+	—	—
GM8437 (XP2YO)	XPF	+++	+	—	—
CHO 43-3B	ERCC1	+++	+	—	—
LBL463 (XP3BR)	XPG	++	—	—	—
XPG83 (XP125LO)	XPG	++	—	—	—
AG08802 (XP20BE)	XPG	+	—	—	—
XPG415A (XP2BI)	XPG	++	—	—	—
TTD1BR	(TTD-A)	n.d.	±	—	+

n.d., not determined.

HeLa cell extract by anti-XPG antibody permits correctly-positioned 5' incisions (J.G.Moggs, unpublished data). Consistent with these observations, we found that various XPG-defective extracts induced permanganate sensitivity even when 3' incision activity was missing, but that this pattern was consistently enhanced by adding functional XPG protein to the reaction. This effect was most pronounced in extracts of cells from the severely affected patient XP20BE (Moriwaki *et al.*, 1996), which do not catalyze detectable dual incisions *in vitro* and lack detectable full-length XPG protein. Adding XPG to these extracts reproducibly enhanced the sensitivity of the 5' residues (Figure 3) which may represent increased turnover of repair complexes by allowing dual incision, or may represent a structural modification of the open pre-incision complex induced by the binding of XPG.

XPC protein also plays an essential role in promoting open complex formation. Both XPC-defective extracts tested were completely defective in opening and displayed the same inability to induce hypersensitivity of the T[–2] site. This hypersensitivity has been observed in repair-proficient extracts even when repair is inhibited by the omission of ATP from the reaction, as well as in most other incision-defective extracts including those from XP-A, XP-G, XP-F and ERCC1 mutant cell lines, and HeLa extracts depleted of RPA. This suggests that XPC has a role in modulating DNA structure immediately surrounding a lesion and may perform an initial step that allows the further action of other factors to promote opening and dual incision. Such a role may not be required in the context of a stalled RNA polymerase complex during transcription-coupled repair (van Hoffen *et al.*, 1995; Mu and Sancar, 1997).

XPA binds to damaged DNA *in vitro* (Jones and Wood, 1993), interacts with TFIIH (Park *et al.*, 1995), RPA (He *et al.*, 1995; Li *et al.*, 1995) and ERCC1 (Li *et al.*, 1994; Nagai *et al.*, 1995), and may help recruit these repair factors to the site of a lesion prior to or during open complex formation. RPA also binds preferentially to dam-

aged DNA *in vitro* (Clugston *et al.*, 1992) and this binding is increased synergistically in the presence of XPA (He *et al.*, 1995). RPA is able to unwind duplex DNA to some extent (Georgaki *et al.*, 1992) and binds in modes of 8–10 and 30 nt (Blackwell *et al.*, 1996). We found that depletion of RPA from a HeLa extract inhibited the NER incision reaction and reduced permanganate sensitivity around the Pt adduct, but that T residues toward the 5' incision remained somewhat sensitive. This pattern of sensitivity in the absence or presence of repair appeared similar to that observed in GM2345 XP-A cell extracts (see Figure 3). GM2345 cells harbor no detectable XPA protein and catalyze no NER. These results are consistent with the close cooperation of RPA and XPA during the reaction. The remaining permanganate sensitivity in extracts depleted of either factor suggests that other NER components may be involved in the initial steps of damage recognition.

Mechanism of opening and incision during mammalian NER

A survey of the incision activity in cells defective in core NER proteins reveals deficiencies in either the 5' incision or both cleavages together but not in the 3' incision reaction only (Table I). This suggests a sequential cleavage mechanism during NER with 3' incisions being made first (O'Donovan *et al.*, 1994; Aboussekhra and Wood, 1995; Mu *et al.*, 1996). Although some uncoupled incisions occur in repair-proficient cell extracts the majority of incision events around the Pt-GTG adduct are tightly coupled, leading to excision of the characteristic 24 to 32-mer oligonucleotides (Figure 1). Some uncoupled 3' incisions were found to precede dual incision during repair of a single pyrimidine dimer in a reconstituted system (Mu *et al.*, 1996). Uncoupling may be more readily observed during repair of thymine dimers due to their much slower repair relative to 1,3-intrastrand d(GpTpG)-cisplatin crosslinks. Based on their location and their inhibition by neutralizing antibodies, it is clear that 3'

Fig. 7. (A) Domain structure and positions of relevant mutations in the XPB protein subunit of TFIIH. (B) Model of open complex formation leading to dual incision around the cisplatin crosslink during human nucleotide excision repair.

uncoupled incisions are mediated by XPG at the correct position, but they may not be normal intermediates leading to dual incision. Indeed, if 5' and 3' incisions are usually tightly coupled, uncoupled incisions may represent an abortive reaction product. This view is supported by the fact that complementation of ERCC1, XP-F or GM2252 extracts defective in 5' cleavage restores dual incision formation, but does not reduce the number of uncoupled 3' incisions detected (Figures 4B and 6B).

The complete insensitivity of residues flanking the Pt-GTG crosslink in XPC and most TFIIH-defective extracts suggests that these two factors play a fundamental role in initiating the opening that leads to dual incision formation. One possible model (Figure 7B) is that TFIIH-associated DNA helicases initially catalyze limited opening between the lesion and the 3' incision site followed by binding of RPA to an 8–10 nt open intermediate, facilitating further ATP-driven opening by a combination of factors which finally targets nuclease cleavage. This interpretation suggests that opening catalyzed by TFIIH during repair may be mechanistically related to the limited strand opening observed during RNA polymerase II transcription initiation, where the formation of a ~10 bp bubble around the promoter depends on TFIIH and ATP hydrolysis (Jiang and Gralla, 1995; Dvir *et al.*, 1996; Holstege *et al.*, 1996). Finally, following the promotion of an open complex during NER, it appears that TFIIH has a further role in events leading to the 5' incision. Our data suggest that the C-terminal domain of XPB might be involved in recruitment of the XPF-ERCC1 endonuclease to the 5' incision site or may interact with another NER factor which facilitates the 5' cleavage reaction.

Materials and methods

Human cell extracts and purified repair proteins

Whole cell extracts were prepared as described previously (Wood *et al.*, 1995) from the cell lines listed in Table I. The presence of the XP11BE mutation in GM2252 lymphoblastoid cells was confirmed by G. Weeda (Erasmus University, Rotterdam). Fractionation of HeLa whole cell extract on phosphocellulose to obtain CFII was as described (Shivji *et al.*, 1992). Preparation of purified XPA, XPC-hHR23B, XPG, RPA and TFIIH (Heparin-5PW fraction) proteins was as described (Aboussekhra *et al.*, 1995; Hwang *et al.*, 1996; Evans *et al.*, 1997). The expression in *Escherichia coli* and purification of recombinant XPF-ERCC1 protein (R.Ariza, M.Biggerstaff and R.D.W.) will be presented elsewhere.

DNA containing a 1,3-intrastrand d(GpTpG)-cisplatin crosslink

Covalently closed circular DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink (Pt-GTG) or lacking damage (Con-GTG) was produced as described, using M13mp18GTGx bacteriophage DNA (Moggs *et al.*, 1996). For chemical footprinting of repair reactions, closed circular DNA was cleaved at the *Ava*II site 140 bp away from the platinum lesion and radiolabeled uniquely at the 3'-end of the adducted strand prior to incubation with cell extract.

In vitro repair

Reaction mixtures (50 μ l) contained 200 ng of the platinated or control DNA substrate and whole cell extract protein (100 μ g unless otherwise indicated) in a buffer containing 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μ M each of dATP, dCTP, dGTP and TTP, 22 mM phosphocreatine (di-Tris salt), 2.5 μ g creatine phosphokinase, 3.4% glycerol and 18 μ g bovine serum albumin (Wood *et al.*, 1995). Some experiments used repair buffer without dNTPs, ATP or an ATP-regenerating system and were supplemented with ATP, ATP- γ -S or AMP-PNP (Fluka) as indicated. Cell extract was pre-incubated with buffer at 30°C for 5 min, DNA

substrate was added and reactions incubated at 30°C for 30 min or as indicated.

Analysis of DNA repair reaction intermediates

Incisions 3' and 5' to the lesion were detected by Southern hybridization as described previously (Moggs *et al.*, 1996; Sijbers *et al.*, 1996). Briefly, purified reaction products were cleaved with *Hind*III and *Xho*I, separated in denaturing 12% polyacrylamide gels, transferred to charged nylon membrane and hybridized with a ³²P-labelled oligonucleotide probe complementary to the DNA sequence surrounding the cisplatin-DNA adduct. Chemical footprinting of single-stranded DNA was performed as described (Evans *et al.*, 1997). After 15 min of repair, 6 mM KMnO₄ was added to reactions for 1 min to oxidize unpaired (primarily thymine) bases, followed by the quenching of oxidation with 1 M 2-mercaptoethanol. Oxidized residues were cleaved with 1 M piperidine and DNA was separated on denaturing 6% polyacrylamide gels. Maxam-Gilbert sequencing ladders of 3' end-labeled, non-adducted M13 DNA were prepared as markers. Gels were fixed, dried and visualized by autoradiography or the phosphorimager. In some cases, parallel reactions were done using unlabeled, *Ava*II-cleaved Pt-GTG DNA and the products analyzed for dual incision formation to confirm that complementation of repair proceeded as expected on the linear template.

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